THE GENETICS OF MYCOLIC ACIDS BIOSYNTHESIS IN *CORYNEBACTERIUM GLUTAMICUM*

By

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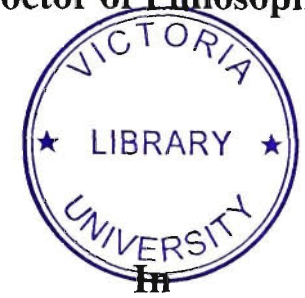
A thesis submitted for the degree of

Doctor of Philosophy



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DECLARATION

I, Sayyada Amatul Hafeez, declare that the PhD thesis entitled *The genetics of mycolic acid biosynthesis in Corynebacterium glutamicum* is no more than 100,000 words in length, exclusive of tables, figures, appendices, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work.

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ACKNOWLEDGEMENT

Many people contributed towards the completion of this study. First of all I would like to thank *AusAID,* Centre for Bioprocess and Food Technology for providing financial support.

My greatest acknowledgement is to my supervisor, Prof. Margaret Britz who encouraged me to undertake this study. I am thankful to her for the faith that she placed in me as a researcher, her timely advices clear guidance and encouragement throughout the course of this work.

I would like to recognise the assistance and concern of Dr. Maria Serificia for her role as co-supervisor.

Sincere and appreciative acknowledgment is made to everyone who contributed to the thesis and this includes: Prof. Akhter Kalam Deputy Dean; former staff, Nikola Popovic for his technical assistance, Mr Vilnis Ezerniks for assistance in GC work, Ivana Radojevic, Shin-Jae Change, Dr. Ki-Hyo Jang and other friends n the laboratory.
Special thanks must go to the technical and professional staff of the Centre for Bioprocess and Food Technology, Dale Tomilson for his technical assistance.

My special thanks are extended to Dr. Sara Fraser for her warm friendship, encouragement valuable assistance and advice through out my stay at VUT. I must express my appreciation for the friendly guidance and support of my Lab friends Jang, Aslam Khan, and Mathiew Knight.

This acknowledgement would be incomplete if I do not mention two silent supporters - my parents - whose prayers are source of my strength. I wish my father to be there he had great love for knowledge.

At the end I would like to dedicate this humble effort, the fruit of my thoughts and studies to my family, my husband and my little daughter Zuhaa for their love and support.

SUMMARY

Because of the great importance of *C. glutamicum*, and *Brevibacterium* in fermentation Industry and its potential for application in foreign protein expression, research has been focused mainly on strain improvement and development of technique to overcome the barriers that effect successful introduction of foreign DNA into these corynebacteria. Corynebacterial cell wall structures, which include corynemycolic acids, act as a physical barrier for DNA transformation into the cell. While investigating the physical and biochemical barrier for gene manipulation to these organism Britz and colleague isolated a series of auxotrophic mutants of *C. glutamicum* strain 13059, which were later found to be cell surface structure mutants.

The research reported in this thesis focuses mainly on understanding the nature of physical barrier including: (i) determining the effect of growth medium containing glycine and INH on the mycolic acids and fatty acids composition of cell wall and compare this with the whole cell mycolates of wild-type and mutants of *C. glutamicum.* (ii) investigating the genetics of mycolic acids biosynthesis this involve identification of presumptive *inhA* gene involve in mycolic acid synthesis using various molecular biology approaches in *C. glutamicum* and *Brevibacterium* species, sequencing the *inhA* gene in several corynebacteria strains and to define any genetic changes, which may have occurred in INH-hypersensitive mutants through sequence analysis of the InhA protein in mutants in comparison the sequence, determined for the wild type *C. glutamicum.* (iii) determining the function of presumptive *inhA* gene in corynebacteria by inactivation using homologous recombination and analysis of the resulting mutants to deduce correlation between genetic and physiological changes.

Corynebacterial strains used in this study included *C. glutamicum* AS019 (rif^r mutant ATCC 13059) and auxotrophic mutants MLB133 and MLB194, *csp*1 inactivated (*csp*1 gene responsible for S-layer formation) mutant strain of *C. glutamicum*, restriction

deficient mutant RM4 of ATCC 13032. Two *Breibacterium* species *B. lactofermentum* and *B. flavum* were also included in this study.

To study the effect of some of the potential growth modifier like glycine, INH on lipids profile of cell wall, MICs of INH and ETH its structural analog were determined. The present work showed that corynebactereia is less sensitive to INH than *Mycobacterium* However, distinct differences in INH MICs between the corynebacterial species used were observed. AS019 and BF4 were more resistant whereas BL1 and RM4 were more sensitive to INH. Similarly, *csp*1 disrupted mutant of *C. glutamicum* behaved similar to AS019. The two mutants MLB133 and MLB194 exhibited greater sensitivity towards INH relative to the parent type strain AS019. MICs of ETH showed that *C. glutamicum* was more susceptible to ETH thus confirming that ETH was more effective than INH in corynebacteria.

Cell wall of all strains of corynebacteria tested contained five major types of mycolic acids ($C_{32:0}$, $C_{34:0}$, $C_{34:1}$, $C_{36:2}$, $C_{36:1}$). In the cell wall fraction the relative proportions of unsaturated mycolic acid ($C_{34:1}$ and $C_{36:2}$ was higher and $C_{32:0}$ was lower). In the AS019 family, the proportion of $C_{32:0}$ was in decreasing order of AS019, MLB194 and MLB133. After the addition of 2% glycine in growth media the proportion of unsaturated MAMEs in the cell wall fractions ($C_{34:1}$, $C_{36:2}$, $C_{36:1}$) decreased significantly (from 60.0% in LBG to 30.3% in LBG-G for AS019 and 63.3% to 30.5% for MLB133) compared to growth in LBG whereas $C_{32:0}$ was proportionately increased. The mycolic acids profile of the strains tested showed little quantitative variation in the mycolic acids for both cells and cell wall fractions. For all strains tested palmitic acid ($C_{16:0}$) and oleic acid ($C_{18:1}$) were the major fatty acids in both cells and cell walls. There was a trend towards slight decreases in FAMEs after growth in LBG-G and LBG-I suggesting that these chemicals inhibited fatty acids synthesis.

Results described in this thesis further report the identification, cloning and sequencing of the *inhA* gene homologue in several corynebacterial strains. This gene was first reported

in mycobacterial species where it was believed to be involved in mycolic acids biosynthesis and also one of the targets for INH. This data is the first documentary report on the genetics of mycolic acids biosynthesis in corynebacteria. Southern hybridisation profiles of AS019, MLB133, MLB194 indicates the presence of multiple copies of the *inhA* gene on the chromosome compared with *M. smegmatis*. The translation product of the *inhA* gene was found to 2-enoyl-acyl-carrier protein reductase, which showed significant homology with enzyme involved in bacterial and plant fatty acids biosynthesis. The highest identity score was 98% with *M. smegmatis* strongly suggesting its involvement in lipid biosynthesis. Sequence comparison of AS019 and two mutants with *M. smegmatis* indicates that two mutants were similar to *M. smegmatis* compared with their parent strain AS019. The *inhA* gene was successfully cloned and sequenced in two *Brevibacterium* strains BL1 and BF4 using PCR approaches. The InhA proteins of two *Brevibacterium* species showed amino acids variations at several points. Amino acid comparison of InhA proteins of *C. glutamicum* strain AS019 with two *Brevibacterium* species showed that *B. flavum* is similar to AS019.

Finally, the *inhA* gene was inactivated in *B. lactofermentum* using homologous recombination. Results showed that the emerging mutants failed to grow ordinary rich media implying they have altered cell wall. The mutant had similar MICs values as seen for the parents indicating that the *inhA* inactivation had no effect on INH resistance and hence InhA protein is not the target for INH in corynebacteria. Lipids profile analysis showed the absence of mycolic acids in transconjugants, fatty acids however, were detected in lower proportion as compare to parents.

ABBREVIATIONS USED IN THIS THESIS

A_{600}	Absorbance at 600 nm
ANGIS	Australian National Genomic Information Service
Amp	Ampicillin
ATCC	American Type Culture Collection, Rockville, U.S.A.
bp	Base pair
BAP	Bacterial alkaline phosphatase
BSA	Bovine serum albumin
°C	Degree Celsius
$C_{14:0}$	Myristic acid
$C_{15:0}$	Pentadecanoic acid
$C_{16:0}$	Palmitic acid
$C_{17:0}$	Hepatadecanoic acid
$C_{18:0}$	Stearic acid
$C_{18:1}$	Oleic acid
C_{25}	Lignoceric acid methyl ester
$C_{32:0}$	Corynemycolic acid
$C_{34:1}$	Corynemycolic acid
$C_{34:0}$	Corynemycolic acid
$C_{36:2}$	Corynemycolic acid
$C_{36:1}$	Corynemycolic acid
FSAQ	Food Safety Authenticity and Quality Unit
c.f.u	Colony forming unit
Cm	Chloramphenicol
CsC1	Caesium chloride
EtBr	Ethidium bromide
Cyd	Cytidine
CTAB	Hexadecyltrimethylammonium bromide
D	Dalton
dNTPs	Dinucleotide triphosphate
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DSM	Deutsche Sammlung von Microorganism, Gottingen, Germany
DTT	Dithiothreitol
EMS	Ethyl methanesulphonate
EtOH	Ethanol
FAMEs	Fatty acid methyl esters
FID	Flame ionisation detector
G+	Gram
GC	Gas chromatography

HPLC	High Performance Liquid Chromatography
INH	Isonicotinic acid hydrazide
IPTG	Isoproylthio-β-D-galactoside
ileu	Isoleucine
K	Kilo
Km	Kanamycin
LA	Luria agar
LAG	Luria agar with glucose
LB	Luria broth
LBG	Luria broth with glucose
LBG-G	LBG supplemented with glycine
LBG-1	LBG supplemented with INH
LBG-G	LBG supplemented with glycine and INH
Leu	Leucine
l	Litre
MAMEs	Mycolic acid methyl esters
MIC	Minimal inhibitory concentration
min	Minute
mM	Millimolar
MS	Mass spectormetry
μg	microgram
μl	microlitre
μ	Cell specific growth rate
M.W	Molecular weight
NA	Nutrient agar
NAG	Nutrient agar with glucose
Nx	Nalidixic acid
NCIB	National Collection of Industrial Bacteria, Aberden, Scotland.
NADH	β-nicotinamide adenine dinucleotide
Neo	Neomycin
NTG	N-methyl-N-nitro-N-nitrosoguanidine
OC DNA	Open circular deoxyribonucleic acid
o.p.m.	Orbits per minute
ORFs	Open reading frames
PEG	Polyethylene glycol
Rf	migration distance relative to front
Rif	Rifampicin
RM system	Restriction and modification system
RNase	Ribonuclease
Sec	Second
SDS	Sodium dodecyl sulphate
SSC	0.15 M Nacl, 0.15 M sodium citrate

Str	Streptomycin
SD	Standard deviation
Tc	Tetracycline
TE	Transformation efficiency
TF	Transformation frequency
TLC	Thin layer chromatography
TMS	Trimethylsily1
Tris	Tris (hydroxymethyl) aminomethane
Tween 80	Polyoxyethlene sorbitan monooleate
U	Unit
V	Volt
v	Volume
v/v	Volume per volume
w/v	Weight per volume
wt	Weight
X-gal	5-Bromo-4-chloro-3-indolyl-β-D-galactoside
VUT	Victoria University of Technology

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Chapter 1

Introduction

1.1 GENERAL INTRODUCTION

Coryneform bacteria are a taxonomically ill-define group of Gram-positive bacteria with a rod or club shape. The term coryneform, originally used to describe the club or wedge-shape of *Corynebacterium diphtheriae* and related animal pathogens, is now widely used to describe Gram-positive, non-mycelial, non-sporing bacteria. The group includes many animal pathogens (Barksdale, 1981), plant pathogens (Carlson and Vidauer, 1982), and saprophytic (non-pathogenic) soil organisms used for the industrial production of amino acids such as arginine, glutamic acid, isoleucine, leucine, lysine, ornithine, proline, threonine, tryptophane and valine (Hirose *et al.*, 1985; Kinoshita, 1985). Other strains synthesise emulsifying agents (Duvnjak and Kosarie, 1981; Zajic *et al.*, 1977), degrade hydrocarbons (Cardini and Justshuk, 1970; Cooper *et al.*, 1979), produce anti-tumour activity (Milas and Scott, 1978) or carry out steroid conversion (Constantinides, 1980; Yamada *et al.*, 1985). Among the explanations advanced for the numerical predominance of some of these bacteria in soil are their extreme resistance to drying and to starvation and the nutritional versatility of the commonly occurring species (Jones and Keddie, 1981). The group also contains various degraders of chlorophenol (McAllister and Trevors, 1996), cyanide (Dubey and Holmes, 1995), or diphenyls (Higson, 1992) and is therefore of environmental and biotechnological interest. The group comprising plant pathogenic corynebacteria were renamed *Clavibacteria* (Davis *et al.*, 1984).

Saprophytic species of corynebacteria used for the industrial production of amino acids include *Corynebacterium glutamicum, Brevibacterium* and related genera *Arthrobacter* and *Microbacterium* (Archer *et al.*, 1989; Batt *et al*; 1985a, Eikmanns *et al.*, 1993; Kinoshita, 1985; Liebl 1991; Wohlleben *et al.*, 1993). In the early 1990s, studies including DNA-DNA hybridisation, chemotaxonomy and biochemical studies revealed only minor differences between the *Brevibacterium flavum*, *Brevibacterium lactofermentum* and *C. glutamicum* species (Eikmanns *et al.*, 1991; Liebl *et al.*, 1991),

and therefore Liebl *et al.* (1991) proposed that these species be considered as *C. glutamicum* species. However, the controversy about the relationship between these two corynebacteria groups used routinely as amino acid producing strains, *B. lactofermentum* and *C. glutamicum,* remained unresolved (Liebl *et al.*, 1991; Correia *et al.*, 1994). Although there was a relatively high degree of similarity (RFLP studies), there were enough clear differences to allow separation of *B. lactofermentum* from *C. glutamicum* (Correia *et al.*, 1994). The cloning of the rRNA operons (*rrn*) provided a very useful instrument to settle the polemic about the taxonomic relatedness of these two glutamic acid producing corynebacteria. Characterisation of an operon from *B. lactofermentum*, including the complete sequence of the 16S rRNA gene and upstream regulatory region and transcriptional analysis, has been recently reported by Amador *et al.* (1999). Phylogenetic studies using complete 16S rRNA sequence analysis showed that '*B. lactofermentum*' is closely related to several species of the genus *Corynebacterium* but only distantly related to the type species *Brevibacterium linens* and therefore the authors suggested that it should be reclassified as *Corynebacterium lactofermentum.* Analysis of the *rrnD* sequence has provided information not only on the phylogeny of '*B. lactofermentum*' but also on the molecular control of macromolecule gene expression.

The largest market for amino acids is in food ingredients and nutritional supplements for human and animal use. The world-wide production in 1992 of the flavour enhancer mono-sodium glutamate (MSG) and the food supplement lysine by *C. glutamicum* was estimated at approximately 800,000 and 400,000 metric tons respectively (Jetten and Sinskey, 1995; Randell and Andreas, 1992). Australia imports over $40 million worth of lysine, an essential amino acid, *per annum* for incorporation into products made from grains (particularly bread) and into pig feed (growth stimulant, improves fertility). Strain improvement for amino acid production has been based traditionally on mutation and selection, which was particularly effective in this group of bacteria because of their inherent ability to overproduce glutamate and the relatively simple metabolic regulatory mechanisms present. Furthermore their physical traits made *C. glutamicum* and related species particularly suitable for large-scale industrial fermentations: strains grow well on simple defined media; secretion of amino acids is achieved by manipulating the cell

surface structures by growth in biotin-limited media and exposure to ethanol or penicillin G, so that high intracellular concentrations of metabolic intermediates, which may otherwise limit production, does not occur; and corynephages are relatively rare so that fermentation failure due to bacteriophage infection is minimal (Britz and Demain, 1985). Because of their industrial importance, the genetics and enzymology of amino acid synthesis by *C. glutamicum* has been studied extensively (Abe and Takayama, 1972; Fazal *et al.*, 1980; Hagino *et al.*, 1975). Extensive "classical" mutagenesis of some coryneform bacteria (*C. glutamicum, B. lactofermentum* and *B. flavum*) was used to obtain improved strains for the production of lysine, glutamic acid, threonine, tryptophane and a variety of other amino acids (Shiio, 1983), and the flavour enhancing nucleotides. A successful approach to obtaining high yielding mutants has been selection of variants resistant to amino acid analogs such as 5-fluorotryptophane, S-aminoethylcysteine, and α-amino-β-hydroxyvaleric acid. Resistance to amino acid analogs frequently results in deregulation of the corresponding amino acid biosynthetic pathway due to a change in the structural gene of an enzyme(s) that is the target of inhibition, or mutation in regulatory sequences so that transcription and/or translation is increased. Another successful strategy to increase amino acid production was to raise the metabolic flow through a pathway by blocking branch pathways through auxotrophic or regulatory mutagenesis (Martin *et al.*, 1987). A further increase in production by such traditional techniques is rather limited because the cumulative effect of the mutations previously introduced can result in almost complete physiological deregulation. However, further increase in yields were often necessary to make the fermentation route to production of particular amino acids commercially feasible.

A new strategy to strain improvement was initiated in the early 1980s by developing cloning systems, so that techniques such as the construction of high expression and promoter-probe vectors and site-directed mutagenesis could be applied to corynebacteria. The first reports on the metabolic engineering of *C. glutamicum* appeared in 1984 (Miwa *et al.*, 1984; Santamaria *et al.*, 1985). Since that time, several research groups have independently initiated research programs focusing on the development of metabolic engineering tools for *Corynebacterium* species (for example see Jetten *et al.*, 1993;

Martin *et al.*, 1987; Yoshihama *et al.*, 1985; Wohlleben *et al.*, 1992). The approach normally taken has involved using natural plasmids (Davis *et al.*, 1987; Katsumata *et al.*, 1984) or constructing shuttle vectors which function in *E. coli* and the corynebacterial host and which can be transferred using protoplast transformation (Katsumata *et al.*, 1984; Ozaki *et al.*, 1984; Thierbach *et al.*, 1988; Yoshihama *et al.*, 1985), electroporation (Haynes and Britz, 1990; Liebl *et al.*, 1989; Vertes *et al*; 1994b., Wolf, 1989) or conjugation (Schäfer *et al.*, 1990). The application of recombinant DNA techniques has allowed expression of foreign genes and production of recombinant proteins. Foreign genes successfully expressed by *C. glutamicum* include α-amylase of *Bacillus amyloliquifaciens* (Smith *et al.*, 1986), *Cellulomonas fimi* nuclease (Paradis *et al.*, 1987), bacterial genes involved in amino-acid biosynthesis (Patek *et al.*, 1989), *Staphylococcus aureus* nuclease (Liebl *et al.*, 1992) and ovine gamma interferon (Billman-Jacobe, 1994).

It has been reported that *C. glutamicum* grown in rich medium secretes two main proteins, PS1 and PS2 (Joliff *et al.*, 1992). However, a large fraction of PS2 remains associated with the cell wall. The existence of two distinct major proteins which are abundantly secreted into the *C. glutamicum* culture supernatant shows that *C. glutamicum*, well known for metabolite excretion, is also able to secrete significant amounts of proteins. The deduced N-terminal region of PS1 of *C. glutamicum* encoded by *csp1* gene is similar to the antigens 85 complex of *Mycobacterium tuberculosis,* act as a mycoloyl tranferase catalysing the transfer of mycolic acids not only to arabinogalactan, but also to trehalose monomycolate (Puech *et al.*, 2000). In addition to *csp1* (renamed *cmytA*), five new *cmyt* genes (*cmytB-F*) were identified in the two strains of *C. glutamicum* and three *cmyt* genes in *C. diphtheriae* (De Sousa *et al.*, 2003, Kacem *et al.*, 2004). In silico analysis showed that each of the putative cMyts contains the esterase domain, including the three key amino acids necessary for the catalysis.

Most recently Portevin *et al.* (2004) have identified a new protein pks13, an enzyme which catalyses the condensation of two fatty acids to form mycolic acids both in Corynebacteria and Mycobacteria. They showed that a *C. glutamicum* strain with a deletion in pks13 gene was deficient in mycolic acid production but was able to produce

fatty acids precursors. This mutant strain had an altered cell envelope structure. In 1995, Billman-Jacobe *et al.* reported that *C. glutamicum* can express and secrete foreign exoproteins originating from Gram-positive and Gram-negative bacteria and that this species has the potential to be developed as a host for foreign gene expression and secretion. This conclusion was based on their study of cloning and expression of genes encoding the basic protease of *Dichelobacter nodosus* (*bprV*) and the *Bacillus subtilis* (*aprE*) in *C. glutamicum*. In each case, enzymatically active protein was detected in the supernatants of liquid cultures. More recently Salim *et al.* (1998) have reported the use of *C. glutamicum* as an alternative host for the functional heterologous expression of the *Mycobacterium smegmatis* 85A gene.

Barriers to successful introduction of foreign genes into corynebacteria include the presence of restriction-modification systems (Jang *et al.*, 1996; Katsumata *et al.* 1984; Schäfer *et al.* 1994b; Tauch *et al.* 1994), which preclude survival of heterologously derived plasmid DNA and cause low transformation efficiency (Haynes and Britz, 1990; Serwold-Davis *et al.*, 1987;), and the physical barrier of a complex cell wall structure (Haynes and Britz, 1990).

The cell wall of Gram-positive bacteria of the genera *Corynebacterium, Brevibacterium, Rhodococcus* and *Nocardia* have similar overall architecture, which includes a thick peptidoglycan layer covalently bound to arabinogalactan, and they possess mycolic acids linked to the polysaccharides (Barksdale and Kim, 1977; Collins *et al.*, 1982a; Chevalier *et al.*, 1988; Tomiyasu, 1982). Indeed, mycolic acids are a major component of the cell wall structure of this group of bacteria and mycobacteria where, for instance, up to 60% by weight of the mycobacterial cell is composed of mycolic acids (Jarlier and Nikaido, 1994). Mycolic acids are α-alkyl, β-hydroxy fatty acids with up to 90 carbons in mycobacterial species and 28-50 carbons in corynebacteria. It is believed that mycolic acids have a structural role which gives rise to the resistance of the cell to harsh environments and causes the acid fastness of mycobacteria; they probably play an important role in the restricted permeability of these microbes to water-soluble molecules (Jarlier and Nikaido, 1990). Little is known about the assembly of the cell wall of *C.*

glutamicum, thus it is not clear whether it acts in the same way as the permeability barrier in mycobacteria. Methods to improve the excretion of particular amino acids have developed which may influence the integrity of the cell wall and/or membranes, such as treatment with penicillin G or amide surfactants. Several workers have reported that mycolic acid composition of corynebacteria was modified by changes in environmental conditions such as growth temperature (Suzuki *et al.*, 1969, Tomiyama *et al.*, 1980, Tomiyasu *et al.*, 1980) and additives such as Tween 80, (Chevalier, 1988), glycine, or isonicotinic acid hydrazide (INH) (Best and Britz, 1986; Jang *et al.*, 1997; Tomiyasu *et al.*, 1984). These changes in the mycolic acid composition reduce the physical barrier to DNA transformation. Best and Britz (1986) also observed mutants of *C. glutamicum*, which were auxotrophic for various branched-chain amino acids, protoplasted more readily than their parent (Best and Britz, 1986), noting that these strains showed higher sensitivity to growth inhibition by glycine. Furthermore preliminary studies also showed that *C. glutamicum* is relatively insensitive to INH when compared to *Mycobacterium* species but the mutants were more sensitive to INH than their parent, suggesting that these mutants had altered cell-surface properties. To understand the nature of these mutations and the mechanism of action of glycine and INH, Pierotti (1987) and Jang *et al.* (1997) analysed the fatty acid and mycolic acid composition of strain ATCC 13059 and selected mutants which had been shown previously to protoplast more readily, with specific reference to the lipid composition following growth in glycine and INH. Their findings showed that glycine and INH enormously affected cell growth, cell wall morphology and the mycolic acid composition of *C. glutamicum*. In addition to the previously reported targeting of the peptidoglycan cross-linking, these results showed that glycine affects mycolic acid attachment to the cell surface of *C. glutamicum*.

Biochemical evidence has suggested that both INH and ethionamide (ETH, α-ethylthioisonicotinamide a structural analog of INH and a second line anti-tuberculosis drug) block mycolic acid biosynthesis in *M. tuberculosis* and other mycobacteria (Winder, 1982 and Winder *et al.*, 1982) at a concentration of the drug which produces >99% reduction of bacterial counts. Banerjee *et al.* (1994) used a genetic approach to identify the gene encoding at least one of the targets of INH and ETH and characterised

the mutation within the gene. The target site of INH in *M. tuberculosis* and several other mycobacterial species was identified as the InhA protein, which is a 2-*trans*-enoyl-acyl carrier protein (ACP) involved in fatty acid synthesis (Dessen *et al.*, 1995).

The present study focused on investigating the genetics of mycolic acid synthesis in *C. glutamicum* by analysing the genetic changes which had occurred in INH hyper-sensitive mutants, specifically through characterisation and sequence analysis of the *inhA* gene homolog. Insertional inactivation approaches were used to determine the role of a presumptive *inhA* gene in mycolic acid biosynthesis. Further analysis on the changes in the cell surface structures of the mutants was also performed. The remainder of the Introduction section provides a literature review on various aspects of the above and defines the subject of this thesis.

1.2 CELL SURFACE MUTANTS OF CORYNEBACTERIA

Attempts have been made to improve various strains of *Corynebacterium* for specific applications. The approach taken has largely been along traditional lines of mutagenesis and screening for mutants which overproduce particular metabolites (Tosaka *et at.*, 1983). As a result of mutation, some mutants gave higher transformation efficiencies than seen for the parent strains. Some of these mutants are presumptive cell surface mutants (Best and Britz, 1986) and others seem to be restriction-deficient strains (Liebl *et al.*, 1989; Ozaki *et al.*, 1984; Schäfer *et al.*, 1990, 1994a). Although many mutant strains of corynebacteria have been reported, only specific examples which are relevant to this thesis will described here.

Best and Britz (1986) isolated several auxotrophic mutants of *C. glutamicum* ATCC 13059 by long-term exposure to ethylmethanesulphonate (EMS) (Fig 1). Many of these protoplasted more easily than the parent type strain. Some mutants, including strains MLB131-135 and MLB194, produced larger cells than other auxotrophs or the parent strain when grown in Luria Broth (LB) and always displayed notably apparent morphological changes during growth in LB-glycine. Using scanning electron

microscopic observation, Britz (1986) found that *C. glutamicum* strain ATCC 13059 grown in LB produced irregular rods, with coccobacilli 1-2 μm in length. Characteristically, this strain forms parallel ridges around its circumference. However, growth in LB containing 2% (w/v) glycine caused cell elongation and the rare appearance of X- and Y- shaped cells. Auxotrophic mutants derived from this strain produced elongated cells with buds in LB. When grown in LB-glycine, X-and Y-shaped cells were seen more frequently, as were branched rods, suggesting that cell division was occurring through budding, branch formation, then septation (Jang *et al.*, 1997). In addition, Britz (1989) examined the effect of glycine and INH on cell growth of a number of corynebacteria, including *C. glutamicum* strains AS019, MLB133 and MLB194, *B. lactofermentum* BL1, *B. flavum* BF4 and *C. ulcerans* (wild type), by measuring the final optical density as 600 nm after 16 h cell growth. The author found that mutant strain MLB133 and MLB194 were more sensitive to inhibition by the presence of INH and glycine during growth relative to the parent strain. In the case of *B. lactofermentum*, which gave higher transformation efficiency than *C. glutamicum* AS019 (Haynes and Britz, 1989), sensitivity to INH was higher than AS019.

In Pierott's work (1987) on strain ATCC 13059, AS019, and mutants MLB133 and MLB194, fatty acid and mycolic acid profiles were quantitatively determined by gas chromatography (GC) analysis following growth under different conditions. An alteration in the cell surface structures of two mutant (MLB133 and MLB194) strains was further supported by the observation that the quantitative fatty acid and mycolic acid compositions were different from the parent-type strain AS019 (Pierotti, 1987). For these experiments, Pierotti (1987) used two different media: LB supplemented with 0.5% (w/v) glucose (LBG) and LBG containing 2% (w/v) glycine, where samples were prepared from several cell growth phases. Pierotti (1987) observed two major spots following TLC, corresponding to mycolic acid methyl esters (MAMEs) and fatty acid methyl esters (FAMEs), and characterised these using GC and this combined with mas spectrometry (GC-MS).

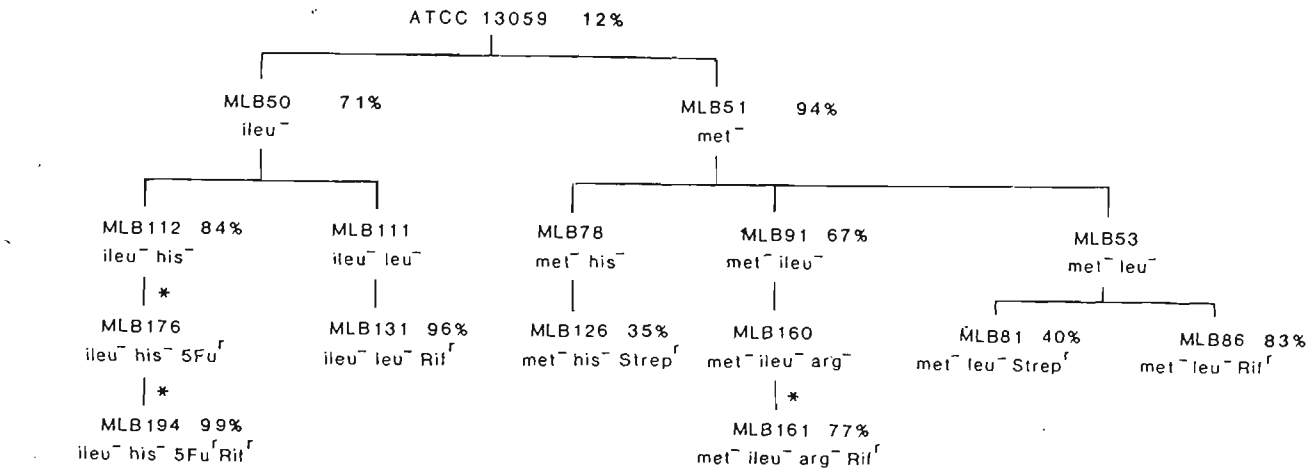


Figure 1 Geneology of mutants derived from *C. glutamicum* strain ATCC 13059 by EMS mutagensis or through selections of strains spontaneously resistant to antimetabolites on selection media (from Best and Britz, 1986). Abbreviations: Rif^r, rifampicin resistant; $Strep^r$, streptomycin resistant; $5\text{-}Fu^r$, 5-florouracil resistant; arg^-, auxotrophic for arginine; his^-, histidine; $ileu^-$, isoleucine; leu^-, leucine; met^-, methionine. Percentages represent protoplasting efficiency.

Analysis of FAMEs showed that, with increasing cultivation time from exponential phase to late stationary phase, the percentage of $C_{18:1}$ in strain MLB133 increased from 1.5% to 6% while the percentage of $C_{18:1}$ in AS019 was not changed. Analysis of MAMEs showed five types of peaks ($C_{32:0}$, $C_{34:1}$, $C_{34:0}$, $C_{36:2}$, $C_{36:1}$) in all of the strains of *C. glutamicum* used, confirming the observation that these strains and the mutants had a mycolic acid composition similar to those seen for *C. glutamicum* NCIB 10025 (Collins *et al.*, 1982a). No significant differences in the quantitative composition of mycolic acids were detected at different cell growth phases for the parent strain of *C. glutamicum*.

Jang (1997) determined the effect of glycine and INH on mycolic acid profiles of whole cells and in spent culture medium. The most important observations regarding his studies were as follows: the mycolic acid profiles in terms of relative proportions of each type in the cells and the culture fluid were quite similar for each sample tested; both mutant strains MLB133 and MLB194 always contained a higher proportion of unsaturated mycolic acids ($C_{32:0}$ lower than the parent, $C_{34:1}$ and $C_{36:2}$ higher); the main effect of glycine (2% w/v) was to increase the proportion of mycolic acids found in extracellular fluids (15.9% for AS019 and 19.3% in MLB133); growth in LBG with INH also increased the proportion of extracellular mycolic acids and changed the proportion of unsaturated mycolic acids; increased proportions of unsaturated mycolic acids corresponded to decreases in $C_{32:0}$ and increases in the proportion of $C_{34:1}$ and $C_{36:2}$; similarly, in the presence of glycine or INH the relative percentage of fatty acids to the total lipids (fatty acids plus mycolic acids) of AS019 decreased from 76.9% (in LBG) to 72.9% (in LBG 2% glycine), and 66.4% in (LBG-8mg/ml INH), implying that glycine and INH in the medium inhibited not only mycolic acid synthesis but also fatty acid synthesis. However, Jang did not investigated the impact of glycine and INH addition in the growth medium on the mycolic acid profiles of cell wall components, as he examined only whole cellular lipids. The present work aimed to determine the profile of lipids bound to the cell wall (both quantitative and qualitative analysis) after growth in the presence of glycine and INH in comparison with the mycolic acid profile of whole cells, to determine whether the observed changes were at the level of the cell surface lipid composition or cellular lipid synthesis.

1.3 INDUSTRIAL IMPORTANCE OF CORYNEFORM

Amino acids are produced *via* chemical synthesis, hydrolysis of natural proteins, and bacterial fermentation (Kinoshita, 1985). Only a limited number of bacterial species are used to obtain production strains for the industrial manufacture of amino acids (Aida *et al.*, 1986) and corynebacteria are the micro-organisms that have been traditionally used for amino acid production. The main use of amino acids is in animal feed supplementation to increase the efficiency of plant proteins that are usually deficient in certain essential amino acids (e.g. lysine, threonine, tryptophane, and methionine) (Flodin, 1993; Howe *et al.*, 1965). In the food industry, amino acids are used as flavour enhancing agents (e.g. glutamate and glycine) (Jetten and Sinskey, 1992). Amino acids are used in the pharmaceutical industry as therapeutic agents in nutritional and metabolic disorders, and in the chemical industries they find use in the manufacture of cosmetics, toothpaste, shampoos and detergents. Amino acid derivatives are also increasingly used in agriculture as plaguicides and plant growth regulators. Another important, although less significant, use of amino acids is in the preparation of intravenous infusions (Yamada *et al.*, 1972).

Lysine, used mainly as a forage additive, is one of the economically most important amino acids produced by *C. glutamicum* (Tosaka and Hirose, 1983). Isoleucine is of commercial interest as a food and feed additive, since mammalian cells are not able to synthesise it. This branched-chain amino acid has been produced on a large scale of about 400 tons per year by extraction of protein hydrolysates (Kleemann *et al.*, 1985) or by fermentation with classically-derived mutants of *Serratia marcescens* (Ikeda and Yoshinaga, 1976; Kisumi and Chibata, 1977) or mutants of *C. glutamicum* (Eggeling *et al.*, 1987; Morbach *et al.*, 1995; Patek *et al.*, 1994). Many natural isolates of glutamic acid producing corynebacteria have been mutated to further increase amino acid production, including *Corynebacterium* species *C. callunae, C. glutamicum, C. herculis* and *C. lilium*; *Brevibacterium* species *B. flavum, B. lactofermentum, B. diverticum* and *Mycobacterium* species *M. ammoniaphilum* (Kinoshta, 1987).

Some corynebacteria have relevance in the cheese production industry while several others are used in bioconversions. For example it is well known that "orange cheese" coryneform bacteria play an important role in the ripening of red smear cheeses, in particular by producing methanethiol (Greez and Hedrick, 1962; Lewis, 1982), a sulfur compound which imparts a typical flavour to this kind of cheese (Law and Sharpe, 1978). Some orange cheese corynebacteria produce antibacterial substances such as bacteriocin, including linencin A and linocin M18, which have been purified and characterised (Kato *et al.*, 1984 and 1991; Valdes and Scherer, 1994). An orange cheese coryneform bacteria isolated from the surface of Gruyere of Comte was identified as *B. linens* and this produces an antimicrobial substance designated linenscin OC2. This compound inhibits Gram-positive food-borne pathogens including *S. aureus* and *Listeria monocytogens* but is not active against Gram-negative bacteria. Linenscin OC2 caused viability loss and lysis of the test organism, *L. innocua* (Sophie *et al.*, 1995). The spectrum of activity of linenscin OC2 is interesting, if not used in food, another application of linenscin OC2 could be as an antibiotic in human therapy, if activity is retained *in vivo*. Other industrial applications of corynebacteria include the conversion of 2,5-diketo-D-gluconic acid into 2-keto-L-gulonate (an intermediate in L-ascorbate synthesis), steroid conversion (hydrocortisone to L-ascorbate) and other oxidations (Anderson *et al.*, 1985; Martin *et al.*, 1987; Yamada *et al.*, 1985).

C. glutamicum is used widely for its ability to produce large amounts of glutamate under particular growth conditions, e.g., biotin limitation. Different research groups have studied glutamate excretion (Bunch *et al.*, 1986; Clement *et al.*, 1986; Gutmann *et al.*, 1992; Hoischen and Kramer, 1989 and 1990), and different hypotheses have been put forward to explain this capacity (Clement *et al.*, 1984). In 1995, Lambert *et al.* attributed glutamate efflux under biotin limitation to the activity of a specific excretion carrier system. Their study showed that *C. glutamicum* can be triggered to excrete glutamate by the addition of local anaesthetics, particularly tetracaine. Glutamate efflux is a carrier-mediated process and not due to unspecific membrane permeabilization. The concentration of local anaesthetic triggering optimal excretion depended on the type and concentration of anaesthetic, ranging from 0.1mM for chlorpromazine, 1.3mM for

tetracaine and 2.6 mM for butacaine to 15 mM for benzocaine, which closely followed the order of anaesthetic efficiencies. The onset of glutamate excretion was not correlated to changes in the viscosity or fluidity of the membrane, nor was it related to an action of the anaesthetic as an uncoupler. Tetracaine-triggered glutamate excretion was not correlated to changes in the transmembrane osmotic gradient. These authors reported that the most possible explanation for the triggering effect is that the action of local anesthetics is not a direct interaction with certain proteins but a general action on the membrane, which triggers the carrier activity. *C. glutamicum* cells, which excrete glutamate without manipulation of the membrane, e.g., biotin-limited cells or glutamate production mutants, were not stimulated by the addition of tetracaine.

Biosynthesis of the aromatic amino acids in micro-organisms proceeds *via* a common pathway to chorismate, from which the pathways to phenylalanine, tyrosine, and tryptophane branch (Umbarger, 1978). Some attempts to genetically engineer existing mutants of *C. glutamicum* which produce the aromatic amino acid resulted in significant improvement in yields. In such studies, the strategy used aimed at amplifying the gene coding for the rate-controlling enzyme, thereby eliminating the bottleneck in the biosynthetic pathway (Ito *et al.*, 1990; Ozaki *et al.*, 1985). Katsumata and Ikeda (1992) reported a tryptophane-producing mutant of *C. glutamicum* which was genetically engineered to produce tyrosine or phenylalanine in abundance. To achieve this, three genes encoding the first enzyme in the common pathway, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase (DS), and the branch-point enzymes chorismate mutase and prephenate dehydratase, were individually cloned from regulatory mutants of *C. glutamicum,* which had either of the corresponding enzymes desensitised to end-product inhibition. These cloned genes were assembled one after another onto a multicopy vector of *C. glutamicum* to yield two recombinant plasmids. One plasmid, designated pKY1, contained the DS and chorismate mutase genes, and the other, designated pKF1, contained all three biosynthetic genes. The enzymes specified by both plasmids were simultaneously over expressed approximately seven-fold relative to the chromosomally encoded enzymes in the parent *C. glutamicum* strain. When transformed with pKY1 or pKF1, tryptophane-producing *C. glutamicum* KY10865, with the ability to

produce 18 g of tryptophane per litre, was altered to produce a large amount of tyrosine (26 g/liter) or phenylalanine (28 g/liter), respectively, because the accelerated carbon flow through the common pathways was redirected to tyrosine or phenylalanine.

1.4 TAXONOMY OF CORYNEFORM BACTERIA

Lehman and Newman (1907) were the first to create the genus *Corynebacterium* to accommodate the diptheria bacillus and a few similar animal parasitic species. The genus was defined mainly on the basis of morphological characteristics (wedge or club shape of the organism) and staining reaction (Gram positive with irregular bar or band formation). Morphological similarity was then generally believed to indicate relatedness and thus organisms from habitats other than human or animal sources were included in this genus. Coryneform bacteria were subsequently recognised as saprophytes in soil, water, milk, dairy products and fish, and as plant pathogens (Keddie, 1978). Keddie concluded that it is convenient to use the term corynebacteria to define "a broad morphological group, sometimes imperfectly, but it does not imply relatedness within it".

The definition of the genera commonly referred to as coryneform (*Corynebacterium*, *Microbacterium*, *Cellulomonas* and *Arthrobacter* [Jensen, 1952]) are equivocal because they are based almost entirely on microscopic appearance and staining characteristics and these are affected by morphological changes during the growth cycle and also by culture conditions (Cure & Keddie, 1972; Jensen, 1993). Furthermore, some *Corynebacterium* species are morphologically similar to *Propionibacterium* and *Brevibacterium* while others in the genus, together with the genera *Arthrobacter* and *Cellumonas*, show a close morphological resemblance to representative of the genera *Mycobacterium* and *Nocardia* (Bowie *et al.*, 1972; Jensen, 1952; Skerman, 1967).

Keddie and Cure (1977) defined legitimate members of *Corynebacterium sensu stricto* as those facultative anaerobes which contain *meso*-diaminopimelic acid (*meso*-DAP), arabinose, galactose and corynemycolic acids in their cell wall. This definition covered organisms with a fairly narrow range of DNA base ratios and included *C. diphtheriae,* most animal pathogens and parasitic species, some saprophytes and *Microbacterium*

flavum, but excluded most saprophytes and all species pathogenic to plants. Saprophytic species included in this group were *C. glutamicum* and several other glutamic acid producing species: *B. divarticum, B. lilium, C. callunae* and *C. herculis.* Abe *et al.* (1967), however, considered that these should be reduced from species level to synonomy with *C. glutamicum.*

In addition to using traditional, taxonomic morphological characteristics to classify coryneform, numerical taxonomy and chemotaxonomic classification have played important roles in determining taxonomic relationships. Chemotaxonomy is defined as the application of chemical techniques which gives information on the presence or absence of specific compounds, such as lipids, amino acids and sugars in the cell surface (Barreau *et al.*, 1993; Cummins, 1962) and is a useful tool for the classification and differentiation of Gram-positive bacteria. In Gram-positive bacteria, since the cell wall structure constitutes up to 40% of the dry cell weight of these bacteria (Dolye, 1992) therefore it is considered as an important indicator in classification of these bacteria. Peptidoglycan is the characteristic cell wall polymer in bacteria. The chemical composition of the cell walls of human and animal pathogenic *Corynebacterium* species was found to be similar to that of *Mycobacterium* and *Nocardia* species (Cumins and Harris, 1956; Cummins 1962). These genera contain *meso*-DAP, arabinose and galactose in the cell wall and could be distinguished from *Streptomyces* species which contain glycine and *meso*-DAP, and *Actinomyces* species, which contain lysine and galactose (Cummins, 1962; Lechevalier, 1970; Mordarska *et al.*, 1972). The peptidoglycan of corynebacteria is composed of 1,4-linked glucosamine and muramic acid units. Chemical and immunological studies suggested that arabinogalactan is a seriological antigen common to mycobacteria, nocardia and corynebacteria (Azuma *et al.*, 1973; Misaki, Seto and Azuma, 1974). The enzymatic degradation of mucopeptides and chemical analysis of their subunits indicated that the tetrapeptide, Ala-Gln-Dpm-Ala, is the principal subunit of the peptide moiety of the mucopeptide portion of cell wall of mycobacteria, nocardia and corynebacteria (Azuma *et al.*, 1973). It was concluded that corynebacteria, mycobacteria, and nocardia were closely related and that strains of these three groups should therefore be taxonomically grouped together. This was supported by Harringston

(1966) and Goodfellow (1967), who made a numerical taxonomic study of coryneform and members of the genera *Mycobacterium* and *Nocardia* and who, as a result of available evidence, proposed that the genera *Corynebacterium, Mycobacterium*, and *Nocordia* be merged to form one genus.

In the last 20 years the taxonomy of this important group of bacteria has much improved. In particular, the careful application of phenotyping methods, used in conjunction with molecular genetic methods (e.g. DNA-DNA hybridisation [Carlotti *et al.*, 1993; Liebel *et al.*, 1991] and 16S rRNA gene sequencing [Pascual *et al.*, 1995; Stackebrandt and Schleifer, 1984]) have resulted in more robust and reliable species identification. In 1989, Ochi and coworkers developed a method for identifying and classifying actinomycetes. This approach is based on the analysis of ribosomal AT-L30 proteins (homologous to *E. coli* L30 protein) which involves determining the electrophoretic mobilities of the AT-L30 proteins and their N-terminal amino acid sequences. In 1995 these authors further extended this study with the objectives (i) to clarify the phylogenetic relationships among the genera *Rhodococcus*, *Nocardia*, *Mycobacterium*, *Corynebacterium* and *Tsukamurella* and (ii) to clarify the taxonomic status of the members of the family *Actinomplanaceae* and actinobacteria as represented by the genera *Actinomyces* and *Micrococcus* within the Gram-positive bacteria. The phylogenetic relationships among 30 mycolic acid-containing, wall chemotype IV, actinomycetes and 12 strains belonging to allied taxa were examined by determining the amino acid sequences of the ribosomal AT-L30 proteins of those organisms. Sequencing 20 N-terminal amino acids of AT-L30 preparations revealed that the members of the mycolic acid-containing actinomycetes formed two clusters: the first cluster contained the genera *Nocardia*, *Rhodococcus*, *Gordona*, and *Tsukamurella*, and second cluster contained the genera *Corynebacterium* and *Mycobacterium*. The phylogenetic clusters identified were entirely consistent with the proposal of Goodfellow that the family *Nocardiaceae* should encompass the mycolate-containing, cell wall type IV actinomycete genera *Nocardia*, *Rhodococcus*, *Gordona*, and *Tsukamurella*. The genera *Actinomycetes* and *Micrococcus* exhibited AT-L30 amino acids sequence characteristics intermediate between those of actinomycetes and those of typical eubacteria. The genera *Rhodococcus* and

Corynebacterium were considered to be taxa that consist of phylogenetically distantly related species. Ochi's results agreed with this classification (Fig 1.1). However, less significant difference between 16S rRNA analysis and Ochi's AT-L30 is that in the 16S rRNA the genera *Nocardia* and *Rhodococcus* occupied separate phylogenetic positions (Klatte *et al.*, 1994; Stackebrandt *et al.*, 1980) whereas Ochi's results imply that among mycolic acids-containing taxa, the genera *Nocardia* and *Rhodococcus* are very closely related.

The taxonomy of mycolate-containing, wall chemotype IV actinomycetes has undergone extensive revision. Ochi's results also agree with this classification as well. *Rhodococcus equi* is an important eqine pathogen and was previously classified in the genus *Corynebacterium.* Although the members of the genus *Rhodococcus* generally contain longer mycolic acids than the members of the genus *Corynebacterium*, several rhodococci, including *R. equi*, have mycolic acids whose lengths overlap the lengths of mycolic acids of corynebacteria. The AT-L30 data support the current taxonomic status of *R. equi* (Fig 1.1). *Actinomyces pyogens*, which was previously classified in the genus *Corynebacterium* (Reddy *et al.*, 1982), is also an important veterinary pathogen. *C. glutamicum*, which was previously classified in the genus *Micrococcus*, is an important organism in the glutamic acid fermentation industry. According to these authors, AT-L30 data provides strong support for the current taxonomic status of *Actinomyces pyogens* and *C. glutamicum* (Fig 1.2). It is especially interesting that *C. amycolatum*, a relatively new species proposed by Collins *et al.*, (1988), differs from other corynebacteria by lacking mycolic acids. Despite such a significant difference, inclusion of this organism in the genus *Corynebacterium* is strongly supported not only by the high level of amino acid sequence similarity to other *Corynebacterium* species, but also by a distinctive characteristic of this genus, a deletion of the amino acid at position two in the AT-L30 protein (Fig 1.1). Finally these authors suggested that the AT-L30 sequence method appears to be useful for bacterial taxonomy not only at the generic level but also at species level.

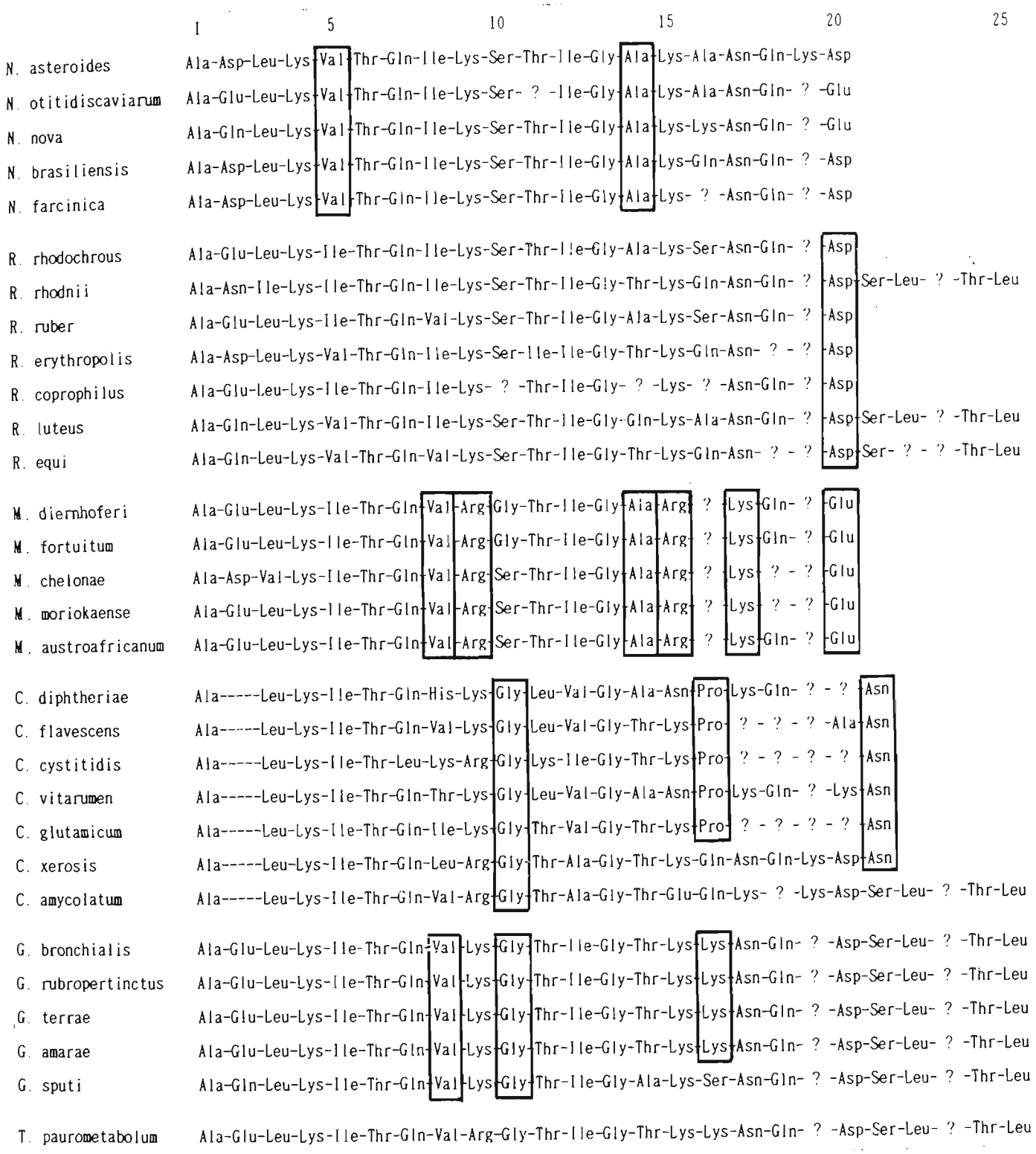


Figure 1.1 Primary structure of the N-terminal of AT-L30 proteins from various species of the genera belonging to mycolic acid-containing, cell wall type IV actinomycetes. The question mark indicates amino acids that were not determined. The dotted lines indicate deletions The amino acids which characterise each genus are enclosed in boxes. Abbreviations: N, *Nocardia*; R; *Rhodococcus*; M, *Mycobacterium*; C, *Corynebacterium*; G, *Gordona*; T, *Tsukamurella* . (Ochi, 1995).

However, identification at the species level has to be confirmed by testing more species and more than one strain per species. Also, this technique is limited for species identification, as *Gordona* species could not be differentiated (Fig 1.1 and 1.2).

Studies of glutamic acid producing bacteria, including *C. glutamicum, B. lactofermentum* and *B. flavum*, indicated that these species share many common characteristics, such as cellular fatty acids composition, the presence of mycolic acids and peptidoglycans containing *meso*-DAP in the cell wall (Suzuki & Komagata, 1983), similar nucleotide sequence of genes encoding the same enzymes in both species, G+C content in the range 54-56% (typical of true members of the genus *Corynebacterium*) and high degree of similarity in DNA-DNA hybridisation studies (Liebel *et al.*, 1991). Based on these data, the transfer of *B. lactofermentum* to the genus *Corynebacterium* has been proposed (Liebl *et al.*, 1991). Differentiation of strains classified as *C. glutamicum* and *B. lactofermentum* has been made by analysis of rDNA restriction patterns (Liebl *et al.*, 1991) and by restriction fragment polymorphisms around *hom* genes (Eikmanns *et al.*, 1991). *B. linens*, the type species of genus *Brevibacterium* (usually isolated from cheese sources), has a higher G+C content than *B. lactofermentum* and *C. glutamicum*. One strain, *B. linens* ATCC 19391, is a patented L-lysine producer. This strain belongs to the DNA homology groups of the type species, *B. linens* ATCC 9172 (Fiedler *et al.*, 1981).

Similarly, studies on the genome of these two corynebacteria groups using pulsed-field gel electrophoresis (PFGE) revealed difference in patterns of chromosomal DNA (Correia *et al.*, 1994). Using *Pac*1 and *Swa*1 endonucleases, the genome of *B. lactofermentum* ATCC 13869 (genome size 3,052kb) was consistently cut into 26 and 20 bands, respectively, and the genome of *C. glutamicum* ATCC 13032 (2,987kb) yielded 27 and 26 fragments respectively (Correia *et al.*, 1994). Bathe *et al.* (1996) constructed a physical and genetic map of the *C. glutamicum* ATCC13032 chromosome using PFGE and hybridisation with cloned genes.

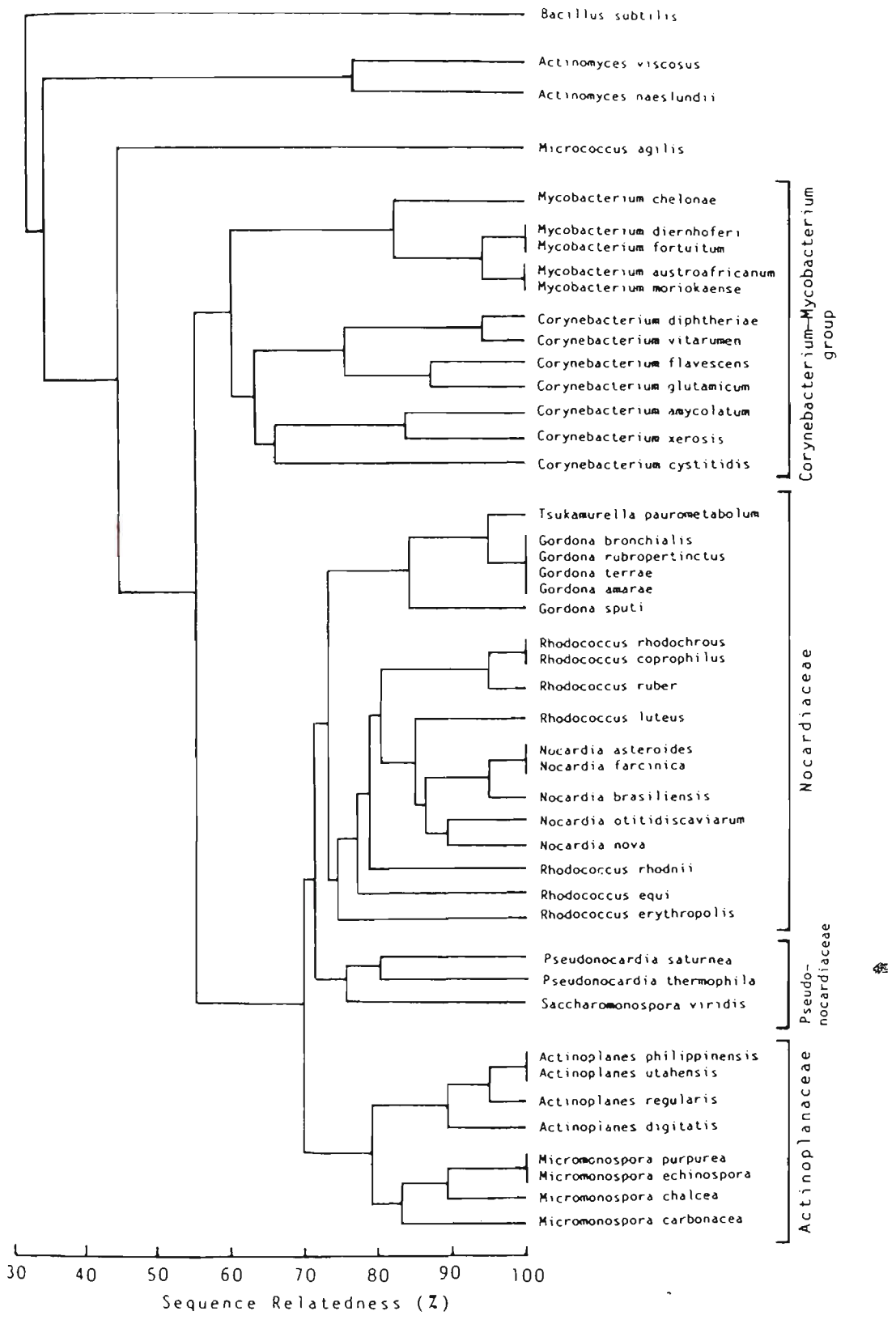


Figure 1.2 Clustering based on AT-L30 protein SAS values (quantitative expression of the levels of similarity of the amino acids sequences, Ochi, 1995).

Furthermore, the pattern of PCR (polymerase chain reaction) amplification of chromosomal DNA using random primers showed that PCR patterns of two strains of *C. glutamicum*, ATCC13032 and AS019, were almost identical but that these were clearly different from that of *B. flavum* BF4 (Webster, 1995).

More recently, *rrn* operons of amino acid producing corynebacteria were studied as a way to understand growth regulation and the mechanism that triggers amino acid accumulation under nutritional starvation (Amador *et al.*, 1999). These authors by hybridisation studies found five rRNA operons (*rrn*) in the genome of *B. lactofermentum* ATCC 13869 and *C. glutamicum* ATCC 13032. *'B. lactofermentum'* DMS 20412 differed from the other corynebacteria tested showing six hybridisation bands. Two of the *rrn* operons (*rrnD* and *rrnE*) were located in a single cosmid. Sequencing of the *rrnD* operon showed that it contains a complete 16S rRNA-23S RNA-5S rRNA gene cluster. Analysis of *rrnD* sequence has provided information on the phylogeny of *'B. lactofermentum'* and on the molecular control of macromolecule gene expression. Based on their 16S RNA analysis, these authors proposed that *B. lactofermentum* ATCC 13869 should no longer be considered as a member of the genus *Brevibacterium* and that it should be transferred to the genus *Corynebacterium* in agreement with the proposal of Liebl *et al.* (1991). However, their studies showed that the divergence between *B. lactofermentum* and *C. glutamicum* was higher than that between different species of *Corynebacterium*, e.g., *C. fastidiosum, C. segmentosum, C. accolens and C. pseudotuberculosis*, which indicated that *'B. lactofermentum'* was a different species of the genus *Corynebacterium*. These researchers therefore suggested that *'B. lactofermentum'* should be changed to *C. lactofermentum*, thus maintaining the name of the species. *'B. lactofermentum'* was clearly different from the glutamic acid producer *C. ammoniagenes* (previously *B. ammoniagenes*). It would be interesting to speculate whether if similar conclusions apply to other glutamic acid producing corynebacteria such as *B. flavum*, but no data are available on their *rrn* genes.

The genome mass of *C. glutamicum* is approximately 1.7 $X10^9$. Similar values have been established in *B. ammoniagenes* (1.9 X 10^9) and other corynebacteria (Bak *et al.*,

1970; Crombach, 1978). This size is smaller than the genome size of *E. coli* (4,000 kb) and of streptomyces (10, 000 kb) as estimated by Benigni *et al.* (1975).

The genus *Corynebacterium* contains many species of clinical importance (Collins & Cummins, 1986). According to previous studies, mycolic acids are of particular importance in the definition of authentic members of the genus *Corynebacterium* (Athalye *et al.*, 1984; Corina and Sesardic, 1980; Carlotti *et al.*, 1993; Keddie and Cure, 1977). Therefore it can be argued that any bacterium devoid of mycolic acids cannot be classified in this genus. However, Barreau *et al.*, (1993) collected clinical isolates that possess all of the phenotypic characteristics of coryneform bacteria but did not contain mycolic acids. All of these strains were identified as *Corynebacterium spp.* by standard culture and biochemical tests. Many of these strains were then identified by a new commercial kit (Frenny *et al.*, 1991; Gavin *et al.*, 1992) as *C. minutissimum*, *C. striatum*, or related organisms of CDC group I.

As a result of polyphasic approaches, and growing awareness of corynebacteria as opportunistic pathogens, a plethora of new species from humans have been described in the past few years (Funke *et al.*, 1977, 1995, 1997; Reigel *et al.*, 1993, 1995). In contrast, the potentially pathogenic corynebacteria in veterenary clinical microbiology have received much less attention, although these organism are the probable cause of, or are associated with, diseases in animals. This situation is evident in the case of subclinical mastitis. Staphylococci are traditionally identified to be the most important aetiological agents of sub-clinical mastitis (Honkanen-Buzalski *et al.*, 1996; Marco, 1994; Serrano *et al.*, 1994), although corynebacteria are emerging as pathogens of clinical significance in this pathology (Fernandez *et al.*, 1997; Honkanen *et al.*, 1996). Recently Fernandez *et al.* (1998) reported the isolation of a non-lipophilic coryneform bacterium biochemically similar to *C. jeikeium* and *C. coyleae* from sheep suffering from sub-clinical mastitis. Based on polyphasic taxonomic analysis, the authors proposed a new *Corynebacterium* species, *C. camporealensis*, for this coryneform from sheep.

1.5 OVERVIEW OF THE LIPIDS FOUND IN CORYNEBACTERTIA AND RELATED SPECIES

1.5.1 The nature of the lipids found in mycolic acid containing bacteria

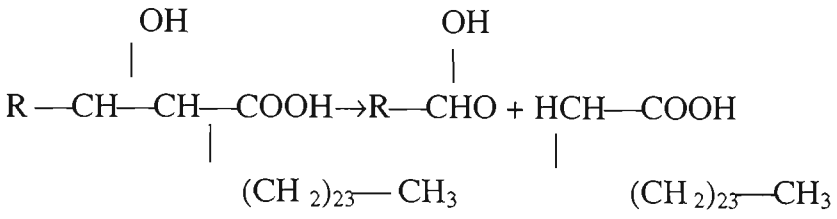
Lipids are natural products which can be isolated from biological materials by extraction with organic solvents and which are usually poorly soluble in water. Lipids are divided into two subgroups: polar and non-polar, the most common type of polar lipids found in corynebacteria are phospholipids, glycolipids and ornithine or lysine-amides. Amphipathic polar lipids consist of hydrophilic head groups usually linked to two hydrophobic aliphatic chains. Non-polar lipids are fatty acids, mycolic acids, isoprenoid quinones and carotenoid pigments. Long-chain fatty acids are released when bound lipids are subjected to chemical degradation. Lipid markers have been traditionally used in the classification and identification of coryneform and related taxa (Minnikin *et al.*, 1978).

Isoprenoid quinone analysis has also been proposed as a means of classification of coryneform and related taxa (Collins *et al.*, 1979; Yamada *et al.*, 1976). Isoprenoid quinones are a class of terpenoid lipids widely distributed in bacteria and two structural groups can be recognised, menaquinones (vitamin K) and ubiquinones (coenzyme Q). They are located in the plasma membranes and are principally involved in electron transport and respiration (Redfearn, 1966). Menaquinones and 2-methyl-3-polyprenyl-1-4-napthoquinones display structural variations which include the length and degree of hydrogenation of the polyprenyl side-chain, hydroxylation (Allen *et al.*, 1967) and possibly epoxidation (Friis *et al.*, 1967). Coryneform and related bacteria can be divided into several groups on the basis of menaquinone composition (Collins *et al.*, 1977; Yamada *et al.*, 1976). Thus human and animal parasitic corynebacteria contain predominantly dihydromenaquinone with eight isoprene units, MK-8(H_2), whereas *C.*

bovis, C. glutamicum and *Mycobacterium* strains have dihydromenaquinones with nine-isoprene units MK-9(H_2) as major components.

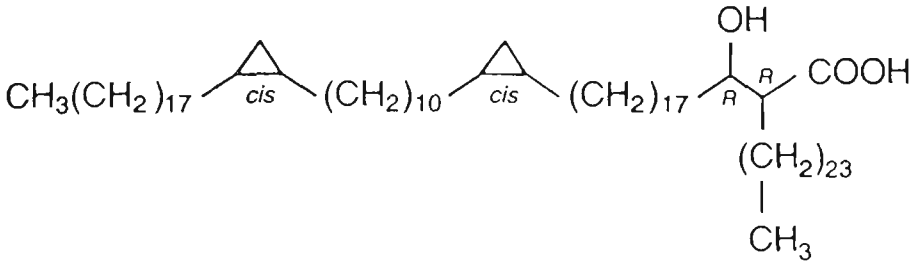
Fatty acids commonly occur as the hydrophobic chains of the membrane phospholipids although they can also be present as free fatty acids. They may be straight chain, unsaturated, branched chain or contain cyclopropane. The fatty acids from corynebacteria have been extensively studied (Collins *et al.*, 1982b; Minnikin *et al.*, 1978) they have chain lengths between ten and twenty carbon atoms and include mainly saturated and unsaturated forms (Collins *et al.*, 1982b; Pierotti, 1987). The cellular fatty acid composition made up from the four types of fatty acids appear to be distinct and do not overlap for distinct groups of corynebacteria, even when the culture conditions are changed. Type I (normal unsaturated) fatty acid composition is found in all coryneform bacteria that have *meso*-DAP and arabinoglactan in their cell wall. Collins *et al.* (1982b) showed that *C. glutamicum* and related saprophytic strains, such as *B. lactofermentum*, and *B. flavum,* and animal-associated strains predominantly contain palmitic acid ($C_{16:0}$) and oleic acid ($C_{18:1}$). In *C. glutamicum*, myristic acid ($C_{14:0}$) petadecanoic acid ($C_{15:0}$), palmitoleic acid ($C_{16:1}$), heptadecanoic acid ($C_{17:0}$) and stearic acid ($C_{18:0}$) were also detected as minor components. According to Pierotti (1987), about 50% of the fatty acids in the cells are unsaturated and that with increasing cultivation time, from exponential phase to late stationary phase, the percentage of $C_{18:1}$ in AS019 was less than seen for cell-surface mutant strains of *C. glutamicum*. Duferene *et al.* (1997) reported that the surface of corynebacteria (*Corynebacterium* strains DMS 44016 and DMS 6688) was rich in hydrocarbon-like compounds including mycolic acids. Jang (1997) showed that *C. glutamicum* also contains a $C_{18:3}$ fatty acid which was particularly noticeable in extracellular culture fluids.

The name mycolic acid was given to a high molecular weight hydroxy, methoxy fatty acid from *M. tuberculosis* which released hexacosanoic acid on pyrolysis (Anderson, 1941; Stodola *et al.*, 1938). The pyrolytic cleavage of mycolic acids was shown to be due to the characteristic 3-hydroxy-2-branched structure (Asselineau, 1950; Asselineau and Lederer, 1950). The pyrolysis of mycolic acid was as follows:

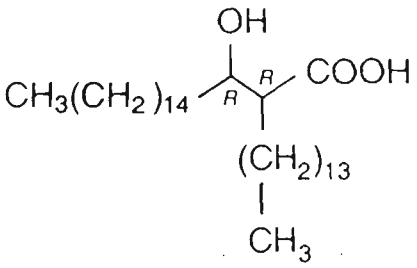


Asselineau and Lederer (1950) were thus able to define mycolic acids as "high molecular weight β-hydroxy fatty acids with a long α-side chain". The chain from the β-carbon can be up 60 carbons long in mycobacteria and may consist of a wide variety of chemical structures, such as cyclopropane rings, methyl branches, combinations of *cis* and *trans* double bonds (unsaturated chains) and hydroxyl, methoxy and other functional groups (Brennan and Nikaido, 1995; Minnikin *et al.*, 1982). Mycolic acids vary considerably in structure, ranging from the relatively simple mixture of saturated and unsaturated acids in corynebacteria to the very complicated mixtures characteristic of mycobacteria (Figure 1.3) (Minnikin *et al.*, 1975, 1984a, b, 1985). Mycolic acids of *Corynebacterium* and *Nocardia* contain up to about 40 and 60 carbon atoms, respectively, but those from *Mycobacterium* usually contain 70-90 carbon atoms (Chevalier *et al.*, 1988; Collin *et al.*, 1982a; Jarlier and Nikaido, 1994; Stodola *et al.*, 1938; Stakebrandt, 1988). The corynemycolic acids are attached by an ester linkage to the 5-hydroxy group of the D-arabinofuranoside residue of arabinogalactan (AG) (Azuma, Yamamura and Fukushi, 1968). In addition, several others lipid species, many of them with unusual structures, are known to exist in the mycobacterial cell wall as "free" lipids, which are solvent-extractable lipids that are not covalently linked to the AG-peptidoglycan complex (Brennan, 1988,1989; Minnikin and Goodfellow, 1980; Minnikin, 1982). The list of such extractable lipids is becoming longer with the addition of trehalose-based lipopolysaccharides (LOSs) (Hunter *et al.*, 1985).

Walker *et al.* (1973) reported that in *C. diphtheriae* the mycolic acids were present in the form of 6% acid-extractable lipids. The bound lipid fraction includes corynemycolic acids in salt form, trehalose esters (trehalose-6, 6'-dimycolate: cord factor) and glucose esters when these bacteria are grown on a glucose-containing medium.



α-Mycolic acid
(Mycobacterium)



Corynomycolic acid
(Corynebacterium)

Figure 1.3 Structure of a representative α-mycolic acid from *M. tuberculosis* and corynemycolic acid from *C. matruchotti* (Minnikin, 1982).

The cell walls of both pathogenic and non-pathogenic strains is coated with salts of corynemycolic acid and "cord factor" which are extractable from cells by treatment with light petroleum (Walker *et al.*, 1973). In 1987 Pierotti studied covalently bound mycolic acids of *C. glutamicum* and showed that approximately 50% of the mycolic acids existed as the bound form and there was little difference in the relative proportion of mycolic acids found in the covalently bound or the extractable lipids. Mycolic acids are a major component of the cell walls of these organisms and the synthesis of these is the subject of this thesis and the biochemistry and genetics of mycolic acid synthesis is described in more detail in section 1.6.

1.5.2 Physical organisation of cell wall lipids

Minnikin (1982) proposed a chemical model for the arrangement of the mycobacterial cell wall and some of the wall-associated lipids, in which the mycolic acids formed a monolayer near the outer surface of the wall, with fatty acyl chains of various wall-associated lipids intercalated with the mycolate chains. It was also proposed that this mycolic acid inner leaflet was composed of extractable lipids, the whole structure thus producing an asymmetric lipid bilayer. There is some experimental evidence to support this model. Nikaido *et al.* (1993) calculated that the amount of mycolic acid in the cell wall of the *M. bovis* BCG was sufficient to form a monolayer with an area similar to that of a mycobacterial cell. In the same paper these authors produced the X-ray diffraction pattern of an aqueous suspension of purified walls of *M. chelonae*. The X-ray diffraction studies showed very clearly that much of the hydrocarbon chains in the cell wall exist in a tightly packed, parallel, quasi-crystalline array, and that they occur directed mainly in a direction perpendicular to the plane of the cell wall.

Based on these results, Nikaido proposed a tentative model similar to that described by Minnikin (1982). Since a monolayer arrangement of mycolic acid molecules will produce a large hydrophobic surface, the model assumes the presence of an outer leaflet, composed of other lipids. However, with the bilayer arrangement proposed, presumably the unusual structure of the mycolic acids can produce a barrier of extraordinarily low fluidity and very high stability. In the apparent variations in the ratio of mycolate to AG

in various mycobacterial species there seems a need for further quantitative investigation of mycolates in the walls of actively growing mycobacteria. To indicate the proposed arrangement of lipids in the mycobacterial model, a recent variant of these models by Liu *et al.* (1995) is shown in Fig 1.4. These authors pointed out that a dominant wall-associated lipid in mycobacteria is triglyceride with C_{14}-C_{18} fatty acids. They suggested that there is more than enough of this to intercalate with the mycolic acids in the cell wall. This explains their findings about the fluidity of the lipids in the wall of *M. chelonae*, which is low in general but higher in outer parts which are accessible to spin-labelled probes. Their proposed model consists of a layer of close-packed mycolic acids with a thermal transition temperature to fluidity well above the growth temperature of the organisms, intercalated with triglyceride. It follows that the distribution of lipids such as phenolic glycolipids (PGL) and α, α- trehalose dimycolate (TDM) is not crucial to prove the Minnikin model: if these lipids are part of the wall, then they could be accommodated in the bilayer, but if they are mainly the capsular components, then their low concentration in the wall compartment does not negate the Minnikin model. The model of Liu *et al.* (1995) includes an outer membrane leaflet composed of wall-associated glycolipids, similar to that proposed by Rastogi (1991), and they point out that there is sufficient glycopeptidolipids (GPL) in the wall of *M. chelonae* to form such a membrane leaflet, and that the presence of a leaflet explains the measured high mobility of the lipids in the outermost part of the wall. However, this still leaves unexplained the nature of the outer leaflet in mycobacterial species that do not produce GPL, and this model is open to the same criticism as the Rostogi model that there is no clear sign of a lipid bilayer in electron microscope sections. Barry and Mdluli (1996) also favour a bilayer model, but locate the region of relative disorder in the outer part of the mycolate model. Although great progress has been made in understanding the arrangement of the lipids of the cell wall and the wall-associated lipids, there are still some important unsettled issues regarding the physical arrangement of this structure.

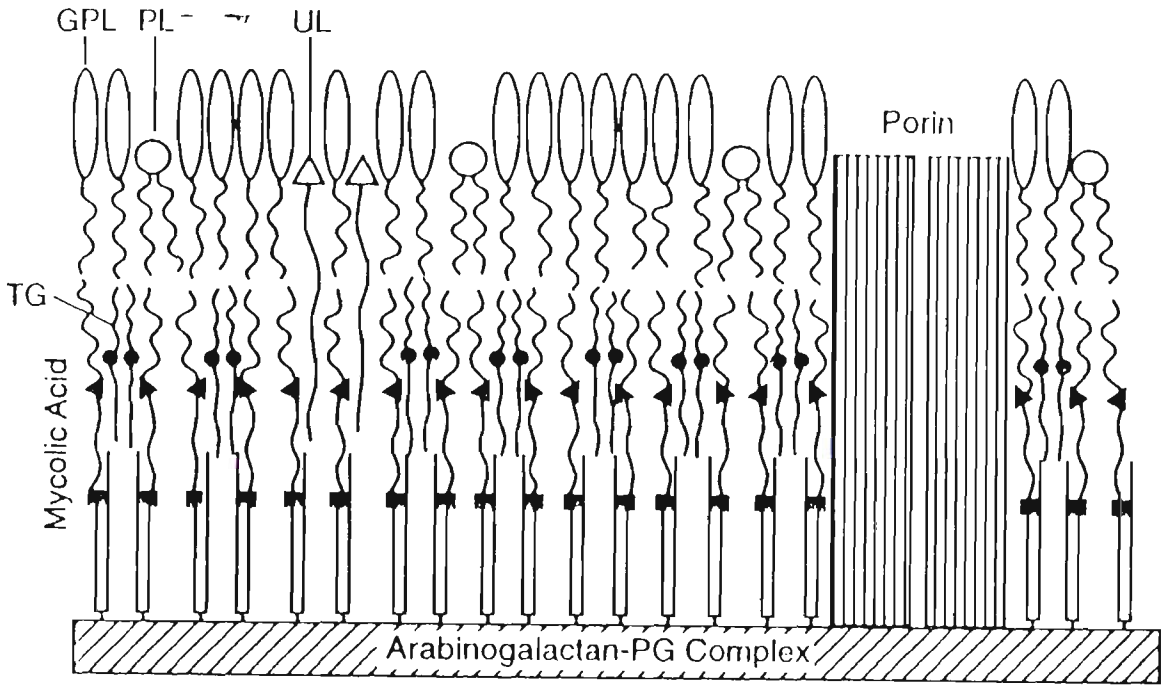


Figure 1. 4 Model of the cell wall of *M. chelonae* (Liu *et al.*, 1995). The proposed arrangement of mycolic acids and associated lipids forming the outer permeability barrier of the mycobacterial envelope is shown, indicating the assumed position of the mycobacterial porin-like protein. A similar arrangement presumably occurs in all mycobacterial walls, though the mycobacterial acid types and the range of other lipids involved would differ. GPL, glycopeptidolipid; PL, glycophospholipid; UL, lipids of unknown structure; TG, triglyceride; PG, peptidoglycan. The solid triangles and squares indicate double bonds in the mycolic acids.

Mycobacterium tuberculosis secreted a series of major exported proteins and protective antigens which are a triad of related gene products called antigen 85 (Ag85) complex. These proteins have been implicated in disease pathogenesis through its fibronectin-binding properties. Belisle *et al.* (1997) discovered a carboxylesterase domain within the amino acid sequences of Ag85A, B, and C, by direct enzyme assay and site-directed mutagenesis he further shown that each protein acted as a mycolyltranferase involved in the final stages of mycobacterial cell wall assembly. It has been demonstrated by Puech *et al.*, 2000 that PS1 which is homologous to mycobacterial antigen 85 act as mycoloyltrasferase. Over expression of PS1 in the wild type strain of *C. glutamicum* suggested the involvement of this protein in the transfer of corynomycolates, evidenced by an increase esterification of the cell wall arabinogalactan with corynomycolic acid residues and an accumulation of trehalose dicorynomycolates. PS1 was present in most strains tested (Puech *et al.*, 2001) and its deletion leads to a considerable decrease in the amount of covalently linked mycolic acids in the cell wall (Puech *et al.*, 2000).

Corynebacterium has a cell wall structure similar to that of *Mycobacterium*, which consists of mycolic acids arabinoglactan, and peptidoglycan (Barksdale and Kim, 1977; Lederer, 1971; Bordet, 1976). X-ray photoelectron spectroscopy studies of Duferene *et al.* (1997) have indicated that for coryneform bacteria peptidoglycan is an important cell wall component and contributes about 23 to 31% to the cell dry weight: the protein content is in the range of 7 to 14%. It was further reported that the surface of corynebacteria (*Corynebacterium* species strain 44016 and DMS 6688) contain about 40% hydrocarbon (Dufrene *et al.*, 1997).

Chami *et al.* (1995) indicated that the surface of *C. glutamicum* grown on solid medium was totally covered with highly ordered (hexagonal) surface layer and reported that *C. glutamicum* is one of the few known examples (Adachi *et al.*, 1991) where the S-layer protein is continuously released from the cell wall, implying a continuous synthesis of the protein. Like mycobacteria, the presence of ion permeable channels has also been reported by Niederweis *et al.* (1995) in the cell wall of *C. glutamicum*. Furthermore it was found that *C. glutamicum* has aqueous channels with little or no interaction between the channel wall and ions. *C. glutamicum* has also been shown to contain both free

mycolic acids (Goodfellow *et al.*, 1976; Keddie and Cure, 1977) and "Cord factor" type lipids (Pierotti, 1987). However, the physical organisation of these lipids in the cell wall has yet to be elucidated.

A unique model has been proposed by Puech *et al.* (2001) for corynebacteria using a combination of molecular compositional analysis, ultrastructural appearance and freeze-etch electron microscopy studies. This study showed that the ultrastructural appearance of the cell walls of both corynebacteria and mycobacteria are similar. The similar features included the plasma membrane bilayer, a thick electron dense layer (EDL), an electron-transparent layer (ETL) and an outer layer (OL). The EDL contains cell wall peptidoglycan which bound to the metallic stains during electron microscopy. The EDL is surrounded by a thin ETL which is traditionally considered to consist of mycolic acids residues because of their transparent structure under electron microscopy. However, this layer was also seen in *C. amycolatum*, which is devoid of corynomycolates (Fig 1.5). Furthermore the analysis of the purified cell wall also revealed the absence of fatty acyl substituents indicating that ETL was not synonymous with the lipid layer. The OL stained heavily with ruthenium red like mycobacteria (Rastogi *et al.*, 1986). This layer consists primarily of polysaccharides and contains protein. It is evident from this study that outermost carbohydrates of corynebacteria like mycobacteria (Ortalo-Magne *et al.*, 1995; Lemassu *et al.*, 1996) were found to be exclusively neutral substances.

In corynebacteria the cell-wall-linked mycolates and corynomycolates certainly contribute to this barrier, since the disruption of genes that code for mycoloyl-transferases causes a decrease in the amount of the cell wall-bound mycolates and corynomycolates, and affects the permeability of the envelope of the mutants (Puech et al., 2000). This outer permeability barrier also includes non-covalently linked lipids which are probably arranged to form a bilayer with the corynomycolol residues as proposed for mycobacteria (Rastogi, 1991; Liu et al., 1995). Freeze-etch electron microscopy showed that corynomycolate-containing strains exhibited a main fracture plane in their cell wall and contained low-molecular-mass porins, while the fracture occurred within the plasma membrane of strains devoid of both corynomycoate and pore-forming proteins.

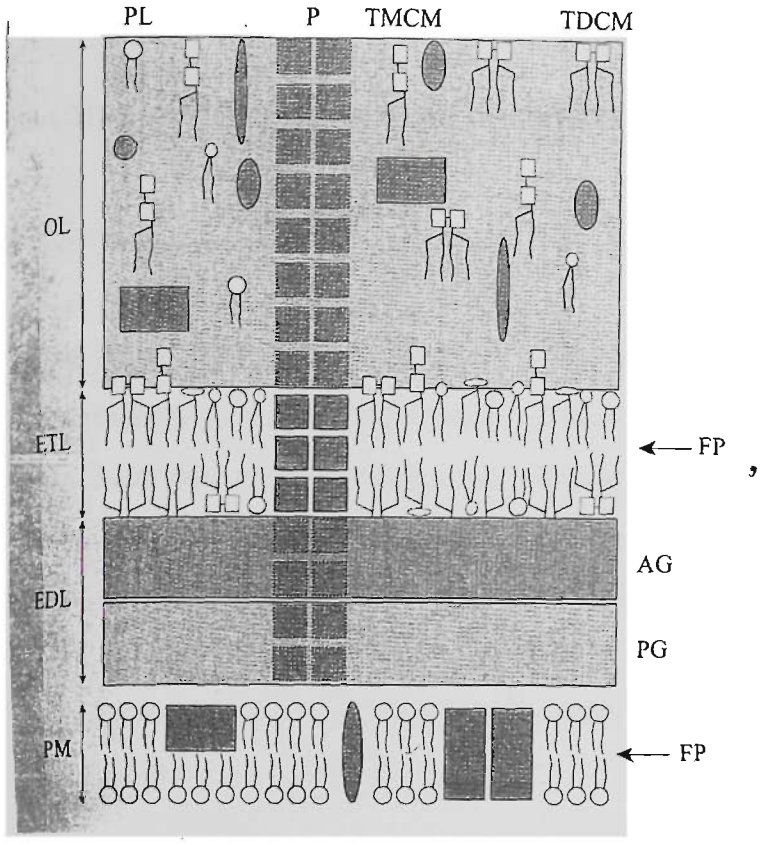


Figure 1.5 A tentative model for the cell envelope of corynomycolate-containing corynebacteria, e.g. *C. glutamicum*. From the cytoplasmic to the external side of the bacteria the cell envelope is composed of plasma membrane (PM), a complex wall that is composed of a plasma membrane (PM), a complex wall that is seen in thin section as an electron-dense layer (EDL) and an electron-transparent layer (ETL), and an outer layer (OL). The plasma membrane is typical bilayer of proteins (dark rectangles and oval spots) and phospholipids (PL, empty oval symbols). The EDL consists of thick peptidoglycan (PG) covalently linked to the heteropolysaccharide arabinogalactan (AG); some of the arabinosyl termini of this polysaccharide are esterified by C_{32-36} corynomycolic acids (thin parallel bars). Because the amount of these fatty acids in most corynebacteria is not sufficient to cover the bacterial surface (see text) covalently bound corynomycoloyl residues are probably arranged to form with other non-covalently linked lipids, e.g. trehalose dicorynomycolate (TDCM, a pair of empty squares with two pairs of thin parallel bars), and trehalose monocorynomycolates (TMCM, a pair of empty square with one pair of thin parallel bar), the inner leaflet of symmetric bilayer that represents a virtually outer barrier obstructing ace of hydrophilic substance; the outer leaflet I composed of non-covalently linked lipid which assemble themselves into a monolayer. In addition, like that of Gram-negative bacteria, the cell envelope of corynebacteria contains proteins, including one with pore forming ability (P, grey squares) which are present in all the cell envelope compartment, except the PM. In freeze-fractured and deep-etched preparations of most corynomycolate-containing strain the major fracture plane (FP) is seen within the cell wall, presumably located between the two leaflet of the outer membrane (arrow); in strains devoid of corynomycolates and porins, e.g. *C. amycolatum*, the FP occurs within the PM (arrow). The different non-covalently linked lipid is also present in OL, which consists primarily of polysaccharides and contains proteins.

1.6 MYCOLIC ACIDS: OCCURRENCE, BIOSYNTHESIS AND GENETICS

1.6.1 Analytical methods

Physiochemical analysis of mycolic acid composition and molecular weight distributions have been used extensively in taxonomic studies of bacteria that contain these acids. Structural studies indicate that mycolic acids of mycobacteria are a complex mixture of high molecular weight (C_{60} to C_{90}), whereas those from nocardiae and rhodococci are smaller (C_{36}-C_{66}) (Alshamony *et al.*, 1976 a, b; Minnikin and Goodfellow, 1980). The mycolic acids from corynebacteria contain homologous mixtures of saturated and unsaturated components containing between 20 to 36 carbon atoms (Collin *et al.*, 1982a; Herrera-Alcaraz *et al.*, 1993; Pierotti, 1987).

A number of methods have also been used in studying mycolic acids including the production of multispot mycolic acid patterns by two-dimensional thin layer chromatography (TLC) (Barreau *et al.*, 1993; Goodfellow *et al.*, 1976; Hamid *et al.*, 1993; Minnikin *et al.*, 1975). Cleavage products of mycolic acids detected by gas chromatography (GC) and GC combined with mass spectrometry (GC-MS) provide characteristic profiles of mycolic acid composition useful in chemotaxonomic identification schemes (Collins *et al.*, 1982a; Chevalier *et al.*, 1988; Pierotti, 1987). High performance liquid chromatography (HPLC) has also been used to fractionate mycolic acids (Butler *et al.*, 1991; Thibert *et al.*, 1993).

Mycolic acids have been studied using many extraction and derivatisation methods. In these methods alkaline hydrolysis is followed by conversion to methyl esters with diazomethane (Asselineau, 1966; Etemadi, 1967). Apart from the toxicity and explosive nature of diazomethane this method has a number of disadvantages. A more convenient method involves acid methanolysis, which directly produces methyl esters (MAMEs) (Jang *et al.*, 1997; Minnikin *et al.*, 1975; Minnikin *et al.*, 1980; Pierotti *et al.*, 1987). In this method, covalently bound mycolic acids and fatty acids are extracted from the cell wall by treating the cells with a mixture of toluene, methanol and sulphuric acid (30:15:1) followed by incubation at 80°C overnight. The released MAMEs and fatty acid methyl

esters (FAMEs) are separated from other cellular components using petroleum extraction, MAMEs and FAMEs move into the petroleum layer leaving other components in the aqueous layer. The presence or absence of MAMEs can be demonstrated by TLC of methanolysates, in which MAMEs are separated from FAMEs due to their slower chromatographic mobility, using petroleum ether and acetone (95:5, v/v) as solvent. R*f* values <0.5 correspond to MAMEs where as R*f* values of >0.7 correspond to FAMEs (Jang, 1997; Pierotti, 1987). Resulting MAMEs can be analysed by GC (Asselineau, 1966). Since mycolic acids are high molecular weight compounds therefore GC analysis is performed at very high temperatures which causes thermal cleavage to yield FAMEs and aldehydes (Asselineau, 1966). To avoid this problem pyrolysis GC has been used to study mycolic acids from various bacteria. Mycobacterial mycolic acids release C_{22} to C_{26} FAMEs, whereas *Nocardia* yield C_{12} to C_{18} FAMEs (Collins *et al.*, 1982a; Lechevalier, 1976; Minnikin *et al.*, 1978). Alternatively, hydroxyl group of mycolic acids can be derivatized to trimethylsilyl (TMS) ethers and these can be analysed as intact molecules by GC, this can avoid pyrolytic cleavage. GC followed by mass spectrometry (MS) have been used to fractionate intact molecules of silylated mycolic acids from corynebacteria (Corina and Sesardic, 1980; Gaily *et al.*, 1982; Pierotti, 1987; Welby-Giesse *et al.*, 1970), and other mycolic acid containing bacteria (Yano *et al.*, 1972). Mycolic acids have also been studied using reversed-phase HPLC (Buttler *et al.*, 1991; Thiebert and Lapierre, 1993). Experimentally, mycolic acids were modified using saponification techniques and derivatising acids to their *p*-bromophenylacyl esters. The resulting products formed distinct pattern types depending on the species examined and these patterns were also used as a means of species differentiation (Butler *et al.*, 1991).

1.6.2 Types of mycolic acids found in corynebacteria.

The mycolic acid composition of the glutamic acid producing coryneform bacteria has been studied previously (Bousfield & Goodfellow, 1976; Collin *et al.*, 1979a; Pierotti, 1987), and they were found to contain mycolic acids with carbon chain length of C_{28} to C_{36} with saturated mono- and di-unsaturated components. Differences in mycolic acid

composition for three corynebacteria have been reported, where the three species contained: *C. glutamicum* NCIB 10025, C_{30}-C_{36}, *B. flavum* NCIB 9565, C_{28}-C_{36} and *B. lactofermentum* NCIB 9567, C_{32}-C_{36}. The main mycolic acid lipids were $C_{32:0}$ and $C_{34:1}$ in all the three species tested. Furthermore, in our laboratories (Jang *et al.*, 1997), studies were performed on the quantitative determination of mycolic acid profiles of *C. glutamicum* strain ATCC 13059, AS019 and mutants MLB133 and MLB 194 under different growth conditions. Results showed that all strains had five major types of mycolic acid ($C_{32:0}$, $C_{34:0}$, $C_{34:1}$, $C_{36:1}$, $C_{36:2}$) with $C_{32:0}$ and $C_{34:1}$ the dominant types present.

It was observed that pathogenic corynebacteria such as *C. diphtheriae*, *C. ulcerans* and *C. urealyticum* have different mycolic acid composition (Corina and Sesardic, 1980; Herrera-Alcarzar *et al.*, 1993; Yano and Saito, 1972). *C. ulcerans* (Yano & Saito, 1972) possesses shorter chain length mycolic acids, C_{20} to C_{32}, while *C. diphtheriae* possess mycolic acids ranging from C_{20} to C_{35}. *C. urealyticum* exhibited mycolic acids with lipids ranging from C_{26} to C_{30}. A summary of mycolic acids found in different corynebacterial species is shown in Table 1.1.

1.6.3 Role of mycolic acids as a permeability barrier

Cell wall-containing eubacteria have traditionally been divided into two main groups, Gram-negative and Gram-positive bacteria on the basis of the structure of their cell walls. The Gram-negative group has a cell wall with a thin peptidoglycan layer and an asymmetric outer membrane. Small hydrophilic compounds can diffuse through the outer membrane by the porin pathway (Benz, 1988; Nikaido *et al.*, 1985). Gram-positive bacteria like mycobacteria and corynebacteria are unique because they contain, in addition to the thick peptidoglycan layer, a large amount of lipid in the form of mycolic acids in the cell wall. Mycolic acids have structural roles which give rise to the resistance of cells to harsh environments and cause the acid fastness of mycobacteria, they probably play an important role in the restricted permeability of these microbes to the water

Table 1.1 Examples of the distribution of mycolic acids in corynebacteria (Jang , 1997).

Organism	Mycolic acids	Reference
Pathogenic corynebacteria		
C. diphtheria	C_{20} to C_{25}	Corina and Sesadic, 1980
C. ulcerans	C_{20} to C_{32}	Yano and Saito, 1972
C. urealyticum	C_{26} to C_{30}	Herrera-Alcaraz *et al.*, 1993
Non-pathogenic bacteria		
B. lactofermentum	C_{32} to C_{36}	Collins *et al.*, 1982a
B. flavum	C_{28} to C_{36}	Collins *et al.*, 1982a
C. glutamicum	C_{30} to C_{36}	Collins *et al.*, 1982a
C. lilium	C_{32} to C_{36}	Collins *et al.*, 1982a

soluble molecules (Jarlier and Nikaido, 1990). The strong permeability barrier of mycobacterial cell wall due to the presence of lipid layer is one of the major factor governing the resistance of pathogenic mycobacteria to otherwise potent antibiotics (Jarlier and Nikaido, 1991).

The mycobacterial cell wall shows an unusually low degree of permeability to hydrophobic solutes. Jarlier and Nikaido (1990) experimentally determined the permeability of *M. chelonae* cell wall to hydrophilic solutes using cephalosporins. The rate of hydrolysis of cephalosporin by intact mycobacterial cells was measured, and the cell wall permeability was calculated by assuming that drug molecules first diffuse through the cell wall (following Fick's first law of diffusion) and then are hydrolysed by periplasmic β-lactamase (following Michaelis-Menten kinetics). The wall permeability measured was indeed low: about three orders of magnitude lower than seen for the *E. coli* outer membrane and 10 times lower than the permeability of the notoriously impermeable *Pseudomonas aeruginosa* outer membrane. Permeation rates had low temperature coefficients and did not increase when more lipophilic cephalosporins were used, indicating that the permeation occurred mainly through aqueous channels (Jarlier and Nikaido, 1990). Recently, channels have been identified in the cell walls of *M. chelonae* and *M. smegmatis* and it became clear that the low permeability of mycobacteria is because of two factors. Firstly, *M. chelonae* porin is a minor protein of the cell wall and, secondly, it produces a permeability far lower than that produced by an equal weight of *E. coli* porin (Trias and Jarlier, 1992). Likewise, Niederweis *et al.* (1995) identified the hydrophilic pathway through the mycolic acid layer of *C. glutamicum*. Their results are in agreement with the assumption that the channel from the cell wall of *C. glutamicum* is an aqueous channel with little or no interaction between the channel wall and ions. Furthermore, it is clear that these channels can be present only in the cell wall and not in cytoplasmic membranes. Otherwise, the presence of these high conducting channels would result in cell death. Several procedures widely used in biotechnology to improve amino acid secretion by *C. glutamicum* are likely to affect the state of corynebacterial cell wall (Kramer, 1994). However, it remains to be elucidated

whether the ability of corynebacteria to excrete amino acids effectively under certain conditions is related to the channel forming activity identified or to other properties of cell wall or a secretary mechanism (Lambert *et al.*, 1995).

Haynes and Britz (1990) speculated that the cell surface of *C. glutamicum* precluded efficient uptake of DNA. The unusual structure of the cell wall makes the corynebacteria resistant to lysozyme unless they have been grown in the presence of cell wall inhibitors, such as penicillin G, glycine or INH, which modify these structures (Best and Britz, 1986; Jang *et al.*, 1997; Katsumata *et al.*, 1984; Santamaria *et al.*, 1984; Thiebach *et al.*, 1988). It has therefore been suggested that cell wall permeability of corynebacteria can be increased by using cell wall inhibitors (Best and Britz 1986; Haynes and Britz 1989; Yoshihama *et al.*, 1985). The action of some of these cell wall acting agents is described in detail in section 1.7.

Several workers have reported quantitative changes in the mycolic acid composition of corynebacteria, nocardia and mycobacteria by varying the environmental conditions such as growth temperature (Toriyama *et al.*, 1980; Tomiyasu *et al.*, 1981) and substrates (Cooper *et al.*, 1979). Tween 80 is often added to enhance the growth rate of some cutaneous corynebacteria in commonly used media (McGinley *et al.*, 1985a, b; Riley *et al.*, 1979). Chevalier *et al.* (1988) observed that the presence of Tween 80 in the growth medium of cutaneous corynebacteria induced variations in double bonds of corynemycolic acids. Tomiyasu (1982) found that mycolic acid composition of *Nocardia* species could be changed by shifting the cultivation temperature at mid-exponential growth phase from 15°C to 50°C, which increased the relative amount of saturated mycolic acids. These results indicate that environmental changes and specific chemicals can alter the mycolic acids compositions of some mycolic acid containing bacteria.

1.6.4 Biosynthesis of mycolic acids

In contrast to the extensive literature on the biochemistry of mycolic acid synthesis in *Mycobacterium* species and the emerging literature on the genetics of this, the models for synthesis of corynemycolic acids are based on relatively few publications and information comes from a diverse range of species (described in the following), reflecting the relatively greater importance of pathogenic mycobacteria in human health. Mycolic acids are thought to result from a unique Claisen-type condensation of two fatty acids moieties; however little is known about this essential step within the mycolic acid biosynthetic pathways. Gastambide-Odier and Lederer (1959) observed that *C. diphtheriae* condensed [1-^{14}C] palmitate when this was supplied in the culture medium to form the corynemycolic acids $C_{32}H_{64}O_3$ which was labelled essentially at C-1 and C-3. Therefore it was postulated that two molecules of palmitic acid condense in a Claisen-type reaction to form the 3-keto derivative, followed by further reaction to yield the corynemycolic acids. It was also proposed that this conversion apparently involved a biotin-dependent carboxylation step (Fig 1.6). However, the formation of the putative tetradecylmalony-CoA, one of the substrates for the subsequent Claisen condensation reaction, proved difficult to demonstrate.

Walker *et al.* (1973) confirmed this reaction in a cell-free system of *C. diphtheriae* and similar condensations were established for the corynemycolic acids of sizes between C_{34} to C_{36} by Shimakata *et al.* (1984). Shimakata *et al.* (1984) demonstrated a complete *in vitro* synthesis of corynemycolic acids ($C_{34:0}$, $C_{34:1}$, $C_{36:0}$ and $C_{36:1}$) from [1-^{14}C] fatty acids by the "fluffy layer" (upper fraction of pellet in homogenised-disrupted cells) fractions prepared from *Bacterionema* (later renamed *Corynebacterium*) *matruchotii* and some characterization of this synthesis. They found that when cells were grown in [1-^{14}C]-stearic acid, the label was incorporated into two major radioactive peaks which were separated by gas chromatography (GC): one corresponding to the peak of ($C_{34:0}$ + $C_{34:1}$) mycolic acids and the other to ($C_{36:0}$ + $C_{36:1}$) mycolic acid. The reaction was dependant

on the pH (optimum at pH 6.4) and required a divalent cation (Mg^{++}, Zn^{++}, Ca^{++} and Mn^{++}). The *in vitro* system utilised myristic, palmitic, stearic and oleic acids (probably *via* their activated forms) as precursors, among which myristic and palmitic acids were more effective than the rest. Cerulenin, a specific inhibitor of β-ketoacyl synthetase in *de novo* fatty acid synthesis, inhibited the reaction at a relatively high concentration. Thin-layer chromatographic analysis of lipids extracted from the reacting mixture without alkaline hydrolysis showed that both exogenous [1-^{14}C] fatty acid and synthesised mycolic acids were bound to an unknown compound by an alkali-labile linkage and this association seemed to occur prior to the condensation of two molecules of fatty acids.

Recently Lee *et al.* (1997) re-investigated the question of a biotin-dependent carboxylation step through the use of 2, 2-[^{2}H]palmitic acid in pulse labelling of whole cells, in the belief that the results would provide clues to the mechanism of the condensation reaction as well as allowing the design of inhibitors targeted against the Claisen condensation reaction, at least in corynebacteria. The model strain chosen for this study was *C. matruchotti,* since the *in vitro* synthesis of corynomycolates from 1,2-[^{14}C] acetate leads to mature corynomycolates and not the intermediate C_{32} β-keto esters, as in the case of *C. diphtheriae.* The presence of a β-keto ester would require additional precautions during chemical analysis, since alkaline hydrolysis results in the liberation of palmitone (Walker *et al.*, 1973). They also reasoned that the Claisen condensation step would be conserved between *C. matruchotti*, *C. diphtheriae* and *Mycobacterium* genus. The results of their studies supported the earlier conclusions of Gastambide-Odier and Lederer (1960), that two molecules of palmitic acid condense together to form the C_{32} corynemycolate. Their results showed that corynomycolate retains two deuterium atoms within the β-chain, at positions C-2 and at C-4. The retention of the deuterium atom at position C-2 clearly supported a pathway whereby two palmitic acids condense without an intermediate carboxylation step in the course of synthesis of the mature mycolic acid. This proposed pathway is demonstrated in Fig 1.7.

The absence of a carboxylation step allows one to propose a mechanism for the condensation reaction involving a highly activated enolate intermediate (Fig 1.8). This evidence allowed the design and synthesis of several structurally related antagonists against the condensation reaction, which were shown to posses potent *in vivo* activity against *C. matruchotti* with complete inhibition of growth on solid medium at concentrations between 1-10 μg/ml (Richard *et al.*, 1997).

Corynebacteria also possess a closely related multifunctional fatty acid synthase complex (FAS-I) similar to mycobacteria which is responsible for the synthesis of medium chain C_{16}-C_{18} acyl-CoA derivatives (Knoche *et al.*, 1991). Experimentally this soluble synthase was found to be insensitive to the fatty acid analogs suspended at 200 μg/ml, suggesting a separate and distinct synthase, that is particulate in nature (EP60), responsible for corynomycolate synthesis, and presumably involving a membrane-bound ACP.

A different model was proposed for mycobacterial mycolic acid biosynthesis. It is postulated that the Claisen condensation reaction of *M. tuberculosis* (Fig 1.9) involves the interaction of an activated C_{56} fatty acid (meromycolate) (I) with an activated C_{26} fatty acid (II) to yield a 3-oxo intermediate of an acyl carrier group (III) which is then reduced to form the mature mycolic acid prior to its transfer to the various cell wall components (IV) (Lee *et al.*, 1997). Lee *et al.* (1997) showed that X was H rather than CO_2. Accordingly, the transition state intermediate of the resulting Claisen condensation reaction could provide a crucial target for antagonists and thus potentially new drugs for application in mycobacterial diseases. Also the inhibition of this step should facilitate

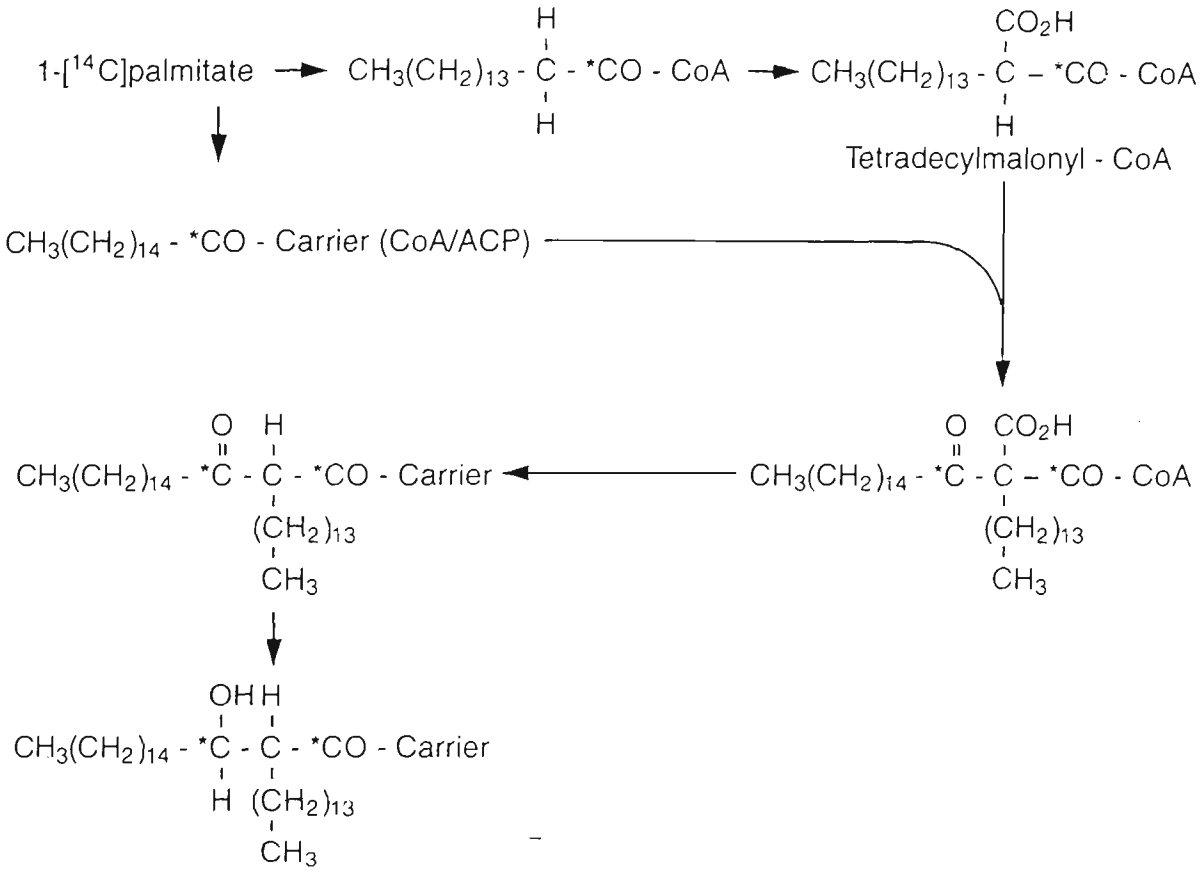


Figure 1.6 Earlier postulated pathway for the synthesis of corynemycolic acids of *C. matruchotti*, involving a carboxylation step (Shimakata *et al.*, 1984; Gastambide-Odier *et al.*, 1960).

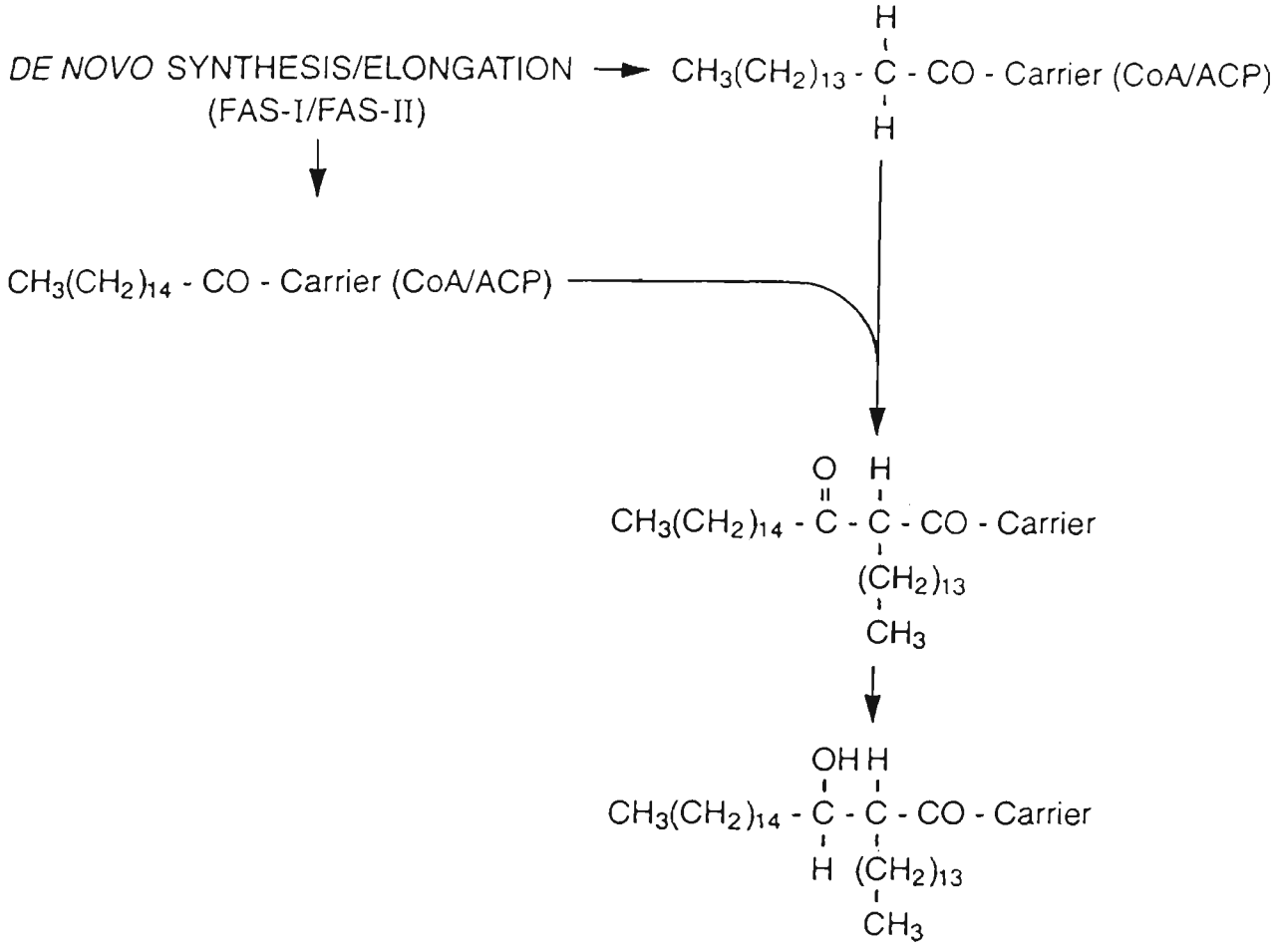


Figure 1. 7 The proposed pathway for corynomycolate synthesis in *C. matruchotti* involving a non-carboxylation dependent step. The nature of the acyl carrier groups is not known but are presumably ACPs (acyl carrier proteins) (Lee *et al.*, 1997).

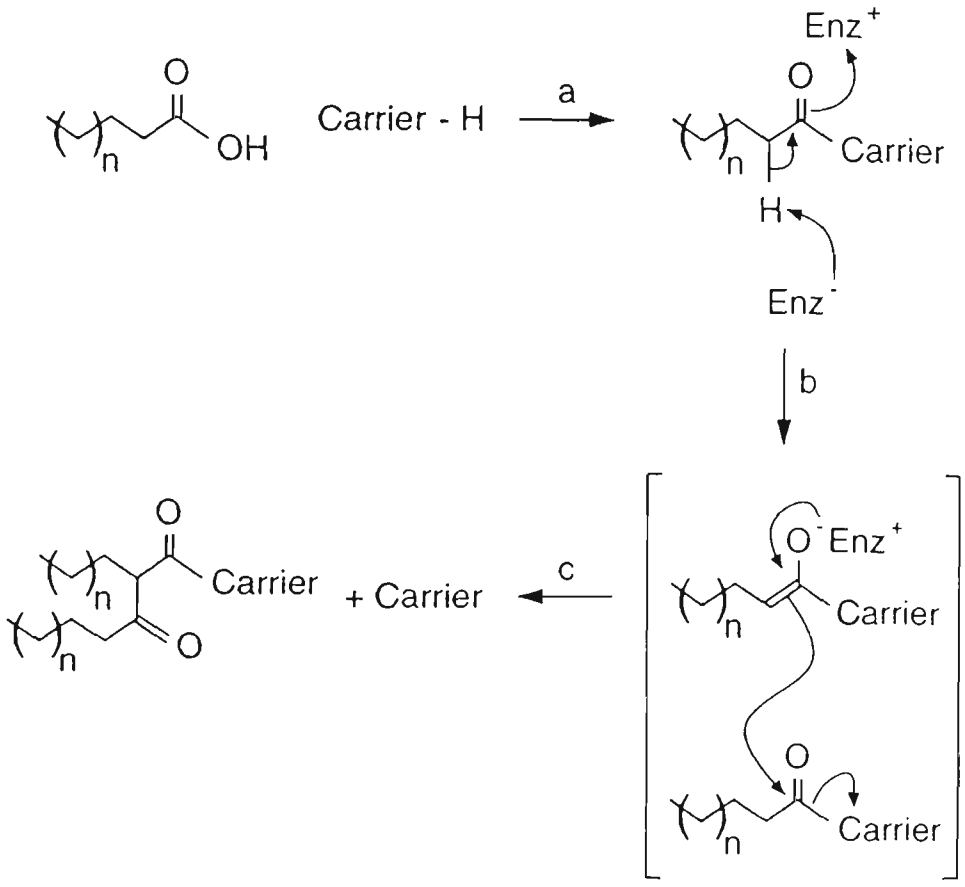


Figure 1.8 The proposed mechanism of Claisen condensation reaction in corynebacteria (Richard *et al.*, 1997).

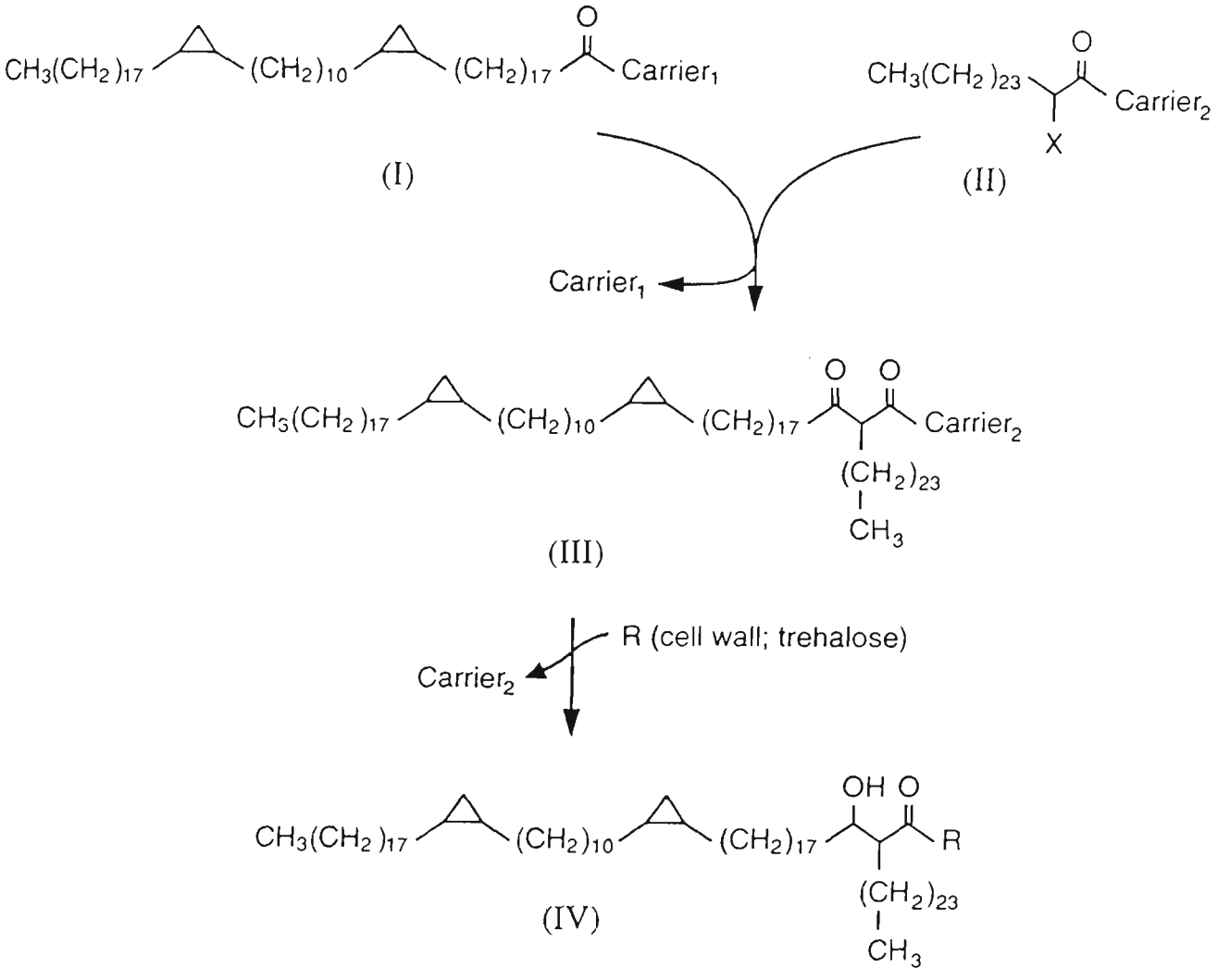


Figure 1. 9 Condensation step in the biosynthesis of the α-mycolates of *M. tuberculosis*. Two fatty acids moieties, a C_{56} meromycolate (I) and a C_{26} saturated fatty acid (II) condense to yeild a 3-oxo ester (III) which is then reduced to the hydroxy form (IV). The nature of the acyl carrier groups are not known but are presumably ACPs (Lee *et al.*, 1997).

identification of the acyl carrier groups involved in the final stages of *de novo* synthesis of mycolic acids. Clearly, these processes are particularly complex in mycobacteria because of the requirement of a C_{56} meromycolate containing within it a variety of functional groups (Minnikin *et al.*, 1982). Information on the synthesis of the meromycolic chain is limited (Daffe *et al.*, 1998; Laneelle, 1989). Previous studies, however, suggest that it would be formed by elongation of a 16- to 24-C precursor by successive additions of 2-C units (Lacave *et al.*, 1990; Lopez *et al.*, 1991).

Based on the results of corynemycolic acids synthesis (Shimakata *et al.*, 1984; Walker *et al.*, 1973), Brennan and Nikaido (1995) speculated that the resulting products are in the form of trehalose mono-mycolates and these esters are thought to be the carriers of the mycolic acid to the mycobacterial cell wall, where it is transferred to the non-reducing terminal D-arabinose residue of arabinoglactan *via* an unknown transacylation reaction in mycobacteria (Brennan and Nikaido, 1995). Early studies on the biosynthesis of mycolic acids have been reviewed by Besra and Chitterjee, 1994. More recently mycolyl-mannosylphosphopolyprenol was identified and it was suggested that mature mycolic acids are formed from β-oxo precursors while attached to a mannosylphosphopolyprenol and are then transported through the membrane for esterification to the arabinan in the wall (Besra *et al.*, 1994; Kolattukudy *et al.*, 1997).

The condensase, the enzyme responsible for the final condensation step in mycolic acid biosynthesis remained an enigma for decades. By in silico analysis of various mycobacterial genomes Portevin *et al.* (2004) has identified an enzyme, pks13, which contain the four catalytic domains required for the condensation reaction. Homologs of this enzyme were found in other *Corynebacterineae* species. They proposed that the substrate of the pks13 condensase are activated by the acyl-CoA synthase, FadD32, and an acyl-CoA corboxylase containing the accD4 protein. These experiments showed that pks13 gene was essential for the survival of *M. smegmatis*.

1.6.5 Genetics of mycolic acids biosynthesis

Knowledge about enzymes that catalyse individual steps that are postulated to be involved in mycolic acid synthesis is limited. Fatty acid elongation and cyclopropanation have been examined. Of the four resolved component enzymes of this very long-chain fatty acid elongation system, only two, the 3-oxo-acyl-CoA reductase, and enoyl-CoA reductase were inhibited by INH, a commonly used antimicrobial drug that is known to inhibit mycolic acid synthesis (Kikuchi *et al.*, 1989). Molecular evidences further suggested that enoyl reductase (InhA, the product of the *inhA* gene) is the target for INH (Dessen *et al.*, 1995). Since the molecular genetics of mycolic acids biosynthesis is the subject of this thesis, InhA and its genetics is the subject of this section.

The major mycolic acid produced by *M. tuberculosis* contains two *cis*-cyclopropanes in the meromycolate chain. The genes involved in the cyclopropanation were found using cosmid cloning of *M. tuberculosis* DNA which, when introduced into *M. smegmatis,* caused cyclopropanation of the double bond in the mycolic acid. A gene, *cma1*, encoding a protein that has 34% identity with the cyclopropanating enzyme of *E. coli*, was found to be responsible for this cyclopropanation (Yuan *et al.*, 1995). Another gene, *cma2*, with 73% identity to *cma1*, was cloned from *M. tuberculosis* by homology to a putative cyclopropane synthase identified from the *M. leprae* sequencing project. This gene cyclopropanated the proximal olefin in the mycolic acids of *M. smegmatis* (George *et al.*, 1995). The methoxymycolate series found in *M. tuberculosis* contains a methoxy group adjacent to the methyl branch, in addition to the cyclopropane in the proximal position. The molecular genetics of this olefin modification were elucidated (Yuan and Barry, 1996). Since cyclopropanation of mycolic acids has not been reported in corynebacteria, this topic is not further reviewed here.

INH was first reported to be an effective antituberculosis drug in 1952, displaying particular potency against *M. tuberculosis* and *M. bovis.* Isoniazid resistant strains were isolated immediately after this antibiotic began to be used therapeutically (Middlebrook, 1952), and about 20% of the *M. tuberculosis* strains in New York City were later

reported as resistant to isoniazid (Frieden *et al.*, 1993). Determining the molecular mechanisms of action of isoniazid, and its cellular targets, is thus crucial both for understanding resistance mechanisms in mycobacteria and for designing more potent anti-mycobacterial agents for controlling tuberculosis and other diseases caused by mycobacteria.

Drug resistance can be caused by many mechanisms, including mutation in the drug target that reduces the binding of the drug or mutations that lead to increased production of the target (Rabussay *et al.*, 1969). The mechanism by which INH inhibits *M. tuberculosis* and its precise target of action are unknown. However, compelling biochemical evidence has suggested that both INH and ethionamide (ETH) block mycolic acid biosynthesis. Previous studies have also shown that in certain cases low-level INH resistance correlated not with the loss of catalase activity but with the coaquisition of ETH resistance (Canetti, 1965), indicating that the mechanism of action of the two drugs may share a common target.

The recent development of molecular genetic techniques for mycobacteria (Jacob *et al.*, 1991) and availability of a cell-free assay system for mycolic acid biosynthesis (Quemard *et al.*, 1991) have allowed advances in the understanding the mechanism of INH action. A novel gene, *inhA*, was identified from *M. tuberculosis*, *M. avium*, *M. smegmatis* and *M. bovis* by characterisation of genetic variants that conferred resistance to both INH and ETH (Banerjee *et al.*, 1994). Resistance to these two anti-tubercular drugs was shown to be mediated by either mutations in the structural gene or the presence of the wild-type gene on a multi-copy plasmid. Sequence analysis revealed that the *inhA* locus consists of two open reading frames (ORFs), designated *orf1* and *inhA*, encoding 25.7-kd and 28.5-kd proteins respectively, which may participate in fatty acid biosynthesis (Dessen *et al.*, 1995). Sub-cloning studies demonstrated that the second *orf* from *M. smegmatis* DNA was sufficient to confer the INH resistance phenotype and was thus named the *inhA* gene. The preferred designation for *orf1* is now *mabA* [mycolic acid biosynthesis] [Musser *et al.*, 1996]). In *M. tuberculosis* and *M. bovis*, the two genes are separated by a short (21 bp) non-coding region that lacks a readily identifiable promoter (Banerjee *et al.*, 1994). It

is thought that *mabA* and *inhA* together constitute a two-gene operon that is transcribed from a promoter located upstream of *mabA*.

Quemard *et al.* (1995) in their steady state kinetic experiments have shown that isoniazid is a poor inhibitor of enzymatic activity (K_{is} ca. 40 mM against 2-*trans*-octenoyl-CoA) suggesting that isoniazid is not the active form of the drug but rather a pro-drug which must be converted to an active form. Earlier observation suggested a link between INH resistance and the loss of mycobacterial catalase–peroxidase activity (Cohn *et al.*, 1954; Middlebrook, 1954). Recent reports have demonstrated that deletion of, or point mutations in, the *M. tuberculosis katG* gene, which encodes a unique catalase–peroxidase, results in the aquisition of INH resistance and that transformation of INH-resistant strains of *M. tuberculosis* with a functional *katG* gene restores sensitivity to the drug (Zhang *et al.*, 1992, 1993). A more recent report has shown that the catalase-peroxidase oxidizes isoniazid to form reactive intermediates, which can be quenched with added nucleophiles (Johnsson and Schultz, 1994).

The suggested targets for the activated drug in mycobacteria have included InhA, an enoyl acyl carrier protein reductase (Banerjee *et al.*, 1994), as well as two components of a type II fatty acid synthase system, AcpM and KasA (Mdluli *et al.*, 1996a, b). This is consistent with the observation that mycolic acid biosynthesis is inhibited by isoniazid and that fatty acids up to 24-26 carbons accumulate in its presence (Quemard *et al.*, 1991). Alternative mechanisms for the *in vivo* activation of isoniazid, which are independent of KatG–dependant oxidation, may additionally generate enoyl-ACP reductase inhibitors, since isoniazid-sensitive strains of *M. tuberculosis* with no catalase–peroxidase activity have been isolated (Heym *et al.*, 1994; Kapur *et al.*, 1995; Stoeckle *et al.*,1993).

The predicted InhA proteins of *M. tuberculosis, M. bovis*, and *M. smegmatis* show some sequence similarity to the EnvM proteins of *Salmonella typhimurium* and *E. coli*. The protein EnvM is thought to be involved in fatty acid biosynthesis (Bergler *et al.*, 1992; Turnowsky *et al.*, 1989) and has been shown to catalyse the reduction of a crotonyl-acyl carrier protein (Bergler *et al.*, 1994), an essential part of fatty acid

elongation. The first open reading frame (*orf1*) also exhibits sequence similarity to several other proteins involved in fatty acid biosynthesis; the highest identity score was 46.5% over 241 amino acids of the 3-keto-acyl carrier protein reductase (*fab*G gene product) from *E. coli*. The InhA protein uses flavin nucleotides as substrates or cofactors, as it has a putative binding site for these molecules (Banerjee *et al.*, 1994). Kinetic analysis suggested that isoniazid resistance is due to a decreased affinity of the mutant protein for NADH. Moreover, examination of the crystalline structure of the target protein (2-*trans*-enoyl-acyl carrier protein) revealed that drug resistance was directly related to perturbation in the hydrogen-bonding network that stabilises β-nicotinamide adenine dinucleotide (NADH) binding.

In two different studies, 25% of the clinical isolates contained mutations within the *inhA* locus, and substitutions within the *inhA* open reading frame have been shown to express the $Ser^{94} \rightarrow Ala^{94}$ (S94A) and $Ile^{16} \rightarrow Thr^{16}$ (116T) InhA enzymes (Heym *et al.*, 1994; Banerjee *et al.*, 1994; Kapur *et al.*, 1995), suggesting that these changes are important for continued activity by the InhA protein.

The identification of an *inhA* gene missense mutation (Ser^{94}-Ala^{94}) that conferred isoniazid and ethionamide resistance in the laboratory, together with the observation that most rifampicin resistant strains had missense mutation in a defined region of *rpoB* (Musser *et al.*, 1995), led to the plausible hypothesis that missense mutations in the *inhA* gene would constitute a major cause of resistance among *M. tuberculosis* strains recovered from patients. However, sequencing of the entire *inhA* gene, including the upstream presumed regulatory region, in 37 isoniazid-resistant organisms revealed that only with one exception, all strains had the identical wild-type *inhA* allele, and none contained the Ser^{94}-Ala^{94} substitution conferring resistance in the laboratory (Kapur *et al.*, 1995). Variation was also examined in the 744-bp *mabA* gene in 24 resistant clinical isolates, and substitutions were identified at two nucleotides flanking a presumed ribosomal binding site in four isolates (Kapur *et al.*, 1995). On the basis of these studies,

it was suggested that the base-pair substitution flanking the presumed *mabA* ribosomal binding site resulted in altered regulation of MabA or InhA (or both) in some *M. tuberculosis*-resistant strains. In another study by Musser *et al.* (1996) it has been shown that *katG* missense mutation virtually never occur in association with a mutation in the putative regulatory region of the *inh*A locus. The most probable explanation proposed by these authors for the failure of these changes occurring together was that either mutation alone is sufficient to confer the resistance phenotype.

The product of the *inhA* gene is characterised as being part of an elongase as it does not reduce the initial *trans*-2-enoyl product, crotoyl-CoA, of fatty acid biosynthesis *de novo*. Its preferred substrates are C_{16} to C_{24} enoyl-ACP. Wheeler *et al.* (1996) did further investigations to find out whether an elongase step in mycolic acid biosynthesis is the primary target of isoniazid. According to them, three lines of evidence identified 24:1 *cis*-5-elongase as the primary isoniazid target. Firstly, 24:1 *cis*-5 fatty acid did not restore isoniazid-inhibited mycolic acid biosynthetic activity in a crude cell-wall preparation, suggesting that the drug acts after the formation of the Δ-5 double bond. Secondly, 24:1 *cis*-5 elongase assay, in which the product is mycolic acid, is completely inhibited by isoniazid. Finally, the only intermediates that accumulate as a result of the addition of isoniazid are fatty acids of 24 carbons. Both 24:0 and 24:1 are observed in a similar ratio whether or not isoniazid is present, even though concomitant mycolic acid biosynthesis is inhibited by isoniazid. The proposed scheme (Wheeler *et al.*, 1996) to demonstrate isoniazid-sensitive reduction of *trans*-2-*cis*-5-tetracosdienoyl-ACP is shown in Fig 1.10. These results are consistent with studies of *M. tuberculosis* InhA by Dessen *et al.* (1995). Although the work of these people adds to the evidence that the target of isoniazid in *Mycobacterium* is a highly susceptible fatty acyl elongase involved in mycolic acid biosynthesis, much work remains to be done in this area. Ultimately the isolation of individual enzymes should allow the correct pathway to be established and the binding of isoniazid or its activated form to its target demonstrated directly.

More recently, the studies of Mdluli *et al.* (1996) on lipid biosynthetic responses to isoniazid treatment of *M. tuberculosis* and *M. smegmatis* suggested that the mode of action of activated INH differs between these two organisms. Transformation of *M. smegmatis* with *inhA* cloned into a plasmid conferred a high level of resistance to INH, while the same construct failed to confer resistance in *M. tuberculosis*. The *inhA* region from two clinical isolates whose resistance had been attributed to changes in the upstream promoter region has been cloned and was not sufficient to impart INH resistance to the level of the parent strain on sensitive *M. tuberculosis*. These putative mutant promoter elements appear to elevate expression levels of gene fusion reporter constructs, suggesting some non-causal connection between observed mutations and the lipid metabolism of the drug-resistant organism.

It remains unclear as to what extent *inhA* mutations co-occur with *katG* mutations in some reports, it is clear that many of the observed mutations occur in strains with very high MICs, consistent with the absence of *katG* (Heym *et al.*, 1995). In other reports, *inhA* promoter mutations occur in the context of multi-drug resistant strains with unspecified levels of INH resistance (Rislow *et al.*, 1995). In such complex backgrounds, it is clearly necessary to establish the function of each mutation individually and in relation to the metabolism of the drug-resistant organism. Perhaps the observed changes in the *inhA* loci may be related to compensatory mutations in lipid metabolism in a *katG* background, similar to the compensatory changes in enzymes of the oxidative stress regulon recently reported (Sherman *et al.*, 1996). Such mutations may even confer enhanced resistance to INH in a *katG*-impaired background, which might not be obvious when tested in wild-type *M. tuberculosis* but which may have clinical significance.

Finally, the combination of previously reported genetic (Banerjee *et al.*, 1994), structural (Dessen *et al.*, 1995), enzymological (Quemard *et al.*, 1995) data and recent studies of Mdluli *et al.* (1996) indicate that InhA is not the major target for isoniazid in *M. tuberculosis*. The most consistent interpretation of the data is that a currently unidentified protein involved in the production of unsaturated 24-carbon fatty acids is directly or indirectly inhibited by *katG*-activated INH in the uniquely INH-sensitive *M. tuberculosis*.

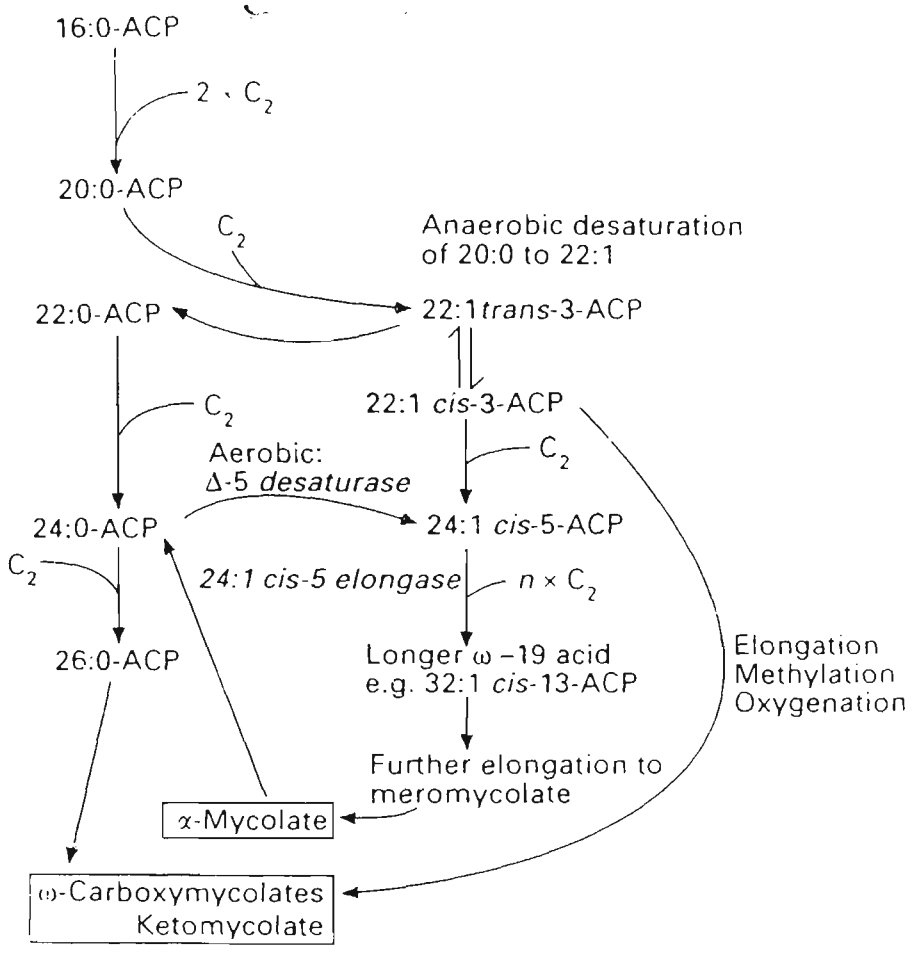


Figure 1.10: Possible pathways for mycolic acids biosynthesis. *M. aurum* and *M. tuberculosis* converts double bonds to cyclopropane rings (Yuan *et al.*, 1995) and elaborates the mycolate class methoxymycolates. InhA is involved in elongation of acyl-ACP, but has low affinity for acyl-CoA. The carrier involved in mycolic acid biosynthesis remains to be identified directly. The target of isoniazid was identified as elongation of the 24:1 *CIS*-5 moiety. Note that trans-2-enoyl-carrier reductases are involved in all C2 elongation steps, but their reaction is shown here only for the elongation of 20:0-ACP to 22:0-ACP (Wheeler and Anderson, 1996).

1.7 MODULATORS OF CELL SURFACE STRUCTURES

The impact of agents like glycine, INH and ETH, which disrupt the physical integrity of the bacterial cell wall, are described in the following sections.

1.7.1 Glycine

It has been known for many years that increasing concentrations of glycine have some inhibitory effect on bacterial growth (Dienes *et al.*, 1950; Gorden and Gorden., 1943). Studies have shown that the morphological effects of glycine are similar to those of penicillin G, i.e., cell elongation and spheroplast formation, therefore it has been assumed that the cell wall is the main site of glycine action. In 1965 Strominger and Birge studied the effect of glycine on *S. aureus* and observed the accumulation of uridine diphosphate-N-acetyl muramic acid (UDP-MurNAc) and other cell wall precursors in which L-alanine was replaced by glycine. The mechanism of action of glycine was studied in detail by Hishinuma *et al.* (1971). These authors proposed that growth inhibition is partly due to the inhibition of the "L-Ala-adding-enzyme".

Studies of Hammes *et al.* (1973) had shown that the structure of peptidoglycan in the Gram-positive cell wall was altered following growth in medium containing glycine, where these authors proposed that glycine substituted for alanine in the peptidoglycan, impairing the cross-linking and weakening the peptidoglycan structure.

Previously, Hopwood *et al.*(1977) used glycine in the growth medium as a preliminary step to protoplasting *Streptomyces* species and later on this approach was applied successfully in *C. glutamicum* species. Yoshihama *et al.* (1985) reported that when *C. glutamicum* cells were grown in medium containing 2% (w/v) glycine before lysozyme treatment, there is 99% increase in the proportion of osmotically sensitive cells (mixture of spheroplast and protoplasts). Best and Britz (1986) also reported that more osmotically sensitive cells are obtained following growth in glycine and lysozyme treatment. *Streptomyces* mycelium subcultured into a medium containing glycine showed some growth retardation and cells became much more sensitive to lysozyme than those grown without glycine (see reviews by Okanishi *et al.*, 1974 and Hopwood, 1981).

Zuneda *et al.* (1984) reported that increasing glycine concentrations from 1% to 2% increased the lag time, slowed down the growth rate and decreased the biomass yield of *S. antibioticus*.

In 1986 Best and Britz studied the effect of presence of glycine in growth medium on cell morphology of *C. glutamicum*. Cells were harvested at mid-exponential phase after growth in medium containing 2% (w/v) glycine, incubated with 2.5 mg/ml lysozyme for 2 h and then with 0.1 M EDTA for 30 min. These researchers observed that after growth in glycine cells became larger, more irregularly shaped when compared to those grown in unsupplemented media. They also found that these effects were less marked in stationary phase cultures. These observations suggested strongly that alterations in cell morphology in *C. glutamicum* were related to the presence of glycine in the growth medium.

Jang *et al.* (1997) also studied the effect of glycine on the cell growth and cell morphology of *C. glutamicum* AS019 and the readily-protoplasting mutants MLB133 and MLB194. They noticed that the specific growth rates of all strains tested decreased as the concentration of glycine in the growth medium increased. Furthermore, growth in glycine caused some cell elongation and the rare appearance of X-and Y-shaped cells. The presence of X-and Y-type cells was more frequent in the cell surface mutants (MLB133), as were branched rods, relative to the parent strain grown under the same conditions, suggesting that cell division here occurs through budding, branch formation, then septation.

A similar observation was also reported in other corynebacteria. When *C. diphtheriae* were grown in the presence of 2% (w/v) glycine, the cells elongated and bulged at the side where the cell wall had apparently been damaged. When cells were treated with lysozyme (2 mg/ml) and washed in hypertonic buffer, the misshapen rods were replaced by spherical forms (Serwold-Davis *et al.*, 1987). The effective concentration of glycine also varied with each strain for the formation of osmotically sensitive cells, *C. ulcerans* required much less glycine than did *C. diphtheriae* (Serwold-Davis *et al.*, 1987).

1.7.2 Isoniazid

For several decades, isoniazid (INH) has been a key component in the treatment of tuberculosis. The INH minimal inhibitory concentration (MIC) for susceptible *M. tuberculosis* strains is usually less than 0.02 to 0.05 μg/ml (reviewed by Musser, 1995). Although neither the precise target of the drug nor its mode of action are known, INH treatment is bactericidal for mycobacteria. Among the many metabolic perturbations which have been observed are altered pigment formation (Youatt, 1961), reduced nucleic acid synthesis (Gangadharam *et al.*, 1963), decreased protein synthesis (Tsukamura and Tsukamura, 1963), reduced lipid content and altered cell wall permeability (Winder and Brennan, 1965; Winder, 1964). It was thougt that many of these effects may be indirect and may simply reflect growth inhibition (Youatt, 1969).

Key informations about the into the mechanism of INH resistance were obtained in 1954 when Middlebrook and colleagues discovered that INH-resistant organisms had decreased catalase activity (Cohn *et al.*, 1954; Middlebrook, 1954). Hedgecock and Faucher (1957) further extended this observation, these authors studied INH-resistant organisms and found an inverse correlation between INH MIC and catalase-peroxidase activity.

In *M. tuberculosis*, resistance is typically associated with at least two independent mechanisms: (1) loss of activation of the pro-drug through either deletion or mutation of *katG* gene (Musser *et al.*, 1996) and (2) mutation or inactivation of the *inhA* gene, which putatively encodes a fatty acid synthetase associated with mycolic acid synthesis (Banerjee *et al.*, 1994). The *katG* gene encodes a catalase-peroxidase (Jackett *et al.*, 1978). Peroxide, in the presence of catalase-peroxidase, converts isoniazid to a variety of intermediates, ultimately leading to isonicotinic acid (Johnsson *et al.*, 1994). It is thought that one of these products binds to the target or complexes with NADH to bind to the target, with isoniazid itself being a poor inhibitor of the *inhA* gene product (Quemard *et al.*, 1995).

In another study Stoeckle and colleagues (1993) found 31 (76%) of 41 isoniazid-resistant strains isolated in New York City contained *katG* sequences. The observation that only a relatively small percentage of INH-resistant *M. tuberculosis* strains are catalase negative, with either gross alterations or missense mutations involving *katG* (Cockerill *et al.*, 1995), led to the conclusion that mechanisms other than catalase activity play an important role in isoniazid resistance among clinical isolates of *M. tuberculosis*. Likewise some data are available to suggest that INH blocks the synthesis of long-chain-branched, β-hydroxy fatty acids, or mycolic acids, characteristics of cell walls of mycolic acid containing bacteria (Banerjee *et al.*, 1994; Davidson and Quemard *et al.*, 1991; Quemard *et al.*, 1995b; Takayama, 1979; Winder and Collins, 1970), resulting in the loss of acid fastness. This is inferred by the observation that INH decreases the amount of mycolic acids and possible intermediates, very long chain fatty acids, in *Nocardia* species (Tomiyasu and Yano, 1984), and INH inhibited the enzymatic biosynthesis of very long chain fatty acids in *M. tuberculosis* $H_{37}Ra$ (Quemard *et al.*, 1995b). Tomiyasu and Yano (1984), working with *Nocardia* species, also found that INH markedly reduced the synthesis of mycolic acids longer than C_{44} or C_{46}, specifically by inhibiting chain elongation or desaturation of the precursor fatty acids longer than C_{28} to C_{30}. However, the exact point of inhibitory action or lethal changes in mycolic acids has not been demonstrated to date. According to the observations of Winder *et al.* (1964) and others (Tomiyasu and Yano, 1984; winder and Collins, 1970), INH treatment also induced morphological changes, such as disappearance of acid-fastness or wrinkled surface structures in *Mycobacteria* and *Nocardia*. The latter also implied that INH impacts on surface structures.

Britz and colleagues (Britz, 1985; Haynes and Britz, 1989; Haynes and Britz, 1990) added INH as a component of the growth medium of coryneform bacteria species in an order to reduce cell surface barriers for DNA transformation, in respect to the known effects of INH on mycolic acid synthesis in mycobacterial species. In their experiment they grew two species of corynebacteria, *C. glutamicum* and *B. lactofermentum*, in LBG containing different concentrations of INH (0-4 mg/ml), harvested at early-exponential

phase, then transformed with homologously isolated plasmid DNA (Haynes and Britz, 1989; 1990). An increase in transformation efficiency was observed with higher concentration of INH in the growth medium, when compared to cells grown in the absence of INH (Haynes and Britz, 1990). Based on this observation, these authors suggested that mycolic acids were probably the principal physical barrier to DNA entering cells. The effect of INH on mycolic acid composition of corynebacteria was studied by Jang *et al.* (1997). These authors noticed that although *C. glutamicum* is insensitive to INH, when this was included in the growth medium at high concentrations (8 mg/ml), all of the strains tested had decreased relative proportions of $C_{32:0}$ with parallel increased proportions of $C_{34:1}$ and $C_{36:2}$ and extracellular mycolates increased to 18-20% for cell-surface mutants (MLB133 and MLB194). These authors therefore suggested that INH inhibited synthesis of shorter chained, saturated mycolic acids in all strains and also further impaired covalent binding of mycolates to the cell surface in the mutants. It was further proposed that unlike mycobacteria, where INH is thought to be converted *in vivo* to an active metabolite by the action of T-catalase, such inactivation may not occur in *C. glutamicum* but trace amount of an active form may be present in media containing INH at the high concentrations used, which causes inhibition of mycolic acid synthesis (Britz personal communication).

1.7.3 Ethionamide (ETH)

Isoniazid and ethionamide are specific tuberculosis drugs. The mode of action of the former has been reviewed several times (Iwainsky, 1988; Krishna, 1975; Takayama *et al.*, 1979; Winder, 1982), while there are only a speculative data for the latter (Winder, 1982).

ETH, presents strong bacteriostatic properties against some mycobacteria and is rather more active against INH-resistant mutants (Rist, 1960). Structurally, it is strikingly analogous to INH and a similar mode of action has been proposed for the two drugs (Winder, 1982). Similar effects on acid fastness and respiration were also recorded

(Dunbar,1957; Schäefer, 1960), and identical decreases in the "alkali-soluble carbohydrates" of the cell envelope were noted (Winder and Rooney, 1970). In both cases, the bacteria double in number before the growth stops (Rist, 1960). Moreover, as observed after INH treatment (Winder *et al.*, 1971), whole ETH-treated cells of *M. bovis* (BCG strain) showed strong inhibition of mycolic acid synthesis

However, no cross-resistance between the two drugs could be established (Winder, 1982). Quemard *et al.* (1992) determined the MICs of ETH on mycolic acid synthesis in whole cells and cell extracts of different mycobacterial species. Their studies showed that there was not a direct relationship between ETH susceptibility and mycolic acid inhibition. The presence of ETH disturbed mycolic acid synthesis in both resistant and susceptible mycobacteria. The production of oxygenated species of mycolic acid was inhibited, while that of di-unsaturated mycolic acids was either slightly altered or even increased. Moreover they found that in the presence of ETH, the unsaturated mycolic acid molecule presented a methyl end different from the usual one. Finally they suggested that the normal unsaturated mycolic acid species are not the precursor of the oxygenated mycolic acids. Moreover, they showed that ETH probably acts early in the pathway leading to oxygenated mycolic acids (Quemard *et al.*, 1991).

1.8 MOLECULAR GENETICS METHODS IN *C. GLUTAMICUM* AND RELATED SPECIES

Currently several methods for the introduction of cloning vectors and exogenous DNA into *Corynebacterium* species are available. These include protoplast transformation, transduction, electroporation, and transconjugation (Jetten and Sinskey, 1995). Early attempts to develop genetic exchange systems in corynebacteria were hampered by two barriers: the unique cell surface structure and the presence of restriction-modification systems. The latter caused low frequency of expression of exogenous DNA, particular if this came from *E. coli* or *B. subtilis* and the restriction barriers between strains of

corynebacteria were also severe (Jang, 1997; Serwold-Davis *et al.*, 1987). As this is not the subject of this thesis, this will not be reviewed further here.

1.8.1 Protoplast transformation

Most published protoplast transformation protocols are based on methods developed for *B. subtilus* and *Streptomyces* species and involve lysozyme treatment followed by PEG-stimulated uptake of DNA. The methods used in these species involved harvesting the culture at the appropriate growth stage (usually, during mid-exponential growth), incubating cells with lysozyme in order to prepare protoplasts, transformation of protoplasts with DNA and regeneration of the protoplasts (Tichy and Landman, 1969). Later glycine was added in the growth medium to make cells more sensitive to lysozyme action (Okanishi *et al.*, 1974). According to a report published by Okanishi *et al.* in 1974 higher reversion of protoplasts to the normal state was observed by incubating the protoplasts in hypertonic medium containing $MgCl_2$ (20 or 50 mM), $CaCl_2$ (50 or 20 mM), phosphate (0.22 or 0.44mM) and casamino acid (0.01%). Subsequently, the polyethylene glycol 6000 (PEG 6000) method was developed for improving DNA uptake based on its high cell fusion-inducing ability and broad applicability for various organisms (Ahkong *et al.*, 1975), including *B. subtilus* (Gabor and Hotchkiss, 1979; Schaeffer *et al.*, 1976).

The reported transformation methods for the corynebacteria are based on the treatment of protoplasts or spheroplasts with DNA and PEG. Protoplasts of *Corynebacterium* species *B. flavum* was first obtained by Kaneko and Sakaguchi (see Komatsu, 1979). After transformation, the protoplasts were regenerated in selective regeneration medium in the presence of adequate concentrations of antibiotics to select clones carrying the antibiotic resistance markers of the vector. A procedure for the production and regeneration of protoplasts of *B. flavum* and the subsequent fusion of protoplasts accompanied by

genetic recombination was initially described by Kaneko and Sakaguchi (1979). Exponentially growing cultures were grown in 0.3 units penicillin G per ml followed by lysozyme treatment in hypertonic medium, resulting in the generation of osmotically sensitive protoplasts. A modification of this method was used by Katsumata *et al.* (1984) for *C. glutamicum* and Santamaria *et al.* (1984) for *B. lactofermentum*. Santamaria *et al.* (1985) reported that the concentration of penicillin G added during growth affected not only the efficacy of protoplast formation and regeneration, but also the PEG induced DNA uptake by protoplasts. They further reported that prolonged lysozyme treatment (16 hours at 30°C) used to obtaining more protoplasts, decreased cell viability and impaired regeneration, leading to low frequencies of transformation. These authors suggested that a solution to the problem of long lysozyme treatment could be the use of mutants that form protoplasts more easily and proposed that such mutants could be isolated on the basis of their hypersensitivity to lysozyme. High frequency transformation of lysozyme-sensitive variants of corynebacteria was reported by Ozaki *et al.* (1984) and by Smith *et al.* (1986) but similar or higher frequencies have been obtained using wild-type strains (Santamaria *et al.*, 1985). Morphologically abnormal strains of *C. glutamicum* which formed protoplasts more readily than the wild type strain (ATCC 13059) have been reported (Best and Britz, 1986) although their sensitivity to lysozyme was not greatly altered.

A variety of parameters affecting the efficiency of protoplast formation, regeneration and uptake of DNA have been optimised. Santamaria *et al.* (1984, 1985) reported an efficient PEG-assisted method for transformation of *B. lactofermentum* protoplasts that used constructed shuttle vectors. Unlike *C. glutamicum* (Katsumata *et al.*, 1984), *B. lactofermentum* protoplasts were formed readily following growth in the presence of penicillin G (0.3 units) and subsequent lysozyme treatment for four hours (Santamaria *et al.*, 1984). Santamaria *et al.* (1985) found that transformation efficiency in *B. lactofermentum* depended on the concentration of DNA, the number of cells used for transformation, and the concentration and type of PEG. Under optimal conditions, 10^5 transformants were obtained per μg of DNA used per 10^9 cells treated.

A different strategy was used by Best and Britz (1984, 1986) and Yoshihama *et al.* (1985) to make *C. glutamicum* cells more sensitive to lysozyme treatment by prior growth in media containing glycine. Yoshihama *et al.* (1985) obtained osmotically sensitive cells of *C. glutamicum* after growth in the presence of 2% glycine, harvesting during stationary growth phase and treating with lysozyme for 90 min. After protoplast formation, cells were transformed in the presence of 50% PEG, regeneration medium was added, and the mixture was incubated for 3 h. Glycine was added into the growth medium and the cells were turned into osmotically sensitive cells by lysozyme treatment. These authors reported that no alterations in cell morphology was seen using light microscopy, suggesting that instead of true protoplasts cells formed spheroplasts, which were expected to have appeared as rounded cells. To obtain real protoplasts by further removal of cell wall structures, cells were further grown in the presence of cell wall biosynthetic inhibitors (cerulenin and INH) at growth-inhibitory concentrations or treating the cells with a lipase in conjunction with lysozyme treatment, but there was no success in context with the increase in transformation efficiency. However, the concentration of INH used were relatively low in comparision those used later to improve protoplast transformation (Best and Britz, 1986).

Best and Britz (1986) used glycine plus INH to produce protoplasts following lysozyme treatment, INH concentrations (5 mg/ml) was relatively high which caused substantial growth inhibition. True protoplasts and spheroplast were differentiated from each other using differential counting, which were seen under phase contrast microscopy after growth in glycine plus INH and prolonged lysozyme treatment (Britz, unpublished observation). Cell were plated on regeneration media, ET, which contained sodium succinate, gelatin, bovine serum albumin, $CaCl_2$, $MgCl_2$, sucrose and a low concentration of phosphate (Best and Britz, 1986). These researchers reported 50-70% increase in recovery of cells using ET media compared to cells regenerated on normal media such as LAG (LB [1% tryptone, 0.5% yeast extract, 0.5% NaCl] supplemented with 1% glucose and solidified with 1.2% agar, pH 7.2) or NAG (Nutrient Broth No. 2 supplemented with 1% glucose and solidified with 1.2% agar). To perform total cell counts protoplast

mixture was diluted in the osmotically protective medium then plated onto ET (protoplast, spheroplasts, damaged cells and normal cells were enumerated) or LAG (spheroplasts, damaged cells and normal cells were enumerated). In parallel, osmotically-sensitive cells were detected by dilution in water, to lyse the spheroplasts, protoplasts, and damaged cells, allowing enumeration of protoplasts from the differences in counts seen between this and the above (Britz 1985, unpublished observation). It was also noted that protoplasts of *C. glutamicum* AS019 prepared following growth in glycine-INH were transformed more readily if cells were harvested earlier in the growth phase and the efficiency fell by 80% if cells came from stationary phase cultures.

Genetic exchange using protoplast fusion has been reported for corynebacteria where PEG proved useful in the fusion of protoplasts. Karasawa *et al.* (1986) described PEG-induced fusion of protoplasts to produce a lysine auxotroph strain of *B. lactofermentum* which over-synthesised lysine and threonine.

Protoplast transformation developed for amino acid-producing *Corynebacterium* strains was based on the PEG-mediated standard procedures used for other Gram-positive bacteria (Katsumata *et al.*, 1985; Santamaria *et al.*, 1985; Yoshihama *et al.*, 1985). Once different parameters had been optimised, transformation efficiencies in the range of 10^4 to 10^6 transformants per microgram of DNA were routinely obtained. These transformation efficiencies are high enough to allow direct cloning in corynebacteria even if a 10 to 100-fold reduction in transformation occurs when using cloning vectors containing foreign DNA fragments.

1.8.2 Transduction

Tranduction was first reported in *S. typhimurium* by Zinder and Lederberg in 1952, since then tranduction has been observed for both Gram-positive and Gram-negative bacteria and their phages. Several review papers (Low and Porter, 1978; Susskind and Botstein, 1978) has been written on generalised transduction. Phage-based transduction of corynebacteria have also been the subject of few reports (Momose *et al.*, 1976; Ozaki

et al., 1984; Patek *et al.*, 1988). Momose *et al.* (1976) isolated temperate phages from coryneform bacteria. One of these phages named CP-123, transduced the *trp* marker at a frequency of approximately 10^{-6} when 1.2 M $MgCl_2$ was added to the reaction mixture. One report appeared about the construction of a functional vector system based on phages (Miwa *et al.*, 1985). These researchers constructed a cosmid vector using a DNA fragment containing the cohesive ends of a *Brevibacterium* phage. *E. coli-Corynebacterium* shuttle vectors carrying the *cos* fragment were transduced into *B. lactofermentum* and *C. glutamicum* using the intact phage. Although this system was described as being suitable for cloning genes into *C. glutamicum* and *B. lactofermentum*, but no further report about the use of this system for gene transfer has been reported.

Protoplasts were also used for transduction of *Corynebacterium* species (Ozaki *et al.*, 1984; Smith *et al.*, 1986) and *B. lactofermentum* (Karasawa *et al.*, 1986; Smith *et al.*, 1986) strains. It was reported by Ozaki *et al.* (1984) that protoplasts could be used for gene transformation. They reported the successful introduction of an *E. coli-C. glutamicum* shuttle vector into *E. coli* and protoplasts of *C. glutamicum* by transduction. The shuttle vector contained ampicillin, kanamycin and tetracycline resistance genes. The resistance phenotype, except for ampicillin, was expressed in *C. glutamicum*. Similarly, Smith *et al.* (1986) reported that protoplasts of lysozyme-sensitive *C. lilium* strains were efficiently transfected with lytic phage CS1 DNA at a frequency between 10^5 and 10^6 transfected cells per μg of CS1 DNA. However, they could not obtain transfectants when lysozme treatment was omitted.

Petek *et al.* (1988) used extended protoplast-based transfection for *B. flavum*. They prepared osmotically sensitive cells when grown in medium containing penicillin G (0.4 unit/ml), followed by lysozyme treatment (3 mg/ml). Under these conditions, they found the efficiency of transfection varied with the period of lysozyme treatment, PEG concentration and the number of competent cells used. Based on these observations, they suggested that the most suitable recipient for the transfer of phage or plasmid DNA in competent cultures of cornybacteria are cells with a partially degraded cell wall.

1.8.3 Electroporation

Instead of protoplast transformation, electroporation and transconjugation are now used as more convenient and much more efficient methods for transformation. Electroporation involves the use of a high voltage and high current to cause reversible local disorganisation and transient breakdown of the cell membrane, which allows DNA to enter the bacterial cell through the "pores" in the membrane and bring about transformation (Chang, 1992).

Transformation using electroporation has now been reported for a wide range of Gram-positive (Berthier *et al.*, 1996; Chassy and Flickinger, 1987; Foley-Thomas *et al.*, 1995; Powell *et al.*, 1988) and Gram-negative bacteria (Dower *et al.*, 1988). An early example was the stimulation of trasformation of *B. cereus* protoplasts by electric field pulses (Shivarova *et al.*, 1983) since then the electroporation technique was successfully applied to transform *Streptococcus thermophilus* (Somkuti and Steinberg, 1987), *S. lividans* protoplasts (MacNeil, 1987), *Lactobacillus casei* (Chassy and Flicking, 1987), *Campylobacter jejuni* (Miller *et al*, 1988), different strains of lactic streptococci (Powell *et al.*, 1988), *Enterococcus faecalis* protoplasts, *Pseudomonas putida* and *E. coli* (Fiedler and Wirth, 1988; Taketo, 1988).

Various groups have developed their own procedures for electroporation in *C. glutamicum* (Bonamy *et al.*, 1990; Bonnassie *et al.*, 1990; Dunican and Shivnam, 1990; Follettie *et al.*, 1993; Haynes and Britz, 1989, 1990). Normally, cells used for electroporation are grown in rich medium for 3 to 5h, harvested at early logarithmic phase, washed extensively with low-salt buffers containing 5 to 20% glycerol, and concentrated to approximately 10^{10} cells per millilitre before use. The physiological state of the cells being pulsed appears to be important. In general, cells harvested during exponential growth are electrotransformed more efficiently.

Several compounds (penicillin G, ampicillin, glycine, INH, or Tween 80) are reported to increase the efficiency of electroporation, addition of these agents in the growth medium can make the cells more permeable for exogenous DNA (Bonassie *et al.*, 1990; Follettie *et al.*, 1993; Haynes and Britz, 1990; Wolf *et al.*, 1990). A reasonably good

transformation efficiencies of 7 X 10^7 transformants per μg of DNA with *C. glutamicum* strain AS019 and plasmid pHY416 without using any cell wall-weakening agents has been reported by Dunican and Shivnan (1989). In an experiment, they harvested cells at an A_{600} of 0.6, washed with 10% glycerol twice, suspended in 10% glycerol before storage at -70°C. Subsequently 40 μl aliquots of the cells were mixed with 0.1 to 1μg of DNA and subjected to field strengths of 12.5 kV/cm for about 3 to 8 msec. Thereafter the cells were immediately mixed with 0.9 ml of an isotonic medium, incubated for 1 to 1.5 h then plated onto appropriate media. Transformants generally appear within 48 h. Freezing prior to electroporation may have altered cell surface integrity. However, many other workers reported that such a high transformation efficiency is not normally obtained when cells are grown without using cell wall-weakening agents, and addition of glycine (2-2.5 %, w/v) to cultures improved the transformation efficiency about 10 to 100 times (Haynes and Britz, 1990; Noh *et al.*, 1990).

Haynes and Britz (1989) electroporated *C. glutamicum* strain AS019 after growth in LBG supplemented with 2.5%, w/v, glycine plus 4mg/ml INH plus 0.1% Tween 80 and obtained 4 x 10^7 transformants per ug DNA (using small BioRad cuvettes) per 10^9 cells electroporated, transformants were recovered on ET media. This was about four orders of magnitude higher than efficiencies obtained for cells grown in LBG. In their attempts of improving electrotransformation efficiencies, Haynes and Britz (1990) reported a transformation frequency of 5 x 10^5 transformants per μg DNA with *C. glutamicum* strain AS019 when grown in LBG supplemented with 2.5% (w/v) of glycine and 4 mg/ml of INH (using large BioRad cuvettes). There was 10 to 100-fold improvements on using the protoplast transformation of the same strain of *C. glutamicum* grown similarly. In the presence of INH alone, considerable improvement in electrotransformation efficiencies were noticed only when concentrations were very high (>5mg/ml). Haynes and Britz (1990) further reported that the recovery medium on which the transformants were isolated and the diluents used following electroporation had a significant effect on the number of transformants obtained, as observed for protoplast transformation (Britz, unpublished, 1985). The osmotically-protective medium (ET) gave at least 70% higher numbers of transformants (in terms of transformation efficiency) than those found on

non-osmotically protective medium after using hypertonic diluents following electroporation, suggesting that electroporation after growth in glycine/INH caused cell damage and made them osmotically fragile.

Bonnassie *et al.* (1990) electroporated on *B. lactofermentum* after growing in LB, incubating to early-exponential growth phase before adding ampicillin (0.5 to 1.5 μg/ml) then harvesting at A_{540} of 0.3 to 0.5. Subsequently, cells were electroporated with homologously-isolated DNA at 6.25 kV/cm (25μF capacitor). Pre-treatment with ampicillin allowed electrotransformation of the cell whereas no transformants were obtained without pre-treatment. Haynes and Britz (1989) reported the improved electrotransformation frequencies of *B. lactofermentum* after growth in Tween 80 alone or in combination with glycine and INH.

Electroporation was used to transform (Noh *et al.* 1991) *C. glutamicum* JS231, a strain derived from ATCC 13032, these workers grew the cells in LG medium (1% w/v, tryptone, 0.5%, v/v, yeast extract, 0.5 w/v, glucose 1%, w/v, NaCl, pH 7.0) supplemented with 0.3 U/ml of penicillin G and showed about 10 times higher transformation efficiencies than those for cells grown in medium without penicillin G. This approach paralleled the earlier work of Kaneko and Sakaguchi (1979) in *B. lactofermentum*.

1.8.4 Conjugation

Another very efficient means for the introduction of DNA into *Corynebacterium* is by transconjugation. Conjugation is a mean of DNA transfer from donor to recipient bacteria by a mechanism involving cell to cell contact. This process is usually mediated by conjugative plasmids, which have been isolated from a diverse range of Gram-positive and Gram-negative bacteria. Plasmids of some incompatibility groups are capable of conjugal transfer or immobilization and stable maintenance in almost all Gram-negative and even Gram-positive bacteria. They are called broad host range plasmids and belong mainly to the compatibility groups IncC, IncN, IncP, IncQ and IncW (Datta and Hedges, 1972; Olsen and Shipley, 1973; Wilkins, 1990).

Conjugal transfer of broad-host-range IncP-type resistance plasmids within the Gram-negative bacterial species is well known (Beringer, 1974; Datta and Hedges, 1972; Olson and Shipley, 1973). Non-self-transmissible plasmids carrying the appropriate origin of transfer (*oriT*) can be mobilised by IncP plasmids (Willetts and Crowther, 1981). Recent studies have shown that conjugation is a non-specific process and accounts for most horizontal gene transfer between even phylogenetically remote organisms (Buchanan *et al.*, 1987; Mazodier *et al.*, 1989; Trieu-Cuot *et al.*, 1987, 1988). Conjugation mediated by the broad-host-range (bhr) plasmid RP4 (Datta and Hedges, 1971) is suitable for transfer of plasmid DNA from *E. coli* to a wide range of Gram-negative bacteria and also at high efficiency into several Gram-positive genera, like *Streptococcus*, *Bacillus*, *Staphylococcus*, *Mycobacterium*, *Streptomyces*, *Corynebacterium*, *Brevibacterium*, *Arthrobacter* or *Rhodococcus* .

Unlike transformation and electroporation, conjugation is a highly efficient way to introduce foreign DNA into wild-type *C. glutamicum* strains, which seem to have a rather strong restriction system (Martin *et al.*, 1987; Schäfer *et al.*, 1990; Thierbach *et al.*, 1988). Schäfer *et al.* (1990) reported the mobilization of shuttle plasmids from Gram-negative *E. coli* to Gram-positive corynebacteria by P-type transfer functions. This strategy is based on the *oriT* and plasmid transfer (*Tra*) function of IncP-type broad-host-range plasmid RP4 (Datta *et al.*, 1971) and consists of *E. coli* mobilising strains (*E. coli* S17-1) and derivatives of conventional *E. coli* vectors (pSUP vectors[Simon and Pühler, 1983; Simon *et al.*, 1986]). Conjugation between *E. coli* and corynebacteria is performed using nitrocellulose filters to concentrate cells. This system was applicable to a wide range of different *C. glutamicum* strains (Schäfer *et al.*, 1990). Transfer frequencies up to 10^{-2} per final donor colony, with 10^{7} to 10^{8} transconjugants per mating assay, were obtained. Restriction systems in coryneform bacteria impairing intergeneric conjugation have been shown to be stress sensitive and can be inactivated by short stress treatments (Schäfer *et al.*, 1990 and 1994). The recipient cells can be rendered more fertile by applying heat treatment, which consisted of incubating *C. glutamicum* ATCC 13032 at 49°C for approximately 9 min (Schäfer *et al.*, 1994a). Thereafter, non-growing cultures remained in a "competent state" for several days. When transferred to fresh

medium and incubated at optimal growth temperature and aeration, the cells lost their high fertility within a few hours. Conjugation frequency was increased by 10^4-fold after heat treatment of host cells. Schäfer *et al.* (1996) observed high frequency plasmid transfer when the restriction system of the recipient was mutated. This problem can also be overcome by the use of restriction-deficient host strains, or exposing the cells to environmental stress such as pH, ethanol or SDS (Bonassie *et al.*, 1990; Follettie *et al.*, 1991; Liebel, 1991; Schäfer *et al.*, 1990, 1994).

Vertes *et al.* (1994a) described the presence in *C. glutamicum* of the element IS*31831* from ATCC 31831 and later used it to construct two artificial transposons (Tn*31831* and mini-Tn*31831*) for mutagenesis of corynebacteria by conjugation. Resulting mutagenesis produced a variety of mutant strains of *B. flavum* at an efficiency of 4.3 X 10^4 mutants per μg DNA. A new IS element (IS*1206*) from *C. glutamicum* was described by Bonamy *et al.* (1994), and a different one (IS*13869*) has been isolated from *B. lactofermentum* (Correia and Martin, 1996). In addition, an extensive survey on the occurrence of IS elements in coryneform bacteria, conducted with an IS entrapment vector based on the *B. subtilis sacB* gene, indicated that at least three different classes of IS elements are present in coryneform bacteria (Jager and Pühler, 1995). A transposable element from *C. xerosis* was also shown to be active in *C. glutamicum* after introduction of the delivery vector by intergeneric conjugation (Tauch and Pühler, 1995).

Numerous observations made during transformation and transconjugation experiments indicate the presence of restriction or modification system in *Corynebacterium* strains (Follettie *et al.*, 1991; Liebl, 1991; Wohlleben *et al.*, 1992). The problems caused by these systems for cloning experiments can be overcome to some extent by the use of restriction-deficient host strains, or by stress (Bonassie *et al.*, 1990; Follettie *et al.*, 1991; Liebl, 1991; Schäfer *et al.*, 1990, 1994)

1.8.5 Mobilisable vectors: gene disruption and replacement

A special class of integration vectors has been developed and is now very frequently used for gene disruption and gene replacement experiments in *Corynebacterium* (Schäfer *et al.*, 1990; Schwarzer and Pühler, 1991). These so-called suicide vectors lack a functional

replicon, and can therefore only be maintained when integration occurs *via* homologous recombination into the chromosome. To employ these vectors, very efficient DNA transfer *via* transconjugation or electroporation is required.

Disruption of a chromosomal gene by integration of a vector plasmid with an internal fragment of a well characterised or sequenced gene is a rapid way to analyse its function. Three methods provide a means of constructing a mutation *in vitro* in a cloned gene and reintroducing this mutation at the correct chromosomal site. These techniques are: integrative disruption, one-step gene disruption and transplacement.

Integrative disruption (Shortle *et al.*, 1982) generates a deletion in the chromosomal copy of a cloned gene. In this method, an internal fragment of the target gene is used. Disruption of a cloned gene is introduced into the chromosome on an integrating plasmid. This generates a gene duplication, but neither copy of the gene is intact copy: one copy is missing the 3′ end of the gene and one copy is missing the 5′ end.

Like integrative disruption one-step gene disruption (Rothstein, 1983) generates a gene disruption in one step *via* transformation, using a fragment of DNA containing a cloned gene that is disrupted by a selectable genetic marker. Homologous recombination between free DNA ends, which are highly recombinogenic, and homologous sequences in the genome result in replacement of the wild-type gene by the disrupted copy. The disrupted gene can contain either a simple insertion (of the selectable marker) or a deletion/insertion mutation. Introduction of this disruption into the genome can be achieved in a single step, resulting in stable, non-reverting mutations.

The third technique, transplacement is more generally applicable: it can be used to introduce insertion or deletion mutations containing a selectable marker, but it can also be used to introduce non-selectable mutations, such as conditional lethal mutations in an essential gene.

Gene disruption and replacement have been described for a wide range of prokaryotes such as Gram-negative *Rhizobia* (Simon *et al.*, 1983), Gram-positive *B. subtilis* (Ferrari *et al.*, 1983) and *Streptomycetes* species (Charter and Bruton, 1983). Several systems

were developed to force homologous recombination between vector-born and chromosomally located gene copies using non-replicative plasmids (Simon *et al.*, 1986), unstable replicating vectors (Weber and Losick *et al.*, 1988), plasmids with temperature-sensitive replicons (Muth *et al.*, 1989), an attachment site deleted phage or plasmid curing by protoplast formation (Anzai *et al.*, 1988). Insertional mutagenesis by illegitimate recombination was observed in *R. fascians* (Desomer and Montagu, 1991) and in mycobacteria (Kalpana *et al.*, 1991). However, in both cases, the plasmids used contained host DNA fragments. Marklund *et al.* (1995) described the experimental system used to study DNA recombination events in the *M. intracellulare*. First, an integrative plasmid was introduced into *M. intracellulare* 1403. A non-replicative, non-integrative plasmid having homology with the integrated plasmid was then introduced and the resultant recombinants were analysed to distinguish between the events of homologous and illegitimate recombination. No illegitimate recombination occurred; in all recombinants, a single crossover between homologous regions of the two plasmids was noted. During subsequent growth of a recombinant clone, a spontaneous deletion occurred that resulted in a gene replacement on the chromosome of *M. intracellulare* 1403. This gene-exchange protocol can be used for knockout mutagenesis, site-specific gene manipulation, or targeted introduction of a reporter gene such as *lux* (e.g. see Barletta *et al.*, 1992, and Boyer *et al.*, 1969).

Recently a special class of integration vectors has been developed for corynebacteria, where these are called suicide vectors as they lack a functional corynebacterial replicon, and can therefore only be maintained when integration occurs through homologous recombination into the chromosome.

During plasmid transfer experiments carried out to evaluate the applicability of known transposons in coryneform strains, it was discovered that the *E. coli* mobilizable plasmids pSUP 1021, pSUP2021 (Simon *et al.*, 1986), pSUP301 (Simon *et al.*, 1983) and pK18mob (Schäfer *et al.*, 1994), all of which are based on classical narrow-host range plasmids and carry the *oriT* of plasmid RP4, become integrated into the genomes of different corynebacteria. These plasmids are mobilised by *E. coli* S17-1 (Fig 1.11),

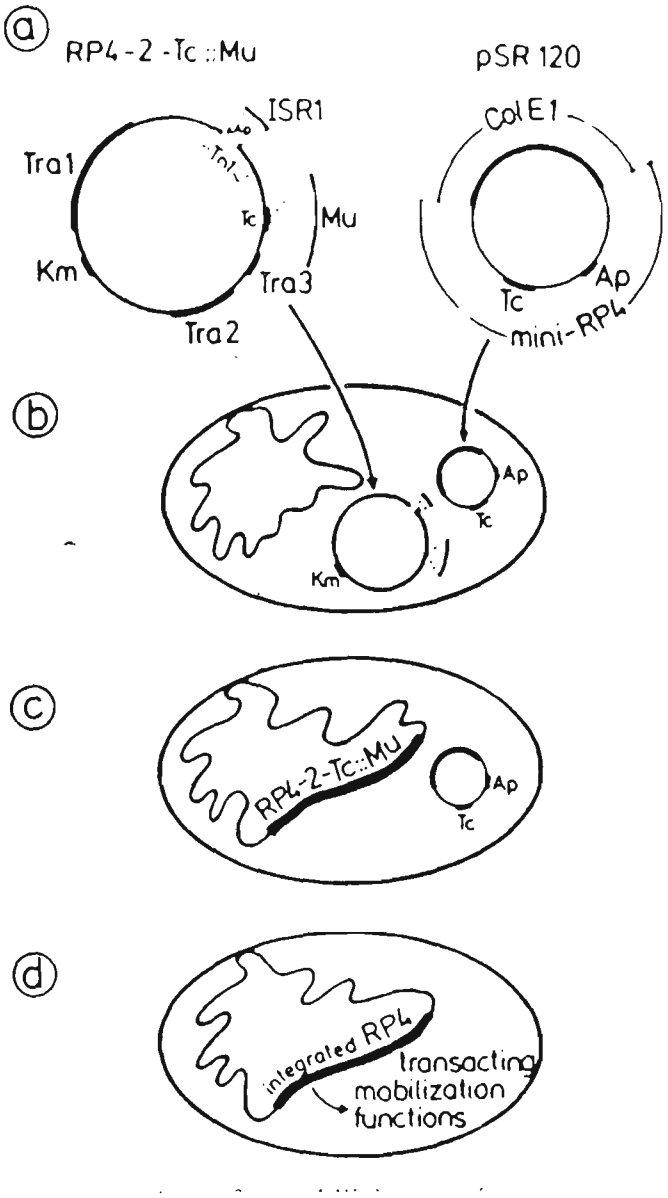


Figure 1.11 Construction of mobilizable strains of *E. coli* S17-1 (a) RP4-2-Tc::Mu is kanamycin resistant (Km^r) and self-transmissible (Tra^+) (Simon and Pühler *et al.*, 1983). It carries the insertion element ISR1 within the transposon $Tn1^{35}$ deletion resulting in the ampicillin sensitive (Ap^s) phenotype. The Tc resistance is inactivated by insertion of the bacteriophage Mu genome. The plasmid pS120 is a fusion product between two functional replicons, ColE1 (thick line) and a mini-RP4 (thin line). The mini-RP4 is devoid of the Km resistance and most of the Tra genes, but it still confer resistance to Ap and Tc. (b) The two incompatible RP4 derivatives were introduced into a *recA*$^-$ cell of *E. coli* by transduction. (c) Co-selection for Km and Tc resistance led to spontaneous integration of the RP4-2-Tc::Mu into the chromosomes. (d) The plasmid-free mobilizing strain was isolated in curing experiments.

which harbours a derivative of the self-transmissible plasmid RP4 integrated into the chromosome. The natural resistance of *C. glutamicum* to nalidixic acid and the reliable loss of the vector, which is unable to replicate in the new host, facilitates the identification of the new host, with the foreign DNA integrated into their genome *via*homologous recombination (Schäfer *et al.*, 1994). Special strains with defined deletion mutations, which lack all vector and antibiotic resistance marker sequences can be constructed by combining the techniques of gene disruption and replacement. Strains constructed in this manner are very likely to be considered safe with respect to environmental release or industrial use. Several examples of the application of these techniques are summarised in Table 1.2 (Jetten and Sinskey, 1995).

Mateos *et al.* (1996) reported the conjugative transfer of mobilizable derivatives of the *E. coli* narrow-host-range plasmids pBR322, pBR325, pACYC177, and pACYC184 from *E. coli* to species of Gram-positive genera *Corynebacterium* and *Brevibacterium,* resulted in the integration of the plasmids into the genomes of the recipient bacteria. Transconjugants appeared at low frequencies and reproducibly, with a delay of two to three days in recovering transconjugants compared with matings using replicative vectors. Southern analysis of corynebacterial transconjugants and nucleotide sequences from insertion sites revealed that integration occurred at different locations and different parts of the vector were involved in the process. Integration was not dependent on an indigenous insertion sequence element but resulted from recombination between very short homologous DNA segments (8 to 12 bp) present in the vector and host DNA. Classical *recA*$^-$ mediated recombination and site-specific integration were not possible because integration had occurred at different sites. Therefore these authors postulated that integration was mediated by an enzyme that recognises very short homologous sequences.

Schwarzer and Pühler (1991), developed a system for the genetic manipulation of the amino acid-producing *C. glutamicum*. Gene disruption and replacement was achieved by introducing, *via* conjugation, *E. coli* vector plasmids carrying the manipulated *C. glutamicum* DNA fragments. Using this system, stable mutants were obtained in which the chromosomal *lysA* gene, encoding *meso*-diaminopimelate decarboxylase, was

interrupted by a chloroamphenicol resistance cartridge, or one in which an essential internal part of the *lysA* gene was deleted. The deletion mutants retained neither antibiotic resistance marker nor vector sequences. This strategy is generally applicable to the construction of industrial strains to be used in the fermentation industry.

By using the plasmid pK18*mobsacB*, Schäfer *et al.* (1994) created deletions of the *hom-thrB* genes in the chromosome of *C. glutamicum*. The genes encoding enzymes homoserine dehydrogenase (*hom*) and homoserine kinase (*thrB*) involved in the biosynthesis of threonine and methionine constitute an operon. A 3-kb fragment containing the *hom-thrB* operon was cloned into pK*18mobsacB*. A central one Kb *PstI* fragment comprising the C-terminal 236 amino acid of *hom* and the 113 N-terminal amino acids of *thrB* was deleted and the resulting plasmid pAT20 was transferred by intergeneric conjugation from *E. coli* S17-1 to *C. glutamicum*. Since pAT20 is unable to replicate in *C. glutamicum*, transconjugant could only arise after integration of pAT20 into the chromosome by homologous recombination.

Moreover, studies of Schäfer *et al.* (1990) on the transfer of shuttle vectors to various strains of coryneform bacteria revealed efficient plasmid transfer into several related species of *C. glutamicum*. Preliminary experiments showed that gene disruption experiments with p301 *lys-int* led to lysine defective mutants in the closely related strains *B. flavum* DSM 20411 and *A. albidus* DSM 20128 (Schwarzer and Pühler, 1991).

Table: 1.2 Genes disrupted or integrated *via* transconjugation in *C. glutamicum* (Jetten and Sinskey, 1995).

Target gene	Disrupted or Integrated	Phenotype	Ref
ask	Integrated	Increased internal lysine	Broer and Kramer, 1983
	Disrupted	No lysine production	Schrumpf *et al.*, 1992
	Integrated	Increased lysine production	Jetten *et al.*, 1994a
cspA	Disrupted	No S-layer formation	Payert *et al.*, 1993
dapE	Disrupted	Decreased lysine production	Wehrman *et al.*, 1983
ddh	Disrupted	Decreased lysine production	Schrumpf *et al.*, 1991
dgh	Disrupted	No apparent effect	Labarre *et al.*, 1993
gltA	Disrupted	No apparent effect	Reyes *et al.*, 1991
hom^{dr}-*thrC*	Integrated	Increased threonine production	Reinscheid *et al.*, 1994
ilvA	Disrupted	Isoleucine auxotrophy	Boles *et al.*, 1993
	Disrupted	No threonine degradation	Jetten, Unpublished
lysA	Disrupted	Lysine auxotrphy	Schwarzer and Phueler, 1991
ppc	Disrupted	No apparent effect	Gubler *et al.*, 1994a
pyK	Disrupted	Decreased lysine production	Gubler, 1994

1.9 AIMS AND OBJECTIVES OF THE THESIS

Because of the great importance of *C. glutamicum* in fermentation industry and its potential for application in foreign protein expression, research has been focussed mainly on strain improvement and development of techniques to overcome the barriers which affect successful introduction of foreign DNA into these corynebacteria. While investigating the physical and biochemical barriers to efficient gene transfer in these organisms, a series of auxotrophic mutants of *C. glutamicum* strain ATCC 13059 were isolated which protoplasted more readily than their parent strain and which showed morphological changes (Best and Britz, 1986) and hypersensitivity to both glycine and INH. Previously in our laboratory, studies were focussed on the analysis of lipids under standard growth conditions by these mutants (Jang *et al.*, 1997). A key finding of these studies was that the proportion of extracellular mycolic acids, and solvent-extractable mycolates, was higher for the mutants and growth in glycine and INH greatly increased the proportion of extracellular mycolates for the mutants and the parent. These results suggested that these agents exert effects in addition to those previously reported, where these may be related to the attachment of the mycolates to the cell surface. This project is concerned with defining the nature of the mutations which give rise to hypersensitivity to INH and glycine by studying cell wall composition, and gaining insight into the genetic and biochemical mechanisms of mycolic acid synthesis in *C. glutamicum*, noting that this pathway has not been characterised for this species previously and similar pathways in other genera are still largely described only in models.

The specific aims of the projects were

1- To determine whether an *inhA* gene equivalent occurs in *C. glutamicum,* and characterise this gene through sequence analysis.

2- To define any genetic changes which may have occurred in INH-hypersensitive mutants through sequence analysis of the *inhA* gene in these mutants in comparison with the sequence determined for the wild-type *C. glutamicum*.

3- To characterise and analyse the sequence of the *inhA* gene in closely related species of *C. glutamicum* such as *B. lactofermentum* and *B. flavum,* and to compare of the sequence with the *C. glutamicum* gene.

4- To compare the *inhA* gene sequence of *C. glutamicum* and related species with those of *Mycobacterium* species.

5- To determine the role of the presumptive *inhA* gene in mycolic acid biosynthesis in *C. glutamicum* using insertional inactivation approaches and evaluating the impact on fatty acid and mycolic acid production, for both cell-associated and extracellular mycolic acids.

6- To examine the effect of growth medium containing glycine, INH or glycine plus INH on the mycolic acid and fatty acid composition of the cell wall and compare this with the whole cell mycolates of wild-type and mutants of *C. glutamicum.*

Chapter 2

Materials and methods

2.1 MATERIALS

2.1.1 Bacterial strains and plasmids

The corynebacteria, mycobacteria and *E. coli* strains and plasmids used in this study are shown in Tables 2.1 and 2.2.

2.1.2 Chemicals

The sources of all chemicals and media are listed in Appendix 1 and abbreviations used for these are listed on page. All reagents, where not specified, were analytical grade and supplied by BDH (UK) or Sigma (USA). Distilled water was prepared using the Milli-RO Water Purification System or deionised water using a Milli-Q Ultrapure Water System. Amino acids and vitamin stock solutions were prepared at 50 mM in sterile distilled water and sterilised by filtration (0.45 μm, Millipore). Sources of other materials and media are otherwise given in the text describing their use or preparation or in appendices.

2.1.3 Buffers

All buffers were made up as previously described by Dawson *et al.* (1986), unless otherwise stated. The pH of each buffer was checked on an Orion model 410A pH meter and adjusted with appropriate solutions of salts, acid (10 M HCl) or base (10 M NaOH) as needed before use. All buffers were prepared in glass with Milli-Q (Millipore) water and sterilised by autoclaving at 121°C for 20 min unless otherwise stated. Sugar components in buffers were added from stock solutions of 50% (w/v) sucrose and 20% (w/v) glucose sterilized by autoclaving at 109°C for 25 min.

Table 2.1 Bacterial strains used in this work

Strains	Relevant characteristics[a]	Source or Reference
C. glutamicum		
AS019	Spontaneous Rif^r mutant of ATCC 13059	Yoshihama *et al.*, 1985
MLB133	Mutants of ATCC 13059, ilu$^-$, leu$^-$, Rif^r	Best and Britz, 1986
MLB194	Mutants of ATCC 13059, ileu$^-$, leu$^-$, Rif^r	Best and Britz, 1986
RES467	*csp*1 gene knock out	Dr. Kalinowski
B.lactofermentum[c]	ser$^-$	ATCC 21789[b]
BL1		
B. flavum		
BF4	met$^-$, thr$^-$	Rogers, University of New South Wales, Australia
M. smegmatis		
mc^{2}155		Jacob *et al.*, 1994
E. coli strains		
JM109	*recA1 supE44 endA1 hsdR17 gyrA96 rel A1 thi* Δ(*lac-proAB*)F'(*traD36 proAB*$^+$ *lacI*^{q} *ΔM15*)	Promega, Yanisc-Perron *et al.*, 1985
DH5α	F$^-$ *endA1 hsdR17*($r_k^-m_k^+$) *supE44 thi-1recA1 gyrA1 relA*1 80d*lacZ*ΔM15 Δ(*lacZYAargF*)*U169*	Bethesda Research Laboratories, Gaithersburg, Md, USA
S17-1	[*rec*$^-$, *m*$^+$][mobilizing donor strain MM294*recA* with an RP4 derivative integrated into the chromosome]	Simon *et al.*, 1991
Top10F'	F' {*lacI*^{q} Tn10 (Tet^R)} *mac*A Δ (mrr-*hsd***RMS**-*mcr***BC**) Φ80*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rsp*L (Str^R) *endA*1 *nup*G	Invitrogen

[a] leu$^-$ (leucine), ileu$^-$ (isoleucine), met$^-$ (methionine), ser$^-$ (serine), thr$^-$ (threonine) auxotrophy;

Km^r (Kanamycin), Rif^r (rifampicin) resistance.

[b] ATCC = American Type Culture Collection, Rockville, U. S. A.

[c] *B. lactofermentum* was renamed as *C. lactofermentum* by Amador *et al.*, (1999) based on their 16S rRNA studies but in this thesis the name *B. lactofermentum* will continue to be used as originally obtained by ATCC culture collection.

Table 2.2 List of plasmids used in this study

Plasmid	Characteristics[a]	Source or Refrence
pBluescript II SK+/-	3.9 kb Amp^r cloning vector	Stratagen
PCR ®2.1	3.9 kb TA Km^r cloning vector	Invitrogen
PECMΔ	*B. ammoniagenes* suicide vector derived from pECM1 by elimination of a 2.4 kb *Sph*1 fragment. Km^r, Cm^r	Shin-Jae Ph.D student in the University,of Melbourne, Australia
pSUP301	pACYC177-mob, Km^R, Amp^R	Simon *et al.*, 1994
p301*lysA*-int	pSUP301 plasmid containing a 990 bp *Hinc*II-*Eco*RV fragment of pTG1225 carrying an internal part of *C. glutamicum lysA* gene into *Sca*I site.	Schwarzer *et al.*, 1991
pK18*mob*	3.8 kb *E. coli* mobilizable vector (*mob* of plasmid RP4); pBR325 replicon; Km^r *lacZ*	Schäfer *et al.*, 1994

[a] Resistance to particular antibiotics: Amp^r (ampicillin), Km^r (Kanamycin), Cm (chloroamphenicol)

Lysis buffer (Best and Britz, 1986), pH 8.0, was used for plasmid and chromosomal DNA extractions in corynebacteria and contained 10 mg lysozyme/ml, 10 mM Tris pH 8.0, 0.7 M sucrose, 10 mM $MgCl_2$. $6H_2O$, 10 mM $CaCl_2$, 0.05% KH_2PO_4, 0.01% bovine serum albumin (BSA). BSA and lysozyme solutions were prepared freshly before use and filtered sterilized (0.25 μm, Millipore filters).

SMMC buffer (Yoshihama *et al.*, 1985), pH 6.5, was used as diluent for viable cell counts before and after electroporation and contained 0.7 M sucrose, 50 mM maleic acid, 10 mM $MgCl_2.6H_2O$ and 10 mM $CaCl_2$.

Veronal buffer was prepared by dissolving 572 mg veronal, 375 mg sodium veronal, 27 mg $CaCl_2$ $2H_2O$ and 109 mg $MgSO_4$ $7H_2O$, in 1L distilled water and pH adjusted to 7.5.

Maleic acid buffer contained 0.1 M maleic acid, 0.15 M NaCl and the pH adjust with NaOH (solid or 10 M stock solution) to pH 7.5 (20°C) and stored at room temperature.

The following buffers were prepared as described by Sambrook *et al.* (1989).

Denatured salmon sperm DNA was prepared in sterile H_2O at a concentration of 1 mg/ml denatured at 95°C for 10 min and chilled on ice prior to use.

20X SSC solution was made by dissolving 175.3 g of sodium chloride and 88.2 g of sodium citrate in one litre of H_2O. The pH was adjusted to 7.0 with the addition of NaOH. The stock solution was stored at room temperature.

TAE buffer (50X), pH 7.8, contained 200 mM Tris, 100 mM sodium acetate (NaOAc) and 10 mM ethylenediamine tetraacetic acid (EDTA). A 50X concentrated stock was diluted in distilled water to 1X TAE prior to use in agarose gel electrophoresis.

TE buffer, pH 8.0, contained 10 mM Tris and 1 mM EDTA. This was used for dissolving, diluting and storing DNA, particularly for *E. coli* DNA.

Two types of restriction enzyme buffers were used from 10X stock solutions as follows: One-Phor-All buffer (Pharmacia) and specific commercial buffers from suppliers of restriction enzymes. 10X One-Phor-All buffer, pH 7.5, contained 100 mM Tris-acetate, 100 mM magnesium acetate and 100 mM potassium acetate.

Protein gel loading buffer (2X) contained 0.3 g of dithiothreitol (DTT, Sigma), 4.0 ml of 10% sodium dodecyl sulphate (SDS), 1.6 ml of 1.25 M Tris-HCl (pH 6.8), 2.5 ml of 87% glycerol, 0.5 mg of bromophenol blue and was made up to 20 ml with Milli-Q water. This was stored at $-20^{\circ}C$ in 1ml aliquots.

Electrode buffer (10X) for SDS-polyacryamide gel electrophoresis (SDS-PAGE) contained 30.3 g of Tris base, 144.2 g of glycine and 10.0 g of SDS and was made up to 1 litre with Milli-Q water. The pH was not adjusted and the solution was stored at room temperature. The 10X concentrated stock solution was diluted in Milli-Q water to 1X electrode buffer before use in SDS-PAGE.

2.1.4 Instrumentation

An Eppendorf centrifuge was used for samples of volume less than 1.5 ml. For samples greater than 1.5 ml, a Beckman model J2-HS refrigerated centrifuge was used. For higher speeds, a Beckman model L-70 ultracentrifuge with 70.1 Ti rotor was used. An Ultraspec model spectrophotometer (LKB) was use to measure absorbance in the range between 260 and 680 nm. Liquid cultures were grown in orbital shaker incubators (model 013422, Panton Scientific Pty, Ltd). A Dynavac mini-freeze dryer was used for lyophilising samples. Other general analytical instrumentation is described in the specific methods sections.

2.2 MICROBIOLOGICAL METHODS

2.2.1 Media

Unless specified, all media were sterilised by autoclaving for 15 min at 121°C. Media additives were stored in brown-coloured bottles at room temperature. Supplements, such as antibiotics and amino acids, were added aseptically from stock solutions to the medium at 50°C, immediately prior to dispensing the medium.

Luria Broth (LB) (Miller, 1972), pH 7.2, was used for growth of *E. coli* strains and contained 10 g of tryptone, 5 g of yeast extract and 5 g of sodium chloride per litre. For plates, LB was solidified with 15 g/l of agar (LA).

Luria broth with glucose (LBG) was routinely used for corynebacterial growth (Best and Britz, 1986). LB was supplemented with 0.5% (w/v) glucose from a 20% (w/v) stock solution. For plates, LBG was solidified with 15 g/l of agar (LAG).

LBG supplemented with glycine (LBG-G) was used for DNA isolation, electrotransformation, and qualitative analysis of mycolic acid composition from corynebacteria (Haynes and Britz, 1989). Due to differential sensitivities of strains to glycine, the final concentrations of glycine in LBG-G varied from experiment to experiment depending on the strain used but usually the concentration was 2% (w/v). For the LBG-G (2% glycine), 2X LB was supplemented with 0.5% glucose and glycine from a sterile stock solution (20% w/v glucose and 20% w/v glycine) and the volume was adjusted with sterile water to produce 1X LB. Glycine stock solution often precipitated during storage and therefore crystals were redissolved by warming at 65°C prior to use.

LBG supplemented with INH (LBG-I) was used for qualitative analysis of mycolic acid composition of corynebacteria (Haynes and Britz, 1989). Final concentrations of INH in the LBG-I varied from experiment to experiment but usually the concentration was 4% (w/v). For LBG-I (4 mg/ml INH), 2X LB was supplemented with 0.5% glucose and INH from sterile stock solutions (20% [w/v] glucose and 100 mg/ml INH) and the volume was adjusted with sterile water to produce 1X LB.

LBG supplemented with glycine plus INH (LBG-GI) was used for quantitative analysis of mycolic acid composition of corynebacteria. Corynebacteria were grown in this medium before electroporation, conjugation and for DNA isolation. Final concentrations of glycine and INH in LBG-GI varied from experiment to experiment but usually the concentration was 2% (w/v) glycine and 4 mg/ml INH and this was prepared as described above for LBG-G and LBG-I.

M-ADC-TW broth was used routinely for the growth of mycobacteria and contained 4.7 g of Middlebrook 7H9 broth (Difco) base and 2 ml of glycerol dissolved in 900 ml of deionised water and autoclaved for 20 min. After cooling, 100 ml of albumin-dextrose complex (ADC) enrichment and 2.5 ml of 20% Tween 80 solution were added. ADC enrichment was made by dissolving 2 g glucose, 5 g BSA (Sigma), and 0.8 g of NaCl in 100 ml deionised water. This solution was filtered sterilised and stored at 4°C. Tween 80 solution was made by dissolving 20 ml of polyoxyethylene sorbitan monooleate to 80 ml deionised water, heating at 55°C for 30 min to dissolve completely, sterilising by filteration (0.45 μm, Millipore).

Middlebrook 7H10 agar was used for growth of mycobacteria on plates. This was made by dissolving 19 g Middlebrook 7H10 agar in 900 ml deionised water, autoclaving for 20 min and allowing to cool to 55°C. ADC enrichment (100 ml) was added then cycloheximide was added to give a concentration of 50 μg/ml (to inhibit mold contamination) before the media was poured as plates. Plates contained 25-30 ml of medium.

Nutrient agar (NA) with glucose (NAG) was prepared by supplementing sterile NA (25g Oxoid Nutrient Broth no. 2 and 15 g agar per litre) with 0.5% (w/v) glucose.

ET medium (Best and Britz, 1986) was used for viable counts of corynebacteria and this had the following composition (per litre): mixture A in 590 ml solution (yeast extract, 5g; tryptone, 5g; K_2SO_4, 0.25g; gelatin, 5g; agar, 12g); mixture B in 300 ml solution (succinic acid, 35.7 g, pH adjusted to 7.2 with 5N NaOH before sterilising); Tris base, 3.94 g (pH adjusted to pH 7.2 with HCl); glucose 10 g; $CaCl_2$, 1.47 g; $MgCl_2.6H_2O$, 2.03

g; KH_2PO_4, 50 mg; BSA, 0.1 g (prepared as a 2% solution, filter sterilised); trace elements solution (which contained per litre: $ZnSO_4.7H_2O$, 8.8 g; $FeCl_3.6H_2O$, 0.97g; $CuSO_4.5H_2O$, 0.27g; $Na_2B_4O_7.10H_2O$, 88 mg; $(NH_4)_6Mo_7O_2$. H_2O, 37 mg; $MnCl_2.4H_2O$, 72 mg). To prepare ET medium, sterile mixture A and B were combined with gentle mixing and supplemented with the following sterile stock solutions in the following order: 25 ml of Tris (1M); 50 ml of 20% (w/v) glucose; 5 ml of 2% (w/v) BSA; 2.5 ml of 2% (w/v) KH_2PO_4; 10 ml of 1M $MgCl_2.6H_2O$; 10 ml of 1M of $CaCl_2.2H_2O$; 1 ml of trace elements.

Minimal medium for corynebacteria (Britz, personal communication) contained the following composition (per litre): $(NH_4)_2SO_4$, 1.5 g; urea, 1.5; KH_2PO_4, 1.0 g; K_2HPO_4, 3.0 g; $MgSO_4.7H_2O$, 0.1 g; $CaCl_2.2H_2O$, 1.0 mg; biotin, 100 µg; thiamine hydrochloride, 100 µg; trace elements solution (as for ET), 1.0 ml, agar 15 g. After sterilisation at 121°C for 15 min, the basal medium was supplemented with appropriate amino acids (100 mg/l) before dispensing as plates.

X-Gal medium was prepared in LA containing ampicillin/IPTG/X-Gal. One hundred µl of 100mM IPTG (isopropyl-β-D-thiogalactoside) and 20 µl of 50 mg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-galactoside, dissolved in 1 ml of N, N'-dimethyl formamide) was spread over the surface of LA containing 100 µg/ml ampicillin and allowed to absorb for 30 min prior to use.

SOB (Sambrook *et al.*, 1989) was used for DNA transformation into *E. coli* and one litre of SOB contained 20 g of tryptone, 5 g of yeast extract, 0.5 g NaCl, 0.186 g KCl. After autoclaving, 1 g of $MgCl_2$ were added from stock solutions.

SOC consisted of SOB supplemented with 20 mM glucose (Sambrook *et al.*, 1989) and was used as the recovery medium for *E. coli* cells after transformation.

Recovery medium (Britz, personal communication) was used to recover transformants of *C. glutamicum* after electroporation and contained LBG supplemented with 10 mM $CaCl_2$ and 10 mM $MgCl_2.6H_2O$.

2.2.2 Growth of bacteria

2.2.2.1 Growth of corynebacteria on plates

LAG inocula were prepared on LAG plates, where corynebacteria glycerol stocks stored at –20°C were transferred onto LAG plates with a sterile loop and then allowed to grow at 30°C for about 16 h before being used as inocula. NAG was often used for sub-culturing of corynebacteria instead of LAG. MacCartney bottles (25 ml capacity) containing 10 ml of sterile LBG were inoculated with single colonies from LAG plates. The cultures were incubated overnight at 200 oscillations per min (o.p.m.) and 30°C and cultures used as inocula for large-scale cultures, including determination of growth rates by measuring absorbance at 600 nm.

Larger volume cultures were performed to compare mycolic acid composition in cell wall fractions for cells grown in Erlenmeyer flasks of two litre capacity containing one litre of the following media: LBG, LBG containing 2% (w/v) glycine, LBG containing 4 mg/ml or 8 mg/ml of INH (AS019, MLB194, MLB133), or LBG containing 2% glycine plus 0.4 mg/ml or 4 mg/ml (BL1, BF4, RM3, RM4) INH, harvesting cells at early stationary phase.

2.2.2.2 Growth of mycobacteria

Mycobacteria stored at –80°C in glycerol were transferred onto a thick poured Middlebrook 7H10 agar plate and wrapped with parafilm both to prevent desiccation of the media and to reduce possible risks of mold contamination. The plates were then allowed to grow at 37°C for three to four days before being used. Small flasks (250 ml) containing 25 ml of sterile M-ADC-TW broth were inoculated with single colonies from Middlebrook 7H9 plates. The cultures were maintained at 37°C at 100 o.p.m for four days, and 0.1 ml of these cultures were used subsequently as inoculum into 100 ml of M-ADC-TW broth.

2.2.2.3 Growth of *E. coli*

MacCartney bottles containing 10 ml of sterile LB media supplemented when required with appropriate antibiotics (50 μg/ml kanamycin or 100 μg/ml of ampicillin) were inoculated with single colonies from LA plates of *E. coli*. The cultures were maintained at 37°C overnight and 200 o.p.m. and subsequent cultures were used as inoculum for larger volume cultures.

2.2.2.4 Growth curves, viable counts and drug sensitivity tests

Growth was monitored by measuring absorbance at 600 nm using an LKB Ultraspec spectrophotometer; when A_{600} exceeded 0.8, cultures were diluted in the medium used for growth and A_{600} recorded against medium blanks. Viable counts were performed using 10-fold dilutions of cultures in SMMC buffer. Dilutions were made using 0.9 ml volumes of diluent in 1.5 ml capacity capped tubes with 0.1 ml samples transferred using a 200μl Gilson pipette and sterile tips. After vortexing tubes, samples of 0.1 ml were taken from tubes and spread onto appropriate plates (usually ET and LAG) using a sterile glass spreader. Samples were allowed to dry then plates were incubated at appropriate temperatures for up to seven days. All counts were performed in triplicate.

Drug sensitivity tests were performed by incorporating antimicrobial agents into the plates. The method for corynebacteria involved preparing various concentrations of the drugs, ranging between 0.1 to 20 mg/ml. Plates were prepared on the day of use and dried at 37°C. Overnight cultures of coryebacteria were diluted in SMMC buffer or peptone water. Different dilutions of each strain were plated onto agar plates containing drugs with a multiprong replicator. The spots were allowed to dry and plates incubated at 30°C for 24 h. Plates without drugs were inoculated to ensure that viable organisms were present throughout the experiment. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of the drug that completely inhibited growth, after 48 h incubation at 30°C.

2.2.3 Storage of strains

Freeze-dried strains of corynebacteria and *E. coli* were obtained from frozen ampoules and streaked out onto LA (for *E. coli* strains) or LAG (for corynebacteria). The working stock cultures were prepared by streaking single colonies of cells onto the entire surface of LA plates (for *E. coli*) or LAG plates (for corynebacteria). After 16 h incubation, plates were taken into a lamina flow hood and growth harvested into glycerol storage broth, using a sterile stainless-steel wire loop. Glycerol storage broths contained 50% glycerol in LB (for *E. coli*) or LBG (for corynebacteria), supplemented with antibiotics (50 μg/ml as final concentration) if strains contained plasmid DNA, and were dispensed in 2-3 ml aliquots into 5ml capacity glass bottles. Sealed bottles were stored at –20°C (for working stocks) and –80°C (for long term storage stocks).

2.3 MOLECULAR BIOLOGY METHODS

2.3.1 Preparation and analysis of DNA

Standard recombinant DNA methods, including restriction digestion, agarose gel electrophoresis and plasmid purification, were performed using standard apparatus and methods (Sambrook *et al.*, 1989; Ausubel *et al.*, 1992), unless otherwise stated, and these are briefly described in the following.

2.3.1.1 Small-scale plasmid DNA isolation

The following is the protocol for rapid isolation of plasmid DNA without column purification for small-scale preparation or for large-scale preparation using CsCl gradients (Sambrook *et al.*, 1989). *E. coli* strains (for example, S17-1 and DH5α containing pK18*mob-inhA* and p301*lysA*-int) were grown overnight in 250 ml of LB containing suitable antibiotics (such as 50 μg/ml Km). The cells were harvested at 4,000 r.p.m., 4°C, 10 min, using a JH-2S refrigerated centrifuge (Beckman). The pellet was resuspended in 6 ml of freshly prepared lysis solution (25 mM Tris-HCl, pH 7.5, 10 mM EDTA, 15% sucrose, 2 mg/ml lysozyme) by pipetting cells up and down thoroughly

using a 10 ml pipette. The resuspended cells were incubated in ice water for 20 min then 12 ml of a solution containing 0.2 M NaOH and 1% SDS was added. After mixing by inversion and incubating in an ice water bath for 10 min, 7.5 ml of 3 M sodium acetate, pH 4.6, was added, mixed by inversion and incubated in an ice water for 20 min. The sample was centrifuged at 15,000 r.p.m. for 25 min. The supernatant was collected and treated with 60 µl of DNase-free RNase (1 mg/ml) for 30 min at 37°C. The samples were extracted twice with phenol:chloroform (1:1), then two volumes of ethanol added to precipitate the DNA at –20°C overnight. The sample was centrifuged at 10,000 r.p.m. for 10 min at 4°C. The supernatant was removed and the pellet was washed with 70% ethanol. The DNA was dissolved in sterile Milli-Q water or TE buffer (pH 7.5) and stored at –20°C.

Alternatively, plasmid purification was achieved using a Plasmid Mini kit (QIAGEN) according to the manufacturer's instruction. Briefly, *E. coli* JM 109 and X-cell blue transformants, containing *inhA* gene PCR products or *PstI* digested genomic DNA fragments from corynebacteria ligated in the PCR2.1® vector and pBlueScript II (Invitrogen, Stratagen), were grown overnight at 37°C in LB (10 ml) containing 100 µg/ml ampicillin. Cultures samples (2 ml) were centrifuged (Eppendorf) for 30 sec and the cells resuspended in the supplied resuspension (200 µl) and lysis (250 µl) solutions, mixing by gentle inversion and then adding the supplied neutralisation solution (250 µl) and mixing by gentle inversion. The white precipitate was collected by centrifugation (8,000 r.p.m.) for 10 min and the collected supernatant transferred to the supplied Spin filter then centrifuged for 30 sec. The column was then washed once with the supplied washing solution and finally with the ethanol wash solution by centrifugation (30 sec and 1min). Sterile Milli-Q water (100 µl) was added and the plasmid DNA was eluted by centrifugation for 30 sec. The eluted plasmid DNA was analysed by gel electrophoresis then aliquoted and stored at –20°C.

Plasmid DNA from corynebacteria was isolated using an alkaline lysis method (Best and Britz, 1986). This procedure was used with minor modification as described in the next section. To increase the efficiency of cell lysis, cells were treated with 10 mg/ml

lysozyme in lysis buffer at 37°C for at least three hours. Plasmid DNA was also extracted from larger volumes of cultures.

2.3.1.2 Large-scale plasmid DNA isolation

Plasmid DNA from corynebacteria was isolated after growth in 400 ml of LBG-G (2% glycine) with Km (50 μg/ml). The procedure involved preparation of osmotically sensitive cells by growing cells in the presence of glycine and followed by lysozyme treatment, breaking the cell surface structure using SDS treatment, removing chromosomal DNA and proteins from plasmid DNA using ultracentrifugation through CsCl-ethidium bromide gradients. When the A_{600} of cells reached greater than 1.0 (ie. late exponential growth), cells were collected by centrifugation (5,000 X g, 10 min, 4°C) then resuspended in 50 ml lysis buffer supplemented with lysozyme (10 mg/ml). Cells were incubated at 37°C for three h before adding 12.5 ml of 0.5M EDTA (pH 8.0) and incubating for one h. This was followed by the addition of 7 ml of freshly prepared lysis solution (10% SDS, 1 M NaOH) pre-heated at 55°C. Cells were heated at 55°C for 10 min in a water bath. If lysis did not occur, then another 7 ml of lysis solution was added and heated at 55°C. Subsequently, 35 ml of ice-cold acetate solution (200 ml of this contained 120 ml of 5 M potassium acetate; 23 ml of acetic acid; and 57 ml of dH_2O) [Sambrook *et al.*, 1989]) was added. The mixture was chilled at -20°C for 30 min and the precipitate removed by centrifugation (3,000 X g, 15 min, 4°C). The supernatant fluid were collected and filtered through cheese cloth. Plasmid DNA was precipitated by addition of 0.6 volumes of isopropanol and nucleic acids collected by centrifugation (14,000 X g, 30 min). The pellet was washed with 70% ethanol, collected by centrifugation and dried. The DNA was resuspended in 9 ml of water and applied to CsCl density gradient centrifugation.

2.3.1.3 Preparation of caesium chloride-ethidium bromide (CsCl-EtBr) gradients

CsCl was finely powdered and nine grams amount added to 15 ml sterile, capped plastic centrifuge tubes. DNA mixture in water (9 ml) was added with 0.92 ml of ethidium

bromide (10 mg/ml stock) and the CsCl dissolved by repeating gentle inversion. The DNA-CsCl-EtBr mixture was centrifuged (2,000 X g, 15 min, 4°C) and the clear red fluid decanted into a quick-seal ultracentrifuge tube (16 X 76 mm, Beckman), leaving behind a buoyant red precipitate. Tubes were filled with light mineral oil (Sigma), weighed for balancing, and sealed with a Beckman tube topper. The tubes were centrifuged (Beckman L-70 ultracentrifuge, with a Beckman 70 Ti/70.1 rotor) at 20°C and 60,000 r.p.m. (approximately 250,000 X g) for 16 h and the rotor allowed to stop without braking. Usually, two bands (the top band corresponding to the chromosomal DNA) were formed after centrifugation and the plasmid (lower band) was collected under long wave (λ= 302 nm) light (LKB 2011 Macrovue Transluminator). Depending on the amount of residual chromosomal DNA, this was done either by piercing the side of the tube with a syringe, removing fluorescent material in the plasmid band through a 21 gauge needle, or by gently removing liquid above this band using a Pasteur pipette after opening the top of the tube using a sterilised blade, then collecting the plasmid band with a fresh pipette. Ethidium bromide was removed from the DNA solution by several extractions with equal volumes of isopropanol until the red coloured had disappeared from the DNA solution. Two volumes of cold ethanol were added to the DNA solution, mixed, then stored at –20°C for 30 min. The DNA was collected by centrifugation (14,000 X g, 20 min, 4°C) and washed with 70% ethanol prior to centrifugation. After removing the ethanol, DNA was resuspended in a minimum volume of distilled water, aliquoted, and then stored at -20°C.

2.3.1.4 Isolation of chromosomal DNA

The method used to extract chromosomal DNA from *E. coli* was described by Ausubel *et al.* (1987) and used as follows. Broth cultures (100 ml) of *E. coli* strains were grown to saturation at 37°C overnight and harvested by centrifugation (5,000 X g, 4°C, 10 min). The pellet was resuspended in a mixture containing 9.5 ml of TE buffer, 0.5 ml of 10% SDS and 50 µl of 20 mg/ml proteinase K and incubated at 37°C for one h. Sodium chloride (5 M) was added to a final concentration of 1 M and the mixture was shaken thoroughly at room temperature before adding 1.5 ml of CTAB/sodium chloride solution

(10% ceyl-trimethylammonium bromide/0.7 M NaCl). The sample was incubated for 20 min at 65°C in a water bath. This was followed by a chloroform/isoamyl alcohol (24:1) extraction and the aqueous phase transferred to a new tube using a Pasteur pipette. Isopropanol (0.6 volumes) was added and incubated for 10 min at room temperature. The nucleic acids were collected by centrifugation (14,000 X g, 20 min, 20°C), resuspended in 9 ml of water and used to prepare CsCl gradients for further separation of chromosomal DNA.

The method used to extract chromosomal DNA from coryneform bacteria was based on one described by Britz and Best (1986) and which was based on the earlier method of Marmur (1961). Cells from 100 ml LBG-G broth cultures were pelleted (5,000 r.p.m., 10 min, 4°C) after 16 h growth and washed once with 100 ml of lysis buffer (without lysozyme), then cells collected by centrifugation and resuspended in 5 ml of lysis buffer containing lysozyme (final concentration of 10 mg/ml) followed by incubating for 16 h at 37°C using a orbitary shaker incubator (200 o.p.m.). EDTA (10 mM in 10 mM Tris buffer, pH 8.0) was added and the mixture incubated at 37°C and 200 r.p.m. for one h. Osmotically sensitive cells were collected by centrifugation (1,200 X g, 10 min, 4°C) in a JA-20 Beckman rotor. Cells were washed with buffer without lysozyme, resuspended in 20 ml of lysis buffer (containing 2 mg/ml of lysozyme in 10 mM-Tris pH 8.0, 0.7% sucrose, 10 mM $CaCl_2$, 10 mM $MgCl_2$.$6H_2O$, 0.05% KH_2PO_4, 0.01% BSA) and incubated at 37°C for 2-3 h. Cells were lysed by the addition of 8 ml of SDS solution (1% w/v, in water) followed by heating at 55°C for 10 min. Proteinase K (0.2 mg/ml) was added and the tube was incubated at 37°C for one h. Sodium perchlorate was added to a final concentration of 1 M and the mixture shaken at room temperature in an equal volume of phenol and extraction was performed at 30°C with slow shaking (100 r.p.m.). The emulsion was separated (5,000 X g, 10 min, 20°C) and the upper aqueous layer transferred into a new tube using a Pasteur pipette. Isopropanol (0.6 volumes) was added and the mixture was incubated for 10 min at room temperature. The nucleic acids were collected by centrifugation (14,000 X g, 20 min, 20°C), resuspended in 9 ml of water and used to prepare CsCl gradients for further separation of chromosomal DNA.

2.3.1.5 Isolation of high molecular weight mycobacterial chromosomal DNA

Chromosomal DNA from *M. smegmatis* mc^2155 was prepared according to the method of Jacob *et al.* (1991). Cells were grown in 100 ml M-ADC-Tw broth and collected after overnight culture by centrifugation (5,000 X g for 10 min at 4°C). The resulting pellet was resuspended in 10 ml of homogenisation buffer (0.3 M Tris, pH 8, 0.1M NaCl, 6mM EDTA). The resuspended cells were transferred to a conical screw-cap vial which was filled to one quarter to one half with 0.1-0.11mm, acid-wash sterile glass beads (B. Braun, Germany). The vial was then placed in a MSK cell homogeniser (B. Braun) and homogenised for a total of 60 sec with the temperature of the homogenisation medium maintained at 4°C by a flow of CO_2. The homogenised suspension was extracted twice with an equal volume of phenol-chloroform (1:1) then once with chloroform-isoamyl alcohol (24:1). The nucleic acids were precipitated with 1/10 volume of 3M sodium acetate, pH 5.2, and two volumes of cold ethanol by incubating at –20°C overnight and collecting DNA by centrifugation (14,000 X g, 20 min, 20°C). The pellet was washed with 70% ethanol, dried and resuspended in 1 ml of TE (10 mm Tris-1mM ETA) buffer. RNA was digested by adding RNase A (100 µg/ml from 10 mg/ml boiled stock) and incubating at 37°C for 30 min. After RNA digestion, the suspension was extracted twice with an equal volume of phenol-chloroform then once with an equal volume of chloroform-isoamyl alcohol as before. Finally, DNA was pelleted with two volumes of cold ethanol and 1/10 volume of 3 M sodium acetate, dried and resuspended in 1 ml of TE.

2.3.1.6 Estimation of DNA concentration

DNA concentrations were determined using the spectrophotometric and ethidium bromide/agarose plate methods as described by Sambrook *et al.* (1989). Amounts of synthesised oligonucleotides were measured using spectrophotometric methods. Isolated nucleic acid samples and synthesised oligonucleotides were diluted 1:100 in distilled water and absorbance readings were taken at wavelengths of 260 nm and 280 nm, and the

A_{260}/A_{280} ratio provided an estimation of the purity of samples. Nucleic acid concentrations were determined according to the equation: 1 A_{260} unit (1cm light path) = 50 µg/ml double stranded DNA and 33 µg/ml for single-stranded oligonucleotides (Sambrook *et al.*, 1989).

2.3.1.7 Agarose gel electrophoresis

Electrophoresis of DNA was usually performed with horizontal 0.8% (w/w) agarose slab gels measuring 10.5 X 8 cm (Gel Electrophoresis Apparatus GNA-100, Pharmacia, Sweden). For good separation of small DNA fragments, different concentrations (1.0%, 1.5%, or 2%) of agarose slab gels were used. Large gels (20 X 20 cm, GNA-200, Pharmacia, Sweden) were used for endonuclease digested genomic DNA. Electrophoresis was carried out using 1X TAE buffer running normally at 80 volts for 1-2 h or until the loading dye ran near the bottom of the gel, whilst 25 or 35 volts were used for large gels in 1X TAE buffer, running overnight. Ethidium bromide staining solution was added to the agarose gel before electrophoresis and to the 1X TAE tank buffer at a final concentration of 0.5 µg/ml. The DNA bands were visualised on transilluminator (λ = 302 nm) (LBK 2011 Macrovue transluminator). *EcoR1* and *HindIII* digested lambda DNA preparations (Progen) containing eleven fragments (21.1, 5.1, 4.3, 3.5, 2.0, 1.9, 1.6, 1.4, 0.9, 0.8, 0.6 and 0.1 kb) was used as molecular markers for the digested genomic DNA. Alternatively Spp1/*EcoR1* markers (Promega) containing 15 fragments (8.5, 7.3, 6.1, 4.8, 3.5, 2.8, 1.9, 1.8, 1.5, 1.3, 1.1, 0.98, 0.72, 0.48, 0.36) were also used for size determination. Standards used for PCR products or digested PCR products were a 100 bp DNA ladder (Promega) containing 11 fragments (1,500, 1,000, 900, 800, 700, 600, 500, 400, 300, 200, and 100 bp) or Probase 50™ (Progen) containing 16 fragments (3,147, 2,647, 900, 800, 700, 600, 500, 450, 400, 350, 300, 250, 200, 150, 100, and 50 bp). Approximately 0.5-1.0 µg of the size markers was used per gel well.

The migration distances of linear fragments of plasmid DNA on agarose gels were related to their molecular weights from a plot of the logarithm of the relative migration distances of standards against logarithm of molecular weights, which gave linear curves.

2.3.1.8 Photography

Gel photography was performed using a Polarid MP-4 Camera if required. Typically, Type 667 black and white film was used for documentation at a shutter speed of 1/60 to 1/30 sec with an aperture of F16. Type 665 positive/negative film was used at a shutter speed 1/4 to 1/12 sec and an aperture of F16. If the picture was too light, the shutter speed was increased or a higher F number was set or altered inversely when the picture was too dark (Ausubel *et al.*, 1992). The MICs plates were photographed with a top lighting black paper on white light box with a shutter speed of 1/30 sec and an aperture of F22..

2.3.1.9 Restriction enzyme digestion of chromosomal DNA

Chromosomal DNA from corynebacteria and mycobacteria strains was digested with various restriction endonucleases. Endonucleases (ENase) *Eco*RI, *Eco*RV, *Kpn*1, *Pst*1, *Bam*HI, *Hind*III, and *Sal*1 were purchased from Promega. Incubation mixtures for digestion (30 µl) contained 2µg of DNA, 3µl of the 10X buffer recommended by the manufacturer and 5-15 units of ENase to cleave the DNA for one h at 37°C. For each gel well, 15 µl (out of 36 µl) of mixture was loaded and the remainder stored at –20°C. Phage lambda DNA (0.35µg/µl) and Spp1/*Eco*RI (0.25µg/µl) (Promega) were used as the molecular weight standard and 2µl was loaded on each gel.

2.3.1.10 Recovery of DNA fragments from agarose gels

DNA fragments were recovered from agarose gels (0.6%) and used for different purposes, depending upon the need. After electrophoresis, gels were photographed and DNA bands corresponding to the desired size was excised from the gel and isolated using a Gene clean kit (Bio 101 Inc) as recommended by the manufacturer.

2.3.1.11 Synthesis and preparation of the oligonucleotides

In the beginning of this research oligonucleotides were synthesised by Ms. U. Manueplillai (PhD student, CBFT, VUT) with an Applied Biosystem Model 391 DNA

synthesiser (Perkin-Elmer) but later on purchased from Pacific Oligos Pty. Ltd. For the synthesis of oligonucleotides chemicals and columns were obtained from Perkin-Elmer and the primers were processed as recommended by the supplier. After synthesis, cleavage from the column, deprotection, dehydration and precipitation of the oligonucleotides were carried out as described in the instruction manual provided by the supplier (Applied Biosystem).

For the cleavage of oligonucleotides from synthesis columns, 5-7 ml of concentrated ammonia solution (30-35%) was dispensed into a clean glass bottle (MacCartney). The tubes were capped tightly. The tip of a sterile 1ml syringe was inserted into one end of the column. The syringe was fitted snugly with a plunger at the needle end and 0.75 ml concentrated ammonia was drawn into another 1 ml syringe. Any air bubbles at the tip were removed. The second syringe was inserted into the other end of the column. The syringe-column-syringe unit was held horizontally. The plunger of the syringe containing ammonia was pushed gently and the ammonia was allowed to pass thorough the column into the opposite syringe. Pushing the ammonia back and forth between the syringes was repeated 4-5 times. The syringe-column-syringe unit was laid down on a flat surface and incubated for 15 min at room temperature, making sure that there was ammonia solution inside the column. The ammonia solution was drawn into one of the syringes, which was then removed from the column. The ammonia was expelled into a pre-cleaned 4 ml vial, and the vial was tapped tightly with a teflon-lined screw cap. These vials were then placed at 55°C overnight for deprotection of the oligonucleotides.

The deprotected oligonucleotide was incubated in a waterbath at 40°C for 16 h, with the vial capped loosely so that the ammonia evaporated, then frozen at –70°C before dehydration. The frozen oligonucleotides were dehydrated overnight under vacuum in a freeze drier.

The freeze-dried oligonucleotides were dissolved in 600µl of water and 100 µl of 3M sodium acetate (pH 5.2); these volumes were calculated on the basis that expected optical density of oligonucleotides would be 20 and 30µl of water plus 5 µl of 3 M sodium

acetate were added per one unit of optical density according to the supplier's instructions. Two ml of ethanol was added to the oligonucleotide solutions then vortexed briefly. The mixture was stored at –20°C for 30 min, then centrifuged at 14,000 rpm in a microcentrifuge for 5 min at 4°C. The supernatant was removed and the pellet was washed using 70% ethanol. Finally, the oligonucleotide pellet was dried under vacuum and dissolved in 300 µl of distilled water. The oligonucleotide concentration was determined according to the formula: 1 A_{260} unit = 32 µg DNA/ml (Sambrook *et al.*, 1989).

2.3.1.12 Polymerase Chain Reaction (PCR) amplification

The PCR amplification of the presumptive *inhA* genes were carried out using the QIAGEN PCR kit and AmliTaq DNA polymerase (Perkin-Elmer) and used according the supplier's instructions. A typical PCR mixture contained PCR buffer (1X), Soln Q, deoxynucleoside triphophosphate (final concentration 200 µM of each dNTP), PCR primers (final concentration 0.5 µM of each), 2 mM $MgCl_2$, 100 ng of DNA, 2.5 units of enzyme, with water added to a final volume of 100 µl. All reactions included negative (sterile distilled water) controls. Amplification was performed on the DNA Thermal Cycler-480 (Perkin-Elmer) or Peltcer Thermal Cycler (PTC-200) (Bresatec) using the protocol described by the manufacturer. For genomic DNA and plasmid DNA, PCR temperature profiles were varied and this is described in detail in Table 4.2.

The PCR amplification of the 16S rDNA was performed according to the Expand™ Long Template PCR system (Boehringer Mannheim) protocol. The PCR mixtures (50 µl) contained 20 pmol of each appropriate primer, and approximately 500 ng of genomic DNA. Briefly, two mixtures (25 µl of master mix I [1.75 µl each of 10 mM dNTP, downstream and upstream primer and template DNA] and 25 µl of master mix II [5 µl of 10 times PCR buffer with system II and 0.75 µl of amplify enzymes]) were prepared and mix shortly before cycling. The cycle profile was as follows: after initial denaturation at 95°C for 2 min, samples were chilled on ice briefly and enzyme was added then cycling

were perfoprmed at 95°C 1 min; 54°C 1 min; 72°C 2 min for 35 rounds followed by a extension at 72°C for 5 min. Following PCR, 5 µl of the amplified products was electrophoresed in 1% agarose gels containing ethidium bromide (0.5 mg/ml) with 1X TAE buffer and viewed on a transilluminator.

2.3.1.13 Purification of PCR Products

The PCR products of expected sizes were purified using two methods, depending on wether the PCR products were in solution or prepared following separation on agarose gels.

Wizard™ Minicolumns (Promega)

PCR products were purified using the Wizard™ PCR Preps DNA Purification System (Promega) following the manufacturer's instructions. For each complete PCR reaction, the entire contents (approximately 95 µl) were added to a 1.5 ml microcentrifuge tube containing 100 µl of Direct Purification buffer, and then vortexed briefly to mix. Subsequently, 1 ml of PCR Preps DNA Purification Resin was added and the samples were then vortexed briefly three times over a one minute period. One Wizard™ Minicolumn was prepared for each PCR reaction. The 3-ml disposable syringe barrel was attached to the Luer-Lock R extension of each minicolumn while the syringe plunger was set aside. The DNA/resin mixtures were put into the syringe barrel and then the syringe plunger was inserted slowly and gently to push the DNA slurry into the minicolumn. Subsequently, the column was washed with 2 ml of 80% isopropanol. The column was centrifuged for 20 sec at 12,000 X g to dry the resin. To remove DNA, 50 µl of sterile distilled water was added to the column, which was left for two min. The column was centrifuged for 20 sec at 12,000 X g to elute the bound DNA fragments, and the purified DNA solution was stored at –20 °C.

BANDPURE ™ DNA Purification kit (Progen)

Alternatively, PCR products were firstly separated on agarose gels and the bands excised under UV light. At least 2.5 volumes of the NaI binding buffer was added to the DNA

slice and melted at 55°C for 5 min. The BANDPURE silica matrix suspension was vortexed until it was resuspended thoroughly and then 5µl of it was added to the DNA solution. For amounts of DNA greater then 5 µg, an additional 1 µl of silica matrix suspension was added for each 1 µg of DNA. The melted gel-silica matrix suspension was incubated at room temperature for 5 min with frequent gentle mixing, then centrifuged for about 30 sec to pellet the silica matrix with DNA bound to it. The supernatant was discarded. The pellet was washed by adding 500 µl of ice-cold ethanol wash solution to the pellet. The pellet was resuspended and centrifuged for about 30 sec to pellet the matrix, and the ethanol wash solution was discarded. This step was repeated three times, retaining the pellet each time. The DNA was eluted from the pellet by adding 30-50 µl of water, incubating at 55°C for 5 min, then centrifuging for 30 sec to collect the supernatant containing the DNA. The approximate DNA concentration was determined on agarose gels, using the intensity of one band of the 50 base or 100 base PCR markers for comparison, according to instructions of the supplier (Promega). The PCR products purified by either method were cloned into vectors, used directly for sequencing or were used to prepare probes for Southern hybridisation.

2.3.1.14 16S ribosomal RNA (rRNA) gene analysis

In order to check the purity of DNA samples 16S rRNA sequence analysis was performed. Approximately, 1300 bp of a consensus (16S RNA) sequence was amplified from DNA templates of six corynebacterial strains using two primers 63f (5'CAG, GCC,TAA, CAC, ATG, CAA, GTC 3') (Marchesi *et al.*, 1998) forward primer, and 1387r reverse primer (5' GGG, CGG,TGT, GTA, CAA, GGC) (Pharmacia; Marchesi *et al.*, 1998). The primers were purchased from Pacific Oligos.

Expand long template PCR system (Boehringer Mannheim) was used for PCR amplification. In each reaction 500ng of genomic DNA was used as the template. The PCR products were sequenced directly using the same PCR primers, 63f and 1387r.

2.3.2 Southern hybridisation

Genomic DNA was digested with restriction enzyme and run on an agarose gel in preparation of Southern blotting. Gels were placed in denaturing solution for 45 min at room temperature with gentle agitation. Gels were transferred to neutralising solution and agitated for 20 min. Excess solution was removed by blotting the gels on filter paper. The gels were transferred to perspex boards (DNA side up) and precut nitrocellulose membranes (Hybond, Amersham) were placed on top. Air bubbles were removed by rolling the membrane with a 10 ml pipette. Three layers of filter paper (Whatman 3 mm Chromatographic paper) were applied to the membranes plus approximately 4 cm absorbent paper towelling. A second perspex board was applied on top of the paper towel and 1Kg weight placed on top. Gels were blotted overnight. After blotting the membranes were washed in 2XSSC for 5 min, blotted on filter paper to remove excess solution and wrapped in glad wrap. Membranes were exposed to UV light (LKB 2011 Macrovue transIluminator, Bromma) for five min to fix the DNA to the membrane then stored at room temperature.

2.3.2.1 Use of DIG-labelled probes

Prehybridisation and hybridisation of membrane-bound nucleic acid to DIG-labelled λDNA was performed according to the manufacturer's instructions (Boehringer Mannheim). Membranes were prehybridised in a sealed bag which contained 20 ml of hybridisation buffer (5X SSC and blocking reagents; 0.01 % w/v, N-laurosarcosine; 0.02%, w/v, SDS) at 68°C for three h.

In order to prepare the probe, 190-210 ng of template DNA was added to sterile water (Milli-Q) to a final volume of 40 µl in a 1.5 ml Eppendorf tube. The DNA was denatured by heating in a boiling water bath for 10 min and chilled quickly on ice. DIG-High Prime™ (4µl, Boehringer Mannheim) was added into aqueous DNA then mixed by centrifuging (Eppendorf) briefly. The probe was incubated overnight at 37°C. The reaction was stopped by adding 5 µl of 0.2 M EDTA (pH 8.0) and stored at −20°C until used.

The membrane was then transferred to a fresh bag containing the same buffer plus 5 µl of DIG-labelled λ DNA or SPP1/*Eco*R1 DNA markers and 20 µl of specific probe in the same buffer. The probes had been denatured in boiling water for 10 min, chilled on ice, diluted in hybridisation solution and filtered through a 0.45 µm filter (Millipore, GS) after pre-warming at 40°C before adding to the membrane. After hybridisation at 68°C overnight, blots were washed at room temperature twice each for 15 min with 100 ml of 2X SSC plus 0.1% SDS, followed by one wash in 0.1X SSC plus 0.1% SDS at 68°C.

2.3.2.2 Detection of hybridisation signal

Bound probes were detected by enzyme-linked immuno-assay using an antibody conjugate (antidioxigenin-alkaline phosphatase conjugate) and visualised with the chemiluminence substrate CSPD™ supplied by the kit manufacturer (Boehringer Mannheim). Enzymatic dephosphorylation of CSPD™ by alkaline phosphatase leads to light emission at a maximum wavelength of 477 nm which is recorded on X-ray films. This procedure was described by the manufacturer and used without modification. Membranes were washed briefly (1 to 5 min) in washing buffer (maleic acid buffer plus 0.3% [v/v] Tween 20) and incubated for 30 min with 100 ml of blocking solution (10x supplied blocking solution diluted in maleic acid buffer 1:10). The membrane was then incubated for 30 min with anti-DIG-AP conjugate (diluted to 75 mU/ml [1:10,000] in blocking solution). The membrane was washed twice for 15 min each in 100 ml washing buffer. The membrane was equilibrated for 2 min in 20 ml detection buffer (100 mM Tris HCl, 100 mM NaCl, 50 mM $MgCl_2$; pH 9.5). The membrane was placed into a hybridisation bag and about 20 drops (1 ml) of the supplied CSPD™ was added. Following this, membranes were placed on a development folder and incubated at 37°C for 15 min. Finally membranes were exposed to X-ray films (Amersham, Hyerplus) for 15-25 min at room temperature.

2.3.2.3 Radiolabelling of the DNA probes

Hybridization buffer was prepared as follows: Denhardt's (5X) solution contained 2% Ficoll (type 400, Pharmacia), 2% polyvinylpryrrolidone (PVP), 2% BSA (FractionV, Sigma), and was sterilised by filtration (0.22 μm, Millipore, GS) then stored at –20°C. Prehybridisation solution (9.0 ml) was prepared by mixing 4 ml of 6X SSC, 4 ml of 5X Denhardt's solution, 0.5 ml of 10% SDS (mixed together and preheated at 55°C for 10 min before use), and 0.5 ml of denatured salmon sperm DNA (denatured at 95°C for 10 min using a water-bath, and placed on ice immediately). This solution (10-20 ml) was placed into a sealable plastic blender bag containing the membrane. The hybridisation bag was sealed and placed on a glass tray or into a suitable container and prehybridised at 65°C for up to 8 h.

Probes were labelled randomly with α-P^{32} dCTP (sp. act. > 3000 ci/nmole, Brestec) using a High Prime Labelling Kit (Boehringer Mannheim). Reaction mixtures of 25 μl contained 25 ng of denatured probe DNA mixed with 6 μl of high prime labelling mix, 2 μl of α-P^{32} dCTP and 7 μl of sterile distilled water. The mixture was incubated at 37 °C for 15 min. The reaction was terminated by the addition of 2 μl of 0.25 mM EDTA. A G-50 Sephadex column was prepared to remove the unincorporated P^{32} nucleotide. The column was washed with 100 μl of cold STE buffer and then 100 μl of TE. Prior to passing the probe through the column, 50 μl of TE buffer and 2 μl of tRNA (Gibco, BRL) (5 μg/ml) was added to the probe, then this loaded onto the column. The probe was collected by centrifuging for 30 sec and 100 μl of TE buffer was added before it was denatured at 95°C (10 min). After denaturation, the probe was mixed with 200 μl of TE buffer and placed on ice immediately. The probe was then added to the membrane, then this was allowed to hybridise for 24 h at 65°C. The membranes were washed twice in 2X SSC plus 0.1% SDS at room temperature for 10 min and once in 0.2X SSC plus 0.1 SDS at 65°C for 5 min with gentle agitation. The probe emitted radioactivity of 5-10 cps when measured by a Geiger Muller radiation monitor. The membrane was exposed to X-ray film (Kodak XAR-5) in a Kodak intensify cassette for 48 h at –80°C.

2.3.2.4 Development of the X-ray films

Films were developed manually as follows: 5 min in developer solution (phenol-X-ray developer supplied), 1 min in the 3% acetic acid, 5 min in the fixer solution (Agfa, Gevaert-X ray fixing bath). Finally, the films were washed in running tap water for 15 min and dried by hanging at room temperature. The probes were washed off from membranes following the Amersham protocol for use with DNA blots only. Membranes were incubated at 45°C in 0.4 M NaOH for 30 min and then transferred into 0.1X SSC, 0.1% w/v SDS, 0.2 M Tris HCl pH 7.5 then incubated for another 15 min. The membranes were air dried on Whatman filter paper, sealed in a plastic bag and stored in a cool, dry place at room temperature.

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2.3.3 Construction of subgenomic libraries

2.3.3.1 Preparation of the insert

Chromosomal DNA from strains of *C. glutamicum* and related species was subjected to *Pst1* restriction digestion, where the 30 µl reaction mixture contained 2 µg of DNA, 3µl of restriction buffer and 10 units of enzyme, with the reaction carried out at 37°C for 3 h. After incubation, 4 µl of reaction mixture was loaded on the gel and run for 1 h to check the extent of digestion and the reaction was then terminated by heating at 65°C for 10 min. The DNA was extracted with an equal volume of phenol-chloroform (24:1), precipitated with two volumes of cold ethanol and 1/10 volume of 3M sodium acetate, then washed with 70% ethanol. The pellet was dissolved in 10 µl of sterile distilled water, 1 µl of the sample was run on an agarose gel to determine the concentration. The DNA was stored at –20°C until used.

2.3.3.2 Preparation of the vector

Plasmid pBluescript II SK+/- was completely digested with *Pst*1 restriction endonuclease at the multi-cloning site. The reaction mixture contained 5 µg of DNA, 5 µl of 10X buffer, 25 units of enzyme and 40 µl of deionised water. The mixture was incubated at 37°C for 3 h. The digested DNA was treated with calf intestinal alkaline phosphatase (CIAP) as described by the supplier (Promega) to remove 5' phosphate groups and thus prevent recircularization of the vector during ligation. After CIAP treatment, the DNA was purified with phenol-chloroform and precipitated by addition of 1/10 volume of 3M sodium acetate and two volumes of ethanol as above. The pellet was washed with 70% ethanol and resuspended in 10 µl of nuclease-free water.

2.3.3 3 Ligation of vector and insert DNA

To obtained the optimal ratio of the vector to insert DNA, molar ratios of 1:1 or 3:1 insert to vector were tried. Ligation mixtures contained 100 or 300 ng of insert DNA, 100 ng of vector DNA, 1 µl of T4 DNA ligase (4 Weiss units), 1 µl of 10X ligase buffer and deionised water to a total volume of 10 µl. The reaction was carried out at 15°C overnight. At the completion of the reaction, tubes were kept at –20°C until used.

2.3.3.4 Preparation of *E. coli* cells for transformation

E. coli strains XL1-blue and JM109 (Promega) were used for preparation of competent cells by the $CaCl_2$ treatment method, as described by Sambrook *et al.* (1989). Bacterial cells were streaked on M-9 plates containing 5mg/ml of thiamine-HCl and incubated at 37°C overnight. A single colony was picked to inoculate 10 ml of SOB broth (starter culture). SOB broth (100 ml) was inoculated with an overnight starter culture to give a starting A_{600} of 0.1 and incubated at 37°C, 200 o.p.m. for 2-3 h until growth reached an A_{600} of 0.4. Cells were then harvested by centrifugation (2, 000 x g, 4°C, 10 min) and resuspended with 10 ml of ice-cold 0.1 M $CaCl_2$; all subsequent steps were performed with sterile solutions held on ice and centrifugation were performed at 4°C. After 10 min

on ice, cells were pelleted and resuspended in 2 ml of ice-cold 0.1 M $CaCl_2$. Using a chilled, sterile pipette tip, 200 µl of each suspension of competent cells were transferred into Eppendorf tubes, frozen on dry ice and stored at –70°C.

Alternatively, for electroporation a single colony was used to inoculate 25 ml of LB medium and the culture was incubated at 30°C overnight with vigorous shaking. Using a 2.8 litre flask, 500 ml of LB medium was inoculated with 5 ml of cells from the overnight culture and shaken at 150-200 o.p.m. at 30°C until the A_{600} reached 0.45-0.55. The cells were chilled in ice water for 2 h and centrifuged at 2,500 X g for 15-20 min at 4°C. The cells were washed twice with 15% glycerol (W/V) at 4°C. Finally, the cells were pooled then 80% glycerol was added dropwise with gentle swirling to a final concentration of 15% (v/v) and the suspension aliquoted in 0.2-1 ml quantities, frozen on dry ice and stored at –70°C.

2.3.3.5 Transformation of competent *E. coli* cells

The transformation of competent cells of *E. coli* XL1-blue and JM109 were carried out as per the protocols provided with their respective kits. The competent cells stored at –70 °C were thawed on ice and an appropriate volume of ligation mixture was added to the tubes and these were left on ice for 30 min. The cells were then heat shocked for 90 sec at 42°C, then allowed to recover for five min on ice before the addition of 800 µl SOC medium. After incubating at 37°C for 45 min, 100 µl of the transformation mixture was plated onto LBG X-gal plates containing 100 µg/ml of ampicillin and plates incubated at 37°C overnight. Alternatively, 2 µl of 0.5 M β-mercaptoethanol was also added to the competent cells prior to mixing with cloned DNA to enhance the transformation efficiency (Invitrogen).

2.3.3.6 Identification of positive clones

Both pBluescript (pBSK+/-) and PCR 2.1 vectors contained the *lacZ* gene, which provides α-complementation of the β-galactosidase subunit encoded by the plasmid, with

that encoded by the appropriate host cells. This allows colour selection of the recombinant phagemids (colourless colonies) and non-recombinant phagemids (blue colonies) on plates containing X-gal and IPTG. The white colonies were identified as 'positive' for the successful insertion of PCR products. The efficiency of transformation was calculated as the number of white colonies per μg DNA used. A number of independently transformed colonies were picked and grown in small-scale culture containing 100 μg/ml ampicillin at 37°C overnight with vigorous shaking. Plasmids were isolated from each one of them using the QUAGEN miniprep protocol according to the manufacturer's instructions.

2.3.3.7 Screening of the Genomic library for the *inhA* gene

The positive clones were identified by three approaches.

Plasmids isolated from transformed colonies were analysed by digestion with restriction enzymes with cutting sites on either side of the insert then the size was determined by gel electrophoresis as described in section 2.3.1.7. The plasmids showing appropriate size inserts were further analysed by Southern hybridisation and by PCR.

The selected plasmids were digested with appropriate enzymes, DNA was transferred to the nylon membrane and hybridised with PCR-amplified *inhA* product from *M. smegmatis* or *C. glutamicum*. The clones containing inserts that showed hybridisation signal with *inhA* probe were further analysed by PCR.

Several sets of primers were designed based on the reported *inhA* gene sequence of *M. smegmatis* (Banerjee *et al.*, 1994) and the conditions used are described in Table 4.2. Clones generating PCR products of the size expected were chosen for sequence analysis as described in section 2.3.4.

2.3.4 Determination of DNA sequences

2.3.4.1 Primers for sequencing reaction

Both purified PCR products and plasmid DNA were used in sequencing reactions. In addition to the primers used for amplification of the *inhA* gene, T7, T3 and M13 universal primers were also used in sequencing reactions. To sequence the whole clones containing the *PstI* fragments of genomic DNA from *C. glutamicum* strains, the sequencing strategy started with T7 and T3 primers, then as the sequencing data accumulated, additional internal primers were designed based on this data, to confirm the sequences and obtain overlaps, to allow the reconstruction of the contiguous sequences. The primers used are described in the appropriate results sections.

2.3.4.2 Ligation and transformation of PCR products for sequencing

PCR products were cloned for sequencing purposes using the TA cloning kit from Invitrogen following the manufacturer's instructions. Fresh PCR products were WIZARD purified and the amount of PCR product needed to ligate with 50 ng (20 f moles) of PCR was calculated using the formula below

$$x \text{ ng of PCR product} = \frac{(y \text{ bp PCR product})(50 \text{ ng PCR } 2.1 \text{ vector})}{(\text{size in bp of the PCR } 2.1 \text{ vector}=3900)}$$

Where x ng is the amount of PCR product of y base pairs to be ligated for a 1:1 (vector: insert) molar ratio using the concentration previously determined for the PCR products. The ligation reaction was as follows: x µl of PCR product, 1 µl of 10X ligation buffer, 2 µl PCR 2.1 vector (25 ng/µl), 1 µl of T4 DNA ligase (4.0 Weiss units) and sterile water to a total volume of 10 µl. Ligation was performed at 16°C overnight and, after completion, the reaction mixture was stored at –20°C until used. Transformation was done using Top10F′ competent cells according to the manufacturer's instruction. Transformants were selected as described previously, plasmid DNA isolated and inserts were sequenced using T7 and T3 primers.

Table 2.3 Composition of sequencing reactions

Composition	Standard FS Kit	BigDye Kit
DNA sample	30-90 ng PCR products or 300-500 ng plasmid DNA	30-90 ng PCR products or 300-500 ng plasmid DNA
Primer	1 µl (3-5 pmol)	1 µl (3-5 pmol)
Premixed reagents*	8 µl	6 µl
Total Volume	20µl	16 µl

*Premixed reagents were provided by Perkin-Elmer

2.3.4.3 Sequencing reaction

Two kits (Perkin Elmer) were used to carry out DNA sequencing reactions: the ABI PRISM ™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Standard FS) and ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit. The composition of reaction mixes is given in Table 2.3. The cycle sequencing reaction was performed in 20 µl reaction volumes for 25 cycles, each cycle consisting of step 1 (96°C for 30 sec), step 2 (50°C for 15 sec) and step 3 (60°C for 4 min), on the DNA Thermal cycler-480 (Perkin-Elmer) and Peltcer Thermal Cycler (PTC-200) (Bresatec). The extension products were then purified using ethanol precipitation. A 1.5-ml microcentrifuge tube containing 2.0 µl of 3 M sodium acetate, pH 4.6 and 50 µl of 95% ethanol, was prepared for each reaction. The entire 20 µl contents of the reaction was transferred into a microcentrifuge tube containing the ethanol solution. The tube was then vortexed and placed on ice for 10 min before being centrifuged at maximum speed (14,000 X g) for 30 min. The ethanol solution was carefully aspirated using a micropipettor. The pellet was rinsed by adding 250 µl of 70% ethanol and centrifuged at maximum speed for 10 min.

The ethanol solution was carefully removed and the pellet was dried in a vacuum centrifuge. The DNA sequences were determined using an ABI 373-Automated DNA sequencer (Perkin-Elmer) at the Department of Microbiology, Monash University, Melbourne.

2.3.4.4 Analysis of *InhA* gene sequences and deduced proteins

Sequence editing, assembly, translation and alignment were carried out using the Australian National Genomic Information Service (ANGIS) on the WebANGIS interface (Greta 1997), as described briefly below.

Editing and analysis of DNA sequences

The Ereverted programme was used to reverse the sequence data determined by the reverse primers into the forward direction for further analysis. The Ecomposition

program was used to analyse A, T, G, and C composition of the DNA sequnces. *InhA* gene sequences were compared with all currently accessible nucleotide sequences from the SWISS-PROT, PIR, and GenBank databases that are combined in the non-redundant sequence database at the National Centre for Biotechnology (NCBI) using the program BLASTN.

Comparison of two or more sequences

The Bestfit program was used to determine the extent of identity between two DNA sequences and the extent of identity or similarity in amino acid sequences of any two proteins. The conventional conditions set for this comparison were used for gap weight 5.00, length weight 0.30, average match 1.00 and mismatch -0.90. Any two sequences were compared by the Prettybox program to exhibit the locations of the altered nucleotides or amino acids.

Simultaneous alignment of DNA sequences of more than two genes or amino acid sequences of more than two proteins were performed by the Pileup program of ANGIS. The conventional conditions were gap weight 3.00 and length weight 0.10. The multiple sequence alignments were also analysed further with the Readseq and Prettybox programs to identify the location of variable sequences. DNA sequences of the *inhA* genes from several *C. glutamicum* strains and related species (*M. smegmatis etc.*) were analysed by the Mapping program to search for the sites of 6-base cutter restriction enzymes within these genes. The maps for any unique restriction enzyme sites present in these genes were also developed. The *inhA* genes were translated into putative amino acid sequences of their respective proteins using the Etranslation program. The BLASTP program was used to screen the amino-acid sequence database, and the BLASTX program was used to screen the conceptual translation of the nucleotide sequence database in six reading frames (Altschul *et al.*, 1990, 1994).

The putative amino acid sequences of the InhA proteins were analysed by the Pepstats program for the physical properties including molecular weight, isoelectric point and composition of amino acids.

2.3.5. Introduction of plasmid DNA into corynebacteria strains

2.3.5.1 Electroporation

Transformation by electroporation was based on the method developed by Haynes and Britz (1990), and this was carried out using the Gene-Pulser system (Bio-Rad Laboratories, Richmond, CA). The system was composed of the Gene Pulser apparatus, pulse controller unit and Gene-Pulser cuvettes of 0.2 cm electrode gap.

To prepare the cells, a single colony was cultured in 10 ml LBG. Next morning, 5 ml of the culture was diluted into 100 ml of LBG-GI (2% glycine, plus 4 mg/ml INH for AS019, MLB133, MLB194 and 2% glycine plus 0.4 mg/ml INH for BL1, RM3 and RM4). Cells (100 ml) were harvested by centrifugation (2,000 X g, for 10 min, 4°C) (Beckman J2-HS centrifuge with rotor JA-20) at A_{600} between 0.35 and 0.45, the pellet was washed twice with 100 ml of 15% (w/v) of glycerol at 4°C, cells evenly dispensed before use, and then finally resuspended in 2 ml of cold 15% (v/v) glycerol.

The following procedure was performed in a lamina flow to ensure aseptic conditions. Forty µl samples of suspension (approximately 10^9 cells) were mixed with 2 to 5 µg of plasmid DNA of pK18mob-*inhA*, p301*lysA*-int and PCR2.1-*csp1*. The total volume was adjusted to 72 µl with distilled water and 100% v/v glycerol was added to a final concentration of 15% glycerol. Subsequently the contents of the tubes were mixed thoroughly, stored on ice for 5 min and then transferred by pipetting into a pre-cooled 0.2 cm cuvette. The cuvette was then placed in a pre-cooled pulse chamber, and the cells were exposed to a single pulse (2.5KV, 25µF). The output of the pulse generator was directed through a Pulse Controller unit containing two ohm resistors in series and two 100 ohm resistors in parallel circuit with the sample. Time constants were recorded for each experiment and these were normally 4.0-4.9 msec. If arching occurred during the pulse, the samples were discarded. Following the pulse, the cells were immediately removed from the electrodes and stored on ice for 5 min. Subsequently, the cells were

transferred by pipetting into an Eppendorf tube and mixed with 1 ml of recovery medium. The cells were incubated, without shaking for one h at 30°C. At the end of this recovery period, 10-fold serial dilutions were made in SMMC and appropriate dilutions spread onto LAG, NAG or ET, (to determine the number of survivors), and onto ET plates containing 50 μg/ml of kanamycin (ET-Km), NAG-Km or LAG-Km (to enumerate transformants). For the selection of transformants, the recovery broth was not diluted. To determine the number of cells used for electroporation, the concentrated cell suspensions stored in 15% (v/v) glycerol were diluted in SMMC and appropriate dilutions spread onto LAG and ET media. Enumeration of transformants was done using triplicate plates. Controls included untreated cells plated onto ET-Km medium. Transformants were scored initially after 2 to 3 days at 30°C, then after 4 days of growth, triplicate samples were scored and means calculated.

2.3.5.2 Conjugational transfer into *C. glutamicum*.

Conjugational plasmid transfer from *E. coli* S17-1 to *C. glutamicum* was performed according to the method of Schäfer *et al.* (1990). Two types of suicide vectors were chosen for this purpose, PECM1 and pK18mob. Suicide vector pK18mob carries unique MCS sites and plasmids carrying insertions can be easily identified using the *lac* colour reaction. A 700bp PCR amplified fragment of *inhA* gene obtained from *C. glutamicum* strains AS019 using a primer set CGP1 and CGP4 was ligated into the *Pst*1 digested pK18mob and *EcoR1* digested PECM1 suicide vectors. These primers are designed to

Table 2.4 Antibiotics stock solutions (Sambrook *et al.*, 1989; Ausubel *et al.*,1992)

Antibiotics	Stock solution	Mode of resistance
Ampicillin	50 mg/ml in water	β-lactamase hydrolyzes ampicillin before it enters the cell
Carbeneicillin	50 mg/ml in water	in place of ampicillin
Chloramphenicol	10 mg/ml in methanol	Chloramphenicol acetyltransferase inactivates chloramphenicol
Kanamycin	50 mg/ml in water	Aminoglycoside phosphotransferase inactivates kanamycin
Nalidixic acid	PH to 10 mg/ml in 1N NaOH 11 with NaOH	Mutation in the host DNA gyrase prevent nalidixic acid from binding
Rifampicin	34 mg/ml in methanol	Mutation in the β subunit of the RNA polymerase prevents rifampicin from complexing

contain the *EcoR1* restriction site on one end. Finally, the suicide vector containing the insert was transformed into S17-1 *E. coli* strains using standard protocols (Sambrook *et al.*, 1989). The presence of insert in pK18mob was confirmed using the standard M13 sequencing primer.

For mating experiments, plasmids (pECM1Δ-*inhA*, pK18mob-*inhA*, p301*lysA*-*int*) were introduced by transformation into mobilising strain *E. coli* S17-1. *E. coli* S17-1 carries an RP4 derivative integrated into the chromosome which provides the transfer functions necessary for mobilization. Preparation of the competent cells and transformation was done as described by Sambrook *et al.* (1989). Transformants were selected on LB plates containing Km (50 μg/ml). For conjugational transfer of plasmids (pECMΔ-*inhA*, pK18mob-:*inhA*, p301*lysA*-int) into *C. glutamicum* AS019, MLB133, BL1 and RM3 recipient strains, donor strains S17-1 from a single colony containing these plasmids were grown overnight at 37°C in 5 ml of LB medium supplemented with appropriate antibiotics. Next day the culture was diluted 1/5 in LB and grown up to an optical density of 1 to 1.5 (580 nm) in a rotary shaker. Recipient strains were grown in LBG medium containing 0.4mg/ml INH and 2% glycine at 30°C in rotary shaker (120 r.p.m). When OD_{580} reached 0.4 to 0.6, ten ml of culture was transferred to a test tube and placed in a 49°C water bath for 9 min. The donor (*E. coli*) and recipient (*C. glutamicum*) cells were mixed in a ratio of 1:1 (5 x 10^9 cells), centrifuged (2,000 r.p.m for 2 min), the supernatant discarded and cells resuspended in residual medium. The mating mixture was then placed onto a nitrocellulose filter (2.5 cm diameter and 0.45-um-pore size) (Millipore Corp., Bedford, MA.) and this placed on prewarmed LB plates, dried and incubated for 20 h at 30°C, the cells were washed off from the filter with 0.9 ml of LB medium. Transconjugants were plated both on LBG and ET plates containing 30 μg of Km and 50 μg of nalidixic acid (Nx) per ml and plates were incubated at 30°C for two to three days. Transconjugates were further subcultured onto ET plates containing higher concentrations (80 and 100 μg/ml) of Km and Nx.

To confirm the integration of the mobilizable plasmids carrying the insert, total DNAs of transconjugant clones were isolated and digested with selected restriction enzymes.

Restriction fragments were separated by agarose gel electrophoresis and transferred to a nylon membrane (Amersham) and probed with PCR amplified *inhA* gene from *C. glutamicum*. The plasmid insertion is expected to destroy the *inhA* gene by disrupting it into two halves. The proximal ends of both halves contain a duplicate *inhA* sequence from the recombinational event. Therefore in Southern hybridisation the *inhA* gene will appear as two new bands or there will be a shift in the respective *inhA* restriction fragment to the higher-molecular-mass position in the gel. Alternatively the suicide vector was also used as a probe to confirm the integration of this into the genomic DNA of the host. Southern hybridisation was performed with the ^{32}P see section (2.3.2.3).

2.4 BIOCHEMICALS METHODS

2.4.1. Analysis of the proteins and enzymes

2.4.1.1 Assay of NADH Oxidase activity

NADH Oxidase activity was calculated according to Osborn *et al.*, 1972. Incubation mixtures contained 50 mM Tris-HCl, pH 7.5, 0.12 mM NADH, 0.2mM dithiothreitol, and 3 to 100 µg of protein in a total volume of 1.0 ml. Cuvettes containing reaction mixtures were placed in the spectrophotometer and cell fractions added quickly to initiate reactions. Control tubes contained Tris buffer, NADH and reaction mixture without enzyme. The rate of decrease in absorbance at 340 nm was measured at 22°C in a Gilford 2400 recording spectrophotometer.

2.4.1.2 Protein concentration assays

Bacterial cells were harvested from cultures, disrupted mechanically by homogenisation and protein concentrations were determined according to the method described by Lowry and co-workers (1951). All buffers and reagents used for protein concentration assays are described in Appendix 3. Bovine serum albumin BSA, was used as a protein standard, (0-100 µg in the sample volume.) to quantify tests in all analyses. To 0.5 ml of

protein sample, 2.5 ml of the alkaline-copper reagent (Lowry C reagent) was added, followed by vortexing and then standing at room temperature for 10 min. After adding 0.25 ml of diluted Folin-Ciocalteau reagent (BDH) (2:3 in deionised water), the contents were mixed thoroughly and allowed to stand at room temperature for another 30 min. The absorbance at wavelength of 750 nm was read for each sample. The amount of protein was determined from a linear standard curve ($R^2 > 0.99$) for 0-100 µg of BSA.

2.4.1.3 Polyacyamide gel electrophoresis

Proteins were resolved by polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). Samples were prepared for electrophoresis by mixing two parts of the sample to one part of the sample buffer and boiling for five min, then immediately transferring onto ice. The preparation was centrifuged for 10 sec to remove debris. About 20 µl of protein sample were applied in each well in 4% stacking gel over a 12% separating gel. Electrophoresis was performed with a discontinuous buffer system in a Mini-Protein-II slab cell (Bio-Rad). The gel was run at a current of 15-30 mM current until the bromophenol blue marker had reached the bottom. Low-range prestained SDS-PAGE standards (Bio-Rad) were used as markers. The polyacrylamide gel was visualised by either Coomassie blue or silver staining and photographed.

A 0.5 mg/ml solution of Coomassie brilliant blue R-250 (Sigma) was prepared by dissolving the dye in five parts methanol before addition of one part acetic acid and four parts Milli-Q water. Gels were stained for 30 min at room temperature with gentle shaking. The gels were rinsed in Milli-Q water and transferred to destaining solution (one part acetic acid, four parts ethanol and five parts Milli-Q water) then gently shaken at room temperature until blue bands and a clear background were obtained. Fresh destaining solution was added if required. The gels were kept in Milli-Q water overnight after destaining, then dried using a Gel Air dryer (Bio-Rad).

The silver staining protocol was followed according to the instruction manual (Pharmacia) as follows. The gel was immersed in the fixing solution (one part acetic

acid, four parts ethanol, and five parts Milli-Q water) for at least 30 minutes. The gel was placed in the incubation solution (60 ml of ethanol, 10.25 g of sodium acetate, anhydrous, 1.04 ml of glutaraldehyde, 0.4 g of sodiumthiosulfate and made up to 200 ml with Milli-Q water) for at least 30 min. The gel was washed three times, each time for 15 min, in Milli-Q water. The gel was then put into the silver solution (0.2 g of silver nitrate, 40 µl of formaldehyde and made up to 200 ml with Milli-Q water) for 40 min. The gel was placed into developing solution (5 g of sodium carbonate, 20 µl of formaldehyde and made up to 200 ml with Milli-Q water) for 5-10 min. The gel was washed in Milli-Q water with one change for 5-10 min then preserved in a GelBond film (Pharmacia) as recommended by the manufacturer.

2.4.2. Isolation and analysis of fatty acids and mycolic acids from cell wall and whole cells

2.4.2.1 Preparation of cell walls

Method 1

Cell walls were initially prepared from *C. glutamicum* strains using the method described by Nikaido *et al.* (1993). The cells were cultured at 30°C overnight in a rotary shaker in multiple one litre flasks. Cells (from 2L culture) were chilled to 5°C, harvested by centrifugation at 2,000 X g for 10 min, and washed once with 60 mM sodium phosphate buffer, pH 7.5, containing 0.1 M NaCl with 1mM phenylmethylsulfonyl flouride, by resuspension and centrifugation. The same buffer was added to the pellet (40 ml buffer per 10 g wet weight), and the resuspended cells were broken by homogenisation for a total of 60 sec using a MSK cell homogeniser (B. Braun, Germany) and 0.1mm glass beads. After the addition of pancreatic deoxyribonucleases and ribonuclease (Promega) (10 µg/ml), the cellular fractions were left at 4°C overnight. The extract was centrifuged at 1,000 X g for 10 min in a Sorvall GSA rotor, and the supernatant was centrifuged again under the same conditions. The supernatant was diluted two-fold with the same phosphate buffer, and centrifuged at 2,500 X g for 40 min. The supernatant thus obtained

was centrifuged at 5,000 X g for 40 min, in a Beckman centrifuge to sediment the cell walls. The pellet was washed twice in 1M NaCl, and once in water, by resuspension and centrifugation. The pellet containing the cell envelops (cytoplasmic membranes and cell wall) were resuspended in 2 ml of water, and was layered onto sucrose step gradient consisting of 15 (1 ml), 30 (2 ml), 40 (4 ml) and 70 (3 ml). The gradient was centrifuged in an SW28 rotor at 27,000 r.p.m for 2 h. The three fractions of the gradients were collected, analysed for protein content by SDS-PAGE and for the presence of NADH-oxidase activity by the method of Osborn *et al.* (1972). These fractions were kept as the 'purified cell wall' fractions at –70°C, after recovery by centrifugation and washing in water.

Method 2

Cell wall fractions of *C. glutamicum* were prepared according to the method of Fujika *et al.* (1985) as follows. The cells were cultured at 30°C overnight in a rotary shaker in multiple 500 ml flasks. After cooling to below 10°C, the cells (from 1 L of culture) were harvested by centrifugation and washed with distilled water. The cells were suspended in distilled water and disrupted mechanically by homogenisation for a total of 60 sec using a MSK cell homogeniser and 0.1mm glass beads. The disrupted cell fraction was mixed with 400 ml veronal buffer pH 7.5, assayed for NADH-oxidase activity and then mixed with 10 mg deoxyribonuclease and 15 mg ribonuclease and stirred at room temperature for 30 min. The enzyme-treated cell suspension was centrifuged at 1,000 X g for 10 min in a Sorvall GSA rotor (twice), and the supernatant thus obtained was diluted two-fold with the same veronal buffer and was then centrifuged at 2,500 X g for 30 min. The supernatant thus obtained was centrifuged at 5,000 X g for 40 min, to sediment the cell wall. This fraction was further washed with acetone, 20% (v/v) Triton X-100 solution and ethanol/distilled water (2:3, v/v), successively. The residue was collected by centrifugation at 16,950 X g, resuspended in distilled water, and the pH adjusted to 5.5 with 6 M HCl. After stirring for 60 min, the suspension was centrifuged at 16,950 X g to obtained crude cell walls. The walls were then incubated with 0.25 g protease (Sigma) at room temperature for 24 h in veronal/HCl buffer pH 9.5 (0.1 M veronal sodium solution

was adjusted to pH 9.5 with 1 M HCl). The walls were sedimented at 16,950 X g, washed with distilled water and 1,2-dichloroethane/ethanol (1:2,v/v), and finally freeze-dried in a Dynavac freeze-dryer unit for 16 h. This material was disrupted using a sterile pessel and mortal and designated as Coryne-CWS.

2.4.2.2 Use of internal standard

Lignoceric acid methyl ester (LAME) $C_{25}H_{50}O_2$, was added at 2 mg/ml into each sample before extraction of mycolic acids. The peak corresponding to the internal standard in gas chromatography (GC) chromatograms appeared between-the last peak of the fatty acids and the earliest peak of the mycolic acids, and did not interfere with the interpretation of data for both fatty and mycolic acid analyses. Three other chemicals (nonacosanoic acid methyl ester, $C_{30}H_{60}O_2$, tricontanoic acid methyl ester, C_{31} H_{62} O_2, hentriacontanoic acid methyl ester, C_{32}) were also tested as internal standard. As reported by Jang (1997), peaks of these compounds appeared between the peaks of either fatty acids or peaks of mycolic acids in the GC chromatogram, but were closer to the peaks corresponding to mycolic acids than seen for LAME (data not shown). For this reason lignoceric acid methyl ester was normally added as internal standard to each sample prior to extraction and all analysis standardised using the area obtained following extraction and derivatisation of LAME.

2.4.2.3 Extraction of long-chained lipid components from whole cells and cell wall preparations

Cells and Coryne-CWS were subjected to acid methanolysis as described by Jang (1997). This procedure was described previously by Minnikin *et al.* (1980), modified by Pierotti (1987) and finally modified by Jang (1997). Cells (100-250 mg, wet weight, depending on the medium) were harvested at stationary phase by centrifugation (2,000 X g, 4°C, 10 min); the cells were stored at –20°C until analysis. Stored cells were lyophilised using a Dynavac freeze-dryer for 16 h. Dried samples of cells were weighed and about 50 mg of whole cells and 200 mg of Coryne-CWS samples were transferred into 5 ml glass tubes

(Wheaton). Since results reported by Jang (1997) showed that both FAME and MAME appeared in GC chromatograms and the internal standard (LAME) also appeared between FAME and MAME peaks (which enable comparison between the amount of FAMEs and MAMEs from the cells) 2 mg/ml of internal standard (LAME) was added into each sample prior to extraction.

Dried cells, cell walls and internal standard were subjected to acid methanolysis as previously described by Minnikin *et al.* (1980). Samples were vortexed after the addition of 3 ml of methanol:toluene:H_2SO_4 (30:15:1, vol/vol/vol) then incubated at 80°C for 16 h in sealed tubes to disrupt the cell wall structure for methanolysis. The reaction mixture was cooled to room temperature and liberated mycolic acid methyl esters (MAMEs) and fatty acid methyl esters (FAMEs) were extracted using petroleum ether (b.p. 60-80°C, 2 ml). After vortexing for 30 sec, the emulsion was allowed to separate for 10 min, then the top layer (petroleum ether) was collected with a Pasteur pipette and neutralised by pipetting directly onto a 1 cm column of ammonium hydrogen carbonate (BDH) (prepared dry in a Pasteur pipette with a cotton wool and pre-washed with diethyl ether [BDH]). This procedure was repeated twice by adding two aliquots of 2 ml of petroleum ether and proceeding as described above. The eluent were combined into 5 ml glass tubes and concentrated under a stream of nitrogen and stored at –20 °C. In his PhD thesis, Jang (1997) had shown that the inclusion of a TLC step to separate MAMES and FAMES was not necessary when analysing TMS derivatives and this gave better quantification of MAMES and FAMES, this step was also omitted here before GC analysis. The control was 100 µl (2 mg/ml) of LAME, which was derivatised and extracted as above. The area of LAME in each sample was compared to that of the control and extraction yields of each sample obtained, peaks were identified by comparison with the retention time and peak area of the internal standards (Sigma 189-6, 189-17) for FAMES.

2.4.2.4 Derivatising and analysis of trimethylsilyl (TMS) derivatives of MAMEs and FAMEs

Purified MAMEs and FAMEs were derivatised to trimethylsilyl (TMS) ethers by dissolving in 400 µl of trimethylsilylimidazole (Tri-sil Z, pierce) at 60°C for 20 min. The TMS derivatives of MAMEs were analysed by GC using a flame ionisation detector (FID) or by GC-MS. The conversion of the MAMEs to TMS ethers protects the molecules from pyrolysis and makes them amenable to conventional GC at high temperatures (Yano *et al.*, 1972).

2.4.2.5 Gas chromatography (GC)-flame ionization detection of TMS ethers of MAMEs and FAMEs

The TMS ethers of MAMEs and FAMEs were analysed by GC using a Varian Star 3400 *CX* gas chromatograph fitted with FID. One µl of the solution was injected onto a 25 m non-polar BPX5 (0.22 mm i.d.; 0.33 mm o.d., fused silica, SGE, Scientific Pty, Ltd, Australia) with split ratio set at 85:1. The oven temperature program was set at 150°C isothermally for one min, increased by 5°C/min to 165°C, then increased to 185°C at 0.3 °C/min. The column temperature was increased to 260°C by 6°C/min, increased by 2 °C/min to 320°C and held at 320°C for 6 min and 50 sec before analysis of the next sample. The injection temperature and the detector temperature were 300°C. Nitrogen gas was used as carrier gas (0.9 ml/min). Injection of samples was carried out by an autosampler (Varien 8200) (needle was washed by nitrogen between injections). The identity of individual TMS ethers of FAMEs was established by comparison of the retention time with those of standard TMS ethers of FAMEs (Sigma) containing known amounts of saturated and unsaturated FAMEs (C_{12}-C_{20}, Sigma). The relative peak proportions of TMS ethers of FAMEs were determined by comparing peak area. All analyses were standardised using the area obtained for the C_{25} internal standards, which was added prior to extraction and derivatisation

Chapter 3

Analysis of the mycolic acid and fatty acid composition of the cell wall of *C. glutamicum* and comparison between parents and mutant strains

3.1 INTRODUCTION

Previous studies have shown that transformation efficiency in corynebacteria remained low even with homologously derived DNA, implying that the cell wall structure of this species could be one of the key parameters in DNA transformation in addition to restriction and modification barriers (Jang *et al.*, 1997). Several workers had shown that growth in the presence of agents which, presumably, modified cell surface structures of coryneform bacteria (penicillin G, glycine, INH, Tween 80) (Haynes and Britz, 1989, 1990; Katsumata *et al.*, 1984; Noh *et al;* Yoshihama *et al.*, 1985) improved transformation of plasmid DNA into these bacterial species. Since INH and glycine are known to alter the mycolic acid composition in mycobacteria and the peptidoglycan structure of the Gram positive cell wall respectively, these chemicals were selected for addition into the growth medium to study the impact of these chemicals on cellular mycolic acid composition and growth rates (Jang *et al.*, 1997).

Prior growth of strain AS019, a rifampicin-resistant derivative of *C. glutamicum* ATCC 13059, in either glycine or INH (at high concentrations) or combinations of these had been shown to increase electroporation frequencies significantly (Haynes and Britz, 1989; 1990). Furthermore, a series of auxotrophic mutants of strain ATCC 13059 had been isolated which protoplasted more readily: two of these mutants, strains MLB133 and MLB194, were studied in the present work. Because of the observed morphological changes seen for MLB133 and MLB194, they were thought to be cell-surface structure mutants (Best and Britz, 1986; Pierotti, 1987). Two species of *Brevibacterium*, *B. lactofermentum* strain BL1 and *B. flavum* BF4, were also included in the study to enable comparisons between different sources of *C. glutamicum,* noting that it has been proposed to consider them as one *C. glutamicum* species (Liebl *et al.*, 1991).

As indicated above, when glycine and INH were added to LBG cultures of AS019, MLB133 or MLB194, Jang *et al.* (1997) showed that several changes in lipid composition occurred: both mutant strains MLB133 and MLB194 contained decreased proportions of $C_{32:0}$ relative to their parent, there was an increase in the proportion of extracellular mycolic acids, the relative percentage of fatty acids to the total lipids also decreased, and the cell wall became thinner (Jang, 1997; Jang and Britz, 2000).

The goal of the research described in this chapter was to obtain an understanding of how growth in the presence of cell wall modifiers (glycine and INH) affected cell wall composition of *C. glutamicum*. Previous studies had noted that the fatty acid composition of *C. glutamicum* AS019 altered when grown in different media but this was not studied in detail. Furthermore, these studies did not differentiate between cell wall and total cellular lipids as analysis was performed on whole cells. So that it was not clear whether fatty acid or mycolic acid synthesis had been altered by glycine and INH.

A method to prepare cell walls of mycobacteria had been described, where this involved differential centrifugation to prepare cell wall fractions free of cell membranes. Therefore a major aim of this chapter was to try this method with *C. glutamicum* and obtain some structural analysis of fatty acid composition of the cell wall. An important feature of this method when used with mycobacteria was tracking the protein profile of both the cell wall and cell membrane. NADH oxidase is a cell membrane protein which should be enriched in cell membrane or whole cell fractions but not in the cell wall (Nikaido *et al.*, 1994). This assay was therefore used to determine the success of the fractionation procedure in *C. glutamicum*. Cell wall fractions were also washed with several detergent solutions and finally digested with proteinase to solubilize the cell membrane so that only cell wall bound lipids would remain for analysis.

Knowing that the cell wall fatty acids had changed would have been useful in preparing models of how the mutants have changed. Our preliminary experiments included determining the minimal inhibitory concentrations of INH and ETH, a structural analogue of INH, for C. *glutamicum* strains and *Brevibacterium* species. This was undertaken to

validate the phenotypes of the strains under study using solid media (noting that the previous MICs were determined in LBG) and to test whether the previously observed differences in INH sensitivity were also seen for ETH.

3.2 DETERMINATION OF MICs FOR INH AND ETH for *C. GLUTAMICUM* AND RELATED SPECIES

MICs were determined by using a multi-prong replicator to transfer small drops of overnight cultures of all isolates onto NA plates containing INH at concentrations of 0 to 18 mg/ml at increments of 1mg INH/ml. Cultures were used either undiluted ("heavy inocula") or diluted up to 10^{-9} in 0.1% tryptone water ("light inocula") to determine the MICs for whole cultures and single cells. Tests were performed in duplicate. Examples of the tests were photographed and these are shown in Figures 3.1A and 3.1B.

Plates were observed after 24 to 48 h incubation at 30°C and growth was arbitrarily scored and recorded (Table 3.1): MICs were defined as the lowest concentration of INH which first significantly decreased growth of single colonies (light inocula). The following observations were made:

- The parent strain of *C. glutamicum*, AS019, had the highest MICs for INH among all of the strains tested;
- The two mutants strains MLB133 and MLB194 both had lower INH MICs relative to the AS019, confirming their known phenotype;
- *csp1* disrupted mutant of *C. glutamicum* (strain RES467) also showed lower MIC, and in this respect was more like MLB133 and MLB194;
- The RM4 (restriction deficient mutant) showed more sensitivity to INH relative to AS019;
- other *C. glutamicum* strains, CG2 and ATCC 13032, were more like MLB133 and MLB194, in that they had lower MICs then AS019;

- BL1 showed the highest sensitivity to INH, however, BF4 showed less sensitivity to INH and the MICs determined were more similar to AS019;
- The above results were consistent both with previously recorded tests in liquid media and with the observed ease of protoplasting and transformation seen for these strains (Jang and Britz 2001).

MICs for ETH were determined similarly using NA plates which contained ETH at concentrations of 0.1 to 10 mg/ml. The amount of drug increased at a concentration of 0.2 mg/ml up to 1mg/ml and then at increments of 1 mg/ml to 10mg/ml. Examples of some of the tests were photographed and these are shown in Figure 3.2. MICs were defined as the lowest concentration of the ETH that first significantly inhibited the growth of light inocula of the *C. glutamicum* strains tested. MICs varied from strain to strain and important observations are summarised below.

For all of the strains tested, growth in the presence of ETH was inhibited at lower concentrations than seen for INH. MICs were therefore repeated using plates prepared with concentrations at smaller increments and over a lower concentration range: data for several strains is summarised in Table 3.2. In contrast to the data seen for INH, MICs for all of the strains were similar. This meant that the differential sensitivity seen when using INH could not be detected when using ETH, which was reported as a more potent drug when tested using mycobacteria.

- AS019 had the highest MICs for ETH of the strains tested, followed by BF4;
- MLB194 and MLB133 both had slightly lower ETH MICs relative to AS019;

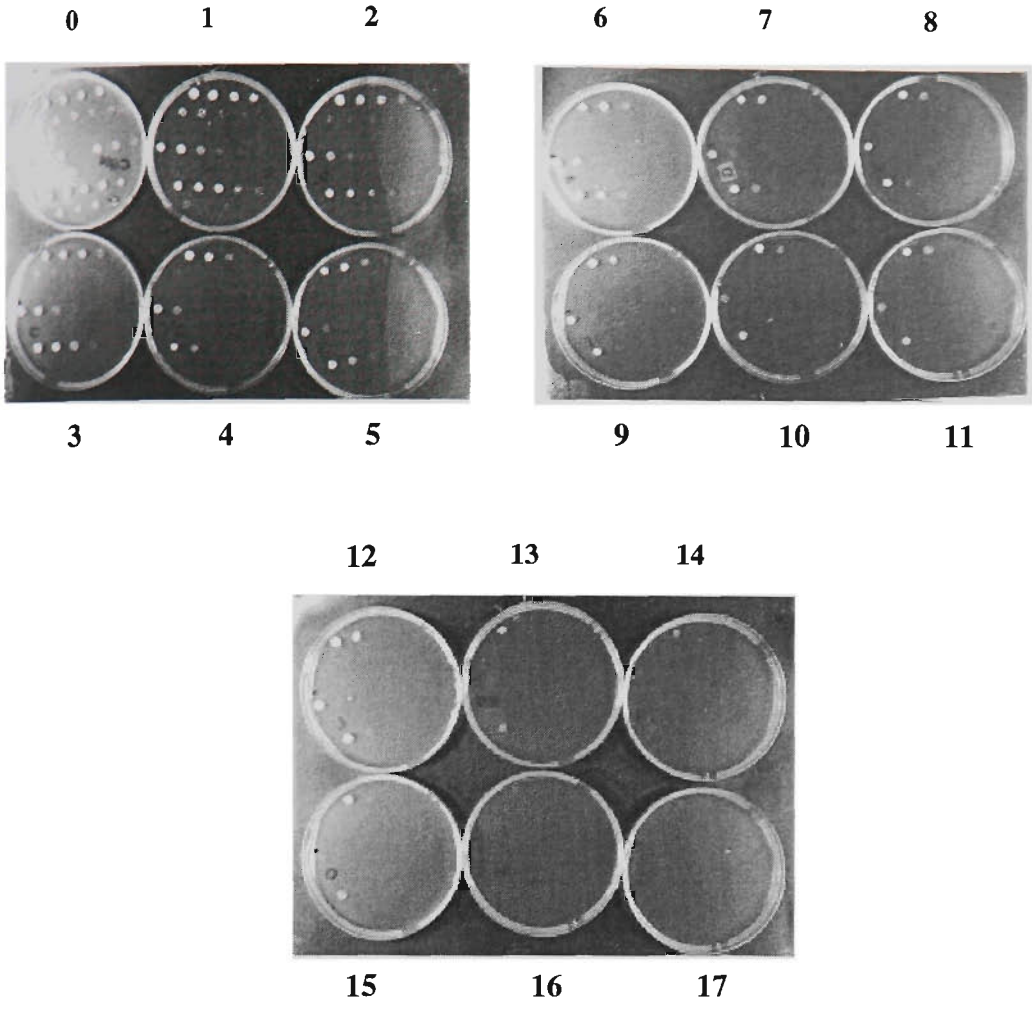

Figure 3.1A Photograph of the MICs plates for AS019, MLB194, MLB133, where a multi-prong replicator was used to transfer small drops of overnight culture and dilutions onto INH-containing NA plates. These plates have increased INH concentration from 0 to 17 mg/ml and were photographed after 24 h at 30°C. Bacterial isolates were as follows (identical on each plate).

Row 1-2 Strain AS019 undiluted then diluted 10^{-2} to 10^{-9}.

Row 3 Strain MLB 194 undiluted then diluted 10^{-2} to 10^{-6}

Row 4-5 Strain MLB133 undiluted then diluted 10^{-2} to 10^{-9}

Photograph 1, 2, 3, Top rows of plates contained 0, 1, 2; 6, 7,8; 12, 13, 14 mg/ml INH

Photograph 1, 2, 3, 2nd row of plates contained 3, 4, 5; 9, 10, 11; 15, 16, 17 mg/ml INH

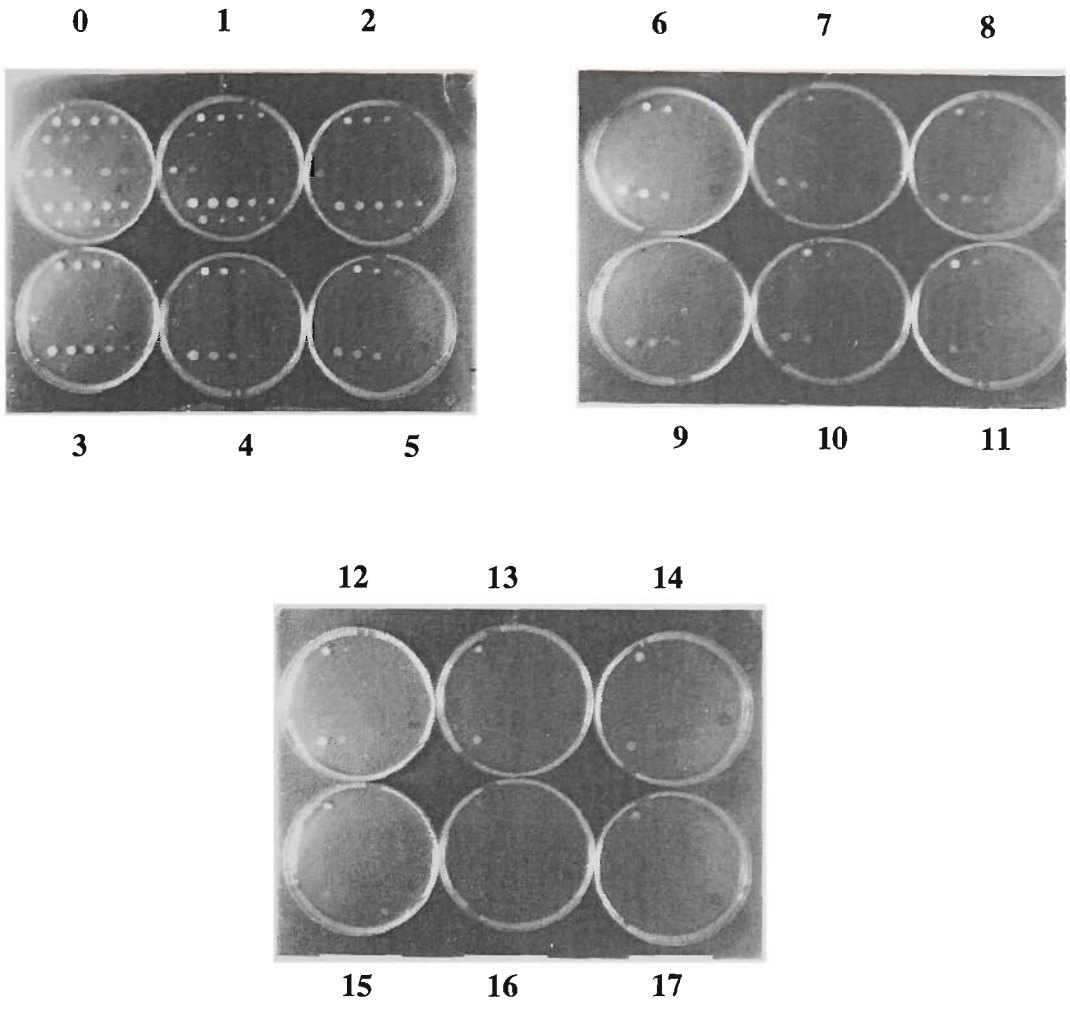

Figure 3.1B Photograph of MICs plates for AS019, BL1 and BF4, where a multiprong replicator was used to transfer small drops of overnight culture onto INH containing NA plates. The plates have increasing INH concentrations from 0 to 17 mg/ml. Bacterial strains used were as follows (identical on each plate). From left to right;

Row 1-2 Strain AS019, undiluted, then diluted 10^{-2} to 10^{-9}

Row 3, Strain BL1 undiluted then dilution 10^{-2} to 10^{-6}

Row 4-5 Strain BF4 undiluted then dilution 10^{-2} to 10^{-9}.

Photograph 1, 2, 3, Top rows of plates contained 0, 1, 2; 6, 7,8; 12, 13, 14 mg/ml INH

Photograph 1, 2, 3, 2nd row of plates contained 3, 4, 5; 9, 10, 11; 15, 16, 17 mg/ml INH

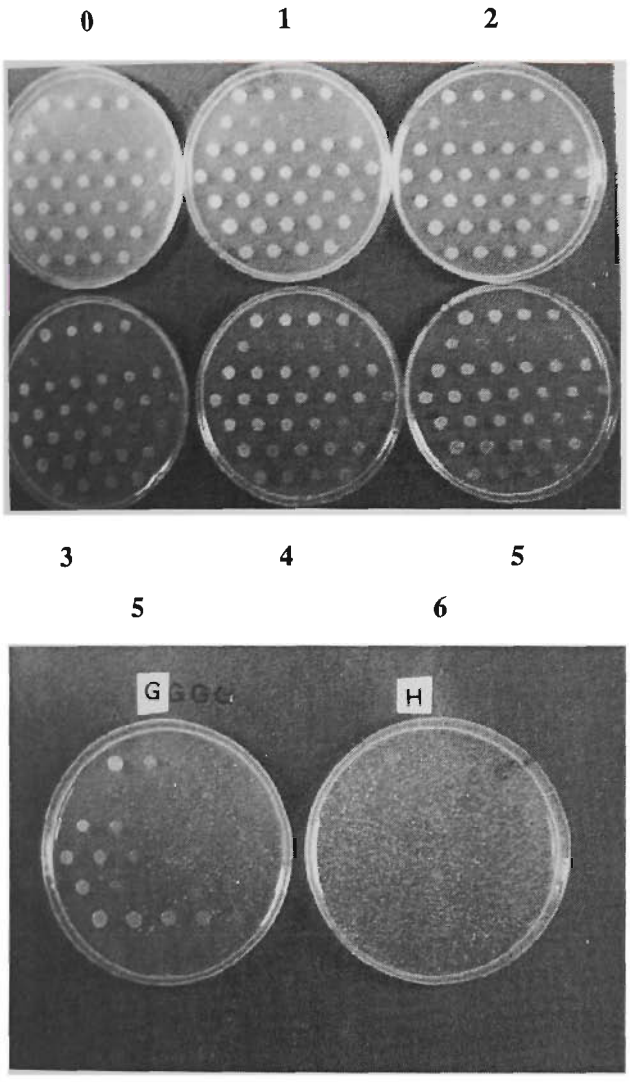

Figure 3.2 Photograph of ETH MICs plates for several *C. glutamicum* strains where a multi-prong replicator was used to transfer small drops of overnight culture onto ETH-containing plates. The plates have increasing ETH concentrations (from 0 to 4 mg/ml). Bacterial strains used were as follows (identical on each plate). From left to right:

Row 1, 2 Strain AS019 undiluted then diluted 10^{-2} to 10^{-9}

Row 3 Strain MLB194 undiluted then diluted to 10^{-2} to 10^{-6}

Row4 Strain MLB133 undiluted then diluted 10^{-2} to 10^{-7}

Row 5 Strain BL1 undiluted then diluted 10^{-2} to 10^{-6}

Row 6-7 Strain BF4 undiluted then diluted 10^{-2} to 10^{-9}

Photograph 1, Top rows of plates contained 0, 0.4, 0.8

Bottom rows of plates contained 1, 2, 3 mg/ml of ETH

Photograph 2 contained 4 and 5 mg/ml ETH respectively.

Table 3.1 INH MICs for *C. glutamicum* strains.

Strain	INH concentration in mg/ml																				MICs mg/ml[a]
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
AS019	[b]+++	+++	+++	+++	++	++	++	++	++	++	++	+	+	+	+	+	....	.....	–	–	11
MLB194	+++	+++	+++	++	++	++	+	+	+	+	+	+	+	..	–	–	–	–	–	–	6
MLB133	+++	+++	+++	++	++	+++	++	+	+	+	+	+	–	–	–	–	–	–	–	–	7
RM4	++	++	++	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	3
Csp1 mutant	+++	+++	+++	+++	++	++	++	++	++	++	+	+	+	–	–	–	–	–	–	–	10
CG2	+++	+++	+++	++	++	++	++	++	++	+	+	+	+	+	–	–	–	–	–	–	9
ATCC13032	+++	+++	+++	+++	++	++	+	+	+	+	+	+	+	+	+	+	..	–	–	–	7
BL1	+++	++	++	+	+	..	·	–	–	–	–	–	–	–	–	–	–	–	–	–	4
BF4	+++	+++	+++	+++	+++	+++	++	++	++	++	+	+	+	+	+	+	..	–	–	–	10

[a] Data for MICs determines using light inocula, averages for replicate plates.

[b] Symbols used +++ = good growth, ++, +=less growth, ·· = growth just visible, –no growth

Table 3.2 MICs of *C. glutamicum* on medium containing ETH (ethionamide)

Strains	ETH Concentration in mg/ml																			MICs
	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1	2	3	4	5	6	7	8	9	10	mg/ml[a]
AS019	[b]+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+	+	+	–	–	–	–	3
MLB194	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+	–	–	–	–	–	–	–	2
MLB133	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+	–	–	–	–	–	–	–	2
CG2	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+	–	–	–	–	–	–	–	2
ATCC13032	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+	–	–	–	–	–	–	–	2
BL1	+++	+++	+++	+++	+++	+++	+++	++	++	+++	+	+	–	–	–	–	–	–	–	1
BF4	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+	–	–	–	–	–	–	3

[a] Data for MICs determines using heavy inocula

[b] Symbols used +++ = good growth, ++, +=less growth, ··= growth just visible, ⁻ no growth

- BL1 was the most sensitive strain, which was consistent with its relative sensitivity to INH.

3.3 EVALUATION OF THE METHODS TO PREPARE CELL WALL FRACTIONS OF *C. GLUTAMICUM*.

Results reported by Jang *et al.* (1997) had concentrated on the effects of INH and glycine on the mycolic acid composition and location (cellular and extracellular) for two families of *C. glutamicum* strain, ATCC 13059 and ATCC 13032, and their mutants. However, these studies could not differentiate between changes in the lipids at the cell wall in relationship to changes in whole cellular lipids as no sub-cellular fractionation was attempted. Changes in the mutants obtained from ATCC13059 could have occurred at different parts of the mycolic acid biosynthesis pathways:

(a) Biosynthesis of the fatty acids, destined for incorporation into both mycolic acids and membrane lipid structures;
(b) Assembly of the fatty acids into mycolic acids;
(c) Attachment of the mycolic acids to arabinogalactan at the cell surface, noting that extracellular mycolic acids increased for the mutants and after growth in glycine/INH;
(d) Cross-linking between mycolic acids;
(e) Other interactions between free and bound mycolic acids and free fatty acids at the cell surface.

Consequently, obtaining further information on the composition of the cell wall, in relation to analysis of whole cells, may have provided further insight into the nature of the mutations in MLB133 and MLB194, and the site of action of glycine and INH in altering cell wall composition.

Several methods for preparing cell walls had been described for mycobacteria, nocardia and rhodococcus species (Fujioka *et al.*, 1985; Shigetaka *et al.*, 1986; Nikaido *et al.*, 1991). These involved various fractionation procedures, often with differential

centrifugation, to obtained cell wall fractions free from cytoplasmic membrane. The methods described by Nikaido *et al.* (1991) and Fujioka *et al.* (1985) were tested here with *C. glutamicum.*

3.3.1 Preparation of cell wall

Experiment 1: Preparation of cell wall using Nikaido *et al.* (1993) method.

First a method described by Nikaido *et al.* (1993) was used without modification for the preparation of cell wall fractions (see section 2.4.2.1 for detail). Crude cell walls were obtained after three successive low-speed centrifugation steps were washed with distilled water and layered onto a sucrose step gradient using 15, 30, 40, 70 % (w/v) steps. Instead of two bands as anticipated in Nikaido's experiments with mycobacterial walls, four to five bands were seen with corynebacterial walls. The top brown and two bottom light brown fractions were collected for assay of NADH oxidase activity using the method of Osborn *et al.* (1970) (data not shown) and protein content were analysed by SDS-PAGE. The results of the experiment are briefly summarised in Fig 3.3 (A, B). The top fractions showed maximum NADH oxidase activity and complex protein profiles (cytoplasmic membranes) while the two bottom fractions showed almost negligible activity. However, the yields of cell wall in these fractions were too low and did not meet my requirements which involved analysing the mycolic acid and fatty acid composition of cell wall fractions by GC. Therefore this method was not considered to be a good one for *C. glutamicum* despite its use by many workers (Benz *et al.*, 1993, 1994; Nikaido *et al.*, 1994) for mycobacteria. This method was not further used and other methods which involve the use of detergents were tested, as these were reported to give better yields Fujioka *et al.* (1985)..

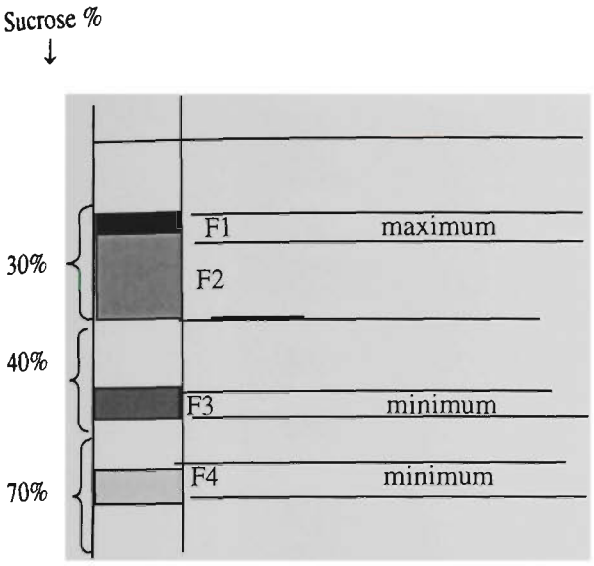

Figure 3.3 A Fractions formed in a sucrose-step gradient of the cell envelope from *C. glutamicum* strain AS019. The sucrose concentration before centrifugation is indicated to the left of the tube. The different zones formed in the tube after centrifugation were clearly distinguishable by their colours. Only three fractions were collected and NADH-oxidase activity was measured. The F1 layer showed miximum NADH oxidase activity, and F3 and the lower bottom layer showed minimum NADH oxidase activities.

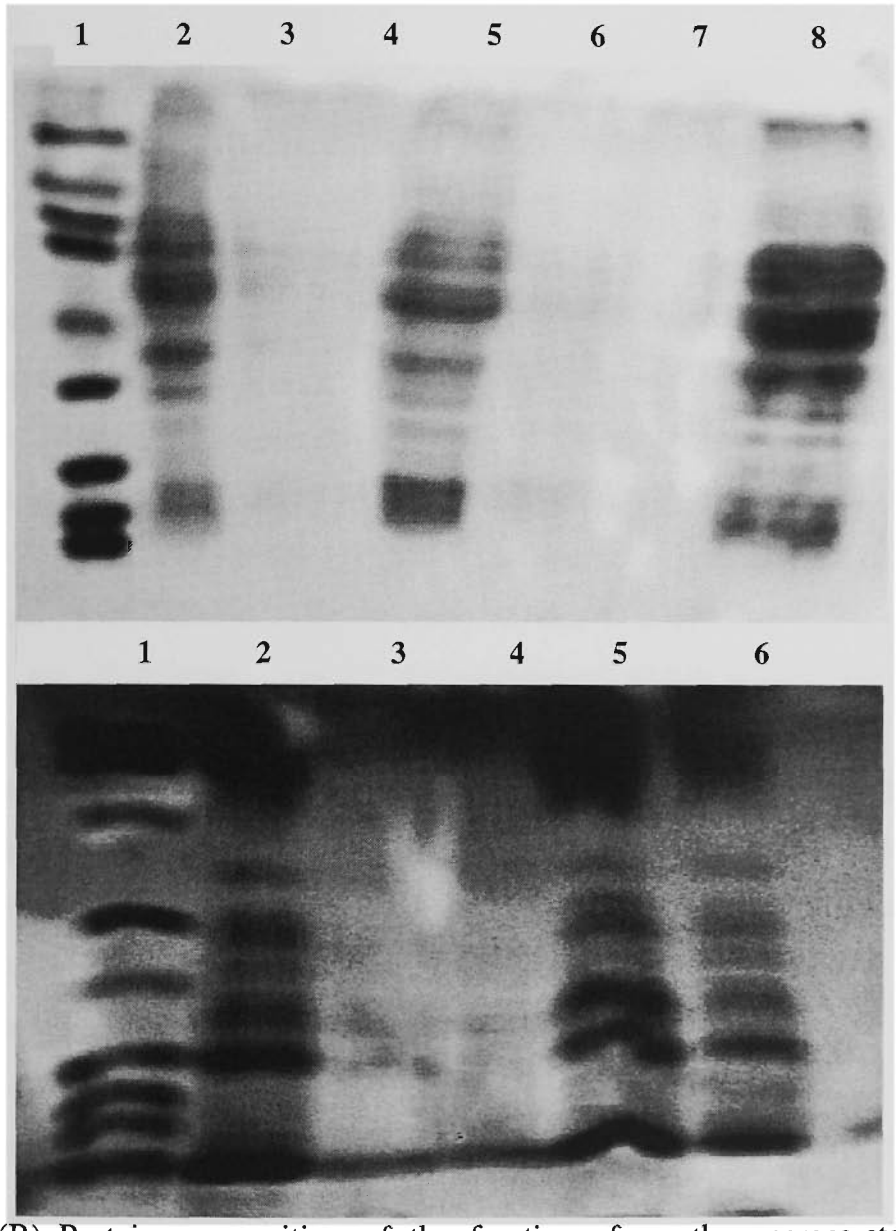

Figure 3.3 (B) Protein composition of the fractions from the sucrose-step gradient centrifugation.

Top gel: Lane 1 Broad-range SDS-PAGE standards (209, 124, 80, 49, 34.8, 28.9, 20.6, 7.1); lanes 2,3,4 contain fractions F1, F3, F4 from AS019; Lanes 5,6,7 fractions F1, F3, F4 from MLB194; lane 8 fraction F1 from MLB133.

Bottom gel: Lane 1 Broad-range SDS-PAGE standards (209, 124, 80, 49, 34.8, 28.9, 20.6, 7.1); lanes 2,3,4 fractions F1, F3, F4 from MLB133; lanes 5, 6 fractions F1 from AS019 and MLB194.

Experiment 2: Preparation of cell wall fractions using a method modified from Fujioka *et al.* (1985).

A method was developed (see section 2.4.2.1) which utilised the same initial steps as in Nikaido's method, until the preparation of crude cell wall. Briefly, AS019 cells were chilled to 5°C, harvested and homogenised, then protein concentrations and NADH-oxidase activities were determined. The fractions were then treated with RNase and DNase and subjected to three low-speed centrifugation steps and finally crude cell-wall fractions were obtained by sedimentation. It has been found previously that low speed centrifugation steps removed unbroken cells completely with minimal sedimentation of the cell wall (Nikaido *et al.*, 1993). The cell wall fractions is then obtained as a pellet after centrifugation at 5,000 x g for 40 min, leaving behind most of the cytoplasmic membrane in the supernatant. Despite the significant enrichment in cell walls, this fraction is normally contaminated with cytoplasmic membrane, as shown by the level of NADH oxidase activity, although the levels present were much less than found in the supernatant fraction (Table 3.3 A, B. The results showed that after several low speed centrifugations steps the crude cell wall fractions had reduced level of NADH oxidase activity, indicating that a preparation of the cytoplasmic membrane have been removed when compared with the crude cell lysate). These fractions were further purified as described by Fujioka *et al.* (1985). The use of detergent in this method solubilised the cytoplasmic membrane and SDS-PAGE was also performed to detect residual proteins during the preparation of the cell wall fraction (Fig 3.4). The results are shown in Fig 3.4 but since no control has been run it is difficult to predict how much protein was removed from the cell wall during subsequent washing in detergent. These fractions were further subjected to protease treatment which completely remove the protein component of the cell wall, leaving behind lipids and other components. The walls were sedimented after a high speed centrifugation step and washed with 1,2-dichloroethane/ethane and finally freeze-dried. This material was disrupted using a mortar and pestle, and designated Coryne-CWs (coryne-cell walls). Mycolic acid extraction from the cell wall was done initially using the extraction procedure described by Fujioka *et al.*, (1986) but later on the

Table 3.3 NADH-oxidase in cellular fractions of *C. glutamicum* strains.

a-NADH-Oxidase activity of supernatant obtained after centrifugation at 5,000 x g for 40 min.

Strain	NADH-Oxidase activity µmol/min/mg of protein
AS019	40.92
MLB194	25.31
MLB133	33.10

b NADH-oxidase activity of crude cell wall fractions (pellet obtained after centrifugation at 5,000 x g for 40 min.)

Strain	NADH-Oxidase activity µmol/min/mg of protein
AS019	9.73
MLB194	4.08
MLB133	11.15

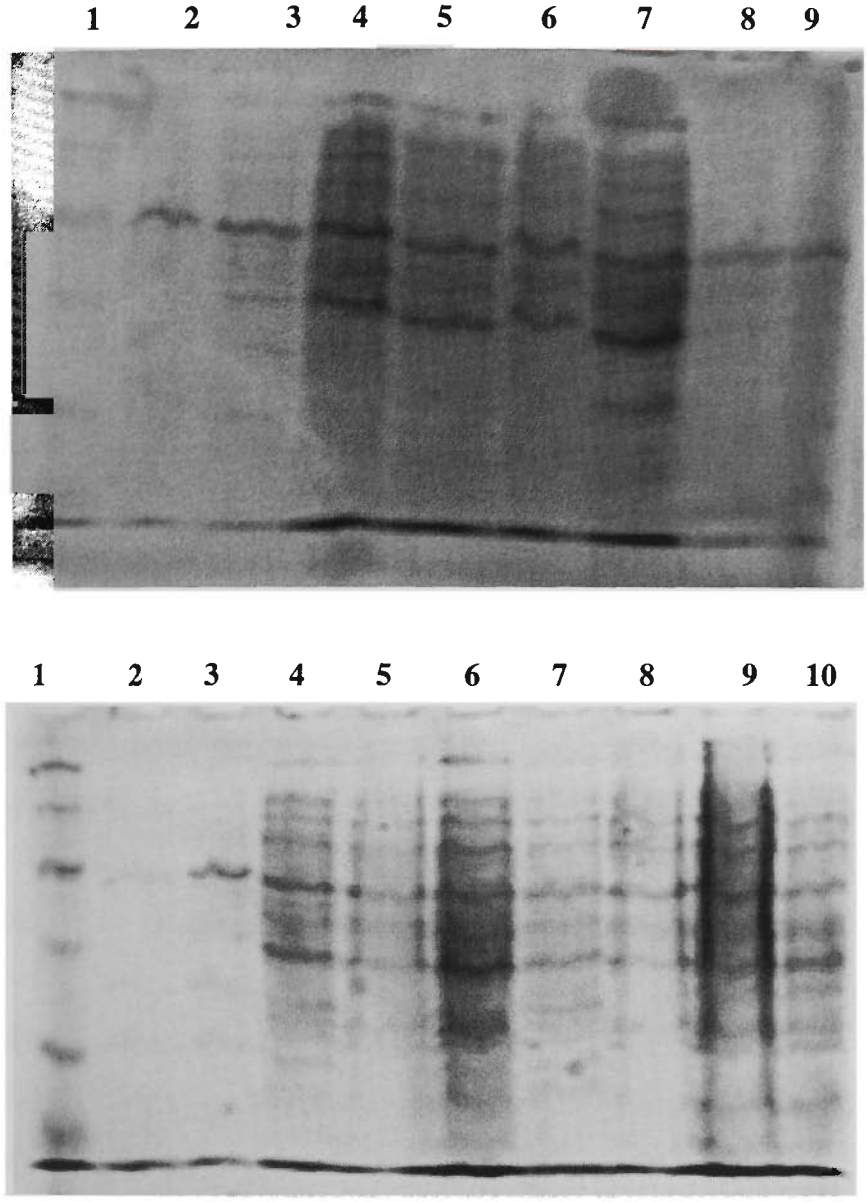

Figure 3.4 SDS-PAGE analysis of crude CW proteins (after 3 low speed centrifugation steps).

A; Lane 1 Low-range prestained SDS-PAGE standards (103, 77, 50, 34.3, 28.8, 20.7); Lanes 2, 3, 4 AS019 (LBG-I, LBG-G, and LBG); Lanes 5, 6, 7 BL1 (LBG-I, LBG-G, and LBG); Lanes 8, 9 BF4 (LBG-I, LBG-G).

B; Lane 1; Low-range prestained SDS-PAGE standards (103, 77, 50, 34.3, 28.8, 20.7); Lanes 2, 3, 4 AS019 (INH, glycine, LB); Lanes 5, 6, 7 MLB133 (LBG-I, LBG-G, and LBG); Lanes 8, 9, 10 MLB194 (LBG-I, LBG-G, and LBG).

extraction procedure developed by Jang (1997) was used as this gave better quantification of mycolic acids and omitted purification of these by TLC, shortening the procedure.

3.3.2 Standardisation of quantitative methods

3.3.2.1 Quantification of mycolic acids by gas chromatography (GC)

In order to determine the relationship between the amount of mycolic acids injected and peak areas detected, several injections were carried out using mycolic acids extracted from cells and samples of several dilutions injected (Fig 3.5). Samples were diluted with pyridine because the derivatizing agent (Tri-Sil-Z) was solubilised in pyridine. As shown in Fig. 3.5, the relationship between the peak area and amount of TMS ethers of MAMEs applied was linear, with peak area ranging between <20,000 to 120, 000. In most cases, samples described in the present study contained amounts of mycolic acids within this range of peak area.

3.3.2.2 Reproducibility of the analysis between experiments

Analysis of the extracts prepared from the cells of strain AS019 (grown in LBG) was performed twice to determine variations in quantification between samples. Three separately grown cultures were harvested in early stationary phase and mycolic acids were separately prepared from the cells using the procedure described in section 2.4.2.3. The qualitative mycolic acid profile obtained in the two analyses were the same for the five types of mycolic acid, but there were quantitative variations, related to the relative proportion of each mycolic acid detected (Table 3.4). Variation was found for the compound $C_{32:0}$ (62.53 to 64.38), $C_{34:1}$ (18.45 to 19.79), $C_{34:0}$ (8.35 to 10.90) $C_{36:2}$ (3.08 to 3.60) and $C_{36:1}$.(2.76 to 3.10). In the case of repeated injections (twice using the same sample), the variation in peak area was less than ±2 % between injections.

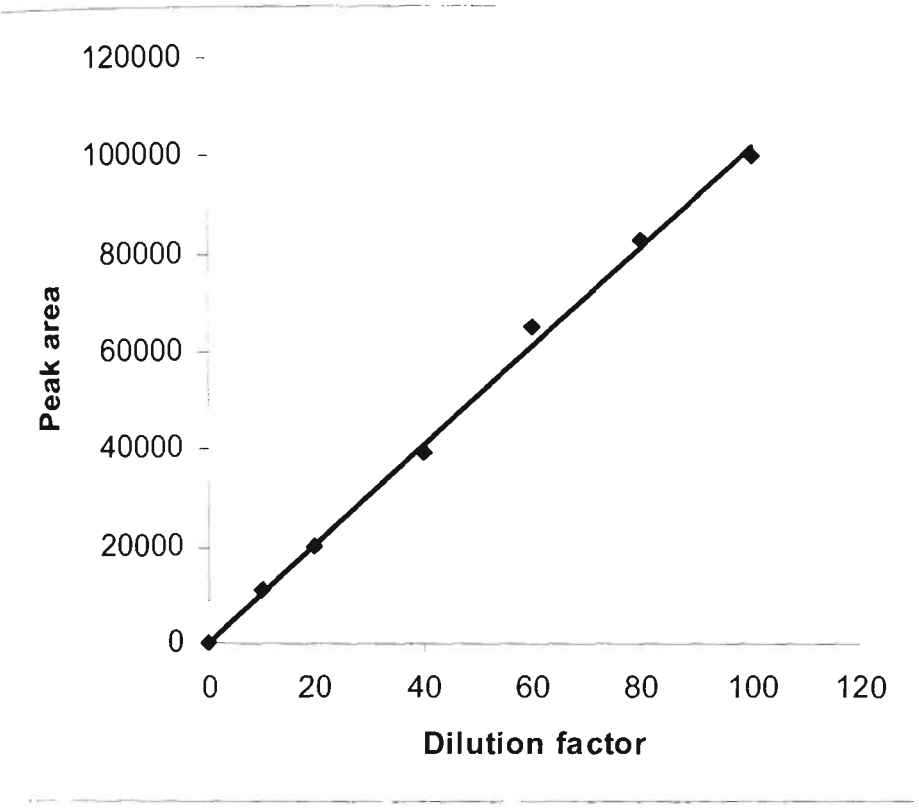

Figure 3.5 Relationship between the amount of mycolic acid from whole cells and peak area detected using gas chromatography. Cells of *C. glutamicum* AS019 were grown in LBG and whole cells extracted as described in section 2.4.2.3. The liberated mycolic acids were converted to TMS ethers as described in section 2.4.2.4. The MAMEs were diluted from samples with dilution factors of 5, 10, 50, 100 and 1,000 and analysed by GC. The same amount of each sample from each dilution was injected onto the column.

Table 3.4 Evaluation of reproducibility of quantitative analysis of mycolic acids composition between different experiments using *C. glutamicum* AS019 grown in LBG.

Cultures containing 500 ml of media were grown in 1litre flask at 30°C and harvested at early stationary phase. Mycolic acids were extracted from dried cells and from the cell wall fractions prepared as described in section 2.4.2.1. After acid methanolysis of the dried cells, samples were extracted three times using petroleum ether, dried under N_2 gas, silylated to TMS ethers and then analysed by GC.

	Mycolic acids type[b]				
N[a]	$C_{32:0}$	$C_{34:1}$	$C_{34:0}$	$C_{36:2}$	$C_{36:1}$
Whole cell					
1	62.53	19.79	10.9	3.60	3.10
2	63.77	18.85	9.30	3.25	2.76
3	64.38	19.95	8.35	3.08	3.10
Average	63.56	19.53	9.51	3.31	2.99
Standard deviation	0.43	0.59	1.31	0.26	1.96
CCW					
1	30.16	41.95	7.41	15.95	4.1
2	31.16	44.51	5.95	15.11	3.25
3	30.58	44.71	5.95	15.39	3.34
Average	31.73	43.7	6.43	15.48	3.56
Standard deviation	0.50	1.53	0.84	0.59	0.60

a N is experiment number. Data was from three separately grown cultures and mycolic acids were separately prepared.

b Proportion of each TMS derivatives of the MAMEs was calculated as a % of the total in terms of peak area detected, correcting for variation in injection using the internal standard and using three extractions with petroleum ether.

3.3.3 Preparation of mycolic acids using Fujioka *et al.*, (1986)

Initially the method of Fujioka *et al.* (1986) with some modification was used to extract the mycolic acids from the cell wall fractions. About 200 mg of cell wall of *C. glutamicum* strain AS019, prepared after growth in LBG, was mixed with and hydrolysed with 5 ml of KOH in methanol at 100 °C for 3 hrs in a sealed tube. After acidification with 10 ml 1M HCL, the sample was extracted three time with 25 ml diethyl ether. The combined ethereal solution was rinsed twice with water and then evaporated to dryness. The TLC step for the further purification was omitted and the dried extract was silylated as described by Jang (1997) and mycolates were analysed by GC. Using the GC conditions described in section 2.4.2.5, FAMEs and MAMEs were identified by comparison with the retention time and peak area of the external standards (Sigma 189-6, 189-17). Similar FAMEs and MAMEs patterns were observed both for cell wall and cells, however, the yield was very low (data not shown). Among the five major mycolic acids, two components of the mycolic acids fraction ($C_{32:0}$ and $C_{34:1}$) made up to 72.01 to 76.91% of the total composition in the cell wall of AS019. This method was not very convenient and losses may have occurred due to the many steps used. Therefore it was decided to use the method described by Jang (1997) as it was simple, more accurate and gave 95% recovery yield of MAMEs.

3.3.4 Preparation of mycolic acids by the method of Jang (1997)

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After the crude cell wall was obtained from AS019 grown in LBG by the method of Fujioka *et al.* (1985), the wall was incubated with protease at room temperature for 24 hrs in veronal/HCl buffer pH 9.5 [0.1 M sodium solution was adjusted to pH 9.5 with 1M-HCl]. The wall was sedimented at 16,950 x g, washed with distilled water and 1,2-dicholoroethane/ethanol (1:2, v/v), and finally freeze dried. Cell wall (Coryne-CWs) fractions were disrupted mechanically using a mortar and pestle and were further processed for mycolic acids extractions according to the method as described by Jang (1997).

Due to the frequent use of the GC column by several people, the column had to be completely cleaned before analysis to avoid any chances of contamination by the presence of chemicals in the column from previous analysis by others. The temperature gradient was adjusted (section 2.4.2.5) in such a way that both FAMEs and MAMEs appeared in the same chromatogram. The first peak from TMS ethers of MAMEs was eluted at above 280°C and all the peaks corresponding to FAMEs appear before MAMES. In most cases, there were no or few background peaks detected above 280°C.

3.4 MYCOLIC ACID COMPOSITION IN WHOLE CELLS AND CELL WALL FRACTIONS FOLLOWING GROWTH IN DIFFERENT MEDIA

The procedure used for the extraction of mycolic acids and fatty acids from whole cells and the cell wall fractions is described in detail in section 2.4 2.3. Although the nature of the two samples were different, once the samples were freeze dried, the same procedure was used for the extraction of mycolic and fatty acids from both whole cells and cell walls.

3.4.1 LBG

Mycolic acid profiles of whole cells and cell wall fractions from several corynebacterial strains after growth in LBG are shown in Table 3.5. Among the five major mycolic acids seen in all of the strains, two components $C_{32:0}$ and $C_{34:1}$ made up to 73.1 to 86.4% of the total composition in all strains. Although the type of mycolic acids detected were the same for all strains, difference in the relative amounts of mycolic acids were seen: the abundance of $C_{32:0}$ was higher in strain AS019 (63.5%) than in mutant strains MLB133 and MLB194, which agreed with data reported previously by Jang (1997) for whole cells. In terms of relative proportions of unsaturated to saturated mycolic acids for AS019 and its mutants, significant differences were observed: strain AS019 had relatively low proportions of unsaturated mycolic acids (27.2 %), whereas the two mutants had higher proportions of unsaturated mycolic acids, ranging from 40.6% in MLB133 to 39.1% in

MLB194 in whole cells. The two *Brevibacterium* strains showed mycolic acids profiles similar to MLB133.

The relative proportions of the different types of mycolic acids found in the cells and cell walls were quite different for all the strains tested: the proportion of unsaturated mycolic acids found in the cell wall fraction was 60.0% for AS019 and 63.3% for MLB133. In the cell wall fractions, the proportion of $C_{32:0}$ was relatively lower, which corresponded to an increase in the relative proportion of unsaturated mycolic acids compared to whole cells. The abundance of $C_{32:0}$ was higher in the cell wall fractions of MLB194 (39.6 %) than seen for AS019 (31.7%) and MLB133 (30.6%). The two *Brevibacterium* strains showed similar proportions of cell wall mycolic acids, where the level of unsaturated mycolic acids ranged from 56.4% in BL1 to 63.3% in BF4; the level of $C_{32:0}$ in BL1 and BF4 was 33.4% to 33.7% respectively. The differences in the relative proportion of certain mycolic acids in whole cells compared with cell walls showed that treatment with solvents, detergent and enzymes removed the free extractable mycolic acids, leaving behind mycolic acids in bound form. These results suggest that differences in MA composition between strains arise largely due to differences in the non-bound MA.

3.4.2 LBG-glycine

In order to determine if growth in 2% glycine had any affect on the mycolic acid profiles of the strains, cells were grown in the presence of glycine and samples prepared as above. The results of analysis of mycolic acids in whole cells and the cell wall fractions are shown in Table 3.6. The relative proportions of unsaturated mycolic acids in whole cells ranged between 28.3% for AS019 to 38.3% for MLB133. The results showed that growth in LBG-glycine had little impact although the mutant strain on the relative proportion of the mycolic acids detected in whole cells of AS019, MLB133 exhibited a small increase in the proportion of $C_{32:0}$ when compared to cells grown in LBG (Table 3.5). The data in agreement with results previously reported by Jang (1997), which show that glycine mainly increased the proportion of extracellular mycolic acids.

Table 3.5 Mycolic acid composition of whole cells and cells wall fractions following growth in LBG.

Strains	Mycolic acids[a]					% of UM to TM[b]
	$C_{32:0}$	$C_{34:1}$	$C_{34:0}$	$C_{36:2}$	$C_{36:1}$	
Whole cells						
AS019	63.6	20.9	9.5	3.3	3.0	27.2
MLB133	48.5	28.8	6.4	7.9	3.9	40.6
MLB194	54.8	31.6	6.1	5.7	3.3	39.2
BL1	45.2	30.0	10.9	9.74	3.7	43.5
BF4	41.3	31.8	11.3	9.2	3.1	44.11
Cell Wall						
AS019	31.7	40.9	7.4	15.0	4.1	60.0
MLB133	30.6	41.8	6.1	17.9	3.7	63.3
MLB194	39.6	40.2	8.6	13.0	4.5	57.5
BL1	33.4	41.8	9.2	11.4	4.3	56.4
BF4	33.7	38.7	9.4	13.2	4.5	63.3

[a] Proportion % of each mycolic acids found in whole cells and the cell wall fractions. The proportion of each mycolic acids was calculated as a % of the total in terms of peak area detected.

[b] The ratio of UM (unsaturated mycolic acids, $C_{34:1}$, $C_{36:2}$, $C_{36:1}$) to TM (sum of saturated and unsaturated mycolic acids) was determined by dividing the sum of the peak areas obtained for unsaturated mycolic acids by the sum of the total mycolic acids.

Table 3.6 Mycolic acid composition of whole cells and cell wall fractions following growth in LBG-glycine (2%).

strain	Mycolic acids[a]					% UM to TM[b]
	$C_{32:0}$	$C_{34:1}$	$C_{34:0}$	$C_{36:2}$	$C_{36:1}$	
Whole cells						
AS019	58.4	22.6	13.2	2.8	2.9	28.3
MLB133	54.6	27.4	8.7	7.1	3.7	38.3
MLB194	51.0	20.9	16.3	3.4	3.4	27.8
Cell Wall						
AS019	55.7	26.1	13.8	3.0	3.3	30.3
MLB133	55.9	25.4	13.7	2.8	2.5	30.5
MLB194	47.9	25.6	11.0	11.3	4.2	42.7

[a] Proportion (%) of mycolic acids found in whole cells or the cell wall fractions. The proportion of each mycolic acid was calculated as a % of the total in terms of peak area detected.

[b] The ratio of unsaturated mycolic acid, ($C_{34:1}$, $C_{36:2}$, and $C_{36:1}$) to TM (sum of saturated and unsaturated mycolic acids) was determined by dividing the sum of the unsaturated mycolic acids values by the sum of the total mycolic acids.

The relative proportions of five types of mycolic acids found in the cell wall fraction for all strains tested were quite similar to those seen in whole cells, in contrast to seen for cells grown in LBG. The proportion of unsaturated mycolic acids ranged between 30.3% in AS019 to 42.7% in MLB194, and 30.5% in MLB133 after growth in LBG-G. This represented a significant decrease in the relative proportion of unsaturated mycolic acids in the cell wall fractions of the cells grown in 2% glycine compared to cells were grown in LBG. The proportions of unsaturated mycolic acids decreased from 60.0% in LBG to 30.3% in LBG-G for AS019, 63.3% to 30.5% for MLB133 and 57.5 % to 42.7% for MLB194. For the three strains tested, the cell wall fractions contained proportionately more $C_{32:0}$ after growth in glycine as compared with Coryne-CW from LBG-grown cells.

3.4.3 LBG-INH (4 mg/ml INH)

The *C. glutamicum* strains were cultured in LBG containing 4 mg/ml INH and cells harvested in early stationary phase; the specific growth of the three strains in the AS019 family were reduced by 40% as previously reported by Jang *et al.* (1997). The analysis of mycolic acids in whole cells and cell wall fractions is shown in Table 3.7. The relative proportion of unsaturated mycolic acids was 36.8% in AS019, 43.6% in MLB194 and 40.1% in MLB133, and similar trends were seen in both whole cells and cell wall fractions. However, MLB133 showed a small decrease in $C_{32:0}$ and increase in $C_{34:1}$ and $C_{36:2}$ in the cell wall fraction compared to the whole cells. The relative proportions of unsaturated to saturated mycolic acids ranged between 39.9% AS019 and 48.6% in the cell wall. Growth in the presence of 4 mg/ml INH had little impact on whole cell mycolic acids when compared to LBG. This is consistent with results reported by Jang *et al.* (1997), who showed that INH at 4mg/ml had little impact on whole cell mycolic acids but at 8mg/ml the proportion of extracellular mycolates increased. However, analysis of the cell wall fractions showed a significant decrease in the proportion of unsaturated mycolic acids relative to these in cells grown in LBG (Table 3.5), similar to the trends seen for cell wall fractions following growth in LBG-G.

3.4.4 LBG containing 2% glycine and 4 mg/ml INH.

The cumulative effects of glycine plus INH were also evaluated, where strains AS019 and the mutants (MLB133 and MLB194) were grown in LBG cotaining 2% glycine plus 4 mg/ml INH. The results for mycolic acid analysis of whole cells and cell wall fractions are shown in Table 3.8 for cultures harvested at early stationary phase. In whole cells, the relative proportions of unsaturated to saturated mycolic acids was 31.4 % in AS019 and 44.6 % in MLB133 and for cell fractions was 24.7 % in AS019, and 50% in MLB133. Changes caused by the presence of this combination were similar to those seen for LBG-G for whole cells and cell walls. The relative proportions of mycolic acids found in the cell walls and whole cells were similar for AS019 and MLB194. However, in the cell wall of MLB133 the relative proportion of unsaturated to saturated mycolic acids showed an increase after growth in LBG-INH-glycine compared to growth in LBG-2% glycine: 49% in LBG-INH-glycine and 30.5% after growth in LBG-2% glycine. Little changes were observed for AS019, while MLB194 and MLB133 showed a small decrease in the relative proportion of $C_{32:0}$. The previously reported results by Jang (1997) also showed that 4 mg/ml INH alone or in combination with glycine had little further impact on the mycolic acids composition of the AS019 family of strains foe whole cells. The most important impact was the increase in extracellular mycolates.

Table 3.7 Mycolic acids composition of whole cell and cell walls fractions following growth in LBG-INH (4 mg/ml).

strains	Mycolic acids[a]					% of UM to TM[b]
	$C_{32:0}$	$C_{34:1}$	$C_{34:0}$	$C_{36:2}$	$C_{36:1}$	
Whole cell						
AS019	52.8	28.7	10.6	4.8	2.3	36.8
MLB133	46.3	31.7	10.1	8.1	3.6	43.6
MLB194	48.5	29.6	11.1	6.7	3.8	40.1
Cell Wall						
AS019	50.1	28. 9	11.8	5.6	3.5	39.9
MLB133	40.1	38.1	8.8	9.6	3.4	48.6
MLB194	47.1	31.9	9.8	7.2	3.8	39.3

[a] Proportion (%) of each mycolic acid found in whole cell or culture fluids. The proportion of each mycolic acid was calculated as a % of the total in terms of peak area detected.

[b] The ratio of UM (unsaturated mycolic acids, $C_{34:1}$, $C_{36:2}$, $C_{36:1}$) to TM (sum of saturated and unsaturated mycolic acids) was determined by dividing the sum of the unsaturated mycolic acids values by the sum of the total mycolic acids.

Table 3.8 Mycolic acids composition of whole cell and cell wall of AS019 family of *C. glutamicum* following growth in LBG containing 2% glycine and 4 mg/ml.

Strains	Mycolic acids[a]					% of UM to TM[b]
	$C_{32:0}$	$C_{34:0}$	$C_{34:1}$	$C_{36:2}$	$C_{36:1}$	
Whole cell						
AS019	57.6	18.1	11.6	10.5	2.8	31.4
MLB133	44.3	30.8	11.0	8.8	5.1	44.6
MLB194	43.4	33.5	6.4	12.3	3.6	44.6
Cell wall						
AS019	60.0	17.7	15.3	3.9	2.1	24.7
MLB194	38.9	36.9	9.6	10.5	4.4	50.0
MLB133	40.3	36.5	8.8	9.6	3.4	49.0

[a] Proportion (%) of each mycolic acid found in whole cells and cell wall. The proportion of each mycolic acid was calculated as a % of the total in terms of peak area detected.

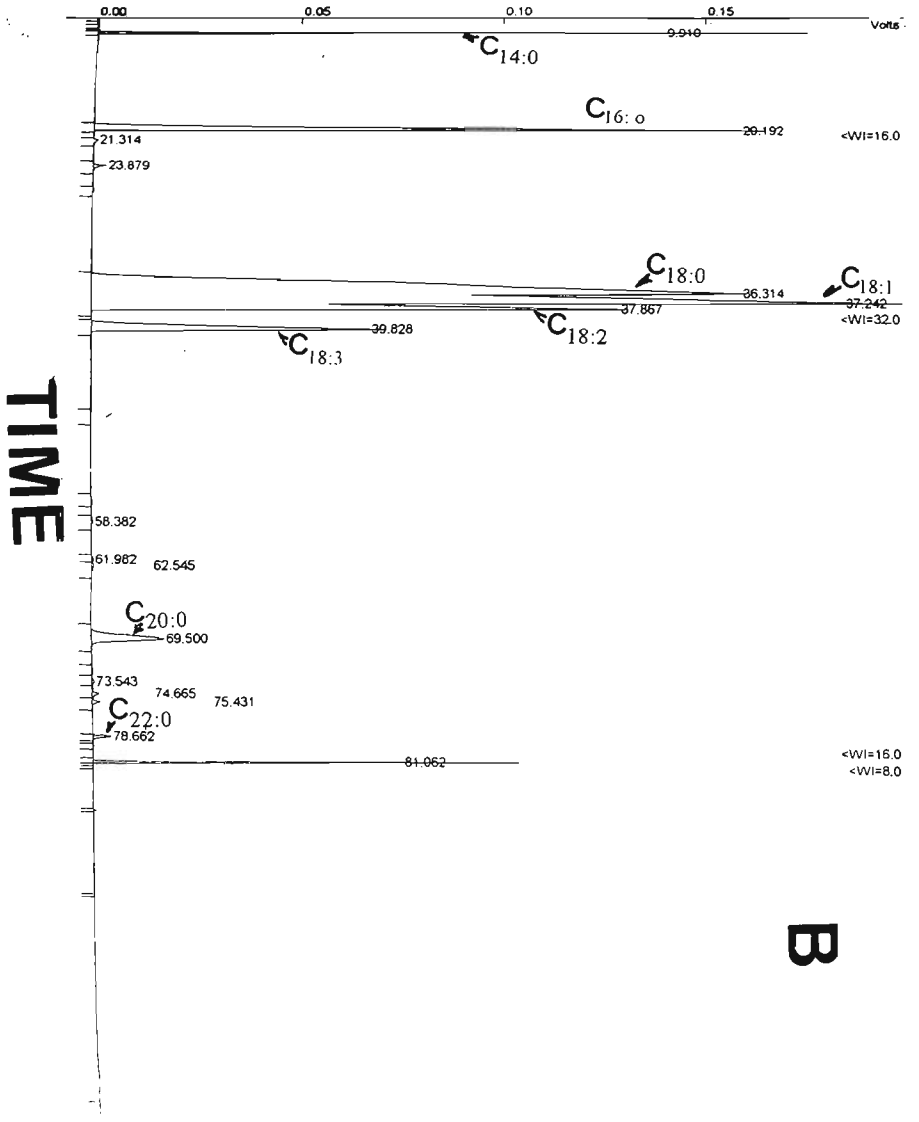
[b] The ratio of UM (unsaturated mycolic acids, $C_{34:1}$, $C_{36:1}$ and $C_{36:2}$) to TM (sum of saturated and unsaturated mycolic acids) was determined by dividing the sum of the mycolic acids values by the sum of the total mycolic acids.

3.5 FATTY ACIDS COMPOSITION OF *C. GLUTAMICUM* STRAINS FOLLOWING GROWTH IN DIFFERERENT MEDIA

The composition of fatty acids along with mycolic acids was analysed for whole cells and cell wall fractions for different media (LBG, LBG containing 2% glycine, LBG containing 4 mg/ml INH and LBG containing 2% glycine and 4 mg/ml of INH). The procedure developed by Jang (1997) was used as described in section 2.4.2.3 and this allowed comparison of the relative proportions of the fatty acids to mycolic acids from the peak areas obtained in the same GC profile.

Fatty acids were fractionated using a 25m non-polar BPX5 column. Hexadecanoic acid ($C_{16:0}$) and octadecanoic acids ($C_{18:1}$) were the major fatty acids found in all of the strains tested when the samples were obtained from whole cells. These results were in agreement with data reported by Jang (1997). Myristeric acid ($C_{14:0}$), steric acid ($C_{18:0}$), pentadecanoic acids, palmitoleic acids ($C_{16:1}$) and some other fatty acids were also detected in very small amounts, so that these fatty acids were not taken into consideration when calculating relative proportions of lipids. The identity of the fatty acids were confirmed by co-injecting purified FAMEs plus external standards (FAMEs, Sigma). The GC profiles are shown in Fig 3.6.

Several repeat analysis were performed to check the reproducibility of the methods used (Table 3.9). Cells of AS019 were cultivated in LBG and harvested in early stationary phase. Dried samples of whole cells and cell wall fractions were prepared, then 2 mg/ml of internal standard was added, and lipids extracted as described in sections (2.4.2.3). FAMEs and MAMEs were analysed by GC. The quantitative fatty acid profile obtained from the cell walls of four samples were the same in terms of relative percentages of fatty acids detected. However, there was a significant variation between experiments in terms of the ratio between FAMEs to total lipids (FAMEs plus MAMEs) detected. All experiments were performed at least in duplicate and the average % of FAMEs for AS019 across several experiments was similar to that prepared in Table 3.9.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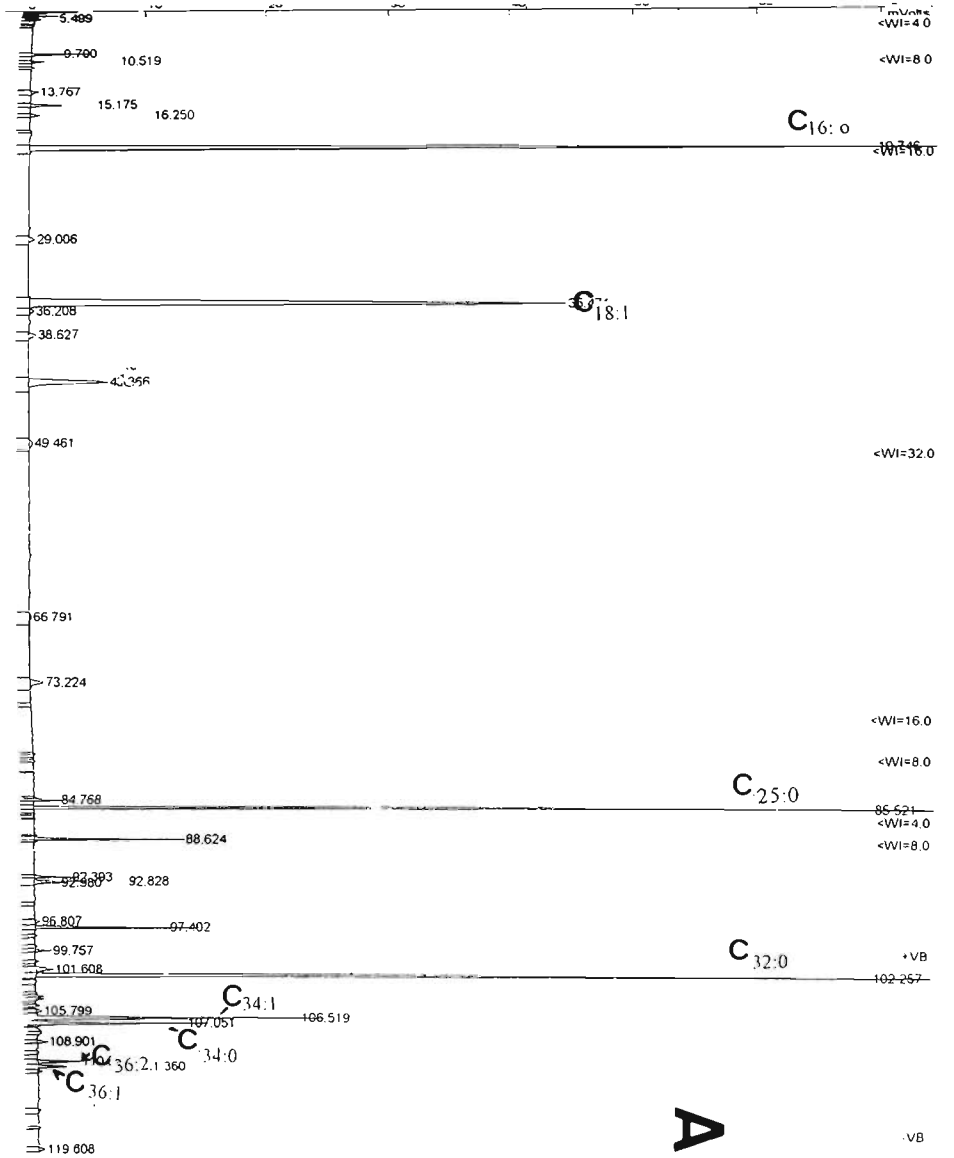
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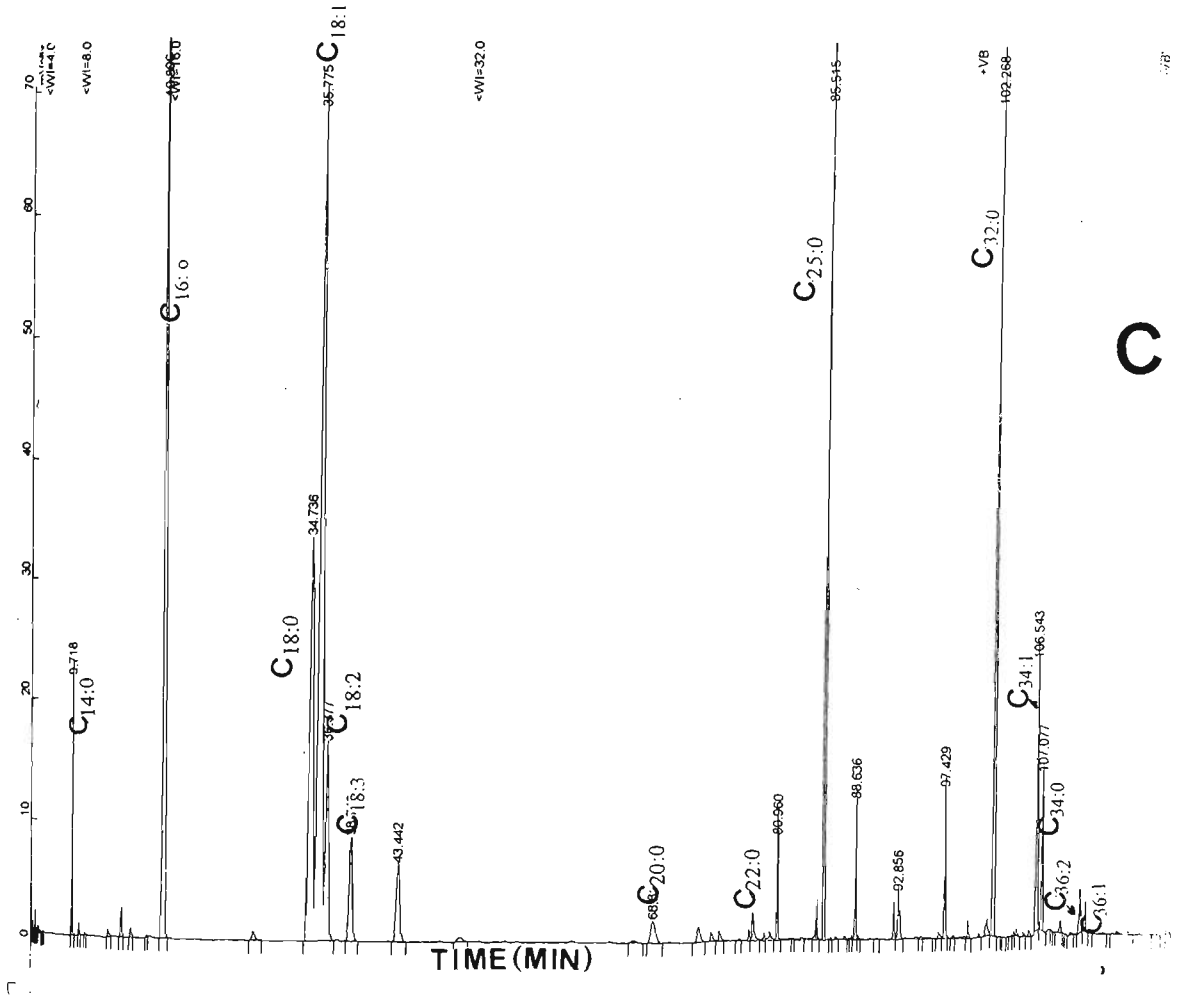

Figure 3.6 GC analysis of FAMEs on a 25-m non-polar BPX column. (A) Cellular FAMEs and MAMEs from *C. glutamicum* AS019 after growth in LBG. (B) Standard FAMEs mixture-Sigma 189-17. (C) 189-17 plus FAMEs and MAMEs from strain AS019

Table 3.9 Evaluation of reproducibility of quantitative analysis of fatty acids between different experiments using *C. glutamicum* strain AS019 cell wall fractions prepared after growth in LBG.

N[a]	Fatty acids[b]			Total peak area of FAMEs[c]	Mycolic acids[d]					Total peak area of MAMEs[e]	% FAMEs[f]
	$C_{16:0}$	$C_{18:1}$	$C_{18:3}$		$C_{32:0}$ $_{36:1}$	$C_{34:1}$	$C_{34:0}$	$C_{36:2}$	C		
1	49.25	45.94	4.80	379123	29.77	42.36	8.03	15.83	3.99	100103	79.11
2	46.12	48.48	5.38	121844	31.16	44.51	5.95	15.11	3.25	77241	71.01
3	47.02	43.93	9.04	217166	30.96	42.46	6.05	16.92	3.59	92993	70.00
4	46.43	48.11	5.45	137529	30.50	44.45	5.91	15.86	3.38	73938	63.74
average	47.20	46.11	6.16		30.59	43.44	6.48	15.93	3.54		70.96
±SD	1.6	2.3	2.3		0.75	1.2	1.2	0.91	0.53		4.99

[a] Experiment number. Data for each experiment was from four separately grown cultures and samples (fatty acids plus mycolic acids) were separately prepared.

[b] Proportion of each TMS derivative of the FAMEs was calculated as a % of the total for FAMEs in terms of peak area detected.

[c] Sum of the peak area of FAMEs detected.

[d] Proportion of each TMS derivative of the MAMEs was calculated as a % of the total for MAMEs in terms of peak area detected.

[e] Sum of the peak areas of MAMEs detected.

[f] The proportion of FAMEs, which was obtained from the equation of $[c/(c+e)] \times 100$.

Table 3.10 and 3.11 showed the relative proportion of fatty acids of three strains of *C. glutamicum* (AS019, MLB133, MLB194) after growth in three different media. All three strains showed approximately the same proportions of $C_{16:0}$ and $C_{18:1}$ when cells were grown in LBG. The relative percentage of total FAMEs to total esters (FAMEs plus MAMEs) were 67.92 % for AS019, 69.78 % for MLB133 and 69.44% for MLB194, for cells grown in LBG.

In the presence of glycine or INH, the relative percentage of the fatty acids of AS019 declined very slightly from 67.92 % in LBG to 66.83% in LBG-2% glycine and 61.37% in LBG-4 mg/ml INH in whole cells. Similar decreases in the relative proportion of cellular fatty acids were also observed in MLB133 and MLB194 (Table 3.9 and 3.10). This data is in agreement with the results previously reported by Jang (1997). The major cell wall fatty acids were $C_{16:0}$ and $C_{18:1}$ but the fatty acid $C_{18:3}$ and some other fatty acids were also detected in small amounts in the cell wall in addition to these two fatty acids. The relative proportions of total fatty acids in the cell wall of the three strains grown in LBG were 71.29 % for AS019, 68.39 % for MLB133 and 70.97% for MLB 194. In the presence of 2% glycine or 4 mg/ml INH, the relative proportion of the fatty acids decreased, as seen for whole cells. The fatty acid $C_{18:3}$ was too low to detect in whole cells, because in cell wall the $C_{18:3}$ is a minor fatty acid. This fatty acid had only been seen previously in extracellular fluids of *C. glutamicum* (Jang, 1997). These results suggest that the total fatty acids in the cell wall fractions decline following growth in LBG-G or LBG-I so that the $C_{18:3}$ is detected as a relatively major component. This is consistent with the increased release of all lipids into the culture fluids during growth in glycine and INH (Jang, 1997).

Table 3.10 Quantitative analysis of fatty acids of two strains of *C. glutamicum* in different media.

Strain	Medium [a]	Fatty acids [b]			%FAMEs [c]
		$C_{16:0}$ $C_{18:3}$		$C_{18:1}$	
Whole cells					
AS019	LBG	53.29	46.50		67.92
AS019	LBG	52.01	47.82		66.83
AS019	LBG-I	53.50	46.38		61.37
MLB133	LBG	54.07	45.91		69.78
MLB133	LBG-G	53.45	46.45		63.74
MLB133	LBG-I	53.27	46.72		56.46
Cell wall					
AS019	LBG	47.20	46.61	6.16	71.29
AS019	LBG-G	46.43	48.10	5.45	61.57
AS019	LBG-I	50.00	40.25	9.73	59.62
MLB133	LBG	47.94	43.53	8.48	68.39
MLB133	LBG-G	48.40	42.67	8.87	68.64
MLB133	LBG-I	49.51	43.51	6.96	61.29

[a] Data for each experiment was from two separately grown cultures and sample (fatty and mycolic acids) preparations.

[b] Proportion of each TMS derivative of the FAMEs was calculated as a % of the total in terms of peak area detected.

[c] The proportion of FAMEs, which was obtained from equation of [c/(c+e)] x 100

Table 3.11 Quantitative analysis of fatty acids composition of whole cells and cell wall of *C. glutamicum* strain MLB194 after growth in three different media.

Strain	Medium	Fatty acids[a]			% FAMEs [b]
		$C_{16:0}$	$C_{18:1}$	$C_{18:3}$	
Whole cells					
194	LBG	60.86	39.13	-	69.44
194	LBG-G	51.17	48.22	-	62.19
194	LBG-I	45.65	53.94	-	59.50
Cell Wall				-	
194	LBG	57.20	37.34	5.45	70.97
194	LBG-I	49.62	38.23	12.12	53.78

[a] Proportion of each TMS derivative of the FAMEs was calculated as a % of the total in terms of peak area detected.

[b] The proportion of FAMEs, which was obtained from equation of [c/(c+e)] x 100

3.6 MYCOLIC ACID COMPOSITION OF *CSP1* DISTRUPTED MUTANTS OF *C. GLUTAMICUM*

The results of mycolic acids profiles in whole cells for the *csp1* disrupted mutant strain of *C. glutamicum,* which lacks the S-layer, after growth in four different media are presented in Table 3.12. The mycolic acids composition was similar to the AS019 family in terms of qualitative profiles; however, quantitative profiles differ significantly, indicating a difference in the cell surface structure of this mutant relative to AS019. Among the five major types of mycolic acids found in the whole cells of this mutant, the mycolic acid $C_{32:0}$ and $C_{34:1}$ made up to 75.11 to 85.11% of the total composition. However, the relative proportion of $C_{32:0}$ (35.20%) was lower than the AS019 family while the proportion of $C_{34:1}$ and $C_{36:2}$ was slightly higher (41.21% and 12.85%). Similar mycolic acids profiles were observed by Jang (1997) for restriction deficient mutants of *C. glutamicum* RM3 and RM4 after growth in LBG.

After growth in glycine there was an increase in the proportion of $C_{32:0}$ with parallel decreased in the relative proportion of $C_{34:1}$ and $C_{36:2}$ in the *csp1* mutants. These results showed that the *csp1* disrupted mutant has a mycolic acid profile similar to those previously reported for strains RM3 and RM4 by Jang (1997), where he observed lower proportions of $C_{34:1}$ and $C_{36:2}$ after growth in 2% glycine. Following growth in LBG plus glycine, there was a relative increase in the percentage of $C_{32:0}$ (from 35.20 to 44.56%) with the parallel decrease in the relative proportion of unsaturated mycolic acids (34.99 and 9.15%). The addition of INH (3 mg/ml) had similar impacts on the relative proportion of saturated and unsaturated mycolic acids: % of unsaturated mycolic acid decreased in LBG-I and LBG-GI. The cumulative effect of 2% glycine and INH was also studied. The results showed impacts on the relative proportions of mycolic acids when compared with LBG and LBG plus 2% glycine. There was a further increase in the relative proportion of $C_{32:0}$ (47.17%), with decreases in the relative proportion of unsaturated mycolic acids (35.64% $C_{34:1}$ and 5.81% $C_{36:2}$). The specific growth rate of

Table 3.12 Fatty acid and mycolic acid composition of whole cells of the *csp1* disrupted mutant of *C. glutamicum*

Medium	Mycolic acids[a]					% of UM to TM[b]	Fatty acids[c]			% FAMEs[d]	% FAMEs$_e$
	$C_{32:0}$	$C_{34:1}$	$C_{34:0}$	$C_{36:2}$	$C_{36:1}$		$C_{16:0}$	$C_{18:1}$	$C_{18:3}$		
LBG	35.20	41.21	7.21	12.85	3.46	57.53	47.41	40.42	12.43	76.89	68.79
LBG-G	44.56	34.99	9.28	9.15	3.53	45.56	45.45	37.26	18.93	72.46	59.13
LBG-I	42.62	36.75	7.83	8.96	3.79	49.36	45.9	37.25	16.83	78.92	65.58
LBG-GI	47.17	35.64	8.59	5.81	2.78	44.23	36.86	47.67	15.44	81.17	69.15

[a] Proportion (%) of each mycolic acid found in whole cells. The proportion of each mycolic acid was calculated as a % of the total in terms of peak area detected.

[b] The ratio of UM (unsaturated mycolic acids, $C_{34:1}$, $C_{36:2}$, and $C_{36:1}$) to TM (sum of saturated and unsaturated mycolic acids) was determined by dividing the sum of the unsaturated mycolic acid values by the sum of the total mycolic acids.

[c] Proportion of each TMS derivative of FAMEs was calculated as a % of the total in terms of peak area detected.

[d] The proportion of FAMEs ($C_{16:0}$, $C_{18:1}$, $C_{18:3}$), which was obtained from the equation [c/(c+e)] x 100

[e] The proportion of FAMEs ($C_{16:0}$, $C_{18:1}$) which was obtained from the equation [c/(c+e)] x 100

these mutants was significantly affected by the combination of INH plus glycine at the concentrations used (3mg/ml).

Table 3.12 also showed the fatty acid composition for *csp1* mutants along with mycolic acids. The results showed that, like AS019 family, significant changes were observed in the relative percentage of fatty acids to total esters (fatty acids plus mycolic acids) when growth was in the presence of glycine or glycine plus INH. In the presence of glycine or INH, or the combination of the two, the relative percentage of fatty acids also changed.

3.7 DISCUSSION

3.7.1 Drug sensitivity analysis

It is difficult to find reliable data in the literature concerning MICs, because experimental conditions are so different so that it may be erroneous to compare published values. INH inhibits mycolic acid synthesis in mycobacteria and nocardia at concentrations of <50 μg/ml (Tomiyasu and Yano, 1984: Winder and Collins 1970; Quemard *et al.*, 1995b). Tables 3.1 and 3.2 described the MICs in detail for different strains of *C. glutamicum*. The presented work showed that corynebacteria is less sensitive to INH than *Mycobacterium* and nocardia species. However, there are distinct differences in INH MICs between the corynebacterial species used, where *C. glutamicum* strains AS019, *B. flavum* and *B. lactofermentum* exhibited different levels of MICs. MICs were defined as lowest concentration of drug required to produce 85% end point. AS019 and BF4 were relatively more resistant to INH (MICs 11 and 10 mg/ml) and growth was completely inhibited at the concentrations of 16 mg/ml and 17 mg/ml. In contrast, BL1 was more sensitive and complete inhibition of growth occurred at lower concentrations of INH (MICs 5-6 mg/ml) indicating different levels of drug resistance in this important group of corynebacteria. It has been argued that *B. lactofermentum* should be re-classified as *C. glutamicum* (Abe *et al.*, 1967; Liebel *et al.*, 1991) but their different sensitivity to INH indicates physiological differences which may argued against this. Similar results were observed by Jang (1997) when he noted the effect of INH and glycine on specific growth rates. For glycine medium, BF4 was less inhibited than AS019 and BL1 and when

glycine was replaced by INH, AS019 was found to be the least sensitive of the strains tested. However, our method was different from that used by Jang (1997), who observed the effect of INH added directly into broth cultures at different stages of growth. However, the trends in results were quite similar.

The two mutants MLB133 and MLB194 exhibited greater sensitivity towards INH relative to the parent type strain AS019. MIC values for MLB194 and MLB133 were 6 and 8 mg/ml, where the decline in growth started and 100% inhibition was observed at a concentration of 14 and 13 mg/ml of INH. The present study also showed that at concentrations of 6, and 8 mg/ml INH, the inocula prepared from dilutions of 10^{-1}, 10^{-2}, 10^{-3} (AS019), 10^{-1}, 10^{-2} (MLB133 and MLB194) survived and as the concentration of the drug increased only "heavy" inocula survived, indicating the impact of inoculum size on determining MICs of corynebacteria.

The sensitivity of a *csp1* mutant strain of *C. glutamicum*, in which the *csp1* gene responsible for S-layer formation has been inactivated, and restriction deficient strain RM4, was also determined. The results showed that, like BL1, RM4 was much more sensitive to INH. Growth started declining at 3 mg/ml and was completely inhibited at 10 mg/ml of INH. Whereas *csp*1 disrupted mutants behaved similar to AS019 (MICs 10 mg/ml). However, in the later experiment when INH was used at 4 mg/ml in combination with glycine the growth was extremely slow so the concentration of INH was reduced to 2 mg/ml. This suggests that this strain is more sensitive to the combined inhibitory effect of glycine and INH relative to AS019.

ETH, α-ethylthioisonicotinamide, exhibits strong bacteriostatic properties against some mycobacteria and is rather more active against INH-resistant strains (Rist 1960). Structurally it is strikingly analogous to INH and a similar mode of action has been proposed for the two drugs (Winder 1982). In this study, MICs of ETH were determined for different corynebacterial species. *C. glutamicum* was more susceptible to ETH compared to INH. In contrast to data for INH, the differential sensitivity between strains seen when using INH could not be detected for ETH, with the exception that BL1 was most sensitive. No growth was seen beyond 6 mg/ml in almost all of the strains tested,

confirming that ETH was more effective than INH in corynebacteria, which is similar to the results reported for mycobacteria (Rist, 1960).

3.7.2 Lipids composition of the cell wall of *C. glutamicum*

The cell walls of *Corynebacterium, Mycobacterium* and *Nocardia* have a common construction consisting of mycolic acid, arabinoglactan and peptidoglycan (Lederer, 1971; Michel and Bordet, 1976; Barksdale and Kim 1977). Mycolic acid composition and the effect of agents like glycine or INH on cellular mycolic acids have been studied in detail (Collins *et al.*, 1982, Collins *et al.*, 1982b, Pierotti, 1987; Jang, 1997). In this thesis the comparative analysis of lipid composition (both mycolic acids and fatty acids) of cell wall plus whole cells has been presented to provide a better understanding about the arrangement of lipids on the cell surface. The cellular mycolic acid profiles of the strains included in the present study are in full agreement with the previously reported results (Collins *et al.*, 1982, Collins *et al.*, 1982b, Pierotti, 1987; Jang, 1997). Table 3.18 summarises results for the AS019 series of strains to allow comparison.

These results suggested that in LBG-grown cells, the cell wall was richer in unsaturated mycolic acids compared with the whole cells, indicating the different nature of the cellular fractions used for mycolic acid analysis. The method used for the preparation of the cell wall fractions included several washing steps that solubilised the cytoplasmic membrane and also washed off the free and loosely bound mycolic acids. The difference in the mycolic acid proportions of the cell wall fraction and whole cells further support the previously reported Pierotti (1987) observation that approximately 45 to 48 % of the *C. glutamicum* mycolic acids were solvent-extractable.

The mutants of *C. glutamicum* examined here, MLB133 and MLB194, had similar qualitative profiles to the parent type strain AS019, but differed from this strain in that they had higher proportions of $C_{34:1}$ and $C_{36:2}$. Similarly, in the cell wall fraction the relative proportions of $C_{34:1}$ and $C_{36:2}$ was further increased and a decrease in the

Table 3.13 Comparison of mycolic acids found in cell wall and whole cells of *C. glutamicum* strains grown in four different media. Data were obtained from Tables 3.5, 3.6, 3.7, 3.8. Abbreviations: LBG-G, LBG supplemented 2% (w/v) glycine; LBG-I, LBG supplemented with 4 mg/ml INH. LBG-GI, LBG supplemented with 2% glycine and 4 mg/ml INH.

Mycolic acids	AS019				MLB133				MLB194			
	LBG	LBG-G	LBG-I	LBG-GI	LBG	LBG-G	LBG-I	LBG-GI	LBG	LBG-G	LBG-I	LBG-GI
Whole cell												
$C_{32:2}$	63.6[a]	58.4	52.8	57.6	48.5	54.6	46.3	44.3	54.8	51.0	48.5	43.4
$C_{34:1}$	20.9	22.6	28.7	18.1	28.8	27.4	31.7	30.8	31.6	20.9	29.6	33.5
$C_{34:0}$	9.5	13.2	10.6	11.6	6.4	8.7	10.1	11.0	6.1	16.3	11.1	6.4
$C_{36:2}$	3.3	2.8	4.8	10.5	7.9	7.1	8.1	8.8	5.7	3.4	6.7	12.3
$C_{36:1}$	3.0	2.9	2.3	2.8	3.9	3.7	3.6	5.1	3.3	3.4	3.8	3.6
% of UM TO TM[b]	27.2	28.3	36.8	31.4	40.6	38.3	43.6	44.7	39.2	27.8	40.1	44.7
Cell Wall												
$C_{32:0}$	31.7	55.3	50.1	60.0	30.6	55.9	40.1	40.3	39.6	47.9	47.1	38.9
$C_{34:1}$	40.9	26.1	28.9	17.7	41.8	25.4	38.0	36.5	40.2	25.6	31.9	36.9
$C_{34:0}$	7.4	13.8	11.8	15.3	6.1	13.7	8.8	8.8	8.6	11.0	9.8	9.6
$C_{36:2}$	15.0	3.0	5.6	3.9	17.9	2.8	9.6	9.6	13.0	11.3	7.2	10.6
$C_{36:1}$	4.1	3.3	3.5	2.1	3.7	2.5	3.4	3.4	4.5	4.2	3.8	4.4
% of UM to TM	60.0	30.3	39.9	24.7	63.3	30.5	48.6	49.0	57.5	42.7	39.3	50.0

[a] Proportion (%) of each mycolic acid found in whole cells or cell walls. The proportion of each mycolic acid was calculated as a % of the total in terms of peak area detected.

[b] The ratio of UM to TM was determined by dividing the sum of the unsaturated mycolic acids values by the sum of the total mycolic acid

proportion of $C_{32:0}$ was observed. In the AS019 family, the proportion of $C_{32:0}$ was in decreasing order of AS019, MLB194, and MLB133. Both *Brevibacterium* species BF4 and BL1 showed similar patterns of mycolic acids both in quantitative and qualitative compositions, in that these strains exhibited the presence of five mycolic acids in both whole cells and cell wall fractions.

After the addition of 2% glycine in growth media there was a small decline in the relative proportion of cellular unsaturated mycolic acids for AS019, MLB133, MLB194. However, in the cell wall fractions the proportion of unsaturated MAMEs ($C_{34:1}$, $C_{36:2}$ and $C_{36:1}$) showed a significant decrease compared to growth in LBG, whereas $C_{32:0}$ was proportionately increased. This observation that the level of unsaturated mycolic acids declined dramatically in the presence of 2% glycine in the growth media further adds to the observation that the cell wall becomes thinner after growth in 2% glycine (Jang, 1997).

Previous studies have shown that glycine interferes with several steps of peptidoglycan synthesis (Hammes *et al.*, 1973). The replacement of L-alanine residues (Strominger *et al.*, 1965) and the inhibition of L-alanine adding enzymes (Hishinuma *et al.*, 1971) are only two of several possible impacts and glycine may act as an analogue of either of L-alanine or of D-alanine. Hammes *et al.* (1973) showed that in *Corynebacterium* sp., *C. callunae*, *L. pantarum* and *L. cellobiosus* most of the D-alanine replaced by glycine occurs in the C-terminal position. Based on their studies, two mechanisms leading to a more loosely cross-linked peptidoglycan and morphological changes of the cells were considered. Firstly, the accumulation of glycine-containing precursors may lead to a disruption of the normal balance between peptidoglycan synthesis and the control of enzymatic hydrolysis during growth. Secondly, the modified glycine-containing precursors may be incorporated. Since these are poor substrates in the transpeptidation reaction, a high percentage of muropeptides remains uncross-linked.

The studies of Jang (1997) also showed that extracellular mycolic acid levels increased following growth in glycine, suggesting that glycine exerted an effect in addition to those previously reported, where this may be related to the attachment of mycolates to the cell surface. Since the knowledge about the synthesis, assembly and transportation of mycolic acids is limited in corynebacteria, the mechanism of how glycine promotes the leakage of extracellular mycolic acids cannot be explained with any certainty. It is possible that glycine may interfere directly with the translocation of corynemycolic acids from presumptive, yet unidentified carriers *via* transacylation to arabinose units in the arabinogalactan. Alternatively, destabilisation of peptidoglycan cross-linking by glycine incorporation could impair arabinogalactan linking to the altered backbone, leading to a looser external structure which resulted in "leakage" of mycolic acids-arabinogalactan structure into the culture fluids. Changes observed in the mycolic acid composition of cell wall, however, suggest that glycine had impaired cross-linking and weakened the peptidoglycan structure. Some of the mycolic acids in the cell wall are bound to the arabinose residue of arabinogalactan by an ester linkage in mycobacteria, while the nature of linkage between arabinogalactan and peptidoglycan remains unknown in *C. glutamicum*. A glycosidic linkage between the arabinogalactan and peptidoglycan has been demonstrated in the cell wall of *Mycobacterium* (Kanetsuna and San Blas, 1970) and also for the first time in the cell wall of *N. rubra* AN-115 (Fujioka *et al.*, 1985). The nature of the linkage between peptidoglycan and arabinoglactan needs to be revealed to allow a better understanding of the action of glycine in *Corynebacterium*, particularly in relation to the attachment of mycolic acids.

The overall mycolic acid profile for the relative proportions of each mycolic acid in cells and cell walls were quite similar for each sample tested indicating that a particular mycolic acid is not being released into the culture fluid and hence affecting the cellular mycolic acids profile. Analysis of the whole cell MAMEs confirmed that strains MLB133 and MLB194 could be differentiated from AS019 following growth in LBG-glycine/INH, confirming their phenotype for later studies.

The mycolic acid profile of the strains tested showed little change following growth in 4 mg/ml of INH, as there was little quantitative variation in the mycolic acids for both cells and cell wall fractions. This is probably due to the relatively low concentrations of INH in the growth medium, which was well below a lethal level. However, the two mutants showed higher sensitivities towards the presence of INH into growth medium and there was slight decrease in the proportion of saturated mycolic acids in whole cells and unsaturated mycolic acids of cell wall as compare to growth in LBG. After growth in 4 mg/ml of INH there was decrease in the proportion of $C_{34:1}$ and $C_{36:2}$ in the cell wall of mutants with parallel increases in the proportion of $C_{32:0}$ and $C_{34:0}$. This study did not include investigating the effect of increasing INH concentration on the cell wall mycolic acids, however, studies of Jang (1997) indicated that after the addition of 2% glycine or 8 mg/ml of INH, the level of extracellular mycolic acids increased to 18-21% for the mutants. He suggested that INH inhibited the synthesis of shorter chained mycolic acids in all strains and also further impaired covalent binding of mycolates to the cell surface in the mutants.

The effect of different growth media on the mycolic acid compositions of the *csp1* disrupted mutant of *C. glutamicum* which lacks the S-layer are shown in Table 3.12. The results showed a decrease proportion of $C_{32:0}$ and an increase proportion of $C_{34:1}$ and $C_{36:2}$ after growth in LBG. Results previously reported by Jang (1997) showed that a similar profile was observed for two restriction-deficient mutants strains RM3 and RM4, where $C_{32:0}$ was less than the unsaturated mycolic acids. His studies further showed that following growth in LBG plus 2% glycine there was a decrease in the proportion of $C_{34:1}$ and $C_{36:2}$ with parallel increase in the proportion of $C_{32:0}$. Similar results were also observed for the *csp1* mutant after growth in LBG-G, where the relative proportion of $C_{32:0}$ increased with a parallel decrease in the proportion of $C_{34:1}$ and $C_{36:2}$. Growth in the combination of INH and glycine also had similar impacts on lipids. It has been reported (Bardou *et al.*, 1996) that the important decrease in cell wall mycolic acid content of mycobacteria selectively induces an overproduction of secreted proteins about which little is known. Whether the action of INH also leads to the overproduction of

extracellular proteins in corynebacteria needs to be investigated. These studies should include the S-layer proteins and their fate following growth in INH and glycine.

In the present study, the fatty acid composition of cells and cell walls was also studied. For all strains tested, palmitic acid ($C_{16:0}$) and oleic acid ($C_{18:1}$) were the major fatty acids in both cells and cell walls. There was a trend towards slight decreases in FAMEs after growth in LBG-G, LBG-I implying that including glycine and INH in the growth medium not only affected mycolic acids but also impacted on the fatty acids composition. The main effect of the addition of the cell wall modifiers was to decrease the level of FAMEs as compared to MAMEs. Similar trends were seen in cell wall fractions where the proportion of FAMEs decreased after growth in glycine and INH medium, implying that INH affects FAMEs of the cell wall. The previously reported results of Jang (1997) showed the presence of extracellular fatty acids in culture fluids. He suggested that fatty acids were probably located at the cell surface in unbound form and leaked out during growth as the integrity of the cell wall structure was affected by glycine and INH. In the present study, since the starting material was cell wall fractions it can said that INH caused changes in the relative proportion of fatty acids at the cell surface or membrane level. This study further shows that fatty acids are present in a bound form in the cell wall fraction, as they were not extracted during the procedures used to prepare cell wall fractions.

3.7.3 Models for the cell wall structure of *C. glutamicum*

In mycobacteria, it has been shown that some changes in the cell envelope occur before the bacteriostatic effect of INH, which appears one generation after the addition of the drug (Takayama *et al.*, 1974). These changes include the increase in respiration compatible with the increase in the permeability of the cell envelope (Schäefer *et al.*, 1960), the fragility of the cell wall and increased loss of the cellular material into the medium. Similar studies in *C. glutamicum* revealed that addition of 2% glycine and INH has an effect on the morphology of corynebacteria. These changes included reduction in

size of cell (after growth in INH) budding and branching following growth in LBG plus 2% glycine (Best and Britz, 1986; Jang, 1997) and increased loss of cellular lipids into the medium. The present study further adds to the observation that INH and glycine affect the cell wall by altering the lipid composition of the cell wall. In mycobacterial cell walls, mycolic acids are quantitatively the major component (ca. 40%); inhibition of their formation may explain, on one hand, the narrow specificity of INH for mycobacteria and, on the other hand, death of the cells as a consequence of cell wall structure damage. This study and previously reported results of Jang (1997) support the idea that INH inhibited production of the shorter chained lipids and, if fatty acids are used as a substrate for mycolic acid synthesis, the presence of glycine or INH in the medium inhibit not only MA synthesis but also fatty acid synthesis. Studies in mycobacteria also showed that the target of INH is shorter chained length lipids (C_{16}-C_{22} carbon containing molecules), noting that mycobacteria utilize these to make complex mycolic acid (Banerjee *et al.*, 1994).

Models for the cell surface structures of mycobacteria are described in detail in Chapter 1 (section 1.6.4) and these include the presence of bound plus free mycolic acids and fatty acids. According to Pierotti's work (1987), there are at least two different mycolic acids: extractable (free mycolic acids, approximately 45-48% of total mycolic acids) and covalently bound mycolic acids.

If the physical organisation of the lipids in the cell wall of *C. glutamicum* is similar to that of proposed model system in mycobacteria, then the cell wall structure of *C. glutamicum* is basically a highly asymmetric lipid bilayer. In this bilayer model the bound lipids must form the inner leaflet of cell wall as they are covalently bound mycolic acid to the arabinoglactan which in turn are cross-linked (presumably by ester linkage) to the underlying peptidoglycan. The free and loosely bound lipids will serve as a good candidate for the outer leaflet. This study showed the presence of fatty acids (C_{14}-C_{18}) in bound form attach to the cell wall, whereas the C_{16} and $C_{18:1}$ were the dominant fatty acids and the proportion of FAMEs (as calculated by the equation [(c/c+e) x 100]) were more than the MAMEs. Therefore it can be assumed that there are more than enough of

these to intercalate with the mycolic acids in the cell wall. The cell wall presumably contains proteins and peptides, and an ion channel (proposed model of cell wall structure of *C. glutamicum* is presented in Fig 3.6).

Since the results showed that cell wall was richer in unsaturated mycolic acids ($C_{34:1}$, $C_{36:2}$, $C_{36:1}$) it appears that these lipids are forming part of the inner leaflet and, if this model is true, it can explain the low permeability of *C. glutamicum*. In the proposed model, the inner leaflet consisted of unsaturated MAs they will form the effective permeability barrier by producing lipid environments of exceptionally low fluidity as (i) the arms of mycolic acid residues are quite long (18 carbons) and (ii) they contain few double bonds (1 to 2). Nikaido *et al.* (1994) in the proposed model for the mycobacterial cell wall explained that the inner leaflet containing mycolic acids was expected to produce a very effective barrier for permeation.

Liu *et al.* (1995) also reported similar findings about the fluidity of lipids in the cell wall of *M. chelonae*, which was low in general but higher in the outer part, and was accessible by spin-labelled probes. Their proposed model consisted of a layer of close-packed mycolic acids with a thermal transition temperature to fluidity well above the growth temperature of the organism, intercalated with triglycerides. However, experimental support such as x-ray chrystallographic studies and spin-labelled probes analysis are needed to prove the validity of this model.

Puech *et al.*, 2001 analysed several corynebacterial species and compared their cell envelope constituents before drawing a model, this model has been described in detail in chapter 1 (section 1.5.2). According to their model the corynebacterial cell envelopes consist of (i) an outer layer composed of polysaccharides, proteins, which include the mycoloyltranferase PS1and lipids; (ii) a cell wall glycan core of peptidoglycan-arabinogalactan which may contain other sugar residues and was usually esterified by corynomycolic acids; and (iii) a typical plasma membrane bilayer. With freezed-etch electron microscopy they showed that most corynomycolate-containing strains have a main fracture plane in their wall and contained low-molecular mass porins. The fracture

plane seen within the plasma membrane of those strains was devoid of both corynomycolate and pore-forming protein. They found that most strains the amount of cell wall-linked corynomycolates was not sufficient to cover the bacterial surface; and that the occurrence of a cell wall fracture plane correlated with the amount of non-covalently bound lipids of the strains. They proposed that these lipids spontaneously form liposomes, indicating their participation in bilayer structure. In conclusion, they suggested that the cell wall permeability barrier in corynebacteria involved both covalently linked corynomycolates and non-covalently bound lipids of their cell wall.

The model proposed in this study is based only on the mycolic acids composition of whole cell, cell wall culture fluid extract (free mycolic acids) and involved few corynebacterial species and cell surface mutants. Thus this model is similar to that proposed by Puech *et.al.* (2001). It involved a study of both covalently bound lipids and unbound lipids. The bound lipids were proposed to be part of inner leaflet of bilayer cell wall since the (i) permeability of envelope is low towards inner side of the membrane and (ii) the proportion of MAMEs were less than FAMEs. The free and loosely bound lipids will form the outer leaflet.

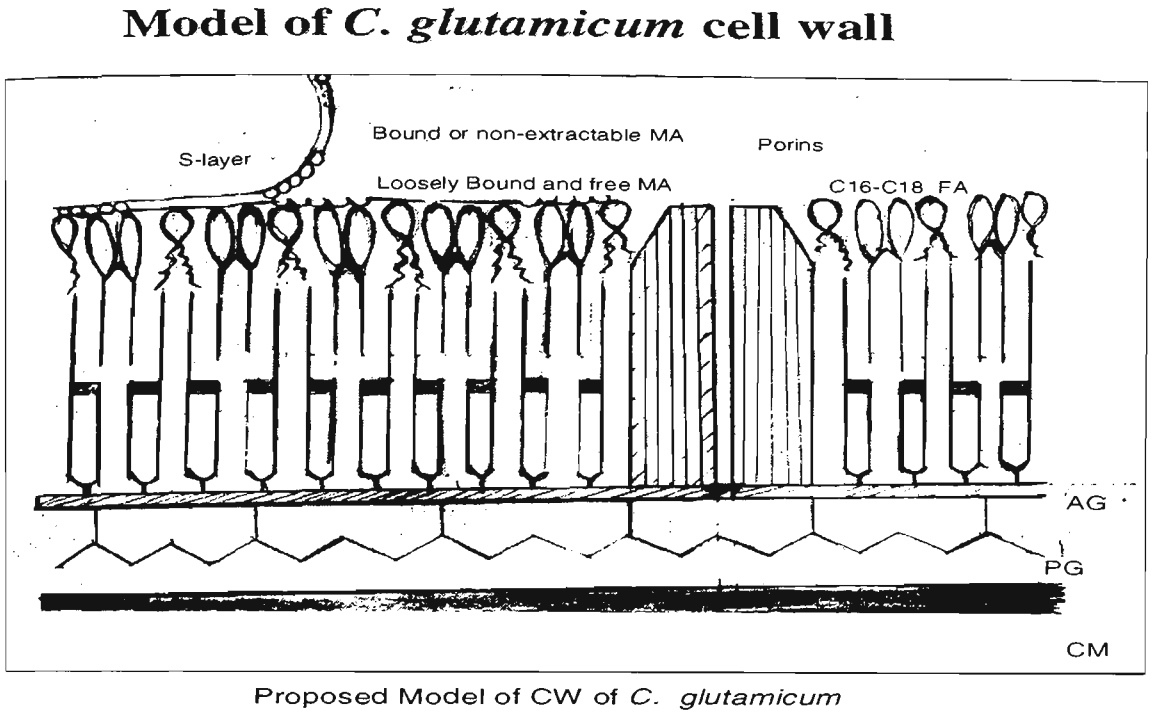

Figure 3.7 Proposed model of the structure of *C. glutamicum* cell wall, redrawn from Jang. (1997) with permission, showing the proposed arrangement of mycolic acids and associated lipids forming the outer permeability barrier of the mycobacterial envelope and indicating the assumed position of the porins and S-layer-like proteins. A similar arrangement presumably occurs in all mycobacterial walls, though the mycolic acid types and the range of other lipids involved would be different.

Chapter 4

Cloning, sequencing and sequence analysis of an *inhA* gene homologue in *C. glutamicum* wild type strain AS019

4.1 INTRODUCTION

The results reported in the previous chapter showed that *C. glutamicum* is naturally more resistant to INH and ETH (MICs of 12-16 mg ml^{-1} and 4-8 mg ml^{-1} respectively) on plates relative to reported sensitivity of mycobacterial species. Previous studies have indicated that growth of strain AS019, a rifampicin-resistant derivative of *C. glutamicum* ATCC 13059, in either glycine or INH (high concentrations) or combinations of these increased electroporation frequencies significantly (Haynes and Britz, 1989, 1990).

Previous results had further shown that significant differences in cell growth rate, transformation efficiency and mycolic acid composition appeared when the concentration of INH was increased in the growth medium, particularly when this was in the presence of glycine (Jang *et al.*, 1997). At high concentrations of INH (8 mg ml^{-1}), all of the *C. glutamicum* strains in the AS019 series tested had decreased relative proportions of $C_{32:0}$ and $C_{34:0}$ with paralleled increased proportions of $C_{34:1}$ and $C_{36:2}$. This suggested that INH inhibited synthesis of shorter chained saturated mycolic acids. The relative proportion of total fatty acids to total lipids (fatty acids plus mycolic acids) following growth in LBG was higher than following growth in LBG plus 8mg ml^{-1} INH, as INH decreased the ratio of fatty acids significantly. This indicated that INH might inhibit some stage of fatty acid synthesis in addition to the mycolic acid synthesis.

The mechanism of INH resistance and genetics of mycolic acid biosynthesis is not known in corynebacteria. However, substantial biochemical evidence has suggested that both INH and ETH block mycolic acid biosynthesis in closely related mycobacterial species. The emerging literature over the last few years showed that one target site of INH in *M. tuberculosis*, and several other mycobacterial species, was identified as the InhA protein,

the product of the *inhA* gene, which is a 2-*trans* enoyl acyl carrier protein involved in fatty acid synthesis (Dessen *et al.*, 1995). This enzyme preferentially reduces C_{16} derivatives, consistent with its involvement in mycolic acid biosynthesis (Quemard *et al.*, 1995b). Since mycolic acids are the unique characteristic of corynebacteria and mycobacteria, it was reasoned that the drug might have similar impacts on mycolic acid biosynthesis in corynebacteria as seen in mycobacteria.

The work reported in this chapter was carried out with the following objectives:

- To determine whether there is an *inhA* gene equivalent in *C. glutamicum* strain AS019;
- If a *inhA* gene was found the second aim was to sequencing the *inhA* gene in *C. glutamicum* and comparie this with the reported *inhA* gene sequences in mycobacterial species.

4.2 CLONING OF THE *inhA* GENE IN *C. GLUTAMICUM* STRAIN AS019

4.2.1 Verification of the strains

The corynebacterial strains used in this study were recovered from glycerol stock solution as described in section 2.2.3. The identity of all corynebacterial strains were checked by 16S rRNA partial sequencing. The target sequences were amplified using PCR in *C. glutamicum* strain AS019, two mutants MLB194, MLB133 and related species *B. lactofermentum* (BL1). Approximately 1,300 bp of consensus 16S rRNA gene was amplified using two primers 63f and 1387r (Pharmacia; Marchesi *et al.*, 1998) as described in section 2.3.1.14.

The PCR products were sequenced directly using the same primers, used for amplification (63f and 1387r). The sequence analysis was performed on DNA samples for all coryne strains using the BLASTN programme of the WebANGIS and showed 99-100% similarity with known 16S rRNA gene of the *C. glutamicum*. The 16S rRNA

sequencing data for corynebacterial strains and their BLASTN similarity results are shown in appendix 4.

Based on partial 16S rRNA sequence information Southern blot analysis of genomic DNA digest was performed to provide some information on the number of copies of the gene and to compare the "ribotype" of the mutants relative to the parent. Southern hybridisation was performed using ^{32}P-labelled PCR amplified 16S rRNA gene probe from *C. glutamicum* strain AS019 against the genomic DNA digest of corynebacterial strains with various enzymes. The results of Southern hybridisation are shown in appendix 5 (Fig 4I, 4II, 4II). The results showed common restriction pattern of 16S rRNA gene between parents and mutants. The Southern results indicate several bands depending on the restriction enzyme used.

4.2.1.1 Construction of the primers to amplify the *inhA* gene from *C. glutamicum* strain AS019

On the basis of the published *inhA* gene sequence of *M. smegmatis* (Banerjee *et al.*, 1994), several sets of degenerate primers were designed to amplify the *inhA* gene in corynebacteria. The *inhA* gene from AS019 was amplified as five overlapping fragments which together cover the area from upstream of the start codon to the stop codon, as deduced from the known sequence of the mycobacterial *inhA* gene. All primers were at least 22 bases long to allow specific binding to the templates, with no self-complementary regions and they had similar annealing temperatures (58-65°C). Table 4.1 provides the list of all the primers used for amplification and sequencing of the *inhA* gene. All primers were used for PCR and sequencing.

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Table 4.1 List of the primers used in the amplification and sequencing of the *inhA* gene from *C. glutamicum* and related species (*B. lactofermentum* and *B. flavum*). All the primers were derived from the reported *inhA* gene sequence of *M. smegmatis* (MSU02530) (Banerjee *et al.*, 1994).

	SEQUENCE (5'.....3')	SIZE[a]	TM(°C)[b]
CGP1	ATC ATC ACC GAT TCG TCG ATC GCG T	25	76
CGP2	CT T GGC GAC GGT CAT CCA GTT GT	24	72
CGP3	CTC GAA TCT CGT TGC GGC AGGA	22	70
CGP4	ACA GCA GTG CGC ACA CGG TCTT	22	70
CGP5	ATG ACA GGC CTA CTC GAA GGC A	22	68
CGP6	ATC CGG TCG GCC AGA GTC GAC A	22	72
CGP8	CAT CCA GTT GTA GGC CGG CAT C	22	70
INH1	CCG GAC ACA CAA GAT TTC TCG CTC	24	77
INH2	ATC ACA ACA GCT GCG TGC TGG CGC	24	78

[a] Size=number of bases

[b] TM=melting temperature

4.2.2 Optimisation of PCR conditions and use of Soln Q

DNA from the genus *Corynebacterium* has very high G+C content (54%) and therefore was a difficult template for PCR amplification. In attempts to optimise the conditions for amplification of the *inhA* gene, several kits were tried: PCR products of expected size were obtained using the QIAGEN Kit and Soln Q and AmpliTaq DNA polymerase. According to the suppliers, Soln Q is designed to change the melting behaviour of the template DNA and can be used for those PCR systems which do not work under standard conditions. In this work it was found that Soln Q was one of the essential requirements for successful PCR. For maximum specificity and yield, temperature and cycling times were optimised for each new primer pair. Table 4.2 shows the respective PCR profile for each pair of primers and their anticipated products. Amplification of the *inhA* gene from *M. smegmatis* was simple and was done using the published PCR profile (step1 94°C 3 min; step 2 94°C 1min; step 3 71°C 1min; step 4 72°C 1min; step 2-4 35 cycles; step 5 72°C 5min) as well as the conditions used to amplify *inhA* fragments from *C. glutamicum*.

4.2.3 Sequencing of PCR fragments of the *inhA* gene of strain AS019 and *M. smegmatis*.

Figure 4.1 shows the PCR amplification products obtained from genomic DNA of *C. glutamicum* strain AS019 using the selected primers listed in Table 4.1 and conditions in Table 4.2. Figure 4.2 shows the PCR amplification products of the *inhA* gene from *M. smegmatis* using the same sets of primers but using the PCR protocols mentioned in Table 4.2 without the SolnQ in the reaction mixture. The figures show:

(a) using primer sets INH1-CGP4, CGP5-CGP2 and CGP3-INH2 the fragments obtained from both *M. smegmatis* and *C. glutamicum* DNA were the same for both species and approximately the size expected for the primers used, however, additional minor bands were also seen for AS019 in some experiments.

(b) when using primer set CGP3-CGP4, *M. smegmatis* produced a fragment measuring approximately 255 bp which was longer than the expected size of 184 bp. However, sequence read out of this fragment and the CGP3-INH2 amplified fragment using CGP3

Table 4.2 Primer pairs used for the amplification of the *inhA* gene from *C. glutamicum*, its mutant strains and related species (*B. lactofermentum* and *B. flavum*), their respective PCR profile and expected product size.

PRIMER PAIRS	PCR PROFILE FOR PLASMID DNA	PCR PROFILE FOR GENOMIC DNA	PRODUCT SIZE
INH1-CGP4 (1-778)	**1-95°C/2 min** **2-94°C/1 min** **3-68°C/1min** **4-72°C/1min** **step 2-4 35 cycles** **5-72°C/5min**	**1-95°C/4 min** **2-94°C/1 min** **3-69°C/3 min** **step 2-3 35 cycles** **4-72°C/7 min**	**778 bp**
CGP5-CGP2 (511-535)	**As above**	**As above**	**494 bp**
CGP1-CGP2 (82-535)	**As above**	**As above**	**443 bp**
CGP5-CGP6 (39-272)	**As above**	**As above**	**234 bp**
CGP3-CGP4 (594-779)	**1-95°C/2 min** **2-94° C/1 min** **3-55°C/1 min** **4-72° C/1 min** **step 2-4 35 cycles** **5-72°C/5 min**	**As above**	**184 bp**
CGP3-INH2 (594-850)	**1-95°C/2 min** **2-94°C/1 min** **3-70°C/3 min** **step 2-3 35 cycles** **4-72°C/5 min**	**1-95°C/4 min** **2-94°C/1 min** **3-70°C/3 min** **step 2-3 35 cycles** **4-72°C/5 min**	**256 bp**

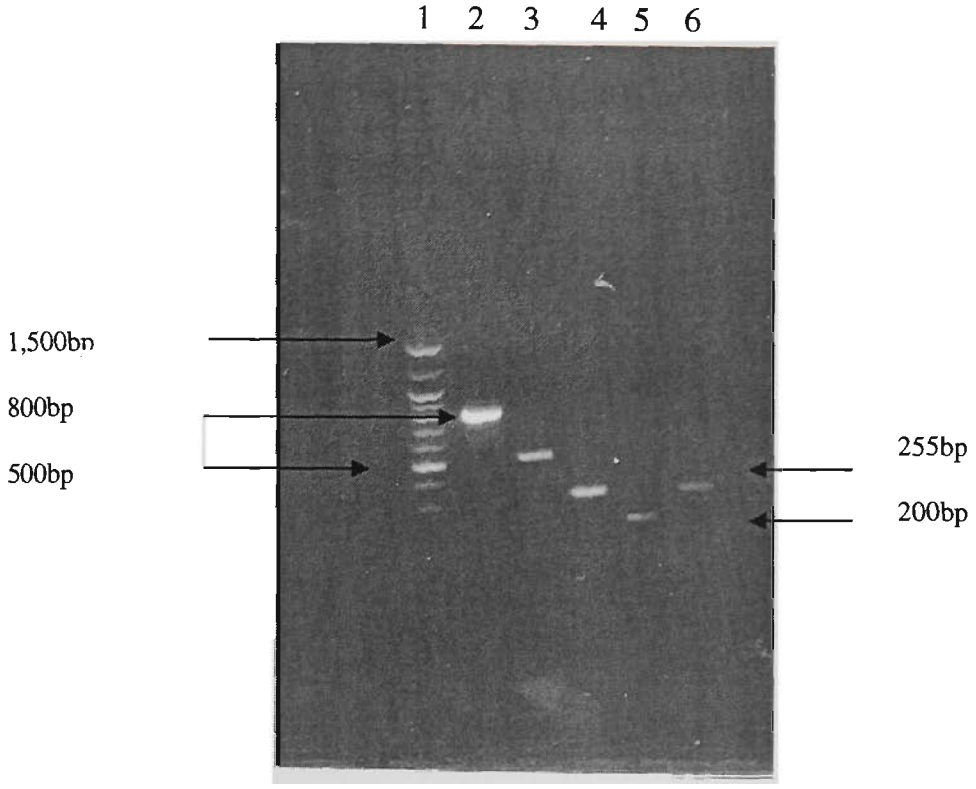


Figure 4.1 PCR amplification of *inhA* gene from genomic DNA of *C. glutamicum*. Lane 1, 100 bp DNA ladder (1,500, 1,200, 1,000, 900, 800, 700, 600, 500, 400, 300, 200 and 100bp); 2, *inhA* gene fragment (800 bp of INH1-CGP4 primers); 3, *inhA* gene fragment (496 bp CGP5 -CGP2 primers); Lane 4, *inhA* fragment (255 bp of CGP3-INH2 primers); Lane 5, *inhA* fragment (200 bp of CGP3-CGP4 primer pairs); Lane 6, *inhA* fragment (250 bp of CGP5-CGP6 primers).

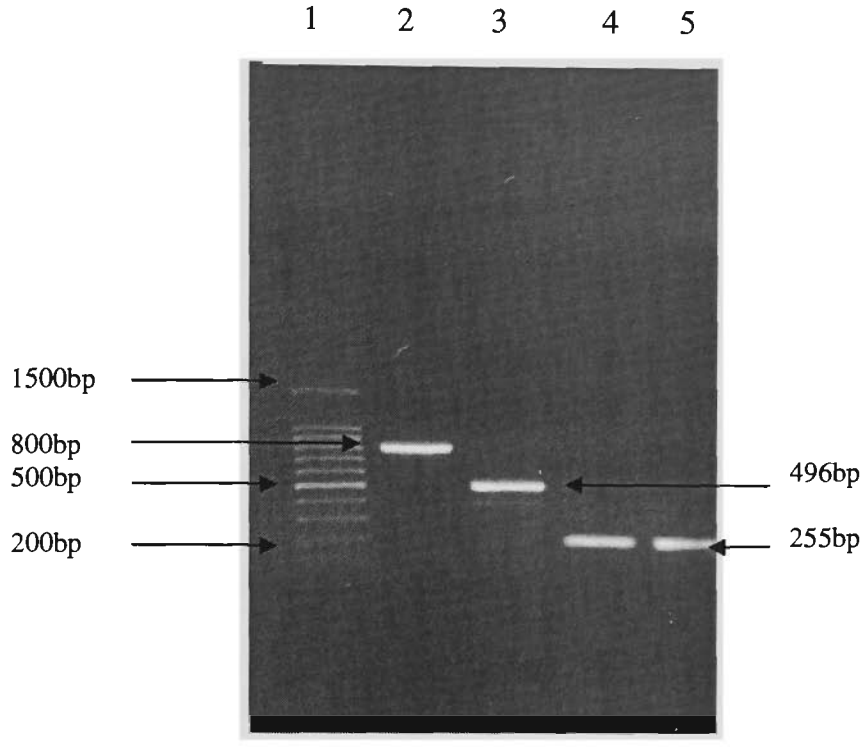


Figure 4.2 PCR amplification of the *inhA* gene from genomic DNA of *M. smegmatis* mc^2 155. Lane 1, 100 bp DNA ladder (1,500, 1,000, 900, 800, 700, 600, 500, 400, 300, 200 and 100bp); Lane 2, *inhA* gene fragment (800bp from INH1-CGP4 primers); Lane 3, *inhA* fragment (496 bp product of CGP5-CGP2); Lane 4, *inhA* fragment (255 bp of CGP3-INH2 primers); Lane5, *inhA* fragment (200bp of CGP3-CGP4 primers).

(forward) primer was exactly the same as the sequence obtained for CGP3-CGP4 products, as expected.

In this experiment *M. smegmatis* was used as a control therefore only four primer sets were used for amplification of the *inhA* gene. For *C. glutamicum*, extra primer pairs (e.g CGP5-CGP6, CGP1-CGP2, CGP5-CGP4) were also used to get more overlapping fragments to improve the accuracy of sequencing the entire gene.

4.2.4 Sequence analysis of the amplified products

PCR amplified fragments of the *inhA* gene from *M. smegmatis* and *C. glutamicum* strain AS019 obtained using the CGP1-CGP2 and CGP3-CGP4 primer sets were sequenced using the same primers. A sequence similarity search based on the NCBI was performed using the National Genomic Information Service (ANGIS) and BLASTN (nucleotide sequence against nucleotide sequence database similarity search) programs. The polypeptides deduced for the fragments amplified from AS019 were compared to all known proteins in the database using the BLASTX (Altschul *et al.*, 1990) and FASTA (Pearson and Lipman, 1988) programs. Tables 4.3, 4.4 and 4.5 show BLASTN, BLASTX and FASTA homology searches for these products. These data showed that the highest similarity was with mycobacterial proteins and confirmed the presence of an *inhA* gene homologue in *C. glutamicum*. Sequence assembly of this is reported in section 4.3.

4.2.5 Southern hybridisation and identification of the *inhA* gene in *C. glutamicum* strain AS019

The PCR amplified products of the CGP3-INH2 primer set from *C. glutamicum* AS019 were labelled using two different labelling systems: DIG labelled, using the DIG High Prime labelling kit and radiolabelled, using αdCTP and the High Prime labelling kit. The two probes were then used to hybridise the blots containing the genomic DNA of *C. glutamicum* and *M. smegmatis* digested with various enzymes. Figures 4.3 and 4.4 show the results of Southern hybridisation with non-radioactive and radioactive labelling systems.

Table 4.3 BLASTN sequence analysis of PCR amplified products of *M. smegmatis* mc^2155 and *C. glutamicum* using CGP3-CGP4 and CGP1-CGP2 primers sets.

Sequence	% homology				
	M. smegmatis inhA gene	*M. tuberculosis M. bovis inhA* gene	*M. tuberculosis* mutant NADH dependant *inhA*	*M. tuberculosis* H37RV	Sequence 3 from patent US 5686590
M3P3[a]	91	79	79	79	91
M1P1[a]	96	82	82	82	90
AS019 P1[b]	98	83	83	83	–[d]
AS019 P2[b]	98	82	82	82	–
AS019 P3[c]	98	84	84	84	98
AS019 P4[c]	94	82	82	82	94

[a]M3P3 and M1P1 are PCR products of CGP3-CGP4 and CGP1-CGP2 primer sets from *M. smegmatis* genomic DNA and sequenced by CGP3 and CGP1 primers respectively.

[b]AS019 P1 and AS019 P2 are PCR products of CGP1-CGP2 primers sets sequenced by the same respective primers.

[c]AS019 P3 and AS019 P4 are PCR products of CGP3-CGP4 primers sets sequenced by the same primers.

[d] – no data was obtained.

Table 4.4 BLASTX sequence analysis results of PCR amplified products from *M. smegmatis* mc^2 155 and *C. glutamicum* genomic DNA using the CGP3-CGP4 and CGP1-CGP2 primers sets.

Sequence	% homology				
	M. smegmatis Enoyl-ACP reductase	*M. tuberculosis* Enoyl-ACP reductase	*M. avium* Enoyl-ACP reductase	Enoyl-ACP (fragment)	Putative Enoyl-ACP reductase
M3P3[a]	81.4	69	69	76	–[d]
M2P1[a]	94	78	76	94	50
AS019 P1[b]	99	83	85	85	46
AS019 P2[b]	96	79	–	–	–
AS109 P3[c]	98	84	63	84	–
AS019 P4[c]	96	81	63	81	–

[a]M3P3 and M2P1 are PCR products of CGP3-CGP4 and CGP1-CGP2 primer sets from *M. smegmatis* and sequenced by CGP3 and CGP1 primers respectively.

[b]AS019 P3 and AS019 P4 are PCR products of CGP3-CGP4 primers sets from AS019 and sequenced by the same primers.

[c]AS019 P1 and AS019 P2 are PCR products of CGP1-CGP2 primers sets from AS019 and sequenced by the same respective primers.

[d]– no data obtained

Table 4.5 FASTA sequence analysis results of PCR amplified products of *M. smegmatis* mc^2 155 and *C. glutamicum* using CGP3-CGP4 and CGP1-CGP2.

Sequence	%homology			
	M. smegmatis isoniazid	*M. tuberculosis* H37RV *inhA*	*M. bovis* putative keto acyl-ACP reductase	*M. avium* GIR10 transcript
M3P3[a]	89.24	77.21	77.21	73.41
M2P1[a]	94.42	79.94	79.99	79.93
AS019 P1[b]	98.22	83.33	83.33	82.60
AS019 P3[c]	91.51	79.59	79.59	74.51

[a]M3P3 and M1P1 are PCR products of CGP3-CGP4 and CGP1- CGP2 primer sets from *M. smegmatis* and sequenced by CGP3 and CGP1 primers respectively.

[b]AS019 P1 is PCR products of CGP3-CGP4 primers sets sequenced by the same primers.

[c]AS019 P3 is PCR products of CGP1-CGP2 primers sets sequenced by the same respective primers.

The sizes of the different hybridisation signals obtained using both approaches were determined and shown in Table 4.6. These results showed that the probe bound strongly to 3kb *PstI* fragments of *M. smegmatis* genomic DNA, confirming the previously reported observation of Banerjee *et al.* (1994). In contrast, a completely different and unique hybridisation pattern appeared for strain AS019, where the probe bound to several fragments of different sizes for all of the various enzymes used. The Southern hybridisation pattern obtained using the two labelling systems were quite similar. However, some additional minor bands were also observed using DIG labelled probes along with the major bands. For example, *PstI* restriction digestion of *C. glutamicum* DNA showed three major bands plus another very strong signal which corresponded to the probe binding to a very small molecular weight (0.25kb) fragment of genomic DNA.

It is interesting to note that the two gels for Figs. 4.3 and 4.4 were run simultaneously and particular enzymatic digestion mixtures were loaded on each gels from one tube. However, additional minor and major bands were seen in DIG system which did not appear with ^{32}P probe, suggesting that DIG system was more sensitive than ^{32}P but may also have shown less specificity.

The Southern blot analysis indicated the putative presence of an *inhA* gene homologue in *C. glutamicum*. The multiple signals may have arisen due to the presence of several gene copies, cutting sites for the enzymes used within the gene or to non-specific binding of the probe to similar sequences. To confirm the identity of the *inhA* gene two approaches were used subsequently: construction of a genomic library of AS019 (section 4.2.6) and sequencing the gene from the cloned DNA plus assembling the sequence directly from PCR products of genomic DNA (section 4.3).

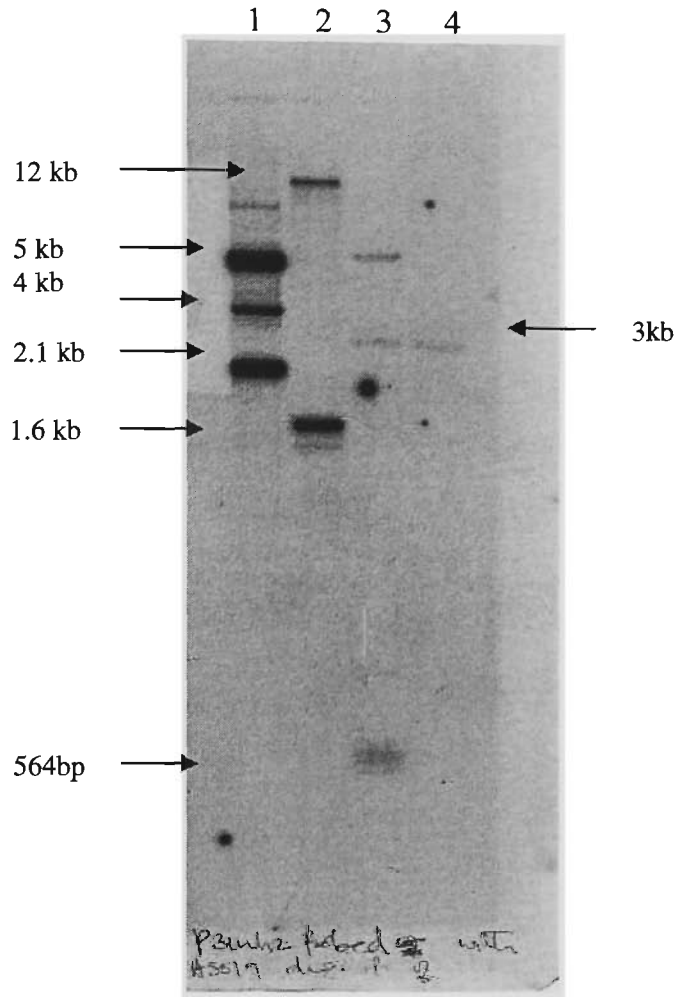


Figure 4.3 Southern hybridisation of genomic DNA digest of *C. glutamicum* strain AS019 and *M. smegmatis* mc^2155 probed with the 256 bp PCR amplified fragment from *C. glutamicum* using CGP3-INH2 primer pairs using the radioactive labelling system. Lanes 1, 2, 3, *BglII*, *HindIII*, *PstI* digested genomic DNA of *C. glutamicum* strain AS019. Lane 4, *PstI* digested genomic DNA of *M. smegmatis* mc^2 155.

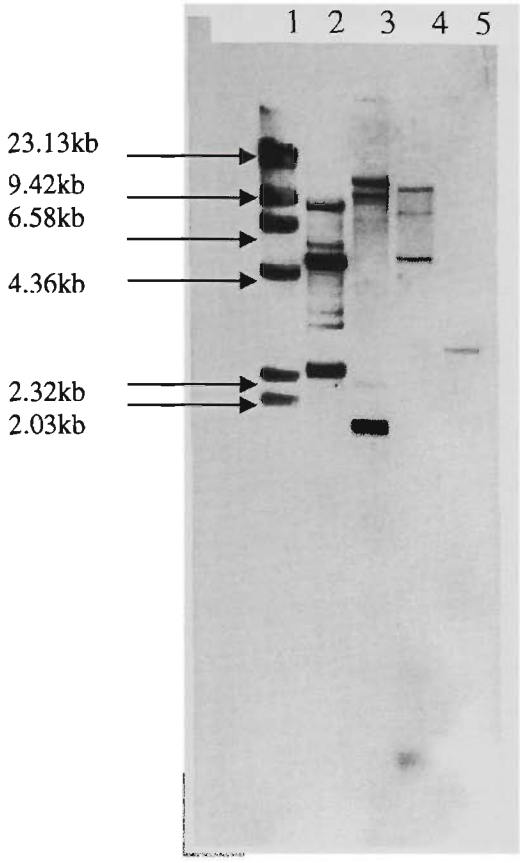


Figure 4.4 Southern blot hybridisation of genomic DNA digest of *C. glutamicum* strain AS019 and *M. smegmatis* mc^2 155 probed with a 256bp PCR amplified *inhA* fragments from *C. glutamicum* of CGP3-INH2 primers pairs, using the DIG labelling system. Lane 1, Lambda phage DNA *HindIII* digests (size markers); Lanes 2, 3, 4 , *BglII*, *HindIII*, *PstI* digested genomic DNA of *C. glutamicum* strain AS019. Lane 5, *PstI* digested genomic DNA of *M. smegmatis* mc^2 155

Table 4.6 Molecular weights of the hybridisation fragments obtained when the CGP3-INH2 probe was used against genomic DNA from AS019 was digested with various enzymes.

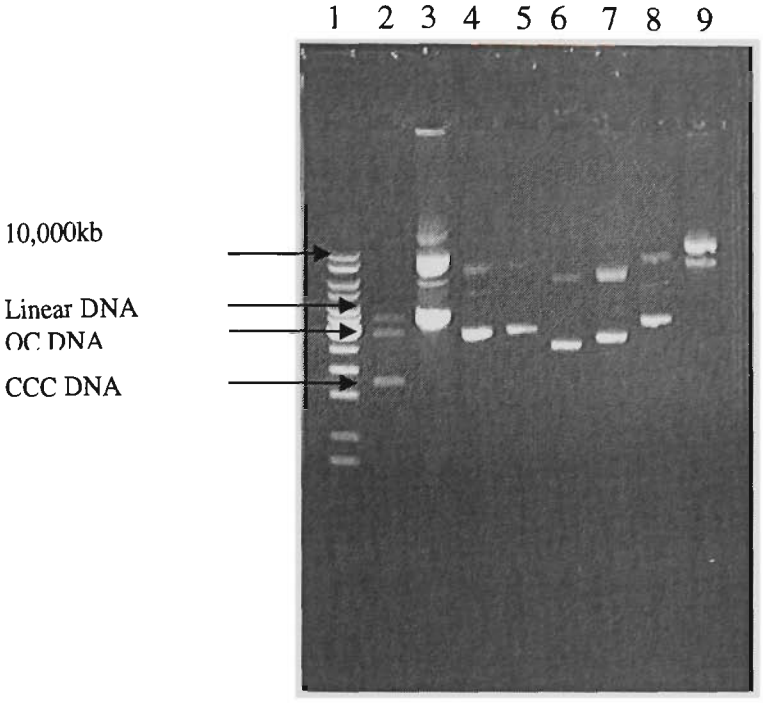
MW (kb) of the bands detected						
Dig Labelling			**^{32}P Labelling**			
λ*Hind*III	***Bgl* II**	***Hind*III**	***Pst*1**	***Bgl*II**	***Hind*III**	***Pst*1**
23,130	**8-9**[a]	**12**	**9.2**	9	**12**	9.2
9,416	6	**10**	6.5	**5**	10	
6,557	**5**		**5.5**	-		**5.2**
4,322	4		3.5	**4**		**3.5**
2,322	3.5	2.2				
2,027	**2.1**	**1.6**		**2.1**	**1.6**	
564			0.25			
130			0.12			0.12

[a] Numbers in bold correspond to major bands seen. The band sizes not in bold type were minor bands.

4.2.6 Cloning of the entire *inhA* gene in *C. glutamicum* AS019 and verifying that clones contain the *Pst*1 fragment.

*Pst*1 digestion of *M. smegmatis* DNA contained the entire *inhA* gene (Banerjee *et al.*, 1994). Although *C. glutamicum* DNA showed a different pattern, the three major bands obtained were >3kb so it was likely that the fragments would contained the entire *inhA* gene from *C. glutamicum*. Both DIG and ^{32}P labelled probes, however, bound to a 3.5kb fragment, which is slightly bigger when compared to the 3kb *Pst*1 fragment of *M. smegmatis* and this appeared to be very feint compared to the other stronger bands. Due to the phylogenetic similarity of the two species, it was likely that 3.5kb fragment also contained the gene. Moreover, the size of this fragment could easily be cloned into the pBluescript SKII vector, therefore *Pst*1 digested genomic DNA of AS019 was randomly cloned into pBluescript SKII vector using *Pst*1 digestion and a subgenomic library of AS019 DNA was constructed in *E.coli* JM109 (as described in section 2.3.1.1). The second largest fragment (5.2kb) was still small enough to be successfully cloned using the pBluescript SKII vector (Sambrook *et al.*, 1989).

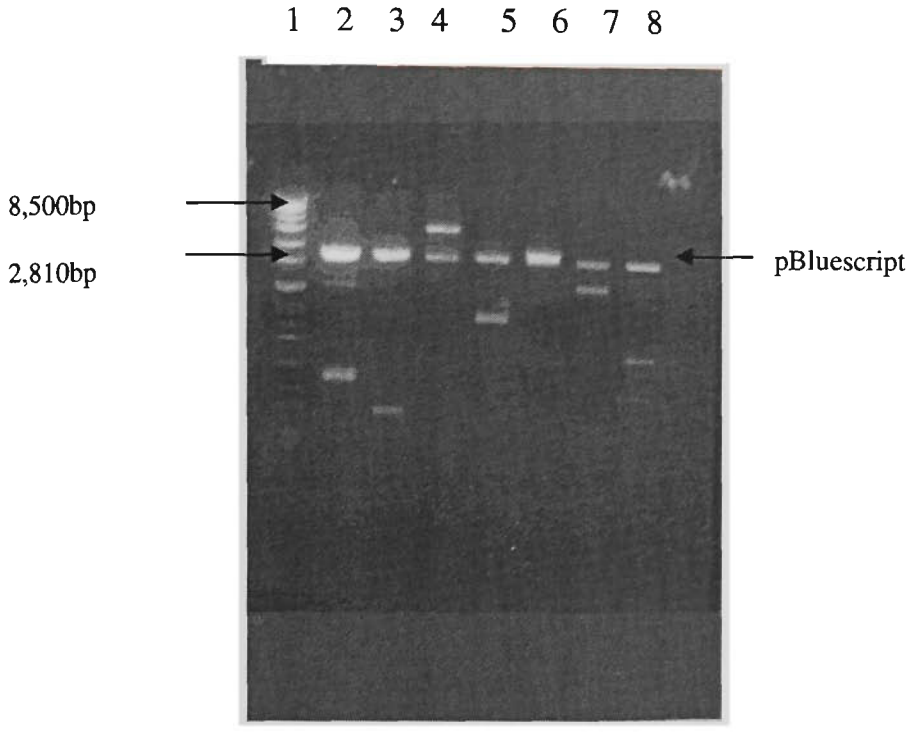
Of the resulting transformants, approximately fifty white colonies were grown in LBG with suitable antibiotic and plasmid DNA was isolated and run on agarose gels. About 30 putative clones were randomly chosen and, based on the relative mobility of the plasmids, selected for screening. Figure 4.5a shows the relative mobility of plasmids from various clones with respect to each other and pBluescript SKII vector. The circular plasmid DNA from the putative clones which were larger than the pBluescript SKII vector were digested with *Pst*1. Unfortunately, most of the clones generated more than one *Pst*1 fragment and therefore only a few clones which generated a single *Pst*1 fragment of sizes between 3kb to 3.5kb and above were chosen for further study. Figure 4.5b shows the *Pst*1 restriction digestion profiles of some of the selected clones. The digest shown in lane 6 in Fig 4.5b contained a doublet, indicating that the vector (3.5kb) and insert (2.99kb).are not well separated.



a

Figure 4.5 Analysis of *E. coli* clones containing pBluescript SKII with inserts of *C. glutamicum* AS019 genomic DNA.

a Lane 1, 1kb DNA ladder (10,000, 8,000, 6,000, 5,000, 4,000,3,500, 3,000, 2,500, 2,000, 1,500, 1,000, 750, 500, 250bp respectively); Lane 2, pBluscript SKII; Lanes 3, 4, 5, 6 ,7, 8 and 9 are AS019 clones A29, A30, A36, A37, A38, A26, A27 respectively. The three configurations of closed covalently circular (ccc), open circular (oc) and linear DNA are shown for the pBluescript SKII vector only.



b *Pst*1 restriction digestion of some of the selected clones. Lane 1 SPP1/*Eco*R1markers (8,500, 7,350, 6,100, 4,840, 3,590, 2,810, 1,950, 1,860, 1,510, 1,390, 1,160, 980, 720, 480, 360bp); Lanes 2, 3, 4, 5, 6, 7, 8 are clones A1, A2, A29, A28, A30, A36, A37. Note the two bands in lane 6 are not well separated.

4.2.7 Sequencing of the *inhA* gene from clones.

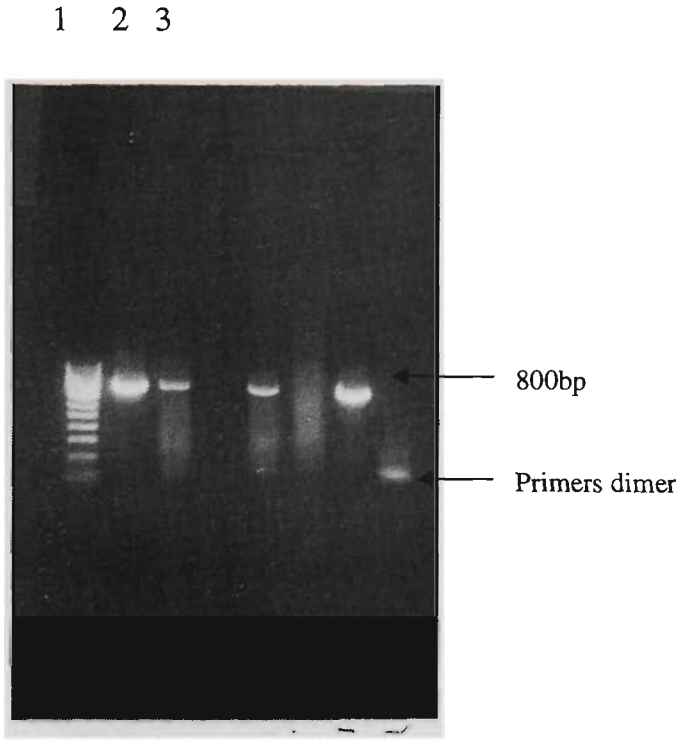
The fifteen clones containing inserts of suitable size were subjected to PCR amplification using *inhA* gene-specific primers. Finally two clones designated as AS29 and AS30 were chosen for further studies. Figure 4.6 shows the PCR amplified *inhA* fragments from some positive clones using primers sets INH1-CGP4 and CGP3-INH2. Clones AS29 and AS30 were selected for sequencing because the amplification products were the expected size for each of the primer pairs used.

Initially, attempts were made to sequence the entire clone for characterisation in order to identify sequences in the vicinity of the *inhA* gene as well as obtaining sequence data for the cloned *inhA* gene. The M13 and T7 universal primers were used as pBluescript had the sites for these sequencing primers in the poly-cloning region. As the sequencing data accumulated (see below), further nested primers were made to "walk up and down" on both sites of the insert. Unfortunately, *C. glutamicum* is a notoriously difficult template for sequencing and the primer walking approach failed. Two new nested primers were designed and sequencing reaction was undertaken with changed reaction conditions but the reactions failed. This may have been due to high GC content, or because of secondary loops inside the template so the primers failed to bind. It was then decided to verify the *inhA* gene sequence by means of PCR amplification from the clones. For the PCR amplified fragments from the clones (A29, A30) as shown in Fig 4.6a and b respectively, each of the PCR products was used as a template for automated DNA sequencing using the two primers (INH1-CGP4 and CGP3-INH2) as sequencing primers.

The identity of the sequences was determined by a BLASTN similarity search by accessing ANGIS. Results of the identity search revealed that the DNA sequences obtained were 92-99% similar to the *M. smegmatis inhA* gene confirming the presence of this gene in these clones.

The nested primers used to sequence the clone A30 in the T7 direction are shown in Table 4.7. The sequencing data obtained from both ends of the insert in clone A30 with M13 and T7 primers are shown in Figures 4.7a and b respectively. A linear restriction map of the generated sequence was obtained through the MAP program of the WebANGIS and is shown in Figure 4.8. According to the map, restriction enzymes *Eco*RV, *Bgl*II, had single cutting sites and *Hind*III had two (one in poly-cloning region and at position 930 within the sequence). The clones were digested singly and doubly with these enzymes to validate the sequence. Figure 4.9 shows the restriction digestion of clone A30 using various restriction enzymes. The restriction digestion with *Eco*RV and *Bgl*II generated single fragments of sizes between 6.5 and 7kb whereas with *Hind*III gave two fragments of sizes 5.5 and 0.93kb. Similarly, double digestion of the clone with *Hind*III/*Bgl*II, *Hind*III/*Eco*RV, *Hind*III/*Pst*1 generated two and three fragments of expected sizes. The figures showed that restriction digestion profiles of the clone was in accordance to the linear restriction map which was obtained accessing MAP programme of the WebANGIS. This implies that although the exact position of the gene within the clone was not mapped, the clone was sequenced in the right direction. The sequence readout with M13 primer was limited to only 450 bp. No further success in that direction was made because of the failure of reaction with the nested primers designed from this sequence.

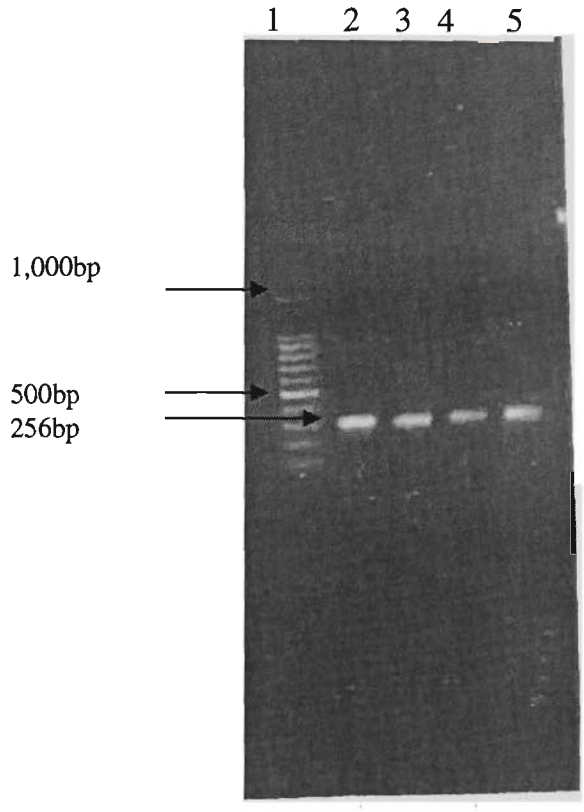
A sequence similarity search was performed using the BLASTN program of ANGIS. More recently, the whole genome of *C. glutamicum* had been published The results of the identity search showed that the DNA sequence obtained from clone A30 had similarity with published *C. glutamicum* and mycobacterial genomes. The results showed 99 to 85% similarity with *C. glutamicum* ATCC13032 and *C. effeciens* YS-314 DNA, similarity scores with mycobacterial genomes were, *M. tuberculosis* 72%, *M. leprae* cosmid Ml496 69%, *Streptomyces coelicolor* cosmid 70% etc. The polypeptides deduced from the sequences of these clones were also compared to that of all known proteins in the database using the BLASTX and FASTA programs. The results showed maximum homology 87% with 94.2 kDa protein RV146, hypothetical 95.7 kDa protein B1496, *M. leprae* cosmid L536 87%, *M. tuberculosis* complete genome H37RV 81%, *Pyrococcus furiosus* putative thiamine biosynthesis protein gene 74%, *Erwinia chrysanthemi* ATPase operon 66% and a lesser degree of similarity with *Bacillus halodrans* genomic DNA 45%. Sequencing data for clone A29 was limited to only 500bp and the identity search results performed by accessing BLASTN showed 99% similarity with *C. glutamicum* ATCC13032 DNA, 65% similarity with *M. tuberculosis* H37 RV and *M. tuberculosis* sequence VO44. Figures 4.10 a, b and c showed the nucleotide sequence, linear restriction map and restriction digestion profile of clone A29 respectively.



a

Figure 4.6 PCR amplified *inhA* fragments for selected clones using primer sets INH1-CGP4 (a) and CGP3-INH2 (b).

a Lane 1 100bp ladder (1,000, 900, 800, 700, 600, 500, 400, 300, 200, 100); Lanes, 2,3,5,6,7 contain A28, A29, A30, A37, respectively; Lane, 8 negative control.



b

b PCR amplified fragments for selected clones using primers sets CGP3-INH2. Lane 1 100bp ladder (1,000, 900, 800, 700, 600, 500, 400, 300, 200, 100); Lanes, 2,3,5,6,7 contain DNA from clones A28, A29, A30, A37.

Table 4.7 List of nested primers designed to sequence clone A30.

PRIMERS	SEQUENCE (5'.....3')	SIZE[a]	TM (^{O}C)[b]	POSITION
T7	CCC TAT AGT GAG TCG TAT TA	20	56	
AS30T7	ATG CTC AGG GCC TTG AGG CGC T	24	72	473-494
AST7	GAA GCA TCC ATT CTG GCT CGC TCT	24	74	498-522
AST2	ATG CTG GCA GAG TTG CAA CCC GT	23	72	870-792
AST3	AGC AAG GGA ATC ATA TTC GTG G	22	64	1091-1111
M13	CGT GAC TCG GAA AAC	15	46	

[a]Size=number of bases
[b]TM=melting temperature

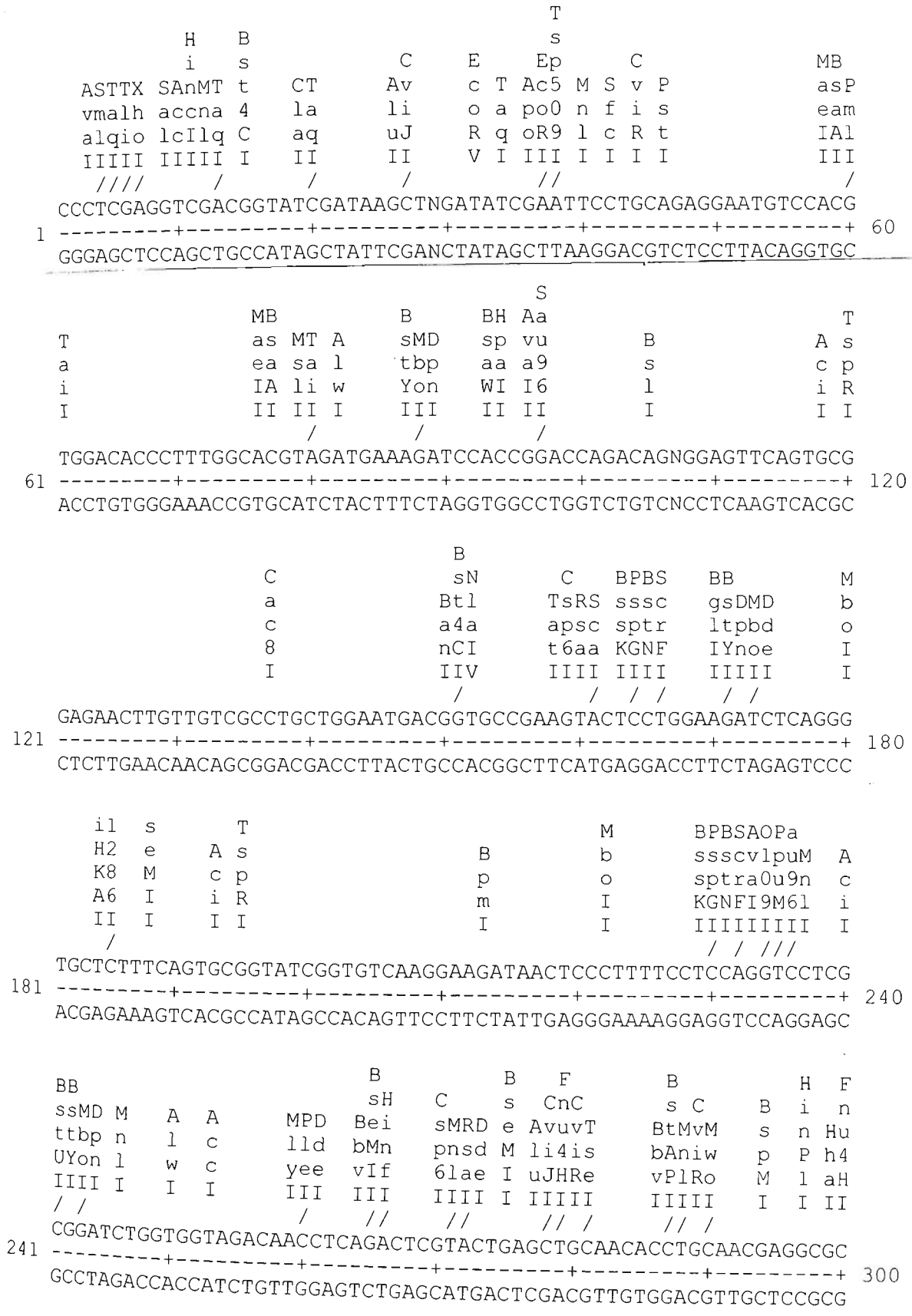
Figure 4.7a Sequence of the DNA insert in clone AS30 using primer walking from the T7 promoter region. of the pBluescript vector. The first 40 bases (1-40) are part of the vector and sequence in the insert start from 41.

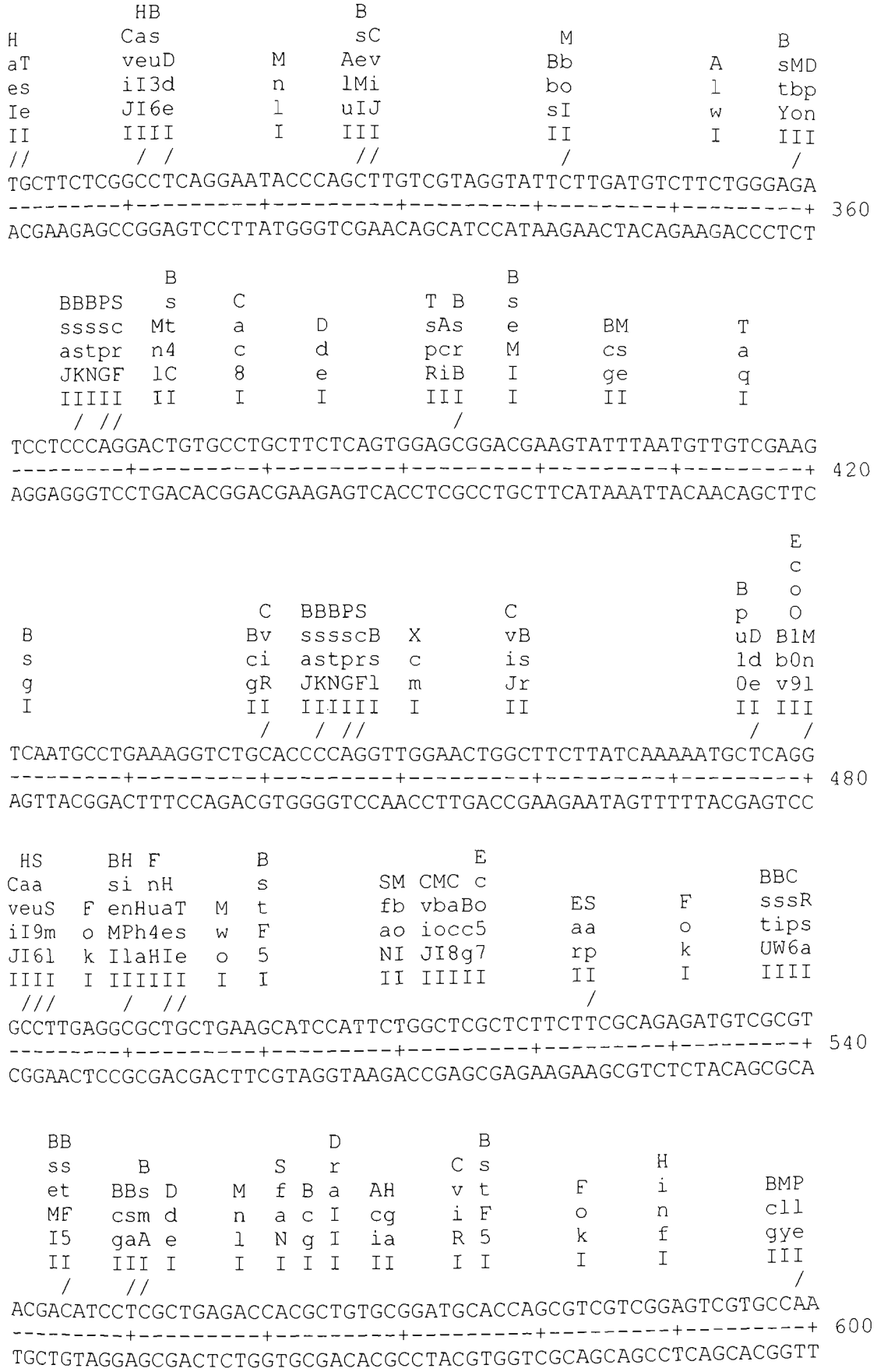
```
   1  CCCTCGAGGT CGACGGTATC GATAAGCTNG ATATCGAATT CCTGCAGAGG
  51  AATGTCCACG TGGACACCCT TTGGCACGTA GATGAAAGAT CCACCGGACC
 101  AGACAGNGGA GTTCAGTGCG GAGAACTTGT TGTCGCCTGC TGGAATGACG
 151  GTGCCGAAGT ACTCCTGGAA GATCTCAGGG TGCTCTTTCA GTGCGGTATC
 201  GGTGTCAAGG AAGATAACTC CCTTTTCCTC CAGGTCCTCG CGGATCTGGT
 251  GGTAGACAAC CTCAGACTCG TACTGAGCTG CAACACCTGC AACGAGGCGC
 301  TGCTTCTCGG CCTCAGGAAT ACCCAGCTTG TCGTAGGTAT TCTTGATGTC
 351  TTCTGGGAGA TCCTCCCAGG ACTGTGCCTG CTTCTCAGTG GAGCGGACGA
 401  AGTATTTAAT GTTGTCGAAG TCAATGCCTG AAAGGTCTGC ACCCCAGGTT
 451  GGAACTGGCT TCTTATCAAA AATGCTCAGG GCCTTGAGGC GCTGCTGAAG
 501  CATCCATTCT GGCTCGCTCT TCTTCGCAGA GATGTCGCGT ACGACATCCT
 551  CGCTGAGACC ACGCTGTGCG GATGCACCAG CGTCGTCGGA GTCGTGCCAA
 601  CCATAGTTGT ACGGACCGAT GGATTCAATG ATCTGGTCAT CGGTCAAGGG
 651  CTCGTTAACC CCTGGGTTCG TCGTTGCCGA AGTCATGATC CGCTCCTTTC
 701  ATCAGGAGTG TTTGATGGGT GTCAATGGAA TATTTGTTGT GCAGATGCCG
 751  TGGCCGTCCG CGATTGTTGC CAATGGTTGC GTGTGCTGCC CCAAAAGTTC
 801  TGAGACTGCT TGATGCTCTG CCTCACACAG TTCCGGAAAT TCCGTGGCGA
 851  CTGTAGATAT TGGACAGTGA TGCTGGCAGA GTTGCAACCC GTTTCGAGTG
 901  GCATCGACAG TTGCTGCATA ACCATGCCGA CTAAAAGCTT CAACTAAAGA
 951  TTTGGCTGTA TCTTCGATTG ATTGATCTGT GACATCTGCT GGGGTAATAC
1001  CCTCAACAAT TGTTTCGATC CGCTTTCTAG CAAATTGCCT TACTGCATCA
1051  TCTCCGCCGA CCTCTCGAAG AGTGGCTAGA GCTGCCGCAG CAAGGGAATC
1101  ATATTCGTGG CCGAAGATTG AGCGACCTTT ATCAGTAAGC CGATAAGTTT
1151  TTGCTGGCCT ACCGCGCATT TTGGGCTCAT ATGGGTTCTG GCGCGGATTT
1201  GCCGCCTCCG CCAGATTTTC TTCAACCAAG TTGTCTAGGT GCCTGCGCAC
1251  TCCCACAGTT GAAAGCTGAA GCTGTTCAGC AATATCTGAA GCGATCACCG
```

Figure 4.7b Sequence of the DNA insert in clone AS30 from the M13 promoter site using M13 as a sequencing primer.

```
  1  CCGGGCTGCA GCATTCGGCT CATGAAGTTT CCGCCAGGGA AATCCACATA
 51  CGGATTATTC ACCACCGGAA GTGCACCCGC GCCCAGCGCA CCAAACGCGA
101  TCATTAACGC ACCCATGGTG CCGATCAGGC GCAGCCATGC AAATCGGCGA
151  ATCTTTTCAG GCTCAAGCCC TGAGGCGTCG GTATCGCGGT CGTTGATGGA
201  GCTCGCGCCG GTCGCGACGT CGAAAAGCGT TGCCCCTAAA GGGCTCTGCT
251  CGTCTTCCAC GTGGAGGGTC GCTGATCTGG AACCAGCGGT GCCGATGCGC
301  GGCAAAGTGT CGAGAAATTG GCGTAGGGGA CGAGGTGCCC GAGGAAACCA
351  TTTTCTGTTT TCAGGGGCGT TCGAATTGGG TTGAATCTGC TCGTCCTTCG
401  TCACTCGCAT CATTCTACGC AAGGGAGCGG AGAACATTTA CCTCGCATCA
451  GAGTCTGGTG GTGACCCGAA GGGGGATAGT GTGAGCTAAA TCTCAAATTA
501  TATTCATTTT CGG
```

Figure 4.8 Linear restriction map of clone A30 obtained by accessing the MAP program of WEB ANGIS.

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361
420
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TCAATGCCTGAAAGGTCTGCACCCCAGGTTGGAACTGGCTTCTTATCAAAAATGCTCAGG
421
480
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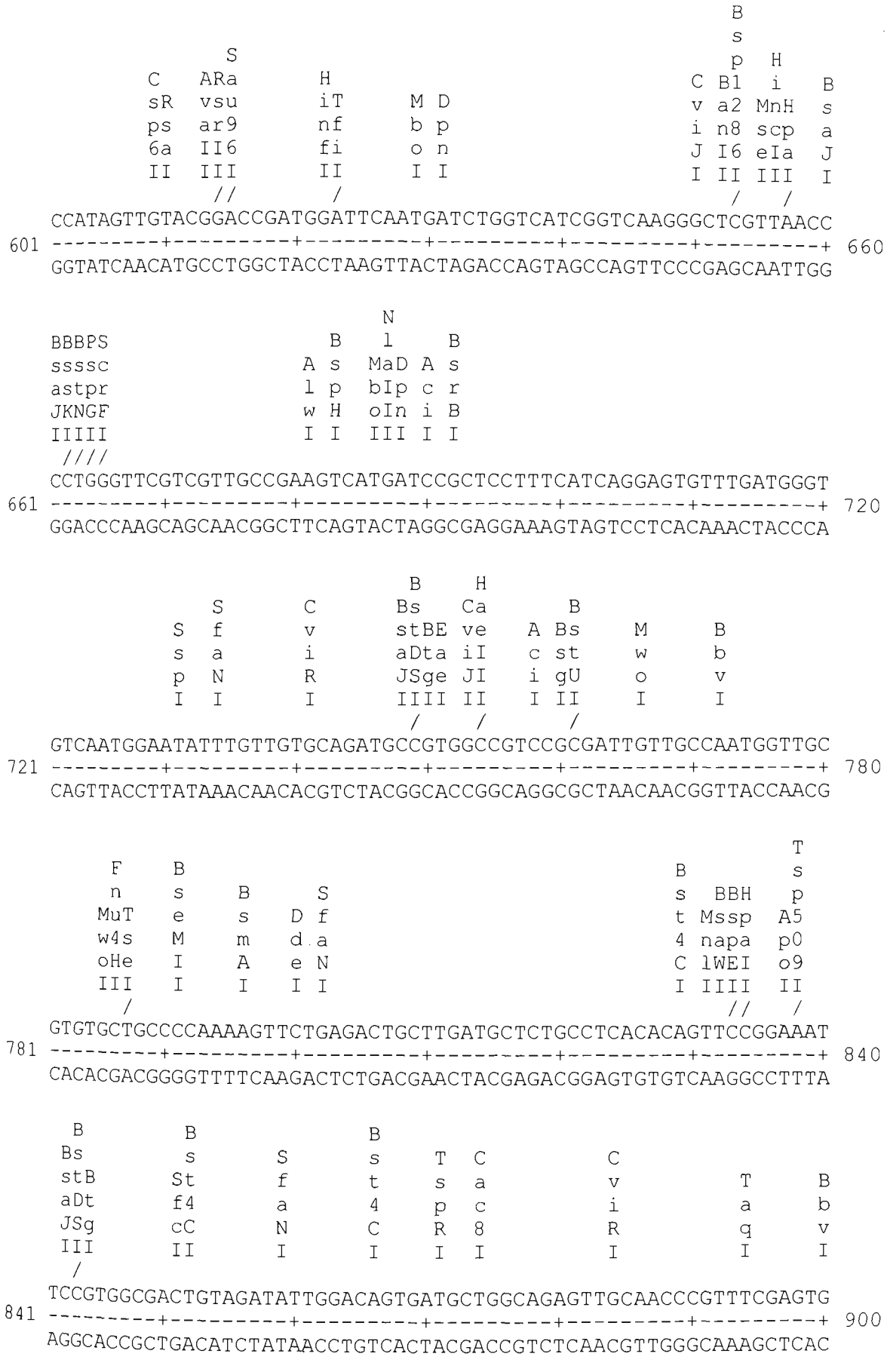
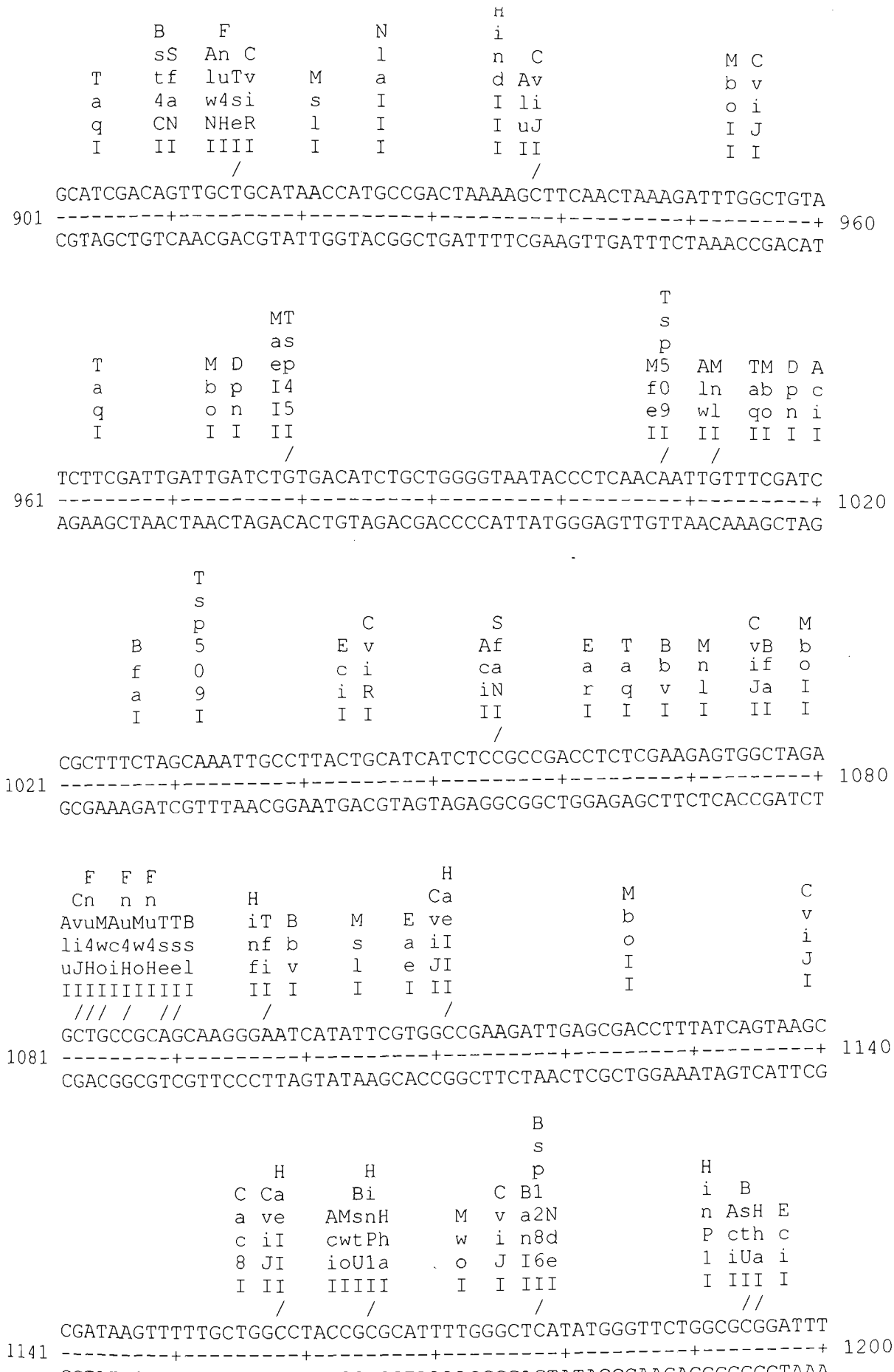
GCCTTGAGGCGCTGCTGAAGCATCCATTCTGGCTCGCTCTTCTTCGCAGAGATGTCGCGT
481
540
CGGAACTCCGCGACGACTTCGTAGGTAAGACCGAGCGAGAAGAAGCGTCTCTACAGCGCA
ACGACATCCTCGCTGAGACCACGCTGTGCGGATGCACCAGCGTCGTCGGAGTCGTGCCAA
541
600
TGCTGTAGGAGCGACTCTGGTGCGACACGCCTACGTGGTCGCAGCAGCCTCAGCACGGTT

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901 960
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961 1020
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CGCTTTCTAGCAAATTGCCTTACTGCATCATCTCCGCCGACCTCTCGAAGAGTGGCTAGA
1021 1080
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GCTGCCGCAGCAAGGGAATCATATTCGTGGCCGAAGATTGAGCGACCTTTATCAGTAAGC
1081 1140
CGACGGCGTCGTTCCCTTAGTATAAGCACCGGCTTCTAACTCGCTGGAAATAGTCATTCG
CGATAAGTTTTTGCTGGCCTACCGCGCATTTTGGGCTCATATGGGTTCTGGCGCGGATTT
1141 1200
GCTATTCAAAAACGACCGGATGGCGCGTAAAACCCGAGTATACCCAAGACCGCGCCTAAA

```
      F                                                 H            B
      n         M                                    N  C i            s
     Au    MA   b    M                          B  B l  a nFH          t
     c4    wc   o    n                          f  a a  c Psh          4
     iH    oi   I    l                          a  n I  8 lpa          C
     II    II   I    I                          I  I V  I III          I
      /
     GCCGCCTCCGCCAGATTTTCTTCAACCAAGTTGTCTAGGTGCCTGCGCACTCCCACAGTT
1201 ---------+---------+---------+---------+---------+---------+ 1260
     CGGCGGAGGCGGTCTAAAAGAAGTTGGTTCAACAGATCCACGGACGCGTGAGGGTGTCAA
```

Enzymes that do cut:

AccI	AciI	AluI	AlwI	AlwNI	ApoI	AvaI	AvaII
BanI	BanII	BbsI	BbvI	BcgI	BfaI	BglII	BpmI
Bpu10I	BsaI	BsaAI	BsaJI	BsaWI	BseMII	BsgI	BsiHKAI
BsiWI	BslI	BsmAI	Bsp1286I	BspEI	BspHI	BspMI	BsrI
BsrBI	BssKI	BstAPI	Bst4CI	BstDSI	BstF5I	BstNI	BstUI
BstYI	Bsu36I	BtgI	Cac8I	ClaI	Csp6I	CviJI	CviRI
DdeI	DpnI	DraIII	EaeI	EarI	EciI	Eco57I	EcoO109I
EcoRI	EcoRV	Fnu4HI	FokI	FspI	HaeII	HaeIII	HgaI
HhaI	HinP1I	HincII	HindIII	HinfI	HpaI	HpaII	HphI
MaeII	MaeIII	MboI	MboII	MfeI	MlyI	MnlI	MseI
MslI	MwoI	NdeI	NlaIII	NlaIV	PleI	PmlI	PpuMI
PspGI	PstI	RsaI	RsrII	SalI	SapI	Sau96I	ScaI
ScrFI	**SfaNI**	**SfcI**	**SmlI**	**SspI**	**TaiI**	**TaqI**	**TatI**
TfiI	TliI	TseI	Tsp45I	Tsp509I	TspRI	XcmI	XhoI
XmnI							

Enzymes that do not cut:

AatII	Acc65I	AclI	AfeI	AflII	AflIII	AgeI	AhdI
ApaI	ApaLI	AscI	AseI	AvrII	BaeI	BaeI	BamHI
BbeI	BbvCI	BciVI	BclI	BglI	BlpI	BmrI	BplI
BsaBI	BsaHI	BseRI	BseSI	BsiEI	BsmI	BsmBI	BsmFI
BsrDI	BsrFI	BsrGI	BssHII	BssSI	BstBI	BstEII	BstXI
BstZ17I	BtrI	BtsI	DraI	DrdI	EagI	Ecl136II	EcoNI
FauI	FseI	KasI	KpnI	MluI	MscI	MspA1I	NaeI
NarI	NciI	NcoI	NgoMIV	NheI	NotI	NruI	NsiI
NspI	PacI	PciI	PflMI	PmeI	Ppu10I	PshAI	PsiI
PspOMI	PvuI	PvuII	SacI	SacII	SanDI	SbfI	SexAI
SfiI	SfoI	SgfI	SgrAI	SmaI	SnaBI	SpeI	SphI
SrfI	StuI	StyI	SwaI	Tth111I	XbaI	XmaI	

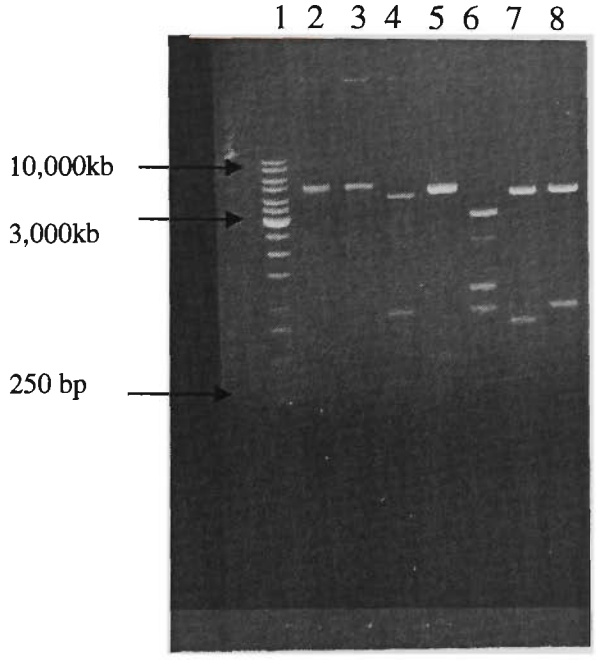
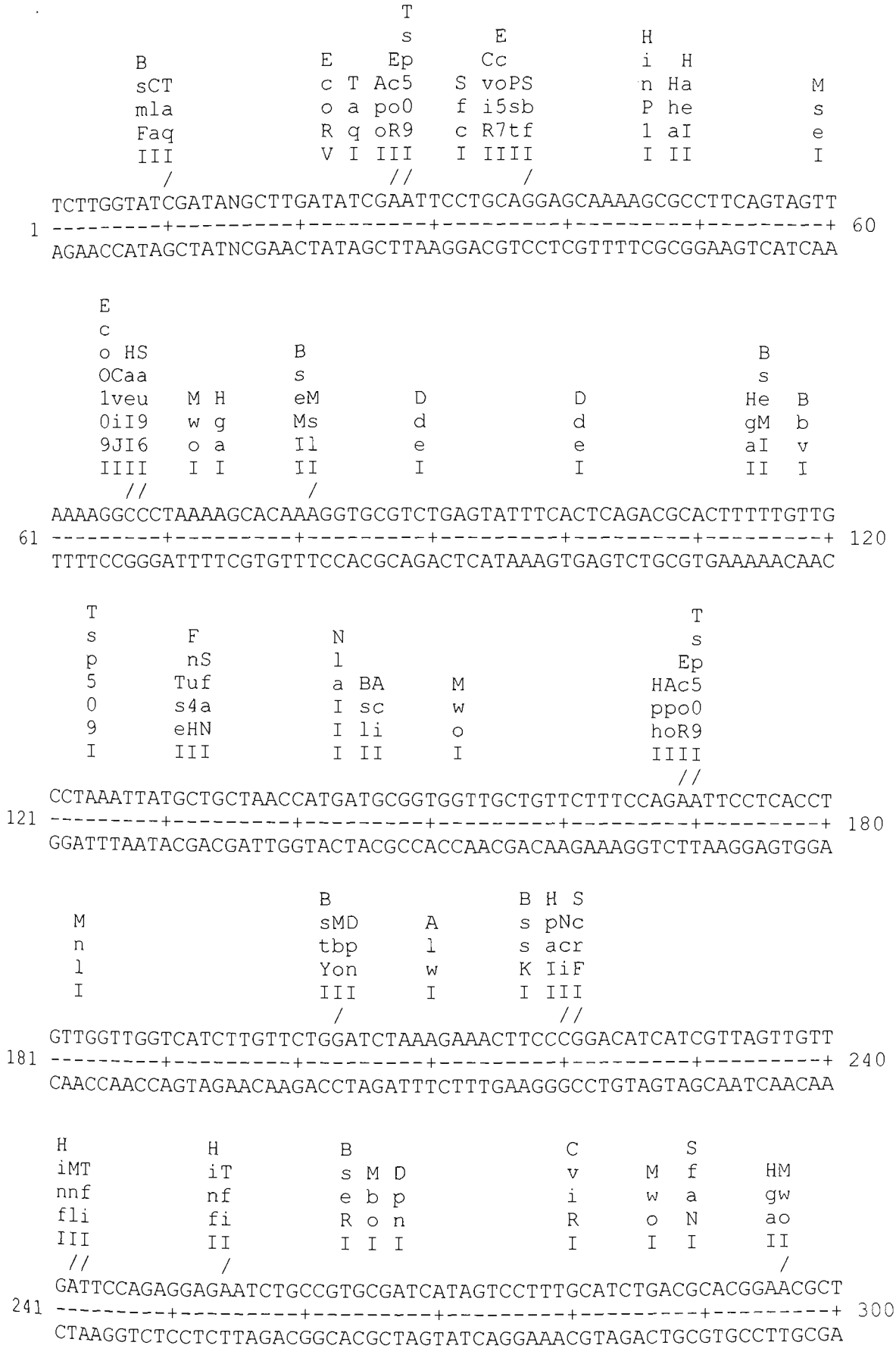


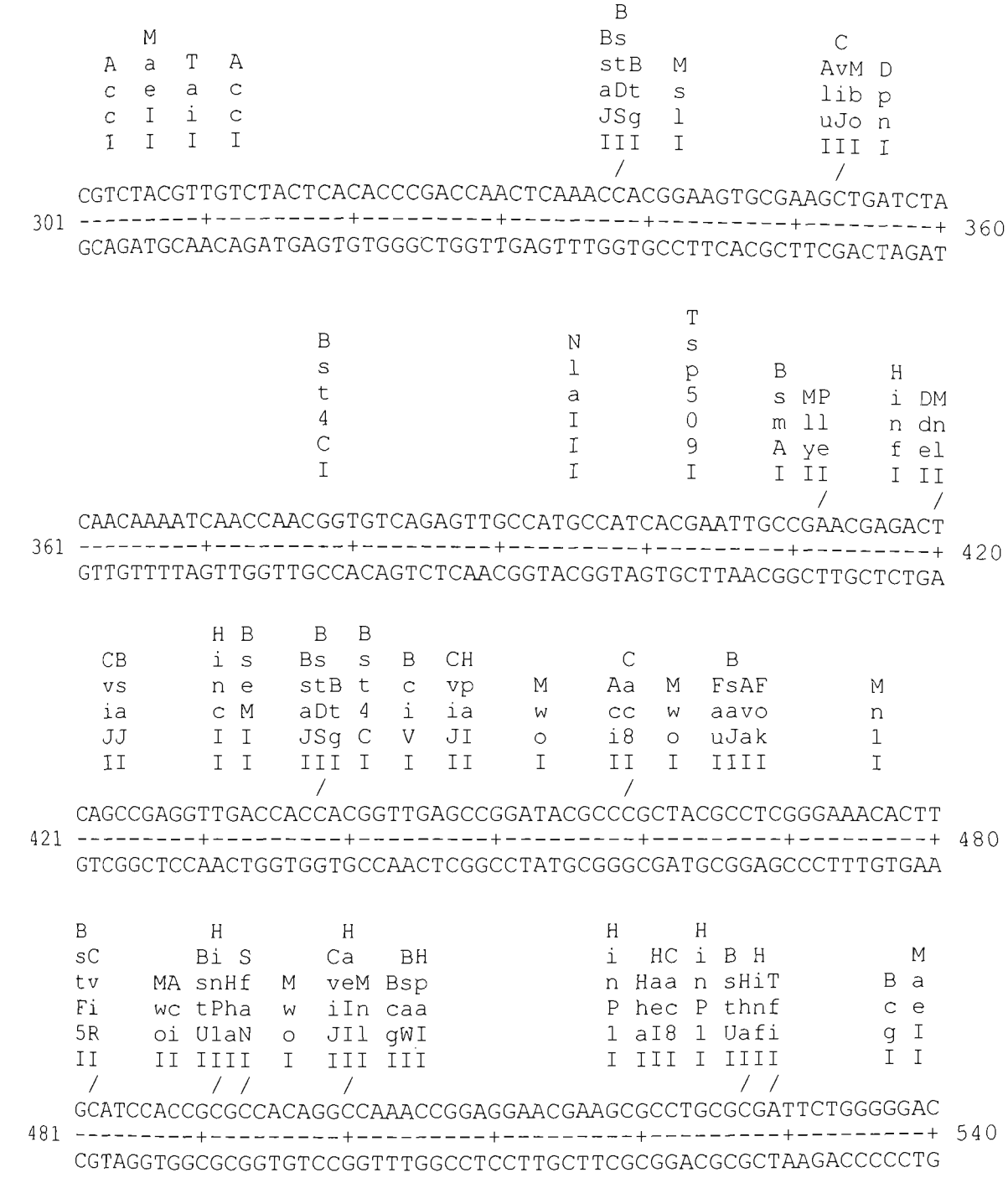
Figure 4.9 Restriction pattern of plasmid DNA from clone A30 containing the *inhA* gene from *C. glutamicum* strain AS019. Lane1, 1kb DNA ladder (10,000, 8,000, 6,000, 5000, 4,000, 3,500, 3000, 2,500, 2,000, 1,500 1,000, 750, 500, 250); Lanes 2,3,4, 5, contained *Eco*RV, *Bgl*II, *Hind*III, *Eco*01091; Lanes 6, 7 and 8 contained double digestion of A30 with: *Pst*1*/HindIII; Hind*III*/Bgl*II, *Hind*III*/ Eco*RV.

Figure 4.10a Sequence of the DNA insert in clone A29 using the T7 primer.

```
  1  TCTTGGTATC GATANGCTTG ATATCGAATT CCTGCAGGAG CAAAAGCGCC
 51  TTCAGTAGTT AAAAGGCCCT AAAAGCACAA AGGTGCGTCT GAGTATTTCA
101  CTCAGACGCA CTTTTTGTTG CCTAAATTAT GCTGCTAACC ATGATGCGGT
151  GGTTGCTGTT CTTTCCAGAA TTCCTCACCT GTTGGTTGGT CATCTTGTTC
201  TGGATCTAAA GAAACTTCCC GGACATCATC GTTAGTTGTT GATTCCAGAG
251  GAGAATCTGC CGTGCGATCA TAGTCCTTTG CATCTGACGC ACGGAACGCT
301  CGTCTACGTT GTCTACTCAC ACCCGACCAA CTCAAACCAC GGAAGTGCGA
351  AGCTGATCTA CAACAAAATC AACCAACGGT GTCAGAGTTG CCATGCCATC
401  ACGAATTGCC GAACGAGACT CAGCCGAGGT TGACCACCAC GGTTGAGCCG
451  GATACGCCCG CTACGCCTCG GGAAACACTT GCATCCACCG CGCCACAGGC
501  CAAACCGGAG GAACGAAGCG CCTGCGCGAT TCTGGGGGAC GT
```

Figure 4.10b Linear restriction map of the DNA insert in clone A29 using MAP program of WEB ANGIS.

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ClaI CviJI CviRI DdeI DpnI Eco57I EcoO109I EcoRI
EcoRV FauI Fnu4HI FokI HaeII HaeIII HgaI HhaI
HinP1I HincII HinfI HpaII HphI MaeII MboI MlyI
MnlI MseI MslI MwoI NciI NlaIII PleI PstI
Sau96I SbfI ScrFI SfaNI SfcI TaiI TaqI TfiI
TseI Tsp509I

Enzyme that do not cut:

AatII	Acc65I	AclI	AfeI	AflII	AflIII	AgeI	AhdI
AlwNI	ApaI	ApaLI	AscI	AseI	AvaII	AvrII	BaeI
BaeI	BamHI	BanI	BanII	BbeI	BbsI	BbvCI	BclI
BfaI	BglI	BglII	BlpI	BmrI	BplI	BpmI	Bpu10I
BsaI	BsaAI	BsaBI	BsaHI	BseSI	BsgI	BsiEI	BsiHKAI
BsiWI	BsmI	BsmBI	Bsp1286I	BspEI	BspHI	BspMI	BsrI
BsrBI	BsrDI	BsrFI	BsrGI	BssHII	BssSI	BstAPI	BstBI
BstEII	BstNI	BstXI	BstZ17I	Bsu36I	BtrI	BtsI	Csp6I
DraI	DraIII	DrdI	EaeI	EagI	EarI	EciI	Ecl136II
EcoNI	FseI	FspI	HindIII	HpaI	KasI	KpnI	MaeIII
MboII	MfeI	MluI	MscI	MspA1I	NaeI	NarI	NcoI
NdeI	NgoMIV	NheI	NlaIV	NotI	NruI	NsiI	NspI
PacI	PciI	PflMI	PmeI	PmlI	Ppu10I	PpuMI	PshAI
PsiI	PspGI	PspOMI	PvuI	PvuII	RsaI	RsrII	SacI
SacII	SalI	SanDI	SapI	ScaI	SexAI	SfiI	SfoI
SgfI	SgrAI	SmaI	SmlI	SnaBI	SpeI	SphI	SrfI
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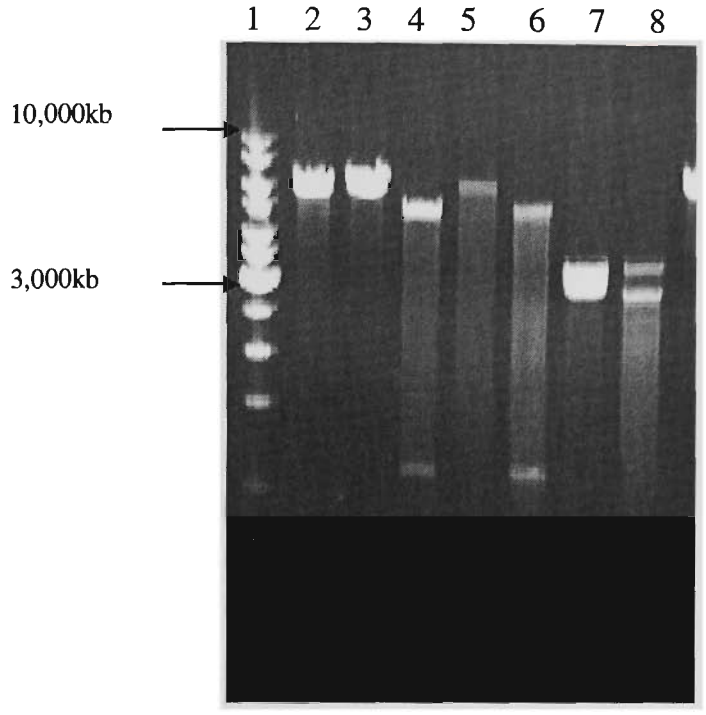


Figure 4.10c Restriction digestion pattern of plasmid DNA from clone A29 containing the *inhA* gene from *C. glutamicum* strain AS019. Lane1, 1kb DNA ladder (10,000, 8,000, 6,000, 5000, 4,000, 3,500, 3000, 2,500, 2,000, 1,500 1,000, 750, 500, 250); Lanes 2,3,4, 5, contained *Xho*I. *Bgl*II, *Eco*RV, *Eco*01091; Lanes 6, 7, 8 and 9 contained double digestion of A29 with *XhoI/Eco*RV, *Pst*1*/HindIII; Hind*III*/Bgl*II, and *Hind*III.

4.3 SEQUENCE ANALYSIS OF THE *inhA* GENE OF AS019

4.3.1 Sequence assembly of the *inhA* gene in AS019 using fragments generated by PCR

Putative *inhA* gene sequences were amplified from the genomic DNA of AS019 as well as from clones (data in Appendix 4) using the *inhA* specific primers and conditions described in detail in Table 4.1. Although primer sets INH1-CGP4 and CGP3-INH2 were able to amplify 100% of the *inhA* gene of *C. glutamicum* and *M. smegmatis* (total gene length of approximately 850 bp for *M. smegmatis*), good sequencing data could only be obtained for up to 300-400 bp due to instrumentation limitations, quality of the DNA templates, primers and reagents. Assembly of the DNA fragments obtained from INH1-CGP4 and CGP3-INH2 into one continous sequence was not achieved with these two primers sets alone. In order to join these fragments and to unambiguously determine the *inhA* gene sequence from *C. glutamicum* strains, several sets of degenerate primers were used to amplify internal regions of the gene. Finally, five PCR products (Figure 4.1 [800bp using INH1 and CGP4, 500 bp using CGP5 and CGP2, 256 bp using CGP3 and INH2, 200 bp using CGP3 and CGP4, and 255-bp using CGP5 and CGP6]) were chosen for sequencing. The longer PCR fragments were cloned into the PCR 2.1 vector using the TA cloning kit and read using M13 and T7 primers. The smaller fragments were read directly using the relevant PCR primers. All sequences were analysed for their overlap portions and then assembled both manually and using the GCG program of ANGIS. Sequences obtained for strain AS019 were from positions 1-495, 107-476, 286-773, 628-850, 645-850 for primers M13, CGP5, CGP4, CGP3, etc. The assembled *inhA* gene sequence of *C. glutamicum* strain AS019 was 100% complete from positions 1-850 bp. The DNA sequence was deposited to GenBank under the accession number (NCBI_REF566285). Figure 4.11 shows the nucleotide sequence of the *inhA* gene of AS019 and the relationship between the PCR products used to obtained this sequence. A linear restriction map of the *inhA* gene was also constructed using the MAP program of WEB ANGIS and this is shown in Figure 4.12. The similarity of the *inhA* gene sequence was determined using BLASTN similarity search. Figure 4.13 shows the BESTFIT of the *inhA* gene sequence of AS019 with that of *M. smegmatis*. The top four high scoring pairs of *inhA* sequences obtained

Figure 4.11a The nucleotide sequence of the *inhA* gene of *C. glutamicum* strain AS019 (position 1-850) [NCBI_REF 566285].

```
  1  CCGGACACAC AAGATTTCTC GCTCACAAGG AGTCACCAAA TGACAGGCCT
 51  ACTCGAGGGC AAGCGCATCC TCGTCACGGG GATCATCACC GATTCGTCGA
101  TCGCGTTCCA CATCGCCAAG GTCGCCCAGG AGGCCGGCGC CGAACTGGTG
151  CTGACCGGTT TCGACCGCCT GAAGTTGGTC AAGCGCATCG CCGACCGCCT
201  GCCCAAGCCG GCCCCGCTGC TGGAACTCGA CGTGCAGAAC GAGGAGCACC
251  TGTCGACTCT GGCCGACCGG ATCACCGCCG AGATCGGTGA GGGCAACAAG
301  ATCGACGGTG TGGTGCACTC GATCGGGTTC ATGCCGCAGA GCGGTATGGG
351  CATCGACCCG TTCTTCGACG CGCCGTACGA GGATGTGTCC AAGGGCATCC
401  ACATCCCGGC GTACTCGTAC GCCTCGCTCG CCAAAGCCGT TCTGCCGATC
451  ATGAATCCGG GCGGCGGCAT CGTCGGCATG GACTTCGACC CCACGCGCGC
501  GATGCCCGGC TACAACTGGA TGACCGTCGC CAAGAGCGCG CTCGAATCGG
551  TCAACCGGTT CGTCGCGCGT GAGGCGGGCA AGGTGGGCGT GCGCTCGAAT
601  CTCGTTGCGG CAGGACCGAT CCGCACGCTG GCGATGAGCG CAATCGTGGG
651  CGGTGCGCTG GGCGACGAGG CCGGCCAGCA GATGCAGCTG CTCGAAGAGG
701  GCTGGGATCA GCGCGCGCCG CTGGGCTGGA ACATGAAGGA CCCGACGCCC
751  GTCGCCAAGA CCGTGTGCGC ACTGCTGTCG GACTGGCTGC CGGCCACCAC
801  CGGCACCGTG ATCTACGCCG ACGGCGGCGC CAGCACGCAG CTGTTGTGAT
```

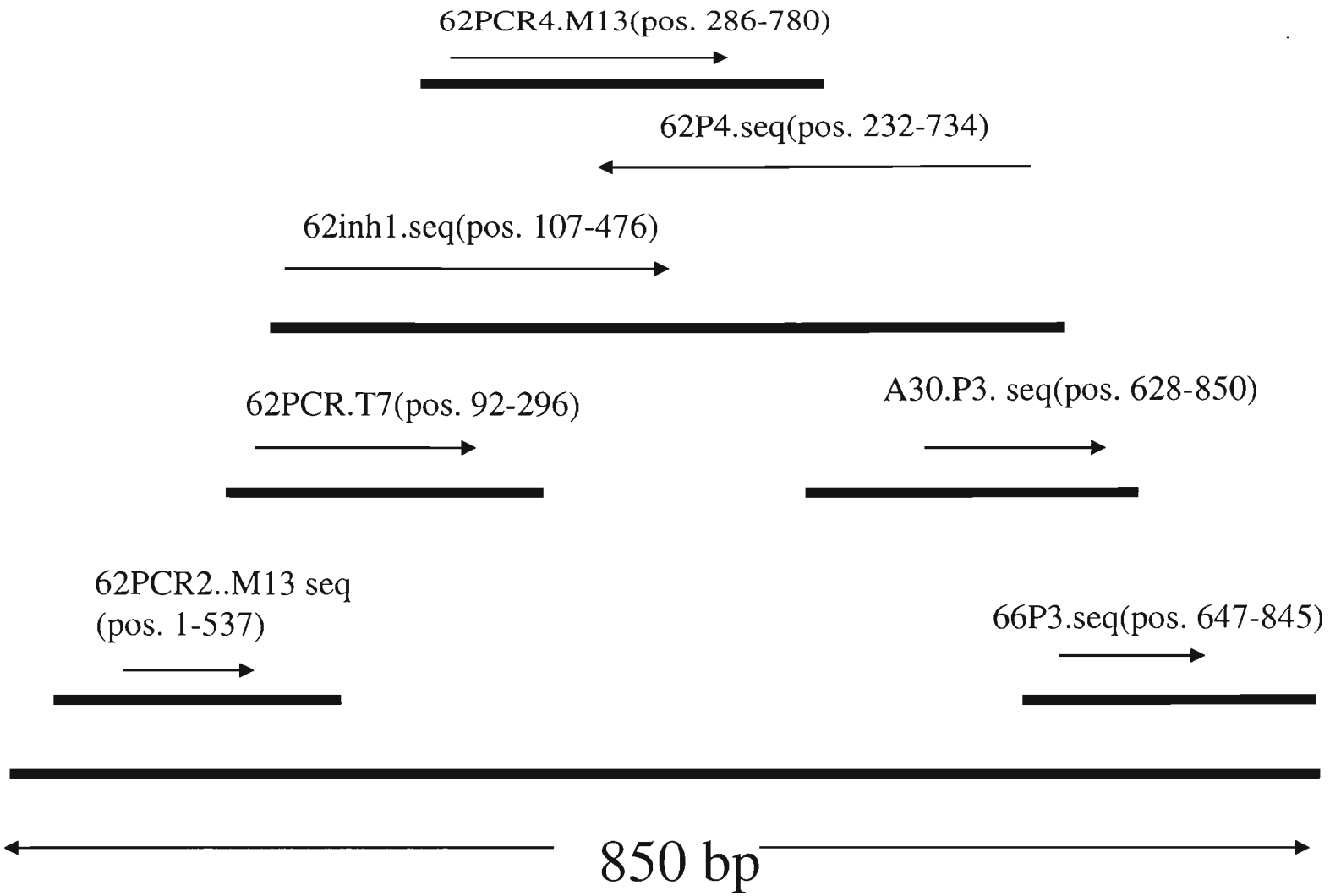
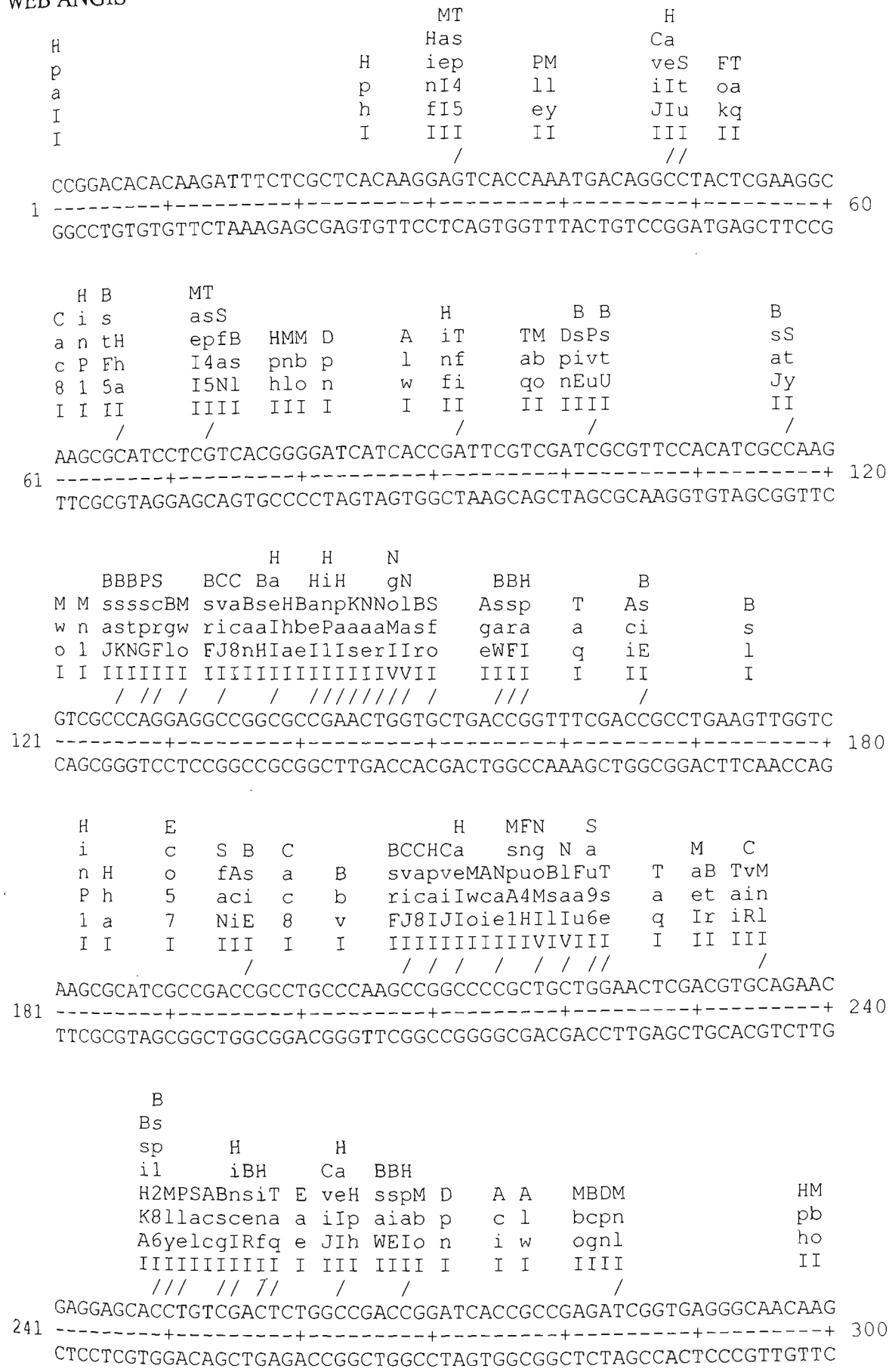
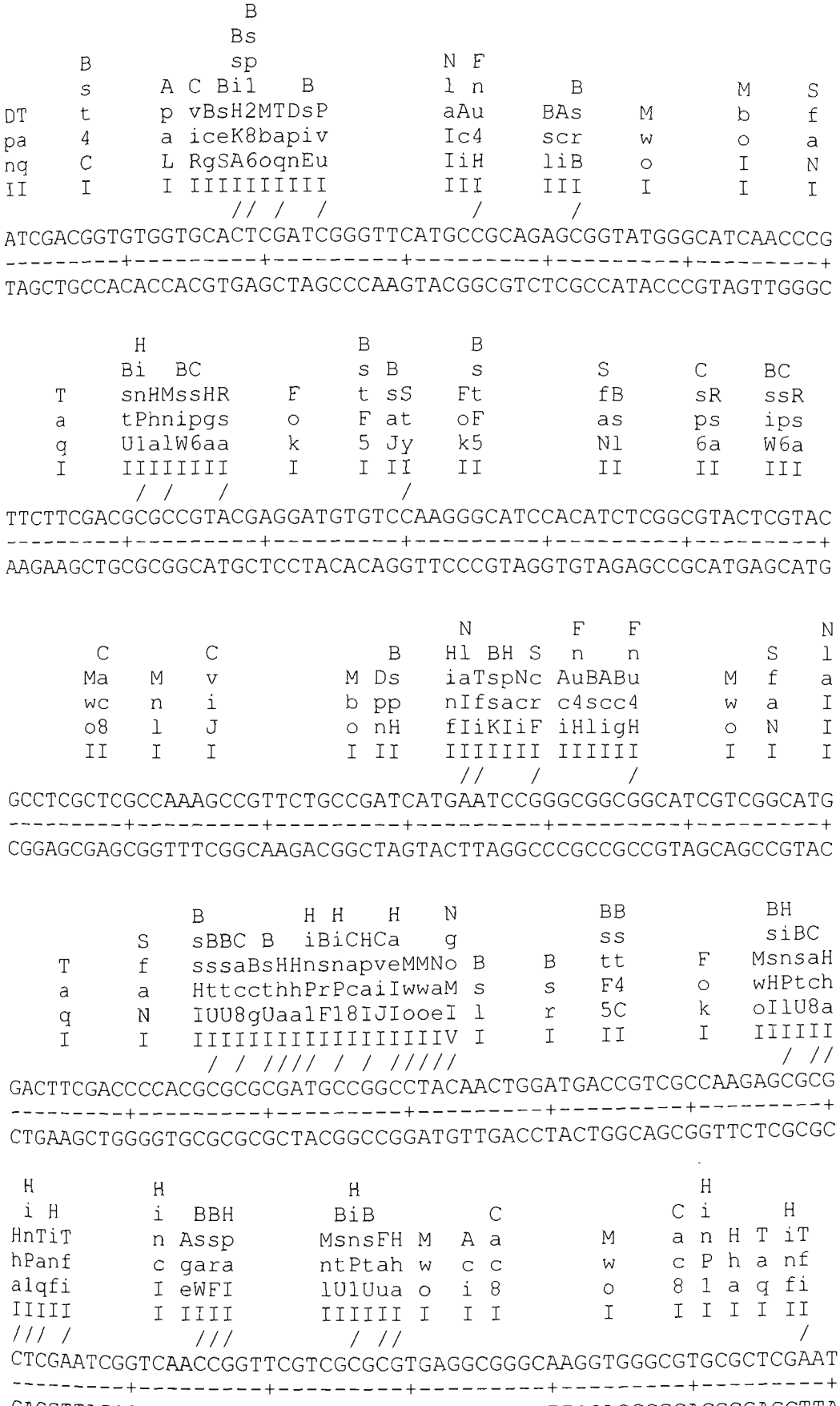
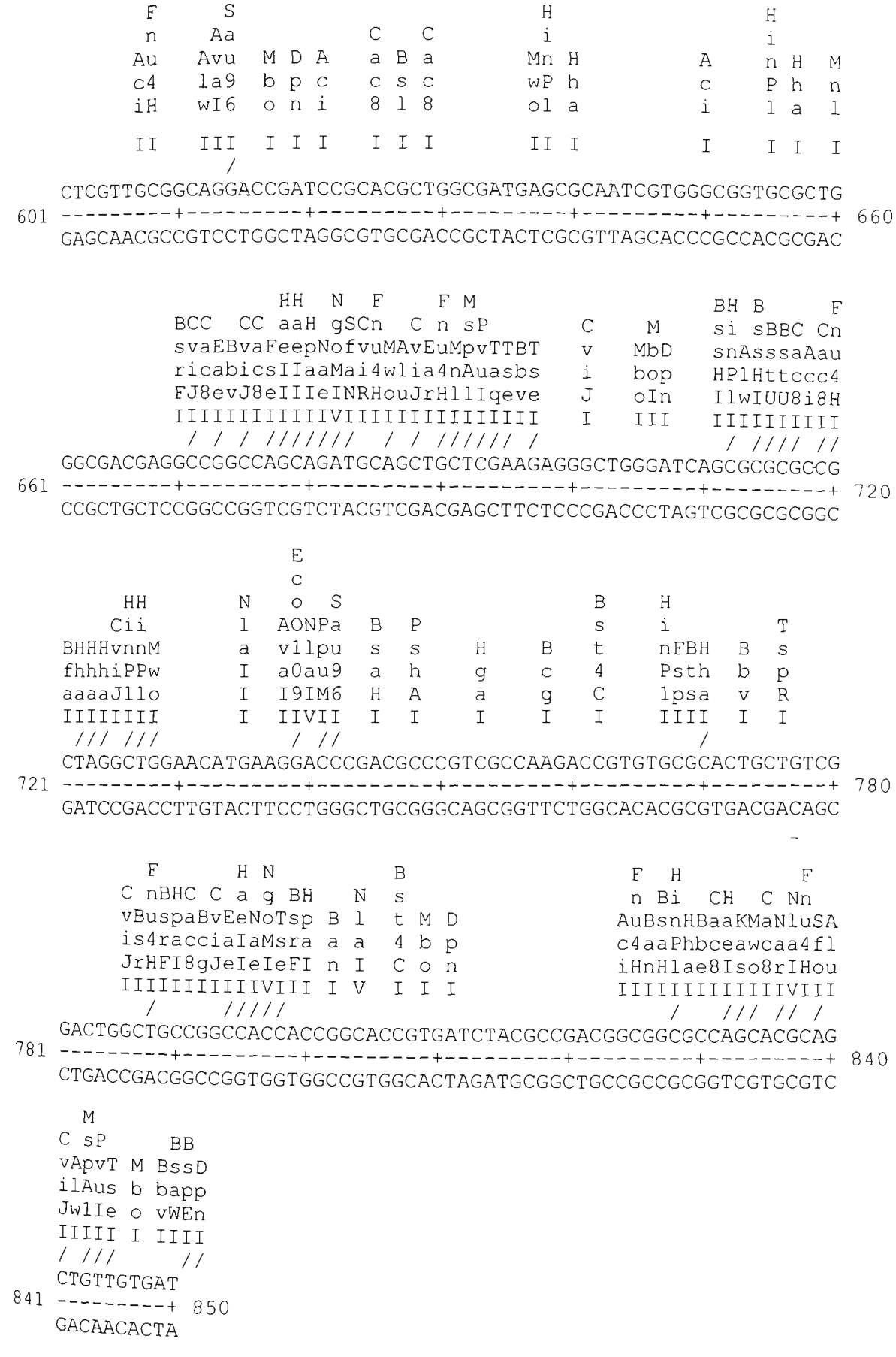


Figure 4.11b Schematic illustration of the *inhA* gene sequence assembly for *C. glutamicum* strain AS019. The list of the primers and completed sequence of the *inhA* gene for strain AS019 are shown in Table 4.1 and Figure 4.11a respectively.

Figure 4.12 A linear restriction map of the *inhA* gene of AS019 using MAP program of WEB ANGIS







Enzyme that do cut:

AccI	AciI	AgeI	AluI	AlwI	ApaLI	AvaII	BanI
BbeI	BbvI	BcgI	BfaI	BglI	BsaHI	BsaJI	BsaWI
BseRI	BseSI	BsgI	BsiEI	BsiHKAI	BsiWI	BslI	Bsp1286I
BspEI	BspHI	BsrI	BsrBI	BsrFI	BssHII	BssKI	Bst4CI
BstF5I	BstNI	BstUI	BtrI	BtsI	Cac8I	Csp6I	CviJI
CviRI	DpnI	EaeI	EarI	Eco57I	EcoO109I	FauI	Fnu4HI
FokI	FseI	FspI	HaeII	HaeIII	HgaI	HhaI	HinP1I
HincII	HinfI	HpaII	HphI	KasI	MaeII	MaeIII	MboI
MboII	MlyI	MnlI	MspA1I	MwoI	NaeI	NarI	NciI
NgoMIV	NlaIII	NlaIV	PleI	PpuMI	PshAI	PspGI	PvuI
PvuII	RsaI	SalI	Sau96I	ScrFI	SfaNI	SfoI	StuI
StyI	TaiI	TaqI	TfiI	TseI	Tsp45I	TspRI	

Enzyme that do not cut:

AatII	Acc65I	AclI	AfeI	AflII	AflIII	AhdI	AlwNI
ApaI	ApoI	AscI	AseI	AvaI	AvrII	BaeI	BaeI
BamHI	BanII	BbsI	BbvCI	BciVI	BclI	BglII	BlpI
BmrI	BplI	BpmI	Bpu10I	BsaI	BsaAI	BsaBI	BseMII
BsmI	BsmAI	BsmBI	BsmFI	BspMI	BsrDI	BsrGI	BssSI
BstAPI	BstBI	BstDSI	BstEII	BstXI	BstYI	BstZ17I	Bsu36I
BtgI	ClaI	DdeI	DraI	DraIII	DrdI	EagI	EciI
Ecl136II	EcoNI	EcoRI	EcoRV	HindIII	HpaI	KpnI	MfeI
MluI	MscI	MseI	MslI	NcoI	NdeI	NheI	NotI
NruI	NsiI	NspI	PacI	PciI	PflMI	PmeI	PmlI
Ppu10I	PsiI	PspOMI	PstI	RsrII	SacI	SacII	SanDI
SapI	SbfI	ScaI	SexAI	SfcI	SfiI	SgfI	SgrAI
SmaI	SmlI	SnaBI	SpeI	SphI	SrfI	SspI	SwaI
TatI	TliI	Tsp509I	Tth111I	XbaI	XcmI	XhoI	XmaI
XmnI							

Figure 4.13 Nucleotide sequence similarity alignment of the *inhA* genes of AS019 and *M. smegmatis* using the GCG/BESTFIT program. Identical nucleotides are indicated by vertical lines. Gaps are introduced by the program to maximise similarity scores. The *inhA* of AS019 is shown on top and of *M. smegmatis* is shown at the bottom.

```
  1 CCGGACACACAAGATTTCTCGCTCACAAGGAGTCACCAAATGACAGGCCT 50
    ||||||||||||||||||||||||||||||||||||||||||||||||||
  1 CCGGACACACAAGATTTCTCGCTCACAAGGAGTCACCAAATGACAGGCCT 50

 51 ACTCGAGGGCAAGCGCATCCTCGTCACGGGGATCATCACCGATTCGTCGA 100
    |||||| ||||||||||||||||||||||||||||||||||||||||||
 51 ACTCGAAGGCAAGCGCATCCTCGTCACGGGGATCATCACCGATTCGTCGA 100

101 TCGCGTTCCACATCGCCAAGGTCGCCCAGGAGGCCGGCGCCGAACTGGTG 150
    ||||||||||||||||||||||||||||||||||||||||||||||||||
101 TCGCGTTCCACATCGCCAAGGTCGCCCAGGAGGCCGGCGCCGAACTGGTG 150

151 CTGACCGGTTTCGACCGCCTGAAGTTGGTCAAGCGCATCGCCGACCGCCT 200
    ||||||||||||||||||||||||||||||||||||||||||||||||||
151 CTGACCGGTTTCGACCGCCTGAAGTTGGTCAAGCGCATCGCCGACCGCCT 200

201 GCCCAAGCCGGCCCCGCTGCTGGAACTCGACGTGCAGAACGAGGAGCACC 250
    ||||||||||||||||||||||||||||||||||||||||||||||||||
201 GCCCAAGCCGGCCCCGCTGCTGGAACTCGACGTGCAGAACGAGGAGCACC 250

251 TGTCGACTCTGGCCGACCGGATCACCGCCGAGATCGGTGAGGGCAACAAG 300
    ||||||||||||||||||||||||||||||||||||||||||||||||||
251 TGTCGACTCTGGCCGACCGGATCACCGCCGAGATCGGTGAGGGCAACAAG 300

301 ATCGACGGTGTGGTGCACTCGATCGGGTTCATGCCGCAGAGCGGTATGGG 350
    ||||||||||||||||||||||||||||||||||||||||||||||||||
301 ATCGACGGTGTGGTGCACTCGATCGGGTTCATGCCGCAGAGCGGTATGGG 350

351 CATCGACCCGTTCTTCGACGCGCCGTACGAGGATGTGTCCAAGGGCATCC 400
    |||| ||||||||||||||||||||||||||||||||||||||||||||
351 CATCAACCCGTTCTTCGACGCGCCGTACGAGGATGTGTCCAAGGGCATCC 400
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401 ACATCCCGGCGTACTCGTACGCCTCGCTCGCCAAAGCCGTTCTGCCGATC 450

401 ACATCTCGGCGTACTCGTACGCCTCGCTCGCCAAAGCCGTTCTGCCGATC 450

451 ATGAATCCGGGCGGCGGCATCGTCGGCATGGACTTCGACCCCACGCGCGC 500

451 ATGAATCCGGGCGGCGGCATCGTCGGCATGGACTTCGACCCCACGCGCGC 500

501 GATGCCCGGCTACAACTGGATGACCGTCGCCAAGAGCGCGCTCGAATCGG 550

501 GATGCCGGCCTACAACTGGATGACCGTCGCCAAGAGCGCGCTCGAATCGG 550

551 TCAACCGGTTCGTCGCGCGTGAGGCGGGCAAGGTGGGCGTGCGCTCGAAT 600

551 TCAACCGGTTCGTCGCGCGTGAGGCGGGCAAGGTGGGCGTGCGCTCGAAT 600

601 CTCGTTGCGGCAGGACCGATCCGCACGCTGGCGATGAGCGCAATCGTGGG 650

601 CTCGTTGCGGCAGGACCGATCCGCACGCTGGCGATGAGCGCAATCGTGGG 650

651 CGGTGCGCTGGGCGACGAGGCCGGCCAGCAGATGCAGCTGCTCGAAGAGG 700

651 CGGTGCGCTGGGCGACGAGGCCGGCCAGCAGATGCAGCTGCTCGAAGAGG 700

701 GCTGGGATCAGCGCGCGCCGCTAGGCTGGAACATGAAGGACCCGACGCCC 750

701 GCTGGGATCAGCGCGCGCCGCTGGGCTGGAACATGAAGGACCCGACGCCC 750

751 GTCGCCAAGACCGTGTGCGCACTGCTGTCGGACTGGCTGCCGGCCACCAC 800

751 GTCGCCAAGACCGTGTGCGCACTGCTGTCGGACTGGCTGCCGGCCACCAC 800

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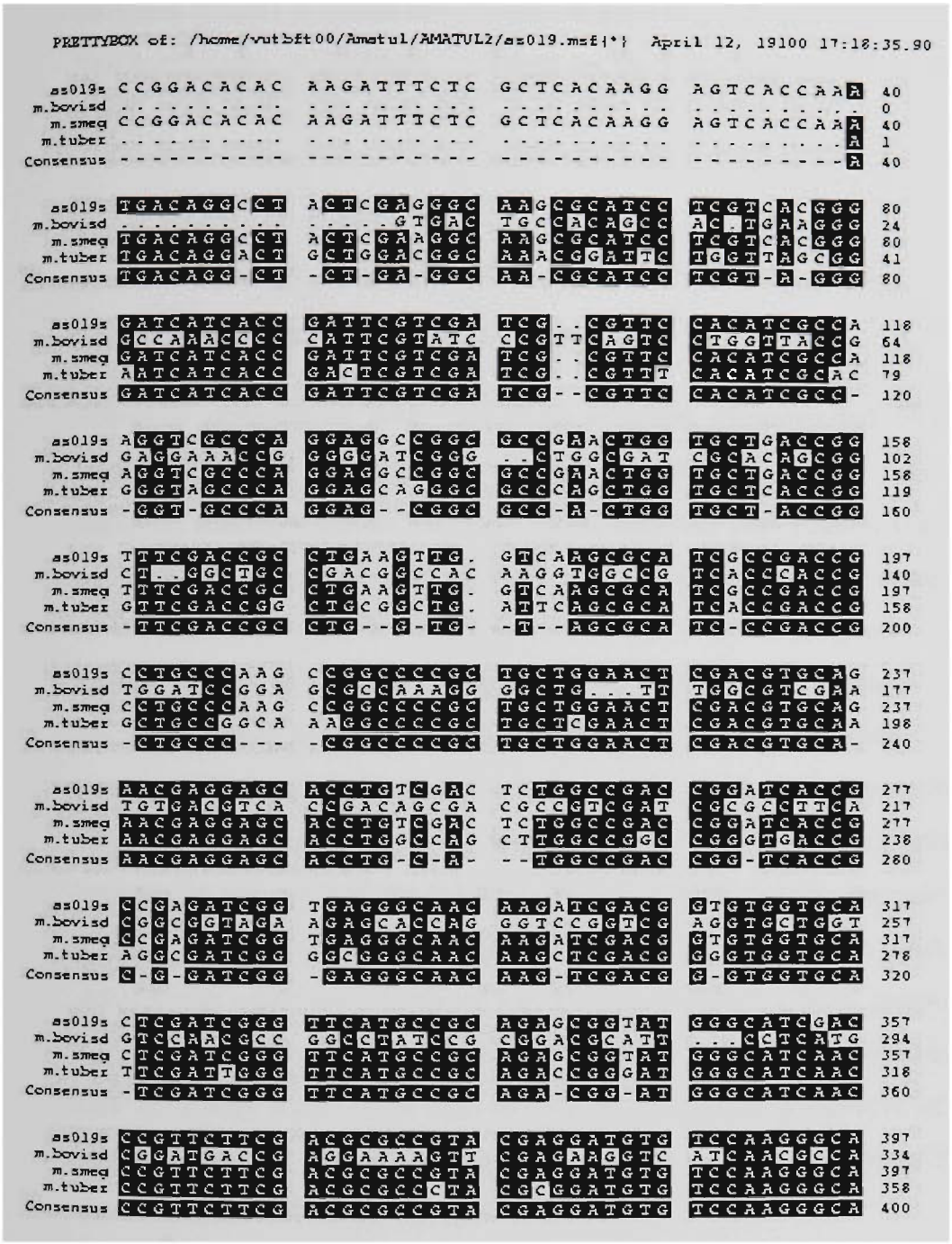


Figure 4.14 Alignment of the *inhA* sequence of AS019 with that of mycobacterial species using the PRETTYBOX program. Identical bases are shown in black boxes and similar bases in grey boxes (light or deep grey according to the similarity). Gaps are introduced to maximise similarity scores. The *inhA* sequences shown are the following order: *inhA* of *C. glutamicum* strain AS019, *M. bovis*, *M. smegmatis*, *M. tuberculosis*, then the consensus sequence.

From the BLASTN search results were aligned using the PRETTYBOX program. The high degree of similarity is shown by the black highlighted sections (Figure 4.14).

4.3.2 Homology search results of assembled sequence

Sequence comparison with the GCG program demonstrated 98% similarity between the *inhA* genes of *C. glutamicum* AS019 and *M. smegmatis*. A sequence similarity search based on NCBI data was performed using ANGIS and the BLASTN programs. The deduced protein from AS019 was compared to that of all known proteins in databases using the FASTA programs (Pearson and Lipman, 1988) and BLAST (Altschul *et al.*, 1990) and similarity scores were calculated using the GCG/BESTFIT and GCG/GAP (Devereux *et al.*, 1984) programs. The most striking observation was the similarity range of 92-98% with the *inhA* gene of mycobacterial species. Figure 4.15 shows the BESTFIT of the amino acid sequence of the InhA protein of AS019 and *M. smegmatis*. Figure 4.16 shows the multiple sequence alignments of the deduced InhA protein of AS019 with other mycobacterial species using PRETTYBOX program. Tables 4.8, 4.9 and 4.10 show BLASTN, BLASTX, FASTA results for strain AS019. Low homology scores were seen for non-mycobacterial proteins of other species, including the *fabI* gene of product of *P. aeruginosa*, the EnvM of *E. coli* and short chain alcohol dehydrogenase of *E. coli*.

```
  1 PDTQDFSLTRSHQMTGLLEGKRILVTGIITDSSIAFHIAKVAQEAGAELV 50

  1 PDTQDFSLTRSHQMTGLLEGKRILVTGIITDSSIAFHIAKVAQEAGAELV 50

 51 LTGFDRLKLVKRIADRLPKPAPLLELDVQNEEHLSTLADRITAEIGEGNK 100

 51 LTGFDRLKLVKRIADRLPKPAPLLELDVQNEEHLSTLADRITAEIGEGNK 100

101 IDGVVHSIGFMPQSGMGIDPFFDAPYEDVSKGIHIPAYSYASLAKAVLPI 150

101 IDGVVHSIGFMPQSGMGINPFFDAPYEDVSKGIHISAYSYASLAKAVLPI 150

151 MNPGGGIVGMDFDPTRAMPGYNWMTVAKSALESVNRFVAREAGKVGVRSN 200

151 MNPGGGIVGMDFDPTRAMPAYNWMTVAKSALESVNRFVAREAGKVGVRSN 200

201 LVAAGPIRTLAMSAIVGGALGDEAGQQMQLLEEGWDQRAPLGWNMKDPTP 250

201 LVAAGPIRTLAMSAIVGGALGDEAGQQMQLLEEGWDQRAPLGWNMKDPTP 250

251 VAKTVCALLSDWLPATTGTVIYADGGASTQLL 282

251 VAKTVCALLSDWLPATTGTVIYADGGASTQLL 282
```

Figure 4.15 Amino acid sequence similarity alignment of the InhA protein of AS019 and *M. smegmatis* using the GCG/BESTFIT program. Identical amino acids are indicated by vertical lines, and similar amino acids are indicated by two dots or a single dot. Gaps are introduced by the program to maximise similarity scores. The InhA proteins of AS019 is shown on the top and that of *M. smegmatis* is shown on the bottom.

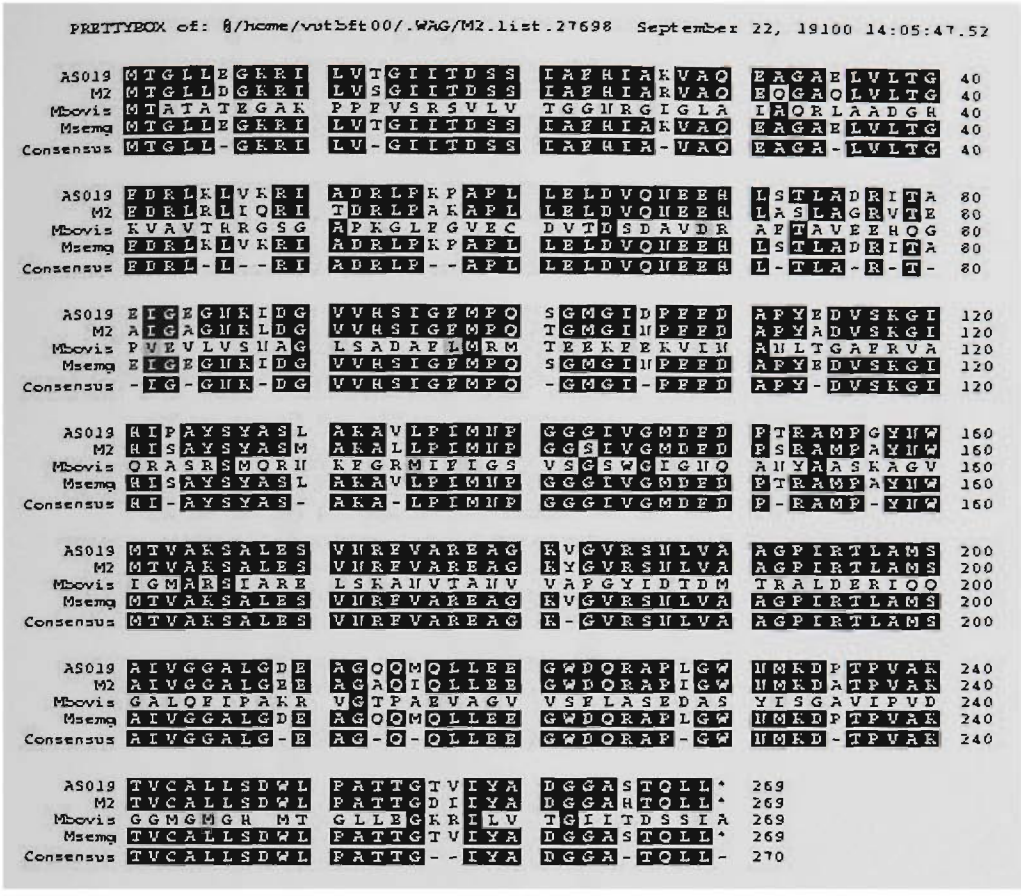


Figure 4.16 Alignment of the InhA protein sequence of *C. glutamicum* AS019 with InhA proteins of mycobacterial species from data bases (SwissProt, Pir, GenEmbl) using program PRETTYBOX. Identical amino acids are shown in black boxes and similar amino acids in grey boxes (light or deep grey according to the similarity). Gaps are introduced to maximise similarity scores. The protein sequences shown are the following: InhA of *C. glutamicum* strain AS019, *M. tuberculosis M. bovis*, *M. smegmatis* and the consensus sequence..

Table 4.8 BLASTN homology search results for the *inhA* gene of AS019 using the NCBI database.

	% similarity of *inhA* gene of AS019 to similar genes on NCBI database						
	AS019	*M. smegmatis*	*M. tuberculosis* *M. bovis*	*M. avium* strainGIR10	*P. aeruginosa*	*S. typhimurium*	*E. coli*
AS019	100	99	82	82	58	55	55
M. smegmatis		100	83	83	59	56	56
M. tuberculosis *M. bovis*			100	85	59	-	-
M. avium strain GIR10				100	-	-	-
P. aeruginosa					100	65	64
S. typhimurium						100	86
E. coli							100

Table 4.9 BLASTX similarity search results for the InhA protein of *C. glutamicum* strain AS019 from the NCBI database.

	% similarity of NADH-dependant Enoyl-ACP reductase of AS019 with similar proteins from other organisms						
	AS019	*M. smegmatis*	*M. tuberculosis*	*P. aeruginosa*	*S. typhimurium*	*E. coli*	*B. napus*
AS019	100	98	86	54	54	54	32
M. smegmatis		100	87	54	54	54	34
M. tuberculosis			100	54	54	54	43
P. aeruginosa				100	70	70	43
S. typhimurium					100	97	41
E. coli						100	41
B. napus							100

Table 4.10 FASTA homology search results for the *inhA* gene of *C. glutamicum* strain AS019 from the NCBI database.

Organism	Gene	% Similarity
M. smegmatis	*inhA*	99.29
M. tuberculosis	*inhA*	82.76
M. bovis	*inhA*	82.76
M. avium	*inhA*	83.73
Pseudomonas aeruginosa	*fabI*	52.89
E. coli	*envM*	52.71
H. influenza	Short chain alcohol dehydrogenase	59.00
E. coli	Short chain alcohol dehydrogenase	52.71
H. pylori	Enoyl acyl carrier protein	38

4.4 DISCUSSION

The results presented in this chapter suggest that the *inhA* gene is present in *C. glutamicum* strain AS019 and its two mutants. Morover, the results suggest that *inhA* gene is also present in related species *B. lactofermentum* and *B. flavum.* At the time of this study several lines of evidence support the existence of the *inhA* gene in these strains.

1 *InhA* specific primers could be used to amplify the *inhA* gene specific fragments directly from genomic DNA and also from individual clones on a genomic library of *C. glutamicum*

2 An *inhA* specific probe which correctly identified the predicted *Pst*1 derived fragment on *M. smegmatis* genomic DNA identified more than one band in *C. glutamicum* species under the same conditions.

At the time of this study there was no reason to doubt the validity of these findings and problems in direct sequencing had been attributed to the high GC contents of this species. Indeed this data was critically reviewed and sequences were submitted to the Gene Bank. However, since this work was completed in 2002 there have been two published reports which do not support the findings of this study on the discovery of a *inhA* gene in *C. glutamicum.* The first report was published, on the genome of *C. efficiens* YS-314 by Kawarabyasi *et al* (2002). *C. efficiens* is the closest relative of *C. glutamicum* but unlike *C. efficiens* can grow above 40°C and it has a high GC content. The complete genome sequence analysis was performed by Kawarabyasi *et al.* (2002) to investigate the basis of its thermostability by comparing its genome with that of *C. glutamicum.* Their comparative genomic study showed tremendous bias in amino acids substitutions in all orthologous ORFs. The other report was about the complete genome sequence of *C. glutamicum* strain ATCC 13032 by Nakagawa *et al.* (2002). It is important to mention here that shotgun-cloning method (Fleischmann *et al.*, 1995) was used to construct a subgenomic library for AS019 and MLB194 in pBluescript. Kawarabayasi *et al.*, 2003 also used the shotgun method to construct genomic libraries of *C. efficiens* in JCM

44549 (strain YS-314) for sequencing purpose. Moreover, it has been also mentioned in this paper that the genome sequence itself was used for training.

The following discussion is based on our findings although at the end of the chapter we have addressed some of the reasons for the apparent conflicts between our results and those of Kawarabyasi *et al.* (2002) and Nakagawa *et al.* (2002). This chapter describes the identification, cloning and sequencing of the putative *inhA* gene homologue from *C. glutamicum* strain AS019 and its comparison with the mycobacterial *inhA* gene.

The findings in this study of the presence of the *inhA* gene in *C. glutamicum* were based on the assumption that the *C. glutamicum* strain AS019 was correctly verified. These verification experiments included the sequencing of 16S rRNA from a stored strain of *C. glutamicum* and Southern blotting of a ^{32}P labelled PCR amplified 16S rRNA to determine the copy number and to compare the "ribotype" of the mutants compared with the parent (AS019). The results from these verification experiments indicated that the parents and mutants have same restriction pattern. The BLASTN sequence similarity results indicated 99-100% homology with the published *C. glutamicum* 16S rRNA gene (appendix 5).

This putative *C. glutamicum* strain AS019 was then used in a study to identify the *inhA* gene using PCR and Southern blotting. Initial detection of the presence of an *inhA* gene homology from the putative *C. glutamicum* was obtained through PCR amplification of fragments using primers designed on the basis of mycobacterial sequences. *C. glutamicum* DNA was found to be an extremely difficult template for PCR because of the high (G+C) content in this organism. This problem was overcome by using the "Soln Q" denaturing solution supplied with the QIAGEN kit. This PCR method should thus be applicable to identification and amplification of different genes from corynebacterial species, if they are significantly similar to the known gene. SolnQ was found to be highly useful in this regard.

To unambiguously determine the sequence of the *inhA* gene from the putative *C. glutamicum*, several overlapping *inhA* gene fragments were amplified from the genomic DNA of AS019 using various primer combinations. Sequence data from these confirmed the presence of the *inhA* gene.

The identity of *inhA* gene was further confirmed by Southern hybridisation by using two different labelling systems. With the exception of a few extra minor bands seen when using the DIG method, the Southern patterns obtained using the two approaches were similar and mainly confirmatory. Another key finding of this study is that multiple copies of the gene are present on the chromosome of the putative *C. glutamicum*, as indicated initially from DNA-DNA hybridisation profiles of strain AS019 when compared with *M. smegmatis*. In *M. smegmatis*, the probes used always bound to a 3Kb *PstI* fragment, implying there is one copy of the 0.85 kb gene. The genomic DNA of the putative *C. glutamicum* was digested with three restriction enzymes, *BglII*, *HindIII*, and *PstI*, with all of these three-restriction digestion producing two to three strong signals, plus some minor bands were also seen. The high temperature (65°C to 68°C) used during the incubation with probes plus high stringency washes of the blots, minimised non-specific binding.

The *inhA* gene sequence was assembled from different overlapping PCR fragments of the putative strain AS019 that covered the total length of 850bp, for the positive strand. A linear restriction map analysis was performed on the *inhA* gene sequence *via* MAP program of the WebANGIS in order to locate any internal cutting site for the above three enzymes used for Southern analysis. This analysis showed there are no internal cutting sites for *Pst*1, *Bgl*II and *Hind*III enzymes within the sequence. This observation indicated that binding of probe to multiple fragments following enzymatic digestion of genomic DNA must be due to the presence of multiple copies of the gene. However, Southern results of *PstI* restriction digestion were contradictory. Apart from three main signals, two small (250 and 150 bp) bands were also seen, indicating a significantly prominent signal: the origin of these bands is unknown, and the restriction map does not show any internal cutting sites within the *inhA* gene for *Pst*1. This could have arisen from non-

specific interactions between the probe used and either other DNA in the chromosome or protein. In the restriction digestion of the genomic DNA with *Pst*1, BSA was used. The signal seen at the bottom of the gel may be due to the non-specific binding of protein and nucleic acids, which occurs quite often. This phenomenon was not further investigated.

The nucleotide sequence data obtained here revealed that variability in the coding region was much smaller between corynebacteria and mycobacterial species than for other species. Sequence similarity search based on the NCBI database revealed a 85-98% nucleotide sequence similarity between the *inhA* gene of *C. glutamicum* AS019 and mycobacterial species.

From the deduced amino acid sequence, it appears that the *inhA* gene of AS019 is a 2-*trans*-enoyl acyl carrier protein, and when compared with sequences from the SWISS PROT databases, significant homologies with enzymes involved in bacterial and plant fatty acid biosynthesis were also seen. The highest identity score was 98% with the *Mycobactetrium* family (98% with *M. smegmatis*, 85% with *M. tuberculosis*, 85% with *M. bovis* and 84% with *M. avium*). The InhA protein of *C. glutamicum* also showed 59% similarity with a short chain alcohol dehydrogenase of (EnvM) from *Haemophilus influenza*), 54% with EnvM from *S. typhimurium*, 54% with EnvM of *E. coli*, 40% with an enoyl acyl carrier protein of *Helicobacter pylori* and 38% identity with a *Brassica napus* Enoyl-ACP reductase. The high degree of similarity of *C. glutamicum* InhA protein with the mycobacterial InhA and other proteins involved in fatty acids biosynthesis suggest that InhA might also be involved in lipid biosynthesis.

Initially the project aimed to get some sequencing information outside the coding regions of *inhA* to indicate what other genes are located near the *inhA* gene. A subgenomic DNA library of AS019 was constructed for this purpose using the *Pst*1 site of pBluescript. Our results showed that shotgun or random cloning approach was not a good method because of self-ligation of *Pst*1 fragments, as many *inhA* positive clones were rejected because they generated multiple *Pst*1 fragments following digestion with this enzyme. Only two *inhA* positive clones were used for sequencing but a read-out of only 1200bp could be

obtained because of the reaction failure probably due to the high GC content. A linear restriction map of this sequence was created, accessing the MAP programme and confirmation was made by restriction digestion of the clones with several enzymes. The restriction digestion profiles were in accordance with the created theoretical map thus indicating that the clone was sequenced in the right directions. Finally the sequence of the *inhA* gene was validated by amplification and sequencing of the *inhA* fragments from these clones. Although the *inhA* gene was amplified from these clones, the exact position of the gene within the cloned DNA was not located. The minimum sequencing data from two clones was compared with BESTFIT program and results showed no match between the two. Although the sequencing information is not conclusive because of failure to reach the far end of the *inhA* gene, it can be predicted that the two clones contain insert from different parts of the genome. Moreover, the sequence similarity search performed on the non-*inhA* sequencing data obtained from both clones showed 72-87% homology with various mycobacterial proteins whose functional properties are not known. The location of the *inhA* gene and its function with possible operon needs further investigation.

The evidence that InhA is one target for activated INH in *M. smegmatis* is compelling. Both genetic selection (Banerjee *et al.*, 1994) and *in vitro* assays (Johnsson *et al.*, 1995, Quemard *et al.*, 1996) have established that InhA is inhibited by a metabolite derived from activation of INH by *KatG*. No such studies have been documented in corynebacteria therefore the mechanism of INH action is not understood. Studies reported to date showed that *C. glutamicum* was relatively insensitive to the drug and significant differences in cell growth rate, transformation efficiency and mycolic acid composition of *C. glutamicum* were only obvious at high concentrations of INH (8mg/ml) (Jang *et al.*, 1997). The present study indicates the presence of a putative target gene for INH in corynebacteria. The observed INH resistance in *C. glutamicum* may arise due to several possible mechanisms. For example, the conversion of INH into an active metabolite might not occur in corynebacteria but trace amounts of an active form may be present in the INH added to the media at the high concentrations used, which then causes inhibition of mycolic acid synthesis. Alternatively, the presence of more than one

copy of the *inhA* gene in *C. glutamicum* may also explain the insensitivity of this species to high concentrations of INH. This subject will be further discussed in later chapters.

The InhA protein, which is *trans*-2-enoylacyl carrier protein (ACP) reductase (Quemard *et al.*, 1995a), is one of the enzymes that constitutes fatty acids synthase or elongase-type complexes. This finding is consistent with the observation that mycolic acid biosynthesis is inhibited by INH with the subsequent accumulation of fatty acids of up to 24-26 carbons (Quemard *et al.*, 1995b, Takayama *et al.*, 1972). The presence of an *inhA* gene in *C. glutamicum* and its considerably high similarity with the mycobacterial *inhA* gene enables speculation that the InhA protein might catalyse similar functions to those seen in mycobacteria. The existence of multiple copies of the gene in corynebacteria, however, might suggest that the InhA protein plays a slightly different role in *C. glutamicum* despite its similarity to the mycobacterial gene. Studies of Jang *et al.* (1997) also showed that INH disrupts the attachment of mycolic acids to the cell surface, an observation that had not been reported previously in mycobacteria. The following chapters will explore the functional properties of the *inhA* gene in *C. glutamicum* and provide further data on sequence analysis in other related species.

The reason for this apparent discrepancy between our results and those of Kawarabyasi *et al.* (2002) and Nakagawa *et al.* (2002) is not apparent. The Southern hybridisation experiment in particular is difficult to rationalize. In these Southern hybridisation experiments the PCR amplified *inhA* gene probe from *M. smegmatis* and *C. glutamicum* was used against the genomic DNA digest of *C. glutamicum*. Hybridisation was carried out at high temperature (68°C) to avoid any cross contamination, high stringency wash conditions were used to clean the blots further minimising any unnecessary binding of the probe. Two different hybridisation methods (radioactive-labelling and non radioactive-labelling) were used which produced similar results. In this study a comparative DNA-DNA hybridisation analysis of *C. glutamicum* and *M. smegmatis* revealed two different Southern patterns. In our experiment involving *M. smegmatis* the probe bound singly to 3Kb *Pst*1 fragment which appeared to verify the results of Banerjee *et al.*, (1994). The *C.*

glutamicum results, however, behaved differently showing multiple banding patterns. In addition to *Pst*1, two other enzymes *Bgl*II and *Hind*III used to digest the genomic DNA of three corynebacterial strains (AS019, MLB194, MLB133), two *Brevibacterium* species (*B. lactofermentum*, *B. flavum*) and *M. smegmatis* using Southern blotting analysis. The results were again consistent showing different Southern pattern for *Corynebacterium* and *Mycobacterium* species. In the case of *M. smegmatis* no signal appeared with *Bgl*II and *Hind*III indicating the presence of *inhA* gene only on 3Kb *Pst*1 fragment whereas multiple bands were observed with *C. glutamicum* and *Brevibacterium* restriction digests. The high degree of similarity of the sequencing data generated from the clones with that of published *Corynebacterium* sequences reported to NCBI indicates the presence of *inhA* gene in *C. glutamicum*.

However as a result of some preliminary experiments there now appears to be some doubt regarding the authenticity of the *C. glutamicum* strain that was used for the study although as indicated in the methods section of this chapter considerable effort was made at the start of the project to verify the purity of the *C. glutamicum* DNA used for this study. The verification of the purity involved the determination of the sequence analysis of 16S rRNA, which showed 99-100% similarity with 16S rRNA gene of *C. glutamicum* as expected. In addition a Southern blot analysis of different corynebacterial strains with ^{32}p labelled probe from parent strain AS019 was undertaken which again produce results that were consistent with the PCR results.

A number of procedures were taken to avoid any possible contamination of the different strains of bacteria. These included a storage procedure, which included holding the strains of *C. glutamicum* with *M. smegmatis* in separate containers. All buffers for each experiment were made up fresh before each experiment and sterile water was used as a positive control, which did not show any amplification product. For the PCR reaction hybridisation was undertaken at an elevated temperature to reduce the chances of cross contamination. The isolation of genomic DNA from *M. smegmatis* and *C. glutamicum* were carried out at different time periods. The DNA vials of *M. smegmatis* and *Corynebacterium* were kept with two separate PCR reagents in two separate boxes. PCR

reactions with *M. smegmatis* DNA were carried out at different times. Moreover, the sequencing data generated from the four clones two from *C. glutamicum* strain AS019 genomic library and two (1C, 1F) from MLB194 library using T3 and T7 sequencing primers was analysed using BLASTN search programme showed 96-99% homology with the relevant parts of the published genomic DNA sequence of *Corynebacterium* species proving the purity of genomic DNE in these clones. The sequencing data obtained from clones 1C and 1F was not included in the thesis therefore it is attached as appendix 5.

However, despite these precautions mentioned above the possibility remains that there was an accidental contamination of the *C. glutamicum* with *M. smegmatis*. This contamination may have arisen from the initial allocation of the two different strains of bacteria including *C. glutamicum* with *M. smegmatis* since a number of people were involved in running the laboratory including a postdoctoral research assistant and a number of other postgraduate students. Given the earlier verification experiments that were undertaken at the commencement of the study no further verification was undertaken once the project had commenced. It was only after receiving reports from some overseas investigators that had that the possibility of cross-contamination was raised which occurred too late for the work to be repeated. The reasons for the discrepancy between our results and those of Kawarabyasi *et al* (2002) and Nakagawa *et al.* (2002) are still to be resolved but could not be attempted because the scholarship I was on could not be extended.

Chapter 5

Comparison of *inhA* gene of parent strain AS019 with two mutants MLB133 and MLB194.

5.1 INTRODUCTION

The results reported in Chapter 3 showed significant differences in the INH MICs for strain AS019 and two auxotrophic mutants, MLB133 and MLB194. Previous studies have shown that these mutants protoplasted more readily than the parent strain and this plus the observed morphological changes seen for MLB133 and MLB194 suggested that these strains were cell-surface structure mutants (Best and Britz, 1986: Jang *et al.*, 1997: Pierotti 1987). Mutants MLB133 and MLB194 were more sensitive to growth inhibition by INH and glycine, which caused budding and branching (Jang *et al.*, 1997).

Further studies on mycolic acid composition following growth in LBG and 8mg/ml of INH showed increased proportions of extracellular mycolic acids for the mutants (MLB133 and MLB194 had 18.8% and 21.2% whereas AS019 had 8.5%) and the proportion of unsaturated mycolic acids increased. This corresponded to decreases in $C_{32:0}$ relative to increases in the proportion of $C_{34:1}$ and $C_{36:2}$. These results suggested that the mutations in strains MLB133 and MLB194 were associated with synthesis of specific mycolic acids (e.g $C_{32:0}$) and possibly attachment of mycolic acids to the cell surface, both of which are likely target sites for glycine and INH action for cell surface modifications.

InhA is the product of the *inhA* gene, which catalyses the NADH-specific reduction of the ACP. It has been documented that single amino acid substitutions (Ser to Ala or Ile to Thr) caused resistance to INH in mycobacteria in laboratory strains (Dessen *et al.*, 1995). Moreover changes in the regulatory region upstream of the *inhA* locus have also been observed in resistant clinical isolates of mycobacteria.

After the discovery of an *inhA* gene in strain AS019, it was decided to extend this study towards the comparative genetic analysis of the *inhA* gene in the parent represented by AS019 and the above mutants. This may have provided a better understanding of the mechanism of INH action and the observed biochemical changes in mycolic acid composition. In this chapter, the *inhA* gene of these INH hypersensitive mutants (MLB133 and MLB194) were cloned and sequenced, including regions upstream from the open reading frame. Computer analysis of the resulting nucleotide and amino acid sequences were compared with known *inhA* gene sequences to identify sequence differences in the structural gene or in its regulatory region, which may correlated with the phenotypes.

5.2 CLONING OF THE *inhA* GENE FROM MLB194

5.2.1 PCR products of the *inhA* gene from MLB194

PCR amplification products of the *inhA* gene from strain MLB194 genomic DNA are shown in Figure 5.1. The same strategy was used to sequence the *inhA* gene from strain MLB194 as was used for AS019. The *inhA* gene from MLB194 genomic DNA was amplified as five overlapping fragments using the same sets of primers and same sets of PCR conditions as mentioned in Tables 4.1 and Table 4.2 respectively. Using the primer sets INH1-CGP4, CGP1-CGP2, CGP5-CGP2 and CGP3-INH2, the sizes of the bands obtained were in accordance with the expected sizes for each respective primer set.

5.2.2 Sequence analysis of the amplified products

PCR amplified fragments of the *inhA* genes from *M. smegmatis* and *C. glutamicum* strain MLB 194 obtained using the CGP1-CGP2 and CGP3-CGP4 primer sets were sequenced using the *inhA* specific primers.

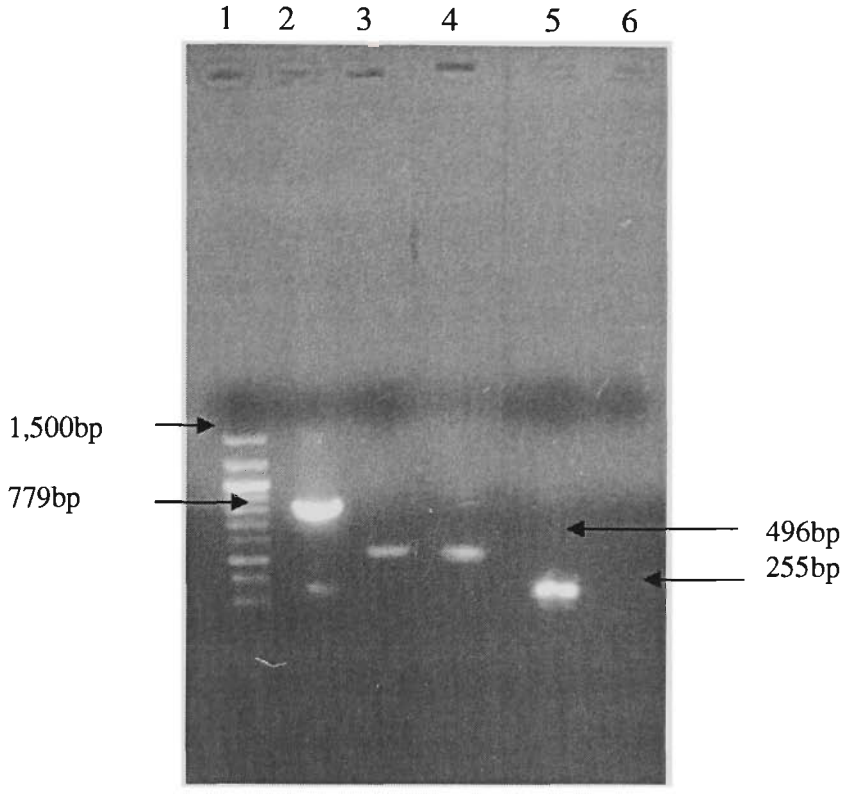


Figure 5.1 PCR amplification of the *inhA* gene from genomic DNA of MLB194. Lane 1, PCR markers (100 bp DNA ladder, 1,500, 1,200, 1,000, 900, 800, 700, 600, 500, 400, 300, 200, 100 bp); Lanes 2 to 5 show amplification products for primers INH1-CGP4; CGP1-CGP2; CGP5-CGP2; and CGP3-INH2.

Table 5.1 BLASTN sequence analysis of PCR amplified products of *M. smegmatis* mc^2 155 and *C. glutamicum* strain MLB194 using CGP3-CGP4 and CGP1-CGP2 primers sets.

Sequence	% Homology				
	M. smegmatis inhA gene	*M. tuberculosis* *M. bovis inhA* gene	*M. tuberculosis* mutant NADH dependant *inhA*	*M.tuberculosis* H37RV	Sequence 3 from patent US 5686590
M3P3[a]	91	79	79	79	91
M1P1[a]	96	82	82	82	90
194 CGP1[b]	98	83	83	83	-
194 CGP2[b]	98	82	82	82	
194 CGP3[c]	90	77	77	77	90
194 CGP4[c]	92	80	80	80	90

[a] M3P3 and M1P1 are PCR products of CGP3-CGP4 and CGP1-CGP2 primer sets from *M. smegmatis* mc^2 155 and sequenced by CGP3 and CGP1 primers respectively.

[b] 194 CGP1 and 194 CGP2 are PCR products of CGP1-CGP2 primers sets sequenced by the same respective primers.

[c] 194 CGP3 and 194 CGP4 are PCR products of CGP3-CGP4 primers sets sequenced by the same primers.

Table 5.2 BLASTX sequence analysis results of PCR amplified products from *M. smegmatis* mc^2 155and *C. glutamicum* strain MLB194 genomic DNA using the CGP3-CGP4 and CGP1-CGP2 primers sets.

Sequence	% Homology				
	M. smegmatis Enoyl-ACP rreductase	*M. tuberculosis* Enoyl-ACP reductase	*M.avium* Enoyl-ACP reductase	Enoyl-ACP (fragment)	Putative Enoyl-ACP reductase
M3P3[a]	81.4	69	69	76	
M2P1[a]	94	78	76	94	50
194 CGP1[b]	99	83	85	85	46
194 CGP2[b]	96	79			
194 CGP3[c]	81	75	63	81	
194 CGP4[c]	81	73	63	81	

[a] M3P3 and M1P1 are PCR products of CGP3-CGP4 and CGP1-CGP2 primer sets from *M. smegmatis* and sequenced by CGP3 and CGP1 primers respectively.

[b] 194 CGP1 and 194 CGP2 are PCR products of CGP1-CGP2 primers sets sequenced by the same respective primers.

[c] 194 CGP3 and 194 CGP4 are PCR products of CGP3-CGP4 primers sets sequenced by the same primers.

Table 5.3 FASTA sequence analysis results of PCR amplified products of *M. smegmatis* mc^2 155 and *C. glutamicum* MLB194 using CGP3-CGP4 and CGP1-CGP2.

Sequence	% Holmology			
	M.smegmatis isoniazid	*M.tuberculosis* H37RV *inhA*	*M.bovis* putative keto acylACP	*M.avium* GIR10 transcript
M3P3[a]	89.24	77.21	77.21	73.41
M2P1[a]	94.42	79.94	79.99	79.93
194 CGP1[b]	98.22	83.33	83.33	82.60
194 CGP3[c]	91.51	79.59	79.59	74.51

[a] M3P3 and M1P1 are PCR products of CGP3-CGP4 and CGP1-CGP2 primer sets from *M. smegmatis* and sequenced by CGP3 and CGP1 primers respectively.

[b] 194 CGP1 is PCR products of CGP1-CGP2 primers sets sequenced by the same respective primers.

[c] 194 CGP3 is PCR products of CGP3-CGP4 primers sets sequenced by the same primers.

A sequence similarity search based on the NCBI was performed *via* the National Genomic Information Service (ANGIS) using the BLASTN programs. InhA deduced protein from MLB194 was compared to that of all known proteins in databases using the FASTA (Pearson and Lipman, 1988) and BLAST (Altschul *et al.*, 1990) programs. Tables 5.1, 5.2, 5.3 show the results of BLASTN, BLASTX and FASTA homology searches for these products. The data showed that the highest similarity was with the mycobacterial *inhA* genes (82-97%). Sequence assembly of this is reported in section 5.4.1

5.2.3 Southern hybridisation and identification of the *inhA* gene in *C. glutamicum* strain MLB194.

The PCR amplified products of CGP3-CGP4 primer sets from *C. glutamicum* strain MLB194 were labelled using radioactive ^{32}P and non-radioactive (DIG) labelling systems. The two probes were then used to hybridise the blots containing the genomic DNA of *C. glutamicum* strain MLB194 and *M. smegmatis* digested with various enzymes.

Figure 5.2 a and 5.2 b showed the results of Southern hybridisation using ^{32}P and DIG labelling systems respectively. Similar to Southern hybridisation results for AS019 genomic DNA, binding of the probe with multiple fragments of strain MLB194 genomic DNA digest with various restriction enzymes was observed. The Southern hybridisation pattern obtained using the two systems were identical to those observed for AS019. Similarly, some additional minor bands were obtained for DIG labelling system, which were not seen with the ^{32}P labelled probes. The probe showed strong hybridisation signals with a 3kb *Pst*1 fragment of *M. smegmatis* DNA thus confirming the previously reported results of Banerjee *et al.* (1994). In contrast to *M. smegmatis*, the phenomenon of probe binding to multiple fragments of different molecular weights was common both in MLB194 and AS019. For example, *Pst*1 restriction digestion of MLB194 (Figure 5.2 a, lane 2) using ^{32}P labelled probe in addition to the two major bands, the probe strongly hybridise to two small molecular wteigh fragments (0.25 and 0.1kb) at the bottom of the gel.

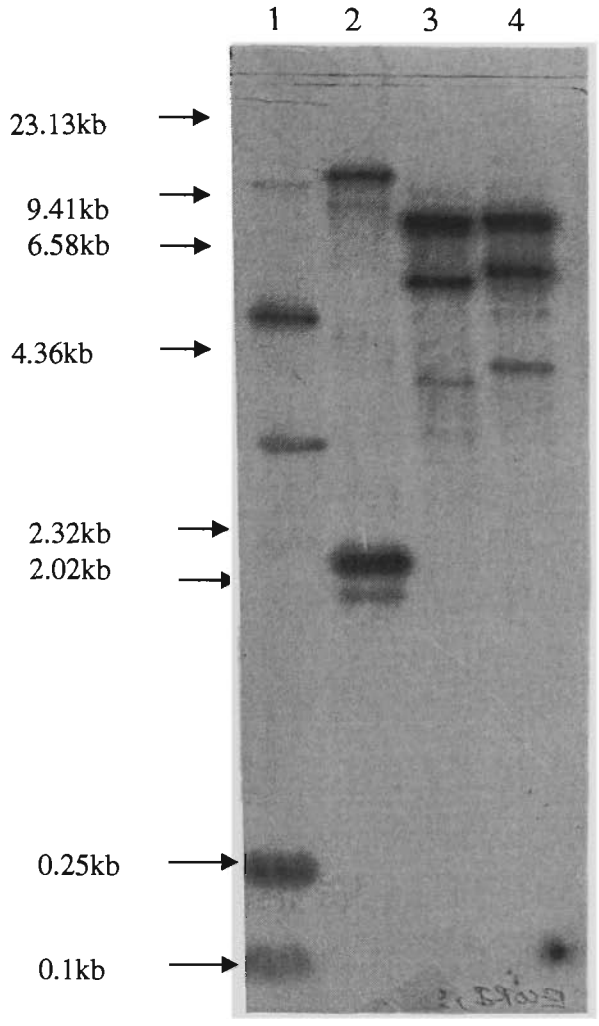
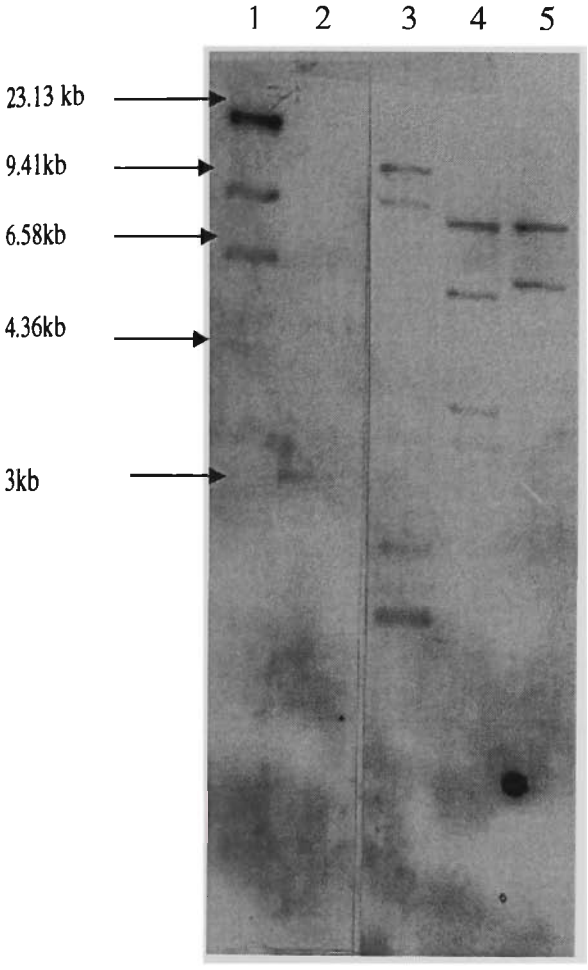


Figure 5.2 Southern blot hybridisation analysis of genomic DNA from *C. glutamicum* strain MLB194 and *M. smegmatis* mc^2 155 following digestion with different restriction enzymes and probed with the 256 bp PCR fragment obtained from *C. glutamicum* using CGP3-INH2 primers pairs

a Analysis using ^{32}P radioactive labelling system. Lane 1, Lambda phage DNA *Hind*III digest (size markers); Lanes 2 to 5 contained *Pst*1, *Hind*III, *EcoR*1, *Sal*1 digested total genomic DNA of strain MLB194.



b Analysis using DIG labelling system. Lane 1, Lambda DNA *Hind*III digest (size markers); Lane 2, *Pst*1 digested genomic DNA of *M. smegmatis;* Lanes 3, 4, 5, *Hind*III, *EcoR*1, *Sal*1, digest of genomic DNA of *C. glutamicum* strain MLB194.

Similarly, lanes 3 in Figures 5.2 a and b contained *HindIII* digest of MLB194 and the results were similar to AS019 when using the two types of labelled probes. Table 5.4 shows the calculated molecular weights of all the bands obtained in Southern hybridisation using the two labelling systems.

To further confirm the identity of the *inhA* gene, two approaches were used subsequently: construction of a genomic library of MLB194 and sequencing the gene from the cloned DNA plus assembling the sequence directly from PCR products amplified from genomic DNA.

5.3 CLONING AND ANALYSIS OF THE *inhA* GENE OF MLB194.

Results of Southern hybridisation had shown one hybridisation fragment for *M. smegmatis* and more than one hybridisation fragment for *C. glutamicum* strain MLB194 when various restriction enzymes were used. The Southern pattern obtained for MLB194 was similar to that seen for AS019: when using *Pst*1, the two major signals seen in MLB194 Southern were greater than 3kb. Therefore, because AS019 and MLB194 were derived from the same parent strain and the two strains had similar genomes, it was anticipated that these fragments might contain the entire *inhA* gene.
Subsequently, genomic DNA from MLB194 was digested with *Pst*1 and randomly cloned into the *Pst*1-digested pBluscript SK II vector and a subgenomic library of MLB194 was constructed in *E. coli* XL1 Blue (as described in section 2.3.1.1).

The library was screened for the presence of the *inhA* gene by the following means:

1 Screening by restriction digestion and Southern hybridisation analysis of the *Pst*1 digested recombinant clones and
2 By PCR amplification using *inhA* gene specific primers.

Table 5.4 Molecular weights of hybridisation fragments obtained when the CGP3-INH2 probe was used against genomic DNA digests of strain MLB194 using various enzymes.

MW of the bands detected							
DIG labelling				^{32}P labelling			
λDNA	*Hind*III	*Eco*R1	*Sal*1	*Pst*1	*Hind*III	*Eco*R1	*Sal*1
23.123	**12.2**[a]	**8.8**	**8.8**	11	**12.2**	**8.8**	**8.8**
9.41	10	**6.8**	**6.7**	**5.2**	10	**6.8**	**6.8**
6.58	2.2	4.1	4.4	**3.5**	**2.2**	4.1	**4.4**
4.36	**1.6**	3.6		**0.25**	1.6	3.6	
2.32				0.1			
2.02							

[a]Numbers in bold correspond to the major bands seen. The band sizes not in bold type were minor bands.

5.3.1 Screening by restriction digestion

Of the resulting transformants, one hundred white colonies were grown in LBG with suitable antibiotics, then plasmid DNA was isolated from the recombinant clones and run on an agarose gel. About fifty clones were randomly chosen based on the relative mobility of the plasmids, and selected for screening. The circular plasmids of molecular weight in the ranges between 3.5 to 4kb and above (judged relative to the mobility of pBluescript ccc DNA) were subjected to *Pst*1 restriction digestion. Figure 5.3 shows the restriction digestion patterns of some of the selected clones. Clones which generated *Pst*1 fragments of sizes between 3 to 4kb and greater were selected for further sequence analysis.

Furthermore, the DNA from this gel which shows *Pst*1 restriction digestion of clones was transferred to the nylon membrane and subjected to Southern hybridisation. The probe was the PCR amplified product of the CGP3-INH2 primer set and radiolabelled with ^{32}P (Figure 5.4). Clone 1C, 1F, C22, C1, C2 showed positive signals with the CGP3-INH2 probe. Three clones, 1C, IF, C22 which contained inserts greater than 3kb, were chosen for further analysis.

5.3.2 Southern hybridisation and PCR screening of the recombinant clones

Recombinant clones, which contained inserts of sizes between 3.5 to 4.5kb and which also showed positive signals with the *inhA* probe, were further screened by PCR amplification using *inhA*-specific primers. Ten clones generated positive signals, then clones 1C, 1F, C22 were chosen based on the sizes of their insert for sequencing purpose. Figure 5.5 a, b, c shows the sizes of the PCR fragments obtained for clones 1F and C22 using the INH1-CGP4 and CGP3-INH2 primers. Although clones 1C and 1F generated successful PCR products and also showed positive signals in Southern hybridisation analysis, they were not chosen for further study because they generated more than one

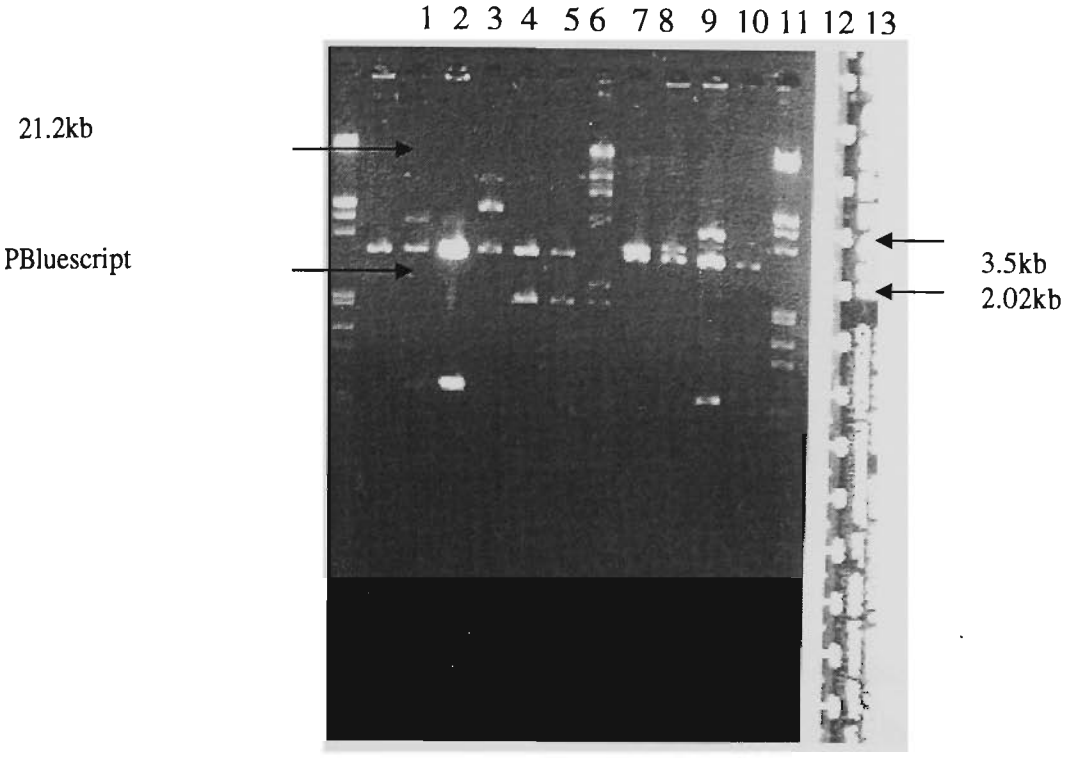


Figure 5.3 Restriction digestion of some of the recombinant clones produced in pBluescript following *Pst*1 digestion of MLB194 DNA. Lanes 1 and 13, lambda DNA digested with *Hind*III/*Eco*R1(size markers); Lane 6, *Hind*III); Lanes 2, 3, 4, 5, 7, 8, 9, 10, 11, 12 pBluescriptSK DNA; clones1C, 1F, C22, C2, C3, C4, 2C, 2D, 2E digested with *Pst*1. DNA from this gel was transferred to nylon membrane and subjected to Southern hybridisation.

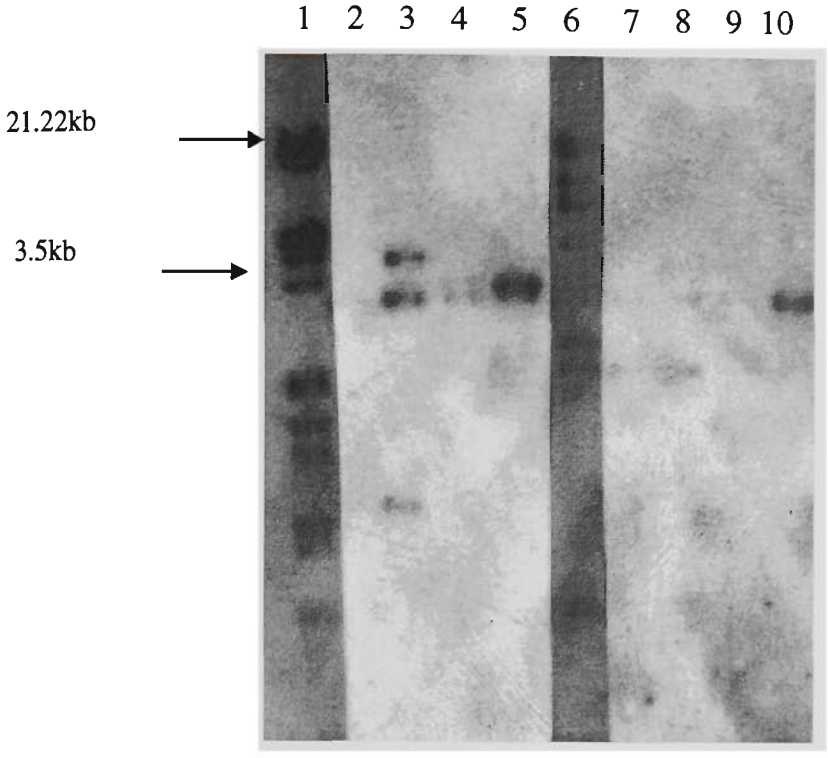


Figure 5.4 Southern blot hybridisation of some *Pst*1 digested clones containing MLB194 DNA using the 255 bp PCR amplified *inhA* probe (CGP3-INH2) from *C. glutamicum*. Lanes 1, and 6 represent *Hind*III/*Eco*R1, and *Hind*III digested Lambda DNA size markers respectively. Lane 2, *Pst*1 digested pBluscript SKII; Lanes, 3, 4, 5, 7, 8, 9, 10 represent *Pst*1 digestion profiles of clones 1C, 1F, C22, C2, C3, C4, 2C, respectively.

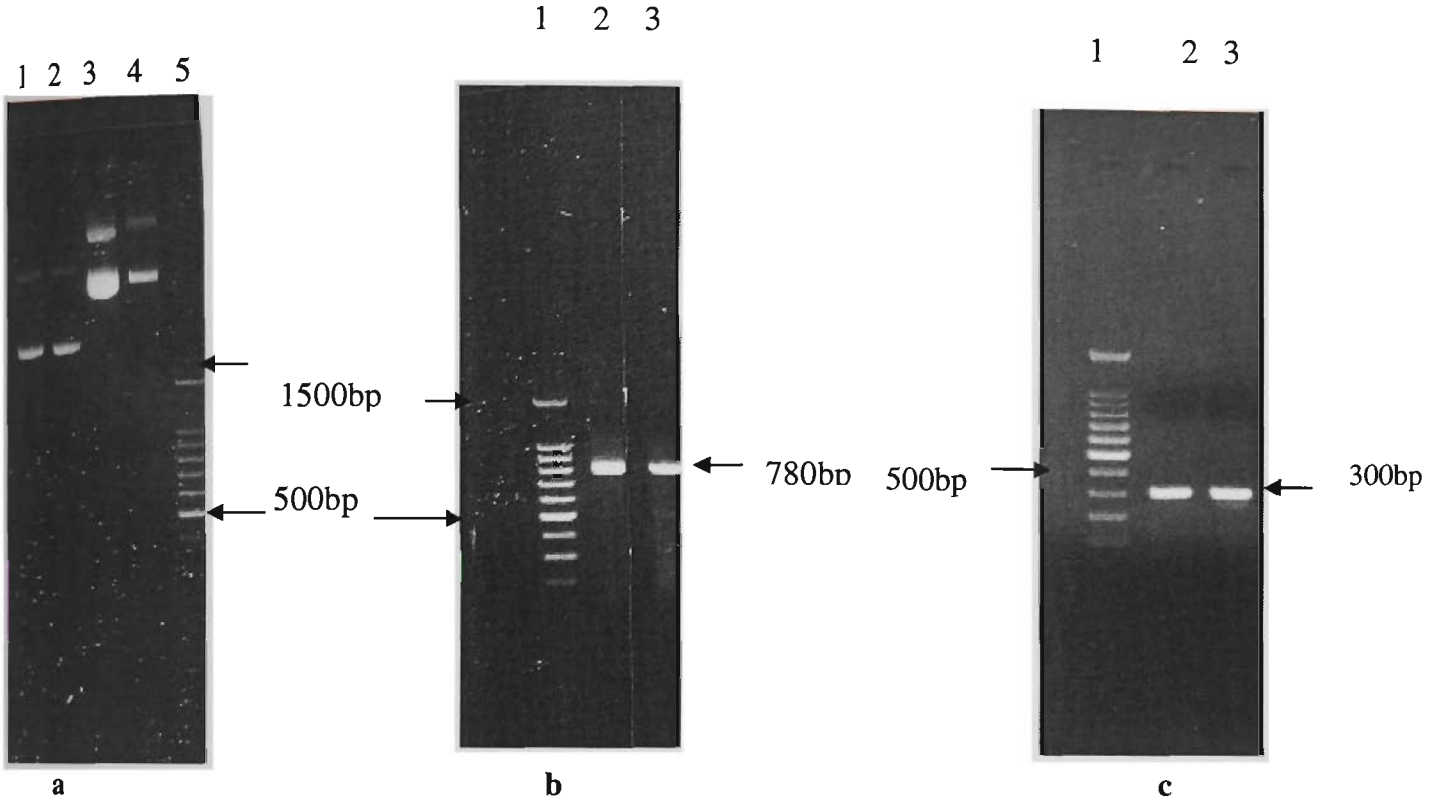


Figure 5.5 PCR analysis of pBluscript clones containing DNA from MLB194.

a Uncut recombinant clones of MLB194. Lanes 1, and 2, clone 1F; Lanes 3, and 4, clone C22; Lane 5, PCR size markers.

b PCR amplification of the *inhA* gene from some of the Clones C22 and 1F using INH1-CGP4 primers sets. Lane1, PCR size markers; Lane 2, *inhA* gene fragment from C22; Lane 3, *inhA* fragment from 1F.

c PCR amplification of *inhA* gene from Clones C22 and 1F using CGP3-INH2 primers sets. Lane 1, PCR size markers; Lane 2, *inhA* fragment from clone C22; Lane 3, *inhA* fragment from clone 1F.

*Pst*I fragment. Finally, the clone designated as C22, which contained a slightly bigger fragment, was chosen for further sequencing and analysis.

5.3.3 Sequencing of the *inhA* gene from clones

In the beginning, the insert in the chosen clone was sequence in both directions using T3 and T7 universal primers. As the sequencing data accumulated, several internal primers were designed to walk up the DNA. Table 5.5 shows the nested primers used to sequence clone C22. Unfortunately, after 1,250 bp read out (Fig 5.6) further sequencing reactions failed and no further data accumulated.

The sequencing data obtained for clone C22 is shown in Figure 5.6 where the first forty bases correspond to the vector including the regenerative *Pst*I site used for cloning. A linear restriction map of the generated sequence was obtained through the MAP program of the WebANGIS. The linear restriction map of clone C22 and restriction digestion pattern is shown in Figures 5.7 a and b respectively. This showed that restriction digestion of the clone matches with the linear restriction map, as expected. A sequence similarity search based on the BLASTN was performed on this generated sequence. The results of identity search revealed 61% homology with *Rhizobium meliloti* C4-dicarboxylate transport protein (*dcTA, dcTB, dcTD*) genes, 61% with *Ralstonia KNI* genes for regulator protein, phenol hydroxylase component, ferredoxin-like protein and catechol 2,3-dioxygenase, partial and complete genome sequence. A lower degree of similarity was observed for Trans-Acting Transcriptional protein ICPO (immediately early protein) 53%, N-tabacum cysteine-rich extension-like protein-1 gene, complete cds 35%, Transcription initiation factor Tfiid 135kDa subunit (TAFII135) 31%. It was then decided to verify the *inhA* sequence from the clones. PCR amplified fragment from clone C22 are shown in Figure 5.8a. Each of the PCR products was used as a template for automated DNA sequencing using the relevant primers. The identity of the sequence was determined by a BLASTN similarity search, which was run by accessing ANGIS. Results of the identity search revealed that DNA sequences obtained were 90-99% similar to the mycobacterial *inhA* genes, confirming the presence of this gene in these clones.

Table 5.5 List of the nested primers constructed to sequence clone C22.

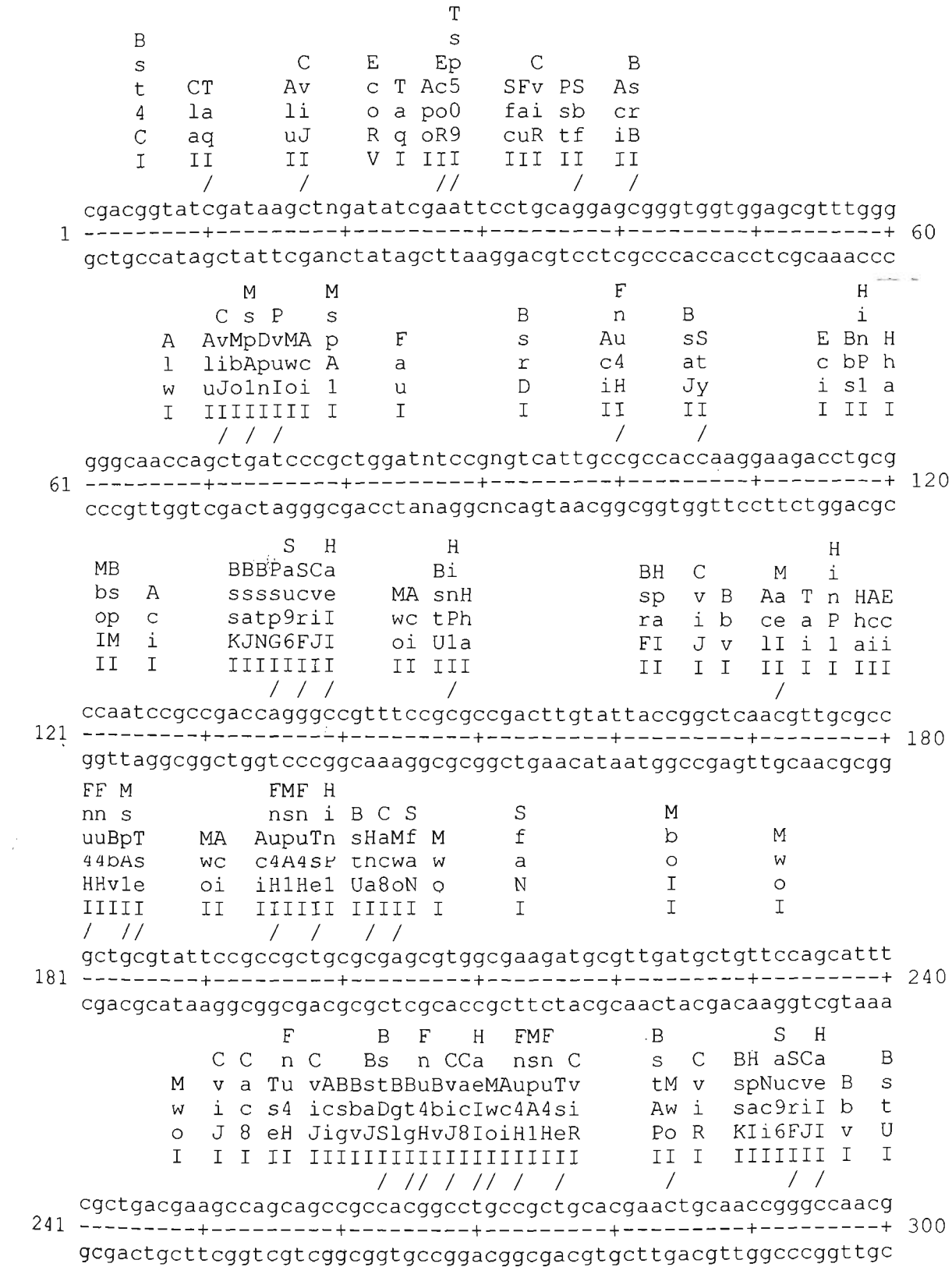
Primer	Sequence	Position	TM (°C)
T7	CCC TAT AGT GAG TCG TAT TA	351-373	56
CT22F	ACG CCG CCG AAC GCT TTG CCC T	362-385	74
CTA22C	ACG CTT TGC CCT CGG CCT GGA ACT	672-694	78
cta22cf1	GTC CTT CAC CAT GAC ATC CCC C	887-909	70
cta22cf2	ATTCAG GAA GGG CAG ATC AAG TTA	1167-1187	68
Ct22t4	TTC ACC GGC CAA TAC GTC CT		62

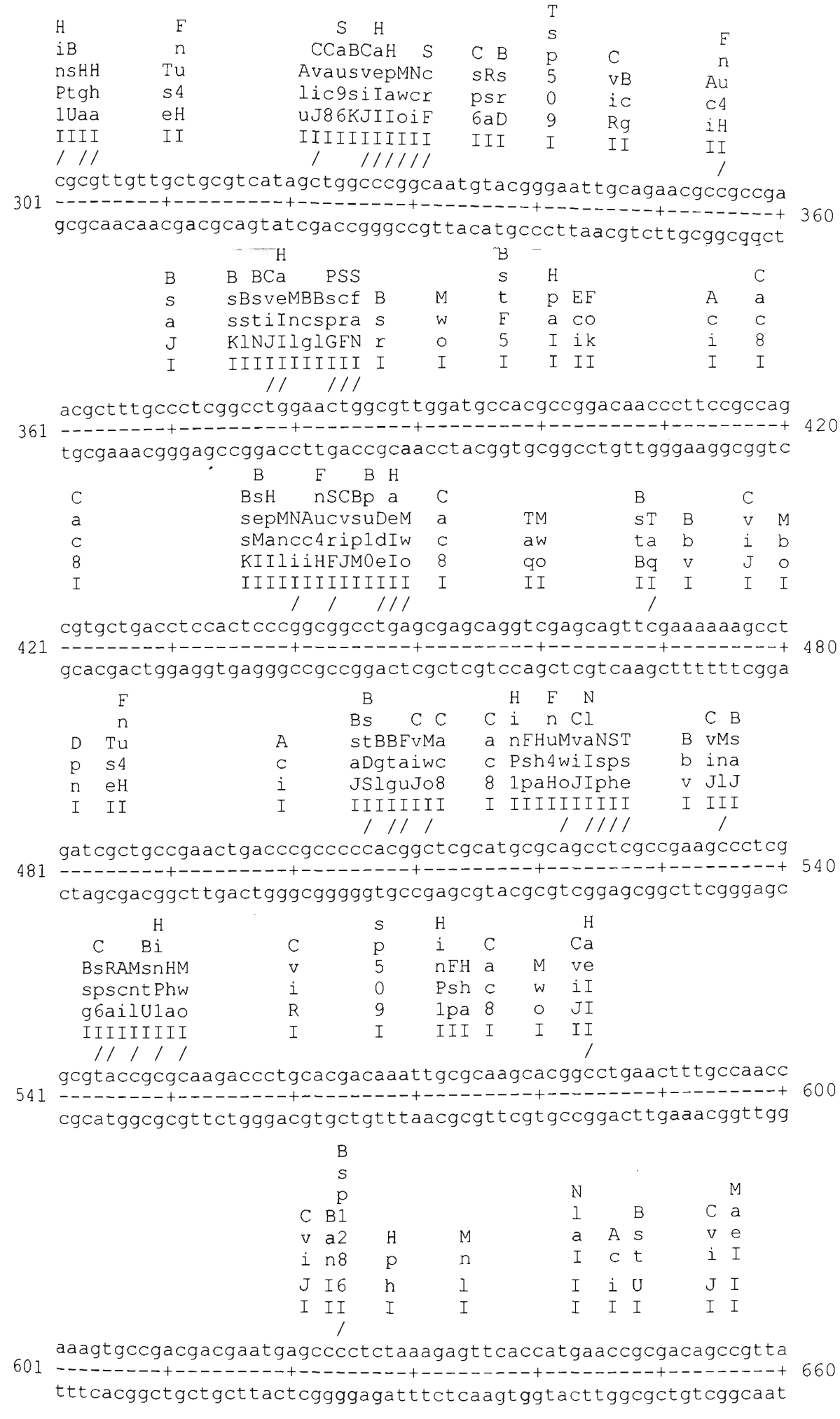
Figure 5.6 Sequence of the clone C22 using primer walking from T7 region of the pBluescript.

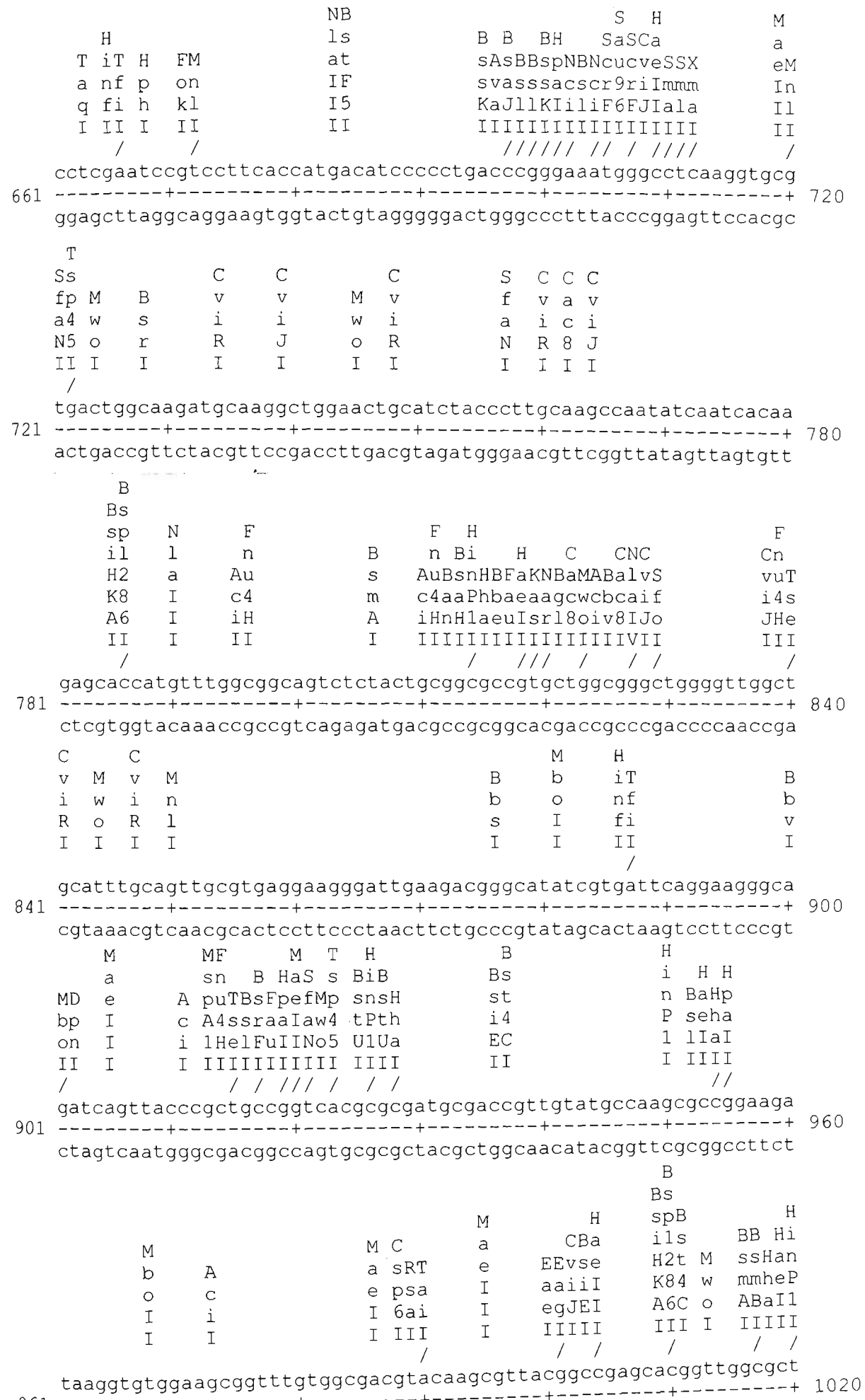
```
   1  cgacggtatc gataagctng atatcgaatt cctgcaggag cgggtggtgg
  51  agcgtttggg gggcaaccag ctgatcccgc tggatntccg ngtcattgcc
 101  gccaccaagg aagacctgcg ccaatccgcc gaccagggcc gtttccgcgc
 151  cgacttgtat taccggctca acgttgcgcc gctgcgtatt ccgccgctgc
 201  gcgagcgtgg cgaagatgcg ttgatgctgt tccagcattt cgctgacgaa
 251  gccagcagcc gccacggcct gccgctgcac gaactgcaac cgggccaacg
 301  cgcgttgttg ctgcgtcata gctggcccgg caatgtacgg gaattgcaga
 351  acgccgccga acgctttgcc ctcggcctgg aactggcgtt ggatgccacg
 401  ccggacaacc cttccgccag cgtgctgacc tccactcccg gcggcctgag
 451  cgagcaggtc gagcagttcg aaaaaagcct gatcgctgcc gaactgaccc
 501  gcccccacgg ctcgcatgcg cagcctcgcc gaagccctcg gcgtaccgcg
 551  caagaccctg cacgacaaat tgcgcaagca cggcctgaac tttgccaacc
 601  aaagtgccga cgacgaatga gcccctctaa agagttcacc atgaaccgcg
 651  acagccgtta cctcgaatcc gtccttcacc atgacatccc cctgacccgg
 701  gaaatgggcc tcaaggtgcg tgactggcaa gatgcaaggc tggaactgca
 751  tctacccttg caagccaata tcaatcacaa gagcaccatg tttggcggca
 801  gtctctactg cggcgccgtg ctggcgggct ggggttggct gcatttgcag
 851  ttgcgtgagg aagggattga agacgggcat atcgtgattc aggaagggca
 901  gatcagttac ccgctgccgg tcacgcgcga tgcgaccgtt gtatgccaag
 951  cgccggaaga taaggtgtgg aagcggtttg tggcgacgta caagcgttac
1001  ggccgagcac ggttggcgct ggagacgtgg attgtgaatg agggcagcga
1051  ggagcgggca gtagccttca ccggccaata cgtcctgcac cgctagaagc
1101  cttcacacaa aaccaaatgt gggagg
```

Figure 5.7 Analysis of clone C22 by restriction digestion and sequence analysis of the insert using T7 primers.

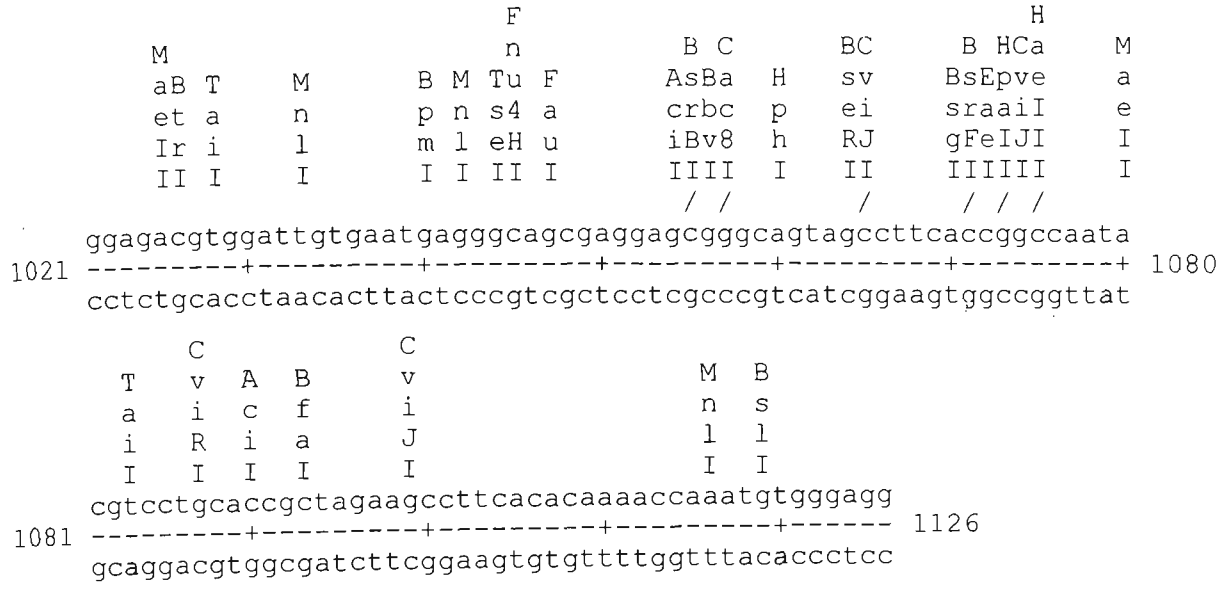
a A linear restriction map of clone C22 of MLB194 obtained using the MAP program of WEB ANGIS





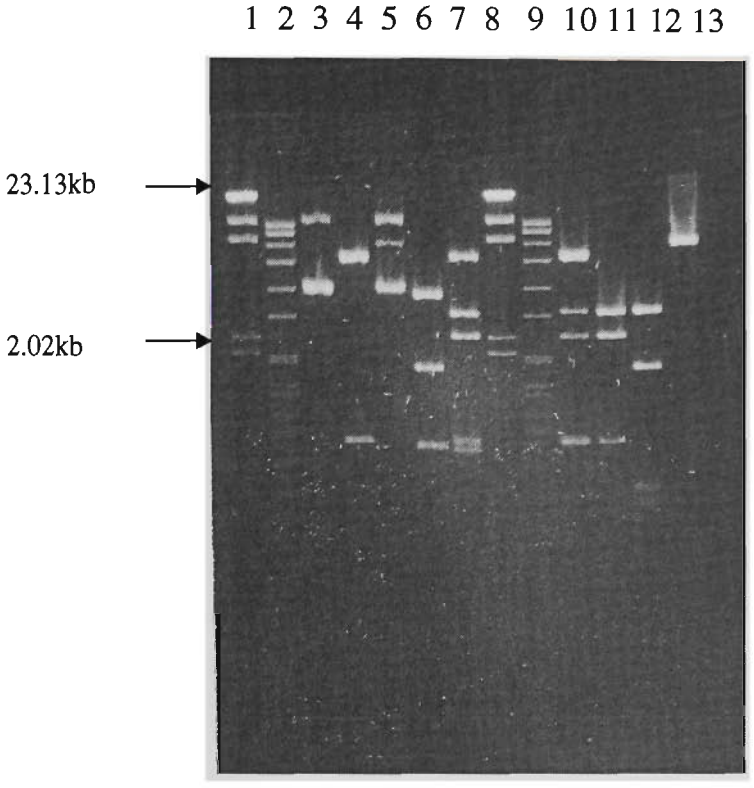


256



Enzymes that do cut

AciI	AclI	AluI	AlwI	ApoI	AvaI	BanI	BanII
BbeI	BbsI	BbvI	BcgI	BfaI	BglI	BpmI	Bpu10I
BsaHI	BsaJI	BseMII	BseRI	BsgI	BsiEI	BsiHKAI	BslI
BsmAI	BsmBI	Bsp1286I	BspMI	BsrI	BsrBI	BsrDI	BsrFI
BssKI	BstAPI	BstBI	Bst4CI	BstDSI	BstF5I	BstNI	BstUI
BtgI	BtrI	Cac8I	ClaI	Csp6I	CviJI	CviRI	DdeI
DpnI	EaeI	EagI	EciI	EcoRI	EcoRV	FauI	Fnu4HI
FokI	FspI	HaeII	HaeIII	HgaI	HhaI	HinPlI	HinfI
HpaII	HphI	KasI	MaeII	MaeIII	MboI	MboII	MnlI
MspAlI	MwoI	NarI	NciI	NlaIII	NlaIV	NspI	PspGI
PstI	PvuII	RsaI	Sau96I	SbfI	ScrFI	SfaNI	SfcI
SfoI	SmaI	SmlI	SphI	StyI	TaiI	TaqI	TfiI
TseI	Tsp45I	Tsp509I	XmaI				



b Restriction digestion pattern of clone C22 containing the complete *inhA* gene on a 4.2 kb *Pst*1 insert from strain MLB194 with various restriction enzymes. Lane 1, Lambda DNA digested with *Hind*III (size markers); 2, SPP1/*Eco*RI markers; 3, Uncut C22; 4, *Sac1*; 5, *Sal1*; 6, *EcoRV* 7, *Sac*1/*Kpn*1 doubly digested; 8, Lambda DNA digested with *Hind*III (Size markers); 9, SPP1/*Eco*RI size markers; 10, *Sac*1/*Sal*1; 11, *Sac*1/*Dra*II; 12, *Sac*1 and *Eco*RI double digestion of clone C22; Lane 13, *Dra*II single digestion of clone C22.

5.4 SEQUENCE ANALYSIS OF THE *inhA* GENE OF STRAIN MLB194

5.4.1 Sequence assembly of the *inhA* gene in MLB194 using fragments generated by PCR

Putative *inhA* sequences were amplified from the positive clone C22 and also from the genomic DNA of MLB194 as several overlapping PCR fragments using the same primer sets and conditions as described previously in Chapter 4. Figure 5.8 a shows the PCR amplified fragments from clone C22.

All of the seven PCR fragments shown in Figure 5.8 a (778 bp using INH1-CGP4, 535 bp INH1-CGP2, 495 bp CGP5-CGP2, 443 bp of CGP1-CGP2, 184 bp of CGP3-CGP4, 255 bp CGP3-INH2 and 134 bP of CGP5-CGP6) were sequenced directly using the relevant primers. However, the longer PCR fragments were cloned into the PCR 2.1 vector using the TA cloning kit and read using M13 and T7 primers. Restriction digestion of recombinant (PCR2.1) clones containing *EcoR1* fragments of the *inhA* gene are shown later in this chapter. The map of PCR 2.1 vector is shown in Appendix 4.

Figure 5.8 b summarises the entire sequence assembly obtained for strain MLB 194 from genomic DNA and clone C22. Sequences obtained for strain MLB194 were from position 1-404, 422-778, 125-570, 144-525, 80-252, 618-774, 644-845 with M13, INH1, CGP1, CGP5 and CGP3 primers, and from position 485-155, 282-90, 735-419, 755-595 with CGP2, CGP6, and CGP4 primers. After sequence assembly, the sequence of the *inhA* gene from positions 1 to 845 bp was obtained from clone C22, which contained a 4.6kb single *Pst1* insert. This sequence was 99.4% complete and the strand that was sequenced was the positive strand. Figure 5.9 shows the nucleotide sequence for strain MLB194. The DNA sequence was deposited to GeneBank under accession number (AF050109). A linear restriction map of the *inhA* gene from strain MLB194 was constructed using the MAP program of WEB ANGIS and is shown in Figure 5.10. The nucleotide sequence of the *inhA* gene was translated using the Etranslate program of WEB ANGIS.

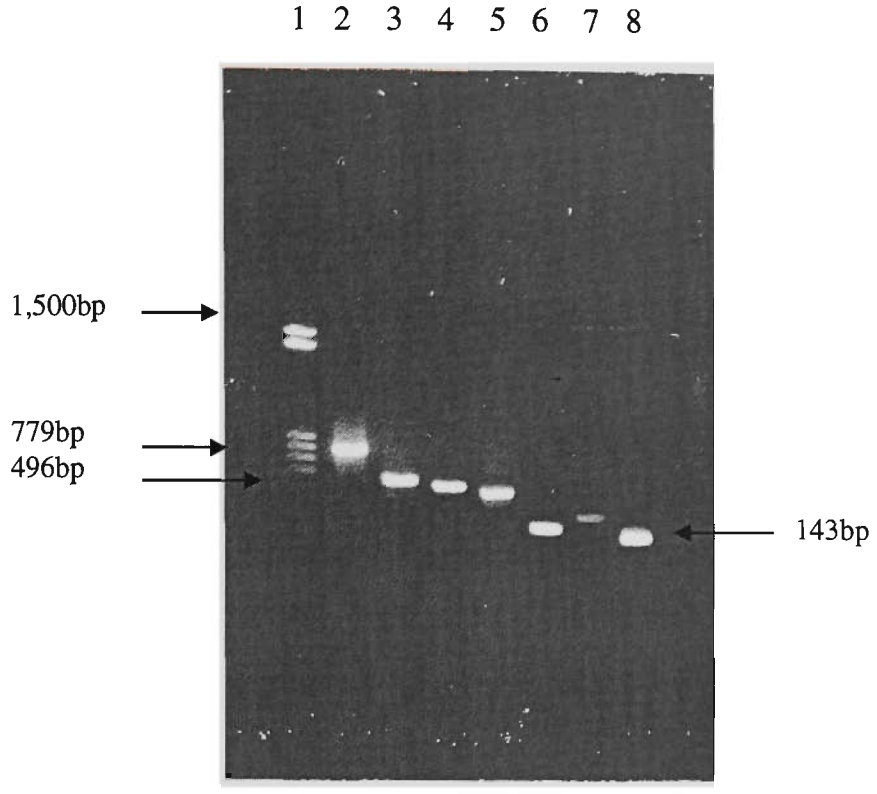


Figure 5.8 a PCR amplification of the *inhA* gene from clone C22 for strain MLB194. Lane 1, 100bp DNA ladder (1, 500, 1,200, 1,000, 900, 800, 700, 600, 500, 400, 300, 200, 100); Lane 2, *inhA* gene fragment (779 bp INH1-CGP4 primers); Lane 3, *inhA* fragment (535 bp INH1-CGP2); Lane 4, i*nhA* fragment (494 bp CGP5-CGP2); Lane 5, *inhA* fragment (443 bp CGP1-CGP2); Lane 6, *inhA* fragment (184 bp CGP3-CGP4); Lane 7, *inhA* fragment (255 bp CGP3-INH2); Lane 8, i*nhA* fragment (134 bp CGP5-CGP6).

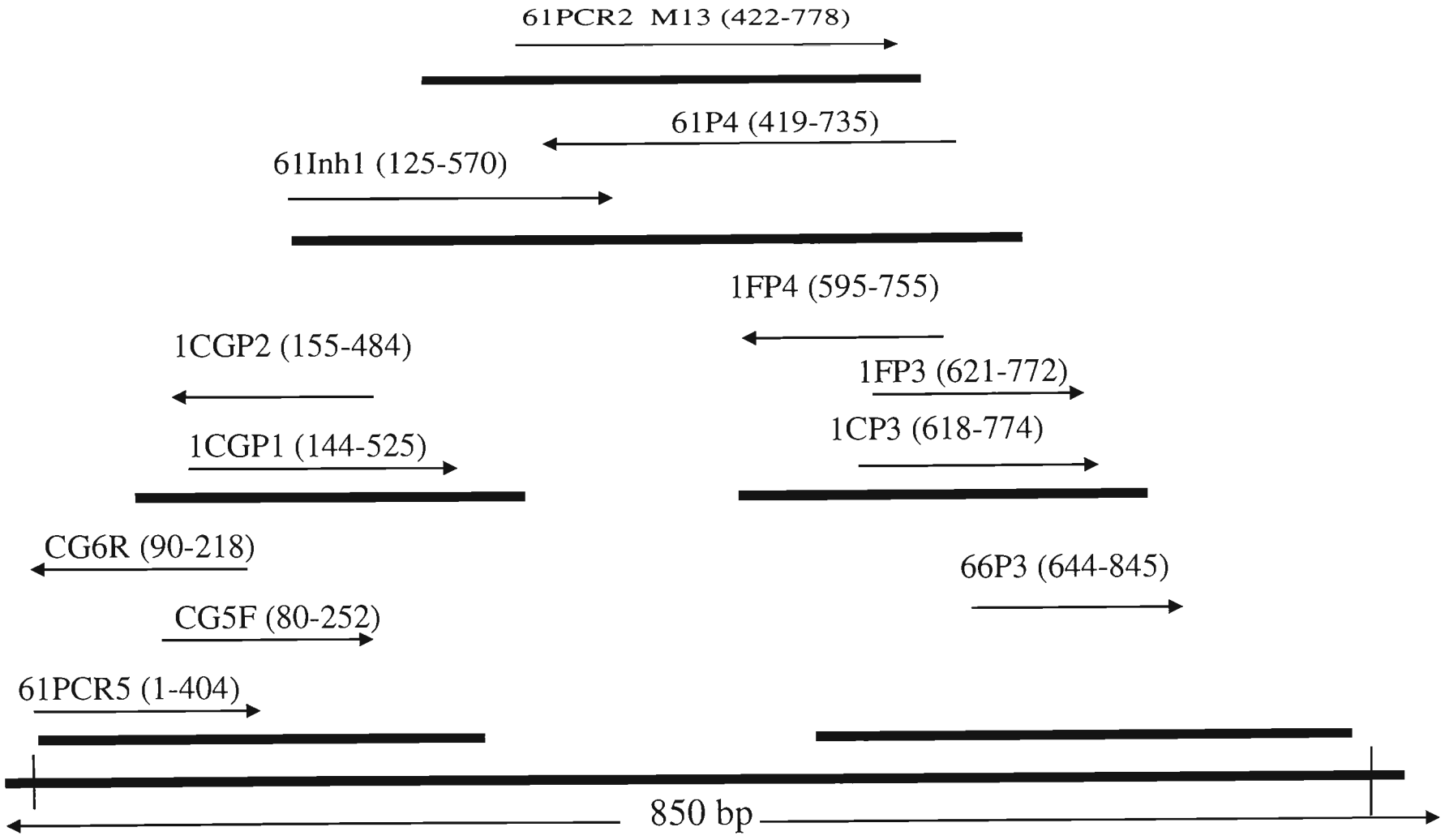
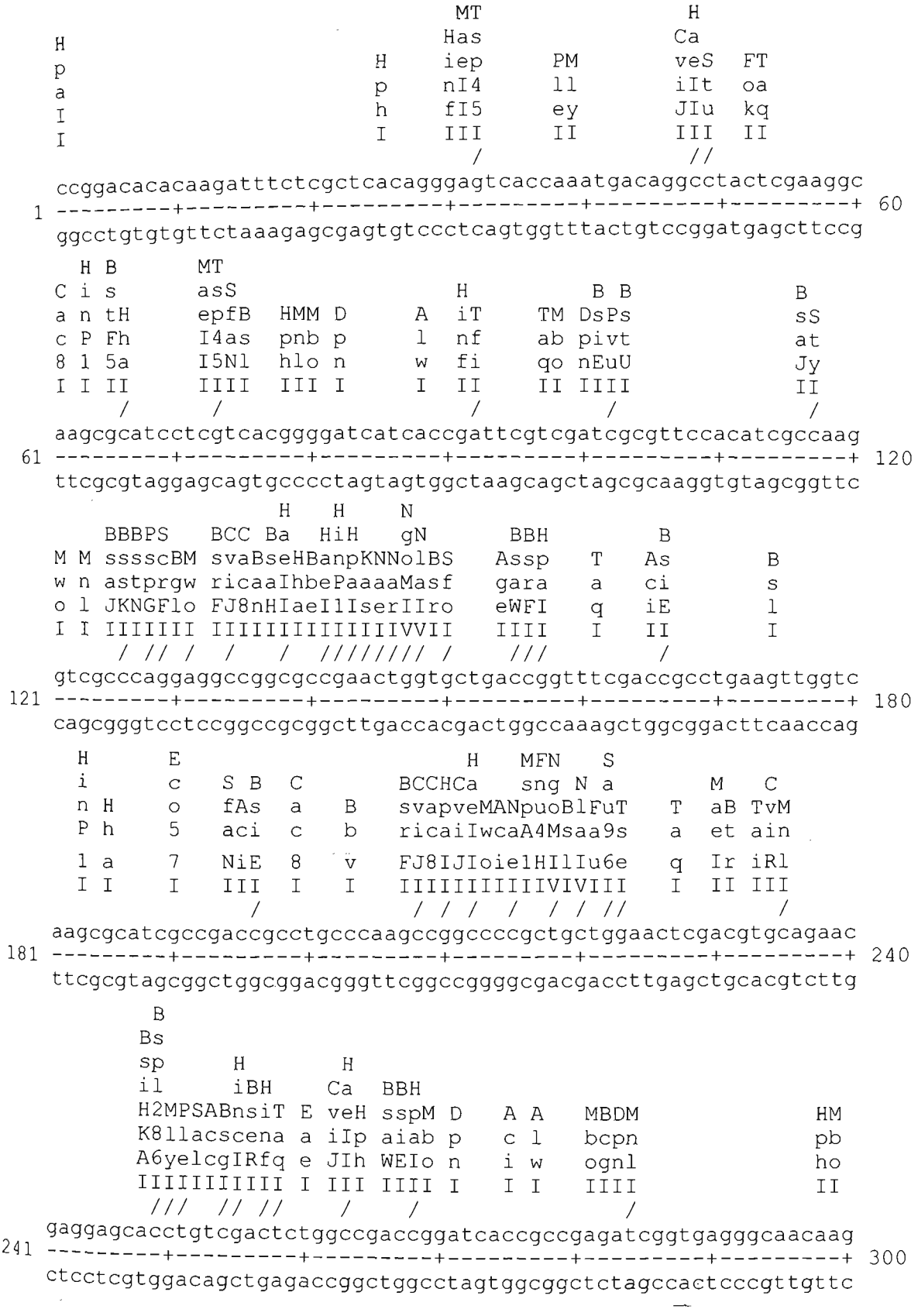


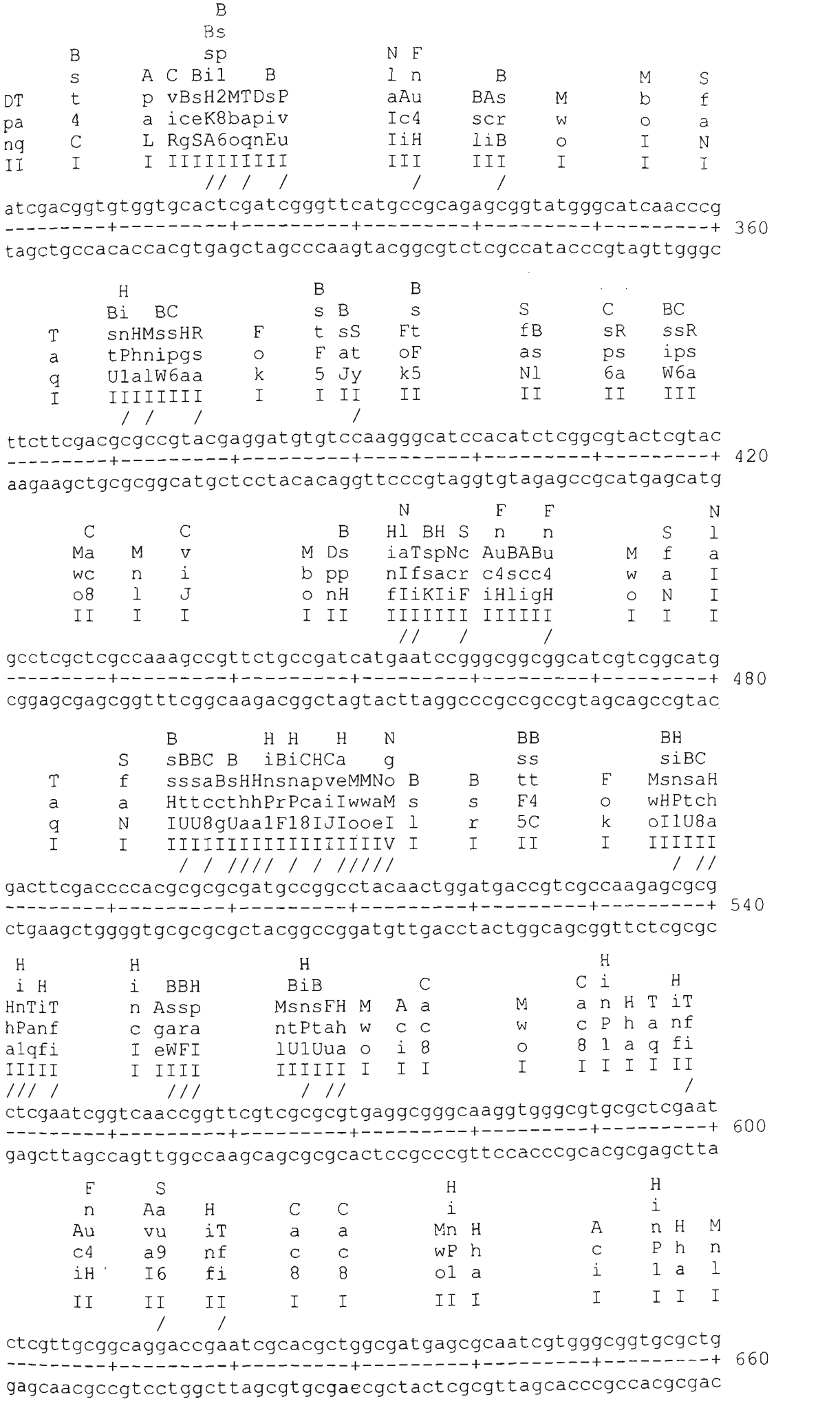
Figure 5.8b Schematic illustration of the *inhA* gene sequence assembly for *C. glutamicum* strain MLB 194. PCR amplified products from genomic DNA and from clones (designated 1C and 1F) were assembled togather on the bases of BLASTN analysis and using the program Sequencer.

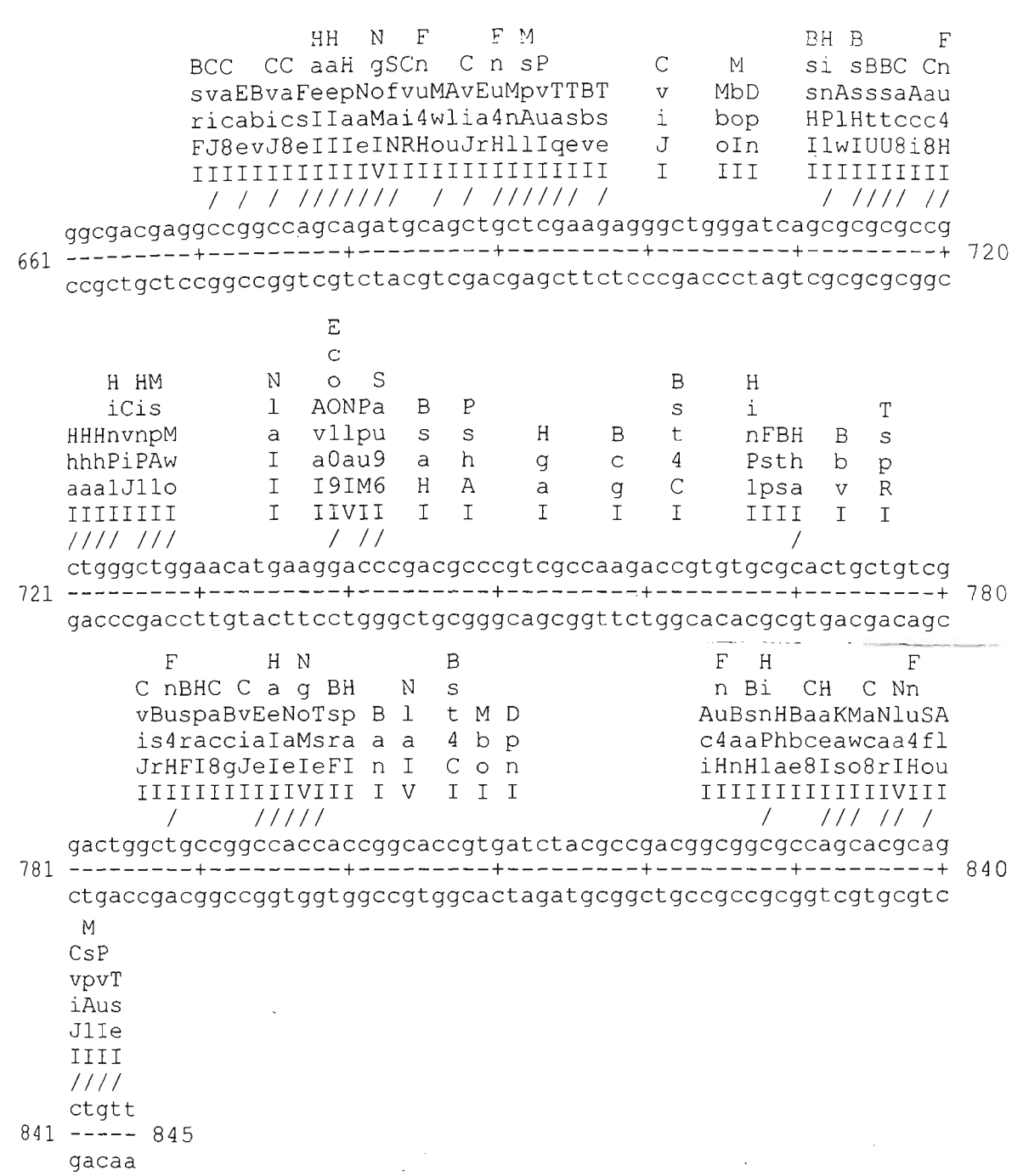
```
   1 ccggacacac aagatttctc gctcacaggg agtcaccaaa tgacaggcct
  51 actcgaaggc aagcgcatcc tcgtcacggg gatcatcacc gattcgtcga
 101 tcgcgttcca catcgccaag gtcgcccagg aggccggcgc cgaactggtg
 151 ctgaccggtt tcgaccgcct gaagttggtc aagcgcatcg ccgaccgcct
 201 gcccaagccg gccccgctgc tggaactcga cgtgcagaac gaggagcacc
 251 tgtcgactct ggccgaccgg atcaccgccg agatcggtga gggcaacaag
 301 atcgacggtg tggtgcactc gatcgggttc atgccgcaga gcggtatggg
 351 catcaacccg ttcttcgacg cgccgtacga ggatgtgtcc aagggcatcc
 401 acatctcggc gtactcgtac gcctcgctcg ccaaagccgt tctgccgatc
 451 atgaatccgg gcggcggcat cgtcggcatg gacttcgacc ccacgccgcg
 501 atgcccggcc tacaactgga tgaccgtcgc caagagcgcg ctcgaatcgg
 551 tcaaccggtt cgtcgcgcgt gaggcgggca aggtgggcgt gcgctcgaat
 601 ctcgttgcgg caggaccgaa tcgcacgctg gcgatgagcg caatcgtggg
 651 cggtgcgctg ggcgacgagg ccggccagca gatgcagctg ctcgaagagg
 701 gctgggatca gcgcgcgccg ctgggctgga acatgaagga cccgacgccc
 751 gtcgccaaga ccgtgtgcgc actgctgtcg gactggctgc cggccaccac
 801 cggcaccgtg atctacgccg acggcggcgc cagcacgcag ctgtt
```

Figure 5.9 Nucleotide sequence of the *inhA* gene for strain MLB 194 obtained from positions 1 to 845. The sequence was submitted to GenBank (accession number AF0501).

Figure 5.10 A linear restriction map of the *inhA* gene of MLB194 obtained using the MAP program of the WEB ANGIS.







Enzymes that do not cut:

AatII	Acc65I	AclI	AfeI	AflII	AflIII	AhdI	AlwNI
ApaI	ApoI	AscI	AseI	AvaI	AvrII	BaeI	BaeI
BamHI	BanII	BbsI	BbvCI	BciVI	BclI	BfaI	BglII
BlpI	BmrI	BplI	BpmI	Bpu10I	BsaI	BsaAI	BsaBI
BseMII	BsmI	BsmAI	BsmBI	BsmFI	BspEI	BspMI	BsrDI
BsrGI	BssSI	BstAPI	BstBI	BstDSI	BstEII	BstXI	BstYI
BstZ17I	Bsu36I	BtgI	ClaI	DdeI	DraI	DraIII	DrdI
EagI	EciI	Ecl136II	EcoNI	EcoRI	EcoRV	HindIII	HpaI
KpnI	MfeI	MluI	MscI	MseI	MslI	NcoI	NdeI
NheI	NotI	NruI	NsiI	NspI	PacI	PciI	PflMI
PmeI	PmlI	Ppu10I	PsiI	PspOMI	PstI	RsrII	SacI
SacII	SanDI	SapI	SbfI	ScaI	SexAI	SfcI	SfiI
SgfI	SgrAI	SmaI	SmlI	SnaBI	SpeI	SphI	SrfI
SspI	SwaI	TatI	TliI	Tsp509I	Tth111I	XbaI	XcmI
XhoI	XmaI	XmnI					

Figure 5.16 shows the comparison between the amino acid sequence of the InhA protein for strain AS019 and MLB 194 using the program GCG/PRETTYBOX *via* the WEB ANGIS).

5.4.2 Homology search results of the assembled sequence (FASTA, BASTN, BLASTX)

The nucleotide similarity of the entire *inhA* gene sequence of MLB194 was determined using BLASTN similarity search on the NCBI database. A high degree of similarity was seen with the *inhA* gene of mycobacterial species. The results of the BLASTN search are shown in Table 5.6.

The similarity scores between InhA deduced amino acid sequences of MLB194 and AS019 were calculated using GCG/BESTFIT and GCG/GAP programs. The results showed the two proteins were 98% identical and the similarity score between the InhA protein of MLB194 and *M. smegmatis* was 99.62%.

Tables 5.7 and 5.8 summarise the results of BLASTX and FASTA homology searches. The highest similarity score was 83-99% with that of mycobacterial species. The deduced protein from MLB194 was compared to that of all known proteins in databases using BLASTP program. As seen for AS019, the highest similarity was observed with that of mycobacterial proteins and low homology scores were seen for non-mycobacterial proteins of other species, including the *fabI* (*P. aeruginosa*), EnvM and short chain alcohol dehydrogenase of *E. coli*, Enoyl-ACP reductase of (*Brassica napus*) etc.

Table 5.6 BLASTN homology search results for the *inhA* gene of *C. glutamicum* strain MLB194.

	inhA MLB194	*inhA* *M. smegmatis*	*inhA* of *M bovis* *M. tuberculosis*	*inhA* of *M. avium* strain GIR10	*fabI* *P. aeruginosa*	*envM* *S. typhimurium*	*envM* *E. coli*
MLB194	100	99	83	83	59	59	59
M. smegmatis		100	83	83	59	56	56
M. tuberculosis *M. bovis*			100	85	59	-	-
M..avium strain GIR10				100	-	-	-
P. aeruginosa					100	65	64
S. typhimurium						100	86
E. coli							100

Table 5.7 BLASTX similarity search results for the InhA protein of *C. glutamicum* strain MLB194 from the NCBI database.

	% similarity of NADH-dependant Enoyl-ACP reductase of MLB194 with similar proteins from other organisms						
	MLB194	*M. smegmatis*	*M. tuberculosis*	*P. aeruginosa*	*S. typhimurium*	*E. coli*	*B. napus*
MLB194	100	98	85	54	54	54	38
M. smegmatis		100	87	54	54	54	34
M. tuberculosis			100	54	54	54	43
P. aeruginosa				100	70	70	43
S. typhimurium					100	97	41
E. coli						100	41
B. napus							100

Table 5.8 FASTA homology search results for the *inhA* gene of MLB194.

Organism	Gene	% homology
M. smegmatis	*inhA*	99.40
M. tuberculosis	*inhA*	82.88
M bovis	*inhA*	82.88
M. avium	*inhA*	83.84
M. bovis	NADH dependent malat	53.56
P. aeruginosa	*fabI*	52.80
Actinamadura hisca	polyketide synthase	53.87
E. coli	*envM*	53.02
E. coli	Short chain alcohol Dehydrogenase	53.02

5.5 CLONING SEQUENCING AND SEQUENCE ANALYSIS OF THE *inhA* GENE IN MLB133.

The same strategy used for MLB194 to sequence the *inhA* gene was used for strain MLB133, except that a library was not constructed for MLB133. PCR fragments were amplified directly from the genomic DNA using the same sets of primers and same sets of PCR conditions as described in Table 4.1 and 4.2 respectively. PCR amplification products of the *inhA* gene for strain MLB133 genomic DNA are shown in Figure 5.11. The expected sizes of the PCR products were obtained for each set of primers.

5.5.1 Sequencing of the *inhA* gene cloned from MLB133

Four PCR products (Figure 5.11) (i.e. 800bp, 500bp, 255bp and 184bp obtained using the INH1-CGP4, CGP5-CGP2, CGP3-INH2 and CGP3-CGP4 primer sets) were chosen for sequencing. Each of the PCR products was used as a template for automated DNA sequencing using the PCR primers. The identity of the sequences was determined by a BLASTN similarity search program. The smaller PCR fragments were sequenced directly using the relevant primers. The longer PCR fragments obtained from the primer sets INH1-CGP4 were sequenced directly from the PCR products using specific primers. About 400 bp of sequence was generated from each primers (INH1 and CGP4). To obtain better readout, the longer fragments of the INH1-CGP4 primer sets were cloned into PCR 2.1 vector using the TA cloning kit. The putative *inhA* clones were screened using standard methods (PCR amplification using INH1-CGP4 primers and *Eco*RI restriction digestion to check the insert size).

Figure 5.12 shows the PCR screening and *EcoRI* restriction digestion patterns of the putative PCR 2.1 clones. The *inhA*-positive clones were sequenced using M13 and T7 primers. A sequence similarity search based on NCBI was performed through ANGIS using BLASTN, BLASTX, and FASTA programs for the sequences obtained for each set of primers. Results of the identity searches revealed 90-98% similarity to the *M. smegmatis inhA* gene.

5.5.2 Sequence assembly for MLB133

Sequences data of four overlapping fragments of the MLB133 *inhA* gene were used to assemble one continuos sequence for the *inhA* gene. The sequences were assembled both manually and by using GCG program of ANGIS. Figure 5.13a summarises the sequencing strategy. After sequence assembly the sequence of *inhA* gene obtained was from position 1-810. The nucleotide sequence of the *inhA* gene of MLB133 is shown in Figure 5.13b. In both strains MLB194 and MLB133 the strand that was sequenced was the positive strand. The assembled DNA sequence of MLB133 was 95% complete and only 40 bp need to be sequenced to obtained a full length *inhA* gene. The DNA sequences were submitted to the GeneBank. A linear restriction map of the *inhA* gene for MLB133 was also created using the MAP program of the WEB ANGIS and this is shown in Fig 5.13c.

5.5.3. Sequence analysis of the assembled *inhA* gene for MLB133

The nucleotide sequence and deduced InhA protein from MLB133 was analysed using BLASTN, BLASTX, FASTA programs (Tables 5.9, 5.10, and 5.11). The *inhA* gene of MLB133 showed the highest similarity score of 81-96 % with that of mycobacterial species. Lowest homology was observed with that of other proteins like EnvM, Short chain alcohol dehydrogenase of (*E. coli*), ACP protein reductase of (*Brassica napus*), and *fabI* of *P. aeruginosa*. Amino acids sequence comparison and calculated similarity score of the deduced InhA protein for strain MLB133 and *M. smegmatis* using (GCG/BESTFIT and GCG/GAP) programs ranged between 94.32 to 97%.

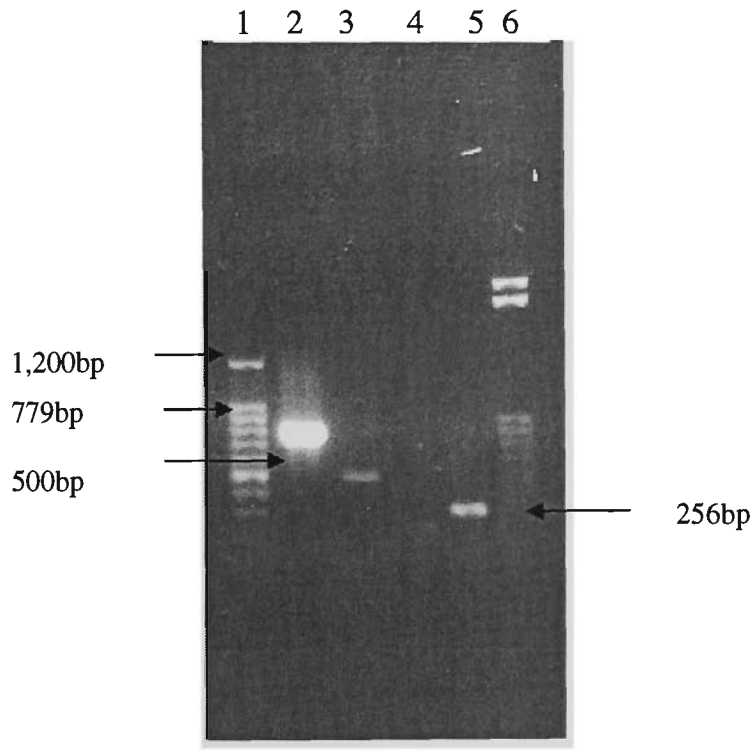


Figure 5.11 PCR amplification of the *inhA* gene from the genomic DNA of strains MLB133. Lane 1 100 bp DNA ladder; Lane 2 to 5 contained *inhA* gene fragment of INH1-CGP4, CGP5-CGP2, CGP5-CGP6, and CGP3-INH2primers sets. Lane 6 contained probase PCR markers.

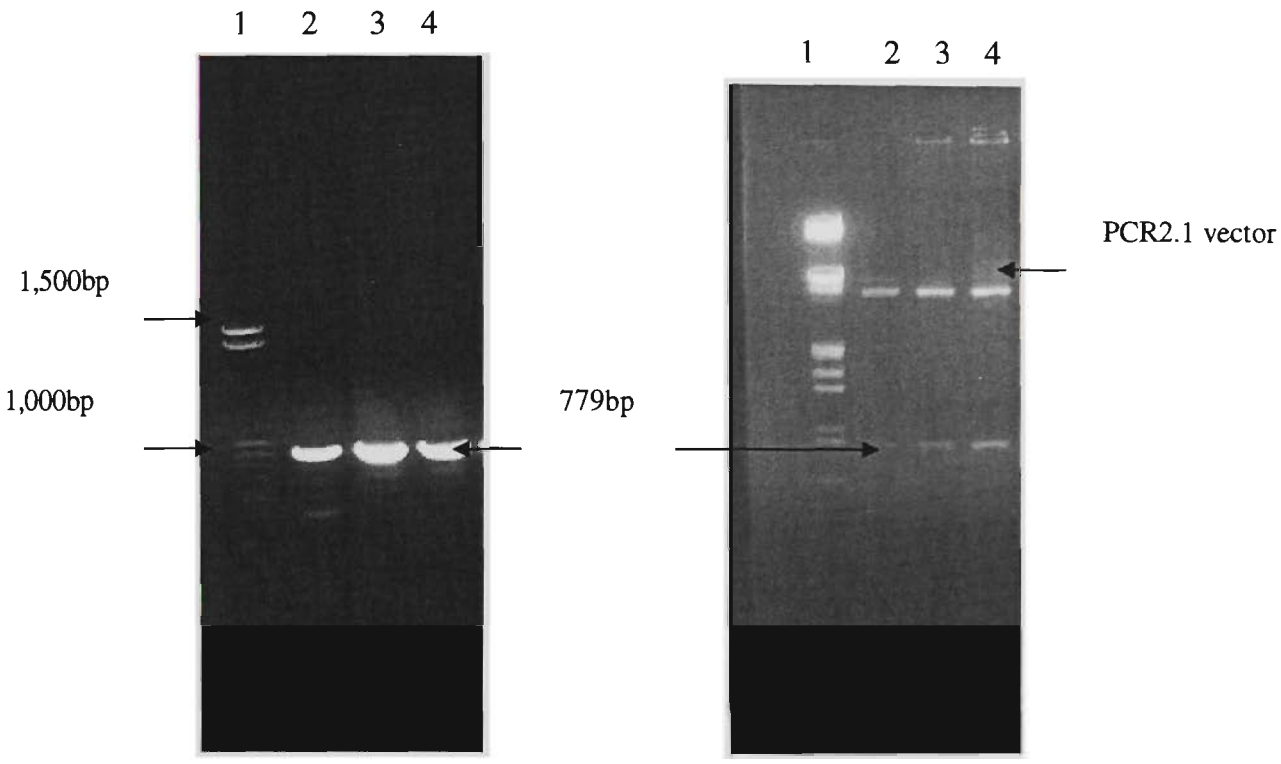


Figure 5.12 Analysis of plasmid DNA from clones generated in the PCR 2.1 vector by insertion of PCR amplification products from genomic DNA of *C. glutamicum* strain MLB133.

a PCR products obtained using primers INH1-CGP4. Lane 1 PCR markers.(Probase, 1,500, 1,200, 1,000, 900, 800, 700, 600, 500, 400, 300, 200, 100), lanes 2 to 4 contained *inhA* gene from *C. glutamicum* strains AS019, MLB194 and MLB133.

b Restriction digestion of PCR2.1 clones containing a 779 bp PCR amplified gene using primers sets INH1 and CGP4 from *C. glutamicum* strains AS019, MLB194 and MLB133 with *Eco*R1 restriction enzyme. Lane 1 *Hind*III digested Lambda DNA size markers; Lanes 2, 3, 4 AS019, MLB194, MLB

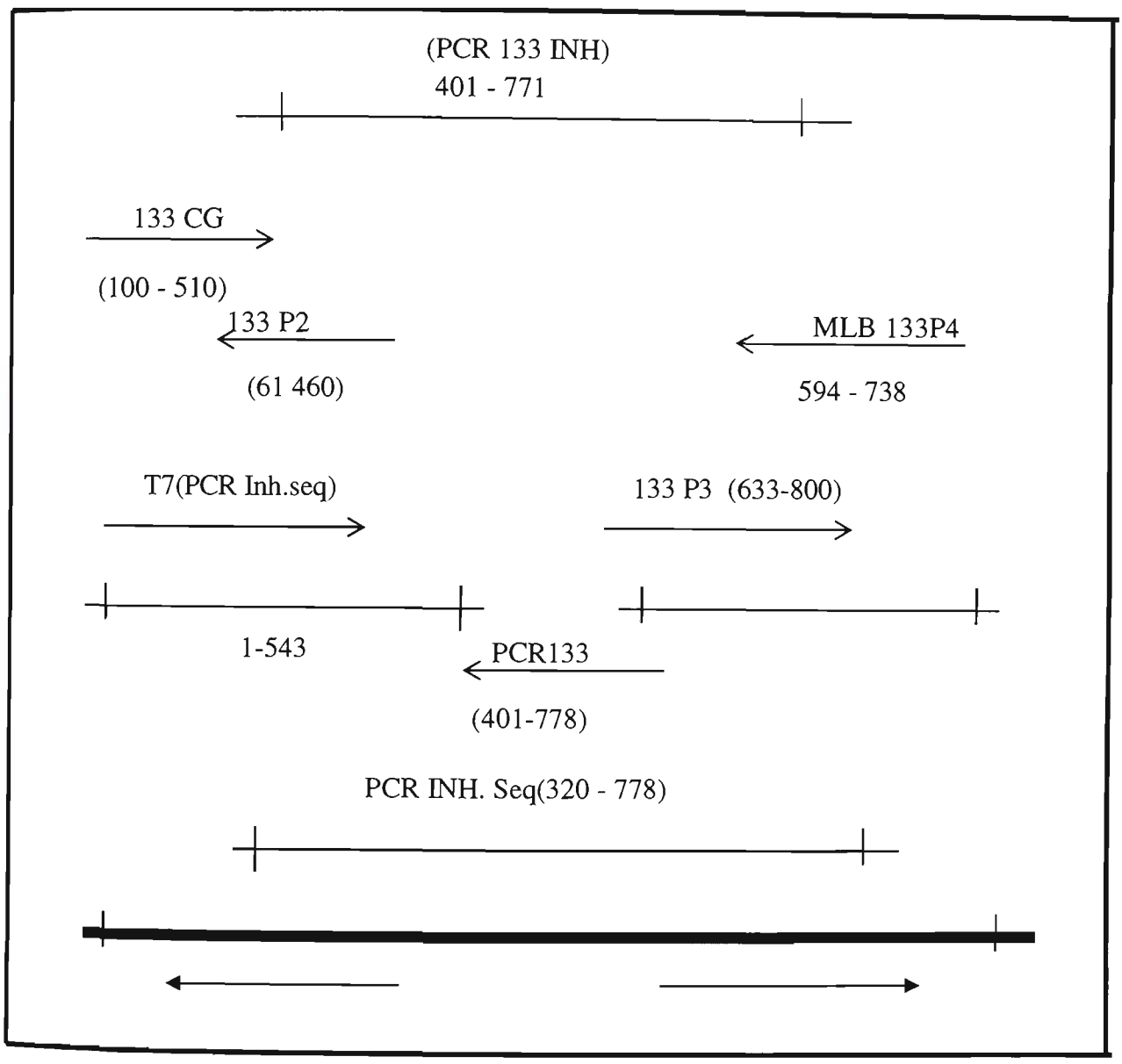
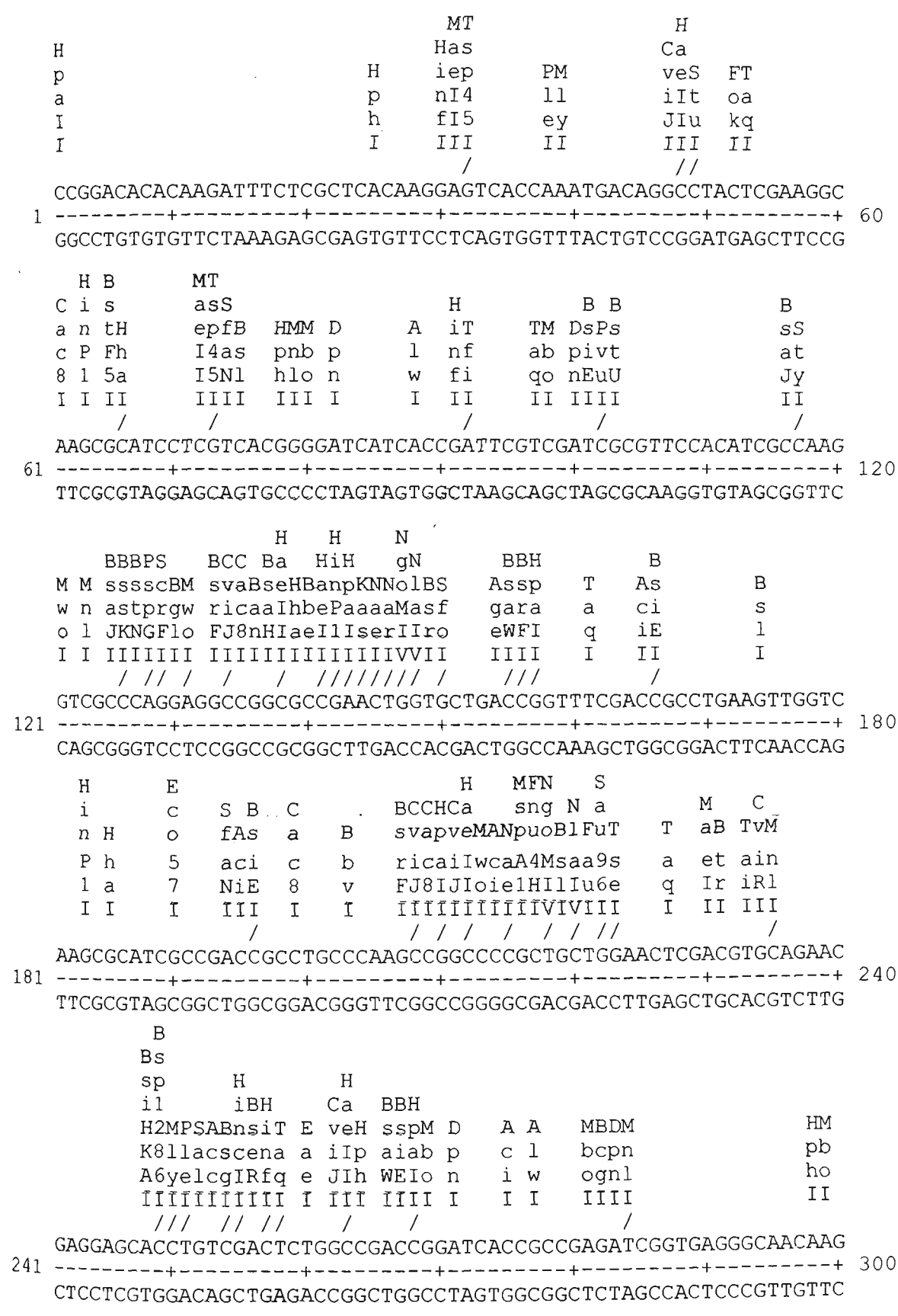


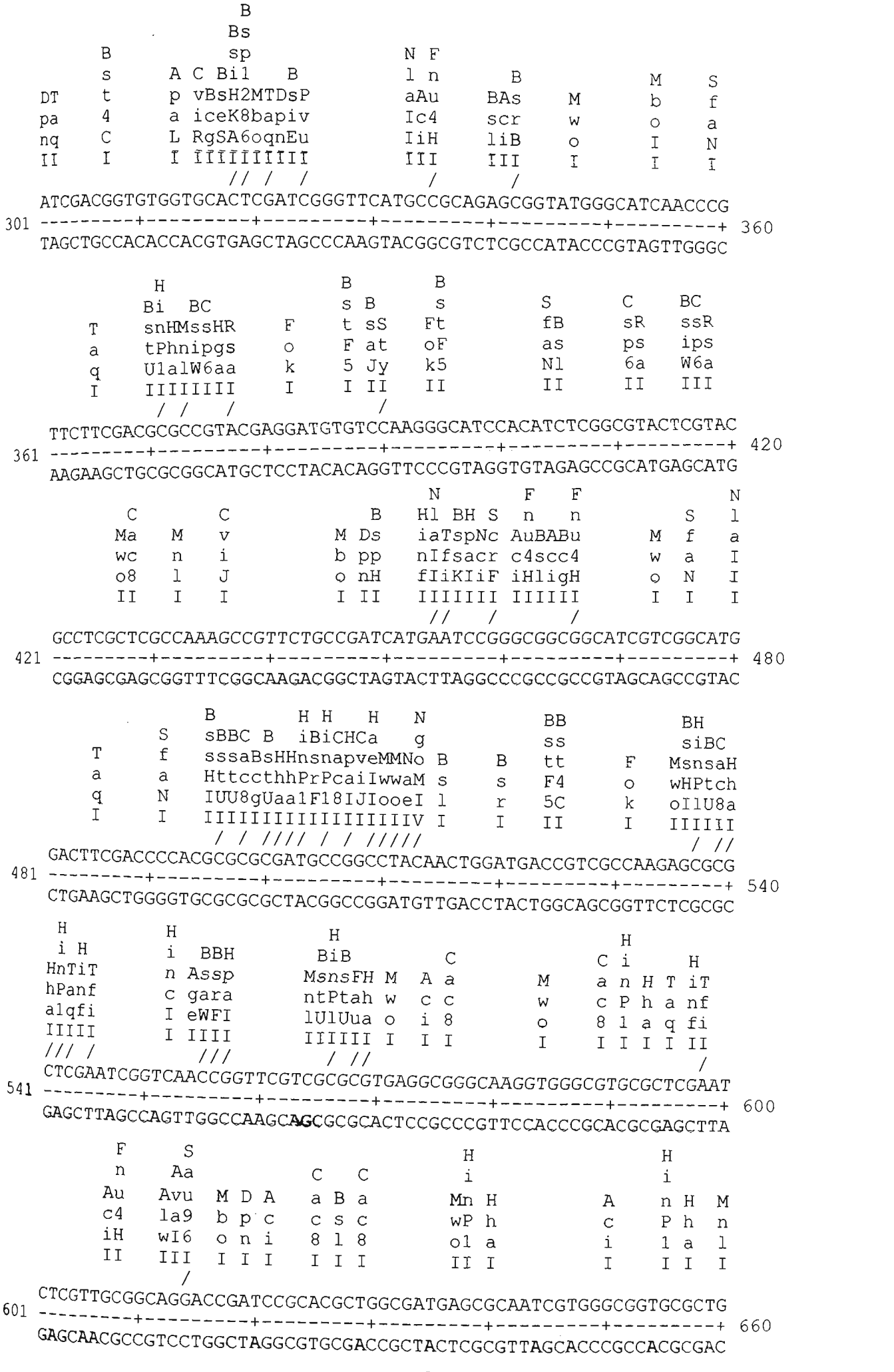
Figure 5.13a Schematic illustration of the *inhA* gene sequence assembly for *C. glutamicum* strain MLB133. Primers used and completed sequence of the *inhA* gene for this strain are shown in Table 4.1 and Fig 5.13 b respectively.

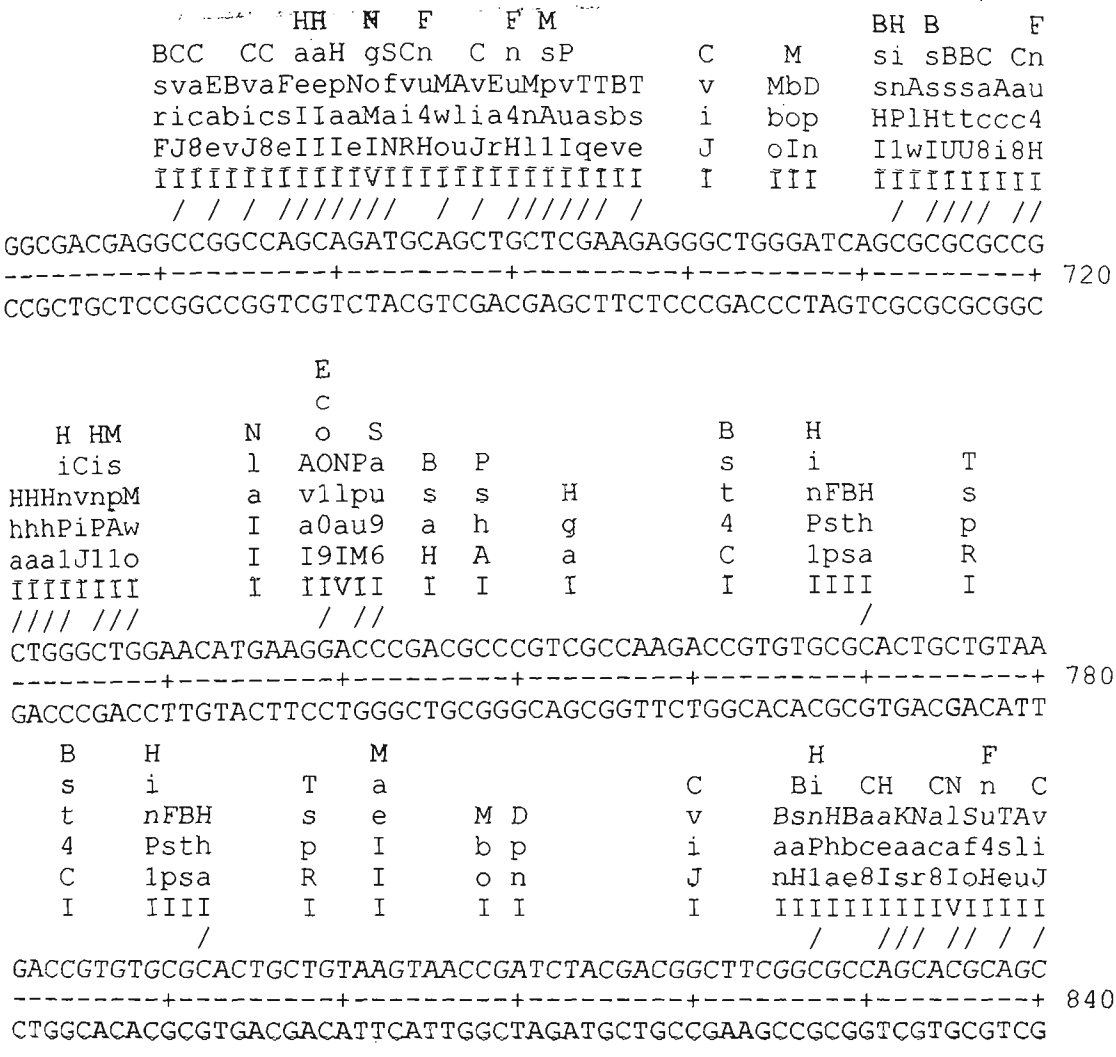
```
  1  CCGGACACAC AAGATTTCTC GCTCACAAGG AGTCACCAAA TGACAGGCCT
 51  ACTCGAAGGC AAGCGCATCC TCGTCACGGG GATCATCACC GATTCGTCGA
101  TCGCGTTCCA CATCGCCAAG GTCGCCCAGG AGGCCGGCGC CGAACTGGTG
151  CTGACCGGTT TCGACCGCCT GAAGTTGGTC AAGCGCATCG CCGACCGCCT
201  GCCCAAGCCG GCCCCGCTGC TGGAACTCGA CGTGCAGAAC GAGGAGCACC
251  TGTCGACTCT GGCCGACCGG ATCACCGCCG AGATCGGTGA GGGCAACAAG
301  ATCGACGGTG TGGTGCACTC GATCGGGTTC ATGCCGCAGA GCGGTATGGG
351  CATCAACCCG TTCTTCGACG CGCCGTACGA GGATGTGTCC AAGGGCATCC
401  ACATCTCGGC GTACTCGTAC GCCTCGCTCG CCAAAGCCGT TCTGCCGATC
451  ATGAATCCGG GCGGCGGCAT CGTCGGCATG GACTTCGACC CCACGCGCGC
501  GATGCCGGCC TACAACTGGA TGACCGTCGC CAAGAGCGCG CTCGAATCGG
551  TCAACCGGTT CGTCGCGCGT GAGGCGGGCA AGGTGGGCGT GCGCTCGAAT
601  CTCGTTGCGG CAGGACCGAT CCGCACGCTG GCGATGAGCG CAATCGTGGG
651  CGGTGCGCTG GGCGACGAGG CCGGCCAGCA GATGCAGCTG CTCGAAGAGG
701  GCTGGGATCA GCGCGCGCCG CTGGGCTGGA ACATGAAGGA CCCGACGCCC
751  GTCGCCAAGA CCGTGTGCGC ACTGCTGTAA GACCGTGTGC GCACTGCTGT
801 AAGTAACCG   ATCTACGACG GCTTCGGCGC CAGCACGCAG CTGTTGTGAT
```

Figure 5.13b Nucleotide sequence of the *inhA* gene for strain MLB133, based on primers derived from *M. smegmatis inhA* gene sequences (INH1, INH2, CGP2, CGP3, CGP4, CGP5, CGP6).

Figure 5.13c A linear restriction map of the *inhA* gene of MLB133 created by accessing MAP program of the WEB ANGIS.




ATCGACGGTGTGGTGCACTCGATCGGGTTCATGCCGCAGAGCGGTATGGGCATCAACCCG
301
TAGCTGCCACACCACGTGAGCTAGCCCAAGTACGGCGTCTCGCCATACCCGTAGTTGGGC
360
TTCTTCGACGCGCCGTACGAGGATGTGTCCAAGGGCATCCACATCTCGGCGTACTCGTAC
361
AAGAAGCTGCGCGGCATGCTCCTACACAGGTTCCCGTAGGTGTAGAGCCGCATGAGCATG
420
GCCTCGCTCGCCAAAGCCGTTCTGCCGATCATGAATCCGGGCGGCGGCATCGTCGGCATG
421
CGGAGCGAGCGGTTTCGGCAAGACGGCTAGTACTTAGGCCCGCCGCCGTAGCAGCCGTAC
480
GACTTCGACCCCACGCGCGCGATGCCGGCCTACAACTGGATGACCGTCGCCAAGAGCGCG
481
CTGAAGCTGGGGTGCGCGCGCTACGGCCGGATGTTGACCTACTGGCAGCGGTTCTCGCGC
540
CTCGAATCGGTCAACCGGTTCGTCGCGCGTGAGGCGGGCAAGGTGGGCGTGCGCTCGAAT
541
GAGCTTAGCCAGTTGGCCAAGCAGCGCGCACTCCGCCCGTTCCACCCGCACGCGAGCTTA
600
CTCGTTGCGGCAGGACCGATCCGCACGCTGGCGATGAGCGCAATCGTGGGCGGTGCGCTG
601
GAGCAACGCCGTCCTGGCTAGGCGTGCGACCGCTACTCGCGTTAGCACCCGCCACGCGAC
660



Enzymes that do not cut

AatII	Acc65I	AclI	AfeI	AflII	AflIII	AhdI	AlwNI
ApaI	ApoI	AscI	AseI	AvaI	AvrII	BaeI	BaeI
BamHI	BanII	BbsI	BbvCI	BciVI	BclI	BfaI	BglII
BlpI	BmrI	BplI	BpmI	Bpu10I	BsaI	BsaAI	BsaBI
BseMII	BsmI	BsmAI	BsmBI	BsmFI	BspEI	BspMI	BsrDI
BsrGI	BssSI	BstAPI	BstBI	BstDSI	BstEII	BstXI	BstYI
BstZ17I	Bsu36I	BtgI	ClaI	DdeI	DraI	DraIII	DrdI
EagI	EciI	Ecl136II	EcoNI	EcoRI	EcoRV	HindIII	HpaI
KpnI	MfeI	MluI	MscI	MseI	MslI	NcoI	NdeI
NheI	NotI	NruI	NsiI	NspI	PacI	PciI	PflMI
PmeI	PmlI	Ppu10I	PsiI	PspOMI	PstI	RsrII	SacI
SacII	SanDI	SapI	SbfI	ScaI	SexAI	SfcI	SfiI
SgfI	SgrAI	SmaI	SmlI	SnaBI	SpeI	SphI	SrfI
SspI	SwaI	TatI	TliI	Tsp509I	Tth111I	XbaI	XcmI
XhoI	XmaI	XmnI					

Table 5.9 BLASTN homology search results for the *inhA* gene of *C. glutamicum* strain MLB133.

	inhA MLB133	*inhA* *M. smegmatis*	*inhA* of *M bovis* *M. tuberculosis*	*inhA* of *M. avium* strain GIR10	*fabI* *P. aeruginosa*	*envM* *S. typhimurium*	*envM* *E. coli*
MLB133	100	96	81	79	59	56	56
M. smegmatis		100	83	83	59	56	56
M. tuberculosis *M. bovis*			100	85	59	-	-
M..avium strain GIR10				100	-	-	-
P. aeruginosa					100	65	64
S. typhimurium						100	86
E. coli							100

Table 5.10 BLASTX similarity search results for the InhA protein of *C. glutamicum* strain MLB133 using the NCBI database.

	% similarity of NADH-dependant Enoyl-ACP reductase of MLB194 with similar proteins from other organisms						
	MLB133	*M. smegmatis*	*M. tuberculosis*	*P. aeruginosa*	*S. typhimurium*	*E. coli*	*B. napus*
MLB133	100	95	83	50	54	54	34
M. smegmatis		100	87	54	54	54	34
M. tuberculosis			100	54	54	54	43
P. aeruginosa				100	70	70	43
S. typhimurium					100	97	41
E. coli						100	41
B. napus							100

Table 5.11 FASTA homology search results for the *inhA* gene sequence of MLB133.

Organism	Gene	% homology
M. smegmatis	*inhA*	97.17
M. tuberculosis	*inhA*	81.06
M. bovis	Putative Keto-acyl ACP	81.08
M. avium	*inhA*	81.62
P. aeruginosa	*fabI*	52.74
E. coli	envM protein gene	53.02
E. coli	Short chain alcohol dehydrogenase	53.02

5.6 COMPARISON OF THE *inhA* GENE AND GENE PRODUCTS IN *C. GLUTAMICUM* STRAINS AS019, MLB133 AND MLB194

The *inhA* gene sequences of C. glutamicum strains AS019, MLB133, and MLB194 were deposited to the GenBank. Table 5.12 showed the asscession number for these genes.

5.6.1 Southern hybridisation patterns

Genomic DNA of each strain was digested with restriction enonucleases, transferred to the nylon membrane and probed with a 800 bp PCR amplification products from the *inhA* of AS019 which was DIG labelled. Since the small part of the *inhA* gene was used as a probe previously, the possibility that a particular sequence was repeated in the genome could not be ruled out. Therefore the main purpose of this experiment was to see how many copies of the gene were present in the genome and to validate if there is any consistency in the results when small and large probes were used. Figure 5.14a and 5.14b show the results of Southern blot hybridisation of genomic DNA of the four different strains of *C. glutamicum* (AS019, MLB194, MLB133, and ATCC 13032) digested with *Pst*1, and *Kpn*1/*Eco*R1. The results showed that the Southern hybridisation pattern obtained with the larger probe was similar with the Southern pattern obtained with the small probe, when ^{32}P labelled major bands of 9.2, 5.2, 3.5 kb were obtained whereas using the DIG system major bands of 6.5, 5.2, and 3.5 kb were common with both probes (CGP3-INH2 and INH1-CGP4). The two small bands seen previously in *Pst*1 Southern hybridisation were not seen this time. One possible explanation is that *Pst*1 enzyme was from a different source and digestion was accomplished with the addition of BSA each time. The variations in the results with two approaches (DIG and ^{32}P) can be explained by the fact that the DIG system uses 300-1,000 ng of probe DNA whereas the ^{32}P system uses only 25 ng of DNA. Therefore the DIG system is more sensitive than ^{32}P system. Table 5.13 shows the molecular weights of the hybridisation signals. The three strains showed similar patterns as evident from the hybridisation signals for each enzyme used, demonstrating similarity between the strains. The Southern pattern obtained with *Kpn*1/*Eco*R1 double digested genomic DNA with large probe also showed multiple signals thus confirming the previous observation with the smaller probe.

Table 5.12 Accession numbers for the *inhA* gene sequences of the three corynebacterial strains were submitted to the Gene bank.

ORGANISM	ACCESSION NUMBER
Corynebacterium glutamicum AS019	AF 139472
Corynebacterium glutamicum MLB133	AF145898
Corynebacterium glutamicum MLB194	AF050109

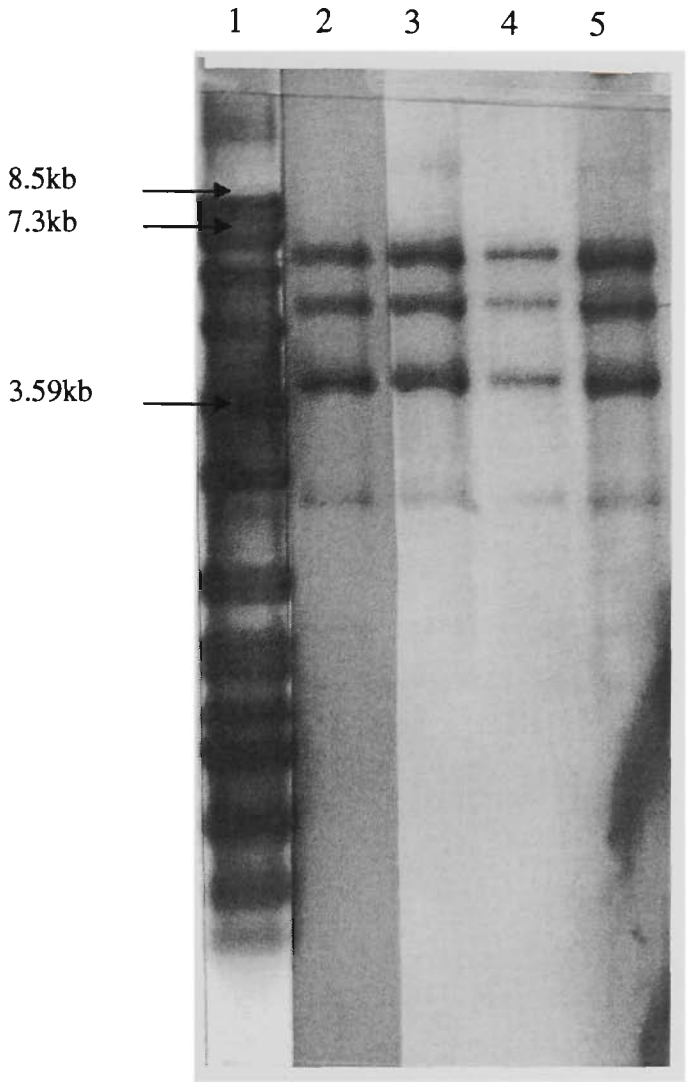
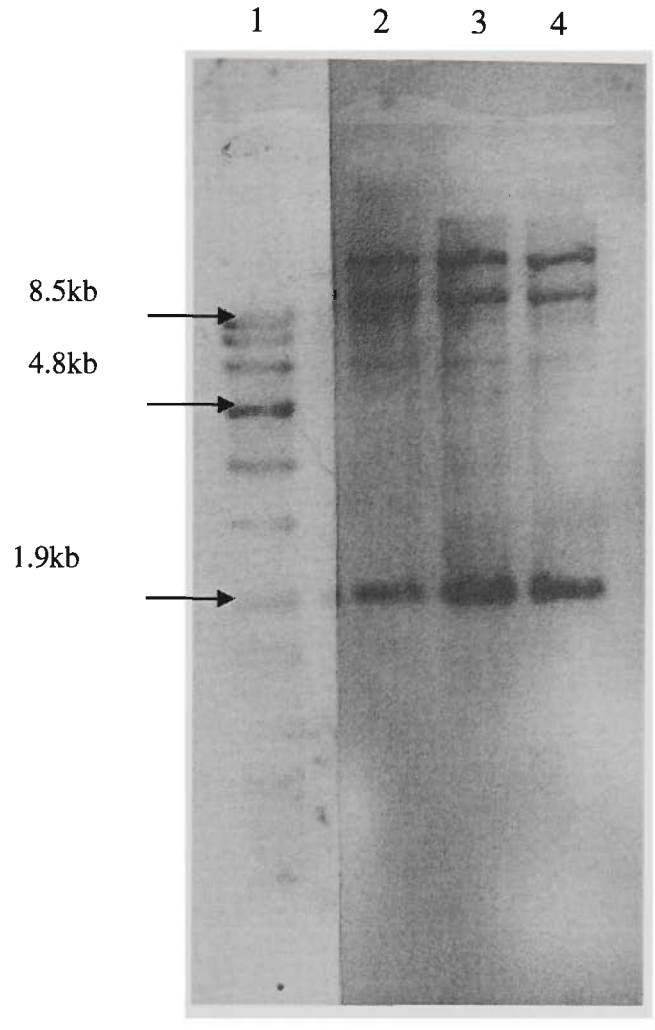


Figure 5.14 Southern hybridisation analysis of digested genomic DNA of *C. glutamicum* strains AS019, MLB194, MLB133 probed with the DIG-labelled PCR amplification products obtained from AS019 using the INH1-CGP4 primers sets.

a *Pst*1 digested genomic DNA. Lane 1, SPP1/*Eco*R1 DNA size markers; Lanes, 2 to 5 contained *C. glutamicum* strains AS019, MLB194, MLB133, ATCC 13032 respectively.



b *Kpn*1/*Eco*R1 double-digested genomic DNA. Lane 1 SPP1/*Eco*R1 DNA size markers, Lanes 2, 3, and 4, *C. glutamicum*, strains AS019, MLB194, MLB133.

Table 5.13 Molecular weights of the genomic DNA fragments obtained after Southern hybridisation for AS019, MLB133, and MLB194 using a DIG-labelled probe (800bp PCR product from AS019 DNA).

SPP1/*Eco*R1 Markers	***Kpn*1/*Eco*RV**	***Pst*1**
8.5	8.5	6.5
7.35	6.1	5.2
6.10	1.95	3.5
4.84		2.5
3.59		
2.81		
1.95		
1.86		
1.51		
1.39		
1.16		
0.98		
0.72		
0.48		
0.36		

5.6.2 Sequence alignment of the *inhA* genes of AS019, MLB133, and MLB194

Alignment of sequence data (Fig.5.15a) for the three *inhA* genes of *C. glutamicum* strains AS019, MLB133 and MLB194 with *M. smegmatis inhA* gene (Banerjee *et al.*, 1994) showed that the three genes aligned almost perfectly, as expected from the similarity scores. The alignment indicated that the translation initiation codon (ATG) and termination codon were highly conserved and present at the corresponding positions in all three genes of corynebacteria and *M. smegmatis*.

However, nucleotide variations (single base changes) were observed at several points. Nucleotide sequences were verified several times by PCR amplification directly from the genomic DNA and also from the clones thus minimizing the risk of any sequencing error that have occurred. The nucleotide variations at different points are shown by red letters in Figure 5.15 b. The sequences of MLB133 and MLB194 were more similar to *M. smegmatis*: the variations were observed mostly in AS019 and *M. smegmatis* sequences. The common nucleotide substitutions were observed between AS019 and *M. smegmatis* and AS019 and MLB194 and MLB133. For example, A (adenine) at position 115 is replaced by G in AS019; similarly, at position 120, T is replaced by C in AS019 whereas in MLB133 and MLB194 these two bases are (A, T), similar to *M. smegmatis*.

Figure 5.15a Multiple sequence alignment of the *inhA* genes of *C. glutamicum* strains AS019, MLB133 and MLB194, with the *inhA* gene of *M. smegmatis* using the Eclustaw program of WEBANGIG. Asterisk represents similar nucleotides and a gap represents a difference in nucleotide at that position.

```
                10         20         30         40         50         60
194d            CCGGACACACAAGATTTCTCGCTCACAGGGAGTCACCAAATGACAGGCCTACTCGAAGGC
AS019d          CCGGACACACAAGATTTCTCGCTCACAAGGAGTCACCAAATGACAGGCCTACTCGAGGGC
M.smegD         CCGGACACACAAGATTTCTCGCTCACAAGGAGTCACCAAATGACAGGCCTACTCGAAGGC
133.seq         CCGGACACACAAGATTTCTCGCTCACAAGGAGTCACCAAATGACAGGCCTACTCGAAGGC
                ***************************** ***************************** ***

194d            AAGCGCATCCTCGTCACGGGGATCATCACCGATTCGTCGATCGCGTTCCACATCGCCAAG
AS019d          AAGCGCATCCTCGTCACGGGGATCATCACCGATTCGTCGATCGCGTTCCACATCGCCAAG
M.smegD         AAGCGCATCCTCGTCACGGGGATCATCACCGATTCGTCGATCGCGTTCCACATCGCCAAG
133.seq         AAGCGCATCCTCGTCACGGGGATCATCACCGATTCGTCGATCGCGTTCCACATCGCCAAG
                ************************************************************

194d            GTCGCCCAGGAGGCCGGCGCCGAACTGGTGCTGACCGGTTTCGACCGCCTGAAGTTGGTC
AS019d          GTCGCCCAGGAGGCCGGCGCCGAACTGGTGCTGACCGGTTTCGACCGCCTGAAGTTGGTC
M.smegD         GTCGCCCAGGAGGCCGGCGCCGAACTGGTGCTGACCGGTTTCGACCGCCTGAAGTTGGTC
133.seq         GTCGCCCAGGAGGCCGGCGCCGAACTGGTGCTGACCGGTTTCGACCGCCTGAAGTTGGTC
                ************************************************************

194d            AAGCGCATCGCCGACCGCCTGCCCAAGCCGGCCCCGCTGCTGGAACTCGACGTGCAGAAC
AS019d          AAGCGCATCGCCGACCGCCTGCCCAAGCCGGCCCCGCTGCTGGAACTCGACGTGCAGAAC
M.smegD         AAGCGCATCGCCGACCGCCTGCCCAAGCCGGCCCCGCTGCTGGAACTCGACGTGCAGAAC
133.seq         AAGCGCATCGCCGACCGCCTGCCCAAGCCGGCCCCGCTGCTGGAACTCGACGTGCAGAAC
                ************************************************************

194d            GAGGAGCACCTGTCGACTCTGGCCGACCGGATCACCGCCGAGATCGGTGAGGGCAACAAG
AS019d          GAGGAGCACCTGTCGACTCTGGCCGACCGGATCACCGCCGAGATCGGTGAGGGCAACAAG
M.smegD         GAGGAGCACCTGTCGACTCTGGCCGACCGGATCACCGCCGAGATCGGTGAGGGCAACAAG
133.seq         GAGGAGCACCTGTCGACTCTGGCCGACCGGATCACCGCCGAGATCGGTGAGGGCAACAAG
                ************************************************************
```

```
194d          ATCGACGGTGTGGTGCACTCGATCGGGTTCATGCCGCAGAGCGGTATGGGCATCAACCCG
AS019d        ATCGACGGTGTGGTGCACTCGATCGGGTTCATGCCGCAGAGCGGTATGGGCATCGACCCG
M.smegD       ATCGACGGTGTGGTGCACTCGATCGGGTTCATGCCGCAGAGCGGTATGGGCATCAACCCG
Pcrp4.seq     ATCGACGGTGTGGTGCACTCGATCGGGTTCATGCCGCAGAGCGGTATGGGCATCAACCCG
              ************************************************************

194d          TTCTTCGACGCGCCGTACGAGGATGTGTCCAAGGGCATCCACATCTCGGCGTACTCGTAC
AS019d        TTCTTCGACGCGCCGTACGAGGATGTGTCCAAGGGCATCCACATCCCGGCGTACTCGTAC
M.smegD       TTCTTCGACGCGCCGTACGAGGATGTGTCCAAGGGCATCCACATCTCGGCGTACTCGTAC
Pcrp4.seq     TTCTTCGACGCGCCGTACGAGGATGTGTCCAAGGGCATCCACATCTCGGCGTACTCGTAC
              ********************************************** **************

194d          GCCTCGCTCGCCAAAGCCGTTCTGCCGATCATGAATCCGGGCGGCGGCATCGTCGGCATG
AS019d        GCCTCGCTCGCCAAAGCCGTTCTGCCGATCATGAATCCGGGCGGCGGCATCGTCGGCATG
M.smegD       GCCTCGCTCGCCAAAGCCGTTCTGCCGATCATGAATCCGGGCGGCGGCATCGTCGGCATG
133.seq       GCCTCGCTCGCCAAAGCCGTTCTGCCGATCATGAATCCGGGCGGCGGCATCGTCGGCATG
              ************************************************************

194d          GACTTCGACCCCACGCGCGCGATGCCGGCCTACAACTGGATGACCGTCGCCAAGAGCGCG
AS019d        GACTTCGACCCCACGCGCGCGATGCCCGGCTACAACTGGATGACCGTCGCCAAGAGCGCG
M.smegD       GACTTCGACCCCACGCGCGCGATGCCGGCCTACAACTGGATGACCGTCGCCAAGAGCGCG
133.seq       GACTTCGACCCCACGCGCGCGATGCCGGCCTACAACTGGATGACCGTCGCCAAGAGCGCG
              *************************** * ******************************

194d          CTCGAATCGGTCAACCGGTTCGTCGCGCGTGAGGCGGGCAAGGTGGGCGTGCGCTCGAAT
AS019d        CTCGAATCGGTCAACCGGTTCGTCGCGCGTGAGGCGGGCAAGGTGGGCGTGCGCTCGAAT
M.smegD       CTCGAATCGGTCAACCGGTTCGTCGCGCGTGAGGCGGGCAAGGTGGGCGTGCGCTCGAAT
133.seq       CTCGAATCGGTCAACCGGTTCGTCGCGCGTGAGGCGGGCAAGGTGGGCGTGCGCTCGAAT
              ************************************************************
194d          CTCGTTGCGGCAGGACCGAATCGCACGCTGGCGATGAGCGCAATCGTGGGCGGTGCGCTG
AS019d        CTCGTTGCGGCAGGACCGATCCGCACGCTGGCGATGAGCGCAATCGTGGGCGGTGCGCTG
M.smegD       CTCGTTGCGGCAGGACCGATCCGCACGCTGGCGATGAGCGCAATCGTGGGCGGTGCGCTG
133.seq       CTCGTTGCGGCAGGACCGATCCGCACGCTGGCGATGAGCGCAATCGTGGGCGGTGCGCTG
              ******************  ***************************************

194d          GGCGACGAGGCCGGCCAGCAGATGCAGCTGCTCGAAGAGGGCTGGGATCAGCGCGCGCCG
AS019d        GGCGACGAGGCCGGCCAGCAGATGCAGCTGCTCGAAGAGGGCTGGGATCAGCGCGCGCCG
M.smegD       GGCGACGAGGCCGGCCAGCAGATGCAGCTGCTCGAAGAGGGCTGGGATCAGCGCGCGCCG
133.seq       GGCGACGAGGCCGGCCAGCAGATGCAGCTGCTCGAAGAGGGCTGGGATCAGCGCGCGCCG
              ************************************************************
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```
194d            CTGGGCTGGAACATGAAGGACCCGACGCCCGTCGCCAAGACCGTGTGCGCACTGCTGTCG
AS019d          CTAGGCTGGAACATGAAGGACCCGACGCCCGTCGCCAAGACCGTGTGCGCACTGCTGTCG
M.smegD         CTGGGCTGGAACATGAAGGACCCGACGCCCGTCGCCAAGACCGTGTGCGCACTGCTGTCG
133.seq         CTGGGCTGGAACATGAAGGACCCGACGCCCGTCGCCAAGACCGTGTGCGCACTGCTGTAA
                ** **************************************************

194d            GACTGGCTGCCGGCCACCACCGGCACCGTGATCTACGCCGACGGCGGCGCCAGCACGCAG
AS019d          GACTGGCTGCCGGCCACCACCGGCACCGTGATCTACGCCGACGGCGGCGCCAGCACGCAG
M.smegD         GACTGGCTGCCGGCCACCACCGGCACCGTGATCTACGCCGACGGCGGCGCCAGCACGCAG
133seq          GACCGTGTGCGCACTGCTGT----------------------------------------
                *** *  ***   *  *

194d            CTGTT-----
AS019d          CTGTTGTGAT
M.smegD         CTGTTGTGAT
133.seq         ----------
```

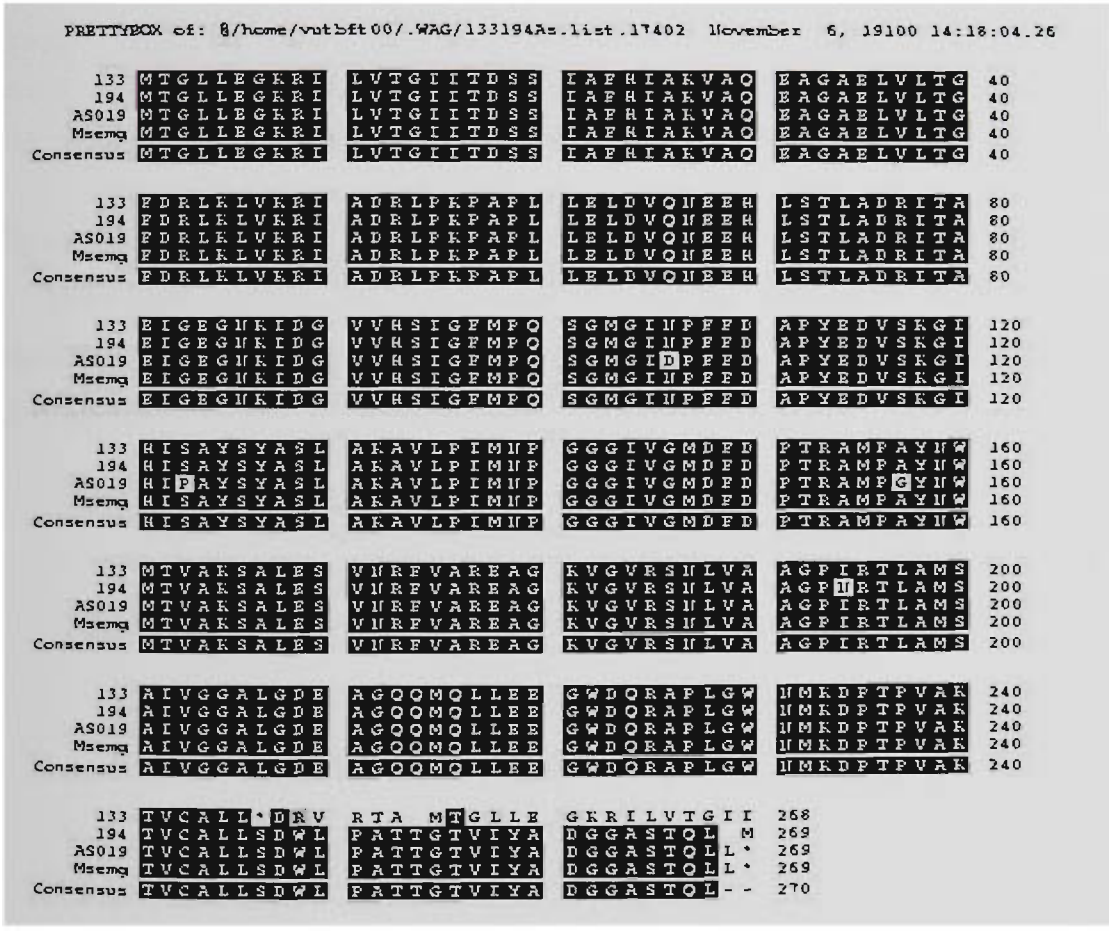
5.6.3 Sequence comparision of InhA proteins of AS019, MLB133 and MLB194

The DNA sequences of the three *inhA* genes from *C. glutamicum* wild type strain AS019 and two mutants MLB133 and MLB194 were translated into putative sequences of their respective InhA proteins using the Etranslation program. InhA proteins of these three strains were further aligned using the PRETTYBOX program. Figure 5.17 shows the extent of identity of these InhA proteins from three strains of corynebacteria with the same protein from *M. smegmatis*.

The results showed that the three proteins aligned almost perfectly as was obvious from the sequencing data. The InhA protein is highly conserved among the different *C. glutamicum* strains: AS019, MLB133 and MLB194, had >95% identity with *M. smegmatis* InhA. The position where the variation in the protein sequence occurred is indicated in the white boxes (Fig. 5.16). The structure of the InhA protein had also been determined in *M. smegmatis* using X- ray chrystallography. Based on the similarity seen between this and corynebacterial proteins it is possible to predict that the InhA protein of *C. glutamicum* might have a similar structure.

A putative binding site in InhA for nicotinamide or flavin nucleotide is shown in Figure 5.17). The points of difference are indicated with red letters. Variations in the amino acids sequence occurred at position 106, 123, 157 in AS019 relative to *M. smegmatis* MLB133 and MLB194, whereas the two sequences MLB194 and MLB133 vary at position 194.

Figure 5.16 Alignment of the InhA protein sequence of *C. glutamicum* and *M. smegmatis* using the PRETTYBOX program of ANGIS. Identical amino acids are shown in black boxes and similar amino acids in grey boxes light or deep grey according to the similarity. Gaps are introduced to maximise similarity scores. The protein sequences shown are the following: InhA of the *C. glutamicum* strain As019, MLB133, MLB194 and *M. smegmatis*.



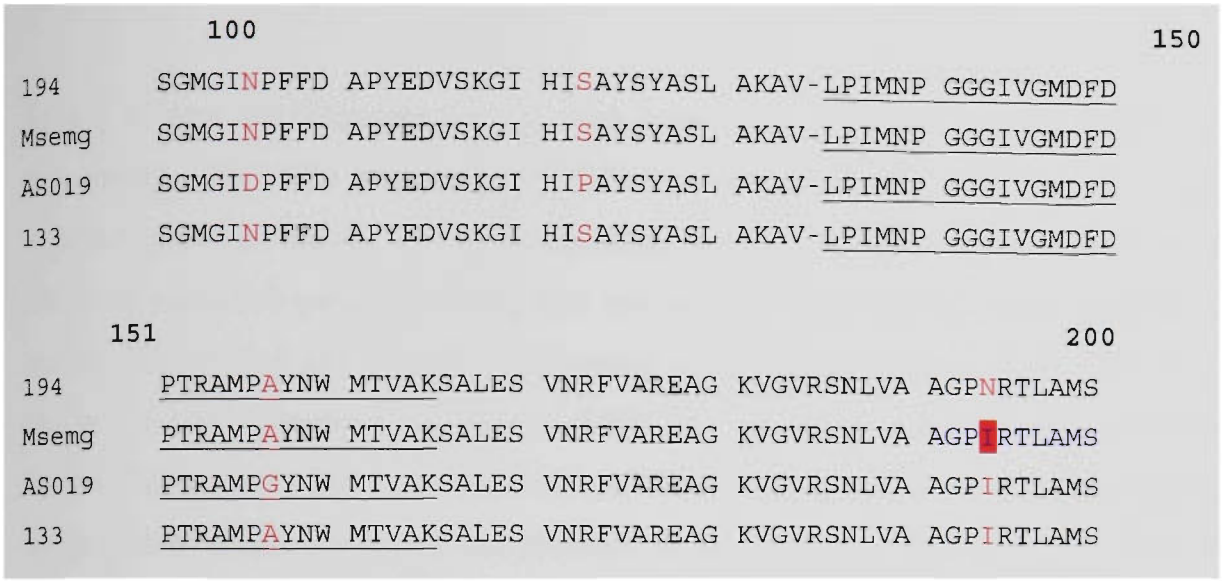


Figure 5.17 Alignment of amino acid sequences of InhA proteins of the three strains of *C. glutamicum* (AS019, MLB194, MLB133) and *M. smegmatis* showing a putative binding site in InhA for nicotinamide or flavin nucleotide. Red letters indicates the points of variations.

5.7 GENERAL DISCUSSION

In this chapter the sequence of the *inhA* gene from MLB133 and MLB194 was determined. The *inhA* sequence of MLB194 was determined following the same approach used for AS019. The *inhA* sequences were amplified from genomic DNA of MLB194 and a sub-genomic library was constructed. The identity of the sequences was further verified after being amplification and sequencing from clone C22. The identity scores calculated using the BEST/FIT program revealed 98% identity between *inhA* from AS019 and MLB194 and 99% with *M. smegmatis*. The Southern hybridisation results confirmed the presence of the *inhA* gene and the appearance of more than one signal confirmed the existence of multiple copies of the gene in the genome of MLB194.

The approach used to analyse the *inhA* gene homologue in MLB133 was limited to only PCR amplification directly from the genomic DNA and sequencing of the amplified fragments. Since the conditions were already optimised for AS019, PCR fragments of expected sizes were obtained without any difficulty. The sequence of the *inhA* gene for this strain was 96% complete and the last 40 bases need to be sequenced to achieve the total length of 850 bases. The sequence data obtained through the PCR fragment produced using the CGP3-INH2 primer set was not reliable and therefore, to determine the sequence this PCR fragment unambiguously the fragment was also cloned into the PCR2.1 vector. The readout obtained using T3 and M13 primers was acceptable but the sequence of last 40 bases could not be achieved. DNA-DNA hybridisation profiles strongly suggest that gene is highly conserved in the parent type strains and mutants, and multiple copies of the gene are present in the genome. The comparative analysis of AS019 and the two mutants showed 85-96% homology among themselves and greater than 95% with *M. smegmatis*. However, the sequence alignment showed some differences in the sequences for AS019, MLB133 and MLB194 at various points.

The deduced InhA protein product of the *inhA* gene of AS019, MLB133, MLB194 is an Enoyl-ACP, an enzyme essential for mycolic acid biosynthesis, which shows

significant homologies with other enzymes involved in bacterial (mycobacteria) and plant fatty acid biosynthesis. Besides significant homologies with the *Mycobacterium* family (84 to 98%) InhA protein, the protein in of *C. glutamicum* also show some identity with EnvM from *H. influenza*, *S. typhimurium* and *E. coli*, and with Enoyl-ACP of *H. pylori* and *B. napus*.

The alignment of the two proteins from *C. glutamicum* and *M. smegmatis* indicates amino acid variations at four different positions: aspartic acid at position 106 in AS019 is hydrophobic instead of asparagine, which is neutral; residue 123 is proline while in *M. smegmatis* this is serine. Proline is often used to produce a bend in the protein chain. Residue 157 in the putative NADH binding site is glycine instead of alanine. This suggests that the three dimensional structure of the two InhA proteins (AS019 and *M. smegmatis*) might be different. Our results (see chapter 3 Table 3.1 & 3.2) showed the two mutants of *C. glutamicum* MLB194, and MLB133 are more sensitive to inhibition by INH and ETH than their parent strain AS019, suggesting that structural changes in these mutants may be associated with the target of INH activity. Subsequent studies on sequence analysis of the putative translation product revealed that the amino acids substitution between AS019 and mutants have occurred at the same points as in AS019 and *M. smegmatis*. Residues 106, 123, 157 are replaced by asparagine, serine and alanine in two mutants. There is single amino acid substitution between MLB194 and *M. smegmatis* and also between MLB194 and MLB133 isoleucine 194 is replaced by asparagine in MLB194 which is a neutral amino acid but because of its polarity it is often classified as external whereas isoleucine is uncharged and therefore constitutes internal side chain. These amino acids mutations lie in the putative NADH binding site. In other words MLB194 and MLB133 are more similar to *M. smegmatis* than its parent AS019, the only difference between MLB194 and *M. smegmatis* is at position 194.

Several studies showed that about 25% of the clinical isolate of *M. tuberculosis* contains mutations within the promoter or structural regions of the *inhA* gene, and no *inhA* mutations have been identified in isoniazid sensitive isolates (Telenti *et al.*, 1997; Musser *et al.*, 1996; Rouse *et al.*, 1995; Kapur *et al.*, 1995). Four different clinical isolates have been identified where mutation have occurred due to single amino acid substitution within or near the NADH binding region of InhA. In mycobacteria the location of S94A mutation is also within the NADH binding region.

Studies of Dessen *et al.* (1995) showed that the S94A mutant of mycobacteria displays reduced hydrogen bonding pattern between NADH and the enzyme. Similarly, Quemard *et al.*, 1996 have also shown that isoniazid-dependent inhibition of wild-type InhA requires the presence of NADH, and inhibition of the S94A mutant occurs only when the concentration of NADH is increased. Furthermore, acyl-ACP substrates can prevent isoniazid-dependent inhibition of InhA, suggesting that activated isoniazid interacts with the substrate-binding region of InhA.

Data from X-ray crystallography and mass spectrometry also reveal that mechanism of isoniazid action against InhA is covalent attachment of the activated form of the drug to the nicotinamide ring of nicotinamide adenine dinucleotide bound within the active site of InhA (Rozwarski *et al.*, 1998). There are two possible scenarios for the formation of isonicotinic acyl-NADH inhibitor of InhA as shown in (Figure 5.18), in which an activated form of isoniazid (isonicotinic acyl anion or radical) covalently attaches to a form of NADH (NAD^+ or NAD radical) within the active site of InhA, while retaining a tetrahedral carbon at position four of the the nicotinamide ring. The free radical pathway is more likely as isoniazid dependant inhibition of InhA occurs at a faster rate with NADH than with NAD^+ (Johnson *et al.*, 1995; Basso *et al.*, 1996).

As reported in Chapter 3, both glycine and INH inhibit not only mycolic acid synthesis but also fatty acids biosynthesis. The presence of an *inhA* gene homology in *C. glutamicum* and its analogy with the mycobacterial *inhA* gene strengthen the hypothesis that InhA might be the target for INH in corynebacteria. Furthermore, the fact that extracellular mycolic acid level increases following growth of *C. glutamicum* in INH suggest that drug might disrupt the attachment of mycolic acid from cell surface. This also suggests that beside its involvement in mycolic acid biosynthesis InhA is playing slightly different role in *Corynebacterium*. However, this hypothesis is controversial, because of the observed insensitivity of corynebacteria to INH as compare to mycobacteria (MIC 14-16 mg/ml).

It is likely that InhA protein of *C. glutamicum* may use nicotinamide or flavin nucleotide as substrate, as it has a putative binding site for these molecules (Figure 5.17).

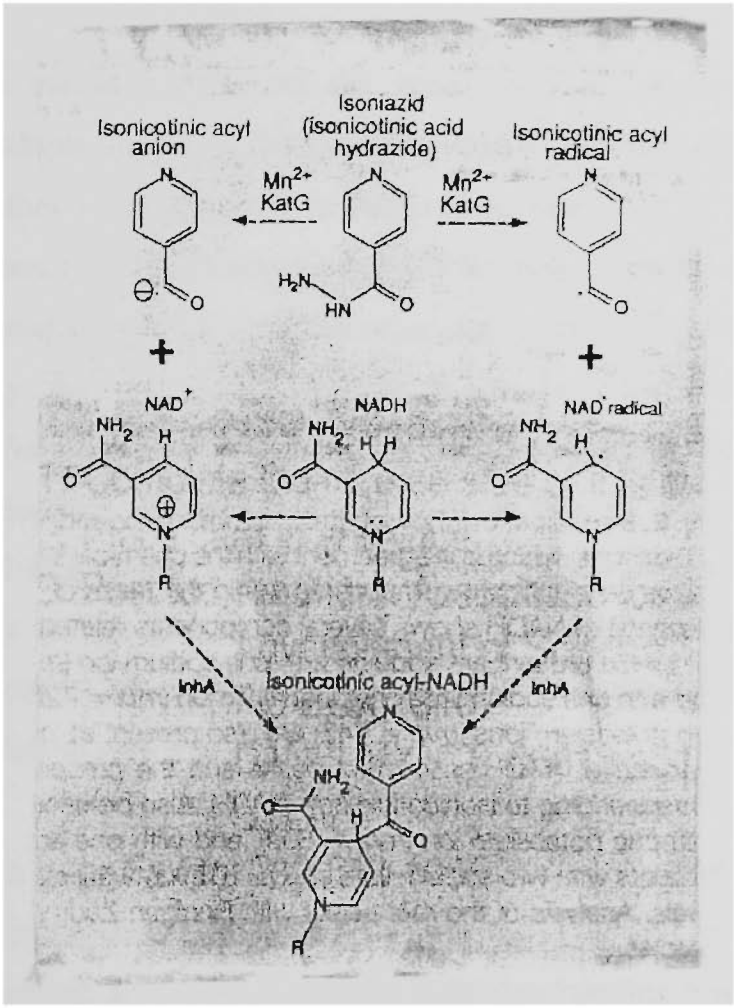


Figure 5.18 Proposed pathway for the formation of isonicotinic acyl-NADH inhibitor of InhA.

Since the free radical pathway have been much favoured (Rozwarski *et al.*, 1998) because the isoniazid dependant inhibition of InhA occurs at faster rate with NADH than with NAD^+ (Figure 5.18), it is possible that structure of InhA in *C. glutamicum* might be such to allow decrease in molecular contact between isonicotinic acyl-NADH and the active site of InhA and therefore higher concentrations of INH are required to produce a significant change in mycolic acid composition.

In corynebacteria multiple copies of the gene has been observed by Southern hybridisation. Multiple copies of the gene may lead to the over expression of InhA protein and therefore more substrate is required to inactivate or inhibit the InhA protein. The existence of more than one copy of the gene might be an explanation of a naturally high level of resistance of this organism against isoniazid, and this might also explain why the significant changes in the mycolic acids composition, transformation efficiency appear when higher concentrations of the drug are added into the growth medium. In addition to the genetic data, the fact that extracellular mycolic acid level increases following growth of *C. glutamicum* in INH suggest that drug might disrupt the attachment of mycolic acids from the cell surface. This also suggests that besides its involvement in mycolic acids biosynthesis InhA is playing a slightly different role in corynebacteria.

Molecular contacts between isonicotinic acyl-NADH and the active site of InhA has also been studied in mycobacteria (Figure 5.19) it show very clearly that the nitrogen atom of the isonicotinic acyl group is within hydrogen-bonding distance of a buried water molecule held by the side chain of Met155. The putative translation product of the *inhA* gene of *C. glutamicum* strains MLB133 and MLB194 (Figure 5.16) are much more similar to *M. smegmatis* as compared to AS019. There is a single amino acid difference or substitution between *M. smegmatis* and MLB194 as compared to AS019. This is not surprising as our biochemical data shows that the two mutants (particularly MLB133) are more sensitive to INH inhibition while the parent strain AS019 is more resistant to INH.

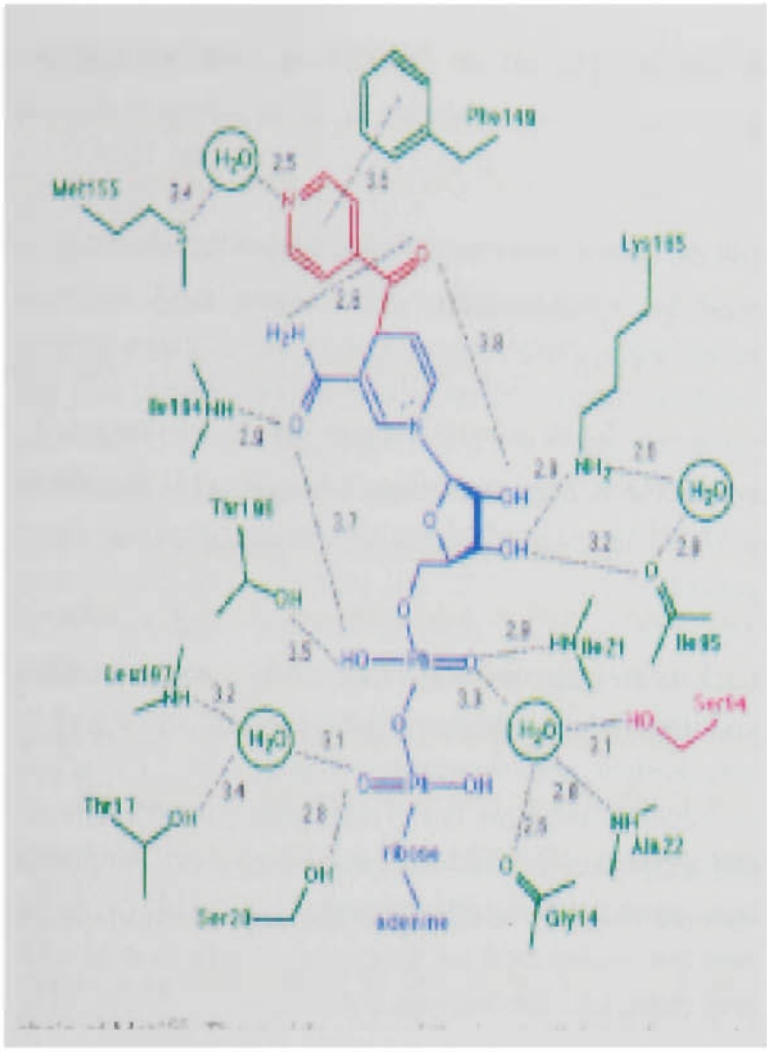


Figure 5.19 Molecular contact between isonicotinict acyl-NADH and the active site of InhA. The isonicotinic acyl group derived from isoniazid is red, the NADH portion of the analog is blue, the side chains of InhA are green, and Ser94, the residue that causes isoniazid resistance when converted into Ala is magenta. Numbers represent the distance in angstroms between selected atoms. The orientation of the isonicotinic acyl group with respect to the NADH portion of inhibitor is such that its carbonyl oxygen is positioned about halfway between two hydrogen donors, the amide nitrogen of the nicotinamide ring, and the 2-hydroxyl oxygen of the nicotinamide ribose ring. In addition, the nitrogen atom of isonicotinic acyl group is within hydrogen-bonding distance of a buried water molecule held by the side chain of Met^{155}, The pyridine ring of the isonicotinic acyl group is surrounded by hydrophobic residues, which include Phe^{149}, Gly^{192}, Pro^{193}, Leu^{218}, Tyr^{158} and Trp^{222}.

In *Mycobacterium*, studies were performed on the crystal structure of isoniazid-inhibited InhA, these studies provide an explanation for the exquisite specificity of activated isoniazid for InhA. Although KatG is not the activator of ethionamide (Basso *et al.*, 1996), ethionamide also requires activation and, by analogy to isoniazid it is proposed that activated ethionamide inhibits InhA by becoming covalently attached to position four of the nicotinamide ring of NADH by a 2-ethyl isonicotinic thioacyl group. Comparison of the crystal structures of InhA with bound NADH (Dessen *et al.*, 1995) and with bound isonicotinic acyl-NADH reveals that the only significant difference in the protein is location of the side chain of Phe^{149}. When isonicotinic acyl-NADH is bound, the side chain of Phe^{149} has rotated ~90° and forms an aromatic ring-stacking interaction with pyridine ring of isonicotinic acyl group. The authors proposed that such structural arrangement would increase the affinity over NADH alone. Similarly, mutations with decreased affinity for NADH are likely to decrease the affinity for isonicotinic acyl-NADH. By analogy with *M. smegmatis* it can be presumed that similar reaction takes place between isoniazid and active side of InhA. Since counter studies related to the comparative crystal structure of InhA with bound NADH and with bound isonicotinic acyl-NADH are lacking it is difficult to draw any conclusion at this stage. However, it is likely that although the coryne-InhA protein show 98 % sequence homology with mycobacteria but the structure InhA would be such to decrease the affinity for NADH.

Furthermore, kinetic isotope analysis of InhA has demonstrated that the binding sequence of NADH and long-chain acyl-ACP substrates is not strictly ordered, but there is a preference for the NADH binding first (Quemard *et al.*, 1995). This preference would leave most of the wild-type enzyme in the NADH-bound form, available for attack by activated isoniazid. If the wild-type InhA cannot release significant amount of isonicotinic acyl-NADH, this will effectively create a permanent inhibition of the enzyme and prevent mycolic acid biosynthesis. In contrast, the decreased affinity of the S94A mutant for NADH would promote acyl-ACP substrates binding before NADH, thereby protecting most of the enzyme from activated isoniazid. When the isonicotinic acyl-NADH is formed on the mutant enzyme, the lowered affinity for NADH promotes release of isonicotinic acyl-NADH,

allowing normal substrate catalysis to proceed and resulting in isoniazid-resistant tuberculosis.

InhA from *C. glutamicum* also displays 38% amino acid identity with enoyl-acyl carrier protein (ACP) reductase of *B. napus* and 54% identity with enoyl-reductase (ENR) of *E. coli* (NCBI data base similarity search). The structure of the *E. coli* enzyme show resemblance to its mycobacterial counterpart (40% sequence identity), particularly in the region of the active site, and also to that of *B. napus* ENR (33% sequence identity). Examination of the structures of the *E. coli* and *M. tuberculosis* ENRs showed that respective Gly^{93}→Ser (G93S) and Ser^{94}→Ala mutations, which lead to diazaborine (Bergler *et al.*, 1992) and isoniazid (Banerjee *et al.*, 1994) resistance, map to the region close to the nucleotide-binding site.

In addition to the difference in the InhA proteins and existence of multiple copies of gene it is likely that in *C. glutamicum* resistance to INH may arise from other sources. For example lack of a *katG* gene or inactivation of this resulting in no or low activity may be an alternative explanation. The observed hypersensitivity of the two mutants relative to their parent strain could be explained by the differences in the two InhA proteins relative to AS019 making these more similar to the mycobacterial InhA protein. If we assume that the mechanism of action of INH is similar to that proposed for mycobacteria, it is possible that in the mutants InhA cannot release significant amounts of isonicotinic acyl-NADH, which will create a permanent inhibition of the enzyme and prevent mycolic acid biosynthesis, however, this hypothesis needs to be explored further experimentally. Since other studies have shown that the target of the InhA protein is short-chain C_{16} fatty acids it is also possible that elongation or condensation of C_{16} and C_{18} fatty acids to their counterpart corynemycolates is impaired, giving rise to decreased synthesis of $C_{32:0}$ and accumulation of these fatty acids normally during growth in the presence of INH. Moreover due to difference in the peptide sequences of InhA proteins in the two species the overall structure will be different and mechanism of action is different from that proposed in the *Mycobacterium tuberculosis*.

The mechanism of drug resistance has been studied in detail in *Mycobacterium* where it is assumed that resistance to INH is associated with a variety of mutations affecting one or more genes such as those encoding catalase-peroxidase (*katG*) (Zhang *et al.*, 1992), the enoyl-acyl carrier protein reductase involved in mycolic acid biosynthesis (*inhA*) (Banerjee *et al.*, 1994), and the recently described alkyl-hydroperoxide reductase (*ahpC*), which is involved in the cellular response to oxidative stress (Deretic *et al* ., 1995; Wilson *et al.*, 1996; Sherman *et al.*, 1995). However, the most recent study on the crystal structure of complex between isonicotinic acyl-NADH and InhA provides the basis for the design of agents that inhibits InhA without the involvement of KatG activation. The pathway of mycolic acid biosynthesis is essential to corynebacteria, therefore the detail study of InhA is very crucial. Therefore, cost and time efficient investigation of INH resistance in *Corynebacterium* will require a strategy for targeted mutation analysis, which is beyond the scope of this thesis.

Chapter 6

Sequencing and sequence analysis of the *inhA* gene in *Brevibacterium* species and comparison with *C. glutamicum*.

6.1 INTRODUCTION

Previous studies have shown that *C. glutamicum* is much more difficult to protoplast and transform than *B. lactofermentum* and *B. flavum* and subsequently most workers have concentrated their effort on the latter species in the saprophytic corynebacteria group (Liebl and Sinskey *et al.*, 1987). Studies of Britz and colleagues (1989) with different *Corynebacterium* species using cell wall modifier (Tween 80, INH and glycine) in growth medium showed that Tween 80 had significant effects on the electroporation frequency of *B. lactofermentum* in particular. *B. lactofermentum* based on 16S rRNA sequence analysis, has been transferred to *Corynebacterium* species and a new name *C. lactofermentum* has been proposed for this species. Similarly INH also had a significant effect on the electrotransformation frequency, which increased 10-100 fold for some strains. These reports, however, showed that *B. lactofermentum* is much more sensitive than *C. glutamicum* to growth inhibition by INH and that growth in the presence of INH did not significantly improve electrotransformation frequency of *B. lactofermentum*. Studies of Jang *et al.* (1997) as well as the present studies on the effect of INH on the growth of different corynebacterial species further showed that *B. lactofermentum* behaves differently to INH-containing media compared with *C. glutamicum* and *B. flavum*. It is not clear why *B. lactofermentum* is more sensitive to INH than *C. glutamicum* but this may suggest a significant difference in cell wall composition of these species. Studies on the mycolic acid composition showed that both *Brevibacterium* strains, BF4 and BL1 showed similar patterns of mycolic acids to each other in terms of both quantitative and qualitative composition. It is for this reason that the present study focussed on exploring the *inhA* genes involved in mycolic acid biosynthesis of these strains, particularly the gene dose of this gene.

In this chapter we report the presence of an *inhA* gene equivalent in these two species, *B. lactofermentum* BL1 and *B. flavum* BF4, plus the cloning and sequencing of this gene. This chapter also focussed on the comparative analysis of amino acid sequences of the InhA proteins of *C. glutamicum* and the two *Brevibacterium* species BL1 and BF4 in order to determine wether a relationship exists between the ease of protoplasting and changes in the cell surface chemistry of these three corynebacterial species at a genetic level. The deduced biochemical and physical properties of three InhA proteins, the molecular basis of physiological differences observed and differences in the predicted secondary structures of these proteins are analysed and discussed.

6.2 CLONING AND SEQUENCING OF THE *inhA* GENE IN *B. LACTOFERMENTUM* STRAIN BL1 BY PCR

The strategy used to sequence the *inhA* gene from BL1 was based primarily on PCR amplification directly from genomic DNA. Since the PCR conditions were optimized and gene was sequenced previously in three strains of *C. glutamicum*, there was no need of constructing a separate genomic library.

6.2.1 PCR amplification of the *inhA* gene in *B. lactofermentum*

PCR amplified fragments from BL1 are shown in Figures 6.1 and 6.2. PCR fragments were amplified from the genomic DNA of BL1 using primers sets mentioned in Table 4.1 under similar PCR conditions used to amplify the *inhA* gene from three corynebacterial strains (Table 4.2). The *inhA* gene from BL1 was amplified as four overlapping fragments and AS019 genomic DNA was used as a control in this experiment to confirm the sizes of the bands. The sizes of the PCR fragments obtained from BL1 using the primers sets INH1-CGP4, CGP5-CGP2, CGP3-CGP4, CGP3-INH2 were similar to those expected for each primer set. To further confirm the identity of the *inhA* gene in *Brevibacterium*, Southern hybridisation was performed using the *inhA* probe from AS019. The same strategy was used to sequence the *inhA* gene from *Brevibacterium* as was used for *C. glutamicum* strain MLB133. Sequence assembly of this is reported in section 6.2.2.1.

6.2.2 Southern hybridisation and identification of *inhA* gene in *B. lactofermentum*.

The PCR amplified products of CGP3-INH2 primers set from *C. glutamicum* strain AS019 were radiolabelled and probed against the genomic DNA digest of BL1. Figure 6.3 shows the results of Southern hybridisation of the genomic DNA digest of BL1 with various restriction enzymes. Table 6.1 summarizes the calculated molecular weight of the different hybridization bands obtained. The hybridisation pattern obtained for BL1 was quite different from that seen for AS019, however, the observation of the occurrence of multiple copies of the gene was common in the two *Brevibacterium* species. For example, with *Hind*III digestion, the probe showed one strong signal of 8.5 kb and other feint signals were also observed. With *Eco*R1 digestion, two major and other minor signals were seen. The minor signal could have arisen due to non-specific binding of the probe. With *Pst*1 digestion, of BL1 apart from the three main signals, the probe also showed very strong binding with two very small molecular weight (250, 100 bp) fragments (as seen for AS019) of unknown origin. The identity of these fragments is not known: they could have originated due to the non-specific binding of the probe with BSA which was added in the digestion mixture, as *Pst*1 was shown not to cut in the *inhA* sequence (see Figure 6.6b). The hybridisation experiment confirmed the presence of the *inhA* gene in BL1, which was further confirmed by cloning and sequencing of the PCR fragments.

6.2.3 Sequencing and cloning of the PCR amplified *inhA* gene fragments from BL1.

PCR fragments obtained from the BL1 genomic DNA were WIZ purified. Each of the PCR fragments was used as template in automated DNA sequencing, using the relevant PCR primers as sequencing primers. Sequencing data for each of these PCR amplified fragments was analysed using BLASTN, FASTA and BLASTX homology search programs *via* NCBI. The data showed highest similarity with mycobacterial proteins and confirmed the presence of the *inhA* gene in *B. lactofermentum*.

To unambiguously determine the sequence and to get more sequence data at the end, the longer PCR fragment obtained using the INH1-CGP4 primer set was directly sequenced with the respective primer and cloned into PCR 2.1 vector. The *inhA* positive clones were screened by PCR amplification using specific primers (INH1-CGP4) and the presence of insert was further confirmed by *Eco*R1 restriction digestion. The cloned fragments were sequenced using the T7 and M13 primers. Figure 6.4 a and b respectively showed the PCR and *Eco*R1 restriction digestion pattern of selected *inhA* positive PCR 2.1 clones.

6.2.4 Sequence assembly for BL1

Sequences data obtaining for the four PCR fragments from BL1 (as shown in Figures 6.1 and 6.2) were used to build one continuous *inhA* gene sequence. All sequences were analysed for their overlap portions and then assembled manually as well as using the GCG program of the WEB ANGIS. Figure 6.5 summarises the entire sequence strategy. Complete sequence of the *inhA* gene of BL1 from position 1-850 was obtained after sequence assembly (Figure 6.6). The nucleotide sequence of the *inhA* gene of BL1 was deposited in the GenBank.

6.2.5 Sequence analysis of assembled *inhA* gene for BL1

The entire *inhA* gene sequence of BL1 was analysed for the extent of identity with mycobacterial genes and other genes in the databases using BLASTN and FASTA programs. The BLASTN, BLASTX and FASTA search results of the completed *inhA* gene for BL1 are summarised in Tables 6.2, 6.3 and 6.4. The highest homology score was found to be with *inhA* gene of corynebacterial (95%) and mycobacterial species (80-95%). Less homology score was recorded with other proteins for example 41% identity with envM proteins of *S. typhimurium* and *E. coli* short chain alcohol dehydrogenase. The nucleotide sequence of *inhA* gene was translated using Etranslate program of the WEBANGIS. Table 6.3 summarises the results of BLASTX homology search for InhA deduced protein of BL1. Amino acids comparison and calculated similarity scores of InhA deduced protein for strain BL1 and *M. smegmatis* and BL1 *vs* AS019 using the GCG/BESTFIT and GCG/GAP program was 92.9%.

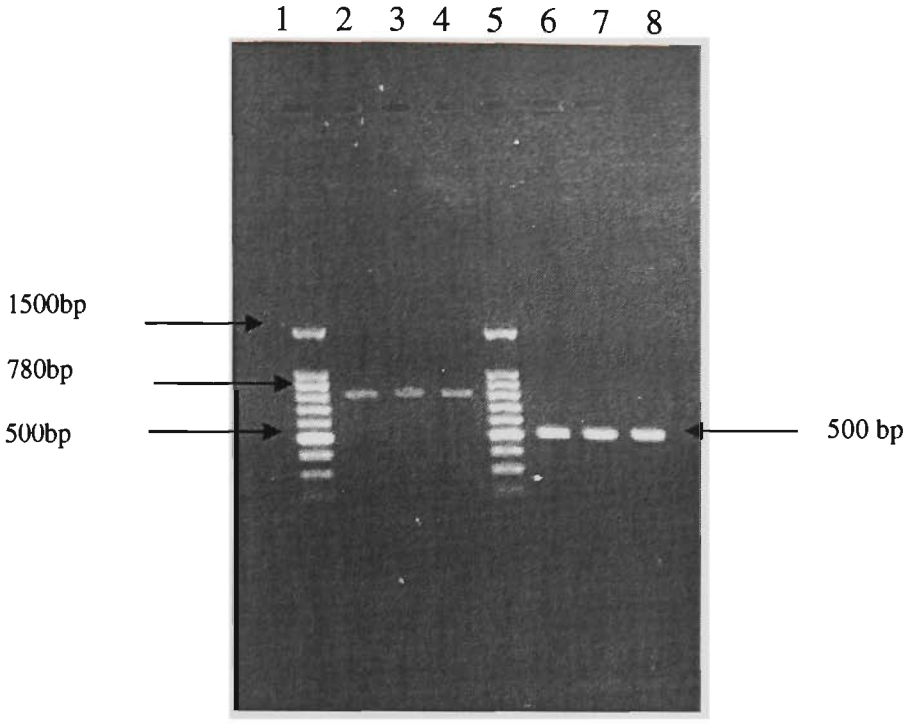


Figure 6.1 PCR amplification of the *inhA* gene from genomic DNA of *Brevibacterium* and *Corynebacterium* species. Lanes 1 and 5 contained the 100 bp DNA ladder; Lanes 2 to 4 contained *inhA* gene fragment obtained using the INH1-CGP4 primer set with templetes from AS019, BL1 and BF4; Lanes 6 to 8 contained, *inhA* gene fragment using CGP5-CGP2 primer set from AS019, BL1 and BF4.

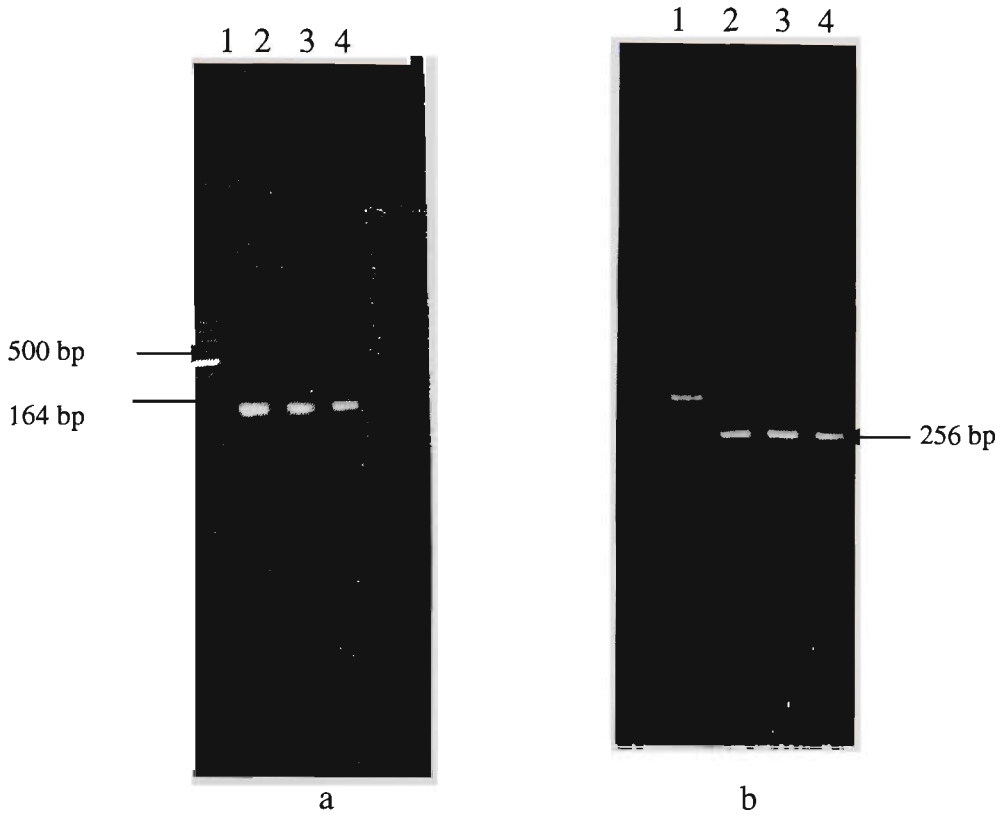


Figure 6.2 PCR amplification of the *inhA* gene from genomic DNA of *Brevibacterium* and *Corynebacterium* species.

a PCR amplification using the primer sets CGP3-CGP4. Lane 1 contained the 100 bp DNA ladder; Lanes 2 to 4 contained AS019, BL1 and BF4.

b PCR amplification of *inhA* gene using the primers set CGP3-INH2. Lane 1 contained the 100 bp DNA ladder; Lanes 2 to 4 contained AS019, BL1 and BF4.

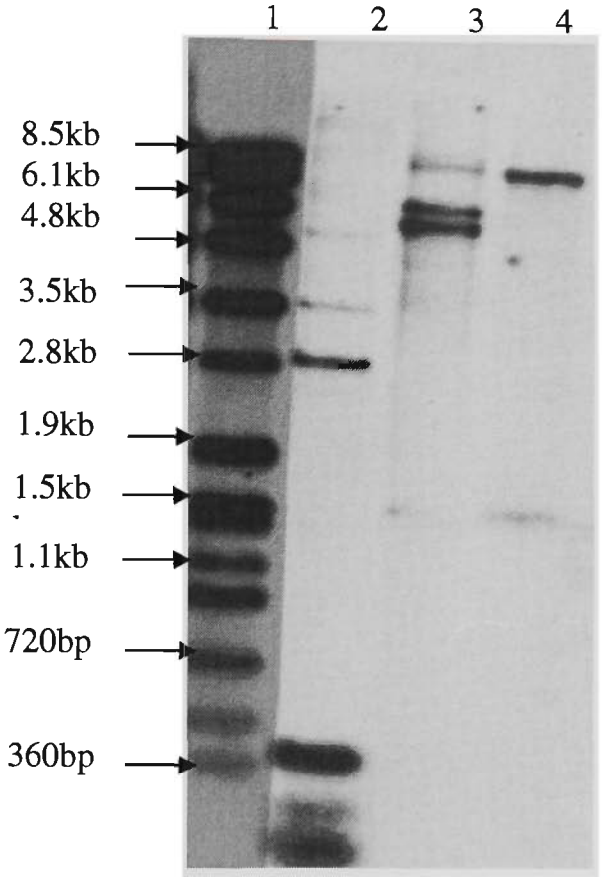


Figure 6.3 Southern blot hybridisation of genomic DNA restriction digests of *B. lactofermentum* probed with a 250 bp PCR product obtained using primer set CGP3-INH2 and labelled using ^{32}P. Lane 1, SPP1/*Eco*R1 size markers; Lanes 2 to 4 contained *Pst*1, *Eco*R1 and *Hind*III digested genomic DNA of *B. lactofermentum* strain BL1.

Table 6.1 Molecular weights of the hybridisation fragments obtained when the *inhA* gene probe was used against the genomic DNA from *B. lactofermentum* strain BL1 with various enzymes.

	MW in (kb) of the bands detected		
	***Pst*1**	***Eco*R1**	***Hind*III**
SPP1/*Eco*R1 markers	5.2	**6.1**	**8.3**
8.5	3.5	**5**	1.5
7.3	**2.8**		
6.1	**0.36**		
4.8	0.25		
3.5	0.1		
2.8			
1.9			
1.8			
1.5			
1.3			

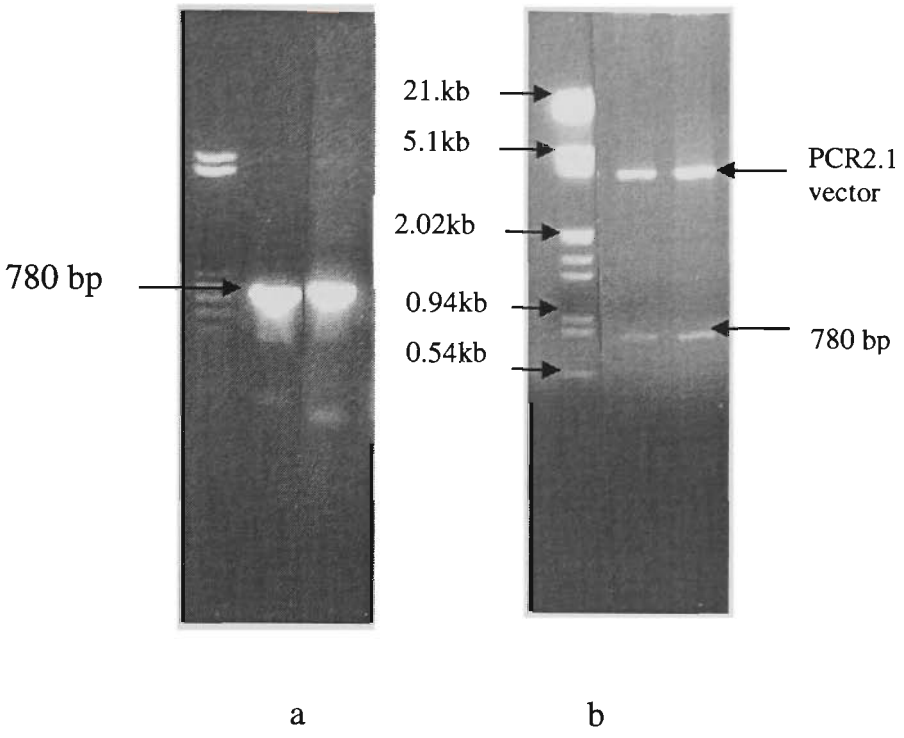


Figure 6.4 PCR amplification and restriction digestion analysis of recombinant PCR 2.1 clones containing PCR amplified *inhA* gene from *B. lactofermentum* and *B. flavum.*

a PCR amplification and of *inhA* gene using INH1-CGP4 primers sets. Lane 1; PCR size markers; Lanes 2 to 3 contained BL1 and BF4 respectively.

b Restriction digestion of clone PCR2.1 with *Eco*R1 restriction enzymes. Lane 1 *Eco*R1/*Hind*III digested Lambda DNA size markers; Lanes 2 to 3 contained BL1, BF4 respectively.

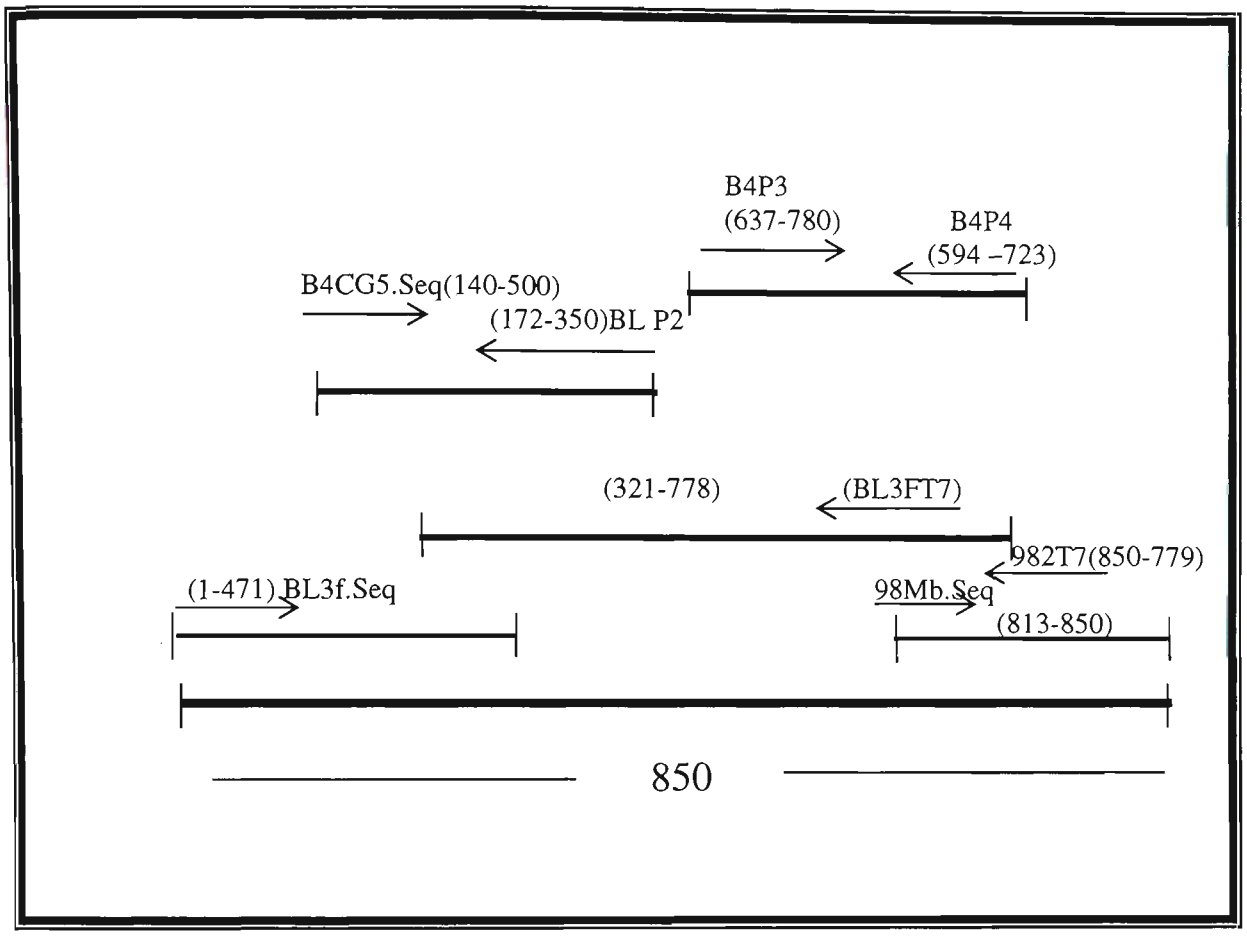
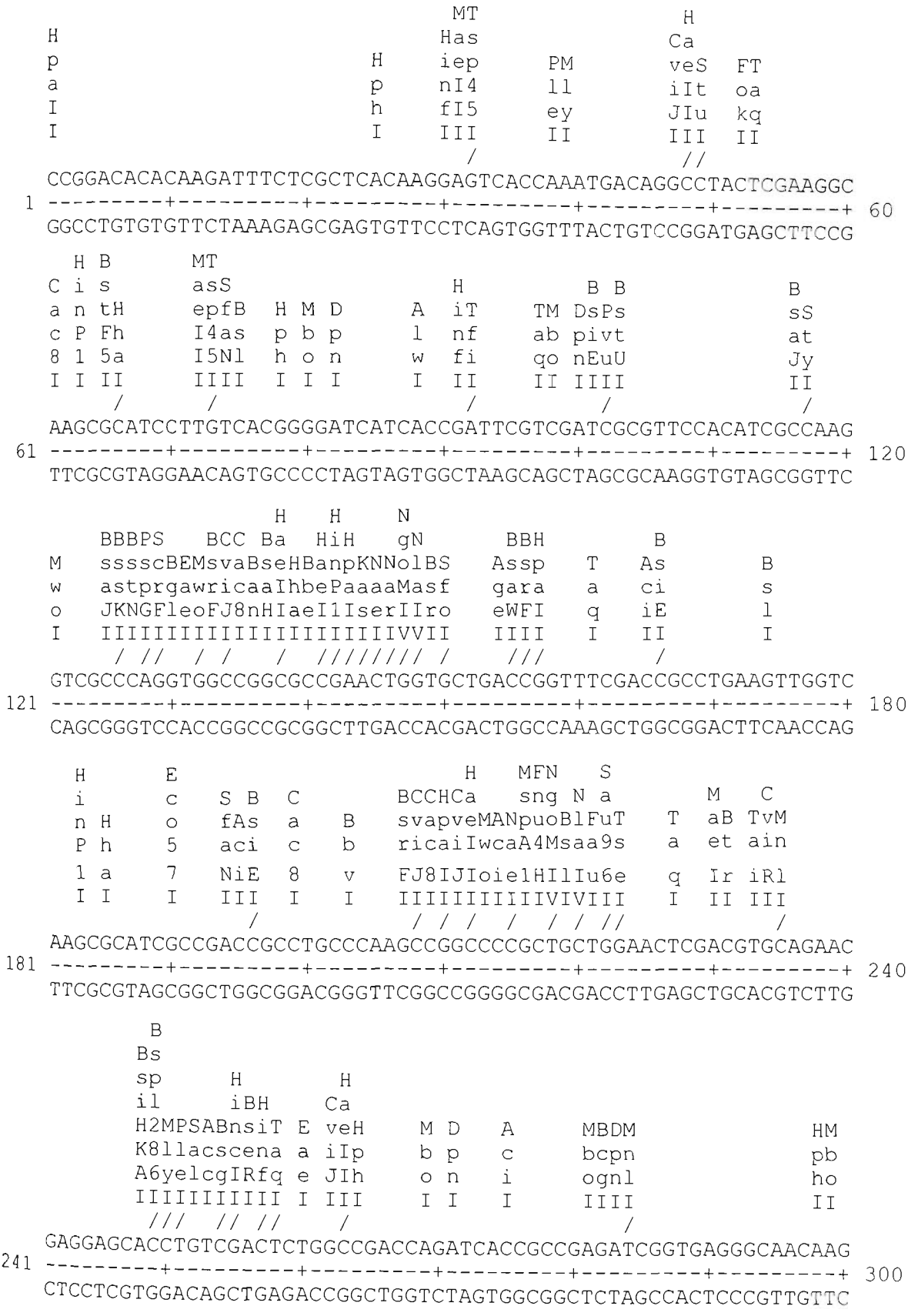


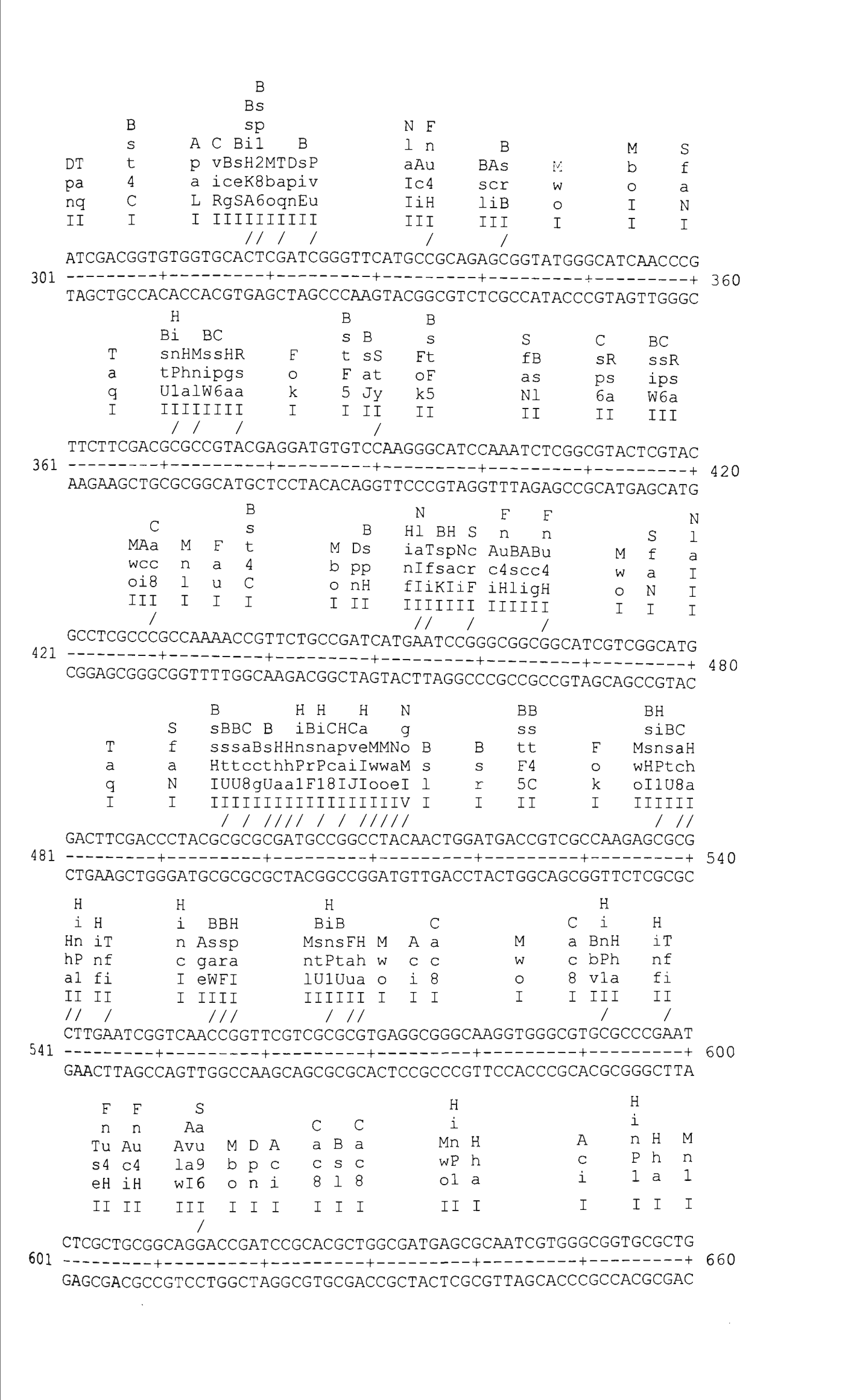
Figure 6.5 Schematic illustration of *inhA* gene sequence from *B. lactofermentum* (BL1).

```
  1  CCGGACACAC AAGATTTCTC GCTCACAAGG AGTCACCAAA TGACAGGCCT
 51  ACTCGAAGGC AAGCGCATCC TTGTCACGGG GATCATCACC GATTCGTCGA
101  TCGCGTTCCA CATCGCCAAG GTCGCCCAGG TGGCCGGCGC CGAACTGGTG
151  CTGACCGGTT TCGACCGCCT GAAGTTGGTC AAGCGCATCG CCGACCGCCT
201  GCCCAAGCCG GCCCCGCTGC TGGAACTCGA CGTGCAGAAC GAGGAGCACC
251  TGTCGACTCT GGCCGACCAG ATCACCGCCG AGATCGGTGA GGGCAACAAG
301  ATCGACGGTG TGGTGCACTC GATCGGGTTC ATGCCGCAGA GCGGTATGGG
351  CATCAACCCG TTCTTCGACG CGCCGTACGA GGATGTGTCC AAGGGCATCC
401  AAATCTCGGC GTACTCGTAC GCCTCGCCCG CCAAAACCGT TCTGCCGATC
451  ATGAATCCGG GCGGCGGCAT CGTCGGCATG GACTTCGACC CTACGCGCGC
501  GATGCCGGCC TACAACTGGA TGACCGTCGC CAAGAGCGCG CTTGAATCGG
551  TCAACCGGTT CGTCGCGCGT GAGGCGGGCA AGGTGGGCGT GCGCCCGAAT
601  CTCGCTGCGG CAGGACCGAT CCGCACGCTG GCGATGAGCG CAATCGTGGG
651  CGGTGCGCTG GGCGACGAGG CCGGCCAGCA GATGCAGCTG CTCGAAGAGG
701  GCTGGGATCA GCGCGCGCCG CTGGGCTGGA ACATGAAGGA CCCGACGCCC
751  GTCGCCAAGA CCGTGTGCGC ACTGCTGTCC GAGTGGGGAC TGCTCGCGGT
801  CGGTCTCAAG ATCTGGGTTC TCGGAAGCGC CAGCACGCAG CTGTTGTGAT
```

Figure 6.6a Nucleotide sequence of *inhA* gene for *B. lactofermentum* strain BL1 based on the designed primers (INH1, INH2, CGP2, CGP3, CGP4, CGP5, and CGP6.) derived from the *M. smegmatis inhA* gene sequence.

Figure 6.6b Linear restriction map of the *inhA* gene of BL1 using MAP program of WEB ANGIS





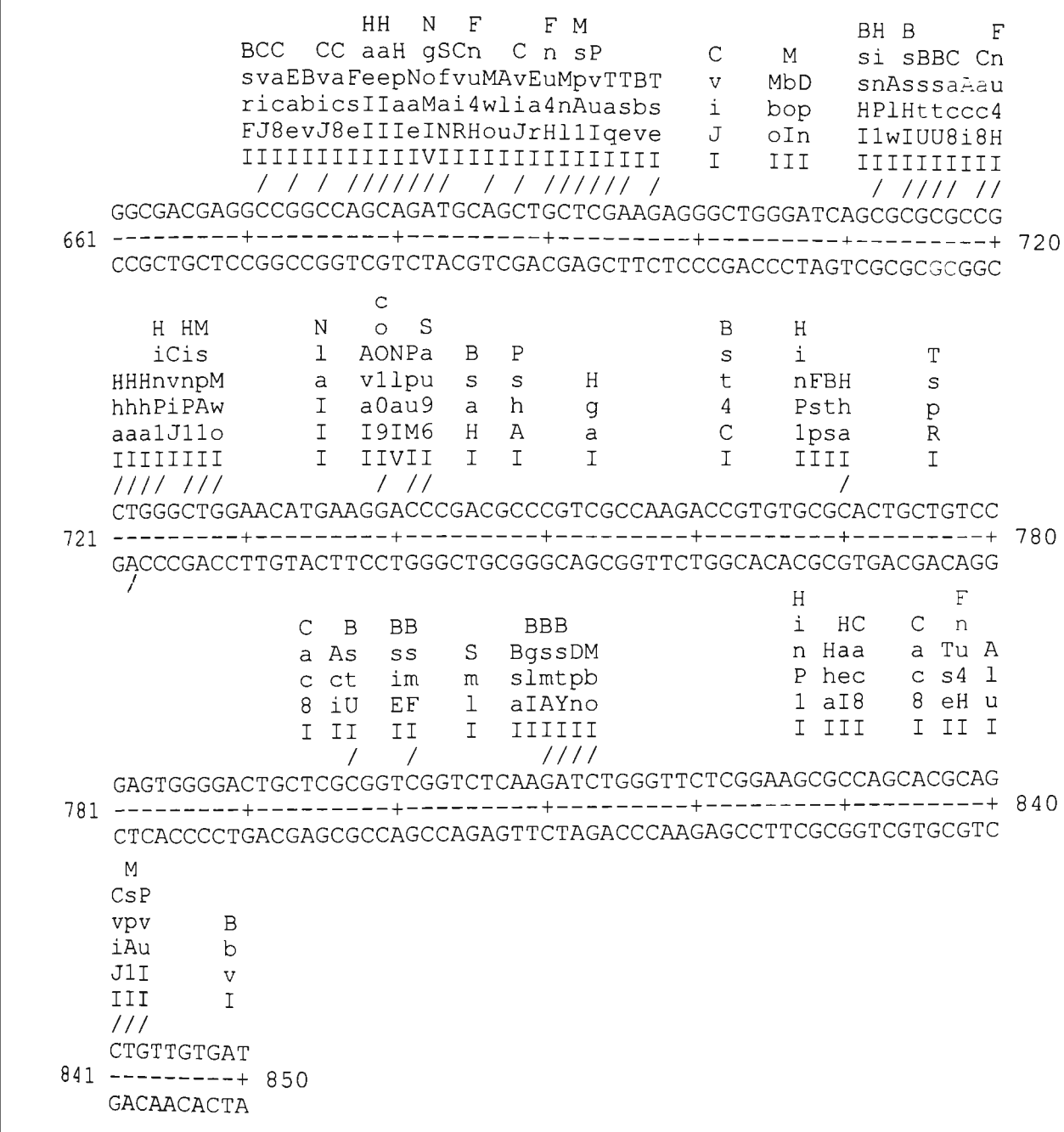


Table 6.2 BLASTN homology search result of *inhA* gene of *B. lactofermentum* strain BL1.

	inhA BL1	*inhA* *M. smegmatis*	*inhA* *M. tuberculosis* *M. bovis*	*inhA* *M. avium* strain GIR10	*fabI* *P. aeroginosa*	*EnvM* *S. typhimurium*	*EnvM* *E. coli*
inhA BL1	100	95	80	78	59	-	-
M. smegmatis		100	83	83	59	56	56
M.tuberculosis			100	85	59	-	-
M. bovis							
M. avium strain GIR10				100	-	-	-
P. aeruginosa					100	65	64
S. typhimurium						100	86
E. coli							100

Table 6.3 BLASTX homology search results of BL1 on NCBI database.

	BL1 NADH-dependant Enoyl-ACP reductase	*M. smegmatis* NADH-dependant Enoyl-ACP	*M. smegmatis* *M. bovis* NADH dependant Enoyl reductase	*M. avium* Enoyl reductase	*P.aeruginosa* Enoyl-ACP reductase	*S.typhimurium* EnvM protein	*E. coli* EnvM	*B. napus* Enoyl-ACP
BL1 NADH-dependant Enoyl-ACP reductase	100	93	81	77	52	54	54	38
M. smegmatis NADH-dependant Enoyl-ACP		100	87	86	54	54	54	34
M. smegmatis *M. bovis* NADH dependant Enoyl reductase			100	87	54	54	54	43
M. avium Enoyl reductase				100	54	56	56	_
P. aeruginosa Enoyl-ACP reductase					100	70	70	43
S. typhimurium						100	97	41
E. coli							100	41
B. napus								100

Table 6.4 FASTA homology search results of *inhA* gene of BL1

Organism	Gene	% homology
M. smegmatis	*inhA* gene	95.88
M. tuberculosis	*inhA* gene	80.53
M. bovis	putative ketoacyl ACP	83
M. tuberculosis mutant	NADH-dehdrogenase	80.64
M. avium strain GIR	transcriptase	80.64
P. aeruginosa	Enoyl-ACP reductase (fabI)	52.30
E. coli	Short chain alcohol dehydrogenase	53.32
E. coli	*envM* gene	53.32

6.3 CLONING AND SEQUENCING OF THE *inhA* GENE IN *B. FLAVUM* STRAIN BF4 BY PCR

6.3.1.1 PCR amplification of *inhA* gene in *B. flavum* BF4

The PCR amplified fragments from BF4 are shown in Figure 6.1 and 6.2. Based on the strategy adopted for strain BL1, PCR products were amplified directly from the genomic DNA of *B. flavum* as four overlapping fragments obtained using the same sets of primers and same PCR profiles as described in Tables 4.1 and 4.2.

6.3.1.2 Southern hybridisation using the *B. flavum* genomic DNA probed with PCR fragments.

Figure 6.7 shows the results of Southern hybridisation of the genomic DNA digestion of BF4 with various restriction enzymes and probed with the 256 bp PCR amplified product of *inhA* gene from AS019. The hybridisation pattern obtained for BF4 was slightly different from BL1. After confirming the presence of *inhA* in BF4, attempts were made to determine the sequence of this gene.

6.3.1.3 Sequencing of the *inhA* gene from BF4

PCR fragments of *inhA* gene amplified from BF4 were sequenced using their relevant primers. Sequencing data of each genomic fragment obtained by PCR amplification was analysed individually using BLASTN, FASTA and BLASTX homology search programs *via* NCBI.

The longer PCR fragments of INH1-CGP4 primers sets were sequenced with their respective primers and further cloned into the *Eco*R1 site of the PCR 2.1 vector to get more reliable sequence data at the end. The putative clones containing the *inhA* fragments were identified by mean of PCR amplification using the specific primers and by *Eco*R1 restriction digestion. Figure 6.4 a and b shows the PCR and *Eco*R1 restriction digestion patterns of *inhA* positive PCR2.1 clones. T7 and M13 primers were used to sequence the cloned fragments.

6.3.1.4 Sequence assembly for BF4

Sequence data of four overlapping PCR fragments obtained from the BF4 *inhA* gene were joined together to obtained one continuous sequence: Figure 6.8 summarises the amplification strategy for *inhA* gene of *B. flavum*. The complete sequence of the *inhA* gene of BF4 from position 1-850 was obtained after sequence assembly (Figure 6.9). The nucleotide sequence and deduced InhA protein from BL1 was analysed for homology through NCBI database. Tables 6.5, 6.6 and 6.7 summarises the results of BLASTN, BLASTX and FASTA homolgy for BF4.

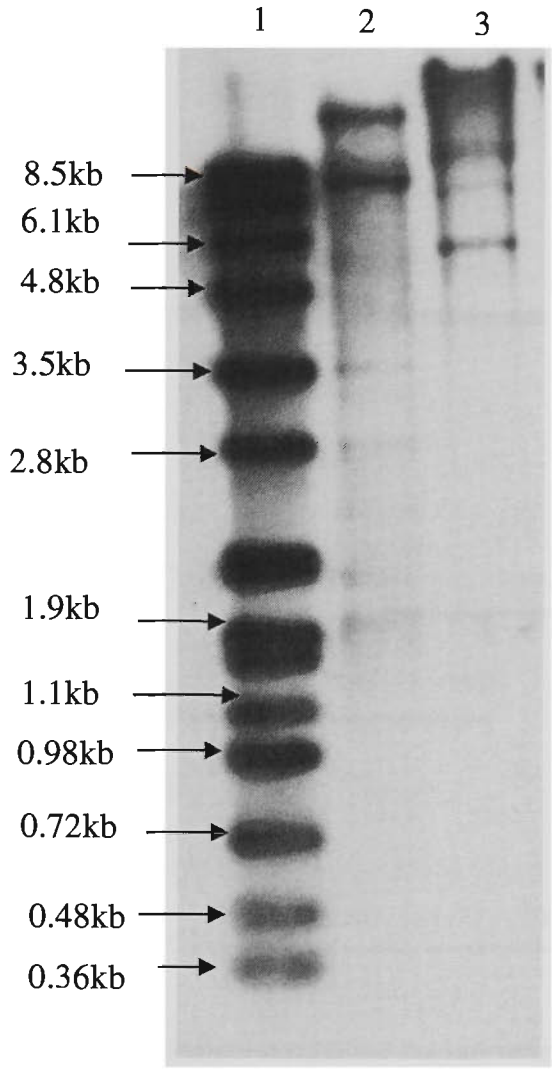


Figure 6.7 Southern blot hybridisation of restriction enzyme digested genomic DNA of *B. flavum* strain BF4 probed with a 250 bp PCR product obtained using primer set CGP3-INH2 from AS019 and labelled using ^{32}P. Lane 1, SPP1/*Eco*R1 size markers; Lane 2 to 3 contained *Hind*III, *Pst*1 digested genomic DNA of *B. flavum* strain BF4.

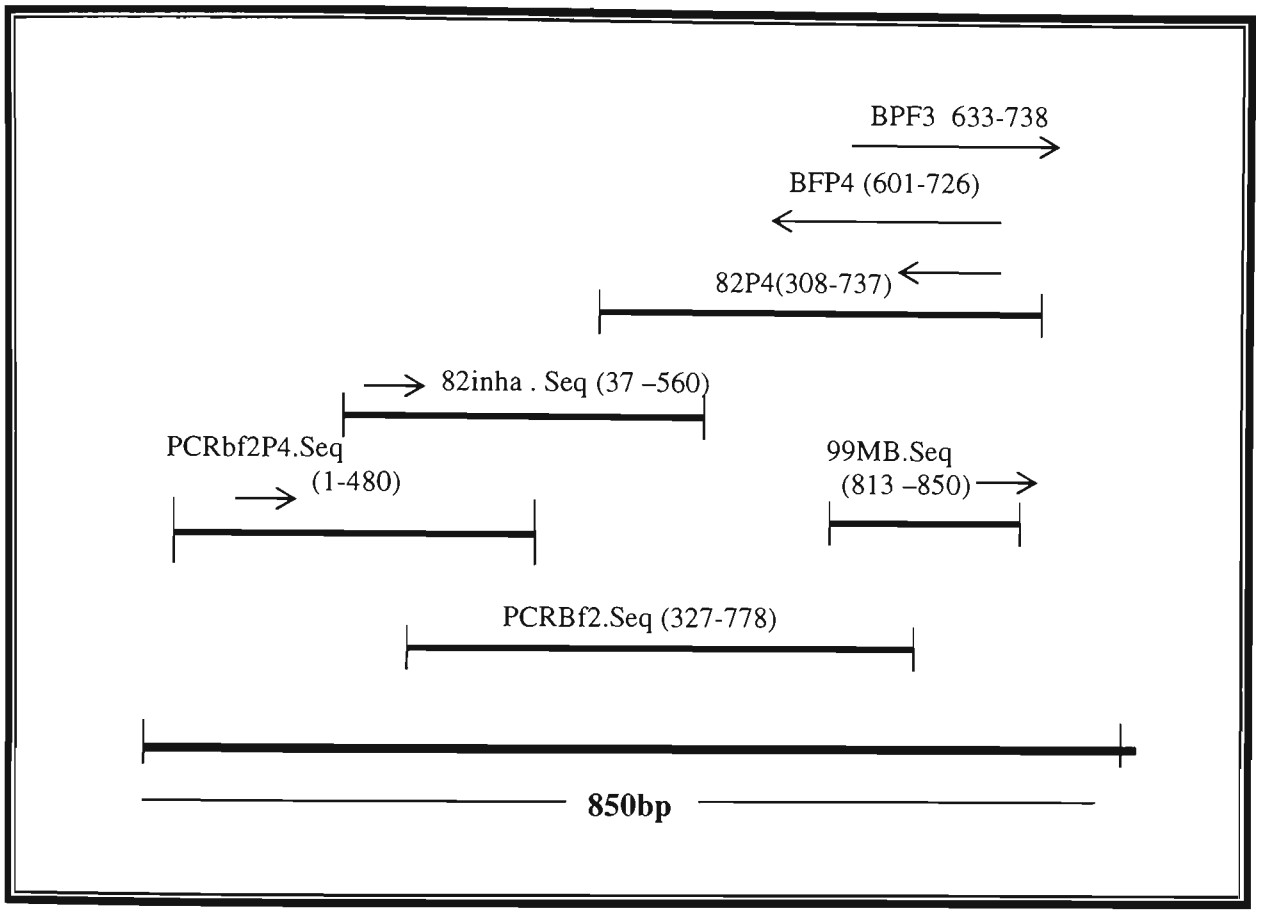
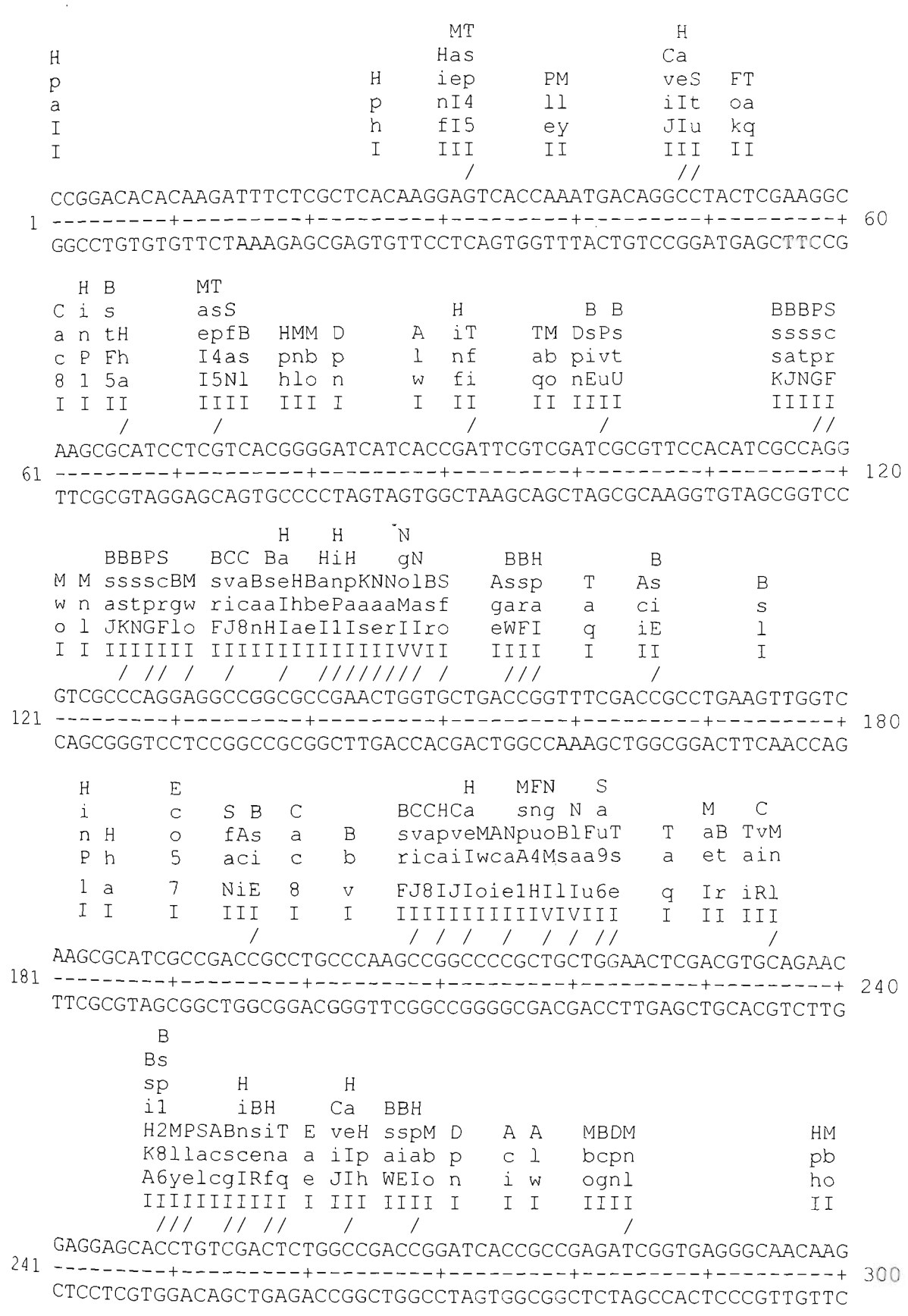


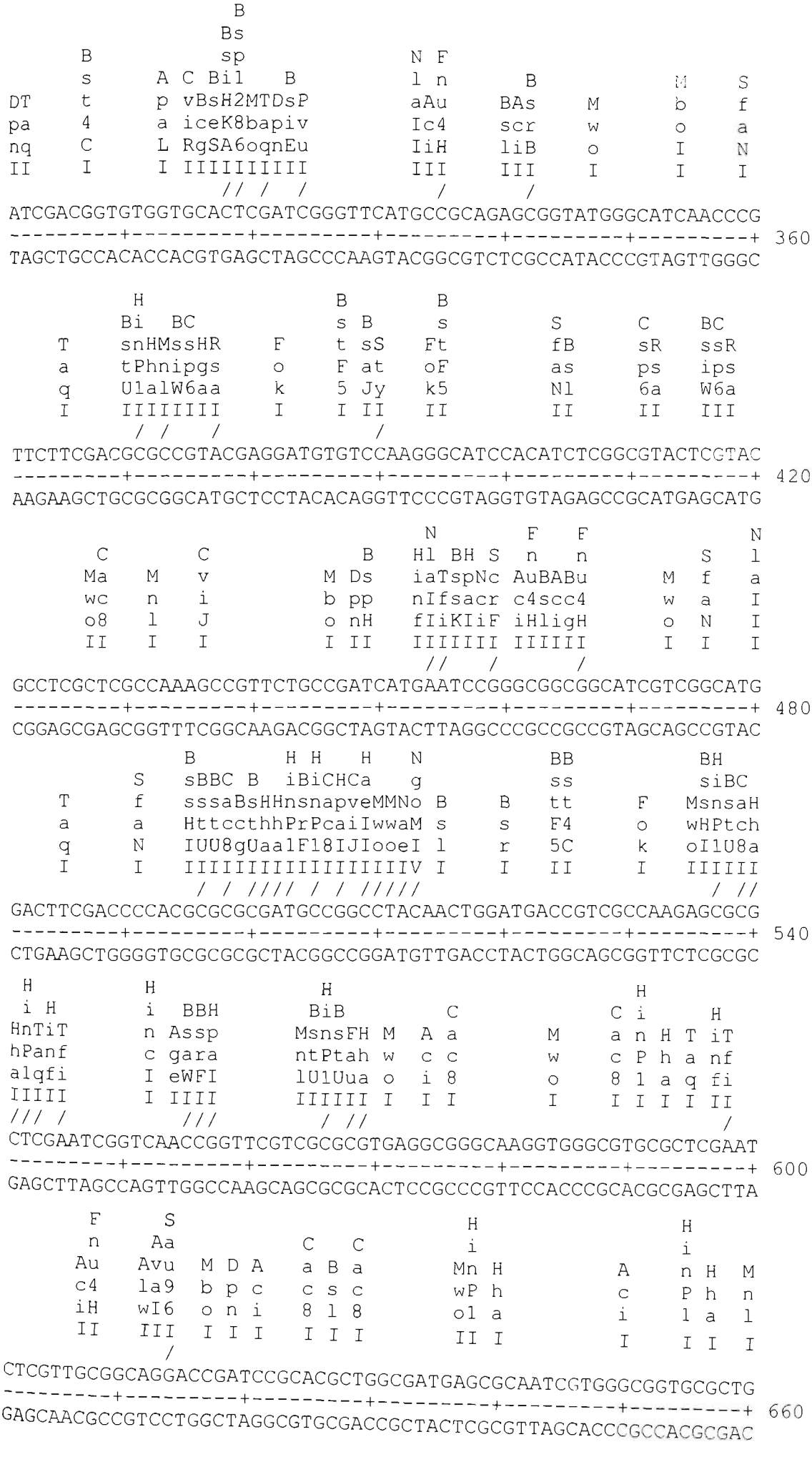
Figure 6.8 Schematic illustration of the sequencing assembly for *B. flavum* (BF4).

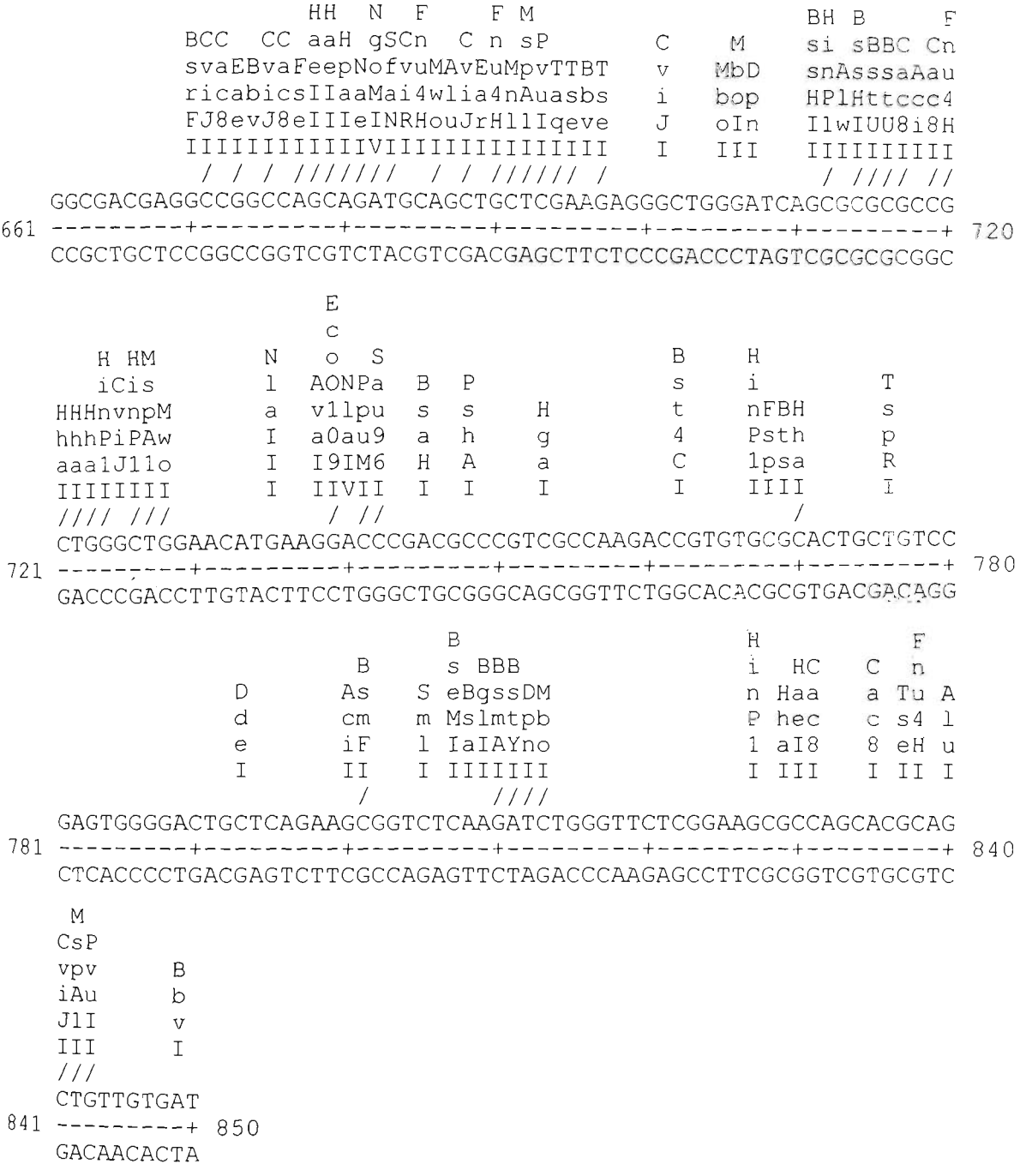
```
  1  CCGGACACAC AAGATTTCTC GCTCACAAGG AGTCACCAAA TGACAGGCCT
 51  ACTCGAAGGC AAGCGCATCC TCGTCACGGG GATCATCACC GATTCGTCGA
101  TCGCGTTCCA CATCGCCAGG GTCGCCCAGG AGGCCGGCGC CGAACTGGTG
151  CTGACCGGTT TCGACCGCCT GAAGTTGGTC AAGCGCATCG CCGACCGCCT
201  GCCCAAGCCG GCCCCGCTGC TGGAACTCGA CGTGCAGAAC GAGGAGCACC
251  TGTCGACTCT GGCCGACCGG ATCACCGCCG AGATCGGTGA GGGCAACAAG
301  ATCGACGGTG TGGTGCACTC GATCGGGTTC ATGCCGCAGA GCGGTATGGG
351  CATCAACCCG TTCTTCGACG CGCCGTACGA GGATGTGTCC AAGGGCATCC
401  ACATCTCGGC GTACTCGTAC GCCTCGCTCG CCAAAGCCGT TCTGCCGATC
451  ATGAATCCGG GCGGCGGCAT CGTCGGCATG GACTTCGACC CCACGCGCGC
501  GATGCCGGCC TACAACTGGA TGACCGTCGC CAAGAGCGCG CTCGAATCGG
551  TCAACCGGTT CGTCGCGCGT GAGGCGGGCA AGGTGGGCGT GCGCTCGAAT
601  CTCGTTGCGG CAGGACCGAT CCGCACGCTG GCGATGAGCG CAATCGTGGG
651  CGGTGCGCTG GGCGACGAGG CCGGCCAGCA GATGCAGCTG CTCGAAGAGG
701  GCTGGGATCA GCGCGCGCCG CTGGGCTGGA ACATGAAGGA CCCGACGCCC
751  GTCGCCAAGA CCGTGTGCGC ACTGCTGTCC GAGTGGGGAC TGCTCAGAAG
801  CGGTCTCAAG ATCTGGGTTC TCGGAAGCGC CAGCACGCAG CTGTTGTGAT
```

Figure 6.9 Nucleotide sequence of the *inhA* gene for *B. flavum* for strain BF4, based on the designed primers (INH1, INH2, CGP2, CGP3, CGP4, CGP5) derived from *M. smegmatis inhA* gene sequences.

Figure 6.9b A linear restriction map of the *inhA* gene of *B. flavum* strain BF4 using MAP program of the WEB ANGIS.







Enzyme that do not cut:

AatII	Acc65I	AclI	AfeI	AflII	AflIII	AhdI	AlwNI
ApaI	ApoI	AscI	AseI	AvaI	AvrII	BaeI	BaeI
BamHI	BanII	BbsI	BbvCI	BciVI	BclI	BfaI	BlpI
BmrI	BplI	BpmI	Bpu10I	BsaAI	BsaBI	BsmI	BsmBI
BspEI	BspMI	BsrDI	BsrGI	BssSI	BstAPI	BstBI	BstDSI
BstEII	BstXI	BstZ17I	Bsu36I	BtgI	ClaI	DraI	DraIII
DrdI	EagI	EciI	Ecl136II	EcoNI	EcoRI	EcoRV	HindIII
HpaI	KpnI	MfeI	MluI	MscI	MseI	MslI	NcoI
NdeI	NheI	NotI	NruI	NsiI	NspI	PacI	PciI
PflMI	PmeI	PmlI	Ppu10I	PsiI	PspOMI	PstI	RsrII
SacI	SacII	SanDI	SapI	SbfI	ScaI	SexAI	SfcI
SfiI	SgfI	SgrAI	SmaI	SnaBI	SpeI	SphI	SrfI
SspI	SwaI	TatI	TliI	Tsp509I	Tth111I	XbaI	XcmI
XhoI	XmaI	XmnI					

Table 6.5 BLASTN homology search result of *inhA* gene of *B. flavum* strain BF4.

	inhA BF4	*inhA M.smegmatis*	*inhA M.tuberculosis M. bovis*	*inhA M.avium* strain GIR10	*fabI P. aeruginosa*	*EnvM S. typhimurium*	*EnvM E. coli*
BF4 *inhA*	100	97	81	79	59	56	56
M.smegmatis inhA		100	83	83	59	56	56
M.tuberculosis M. bovis inhA			100	85	59	-	-
M. avium strain GIR10 *inhA*				100	-	-	-
P. aeruginosa fabI					100	65	64
S. typhimurium EnvM						100	86
E. coli EnvM							100

Table 6.6 BLASTX homology search results of BF4 on NCBI database.

	BF4 NADH-dependant Enoyl-ACP reductase	*M. smegmatis* NADH-dependant Enoyl-ACP	*M. smegmatis* *M. bovis* NADH dependant Enoyl reductase	*M. avium* Enoyl reductase	*P. aeruginosa* Enoyl-acyl Carrier protein reductase	*S. typhimurium* EnvM protein	*E. coli* envM	*B. napus* Enoyl-ACP
BF4 NADH-dependant Enoyl-ACP reductase	100	93	81	77	52	54	54	38
M. smegmatis NADH-dependant Enoyl-ACP		100	87	86	54	54	54	34
M. smegmatis *M. bovis* NADH dependant Enoyl reductase			100	87	54	54	54	43
M. avium Enoyl reductase				100	54	56	56	_
P. aeruginosa Enoyl-ACP reductase					100	70	70	43
S. typhimurium						100	97	41
E. coli							100	41
B. napus								100

Table 6.7 FASTA results for *B. flavum*

Organism	Gene	% Homology
M. smegmatis	*inhA*	97.05
M. tuberculosis	*inhA*	81.31
M. bovis	Putative keto acylACP	81.31
M. avium strain GIR10	transcript	81.62
P. aeruginosa	enoyl-ACP-reductase (*fabI*)	52.74
E. coli	envM protein gene	53.47
E. coli	Short chain alcohol dehydrogenase	53.47
S. typhimurium	envM protein gene	53.38
Actinomadura hibisca	Polyketide synthase	54.13

6.4 SEQUENCES OF THE *inhA* GENE OF *BREVIBACTERIUM* SPECIES

By comparison with the nucleotide sequence of the *inhA* gene of *M. smegmatis* (Banerjee *et al.*, 1994) and *C. glutamicum* strain AS019, the translation initiation codon (ATG) and termination codon (TGA) of *inhA* genes of *B. lactofermentum* and *Brevibacterium* strains BL1 and BF4 were found to be at sites homologous to that of *M. smegmatis inhA* gene, as expected. Therefore the *inhA* genes cloned here consisted of 850 bp.

Amino acid sequences of InhA proteins of *Brevibacterium* species are aligned with that of *M. smegmatis* and AS019 through the PRETTYBOX program (Fig 6.10a). Alignments of sequences of the four proteins showed that all four proteins aligned almost perfectly as expected, however, the InhA protein of BL1 showed several amino acid differences at various points. A putative NADH binding site in InhA for nicotinamide or flavin nucleotide is underlined for the four strains ie AS019, BL1, BF4 and *M. smegmatis* (Fig 6.10).

6.4 1 Submission of the *inhA* gene sequence to the GenBank

The inhA gene sequences of *B. lactofermentum* and *B. flavum* strains BL1 and BF4 were deposited to the GenBank. These sequences were assigned accession numbers as shown in Table 6.8.

Table 6.8 Accession numbers of the *inhA* gene sequences of *Brevibacterium* strains submitted to the GenBank.

ORGANISM	ACCESSION NUMBER
B. lactofermentum (BL1)	AF139473
B. flavum (BF4)	AF145897

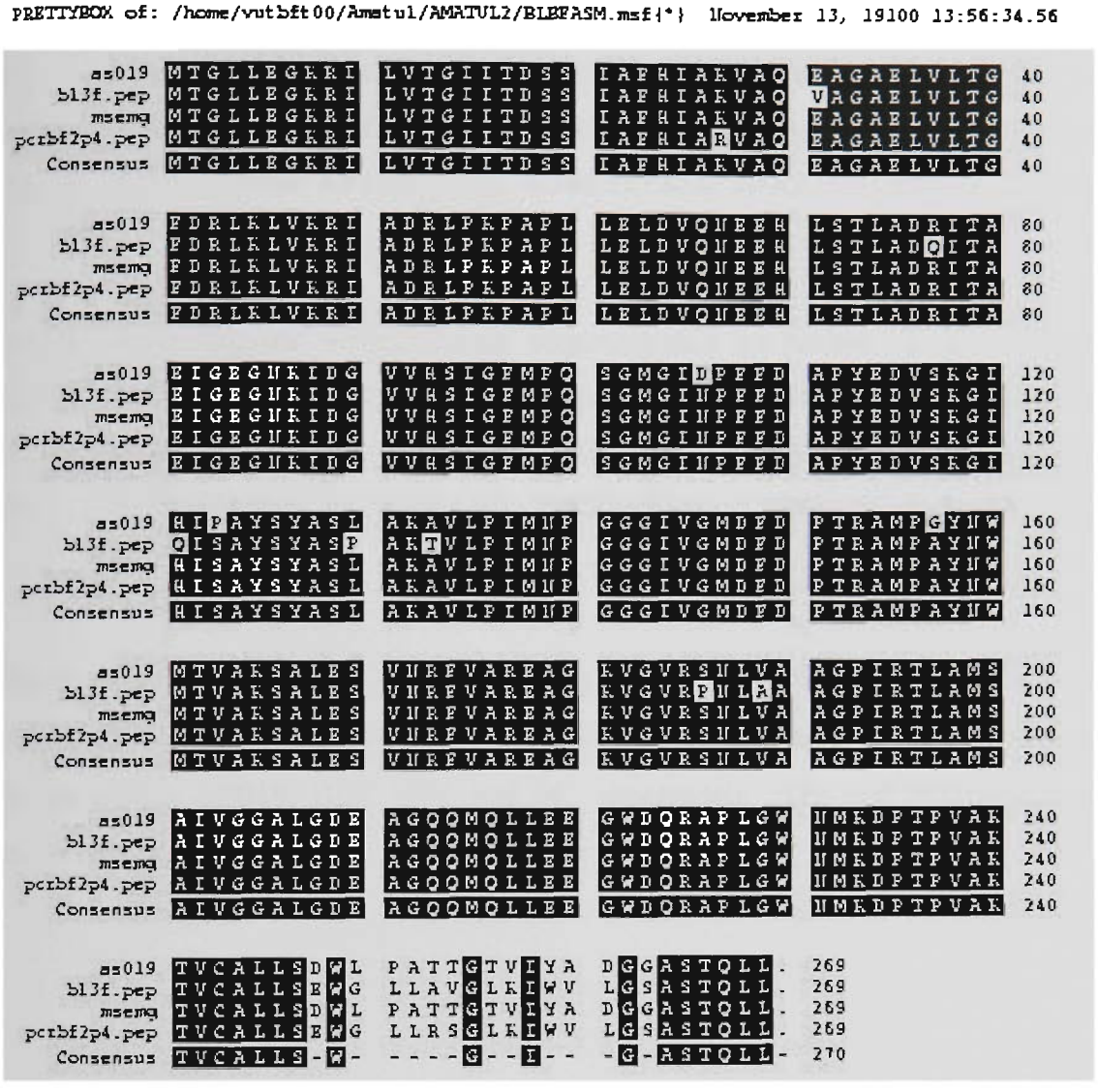


Figure 6.10 a Alignment of the InhA proteins of *C. glutamicum* strain AS019 and *M. smegmatis* (Msmeg) with two *B. lactofermentum* (BL3f) and *B. flavum* (pcrbf2p4) using the program PRETTYBOX. Identical amino acids are shown in black boxes. Gaps are introduced to maximise similarity.

```
        101                                                        150
AS019   SGMGIDPFFD APYEDVSKGI HIPAYSYASL AKAV-LPIMNP GGGIVGMDFD
BL1     SGMGINPFFD APYEDVSKGI QISAYSYASP AKTV-LPIMNP GGGIVGMDFD
Msemg   SGMGINPFFD APYEDVSKGI HISAYSYASL AKAV-LPIMNP GGGIVGMDFD
BF4     SGMGINPFFD APYEDVSKGI HISAYSYASL AKAV-LPIMNP GGGIVGMDFD

        151                                                        200
AS019   PTRAMPGYNW MTVAKSALES VNRFVAREAG KVGVRSNLVA AGPIRTLAMS
BL1   . PTRAMPAYNW MTVAKSALES VNRFVAREAG KVGVRPNLAA AGPIRTLAMS
Msemg   PTRAMPAYNW MTVAKSALES VNRFVAREAG KVGVRSNLVA AGPIRTLAMS
BF4     PTRAMPAYNW MTVAKSALES VNRFVAREAG KVGVRSNLVA AGPIRTLAMS
```

Figure 6.10 b A putative NADH binding site in InhA for nicotinamide or flavin nucleotide is underlined in the four proteins of *C. glutamicum, B. lactofermentum, B. flavum* strains AS019, BL1, BF4 and *M. smegmatis*. The red letters indicate the points of variation.

6.4.2 Comparison of *inhA* gene of *C. glutamicum* and *Brevibacterium*

6.4.2.1 Southern hybridisation analysis

Figure 6.11 and 6.12 and 6.13 show the Southern hybridisation patterns obtained when using an *inhA* gene probe from *C. glutamicum* strain AS019 against *Hind*III, *Bgl*II and *Eco*R1 genomic digest of *Corynebacterium* strains (AS019, MLB194, MLB133) and two *Brevibacterium* strains (BL1, BF4). Tables 6.9, 6.10 and 6.11 summarise the sizes of different hybridization bands obtained. The hybridisation pattern for corynebacterial and brevibacterial species is consistent with the previously observed Southern results for each species individually, however, minor visual differences can be explained due to different experimental conditions each time (e.g % of agarose, voltage, different volumes of digestion mixture and different running conditions etc). Two different hybridisation patterns can be seen for *C. glutamicum* strains and *Brevibacterium* strains.

The results show that the three strains of *C. glutamicum* (AS019, MLB194, MLB133) show similar hybridisation patterns among themselves with three enzymes, two *Brevibacterium* strains BL1 and BF4 however, have similar patterns with *Bgl*II but a slightly varied pattern was observed with *Hind*III genomic DNA digestion. These results, however, reveal the presence of more than one copy of the gene in different *Corynebacterium* species.

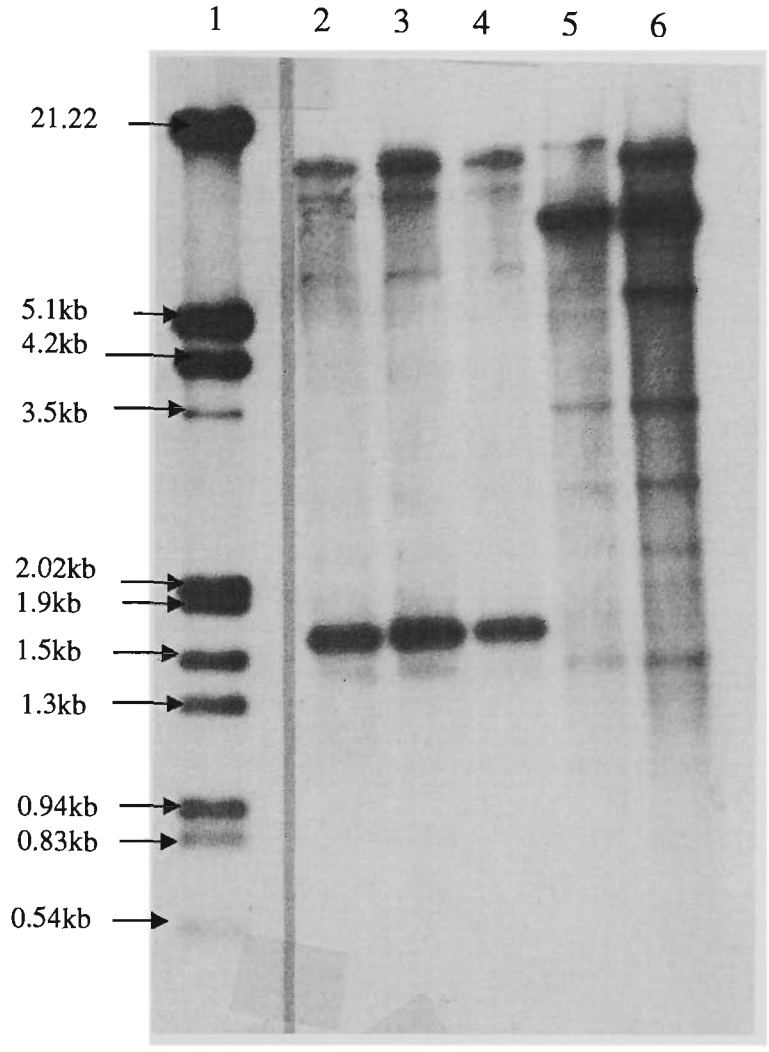


Figure 6.11 Southern blot hybridisation analysis of *Hind*III digested genomic DNA of different strains of *C. glutamicum, B. lactofermentum* and *Brevibacterium* probed with a 250 bp PCR product obtained using the primer sets CGP3-INH2 using the ^{32}P radioactive labelling system. Lambda DNA *Hind*III/*EcoR1* size markers Lane 2, 3, 4, 5, 6, *C. glutamicum* strains AS019, MLB194, MLB133, *B. lactofermentum* (BL1), *B. flavum* (BF4).

Table 6.9 Molecular weights of the hybridisation fragments obtained when CGP3-INH2 probe was used against the genomic DNA digest of various corynebacterial species digested with *Hind*III.

λDNA markers	**MW (kb) of the bands detected**		
	C. glutamicum **strains AS019, MLB194, MLB133**	***B. lactofermentum*** **strain BL1**	***B. flavum*** **strain BF4**
21.22	**12.2**	13	12.2
5.1	10	**8.5**	**8.5**
4.2	**1.6**	3.5	3.5
3.5		1.5	1.5
2.02			
1.9			
1.5			
1.3			
0.94			
0.54			

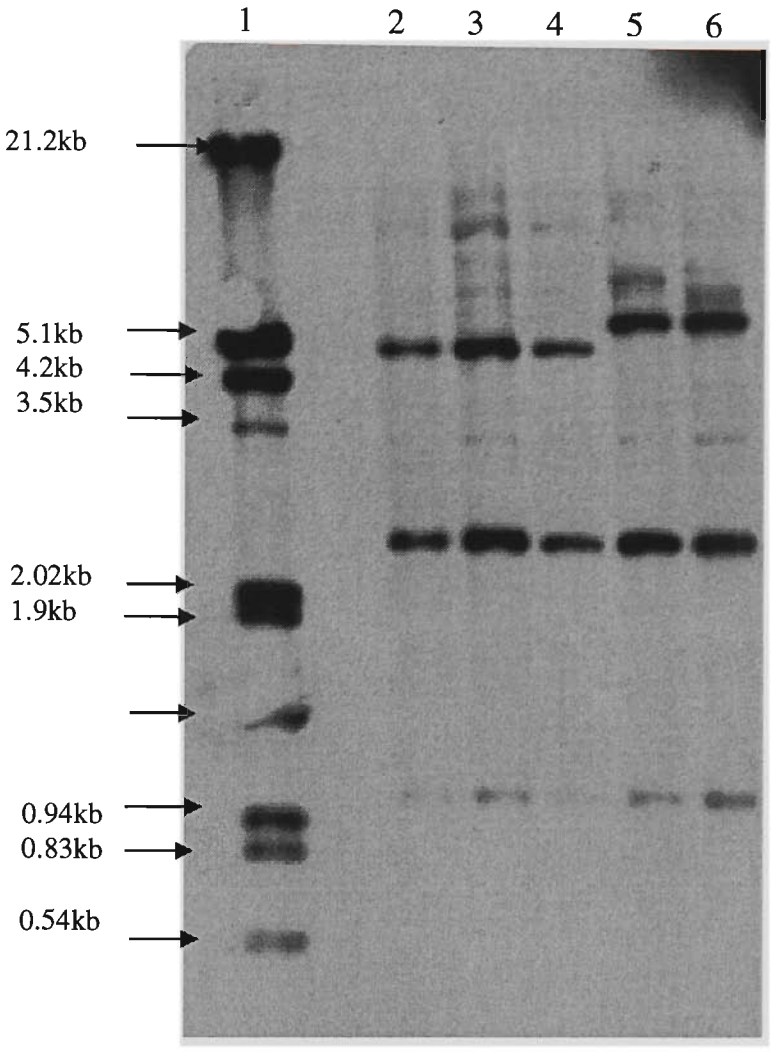


Figure 6.12 Southern blot hybridisation analysis of *Bgl*II digested genomic DNA of various species of *C. glutamicum* with a 250 bp PCR amplified product obtained from *C. glutamicum* strain AS019 using primers sets CGP3-INH2. Lane 1 Lambda DNA digested *Hind*III/*Eco*R1 size markers. Lanes 2, 3, 4, *C. glutamicum* strains AS019, MLB194, MLB133, and Lanes 5, 6 *B. lactofermentum* and *B. flavum* strains BL1, BF4.

Table 6.10 Molecular weights of the hybridizaton fragments obtained when CGP3-INH2 probe was used against the genomic DNA digest of various corynebacterial species digested with *Bgl*II.

λDNA markers	MW (kb) of the bands detected		
	C. glutamicum strains AS019, MLB194, MLB133	*B. lactofermentum* strain BL1	*B. flavum* strain BF4
21.22 5.1 4.2 3.5 2.02 1.9 1.5 1.3 0.94 0.54	8-9 **5.1** **2.3** 1.2	6.6 **6.0** **2.3** 1.2	6.6 **6.0** **2.3** 1.2

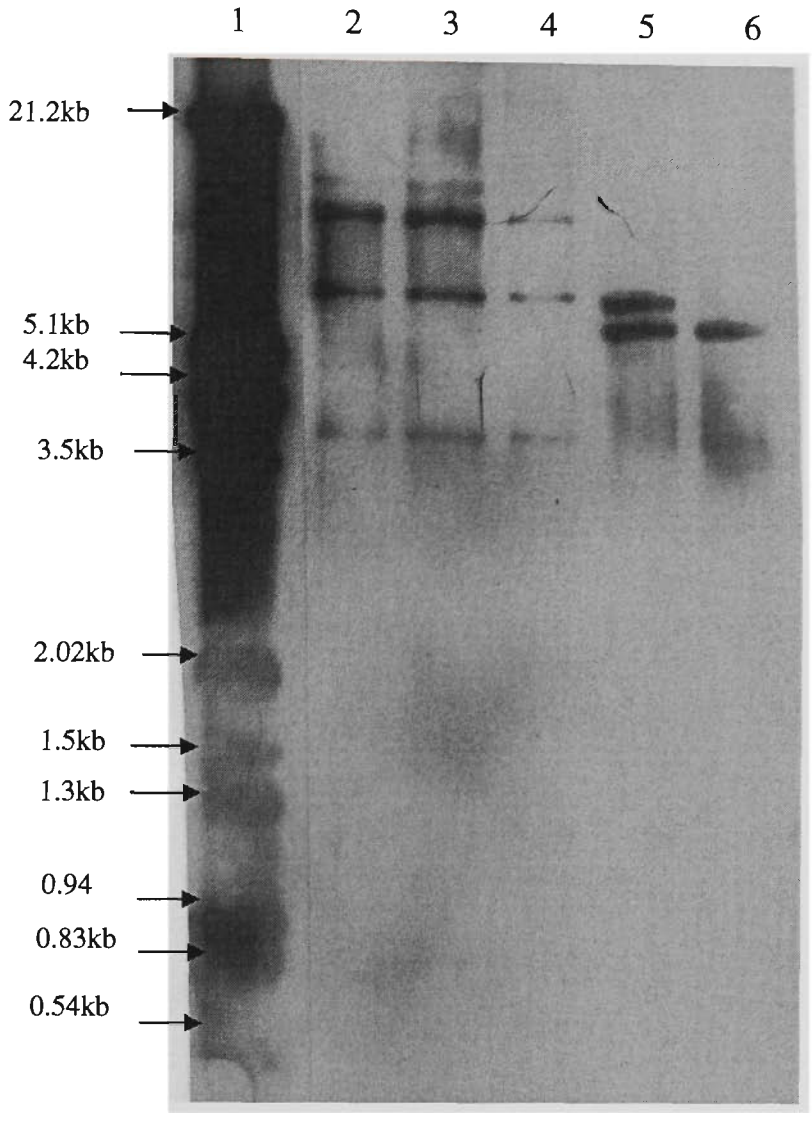


Figure 6.13 Southern blot hybridisation analysis of *Eco*R1 digested genomic DNA of various species of *C. glutamicum* with a 250 bp PCR amplified product obtained from *C. glutamicum* strain AS019 using primers sets CGP3-INH2. Lane 1 Lambda DNA digested *Hind*III/*Eco*R1 size markers. Lanes 2, 3, 4; *C. glutamicum* strains AS019, MLB194, MLB133, Lanes 5, 6; *B. lactofermentum* and *B. flavum* strains BL1, BF4.

Table 6.11 Molecular weights of the hybridisation fragments obtained when CGP3-INH2 probe was used against the genomic DNA digest of various corynebacterial species digested with *EcoRI*.

λDNA markers	MW (kb) of the bands detected		
	C. glutamicum strains AS019, MLB194, MLB133	*B. lactofermentum* strain BL1	*B. flavum* strain BF4
21.22 5.1 4.2 3.5 2.02 1.9 1.5 1.3 0.94 0.54	10 8-9 6.2	6.2 5.1	5.1

6.4.2.2 Base composition of *inhA* genes in *C. glutamicum* and *Brevibacterium*

By comparison of the base composition (Table 6.12) of the gene sequences between the initiation codon (ATG) and termination codon (TGA), it was found that there was a marked GC bias in the *inhA* genes of different *Corynebacterium* and *Brevibacterium* species. The GC contents of the five *inhA* genes of three *Corynebacterium* strains and two *Brevibacterium* species was 66.12% and 64.46, 64.70 % respectively, similar to that of the corresponding *inhA* gene of *M. smegmatis* (65.50 %).

6.4.2.3 Sequence comparison of *inhA* genes of *C. glutamicum* and *Brevibacterium* with mycobacterial *inhA* gene

The *inhA* gene is highly conserved in different corynebacterial species indicating its unique involvement in mycolic acid biosynthesis. The *inhA* genes of *Corynebacterium* and *Brevibacterium* exhibited the best identity with each other and with mycobacterial *inhA* genes. The percentage identity between *C. glutamicum* strain AS019 and *M. smegmatis* is 99.62, the two mutants MLB194 and MLB133 exhibited 98.507 and 96.28 % identity with AS019. Similarly the two *Brevibacterium* species *B. lactofermentum* and *B. flavum* exhibited 97.16 % identity with each other and 94.42 to 96.65 % with *C. glutamicum* (AS019) and *M. smegmatis* respectively.

Table 6.12 Base composition (%) of the *inhA* genes of different *Corynebacterium* species.

	C. glutamicum		*B. lactofermentum*	*B. flavum*	*M. smegmatis*
Contents	AS019	MLB194	BL1	BF4	mc2
A	18.58	18.69	18.94	18.94	18.70
G	32.82	33.01	32.70	32.94	32.70
C	32.94	32.66	31.76	31.76	32.82
T	15.64	15.62	16.58	16.35	15.76

The base composition of the different *inhA* gene sequences was determined using the Ecomposition ANGIS and then converted to % of each base out of total.

6.4.3 Analysis of the deduced amino acids sequences of the InhA proteins

The amino acid sequences of the five InhA proteins, AS019, MLB194, MLB133, BL1, BF4, were deduced from their respective gene sequences. The deduced biochemical and physical properties, the molecular basis of electrophoretic differences observed and differences in the predicted secondary structures of these InhA proteins are analysed and discussed below.

6.4.3.1 Sizes of the InhA proteins

The *inhA* genes of *Corynebacterium* and *Brevibacterium* had the same total length (850 bp) from the translation initiation codon (ATG) to the termination codon (TGA) and encoded 269 amino acid of proteins. InhA proteins deduced from the sequences of *inhA* genes had calculated molecular weights ranging from 28.41 kd to 30.04 kd. Table 6.13 shows the comparision of InhA proteins of *C. glutamicum* strains AS019, MLB194 and MLB133 with BL1, BF4, *M. smegmatis* and *M. tuberculosis*. These results are in complete agreement with InhA proteins deduced from the sequences of respective genes in mycobacterial species.

6.4.3.2 Biochemical properties of the InhA proteins

InhA proteins of different corynebacterial and brevibacterial strains (AS019, MLB194, MLB133 and BL1, BF4) were composed of 269 amino acids very similar to those of *M. smegmatis*. All of these proteins have acidic deduced isoelectric points ranging from pI 5.03 of AS019 to pI 6.16 of BF4 and net negative charges (-7 of AS019 to -3 of BF4) compared to mycobacterial species which had pI in the ranges of pI 5.19 for *M. smegmatis* to pI 6.04 for *M. tuberculosis* and net negative charges of –6 and –4 respectively.

Table 6.13 Comparison of InhA proteins of *Corynebacterium* and *Brevibacterium* species

Proteins	Number of A.As	MW	pI	Charge	Average residue weight
InhA AS019	269	28.52	5.03	-7	106.036
InhA 194	268	28.41	5.19	-6	106.016
InhA 133	259	27.79	5.17	-5	107.312
InhA BL1	269	28.55	5.44	-4	106.171
InhA BF4	269	28.73	6.16	-3	106.171
M. smegmatis	269	28.52	5.19	-6	106.047
M. tuberculosis	269	28.52	6.04	-4	106.050

The molecular weights (MW), isoelectric points (pI) and charges of different InhA proteins were calculated using the Pepstat program of ANGIS. InhA of AS019, MLB194, MLB133, BL1 and BF4 were deduced from their respective sequences. Sources of InhA protein data for *M. smegmatis* and *M. tuberculosis* is Banerjee *et al* (1994).

6.4.3.3 Differences in the amino acid sequences of InhA proteins of corynbacterial and mycobacterial species

All amino acids substitutions between InhA proteins of *C. glutamicum* and *Brevibacterium* were caused by single nucleotide differences. DNA sequences responsible for amino acids substitution in the InhA proteins are shown in Table 6.10. and 5.11. Table 6.10 shows the amino acid differences in three InhA proteins of *C. glutamicum* (AS019, MLB194, MLB133) and *M. smegmatis*. There are three amino acids substitutions in AS019 and *M. smegmatis*, asparagine, serine and alanine at position 106, 123 and 157, are replaced by aspartic acid, proline and glycine. MLB194 and MLB133 are very similar to *M. smegmatis* except for position 194 where isoleucine in MLB194 is replaced by asparagine.

There are 10 amino acid variations between these in *B. lactofermentum* and *B. flavum* at positions 27, 31, 77, 121, 130, 186, 189, 253 and 254 respectively, these substitutions are similar as seen in *C. glutamicum* strain AS019 and *B. lactofermentum* except for position 27 and 123 where there are arginine and serine in BF4. The two proteins of BL1 and BF4 are similar towards the end but show greater degree of variations with the InhA potein of AS019 in terms of amino acid composition (see Table 6.14 for details).

6.4.3.4 Composition of amino acid of the InhA proteins of *Corynebacterium*

Although the deduced biochemical properties of the various corynebacterial InhA proteins were quite similar (Table 6.13) there were subtle differences to be found in proportions of various amino acids groups (Table 6.15). For example, composition of charged amino acids in the D+E+H+K+R groups in InhA proteins of *C. glutamicum* strains AS019, MLB194, MLB133 and *Brevibacterium* strains BL1 and BF4 were 21.190, 20.896 and 19.703, 21.190 respectively. However, the apparently minor alterations might have relatively large effect on the predicted secondary structures of these InhA proteins.

Table 6.14 Differences in amino acid sequences of InhA proteins of two *Brevibacterium* species and its comparison with the InhA of *C. glutamicum* (strain AS019).

AS019			*B. flavum*		*B. lactofermentum*	
	Amino acids	Codon	Amino acids	Codon	Amino acids	Codon
27	K Lysine	AAG	R Arginine	AAG	K Lysine	AAG
31	E Glutamic acid	GAG	-	-	V Valine	GTG
77	R Arginine	CGG	-	-	Q Glutamine	CAG
121	H Histidine	CAC	-	-	Q Glutamine	CAA
123	P Proline	CCG	S Serine	TCG	S Serine	TCG
130	L Leucine	CTC	-	-	P Proline	CCC
133	A Alanine	GCC	-	-	T Threonine	ACC
186	S Serine	TCG	-	-	P Proline	CCG
189	V Valine	GTT	-	-	A Alanine	GCT
248	D Aspartic acid	GAC	E Glutamic acid	GAG	E Glutamic acid	GAG
250	L Leucine	CTG	G Glycine	GGA	G Glycine	GGA
251	P Proline	CGG	L Leucine	CTC	L Leucine	CTG
252	A Alanine	GCC	L Leucine	CTC	L Leucine	CTG
253	T Threonine	ACC	R Arginine	AGA	A Alanine	GCG
254	T Tyrosine	ACC	S Serine	AGC	V Valine	GTC
256	T Threonine	ACC	L Leucine	CTC	L Leucine	CTC
257	V Valine	GTG	K Lysine	AGA	K Lysine	AAG
259	Y Tyrosine	TAC	W Tryptophan	TGG	W Tryptophan	TGG
260	A Alanine	GCC	V Valine	GTT	V Valine	GTT
261	D Aspartic acid	GAC	L Leucine	CTC	L Leucine	CTC
263	G Glycine	GGC	S Serine	AGC	S Serine	AGC

Table 6.15 Amino acid composition of InhA proteins of *Corynebacterium* and *Brevibacterium* species.

Types of amino acids		*C. glutamicum*						*B. lactofermentum*		*B. flavum*	
		AS019		MLB194		MLB133		BL1		BF4	
		Number	%	Number	%	Number	%	Number	%	Number	%
Small	A+G	60	22.305	59	22.015	54	20.849	59	21.933	58	21.561
Hydroxyl	S+T	29	10.781	30	11.194	28	10.811	28	10.409	29	10.781
Acidic	D+E	30	11.152	29	10.821	29	11.197	27	10.037	28	10.409
Acid/Amide	D+E+N+Q	45	16.726	46	17.164	46	17.761	45	16.729	44	16.357
Basic	H+K+R	27	10.729	27	10.075	29	11.197	26	9.665	29	10.781
Charged	D+E+H+K+R	57	21.191	56	20.896	58	22.394	53	19.703	57	21.996
Small hydophob	I+L+M+V	75	27.881	72	26.866	70	27.027	78	28.996	78	28.996
Aromatic	F+W+Y	16	5.948	16	5.970	16	6.178	16	5.948	16	5.948

Amino acids of the InhA proteins of different corynebacterial species were grouped using the Pepstat program of ANGIS. Number and % indicate the number of amino acids in each group and its percentage to the total number of amino acids of the InhA proteins, respectively.

Table 6.16 Differences in the amino acid sequences of the InhA proteins of *C. glutamicum* parent and mutants and comparison with *M. smegmatis*

AS019			*M. smegmatis*		MLB133		MLB194	
Site	Amino acid	Codon	Amino acid	Codon	Amino acid	Codon	Amino acid	Codon
106	D Aspartic acid	GAC	N Aspartic acid	AAC	N Asparagine	AAC	N Asparagine	AAC
123	P Proline	CCG	S Serine	TCG	S Serine	TCG	S Serine	TCG
157	G Glycine	GGC	A Alanine	GCC	A Alanine	GCC	A Alanine	GCC
153	**R Arginine**	CGC	**A Arginine**	CGC	**R Arginine**	CGC	**P Proline**	CCG
154	A Alanine	GCG	A Alanine	GCG	A Alanine	GCG	R Arginine	CGA
155	M Methionine	ATG	M Methionine	ATG	M Methionine	ATG	C Cysteine	TGA
157	G Glycine	GGC	A Alanine	GCC	A Alanine	GCC	A Alanine	GCC

Amino acids differing in InhA proteins of *C. glutamicum* strains AS019, MLB133, MLB194 and *M. smegmatis*. Codons for these variable sequences of were obtained from DNA sequences of the respective *inhA* genes.

6.4.3.5 Comparison of the InhA proteins of different corynebacterial species with other bacterial and plant proteins catalysing similar function.

When the Swiss-PROT databases were searched for the text strings associated with InhA proteins, 41 sequences satisfied the expectation threshold parameter value of 10. InhA proteins of mycobacterial species were amongst those showing high degree of homology besides a few plants and bacterial proteins which are supposed to be involved in fatty acid biosynthesis. These latter proteins showed significant homology (59%) with corynebacterial InhA proteins when alignment was performed using the Pileup program of WEBANGIS (Fig. 6.14).

Amino acid sequences of InhA proteins from AS019, MLB133, MLB194, and *Brevibacterium* strains BL1 and BF4 showed that the InhA proteins are highly conserved among corynebacterial strains. The average identity between InhA of *Mycobacterium* and *C. glutamicum* was > 95%. The various envM proteins were also highly conserved among themselves. A putative binding site in InhA for nicotinamide or flavin is underlined (Fig. 6.14).

Fig. 6.14 Alignment of deduced sequences of InhA proteins from *C. glutamicum* strains, AS019, MLB194, MLB133, *B. lactofermentum*, (BL1) and *B. flavum* (BF4) with InhA from *M. smegmatis*, *M. tuberculosis* (M2), envM proteins from *E. coli* (E), *S. typhimurium* (S), Enoyl ACP reductase from *P. aeruginosa* (P) and *B. nappus* (Brassica). The amino acid sequences are aligned using the Pileup program of ANGIS.

```
  AS019  ..........  .MTGLLEGKR ILVTGIITDS SIAFHIAKVA QEAGAELVLT    39
  Msemg  ----------  ---------- ---------- ---------- ----------    50
    133  ----------  ---------- ---------- ---------- ----------    50
    194  ----------  ---------- ---------- ---------- ----------    50
    BF4  ----------  -.-------- ---------- -------R-- ----------    49
    BL1  ----------  ---------- ---------- ---------- -V--------    50
     M2  ----------  ------D--- ---S------ -------R-- --Q--Q----    50
      E  ----------  --GF.-S--- -----VASKL ---YG--QAM HRE-------    46
      S  ----------  --GF.-S--- -----VASKL ---YG--QAM HRE-------    46
      P  AEN*VADKER  T-GF.-T--- A-IV-VASKL ---SG--AAM HRE-------    46
Brassica SESSESKASS  GLPID-R--- AFIA--AD-N GYGWAV--SL AA----ILVG    50

  AS019  GF........  DRLKLVKRIA DRLPKPAPLL EL........ ..........    63
  Msemg  ----------  ---------- ---------- ---------- ----------   100
    133  ----------  ---------- ---------- ---------- ----------   100
    194  ----------  ---------- ---------- ---------- ----------   100
    BF4  ----------  ---------- ---------- ---------- ----------    99
    BL1  ----------  ---------- ---------- ---------- ----------   100
     M2  ----------  ---R-IQ--T ----AK---- ---------- ----------   100
      E  ..--AFTYQN  -K-------. ......GRVE -FAAQLGS-- DIVLQCDVAE    82
      S  ..--AFTYQN  -K-------. ......GRVE -FAAQLGS-- SIVLPCDVAE    82
      P  ..--AFTYQN  -K-R.....  ......GRVE -FASGWGSRP ELCFPCDVAD    82
Brassica TWVPALNIFE  TS-RRG-FDQ S-VLPDGS-M -IKKVYPLDA VFDNPEDVPE   100

  AS019  DVQNEEHLST  LADRITAEIG EG.....NKI DGVVHSIGFM PQSGMGIDPF   108
  Msemg  ----------  ---------- ---------- ---------- -------N--   150
    133  ----------  ---------- ---------- ---------- -------N--   150
    194  ----------  ---------- ---------- ---------- -------N--   150
    BF4  ----------  ---------- ---------- ---------- -------N--   149
    BL1  ----------  ---Q------ ---------- ---------- -------N--   150
     M2  --------AS  --G-V-EA-- A--------L ---------- --T----N--   150
      E  -ASIDTMFAE  -GK.......  ..---VWP-F --F------A -GDQLDG-YV   123
      S  -ASIDAMFAE  -GN.......  ..---VWP-F --F------A -GDQLDG-YV   123
      P  -S-I-AVFAA  -GK.......  ..---HWDGL -II---V--A -GDQLDG-FT   123
Brassica --KANKRYAG  SSNWTVQ-AA -CVRQDFGS- -IL---..LA NGPEVSKPLL   148
```

```
AS019      FDAPYEDVSK GIHIPAYSYA SLAKAVLPIM .NPGGGIVGM DF.DPTRAMP  156
  Msemg    ---------- ----S----- ---------- ---------- ----------  200
    133    ---------- ----S----- ---------- ---------- ----------  200
    194    ---------- ----S----- ---------- ---------- ------PRC-  200
    BF4    ---------- ----S----- ---------- ---------- ----------  199
    BL1    ---------- --Q-S----- -P--T----- ---------- ----------  200
     M2    -----A---- ----S----- -M---L---- -----S---- -----S----  200
      E    NAVTR-GFKI AHD-SS--FV AM---CRSML ----SALLTL SYLGAE--I-  173
      S    NAVTR-GFKV AHD-SS--FV AM---CRTML ----SALLTL SYLGAE--I-  173
      P    AVTTR-GFRI AHD-S---FI A----GREM- KGRN-SLLTL SYLGAE-T--  173
Brassica   E.TSRKGYLA A-SASS--FV --LSHF---- -----ASISL TYIASE-II-  197

 AS019  GY.NWMTVAK SALESVNRFV AREAGKV.GV RSNLVAAGPI RTLAMSAIVG  204
  Msemg A--------- ---------- ---------- ---------- ----------  250
    133 A--------- ---------- ---------- ---------- ----------  250
    194 A--------- ---------- ---------- ---------N ----------  250
    BF4 A--------- ---------- ---------- ---------- ----------  249
    BL1 A--------- ---------- ---------- -P--A----- ----------  250
     M2 A--------- ---------- ------Y--- ---------- ----------  250
      E N---V-GL-- AS--ANV-YM -NAM-P.E-- -V-AIS---- ----A-G-..  220
      S N---V-GL-- AS--ANV-YM -NAM-P.E-- -V-AIS---- ----A-G-..  220
      P N---V-GM-- AS--AGV-YL -GSL-A.E-T -V-A-S---- ----A-G-..  220
Brassica --GGG-SS-- A----DT-VL -F---RKQNI -V-TIS---L GSR-AK--..  245

   AS019  GALGDEAGQQ MQLLEEGWDQ RAPLGWNMKD PTPVAKTVCA LLSDWLPATT  254
   Msemg  ---------- ---------- ---------- ---------- ----------  300
     133  ---------- ---------- ---------- ---------- --*-RVRTA.  299
     194  ---------- ---------- ---------- ---------- ----------  300
     BF4  ---------- ---------- ---------- ---------- ---E-GLLRS  299
     BL1  ---------- ---------- ---------- ---------- ---E-GLLAV  300
      M2  ----E---A- I--------- ---I------ A--------- ----------  300
       E  ........KD FRKMLAHCEA VT-IRRTV.T IED-GNSAAF -C--LSAGIS  261
       S  ........KD FRKMLAHCEA VT-IRRTV.T IED-GNSAAF -C--LSAGIS  261
       P  ........KS FRKMLAANER QT--RR-V.T IEE-GNAGAF -C--LASGIS  261
 Brassica ........GF IDTMI-YSYN N--IQKTL.T ADE-GNAAAF -V-PLAS-I-  286
```

```
   AS019  GTVIYADGGA STQLL*.... .......... .....      270
   Msemg  ---------- ---------- ---------- -----      335
     133  .......... ......---- ---------- -----      318
     194  ---------- ----..---- ---------- -----      333
     BF4  -LK-WVL-S- ---------- ---------- -----      334
     BL1  -LK-WVL-S- ---------- ---------- -----      335
      M2  -DI------- H--------- ---------- -----      335
       E  -E-VHV---F -I..AAMNEL ELK------- -----      294
       S  -E-VHV---F -I..AAMNEL ELK------- -----      294
       P  -EIL-V---F N-..TAMGPL DDD*SPRP*K MAASS      294
Brassica  -AT--V-N-L NSMGVALDSP VFKDLDK--- -----      321
```

6.5 DISCUSSION

In this chapter we report that the *inhA* genes of two *Brevibacterium* species BL1 and BF4 have been successfully cloned and sequenced using PCR approaches. The sequences of the two *inhA* genes of *Brevibacterium* species and their deduced InhA proteins showed amino acid variations at several points, when compared to other known InhA proteins of *Mycobacterium* species. The *inhA* genes of the two *Brevibacterium* species and their deduced proteins were compared with the corresponding genes in *C. glutamicum* and number of interesting observations were made which will be discussed in the following.

6.5.1 Differences in the Southern hybridisation pattern of *C. glutamicum* and *Brevibacterium*

Southern hybridisation experiments reveals two unique patterns for the three-cornebacterial species tested. In one experiment, where *Bgl*II digested genomic DNA of three corynebacterial species was probed with an *inhA* probe from AS019, differences were seen in the banding pattern of the three (Fig. 6.13). Three *C. glutamicum* strains AS019, MLB133 and MLB194 showed similar hybridisation patterns indicating the homologous nature of genome, as expected for strains derived from the same parent. The two *Brevibacterium* strains BL1 and BF4 generated similar patterns among themselves but slightly different from the *C. glutamicum* strains. In the second experiment, where the same *inhA* probe was used against the *Hind*III digested genomic DNA of these three corynebacterial species, three patterns were seen (Fig. 6.12). The three *C. glutamicum* strains AS019, MLB133 and MLB194 generated one specific pattern showing more than one copy of the *inhA* gene, the two *Brevibacterium* species, however, showed two patterns that had no similarity with each other nor with *C. glutamicum*. For *B. lactofermentum* one strong signal was obtained with *Hind*III but other enzymes like, *Pst*1, *Bgl*II, *Eco*R1 showed multiple fragments, therefore the controversy still remained unsolved whether it contained a single copy or multiple copies of the gene. Similarly, for *B. flavum* one signal was obtained with *Eco*R1 and more than one signal were seen with other enzymes. In both of these experiments, the gels included the *M. smegmatis* genomic

DNA digest as well but no signal was obtained with either of the two enzymes used, only *Pst*1 digested genomic DNA of *M. smegmatis* showed binding of the probe with a single 3 kb fragment. From these results two conclusions can be drawn.

1 Firstly the two *Brevibacterium* species and to a lesser degree the *C. glutamicum* strains are closely related from the point of view of biochemical and morphological characteristics, but they represent a heterogenous family of isolates with different genome structures. Whether this diversity results from deep chromosome rearrangements will be clarified by the fine mapping and determination of relative gene positions on the genome of these strains.

2 Secondly, although the hybridisation results varied from species to species they emphasise the presence of more than one copy of the *inhA* gene in the different corynebacterial species. In *Mycobacterium*, the probe binds only to a 3 kb *Pst*1 fragment, indicating the presence of one copy of the gene in this species.

6.5.2 The comparison of the deduced InhA proteins of different corynebacterial species.

The InhA proteins of the different corynebacterial species AS019, BL1 and BF4, deduced from their respective gene sequences, had the same number of amino acids, but exhibited slight differences in their calculated molecular weights and isoelectric points (Table 5.9). There are ten amino acid substitutions in a total of 269 amino acid of the two *Brevibacterium* species BL1 and BF4 at positions 27, 31, 77, 121, 130, 133, 186, 189, 253. The most important is arginine (MW=174 Da, pI 10.76) at positions 77 and 253 in BF4 which is replaced by lysine and alanine in BL1. Another important amino acid is proline, which is supposed to have a pronounced effect on the three dimensional structure of these protein.

Overall the composition of the InhA proteins of *Corynebacterium* and *Brevibacterium* species showed significant differences among the three strains examined. The lower portion of the InhA proteins (from position 248-269) of BL1 and BF4 is almost identical (except at position 253 and 254) but this region is different for AS019 and the mutants MLB133 and MLB194. In the upper region (from position 1-247) of the

InhA proteins of *C. glutamicum* strains AS019 and *B. flavum* BF4 show two differences: lysine and proline at position 27 and 123 in AS019 are replaced by arginine and serine in BF4.

Similarly in *C. glutamicum* (strain AS019) and *B. lactofermentum* there are eight differences (from position 1-247), glutamic acid, arginine, histidine, proline, leucine, alanine, serine, valine, at position 31, 77, 121, 123, 133, 186, 189 in *C. glutamicum* strain AS019, are replaced by valine, glutamine, glutamine, serine, proline, threonine, proline, and alanine in *B. lactofermentum.* It is very interesting to note that exactly the same substitutions have occurred at these positions with the same amino acid between BF4 and BL1. In other words amino acids composition of BF4 is more similar to that of AS019. These results are consistent with the previous observations, which indicate that

1 Among the three corynebacterial species tested *C. glutamicum* strain AS019 and *B. flavum* BF4 were less sensitive to inhibition by INH and ETH (similar values of MICs 16mg/ml for INH) where as *B. lactofermentum* BL1 was more sensitive to inhibition by these two analogs (MICs 4mg/ml for INH). This may indicate the close relatedness between the cell structure of the two strains, AS019 and BF4, but at the same time it may also reflect the role or activity of the InhA proteins in these species.
2 Studies of the Jang *et at.*(1997) on the restriction modification barriers also indicated that methylation patterns in *B. flavum* strain BF4 are similar to those of *C. glutamicum* strain AS019, whereas *B. lactofermentum* showed different methylation patterns on genomic DNA..

Two possibilities could explain the differences in amino acid sequences between these corynebacterial species. Firstly, a few point mutations in InhA proteins have arisen which could have lead to the differential activity of the InhA proteins. This possibilty is supported by our results, which indicate that point mutations could result in distinguishable differences between AS019, BF4 and BL1.

Secondly, genetic polymorphism might exist between different corynebacterial species used in this study.

Our results indicate the presence of one copy of the *inhA* gene in *M. smegmatis* but more than one copy of this *inhA* gene in *C. glutamicum*. Certainly more copies of the gene will lead to more enzymatic activity and hence more drug would be needed to inactivate the organism, which may unravel the mystery of why *C. glutamicum* is naturally more resistant to INH compared to *Mycobacterium*. Alternatively, mutations that cause the over-expression of InhA (for example, in the *inhA* gene promoter or in a regulatory gene) or gene amplification could also mediate the increase level of resistance to the drug.

The hypersensitivity of *B. lactofermentum* to INH and ETH relative to *C. glutamicum* can also be explained by a lower copy number of the *inhA* gene in BL1 but evidence for this is not strong because Southern results with *Hind*III genomic DNA digestion showed one major band and few minor bands in BL1 but *Bgl*II genomic DNA digestion showed a similar pattern for BF4 and BL1, however, *Bgl*II digests indicated the same copy number in the three corynebacterial species tested. This variation can simply be explained as a result of heterologous nature of genome of the three species. However, these data indicate the presence of multiple gene copies in these species, suggesting that INH MICs and gene copy may not be related, although all of the corynebacterial species were more highly resistant to INH then *Mycobacterium* species.

6.5.3 Properties of amino acid sequences of InhA proteins

In the previous two chapters, a subgenomic library was constructed for two corynebacterial strains, AS019 and MLB194, and an unsuccessful attempt was made to sequence the entire *inhA* positive clone. Although the *inhA* sequences were amplified from these clones, the exact position of the gene in these clones was not located so this study failed to determine what other genes are located in upstream and downstream regions of *inhA*. However, the results indicated that the deduced amino acid sequences of InhA proteins have high homology with InhA proteins of mycobacterial species, envM proteins of *E. coli* and *S. typhimurium*, Enoyl acyl carrier proteins of *B. napus*, and *P. aeruginosa*. All these proteins are thought to participate in fatty acid biosynthesis. The location of the *inhA* gene copies in the

corynebacterial genomes may help to determine the role of this gene in fatty acid and mycolic acid synthesis including the regulation of this gene in cognate operons. This is the subject of another Ph.D thesis in our laboratory.

The similarities of InhA proteins with several other bacterial and plants proteins involved in fatty acid biosynthesis is consistent with the Southern results which indicated that multiple copies of the gene are present in different corynebacterial species. *Mycobacterium* and *Corynebacterium* are two closely related species in terms of the presence of the mycolic acids; however, mycolic acid composition of the two is different, where the latter has relatively simple mycolic acids. Although the fact that more copies of the *inhA* gene are present in corynebacteria explains the different behaviour of two bacteria towards INH but at the same time may reflect a possible different role of InhA proteins in *Mycobacterium* and *Corynebacterium*. Although the present study did not attempt to assay InhA activity, this should be done in future work to determine the difference between gene dose, expression and enzyme activity.

Chapter 7

Disruption of *inhA* gene by mean of homologous recombination and characterization of mutants

7.1 INTRODUCTION

The studies reported in previous chapters identified the presence of a gene encoding for an *Enoyl*-acyl carrier protein (ACP) reductase in *C. glutamicum* and related corynebacterial species. The gene was sequenced and homology searches results showed clearly its relationship to *inhA* genes of the mycobacterial family. In mycobacteria *Enoyl*-ACP reductases catalyses the nucleotide (NADH)-dependant reduction of the double bond at position two of a growing fatty acid chain linked to ACP, an enzymatic activity common to all fatty acid biosynthetic pathways. Mycobacteria utilise the products of InhA catalysis to create mycolic acids, which are important components of their cell wall. The taxonomic relatedness of mycobacteria to corynebacteria in particular has already been mentioned in chapter1 (section 1.4). The deduced InhA protein in corynebacteria are likely to play a similar role in mycolic acid biosynthesis, but the nature of this role has not been determined previously in *Corynebacterium* species. However, several differences were observed in terms of copy number of the *inhA* gene and different levels of INH resistance seen between *C. glutamicum* and two *Brevibacterium* species. Therefore it was important to investigate the functional role of the *inhA* gene, which shows extremely high similarity to mycobacterial *inhA* genes. Elucidation of the function of such *inhA* genes will require multiple approaches, including classical biochemical approaches such as enzymatic assays with InhA proteins, as well as molecular genetic approaches including cloning of the genes, targeted gene disruption and testing of biochemical and physiological consequences. An enzymatic assay with InhA protein is not possible unless complete functional enzyme is isolated. Therefore, our intention was to inactivate the *inhA* gene in *C. glutamicum* and analyse the resulting mutants to deduced correlation between genetic and physiological changes. This chapter describes several approaches to gene inactivation and the resulting mutants obtained.

7.2 ESTABLISHING A METHOD FOR DISRUPTION OF THE *inhA* GENE IN *C. GLUTAMICUM*

The first part of this work was done to establish parameters necessary for introducing genes: determining antibiotic sensitivity to select which marker genes could be used in this background and establishing gene transfer systems such as conjugation / electroporation with the strains under study.

7.2.1 MICs

The MICs of the antibiotics were determined prior to introduction of plasmids into *C. glutamicum* strains. The genetic exchange methods for *C. glutamicum* employing bi-parental matings and electroporation were performed in sections 2.3.5. 1. The MICs for antibiotics used in counter selection of transconjugants or transformants were determined using LAG (Luria agar) supplemented with 2% glucose, BHIA and ET at 30°C using a range of concentrations of selected antibiotics (5 to 100 µg/ml) (Table 7.1). *C. glutamicum* displayed higher MICs for all strains when using BHIA and ET compared to LA and the concentrations used to select transconjugant strains were chosen on the basis of these antibiotic sensitivity patterns. The *C. glutamicum* strains showed strong resistance to nalidixic acid and sensitivity to kanamycin and chloramphenicol, as expected from their known phenotypes and lack of plasmids. The *E. coli* strains showed sensitivity to nalidixic acid with few colonies appearing at 10 µg/ml. The experimental results indicated that 50 µg/ml of Km and 100 µg/ml of nalidixic acid could be used to select transconjugants in rich media and to suppress the growth of parental strains after conjugation or electroporation for the tranconjugants (Table 7.2).

Table 7.1 Determination of MICs of selected antibiotics. The antibiotics were used at concentrations of 5, 10, 20, 30, 40, 50, 100 μg/ml in LAG plates supplemented with 2% glucose and results recorded after incubation at 30°C for up to 48-72 h and the numbers shown are MICs.

Antibiotics μg/ml	AS019	MLB133	BL1	RM4	*E. coli* S-17-1	S17-1 with pK18*mob*
Kanamycin	–[a]	–	–	–	–	100
Rifampicin	50	30	–	–	25	25
Chloramphenicol	–	–	–	–	–	20
Nalidixic acid	100	90	100	100	10	10

[a]– means MIC < 5 μg/ml

Table 7.2 Selection of antibiotic markers to introduce mobilizable plasmids carrying internal fragments of the *inhA* gene. The symbols indicated that (+) for growing and (-) for non-growing status.

Strains	Nalidixic acid[a]	Kanamycin	Chloramphenicol	Kanamycin+ Nalidixic acid
E. coli S17-1 pK18*mob*::*inhA*	–	+	–	–
E.coli LysA::int	–	+	+	–
C. glutamicum	+	–	–	–
Transconjugant	+	+	+	+

[a] Antibiotics concentrations (μg/ml): Nalidixic acid (50-70), Km (50), Cm (7.5)

7.2.2 Failed attempts and strategies for *inhA* gene disruption

This section describes the use of methods and selection media adopted by different research groups for the genetic engineering of *C. glutamicum* by mean of gene disruption and their application in attempt ion to obtain mutants with the *inhA* gene disrupted..

7.2.2.1 Construction of mobilizable pK18*mob-inhA*

Figure 7.1 shows the restriction map of suicide vector pK18*mob*. This plasmid was supplied from Schäfer *et al.* (1994) and was used because of the following advantages

1. it carries the Tn5 Km^r marker;
2. it is small and offers a higher cloning capacity;
3. the pK vector carries unique multiple cloning sites (MCS) and plasmids carrying insertions cloned into the MCS can easily be identified using the Lac colour reaction;
4. Inserts can be directly sequenced using the standard M13 sequence primers.

For gene disruption experiments, a 700 bp internal fragment of the *inhA* gene was amplified from chromosomal DNA of AS019. The PCR product was ligated into mobilizable vector pK18*mob*, which was previously digested with *Pst*1 (cutting in the MCS). The ligation mixture was transformed into *E. coli* S17-1 (Simon *et al.*, 1983), a strain with an RP4 derivative in the chromosome, which provides the transfer functions necessary for mobilization. Transformants were selected on LAG agar plates containing X-gal, IPTG and 50 μg/ml of Km to allow blue and white colony screening. The positive clones were checked for the presence of insert by

1. *EcoR1* restriction digestion
2. By sequencing using M13 primers.

The positive clones carrying *inhA* internal fragments were designated pK18*mob-inhA*, which carries an *inhA* gene internal fragment and are efficiently mobilisable by *E. coli* S17-1.

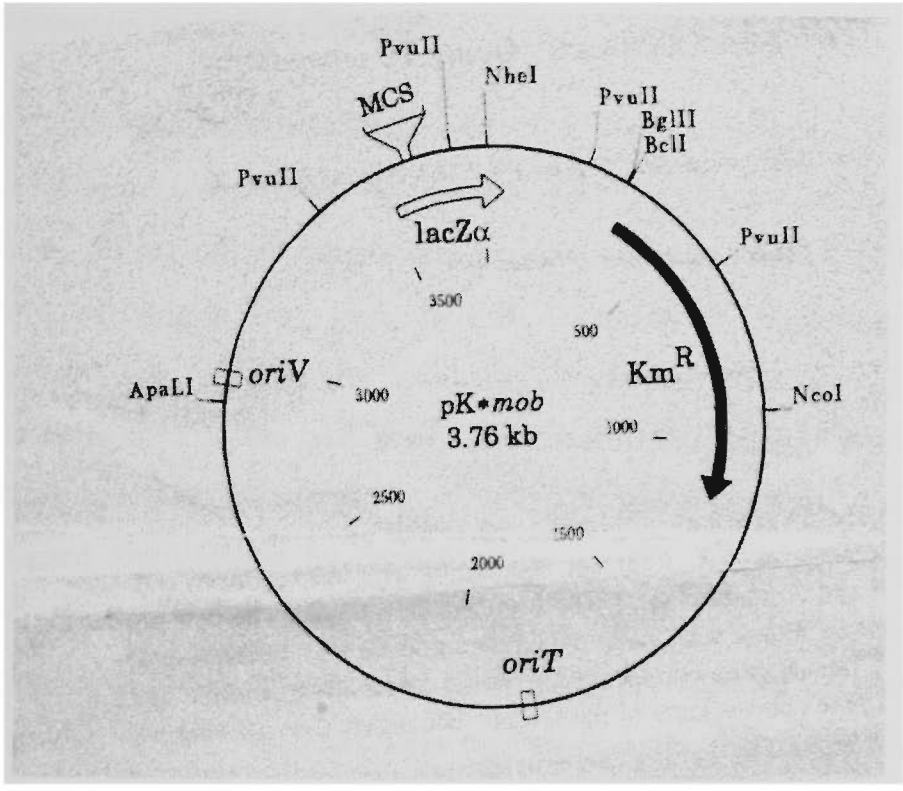


Figure 7.1 Detailed physical map of the mobilizable pK18*mob* Schäfer *et al.* (1994). The Km^R gene, and the *lacZ* fragment are represented by arrows indicating the direction of transcription. The *oriV* and the *oriT* (as the part of RP4mob) are shown as small boxes. The asterisk indicates the plasmid labels 18 or 19 respectively. Unique restriction sites for cloning purposes in the MCS of pK18*mob* are: *EcoR1*, *SmaI*, *BamHI*, *XbaI*, *Sal*1, *Pst*1, *Sph*I and *Hind*III. The universal primer-binding site is located downstream from the *Hind*III site and the reverse primer binds upstream the *Eco*R1-site of the MCS in the *lacZ*α fragment. The plasmid pK18*mob* is efficiently mobilizable by *E. coli* S17-1.

7.2.2.2 Transconjugation

For *C. glutamicum* and related species, RP4-mediated intergeneric conjugation is the most efficient system to introduce foreign DNA (Schäfer *et al.*, 1990). Initially attempts were made to transfer the pK18*mob-inhA* into strains *C. glutamicum* and *B. lactofermentum* by transconjugation.

For conjugal transfer of pK18*mob-inhA* to coryneform recipient strains, donor strain *E. coli* S17-1 pK18*mob-inhA* were grown to the late exponential phase in LB medium containing 50 µg/ml of kanamycin per ml. In the control experiment plasmid pSUP301 carrying a *lysA* gene internal fragment (the resulting vector is called p301*lysA*-int) was mobilized by S17-1 into *C. glutamicum* strain AS019 to disrupt the chromosomal copy of the *lysA* gene. Recipient strains were grown in LB medium to an optical density at 580 nm of 3 to 4. About 5 x 10^8 donor and 3.5x10^9 recipient cells were mixed and pelleted by centrifugation at 20°C for a short time. The mating mixture was then resuspended in residual LB medium and carefully spread onto 0.45 µm pore size cellulose acetate filter placed on a pre-warmed LA plates. After 20 hrs of incubation at 30°C, the cells were washed from the filter with 1ml of LB medium and mechanical agitation. Transconjugants were selected by plating onto LAG containing 25 µg/ml of kanamycin and 50 µg/ml of nalidixic acid. Alternatively, the mixture was also plated on BHIA and LAG plus fatty acids and mycolic acids extract from normal cells. Since the *inhA* gene is supposed to inhibit mycolic acid biosynthesis it was likely that its inactivation might be lethal for cells and resulting transconjugants might need some mycolic acids or fatty acid supplement into the growth media for their survival. Mycolic acids are not available commercially therefore mycolic acids and fatty acids extract was prepared from *C. glutamicum* strains as described in section 2.4.2.3 and spread on BHIA and LAG plates containing antibiotic.

Recipient cells of *C. glutamicum* strain AS019 was reported to have low fertility in intergeneric mating (Schäfer *et al.*, 1990) because of the presence of an effective restriction system. Therefore the cells were subjected to heat treatment for 9 mins at 49°C to inactivate the restriction barrier. It was reported that *B. lactofermentum* strains showed satisfactory mating with *E. coli* for insertional mutagenesis of the

desired genes using internal fragments as mutagenic tools (Chater and Burton, 1983) therefore these cells were not heat-treated.

7.2.2.3 Results of the Transconjugation

The transconjugation mixture for pK18*mob-inhA* was plated on LAG and BHIA plates as above. Parental strains did not appear on selection media but also no transconjugants colonies were isolated after 36-72 h on antibiotic plates. Owing to the fact that pK18*mob* does not contain a corynebacterial origin of replicon, transconjugants can only be recovered after a single recombination event between the plasmid borne *inhA* gene and the chromosomal gene. Failure to obtain any viable colonies indicated that the integration event has not taken place. However, in the control experiment using S17-1 (p301*lysA*-int) donor and *C. glutamicum* recipient cells, several transconjugant colonies appeared. Plasmid p301*lysA*-int is not able to replicate into *C. glutamicum* but a single recombination event between the plasmid borne *lysA* gene fragment and the chromosomal copy would lead to incorporation of the vector into the genome. As a result of integration, the *lysA* gene will be split into two incomplete genes lacking either 5′ or 3′ terminus. The transconjugants appeared at frequencies of $5x10^2$. The number of transconjugants obtained with *B. lactofermentum* was 5-10 fold higher than with AS019 after heat treatment. Transfer frequencies were expressed as the number of transconjugants per final donor colony. The transconjugants were grown on minimal media and supplemented with (10 µg/ml) kanamycin and lysine (0.3 mM). Transconjugants were resistant to kanamycin and auxotrophic for lysine. Free plasmid DNA was not detected after alkaline lysis of few randomly chosen clones on agarose gel electrophoresis.

7.2.2.4 Southern hybridisation to confirm integration of *lysA*

Southern hybridisation was carried out to confirm the integration of p301*lysA*-int in the genome of AS019. Two transconjugants T1 and T2 were selected. Genomic DNAs were purified using Nucleospin kit as per the manufacturer's instruction, digested with *Pst*1 and transferred to a nitrocellulose membrane as described in section 2.3.7. Probe pSUP301 DNA and p301*lysA*-int plasmid DNA were radiolabelled with α^{32}PdCTP (3,000 C1/mmol, Brestech and hybridisation was

performed) as described in section 2.3.7. The membrane was washed with SSPE buffer containing 0.1% SDS at room temperature and exposed to Kodak X-ray film at -80°C and developed as described in section 2.3.3. As seen in Fig 7.2, the *Pst*1 cleaved chromosomal DNA of transconjugants showed two signals (MW 7.3 and 1.6 kb), which is in accordance with the previously reported results of Schwarzer and Pühler (1991).

These results demonstrated that p301*lysA*-int had successfully integrated into the genome of *C. glutamicum* by homologous recombination thus splitting the chromosomal copy of *lysA* into two parts. This control experiment provides substantial evidence that the transconjugation method had worked efficiently for *lysA* but not for introduction of *inhA*.

The transconjugation process failed to isolate transconjugants with *inhA* disruption although all the necessary parameters reported by other researchers were taken into consideration. This failure would be attributed to several factors including.

- No integration had occurred because of the unsuccessful introduction of plasmid DNA into the host system, possibly, due to restriction barriers or cell-to-cell contact during mating failed.

- Integration had occurred and disruption of the *inhA* gene leads to the occurrence of a lethal mutation. If InhA is involved in mycolic acids biosynthesis as occurs in *Mycobacterium* species, then transconjugants might lack mycolic acids and therefore ordinary media cannot support their growth.

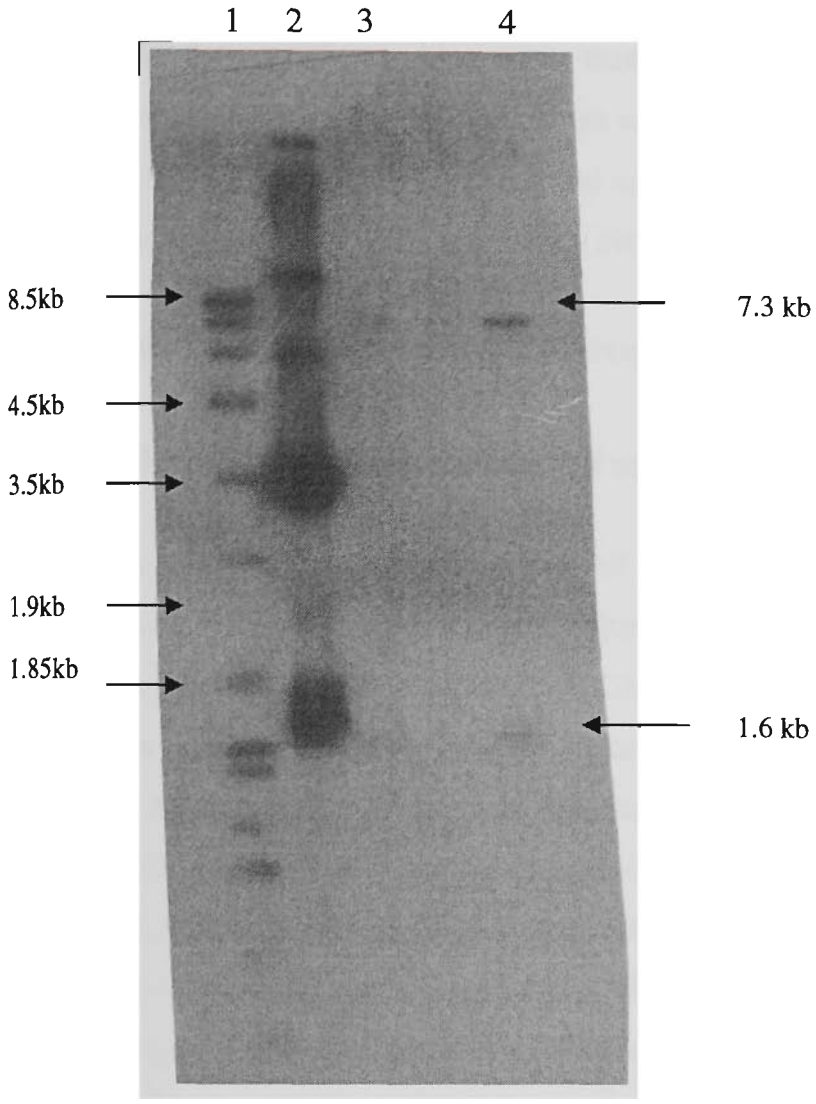


Figure 7.2 Southern analysis of the *lysA* disrupted mutants of AS019. Lane 1: Spp1/*Eco*R1 markers; Lane 2: Plasmid pSUP301 containing *lysA* gene; Lanes 3 to 4; Total DNA isolated from the AS019 *lysA*$^-$ mutants T1 and T2. digested with restriction enzyme *Pst*1 and probed with radio-labelled p301*lysA*-int. Two signals corresponding to the MWs of 7.3 and 1.6kb appeared in mutants confirming the previously reported results of Schwarzer *et al.* (1991).

Attempts to obtained transconjugants of *C. glutamicum* showed limited or no success, as no transconjugants were obtained for *inhA* inactivation on LAG, BHIA or various other selective media. Also few transconjugants were produced in control experiments and very poor efficiency was seen when AS019 was used as recipient for *lysA* and *inhA* disruption. This promoted an examination of using electroporation and ET media developed by Britz and Best, (1986).

7.2.3 Gene disruption using electroporation

7.2.3.1 Electroporation and selection of mutants

Electroporation is an efficient mean of introduction of plasmid DNA into *C. glutamicum*. ET is the regeneration medium designed for the recovery of protoplasts based on similar media described by Chang and Cohen (1979) and Gabor and Hotchkiss (1979). Using various combinations and concentrations of the constitutes Best and Britz (1986) showed that lowering phosphate levels and $CaCl_2$ resulted in more rapid regeneration of protoplasts. Sodium succinate, the osmotic stabiliser, was used at 0.3 M as higher concentrations, inhibited growth of protoplasts and, indeed, untreated cells of these particular strains. This media was also used for the selection and facilitated recovery of electrotransformants Hynes and .Britz (1990).

For electroporation cells of several different strains of corynebacteria were grown at 30°C in LBG containing appropriate concentrations of INH and glycine to the desired A_{600} (0.2 to 0.4) and harvested by centrifugation. The recipient cells were washed, concentrated 10-fold and finally resuspended in 15% glycerol. The concentrated cells (40 µl) containing approximately 10^9 cells were transferred to a cold polypropylene tube. One to five 5 µg of DNA were mixed with the cell suspension and electroporation was performed as described in section 2.3.4. A single pulse (2.5kv, µF) was applied and then the cells were diluted with recovery media (LBG+$CaCl_2$+$MgCl_2$) described in section 2.2.2. After incubating at 30°C, the mixture was plated onto ET, LAG, BHIA, LAG plus fatty acid and mycolic acids media containing Km+Nx (25 and 50µg/ml) and incubated at 30°C for 2 to 3 days. In the control experiment, plasmid PCR2.1 containing the *csp1* fragment from *C.*

glutamicum was mixed similarly and electroporation performed as described for pK18mob-*inhA* plasmid.

After 72 hour of incubation, plates were examined and transformants appeared for both control and for the experimental gene. However, it was interesting to note that for the control (i.e. *csp1*) mutants appeared on LAG, BHIA and ET plates but *inhA* mutants appeared only on ET plates. Since ET is a rich media and it increases the MICs, exclude the possibility of contaminants transformants were subcultured on ET plates containing higher concentrations of antibiotics (50 µg/ml of Km and 80 µg/ml of Nx). Transformants of strains AS019, RM3, RM4 and BL1 grew on successive subculture on Km-Nx-ET indicating that integration of the plasmid pK18*mob-inhA* has probably integrated into the chromosome by homologous recombination.

7.2.3.2 Proof of integration

The integration event is explained in Figure 7.3. When insertional mutation by homologous recombination occurs successfully, the suicide vector used is integrated into the region that is homologous between the insert of the suicide vector and the chromosomal DNA of recipient strains. As a result of the integration of a suicide vector, the gene will generate two incomplete genes lacking either 5′ or 3′ terminus of the coding region. Transformants were Km resistant and Gram positive. Free plasmid DNA was not detected after alkaline lysis from 14 randomly chosen clones of BL1 transformants named mob 4, 5, 7, 8, 13, 14 for Southern analysis. "Although *inhA* disrupted mutants were isolated from *C. glutamicum* strains AS019, RM4 and MLB133 and some preliminary characterization was undertaken, these mutants did not survive long-term storage in glycerol broth. Mutants of BL1 did survive so that all of the analysis was performed using these mutants."

To confirm the presence of relative location of pK18*mob-inhA* integration onto chromosome Southern blot hybridisation and several other biochemical test were performed. The genomic DNA from transformants and wild type BL1was isolated as described in section 2.4.4, digested with *Pst*1 and other enzymes, subjected to agarose gel electrophoresis, transferred to nylon membranes and probed with a radio-labelled *inhA* gene or pK18*mob-inhA*. Figure 7.4 a, b and c show *Pst*1, *Hind*III and *Eco*R1 digests of mob 4, 5, 6, 7, 8, 13, 14 probed with pK18*mob-inhA*.

Vector Construction

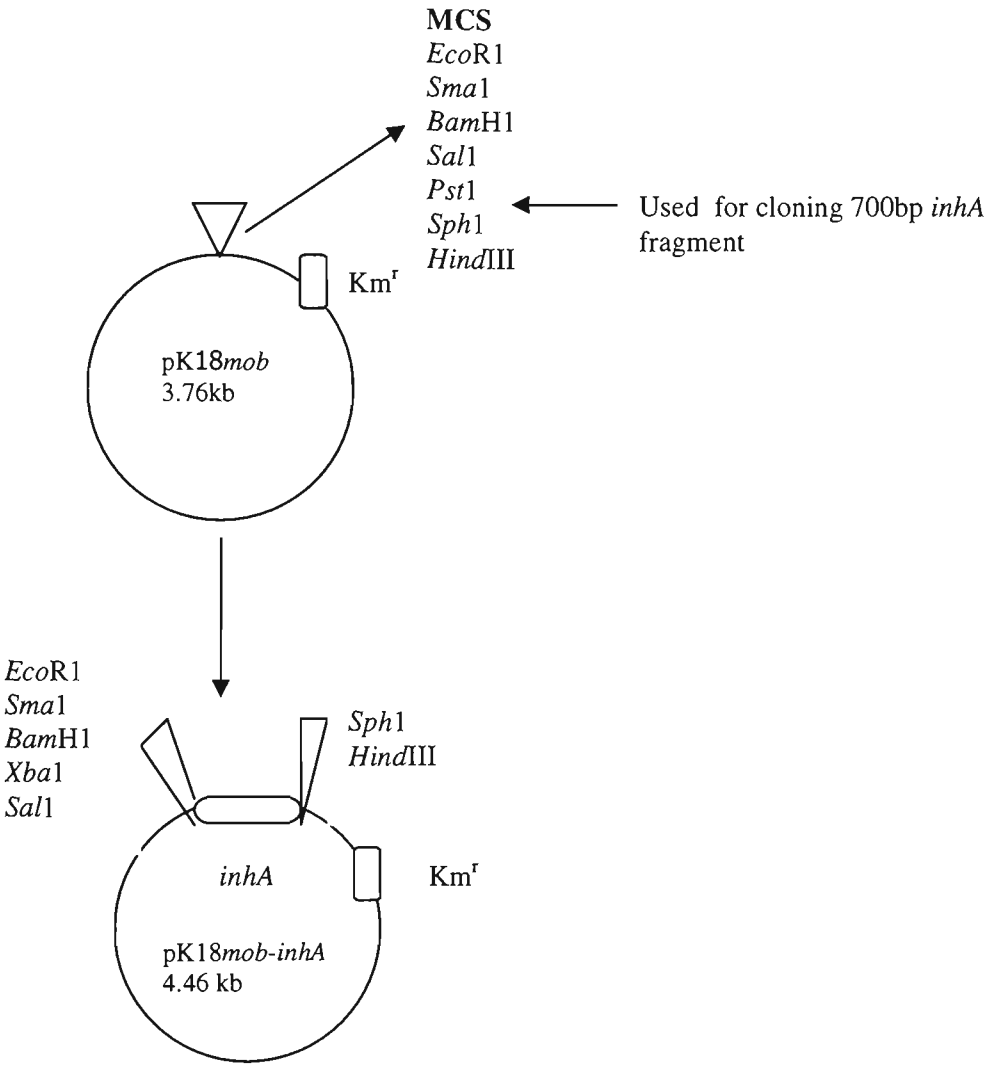
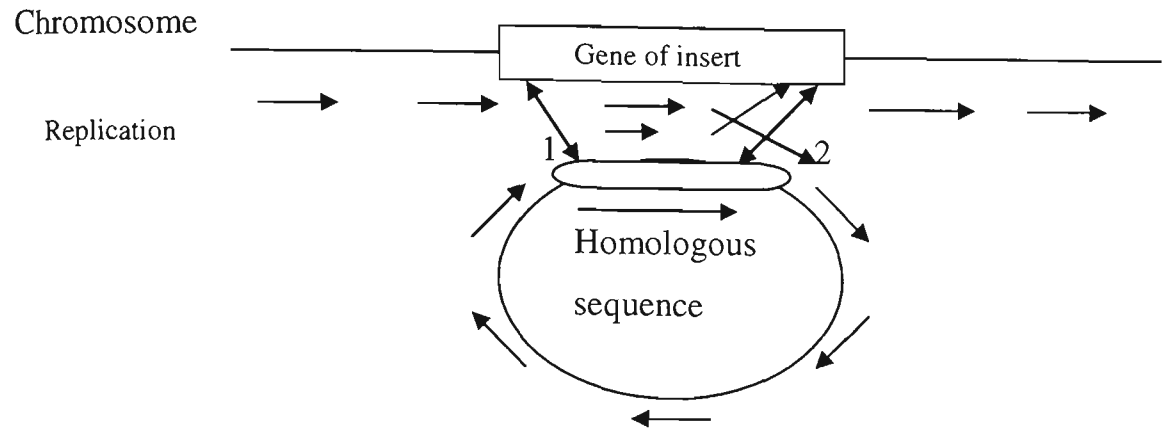


Figure 7.3 a Construction of mobilizable shuttle vector pK18*mob-inhA*. This vector was derived from mobilizable pK18*mob* . Plasmid pK18*mob* was digested with *Pst*1 at MCS and 700 bp internal fragment of the *inhA* gene was ligated into the MCS.

I



II

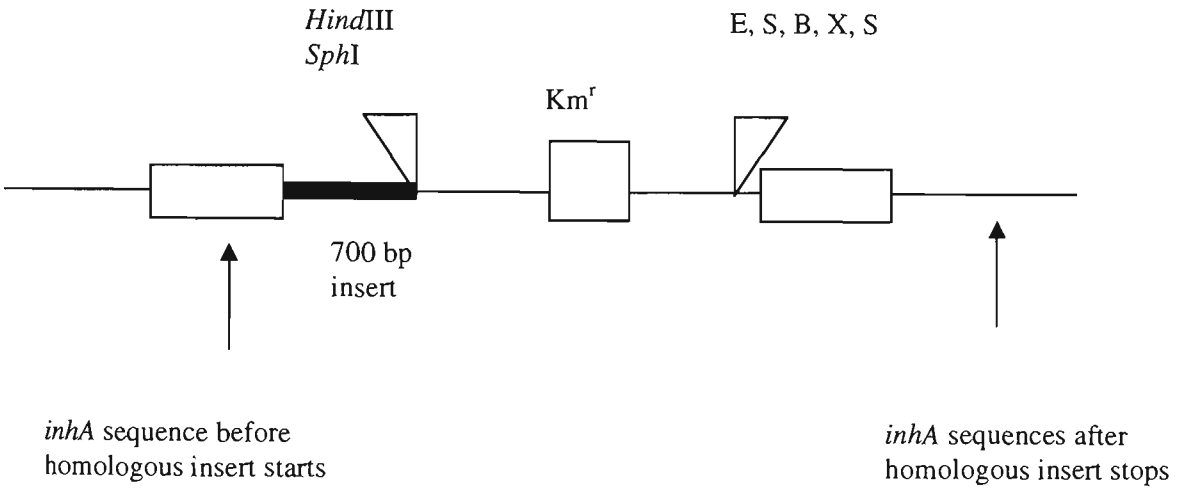


Figure 7.3 .b Scheme illustrating the recombinational integration and disintegration of *inhA* containing plasmids into the chromosomal DNA. **I** Homologous recombination between the *inhA* sequence in suicide vector and the chromosomal DNA. The arrows show the direction and course of DNA replication. Replication proceeds around vector from first point of homology 1 to 2 then back to the chromosome. **II**. The resulting structure of *inhA* gene inserted with 3.76 kb. MCS is introduced into the chromosome. Km is the kanamycin resistance gene.

To confirm the presence of relative location of pK18*mob-inhA* integration onto chromosome Southern blot hybridisation and several other biochemical test were performed. The genomic DNA from transformants and wild type BL1was isolated as described in section 2.4.4, digested with *Pst*1 and other enzymes, subjected to agarose gel electrophoresis, transferred to nylon membranes and probed with a radio-labelled *inhA* gene or pK18*mob-inhA*. Figure 7.4 a, b and c show *Pst*1, *Hind*III and *Eco*R1 digests of mob 4, 5, 6, 7, 8, 13, 14 probed with pK18*mob-inhA*.

The *Pst*1 digest of wild type BL1 strain of *B. lactofermentum* showed a 2.8kb major band with *inhA* gene. If integration has occurred at that position then in principle the size of the resulting *inhA* gene would be 2.8kb plus 4.45kb (3.8kb vector plus 0.7kb insert) add up to 7.3kb. In Southern hybridisation *Pst*1 digested genomic DNA of mutants (0.7kb PCR amplified *inhA* gene was ligated into *Pst*1 digested pK18*mob*, one *Pst*1 site is conserved) should show two hybridisation fragments with pk18mob-*inhA* probe and sum of expected DNA fragment should be 7.3kb. Fig 7.4a showed the results of *Pst*1 digested mutants lanes 3, 4, 8, 9 (mutants mob 4, 5, 13, 14 respectively). Lanes 3 and 4 showed only two signals corresponding to 2.8kb which showed no integration in mob 4 and 5 whereas in lane 8 and 9 (mob 13 and 14) two and three signals appeared. According to the Fig 7.4a the calculated mol.wt of the two divided *inhA* gene signals which appeared in mutants (mob13, 14 lane 8, 9) is 2.8 +1.6 kb and 2.8+ 2+1.6kb which adds up to 4.6 and 6.6 kb respectively. From this result it can be predicted that integration has occurred in mutants mob13 and 14 however, the sum of sizes of the two divided *inhA* gene signals is smaller than the expected 7.3 kb fragment.

As predicted in Fig 7.3b integration of vector would introduce MCS into the BL1 genome along with Km^r and this would be intact. If integration has occurred then in Southern hybridisation analysis of *Hind*III and *Eco*R1 genomic DNA digests of mutants two bans should be detected. With *Eco*R1 the genomic digest of BL1 showed two major bands of 6.1 and 5.2kb respectively. In mutants the two bands (6.1+4.4=10.7 or 5.1+4.5=9.6kb) depending on where integration has occurred should be seen. Similarly, *Hind*III digestion of BL1 genomic DNA showed one major band of 8.5kb with the *inhA* gene probe. And in mutants two fragments 8.5 plus 4.5kb that adds up to 13.5kbshould be seen.

Fig 7.4b showed the results of *Hind*III digestion of mutants. Only one mutant mob14 (lane 9) showed presence of integration where two bands can be seen. The band corresponding to 8.5kb is at higher position. This can be explained by that the mutants were growing on ET plates for DNA isolation moreover DNA is not digested properly.

Similarly, with *Eco*R1 the genomic DNA of mutants was not digested. As can be seen in Fig 7.4c transformant mob14 (lane 9) showed double band which are not separated so their sizes can not be measured

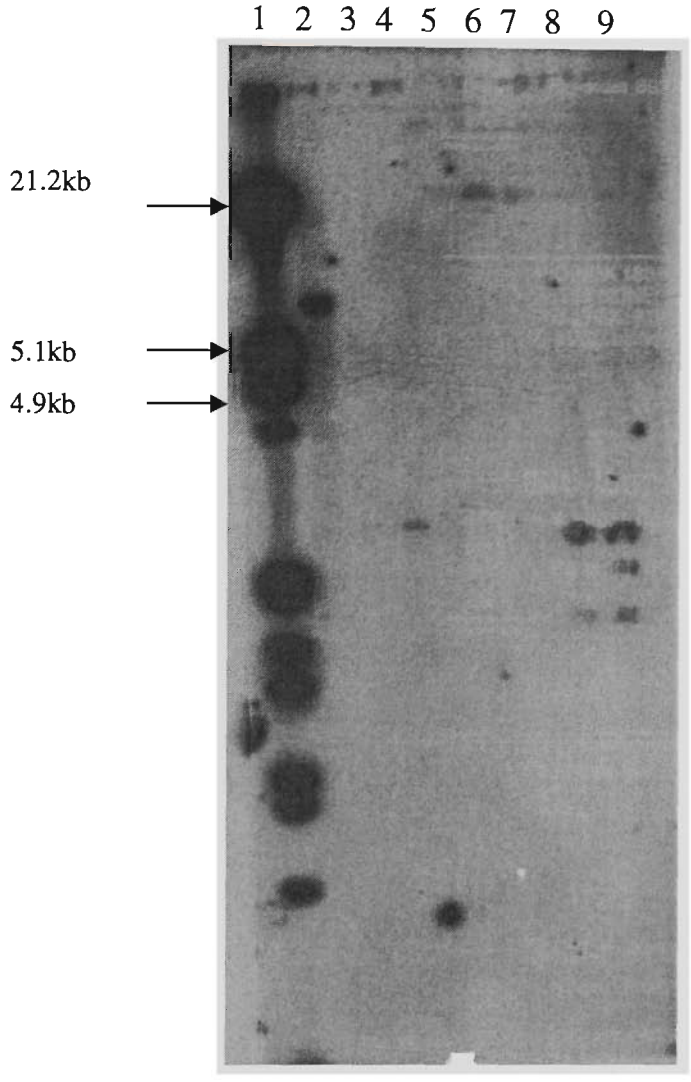


Figure 7.4 a Southern analysis of the putative *inhA*-disrupted mutants of BL1. Lane 1, Lambda *Hind*III/*Eco*RI markers; Lane 2, *Eco*R1 digested total genomic DNA of BL1; Lanes 3, 4, 8, 9 contained total genomic DNA of transconjugants mob 4, 5, 13, 14 digested with *Pst*1. Lanes 5, 6, 7 contained genomic digest of transconjugants mob 6, 7, 8, digested with *Eco*R1 restriction enzyme and probed with labelled pK18*mob-inhA* suicide plasmid.

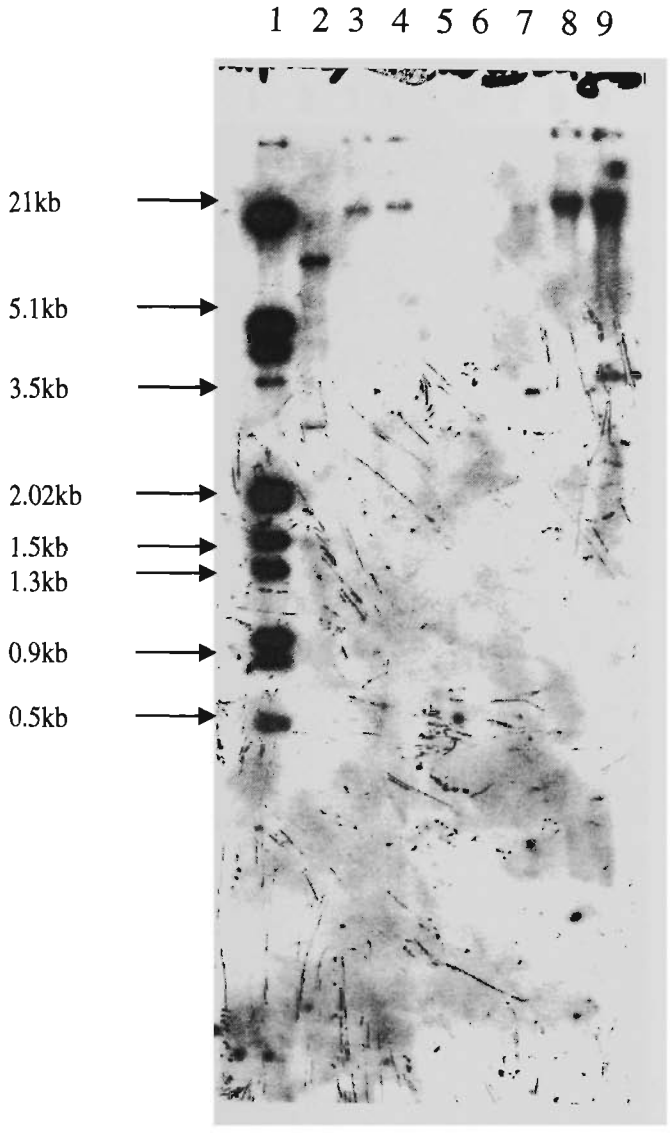


Figure 7.4b Southern analysis of the constructed mutants. Lane 1, Lambda *Hind*III/*Eco*R1 markers; Lane 2, *Hind*III digested total genomic DNA of BL1; Lanes 3 to 9 contained total genomic DNA of transformants mob 4, 5, 6, 7, 8,13, 14 digested with *Hind*III restriction enzyme and probed with labelled pK18*mob-inhA* suicide plasmid containing corynebacterial *inhA* gene.

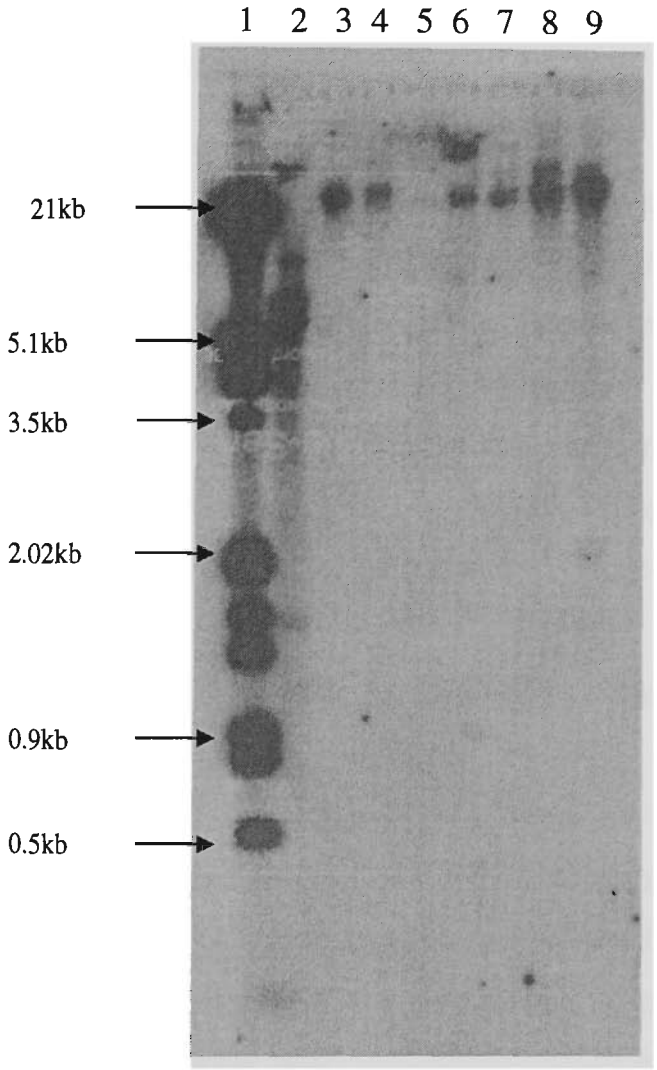


Figure 7.5c Southern analysis of the putative *inhA*-disrupted mutants. Lane 1, Lambda *Hind*III/*Eco*R1 markers; Lane 2, *Eco*R1 digested total genomic DNA of BL1; Lanes 3 to 9 contained total genomic DNA of transconjugants mob 4, 5, 6, 7, 8, 13, 14 digested with *Eco*R1 restriction enzyme and probed with labelled pK18*mob-inhA* suicide plasmid containing corynebacterial *inhA* gene.

7.3 PRELIMINARY CHARACTERISTICS OF THE TRANSFORMANTS

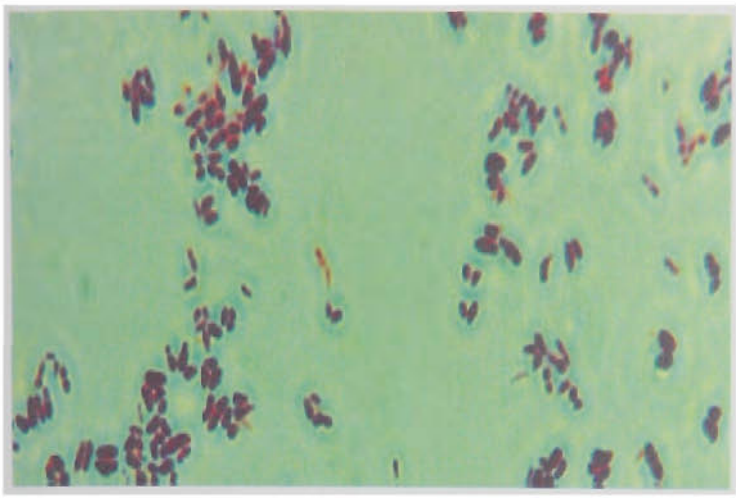
7.3.1 Gram staining

Both parents and transformants were Gram stained to compare them with respect to their morphology. To get an overall view of cells, samples were examined (using a light microscope) and photo taken. The morphology of transformants after growth on ET plates was found to be a mixture of round, oval and irregular shaped.cells compared with their parents, which are a mixture of round, short and long rods. Change in morphology of the mutants strongly suggests that gene disruption has a profound effect on cell wall formation. These mutants have defective cell wall.

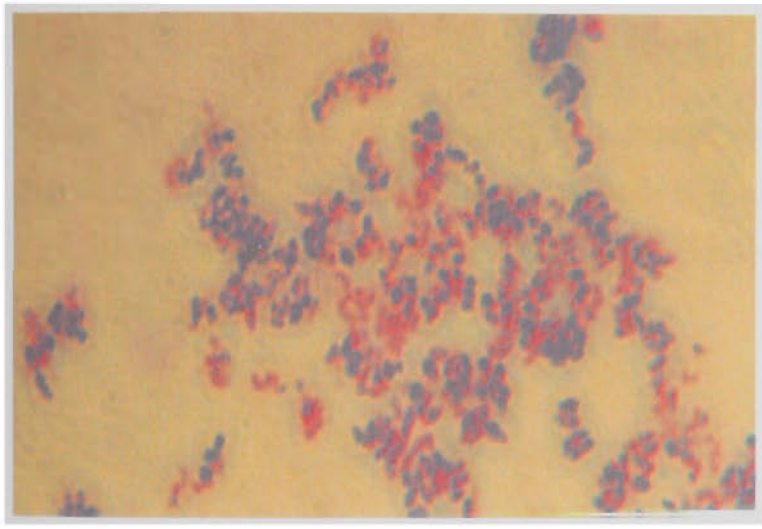
7.3.2 Inability of the mutants to grow on normal media

The use of media emerged as an issue for the selection of transformants in early experiments and success occurred due to the use of osmotically protective media. Therefore it was important to study the growth of these mutants on different media for their characterisation. Both parents and mutants were streaked onto LBG, BHI, NA and Baired Parker agar. On Baired Parker agar, the *C. glutamicum* strains formed black colonies, the mutants failed to grow but little black spots at the points of streaking indicated that these mutants tried to grow but failed. Similarly, other media, which supplement the growth of normal cells failed to support the growth of these mutants indicating they have altered cell wall surfaces and are osmotically sensitive, therefore these mutants need osmotically protective media to grow. The sodium succinate, $CaCl_2$ and $MgCl_2$ in the ET media provides osmotic protection and helps growth. Figure 7.6 showed the growth of parents and selected transconjugants on LAG and ET media.

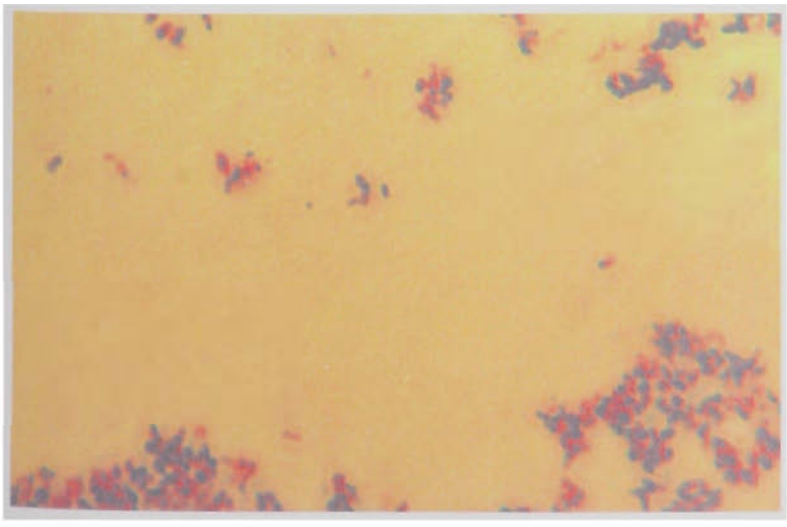
a



b



c



d

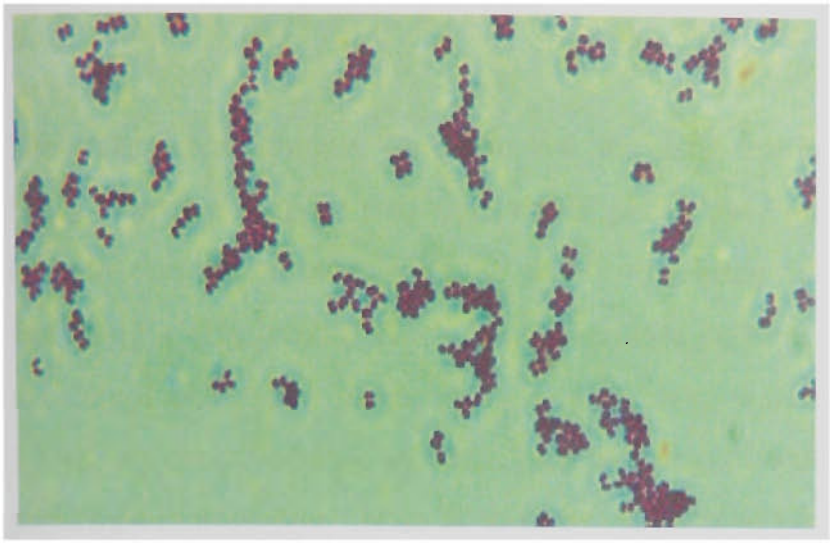


Figure 7.5 Gram staining of parents and mutant strains of *B. lactofermentum* BL1 following growth on different media. Plates a, b and c show BL1 grown on LAG, LAG plus 2% glycine and Baired Parker agar, respectively. Plate d shows mutant strain mob13 grown on ET agar.

7.3.3. MICs of mutants for INH and ETH

The constructed mutants were checked for their sensitivity to INH by performing MICs. For that purpose two types of ET plates were prepared: ET plates containing different concentrations of INH, ET plates containing different concentrations of INH and 50 μg + 80 μg/ml of Km + Nx. Overnight cultures of parents and mutants were transferred onto these plates using a multiprong replicator (as described in section 2.3.4). As described in Chapter 3, light and heavy inocula were used and tests were performed in duplicate. After 24 to 48 hrs, growth was recorded. Table 7.3 shows the MIC results of both parents and mutants. It is very interesting to note that mutants showed similar MIC values for INH and ETH as seen for the parent BL1 and no variations were observed as seen between AS019 parent and mutants MLB133 and MLB194. These mutants in addition to resistance to Km also showed resistance to INH higher than the parent BL1. Photographs of MICS plates are taken and are shown in Figure 7.6.

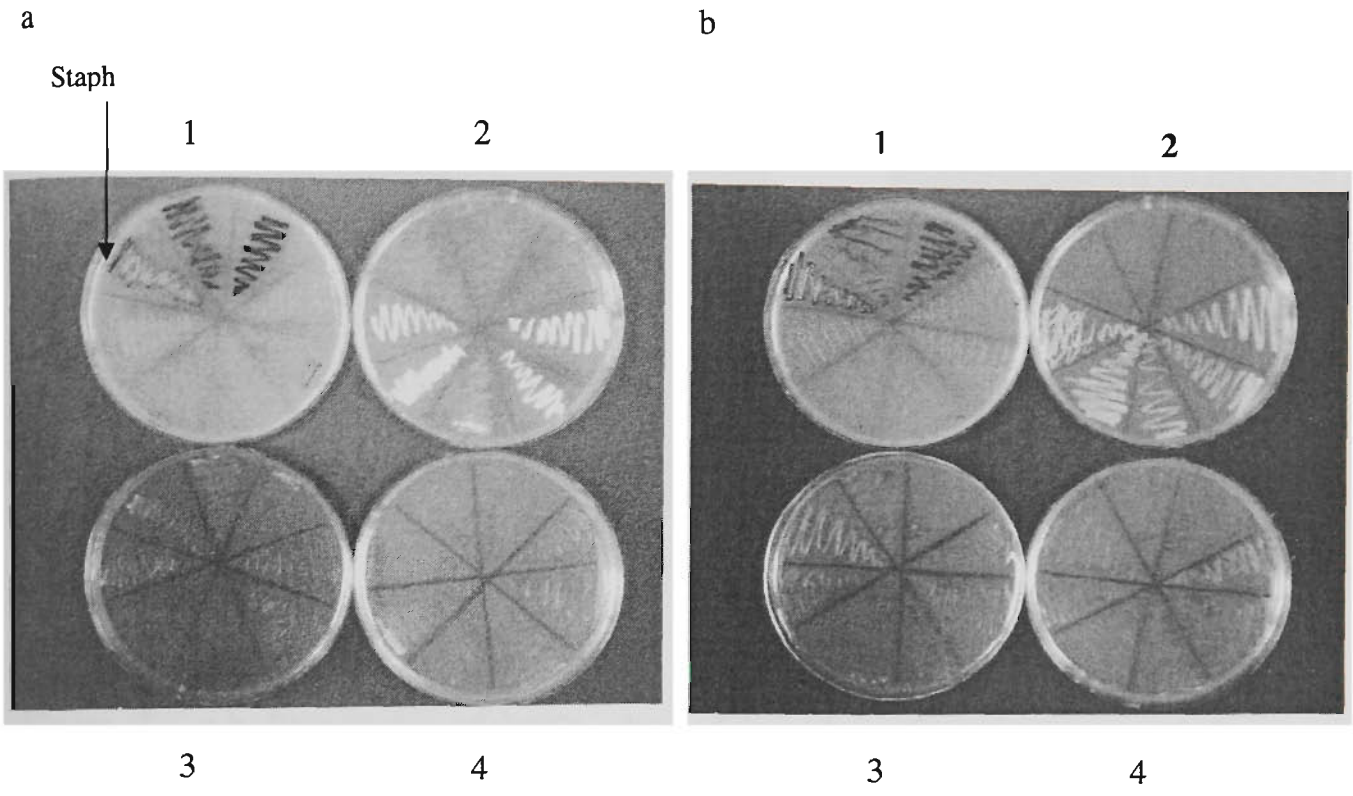


Figure 7.6 Growth of *Staphylococcus, C. glutamicum* strain AS019, *B. lactofermentum,* and mutants of BL1 (clock wise) on different media.

a Staph, AS019, Bl1, mutants mob 4,5 6, 7, 8.

b staph, AS019, BL1, mob 4,5, 13, 14, PE11, PE1.

Plate 1.Growth on Baired Parker agar AS019, BL1 formed black colonies. *Staphlococcus* can be differentiated by dark shinny colonies. Clear areas represent no growth. tranformants failed to grow on Baired Parker agar.

Plate 2.Growth on ET plates containing 80 µg/ml Km and 100 µg/ml Nx.

Plate 3.Growth on LAG Plates containing 80 µg/ml Km and 100 µg/ml Nx.

Plate 4.growth on BHI brain heart infusion media containing 80 µg/ml Km and 100 µg/ml Nx.

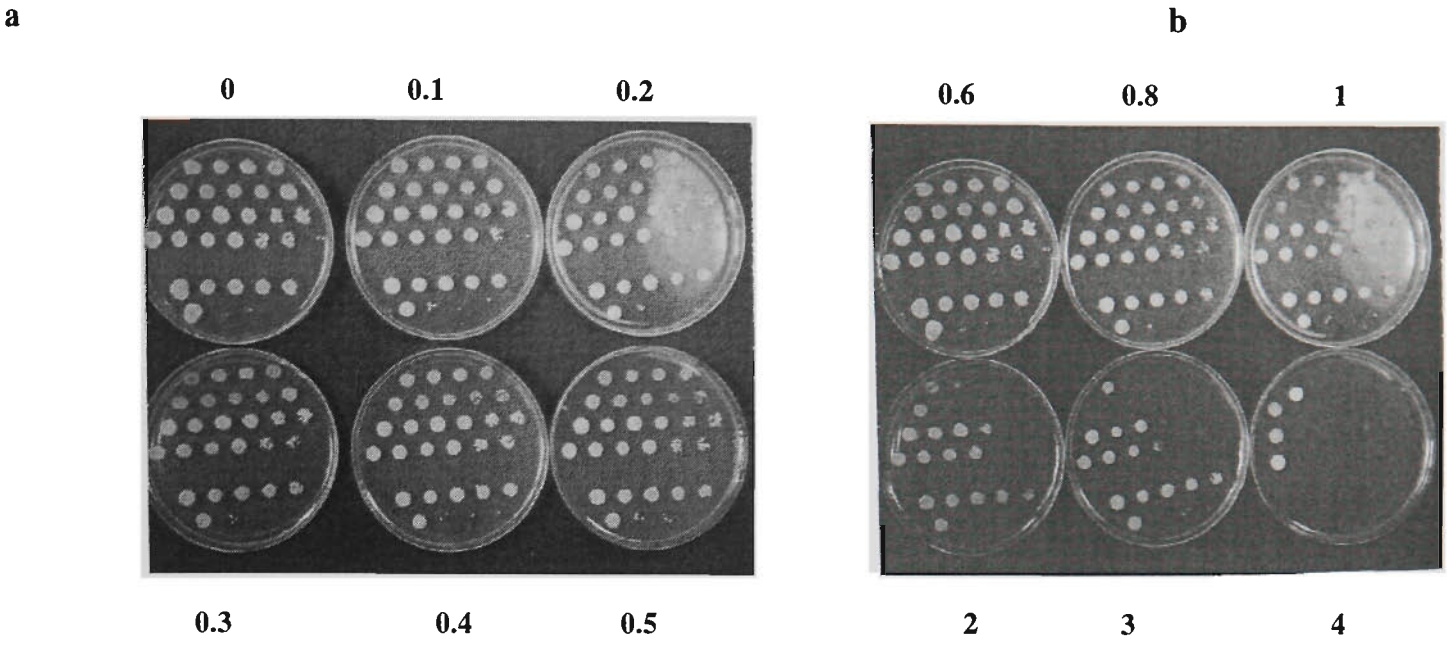


Figure 7.7 a,b Photograph of MICs plates for *inhA*-inactivated mutants of *B. lactofermentum* (mob 4, 6, 8) and parent strains BL1. A multiprong replicator was used to transfer small drops of overnight cultures and dilution onto INH-containing ET plates. The control plate (0) did not contain any INH. These plates have increased INH concentration from (0-4 mg/ml). Bacterial isolates were as follow (identical on each plate).

Row 1-2 BL1 undiluted, then dilution 10^{-2} to 10^{-4}

Row 3 mob4 undiluted, then diluted 10^{-2} to 10^{-5}

Row 4 mob6 undiluted, then diluted 10^{-2} to 10^{-6}

Row 6-7 mob8 undiluted, then diluted 10^{-2} to 10^{-6}

Table 7.3 INH MICs for *inhA* inactivated mutants of *C. glutamicum* in ET medium.

Strains	INH concentration in mg/ml															*MICs mg/ml*
	0.8	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
BL1	+++[a]	++	++	++	+	..	..	_								4
RM4	+++	++	++	++	+	..	..	_	_	_	_	_	_	_	_	4
Mob6	+++	++	++	++	+	..	..	_	_	_	_	_	_	_	_	4
Mob8	+++	++	++	++	+	..	..	_	_	_	_	_	_	_	_	4
Mob13	+++	++	++	++	+	..	..	_	_	_	_	_	_	_	_	4
mob14	+++	++	++	++	+	..	..	_	_	_	_	_	_	_	_	4

[a] Data for MICs determined using heavy inocula

b Symbols used +++ = good growth, ++, + = less growth, ..growth just visible, _ no growth

Table 7.4 ETH MICs of *inhA* inactivated mutants and parents strains of *C. glutamicum* in ET medium

Strains	ETH concentration in mg/ml										MICs in mg/ml
	0.1	0.2	0.4	0.8	1	2	3	4	5	6	
BL1	+++[a]	++	++	+	+	+	_	+	_	_	2
RM4	+++	++	++	+	+	+	_	+	_	_	2
Mob4	+++	++	++	+	+	+	_	+	_	_	2
Mob7	+++	++	++	+	+	+	_	+	_	_	2
Mob8	+++	++	++	+	+		_	+	_	_	2
Mob13	+++	++	++	+	+	+	_	+	_	_	2

[a] Data for MICs determines using heavy inocula

b Symbols used +++ = good growth, ++ = less growth, _ no growth

Similar to INH both parents and mutants showed similar MICs values; however, both parents and mutants are more sensitive to ETH.

7.3.4 Analysis of lipids

Results reported in the previous chapters indicated the presence of multiple copies of the *inhA* gene in *Corynebacterium* in contrast to its neighbour mycobacterial species. The biochemical approaches used to study the function of *inhA* gene in *Mycobacterium* clearly showed the involvement of InhA protein in mycolic acid biosynthesis. To study the counter function of this gene in *C. glutamicum* a different approach was used which involves gene disruption and inactivation. The Southern results showed the disruption of one chromosomal copy in BL1.

To see whether this disruption had some effect on mycolic acid biosynthesis or not, analysis of lipids of whole cells of the Km resistant mutants was performed. For this purpose 50 µg dried cells of mutants and parent strain BL1 were transferred to a glass tube, 2 mg/ml of internal standard (LAME) was added into each sample and mycolic acids were prepared as described previously in Chapter 2 (section 2.4.2.3). Each extraction was dried separately, silylated and analysed by GC. The control was 100 µl (2 µg/ml) of LAME which was derivatised and extracted as above. The area of LAME in each sample was compared to that of the control and the extraction yield of each sample was obtained. The peaks were identified by comparison with the retention time of the external standards (Sigma189-6, 189-17).

The results are shown in Table 7.5. These showed the presence of hexadecanoic $C_{16:0}$, octadecenoic $C_{18:1}$ and stearic $C_{18:3}$ acids peaks and internal standard peak appeared but no peak was seen after 86 minutes for mutants compared to the parents strains. The presence of these three fatty acids peaks were also confirmed by co-injecting purified FAMEs plus external standards (FAMEs, Sigma). Since all peaks corresponding to the mycolic acids appeared at above 280°C it was therefore assumed that these cells have no mycolic acids or that the mycolic acid concentration was too low to be detected by GC.

Table 7.5 Fatty acid composition of selected *inhA* disrupted mutants of BL1.

Strains	Fatty acids		
	$C_{16:0}$	$C_{18:1}$	$C_{18:3}$
BL1	49.34	40.65	9.99
13mob	28.41	55.07	16.50
8mob	30.24	54.97	13.46
7mob	46.51	47.7	13.55
6mob	24.84	50.62	24.53
4mob	31.56	56.14	13.66

Therefore, in a second experiment about 100 mg of dry cells were subjected to lipid extraction the results were similar as seen when 50 mg of the dried cells were used. The results showed that fatty acids remained the same even with 100mg of cells and no visible peak corresponding to mycolic acids appeared at above 280°C. This showed that disruption of the *inhA* gene had significant impacts on mycolic acids biosynthesis.

7.3.5 16S ribosomal RNA (rRNA) gene analysis of the mutants

Since the tranformants had changed morphology from the parent strains and they failed to grow on ordinary media 16S rRNA gene analysis was performed to prove the identity of these strains.

7.3.5.1 16S rRNA gene amplification

The analysis of 16S rRNA genes was aided by using PCR to amplify target sequences in transformants mob4, 7, 8, 13, and 14. Two primers were used to amplify approximately 1,300 bp of a consensus 16S RNA (Figure 7.8): forward primer 63f (5'-CAG GCC TAA CAC ATG CAA GTC-3') (Marchesi *et al.*, 1998) and reverse primer 1387r (5'-GGG CGG TGT GTA CAA GGC-3') (Pharmacia; Marchesi *et al.*, 1998) were purchased from Pacific Oligos. PCR amplification was performed according to Expand Long Template PCR system (Boehringer Mannheim) and 500ng of genomic DNA was used as the template.

7.3.5.2 16S rDNA sequences

The PCR products were sequenced directly using the same primer, used for amplification (63f and 1387r). Sequence similarity was checked against the 16S rDNA sequences of other bacteria in the GenBank database using BLASTN program, ANGIS. The sequence data showed 96 % similarity to known strains of these species, 93% similarity with *C. glutamicum* 16S DNA operon and various other similar strains.

7.3.5.3 rRNA operon copy number

Based on partial rRNA sequence information, Southern blot analysis of genomic DNA digests was performed to provide some information on rRNA copy number and to compare the "ribotype" of the mutants relative to the parent. Southern blotting was performed using a PCR ^{32}P-labelled probe constructed from strain *B. lactofermentum* genomic DNA with two rRNA primers: 63f and 1387r. The probe was hybridised to restriction enzyme digested genomic DNA of the transconjugants. The restriction sites in the 16S rRNA gene sequences obtained were analysed to determine which restriction enzyme did or did not cut within the fragment. The digests for strains showed several bands, depending on the restriction enzyme used. The blot results may propose a copy number for the rRNA operon in these strains. The Southern blots shown in Figure 7.9 indicated two or three bands from *Hind*III, *Pst*1 digest etc. From these observations, it is proposed that the copy number for the 16S rRNA gene of strain is two or three. Mob 14 showed identical patterns for restriction digestion of the 16S rRNA gene, proving that this mutant was *B. lactofermentum.*

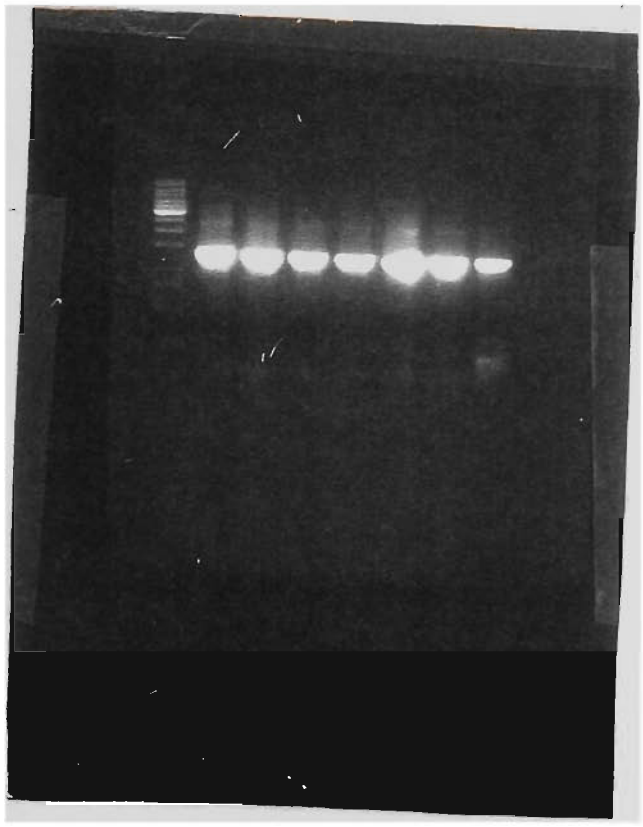


Figure 7.8. Agarose gel electrophoresis of 16S rRNA gene PCR amplification products.

Lane 1, 1KB DNA ladder consisted of 13 fragments with 10,000, 8,000, 6,000, 5,000, 4,000, 3,000, 2,500, 2,000, 1,500, 1,000, 750, 500, 250 bp. Lanes 2-7 contained mob 4,8, 13, 14, PE1, PE11; Lane 8 BL1.

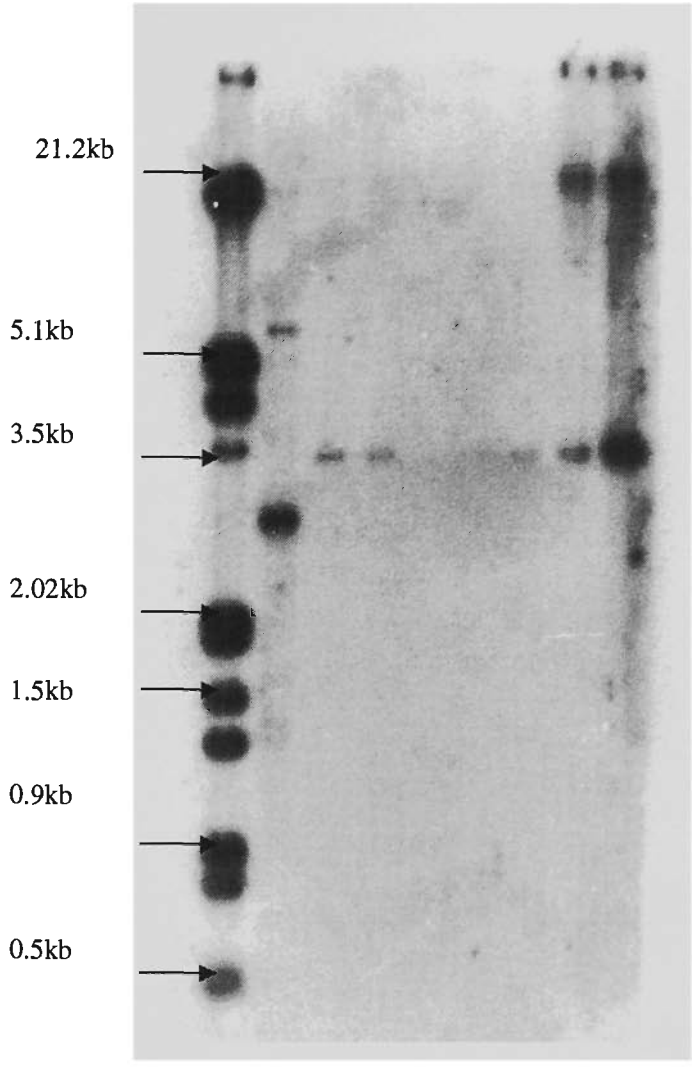
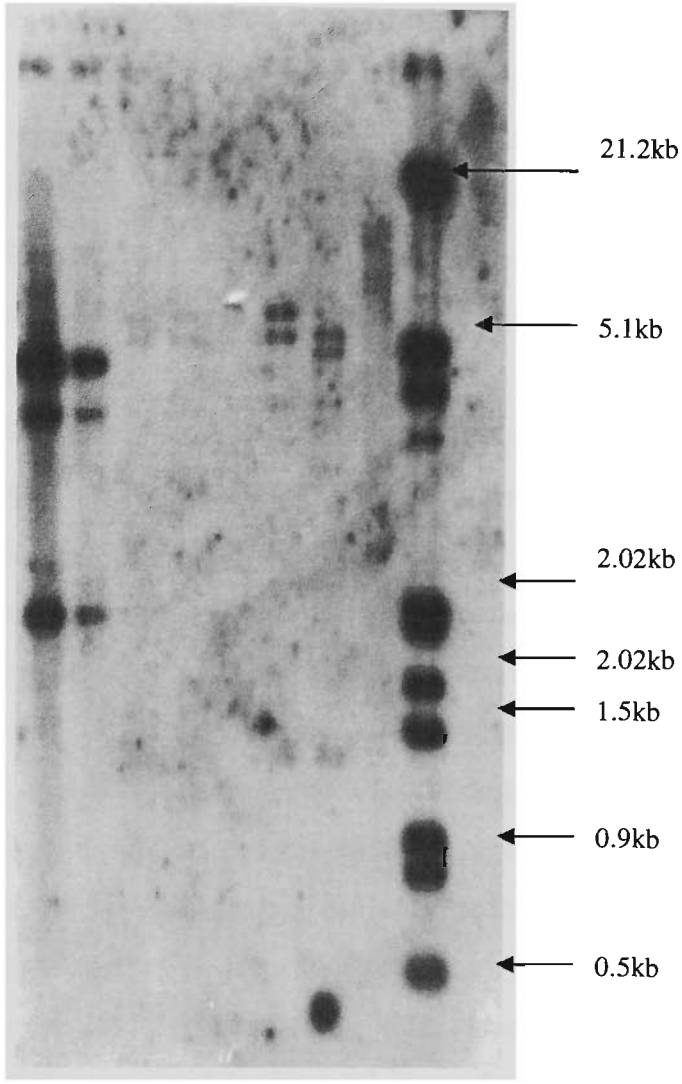


Figure 7.9 a Analysis of 16S rRNA gene of transformants by Southern hybridisation of genomic DNA digests, probing with an amplified 16S rRNA PCR product.

a The genomic DNA was digested with selected restriction enzymes at appropriate temperatures and 2μg of DNA were resolved on large agarose gels by electrophoresis. Hybridisation was performed by ^{32}P labelling protocols as described previously. Lane 1 λ*Hind*III/*Eco*R1 markers; Lanes 2 to 8 genomic DNA of transformants mob 4, 5, 8, 13, 14 digested with *Hind*III; Lane 9, *Hind*III digest genomic DNA of BL1.



b The genomic DNA was digested with selected restriction enzymes at appropriate temperatures and 2µg of DNA were resolved on large agarose gels by electrophoresis. Hybridisation was performed by ^{32}P labelling protocols as described previously. Lane 1, λ*Hind*III/*Eco*R1 markers; Lanes 2 to 8 genomic DNA of transconjugants mob 4, 5, 8, 13, 14 digested with. *Pst*1; Lane 9, *Pst*1 digested genomic DNA of BL1 (Numbering starts from right to left).

7.4 DISCUSSION

Disruption of a chromosomal gene by integration of a vector with an internal fragment of a well-characterised or sequenced gene is a rapid way to analyse its function. This approach was taken to elucidate the function of the *inhA* gene in *C. glutamicum*. This chapter described the means and methods adapted to get *inhA* disrupted mutants and physiological and biochemical consequences of this phenomena on the emerging mutants. Unlike previous systems, which were largely based on well-established media for the selection of transformants, part of the work reported in this chapter involved the choice and development of suitable media for the selection and proper maintenance of these unique mutants. Preliminary drug tests were performed, to determine the MICs in LAG plates with concentration ranges from 0 to 100 μg/ml for donor and recipients. Based on antibiotic sensitivity, kanamycin and nalidixic acid were chosen to suppress the parental strains. The transconjugation experiments were carried out using the method described by Schäfer *et al.* (1990). In control experiments *lysA* transconjugants were successfully recovered on LA plates supplemented with 2% glucose while *inhA* transconjugants failed to appear, indicating that a lethal mutation may have occurred or that ordinary media could not support mutant growth. Since InhA is known to block mycolic acid biosynthesis in mycobacteria, similarity with mycobacterial counterpart strongly suggested its involvement in mycolic acids biosynthesis. To elucidate the function of the *inhA* gene in corynebacteria gene inactivation was carried out. Inactivation of the *inhA* gene may lead to the inhibition of mycolic acid biosynthesis in corynebacteria and hypothetically, the resulting mutants might have defective cell walls resulting in lethal effects on the emerging transconjugants.. Therefore this study was focussed on the design of media for the recovery of transconjugants or transformants that may have defective cell surfaces. There are reports in the literature showing the use of ET media for the recovery of spherolplasts. One of the important components in ET media is $CaCl_2$ and $MgCl_2$ both of these component help in the attachment of peptidoglycan and arabinogalactan and help in the cell wall formation. Therefore this medium was used to recover potentially damaged cell. Transformants failed to grow on LAG, and all other media tested for this purpose indicating the requirement of

osmotically protective media. ET media was used previously for the recovery of electrotransformants and spheroplasts. Failure of the transconjugants to grow on any other media except ET strongly suggested that these mutants might have altered cell surface structures.

Results of the integration are shown in Fig 7.4 a, b. The insert was located in the *Pst*1 site of cloning vector as seen in Fig. 7.4a where mutants were digested with this enzyme (Fig 7.4a) and probed with *inhA* fragment two signals appeared indicating the bifurcation of gene into two haves after integration. Schwarzer and Phüler (1991) also identified the structure of *lysA* gene fragment integrated with the suicide vector using the same strategy.

Lipids profiles of the mutants showed that mycolic acids were not present, as no mycolic acid peak was detected, except for one mutant where a small peaks was detected which have the same retention time as was seen for the parent strains. This indicated the presence of small amounts or no mycolic acids in the mutants. The inability of the mutants to grow on any other media except ET and the absence of mycolic acids suggests that the mutants were osmotically fragile. Previous studies in *C. glutamicum* showed enzymatic removal of the cell wall, leaving behind sepheroplast, which could be propagated. Although the mutants lacking mycolic acids as determined by GC analysis but fatty acids $C_{16:0}$, $C_{18:1}$, $C_{18:3}$ were detected. The presence of $C_{18:1}$ is interesting as this fatty acid is found rarely but appears to be characteristic of corynebacteria.

The most recently described model for the synthesis of corynemycolic acids is described in detail in Chapter 1 (section 1.6.4) where two molecules of palmitic acid condense without an intermediate carboxylation step in the course of the synthesis of mature mycolic acids. This condensation reaction involves a highly activated enolate intermediate. However, the nature of the acyl carrier groups is not known but is presumably an ACP (Lee *et al.*,1997). The deduced InhA proteins of coryne *inhA* genes are enoyl-ACPs: this protein in *Mycobacterium* is believed to catalyses the NADH-dependant reduction of the double bond at position two of a growing fatty acid chain linked to ACP, an enzymatic activity common to all known fatty acids biosynthetic

pathways. InhA preferentially reduces long-chain substrates (those containing 16 or more carbon atoms). *Mycobacterium* utilizes the products of InhA catalyses to create long chained (C_{40} to C_{60}) α-branched fatty acids. The present study supports this hypothesis since disruption of *inhA* gene (deduced protein 2-*trans*-enoyl ACP) lead to failure of the mutants to produce mycolic acids. The nature of the presumed ACP in *C. glutamicum* and related species must be the InhA protein. However, biochemical studies to elucidate the functional role of this protein are needed. This study is first step towards understanding the genetics of mycolic acid synthesis in these species.

Results of Jang (1997) reported that INH causes the disruption of mycolic acids attachment, which indicates that INH function differently in coryne and mycobacteria. Another important point to be consider is that mycobacteria have a complex lipid structure and large number of enzymes are involved in its biosynthesis. Recently, the *mas* gene was disrupted in *M. bovis* BCG using a construct in which an internal 2kb segment in the *mas* gene was replaced with the hygromycin-resistant gene (*hyg*) of approximately the same size, such that the *hyg* was flanked by 4.7 and 1.4kb segments of *mas* (Azad *et al.*, 1996). The gene-disrupted mutant was not able to synthesise mycoside B. Obviously, mycocerosic acids that constitute the major acyl moieties in mycoside B cannot be substituted thus mycoside could not be produced by the mutants in the absence of mycocerosic acids. On the other hand, the acyl portion of the wax-type esters is composed of methyl-branched acids shorter than mycocerosic acids and some mycocerosic acids. The *mas*-disrupted mutants produced phthiocerol esters, which contained only the shorter methyl-branched acids. Obviously, these branched acids are produced by enzymes other than MAS. In fact, a protein fraction that catalysed the synthesis of these specific branched acids was obtained from the extracts of a *mas*-disrupted mutants (Kolattkudy *et al.*, 1997), thus clearly distinguished two enzymes involved in branched fatty acids synthesis. Similarly, in corynebacteria although the biochemical consequences of *inhA* disruption were clear, the functional consequences are not yet known.

Secondly InhA proteins play a slightly different role in two phylogenetically similar genera because of the different pathways of mycolic acid biosynthesis, wherein mycobacteria it is a complicated because of the involvement of several enzymes and in *Corynebacterium* it is mere condensation of two molecules with the need of an important ACP.

The results presented further showed there is no direct or indirect interactions between isoniazid and InhA proteins, as *inhA* disrupted mutants showed similar MICs values to the parents. However, further studies of the mutants (like effect of INH on specific growth, protoplasting and transformation efficiencies) need to be elucidated. In mycobacteria, it has been proven that INH indirectly interacts with InhA. Several lines of evidence prove the theory that, to inhibit InhA, isoniazid requires conversion to an activated form of the drug and that a catalase-peroxidase (*katG*) participates in isoniazid activation. This may indicate that the two protein although showing high homology, still different functional role in two closely related species.

APPENDIX 1

MEDIA, REAGENTS AND SUPPLIERS OF CHEMICALS, MEDIUM CONSTITUENTS AND ACCESSORIES

A. Preparation of chemicals and medium

Antibiotic solutions were prepared from Sigma chemicals as follows: kanamycin, (50mg/ml) neomycin (10mg/ml), ampicillin (50mg/ml), and streptomycin (10mg/ml) were prepared aseptically in sterile distilled water and filtered through 0.22 μm filter (Millipore, GS). Nalidixic acid (50mg/ml) in 1N NaOH. Chloramphenicol (50 mg/ml) was prepared in ethanol and tetracycline (5 mg/ml) and rifampicin (10 mg/ml) in methanol.

Derivatising agent ('Tri-Sil Z') for preparation of trimethylsilyl (TMS) was from Pierce Chemical. Co., Rockford, Illinois, U.S.A.

Fatty acids were identified by gas chromatography by comparing retention times of their methyl ester derivatives with the sigma "standard" methyl esters (Sigma 189-17). This standard contained the following weight percentage of each methyl ester: for 100 mg of fatty acids standard 1 (Sigma 189-17); Myristic acid methyl ester ($C_{14:0}$), 4%; Palmitic acid methyl ester ($C_{16:0}$), 10%; Stearic acid methyl ester ($C_{18:0}$), 6 %; Oleic acid methyl ester ($C_{18:1}$ *cis*-9), 25%; Elaidic acid methyl ester ($C_{18:1}$ *trans*-9), 10%; Linoleic acid methyl ester ($C_{18:2}$, *cis*-9, 12), 34%; Linolelaidic acid methyl ester ($C_{18:2}$, *trans*-9, 12), 2% ; Linoleic acid methyl ester methyl ester ($C_{18:3}$ *cis*-9, 12, 15), 5%; Arachidic acid methyl ester ($C_{20:1}$), 2%; Behenic acid methyl ester ($C_{22:0}$), 2%. Lignoceric acid methyly ester, $C_{25}H_{50}O_2$, Sigma L6766 was used as an internal standard.

Isonicotinic acid hydrazide (INH) was obtained from Sigma (catalogue number I-3377) and stock (100 mg/ml prepared in deionised water) was sterilised at 109°C for 25 min and stored at room temperature in brown bottles.

Lysozyme was from hen egg white and had an activity of 250,000 units/mg, Boehringer Mannheim.

Phenol was from Wako (Tokyo, Japan).

Polaroid films (type 665 and type 667) were from Polaroid

DNA standards were obtained from Promega, MBI and Sigma.

Other chemicals and medium constituents are also listed below.

Reagent	Source
Acetic acid	BDH
Agar	Oxoid
Bromophenol	BDH
BSA (for protein assay)	Sigma
Casoamino acid	Difco
Caesium chloride	Boehringer Mannheim
Chloroform	BDH
Diethyl ether	BDH
Dithiothreitol	Sigma
DNA grade agarose	Progen
EDTA (di-sodium salt)	Sigma
Folin-Ciocalteu's reagent	AJAX
Glucose	BDH
Glycerol	AJAX
Hexane	BDH
Isopropanol	BDH
Isoamylalcohol	BDH
Maleic acid	Sigma
Methanol	BDH
Middlebrook 7H9	Difco
Peptone	Oxoid
Petroleum ether	BDH
Potassium accetate	Sigma
SDS	Pierce
Sucrose	BDH
α- ^{32}P Deoxycytidine 5′- Triphosphate	Bresatec
Tris	Sigma
Tween 80	Promega
Tween 40	Promega
Urea	Promega

Yeast extract	Oxoid

B. SUPPLIERS and ADRESSES

AJAX chemicals Ltd	18 Hamlet St. Cheltenham, Vic 3192
AldrickSigma-Aldrick Pty Ltd	Unit 2, 10 Anella Ave, Castle Hill, NSW 2154.
B. Braun Melsunger AG, Australia	10 Arcae Rd, Box Hill Nth, Vic 3153
Beckman instruments P/L	24 College St, Gladesville, NSW 2111
BDH Laboratories supplies	Poole, BH15 HD, England
Bio-Rad Laboratories P/L	Unit 11, 112-118 Talavera Rd, Nth Ryde, NSW 2113
Boehringer Mannheim Biochemica Australia P/L	26-28 Ellingworth Parade, Box Hill, Vic 3128
Disposable Produce P/L	16 Park Way, Technology Park, S.A. 5095
Essendon Produce P/L	16 Rusell St, Essendon, Vic 3040
FSE Du Pont Australia Ltd	Medical products Dept. Healthcare Block C, Centre Court Industrial Estate, 25-27 Paul St, Nth Northside, NSW 2113
Merck (BDH distributor)	207 Colchester Rd, Kilsyth, Vic 3137
Millipore	PO Box 721, Richmond, Vic 3121
Oxoid	PO Box 220, West Heidelberg, Vic 3081
Pharmacia Australia P/L	4 Byfeild St, Nth Ryde, NSW 2113
Pierce	PO Box 117, Rockford, IL U.S.A 61105
Polaroid Australia Pty Ltd	13-15 Lyon Park Road, North Ryde NSW 2113
QIAGEN Pty Ltd	PO Box 25 Clifton Hill Victoria 3068
Selby Scientific Laboratory Equipment	368 Ferntree Gully Rd, Notting Hill, Vic 3168
Sigma-Aldrick Pty Ltd	Unit 2, 10 Anella Ave, Castle Hill, NSW 2154
Varian Australia P/L	679 Springvale Rd, Mulgrave, Vic 3170
Wako/Novachem Pty. Ltd	50 Garden street, South Yarra VIC 3141 Australia

APPENDIX 2

BUFFERS, REAGENTS AND ENZYMES FOR MOLECULAR WORK

All media, buffers, reagents and enzyme preparations described in this section are taken from Sambrook and Maniatis (1989).

5.0 M Ammonium Acetate
Dissolve 385.4g-ammonium acetate in 1 litre of distilled water

Denhardt's Solution (50X)
Dissolve the following ingredients in succession in 500 ml of H_2O: 5g of Ficoll^{TM} 5g of polyvinylpyrrolidone (PVP), and 5g of bovine serum albumin. Filter sterilise using 0.45μm filters Millipore and store at –20°C.

Denaturing Solution
The composition of the denaturation solution was 1.5 M NaCl and 0.5 M NaOH. The solution was freshly prepared each time prior to use.

0.5 M EDTA (pH8.0)
Add 186.1g of disodium ethylenediaminetetra-acetate-2H_2O to 800ml of H_2O. Stir on a magnetic stirrer. Adjust the pH to 8.0 with NaOH (approximately 20g of NaOH pellets). Dispense into aliquots and sterilise by autoclaving.

Ethidium Bromide Solution
Add 1g of ethidium bromide to 100 ml of distilled H_2O. Stir on a magnetic stirrer for several hours to ensure that the dye has dissolved. Wrap the container in aluminium foil or keep the solution in a dark bottle and store at 4°C..

Gel-Loading Buffer

DNA sample loading dye (6X) was used for agarose gel electrophoresis and contained 0.25% bromephenol blue, 0.25% xylene cyanol FF, and 40%(w/v) sucrose in water

Hybridisation Wash (Low Strengency)
This washing solution consist of 0.2X SSC and 0.1% SDS. The hybridisation wash solution was prepared by combining stock solution of respective constitutes and diluting with appropriate volume of H_2O. The stock solution was stored at room temperature and heated to 65°C prior to use.

Hybridisation Wash (High Strengency)
This solution contained 2X SSC and 0.1% SDS. The hybridisation wash solution was prepared by combining stock solution of respective constitutes and diluting with

appropriate volume of H_2O. The stock solution was stored at room temperature and heated to 65°C prior to use.

IPTG, 0.1 M Stock Solution
To 1.2 g IPTG (Sigma Cat # V3955) 50 ml of water was added. The solution was filter sterilised through a 0.2 μm filter unit and store at 4°C.

Luria Broth (LB)
LB was used to culture bacteria for molecular biology-based studies. Medium contained per litre: 10g of tryptone, 5g of yeast extract, and 10g of sodium chloride. pH was adjusted to 7.0 with 5 N NaOH, and sterilised by autoclaving for 20 min at 121°C.

Lysozyme
Prepare a stock solution of lysozyme at a concentration of 10 mg/ml in water. Dispense into aliquots and store at -20 °C. Discard each after use.

Neutralising Solution
1.0 M Ammonium acetate, 0.2 M NaOH.

0.4 N NaOH
Dissolve 16.0g of NaOH in distilled H_2O. Dispense into aliquots and sterilise by autoclaving.

5.0 M NaCl
Dissolve 292.2g NaCl in 800 ml of H_2O. Adjust the volume to 1 litre with H_2O. Dispense into aliquots and sterilise by autoclaving.

Peptone solution

This solution was made by dissolving peptone in water at a final concentration of 50 mg/ml and sterilized by autoclaving. The stock solution was stored at room temperature.

1.0 M Potassium Acetate (pH 7.5)
Dissolve 9.82g of potassium in 90 ml of H_2O (Milli-Q or equivalent). Adjust the pH to 7.5 with 2.0 M acetic acid. Add pure H_2O to 100 ml. Divide the solution into aliquot and store at –20°C

Prehybridisation /Hybridisation Solution Hybridization solutions consisted of 5X Denhard's reagent, 6X SSC, 0.5% SDS, and 1.0 mg/ml denatured salmon sperm DNA. Solutions were prepared by combining stock solutions of the respective constituents. Hybridisation solution was stored at 4°C.

Probe Washing Solution.
Per 100 ml of probe wash solution: 0.5 ml of 20X SSC, 1.0ml of 10% SDS, 10.0 ml of 2.0 M Tris.HCl (pH 7.5). Adjust the volume to 100 ml by adding distilled water.

Proteinase K (stock solution 20 mg/ml in H_2O)

Dissolve 100 mg of Proteinase K was dissolved in TE (pH 8.0) to achieve the final concentration of 20 mg/ml and stored at -20°C.

RNase (DNase free)
Dissolve 100 mg pancreatic RNase (RNase A) in 10 ml of sterile TE buffer, pH 8.0. Heat in boiling water bath for 5 minutes, then switch off the water bath and leave the solution for another 10-15 min. Allow cooling slowly at room temperature. Dispense into aliquots and store at -20°C.

20X SSC
Dissolve 175.3 of NaCl and 88.2g of sodium citrate in 800ml of H_2O. Adjust the pH to 7.0 with a few drops 10N solution of NaOH. Adjust the volume to 1 litre with H_2O.Dispense into aliquots. Sterilise by autoclaving.

10 % Sodium Dodecyl Sulphate (SDS)
Dissolve 100g of electrophoresis grade SDS in 900ml of H_2O. Heat to 60°C to assist dissolution Adjust the pH to 7.2 by adding a few drops of concentrated HCl. Adjust the volume to 1 litre with H_2O. Dispense into aliquots.

3.0 M Sodium Acetate (pH 5.2)
Dissolve 408.1g of sodium acetate in 800 ml of H_2O. Adjust the pH to 5.2 with glacial acetic acid and make up the volume to 1 litre with H_2O. Dispense into with aliquots and sterilise by autoclaving.

STE Buffer
The composition of STE was 0.1 M NaCl, 10 mM Tris.HCl (pH 8.0), 1.0 mM EDTA (pH 8.0). The stock solution was stored at room temperature.

TE Buffer, pH 8.0
TE buffer consist of 10mM Tris.HCl (pH 8.0) and 1 mM EDTA (pH 8.0). TE buffer was made by combining stock solutions of the respective constituents and diluting with appropriate volume of H_2O.

1.0 M Tris
Dissolve 121.14g of Tris base in 800 ml of H_2O. Adjust the pH to the desired value by adding concentrated HCl. Allow the solution to cool to room temperature before making the final adjustments to the pH. Adjust the volume of the solution to 1 litre with H_2O. Dispense into aliquots and sterilise by autoclaving at 121°C.

X-gal
Stock solution 20 mg/ml was prepared by dissolving X-gal in dimethylforamide.

APPENDIX 3

BUFFERS AND REAGENTS FOR MEMBRANE PREPARATION AND FOR PROTEIN ASSAY

Bovine Serum Albumin (BSA)
The stock solution of BSA (5.0 %) was diluted in 0.45 N NaHPO4 to achieve the final concentration of 100ug/ml. The BSA solution was stored at 4°C.

Folin Ciocalteau Reagent (Diluted)
Dilute Folin reagent was prepared by mixing 2.0 ml of concentrated Folin Ciocalteau reagent with 3.0 ml of deionised water. The reagent was prepared immediately before protein assay.

Lowery Reagent A

Reagent A consisted of 20.0 g of Na_2CO_3 in 1 litre of 0.1 N NaOH. The solution was stored at room temperature.

Lowery Reagent B
Reagent B consisted of 0.5 g of $CuSO_4.5H_2O$ in 100 ml of 1.0 % (w/v) aqueous solution of K-Na-tartrate. The pH was adjusted to 9.00 by and the solution was stored at 4°C.

Lowery Reagent C
Reagent C was prepared by mixing 50.0 ml of reagent A and 1.0 ml of reagent B. Reagent C was prepare just before use and discarded after one day.

0.45M NaH2PO4 Buffer
Dissolve 54.0 g of NaH_2PO_4 in 1itre of distilled water. Sterilise by autoclaving at 121°C for 15 minutes.

4.6 N NaOH
Dissolve 184.0 g of NaOH in 1 litre of distilled water.

0.N NaOH
Dissolve 4.0 g of NaOH in 1 litre of distilled water. Sterilise by autoclaving at 121°C for 15 min
DNA sample loading dye (6X) was used for agarose gel electrophoresis and contained 0.25% bromophenol blue and 40% (w/v) sucrose in water.

Triton X-100 Solution
20% v/v triton X-100 solution was prepared by dissolving 20 ml of tritonX-100 in 80 ml of water and heating 60-80°C.

Veronal Buffer

Veronal buffer consisted of 572 mg Veronal, 375 mg Veronal sodium, 27 mg $CaCl_2$. $2H_20$ and 109 mg $MgSO_4$. $7H_2O$, in 1000 ml distilled water. The solution was stored at 4^oC.

Veronal /HCl Buffer (pH 9.5)

Veronal /HCl buffer consisted of 0.1 M Veronal sodium solution and pH was adjusted to 9.5 with 1 M-HCl

Appendix 4

Sequence of the 16sRNA for strain AS019 using 63F primer

```
  1  TTTCCAATNC CCTCGCTNTN GNGGATGAGT GGCGAACGGG TGAGTAACAC
 51  GTGGGTGATC TGCCCTACAC TTTGTGATAN NCCTGGGAAA CTGGGTCTAA
101  TNCCGAATAT TCACACCACC GTAGGGGTGG TGTGGAAAGC NTTATGCGGT
151  GTGGGATGAG CCTGCGGCCT ATCAGCTTGT TGGTGGGGTA ATGGCCTACC
201  AAGGCGTCGA CGGGTAGCCG GCCTGAGAGG GTGTACGGCC ACATTGGGAC
251  TGAGACACGG CCCAGACTCC TACGGGAGGC AGCAGTGGGG AATATTGCAC
301  AATGGGCGCA AGCCTGATGC AGCGACGCCG CGTGGGGGAT GAAGGCCTTC
351  GGGTTGTAAA CTCCTTTCGC TAGGGACGAA GCCTTATGGN GACGGNACCT
401  GGAGAAGAAN CACCGGCTAA CTACNTGCCN NCAGCCGCGG NAATACGTAN
451  GGNGCGAGCG TTGTCCGGAA TTACTGGGCG TAAAGAGCTC GTANGTGGNT
501  TGTCGCGTCN TCTGTGAAAT CCCGGGGCTT AACTTCGGGC GTGCACGCGA
551  TACGGGCATA ACTTGAGTGC TGTAGGGGAG ACTGGAATTN CTGGTGTAGC
601  CGGTGAAATN CCGCACATAT CANGAGGAAC ACCAATGGCG AAAGCAGGTC
651  TNTGGGCAGT AACTGACCTG AAGAGCNAAA CATGGGTAGT GNACAAGATT
701  ANATACCCTG GTAGTCCATG CCNTAAACCG NGGGGCGCTA GGTGTANGGG
751  TCTTTCACCA CTTTTGGGCC GCACTTACCC ATTAAACGCC CCCCTGGGGA
801  GTTCCNGCNC ANGGNTTTAA ACTNCAAAGG NATTTGACCC GGGGGCCCCA
851  CAAANCCGGN GGGGCATTGT NGGTTTAAAT TNATTNCAAC CCNAAAAAAA
901  CCTTANCNCG GGCTTGGACA TTGGANCCCG ATNNGGCGGG ANAAANACCN
951  TTTTCCCTTT TTNGGNTCNG GTCCACCAGG GGGGGGCCT
```

Sequence of the 16sRNA.for strain AS019 using 1387R primer.

```
1  TTCCATCTCC NACACCANTG ATGTTGATCT GCGATTNCTA GCGACTCCGA
 51  CTTCATGGGG TCGAGTTGCA GACCNNAATN TTAACTGAGG CCGGCTTTAA
101  GAGNTTAGNT CCACCTCACG GTGTGGCAAC TCGCTGTACC GACCATTGTA
151  GCATGTGTGA AGCCCTGGAC ATAAGGGGCA TGATGATTTG ACGTCATCCC
201  CACCTTCCTC CGAGTTAACC CCGGCAGTCT CTCATGAGTA CCCACCANAA
251  TGTGCTGGCA ACATAAGACA AGGGTTGCGC TCGTTGCGGG ACTTAACCCA
301  ACATCTCACG ACACGAGCTG ACGACAACCA TGCACCACCT GTGAACCGAC
351  CACAAGGGAA AACGTATCTC TACGCCGATC CGGTCCATGT CAAGCCCAGG
401  TAAGGTTCTT CGCGTTGCAT CGAATTAATC CACATGCTCC GCCGCTTGTG
451  CGGGCCCCCG TCAATTCCTT TGAGTTTTAG CCTTGCGGCC GTACTCCCCA
501  GGCGGGGCGC TTAATGCGTT AGCTGCGGCA CAGAAGTCGT GGAAGACCCC
551  TACACCTAGC GCCCACCGTT TACGGCATGG ACTACCAGGG TATCTAATCC
601  TGTTCGCTAC CCATGCTTTC GCTCCTCAGC GTCAGTTACT GCCCAGAGAC
651  CTGCCTTCGC CATTGGTGTT CCTNCTGATA TCTGCGCATT TCACCGCTAC
701  ACCAGGAATT NCAGTCTTCC CTACAGCACT CAAGTTATGC CCGTATCGCC
751  TGCACGCCCC GAAGTTAAAC CCCNGGATTT CACAAGACAA CGCGACAAAC
801  CACCTACGAG CTNTTTACGC CCAATAATTN CGGACAACGC TTGGACCCTT
851  ACGTATTACC CGCGGGTGNT GGCACGTAAT TAAGCCGGNG CTTNTTTTTC
901  AAGGACCCGC ACCATAAAGG TTTTGNCCTT ACCGAAAGGG GGTTACAACC
951  CCNAAGGCTT TATTCCCCCC CCG
```

Table: BASTN Homology Saearch Results for the 16S rRNA gene of strain AS019 from NCBI database.

DNA Sequence	Organism	% Homology for 63 f and 1387r	
16S rRNA gene	*C. glutamicum*	96%	95%
16S rRNA gene	*C. acetoacidophilum*	96%	96%
16S rRNA gene	*Corynebacterium* species T14433	96%	94%
rrnE operon	*C. glutamicum*	96%	95%
16S rRNA	*C. efficiens*	93%	94%
16S rRNA gene	*C. pseudotuberculosis*	92%	
16S rRNA gene	*Corynebacterium* sp ATCC43833	96%	
16S ribosomal gene	*Corynebacterium bovis*	93%	95%

Sequence of the 16sRNA. Strain MLB133 using 1387R primer.

```
   1  TTNNTTTNTA CCCAGNGTNG CTGATCTGCG ATACTAGCGA CTCCGACTTC
  51  ATGGGGTCGA GTTGCAGACC NTATTTTAAC TGAGGCCGGC TTTAAGAGNT
 101  TAGGTCCANN TCACGGTGTG GCAACTCGCT GTACCGACCA TTGTAGCATG
 151  TGTGAAGCCC TGGACATAAG GGGCATGATG ATTTGACGTC ATCCCCACCT
 201  TCCTCCGAGT TAACCCCGGC AGTCTCTCAT GAGTACCCAC CANAATGTGC
 251  TGGCAACATA AGACAAGGGT TGCGCTCGTT GCGGGACTTA ACCCAACATC
 301  TCACGACACG AGCTGACGAC AACCATGCAC CACCTGTGAA CCGACCACAA
 351  GGGAAAACGT ATCTCTACGC CGATCCGGTC CATGTCAAGC CCAGGTAAGG
 401  TTCTTCGCGT TGCATCNAAT TAATCCACAT GCTCCGCCGC TTGTGCGGGC
 451  CCCCGTCAAT TCCTTTGAGT TTTAGCCTTG CGGCCGTACT CCCCAGGCGG
 501  GGCGCTTAAT GCGTTAGCTG CGGCACAGAA GTCGTGGAAG ACCCCTACAC
 551  CTAGCGCCCA CCGTTTACGG CATGGACTAC CANGGTATCT AATCCTGTTC
 601  GCTACCCATG CTTTCGCTCC TCANCGTCAG TTACTGCCCA GAGACCTGNC
 651  TTCGCCATTG GTGTTNCCTC CTGATATCTG CCCATTTNAC CGCTACACCC
 701  ACGAATTCCA NGTNTTCCCC TACNNCCTCA ANNTATTGCC CNTATCNCCT
 751  GCNCGCCCNA NNTAAACCCC CGGGATTTAC ANACGACTCN ACCAACCCAC
 801  CTACNAGCTT NTTTACCCCC CNTAATTTCC GNGACCANCC GNTTTCGAAC
 851  CCCTACCTNN TTAACCNCCC GNNGCNTGGC CNCCNTAANC TTAANCCNNG
 901  CNGCNTTCCT TTTTTCACGG GCCCCCNCNN CCCATTAANG GNCTTTTNNC
 951  CCCTTNCCCN CNNNGGNGNT TTNCCNNCCC CNCANNGGGC CCCTATTTCC
1001  CCCCCCNCCT
```

Sequence of the 16sRNA gene for strain BL1 using 1387R primer.

```
1   ACGTAAGATT ACCGAGCGTT GCTGATCTGC GATACTAGCA CTCCGACTTC
  51  ATGGGGTCGA GTTGCAGACC CAATCCGAAC TGAGGCCGGC TTTAAGAGAT
 101  TAGCTCCACC TCACGGTGTG GCAACTCGCT GTACCGACCA TTGTAGCATG
 151  TGTGAAGCCC TGGACATAAG GGGCATGATG ATTTGACGTC ATCCCCACCT
 201  TCCTCCGAGT TAACCCCGGC AGTCTCTCAT GAGTACCCAC CACAATGTGC
 251  TGGCAACATA AGACAAGGGT TGCGCTCGTT GCGGGACTTA ACCCAACATC
 301  TCACGACACG AGCTGACGAC AACCATGCAC CACCTGTGAA CCGACCACAA
 351  GGGAAAACGT ATCTCTACGC CGATCCGGTC CATGTCAAGC CCAGGTAAGG
 401  TTCTTCGCGT TGCATCGAAT TAATCCACAT GCTCCGCCGC TTGTGCGGGC
 451  CCCCGTCAAT TCCTTTGAGT TTTAGCCTTG CGGCCGTACT CCCCAGGCGG
 501  GGCGCTTAAT GCGTTAGCTG CGGCACAGAA GTCGTGGAAG ACCCCTACAC
 551  CTAGCGCCCA CCGTTTACGG CATGGACTAC CAGGGTATCT AATCCTGTTC
 601  GCTACCCATG C
```

BL1 and1387R primer

```
1   ACTATTACCG AGCGTTGCTG ATCTGCGATA CTAGCGACTC CGACTTATGG
  51  GGTCGAGTTG CAGACCCCAA TCCGAACTGA GGCCGGCTTT AAGAGATTAG
 101  CTCCACCTCA CGGTGTGGCA ACTCGCTGTA CCGACCATTG TAGCATGTGT
 151  GAAGCCCTGG ACATAAGGGG CATGATGATT TGACGTCATC CCCACCTTCC
 201  TCCGAGTTAA CCCCGGCAGT CTCTCATGAG TACCCACCAC AATGTGCTGG
 251  CAACATAAGA CAAGGGTTGC GCTCGTTGCG GGACTTAACC CAACATCTCA
 301  CGACACGAGC TGACGACAAC CATGCACCAC CTGTGAACCG ACCACAAGGG
 351  AAAACGTATC TCTACGCCGA TCCGGTCCAT GTCAAGCCCA GGTAAGGTTC
 401  TTCGCGTTGC ATCGAATTAA TCCACATGCT CCGCCGCTTG TGCGGGCCCC
 451  CGTCAATTCC TTTGAGTTTT AGCCTTGCGG CCGTACTCCC CAGGCGGGGC
 501  GCTTAATGCG TTAGCTGCGG CACAGAAGTC GTGGAAGACC CCTACACCTA
 551  GCGCCCACCG TTTACGGCAT GGACTACCAG GGTATCTAAT CCTGTTCGCT
 601  ACCCATGCTT TCGCTCCTCA ACGTCAGTTA CTGCCCAGAG ACCTGCTTCG
 651  CCATTGGTGT TCCT
```

Table: BASTN Homology Saearch Results for the 16S rRNA gene of strain MLB133 from NCBI database.

DNA Sequence	Organism	% Homology for 63 f and 1387r
16S rRNA gene	*C. glutamicum*	97%
16S rRNA gene	*C. acetoacidophilum*	97%
16S rRNA gene	*Corynebacterium* species T14433	97%
rrnE operon	*C. glutamicum*	97%
16S ribosomal DNA	*C. glutamicum*	97%
rrn gene for 16S rRNA	*Corynebacterium* spp	93%

Table: BASTN Homology Saearch Results for the 16S rRNA gene of strain BL1 from NCBI database.

DNA Sequence	Organism	% Homology for 63 f and 1387r
16S rRNA gene	*C. glutamicum*	100%
16S rRNA gene	*C. acetoacidophilum*	100%
16S rRNA gene	*Corynebacterium* species T14433	99%
rrnE operon	*C. glutamicum*	99%

Figure Sequencing data obtained from 1F clone

T3 as a sequencing primer on 1f clone.

```
  1  attnctttat ttttgacccg tttaaacgtt taaaatagtg cctccngccg
 51  nctgcatcca ttcaancgat catccttcaa gctcaataac tcaaacagaa
101  gtgtcttttg tagaatttca taatctgaac ttttgtttga actctnttcg
151  gcatcaccca cgtgccgcgt ccgaattatt aacacctana aacctgtgga
201  nganana aaaa ccatggcaac cacnttgctg gacctcacca aacttatcga
251  tggcatcctc aanggctctg cccagggcnt tcccgctcnc tcagtacggg
301  aacaatcaat cccggctatn ggtcttgant cntccnnctt acctacctcn
351  nacnctattt ttgctgcang aattctatat caagcttatt gataccgntc
401  cacctctang gggggcncgg tanccaactc nccctatatt nagtcttatt
451  acgctcgcnc accngccntc ntttttncta cttcnttgac tgggaaanan
501  cctnncgttn cccntcttaa tccncttttc ttnncatctc tctnttccct
551  tctttgtctn nttatctatt aagctntntc tctattctcn ctt
```

Figure T7 as a sequencing primer on 1f clone.

```
1  ttattntctt tttnggcccg ttggnnccnt tttaacnacn ggncccatgg
  51  gccngatatc naattcctgc attaaaaata gcgtccgagg tatgtaagct
 101  ggaggagtca ataccaatag ccgcgantgc ttgttccct actgcgtgag
 151  cgggaacgcc ctgggcanag cccttgagga tgccatcgat aagtttggtg
 201  aggtccanca acgtggttgc catggttttc tctcctccac aggtttctag
 251  gtgttaataa ttcggacncg gcacgtgggt gatgccgaaa agagttcaaa
 301  caaaatttca cattatgaaa ttctacaaaa nacacttctg tttgagttat
 351  tgancttgaa ngatgatcgg ttctgtggct gcngcccggg ggatccacta
 401  tttctatanc ggccgccacc gcngtgganc tccacctttt gttcccttta
 451  ntganggtta attgcgcnct tggcgtattc ntggtcatag ctgnttccng
 501  tgtgaaattg taacccgctc ccaattcccc ctcaacnnta ctatcctcga
 551  tnccatanag tgtttaagcc ntgttggtnc ctattnatt
```

Table: BLASTN Homology search results for DNA sequencing data generated for clone 1F selected from MLB194 genomic libraries

DNA Sequence	***Organism***	% Homology
1F Clone	*C. glutamicum* ATCC13032 DNA complete	93%
T3 as a sequencing primer	*C. glutamicum* ftsI, murE, murF, gene penecillin-binding protein, UDP-N-acetylmuramoyl-L-alanyl-D-glutamate, UDP-N-acetylmuramoyl-L-alanyl-D-glutamyl-meso-diaminopimelate, complete and partial cds length	92
T7 as a sequencing primer on 1F	*C. glutamicum* ATCC13032 DNA complete	96%
	C. glutamicum ftsI, murE, murF, gene penecillin-binding protein, UDP-N-acetylmuramoyl-L-alanyl-D-glutamate, UDP-N-acetylmuramoyl-L-alanyl-D-glutamyl-meso-diaminopimelate, complete and partial cds length	95%%

Figure Sequencing data obtained from 1C clone

```
   1  cgctctagaa atagngnatc ccccgggctg cagaaatact agagattgtg
  51  gccaagaaca ccctgaggtg cacctcgagc gcgaattcct ccaaaacacc
 101  gtggagcttg tcaccggagt gtgcgacacc gtccccgaag cggtggcaga
 151  gctttcccac gatctagatg cgctgaaaga agcagcggat tctctcgggc
 201  ttcggttgtg gacctctgga tcccacccat tttcggattt ccgcgaaaac
 251  ccagtatctg aaaaaggctc ctacgacgag atcatcgcgc gcacccaata
 301  gtggggaaac cagatgttga tttggggcat tcacgtccac gtgggcatca
 351  gccatgaaga tcgcgtgtgg ccgatcatca atgcgctgct gacaaattac
 401  ycrsacctgt tggcactttc tgcaagctct ccagcatggg acggacttga
 451  taccggttat gcctccaacc ggacgatgct ctaccaacag ctgcctacag
 501  ccggactgcc ataccaattc caaagctgga tgaatggtgc aactacatgg
 551  cggatcaaga taaatccggt gtcatcaacc acaccggatc catgcacttt
 601  gatatccgcc ccgcatccaa atggggaacc atcgaagtcc gcgtggccga
 651  ttctacctcc aacmtgcggg aactgtctgc catcgtggcg ttgacccact
 701  gtctcgtggt gcactacgac cgcatgatcg acgctggcga agagcttccc
 751  tccctgcaac aatggcacgt ttcggaaaat aaatggcgcg cggctaggta
 801  tggtctggat gccgaaatca tcatttccag agacaccgat gaagcgatgg
 851  ttcaagacga actcccgccg actagtagcg caattgatgc ctcttagccc
 901  aacgaactcg gctgcgctcg tgagcttgaa cttgtttttg trtwggaaat
 951  ccctggaacg ttggtgggtg gattacgaaa cgcccacgca gagtgtttaa
1001  agaaactggc agttggaaag ctgcaggaat tcgatattca ngc
```

Table: BLASTN Homology search results for DNA sequencing data generated for clone 1C selected from MLB194 genomic libraries.

DNA sequence	Organism	% Homology
1C sequence generated from t3 primer walk	*C. glutamicum*ATCC13032 IS fingertype 4-5	96%
	*C glutamicum*ATCC13032 DNA, complete genome, sect	96%
	C. effeciens YS-314 DNA complete genome, section 9	82%
	C. diphtheria gravis NCTC13129, complete genome segment 7/8	78%

Appendix 5

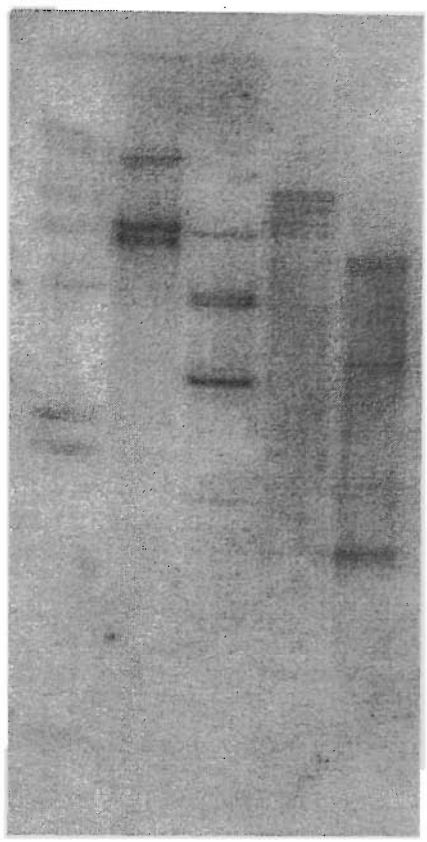


Figure 4.1 Analysis of the 16S rRNA gene of the *C. glutamicum* strain AS019 by Southern hybridization of genomic DNA digest, probing with an amplified 16S rRNA PCR product.

The genomic DNA was digested with selected restriction enzymes at appropriate temperature and 1µg of DNA was resolved on large agarose slab gel by electrophoresis. Hybridisation was performed by ^{32}P radioactive labeling system. Lane 1, Lambda phage DNA *HindIII* digest (size markers); Lanes 2, 3, 4, *BglII*, *HindIII*, *PstI* digested genomic DNA of *C. glutamicum* strain AS019. Lane 5, *PstI* digested genomic DNA of *M. smegmatis* mc^2 155.

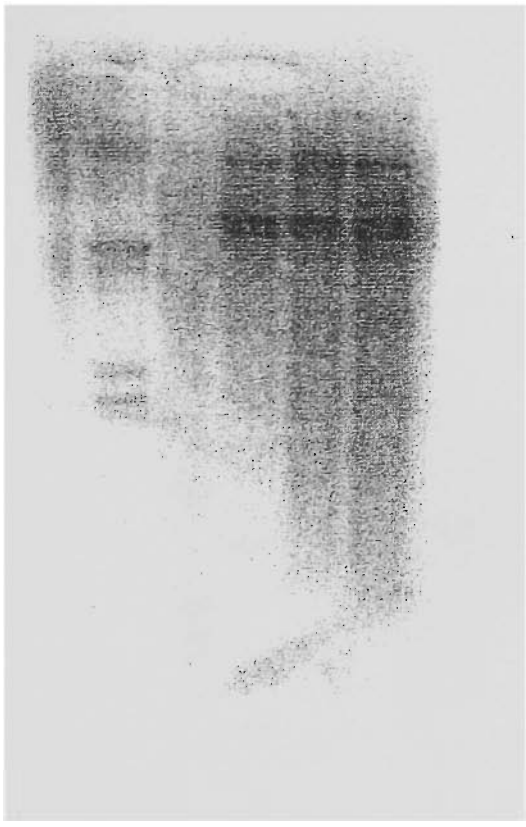


Figure 4 III Analysis of the 16S rRNA gene of the *B. lactofermentum* strain BL1 by Southern hybridization of genomic DNA digest, probing with an amplified 16S rRNA PCR product.

The genomic DNA was digested with selected restriction enzymes at appropriate temperature and 1μg of DNA was resolved on large agarose slab gel by electrophoresis. Hybridisation was performed by ^{32}P radioactive labeling system. Lane 1, Lambda phage DNA *HindIII /EcoR*1 digest (size markers); Lanes 3, 4, and 5 *BglII*, digested genomic DNA of *C. glutamicum* strain AS019 *B. lactofermentum* (BL1) and *B. flavum* (BF4)

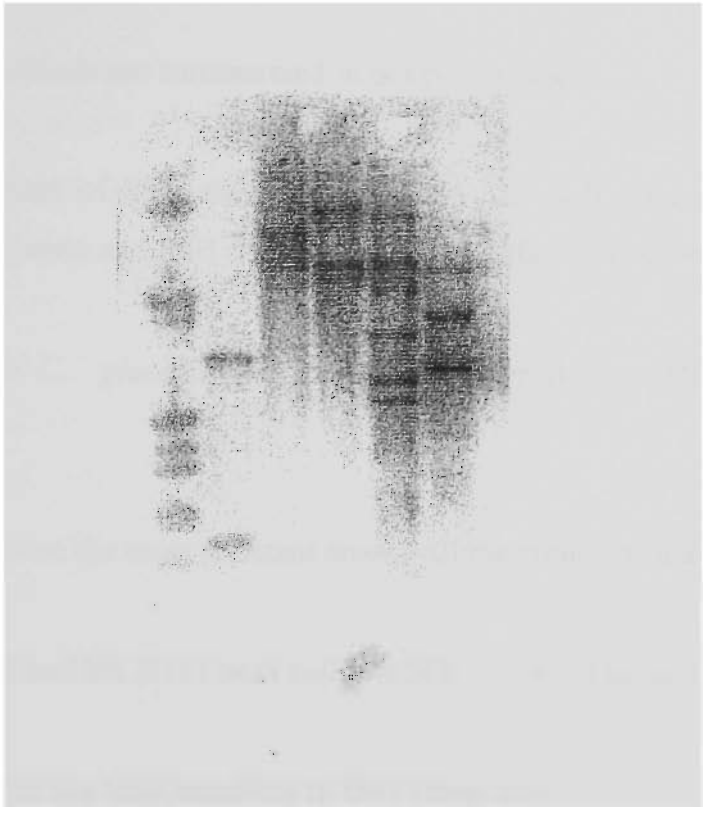


Figure 4 II Analysis of the 16S rRNA gene of strain MLB133 by Southern hybridization of genomic DNA digests, probing with an amplified 16S rRNA PCR product.

The genomic DNA was digested with selected restriction enzymes at appropriate temperatures and 2 μg of DNA were resolved on large agarose slab gels by electrophoresis. Hybridisation was performed by ^{32}P labelling protocol as described in section 2.4. Lane 1, *HindIII/EcoR*1 markers. Lane 2, *Pst*1 digested *M. smegmatis* mc^2, Lanes 3,4, 5 and 6 *Pst*1, *BglII*, *EcoR*1 and *HindIII* digested genomic DNA of *C. glutamicum* strain MLB133

APPENDIX 6

Conclusions and further recommendations

Major findings of the thesis are summarized in dot points below.

MICs studies and effect of INH and glycine on the cell wall lipids composition after growth in INH and glycine and INH plus glycine are briefly summarized.

- Our studies show *C. glutamicum* is relatively insensitive to INH compared with mycobacteria.

- AS019 and BF4 were the most resistant among all the strains tested (16 -17mg/ml).

- Mutants MLB194 and MLB133 both had low MICs relative to their parent.

- BL1 and RM4 were the most sensitive to INH (4mg/ml).

- ETH (ethionamide), which is an analog of INH are assumed to share a common target however, MIC results for these were not the same. Differential sensitivity as seen with INH was not observed with ETH and ETH was found to be a more potent drug.

- Analysis of cell wall lipids showed that it was richer in unsaturated MA compared with the whole cells when grown in LBG.

- In 2% glycine the level of unsaturated MA ($C_{34:1}$, $C_{36:2}$) decreases with the relative increase in the level of saturated MA ($C_{32:0}$) compared with whole cells

- Growth in INH and INH+glycine also had a similar effect, that is a decrease in unsaturated and increase in saturated MA.

- Studies also indicate the presence of FA in cell wall. There was a trend towards decrease in FAMEs after growth in LBG-G, LBG-I implying that including glycine and INH in the growth medium not only affected mycolic acids but also impacted on fatty acids composition.
- If the FA are used as precursor for MA then decrease in their level might impairs MA synthesis (decrease in MA level) (Jang *et al.*, reported that the cell wall become thiner after growth in INH and glycine).

Identification of an *inhA* homologue in corynebacteria

- Southern hybridization was used as a mean to identify an *inhA* gene homologue in *C. glutamicum* strain AS019. PCR amplified *inhA* fragment from *M. smegmatis* was probed against the genomic DNA digest of AS019 with various restriction enzymes to detect the *inhA* gene. Southern hybridization results confirmed the existence of an *inhA* gene in *C. glutamicum.* DNA-DNA hybridization results further indicate the presence of several copies of the gene in the genome.
- To amplify putative *inhA* gene from AS019, various sets of primers were designed based on the published *inhA* sequences of *M. smegmatis*. To confirm the identity of the *inhA* gene, two approaches were used subsequently: construction of the sub-genomic library in pBluescript and sequencing the gene directly from PCR products of genomic DNA.

- Gene was sequenced using the PCR primers, and assembled. A restriction map of the gene created *via* WEBANGIS did not show any restriction site for the enzymes used in Southern hybridization. This confirmed the phenomena of the existence of multiple copies of an *inhA* gene in the genome.

- Nucleotide similarity searches showed 85% to 98% (*M. tuberculosis* to *M. smegmatis*) sequence similarity with mycobacterial species.

- From the deduced amino acid sequence it appeared that the *inhA* gene of AS019 is a 2-enoyl-acyl-carrier reductase, which showed significant homologies with enzymes involved in bacterial and plant fatty acids biosynthesis. The highest identity score was 98% with the *Mycobacterium* family. This high degree of similarity of *C. glutamicum* InhA protein with the mycobacterial InhA and other proteins involved in fatty acids biosynthesis strongly suggest its involvement in lipid biosynthesis.

- The DNA-DNA hybridization profile strongly suggests that the gene is highly conserved in parent and mutants and multiple copies of the gene are present.

- Multiple sequence alignment of InhA proteins of AS019 with two mutants MLB194 and MLB133 with that of *M. smegmatis* indicates that two mutants MLB194 and MLB133 are more similar to *M. smegmatis* compared with their parent strain AS019.

- It can be speculated that the InhA protein of *C. glutamicum* might use nicotinamide or flavin nucleotide as it has putative binding site for these molecules. It is also possible that the structure of the InhA in *C. glutamicum* might be such to allow decrease in molecular contact between isonicotinic acyl-NADH and the active site of InhA and therefore higher concentrations of INH are required to produce a significant change in MA composition.

- The *inhA* gene was successfully cloned and sequenced in two *Brevibacterium* species namely, *B. lactofermentum* and *B. flavum*, using PCR approaches. The InhA proteins of the two *Brevibacterium* species showed amino acids variations at several points.

- Amino acid comparison of InhA proteins of *C. glutamicum* strain AS019 with two *Brevibacterium* species showed that *B. flavum* is similar to AS019. In the upper region of the two InhA proteins (1-247) the only difference is at position 27

and 123 where lysine and proline in AS019 are replaced by arginine and serine in BF4. Whereas in the lower portion of the InhA (247-269) several differences are observed.

- Same differences are observed between *M. smegmatis* and BF4 at these positions.

- InhA proteins of two *Brevibacterium* species *B. lactofermentum* and *B. flavum* differed at eight points (from pos. 1-247) in terms of amino acid substitution. These are at positions 31, 77, 121, 123, 133, 186, 189. In BL1 these amino acid are replaced by valine, glutamine, glutamine, serine, proline, threonine, serine and alanine instead of glutamic acid, arginine, histidine, leucine, alanine, serine and valine in BF4. At position 33 there is lysine in BL1 instead of arginine in BF4. Both BF4 and AS019 share the common points of amino acid variation with BL1 except at position 123, where serine in BL1 and BF4 occur instead of proline in AS019.

- The lower region of the two InhA proteins (247-269) of BL1 and BF4 are similar except position 253 where arginine in BF4 is replaced by alanine in BL1. InhA proteins of AS019 and BF4 vary significantly from position 247-269.

- Amino acid sequence homology of two InhA proteins (AS019 and BF4) is consistent with the INH MICS results. This might suggest the similar structure of two InhA proteins and further indicates the possibility of same biochemical function in the two species of *C. glutamicum* and *B. flavum*. Previously, studies reported by Jang *et al.* (1997) on restriction modification barrier also indicates similar methylation pattern in the above two species.

- Southern hybridization experiments revealed two unique patterns for three corynebacterial species tested. In one experiment where *Bgl*II was used in Southern blotting, the patterns of *B. flavum* and *B. lactofermentum* were similar. However, linear restriction map of *inhA* gene of both BL1 and BF4 showed an

internal cutting site for *Bgl*II (At position 810) whereas three corynebacterial strains AS019, MLB194, MLB133 did not show any cutting site for *Bgl*II.

- Southern hybridization with *Hind*III and *Eco*R1 generated two types of pattern. Three *C. glutamicum* strains, AS019, MLB194 and MLB133, showed one specific pattern whereas BL1 and BF4 showed a different pattern from each other and also different from *C. glutamicum*. This showed heterogonous nature of three genomes. For *B. lactofermentum*, *Hind*III showed one major band whereas other enzyme, *Eco*R1 and *Pst*1, showed multiple signals. Similarly, BF4 showed one major signal with *Eco*R1 but multiple bands with *Pst*1 and *Hind*III. Restriction map of both BL1 and BF4 does not indicate any internal cutting site for these enzymes (*Pst*1, *Eco*R1, *Hind*III) in BL1 and BF4. So ambiguity still remains about the existence of multiple copies of *inhA* in *Brevibacterium*. Therefore low level of resistance to INH in *B. lactofermentum* cannot be attributed to low copy number of *inhA* genes.

- To elucidate the functional properties of the *inhA* gene in corynebacteria, gene inactivation by homologous recombination was carried out. Only *B. lactofermentum* strains were used for inactivation experiments. Hence this study does not answer what role InhA is playing in *C. glutamicum* and the differences that appeared at genetic level in parents and mutants affect its function.

- Results showed that the emerging mutants or transconjugants failed to grow on ordinary rich media indicating the occurrence of lethal effect because of *inhA* inactivation.

- Inability of these mutants to grow only on osmotic protective media such as ET also points to the presence of altered cell walls or its absence altogether.

- The results showed similar MICs values of 4mg/ml for parents and transconjugants indicating that destruction of the *inhA* gene had no effect on INH resistance. This fact implies that InhA protein is not the target for INH (isoniazid) in corynebacteria and hence some other resistance mechanism is present. Other reports showed that InhA is not the major target in mycobacteria Mdluli *et al.* (1996).

- Lipids profile analysis showed the absence of mycolic acids in transconjugant. Fatty acids, however, were detected in lower proportion as compare to parents. Recent studies in corynebacteria postulates the condensation of two palmitic acids molecules involving highly activated enolate intermediate most presumably ACPs (Lee *et al.*, 1997). The deduced InhA proteins of coryne-*inhA* genes is trans-2-enoyl-ACP-reductse, this protein in *Mycobacterium* is believed to catalyze the NADH-dependant reduction of the double bond at position two of growing fatty acids chain linked to ACP, an enzymatic activity common to all known fatty acids biosynthetic pathways. Since the present studies showed the transconjugants lacking mycolic acid, it can be speculated that the highly activated enolate intermediate step in corynebacteria must be catalyzed by the InhA enzyme. However, biochemical studies like purification of InhA proteins and *in vitro* assays to study its involvement in condensation reaction is needed.

Recommendations

- InhA gene was cloned and sequenced from the *C. glutamicum* strains and two *Brevibacterium* strains. Initially, the project aimed to map the exact position of gene within the chromosome and to see if it is located in an operon and. A sub-genomic library was constructed for that purpose but project failed to provide such information. Therefore the location of the *inhA* gene with its possible operon needs further investigation.

- MIC studies of transconjugants indicated that *inhA* disruption have no effect on INH resistance of these bacteria, indicating there is no direct interaction between InhA and isoniazid. In mycobacteria it has been proven that drug indirectly interacts with InhA protein. INH resistance has been associated with a variety of mutations affecting one or more genes such as those encoding catalase-peroxidase (*katG*), the *inhA* and alkyl-hydroperoxide reductase (*ahpc*). In corynebacteria only *inhA* has been studied since the exploration of other genes like *katG, ahpc* was beyond the scope of this thesis. Therefore, studies to identify these genes and elucidate their functional properties are necessary to understand the mechanism of high level INH resistance in corynebacteria.

- The *inhA* gene has recently been shown to encode one of the enzymes of a fatty acyl elongase complex (involve in FASII system Velchesze *et al.*, 2000). Therefore studies like cloning of cryne-*inhA* gene in some expression vector, large scale purification of InhA protein and enzyme assay with 2-enoyl-CoAs of chain length 12-20 carbons should be conducted to find its target in corynebacteria. Studies with InhA in mycobacteria showed it preferred primers are C16 to C24 enoyl-ACP. These studies are important because the emerging reports showed the involvement of C_{16} to C_{18} fatty acids for mycolic acids biosynthesis in corynebacteria.

REFERENCES

Abe, S., Takayama, K.-I. and Kinoshita, S. 1967. Taxonomical studies on glutamic acid-producing bacteria. J. Gen. Appl. Microbiol. **13**: 279-301.

Abe, S. and K. Takayama. 1972. Amino acid-producing microorganisms: variety and classification, p. 3-38. *In* Yamada, K., Konoshita, S., Tsunodo, T., K. Aida (ed.), The microbial production of amino acids. Tokyo: Kodansha Ltd.

Adachi, T., Yamagata, H., Tsukagoshi, N., S. Udaka. 1991. Repression of the cell wall protein gene operon in *Bacillus brevis* 47 by magnesium and calcium ions. J. Bacteriol. **173**: 4243-4245.

Ahkong, Q. A., Howell, J. I., Lucy, J. A., Safwat, E., Davy, M. R. and E. C. Cockinh. 1975. Fusion of hen erythrocytes with yeast protoplasts by polyethylene glycol. Nature. **255**: 66-67.

Aiba, S., Imanaka, T. and H. Tsunekawa. 1980. Enhancement of tryptophane production by *Escherichia coli* as an application of genetic engineering. Biotechnol. Lett. **2**: 525.

Aida, K., Chibita, L., Nakayama, K., Takinami, K. and H. Yamada. 1986. Biotechnology of amino acids production. Elsevier, Amsterdam, The Netherlands.

Alshamaony, L., Goodfellow, M., Minnikin, D. E. and H. Mordarkska. 1976a. Free mycolic acids as criteria in the classification of *Gordona* and "R*hodochrous*" complex. J. Gen. Microbiol. **92:** 183-187.

Allen, S. P. and Blaschek, H. P. 1988. Electroporation-induced transformation of intact cells of *Clostridium perfringer*. Appl. Environ. Microbiol. **54**: 2322-2324.

Altschul, S. F., Bogski, M. S., Gish, W. and J. C. Wooton. 1994. Issues in searching molecular sequence databases. Nat. Genet. **6**: 119-129.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and D. J. Lipman. 1990. Basic local alignment search tool. J. Mol. Biol. **215**: 403-410.

Amador, E., Castro, Jose M., Correia, A. and Juan F. Martin. 1999. Structure and organisation of the *rrnD* operon of '*Brevibacterium lactofermentum*' analysis of the 16S rRNA gene. Microbiol. **145**: 915-924.

Anderson, R. J. 1941. Structural peculiarities of acid fast bacterial lipids. Chem. Rev. **29**: 225-243.

Anderson, S., Marks, C. B., Lazarus, R., Miller J., Stafford, K., Seymour, S., Light. D., Rastetter, W., D. Estell. 1985. Production of 2-keto-L-gluconate, an intermediate in L-

ascorbate synthesis by a genetically modified *Erwinia herbicoba.* Science. **230**: 144-149.

Asselineau, J. 1950. Surla structure chimique des acides mycoliques isoles de *Mycobacterium tuberculosis* var. Hominis; determination de la position de I'hydroxyle. Compte Redu hebdomadaire des Seances de I'Academie des Sciences. **230**: 1,620-1,622.

Asselineau. J. and Lederer. E. 1950. Structure of the mycolic acids of mycobacteria. Nature. London. **166:** 782-783.

Assellineau, J. 1966. The bacterial lipids. Hermann: Paris.

Archer, J. A. C., Follettie, M. T. and Sinskey, A. J. 1989. Biology of *Corynebacterium glutamicum:* a molecular approach, p. 27. *In* Hershberger,C. L., Queenner, S. W. and G. Hegeman (eds.), Genetics and molecular biology of industrial microorganisms. ASM, Washington, D. C.

Athalye, M., Noble, W. C., Mallet, A. I. and D. E. Minnikin. 1984. Gas chromatography-mass spectrometry of mycolic acids as a tool in the identification of medically important coryneform bacteria. J. Gen. Microbiol. **130**: 513-519.

Aushel, F. M., Brent, R., Kingston, R. E., Moore, R. E., Seidman, J. G., Smith, J. A. and K. Struhl. 1987. Current protocol in molecular biology. John wiley: New York.

Azuma, I., Yamamura, Y. and K. Fukushi. 1968. Fractionation of mycobacterial cell wall. Isolation of arabinose mycolate and arabinogalactan from cell wall fraction of *Mycobacterium tuberculosis* strain Aoyama B. J Bacteriol. **96**: 1,885-1,887.

Azuma, I., Kanetsuna, F., Tanaka, Y., Mera, M., Yanagihara, Y., Mifuchi, I. and Y. Yamamura. 1973. Partial chemical characterisation of cell wall of *Nocardia asteroides* strain 131. Jap. J. Microbiol. **17**: 154-159.

Azuma, I., Yamamura, Y. and E. E. Ribi. 1974. Preparation of adjuvant-active, tuberulin-free peptidoglycolipids from human tubercle bacilli. Jap. J. Microbiol. **18**: 327-332.

Bake, A. L., Christiansen, L. and Staudrup, A. 1970. Bacterial genome sizes determined by DNA renaturation studies. J. Gen. Microbiol. **64**: 377-380.

Banerjee, A., Dubnau, E., Quemard, A., Balasubramaniau, V., Um, K-S., Wilson, T., Collins, D., de Lisle, G. and Jr. W. R. Jacobs. 1994. *inhA*, a gene encoding a target for isoniazid and ethionamide in *Mycobacterium tuberculosis*. Science. **263**: 227-230.

Bardou, F., Quemard, A., Dupont, M.-A., Horn, C., Marchal, G., and Daffe, M. 1996. Effects of isoniazid on the ultra-structure of *Mycobacterium aurum* and *Mycobacterium*

tuberculosis and on a production of secreted proteins. Antimicrob. Agents and Chemother. **40**: 2459-2467.

Barksdale, L. and K. S. Kim. 1977. *Mycobacterium*. Bacteriological Review **41**: 217-372.

Barksdale, L. 1981. The genus *Corynebacterium*, p. 1,827-1,837. *In* Starr, M. P., Stolp, H., Truper, H. G., Balows, A. and H. G. Schlegel (ed.), The prokaryotes. Springer Verlag, Berlin.

Barreau, C., Bimet, F., Kiredjian, M., Roullin, N. and C. Bizet. 1993. Comparative chemotaxonomic studies of mycolic acid-free coryneform bacteria of human origin. J. Clin. Microbiol.**31:** 2,085-2,090.

Barletta, R. G., Kim, D. D., Snapper, S. P., Bloom, B. R. and Jr. W. R. Jacobs,. 1992. Identification of expression signals of the mycobacteriophages, Bxb1, L1 and TM4 using *Escherichia-Mycobacterium* shuttle plasmids pYUB75 and pYUB76 designed to create translational fusion to the *lacZ* gene. J. Gen. Microbiol. **138:** 23-30

Barry, C. E. and K. Mdluli. 1966. Drug sensitivity and environmental adaptation of mycobacterial cell wall components. Trends Microbiol. **4**: 275-281.

Basso, L. A., Zheng, R., J. S Blanchard. 1996. Kinetics of inactivation of WT and C243S mutant of *Mycobacterium tuberculosis* enoyl reductase by activated isoniazid. J. Am. Chem. Soc. 118:11301-2.

Batt, C. A., Follettie, M. T., Shin, H. K., Yeh, P. and A. J Sinskey. 1985. Genetic engineering of coryneform bacteria, Trends Biotechnol. **3**: 305-310.

Bathe, B., Kalinowski, J. and A. Pühler. 1996. A physical and genetic map of the *Corynebacterium glutamicum* ATCC 13032 chromosome. Mol. Gen. Genet. **252**: 255-265.

Beppu, T. 1986. Application of recombinant DNA technology in breeding of amino acid producing strains, p. 24. *In* Aida, K., Chibita, L., Nakayama, K., Takinami, K., and H. Yamada (eds.), Biotechnology of amino acids production. Elsevier, Amsterdam,

Benigni, R., Antonov, R. P., and A. Carere. 1975. Estimate of the genome size by renaturation studies in *Streptomyces*. Appl. Microbiol. **30**: 324-326.

Bendinger, B., Kroppenstedt, R. M., Klatte, S. and K. Altendorf. 1992. Chemotaxonomic differentiation of coryneform bacteria isolated from biofilters. Int. J. Syst. Bacteriol. **42**: 474-486.

Benz, R., and K. Bauer. 1988. Permeation of hydrophilic molecules through the outer membrane of gram-negative bacteria. Eur. J. Biochem. **176**: 1-19.

Besra, G. S. and D. Chatterjee. 1994. Lipids and carbohydrates of *Mycobacterium tuberculosis*, p. 285-306. *In* B. R. Bloom. (ed.), Pathogenesis, protection, and control. Washington, DC: American Society for Microbiology,.

Besra, G. S., Sievert, T., Lee, R. E., Slayden, R. A., Brennan, P. J. and K. Takayama. 1994. Identification of the apparent carrier in mycolic acid synthesis. Proc. Natl. Acad. Sci. USA **91**: 12,735-12,739.

Best, G. R., and M. L. Britz. 1984. Generation of chloramphenicol resistant strains of *Corynebacterium glutamicum* after exposing protoplasts to pC194 plasmid DNA from *Bacillus subtilis*. VII International Biotechnology Symposium, New Delhi, India. pp. 404-405.

Best, G. R. and M. L. Britz. 1986. Facilitated protoplasting in certain auxotrophic mutants of *Corynebacterium glutmicum*. App. Microbiol. Biotechnol. **23**: 288-293.

Bergler, H., Wallner, P., Ebeling, A., Leitinger, B., Fuchbichler, S., Aschauer, H., Kollenz, G., Hogenauer, G. and F. Turnowsky. 1994. Protein of EnvM is the NADH-dependant enoyl-ACP reductase (Fabl) of *Escherichia coli*. J. Biol. Chem. **269:** 5493-5496.

Bergler, H., Högenauer, G. and F Turnowsky. 1992. Sequence of the *envM* gene and two mutated alleles in *Escherichia coli*. J. Gen. Microbiol. **138**: 2,093-2,100.

Billman-Jacobe, H., Hodgson, A. L. M., Lightowlers, M., Wood, P. R., and A. J. Radford. 1994. Expression of ovine gamma interferon in *E. coli* and *C. glutamicum*. Appl. Environ. Microbiol. **60**: 1,641-1,645.

Bligh, E. G. and W. J. Dryer. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37**: 911-917.

Bonamy, C., Guyonvarch, A., Reyes, O., David, F. and G. Leblon. 1990. Interspecies electro-transformation in corynebacteria. FEMS Microbiol. Lett. **66**: 263-270

Bonnassie, S., Burini, J.-F., Oreglia, J., Trautwetter, A., Patte, J.-C. and A. M. Sicard. 1990. Transfer of plasmid DNA to *Brevibacterium lactofermentum* by electroporation. J. Gen. Microbiol. **136**: 2107-2112.

Bousfield, I. J. 1972. A taxonomic study of some coryneform bacteria. J. Gen. Microbiol. **71**, 441-455.

Bousfield, I. J. and A. G. Galley. 1978. Coryneform bacteria. Acedemic Press London.

Boyer, H. W. and D. Roulland-Dussoix. 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41**: 459-472.

Boramann, E. R., Eikmanns, B. J. and Sahm, H. 1992. Molecular analysis of the *Corynebacterium glutamicum gdh* gene encoding glutamate dehydrogenase. Mol. Microbiol. **6**: 317

Britz, M. L and A. L. Demian. 1985. Regulation of metabolite synthesis, p. 617-636. *In* Moo-Young, M. (ed), Comprehensive Biotechnology. Vol1. Pergamon Press. New York.

Brennan, P. J. 1989. Structure of mycobacteria: Recent developments in defining cell wall carbohydrates and proteins. Rev. Inf. Dis. **11**:420-430.

Brennan, P. J. 1988. *Mycobacterium* and other actinomycetes, p. 251-278. *In* Ratledge, C. and S. G. Wilkinson (eds.), Microbial lipids. Academic Press, London.

Brennan, P. J. and Nikaido, H. 1995. The envelope of mycobacteria. Annu. Rev. Biochem. **64**: 29-63.

Broer, S., Eggeling, L. and R. Kramer. 1993. Strains of *Corynebacterium glutamicum* with different lysine productivities may have different lysine excretion systems. Appl. Environ. Microbiol. **59**: 316.

Bunch, A. W., and R. E. Harris. 1986. The manipulation of micro-organisms for the production of secondary metabolites. Biotechnol. Genet. Eng. Rev. **4**: 117-144.

Buchanan-Wollaston, V., Passiatore, J. E. and F. Cannon. 1987. The *mob*- and *ori*T mobilisation functions of a bacterial plasmid promote its transfer to plants. Nature (London) **328**: 172-175.

Butler, W. R., Jost, Jr. C. and J. O. Kilburn. 1991. Identification of mycobacteria by high-performance liquid chromatography. J. Clin. Microbiol. **29**: 2468-2472.

Cardini, G., and P. Jurtshuk. 1970. The enzymatic hydroxylation of n-octane by *Corynebacterium* sp. strain 7E1C. J. Biol. Chem. **245**: 2789-2796.

Carlotti, A., Meugnier, H., Pommier, M. T., Villard, J. and J. Freney. 1993. Chemotaxonomy and molecular taxonomy of some coryneform clinical isolates. Zbl. Bakt. **278**: 27-33

Carlson, R. R. and A. K. Vidaver. 1982. Taxonomy of *Corynebacterium* plant pathogens including a new pathogen of wheat, based on polyacrylamide gel electrophoresis of cellular proteins. Int. J. Syst. Bacteriol. **32**:315-326.

Charter, K. F. and C. J. Bruton. 1983. Mutational cloning in *Streptomyces* and the isolation of antibiotic production genes. Gene **26**: 67-78.

Chevalier, J., Pommier, M-T., Creamier, A. and G. Mice. 1988. Influence of Tween 80 on the Mycolic acid composition of three cutaneous Corynebacteria. J. Gen. Microbiol. **134**: 2457-2461.

Chang, D. C. 1992. Design of protocols for electroporation and transfusion: selection of electric parameters, p. 429-455. *In* Chang, D. C., Chassy, B. M., Saunders, J. A. and A. E. Sowers (ed.), Guide to electroporation and electrofusion, Academic Press, San Diego.

Chassy, B. M. and J. L. Flickinger. 1987. Transformation of *Lactobacillus casei* by electroporation. FEMS Microbiol. Lett. **44**: 173-177.

Clement, Y., Escoffier, C., Trombe, M. C. and G. Laneelle. 1984. Is glutamate excreted by its uptake system in *Corynebacterium glutamicum*? a working hypothesis. J. Gen. Microbiol. **130**: 2,589-2,594.

Clement, Y. and G. Laneelle. 1986. Glutamate excretion mechanism in *Corynebacterium glutamicum*: triggering by biotin starvation or by surfactant addition. J. Gen. Microbiol. **132**: 925-929.

Cockerill, III, F. R., Uhl, J. R., Temesgen, Z., Zhang, Y., Stochman, L., Roberts, G. D., Williams, D. L. and B. C. Kline. 1995. Rapid identification of a point mutation of the *Mycobacterium tuberculosis* catalase-peroxidase (*KatG*) gene associated with isoniazid resistance. J. Infect. Dis. **171**: 240-245

Cohn M. L., Kovitz, C., Oda, U. and G. Middlebrook. 1954. Studies on isoniazid and tubercle bacilli. II. The growth requirements, catalase activities, and pathogenic properties of isoniazid-resistant mutants. Am. Rev. Tuberc. **70**: 641-644.

Collins, M. D., Pirouz, T., Goodfellow, M. and D. E. Minnikin. 1977. Distribution of menaquinones of actinomycete and related bacteria. J. Gen. Micobiol. **100**: 221-230.

Collins, M., Goodfellow, M. and D. E. Minnikin. 1979. Isoprenoid quinones in the classification of coryneform and related bacteria. J. Gen. Microbiol. **110**: 127-136.

Collins, M. D., Goodfellow, M. and D. E. Minnikin. 1982a. A survey of the structure of mycolic acids in *Corynebacterium* and related taxa. J. Gen. Microbiol. **128**: 129-149.

Collins, M. D., Goodfellow, M. and D. E. Minnikin. 1982b. Fatty acid composition of some mycolic acid-containing coryneform bacteria. J. Gen. Microbiol. **128**: 2,503-2,509.

Collins, M. D., and C. S. Cummins. 1986. *In* Sneath, P. H. A., Mair, N. S., Sharpe, M. E. and J. E. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 2, p. 1,266-1,276. The Williams and Wilkins Co., Baltimore.

Collins, M. D., Burton, R. A. and D. Jones. 1988. *Corynebacterium amycolatum* sp. nov. a new mycolic acid-less *Corynebacterium* species from human skin. FEMS Microbiol. lett. **49**: 349-352.

Corina, D. L. and D. Sesardic. 1980. Profile analysis of total mycolic acids from skin corynebacteria and from named *Corynebacterium* strains by gas-liquid chromatography/Mass spectrometry. J. Gen. Microbiol. **116**: 61-68.

Correia, A., Martin, J. F. and J. M. Castro. 1994. Pulsed field electrophoresis analysis of the genome of amino acid producing corynebacteria: chromosome sizes and diversity of restriction patterns. Microbiol. **140**: 2,841-2,847.

Constantinide, A. 1980. Steroid transformation at high substrate concentrations using immobilised *Corynebacterium simplex* cells. Biotechnol. Bioeng. **22**: 119-136.

Cooper, D. G., Zijic, J. E. and D. E. F Gracey. 1979. Analysis of corynemycolic acids and other fatty acids produced by *Corynebacterium lepus* grown on kerosene. J. Bacteriol. **137**: 795-801.

Crombach, W. H. J. 1978. DNA base ratios and DNA hybridisation studies of coryneform bacteria, mycobacteria and nocardiae, p. 161-179. *In* Bousfield, I. J. and A. G. Calley (ed.). Coryneform bacteria. London: Academic Press.

Cummins, C. S. and H. Harris. 1956. The chemical composition of the cell-wall in some Gram-positive bacteria and its possible value as a taxonomic character. J. Gen. Micobiol. **14**: 583-600.

Cummins, C. S. 1962. Chemical composition and antigenic structure of *Corynebacterium, Mycobacterium, Nocardia, Actinomyces* and *Arthrobacter.* J. Gen. Microbiol. **28**: 35-50

Datta, N., Hedges, R. W., Shaw, E. J., Sykes, R. B. and M. H. Richmond. 1971. Properties of an R factor from *Pseudomonas aeroginosa.* J. Bacteriol. **108**: 1,244-1,249.

Datta, N., and R. W. Hedges. 1972. Host ranges of R factors. J. Gen. Microbiol. **70**: 453-460

Davis, M. J., Gillespie Jr, A. G., Vidaver, A. K. and R. W. Harris. 1984. *Clavibacter:* a new species containing some phytopathogenic coryneform bacteria, including *Clavibacter xyli* subsp. *Xyli* sp. nov., subsp. nov. and *Clavibacter xyli* subsp. *Cyanodoutis* subsp. nov., pathogens that cause ratoon stunting disease of sugar-cane and Bermudagrass stunting disease. Int. J. Syst. Bacteriol. **34:** 107-117

Dawson, R. M. C., Ellioh, W. H. and K. M. Jones. 1986. PH, buffers and physiological media, p. 417-488. *In* Dawson, R. M. C., Ellioh, D. C., Ellioh, W. H. and K. M. Jones (ed.), Data for biochemical research -3rd Edition. Oxford University Press. New York.

Debadov, J. 1983. Construction of strains producing L-threonine, p. 254-. *In* Ikeda, Y. and Beppu, T. (eds.), Genetics of industrial microorganism. Kodansha, Tokyo.

Deretic, V., Philipp, W., Dhandayuthapani, S. 1995. *Mycobacterium tuberculosis* is a natural mutant with an activated oxidative-strees regulatory gene: implication for sensitivity to isoniazid. Mol. Microbiol. **1**: 889-900.

De Sousa-D'Auria, C., Kacem, R., Puech, V., Tropis, M., Lellon, G., Houssin, C., M. Daffe'. 2003. New insights into the biogenesis of the cell envelope of corynebacteria: identification and functional characterization of five new mycoloyltransferase genes in *Corynebacterium glutamicum*. FEMS Mic. Letts. **224**: 35-44.

Desomer, J., Crespi, M. and M. Van Montagu. 1991. Illegitimate integration of nonreplicative vectors in the genome of *Rhodococcus fascians* upon electrotransformation as an insertional mutagenesis system. Mol. Microbiol. **5**: 2,115-2,124.

Dessen, A., Quemard, A., Blanchard, J. S., Jacobs Jr, W. R. and J. C. Sacchettini. 1995. Crystal structure and function of the isoniazid target of *Mycobacterium tuberculosis*. Science. **267**: 1,638-1,641.

Devereux, J. Haberli, P. and O. Smithies. 1984. Nucleic Acids Res. **12**: 387-395.

Dienes, L., Weinberger, J. H. and S. Madoff. 1950. The transformation of typhoid bacilli into L-forms under various conditions. J. Bacteriol. **59**: 755-764.

Dolye, R. J. 1992. Cell walls of bacteria, p. 479-493. *In*, Lederberg, J., Alexander, M., Hopwood, D. A., Iglewski, B. H. and A. I. Laskin (ed), Encyclopedia of Micobiology Vol. 1, Academic Press, New York.

Dower, W. J., Miller, J. F. and C. W. Ragsdale. 1988. High efficiency transformation of *E. coli* by high voltage electroporation. Nucleic Acids Research. **16**: 6,127-6,145.

Dubey, S. K. and D. S. Holmes. 1995. Biological cynide destruction mediated by microorganisms. Word J. Microbiol. Biotechnol. **11**: 257-265.

Dufrene, Y. F., van der Wal, A., Norde, W. and P. G. Rouxhet. 1997. X-ray photoelectron spectroscopy analysis of whole cell walls of Gram-positive bacteria: comparison with biochemical analysis. J. Bacteriol. **179**: 1,023-1,028.

Dunbar, J. M. 1957. L'apparition de formes non-acidoresistantes de *Mycobacterium tuberculosis* en presence d'isoniazide, de cycloserine et de thioamide de l'acide α-ethyl isonicotinique. Ann. Inst. Pastuer. **92**: 451-458.

Dunican, L. K. and E. Shivnan. 1989. High frequency transformation of whole cells of amino acid producing coryneform bacteria using high voltage electroporation. Biotechnol. **7**: 1,067-1,070.

Eggeling, I., Cordes, C., Eggeling, L. and H. Sahm. 1987. Regulation of acetohydroxy acid synthase in *Corynebacterium glutamicum* during fermentation of α-ketobutyrate to L-isoleucine. Appl. Microbiol. Biotechnol. **25**: 346-351.

Eikmanns, B. J., Kircher, M. and D. J Reinscheid. 1991. Discrimination of *Corynebacterium glutamicum*, *Brevibacterium flavum* and *Brevibacterium lactofermentum* by restriction pattern analysis of DNA adjacent to *hom* gene. FEMS Microbiol. Lett. **82**: 203-208.

Eikmanns, B. J., Eggeling, L. and H. Sahm. 1993. Molecular aspects of lysine, threonine, and isoleucine biosynthesis in *Corynebacterium glutamicum*. Antonie van Leeuwenhock, **64:** 145-164

El-Yas, A. H., Nashed, S. M. and A. H. Yas. 1988. Bacteriological studies on mastitis in ewes and she-goats. J. Assiut. Vet. Med. **20**: 37-42.Etemadi, A. H., Pinte, F., J. Markovits. 1966. Sur la structure des precurseurs eventuels des acides α-smegmamycoliques. C. R. Acad. Sci. Paris. **262**: 1,343-1,346.

Etemadi, A. H. 1967. Isomerisation die methyle en milieu alcalin. Chem. Phys. Lipids. **1**: 165-175.

Fazal, A. M. and R. A. Jensen. 1980. Regulation of prephenate dehydratase on coryneform species of bacteria by L-phenylalanine and by remote effectors. Arch. Biochem. Biophys. **200**: 165-176.

Fernandez-Garazabal, J. F., Collins, M. D., Hutson, R. A., Gonzalez, I., Fernadez, E. and L. Domigguez. 1995. Int. J. Syst. Bacteriol. **48**: 463-468.

Ferrari, F. A., Nguyen, A., Lang, D. and J. A. Hoch. 1983. Construction and properties of an integrable plasmid for *Bacillus subtilis*. J. Bacteriol. **154**: 1,513-1,515.

Fiedler, F., Schaffler, M. J. and E. Stackbrandt. 1981. Biochemical and nucleic acid hybridisation studies on *Brevibacterium linens* and related strains. Arch. Microbiol. **129**: 85-93

Fiedler S. and R. Writh. 1988. Transformation of bacteria with plasmid DNA by electroporation. Anal. Biochem. **170:** 38-44.

Foley-Thomas, E. M., Whipple, D. L., Breudez, L. E. and R. G. Barletta. 1995. Phage infection, transfection and transformation of *Mycobacterium avium* complex and *Mycobacterium paratuberculosis*. Microbiol. **141:** 1,173-1,181.

Follettie, M. T. and A. J. Sinskey. 1986. Recombinant DNA technology for *Corynebacterium glutamicum*. Food Technol. **40**: 88-94.

Freney, J., Duperron, M. T., Courtier, C., Hanse, W., Allard, F., Boeufgras. J. M., Monget, D. and J. Fleurette. 1991. Evaluation of API-Coryne in comparison with conventional methods for identifying coryneform bacteria. J. Clin. Microbiol. **29**: 38-41.

Frieden, T. R., Sterling, T., Pablos-Mendez, A., Kilburn, J. O., Cauthen, G. M., S. W. Dooley. 1993. The emergence of drug-resistant tuberculosis: New York City. N Engl J. Med. **328**: 521-526.

Fujioka, M., K. Shigetaka and M. Yukiyoshi. 1985. Novel glycosidic linkage between arabinogalactan and peptidoglycan in the cell wall skeleton of *Nocardia rubra* AN-115. J. Gen. Microbiol. **131**: 1323-1329.

Funk, G., Hutson, R. A., Hilleringmann, M., Heizmann, W. R. and M. D. Collin. 1977. *Corynebacterium lipophiloflavum* sp. nov. isolated from a patient with bacterial vaginosis. FEMS Microbiol. Lett. **150**: 219-224.
Funk, G., Lawson, P. A. and M. D. Collins. 1995. Heterogeneity within Centres for Disease Control and Prevention coryneform group ANF-1 like bacteria and description of *Corynebacterium* auris sp. nov. Int. J. Syst. Bacteriol. **45**: 735-739.

Funke, G., Pascual, C. and M. D. Collins. 1997. *Corynebacterium coyleae* sp. nov., isolated from human clinical specimens. Int. J. Syst. Bacteriol. **47**: 92-96.

Gabor, M. H. and R. O. Hotchkiss. 1979. Parameters governing bacterial regeneration and genetic recombination after fusion of *Bacillus subtilus* protoplasts. J. Bacteriol. **137**: 1,346-1,353.

Gailly, C., Sandra, P., Verzele, M. and C. Cocito. 1982. Analysis of mycolic acid from a group of corynebacteria by capillary gas chromatography and mass chromatography. Eur. J. Biochem. **125**: 83-94.

Gavin, S. E., Leonard, R. B., Briselden, A. M. and M. B. Coley. 1992. Evaluation of rapid CORYNE identification system for *Corynebacterium* species and other coryneforms. J. Clin. Microbiol. **30**: 1,962-1,965.

Gastambide-Odier, M. and E. Lederer. 1959. Biosynthesis of corynemycolic from two molecules of palmitic acid. Nature. **184**: 1,563-1,564.

Gastambide-Odier, M. and E. Lederer. 1960. Biosynthesis de l'acide corynemycolique a partir de deux molecules d'acide palmitique. Biochem. Zeitsch. **333**: 285-295.

Gangadharam, P. R. J., Harold, F. M. and Schaefer, W. B. 1963. Selective inhibition of nucleic acid synthesis in *Mycobacterium tuberculosis* by isoniazid. Nature (Lond.). **198**: 712-714.

George, K. M., Yuan, Y., Sherman, D. R. and C. E. Barry, III. 1995. The biosynthesis of cyclopropanated mycolic acids in *Mycobacterium tuberculosis*. Identification and functional analysis of CMAS-2. J. Biol. Chem. **270**: **45**. 27,292-27,298.

Goodfellow, M. 1967. Numerical taxonomy of some named bacterial cultures. Can. J. Microbiol. **13**: 1365-1375.

Goodfellow, M., Collins, M. D. and D. E. Minnikin. 1976. Thin layer chromatographic analysis of mycolic acid and other long-chain components in whole-organism methanolysates of coryneform and related taxa. J. Gen. Microbiol. **96**: 351-358.

Gordon, J. and M. Gordon. 1943. Involution forms of the genus *Vibro* produced by glycine. J. Pathol. Bacteriol. **55**: 63-68.

Greez, N., Dack, D. M. and L. R. Hedrick. 1962. Antimicrobial agents of surface ripened cheese. II. Source and properties of active principles. J. Food. Sci. **27**: 335-342.

Gubler, M. E., Park, S. M., Jetten, M. S. M., Stephanopoulos, G. and A. J. Sinskey. 1994a. Effects of phosphoenol pyruvate carboxylase deficiency on metabolism and lysine production of *Corynebacterium glutamicum*. Appl. Microbiol. Biotech. **40**: 857.

Gubler, M. E., Jetten, M. S. M., Lee, S. H. and A. J. Sinskey 1994b. Cloning of pyruvate kinase gene (*pyk*) *of Corynebacterium glutamicum* and the site-specific inactivation of *pyK* in a lysine producing *Corynebacterium lactofermentum* strain. Appl. Environ. Microbiol. **60**: 2,469-2,500.

Gutmann, M., Hoischen, C. and R. Kramer. 1992. Carrier mediated glutamate secretion by *Corynebacterium glutamicum* under biotin limitation. Biochem. Biophys. Acta. **1112**: 115-123.

Hagino, H. and K. Nakayama. 1975a. L-Tryptophan production by analog-resistant mutants derived from a phenylalanine and tyrosine double auxotroph of *Corynebacterium glutamicum*. Agric. Biol. Chem. **39**: 343-349.

Hagino, H. and K. Nakayama. 1975b. The biosynthetic control in aromatic amino acid producing mutants of *Corynebacterium glutamicum*. Agric. Biol. Chem. **39**: 351-361.

Hamid, M. E., Minnikin, D. E., Goodfellow, M. and M. Ridell. 1993. Thin layer chromatographic analysis of glycopeptids and mycolic acids from *Mycobacterium senegalense* and related taxa. Zbl. Bakt. **279**: 354-367.

Hamid, M. E., Minnikin, D. E. and M. Goodfellow. 1993. A simple chemical test to distinguish mycobacteria from other mycolic-containing actinomycetes. J. Gen. Microbiol. **139**: 2, 203-2,213.

Hammes, W., Schleifer, K. H. and O. Kandlers. 1973. Mode of action of glycine on the biosynthesis of peptidoglycan. J. Bacteriol. **116**: 1,029-1,053

Harringston, B. J. 1996. A numerical taxonomic study of some corynebacteria and related organism. J. Gen. Microbiol. **45:** 31-40.

Hartmans, S. and J. A. M. De Bont. 1992. The genus *Mycobacterium*—nonmedical, p. 1,214-1,237. *In* Balows, A., Truper, H. G., Dworkin, M., Harder, W. and K. H. Schleifer (ed.), The Prokaryotes, 2nd ed. Spring-Verlag, New York.

Haynes, J. and M. L. Britz. 1989. Electransformation of *Brevibacterium lactofermentum* and *Corynebacterium glutamicum*: growth in Tween 80 increases transformation frequencies. FEMS Microbial. Lett. **61**: 329-334.

Haynes, J. A. and M. L. Britz. 1990. The effect of growth conditions of *Corynebacterium glutamicum* on the transformation frequency obtained by electroporation. J. Gen. Microbiol. **136**: 255-263.

Hedgecock, L. W., O. I. Faucher. 1957. Relation of pyrogallol-peroxidase activity to isoniazid resistance in *Mycobacterium tuberculosis*. Am. Rev. Tuberc. **75**: 670-674.

Hendrik, C. A., Haskin, W. P. and A. K. Vidaver. 1984. Conjugative plasmid in *Corynebacterium flaccumfaciens* subsp. *ooriti* that confers resistance to arsenite and antimony (III). Appl. Environ. Microbiol. **48**: 56-60.

Herrera-Alcarzar, E., Valero-Guillen, P., Martin-Luengo, F. and M. Canteras-Jordana. 1993. Numerical analysis of fatty and mycolic acid profiles of *Corynebacterium urealyticum* and other related corynebacteria. Microbiologia. **9**: 53-62.

Heym, B., Honore, N., Truffot-Pernot, C., Banerjee, A., Schurra, Jr, C. W. R. J., J. D. A. V. Embden,. 1994. Implications of multidrug resistance for the future of short-course chemotherapy of tuberculosis: a molecular study. Lancet. **344**: 293-298.

Heym. B., Alzari, P. M., Honore, N., S. T. Cole. 1995. Missense mutations in the catalase-peroxidase gene, *KatG*, are associated with isoniazid resistance in *Mycobacterium tuberculosis*. Mol. Micobiol. **15**: 235-245.

Hirose, Y., Enei, H. and H. Shibai. 1985. L- Glutamic acid fermentation, p. 593-605. *In* M. Moo-Young (ed), Comprehensive Biotechnology. Vol 3. Pergamon press. Oxford, New York, Toronto, Sydney, Frankfurt.

Hishinuma, F., Izaki, K. and H. Takahashi. 1969. Effects of glycine and D-amino acids on growth of various microorganisms. Agr. Biol. Chem. **33**: 1,577-1,586.

Hishinuma, F., Izaki, K. and H. Takahashi. 1971. Inhibition of L-alanine adding enzymes by glycine. Agr. Biol. Chem. **35**: 2,050-2,058.

Hoischen, C. and R. Kramer. 1989. Evidence for an efflux carrier system involved in the secretion of glutamate by *Corynebacterium glutamicum*. Arch. Microbiol. **151**: 342-347.

Hoischen, C. and R. Kramer. 1990. Membrane alteration is necessary but not sufficient for effective glutamate secretion in *Corynebacterium glutamicum*. J. Bacteriol. **172**: 3,409-3,416.

Honkanen-Buzalski, T., Myllys, V. and L. Kulkas. 1996. Prevalence of bovin mastitis in Finland. Soum Elainlakaaril. **102**: 191-197.

Hopwood, D. A., Wright, H. M., Bibb, M. J. and S. N. Cohn. 1977. Genetic recombination through protoplast fusion in *Streptomyces*. Nature. **268**: 171-174.

Hopwood, D. A. 1981. Genetic studies with bacterial protoplasts. Annu. Rev. Microbiol. **35**: 237-272.

Howe, E. E., Jensen, G. R. and E. W. Gilfillan. 1965. Amino acid supplementation of cereal grains as related to the food supply. Am. J. Clin. Nutr. **16**: 315-320.

Hunter, S. W., Murphy, R. C., Clay, K., Goren, M. B., P. J. Brennan. 1985. J. Biol. Chem. **258**: 10,481-10,487.

Ikeda, S., Fujita, I. and F. Yoshinaga. 1976. Screeninmg of L-isoleucine producers among ethionine resistant mutants of L-threonine producing bacteria. Agric. Biol. Chem. **40**: 511-516.

Ikeda, M. and A. Katsumata. 1992. Metabolic engineering to phenylalanine in a tryptophane-producing *Corynebacterium glutamicum* strain. App. Environ. Microbiol. **58**: 781-785.

Ito, H., Sato, K., Matsui, K., Sano, H. Enei. and Y. Hirose. 1990. Molecular breeding of *a Brevibacterium lactofermentum* L-phenylalanine producer using a cloned prephenate dehydratase gene. Appl. Microbiol. Biotechnol. **33:** 190 195.

Iwainsky, H. 1988. INH, mode of action, p. 476-508. *In* K. Bartmann (eds), Handbook of experimental pharmacology, vol. 84. Spring-Verlag, Berlin.

Jackett, P. S., Aber, V. R. and D. B. Lowrie. 1978. Virulence and resistance to superoxide, low pH and hydrogen peroxide among strains of *Mycobacterium tuberculosis*. J. Microbiol. **104**: 37-45.

Jacobs Jr, R. W., Ganjam, V. K., Jeffery, D. R., Lisa, P., Scott, B. S., Rupa, A. U., Wilber Jones, Raul G. B. and Barry R. Bloom. 1991. Genetic systems for mycobacteria. Methods Enzymol. **204**, 537-556.

Jäger, W., Schäfer, A., Kalinowski, J. and A. Pühler. 1995. Isolation of insertion element from Gram-positive *Brevibacterium*, *Corynebacterium*, and *Rhodococcus* strains using the *Bacillus subtilis sacB* gene as a positive selection marker. FEMS Microbial. Lett. **126**: 1-6

Jarlier, V. and H. Nikaido. 1990. Permeability barrier to hydrophilic solutes in *Mycobacterium chelonei*. J. Bacteriol. **172**: 1418-1423.

Jarlier, V., Gutmann, L. and H. Nikaido. 1991. Interplay of cell wall barrier and β-lactamase activity determines high resistance to β-lactam antibiotics in *Mycobacterium chelonae*. Antimicrob. Agents. Chemother. **35**: 1937-1939.

Jarlier, V. and H. Nikaido. 1994. Mycobacterial cell wall: structure and role in natural resistance to antibiotics. FEMS Microbiol. Lett. **123**:11-18.

Jang, K. H., Chambers, P. J. and M. L. Britz. 1996. Analysis of nucleotide methylation in DNA from *Corynebacterium glutamicum* and related species. FEMS Microbiol. Lett. **136**: 309-315.

Jang, K. H., Pierotti, D., Kemp, G. W., Best, G. R. and M. L. Britz. 1997. Mycolic acid composition of *Corynebacterium glutamicum* and its cell surface mutants: effects of growth with glycine and isonicotinic acid hydrazide. Microbiol. **143**: 3209-3221.

Jang, K. H. 1997. Identification and characterisation of the cell surface and enzymatic barriers to plasmid transformation in *Corynebacterium glutamicum* and related species. Ph. D. thesis. Victoria University of Technology. Australia.

Jensen, H. L. 1952. The coryneform bacteria. Annu. Rev. Microbiol. **6**: 77-90.

Jetten, M. S. M., Gubler, M. E., McCormick, M. M., Colon, G. E., Follettie, M. T. and A. Sinskey 1993. Molecular organisation and regulation of the biosynthetic pathway for aspartate derived amino acids in *Corynebacterium glutamicum*, p. 97. *In* Baltz, R., Hedgeman, G. and P. L. Skatrud (eds.), Industrial Microorganisms: Basic and Applied Molecular Genetics, American Society for Microbiology, Washington, D. C.

Jetten, M. S. M., Follettie, M. T. and A. J. Sinskey. 1994. Metabolic engineering of *Corynebacterium glutamicum*, p. 754. *In* Bajpai, R. and A. Prokop (eds.), Recombinant DNA Technology II, Annals of the New York Academy of Science, New York.

Jetten, M. S. M., Follittie, M. T. and A. J. Sinskey 1995. Effect of different levels of aspartokinase on the lysine production of *Corynebacterium lactofermentum*, Appl. Microbiol. Biotechnol.

Johnsson, K. and P. G. Schultz. 1994. Mechanism of the oxidation of isoniazid by the catalase peroxidase from *Mycobacterium tuberculosis*. J. Am. Soc. **116**: 7,425 -7,426.

Joliff, G., Mathieu, L., Hahn, V., Bayan, N., Duchiron, F., Renaud, M., Shechter, E., G. Leblon. 1992. Cloning and nucleotide sequence of the *csp1* gene encoding PS1, one of the two major secreted proteins of *Corynebacterium glutamicum*: the deduced N-terminal region of PS1 is similar to the *Mycobacterium* antigen 85 complex. Mol. Microbiol. **6**: 2,349-2,362.

Jones, D. and R. M. Keddie. 1981. The genus *Arthrobacter*, p. 1,281-1,299. *In* Stolp, M. P., Truper, H. G., Balows, A. and H. G. Schlegel (ed.), The prokaryotes. A handbook on habitates, isolation and identification of bacteria. Springer-Verlag, Berlin, Germany.

Jones, D. and M. D. Collins. 1986. Irregular non-sporing Gram positive rods, p. 1,261-1,264. *In* Williams, S. T., Sharpe, M. E. and J. Holt (ed.), Bergey's Manual of Systematic Bacteriology, vol. 2, Williams and Wilkins: Baltimore.

Kacem, K., De Sousa-D' Auria, C., Tropis, M., Chami, M., Gounon, P., Leblon, G., Houssin, C., M. Daffe. 2004. Importance of mycoloyltransferases on the physiology of *Corynebacterium glutamicum*. Microbiol. 150: (Pt 1): 73-84.

Kalpana, G. V., Bloom, B. R. and W. R. Jacobs, Jr. 1991. Insertional mutagenesis and illegitimate recombination in mycobacteria. Proc. Natl. Acad. Sci. USA. **88**: 5,433-5,437.

Kaneko, H. and K. Sakaguchi. 1979. Fusion of protoplast and genetic recombination in *Brevibacterium flavum*. Agric. Biol. Chem. **43**: 1,007-1,013.

Kapur, V., Li, L. L. and M. R. Hanrick., 1995. Rapid *Mycobacterium* species assignment and unambiguous identification of mutations associated with antimicrobial resistance in *Mycobacterium tuberculosis* by automated DNA sequencing. Arch. Pathol. Lab. Med. **119**: 131-138.

Karasawa, M., Tosaka, O., Ikeda, S. and H. Yoshii. 1986. Application of protoplast fusion to the development of L-threonine and L-lysine producers, Agric. Biol. chem., **50**, 339.

Kato, F., Yoshimi, M., Araki, K., Motomura, Y., Mastsufune, Y., Nobunaga, H. and A. Murata. 1984. Screening of bacteriocins in amino acid or nucleic acid producing bacteria and related species. Agric. Biol. Chem. **48**: 193-200.

Kato, F., Eguchi, Y., Nakano, M., Oshima, T. and A. Murata. 1991. Purification and characterization of linencin A, a bacteriocin of *Brevibacterium linens*. Agric. Biol. Chem. **55**:161-166.

Katsumata, R, Ozaki, A., Oka, T. and A. Furuya. 1984a. Recombinant DNA technology for *Corynebacteria* and *Brevibacteria*. Abstracts, VII Int. Biotech. Symp. New Delhi, India, p 61.

Katsumata, R., Ozaki, A., Oka, T. and A. Furuya. 1984 b. Protoplast transformation of L-glutamate-producing bacteria with plasmid DNA. J. Bacteriol. **159**: 306-311.
Keddie, R. M. and G. L. Cure. 1977. The cell wall composition and distribution of free mycolic acids in named strains of corynebacteria and in isolates from various natural sources. J. Appl. Bacteriol. **42**: 229-252.

Keddie, R. M. 1978. What do we mean by coryneform bacteria? *In* Bousfield, I. J. and A. G. Calley (ed.), Coryneform Bacteria,. Acedemic Press: London.

Kinoshita, S. 1985. Glutamic acid bacteria, p.15-142. *In* Demain, A. L. and N. A. Solomon (eds.), Biology of industrial microorganisms. Butterworth publication, Stoneham, Mass.

Kinoshita, S. 1987. Amino acids and nucleotide fermentations: from their genesis to the current state. J. Ind. Microbiol. Supp. **1**:1-12.

Kikuchi, S., Takeuchi, T., Yasui, M., Kusaka, T. and P. E. Kaolattukudy. 1989. A very long-chain fatty acid elongation system in *Mycobacterium avium* and a possible mode of action of isoniazid on the system. Agric. Biol. Chem. **53**: 1,689-1,698.

Kikuchi, S., Rainwater, D. L. and P. E. Kolattukudy. 1992. Purification and characterisation of an unusually large fatty acid synthase from *Mycobacterium tuberculosis* var. *bovis* BCG. Arch. Biochem. Biophys. **295**: 318-326.

Kisumi, M., Komatsubara, S. and I. Chibata. 1977. Enhancement of hydroxamate-mediated growth inhibition and improvement of isoleucine-producing strains of *Serratia marcescens*. Appl. Environ. Microbiol. **34**: 647-653.

Klatte, S., Rainey, F. A., and R. M. Kroppenstedt. 1994. Transfer of *Rhodococcus aichiensis* Tsukamura 1982 and *Nocardia amarae* Lechevalier and Lechevalier 1974 to the genus *Gordona* as *Gordona aichiensis* comb. nov. and *Gordona amarae* comb. nov. Int. J. Syst. Bacteriol. **44**: 769-773.

Kleemann, A., Leuchtenberger, W., Hoppe, B. and H. Tanner. 1985. Amino acids, p. 57-97. *In* Gerhartz, W (ed.), Ullmanns' encyclopedia of industrial chemistry, vol. 2A. VCH Verlagsgesellschaft mbH, Weinheim, Germany.

Kolattukudy, P. E., Norvin, D. F., Azad, A. K., Fitzmaurice. A. M. and D. S. Tatiana 1997. Biochemistry and molecular genetics of cell-wall lipid biosynthesis in mycobacteria. Mol. Microbiol. **24**: 163-270.

Kramer, R. 1994. Secretion of amino acids by bacteria: physiology and mechanism, FEMS Microbiol. Rev. **13**, 75.

Krisna Murti, C. R. 1975. Isonicotinic acid hydrazide, p. 623-652 . In Corcoran. J. W. and F. E. Hahn (ed.), Antibiotics III. Springer-Verlag, Berlin.

Labarre, J., Reyes, O., Guyonvarch, A., and G. Leblon. 1993. Gene replacement integration, and amplification at the *gdhA* locus of *Corynebacterium glutamicum*. J. Bacteriol. **175**: 1,001-1,007

Lacave, C., Laneelle, M. A. and G. Laneelle. 1990a. Mycolic acid synthesis by *Mycobacterium aurum* cell-free extracts. Biochim. Biophys. Acta. **1,042:** 315-323.

Lacave, C., Quemard, A. and G. Laneelle. 1990b. Cell free synthesis of mycolic acid in *Mycobacterium aurum:* radioactivity distribution in newly synthesised acids and presence of cell wall in the system. Biochem. Biophys. Acta. **1045**: 58-68.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**: 680-685.

Lambert, C., Erdmann, A., Eikmanns, M. and R. Kramer. 1995. Triggering glutamate excretion in *Corynebacterium glutamicum* by modulating the membrane state with local anaesthetics and osmotic gradients. Appl. Environ. Microbiol. **61**: 4,334-4,342.

Laneelle, G. 1989. Mycolic acid metabolism. Acta. Leprol (suppl. 1), **7**: 65-73.

Law, B. A., and M. E. Sharpe. 1978. Formation of methanethiol by bacteria isolated from raw milk and cheddar cheese . J. Dairy Res. **45**: 267-275.

Lee. E. R, Armour, J. W., Takayama, K., Brennan, P. J., G. S. Bersa. 1997. Mycolic acid biosynthesis: definition and targeting of the Claisen condensation step. Bioch. Bioph. Acta. **1346**: 275-284.

Lechevalier, M. P. and H. A. Lechevalier. 1970. Chemical composition as a criterion in the classification of aerobic *Actinomycetes*. Int. J. Syst. Bacteriol. **20**: 435-443.

Lechevalier, M. P. 1976. The taxonomy of the genus *Nocardia:* Some light at the end of the tunnel? p. 1-38. *In* Goodfellow, M., Brownell, G. H., and J. A. Serrano (ed.), The Biology of Nocardiae, Academic Press: London.

Lederer, E. 1971. The mycobacterial cell wall. Pure. Appl. Chem. **25**: 135-164.

Lehmann, K. B. and R. O. Neumann. 1907. Atlas und grundriss der bakteriologichen diagnostik. J. F. Lehmann Munich.

Lemassu, A., Ortalo-Magne', A., Bardou, F., Silve, Laneelle, M.-A.G and Daffe',M. 1996. Extracellular and surface-exposed poly-saccharides of non tuberculous mycobacteria. Microbiol: 142: 1513-1520.

Lewis, B. A. 1982. Correlation between thiol production and antifungal activity by *Brevibacterium linens*. Microbios Lett. **21**: 75-78.

Liebl, W., Bayerl., A., Stillner, U. and K. H. Scheilfer. 1989. High frequency electroporation of intact *Corynebacterium glutamicum* cells. FEMS Microbiol. Lett. **65**: 299-304.

Liebl, W., Ehrmann, M. Luwig, W. and K. H. Schleifer. 1991. Transfer of *Brevibacterium diverticatum* DSM 20297, *Brevibacterium flavum* DSM 20411, *Brevibacterium lactofermentum* DSM 20412 and DSM 1412, and *Corynebacterium lilium* DSM20317 to *Corynebacterium glutamicum* and their distinction by rDNA gene restriction patterns. Int. Syst. Bacteriol. **41**: 255-260.

Liebl, W., Sinskey, A. J. and K. H. Schleifer. 1992. Expression, secretion, and processing of staphylococcal nuclease by *Corynebacterium glutamicum*. J. Bacteriol. **174:** 1,854-1,861.

Liebl, W. 1992. The genus *Coryneacterium*-nonmedical, p. 1,157-1,171. *In* A. Balows, H. G. Truper, M. Dworkin, W. Harder, and K. H. Schleifer (ed.), The Prokaryotes, 2nd ed. Springer-Verlag, Berlin.

Liu, J., Rosenberg, E. Y. and H. Nikaido. 1995. Fluidity of the lipid domain of cell wall from *Mycobacterium chelonae*. Proc. Natl. Acad. Sci. USA. **92:** 11,254-11,258.

Lopez-Marin, L. M., Quemard, A., Laneelle, G. and C. Lacave. 1991. Ethylenic mycolic acid biosynthesis: extension of the biosynthetic model using cell-free extracts of *Mycobacterium aurum* and *Mycobacterium smegmatis*. Biochem. Biophys. Acta **1086**: 22-28.

Low, K. B. and D. D. Porter. 1978. Modes of action of gene transfer and recombination in bacteria. Annu. Rev. Genet. **12**: 249-287.

MacNeil, D. G. 1987. Introduction of plasmid DNA into *Streptomyces lividans* by electroporation. FEMS Microbiol. Lett. **42**: 239-244.

Maniatis, T., Fritsch, E. F., and J. Sambrook. 1982. Molecular cloning. Cold spring Harbor Laboratory, New York.

Marklund. B. I., Speert, D. P. and R. W. Stokes. 1995. Gene replacement through homologous recombination in *Mycobacterium intracellulare*. J. Bacteriol. **177**: 6,100-6,105.

Marco, J. C. 1994. Mastitis en la oveja Latxa: epidemiología, diagóstico y control. PhD thesis, University of Zaragoza.

Martin, J. F., Santamaria, R., Sandoval, H., del Real, G., Mateos, L. M., Gil, J. A. and A. Aguilar. 1987. Cloning systems in amino acid-producing Corynebacteria. Biotechnol. **5**: 137-146.

Marrakchi, H., Ducasse. S., Labesse. G., Montrozier, H., Margeat, E., Emorine, L., Charpentier, X., Daffe', M., A. Quemard. 2002. MabA(FabGI), a *Mycobacterium tuberculosis* protein involved in the long-chain fatty acid elongation system FAS-II. Microbiol. **148**(Pt 4): 951-60.

Martin, J. F., Cadenas, R. F., Malumbres, M., Mateos, L. M., Guerrero, C. and J. A. Gil. 1990. Construction and utilisation of promoter-probe and expression vectors in corynebacteria. Characterisation of promoters, p. 283-292. *In* Heslot, H., Davies, J., Florent, J., Babichon, L., Durant, G. and L. Penasse (ed), Proceedings of the 6th International Symposium of industrial Microorganisms. Société Francaise de Micobiologie, Strasbourg, France.

Mateos. M. L., Schäfer, A., Kalinowski, J., Martin, J. F. and A. Pühler. 1996. Integration of narrow-host-range vectors from *Escherichia coli* into the genomes of amino acid-producing Corynebacteria after integeneric conjugation. J. Bacteriol. **178**: 5,768-5,775.

Mazodier, P., Petter, R. and C. Thompson. 1989. Intergeneric conjugation between *Escherichia coli* and *Streptomyces* species. J. Bacteriol. **171**: 3,583-3,585.

McAllister, K. A., Lee, H. and J. T. Trevors. 1996. Microbiol deregulation of pentachlorophenol. Biodegradation. **7**: 1-40.

McGinely, K. J., Labows, J. N., Zechman, J. M., Nordstorm, K. M., Webester, G. F. and J. J. Leyden. 1985. Analysis of cellular components, biochemical reactions and habitat of human cutaneous lipophilic diphtheroids. J. Invest. Dermatol.. **85**: 374-377.

McNeil, M., Daffe, M. and P. J. Brennan. 1990. Evidence for the nature of link between the arabinogalactan and peptidoglycan of mycobacterial cell wall. J. Biological. Chem. **265**: 18,200-18,206.

McNeil, M. R. and P. J. Brennan. 1991. Structure, function and biogenesis of the cell envelope of mycobacteria in relation to bacterial physiology, pathogenesis and drug resistance; some thoughts and possibilities arising from recent structural information. Res. Microbiol. **142**: 451-463.

Mdluli, K. and C. E. Barry, III. 1996. Drug sensitivity and environmental adaptation of mycobacterial cell wall components. Trend Microbiol. **4**: 275-281.

Mdluli, K., Sherman, D. R., Hickey, M. J., Kreiswrith, N. B., Morris, S., Stover, C. K., C. E. Barry, III. 1996. Biochemical and genetic data suggest that InhA is not the primary target for activated isoniazid in *Mycobacterium tuberculosis*. J. Infect. Dis. **174**: 1,085-1,090.

Michel, G. and C. Bordet. 1976. Cell walls of *Nocardiae*, p. 141-159. *In* Goodfellow. M., Brownell, G. H. and J. A. Serrano (ed.), The Biology of *Nocardiae*. London: Academic Press.

Middlebrook, G. 1952. Sterilisation of tubercle bacilli by isonicotinic acid hydrazide and the incidence of variants resistant to the drug *in vitro*. Am. Rev. Tuberc. **65**: 765-767.

Middlebrook, G. 1954. Isoniazid-resistance and catalase activity in tubercle bacilli. Am. Rev. Tuberc. **69**: 471-472.

Middlebrook, G., Cohn, M. L., W. B. Schäefer. 1954. Studies on isoniazid and tuberculosis bacilli. III. The isolation, drug susceptibility, and catalase testing of tubercle bacilli from isoniazid-treated patients. Am. Rev. Tuberc. **70**; 852-872.
Miller, J. F., Dower, W. J. and L. S. Tompkins. 1988. High-voltage electroporation of bacteria: genetic transformation of *Campylobactor jejuni* with plasmid DNA. Proc. Natl. Acad. Sci. USA. **85**: 856-860.

Milas, L. and M. T. Scott. 1978. Antitumor activity of *Corynebacterium parvum*. Adv. Cancer Res. **26**: 257-306.

Minnikin, D. E., Alshamaony, L. and M. Goodfellow. 1975. Differentiation of *Mycobacterium, Nocardia* and related taxa by thin layer chromatography analysis of whole-organism methanolysates. J. Gen. Micobiol. **88:** 200-204.

Minnikin, D. E., Patel, P. V., Alshamaony, L. and M. Goodfellow. 1977. Polar lipids composition in the classification of *Nocardia* and related bacteria. Int. J. Syst. Bacteriol. **27:** 104-117.

Minnikin, D. E., Goodfellow, M. and M. D. Collins. 1978. Lipids composition in the classification and identification of coryneform and related taxa, p. 85-160. *In* Bousfield, I. J. and A. G. Callely (ed.), "Coryneform Bacteria"Academic Press: London.

Minnikin, D. E. and Goodfellow. 1980. Lipid composition in the classification and identification of acid-fast bacteria, p. 189-256. *In* M. Goodfellow and R. G. Board (ed.), Microbiological classification and identification. Academic Press. London.

Minnikin, D. E., Hutchinson, I. G., Caldicott, and M. Goodfellow. 1980. Thin layer chromatography of methanolysates of mycolic acid-containing taxa. J. Chromatogr. **188**: 221-233.

Minnikin, D. E. 1982. Lipids: Complex lipids, their chemistry, biosynthesis and roles, p. 95-184. *In* C. Ratledge, J. L Stanfords (eds.), The Biology of the Mycobacteria, Vol.1.44. Physiology, Identification and Classification. Academic Press, London.

Minnikin, D. E. and A. G. O'Donnell. 1984. Actinomycete envelope lipids and peptidoglycan composition, p. 337-388. *In* Goodfellow, M., Mordarski, M. and S. T. Williams (eds), The Biology of the *Actinomycete*. Academic Press. London.

Misaki, A., Seto, N. and I. Azuma. 1974. Structure and immunological properties of D-arabino-D-galactans isolated from cell-walls of *Mycobacterium* species. J. Biochem. **76**: 15-27.

Miwa, K., Tsuchida, T., Kurahashi, D., Nakamori, S., Sano, K. and H. Momose. 1983. Construction of L-threonine over producing strains of *Escherichia coli* K-12 using recombinant DNA techniques. Agric. Biol. Chem. **47**: 2,329,

Miwa, K., Matsui, H., Terabe, M., Ito, K., Ishida, M., Takagi, H., Nakamori, S. and K. Sano. 1985. Construction of novel shuttle vectors and cosmid vector for the glutamic acid producing bacteria *Brevibacterium lactofermentum* and *Corynebacterium glutamicum.* Gene. **39**: 281-

Momose, H., Miyashiro, S. and M. Oba. 1976. On the transducing phages in glutamic acid producing bacteria. J. Gen. Appl. Microbiol. **22**: 119-

Morbach, S., Sahm, H. and L. Eggeling. 1995. Use of feedback-resistant threonine dehydratases of *Corynebacterium glutamicum* to increase carbon flux towards L-isoleucine. Appl. Environ. Microbiol. **61:** 4,315-4,320.

Mordarska, H., Mordarski, M. and M. Goodfellow. 1972. Chemotaxonomic characters and classification of some nocardioform bacteria. J. Gen. Microbiol. **71**: 77-86.

Mordarski, M., Goodfellow, M., Tkaez, A., Pulverer, G. and K. P. Schaal. 1980. Ribosomal ribonucleic acid similarities in the classification of *Rhodococcus* and related taxa. J. Gen. Microbiol. **118**: 313-319

Musser, J. M. 1995. Antimicrobial agent resistance in mycobacteria: molecular genetic insights. Clin. Microbiol. Rev. **8**: 496-514.

Musser, J. M., Kapur, V., William, D. L., Kreiswirth, B. N., van Soolingen, D. and J. D. A. van Embden. 1996. Characterisation of the catalase-peroxidase gene (*KatG*) and *inhA* locus in isoniazid-resistant and susceptible strains of *Mycobacterium tuberculosis* by

automated DNA sequencing: restricted array of mutations associated with drug resistance. J. Infect. Dis. **173:** 196-202.

Niederweis, M., Maier, E., Lichtinger, T., Benz, R. and R. Krämer. 1995. Identification of channel-forming activity in the cell wall of *Corynebacterium glutamicum*. J. Bacteriol. **177**: 5,716-5,718.

Nikaido, H. and M. Vaara. 1985. Molecular basis of bacterial outer membrane permeability. Microbiol. Rev. **49**: 1-32.

Nikaido. H., Kim, S.-H. and E. Y Rosenberg. 1993. Physical organisation of lipids in the cell wall *of Mycobacterium chelonae*. Mol. Mirobiol. **8**: 1,025-1,030.

Noh, K-S., Kim, S-J., Lee, H-H., Hyun, H-H. and J-H. Lee. 1991. High frequency electroporation-transformation of coryneform bacteria grown in the penicillin-G. Korean J. Biotechnol. Bioeng. **6**: 223-230.

Osborn, M. J., Gander, J. E., Parisi, E. and J. Carlson. 1972. Mechanism of assembly of the outer membrane of *Salmonella typhimurium*. Isolation and characterisation of cytoplasmic and outer membrane. J. Biol. Chem. **247**: 3,962-3,972.

Ochi, K. 1989. Heterogeneity of ribosomal proteins among *Streptomyces* species and its application to identification. J. Gen. Microbiol. **135**: 2,635-2,642.
Ochi, K. 1995. Phylogenetic analysis of mycolic acid-containing Wall-Chemotype IV *Actinomycetes* and allied taxa by partial sequencing of ribosomal protein AT-L30. Int. J. Syst. Bacteriol. **45**: 653-660.

Olsen, R. H. and P. Shipley. 1973. Host rang properties of the *Pseudomonas* R factor R1822. J. Bacteriol. **113:** 772-780.

Ortalo-Magne', A., Dupont, M.-A., Lemassu, A.,Andersen, A. B., Gounon, P. and Daffe',M. 1995. Molecular composition of the outermost capsular material of the tubercle bacillus. Microbiol. **141**: 1609-1620.

Okanishi, M., Suzuki, K., and H. Umezawa. 1974. Formation and reversion of *Streptomycete* protoplasts: cultural conditions and morphological study. J. Gen. Microbiol. **80**: 389-400.

Ozaki, A., R. Katsumata, T. Oka. and A. Furuya. 1985. Cloning of the genes concerned in phenylalanine biosynthesis in *Corynebacterium glutamicum* and its application to breeding of phenylalanine producing strain. Agric. Biol. Chem. **49**: 2,925-2,930.

Ozaki, A., Katamata, R., Oka, T. and A. Furuya. 1984. Functional expression of the genes of *Escherichia coli* in Gram positive *Corynebacterium glutamicum*. Mol. Gen. Microbiol. **196**: 175-178.

Paradish, F. W., Warren, D. G., Kilburn, D. G. and R. C. Miller, Jr. 1987. The expression of *Cellulomonas fimi* cellulase genes in *Brevibacterium lactofermentum*. Gene **61:** 199-206.

Patek, M., Ludvik, J., Benada, O., Hochmannova, J. and J. Stockrova. 1988. Transfection of *Brevibacterium flavum* with bacteriophage BFB10 DNA. Folia Microbiol. **333**: 247-254.

Patek, M., O. Navratil., J. Hochmanova., J. Nesvera., V. Krumphanzl. and M. Bucko. 1989. Expression of the threonine operon from *E. coli* in *B. flavum* and *C. glutamicum*. Biotechnol. Lett. **11**: 231-236.

Pearson, W. R., and D. J. Lipman. 1988. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. U. S. A. **85**, 2,444-2,448.

Peterswendisch, P. G., Eikmanns, B. J., Thierbach, G., Bachmann, B. and H. Sahm, 1993. Phosphoenol pyruvate corboxylase in *Corynebacterium glutamicum* is dispensable for growth and lysine production. FEMS Microbiol. Lett. **112**: 269

Pierotti, D. 1987. An investigation of the relationship between cell surface chemistry and efficiency of protoplast formation in *Corynebacterium glutamicum*. Master thesis. Royal Melbourne Institute of Technology, Australia.

Portevin, D., deSousa-D'Auria C., Montrozier, H., Hussin, C., Stella A., Laneelle, M. A., Bardou, F., Guilhot, C., Daffe, M. 2005. The acyl-AMP ligase Fad32 and AccD4-containing acyl-CoA carboxylase are required for the synthesis of mycolic acids and essential for mycobacterial growth: identification of the carboxylase components. J. Biol. Chem. Mar. **11**;280(10):8862-74.

Portevin, D., deSousa-D'Auria C., Hussin, C., Grimmaldi. C., Chami. M., Daffe, M., C. Guilhot. 2004. A polyketide synthase catalyzes the last condensation step of mycolic acid biosynthesis in mycobacteria and related organisms. Proc. Natl. Acad. Sci. U. S. A..Jan 6; **101** (1): 314-319.

Puech, V., Chami, M., Lemassu, A., Laneelle, M. A., Schiffler, B., Gounon, P., Bayan, N., Benz, R., M. Daffe'. 2001. Structure of cell envelope of corynebacteria: importance of the non-covalently bound lipids in the formation of the cell wall permeability barrier and fracture plane. Microbiol. **147**. 1365-1382.

Puech, V.,Bayan., Salim, K., Leblon, G., and M. Daffe'. 2000. Characterization of the *in vivo* acceptors of the mycoloyl residues transferred by the corynebacterial PS1 and the related mycobacterial antigens 85. Mol. Microbiol. **35**: 1026-1041.

Powell, I. B., Achen, M. G., Hillier, A. J. and B. E. Davidson. 1988. A simple and rapid method for genetic transformation of lactic streptococci by electroporation. Appl. Environ. Microbiol. **54**: 655-660.

Quemard, A., Mazeres. S., Sut. A., Laneele, G. and C. Lacave. 1995. Certain properties of isoniazid inhibition of mycolic acid synthesis in cell-free systems of *M. aurum* and *M. avium*. Biochim. Biopyhs. Acta. **1254**: 98-104.

Quemard, A., Lacave, C. and G. Laneelle. 1991. Isoniazid inhibition of mycolic acid synthesis by cell extracts of sensitive and resistant strains of *Mycobacterium aurum*. Antimicrob. Agents. Chemother. **35**: 1,035-1,039.

Quemard, A., Dessen. A., Sogantio. M., Jacobs WR Jr., Sacchettini JC. 1996. Binding of the catalase-peroxidase-activated isoniazid to wild type and mutant *Mycobacterium tuberculosis* enoyl-ACP reductases. J. Am. Chem. Soc. **118**: 1,561-1,562.

Rastogi, N. 1991. Recent observations concerning structure and function relationships in the mycobacterial cell envelope: elaboration of a model in terms of mycobacterial pathogenicity, virulence and drug-resistance. Res. Microbiol. **142**: 464-476.

Randall, J. R. and D. O. Andreas. 1992. in Archer Daniels Midland Annual Report, ADM, Decatur, IL, 16

Rastogi, N., Frehel, C. and L. H. Davis. 1986. The triple-layered structure of mycobacterial cell wall: evidence for the existence of a polysaccharide-rich outer layer in 18 mycobacterial species. Curr. Microbiol. **13**. 237-242.

Redferarn, E. R. 1966. Mode of action of ubiquinones (coenzyme Q) in electron-transport systems. Vitam. Horm. **24**: 465-468.

Reddy, C. A., Cornell, C. P. and A. M. Fraga. 1982. Transfer of *Corynebacterium pyogenes* (Glage) Eberson to the genus *Actinomyces* as *Actinomyces pyogenes* (Glage) comb. nov. Int. J. Syst. Bacteriol. **32**: 419-429.

Riegel, P., Ruimy, R., de Brial, D., Prevost, G., Jehl, F., Bimet, F., Christen, R. and H. Monteil. 1995. Genomic diversity and relationships among lipids-requiring diptheroids from human and characterisation of *Corynebacterium macginleyi* sp. nov. Int. J. Syst. Bacteriol. 45:128-133.

Riegel, P., de., Briel, D., Prevost, G., Jehl, F., Monteil, H. and , R. Minch. 1993. Taxonomic study of the *Corynebacterium* group ANF-1 strains: proposal of *Corynebacterium afermentans* sp. nov. containing the subspecies *C. afermentans* subsp. *afermentans* subsp. nov. and *C. afermentans* subsp. *lipophilum* subsp. nov. and Int Syst Bacteriol. **43**: 287-292.

Riegel, P., Ruimy, R., de Briel, D., Prevost, G., Jehl, F., Bimet, F., Christen, R. and H. Monteil. 1995. *Corynebacterium argentoratense* sp. nov. from human throat. Int J Syst. Bacteriol. **45**: 533-537.

Riegel, P., Ruimy, R., de Briel, D., Prevost, G., Jehl, F., Bimet, F., Christen, R. and H, H. Monteil. 1995. *Corynebacterium seminale* sp. nov., a new species associated with genital infections in male patients. J Clin Microbiol. **33**: 2,244-2,249.

Riley, P. S., Hollis, D. G., Utter, G. B., Weaver, R. E. and C. N. Baker. 1979. Characterisation of 95 dipheroid (JK) cultures isolated from clinical specimens. J. Clin. Microbiol. **9**: 418-427.

Richard E. Lee., Armour, K., Takayama., P. J. Brennan., G. S. Bersa. 1997. Mycolic acid biosynthesis: definition and targeting of the Claisen condensation step. Bioch. Bioph. Acta. **1346**: 275-284.

Rist, N. 1960. L'activite antituberculeuse de I'ethionamide. Adv. Tuberculosis Res. **10:** 69-126.

Rislow, M., Moblig. M., Rifai. M., Schatz, H., Fieldman. K., A. Pfeiffer. 1995. A new isoniazid/ethionamide resistance gene mutation and screening for multidrug-resistant *Mycobacterium tuberculosis* strains. Lancet. **310**: 502-3

Rouse, D. A., Li, Z., Bai, G. H., L. S. Morris. 1995. Characterization of the *katG* and *inhA* genes of isoniazid resistant clinical isolates of *Mycobacterium tuberculosis*. Antimicrob. Agents Chemother. **39**: 2472-2477.

Rozwarski, D. A., Grant. G. A., Barton, D. H.R., Jacobs, W. R. Jr., J. C. Sacchettini. 1998. Modification of the NADH of the isoniazid target (*inhA*) from *Mycobacterium tuberculosis*. Science. **279**:98-102.

Salim, K., Haedens. V., Content, J., Leblon. G. and K. Huygen. 1997. Heterologous expression of the *Mycobacterrium tuberculosis* gene encoding antigen 85A in *Corynebacterium gulatmicum*. App. Environ. Microbiol. **63**: 4392-440.

Sambrook, J., Fritsch, E. F. and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbour Laboratory Press, Cold Spring Harbor, N. Y.

Santamaria, R., Mesa, J. M., J. F. Martin. 1982. Genetic recombination by protoplast fusion in coryneform bacteria. Abstracts, 4th International symposium on Genetics of Industrial Microorganisms, Kyoto, Japan, p. 60.

Santamaria, R., Gil, J. A., Mesas, J. M. and J. F. Martin 1984. Characterisation of an endogenous plasmid and development of cloning vectors and a transformation system in *Brevibacterium lactofermentum*. J. Gen. Microbiol. **130**: 2,237-2,246.

Santamaria , R., Gil, J. A. and J. F. Martin. 1985. High-frequency transformation of *Brevibacterium lactofermentum* protoplasts by plasmid DNA. J. Bacteriol. **162**: 463-467.

Schleifer, K. H., and Kandler. 1972. Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriol. Rev. **36**:407-477.

Schwarzer, A. and A. Pühler. 1991. Manipulation of *Corynebacterium glutamicum* by gene disruption and replacement. Biotechnol. **9**: 84-87.

Schaeffer, P., Cami, B. and R. D. Hotchkiss. 1976. Fusion of bacterial protoplasts. Proc. Natl. Acad. Sci. USA. **73**: 2,151-2,155.

Schäfer, A., Kalinowski, J., Simon, R., Seep-Feldhaus, R. A. and A. Pühler. 1990. High-frequency conjugal transfer from Gram-negative *Escherichia coli* to various Gram-positive coryneform bacteria, J. Bacteriol. **172**: 1,663-1,666.

Schäfer, A., Kalinowski, J. and A. Pühler. 1994a. Increased fertility of *Corynebacterium glutamicum* recipients in intergeneric matings with *Escherichia coli* after stress exposure, Appl. Environ. Microbiol., **60**: 756-759.

Schäfer, A., Kalinowski, J. and A. Pühler. 1994b. Cloning and characterisation of a DNA region encoding a stress-sensitive restriction system from *Corynebacterium glutamicum* ATCC 13032 and analysis of its role in intergeneric conjugation with *Escherichia coli*. J. Bacteriol. **176**. 7309-7319

Schäfer, A., Tauch, A., Jager, W., Kalinowski, J., Thierbach, G. and A. Pühler. 1994. Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. Gene. **145**: 69-73.

Serwold-Davis, T. M., Groman, N. and M. Rabin. 1987. Transformation of *Corynebacterium diphtheria*, *Corynebacterium ulcerans*, *Corynebacterium glutamicum*, and *Escherichia coli* with *C. diphtheria* plasmid PNG2. Proc. Natl. Acad. Sci. USA. **84**: 4964-4968

Shiio, I. 1983. Biochemical conditions for amino acid overproduction, *In*: I. Ikeda, T. Beppu. (eds.), Genetics of industrial organisms. p. 242-246. Kotdansha Ltd., Tokyo.

Sherman, D. R., Mudluli. K., Hickey. M. J., Arain. T. M., Morris. S. L., C. E. Barry. III.. 1996. Compensatory *ahpC* gene expression in isoniazid-resistant *Mycobacterium tuberculosis*. Science. **272**: 1641-1643.

Sherman, D. R., Sabo, P. J., M. J. Hickey *et al.* 1995. Disparate responses to oxidative stress in saprophytic and pathogenic mycobacteria. Proc. Natl. Acad. Sci. U.SA **92**: 6625-6629.

Shimakata, T., Iwaka, M. and T. Kusaka. 1984. *In vitro* synthesis of mycolic acids by the fluffy layer fraction of *Bacterionema matruchotii*. Arch. Biochem. Biophys. **229**: 329-339.

Shivarova, N., Forster, W., Jacob, H-E. and R. Grigorova. 1983. Microbiological implications of electric field effects VII. Stimulation of plasmid transformation of *Bacillus cereus* protoplasts by electric field pulses. Z. Allg. Mikrobiol. **23**: 595-599.

Simon, R., U. Priefer and A. Pühler. 1983. A broad host range mobilisation system for *in vivo* genetic engineering: transposon mutagenesis in Gram-negative bacteria. Biotechnol. **1**: 784-794.

Simon, R., O'Connell, M., Labes, G. and A. Pühler. 1986. Plasmid vectors for the genetic analysis and manipulation of *Rhizobia* and other Gram-negative bacteria. Methods Enzymol. **118**: 640-659.

Sophie, M. P. and J. Richard. 1995. Activity and purification of linenscin OC2, an antibacterial substance produced by *Brevibacterium linens* OC2, an orange cheese coryneform bacteria. Appl. Envir. Microbiol. **61**:1,847-1,852.

Somkuti, J. A. and D. H. Steinberg. 1978. Genetic transformation of *Streptococcus thermophilus* by electroporation. *In*: Neijssel, O. M., Van der Meer, R. R, Luyben KCHAM (eds.), p. 412 Proc. 4th Eur. Cong. Biotech, Vol1, Elsevier, Amsterdam.

Smith M, D., Flickinger, J. L., Lineberger, D. W. and B. Schmidt 1986. Protoplast transformation in coryneform bacteria and introduction of an α-amylase gene from *Bacillus amyloliqefaciens* into *Brevibacterium lactofermentum*. Appl. Environ. Microbiol. **51**: 636-639.

Stackebrandt, E., Lewis, B. J. and C. R. Woese. 1980. The phylogenetic structure of the coryneform group of bacteria. Zentralbl. Bakteriol. Mikrobiol. Hyg. Abt. 2 Orig. Reihe C **1**: 137-149.

Stakebrandt, E. and K. H. Schleifer. 1984. Molecular systematics of *Actinomycetes* and related organisms, p. 485-504. *In* Bojalil, L. F. (ed), Biological, biochemical and biomedical aspects of *Actinomycetes*. Academic Press. London.

Stackebrandt, E. and J. Smida. 1988. The phylogeny of the genus *Mycobacterium* as determined by 16S rRNA sequences, and development of DNA probes, p. 244-250. *In* Biology of *Actinomycetes*. Japan Scientific Societies press, Tokoyo.

Stoeckle, M. Y., Guan, l., Riegler, N., Weitzman, I., Kreiswrith, B., J. Kornblum. 1993. Catalase-peroxidase gene sequences in isoniazid-sensitive and –resistant strains of *Mycobacterium tuberculosis* from New York City. J. Infect. Dis. **168**: 1,063-1,065.

Stodola, F. H., Lesuk, A. and R. J. Anderson. 1938. The chemistry of lipids of tubercle bacilli. LIV. The isolation and properties of mycolic acid. J. Biol. Chem. **126**. 505-513.

Strominger. J. L., and C. H. Birge. 1965. Nucleotide accumulation in *Staphylococcus aureus* by glycine. J. Bacteriol. **89**: 1,124-1,127.

Susskind, M. M. and D. Botstein. 1978. Molecular genetics of bacteriophage p22. Microbiol. Rev. **42**: 385-413.

Suzuki, T., Tanaka, K., Matsubra, I. and S. Kinoshita. 1969. Trehalose lipids and α-hydroxy β-hydroxy fatty acid formed by bacteria grown on n-alkanes. Agric. Biol. Chem. **33**: 1,619-1,627.

Suzuki, K. and K Komagata. 1983. Taxonomic significance of cellular fatty acid composition in some coryneform bacteria. Int. J. Syst. Bacteriol. **33**: 188-200.

Takayama, K. and L. A. Davidsons. 1979. Isonicotinic acid hydrazide, p. 98-119. *In* F. E. Holms (ed), Antibiotics V, part 1. Spring-verlag KG, Berlin.

Tauch, A., Kirchner, O., Wehmeier, L., Kalinowski, J. and A. Pühler. 1994. *Corynebacterium glutamium* DNA is subjected to methylation-restriction in *Escherichia coli*. FEMS Microbiol. Lett. **123**: 343-348.

Telenti, A., Honore, N., Bernasconi, C. *et al.* 1998. Genotypic assessment of isoniazid and rifampicin resistance in *Mycobacterium tuberculosis*: a blind study at refrence laboratory level. J. Clin. Microbiol. 35: 719-723.

Thierbach, G., Schwarzer, A. and A. Pühler. 1988. Transformation of spheroplasts and protoplasts of *Corynebacterium glutamicum*. Appl. Microbiol. Biotechnol. **29**: 356-362.

Thibert, L. and S. Lapierre. 1993. Routine application of high performance liquid chromatography for identification of mycobacteria. J. Clin. Microbiol. **31**: 1759-1763.

Tichy, P. and O. E. Landman. 1969. Transformation in quasi spheroplasts of *Bacillus subtilis*. J. Bacteriol. **97:** 42-51.

Tsukamura, M. and S. Tsukamura. 1963. Isotopic studies on the effect of isoniazid on protein synthesis of mycobacteria. Jap. J. Tuberc. **11**: 14.

Tosaka, O., Enei, H. and Y. Hirose. 1983. The production of L-lysine by fermentation. Trend. Biotechnol. **1**: 70-74.

Tomiyasu, I., Toriyama, S., Yano, I. and M. Masui. 1981. Changes in molecular species composition of nocardomycolic acids in *Nocardia rubra* by the growth temperature. Chemistry and Physics of Lipids. **28**: 41-45.

Tomiyasu, I. 1982. Mycolic acid composition and thermally adaptive changes in *Nocardia asteroides*. J. Bacteriol. **151**: 828-837.

Tomiyasu, I. and I. Yano. 1984. Isonicotinic acid hydrazide induced changes and inhibition in mycolic acid synthesis in *Nocardia* and related taxa. Arch. Microbiol. **137:** 316-323.

Toriyama, S., Yano, I., Masui, M., Kusunose, E., Kusunose, M. and N. Akimori. 1980. Regulation cell wall mycolic acid biosynthesis in acid-fast bacteria. I. Temperatures induced changes in mycolic acid related compounds in *Mycobacterium phli.* J. Biochem. **88**: 211-221.

Trias, J., Jarlier, V. and R. Benz. 1992. Porins in the cell wall of mycobacteria. Science. **258**: 1,479-1,481.

Trieu-Cout, P., Carlier, C. and P. Courvalin. 1988. Conjugative plasmid transfer from *Enterococcus faecalis* to *Escherichia coli.* J. Bacteriol. **29**: 356-362.

Trieu-Cout, P., Carlier, C., Martin, P. and P. Courvalin. 1987. Plasmid transfer by conjugation from *Escherichia coli* to Gram-positive bacteria. FEMS Microbiol. Lett. **48**: 289-294.

Turnowsky, F., Fuchs, K., Jeschek, C. and G. Hogenauer. 1989. *envM* genes of *Salmonella typhimurium* and *Escherichia coli.* J. Bacteriol. **171:** 6,555-6,565.

Valdes-Stauber, N. and S. Scherer. 1994. Isolation and characterisation of linocin M18, a bacteriocin produced by *Brevibacterium linens.* Appl. Environ. Microbiol. **60**: 3,809-3,814.

Vertes, A. A., Inui, M., Kobayashi, M., Kurusu, Y. and H. Yukawa. 1994a. Isolation and characterisation of *IS*31831, a transposable element from *C. glutamicum.* Mol. Microbiol. **11**: 739-746.

Vertes, A. A., Asai, Y., Inui, M., Kobayashi, M., Kurusu, Y. and H. Yukawa. 1994b. Transposon mutagenesis of coryneform bacteria. Mol. Gen. Genet. **245**: 397-405.

Vertes, A. A., Asai, Y., Inui, M., Kobayashi, M., Kurusu, Y. and H. Yukawa. 1994b. Transposon mutagenesis of coryneform bacteria. Mol. Gen. Genet. **245**: 397-405.

Walker, R. W., Prome, J. C. and C. S. Lacave. 1973. Synthesis of mycolic acids. Formation of a C_{32}-β-ketoester from palmitic acid in a cell free system of *Corynebacterium diphtheriae.* Biochem. Biophys. Acta. **326**: 52-62.

Weber, J. M. and R. Losick. 1988. The use of chromosome integration vector to map erythromycin resistance and production genes in *Saccharopolyspora erythraea* (*Streptomyces erythraeus*). Gene. **68**: 173-180.

Webster, M. 1995. Identification and differentiation of species and strains of bifidobacteria and *Lactobacillus acidophilus* by the RAPD method of DNA profiling. Honours thesis. Victoria University of Technology.

Wehrmann. A., Ruether, K., Eggleling, L. and H. Sahm. 1993. Role of split pathway in lysine synthesis of *Corynebacterium*: a genetical and physiological approach. BioEngin: **9**: 33.

Welby-Gieusse, M., Lanéele, M-A. and J. Asselineau. 1970. Structure des acides corynomycoliques de *Corynebacterium hofmanii* et leur implication biogénétique. Europ. J. Biochem. **13**: 164-167.

Welsh, M. and P. Osterrieth. A comparison of the transformation of Gram-negative rod "protoplast" under the influence of pencillin G and glycine. Antonie Van Leeuwenhoek . J. Microb. **24**: 257-272.

Wheeler, P. R. and P. M. Anderson. 1996. Determination of the primary target for isoniazid in mycobacterial mycolic acid biosynthesis with *Mycobacterium aurumA+*. Biochem. J. **318**: 451-457.

Willetts, N. and C. Crowther. 1981. Mobilisation of the non conjugative IncQ plasmid RSF1010. Genet. Res. **37**: 311-316.

Wilkins, B. M. 1990. Factors influencing the dissemination of DNA by bacterial conjugation, p. 22-30. *In* Fry. J. C. and M. J. Day (eds.), Bacterial genetics in natural environments. Chapman and Hall. London. New York.

Wilson, T. M., M. D. Collins. 1996. *ahpC*, a gene involved in isoniazid resistance of the *Mycobacterium tuberculosis* complex. Mol. Microbiol. **19**: 1025-1034.

Winder, F. 1964. The antibacterial action of streptomycin, isoniazid and PAS, p. 111. *In* V. C. Barry, (ed.), Chemotherapy of Tuberculosis. London: Butterworth,

Winder, F. G. and P. Brennan. 1965. Effect of isoniazid on lipid metabolism of *Mycobacterium tuberculosis*. Biochem. J. **96**: 77.

Winder, F. G. and P. B Collins. 1970. Inhibition by isoniazid of synthesis on *Mycobacterium tuberculosis*. J. Gen. Microbiol. **63**: 41-48.
Winder, F. G. and S. A. Rooney. 1970. The effects of isoniazid on the carbohydrates of *Mycobacterium tuberculosis* BCG. Biochem. J. **177**: 335-368.

Winder, F. G., Collins, P. B. and D. Whelan. 1971. Effects of ethionamide and isoxyl on mycolic acid synthesis in *Mycobacterium tuberculosis* BCG. J. Gen. Microbiol. **66**: 379-380.

Winder, F. G. 1982. Mode of action of the antimicrobial agents and associated aspects of the molecular biology of the mycobacteria, p. 353-438. *In* Ratledge, C. and Stanford (eds.), The Biology of Mycobacteria, vol1. Academic Press, New York.

Wohlleben, W., Muth, G. and J. Kalinowski. 1993. Genetic engineering of Gram positive bacteria. p. 477. *In* Rehm, H. H., Reed, G., Pühler, A. and H. Sahm (eds.), Biotechnology genetics and fundamentals of genetic engineering. VCH Publishers, Weinheim, Germany.

Wolf, H., Pühler, A. and E. Neumann. 1989. Electrotransformation of intact and osmotically sensitive cells of *Corynebacterium glutamicum*. Appl. Microbiol. Biotechnol. **30**: 283-362.

Umbarger, H. E. 1978. Amino acid biosynthesis and its regulation. Annu. Rev. Biochem. **47**: 533-606.

Yano, I. and K. Saito. 1972. Gas chromatographic and mass spectrometric analysis of molecular species of corynemycolic acids from *Corynebacterium ulceran*s. FEBS Lett. **23:** 352-356.

Yano, I., Saito, K., Furukawa, Y. and M. Kusunose. 1972. Structural analysis of molecular species of nocardomycolic acids from *Nocardia erythropolis* by the combined system of gas chromatography and mass spectrometry. FEBS Lett. **23**: 215-219.

Yamada, K., Kinoshita, S., Tsunoda, T. and K. Aida. 1972. The Microbial Production Of Amino Acids. Wiley, New York.

Yamada, Y., G. Inouye., Y. Tahara. and K. Kondo. 1976. The menaquinone system in the classification of coryneform and nocardioform bacteria and related organisms. J. Gen. Appl. Microbiol. **22**: 203-214.

Yamada, K. 1977. Bioengineering report: recent advances in industrial fermentations in Japan. Biotechnol. Bioeng. **19**: 1563-1621.
Yamada, Y., Won Seo, G. and H. Okada. 1985. Oxidation of acyclic terpenoids by *Corynebacterium* sp. Appl. Environ. Microbiol. **49**: 960-963.

Youatt, J. 1961. The formation of 4-pyridylmethanol from isonicotinic acid hydrazide (isoniazid) by mycobacteria. Aust. J. Chem. **14**: 308-311.

Youatt, J. 1969. A review of the action of isoniazid. Am. Rev. Respir. Dis. **99**: 729-749.

Yoshihama, M., Higashiro, K., Rao, E. A., Akdo., M., Shanabruch, W. G., Follittie, M T., Walker, G. C. and A. J. Sinskey. 1985. Cloning vector system for *Corynebacterium glutamicum*. J. Bacteriol. **162**: 591-597.

Yuan, Y., Lee, R. E., Bersa, G. S., Belisle, J. T. and C. E. Barry, III. 1995. Identification of a gene involved in the biosynthesis of cyclopropanated mycolic acids in *Mycobacterium tuberculosis*. Proc. Natl. Acad. Sci. USA **92**: 6,630-6,634.

Yuan, Y., and C. E. Barry, III. 1996. A common mechanism for the biosynthesis of methoxy and cyclopropyl mycolic acids in *Mycobacterium tuberculosis*. Proc. Natl. Acad. Sci. USA **93**: 12828-12833.

Zhang, Y., Heym, B., Allen, B., Young, D. and S. Cole. 1992. The catalase-peroxidase gene and isoniazid resistance of *Mycobacterium tuberculosis*. Nature. **358**: 591-593.

Zhang, Y.,Garbe, T. and D. Young. 1993. Transformation with *katG* restores isoniazid-sensitivity in *Mycobacterium tuberculosis* isolates resistant to a range of drug concentrations. Mol. Microbiol. **8**: 521-524.

Zajic, J. E., H. Guignard, and D. F. Gerson. 1977. Emulsifying and surface active agents from *Corynebacterium hydrocorboclastum*. Biotechnol. Biioeng. **19:** 1295-1301.

Zinder, N. D. and Lederberg. 1952. Genetic exchange in *Salmonella*. J. Bacteriol. **64:** 679-699.

Zuneda, M. C., Guillenea, J. J., Dominguez, J. B., Prado, A. and F. M. Goni. 1984. Lipid composition and protoplast forming activity of *Streptomyces antibioticus*. Lipids. **19**: 223-228.