IDENTIFICATION AND CHARACTERISATION OF THE CELL SURFACE AND ENZYMATIC BARRIERS TO PLASMID TRANSFORMATION IN *CORYNEBACTERIUM GLUTAMICUM* AND RELATED SPECIES

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Identification and
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DECLARATION

I certify that unless otherwise stated, the work presented in this is solely my work, with the exception of some experiments reported in Chapter 3 and Chapter 4. Sample preparation and electron microscopy operations were performed by Ms. Anne Martsi McClintock (Dept. of Physiology, Monash University). Oligonucleotides for PCR and DNA sequencing were synthesised by Ms. U. Manuelpillai (CBFT, VUT) and subsequent automated DNA sequencing was performed at Dept of Microbiology, Monash University using the VUT-Monash joint sequencing facility. The results detailed in Chapter 3 and Chapter 4 have been previously presented or published and details appear in the front of each results section.

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ABBREVIATIONS

The following abbreviations have been used throughout this thesis:

A_{600}	Absorbance at 600 nm
Ado	Adenosine
Amp	Ampicillin
ATCC	American Type Culture Collection, Rockville, U.S.A.
bp	Base pair
BAP	Bacterial alkaline phosphatase
BSA	Bovine serum albumin
$C_{14:0}$	Myristic acid
$C_{15:0}$	Pentadecanoic acid
$C_{16:0}$	Palmitic acid
$C_{17:0}$	Heptadecanoic 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
CBFT	Centre for Bioprocessing and Food Technology
c.f.u.	Colony forming unit
CCC DNA	Covalently closed circular deoxyribonucleic acid
Cm	Chloramphenicol
CsCl-EtBr	Caesium chloride - ethidium bromide
Cyd	Cytidine
CTAB	Hexadecyltrimethylammonium bromide
D	Dalton
dAdo	Deoxyadenosine
dCyd	Deoxycytidine
dGuo	Deoxyguanosine

DAP	Diaminopimelic acid
DIG	Digoxigenin
DNase	Deoxyribonuclease
DSM	Deutsche Sammlung von Mikroorganism, Gottingen, Germany
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid (disodium salt)
Em	Erythromycin
EMS	Ethyl methanesulphonate
ENase	Endonuclease
EtOH	Ethanol
FAMEs	Fatty acid methyl esters
FID	Flame ionisation detector
GC	Gas chromatography
Guo	Guanosine
h	hour
HPLC	High Performance Liquid Chromatography
INH	Isonicotinic acid hydrazide
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-I	LBG supplemented with INH
leu	Leucine
m^5dCyd	N^4-methyl deoxycytidine
m^4dCyd	5-methyldeoxycytidine
m^6dAdo	6-methyl deoxyadenosine
MAMEs	Mycolic acid methyl esters
MIC	Minimal inhibitory concentration
min	Minute
MTase	Methyltransferase

MS	Mass spectrometry
μ	Cell specific growth rate
M.W.	Molecular weight
NA	Nutrient agar
NAG	Nutrient agar with glucose
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
PCR	Polymer chain reaction
PEG	Polyethylene glycol
Rf	migration distance relative to front
Rif	Rifampicin
RM system	Restriction and modification system
RNase	Ribonuclease
RP-HPLC	Reversed phase-HPLC
SAM	S-adenosyl-*L*-methionine
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
Thd	Thymidine
TLC	Thin layer chromatography
TMS	Trimethylsilyl
Tris	Tris (hydroxymethyl) aminomethane
U	Unit
Urd	Uridine

V	Volt
Vol	Volume
VUT	Victoria University of Technology

SUMMARY

Non-pathogenic corynebacteria including *Corynebacterium glutamicum*, *Brevibacterium flavum*, and *B. lactofermentum* have been used traditionally for industrial production of amino acids. Recently, these strains have been also studied using molecular biology or recombinant DNA approaches. However, a major early limitation in the application of this technology was poor transformation efficiency due to the presence of barriers which prevented entry or survival of incoming DNA. These barriers are both physical (cell surface structures) and enzymatic (restriction and modification) barriers. The research described in this thesis involved investigating the nature of both the physical and enzymatic barriers, includings: (i) determining the effect of the presence of glycine and/or isonicotinic acid hydrazide (INH) and resulting lipid profiles (mycolic acids and fatty acids) of several strains of corynebacteria. (ii) examining the enzymatic barriers to transformation of these corynebacteria with specific reference to methyltransferase (MTase) activity and the sites of methylation. Strains used in this work included: *C. glutamicum* AS019 (rif^r mutant ATCC 13059) and auxotropic mutants MLB133 and MLB194 (Best and Britz, 1986); ATCC 13032 and its restriction deficient mutants, RM3 and RM4 (Schäfer *et al.*, 1994a). Two *Brevibacterium* strains, *B. flavum* and *B. lactofermentum*, and *Corynebacterium ulcerans* were included for reference.

The impact on growth of several potential cell wall modifying chemicals was examined using the following combinations: glycine, 0-10% (w/v); INH, 0-10 mg/ml; Tween 80, 0-0.9% (w/v). These were selected for use because growth of cells in the presence of these chemicals prior to electroporation was known to improve transformation efficiencies (Haynes and Britz, 1990). The combined effect of these chemicals on cell growth was also tested. Among the three cell wall modifiers used at the above concentration ranges, glycine and INH showed significant impact on the specific growth rates of corynebacteria or on the length of lag phase, whilst little impact was seen with Tween 80. The two mutant strains, MLB133 and MLB194, were more sensitive to inhibition by glycine than AS019. For the ATCC 13032 family, RM4 showed higher sensitivity to the presence of glycine in the growth medium than ATCC 13032. Of the 10 strains examined, AS019 was most resistant to growth inhibition by INH whilst *C. ulcerans* was the most sensitive to INH. The two mutant strains, MLB133 and MLB194, were more sensitive to inhibition by INH than AS019. For the ATCC 13032 family, two mutants were more sensitive than their parent strain to inhibition by INH. As a group, the corynebacteria were relatively more resistant to INH than mycobacteria.

Growth in glycine and INH improved electrotransformation efficiencies by 10^2 to 10^3 fold relative to cells harvested from LBG medium, indicating that the structure of the cell wall can act as a physical barrier for DNA transformation into the corynebacteria. Strain MLB133 showed the highest transformation efficiency of all of the strains tested, following growth of this strain in 2% glycine and 4 mg/ml INH and using homologously-derived DNA. Strains RM3 and RM4 showed similar transformation efficiency whatever the source of DNA used, reflecting their known restriction minus nature.

All strains of corynebacteria tested had five major types of mycolic acids ($C_{32:0}$, $C_{34:0}$, $C_{34:1}$, $C_{36:2}$, $C_{36:1}$) in whole cells and culture fluids, but the relative proportions of each varied with the strain and medium composition. 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). Among the ATCC 13032 family, differences in proportions of mycolic acids were also seen, where $C_{32:0}$ was higher in ATCC 13032 and RM4 relative to RM3, and $C_{36:2}$ was very much higher in RM3 (28.8%). The relative proportion of each mycolic acid species in profiles for cells and culture fluids was quite similar for each sample tested. In LBG medium, extracellular mycolic acids ranged between 3.5% (in ATCC 13032) to 7.8% (in MLB133). The main effect of addition of glycine (2%, w/v) or 8 mg/ml INH was to increase the proportion of mycolic acids found in extracellular fluids (15.9% [glycine medium] and 9.6% [INH medium] in AS019, and 19.3% [glycine medium] and 15.1% [INH medium] in MLB133). All strains tested had two major cellular types of fatty acids, palmitic ($C_{16:0}$) and oleic ($C_{18:1}$) acids. In the presence of 2% glycine or 8 mg/ml INH, the relative percentage of the fatty acids of AS019 to the total lipids (mycolic acids plus fatty acids) was decreased from 76.9% (in LBG) to 72.9% (in LBG-2% glycine), and 66.4% (in LBG-8 mg/ml INH), suggesting that these chemicals inhibited fatty acids synthesis.

Mutant strain MLB133 had a relatively thin cell wall structure after growth in LBG when compared with the parental-type strain, AS019, and cell wall thickness was further reduced following growth in the presence of 2% glycine or 4 mg/ml INH.

The above results suggest that the mutations in strains MLB133 and MLB194 were associated with synthesis of specific mycolic acids (e.g. $C_{32:0}$) and their attachment to the cell surface, both of which are likely target sites for glycine and INH action for cell-surface modifications. Improved synthesis or attachment of mycolic acids by strain MLB133 is consistent with the observed thinner

cell wall and with the observation that this strain was transformed more efficiently even when not grown in glycine or INH.

Cell-free extracts of corynebacteria showed several nuclease activities, including endonuclease and exonuclease which linearised and degraded plasmid DNA. The MTase activity of *C. glutamicum* and *B. flavum* was characterised using four approaches: HPLC analysis of DNA to determine the methylated base content of chromosomal and plasmid DNA from corynebacteria; transformation using *B. flavum*-derived plasmid DNA and a range of *E. coli* strains with different restriction and modification backgrounds; analysis of chromosomal and plasmid DNA from corynebacteria for susceptibility to digestion by restriction enzymes which specifically cleave DNA at either methylated or unmethylated recognition sites; sequence analysis of a methylated target site of the MTase using a bisulphite modification method.

HPLC analysis of nucleotides in corynebacteria-derived plasmid DNAs and chromosomal DNA showed the presence of methylated adenosine and methylated cytidine for three corynebacterial DNAs, suggesting the presence of adenosine MTase and cytidine MTase in the three corynebacterial species, with latter the dominant type. Using plasmid DNA isolated from *B. flavum*, it was found that a MTase in *B. flavum* recognised the GC sequence. *Mcr*BC enzyme digestion confirmed that DNAs derived from *C. glutamicum* and *B. flavum* contained methylated cytidine on the GC DNA sequences.

Restriction enzymes which are inhibited by the presence of methylated cytidine in their recognition sequence failed to cut pCSL17 from *C. glutamicum* and *B. flavum*, whereas enzyme which require methylation at adenosine in GATC sequences failed to cut. Failure of *Hae*III to cut two specific sites of *C. glutamicum*-derived pCSL17 identified the first cytidine in the sequence GGCCGC as one target of methylation in this species, which contains the MTase recognition sequence. *Fnu*4HI and *Tse*I enzyme digestion showed that *C. glutamicum*- and *B. flavum*-derived DNA contained methylated cytidine in the GCSGC sequence (where, S is either G or C). Bisulphite DNA modification and subsequent sequencing showed that the first cytidine was methylated at the GCCGC sequence for DNA obtained from *C. glutamicum*.

These result showed that the target sequence for MTase activity in *C. glutamicum* and *B. flavum* is GCCGC, while, at least for the strain tested, *B. lactofermentum* MTase recognises a different sequence.

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Chapter 1 INTRODUCTION

1.1 OVERVIEW OF INTRODUCTION

Corynebacteria or coryneform bacteria are a taxonomically ill-defined group of Gram-positive bacteria which are rod or club shaped. 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. Two subgroups are distinguished in the taxonomy of corynebacteria: human and animal pathogens (Barksdale, 1981) and saprophytic soil bacteria, which are capable of producing several amino acids such as arginine, glutamic acid, isoleucine, leucine, lysine, ornithine, proline, threonine, tryptophan and valine (Hirose *et al.*, 1985; Nakayama *et al.*, 1985). The group formerly comprising the plant pathogenic corynebacteria has been renamed *Clavibacter* (Davis *et al*, 1984). Saprophytic species of corynebacteria include *C. glutamicum*, *Brevibacterium lactofermentum* and *Brevibacterium flavum*; these species are important industrially for production of amino acids (Hirose *et al.*, 1985; Martin *et al.*, 1987). For example, 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). *Escherichia coli* and related enteric organisms have been also used for constructing amino acid-producing strains, since this bacterium is amenable to genetic manipulation and their biosynthetic pathways are well understood. However, use of *E. coli* for amino acid production industrially poses certain problems, including potential pathogenicity, production of endotoxins and susceptibility to bacteriophage infection during fermentation (Britz and Demain, 1985). A major advantage of using *Corynebacterium* species for production of food additives or other products which will be consumed by animals and/or humans is that *Corynebacterium* species do not produce endotoxins and corynebacterial lytic bacteriophage are relatively rare. In addition, *Corynebacterium* species have been used for over 50 years in industry, the primary metabolism of selected species has been well characterised, and metabolic regulatory mechanisms are relatively

simple when compared to *E. coli* (Jetten and Sinskey, 1995; Martin *et al.*, 1987).

Hence, in order to increase the ability of amino acid production by corynebacteria, extensive classical mutation and strain selection has been carried out. Selection of antimetabolite-resistant and auxotrophic mutants led to the isolation of high producer mutants which are used for the industrial production of a variety of amino acids. However, achieving further increases in production by such traditional techniques is limited because the accumulated mutations introduced almost completely remove feed-back regulation in the amino acid biosynthesis pathways (Sandoval *et al.*, 1984). Consequently, over the last 10 to 15 years, recombinant DNA methodologies have been developed to assist in strain improvement and provide tools for studying the basic genetics of this group of bacteria. The approach normally taken has involved using natural plasmids (Katsumata *et al.*, 1984; Serwold-Davis *et al.*, 1987) 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; Vértes *et al.*, 1994b; Wolf *et al.*, 1989) or conjugation (Schäfer *et al.*, 1990, 1994b). The application of recombinant DNA techniques to *C. glutamicum* has demonstrated that this bacterium can express foreign genes and produce functional, recombinant proteins. Foreign genes successfully expressed in *C. glutamicum* and related bacteria include heterologous α-amylase (Smith *et al.*, 1986), nuclease (Liebl *et al.*, 1992) and protease (Billman-Jacobe *et al.*, 1995). Several amino acid biosynthesis genes have been isolated and characterised (see review papers by Follettie and Sinskey [1986] and Jetten and Sinskey [1995]).

Although these foreign genes have been transformed into *C. glutamicum* successfully, several workers reported poor transformation efficiency when using heterologously-derived DNA (Haynes and Britz, 1990; Ozaki *et al.*, 1984; Serwold-Davis *et al.*, 1987; Yoshihama *et al.*, 1985).

Barriers to efficient transformation include restriction-modification systems involving restriction and modification enzyme(s) (Schäfer *et al.*, 1994b; Tauch *et al.*, 1994), as well as the structure of the cell wall itself, which can act as a physical barrier for DNA transformation into the cell (Haynes and Britz, 1990).

The cell wall of *Corynebacterium*, *Brevibacterium*, *Nocadia*, *Mycobacterium* and *Rhodococcus* species includes three major components: peptidoglycan (a branched and cross-linked glycopeptide), arabinoglycan (a branched polysaccharide) and mycolic acids. Mycolic acids occur in only a few bacterial genera and they are a major component of the cell wall structure of these bacteria (Alshamaony and Goodfellow, 1976; Barksdale and Kim, 1977; Chevalier *et al.*, 1988; Collins *et al.*, 1982a; Etémadi, 1967; Stodola *et al.*, 1938; Tomiyasu, 1982). For instance, up to 60% of the weight of the mycobacterial cell wall is occupied by lipids that are mainly composed of unusually long-chain fatty acids containing 60 to 90 carbons in their carbon chains, the mycolic acids (see Minnikin, 1982). Further observations showed that mycolic acids are structural components which probably play an important role in the restricted permeability of these microbes to water-soluble molecules (Jarlier and Nikaido, 1990).

Studies on the mycolic acid composition of mycolic acid-containing bacteria revealed that mycolic acid composition varied depending on the bacterial species and that mycolic acid composition could be altered by environmental conditions (Chevalier *et al.*, 1988; Tomiyasu *et al.*, 1982). In order to decrease the physical barrier in mycolic acid-containing bacteria, several compounds, including glycine, penicillin and isonicotinic acid hydrazide (INH), have been used during growth. Several compounds, including Tween 60, C_{16} to C_{18} saturated fatty acids and penicillin, have been used in industrial fermentations to increase secretion of amino acids (Hirose *et al.*, 1985). Best and Britz (1986) also observed that mutant strains of *C. glutamicum* which were auxotrophic for isoleucine/leucine protoplasted more readily, noting that these strains showed higher sensitivity to

Table 1.1 Examples of biotechnological processes performed by corynebacteria [a]

Process	Organism	Reference
Production of amino acids	*Corynebacterium glutamicum*	Martin *et al.*, 1987
Production of nucleotides	*Brevibacterium ammoniagenes*	Ogata *et al.*, 1976
Production of antibiotics	*Corynebacterium* species	Suzuki *et al.*, 1972
Production of surfactants	*Corynebacterium hydrocarboclastum*	Zajic *et al.*, 1977
Vitamin C precursors	*Corynebacterium* species	Anderson *et al.*, 1985
Cheese ripening	*Brevibacterium linens*	Lee *et al.*, 1985
Bioconversion of steroids	*Corynebacterium simplex*	Constantinides, 1980

[a] Information from Wohlleben *et al.* (1993)

growth inhibition by glycine and INH. These presumptive cell surface mutants were then investigated by Pierotti (1987) in terms of cell surface structures. Pierotti (1987) found that these mutants contained a higher relative proportion of unsaturated mycolic acids when compared to the parent strain. Diong (1989) found that *C. glutamicum* and several related species contained endonuclease activity, which could convert the circular form of plasmid DNA to the open circular then linear form of DNA. Furthermore, during the course of this PhD study, Tauch *et al.* (1994) presented evidence that DNA isolated from *C. glutamicum* contained methylated cytidine probably in sequences containing GC bases.

The subject of this thesis is an investigation into the barriers to transformation of corynebacteria, including both enzymatic restriction and modification systems plus physical barriers associated with the nature of the cell wall structures present. The remainder of the Introduction sections provides a literature review on various aspects of the above and defines the subject of this thesis.

1.2 THE IMPORTANCE OF CORYNEBACTERIA IN INDUSTRY

Members of the "corynebacteria" perform a broad spectrum of biotransformations and are used in a variety of biotechnological processes (Table 1.1). Industrially, the most important feature of corynebacteria is the fermentative production of amino acids. Amino acids are used as flavour-enhancing agents (e.g. glutamate and lysine) (Jetten and Sinskey, 1995). These are also used in the pharmaceutical industry as therapeutic agents in nutritional or metabolic disorders, and in the chemical industries they find use in the manufacture of cosmetics, toothpaste, shampoos and detergents. For instance, glutamic acid, which has been studied for over 50 years, is used as a starting material for the synthesis of various kinds of speciality chemicals. *N*-Acylglutamate is commercially available as a biodegradable surfactant with low skin irritation properties, which is valued as an additive in cosmetics, soaps and shampoos. Oxopyrrolidinecarboxylic acid, another

derivative of glutamic acid, is used as a natural moisturising factor in cosmetics, playing an important role in maintaining water in the cornified layer of skin, acting synergistically with glycine, threonine, alanine, aspartic acid, glutamic acid and serine. Amides of acylglutamate are utilised as gelatinisation agents (Hirose *et al.*, 1985). Amino acid derivatives are also increasingly used in agriculture as plagucides and plant growth regulators (Martin *et al.*, 1987).

A number of natural isolates of corynebacteria produce glutamic acid and these 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* and *B. divaricatum*; and *Microbacterium* species *M. ammoniaphilum* (Kinoshita, 1987).

Kinoshita (1987) reviewed the development of amino acid production, particularly for glutamic acid and lysine. According to this paper, Kinoshita and his colleagues initially screened bacteria which were able to produce glutamic acid in the presence of biotin; one of these strains produced 10.3 mg/ml of glutamic acid in broth cultures. Subsequently, other amino acids were produced by *C. glutamicum* by blocking the glutamic acid biosynthesis pathway. As a result, it was found that lysine instead of glutamic acid was produced in large quantities and that the production of lysine and glutamic acid was modulated by the biotin levels supplied. When biotin was limited to a suboptimal level for growth, glutamic acid was produced, whereas overproduction of lysine occurred in medium that was rich in biotin when limiting concentrations of homoserine (a precursor of lysine) were supplied. Based on this approach, improved productivity was achieved by altering the genetic or biochemical control of enzymes regulating the amino acid biosynthetic pathways. Consequently, glutamic acid and lysine were produced at over 100 g/l (Kinoshita, 1987). A great number of papers have been published on the mutant strains which showed improved productivity of various amino acids (see review articles, Hirose *et al.*, 1985; Nakayama, 1985). However as the focus of this thesis is not on amino acid production by corynebacteria,

this subject will not be reviewed further here.

1.3 TAXONOMY OF THE GENUS *CORYNEBACTERIUM* AND RELATED SPECIES

The genus *Corynebacterium* was created by Lehmann and Newmann (1907) to accommodate the diphtheria bacillus and a few similar animal parasitic species. The genus was defined mainly on the basis of a morphological characteristic (wedge or club shape of the organism) and staining reaction (Gram-positive with irregular bar or band formation). Morphological similarity was at that time generally believed to be a primary indicator of relatedness and thus organisms from habitats other than the human or animal sources were included in the genus. Coryneform organisms were subsequently recognized as saprophytes in soil, water, milk, dairy products and fish, and as plant pathogens (see Keddie, 1978).

Because of the poor definition of the characteristics defining the genus *Corynebacterium*, several isolates were incorrectly placed in this genus or incorrectly named as separate genera (e.g. *Micrococcus glutamicus*). Conn (1947) deplored the misuse of the generic name *Corynebacterium* which led to the inclusion within the genus of almost any non-sporing Gram-positive organism from any habitat. Jensen (1952, 1966) suggested that the genus *Corynebacterium sensu stricto* be restricted to "animal parasitic" bacteria and that all other corynebacteria be called *Corynebacterium sensu lato*. However, the lack of good differential tests made this distinction difficult. Developments in the succeeding decade enabled Keddie and Cure (1978) to define *Corynebacterium* (*sensu stricto*) as a facultatively anaerobic organism with cell walls containing *meso*-diaminopimelic acid, arabinose and galactose and lipid components considered to be corynemycolic acids. This definition covered organisms with a fairly narrow range of DNA base ratios and parasitic species, some saprophytes and all species pathogenic to plants. Prominent

among the saprophytic species included in this definition are *Corynebacterium glutamicum* and a number of very similar glutamic acid-producing species: *Brevibacterium divaricatum*, *B. flavum*, *B. lilium*, *C. callunae* and *C. herculis*.

Morphologically, coryneforms are very similar to mycobacteria and nocardia. Acid fastness, which had been a characteristic considered to be typical for *Mycobacterium*, was also observed in *Corynebacterium* (e.g., *C. equii*, Jensen 1953) and in several *Nocardia* species (Cummins and Harris, 1956). Further similarity in taxonomy in these species was obtained by chemotaxonomic studies. Chemotaxonomy is the application of chemical techniques which give information on the presence or absence of specific components, such as lipids, amino acids and sugars in the cell surface (Barreau *et al.*, 1993; Cummins, 1962) and this has been useful approach to classifying and differenciating Gram-positive bacteria. In particular, the structure of cell walls of Gram-positive bacteria is considered as an important indicator in classification, since cell wall structures constitute up to 40% of the dry weight of these bacteria (Doyle, 1992). The characteristic cell wall polymer in bacteria is peptidoglycan. The chemical composition of the cell walls of human and animal pathogenic *Corynebacterium* species was found to be identical to that in *Mycobacterium* and *Nocardia* species (Cumins and Harris, 1956; Cummins, 1962). All of these genera contain *meso*-diaminopimelic acid (*meso*-DAP), arabinose and galactose in the cell wall and could be distinguished from *Streptomyces* species, which contain glycine and *meso*-diaminopimelic acid (*meso*-DAP), and *Actinomyces* species, which contain lysine and galactose (Cummins, 1962; Lechevalier and Lechevalier, 1970; Mordarska *et al.*, 1972). As a result of studies on cell wall composition and serological specificity, Cummins (1962) 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 coryneforms and members of the genera *Mycobacterium* and *Nocardia* and who, as a result of available evidence, proposed that the

Table 1.2 Differential characteristics of the genus *Corynebacterium* and other actinomycete and coryneform taxa [a]

Species	Major peptidoglycan type [b]	Mycolic acids [c]	Fatty acid type [d]	Major menaquinone isoprenologue(s) [e]
Agromyces	DAB	-	S,A,I	MK-12
Arthrobacter	L-Lysine	-	S,A,I	MK-9(H_2)
Brevibacterium	*meso*-DAP	-	S,A,I	MK-8(H_2)
Caseobacter [f]	*meso*-DAP	30-36	S,U,T	MK-9(H_2), MK-8(H_2)
Cellulomonas	L-Ornithine	-	S,A,I	MK-9(H_4)
Corynebacterium	*meso*-DAP	22-36	S,U,T	MK-9(H_2), MK-8(H_2)
Plant pathogenic *Corynebacterium*	DAB	-	S,A,I	MK-9, MK-10
Curtobacterium	D-Ornithine	-	S,A,I	MK-9
Microbacterium	L-Lysine	-	S,A,I	MK-9
Mycobacterium	*meso*-DAP	60-90	S,U,T	MK-9(H_2)
Nocardia	*meso*-DAP	46-60	S,U,T	MK-8(H_4)
Rhodococcus	*meso*-DAP	30-64	S,U,T	MK-9(H_2), MK-8(H_2)

[a] Data from Bergey's Manual of Systematic Bacteriology (Jones and Collins, 1986).

[b] DAB; diaminobutyric acid, DAP, diaminopimelic acid.

[c] "-" means that 90% or more of strains examined lacked mycolic acids. Numbers indicate total number of carbon atoms in the structure.

[d] S, straight-chained saturated; A, *anteiso*-methyl-branched; U, monounsaturated; T, 10-methyl-branched acids.

[e] MK-8, dihydromenaquinones with eight isoprene units. Numbers indicate number of isoprene units.

[f] Caseobacteria are similar to corynebacteria. However, caseobacteria are considered obligately aerobic and possess a higher G+C content (65-67%) than that of corynebacteria (48-59%).

genera *Corynebacterium*, *Mycobacterium*, and *Nocardia* be merged to form one genus.

Numerical taxonomy (Bousfield, 1972; Jones, 1975) and chemotaxonomic studies (Goodfellow *et al.*, 1976; Keddie and Cure, 1977; Schleifer and Kandler, 1972) have shown that, contrary to these earlier opinions, the various members of the coryneform group are taxonomically related but that they display sufficient differences to warrant separate status as genera (Table 1.2). As information accumulated, the genus *Corynebacterium* was distinguished from *Mycobacterium* and *Nocardia* on the basis of G+C content, and the structure of the cell surface. The DNA base composition of 14 mycobacterial species was studied by Wayne and Gross (1968). The G+C ratio covered a range of 64-70%. Since G+C content for *Corynebacterium* species had been reported by Hill (1966) to be 48-59%, the authors were of the opinion that this provided strong evidence for maintaining the separation between the genera *Corynebacterium* and *Mycobacterium*. The G+C content in nocardia DNA was found 64-70% (Hill, 1966).

The classification of corynebacteria has been more advanced by mycolic acid analyses (Athalye *et al.*, 1984; Carlotti *et al.*, 1993; Corina and Sesardic, 1980; Keddie and Cure, 1977), DNA base composition determination (Bousfield, 1972), DNA-DNA hybridisation (Carlotti *et al.*, 1993; Liebl *et al.*, 1991) and 16S RNA sequencing (Stackebrandt and Schleifer, 1984).

Studies on the cell surface structure of glutamic acid-producing bacteria, including *C. glutamicum*, *B. lactofermentum* and *B. flavum*, showed that all three species contained mycolic acids which are not commonly seen in the majority of bacterial species (Collins *et al.*, 1982a). Furthermore, DNA-DNA hybridisation indicated a strong close relationship between these strains (Liebl *et al.*, 1991). Liebl *et al.* (1991) showed that the G+C content of tested strains (*C. glutamicum* DSM 20300, *B. lactofermentum* DSM 1412, and *B. flavum* DSM 20411) was in the narrow range from 52.8 to 56.0 mol %. This range is similar to the G+C content of true *Corynebacterium* strains

but substantially lower than the G+C content typical of true *Brevibacterium* species (60 to 67 mol %). The G+C content of the DNA from other glutamic acid-producing bacteria such as *C. lilium*, *C. callunae*, *B. divaricatum* and *B. linens*, were within a range 52.8 to 63.8%, mostly around 54% (Liebl *et al.*, 1991). Furthermore, Collins *et al.* (1980) and Carlotti *et al.* (1993) demonstrated the essential features of the genus *Brevibacterium* are the presence of *meso*-diaminopimelic acid (*meso*-DAP), arabinogalactan and mycolic acids in the cell wall, the occurrence of major amounts of dihydrogenated menaquinones with eight isoprene units and DNA with a mol % G+C of 60-70. Therefore, some authors have suggested that *B. lactofermentum* and *B. flavum* should be reclassified into the *Corynebacterium* genus (Abe *et al.*, 1967; Archer and Sinskey, 1993; Keddie and Cure, 1977; Liebl *et al.*, 1991).

In contrast, studies on the genome of two corynebacteria using pulsed-field gel electrophoresis (PFGE) revealed difference in banding patterns of chromosomal DNA (Correia *et al.*, 1994). Using *Pac*I and *Swa*I endonucleases, the genome of *B. lactofermentum* ATCC 13869 (genome size 3,052 kb) was consistently cut into 26 and 20 bands respectively and the genome of *C. glutamicum* ATCC 13032 (2,987 kb) yielded 27 and 26 fragments respectively. Bathe *et al.* (1996) also obtained similar banding patterns of result *C. glutamicum* ATCC 13032 chromosomal DNA. Bathe *et al.* (1996) constructed a combined physical and genetic map of *C. glutamicum* ATCC 13032 chromosome, using PFGE and hybridisations with cloned gene probes. Furthermore, the pattern of PCR (polymer chain reaction) amplification of chromosomal DNA using random primers showed that the PCR patterns of two strains of *C. glutamicum*, ATCC 13032 and AS019, were almost identical but that these were clearly different from that of *B. flavum* BF4 (Webster, 1995).

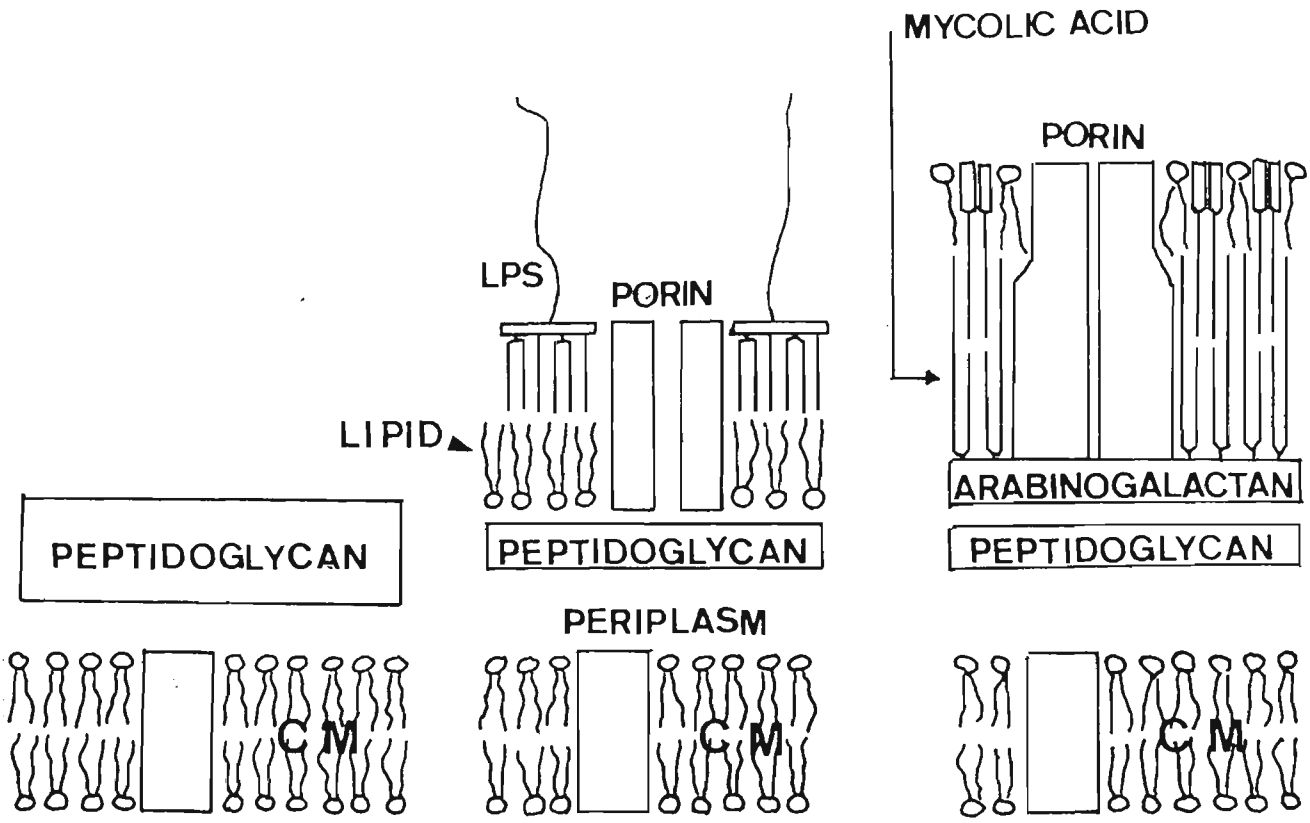


Fig. 1.1 Cell envelopes of bacteria. (a) Gram-positive bacteria, (b) Gram-negative bacteria, (c) mycolic acid-containing Gram-positive bacteria (from Nikaido, 1994). CM = cell membrane.

1.3.1 Peptidoglycan component of mycolic acid-containing bacteria

In the review of Nikaido (1994) on cell envelopes of bacteria, it was noted that most of the Gram-positive bacteria are covered by a porous peptidoglycan layer, which does not exclude most antimicrobial agents [see (a) in Fig. 1.1]. In contrast, Gram-negative bacteria are surrounded by an outer membrane, which functions as an efficient permeability barriers because it contains lipopolysaccharide [see (b) in Fig. 1.1], while mycolic acid-containing bacteria produce an unusual bilayer, which functions as an exceptionally efficient barrier to enzymatic and chemical attack outside the peptidoglycan layer [see (c) in Fig. 1.1]. The characteristic cell wall polymer in bacteria is peptidoglycan. Peptidoglycan of corynebacteria appears to be of the common type, based on a linear glycan composed of alternating 1,4-linked glucosamine and muramic acid units. These glycan chains are cross-linked by peptide subunits which, through variations in their amino acid composition and mode of cross-linkage, account for the majority of differences in peptidoglycan structures (Minnikin and O'Donnell, 1984) (see Fig. 1.1). The genera, corynebacteria, contain *meso*-diaminopimelic acid (*meso*-DAP), arabinose and galactose in the cell wall.

1.3.2 Lipids component of mycolic acid-containing bacteria

Lipids are natural products which can be isolated from cells by extraction with organic solvents and which are usually insoluble in water. Lipids may be broadly divided into polar and non-polar subgroups; the common polar lipids in corynebacteria are phospholipids, glycolopids and ornithine or lysine amides (Hamid *et al.*, 1993; Minnikin *et al.*, 1977). Non-polar lipids include fatty acids, mycolic acids, isoprenoid quinones and carotenoid pigments. Phospholipids occur commonly in many bacteria but certain actinomycetes and corynebacteria contain very characteristic phospholipids, the phosphatidyl inositol mannosides. Chemical degradation of

covalently bound lipids, which are extracted using either acid or alkali to cleave the linkage to polymers of protein or polypeptides, leads to the release of long-chain fatty acids and mycolic acids (Minnikin *et al.*, 1977; Pierotti, 1987; Tomiyasu and Yano, 1984). Lipid structure studies of corynebacteria involving analyses of mycolic acid and isoprenoid quinones revealed that this was a very powerful chemotaxonomic tool in coryneform taxonomy, since the strains tested gave distinct patterns depending on the species used (Collins *et al.*, 1977; 1979) (Table 1.2).

Isoprenoid quinones are a class of terpenoid lipids in the cytoplasmic membranes of many bacteria. Isoprenoid quinones are widely distributed in bacteria and there are two major structural groups: menaquinones (vitamin K) and ubiquinones (coenzyme Q). They play important roles in electron transport and oxidative phosphorylation (Jones and Krieg, 1986). Collins *et al.* (1977; 1979) showed that isoprenoid quinone analyses provided good markers for the classification of corynebacteria and related bacteria and could be used to divide corynebacteria into several groups on the basis of menaquinone composition. Thus, while human and animal pathogenic corynebacteria contain predominantly dihydromenaquinones with eight isoprene units, *Mycobacterium* and glutamic acid-producing strains including, *C. glutamicum*, *C. lilium*, *B. flavum* and *B. lactofermentum*, have dihydromenaquinones with nine isoprene units (Table 1.2).

Fatty acids commonly occur as the hydrophobic chains of the membrane phospholipids although they can also be present as free fatty acids. The fatty acids from corynebacteria have chain lengths of between 10 and 20 carbon atoms and were mainly saturated and monounsaturated (Collins *et al.*, 1982b; Pierotti, 1987). Furthermore, 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 ($C_{16:0}$) and oleic acid ($C_{18:1}$). In *C. glutamicum*, myristic acid ($C_{14:0}$), pentadecanoic 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. Approximately 50% of total fatty

acids in the cell are unsaturated (Pierotti, 1987), but fatty acid composition can change with changes in growth medium or the age of culture (Pierotti, 1987). Pierotti (1987) observed that, with increasing cultivation time from exponential phase to late stationary phase, the percentage of $C_{18:1}$ was less than the parent strain compared to cell surface mutant strains of *C. glutamicum*. Since the existence of mycolic acids is unusual except for a few genera, these lipids are described separately in the next section.

1.3.3 Mycolic acids

1.3.3.1 Mycolic acid structure

Mycolic acids are long-chain, α-alkyl branched, β-hydroxy fatty acids. The alkyl chain is normally composed of up to 22 or 24 carbons in mycobacteria. The chain from the β carbon can be up to 60 carbons long 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 (see review papers by Brennan and Nikaido [1995] and Minnikin *et al.* [1982]). Mycolic acids are only found in bacteria such as *Corynebacterium*, *Mycobacterium*, *Nocardia* and *Rhodococcus* species (Chevalier *et al.*, 1988; Collins *et al.*, 1982a; Jarlier and Nikaido, 1994; Stodola *et al.*, 1938). These bacteria have a common cell wall architecture consisting of covalently-bound peptidoglycan-arabinogalactan-mycolic acids (Barksdale and Kim, 1977; Brennan and Nikaido, 1995; Jarlier and Nikaido, 1994; Michel and Bordet, 1976; Minnikin and O'Donnell, 1984), which require treatment with an acid or alkali to cleave the linkage (Tomiyasu and Yano, 1984) and a non-covalently bound fraction which can be extracted using organic solvents (Minnikin *et al.*, 1977) (Fig. 1.2). According to the review paper on mycobacteria by Brennan and Nikaido (1995), the covalently connected structure of the cell wall is made of peptidoglycan, to which arabinogalactan is linked *via* a

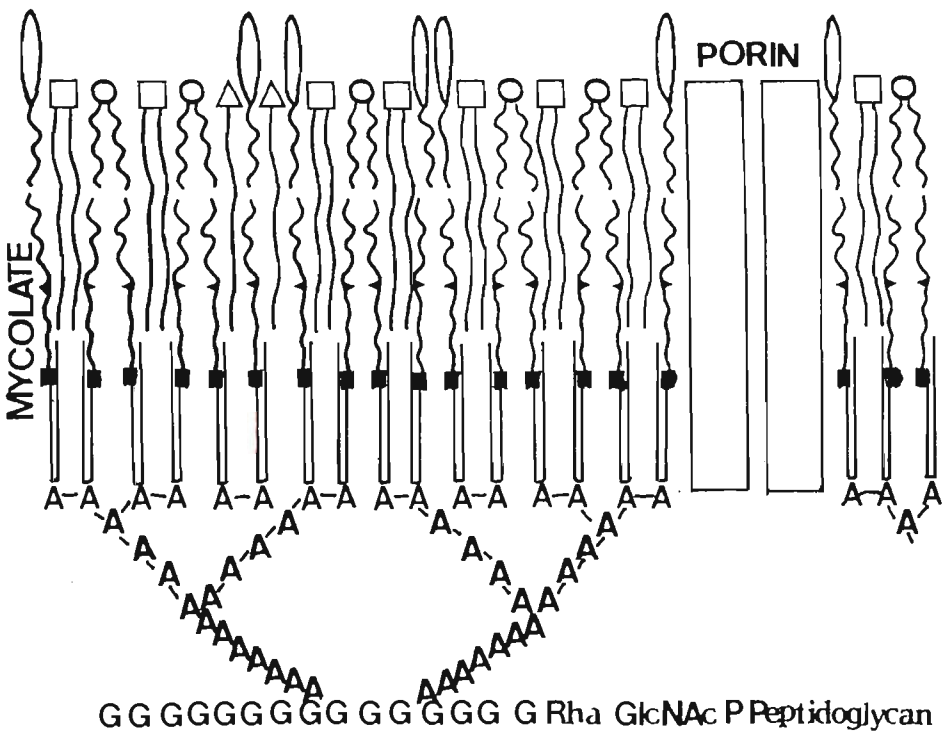


Fig. 1.2 A model of the mycobacterial cell wall (from Brennan and Nikaido, 1995). Legend: (■) cis- or trans-Double bond, or cis- or trans-cyclopropane group; (▲) cis-double bond, cis-cyclopropane group, or oxygen-containing groups; (□) extractable lipid with intermediate chain-length hydrocarbons; (Δ) extractable lipid with C_{14}-C_{18} fatty acids. The lipids composing the outer leaflet differ from species to species. Those containing short-chain (C_{16}-C_{18}) fatty acids include glycerophospholipids and glycopeptidolipids and others. Those containing intermediate chain-length hydrocarbons include phenolic glycolipids, dimycocerosate, and others. Abbreviations: A; arabinose, G; galactan.

phosphodiester bridge (Fig. 1.2). About 10% of the arabinose residues in arabinogalactan are, in turn, bound to mycolic acid (McNeil and Brennan, 1991; Minnikin, 1982). In this model, mycolic acid chains are packed side-by-side in a direction perpendicular to the plane of the cell surface (Minnikin, 1982), an hypothesis which is supported by X-ray diffraction data of purified *M. chelonae* cell walls (Nikaido *et al.*, 1993).

In a review by Walker *et al.* (1973), it was demonstrated that the mycolic acids of *C. diphtheriae* are comprised of as much as 6% acid-extractable lipid. The bound lipid fraction includes corynemycolic acids in the salt form, trehalose esters (trehalose-6,6´-dimycolate: "cord factor") and glucose esters when bacteria are grown on a glucose-containing medium. Salts of corynemycolic acid and "cord factor" have been shown to coat the cell walls of both pathogenic and non-pathogenic strains and are extractable from cells by treatment with light petroleum (Walker *et al.*, 1973). Covalently bound mycolic acids of *C. glutamicum* were studied by Pierotti (1987), who 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 fraction or the extractable lipids.

Studies on the biosynthesis pathway for mycolic acids of corynebacteria and other mycolic acid-containing bacteria have been reported by several authors (Besra *et al.*, 1994; Brennan and Nikaido, 1995; Gastambide-Odier and Lederer, 1959; Lacave *et al.*, 1990; Qureshi *et al.*, 1984; Shimakata *et al.*, 1984; Walker *et al.*, 1973; Wheeler *et al.*, 1993). Gastambide-Odier and Lederer (1959) found that when [1-^{14}C] palmitate was added to a culture of *C. diphtheriae*, the label was incorporated into positions C-1 and C-3 of the isolated corynemycolic acid ($C_{32}H_{64}O_3$) and 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 acid. Similarly, biosynthesis of corynemycolic acid ($C_{32}H_{64}O_3$) was also reported by Walker *et al.* (1973).

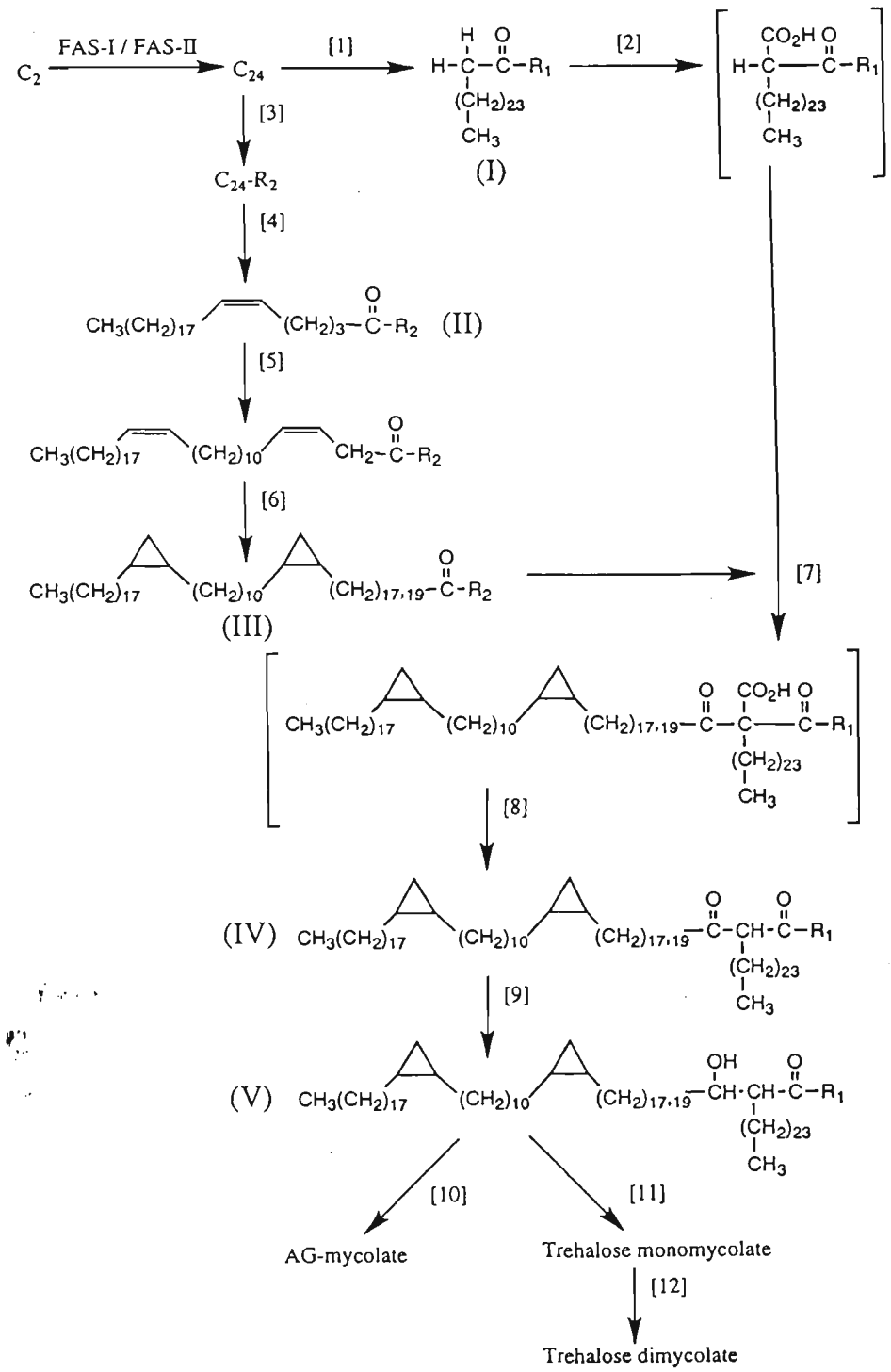


Fig. 1.3 Proposed biosynthesis pathway of mycolic acids in *M. tuberculosis* (from Brennan and Nikaido, 1995). The reactions are identified as follows: [1] elongation and introduction of carrier group R1, [2] carboxylation, [3] introduction of carrier group R2, [4] Δ-5-desaturation, [5] elongation and Δ-3-desaturation, [6] introduction of cyclopropane rings and elongation, [7] Claisen-type condensation, [8] decarboxylation, [9] reduction, [10] mycolate transfer to arabinogalactan, [11] mycolate transfer to trehalose and [12] trehalose mycolyltransferase. Carrier group R1 is believed to be the β-D-mannopyranosyl-monophosphoryl-polyisoprenol, and R2 may be either CoA or an acyl carrier protein.

Experimentally, cells of *C. diphtheriae* were grown in [1-^{14}C] palmitic acid. The label was incorporated into derivatives of 2-tetradecyl-3-keto-octadecanoic acid, which was reduced with $NaBH_4$ to yield a mixture of stereoisomers of corynemycolic acid. Based on this observation, they postulated that the 2-tetradecyl-3-keto-octadecanoic acid could be formed by condensation between labelled palmitate and an endogenous metabolite synthesised through biotin-dependent reactions from naturally occurring constituents of the cell-free extract. They also further suggested the carboxylase carboxylates a palmityl derivative to a tetradecylmalonyl derivative.

Similar condensations were established for the corynemycolic acids of sizes between C_{34} to C_{36} (Shimakata *et al.*, 1984). Shimakata *et al.* (1984) demonstrated biosynthetic activity for corynemycolic acids ($C_{34:0}$, $C_{34:1}$, $C_{36:0}$ and $C_{36:1}$) in the fluffy layer fraction of *Corynebacterium matruchotti* (formerly called *Bacterionema matruchotti*). 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 corresponded to the peak of ($C_{34:0}$ + $C_{34:1}$) mycolic acids and the other to ($C_{36:0}$ + $C_{36:1}$) mycolic acid. This reaction was dependant on the pH (optimum at pH 6.4) and required a divalent cation (Mg^{++}, Zn^{++}, Ca^{++} and Mn^{++}). Cerulenin, a specific inhibitor of β-ketoacyl synthetase in *de novo* fatty acid synthesis, inhibited the reaction at a relatively high concentration.

In *Mycobacterium* species, a different mycolic acid synthesis procedure was suggested, since the structure of mycolic acids in mycobacteria are much larger than corynemycolic acids; this proposed pathway in *M. tuberculosis* H37Ra involved elongation of fatty acids and introduction of a carrier group, desaturation and decarboxylation (Fig. 1.3). The Claisen-type condensation of the C_{26} fatty acid with meromycolic acid to yield the ketomycolyl-R1 intermediate (see [7,8] in Fig. 1.3) has not been demonstrated in a cell-free system. However, based on the results of corynemycolic acids (Shimakata *et al.*, 1984; Walker *et al.*, 1973), Brennan and Nikaido (1995)

speculated that resulting products are in the form of trehalose mono-mycolates and these esters are thought to be carriers of the mycolic acid to the mycobacterial cell wall, where it is transferred to the non-reducing terminal D-arabinose residue of arabinogalactan *via* an unknown transacylation reaction in mycobacteria (Brennan and Nikaido, 1995).

1.3.3.2 Mycolic acid composition in mycolic acid-containing bacteria

The formulation of mycolic acids varies from species to species and these differences are used as a useful taxonomic marker, as mentioned earlier. Mycobacterial mycolic acids are the largest in size (containing 60-90 carbon atoms in total size) and the most abundant constituents (40% by weight) of cell walls of mycobacteria (Minnikin, 1982). The genera *Nocardia* and *Rhodococcus* have mycolic acids with 36-66 carbon atoms which have from zero to four double bonds (Alshamaony *et al.*, 1976; Minnikin and Goodfellow, 1976; Tomiyasu and Yano, 1984). The mycolic acids from corynebacteria contain homologous mixtures of saturated and unsaturated components containing between 20 and 36 carbon atoms (Collins *et al.*, 1982a; Herrera-Alcaraz *et al.*, 1993; Pierotti, 1987).

Studies on mycolic acids have been carried out using either thin layer chromatography (TLC) (Barreau *et al.*, 1993; Goodfellow *et al.*, 1976; Hamid *et al.*, 1993; Minnikin *et al.*, 1975), or gas chromatography combined with mass spectrometry (GC-MS) (Collins *et al.*, 1982a; Chevalier *et al.*, 1988; Pierotti, 1987), or high performance liquid chromatography (HPLC) (Butler *et al.*, 1991; Thibert *et al.*, 1993).

A number of extraction and derivatisation methods have been used for studying mycolic acids. Early methods involved alkaline hydrolysis followed by conversion to methyl esters with diazomethane (Asselineau, 1966; Etémadi, 1967). This method had a number of disadvantages,

not the least of which was the toxic, explosive nature of diazomethane. A more convenient method involves acid methanolysis which directly produces mycolic acid methyl esters (MAMEs) (Minnikin *et al.*, 1980; Pierotti, 1987). In this acid methanolysis procedure, covalently bound mycolic acids and fatty acids are extracted from the cell wall by treating cells with a mixture of toluene, methanol and sulfuric acid followed by incubating at 80°C overnight. The MAMEs and fatty acid methyl esters (FAMEs) produced are then separated from other cellular components using petroleum extraction, in which MAMEs and FAMEs move into the petroleum layer while other components are located in the aqueous layer. The presence or absence of MAMEs can be demonstrated by TLC of methanolysates, in which MAMEs was separated from FAMEs due to the slower chromatographic mobility of MAMEs, using petroleum ether and acetone (95:5, v/v) as solvent. R*f* values of below 0.5 usually correspond to MAMEs while R*f* values of above 0.7 correspond to FAMEs (Pierotti, 1987). Resulting MAMEs can be analysed by GC (Asselineau, 1966). However, due to the high molecular weight of mycolic acids, high temperatures required for the GC analysis caused thermal cleavage to yield FAMEs and aldehydes (Asselineau, 1966). 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, mycolic acids can be protected from pyrolytic cleavage by derivatisation of the hydroxyl group to a trimethylsilyl (TMS) ether and thus be analysed as intact molecules by GC. Intact molecules of silylated mycolic acids from corynebacteria (Corina and Sesardic, 1980; Pierotti, 1987; Yano and Saito, 1972) and other mycolic acid-containing bacteria (Yano *et al.*, 1972) have been fractionated and identified by GC-MS.

Mycolic acids have also been studied using reversed-phase HPLC (Butler *et al.*, 1991; Thibert and Lapierre., 1993). Experimentally, mycolic acids were modified using saponification techniques

Table 1.3 Examples of distribution of mycolic acid in corynebacteria

Organism	Mycolic acids	Reference
Pathogenic corynebacteria		
C. diphtheriae	C_{20} to C_{35}	Corina and Sesardic, 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

and derivatising the acids to their *p*-bromophenacyl 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).

The mycolic acids and fatty acids of some non-pathogenic corynebacteria species have been investigated previously (Collins *et al.*, 1982a; Collins *et al.*, 1982b; Pierotti, 1987). Collins *et al.* (1982a) prepared MAMEs from late stationary phase (three days) of growth for cells grown on nutrient agar. Qualitative differences in mycolic acid composition for three corynebacteria were 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 three species tested, while $C_{36:1}$ existed as a minor component.

The overall composition of mycolic acids from the three saprophytic species was similar but this was different to that of pathogenic corynebacteria, such as *C. diphtheriae*, *C. ulcerans* and *C. urealyticum* (Corina and Sesardic, 1980; Herrera-Alcaraz *et al.*, 1993; Yano and Saito, 1972) (Table 1.3). *C. ulcerans* (Yano and Saito, 1972) possesses mycolic acids with exceptionally low carbon numbers (C_{20} to C_{32}) while *C. diphtheriae* (Corina and Sesardic, 1980) possesses mycolic acids with C_{20} to C_{35}. *C. urealyticum* exhibited mycolic acids with lipids ranging from C_{26} to C_{30}, and this species is characteristically typified by its content of $C_{30:3}$ and $C_{28:1}$ mycolic acids (Herrera-Alcaraz *et al.*, 1993).

1.3.3.3 The role of mycolic acids as a permeability barrier

Several papers have demonstrated that the cell wall in mycolic acid-containing bacteria constitutes an efficient permeability barrier and several properties of mycobacteria, such as acid fastness, the slow rates of growth and the natural resistance to a wide range of antibiotics, are often thought to

be at least partially related to the poor penetration of solutes across the cell wall (Hui *et al.*, 1977; Jarlier and Nikaido, 1990; Katsumata *et al.*, 1984).

Haynes and Britz (1990) speculated that the cell surface of *C. glutamicum* precluded efficient uptake of DNA. Jarlier and Nikaido (1990) quantitatively measured the permeability of the mycobacterial cell wall and found that this formed a permeability barrier to the uptake of hydrophilic molecules. Experimentally, the rate of hydrolysis of cephalosporins 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 were hydrolysed by periplasmic β-lactamase (following Michaelis-Menten kinetics). The cell wall permeability measured was very low, being about three orders of magnitude lower than seen for the *E. coli* outer membrane and ten 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. Permeability to hydrophilic nutrient molecules, such as glycerol and glucose, was also very low (Jarlier and Nikaido, 1990).

In corynebacteria, Katsumata *et al.* (1984) demonstrated that the cell wall structure of *C. glutamicum* reduced access of lysozyme to the peptidoglycan, which is located inside the mycolic acid layer, and longer lysozyme treatment (16 h) was required to form protoplasts. When the cell wall was efficiently modified following growth in the presence of known cell wall inhibitors or modifiers, such as glycine, isonicotinic acid hydrazide (INH), or penicillin G, and cells were treated with approximately the same amounts of lysozyme, workers have obtained high proportions of osmotically sensitive cells within 1 to 2h incubation (Santamaria *et al.*, 1984; Best and Britz, 1986; Thierbach *et al.*, 1988). Based on this observation, it was suggested that the low cell wall permeability plays an essential role in making corynebacteria resistant to many molecules

and also that cell wall permeability could be reduced by using cell wall inhibitors (Best and Britz, 1986; Haynes and Britz, 1989; Katsumata *et al.*, 1984; Santamaria *et al.*, 1984; Thierbach *et al.*, 1988; Yoshihama *et al.*, 1985). Furthermore, it has been reported that sensitivity to antibiotics is increased when the cell wall of mycobacteria is altered by growth in the presence of a detergent, such as Tween 80 (Hui *et al.*, 1977) or by the removal of cell wall material through spheroplasting (David *et al.*, 1987).

Although it has been suggested that the mycolic acid profile is a form of finger-print for a single bacterial species (Gailly *et al.*, 1982), quantitative changes in mycolic acid composition have been reported under different environmental conditions, including growth in media containing Tween 80 (Chevalier *et al.*, 1988) and growth at different temperatures (Tomiyasu *et al.*, 1982). 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 acid. 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 indicated that environmental temperature and the presence of specific chemicals could change the structure of mycolic acids of some mycolic acid-containing bacteria.

1.4 MODULATORS OF CELL SURFACE STRUCTURES

The impact of chemicals, including glycine, INH, Tween 80 and penicillin G, which are known to modify cell wall structure in Gram-positive species and mycobacteria are described in the following sections.

1.4.1 Glycine

The inhibitory effect of glycine on bacterial growth has been known for a long time (Dienes *et al.*, 1950; Gordon and Gordon, 1943). In the Gram-positive bacteria, it was reviewed that *Streptomyces* mycelium subcultured into a medium containing enough glycine to cause some growth retardation of cells was much more sensitive to lysozyme than mycelium grown in the absence of added glycine (Okanishi *et al.*, 1974; Hopwood *et al.*, 1977; 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*.

Studies on the effect of glycine on *Staphylococcus aureus* revealed that accumulation of uridine diphosphate-*N*-acetyl muramic acid and other cell wall precursors in which L-alanine was replaced by glycine occurred (Strominger and Birge, 1965). Hishinuma *et al.* (1971) found that the enzyme responsible for incorporation of L-alanine was inhibited by glycine. A more detailed study of glycine action on the biosynthesis of peptidoglycan of several Gram-positive bacteria, *S. aureus*, *Lactobacillus cellobiosus* and *C. callunae,* was carried out by Hammes *et al.* (1973). Hammes *et al.* (1973) investigated whether glycine was incorporated into the nucleotide-activated glycine precursors and found that glycine could replace D-alanine residues in positions 4 and 5 of the peptide subunit. As a result, cells were more susceptible to lysozyme treatment. Best and Britz (1984) introduced this approach to forming protoplasts of *C. glutamicum*. Similarly, Yoshihama *et al.* (1985) also found that when cells of *C. glutamicum* were grown in the medium containing 2% (w/v) glycine before lysozyme treatment, the proportion of osmotically sensitive cells (mixture of spheroplasts plus protoplasts) was increased to 99%.

Using microscopic observation, Best and Britz (1986) reported the effect of glycine in the growth medium on cell morphology of *C. glutamicum*. Experimentally, 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. Using these conditions, Best and Britz (1986) observed that growth in glycine resulted in larger, more irregularly shaped cells when compared to those grown in unsupplemented media. Also, they found that these effects were less marked in stationary phase cultures. These observation implied strongly that alterations in cell morphology in *C. glutamicum* were related to the presence of glycine in the growth condition.

A similar observation was later reported in other corynebacteria. When *C. diphtheriae* was grown in the presence of 2% (w/v) glycine, the cells elongated and bulged at sites where the cell wall had apparently been damaged. When cells were treated subsequently with lysozyme (2 mg/ml) and washed in hypertonic buffer, the mis-shapen rods were replaced by spherical forms (Serwold-Davis *et al.*, 1987). The effective concentration of glycine also varied with each strain (Serwold-Davis *et al.*, 1987); for the formation of osmotically sensitive cells, *C. ulcerans* required much less glycine than did *C. diphtheriae*. Growth of *C. glutamicum* in glycine was used prior to plasmid transformation (Haynes and Britz, 1990; Schäfer *et al.*, 1994b; Yoshihama *et al.*, 1985) and some specific examples of DNA transformation methods will be described in section 1.5.2.

1.4.2 Isonicotinic acid hydrazide (INH)

INH (trivial name, Isoniazide) is a potent anti-tubercular agent which has been used for over 40 years for the chemotherapy of tuberculosis, caused by *Mycobacterium* species. (Barclay *et al.*, 1953; Musser, 1995). In the past four decades, numerous reports have appeared on the mechanism of the anti-tuberculosis action of this compound in mycobacteria, which indicate antagonistic action towards pyridoxal or pyridine nucleotides and their analogues (Barclay *et al.*, 1953; Gayathri *et al.*, 1972; Yoneda *et al.*, 1952). 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).

However, neither the bacterial target nor the mode of INH is totally understood. Potential insight into the mechanism of INH resistance was elucidated in 1954 when Middlebrook and colleagues discovered that INH-resistant organisms had decreased catalase activity (Middlebrook, 1954; Cohn *et al.*, 1954). This observation was extended by Hedgecock and Faucher (1957), who studied INH-resistant organisms and noted an inverse correlation between INH MIC and catalase-peroxidase activity.

Based on reported information to date, resistance of mycobacteria to INH is now known to be associated with at least two independent mechanisms: (1) mutation or inactivation of the *katG* gene encoding the HPI-type catalase-peroxidase (Heym *et al.*, 1993; Heym *et al.*, 1995; Zhang *et al.*, 1992) 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 observation that only a relatively small percentage of INH-resistant *M. tuberculosis* strains are catalase negative, with either gross alterations (Stoeckle *et al.*, 1993) or missense or mutations involving *katG* (Cockerill *et al.*, 1995), indicated that other molecular mechanisms are also involved in INH resistance. Furthermore, a link between INH and catalase-peroxidase systems is not sufficient to explain the observation that INH only affects the growth of mycobacteria and related bacteria containing mycolic acids. Although the exact mechanism by which INH exerts its effect is unknown, one working hypothesis postulates that INH or an INH metabolite blocks the synthesis of mycolic acids in mycolic acid-containing bacteria (Banerjee *et al.*, 1994; Davidson and Takayama, 1979; Quémard *et al.*, 1991; Quémard *et al.*, 1995b; Takayama *et al.*, 1972; Tomiyasu and Yano, 1984; Winder *et al.*, 1964; Winder and Collins, 1970). This is inferred by the observation that INH decreases the amount of mycolic acids and possible intermediates, very

long-chain non-polar fatty acids, in *Nocardia* species (Tomiyasu and Yano, 1984), and INH inhibited the enzymatic biosynthesis of very long chain fatty acids in *M. tuberculosis* $H_{37}Ra$ (Quémard *et al.*, 1995b). Tomiyasu and Yano (1984), working with *Nocardia* species, also found that INH markedly affected both 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} or C_{30}. However, the exact point of inhibitory action or the actual 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.

As a consequence, there has been a concerted effort by several groups to isolate additional genes involved in INH resistance. Banejaree *et al.* (1994) identified a locus containing two continuous open reading frames (ORFs) (designated *orf1* and *inhA*) coding for products that may participate in resistance to both INH and ethionamide (ETH). ETH is a structural analog of INH that is thought to inhibit mycolic acid synthesis in mycobacteria (Winder *et al.*, 1971). DNA sequence analysis from INH sensitive strains of mycobacteria including *M. smegmatis*, *M. bovis* and *M. tuberculosis*, as well as INH resistant strains of *M. bovis* and *M. smegmatis*, revealed that the ORFs encode a 29-KD protein followed by a 32-KD protein. DNA sequencing analysis showed that the resistant organisms of *M. smegmatis* had a single base change in the *inh*A gene, which encodes the 29-KD protein.

The inferred proteins made by *inhA* of *M. tuberculosis* (and *M. bovis* and *M. smegmatis*) have more than 40% sequence identity over a stretch of 203 amino acids with the EnvM proteins of *E. coli* and *Salmonella typhimurium*. EnvM is thought to participate 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. Subsequent subcloning studies demonstrated that changes in this ORF from *M. smegmatis* were sufficient to confer the INH resistance phenotype, in that a mutation within the gene resulted in resistance to INH. Recently, Dessen *et al.* (1995) reported that an INH resistant strain of *M. tuberculosis* contained altered InhA protein where serine 94 was replaced by alanine. 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-binding network that stabilises β-nicotinamide adenine dinucleotide (NADH) binding. One can only speculate about the role of the catalase/peroxidase system in this activation process, although conversion of INH to an active form by catalase/peroxidase activity is an alternative model.

Britz and colleagues (Britz, 1985; Haynes and Britz, 1989; Haynes and Britz, 1990) used INH as a component of the growth medium of coryneform bacteria species in an attempt to reduce cell surface barriers for DNA transformation, in context of the known effects of INH on mycolic acid synthesis in mycobacterial species. Experimentally, two species of corynebacteria, *C. glutamicum* and *B. lactofermentum,* were grown in LBG containing different concentrations of INH (0-12 mg/ml), harvesting at early-exponential phase then transforming with homologously-isolated plasmid DNA (Haynes and Britz, 1989; 1990). As the concentration of INH increased in the growth medium, transformation efficiency also increased when compared to cells grown in the absence of INH (Haynes and Britz, 1990). Based on this result, these workers suggested that mycolic acids were probably the principal a physical barrier to DNA entering cells. However, the effect of INH on mycolic acid composition in these corynebacteria was not reported in these publications.

1.4.3 Tween 80

Tween 80 (polyoxyethylene-sorbitan-monooleate) is the polyoxyethylene derivatives of fatty acid monoesters of sorbitan, where the "80" indicates the presence of esters of oleic acid which are emulsifying and dispersing agents (Nieman, 1954). Tween 80 itself is stimulatory to the growth of cells because it is a derivative of oleic acid [the detoxication of Tween 80 is achieved by its incorporation]. This has been used as a growth medium supplement for mycobacteria (Dubos, 1947), *Clostrium sporogenes* (Thoma and Peterson, 1950), and lactic acid bacteria (LAB) where it is a constituent of the standard medium used for this group of bacteria, MRS (de Man *et al.*, 1960). The presence of Tween 80 affects the fatty acid composition in LABs: Johnson *et al.* (1995) found that, following cultivating in Tween 80, LABs had a different fatty acid pattern and two new fatty acids appeared in the profile. Similar observations have been reported for cutaneous corynebacteria (Chevalier *et al.*, 1988). Several *Corynebacterium* strains were able both to hydrolyse Tween 80 and to use its oleic acid component for synthesis of fatty acids. Furthermore, the mycolic acid composition of cells grown in the presence of Tween 80 was altered (Chevalier *et al.*, 1988). Although the effect of Tween 80 on cell surface components of corynebacteria was not understood clearly, Tween 80 was also used by Haynes and Britz (1989) as a medium component to test its effect on reducing the physical barriers to transformation. These researchers found that growth in LBG medium supplemented with 0.1% or 0.5% Tween 80 had a significant effect on the electroporation frequency of *B. lactofermentum*, where an increase of approximately 20-fold was seen, which also suggested that growth in Tween 80 affected cell surface structures to improve DNA entry into cells.

1.4.4 Penicillin G

Penicillin is known to prevent the cross-linking reaction of the mucopeptidase involved in cell wall

synthesis (Wise and Park, 1965) and was found to be an inhibitor of cell wall synthesis for protoplasts of *Bacillus megaterium* when cells were grown in the presence of penicillin (Kusaka, 1971). Penicillin treatment is also used industrially in the production of glutamic acid, to facilitate the excretion of glutamic acid (Hirose *et al.*, 1985). Addition of penicillin G to an exponential growth phase culture of *C. glutamicum* resulted in a rapid decrease in cell viability accompanied by rapid excretion of glutamate. The cells continued to produce glutamate for 40 to 50 h after penicillin addition, with no lysis throughout the entire fermentation. Penicillin G has been used in corynebacterial transformation for both protoplast formation (Kaneko and Sakaguchi, 1979; Katsumata *et al.*, 1984; Santamaria *et al.*, 1984) and electroporation (Noh *et al.*, 1991; Satoh *et al.*, 1990) which resulted in increases in efficiency of transformation; specific examples of this are described in section 1.5.2.

1.5 DNA TRANSFER VEHICLES AND METHODS

1.5.1 Corynebacterial plasmids and cloning vectors

Until quite recently, corynebacteria have been relatively refractory to the introduction of foreign DNA into cells and suitable corynebacterial cloning vectors have only been developed relatively recently. However, the recent development of efficient genetic tools for the corynebacteria, such as endogenous plasmid systems (Miwa *et al.*, 1984; Katsumata *et al.*, 1984; Santamaria *et al.*, 1984; Yoshihama *et al.*, 1985), the development of efficient electroporation methods (Haynes and Britz, 1989; 1990; Liebl *et al.*, 1989; Wolf *et al.*, 1989) and gene disruption and replacement techniques (Labarre *et al.*, 1993; Schwarzer and Pühler, 1991), have enabled the construction of more versatile strains for the biotechnology industry (see review articles, Jetten and Sinskey, 1995; Wohlleben *et al.*, 1993).

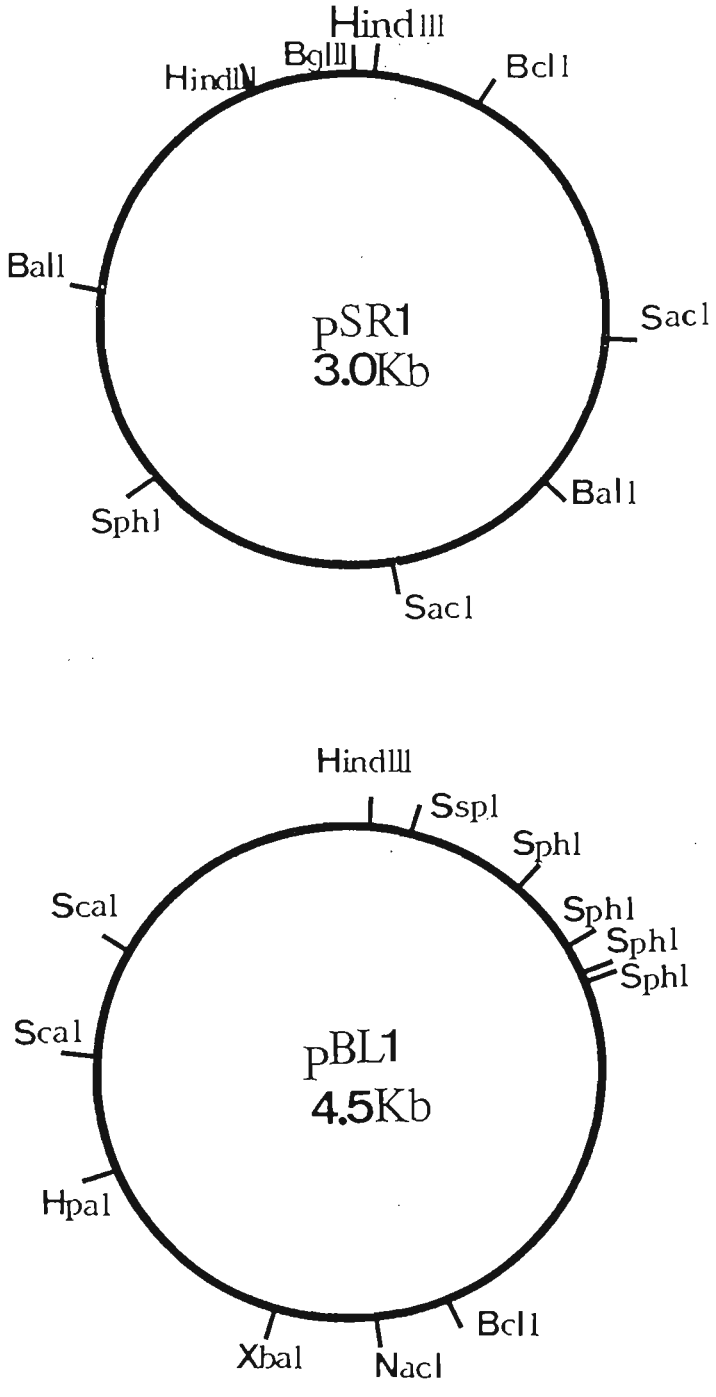


Fig. 1.4 Restriction maps of the coryneform plasmids pBL1 (Martin *et al.*, 1987; Santamaria *et al.*, 1984;) and pSR1 (Archer and Sinskey, 1993; Yoshihama *et al.*, 1985).

As noted by Jetten and Sinskey (1995), some of the endogenous plasmids are very similar and many shuttle vectors for corynebacteria have an origin of replication from the cryptic plasmids of *B. lactofermentum* or *C. glutamicum* plus an origin of replication which functions in *E. coli* or other species. The plasmid from *B. lactofermentum* ATCC 21798, pBL1, is 4.4 kb and has a copy number of 10-30 (Fig. 1.4) (Martin *et al.*, 1987; Santamaria *et al.*, 1984). The pSR1 is a 3.0 kb plasmid isolated from *C. glutamicum* ATCC 13032 (Fig. 1.4) (Archer and Sinskey, 1993; Yoshihama *et al.*, 1985). These two cryptic plasmids were used to construct shuttle vectors such as pHY416 (Follettie and Sinskey, 1986) and pCSL17 (Hodgson *et al.*, 1989). For pHY416, pSR1 was ligated into the gene encoding erythromycin-resistance in the *B. subtilis* vector pBD10 (kanamycin-, chloramphenicol-, and erythromycin-resistant) to give the 9.4 kb plasmid pHY416. The kanamycin gene was expressed strongly in *C. glutamicum* (resistance >100 μg/ml) but the cells were scarcely more resistant to chloramphenicol (Cm) than the parent (Yoshihama *et al.*, 1985). Britz and Best (1986) later showed that Cm-resistance is not expressed unless a deletion occurs which allows expression of Cm-acetyltransferase presumably under the control of the Km-resistance promoter. Therefore, pHY416 was able to transform into *B. subtilis* and *C. glutamicum*, using Km-resistance as the selection marker. The vector pCSL17 (7.2 kb) was constructed using a derivative of pBR322, the Tn*903* kanamycin gene and the cryptic plasmid from *C. glutamicum* (pSR1). This has replication origins for cloning in *E. coli*, where the ampicillin and kanamycin resistance markers can be selected, as well as in *C. glutamicum*, where kanamycin resistance is selectable (Hodgson *et al.*, 1989). Kanamycin resistance is expressed in corynebacteria, such as *C. glutamicum*, *B. lactofermentum*, and *B. flavum*, and *E. coli*, whilst ampicillin resistance is expressed only in *E. coli*. A natural plasmid pNG2, isolated from *C. diphtheriae*, can replicate in several corynebacteria species, *E. coli* and *Mycobacterium* (Serwold-Davis and Groman, 1986, 1990). Compatibility tests indicated that at least pHM1519, pBL1, pCG1 and pGA1 can coexist stably within one cell (Sonnen *et al.*, 1991) (Table 1.4).

Table 1.4 Examples of natural and chimeric plasmids which replicate in *Corynebacterium* strains and related species

Group	Plasmid	Source/Characteristics	Size	Marker	Reference
Natural plasmid					
pCC1	pCC1	*C. callunae* 2243	4.3	Cryptic	Sandoval *et al.*, 1984
	pCC100	NCIB 10318	4.2	Cryptic	Shaw, 1989
pBL1	pBL1	*B. lactofermentum* ATCC 21798	4.4	Cryptic	Santamaria *et al.*, 1984
	pAM330	ATCC 13869	4.5	Cryptic	Miwa *et al.*, 1984
	pWS101	ATCC 13869	4.4	Cryptic	Yoshihama *et al.*, 1985
	pGX1901	ATCC 13869	4.5	Cryptic	Smith *et al.*, 1986
	pX18	ATCC 21086	4.4	Cryptic	Yeh *et al.*,1986
	pBL100	ATCC 13869	4.5	Cryptic	Shaw and Hartley, 1988
pHM1519	pHM1519	*C. glutamicum* ATCC 13058	3.0	Cryptic	Miwa *et al.*, 1984
	pCG1	ATCC 31808	3.2	Cryptic	Ozaki *et al.*, 1984
	pSR1	ATCC 13032	3.1	Cryptic	Yoshihama *et al.*, 1985
	pCG100	ATCC 13058	3.0	Cryptic	Shaw and Hartley, 1988
pGA1	pGA1	*C. glutamicum* Lp-6	4.9	Cryptic	Sonnen *et al.*, 1991
pNG2	pNG2	*C. diphtheriae* C71	4.4	Em^r	Serwold-Davis *et al.*, 1986
Chimeric plasmid					
	pUL340	*B. lactofermentum* vector carrying the Km^r gene from *Tn5*	5.8	Km^r	Santamaria *et al.*, 1985
	pHY416	*B. subtilis-C. glutamicum* vector	9.4	Km^r	Follettie and Sinskey, 1986
	pCSL17	*E. coli-C. glutamicum* shuttle vector	7.2	Km^r	Hodgson *et al.*, 1989
	pWJ5	Mobilisable *E. coli-C. glutamicum* vector	11.8		Schwazer and Pühler, 1991
	pEBM3	Mobilisable *E. coli-C. glutamicum* vector	9.6	Km^r	Schäfer *et al.*, 1994b

NCIB, National Collection of Industrial Bacteria, Aberdeen, Scotland. ATCC, American Type Culture Collection, Rockville, MD, U.S.A.
Abbreviations: Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance; Em^r, erythromycin resistance.

In a review by Wohlleben *et al.* (1993), it was noted that the majority of these plasmids are stable and are retained in >90% of the cells, after 25 generations without selective pressure.

Only a few endogenous antibiotic resistance genes, which can be used as marker genes, have been found in amino acid-producing corynebacteria: resistance to streptomycin (Katsumata *et al.*, 1984), chloramphenicol (Zhen *et al.*, 1987) and tetracycline (Takeda *et al.*, 1990). However, antibiotic resistance markers from other organisms were expressed well in *C. glutamicum* and related species. These include resistance to kanamycin from *B. subtilis* (Yoshihama *et al.*, 1985), resistance to erythromycin from *C. diphtheriae* (Serwold-Davis, 1990), resistance to erythromycin, chloramphenicol, kanamycin and tetracycline from *Corynebacterium xerosis* (Kono *et al.*, 1983; Tauch *et al.*, 1995). Using these genes, a variety of cloning vectors were constructed, some of which have specific applications such as promoter probing (Bardonnet and Blanco, 1991; Eikmanns *et al.*, 1991) and integration into the chromosome (Schwazer and Pühler, 1991).

Natural phages in corynebacteria have been reported; a number of them are active on *C. glutamicum* (Miwa *et al.*, 1985; Patek *et al.*, 1985; Trautwetter *et al.*, 1987b). Some natural- and chimeric plasmids are summarised in Table 1.4.

1.5.2 DNA transfer methods

Several methods for transforming corynebacteria have been explored including the use of protoplasts transformation, transduction, conjugation, and electroporation. (see reviews by Jetten and Sinskey [1995] and Martin *et al.*, [1987].

1.5.2.1 Protoplast transformation

Successful introduction of DNA using protoplast transformation depends on the ability to generate protoplasts, introduce DNA into protoplasts, then regenerate these. Lysozyme treatment of cells in hypertonic buffers had been used in a number of Gram-positive genera such as *B. subtilis* and *Streptomyces* species as a preliminary to introduction of DNA. The methods used in *Bacillus* 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). For the *Streptomyces* species, a similar procedure was applied (Romano and Nickerson, 1956) and later glycine was often added to the growth medium to make cells more sensitive to lysozyme action (Okanishi *et al.*, 1974). Okanishi *et al.* (1974) reported that a higher reversion of protoplasts to the normal state was obtained by incubating the protoplasts on hypertonic medium containing $MgCl_2$ (20 or 50 mM), $CaCl_2$ (50 or 20 mM), phosphate (0.22 or 0.44 mM) and casamino acid (0.01%). Subsequently, the polyethylene glycol 6000 (PEG 6000) method was developed for improving uptake of DNA based on its high cell fusion-inducing ability and broad applicability with various organisms (Ahkong *et al.*, 1975) including *B. subtilis* (Gabor and Hotchkiss, 1979; Schaeffer *et al.*, 1976).

In order to develop an efficient transformation system using *C. glutamicum* protoplasts, several workers have investigated the effect of various parameters on transformation efficiency. Factors analysed have included: growth conditions, washing and transformation solutions, lysozyme concentration and incubation time, concentration of PEG, expression or resuscitation medium, DNA size and concentration.

Procedures, similar to those above involving PEG-induced DNA uptake by protoplasts and

subsequent regeneration of the bacterial cell wall, were also applied to corynebacteria (Katsumata *et al.*, 1984; Kaneko and Sakaguchi, 1979; Ozaki *et al.*, 1984; Yoshihama *et al.*, 1985). 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*. Katsumata *et al.* (1984) found that penicillin G treatment of cells affected the ability of the resultant protoplasts to take up DNA and to regenerate and suggested that the efficiency of transformation was dependant on the composition of regeneration medium and source of DNA used. They also reported that when lysozyme treatment was prolonged to 16 h at 30°C, this long period of treatment led to increased protoplast formation but caused decreases in cell viability so that low frequencies for transformation were obtained. These observations indicated that the methods reported had not yet been optimised and they reflected the resistance of the coryneform cell surface. The formation of protoplasts was monitored under a phase-contrast microscope by counting spherical cells (Kaneko and Sakaguchi, 1979; Katsumata *et al.*, 1984). Generally, the regeneration and osmotic sensitivity of the lysozyme-treated preparations was examined by serial dilution in distilled water and in hypertonic buffer. Subsequently, samples were plated to regeneration agar plates, incubated and then colonies were counted. Under the conditions of the protocol described above, the difference in the numbers of colonies after dilution in hypertonic buffer and that of colonies after dilution in distilled water was ascribed to the presence of osmotically-sensitive cells.

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

Table 1.5 Transformation efficiency of corynebacteria protoplasts

Strains	Plasmids	Plasmid (ng)	TE [a]	Supplements in medium	References
Non-pathogenic corynebacteria					
B. flavum					
MJ-233	pCRY3	1000	$5X10^4$ [b]	penicillin G (0.2 u/ml)	Satoh *et al.*, 1990
B. lactofermentum					
	pUL61	1000	10^2 [b]	penicillin G (0.3 u/ml)	Santamaria *et al.*, 1984
BLR31	pUL330	10	10^5	penicillin G (0.3 u/ml)	Santamaria *et al.*, 1985
GX2548	pGX1415	1000	10^5	— [c]	Smith *et al.*, 1986
C. glutamicum					
T106	pCG4	100	$6X10^5$	penicillin G (0.5 u/ml)	Katsumata *et al.*, 1984
AS019	pHY416		10^4 [b]	2% glycine	Yoshihama *et al.*, 1985
AS019	pCSL17	1260	$2X10^2$	2% glycine + 5 mg/ml INH	Britz, 1985
AS019	pCSL17	1260	$1X10^3$	2.5% glycine + 5 mg/ml INH	Britz, 1985
CG2	pCSL17	1260	$6X10^3$	2% glycine + 5 mg/ml INH	Britz, 1985
CG2	pCSL17	1260	$2X10^2$	2.5% glycine + 5 mg/ml INH	Britz, 1985
ATCC 13032	pNG2		$3X10^3$ [b]	2% glycine	Serwold-Davis *et al.*, 1987
ATCC 13032	pUL330	1000	$7X10^5$	0.5% glycine	Thierbach *et al.*, 1988
ATCC 13059	pC7		10^2	2% glycine	Kim *et al.*, 1988
Pathogenic corynebacteria					
C. diphtheriae					
1030	pNG2		$2X10^3$ [b]	2% glycine	Serwold-Davis *et al.*, 1987
C. ulcerans					
712	pNG2		$3X10^3$ [b]	2% glycine	Serwold-Davis *et al.*, 1987

a TE, transformation efficiency, transformants per μg DNA per 10^9 cells treated.

b Transformation efficiency was reported as transformants per μg DNA.

c No supplement was used in the growth medium.

A blank indicates that no information was available.

the presence of penicillin G (0.3 units) and subsequent lysozyme treatment for 4 h (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, the concentration and type of PEG. Under optimal conditions, they obtained 10^5 transformants per μg of DNA used per 10^9 cells (Table 1.5).

Best and Britz (1984, 1986) and Yoshihama *et al.* (1985) used a different strategy to render *C. glutamicum* cells more sensitive to lysozyme by prior growth in media containing glycine. Yoshihama *et al.* (1985) obtained osmotic sensitive cells of *C. glutamicum* when cells were grown in the presence of 2% glycine, harvested during stationary growth phase and treated 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. By adding the glycine in the growth medium, cells were turned into osmotically sensitive cells by lysozyme treatment. However, the researchers reported that there were no noticeable alterations in cell morphology seen using light microscopic observation, suggesting that cells formed spheroplasts rather than true protoplasts, which would have appeared as rounded cells. Subsequently, in order to obtain real protoplasts by further removal of cell wall structures, they grew cells in the presence of cell wall biosynthetic inhibitors (cerulenin and INH) at growth-inhibitory concentrations or treating the cells with lipase in conjunction with lysozyme treatment, but they could not obtained any increase in transformation efficiency.

Best and Britz (1986) reported using glycine and INH to produce protoplasts following lysozyme treatment, where INH was used at relatively high concentrations (5 mg/ml) which caused substantial growth inhibition. Differential counting was used to differentiate between spheroplasts and true protoplasts, which were seen under phase contrast microscopy after growth in glycine plus INH and prolonged lysozyme treatment (M.L. Britz, unpublished observation). The

regeneration media, ET, contained sodium succinate, gelatin, bovine serum albumin, $CaCl_2$, $MgCl_2$, sucrose and a low concentration of phosphate (Best and Britz, 1986). In the same paper, these authors reported an increase of more than 50-70% in the number of cells recovered using ET media when 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). Total cell counts were performed by diluting the protoplast mixture in osmotically protective medium then plating on ET (protoplasts, 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 spheroplasts, protoplasts, and damaged cells, allowing enumeration of protoplasts from the differences in counts seen between this and the above. Britz (1985, unpublished observation) noted that protoplasts of *C. glutamicum* AS019 prepared following growth in glycine-INH were transformed more efficiently if cells were harvested earlier in the growth phase and the efficiency fell by 80% if cells came from stationary phase cultures. Mutants which protoplasted more readily after growth in 2-3% glycine and 4 mg/ml INH (Best and Britz, 1986) were also transformed more efficiently (M. L. Britz, unpublished observation).

To increase efficiency of cell regeneration after transformation, several osmotic stabiliser have been used such as succinate, glycerol, sucrose, mannitol, sorbitol, lactose, as part of the buffer composition or part of the media composition (Best and Britz, 1986; Dunn *et al.*, 1987; Jandova and Tichy, 1987; Katsumata *et al.*, 1984; Kaneko and Sakaguchi, 1979).

Thierbach *et al.* (1988) developed a protoplast transformation procedure for *C. glutamicum* ATCC 13032 and obtained 10^5 to 10^6 transformants per 10^9 cells per μg of plasmid used. They obtained protoplasts by treating cells with the lytic enzyme, achromopeptidase, and lysozyme (10 mg/ml in

the sucrose-$MgSO_4$) after growing cells in medium containing 0.5% glycine. They also prepared spheroplasts by incubating cells with lysozyme alone after growing cells in the presence of 0.5% glycine. Using spheroplasts, they obtained 10^3 to 10^4 transformants per 10^9 cells treated per μg of DNA used.

Transformation efficiency has been reported by authors in different ways including: the number of transformants obtained per μg of DNA, or the number of transformants obtained per μg of DNA per number of cells protoplasted or cells treated or surviving. High transformation efficiency has been reported when large numbers of protoplasts were used for transformation, before saturation of cells occurred. To enable comparison between reports, transformation efficiency will be described here as the number of transformants obtained per μg of DNA per number of cells used initially, unless this information was not available in the publications.

Using the protoplast technique reported transformation frequencies have varied between 10^2 and 10^6 transformants per 10^9 cells transformed per μg DNA in corynebacteria (Table 1.5) (Katsumata *et al.*, 1984; Kim *et al.*, 1988; Santamaria *et al.*, 1985; Smith *et al.*, 1986; Thierbach *et al.*, 1988; Yoshihama *et al.*, 1985). Differences in transformation efficiency reflect the differences in strains used, their easy of protoplasting and possibly their cell surface structures.

Another important factor, which greatly affects the efficiency of transformation, is the source of plasmid DNA used (Serwold-Davis *et al.*, 1987; Thierbach *et al.*, 1988; Yoshihama *et al.*, 1985) and is described in section 1.6.4.

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

auxotroph. By selecting for resistance to two different amino acid analogs, one of which was carried by each of the parent strains, fusants were found which displayed lysine auxotrophy and produced threonine. Due to its lysine requirement, the fusant strain produced significantly more threonine than its parent. Protoplast fusion can also be induced by electric shock (Rols *et al.*, 1987), with maximum frequencies of 10^{-3} to 10^{-4} fusants per viable cell.

1.5.2.2 Transduction

Since Zinder and Lederberg (1952) described transduction of *S. typhimurium*, transduction has been observed for both Gram-positive and Gram-negative bacteria and their phages. Generalised transduction has been the subject of several reviews (Low and porter, 1978; Susskind and Botstein, 1978). A few reports have appeared that described phage-based transduction of corynebacteria (Momose *et al.*, 1976; Ozaki *et al.*, 1984; Patek *et al.*, 1988). Momose *et al.* (1976) found 24 temperate phages harbored by coryneform bacteria. One of these phages, phage 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 exists of the establishment of a functional vector system based on phages (Miwa *et al.*, 1985). These authors 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*, no further application using this system for gene transfer has been reported.

Protoplasts were also used for transduction of *Corynebacterium* (Ozaki *et al.*, 1984; Smith *et al.*, 1986) and *B. lactofermentum* (Karasawa *et al.*, 1986; Smith *et al.*, 1986) strains. Ozaki *et al.* (1984) found that protoplasts of *C. glutamicum* could be used for gene transformation. They

reported that an *E. coli-C. glutamicum* shuttle vector was successfully introduced into *E. coli* and protoplasts of *C. glutamicum*. The shuttle vector contained resistance genes to kanamycin and tetracycline. They expressed all the *E. coli* resistance phenotypes except ampicillin resistance in *C. glutamicum*. Similarly, Smith *et al.* (1986) reported that protoplasts of the lysozyme-sensitive *C. lilium* strain were efficiently transfected with lytic phage CS1 DNA at frequencies between 10^5 and 10^6 transfectants per μg of CS1 DNA. However, they could not obtain transfectants when lysozyme treatment was omitted.

Extended protoplast-based transfection was also used for *B. flavum* by Patek *et al.* (1988). Patek *et al.* (1988) prepared osmotically sensitive cells when cells were 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 recipients for the transfer of phage or plasmid DNA in competent cultures of corynebacteria are cells with a partially degraded cell wall.

1.5.2.3 Conjugation

Conjugation is the highly specific process whereby DNA is transferred from donor to recipient bacteria by a mechanism involving cell to cell contact. This process is usually encoded by conjugative plasmids, which have been isolated from a diverse range of Gram-positive and Gram-negative bacteria. Sometimes, conjugation functions can also be located on conjugative transposons. Plasmids of some incompatibility groups are capable of conjugal transfer or immobilisation and stable maintenance in almost all Gram-negative and even Gram-positive bacteria. They are called broad host range plasmids and seem to belong mainly to the incompatability 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 Gram-negative bacterial species is well known (Datta and Hedges, 1972; Olsen and Shipley, 1973). Non-self-transmissible plasmids carrying the appropriate origin of transfer (*ori*T) can be mobilised by IncP plasmids (Willetts and Crowther, 1981). Trieu-Cuot *et al.* (1987) described a vector strategy that allowed transfer of plasmid DNA by conjugation from *E. coli* to various Gram-positive bacteria in which conjugation occurred *via* natural competence. They constructed a prototype vector, pAT187, which contained the origins of replication of pBR322 and the broad host range streptococcal plasmid pAM(β)1, a kanamycin-resistance gene known to be expressed in both Gram-negative and Gram-positive bacteria, and the origin of transfer of the IncP plasmid RK2. Using this shuttle plasmid and the self-transferable IncP plasmid, they transferred pAT187 from *E. coli* into several Gram-positive bacteria (*Enterococcus faecalis*, *B. thuringiensis*, *Listeria monocytogenes* and *S. aureus*), at frequencies of 10^{-7} to 10^{-8} transconjugants per donor cell.

Schäfer *et al.* (1990, 1994a) showed that conjugation occurs in corynebacteria. Mobilisable shuttle plasmids were transferred from *E. coli* into several corynebacteria by conjugation, where the system depended on the mobilisation functions of the Gram-negative broad-host-range plasmid RP4 to transfer plasmids containing the RP4 origin of transfer, (*ori*)T, to different corynebacteria by conjugation Schäfer *et al.* (1990). Conjugation between *E. coli* and corynebacteria is performed using nitrocellulose filters to which the bacteria can attach. This system was applicable to a wide range of different *C. glutamicum* strains (Schäfer *et al.*, 1990). When wild-type corynebacteria strains were conjugated, a relatively low transfer frequency of 4 X 10^{-7} transconjugants per donor cell was obtained. In contrast, highly efficient plasmid transfer (displaying a frequency of $>10^{-2}$) was obtained when restriction-deficient mutants were used (Schäfer *et al.*, 1994a).

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 efficiency was increased by 10^4-fold after heat treatment of host cells. Based on this observation, Schäfer *et al* (1994a) suggested that heat treatment impairs the restriction of incoming foreign DNA by recipients and that the presence of a restriction barrier in the recipient cells is the main barrier preventing efficient conjugal transfer between *E. coli* and corynebacteria strains. The fertility of *C. glutamicum* recipients in intergeneric matings was also improved by exposing cells to a number of stresses, including organic solvents, detergents and pH shifts (Schäfer *et al.*, 1994a).

Vertès *et al.* (1994a) isolated the insertion sequence IS *31831* from *C. glutamicum* ATCC 31831 and the same authors (Vertès *et al.*, 1994b) used this to construct artificial transposons 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.

1.5.2.4 Electroporation

Electroporation involves the use of a high voltage, 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).

The first application of electroporation to bacteria was described by Shivarova *et al.* (1983), who transformed *B. cereus* protoplasts. This did not offer any advantage over other methods since protoplasts had to be prepared and regenerated. The first data on the successful electroporation of

whole cells were presented by Harlander, who transformed *L. lactis* (1986). Today, electroporation is used routinely to increase transformation efficiency in a range of Gram-negative (Dower *et al.*, 1988) and Gram-positive bacteria (Berthier *et al.*, 1996; Chassy and Flickinger, 1987; Foley-Thomas *et al.*, 1995; Powell *et al.*, 1988) including corynebacteria (Bonnassie *et al.*, 1990; Haynes and Britz, 1989, 1990; Liebl *et al.*, 1989; Noh *et al.*, 1990; Schäfer *et al.*, 1994b; Wolf *et al.*, 1989).

Reported transformation efficiencies for corynebacteria using electroporation have varied between 10^4 to 10^7 transformants per μg of DNA (Dunican and Shivnan, 1989; Haynes and Britz, 1989, 1990; Liebl *et al.*, 1989; Noh *et al.*, 1990, 1991; Satoh *et al.*, 1990; Wolf *et al.*, 1989). The following factors are consider to be important: strains used, cell growth stage, cell density, the presence of cell wall inhibitors in the growth medium prior to electroporation, the source and concentration of DNA, electric field strength, types of cuvettes used (hence electrical capacity) and the recovery medium (see, Haynes and Britz, 1990; Noh *et al.*, 1991; Wolf *et al.*, 1989).

Normally, cells used for electroporation are grown in rich medium for 3 to 5 h, harvested during exponential growth stage, washed extensively with low-salt buffers containing 5 to 20% glycerol, and concentrated to approximately 10^9-10^{10} cells per ml before use (Haynes and Britz, 1990; Tauch *et al.*, 1994). The stage of growth required for efficient electroporation transformation of *C. glutamicum* appeared to be critical in some strains. Haynes and Britz (1990) found that early exponential phase cells were more easily transformed with pCSL17 DNA and that as the absorbance increased, there was a significant reduction in the number of transformants obtained. In their experiments, the best results were obtained when cells were harvested at A_{600} 0.15-0.25. In contrast, Liebl *et al.* (1989) found that cells harvested at A_{578} 1.1 showed higher electroporation efficiency for the *C. glutamicum* strain used. However, they did not report transformation efficiency for cells harvested below A_{578} 0.6.

Transformation efficiencies (10^4 transformants per μg of DNA per 10^9 cells electroporated) were fairly constant over three orders of magnitude of DNA concentrations used but the number of transformants obtained increased in a log-linear relationship according to the DNA concentration over the range from one ng to one μg of pCSL17 DNA (7.2 kb) (Haynes and Britz, 1990). The recovery of transformants also increased in a log-linear relationship with DNA concentrations for two other plasmids (pHY416 [9.4 kb] and pUL340 [5.8 kb]) tested. Noh *et al.* (1991) also obtained similar results with *C. glutamicum* strain JS231, reporting transformation efficiencies of 1 X 10^8 per μg of DNA over a concentration range of 0.1 ng to 100 ng of DNA. With *E. coli* (Dower *et al.*, 1988), the relationship between DNA concentration and the number of transformants was also linear over several orders of magnitude (from 0.4 pg to 300 ng) and a constant efficiency (2.9 $\pm$ 1.2 X 10^9 transformants per μg of DNA) of transformation was obtained.

Field strength and pulse length greatly affect cell viability and transformation in a variety of species (Dower *et al.*, 1988; Dunican and Shivnan, 1989). In the majority of the reports describing electroporation of *C. glutamicum*, the highest efficiency was achieved using an electric field strength of 12.5 kV/cm, the maximum possible value obtainable with the BioRad Gene Pulser, even though culture conditions of host strains and the procedures used for electroporation were different (Dunican and Shivnan, 1989; Haynes and Britz, 1990; Schäfer *et al.*, 1994b). In contrast, Bannassie *et al.* (1990) obtained a maximum transformation rate for *B. lactofermentum* at 5.7 kV/cm.

To increase the efficiency of electroporation, several compounds such as glycine (Haynes and Britz, 1990; Noh *et al.*, 1990; Schäfer *et al.*, 1994b), INH (Haynes and Britz, 1989, 1990; Schäfer *et al.*, 1994b), Tween 80 (Haynes and Britz, 1989; Schäfer *et al.*, 1994b), ampicillin (Bonnassie *et al.*, 1990) and penicillin G (Noh, *et al.*, 1991), have been added to the growth

medium to make the cells more permeable to DNA or minimise the normal cell surface barriers which prevent DNA uptake. Dunican and Shivnan (1989) achieved fairly good transformation efficiencies of 7 X 10^5 transformants per μg of DNA with *C. glutamicum* strain AS019 and plasmid pHY416 without using any cell wall-weakening agents. Experimentally, 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 of cells were thawed, mixed with 1 ng of plasmid DNA and then electrotransformed (electrical pulses of 12.5 kV/cm). 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). Some specific examples follow.

Haynes and Britz (1989) used electroporation on *C. glutamicum* AS019 grown in LBG supplemented with 2.5%, w/v, glycine plus 4 mg/ml INH plus 0.1% Tween 80 and obtained 4 X 10^7 transformants per μg DNA (using small BioRad cuvettes) per 10^9 cells electroporated when transformants were recovered on ET media. This was about four orders of magnitude higher than efficiencies obtained for cells grown in LBG. When optimising electrotransformation efficiencies, Haynes and Britz (1990) also reported a transformation frequency of 5 X 10^5 transformants per μg DNA using *C. glutamicum* strain AS019 grown in LBG supplemented with 2.5% (w/v) of glycine and 4 mg/ml of INH (using large BioRad cuvettes). This was a 10 to 100-fold improvement on using the protoplast transformation of the same strain of *C. glutamicum* grown similarly. When grown in INH alone, electrotransformation efficiencies were only significantly improved when concentrations were very high (>5 mg/ml). Haynes and Britz (1990) reported that the recovery medium on which the transformants were isolated had a significant effect on the number of

transformants obtained as observed using protoplast transformation (M.L. Britz, unpublished observation, 1985). The osmotically-protective medium (ET) gave at least 70% higher numbers of transformants (in transformation efficiency) than those found on non-osmotically protective medium, suggesting that electroporation damaged cells and made them osmotically fragile.

Bonnassie *et al.* (1990) used electroporation on *B. lactofermentum* grown in LB, incubated 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 cells, whereas no transformants were obtained without pre-treatment. Haynes and Britz (1989) noted that *B. lactofermentum* electrotransformation frequencies were improved by growth in Tween 80 alone or in combination with glycine and INH.

Noh *et al.* (1991) used electroporation to transform *C. glutamicum* JS231, a strain derived from ATCC 13032, following growth in LG medium (1%, w/v, tryptone, 0.5%, w/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 parallelled the earlier work of Kaneko and Sakaguchi (1979) in *B. lactofermentum*.

1.6 ENZYMATIC RESTRICTION AND MODIFICATION SYSTEMS

Literature reviewed in the previous section indicated that one barrier to efficient transformation in corynebacteria is the cell surface structure. A second major obstacle is the enzymatic barriers present which prevent entry or survival of heterologous DNA. This next section reviews the literatures on restriction and modification systems in bacteria, with specific reference to

corynebacteria.

1.6.1 General introduction on restriction and modification systems

Restriction and modification (RM) systems are found in many bacterial species (for example, see Cue *et al.*, 1996; Davis *et al.*, 1993; Grant *et al.*, 1990; Lee *et al.*, 1992; Soper and Reddy, 1994; Wilson, 1988; Wilson *et al.*, 1991). RM systems are mediated by endonucleases (ENases) and methyltransferases (MTases) that generally recognize the same DNA sequences (Burckhardt *et al.*, 1981; Butkus *et al.*, 1985). An ENase recognizes a specific sequence of bases in the DNA as a signal to cleave the DNA. MTase enzyme recognizes and methylates bases at the ENase cleavage site, where methylation normally protects the cleavage site from ENase activity within the cell. Thus ENases do not cleave endogenous DNA, but will cleave, and therefore destroy, any incoming DNA molecule which has a foreign methylation pattern. The enzymes are prevalent and diverse, being found in approximately one quarter of bacterial isolates examined to date (Roberts and Macelis, 1997). Hence, restriction is likely to be a frequently encountered barrier to the flow of DNA between bacterial strains in nature.

Not all DNA ENases in prokaryotes are necessarily involved in RM system; some ENases are considered to be prime participants in the cellular process of DNA replication, genetic recombination, and DNA repair (Glickman and Radman, 1980; Lu *et al.*, 1983; Sternberg, 1985). Evidence, both circumstantial and direct, has accumulated in support of this role. Methylation of eukaryotic DNA has also been proposed to function in many ways, including control of transcription, maintenance of chromosome structure, repair of DNA, establishment of preferred sites for mutation, oncogenic transformation, and, in certain systems, protection of DNA against enzymatic degradation (reviwed by Ehrlich and Wang [1981] and Laird and Jaenisch [1996]. In bacteria, some MTase might be associated with DNA damage or repair. For example, in *E. coli*,

N^6-methyladenosine (m^6dAdo) in GATC sequences (Dam methylation) appears to be involved in strand discrimination during DNA mismatch correction in newly replicated DNA (Glickman and Redman, 1980; Pukkila *et al.*, 1983). Since each of these processes constitutes a study in itself, no attempt will be made here to give a comprehensive review of the current status of research in these fields; rather, the reader is referred to many excellent review articles that have been written on these topics (Bickle and Krüger, 1993; Wilson, 1991).

Some ENases cleave specifically at recognition sequences which contain methylated bases (Brooks and Robert, 1982). In *E. coli*, several systems have been studied. These are McrA, McrBC and Mrr: the McrBC system restricts DNA when cytidine residues in GC sequences are methylated (Noyer-Weidner *et al.*, 1986; Raleigh and Wilson, 1986), whilst the McrA system restricts DNA when cytidine residues in CG sequences are methylated (Raleigh *et al.*, 1988), and the Mrr system restricts DNA containing methylated adenosines (m^6dAdo) in either CAG or GAC sequences (Heitman and Model, 1987; Waite-Rees *et al.*, 1991). McrA and McrBC recognize various modification of cytidine bases, including 5-methylcytidine (m^5dCyd), 5-hydroxymethylcytidine, and, for McrBC, N^4-methylcytidine (m^4Cyd) (Kelleher and Raleigh, 1991). *Dpn*I, isolated from *Diplococcus pneumoniae*, cleaves at GATC sequences when adenosine is methylated (Gottesman *et al.*, 1980; Lacks and Greenberg, 1975, 1977).

MTases catalyse the addition of a methyl group to one nucleotide in each strand of the recognition sequence when S-adenosyl-L-methionine (SAM) is present in prokaryotes to serve as a methyl donor (Wilson and Murray, 1991). Adenosine and cytidine are the only bases known to be methylated (Wilson and Murray, 1991). DNA MTases fall into three classes based on the type of methylation catalysed. Two classes modify exocyclic nitrogens, converting adenosine to m^6dAdo or cytidine to m^4dCyd. The third class methylates the 5-carbon of the pyrimidine ring of cytidine, creating m^5dCyd (Kumar *et al.*, 1994).

RM systems in *E. coli* have been extensively studied and it has been found that there are at least six restriction systems (Bickle and Krüger, 1993; Kelleher and Raleigh, 1991; Raleigh *et al.*, 1988, 1992), including *EcoK* and two modification systems, Dam methyltransferase (MTase) (Hale *et al.*, 1994; Marinus and Morris, 1973) and Dcm MTase (Marinus and Morris, 1973), in addition to McrA, McrBC and Mrr. Among the restriction systems in *E. coli* K-12, only the *EcoK* ENase degrades unmethylated DNA, while the others all restrict specifically methylated DNA. The *Eco*K ENase recognizes the sequence AACNNNNNNGTGC (where N is a purine or pyrimidine base) (Raleigh, 1987) and cleaves DNA unless one of the first adenosines is methylated. In addition, the Dam MTase methylates adenosine bases in the sequence of GATC and the Dcm MTase methylates the second cytidine base in CCAGG or CCTGG sequences.

1.6.2 Occurrence of RM system in the bacterial kingdom

In the early 1950's, several examples of host-controlled modification were found using phage in bacteria, including *E. coli* strains (Bertani and Weigle, 1953; Luria and Human, 1952) and *Salmonella typhi* (Anderson and Fraser, 1956), details of which may be found in a review by Luria (1953). Luria observed that *E. coli* strain B always yielded phage T2 when plated with phage T2 whereas the mutants strains, when plated with moderate amounts of phage T2, gave no plaques. When high concentrations of T2 were plated, a modified phage, T2′ was liberated. This was unable to infect young strain B cells or its mutants but could infect a small fraction of old, starved strain B cell. Based on this observation, Luria (1953) suggested that the supply of some essential component for phage maturation might be involved.

Later on, the mechanism actually used to restrict foreign DNA was proposed by Arber and Dussoix (1962). Using phage lambda they showed that reproduction in a new *E. coli* host is subject to two successive host controls. Either DNA is recognized as incompatible and is

Table 1.6 Some of specific examples of ENases (from Roberts and Macelis, 1997).

ENase	Isoschizomers	Species	Recognition site [1]	Me [2]
Type I enzyme				
*Eco*AI		*Escherichia coli*	GAGNNNNNNGTCA	2(6)
*Eco*DI		*Escherichia coli*	TTANNNNNNGTCY	
*Sty*SJ		*Salmonella typhimurium*	GAGNNNNNNGTRC	
Type II enzyme				
*Bam*HI		*Bacillus amyloliquefaciens*	G*GATCC	5(4)
*Bgl*II		*Bacillus globigii*	A*GATCT	
*Cla*I	*Ban*III, *Bsc*I	*Caryophanon latum*	AT*CGAT	5(6)
*Dpn*I [3]		*Diplococcus pneumoniae*	GmA*TC	
*Dpn*II	*Mbo*I	*Diplococcus pneumoniae*	*GATC	2(6)
*Eco*RI		*Escherichia coli*	G*AATTC	3(6)
*Hae*III	*Pal*I	*Haemophilus aegyptius*	GG*CC	3(5)
I-*Ceu*I		*Chlamydomonas eugametos*	CTATAACGGTCCTAA*GGTAGCGAGG	
I-*Ppo*I		*Physarum polycephalum*	CTATGACTCTCTTAA*GGTAGCCAAA	
Type III enzyme				
*Eco*PI		*Escherichia coli*	AGACC	3(6)
*Hinf*III		*Haemophilus influenzae*	CGAAT	
*Sty*LTI		*Salmonella typhimurium*	CAGAG	4(6)

[1] Recognition sequences are written from 5′ to 3′, and the point of cleavage is indicated by symbol (*). When no sign appears, the cleavage site has not been determined. Abbreviations: R = G or A, Y = C or T, N = A or C or G or T.

[2] The first number shows the base within the recognition sequence that is modified. The number in parentheses indicates the specific methylation involved. (6) = m^6dAdo; (5) = m^5dCyd; (4) = m^4dCyd. A blank indicates that no information was available.

[3] Signifies that *Dpn*I requires the presence of m^6dAdo within the recognition sequence of G^{m}ATC.

degraded, or it is recognized as compatible and is accepted. In the latter case, phage progeny genomes receive host specificity. Arber and Dusssoix (1962) also investigated the fate of phage DNA in restricting hosts. ^{32}P-labelled phage DNA was degraded in *E. coli*. Part of the products were used and part excreted. Using genetic rescue of markers from the restricted phage genome by superinfection of the complex with unrestricted, genetically marked phages, they showed that the injected DNA was available for genetic exchange. The efficiency of rescue was greatest if the restricted phage was added after the superinfecting unrestricted phage; it was lowest for additions in the reverse sequence indicating that degradation and exchange were complete. These observation on the fate of phage DNA suggested that plasmid and bacterial DNA might also be restricted.

A molecular mechanism was proposed by Arber (1965). His concept of two enzymes, a restricting ENase and a modifying MTase, is now accepted as a general phenomenon in bacteria. Although both enzymes (ENase, MTase) recognize the same site, not all ENases cleave at this site. Those that do not are referred to as Type I RM system; the first was isolated from *E. coli* K by Meselson and Yuan (1968). The type II RM system was discovered later, when Smith and Wilcox (1970) isolated the ENase from *H. influenzae* serotype d.

There are at least three major types of RM systems found in bacteria which are distinguished on the basis of where their ENase cleave DNA and the cofactors requirement of both enzymes: Type I, II, and III (see Bickle and Krüger, 1993; Roberts and Macelis, 1997; Wilson and Murray, 1991). According to information from Roberts and Macelis (1997), 2,821 restriction enzymes have been reported, including: 18 fully characterized Type I specificities, 210 different Type II specificities and four different Type III specificities. Some specific examples of these are shown in Table 1.6. Although Type I RM systems are the least useful of the three types, for historical reasons they will be discussed first.

The most complicated of the RM systems is the Type I, which is mediated by three different polypeptides, namely R (restriction), M (modification), and S (specificity), which form a complex that is capable of both cleaving and modifying DNA (Fuller-Pace and Murray, 1986). ATP, Mg^{++} and S-adenosyl-L-methionine (SAM) are required for Type I DNA cleavage (Meselson and Yuan, 1968), which occurs at non-specific sites often kilobases away from the recognition sequence (Horiuchi and Zinder, 1972). In the presence of SAM, modification of one strand is sufficient to protect the DNA from restriction (Meselson and Yuan, 1968). This allows it to replicate semi-conservatively, since one strand of the daughter DNA is always modified and the other is modified shortly after replication (Billen, 1968). Type I RM systems have been well studied using *E. coli* K12 and the three genes involved in this strain are called *hsd*R, *hsd*M and *hsd*S. All three genes are transcribed in the same direction but from two promoters. One of the promoters is located upstream of *hsd*R and the other between *hsd*M and *hsd*R (Sain and Murray, 1980).

Linn and coworkers (Endlich and Linn, 1985; Eskin and Linn, 1972) studied Type I ENase from *E. coli* B. In the presence of SAM and ATP, an *Eco*B enzyme cleaves a sequence of unmodified DNA several thousand nucleotides to one side of the recognition site (Endlich and Linn, 1985). Since *Eco*B catalyses only one strand cleavage, cleavage of the opposite strand is then catalysed by a second enzyme molecule (Eskin and Linn, 1972). Some strains of *E. coli* and *Salmonella* species, including *S. typhimurium* (Fuller-Pace and Murray, 1986), have Type I restriction systems and target sequences of Type I systems have been reviewed by Roberts and Macelis (1997) (see Table 1.6).

Type II systems include those enzymes that have simple cofactor requirements (Mg^{++} for restriction, SAM for modification) and simple sub-unit structures. Type II systems produce two separate enzymes sharing the same DNA sequence specificity: an ENase and a MTase. The

ENase and MTase activities are separate enzymes (Connaughton *et al.*, 1990), encoded by single usually adjacent genes (Howard *et al.*, 1986; Lunnen *et al.*, 1988). Modification by the MTase protects the cell's own DNA from the ENase (Kulakauskas *et al.*, 1995). In contrast to the other groups, Type ll ENases cleave DNA at or near the site to which they bind. This property makes Type ll ENase of enormous value in genetic engineering; these enzymes are the tools we use to cut DNA at specific sites for gene cloning (Anthanasiadis *et al.*, 1990; Bickle and Krüger, 1993; Brooks *et al.*, 1991). The majority of these enzymes recognize four, five or six-bases in sequences. Some Type II ENases are extremely useful for genomic analysis since they recognize numerous cutting sites in DNA (Table 1.6).

More than 2,000 Type ll ENases from 210 different specificites have been identified, and they are produced by every known major group of prokaryotes (Roberts, 1990; Roberts and Macelis, 1997; Wilson, 1991). These ENases are generally dissimilar, suggesting an independent evolution and the absence of a common ancestor (Bickle and Krüger, 1993; Wilson and Murray, 1991). In contrast, extensive similarities occur amongst the MTases (Bickle and Krüger, 1993; Klimasauskas *et al.*, 1989; Wilson and Murray, 1991). MTases can be grouped into three classes corresponding to the modification types: m^4dCyd, m^5dCyd, and m^6dAdo (Kumar *et al.*, 1994; Wilson, 1991; Wilson and Murray, 1991).

Type III systems, which are the least common, contain two non-identical sub-units, one responsible for site recognition and modification (the Mod subunit), and the other (Res) for DNase action. The Res subunit can act only in association with the Mod unit, and the complex can both modify DNA and cleave it, so that cleavage and modification can be completed *in vitro*. DNA cleavage requires both ATP and Mg^{++}, is stimulated by SAM, and occurs at a fixed position near the unmodified recognition site (Roberts 1990). Modification requires only SAM, the methyl donor. However, the factor modulating the choice of action of the complex (cleavage *versus*

modification) are not understood. A few bacterial species have been known to contain the Type III system; *E. coli*, *S. typhimurium*, and *H. influenzae*.

1.6.3 RM barriers to gene transfer in bacteria

RM systems have been shown to affect DNA transfer in many bacteria (Cue *et al.*, 1996; Miller *et al.*, 1988; Moser *et al.*, 1993; Soper and Reddy, 1994). For instance, Miller *et al.* (1988) attributed their successful use of electroporation with *Campylobacter jejuni* to the ability to isolate plasmid DNA directly from this strain. The DNA was therefore suitably protected from the recipient's restriction enzymes and the transformation efficiency was 10^4-fold higher than the transformation efficiency in similar experiments using the same plasmid DNA molecules isolated from *E. coli* HB101.

Moser *et al.* (1993), who were working with *Cyanobacterium* species, investigated host restriction as a barrier to transformation and characterised an ENase so that they were able to identify MTase activity for protecting of DNA against digestion by the endogenous ENase. Heterologously-isolated DNA was modified by MTase from the host cell, used in an electroporation procedure to transform into cyanobacterial cells and this showed transformation frequencies 10^2- to 10^3- fold higher than the corresponding unmodified plasmid. A similar strategy has also been employed recently to protect plasmid DNA from restriction by the methylotropic bacteria, *B. methanolicus* (Cue *et al.*, 1996) and nitrogen-fixing cyanobacterial species in the genus *Cyanothece* (Morrison *et al.*, 1992).

In order to avoid ENase digestion of incoming heterologous DNA, mutation has been used as an alternative approach to characterising the cognate MTase. Mutations in the restriction-modification loci can result in either the loss of both restriction and modification functions or the

loss of restriction functions only. Zhang and Skurnik (1994) isolated a restriction-deficient strain of *Yersinia enterocolitica* serotype 0:8 by insertional mutagenesis. The resulting mutant strain, 8081-res, had lost the ability to produce the *Yen*I ENase, and the mobilisation frequency by conjugation into 8081-res was increased about 10^5 times compared to the wild-type strain.

It has been reported that the partial inhibition of *in vivo* restriction activity can be achieved using heat treatment of the host cell to temporarily inactive RM systems (Bailey and Winstanley, 1986; Engel, 1987). In *Streptomyces* species, it was found that the restriction system in *S. clavuligerus* (at 45°C for 10 min, Bailey and Winstanley, 1986) and *S. tendae* (at 50°C for 30 min, Engel, 1987) could be partially inhibited by treating protoplasts at high temperature; heat treatment of protoplasts increased the efficiency of transduction by 100 fold.

1.6.4 Enzymatic barriers to gene transfer in corynebacteria

The presence of RM system in the saprophytic corynebacteria was found to be a major barrier to the introduction of heterologous DNA using transformation (Haynes and Britz, 1990; Katsumata *et al.*, 1984; Serwold-Davis *et al.*, 1987; Thierbach *et al.*, 1988). These workers found that transformation frequency is enormously affected by the source of DNA used: heterologous DNA transforms with much less efficiency than DNA isolated from the host strain.

Katsumata *et al.* (1984) reported that restriction barriers existed between *C. glutamicum* T106 and *C. glutamicum* T250. The transformation frequencies differed widely according to the source of plasmid DNA. The frequency of *C. glutamicum* T106 with pCG4, isolated from the same host was over 100 times higher than seen with pCG4 from *C. glutamicum* T250. Other workers have also found that restriction barriers exist between *E. coli*, *B. lactofermentum* and *C. glutamicum*. Yeh *et al.* (1986) found that *B. lactofermentum* could be transformed at a frequency of 1.5 X 10^5

Table 1.7 Transformation efficiencies of *E. coli* recipient strains with different restriction backgrounds using *C. glutamicum*- and *E. coli*-derived pCSL17 DNA (from Jang *et al.*, 1996)

	Transformation efficiencies for DNA from: [1]		Restriction system [2]					Reference
			*Eco*K		*Mcr*A	*Mcr*BC	*Mrr*	
E. coli recipient strain	*E. coli* N4830[3]	*C. glutamicum* AS019 [3]	R	M				
MC1061	1.2 X 10^5	2.4X10^5	-	+	-	-	+	Sambrook *et al.*, 1989
RR1	6.7 X 10^4	4.7X10^4	-	-	+	-	-	Sambrook *et al.*, 1989
HB101	1.9 X 10^4	1.9X10^4	-	-	+	-	-	Sambrook *et al.*, 1989
LE392	1.8 X 10^5	2.0X10^3	-	+	-	+	+	Sambrook *et al.*, 1989
ED8654	2.2 X 10^6	380	-	+	-	+		Sambrook *et al.*, 1989
CSR603	2.1 X 10^3	0	+	+		+		Sancar and Rupert, 1978
JM101	7.5 X 10^4	0	+	+	+	+	+	Sambrook *et al.*, 1989

1 Transformation efficiency was calculated as the number of transformants per μg DNA used.

2 Information on the restriction background of the strains used is from Raleigh *et al.* (1988) and Waite-Rees *et al.* (1991). '+' indicates the presence of activity; '-' indicates lack of activity. A blank indicates that no information was available.

3 0.5 μg of pCSL17 DNA derived from either *E. coli* N4830 (Gottesman *et al.* 1980) or *C. glutamicum* AS019 was used for each transformation.

transformants per μg with homologously isolated DNA whereas DNA isolated from *C. glutamicum* only gave 2.8 X 10^1 transformants per μg DNA. Serwold-Davis *et al.* (1987) found that *C. glutamicum* was transformed with homologous DNA at frequency of 10^3 transformants per μg but with heterologous DNA from *C. diphtheriae* and *E. coli* only 10^1 transformants per μg and 10^{-1} transformants per μg DNA were obtained, respectively. Similar observations were also made by other workers (Diong, 1989; Thierbach *et al.*, 1988), affirming the suggestion that *C. glutamicum* strains have enzymatic RM systems.

Tauch *et al.* (1994) provided the first published evidence on the nature of the modification systems in *C. glutamicum*. Experimentally, they isolated the same plasmid DNA from *C. glutamicum* and from *E. coli* DH5α, and transformed these into various *E. coli* strains with different RM backgrounds, including DH5α and DH5αMCR. They obtained a significant difference in the transformation frequencies between homologous and heterologous plasmid DNA for the Mcr-proficient *E. coli* DH5α, where *C. glutamicum* plasmid DNA transformed up to 500-fold less efficiently. However, when *E. coli* DH5αMCR was used as a recipient for electroporation, similar transformation efficiency was seen for homologous- and heterologous-DNA. This experiment was supported by using several other *E. coli* strains which had various restriction backgrounds, which suggested that plasmid DNA isolated from *C. glutamicum* contained methylated cytidine bases in GC sequences.

Simiarly, Britz also performed transformation experiments in 1985 using homologous DNA (pCSL17 DNA from *E. coli*) and heterologous DNA (pCSL17 from *C. glutamicum*). When DNAs were transformed into *E. coli* strains which has various restriction and modification backgrounds (McrA, McrBC and Mrr), Britz obtained similar results to that published by Tauch *et al.* (1994); plasmid pCSL17 isolated from *C. glutamicum* AS019 transformed recipient McrBC$^+$ strains of *E. coli* with lower efficiency than McrBC$^-$ strains. This result was published as part of

paper in 1996 (Jang *et al.*, 1996) and a table from this publication appears as Table 1.7. However, the above approach could not indicate which sequences in *C. glutamicum* were methylated.

In 1989, Diong (an Honours student from Monash University, supervised by Britz), examined restriction and modification activity in corynebacteria, including *C. glutamicum*, *B. lactofermentum*, *B. flavum* and *C. ulcerans*; all of the tested strains contained DNase activity (ENase or nickase, and exonuclease). *C. ulcerans* showed the most abundant DNase activity. When cell-free extracts of *C. glutamicum* were prepared by sonication and incubated in a "core" restriction enzyme buffer (BRL, Bethesda Research Laboratories, Gaithersburg, M.D., U.S.A.) with heterologously-prepared pCSL17 DNA (isolated from *E. coli* strains) for different incubation time, covalently closed circular (CCC) DNA was firstly converted to open circular form which was then converted into linear form before further degradation to small fragments by exonuclease; negative controls (without cell-free extract) did not produce open circular form from CCC pCSL17 DNA. These results suggested that ENase may be present in *C. glutamicum*, since exonuclease activity would not normally be able to convert CCC DNA to open circular or linear form. However, these workers failed to show specific ENase activity. Also, Diong observed S-adenosyl-L-methionine (SAM) MTase activity in cell-free extracts from *C. glutamicum* but these results were only preliminary and needed validation.

As mentioned earlier, when examining conjugation efficiency for plasmid transfer between *E. coli* and *C. glutamicum* species, Schäfer *et al.* (1994a) found that the fertility of *C. glutamicum* recipients in intergeneric matings could be improved by exposing cells to a number of stresses, including organic solvents, detergents and pH shifts. Based on this observation, these workers suggested the presence of a cell surface located, stress-sensitive restriction complex (Schäfer *et al.*, 1994a).

These researchers also reported one of two genes cloned from *C. glutamicum* had homology to known ATPases, but no homology to the known Type II restriction enzyme genes (Schäfer *et al.*, 1994b). Experimentally, they constructed a genomic library of the *C. glutamicum* wild type strain, ATCC 13032, which showed low transformation efficiency with heterologously-isolated DNA. They then transformed plasmids containing fragments of this chromosomal DNA into the restriction-deficient *C. glutamicum* strain RM3. Subsequently, when heterologously isolated DNA from *E. coli* was electrotransformed into RM3 transformants, a few of the recombinants (RM3*) showed transformation efficiencies as low as that seen for ATCC 13032, whilst the majority of recombinants showed high transformation efficiencies for both homologously- and heterologously-isolated DNA. This indicated that the clones contained genes which complemented the mutation in RM3 which was responsible for its RM^- phenotype and that RM3* contained DNA responsible for ENase. The cloned DNA was further characterised. Sequence analysis revealed the presence of two open reading frames (*orf1* and *orf2*) on the complementing DNA fragment. Gene disruption experiments with the wild type showed that *orf1* was essential for complementation. They made primers on the basis of the sequence of *orf1* and *orf2* in *C. glutamicum* and hybridised this to the chromosomal DNA from other saprophytic corynebacteria to see if similar sequences were present. However, hybridisation experiments failed to show similarity between genes from *C. glutamicum* and other glutamic acid-producing bacteria, including *B. lactofermentum* and *B. flavum*. The protein(s) corresponding to the two open reading frames have not been isolated, so that no information on the specificity (size, target site of DNA sequence) of ENase and MTase from *C. glutamicum* is available to date.

1.6.5 Type II RM system in corynebacteria

The ENase and MTase from some *Corynebacterium* species and *Brevibacterium* species have been investigated previously and some of this data is summarised in Table 1.8. As far as I know,

Table 1.8 Endonucleases and methyltransferases from corynebacteria

Name	Bacterial strain	Target sequence [1]	Isoschizomer	Temperature [2] (°C)	Cation or coenzyme required [2]	References
*Bdi*I	*B. divaricatum* FERM 5948	5´-AT*CGAT-3´	*Cla*I	37	100 mM NaCl	Kim and Rho, 1986
M.*Bdi*I [3]	*B. divaricatum* FERM5948	5´-ATCG*AT-3´		37		Jun and Rho, 1987
*Bep*I	*B. epidermis*	5´-CG*CG-3´	*Fnu*II	30	10 mM Mg^{++}	Venetianer and Orosz, 1988a, 1988b
M.*Bep*I [3]	*B. epidermis*	5´-CGCG-3´				Kupper *et al.*, 1988b
*Ceq*I	*C. equii*	5´-GAT*ATC-3´	*Eco*RV		200 mM NaCl	Duda *et al.*, 1987

[1] Recognition sequences are written from 5′ to 3′, and the point of cleavage (or methylation) is indicated by symbol (*). When no sign appears, the cleavage site has not been determined.

[2] A blank indicates that no information was available.

[3] MTase.

biochemical information on restriction or modification enzymes from *C. glutamicum* and related bacteria, such as *B. lactofermentum* and *B. flavum*, have not been published at the time of writing this thesis.

Kupper *et al.* (1988a, 1988b) purified cognate ENase and MTase from *B. epidermidis* and showed that the MTase had sequence similarities with other cytosine MTase. Reported ENases (*Bdi*I, *Beq*I, and *Ceq*I) are Type II ENases and the biochemical properties, such as target sequence, optimum temperature, and salt requirements for activity, are dissimilar, suggesting that each species has a different RM system.

*Ceq*I ENase was purified from *C. equii* and was shown to exist as an aggregation of 12-20 subunits. This molecule partially dissociated in the presence of DNA sequences recognized by *Ceq*I or in the presence of non-ionic detergents. Analysis of the enzyme kinetics revealed a positive co-operative interaction between the subunits of the enzyme (Jobbagy *et al.*, 1992).

Although these strains described above are classified as corynebacteria, expect for *B. divaricatum*, these are distantly related to *C. glutamicum*, in terms of mycolic acid composition and G+C content (Collins *et al.*, 1982a; Collins *et al.*, 1982b; Liebl *et al.*, 1991).

1.7 DETECTION OF DNA METHYLATION

DNA methylation is common in bacteria. The presence of methylated DNA bases is evidence of the presence of MTase and often provides information on the nature of RM systems in bacteria. Several methods have been used for the detection of methylated DNA bases and three methylated DNA bases are known to be products of RM systems in bacteria (Lange *et al.*, 1991; Wilson, 1991). It has long been known that m^6dAdo, m^5dCyd occurs as minor bases in bacterial DNA

(Cue *et al.*, 1996; Kuo *et al.*, 1980; Vanyushin *et al.*, 1968; Zhang *et al.*, 1993) and m^4dCyd has also been found in DNA of thermophilic bacteria (Ehrlich *et al.*, 1985) and mesophilic bacteria (Janulaitis, *et al.*, 1983). In human DNA, m^5dCyd is found as the major modified base (Frommer *et al.*, 1992; Rideout *et al.*, 1991).

1.7.1 Level of methylation in DNA

The DNA of many vertebrates studied has been found to contain 0.7-2.8 mol % m^5dCyd (Ehrlich and Wang, 1981). Relatively higher levels of m^5dCyd are found in the vascular plants, ranging from 3.6 to 7.1% (Hall, 1971). In comparison to eukaryotes, bacteria have methylated bases present in relatively smaller amounts. For example, m^5dCyd is present as a minor component of DNA in *E. coli* (<0.25%)(Vanyushin *et al.*, 1968) and the thermophilic bacterium *Clostridium thermohydrosulfuricum* (<0.02%) (Ehrlich *et al.*, 1985). Similarly, many species of bacteria contain m^6dAdo in their DNA at lower levels, including 0.44% for *E. coli* (Vanyushin *et al.*, 1968), 0.25% for *C. thermohydrosulfuricum* (Ehrlich *et al.*, 1985) and 0.07% for *Bacillus stearothermophilus* (Ehrlich *et al.*, 1985). m^4dCyd is not present in any variety of eukaryotic DNAs and it is known to be more resistant to heat-induced deamination than cytidine or m^5dCyd (Ehrlich *et al.*, 1985). Several thermophilic bacteria contain m^4dCyd (ranged 0.01-0.39%) at levels above m^5dCyd (ranged 0.01-0.09%) (Ehrlich *et al.*, 1985). In the pathogenic corynebacteria, the presence of m^6dAdo was reported for DNA from *C. diphtheriae* (0.84%) and *C. vadosum* (0.58%), whilst no m^5dCyd was detected (Vanyushin *et al.*, 1968).

Many bacterial species contain more than one modified base in their DNA. Using reversed phase (RP) high performance liquid chromatography (HPLC), Ehrlich *et al.* (1987) examined the methylated DNA base composition of 26 strains of bacteria which are sources of widely used ENases. Approximately 35% of the bacterial DNAs contained m^4dCyd, about 60% contained

m^5dCyd, and about 90% had m^6dAdo. In the same paper, they observed that several strains contained two or three different modified base in their DNA. Based on this observation, they suggested that the presence of several modified bases in the same bacteria implied the presence of several MTases. For example, Piekarowicz *et al.* (1988) isolated three DNA MTases from *Neisseria gonorrhaeae* and demonstrated that each recognized a unique nucleotide sequence.

1.7.2 Detection of DNA methylation using chromatographic methods

Several different analytical techniques have been used for determining the major and minor base composition of DNAs. One of the earliest techniques used was paper chromatography of DNA digests with quantification by ultraviolet absorption (Vanyushin and Mazin, 1973). Thin layer chromatographic methods have also been applied to digested DNA because it is simple and fast (Janulaitis *et al.*, 1983; Landry *et al.*, 1989). More advanced chromatographic techniques, including gas-chromatography (Hashiyme and Sasaki, 1968), cation-exchange chromatography (Eksteen *et al.*, 1978; Yuki *et al.*, 1979), RP-HPLC (Gehrke *et al.*, 1984; Kuo *et al.*, 1980) and GC-MS (Singer *et al.*, 1979) have also been applied to analysis of the major, minor or total base composition of DNAs.

Among the methods described above, RP-HPLC was more attractive since this method offers good sensitivity, precision and accuracy for the detection of the DNA bases without the use of harsh hydrolysis conditions, large DNA samples, difficult sample preparation procedures or *in vivo* labelling of DNA (Kuo *et al.*, 1980). The detection limit is about 0.2 nmol per injected DNA base and this method gives a linear response over the range of 50 to 2,500 pmol (Kuo *et al.*, 1980). Normally, protocols for HPLC analysis include the isolation of DNA from target cells, RNase treatment to avoid RNA contamination, heat incubation to denature DNA and mild enzymatic hydrolysis using DNase, Nuclease P1 or bacterial alkaline phosphatase (Ehrlich *et al.*,

1985; Gehrke *et al.*, 1982, 1984; Kuo *et al.*, 1980). Enzymatic hydrolysis of the DNA and RNA to give the major and methylated nucleosides yields a product suitable for RP-HPLC analysis. Under these conditions, not only DNA bases but also RNA bases are produced and this often caused difficulties in differentiating modified DNA bases from RNA bases (Ehrlich *et al.*, 1985; Gehrke *et al.*, 1984). Therefore, excess amounts of DNase-free RNase have been used to remove RNA contamination. Following heat treatment to convert double stranded to single stranded DNA, the DNA is cleaved to 5´-mononucleotides by Nuclease P1, an ENase derived from *Penicillium citrinum*, which quantitatively hydrolyses both DNA and RNA (Fujimoto *et al.*, 1974) and hydrolyses single stranded DNA 200-fold faster than double stranded DNA (Fujimoto *et al.*, 1974).

1.7.3 Detection of DNA methylation using bisulphite genomic sequencing

Despite the efficiency and broad use of the above methods, there are limitations in analysing DNA using HPLC, since HPLC is unable to demonstrate which sequence is specifically methylated by MTase. Recently, a method employing bisulphite modification of deoxycytidine (dCyd) bases has been developed in order to determine the role of dCyd methylation in genomic DNA. This methodology relies on the ability of sodium bisulphite to efficiently convert dCyd bases to uridine in single-stranded DNA, under conditions whereby m^5dCyd remains essentially non-reactive (Clark *et al.*, 1994; Feil *et al.*, 1994; Frommer *et al.*, 1992; Wang *et al.*, 1980). Experimentally, the DNA sequence under investigation is amplified by polymerase chain reaction (PCR) with two sets of strand-specific primers to yield a pair of fragments, one derived from each strand, in which all uridine and thymidine residues have been amplified as thymidine and only m^5dCyd residues have been amplified as dCyd. Resulting PCR products are then sequenced and the band patterns from sequence analysis are compared with non-modified DNA controls. This method for identifying m^5dCyd is therefore a positive one in which the position of each m^5Cyd is given by a

distinct band on a sequencing gel (Clark *et al.*, 1994). According to the observation by Wang *et al.* (1980), sodium bisulphite specifically deaminated more than 96% of the cytosine residues in single-stranded DNA via formation of a 5,6-dihydrocytosine-6-sulfonate intermediate. Although deamination also occurred in m^5dCyd (2-3%), the rate of reaction was very slow compared to that of cytidine. This techniques has been applied to several genomic DNAs including mouse insulin-like growth factor 2 (Feil *et al.*, 1994), mouse L cell genomic DNA (Clark *et al.*, 1994) and human genomic DNA (Frommer *et al.*, 1992), showing that many dCyd bases in CG sequences are methylated. As far as I know, this techniques has been not used for plasmid DNA or bacterial DNA.

1.8 MUTANT STRAINS OF CORYNEBACTERIA

Attempts have been made to improve various strains of *Corynebacterium* species for specific applications. The approach taken has largely been along traditional lines of mutagenesis and screening for mutants which overproduce particular metabolites (Tosaka *et al.*, 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 be described in the following section.

1.8.1 Cell surface mutants of corynebacteria

Best and Britz (1986) isolated several auxotrophic mutants of *C. glutamicum* ATCC 13059 by long-term exposure to EMS (Fig. 1.5). Many of these protoplasted more easily than the parent-type strain. Some mutants, including strains MLB131-135 and MLB194, produced larger cells

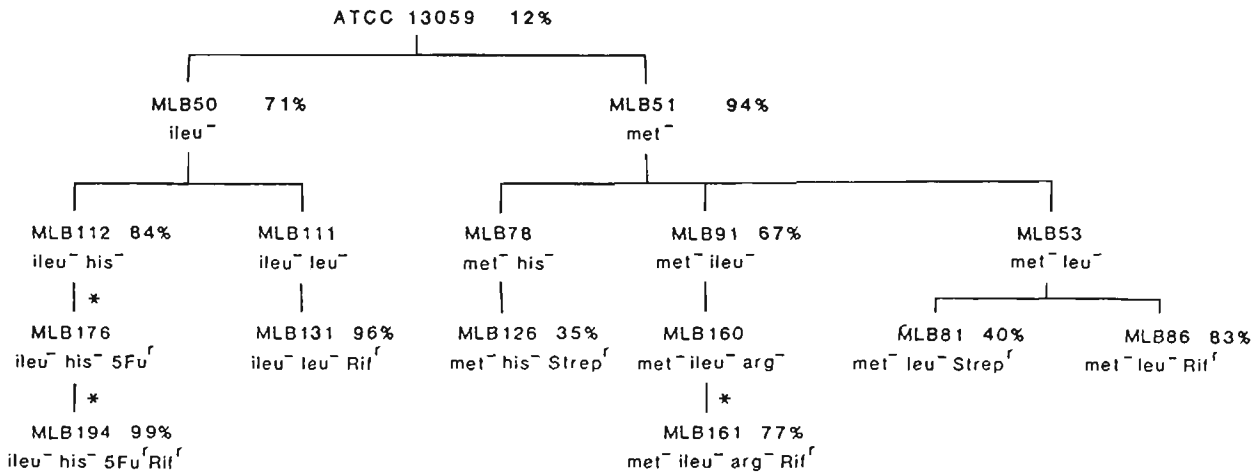


Fig. 1.5 Genealogy of mutants derived from *C. glutamicum* strain ATCC 13059 by EMS mutagenesis or through selection of strains spontaneously resistant to antimetabolites on selection media (from Best and Britz, 1986). Abbreviations: Rif^r, rifampicin resistant; Strep^r, streptomycin resistant; 5-Fu^r, 5-fluorouracil resistant; arg$^-$, auxotrophic for arginine; his, histidine; ileu, isoleucine; leu, leucine; met, methionine. Percentages represented protoplasting efficiency.

than other auxotrophs or the parent strain when grown in LB and always displayed notably more aberrant morphological changes during growth in LB-glycine. Using scanning electron microscopic observation, Britz (1985) found that *C. glutamicum* strain ATCC 13059 grown in LB was an irregular rods, with coccobacilli 1-2 μm in length. Characteristically, this strain forms parallel ridges around its circumference. However, grown 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 here occurs through budding, branch formation, then septation (Jang *et al.*, 1997). In addition, Britz examined the effect of glycine and INH on the 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 mutants strains 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 electrotransformation efficiency than *C. glutamicum* AS019 (Haynes and Britz, 1989), sensitivity to the INH was higher than AS019.

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 his experiments, Pierotti (1987) used two different media: Luria Broth (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 MAMEs and FAMEs, and characterised these using GC and GC-MS. 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 that these strains of *C. glutamicum*, including the mutants, had mycolic acids with carbon lengths 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*. However, changes in mycolic acids were observed with changes in cell growth phases when mutant strains of *C. glutamicum* were analysed. Overall, the trend observed for the mutant strains was towards an increased the proportion of longer chained ($C_{34:1}$) and unsaturated mycolic acids ($C_{34:1}$ and $C_{36:2}$) as the fermentation progressed (Jang *et al.*, 1997; Pierotti, 1987).

1.8.2 Restriction deficient mutants of corynebacteria

To avoid ENase action in corynebacteria, restriction deficient strains were constructed (Bonnassie *et al.*, 1990; Liebl *et al.*, 1989; Ozaki *et al.*, 1984; Schäfer *et al.*, 1994a). Ozaki *et al.* (1984) reported that *C. glutamicum* RR25 was a lysozyme sensitive and restriction minus strain. In 1989, Liebl *et al.* (1989) prepared restriction minus strains of *C. glutamicum* and used these as host strains for plasmid DNA transformation by electroporarion. Experimentally, they mutagenised *C. glutamicum* AS019 with N-methyl-N$^{/}$-nitro-N-nitrosoguanidine (NTG) at 30°C for various times (0-60 min) and isolated restriction minus strains, R127 and R163. Subsequently, workers transformed strain R127 with either heterologously- or homologously-isolated plasmid DNA from *E. coli* and *C. glutamicum*, respectively, using protoplast transformation and electroporation. *C. glutamicum* AS019 was used as the control strain. They found that strain R127 could be transformed equally well with heterologously- or homologously-isolated plasmid DNA, whilst strain AS019 was transformed well with homologously-isolated DNA but poor with heterologously-isolated DNA. Bonnassie *et al.* (1990) reported restriction deficient mutants from

B. lactofermentum, and electrotransformed with homologously- (DNA from *B. lactofermentum*) and heterologously-isolated DNA (from *E. coli*). From this experiment, they obtained transformation efficiency of 1.6 X 10^6 transformants per μg of homologously isolated DNA and 3.0 X 10^5 transformants per μg of heterologously-isolated DNA.

Schäfer *et al.* (1994a) obtained restriction minus mutants (RM3 and RM4) of *C. glutamicum* derived from ATCC 13032 by chemical mutagenesis with NTG, where RM3 and RM4 displayed higher frequencies of transformation or transconjugation with heterologous DNA while ATCC 13032 restricted transformation of foreign DNA. Using electroporation, Schäfer *et al.* (1994b) found that *C. glutamicum* was transformed with homologous DNA at 5 X 10^5 transformants per μg DNA and with heterologous DNA from *E. coli*, 9 X 10^4 transformants per μg DNA were obtained. However, parallel changes in cell surface structures of these strains (R127, R163, RM3 and RM4) have not been reported; two of these strains, RM3 and RM4, were chosen for study of their mycolic acid structures as part of this thesis.

1.9 AIM AND OUTLINE OF THE THESIS

Members of genus saprophytic corynebacteria, including *C. glutamicum*, *B. lactofermentum* and *B. flavum*, are important in industrial amino acid fermentations and have a large potential for application in foreign protein expression. Unfortunately, there are many factors that limit the possibilities for these types of processes since these species contain both enzymatic (restriction and modification) and cell surface barriers to introduction of foreign DNA. To overcome these limitations, several priorities for research and development were proposed (Katsumata *et al.*, 1984; Schäfer *et al.*, 1990; Yoshihama *et al.*, 1985), including: alteration of cell wall structure by the addition of cell wall modifiers in the growth medium; heat inactivation of putative ENase on *C. glutamicum* and use of mutants which transform more readily.

Initially, the study reported in this thesis was based on two hypotheses. Firstly, that part of the observed barrier to efficient transformation stemmed from the physical structure of the cell surface, including mycolic acids. Secondly, cellular ENase(s) could be restricting the incoming heterologous DNA. Therefore, one main part of this work was investigating the nature of the restriction barriers with specific emphasis on defining the nature of the enzyme activities involved. It was originally planned to isolate genes responsible for ENase and MTase. When this project started in 1993, little had been published on this topic. However, during the course of this thesis, it became apparent that a very similar project with the same goals was well advanced (Schäfer *et al.*, 1994b; Tauch *et al.*, 1994). Thus, this work was redirected to concentrate on a study of the nature of methylation activity present in *C. glutamicum* and related species, with emphasis on identifying the target sequence(s) of MTase and ENase. The specific aims of this thesis were:

1) to examine the effect of growth medium containing glycine or INH on the cell growth rate, cell wall morphology, transformation efficiency and composition of mycolic acids of *C. glutamicum* and related species.
2) to investigate the biochemical changes in cell surface structures of readily-protoplasted mutants of *C. glutamicum* (MLB133 and MLB194) in context of their presumptive sensitivity to glycine and INH.
3) to determine the level of methylated base in DNA from corynebacteria to demonstrate the target of MTase activity.
4) to determine the possible methylation sites(s) of the MTase.

This thesis consists of two major sections concerned with the above and provides further insight into the nature of the cell surface structures of *C. glutamicum*.

Chapter 2 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Bacterial strains and plasmids

The list of corynebacteria and *Escherichia coli* strains and plasmid DNAs used in this study is shown in Table 2.1.

2.1.2 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, acids or bases before use. All buffers were prepared in glass with distilled water and sterilised by autoclaving at 121°C for 20 min unless otherwise stated. Sugar components in buffers were added from sterile stock solutions 50%, w/v, sucrose and 20%, w/v, glucose, 109°C for 25 min.

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 in 10 mM Tris, 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). This was prepared freshly before use.

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

Table 2.1 Bacterial strains and plasmid DNAs used in this work

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strain		
C. glutamicum strains		
ATCC 13059	Wild-type strain	ATCC strain
AS019	Spontaneous Rif^r mutant of ATCC 13059	Yoshihama *et al.*, 1985
CG2	Wild-type strain	Queensland University Culture Collection (No. 2740), Australia
MLB133	Mutant of ATCC 13059, $ileu^-$, leu^-, Rif^r	Best and Britz, 1986
MLB194	Mutant of ATCC 13059, $ileu^-$, leu^-, Rif^r	Best and Britz, 1986
ATCC 13032	Wild-type strain	ATCC strain
RM3	Mutant of ATCC 13032	Schäfer *et al.*, 1994a
RM4	Mutant of ATCC 13032	Schäfer *et al.*, 1994a
B. lactofermentum		
BL1	Ser^-	ATCC 21798
B. flavum		
BF4	Met^-, thr^-	Rogers, University of New South Wales, Australia
C. ulcerans CU	Wild-type strain	Commonwealth Serum laboratories, Australia
E. coli strains		
MC1061	hsdR mcrB araD 139Δ(araABC-leu)7679 ΔlacX74 galU galK rpsL thi	Sambrook *et al.*, 1989
RR1	supE44 hsdS20($r_B^-m_B^-$) ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1	Sambrook *et al.*, 1989
HB101	supE44 hsdS20($r_B^-m_B^-$) recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-4 mtl-1	Sambrook *et al.*, 1989
LE392	supE44 supF58 hsdR514 galK2 galT22 metB1 trpR55 lacY1	Sambrook *et al.*, 1989
ED8654	supE supF hsdR metB lacY gal trpR	Sambrook *et al.*, 1989

CSR603	F⁻ thr-1 leuB6 proA2 phr-1 recA1 argE3 thi-1 uvrA6 ara-14 lacY1 galK2 xyl-5 mtl-1 rpsl31 tsx-33 λ⁻ supE44 gyrA98 (nal A98)	Sancar and Rupert, 1978
JM101	supE thi Δ (lac-proAB) F'[traD36 proAB⁺ lacI^q lacZ Δ M15]	Sambrook *et al.*, 1989
N4830	SIΔ8(λΔBAMΔH1)	Gottesman *et al.*, 1980
Plasmid		
pBL1	(4.4kb) Cryptic	Santamaria *et al.*, 1984
pBR322	(4.4kb) Amp^r, Tc^r	Bolivar *et al.*, 1977
pCSL17	(7.2kb) Sub-cloning the *C. glutamicum ori* from pHY416 into pBR322 and inserting Tn903, Km^r	Hodgson *et al.*, 1989
pHY416	(9.4Kb) *B. subtilis-C. glutamicum* shuttle vector, Km^r	Yoshihama *et al.*, 1985

[a] leu⁻ (leucine), ileu⁻ (isoleucine), met⁻ (methionine), ser⁻ (serine), thr⁻ (threonine) auxotroph; Amp^r (ampicillin), Km^r (kanamycin), Rif^r (rifampicin) resistance.
Abbreviation: ATCC, American Type Culture Collection, Rockville, U.S.A.

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

TBE buffer (5X), pH 8.2, contained 89 mM Tris base, 89 mM boric acid and 2.5 mM EDTA. A 5X concentrated stock solution was diluted in distilled water to give the desired concentration and used for agarose gel electrophoresis.

TE buffer, pH 8.0, was used for storage of DNA and contained 10 mM Tris and 1 mM EDTA.

Three types of restriction enzyme buffers were used from 10X stock solutions as follows: TA buffer, One-Phor-All buffer (Pharmacia) and specific commercial buffers from restriction enzyme manufacturers. 10X TA buffer (O'Farell *et al.*, 1980) contained 33 mM Tris-acetate, pH 7.9, 66 mM potassium acetate, 10 mM magnesium acetate and 0.5 mM dithiothreitol and 100 μg/ml BSA. 10X One-Phor-All buffer, pH 7.5, contained 100 mM Tris-acetate, 100 mM magnesium acetate and 500 mM potassium acetate.

2.1.3 Chemicals

The sources of all chemicals and media are shown in Appendix 1, abbreviations used for these are also listed on page iii. All reagents, where not specified, were analytical grade and supplied by BDH (UK) or Sigma (U.S.A.). Distilled water was prepared using the Milli-RO Water Purification System or deionised water using the Milli-Q Ultrapure Water System throughout.

Amino acids, purines, pyrimidines 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.

2.1.4 Instrumentation

An Eppendorf centrifuge was used for samples 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 used 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 was used for lyophilising samples. Other general analytical instrumentation is described in the specific methods section.

2.2 MICROBIOLOGICAL METHODS

2.2.1 Media

Unless specified, all media were sterilised by autoclaving for 15 min at 121°C, except for media used in the 10 litre fermenter for plasmid DNA isolation from *C. glutamicum*. Media additives were stored in brown-coloured bottles at room temperature. For 10 litre fermentations, broth media were transferred into the fermenter first, together with the other accessories, and sterilised for 30 min at 121°C. Supplements, such as drugs, sugars, and amino acids, were added aseptically from stock solutions to the medium at 50°C, immediately prior to dispensing the medium.

2.2.1.1 Luria Broth (LB)

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

2.2.1.2 Luria broth with glucose (LBG)

The medium routinely used for corynebacterial growth was LBG (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).

2.2.1.3 LBG supplemented with glycine (LBG-G)

The medium used for DNA isolation, determination of cell growth rates, electrotransformation, and quantitative analysis of mycolic acids composition from corynebacteria was LBG-G (Haynes and Britz, 1989). Final concentrations of glycine in LBG-G varied from experiment to experiment 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 sterile stock solutions (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 this was redissolved by incubating at 65°C for several hours prior to use.

2.2.1.4 LBG supplemented with INH (LBG-I)

This medium was used for determination of cell growth rates, electrotransformation, and

quantitative analysis of mycolic acids composition from 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 solution (20% [w/v] glucose and 100 mg/ml INH) and the volume was adjusted with sterile water to produce 1X LB.

2.2.1.5 LBG supplemented with Tween 80 (LBG-Tween 80)

The medium used for determination of cell growth rates was prepared as follows (Haynes and Britz, 1989): sterile 2X LB was supplemented with 0.5% glucose and Tween 80 (0.1-1.0%) from sterile stock solutions (20% [w/v] glucose and 20% [w/v] Tween 80) and the volume was adjusted with sterile water to produce 1X LB.

2.2.1.6 Nutrient agar with glucose (NAG)

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

2.2.1.7 ET

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, 5 g; tryptone, 5 g; K_2SO_4, 0.25 g; gelatin, 5 g; agar, 12 g); mixture B (succinic acid, 35.7 g in 300 ml solution (prepared as a solution in 5N NaOH, pH adjusted to 7.2 before sterilising); Tris base, 3.94 g (pH adjusted to 7.2 added from a 1 M stock of Tris buffer); 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 1 ml. The trace elements solution contained per litre: $ZnSO_4.7H_2O$, 8.8 g; $FeCl_3.6H_2O$, 0.97 g; $CuSO_4.5H_2O$, 0.27 g; $Na_2B_4O_7.10H_2O$, 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 (1 M); 50 ml of 20% (w/v) glucose; 5 ml of 2% (w/v) BSA (filter sterilised); 2.5 ml of 2% (w/v) KH_2PO_4; 10 ml of 20.3% (w/v) $MgCl_2.6H_2O$; 1 ml of trace elements; 10 ml of 14.7% (w/v) $CaCl_2$.

2.2.1.8 Minimal medium for corynebacteria

This medium (M.L. Britz, personal communication) contained the following composition (per litre): ammonium sulphate, 1.5 g; urea, 1.5 g; dihydrogen potassium phosphate, 1.0 g; dipotassium hydrogen phosphate, 3.0 g; magnesium sulphate, 0.1 g; calcium chloride, 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.

2.2.1.9 SOB

This medium (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.

2.2.1.10 SOC

SOB was supplemented with 20 mM glucose (SOC) (Sambrook *et al.*, 1989) and used as recovery

medium for *E. coli* cells after transformation.

2.2.1.11 Recovery medium

This medium (M.L. Britz, personal communication) was used as a recovery medium for transformants after electroporation and contained LBG supplemented with 10 mM $CaCl_2$ and 10 mM $MgCl_2.6H_2O$.

2.2.2 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 the lamina flow 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, dispensed in 2-3 ml aliquots into 5 ml capacity glass bottles. Sealed bottles were stored at -20°C (for working stocks) and -80°C (for the long term storage stocks).

2.2.3 Growth of corynebacteria

2.2.3.1 Growth on plates

LAG inocula were prepared on LAG plates, where corynebacteria stock stored at -20°C were transferred onto LAG plates with a sterilised loop and then allowed to grow at 30°C (37°C for *C.*

ulcerans) in the incubator for approximately 16 h before being used as inocula. NAG was often used for sub-culturing of corynebacteria instead of LAG.

2.2.3.2 Growth in MacCartney (25 ml capacity) bottles

MacCartney bottles containing 10 ml of sterile LBG were inoculated with single colonies from LAG plates (see section 2.2.3.1). The cultures were incubated overnight at 200 orbits per min (o.p.m.) and 30°C (or 37°C for *C. ulcerans*) and used as inocula for large scale cultures, including determination of growth rates by measuring absorbance at 600 nm.

2.2.3.3 Small-volume shake-flask fermentation

Erlenmeyer flasks of 250 ml capacity containing 100 ml of appropriate broth were inoculated with one to two ml of LBG storage cultures. Liquid cultures were grown at 30°C (or 37°C for *C. ulcerans*) and 200 o.p.m. The fermentation was performed to examine the effects of cell wall modifiers in the growth medium on the cell growth rate, to isolate chromosomal DNA and to determine whether growth medium affected the mycolic acid composition. For the isolation of plasmid DNA, antibiotics (50-100 μg/ml) (see section 2.3.2) were added to LBG containing 2% glycine.

To test the effects of glycine, INH, and Tween 80 on cell growth rates, strains were inoculated to a starting A_{600} of 0.1-0.2 into 100 ml of LBG in 250 ml flasks supplemented with glycine (0-10%, w/v), or INH (0-10 mg/ml) or Tween 80 (0.1-1%, w/v), and combinations of these, from sterile stock solutions.

For analysis of mycolic acids and fatty acids, cultures were performed in two configurations

depending on the size of samples required, including shake-flasks containing 30 ml or 100 ml of growth medium. To compare mycolic acid composition for whole cells and culture fluids, cells were grown in 30 ml (or 100 ml) of the following medium in 100 ml (or 250 ml) flasks: LBG, LBG containing 2% (w/v) glycine, LBG containing 4 mg/ml or 8 mg/ml of INH, or LBG containing 2% glycine plus 0.4 mg/ml or 4 mg/ml INH, harvesting at early stationary phase.

2.2.3.4 Large-volume shake-flask fermentation

To isolate pCSL17 plasmid DNA from corynebacteria, erlenmeyer flasks each of two litre capacity containing one litre LBG supplemented glycine (2%, w/v) and kanamycin (50 μg/ml) (LBG-G/Km) were incubated at 200 o.p.m.

2.2.3.5 Growth in a ten litre fermenter

Growth of *C. glutamicum* containing pCSL17 plasmid was conducted in a ten litre capacity laboratory fermenter (Applikon, Bio Cansle ADI, Holland). LBG supplemented with 2% (w/v) glycine was firstly sterilised at 115°C for 30 min and then inoculated with 500 ml of LBG-G/Km inoculum to give a total volume of seven litre. The fermentation conditions were as follows: pH controlled (7.2), 30°C, stirring rate 150 r.p.m. The air flow was set at three litres per min. Cells were cultured for 24 h before harvesting for plasmid preparation.

2.2.4 Growth of *E. coli*

MacCartney bottles containing 10 ml of media were sterilised then inoculated with single colonies from plates LA inoculum containing *E. coli*. The cultures were maintained at 37°C overnight and 200 o.p.m., and subsequent cultures were used as inoculum for large scale fermentation.

2.2.5 Growth curves and viable counts

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 appropriate medium being used and A_{600} recorded against medium blanks. Viable counts were performed using 10-fold serial dilutions of cultures in SMMC buffer or water. 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 200 μl 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 for up to seven days. All counts were performed in triplicate.

2.2.6 Effect of cell wall modifiers on growth of coryneform bacteria

To test the effect of cell wall modifiers on growth rates, strains were inoculated into appropriate media, with a starting A_{600} of 0.1-0.2 (section 2.2.3.3). Cell growth rates were compared in three ways: μ value (specific growth rate, μ, h^{-1}), period of lag growth phase and maximum A_{600} obtained. For calculation of μ value, samples (1 ml) were removed hourly for measurement of A_{600}. All samples above 0.8 at A_{600} were diluted with appropriate fresh media. Doubling time was obtained during the exponential growth period from the slope of the curve obtained by plotting the logarithm of A_{600} against time, and cell specific growth rate was calculated as follows (Aiba *et al.*, 1973):

$$\mu \propto 1/X \, . \, dX/dt,$$

where,

X = the cell concentration, t = time,

If the value of μ is constant, the above equation represents exponential growth, where growth is proportional to the mass of cells present. Therefore, the μ value can be expressed as the following equation.

td (doubling time) = $0.693/\mu = \ln 2/\mu$,

$\mu = 0.693/td$

In the presence of high concentrations of cell wall modifiers in the growth medium, growth of some strains were retarded for several hours before starting growth. Therefore, growth inhibition was also evaluated by determining the length of the lag phase, which was defined as the time taken from inoculation to initiation of exponential growth phase, using standard inoculation protocols.

Alternatively, the extent of cell growth was also determined by measuring the final optical density at 600 nm of each samples after 16 h incubation. Freshly prepared cultures (4%, v/v) from overnight cultures were inoculated into MacCartney bottles containing 10 ml of media and incubated at 30°C and 200 o.p.m.

2.2.7 Determination of drug sensitivities

Drug sensitivities were performed using the following antimicrobial agents (abbreviations in parenthesis) and levels: ampicillin (Amp), 20-60 μg; chloramphenicol (Cm), 20-170 μg; kanamycin (Km), 10-50 μg; neomycin (Neo), 10-50 μg; rifampicin (Rif), 10-50 μg; streptomycin (Str), 10-50 μg; tetracycline (Tc), 10-50 μg.

LA plates for *E. coli* and LAG for corynebacteria, which were prepared on the day of use and

dried at 37°C, were inoculated with overnight broth cultures by spreading 100 μl of cultures onto the plates. Plates were allowed to dry before sterile 6 mm discs (Oxoid) were applied. Subsequently, two to three drops of antibiotic solution were applied onto the disc using a sterile Pasteur pipette. Sensitivity patterns were recorded initially after 24 h incubation at 30°C for non-pathogenic corynebacteria or 37°C for pathogenic corynebacteria (*C. ulcerans*) and *E. coli* strains, then 48 h for slowly growing cells. Quantitative analysis was not tried using this method and qualitative drug sensitivity patterns were determined by formation of inhibited growth zones; organisms were clearly resistant if growth occurred up to the disc.

Alternatively, drug sensitivities were also performed by incorporating agents into plates. The method for corynebacteria involved preparing LAG plates with various concentrations of drugs, ranged between 10 to 170 μg/ml. Plates were prepared on the day of use and dried at 37°C. Inocula of 100 μl of overnight cultures were transferred onto plates using a 200 μl Gilson pipette, spread with a glass rod and incubated for 24-48 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 drug that completely inhibited growth, after 48 h incubation at 30°C, except for *C. ulcerans* (37°C). MIC's were performed for *E. coli* strains similarly; LA media was used instead of LAG.

2.2.8 Transmission electron microscopy (TEM)

Cells from fresh cultures (100 ml) grown in LBG, LBG supplemented with glycine (2%, w/v) or LBG supplemented with INH (4 mg/ml) were harvested (1,200 X g, 4°C, 10 min) at A_{600} = 0.4. Cells of *C. glutamicum* AS019 and MLB133 were washed with 50 ml of distilled water, centrifuged and the supernatant removed. Further processing was performed by Ms. Anne Martsi McClintock (Department of Physiology, Monash University) using the formamide technique

described by Hayat (1981) and modified by McClintock. Thin sections of fixed, embedded bacteria were viewed and recorded on a JEOL 100S electron microscope (Japan). Multiple fields were examined using several magnifications and representative cells were photographed. To obtain an overview of cell size distribution after growing cells in different media, cells were magnified by 1,200 times using the electron microscope and photographed. Such magnification showed approximately 300 to 350 cells in one frame (approximately, 20 cm X 25 cm). Three different photos were taken from different areas and the lengths of cells were measured using a millimetre ruler and the real length of cells was calculated by comparing the size of cells to that of known references. To measure cell wall thickness after growing cells in different media, cells were magnified by 120,000 times using the electron microscope and photographed. Such magnification showed approximately 10 to 20 cells in one frame. The thickness of cell walls was measured from at least 40 cells and the average of multiple measurements (3-5 times) from each cell were taken. Standard deviations for each conditions (strains, growth media) were calculated using the Sigma-plot program.

2.3 DNA METHODS

2.3.1 Small scale plasmid DNA isolation

The boiling method (for isolation of pBR322 plasmid) and the alkaline lysis protocol (for isolation of pCSL17 plasmid) described by Sambrook *et al.* (1989) were used for rapid small scale isolations of plasmid DNA from *E. coli* strains.

Plasmid DNA from corynebacteria was isolated using an alkaline lysis method (Santamaria *et al.*, 1984) and modified by Best and Britz (1986). This procedure was used with minor modification and described in section 2.3.2. 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 h. Plasmid DNA was also extracted from larger volumes.

2.3.2 Large scale plasmid DNA isolation

Plasmid DNA used in restriction enzyme mapping, transformation, HPLC analysis of DNA bases and DNA sequencing was extracted using this method. The plasmid DNAs from *E. coli* strains were prepared by the alkaline lysis described by Sambrook *et al.* (1989) after growth in LB-Km. Erlenmeyer flasks of 250 ml capacity containing 100 ml LB/Km were inoculated with 1-2 ml of overnight starter cultures, and incubated until A_{600} reached a value greater than 1.0. The presence of plasmid DNA was confirmed using agarose gel electrophoresis. Preparations of pHY416 plasmid DNA were obtained from VUT stocks (provided by M. L. Britz).

Plasmid DNA from corynebacteria was isolated after growth in 100 ml of LBG-G/Km (2% glycine). The procedure involved preparation of osmotically sensitive cells by growing cells in the presence of glycine and following lysozyme treatment, breaking the cell surface structure using SDS treatment, removing chromosomal DNA and protein from plasmid DNA using (CsCl-EtBr) gradients. When the A_{600} of cells reached a value 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 0.5 M EDTA (pH 8.0) and incubating at 37°C for a further 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 the waterbath. 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 (per 200 ml, 120 ml of 5 M potassium acetate; 23 ml of acetic acid; and 57 ml of water) was added (Sambrook *et al.*, 1989). 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 fluids were collected and filtered through cheesecloth. Plasmid DNA was precipitated by addition of 0.6 volumes of isopropanol and collected by centrifugation (14,000 X g, 30 min, 20°C). 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 (section 2.3.3).

Alternatively, pCSL17 DNA from *C. glutamicum* AS019 was also isolated from 7 litres of culture which were grown in a 10 litre fermenter. Cells from LBG-G/Km were pelleted (5,000 X g, 10 min, 4°C) and washed once in 500 ml of lysis buffer (without lysozyme), collected by centrifugation and resuspended in 500 ml of lysis buffer containing lysozyme (final concentration: 10 mg/ml) followed by incubation (200 o.p.m., 37°C) for 16 h. EDTA (10 mM in 10 mM Tris buffer, pH 8.0) was added and the mixture incubated for one h (200 o.p.m., 37°C). Osmotically sensitive cells were collected by centrifugation. Lysis was achieved by the addition of 400 ml of SDS solution (1%, w/v, in 0.2 M NaOH) followed by heating at 55°C for 10 min. Sixty ml of ice-cold acetate solution was added and the mixture shaken gently several times before the mixture was left on ice for 20 min. The plasmid DNA was separated from cell debris by centrifugation (14,000 X g, 10 min, room temperature). The rotor was allowed to stop without braking. The supernatant was collected and 0.6 volumes of isopropanol added and the mixture left on the bench for 10 min. The precipitated nucleic acids were collected by centrifugation (14,000 X g, 4°C, 20 min), resuspended in 9 ml of water and used in CsCl gradient separation of plasmid DNA (section 2.3.3).

2.3.3 Preparation of caesium chloride-ethidium bromide gradients

CsCl was finely powdered and nine grams amounts added to 15 ml capped plastic centrifuge tubes. DNA mixture in water (9 ml) was added then 0.92 ml ethidium bromide (from 10 mg/ml

stock) and the CsCl dissolved by repeating 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 rotor 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 corresponded 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 transilluminator) 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 sharp 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 isoamyl alcohol until the red colour had disappeared from the DNA solution. Two volumes of cold EtOH was added to the DNA solution, which was 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% EtOH prior to centrifugation. After removing EtOH, DNA was resuspended in a minimum volume of distilled water, aliquoted, and then stored at -20°C.

2.3.4 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 shaken thoroughly at room temperature before adding 1.5 ml of CTAB/sodium chloride solution (10% CTAB [Hexadecyltrimethylammonium bromide] in 0.7 M NaCl). This was followed by a chloroform/isoamyl alcohol extraction and 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 corynebacteria was based on a method described by Britz and Best (1986) and 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 in 100 ml of lysis buffer (without lysozyme), collected by centrifugation and resuspended in 5 ml of lysis buffer containing lysozyme (final concentration: 10 mg/ml) followed by incubating 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 o.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. Lysis was affected 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 the phenol 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 was transferred to 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 (section 2.3.3).

2.3.5 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 1:50 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 concentration were determined according to the equation: 1 A_{260} unit (1 cm light path) = 50 μg/ml DNA and 37 μg/ml single-stranded oligonucleotides (Sambrook *et al.*, 1989).

DNA concentration was also routinely estimated using the fluorimetric method described by Setaro and Morley (1976) as modified by Britz (1978). Diaminobenzoic acid dihydrochloride (DABA) (Sigma) was prepared at 1.32 M by adding sufficient 6N HCl to just cause the diaminobenzoic acid to go into aqueous solution; this obviated the necessity for fresh, highly purified DABA. Samples containing 0.5 to four μg of DNA were dried in acid-washed glass tubes at 150°C in a hot air oven. DABA solution was added (0.1 ml), tubes were shaken vigorously using a vortex mixer, then covered and incubated at 60°C for 30 min. Perchloric acid (0.6 N in distilled water) was added to give a final volume of 2 ml. Fluorescence was measured using a Luminescence Spectrometer LS 50 (Perkin-Elmer), with activating wavelength set at 420 nm and fluorescent wavelength at 520 nm. Salmon sperm DNA (0.05 to four μg) was used as the standard.

2.3.6 Restriction enzyme digestion of pCSL17 plasmid DNA

Restriction endonucleases were purchased from Boehringer Mannheim, BioRad, and Promega. Usually, restriction digests were carried out for three h in 15-30 μl volumes using 5-10 units of

restriction-endonuclease per μg of DNA. In digestion, different endonuclease reactions were performed in 1X TA buffer, One-Phor-All, or as recommended by the enzyme manufacturers. Reactions were terminated by heating at 65°C (or 85°C) for 10 min. Loading dye (one-fifth volume of sample) was added to each reaction digest prior to loading onto an agarose gel for electrophoresis. DNA separation and visualisation were performed with 0.8% (w/v) agarose gels (see section 2.3.8). When DNA fragments were too small to differentiate with 0.8% (w/v) agarose gels, the concentration of agarose was increased up to 2% (w/v).

For the precise determination of restriction enzyme mapping of pCSL17 DNAs, twenty endonucleases were used, alone or in combinations. For the double digestions by restriction endonucleases, the first restriction endonuclease which required low ionic strength was incubated at 37°C for three h and heat-inactivated at 65°C or 85°C for 5 min. After the tube was cooled to room temperature, if required, buffer composition was adjusted by the addition of enzyme buffer and the second enzyme was added. After three h incubation, reaction was terminated by heating the tube as above.

2.3.7 Restriction enzyme digestion of chromosomal DNA

This experiment was carried out to determine whether any restriction endonucleases failed to cut the chromosomal DNAs completely or only partially cut them, to indicate whether methylation had occurred in specific sites on the DNA. Chromosomal DNA from corynebacteria strains were digested with various restriction endonucleases using phage lambda DNA (0.39 μg/μl) (Promega) as a positive control. ENases *Dpn*I, *Dpn*II *Bcl*I, *Cla*I, *Eco*RI, *Eco*RV, *Hae*III, *Kpn*I, *Pst*I, *Xba*I, and *Spe*I were obtained from Biolabs, *Bam*HI, *Bgl*II, *Hind*III, *Sal*I, *Sau*3AI, *Sma*I and *Xho*I were purchased from Promega, and *Pvu*I and *Pvu*II were obtained from Boehringer Mannheim. Incubation mixtures for digestion (30 μl) contained 3 μg of DNA, 3 μl of 10X One-Phor-All

buffer or buffer recommended by manufacturers and 5-15 units of ENase to cleave the DNA for one h at 37°C (50°C for *Bcl*I digestion). For each gel well, 15 μl (out of 36 μl) of mixture was loaded and the remainder stored at -20°C.

2.3.8 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 gels were used. Large gels (20 X 20 cm) was used for double digestion experiments (GNA-200, Pharmacia). Electrophoresis was carried out using 1X TAE buffer running normally at 65 volts, 1-2 h or until the loading dye neared the bottom of the gel, whilst 25 or 35 volts were used for large gels in 1X TAE buffer, running overnight. EtBr staining solution was added to the agarose gel before electrophoresis and to the 1X TAE at a final concentration of 0.5 μg/ml. The DNA bands were visualised under a UV transilluminator (λ = 302 nm) (LKB 2011 Macrovue transilluminator). *Hind*III digested lambda DNA fragments containing eight fragments (23.1, 9.4, 6.6, 4.4, 2.3, 2.0, 0.6 and 0.1 kb) were used as size markers. Alternatively, a PCR marker (Promega) containing 6 fragments (1000, 750, 500, 300, 150, 50 bp) was used for size determination. Approximately, 0.5-1 μg of size markers were used.

The migration distances of linear fragments of plasmid DNA on agarose gels were related to their molecular weights such that a plot of the logarithm of the relative migration distances against logarithm of molecular weights gave linear curves. Relative migration distances were measured by millimetre ruler from photographs and DNA band intensities were calculated by using an Ultra Scan™ XL laser densitometer (Pharmacia) with Gel Scan™ XL evaluation software.

2.3.9 Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was performed using the BioRad Protein II unit without sodium dodecyl sulfate, in order to obtain better resolution of small DNA fragments. The electrophoresis runs and the staining of the separated DNA bands were performed in accordance to the procedures described by Sambrook *et al.* (1989). DNA samples (approximately 0.5 to 1.0 μg of DNA in the 20 μl of solution after *Hae*III restriction ENase digestion) were mixed with one-fifth volume of sample loading dye. Tubes were centrifuged at 12,000 X g for 15 sec in an Eppendorf centrifuge before samples (12 μl) were applied. PCR markers (Promega) were run in tandem with the samples. The gels (8% polyacrylamide) were run for 3-4 h at a constant voltage of 100 V, using 1X TBE buffer (0.09 M Tris-borate; 0.002 M EDTA) (Sambrook *et al.*, 1989). After separation of the fragments, the gels were transferred into water containing 0.5% (w/v) EtBr. Staining was performed for one h before washing and visualising bands at 302 nm.

2.3.10 Photography

A record of the distribution of DNA, as seen under UV light, was obtained using a Polaroid MR-4 land camera assembly loaded with Polaroid 667 black and white film or Polaroid 665 film. Exposures were between 1/4 to 1 sec (for 667 film), using a yellow filter. The negative provided with the 665 film was exposed for 20-90 sec and scanned using a densitometer.

2.3.11 Recovery of DNA fragments from the agarose gel

DNA fragments were recovered from agarose gels (0.6%) and used either in digestions to detect restriction activities in cell-free extracts (section 2.5.5) or for preparation of DNA samples for

PCR reaction (section 2.5.4). For the former experiment, *E. coli*-derived pCSL17 DNA (25 μg) was incubated with 190 μl of cell-free extract of *C. glutamicum* AS019 and 20 μl 10X One-Phor-All buffer at 37°C for three h. After electrophoresis, the gel was stained and visualised under UV light. DNA bands corresponding to the linear form of plasmid DNA were excised and trimmed using a sterile scalpel blade. The gel fragment of interest was isolated using a Gene Clean kit (Bio 101 Inc.) as recommended by the manufacturer. For the latter experiment, samples were loaded onto the gel and bands later cut from the gel, in order to purify the desired PCR product away from non-specific amplification products. The DNA was eluted from the gel using a Wizard PCR Preps kit (Promega), as recommended by the manufacturer.

2.3.12 Southern hybridisation

2.3.12.1 DNA transfer to membrane

After electrophoresis, agarose gels were capillary blotted to nitrocellulose membranes (Boehringer Mannheim) (Sambrook *et al.*, 1989) as follows. DNA in the gel was denatured by soaking the gel for 45 min in several volumes of 1.5 M NaCl and 0.5 N NaOH, with constant agitation. The gel was rinsed briefly in distilled water and neutralised (1 M Tris, pH 7.4, 1.5 M NaCl) for 30 min, with occasional shaking of the tray from side to side. DNA transfer from the gel to the nylon membrane was performed by capillary action and blotting was carried out for 16 h using 20X SSC (3 M sodium chloride, 0.3 M sodium citrate).

2.3.12.2 DIG DNA labelling

λ DNA were labelled in a random primed fashion with digoxigenin-labelled deoxyuridine-triphosphate (DIG-dUTP) (Boehringer Mannheim). Reaction mixtures in 20 μl contained 3 μg of

DNA, 4 μl of hexanucleotide mixture, 4 μl dNTP labelling mixture (1 mM/litre of dATP, dCTP, dGTP; 0.65 mM/litre of dTTP; 0.35 mM/litre DIG-dUTP, pH 7.5), and 2 μl Klenow enzyme (2 U/ml). After incubation overnight at 37°C, the reaction was terminated by adding 4 μl of 0.2 M EDTA (pH 8.0) then 5 μl 4 M LiCl and 150 μl of ethanol were added. The precipitate was collected by centrifugation and the pellet was rinsed with 70% ethanol, dried at room temperature for one h before resuspending in 50 μl of TE buffer.

2.3.12.3 Hybridisation of membrane-bound nucleic acids to DIG-labelled λDNA

Prehybridisation and hybridisation of membrane-bound nucleic acid to DIG-labelled λDNA were performed according to the manufacturer's instructions (Boehringer Mannheim). Membranes were dried at 80°C for two h, prehybridised in a sealed bag which contained 20 ml of hybridisation buffer (5X SSC; blocking reagent; 0.01%, w/v, N-lauroylsarcosine; 0.02%, w/v, SDS) at 68°C for two h. The membrane was then transferred to a fresh bag and hybridised at 68°C overnight with DIG-labelled λ DNA (5 μl) in 3 ml of the same buffer. Blots were washed at room temperature for 5 min with 100 ml of 2X SSC; 0.1% SDS, followed by a wash at 68°C in 0.1X SSC; 0.1% SDS, for 15 min. All washes were carried out in duplicate with agitation.

2.3.12.4 Detection of hybridisation signal

DNA on membranes was detected by enzyme-linked immunoassay using an antibody-conjugate (antidigoxigenin-alkaline phosphatase conjugate) and subsequently colour developed using 5-bromo-4-chloro-3-indolyl phosphate (X-phosphate) and nitro blue tetrazolium salt (NBT). This procedure was described by the manufacturer and used without modification. Membranes were washed in buffer 1 (100 mM maleic acid, 0.15 M NaCl, pH 7.5) and incubated for 30 min with 100 ml buffer 2 (1:10 of blocking reagent in buffer 2). They were then incubated with anti-DIG-

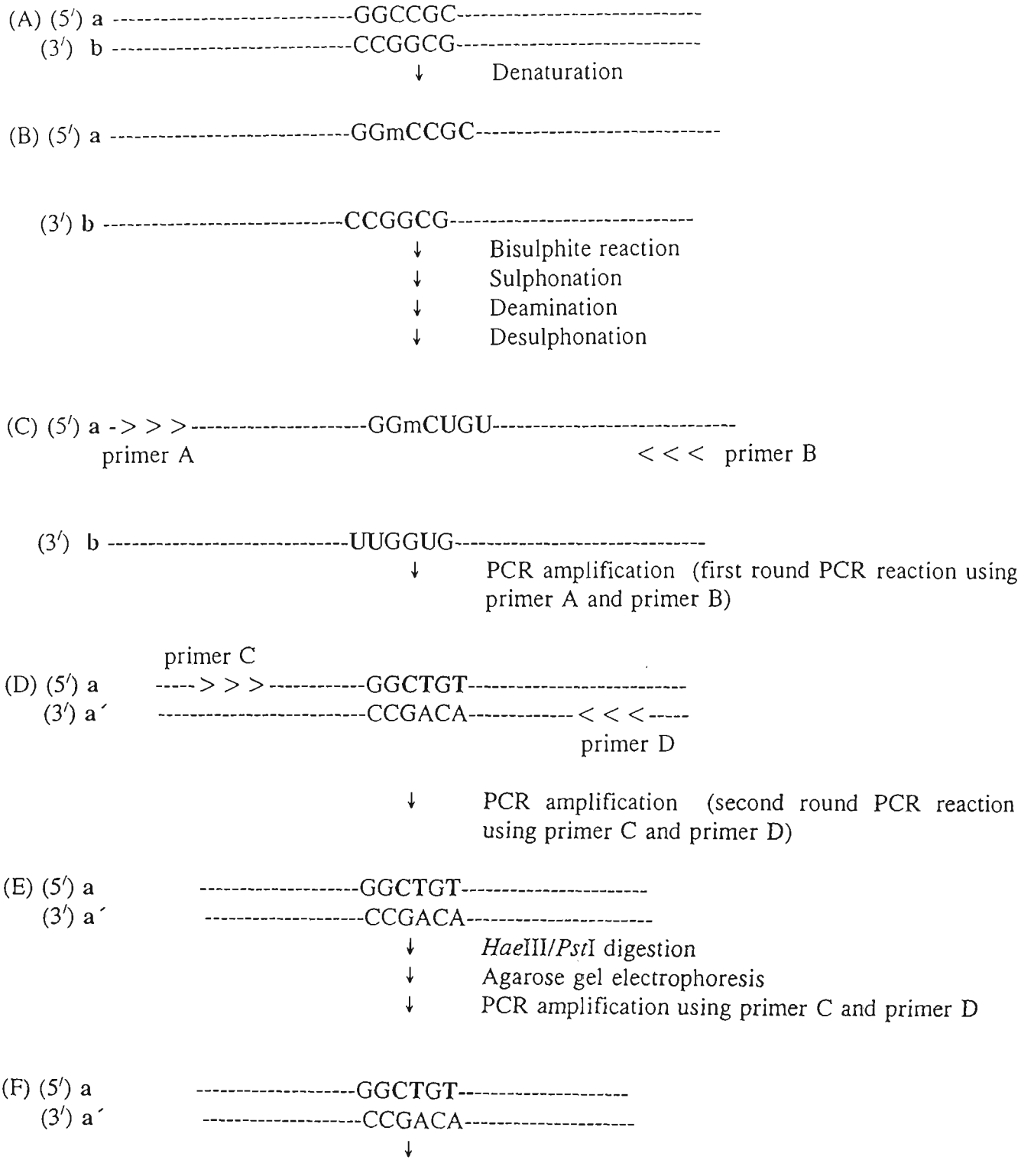


Fig. 2.1 Bisulphite genomic sequencing procedure (from Clark *et al.*, 1994). Strands in the original DNA are labelled **a** and **b**. For the present work, only one strand (a) was examined and cytosine residue and their corresponding uracil and thymine conversion procedures are shown in bold type. It should be noted, after the bisulphite reaction, the two DNA strands (a) and (b) are no longer complementary and therefore can be amplified independently.

alkaline phosphatase for 30 min and washed twice in buffer 1 before incubation in buffer 3 (100 mM Tris. Cl, 100 mM NaCl, 50 mM $MgCl_2$; pH 9.5) for 2 min. Following this, membranes were placed in a colour substrate solution consisting of NBT and X-phosphate in buffer 3.

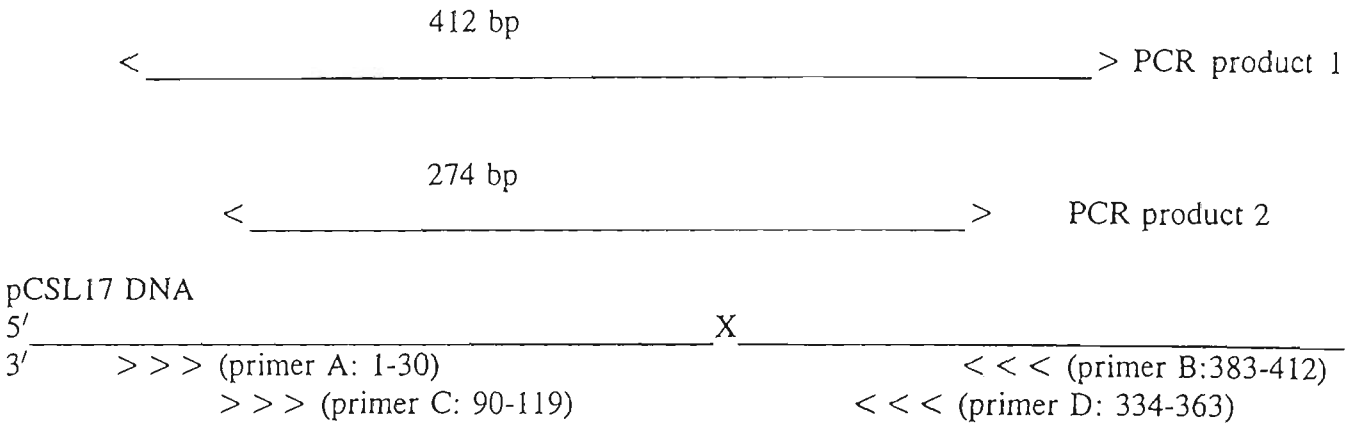
2.3.13 Determination of methylated bases in DNA using bisulphite modification

The procedure used here was obtained from Dr. Susan Clark (Kanematsu Laboratories, NSW, Australia).

2.3.13.1 Sodium bisulphite modification

This procedure has been used for detection of m^5dCyd in the mammalian DNA and was used here for detection of methylation of dCyd base in bacterial DNA sequences. This method contained the following procedures (Fig. 2.1)

Plasmid pCSL17 (2 μg) derived from *C. glutamicum* AS019 and *B. lactofermentum* was digested in 20 μl by 10 units of *BglII* restriction enzyme at 37°C for three h, and denatured by adding freshly prepared 3 M NaOH to a final concentration of 0.3 M then incubated for 15 min at 37°C. At this stage, solutions of 10 mM hydroquinone (BDH) and 2 M sodium metabisulphite (BDH) were prepared and adjusted to pH 5.0 with 10 M NaOH. The solution of bisulphite was achieved by gently inverting the reagent/water mixture, with minimum mixing and aeration, and the pH adjustment before all solids were dissolved. Aliquots of 208 μl of sodium metabisulphite, and 12 μl hydroquinone were added to each reaction tube containing denatured DNA to a final concentration of 0.5 mM. The reaction mixtures were then overlayed with 40 μl mineral oil and incubated at 55°C, in the dark, for 16 h. The bisulphite-treated DNA was recovered from under



primer A	
Original	5′ CTC CAG ATT TAT CAG CAA TAA ACC AGC CAC
Converted	5′ TTT TAG ATT TAT TAG TAA TAA ATT AGT TAT
primer	5′ TTT TAG ATT TAT TAG TAA TAA ATT AGT TAT
primer B	
Original	5′ CTT ACT GAA CCA ACT CAT GAG TGG TCA GTG
Converted	5′ TTT ATT GAA TTA ATT TAT GAG TGG TTA GTG
primer	3′ AAA TAA CTT AAT TAA ATA CTC ACC AAT CAC 5′
primer C	
Original	5′ ATT GTT GCC GGG AAG CTA GAG TAA GTA GTT
Converted	5′ ATT GTT GTT GGG AAG TTA GAG TAA GTA GTT
primer	5′ ATT GTT GTT GGG AAG TTA GAG TAA GTA GTT
primer D	
Original	5′ CCG TAC TGT CAT TCT CTT AAT ACG TCA CGA
Converted	5′ TTG TAT TGT TAT TTT TTT AAT ATG TTA TGA
primer	3′ AAC ATA ACA ATA AAA AAA TTA TAC AAT ACT 5′

Fig. 2.2 Schematic diagram of the nested primer set designed for amplification of a segment of the pCSL17 DNA derived from *C. glutamicum* or *B. lactofermentum*. Numbers in parentheses give the nucleotide positions of each primer. Numbering is started from the beginning of primer A. Target site (GCCGC) is indicated by X and located between 305 and 309. Primer A-D were; original, DNA sequence before bisulphite treatment; converted; DNA sequence after bisulphite treatment.

the oil layer by pipetting and free bisulphite removed by passing the sample through a desalting column (Wizard DNA Clean-Up System, Promega) and eluted in 50 μl of deionised water. Freshly prepared NaOH (3 M) was added to a final concentration of 0.3 M and the sample incubated at 37°C for 15 min after vortexing. The solution was neutralised by addition of 33 μl of 5 M ammonium acetate (pH 7.0) and the modified DNA was collected by ethanol precipitation (330 μl). DNA was dried, resuspended in 100 μl of TE buffer and stored at -20°C.

2.3.13.2 PCR amplification

Oligonucleotides were synthesised by Ms. U. Manuelpillai (Ph.D student, CBFT, VUT) with an Applied Biosystems model 391 DNA synthesizer (Perkin-Elmer). Chemicals and columns were obtained from Perkin-Elmer, and the primer was used as recommended by the manufacturer's instructions. The PCR kit (Gene Amp^R PCR core reagent) was obtained from Perkin-Elmer and contained all components used for PCR reactions except for DNAs and primers. One set of PCR primers were prepared to amplify the unmodified DNA while two sets of primers were prepared to amplify the modified DNA site. In the latter case, these primers sets will be referred to as the outer (primer A and primer B in Fig. 2.2) and inner (primer C and primer D in Fig. 2.2) primer sets. Initially, the outer primer sets were used to PCR amplify the target DNA. Once completed, the target DNA was again amplified using the inner primer set. Also, a combination of the inner and outer primer sets were used to PCR amplify the target DNA. The PCR amplification in 100 μl reaction mixtures contained 5 μl of bisulphite-treated DNA, 200 μM dNTPs, 0.5 μM primers, 3 mM $MgCl_2$, 50 mM KCl, 10 mM Tris, pH 8.3, 0.5 μl (2.5 units) *Taq* DNA polymerase, under the following conditions: 94°C/2 min X 1 cycle; 94°C/1 min, 50°C/2min, 72°C/3 min, 5 cycles; 94°C/0.5 min, 50°C/2min, 72°C/1.5 min, X 25 cycles; 72°C/6 min X 1 cycle, in a Perkin-Elmer DNA Thermal Cycler 480.

2.3.13.3 Sequencing

Sequencing reactions were prepared using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer). This was performed following the manufacturer's instructions. Sequence analysis was performed at Monash University (Department of Microbiology) on the ABI 373-Automated DNA Sequencer (Perkin-Elmer) (VUT joint facility).

2.4 TRANSFORMATION OF DNA

2.4.1 Electrotransformation with coryneform bacteria

2.4.1.1 Instrumentation

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

2.4.1.2 Preparation of plasmid DNAs

pCSL17 DNA was prepared by an alkaline lysis method and further purified with CsCl-EtBr density gradient centrifugation. *E. coli* strain LE392-derived pCSL17 DNA was isolated and used as the source of heterologous DNA, while *C. glutamicum* strain AS019-derived pCSL17 was used as the source of homologous DNA. The preparations contained a mixture of supercoiled and relaxed circles, as seen by agarose gel electrophoresis. The plasmids were resuspended in distilled water and stored in aliquots at -20°C. Because transformation efficiencies were dependent

on accurate DNA quantification, the DNA concentrations of these stocks were measured in two ways (see section 2.3.5): spectrophotometric measurement and spectrophotofluorimetric measurement.

2.4.1.3 Preparation of cells

Three media were used differentially for growth: LBG, LBG-GI ([2% glycine plus 4 ml INH, for AS019, MLB133 and MLB194] and [2% glycine plus 0.4 mg/ml INH for ATCC 13032, RM3 and RM4]). To obtain growth in liquid media, LBG starter cultures were grown overnight as 10 ml cultures in 20 ml McCartney bottles (30°C and 200 r.p.m.). Cells (100 ml) were harvested by centrifugation (2,000 X g, for 10 min, 4°C) on a bench top centrifuge (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 cold 15% (v/v) glycerol. These concentrated cells were used directly for electroporation.

2.4.1.4 Electroporation

The following procedures were performed in the lamina flow to ensure aseptic conditions. Forty μl samples of cell suspension (approximately 10^9 cells) were mixed in Eppendorf tube (1.5 ml capacity) with various amounts of DNA and up to 5 μg of water. The total volumes was adjusted to 72 μl with distilled water and 100% (v/v) glycerol, and the final concentration of glycerol in the mixture was 15% (v/v). Subsequently, the contents of the tubes were mixed thoroughly, stored on ice for 5 min, and then transferred by pipetting to a pre-cooled 0.2 cm cuvette (BioRad); the cuvette was then placed in a pre-cooled pulse chamber, and the cells were exposed to a single pulse (2.5 kV, 25 μF). 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 arcing occurred during the pulse, the sample was 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 to 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 of cells were made in SMMC and appropriate dilutions spread onto LAG, NAG or ET media (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). 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, NAG or ET media. Enumeration of transformants was done using triplicate plates. Controls included untreated cells plated onto ET-Km medium.

2.4.1.5 Characterisation of transformed cells

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. The presence of plasmid DNA in presumptive transformants was isolated by the alkaline lysis method, digested by restriction ENases and fragment patterns confirmed by agarose gel electrophoresis. To account for variation in the number of cells treated, transformation efficiencies were calculated as transformants per μg of DNA per number of cells pulsed. Alternatively, transformation frequencies were counted as transformants per number of cells which survived.

2.4.2 Transformation with *E. coli*

Competent *E. coli* cells were prepared by $CaCl_2$ treatment as described by Sambrook *et al.*

(1989). SOB broth (100 ml) was inoculated with an overnight 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 tubes. Plasmid DNAs of 100 ng (pCSL17 DNA isolated from *E. coli* LE392 or pCSL17 DNA isolated from *B. flavum*) were 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 the tubes transferred to an ice bath. After 2 min on ice, SOC medium (800 μl) was added, then tubes incubated at 37°C for 45 min. Serial dilutions of cells were plated in triplicate onto SOB and SOB containing 50 μg/ml kanamycin and incubated overnight at 37°C. The presence of plasmid DNA in transformants was confirmed using agarose gel electrophoresis of alkaline lysates. Transformation efficiency was calculated as colony forming units (c.f.u.) per μg of plasmid DNA.

2.4.3 Effect of methylation of DNA by *Hae*III on efficiency of electroporation

These experiment were performed to test whether or not methylation of *E. coli*-derived pCSL17 by *Hae*III MTase increased efficiency of electroporation into *C. glutamicum* strains. Reaction mixtures in 60 μl contained 15 μg of pCSL17 DNA derived from *E. coli* LE392, 80 μM S-adenosylmethione, 6 μl of 10X MTase buffer (500 mM NaCl, 50 mM Tris, pH 8.5, 10 mM DTT), 40 μl of deionised water, and 50 units of *Hae*III MTase (10 U/μl, Biolabs) and incubated at 37°C for three h. Successful methylation of DNA was checked by the addition of 1 μl of 10X reaction enzyme buffer (NE buffer 2 from Biolabs), 0.4 μg of methylated DNA, and 5 units of *Hae*III ENase in a 10 μl reaction mixture. Incubation for three h at 37°C was followed by analysis an agarose gels (1.2%). In the restriction digestion, unmethylated pCSL17 DNA derived

from *E. coli* LE392 was used as a positive control. Methylated DNA was also incubated with *Hind*III ENase using similar condition described as above; in order to check if methylated DNA could be cleaved by an other restriction enzyme.

For electroporation transformation, methylated pCSL17 DNA isolated from *E. coli* LE392, pCSL17 DNA isolated from *E. coli* LE392 and pCSL17 from *C. glutamicum* AS019 were used as sources of heterologous and homologous DNA. The three sources of pCSL17 DNAs (0.5 μg) were electrotransformed into two *C. glutamicum* strains by protocols described in section 2.4.1. Except for the source of plasmid DNA, all conditions used for electroporation were identical.

2.5 BIOCHEMICAL METHODS

2.5.1 Isolation and analysis of fatty acids and mycolic acids from whole cells and culture fluids

2.5.1.1 Extraction of long-chained lipid components from whole cells and culture fluids

Cells were subjected to acid methanolysis as previously described (Minnikin *et al.*, 1980) and the liberated methyl esters of mycolic acids studied by thin-layer chromatography (TLC), gas chromatography (GC) and mass spectrometry (MS) techniques (Collins *et al.*, 1982a; Pierotti, 1987). Cells (50-250 mg, wet weight, depending on the culture) were harvested at stationary phase by centrifugation (2,000 X g, 4°C, 10 min); the supernatant was collected and used as the source of mycolic acids and fatty acids in the culture fluids. Both samples (culture fluids and cells) were stored at -20°C until analysis. Stored cells were lyophilised using a Dynavac freeze-

dryer unit for 16 h. Dried samples of cells were weighed and about 50 mg portions were transferred into 5 ml glass tubes (Wheaton). In early experiments, no internal standard was used but in later experiments, which aimed at quantifying rather than detecting mycolic acids in different fractions, several potential internal standards were tested using GC for their suitability. These were lignoceric acid methyl ester (C_{25}), nonacosanoic acid methyl ester (C_{30}), triacontanoic acid methyl ester (C_{31}) and hentriacontanoic acid methyl ester (C_{32}). Since lignoceric acid methyl ester appeared in the GC chromatogram earlier than any of these compounds tested and its detection did not interfere with the analysis of mycolic acids peaks, it was used as the internal standard and added to the samples as follows. At the beginning of sample extraction, 100 μl of lignoceric acid methyl ester was added as an internal standard to each sample and all analyses standardised using the area obtained following extraction and derivatisation of the C_{25} internal standard.

Dried cells and internal standard were subjected to acid methanolysis (methanol/toluene/H_2SO_4, 30:15:1, v/v/v, 3 ml) in sealed tubes for 16 h at 80°C as previously described by Minnikin *et al.* (1980). The reaction mixture was cooled to room temperature and the liberated mycolic acid methyl esters (MAMEs) and fatty acid methyl esters (FAMEs) were extracted using petroleum ether (b.p. 60-80°C, 2 ml). The upper 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 cotton wool and prewashed with diethyl ether [BDH]). The procedure was repeated twice by adding two aliquots of 2 ml of petroleum ether and proceeding as described above. The eluant were combined into a 5 ml glass tube and concentrated under a stream of nitrogen and stored at -20°C. Initially, dried extracts of samples were further analysed using TLC after dissolving in 50 μl of petroleum ether, which separated MAMEs and FAMEs. In later experiments, analysis of mycolic acids was performed without TLC procedure after derivatising as described in the section 2.5.1.3 and 2.5.1.4. In cases of

quantitative analysis of MAMEs and FAMEs, TLC procedure was not applied in order to minimise sample loss during MAMEs and FAMEs preparations from both whole cells and culture fluids samples.

2.5.1.2 Thin-layer chromatography of acid methanolysates

Dried extracts of samples were reconstituted in 50 μl of petroleum ether, vortexed and then this was spotted onto aluminium-baked silica-gel plates (Merck). The plates were developed in petroleum ether-acetone (95:5, v/v). The separated MAMEs and FAMEs were detected by spraying with a 10% (w/v) ethanolic solution of molybdophosphoric acid (BDH) in ethanol followed by heating in an oven at 120°C for 15 min (Gunstone and Jacobsberg, 1972). Areas corresponding to R*f* values of MAMEs and FAMEs were scraped from the silica gel plate and then transferred into a glass tube. Two ml of diethyl ether was used for extraction. After vortexing, the solvent was collected, and passed through diethyl ether-washed cotton wool in a Pasteur pipette. The eluant was collected into a test tube and reduced to dryness under a stream of nitrogen. Dried MAMEs were then resuspended with 1 ml diethyl ether, vortexed thoroughly, and transferred into an autosampler vial (Alltech). After rinsing the test tube with 1 ml diethyl ether, the solvent was collected in the vial then the solvent removed under a stream of nitrogen. MAMEs and FAMEs were derivatised as described in the next section.

2.5.1.3 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).

2.5.1.4 Gas chromatography of TMS ethers of MAMEs

The TMS ethers 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 the split ratio set at 85:1. The BPX5 column was used for both GC and GC-MS. The oven temperature program was 260°C isothermally for one min, then increasing by 4°C/min to 300°C, then increasing to 320°C at 1°C/min. Finally, the column temperature was held at 320°C for four min before analysis of the next sample. The injection and detector temperatures were 300°C. Nitrogen was used as the carrier gas (0.6 ml/min). Injection of samples was carried out by an autosampler (Varian 8200) (needle was washed by nitrogen) between each injections.

2.5.1.5 Mass spectrometry of TMS ethers of MAMEs

The identity of the fractionated TMS ether of MAMEs obtained by GC was determined by GC-MS using a Varian Saturn II GC/MS equipped with a direct insertion probe. For GC-MS analysis, the column (25 m non-polar BPX5) was directly coupled to the ion source which was held at 300°C and helium (0.6 ml/min) was used as carrier gas. The oven temperature was programmed at 260°C for one min, then increasing by 10°C/min to 300°C, and increasing from 300°C to 320°C at a rate of 1°C per min. Finally, column temperature was held at 320°C for four min. The injection temperature was set at 300°C. Electron impact ionisation was used and the ionisation potential was 70 eV.

2.5.1.6 Assay of TMS ethers of MAMEs in culture fluids

To analyse mycolic acid composition of culture fluids, supernatant fluids were collected by centrifugation (see section 2.5.1.1) and the total volume filtered (0.22 μm, Millipore) before the application of one of two procedures used to extract and concentrate mycolic acids.

(i) Sample concentration by evaporation

MAMEs and FAMEs were also prepared from culture fluids by heating samples at 80°C for 16 h to concentrate. Cells were grown in the appropriate media (30 ml), harvested, filtered and then transferred into a 100 ml beaker. The beakers were heated at 80°C for several hours to reduce the initial volume then this was transferred into 20 ml MacCartney bottles and samples completely dried at 80°C in a water bath. After 16 h evaporation, dried samples were subjected to acid methanolysis, extracted by petroleum ether then concentrated under a stream of nitrogen. Finally, the mixture of MAMEs and FAMEs were silylated.

To quantify losses of mycolic acids found in the various growth media and following the extraction procedure described above, dried cells (250 mg) were weighed and dispensed in four 50 mg lots in four tubes. One of these was stored at -20°C without the addition of liquid while the other three were incubated at 80°C after the addition of 3 ml of water or 3 ml of growth medium; water, LBG and LBG containing 2% glycine plus 4 mg/ml INH. After 16 h incubation, which allowed evaporation of all liquid from the tubes, the four samples were subjected to acid methanolysis, extracted by petroleum ether, then concentrated under a stream of nitrogen. Finally, the mixture of MAMEs and FAMEs was silylated. After injection into the GC, samples were stored at -20°C for 30 days and then reanalysed using the same GC conditions, in order to check reproducibility of analysis and stability of TMS ethers of MAMEs at -20°C during storage.

(ii) Sample preparation by solvent extraction

Mycolic acids were also obtained from culture fluids using extraction methods which had been used for short chain fatty acids but had not been used previously for extraction of mycolic acids from culture fluids. Therefore, preliminary optimisation was required before applying these approaches to the experimental samples. Two different solvent systems were tested: (1) methanol-toluene-H_2SO_4 followed by petroleum ether and (2) chloroform-methanol followed by following chloroform-water. These were examined for their efficiency in extracting mycolic acids from culture fluids (30 ml) of LBG containing 2% glycine plus 4 mg/ml of INH. For the first solvent system (Bligh and Dyer, 1959), the culture fluids after filtration were shaken at 100 o.p.m. with 100 ml of methanol-toluene-H_2SO_4 (30:15:1, vol/vol/vol) for one h in a 500 ml glass bottle using a rotary shaker incubator and then samples were held stationary for four h. Subsequently, 60 ml of petroleum ether was added, and the mixture shaken for one h before being and then this transferred into a separation funnel. This was kept on the bench for 16 h to allow phase separation. After the collection of the top petroleum ether layer, it was transferred into a round-bottomed flask and the organic solvent was removed using a rotary evaporator (Eyela) set at 40°C. After solvents were evaporated to dryness, the extracts were transferred to GC vials by the addition of 2-3 ml of chloroform and the round bottomed-bottle was washed with chloroform twice and the washes collected into the vial. Samples were concentrated by removing chloroform under a nitrogen stream. Mycolic acids and fatty acids were converted to MAMEs and FAMEs respectively by acid methanolysis, extracted by petroleum ether then concentrated under nitrogen. Finally, MAMEs and FAMEs were silylated.

The method used for the second solvent system was described by Bligh and Dyer (1959) and modified by by Juhasz (Ph.D student, CBFT, VUT) (Honours thesis, 1991). The culture fluids (30 or 100 ml) after filtration were incubated with 3.3 volumes of chloroform/methanol (1:2, v/v)

for one h using a rotary shaker (100 o.p.m) at 30°C and then held stationary for four h. Subsequently, one volume of chloroform and one volume of deionised water were added, the mixture incubated for one h using a rotary shaker (100 o.p.m), and transferred into a separation funnel, which was kept on the bench for 16 h to allow phase separation. After collecting the bottom organic layer, the solvent was removed by rotary evaporation at 40°C, and the residue derivatised by acid methanolysis and extracted by petroleum ether. Subsequently, samples were concentrated under nitrogen and silylated, as described previously.

2.5.1.7 Gas chromatography of TMS ethers of FAMEs extracted from cells

FAMEs from cells were prepared as described in section 2.5.1.1 and 2.5.1.3. The TMS ethers of 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 the 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 was used as the carrier gas (0.9 ml/min). Injection of samples was carried out by an autosampler (Varian 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 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 standard, which was added prior to extraction and derivatisation.

2.5.1.8 Assay of TMS ethers of FAMEs in culture fluids

FAMEs from culture fluids were prepared by extracting FAMEs with chloroform-methanol (section 2.5.1.6) and analysed by GC (section 2.5.1.7).

2.5.2 Separation of DNA bases by high performance liquid chromatography

2.5.2.1 Reagents and sample preparation

The purity of the reagents used for preparation of buffers was crucial. All glassware was washed with chromic acid, rinsed with water, and then dried in a 150°C oven for 16 h.

All solvents were filtered to remove all particles larger than 0.45 μm (cellulose nitrate, diameter 47 mm, Whatman), and then degassed. The HPLC buffer was prepared as follows: a stock buffer concentrate was prepared as 1 litre of a 1.0 M solution of KH_2PO_4. This concentrate was then sterilised by filtering through a cellulose nitrate filter (Whatman) and stored in a brown-coloured glass bottle at room temperature. A 1 litre volume of the working buffer was prepared daily by diluting an aliquot of the buffer concentrate in ca. 200 ml of deionised water, adding the appropriate volume of methanol, diluting the solution to 1 litre with deionised water and filtering through a cellulose nitrate filter. Buffers were stored in a cold room at 4°C and discarded after three days. The methanol was HPLC grade. Salmon sperm DNA (10 mg/ml) was from Bethesda Research Laboratories. Plasmid (see section 2.3.2) and chromosomal DNA (see section 2.3.4) were isolated from cells and then further purified using CsCl gradient (section 2.3.3). Since both DNA and RNA nucleosides were detectable using the protocol mentioned in this section, DNA samples were treated with RNase A in order to remove RNA nucleosides from DNA samples. After phenol extraction to remove RNase A from DNA samples, 2.5 M sodium acetate (pH 5.3)

was added to give a final concentration to 0.3 M. DNA was precipitated by isopropanol, and washed with 70% EtOH. After washing, DNA were resuspended in 100 μl of water and 5-150 μg of DNA was used for enzyme digestion. DNA was digested to nucleosides with nuclease P1 (Sigma) and bacterial alkaline phosphatase (BAP, Sigma), as originally described by Gehrke *et al.* (1984).

Samples (100 μl) containing 5-150 μg of the DNA solution were pipetted into 1.5-ml centrifuge tubes and heated for 2 min in boiling water to denature DNA. The samples were immediately chilled in ice-water to prevent renaturation during cooling. Sodium acetate (10 μl of 300 mM solution, pH 5.3) was added to each sample, followed by 5 μl of 20 mM zinc sulphate. The tube was centrifuged for 5 sec at 14,000 X g to mix all components before 20 μl of nuclease P1 (1 mg/ml, 200 units per mg in 30 mM sodium acetate, pH 5.3) was added. Nuclease P1 is an ENase which quantitatively hydrolyses both DNA and RNA to 5′-mononucleotides with little or no specificity for the type of base (Gehrke *et al.*, 1984). The tube was incubated for at least four h at 37°C after centrifugation for 5 sec. The pH was adjusted to 8.5 by the addition of 40 μl of 0.5 M Tris and then centrifuged for 5 sec. After adding 20 μl of BAP in 50 mM Tris, pH 8.5, 150 units per ml, tubes were incubated for at least four h at 37°C after centrifugation for 5 sec. Since some BAP may be contaminated with deoxyadenosine deaminase which forms deoxyinosine, the BAP was heated at 95°C, pH 8.0, for 5 min and centrifuged (14,000 X g for 20 min) to remove the denatured protein. The final volume of the digested DNA sample was adjusted with deionised water to 400 μl and stored at -20°C.

2.5.2.2 Conditions for HPLC analysis

Quantification and identification of m^5dCyd, m^6dAdo and the major deoxynucleosides was performed using C^{18} reversed phase columns. Earlier work was carried out using Resolve™ C^{18}

column (90Å , 5 μm), with 3.9 mm X 30 cm internal diameter (Waters). When the following column was available, experiments was carried out using a C^{18} reversed phase column with 4.6 mm x 25 cm internal diameter (bondpack, Varian), where the system was fitted with a Waters Resolve C18 guard column with a particle size of 10 μm and pore size of 90Å, and a two-solvent systems at a flow rate of 1.0 ml per min. Gradient elution was performed using two solvents: solvent A (2.5%, v/v, methanol, 0.05 M KH_2PO_4, pH 4.0) (freshly prepared on the day of use, filtered and degassed) and solvent B (methanol) starting with 95% A and 5% B. A final ratio of 50% A and B was achieved by operating a continuous gradient over one h. A Varian HPLC system integrated by a 486 computer was used. Analyses were performed using a Varian 9012 solvent delivery system equipped with a Varian 9050 UV-VIS detector set at 254 nm and Varian 9100 autosampler (CA, USA). The solvent delivery system featured a single reciprocating pump and was ported to accommodate three proportioning valves. The injection system on the automatic sampler was an automatic injector, equipped with a 50 μl sample loop. A Varian 9065 UV-VIS detector was used to scan eluants from 220-320 nm, to differentiate nucleosides with similar volumes and identify m^5dCyd. The signal output from HPLC systems was integrated using the Varian Star Chromatography Software 4.01 package which was read through the ADC board of the 486 computer.

The concentrations of nucleosides were calculated from the areas under peaks compared to known nucleoside standards. Samples of 20 μl (or 50 μl) were injected for all tests and standards. External standards were obtained from Sigma and contained the following DNA and RNA nucleosides: DNA nucleosides [(deoxycytidine, dCyd), (deoxyguanosine, dGuo), (thymidine, Thd), (deoxyadenosine, dAdo), (m^5-methyl-deoxycytidine, m^5dCyd), (N^6-methyl-deoxyadenosine, m^6dAdo)]; and RNA nucleosides [(cytidine, Cyd), (uridine, Urd), (guanosine, Guo), (adenosine, Ado). A known amount of DNA nucleoside standard (50 μM-1 mM) was dissolved with deionised water and injected into HPLC and mol. concentration of each DNA base was calculated

by comparing the area of DNA samples with the area from their external nucleosides. Details of these procedures appear in the results section (see section 4.3.1, section 4.3.2 and section 4.3.3).

2.5.3 Preparation of cell-free extracts for characterising DNase activity

Cell-free extracts of coryneform strains were initially prepared by sonication. However, when a homogeniser became available, this was used instead since the time required for cell disruption was less. Cells which were grown in 400 ml of LBG containing 2% (w/v) glycine plus 4 mg/ml of INH were harvested by centrifugation (5,000 X g, 10 min, 4°C) and washed once with distilled water. The cells were pelleted and resuspended in 10 ml of protein extraction buffer containing 40 mM Tris, pH 8.0, 1 mM EDTA, 1 mM DTT. The cells were then left on ice for 5 min; all subsequent steps were performed with solutions held on ice and centrifugation performed at 4°C. Cells were disrupted by sonication using a Branson B-12 sonifer at 100 watts for a total of 10 min (several treatments of 20 sec with a large probe, output set on 8). The probe and the cells were kept chilled on ice throughout the procedure to prevent heat inactivation of enzymes in the cell-free extract. Cell debris and whole cells were then removed by centrifugation (14,000 X g, 10 min, 4°C). The supernatant fluids were removed from the pellet and stored at -20°C. The protein content of the cell-free extract was determined by the modified Lowry assay (section 2.5.4).

Cells from 1 l of LBG broth were harvested by centrifugation (5,000 X g, 4°C, 10 min) at stationary growth phase and the resulting cell pellet resuspended in 10 ml of beads (0.1-0.11 mm, B. Braun, Germany) and two volumes of protein extraction buffer (40 mM Tris, pH 8.0, 1 mM EDTA, 1 mM DTT). Cells were disrupted by homogenisation for a total of 3 min using a MSK cell homogeniser (B. Braun); the temperature of the homogenisation medium was maintained at 4°C by a flow of CO_2. All the purification steps were performed at 4°C, unless stated otherwise. The homogenised suspension was centrifuged (14,000 X g, 20 min) and the resulting supernatant

transferred into tubes and used for assay of DNase activity.

2.5.4 Estimation of protein concentration

The method was modified from the original described by Lowry *et al.* (1951). The sample volume was 0.5 ml, contained 0 to 100 μg of protein, to which was added 0.5 ml of solution A (0.1 ml of 5%, w/v, $CuSO_4$, 0.9 ml of Na_2CO_3 in 0.5 M NaOH). After 10 min at 37°C, 1.5 ml of solution B (one ml Folin-Ciocalteu's reagent plus 10 ml deionised water) was added and the solutions immediately vortexed. Absorbance at 680 nm was recorded after incubation at 52°C for 20 min. Standards containing 0 to 100 μg of protein were prepared from two mg/ml BSA solution, in the 50 mM Tris buffer (pH 7.2). Reagents A and B were prepared immediately before use from the stock solutions.

2.5.5 Estimation of deoxyribonuclease (DNase) activity

DNase was determined using two methods, these being detection methods using densitometer scans of photographic negatives and direct assay of activity in cell-free extracts. For the analysis of DNase activity using densitometry, cells were harvested at various growth stages, disrupted by either using sonication as described in section 2.5.3. DNA activity in cell-free extracts was determined by incubating these with CCC DNA and estimating the percentage of conversion in DNA form from CCC DNA to the open circular DNA or linear form of DNA. Reaction mixtures of 20 μl contained 15 μl of cell-free extracts, 2 μl of 10X One-Phor-All buffer (or 10X TA buffer), 2 μl of DNA solution (0.5-1 μg) and 1 μl of deionised water. Several identical reaction mixtures were prepared as above and incubated at 30°C. Reactions sampled over the different time courses (usually, 0, 5, 20, 40, 60, 120, 180, 240, 300, 360 min, and overnight) were terminated by transferring the tubes to a 65°C waterbath and incubating for 5 min. For the

negative controls, reaction mixtures were prepared as above but without DNA or cell-free extract. The total volume of negative controls were adjusted to 20 μl with deionised water and incubated at 37°C overnight. No change in the DNA forms from negative controls were observed during the incubation periods.

Chapter 3

The effect of cell wall modifiers on cell growth, electrotransformation, mycolic acid composition and cell wall physiology of selected species of coryneform bacteria

Part of work described in this chapter has been submitted to or accepted to the journals for publication as follows.

1. **Jang, K.H., Pierotti, D., Kemp, G.W., Best, G.R., and Britz, M.L.** (1997) Mycolic acid composition of *Corynebacterium glutamicum* and its cell surface mutants: effects of growth with glycine and isonicotinic acid hydrazide. Microbiology. 143: 3209-3221.

2. **Jang, K.H., and Britz, M.L.** Factors affecting electrotransformation frequencies of *Corynebacterium glutamicum* strain AS019 and its INH-sensitive mutant, MLB133. Biotechnology Letters. (in preparation).

3.1 INTRODUCTION

Previous publications 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; Haynes and Britz, 1990; Katsumata *et al.*, 1984; Noh *et al.*, 1991; Yoshihama *et al.*, 1985) improved transformation of plasmid DNA into these bacterial species.

The goal of the research described in this section was to obtain an understanding of how growth in the presence of cell wall modifiers (glycine and INH) affected cell wall structure of *C. glutamicum* and how this influenced introduction of plasmid DNA by electroporation.

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 increased 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, MLB133 and MLB194, were studied in this thesis. 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). Strains RM3 and RM4 were derivatives of *C. glutamicum* ATCC 13032 and are restriction deficient strains (Schäfer *et al.*, 1994a). The ATCC 13032 series were included for study to enable comparisons between different sources of *C. glutamicum*.

Typically, transformation efficiency in coryneform bacteria remains low when cells are grown in the absence of cell wall modifiers, even with homologously-derived DNA, implying that the cell wall structure of this species could be one of the key parameters in DNA transformation. Since INH and glycine are known to alter the mycolic acid and peptidoglycan structures of the cell wall

respectively, these chemicals were added to the growth media and the impacts of these chemicals on the cell physiology and cell wall structure were studied. Preliminary experiments included determining the inhibitory action of these compounds on growth of *C. glutamicum* strains, *Brevibacterium* species and some selected pathogenic coryneform bacteria. The effects of these cell wall modifiers on cell morphology, cell wall thickness and DNA transformation were also studied. The cell morphology investigation involved a comparative analysis of the cell wall thickness of MLB133, which was known to form protoplasts more readily, and AS019, a parental-type strain of *C. glutamicum*, which protoplasted poorly. Since mycolic acids are a major component of the cell wall of coryneform bacteria, part of this study involved the structural and quantitative analysis of these compounds in whole cells in extracellular fluids. The latter was pursued because earlier data of Pierotti (1987) suggested that the cell surface mutants of ATCC 13059 produced high levels of extracellular mycolic acids.

3.2 INFLUENCE OF CELL WALL MODIFIERS ON CELL GROWTH RATE

Cell growth of a number of coryneform bacteria in different concentrations of glycine, INH and Tween 80, in combination or alone, was compared to identify any differences in sensitivity to these additives. Initially, cell growth of coryneform bacteria was compared by determining the final A_{600} after 16 h incubation under standard incubation and growth conditions. However, as this approach provided no information in the kinetics of growth, sensitivity to cell wall modifiers was also compared using specific growth rates. For the latter case, since cell growth rates were reduced at the high concentrations of compounds used and long lag periods occurred before exponential phase commenced, monitoring growth was often extended to the next day or, indeed, over several days.

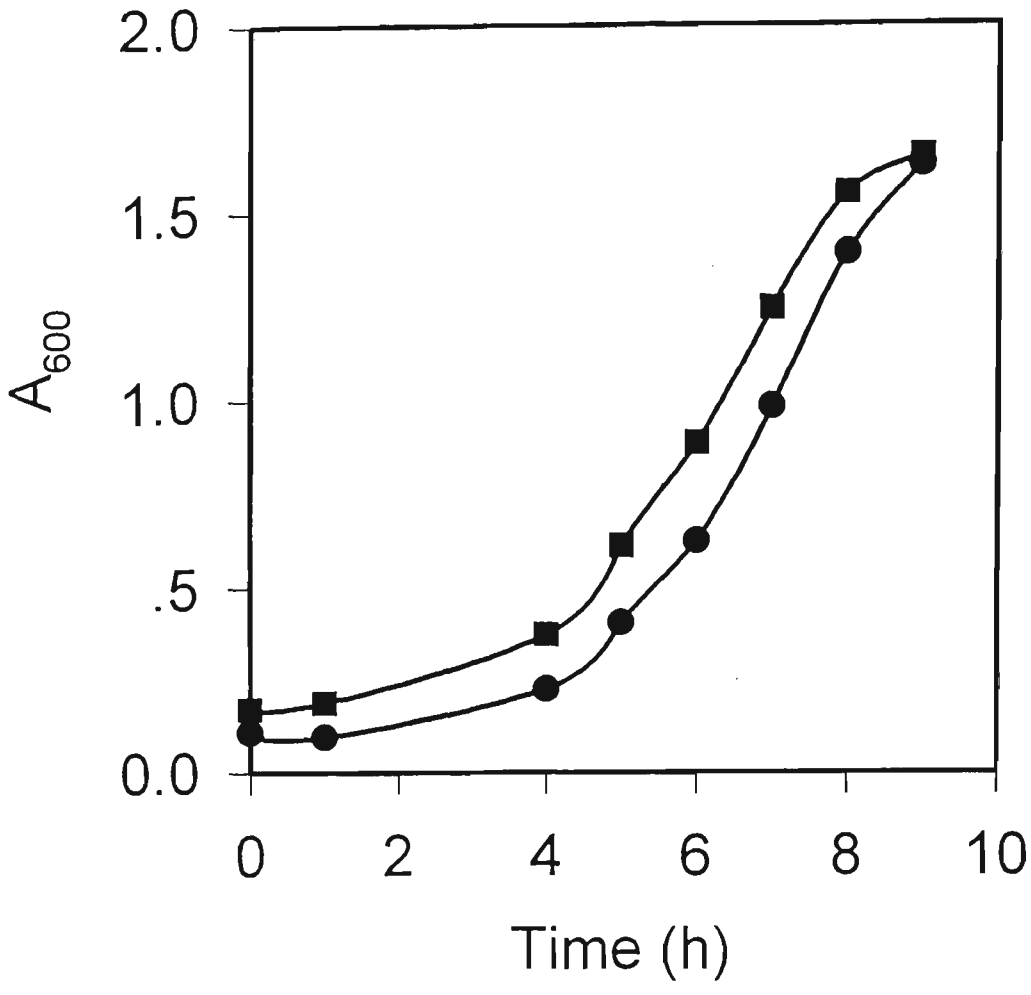


Fig. 3.1 Growth curves of *C. glutamicum* AS019 with different inoculum sizes. Cells of AS019 were inoculated to LBG medium from freshly prepared overnight cultures. Two sets of flasks (inocula 4%,●, and 8%,■) were prepared and incubated at 30°C, 200 o.p.m. Absorbance of cultures shown here were measured without dilution. Abbreviation: AS019, *C. glutamicum* AS019.

3.2.1 Preliminary experiments: influence of inoculum size on growth rate and relationships between A_{600} and viable cell count

Overnight, LBG starter cultures were used to inoculate 100 ml of LBG in 250 ml flasks to give 4% or 8% (vol/vol) inocula. As seen in Fig. 3.1, little or no difference in the growth curve pattern was observed during exponential phase with the different inoculum sizes. Based on this result, further experiments were carried out with starting A_{600} of 0.1 to 0.25, equivalent to an inoculum of approximately 4%.

Fig. 3.2 shows the relationship between A_{600} values and number of viable cells of AS019 during growth in LBG. Slightly higher numbers of cells were obtained when samples were diluted in SMMC buffer rather than diluted in water. Approximately 2-3 X 10^8 c.f.u./ml was counted at early exponential phase (A_{600} = 0.4) and the number of cells increased up to 2-3 X 10^9 c.f.u./ml by early stationary phase (A_{600} = 4).

3.2.2 Determining inhibition by glycine or INH from maximum absorbances obtained after overnight growth

As a preliminary screening method to determine the concentration ranges of glycine and INH which inhibited growth of several coryneform strains, the effect of these compounds on the final A_{600} reached after 16 h incubation was determined (Fig. 3.3). These data showed that the species and strains displayed different levels of sensitivity: under these test conditions, strain BF4 was relatively less sensitive to inhibition by glycine although both of the *Brevibacterium* species were more sensitive to INH. As seen in Fig. 3.3, the final A_{600} of all cultures tested decreased as the concentration of glycine increased. At glycine concentrations higher than 4% (w/v), cell

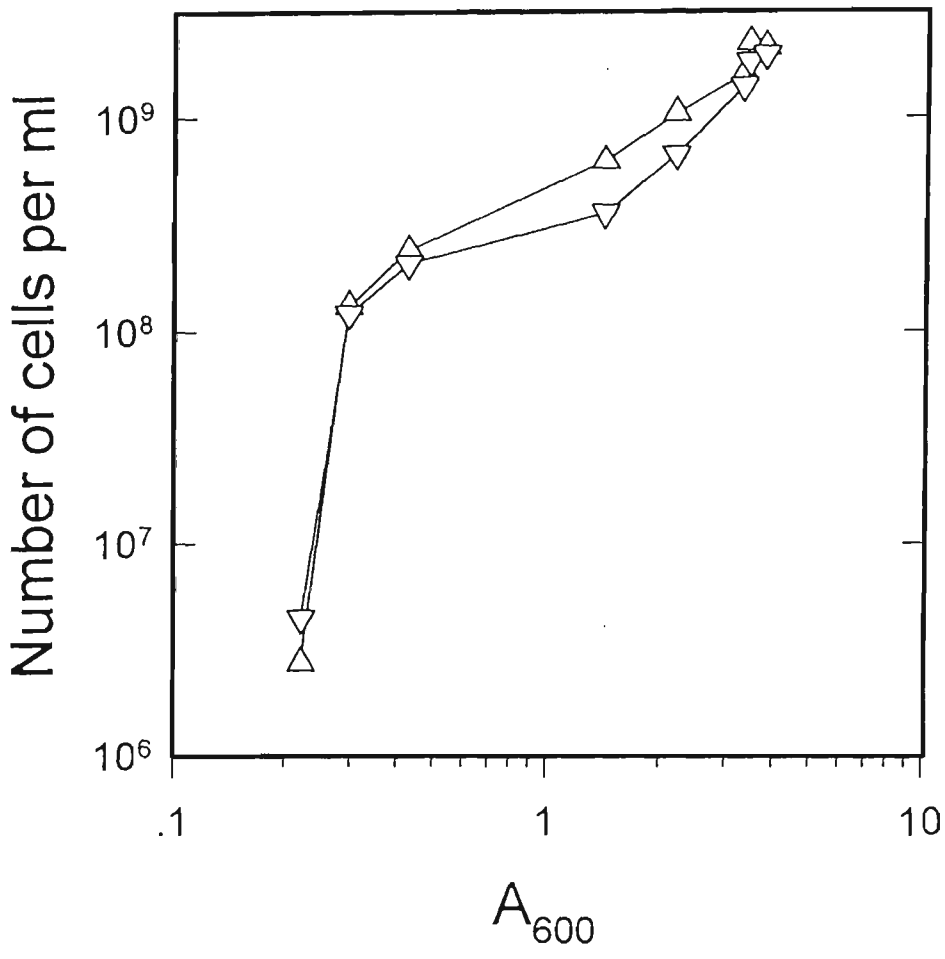


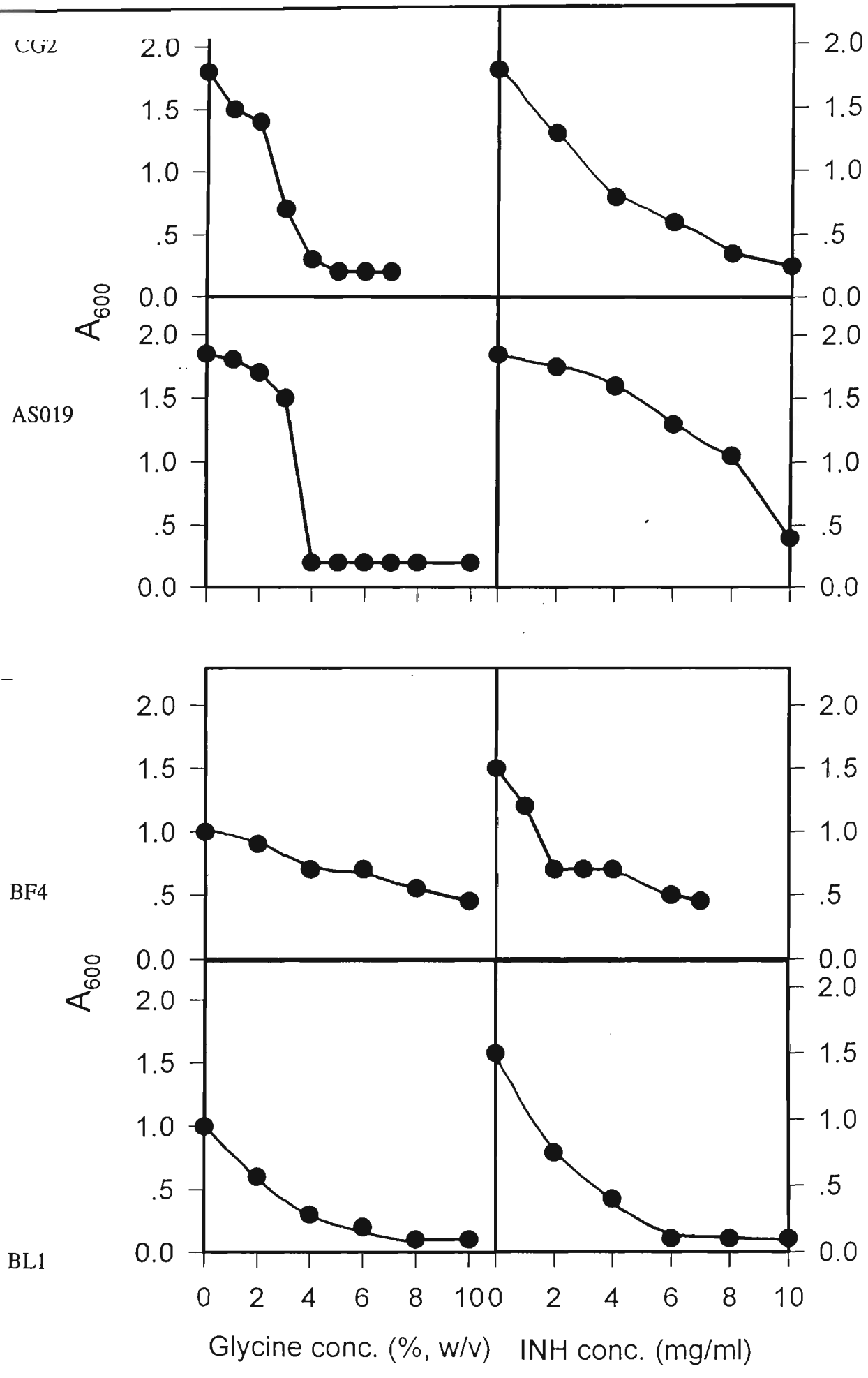
Fig. 3.2 Relationship between A_{600} and viable cells of *C. glutamicum* AS019 during cell growth. Cultures were grown in LBG medium and A_{600} values were measured hourly. Viable counts were performed using 10-fold serial dilutions of culture in SMMC (△) or water (▽). Samples of 0.1 ml were taken from appropriate dilutions and spread onto LAG. Absorbance readings of cultures were measured following dilution in LBG once readings were above A_{600} of 0.8. Cell counts were performed in triplicate and average numbers are shown.

grown of AS019, CG2 and BL1 strains was almost completely inhibited.

3.2.3 Effect of glycine on growth kinetics

The specific growth rates of strains grown in different concentrations of glycine were also determined. Each experiment contained the same media composition except for the concentration of cell wall inhibitor, noting that the medium was prepared using 2X LB and the volume was adjusted with distilled water following supplementation to give 1X LB. For each set of experiments, growth was also performed in the LBG medium without supplementation, and this used as the control. Glycine affected the rate of growth and the lag period seen before exponential growth commenced, depending on the strain. This was seen from the growth curves for AS019 (Fig. 3.4) and BL1 (Fig 3.5). For AS019, as glycine concentration increased, growth rate was decreased whilst no influence on lag phase was seen. BL1 was more sensitive to growth inhibition than AS019 (Fig. 3.5), as seen from preliminary experiments (Fig. 3.3). The growth rate of BL1 was inhibited little when grown in the presence of 1-2% (w/v) glycine and further increases in glycine concentration caused significant decreases in the specific growth rates; above 2-3% glycine, cell growth was completely inhibited for more than 10 h before growth started. Typical results from duplicate experiments are shown in Fig. 3.5. When no cell wall modifiers were added to the growth medium, the doubling time during the mid-exponential phase of growth differed for each strain, ranging from $0.35h^{-1}$ for RM4 to $0.75h^{-1}$ for BL1. The specific growth rates (μ values, h^{-1}) seen for the strains tested were: 0.60-0.70 (AS019), 0.60-0.65 (MLB133), 0.55-0.65 (MLB194), 0.40-0.50 (ATCC 13032), 0.55-0.65 (RM3), 0.30-0.35 (RM4), 0.50-0.60 (CG2), 0.60-0.65 (CU), 0.50-0.55 (BF4) and 0.65-0.75 (BL1). Since some strains (RM4, ATCC 13032, BF4) grew much more slowly in LBG than the other strains, growth rates were expressed as percentage values relative to controls in LBG to enable comparison between strains (Fig. 3.6). The specific growth rates of all strains decreased as the concentration of glycine in the growth

Fig. 3.3 Final absorbance at 600 nm of coryneform bacteria after growth in LBG with or without glycine or INH in the growth medium. Cultures (100 ml) of four corynebacteria strains were grown in the 250 ml flask containing LBG with either 0 to 10% (w/v) glycine or 0 to 10 mg/ml INH (0 to 7 mg/ml for BF4), with starting A_{600} = 0.1-0.25. Cultivation temperature and agitation speed were 30°C and 200 o.p.m. Results of final absorbance values after 16 h incubation were measured without dilution and represented the average values of three experiments. Abbreviations: CG2, *C. glutamicum* CG2; AS019, *C. glutamicum* AS019; BF4, *B. flavum* BF4; BL1, *B. lactofermentum*.


CG2
AS019
BF4
BL1
A_{600}
2.0
1.5
1.0
.5
0.0
0 2 4 6 8 10
0 2 4 6 8 10
Glycine conc. (%, w/v)
INH conc. (mg/ml)

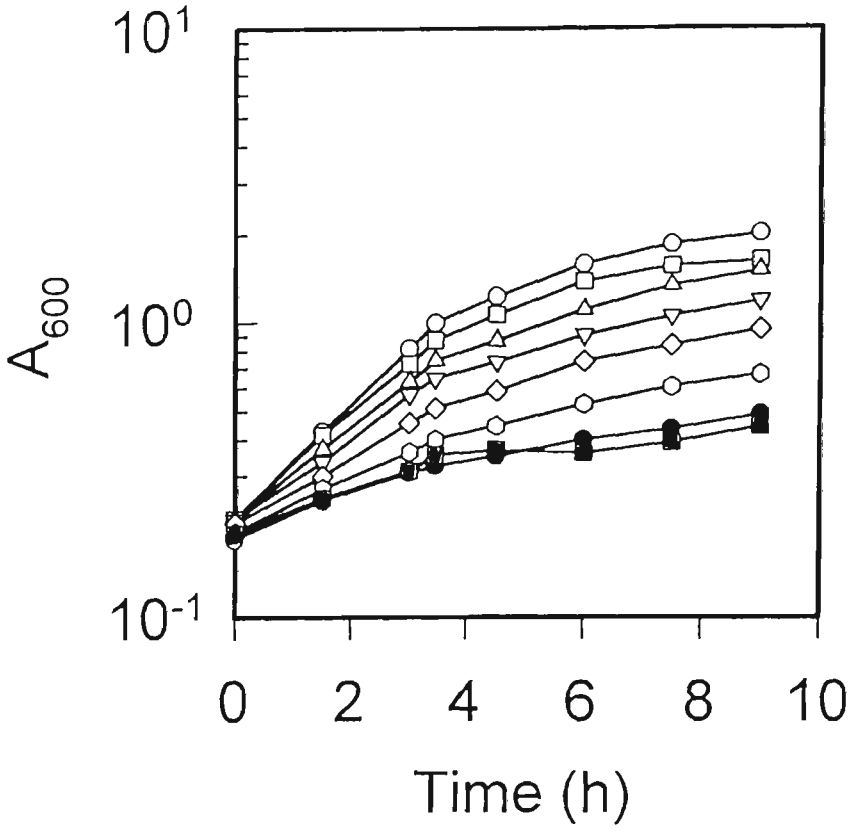


Fig. 3.4 Effect of glycine in the medium on growth of *C. glutamicum* AS019. Freshly prepared overnight starter cultures were inoculated into 250 ml flasks containing 100 ml of LBG plus glycine (0 to 10%, w/v) to give starting A_{600} of 0.2-0.25. Cultivation temperature and agitation speed were 30°C and 200 o.p.m. For the control, AS019 was grown in LBG. Two separate replicate experiments were performed where the results showed similar trends: results of one of these experiments is represented here.

Symbol	Glycine concentration (%, w/v) in LBG medium
(○)	0
(□)	1
(▽)	2
(△)	3
(◇)	4
(⬡)	6
(●)	8
(■)	10

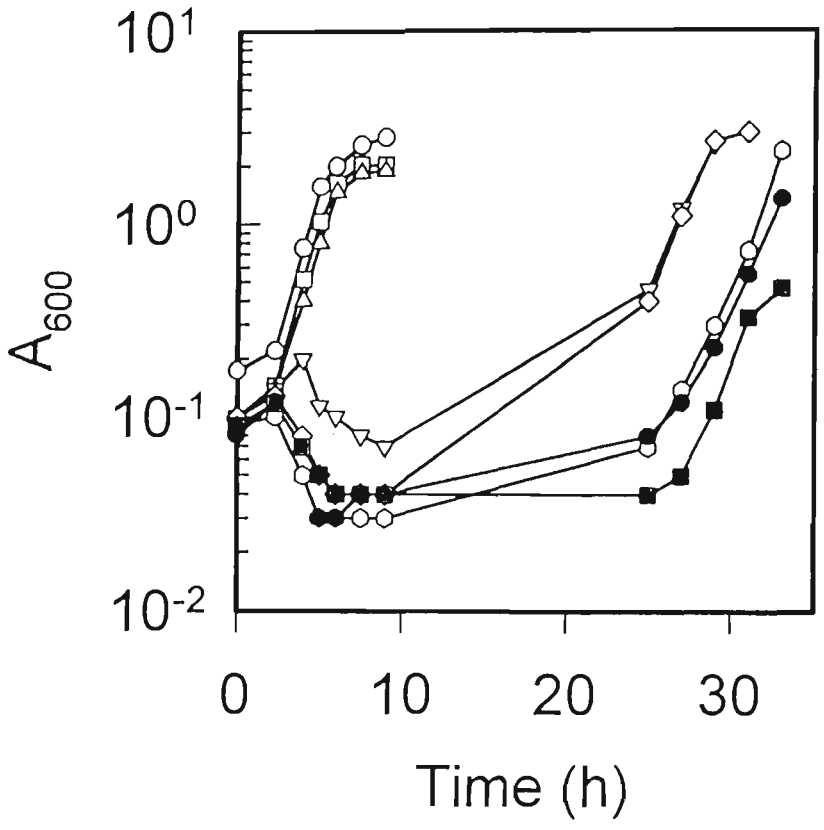
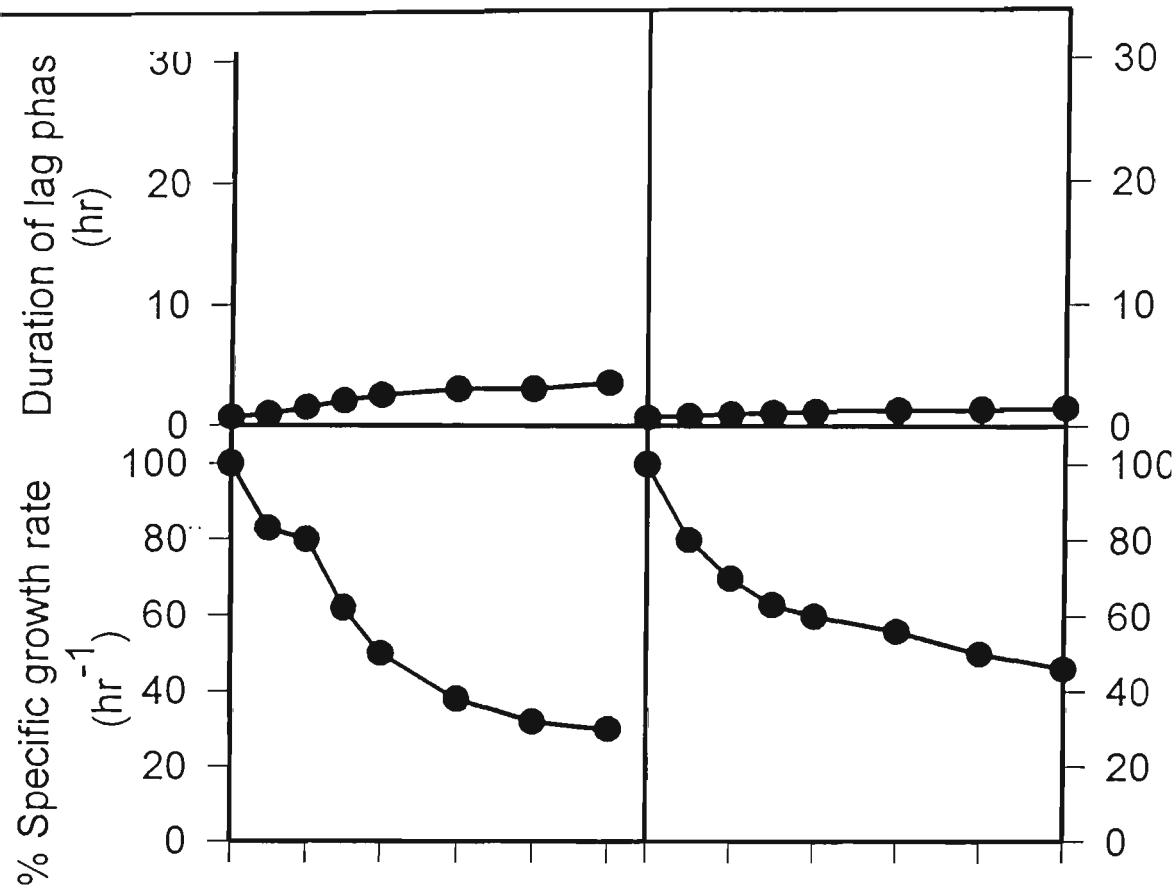


Fig. 3.5 Effect of glycine in the medium on cell growth of *B. lactofermentum* BL1. Freshly prepared overnight starter cultures were inoculated into 250 ml flasks containing 100 ml of LBG plus glycine (0 to 4%, w/v) to give starting A_{600} of 0.08-0.18. Cultivation temperature and agitation speed were 30°C and 200 o.p.m. For the control, BL1 was grown in LBG. At concentrations above 2% (w/v) glycine, absorbances were not measured during the period 9 to 24 h of incubation. Two separate replicate experiments were performed where the results showed similar trends: results of one of these experiments is represented here.

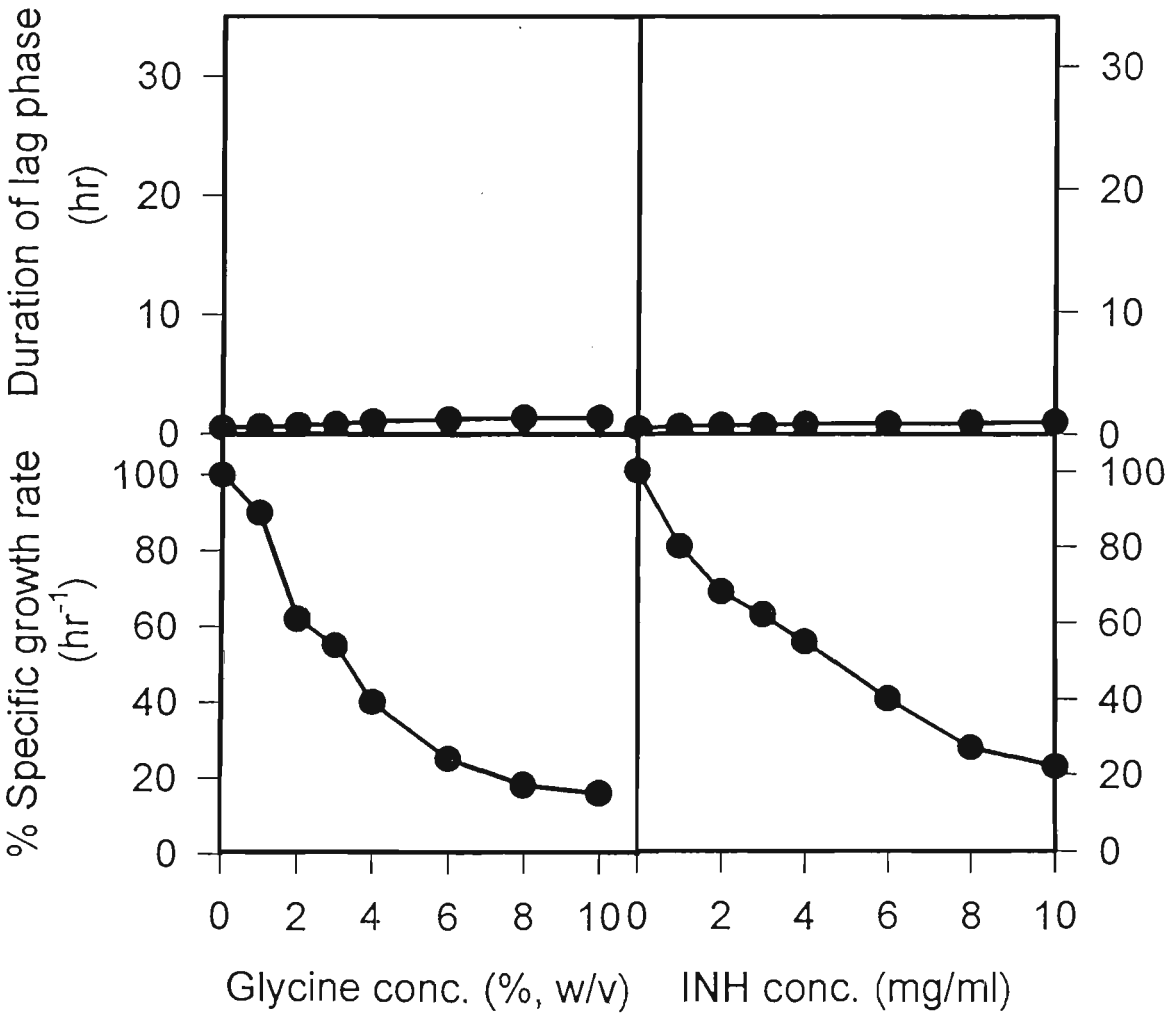
Symbol	Glycine concentration (%, w/v) in LBG medium
(○)	0
(□)	1
(△)	1.5
(▽)	2
(◇)	2.5
(●)	3
(⬡)	3.5
(□)	4

Fig. 3.6 Effect of glycine or INH in the medium on the specific growth rates and duration of lag phase of the coryneform bacteria tested. Freshly prepared overnight starter cultures were inoculated into 250 ml flasks containing 100 ml of LBG with glycine (0 to 10%, w/v) or INH (0 to 10 mg/ml), with starting A_{600} of 0.1-0.25. Cultivation temperature and agitation speed were 30°C and 200 o.p.m. For controls, cells were grown in LBG. Specific growth rates are presented as percentages relative to growth rates seen in LBG. Duration of lag phase was the time taken before exponential growth started. Abbreviations: AS019, *C. glutamicum* AS019; MLB133, *C. glutamicum* MLB133; MLB194, *C. glutamicum* MLB194; ATCC 13032, *C. glutamicum* ATCC 13032; RM3, *C. glutamicum* RM3; RM4, *C. glutamicum* RM4; CG2, *C. glutamicum* CG2; CU, *C. ulcerans*; BF4, *B. flavum* BF4; BL1, *B. lactofermentum* BL1.

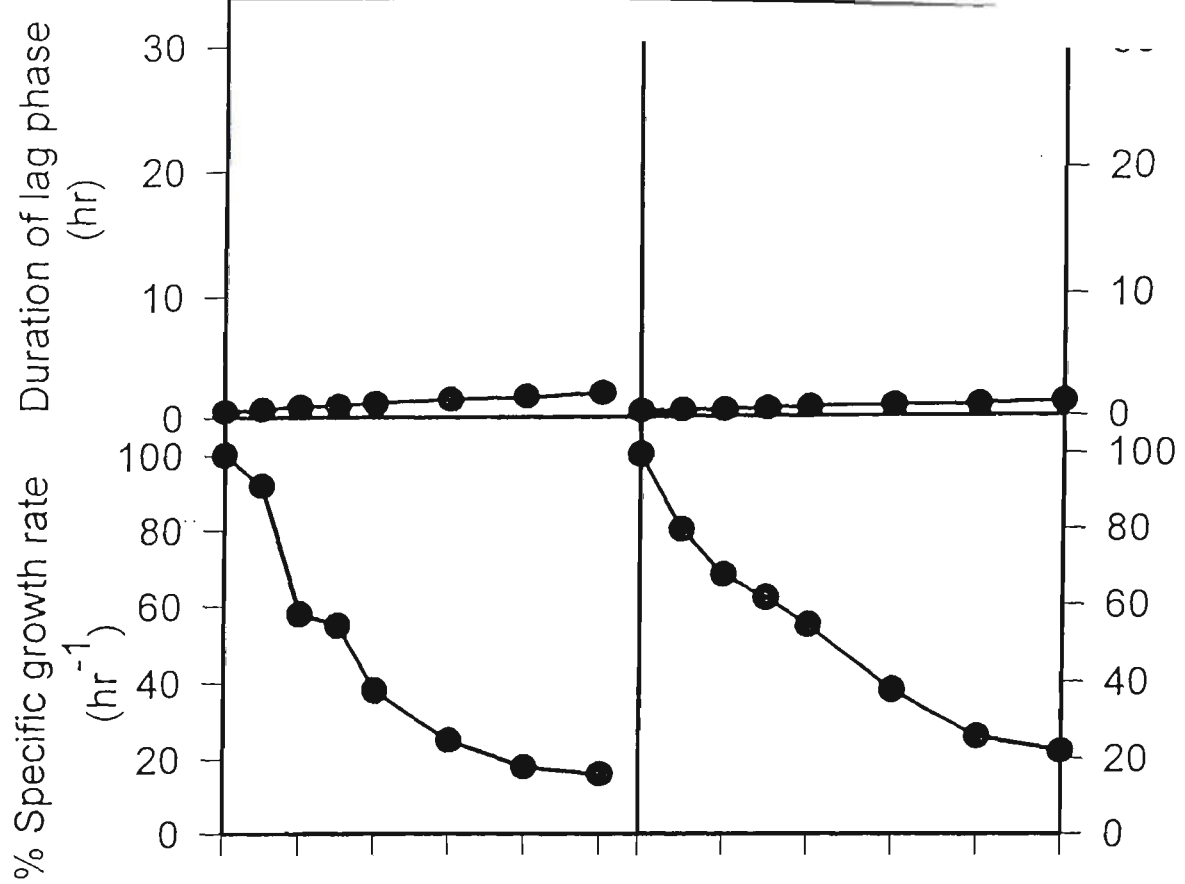
AS019



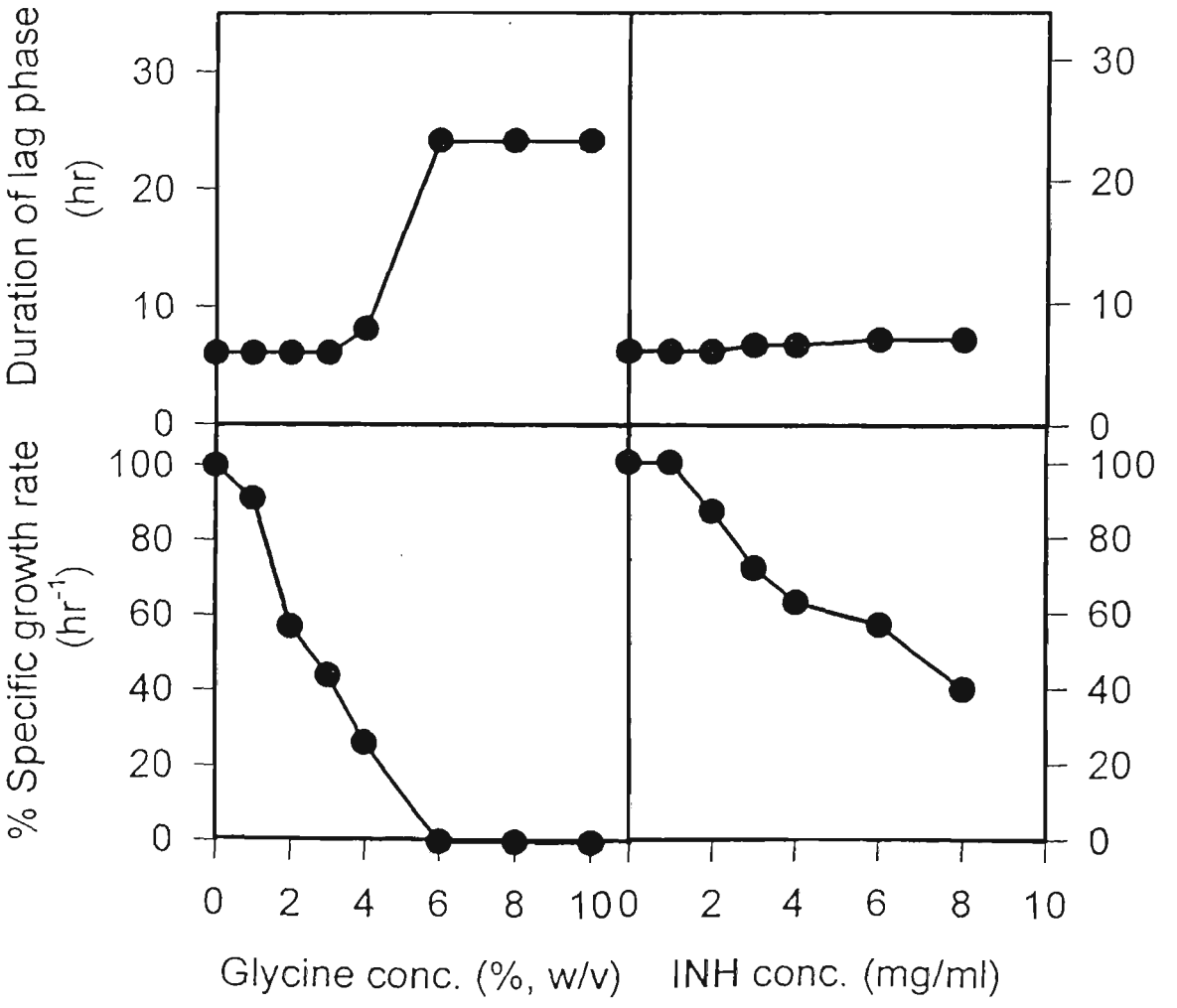
MLB133



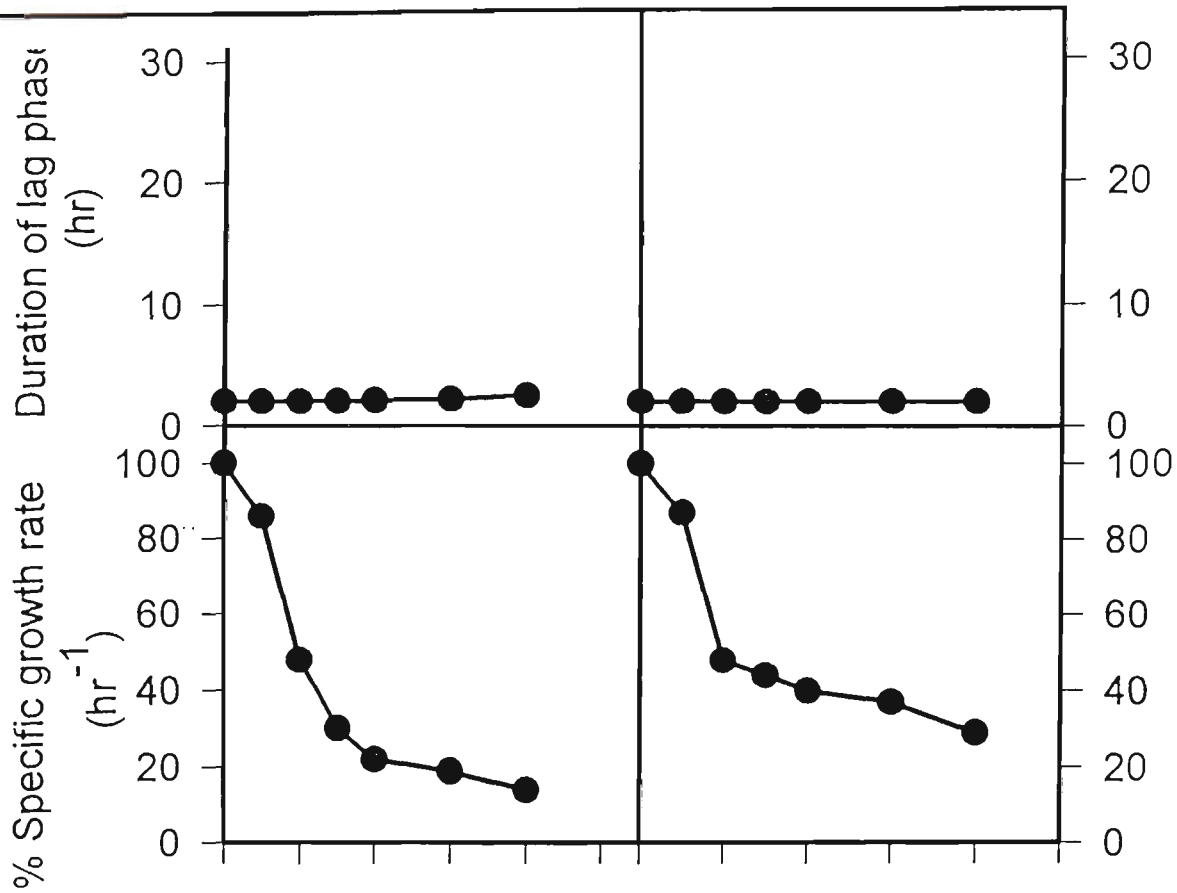
MLB194



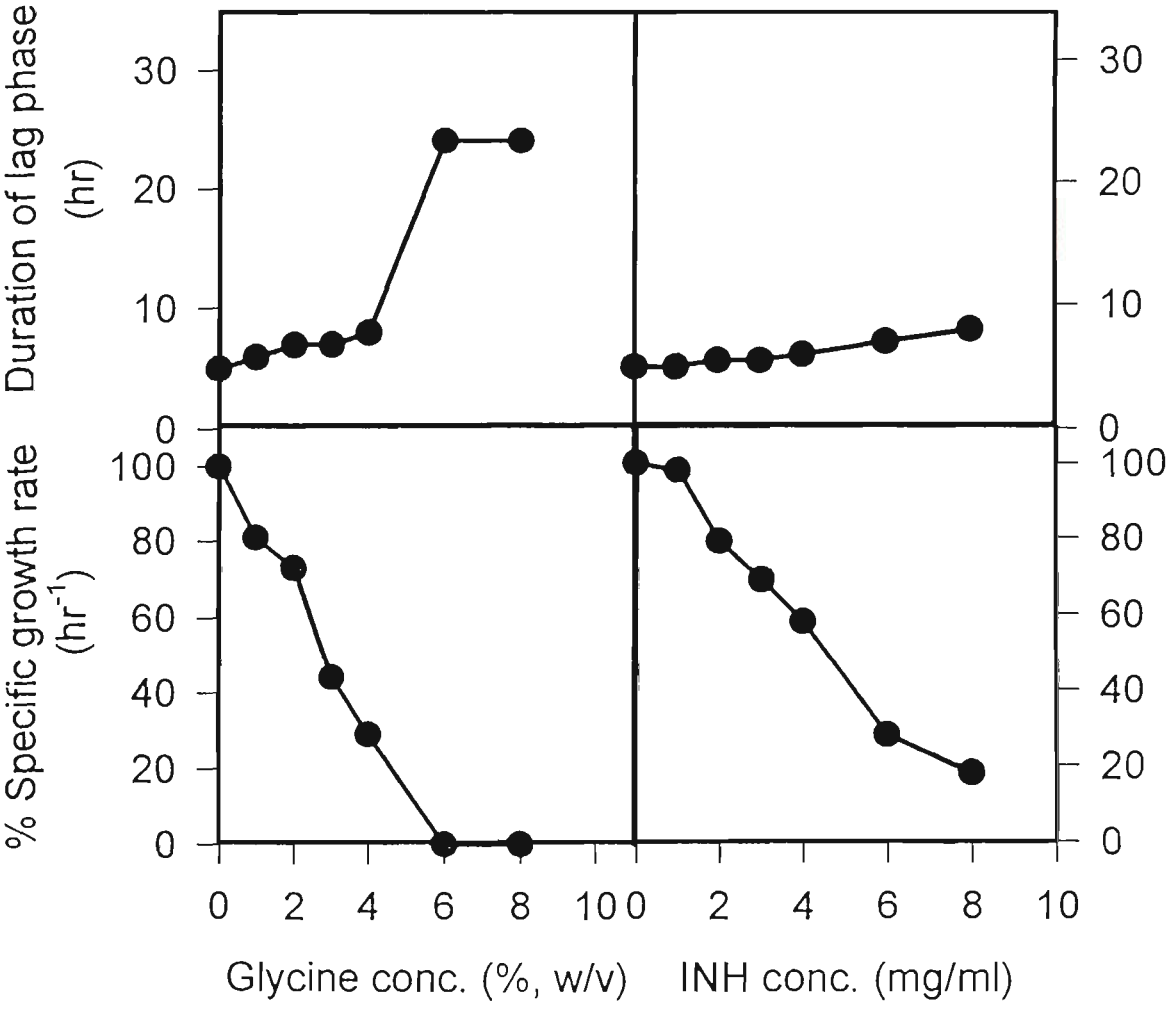
ATCC 13032



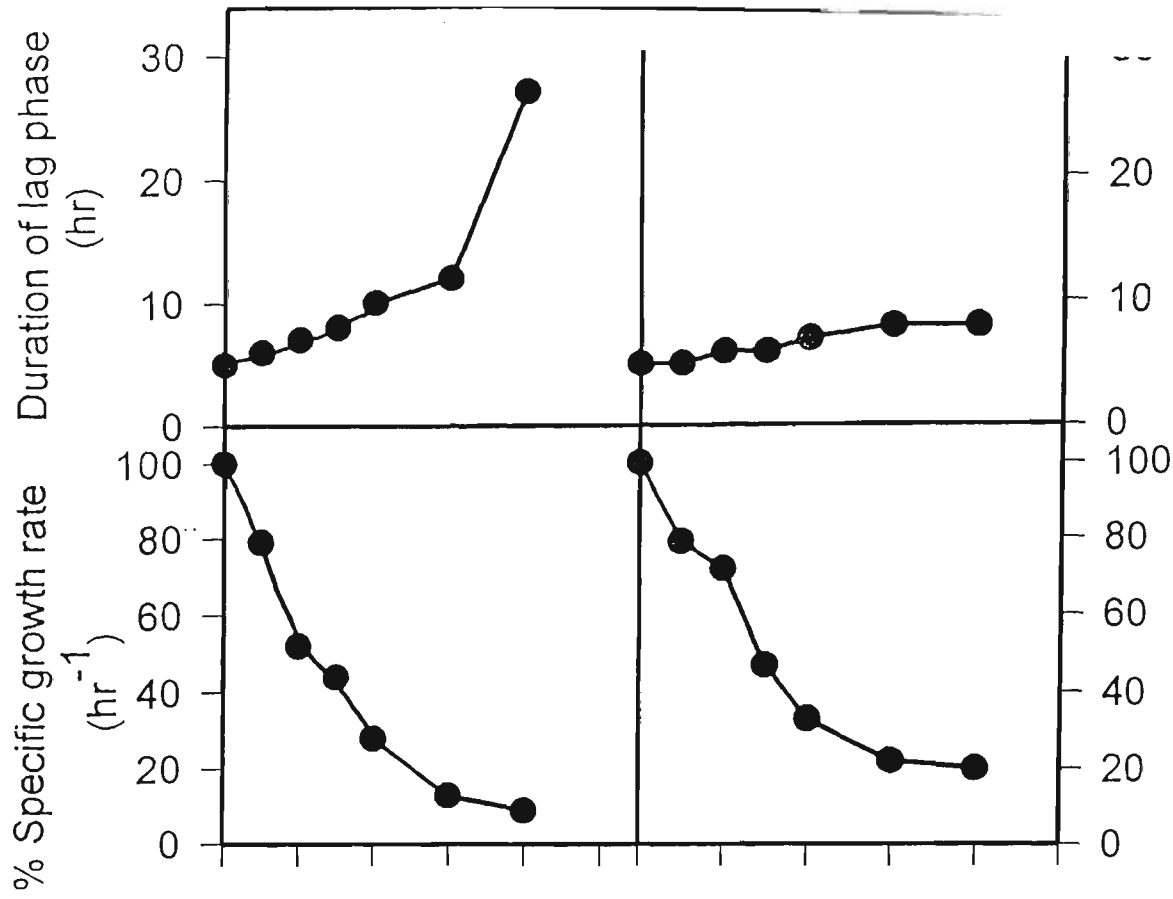
RM3


Duration of lag phas
(hr)
30
20
10
0
% Specific growth rate
(hr-1)
100
80
60
40
20
0

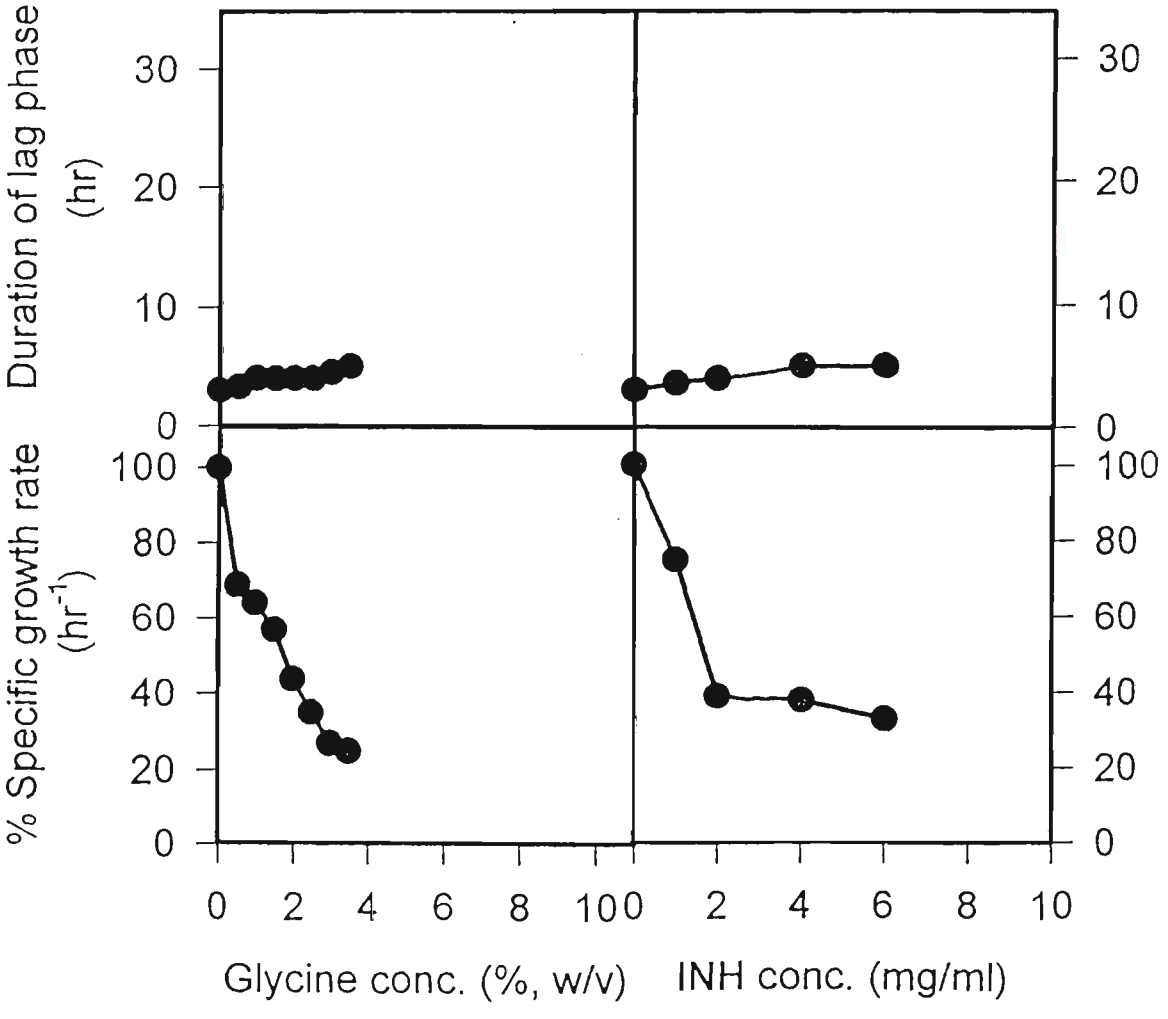
RM4


Duration of lag phase
(hr)
30
20
10
0
% Specific growth rate
(hr-1)
100
80
60
40
20
0
0 2 4 6 8 10
0 2 4 6 8 10
Glycine conc. (%, w/v)
INH conc. (mg/ml)

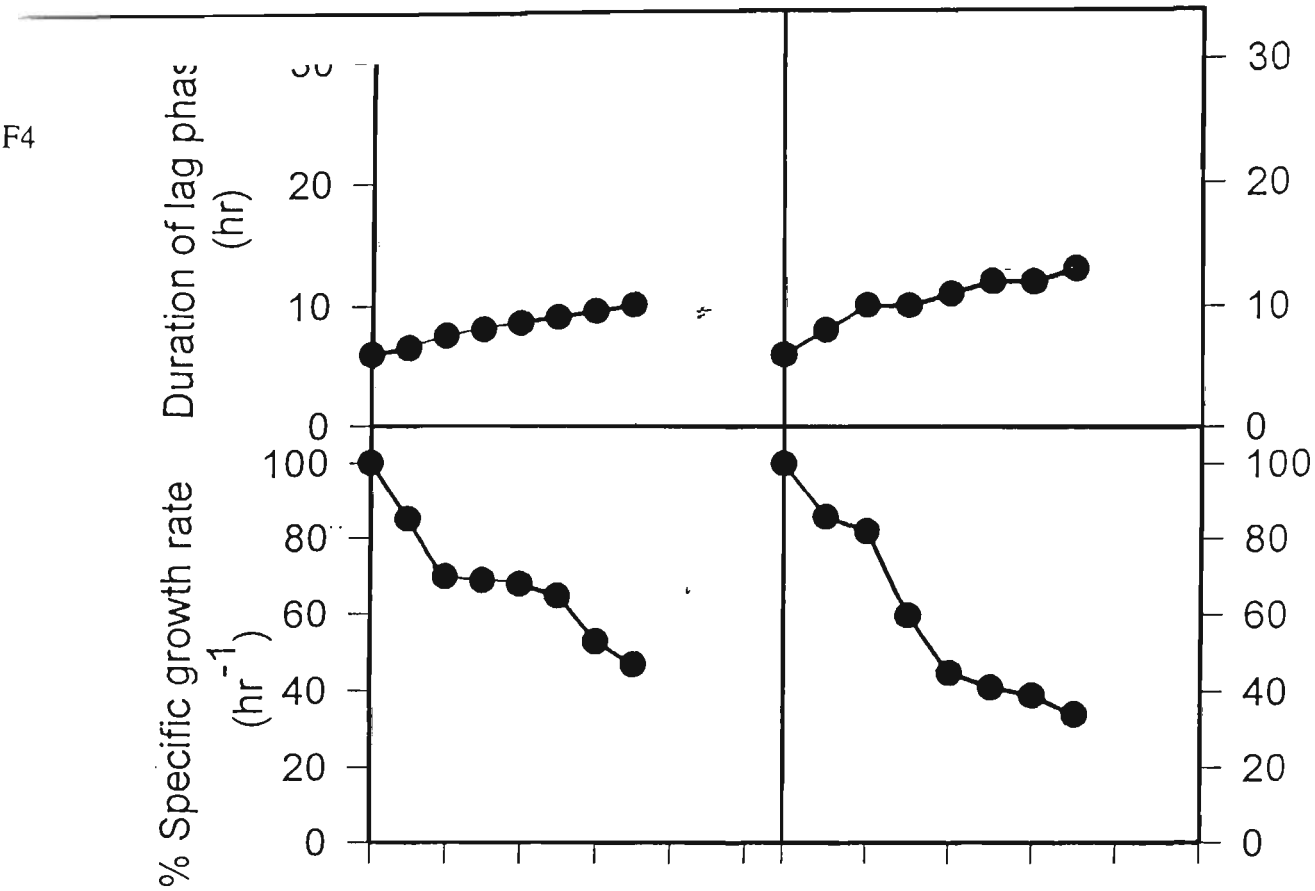
CG2


Duration of lag phase (hr)
% Specific growth rate (hr-1)
0
10
20
30
40
60
80
100

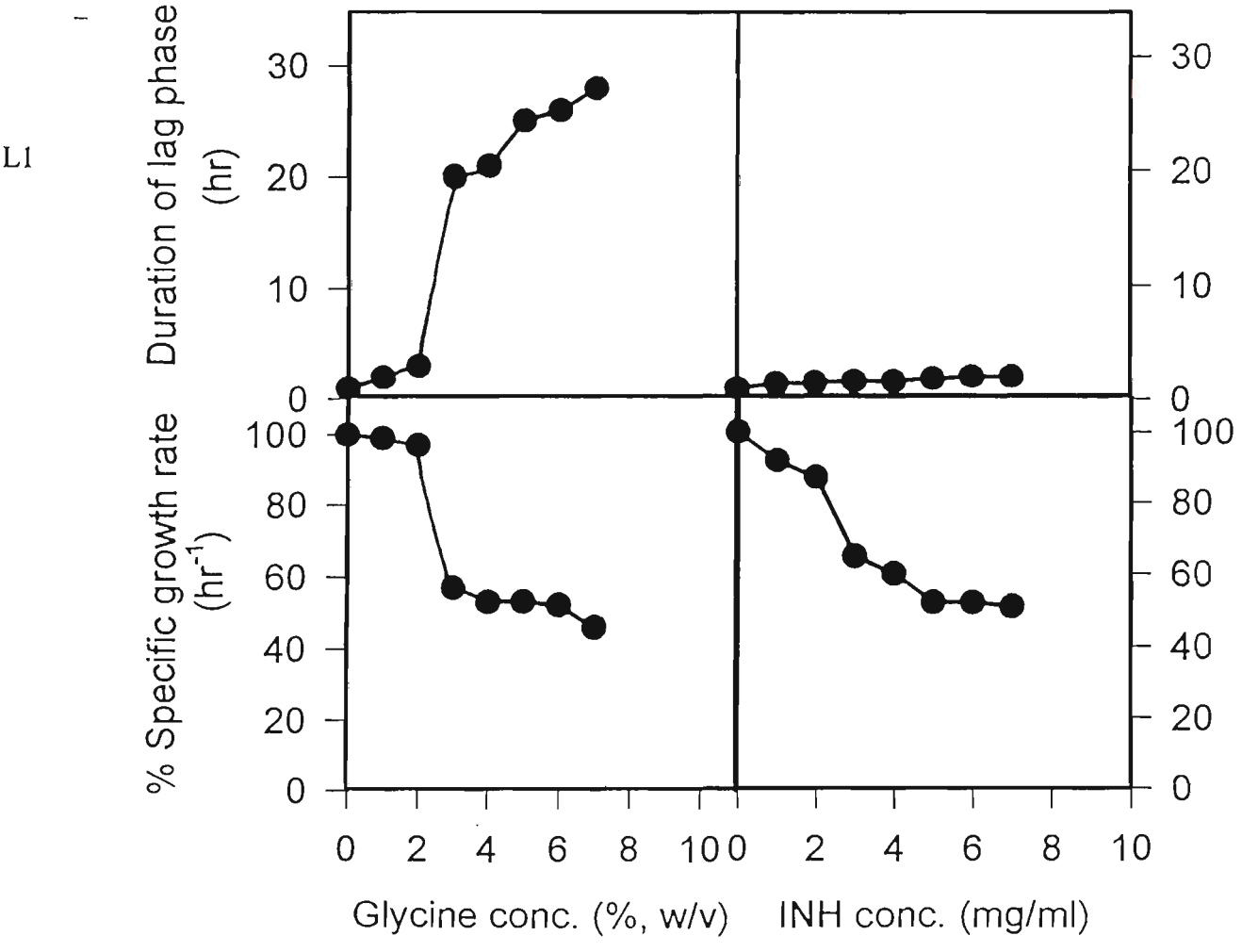
CU


Duration of lag phase (hr)
% Specific growth rate (hr-1)
0
10
20
30
40
60
80
100
0 2 4 6 8 10
0 2 4 6 8 10
Glycine conc. (%, w/v)
INH conc. (mg/ml)

BF4


Duration of lag phas
(hr)
% Specific growth rate
(hr-1)
0
10
20
30
40
60
80
100

BL1


Duration of lag phase
(hr)
% Specific growth rate
(hr-1)
0
10
20
30
40
60
80
100

0 2 4 6 8 10
0 2 4 6 8 10
Glycine conc. (%, w/v)
INH conc. (mg/ml)

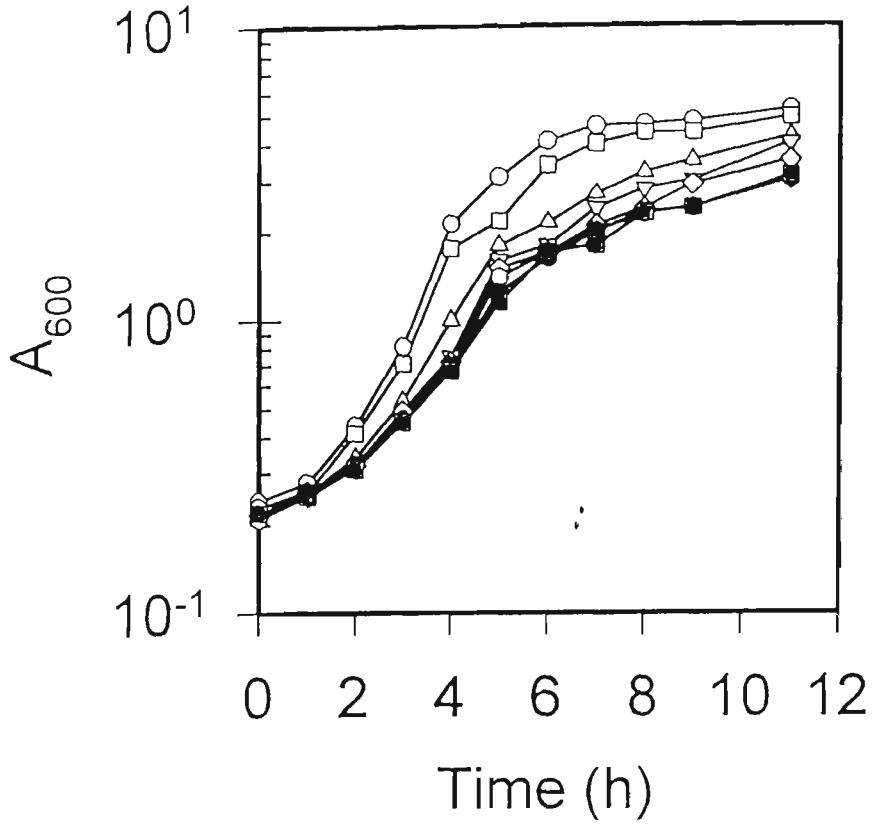


Fig. 3.7 Effect of the presence of INH in the medium on cell growth of *C. glutamicum* AS019. Freshly prepared overnight starter cultures were inoculated into 250 ml flasks containing 100 ml of LBG plus INH (0 to 10 mg/ml) to give starting A_{600} of 0.2-0.25. Cultivation temperature and agitation speed were 30°C and 200 o.p.m. For the control, AS019 was grown in LBG. Two separate replicate experiments were performed where the results showed similar trends: results of one of these is represented here.

Symbol	INH concentration (mg/ml) in LBG medium
(○)	0
(□)	1
(△)	2
(▽)	3
(◇)	4
(●)	6
(◎)	8
(■)	10

medium increased. As seen in preliminary experiments (Fig. 3.3), BF4 was less inhibited in the presence of glycine than the other strains tested (Fig. 3.6 and Table 3.1). For 2% glycine in the growth medium, the specific growth rate of the ATCC 13059 family (AS019, MLB133 and MLB194) was inhibited by 20 to 50%. In comparison, the specific growth rate of the ATCC 13032 family (ATCC 13032, RM3 and RM4) was inhibited by 30 to 50% by growth in the presence of 2% glycine. Of the *C. glutamicum* strains examined, AS019 was most resistant to growth inhibition to glycine and, at the concentration used increased, glycine did not alter the lag period. In contrast, ATCC 13032, RM4 and CG2 showed longer lag periods as the concentration of glycine increased, behaving like BL1 in this respect. The two mutant strains, MLB133 and MLB194, were more sensitive to inhibition by glycine than AS019. The difference in sensitivity was more significant at higher concentrations: the concentrations of glycine which caused 50% inhibition of the specific growth rate were 4% glycine for AS019 and 2% glycine for MLB133 and MLB194. For the ATCC 13032 family, RM4 showed higher sensitivity to glycine in the growth medium than ATCC 13032. Although RM3 was less inhibited by glycine in the growth medium than ATCC 13032, it showed much higher sensitivity than AS019 and BF4. The pathogenic coryneform bacterium, *C. ulcerans*, was highly sensitive to glycine: 50% inhibition of cell growth rate occurred at 1% glycine. When BL1 was grown in medium containing above 2-3% glycine, cell growth was completely inhibited for more than 10 h before growth commenced.

3.2.4 Influence of INH on cell growth

As shown in Fig. 3.7, AS019 grown in the presence of INH showed increasing inhibition of growth as the concentration of INH increased although the lag period was not greatly affected. Similar kinetics of growth were seen for all strains tested, as INH did not significantly alter the lag phase for any of these strains (with the possible exception of BF4), which is in contrast to the results for growth in glycine. Data presented in Fig. 3.6 suggested that there were differences in

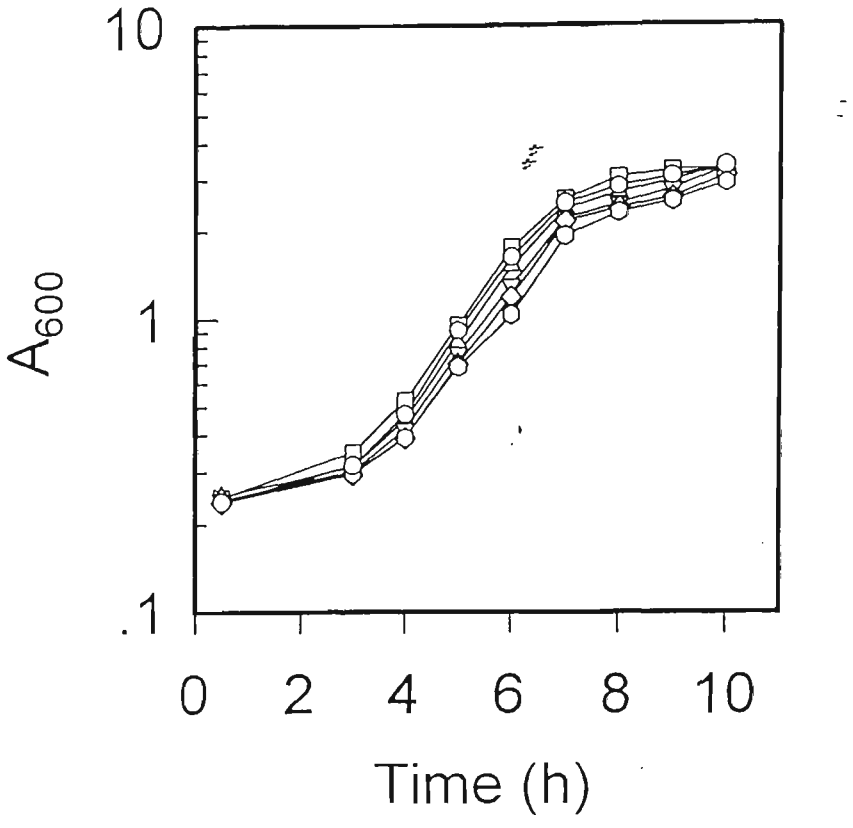


Fig. 3.8 Effect of the presence of Tween 80 in the medium on cell growth of *C. glutamicum* AS019. Freshly prepared overnight starter cultures were inoculated into 250 ml flasks containing 100 ml of LBG with Tween 80 (0 to 0.9%, w/v) to give starting A_{600} 0.2-0.25. Cultivation temperature and agitation speed were 30°C and 200 o.p.m. For the control, AS019 was grown in LBG. When A_{600} values were above 0.8, cultures were diluted in appropriate fresh media.

Symbol	Tween 80 concentration (%, w/v) in LBG medium
(□)	0
(○)	0.1
(△)	0.3
(▽)	0.5
(◇)	0.7
(⬡)	0.9

sensitivity to INH; AS019 was less inhibited than the other strains tested (CG2, BF4 and BL1). For the ATCC 13059 family, the two mutants were more inhibited by INH than the parent-type strain AS019: at 4 mg/ml INH, the relative specific growth rate was inhibited by 40% for both AS019 and the mutant strains. However, at 8 mg/ml of INH, the growth rate of AS019 was inhibited by 50% whereas the growth rates of strains MLB133 and MLB194 were inhibited by 70%. For the ATCC 13032 family, the two mutants RM3 and RM4 were more sensitive than their parent strain to inhibition by INH: 6-8 mg/ml of INH inhibited the growth rate of ATCC 13032 by 50% whereas this occurred at 2-4 mg/ml of INH for RM3 and RM4. The pathogenic strain *C. ulcerans* was the most sensitive to INH among the strains tested: at 2 mg/ml of INH, the growth rate was decreased by 60%. However, this and the other strains of coryneform bacteria tested were not inhibited by concentrations of INH (0.1 mg/ml) known to completely inhibit growth of *Mycobacterium*, *Nocardia*, *Rhodococcus* (Tomiyasu and Yano, 1984). As a group, the corynebacteria were relatively more resistant to INH then the other nocardioform group genera.

3.2.5 Influence of Tween 80 on cell growth

Prior growth in Tween 80 had been shown to increase transformation efficiencies in both *C. glutamicum* AS019 and *B. lactofermentum* BL1 (Haynes and Britz, 1989). However, since there had been no systematic study on the relative sensitivity of corynebacteria to Tween 80 this was undertaken here for selected saprophytic strains. Fig. 3.8 shows typical growth curves obtained for the strains tested: the lag period was not altered but the specific growth rate declined slightly with increasing concentrations of Tween 80. Four strains of coryneform bacteria (AS019, CG2, BF4, CU) were grown in LBG containing Tween 80 up to 0.9% (Fig. 3.9). However, over the concentration range used, growth was not greatly inhibited for any strain: growth of AS019 and CG2 was inhibited by 20% at the highest concentration of Tween 80 used, 0.9%. Although little

growth inhibition was seen here, even relatively low concentrations of Tween 80 significantly improved electrotransformation frequencies of AS019 and BL1, especially when used in combination with glycine (Haynes and Britz, 1989).

3.2.6 Effects on growth kinetics of combinations of cell wall modifiers

The effects of combinations of cell wall modifiers (glycine, INH and Tween 80) were also investigated using the following combinations: 2% glycine and various concentrations of INH (0 to 6 mg/ml) (Fig. 3.10); 2% glycine and various concentrations of Tween 80 (0.1 or 0.5%, w/v) (Fig. 3.11); 4 mg/ml of INH and various concentrations of Tween 80 (0.1 or 0.5%) (Fig. 3.12); 2% glycine plus 4 mg/ml INH plus various concentrations of Tween 80 (0.1 or 0.5%) (Fig. 3.13). Rather than designing experiments to cover a full matrix of possible combinations of concentrations, fixed concentration of cell wall inhibitors were chosen selectively and the results compared to appropriate controls (including growth in the absence of cell wall modifiers). Since strains grew much more slowly in media containing several inhibitors, the fermentations were started early in the day then continued throughout the entire growth cycle when possible (Fig. 3.11-3.13). Monitoring was not carried out for some experiments during the night but this was recommenced early the next day. Consequently, no measurements were made during 12-28 h incubation for Fig. 3.11-3.13. When 2% of glycine and various concentrations of INH were present in the growth medium, as the concentration of INH increased, the specific growth rate of the strains tested (AS019 and CG2) decreased and the duration of the lag phase also increased (Fig. 3.10). At 2% glycine and 4 mg/ml of INH, the relative growth rate of AS019 and CG2 was inhibited by approximately 80%. When 4 mg/ml of INH was present alone in the growth medium, the specific growth rate was inhibited by about 40% for AS019 and 60% for CG2 (Fig. 3.6). Similarly, further reductions in specific growth rates for the strains tested were seen for other combinations of cell wall inhibitors relative to cultures grown in the presence of a single cell

Fig. 3.9 Effect of the presence of Tween 80 in the growth medium on growth rates and duration of lag phase of four coryneform bacteria. Freshly prepared overnight starter cultures were inoculated into 250 ml flasks containing 100 ml of LBG with Tween 80 (0 to 0.9%, w/v) to give starting A_{600} of 0.1-0.25. CU was tested using 0 to 0.5% (w/v) Tween 80 only. Cultivation temperature and agitation speed were 30°C and 200 o.p.m (CU, 37°C). For the control, cells were grown in LBG. Specific growth rate are presented as percentages relative to growth rates seen in LBG. Duration of lag phase was the time taken before exponential growth started. Abbreviations: AS019, *C. glutamicum* AS019; CG2, *C. glutamicum* CG2; BF4, *B. flavum* BF4; CU, *C. ulcerans*.

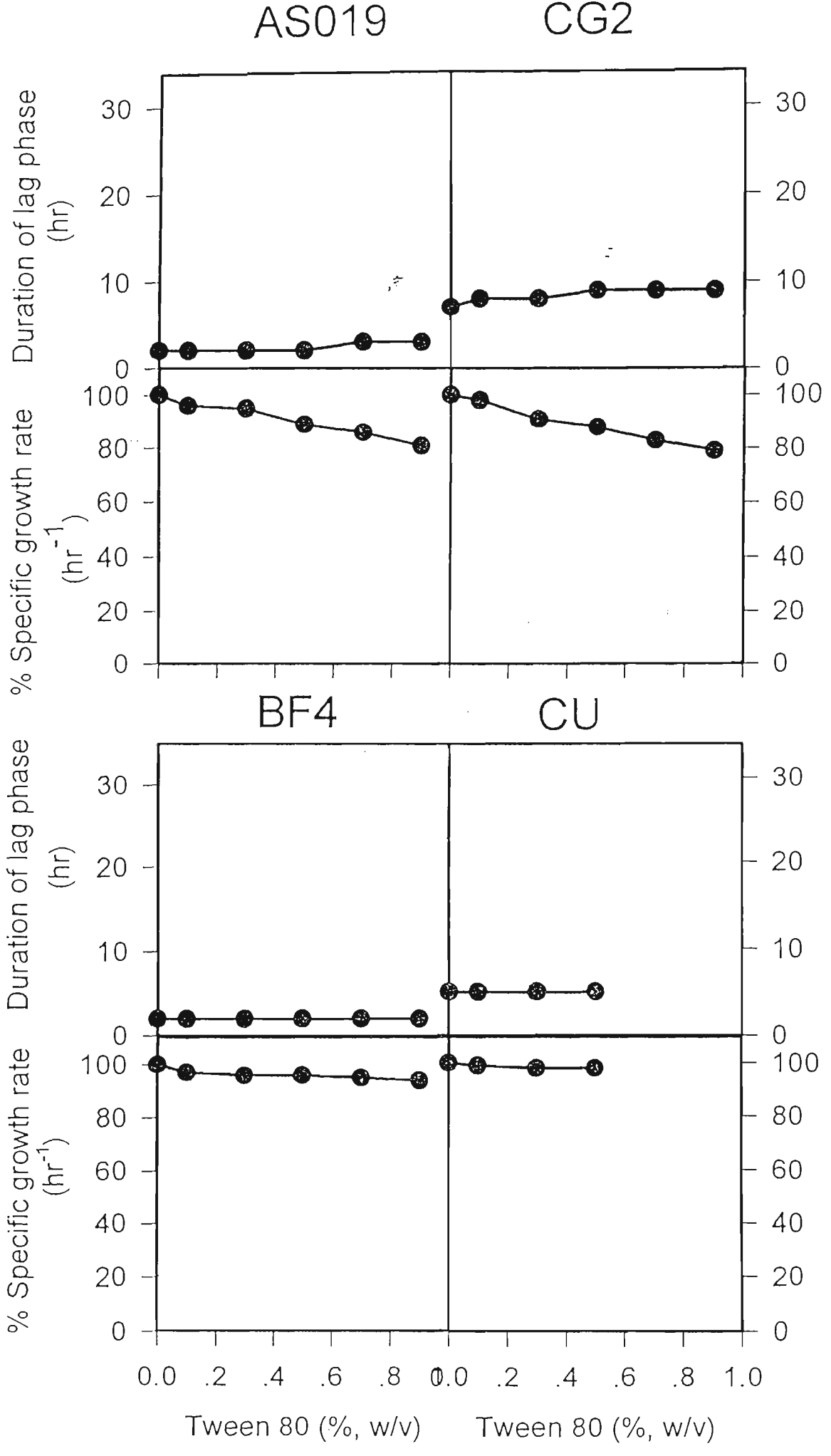
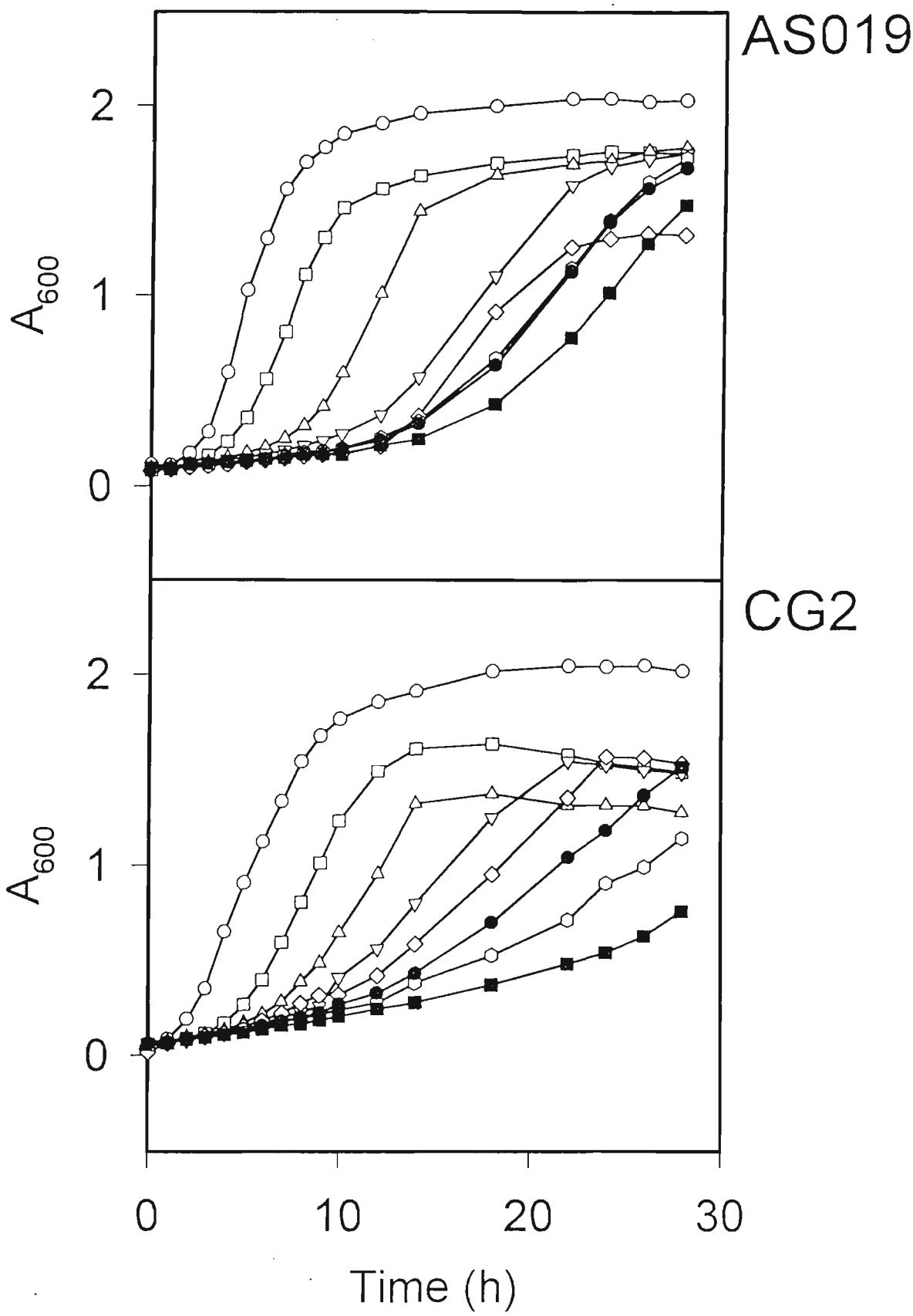

AS019
CG2
BF4
CU
Duration of lag phase (hr)
% Specific growth rate (hr-1)
0
10
20
30
40
60
80
100
0.0
.2
.4
.6
.8
1.0
Tween 80 (%, w/v)
Tween 80 (%, w/v)

Fig. 3.10 Effects of combinations of glycine plus INH on the growth of two *C. glutamicum* strains. Cultures of two coryneform bacteria from overnight cultures were inoculated into 250 ml flasks containing 100 ml of LBG containing 2% (w/v) glycine and various concentrations of INH (0 to 6 mg/ml) as below. Values of A_{600} were obtained by measuring absorbances of cultures without dilution. Abbreviation: LBG-G, LBG containing 2% glycine.

Symbol	Growth medium
(○)	LBG
(□)	LBG-G
(△)	LBG-G plus 1 mg/ml INH
(▽)	LBG-G plus 2 mg/ml INH
(◇)	LBG-G plus 3 mg/ml INH
(●)	LBG-G plus 4 mg/ml INH
(◎)	LBG-G plus 5 mg/ml INH
(■)	LBG-G plus 6 mg/ml INH


AS019
CG2
A_{600}
A_{600}
0
1
2
0
1
2
0
10
20
30
Time (h)

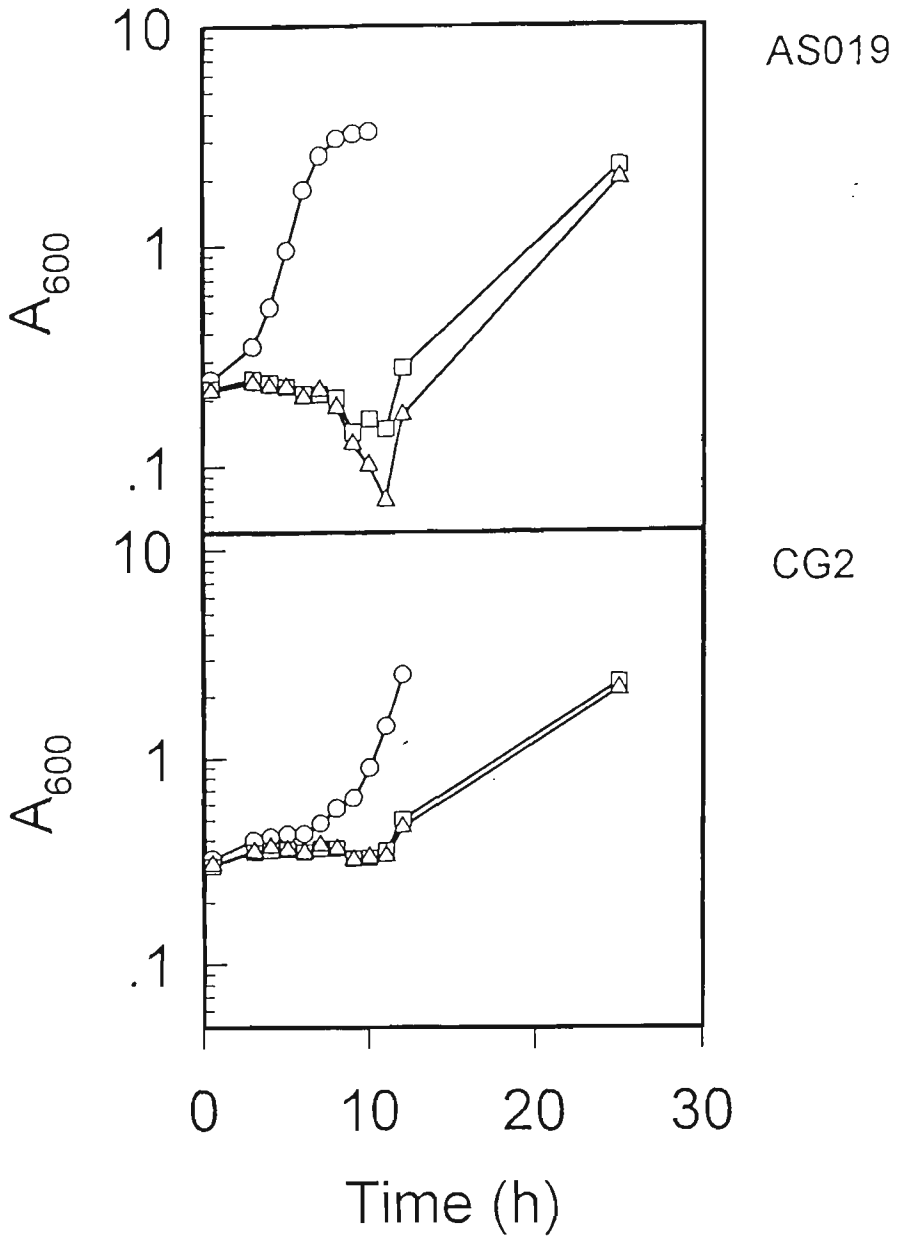


Fig. 3.11 Effects of combinations of glycine plus Tween 80 on cell growth of two *C. glutamicum* strains. Cultures of two coryneform bacteria from overnight cultures were inoculated into 250 ml flasks containing 100 ml of LBG containing 2% (w/v) glycine and various concentrations of Tween 80 (0.1 or 0.5%, w/v) as below. Values of A_{600} were obtained by measuring absorbance of cultures with dilution in appropriate media when A_{600} reached 0.8. No sample was measured during 12-26 h in incubation. Abbreviation: LBG-G, LBG containing 2% glycine.

Symbol	Growth medium
(○)	LBG
(□)	LBG-G plus 0.1% Tween 80
(△)	LBG-G plus 0.5% Tween 80

wall modifier (Fig. 3.11-3.13). For strain AS019, the growth rate was decreased by 50% (in the 2% glycine and 0.5% Tween 80), 40% (in the 4 mg/ml INH plus 0.5% Tween 80), and 85% (in the 2% glycine and 4 mg/ml INH and 0.5% Tween 80). For strain CG2, growth rate was decreased by 70% (in the 2.0% glycine and 0.5% Tween 80), 70% (in the 4 mg/ml INH plus 0.5% Tween 80), and 85% (in the 2.0% glycine and 4 mg/ml INH and 0.5% Tween 80). Under these conditions, the period of lag phase for these two strains was also affected. As seen in Fig. 3.13, the presence of mixtures of glycine (2%) plus 4 mg/ml INH plus Tween 80 (0.5%) caused an extremely extended lag phase (more than 20 h). It is interesting to note that, although growth of the two strains of *C. glutamicum* was retarded for extended periods when the medium contained mixtures of cell wall modifiers, the final A_{600} of cultures remained almost the same as seen for control cultures grown in the absence of cell wall modifiers (Fig. 3.11, Fig. 3.12). Similar observations were also obtained from *B. lactofermentum* BL1 (data not shown). Another point to be noted is the viable cell numbers of AS019 during growth, in particular, in the early stages of the fermentation period. When cells of AS019 were grown in the presence of 2% glycine and 4 mg/ml of INH, the values of A_{600} were often seen to fluctuate; A_{600} increased for several hours before decreasing and, after lag phase, increased continuously until stationary phase (Fig. 3.14). When samples of cultures were taken from several points and viable cells counted using two different media (LAG to recover normal cells, and ET to recover normal cells and fragile cells), the same results were obtained (Fig. 3.14); viable cells were initially 5-10 X 10^6 c.f.u./ml, increased up to 8 X 10^7 c.f.u./ml at the 6 h in incubation, and thereafter decreased by 5 to 10-fold. After lag phase, the number of viable cells continuously increased until stationary phase. These observations indicate that combinations of cell wall modifiers were toxic to cells during lag phase but, once growth started, cells could cope with these concentrations. Mechanisms of cell adaptation were not further investigated here nor was there any attempt to analyse the changes in cell surface composition (see section 3.4), although the latter analysis may have provided some insight into the mechanisms concerned here.

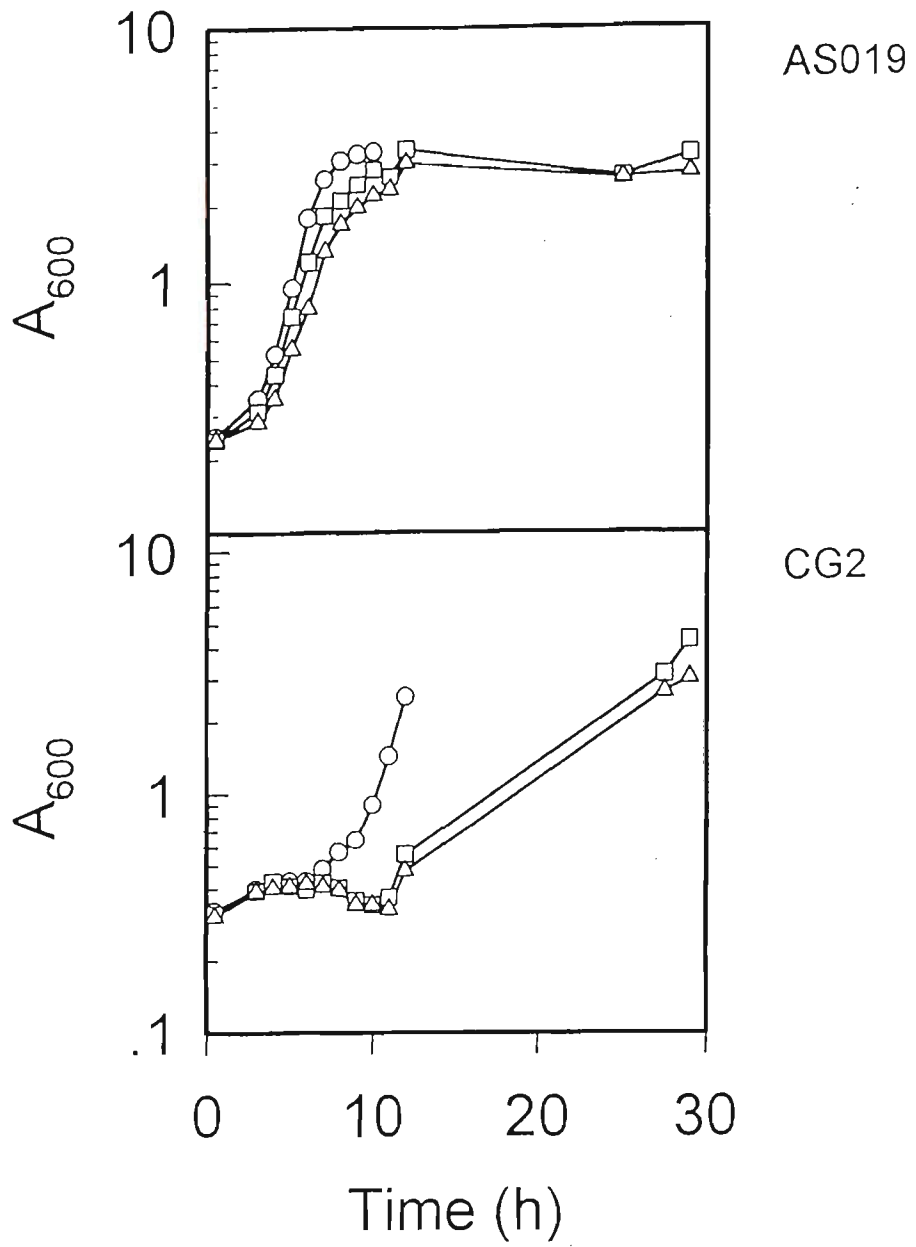


Fig. 3.12 Effects of combinations of INH plus Tween 80 on cell growth of two *C. glutamicum* strains. Cultures of two coryneform bacteria from overnight cultures were inoculated into 250 ml flasks containing 100 ml of LBG containing 4 mg/ml of INH and various concentrations of Tween 80 (0.1 or 0.5%, w/v) as below. Values of A_{600} were obtained by measuring absorbance of cultures with dilution in appropriate medium when A_{600} reached 0.8. No sample was measured during 14-28 h in incubation. Abbreviation: LBG-I, LBG containing 4 mg/ml INH.

Symbol	Growth medium
(○)	LBG
(□)	LBG-I plus 0.1% Tween 80
(△)	LBG-I plus 0.5% Tween 80

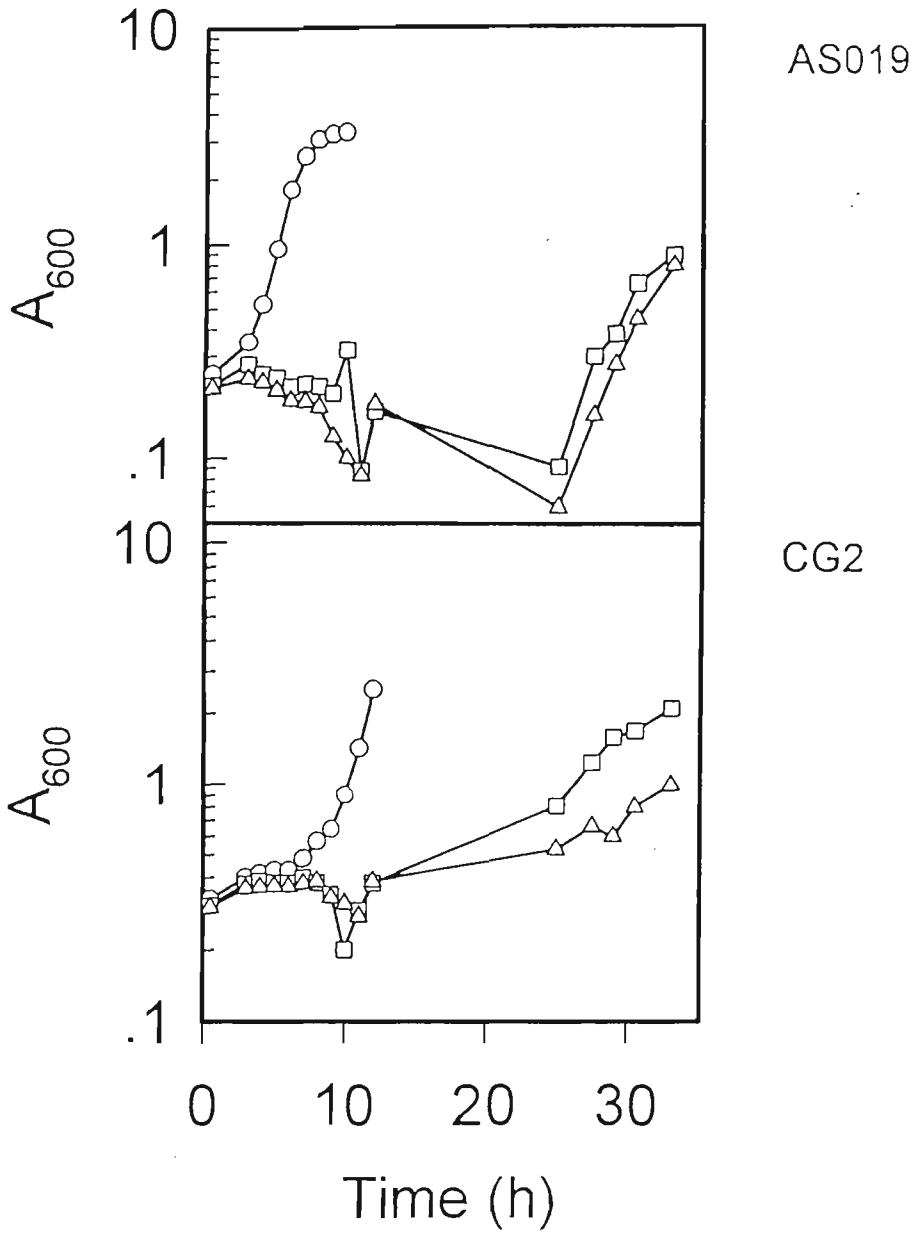


Fig. 3.13 Effects of combinations of glycine plus INH plus Tween 80 on cell growth of two *C. glutamicum* strains. Cultures of two coryneform bacteria from overnight cultures were inoculated into 250 ml flasks containing 100 ml of LBG containing 2% glycine plus 4 mg/ml INH plus various concentrations of Tween 80 (0.1 or 0.5%, w/v) as below. Values of A_{600} were obtained by measuring absorbance of cultures with dilution in appropriate media when A_{600} reached 0.8. No sample was measured during 12-25 h in incubation. Abbreviation: LBG-GI, LBG containing 2% glycine plus 4 mg/ml INH.

Symbol	Growth medium
(○)	LBG
(□)	LBG-GI plus 0.1% Tween 80
(△)	LBG-GI plus 0.5% Tween 80

Fig. 3.14 Effects of combinations of glycine and INH on the cell growth as determined by A_{600} (A) and the number of viable cells (B) of *C. glutamicum* AS019 during the fermentation process. Cultures of cells were grown in either LBG or LBG containing 2% glycine plus 4 mg/ml of INH (LBG-GI). The top panel represents values of A_{600} of *C. glutamicum* AS019 grown in LBG and LBG-GI. For viable counts (bottom panel), cells grown in LBG-GI were diluted in either SMMC buffer or water, and plated onto LAG or ET plate.

(A)

Symbol	Growth medium
(△)	LBG
(□)	LBG-GI

(B)

Symbol	Diluent	Solid medium
(○)	SMMC	ET
(△)	Water	ET
(□)	SMMC	LAG
(▽)	Water	LAG

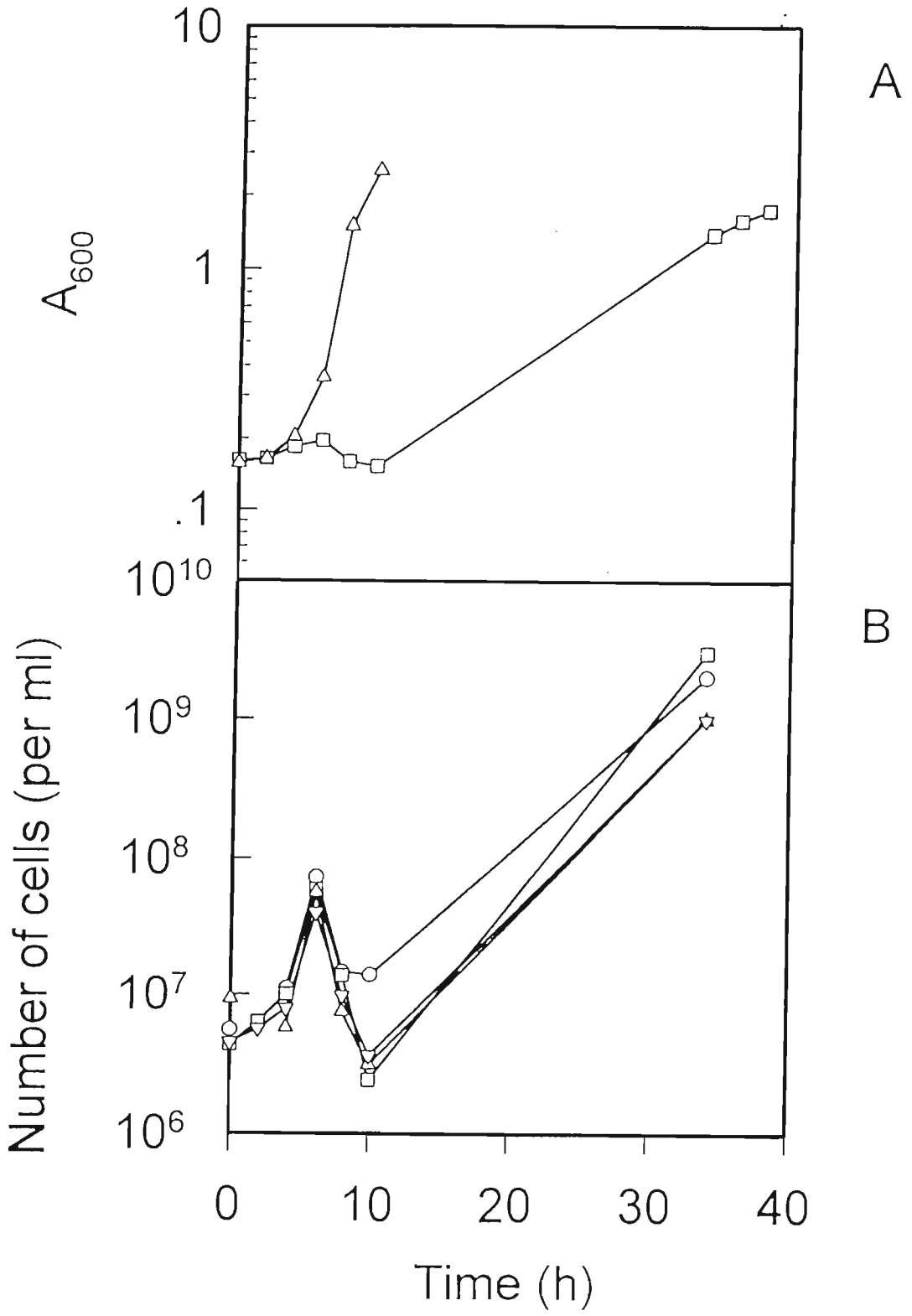

10
1
.1
A_{600}
A
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
B
Number of cells (per ml)
0
10
20
30
40
Time (h)

Table 3.1 Summary on the effects of cell wall inhibitors on cell specific growth rate and duration of lag phase of the corynebacteria tested.

Strain	μ values (h^{-1}) or duration of lag phase	Cell wall inhibitors in LBG									
		glycine (%, w/v)		INH (mg/ml)		Tween 80 (%, w/v)		2 % glycine + 4 mg/ml INH	2% glycine + 0.5% Tween 80	4 mg/ml INH + 0.5 % Tween 80	2% Glycine + 4mg/ml INH + 0.5% Tween 80
		2	4	4	8	0.1	0.5				
AS019	μ value (h^{-1})	20%	50%	40%	50%	5%	10%	80%	50%	40%	85%
	lag phase (h)	1h	1.5h	1h	1h	0.5h	1h	10h	>14h	1h	>24h
MLB133	μ value (h^{-1})	40%	60%	40%	75%						
	lag phase (h)	1h	1.5h	1h	1h						
MLB194	μ value (h^{-1})	40%	60%	40%	75%						
	lag phase (h)	1h	1.5h	1h	1h						
ATCC	μ value (h^{-1})	40%	70%	40%	60%			80%			
13032	lag phase (h)	1h	1h	1h	1.5h			4h			
RM3	μ value (h^{-1})	50%	80%	60%	70%			90%			
	lag phase (h)	1h	1h	1h	1.5h			10h			
RM4	μ value (h^{-1})	30%	70%	40%	80%			85%			
	lag phase (h)	2h	3h	1h	1.5h			7h			
CG2	μ value (h^{-1})	50%	70%	65%	80%	5%	10%	80%	70%	70%	85%
	lag phase (h)	2h	5h	1.5h	2h	1h	2h	12h	>14h	8h	>24h
CU	μ value (h^{-1})	60%	>80%	60%		2%	5%				
	lag phase (h)	1h	2h	1h		0.5h	1h				
BF4	μ value (h^{-1})	30%	35%	50%		5%	5%				
	lag phase (h)	2h	3h	3h		0.5h	1h				
BL1	μ value (h^{-1})	10%	50%	40%				65%			
	lag phase (h)	2h	>20h	1h				>24h			

Table 3.1 summarises the impact of individual and combined additives to LBG on the growth of different corynebacteria.

3.3 EFFECTS OF GROWTH IN GLYCINE AND INH ON PLASMID TRANSFORMATION USING ELECTROPORATION

3.3.1 Growth conditions, plasmid DNA and *C. glutamicum* strains used for electroporation

Experiments described in this section were mainly carried out with two wild-type *C. glutamicum* strains, AS019 (rif^r mutant of wild-type parent, ATCC 13059) and ATCC 13032, and mutants derived from these strains. All of these strains normally did not contain plasmid DNA, which was confirmed by DNA isolation procedures and following agarose gel electrophoresis (data not shown). In order to compare the effects of growth in glycine and INH on the transformation efficiencies obtained, attempts were made to grow cultures using LBG and LBG-GI (2% glycine and 4 mg/ml INH). As observed in section 3.2.6, the presence of glycine and INH in the growth medium inhibited cell growth but sensitivity to inhibition by these compounds varied from strain to strain. For example, the presence of 2-5% of glycine and 4-8 mg/ml of INH in the growth medium significantly reduced growth of strains RM3 and RM4 to an extent that it was not practical to use these concentrations experimentally. It was therefore necessary to alter the combination of concentrations of glycine and INH used in media to allow a basis for comparison to be made. Previous work by Haynes and Britz (1990) showed that the highest transformation efficiency of AS019 was obtained following growth in 2% glycine plus 4 mg/ml INH. Thus, two different combinations of glycine and INH were applied to growth of the six strains of *C. glutamicum* tested: LBG-GI (2% glycine and 4 mg/ml INH) for AS019, MLB133 and MLB194,

LBG-GI (2% glycine and 0.4 mg/ml INH) for ATCC 13032, RM3 and RM4.

According to the earlier observations of Haynes and Britz (1990), it was shown that pCSL17 plasmid gave slightly higher or equivalent transformation frequencies when compared with other available plasmids, some of which were smaller than pCSL17, and pCSL17 was able to transform into both coryneform bacteria and *E. coli* strains, having the *ori* of both species. For these reasons, pCSL17 DNA was used in the present set of experiments. Plasmid pCSL17 DNA has two antibiotic resistance markers, kanamycin and ampicillin, and kanamycin resistance is expressed in both *C. glutamicum* and *E. coli*. *C. glutamicum* strains containing pCSL17 DNA were able to grow in the presence of 100 μg/ml kanamycin in the growth medium, whilst no growth were seen when cells did not contain pCSL17 DNA (data not shown).

3.3.2 Reproducibility and analysis of information

Reproducibility was determined by triplicates in any one experiment and by running separate experiments using the same or different batches of cells. The procedure was readily reproducible; a maximum of two to three fold variation in transformation efficiency was observed when different aliquots of the same batch of cells were transformed with the same plasmid, and a five-fold variation with transformation of aliquots of different batches. These differences probably reflected subtle differences between batches of cells due to harvesting cells at different growth phases, as this parameter was known to be important in determining both protoplast transformation (M. L. Britz, unpublished observation) and electrotransformation efficiencies (Haynes and Britz, 1990). Therefore, repeated experiments were conducted using DNA from the same batch and different batches of cells grown similarly and harvested at approximately the same absorbance, and average values were calculated for all data presented. To minimise variations, the same batch of cells and DNA from the same batch were used for determining the relationship

between number of transformants and amount of DNA used. In some cases, the number of survivors obtained varied after electroporation and this caused errors in the interpretation of the data. Therefore, the yield of transformation was calculated in two ways. Transformation efficiency was defined as the number of transformants per μg of DNA per 10^9 cells treated. Transformation frequency was defined as the number of transformants per number of survivors after electroporation per μg DNA; this was normally multiplied by 10^6 for graphical representations. Plasmid pCSL17 DNA was isolated using an alkaline lysis method and its restriction enzyme map confirmed by restriction enzyme digestion (see section 4.4.1 for restriction enzyme map of pCSL17). Results of MIC estimation showed that the six strains of *C. glutamicum* tested were all sensitive to ampicillin, chloramphenicol, kanamycin, neomycin, streptomycin, and tetracycline. Two strains, including AS019 and MLB194 were resistant to rifampicin (50 μg) (data not shown).

3.3.3 Optimisation of electrotransformation of *C. glutamicum*

Several factors (such as cell harvesting phase, cell density, composition of buffer, growth medium, regeneration medium, electric field strength, capacitance, *etc.*) can influence electrotransformation frequencies (Chang *et al.*, 1992). The present work was based on the conditions used by Haynes and Britz (1990), with minor modifications, such as concentration of glycine and INH, concentration of plasmid DNA used and cell harvesting phase. Electroporation conditions are described in Materials and Methods (section, 2.4.1). In most of experiments, between 5 to 20 X 10^8 cells and 0.5 or 1.76 μg of DNA were used per transformation since this range of DNA (0.5 - 2 μg) gave a linear relationship between the number of log transformants and amounts of log DNA (see Fig. 3. 15).

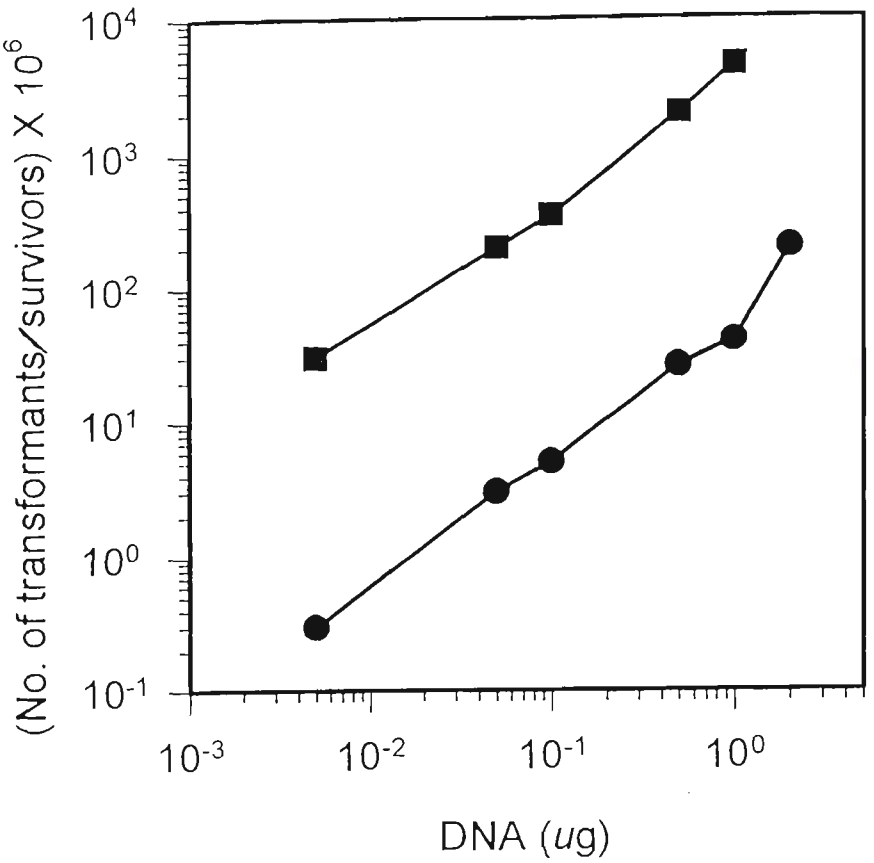


Fig. 3.15 Effect of increasing amount of DNA on the transformation frequency of two *C. glutamicum* strains. *C. glutamicum* MLB194 (•) was grown in LBG, and *C. glutamicum* MLB133 (■) was grown in LBG supplemented with 2% glycine and 4 mg/ml INH. Cells were harvested at an A_{600} of approximately 0.4. pCSL17 DNA (5 ng to 2 μg) was derived from *C. glutamicum* AS019 and added to 40 μl cells suspended in 15% glycerol and the final concentration of glycerol adjusted to 15% in a final volume of 75 μl by adding 100% glycerol and water, prior to pulsing. The recovery period was for 1 h at 30°C in LBG supplemented with 10 mM $MgCl_2$ plus 10 mM $CaCl_2$. Cell counts (from ET and ET-Km medium) were performed in triplicate and average numbers were taken.

3.3.3.1 DNA preparation

The DNA used for electroporation was isolated from two different bacterial sources; *C. glutamicum* AS019 and *E. coli* LE392 (see section 4.5 for details in restriction and modification background of this strain). The presence of a robust cell surface structure in *C. glutamicum* and low copy number of pCSL17 DNA (2-3 copies per chromosome, Haynes and Britz, unpublished data) caused difficulties in preparation of plasmid DNA from *C. glutamicum*. Often, there was not enough DNA to visualise two DNA bands (top band for chromosomal DNA and bottom band for plasmid DNA) expected after CsCl-gradient centrifugation, therefore, the faint band corresponding to the bottom band was collected from several centrifuge tubes, samples combined, and the pooled DNA centrifuged again on CsCl-EtBr gradients. Usually, this further step in DNA purification formed two clear bands in the tube. The purity of DNA was checked by agarose gel electrophoresis and the purity of DNA was also measured using absorbance readings at 260 nm and 280 nm. The ratio of A_{260}/A_{280} was always above 1.8 (Sambrook *et al.*, 1989). Since transformation efficiency and frequency are dependent on DNA concentration, accurate measurement of DNA concentration was required. DNA concentration was determined using the fluorimetric method (described in section 2.3.5), which showed a linear relationship between fluorescence at 420 nm excitation and amount of DNA over the range of 0.05-4 μg.

3.3.3.2 Effect of DNA concentration on transformation frequency

To determine the relationship between the amount of DNA supplied and the number of transformants, various amounts of CsCl-purified pCSL17 DNA from 5 ng to 2 μg in 35 μl were added to 40 μl portions of cell suspensions of either MLB133 or MLB194. DNA/cell mixtures were electroporated under the conditions described in the section 2.4.1. As mentioned above, the relationship between DNA concentrations and number of transformants recovered for homologous

pCSL17 was linear (Fig. 3.15). The transformation frequencies ([number of transformants per number of survivors per μg DNA] X 10^6) for these points were constant within experimental variation; means $\pm$ SD were MLB133, 4.4 $\pm$ 0.9 X 10^3; MLB194, 5.6 $\pm$ 0.5 X 10^1 (MLB194 was grown in LBG but MLB133 was grown in LBG-GI). In the case of electroporation of strain MLB194, two separate experiments were conducted and a linear relationship obtained again, although transformation frequencies were 5-fold lower. From these data, the amount of DNA usually used for electroporation was 0.5 μg for further experiments.

3.3.3.3 Effect of growth phase on transformation frequency obtained

The efficiency of transformation in bacteria is typically dependent on the growth phase at which recipient cells are harvested, possibly indicating differences in the cell surface structure or state of restriction and modification systems. In order to gain some insight into how growth phase may influence the former, cells were harvested from *E. coli* LE392 and several strains of corynebacteria at different growth phases and the kinetics of protein release during sonication determined. Since the number of cells in the culture increased with increases in fermentation time, the number of cells used for sonication was adjusted by reducing the volume of cultures harvested as growth progressed. After centrifugation of culture fluids, cells were resuspended with the same amount of buffer and used for sonication. With increasing sonication time, the amount of protein released from *E. coli* cells increased but the time taken to completely disrupt cells was much less than for the corynebacteria tested (Fig. 3.16). This trend was seen throughout all cell growth phases tested, indicating that corynebacteria have much stronger cell surface structures than *E. coli*. Release of protein from BL1 was faster than seen for the other corynebacteria, which is consistent with its greater ease of protoplasting and electroporation. The rate of release of protein by sonication was slightly slower later in growth (Fig. 3.16C). There was no clear relationship between rate of protein release by sonication and stage of growth of

Fig. 3.16. Relationship bwteen the amount of protein levels from corynebacteria and increasing sonication time. Cultures of 3-4 strains of corynebacteria and *E. coli* LE392 from overnight cultures were incubated at 37°C (or 30°C for LE392), 200 o.p.m. Various volumes of LBG were harvested (750 ml for A; 375 ml for B; 150 ml for C) at different growth stages. After sonication (power, 340 Watt), 0.5 ml of samples were taken from different sonication times and 50 μl of samples was used for protein analysis using Lowry assay (Lowry *et al.* 1951).

A

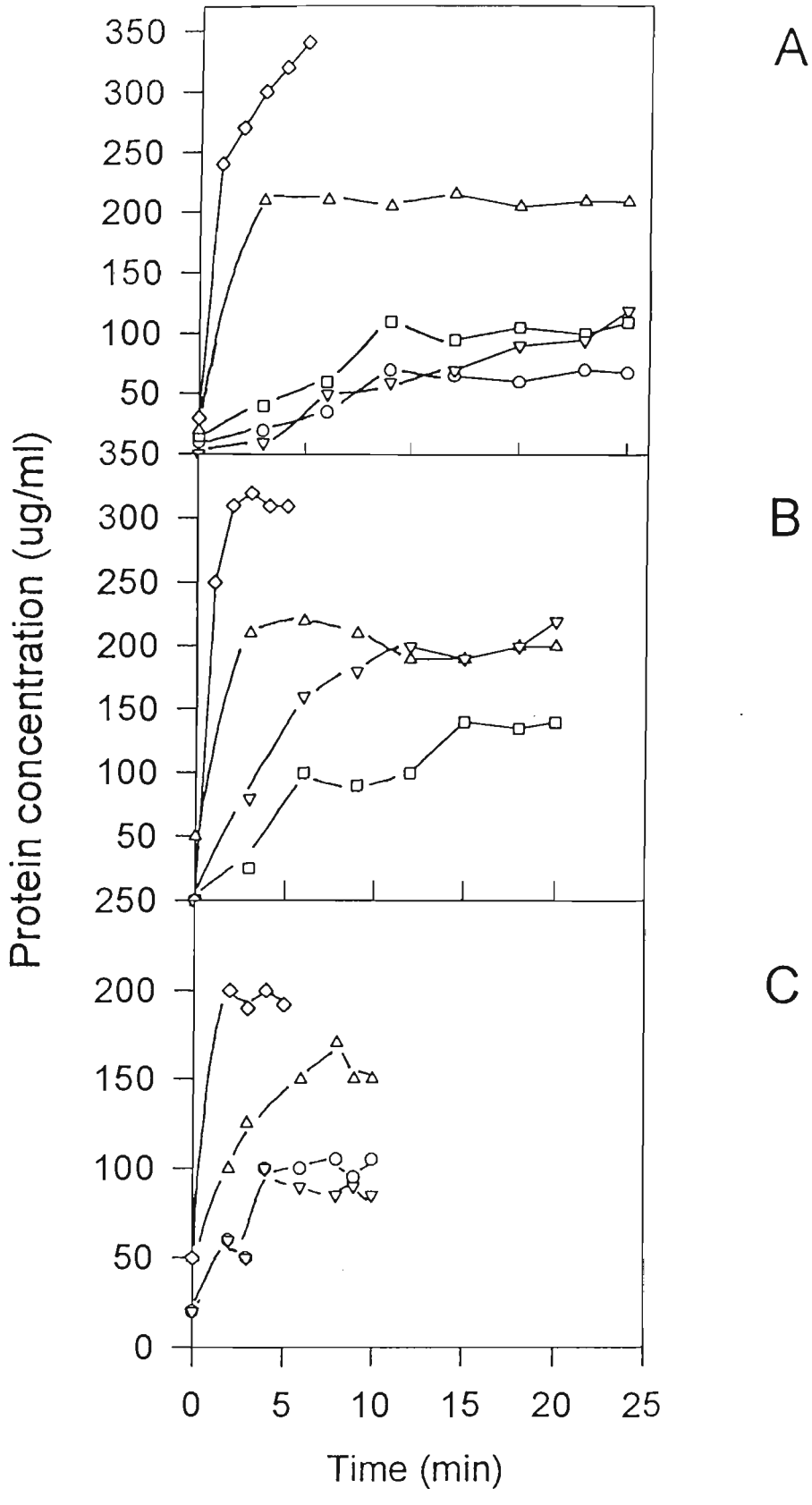
Symbol	strain	A_{600}
(○)	AS019	0.43
(□)	CG2	0.44
(▽)	BF4	0.48
(△)	BL1	0.68
(◇)	LE392	0.45

B

(□)	CG2	0.77
(▽)	BF4	0.84
(△)	BL1	0.86
(◇)	LE392	0.87

C

(○)	AS019	1.16
(▽)	BF4	1.2
(△)	BL1	1.1
(◇)	LE392	1.29


A
B
C
Protein concentration (ug/ml)
350
300
250
200
150
100
50
350
300
250
200
150
100
50
250
200
150
100
50
0
0
5
10
15
20
25
Time (min)

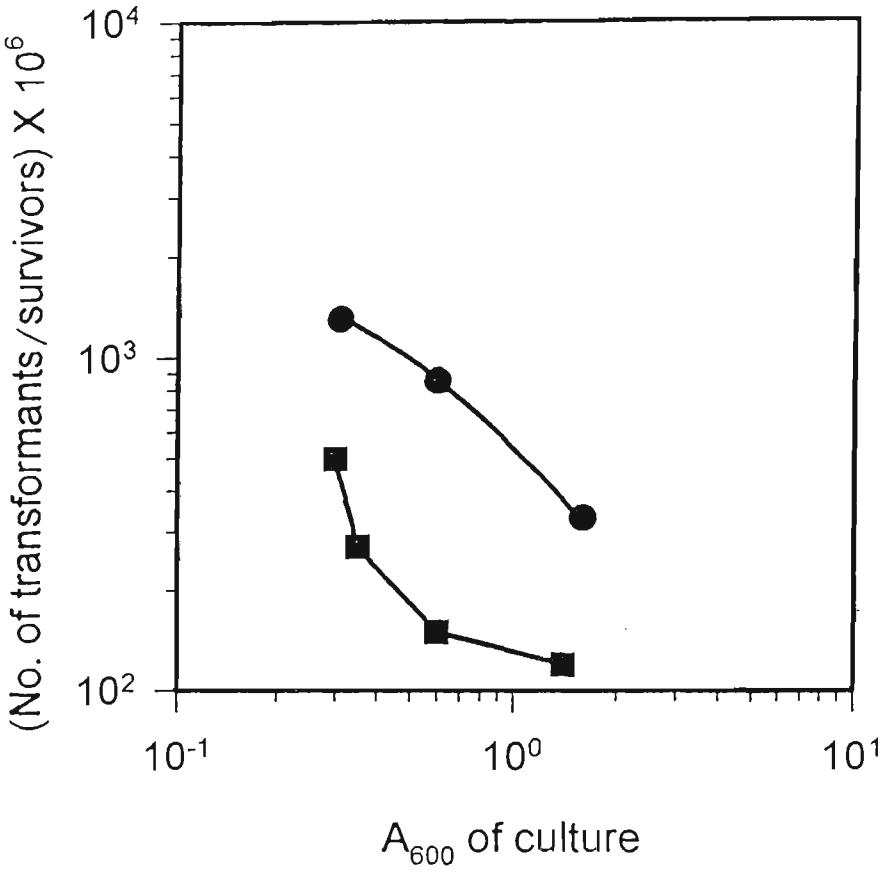


Fig. 3.17 Effect of harvesting cultures at different growth phases on the number of transformants obtained using electroporation. *C. glutamicum* AS019 (■) was grown in LBG supplemented with 2% glycine and 4 mg/ml INH, and *C. glutamicum* ATCC 13032 (●) was grown in LBG supplemented with 2% glycine and 0.4 mg/ml INH. pCSL17 DNA (0.5 μg) was derived from AS019 was added to 40 μl of cells suspended in 15% glycerol and final concentration of glycerol adjusted to 15% in the final volume of 75 μl, prior to pulsing. The recovery period was for 1 h at 30°C in LBG supplemented with 10 mM $MgCl_2$ plus 10 mM $CaCl_2$. Cell counts (from ET and ET-Km medium) were performed in triplicate and average numbers were taken.

harvesting cells for the other corynebacteria so that further tests were not performed. In order to determine the effect of growth phase of *C. glutamicum* ATCC 13032 and AS019 on transformation efficiency, cells were harvested at different A_{600} values prior to electroporation. Cell suspensions were then adjusted to a final concentration of approximately 5 X 10^{10} c.f.u/ml in 15% glycerol, equivalent to an A_{600} 0.4, by concentration or by dilution. Aliquots were mixed with homologously-derived pCSL17 DNA and the mixtures were immediately used for electroporation. Approximately 50 to 70% of the cells were killed during the electric pulse, noting that the recovery period, dilution in SMMC and plating on ET media assisted in recovering damaged cells (Haynes and Britz, 1990). Early-exponential phase cells were more easily transformed and, as the A_{600} increased there was a significant reduction in the number of transformants obtained (Fig. 3.17). This data indicates that the growth phase did influence the efficiency observed significantly and confirmed early results of Haynes and Britz (1990) for AS019. Cells to be electroporated were subsequently harvested at A_{600} values between 0.35 and 0.45.

3.3.3.4 Effect of recovery medium on transformation efficiency

Often, the regeneration media used after transformation of protoplasts is a key factor in recovering damaged cells and this also determines the number of cells surviving *in toto*. This is also known to be important in electrotransformation for at least strain AS019 (Haynes and Britz, 1990) so the effect of recovery medium was examined here with several strains of *C. glutamicum*. After electroporation, the cells were incubated in the recovery medium at 30°C for 1 h, diluted in SMMC buffer, and spread onto two selective media: ET-Km, NAG-Km. Conditions used for the present experiments had been optimised by Haynes and Britz (1990), who tested several diluents including PBS, LB, SMMC, water and had found that SMMC was best in terms of recovering the highest number of cells. Also, they found that incubation of the cells in the presence of Ca^{++} and

Table 3.2 Effect of recovery medium following electroporation on the number of transformants of *C. glutamicum* obtained.

Strain [a]	Growth medium	Source of DNA [b]	Transformants (μg DNA)$^{-1}$ [c]	
			NAG-Km	ET-Km
AS019	LBG	homologous	5.0×10^3	7.5×10^3
AS019	LBG	heterologous	2.8×10^2	3.0×10^2
AS019	LBG-GI	homologous	1.1×10^5	1.5×10^5
MLB133	LBG	homologous	7.4×10^3	1.0×10^4
MLB194	LBG	homologous	6.2×10^3	8.1×10^3
MLB194	LBG	heterologous	3.5×10^2	4.6×10^2
MLB194	LBG-GI	heterologous	4.3×10^2	9.8×10^2
13032	LBG	homologous	1.0×10^5	1.7×10^5
13032	LBG	heterologous	< 150	1.8×10^2
13032	LBG-GI	homologous	3.1×10^6	7.0×10^6
13032	LBG-GI	heterologous	3.5×10^2	8.0×10^2
RM3	LBG	homologous	8.5×10^3	9.7×10^3
RM3	LBG	heterologous	4.2×10^3	7.5×10^3
RM3	LBG-GI	homologous	1.7×10^4	1.6×10^4
RM3	LBG-GI	heterologous	2.3×10^4	2.8×10^4
RM4	LBG	homologous	4.2×10^3	5.3×10^3
RM4	LBG	heterologous	1.3×10^3	3.5×10^3
RM4	LBG-GI	homologous	1.1×10^5	1.5×10^5
RM4	LBG-GI	heterologous	5.0×10^4	5.8×10^4

[a] Cells were grown to an A_{600} of 0.4 in either LBG or LBG-GI (LBG containing 2% glycine plus 0.4 mg/ml INH for ATCC 13032, RM3 and RM4, or 4 mg/ml INH for AS019, MLB133 and MLB194) harvested, and then electroporated in the presence of 0.5 or 1.76 μg of pCSL17 DNA.

[b] Homologous, pCSL17 DNA-derived from *C. glutamicum* AS019; heterologous, pCSL17 DNA-derived from *E. coli* LE392.

[c] Cell counts were performed in triplicate and average numbers were taken. Cells were diluted in SMMC.

Mg^{++} in the expression medium caused increases in the number of cells recovered.In addition, it was also found that when cells were grown in the presence of glycine and INH, and spread onto ET medium, ET gave better recovery than NAG medium. The present experiments were carried out to validate the effect of ET medium specifically, for all of the strains used here. Although the degree of superiority of ET-Km over NAG-Km showed some variability from experiment to experiment, counts on ET-Km usually were 30 to 70% higher than those on NAG-Km (Table 3.2). This was observed for all strains when using different sources of DNA, and when different media were used for cell growth prior to electroporation. These results indicated that transformants arising following electroporation were osmotically or electrochemically fragile, confirming observations made by Haynes and Britz (1990). From these observations, cell counts were conducted using ET and ET-Km media for further experiments.

3.3.4 Effect of prior growth in the presence of glycine and INH on electrotransformation efficiency and frequency

Cells were grown to an A_{600} of approximately 0.4 in LBG-GI, and in non-supplemented LBG broth as a control. Electroporation was conducted using both heterologously- or homologously-isolated pCSL17 DNA. Figs. 3.18 and 3.19 show results for six strains in terms of transformation efficiency and transformation frequency respectively. Trends appear to be basically similar in that relativities are the same: for instance, the presence of glycine and INH in the medium increased both transformation efficiency and transformation frequency of all strains tested.

When homologously-derived DNA was used, both transformation efficiency and transformation frequency were usually higher than those with heterologously-derived DNA, with exception of RM3 and RM4. When cells were grown in LBG and electrotransformed with homologously-

derived DNA, transformation efficiencies were relatively low, with variations in transformation efficiencies seen from strain to strain depending on the nature of the strain. However, when glycine and INH were included in the growth medium and transformed with homologously-derived DNA, the number of transformants increased for all strains with the increases in transformation efficiency ranging from 7.2 X 10^3 (10^9 cells electroporated X μg DNA)$^{-1}$ (for RM3) to 5.8 X 10^6 (10^9 cells electroporated X μg DNA)$^{-1}$ (for MLB133) (Fig 3.18).

For strains of the ATCC 13032 family, transformation efficiencies ranged from 30 (in ATCC 13032) to 5 X 10^3 (in RM4) when cells were grown in LBG and transformed with heterologously-derived DNA. Of the three strains, ATCC 13032 showed the highest levels of restriction barrier, where homologously-derived DNA was transformed at a much lower frequency than homologously-derived DNA. In contrast, as anticipated, strains RM3 and RM4 showed high transformation efficiencies whatever the source of DNA, reflecting their restriction minus nature (Liebl *et al.*, 1989; Schäfer *et al.*, 1994b); there was little or no difference in transformation efficiency with different sources of DNA. Transformation efficiency on RM3 grown in the LBG was 3.7 X 10^3 transformants per 10^9 cells per μg DNA with heterologously-derived DNA and 4.6 X 10^3 transformants per 10^9 cells per μg DNA with homologously-derived DNA. Similar results were obtained for strain RM4. Transformation efficiency on ATCC 13032 grown in the LBG was only 31 transformants per 10^9 cells per μg DNA with heterologously-derived DNA.

In contrast, when cells were grown in the LBG and transformed with homologously-derived DNA, transformation efficiency on ATCC 13032 was higher than those of RM3 and RM4; transformation efficiencies on ATCC 13032, RM3 and RM4 were 5.1 X 10^4, 4.6 X 10^3 and 2.0 X 10^4 transformants per 10^9 cells per μg DNA, respectively. This indicates that cell surface structure barriers to electroporation of ATCC 13032 was lower than those seen for the two mutant strains.

Fig. 3.18 Transformation efficiencies of *C. glutamicum* strains with either homologous- or heterologous DNA following growth in LBG or LBG-GI broths. Cells were harvested when the A_{600} was 0.35-0.45. pCSL17 DNA (0.5 μg) isolated from *E. coli* LE392 (source of heterologous DNA) or *C. glutamicum* AS019 (source of homologous DNA) was added to 40 μl cells suspended in 15% glycerol and final concentration of glycerol adjusted to 15% in the final volume of 75 μl, prior to pulsing. The recovery period was for 1 h at 30°C in LBG supplemented with 10 mM $MgCl_2$ plus 10 mM $CaCl_2$. Cell counts (from ET and ET-Km medium) were performed in triplicate and average numbers were taken. Transformation efficiency was calculated as the number of transformants (10^9 cells electroporated X μg DNA)$^{-1}$. Data presented are averages of 2 to 7 repeats of separated experiments. Abbreviations: LBG-GI, LBG containing 2% glycine plus 0.4 mg/ml INH (for ATCC 13032, RM3 and RM4), or LBG containing 2% glycine plus 4 mg/ml INH (for AS019, MLB194 and MLB133).

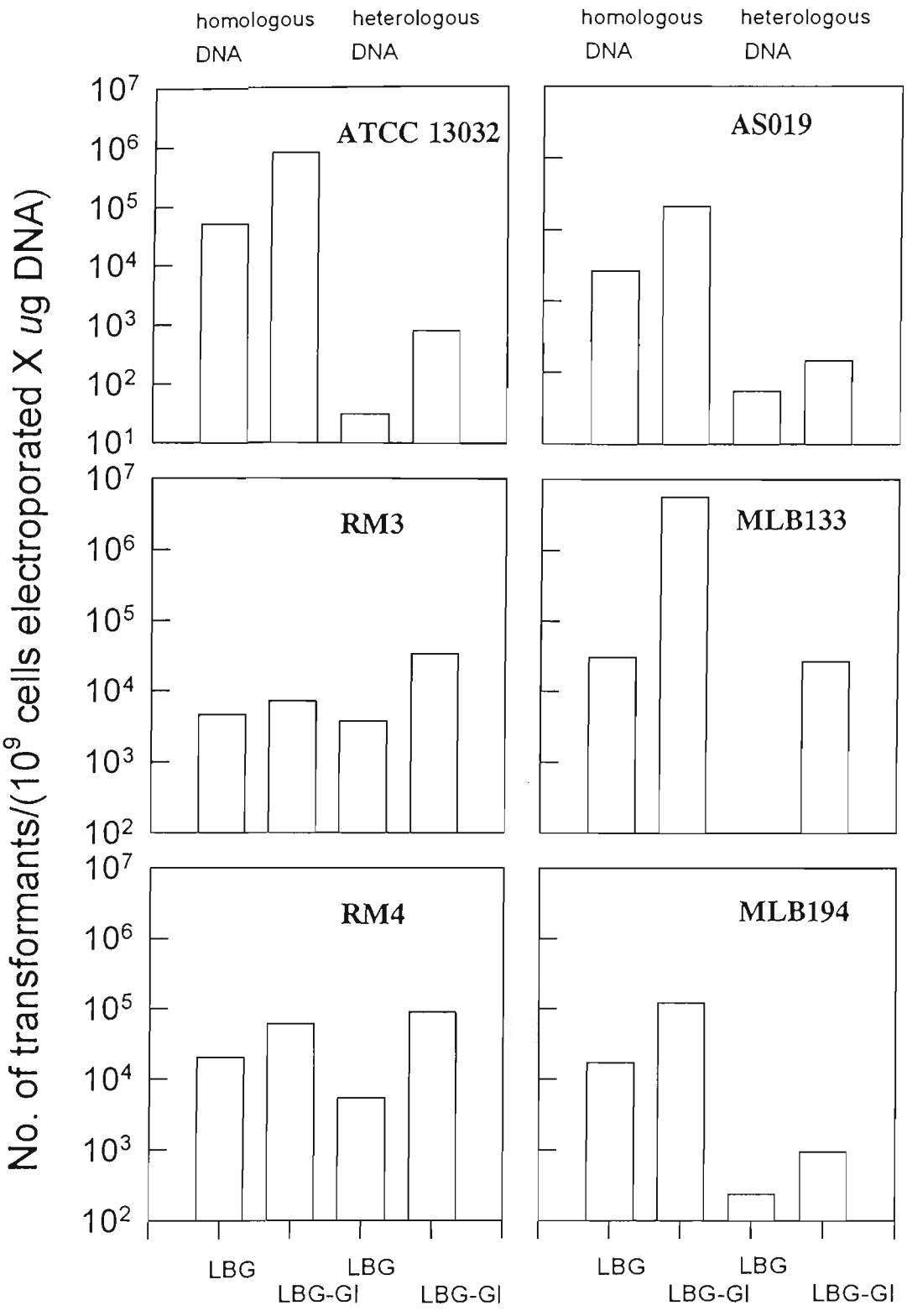
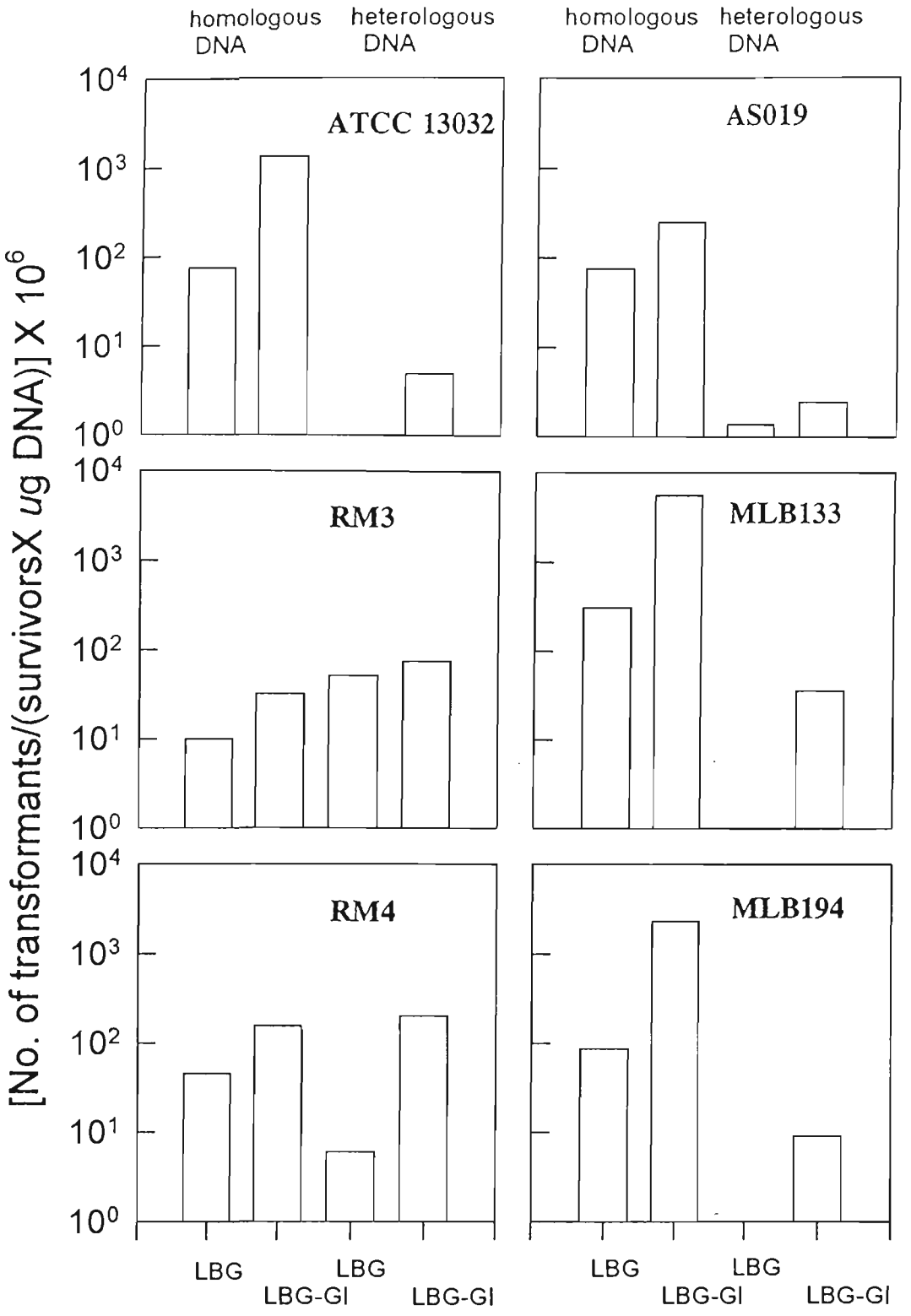

homologous DNA
heterologous DNA
homologous DNA
heterologous DNA
ATCC 13032
AS019
RM3
MLB133
RM4
MLB194
No. of transformants/(10^9 cells electroporated X ug DNA)
10^7
10^6
10^5
10^4
10^3
10^2
10^1
LBG
LBG
LBG-GI
LBG-GI
LBG
LBG
LBG-GI
LBG-GI
Medium

Fig. 3.19 Transformation frequencies of *C. glutamicum* strains with either homologous- or heterologous DNA following growth in LBG or LBG-GI broths. Cells were harvested when the A_{600} was 0.35-0.45. pCSL17 DNA (0.5 μg) isolated from *E. coli* LE392 (source of heterologous DNA) or *C. glutamicum* AS019 (source of homologous DNA) was added to 40 μl cells suspended in 15% glycerol and final concentration of glycerol adjusted to 15% in the final volume of 75 μl, prior to pulsing. The recovery period was for 1 h at 30°C in LBG supplemented with 10 mM $MgCl_2$ plus 10 mM $CaCl_2$. Cell counts (from ET and ET-Km medium) were performed in triplicate and average numbers were taken. Transformation frequency was calculated as [number of transformants (survivors X μg DNA)$^{-1}$] X 10^6. Data presented are average of 2 to 7 repeats of separated experiments. Abbreviations: LBG-GI, LBG containing 2% glycine plus 0.4 mg/ml INH (for ATCC 13032, RM3 and RM4), or LBG containing 2% glycine plus 4 mg/ml INH (for AS019, MLB194 and MLB133).


homologous DNA
heterologous DNA
homologous DNA
heterologous DNA
ATCC 13032
AS019
RM3
MLB133
RM4
MLB194
[No. of transformants/(survivorsX ug DNA)] X 10^6
10^4
10^3
10^2
10^1
10^0
LBG
LBG
LBG-Gl
LBG-Gl
LBG
LBG
LBG-Gl
LBG-Gl
Medium

For the AS019 family, when cells were grown in LBG, all three strains tested showed a low level of transformation efficiencies, ranging from less than 10 to 10^3 (10^9 cells X μg DNA)$^{-1}$ for heterologously-derived DNA; no transformants were found for MLB133 when cells grown in the LBG were then transformed with heterologously-derived DNA. Similarly, only a few transformants were found from strain AS019 and MLB194 under the above conditions. In contrast, when cells were grown in the LBG then transformed with homologously-derived DNA, transformation efficiencies on three strains were much higher than those with heterologously-derived DNA; transformation efficiencies on AS019, MLB133 and MLB194 were 2.7 X 10^4, 3.1 X 10^4 and 1.7 X 10^4 transformants per 10^9 cells per μg DNA, respectively. This indicates that these strains contain restriction barriers, which can restrict the incoming heterologouly-derived DNA.

It is interesting to note that the level of transformation seen for *C. glutamicum* ATCC 13032 was normally higher than seen for AS019 for all circumstances, although pCSL17 DNA was isolated from AS019. Routinely, a three- to seven-fold higher transformation frequency was obtained with ATCC 13032 when cells were grown in the presence of LBG-GI. Similar results were obtained when cells were grown in LBG, although the difference was less marked.

For all circumstances, the presence of glycine and INH in the growth medium improved both transformation efficiency and transformation frequency. When cells were grown in LBG plus glycine and INH then transformed with DNA, transformation efficiencies on 6 strains ranged from 7.9 X 10^2 (for ATCC 13032) to 8.9 X 10^5 transformants per 10^9 cells per μg DNA (for RM4) for heterologously-derived DNA and from 7.2 X 10^3 (for RM3) to 5.8 X 10^6 transformants per 10^9 cells per μg DNA (for MLB133) for homologously-derived DNA. Similar improvement in transformation frequencies on all six strains was also seen by the presence of glycine and INH in the medium. For instance, when MLB133 was grown in the LBG-GI then transformed with

homologously-derived DNA, transformation efficiency was 5.8 X 10^6 transformants per 10^9 cells per μg DNA, which is more than 100-fold higher than that seen for LBG.

Differences in transformation efficiency were evident among the three strains of the AS019 family; when cells were grown in LBG-GI then transformed with homologously-derived DNA, strain MLB133 showed relatively high transformation efficiency which was at least 10-fold higher than seen for AS019 and MLB194. Similar results were obtained following growth in both LBG and LBG-GI.

In the case of the ATCC 13032 family, when cells were grown in LBG-GI then transformed with homologously-derived DNA, a higher transformation efficiency was obtained for ATCC 13032 when compared with mutant strains, RM3 and RM4. The presence of 2% glycine and 0.4 mg/ml of INH in the growth medium was also found to be important when heterologously-derived DNA was transformed into strains of the ATCC 13032 family; although RM3 and RM4 gave reasonable transformants in the absence of glycine and INH in the growth medium, the presence of glycine and INH lead to approximately 10-fold increases in the transformation efficiencies.

Since the transformation efficiency for ATCC 13032 is not lower than that for AS019, it is possible to compare transformation efficiency across the two classes of mutants. Transformation efficiencies from the two restriction deficient mutants, RM3 and RM4, were lower than those for MLB133 and MLB194 when cells grown in medium containing LBG-GI and transformed with homologously-derived DNA. However, the concentrations of glycine and INH which were optimum for transformants of RM3 and RM4 were not determined and high frequency may be seen with other combinations of glycine plus INH.

Of six strains tested and the growth conditions used, the highest number of transformants was obtained when MLB133 cells were grown in the presence of 2% glycine and 4 mg/ml INH, so that a frequency of 5.8 X 10^6 transformants (10^9 cells treated X μg of DNA)$^{-1}$ was seen. This indicates that mutation in MLB133 is significant and this contributes to improved uptake of DNA. The results presented here also indicate that the growth media for *C. glutamicum* are important and the presence of glycine and INH in the growth medium is useful to reduce cell surface barriers in electrotransformation of *C. glutamicum*.

3.4 EFFECTS OF GLYCINE AND INH IN THE GROWTH MEDIUM ON THE MYCOLIC ACID COMPOSITION OF *C. glutamicum*

In the MSc thesis of Pierotti (1987), it was shown that there were quantitative differences in the relative proportions of mycolic acids made by *C. glutamicum* AS019 and the presumptive cell surface mutants MLB133 and MLB194 when grown in LB broth. Furthermore, preliminary evidence was presented that at least mutants of the MLB130 - 133 family, represented by strain MLB133, had a relatively higher proportion of extractable and extracellular mycolic acids. However, quantification of the mycolic acids was on the basis of material recovered through a number of extraction procedures and this was performed without validating the efficiency of extraction or derivatisation methods. The aim of the work described in this section was to determine the importance of growth in the presence of glycine, INH or combinations of these on the mycolic acid composition and location for the ATCC 13059 and ATCC 13032 family of strains and their mutants. Part of this work involved developing extraction procedures which may have minimised loss of lipids at different preparation or extraction steps, to allow better quantification of mycolic acids which would then allow comparisons across strains to be made. In the absence of commercially-available corynemycolic acids, efficiency of extraction was evaluated where possible by inclusion of lignoceric acid methyl esters as a standard.

Data presented in this section were therefore obtained using various extraction methods for mycolic acids and fatty acids from either whole cells or culture fluids. Methods used by Pierotti (1987) were initially applied in this work, but some of the procedures required modification. Earlier extraction methods for cells contained a TLC step before further extraction of mycolic and fatty acids. Later on, the TLC step was found to be not necessary and was omitted. Therefore, when different extraction procedures for mycolic and fatty acids were used for different experiments, these are mentioned with each data set presented. In the case of extraction procedures applied to culture fluids, the method used by Pierotti (1987) was also modified because a working large-scale freeze-dryer was not available locally to concentrate culture filtrates. Instead of using freeze-drying, mycolic and fatty acids were initially concentrated from culture fluids by evaporation, which involved heating the culture fluids at 80°C (see section 2.5.1.6 and section 3.4.3.3). Alternatively, solvent extraction (section 2.5.1.6 and section 3.4.3.4) was applied and this gave more reliable results. For the latter case, each step of the extraction procedure step was evaluated for efficiency and this procedure optimised for recovery of mycolic acids, which is described in section 3.4.3.4.

3.4.1 Analysis of mycolic acids using separation of MAMEs and FAMEs by TLC

Non-polar lipids were extracted from dried cells by acid methanolysis and separated by TLC as shown in Fig. 3.20. Two major components were observed in all the extracts. The component with the highest chromatographic mobility ($Rf > 0.5$) corresponds to fatty acid methyl esters (FAMEs), while the lower mobility corresponds to mycolic acid methyl esters (MAMEs) (Minnikin *et al.*, 1980; Pierotti, 1987). All the extracts produced single spots for the FAMEs and MAMEs. The solvent blank also produced spots on the TLC plates (see track 1 in Fig. 3.20B) and the absence of mycolic acids in these spots was confirmed by mass-spectrometric analysis of

Fig. 3.20 Thin layer chromatography of whole cell methanolysates of strains of *C. glutamicum*.

Solvent system: single run in petroleum ether (b.p. 60-80°C)-acetone (95:5, v/v). Abbreviations: FAMEs, fatty acid methyl esters, MAMEs, mycolic acids methyl esters. LBG-GI, LBG containing 2% glycine and 4 mg/ml INH (for ATCC 13032, 0.4 mg/ml INH).

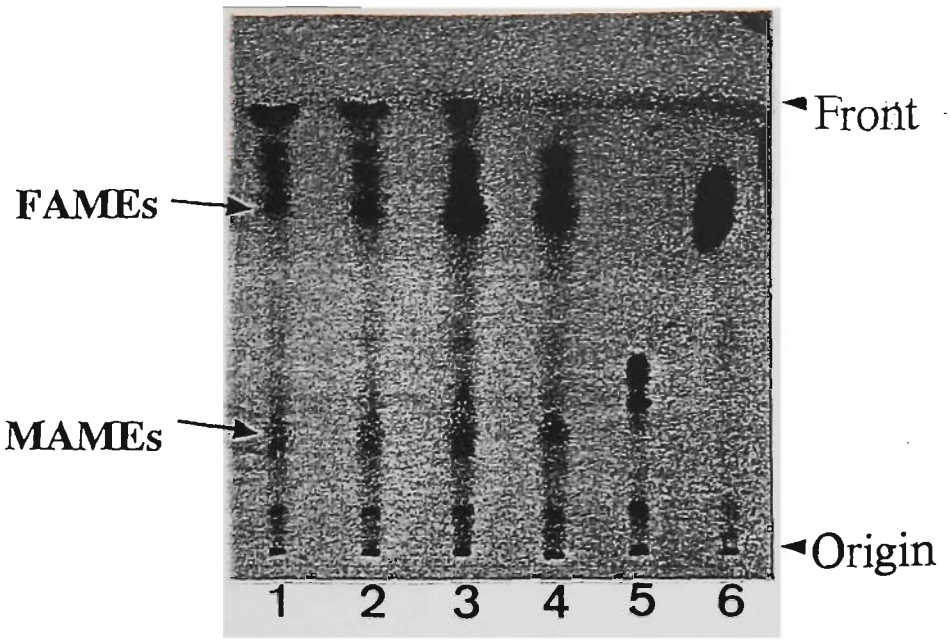
(A)

Track	Source of sample
1	MLB194 (LBG-GI)
2	ATCC 13032 (LBG-GI)
3	AS019 (LBG-GI)
4	MLB133 (LBG-GI)
5	Mycolic acid standard from *Mycobacterium* species (Sigma, M4537)
6	Fatty acid standard (Sigma, 189-6)

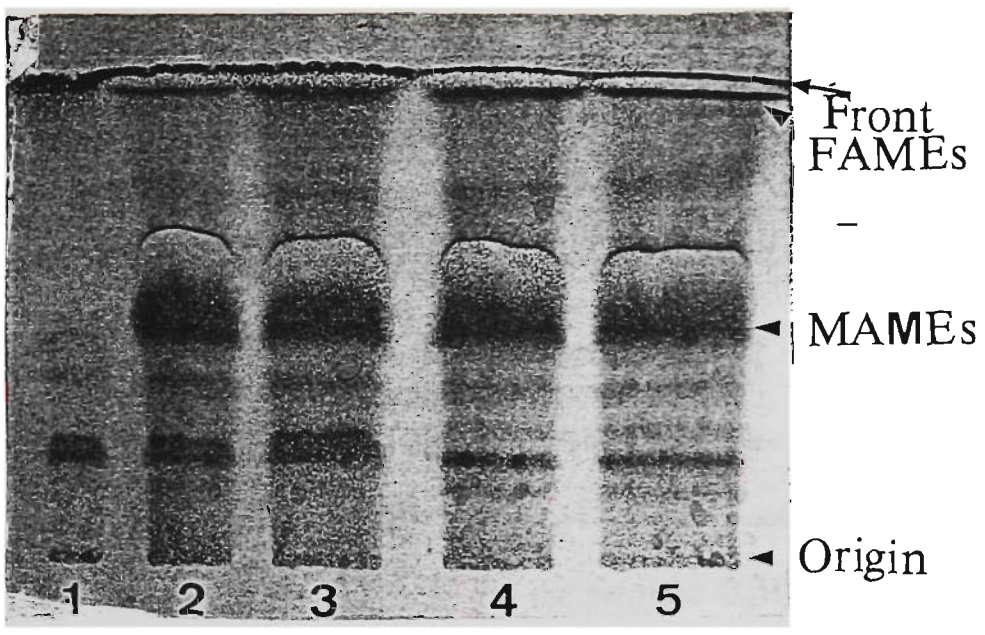
(B)

Track	Source of sample
1	Reagent blank
2	MLB194 (LBG)
3	MLB194 (LBG-GI)
4	MLB133 (LBG)
5	MLB133 (LBG-GI)

A


Front
FAMEs
MAMEs
Origin
1
2
3
4
5
6

B


Front
FAMEs
MAMEs
Origin
1
2
3
4
5

fractions collected from the TLC plates and the two major spots (FAMEs and MAMEs) were not detected when samples were prepared without cells. There were small variations in the R*f* values of the spots corresponding to the FAMEs and MAMEs between experiments.

3.4.2 Mass-spectrometric analysis of TLC-derived MAMEs

MAMEs purified from TLC plates were derivatised, then fractionated by GC. Further analysis to identity each peak was carried out by combined GC-MS as shown in Fig. 3.21. The overall structure of the mycolates was determined from peaks corresponding to the loss of a methyl group (M-15$^+$) and the loss of the trimethylsilyl group (M-90$^+$). The R1 group and R2 group were determined from the resulting fragments A and B, respectively. Peak A was commonly observed corresponding to B-29 (from ethyl groups) and this assists in identifying the R2 side chain (Fig. 3.22). By this method, mass spectra of each peak from GC were analysed (Fig. 3.21) and summarised in Table 3.3. Five major types of mycolic acids were identified ($C_{32:0}$, $C_{34:0}$, $C_{34:1}$, $C_{36:1}$, $C_{36:2}$). These structure were in accord with the observations reported by Pierotti (1987) and Collins *et al.* (1982a).

The first component eluted from the column was a $C_{32:0}$ mycolate, which showed the characteristic peak at *m/z* 567 (M-15$^+$) (Fig. 3.23A). Peaks at *m/z* 313, 371 and 342 corresponded to fragments A, B and B-29 respectively and showed that the R1 chain was the saturated $C_{15}H_{31}$ and the R2 group was $C_{14}H_{29}$.

The second component fractionated was a $C_{34:1}$ mycolate, which gave fragments at *m/z* 593 (M-15$^+$) and 518 (M-90$^+$) (Fig. 3.23B). This peak consisted of two structural isomers, one form bearing an unsaturated $C_{17}H_{33}R_1$ chain and a saturated $C_{14}H_{29}R_2$ side chian, the other bearing a saturated $C_{15}H_{31}R_1$ chain and an unsaturated $C_{16}H_{31}R_2$ side chain.

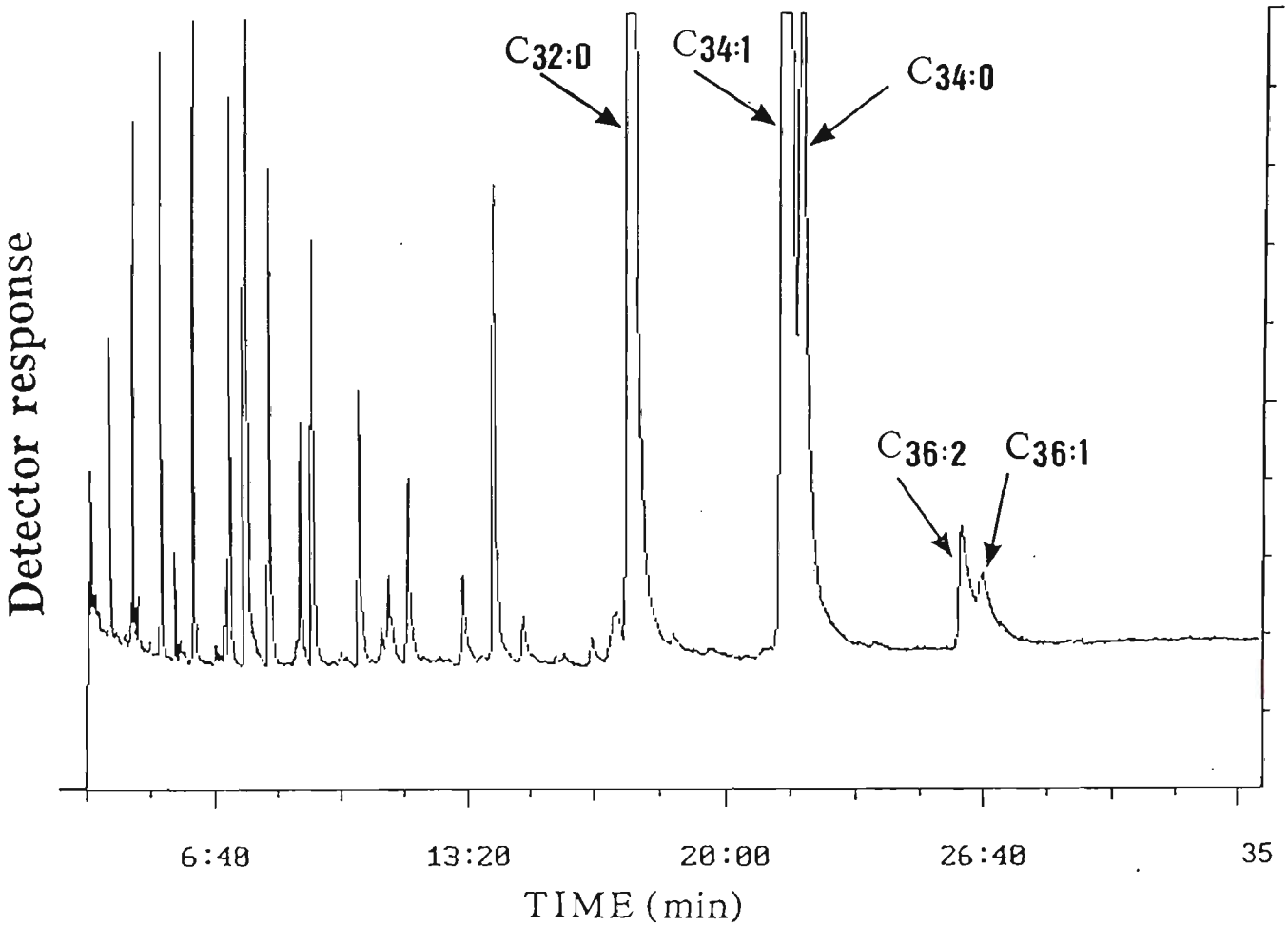


Fig. 3.21 GC analysis of TMS ethers of MAMEs on a non-polar BPX5 fused silica capillary column. Samples were prepared from stationary phase cells of *C. glutamicum* strain AS019 following growth in LBG-GI (2% glycine and 4 mg/ml INH). A mass spectrometry detector was used. For each peak, the first number indicates the number of carbon atoms and the second indicates the number of double bonds.

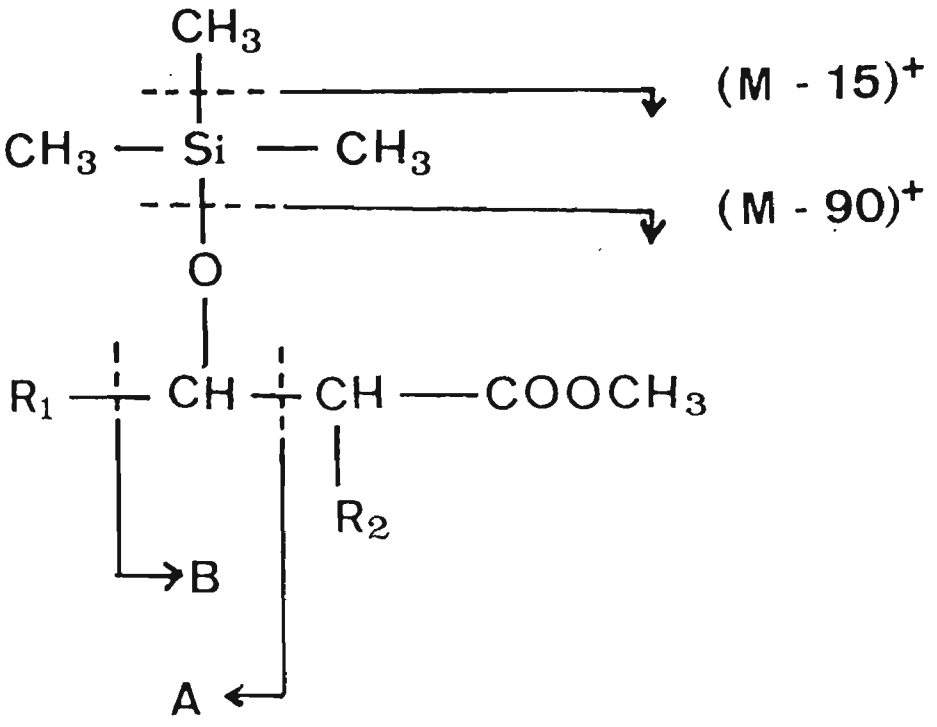
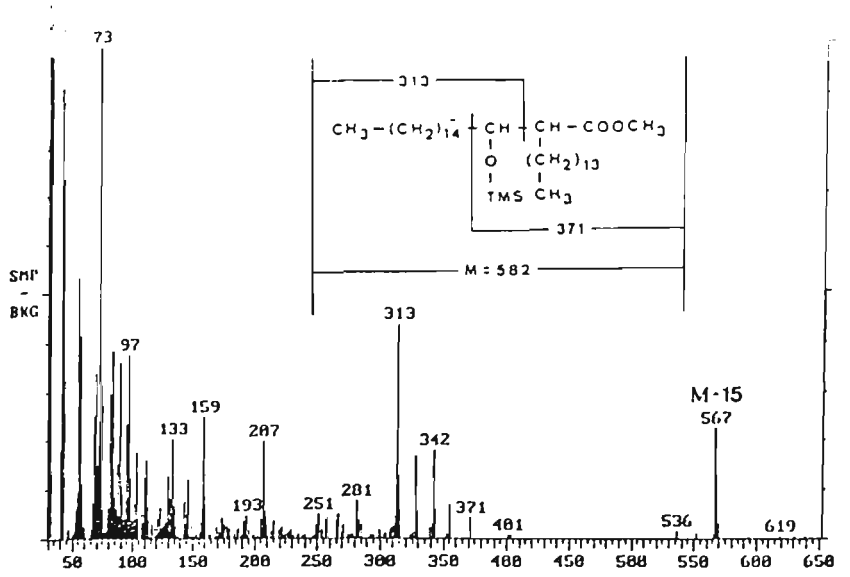
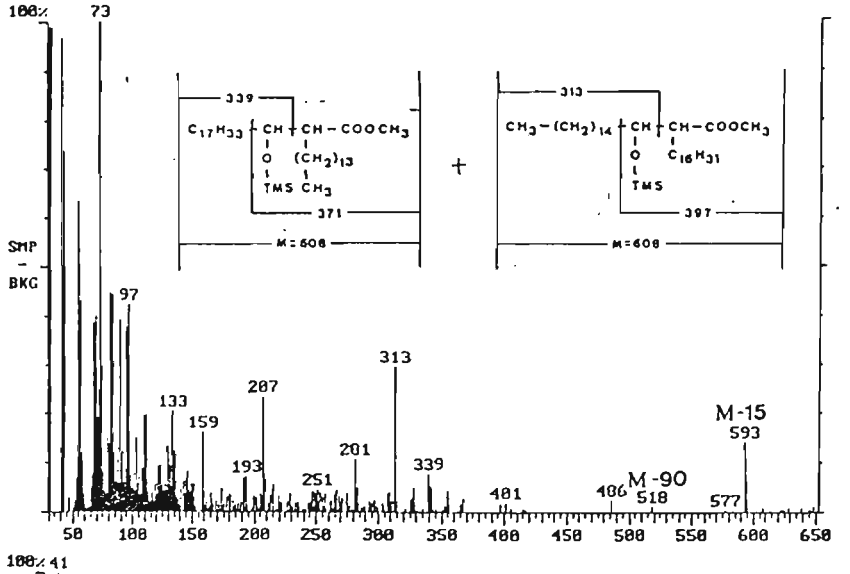


Fig. 3.22 The fragmentation scheme for TMS ethers of MAMEs of mycolic acids from *C. glutamicum* AS019. Information is from Yano *et al.* (1972).

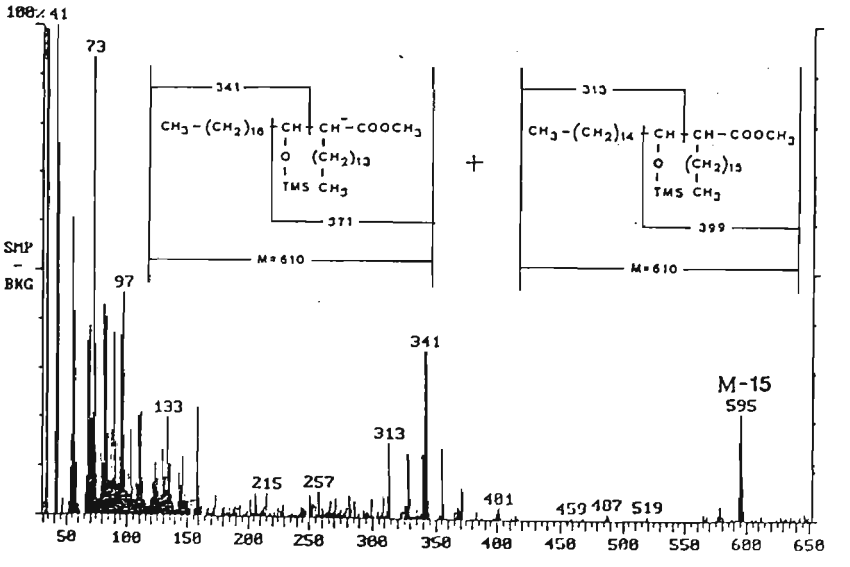
A


73
313
CH3-(CH2)14-CH-CH-COOCH3
O (CH2)13
TMS CH3
371
M=582
SMP
BKG
97
159
133
207
313
342
M-15
567
193
251
281
371
401
536
619
50 100 150 200 250 300 350 400 450 500 550 600 650

B


100%
73
339
C17H33-CH-CH-COOCH3
O (CH2)13
TMS CH3
371
M=608
+
313
CH3-(CH2)14-CH-CH-COOCH3
O C16H31
TMS
397
M=608
SMP
BKG
97
133
159
207
313
193
201
251
339
401
406
M-90
518
M-15
593
577
50 100 150 200 250 300 350 400 450 500 550 600 650

C


100%
41
73
341
CH3-(CH2)16-CH-CH-COOCH3
O (CH2)13
TMS CH3
371
M=610
+
313
CH3-(CH2)14-CH-CH-COOCH3
O (CH2)13
TMS CH3
399
M=610
SMP
BKG
97
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341
313
M-15
595
215
257
401
459
487
519
50 100 150 200 250 300 350 400 450 500 550 600 650

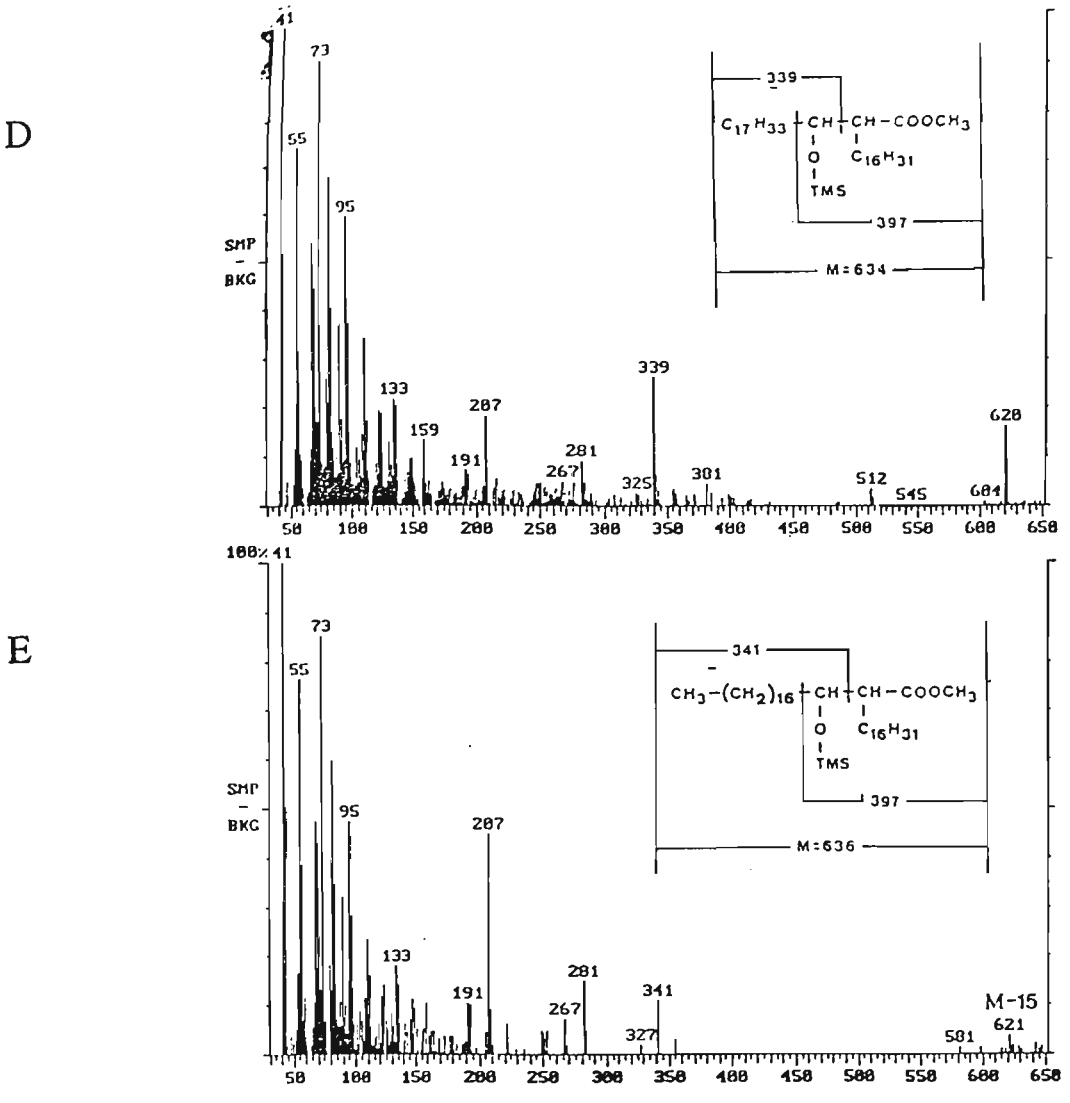


Fig. 3.23 Mass spectra of TMS ethers of MAMEs of *C. glutamicum* AS019. The five major peaks detected by GC for the TMS derivatives were identified by GC-MS: the ordinate axis gives the relative fragment abundance (%). A = $C_{32:0}$, B = $C_{34:1}$, C = $C_{34:0}$, D = $C_{36:2}$, E = $C_{36:1}$, where the first number in the subscripts indicates the number of carbon atoms and the second number indicates the number of double bonds in the compound.

Table 3.3 Mycolic acid structures of *C. glutamicum* [a].

See Fig. 3.22, for the overall structure of mycolic acids.

Fragment ions (*m/z*)			Formula of acids	Side chain		No. of double bonds
M	*M*-15	*M*-90		R1	R2	
582	567	492	$C_{32}H_{64}O_3$	$C_{15}H_{31}$	$C_{14}H_{29}$	0
608	593	518	$C_{34}H_{66}O_3$	$C_{17}H_{33}$ or $C_{15}H_{31}$	$C_{14}H_{29}$ $C_{16}H_{31}$	1
610	595	520	$C_{34}H_{68}O_3$	$C_{17}H_{35}$ or $C_{15}H_{31}$	$C_{14}H_{29}$ $C_{16}H_{33}$	0
634	619	544	$C_{36}H_{68}O_3$	$C_{17}H_{33}$	$C_{16}H_{31}$	2
636	621	546	$C_{36}H_{70}O_3$	$C_{17}H_{35}$	$C_{16}H_{31}$	1

[a] Identical structures were found for all strains tested.

The third component fractionated was a $C_{34:0}$ mycolate, which also consisted of two structural isomers (Fig. 3.23C). One form was a saturated $C_{17}H_{35}R_1$ chain and a saturated $C_{14}H_{29}R_2$ side chain, the other a saturated $C_{15}H_{31}R_1$ chain and a saturated $C_{16}H_{33}R_2$ side chain.

The fourth component fractionated was a $C_{36:2}$ mycolate (Fig. 3.23D), which did not give the expected fragment at *m/z* 619 (M-15$^+$). The absence of *m/z* values of 619 may be due to self-ionisation in the chamber (K. J. Voorhees, personal communication).

The fifth component fractionated was a $C_{36:1}$ mycolate which gave a fragment at *m/z* 621 (M-15$^+$) (Fig. 3.23E).

3.4.3. Standardisation of methods for quantitative analysis of mycolic acids in cells and culture fluids

3.4.3.1 Standard curves for quantification of mycolic acid

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. 24). Samples were diluted with pyridine because the derivatising agent (Tri-sil Z) was solubilised in pyridine. As shown in Fig. 3.24, the relationship between the peak area and the amount of TMS ethers of MAMEs applied was linear, with peak area ranging between $<$2,000 to 65,000. In most cases, samples described in the present study contained amounts of mycolic acids within this range of peak area.

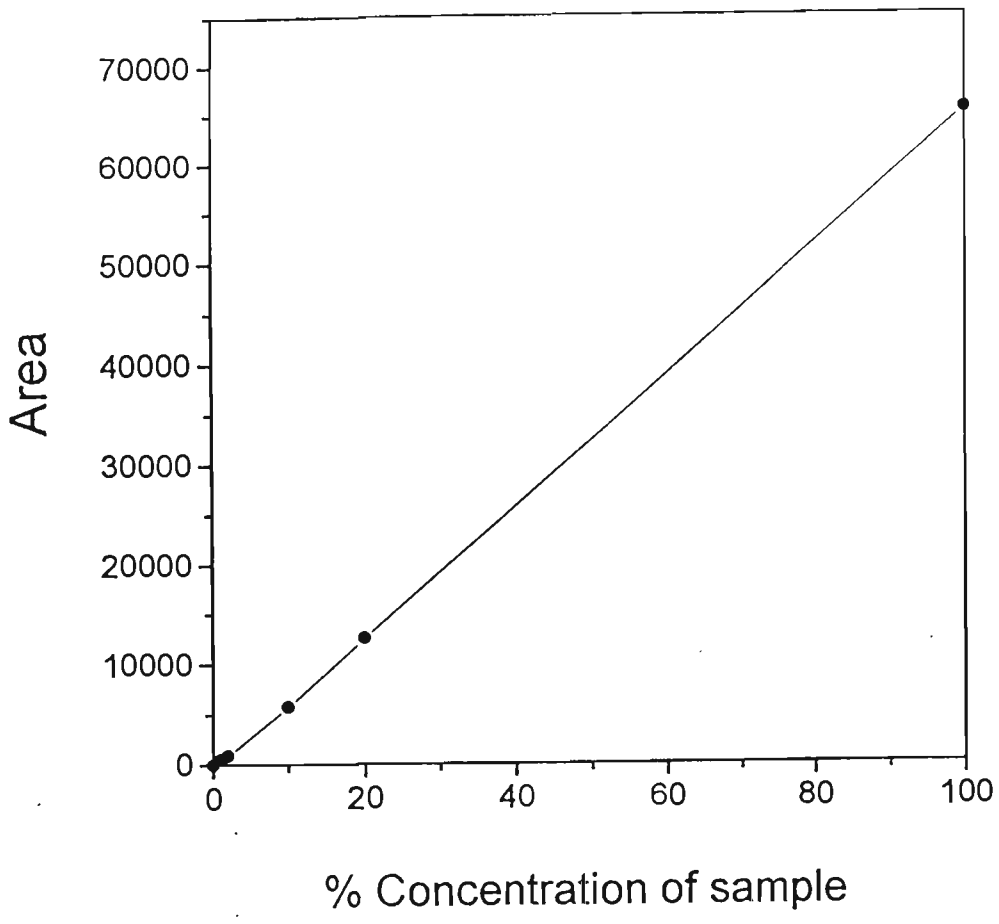


Fig. 3.24 Relationship between amount of mycolic acids from whole cells and peak area detected from GC. Cells of *C. glutamicum* AS019 were grown in LBG were extracted from whole cells as described in section 2.5.1, omitting the TLC step. MAMEs were diluted from samples with dilution factors of 5, 10, 50, 100 and 1,000 and analysed by GC. The same amount of each sample from each dilution was injected into the column.

3.4.3.2. Use of internal standard

Due to the difference in concentration of mycolic acids in cell and culture fluid samples, the concentration of the internal standard (lignoceric acid methyl ester, LAME, $C_{25}H_{50}O_2$, 100 μl) was reduced from 5 mg/ml to 2 mg/ml when samples were prepared from culture fluids. The peak corresponding to the internal standard in GC chromatograms appeared between the last peak from fatty acids and the earliest peak from mycolic acids, and did not interfere with the interpretation of data for both fatty and mycolic acids 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, $C_{32}H_{64}0_2$) were also tested as possible standards. These eluted between the peaks of either fatty acids or peaks of mycolic acids in the GC chromatograms, 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 an internal standard to each sample prior to extraction and all analyses standardised using the area obtained following extraction and derivatisation of this LAME internal standard.

3.4.3.3 Evaluation of using evaporation at 80°C to concentrate mycolic acids in culture fluids

Mycolic acids in culture fluids were concentrated to dryness by heating samples at 80°C for 24 h. In order to determine whether mycolic acids were stable at this temperature during this procedure, AS019 was firstly cultivated in LBG. After harvesting, dried samples from whole cells were divided into four equal portions and 100 μl of the internal standard, LAME (5 mg/ml), added. Three portions of dried cells were resuspended with the same amount of either water, or LBG, or LBG containing 2% glycine and 4 mg/ml INH (LBG-GI), respectively. These was incubated at 80°C for 24 h, while the other portion was stored at -20°C without heat treatment and used as the

Table 3.4 Recovery of TMS ethers of MAMEs from solutions following heating samples at 80°C in a water bath for 24 h.

Cultures of AS019 containing 150 ml of LBG media were grown in a 500 ml flask at 30°C, and harvested at early stationary growth stage. Cells were dried using a freeze-dryer and divided into four equal portions. Subsequently, three portions of dried cells were resuspended with each solvent described below and incubated at 80°C for 24 h to remove any solution from the cells. Mycolic acids were extracted from dried cells as described in sections (2.5.1.1, 2.5.1.3 and 2.5.1.4), omitting TLC purification. After acid methanolysis of the dried cells, samples were extracted using petroleum ether, dried under N_2 gas, silylated to TMS ethers and then analysed by GC.

Days [a]	Solvent/Temperature [b]	$C_{32:0}$	$C_{34:1}$	$C_{34:0}$	$C_{36:2}$	$C_{36:1}$	Total [c] peak area
1	Control/-20°C	52.7 [d]	31.3	8.5	5.3	2.2	493,749
	Water/80°C	53.7	30.8	8.7	4.9	2.0	518,774
	LBG/80°C	49.2	32.9	9.1	6.4	2.5	384,282
	LBG-GI/80°C	57.4	28.2	7.8	5.2	1.5	89,114
20	Control/-20°C	54.3	29.9	8.2	5.3	2.4	214,456
	Water/80°C	55.3	29.3	8.1	5.0	2.4	179,792
	LBG/80°C	55.4	29.4	8.1	5.1	2.0	116,544
	LBG-GI/80°C	58.1	28.0	7.7	4.6	1.6	21,565
30	Control/-20°C	54.5	29.9	8.5	5.1	2.0	161,505
	Water/80°C	55.5	29.6	8.4	4.6	1.9	152,097
	LBG/80°C	55.0	29.6	8.2	5.0	2.0	94,102
	LBG-GI/80°C	56.6	29.1	8.3	4.1	2.0	23,920
	Average of MAMEs %	54.8	29.8	8.3	5.1	2.0	
	Standard deviation	±2.3	±1.3	±0.4	±0.6	±0.3	

[a] Duration of sample (derivatised TMS ethers of MAMEs) storage at -20°C after various treatments

[b] For controls, no solvent was added and samples stored at -20°C. For other samples, three portions of dried cells were resuspended with water, LBG, or LBG containing 2% glycine and 4 mg/ml INH, and incubated at 80°C for 24 h.

[c] The peak areas for the MAMEs were corrected for variations in the area obtained for the internal standard to account for loss during extraction procedures.

[d] Proportion of each mycolic acid was calculated as a % of the total in terms of peak area detected.

control. After incubation at 80°C when the extracts were dry, the four samples were treated as described in section 2.5.1.6 for dry cell extraction and mycolic acids analysis by GC.

The peak areas obtained for the MAMEs were corrected for variations in the area obtained for the internal standard to account for loss of esters during extraction procedures. The qualitative mycolic acid profiles obtained from four samples were almost identical in terms of the relative percentage of mycolic acids detected, but there were quantitative variations (Table 3.4). When samples were resuspended in water and evaporated by heating at 80°C, there was little difference in the total peak area detected using fresh material (Day 1 analysis) compared to from control (dry cells, not heated). This indicated that mycolic acids were stable during the heating procedure when cells were in water. However, when cells were resuspended with LBG or LBG-GI and evaporated, the amount of mycolic acids detected by GC analysis decreased by 20% in LBG and 80% in LBG-GI, respectively. This was presumably due to the presence of sugar and amino acid components of the media, such as glucose, yeast extract, and glycine, which formed sticky complexes during the evaporation procedure and decreased extraction yield during the following solvent extractions.

Above results indicated that using 80°C heating to concentrate mycolic acids in culture fluids was probably reliable for determining the relative proportion of the mycolic acids present but this method could not be used reliably for quantitative analysis of mycolic acids from cells plus culture fluids. Consequently, an alternative approach to quantifying mycolic acids in cells plus culture fluids was sought, based on solvent extraction to remove mycolic acids from culture fluids immediately (see section 2.5.1.6).

3.4.3.4 Evaluation of solvent extraction methods for concentrating mycolic acids in culture fluids

Using heating at 80°C lead to the formation of a dried sample which was sticky and hard to redissolve in solvents used for subsequent derivatisation. As suggested above, although this method probably resulted in a reasonable estimation of the preparation of mycolic acids present extracellularly, alternative methods were sought using solvent extraction to avoid concentration of media components at the same time. Initially, two solvent systems were investigated in terms of recovery efficiencies. For the first solvent extraction system, the filtered culture fluids (30 ml) of AS019 grown in LBG were mixed with the LAME (100 μl, 2 mg/ml) and 2.5 volumes of chloroform/methanol (1:2, vol/vol) then the emulsion held stationary for 4 h. Subsequently, one volume of chloroform and one volume of deionised water were added, incubated for 1 h at 100 o.p.m, then the mixture transferred to a separating funnel to allow separation for 16 h. The bottom layer (containing mycolic acids) was collected (Bligh and Dyer, 1959 and see section 2.5.1.6).

Conditions used for the second solvent extraction method were the same as conditions used for the first solvent extraction method described as above, except that the solvents were replaced as follows: methanol/toluene/H_2SO_4 (30:15:1, vol/vol/vol) was used instead of chloroform/methanol and petroleum ether was subsequently used instead of chloroform/water. The top layer (potentially containing mycolic acids) was collected. Collected extracts were dried under N_2 gas, derivatised by acid methanolysis, MAMEs extracted using petroleum ether then concentrated under nitrogen before trimethylsilylation as described in section 2.5.1.6. Samples were then immediately analysed by GC.

Table 3.5 Recovery of TMS ethers of MAMEs from solutions following solvent extractions.

Cultures of AS019 containing 150 ml of LBG media were grown in the 500 ml flask at 30°C, and harvested at early stationary growth stage. Culture fluids were filtered then divided into the five equal portions. Three portions of these were used for mycolic acids extraction from culture fluids with the first solvent extraction system (see section 2.5.1.6), while two portions of these were used for mycolic acids extraction from culture fluids with the second solvent extraction system. Mycolic acids were extracted from culture fluids as described in the text, omitting TLC purification. After acid methanolysis of the dried cells (see section 2.5.1.1), samples were extracted using petroleum ether, dried under N_2 gas, silylated to TMS ethers and then analysed by GC.

Solvent mixture [a]	N [b]	$C_{32:0}$	$C_{34:1}$	$C_{34:0}$	$C_{36:2}$	$C_{36:1}$	Total peak area [d]	Area of internal standard [e]	STD [f]
Chloroform/methanol	1	51.7 [c]	31.9	9.1	5.0	2.3	57,698	179,120	0.322
(Chloroform/water)	2	49.7	33.8	9.0	5.3	2.3	85,822	253,070	0.339
	3	47.4	33.5	9.0	7.1	3.1	38,913	100,370	0.388
Average		49.6	33.1	9.0	5.8	2.6			
Standard deviation		±2.3	±1.3	±0.4	±0.6	±0.3			
Methanol/toluene/H_2SO_4 [g]	1							19,195	
(Petroleum ether)	2							27,675	

[a] Two combinations of solvents were used for extraction of mycolic acids. Solvent in the parenthesis was used later.
[b] Serial number of experiment for each method
[c] The proportion of each mycolic acid was calculated as a % of the total.
[d] Sum of peak area of mycolic acid derivatives.
[e] Area of internal standard.
[f] Standardised sum of peak area from mycolic acids, which was obtained from the ratio of d/e.
[g] Where in blank, peaks corresponding to mycolic acids were mixed with strong background and information was not available.

Data in Table 3.5 indicated that mycolic acids were able to be extracted from culture fluids using the first solvent system and that this procedure was reliable. Although the total peak area seen for mycolic acids varied between extractions, corresponding changes in the area of the internal standard could be used to account for these differences, as the ratio between the total area for MAMEs and the area detected for the TMS ether of LAME remained relatively constant. The average for the relative percentage of each mycolic acid in this culture fluids sample had small standard deviation, indicating that this method could be reliably used to detect the relative proportion of mycolic acids. When samples were prepared from the culture fluids using the first solvent system, there were no or few background peaks detected above 280°C. In contrast, the second solvent system was found to be not suitable for this purpose as it failed to extract significant amounts of mycolic acids.

3.4.3.5 Evaluation of the efficiency of extracting MAMEs and FAMEs using petroleum ether

Once samples (whole, dried cells or solvent extracted from culture fluids) were collected, the following procedure was used for extraction of mycolic acids which was previously described by Minnikin *et al.* (1980) and modified by Pierotti (1987). Samples were transferred to glass tubes and 100 μl of the LAME (5 mg/ml internal standard for samples from cells) or 2 mg/ml LAME for samples obtained from culture fluids was added. 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 to disrupt cell wall structures and for methanolysis. Normally, this mixture was extracted using petroleum ether. To evaluate the efficiency of extraction, three successive extractions were performed using 2 ml of petroleum ether. After vortexing for 30 seconds, the emulsion was allowed to separate for 10 min, then the top layer was collected and transferred to a new tube then this procedure repeated twice. Each extraction was dried separately under N_2 flow, silylated and analysed by GC

Table 3.6 Recovery of mycolic acids from the cell wall of ATCC 13032 using acid methanolysis and following repeated petroleum ether extractions.

Cultures of ATCC 13032 containing 30 ml of media were grown in the 250 ml flask at 30°C, and harvested at early stationary growth stage. Cells were dried using a freeze-dryer and approximately 50 mg of dried cells were used in methanolysis then three successive extractions using petroleum ether were performed as described in the text, omitting TLC purification. Abbreviations: LBG-G, LBG containing 2% glycine, LBG-I, LBG containing 4 mg/ml INH, LBG-GI, LBG containing 2% glycine and 0.4 mg/ml INH.

Growth medium	Total peak area [a]			Area of Internal standard [b]		
	1st	2nd	3rd	1st	2nd	3rd
LBG	72,842 (69.9)	29,247 (28.1)	2,156 (2.1)	86,691 (82.7)	16,643 (15.9)	1,521 (1.5)
LBG-G	69,868 (79.6)	17,924 (20.4)	Not detected	89,048 (86.0)	14,107 (14.0)	Not detected
LBG-I	50,978 (71.6)	19,276 (27.1)	958 (1.3)	68,923 (60.0)	45,649 (39.4)	1,262 (1.0)
LBG-GI	51,028 (69.8)	20,822 (28.5)	1,294 (1.8)	66,536 (77.5)	17,557 (20.5)	1,732 (2.0)

[a] Sum of peak area of five mycolic acids from each extraction procedures. Figures in parenthesis are the proportion of mycolic acids recovered in each extraction, based on the total peak area obtained for all three extractions.

[b] Peak area of internal standard (LAME) from each extraction procedures. Figures in parenthesis are the proportion of LAME recovered in each extraction, based on the total peak area obtained for all three extractions.

without prior separation by TLC (Table 3.6). 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.

Results presented in Table 3.6 showed that the extraction yield of the internal standard was 60-85% in the first extraction, up to 90-98% by the second extraction and more than 99% by the third extraction, when comparing the area obtained relative to the control. When cells of ATCC 13032 were grown in various growth media and mycolic acids were prepared using the procedure described above, the recovery of mycolic acids from each extraction was 70-80% in first extraction, 97-99% by the second extraction, and more than 99% total by the third extraction. Based on this data, petroleum ether extraction after methanolysis was performed in triplicate for all further experiments.

3.4.3.6 Reproducibility of results between experiments

Analyses of extracts prepared from cells of strain AS019 (growth in LBG) were performed six times to determine variations in quantification between samples. Six separately grown cultures were harvested from early stationary growth phase and mycolic acids were separately prepared from the cells using the procedure described in section 2.5.1. The qualitative mycolic acid profiles obtained in the six analyses were the same for the five types of mycolic acid, but there were but there were quantitative variations, related to the relative proportion of each mycolic acid detected (Table 3.7). Variation was found for the compound $C_{32:0}$ (49.2 to 58.2%), $C_{34:1}$ (27.3 to 34.2%), $C_{34:0}$ (7.2 to 10.3%), $C_{36:2}$(3.2 to 7.4%) and $C_{36:1}$ (1.9 to 4.1%). In the case of repeated injection (twice using same sample), variation was less than $\pm$2% between injections.

Table 3.7 Evaluation of reproducibility of quantitative analysis of mycolic acid composition between different experiments using *C. glutamicum* AS019 grown in LBG.

Cultures containing 30 ml (or 100 ml) of media were grown in 250 ml flasks at 30°C and harvested at early stationary growth phase. Mycolic acids were extracted from dried cells as described in section 2.5.1, omitting TLC purification. 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.

N [a]	$C_{32:0}$	$C_{34:1}$	$C_{34:0}$	$C_{36:2}$	$C_{36:1}$
1	58.2 [b]	28.4	7.2	4.2	1.9
2	56.1	27.3	9.0	5.0	2.5
3	52.7	31.3	8.5	5.3	2.2
4	51.0	30.1	9.4	6.4	3.2
5	50.8	34.2	8.5	3.2	3.2
6	49.2	29.0	10.3	7.4	4.1
Average	53.0	30.0	8.8	5.3	2.9
Standard deviation	±3.4	±2.4	±1.0	±1.5	±0.8

[a] N is experiment number. Data was from six separately grown cultures performed 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.

3.4.3.7 Formulae used

After GC analysis of each group of samples, the average area of the internal standard was derived by dividing the total sum of the area from internal standard peaks by the number of analyses performed in that group. Different formula were used for cells and culture fluids, which reflected the different nature of the samples analysed; only part of the dried cells were used for analysis whilst, in the most cases, all the culture fluids were used following solvent extraction to concentrate. Standardisation of mycolic acids or fatty acids from cells and culture fluids was calculated using the following formulae.

For mycolic acids or fatty acids from dried cells,

$$\text{Relative area} = \frac{\text{SA x Weight TDC x Aver IS}}{\text{IS X Weight DC}}$$

where,

SA = peak area of sample

Weight TDC = total dry weight of cells

IS = area of internal standard for each injection

Aver IS = average area of internal standard

Weight DC = dry weight of cells used for extraction

For mycolic acids or fatty acids from culture fluids,

$$\text{Relative area} = \frac{\text{SA x CF x Aver IS}}{\text{IS}}$$

where,

SA = peak area of sample

CF = conversion factor for internal standard (= 5 mg/ml/2 mg/ml)

Aver IS = average area of internal standard

IS = area of internal standard for each injection

Since a smaller amount of internal standard was used for sample extraction of culture fluids than for whole cells, the CF was required to allow comparison of sample area for cells and culture fluids.

3.4.4 Mycolic acid composition following growth in LBG

Results presented in this section and the three following sections (section 3.4.5, section 3.4.6, section 3.4.7) were obtained from two different sets of procedures for cells and culture fluids.

For analysis of mycolic acids from the cells, one procedure (described as Experiment 1 in each section) contained a TLC step to separate FAMEs and MAMEs whilst the other procedure did not contain this TLC step and lipids were solvent extracted, derivatised and analysed, as described in the section 2.5.1.3 and section 2.5.1.4 (Experiment 2 in each section).

Two different approaches were performed in order to obtain mycolic acids and fatty acids from the culture fluids as follows; evaporation of samples to dryness in a waterbath at 80°C (results of these are shown in Experiment 1), or solvent extraction procedure (results of these are shown in Experiment 2).

For the first approach (results from this are shown in Experiment 1), the following procedures were applied to the samples of whole cells and culture fluids. The mycolic and fatty acids were

obtained from whole cells using the procedure containing a TLC step, as described in section 2.5.1.2. For sample preparation of culture fluids, acids were concentrated using evaporating samples to dryness in a waterbath at 80°C as described in the section 2.5.1.6 (i). In the case of this approach, because mycolic acid standards were not available for TLC separation, the internal standard (LAME) was not added to the samples from the cells (although LAME was added to the samples from culture fluids) to check recovery of mycolic acids so that analyses could not be corrected to account for errors generated during collection of MAMEs. It was therefore not possible to obtain reliable data for the relative distribution of mycolic acids in culture fluids and cells, although it was possible to compare the relative mycolic acid composition (% of each type of mycolic acids) within fractions prepared similarly and between strains. Mycolic acids are not stable at high temperature but, when mycolic acids are derivatised to MAMEs, MAMEs can be protected from pyrolytic cleavage by derivatisation of the hydroxy group to a TMS ether which is stable at high temperatures (>80°C). Since the release of mycolic acids into culture fluids was found to be highest at stationary phase (Pierotti, 1987), cells were harvested at stationary phase in order to determine the composition of mycolic acids in the cells and the culture fluids. Culture fluids were collected, filtered, dried and then derivatised, and extracted as for the dried cells, prior to GC analysis.

Later on, different approaches (results of these are shown in Experiment 2) were performed as follows. The mycolic acids were obtained from whole cells using a procedure which omitted the TLC step, as described in section 2.5.1.1. For sample preparation of culture fluids, mycolic and fatty acids were obtained using the solvent extraction method as described in section 2.5.1.6 (ii). For this procedure, the internal standard (LAME) was added to the samples at the beginning of the extraction of culture fluids or treatment of dry cells. The levels of extracellular mycolic acids and fatty acids in culture fluids plus cells was determined from these approach. For the analysis of the composition and distribution of fatty acids, GC conditions were changed in order to get

better resolution in FAMEs peaks, where the chromatograms of FAMEs and the results of FAMEs analysis are presented in section 3.4.8.

Due to the frequent use of the GC column by several people, the column had to be completely cleaned before analysis to avoid any chance of contamination by the presence of any chemicals in the column from previous analyses by others. Temperature gradient for TMS ethers of MAMEs analysis were from 260°C to 320°C for 60 min. The first peak from TMS ethers of MAMEs was eluted at above 280°C (approximately 15 min of analysis), while the majority of background peaks corresponding to the other compounds including FAMEs, solvent, and other chemicals, were eluted within 5 min of analysis, well before the first MAMEs. In most cases, there were no or few background peaks detected above 280°C when mycolic acids were absent in the sample solution (data not shown).

Experiment 1. Isolation and analysis of mycolic acids from cells and culture fluids using procedure 1.

The methods used by Pierotti (1987) were used without modification, to allow comparison with his data for ATCC 13059, MLB133 and MLB194. Mycolic acids and fatty acids were converted to the MAMEs and FAMEs by acid methanolysis. Following extractions using petroleum ethers, MAMEs and FAMEs were collected separately from a TLC plate (section 2.5.1.1 and section 2.5.1.2) then further derivatised for GC analysis (see sections 2.5.1.3 and 2.5.1.4). Using GC conditions described in the section 2.5.1.4, FAMEs eluted at 2.64 min for $C_{16:0}$ and 3.13 min for $C_{18:1}$ and the peaks were identified as being FAMEs by comparison with the retention time and peak area of external standards (Sigma 189-6, 189-17). Pierotti (1987) had shown that the composition of FAMEs of *C. glutamicum* AS019 and its mutants did not significantly depend on the growth phase of harvesting cells so that analyses of all samples here were from stationary

phase cultures.

After growth in LBG, the composition of MAMEs was analysed. Among five major mycolic acids, two components of the mycolic acids fraction ($C_{32:0}$ and $C_{34:1}$) made up 80-90% of the total composition in all strains (analysis of mycolic acids in whole cells and culture fluids is shown in Table 3.8A). All the strains tested exhibited similar mycolic acid compositions in terms of types of mycolic acids detected when grown in LBG. However, differences in the relative amounts of mycolic acids were seen; the abundance of $C_{32:0}$ was higher in strain AS019 than in mutant strains MLB133 and MLB194, which agreed with data reported previously by Pierotti (1987). In terms of the relative proportions of unsaturated to saturated mycolic acid for AS019 and its mutants, significant difference were observed: strain AS019 had low proportions of unsaturated mycolic acid (26.2%), whereas both mutant strains had higher proportions of unsaturated mycolic acids (41.8% in MLB133 and 37.9% in MLB194).

Differences in mycolic acid composition were also seen between the two parent-type strains of AS019 and ATCC 13032: strain ATCC 13032 showed relatively low proportions of $C_{32:0}$ which were approximately 20% lower than seen for AS019. Previously, it was seen that the level of transformation seen for ATCC 13032 was normally higher than seen for AS019 (see section 3.3.4). This difference in transformation efficiency may be explained by the difference in mycolic acid composition. The mycolic acid composition of ATCC 13032 was more similar to that of MLB133. It is interesting to note that both strains showed high transformation efficiencies when cells were transformed with homologously-derived DNA (see section 3.3.4). In the case of the ATCC 13032 family, both mutant strains (RM3 and RM4) showed very different mycolic acid compositions; the relative proportions of $C_{32:0}$ from the two mutants were lower than ATCC 13032, whilst the relative proportion of $C_{36:2}$ was slightly higher than ATCC 13032. In terms of mycolic acids composition, two *Brevibacterium* strains of BL1 and BF4 looked similar to ATCC

13032.

The proportions of unsaturated mycolic acid from the eight strains tested ranged between 26% (in AS019) to 65% (in RM4), showing significant differences in the cell surface structures of strains tested.

This published standard procedure is considered to be a good one; it has been used by many other workers to quantify the relative proportions of mycolic acids (Collins *et al.*, 1982a; Pierotti, 1987). However, my needs were different and involved quantifying the relative proportions of mycolic acids between fractions rather than within fractions, to enable comparison in relative amounts of extracellular mycolic acid. The published methods were not suitable for this purpose due to the absence of internal standards in the samples. Therefore, I decided to explore other methods which allowed direct extraction of mycolic acids to minimise loss at the TLC step. When the TLC step was omitted from the above procedure, both FAMES and MAMEs appeared in the GC chromatogram and the internal standard (LAME) also appeared between FAMEs and MAMEs peaks, which enabled comparisons between the amount of FAMEs and MAMEs from either cells or culture fluids. Results from the latter approaches are shown in Experiment 2. This indicated that the TLC step in the extraction procedure is not necessary.

Results reported by Pierotti (1987) indicated that mycolic acids were found in culture fluids of *C. glutamicum* strains, where these were prepared for analysis be freeze-drying. Because the experimental design of this thesis planned to verify Pierotti's results, which involved a large number and volume of samples, an alternative to freeze-drying for concentrating the culture fluids was sought, which involved evaporating samples to dryness in a waterbath at 80°C. Heating cellular mycolic acids from strain AS019 caused little change in composition (in terms of relative proportions) of mycolic acids extracted (see section 3.4.3.3). For analysis of extracellular

mycolic acids, culture fluids were collected, filtered, dried and then extracted as for the dried cells, and derivatised, prior to GC analysis (Fig. 3.8A).

The relative proportions of the different types of mycolic acids found in the cells and culture fluids were quite similar for all the strains tested: the proportions of unsaturated mycolic acids found in culture fluids ranged between 29% (in AS019) and 54% (in RM4). The abundance of $C_{32:0}$ was higher in culture fluids from strain AS019 than from strains of MLB133 and MLB194. In the case of RM3 and RM4, $C_{32:0}$ was relatively low (45% for RM3 and 42% for RM4) and $C_{36:2}$ was more abundant than in the parent strain ATCC 13032. The proportions of $C_{32:0}$ from AS019 (63%) was always higher than seen for ATCC 13032 (53%). Furthermore, two *Brevibacterium* strains BL1 and BF4, and two *C. glutamicum* strains, ATCC 13032 and MLB133 show similar profiles for mycolic acids found in culture fluids. These results show that mycolic acid profiles in the cell and culture fluids are similar. Results from LBG medium are summarised in Table 3.18 (in discussion section) to allow comparisons with results obtained for other growth media.

Data from culture fluids in Experiment 1 were obtained using the evaporation procedure, as described in section 2.5.1.6. This approach was found to be a good one for qualitative analysis of MAMEs but not for quantitative analysis of MAMES, due to the low extraction yield (see section 3.4.3.3). The solvent extraction procedure was alternatively applied for the extraction of MAMEs and FAMES from culture fluids since this procedure gave above 95% recovery yields for MAMEs. Results from this approach are seen in Experiment 2.

Table. 3.8 Mycolic acids composition of whole cells and culture fluids of eight strains of corynebacteria following growth in LBG using 80°C heating to concentrate and TLC purification (A) or solvent extraction without TLC purification (B).

A

Strains	Mycolic acids [a]					% UM to TM [b]
	$C_{32:0}$	$C_{34:1}$	$C_{34:0}$	$C_{36:2}$	$C_{36:1}$	
Mycolic acids from whole cells						
AS019	68.3±1.3	22.5±1.0	6.1±0.3	2.8±0.4	0.9±0.1	26.2
MLB133	53.2±1.1	34.6±0.6	4.9±0.1	5.8±0.3	1.4±0.2	41.8
MLB194	57.1±0.9	31.2±0.3	5.2±0.3	5.5±0.4	1.2±0.1	37.9
ATCC 13032	49.0±0.2	38.0±0.1	3.5±0.1	8.2±0.1	1.5±0.1	47.7
RM3	40.9	41.3	3.1	13.0	1.7	56.0
RM4	34.0	45.2	1.2	19.0	0.6	64.8
BL1	50.7±0.6	36.9±0.2	3.8±0.1	7.4±0.4	1.5±0.1	45.8
BF4	51.3±0.8	31.4±0.2	9.2±0.1	5.5±0.5	2.7±0.2	39.6
Mycolic acids from culture fluids						
AS019	62.6±0.5	26.3±1.2	8.2±0.4	2.9	-	29.2
MLB133	49.4	33.8	8.1	6.2	2.5	42.5
MLB194	52.7	34.3	7.3	5.6	-	39.9
ATCC 13032	52.9±0.2	34.8±0.1	5.5±0.6	7.1±0.7	-	41.9
RM3	45.0	40.3	4.6	10.1	-	50.4
RM4	41.8	39.7	4.3	12.3	1.9	53.9
BL1	50.8	35.3	5.6	6.1	2.3	43.7
BF4	49.1	33.7	11.2	5.0	1.1	39.8

B

Strains	Mycolic acids [a]					% UM to TM [b]	% extracellular mycolic acids [c]
	$C_{32:0}$	$C_{34:1}$	$C_{34:0}$	$C_{36:2}$	$C_{36:1}$		
Mycolic acids from whole cells							
AS019	53.0±3.4	30.0±2.4	8.8±1.0	5.3±1.5	2.9±0.8	38.2	
MLB133	42.1±6.2	38.1±3.1	7.1±1.4	9.4±2.7	3.3±1.1	50.8	
MLB194	46.1±5.5	34.2±4.1	8.5±0.7	8.1±1.7	3.2±0.7	45.5	
ATCC 13032	40.8±1.4	39.1±2.8	7.2±3.8	9.8±1.4	3.0±1.6	51.9	
RM3	28.9±7.1	37.7±1.7	8.0±2.4	20.4±11.9	5.0±0.1	63.1	
RM4	47.4±2.2	34.6±1.3	8.5±0.4	6.9±0.6	3.2±0.2	44.7	
Mycolic acids from culture fluids							
AS019	54.2±5.7	30.4±3.8	7.6±0.9	6.1±0.6	2.1±1.0	38.6	4.5±1.7
MLB133	46.0±5.0	37.4±5.5	7.4±1.4	7.2±1.2	2.3±0.2	46.9	7.2±2.1
MLB194	47.5±8.8	34.1±4.3	8.4±1.3	7.2±2.7	2.9±1.9	44.2	7.8±2.5
ATCC 13032	51.0	37.9	4.0	7.2	-	45.1	3.5
RM3	36.8±5.9	40.9±13.0	10.4±4.0	6.0±1.7	4.9±0.1	51.8	4.1
RM4	48.6	29.9	10.1	11.5	-	41.4	5.4

[a] Proportion (%) of each mycolic acid found in whole cells or culture fluids. The proportion of each mycolic acids was calculated as a % of the total in terms of peak area detected. Dash represents no peak detected under the condition used.

[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 unsaturated mycolic acids values by the sum of the total mycolic acids.

[c] Proportion (%) of mycolic acids found in culture fluids relative to total mycolic acids detected (whole cells plus culture fluids).

Experiment 2. Isolation and analysis of mycolic acids from cells and culture fluids using procedure 2.

FAMEs and MAMEs were prepared using the procedure described in sections (section 2.5.1.1, section 2.5.1.3 and section 2.5.1.4) for samples from cells and sections (section 2.5.1.6 ii, section 2.5.1.3 and section 2.5.1.4) for samples from culture fluids. Similar results to above were obtained for the mycolic acid profiles when samples were extracted by solvent rather than TLC purified. The major components of the mycolic acids fraction were $C_{32:0}$ and $C_{34:1}$, making up 65-80% of the total mycolic acid composition in all strains (Table 3.8B). However, the proportions of $C_{32:0}$ was usually lower than seen for TLC fractionation of MAMEs. The relative proportions of unsaturated to saturated mycolic acids found in the cells ranged between 38% (in AS019) to 63% (in RM3). All of the strains tested showed similar mycolic acid profiles to those seen in Experiment 1 in this section, except for RM4. In RM4, the proportions of $C_{32:0}$ (47.4%) and $C_{34:0}$ were higher than those seen in Experiment 1 (34.0% in $C_{32:0}$). Trends similar to Experiment 1 were observed for the AS019 family, where the relative abundance of $C_{32:0}$ was higher in strain AS019 than MLB133 and MLB194. When the two parent strains were compared, strain ATCC 13032 again showed relatively lower proportions of $C_{32:0}$ than seen for AS019. Strains MLB133 and ATCC 13032 showed very similar mycolic acid profiles, confirming the results seen using procedure in Experiment 1. RM3 contained lower proportions of $C_{32:0}$ and higher levels of $C_{36:2}$, compared to the parent strain, ATCC 13032.

Mycolic acids were extracted from culture fluids using solvent extraction procedures after harvesting cells from stationary phase in order to determine the composition both qualitatively and quantitatively. The internal standard (LAME) was added into culture fluids prior to extraction. After addition of internal standard to the filtered culture fluids, mycolic and fatty acids were extracted with a mixture of chloroform/methanol (1:2, vol:vol) (section 3.4.3.4). Mycolic and

fatty acids were transferred into the chloroform layer (top layer) after the addition of a mixture of chloroform/water (1:1, vol:vol) and further incubation. Samples were derivatised by acid methanolysis and extracted as for the dried cells prior to GC analysis. Although the proportion of $C_{32:0}$ was usually higher than seen for whole cells, the relative proportions of the different types of mycolic acids found in the culture fluids were quite similar to those seen for the whole cells, for all the strains tested (Table 3.8B).

The distribution of mycolic acids between cells and culture fluids was also determined (see Table 3.8B). For the parent-type strain AS019, a relatively small proportion (4.5%) of the total mycolic acids was found in the culture fluids and the mycolic acid profiles for whole cells and culture fluids were similar, confirming previous observations by Pierotti (1987) when using TLC purification of mycolic acids and freeze-drying of culture fluids. Both mutant strains MLB133 and MLB194 showed a higher proportion of extracellular mycolic acids than the parent strain AS019 at early stationary growth phase and the amount of mycolic acids in culture fluids were 7.2% for MLB133 and 7.8% for MLB194. In the case of the ATCC 13032 family, extracellular mycolic acids were found to be 3.5%, 4.1%, 5.4% for ATCC 1032, RM3 and RM4, respectively.

3.4.5 Mycolic acids composition following growth in LBG-glycine

Experiment 1. Isolation and analysis of mycolic acids from cells and culture fluids using procedure 1.

In order to determine if growth in 2% glycine had any affect on the mycolic acid profiles of the strains, cells were grown as 30 ml (or 100 ml) cultures in the presence of glycine and samples prepared as described in sections 2.5.1.1, 2.5.1.2 and 2.5.1.4. Results of analysis of mycolic

acids in whole cells and culture fluids are shown in Table. 3.9A. When compared with cells grown in LBG, the presence of 2% glycine in the growth medium caused changed in mycolic acid profiles of several strains of *C. glutamicum* tested. The relative proportions of unsaturated to saturated mycolic acids ranged between 25% (in AS019) to 47% (in MLB194). No or little changes in the relative proportions of the mycolic acid profile were seen in AS019, MLB133, ATCC 13032, BL1 and BF4, compared to those seen in LBG. The trends with strains of MLB194 was towards a decreased proportion of $C_{32:0}$, whilst two strains RM3 and RM4 grown in the presence of 2% glycine in medium showed an increase in the relative proportion of $C_{32:0}$ (52.8% for RM3 and 60.9% for RM4) and in the relative proportion of $C_{36:2}$. The relative proportions of the five types of mycolic acids found in the culture fluids for all the strains tested were quite similar to those seen in cells; the proportions of unsaturated mycolic acids ranged between 25% (in AS019) and 49% (in MLB194). No or little difference in the relative proportion of mycolic acids was seen when five strains (AS019, MLB133, ATCC 13032, BL1 and BF4) were grown in LBG containing 2% glycine, compared to those seen for LBG. Mycolic acid composition of ATCC 13032 was similar to that of MLB133, which was also similar to profiles seen in LBG medium. The two mutants RM3 and RM4 exhibited significant increases in the relative proportion of $C_{32:0}$ and decreases in $C_{34:1}$ and $C_{36:2}$.

Experiment 2. Isolation and analysis of mycolic acids from cells and culture fluids using procedure 2.

Growth in LBG-glycine had little impact on the relative proportion of mycolic acids detected in AS019 (Table 3.9B), which was also seen in Experiment 1 in this section. The mutant strain MLB133 exhibited a small increase in the proportion of $C_{32:0}$, whilst MLB194 showed a significant decrease in the proportion of $C_{32:0}$, which was in agreement with observations from Experiment 1. In the case of the ATCC 13032 family, a small increased in $C_{32:0}$ was seen in

Table. 3.9 Mycolic acids composition of whole cells and culture fluids of eight strains of corynebacteria following growth in LBG-glycine (2%) using 80°C heating to concentrate and TLC preparation (A) or solvent extraction without TLC purification (B).

A

Strains	Mycolic acids [a]					% UM to TM [b]
	$C_{32:0}$	$C_{34:1}$	$C_{34:0}$	$C_{36:2}$	$C_{36:1}$	
Mycolic acids from whole cells						
AS019	68.1±0.5	21.2±0.3	7.1±0.1	2.6±0.2	1.1±0.1	24.9
MLB133	54.6±0.2	33.4±0.1	5.6±0.2	5.1±0.1	1.5±0.1	40.0
MLB194	46.6±0.6	37.2±0.2	6.7±0.1	7.3±0.3	2.4±0.1	46.9
ATCC 13032	51.9	36.5	3.7	6.6	1.4	44.5
RM3	52.8	35.0	3.6	7.0	1.6	43.6
RM4	60.9	30.3	3.2	4.9	0.9	36.1
BL1	50.7±0.1	36.3±0.5	3.7±0.1	8.1±0.7	1.3±0.1	45.7
BF4	49.1±0.3	31.6±1.2	12.8±0.1	5.4±2.1	2.339.3	
Mycolic acids from culture fluids						
AS019	63.9	21.7	11.0	3.4	-	25.1
MLB133	50.7±0.7	35.8±0.5	7.8±0.1	5.8±0.2	-	41.6
MLB194	43.1	38.0	8.7	7.4	2.8	48.2
ATCC 13032	52.7	32.7	8.9	5.8	-	38.5
RM3	55.3	33.7	5.8	5.2	-	38.8
RM4	63.2	28.3	5.1	3.4	-	31.7
BL1	50.4	36.2	5.7	7.7	-	43.5
BF4	51.4	29.9	10.9	6.2	1.5	37.6

B

Strains	Mycolic acids [a] $C_{32:0}$	$C_{34:1}$	$C_{34:0}$	$C_{36:2}$	$C_{36:1}$	% UM to TM [b]	% extracellular mycolic acids [c]
Mycolic acids from whole cells							
AS019	52.7±3.8	27.4±4.5	10.8±1.0	6.3±2.8	2.9±0.2	36.6	
MLB133	48.3±8.3	32.9±4.8	8.6±5.1	7.6±1.4	2.6±1.3	43.1	
MLB194	41.1±2.3	37.1±1.1	9.1±0.3	8.5±0.9	4.1±0.6	49.7	
ATCC 13032	48.4±0.2	34.6±0.4	8.6±0.2	6.2±0.2	2.5±0.1	43.3	
RM3	45.3±7.5	33.4±3.6	10.4±0.4	7.2±2.2	3.6±1.2	44.2	
RM4	62.2±2.6	26.8±1.4	5.7±0.5	3.8±0.5	1.3±0.2	31.9	
Mycolic acids from culture fluids							
AS019	54.1	28.2	10.3	4.5	2.9	35.6	15.9±4.9
MLB133	45.2	35.5	8.7	7.8	2.9	46.2	19.3±4.1
MLB194	58.7±2.1	29.3±1.0	6.9±2.1	4.8±0.1	-	34.1	10.1±2.6
ATCC 13032	54.0	32.0	7.8	4.8	1.8	38.6	5.5
RM3	62.7	25.4	8.3	3.7	1	30.1	8.1
RM4	64.3	25.1	8.1	2.6	-	27.7	9.5

[a] Proportion (%) of each mycolic acid found in whole cells or culture fluids. The proportion of each mycolic acids was calculated as a % of the total in terms of peak area detected. Dash represents no peak detected under the conditions used.

[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 unsaturated mycolic acids values by the sum of the total mycolic acids.

[c] Proportion (%) of mycolic acids found in culture fluids relative to total mycolic acids detected (whole cells plus culture fluids).

ATCC 13032, whilst the two mutants RM3 and RM4 exhibited significant decreases in the relative proportion of $C_{34:1}$ and $C_{36:2}$, and increases in the relative proportion of $C_{32:0}$, which increased from 40.8% in LBG to 48.4% in LBG-G for ATCC 13032, 28.9% to 45.3% for RM3, and 47.4% to 62.2% for RM4. These observations confirmed those made in Experiment 1 in this section. The relative proportions of the different types of mycolic acids found in cells and culture fluids were similar to results seen in Experiment 1 for all strains tested; no or little variation in mycolic acid profiles were seen in AS019, MLB133 and ATCC 13032, whereas visual variations were observed for MLB194, RM3 and RM4 (Table 3.9B). The most obvious impact of growth in the presence of 2% (w/v) glycine was an increase in the extracellular mycolic acids, which increased to 15.9% for AS019, 19.3% for MLB133 and 10.1% for MLB194. Smaller increases in extracellular mycolic acids were seen for strains of the ATCC 13032 family after growth in glycine, with 5.5% for ATCC 13032, 8.1% for RM3 and 9.5% for RM4 of the mycolic acids found in the culture fluids.

3.4.6 Mycolic acids composition following growth in LBG-INH (4 mg/ml INH)

Experiment 1. Isolation and analysis of mycolic acids from cells and culture fluids using procedure 1.

Previous cell growth studies showed that the presence of 4 mg/ml of INH caused changes in the specific growth rates of all strains of *C. glutamicum* tested (see section 3.2.4). To evaluate any changes in mycolic acid synthesis in *C. glutamicum*, strains were cultured in LBG containing 4 mg/ml INH, harvesting cells in early stationary phase: analysis of mycolic acids in whole cells and culture fluids is shown in Table 3.10A. The relative proportions of total unsaturated to saturated mycolic acids for the eight strains tested ranged between 30% (in AS019) and 52% (in

Table. 3.10 Mycolic acids composition of whole cells and culture fluids of eight strains of corynebacteria following growth in LBG-INH (4 mg/ml) using 80°C heating to concentrate and TLC (A) or solvent extraction without TLC purification (B).

A

Strains	Mycolic acids [a]					% UM to TM [b]
	$C_{32:0}$	$C_{34:1}$	$C_{34:0}$	$C_{36:2}$	$C_{36:1}$	
Mycolic acids from whole cells						
AS019	63.3	25.4	6.9	3.1	1.3	29.8
MLB133	54.2±1.5	32.7±0.6	5.5±0.4	6.1±0.4	1.6±0.7	40.4
MLB194	59.6±0.1	28.6±0.1	6.2±0.1	4.3±0.1	1.4±0.1	34.3
ATCC 13032	45.9±0.2	38.7±0.1	4.8±0.1	8.8±0.1	1.9±0.1	49.4
RM3	47.1	37.2	5.6	8.1	2.0	47.3
RM4	44.1	40.8	4.2	9.2	1.8	51.8
BL1	52.5	34.6	4.8	6.6	1.6	42.8
BF4	54.0±0.6	30.9±0.1	7.8±0.6	4.4±0.5	2.1±0.3	37.4
Mycolic acids from culture fluids						
AS019	56.0±0.1	29.0±1.4	10.5±0.3	3.5	-	32.5
MLB133	51.7±1.9	33.1±1.8	7.1±0.4	6.2±0.1	2.1	41.4
MLB194	52.0	33.8	8.4	5.8	-	39.6
ATCC 13032	51.3	34.9	7.5	6.4	-	41.3
RM3	49.0	36.8	7.5	6.8	-	43.6
RM4	47.5	38.5	6.4	7.5	-	46.0
BL1	48.3	37.0	7.0	7.6	-	44.6
BF4	50.0	35.5	7.3	7.2	1.8	44.5

B

Strains	Mycolic acids [a] $C_{32:0}$	$C_{34:1}$	$C_{34:0}$	$C_{36:2}$	$C_{36:1}$	% UM to TM [b]	% extracellular mycolic acids [c]
Mycolic acids from whole cells							
AS019	49.1±0.7	32.9±2.5	9.0±3.2	6.2±1.7	2.9±0.7	42.0	
MLB133	38.9±1.7	39.9±1.0	7.5±2.0	10.5±1.7	3.3±0.5	53.7	
MLB194	44.3±4.8	36.9±3.0	7.7±2.1	8.3±3.2	2.9±0.4	48.1	
ATCC 13032	43.1±1.0	36.2±0.1	9.5±0.5	7.8±0.3	3.4±0.4	47.4	
RM3	35.8±5.2	38.6±1.8	10.0±0.2	10.8±2.3	4.5±0.8	53.9	
RM4	51.7	34.6	5.5	6.4	1.9	42.9	
Mycolic acids from culture fluids							
AS019	53.6±0.5	31.9±1.0	7.5±1.4	5.0±0.7	2.1±0.2	39.0	4.2
MLB133	41.8±2.8	38.4±1.4	6.8±1.3	10.2±2.4	3.0±0.4	51.6	2.5
MLB194	46.1±7.3	36.4±3.4	6.7±1.0	9.0±4.1	2.1±1.3	47.5	8.6
ATCC 13032	47.1	35.0	8.8	6.5	2.5	44.0	4.9
RM3	46.5	36.3	10.5	6.7	-	43.0	7.7
RM4	51.2±2.0	35.9±1.0	8.1±0.4	4.8±0.6	-	40.7	4.8

[a] Proportion (%) of each mycolic acid found in whole cells or culture fluids. The proportion of each mycolic acids was calculated as a % of the total in terms of peak area detected. Dash represents no peak detected under the conditions used.

[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 unsaturated mycolic acids values by the sum of the total mycolic acids.

[c] Proportion (%) of mycolic acids found in culture fluids relative to total mycolic acids detected (whole cells plus culture fluids).

RM4). Growth in the presence of 4 mg/ml INH had little impact on the relative proportions of mycolic acids compared to growth in LBG. Similar trends were seen in both cells and culture fluids. However, RM3 and RM4 showed a small increase in the relative proportions of $C_{32:0}$ and $C_{34:0}$, and decreases in $C_{34:1}$ and $C_{36:2}$.

Experiment 2. Isolation and analysis of mycolic acids from cells and culture fluids using procedure 2, following growth in LBG containing 4 mg/ml INH.

The trends seen for the relative proportions of the different types of mycolic acids found in cells and culture fluids were similar to cells were grown in LBG (Table 3.10B) for all strains tested. The relative proportion of total unsaturated to saturated mycolic acids for the eight strains tested ranged between 42% (in AS019) and 54% (in RM3) for samples prepared from cells. Growth in medium containing 4 mg/ml INH had little affect on the relative proportion of mycolic acids detected in all strains (except for RM3). In the case of RM3, the presence of INH in the medium caused an increase in the relative proportion of $C_{32:0}$, when samples were obtained from both cells and culture fluids. The amount of extracellular mycolic acids of the six strains tested ranged between 2.5% (in MLB133) and 9.0% (in MLB194), which were similar to those seen following growth in LBG medium, indicating that growth in medium containing 4 mg/ml INH had little impact on the cellular location of mycolic acids.

Experiment 3. Isolation and analysis of mycolic acids from cells and culture fluids after growth in LBG containing 8 mg/ml of INH.

Although prior growth in the presence of INH alone had been shown to improve electroporation frequencies in AS019 (Haynes and Britz, 1990), this effect was only significant at very high concentrations (>5 mg/ml) of INH, data which was consistent with the observation that growth in

Table. 3.11 Mycolic acids composition of whole cells and culture fluids for three strains of *C. glutamicum* following growth in LBG-INH (8 mg/ml) using solvent extraction without TLC purification.

Strains	Mycolic acids [a]					% UM to TM [b]	% extracellular mycolic acids [c]
	$C_{32:0}$	$C_{34:1}$	$C_{34:0}$	$C_{36:2}$	$C_{36:1}$		
Mycolic acids from whole cells							
AS019	42.6±2.7	39.3±0.8	5.9±0.4	9.2±2.5	3.1±0.2	51.6	
MLB133	32.9±2.9	41.5±1.9	7.0±1.6	14.3±2.6	4.4±0.5	60.2	
MLB194	31.4	42.5	5.8	16	4.3	62.8	
Mycolic acids from culture fluids							
AS019	43.4	39.5	7.2	10.0	-	49.5	9.6±1.6
MLB133	38.8	50.3	6.7	4.2	-	54.5	15.1±5.9
MLB194	30.8	42.4	6.4	16.2	-	62.8	21.2

[a] Proportion (%) of each mycolic acid found in whole cells or culture fluids. The proportion of each mycolic acids was calculated as a % of the total in terms of peak area detected. Dash represents no peak detected under the conditions used.

[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 unsaturated mycolic acids values by the sum of the total mycolic acids.

[c] Proportion (%) of mycolic acids found in culture fluids relative to total mycolic acids detected (whole cells plus culture fluids).

4 mg/ml INH had little impact on the type or position of mycolic acids in the strains tested (see Experiments 2 in this section). In the study described in section 3.2.4, the specific growth rates of three strains of the AS019 family were reduced by 40% in the presence of 4 mg/ml INH, whilst 8 mg/ml INH decreased growth rates by 50% (in AS019) and 70% (in both mutants) (section 3.2.4). To determine whether higher concentrations of INH affected mycolic acid synthesis, AS019 and the mutants were grown in LBG supplemented with 8 mg/ml, harvested at late stationary phase and mycolic acids analysed for whole cells and culture fluids (Table 3.11). The relative proportions of unsaturated to saturated mycolic acids for three strains tested ranged between 52% (in AS019) and 63% (in MLB194). Trends for changes seen in the relative proportions of mycolic acids in all three strains tested were similar: following growth of cells in 8 mg/ml, the relative proportion of $C_{32:0}$ was decreased, $C_{34:1}$ and $C_{36:2}$ increased, and the total unsaturated mycolic acids increased relative to the control cultured in parallel.

When cultures in LBG-I with 8 mg/ml of INH were harvested at stationary phase, strains MLB133 and MLB194 had 15.1% and 21.2% extracellular mycolic acids, with a significant increase in the relative proportion of unsaturated mycolic acids (Table 3.11). In comparison, AS019 showed a lesser increase in extracellular mycolic acids (9.6%) but a small decrease in $C_{32:0}$ relative to increases in the proportion of $C_{34:1}$ and $C_{36:2}$. Also, the total unsaturated mycolic acids in AS019 were increased relative to the control (LBG) cultured in parallel.

3.4.7 Mycolic acids composition following growth in LBG-glycine/INH

For the ATCC 13032 family, LBG containing 2% glycine plus 0.4 mg/ml INH was used as the growth medium, whilst LBG containing 2% glycine plus 4 mg/ml INH was used for the AS019 family, because of the greater sensitivity of the former to INH. Therefore comparison across the two families is not possible, but comparison between family members can be made.

3.4.7.1 Mycolic acid composition of the AS019 family following growth in LBG containing 2% glycine and 4 mg/ml INH

Experiment 1. Isolation and analysis of mycolic acids from cells and culture fluids using procedure 1.

The cumulative effects of glycine plus INH were also evaluated, where strain AS019 and the mutants (MLB133 and MLB194) were grown in LBG containing 2% glycine plus 4 mg/ml INH. All cultures were harvested at early stationary phase and mycolic acids analysed in whole cells and culture fluids. The relative proportions of unsaturated to saturated mycolic acids ranged between 29% (in AS019) and 42% (in MLB133) in the cells, and between 30% (in AS019) and 46% (in MLB133) in culture fluids (Fig. 3.12A). Changes caused by the presence of the above combination of glycine and INH in the growth medium were similar to these seen for LBG-2% glycine. The relative proportions of mycolic acids found in whole cells and culture fluids were similar and trends were similar to those seen for growth in LBG-2% glycine. Little changes were seen in AS019 and MLB133, whilst MLB194 showed a small decrease in the relative proportion of $C_{32:0}$.

Experiment 2. Isolation and analysis of mycolic acids from cells and culture fluids using procedure 2.

The relative proportions of unsaturated to saturated mycolic acids ranged between 34% (in AS019) and 53% (in MLB133) in the cells and ranged between 33% (in AS019) and 47% (in MLB133) in culture fluids, showing similar mycolic acid composition in the cells and culture fluids (Fig. 3.12B). The addition of glycine and INH to the growth medium resulted in no significant changes in the relative proportions of the mycolic acids in all the strains used, relative to LBG cultures.

Table. 3.12 Mycolic acids composition of whole cells and culture fluids of AS019 family of *C. glutamicum* following growth in LBG containing 2% glycine and 4 mg/ml INH using 80°C heating to concentrate and TLC (A) or solvent extraction without TLC (B).

A

Strains	Mycolic acids [a]					% UM to TM [b]
	$C_{32:0}$	$C_{34:1}$	$C_{34:0}$	$C_{36:2}$	$C_{36:1}$	
Mycolic acids from whole cells						
AS019	64.5	23.9	6.6	3.5	1.5	28.9
MLB133	53.6	33.3	4.8	6.7	1.6	41.6
MLB194	54.2	32.4	6.1	5.5	1.9	39.8
Mycolic acids from culture fluids						
AS019	59.7	25.0	10.3	4.9	-	29.9
MLB133	49.3	34.5	7.5	6.2	-	43.2
MLB194	47.0	37.8	6.8	8.4	-	46.2

B

Strains	Mycolic acids [a]					% UM to TM [b]	% extracellular mycolic acids [c]
	$C_{32:0}$	$C_{34:1}$	$C_{34:0}$	$C_{36:2}$	$C_{36:1}$		
Mycolic acids from whole cells							
AS019	55.9±2.1	27.9±0.9	10.6±0.5	4.1±0.6	2.4±0.3	34.4	
MLB133	39.0±0.9	39.2±0.6	8.6±0.1	9.5±0.1	3.8±0.1	52.5	
MLB194	50.8±5.3	33.0±3.0	6.2±1.2	8.0±3.3	2.1±0.1	43.1	
Mycolic acids from culture fluids							
AS019	57.4	27.9	9.3	3.7	1.6	33.2	15.9
MLB133	45.8	35.8	7.3	8.6	2.6	47.0	37.0
MLB194	48.6±4.7	34.9±1.9	7.6±0.3	8.6±2.5	1.7±0.1	45.2	18.5

[a] Proportion (%) of each mycolic acid found in whole cells or culture fluids. The proportion of each mycolic acids was calculated as a % of the total in terms of peak area detected. Dash represents no peak detected under the conditions used.

[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 unsaturated mycolic acids values by the sum of the total mycolic acids.

[c] Proportion (%) of mycolic acids found in culture fluids relative to total mycolic acids detected (whole cells plus culture fluids).

The presence of glycine plus INH in the medium increased the level of extracellular mycolic acids, which ranged between 15.9% (in AS019) and 37.0% (in MLB133).

3.4.7.2 Mycolic acid composition of the ATCC 13032 family following growth in LBG containing 2% glycine and 0.4 mg/ml INH

Experiment 1. Isolation and analysis of mycolic acids from cells and culture fluids using procedure 1.

The presence of 2% glycine and 0.4 mg/ml caused small changes in ATCC 13032, but trends for all strains were the same: increases in $C_{32:0}$ and decreases in $C_{34:1}$ and $C_{36:2}$, compared to results seen following growth in LBG (Table 3.13A). All three strains (ATCC 13032, RM3 and RM4) exhibited similar mycolic acid profiles in quantitative terms compared to cells prepared from LBG-glycine (Table 3.9A).

Experiment 2. Isolation and analysis of mycolic acids from cells and culture fluids using procedure 2.

The relative proportions of total unsaturated to saturated mycolic acids for the three strains tested ranged between 29% (in RM4) and 67% (in RM3) in the cells, and between 26% (in RM4) and 52% (in RM3) in the culture fluids (Table 3.13B). Two strains, ATCC 13032 and RM4, exhibited very similar mycolic acids profiles, compared to those seen in LBG containing 2% glycine, indicating that combinations of glycine (2% glycine) and INH (0.4 mg/ml) used here did not change the mycolic acid compositions of the strains tested. In contrast, although little changes were seen in Experiment 1 in this section, RM3 showed significant changes in the presence of the above combinations of glycine and INH when compared to those seen in LBG containing 2%

Table. 3.13 Mycolic acids composition of whole cells and culture fluids of the ATCC 13032 family of *C. glutamicum* following growth in LBG containing 2% glycine plus 0.4 mg/ml INH using 80°C heating to concentrate and TLC (A) or solvent extraction without TLC purification (B).

A

Strains	Mycolic acids [a]					% UM to TM [b]
	$C_{32:0}$	$C_{34:1}$	$C_{34:0}$	$C_{36:2}$	$C_{36:1}$	
Mycolic acids from whole cells						
ATCC 13032	53.7	36.6	4.0	5.7	-	42.3
RM3	51.9	32.7	7.3	5.9	2.3	40.9
RM4	58.4	31.0	4.5	5.0	1.1	37.1
Mycolic acids from culture fluids						
ATCC 13032	52.0	33.4	8.8	5.8	-	39.2
RM3	53.4	31.6	9.4	6.8	-	38.4

B

Strains	Mycolic acids [a]					% UM to TM [b]	% extracellular mycolic acids [c]
	$C_{32:0}$	$C_{34:1}$	$C_{34:0}$	$C_{36:2}$	$C_{36:1}$		
Mycolic acids from whole cells							
ATCC 13032	43.5±2.3	35.5±0.3	9.3±0.6	7.8±0.7	3.9±1.0	47.2	
RM3	30.5±3.1	43.2±0.6	2.6±0.1	21.6±1.1	2.2±0.5	67.0	
RM4	66.6±3.4	24.9±2.0	4.5±0.5	3.3±0.8	1.0±0.1	29.2	
Mycolic acids from culture fluids							
ATCC 13032	52.0	34.1	8.3	5.6	-	39.7	7.2
RM3	48.5	37.7	-	13.8	-	51.5	10.5
RM4	68.9±1.7	22.8±1.2	5.6±0.6	2.8±0.1	-	25.6	11.9

[a] Proportion (%) of each mycolic acid found in whole cells or culture fluids. The proportion of each mycolic acids was calculated as a % of the total in terms of peak area detected. Dash represents no peak detected under the conditions used.

[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 unsaturated mycolic acids values by the sum of the total mycolic acids.

[c] Proportion (%) of mycolic acids found in culture fluids relative to total mycolic acids detected (whole cells plus culture fluids).

glycine. If the differences seen here are real (noting that only one set of data was collected), then this is consistent with observations seen for the relative sensitivity of these strains to INH: the specific growth rate of RM3 was significantly affected by the presence of INH, whilst strains ATCC 13032 and RM4 were relatively less affected (see section 3.2.4).

The amount of extracellular mycolic acid for three strains were 7.2% for ATCC 13032, 10.5% for RM3 and 11.9% for RM4 respectively. These are almost two-fold higher than levels seen in LBG medium.

3.4.8 Fatty acids composition of three strains of *C. glutamicum* following growth in three different media

Previously, Pierotti (1987) analysed the fatty acids composition of several strains of *C. glutamicum* during the fermentation and demonstrated that the major fatty acids found for all strains tested were similar to observations by Collins *et al.* (1982b). He found that, for all strains tested, hexadecanoic ($C_{16:0}$) and octadecenoic acids ($C_{18:1}$) accounted for 92-98% of the total fatty acids extracted from cells throughout the growth cycle. Experimentally, he collected fatty acids using a procedure containing TLC steps (similar to a procedure used in section 2.5.1.2 in this thesis), then silylation of fatty acids to FAMEs. However, no information on the composition and amount of extracellular fatty acids was obtained. Since it has been reported that the fatty acid play an important role in mycolic acid biosynthesis in corynebacteria (Shimakata *et al.*, 1984; Walker *et al.*, 1973), quantitative analysis of fatty acids in relation to mycolic acids was of interest in the present study. The composition of fatty acids along with mycolic acids was analysed for cells and culture fluids after growth in three different media (LBG, LBG containing 2% glycine, and LBG containing 8 mg/ml INH). For quantitative analysis of both fatty and mycolic acids, procedures used by others were not appropriate because these methods contained a

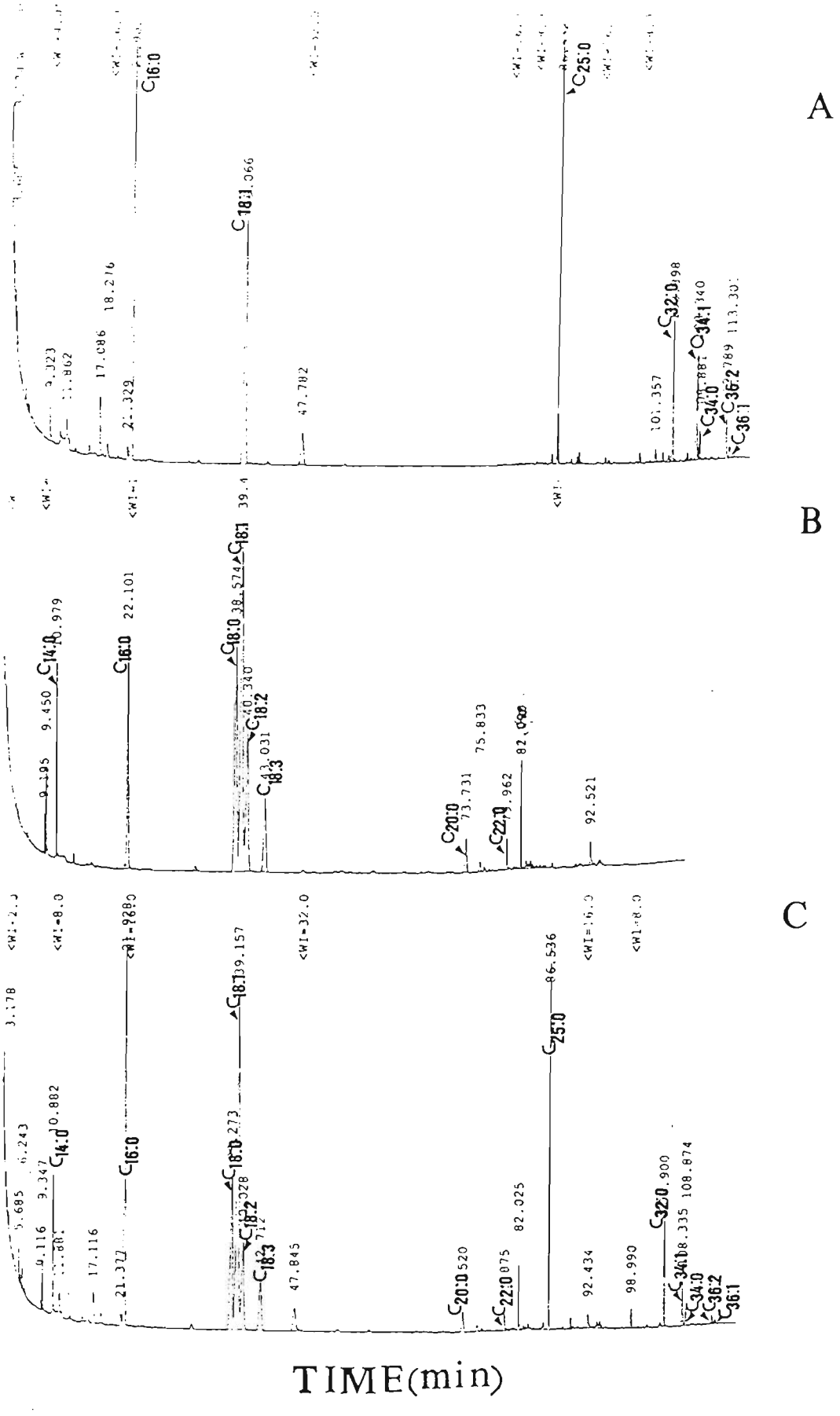


Fig. 3.25 GC analysis of FAMEs on a 25 m non-polar BPX5 column. (A) Cellular FAMEs and MAMEs from *C. glutamicum* AS019 after growth in LBG. (B) Standard FAMEs mixture - Sigma, 189-17. (C) 189-17 plus FAMEs and MAMEs from strain AS019 (A).

TLC step, which separated fatty acids from mycolic acids. Consequently, a procedure used for Experiment 2 in section 3.4.7 in this thesis was applied, so that both fatty acids and mycolic acids were collected together and appeared in the same GC profile. In this way, comparison of the relative proportions of fatty acids to mycolic acids was possible. Results of fatty acid analysis are shown in this section.

Fatty acids were fractionated using a 25 m non-polar BPX5 column (section 2.5.1.7) as shown in Fig. 3.25. Hexadecanoic ($C_{16:0}$) and octadecenoic acids ($C_{18:1}$) were the major fatty acids found in all of the strains tested when samples were obtained from whole cells. Myristic ($C_{14:0}$), pentadecanoic ($C_{15:0}$), palmitoleic ($C_{16:1}$), heptadecanoic ($C_{17:0}$), and stearic acid ($C_{18:0}$) were also detected as trace components (less than 0.5% in total), so that these fatty acids were not taken into account for consideration in this study. In contrast, when fatty acids were obtained from culture fluids, a new fatty acid, $C_{18:3}$, was seen, which was not seen in samples from cells. The presence of this ($C_{18:3}$) and two other fatty acids ($C_{16:0}$ and $C_{18:1}$) was confirmed by co-injecting purified FAMEs plus external standards (FAMEs, Sigma). The GC profiles are shown in Appendix 2.

In order to evaluate this procedure, several analyses were performed. Cells of AS019 were cultivated in LBG and harvested. Dried samples were prepared and 100 μl of LAME (5 mg/ml, internal standard) were added, and then this treated as described in sections 2.5.1.1, 2.5.1.3 and 2.5.1.4 for samples from cells and sections 2.5.1.6, 2.5.1.3 and 2.5.1.4 for samples from culture fluids, FAMEs and MAMEs analysed by GC (section 2.5.1.7). The quantitative fatty and mycolic acids profile obtained from four samples were similar in terms of the relative percentages of fatty and mycolic acids detected (Table 3.14).

Table 3.15 showed the relative proportion of fatty acids of two strains of *C. glutamicum* AS019 and MLB133 after growth in three different media. The two strains showed approximately the

Table. 3.14 Evaluation of reproducibility of quantitative analysis of fatty acids between different experiments using *C. glutamicum* AS019 grown in LBG

N [a]	Fatty acids [b]		Total peak area of FAMEs [c]	Mycolic acids [d]					Total peak area of MAMEs [e]	% FAMEs [f]
	$C_{16:0}$	$C_{18:1}$		$C_{32:0}$	$C_{34:1}$	$C_{34:0}$	$C_{36:2}$	$C_{36:1}$		
1	53.5	46.5	54,277	49.2	29.0	10.3	7.4	4.1	16,498	76.7
2	50.9	49.1	48,927	52.7	31.3	8.5	5.3	2.2	13,794	78.0
3	50.3	49.7	106,226	50.8	34.2	8.5	3.2	3.2	31,019	77.4
4	50.7	49.3	92,625	51.0	30.1	9.4	6.4	3.2	29,914	75.6
Average of four experiments	51.4	48.7		50.9	31.2	9.2	5.6	3.2		76.9
Standard deviations	±1.5	±1.5		±1.4	±2.2	±0.9	±1.8	±0.8		±1.0

[a] N is 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 peak areas 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 peak areas of MAMEs detected.

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

same amount of $C_{16:0}$ and $C_{18:1}$ when cells were grown in LBG. The relative proportions of the total FAMEs to total esters (FAMEs plus MAMEs) were 76.9% for AS019 and 81.1% for MLB133. In the presence of 2% glycine or 8 mg/ml INH in LBG, fatty acid composition of both strains was little changed: the relative proportions of $C_{16:0}$ of AS019 ranged between 48.9% (in INH medium) to 51.4% (in LBG medium) for AS019, and ranged between 47.4% (INH medium) to 50.6% (in LBG) for MLB133.

The most significant observation was changes in the relative percentage of fatty acids to total esters (fatty acids plus mycolic acids). In the presence of glycine or INH, the relative percentage of the fatty acids of AS019 was decreased from 76.9% in LBG to 72.9% in LBG-2% glycine, and 66.4% in LBG-8 mg/ml INH. Similar decreases in the relative proportion of cellular fatty acids were also observed in MLB133 (Fig. 3.15). This indicated that growth in glycine and INH influenced not only mycolic acid biosynthesis but also the biosynthesis of fatty acids. When fatty acids were obtained from culture fluids, $C_{16:0}$ and $C_{18:1}$ were again the major fatty acids. Surprisingly, $C_{18:3}$ was detected in addition to these two fatty acids, when fatty acids were collected from culture fluids. The relative proportions of total fatty acid in culture fluids of the two strains grown in LBG were 88.2% for AS019 and 84.6% for MLB133, which is higher than seen for cells, reflecting the presence of $C_{18:3}$ in the culture fluids. In the presence of glycine or INH, the relative proportion of the fatty acids in the culture fluids decreased, as seen for the cells. The relative amount of extracellular fatty acids in AS019 showed little change in the presence of glycine or INH. In contrast, extracellular fatty acids in MLB133 significantly increased from 5.6% in LBG to 19.6% in LBG-2% glycine and 15.3% in LBG-INH, trends similar to those seen for mycolic acid analysis (see section 3.4.6).

Table. 3.15 Quantitative analysis of fatty acids of two strains of *C. glutamicum* grown in different media.

Strain	Medium [a]	Fatty acids [b] $C_{16:0}$	$C_{18:1}$	$C_{18:3}$	% FAMEs [c]	% FAMEs [d]	% extracellular FAMEs [e]	% extracellular FAMEs [f]
Fatty acids from cells								
AS019	LBG	51.4±1.5	48.7±1.5	-		76.9±1.0		
AS019	LBG-G	50.0±4.5	50.0±4.5	-		72.9±1.1		
AS019	LBG-I8	48.9±1.5	51.2±1.5	-		66.4±2.7		
MLB133	LBG	50.6±1.5	49.4±1.5	-		81.1±1.0		
MLB133	LBG-G	48.7±1.8	51.4±1.8	-		68.8±0.4		
MLB133	LBG-I8	47.4±0.6	52.5±0.6	-		69.4±2.2		
Fatty acids from culture fluids								
AS019	LBG	39.4±3.1	36.2±2.3	24.5±5.3	88.2±3.2	85.0	10.9±2.2	8.2
AS019	LBG-G	37.4±0.7	42.2±9.4	20.5±2.6	64.7±1.0	58.9	6.2±0.4	5.0
AS019	LBG-I8	39.1±0.2	40.7±0.8	20.3±1.0	66.4±10.2	61.2	10.4±3.2	8.5
MLB133	LBG	40.0±1.8	41.8±2.3	18.9±4.9	84.6±2.9	81.8	5.6±1.6	4.6
MLB133	LBG-G	45.5±0.6	48.8±0.5	5.7±1.1	79.4±2.8	78.4	19.6±3.6	18.7
MLB133	LBG-I8	42.8±0.6	45.0±3.8	13.6±3.2	70.1±3.5	67.3	15.3±8.0	13.7

[a] Data for each experiment was from between 2-6 separately grown cultures and sample (fatty and mycolic acids) preparation.

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

[c] Proportion (%) of FAMEs ($C_{16:0}$, $C_{18:1}$, $C_{18:3}$) relative to total esters (FAMEs plus MAMEs).

[d] Proportion (%) of FAMEs ($C_{16:0}$, $C_{18:1}$) relative to total esters (FAMEs plus MAMEs).

[e] Proportion (%) of FAMEs ($C_{16:0}$, $C_{18:1}$, $C_{18:3}$) found in culture fluids relative to total fatty acids detected (whole cells plus culture fluids).

[f] Proportion (%) of FAMEs ($C_{16:0}$, $C_{18:1}$) found in culture fluids relative to total fatty acids detected (whole cells plus culture fluids).

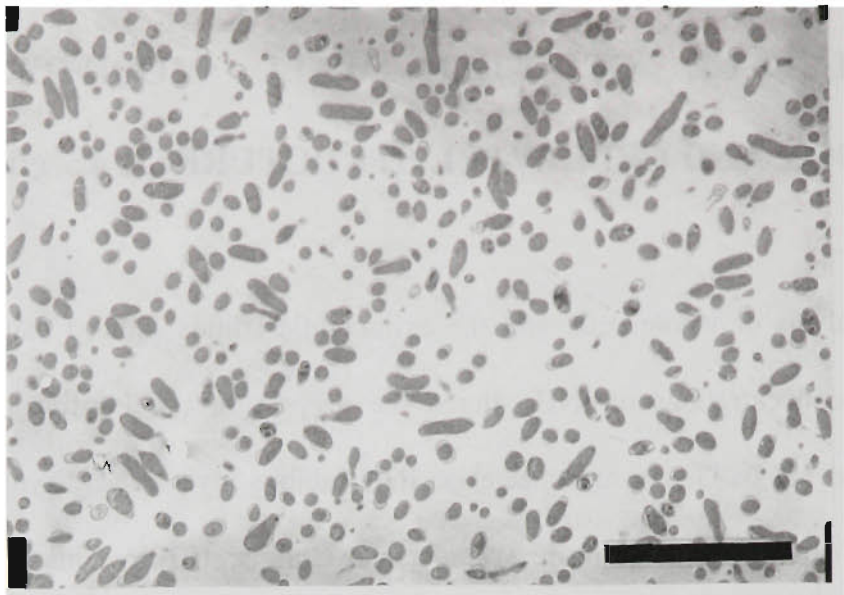
3.5 INFLUENCE OF CELL WALL MODIFIER ON CELL MORPHOLOGY AND CELL WALL STRUCTURE OF *C. glutamicum*

Mycolic acid analysis of whole cells and the extracellular fluids showed that glycine, INH plus glycine or high concentrations of INH altered the proportion and location of mycolic acids in strains AS019 and MLB133. In mycobacteria, INH inhibits mycolic acid synthesis and decreases the total amount of mycolic acids made (Winder and Collins, 1970). This effect may have been occurring in the corynebacteria strains tested, but this was difficult to quantify on a cell dry-mass basis in the absence of standards for each mycolic acid which would allow measurement of the amounts of mycolic acids. One way of approaching the question of whether mycolic acid synthesis was decreased was to look at the thickness of the cell wall, presuming that decreases in mycolic acid production or an increase in leakage to extracellular fluids would lead to a decrease in cell wall thickness. This approach used was to perform TEM on thin sections of AS019 and MLB133 grown in LBG, LBG containing 2% glycine (LBG-G) and LBG containing 4 mg/ml INH (LBG-I).

Two strains, AS019 and MLB133, were compared with respect to their morphology, cell length and cell wall thickness after growth in the above media. Samples were taken from liquid medium at A_{600} = 0.4 by centrifugation then treated, embedded and prepared for TEM using procedures described in Materials and Methods (see section 2.2.8) and observed under an electron microscope.

The results were obtained from a single experiment so that multiple measurements were performed from each strain and each growth medium. To minimise bias and obtain more reliable information, multiple measurements were carried out with the multiple fields examined using several magnifications and cells were randomly photographed by the EM operator. To get an

(A)



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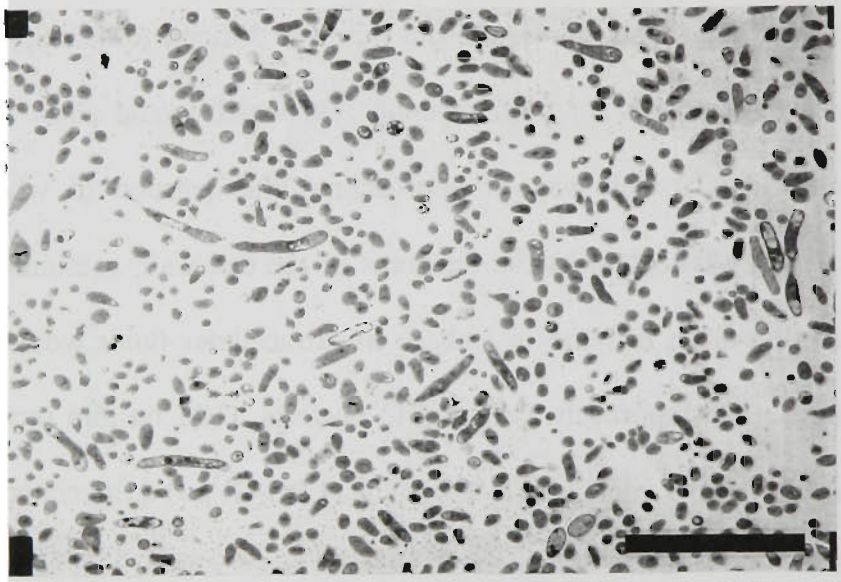

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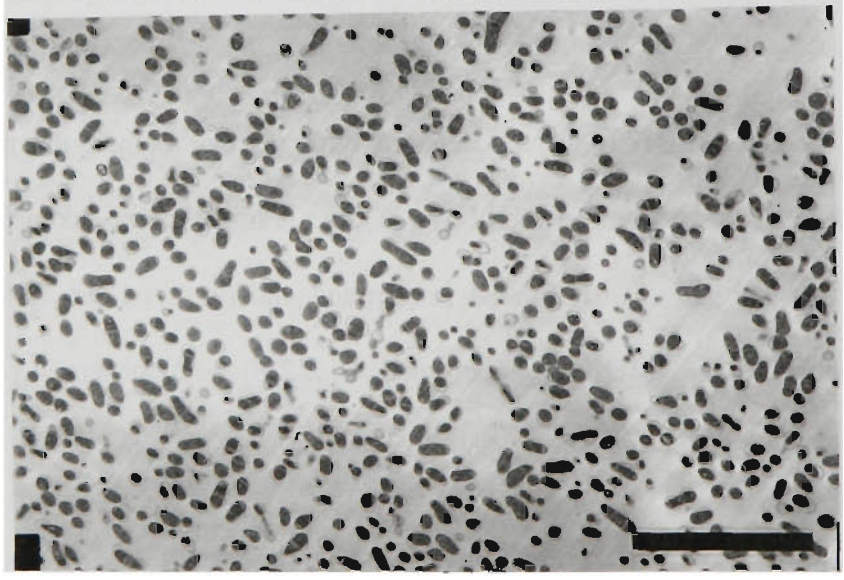


Fig. 3.26 Transmission electron microscopy of *C. glutamicum* strain AS019 following growth in three media: magnification 1,200 X. Cells were grown in liquid media and harvested at A_{600} 0.4. Samples for electron microscopic observation was prepared as described in section 2.2.8 and photos were taken. Bars in photos are equal to 10 μm. (A) LBG; (B) LBG containing 2% glycine; (C) LBG containing 4 mg/ml INH.

overall view of cells, samples were examined and photos taken at a magnification factor of 1,200 (Fig. 3.26). Under the above conditions, approximately 300 to 400 cells were shown in each photograph (Fig. 3.26) and these were used for measuring the cell length (Table 3.16); each data set represents an average distribution in cell length for at least 1,000 cells measured. Some of the cells were sliced across their middle not along their length, therefore data is an average distribution in cell length and this may be smaller than seen in real cells.

The morphology of cells of *C. glutamicum* after growth in LBG was found to be a mixture of short and long rods, which is consistent with descriptions for this group given in Bergey's Manual of Systematic Bacteriology (Jones and Collins, 1986). The distribution of cells length for strain AS019 was 0.5 - 2 μm when cells were grown in LBG (Fig. 3.26 and Table 3.16). Longer cells (above 2.5 μm) comprised 3.1% of total cells and the majority of such cells showed lines where cell divisions had occurred without separation of individual cells. In the presence of 2% glycine, the distribution in length of AS019 was very similar to that of cells grown in LBG. Approximately 92% of cells were less than 2 μm in length and 1.9% of cells were found to be above 2.5 μm. However, unlike cells grown in LBG medium, more than half of the longer cells did not contain septation lines (Fig. 3.27). In addition, some cells grown in LBG-glycine showed elongation and the appearance of Y-shaped and branched cells (Fig. 3.27). This considerable pleomorphism, which resembled branching and filament formation, presumably arose due to modification of cell wall structures or changes in septation caused by growth in glycine. In the presence of 4 mg/ml of INH in the growth medium, cells were normally shorter and more even in size; approximately 99% of cells were less than 2 μm in cell length. The proportion of cells which were longer than 2.5 μm was only 0.2%. No cells were found to be longer than 3.5 μm after growth in LBG-I.

Table 3.16 Distribution of cell length of two strains of *C. glutamicum* grown in three media.

Cell length (μm)	*C. glutamicum* AS019						*C. glutamicum* MLB133					
	LBG [a]		LBG-G		LBG-I		LBG		LBG-G		LBG-I	
	N [b]	% [c]	N	%	N	%	N	%	N	%	N	%
<0.5	99	9.8	228	12.3	111	10.7	197	12.2	120	7.7	270	15.8
0.5 - 1	443	43.9	1023	55.0	571	55.3	742	45.8	706	45.2	915	53.5
1 - 1.5	295	29.2	488	26.2	265	25.7	316	23.2	424	27.1	370	21.7
1.5 - 2	96	9.5	67	3.6	76	7.4	168	10.4	172	11.0	109	6.4
2 - 2.5	46	4.6	24	1.3	9	0.9	84	5.2	64	4.1	37	2.2
2.5 - 3	15	1.5	16	0.9	1	0.1	37	2.3	38	2.4	6	0.4
3 - 3.5	12	1.2	6	0.4	1	0.1	10	0.6	29	1.9	2	0.1
3.5 - 4	3	0.3	3	0.2			4	0.3	7	0.5		
>4	1	0.1	6	0.4			2	0.1	2	0.13		
Total cells [d]	1010		1861		1034		1620		1562		1709	

[a] LBG-G; LBG containing 2% glycine, LBG-I; LBG containing 4 mg/ml INH.
[b] N: Numbers of cells measured.
[c] %: Relative percentage of the total cells. A blank indicates no cells were in that range of cell length.
[d] Total cells: number of cells measured.

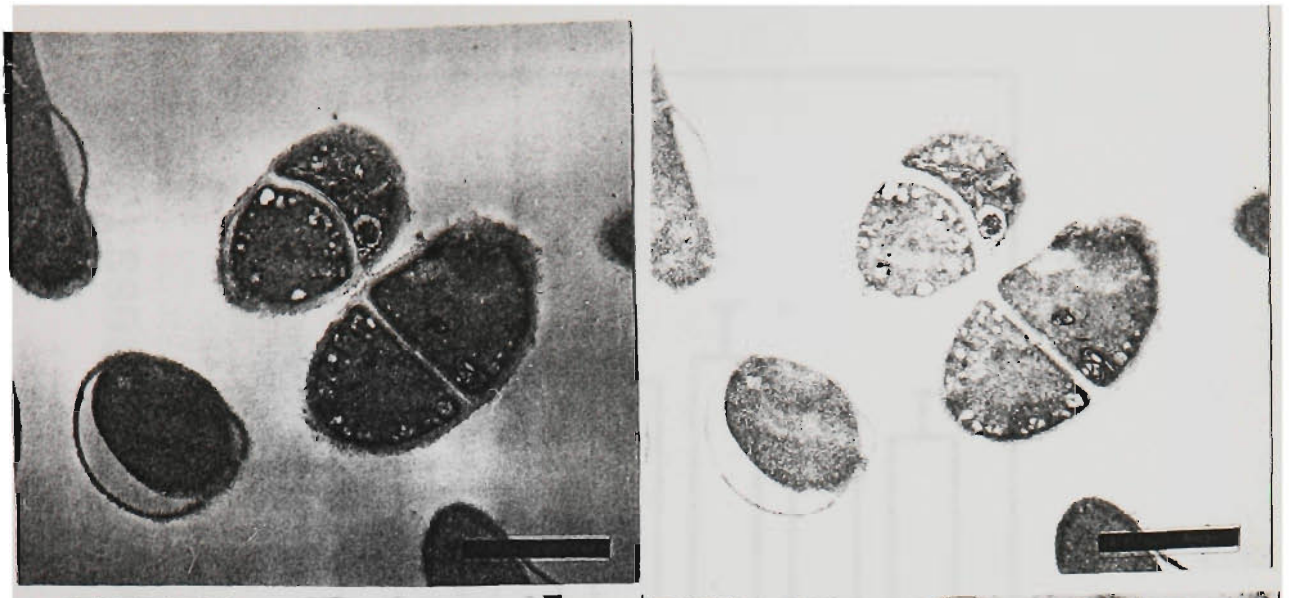
The cell wall structure was clearly visible under higher magnification of the sections (Fig. 3.27). Measurements were made of the thickness of these structures following growth in glycine- and INH- supplemented media to ascertain if these caused changes in cell wall thickness. Multiple cells were randomly photographed at a magnification of 6,000 X. Some cells had lost their cell wall structure during sample preparation and such samples were not taken into account for measurement. In addition, several measurements were made in three to five places for each cell in order to obtain more reliable average measurements. Often, film processing gave different brightness of photos; some of these are darker and others were lighter, which may have varied the visual estimation of where the cell wall structures started and ended. To check whether development of negatives caused any changes in the fidelity of measuring the cell wall thickness, the exposure was varied to obtain darker or brighter prints and the cell wall thickness measured for each. No differences in the measurements was observed with the different exposures (data not shown). Data summarised in Fig. 3.27 and Fig. 3.29 represent approximately 150 total measurements using at least 40 cells and standard deviations were analysed using a statistical program (Sigma-plot).

Cell wall thickness of strain AS019 ranged between 0.032 - 0.044 μm depending on the growth media: when cells were grown in LBG without glycine or INH, the cell wall was thickest (0.044 μm). In the presence of glycine or INH, cell wall thickness was decreased by 20-30% for this strain when compared to cells grown in LBG (Fig. 3.27 and Fig. 3.28).

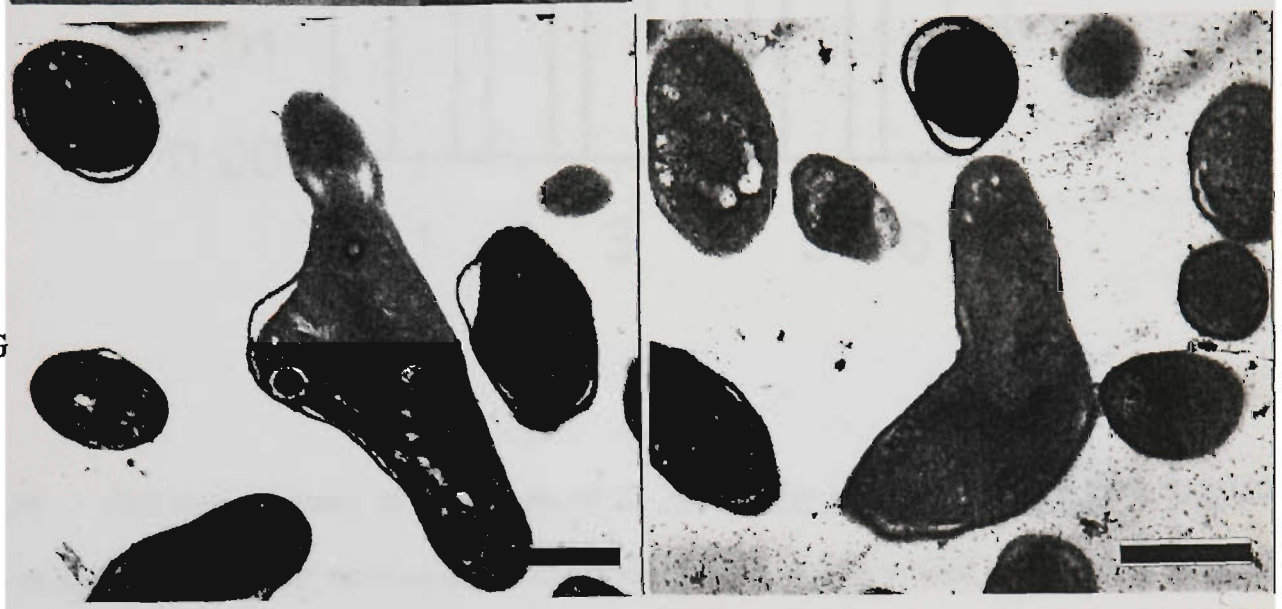
Similar observations to the above were also made for strain MLB133 grown in the three different media (Fig. 3.29). The distribution of cell length was similar to that of strain AS019 grown in LBG (Table 3.16). However, it is interesting to note that the proportion of longer cells (above 2.5 μm) for MLB133 was higher than seen for strain AS019. The differences seen for cell length following growth in LBG medium were small but these were more obvious when MLB133 was cultured in

Fig. 3.27 Effect of growth media on the cell wall thickness of *C. glutamicum* AS019 following growth in three growth media. Cells were grown in liquid media and harvested at A_{600} of 0.4. Samples for electron microscopic observation were prepared as described in section 2.2.8 and photos were taken at a magnification factor of 6,000 X. Bars in photos are equal to 1 μm. Abbreviations: LBG-G, LBG containing 2% glycine; LBG-I, LBG containing 4 mg/ml INH.

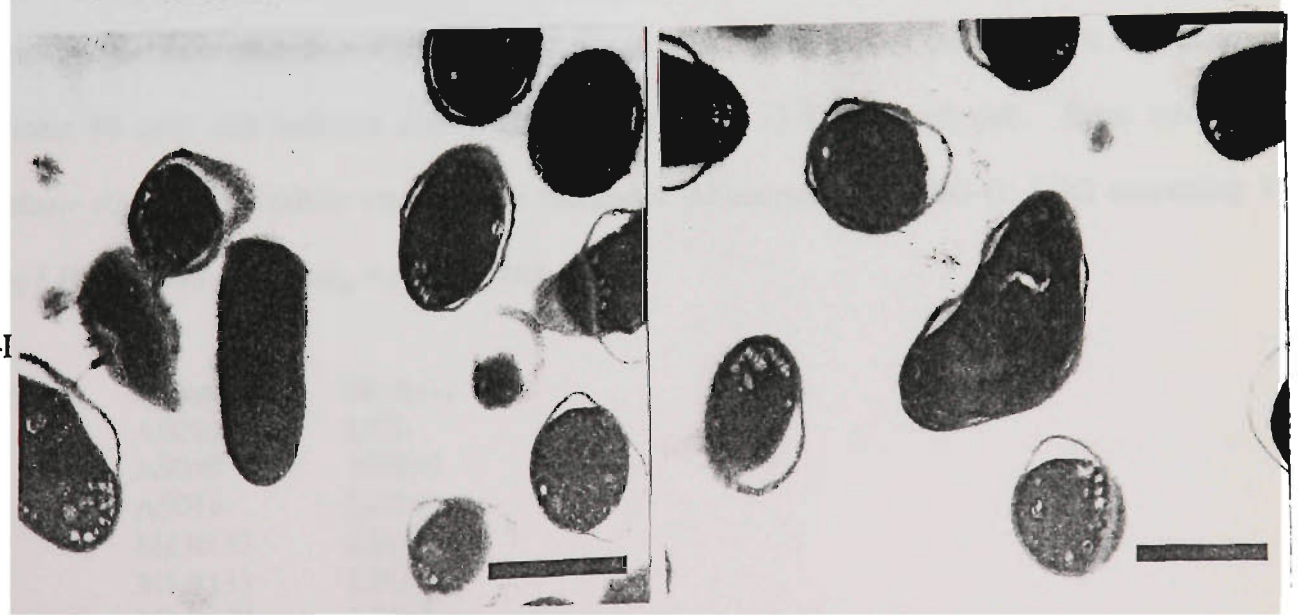
LBG



LBG-G



LBG-I



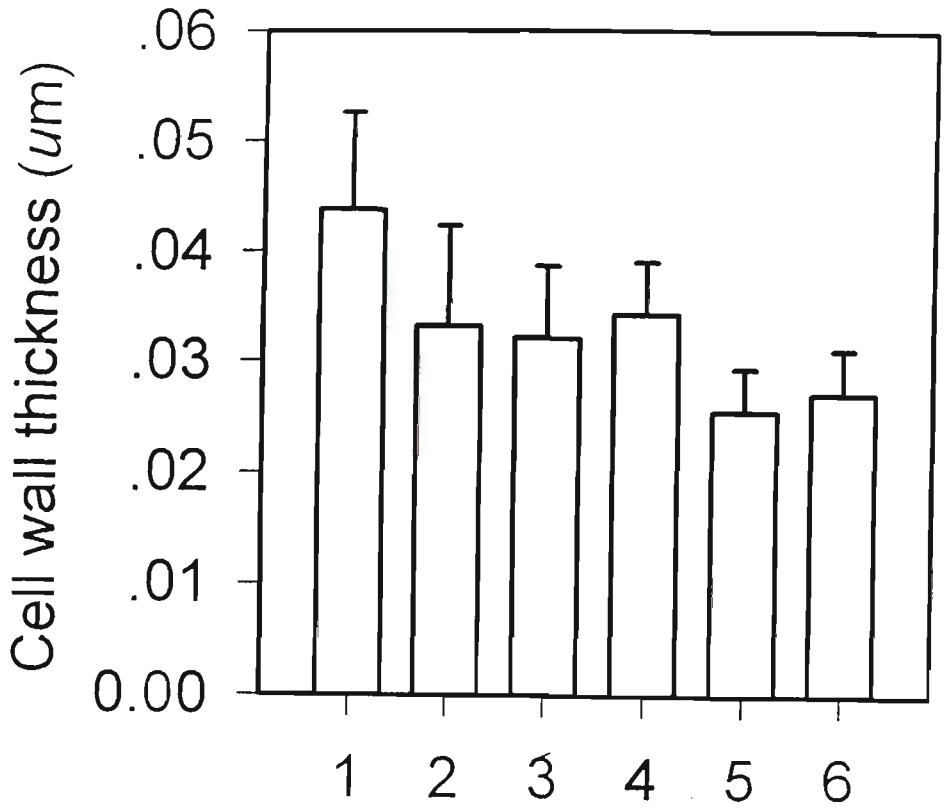


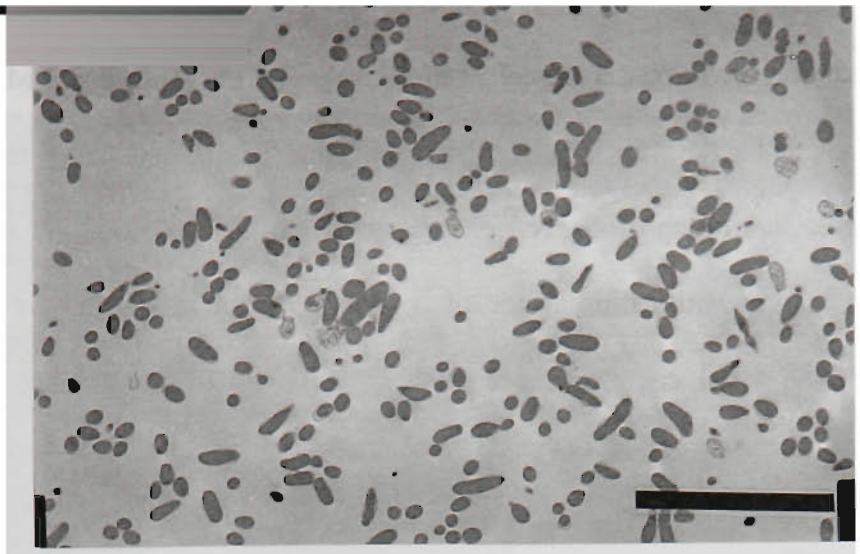
Fig. 3.28 Cell wall thickness of two strains of *C. glutamicum* grown in LBG or LBG containing glycine or INH. Samples for electron microscopic observation were prepared as described in section 2.2.8 and photos were taken (see Fig. 3.27 and Fig. 3.30). Thickness of the cell walls was measured for at least 40 cells and multiple measurements were made (3-5) for each cell. Error bars in the figure show standard deviations for cell wall thickness. Abbreviations: LBG-G, LBG containing 2% glycine; LBG-I, LBG containing 4 mg/ml INH.

Number	Strain	Medium
1	AS019	LBG
2	AS019	LBG-G
3	AS019	LBG-I
4	MLB133	LBG
5	MLB133	LBG-G
6	MLB133	LBG-I

LBG-G or LBG-I. The proportion of longer cells (above 2.5 μm) of strain MLB133 was 3.3% in LBG, 4.9% in LBG-G and 0.5% in LBG-I. This presumably arose due to higher sensitivity of MLB133 to the presence of glycine and INH in growth medium. When MLB133 was grown in LBG-G, Y-shaped cells were seen more frequently, as were branched rods, suggesting that cell division here occurs through budding, branch formation, then septation (Fig. 3.29 and Fig. 3.30). In contrast to earlier SEM (scan electron microscopy) photos, which showed similar shapes, TEM showed that septation did not occur at the branch points.

Cell wall thickness of MLB133 ranged between 0.023 - 0.036 μm depending on the growth media: MLB133 cells had a thinner cell wall structure than AS019 when grown in LBG or the corresponding other media. When cells were grown in LBG without glycine or INH, the cell wall of MLB133 was 0.036 μm. In the presence of glycine or INH, cell wall thickness decreased by 20-30% when compared to cells grown in LBG (Fig. 3.28 and Fig. 3.30), which is similar to result seen for AS019 (Fig. 3.28). These visual results, although arguably subject to interpretation due to potential bias in measurement or sample preparation, indicated that strain MLB133 had a physically thinner cell wall structure than the parental-type strain AS019 and that the presence of either glycine or INH decreased the thickness of the cell surface structures measured by TEM.

(A)



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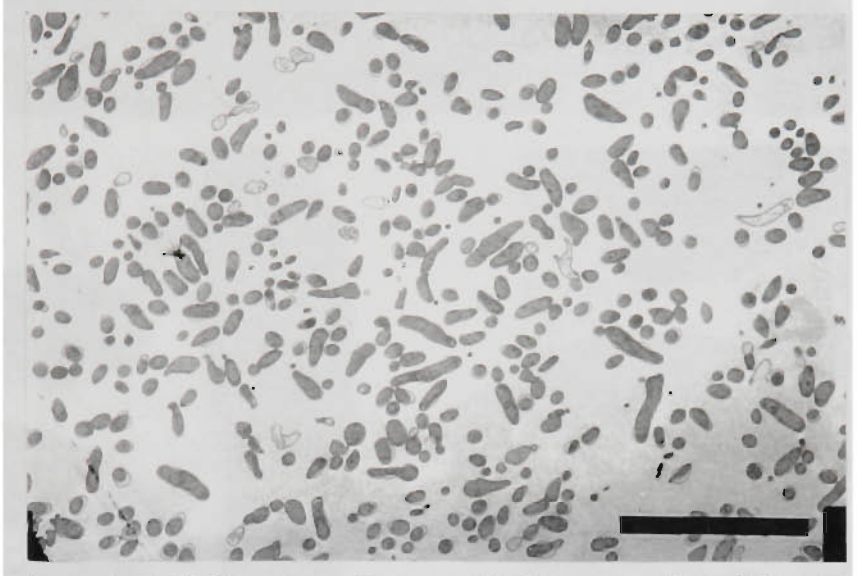

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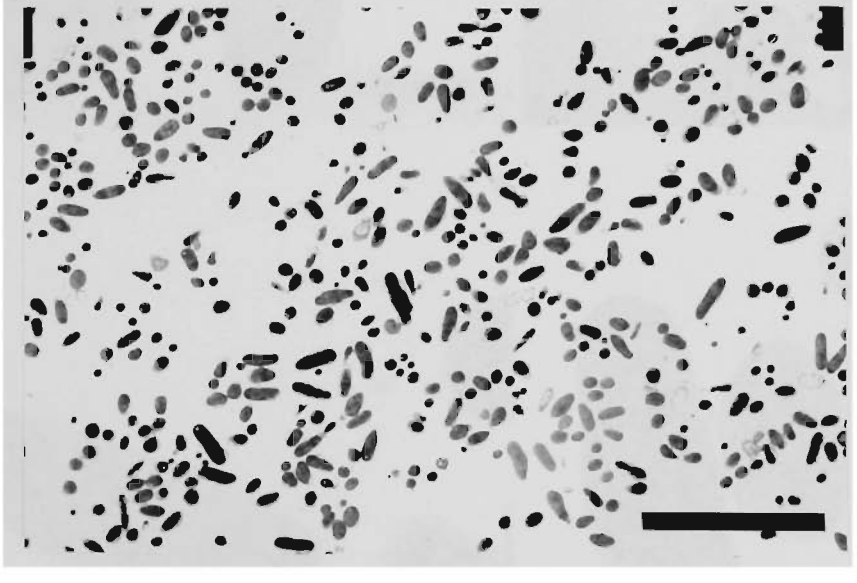
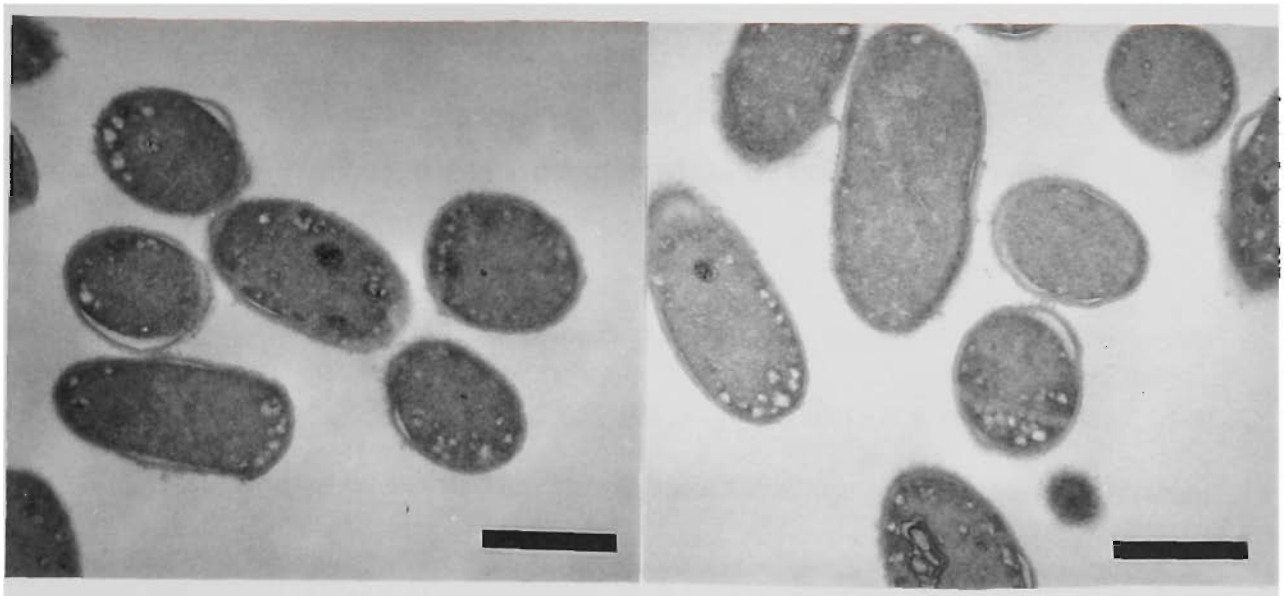


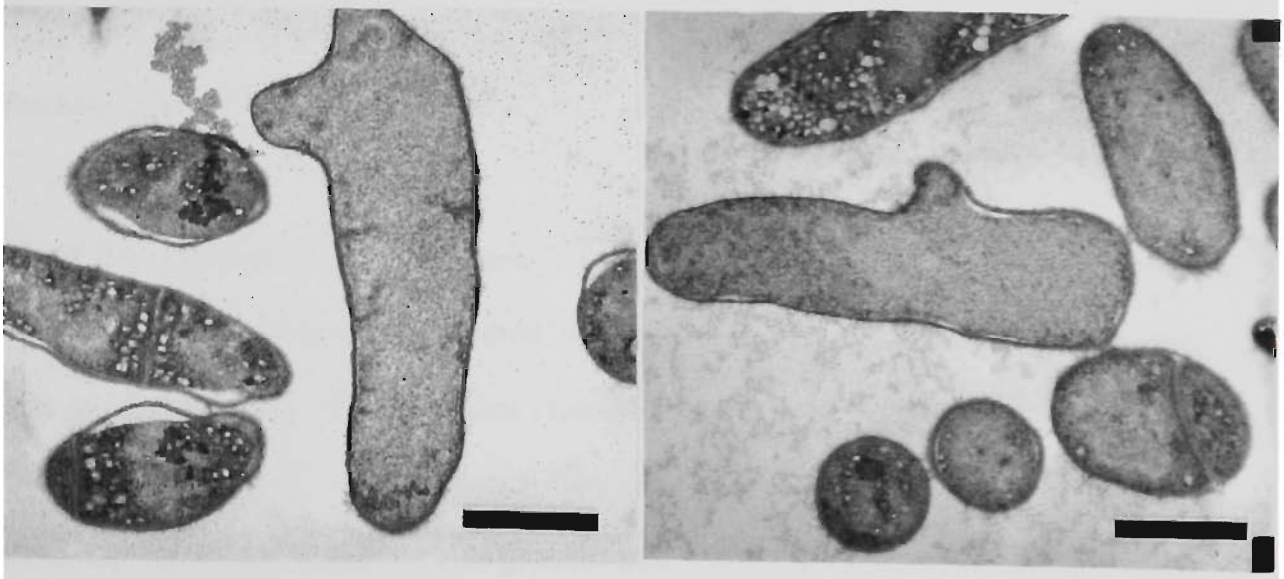
Fig. 3.29 Transmission electron microscopy of *C. glutamicum* strain MLB133 following growth in three media: magnification 1,200 X. Cells were grown in liquid media and harvested at A_{600} 0.4. Samples for electron microscopic observation were prepared as described in section 2.2.8 and photos were taken. Bars in photos are equal to 10 μm. (A) LBG; (B) LBG containing 2% glycine; (C) LBG containing 4 mg/ml INH.

Fig. 3.30 Effect of growth media on cell wall thickness of *C. glutamicum* MLB133 following growth in three growth media. Cells were grown in liquid media and harvested at A_{600} of 0.4. Samples for electron microscopic observation was prepared as described in section 2.2.8 and photos were taken at a magnification factor of 6,000 X. Bars in photos are equal to 1 μm. Abbreviations: LBG-G, LBG containing 2% glycine; LBG-I, LBG containing 4 mg/ml INH.

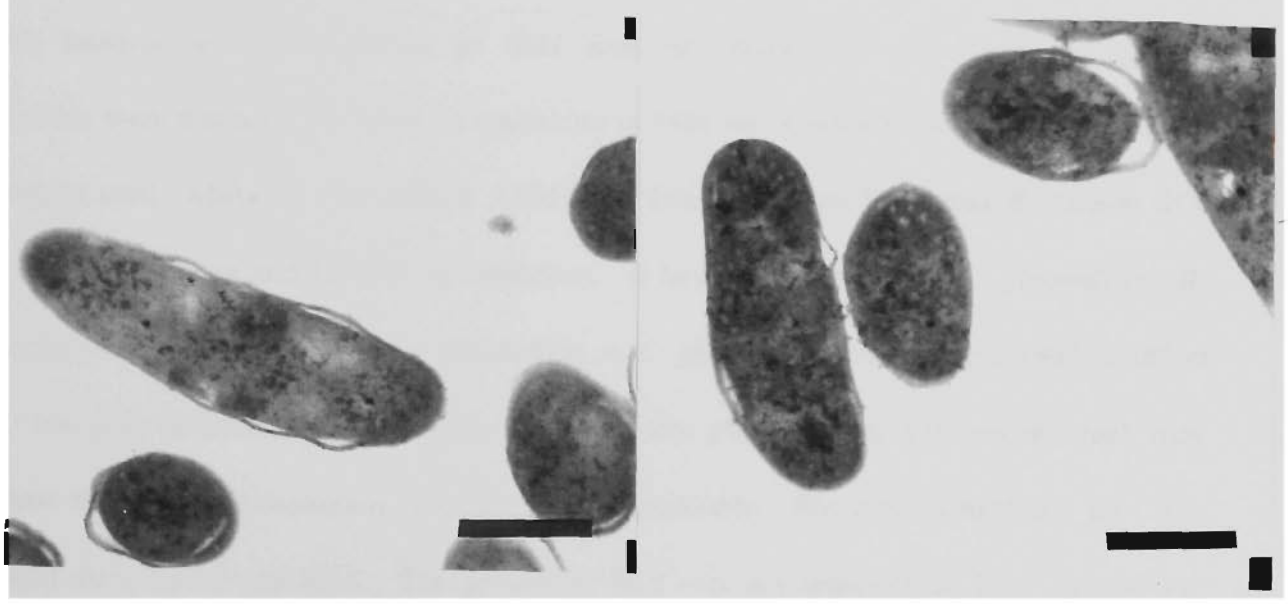
LBG



LBG-G



LBG-I



3.6 DISCUSSION

3.6.1 Influence of cell wall modifiers on coryneform cell growth rates

The inhibitory effects of glycine and INH on specific growth rates varied from strain to strain, reflecting the differences in cell wall composition and structure between these strains. The concentration of glycine required to inhibit cell growth rates significantly was generally between 1-6% (w/v) and this range is similar to that reported for Gram-positive bacteria, including *B. subtilis* (Hishinuma *et al.*, 1969), *Streptomyces* species (Hishinuma *et al.*, 1969), *Corynebacterium* species (Hammes *et al.*, 1973), and previously for *C. glutamicum* strains (Best and Britz, 1986).

The amount of INH required to cause inhibition of *C. glutamicum* strains was very high compared to other mycolic acid-containing bacteria. INH inhibits mycolic acid synthesis in mycobacteria and nocardia at concentrations of < 50 μg/ml (Tomiyasu and Yano, 1984; Winder and Collins, 1970; Quémard *et al.*, 1995b). The presented work showed that only concentrations above 2-8 mg/ml caused any significant impact on cell growth rates. This indicated that these saprophytic coryneform bacteria are less sensitive to INH than *Mycobacterium* and *Nocardia* species. However, there were distinct differences in sensitivity to INH and glycine between the coryneform bacteria strains used, where *C. glutamicum* AS019, *B. lactofermentum* BL1, and *B. flavum* BF4 exhibited different degrees and kinetics of inhibition. It has been argued that *C. glutamicum*, *B. lactofermentum* and *B. flavum* should be reclassified as *C. glutamicum* (Abe *et al.*, 1967; Liebl *et al.*, 1991) but their differential sensitivity to INH indicates physiological differences which may argue against this, if the phenomenon seen here are generalisable. For glycine medium, BF4 was less inhibited than AS019 and BL1. The growth of BL1 was not impaired at 1-2% glycine but this dramatically reduced at concentrations above 3% glycine. When glycine was replaced by INH, AS019 was found to be the least sensitive of the strains tested.

C. ulcerans, a pathogenic strain, showed higher sensitivity towards both glycine and INH, amongst the strains tested. Unlike other strains tested here, this species required 37°C for optimal growth conditions whereas all of the other strains tested required 28-30°C for optimal growth, although AS019 could grow at 37°C (personal communications, M. L. Britz).

Mutants MLB133 and MLB194 exhibited hypersensitivity towards glycine and INH relative to the parent-type strain AS019, which was manifested by slower specific growth rates. At 2-3% glycine or 4-5 mg/ml INH, the cell growth rates of the AS019 family were inhibited by approximately 50%. Surprisingly, susceptibility of mutants RM3 and RM4 was also clearly different to that of the parent strain, ATCC 13032. Both of these mutants, showed hypersensitivity towards INH relative to ATCC 13032. These mutants were isolated by mutagenesis as restriction minus strains, and are not known to have other changed traits (Schäfer *et al.*, 1994b) although other changes could have been introduced at the same time. The same authors (Schäfer *et al.*, 1994b) proposed that the restriction system in *C. glutamicum* was cell surface-located. Because these mutations were isolated on the basis of better transformation frequency, it is possible that the surface could have changed at the same time but this would not have been obvious: the phenotype of restriction minus was proven genetically later (Schäfer *et al.*, 1994b) but this still does not eliminate coincidental changes in surface structures. Clearly, the relationship between changes in cell surface structure and the location and action of the RM system here requires further investigation.

The inhibitory action of Tween 80 was also investigated but, at the concentrations used, was found not to produce visual inhibitory effects on growth, although prior growth in Tween 80 at 0.5% improved electrotransformation frequencies of *C. glutamicum* and *B. lactofermentum* (Haynes and Britz, 1989). However, when this was used with glycine and INH together, it produced a synergistic effect on cell growth rates. When the three compounds were used together

as components of LBG, the cell growth rate was further reduced: initiation of growth was retarded for up to 20-30 h. Under the above conditions, the early stages of cell growth showed fluctuations in A_{600} and viable cell numbers. The number of viable cells in the culture increased for several hours early in growth, but after this, the total cell numbers and absorbance values at 600 nm decreased, so that these were lower than starting levels following inoculation. After 20-30 h, growth started and the growth rate was almost the same as control cells grown in LBG. This observation could be explained by adaptation of a minority of cells to these harsh conditions which inhibited the majority of cells. The cumulative effects of Tween 80, glycine and INH, caused very lengthy incubation periods, therefore these effects were not evaluated further in this study.

If the structural model of *C. glutamicum* is similar to that described for mycobacteria species (Nikaido *et al.*, 1993), then the cell surface would contact mycolic acids and fatty acids in a laminar structure. Tween 80 is known to solubilise fatty acids (Nieman, 1954) and may act at this point to disrupt surface or membrane structures. This may account for the cumulative effect of Tween 80, INH plus glycine, where the former affects fatty acids and the latter affects the mycolic acids to cause a larger change in the surface structures than would be caused by any one additive alone.

3.6.2 Effects of growth in glycine and INH on plasmid transformation using electroporation

The effect of prior growth in glycine and INH on plasmid DNA transformation was evaluated using electroporation. Several factors influenced the number of transformants obtained. There was a linear relationship between the amount of DNA up to 2 μg added and the number of cells transformed. The recovery medium on which the transformants were isolated had a significant

effect on the number of transformants obtained; the osmotically-protective ET-Km medium was better than NAG-Km medium as a recovery medium, suggesting that cells successfully transformed by electroporation were electrochemically or osmotically sensitive, as described by Haynes and Britz (1990).

The age of cultures at harvest was found to be a critical parameter for protoplast formation of AS019 (M. L. Britz, unpublished observation). When cells were harvested from early-exponential phase, the electrotransformation efficiency was high and this gradually reduced with increased fermentation time. This observation from the present work is consistent with data obtained with electoporation (Dower *et al.*, 1988) and observations using protoplast transformation of *C. glutamicum* (M. L. Britz, unpublished observation), confirming that the highest transformation efficiency of *C. glutamicum* using electroporation was obtained between early- to mid-exponential cell growth phase (Haynes and Britz, 1990). A possible explanation for this observation may be that the cells in early growth phase have a less strong structure in the cell wall so that cells are more susceptible to physical, enzymatic or electrical assault. Alternatively, restriction and modification systems may be poorly expressed earlier in the growth cycle, although no supportive data is available.

The frequencies and efficiencies of electrotransformation were comparable with those obtained by other authors using spheroplast and protoplast transformation (Table 3.17) (see Table 1.5 for transformation efficiency using protoplast formation procedure). Using the same strains (AS019) and plasmid (pCSL17), it was found that an increase in transformation efficiency of 100-fold using electroporation, relative to protoplast transformation methods, occurred (M.L. Britz, unpublished observation). Published protoplast-dependent procedures yield transformant colonies after 2-14

Table 3.17 Transformation efficiencies of corynebacteria using electroporation systems.

Strain	Plasmid	Plasmid (ng)	TE [a]	Supplements in medium	Reference
B. lactofermentum					
BLR31	pULRS8	100	$6.7X10^2$	— [b]	Dunican and Shivnan, 1989
BL1	pUL340	100	$4.6X10^7$	2.5% glycine + 4 mg/ml INH + 0.1 % tween 80	Haynes and Britz, 1989
180	pBLA	500	$4X10^6$	ampicillin (1.5 μg/ml)	Bonnassie *et al.*, 1990
CGL2002	pCGL2002	20	$1X10^7$	-	Bonamy *et al.*, 1990
B. flavum					
MJ-233	pCRY3	1000	$4X10^5$	penicillin G (1 u/ml)	Satoh *et al.*, 1990
ATCC 14067	pCGL1017	20	$4X10^5$	-	Bonamy *et al.*, 1990
C. glutamicum					
AS019	pHY416	100	$6.6X10^4$	— [b]	Dunican and Shivnan, 1989
AS019	pHY416	1	$1.0X10^6$	— [b]	Dunican and Shivnan, 1989
ATCC 21850	pHY416	100	$2.6X10^2$	— [b]	Dunican and Shivnan, 1989
ATCC 21850	pHY416	1	$6.5X10^2$	— [b]	Dunican and Shivnan, 1989
AS019	pWST4B	250	$6X10^5$	— [b]	Liebl *et al.*, 1989
R127	pWST4B	250	$1X10^5$	— [b]	Liebl *et al.*, 1989
AS019	pUL340	100	$4.0X10^7$	2.5% glycine + 4 mg/ml INH + 0.1 % tween 80	Haynes and Britz, 1989
AS019	pHY416	1000	$3.9X10^5$	2.5% glycine + 4 mg/ml INH	Haynes and Britz, 1990
AS019	pCSL17	1000	$5.0X10^5$	2.5% glycine + 4 mg/ml INH	Haynes and Britz, 1990
AS019	pUL340	1000	$4.5X10^5$	2.5% glycine + 4 mg/ml INH	Haynes and Britz, 1990
ATCC 13287	pCGL2002	20	$6X10^4$	-	Bonamy *et al.*, 1990
ATCC 14752	pCGL2002	20	$3X10^6$	-	Bonamy *et al.*, 1990
JS231	pECCG1	100	$6.5X10^5$	2% glycine	Noh *et al.*, 1990
ATCC 13032	pECCG1	100	$1.0X10^6$	2% glycine	Noh *et al.*, 1990
ATCC 13032	pUL330	800	$7.0X10^5$	0.5% glycine	Wolf *et al.*, 1990
JS231	pECCG1	100	$3.0X10^6$	penicillin G (0.3 u/ml)	Noh *et al.*, 1991
MLB133	pCSL17	500	$5.8X10^6$	2% glycine + 4 mg/ml INH	This work
MLB133	pCSL17	500	$3.1X10^4$	— [b]	This work

a TE, transformation efficiency, transformants (10^9 cells electroporated X μg DNA)$^{-1}$

b Dash represents that no supplement was used in growth medium.

days on selective plates (Katsumata *et al.*, 1984; Yoshihama *et al.*, 1985) and the procedure were difficult to reproduce (Thierbach *et al.*, 1988). The electroporation procedure described here was simple and rapid, with transformant colonies appearing after 24 h of incubation.

The addition of glycine and INH to the growth medium was found to be an important prerequisites for efficient electroporation. Under these growth conditions, transformation efficiencies obtained were up to $10^5/\mu g$. This was found to be 10^2 to 10^3 times higher than the transformation efficiency obtained with LBG medium. This observation is in contrast to the observations by Dunican and Shivnan (1989), where growth medium containing glycine or L-threonine did not improve the yield of transformants although concentrations of these compounds were not specified. The results presented by these authors are in conflict with several other reports (see Table 3.17). Many other workers reported that such high transformation efficiencies were not normally obtained when cells are grown without cell wall-weakening agents, including glycine and INH. The results presented here confirmed the observation by Haynes and Britz (1990), showing that cells of *C. glutamicum* grown in LBG medium containing glycine and INH can be efficiently transformed by electroporation. A possible explanation for this discrepancy in observation may be that concentrations of glycine or L-threonine used for cell growth were relatively lower than what were required for changes in the cell-surface structure.

The present data confirm that the structure of the cell wall can act as a physical barrier for DNA transformation into the cell. Results presented here and elsewhere showed relatively low transformation efficiencies, ranging between 10^2 to 10^6 transformants per 10^9 cells per μg DNA, when *C. glutamicum* is grown in the absence of cell wall modifiers in the medium. This is far lower than seen in *E. coli* using electroporation techniques, where up to 10^8-10^9 transformants per 10^9 cells per μg DNA are obtained (Dower *et al.*, 1988; Haynes and Britz, 1990). The sonication experiments presented here also showed that the time required to release proteins in corynebacteria

was far longer than that for *E. coli*. Taken together, these results indicated that cell surface structures of corynebacteria are more robust than *E. coli* and this causes lower transformation efficiency in corynebacteria.

Transmission electron microscopic observations showed that when corynebacterial cells were grown in the presence of glycine and INH in the medium, cells had thinner cell walls and better transformation efficiencies than cells grown in LBG. Mycolic acid analysis also showed that when cells were grown in medium containing glycine or INH, higher proportion of extracellular mycolic acids were seen. However, lower concentrations of INH failed to cause changes in compositions and location of mycolic acids, indicating that only very high concentrations of INH had impacts on the mycolic acid composition. Presumably alterations in the cell wall structure in these strains aided uptake of DNA during the electric pulse of electroporation.

Published works from others also indicated that prior growth in supplemented media, including glycine, INH and penicillin G, improved electrically-mediated uptake of DNA. Cells treated in this way were osmotically fragile, but did not require the lengthy regeneration period necessary with protoplasts. Transformation efficiencies obtained from corynebacteria varied from strain to strain but, *B. lactofermentum* gave better transformation efficiencies than seen for other *C. glutamicum* strains, as described by Haynes and Britz (1989).

Transformation efficiencies dropped 10^2- to 10^3-fold if plasmid DNA prepared from *E. coli* was used for electroporation of *C. glutamicum*. This observation indicates the presence of an efficient restriction system in *C. glutamicum* and confirms observations by others (Bonamy *et al.*, 1990; Haynes and Britz, 1990; Katsumata *et al.*, 1984; Thierbach *et al.*, 1988), although the cellular localisation of the restriction enzymes remains unknown. These results suggest that at least in electroporation the DNA modification is one of the major factors determining electroporation

efficiency. In *C. glutamicum*, inhibition of restriction by a temperature shock was observed by Schäfer *et al* (1990; 1994a). Using conjugation procedures, they observed that the introduction of plasmids into *C. glutamicum* was enhanced after heat treatment and, based on this observation, they proposed that restriction enzymes in *C. glutamicum* are more unstable than other cellular components or that ENases are localised at the cytoplasmic membrane (Schäfer *et al.*,1990; 1994b).

The restriction barriers of *C. glutamicum* were overcome using restriction deficient strains, RM3 and RM4: little difference in the transformation efficiencies and frequencies were seen when cells were transformed with homologous- or heterologous DNA, which is in agreement with the results reported by Schäfer *et al.* (1994b). Interestingly, although RM3 and RM4 were isolated on the basis of lower restriction barriers, transformation efficiencies of these two strains were always lower than seen for ATCC 13032 when cells were transformed with homologous DNA. For example, when cells were grown in the presence of 2% glycine and 0.4 mg/ml INH and transformed with homologous DNA, transformation efficiency on strain ATCC 13032 was 10 to 100 times higher than seen for strains RM3 and RM4. One possible explanation for this difference in transformation efficiencies is that the cell surface structures of the two strains were different from that of parent strain, ATCC 13032 (see the next section for analyses of mycolic acids for these three strains). Strain RM3 and RM4 showed higher transformation efficiencies than ATCC 13032 with heterologous DNA and growth in glycine plus INH aided transformation, showing that removal of physical barriers further improved transformation above levels expected for restriction minus strains.

The highest number of transformants was obtained when *C. glutamicum* MLB133 cells were grown in the presence of 2% (w/v) glycine and 4 mg/ml INH, harvested at A_{600} 0.4 and transformed with homologously-isolated DNA, so that a frequency of 5.8 X 10^6 transformants/10^9

cells electroporated/μg DNA was obtained. This transformation efficiency is comparable or better than those reported in the literature (see Table 3.17). Since no attempt was made here to optimise the concentration of glycine and INH in the growth medium for MLB133 and the other strains tested, it may be possible that further increases in the transformation efficiency and frequency could be obtained if cells were grown in medium containing optimised concentrations of glycine and INH. However, optimal concentration of glycine and INH for transformation efficiency may vary from strain to strain. According to the observations by Haynes and Britz (1989), transformation efficiency on *C. glutamicum* AS019 was increased when cells were grown in the presence of glycine (2%, w/v), INH (4 mg/ml), or Tween 80 (0.1%, w/v). In the present studies, the combination effect of three compounds on the transformation efficiency was not further investigated with MLB133.

3.6.3 Effects of glycine and INH on the mycolic acid composition of *C. glutamicum*

The above transformation experiments indicated that prior growth in the presence of glycine or INH increased transformation efficiency, implying that the cell wall structure, was affected. Because mycolic acids are a distinguishing feature of the coryneform group, these observations initiated the analysis of fatty acid and mycolic acids of the *C. glutamicum* strains. The mycolic acids and fatty acids of some of the strains included in the present study have been previously investigated before (Collins *et al.*, 1982a; Collins *et al.*, 1982b; Pierotti, 1987) and results obtained are in full agreement with these results in terms of the qualitative compositions detected.

In Pierotti's work on strains ATCC 13059, AS019, and mutants ML133 and MLB194, fatty acid and mycolic acid profiles were quantitatively determined by GC analysis following growth under different conditions, including sampling stationary phase LBG cultures and determining the ratio

of covalently-bound and solvent extractable, non-covalently bound mycolates, and growth in fermenters under controlled growth conditions, sampling at all stages of growth to late stationary phase, measuring fatty acids and mycolic acids. The most important observations regarding fatty acid profiles were as follow. For all strains tested, palmitic ($C_{16:0}$) and oleic ($C_{18:1}$) acids accounted for 92-98% of the total fatty acids extracted from cells throughout the growth cycle. Strains ATCC 13059 and MLB194 showed similar relative proportions of fatty acid to each other: the proportion of palmitic acid may have been marginally higher on average in MLB194, but this needs verification. In the same study, Pierotti (1987) found that two mutants, MLB133 and MLB194, contained a higher proportion of unsaturated mycolic acids and also showed that changes in the relative proportions of all mycolic acids during growth whereas the parent showed relatively constant proportions throughout growth, for both cell-associated and extracellular mycolic acids. These was same evidence that MLB133 showed slightly higher levels of extracellular mycolic acids. However, Pierotti (1987) did not investigated the effect of glycine and INH in the growth medium on the amount of extracellular mycolic and fatty acids. The present work aimed to determine whether any differences in the quantitative mycolic acid profile was caused by the presence of glycine and INH, or whether mycolic acids leaked from the cell into the culture fluids during cell growth.

For *C. glutamicum* strains, five types of mycolic acid peaks ($C_{32:0}$, $C_{34:1}$, $C_{34:0}$, $C_{36:2}$, $C_{36:1}$) were obtained from the present work, ranging from C_{32} to C_{36}, confirming that these strains of *C. glutamicum* contain mycolic acids with carbon lengths similar to those from *C. glutamicum* NCIB 10025 (Collins *et el*, 1982a). In the same paper, workers reported mycolic acid compositions for three coryneform bacteria: *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}). $C_{32:0}$ and $C_{34:1}$ were the main components in all strains tested, whereas $C_{36:1}$ was a minor component.

Table 3.18 Comparison of mycolic acids found in culture fluids and whole cells of *C. glutamicum* strains grown in four different media using solvent extraction without TLC purification.

Data are obtained from Tables 3.8B, 3.9B, 3.10B and 3.12B. 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 (for ATCC 13032, RM3, and RM4, 0.4 mg/ml INH was used instead of 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
Mycolic acids from whole cells												
$C_{32:0}$	53.0 [a]	52.7	49.1	55.9	42.1	48.3	38.9	39.0	46.1	41.1	44.3	50.8
$C_{34:1}$	30.0	27.4	32.9	27.9	38.1	32.9	39.9	39.2	34.2	37.1	36.9	33.0
$C_{34:0}$	8.8	10.8	9.0	10.6	7.1	8.6	7.5	8.6	8.5	9.1	7.7	6.2
$C_{36:2}$	5.3	6.3	6.2	4.1	9.4	7.6	10.5	9.5	8.1	8.5	8.3	8.0
$C_{36:1}$	2.9	2.9	2.9	2.4	3.3	2.6	3.3	3.8	3.2	4.1	2.9	2.1
% UM to TM [b]	38.2	36.6	42.0	34.4	50.8	43.1	53.7	52.5	45.5	49.7	48.1	43.1
Mycolic acids from culture fluids												
$C_{32:0}$	54.2 [a]	54.1	53.6	57.4	46.0	45.2	41.8	45.8	47.5	58.7	46.1	48.6
$C_{34:1}$	30.4	28.2	31.9	27.9	37.4	35.5	38.4	35.8	34.1	29.3	36.4	34.9
$C_{34:0}$	7.6	10.3	7.5	9.3	7.4	8.7	6.8	7.3	8.4	6.9	6.7	7.6
$C_{36:2}$	6.1	4.4	5.0	3.7	7.2	7.8	10.2	8.6	7.2	4.8	9.0	8.6
$C_{36:1}$	2.1	2.9	2.1	1.6	2.3	2.9	3.0	2.6	2.9	-	2.1	1.7
% UM to TM [b]	38.6	35.6	39.0	33.2	46.9	46.2	51.6	47.0	44.2	34.1	47.5	45.2
% extracellular [c]	4.5	15.9	4.2	15.9	7.2	19.3	2.5	37.0	7.8	10.1	8.6	18.5

Mycolic acids	ATCC 13032				RM3				RM4			
	LBG	LBG-G	LBG-I	LBG-GI	LBG	LBG-G	LBG-I	LBG-GI	LBG	LBG-G	LBG-I	LBG-GI
Mycolic acids from whole cells												
$C_{32:0}$	40.8 [a]	48.4	43.1	43.5	28.9	45.3	35.8	30.5	47.4	62.2	51.7	66.6
$C_{34:1}$	39.1	34.6	36.2	35.5	37.7	33.4	38.6	43.2	34.6	26.8	34.6	24.9
$C_{34:0}$	7.2	8.6	9.5	9.3	8.0	10.4	10.0	2.6	8.5	5.7	5.5	4.5
$C_{36:2}$	9.8	6.2	27.8	7.8	20.4	7.2	10.8	21.6	6.9	3.8	6.4	3.3
$C_{36:1}$	3.0	2.5	3.4	3.9	5.0	3.6	4.5	2.2	3.2	1.3	1.9	1.0
% UM to TM [b]	51.9	43.3	47.4	47.2	63.1	44.2	53.9	67.0	44.7	31.9	42.9	29.2
Mycolic acids from culture fluids												
$C_{32:0}$	51.0 [a]	54.0	47.1	52.0	36.8	62.7	46.5	48.5	48.6	64.3	51.2	68.9
$C_{34:1}$	37.9	32.0	35.0	34.1	40.9	25.4	36.3	37.7	29.9	25.1	35.9	22.8
$C_{34:0}$	4.0	7.8	8.8	8.3	10.4	8.3	10.5	-	10.1	8.1	8.1	5.6
$C_{36:2}$	7.2	4.8	6.5	5.6	6.0	3.7	6.7	13.8	11.5	2.6	4.8	2.8
$C_{36:1}$	-	1.8	2.5	-	4.9	1	-	-	-	-	-	-
% UM to TM [b]	45.1	38.6	44.0	39.7	51.8	30.1	43.0	51.5	41.4	27.7	40.7	25.6
Relative % of total [c]	3.5	5.5	4.9	7.2	4.1	8.1	7.7	10.5	5.4	9.5	4.8	11.9

[a] Proportion (%) of each mycolic acid found in whole cells or culture fluids. The proportion of each mycolic acid was calculated as a % of the total in terms of peak area detected. Dash represent no peak detected under the condition used.

[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 acids.

[c] Proportion (%) of mycolic acids found in culture fluids relative to total mycolic acids detected (whole cells plus culture fluids).

The strains of saprophytic coryneform bacteria tested have distinctly different composition of mycolic acids from pathogenic *Corynebacterium* species (*C. diphtheriae*, *C. ulcerans* and *C. urealyticum*) (Yano and Saito, 1972; Corina and Sesardic, 1980; Herrera-Alcaraz, 1993). It is interesting to note that the strain of *C. ulcerans* used in the present work for reference was highly sensitive to glycine and INH, compared with the other strains tested. Differences in the sensitivity of *C. ulcerans* to these cell wall modifiers could be explained by the different composition of mycolic acids. The two 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 a higher proportion of $C_{34:1}$ and $C_{36:2}$ and a decreased proportion of $C_{32:0}$. In Pierotti's work (1987), the relative proportion of $C_{32:0}$ in MLB194 and MLB133 were 39.2% and 19.3% respectively, whilst the relative proportions in the present study were 38.2% in MLB194 and 33.7% in MLB133. The differences seen are probably due to the differences in fermentation conditions between the flask cultures in the present study and the ten-litre fermentations performed in Pierotti's work, which involved not only differences in media volumes but also differences in aeration, agitation and pH control. However, the trends seen in the proportion of mycolic acids were reproducible.

Amongst the AS019 family, the proportion of $C_{32:0}$ was in the decreasing order of AS019, MLB194, MLB133 and this order was always the same for repeated experiments (Table 3.18). Amongst the ATCC 13032 family, differences in the proportion of mycolic acid were also seen, where $C_{32:0}$ was higher in ATCC 13032 and RM4 relative to RM3, and $C_{36:2}$ was considerably higher in RM3 (28.8%). Both *Brevibacterium* species, BF4 and BL1, showed similar patterns of mycolic acids both in quantitative and qualitative compositions; these strains exhibited the presence of five MAMEs both in the cells and culture fluids.

When used at a concentration of 2%, glycine had little impact on the cellular mycolic acid profiles

of AS019, MLB133 and MLB194 except for a possible small decline in the relative levels of unsaturated mycolic acids. This is not surprising, considering that the presumptive target for glycine action in *C. glutamicum* and other Gram positive bacteria is the peptidoglycan (Hammes *et al.*, 1973). However, the most obvious impact of including glycine was a large increase in the extracellular mycolic acids for strains AS019 and MLB133, suggesting that glycine impacts either directly or indirectly with the attachment of corynemycolic acids to the cell surface. A similar trend was also observed from strains ATCC 13032, RM3 and RM4, but with more significant variation in the mycolic acid composition. Following growth in glycine medium, the proportion of $C_{34:1}$ and $C_{36:2}$ were decreased, so that the unsaturated mycolic acids were decreased. The mycolic acid profile for the relative proportions of each mycolic acid in the cells and the culture fluids 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 acid profile.

The cellular mycolic acids profiles of the strains tested showed little change following growth in the presence of 4 mg/ml INH and there were also little quantitative variation in extracellular mycolic acids. This is probably due to the relatively low concentration of INH in the growth medium. When the concentration of INH was increased to 8 mg/ml, the proportion of $C_{32:0}$ and $C_{34:0}$ decreased for all three strains tested (AS019, MLB133 and MLB194), and the amount of extracellular mycolic acids increased. Again, the two mutants showed higher sensitivities to the presence of INH in the growth medium and they showed greater variation in mycolic acid composition: 8 mg/ml INH caused an increase of more than 15% in the proportion of unsaturated mycolic acid for both cells and culture fluids.

3.6.4 Influence of cell wall modifiers on cell morphology and cell wall structure of *C. glutamicum*

Differences in transformation efficiencies could be explained by differences in cell wall thickness although it has been argued that the resolution and contrast available by electron microscopy of thin sections are insufficient to observe substructures in the surface layer (Bardou *et al.*, 1996). Observation by others and results from the present work indicated that the two mutants (MLB133 and MLB194) were more susceptible to glycine and INH, suggesting that these mutants had altered cell surface structures. In mycobacteria, treatment of cells with INH at the MIC resulted in different morphological changes that were visualised by electron microscopy (Bardou *et al.*, 1996). This data provides evidence which strongly suggested that INH affect the cell envelope of mycobacteria. This led me to initiate a study looking at the cell surface structures of *C. glutamicum* by TEM.

Approximately 90% of cells measured were less than 2 μm in cell length after growth in LBG. This was slightly smaller than the cell length of *C. glutamicum* described in Bergey's Manual of Systematic Bacteriology (Jones and Collins, 1986). The difference was due to the sample preparation, where cells were fixed then sectioned. Because such cleavage of immobilised cells could occur at any position in cells, the distribution of cell length in the photos was smaller than the length of the cells prepared due to non-longitudinal sectioning.

Although differences were small, the proportion of longer cells in MLB133 was higher than AS019, which was in agreement with the observations using phase contrast microscopy (Best and Britz, 1986). The morphology of cells from any of the growth media was found to be a mixture of short and long rods, which is typical of *C. glutamicum,* as described in Bergey's Manual of Systematic Bacteriology (Jones and Collins, 1986).

However, both strains (AS019 and MLB133) often showed greater budding and branching following growth in LBG containing 2% glycine, morphology which is more characteristics of other nocardioform species. This is also in agreement with observation by Best and Britz (1986). They noted that growth in the presence of glycine produced larger, more irregularly shaped cells for both strains MLB133 and AS019, and that the MLB133 displayed considerably more pleomorphism during growth in the presence of glycine than the AS019. TEM studies confirmed the light microscopy observations (Jang *et al.*, 1997) and the TEM images showed that septation did not occur at the branch points, which was an important observation for understanding how cell growth occurs.

In this work, the most important finding was in measurements of the cell wall thickness of the two strains, both in the absence and presence of glycine or INH. Strain MLB133 had a thinner cell wall after growth in LBG, relative to AS019. When the growth medium was replaced with 2% glycine or 4 mg/ml INH, the cell wall thickness decreased by approximately 30% for both strains. This indicated that the mutant strain contains a thinner cell wall structure normally and its cell wall thickness could be further reduced by the presence of appropriate concentrations of glycine or INH. These observation are consistent with those for higher transformability and the higher release of mycolic acids into the extracellular fluids following growth in glycine and INH.

3.6.5 Cell wall structure of *C. glutamicum*

In mycobacteria, up to 60% of the weight of the cell wall is occupied by lipids that consist mainly of long-chain mycolic acids containing 60 to 90 carbons (Minnikin, 1982). The covalently connected structure of the cell wall is made of peptidoglycan, to which arabinogalactan is linked via a phosphodiester bridge (Brennan and Nikaido, 1995; Jarlier and Nikaido, 1994; Minnikin, 1982). About 10% of the arabinose residues in arabinogalactan are in turn substituted by mycolic

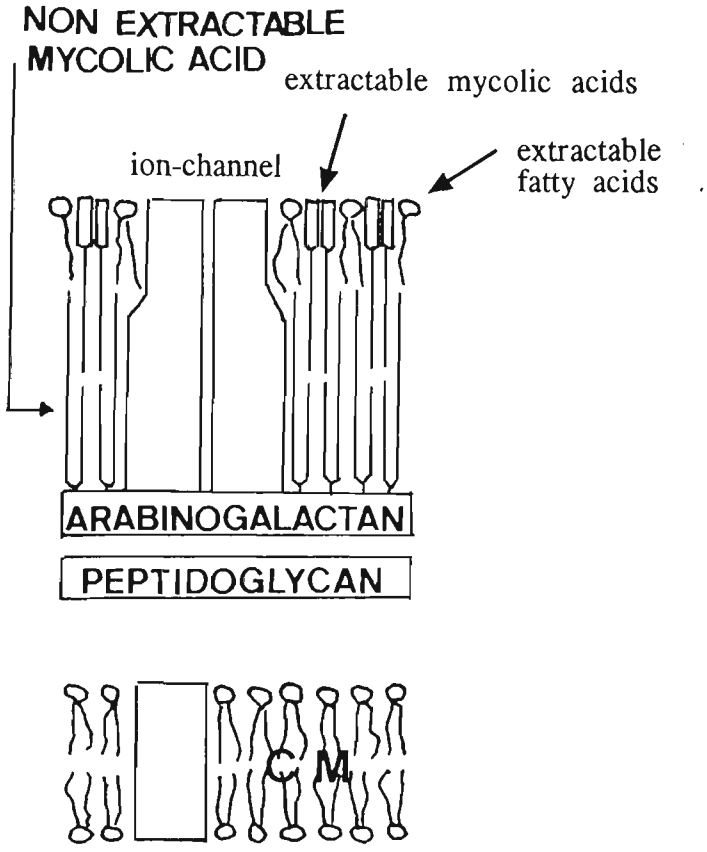


Fig. 3.31 Proposed model for structure of corynebacterial cell wall. The model is based on the model of the mycobacterial cell wall (Nikaido *et al.*, 1993). Unlike the mycobacterial model, the presence of solvent-extractable mycolic acids (approximately 50% of total cellular mycolic acids) has been confirmed (Pierotti, 1987) and the present work indicated the presence of fatty acids in the cell wall, therefore, both extractable mycolic and fatty acids are included in this model. The model also shows the presence of an ion-channel, which was recently established by Niederweis *et al.*, 1995.

acid (McNeil and Brennan, 1991). Also, the mycobacterial cell wall contains several types of extractable lipids that are not covalently linked to this basal skeleton and could be extracted with hydrocarbon solvents; these include trehalose-containing glycolipids, phenol-phthiocerol glycosides, and glycopeptidolipids (Jarlier and Nikaido, 1994; McNeil and Brennan, 1991; Minnikin, 1982). In addition, the cell wall also contains proteins (Brennan, 1989; Nikaido *et al.*, 1993) and porin protein (Trias *et al.*, 1992).

Similar to mycobacteria, corynebacteria have a thick peptidoglycan layer covalently bound to arabinogalactan and they posses mycolic acids linked to the polysaccharides (Barksdale, 1981; Goodfellow and Minnikin, 1977). Dufréne *et al.* (1997) reported that the surface of corynebacteria (*Corynebacterium* species strain DSM 44016 and DSM 6688) was rich in hydrocarbon-like compounds (about 40%), including mycolic acids. Both strains of corynebacteria were found to have 23-27% (dry weight percentage of cell wall constituents) of peptidoglycan and 14% protein.

The presence of cell surface layer in *C. glutamicum* has been reported, indicating the presence of proteins (Chami *et al.*, 1995). They found that when cells were grown on a solid medium, the cells were surrounded by a continuous surface layer of highly ordered protein, which interacted with some cell wall material. In addition to this, the presence of an ion-permeable channel in the cell wall structure in *C. glutamicum* was reported (Niederweis *et al.*, 1995). Therefore, the cell wall structure of *C. glutamicum* is a quite complicated structure which contains peptidoglycan, arabinogalactan, free mycolic acids, covalently-bound mycolic acids, free or covalently-bound fatty acids (not yet experimentally studied), proteins and peptides, and an ion channel (proposed structure of cell wall structure of *C. glutamicum* are presented in Fig. 3.31). For the latter structure, it was suggested 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 (Niederweis *et al.*, 1995).

C. glutamicum has been shown to contain both free mycolic acids (Goodfellow *et al.*, 1976; Keddie and Cure, 1977) and "cord-factor" type lipids (Pierotti, 1987). This would explain why these bacteria are so difficult to protoplast because the linked lipid and sugar-rich layers would interfere with the action of lysozyme on the peptidoglycan. The presumptive targets for glycine and INH action in *C. glutamicum* and other Gram-positive bacteria are peptidoglycan and mycolic acids, respectively.

In the other Gram-positive species, glycine is thought to substituted for alanine in the peptidoglycan, impairing cross-linking and weakening the peptidoglycan structure. The observation that extracellular mycolic acid levels increased following growth in glycine suggest that glycine exerts effects in addition to those previously reported, where these may be related to the attachment of the mycolates to the cell surface.

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 structures into the culture fluids. Detecting the presence of such structures in culture filtrates of AS019 or the mutants remains experimentally untested and acid methanolysis used in preparing MAMEs for analysis in the present work would have disrupted the covalent bonds. Alternatively, glycine may interfere directly with translocation of corynemycolic acids from presumptive, yet unidentified, carriers *via* transacylation to arabinose units in the arabinogalactan.

At the 4 mg/ml of INH used here, no visual difference in the cell shape was seen using electron microscopic observation, except that cells became shorter. This could be due to the relatively low concentration of INH used, which was not enough to create a visual difference. In mycobacteria, it has been reported that some changes in the cell surface occurred due to the presence of INH.

Bardou *et al.* (1996) observed that when mycobacteria was treated with subinhibitory concentrations of INH, this caused a decrease of 20 to 35% (by weight) of their mycolic acid content and an increase of extracellular materials was seen.

Although *C. glutamicum* is relatively insensitive to INH, when this was included in growth media at high concentrations (8 mg/ml), all of the *C. glutamicum* strains tested had decreased relative proportions of $C_{32:0}$ and $C_{34:0}$ with parallelled increased proportions of $C_{34:1}$ and $C_{36:2}$, and extracellular mycolates increased to 18-21% for the mutants. These results 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. Disruption of attachment of mycolic acids following growth in the presence of INH has not been reported previously for corynebacteria.

Results presented here showed the presence of extracellular fatty acids in the culture fluids (5 to 19% of total detected) in *C. glutamicum*. Analysis of the extracellular fatty acids suggested that some fatty acids of *C. glutamicum* are probably located near the cell surface and leak out during growth. All strains of *C. glutamicum* tested had two major types of fatty acids ($C_{16:0}$ and $C_{18:1}$) and showed little changes in the relative proportions of all fatty acids after growth in the presence and absence of glycine or INH, for both cell-associated and extracellular fatty acids. In mycobacteria, fatty acids are found in both the cell wall and cytoplasmic membrane (Nikaido et al., 1993). In the present study of fatty acids in *C. glutamicum*, since the starting material was obtained from whole cells rather than the cell wall, it is impossible to know with any certainty whether changes caused by the presence of INH or glycine in the relative proportion of fatty acids were at the cell surface or membranes. Therefore, analysis of fatty acids both qualitatively and quantitatively should be performed after preparation of fatty acids from the cell wall.

However, the main effect of addition of glycine (2%) or INH (8 mg/ml) was to decrease the proportion of fatty acids relative to mycolic acids found in both cell-associated and extracellular fractions. If these two major fatty acids are used as substrates for biosynthesis of mycolic acids, it could be possible to postulate that the presence of glycine or INH in the medium inhibits not only mycolic acid synthesis but also fatty acid synthesis. In the latter case, the inhibitory mode seems more significant. This observation indicates indirectly that fatty acids are present in the cell wall, although the presence of fatty acids on the cell surface has not been studied directly. However, before any conclusions can be drawn, more information on the quantitative changes in fatty acids and mycolic acids needs to be accumulated.

In mycobacteria, INH concentrations of 1-3 μg/ml exerted selective inhibition over the biosynthesis of mycolic acids compared with fatty acids synthesised in the same assay mixture (Wheeler and Anderson, 1996). The intermediates accumulated by the action of isoniazid were 24-carbon atoms acids. The ratio of unsaturated to saturated fatty acid was not effected by isoniazid. Based on these observations, it has been suggested that isoniazid acts on the elongation of C_{24} acids in mycobacteria rather than the desaturation of $C_{24:0}$ and also that isoniazid might block the pathway from mycolic acids biosynthesis at a later stage than 24-carbon chains (Wheeler and Anderson, 1996).

In contrast, the present work demonstrated that, in *C. glutamicum*, INH may have inhibited not only the mycolic acid synthesis but also fatty acid synthesis. The relative proportion of total fatty acids to total lipids (fatty acids plus mycolic acids) following growth in LBG was higher than following grown in LBG-8 mg/ml INH, as INH decreased the ratio of fatty acids significantly. This indicated that INH may inhibit the early stage of fatty acid synthesis.

INH and glycine could be impacting on fatty acid and mycolic acid synthesis in *C. glutamicum* at

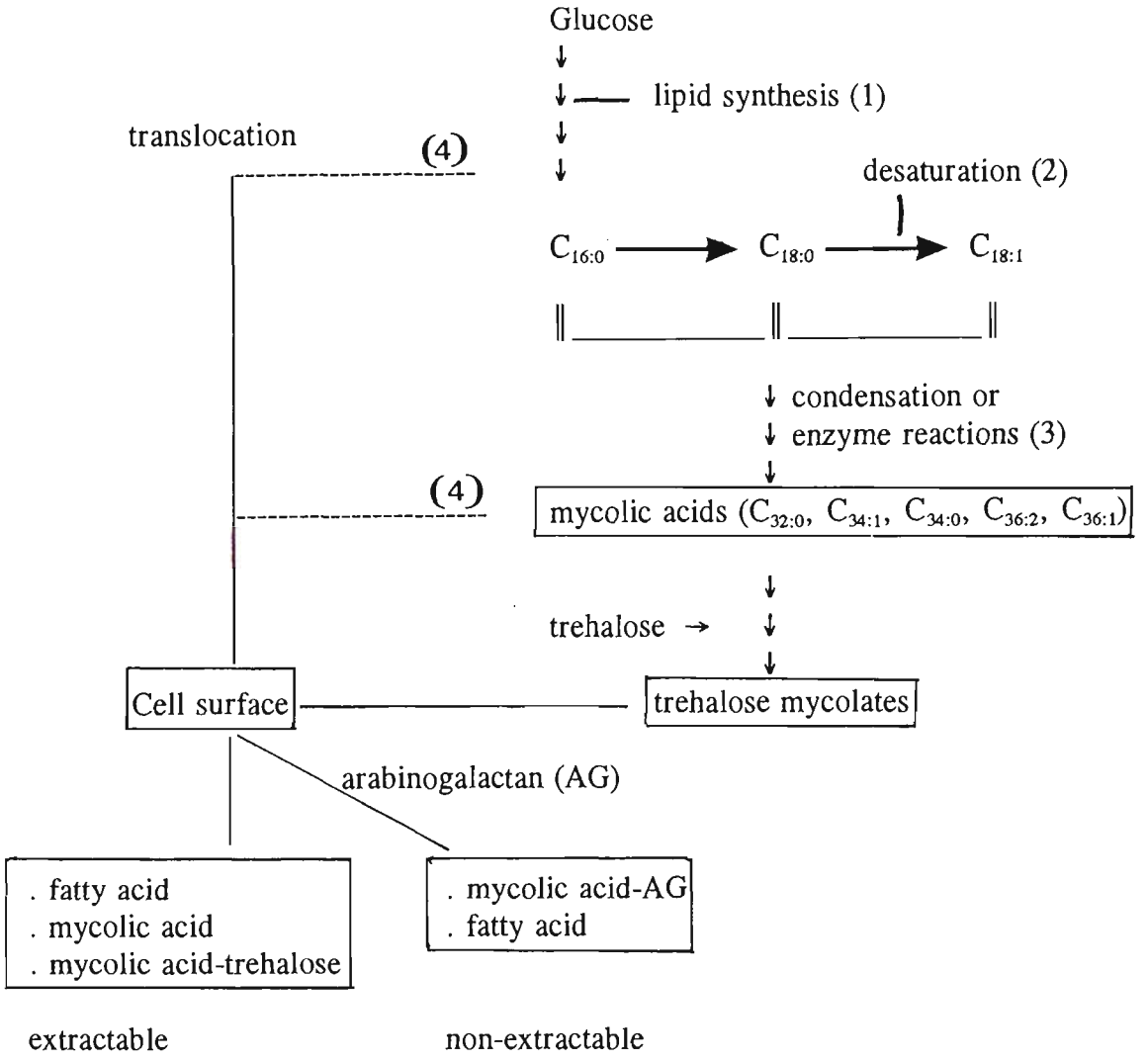


Fig. 3.32 The proposed pathway of mycolic acid synthesis in *C. glutamicum*.

(1) fatty acid synthesis ($C_{16:0}$) and to $C_{18:0}$.
(2) desaturation.
(3) chemical condensation or enzymatic reaction.

$C_{16:0} + C_{16:0} = C_{32:0}$
$C_{16:0} + C_{18:0} = C_{34:0}$
$C_{16:0} + C_{18:1} = C_{34:1}$
$C_{18:0} + C_{18:1} = C_{36:1}$
$C_{18:1} + C_{18:1} = C_{36:2}$

(4) translocation of mycolic acids and/or fatty acids to the cell surface.

several points (see Fig. 3.32), for example: (1) synthesis of fatty acid precursors of mycolic acids (2) enzymatic or natural condensation of fatty acids to produce β-oxymycolates (3) translocation through the cell wall membrane and conversion to free mycolic acid, covalently bound mycolic acid, trehalose monomycolate and trehalose dimycolates and (4) deposition onto arabinose units of arabinogalactan or (5) synthesis of arabinogalactan. Alternatively, at step (3) not only β-oxymycolates but also fatty acids (possibly in combined structures with other cell wall components) are translocated through the cell membrane and wall.

3.6.6 Model for biosynthesis of mycolic acids in *C. glutamicum*

For modelling the physical organisation of the lipids in mycobacteria, Minnikin (1982) proposed that the lipids may be arranged as a bilayer, and Nikaido *et al.* (1993) further developed this model by using X-ray diffraction studies of purified *M. chelonae* cell wall and suggested that the physical organisation of lipids in the cell wall of *M. chelonae* has an asymmetric bilayer. Although a monolayer, of one lipid component (dimycolytrehalose) was suggested (Durand *et al.*, 1979), it seems that a bilayer model is more logical. According to these workers, since a monolayer arrangement of mycolic acid residues will produce a large hydrophobic surface, the model assumes the presence of an outer leaflet, composed of other lipids (including fatty acid) (Nikaido *et al.*, 1993). The model predicts that the amount of mycolic acid present in each cell must be enough to cover the cell surface area. Based on calculations from Nikaido *et al.* (1993), 0.085 μmol of mycolic acid exists in 1 mg (dry weight) of cells of mycobacteria strains, an amount which would cover about 240 cm^2 of cell surface, based on the value of the surface area per mycolic acid, determined with the monolayer method (Duran *et al.*, 1979). Since 1 mg of mycobacterial cells is estimated to have about 230 cm^2 of cell surface (assumptions: cell width, 0.6 μm; cell length, 2 μm; cell volume, 3 μl per mg dry weight), there amount of mycolic acids its sufficient to cover the cell surface as a monolayer.

The bilayer model demands that the cell wall contains enough of other lipids to form the outer leaflet. Minnikin (1982) suggested solvent-extractable lipids could served as candidates for these lipids, but this was criticised because the presence of these lipids was not described in some saprophytic species, including *M. chelonae* (Nikaido *et al.*, 1993). In contrast, Nikaido *et al.* (1993) demonstrated that the amount of cell wall-located fatty acids (C_{14}-C_{18}) is more than one mole per mole of mycolic acids (Nikaido *et al.*, 1993) and this could serve as an outlayer leaflet.

The presence and types of mycolic acids in *C. glutamicum* have been reported including the present thesis work: $C_{32:0}$, $C_{34:1}$, $C_{34:0}$, $C_{36:2}$, $C_{36:1}$ (Collins *et al.*, 1982a; Pierotti, 1987). According to Pierotti's work (1987), there are at least three different mycolic acids: extracellular mycolic acids (approximately, 5-10% of the total mycolic acids), solvent extractable mycolic acid (= free mycolic acid, approximately 45-48% of the total mycolic acids), and covalently-bound mycolic acids (approximately 45-48% of the total mycolic acids). The present work in this thesis showed that when cells were grown in the presence of 2% glycine or 8 mg/ml INH, the amount of extracellular mycolic acid in the culture fluids could be increased by two to three-fold. If the physical organisation of lipids in the cell wall of *C. glutamicum* is similar to that of the proposed model system in mycobacteria, the cell wall structure of *C. glutamicum* may contain extractable mycolic acids (or mixtures of extractable mycolic acids-fatty acids) as the outlayer leaflet.

The transmission electron micrographs presented here indicated that when *C. glutamicum* strains were grown in the presence of glycine (2%, w/v) or INH (4 mg/ml), the thickness of the cell surface structure of two strains became thinner (by 20-30%) than seen in LBG. Glycine and INH caused alterations in the cell wall of *C. glutamicum* resulting in the disorganisation and loss of structural integrity of the outer layer. Electron micrographs revealed that at a concentration of 2%, glycine dismantled the *C. glutamicum* outer layer where parts of this were lost into the surrounding medium without causing bacterial lysis. Subsequently, this resulted in a lesser

amount of mycolic acids left at the cell surface, which may be not enough to cover the cell surface in a continuous, protective layer. Alternatively, reduction in the amount of mycolic acids may have meant that the spatial arrangement was insufficient to allow interactions between bound mycolic acids and other mycolic or fatty acid, as was able to occur for LBG-grown cells, so that the cell wall became a looser structure. The removal of the out-most components of the cell wall may facilitate transformation of DNA into the interior and increase its permeation ratio.

Models for synthesis of mycolic acids in mycobacteria and related species propose that cellular fatty acids undergo chain elongation and modification (carboxylation, introduction of carrier groups, desaturation) to produce the two forms of mycolates which are either bound to arabinogalactan or occur as trehalose dimycolates (Brennan and Nikaido, 1995). Evidence for involvement of fatty acids as precursors for corynemycolic acids includes the incorporation of [1-^{14}C]-palmitate into C_{32} mycolates of *C. diphtheriae* (Gastambide-Odier and Lederer, 1959; Walker *et al.*, 1973) and [1-^{14}C]-stearic acid into $C_{34:0}/C_{34:1}$ and $C_{36:0}/C_{36:1}$ in *Bacterionema* (now *Corynebacterium*) *matruchotti* (Shimakata *et al.*, 1984).

Recently, 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 involved in fatty acid synthesis (Dessen *et al.*, 1995): the product of the *inhA* gene has greater than 40% sequence identity over 203 amino acids with the EnvM proteins of *E. coli* and *Salmonella typhimurium* and EnvM is thought to participate in fatty acid biosynthesis in these species (Bergler *et al.*, 1992; Turnowsky *et al.*, 1989). It is interesting to note that this enzyme preferentially reduces C_{16} derivative, consistent with its involvement in mycolic acid biosynthesis (Quémard *et al.*, 1995b).

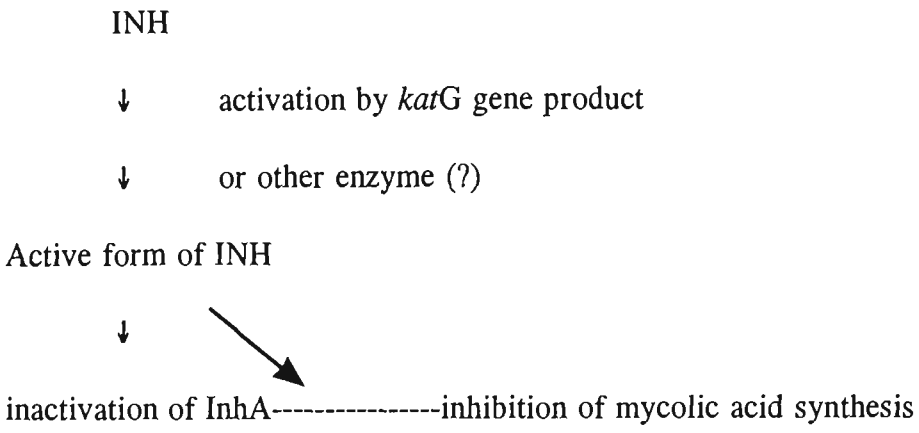


Fig. 3.33 The proposed mechanism of action of INH in *C. glutamicum*. Similar mechanism are suggested in mycobacteria (see Blanchard, 1996). The presence of a *kat*G equivalent has not been investigated in this bacterium.

The presence of the *inh*A gene in corynebacterial DNA library in *E. coli* has been identified, by A. Sayyada and M. Serafica in CBFT at VUT (unpublished data), where the cloned genes showed 90% sequence homology with the corresponding fragment in mycobacteria. Sequence homology with the *inh*A gene in mycobacteria enable speculation that an InhA product occurs in corynebacteria and this catalyses a similar reaction as seen in mycobacteria. Although the above observation showed the presence of a putative target gene for INH in corynebacteria, it is not known why *C. glutamicum* is relatively insensitive to INH, compared to mycobacteria. Results presented here showed that *C. glutamicum* was relatively insensitive to INH and significant differences in cell growth rate, transformation efficiency, and mycolic acid composition of *C. glutamicum* were only obvious at high concentrations (8 mg/ml). The reason for this significant differences in the concentration of INH which inhibits *C. glutamicum* and mycobacteria may be found in the activation of INH *in vivo*. INH is thought to be converted *in vivo* to an active metabolite in mycobacteria, possibly by the action of a thermolabile catalase-peroxidase (T-catalase) (Quémard *et al.*, 1995a). In mycobacteria, at least three different types of catalase (A, M, T-catalase) are found (see Quémard *et al.*, 1995a), and T-catalase is known to be involved in the INH activation procedure.

Early observations suggested a link between INH resistance and the loss of mycobacterial catalase-peroxidase activity (Cohn *et al.*, 1954; Middlebrook, 1954). Recent studies have demonstrated that deletion of, or point mutations in, the *M. tuberculosis kat*G gene, which encodes a unique catalase-peroxidase, results in the acquisition of INH resistance and that transformation of INH-resistant strains of *M. tuberculosis* with a functional *kat*G gene restores sensitivity to the drug (Zhang *et al.*, 1992). This activation may not occur in *C. glutamicum* but trace amounts of an active form may be present in INH added to media at the high concentrations used, which caused inhibition of mycolic acid synthesis (Fig. 3.33). This needs exploring. Alternatively, the *inh*A gene product from *C. glutamicum* may be different from the gene product of mycobacteria species

(i.e. substrate-binding site in the enzyme). This also requires further studies at levels of both DNA sequence analysis and structural-functional analysis of the protein concerned.

3.6.7 Comparison between cell surface mutants and the parent strain of *C. glutamicum*

The results from this study suggest that there is no simple explanation of the cell-surface changes the mutant strains MLB133 and MLB194, which are transformed much more easily than the parent strain. This is not surprising, considering the complexity of the cell surface of these organisms and the lack of detailed chemical research on their cell walls.

There were differences in the sensitivity to the presence of glycine and INH in the growth medium, mycolic acid profiles, and transformation efficiencies of the two mutant strains MLB133 and MLB194, compared to the parent strain. The cell wall thickness of the mutants also varied considerably in the presence of glycine or INH in the growth medium compared to the parent strain. The mutant strains also gave high electrotransformation efficiencies in the presence of glycine and INH in the growth medium. Analysis of the lipids produced under standard growth conditions indicated that the mutants had altered fatty acids and mycolic acids profile. The proportion of extracellular mycolic acids, and solvent-extractable mycolic acids (Pierotti, 1987), was higher for the mutants and growth in glycine and INH greatly increased the proportions of extracellular mycolic acid for mutants and the parent strain.

These sets of results individually do little to explain the differing protoplasting efficiencies but together suggest that the mutants strains have a greater degree of volatility in their cell surface chemistry, that is, they may have mutations which affect the biosynthesis and assembly of the complex cell surface.

However, two other mutant strains (RM3 and RM4) were restriction deficient, since there were no difference in transformation efficiencies whatever the source of DNA used for electroporation. The results from the present work also suggested that these were also cell surface mutant strains. These mutants showed different sensitivities to glycine, and were more severely affected by the presence of INH in the growth medium than the parent strain. The mycolic acid profiles of the mutant strains grown in glycine also showed considerable differences, whilst the parent strain showed little variation in the proportion of unsaturated mycolic acids. Taken together, these results make it probable that the two mutants were also cell surface mutants as well as restriction deficient mutants. One possible explanation is that RM3 and RM4 were cell surface mutants where the surface-located restriction enzyme was dislocated or inactivated due to the surface changes. RM3 and RM4 were complemented by the cloned gene with sequence homology to known restriction systems, but the location of the gene product was not known. This was not incompatible with the mutations in RM3 and RM4 bearing associated with surface changes, considering the hypothesised location of this restriction system.

One explanation for the observed hypersensitivity to INH in the two mutants relative to their parental-type strain AS019 may be that (1) difference in DNA sequence of *inh*A or putative *kat*G genes, causing differences in production of the active form of INH (2) 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 (3) the bonds between mycolic acids and other cell wall components are impaired or (4) some or all of the above assumptions could occur together. How this would affect the extracellular distribution of mycolates remains subject to speculation.

3.6.8 Recommendations for further development

It is obvious that more work has to be performed on the cell surfaces of these organism to understand the reasons behind the increased transformation efficiencies of the mutant strains of *C. glutamicum* and equally important to learn more about the complex cell surface of these organisms. The logical extension of this work would be to focus on the target site of glycine and INH on the cell wall structure of *C. glutamicum* and its related bacteria and also to understand the biosynthetic pathway of mycolic acids, arabinogalactan and peptidoglycan. In particular, the following information is not yet available.

1. the fatty acid composition of the cell wall of corynebacteria, as opposed to the composition found in whole cells. Data presented in the present study was based on analysis of whole cells so that fatty acids found in the cell membrane were included in analysis. It is necessary to understand the composition of the cell wall of corynebacteria to form a model of this structure which can be related to the current models promulgated for mycobacteria species (Nikaido *et al.*, 1993).

2. the form of fatty acids (free, cell wall-bound) on cells (cell wall and membrane) or in culture fluids should be determined in both qualitative and quantitative ways. Results shown in this thesis suggested that the presence of glycine or high concentrations of INH (8 mg/ml) inhibited normal fatty acid synthesis ($C_{16:0}$, $C_{18:1}$) in *C. glutamicum*.

3. the form of mycolic acids (free, trehalose- or arabinose-bound) on cells or in culture fluids is not known (i.e. the relative proportions or presence of each type). Quantification of mycolic acids was a problem, in the absence of commercially-available corynemycolic acid standards. I used C_{25} lipid (lignoceric acid methyl ester) as an internal standard to quantify the efficiency of

recovery of mycolic acids through extraction (acid methanolysis to fatty acid methyl esters and mycolic acid methyl esters, petroleum ether extraction) and derivatisation (trimethylsilylation) prior to GC or GC-MS analysis (Athalye *et al.*, 1984; Minnikin *et al.*, 1980; Yano and Saito, 1972) in the present work. The relative proportions measured, however, were in terms of peak areas rather than in molar ratios. Therefore, to assist in quantification, mycolic acid standards should be prepared from large volumes of culture and each of the five mycolic acids could be prepared by preparative HPLC (Butler *et al.*, 1996). Quantification of fatty acids could be performed using commercially-available sources of lipids.

4. the presence and amount of arabinogalactan or peptidoglycan fragments in the extracellular fluids (which addresses the question of gross destabilisation of the cell surface and linkages between arabinogalactan and peptidoglycan). For this experiment, cells could be grown in LBG as a control. The amount of arabinogalactan or peptidoglycan fragments in the extracellular fluids after growth in LBG media containing cell wall modifiers (glycine, INH and glycine plus) could then be compared to those in LBG. In particular, the presence of mycolic acid-arabinogalactan complexes in the culture fluids would be of interest, since arabinogalactan is also one of the important cell wall components to which mycolic acids connect directly (Takayama and Kilburn, 1989). The same authors suggested that inhibition by ethambutol depletes the sites for transfer and incorporation of D-arabinose into cell wall arabinogalactan. Therefore, ethambutol should be tested in parallel with glycine and INH, which may help identify the nature of the mutations in *C. glutamicum* MLB133 and MLB194 in terms of impacts on mycolic acids.

5. clarification of the role of fatty acids in synthesis of mycolic acids by *C. glutamicum*. Incorporation of specific fatty acids into mycolic acids in whole cells and in a cell-free biosynthesis system should be performed, as described previously for corynebacteria and mycobacteria (Besra *et al.*, 1994). Demonstrating the incorporation of radio-labelled fatty acids,

glucose or acetate into cell surface structures of *C. glutamicum in vivo* will provide a useful tool for showing that specific radio-labelled substrates (fatty acids, glucose or acetate) are precursors of specific mycolic acid end-products and other cell wall components (providing that a cell free assay system can be developed).

6. investigation of the *inh*A gene in corynebacteria. Although the presence of this gene in *C. glutamicum* is currently being studied in our laboratory, the role of this gene in corynebacteria has not been determined. Therefore, whether this gene product catalyses a similar reaction as occur in mycobacteria should be investigated. This and the following experiment (identification and characterisation of *kat*G gene) may provide clues for understanding inhibitors of the enzyme activities and also give some understanding of why *C. glutamicum* is insensitive to INH when compared with mycobacteria. Other students in CBFT, VUT are currently pursuing further information on the biochemistry of mycolic acid synthesis in *C. glutamicum* and the properties of the *inhA* gene equivalent in this species to provide sufficient background to understand the nature of the genetic changes in these mutants and to provide some insight into mycolic acid synthesis in this species.

7. the presence of *kat*G gene in *C. glutamicum* and other corynebacteria species. INH is thought to be converted *in vivo* to an active metabolite in mycobacteria, possibly by the action of T-catalase (Quémard *et al.*, 1995b). The presence of *kat*G in corynebacteria has not been studied. If *kat*G exists in corynebacteria, the sequence of this gene can be determined by using several sets of PCR primers based on the conserved sequences of the mycobacterial *kat*G gene (Zhang *et al.*, 1992). Since the mycobacterial catalase-peroxidase converts INH to reactive intermediates, the presence of *kat*G (or a similar gene) in *C. glutamicum* should be determined. In addition, in mycobacteria, it has been suggested that levels of KatG would result in increased resistance to INH and increased levels of KatG should result in increased sensitivity to INH (Dubnau *et al.*,

1996). Therefore, it would be interesting to know whether or not this occurs in *C. glutamicum*.

In mycobacteria, there are at least three different types of catalase (A, T, M-catalase) and T-catalase is known be involved in the INH activation step (Quémard *et al.*, 1995a). Therefore, it is necessary to determine what type of catalase is present in both the parent and mutant strains of *C. glutamicum*.

8. effect of high concentrations (such as 8 mg/ml or above) of INH on the cell morphology of *C. glutamicum*. Up to now, there has been little knowledge gathered about cell wall structures and wall thickness in *C. glutamicum*. The present study failed to show modified cell morphology after growth in 4 mg/ml INH, whilst this was seen in 2% glycine medium, confirming observation by others (Haynes and Britz, 1990). In mycobacteria, the presence of subinhibitory concentration of INH in the medium caused the presence of aberrant and deformed cells (Bardou *et al.*, 1996). Similar effects may occur in *C. glutamicum* when high concentrations of INH are used but this was not investigated in the present study. It would be interesting to determine any effects using TEM.

Chapter 4

Investigation of some properties of enzymatic restriction and modification systems in *C. glutamicum* and related species

Part of the work described in this chapter has been published (or will submitted to the journal) and their lists are as follows.

1. Jang, K.H., Chambers, P.J., and Britz, M.L. (1996) Analysis of nucleotide methylation in DNA from *Corynebacterium glutamicum* and related species. FEMS Microbiology Letters. **136**:309-315.

2. Jang, K.H., Chambers, P.J., and Britz, M.L. Identification of sequence containing methylated cytidine in *Corynebacterium glutamicum* and *Brevibacterium flavum* strain BF4 using bisulphite DNA sequencing technique. Applied and Environmental Microbiology (in preparation).

4.1 INTRODUCTION

Results from electroporation experiments described in Chapter 3 indicated that strains of *C. glutamicum* (ATCC 13032, AS019, MLB133 and MLB194) have higher frequencies of transformation for homologously-isolated DNA, which implies that *C. glutamicum* must be able to cleave DNA from heterologous hosts by ENase activity. This also suggested that this species must be able to modify their DNA to protect it against degradation by ENase. When I commenced the current project, no information on the specificity of ENase or MTase in *C. glutamicum* had been reported. Therefore, the starting point of the present study was to prove the presence of enzymatic barriers in this species and characterise this activity. For the present study, a coryneform bacteria-*E. coli* shuttle vector, pCSL17 DNA, was used since this plasmid was capable of replication in *C. glutamicum*, *B. lactofermentum*, *B. flavum* strains. *B. lactofermentum* strain BL1 contains a natural plasmid, pBL1. Initially, the strategy to characterise the restriction and modification systems in coryneform bacteria included isolation of enzymes (ENase and MTase) and their corresponding genes. However, in 1994, information on the putative ENase genes in *C. glutamicum* was published (Schäfer *et al.* 1994b) and the future work of these authors was to characterise the gene of the cognate MTase (A. Schäfer, personal communication). Consequently, the objective of the present study was changed to determine how heterologously-isolated plasmid DNA is modified in coryneform species to protect it from the principal restriction endonuclease present in these species. Diong (1989) had observed several types of DNase activity in *C. glutamicum*, such as putative ENase, "nickase" and exonuclease activities: the presence of these activities was confirmed in the present work but further characterisation was not pursued.

The approaches used to explore the putative RM systems in the saprophytic coryneform bacteria was four-fold. Firstly, transformation experiments were performed using corynebacteria-derived plasmid DNA and the same DNA isolated from a range of *E. coli* strains with different RM

backgrounds. Britz (unpublished observation, 1985) had observed that plasmid DNA (pCSL17) isolated from *C. glutamicum* transformed recipient McrBC$^+$ strains of *E. coli* with lower efficiency than McrBC$^-$ strains, which inferred that *C. glutamicum* DNA contains methyl-deoxycytidine (see section 4.5). In *C. glutamicum* strains, the presence of restriction barrier was also reported by Tauch *et al.* (1994). Furthermore, it was also found that *B. lactofermentum* BL1-derived pCSL17 DNA was not restricted by McrBC$^+$ strains of *E. coli* (unpublished observation, M. L. Britz, 1985), indicating that the methylation patterns in *B. lactofermentum* were different from that of *C. glutamicum*. In the present work, a similar approach was also applied to *B. flavum* to gain some insight into the methylation patterns in this species. Secondly, HPLC analysis was used to determine the methylated base content of chromosomal and plasmid DNA isolated from several corynebacteria species. Thirdly, analysis of chromosomal and plasmid DNA from coryneform bacteria was performed to determine susceptibility to digestion by restriction enzymes which cleave DNA specifically at either methylated or unmethylated recognition sequences, in order to identify how MTase activity in coryneform bacteria affects DNA. Finally, one of the target sites of MTase in *C. glutamicum* AS019 was examined using a technique which chemically modifies only unmethylated cytidine followed by DNA sequencing to determine the changes caused, so identifying one sequence which is methylated in *C. glutamicum*.

4.2 DEMONSTRATION OF DNASE ACTIVITY IN CORYNEFORM BACTERIA CELL-FREE EXTRACTS

Preparation of *C. glutamicum* strain AS019 cell lysates for the analysis of DNase activity is described in section 2.5.5. DNase activity was examined in cell-free extracts without further purification. During initial examinations, cell-free extracts were tested for sequence-specific cleavage of lambda DNA or covalently closed circular (CCC) plasmid DNA isolated from *E. coli* LE392 using different buffer compositions. These reactions were performed as follows: 5-10 μl

of cell-free extracts were mixed with 0.5-1 μg of either pCSL17 (CCC form) or lambda DNA (linear form) in a 20 μl reaction mixture containing various buffers. The composition of buffers tested were: One-Phor-All buffer (Promega) and NE buffers I, II, III, IV (Biolabs). The reaction mixtures were incubated at 30°C for up to 16 h. Some typical results are shown in Fig. 4.1 using One-Phor-All buffer: similar results were obtained for the other buffers. pCSL17 DNA-derived from *E. coli* LE392 was present in CCC, open circular (OC) and multimer form (MF) initially and did not degrade after overnight incubation, when cell-free extracts was absent in the reaction mixture. The position of linear DNA was confirmed by subjecting the DNA to digestion with *Pst*I which has a single recognition site on pCSL17 (Fig. 4.1C).

For all strains tested in Fig. 4.1, cleavage of *E. coli* LE392-derived pCSL17 DNA occurred and CCC plasmid was initially nicked and converted to OC form before being further degraded. Once the plasmid DNA is in the OC form, it should be more susceptible to further ENase attack or spontaneous strand breakage: as time progressed, the proportion of OC increased and linear form (LF) was clearly detected, which was not seen in the control incubated in buffer without cell-free extracts. After overnight incubation, the total amount of plasmid DNA had decreased in all cases and the amount of CCC DNA had also significantly declined (see lanes 12 and 21 in Fig. 4.1A and Fig. 4.1B).

It is interesting to note that the nuclease activity in the two mutant strains RM3 and RM4 also degraded supercoiled pCSL17 DNA in a similar fashion to the parent (Fig. 4.1), although results from electrotransformation supported the absence of significant enzymatic barriers in these strains.

These results suggest that the presence of ENase(s) activity in cell-free extracts of strains tested. This was observed for all strains tested, including *C. glutamicum* AS019, ATCC 13032, RM3, RM4, and *B. flavum* BF4.

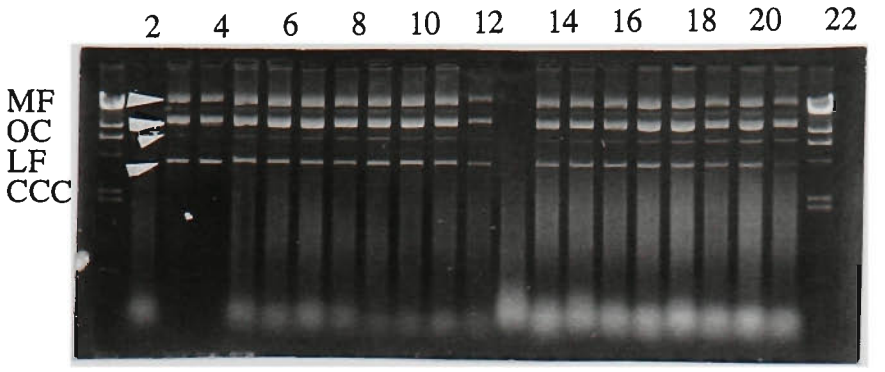
Fig. 4.1 Demonstrating DNase activity in cell-free extracts of *C. glutamicum* strains. Extracts of strains AS019, ATCC 13032, RM3 and RM4 were incubated with *E. coli* LE392-derived pCSL17 DNA for different time intervals then analysed using agarose gel electrophoresis. *E. coli* LE392-derived pCSL17 DNA was exposed to cell-free extracts for time intervals of 5, 20, 40, 60, 120, 180, 240 min and overnight at 30°C. Reactions were terminated at specific times by heat inactivation of cell-free extracts at 65°C for 10 min. The mixture was then stored at -20°C before analysis. Reaction mixtures (20 μl) contained 0.5 μg of DNA, 2 μl of 10X One-Phor-All buffer, and various volumes of cell-free extracts (harvested at OD_{600} = 0.2-0.8 [0.2 for AS019, 0.8 for ATCC 13032, 0.4 for RM3, and 0.6 for RM4] and contained 0.5 μg of protein in the total reaction volume). Results from strain AS019 (lanes 2-12) and ATCC 13032 (lanes 13-21) are shown in **(A)**. Results from strain RM3 (lanes 2-12) and RM4 (lanes 13-21) are shown in **(B)**. For **(C)**, lane 1, *Hind*III-digested λ DNA; lanes 2-5, E. coli LE392-derived pCSL17 DNA (0.25, 0.5, 1.25, 2.0 μg, respectively); lanes 7-10, *Pst*I-linearised *E. coli* LE392 derived pCSL17 DNA (0.25, 0.5, 1.25, 2.0 μg, respectively). Abbreviations: CCC, covalently closed circular form; OC, open circular form; LF, linear form; MF, multimer form DNA.

Lanes 1 and 22: *Hind*III-digested λ DNA
Lanes 2 and 13: Cell-free extracts without plasmid DNA, time zero
Lane 3: DNA only at time zero
Lane 4: DNA incubated overnight in One-Phor-All (OPA) buffer, without cell-free extracts

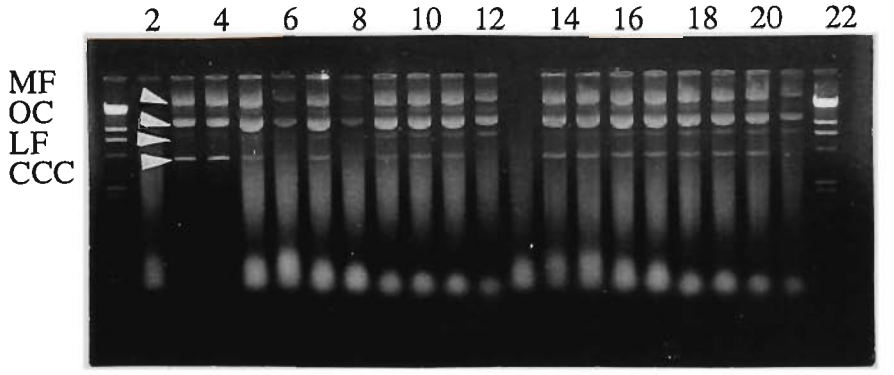
The following were DNA incubated in OPA buffer with cell-free extracts for the times indicated.

Lanes 5 and 14: 5 min
Lanes 6 and 15: 20 min
Lanes 7 and 16: 40 min
Lanes 8 and 17: 60 min
Lanes 9 and 18: 120 min
Lanes 10 and 19: 180 min
Lanes 11 and 20: 240 min
Lanes 12 and 21: overnight

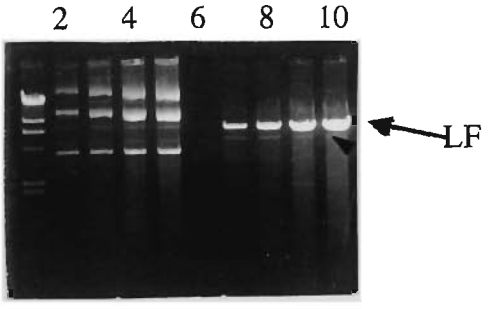
A


2 4 6 8 10 12 14 16 18 20 22
MF
OC
LF
CCC

B


2 4 6 8 10 12 14 16 18 20 22
MF
OC
LF
CCC

C


2 4 6 8 10
LF

After preparation of cell-free extracts of *C. glutamicum* AS019, these were assayed quantitatively for DNase activity by incubating 0.25-1 μg (protein) of cell-free extracts at 30°C with 0.5-1 μg of plasmid pCSL17 DNA-derived from *E. coli* LE392 for various incubation times (Fig. 4.2). Similar trends were observed from all strains (ATCC 13032, RM3, RM4 and BF4) tested although some typical data for AS019 and BF4 only are shown in Fig. 4.2 - Fig. 4.4: the substrate DNA was nicked, converted to OC form and then changed to linear DNA before being further degraded by exonuclease. The last activity was demonstrated by the diffuse streak of EtBr-interacting material at the lower portion of the gels, which is indicative of small MW fragments generated by typical exonuclease activity. For quantitative analysis, samples were taken at different incubation periods and electrophoresed. The intensity of each band (from top to bottom, MF form, OC form, linear form and CCC form DNA) on the negatives (not the photos) analysed using a densitometer. The bands between MF and OC were of unknown nature and these were not considered in the quantification. Therefore, intensity of bands from MF, OC, linear form and CCC form at zero time were considered as 100%. Fig. 4.2 showed that at the zero incubation time, the MF, OC, and CCC forms of DNA composed more than 95% of the total substrate. By 20 min incubation (Fig. 4.2A), all of the CCC form of DNA had disappeared and the proportion of OC had increased; with further incubation time to 6 h, the OC form of DNA decreased gradually from 80% to 50%, with the linear form of DNA increasing from 10% to 50%. No sample was collected between 6 and 16 h of incubation. After 16 h of incubation, the substrate DNA was completely digested and no bands were seen, indicating that the presence of exonuclease activity in the cell-free extracts had degraded linear forms to small MW fragments.

Previously, Schäfer *et al.* (1994a) observed that when recipient cells of *C. glutamicum* ATCC 13032 were incubated at 49°C for 9 min prior to the transformation, efficiency of transformation with heterologously-isolated DNA was increased by 10^4 times. Based on this observation and the

observation that treatment with surface-active compounds, including EtOH and SDS, the authors suggested that DNase activity in strain ATCC 13032 was located at the cell surface and may be temperature sensitive. To test the effect of temperature on nuclease activity in cell-free extracts, these extracts were incubated at 49°C for 9 min prior to assay (data for AS019 shown in Fig. 4.2 and Fig. 4.3 using two different plasmids and sources of DNA). For unheated cell-free extracts of AS019, CCC could not be detected after 5 min incubation, whilst CCC DNA incubated with heated cell-free extracts of AS019 was still visible after 20 min. In the case of heated cell-free extracts of AS019, the appearance of LF was observed later than seen for the control. After 6 h incubation, the DNA sample with heated cell-free extracts still had LF and OC although exonuclease was clearly seen, whilst control samples had little of both left at this time. Similar results were also obtained with different substrate DNA (homologously-derived pHY416) and less amount of proteins were used (Fig. 4.3); again the appearance of LF was later when heated cell-free extracts were used than seen for controls (without heat treatment of the cell-free extracts) (Fig. 4.3). DNA activity which converted CCC form to OC decreased and after 6 h incubation, CCC form of DNA was still detected. This lower level of enzymatic activity seen here may be due to the lesser amount of cell-free extracts used. When cell-free extracts were prepared from BF4 and incubated with *E. coli* N4830-derived pCSL17, very similar results were also seen, with relatively lower DNases activity than seen for AS019 (Fig. 4.4).

From these results, it was concluded that the cell-free extracts of these bacteria contain several DNases (possibly sequence-specific endonuclease, nickase and exonuclease) which coexisted and that exonuclease activity was probably dominant. Furthermore, it could be concluded that pre-incubation of cell-free extracts partially decreased DNase activity, including linearising activity, which is consistent with Schäfer *et al.* (1994a), although enzymatic activity was obtained from cell-free extracts rather than the cell wall.

Fig. 4.2 Demonstrating DNase activity in cell-free extracts of *C. glutamicum* AS019 incubated with *E. coli* LE392-derived pCSL17 DNA for different time intervals, using agarose gel **(A)** and scanning negatives using a densitometer **(B)** and **(C)**. pCSL17 DNA was exposed to cell-free extracts for different time intervals at 30°C and reactions terminated by heat inactivation at 65°C for 10 min. The mixture was then stored at -20°C before analysis by agarose gel electrophoresis. Reaction mixtures (20 μl) contained 0.5 μg of DNA, 2 μl of 10X One-Phor-All buffer, and 15 μl of cell-free extracts (harvested at A_{600} 0.43 and contained 1 μg of protein). For lanes 13-21, cell-free extracts were pre-incubated at 49°C for 9 min. Relative proportions are percentage (%) of each form of MF, OC, LF and CCC in **(B)** and **(C)**.

For **(A)**

Lanes 1 and 22: *Hind*III-digested λ DNA

Lanes 2 and 13: Cell-free extracts with plasmid DNA in One-Phor-All (OPA) buffer, time zero

Lane 3: DNA incubated overnight in OPA buffer, without cell-free extracts

Lane 4: Cell-free extracts only, time zero

The following were DNA incubated in OPA buffer with cell-free extracts for the times indicated.

Lanes 5 and 14: 5 min

Lanes 6 and 15: 20 min

Lanes 7 and 16: 40 min

Lanes 8 and 17: 60 min

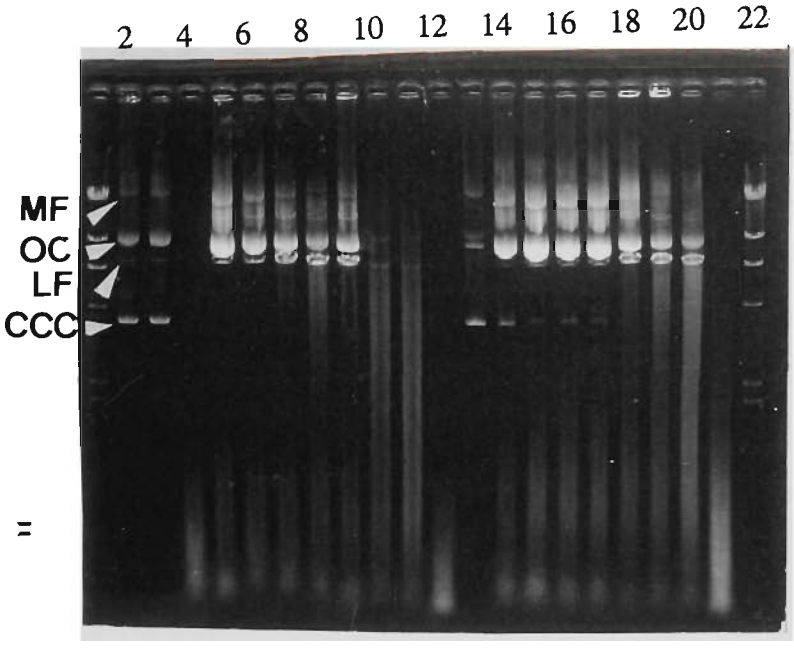
Lanes 9 and 18: 120 min

Lanes 10 and 19: 180 min

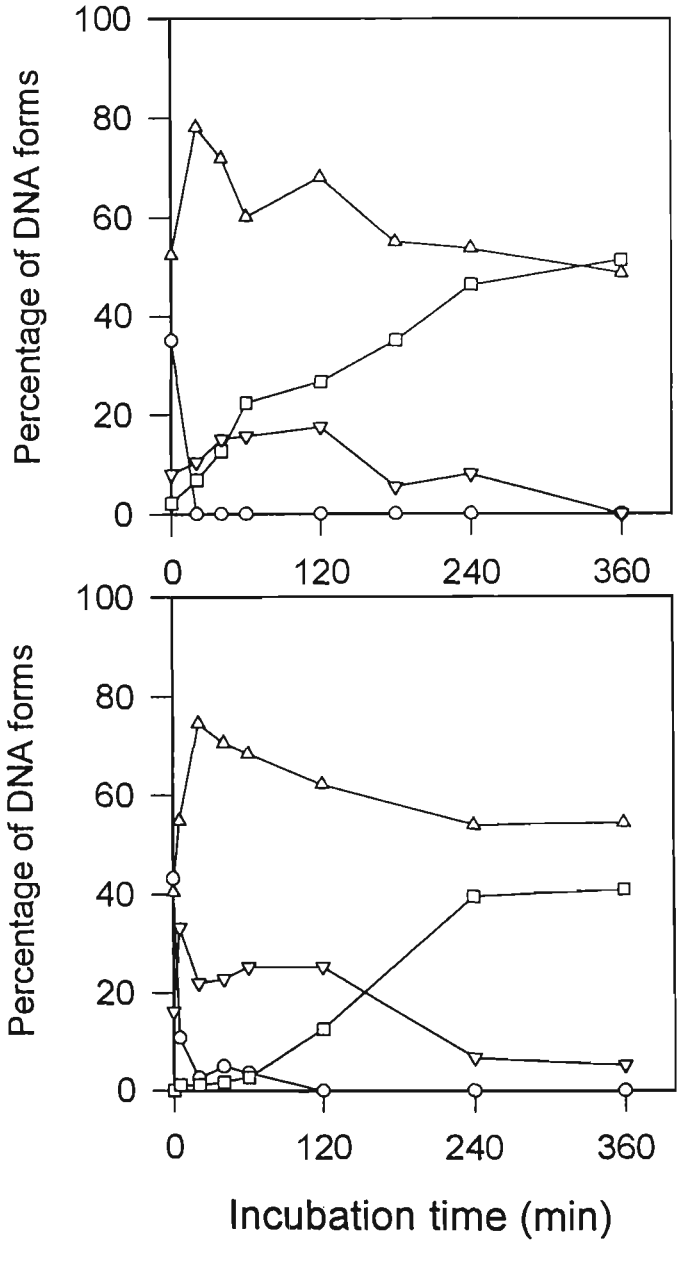
Lanes 11 and 20: 240 min

Lanes 12 and 21: overnight

For **(B)**, data are from lanes 2-12 in **(A)**. For **(C)**, data are from lanes 13-21 in **(A)**. Abbreviations: CCC, covalently closed circular form (○); OC, open circular form (△); LF, linear form (□); MF, multimer form DNA (▽).


2 4 6 8 10 12 14 16 18 20 22
MF
OC
LF
CCC

A


100
80
60
40
20
0
Percentage of DNA forms
0
120
240
360
100
80
60
40
20
0
Percentage of DNA forms
0
120
240
360
Incubation time (min)

B

C

Fig. 4.3 Demonstrating DNase activity in cell-free extracts of *C. glutamicum* AS019 incubated with *Bacillus subtilis* EMG52-derived pHY416 DNA for different time intervals, using agarose gel **(A)** and scanning negatives using a densitometer **(B)** and **(C)**. pHY416 DNA was exposed to cell-free extracts for different time intervals at 30°C and reactions terminated by heat inactivation at 65°C for 10 min. The mixture was then stored at -20°C before analysis by agarose gel electrophoresis. Reaction mixtures (20 μl) contained 0.5 μg of DNA, 2 μl of 10X One-Phor-All buffer, and 15 μl of cell-free extracts (harvested at A_{600} 0.43 and contained 0.35 μg of protein). For lanes 13-21, cell-free extracts were pre-incubated at 49°C for 9 min. Relative proportions are percentage (%) of each form of MF, OC, LF and CCC in **(B)** and **(C)**.

For **(A)**

Lanes 1 and 22: *Hind*III-digested λ DNA

Lanes 2 and 13: Cell-free extracts with plasmid DNA in One-Phor-All (OPA) buffer, time zero

Lane 3: DNA incubated overnight in OPA buffer, without cell-free extracts

Lane 4: Cell-free extracts only, time zero

The following were DNA incubated in OPA buffer with cell-free extracts for the times indicated.

Lanes 5 and 14: 5 min

Lanes 6 and 15: 20 min

Lanes 7 and 16: 40 min

Lanes 8 and 17: 60 min

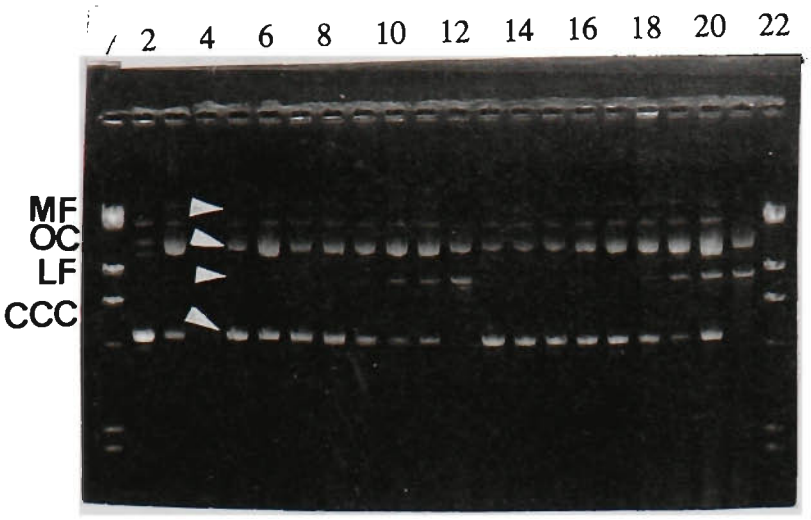
Lanes 9 and 18: 120 min

Lanes 10 and 19: 240 min

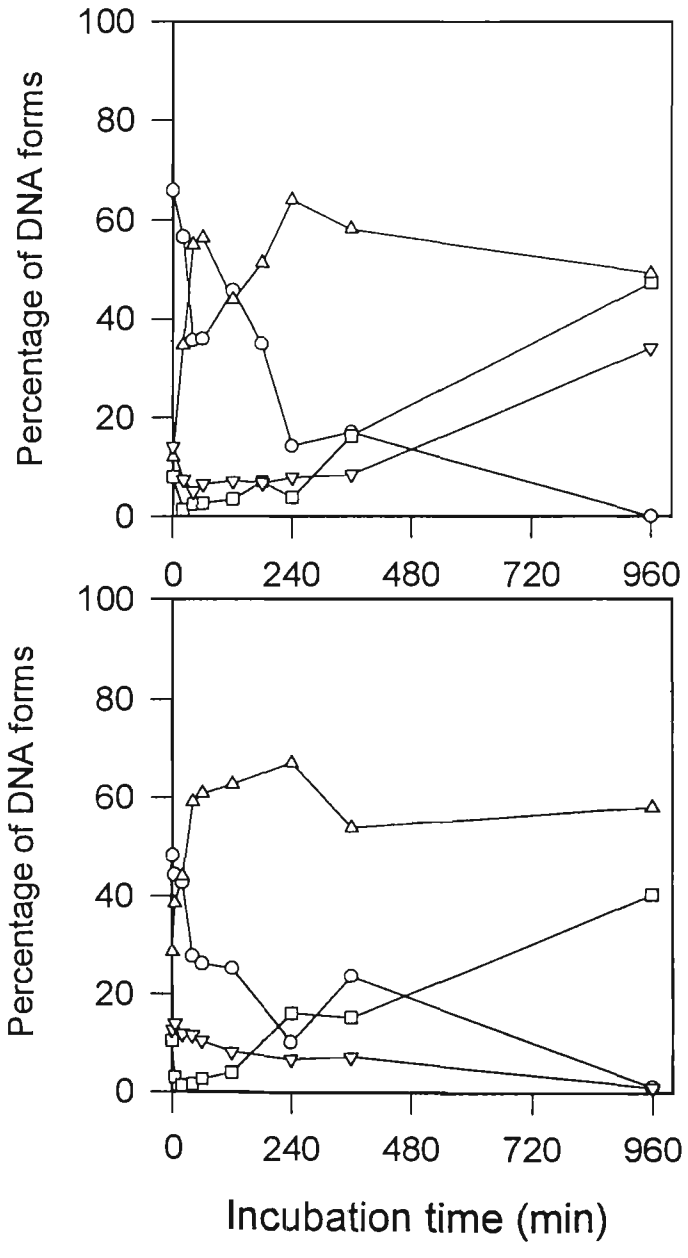
Lanes 11 and 20: 360 min

Lanes 12 and 21: overnight

For **(B)**, data are from lanes 2-12 in **(A)**. For **(C)**, data are from lanes 13-21 in **(A)**. Abbreviations: CCC, covalently closed circular form (○); OC, open circular form (△); LF, linear form (□); MF, multimer form DNA (▽).


/ 2 4 6 8 10 12 14 16 18 20 22
MF
OC
LF
CCC

A


Percentage of DNA forms
100
80
60
40
20
0
0 240 480 720 960
Percentage of DNA forms
100
80
60
40
20
0
0 240 480 720 960
Incubation time (min)

B

C

Fig. 4.4 Demonstrating DNase activity in cell-free extracts of *B. flavum* BF4 incubated with *E. coli* N4830-derived pCSL17 DNA for different time intervals, using agarose gel **(A)** and scanning negatives using a densitometer **(B)** and **(C)**. pCSL17 DNA was exposed to cell-free extracts for different time intervals at 30°C and reactions terminated by heat inactivation at 65°C for 10 min. The mixture was then stored at -20°C before analysis by agarose gel electrophoresis. Reaction mixtures (20 μl) contained 1 μg of DNA, 2 μl of 10X One-Phor-All buffer, and 7 μl of cell-free extracts (harvested at A_{600} 1.2 and contained 0.5 μg of protein). For lanes 13-21, cell-free extracts were pre-incubated at 49°C for 9 min. Relative proportions are percentage (%) of each form of MF, OC, LF and CCC in **(B)** and **(C)**.

For **(A)**

Lanes 1 and 22: *Hind*III-digested λ DNA

Lanes 2 and 13: Cell-free extracts with plasmid DNA in One-Phore-All (OPA) buffer, time zero

Lane 3: DNA incubated overnight in OPA buffer, without cell-free extracts

Lane 4: Cell-free extracts only, time zero

The following were DNA incubated in OPA buffer with cell-free extracts for the times indicated.

Lanes 5 and 14: 5 min

Lanes 6 and 15: 20 min

Lanes 7 and 16: 40 min

Lanes 8 and 17: 60 min

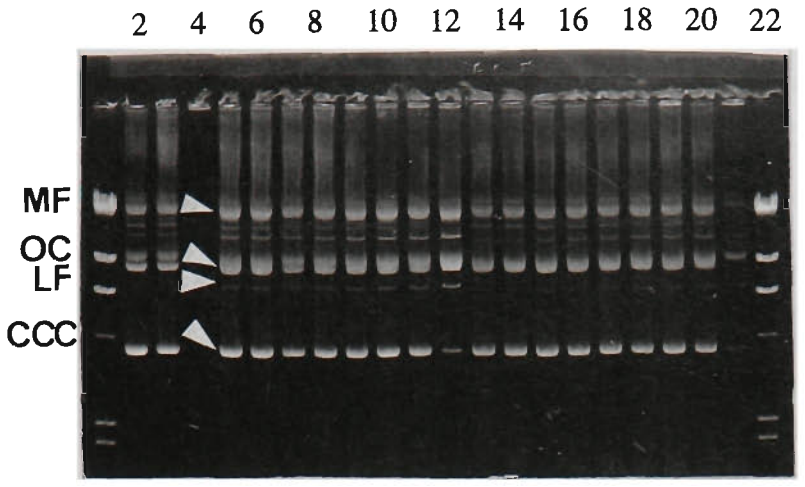
Lanes 9 and 18: 120 min

Lanes 10 and 19: 240 min

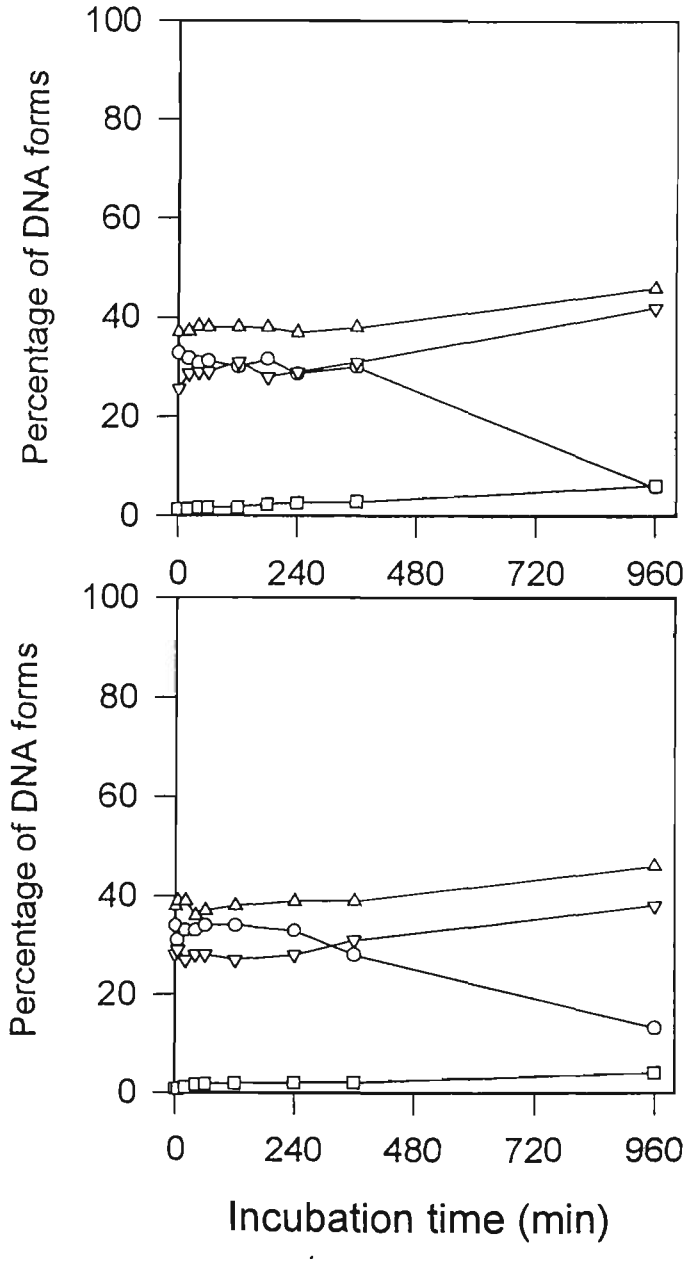
Lanes 11 and 20: 360 min

Lanes 12 and 21: overnight

For **(B)**, data are from lanes 2-12 in **(A)**. For **(C)**, data are from lanes 13-21 in **(A)**. Abbreviations: CCC, covalently closed circular form (○); OC, open circular form (△); LF, linear form (□); MF, multimer form DNA (▽).


2 4 6 8 10 12 14 16 18 20 22
MF
OC
LF
CCC

A


Percentage of DNA forms
100
80
60
40
20
0
0 240 480 720 960
Percentage of DNA forms
100
80
60
40
20
0
0 240 480 720 960
Incubation time (min)

B

C

Fig. 4.5 Demonstrating sequence specific DNase activity in cell-free extracts of two strains of C. glutamicum incubated with linearised DNA for different time intervals. Cell-free extracts were prepared from strain AS019 and CG2. To prepare linearised DNA, DNAs (0.5 μg) were incubated at 37°C for 1 h with following enzymes (10-20 units) in TA buffer (see section 2.1.2 for compositions): *Eco*RV, *Nde*I for *E. coli* DH5α-derived pBR322, *Pst*I, *Bgl*II for *E. coli* LE392-derived pCSL17, and *Hind*III, *Xho*I for λ DNA. Reactions were terminated by heat inactivation at 65°C, cooled down to the room temperature, and then used for sources of linearised DNA for cell-free extracts digestion. Reaction mixtures (20 μl) contained 0.5 μg of linearised DNA, 2 μl of 10X TA buffer, and 5 μl of cell-free extracts (harvested at A_{600} 0.45 for AS019 and 0.56 for CG2, and contained 0.4 μg of protein).

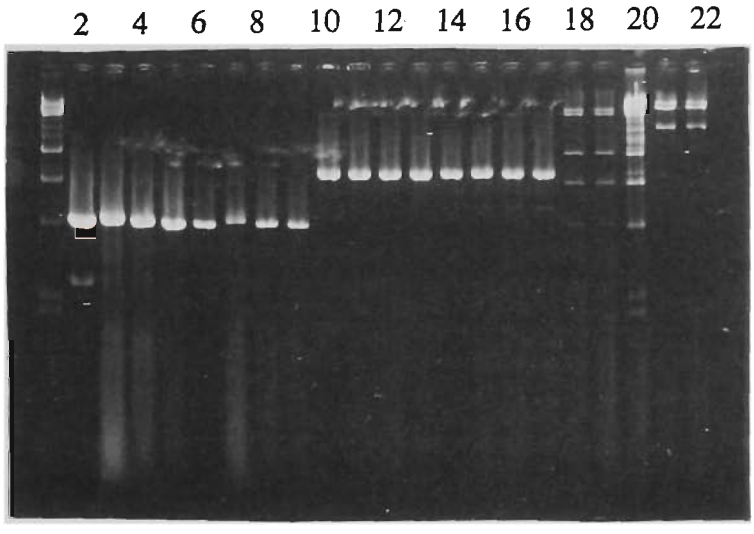
Lane 1: *Hind*III-digested λ DNA

Lane 2: *Eco*RV-linearised pBR322 DNA only, time zero
Lane 3: Lane 2 plus cell-free extracts at time zero
Lane 4: Lane 2 plus cell-free extracts at 15 min incubation
Lane 5: Lane 2 plus cell-free extracts at 60 min incubation
Lane 6: *Nde*I-linearised pBR322 DNA only, time zero
Lane 7: Lane 6 plus cell-free extracts at time zero
Lane 8: Lane 6 plus cell-free extracts at 15 min incubation
Lane 9: Lane 6 plus cell-free extracts at 60 min incubation

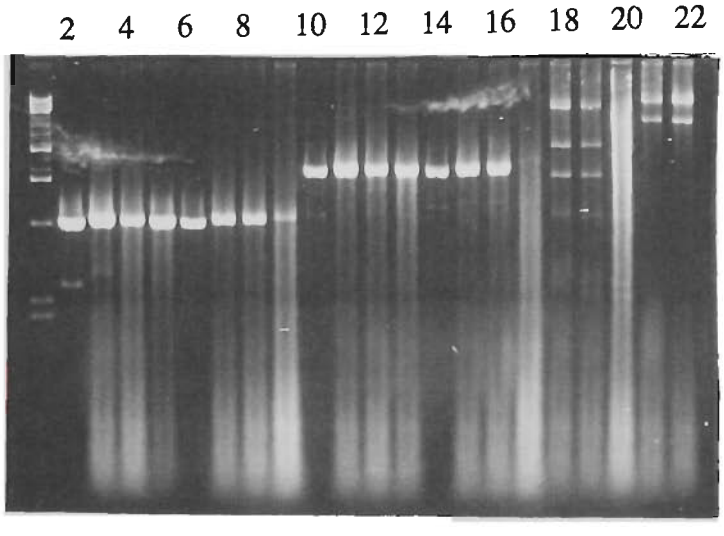
Lane 10: *Pst*I-linearised pCSL17 DNA only, time zero
Lane 11: Lane 10 plus cell-free extracts at time zero
Lane 12: Lane 10 plus cell-free extracts at 15 min incubation
Lane 13: Lane 10 plus cell-free extracts at 60 min incubation
Lane 14: *Bgl*II-linearised pCSL17 DNA only, time zero
Lane 15: Lane 15 plus cell-free extracts at time zero
Lane 16: Lane 15 plus cell-free extracts at 15 min incubation
Lane 17: Lane 15 plus cell-free extracts at 60 min incubation

Lane 18: *Hind*IIII-linearised λ DNA only, time zero
Lane 19: Lane 18 plus cell-free extracts at time zero
Lane 20: Lane 18 plus cell-free extracts at 15 min incubation
Lane 21: *Xho*II-linearised λ DNA plus cell-free extracts, time zero
Lane 22: *Xho*II-linearised λ DNA plus cell-free extracts at 15 min incubation

AS019



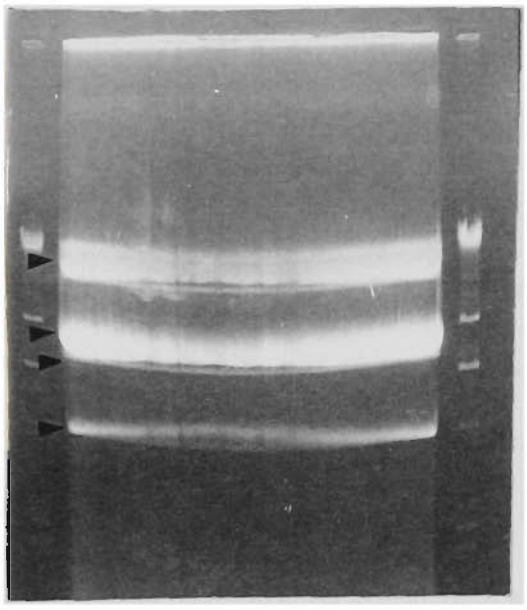
CG2



An attempt was made to characterise the linearising activity in the *C. glutamicum* cell-free extract in terms of where this cut linearised DNAs (pBR322 [4.4kb], pCSL17 [7.2kb], lambda DNA [48.5 kb]) over time courses (0, 15 and 60 min). To generate linearised DNAs, either *Eco*RV or *Nde*I were use for *E. coli* DH5α-derived pBR322, whilst either *Pst*I or *Bgl*II were used for *E. coli* LE392-derived pCSL17 DNA. Since λ DNA was large in size, this was digested with *Hind*III to generate smaller linearised DNA fragments. Cell-free extracts were obtained from two strains of *C. glutamicum*, AS019 and CG2, and these results are shown in Fig. 4.5 and Fig. 4.6. After linearising the substrate DNAs by specific ENases, activity were terminated by heat-inactivation at 65°C (10 min), prior to incubation with cell-free extracts. ENases digestion using *Eco*RV, *Nde*I, *Bgl*II and *Pst*I, resulted in complete digestion which is seen in the controls (see lanes 2, 6, 10 and 14 in Fig. 4.5). As incubation time was increased, the intensity of bands decreased, particularly in cell-free extracts of AS019, indicating the presence of exonuclease activity in the cell-free extracts. In the presence of cell-free extracts of AS019, a new bands appeared of approximately 3.0kb with pBR322, 5.1kb with *Pst*I linearised pCSL17 DNA, and 4.4 kb with *Bgl*II linearised pCSL17 DNA. This change in apparent size was likely to be caused by the cell-free extract, since running of linearised fragments in the cell-free extracts was the same as seen for the control (without cell-free extract). These results suggest that the AS019 cell-free extracts contain sequence specific DNase. However, this was not further investigated using the above approach due to the lower intensity of the bands and the presence of exonuclease activity in the cell-free extracts.

Two different approaches were further used to characterise sequence-specific ENase activity in the cell-free extracts of *C. glutamicum* AS019. The first approach was performed as follows: *E. coli*-derived pCSL17 DNA (25 μg) was digested with cell-free extracts of *C. glutamicum* AS019 and the resulting products were electrophoresed on agarose gels. The band corresponding to the linearised DNA was eluted from the agarose gel using the Gene Cleaning Kit procedure described in section 2.3.11. The resulting DNA was then used as a substrate for endonuclease digestion

(A)



(B)

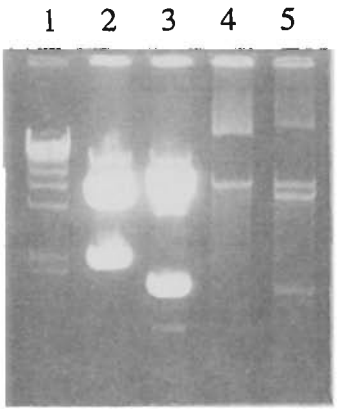


Fig. 4.6 Detecting sequence specific DNase activity in *C. glutamicum* AS019 cell-free extracts using heterologously-derived pCSL17 from *E. coli* LE392. **(A)**, DNA with cell-free extracts; **(B)**, linear form of DNA (in **A**) was derived from the agarose gel and eluted using Gene Cleaning Kit before digestion with *Bam*HI or *Sma*I. Abbreviations: CCC, covalently closed circular form; OC, open circular form; LF, linear form; MF, multimer form DNA.

For **(A)**, reaction mixtures (240 μl) contained 24 μg of DNA, 24 μl of 10X One-Phor-All buffer, and 22 μl of cell-free extracts (harvested at A_{600} 0.69 and contained 19 μg of protein). The mixture was incubated at 30°C for 3 h and reactions terminated by heat inactivation at 65°C for 10 min.

For **(B)**, LF form DNA in **(A)** was eluted from the agarose gel, as described in section 2.3.11. After that, DNA was pooled in 40 μl of water and used as the source of LF DNA. 20 μl of LF DNA was digested with either *Bam*HI or *Sma*I in TA buffer, at 37°C and 3 h.

Lane 1, *Hind*III-digested λDNA

Lane 2, 20 μl of DNA in **(A)** digested with *Sma*I

Lane 3, 20 μl of DNA in **(A)** digested with *Bam*HI

Lane 4, 20 μl of LF DNA digested with *Sma*I

Lane 5, 20 μl of LF DNA digested with *Bam*HI

using *Sma*I or *Bam*HI, which have two recognition sites on this plasmid DNA. The idea was that, if the linearised DNA was formed by an ENase which cleaved at a specific site, then subsequent cleavage with the ENase should produce a characteristic banding pattern on agarose gels. Furthermore, identifying the position of presumptive *C. glutamicum* ENase cleavage relative to *Bam*HI or *Sma*I would assist in identifying the cleavage site subsequently. In contrast, if the linearised DNA was formed by the activity of nickase, where the cleavage could occur randomly, subsequent strand breakage to form linear DNA followed by digestion with *Sma*I and *Bam*HI would not produce a defined banding pattern. Results from this experiment showed that no additional bands was formed by *Bam*HI, indicating that linearised DNA is formed by the activity of random nicking (possibly a mixture of nickase and sequence-specific ENase) (Fig. 4.6). Fig. 4.6 shows the results of enzymatic digestion of *E. coli* LE392-derived pCSL17 DNA by *C. glutamicum* AS019 cell-free extracts. The DNA was partially degraded by cell-free extracts, so that several forms of DNA (from top to bottom, MF, OC, LF and CCC form) are seen, indicating that incubation time should be extended to maximise the amount of LF DNA. The LF form DNA (would contained OC form as well) was obtained from the agarose gel and digested using *Sma*I (see lane 4 in Fig. 4.6B) or *Bam*HI (see lane 5 in Fig. 4.6B). Enzyme digestion of control DNA (lane 2 for *Sma*I and lane 3 for *Bam*HI), produced two major fragments, which was as expected. When DNA fragments from lanes 4 and 5 were compared to those in lanes 2 and 3 in Fig. 4.6B, no additional bands were seen. Failure in this approach could be due to the low yields of the LF DNA, which was too low to generate visual fragments following ENase digestions.

The second approach involved incubating 15 μl of cell-free extracts with 50 μg of sonicated salmon sperm DNA at 30°C for 15 min. Subsequently, 1 μg of lambda DNA was added to the same tube and further incubated for up to 4 h. Small volumes of samples were taken during the incubation period and analysed by agarose gel electrophoresis. The majority of sonicated salmon sperm DNA were located on the agarose gel in the size range between 0.5 to 4 kb (data not

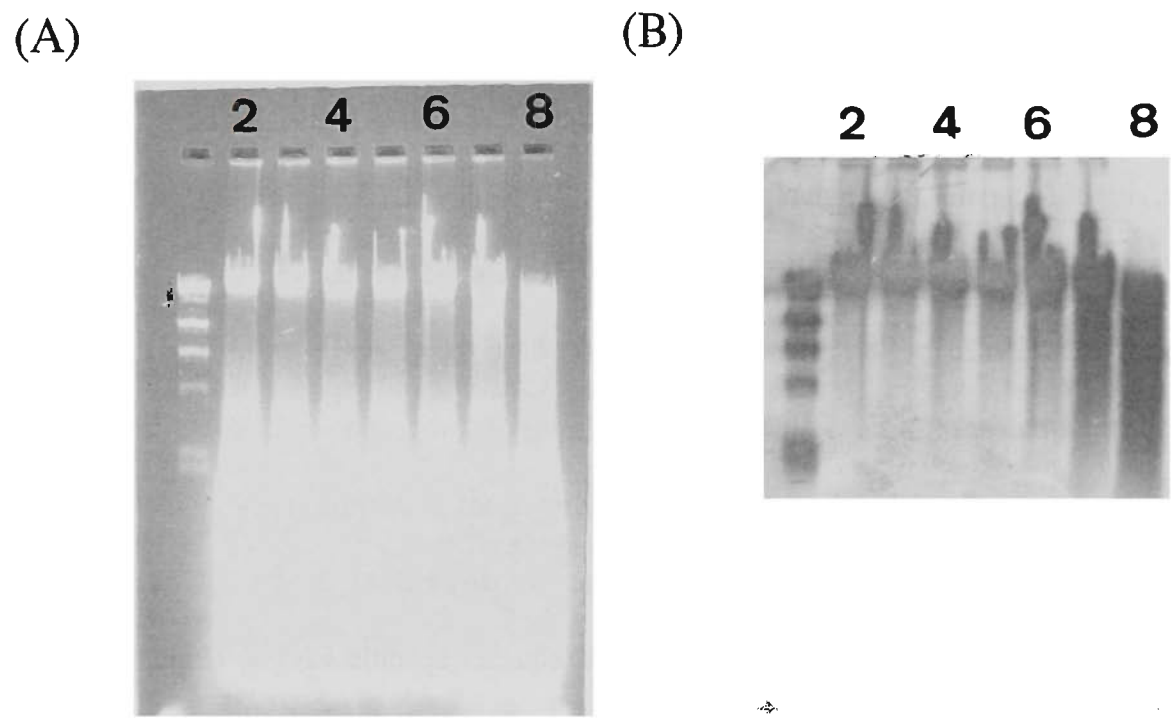


Fig. 4.7 Southern blots of DIG-labelled λDNA after incubating DNA with cell-free extracts of *C. glutamicum* AS019. DNA was exposed to cell-free extracts for time intervals at 30°C. Reactions were terminated at specific times by heat inactivation at 65°C for 10 min. The mixture was then stored at -20°C before analysis by agarose gel electrophoresis. Reaction mixtures (25 μl) contained 50 μg of sonicated salmon sperm DNA, 1.5 μl of 10X One-Phor-All buffer, and 15 μl of cell-free extracts (harvested at A_{600} = 0.43 and contained 0.35 μg of protein). After incubation for 15 min, 1 μg of DIG-labelled λ DNA was added to the tube. Lane 1, λ DNA digested with *Hind*III; 2-8, DNA & cell-free extracts at 0, 5, 15, 30, 60, 120 and 240 min. After electrophoresis (A), DNA was transferred from the agarose gel to the membrane (B) and visualised using the procedure described in section 2.3.12.

shown) but specifically-cleaved lambda DNA fragments were not seen. The idea was that in the presence of excess amounts of linearised salmon sperm DNA, exonuclease activity in the cell-free extracts could have an alternative substrate for degradation so that a small proportion of the specifically-cleaved lambda DNA would remain undigested. This was not observed.

Due to the high concentration of salmon sperm DNA in the reaction mixture, analysis of DNase activity was difficult, therefore lambda DNA was labelled by using the DIG DNA labelling method described in section 2.3.12. Using this procedure, only lambda DNA was visualised subsequently. However, no specific bands were seen on the gel, indicating that this approach was not useful for characterising sequence-specific ENase activity (Fig. 4.7). After these approaches, I had two options to characterise restriction and modification (RM) system in *C. glutamicum*. One possible approach was to purify ENase activity from cell-free extracts containing mixtures of ENase, nickase and exonuclease, and characterise specific recognition sites. The other possible approach was to investigate methylation patterns in the *C. glutamicum*-derived DNA, since ENase and MTase recognised the same sequence. Therefore, if the specific site of MTase was determined this would, by inference, identify the cleavage site of the cognate ENase. I chose the second option in view of the above results and the coincidental publication of the sequences of two presumptive ENase genes from *C. glutamicum* (Schäfer *et al.*, 1994b). Results will be described in the following result sections.

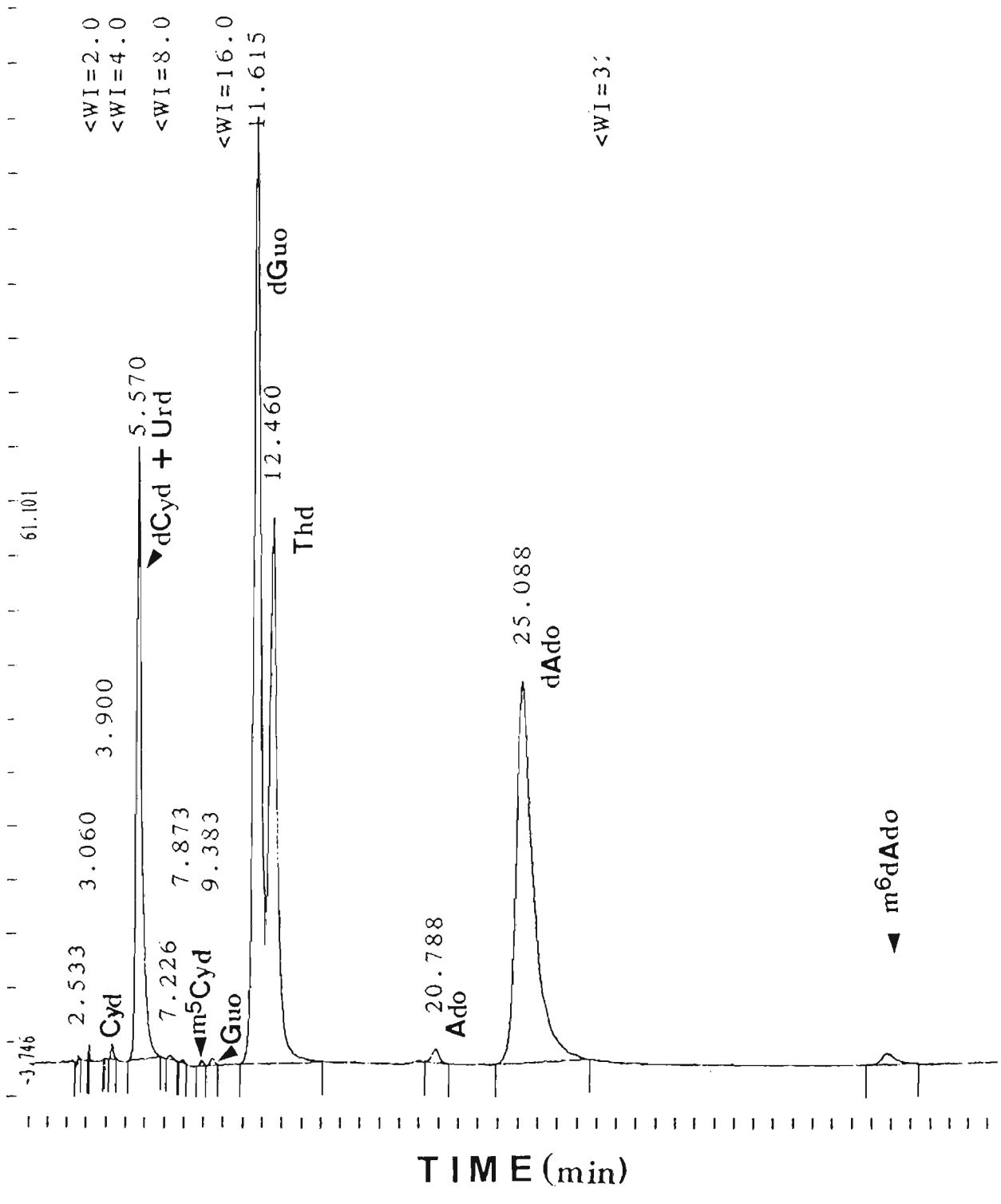


Fig. 4.8 HPLC analysis of *E. coli* LE392-derived pCSL17 digested with nuclease P1 and bacterial alkaline phosphatase (BAP). The eluent was monitored at 254 nm and peaks were identified by their retention time and heights at various absorbances (Gehrke *et al.*, 1984). Abbreviations: Cyd, cytidine; dCyd, deoxycytidine; Urd, uridine; m^5Cyd, 5-methyl-deoxycytidine; Guo, guanosine; dGuo, deoxyguanosine; Thd, thymidine; Ado, adenosine; m^6dAdo, 6-methyl-deoxyadenosine.

4.3.1 Validation of methods

The nucleoside content of both chromosomal and plasmid DNA from four coryneform bacteria (*C. glutamicum* AS019, *C. glutamicum* ATCC 13032, *B. lactofermentum* BL1 and *B. flavum* BF4) and *E. coli* strains was analysed using HPLC to determine the methylated base content. Initially a published method from Gehrke *et al.* (1984), which used a mobile phase using two solvents (solvent A [2.5%, vol/vol, methanol, 0.05M KH_2PO_4, pH 4.0] and solvent B [8.0% vol/vol, methanol, 0.05 M KH_2PO_4, pH 4.0]), was performed without modification. The total run time was approximately 60 min. This method allowed direct injection of the enzymatically-derived hydrolysates onto the column without removal of protein or any other sample preparation. Under these conditions, DNA and RNA bases were detected and this caused difficulties in differentiating between nucleosides. In particular, elution of m^5dCyd was too close to that of guanosine (RNA nucleoside), therefore a solvent gradient needed to be changed to give better separation between these two nucleosides. Analysis was performed using two solvents (95% solvent A [2.5%, vol/vol, methanol, 0.05M KH_2PO_4, pH 4.0] and 5% solvent B [methanol]) as the mobile phase. The presence of nucleosides was identified using external standards (Sigma), alone or in combinations. A typical analysis using DNA samples is shown in Fig. 4.8. The order of elution under these conditions was cytidine (Cyd), uridine (Urd), deoxycytidine (dCyd), methylated-deoxycytidine (m^5dCyd), guanosine (Guo), deoxyguanosine (dGuo), thymidine (Thd), adenosine (Ado), deoxy adenosine (dAdo), and N^6-methyl-deoxyadenosine (m^6dAdo) and this was confirmed using external standards. Also, the presence of each nucleoside base was confirmed using co-injection, where DNA samples were mixed with external standards prior to injection.

Table. 4.1 Relationship between the amount of nucleosides from *E. coli* LE392 and the peak area detected from HPLC analysis. Chromosomal and pCSL17 DNA were extracted from *E. coli* LE392 as described in sections 2.3.2, 2.3.3 and 2.3.4, and subjected to enzymatic digestion (nuclease P1 and BAP). Nucleosides were diluted from samples with various dilution factors and analysed by HPLC. The same amount of each samples (50 μl) from each dilution were injected onto the column.

Type	Dilution	Conc. (μM) [a]	Mole% [b]					
			dCyd	m^5dCyd	dGuo	Thd	dAdo	m^6dAdo
Chromosomal DNA	1	1804	32.60	0.09	22.45	22.74	22.19	0.25
	1/2	956	27.10	0.08	21.30	30.75	20.70	0.22
	1/4	426	29.79	0.14	23.91	23.20	22.8	0.23
	1/8	205	29.90	-	24.31	23.70	21.90	0.17
Average			29.75	0.10	22.99	25.07	21.88	0.22
Standard deviation			±2.13	±0.03	±1.38	±3.74	±0.87	±0.03

[a] Total concentration of nucleosides was calculated as the sum of mole concentration of each compound as determined from peak areas of the external standards.

[b] See Fig. 4.8, for abbreviations. Each value is a percentage of the total base content detected. A dash represents no peak detected for the dilution used.

When samples were mixed with each external standard (50-1,000 μM) and analysed using HPLC, the presumptive nucleoside was eluted at the same time as the corresponding nucleoside but with increased peak area, showing identity between test and standard.

Samples for the analysis of the nucleosides were stored at -20°C for up to one month. The retention time of each peak and the proportion of each DNA base did not vary significantly with storage (data not shown).

4.3.2 Relationship between amount of DNA bases and the peak area detected from HPLC analysis

In order to determine the relationship between the amount of nucleoside and peak area, several injections were carried out using samples from chromosomal DNA derived from *E. coli* LE392 and several dilutions of this. Samples were diluted with water and the same volume (20 μl) of each sample was injected into the HPLC column using the autosampler. As shown in Table 4.1, there was a linear relationship, ranging between 205 μM to 1.8 mM, between the peak area and the amount of nucleoside applied. In most cases, sample concentrations described in the present study were within this range.

4.3.3 Reproducibility of the results

Analysis of pCSL17 prepared from *E. coli* LE392 was performed quantitatively to determine variations in results both between injections and between preparations. In the case of duplicated injection using the same samples, variation was less than SD$\pm$3 % (data not shown). Four separately prepared samples were injected onto the HPLC column (Table 4.2). The qualitative

Table. 4.2 Nucleosides composition of pCSL17 DNA-derived from *E. coli* LE392: reproducibility of HPLC analysis between preparations.

DNA was isolated from *E. coli* LE392 using alkaline lysis methods (section 2.3.2) and following CsCl-gradient ultracentrifugation (section 2.3.3). DNA (10-50 μg) was incubated with RNase A (200 units, 10 mg/ml) at 37°C for 16 h and subsequently subjected to enzymatic digestion (nuclease P1 and BAP).

N [a]	Mole% [b]					
	dCyd	m^5dCyd	dGuo	Thd	dAdo	m^6dAdo
1	29.36	0.09	26.01	21.76	22.46	0.40
2	28.39	0.09	27.06	22.35	21.78	0.38
3	25.75	0.21	25.70	23.28	24.03	0.79
4	25.65	0.25	26.00	24.55	22.70	0.88
Average	27.29	0.16	26.19	22.99	22.74	0.61
Standard deviation	±1.88	±0.08	±0.60	±1.22	±0.93	±0.26

a N is the serial number of the experiment

b See Fig. 4.8 for abbreviations. Each value is a percentage of the total base content.

nucleosides profiles obtained in the four analyses were consistent with little quantitative variation between samples.

Often, because both RNA and DNA bases were in the sample, it was difficult to quantify m^5dCyd which eluted coincidently with Guo. Therefore excess amounts of RNase A (10 mg/ml) were added into DNA solutions after CsCl-gradient ultracentrifugation in order to decrease the amount of RNA bases contaminating the DNA sample. After EtOH precipitation, DNA samples were treated using the procedure described in section 2.5.2.1, and injected onto the HPLC column. The presence of m^5dCyd was also confirmed as follows. A Varian 9065 UV-VIS detector was used to scan eluents across a range of wavelengths from 220 to 320 nm, to differentiate nucleosides with similar elution volumes and identify m^5dCyd. The HPLC chromatogram was obtained with a detector setting at 254 nm. After running the HPLC, new HPLC chromatograms were generated at different wavelengths from information stored during scanning. After scanning, various wavelengths were selected and their chromatograms obtained at 224, 234, 244, 254, 268, 282, and 292 nm. These chromatograms showed variations in peak heights for each nucleoside (Table 4.3). For example, at 254 nm wavelength, the peak height of m^5dCyd and Guo were 2.4 and 10.4 cm respectively and at 292 nm, peak height of m^5dCyd and Guo changed to 9.5 and 2.8 cm, respectively reflecting the difference in their absorption optimum. Differences in the peak heights of two nucleosides were measured at 254 and 292 nm, and the presence and amount of methylated cytidine (m^5dCyd) was determined.

Analyses of nucleoside bases found in *C. glutamicum* and related species are shown in Fig. 4.9 (a typical chromatogram) and Table 4.4 (a summary of the mole % for each base). The mole% G+C contents were found to be in reasonable agreement with published values for *C. glutamicum* ATCC 13032 (54.6%), *B. lactofermentum* DSM 20412 (54.8%) and *B. flavum* DSM 20411 (54.4%) (Liebl *et al.*, 1991). Chromosomal and plasmid (pCSL17) DNA from *C. glutamicum*

Table 4.3 Determination of the relationship between peak heights and wavelengths for RNA- and DNA-derived nucleosides.

Wavelength (nm)	Cyd [a]	Urd	dCyd	m^5dCyd	Guo	dGuo	Thd	Ado	dAdo	m^6dAdo
224	13.2 [b]	5.4	9.5	6.2	2.9	1.6	3.1	1.1	0.3	1.2
234	13.2	6.8	8.9	5.1	8.1	5.0	2.2	2.0	0.9	1.4
244	10.9	12.2	7.2	3.5	13.4	8.4	3.7	4.2	2.0	3.4
254	8.6	13.3	6.1	2.4	10.4	6.5	4.5	4.5	2.3	4.6
268	12.9	12.0	9.8	4.2	6.7	4.2	5.4	3.1	1.6	5.6
282	12.4	3.8	9.8	6.5	5.1	3.2	3.1	0.4	0.2	2.9
292	10.1	-	8.1	9.5	2.8	1.8	1.4	-	-	1.4
320	-	-	-	-	-	-	-	-	-	-

[a] See Fig. 4.8 for abbreviations

[b] Data are from peak heights (cm)

20 μl of sample mixture containing 100 μM of each nucleoside was injected onto the HPLC column and eluted as described in section 2.5.2.2. A dash indicates that no detectable peak at that wavelength was observed.

contained low (0.06%) or undetectable levels of methylated adenosine, with almost identical results obtained for *B. lactofermentum* and *B. flavum*. In contrast, methylated adenosine was readily obtained by HPLC analysis of chromosomal and pCSL17 DNA from *E. coli* LE392, which contained 0.3% and 0.52% methylated adenosine respectively (Fig. 4.9, Table 4.4). Although the level of m^6dAdo is low, the presence of m^6dAdo indicates the presence of MTase with this specificity. For example, although m^6dAdo is only a small percentage of the methylated adenosines (0.06%), it is sufficient to account for methylation of 6 base pairs in pCSL17 (many ENase and MTase recognise 4 or 6 bp) as follows. For *C. glutamicum* DNA, e.g. with a G+C content of 54.6%, there is a 22.7% probability for a randomly chosen base to be an A or a T and a 27.3% chance for it to be a G or a C. If the recognition sequence contains one A, such as xxATxx (x = G or C), the probability of appearance of such a sequence is the product of the above values, i.e. 0.227^2 X 0.273^4 = 0.000286 = 0.0286%. The reverse value of this probability yields the average distance separating two such sites: 3,494 base pairs. Using the above calculation, the probability of a sequence which has two A residues in 6 base pairs is 0.227^4 X 0.273^2 = 0.0001979 (= 0.0198%). The reverse of this probability yields the average of separation of two such sites: 5,053 base pairs. If there are three A bases in a 6 bp sequence, such as AAATTT, the probability of a sequence which has three A is 0.227^6 = 0.0001368 (= 0.0137%). Such a sequence could appear in DNA every 7,308 base pairs. The above calculations indicate that, if an adenosine MTase exists in the cells and this methylates adenosine in 6 bp sequences, the amount of methylated adenosine which is required for methylation of MTase recognition sequence is 0.014-0.029%. Since the content of m^6dAdo seen in *C. glutamicum* ATCC 13032 is 0.06, this is enough to account for the methylation of adenosine in MTase which recognise 6 bp. Similarly, the same conclusion could be applied to strain BL1. If adenosine MTase recognises a 5 bp sequence, the probability for one A, two As or three As in the 5 bp sequence are 0.1048, 0.0725, 0.0602%, respectively. If adenosine MTase recognises a 4 bp sequence, the probability of one A and two As in the sequence are 0.384% and 0.266%, respectively.

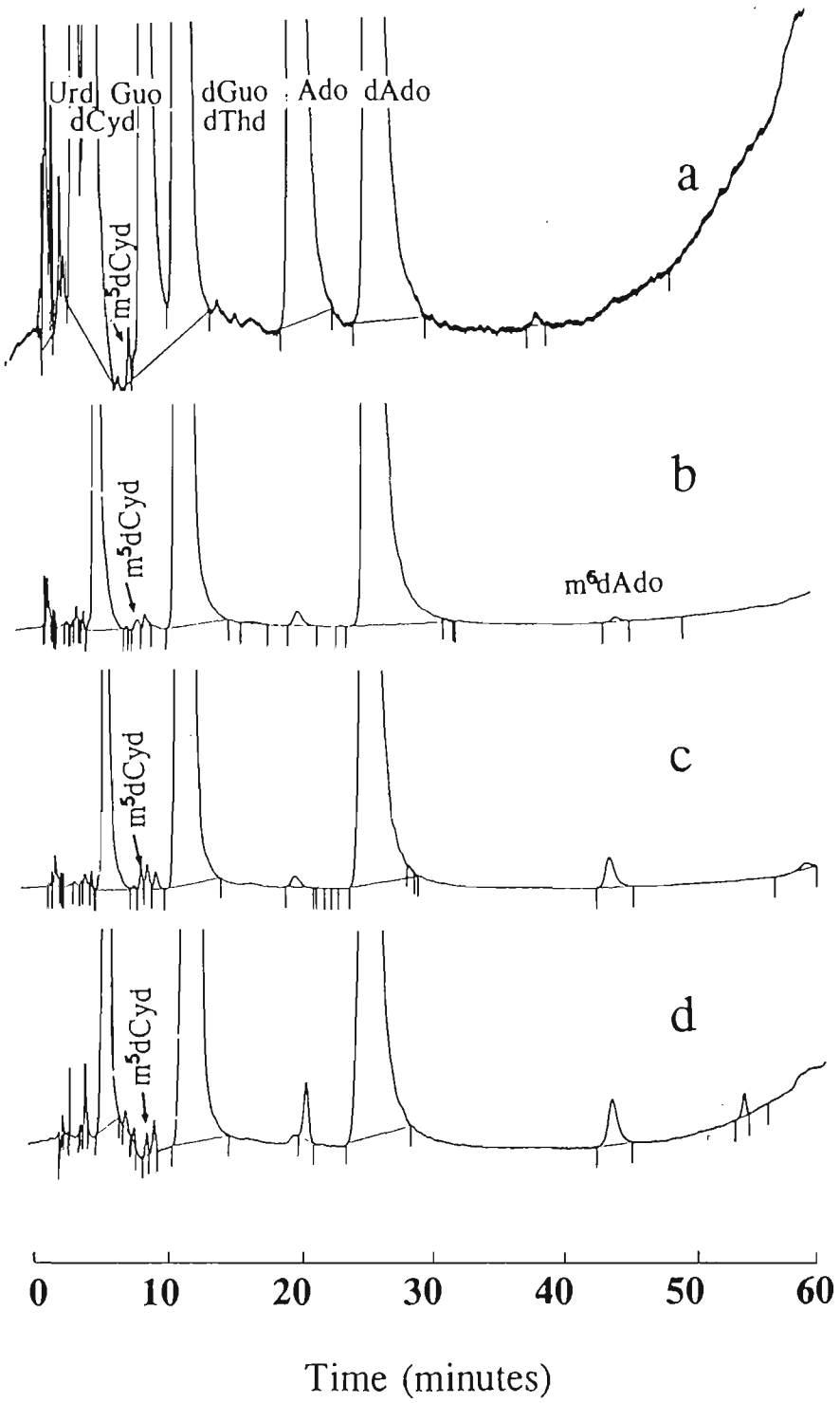


Fig. 4.9 HPLC analysis of *C. glutamicum* and *E. coli* DNA digested with nuclease P1 and BAP. DNA samples of *C. glutamicum* AS019-derived pCSL17 DNA (a), *C. glutamicum* ATCC 13032 chromosomal DNA (b), *E. coli* LE392 chromosomal DNA (c), and *E. coli* LE392-derived pCSL17 DNA (d) were prepared as described in section 2.5.2.1. The eluent was monitored at 254 nm and peaks were identified by their retention time and height at various absorbances. See Fig. 4.8 for abbreviations.

Table. 4.4 The percentage base composition of DNA from various strains of *E. coli* and coryneform bacteria.

Source of DNA	Type of DNA	N [a]	Mole% [b]						G+C
			dCyd	m^5dCyd	dGuo	Thd	dAdo	m^6dAdo	content
C. glutamicum ATCC 13032	Chromosomal DNA	2	28.43	0.17	25.80	22.86	22.50	0.06	54.40
B. lactofermentum BL1	Chromosomal DNA	2	27.43	0.32	27.26	23.01	22.06	0.06	54.98
B. flavum BF4	Chromosomal DNA	2	27.10	0.17	26.35	24.05	22.40	-	53.62
E. coli LE392	Chromosomal DNA	6	27.09	0.12	24.80	24.62	22.74	0.30	52.01
E. coli GM48	Chromosomal DNA	2	27.01	0.78	23.75	24.21	23.80	0.45	51.54
C. glutamicum AS019	pCSL17 DNA	3	27.69	0.47	25.40	23.54	22.86	-	53.56
B. lactofermentum BL1	pCSL17 DNA	2	27.02	0.11	25.40	24.35	23.25	0.05	52.63
E. coli LE392	pCSL17 DNA	6	27.39	0.16	25.41	23.40	23.10	0.52	52.96

a N times tested.

b See Fig. 4.8 for abbreviations. Each value is a percentage of the total base content. A dash indicates that no peak was detected under the conditions used.

Table. 4.5 Theoretical probability of the appearance of target sequences for cytidine MTase in *C. glutamicum* ATCC 13032 [a].

Number of bp recognised by MTase	Number of cytidines in the target sequence	Probability (%)
4	1	0.3840
4	2	0.5554
5	1	0.0872
5	2	0.1261
5	3	0.1516
6	1	0.0199
6	2	0.0286
6	3	0.0414

[a] Calculation are based on DNA G+C contents of 54.6% obtained from published information (Liebl *et al.*, 1991). Probabilities of the appearance of target sequence for cytidine MTase in *C. glutamicum* ATCC 13032 given are based on G or C (27.3%) or A or T (22.7%) as follows. For *C. glutamicum* DNA, e.g., with a G+C content of 54.6%, there is a 22.7% probability for a randomly chosen base to be an A or a T and a 27.3% chance for it to be a G or a C. If the recognition sequence contains one C in 5 base pairs recognised by MTase, such as CxxxG (x = A or T), the probability of appearance of such a sequence is the product of the above values, i.e., $0.227^3 \times 0.273^2 = 0.000872$ (= 0.0872%).

As shown in Table 4.5, *C. glutamicum* ATCC 13032 DNA has a content of m^6dAdo (0.06% of the total base content) which is not enough to account for the methylation of As in the MTase recognition sequence containing 4 bp, which would give a mole% of 0.27-0.38% if this occurred. The possibility that there are more than two adenosine MTases which recognise 6 bp or more than 7 bp cannot be excluded.

In the present work, in contrast to the methylated adenosine (less than 0.05% for all three tested corynebacteria-derived DNA), m^5dCyd was readily detected in the three coryneform strains as well as *E. coli* LE392. Chromosomal and plasmid DNA from *C. glutamicum* contained 0.17% or 0.47% of methylated cytidine. Similar calculations used for adenosine MTase were used for the cytidine MTase (Table 4.5). The level of cytidine methylation seen is consistent with a target sequence of 5 or more than 5 bp.

4.4 DETERMINING METHYLATION SITES IN DNA FROM CORYNEBACTERIA BY RESTRICTION MAPPING

4.4.1 Mapping of pCSL17

pCSL17 DNA is a coryneform bacteria-*E. coli* shuttle vector and could be transformed into coryneform bacteria, including *C. glutamicum*, *B. lactofermentum*, *B. flavum*, and *E. coli* strains. Although this plasmid DNA was constructed using known plasmids (pBR322 and pSR1) and the Tn*903* kanamycin gene, some DNA sequence data was not available (Hodgson *et al.*, 1989). For this reason, various restriction enzymes were used to treat *E. coli*- and *C. glutamicum*-derived pCSL17 DNA to gain more information on this plasmid DNA. Both *E. coli* LE392- and *C. glutamicum*-derived pCSL17 DNA were incubated with more than 20 restriction enzymes, alone

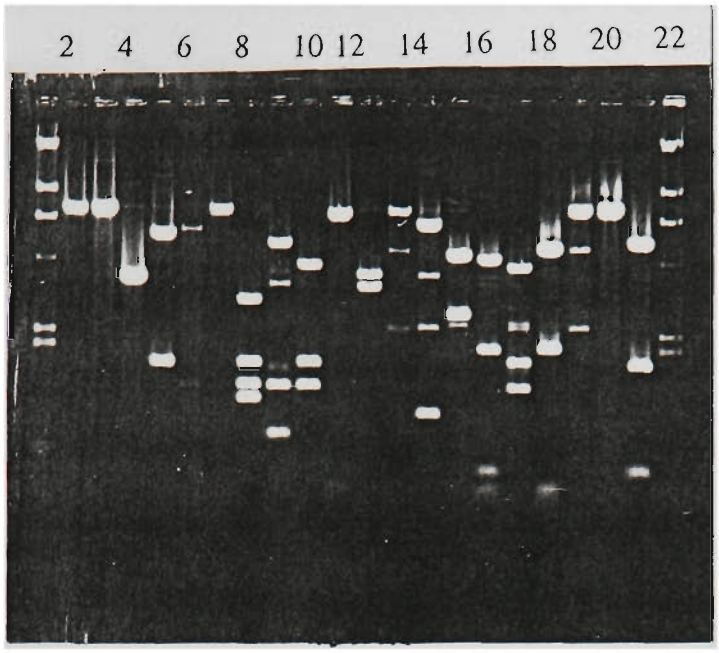


Fig. 4.10 Restriction endonuclease digestion and agarose gel electrophoresis of *E. coli* LE392-derived pCSL17 DNA

Reaction mixtures (30 μl) contained 3 μg of DNA and 3 μl of One-Phor-All buffer or buffers recommended by manufacturers for specific enzymes and 5-10 units of restriction endonuclease, incubating for 3 h at 37°C. For double digestions by restriction enzymes, reactions for first enzyme were terminated by heat-inactivation at 65°C for 10 min, cooled to room temperature then incubated further after adding the second enzyme, as described in the section 2.3.6. For each gel well, 15 μl (out of 36 μl) of the mixture was loaded. For lanes 2 to 21, the following restriction enzymes were used. Lanes 1 and 22 contained λDNA digested with *Hind*III.

Lane	Restriction endonuclease	Lane	Restriction endonuclease
2	*Bgl*II	12	*Xba*I/*Pst*I
3	*Pst*I	13	*Cla*I
4	*Bgl*II/*Pst*I	14	*Cla*I/*Pst*I
5	*Bam*HI	15	*Cla*I/*Bgl*II
6	*Eco*RI	16	*Cla*I/*Hind*III
7	*Xba*I	17	*Cla*I/*Eco*RI
8	*Eco*RI/*Bam*HI	18	*Hind*III
9	*Eco*RI/*Pst*I	19	*Cla*I/*Eco*RV
10	*Eco*RI/*Xba*I	20	*Pst*I/*Eco*RV
11	*Xba*I/*Bgl*II	21	*Bam*HI/*Pst*I

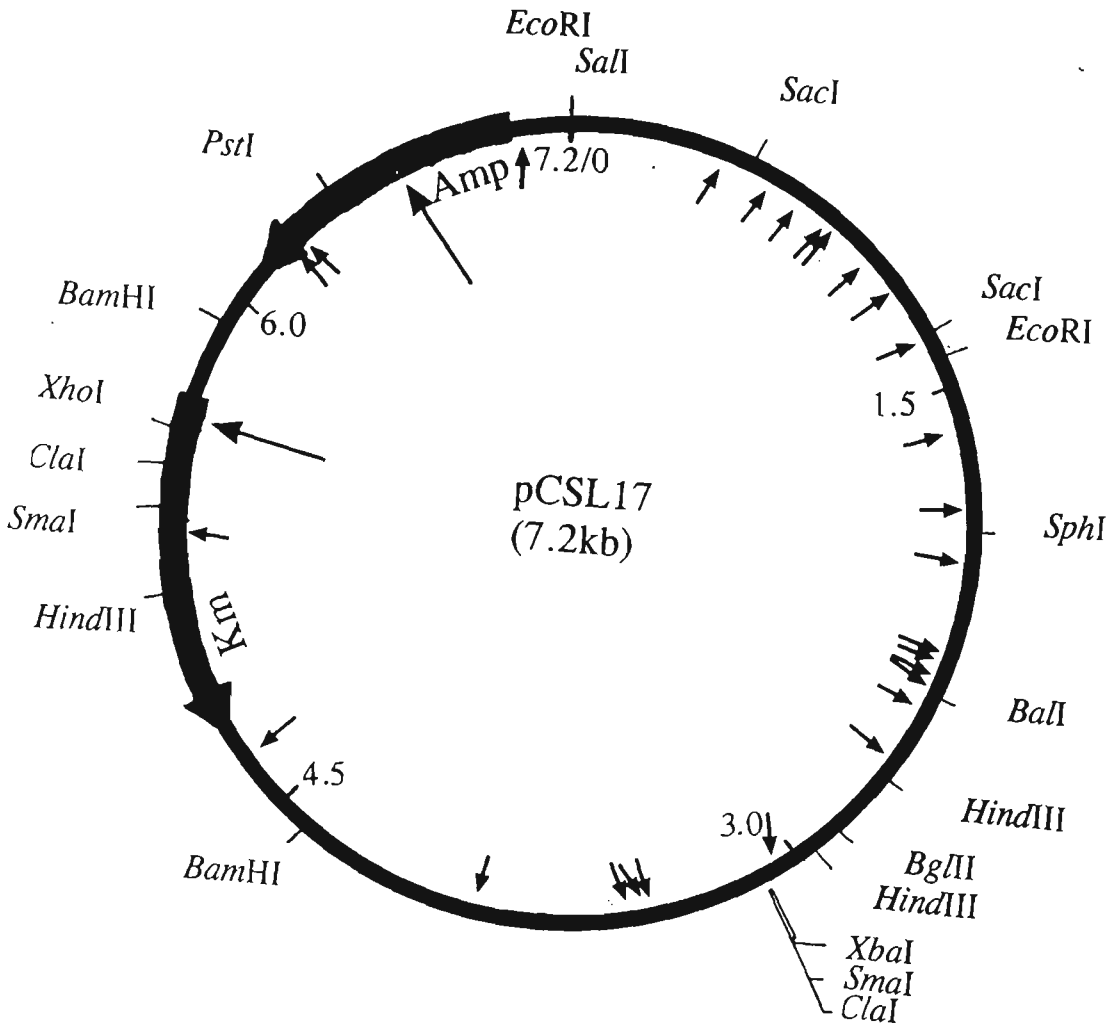


Fig. 4.11 Restriction map of the *E. coli-C. glutamicum* shuttle vector pCSL17. *Hae*III sites are indicated by arrows, based on restriction mapping and known sequences in the components of the plasmid. Large arrows indicate *Hae*III sites which are not cleaved by *Hae*III when DNA is prepared from *C. glutamicum* and *B. flavum*.

or in combinations, and some of the results from these digestions are shown in Fig. 4.10. The resulting restriction enzyme map of pCSL17 DNA is shown in the Fig. 4.11, highlighting enzyme sites relevant to this study. The following restriction enzymes did not cleave pCSL17 DNA: *Kpn*I, *Eco*RV. DNA was linearised with the following enzymes: *Pst*I, *Bgl*II, *Sal*I, *Sph*I, *Xba*I.

4.4.2 Restriction enzyme digestion of pCSL17 DNA to detect methylated sites

When pCSL17 was being mapped, it was observed that different cleavage patterns occurred depending on whether the plasmid was isolated from *E. coli* or *C. glutamicum*. Because one possible cause of this observation was differential methylation at these cleavage sites, pCSL17 from coryneform bacteria and *E. coli* backgrounds were digested with enzymes which either require specific methylation in the cleavage site or were inhibited by specific methylation.

4.4.2.1 Enzymatic digestion at the sequence of ATCGAT (*Cla*I site)

E. coli LE392 and coryneform bacteria-derived pCSL17 DNA showed different restriction enzyme cleavage patterns with *Cla*I (see lanes 7-9 in Fig. 4.12A and lanes 14-19 in Fig. 4.13). pCSL17 has two *Cla*I cleavage sites (ATCGAT); however, one of these sites overlaps with a Dam MTase recognition site (GATC) such that the two share an adenosine which is methylated by Dam MTase (ATCG^{m}ATC). When the shared adenosine is methylated, *Cla*I will not be cleave at this site. Thus pCSL17 derived from *E. coli* LE392, which is Dam^{+}, is cut only once by *Cla*I. However, pCSL17-derived from the three coryneform bacteria and two mutant strains of *C. glutamicum* strains, MLB133 and MLB194, was found to be cleaved at both recognition sites with no partial digests (Fig. 4.12 and Fig. 4.13), suggesting that coryneform bacteria do not methylate adenosine residues in DNA, at least at the *Cla*I cleavage site.

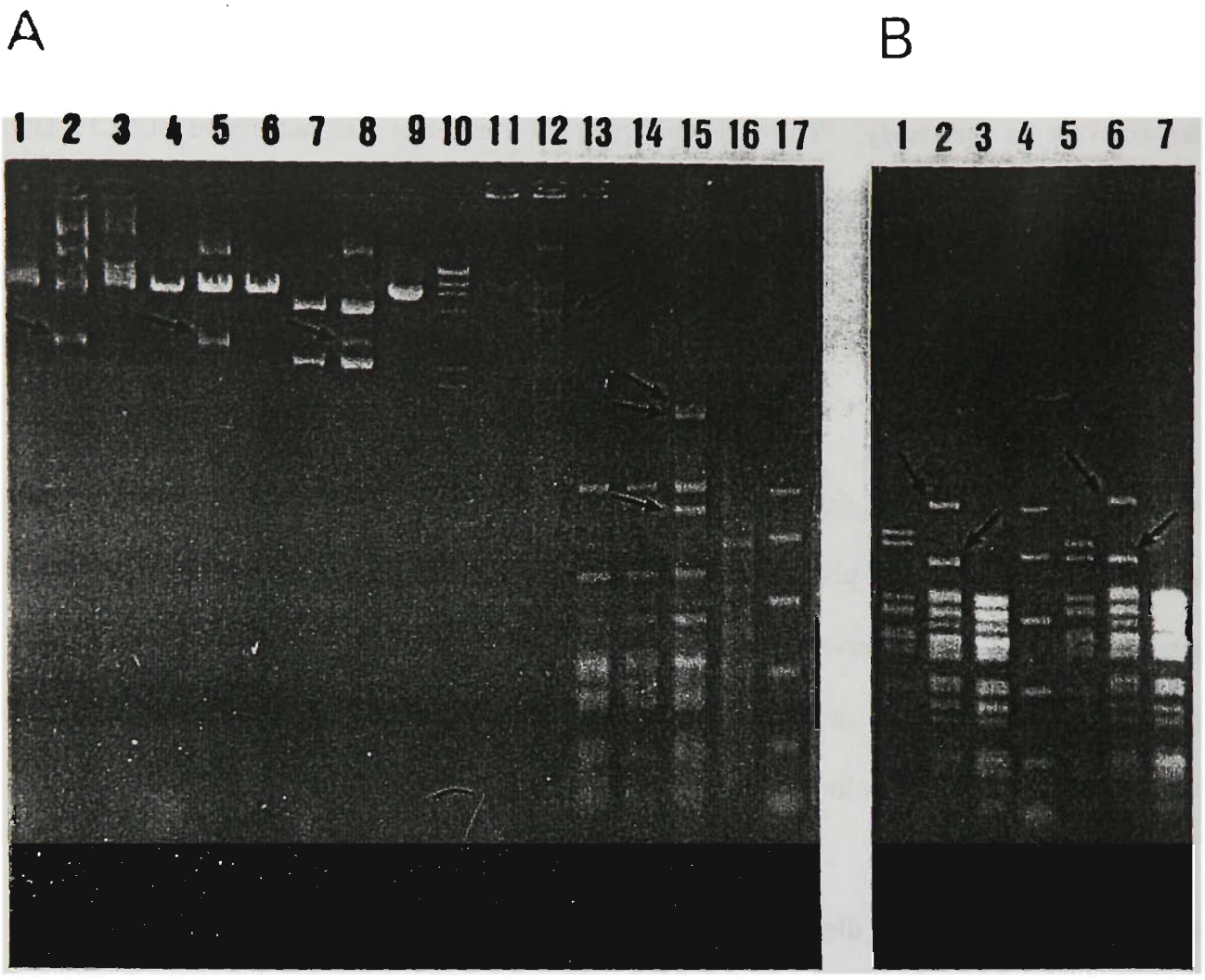


Fig. 4.12 Agarose gel electrophoresis of corynebacteria- and *E. coli*-derived pCSL17 DNA treated with various restriction endonucleases. pCSL17 was purified from *C. glutamicum* AS019, *B. lactofermentum* BL1 and *E. coli* LE392, which appear in the same order for the different digestions with ENases. Two sets of experiments (**A** and **B**) were performed separately and results of these are shown here. (A) Lanes: 1-3, undigested; 4-6, digested with *Pst*I; 7-9, *Cla*I; 10, λ DNA digested with *Hind*III; 11-13, *Dpn*I; 14-16, *Dpn*II; 17, PCR size markers. **(B)** Lanes: 1-3, pCSL17 DNA with *Hae*III; 4, PCR size markers; 5-7, *Pst*I plus *Hae*III. PCR size markers contained the following DNA fragments: 50, 150, 300, 500, 750, 1000 bp. Arrows indicate the endogenous *B. lactofermentum* plasmid, pBL1 (Martin *et al.*, 1987), which was purified with pCSL17 in DNA extracts, and digestion fragments of this.

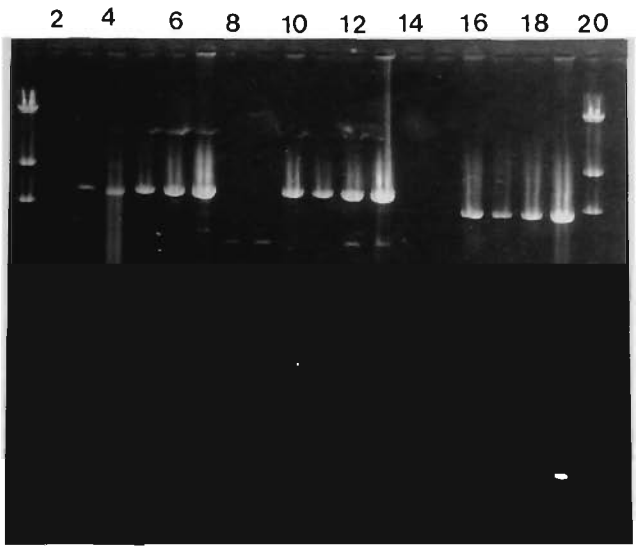


Fig. 4.13 Agarose gel electrophoresis of *C. glutamicum*- and *E. coli*-derived pCSL17 DNA treated with various restriction endonucleases. *C. glutamicum* MLB133, *C. glutamicum* MLB194, *E. coli* ED8654, *E. coli* RR1, *E. coli* HB101, and *E. coli* N4830, which appear in the same order for the different digests, were digested with restriction endonucleases. Lanes: 1 and 20, λ DNA digested with *Hind*III; 2-7, *Pst*I; 8-13, *Cla*I; 14-19, *Cla*I plus *Pst*I.

4.4.2.2 Enzymatic digestion of the sequence GATC

pCSL17 DNA derived from the three coryneform bacteria and from *E. coli* LE392 was digested with the *Dpn*I and its isoschizomers, *Dpn*II, *Sau*3AI, and *Mbo*I. These four enzymes recognise and cleave the sequence GATC (Fig. 4.12 and Fig. 4.14). However, *Dpn*I activity requires that adenosine in the recognition sequence is methylated, whereas the other three enzymes do not require this. *Dpn*I will not cleave at GATC sequences when the adenosine is unmethylated or when the cytidine is methylated. *Mbo*I and *Dpn*II will not cleaved at this site when the adenosine is methylated. Unlike these restriction enzymes, *Sau*3AI will not cleave GATC sequences when the cytidine is methylated. Results shown in Fig. 4.15 indicate that corynebacteria-derived pCSL17 was cleaved by *Dpn*II, *Mbo*I, and *Sau*3AI but not by *Dpn*I, whilst *E. coli*-derived pCSL17 was cleaved by *Dpn*I and *Sau*3AI but not by *Dpn*II and *Mbo*I. These products were also examined by high-resolution polyacrylamide gel electrophoresis to resolve the low-molecular-weight fragments (see section 2.3.8) (Fig. 4.14). The restriction patterns of *Dpn*II, *Mbo*I, and *Sau*3AI were identical.

It was also found that chromosomal DNA from corynebacteria cannot be cleaved by *Dpn*I but is cleaved by *Dpn*II. Other than *Dpn*II, 19 restriction enzymes cleaved chromosomal DNA from *C. glutamicum* (Fig. 4.15). Similarly, chromosomal DNAs from *C. glutamicum* strains RM3 and RM4 were not cleaved by *Dpn*I (data not shown). These data indicate that these strains of corynebacteria do not methylate adenosines in GATC sequences. In contrast, these data indicate that *E. coli* LE392 contained methylated adenosine in GATC sequences. Similar results were also obtained when pCSL17 DNA was derived from *E. coli* ED8654, RR1, N4830, and HB101 and digested with these enzymes (data not shown).

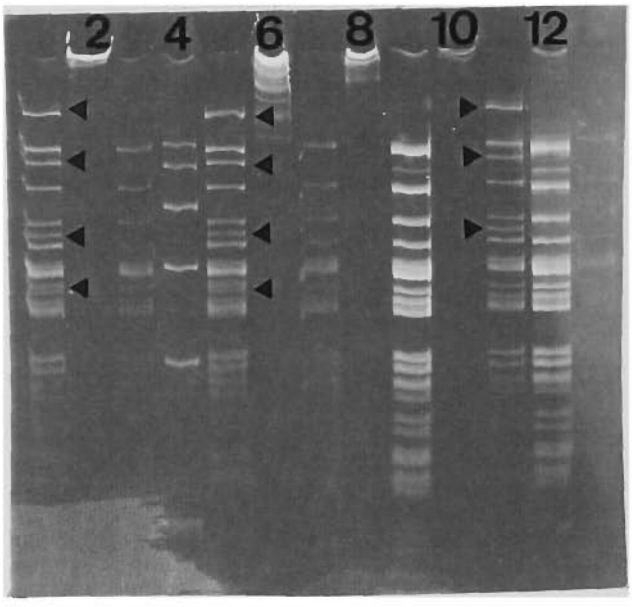


Fig. 4.14 Polyacrylamide slab gel electrophoresis of corynebacteria- and *E. coli*-derived pCSL17 digested with four restriction endonucleases. pCSL17 was purified from *B. lactofermentum* BL1, *E. coli* LE392, and *C. glutamicum* AS019, which appear in the same order for the different digestions with ENases. Lanes: 1-3, digested with *Mbo*I; 4, PCR size markers (50, 150, 300, 500, 750, 1000 bp); 5-7, digested with *Dpn*II, 8-10, digested with *Dpn*I, 11-13, digested with *Sau*3AI. DNA fragments indicated by arrows are from the endogenous *B. lactofermentum* plasmid, pBL1 (Martin *et al.*, 1987).

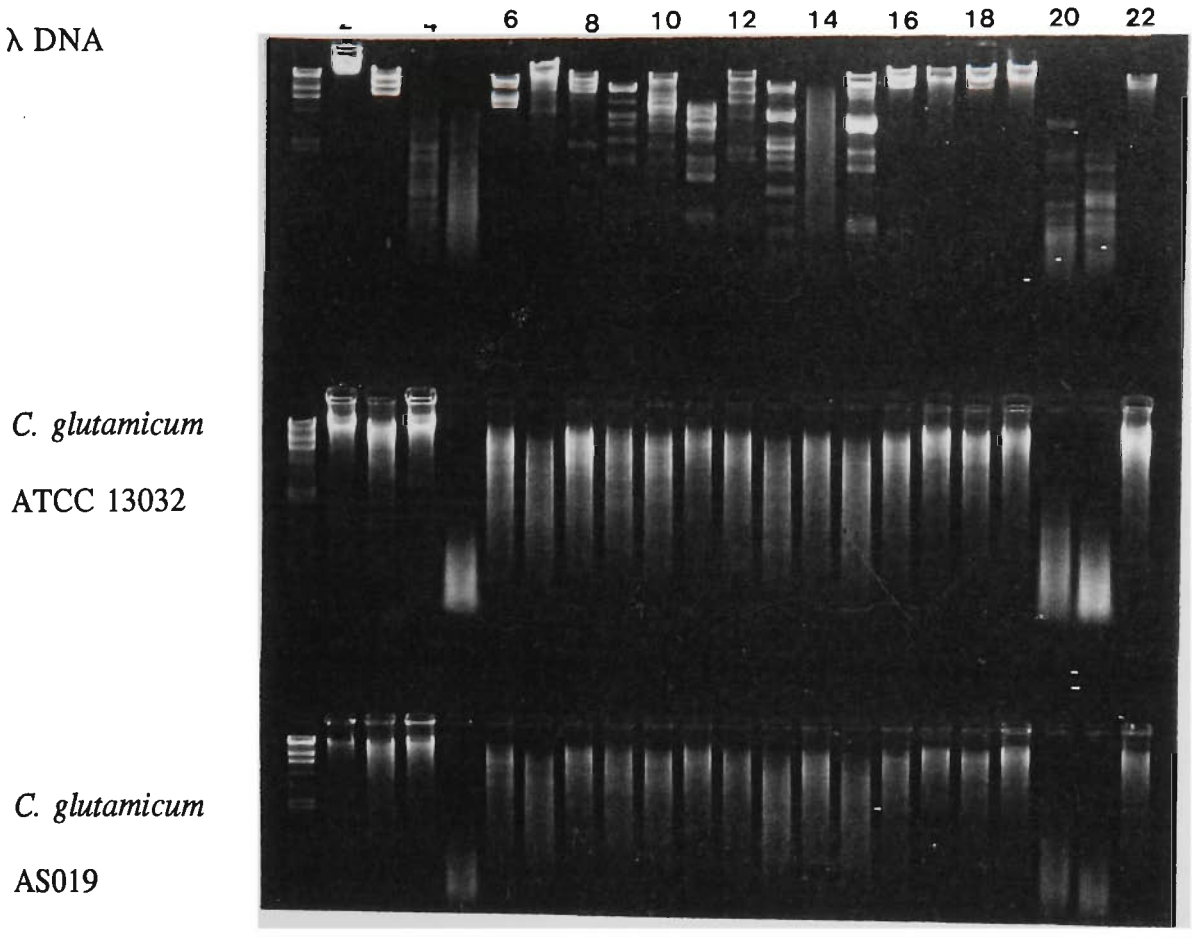


Fig. 4.15 Restriction endonuclease digestion and agarose gel electrophoresis of chromosomal DNA and λDNA from two strains of *C. glutamicum*. Reaction mixtures (30 μl) contained 3 μg of DNA and 3 μl of One-Phor-All buffer or buffer recommended by manufacturers and 5-10 units of restriction endonuclease, incubating for 3 h at 37°C (or 25°C for *Sma*I or 50°C for *Bcl*I). Restriction digestion was carried out as described in the section 2.3.6. Enzymes reactions were terminated by heat-inactivation at 65°C for 10 min. For each gel well, 15 μl (out of 36 μl) of the mixture was loaded. Lane 1 contained λDNA digested with *Hind*III. In this experiment, *Pvu*I did not work. Following restriction enzymes were used.

Lane	Restriction endonuclease	Lane	Restriction endonuclease
2	undigested DNA	12	*Hind*III
3	*Sma*I	13	*Pst*I
4	*Dpn*I	14	*Pvu*I
5	*Dpn*II	15	*Pvu*II
6	*Bam*HI	16	*Sal*I
7	*Bcl*I	17	*Xba*I
8	*Bgl*II	18	*Xho*I
9	*Cla*I	19	*Spe*I
10	*Eco*RI	20	*Hae*III
11	*Eco*RV	21	*Sau*3AI
		22	*Kpn*I

4.4.2.3 Enzymatic digestion of the sequence CCGG

pCSL17 plasmid DNA derived from *E. coli* LE392 and three corynebacteria strains were also analysed using *Hpa*II and *Msp*I digests (Fig. 4.16). These two restriction endonucleases are isoschizomers which recognise and cleave CCGG nucleoside sequences. However, *Hpa*II will not cleave this sequence if cytidine is methylated at either the 4 or 5 position and *Msp*I will not cleave when the first cytidine is methylated in the 5 position. Both enzymes had several cutting sites in pCSL17 DNA and the digestion patterns for both enzymes using coryneform bacteria- and *E. coli*-derived pCSL17 DNA were identical, indicating that the CCGG sequence is not a target for methylation by the presumptive MTase in the coryneform bacteria strains examined.

4.4.2.4 Enzymatic digestion of the sequence *McrBC*

McrBC is a methylation-dependent ENase which recognise the sequence $Pu^{m}C(N_{40\text{-}2,000})Pu^{m}C$ (where Pu indicates A and G bases and N indicates either G, C, T, or A) and acts upon DNA containing methylcytidine on one or both strands of the DNA. *McrBC* will not act on unmethylated DNA. According to the supplier's notes (Biolabs), the enzyme makes one cut between each pair of half-sites and cleavage positions are distributed over several base pairs approximately 30 bp from the methylated base. Therefore the enzyme does not produce defined DNA ends upon cleavage. Also, when multiple target sites are present in DNA, the flexible nature of the recognition sequence results in an overlap of sites so that a smeared, rather than a sharp banding pattern, is produced.

pCSL17 plasmid DNA derived from *E. coli* LE392 and three corynebacteria strains were analysed by *McrBC* digestion (the plasmid DNAs used here were contaminated with small portions of their chromosomal DNA). Chromosomal DNA derived from *E. coli* LE392 and *C. glutamicum* AS019

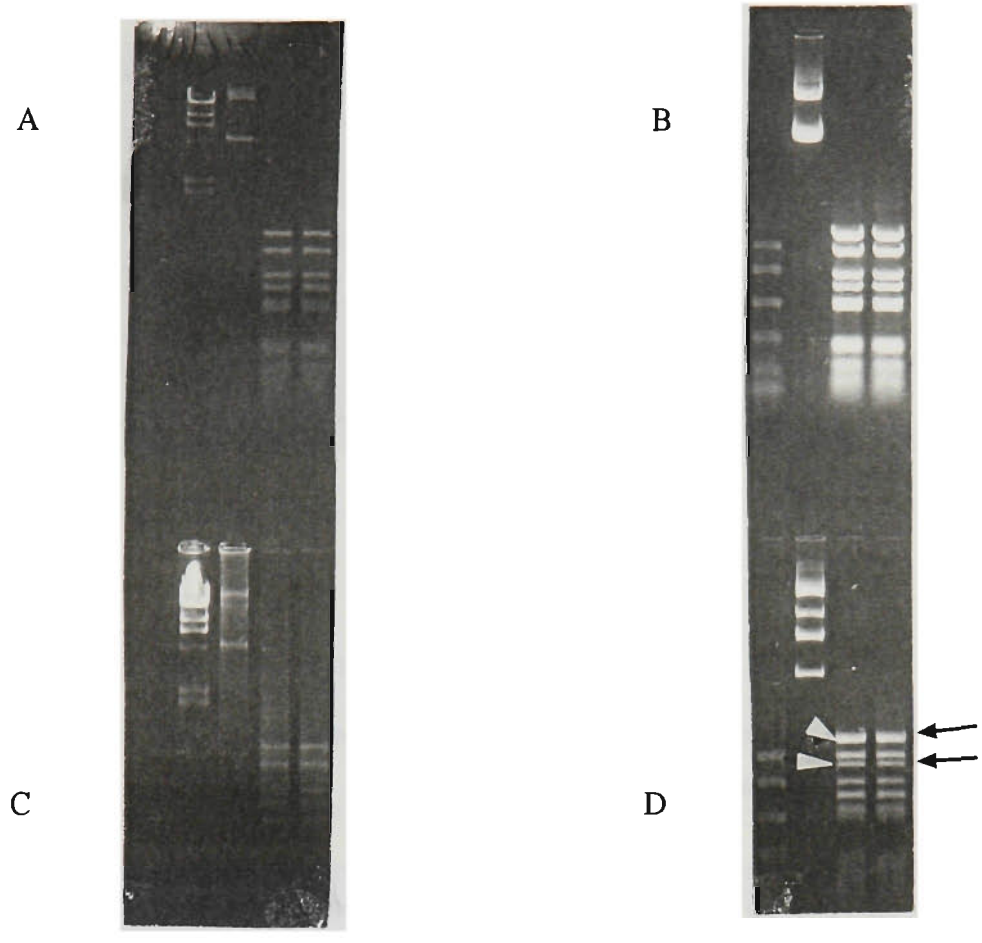


Fig. 4.16 Agarose gel electrophoresis of corynebacteria- and *E. coli*-derived pCSL17 DNA treated with various restriction endonucleases. **(A)** *C. glutamicum* AS019; **(B)** *E. coli* LE392; **(C)** *B. flavum* BF4; **(D)** *B. lactofermentum* BL1. For each panel, the order of lanes are as follows: Lane: 1, size markers (for **A** and **C**, λ DNA digested with *Hind*III, and for **B** and **D**, PCR size markers); 2, undigested plasmid; 3, digested with *Hpa*II; 4, *Msp*I. PCR size markers contained the following DNA fragments: 50, 150, 300, 500, 750, 1000 bp. Arrows indicate the endogenous *B. lactofermentum* plasmid, pBL1 (Martin *et al.*, 1987), which is purified with pCSL17 in DNA extracts, and digestion fragments of this.

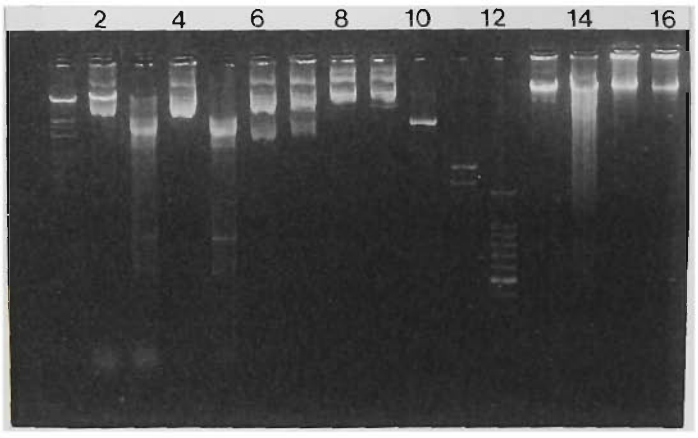


Fig. 4.17 Agarose gel electrophoresis of three corynebacteria- and *E. coli*-derived pCSL17 DNA treated with *McrBC* restriction endonucleases.

Two different size markers were used, λ DNA digested with *Eco*RI/*Hind*III for lane 1, and PCR marker for lane 12 (100 bp DNA Ladder, Promega, used here consisted of 11 fragments with sizes of 100, 200, 300, 400, 500, 600, 700, 800, 900, 1,000, 1,500 bp).

Lane	Strain	Source of DNA	*McrBC* enzyme [a]
2	*C. glutamicum* AS019	pCSL17 DNA	No
3	*C. glutamicum* AS019	pCSL17 DNA	Yes
4	*B. flavum* BF4	pCSL17	No
5	*B. flavum* BF4	pCSL17	Yes
6	*B. lactofermentum* BL1	pCSL17	No
7	*B. lactofermentum* BL1	pCSL17	Yes
8	*E. coli* LE392	pCSL17	No
9	*E. coli* LE392	pCSL17	Yes
10		methylated control plasmid DNA	No
11		methylated control plasmid DNA	Yes
12			
13	*C. glutamicum* AS019	chromosomal DNA	No
14	*C. glutamicum* AS019	chromosomal DNA	Yes
15	*E. coli* LE392	chromosomal DNA	No
16	*E. coli* LE392	chromosomal DNA	Yes

[a] Yes, the *McrBC* enzyme was present in the reaction sample; No, the *McrBC* enzyme was not present in the sample.

were also tested. For comparison, a 4.3 kb linear, methylated control plasmid DNA was obtained from the supplier (Biolabs) and also used as a positive control for *McrBC* digestion. The control DNA is known to produce several fragments of between approximately 700 bp and 2.3 kb following *McrBC* digestion. *McrBC* cleaved both plasmid DNA- and chromosomal DNA-derived from *C. glutamicum* AS019 (lanes 2 and 14) and *B. flavum* BF4 (lane 4), and the control DNA (lane 11) (Fig. 4.17). However, the ENase failed to cleave DNA-derived from *B. lactofermentum* BL1 and *E. coli* LE392. These data confirmed that *C. glutamicum* and *B. flavum* contain methylated cytidines in the target sequence of *McrBC* sequences in both plasmid and chromosomal DNA but that BL1 contains a different methylation pattern.

4.4.2.5 Enzymatic digestion of the sequence GGCC (*Hae*III site)

pCSL17 DNA derived from *E. coli* LE392, *C. glutamicum* AS019, *B. lactofermentum* BL1, and *B. flavum* BF4 were analysed by *Hae*III digestion (data for the first three strains shown in Fig. 4.12 and data for the *B. flavum* BF4 is shown in Fig. 4.18). The target sequence for *Hae*III is GGCC; however, this sequence is not cleaved when the first cytidine is methylated ($GG^{m}CC$). In the case of pCSL17 purification from *B. lactofermentum* BL1, the natural plasmid, pBL1 (4.45 kb) was purified with pCSL17 in DNA extracts. According to published sequence information, pBL1 DNA has nine *Hae*III sites and digestion with this enzyme could produce fragments of the following sizes: 1,020, 733, 726, 594, 414, 384, 321, 183, 77 bp (Martin *et al.*, 1987). The banding patterns in the resulting gels indicated that *C. glutamicum*-derived plasmid DNA was cleaved at fewer sites than *E. coli*- or *B. lactofermentum*-derived plasmids, since there are two large bands (0.87 and 0.81 kb), and fewer small bands in digests of the *C. glutamicum*-derived plasmid. Inhibition of cleavage of other sites may have occurred also, but the fragment sizes would have been too small to resolve by the agarose gel electrophoresis system used. *B. flavum*- and *C. glutamicum*-derived pCSL17 DNA showed identical *Hae*III digestion patterns.

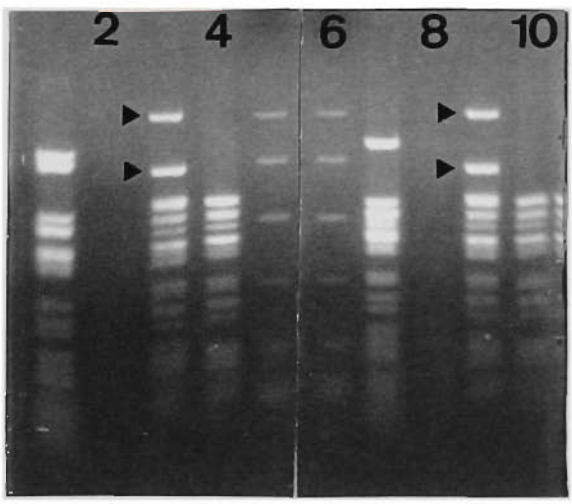


Fig. 4.18 Agarose gel electrophoresis of corynebacteria- and *E. coli*-derived pCSL17 DNA treated with *Hae*III and *Xho*1. pCSL17 purified from *C. glutamicum* AS019, *B. lactofermentum* BL1, and *E. coli* LE392, which appear in the same order for the different digests, were digested with restriction endonucleases. Lanes: 1, 3, 4, pCSL17 with *Hae*III; 5, PCR size markers (50, 150, 300, 500, 750, 1000 bp); 6, PCR size markers; 7, 9, 10, pCSL17 with *Hae*III plus *Xho*I. Arrows indicate the endogenous *B. lactofermentum* plasmid, pBL1 (Martin *et al.*, 1987), which is purified with pCSL17 in DNA extracts, and digestion fragments of this.

These data suggest that *C. glutamicum* and *B. flavum* contain a cytidine MTase which specifically methylates the first cytidine in the GGCC sequence. In theory, prior methylation of this sequence using *Hae*III MTase should improve transformation frequencies of heterologously-derived DNA into *C. glutamicum*, as this would protect incoming DNA from endogenous ENases. An experiment based on this approach is described in section 4.4.3.

However, several of the *Hae*III sites in pCSL17 (identified during mapping of the plasmid or from published sequence of components of pCSL17) were still cleaved, indicating that not all possible *Hae*III cutting sites were methylated and that the recognition sequence of the presumptive MTase was not identical to the *Hae*III cutting site. The relative positions of the two observed *Hae*III sites which remain uncut in the *C. glutamicum*- or *B. flavum*-derived pCSL17 DNA were located by further restriction analyses, based on the known activity of restriction enzymes which either linearise pCSL17 (*Pst*I, *Bgl*II, *Sal*I, *Sph*I and *Xba*I) or produce two fragments (*Cla*I, *Sma*I, *Bam*HI and *Hind*III). For example, when *C. glutamicum*-derived DNA was digested with *Hae*III and subsequently digested with *Pst*I, the largest band (0.87 kb) was changed to 0.74 kb. This indicated that the largest band contains a *Pst*I site (Fig. 4.12). Similarly, when *Pst*I was replaced by *Cla*I or *Xho*I, the second largest band (0.81 kb) reduced to a smaller fragment (data for the *Xho*I digestion in coryneform bacteria strains AS019, BL1 and *E. coli* LE392 shown in the Fig. 4.18). The mapped locations of these two *Hae*III sites are indicated by the large arrows in Fig. 4.11. Published sequence data for the components of pCSL17 indicated that both of the *Hae*III sites which remain uncut when DNA is extracted from *C. glutamicum* or *B. flavum* contain the sequence GGCCGC, which was confirmed for pCSL17 by directly sequencing around these sites (DNA sequencing result of pCSL17 DNA contained the second largest band [0.81 kb] is seen in Fig. 4.19). From the above data, it was concluded that one site of modification in *C. glutamicum* and *B. flavum* involved recognition and methylation in the GC(G/C)GC sequence.

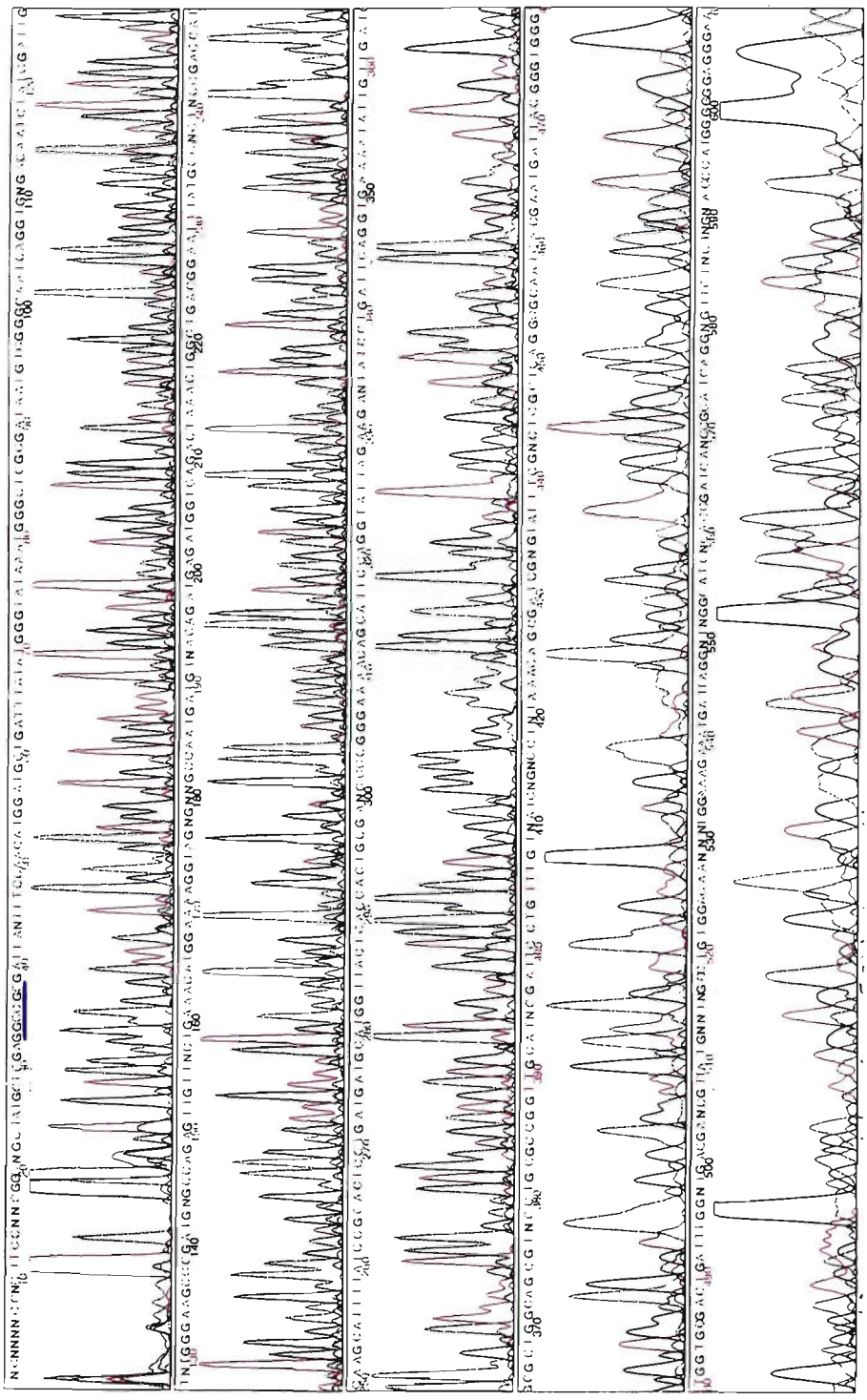


Fig. 4.19 Automated sequencing results of some sequence of pCSL17 DNA derived from *C. glutamicum* AS019 containing *Hae*III sequences. The primer used for this experiment is as follows. The target sequence (GGCCGC) is underlined and located between 32-35.

Primer : GTT ATG AGC CAT ATT CAA CGG GAA (24bp)

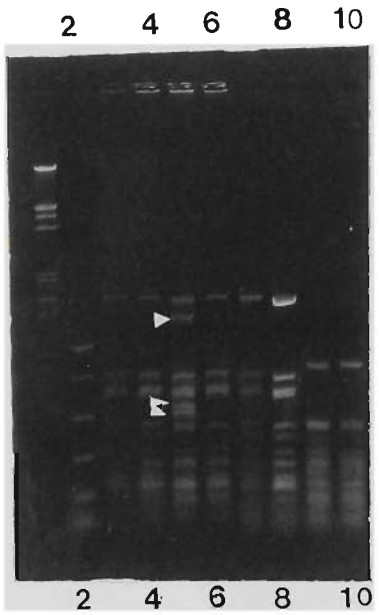


Fig. 4.20 Agarose gel electrophoresis of corynebacteria- and *E. coli*-derived pCSL17 DNA treated with *Tse*I and *Fnu*4H1. pCSL17 purified from *C. glutamicum* AS019, *B. flavum* BF4, *B. lactofermentum* BL1, and *E. coli* LE392, which appear in the same order for the different digests, were digested with restriction endonucleases. Lanes: 1, λ DNA digested with *Eco*R1 and *Hind*III; 2, PCR size markers (50, 150, 300, 500, 750, 1,000 bp); 3-6, pCSL17 with *Tse*I; 7-10, *Fnu*4H1. Arrows indicate the endogenous *B. lactofermentum* plasmid, pBL1 (Martin *et al.*, 1987), which is purified with pCSL17 in DNA extracts, and digestion fragments of this.

4.4.2.6 Enzymatic digestion of the sequence GCNGC

Recently (in 1996), *Tse*I became available from commercial sources (Biolabs). The target sequence for *Tse*I is GCWGC (where, W is either A or T). *Tse*I will cleave at GC(A/T)GC but not at GC(C/G)GC. Another restriction enzyme, *Fnu*4H1, recognises the DNA sequence GCNGC (where N is either A, T, G, or C) (Biolabs). *Fnu*4H1 will cleave at both GC(C/G)GC when the cytidine is not methylated and GC(A/T)GC. Fig. 4.20 shows that both enzymes had multiple cutting sites in pCSL17 and the digestion patterns for *Tse*I using corynebacteria- and *E. coli*-derived pCSL17 were identical, whilst *Fnu*4H1 digestion in *B. lactofermentum*- and *E. coli*-derived pCSL17 gave more fragments than that of *Tse*I. This suggests that all of GCCGC and GCGGC sequences in *C. glutamicum* derived DNA were methylated, so that this sequences of DNA was protected from *Fnu*4H1 digestion.

4.4.3 Effect of methylation of heterologously-derived DNA using *Hae*III MTase on transformation frequency into *C. glutamicum*

E. coli LE392-derived pCSL17 DNA was methylated in GGCC sequences by incubating DNA with *Hae*III MTase. *Hae*III MTase methylates at the first cytidine base in this sequence. Successful methylation of DNA was checked by treating *Hae*III MTase-treated DNA with *Hae*III restriction endonuclease (Fig. 4.21). Methylated DNA was also incubated with *Hind*III endonuclease, in order to test whether the methylated DNA could be cleaved by another restriction enzyme (see lanes 6 and 7 in Fig. 4.21B). The two sources of pCSL17 DNA (derived from *E. coli* and *Hae*III methylated DNA derived from *E. coli*) were cleaved by *Hind*III. *Hae*III digestion cleaved the *E. coli*-derived DNA, but could not cleave the *Hae*III MTase methylated *E. coli*-derived pCSL17 DNA (Fig. 4.21), indicating that *Hae*III MTase methylation was successful. This

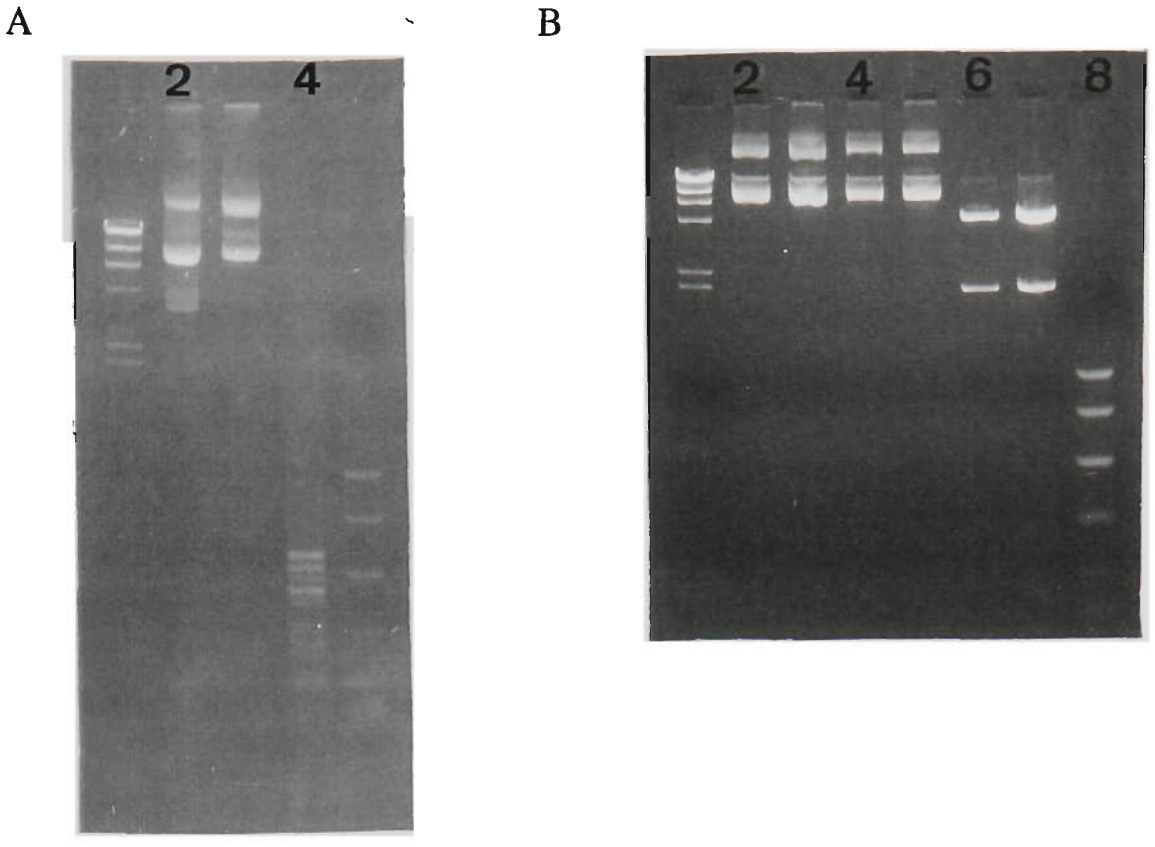


Fig. 4.21 Agarose gel electrophoresis of corynebacteria- and *E. coli*-derived pCSL17 DNA treated with *Hae*III. PCR size markers contained the following DNA fragments: 50, 150, 300, 500, 750, 1,000 bp. For **(B)**, two different batches of *E. coli*-derived pCSL17 DNA were treated with *Hae*III or *Hind*III.

For **(A)**

Lane	Description
1	λ DNA digested with *Hind*III
2	*E. coli* LE392-derived pCSL17 DNA
3	*Hae*III methylated *E. coli*-derived pCSL17 DNA, digested with *Hae*III
4	*E. coli*-derived pCSL17 DNA, digested with *Hae*III
5	PCR size markers

For **(B)**

1	λDNA digested with *Hind*III
2-3	*E. coli* LE392-derived pCSL17 DNA
4-5	*Hae*III methylated *E. coli*-derived pCSL17, digested with *Hae*III
6-7	*Hae*III methylated *E. coli*-derived pCSL17, digested with *Hind*III

DNA was electrotransformed into two strains of *C. glutamicum*, AS019 and ATCC 13032. Previously, these strains showed very low transformation frequencies for heterologously-derived DNA (see section 3.3.4 for transformation frequencies of these strains). For comparison, two source of DNAs (pCSL17 DNA derived from *E. coli* and *C. glutamicum* AS019) were also tested. When methylated, *E. coli*-derived pCSL17 DNA was electroporated into *C. glutamicum* AS019 and transformation frequencies were 1-2.5 X 10^6 (transformants per survivors per μg of DNA), which was four times higher than that seen for *E. coli*-derived pCSL17 DNA (Table 4.6). These results were confirmed by repeating the experiment five times. Similarly, when *C. glutamicum* ATCC 13032 was used as the recipient instead of AS019, transformation frequency increased approximately 4.3-fold (an average of five independent experiments). Therefore, prior methylation using *Hae*III methylation of heterologous DNA did increase transformation frequencies but these were not as high as for homologously-derived DNA. This suggests that other methylation systems may be involved in protecting DNA in *C. glutamicum*.

4.5 CHARACTERISATION OF RESTRICTION BARRIERS IN *B. flavum*

Using corynebacteria-derived plasmid DNA and the same DNA isolated from a range of *E. coli* strains with different RM backgrounds, the presence of restriction barriers in *C. glutamicum* (M. L. Britz, unpublished observation, 1985 and see Table 1.7 in Introduction section 1.6.4) and *B. lactofermentum* (M. L. Britz, unpublished observation; Tauch *et al.*,1994) was studied by means of transformation. In the present work, a similar approach was also applied to *B. flavum* to gain some insight into the methylation patterns in this species. Transformation efficiency of *B. flavum*-derived plasmid DNA was compared with that of *E. coli* LE392-derived plasmid DNA for several different recipient *E. coli* strains with a range of ENase and MTase backgrounds.

Table. 4.6 The effect of *Hae*III methylation of *E. coli*-derived plasmid DNA on transformation frequency of *C. glutamicum*.

Cells of *C. glutamicum* AS019 were grown in LBG-GI (2% glycine and 4 mg/ml INH) and harvested at the A_{600} = 0.35-0.45. pCSL17 DNA (0.5 μg) obtained from either *E. coli* LE392, or *Hae*III methylated *E. coli* LE392, or *C. glutamicum* AS019 was added to 40 μl of cells in 15% glycerol prior to pulsing at 2.5 kV and 25 μF (time constant: 4.5-4.9). Cell counts were performed in triplicate and average numbers were taken.

Host strain	Transformation frequencies for pCSL17 DNA from [a]		
	E. coli LE392	*E. coli* LE392/*Hae*III MTase	*C. glutamicum* AS019
AS019	1.9	8.0	200
ATCC 13032	0.8	3.4	1,100

[a] (Transformants/survivors/μg DNA) X 10^6. Data are from the average of five independent experiments.

Strain LE392 was chosen as the source of *E. coli* DNA as the RM background of this strain was known and also it gave high frequency of transformation with many of the *E. coli* strains used (M.L. Britz, 1985, unpublished observation). Data for the six strains used as recipients in these experiment are shown in Table 4.7. *B. flavum*-derived pCSL17 transformed McrBC$^-$ strains of *E. coli* (MC1061 and HB101) with much the same efficiency as *E. coli* LE392-derived pCSL17 DNA. However, the efficiency of transformation of McrBC$^+$ strains of *E. coli* (LE392, ED8654, CSR603, JM101) using *B. flavum*-derived pCSL17 was at least 100-fold lower than for *E. coli* LE392-derived pCSL17 DNA. Since the McrBC$^+$ background restricts uptake of foreign DNA which has methylated cytidines located at specific GC sites, this data suggests that *B. flavum* has a MTase which methylates cytidines, at least some of which are located in McrBC restriction enzyme recognition sites.

E. coli MC1061 has an Mrr restriction enzyme, an enzyme which restricts DNA containing methylated adenosine in either CAG or GAC sequences. The data presented here shows that *E. coli* MC1061 was transformed at high efficiency by *B. flavum*-derived pCSL17 suggesting that *B. flavum* does not methylate adenosine residues in CAG or GAC sequences. These data indicate that methylation patterns in *B. flavum* strain BF4 are similar to those of *C. glutamicum* strain AS019 (see Table 1.7 for *C. glutamicum* AS019).

4.6 IDENTIFICATION OF METHYLATED CYTIDINE IN DNA FROM *C. glutamicum* USING BISULPHITE DNA SEQUENCING

4.6.1 Optimisation of bisulphite DNA sequencing procedures

Initially, the method applied for determining cytidine methylation in *C. glutamicum*-derived

Table. 4.7 Transformation efficiencies of *E. coli* recipient strains with different restriction backgrounds using *E. coli*- and *B. flavum*- derived pCSL17 DNA.

	Transformation efficiencies for DNA from: [1]		Restriction system [2]					Reference for *E. coli* strains
			EcoK		*McrA*	*McrBC*	*Mrr*	
E. coli recipient strain	*E.coli* LE392 [3]	*B. flavum* BF4 [3]	R	M				
MC1061	1.7 X 10^5	4.5 X 10^4	-	+	-	-	+	Sambrook *et al.*, 1989
HB101	2.3 X 10^4	6.9 X 10^4	-	-	+	-	-	Sambrook *et al.*, 1989
LE392	1.5 X 10^4	5.0 X 10^2	-	+	-	+	+	Sambrook *et al.*, 1989
ED8654	3.1 X 10^3	1.0 X 10^2	-	+	-	+		Sambrook *et al.*, 1989
CSR603	3.0 X 10^2	< 100	+	+	+	+	+	Sancar and Rupert, 1978
JM101	3.2 X 10^4	< 100	+	+	+	+	+	Sambrook *et al.*, 1989

[1] Transformation efficiency was calculated as the number of transformants per μg DNA used. The number presented was obtained from the average values of two experiments.

[2] Information on the restriction background of the strains used is from Raleigh *et al.* (1988) and Waite-Rees *et al.* (1991). '+' indicates the presence of activity; '-' indicates lack of activity. A blank indicates that no information was available.

[3] 0.1 μg of pCSL17 DNA derived from either *E. coli* LE392 or *B. flavum* BF4 were mixed with 200 μl of recipient cell.

pCSL17 was obtained from Dr. S J. Clark (CSIRO Division of Biomolecular Engineering, Sydney Laboratory, Australia). The method of Clark *et al.* (1994) specifically targets 5-methyl-deoxycytidines (m^5dCyd); bisulphite is used to deaminate deoxycytidines (dCyd) thus converting them to uridines which, in subsequent PCR reactions, are amplified as thymidines. m^5dCyd, however, is not deaminated by bisulphite treatment and is therefore amplified as dCyd in PCR reactions. Because methylation protects the dCyd from the reaction, methylated bases can be identified by DNA sequencing following bisulphite treatment.

However, bisulphite treatment is not 100% efficient and many dCyd are not deaminated. To determine which dCyd are methylated in a sequence, it was therefore necessary in the past to clone the PCR products of bisulphite treatment and sequence a number (typically 10-20) of the clones to be confident that any cytidines in the amplified products are due to the presence of a m^5dCyd in the original template DNA, and not simply a failure of the bisulphite treatment to deaminate an unmethylated dCyd. However, a different approach was applied in this study. The site of interest (GGCCGC) contains a *Hae*III recognition sequence (GGCC) which, following successful bisulphite treatment, would be converted into GGTTGT, if there are no methylated cytidines in the sequence. Thus non-deaminated template following bisulphite treatment and PCR amplification can be removed from PCR products of successful deamination by digesting the PCR products with *Hae*III; *Hae*III treatment will leave only bisulphite modified products intact for subsequent rounds of PCR. In addition, because modification reactions target only unmodified cytidine, they produce strands which are no longer complementary. Therefore primers were designed to amplify and produce double-stranded products from only one of the two original strands (Fig. 2.2 and Fig 4.22 for the sequences of primers). After step (C) (see Fig. 2.1 in Materials and Methods section 2.3.13), often no PCR products were obtained and after step (D), a 274 bp PCR product was amplified (Fig. 4.23). When this product was sequenced without further steps using an automated DNA sequencer, signals from bisulphite-modified PCR product were

1	ATT	GTT	GCC	GGG	AAG	CTA	GAG	TAA	GTA	GTT
31	CGC	CAG	TTA	ATA	GTT	TGC	GCA	ACG	TTG	TTG
61	CCA	TTG	CTG	CAG	GCA	TCG	TGG	TGT	CAC	GCT
91	CGT	CGT	TTG	GTA	TGG	CTT	CAT	TCA	GCT	CCG
121	GTT	CCC	AAC	GAT	CAA	GGC	GAG	TTA	CAT	GAT
151	CCC	CCA	TGT	TGT	GCA	AAA	AAG	CGG	TTA	GCT
181	CCT	TCG	GTC	CTC	CGA	TCG	TTG	TCA	GAA	GTA
211	AGT	TGG	CCG	CAG	TGT	TAT	CAC	TCA	TGG	TTA
241	TGG	CAG	CAC	TGC	ATA	ATT	CTC	TTA	CTG	TCA
271	TGC	C								

Fig. 4.22 Sequence data of part of pCSL17 DNA. pCSL17-derived from *C. glutamicim* was constructed by using pBR322, *Tn*903, and pSR1, and sequence presented here are from pBR322 (between 3,541-3,814 in the 4,363 bp total). Sequence information was obtained from GeneBank (accession number X67018). Sequence data from only one strand is presented (strand (a) in Fig. 2.2 in Materials and Methods section 2.3.13). Sequences of primer C and primer D are underlined (==) and the *Tse*I sequence is shown in bold type. The target sequence of GCCGC is underlined (=).

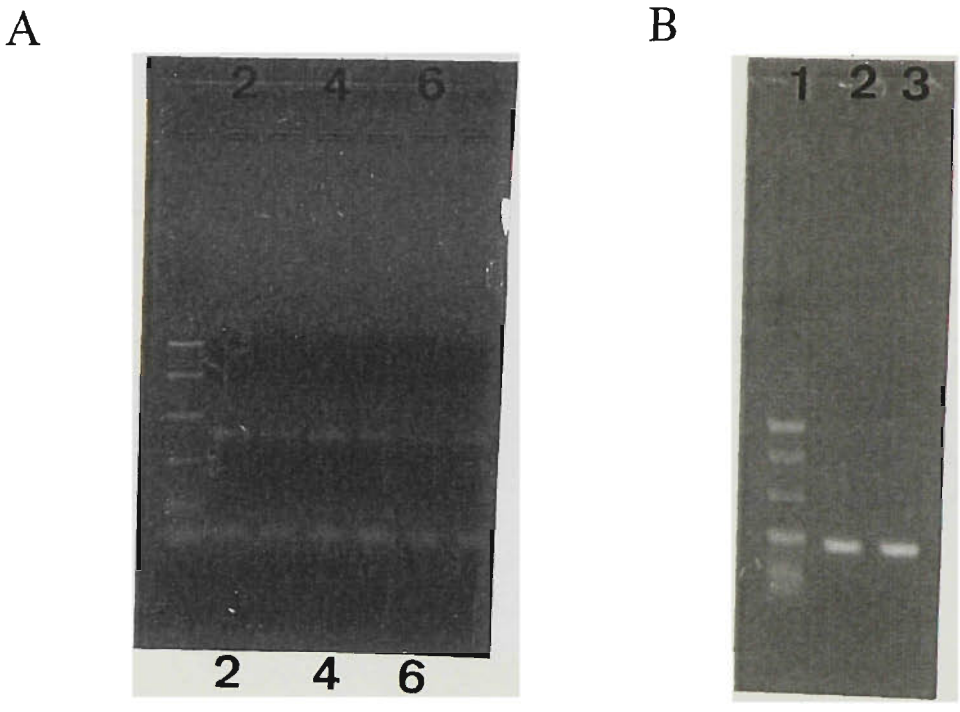


Fig. 4.23. PCR amplification products following restriction enzyme digestion of bisulphite modified DNAs. *Bgl*II-linearised pCSL17 DNA-derived from *C. glutamicum* AS019 and *B. lactofermentum* BL1 were bisulphite modified as described in section 2.3.13, desalted using Wizard PCR product Clean-Up system, and amplified using nested primers (primer A and primer B for first rounds of reaction, primer C and primer D for the second round reaction), designed as shown in Fig. 2.2. For the **(A)**, PCR products were obtained after the first round of PCR reactions and following *Hae*III/*Pst*I digestion at 37°C for 2-3 h. For **(B)**, PCR products in **(A)** were further amplified, without restriction enzyme digestion, using primer C and primer D, and after the second round of PCR reaction, the PCR products were incubated with *Hae*III and *Pst*I. After agarose gel electrophoresis, DNA was eluted from the gel and amplified using primer C and primer D, as described in the text, and then PCR products were incubated with *Hae*III and *Pst*I.

(A), Lanes: 1, PCR size markers (50, 150, 300, 500, 750, 1000 bp); 2-4, PCR product of strain AS019 after first round of PCR; 5-7, PCR product of strain BL1 after first round PCR.
(B), Lanes: PCR size markers (50, 150, 300, 500, 750, 1000 bp); 2, PCR product from strain AS019 after second round of PCR reaction; 3, PCR product from strain BL1 after second round of PCR reaction.

contaminated with signals from PCR products from unmethylated templates, which made analysis of information impossible (data not shown). In order to get rid of unmodified PCR products from the chemically modified PCR product, the mixtures of PCR products were incubated with two restriction enzymes: PCR product (2-3 μg) was digested by 5 units of both *Hae*III and *Pst*I for three hours hr in order to avoid any chance of partial digestion. Approximately 50% of the PCR product was digested by these two restriction enzymes, judging from densitometric analysis (Fig. 4.23), which was indicated that not all of the DNA substrate was changed by the bisulphite treatment. However, the unmodified PCR product was successfully eliminated from the modified PCR product using restriction enzyme digestion. This allowed the collection of the band of interest and the appication of a second round PCR reaction. This produced a PCR product of 274 bp (Fig. 4.22), which was collected, extracted with the Wizard PCR product Clean-Up column and this dissolved in 50 μl deionised water for subsequent sequencing (section 4.6.2).

4.6.2 Detection of DNA methylation in the sequence of GCCGC of pCSL17

The PCR products from the steps described in section 4.6.1 were sequenced in both directions and for pCSL17 DNA derived from both *C. glutamicum* AS019 and *B. lactofermentum* (Fig. 4.24). The DNA sequence without bisulphite treatment was obtained from published information and this is shown in Fig. 4.22 for comparison with data obtained here (Fig. 4.24). From both DNAs, it was found that DNAs were converted. For *C. glutamicum*-derived DNA, the GCCGC sequence was changed to GCTGT and the same sequence was changed to GTTGT in *B. lactofermentum*-derived pCSL17 DNA. This indicated that in *C. glutamicum*-derived DNA, the first cytidine in the sequence of GCCGC is methylated by MTase, whilst no cytidine in the same sequence for *B. lactofermentum* was methylated. This finding was confirmed using a different primer (E), which amplifies in the same direction as primer C but which is closer to the target sequence (see Fig. 4.25 for design of primer E and sequence data).

This sequencing data proved that a MTase in *C. glutamicum* methylate the first cytidine in the sequence of GCCGC. This result also suggested that the putative ENase of *C. glutamicum* also recognises a sequence which includes this methylation site. Only one strand of pCSL17 was analysed, therefore we do not know from these experiments whether the MTase in *C. glutamicum* methylates one or both strands in the sequence of GCCGC and GCGGC. In addition, this PCR product (274 bp) contained one sequence of GCTGC (located between 66-70 bp in Fig. 4.22) and one GCAGC (located between 243-247 bp), which are target sites of *Tse*I. From the sequencing results, none of cytidines in these sequence were found to be methylated in either *C. glutamicum*- or *B. lactofermentum*-derived pCSL17 DNA. This indicated that the cytidine bases in the target sites of *Tse*I are not modified, which is consistent with data presented in section 4.4.2.6.

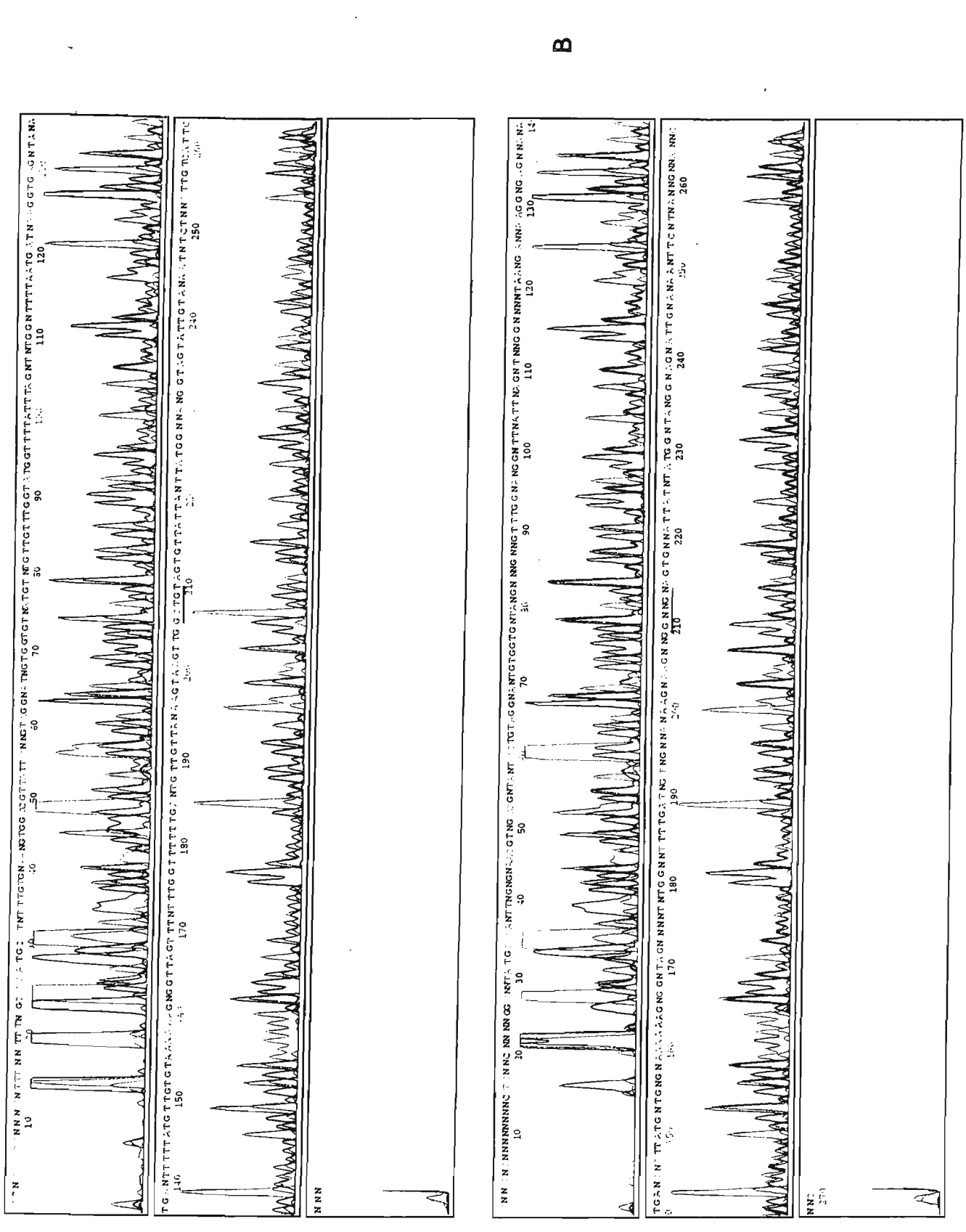


Fig. 4.24 Automated sequencing results of PCR products from the bisulphite-treated pCSL17 DNA derived from *C. glutamicum* AS019 (A) or *B. lactofermentum* BL1 (B). Both PCR products were directly sequenced and only results from one direction are shown here (primer C in Fig. 2.2 was used). The target sequence (GCCGC) is underlined.

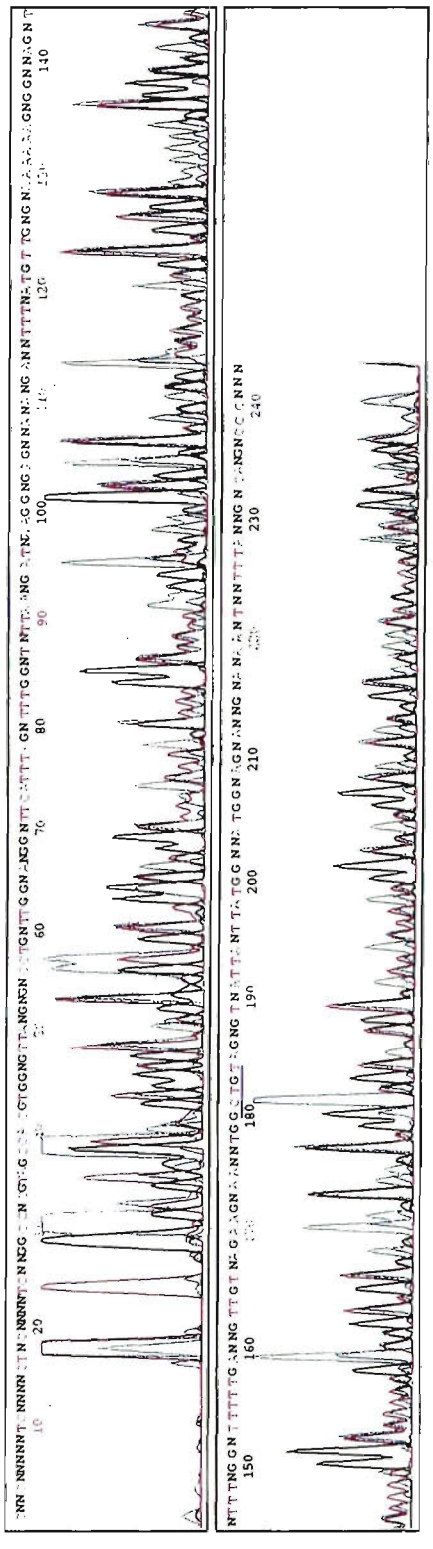


Fig. 4.25 Automated sequencing results of the PCR product from the bisulphite-treated pCSL17 DNA derived from *C. glutamicum* AS019. Primer E (see below) was used for this experiment and located between 35 and 60 (in Fig. 4.22). The target sequence (GCCGC) is underlined. For numbering, see Fig. 4.22

Primer E: AGT TAA TAG TTT GTG TAA TGT TGT TG (26 bp)

4.7 DISCUSSION

The studies presented in this chapter demonstrate that coryneform bacteria contained DNase activity and two coryneform bacteria species studied, *C. glutamicum* and *B. flavum*, contain a cytidine MTase which methylates at the first cytidine on the sequence of GC(G/C)GC.

One of the approaches used in this study involved evaluating the degree and type of methylation present on a plasmid to deduce specific methylated sequences and presumptive target sequences. Similar approaches were used by Wilson (1991). Similar trials were conducted here, using pCSL17 plasmid DNA, which was chosen because this DNA was able to be transformed into *C. glutamicum*, *B. lactofermentum*, *B. flavum* and several strains of *E. coli*. Therefore several different pCSL17 DNAs, which have different methylation patterns on the DNAs, could be prepared depending on the host. However, one of the disadvantages of using pCSL17 was its low copy number in coryneform bacteria. Data on plasmid copy number obtained using the Dupont hybridisation kit indicated that there were 9 copies of pCSL17 in *E. coli*, 4 copies in *C. ulcerans* and *B. flavum*, 3 copies in *B. lactofermentum*, and 2 copies in *C. glutamicum* (M. L. Britz, personal communication). The low plasmid copy number in coryneform bacteria added to the difficulty in isolating plasmid DNA from these strains.

Cell-free extracts from all strains of corynebacteria tested showed ENase and exonuclease activity, a finding similar to that reported by Diong (1989). Although two mutants, RM3 and RM4, were known to be restriction deficient (Schäfer *et al.*, 1994b), the cell-free extracts of these strains showed ENase activity which was able to convert the CCC form of heterologously-derived DNA to open circular form.

The role of the observed activity in RM3 and RM4, and other coryneform strains tested here, is not clear in relation to the reported restriction deficient phenotype of these strains. It is possible that more than one restriction and modification system exists in these species and the observed activity was not the major restriction system in these strains.

The presence of ENase(s) in *C. glutamicum* was further supported by the observation that the fertility of *C. glutamicum* recipients in intergeneric matings can be improved by exposing cells to a number of stresses, including heat, organic solvents, detergents and pH shifts, suggesting the presence of a cell-surface located, stress-sensitive restriction complex (Schäfer *et al.*, 1994a; 1994b). Heat-sensitive restriction enzyme activity was also reported in *Streptomyces* species (Bailey and Winstanley, 1986; Engel, 1987). Results from this work also showed that pre-incubation of cell-free extracts at 49°C for 9 min partially decreased DNase activity, including linearising activity, although in this case enzymatic activity was obtained from total cell-free extracts rather than from cell wall fractions. Subsequently, several approaches were used to characterise sequence-specific ENase activity in cell-free extracts of *C. glutamcium* AS019. These approaches were not useful for characterising sequence-specific ENase activity, due to the presence of highly active exonuclease activity. Further purification steps were required to separate the presumptive sequence specific DNase activity from other DNase activity in crude extracts.

Following publication of the sequences of two presumptive restriction genes in *C. glutamicum* (Schäfer *et al.*, 1994b) and in light of the differences encountered in demonstration ENase in cell-free extracts, methylation patterns in *C. glutamicum*-derived DNA were studied. The presence of methylated DNA bases in corynebacteria were analysed using HPLC. The mol% G+C contents found was in reasonable agreement with published values for *C. glutamicum* ATCC 13032 (54.6%), *B. lactofermentum* DSM 20412 (54.8%) and *B. flavum* DSM 20411 (54.4%) (Liebl *et*

al., 1991). In bacterial DNA, two DNA bases (adenosine and cytidine) are known to be modified by methyltransferases associated with RM systems (Lange *et al.*, 1991; Wilson, 1991). In most cases, m^5dCyd and m^6dAdo are the major modified bases (Vanyushin *et al.*, 1968; Wilson, 1991) and m^4dCyd is found in a few bacterial species such as thermophilic bacteria (Ehrlich *et al.*, 1985) and mesophilic bacteria (Janulaitis *et al.*, 1983). In the present study, the presence of m^4dCyd was not tested because m^4dCyd was not available commercially.

Although the quantities were different for the three corynebacterial DNAs tested, they all showed the presence of adenosine methyltransferase and cytidine methyltransferase. Methylated cytidine was found to be present in higher amounts than methylated adenosine in all three species, indicating that cytidine MTase may be dominant in the corynebacterial RM system.

The proportion of m^5dCyd in all three corynebacterial DNA was well in excess of what would be expected from a MTase, which recognises 5 bp or 6 bp sequences. However, the measured amount of m^5dCyd was below the amount expected if the MTase methylated in 4 bp sequences. This indicates that a putative cytidine MTase in corynebacteria recognises 5 or 6 bp sequence. The possibility that there are other MTase, which recognise 7 bp or 8 bp and so on, cannot be excluded. However, even though such MTases may exist in these bacteria, these may be not so important for limiting plasmid-based DNA transformation, due to the lower likelihood of existence such sequences in plasmid DNA.

Generally, plasmids which have been used for DNA transformation in coryneform bacteria have mainly ranged between 2 kb and 10 kb in size (see Table 1.4 for plasmid DNAs capable for transformation into coryneform bacteria). Based on theoretical calculations, the probability of the existence of a cytidine MTase which recognises 7 bp sequence is at most 0.03735% (0.273^1 X 0.227^6 = 0.00003735). The reverse of this probability yields the average distance separating two

recognition sites: 26772 bp. Therefore the possibility is that such sequences would not occur in most plasmid DNAs, and therefore, if there are ENase in coryneform bacteria which recognise 7 bp, such ENase would not find target sequences in plasmid DNA.

The presence of sequence-specific ENase activity in corynebacteria could be detected using transformation experiments, by comparing transformation efficiencies used homologously-derived DNA to those seen with heterologously-derived DNA (Haynes and Britz, 1990; Katsumata *et al.*, 1984; Thierbach *et al.*, 1988). Results from electroporation experiments described in this thesis (see Chapter 3) showed that significant differences in transformation efficiency of *C. glutamicum* strains were seen with different sources of DNA. When homologously-derived DNA was used, transformation efficiencies were much higher than for heterologously-derived DNA, with the exceptions of strains RM3 and RM4, which confirmed previous observations by Haynes and Britz (1990), which suggested the presence of sequence-specific ENase in *C. glutamicum*.

The presence of methylated bases in DNA from *B. flavum* strain BF4-derived DNA was indicated using a transformation experiments and the presence of methylated cytidine in GC sequences was confirmed using *McrBC* ENase. The results showed that *B. flavum* DNA contains methylated cytidines, some of which are part of the recognition sequence of the *E. coli McrBC* system. Therefore, *B. flavum* DNA was recognised and restricted by the *McrBC*. Similar observations have been described for *C. glutamicum* DNA (Tauch *et al.*, 1994). Tauch *et al.* (1994) found that plasmids derived from *C. glutamicum* AS019 were subjected to restriction in *McrBC*-proficient *E. coli* strains.

Unlike the other species of two corynebacteria, *B. lactofermentum*-derived DNA was not restricted by the *McrBC*+ system in *E. coli*, indicating that *B. lactofermentum* does not contain methylated cytidine in the GC sequence (M. L. Britz, 1985, unpublished observation). In the present work,

McrBC ENase digestion of chromosomal and pCSL17 plasmid DNA showed that two species of coryneform bacteria (*C. glutamicum* and *B. flavum*) contained methylated cytidine in the GC sequence, which is in agreement with results for transformation experiments. In contrast, chromosomal and plasmid DNA from *B. lactofermentum* BL1 were not degraded by *McrBC* ENase. This indicated that this bacterium contained a different modification system to that found in the other corynebacteria strains used.

On the other hand, DNA derived from the three coryneform bacteria strains tested was not affected by the presence of *McrA* or *Mrr* systems in recipient strains of *E. coli* (results for *C. glutamicum* and *B. flavum* are in Table 1.7 and Table 4.7, and conclusion for *B. lactofermentum* was obtained from Britz, unpublished observation, 1985).

However, the above approach could not be used to determine specifically which cytidines in *C. glutamicum* were methylated. Thus, a series of 20 restriction enzymes which specifically cleave DNA at either methylated or unmethylated recognition sequences were used to confirm that corynebacteria contained methylated cytidines and to partially characterise the recognition sequence for the MTase in these bacteria. The plasmid DNA isolated from *C. glutamicum* and *B. flavum* were fully or partially resistant to restriction endonuclease digestion, which could be due to MTase activity preventing cleavage by restriction enzymes.

Restriction enzyme digestion by *Hpa*II and *Msp*I (recognition sequence: CCGG) indicated that DNA derived from the corynebacteria species had the same digestion patterns for both enzymes, indicating that the CCGG sequences was not a target for methylation by the presumptive MTase in the corynebacteria strains tested. This was in agreement with observations from a transformation experiments where it was found that DNAs derived from three corynebacteria were not restricted by *McrA* (which recognise and cleaves ^{m}CG sequence).

Restriction enzyme digestion by *Hae*III (recognition sequence: GGCC) indicated that both *C. glutamicum*- and *B. flavum*-derived DNAs were cleaved at fewer sites than *E. coli*- or *B. lactofermentum*-derived plasmids, since there were two large bands, and fewer small bands in digests of the *B. flavum*- and *C. glutamicum*-derived DNA. This was in agreement with observations from a transformation experiments, where it was found that DNA derived from *B. flavum* was restricted by *McrBC* ENase, indicating that cytidine in the GC sequence was methylated.

When *E. coli*-derived plasmid DNA was methylated by treating DNA with *Hae*III MTase and transformed into *C. glutamicum* strains, transformation efficiency for this modified DNA was a little higher than seen for untreated *E. coli*-derived DNA, but efficiency was still much lower than seen for *C. glutamicum*-derived DNA. This indicated that the *C. glutamicum* MTase was not a homologue of *Hae*III MTase, as treatment with this enzyme was not sufficient to methylate all sites recognised by the cognate *C. glutamicum* restriction system.

Published sequence data for the components of pCSL17 indicated that both of the *Hae*III sites which remained uncut in the *C. glutamicum*- or *B. flavum*-derived pCSL17 DNA contain the sequence GCCGC. Based on these results, further characterisation of the methylation activity in *C. glutamicum* was carried out using *Fnu*4HI and *Tse*I. *Fnu*4HI cleaves at GCSGC (where, S is either G or C) and GCWGC (where, W is either A or T), whilst *Tse*I cleaves at only GCSGC. Therefore, *Fnu*4HI digestion will produce more bands than *Tse*I digestion, unless all of GCSGC sites were methylated so that *Fnu*4HI could not cleave at this sequence (GCSGC). These two enzymes produced identical patterns for the pCSL17 DNA derived from *C. glutamicum* and *B. flavum*, whilst *Fnu*4HI digestion in *B. lactofermentum*-derived pCSL17 gave more fragments than *Tse*I digestion. This confirms that both GCCGC and GCGGC sequences are targets for methylation by a presumptive MTase in corynebacteria and that all of these sites are methylated in

C. glutamicum AS019 and *B. flavum* BF4. MTases which recognise this sequence have been reported in other bacteria, including *Neisseria gonorrhoeae* (*Ngo*BVII MTase, Piekarowicz and Stein, 1995) and *Lactococcus lactis* (*Lla*DII MTase, Josephsen *et al.*, 1996).

The above conclusion was further confirmed by HPLC analysis of DNA from corynebacteria, showing that both chromosomal and plasmid DNA from the two coryneform bacteria (*C. glutamicum* and *B. flavum*) were found to contain 0.17-0.47% m^5Cyd. This level of cytidine methylation in these bacteria is close to the predicted level necessary for methylation of GCSGC sequences in *C. glutamicum*-derived DNA (0.15%, see Table 4.5). Based on these results, it appears that the cytidine MTase which recognises GCSGC sequences is the major cytidine MTases in both *C. glutamicum* AS019 and *B. flavum* BF4.

In a recent publication, Schäfer *et al* (1997) described a cytidine MTase gene in *C. glutamicum* ATCC 13032. These authors cloned the putative MTase (*hsd*M) gene in this species and characterised its sequence. The MTase gene contained 1092 nucleotides and encodes a protein of 363 amino acids with a molecular mass of 40.7kD. They isolated the *hsd*M gene from *C. glutamicum* ATCC 13032 and successfully cloned this into an *E. coli* strain. Expression of the *hsd*M gene in *E. coli* conferred the *C. glutamicum* specific methylation pattern to co-resident plasmids and improved subsequent transformation efficiency into *C. glutamicum*, although efficiency was still below than seen for homologously-derived DNA.

Subsequently, using different *E. coli* strains as recipients and plasmid DNA derived from *E. coli* which harboured the *hsd*M gene, Schäfer *et al.* (1997) demonstrated that *E. coli* DNA, modified by the *hsd*M gene of *C. glutamicum*, was sensitive to *McrBC* nuclease but was not affected by the presence of intact *mcr*A or *mrr* systems. Based on this observation, they suggested that the *hsd*M gene was responsible for the their previous observation, which showed that *C. glutamicum* DNA

was sensitive to *Mcr*BC restriction in *E. coli* (Schäfer *et al.*, 1994b; Tauch *et al.*, 1994). In the same paper, Schäfer *et al.* (1997) demonstrated that the *hsd*M gene is exclusively present in *C. glutamicum* strains including ATCC 13032, ATCC 13058, and AS019, but not in closely related strains including *B. lactofermentum* ATCC 13869 and *B. flavum* ATCC 14067, as judged by Southern analyses. Although they suggested that a gene which they had characterised possibly recognised the GC sequence as part of the target sequence, they have not demonstrated the recognition sequence of the cytidine MTase encoded by the *hsd*M gene or demonstrated activity of the enzyme.

HPLC analysis also indicated the presence of methylated adenosine in the above three coryneform bacteria. Based on theoretical calculations, it could be assumed that these three bacteria could have an MTase activity which methylates an adenosine bases in sequences of 5 bp or more. However, the m^6dAdo content of DNAs of the three coryneform bacteria species was less than 0.05% and this is not sufficient for methylation of a 4 bp sequences, since the probability of the existence of an adenosine MTase which recognises 5 bp ranged between 0.00266 - 0.00384 (0.266-0.384%). If the putative adenosine MTase recognises 6 bp (XYZ Z'Y'X'), the possible combinations are 4^3 (= 64), since the 4th, 5th, and 6th base are dependant on the 1st, 2nd, and 3rd base respectively.

Although some ENases such as *Aci*I (CCGC) and *Rle*AI (CCCACA) recognise nonpalindromic sequences(Roberts and Macelis, 1997), it is not common and therefore these were not taken into consideration here. In addition, if target sequences of 6 bp contains more than two adenosine bases, methylation could occur at either base site. The list of restriction endonucleases used was not exhaustive and it was not feasible to acquire all of the commercially available ENases for detecting methylated bases. Moreover, the DNA sequence(s) which contain methylated bases in these strains of coryneform bacteria may be different from those which are recognised by the

commercially available endonucleases, and hence the presence of any methylated bases in coryneform bacterial DNA would not be detected.

Although information described here is not sufficient to draw definitive conclusions regarding the target site of adenosine MTase in coryneform bacteria, it is worth mentionning that *C. glutamicum*-derived DNA was not methylated in the target site of the *Mrr* ENase (CAG or GAC sequence) and was cleaved by several ENases which recognise 4 or 6 bp but which do not cleave if the target DNA contains m^6dAdo. For example, *Dpn*I and its isoschizomers, *Dpn*II, *Sau*3A1, and *Mbo*I, are commonly used for the detection of adenosine methylation since these restriction enzymes recognise 4 bp sequence, GATC. Restriction enzyme digestion using the enzymes listed indicated that neither adenosine nor cytidine in the sequence of GATC were methylated, confirming results of Diong (1989). This is in contrast to what has been reported for pathogenic coryneform bacteria: *C. tuberculosteacicum* was shown to have the adenosine base on the GATC methylated (Hottat *et al.*, 1988).

As described earlier in this Chapter, *B. lactofermentum* contained m^5dCyd, but the target site for methylation was not determined using the described approaches. Clearly, the cytidine MTase activity differs from that seen in the other two species of coryneform bacteria examined in terms of recognition and methylation sites, which is consistent with the observation that restriction barriers are as great between *C. glutamicum* and *B. lactofermentum* as between these species and *E. coli* (Serwold-Davis, *et al.*, 1987; Haynes and Britz, 1990).

A full description of methylation specificity for an MTase requires identification of both the sequence modified and particular bases within the sequence that is modified. Many procedures can be used to determine the position of the methylated adenosine or cytidine in a target site. Generally, the position of the methylated A or C in a target site can be determined by analysing

partial digestion products of ^{3}H-methylated synthetic oligomer substrates (Greene *et al.*, 1975). Another approach based on the Maxam and Gilbert sequencing method (Maxam and Gilbert, 1980) has been used for characterisation of cytidine MTase (Ohmuri *et al.*, 1978; Korch *et al.*, 1983). In that procedure, m^{5}dCyd in DNA is evidenced by the absence of the corresponding chemical cleavage products. In the present work, the bisulphite genomic sequencing method was applied (Clark *et al.*, 1994; Grigg, 1996) to determine the exact methylation site in the sequence of GCSGC in pCSL17 DNA-derived from *C. glutamicum* AS019. However, the application of the bisulphite sequencing method as described by Clark *et al.* (1994) was unsuccessful in the present work, due to the contamination of chemically modified PCR products with unmodified PCR products; after bisulphite modification and following two rounds of PCR reactions, PCR products were analysed using an automated DNA sequencer and the product was found to be a mixture of modified and unmodified products.

Therefore, the method was modified to eliminate unmodified PCR products. This involved introductory an additional *Hae*III/*Pst*I digestion of the PCR product. Restriction enzyme digestion cleaved and therefore removed only chemically unmodified PCR product, but not the chemically modified PCR product. Automated DNA sequencing of the PCR product (274 bp) showed that only one cytidine was present in the PCR product, and that only one cytidine base must have survived the bisulphite treatment. This cytidine, then, must represent a modified nucleotide in the original template DNA from *C. glutamicum* and was the first cytidine in the sequence GCCGC sequence.

If the target site is GCCGC and one or more of the cytidine bases are methylated, then after bisulphite treatment and PCR amplification, the possible sequences produced would be: GCCGC (if bisulphite treatment failed to convert substrate DNA), GCCGT (if the first cytidines only was methylated), GTCGT (if the second cytidine only was methylated), GTTGT(if no cytidines were

methylated). Chromatogram from *C. glutamicum*-derived pCSL17 DNA show that there are two products: GCTGT (as a major product) and GCCGC (as a minor product). This indicated that a small proportion of cytidine in the original DNA (before bisulphite treatment) was not converted to thymidine by bisulphite treatment and PCR. However, this chromatogram clearly demonstrated that first cytidine in the sequence of GCCGC was methylated and therefore bisulphite treatment could not change this base to thymidine. In the case of *B. lactofermentum*-derived pCSL17 DNA, there were no cytidines remaining after bisulphite treatment.

The complementary strands (strand **a´** in Fig. 2.1) of strand **a** were also sequenced using primer D. Three base types (A,T and C) were expected in strand **a´** since strand **a´** was newly synthesised from strand **a**. Therefore no dGuo were expected in the strand **a´**, unless cytidine in the strand **a** was methylated. In the sequencing result, dGuo base appeared in strand **a´** when DNA was derived from *C. glutamicum*, whilst no dGuo appeared in the sequence in strand **a´** of same DNA but derived from *B. lactofermentum*. This confirmed that the first cytidine in the sequence of GCCGC was methylated in *C. glutamicum* but not *B. lactofermentum*.

From the present work, it was firmly established that the first cytidine in the GCSGC sequence is methylated by the m^5dCyd MTase in *C. glutamicum* AS019 DNA. The work described in this thesis is the first report of this detail defining exactly which cytidine in the sequence is modified in *C. glutamicum* DNA. As far as I know, this is also the first application of bisulphite sequencing for characterising on RM system in bacterial DNAs. The result indicates that the protocol described here could be applied for the detection of a target cytidine bases of cytidine MTases which produce m^5dCyd in the target sequence. In addition, if this method is applied to both strands in the target sequence (strand **a** and **b** in the Fig. 2.1), this technique could determine whether cytidines are methylated on only one strand or on both strands of DNA.

Information from REBASE (which is a comprehensive databases of information about ENases and their associated MTases) showed that two ENases have been reported recently from a *C. glutamicum* strain (Polisson, C. New England Biolabs), namely *Cgl*AI and *Cgl*AII. The author described that *Cgl*AI recognised the sequence of GCATGC, so that *Cgl*AI is an isoschizomer of *Sph*I, while *Cgl*AII recognised the sequence of GTCGAC, so that *Cgl*II is an isoschizomer of *Sal*I. This information has not been published and has only been reported as unpublished observations. However, the strain used (NEB968, home stock of New England Biolabs) of *C. glutamicum* could be very different from what I used for the present study: the incubation conditions for that strain were very different from the saprophytic corynebacteria used in this thesis, as this bacterium was incubated at 37°C (Polisson, 1997). Generally, the optimum temperatures of three strains of saprophytic corynebacteria, including *C. glutamicum*, *B. lactofermentum*, and *B. flavum* are 28-30°C, whilst that of the pathogenic corynebacteria is 37°C. In addition, restriction enzymes *Sal*I and *Sph*I were able to cleave pCSL17 DNA-derived from *C. glutamicum* AS019. An observation also made by other workers: Archer and Sinskey (1993) sequenced pSR1 plasmid DNA isolated from *C. glutamicum* ATCC 19223 (Yoshihama *et al.*, 1985), and showed that several restriction endonuclease, including *Bcl*I, *Bgl*II, *Eco*RI, *Nae*I and *Sph*I, linearised CCC form pSR1.

Ankri *et al.* (1996a; 1996b) reported that, when a PCR products of heterologously-derived DNA, which had no methylated DNA, or heterologous DNA obtained after growth in minimal medium (therefore having no methylated bases or containing less amount of methylated bases) were transformed into *C. glutamicum*, transformation efficiencies of these DNAs were higher than for heterologous DNA obtained from cells grown in rich medium. This indicated that this strain may contain an ENase which recognises and cleaves methylated bases in the target site, like other ENases: *Dpn*I (Brooks and Roberts, 1982), *Mcr*A (Raleigh *et al.*, 1988; Waite-Rees *et al.*, 1991), *Mcr*BC (Raleigh *et al.*, 1988; Waite-Rees *et al.*, 1991), *Mrr* (Raleigh *et al.*, 1988; Waite-Rees *et al.*, 1991). However, this study did not examine whether *C. glutamicum* and other

Brevibacterium species have similar ENase activity. It could be assumed that the strains (*C. glutamicum* ATCC 14752 and CGL1017 which is derived from ATCC 17965) used by these authors contain different RM system compared to *C. glutamicum* strain (AS019, ATCC 13032) used for present study. Ankri *et al.* (1996a; 1996b) demonstrated that total methylation at the *dam* (GATC) and *dcm* (CCWGG, where W is either A or T) sites is the major clue recognised by ATCC 14752 strain DNA immigration control, since this strain is transformed at essentially the same efficiency by DNA issue by a *dam-dcm* mutant, by *in vitro* synthesised DNA or by endogenous DNA. Haynes and Britz (1990) demonstrated that restriction barriers existed between *C. glutamicum* AS019, *B. lactofermentum* BL1 and *E. coli* HB101; the barrier to transformation was as great as between these two species as that seen for *E. coli*-derived DNA. Transformation efficiencies of *C. glutamicum* AS019 using pUL340 (Santamaria *et al.*, 1984) DNA-derived from *B. lactofermentum* BL1 were at least 10^3-fold lower than for *C. glutamicum* AS019-derived pUL340 (Haynes and Britz, 1990). In the present work, it was found that both *C. glutamicum* AS019 and *B. lactofermentum* BL1-derived plasmid DNAs and chromosomal DNAs lack methylated adenosine in the GATC DNA sequence (dam site) (see section 4.4.2.2). This indicates that *dam* site methylation was not a factor causing low transformation efficiency in *C. glutamicum* AS019 using *B. lactofermentum*-derived DNA.

The two of mutants RM3 and RM4 were confirmed as restriction deficient strains using electroporation experiments, as originally described by Schäfer *et al.* (1994b). In addition, the present work indicated that these are also cell surface mutant strains. This is possible to occur if mutation occurred in several positions in the RM3 and RM4 genomic DNA, including genes responsible for the ENase and cell wall synthesis.

Another question which remains unanswered is the number of ENases in *C. glutamicum*. Based on HPLC results presented in this thesis, there is possibly more than one RM system present: one

recognising methylated adenosine bases in the target sequence and the other recognising methylated cytidine bases in the target sequence. If *C. glutamicum* contains several RM systems, like some other bacteria (Piekarowicz and Stein, 1995), the mutations in RM3 and RM4 would be occurred in both ENases in two different RM systems, unless the two RM systems are located side by side and controlled from one promoter (Schäfer *et al.*, 1997). As mentioned previously, possible explanation is that RM3 and RM4 are cell surface mutants where the surface-located restriction enzyme is dislocated or inactivated due to the surface changes.

Alternatively, another possibility is that adenosine MTase does not have a cognate ENase. In bacteria, the action of MTases is not only as part of the RM systems, but also for other cellular function, such as DNA repair systems to the correct strands in newly replicated DNA (Grafstrom *et al.*, 1984). In addition to this, it is possible that the target sequence of the putative adenosine MTase contains more than 6 bp, so that the target sequence would be relatively rare in coming plasmid DNA.

Based on plasmid cloning, transformation and gene disruption studies, Schäfer *et al.* (1997) suggested that the DNA region in the responding to the *C. glutamicum* has the unusual organisation of one MTase gene coupled to two ENase genes. This suggested that *C. glutamicum* must contain more than one RM systems based on HPLC analysis on *C. glutamicum*-derived DNA. Probably, cytidine MTase (recognise GCSGC) is the major MTase activity.

Results reported in this chapter showed, using several experimental approaches, that one target site of methylation in *C. glutamicum* is GCCGC. The genes for at least one MTase and two ENases have been cloned but the enzymes responsible for the activity have not been characterised in terms of their biochemistry or cellular locations. Furthermore, the range of RM systems in the saprophytic corynebacteria is still not clear, considering the apparent differences in activities seen

in different representatives of *C. glutamicum*. This may be classified on the basis of clear phylogenetic classification of these strains, possibly through Pulsed-Field Gel Electrophoresis fingerprinting or other genetic means of speciation. Clearly, there is considerable scope for further research in this area. To understand how enzymatic RM system work, further information are required. Following information is not available from present work and others work.

1. characterisation of DNase activity. Although two mutant strains of *C. glutamicum* RM3 and RM4 showed the absence of ENase activity, they also showed the presence of DNase activity which was able to convert CCC form of heterologously-derived DNA to the OC DNA, in the cell-free extracts. Therefore, it would be interesting to know whether or not, ENase activity seen in cell-free extracts of RM3 and RM4 is sequence specific ENase, the location of the enzyme in the cell, specific activity in relative to other enzyme (exonuclease, MTase). Similar experiment could be performed in other strains of *C. glutamicum*.

2. isolation and characterisation of ENase and MTase enzymes from cell-free extracts of *C. glutamicum* and other two *Brevibacterium* species. So far, no enzyme involved in the RM system in these species have been physically isolated although the present work confirmed the presence of MTase in *C. glutamicum* and *B. flavum*. Therefore, isolation of these enzymes responsible for ENase and MTase in corynebacteria would be very important. HPLC analysis on DNA from three corynebacterial strains indicated that each strain contained at least two different MTase (adenosine MTase and cytidine MTase). The present work failed to show adenosine MTase using the present approaches. Preliminary experiment indicated cell-free extracts of *C. glutamicum* contained significant levels of exonuclease activity in cell-free extracts, which caused difficulty to generate ENase activity, therefore elimination of exonuclease activity from ENase and MTase activity in the cell-free extracts should be noted.

3. characterisation of the ENase and MTase activity. If the above experiments are successful, the properties of the enzymes (including cofactors requirement, molecular weight, optimum temperature, pH of enzyme, recognition site by ENase and MTase, cleavage site by ENase, methylation site on the target sequence by MTase) would be carried out and this result could be compared to that reported in the present study. After protein sequence analysis of enzymes, this would be compared to that of the DNA sequence information for ENase and MTase in *C. glutamicum* (Schäfer *et al.*, 1994b; 1997).

4. protection of heterologous DNA. Present work shows that there are MTases in two species of corynebacteria. Similarly, work by Schäfer *et al.* (1997) also showed that MTase methylation to heterologous DNA increased the transformation efficiency in *C. glutamicum*, and they suggested the target site of this enzyme contained the sequence of GC. It could be postulated that both MTase activity from the present work and Schäfer *et al.* (1997) is most probably the same. If this is true, transformation of heterologous DNA into *C. glutamicum* AS019 and *B. flavum* BF4 recipient could be increased by methylation of heterologous DNA prior to transformation. For this experiment, purification of MTase from *C. glutamicum* may be not necessary, since some of the purified MTase (recognise the sequence of GCNGC, where, N is either G or C or T or A) from various bacteria could be available; for example, *Ngo*BVII MTase (Piekarowicz and Stein, 1995) and *Lla*DII MTase (Josephsen *et al.*, 1996). Hence, modifying DNA *in vitro* with either a cell-free extract of *C. glutamicum* or several MTase described above before introduction into the *C. glutamicum* will overcome the restriction barrier.

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APPENDIX 1

MEDIA, REAGENTS AND SUPPLIERS OF CHEMICALS, MEDIUM CONSTITUENTS AND ACCESSORIES

A. Preparation of Chemicals and medium

Antibiotic solutions were obtained from Sigma and were as follows: neomycin (10 mg/ml), ampicillin (50 mg/ml), kanamycin (50 mg/ml), and streptomycin (10 mg/ml) were prepared aseptically in sterile distilled water. Chloramphenicol (34 mg/ml) were prepared in ethanol and tetracycline (5 mg/ml) and rifampicin (10 mg/ml) in methanol.

Derivatising agent ('Tri-Sil Z') was from Pierce Chemical. Co., Rockford, illinois, U.S.A.

DNA nucleoside standards were obtained from Sigma. N^6-methyl-2-deoxyadenosine, m^6dAdo, M-2389; 2-deoxyadenosine, dAdo, deoxyadenosine, D-7400; 2-deoxycytidine, dCyd, D-3897; 5-methyl-2-deoxycytidine, m^5dCyd, order number, M-2136; 2-deoxyguanosine, dGuo, D-7145; Thymidine, Thd, T-5018).

DNA sample loading dye (6X) was used for agarose gel electrophoresis and contained 0.25% bromophenol blue and 40% (w/v) sucrose in water.

Ethidium bromide (EtBr) stock solution, for agarose gel staining, was made by dissolving 1 g EtBr in 100 ml sterile distilled water (10 mg/ml) and stored at 4°C in the brown bottle.

Ethylenediaminetetraacetic acid, disodium salt (EDTA), pH 8.0, was prepared at 0.5 M and sodium hydroxide was used to adjust the pH of this solution. After sterilisation by autoclaving, stock solution was stored at room temperature.

Fatty acid internal standards were used for gas chromatography and contained following fatty acid methyl esters. For 100 mg of fatty acid standard 1 (Sigma 189-6) contained approximately 20 mg of each following fatty acid derivatives; Tridecanoic acid methyl ester ($C_{13:0}$); Pentadecanoic acid methyl ester ($C_{15:0}$); Heptadecanoic acid methyl ester ($C_{17:0}$); Nonadecanoic acid methyl ester ($C_{19:0}$), Hencicosanoic acid methyl ester ($C_{21:0}$). For 100 mg of fatty acid standard 2 (Sigma 189-17) contained each of the following with the weight percentage of each compounds as indicated; 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%; Linolenic acid 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%.

For the analysis of mycolic acids, several long chained fatty acid methyl ester were obtained from Sigma and used as internal standard. These chemicals were between C_{25} - C_{32} in total carbon numbers and contained following compounds; Lignoceric acid methyl ester, $C_{25}H_{50}O_2$, Sigma L6766; Nonacosanoic acid methyl ester, $C_{30}H_{60}O_2$, Sigma N2762; Triacontanoic acid methyl ester, $C_{31}H_{62}O_2$, Sigma T1902; Hentriacontanoic acid methyl ester, $C_{32}H_{64}O_2$, Sigma H6014.

Isonicontic acid hydrazide (INH) was obtained from Sigma (catalogue number I-3377) and stock (100 mg/ml in deionised water) was sterilised at 109°C for 25 min and stored at room temperature in brown bottle.

Lysozyme was from hen egg white and had an activity of 250,000 units/mg, Boehringer Mannheim, GmbH, Germany.

Phenol was from Wako (Tokyo, Japan).

Polaroid films (type 665 and type 667) were from Polaroid (Cambridge, MA, U.S.A.)

RNA nucleoside standards were obtained from Sigma. (adenosine, Ado, A-9251; Cytidine, Cyd, C-9505; Guanosine, Guo, G-6752; Uridine, Urd, U-3750)].

Spray Reagent (10% molybdophosphoric acid in 95% ethanol) for TLC (Gunstone and Jacobsberg, 1972). Chromatograms were sprayed thoroughly and then heated in an oven at 121°C for 15 minutes. All lipids appear as dark spots on pale green background.

Other chemicals and medium constituents are also listed below.

Reagent	Source
Acetic acid	BDH
Agar	Oxoid
Boric acid	Sigma
Bromophenol	BDH
BSA (for protein assay)	Sigma
Cesium 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
Peptone	Oxoid
Petroleum ether	BDH
Potassium acetate	Sigma
SDS	Pierce
Sucrose	BDH
Tris	Sigma
Tween 80	AJAX
Urea	Promega
Yeast Extract	Oxoid

B. SUPPLIERS

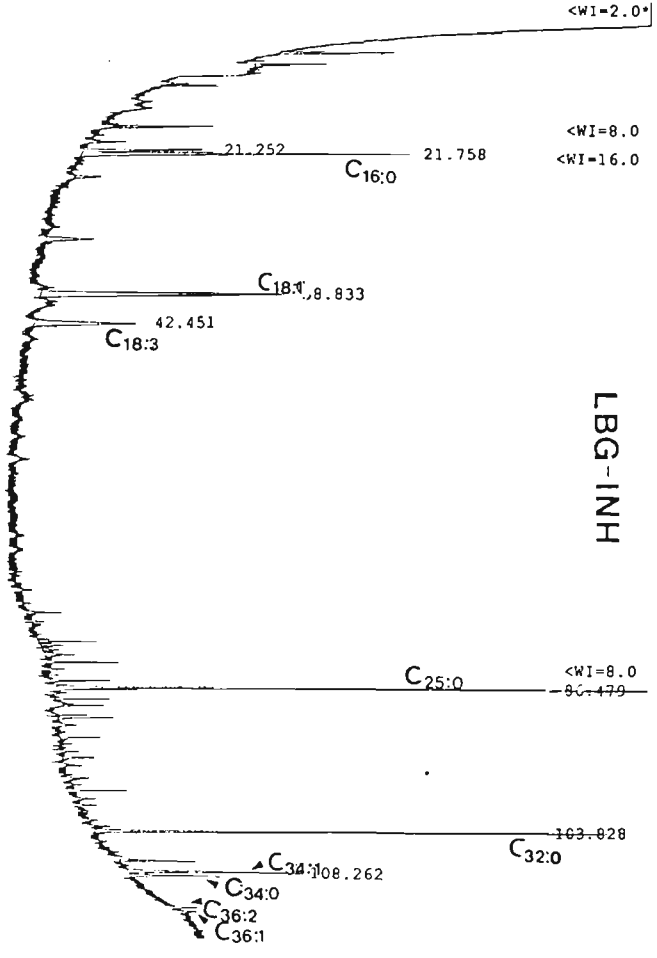
AJAX Chemicals Ltd	18 Hamlet St. Cheltenham, Vic 3192
AldrickSigma-Aldrick Pty Ltd	Unit 2, 10 Anella Ave, Castle Hill, NSW 2154.
B. Braun Melsunger AG, Australia	10 Arcae Rd, Box Hill Nth, Vic 3153.
Beckman instruments P/L	24 College St, Gladesville, NSW 2111
Bio-Rad Laboratories P/L	Unit 11, 112-118 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
Oxid	PO Box 220, West Heidelberg, Vic 3081
Pharmacia Australia P/L	4 Byfield St, Nth Ryde, NSW 2113
Pierce	PO Box 117, Rockford, IL U.S.A. 61105
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

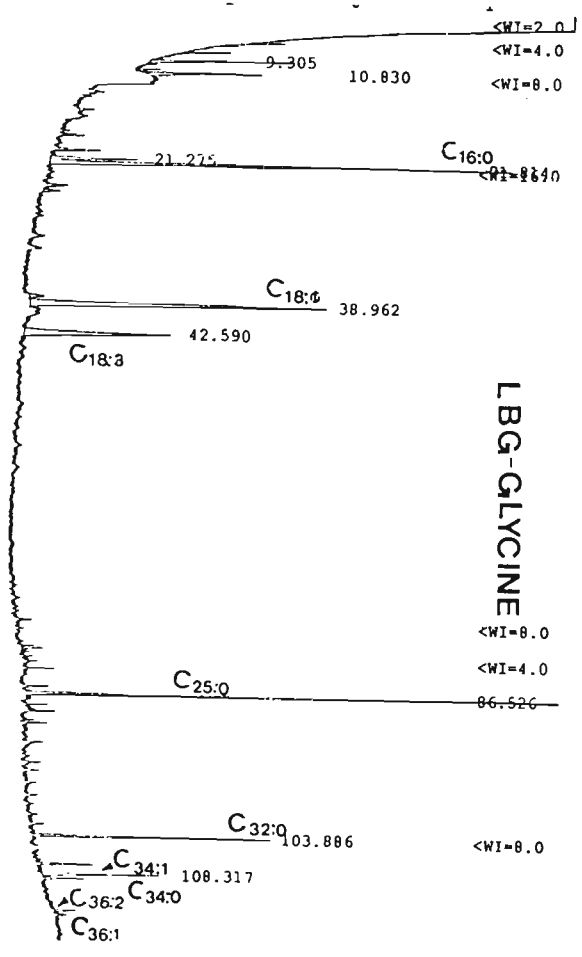
APPENDIX 2

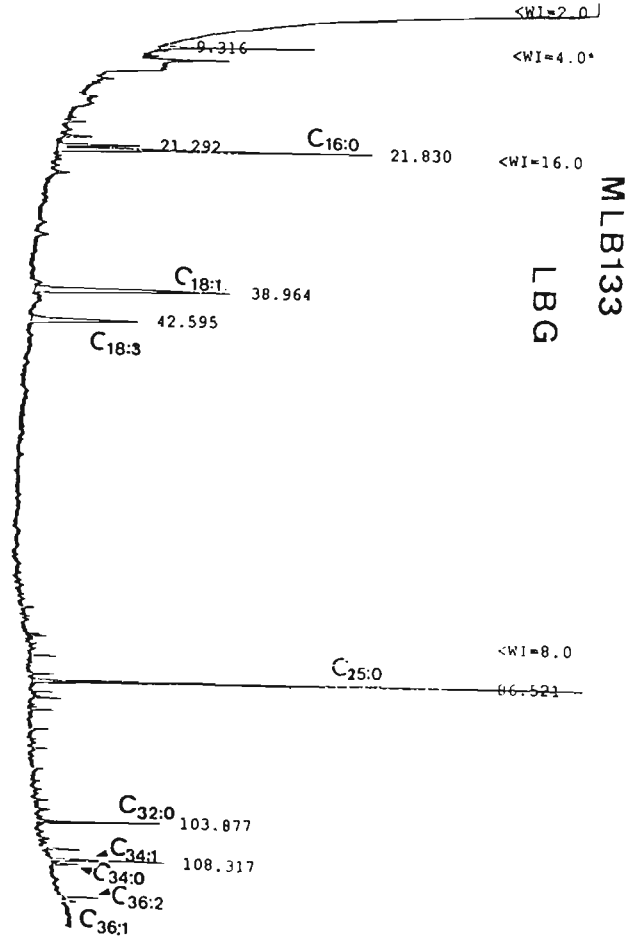
GC PROFILES OF FATTY AND MYCOLIC ACID

GC profiles of fatty and mycolic acids extracted from the cells of the two strains. Chromatographic conditions are given in section 2.5.1.7 and a summary of the results is given in section 3.4.8.

AS019 —LBG
<WI=2.0
<WI=4.0*
<WI=16.0
21.819
C16:0
21.291
C18:1
38.965
C18:3
42.594
<WI=8.0
86.521
C25:0
C32:0
C34:1
C34:0
C36:2
C36:1


LBG-INH
<WI=2.0*
<WI=8.0
<WI=16.0
21.252
21.758
C16:0
C18:1
8.833
42.451
C18:3
C25:0
<WI=8.0
86.479
103.828
C32:0
C34:1
108.262
C34:0
C36:2
C36:1


LBG-GLYCINE
<WI=2.0
<WI=4.0
9.305
10.830
<WI=8.0
21.275
C16:0
C18:1
38.962
42.590
C18:3
C25:0
<WI=8.0
<WI=4.0
86.526
C32:0
103.886
<WI=8.0
C34:1
108.317
C36:2
C34:0
C36:1


MLB133
LBG
<WI=2.0
9.316
<WI=4.0*
21.292
C16:0
21.830
<WI=16.0
C18:1
38.964
42.595
C18:3
C25:0
<WI=8.0
86.521
C32:0
103.877
C34:1
108.317
C34:0
C36:2
C36:1

APPENDIX 3

MAJOR FINDING FROM THIS STUDY

The major finding of Chapter 3 and Chapter 4 are summarised in dot points below.

A Effect of cell wall modifiers on the specific growth rate of corynebacteria cells

- For *C. glutamicum* AS019 cultures with different inoculum sizes, the length of the lag phase decreased with increasing inoculum size but little difference in the growth curve pattern was observed during exponential phase.

- The impact of growth of three potential cell wall modifying chemicals was tested, alone or in combinations, using the following concentrations: glycine (0-10%, w/v), INH (0-10 mg/ml), Tween 80 (0-1%, w/v). Among the three cell wall modifiers used over these concentration ranges, glycine and INH showed significant impact on growth of corynebacteria, whilst little impact was seen with Tween 80.

- The presence of glycine caused decreases in specific growth rate of cells and maximum absorbances reached after overnight incubation but the length of the lag phase increased. Among the three corynebacteria species tested (*C. glutamicum* AS019, *B. flavum* BF4 and *B. lactofermentum* BL1), strain BF4 was relatively less sensitive to inhibition by glycine. The growth of BL1 was inhibited little when grown in the presence of 1-2% glycine and further increases in glycine concentration caused significant decreases in the specific growth rates and increases in the lag period to more than 10 h. The two mutant strains, MLB133 and MLB194 were more sensitive to inhibition by glycine than AS019. For the ATCC 13032 family, RM4 showed higher sensitivity to the presence of glycine in the growth medium relative to ATCC 13032. *C. ulcerans* was the most sensitive to glycine inhibition amongst the 10 strains examined. These results indicate differences in the nature of the cell wall in the different species tested. This results also confirm earlier observation on strains MLB133 and MLB194 by Best and Britz (1986).

- The presence of INH caused decreases in specific growth rates and the maximum absorbances reached after overnight culture but increased the lag period. Of the 10 strains examined, AS019 was most resistant to growth inhibition by INH. The two mutant strains, MLB133 and MLB194 were more sensitive to inhibition by INH than AS019, suggesting that structural changes may be associated with the target of INH activity in *C. glutamicum*. For the ATCC 13032 family, the two restriction and modification mutants were more sensitive than their parent strain to inhibition by

INH, indicating that changes in these strains coincidentally affected their cell surface integrity. *C. ulcerans* was the most sensitive to INH among the strains examined. As a group, the corynebacteria were relatively more resistant to INH a mycobacteria.

- The presence of Tween 80 in the medium at the concentrations tested although Tween 80 was previously shown to improve eletroporation efficiency when used in combinations with glycine and INH (Haynes and Britz, 1989) did not significantly inhibited any strains. For example, growth of AS019 and CG2 was inhibited by 20% at the highest concentration of Tween 80 used, 0.9%.

- In the presence of combinations of cell wall modifiers in the medium, significant decreases in growth rate and increases in the lag period were seen, relative to cultures grown in the presence of a single cell wall modifier. Among the combinations of cell wall modifiers, (2% glycine plus 4 mg/ml INH) and (2% glycine plus 4 mg/ml INH plus 0.5% Tween 80), caused growth rates to decrease by more than 80% (for *Corynebacterium* strains). For the latter combination this caused increases in lag period by more than 24 h. In both cases, growth curve showed long lag periods, but after the lag phase, the both A_{600} values and viable cells continuously increased until stationary phase. This indicated that combinations of cell wall modifiers prevented initiation of cell growth during lag phase but, once growth started, cells could cope with these concentrations.

B. Effects of growth in glycine and INH on plasmid transformation using electroporation

- In confirmation of the report of Haynes and Britz (1990), there was a linear relationship between the amount of DNA added and the number of cells transformed. Early-exponential phase cells of *C. glutamicum* were more easily transformed and, as the A_{600} increased, there was a significant reduction in the numbers of transformants obtained.

- The recovery medium on which the transformants were isolated has a significant effect on the number of transformants obtained, confirming observation by Haynes and Britz (1990). The osmotically-protective (ET-Km) medium was better than LAG-Km as a recovery medium, suggesting that cells successfully transformed by electroporation were electrochemically or osmotically sensitive.

- Using the same strains and plasmids, it was found that transformation efficiency increased 10- to 100-fold using electroporation, relative to spheroplast or protoplast transformation methods (Thierbach *et al.*, 1988; Yoshihama *et al.*, 1985), in agreement with others report (Haynes and Britz, 1990).

- The corynebacterial cell surface structure was found to be more robust than the *E. coli* cell surface structure, and this is probably one of the reasons for low transformation efficiency in corynebacteria, using homologously-derived DNA.

- In contrast with the report of Dunican and Shivnan (1989), this works showed that the presence of glycine and INH in the growth medium is an important prerequisites for efficient electroporation, confirming reports by Haynes and Britz (1989; 1990). Under these growth condition, transformation efficiencies obtained were up to $10^5/\mu g$. This was found to be 10^2 to 10^3 times higher than the transformation efficiency obtained with LBG medium. This results shows that the structure of the cell wall can act as a physical barrier for DNA transformation into the corynebacteria. At the concentrations of glycine and INH used here, these chemicals do not lyse the cells, but they seem to increase transformability. This is probably due to an increase in the fraction of transformable cells in the culture or to an increases possibility of DNA uptake per viable cell. As shown in Table 1.5, several other researchers have used different cell surface modifying agents to improve transformation in coryneform species.

- The above observations are consistent with the observed increase in extracellular mycolic acids following growth in glycine or INH, where high proportions of extracellular mycolic acids were seen when cells grown in the presence of glycine or INH.

- Growth in the presence of glycine and INH caused significant increases in transformation efficiency for six strains of *C. glutamicum*, including AS019, MLB133, MLB194, ATCC 13032, RM3 and RM4. The effects of the presence of these cell wall modifiers in medium on transformation efficiency have not been reported in the earlier work, except for strain AS019 (Haynes and Britz, 1990).

- Four strains of *C. glutamicum* (AS019, MLB133, MLB194 and ATCC 13032) showed the presence of the restriction barriers which exist between *C. glutamicum* and *E. coli*. The presence of restriction barriers in AS019 and ATCC 13032 is in confirmation of report by others (Haynes and Britz, 1990; Schäfer *et al.*, 1994b). In contrast, strains RM3 and RM4 showed similar transformation efficiency whatever the source of DNA used, reflecting their restriction minus nature (Liebl *et al.*, 1989; Schäfer *et al.*, 1994b).

- The highest transformation efficiency of the above strains tested was obtained when *C. glutamicum* MLB133 cells were grown in the presence of 2% glycine and 4 mg/ml and transformed with homologously-derived DNA. Relatively high transformation frequencies were also obtained for heterologously-derived DNA but the presence of restriction barriers were evident. In contrast,

when heterologously-derived DNA was transformed into *C. glutamicum* strains RM3 and RM4, high transformation efficiency was obtained for these restriction minus strains. This indicates that either cell surface or restriction and modification mutant should be used to give relatively high frequencies of *C. glutamicum* electroporation.

- Since no attempts were made here to optimise the concentration of glycine and INH in the medium for RM3 and the other strains examined, it could be possible that further increases in the transformation efficiency could be obtained if cells were grown in medium containing optimised concentrations of glycine and INH.

C. Effects of glycine and INH on the mycolic acid composition of *C. glutamicum*

- All strains of corynebacteria had five major types of mycolic acids ($C_{32:0}$, $C_{34:0}$, $C_{34:1}$, $C_{36:2}$, $C_{36:1}$) in whole cells and culture fluids, but the relative proportions of each varied with the strain and medium composition, confirming reports by Pierotti (1987) and Collins *et al.* (1982a).

- Both mutant strains MLB133 and MLB194 always contained a higher proportions of unsaturated mycolic acids ($C_{32:0}$ lower than the parent, $C_{34:1}$ and $C_{36:2}$ higher), in agreement with reports by Pierotti (1987).

- Among the ATCC 13032 family, differences in the proportion of mycolic acids were also seen, where $C_{32:0}$ was higher in ATCC 13032 and RM4 than that of RM3, and $C_{36:2}$ was very much higher in RM3 (28.8%). Both *Brevibacterium* species, BF4 and BL1, showed similar patterns of mycolic acids to each other both in quantitative and qualitative compositions.

- Solvent extraction procedures for the extraction of MAMEs and FAMEs from culture fluids were evaluated and optimised and these gave above 95% recovery yields for MAMEs.

- The mycolic acid profiles in terms of relative proportions of each species in the cells and the culture fluids were quite similar for each of the samples tested indicating that a particular mycolic acid is not being released into the culture fluid and hence affecting the cellular mycolic acid profile. In LBG medium, extracellular mycolic acids ranged between 3.5% (in ATCC 13032) to 7.8% (in MLB133).

- The main effect of addition 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). This result showed that glycine affected mycolic acid attachment to the cell surface of *C. glutamicum*. This phenomenon

has not been reported in corynebacteria and other mycolic acid-containing bacteria.

- Growth in LBG with 8 mg/ml INH increased the proportion of extracellular mycolic acids (MLB133 and MLB194 had 15.1% and 21.2%, AS019 had 9.6%); the proportion of unsaturated mycolic acids increased, which corresponded to decreases in $C_{32:0}$ relative to increases in the proportion of $C_{34:1}$ and $C_{36:2}$. Disruption of attachment of mycolic acids following growth in the presence of INH has not been reported previously for corynebacteria and other mycolic acid-containing bacteria.

- The impact of glycine plus INH on cell-surface composition showed cumulative effects on extracellular mycolic acids in the culture fluids.

- The above results suggest that the mutations in strains MLB133 and MLB194 are associated with synthesis of specific mycolic acids (e.g. $C_{32:0}$) and attachment of mycolic acids to the cell surface, both of which are likely target sites for glycine and INH action for cell-surface modifications.

- The form of the extracellular mycolic acids was not investigated in this study: the presence of mycolic acids-bound to other cell wall components in the culture fluids needs to be investigated to get further information on the target sites of glycine and INH.

- All strains tested had two major types of fatty acids, palmitic ($C_{16:0}$) and oleic ($C_{18:1}$) acids, in confirmation of reports by Collins *et al.* (1982b) and Pierotti (1987). In contrast, when fatty acids were obtained from culture fluids, $C_{18:3}$ was also seen, which was not seen in samples from cells.

- In the presence of glycine or INH, the relative percentage of the fatty acids to 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-8 mg/ml INH). This implies that glycine and INH in the medium inhibit not only mycolic acid synthesis but also fatty acid synthesis, although absolute amounts of lipids per cell would need to be determined before firm conclusions can be drawn.

D. Influence of cell wall modifiers on cell morphology and cell wall structure of *C. glutamicum*

- Using transmission electron microscopy, cell morphology of two strains of *C. glutamicum* was compared. In the presence of 2% glycine in the medium, cell elongation and the appearance of Y-shapes and branched cells (without septation at the branch points) were seen.

- Mutant strain MLB133 had a relatively thin cell wall structure after growth in LBG, relative to its

parental-type strain, AS019, and cell wall thickness was further reduced by the presence of glycine or INH in the growth medium.

E. Demonstrating DNase activity in corynebacteria cell-free extracts

- Cell-free extracts of *C. glutamicum* and *B. flavum* contained several enzyme activity, including endonuclease and exonuclease, as described previously by Diong (1989). CCC plasmid DNA was initially nicked and converted to OC form before being further degraded.

- Pre-incubation of cell-free extracts at 49°C partially decreased DNase activity, including linearising activity, which is consistent with observation by (Schäfer *et al.*, 1994a).

F. Analysis of methylated bases in corynebacteria using HPLC

- Analysis of nucleotides in both corynebacteria and *E. coli*-derived plasmid DNAs and chromosomal DNA showed the presence of methylated adenosine and methylated cytidine for three corynebacterial DNAs. This suggested the presence of adenosine MTase and cytidine MTase in the three corynebacterial species, with the latter the dominant activity.

G. Characterisation of MTase activity in coryneform bacteria using transformation

- Previous experiments showed that plasmid DNA (pCSL17) isolated from *C. glutamicum* transformed recipient McrBC$^+$ strains of *E. coli* with lower efficiency than McrBC$^-$ strains, suggesting that *C. glutamicum* DNA contains methylated cytidine on the GC DNA sequence (Tauch *et al.*, 1994). Using plasmid DNA isolated from *B. flavum*, the same results was obtained here, implying that a MTase in *B. flavum* also recognises the GC sequence. However, the transformation efficiency of plasmid DNA (pCSL17) isolated from *C. glutamicum* and *B. flavum* was not affected by the presence of McrA or Mrr systems in recipient strains of *E. coli*. *Mcr*BC enzyme was able to cleave the two corynebacterial DNAs, but failed to cleave *B. lactofermentum*-derived DNAs, indicating that DNA derived from *C. glutamicum* and *B. flavum* contained methylated cytidine in the GC DNA sequence. The presence of methylated cytidine in the GC sequence was confirmed by using *Mcr*BC digestion of the two corynebacterial DNAs.

- The above results indicate that cloning of any *C. glutamicum*-derived genes that carry this sequence into *E. coli* hosts that are McrBC$^+$ will be ineffective, that is, the genes will be restricted by *McrBC* ENase and may not be recovered.

H. Characterisation of pCSL17 DNA using restriction endonucleases

- Analysis of chromosomal and plasmid DNA from corynebacteria for susceptibility to digestion by restriction enzymes which specifically cleave DNA at either methylated or unmethylated recognition sites was performed. Restriction enzyme digestion using *Dpn*I and its isoschizomers indicated that corynebacteria do not methylate adenosine in the GATC sequence.

- Failure of *Hae*III and *Fnu*4HI to cut specific sites of *C. glutamicum*- and *B. flavum*-derived pCSL17 indicated the presence of a MTase in both species which recognise the sequence of GCSGC (where, S is either G or C).

I. Detection of DNA methylation of pCSL17 DNA using bisulphite DNA sequencing

- Bisulphite DNA modification followed by sequencing showed that the first cytidine in the GCCGC sequence of *C. glutamicum*-derived DNA, which is consistent with data obtained from *Hae*III digestion.

- The results obtained from this work have significant implications for the genetic manipulation of *C. glutamicum* and *B. flavum*. In the presence of putative ENase, any attempts to introduce foreign DNA that is not properly methylated at the sequence of GCSGC into these bacteria will be subject to failure because of restriction of DNA by ENase.