CHARACTERISATION OF STRESS RESPONSES IN *LACTOBACILLUS PARACASEI* AND *BIFIDOBACTERIUM ANIMALIS* (SYN. *LACTIS*)

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BY

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DECLARATION

I certify, that unless otherwise stated, the work presented in this thesis is solely my own work, and has not been submitted previously, in whole or in part, for any other academic award. The results detailed in Chapters 3 and 4 have been previously presented in the following:

Pepper, S. J., N. Shah and M. L. Britz. 1995. A rapid and simple method for comparative identification of lactic acid bacteria using microtitre plates. Abstracts, International Dairy Lactic Acid Bacteria Conference, Palmerston North, New Zealand. Page 7.13.

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S. J. Pepper. 1997. Use of fatty acid methyl esters as part of the identification of *Lactobacillus acidophilus*. Abstract, Australian Society for Microbiology, as part of workshop entitled 'Approaches to Identification and Classification of Lactic acid bacteria', Adelaide, 1997. Page A131

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ABSTRACT

Characterisation of stress responses in *Lactobacillus paracasei and Bifidobacterium animalis* (syn. *Lactis*)

by

Susan Jessie Pepper

This thesis describes a study of the nature of the responses to stress, particularly acid stress, in *Lactobacillus paracasei* and *Bifidobacterium animalis* (syn. *lactis*). The aims of the study were to validate the identification of the bacteria being studied, to determine the nature of their stress responses and compare them to known responses and to gain information regarding the proteins up-regulated during stress.

Nineteen strains of lactic acid bacteria were tested in terms of their fatty acid methyl ester (FAME), sodium dodecyl sulphate-poly acrylamide gel electrophoresis (SDS-PAGE) and carbohydrate utilisation profiles to provide tools to differentiate these strains. However, these tests allowed only low levels of discrimination. Molecular methods were then used to identify one of the LAB characterised above, VUP 12006, which was later used for detailed analysis of acid stress responses. On the basis of 16S rRNA gene analysis, it was concluded that VUP 12006 was a strain of *Lb. paracasei*. Strain VUP 12006 was found to have an optimum growth temperature range of 37°C ± 0.5 and an optimum pH of 6.3 ± 0.2. Strain VUP 12006 was subjected to pH and temperature stress by culture under conditions that decreased the μ_{max} by 50% (pH 4.3/37°C and pH 6.3/45°C). Under acid stress, a 42 kDa protein, which was later found to be membrane- or cell-surface-associated, was observed to be up-regulated. Under heat stress, two proteins of mass 60 kDa and 70 kDa, which were up-regulated four-fold during growth at 45°C, were shown to cross react with antibodies made against *E. coli* HSP60 and *E. coli* HSP70. The N-terminal sequence of the 42 kDa protein was determined and found to share 100% homology with a protein sequence found in the draft genome of *Lb. casei* ATCC 334. PCR-based techniques were used to determine

the nucleotide sequence of the gene known to encode the 42 kDa AphA protein. The ORF was 1263 nucleotides in length with a G+C content of 47.66% and the translated amino acid sequence of AphA was shown to be rich in serine (15.4%) and alanine (13.3%). A 28 amino acid signal peptide was predicted by SPScan and the cleavage site was confirmed by the N-terminal sequence obtained for this protein. The protein was found to share homology with GSP-781, a glucan B binding protein identified in *Streptococcus mutans*. Although it has been suggested that GSP-781 may be associated with the maintenance of the stability of the cell wall or cell membrane, the biological functions of both GSP-781 and AphA are yet to be determined.

Based on a long history of use as a starter culture, strain VUP Bb 12 was selected for characterisation of stress responses. Identification of the test strain was confirmed as *B. animalis* (syn. *lactis*) through successful PCR amplification. Strain Bb 12 was determined to have an optimum growth temperature range of 42°C ± 0.2 and an optimum pH of 6.3 ± 0.2. The response to acid stress in strain VUP Bb 12 was studied using 1-D and 2-D SDS-PAGE. Strain VUP Bb 12 was cultured at 42°C and pH values fixed at pH 4.0, 5.0, 6.0 or 7.0. Protein profiles for growth at neutral pH (7.0) and mildly acid pH (6.0) were essentially similar. At pH 5.0 an increase was noted in the density of a band of around 33 kDa. At pH 4.0 a protein band of similar size was up-regulated over 10-fold over twenty-four hours growth. This 33 kDa protein was later found to be membrane- or cell surface-associated. The N-terminal sequence of this protein could not be determined experimentally, possibly due to the presence of carbohydrate or lipid. The function of this protein also remains to be determined. Changes in protein profiles during acid stress were observed in cytosolic as well as non-cytosolic proteins. Strain VUP Bb 12 was grown at pH 6.3 and 42°C and challenged by a sudden change in pH (to pH 4.3). 2-D SDS-PAGE gels were compared to confirm the presence of proteins that were up-regulated during growth at pH 4.3. N-terminal sequence data was obtained for four of these, namely Bsb-5, Bsb-6, Bsb-7 and Bsb-8, two of which were identical (Bsb-5 and Bsb-6). A database search revealed a match to two of these proteins whose function was found to be related to ribosome metabolism. The possible function of Bsb-6 was related to elongation factor-P and Bsb-8 to a 50S ribosomal protein L7/L12. The remaining protein (Bsb-7) was a hypothetical protein of unknown function. In conclusion, both species were able to respond to acid stress with novel responses which were physiologically quite different between the species studied.

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ABBREVIATIONS

$\Delta\Psi$	electrical potential
ΔpH	pH gradient
AH	acid habituation
AR	acid resistance
ATP	adenosine triphosphate
ATR	acid tolerance response
bp	base pair
BSA	bovine serum albumin
CBFT	Centre for Bioprocessing and Food Technology
Ci	Curie
CIRCE	controlling inverted repeat of chaperone expression
CSIRO	Commonwealth Scientific and Industrial Research Organisation
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
DNS	3' 5'-dinitrosalicylic acid
dNTP	deoxyribonucleotide
dsDNA	double stranded DNA
DTT	dithiothreitol
EDTA	Ethylenediaminetetra-acetic acid (disodium salt)
F/C	Folin/Ciocalteau
FAME	fatty acid methyl ester
g	acceleration due to gravity
h	hour
HCl	hydrochloric acid
HSP	heat shock protein
IEF	Iso electric focussing
IPG	Immobilised pH gradient
kb	kilobase
kd	kilodalton
kDa	10^3 Daltons
LAB	lactic acid bacteria
L	litre
L/min	litres per minute
L-B	Luria-Bertani medium
mA	milliampere
min	minute
mL	millilitre
mm	millimetre
mol	mole
MRS	de Mann, Rogosa and Sharpe medium

MW	molecular weight
NaOH	sodium hydroxide
NDO	non-digestible oligosaccharides
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
pH	hydrogen-ion exponent
pH_i	internal pH
pH_o	external pH
pI	isoelectric point
PMF	proton motive force
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
rRNA	ribosomal RNA
RS	reducing sugar
s	second
SDS	sodium dodecyl sulphate
SOS	save our souls
T_m	melting temperature
TEMED	N,N,N',N'-tetramethylethylenediamine
u.v.	ultraviolet
V	volt
v/v	volume/volume
VUT	Victoria University of Technology
W	watts
w/v	weight/volume
w/w	weight/weight
X-gal	5-bromo-4-chlor-3-indolyl-β-D-galactopyranoside

Chapter One

LITERATURE REVIEW

GENERAL INTRODUCTION

The objective of this project was to gain a basic understanding of how *Lactobacillus paracasei* and *Bifidobacterium animalis* (syn. *lactis*) respond when challenged with the changes in temperature, pH and oxidation levels which the organisms typically experience during the manufacture and consumption of fermented dairy foods. These organisms, which ferment milk during the production of yoghurt and cheese, are exposed to a rigorous manufacturing process as well as a hostile digestive tract before finally colonising or passing through the lower gastrointestinal tract. This involves growing in or surviving a wide range of pH values from alkaline to highly acidic, and temperature values that vary from refrigeration (2–6°C) up to 45°C. A better understanding of how the organisms respond to stress, particularly at a molecular level, will provide a sounder base for the development of starter cultures for improved manufacturing of fermented food products. This chapter therefore provides a review of the organisms and their use in production of dairy foods, including their role in conferring potential health benefits and the known responses of bacteria to stressful environments.

1.1 Probiotic foods

1.1.1 An introduction to probiotic foods

Lactic acid bacteria (LAB) have been used to ferment a variety of foods for at least 4,000 years. In particular, *Lb. acidophilus* and *Lb. delbrueckii* subspecies *bulgaricus* have been used in the production of these traditional fermented foods.

Table 1.1 Examples of the food, feed products and beverages produced using LAB and the typical strains used in their manufacture (Hammes and Vogel, 1995; Djordjevic *et al.*, 1997; Wood, 1998)

Fermented Product	Example of Product	Examples of the LAB used to ferment these products
Vegetables	Vegetables are fermented worldwide mostly on a small scale and in household amounts, but others are of significant economic importance, particularly olives, sauerkraut (produced from cabbage) and cucumbers.	*Lb. bavaricus, Lb. brevis, Lb. delbrueckii, Lb. plantarum, Lactococcus lactis, Leuconostoc mesenteroides, Pediococcus pentosaceus*
Silage fermentation	Silage is a form of conserved forage that has become an important source of animal feed over winter months.	*Lb. plantarum, Lb. curvatus*
Sourdough bread and related products	A multitude of baked goods can be made using a sourdough process, including panettone, different types of brioche and rye breads.	*Lb. pontis, Lb. sanfrancisco*
Alcoholic and non-alcoholic beverages	These include the production of wine, beer, saké and distilled spirit. In wine manufacture LAB are used to de-acidify the product through malolactic fermentation.	*Lb. saké , Leuconostoc oenos,*
Dairy foods	Fermented milk, yoghurt, cheese, kefir, buttermilk and cultured cream represent about 20% of the value of fermented food production worldwide.	*Lb. acidophilus, Lb. delbrueckii, Streptococcus species, Lb. casei, Lb. kefir, Lb. helveticus, Lactococcus lactis*
Preserved meat	Fermented sausage	*Leuconostoc mesenteroides, Lb. casei, Lb. saké, Lb. curvatus*
Regional foods	Fermented foods particularly from Africa, central Asia, the orient and the Commonwealth of Independent States (CIS) are an important contribution to regional nutrition. Japan produces many fermented products such as koikuchi shoyu and miso in which LAB contribute to the final product while kimchi is a popular and traditional way of preserving vegetables in Korea.	Principally *Lactobacillus species*

This practice has improved flavour and texture without adversely affecting other organoleptic properties of the food (Hull *et al.*, 1992) and it has been noted that consumption of LAB has impacted positively on human and animal health (Vandamme *et al.*, 1996). The food industry currently seeks to offer the benefit of consumption of these bacteria as part of an increasing range of functional foods. Functional foods are similar in appearance to conventional foods but when consumed as part of a normal diet demonstrate physiological benefits and/or reduce the risk of chronic disease beyond basic nutritional functions (Scott and Lee, 1996). LAB have many uses in industry and agriculture and examples of these uses are listed in Table 1.1. They contribute to the long shelf life of these products and confer beneficial health effects on consumers by inhibiting the growth of food spoilage organisms and producing probiotic substances. Many of the attributes are due to the production of lactic acid.

1.1.2 Inhibition of food spoilage organisms

The basis of the ancient art of food preservation is often directly related to the fermentation activity of LAB. These bacteria can inhibit the growth of food spoilage organisms by several means:

- production of hydrogen peroxide by many starter culture bacteria, including *Lactobacillus delbrueckii* ssp. *bulgaricus*, which is considered to be an important food preservative (Yi *et al.*, 1998). The amount of H_2O_2 produced depends upon the strain and its production has been shown to inhibit the growth of *Clostridium* species, *Staphylococcus* species and some psychrotrophs (Wood, 1998);

- production of bacteriocins and bacteriocin-like substances (Tagg *et al.*, 1976; Fooks *et al.*, 1999) which are proteins that are antagonistic to a wide range of Gram-positive and some Gram-negative organisms;

- production of lactic acid and other acids (including acetic and formic acid), which reduce the extra-cellular pH below levels at which most neutrophiles can grow and survive (see section 1.1.6. for further discussion of lactic acid). This leads to the collapse of the proton motive force as un-dissociated acids transport protons across the membrane, which results in the acidification of the cytoplasm of food spoilage organisms (Kashket, 1987, Gonçalves *et al.*, 1997) and

- by competing successfully for nutrients so that the overall level of nutrients available to other bacteria is reduced, causing poor growth of food spoilage and pathogenic bacteria (Saloff-Coste, 1997).

1.1.3 Probiotics: the concept

The concept of probiotics (literally meaning 'for life') is that certain bacterial groups, such as *Lactobacillus* and *Bifidobacterium* species, may have health promoting properties. These bacteria may reduce or prevent disease due to their high numbers and metabolic activities (Gibson and Roberfroid, 1995). This concept was first proposed by Elie Metchnikoff, who lived between 1845 and 1916, one of the first researchers into probiotic organisms and their affect on human health. Metchnikoff (1907) studied a group of Balkan people and concluded that their long life span was in part due to the consumption of large quantities of LAB in fermented foods. He also claimed that lactic acid and other metabolites, present in sour milk fermented by LAB, inhibited the growth and toxicity of the anaerobic, spore-forming bacteria commonly found in the bowel and suppressed "unhealthy fermentation in the intestine". Metchnikoff suggested that the microflora of the large bowel must be detrimental to the host and he attributed premature ageing to the circulation of toxic compounds produced by organisms in the intestinal tract and then absorbed into the blood stream. His advice at the time was to remove the large bowel surgically, which would eliminate the source of

these toxins. However, Metchnikoff also offered an alternative suggestion of administering lactic acid and/or lactic acid-producing bacteria to achieve the same effect. At that time, it was recognised by some scientists that lactic acid produced by LAB inhibited the growth of non-acid tolerant bacteria, thus replacing toxin-producing bacteria with beneficial bacteria (Tannock, 1995). It must be pointed out, however, that there were some commentators who were less than devoted to Metchnikoff's theories. These included Faust (1910), who found that Metchnikoff's work on LAB and lactic acid was "too heuristic and optimistic to be worth much attention".

1.1.4 Probiotics: definitions of probiotic, prebiotic and synbiotic

The term 'probiotic' was originally coined by Lilly and Stillwell (1965) to describe substances produced by one organism that stimulate the growth of another. However, the current use of the term is attributed to Fuller (1992) and is described as "an organism or substance, which contributes to intestinal microbial balance" (Hull *et al.*, 1992). Other authors (Havenaar and Huis in't Veld, 1992) have expanded this description to "a mono- or mixed-culture of live organisms which, applied to man or animal, beneficially affects the host by improving the properties of the indigenous microflora". The latter definition excludes the use of non-digestible oligosaccharides (NDOs) and other food products (nutraceuticals) that have also been shown to exhibit an effect that would be labelled as 'probiotic' under Fuller's definition. More recently Salminen (1999) argued that non-viable cells and cell components should be included in any definition of the term. For the purpose of this thesis, a probiotic is defined as "a microbial cell preparation or components of microbial cells that have a beneficial effect on the health and well being of the host" (Salminen *et al.*, 1999). Other "non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, that have the potential to improve host health" are considered here to be prebiotics (Rowland, 1998; Sako *et al.*, 1999). In addition to the definitions of probiotic and prebiotic, a third term is coming into common use: synbiotic, which

describes the use of a mixture of probiotics and prebiotics (Fooks *et al.*, 1999). For example, synbiotic yoghurt may contain a probiotic organism like *Lb. acidophilus* or *B. bifidum* and a NDO prebiotic such as inulin. If the prebiotic cannot be fermented by the resident microflora, but is readily metabolised by the co-administered probiotic organism, the impact of the live organism is enhanced so that the effect is synergistic (Rowland, 1998). This synbiotic yoghurt would also be classified as a functional food as the sum benefits of its consumption would be greater than the simple nutritional outcome achieved by eating the food.

1.1.5 Probiotics: criteria for selection for use

Marks (1996), Reid (1999), Ouwehand (1999) and Kailaspathy (2000) described the following selection criteria:

- must be totally safe;
- must resist destruction by gastric and intestinal enzymes and by oxygen;
- must show reasonable persistence in the intestinal tract;
- should demonstrate antagonism to harmful bacteria;
- must possess desirable enzyme patterns in that;
    - the enzymes present in a probiotic bacteria should not act to produce toxic metabolites and
    - should be capable of hydrolysing a variety of the compounds found in the diet, which allows fuller use of the nutritional value of food. In particular the enzymes

present in probiotic bacteria should aid the digestion of sugars such as lactose and sucrose (Wood, 1998);

- must meet the requirements for use in large-scale industrial production of food including sensory properties, stability and viability and

- must show demonstrable positive health effects in animal studies.

Marteau and Rambaud (1996) point out that while some endogenous LAB have been responsible for rare cases of local or systemic infection, no cases of illness have yet been demonstrated after ingestion of probiotic LAB.

1.1.6 Potential benefits of the consumption of probiotic foods

Benefits claimed to be associated with the consumption of LAB fermented foods include: reduction in pH within the gastrointestinal tract, improved lactose utilisation, prevention of diarrhoea, competitive exclusion of intestinal pathogens, anti-tumour activity, stimulation of the immune system, and control of serum cholesterol level (Fernandes *et al.*, 1987; Hoier, 1992; Hammes and Tichaczek, 1994; Duncan and Edberg, 1995; Playne, 1995; Mattila-Sandholm *et al.*, 1999; Tannock, 1999). Each of these benefits will be discussed in detail below.

1.1.6.1 Consumption of lactic acid

The consumption of lactic acid, produced by LAB bacteria, is thought to influence human health positively in several ways. Oberman and Libudzisz (1998) claim the following mechanisms, in particular, to be important:

- the accumulation of lactic acid within the intestine may influence the composition of the indigenous microflora by inhibiting the growth of undesirable organisms;

- lactic acid influences the physical properties of food. This may result in easier digestion and improved peristalsis of the food. Additionally, in the elderly, where secretion of gastric juice may be reduced, lactic acid decreases stomach acidity which increases the absorption of calcium and iron and

- the L(+) form of lactic acid is capable of being absorbed through the large intestine where it is utilised as an energy source. D(-) lactic acid is excreted by humans and it is recommended that LAB used in food manufacture produce the L(+) form.

1.1.6.2 Improved lactose utilisation

Lactose, the main carbohydrate in milk, must be digested by the enzyme lactase (β-galactosidase) into its constituent sugars, glucose and galactose, before these are absorbed from the gut. Some individuals have low lactase activity in the small intestine that is associated with age and racial characteristics (this metabolic deficiency is said to be present in half the adult population of the world) and therefore can digest only limited amounts of dairy products containing lactose. Failure to hydrolyse lactose leads to its fermentation in the large intestine, which may result in diarrhoea, flatulence and abdominal pain (Oberman and Libudzisz, 1998). Lactase-intolerant individuals better tolerate foods containing fermented lactose, for example yoghurt, because of the added beta galactosidase in this diet and in addition the fermentation process of yoghurt production converts some of the lactose present in milk to lactic acid.

1.1.6.3 Prevention of diarrhoea

The prevention and control of viral, bacterial and antibiotic-associated diarrhoea has been attributed to the intake of probiotic bacteria, although the evidence to support the use of probiotics in the prevention of all types of diarrhoea is equivocal (Marteau and Rambaud, 1996). Some studies have shown that the duration and severity of rotavirus infection can be reduced through the

administration of *Lb. rhamnosus* GG. There is evidence of protection against "antibiotic associated enteric disorders" but studies, repeated by other authors, have not always been able to replicate the demonstrated effect (Marteau and Rambaud, 1996). Variation in experimental results may be due to poor experimental protocol, the choice of strain selected for each trial and the difficulties in obtaining samples from human volunteers while adhering to ethical considerations (Playne, 1995).

1.1.6.4 Control of intestinal pathogens

Intestinal pathogens may be controlled by competition for nutrients present in the intestine in limited quantities, competition for attachment sites on the mucosal cells, creation of selective environmental conditions and formation of antagonistic compounds (Hull *et al.*, 1992; Fuller, 1993). These are similar to the mechanisms by which LAB inhibit food spoilage organisms, as discussed in section 1.1.2. Studies have shown that lactic acid bacteria in particular *Lb. casei* and *Lb. acidophilus* can inhibit the growth of *Helicobactor pylori*, a cause of stomach ulcers, but the role of probiotic organisms in eradicating *H. pylori* from ulcer sufferers remains to be determined (Canducci *et al.*, 2002; Wendakoon and Ozimek, 2002; Sgouras *et al.*, 2004).

1.1.6.5 Anti-tumour activity and stimulation of the immune system

Claimed anti-cancer properties of probiotic organisms have been attributed to:

- conversion or elimination of procarcinogenic or carcinogenic compounds;
- reduction of the level of faecal enzymes responsible for the conversion of procarcinogens into carcinogens; and
- the stimulation of the immune system (Hammes and Tichaczek, 1994; Tannock *et al.*, 1999)

Faecal enzymes (for example, β-glucuronidase, β-glucosidase, urease, azoreductase and nitroreductase) have been used to monitor mucosal carcinogenesis as they convert procarcinogens to carcinogens. It is believed that higher levels of these enzymes are associated with a higher cancer risk (Oberman and Libudzisz, 1998). The consumption of LAB appears to moderate the effect of these faecal microbial enzymes, possibly by a general reduction in the numbers of organisms producing xenobiotic metabolising enzymes; LAB have low levels of xenobiotic enzymes. LAB also appear to have a role in preventing DNA damage, suppressing pre-neoplastic changes and suppressing tumours, although the mechanisms for these are unclear (Ebringer *et al.*, 1995; Rowland, 1996). Suppression of cancer activity may also be controlled indirectly through modulation of the immune system. It has been shown that whole cells and cell wall fragments of LAB activate macrophages in humans and animals. It has also been found that some probiotic bacteria (*Lb. acidophilus*, *Lb. delbrueckii* subsp. *bulgaricus* and bifidobacteria) influence the *in vitro* regulation of γ-interferon (Oberman and Libudzisz, 1998).

1.1.6.6 Control of serum cholesterol levels

A high cholesterol level is a risk factor for cardiovascular disease in humans and one study (Fernandes *et al.*, 1987) has shown a reduction in serum cholesterol associated with consumption of fermented foods. A study by Taranto *et al.* (1997) suggested that cholesterol reduction is linked to bacterial bile salts hydrolase activity, which causes the release of unconjugated bile acids resulting in cholesterol precipitation.

1.1.6.7 Consumption of bio-active peptides produced during fermentation of milk products

Milk proteins, upon ingestion, are broken down by gastrointestinal enzymes to produce a range of peptides, many of which have been identified as being bioactive. These peptides may also be formed by the action of bacterial enzymes during fermentation of milk by LAB. The biological

significance of these peptides, their impact on human health and production of novel functional food ingredients containing these peptides, are currently the subject of intensive research (Playne *et al.*, 2003). For example, some bioactive peptides that demonstrated an anti-hypertensive effect have been detected in sour milk. These peptides showed an inhibitory effect on angiotensin I-converting enzyme and could only be demonstrated after fermentation of casein by lactic acid bacteria. The same peptides could not be demonstrated after hydrolysis of casein (Yamamoto and Takano, 1999). Use of these bioactive peptides for pharmaceutical or other commercial use, for example as a functional food used to treat borderline hypertension, seems increasingly likely.

1.1.6.8. Treatment of irritable bowel syndrome, ulcerative colitis and Crohn's disease

The results of clinical trials, with respect to treatment of bowel disease, have been encouraging; however, only small numbers of patients have been included in each trial. These results also indicate that in the case of ulcerative colitis, acute pancreatitis and irritable bowel syndrome, available data has demonstrated benefits of probiotic therapy, but in the case of Crohn's disease, the role of probiotics is not yet clearly defined (Kirchgatterer and Knoflach, 2004).

1.2 Lactic acid bacteria

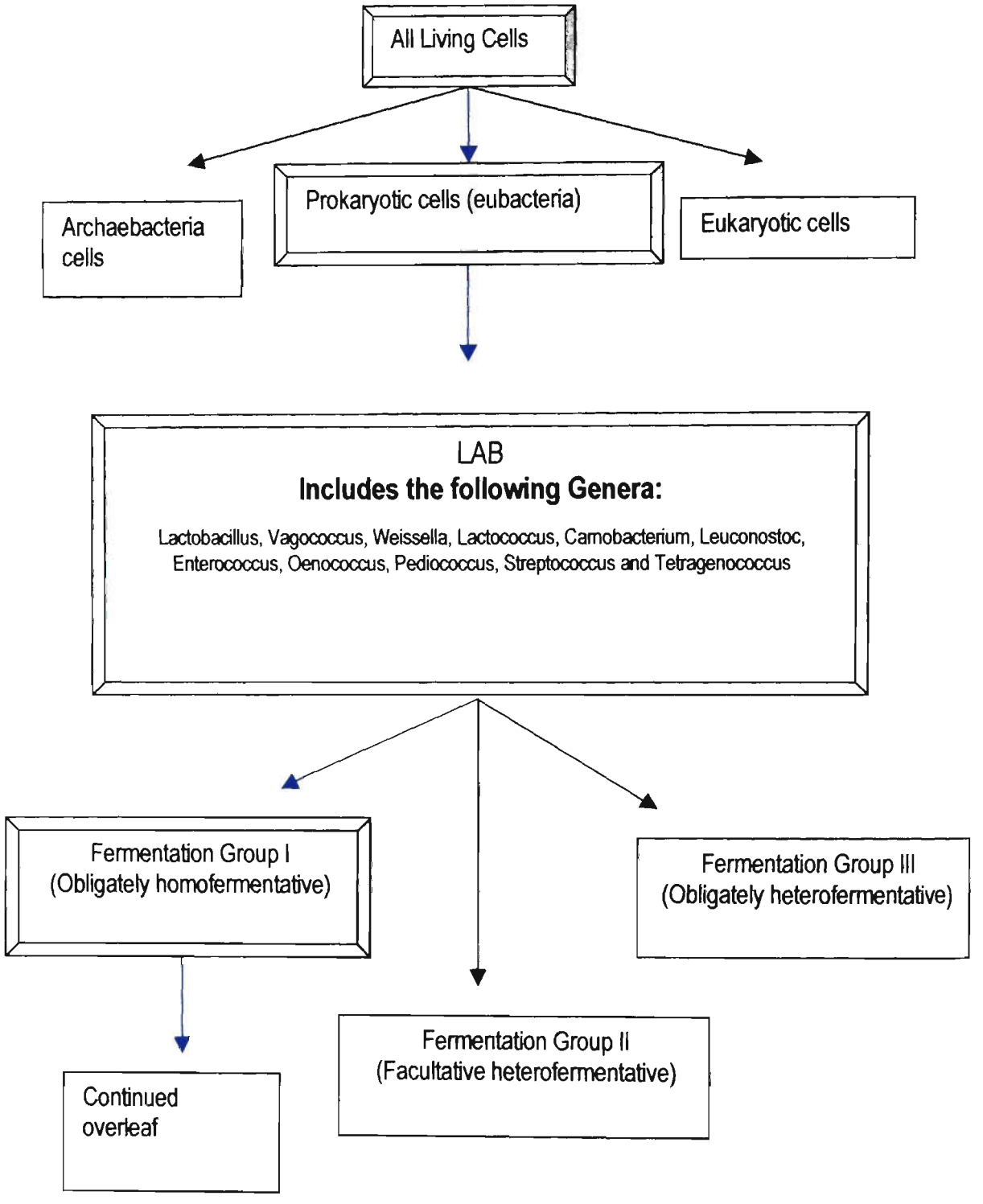
Since the first description of bacteria, scientists have systematically divided them into several groups to aid taxonomic classification. The end-point of classification is the species and it consists of the type strain and all other strains sufficiently similar to be included in the species with it. Unlike higher organisms, bacteria are often similar in nature, resulting in a greater need for methods that separate them into species, sub-species and strains. LAB have been a particular challenge in this regard and in recent years new methods of speciation of lactobacilli have resulted in many changes in classification. Bacterial classification is built on two major kinds of information, phenotypic and phylogenetic. Phenotypic information describes the physical appearance and other direct

observations of bacteria, including size, shape and biochemical traits. Phylogenetic information comes from the developmental history of an organism and is derived from comparison of the structure of highly conserved macromolecules within the cell (DNA and rRNA). Classification systems for LAB based on phenotypic and phylogenetic data are described in detail in the following sections.

1.2.1 Introduction to LAB

LAB are Gram-positive, prokaryotic organisms that are commonly found in a variety of environmental niches; they are commensal colonisers of mammalian gastrointestinal tracts and are found in soils. LAB produce lactic acid as the common end-product of metabolism and can be broadly classified as either homo- or hetero-fermentative, based on the principal end-products made. LAB require a fermentable carbohydrate for growth and those that convert glucose predominantly (more than 90%) to lactic acid are classified as homo-fermentative. Those producing lactic acid plus one or more moles of acetic acid, ethanol and carbon dioxide are classified as hetero-fermentative. Homo-fermentative LAB convert glucose to lactate through the Embden-Meyerhof-Parnas (EMP) pathway by means of a fructose-1,6-diphosphate aldolase.

Hetero-fermentative LAB produce equimolar amounts of lactate, ethanol and carbon dioxide as major end-products through the phosphoketolase pathway by means of a xylulose-5-phosphate phosphoketolase (Saloff-Coste, 1997). There are numerous members of the LAB group including the following genera: *Streptococcus*, *Lactobacillus*, *Lactococcus*, *Carnobacterium*, *Oenococcus*, *Pediococcus*, *Tetragenococcus*, *Vagococcus*, *Enterococcus*, *Weissella* and *Leuconostoc*. The position of these genera in LAB classification can be seen in Figure 1.1.


All Living Cells
Archaebacteria cells
Prokaryotic cells (eubacteria)
Eukaryotic cells
LAB
Includes the following Genera:
Lactobacillus, Vagococcus, Weissella, Lactococcus, Carnobacterium, Leuconostoc, Enterococcus, Oenococcus, Pediococcus, Streptococcus and Tetragenococcus
Fermentation Group I (Obligately homofermentative)
Fermentation Group III (Obligately heterofermentative)
Fermentation Group II (Facultative heterofermentative)
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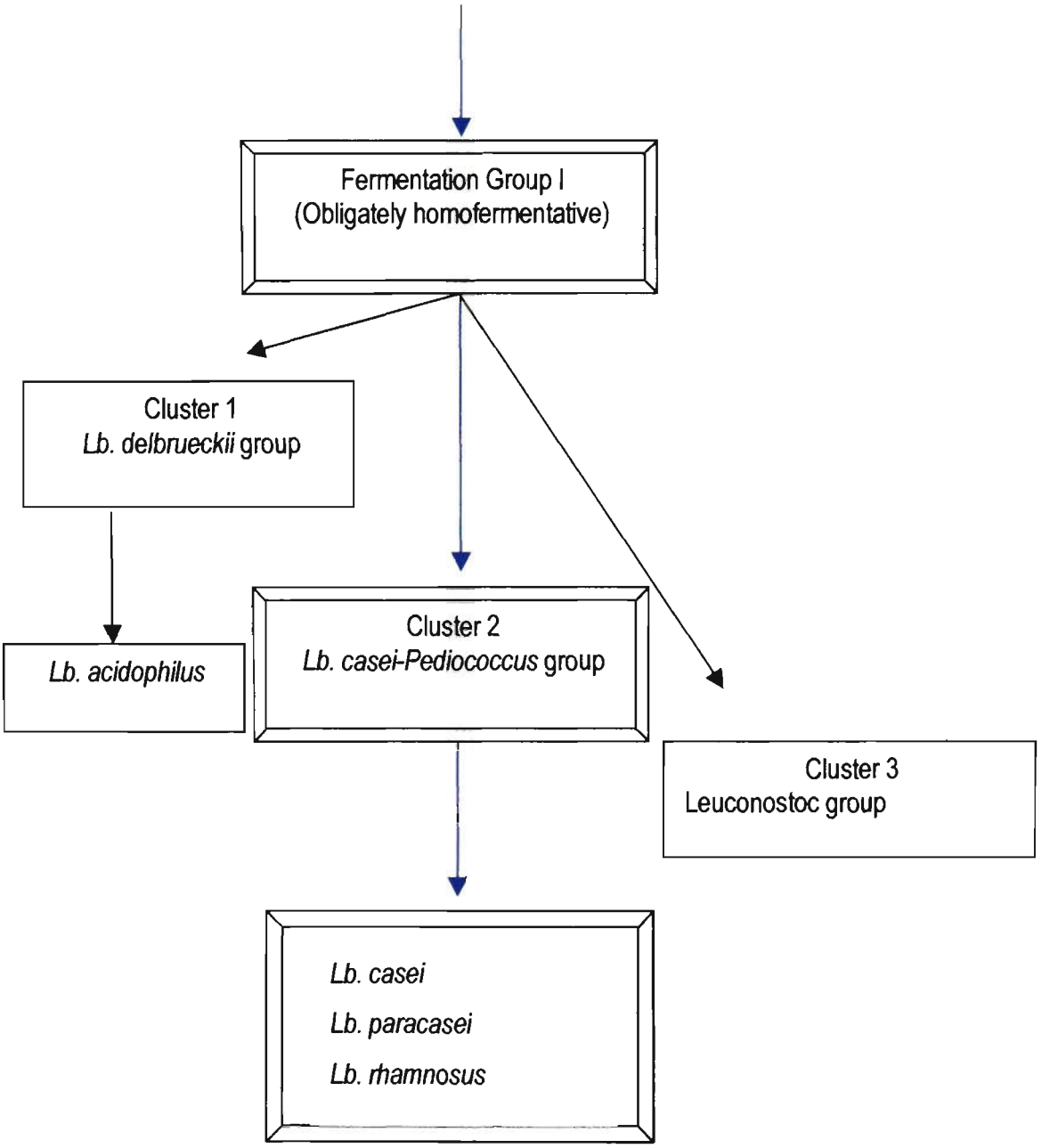


Figure 1.1 Overview of the classification of LAB.

Generally, LAB can be sub-divided into three phenotypic groups according to their fermentation patterns. The groups are further subdivided into three clusters based on similarity of rRNA sequences or DNA-rRNA hybridisations. *Lb. paracasei* is a member of Cluster 2 and fermentation group I.

1.2.2 The genus *Lactobacillus*

There are currently more than 100 members of the genus *Lactobacillus* and they are genetically quite diverse (Hammes and Tichaczek, 1994; Schleifer and Ludwig, 1995; Euzeby, 2003). LAB are divided into three phenotypic groups, which may then be further sub-divided into either three or five phylogenetic clusters according to the classification systems of either Collins or Schleifer (Collins *et al.*, 1991; Hammes and Vogel, 1995; Schleifer and Ludwig, 1995). The three major phenotypic groups are:

- obligate homofermentative lactobacilli, which ferment hexoses to lactic acid;
- facultative heterofermentative lactobacilli, which ferment hexoses to lactic acid only or to lactic acid and acetic acid, ethanol and formic acid under glucose limitation and;
- obligate heterofermentative lactobacilli, which ferment hexoses to lactic acid, acetic acid, ethanol and carbon dioxide, and pentoses to lactic acid and acetic acid (Pot *et al.*, 1994).

However these phenotypic groups do not reflect the classifications based on phylogenetic relationships. The relationships, based on reverse transcriptase sequencing of 16S rRNA, are better described by the three clusters of Collins (1991). The three phylogenetic clusters are:

- Cluster 1: The *Lb. delbrueckii* group. This group, principally consisting of obligately homofermentative LAB, includes *Lb. delbrueckii* and *Lb. acidophilus.*
- Cluster 2: The Casei-pediococcus group. According to Collins (1991) this is the largest group and contains more than thirty *Lactobacillus* species. Included in this group are *Lb. casei, Lb. curvatus* and *Lb. fermentum*

Cluster 3: The *Leuconostoc* group. This group contains members of the genus *Leuconostoc* and obligately hetero-fermentative lactobacilli. Schleifer (1995) proposed a different classification based on an extended data set of 16S rRNA gene sequences and subdivision of Collin's so-called 'Casei-pediococcus group', however there appears to be no clear evidence that allows unambiguous allocation of bacteria to either of the classification systems proposed by either Collins and Schleifer.

The five groups proposed by Schleifer are: the *Lb. acidophilus* group, the *Lb. salivarius* group, the *Lb. reuteri* group, the *Lb. buchneri* group and the *Lb. plantarum* group.

1.2.3 Genus and species characteristics

The general features of the genus *Lactobacillus* are shown in Table 1.2. Historically, identification of *Lb. casei* was based upon a number of characteristics, including:

- colonial morphology;
- growth requirements;
- staining behaviour and
- carbohydrate utilisation tests.

More recently, identification based on analysis of chemical and biochemical traits have been added to these identification methods. These include the characterisation of:

- enzyme profiles such as measurement of peptidase and esterase activity (Tsakalidou *et al.*, 1994) as well as characterisation of lactic dehydrogenase (Gasser, 1970);
- whole cell protein profiles (Pot *et al.*, 1993; Patarata *et al.*, 1994; Tsakalidou *et al.*, 1994); and

Table 1.2 General features of the genus *Lactobacillus* (Hansen and Mocquot, 1970; Collins *et al.*, 1991; Holt *et al.*, 1994; Hammes and Vogel, 1995)

Characteristic	Description
Shape	Rod-shaped
Gram stain	Positive
Spores	Not present
Motility	Rare
G+C content of DNA	32-54 %
Growth environment	Facultative anaerobes
Metabolism	Fermentative and Saccharoclastic
Nitrate	Not reduced
Gelatine	Not liquefied
Catalase	Negative
Cytochrome	Negative
Optimum growth temperature	30-40°C
Normal distribution	Widely distributed including soil, mammal gastrointestinal tracts and food products

- fatty acid methyl esters (FAMEs).

The relative usefulness of each of these methods varies greatly and in general around 17 phenotypic tests are required to identify a *Lactobacillus* isolate (Hammes and Vogel, 1995). Generally, these techniques only help when identifying an organism to genus level but some of the techniques are useful in separating strains. These phenotypic methods are rapidly giving way to molecular techniques, which are fast to perform and increasingly relatively cost effective. Molecular techniques for classification are described in section 1.2.5.

1.2.4 An early history of the identification of *Lactobacillus* species

Orla-Jensen (1919) divided the lactobacilli into three physiological groups: *Betabacterium*, *Streptobacterium* and *Thermobacterium*, the classification being based on physiological and nutritional characteristics as well as the method of fermentation. The identification also took into account the optimum growth temperature and the optical configuration of the lactic acid produced. One of lactobacilli Orla-Jensen classified, *Thermobacteriuim intestinale*, was subsequently noted to have all of the features corresponding to *Lb. acidophilus*. Rogosa and Sharpe (1959) extended this work by including temperature growth ranges and vitamin requirements in the identification procedure (Gasser, 1970).

Moro is believed to be the first person to describe *Lb. acidophilus,* which he did in 1900 under the name *Bacillus acidophilus* (Hansen and Mocquot, 1970). The first use of *Lactobacillus* as a genus is attributed to Beijerinck in 1901 (Vandamme *et al.*, 1996). In 1920, the organism that Moro described was included by Holland in the genus *Lactobacillus,* although it is believed that the organism that was described by Moro was accidentally substituted or destroyed before this time (Johnson *et al.*, 1980; Fujisawa *et al.*, 1992). Hansen and Mocquot were the first to publish the name *Lb. acidophilus*, based on a combination of the name *Bacillus acidophilus* and a bacterium

having the general characteristics of the genus *Lactobacillus* (Hansen and Mocquot, 1970). Hansen and Mocquot gave a complete phenotypic description of the bacterium on which to base identification. According to Hansen, the name *Lb. acidophilus* had been widely used, but no valid description to allow identification had been previously published. Following the recommendations of the International Subcommittee on the Taxonomy of the lactobacilli and closely related organisms, the name as *Lb. acidophilus* was confirmed and ATCC 4356 was designated as the type strain (Hansen and Mocquot, 1970). The identification of lactobacilli based upon differentiation into fermentation groups persisted until the late 1970s, when the first rRNA gene sequencing methods for bacterial speciation became available. This enabled phenotypic and phylogenetic data to be married.

It was also around the early twentieth century that an organism describes by Orla-Jensen as *Lactobacterium casei* was reclassified as *Streptobacterium casei*. The first use of the current name *Lb. casei* is attributed to Hansel and Lessel in 1971 and ATCC 393 was designated the type strain.

1.2.5 Recent history of *Lactobacillus* identification

Research into the classification of all *Lactobacillus* species advanced rapidly with the availability of molecular biology tools such as polymerase chain reaction (PCR) and DNA sequencing. In fact, genus level identification relied on classical methods until 2002 when Dubernet described *Lactobacillus* sp. primers for PCR-based identification. However, molecular biology tools have allowed specific identification of species and differentiation between strains within species. During the past decade, there have been a large number of taxonomic reassignments that have been based principally on DNA-rRNA hybridisation and rRNA sequencing. These have indicated large discrepancies between classical taxonomy and molecular taxonomy (Collins *et al.*, 1991; Decallone *et al.*, 1991; Pot *et al.*, 1993; Rodtong and Tannock, 1993; Dicks *et al.*, 1995; Rementzis and Samelis, 1996).

DNA-DNA hybridisation gives a measure of relatedness across the whole genome but is not particularly useful for identification of an unknown organism, as a large number of known organisms may have to be tested before a close match is found (Schleifer and Ludwig, 1995). DNA-DNA hybridisation has had limited use in differentiating LAB strains, but it has been used to differentiate *Carnobacterium* species (Dicks *et al.*, 1995) and to confirm re-assignment of lactobacilli (Fujisawa *et al.*, 1992). A refinement of this technique, DNA-rRNA hybridisation, introduced in the early 1970s, allows discrimination of organisms at family and genus level. It has been a principal tool used in the re-classification of LAB although this has now been overtaken by the use of ribotyping (Vandamme *et al.*, 1996).

Ribotyping analyses variations in the chromosomal positions or structure of rRNA (Schmidt, 1995). Ribosomal RNA has been conserved through millions of years of evolutionary divergence and most prokaryotes have three rRNAs, called 5S, 16S and 23S. The 5S is usually too small for reliable testing and only 16S and 23S are used for phylogenetic comparison. In prokaryotes, 16S rRNA is primarily used to trace evolution and determines the extent of relatedness of bacteria (Schleifer and Ludwig, 1994; Schleifer and Ludwig, 1995). Meaningful phylogenetic data has been acquired from multiple operons and multiple isolates (Amann *et al.*, 1994). Sequence information so obtained has been used to differentiate fifty-four *Lactobacillus* strains isolated from the intestinal tract of mice (Rodtong and Tannock, 1993). Within the last 20 years, the development of PCR methods has allowed rapid and certain identification of strains of LAB, although some sub-species assays still fail to distinguish between bacteria with closely related 16S sequence homology such as sub-species of *Lb. delbrueckii* (Torriani *et al.*, 1999).

PCR identification of probiotic bacteria has been used to:

- confirm subspecies identities using randomly amplified polymorphic DNA PCR (RAPD-PCR) based on the proline iminopeptidase gene sequence (Torriani *et al.*, 1999);

- detect specific probiotic organisms in animal feed (Castellanos *et al.*, 1996). Castellanos amplified a variable region of 16S rDNA of about 200 bp in *Lb. plantarum, Lb. rhamnosus* and *Lb. fermentum*; and

- identify *Lactobacillus* isolates from the gastrointestinal tract, silage and yoghurt using the 16S-23S intergenic spacer region (Tannock *et al.*, 1999).

The current status of *Lb. casei* has been the subject of much discussion. A number of opinions have been put forward as to whether *Lb. casei* should remain a single species made up of several sub-species (Dellaglio *et al.*, 2002) or whether the former subspecies of *Lb. casei,* except the type strain, should be included within the species *Lb. paracasei* (Collins *et al.*, 1989). The name *Lactobacillus paracasei* subsp. *paracasei* was first proposed by Collins (1989).

In 1991 it was first proposed that the *Lb. casei* type strain, ATCC 393, be reclassified as *Lb. zeae* and replaced by ATCC 334 (Dellaglio *et al.*, 1991). Although that proposal was rejected, it was resubmitted in both 1996 and 2002 (Dicks *et al.*, 1996; Dellaglio *et al.*, 2002). The basis of the proposal was the low levels of DNA homology (30-50%) of the type strain compared with other strains of *Lb. casei* and sequence similarity of a 277 bp recA gene fragment. The status of the type strain is still unresolved.

Although yet to be confirmed by the Judicial Commission and subject to International code of Nomenclature of Bacteria, the current proposed reclassification is that the species name *Lb. paracasei* is rejected and strains previously classified as *Lb. casei* be reunited within the species *Lb. casei*.

1.2.6 The genus *Bifidobacterium*

Bifidobacteria were originally classified within the general 'lactic acid bacteria' group set aside by Orla-Jensen in the 1920s. This was based on the physiological observation that bifidobacteria produce lactic acid as a major product of fermentation. However 16S rRNA profiles show distinct differences to lactobacilli and other LAB (Schleifer and Ludwig, 1995).

Thirty-one members of the genus *Bifidobacterium* are currently recognised with the most recent members, *B. inopinatum* and *B. denticolens* being added in 1996 (Table 1·3) (Crociani *et al.*, 1996). They are usually isolated from human and animal sources, for example from dental caries, intestines and genital tracts (Gomez *et al.*, 2000). However, isolates have also come from wastewater, honey bees and fermented dairy products (Pot *et al.*, 1994). According to Tamine (1999) the species of *Bifidobacterium* found in humans are *B. adolescentis, B. bifidum, B. breve, B. infantis, B. lactis* and *B. longum*. Bifidobacteria are phylogenetically grouped in the actinomycete branch of the Gram-positive bacteria and are characterised by a high G+C content that varies from 54 to 67%. They possess the glycolytic fermentation enzyme, fructose-6-phosphate phosphoketolase, the key enzyme of the 'bifid shunt', which characterises the species. The end-products of glucose metabolism are acetic and lactic acids, which occurs without generation of CO_2 (Gomez and Malcata, 1999). They are saccharolytic organisms and all *Bifidobacterium* isolated from human sources have been able to utilise glucose, galactose and lactose as carbon sources (Gomez and Malcata, 1999). Bifidobacteria are generally characterised as non-spore-forming, non-motile, and catalase-negative anaerobes. Various shapes have been noted from short curved rods to bifurcated Y-shaped rods. According to Gomez and Malacata (1999), *Bifidobacterium* have an optimum growth pH between 6·0 and 7·0 and virtually no growth occurs below pH 4.5-5.0 or above pH 8.0-8.5.

Table 1.3. List of species of the genus *Bifidobacterium,* and origin and year species first reported (Krzewinski, 1997; Krzewinski, 2002)

B. adolescentis	Adult faeces (1963)
B. angulatum	Adult faeces (1974)
B. animalis	Rat, rabbit and calf faeces (1969)
B. asteroides	Bees 1969)
B. bifidum	Child and adult faeces, vagina (1924)
B. boum	Rumen and piglet faeces (1979)
B. breve	Child faeces, vagina (1963)
B. catenulatum	Child and adult faeces, vagina (1974)
B. choerinum	Piglet faeces (1979)
B. coryneforme	Bees (1969)
B. cuniculi	Rabbit faeces (1979)
B. denticolens	Buccal cavity (1996)
B. dentium	Buccal cavity (1974)
B. gallicum	Adult faeces (1990)
B. gallinarum	Chicken faeces (1983)
B. globosum	(unknown)
B. indicum	(unknown)
B. infantis	Child faeces, vagina (1963)
B. inopinatum	Buccal cavity (1996)
B. lactis (subjective synonym of *B. animalis*)	Fermented milk (1997)
B. longum	(unknown)
B. magnum	Rabbit faeces (1974)
B. merycicum	Rumen (1991)
B. minimum	Sewage (1974)
B. pseudocatenulatum	Child faeces (1979)
B. pseudolongum	Animal faeces (1969)
B. pullorum	Chicken faeces (1974)
B. ruminantium	Rumen (1991)
B. saeculare	Rabbit faeces (1991)
B. subtile	Sewage (1974)
B. suis	Piglet faeces (1971)
B. thermophilum	Animal faeces, rumen (1969)

The optimum growth temperature is 37-41°C, with a maximum temperature that permits growth around 43-45°C and virtually no growth below 25-28°C (Gomez and Malcata, 1999). The general features of the genus *Bifidobacterium* are shown in Table 1.4. Identification of *Bifidobacterium* to genus level may be unreliable due to non-specific cultural and physiological characteristics. Identification based on fermentation products is especially difficult as variable amounts of formic acid, acetic acid, succinic acid and ethanol may occur depending on growth conditions (Gomez and Malcata, 1999).

1.2.7 History of the genus *Bifidobacterium*

Bifidobacteria were first described by Tissier in 1899-1900 and the first isolation, *Bacillus bifidus communis*, was from infant faeces (Gomez and Malcata, 1999). The description of the genus came several years later by Orla-Jensen in 1924, when *Bacillus bifidus* was renamed *B. bifidum* although the organism was also referred to as *Lactobacillus bifidus*. After 1960, the genus was enlarged to 16 members and differentiation was based on fermentative and serological features (Pot *et al.*, 1994). With the introduction of identification based on DNA-DNA hybridisation and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), several more species have been described, although taxonomy based on molecular methods has conflicted with phenotypic methods (Miyake *et al.*, 1998).

1.2.8 Species characteristics

*B. animalis (*syn. *lactis)* strain VUP Bb 12, used in this study, was originally known as a strain of *B. bifidum,* but when subjected to molecular biology typing (16S rRNA) it was found that the identification did not fit into any existing species of *Bifidobacterium* and was classified as a novel species called *B. lactis* (Wind, 2001). *B. lactis* was first described as a novel oxygen tolerant species isolated from milk (Meile *et al.*, 1997).

Table 1.4 General features of the genus *Bifidobacterium* (Sgorbati *et al.*, 1995; Krzewinski, 1997; Gomez and Malcata, 1999; Tamine, 1999).

Characteristic	Description
Shape	Rod-shaped including: uniform, branched, bifurcated Y and V forms, spatulate and club shaped rods
Gram stain	Positive
Spores	Not present
Motility	Negative
G+C content of DNA	54-67 %
Growth environment	Strict anaerobes
Metabolism	Fermentative and saccharoclastic
Catalase	Negative
Optimum growth temperature	37-41°C
Normal distribution	Widely distributed including mammal gastrointestinal tracts and food products

This new species name was however subsequently rejected by The International Committee on Systematic Bacteriology (ICSB), Subcommittee on the Taxonomy of Bifidobacterium, Lactobacillus and related organisms (Klein, 2001).

Unpublished work by Chr. Hansen on 16S rRNA and other genetic sequences of *B. lactis* strain Bb 12, shows significant differences between *B. animalis* and *B. lactis* strain Bb 12 (Wind, 2001). Wind suggests, however, that these differences are not sufficient to place strain Bb 12 in a novel group but further research is required to give a definitive identification of the organism. This view is supported by Cai *et al.* (2000), who suggest that *B. lactis* should be considered a junior subjective synonym of *B. animalis*.

Chr. Hansen refer to strain Bb 12 as *B. animalis* (syn. *lactis*) Bb-12, which is line with current accepted nomenclature of ICSB. Researchers from the Nestlé Research Centre analysed the 16S-23S internally transcribed spacer region of strains of *B. lactis* and *B. animalis* (Ventura *et al.*, 2001). Based on that analysis, they concluded that *B. lactis* should not be considered a junior subjective synonym of *B. lactis* but rather be reclassified as a subspecies of *B. animalis.* This proposed reclassification remains unresolved at present. Table 1.5 lists the classical features on which an identification of *B. animalis* (syn. *lactis*) is based. *B. animalis* (syn. *lactis*) strain VUP Bb 12 has been used by the starter culture manufacturer Chr. Hansen since 1986 and is included in several product lines including ABT cultures, single cultures and anti-diarrhoea tablets. ABT cultures contain *Lb. acidophilus*, *S. thermophilus* and *B. lactis* strain Bb 12 (Wind, 2001).

1.3 Probiotic Lactic Acid Bacteria

The minimum basis for selection of probiotic bacteria for human use includes the following:

- that the bacteria produces lactic acid;

Table 1.5 Important characteristics of *B. animalis (Sgorbati et al., 1995; Krzewinski, 1997; Gomez and Malcata, 1999; Tamine, 1999).*

Characteristic (Growth factor or vitamin requirement)	**Usual Finding**		
Riboflavin	+		
Pantothenate	_		
Nicotinic acid	+		
Pyridoxine	_		
Thiamine	_		
Folic acid	+		
Tween 80	+		
Carbohydrates fermented *			
Glucose	+	Fructose	+
Galactose	+	Lactose	+
Maltose	_	Mannose	_
Mannitol	_	Salicin	_
Melibiose	d	Sucrose	d
Trehalose	_	Xylose	_

* Symbols: +, > 90% positive

d, 11-89% positive

-, < 10% positive

- is generally recognised as safe (GRAS); and

- has the ability to colonise/adhere to the gut wall (Playne, 1995).

Kailasapathy (2000) suggests additional factors that are important, including that the organism should survive gastric acidic conditions and be resistant to bile salts and digestive enzymes. These are discussed in sections 1.3.1 and 1.3.2. To be of any value as a probiotic, a microbe must survive two different sets of conditions prior to colonising the intestine: firstly, it must survive (possibly up to several weeks) in the food in which it is contained, then it must survive passage through the upper part of the digestive system, including the stomach, which secretes hydrochloric acid and digestive enzymes.

1.3.1 Survival of LAB in yoghurt

Consumers have indicated their preference for probiotic-containing foods and 20% of yoghurts now sold in Australia contain *Lb. acidophilus* and *B. bifidum* (Playne, 1995). However, the number of live organisms surviving in probiotic foods depends on the manufacturer and length of time since production (Shah *et al.*, 1995; Lankaputhra *et al.*, 1996; Kailaspathy and Rybka, 1997). In particular the survival of *Lb. acidophilus* in yoghurt is extremely variable, with numbers of viable organisms falling quickly after manufacture (Hull *et al.*, 1984; Kailaspathy and Rybka, 1997). A number of causes have been proposed to account for the loss of viability of these organisms, including the effect of hydrogen peroxide produced by starter culture organisms, low pH of storage, and oxygen penetration of storage containers. Condon (1987) claimed that in lactobacilli, toxicity of oxygen exposure varies with the strain, and loss of viability is not significant compared to reduction in viability in the strictly anaerobic *Bifidobacterium* species upon exposure to oxygen. Gilliland and Speck (1977) described the marked effect that hydrogen peroxide had on the survival of *Lb. acidophilus* in yoghurt. Laroia and Martin (1991) found that in frozen dairy desserts with low pHs,

no viable *B. bifidum* could be detected after manufacture. However, increasing the pH of the product resulted in increased survival of the bifidobacteria. Strain selection appears to be important. Clark *et al.* (1993) for example, commented on the variation of survival for each of four bifidobacteria strains (*B. infantis*, *B. longum*, *B. bifidum* and *B. adolescentis*), which were tested for the ability to survive simulated stomach pH conditions. In particular they found that *B. bifidum* did not survive brief exposure to low pH (<3) well and counts dropped several log cycles after only a few minutes. Some recent attempts at improving the viability of *Bifidobacterium* in yoghurt have concentrated on micro-encapsulation of the live bacteria (Adhikari *et al.*, 2000). The study demonstrated some improvement in viability of strains encapsulated in κ-carrageenan, but increased acid content in the product adversely affected sensory characteristics.

1.3.2 Survival of LAB in the digestive tract of humans

Early studies by Draser *et al.* (1969) indicated that the levels and types of intestinal bacteria change constantly. In particular the numbers of lactobacilli and bifidobacteria occasionally diminished to undetectable levels in particular parts of the intestine at certain times during the day. This is probably due to intestinal motility following the stimulation of a meal. They also found that bacteria consumed during meals are able to survive passage through the stomach because food partly neutralises acid levels. Acid-tolerant organisms have an even greater chance of surviving stomach acidity and subsequently surviving in the more alkaline conditions of the small intestine, and thus are more likely to colonise the lower gastrointestinal tract. In order to survive and colonise the GI tract, probiotic organisms must also have a high rate of epithelial attachment, a high growth rate (to prevent being washed out by peristalsis,) and resistance to organic acids and bile salts (Canganella *et al.*, 1996).

1.4 Bacterial responses to growth in stressing environments

1.4.1 Bacterial response to growth in stressed conditions

Stress has been variously described as "a disturbance of the normal functioning of a biological system caused by an environmental factor which is detected by a departure from steady state" and as "an agent which places an organism at a disadvantage as increased energy expenditure is required reducing the chances of its survival" (Hoffmann and Parsons, 1994). In this thesis, a slightly broader definition will be used in which the term 'stress' encompasses an environmental factor (a physical condition) that causes a change that is potentially injurious in a biological system. (Hoffmann and Parsons, 1994). Within this thesis, these environmental factors will be referred to as environmental stressors and any organism experiencing these stressors will be considered to be stressed or under stress. Some of the changes that may happen because of exposure to stress include changes in induction and repression of the synthesis of proteins (enzymes, isoenzymes, structural proteins and heat-shock proteins (HSP)).

1.4.2 Protein synthesis and folding in a normal cellular environment

The purpose of protein synthesis is to create a polypeptide and in all cells this activity occurs at the ribosome. Once formed, the polypeptide must adopt a specific, folded three-dimensional tertiary or native structure in order to become a biologically active protein (Ramakrishnan and White, 1998). Research performed by Anfinsen and Haber in the 1960s found that the amino acid sequence of a protein contained all the information required for an amino acid chain to properly fold into an active protein molecule (Gething and Sambrook, 1992; Ruddon and Bedows, 1997). These and other observations have led to some broad rules for protein folding. The first is that the folding of a polypeptide chain is determined by the amino acid sequence in a given environment. The second is that the structure is thermodynamically controlled and its stability is not very high. The third states that the protein-folding pathway probably involves intermediates, which may evolve into either properly or improperly folded proteins (Creighton, 1995). The amino acid sequence for each protein

specifies only the primary structure, yet the process of folding occurs rapidly after biosynthesis of the polypeptide chain and can even begin before the polypeptide chain is complete. Proteins are compact structures that consist of segments of secondary structure packed tightly together. When they are made up of a large number of residues (>200), they generally contain two or more independent structural units that are referred to as domains. These domains may or may not be identical. Over the last 30 years, a number of theories that model how proteins obtain a native configuration have been put forward although none of them adequately covers all the experimental observations to date. For example Baldwin (1989) described three folding models: (1) the hydrophobic collapse model, (2) the formation of secondary structure model and (3) the specific interaction model (Baldwin, 1989).

1. The hydrophobic collapse model.

This model proposes that the collapse of hydrophobic bonds results in a molten globule state from which the folded protein arises. The molten globule state is one where a protein is neither folded nor unfolded. In this state, the molecule is generally compact and may contain considerable secondary structure but is disordered compared to a fully folded protein.

2. The formation of secondary structure model.

This model suggests that a tertiary structure can originate from the formation of stable secondary structures. The appearance of stable secondary structures produced by random folding may serve as a template for folding the remaining polypeptide chain.

3. The specific interaction model.

This model proposes the formation of specific structures from stabilising interactions. An example is the formation of one correct disulphide bond that leads to the formation of other correct disulphide

bonds (Nilsson, 1991). Creighton (1997) reviewed the significance of protein folding models and highlighted the drawbacks of each. From work on α-lactalbumin, he argued that the molten globule state and other stable intermediates might simply be energetically preferred, but non-productive, forms of the protein. In addition, with regard to the specific interaction model, Creighton pointed out that disulphide bonds probably stabilise proteins only after they have reached a fully-folded conformation and therefore may not contribute greatly to directing correctly folded intermediates to a final conformation. It was suggested at the time that stabilisation of all the weak individual interactions directs the final conformation of the native protein (Creighton, 1997).

Regardless of the exact mechanism, it is suggested that protein folding is enhanced by macromolecular crowding (Ellis, 2001). Macromolecular crowding refers to "the concentration of macromolecules within the cell which is so high that a significant proportion of the cell is physically occupied and hence unavailable to other molecules" (Ellis, 2001). It is considered that molecular crowding has significant effects on reaction rates and equilibria of macromolecular reactions, particularly during the initial collapse of newly synthesised polypeptide chains and also by the association of partly folded chains into non-functional aggregates (Ellis, 2001).

Notwithstanding how proteins are folded, it has been observed that while most small proteins need little assistance to reach a stable, active structure, larger proteins, without assistance, often produce inferior conformations. These are often inactive intermediates or aggregated proteins that must be removed or refolded. It has been seen in denaturation-renaturation experiments that many polypeptides cannot reform native state without the presence of additional proteins (Ruddon and Bedows, 1997). These additional proteins have been termed molecular chaperones. Chaperones derive their name from their function: they prevent illicit interactions between proteins, particularly aggregation, by interacting with them from the time of synthesis on the ribosome until they are properly folded and in their final location within the cell. The principal functions of all molecular

chaperones are: to facilitate folding of nascent proteins to their final native state; to hold substrates in an unstructured form to allow cross membrane transport; and to prevent aggregation of unfolded proteins and allow for their renaturation. During periods of cellular stress, it is the prevention of aggregation and the regeneration of proteins that are particularly important (Boston *et al.*, 1996). Hendrick and Hartl (1993) suggested that there is an additional requirement for classifying proteins as chaperones: they should not be part of the final functional protein structure. According to Jakob and Buchner (1994) there are several properties that characterise proteins as chaperones: "(1) suppression of aggregation during protein unfolding; (2) (it has an influence) on the yield and kinetics of folding; and (3) effects are exerted at near stoichiometric levels". Hendrick (1993) proposed a comprehensive definition of a molecular chaperone. His definition that a chaperone is "a protein that binds to and stabilises an otherwise unstable conformer of another protein and by controlled binding and release of the substrate protein, facilitates its correct fate *in vivo*: be it folding, oligomeric assembly, transport to a particular subcellular compartment, or controlled switching between active/inactive conformations" covers all current observations regarding characterisation of chaperones. These chaperones and another group of proteins called foldases mediate *in vivo* protein folding in both prokaryotic and eukaryotic cells. According to Boston *et al.* (1996), foldases are catalysts that increase the rate of protein folding. It has been proposed that all cellular proteins interact with these proteins at some time during cell life (Gething and Sambrook, 1992). It is not surprising then that chaperones and foldases have been found throughout all cellular compartments, because the need for protein folding which results in a functioning protein is universal.

1.4.3 Stress responses

Bacteria must cope with a wide range of environmental stressors: extremes and sudden changes in pH and temperature, wide variation in nutrient and metabolite concentrations, and the presence of

toxic chemicals such as cadmium, lead and other heavy metals. To enhance the probability of survival, bacteria must possess many ways of rapidly detecting then adjusting to or compensating for these altered conditions. A number of systems have been identified which allow bacterial cells to sense or detect changes in the external medium and subsequently activate regulatory systems (Stock *et al.*, 1989; Tiwari *et al.*, 1996). Furthermore, within the cell, there are a number of mechanisms that protect the cellular contents from lethal stress injuries. These include activation of HSPs which aid in the proper folding and refolding of proteins damaged by growth at temperatures above growth optimum, and induction of enzyme systems that are protective against oxidative, starvation and acid stress (Craig *et al.*, 1993; Parsell and Lindquist, 1993; Boston *et al.*, 1996). HSPs are involved in the production of proteins from the synthesis of nascent chains to the folding of protein complexes; it is for this reason they have also been designated chaperones. However, their role in the cell is far more complex than this simple description implies. These concepts are discussed in further detail in the following sections.

1.4.4 Mechanisms of signalling sub-optimum growth conditions

A number of systems are present within bacterial cells that allow them to detect and cope with environmental changes. These systems also operate during periods of normal cell growth and their function is either enhanced or up-regulated when the cell is stressed. These systems commonly interact with other stress responses through one or more mechanisms that control gene expression.

1.4.4.1 Bacterial signalling systems: the phospho-relay system

One example of bacterial signalling systems is the system of protein pairs in prokaryotic cells, a mechanism that allows cells to signal changes within their cytoplasm and external environment to achieve a rapid adaptive response. The protein pairs form part of a two-component regulatory system, although more than two proteins may be involved (Tiwari *et al.*, 1996), and in some cases

there is more than one kinase or response regulator, or both (Stock *et al.*, 1989). Specific changes that this system may detect include:

- detection and invasion of host cells including situations that lead to symbiosis (Finlay and Falkow, 1997);
- chemotaxis (Stock *et al.*, 1989);
- detection of environmental changes leading to spore formation or production of fruiting bodies (Albright *et al.*, 1989);
- metabolite utilisation (Parkinson and Kofoid, 1992); and
- stress responses to the presence of antibiotics and heavy metals (Parkinson and Kofoid, 1992).

A typical two-component system usually consists of a sensor protein, (e.g. a histidine protein kinase) and a cytoplasmic response regulator (Albright *et al.*, 1989; Stock *et al.*, 1989). The sensor proteins have been found to have similar C-domains while the regulator proteins share homology in their N-domains (Parkinson and Kofoid, 1992). Activation of a signal is based on phosphorylation of the N-domain of the regulatory protein, which often functions as a transcription factor, by the C-domain of the sensor protein. After it has been stimulated by a specific environmental change, the sensor protein transmits a signal to the carboxyl terminal histidine kinase domain resulting in a level of activation of the kinase corresponding to the level of its phosphorylation. Through ATP utilisation and further phosphorylation steps of the receiver domains, gene regulation and expression can occur: this is referred to as the phospho-relay (Wurgler-Murphy and Saito, 1997). Within lactic acid bacteria, several of these two-component signalling systems have been identified, all of which deal

with histidine protein kinases (O'Connell-Motherway *et al.*, 1997). However, a signalling system activating acid tolerance in *Rhizobium meliloti* has been proposed (Tiwari *et al.*, 1996), which suggests that a similar signalling system in other bacterial types may occur.

1.4.4.2 Other signalling systems

The work of Ananthan *et al.* (1985) indicated that the presence of abnormal (denatured) proteins causes increased expression of the heat shock genes in eukaryotic cells. When denatured proteins were injected into *Xenopis laevis* oocytes, activation of an HSP70-β-galactosidase hybrid gene followed. This resulted in an increased ability to degrade these abnormal proteins. With further work on the HSP70 promoter, these authors were able to determine that both heat shock and accumulation of abnormal proteins resulted in activation of HSP genes, which occurred through a common mechanism.

1.4.5 General responses to cell stress

Cells may respond in either specific and/or non-specific ways to stress. In *E. coli* global, non-specific responses to change in the environment have been shown to include the stringent, stationary phase and the SOS responses.

The stringent response is caused by a lack of aminoacetylated tRNAs for protein synthesis (Spira *et al.*, 1995). Protein synthesis requires the presence of all 20 amino acids. When one or more is absent in the growth medium or cannot be made by the cell or when the codon for the missing amino acid is encountered, the process of synthesis stops. Although the missing amino acid is not required for rRNA or tRNA synthesis, their production also stops which conserves cellular energy. The coupling of the synthesis of proteins and RNA is called the stringent response. Microorganisms commonly encounter nutrient limitation during the growth cycle and respond by developing a state that allows the cell to survive long starvation periods, typically as it enters stationary phase. Part of the response may include: changes in cell size and shape, changes in

fatty acid profiles, decrease in protein synthesis and production of distinct sets of proteins. These proteins may also be produced during heat, ethanol, acid, osmotic and oxidative stress (Hartke *et al.*, 1994). The SOS response, one of many global cellular responses, is a mechanism that saves *E. coli* cells from potentially lethal damage. The response was named after the telegraph signal given in the Morse alphabet when a ship is in deadly danger (Save Our Souls) (Janion, 2001). It is specifically triggered by severe damage to DNA or inhibition of DNA replication. A regulatory network that controls approximately twenty genes (the so-called SOS genes) at the transcription initiation level controls the response. The SOS genes are usually repressed by a represser (LexA) protein which binds to operators upstream of the SOS genes, and this prevents their transcription during normal cell growth. When DNA is damaged, single stranded DNA is released which results in a series of binding and cleaving steps. The DNA binds to two proteins in turn, RecA and LexA, causing autocleavage of the represser protein, LexA, which then allows transcription of the SOS genes. This allows DNA repair and other SOS functions to occur until there is a drop in the level of activated RecA. LexA will then accumulate, repressing transcription of the SOS genes until the next episode of DNA damage (Little, 1996). SOS-induced proteins, along with heat stress and stringent response proteins, have been found to be induced after exposure to cadmium stress (Ferianc *et al.*, 1998).

1.4.6 Chaperone families and the cellular response to heat stress

Originally identified by their increased abundance following heat shock (hence the name HSPs) chaperone proteins, in general, recognise exposed hydrophobic surfaces of non-native proteins that are normally within the correctly folded protein (Bukau and Horwich, 1998).

Chaperones were later found to be induced after exposure to a wide range of environmental stresses and are often detectable under normal growth conditions, where they play a role in normal protein folding. The protective nature of HSPs was demonstrated in early thermotolerance

experiments, where cells of *E. coli* that were exposed to a mild heat pre-treatment that induced HSPs, were killed more slowly when shifted to the higher temperature that was lethal to normal cells (Lindquist and Craig, 1988). Some stress proteins, in particular homologues of one particular protein (HSP60), have been identified in bacteria, fungi, plants and mammals (Zeilstra-Ryalls *et al.*, 1991). Some of the stress proteins belong to a group of structurally unrelated proteins called the molecular chaperones as well as a group of ATP-dependent proteases. It is possible for a chaperone to be an ATP-dependent protease and a heat stress protein, but not all stress proteins are chaperones and not all proteases are stress proteins (Boston *et al.*, 1996).

Molecular chaperones are pivotal in mediating protein folding in cells: specifically, these chaperones increase the yield of correctly folded protein by preventing aberrant structure formation and by degradation of mis-folded proteins (Lund, 1995). Degradation of proteins is important for several reasons, particularly because it helps to maintain homeostasis while cell structure is continually rebuilt and because mis-folded proteins are prone to aggregation (Baumeister *et al.*, 1998). The chaperones have been grouped in families according to molecular weight and function: 100, 90, 70, 60 and small HSPs. However, chaperones cannot fully function without co-chaperones and foldases and the function of chaperones, particularly where related to cell stress, are discussed below. Table 1.6 summarises the proteins for which there is evidence of induction during stress.

1.4.6.1 HSP60 and co-chaperone HSP10 (the chaperonins)

HSP60 and HSP10, or more properly chaperonin 60 and chaperonin 10 have been extensively studied in *E. coli* where the best evidence of their function has been gathered. When first isolated from *E. coli*, the two proteins were termed GroEL (HSP60) and GroES (HSP10).

Table 1.6. Stress induced proteins (including chaperone families)

Stress Protein Class	Sub-Class (Alternate names)	Typical Class members	Cell type	Molecular weight (kDa)	Intracellular location	References	SwissProt accession #
Chaperones	CLP proteins (HSP100(110))	Clp A or LOPD Clp B or HTPM Clp X CLPL HSP104	*E. coli* *E. coli* *E. coli* *L. lactis* *S. cerevisiae*	84.2 95.5 46.2 86.0 102.0	nuclear and cytoplasmic	(Gottesman and Maurizi, 1992; Squires and Squires, 1992; Suzuki *et al.*, 1997)	P15716 P03815 P33138 Q06716 P31539
	HSP90	HtpG HSP 82 Grp 94	*E. coli* *S. cerevisiae* *Human*	71.4 81.4 92.4	cytoplasmic cytoplasmic ER	(Lindquist and Craig, 1988; Becker and Craig, 1994; Craig, 1995; Boston *et al.*, 1996)	P10413 P02829 P14625
	HSP70	DnaK Ssa1 HSP70	*E. coli* *S. cerevisiae* *L. lactis*	68.9 69.5 64.9	nuclear/ cytoplasmic / mitochondrial	(Zeilstra-Ryalls *et al.*, 1991; Ellis, 1993; Becker and Craig, 1994; Craig, 1995; Boston *et al.*, 1996; Morimoto *et al.*, 1997)	P04475 P10591 P42368
	HSP60 (Chaperonins)	GroEL Tcp1 TCP1 cpn60	*E. coli* *S. cerevisiae* *Mouse* *L. lactis*	57.1 60.5 60.4 57.2	cytoplasmic	(Ellis, 1993; Becker and Craig, 1994; Craig, 1995; Boston *et al.*, 1996; Morimoto *et al.*, 1997)	P06139 P12612 P11983 P37282
	HSP10	GroES 10 kDa -chaperonin	*E. coli* *L. lactis*	10.4 10.2	cytoplasmic	(Craig *et al.*, 1993; Ellis, 1993; Boston *et al.*, 1996; Hartke *et al.*, 1997)	P05380 P37283

Stress Protein Class	Sub-Class (Alternate names)	Typical Class members	Cell type	Molecular weight (kDa)	Intracellular location	References	SwissProt accession #
	Small HSPs (12-30 kDa.)	IBPA or HtpN IBPB or HtpE HSP20	*E. coli* *E. coli* *S. cerevisiae*	15.8 16.0 23.7	cytoplasmic	(Lindquist and Craig, 1988)	P29209 P29210 P15992
Co-Chaperones	DNAJ	DnaJ DnaJ protein	*E. coli* *L. lactis*	41.1 40.8	cytoplasmic	(Becker and Craig, 1994)	P08622 P35514
	GRPE	GrpE GrpE protein	*E. coli* *L. lactis*	21.8 20.6	cytoplasmic	(Becker and Craig, 1994)	P09372 P42369
Foldases	Protein disulphide isomerase	PDI	*Maize*	57.1	endoplasmic reticulum lumen	(Boston *et al.*, 1996)	P52588
	Peptidyl prolyl isomerase	PPI	*S. cerevisiae*	17.2	cytoplasmic	(Boston *et al.*, 1996)	P14832
Other stress related proteins: *Porter/ Antiporter systems*	Calcium/Proton antiporter	CHAA	*E. coli*	39.2	membrane	(Speelmans *et al.*, 1995)	P31801
	Sodium/Proton antiporter	NHAA or ANT	*E. coli*	41.4	inner membrane	(Speelmans *et al.*, 1995)	P13738
	Cadaverine / lysine antiporter	CADB	*E. coli*	46.7	inner membrane	(Bearson *et al.*, 1997)	P13738
cold shock	cold shock proteins	CSPA	*E. coli*	7.2	cytoplasmic	(Lelivelt and Kawala, 1995; Salotra *et al.*, 1995; Kim and Dunn, 1997)	P15277

Stress Protein Class	Sub-Class (Alternate names)	Typical Class members	Cell type	Molecular weight (kDa)	Intracellular location	References	SwissProt accession #
Eukaryotic only system	Ubiquitin	UBA52 UBB UBC	*Mammals*	8.5	nuclear and cytoplasmic	(Varshavsky, 1997)	P02248
Bacillus related proteins	General stress protein	CTC	*B. subtilis*	21.9	?Ribosomal	(Hecker *et al.*, 1996)	P14194
Structural system	Surface layer proteins	SLPA	*Lb. acidophilus*	46.57	cell wall	(Boot *et al.*, 1993; Boot *et al.*, 1995; Boot *et al.*, 1996)	P35829
acid stress	Acid shock protein	ASR	*E. coli*	18.7	? cytoplasmic	(Heyde and Portalier, 1990; Farber and Pagotto, 1992; Foster, 1995; Garren *et al.*, 1998)	P36560
	Arginine decarboxylase	ADI	*E. coli*	84.4	cytoplasmic	(Melnykovych and Snell, 1958; Auger *et al.*, 1989)	P28629
	Arginine deiminase	ADI	*M. arginini*	46.4	? cytoplasmic	(Casiano-Colón *et al.*, 1988, Curran *et al.*, 1995)	P23793
	ClpP or peptidase family s14	CLPP	*E. coli*	23.2		(Gottesman and Maurizi, 1992; Squires and Squires, 1992; Suzuki *et al.*, 1997)	P19245

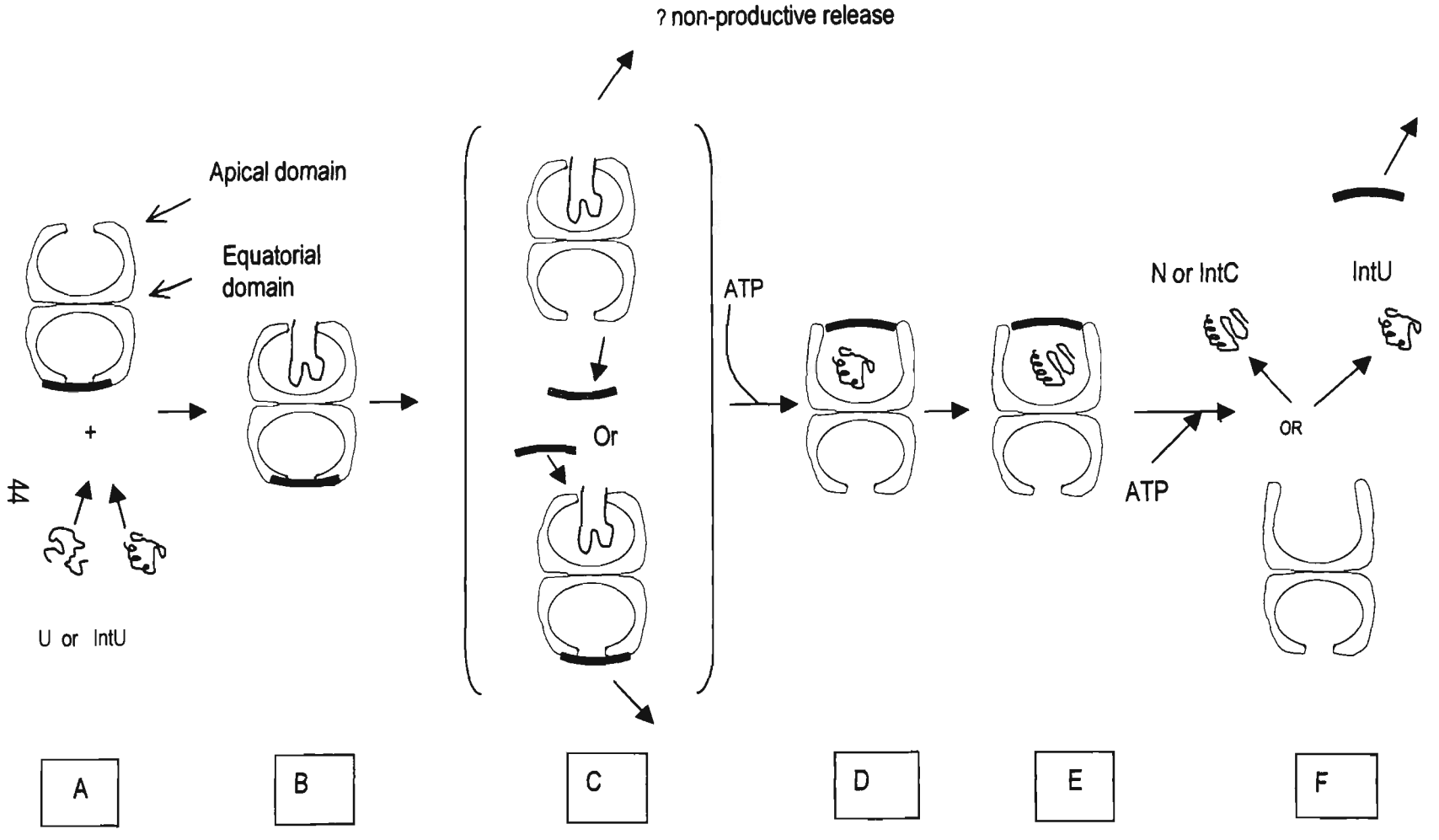
GroEL was initially characterised as a host factor required for bacteriophage assembly (Zeilstra-Ryalls *et al.*, 1991). The *groE* genes were identified through mutation studies with *E. coli*, where mutants of *E. coli* were isolated that could grow in the presence of the bacteriophage that would normally kill the cells. Inside the cells, virus coat particles were produced but they were unable to be assembled into new virus particles and became aggregated. Bacteriophage growth was blocked in *gro*EL mutants at the post-adsorption or injection stages. HSP60 was one of the first proteins to be described as having a chaperone function. Many homologues of HSP60 exist and have been identified in numerous bacteria including, *Streptococcus suis* (Benkirane *et al.*, 1997), *Bacillus subtilis* (Yuan and Wong, 1995), *Lactococcus lactis* (Whitaker and Batt, 1991) and *S. typhimurium* (Foster and Spector, 1995). Many hundreds of HSP60 genes in other species have been cloned and sequenced (Ellis *et al.*, 1996).

The chaperonins have been described as a family of sequence-related molecular chaperones containing two distinctive subclasses. The first is the GroE subclass found in chloroplasts, mitochondria and eubacteria while the second, TCP-1 subclass, is found in archaebacteria and the eukaryotic cytosol (Boston *et al.*, 1996). The TCP-1 class, which does not appear to be heat shock inducible (Lund, 1995) acts without a GroES co-chaperone and indeed may have the GroES function contained within the domain structure (Netzer and Hartl, 1998).

In *E. coli*, the GroEL subclass is encoded on an operon that includes the gene for GroES (Hendrick and Hartl, 1993). The *groE* operon is composed of two genes, *groES* and *groEL*, in that order, and expression of functional proteins of both are required for growth of *E. coli* at all temperatures (Fayet *et al.*, 1989). The genes for the two proteins are transcribed as a single mRNA and are under the control of two promoters. These promoters control expression of the operons $E\sigma^{32}$ and $E\sigma^{70}$ with the $E\sigma^{32}$ controlling expression at higher temperatures (Lund, 1995). Sigma factors are discussed in section 1.5.3.

Biochemical and crystallographic evidence has shown that the denatured molecular weight of GroEL is a 57·1 kDa protein and the active unit is made up of 14 identical sub-units, see Figure 1.2. The sub-units combine to form two heptameric rings with an internal pocket (Hartl, 1994); this complex has a molecular weight of around a million Daltons (Bukau and Horwich, 1998). The two rings stack back-to-back and enclose two non-contiguous cavities (Saibil, 2000). Evidence indicates that opening of the cavities, also referred to as Anfinson's cage (Ellis, 1999) is associated with the binding of ATP and GroES (Bukau and Horwich, 1998). This binding causes movement of the apical domains that results in formation of the cavity, which can hold unfolded proteins (Lorimer, 1997).

The cavity of the ring enlarges around two-fold in volume and the dome shaped GroES ring covers the open end (Bukau and Horwich, 1998). It is postulated that the pocket can hold unfolded proteins of up to 57 kDa (Ellis, 2000), with most substrates being between 10 and 55 kDa (Netzer and Hartl, 1998). Later binding of ATP to the GroEL ring causes ejection of the substrate protein regardless of whether the protein has folded or not (Lorimer, 1997). Evidence suggests that only one of the two GroEL rings can be open at any one time in the 'folding–active state', that is the state in which GroES binds to the GroEL ring in the presence of ATP (Bukau and Horwich, 1998). The asymmetry of the rings appears necessary to allow HSP60 to progress through its reaction cycle. GroES is a single heptameric ring structure of 10 kDa that can form a cap that holds the protein-folding intermediates within the GroEL pocket. Timing for the reaction is set by the ATP hydrolysis cycle, which is 15 seconds at 23°C (Ellis, 1999). This time is said to be insufficient for slow-folding proteins to complete a folding cycle and it is proposed that macromolecular crowding prevents the release of the partly-folded polypeptide chain.


? non-productive release
Apical domain
Equatorial domain
+
4
U or IntU
Or
ATP
N or IntC
IntU
OR
ATP
A
B
C
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KEY

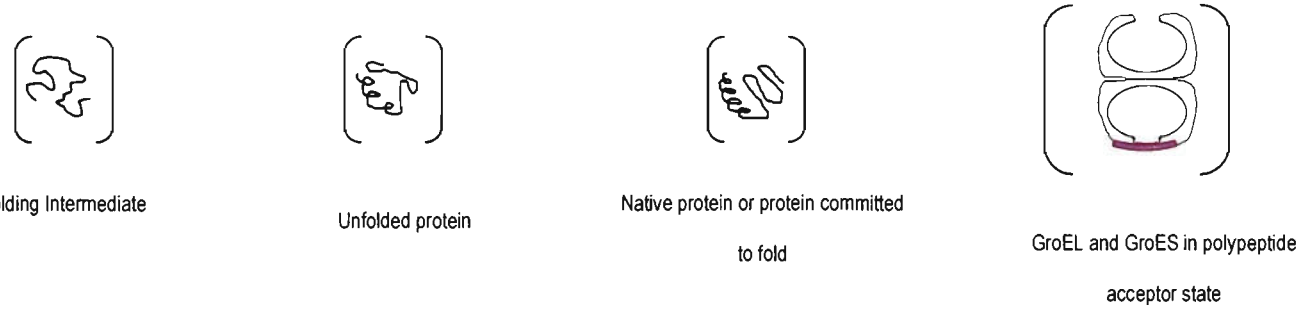


Figure 1.2 Model for GroEL-GroES-mediated folding reaction (after Bukau *et al.*, 1998).

The asymmetric GroEL-GroES complex (Box A) is the most likely state to accept unfolded polypeptides (U) or unstable folding intermediates (IntU) to form a *trans* ternary complex (Box B). Two possible pathways that lead to formation of the *cis* complex are shown in Box C. When GroES binds to the ring containing the polypeptide in the presence of ATP (forming the folding active *cis*-intermediate seen in Box D) major conformational changes occur in the *cis* GroEL ring. This results in release of the polypeptide from the apical binding sites and folding commences. ATP hydrolysis weakens the GroES-GroEL bond while folding continues (Box E). Binding of ATP to the *trans* ring evicts GroES from the *cis* ring and the peptide is released in one of three forms; a native protein (N), a protein committed to fold to a native state (IntC) or an unstable folding intermediate (IntU) (Box F).

This allows rebinding of the polypeptide to the same GroEL oligomer and prevention of exposure of aggregation-sensitive chains in the cytosolic medium (Ellis, 1999). Pulse-chase experiments have shown that larger substrates may remain in contact with the chaperone for an extended time, possibly up to 150 seconds (Lorimer, 1997; Ruddon and Bedows, 1997). Based on the abundance of GroEL, compared to the rate of protein synthesis, it is estimated that GroEL can only be responsible for folding 2-7% of all newly synthesised proteins. However, after a brief heat shock, the fraction of GroEL/peptide interactions can increase to 30% (Netzer and Hartl, 1998). GroES inhibits the ATP activity of GroEL in the absence of unfolded proteins (Bukau and Horwich, 1998).

1.4.6.2 HSP70 (stress–70)

In *E. coli,* HSP70 was originally defined as DnaK and was the product of a host gene required for bacteriophage λ DNA replication (Gething and Sambrook, 1992). The primary action of DnaK appears to be binding of nascent polypeptides as they proceed from the ribosome thus preventing improper folding before synthesis of polypeptides is complete (Ruddon and Bedows, 1997). Secondary actions include transportation of unfolded peptides across membranes, involvement in the re-arrangement of oligomers, and the resolution of protein aggregates. It is believed that all of these general functions are enhanced during periods of cell stress and that protein expression is highly regulated according to current cell conditions (Craig and Gross, 1991).

As a group, heat shock protein 70, HSP70 or 'stress–70' are amongst the most highly conserved proteins known and are found in most cellular compartments (Lindquist and Craig, 1988; Craig *et al.*, 1993). They have a molecular mass of around 70 kDa and have a number of essential roles in protein metabolism including protein folding of newly translated proteins, membrane translocation, degradation of proteins and regulatory roles (Hartl, 1996). Historically HSP70 proteins were identified by induction under conditions of stress. During stress the role of HSP70 is to prevent aggregation and assist refolding of mis-folded proteins (Bukau and Horwich, 1998).

HSP70 proteins have two domains, an amino-terminal ATPase domain of approximately 45 kDa and a carboxyl-terminal peptide-binding domain of approximately 25 kDa. The amino-terminal domain is the most conserved. The most variable region is adjacent to the carboxyl-terminal region and it has been proposed that this may dictate substrate specificity (Boston *et al.*, 1996). The carboxyl-terminal end has been shown to form a substrate binding pocket which is a channel ~5 X 7 Å. In this pocket, DnaK (the name given to *E. coli* HSP70) appears to bind with great affinity to short hydrophobic segments in extended conformation, meaning that the polypeptide must be substantially unfolded (Bukau and Horwich, 1998). It is suggested that the short hydrophobic segments of the un-folded protein, bind approximately seven residues of DnaK; a process which temporarily shields them and reduces their tendency to form aggregates (Ellis, 2000).

For HSP70 proteins, substrate binding is controlled by an ATPase cycle and this represents a switching between the two active states. The first is an ATP-bound state where there is low affinity and fast exchange of substrates and the binding pocket is closed. The second is an ADP-bound state where there is high affinity and slow exchange of substrates and the binding pocket is closed (Bukau and Horwich, 1998). The cycle probably starts by association of DnaJ (a co-chaperone) with a peptide substrate, followed by transfer of the substrate to DnaK-ATP. This transfer is coupled to the coupling of the substrate in the binding pocket of DnaK, which is caused by ATP hydrolysis. DnaJ then leaves the complex to be replaced by GrpE (another co-chaperone), which triggers ADP release from DnaK. ATP may then be bound again releasing GrpE and the substrate from DnaK. This interaction is shown in Figure 1.3 and discussed further in section 1.5 (Interaction of chaperones).

Figure 1.3 The proposed role of chaperone HSP70 (DnaK), co-chaperones DnaJ and GrpE and the chaperonins (HSP60 and HSP10) in protein folding in *E. coli* (Johnson and Craig, 1997; Ruddon and Bedows, 1997).

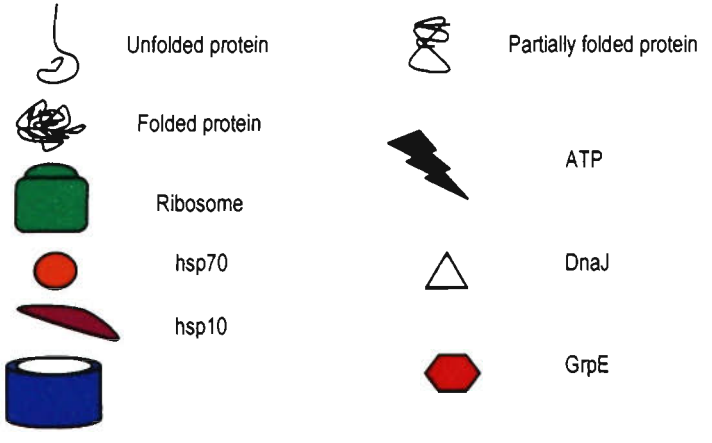
(a) DnaK and DnaJ recognise and bind hydrophobic regions of short peptides in an ATP-utilising step. The binding of DnaK prevents premature folding and aggregation of proteins.

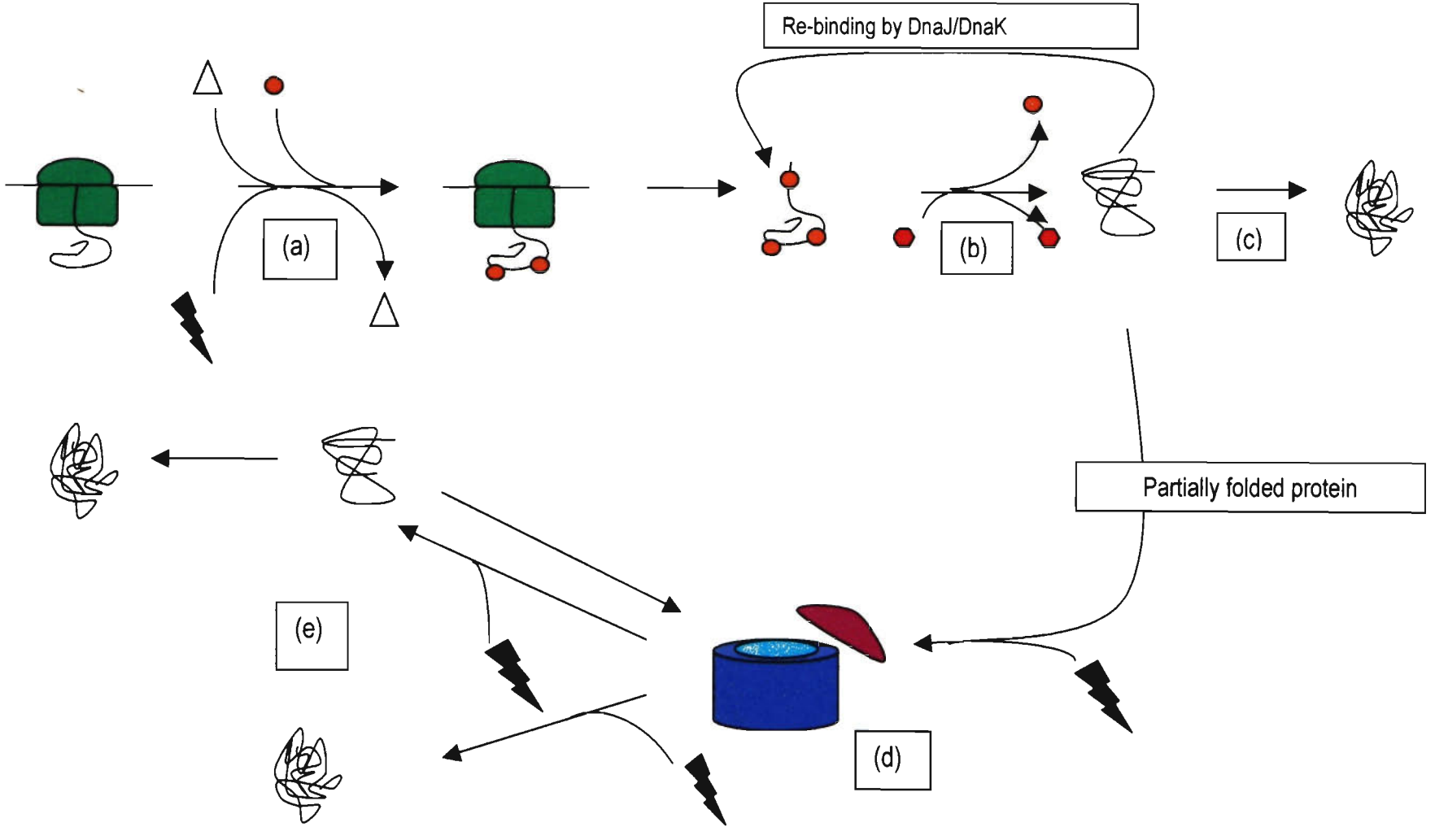
(b) GrpE increases the rate of dissociation of the bound nucleotide to DnaK in a nucleotide exchange step.

(c) Most partially folded proteins complete folding independently of any further chaperone activity, but some are passed to the chaperonin complex for further folding steps.

(d) The chaperonin complex allows completion of folding of those proteins requiring additional assistance to reach a native state.

(e) Proteins may be folded to a fully functional protein after one round of assisted folding by HSP60 and HSP10. Other proteins are ejected by the complex but retain some secondary structure. These proteins may be rebound by the complex or allowed to form native structure in the cytosol without any further chaperone intervention.




Re-binding by DnaJ/DnaK
(a)
(b)
(c)
Partially folded protein
(d)
(e)

The rate at which the chaperone activity of HSP70 is performed is extremely slow, even in the presence of substrates that have been shown to stimulate ATPase activity. A regulatory mechanism has been shown to increase ATP turnover (Bukau and Horwich, 1998). Principally, members of the DnaJ co-chaperone family regulate ATP hydrolysis while the GrpE co-chaperone family accomplishes release of ADP. See section 1.4.6.6.

Although a major function of DnaK is to protect large, slow-folding, thermolabile proteins from aggregating, a condition more likely to occur during heat stress, there is little evidence offered in the literature to support the theory that HSP70 is specifically protective to cellular functions under stress conditions. However, in *in vitro* studies in bacteria, HSP70 has been shown to solubilise aggregated proteins and in eukaryotic cells HSP70 may co-operate with HSP90 to prevent heat-induced protein denaturation (Hartl, 1996).

1.4.6.3 HSP90 (stress-90)

HSP90, a member of the 'stress-90' family, is another highly conserved member of the chaperone proteins. It is an abundant protein under all growth conditions, found primarily within the cytoplasm, but expression levels can be increased by heat shock and other cellular stresses (Lindquist and Craig, 1988; Gething and Sambrook, 1992). HSP90 proteins share a molecular weight of around 90 kDa, with the exception of *E. coli* HtpG (68 kDa), which lacks a highly charged region of around 50 amino acids that is characteristic of other HSP90 homologues (Jakob and Buchner, 1994). Members of this group include HSP82 in yeast and grp 94 in humans, and this group has an affinity for a number of proteins, particularly signal transduction proteins, steroid hormone receptors, actin and tubulin. It is this broad substrate affinity that characterises stress-90 proteins as a molecular chaperones (Hendrick and Hartl, 1993). Whilst the exact function of the HSP90 group is not fully elucidated, it is thought to be related to modulation of protein activity through completion of protein folding, blocking activation and stabilising inactive proteins until they are localised in the correct

cellular compartment. Craig (1993) suggested that stress-90 is essential for correct folding and functional activation of proteins in *S. cerevisiae*. Evidence is increasing that HSP90 plays an important role in receptor regulation (signal transduction) in higher cells, through its effect in modulating protein conformation, for example steroid hormone receptor function (Boston *et al.*, 1996; Morimoto *et al.*, 1997).

1.4.6.4 Clp family including HSP100 (HSP 110) group of proteins

The HSP100/Clp group of proteins is part of a larger group of homologous proteins, the AAA ATPase superfamily, that have diverse roles in biological systems, some of which demonstrate stress tolerance functions but all of which promote dissolution of protein aggregates (Schirmer *et al.*, 1996). In this regard, the function of this family seems to be different from other chaperones. Their principal stress tolerance function is one of dissaggregation plus protein degradation of unfolded or abnormal proteins rather than promotion of protein folding (Boston *et al.*, 1996). They may even cause unfolding of a stably folded protein if it is tagged with a specific recognition sequence (Saibil, 2000).

Some members of the Clp (Caseinolytic protease) proteins found in *E. coli* and named for the ability to promote hydrolysis of casein, have been added recently to the chaperone classes. Originally, the Clp proteins were thought to consist of only three related proteins, Clp A, B and C, but recently the number of members has expanded (Schirmer *et al.*, 1996). The Clp proteins now include eight sub-families, Clp A, B, C, D, M, N, X and Y, and are divided into two major classes, whereas ClpP is a member of the non-homologous s14 family of peptidases (Squires and Squires, 1992; Suzuki *et al.*, 1997). ClpP is discussed separately in section 1.4.10.1.

Because of a connection to heat shock response, four proteins, Clp A, B, C and X, are of particular interest. Clp A, B and C are ATPases and members of Class 1, the class that contains two

nucleotide-binding domains. ClpX belongs to Class 2; this class is shorter and contains only a single nucleotide–binding site. HSP100 chaperones are often found in association with a protease ring: ClpA (a chaperone) is often found in association with ClpP (a non-chaperone protease) (Saibil, 2000). The complex they create is a stack of four rings, with a double-ring protease in the middle, flanked by an ATPase ring at either end that thereby forms a continuous cavity (Saibil, 2000). Proteolysis occurs inside the protease cavity although the cavity is only large enough to accept an unfolded protein, suggesting that the chaperone's task is to unfold proteins and pass them onto the protease (Vale, 2000). This theory was supported by research performed using green fluorescent protein (GFP) tagged with a ClpA recognition sequence at its carboxyl terminus. Not only was the stable protein conformation unfolded but the GFP was also found to be directly inserted into the protease cavity (Langer, 2000; Vale, 2000).

It would appear that HSP100/Clp chaperones are not essential for growth at any specific temperature (low, normal or high) but are highly induced at higher than normal temperatures. They are also induced by other stresses such as alcohol, suggesting that these proteins provide protection of vital cellular components from these stresses (Sanchez *et al.*, 1992). In experiments with *S. cerevisiae* it has been shown that HSP104 mutant cells, in contrast to wild type cells, were not able to clear aggregates of protein accumulated during heat stress (Sanchez *et al.*, 1992). Antibodies against HSP104 from yeast react with both *E. coli* ClpB and mammalian HSP110 suggesting a relationship between them, that is, they all belong to the Clp group of proteins (Squires and Squires, 1992).

1.4.6.5 Small heat-shock proteins

A group of low molecular weight proteins (approx. 16 to 30 kDa) make up the chaperone group called the small HSPs. Often not detected during periods of normal growth, they are significantly induced during periods of heat stress (Boston *et al.*, 1996). The small HSPs are an abundant group

of proteins found in the cytosol of prokaryotic cells and organelles of eukaryotes. Small HSPs have only a low level of shared homology (Jakob and Buchner, 1994) and although their functions have not been fully determined, aside from stress tolerance, they may possibly have a role in cell growth and differentiation (Morimoto *et al.*, 1997). Small HSPs can "selectively recognise and bind to non-native proteins, they suppress non-specific aggregation during folding and unfolding and promote functional folding in an ATP independent manner" (Jakob and Buchner, 1994; Norris *et al.*, 1997). Experiments have shown that over-expression of the small HSPs provided increased thermotolerance to plant cells, but the mechanisms involved are yet to be elucidated (Boston *et al.*, 1996). It is proposed however that proteins bound to small HSPs during cellular stress are released to other chaperone systems when conditions are mitigated (Saibil, 2000). Functions including protection of mRNA during heat shock, overexpression of the protein in differentiating cells and interaction with non-native proteins have also been demonstrated to be associated with small HSPs, but again the controlling mechanisms are unclear (Jakob and Buchner, 1994). However, it is their role in protein folding and unfolding reactions that clearly identifies these proteins as chaperones (Boston *et al.*, 1996).

1.4.6.6 Co-chaperones (DnaJ and GrpE)

Chaperones of the HSP70 class interact with another group of proteins referred to as co-chaperones. Co-chaperones DnaJ and GrpE moderate the activity of DnaK in *E. coli*. Structurally the DnaJ protein consists of an N-terminal conserved domain (called the 'J' domain) of approximately 70 amino acids. There is also a glycine-rich domain and a central domain, which are referred to as the 'G' domain and 'CRR' domain respectively and a C-terminal end.

The function of DnaJ is to stimulate the rate of hydrolysis of DnaK, perhaps by increasing the ATPase activity of DnaK, (the *E. coli* HSP70 homologue) (Kelley, 1998). GrpE appears to increase the rate of dissociation of the bound nucleotide to DnaK although there are observations that it also

increased the binding ability of DnaK. In the presence of both DnaJ and GrpE, DnaK activity can be increased markedly (up to fifty-fold) (Becker and Craig, 1994). No proteins homologous to GrpE have been found in eukaryotic cells with the exception of some similar proteins found in yeast. The effects of DnaJ and GrpE have to be balanced to optimise the equilibrium between the substrate binding and release. Within the cell this is achieved by co-regulation and expression of the genes (Bukau and Horwich, 1998).

1.4.6.7 Foldases

Two classes of foldases have been identified: protein disulphide isomerases (PDI) and peptidyl-prolyl isomerases (PPI), which have a role in protein folding in eukaryotic cells. Although homologues have been identified in prokaryotic cells, PDI is found principally in eukaryotic cells within the lumen of the endoplasmic reticulum and its function is to catalyse the production of disulphide bonds. PDI is important for translocation and folding of proteins within mammalian and yeast systems but the role of the enzyme is yet to be fully elucidated (Boston *et al.*, 1996). Another major group of protein foldases, the PPIases, catalyse isomerisation of peptide bonds around proline residues. This reaction is normally slow in protein folding. These enzymes are found within the cytoplasm of prokaryotic and eukaryotic cells (Boston *et al.*, 1996).

1.4.7 Interactions between chaperone proteins

Individual families of chaperone proteins do not act without collaboration with the other chaperone proteins (Johnson and Craig, 1997). It has been established that the chaperones 'co-operate' in *de novo* protein folding using sequential actions, although it is not obligatory to follow a co-operative pathway (Lund, 1995). The model for co-operation is based on the differential specificity of the chaperones for peptides; for example, DnaK has a specificity for nascent polypeptides while GroEL cross-links to full length proteins. The model is shown in Figure 1.3. A number of observations of chaperone function during protein folding in *E. coli* have led to the formulation of a hypothesis that

DnaK/DnaJ/GrpE and GroEL/GroES cooperate in folding newly synthesised proteins (Hendrick and Hartl, 1993; Frydman and Hartl, 1996; Frydman and Höhfeld, 1997). The model proposes that DnaK and/or DnaJ recognise extended segments of nascent polypeptides, binding to them and preventing their aggregation. As the chain length increases, the polypeptide collapses to a more compact state requiring fewer stabilising molecules until the full-length chain is released, which requires the action of GrpE. While some proteins may have reached a native state, others are transferred to other chaperones for further folding rounds until the native state is reached. It has been suggested that this co-operative chaperone pathway may be bi-directional with proteins being passed back and forth between the chaperonins and HSP70 (Johnson and Craig, 1997). The interactions of chaperones require further study, particularly in the identification of other proteins that have chaperone activities and defining the interactions with those chaperones already detected.

1.4.8 Oxidative stress response

Reactive forms of oxygen can also cause stress responses in bacteria. Under optimum conditions of aerobic growth, cells can convert oxygen to a harmless end-product (water) by transfer of four electrons through the electron transport chain. However, sequential reduction to form active oxygen intermediates is possible and these products can have varying toxicity. These toxic compounds can include superoxide anion radicals, hydrogen peroxide and hydroxyl radicals (Demple, 1991). Denitrification within some bacteria produces nitric oxide, which is an end-product of a particular form of respiration, while a hypohalous acid such as HOCl is generated by the interaction of hydrogen peroxide with phagocyte peroxidases that have been shown to contribute to bio-toxicity (Miller and Britigan, 1997). Furthermore, these intermediates can react in a number of ways. For example, in low pH environments, superoxide can become protonated to form HO_2 that can react with itself to form hydrogen peroxide and, due to its neutral charge, is more membrane permeable

(Miller and Britigan, 1997). Aerobic bacteria, which utilise oxygen as a terminal electron acceptor, must deal with the toxic by-products that are produced during oxidative phosphorylation. Facultative and strictly anaerobic bacteria must also have mechanisms to counter the effects of oxygen exposure during normal growth that arise from transient exposure to air or hydrogen peroxide produced by other organisms. Anaerobic bacteria can be exposed to these compounds when aerobes and anaerobes grow in the same environment. Anaerobes are particularly susceptible to cellular damage because they have fewer mechanisms to cope with the presence of toxic oxidants (Miller and Britigan, 1997). For pathogenic bacteria, phagocytes are often the primary source of oxidants. Phagocytes surround the pathogenic organisms and engulf them with a portion of their cell membrane releasing superoxide and hydrogen peroxide.

Damage resulting from cellular interactions with toxic oxygen compounds can affect all cellular components. Lipid membranes are particularly susceptible and oxidative damage can result in loss of the proton motive force that drives ATP formation and nutrient uptake. Extensive membrane damage can lead to cell lysis (Miller and Britigan, 1997).

The cellular response to oxidative stress consists of two parts. The first involves avoiding contact with oxidants and the second involves preventing lethal injury once oxidants are encountered (Condon, 1987; Demple, 1991; Duwat *et al.*, 1995; Rocha *et al.*, 1996; Miller and Britigan, 1997; Mukhopadhyay and Schellhorn, 1997). Mechanisms for avoidance of phagocytic encounters have been published by Miller (1997) and include the use of a signalling system by *Yersinia* species that interferes with the phagocyte response. The ability of cells to cope with oxidants once they have been encountered, particularly the prevention of lethal injury in lactobacilli due to the exposure to hydrogen peroxide produced by starter bacteria in fermented foods, determines the survival of these cells.

Demple (1991) described three responses to oxidants: hydrogen peroxide response, the superoxide response and a stationary phase response that is protective against oxidative stress injury. These responses are associated with induction of catalase and superoxide dismutase as well as other proteins that are induced when a cell enters the stationary growth phase and during heat shock (Demple, 1991). The broad range of proteins induced to oxidative stress indicates that, unlike heat stress, there are no specific stress response mechanisms to oxidative stress.

1.4.9 Mechanisms of acid tolerance and acid stress responses

Bacteria, because of their uni-cellular nature, can experience significant fluctuations in pH during a normal growth cycle. The response to exposure to acid environments has been principally studied in enteric bacteria, predominantly *E. coli* and *Salmonella* species. This is due to the increase in outbreaks of food poisoning and the link between environmental conditions such as pH and expression of virulence genes (Hill *et al.*, 1995). However, acid stress responses have been studied in both pathogens and non-pathogens, including the following organisms:

- *Aeromonas hydrophila* (Karem *et al.*, 1994);
- *E. coli* (Goodson and Rowbury, 1989; Heyde and Portalier, 1990; Hickey and Hirshfield, 1990; Rowbury *et al.*, 1993; Ohara and Glenn, 1994; Small *et al.*, 1994; Leyer *et al.*, 1995; Lin *et al.*, 1995; Tsai and Ingham, 1997; Rowbury and Goodson, 1999);
- *Lactobacillus* species (Hood and Zottola, 1988; McDonald *et al.*, 1990; Lorca *et al.*, 1998; De Angelis *et al.*, 2001);
- *L. lactis* ssp. *lactis* (Cook and Russell, 1994; Hartke *et al.*, 1995; Hartke *et al.*, 1996; Rallu *et al.*, 1996);

- *Leuconostoc mesenteroides* (McDonald *et al.*, 1990);

- *Listeria monocytogenes* (Farber and Pagotto, 1992; Kroll and Patchett, 1992; Gahan *et al.*, 1996; O'Driscoll *et al.*, 1996; O'Driscoll *et al.*, 1997);

- Oral streptococci (*S. sanguis, S. gordonii, S. mutans,* and *S. oralis*) and other streptococci (*S. bovis*) (Belli and Marquis., 1991; Cook and Russell, 1994; Curran *et al.*, 1995; Quivey *et al.*, 1995; Smith *et al.*, 1996);

- *S. enteritidis* (Humphrey *et al.*, 1993; Humphrey *et al.*, 1993);

- *S. typhimurium* (Foster and Hall, 1991; Foster, 1993; Garcio-Del Portillo *et al.*, 1993; Foster and Bearson, 1994; Foster *et al.*, 1994; Lee *et al.*, 1994; Foster, 1995; Lee *et al.*, 1995; Baik *et al.*, 1996; Leyer and Johnson, 1997); and

- Root nodule bacteria (*Rhizobium loti* and *Rhizobium meliloti*) (Goss *et al.*, 1990; Tiwari *et al.*, 1992; Tiwari *et al.*, 1996; Tiwari *et al.*, 1996; Correa and Barneix, 1997).

Survival at low pH depends on a number of determinants including: growth phase (Gorden and Small, 1993), adaptation response (Foster, 1995), ability to switch to less pH-sensitive metabolic pathways (Miwa *et al.*, 1997), alkalisation of the internal pH (Marshall *et al.*, 1990), as well as expression of sigma factors and pH-dependent genes (Foster and Bearson, 1994). These determinants and the role in pH homeostasis are discussed in the following sections.

1.4.9.1 pH homeostasis

The proton gradient is generally accepted to be inter-convertible with ATP because of a proton ATPase (H^+-ATPase) and, in the absence of a specific pH sensing regulator, is probably the major method of internal pH (pH_i) control (Kashket, 1987). Miwa *et al.* (1997) indicated that not only do

acid tolerant bacteria have higher base levels of H^+-ATPase but they may also have a higher capacity to enhance its production, particularly during the specific conditions of a low pH in the growth medium and a high cellular concentration of intracellular ATP.

Regulation of pH_i in a bacterial cell is related to the permeability of cellular membranes to protons. This is because of a passive transfer of protons across membranes due to an inwardly directed force termed the proton motive force (PMF) (Axelsson, 1993). A PMF is composed of two contributing components, the first being an electrical potential ($\Delta\Psi$) where the inside of the cell is negative. The second is a pH gradient (ΔpH) where internal pH is more alkaline. It is generally considered that biological membranes exhibit low proton permeability because of the impermeability of the lipid bilayer (Booth, 1985; Hutkins and Nannen, 1993). Control of pH_i is more likely to be controlled by an active mechanism, a membrane-bound proton pump. *Lactococcus* and *Streptococcus* species maintain an internal pH that is more alkaline than the external medium but the pH_i decreases as the extracellular pH (pH_o) is acidified during growth and fermentation (Kashket, 1987). Kasket also found that for the particular strain of *Lb. acidophilus* studied (3532), the limiting level of cytoplasmic pH was 4.4, when the external pH was 3.5. Most bacterial cells quickly lose the ability to grow when they are unable to maintain a near neutral pH_i (McDonald *et al.*, 1990). Padan *et al.* (1981) noted that "chemiosmotic processes can adapt so as to maintain a constant pH_i of between 6.5 and 9.5, which appears to be the range permitted by cell physiology". However, as an exception to this, each type of LAB has their own, varying, threshold of pH_i below which all cellular functions are inhibited (Kashket, 1987). When fermentation end-products, such as lactic acid, are in high concentrations, they act as protonophores that perturb membrane phospholipids and increase the inward leak of protons. Weak acids in their uncharged form can diffuse easily across the membrane and dissociate within the cell, lowering the pH_i. The cell cannot maintain the pH gradient against this influx and cytoplasmic re-alkalisation fails, resulting in cell

death (Kashket, 1987). Cook and Russell (1994) have found that *L. lactis* and *Strep. bovis* must decrease their intracellular pH and maintain a low ΔpH to prevent accumulation of fermentation acid anions, which are toxic to the cell. This decrease in intracellular pH potentially restricts growth due to the reduction of other activities, such as biosynthetic reactions, but the organism continues to grow below the limits of other neutrophiles.

Belli and Marquis (1991) demonstrated that when cultures of oral streptococci became acidified the organisms were able to increase the level of ATPase activity. This resulted in removal of protons from the cytoplasm and subsequent protection of pH-sensitive glycolytic enzymes. Lactobacilli in particular seem capable of tolerating a wide range of pH values (Axelsson, 1993) and it has been suggested that the lactate fermentation pathway is less sensitive to low pH than other metabolic pathways (Miwa *et al.*, 1997).

1.4.9.2 Porter- antiporter systems

Cation antiports play a part in the acidification of the cytoplasm during growth in alkaline medium where the antiport exchanges internal cations (Na^+ or K^+) for external protons. A Na^+/H^+ antiporter may serve an important role in control mechanisms and homeostasis by lowering the intracellular Na^+ concentration that prevents Na^+ toxicity and by regulating internal pH. Regulation of cation antiports is probably directly related to internal pH (Speelmans *et al.*, 1995).

1.4.9.3 Growth phase and the relationship to acid resistance (AR)

Acid resistance (AR) is defined as the ability of a bacterial cell to survive a two-hour exposure to pH 2.5. In bacteria AR has been found to be highly dependent on growth phase (Foster and Hall, 1990; Gorden and Small, 1993). Work on AR in enteric bacteria has shown that the acid resistance of a number of *Shigella* and *Salmonella* species, including *S. sonnei* (3421), *S. flexneri* (1a-625), *S. boydii* (14), *S. dysenteriae* (12-31), *S. enteritidis* (3407), *S. typhimurium* (79-80) and *S. typhi* (1231)

was growth phase dependent (Gorden and Small, 1993). This is possibly related to the physiological changes that occur as a cell enters stationary phase, including global regulatory changes that protect the cell from decreased availability of nutrients. Lee *et al.* (1995) found that the stationary phase sigma factor (RpoS) was required for protection of *S. typhimurium* cells from acid stress using a derivative strain of LT2.

1.4.9.4 The role of adaptive acid tolerance (ATR)

Foster and others (Foster and Hall, 1990; Foster, 1991; Foster and Hall, 1991; Farber and Pagotto, 1992; Foster, 1993; Foster and Bearson, 1994; Foster *et al.*, 1994; Foster, 1995; Foster and Spector, 1995) have performed detailed research on the acid tolerance response in *S. typhimurium*. This organism, which is often implicated in cases of food poisoning, may be exposed to a wide range of environmental conditions during its life cycle, including the strongly acid conditions of the stomach. Their work has concluded that ATR in *S. typhimurium* involves a two-stage process. The two stages, designated pre-acid shock and post-acid shock, are an overlapping acid protection system triggered at different levels of acidity. Pre-acid shock is induced at an external pH of approximately 5.8 while the post-acid shock is induced at or below an external pH of 4.0. During post-acid shock a number of acid shock proteins (ASPs) are induced which are important for the subsequent survival of the organism. When a cell is transferred directly from pH 7.0 to 3.3, these ASPs are not synthesised and the cell suffers lethal injury and dies. However, when cells are pre-stressed at pH 5.8 then subsequently transferred to pH 3.3, cells are able to maintain pH homeostasis long enough to synthesise post-shock ASPs. Both stages are required to provide cells with maximum protection against exposure to low pH; however, the pre-shock can be substituted by a brief shock at pH 4.4 for 15 minutes. During this time, a group of ASPs are transcribed, but if cells are held beyond 15 minutes these proteins are no longer detectable and the cells are unable to withstand a subsequent low pH challenge (Foster, 1993). Approximately 50 ASPs are induced

during pre- and post-acid shock and their function is believed to be related to repair or prevention of macromolecular damage. Included in these proteins are several inducible amino acid decarboxylases that include the lysine decarboxylase (CadA) and lysine-cadaverine decarboxylase systems (CadB) (Bearson *et al.*, 1997).

Goodson and Rowbury (Goodson and Rowbury, 1989; Rowbury *et al.*, 1993; Rowbury *et al.*, 1993) found the same effect in *E. coli* (strain 1829 ColV I-K94, a derivative strain of K12), which was exposed to a number of different organic acids. When the test strain was grown at an intermediate pH (5.0) before subsequent exposure to pH 3.5, it gained a survival advantage over the control strain which was grown at pH 7·0 prior to exposure to pH 3.5. However, the extent of growth was also dependent upon the type of acid used. The work of Leyer and Johnson (1993) showed that prior acid adaptation of *S. typhimurium* (LT2) also caused cross protection against other stresses, such as heat and salt. Heyde and Portelier (1990) demonstrated that eight novel proteins were induced when a derivative strain of *E. coli* K12 was shifted from pH 6.9 to pH 4.3. These included heat shock proteins as well as a subset of unknown proteins they called acid shock proteins.

1.4.9.5 The role of arginine utilisation in acid tolerance

As discussed in section 1.4.9.3, the AR of an organism has been found to be dependent on growth phase, however AR also appears to be dependent upon the growth medium. Three complex-medium related AR systems are present in bacteria and their activity depends on whether the cells utilise oxidative or fermentative metabolism. Two of the three systems involve amino acid (arginine and glutamate) utilisation, while the third is dependent upon σ^s and is discussed in section 1.5.2.

The arginine deiminase system (ADS) provides a source of ATP derived from catabolism of arginine to ornithine, CO_2 and NH_3 (Casiano-Colón *et al.*, 1988, Mobley *et al.*, 1989). Marquis (1987) claimed that "the ADS can play an important role in the acid-base physiology of each of the bacteria

(they) studied in that it allowed for the recovery from acid stresses sufficiently severe to stop growth and glycolysis". Arginine catabolism occurs in a variety of bacteria including LAB, *Helicobacter*, *Salmonella* and *Shigella*. Figure 1.4 shows distribution pathways of arginine catabolism.

Marshal *et al.* (1990) have claimed that sufficient ammonia is generated from arginine catabolism to create a micro-environment in which *H. pylori* can survive the normally lethal pH of the human stomach.

1.4.9.6 Microbial ureases

Urease is an enzyme that catalyses the hydrolysis of urea to yield ammonia and carbamate, which spontaneously hydrolyses to form carbonic acid and a second molecule of ammonia resulting in a net increase in pH (Mobley and Hausinger, 1989).

Urease activity has been observed in a wide variety of prokaryotic and eukaryotic organisms with particular interest being paid to pathogens like *H. pylori* and *Y. enterocolitica* which are able to live in environments of extremely low pH (<3). This extreme acid tolerance has been attributed to the ability of these organisms to utilise urea to produce large enough quantities of ammonia to maintain an internal pH compatible with survival (Marshall *et al.*, 1990).

It has been demonstrated that acid resistance was also dependent on the growth phase as well as the presence of urea in the growth medium (de Koning-Ward and Robins-Browne, 1995). LAB appear not to use a urease pathway: evidence suggests that when de-acidifying wine, the arginine deiminase pathway, not an arginine-urease pathway, was used for arginine degradation (Liu *et al.*, 1996). This pathway has also been demonstrated in *L. oenos*, *M. arginine* and *S. lactis* (Poolman *et al.*, 1987; Sugimura *et al.*, 1990; Liu *et al.*, 1996).

Pathway	Organisms
Arginase: urea→CO_2 + NH_3 Arginine↗ ↘ ornithine →glutamate	*Saccharomyces* sp. *Bacillus* sp. *Proteus* sp.
Arginase transamidase: Arginine→ornithine→glutamate	*Klebsiella* sp.
Arginine deiminase: Arginine ↓ NH_3 + citrulline ↓ carbamylphosphate + ornithine ↓ ↓ ATP + NH_3 + CO_2 glutamate	*Streptococci* sp., *Pseudomonas* sp., *Lactobacillus* sp., *Clostridium* sp.
Arginine decarboxylase: Arginine ↙ NH3 + Agmatine→Urea→ Putrecine	*E. coli, Salmonella* sp., *Shigella* sp.

Figure 1.4 Pathways of arginine catabolism (Abdelal, 1979; Poolman *et al.*, 1987)

1.4.10 Proteases

When stressors affect a cell, which results in proteins becoming damaged, there are two possible outcomes. The first is that the molecular chaperones will aid the protein to refold into its correct conformation and the second is that the protein will undergo proteolysis and subsequently be removed. Molecular chaperones may also be involved in this protein degradation and where a protein cannot be refolded they may accelerate its destruction. The ClpP, Lon and the eukaryotic Ubiquitin system are examples of molecular chaperones acting as a protease and they are discussed in the following sections.

1.4.10.1 ClpP

Clp is an endopeptidase of 750 kDa that cleaves peptides in a process requiring ATP hydrolysis; its role in conjunction with the Clp proteins has been mentioned previously in section 1.4.6.1. It is a dimeric protein: ClpP, the proteolytic component, consisting of twelve sub-units. ClpP can cleave peptides when acting alone but this occurs at a slower rate and acts on shorter residue lengths (Gottesman and Maurizi, 1992). The function of ClpP may be that of a master protease which is attracted to different proteins according to the specificity of the ATP regulator, which is usually a chaperone (Gottesman and Maurizi, 1992; Suzuki *et al.*, 1997). The ClpP proteins can be found across prokaryotic and eukaryotic cells.

1.4.10.2 The Lon protease

The Lon (La) ATP-dependent protease is an 89 kDa protein, which was described in 1964 in *E. coli,* making it one of the first HSPs to be identified (Gottesman and Maurizi, 1992). Lon functions as an endo-protease cleaving damaged proteins into peptides of 5-20 amino acids and its association with DnaK accelerates the rate of proteolysis (Parsell and Lindquist, 1993; Missiakas *et al.*, 1996; Suzuki *et al.*, 1997).

1.4.10.3 The ubiquitin (Ub) system

First described in 1975, ubiquitin is a 76-residue protein that is found only in eukaryotic cells. It may be found as either free protein or bound to other cellular proteins. Ubiquitin is an abundant protein, hence the name, that is involved in cell differentiation, signal transduction, DNA repair, vesicular transport and stress responses (Varshavsky, 1997). Damaged or otherwise abnormal proteins can be recognised by the Ub system and are subsequently degraded. A multi-Ub chain becomes linked to the protein after it has recognised a degradation signal. The protein-Ub complex is then degraded by the 26S proteasome. This is possibly because the signals that start the degradation process are usually buried within a normal folded protein are now uncovered in a damaged protein (Suzuki *et al.*, 1997). There is no known prokaryotic equivalent to this protein.

1.4.11 The effect of stressors on cell membrane fatty acid composition

Apart from induction of chaperones, a number of other mechanisms have been proposed which may allow cells to respond to unfavourable changes in the growth medium. These are often associated with the cell wall and/or membrane and include changes to the fluidity of the lipid membrane and a build-up of stress damaged proteins. When challenged by a change in temperature, the major response in membrane composition is an alteration to the fatty acid profile. Adaptive changes in membrane lipid and fatty acyl compositions, that enable bacteria to cope with a wide range of growth temperatures, have been studied in a number of food-poisoning bacteria including clostridia (*Clostridium botulinum* NCIB 4270 and *C. butyricum ATCC 6015*) and *Listeria* species. When *C. botulinum* was subjected to a decrease in growth temperature, from 37°C to 8°C, a number of changes were noted. While the major fatty acids at each growth temperature were 14:0, 16:0 and 16:1, it was found that the proportion of each varied according to the growth temperature. The proportion of 14:0 and 16:1 increased from 16.4 to 37.5% and 10.5 to 22.5% respectively, while the proportion of 16:0 dropped from 40.3 to 19.2%. It was also noted that there

was a decrease in the proportion of cyclopropane fatty acids (15:0cyc and 17:0cyc) from 7.3 to 0.5% (Evans *et al.*, 1998). Similarly, when *L. monocytogenes* was challenged by lowering the growth temperature from 30°C to 7°C, two general changes were noted. Firstly, lipids with a shorter chain length became more abundant and secondly there was a small increase in unsaturated lipids (Russell *et al.*, 1995). These changes make the membrane a less rigid structure at the lower temperature and alteration to the fatty acid profile help the cells maintain both the proton-motive force and uptake of nutrients (Russell *et al.*, 1995).

Apart from changes in temperature, acid habituation has been demonstrated to have an affect on fatty acid profiles. Acid habituation is the phenotypic response that refers to the increased resistance to extreme pH conditions after adaptation to sub-lethal acidic environments. Acid habituation (AH) occurs when *E. coli* cells are grown at pH 5.0. Once habituated they are able to stand a low pH challenge better than a control grown at pH 7.0 (Goodson and Rowbury, 1989; Bearson *et al.*, 1997). According to Bearson (1997) AH, which is a reversible effect, probably involves a number of protein synthesis-dependent and -independent steps as well as DNA repair and the phosphate specific porin, PhoE. The effect of AH on five strains of *E. coli* (MJR, M23, R172, R91 and 0157:H) that exhibited wide variability in their intrinsic tolerance to exposure to low pH was examined by Brown (1997). A marked shift in fatty acid composition was demonstrated after AH. Significant percentages of mono-unsaturated fatty acids were found to be converted to cyclopropane fatty acids or were replaced by saturated fatty acids. It was also found that in a non-habituated, intrinsically acid tolerant strain of *E. coli*, 0157:H a food poisoning strain, had higher levels of cyclopropane fatty acids compared to strains with a low level in intrinsic acid tolerance (Brown *et al.*, 1997). It was concluded that acid tolerance in *E. coli* correlated with the content of cyclopropane fatty acids in the membrane. Cyclopropane fatty acids (CFAs) are found in around thirty-five genera of bacteria including *Bifidobacterium*, *Lactobacillus*, *Salmonella*, *Rhizobium* and

Helicobacter species (Grogan and Cronan, 1997). Three CFAs predominate in bacterial membrane lipids:

- *cis*-9,10-methylene hexadecanoic acid (cyclic 17:0);
- *cis*-11,12-methylene octadecanoic acid (lactobacillic acid or cyclic 19:0 11c); and
- *cis*-9,10-methylene octadecanoic acid (dihydrosterulic acid or cyclic 19:0 9c) (Grogan and Cronan, 1997).

Lactobacillic acid was first demonstrated in 1950 in a culture of *Lb. arabinosus* and is a 19-carbon cyclopropane analog of *cis*-vaccenic acid that has been demonstrated in several *Lactobacillus* and *Lactococcus* species (Johnsson *et al.*, 1995).

CFAs have a number of physiological roles within the cell membrane including membrane fluidity and chemical stability; CFAs also appear to be less reactive to low pH and oxidation than other fatty acids (Grogan and Cronan, 1997). CFAs may reduce the effects of temperature on membrane fluidity by increasing the melting temperature of the membranes and disrupting the acyl chain packing that ensures fluidity of the membrane. This change in rigidity appears to reduce permeability of the membrane to protons (Brown *et al.*, 1997).

1.4.12 General and specific stress response of *Bacillus subtilis*

In *B. subtilis* it has been noted that a variety of stressors, including heat, salt, ethanol and starvation stress, can induce the same set of proteins, designated the general stress proteins or GSPs. In the work of Hecker *et al.* (1996) it was proposed that this general response serves a non-specific protective function allowing adaptation to stress and starvation. In addition to the general response, it was found that each single type of stress also induced a specific set of proteins called the specific stress proteins or SSPs. These proteins were induced by and provided a specific protective

function against a unique stressor such as heat. This protective function is well characterised in *E. coli.*

1.5 Interaction of chaperones, regulation of the stress response and the effect of cross protection on survival

Molecular chaperones of all cells have been conserved through evolution and although there is a great variation between organisms, the way they respond to stress is very similar. However, the methods of regulation of stress responses is not as conserved and it may be concluded that each organism must detect and regulate stress responses according to its own particular environment. Special mention will be made of an inverted repeat, named CIRCE, found in the regulatory regions of many genes coding for heat shock proteins involved in regulation of shock responses at both DNA and mRNA levels.

1.5.1 Regulation of the heat shock response

The heat shock response is characterised by a rapid, stress induced, transcription of genes encoding HSPs after exposure to elevated temperatures. The appearance of misfolded proteins is thought to be the specific stress signal that triggers the transcriptional response (Morimoto *et al.*, 1997).

During heat shock in *E. coli* two regulons are expressed (Missiakas *et al.*, 1996; Missiakas and Raina, 1997). The first, referred to as the 'classical heat shock regulon' is negatively regulated by the presence HSPs, particularly DnaK and is under the control of the σ^{32} transcription factor. The σ^{32} transcription factor is an RNA polymerase sub-unit that encodes products that deal with removal and refolding of damaged proteins. The second encodes proteins that take care of damage in the periplasmic space and is controlled by the σ^{E} transcription factor. However, in Gram positive bacteria, which do appear to contain the Gram negative heat shock specific regulators, the question

of heat shock regulation remains to be fully determined. In *L. lactis* several heat shock promoters are found adjacent to a palindromic sequence called the CIRCE element (Rallu *et al.*, 1996). CIRCE stands for controlling inverted repeat of chaperone expression (Zuber and Schumann, 1994) and consists of 9 bp separated by a 9 bp spacer (TTAGCACTC-N_9-GAGTGCTAA) and is highly conserved within the groEL, dnaK and dnaJ operons (Segal and Ron, 1996). The probable function of CIRCE is as an operator site to which a repressor binds. The protein coded by *orf39*, the first gene in the dnaK operon of *B. subtilis*, was found to bind the inverted repeat at the DNA level and to serve as the repressor in *B. subtilis* (Yuan and Wong, 1995). *Orf39* was recently renamed *hrcA* and homologues are found in several other bacteria (Baldini *et al.*, 1998).

1.5.2 Regulation of stress responses other than heat

1.5.2.1 Regulation of acid stress responses

Expression of a number of bacterial genes, including stress response genes, have been associated with changes in pH. Iriarte *et al* (1995) cloned the *rpoS* gene from *Y. enterocolitica* and demonstrated that the production of an enterotoxin by this organism is partly regulated by pH. The *rpoS* gene is involved in expression of stationary phase proteins in other bacteria and is associated with stress response proteins in *Salmonella* species and acid tolerance in *Shigella* species (Lee *et al.*, 1994; Lee *et al.*, 1995; Waterman and Small, 1996). The connection to acid tolerance was noted in a comparison of virulent strains of *S. typhimurium* (UK1, SL 1344 and SL 14028s) to an avirulent strain of *S. typhimurium* (LT2). It was found the virulent strains were more acid tolerant than the avirulent strain. This was traced to a mutation in the rpoS allele of LT2 and when rpoS alleles were swapped between organisms, virulence was also swapped (Lee *et al.*, 1995). RpoS also controls the synthesis of a set of proteins up-regulated during oxidative stress (Hochman, 1997). In studies of mutants of *E. coli* K12 and *S. typhimurium* LT2, the *cadBA* operon was found to be regulated by external pH and the gene products of this operon increase levels of amino acid

decarboxylases. These products in turn help control internal pH (Auger *et al.*, 1989; Neely *et al.*, 1994; Bearson *et al.*, 1997). Coll *et al* (1994) found in *E. coli* K-12 a gene (*nmpC*), encoding an outer membrane protein that acts as a porin, that was dependent on external pH, while Slonczewski *et al* (1987) reported two genes, which were induced by decreasing internal pH. The relationship between these pH-dependent genes to those responsible for stress protein production in LAB is very unclear, and studies such as those described above are yet to be carried out in LAB. However, it has been suggested that in Gram positive bacteria, particularly the non-pathogenic LAB, regulation AT may be connected to ppGpp, the guanosine tetraphosphate responsible for the stringent response (Grogan and Cronan, 1997).

1.5.2.2 Regulation of stationary phase and starvation response

E. coli and *Salmonella* species have a sigma factor σ^s, a general stress response mediator encoded by the stationary phase gene, *rpoS*. It is induced during stationary phase and nutrient starvation and regulates virulence factors as well as regulating the acid tolerance response, which was discussed in the previous section (Finlay and Falkow, 1997). These different stressors signal their presence to the cell in different ways and differentially affect *rpoS* translation and/or stability. The cellular level of σ^s increases in response to a wide range of stress signals including heat stress and during normal growth is highly unstable and rapidly degraded (Muffler *et al.*, 1997).

CFA formation has been shown to be induced at the same time as the induction of the stringent response, although CFA formation has also been associated with transition to stationary phase. Transcription rates of CFA genes have been found to increase with the accumulation of stationary phase proteins, the products of the *rpoS* gene (Grogan and Cronan, 1997). Synthesis and accumulation of RpoS proteins are determined in part by ppGpp, the guanosine tetraphosphate responsible for the stringent response (Grogan and Cronan, 1997).

1.5.3 The effect of cross protection on survival responses

Induction of stress proteins by one form of stress can protect against other forms of stress. There are many examples of this in the literature: (Farber *et al.*, 1992, Leyer *et al.*, 1993, Hartke *et al.*, 1994, Hartke *et al.*, 1995, Jørgensen *et al.*, 1995, Quivey *et al.*, 1995, Baik *et al.*, 1996, Flahaut *et al.*, 1996, Flahaut *et al.*, 1997, Bearson *et al.*, 1998). A good illustration of this phenomenon can be seen in the results of work performed on *L. lactis* cells. Actively growing cells of strain IL1403 were forced into stationary phase; they were starved by growing the cells in the presence of limited glucose. The cells became significantly more resistant to heat, ethanol, acid and osmotic stress than a control culture exposed to the same stress while in exponential phase (Hartke *et al.*, 1994; Hartke *et al.*, 1995; Hartke *et al.*, 1996). Similar cross protection was induced by prior exposure to UV irradiation (Hartke *et al.*, 1995). It should be noted however, that cross-protection to stress varies markedly between LAB species (van de Guchte *et al.*, 2002).

Leyer and Johnson demonstrated that acid adaptation induces cross protection against a wide range of stressors promoting survival of *S. typhimurium* LT2 and *E. coli* 0157:H7 (Leyer and Johnson, 1992; Leyer and Johnson, 1993; Leyer *et al.*, 1995; Leyer and Johnson, 1997). Kim *et al.* (2001) have also demonstrated a variable response in log-phase cultures of *Lb. acidophilus* pre-exposed to heat, NaCl and bile stress. Their work demonstrated significant improvement in survival of cultures pre-exposed to bile stress when subsequently heat stressed but not salt stressed, and pre-exposure to heat stress did not protect against bile or salt stress.

There have been a number of outbreaks of food poisoning involving *E. coli* 0157:H7 where this pre-adaptation may have led to increased viability of pathogens in under-cooked burgers, cider and salami (Garren *et al.*, 1997; Semanchek and Golden, 1998).

In a study of stress responses in *L. œnos* (now renamed *Oenococcus œnos*) Garbay and Lonvaud-Funel (1996) found that exposure to one stress, either heat, acidity, ethanol or toxic fatty acids, caused a rapid loss of viability. However, the surviving cells were more able than a control to survive in wine and induce malolactic fermentation. These results suggest that any type of stress will adapt this organism for one specific stress, the exposure to the harsh conditions of wine. Additionally it appeared that the profile of proteins up-regulated during each type of stress was similar, regardless of the type of stress (Garbay and Lonvaud-Funel, 1996).

However not all studies support the view that induction of a basic set of stress proteins (pre-adaptation) will protect the cell against all stressors. The reaction of *Enterococcus faecalis* to bile salts, acid and heat shock has been studied by Flahaut (1996; 1998). It was found that exposure to bile salts or heat resulted in cross protection against heat or bile salts but when heat adapted cells were acid stressed no cross protection was found to have occurred. Indeed it was noted that bile salt stressed cells were more sensitive when later exposed to acid stress (Flahaut *et al.*, 1996). In a later study of hydrogen peroxide stress response, it was found that the type of pre-treatment (acid, salt or increased temperature) used to stress *Ent. faecalis* ATCC 19433 resulted in a variation in the strength of the response. Acid pre-stress resulted in the greatest response with 11.8% surviving minute hydrogen peroxide challenge compared to 0.2% survival of the control cells. The least response came from a thermal pre-treatment where only 4.0% survived compared to 1.2% of the control cells. Ethanol and alkaline pre-stress appeared to have no influence on the outcome of the hydrogen peroxide tolerance (Flahaut *et al.*, 1998). Adaptation to stresses other than acid has also not typically induced a high level of acid tolerance. Bacteria, therefore, may recognise some stressors as specific, for example heat stress, invoking induction of a specific set of heat stress proteins while others, such as acid stress, may be recognised as general stress signals (Bearson *et al.*, 1997).

1.6 Aims of this thesis

Previously, the study of stress responses in bacteria has been confined principally to Gram-negative bacteria such as *E. coli* and *S. typhimurium*. While these investigations are far from complete, the directions of these studies have been towards gaining an understanding of the basic biological mechanisms involved in stress tolerance as well as obtaining an insight into the processes that pathogenic bacteria use to invade a host organism. This thesis attempts to increase the understanding of the response to various stressors in a group of non-pathogenic bacteria that are in common use in the dairy industry, by demonstrating that a number of proteins are up-regulated during acid and heat stress.

Specifically the aims of the project were:

- to characterise strains at a biochemical and molecular level to validate taxonomy;
- to determine the nature of the stress response in *Lb. paracasei* and *B. animalis* syn. *lactis* following exposure to heat stress and acid stress;
- to compare these responses to known responses in other bacterial species;
- to characterise the proteins produced by their physical properties; and
- to gain information regarding the proteins in terms of sequence and possible related function.

Chapter Two

MATERIALS AND METHODS

2.1 Materials

2.1.1 Bacterial strains and plasmids

Refer to Table 2.1. for a list of bacterial strains and plasmids used in this work. Strains originally obtained from the Commonwealth Scientific and Industrial Research Organisation (CSIRO) were a gift from Dr Martin Playne. The Australian Starter Culture Centre provided *Lb. acidophilus* type strain ASC 388. Dr Duncan Rouch from The University of Melbourne, Gilbert Chandler Campus, provided *Lb. casei* strain GCRL-163. *Bifidobacterium animals* (syn. *lactis*) strain Bb-12 was a gift from the Chr. Hansen Group, Bayswater, Australia. *E. coli* strains were obtained from commercial sources.

2.1.2 Chemicals

All chemicals used were analytical reagent (AR) grade and supplied by either Crown Scientific Pty Ltd (Australia) or Sigma-Aldrich (Australia) except where another supplier is specified. The sources are shown in Appendix 1. Abbreviations used for these chemicals are listed on page xix. All water used was distilled or deionised. Distilled water was prepared using Milli-RO Water purification system or deionised water using Milli-Q Ultrapure Water System (Millipore).

2.2 General Methods

Unless otherwise stated bacterial cultures and protein extracts were centrifuged at 4°C in a Beckman J2-HS high-speed centrifuge. Other solutions were centrifuged in an Econospin H1323, FSE Du Pont, Australia. Eppendorf tubes were centrifuged in an Eppendorf centrifuge Model 54515C, supplied by Crown Scientific Pty Ltd.

Table 2.1 Source of bacterial strains and plasmids used in this study.

Strain/Plasmid or Victoria University culture collection number	Relevant characteristics	Source/reference.
E. coli		
TG2 (TG2 is stored at VUT as VUN 0105)	SupE hsdΔ5 thi Δ(lac-proAB) Δ(srl-recA)306::Tn10(tet^R) F'[traD36 $proAB^+$ $lacI^q$ lacZΔM15	(Sambrook *et al.*, 1989)
Lactobacillus strains		
C1 VUP12000 *Lb. acidophilus*	Type strain	CSIRO 2400, ATCC 4356
C2 VUP12001 *Lb. acidophilus*		CSIRO 2401 Originally obtained from Chr. Hansen Group.
C3 VUP 153-2345-2		Victoria University culture collection
C4		CSIRO 2403
C5 VUP12002 *Lb. acidophilus*		CSIRO 2404 Mutant of 2401
C6 VUP12003 *Lb. acidophilus*		CSIRO 2405 Mutant of 2401
C7		CSIRO 2406
C8 VUP 153-2457		Victoria University culture collection
C9 VUP 152-2352		Victoria University culture collection
C10 VUP12004 *Lb. acidophilus*		CSIRO 2409 Mutant of 2401
C11 VUP 152-2313-2		Victoria University culture collection
C12 VUP 153-2415		Victoria University culture collection
C13 VUP 12005 *Lb. acidophilus*		CSIRO 2412/ 21 Originally obtained from Chr. Hansen Group
C14 VUP 153-2345-3		Victoria University culture collection

C15		CSIRO 2422
C16 VUP12006 *Lb. paracasei*		CSIRO 2413/46 Originally obtained from Chr. Hansen Group
C17 VUP12007 *Lb. acidophilus*		CSIRO 2415
C18		Bornhofen 200g Acidophilus yoghurt
C19		Yoplait YoPlus yoghurt 'A+B' plain yoghurt
C20		Bornhofen 200g Acidophilus yoghurt
C21		Bornhofen 200g Acidophilus yoghurt
C22		Yoplait YoPlus yoghurt 'A+B' plain yoghurt
C23		Yoplait YoPlus yoghurt 'A+B' plain yoghurt
C24 VUP 152-2313-3		Victoria University culture collection
C25		CSIRO 20097
VUP12012 *Lb. jugurati*		Victoria University culture collection
ASC 388 *Lb. acidophilus*	Type strain alternately sourced compared to VUP 12000	Australian Starter Culture Research Centre
GCRL-163 *Lb. casei*		The University of Melbourne, Gilbert Chandler Research Laboratories,
Bifidobacterium strains		
Bifidobacterium animalis (syn. *lactis*) strain Bb-12		Originally obtained from Chr. Hansen Group
Plasmids		
pBluescript SK+	2958-bp cloning vector derived from pUC 19. Ampicillin resistant	Stratagene
pGEM T-easy		Promega

Optical densities were measured using an Ultraspec III UV/Vis spectrophotometer (Pharmacia). pH was measured using a model PHM220 pH meter, supplied by Radiometer Pacific. Microtiter trays (96 well) were scanned using a Multiscan 8 channel photometer (ELIZA plate reader) supplied by Flow Laboratories, Titertek.

2.2.1 Buffers

Buffers were prepared according to the methods described in Biochemistry LabFax (Chambers, 1993). All other buffers, not described here, are described in the relevant sections.

2.2.1.1 Tris buffer pH 7

Tris(hydroxymethyl)aminomethane (Tris-HCL) buffer solution was prepared from appropriate concentrations of Tris base. The pH was adjusted to 7.0 using HCl.

2.2.1.2 Citrate-phosphate buffer

Stock solutions of citrate phosphate buffer were prepared from 0.2 M citric acid ($C_6H_8O_7$) and 0.2 M sodium dihydrogen phosphate (NaH_2PO_4).

2.3 Microbiological methods

2.3.1 Media

Unless otherwise stated all media was supplied by Oxoid Australia and sterilised by autoclaving at 121°C, 103 kilopascals for 15 minutes. The pH of each batch was checked and if necessary, adjusted immediately before autoclaving.

2.3.1.1 de Man, Rogosa and Sharpe (MRS) broth and agar

MRS broth, developed by de Man, Rogosa and Sharpe to enhance the growth of lactic acid bacteria, was used to support the growth of bacteria used in this study (De Man *et al.*, 1960). MRS agar plates were prepared by the addition of 1% agar to MRS broth prior to autoclaving. Unless otherwise stated, incubation of agar plates was carried out at 37°C under anaerobic conditions.

Anaerobic conditions were obtained using a two litre anaerobic jar with palladium catalyst anaerobic system (Oxoid).

The formula of MRS per litre was: 10g peptone, 10g 'Lab-Lemco' powder, 5g yeast extract, 20g glucose, 1mL Tween 80, 5g sodium acetate, 2g tri-ammonium citrate, 0.05g magnesium sulphate ($MgSO_4.7H_20$), 0.25g manganese sulphate ($MnSO_4.4H_2O$), 2g dipotassium hydrogen phosphate. Water was added to bring the volume to 1 litre. MRS agar was made by addition of 1.5% agar to MRS broth prior to sterilisation. When MRS broth was used to culture *B. animalis (*syn. *lactis)*, 0.05% cysteine hydrochloride was added prior to autoclaving. MRS broth with citrate-phosphate buffer was titrated with 0.2 M citric acid (to adjust to pH 4, 4.3 or 5.3) or 0.2 M sodium dihydrogen phosphate (to adjust to pH 7), according to the final pH required, in double strength MRS broth prior to autoclaving. The pH of the media was confirmed during and after the growth cycle by measuring the pH of aliquots removed from the culture vessel.

2.3.1.2 Lactobacilli fermentation broth (LFB)

LFB was made up from MRS base without Lab-Lemco powder or glucose. The pH was adjusted to 6.3 before the broth was bottled and autoclaved (115°C for 15 minutes). To each 100 mL, final volume, 10 mL of 10% filter sterilised carbohydrate and 2 mL of 0.2 % chlorophenol red was added.

2.3.1.3 Luria-Bertani (L-B) broth

L-B broth was prepared from 10g Tryptone, 5g Yeast extract, 5g NaCl, distilled water to 1 litre. L-B agar was made by addition of 1.5% agar to L-B broth prior to sterilisation.

2.3.1.4 Double strength YT broth

Double strength YT broth contained 3.2 g Tryptone, 2g yeast extract, 1g NaCl made up to 200 mL in distilled water.

2.3.1.5 Biolog Lactic Acid Suspension (BLA™) broth

BLA broth was prepared from 5.6 g BLA broth, 0.16 mL Tween 80 and made up to one litre with distilled water. The formula of BLA broth was not made available by the manufacturer (SPL Diagnostics). BLA was used to study the ability of lactobacilli to utilise various carbohydrate sources.

2.3.1.6 Ampicillin plates

One litre of L-B agar was prepared as described above. After sterilisation, the agar was cooled to 55°C, and then 5 mL of ampicillin (20 mg/mL) was added prior to pouring plates. Ampicillin (20mg/mL) was prepared from 98% Ampicillin-Sodium salt (Aminobenzylpenicillin) (Sigma) and sterilized by filtration through a 0.22 μm filter, Millipore.

2.3.1.7 Colour selection plates

One litre of L-B agar was allowed to cool to 55°C then 5 mL of ampicillin (20 mg/mL), 2.5 mL of X-gal (20 mg/mL) and 1 mL IPTG (200 mg/mL) were added prior to pouring plates. IPTG was prepared from 99% isopropyl-D-thiogalactopyranoside (Sigma). X-GAL was prepared from 95% 5-bromo-4-chloro-3-indolyl N-acetyl-D-galactopyranoside (Sigma).

2.3.1.8 Nutrient broth

Nutrient broth was prepared from Lab-Lemco powder (1.0g), yeast extract (2.0g), peptone (5g), and sodium chloride (5g) made up to 1 L in deionised water. Nutrient agar was made by the addition of 15g of agar to 1 L of nutrient broth.

2.3.2 Preservation of cultures

2.3.2.1 Frozen stocks in glycerol

Stock cultures of LAB were made from the overnight growth on MRS agar plates. Using cotton tipped swabs the organisms were suspended in 2 mL of preserving solution (50% glycerol:50% double strength MRS broth) by gently rotating the swab in the medium. The vials were stored at

–20°C for short-term storage, or –70°C for long-term storage. When the culture was required the vial was removed from the freezer and cotton tipped swabs used to transfer approximately 200 µL of the contents to MRS broth or agar for overnight culture.

2.3.2.2 Frozen stocks using ceramic beads

The 'protect' bead system (Protect Bacterial Preservers) utilises sterile vials that contain a number of porous beads and a cryo-preservative fluid (Technical Service Consultants, Ltd). When microorganisms are inoculated into this fluid they are bound to the porous beads. The following procedure, provided by the manufacturer, was used to prepare frozen culture stocks for use in fermentation experiments.

Overnight growth of LAB were removed from an MRS agar plate using a cotton tipped swab then rotated gently in a 'protect' vial to resuspend the bacteria in the preservative solution and coat the beads with bacteria. The vial was tightly closed and inverted 4-5 times before the preservative solution was removed as completely as possible. The vials were then stored at -20°C for short-term storage or -70°C for long-term storage. When the culture was required the vial was removed from the freezer and held on ice while two beads were removed and transferred to MRS broth for overnight incubation. *E. coli* were preserved using a similar method; however, nutrient broth and agar was substituted for MRS broth and agar.

2.3.3 Gram stain

Glass slides coated with heat fixed bacteria were flooded with crystal violet (10g/L) for one minute. The stain was washed from the slide with gently running tap water then an iodine solution was added for one minute. The iodine solution was prepared from 4 g NaOH dissolved in 25 mL distilled water to which 20 g iodine and 1 g potassium iodide were added and dissolved. This solution was mixed and made up to 1 litre. After washing in tap water, the slide was briefly decolourised with

acetone-alcohol prepared by adding 300 mL of acetone added to 700 mL 95% ethanol. The slide was washed again with water and counterstained with carbol fuchsin (20 g/L), again for one minute. After washing in tap water the slide was air-dried.

2.3.4 Method of biomass determination

Biomass was determined by optical density (OD) and dry biomass. OD was determined by measuring absorbance at 600 nm (A_{600}). Samples were diluted to keep the OD measurements in the linear range between 0.1 and 0.8.

To determine any biomass, ten mL polypropylene screw-capped centrifuge tubes (ProScience) were dried overnight at 55°C then weighed to four decimal places. To each tube exactly 10 mL of test sample was added and then the tubes were centrifuged at 16,000 x g for ten minutes to obtain a firm pellet. The liquid supernatant was decanted and the tube briefly turned upside down on absorbent paper to drain the remaining supernatant. Each tube was then placed upright in a 55°C drying oven overnight. The tubes were examined for complete dryness and then re-capped with its own separately dried cap before being re-weighed to four decimal places. The weight difference was determined against a media only control and this value is referred to as the dry biomass. Biomass studies were performed in replicate.

2.3.5 Carbohydrate utilisation test (rapid test)

This method was developed, as part of the work described in this thesis, to identify *Lb. acidophilus* presumptively and provide a 'metabolic fingerprint' for each of the strains being tested using this method. It was designed to be simple, cheap and rapid. Identification was based upon the ability of organisms to utilise fifteen different carbohydrates (2% w/v) supplied in BLA broth, see Table 2.2. Change in absorbance was determined over six hours in 96-well, flat bottomed, microtitre plates using a microtitre plate reader (Titertek).

Table 2.2 Carbohydrates used in rapid testing.

All carbohydrates were prepared 2% w/v in BLA broth. Carbohydrates that were filter-sterilised rather than autoclaved are marked with an asterisk.

Arabinose	Lactose	Raffinose
Cellibiose	Maltose *	Rhamnose
Fructose	Mannitol	Sorbitol
Galactose	Mannose *	Sucrose
Glucose	Melizitose	Xylose *

2.3.5.1 Carbohydrate substrates

The carbohydrate medium consisted of BLA broth plus one of the fifteen different carbohydrates. Carbohydrates were weighed and added to broth before autoclaving, however some could not be autoclaved and were filter-sterilised using 0.22 μM filters. These are marked with an asterisk in Table 2.2. The media was dispensed in 200 μL aliquots into each well of a 96-well rriicrotitre plate. Only one carbohydrate was tested per plate.

2.3.5.2 Preparation of cultures and testing

Unknown test or known control strains, were grown from a single colony plated on MRS agar. After overnight incubation, a cotton-tipped swab was used to gather 2-3 isolated colonies, which were then added to 2 mL of BLA broth lacking any carbohydrate source. This broth was diluted to achieve an optical density of 0.8 to 1.0 at 600 nm. Using a pre-determined plan of well positions as a guide, the unknown test strains and control strains (50 μL) were used to inoculate a microtitre plate containing the substrate being tested. Samples, standards and controls were run in duplicate.

Controls in each tray included:

- blanks (BLA broth plus inoculum but no carbohydrate source); and
- BLA broth, with carbohydrate but without inoculum, which was used as a sterility control

The tray was placed into a microtitre reader with a 450 nm filter and the instrument was blanked against BLA broth, without carbohydrate source but with inoculum added. The absorbance (A_{450}) of each of the well positions for every tray was then read. A lid was placed over the top of the tray before being wrapped in cling film, this reduced evaporation from the wells. Wrapped plates were placed into an incubator, set at 37°C, for six hours. The plates were passed through the microtitre reader a second time and the change in absorbance calculated. These changes were graded using

a threshold change of 100%. A positive result was a change in absorbance of at least double the starting absorbance, while a negative result was a change in absorbance less than double the starting absorbance. Strain identification was based on comparison of these results to published profiles (Hansen and Mocquot, 1970; Holt *et al.*, 1994; Hammes and Vogel, 1995; Vandamme *et al.*, 1996).

2.3.6 Selection of acid tolerant strains

A microtitre plate method was used to measure the changes in absorbance read at 450 nm (A_{450}) of cultures grown at eight randomly selected pH values (1.6, 2.4, 3.0, 3.8, 4.8, 5.6, 6.3, 7.8 and 8.1). Fifty µL inoculums, standardised to an initial absorbance value of 0.8 at 600 nm (OD_{600}), were added to 200 µL of pH adjusted MRS broth. Growth temperature for all pH values was 37°C. Absorbance was measured at zero, 6 and 24 hours using a microtitre plate reader. The extent of growth was determined by calculating specific growth rates in each time interval, using the formulae described in section 2.3.8.1.

2.3.7 Fermenter operation

One- and two-litre bioreactors (fermenters) manufactured by Braun and Applikon or an in-house system 'Bio-Reactor Under Computer Evaluation' (BRUCE) developed by Dr T. Ueno at Victoria University, were used as a tool to study the growth of *Lb. acidophilus* and *B. animalis* (syn. *lactis)* under optimal and sub-optimal conditions. All the fermenters consisted of a supported glass reaction vessel, either one- or two-litre, which was fitted with a stainless-steel head plate. The head plate had several connections including a pre-mixed gas inlet/outlet, water inlet/outlet, sample collection port, liquid entry port, fixed thermometer pocket and stirrer assembly. Additionally there were access ports for a pH sensor, level control, foam control and a dissolved oxygen sensor. The gas inlet port was used as the inlet through which dissolved oxygen within the fermenter broth was controlled. It was also used to aid in mixing and aeration. The water inlet/outlet, which is a closed

system, was used for heat exchange. The fermenter vessel was filled with culture medium and autoclaved prior to use for 30 minutes at 121°C. When being prepared for operation the fermenter was connected to all cables and tubing and the set points (temperature, pH, dissolved oxygen etc.) adjusted. Water flow, acid/base pumps and gas flow were then switched on and when all parameters had reached the desired set points, the fermenter was inoculated. Standard set points for all fermenter runs included the stirrer rate (150 rpm) and gas flow rate (0.1 L/min).

Innocula were prepared as follows. Two 'Protect' beads coated with the test organism were transferred to 20 mL of MRS broth for 6-8 hours at 37° C. After this time 10 mL of the above culture was transferred to 200 mL MRS broth and incubated overnight at 37°C without mixing. The contents were then centrifuged (10 min at 10,000 x g) and the supernatant decanted leaving approximately 20 mL of pellet and supernatant. During the centrifuge time the fermenter system was flushed with nitrogen and pH-adjusted with either 2M HCl or 2M NaOH to the desired starting set point. Sufficient uninoculated broth was withdrawn and refrigerated to use as a blank during spectrophotometer readings. Using a syringe, the pellet was aseptically transferred through an appropriate inlet port on top of the fermenter reaction vessel so that the contents reached the desired starting optical density (OD_{600} of 0.13 to 0.23).

2.3.8 Determination of growth rates

2.3.8.1 Manual method for the determination of optimum temperature and pH

Absorbance values were used to construct growth curves for *Lb. acidophilus* and *B. animalis (*syn. *lactis)*. Data, from duplicate runs, were collected hourly over a minimum of six hours, from fermenters held at constant temperature (37°C) and pH (2, 3, 4, 5, 6, 7, or 8). Data was also collected from fermenters that were held at constant pH (6.3) and temperature (12, 22, 27, 32, 37, 42, 45, 47, or 51°C). These values were graphed as Ln (absorbance) versus time. The slope of the tangent to the steepest part of the growth curve was used to determine the number of

generations per hour. Specific growth rates (μ) were calculated over two time periods using the formulae below, where X_2 was OD_{600} of sample collected at time (t_2) and X_1 was OD_{600} of sample collected at time (t_1).

$$\frac{\text{Ln } X_2 - \text{Ln } X_1}{t_2 - t_1}$$

The generation time was calculated from the plot of Ln OD_{600} against time and was the time taken for the absorbance to double (McMeekin *et al.*, 1993). The average of the duplicate results was then shown graphically (1/rate versus pH or temperature) and used to determine the optimum growth conditions. The optimum growth conditions were at the peak of the resulting curve.

2.3.8.2 Software based method for the determination of growth rates

A modified computer software program, developed by Viega and Gutiérrez (1991) was used to graphically display growth curves of each batch fermenter run. The program automatically calculated specific growth rates between each time point as well as the maximum specific growth-rate (μ_{max}). These were compared to results obtained using the the above method to determine specific growth rates.

2.3.9 Determination of factors that affect growth

2.3.9.1 Determination of the effect of lactate on the ability to initiate growth

Sodium lactate ($CH_3CH(OH)COONa$) was used to prepare a 10 g/L stock solution of lactate in MRS broth. The stock solution was further diluted in MRS broth to the following concentrations: 5, 2.5, 1.25, 0.63 and 0.31 g/L. MRS broth without added lactate was used as a control. Fermenters inoculated with *Lb. acidophilus* were sampled every hour for at least eight hours and again at 24 hours. Maximum specific growth rates were calculated for each lactate concentration. The graph of μ_{max} versus lactate concentration was used to determine the effect of lactate on the ability of *Lb.*

acidophilus to initiate growth. The pH was controlled at 6.3 during growth and temperature set at 37°C.

2.3.9.2 Determination of acid resistance by challenge at lethal pH values

The ability of *Lb. acidophilus* strains to survive at two lethal pH values was tested using a modification of a method used by Gorden and Small (1993). In the modified method MRS broth was substituted for L-B broth. Acid resistance was determined from strain survival, where if greater than or equal to ten percent of challenged bacteria survived for 2 hours at pH 2.5 the strain was considered acid resistant. As indicated by Gorden and Small "assay conditions were based upon those of a normal fasting stomach, i.e. a pH less than 3.0 and a gastric emptying time of less than two hours".

Acid resistance was determined by taking 50 µL of a twenty-four hour culture of the test strain, which was then added to 10 mL of MRS broth pre-warmed to 37°C. The pH of the test broth was adjusted to either pH 2.0 or 2.5 before the addition of the 50 µL sample, while a control broth was set at pH 6.3. Duplicate 10 µL samples, were taken from each broth at time zero and every 30 minutes for six hours and again at 24 and 48 hours if required. Lawn cultures were prepared from 100 µL taken from serial dilutions of the sample and incubated for 48 hours at 37°C. The colonies on each plate were counted and the percentage of survivors calculated relative to the time zero.

2.3.9.3 Impact of growth phase on pH resistance

Acid resistance in relation to growth phase was determined by preparing an actively growing broth culture from which an aliquot was removed hourly and challenged at pH 2.5 for two hours. Two mL of an overnight culture was used to inoculate 100 mL of MRS broth at 37°C and pH 6.3. Fifty µL of this culture was then added to 10 mL of MRS broth (pH 2.5) pre-warmed to 37°C. Duplicate 10 µL samples, were taken from each broth at time zero and every 30 minutes for six hours and again at

24 and 48 hours if required. Lawn cultures were prepared from 100 µL taken from serial dilutions of the sample and incubated for 48 hours at 37°C. The colonies on each plate were then counted and the percentage of survivors calculated. The process of sampling from the growing culture was repeated hourly for eight hours. The results were compared to determine which phase of growth produced the highest number of survivors.

2.3.9.4 Viable cell counts

Viable cell counts of cultures were performed by preparing serial dilutions of the culture in MRS broth. Aliquots of the dilutions were spread plated onto MRS agar and incubated for 48 hours at 37°C under anaerobic conditions. The colonies on each plate were then counted and plates with between 50 and 500 colonies used to calculate total viable cell counts after correction for dilution factors.

2.4 Analytical methods

2.4.1 Sample collection and treatment

2.4.1.1 Sample collection for optical density, carbohydrate estimation and SDS-PAGE

At specified times 50 mL of fermenter contents was removed from the fermenter and transferred to a screw-capped 50 mL plastic centrifuge tube (Falcon). The mix rate of the fermenters was set to a higher setting for a short time before sampling (300 rpm for 30 seconds). Two mL of this sample was used for estimation of optical density read at 600 nm, while the rest of the sample was immediately placed into an ice filled cooler to stop any further metabolic reactions. When cooled, the tube was centrifuged until a soft pellet was formed (8,000 x g for 4-7 minutes). The supernatant was carefully removed for estimation of carbohydrates and an aliquot was transferred to the freezer (-20°C) until required.

According to the optical density of each sample a calculated volume of Tris buffer (pH 7.0) was added to the pellet to make each sample pellet equivalent to an optical density of 15.0. This pellet was treated as described in section 2.5.1 and frozen for later testing.

2.4.1.2 Collection of samples for analysis of fatty acid methyl esters

This method is based on that used by Decallone *et al.* (1991) where the cultures used in the evaluation of fatty acid methyl esters are in the stationary phase of growth. Unless otherwise stated, ten mL of culture, grown for twenty-four hours, was removed from the fermenter and centrifuged (ten min at 8,000 x g). The pellet was resuspended in 1 mL of 1.2 N NaOH in water:methanol (1:1, v/v) and vortexed before the tubes were placed in a boiling-water bath for thirty minutes. The tubes were then cooled to room temperature before 2 mL of 6 N HCl in methanol was added (325:275, w/v). The tubes were incubated at 80°C for ten minutes then cooled to room temperature before 1.25 mL of ether-hexane (1:1, v/v) was added to each tube. After continuous rotation for ten minutes the tubes were centrifuged (2 min at 8,000 x g) then the upper phase was removed and placed in a second tube. Three mL of NaOH (0.3 N) was added to the second tube and centrifuged (10 min at 8,000 x g) before the organic phase was separated and placed into gas chromatograph sample vials. The vials were frozen (-20° C) until required.

2.4.1.3 Collection and preparation of samples for 2-D SDS-PAGE

Cells from a sample of the fermenter contents were removed, centrifuged and concentrated as described in section 2.4.1.1. This cell suspension was placed, together with an equal volume of glass beads (0.10 to 0.11 mm diameter), into a stainless steel homogeniser bottle (B. Braun) then secured in the homogeniser (B. Braun MSK Cell Homogeniser) and homogenised for thirty seconds. This was repeated after the bottle had been cooled on wet ice for 2 minutes. The temperature of the contents of the bottle was not allowed to rise above 4°C. The homogenate was then centrifuged (10 minutes at 20,000 x g in a refrigerated centrifuge) to obtain a protein rich, cell

free extract, which was then dialysed to remove salts. The samples were concentrated by centrifugation in a 5 kDa molecular weight cut-off filter (Sartorius) then frozen (-20°C) until required.

For later work, this method was modified for use in a Mini-BeadBeater-8™ (BioSpec Products supplied by Daintree Scientific). Up to eight, 2 ml screw-capped tubes replaced the single stainless steel cylinder used in the method described above. Using a volume ratio of 1:1:2 (0.5 g wet weight cell biomass:40mM Tris buffer pH 7:0.10 mm glass beads) each tube was loaded up to 2/3 capacity. The Mini-beadbeater-8 was run according to manufacturers instructions for 2 bursts of one minute. The tubes were cooled on wet ice between bursts. Following treatment the tubes were centrifuged (16,000 x g) and the supernatant removed and frozen until required.

To enhance the extraction of hydrophobic proteins, solvent and detergent assisted extraction of proteins was also used. Tris buffer (200 µL of 40mM, pH 7) was added to each tube containing 0.5 g wet weight cells. The tube was mixed briefly, and then 500 µL of solvent or detergent was added to the tube, which was placed at 4°C for 5 minutes. The contents were homogenised in the Mini-beadbeater-8 as described above. After centrifugation at 16,000 x g, both the aqueous and solvent layers (if present) were carefully removed. The solvent layer was dried down under nitrogen then made up in an equivalent volume of 40mM Tris buffer pH 7. Both the aqueous and solvent extracted samples were frozen for further studies. Solvents tested included: octanol, acetone and chloroform. Detergents tested included CHAPS (4% w/v in 40 mM Tris pH 7) and Triton X-100 (2% w/v in 40 mM Tris pH 7).

2.4.1.4 Dialysis and desalting

Dialysis tubing was prepared by boiling 10 to 15 cm strips for 10 minutes in 3 L of 2% (w/v) $NaHCO_3$ and 1 mM EDTA (di-sodium salts), pH 8.0, then rinsed thoroughly in distilled water and then the strips were boiled again for ten minutes in 1 mM EDTA. The tubing was rinsed inside and

out in distilled water, then stored in distilled water at 4°C. Protein samples were dialysed against two changes of 100 volumes of distilled water at 4°C.

De-salting of small volumes (less than 100 µl) was performed in Micro Bio-Spin P-6 columns (BioRad) according to manufacturer's instructions. These tubes had stated salt retention rates of 99% and a size exclusion limit of 6,000 Daltons.

2.4.1.5 Concentration of samples

Sartorius centrifuge tubes (SM13229) containing a membrane with a 5,000 Dalton molecular weight (MW) cut off were used for concentrating extracts. Two mL samples were transferred to the tube and centrifuged according to the supplier's instructions. The concentrated protein sample was collected after 20 minutes centrifugation at 8,000 x g and 4°C.

2.4.1.6 Samples for protein estimation

One mL of bacterial suspension was removed from the fermenter and centrifuged to obtain a pellet. The pellet was resuspended in an Eppendorf tube in 1.0 mL of 4.6M NaOH and boiled for ten minutes. The suspension was kept at room temperature prior to immediate analysis.

2.4.2 Analysis of carbohydrates and metabolic products

2.4.2.1 Reducing sugar estimation

Reducing sugar (RS) estimation was performed by the dinitrosalicylic acid (DNS) method (Miller, 1959). The DNS reagent contained 3' 5'-dinitrosalicylic acid (10g), phenol (2g), sodium sulphite (0.5g) and sodium hydroxide (10g) made up to 1 L in distilled water. Samples previously collected from a fermenter run were defrosted and diluted appropriately. Sample, water blank or standard (1 mL) was added to 1 mL of DNS reagent, mixed and placed in a boiling water bath for exactly five minutes. The tubes were then placed in a cold water bath and 5 mL of distilled water added. The

absorbance at 570 nm was measured using the zero standard as blank. Standards were prepared from a standard solution (2.0 g/L) and contained from zero to 2.0 g/L of glucose.

2.4.2.2 High performance liquid chromatography (HPLC) estimation of sugar and metabolic products

HPLC was used to determine the extent of glucose utilisation as well as concentration of the metabolites produced during bacterial fermentation. The concentration of the following compounds was measured: glucose, lactate, acetate, maltose, formate and ethanol using a refractive index detector on a Varian HPLC. The system was fitted with an organic acid analysis column (Aminex HPX-87H ion exclusion column 300 x 7.8 mm) supplied by BioRad. The mobile phase was 5 mM H_2SO_4 with a flow rate of 0.60 mL/min and the run time was 24 minutes. Lactate standards (0.1 g/L to 0.5 g/L) were prepared from a stock solution (0.5 g/L) prepared using sodium lactate. Standards (0.2 g/L to 1.0 g/L) for the remaining compounds were prepared from stock solutions of 1.0 g/L.

2.4.3 Estimation of protein concentration (Lowry assay)

This is a modification of the protein assay of Lowry *et al.* (1951). Previously prepared samples of cells were diluted 1/20 (25µL sample, 25 µL 4.6M NaOH, 450µL 0.45M NaH_2PO_4). To each 0.5 mL of diluted sample or standard, 2.5 mL of Lowry reagent was added and mixed by vortex. Lowry reagent was prepared immediately before use by adding 50 mL of 2% Na_2CO_3 in 0.1M NaOH to 1.0 mL 0.5 % $CuSO_4.5H_2O$ in 1% Na-K-Tartrate pH 9. After exactly 10 minutes, 0.25 mL of dilute Folin/Ciocalteau (F/C) reagent (2 mL concentrated F/C plus 3 mL deionised water) was added and the solution immediately vortexed before being allowed to stand at room temperature for 30 minutes. The absorbance was measured at 750 nm. Standards containing zero to 40 µg of bovine serum albumin (BSA) were prepared from a 100 µg/mL standard solution.

2.4.4 Fatty acid methyl ester (FAME) profiles

FAME profiles were obtained using a Varian Gas Chromatograph model 3400, which included a Cx auto-sampler and Star chromatography software, version 4.01. The column was a BPX-70 (25.0 metres x 250 microns supplied by SGE). One μL of stored sample was injected by auto-sampler. GLC reference standards (Sigma) were used to identify unknown peaks by comparison to the retention time of standard peaks. The following fatty acids were used as standards: 8:0, 10:0, 12:0, 14:0, 14:1, 16:0, 16:1, 17:0, 18:0, 18:1, 18:2, 18:3, 19:0, 20:0, 20:1, 20:4, 21:0, 22:0 (FAME standard, Sigma). The run time was 20 minutes. The carrier gas was helium with a flow rate of 1 mL/min. An FID detector was used to detect peaks. Column temperature was ramped from 150°C to 200°C at 2°C/min.

2.5 Protein separation methods

Laemmli was the first to describe an improved method of electrophoresis in 1970 using a detergent (Sodium Dodecyl Sulphate, SDS) and mercaptoethanol, which was the forerunner of the methods now in common use (Laemmli, 1970). Primarily the method described below was used to demonstrate proteins that were up-regulated during cell stress; however, the technique was also used to compare whole cell protein patterns, as studies have shown a high correlation between protein profiles and DNA-DNA hybridisation (Hertel *et al.*, 1993). Samples for identification were harvested at stationary phase after growth in MRS broth at 37°C.

2.5.1 Polyacrylamide gel electrophoresis (PAGE)

One dimension SDS-PAGE of proteins was performed according to the method of Laemmli (1970) using a vertical slab system (BioRad's Modular Mini-PROTEAN II electrophoresis system and PROTEAN II SLAB SYSTEM or C.B.S. Scientific Triple wide system, Silenus Labs). The solutions described in Table 2.3 were prepared as stock solutions.

Table 2.3 Stock solutions for SDS-PAGE.

Stock Solution	Method of preparation
Acrylamide stock solution (29.1% w/v Acrylamide, 0.9% w/v NN'-Methylenebis-acrylamide)	29.1 g Acrylamide 0.9 g NN'-Methylenebis-acrylamide dissolved in 60 mL distilled water then made up to 100 mL with distilled water The solution was stored in an amber coloured bottle at 4°C for up to two weeks.
1.875 M Tris-HCl Buffer pH 8.8	56.8 g Tris dissolved in 150 mL distilled water then carefully adjusted to pH 8.8 with 5N HCl then made up to 250 mL with distilled water. Solution was stored at 4°C for up to four weeks.
1.25 M Tris-HCl Buffer pH 6.8	37.8 g Tris dissolved in 150 mL distilled water then carefully adjusted to pH 6.8 with 5N HCl then made up to 250 mL with distilled water. The solution was stored at 4°C for up to four weeks.
10% Sodium Dodecyl Sulphate (SDS) solution	10.0 g SDS dissolved in 85 mL distilled water then made up to 100 mL with distilled water. The solution was stored at room temperature for up to four months.
10% Ammonium Persulphate solution	Approximately 0.05 g ammonium persulphate made up in ten times volume of distilled water. The solution was made up daily as required.
10X Electrode Buffer Tris-Glycine	144.2 g Glycine 30.3 g Tris 10.0 g SDS dissolved in 800 mL distilled water then made up to one litre with distilled water. Solution was stored at room temperature for up to two months. Single strength buffer was made by diluting 10X buffer one in ten with distilled water.
TEMED	N,N,N',N'-tetramethylethylenediamine 99% pure

Homogenous polyacrylamide gel sandwiches, 12% acrylamide unless otherwise stated, were poured using a clamp assembly held in place on a casting stand. Discontinuous gels were prepared using a stacking and resolving gel. The volume of each of the stock reagents used to prepare minigels is described in Table 2.4. Larger gels were prepared by adjusting volumes according to the system size. The first three solutions used to prepare the resolving gel, (acrylamide, water and pH 8.8 buffer) were gently mixed and degassed under vacuum for 15 minutes. The remaining solutions, SDS, TEMED and ammonium persulphate were added with gentle swirling between additions. The gel was cast immediately into a vertical slab glass sandwich, then layered with butanol water (butan-2-ol: water, 1:1) and left from 30 minutes to 12 hours to polymerise at room temperature. The stacking gel was prepared in the same manner as the resolving gel and cast over the resolving gel after the removal of the butanol water. A 0.75 mm teflon comb was inserted into the acrylamide mixture to form wells (1, 10, 15 or 25 wells) that later held the samples being analysed. The stacking gel was allowed to polymerise for one hour before the gel was fitted into the buffer tank containing single strength electrode buffer and loaded with samples and standards.

2.5.1.1 Sample preparation

Samples for 1D SDS-PAGE were boiled for five minutes with an equal volume of double strength loading buffer. Double strength loading buffer was prepared from 2.5 mL of 1.25 M Tris-HCl pH 6.8 buffer, 1.0 g SDS, 2.5 mL 2-mercaptoethanol, 5.8 mL glycerol (87%) and 5 mg Bromophenol Blue made up to 25 mL in distilled water. The buffer was aliquoted into Eppendorf tubes in one mL amounts and stored frozen for up to 6 months. After boiling, the samples were centrifuged (ten minutes at 16,000 x g) and the protein rich supernatant was then removed and either frozen until required (-20°C) or loaded immediately onto the gel.

Table 2.4 Volume of stock reagents for SDS-PAGE.

Reagent	Resolving gel (volume required)	Stacking gel (volume required)
Acrylamide stock solution	3.2 mL	0.8 mL
Distilled water	3.09 mL	3.6 mL
Tris-HCL pH 8.8	1.6 mL	---
Tris-HCL pH 6.8	---	0.5 mL
10 % SDS	80 µL	50 µL
TEMED	4 µL	9 µL
10% Ammonium Persulphate	27 µL	22 µL

Low weight molecular weight standards, prepared at the same time, were boiled in loading buffer for 45 secondss, according to the manufacturer's (BioRad) instructions. Electrophoresis was performed at 150 V (constant voltage) until the bromophenol blue dye front reached the bottom of the gel sandwich, then the gel was stained by either Coomassie blue or Silver stain as described in section 2.5.3.

2.5.2 Polyacrylamide gel electrophoresis (2-D)

A pre-formed immobilised pH gradient on an 11 cm plastic support strip (Immobiline DryStrip pH 4-7 or pH 3-10, Amrad Pharmacia Biotech) was rehydrated overnight by placing the strip in a sterile screw-capped test tube containing 10 mL of rehydration solution. Rehydration solution was prepared fresh from the following reagents: 24.0 g of urea, 0.25 mL of Triton X-100, 75 mg of dithiothretol (DTT) and 0.10 mL of 1.0 mol/L acetic acid, made up to a final volume of 50 mL of water. The next day the strip was removed from the test tube and the prepared sample applied to the strip. Samples were prepared by defrosting, measuring the protein concentration and diluting to obtain 4-10 μg of protein in sample buffer (1:5). Sample buffer was prepared from 24 g of urea, 1.0 mL of 2-mercaptoethanol, 1.0 mL of pharmalyte 3-10 (Amrad Pharmacia Biotech), 0.25 mL of Triton X-100 and a few grains of bromophenol blue made up to a final volume of 50 mL. The strip was focused in three stages using a Multiphor II electrophoresis unit with 2-D application kit according to the manufacturer's instructions (Amrad Pharmacia Biotech). After focussing, the strip was removed and equilibrated in solutions A and B (ten minutes in each solution). The two equilibration solutions, A and B, were prepared from Tris-HCL stock (Tris 61 g, 300 mL distilled water, adjusted to pH 6.8 with 1.0 mol/L HCl, made up to 1 L in distilled water). Both solutions contained; Tris-HCl stock 20 mL, urea 72 g, glycerol 60 mL, SDS 2 g and were made up to 200 mL with distilled water. Additionally, equilibration solution A contained DTT (25 mg /10 mL) and equilibration solution B contained iodoacetamide (0.45 g/10 mL). Later 2-D SDS-PAGE studies also used both 7 cm and

18 cm strips. These strips were re-hydrated with the sample added to the re-hydration buffer, which improved resolution of the protein spots. Focussing times varied according to the size of the strip, but in general times were set at the high end of the range of times suggested by the manufacturer (Pharmacia). The second dimension of separation was performed by placing the equilibrated strip onto either a precast 8-18% gradient gel (excel gel, Amrad Pharmacia Biotech), or a 12% vertical gel sandwich. Protean II or triple wide gels were prepared as described in 2.5.1, with the exception that volumes were increased for use in the larger systems. The second dimension was run for 120 volt hours (600 V, 40 mA, 10 W) at constant current before the plastic support strip was removed. The gel was then allowed to run until the bromophenol blue dye front had reached the lower edge of the acrylamide gel.

2.5.3 Visualisation of protein bands on polyacrylamide gels

At the completion of 1-D and 2-D electrophoresis, gels were placed in fixing solution (80 mL ethanol, 20 mL acetic acid, 100 mL distilled water) for at least 30 minutes. Depending on the protein concentration of the band or spot of interest, Coomassie blue (milligram quantities of total sample) or silver staining (microgram quantities of total sample) was performed.

2.5.3.1 Coomassie blue stain

Solutions used for Coomassie staining are described in Table 2.5. After fixing, the fixing solution (water:95% ethanol:glacial acetic acid, 5:4:1 v:v) was replaced with staining solution for at least 40 minutes. The gel background was then de-stained in de-staining solution until the bands were clearly visible against the blue background (around one hour). The gel was then transferred to distilled water to complete the destain (12-24 hours). The gel was placed in preserving solution for 20 minutes and then photographed and dried between cellophane sheets using the BioRad gel drying system according to the manufacturer's instructions.

Table 2.5 Solutions for Coomassie blue stain of SDS-PAGE gels.

Solution	Method of preparation
Staining solution	1.5 g Coomassie blue R250 105 mL Ethanol 30 mL Acetic Acid made up to 300 mL with distilled water and mixed overnight
De-staining solution	210 mL Ethanol 60 mL Acetic Acid made up to 600 mL with distilled water
Preserving solution	15 mL Glycerol 53 mL Ethanol 15 mL Acetic Acid made up to 150 mL with distilled water

Table 2.6 Solution used for silver staining of SDS-PAGE gels.

Solution	Method of preparation
Incubation solution	60 mL Ethanol 8.2 g anhydrous Na acetate 1.04 mL 25%w/v Glutaraldehyde $^{\alpha}$ 0.4 g Na thiosulphate made up to 200 mL with distilled water
Silver solution	0.2 g Silver nitrate 40 µL Formaldehyde $^{\alpha}$ made up to 200 ml with distilled water
Developing solution	5.0 g Sodium carbonate 20 µL Formaldehyde $^{\alpha}$ made up to 200 mL with distilled water
Stop solution	2.92 g Sodium EDTA made up to 200 ml with distilled water
Preserving solution	20 mL Glycerol made up to 200 mL with distilled water

$^{\alpha}$these reagents were added immediately before use.

2.5.3.2 Silver staining

After fixing for at least 30 minutes, the fixing solution was replaced with incubation solution for one hour. Solutions for silver staining are described in Table 2.6. The gel was then washed (three washes of twenty minutes) in distilled, deionised water before being placed in silver solution for 40 minutes. The silver solution was then removed and replaced with developing solution. The bands were allowed to develop (usually 2-10 minutes dependent on the protein concentration of the bands of interest). Further development of the bands was stopped by the addition of a stop solution for 5-10 minutes. The gel was washed (2 x 5 minutes) in distilled water then placed in preserving solution for 20 minutes before being photographed and dried between cellophane sheets using the BioRad gel-drying system according to the manufacturer's instructions.

2.5.3.3 Determination of molecular weight and protein band densities

SDS-PAGE gels and Western immunoblots were scanned using an enhanced laser densitometer (UltroscanXL, Pharmacia LKB) or a Gel-Pro analyzer (Gel-Pro version 3.0, Media Cybernetics) to determine the molecular weight of specific protein bands and their density relative to the other bands present.

2.5.4 Western blotting

Solutions used in Western blotting are described in Table 2.7. Detection of specific GroE and DnaK proteins was made using the Western blotting method. After SDS-PAGE, proteins were transferred by electroblot to a nitrocellulose membrane (BioRad) using the following method. The SDS-PAGE gel to be blotted was sandwiched with the nitrocellulose membrane between 6 sheets of 3MM chromatography paper (Whatman). The gel/membrane sandwich was then placed into a transblot transfer cell (BioRad) and filled with transfer buffer. The proteins were transferred to the membrane using the following conditions: 15V/100 mA at 15°C overnight. Following transfer, the gel was stained with Coomassie blue.

Table 2.7 Stock solutions used in Western blotting technique.

Solution	Method of preparation
Tris buffered saline (TBS)	0.05 M Tris pH adjusted to pH 7.5
Tris buffered saline with Tween 20 (TBST)	TBS with 0.02% (v/v) Tween 20
Blocking solution	TBST with 1% (w/v) gelatine
Transfer buffer	39 mM glycine, 48 mM Tris base, 0.037% SDS, 20% (v/v) methanol in 1000 mL water

An absence of bands confirmed the completeness of the protein shift to the membrane. The membrane was then blocked for one hour in blocking solution then washed twice in TBS-T before being incubated for one hour in either a 10^{-5} dilution of a rabbit anti-*E. coli* GroEL (Sigma Immuno Chemicals) or a 10^{-5} dilution of a rabbit anti-*E. coli* Dna-K (DAKO). After two further washes in TBS-T the membrane was incubated for one hour in 10^{-4} diluted goat anti-rabbit antibody (BioRad). Heat shocked *E. coli* cells were used as a positive control. *E. coli* cells were heat-shocked by growth at 42°C for 30 minutes. The blot was visualized using a solution containing 10 mL of methanol, 30 mg of 4-chloro-1-napthol (Sigma Immuno Chemicals) and 35 µL of hydrogen peroxide in 50 mL of TBS according to the supplier's instructions and scanned using an enhanced laser densitometer (UltroscanXL, Pharmacia LKB).

2.5.5 Isoelectric focussing (IEF) using a Rotofor cell

The Rotofor preparative IEF cell was prepared and run according to the manufacturer's instructions (BioRad). The anode was filled with 25 mL acidic electrolyte (0.1 M H_3PO_4) and the cathode was filled with basic electrolyte (0.1 M NaOH). The system was temperature controlled at 4°C during each run by running an external coolant through a central cooling finger.

Crude protein mixtures prepared by homogenisation or bead-beating (section 2.4.1.3) were dialysed against distilled water then mixed with carrier ampholytes to a final concentration of 2% v/v to make a final volume of 55 mL. The protein-ampholyte solution was injected into the sample chamber and large air bubbles removed. Constant power (12 W) was applied to the Rotofor until the current had stabilised for one hour. Each run took approximately four hours. A harvesting box was then connected to the system and the fractions in each of the 20 sample lanes quickly and simultaneously removed by vacuum into 12 x 75 mm glass tubes. The pH of each of the fractions

was determined and then the fractions were analysed by 1-D SDS-PAGE for the presence of the proteins of interest.

2.5.6 Continuous elution electrophoresis

The model 491 Prep Cell (BioRad) was used to purify crude protein mixtures prepared by homogenisation (section 2.4.1.3). Protein samples were electrophoresed through a cylindrical Ornstein-Davis (Tris/chloride/glycine) discontinuous buffer system for native-PAGE where the mobility of proteins depends on both size and charge. As proteins pass through the gel they separate into bands and are eluted from the gel and passed into a collection chamber where they are mixed with an elution buffer and drawn away by the action of a peristaltic pump. The separated proteins then pass into a fraction collector in 1 mL aliquots. A 30% acrylamide/bis stock solution was used to prepare a 10 cm long cylindrical tube of 10% acrylamide. Each 10 mL contained: 4.15 mL deionised water, 2.5 mL resolving gel buffer (1.5 M Tris-HCL 8.8) and 3.33 mL acrylamide/bis 30% stock solution and was polymerised with 50 µL APS and 5 µL TEMED using the method described for preparation of 1-D SDS-PAGE. The resolving gel was topped with a 1cm, 4% stacking gel made up with 6.15 mL deionised water, 2.5 mL resolving gel buffer (0.5 M Tris-HCL 6.8) and 1.33 rnL acrylamide/bis 30% stock solution per 10 mL. The gel was polymerised with 50 µL APS and 10 µL TEMED using the method described for preparation of 1-D SDS-PAGE. Samples were prepared in sample buffer (0.0625 M Tris-Cl, pH 6.8, 25% glycerol, 0.012% bromophenol blue). The electrode running buffer, pH 8.3, was made up of 30.3 g Tris base and 144 g glycine in 1 L deionised water. The preparative cell was run at 10 watt constant power (40 mA and 300 volts) at 4°C. The elution flow rate was set at 0.75 to 100 µl per minute and fractions were collected following the appearance of the dye front. Run time was around 7 hours.

Table 2.8 Solutions used for electro-blotting proteins to PVDF membrane.

Solution	Method of preparation
CAPS stock solution	22.13 g of 3-[cyclohexylamino]-1-propanesulfonic acid in 900 mL water
CAPS transfer buffer for electro-blot cell	200 mL CAPS stock solution added to 200 mL Methanol in 1600 mL water
Amido Black 10B stain	Amido Black 10B 1g Isopropanol 25 mL Acetic acid 10mL in 65 mL water

2.5.7 N-terminal sequencing

Solutions for preparing proteins for sequencing are described in Table 2.8. Protein samples were electrophoresed on 1-D or 2-D SDS-PAGE. Protein bands were electro-blotted using the technique described for Western blotting (2.5.4) to poly vinylidene diflouride (PVDF) membrane using a 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS) buffer and visualised using an Amido Black 10B stain. Bands of interest were excised and sent to either The Australian Proteome Analysis Facility at Macquarie University or LaTrobe University, Department of Biochemistry.

2.5.8 Analysis of protein sequences

Protein sequences were accessed and analysed through the Australian National Genomic Information Service (ANGIS) using the Internet site www.angis.su.org.au and through The Protein Information Resource (PIR) using the Internet site http://www-nbrf.georgetown.edu/pirwww/.

2.6 Molecular Biology methods

Unless otherwise stated molecular biology methods were performed essentially as described in Molecular cloning: a laboratory manual (Sambrook *et al.*, 1989).

2.6.1 Preparation of plasmid DNA from bacterial cells

This method was used for the isolation of plasmid DNA from *E. coli* using an alkaline lysis method. An overnight bacterial culture of plasmid-bearing bacteria, which had been grown overnight in L-B broth containing ampicillin was gently swirled and 1·5 mL transferred into an Eppendorf tube and centrifuged for 2 minutes at 16,000 x g. The supernatant was discarded and another 1.5 mL of the culture poured into the same tube and centrifuged again. The supernatant was discarded. Eighty µL of solution 1 (50 mM glucose, 25 mM Tris-HCl (pH 8·0), 10 mM EDTA (pH 8·0)) was added into each tube and the pellet resuspended by vortexing for a few seconds. Twenty µL of lysozyme solution (Solution 1 containing 10 mg/mL lysozyme) was added then mixed by gentle inversion.

Two hundred μL of solution 2 (0.2 M NaOH, 1% SDS) was added to the tube. The contents were briefly mixed by inversion and stored on ice for 5 minutes. Solution 3 (150 μL of 3M sodium acetate, pH 5·2) was added and the tube mixed by gentle inversion before being placed on ice for 5 minutes. The tube was centrifuged for 5 minutes at 16,000 x g and the supernatant (approximately 400 μL) transferred to a fresh Eppendorf tube. DNAase-free RNAase A solution (5 μL of a 10 mg/mLsolution) was then added to the supernatant and incubated at 37°C for 15 minutes. The tube was transferred to ice and 450 μL of solution 4 (Tris-equilibrated phenol:chloroform:isoamyl alcohol mixture, 25:24:1) which was then mixed by gentle inversion. The tube was centrifuged for 5 minutes at 16,000 x g and the supernatant transferred to another tube leaving the protein interphase behind.

Two volumes of chilled 100% ethanol (stored at -20°C prior to use) was added to the supernatant and gently mixed by inversion then placed at -70°C for ten minutes. The tube was centrifuged for ten minutes at 16,000 x g then the supernatant was decanted and the tube inverted, allowing the tubes to dry over paper towel for a few minutes. The pellet was dissolved in 50 μL of sterile distilled deionised water and stored at -20°C until required.

2.6.2 Quantification of nucleic acids

This technique was used to determine the concentration and purity of nucleic acids. The nucleic acid sample being tested was diluted 1/50 with sterile distilled, deionised water. The sample was gently mixed before being transferred to one of two optically matched quartz cuvettes. The second quartz cuvette was filled with distilled water and the absorbance at 260 nm (A_{260}) set to zero. The absorbance of the sample was then measured. The DNA concentration of the sample was then calculated using the following formula: μg DNA/mL = A_{260} multiplied by 50 multiplied by the dilution factor. 1 A_{260} unit = 50 μg DNA/mL

2.6.3 Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate DNA fragments according to size. Unless otherwise stated 1% agarose gels for electrophoresis were prepared in Tris-acetate buffer (TAE). A stock solution (50 X) of TAE was prepared by mixing 242 g Tris base, 57.1 mL glacial acetic acid, 100 mL 0.5 M EDTA (pH 8.0) and making up the solution to 1 L with water. A working TAE solution was prepared by diluting 1 part in 50 of water.

Agarose particles were melted by heating on 100% power in a microwave oven (Sharp) until the solution just boiled and when cooled (50°C), 3 μL of ethidium bromide solution (10 mg/mL) was added per 50 mL of solution. A gel was formed by pouring the agarose solution into a tray, using an eight-place comb to prepare wells for holding samples (BioRad Miniwide system). After the gel had set (twenty minutes), the comb was removed and samples loaded under running buffer. TBE running buffer was prepared from 10 x TBE stock which contained 108 g Tris Base, 55 g boric acid, 8.3 g EDTA per litre (pH 8.3). The gels were electrophoresed at 110V. Standards run at the same time were selected according to the expected size of DNA bands and supplied by Progen and MBI Fermentas.

2.6.4 Preparation of plasmid vector for cloning

DNA digested by the method described in section 2.6.7.2 was ligated to the plasmid vector pBluescript SK+ using the following method. The plasmid vector was prepared by digestion with the enzyme *BamH1* (MBI Fermentas). The solutions, listed in Table 2.9, were placed into an Eppendorf tube and incubated at 37°C for one hour. After digestion, an equal volume of phenol:chloroform:isoamyl-alcohol (25:24:1) was added to the tube, mixed and then the tube was spun for 5 minutes at 14,000 x g.

Table 2.9 Reagent mixture for preparation of plasmid vector prior to cloning.

Solution	Volume
plasmid DNA	25 μL containing 10 μg of DNA
Distilled water	56.5
10x buffer for *BamH1* enzyme	10 μL
Alkaline phosphatase (1U/μL)	1 μL
BamH1 (10 U/μL)	7.5 μL
Bovine serum albumin 100 mg/mL	7.5 μL

The supernatant was then transferred to another tube and 200 µL of 100% ethanol and 10 µL of sodium acetate (3M at pH 5.2) was then added to precipitate the DNA. The tube was then centrifuged and the DNA pellet dissolved in 15 µL of sterile distilled water.

2.6.5 Preparation of competent bacterial cells

E. coli TG2 cells were treated to induce a 'competence' phase that allowed the cells to take up DNA from the external medium. Two beads of TG2 cells (frozen stock) were transferred to 20 mL of L-B broth and incubated overnight at 37°C. One mL of the overnight culture was transferred into 100 mL of L-B broth and incubated at 37°C on a shaking incubator (200 rpm) until the OD_{600} was 0.40 to 0.45. The culture was centrifuged in sterile 50 mL Falcon tubes for five minutes (8,000 x g at 4°C) and the supernatant discarded. The cells were then resuspended in 25 mL of 100 mM $MgCl_2$ using gentle pipetting then placed on ice for twenty minutes. The cells were centrifuged and resuspended as before in 100 mM $CaCl_2$, then left on ice for one hour. The cells were then ready for immediate use in transformation reactions.

2.6.6 Construction and design of oligonucleotide probes

Oligonucleotide probes were manufactured using an ABI-Perkin Elmer oligonucleotide synthesizer by a service provided by VU or were obtained from Sigma-Genosys (Sigma Aldrich Pty Ltd). Probes were designed using Prime GCG (Angis Biomanager) using the Internet site at www.angis.su.org.au. Netprimer, (Premier Biosoft International), was used to predict the properties of primers designed using Prime GCG. Tables 2.10 and 2.11 show the composition of primers used in this study.

Table 2.10 Composition of oligonucleotide probes used in the identification of LAB.

Name	Oligonucleotide	Tm	Length	Reference
ACI 16S 1	AGCTGAACCAACAGATTCAC	59.3	20	(Walter *et al.*, 2001)
ACI 16S 2	ACTACCAGGGTATCTAATCC	53.6	20	(Walter *et al.*, 2001)
PR 1	CAGACTGAAAGTCTGACGG	58.5	19	(Walter *et al.*, 2001)
CAS 2	GCGATGCGAATTTCTTTTTC	63.4	20	(Walter *et al.*, 2001)
Lm3	CGGGTGCTNCCCACTTTCATG	63.4	21	(Kaufmann *et al.*, 1997)
Lm26	GATTCTGGCTCAGGATGAACG	54.6	21	(Kaufmann *et al.*, 1997)
LbLMA1-rev	CTCAAAACTAAACAAAGTTTC	47.1	21	(Dubernet *et al.*, 2002)
R16-1	CTTGTACACACCGCCCGTCA	62.4	20	(Dubernet *et al.*, 2002)
Ban 2	CATATTGGATCACGGTCC	50.5	18	(Ventura *et al.*, 2001)
23Si	CATTCGGACACCCTGGGATC	62.0	20	(Ventura *et al.*, 2001)
PAF	AGAGTTTGATCCTGGCTCAG	54.0	20	(Yeung *et al.*, 2002)
536R	GTATTACCGCGGCTGCTG	57.5	18	(Yeung *et al.*, 2002)

Table 2.11 Composition of oligonucleotide probes used for amplification of gene products or probing genomic DNA libraries.

Name	Oligonucleotide	Tm	Length	Reference
SP45ASP	GAAACNAA(T/C)GCN(C/T)TN(C/T)C	60.5	17	This study
SP1S	AGATAGCCGAACACGATGCC	66.2	20	This study
SP1AS	CACGACGACAGCAAAGAAAAC	64.6	21	This study
SP2S	AACACAATGCTCTCACGAC	58.8	19	This study
SP2AS	ACGACGACAGCAAAGAAAAC	61.8	20	This study
SP3S	ATGGTGCGGGCAATTAAG	63.4	18	This study
SP3AS	TGAAGATGCGAACACAGAAG	61.6	20	This study
2SP1S	GTCCTTACACGCAGGGTTCT	63.0	20	This study
2SP1AS	CGAACACCCAAACCATCTCT	63.8	20	This study
2SP2AS	CGGCTCAATCATCGTCTTC	63.4	19	This study
2SP3S	CCAGAGATGGTTTGGGTGTT	63.7	20	This study
2SP4S	CGATGGTGCTGGTTGAAGT	63.8	19	This study
2SP4AS	TGAAGACGGAACAGGAAAATG	63.8	21	This study
2SP5S	GCATTTTCCTGTTCCGTCTT	62.9	20	This study
2SP5AS	AAAAGGCTGGCAACTCTGTC	63.3	20	This study
pUC/M13-F	CGCCAGGGTTTTCCCAGTCACGAC	72.5	24	www.promega.com
pUC/M13-R	TCACACAGGAAACAGCTATGAC	55.2	22	www.promega.com
3SP1S	AGACAGCAGCAGAGATAAACAAG	52.9	23	This study
3SP2S	ACAGACAGCAGCAGAGATAAAC	52.6	22	This study
3SP3S	TGCCAAAAAAAGAAGCCACAAG	52.9	22	This study
3SP1AS	CACCATCGCCAACAACACAG	54.4	20	This study
3SP4S	GCCAAATAGCCAAAGCCATAC	52.1	21	This study
3SP2AS	ATCACATCACGATCCCAACC	51.9	20	This study

2.6.7 Construction of representative genomic DNA library

2.6.7.1 Extraction and purification of DNA

This method of extracting DNA from *lactobacilli sp.* was taken from that published by Johnson (1980). The culture to be extracted was grown to stationary phase, and then 1 mL was removed to an Eppendorf tube and spun for two minutes (14,000 x g).

The supernatant was discarded and the pellet resuspended in 100 µl of 50 mM Tris /HCL, 50 mM sodium chloride and 10mM EDTA pH 8. Fifteen µl of 15mg ml^{-1} lysozyme solution was added to the pellet and incubated for 20 minutes at 37°C. Forty µl of 25% (w/v) SDS was then added to the tube and heated to 65°C for 5 minutes before vortexing for 5 seconds. Thirty-three µl of sodium chloride (5 M) was added before the tube was left on ice for one hour.

After centrifugation at high speed (14,000 x g) for fifteen minutes the supernatant fluid was treated with equal volumes of chloroform-isoamyl alcohol (24:1, v/v). The supernatant was re-centrifuged for 5 minutes before the upper phase was taken and the DNA precipitated with ice cold 70% alcohol.

The DNA was resuspended in 150 µl of TE buffer (10mM Tris/HCl, 1mM EDTA, pH 8) and treated with DNAase-free RNAase to a final concentration of 10 µg ml^{-1} by incubation at 37°C for 30 minutes. The DNA was then stored at -20°C until required.

2.6.7.2 Digestion of DNA

Extracted DNA used for preparation of the genomic DNA library was digested with the enzyme *Sau3A 1* and the reaction stopped by a phenol extraction of the enzyme. Sixty µL of a DNA solution containing 100 µg of DNA prepared in section 2.5.8.1 was mixed with 84 µL of enzyme buffer (Promega), 1 µL of bovine serum albumin (Promega) and 0.4 µL (or 1.2 units) of Sau 3A 1

enzyme (Promega). After digestion for 30 minutes at 37°C, an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the tube, mixed and then the tube was spun for 5 rminutes at 14,000 x g. The supernatant was then transferred to another tube and 600 µL of 100% ethanol and 100 µL of sodium acetate (3M at pH 5.2) was then added to precipitate the DNA. The tube was then centrifuged and the DNA pellet dissolved in 50 µL of water.

2.6.7.3 Ligation of insert and vector

One µL of the vector DNA prepared in section 2.6.4 was added to 10 µL of genomic DNA prepared in section 2.6.7.1, 1 µL distilled water 1.5 µL of ligase buffer (Promega) and, 1.5 µL T4 DNA ligase buffer (Promega). The tube was incubated at 20°C for 2 hours then the DNA was transformed into competent cells.

2.6.7.4 Transformation of bacterial cells with plasmids

All of the reaction mixture prepared in 2.6.7.3 was incubated with 200 µL of competent cells prepared as per section 2.6.5. The mixture was left on ice for one hour then incubated at 42°C for 3 minutes. Five hundred µL of double strength YT medium was added to the tube and further incubated for one hour at 37°C before being plated out on colour selection plates. Positive and negative growth controls were prepared from competent cells plated onto both L-B and ampicillin plates. Competent cells with plasmid preparation but no insert were used to check transformation efficiency. Transformed TG2 cells were indicated by white colonies on colour selection plates. These cells were transferred to microtitre plates containing double strength L-B broth and gycerol (1:1) and frozen for later use. TG2 cells without inserts were blue.

2.6.8 Colony blotting

This technique was used to lift colonies containing fragments of the *Lb. acidophilus* genomic DNA sequence so that they could be probed for a particular gene of interest. Circles of Hybond N^+

membrane (Pharmacia) were cut to fit the Petri plates on which transformed TG2 cells, prepared as per section 2.5.7.4, had been grown. Each Petri plate held 47 transformants and one position marker. The transformants had been grown from frozen cultures prepared as described in section 2.5.7.4 and were transferred to a set location on the plate with sterile toothpicks; orientation of the membrane was marked on both plate and membrane relative to the position marker. The membrane was then placed onto the agar surface for one minute before being transferred (colony side up) onto an absorbent filter pad soaked in denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 7 minutes. Next, the membrane was placed onto a pad of absorbent paper soaked in neutralising solution (1.5 M NaCl, 0.5 M Tris-HCl pH7.2, 0.001 M EDTA) for three minutes. This step was repeated with a fresh pad soaked in the same solution.

The membrane was then washed in double strength SSC solution (0.3 M NaCl, 0.03 M Na_3 citrate) before being air-dried colony side up for one hour. After the membrane had dried it was wrapped in plastic wrap and exposed to UV light for three minutes to cross-link the DNA to the membrane. The membrane was then stored at 4°C until required.

2.6.9 Preparation of labelled oligonucleotide probes used in hybridisation procedures

The following reagents were combined in an Eppendorf tube and incubated at 37°C for 30-60 minutes. The oligonucleotide probe was prepared as described in section 2.6.

- 65 µl oligonucleotide probe (100 ng),
- (γ^{32} P) ATP (3000 Ci/mmole @ 10 Ci/mL (volume varied according to time of manufacture),
- 1 µl 10 X T4 polynucleotide kinase (PNK) buffer (Promega) and
- 1 µl T4 PNK (10 units/µl) (Promega) and sterile water added, as required, to bring the final volume to 10 µl.

The reaction was stopped with one µl of 0.5M EDTA. Unincorporated probe was then removed from this reaction mix.

2.6.10 Removal of unincorporated label

A QUIquick™ nucleotide removal kit was used to remove unincorporated radioactive label (QIAGEN). The kit was used according to the manufacturer's instructions. Ten volumes of PN buffer* were added to 1 volume of the reaction rriix, prepared in method 2.6.9, then mixed. A QUIquick™ spin column was placed in a 2-mL collection tube (provided in kit) and the sample applied to the column and centrifuged. (One minute at 4,000 x g). The column was washed by adding 500 µl of PE buffer and centrifuged as before. PE and PN buffers were supplied as part of the kit. The eluent was discarded as radioactive waste and the column washed again with another 500 µl of PE buffer. The flow through was discarded as radioactive waste and the column placed back in the same tube then centrifuged (one minute at 16,000 x g). The column was placed in a clean 1.5 mL centrifuge tube. DNA was eluted by adding 100-200 µl sterile distilled deionised H_2O to the centre of the QIAquick™ column. The column was allowed to stand one minute then centrifuged (one minute at 16,000 x g). The radioactive label was then placed at 65°C for 10 minutes before proceeding to hybridisation.

2.6.11 Hybridisation of oligonucleotide probes

The purpose of the method was to hybridise a radio-labelled probe to specific genomic DNA cross-linked to a membrane prepared according to the method described in section 2.6.8 and the attachment visualised using auto-radiographic techniques. ExpressHyb hybridisation solution was used according to the manufacturer's instructions (ClonTech). The ExpressHyb solution was warmed to 68°C and stirred well to completely dissolve any precipitate. The solution was then equilibrated at 37°C. The colony lift was pre-hybridised in a thick-walled plastic bag (blender bag)

containing a minimum of 15 mL of ExpressHyb solution (37°C for 30 minutes with continuous shaking). Radio-labelled probe was added to 5 mL of fresh ExpressHyb, well mixed, then the ExpressHyb pre-hybridisation solution was replaced with the fresh solution containing the radio-labelled oligonucleotide probe. All air bubbles were removed from the bag and care was taken to distribute the solution evenly. The bag was then incubated with continuous shaking at 37°C for 60 minutes. The blots were then rinsed in wash solution 1 (2 X SSC and 0.05% SDS at room temperature) using several changes of wash solution (total of 30-40 minutes with continuous agitation). The blot was then washed with wash solution 2 (1 X SSC and 0.1% SDS at room temperature) for 40 minutes with one change of liquid. The blots were removed with forceps, excess solution was removed by shaking and the membranes were immediately sealed in plastic blender bags then exposed to X-ray film at -70°C for 1-2 days. Positive reactions as indicated by bright spots on the X-ray film were used to locate colonies containing transformed cells with plasmid inserts that hybridised with the probe, see section 2.6.3. Plasmid preparations were performed on the positive clones and the sequences of the inserts were determined according to section 2.6.14.

2.6.12 DNA amplification

PCR was used to amplify fragments of genes of interest in this study. An MJ Research thermocycler model MPTC-100-96VHB supplied by Geneworks was used according to the manufacturer's instructions. Reaction mixtures and thermal cycling parameters are described in the relevant results sections.

2.6.13 Sequencing of DNA

Preparation of templates for automated sequencing was performed using either a Dye Termination Cycle Sequencing Ready Reaction Kit (Perkin Elmer) or the Beckman CEQ 2000 Dye terminator sequencing protocol and performed according to manufacturer's instructions. Samples were submitted for automated sequencing to a joint VU/Monash University facility at the Department of

Microbiology, Monash University or to the sequencing facility at The University of Melbourne, Gilbert Chandler Research Laboratories.

2.6.14 Analysis of DNA sequences

DNA sequences were analysed using the database similarity search program (BLASTn). BLASTn was accessed through ANGIS and the Computational Biology Program of the Life Sciences Division of Oak Ridge National Laboratory (ORNL) using the internet site at http://genome.ornl.gov/. Other DNA analysis programs accessed through ANGIS included:

- Flip 6 Frames, a Unix C program used to find and translate open reading frames (orf's);
- ClustalW. ClustalW was used to align sets of nucleotide sequences; and
- TACG. TACG was used for the restriction enzyme analysis of DNA.

2.6.15 Bioinformatic analysis of proteins

Bioinformatic analysis of proteins was performed through ANGIS using the following programs:

- BlastP was used to search protein databases;
- SPScan was used to scan protein sequences for the presence of secretory signal peptides;
- Secondary structure was predicted using the Garnier program;
- Isoelectric (GCG) was used to plot charge as a function of pH to determine the iso-electric point and predict molecular weight of a peptide sequence;
- Grease, was used to plot the hydrophobicity profile of a protein using the Kyte-Doolittle hydropathy plot; and

- PrettyBox (GCG) was used to prepare postscript formats of aligned protein sequences.

2.7 Photography

2.7.1 Photography of SDS-PAGE and agarose gels

Photographs of SDS-PAGE and agarose gels were taken with a Polaroid MP4 Land Camera with Black and White positive film (Polaroid Polaplan 667). Agarose gels were placed on a transilluminator (302 nm) (LKB2001 Macrovue) and photographs were taken using an orange filter in the dark. The size of any restriction bands present was deterrnined from comparison with molecular size markers. SDS-PAGE gels were placed on a white light box and photographed without a filter in the dark. Size of any protein bands present was determined according to section 2.5.3.2. 2-D SDS-PAGE gels were scanned on a flat bed scanner (Hewlett Packard) and images were cropped and rotated in the photoeditor software package (Microsoft).

2.7.2 Still Photography

Still photography was performed with a single lens reflex camera (Olympus OM-10) and the film was developed and printed by Prosper Photographics.

2.7.3 Exposure of hybridised membranes to X-ray film

Membranes were exposed to X-ray film (Kodak X AR 5) (Integrated Sciences) in a cassette with intensifying screen (Kodak Bio-Max) between 24 and 72 hours at -70°C. X-ray films were developed in AGFA Gevaert G150 manual X-ray developer (1/5 dilution in water) for 3-5 minutes. Films were then rinsed in running water for one minute followed by fixing (AGFA Gevaert G150 fixer) and rinsing in Kodak Photoflow 600 solution (1/600 dilution in water). A final rinse in running water was performed before films were hung up to dry.

Chapter Three

STRESS RESPONSES IN *LACTOBACILLUS PARACASEI* STRAIN VUP 12006

3.1 Introduction

The direction of the research undertaken in this thesis was, in part, determined from previous projects undertaken in CBFT at VUT investigating the role of stress responses in environmental and industrial bacteria. These research projects had concentrated on characterising changes in protein production by bacteria exposed to harsh industrial-manufacturing processes or environmental pollution, particularly involving heavy metals, such as conditions encountered in fermentations in the food industry and exposure to heavy metals. The proteins of interest in these projects were principally HSP60 and HSP70 and novel stress induced proteins. This research had shown that selected strains of LAB were able to survive in fermented dairy products, such as yoghurt, while others died shortly after manufacture (Shah *et al.*, 1995). The current research was to play a part in a coordinated study to improve the survival of LAB in manufacturing processes and aimed, principally, to determine how probiotic LAB respond to low pH environments. Other changes in the physical environment that may be experienced during dairy manufacturing processes and could affect the survival of bacteria, specifically changes in temperature and gaseous environment, were also to be examined. While a number of methods could have been selected to characterise the response to low pH environments, the principal method chosen was investigation of changes in protein production.

Due to the recognised problems in identification of bacteria used in the dairy industry, the strains selected for examination were initially characterised by phenotypic and phylogenetic characteristics

to confirm their identification. The difficulties in identification were confirmed during this work and a number of tools, including PCR amplification of 16S rRNA genes, had to be used to confirm results. Although initially the research had intended to study only strains of *Lb. acidophilus* these identification methods confirmed misidentification of one of the original strains provided by CSIRO. This strain was subsequently identified as *Lb. paracasei* and was the principal bacterium investigated and reported on in this chapter.

3.2 Identification of test strains

3.2.1 Gram stain and growth on MRS agar

Twenty-five bacterial strains reported to be *Lb. acidophilus,* including type strain ATCC 4356, were obtained from culture collections and commercially available dairy starter cultures. They were given interim identifications of C1 to C25. C1 to C17 were obtained from the CSIRO culture collection, C18 to C23 were isolated from commercially available yoghurts (Bornhofen Acidophilus yoghurt and Yoplait Yoplus A+B brand) and C24 and C25 were obtained from the Victoria University Culture Collection. The bacteria were grown overnight under anaerobic conditions on MRS agar at 37°C then examined for typical colony morphology and Gram stain. Typical colony morphology of lactobacilli was demonstrated by the appearance of creamy-white colonies approximately 2-3 mm in diameter. In a Gram stain the cells appeared as Gram positive bacilli with rounded ends, either as single cells or in short chains. Of the original twenty-five strains only nineteen strains, including the type strain, showed the basic morphology of lactobacilli and these were placed into frozen storage, using ceramic beads and glycerol/MRS broth as described in section 2.3.2. Two cultures failed to grow on MRS agar (C3, C24) and three strains were found to be Gram positive cocci (C7, C21, C23). These frozen stocks were used for additional phenotypic characterisation. Specifically these were: carbohydrate utilisation studies, FAME fingerprinting and protein profiling using SDS-PAGE and the results of these studies are presented in the following sections.

3.2.2 Carbohydrate utilisation

Patterns of carbohydrate utilisation were determined initially for a control strain VUP 12000 (*Lb. acidophilus* strain ATTC 4,356) using a traditional method that utilised lactobacilli fermentation broth in single vials (see section 2.3.1.2). This method did confirm the biochemistry of the test strain, however it was time consuming, expensive (due to the volume of reagent, cost of some of the carbohydrates and the number of vials used) and a quicker technique for examining LAB strains was sought.

Three methods were developed to solve the problem of cheap, rapid and multiple identifications. Initial trials focused on using various combinations of agar bases with 2% of selected carbohydrates. A number of different bases were examined: BLA was eventually chosen because it supported good growth when carbohydrate sources were added but growth was restricted in the absence of carbohydrate. A standard spot inoculation technique was used to transfer 35 standardised concentrations of bacterial suspensions to the surface of each plate containing a single carbohydrate source. Growth characteristics were satisfactory, however there was sufficient nutrient carryover in the inoculum to allow significant growth in the control, which made interpretation of the tests difficult.

The second method sought to compare carbohydrate utilisation using scanning densitometry. Growth on the agar plates, from the first method, was photographed using positive/negative Polaroid film. The negative was scanned using a laser densitometer and an attempt was made to quantitate the growth. The cut-off point for utilisation of a specific carbohydrate was determined for control organisms. Control plates with no carbohydrate were scanned and the difference between the control and the test plate represented the growth on the particular carbohydrate. Although this method was suitable for determining if an organism can or cannot grow on a specific substrate it is not feasible because each negative took around one hour to scan and interpret. In addition the cost

in time and money was excessive. The third method used an ELISA plate reader to quantitate growth in a liquid culture in a microtitre tray. This rapid microtitre plate method was developed to differentiate and presumptively identify LAB strains reliably, based on the results of fermentation tests. This novel method used a microtitre plate reader to quantify growth after six hours in liquid culture in a microtitre tray, which allowed the results to be obtained in one working day. A number of trials were set up to determine the best type of broth, optimal volumes, inoculum densities and incubation times and the final experimental conditions are described in section 2.3.5. These results were presented at the International Dairy Lactic Acid Bacteria Conference (Pepper *et al.*, 1995).

The nineteen stored strains that showed characteristics of being presumptive *Lb. acidophilus* and the type strain were tested for the ability to utilise fifteen different carbohydrate sources. The tests were performed in duplicate within experiments and two separate trials were performed. The seven strains that most closely resembled the typical, published results for the *Lb. acidophilus* type and results for the type strain are listed in Table 3.1. These eight strains designated VUP12000 through VUP 12007, were chosen to undergo acid tolerance screening tests. The type strain was numbered VUP 12000. All eight strains were presumptively identified as *Lb. acidophilus* based on the tests performed, although two strains were unable to utilise galactose, and the majority of *Lb. acidophilus* would normally be expected to utilise galactose. The results for sorbitol utilisation, a test that is normally inconsistent within this species, were variable and it was noted that it could not be utilised by five strains (Hammes *et al.*, 1995).

3.2.3 Fatty acid methyl ester profile

This method was designed to give a fingerprint or profile of the fatty acids produced when the eight strains presumptively identified as *Lb. acidophilus* were grown under a specific set of conditions (see section 2.4.1.2).

Table 3.1 Carbohydrate utilisation pattern in the type strain and 7 putative strains of *Lb. acidophilus* determined using a rapid microtitre plate method.

Ability to utilise a particular carbohydrate was determined from changes in optical density values for growth in 15 different substrates over six hours at 37°C in MRS broth, see section 2.3.5 for experimental details.

		Strain identification (number in bracket indicates strain was given VUP strain identification)																		
	Expected results [a]	C1 (12000)	C2 (12001)	C5 (12002)	C6 (12003)	C10 (12004)	C13 (12005)	C16 (12006)	C17 (12007)	C4	C8	C9	C11	C12	C14	C15	C18	C19	C20	C22	C25
Arabinose	- [b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cellibiose	+ [c]	+	+	+	+	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-
Fructose	+	+	+	+	+	+	+	+	+	-	-	+	-	+	-	+	+	-	+	+	-
Galactose	+	+	+	+	-	+	-	+	+	-	+	-	-	-	-	+	+	+	-	-	+
Glucose	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-

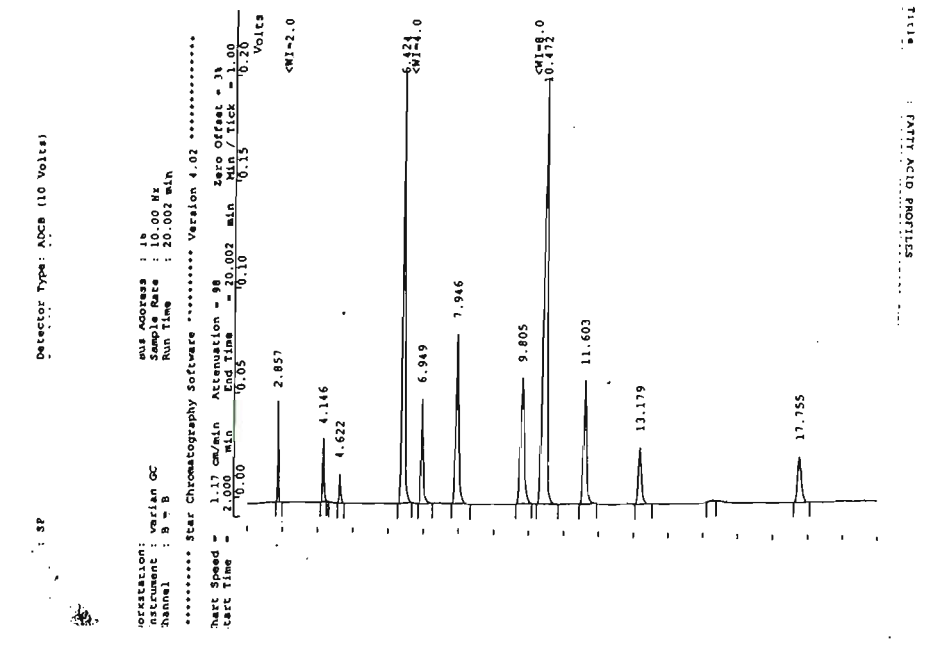
Mannose	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+
Mannitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Melizitose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Raffinose	d [d]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
Sorbitol	d	+	-	-	-	+	+	-	-	-	-	+	-	+	+	+	+	-	+	+	-	
Sucrose	+	+	+	+	+	+	+	+	+	-	+	+	-	-	-	+	+	-	-	-	-	
Xylose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
no carbohydrate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[a] Expected results for *Lb. acidophilus* compiled from published identification Tables (Hansen *et al.*, 1970, Johnson *et al.*, 1980, Holt *et al.*, 1994, Hammes *et al.*, 1995, Schleifer *et al.*, 1995). [b] Negative [c] Positive [d] 11-89 % positive

Long chain fatty acids present in the type strain, 7 presumptive *Lb. acidophilus* strains and 2 other lactobacillus strains (*Lb. jugurati* and *Lb. casei)* were converted into their corresponding methyl esters and and separated using a Varian gas chromatograph with a BPX-70 column. Examples of a standard profile obtained using bacterial fatty acid methyl esters (Sigma) and a FAME profile obtained during testing are shown in Figure 3.1. Only twelve major peaks (1-26% relative abundance) and approximately fifteen minor peaks of less than one percent relative abundance were found to be present using this method. The presence of *cis*-11,12-Methylene-octadecanoic acid (lactobacillic acid) supported the species identification of the strains being studied. The method was not used to differentiate all the fatty acids present completely and as such, the method did not give clear baseline separation for all peaks (section 2.4.4). In particular the peaks with a retention time of 10.4 and 10.6 minutes could not always be separated at the baseline for some tests and samples due to their high relative proportions present (Figure 3.1). Data for these two peaks are presented in Table 3.2 as a combined percentage, relative to the total area, of two fatty acid methyl esters (18:1(9) and 18:3), which is used when using FAME profiles as a form of fingerprint identification (Decallone *et al.*, 1991). The standard run length was 20 minutes; when runs were extended to forty minutes, no further peaks were detected.

Three peaks, designated X1, X2, and X3, could not be identified by comparison to known standards. Table 3.2 shows the relative abundance for the major peaks calculated from data of a typical run (average results of duplicates with standard deviation less than 7%). The results appeared to show that rather than a single profile or fingerprint for all the strains, there were two profiles, designated type 1, (demonstrated by strains VUP 12000 and 12003) and type 2 (demonstrated by strains VUP 12001,12002,12004,12005,12006 and 12007). Differentiation of the two types was based on levels of $C_{10:0}$ and the unidentified fatty acids X1 and X2.

A



B

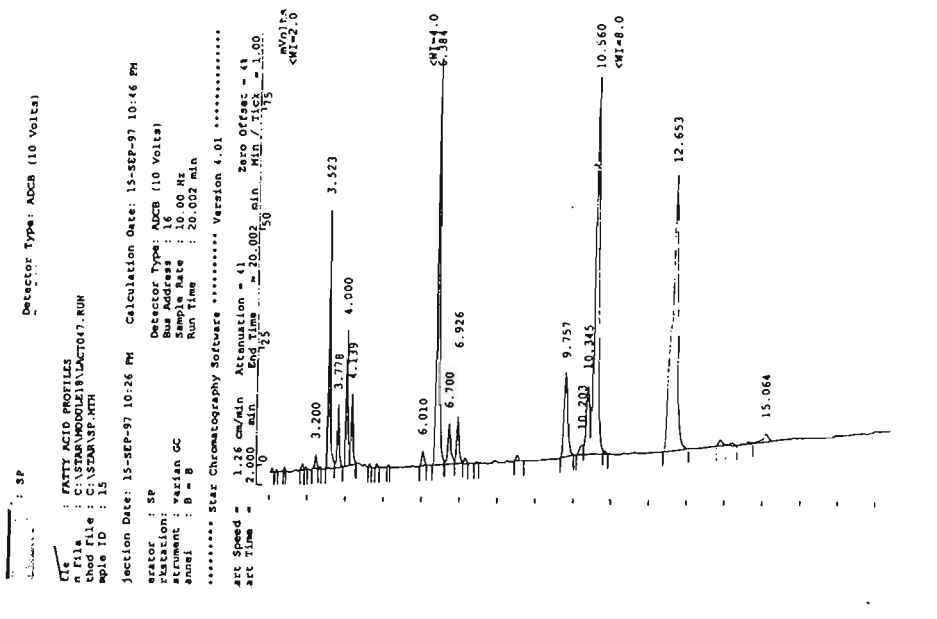


Figure 3.1 Examples of a standard profile obtained using bacterial fatty acid methyl esters (Sigma) and a FAME profile obtained during testing.

Figure A shows standard profile prepared from mixture of Sigma fatty acid standards (189-8, 189-5 and 189-1). Figure B show FAME profile of strain VUP 12006 obtained during testing. Cells of a stationary phase culture were harvested and the long chain fatty acids extracted from these cells were converted into the corresponding methyl ester according to the method described by Decallone *et al.*, (1991) (section 2.4.12). Fame profiles were obtained using a Varian Gas Chromatograph model 3400 using a BPX-70 column (section 2.4.4) and one µL sample injections.

Table 3.2 Fatty acid methyl esters present in the type strain and 7 presumptive *Lb. acidophilus* strains and 2 other lactobacillus strains (*Lb. jugurati* and *Lb. casei*).

Long chain fatty acids present in the type strain, 7 presumptive *Lb. acidophilus* strains and 2 other lactobacillus strains (*Lb. jugurati* and *Lb. casei)* were converted into their corresponding methyl esters and and separated using a Varian gas chromatograph with a BPX-70 column. Twelve major peaks were found to be present. Data below is presented as a percentage of the specific fatty acids present in a particular strain, values for minor peaks are not included. Results were calculated from the area under a specific peak divided by the area under all peaks present in a specific strain and multiplied by 100. The data are means of two independent experiments. Results of interest are highlighted, these are discussed in section 3.2.3.

[a] Methyl ester of fatty acids (%):											
	$C_{10:0}$	$C_{12:0}$	X1	X2	$C_{14:0}$	X3	$C_{16:0}$	$C_{16:01}$	$C_{18:00}$	[b] $C_{18:1\ (9)}$ & $C_{18:3}$	$C_{19:00}$
Retention time (min)	2.05	3.5	3.8	4.0	4.2	5.9	6.3	6.8	9.6	10.2	12.3
Strain No.											
12000	6	0	4	0	3	8	9	2	12	21	8
12001	0	10	2	5	4	1	23	2	5	22	19
12002	0	7	0	4	3	0	26	2	6	22	23

12003	6	0	0	0	1	3	10	3	9	23	11
12004	0	15	0	5	5	0	23	2	4	21	20
12005	0	16	0	8	4	1	10	3	4	20	16
12006	0	8	0	4	5	2	17	2	5	26	26
12007	0	9	0	5	5	0	22	2	6	22	20
Lb. jugurati	0	40	0	14	1	1	9	1	10	5	0
Lb. casei	0	10	0	4	4	1	24	2	5	16	11

[a] Relative proportion of fatty acid methyl ester based on percentage of total area for the peaks detected.

[b] Peaks for fatty acid methyl esters 18:3 and 18:1(9) could not be separated completely at the base line during all runs using the method described in section 2.4.4 and are presented as a combined percentage.

KEY: Methyl decanoate ($C_{10:0}$), Methyl dodecanoate ($C_{12:0}$), Methyl tetradecanoate ($C_{14:0}$), Methyl hexadecanoate ($C_{16:0}$), Methyl *cis*-9-hexadecanoate ($C_{16:1}$), Methyl octadecanoate acid ($C_{18:0}$), Methyl *cis*-9-octadecanoate acid ($C_{18:1(9)}$), *cis*-11,12-Methylene-octadecanoic acid (lactobacillic acid) ($C_{19:0}$)

The three differences noted between the two strains characterised as type 1 and the six strains characterised as type 2 were:

- type 1 strains showed $C_{10:0}$ levels of between 4 and 6 % relative abundance; this fatty acid was not detected in type 2 strains;
- the unidentified fatty acid X1 was not detected in type 1 strains but present between 7 and 16 % of type 2 strains; and
- the unidentified fatty acid X2 was not detected in type 1 strains but present between 4 and 8 % of type 2 strains.

These data suggested that strains VUP12000 and VUP12003 may be different to VUP strains 12001, 12002, 12004, 12005, 12006 and 12007.

Two strains of other *Lactobacillus sp.* were run as a comparison to the presumptive strains of *Lb. acidophilus* tested. The first, *Lb. jugurati,* demonstrated a clearly different FAME GC pattern compared to the presumptive strains of *Lb. acidophilus.* In particular:

- $C_{12:0}$ was present at more than twice the level demonstrated in any of the strains of the presumptive strains of *Lb. acidophilus;*
- the relative abundance of the $C_{18:1}$ and $C_{18:1(9)}$ combined peak was 5%. This was less than a quarter of that demonstrated in the presumptive strains of *Lb. acidophilus* and
- $C_{19:0}$ appeared to be absent in this strain. In the presumptive strains of *Lb. acidophilus* tested, $C_{19:0}$ varied between 8 and 26%.

The second control strain, *Lb. casei*, demonstrated a FAME pattern very similar to the presumptive strains of *Lb. acidophilus* with the exception that the $C_{18:1}$ and $C_{18:1\ (9)}$ combined peak was 16%

relative abundance, 25 % lower than shown in any of the presumptive strains of *Lb. acidophilus* tested.

3.2.4 SDS-PAGE profiles (fingerprinting of whole cell protein patterns)

SDS-PAGE analysis of whole cell proteins was evaluated to determine the utility of this approach for classifying LAB strains. The same growth conditions described for obtaining FAME profiles were used to obtain SDS-PAGE profiles. Visual comparison was made of the banding pattern produced by SDS-PAGE of whole cell proteins (see Figure 3.2). Data presented in a later section (3.4.4) shows that protein profiles taken hourly during growth at pH 6.3 and 37°C show significant changes during the growth cycle. Based on this observation, the test cultures were grown through two complete growth cycles at 37°C in MRS broth without pH control and the cells were collected in stationary phase and treated as described in section 2.4.1.

The protein profiles of all the strains tested, particularly VUP 12006, were dissimilar to the type strain (VUP 12000). In particular, strain VUP 12006 demonstrated fewer major bands and they were of higher density.

The major differences between strains were observed in the protein bands of less than 31 kDa with the exception of strains VUP 12001 and 12002 where no differences were observed. VUP 12002 is a mutant of 12001. This feature allowed differentiation of each of the strains, except VUP 12001 and VUP 12002, based on pattern matching. This method of identification was not pursued further due to the low level of discrimination obtained between strains.

3.2.5 Molecular methods of identification

The initial characterisation of the *Lactobacillus* strains clearly indicated that there were no phenotypic traits that could satisfactorily confirm identification to species level. In fact the classical methods described in previous sections failed to validate the genus of the species being tested.

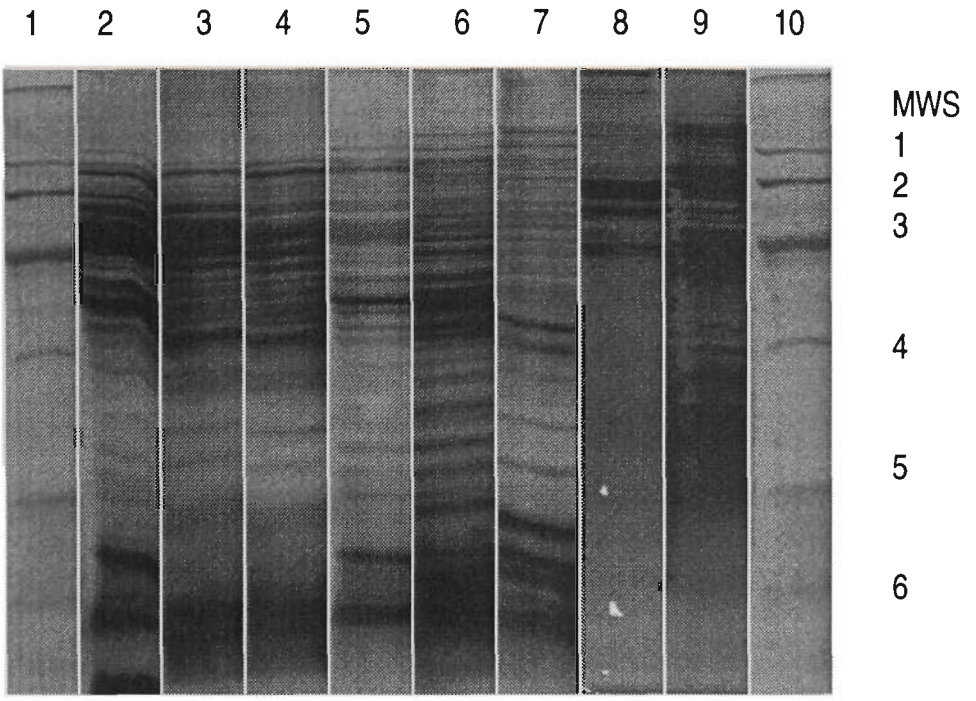


Figure 3.2 1-D SDS-PAGE profiles of presumptive *Lb. acidophilus* strains using whole cells for protein preparation.

Protein samples were prepared by boiling equal volumes of whole bacterial cells with double strength loading buffer as described in section 2.4.1.1 and 2.5.1.1. Approximately 1μg of protein was applied to each well of the SDS-PAGE gel and protein bands were visualised by silver staining.

Lane 1 MW Standard
Lane 2 Lb. acidophilus type strain VUP 12000
Lane 3 Strain VUP 12001
Lane 4 Strain VUP 12002
Lane 5 Strain VUP 12003
Lane 6 Strain VUP 12004
Lane 7 Strain VUP 12005
Lane 8 Strain VUP 12006
Lane 9 Strain VUP 12007
Lane 10 Molecular weight standards

MW Standards (MWS):
1. Phosphorylase B-97.4 kDa.
2. Bovine serum albumin-66.2 kDa
3. Ovalbumin-45 kDa
4. Carbonic anhydrase-31 kDa
5. Trypsin inhibitor 21.5 kDa
6. Lysozyme 14.4 kDa

It was therefore decided to use molecular biological techniques to identify the genus and species of strain VUP 12006, which was selected for characterisation of acid tolerance so that confirmation of identification was required. PCR-based methods were used to determine the identification of strain VUP 12006.

3.2.5.1 Genus level identification using PCR

The method described by Dubernet (2002) was used to identify strain VUP 12006 to the genus level. Total genomic DNA used for all PCR reactions was extracted from stationary phase cells grown overnight in MRS broth at 37°C as described in the method in section 2.6.7.1. Briefly, lysozyme solution was used to treat the cells prior to cell lysis in SDS, which was followed by a chloroform-isoamyl alcohol treatment. The DNA was precipitated following this treatment in ice cold 70% alcohol and made up in TE buffer. The oligonucleotide primer pair, designated R16-1 and LbLMA1-rev, was used to amplify a region between a flanking terminal sequence of the 16S rRNA gene and a lactobacilli-specific intergenic spacer region of the 16S/23S genes. In the study by Dubernet (2002) *Lactobacillus* species generated PCR fragments of approximately 250 bp. The composition of the oligonucleotide primers is described in Table 3.3.

Table 3.3 Composition of the oligonucleotide primers

Name	Sequence	Melting temperature
R16-1	5' CTTGTACACACCGCCCGTCA 3'	62.4
LbLMA1-rev	5' CTCAAAACTAAACAAAGTTTC 3'	47.1

The reaction mixture (50µL) contained 30 pmol of each primer, 200µM each of dATP, dTTP, dCTP and dGTP, approximately 100ng of bacterial DNA, 2.5 U of *Taq* DNA polymerase (Sigma) and PCR buffer with 1.5 mM $MgCl_2$. DNA was amplified as follows: initial denaturation at 95°C for 5 minutes, followed by 30 cycles consisting of denaturation at 95°C for 30 seconds, annealing at 55°C for 30

seconds, extension at 72°C for 30 seconds and a 7 minute final extension step at 72°C. The products were stored at 4°C until required. Aliquots of the reaction mixture (10μL) were separated on a 0.8% agarose gel, stained with ethidium bromide and visualised under UV light. The results are shown in Figure 3.3. Genomic DNA from strain VUP 12006, amplified using R16-1 and LbLMA1-rev, yielded a PCR product of approximately 240 bp. Due to the specificity of the primers for *Lactobacillus* DNA (Dubernet *et al.*, 2002) and the size of the fragments resulting from PCR of DNA extracted from strain VUP 12006, these results were consistent with strain VUP 12006 being placed in the genus *Lactobacillus*.

Two control strains, *Lb. acidophilus* type strain VUP12000 and *Lb. casei* GCRL-163 were run at the same time. It was observed that the fragment sizes of *Lb. casei* GCRL-163 and strain VUP 12006 were identical (≅240 bp) but smaller that obtained for *Lb. acidophilus* type strain VUP12000 (≅250 bp).

3.2.5.2 Species level identification using PCR

The species designation of strain VUP 12006 was determined using information based on PCR products from 3 different primer sets and sequence data from pair 1. The composition of the oligonucleotide primers is shown below in Table 3.4.

Table 3.4 Composition of the oligonucleotide primers

Name	Sequence	Melting temperature
PAF	5′ AGAGTTTGATCCTGGCTCAG 3′	54.0
536R	5′ GTATTACCGCGGCTGCTG 3′	57.5
ACI 16S 1	5′ AGCTGAACCAACAGATTCAC 3′	59.3
ACI 16S 2	5′ ACTACCAGGGTATCTAATCC 3′	53.6
PR 1	5′ CAGACTGAAAGTCTGACGG 3′	58.5
CAS 2	5′ GCGATGCGAATTTCTTTTTC 3′	63.4

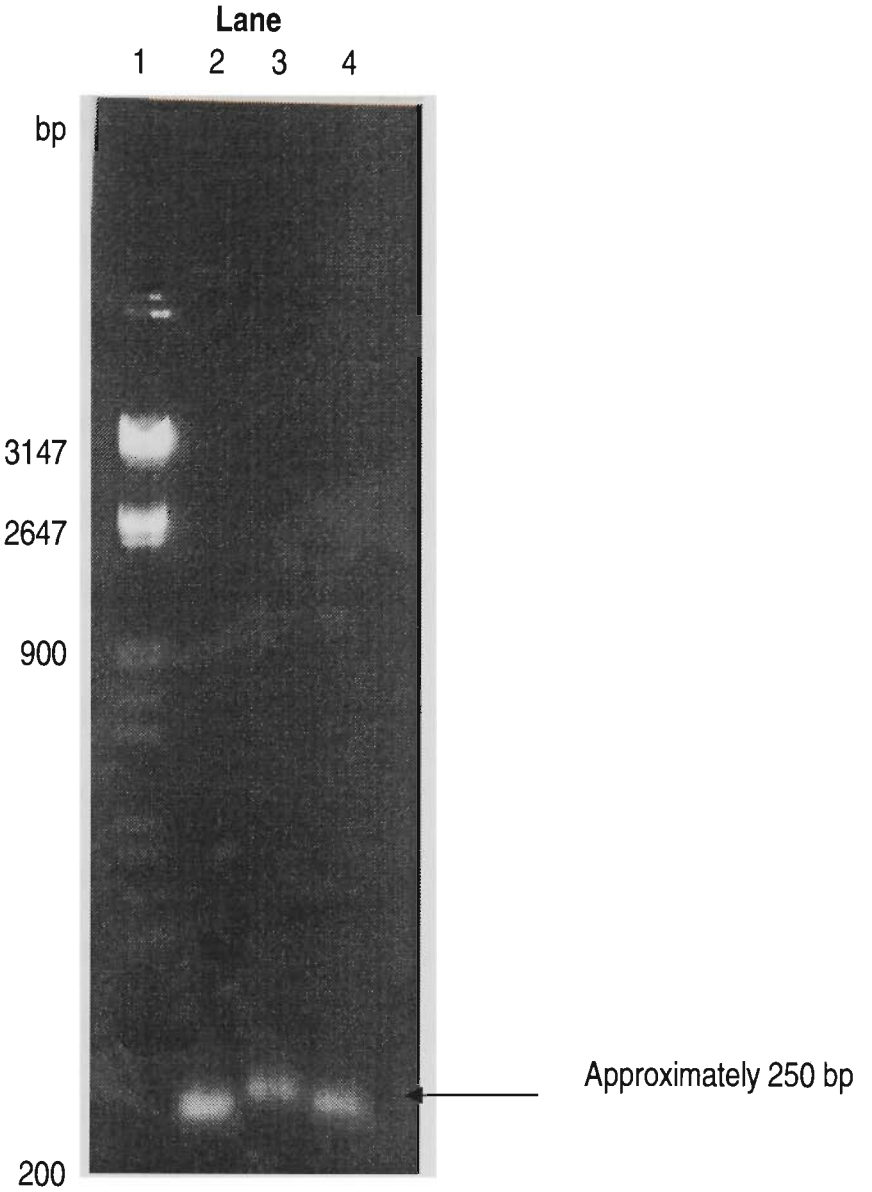


Figure 3.3 PCR amplification of DNA from *Lactobacillus* strain VUP 12006, *Lactobacillus acidophilus* VUP12000 (type strain) and *Lactobacillus casei* GCRL-163 using primers LbLMA1-rev and R16-1.

Lane 1 is marker (EcoRI/Hind III of λ DNA Progen). Lanes 2-4 are PCR amplification products of DNA using primers LbLMA1-rev and R16-1. Products were obtained from the DNA of strain VUP 12006 (lane 2), *Lb. acidophilus* type strain VUP12000 (lane 3) and *Lb. casei* GCRL-163 (lane 4). Products varied in size but were approximately 240-250 bp.

The reaction mixture (50μL) for the first primer pair, PAF/536R contained 30 pmol of each primer, 200μM each of dATP, dTTP, dCTP and dGTP, approximately 100ng of bacterial DNA, 2.5 U of *Taq* DNA polymerase (Sigma) and PCR buffer without $MgCl_2$.

DNA was amplified as follows: initial denaturation at 94°C for 2 minutes, followed by 30 cycles consisting of denaturation at 94°C for 45 seconds, annealing at 55°C for 45 seconds, extension at 72°C for 60 seconds and a 7 minute final extension step at 72°C. The products were stored at 4°C until required. Aliquots of the reaction mixture (10μL) were separated on a 0.8% agarose gel, stained with ethidium bromide and visualised under UV light. The results are shown in Figure 3.4.

Genomic DNA from strain VUP 12006 amplified using species-specific primers PAF/536R yielded a PCR product of approximately 500 bp. Sequences of the PCR products were determined using a CEQ 2000 (Beckman Coulter) according to the manufacturers instructions. The sequences in both the forward and reverse direction were edited in the software package 'bio-edit' to about the first 500 bases and submitted to ANGIS for a BLASTn search (2.6.14). Sequences showing statistically significant alignment are shown in Table 3.5.

In addition the ends of all sequences (forward and reverse separately) were trimmed to the same length and aligned by ClustalW (ANGIS). The alignments are shown in Figure 3.5 (sequence based on forward primer) and Figure 3.6 (sequence based on reverse primer). It can be seen in the alignment that there is significant sequence homology between strain VUP 12006 and *Lb. casei* strain GCRL 163. This data suggested that strain VUP 12006 was more closely related to the *Lb. casei* group and not the *Lb. acidophilus* group, which was originally suggested by carbohydrate utilisation pattern. Ward and Timmins (1999) had suggested that the V1 region of the 16S rRNA genes of *Lb. casei* and *Lb. paracasei*, was important in confirming speciation so alignment of the 50 bases in the V1 region was also performed (Figure 3.7).

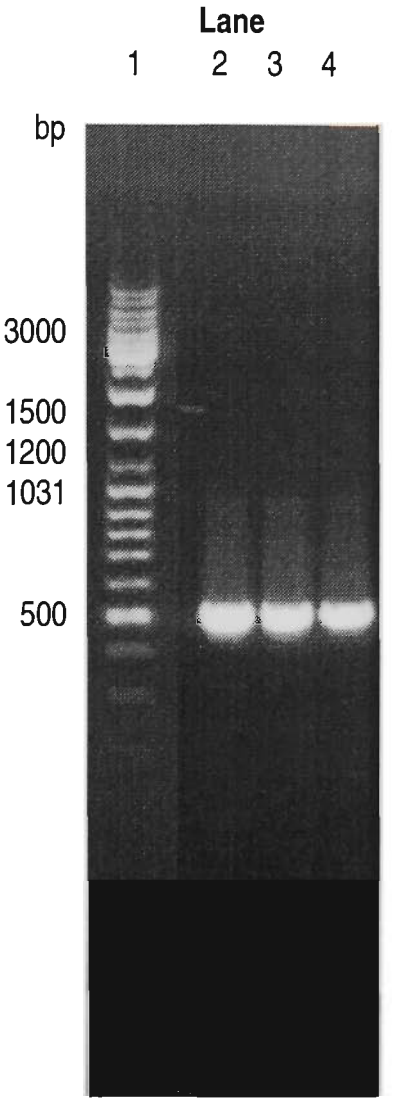


Figure 3.4 PCR amplification of DNA from *Lactobacillus* strain VUP 12006, *Lactobacillus acidophilus* VUP12000 (type strain) and *Lactobacillus casei* GCRL-163 using species-specific primers PAF and 536R.

Lane 1 is marker (generuler, MBI Fermentas). Lanes 2-4 are PCR amplification products of DNA using primers LbLMA1-rev and R16-1. Products were obtained from the DNA of *Lactobacillus* strain VUP 12006 (lane 2), *Lb. acidophilus* type strain VUP12000 (lane 3) and *Lb. casei* GCRL-163 (lane 4). Products were approximately 500 bp.

Table 3.5 Sequences showing significant alignment.

A BLASTn search of databases for strains VUP 12006, VUP12000 and GCRL-163 was performed through ANGIS. Sequences showing significant alignment are shown below.

Strain	Sequence direction	BLASTn Best Hit	Description of Best Hit
VUP 12006	Forward	LBA16SRRNA	*Lb. paracasei* gene for 16S rRNA, partial sequence 6/2002
VUP 12006	Reverse	AY091596.1	*Lb. casei* strain KH-1
VUP12000	Forward	LBARR16SA	*Lb. acidophilus*
VUP12000	Reverse	LBARR16SA	*Lb. acidophilus*
GCRL-163	Forward	LBA16SRRNA	*Lb. paracasei*
GCRL-163	Reverse	AY091596	*Lb. casei* strain KH-1

Figure 3.5 ClustalW alignment of 16S sequences of three strains of LAB (forward primer).

16S sequences of strain VUP12006 (46f), *Lb. acidophilus* strain ASC 388 (Asc388f) and *Lb. casei* strain GCRL-163 (LbcF) were aligned using ClustalW, a multiple alignment program accessed through ANGIS. Sequences were obtained from PCR fragments and amplified using the primer pair PAF and 536R. The ends of all sequences were trimmed to the same length. This sequence is based on data using the forward primer. Nucleotides that match are shaded in black.

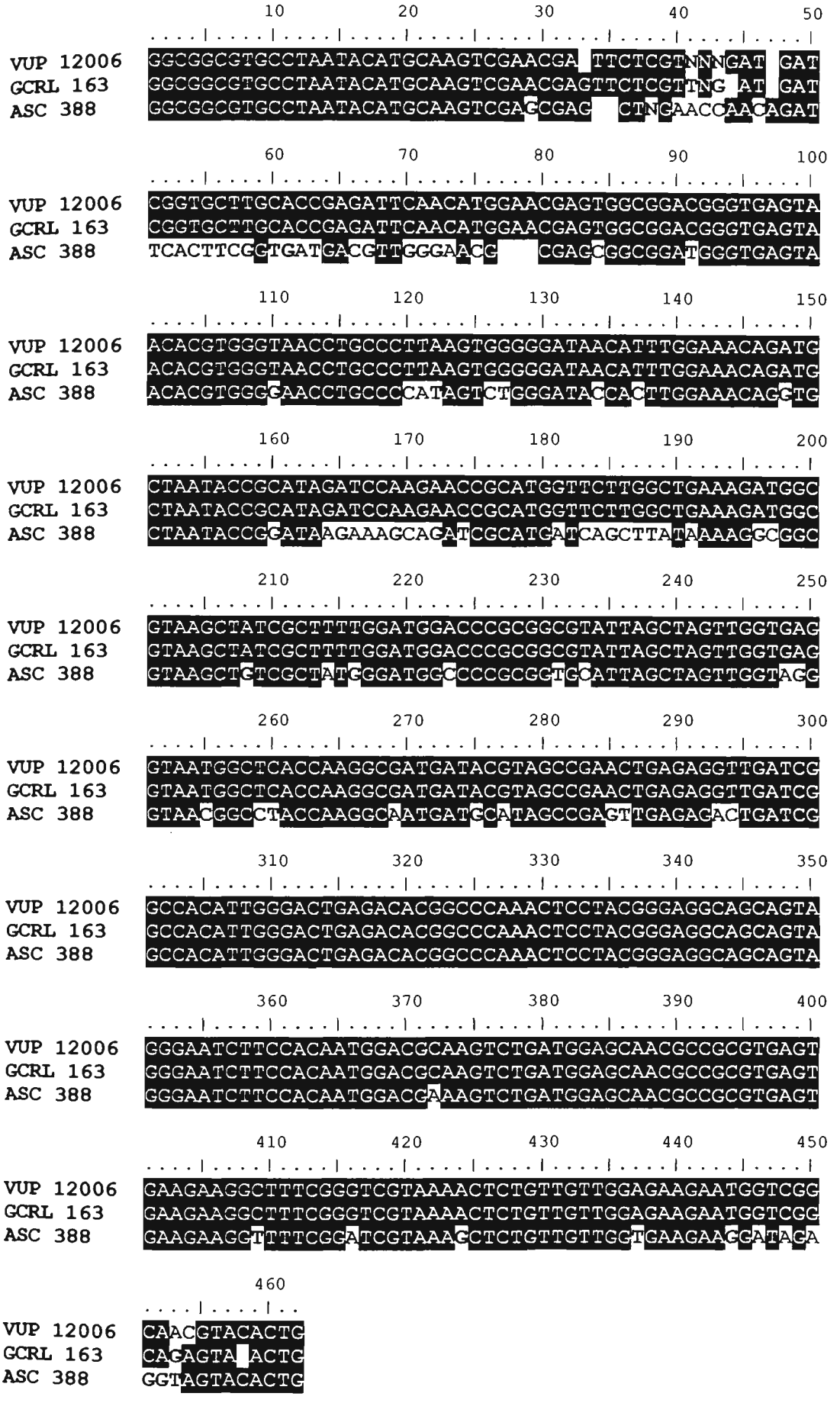
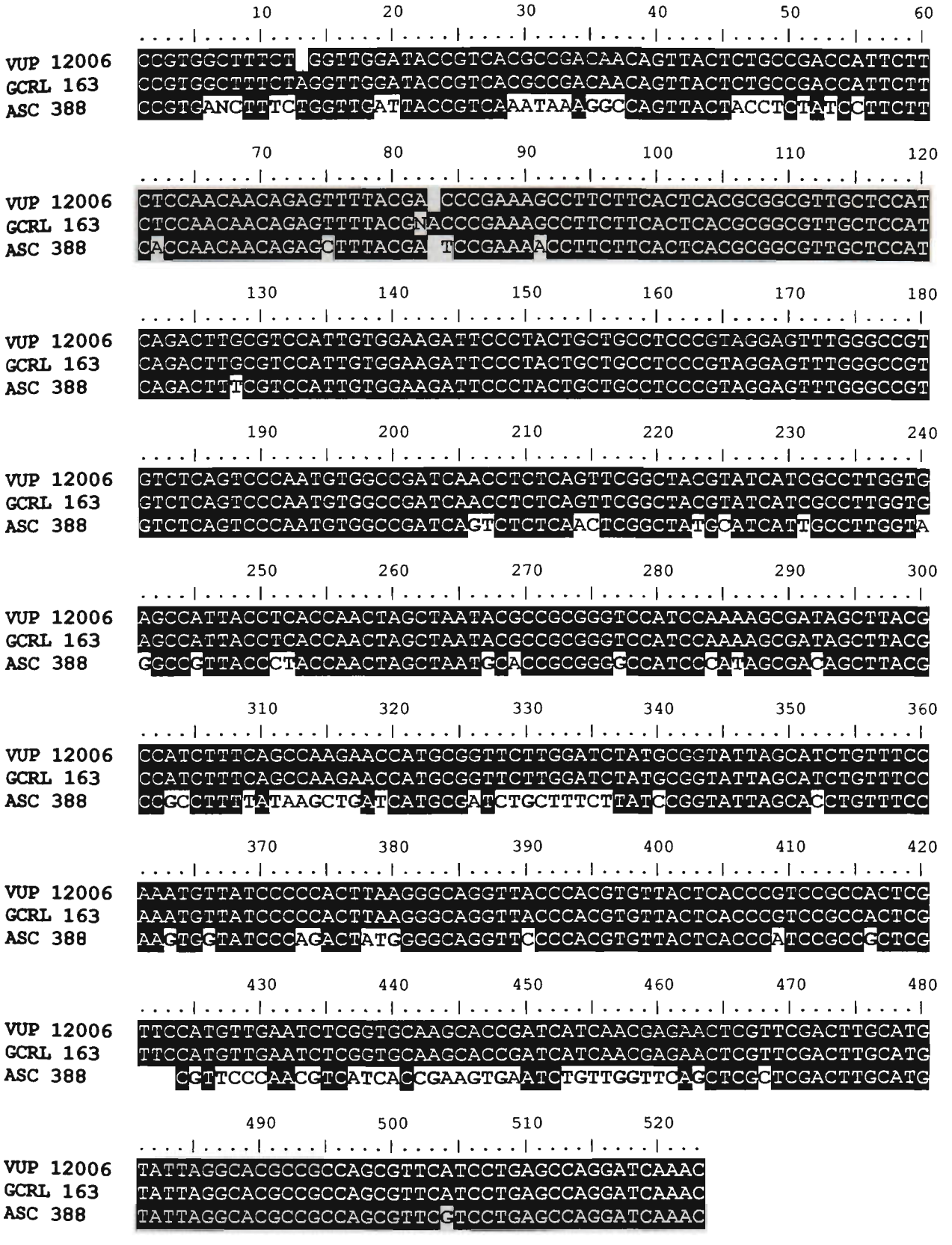

10 20 30 40 50
VUP 12006 GGCGGCGTGCCTAATACATGCAAGTCGAACGA TTCTCGTNNNGAT GAT
GCRL 163 GGCGGCGTGCCTAATACATGCAAGTCGAACGAGTTCTCGTTNG AT GAT
ASC 388 GGCGGCGTGCCTAATACATGCAAGTCGAGCGAG CTNGAACCAACAGAT
60 70 80 90 100
VUP 12006 CGGTGCTTGCACCGAGATTCAACATGGAACGAGTGGCGGACGGGTGAGTA
GCRL 163 CGGTGCTTGCACCGAGATTCAACATGGAACGAGTGGCGGACGGGTGAGTA
ASC 388 TCACTTCGGTGATGACGTTGGGAACG CGAGCGGCGGATGGGTGAGTA
110 120 130 140 150
VUP 12006 ACACGTGGGTAACCTGCCCTTAAGTGGGGGATAACATTTGGAAACAGATG
GCRL 163 ACACGTGGGTAACCTGCCCTTAAGTGGGGGATAACATTTGGAAACAGATG
ASC 388 ACACGTGGGGAACCTGCCCCATAGTCTGGGATACCACTTGGAAACAGGTG
160 170 180 190 200
VUP 12006 CTAATACCGCATAGATCCAAGAACCGCATGGTTCTTGGCTGAAAGATGGC
GCRL 163 CTAATACCGCATAGATCCAAGAACCGCATGGTTCTTGGCTGAAAGATGGC
ASC 388 CTAATACCGGATAAGAAAGCAGATCGCATGATCAGCTTATAAAAGGCGGC
210 220 230 240 250
VUP 12006 GTAAGCTATCGCTTTTGGATGGACCCGCGGCGTATTAGCTAGTTGGTGAG
GCRL 163 GTAAGCTATCGCTTTTGGATGGACCCGCGGCGTATTAGCTAGTTGGTGAG
ASC 388 GTAAGCTGTCGCTATGGGATGGCCCCGCGGTGCATTAGCTAGTTGGTAGG
260 270 280 290 300
VUP 12006 GTAATGGCTCACCAAGGCGATGATACGTAGCCGAACTGAGAGGTTGATCG
GCRL 163 GTAATGGCTCACCAAGGCGATGATACGTAGCCGAACTGAGAGGTTGATCG
ASC 388 GTAACGGCCTACCAAGGCAATGATGCATAGCCGAGTTGAGAGACTGATCG
310 320 330 340 350
VUP 12006 GCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTA
GCRL 163 GCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTA
ASC 388 GCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTA
360 370 380 390 400
VUP 12006 GGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGT
GCRL 163 GGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGT
ASC 388 GGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGT
410 420 430 440 450
VUP 12006 GAAGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTGGAGAAGAATGGTCGG
GCRL 163 GAAGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTGGAGAAGAATGGTCGG
ASC 388 GAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTGGTGAAGAAGGATAGA
460
VUP 12006 CAACGTACACTG
GCRL 163 CAGAGTA ACTG
ASC 388 GGTAGTACACTG

Figure 3.6 ClustalW alignment of 16S sequences of three strains of LAB (reverse primer).

16S sequences of strain VUP12006 (46r), *Lb. acidophilus* strain ASC 388 (Asc388r) and *Lb. casei* strain GCRL-163 (LbcR) were aligned using ClustalW, a multiple alignment program accessed through ANGIS. Sequences were obtained from PCR fragments and amplified using the primer pair PAF and 536R. The ends of all sequences were trimmed to the same length. This sequence is based on data using the reverse primer. Nucleotides that match are shaded in black.


10 20 30 40 50 60
VUP 12006 CCGTGGCTTTCT GGTTGGATACCGTCACGCCGACAACAGTTACTCTGCCGACCATTCTT
GCRL 163 CCGTGGCTTTCTAGGTTGGATACCGTCACGCCGACAACAGTTACTCTGCCGACCATTCTT
ASC 388 CCGTGANCTTTCTGGTTGATTACCGTCAAATAAAGGCCAGTTACTACCTCTATCCTTCTT
70 80 90 100 110 120
VUP 12006 CTCCAACAACAGAGTTTTACGA CCCGAAAGCCTTCTTCACTCACGCGGCGTTGCTCCAT
GCRL 163 CTCCAACAACAGAGTTTTACGNACCCGAAAGCCTTCTTCACTCACGCGGCGTTGCTCCAT
ASC 388 CACCAACAACAGAGCTTTACGA TCCGAAAACCTTCTTCACTCACGCGGCGTTGCTCCAT
130 140 150 160 170 180
VUP 12006 CAGACTTGCGTCCATTGTGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTTTGGGCCGT
GCRL 163 CAGACTTGCGTCCATTGTGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTTTGGGCCGT
ASC 388 CAGACTTTCGTCCATTGTGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTTTGGGCCGT
190 200 210 220 230 240
VUP 12006 GTCTCAGTCCCAATGTGGCCGATCAACCTCTCAGTTCGGCTACGTATCATCGCCTTGGTG
GCRL 163 GTCTCAGTCCCAATGTGGCCGATCAACCTCTCAGTTCGGCTACGTATCATCGCCTTGGTG
ASC 388 GTCTCAGTCCCAATGTGGCCGATCAGTCTCTCAACTCGGCTATGCATCATTGCCTTGGTA
250 260 270 280 290 300
VUP 12006 AGCCATTACCTCACCAACTAGCTAATACGCCGCGGGTCCATCCAAAAGCGATAGCTTACG
GCRL 163 AGCCATTACCTCACCAACTAGCTAATACGCCGCGGGTCCATCCAAAAGCGATAGCTTACG
ASC 388 GGCCGTTACCCTACCAACTAGCTAATGCACCGCGGGGCCATCCCATAGCGACAGCTTACG
310 320 330 340 350 360
VUP 12006 CCATCTTTCAGCCAAGAACCATGCGGTTCTTGGATCTATGCGGTATTAGCATCTGTTTCC
GCRL 163 CCATCTTTCAGCCAAGAACCATGCGGTTCTTGGATCTATGCGGTATTAGCATCTGTTTCC
ASC 388 CCGCCTTTTATAAGCTGATCATGCGATCTGCTTTCTTATCCGGTATTAGCACCTGTTTCC
370 380 390 400 410 420
VUP 12006 AAATGTTATCCCCCACTTAAGGGCAGGTTACCCACGTGTTACTCACCCGTCCGCCACTCG
GCRL 163 AAATGTTATCCCCCACTTAAGGGCAGGTTACCCACGTGTTACTCACCCGTCCGCCACTCG
ASC 388 AAGTGGTATCCCAGACTATGGGGCAGGTTCCCCACGTGTTACTCACCCATCCGCCGCTCG
430 440 450 460 470 480
VUP 12006 TTCCATGTTGAATCTCGGTGCAAGCACCGATCATCAACGAGAACTCGTTCGACTTGCATG
GCRL 163 TTCCATGTTGAATCTCGGTGCAAGCACCGATCATCAACGAGAACTCGTTCGACTTGCATG
ASC 388 CGTTCCCAACGTCATCACCGAAGTGAATCTGTTGGTTCAGCTCGCTCGACTTGCATG
490 500 510 520
VUP 12006 TATTAGGCACGCCGCCAGCGTTCATCCTGAGCCAGGATCAAAC
GCRL 163 TATTAGGCACGCCGCCAGCGTTCATCCTGAGCCAGGATCAAAC
ASC 388 TATTAGGCACGCCGCCAGCGTTCGTCCTGAGCCAGGATCAAAC

	60	70	80	90	100
Lb. casei ATCC 393	cgatgaacgg	tgcttgcact	gagattcgac	ttaaaacgag	tggcggacgg
Lb. casei ATCC 334	tgatgatcgg	tgcttgcacc	gagattcaac	atggaacgag	tggcggacgg
Lb. paracasei ATCC 25302	tgatgatcgg	tgcttgcacc	gagattcaac	atggaacgag	tggcggacgg
Strain VUP12006	tgatgatcgg	tgcttgcacc	gagattcaac	atggaacgag	tggcggacgg

Figure 3.7 Alignment of the V1 region of the 16S rRNA genes of *Lb. casei* ATCC 393 and 334, *Lb. paracasei* ATCC 25302 and strain VUP 12006.

Partial sequence data from *Lb. casei* type strain ATCC 393 was extracted from the 16S ribosomal RNA gene (gi|28194135|gb|AF469172.1|) between positions 62 and 111. Positions 62 to 111 are incorporated in the V1 (variable) region of the gene. Sequence data in this variable region has been shown to assist identification of members of the *casei* group. This data was aligned to similar sequence data from *Lb. casei* strain ATCC 334, *Lb. paracasei* strain ATCC 25302 and strain VUP12006.

Examination of these alignments appears to support the identification of strain VUP 12006 as *Lb. paracasei.* Additionally, when the V1 variable region sequence for strains VUP 12006, *Lb. paracasei* type strain ATCC 25302 and the *Lb. casei* type strain ATCC 393 was submitted to the draft genome of *Lb. casei* strain ATCC 334, it was found that ATCC 334 shared 100% homology with *Lb. paracasei* type strain ATCC 25302 and strain VUP 12006 and only 87% with *Lb. casei* type strain 393.

As strain VUP 12006 was originally identified by CSIRO as *Lb. acidophilus*, two further primer pairs were used to confirm the identification. Using two primer pairs described by Walter (2001), which were shown to be specific for either *Lb. acidophilus* or *Lb. casei*, PCR was performed to support the identification of strain VUP 12006. The reaction mixture (50µL) for the *Lb. acidophilus* primer pair, ACI 16S 1/ ACI 16S 2, contained 30 pmol of each primer, 200µM each of dATP, dTTP, dCTP and dGTP, approximately 500 ng of bacterial DNA, 2.5 U of *Taq* DNA polymerase (Sigma) and PCR buffer with 1.5$MgCl_2$ $MgCl_2$ and 50mM KCl.

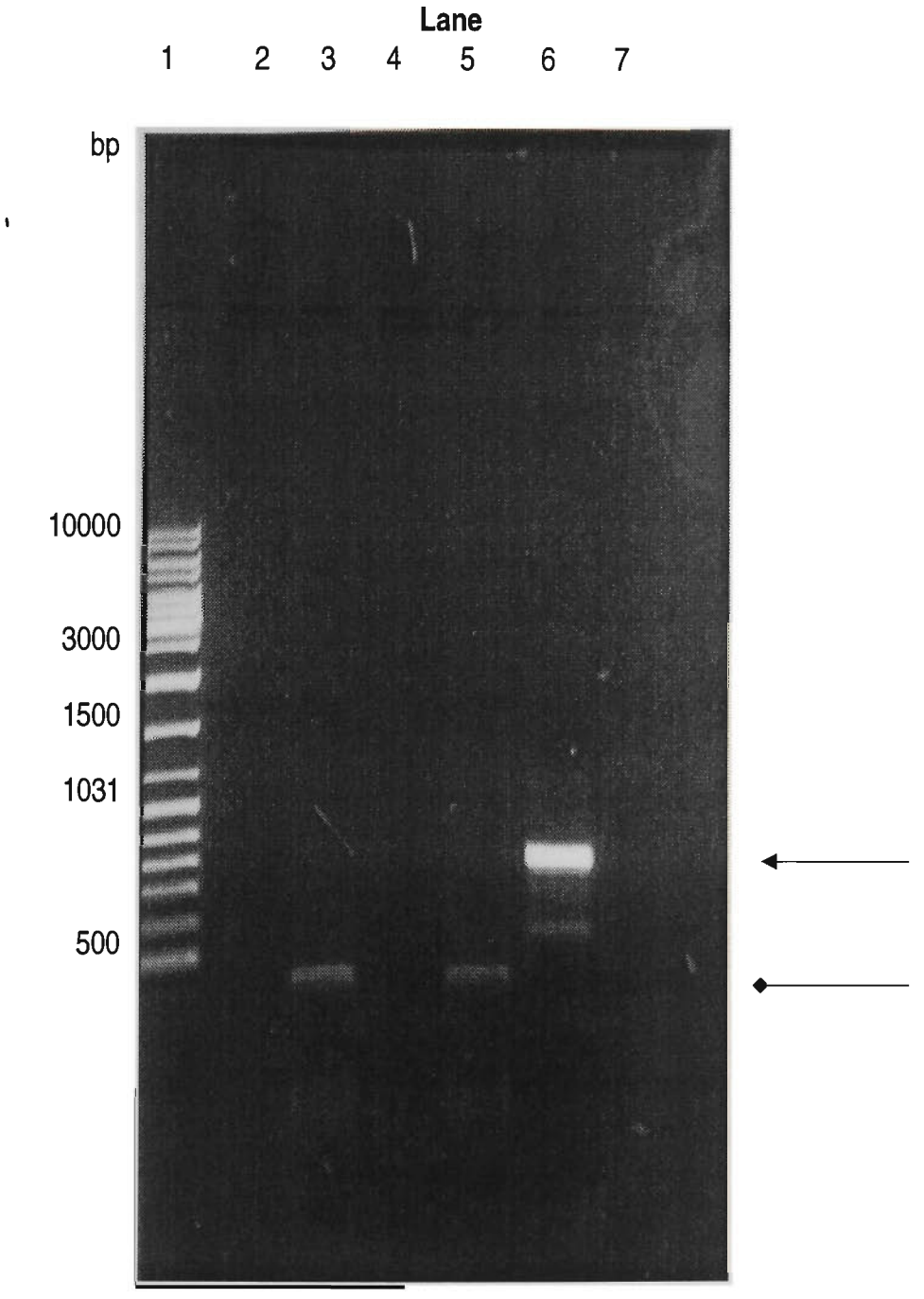
DNA was amplified as follows: initial denaturation at 95°C for 2 minutes, followed by 30 cycles consisting of denaturation at 92°C for 30 seconds, annealing at 62°C for 30 seconds, extension at 72°C for 30 seconds and a 1 minute final extension step at 72°C. Aliquots of the reaction mixture (10µL) were separated on a 0.8% agarose gel, stained with ethidium bromide and visualised under UV light. The results are shown in Figure 3.8.

Genornic DNA from strain VUP 12006 amplified using ACI 16S 1/ ACI 16S 2 failed to produce products while DNA from *Lb. acidophilus* type strain ASC 388 produced an amplicon of approximately 850 bp. *Lb. casei* strain GCRL-163 also failed to produce PCR products.

Figure 3.8 PCR amplification of DNA from Lactobacillus strain VUP 12006, *Lactobacillus acidophilus* VUP12000 (type strain) and *Lactobacillus casei* GCRL-163 using primers pairs ACI 16S 1 and ACI 16S 2 and primers pairs PR 1 and CAS 2.

Lane 1 is marker (generuler, Progen). Lanes 2, 4 and 6 are PCR amplification products of DNA using primers ACI 16S 1 and ACI 16S 2. Products of approximately 850 bp were only obtained from the DNA of *Lb. acidophilus* type strain VUP12000 (lane 6). These are indicated by pointed arrow. Some non-specific PCR products were also observed at approximately 600 bp. No products were detected from DNA of *Lactobacillus* strain VUP 12006 (lane 2) and *Lb. casei* GCRL-163 (lane 4).

Lanes 3, 5 and 7 are PCR amplification products of DNA using primers PR 1 and CAS 2, which were specific for the *casei* group. Products of approximately 450 bp were only obtained from the DNA of *Lactobacillus* strain VUP 12006 (lane 3) and *Lb. casei* GCRL-163 (lane 5). These are indicated by diamond headed arrow. No products were detected from DNA of *Lb. acidophilus* type strain VUP12000 (lane 7).


Lane
1 2 3 4 5 6 7
bp
10000
3000
1500
1031
500

The reaction mixture (50μL) for the *Lb. casei* primer pair, PR 1/ CAS 2, contained 30 pmol of each primer, 200μM each of dATP, dTTP, dCTP and dGTP, approximately 500ng of bacterial DNA, 2.5 U of *Taq* DNA polymerase (Sigma) and PCR buffer with 1.5$MgCl_2$ $MgCl_2$ and 50mM KCl. DNA was amplified as follows: initial denaturation at 92°C for 2 minutes, followed by 30 cycles consisting of denaturation at 95°C for 30 seconds, annealing at 56°C for 30 seconds, extension at 72°C for 30 seconds and a 1 minute final extension step at 72°C. The results are shown in Figure 3.8.

Genomic DNA from strain VUP 12006 amplified using PR 1/ CAS 2 yielded a PCR product of approximately 500 bp. A similar sized product was obtained when DNA from *Lb. casei* strain GCRL-163 was used as the template DNA. *Lb. acidophilus* type strain ASC 388 failed to produce PCR products. After a review of these results, particularly sequence alignments of 16S rRNA, it was determined that the data supported an identification of strain VUP 12006 as *Lb. paracasei*.

3.3 Physiological characterisation of the selected strains

A screening process was used to identify acid tolerant strains for further characterisation as it was anticipated that an acid tolerant strain would exhibit physiological characteristics that contributed towards acid tolerance. Microtitre plates were used to measure changes in OD_{600} in the eight cultures selected for further testing following the previously described carbohydrate utilisation studies. They were grown in MRS broth (37°C, in room air) at eight randomly selected pH values between 1.6 and 8.2 (see section 2.3.6). From the results of this screening test, it was possible to broadly compare general trends in growth rates calculated from the optical density values taken during growth at each pH as described in section 2.3.8. This data was used to determine the strain that had the maximum specific growth rate, μ_{max}, for several pH values.

These growth rates were also used to determine an approximate range over which each of the organisms could initiate and continue growth. Growth rates were calculated from the plot of $\log_{10}$

OD_{600} against time (section 2.3.8). Results are shown in Table 3.6. *Lb. paracasei* strain VUP 12006 demonstrated the highest μ_{max} value of the eight strains tested (0·23 h^{-1}) and the widest apparent growth range (pH 3·1 to 8·2) and this strain was selected for further characterisation. Using one- or two-litre fermenter systems, *Lb. paracasei* (strain 12006) was characterised by growth rate, production of metabolic products, protein profiles (1-D and 2-D) and fatty acid methyl esters profiles at different pHs and temperature, as described in the following sections.

3.3.1 Optimum temperature and pH for growth for strain VUP 12006

For fermenter operation, see section 2.3.7. The optimum conditions for growth of *Lb. paracasei* (strain 12006) in MRS broth were determined by calculating μ_{max} values for duplicate experiments. Optical density values were measured during growth over a range of temperatures (12 to 51°C) and pH values (2 to 8). The pH was fixed at pH 6.3 and the temperature varied between 12 and 51°C and the temperature was fixed at 37°C and the pH varied between 2 to 8. Optical density values were validated as being related to growth by performing dry biomass determinations on growing cultures as described in section 2.3.4.

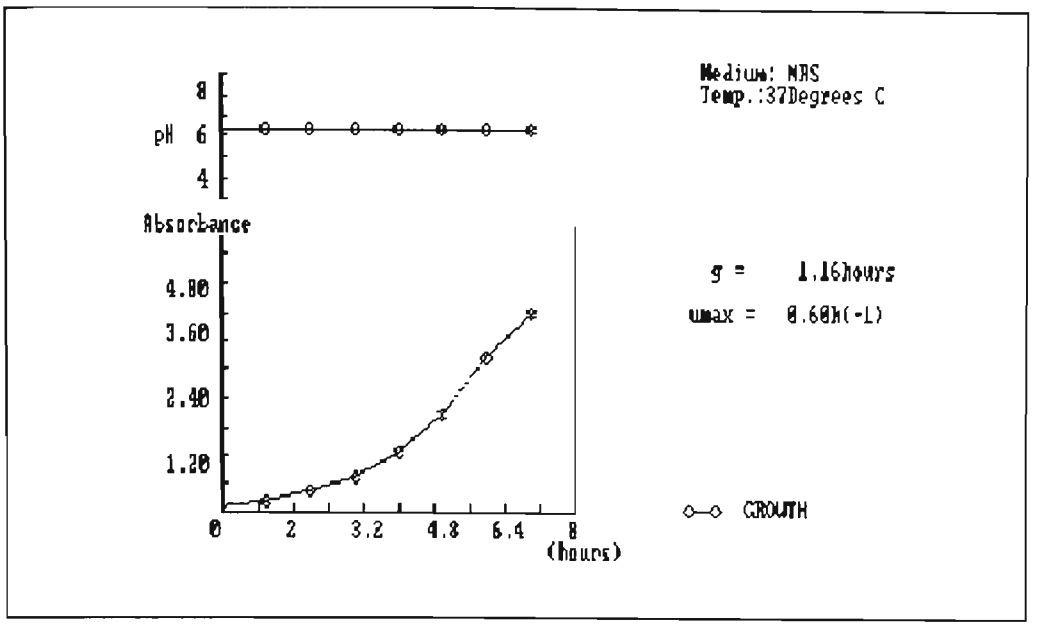
The maximum specific growth rate during each growth cycle was plotted against either pH or temperature and used to determine optimum growth conditions according to the method described in section 2.3.8. The growth rates were calculated using a computer program for the graphic representation of microbial growth curves and batch fermentations. The program, written in BASIC, graphically displayed results from each run (Veiga *et al.*, 1991). A typical example of the results of a single experiment is shown in the screen capture images in Figure 3.9. The results for determination of temperature optimum are presented in Figure 3.10.

Table 3.6 Maximum specific growth rate and pH growth range of test strains determined from growth in microtitre tray cultures.

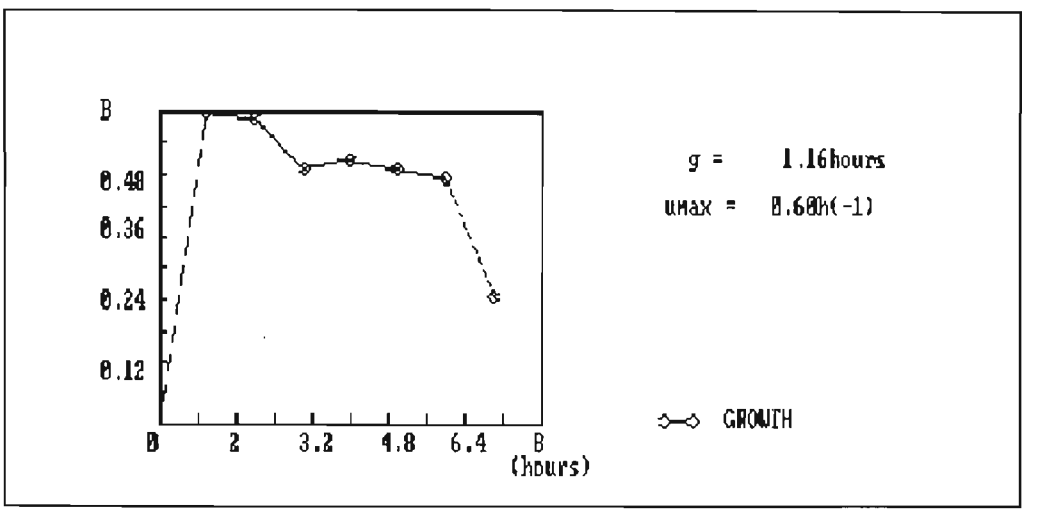
Cultures were grown in MRS broth at 37°C in room air in microtitre trays as described in section 2.3.6. Eight randomly selected pH values (1.6, 2.4, 3.0, 3.8, 4.8, 5.6, 6.3, 7.8 and 8.1) were used to screen bacteria for the ability to grow over a wide pH range and demonstrate satisfactory growth over that range. The pH was adjusted with HCl.

Strain Identification	μ_{max} (h^{-1}) when grown at apparent optimum pH	Highest pH in which the strain was able to initiate growth	Lowest pH in which the strain was able to initiate growth
12000	0.12 (4.9[a])	7.8	3.1
12001	0.04 (5.6)	8.2	4.9
12002	0.06 (5.6)	7.8	3.8
12003	0.12 (6.3)	8.2	4.9
12004	0.18 (6.3)	8.2	3.8
12005	0.12 (5.6)	8.2	4.9
12006	0.23 (5.6)	8.2	3.1
12007	0.12 (5.6)	8.2	3.8

[a] apparent optimum pH is shown in brackets



(A)



(B)

Figure 3.9 Computer screen captured images from the software program used to calculate specific growth rates for *Lb. paracasei* strain VUP 12006 grown in MRS broth at pH 6.3 and 37°C.

Figure 3.9 (A) shows a plot of optical density values over time. For this experiment generation time was calculated as 1.16 hours with a μ_{max} of 0.60 h(-1). Figure 3.9 (B) shows growth rates calculated for each time interval with the maximum specific growth rate occurring at the peak of the plot.

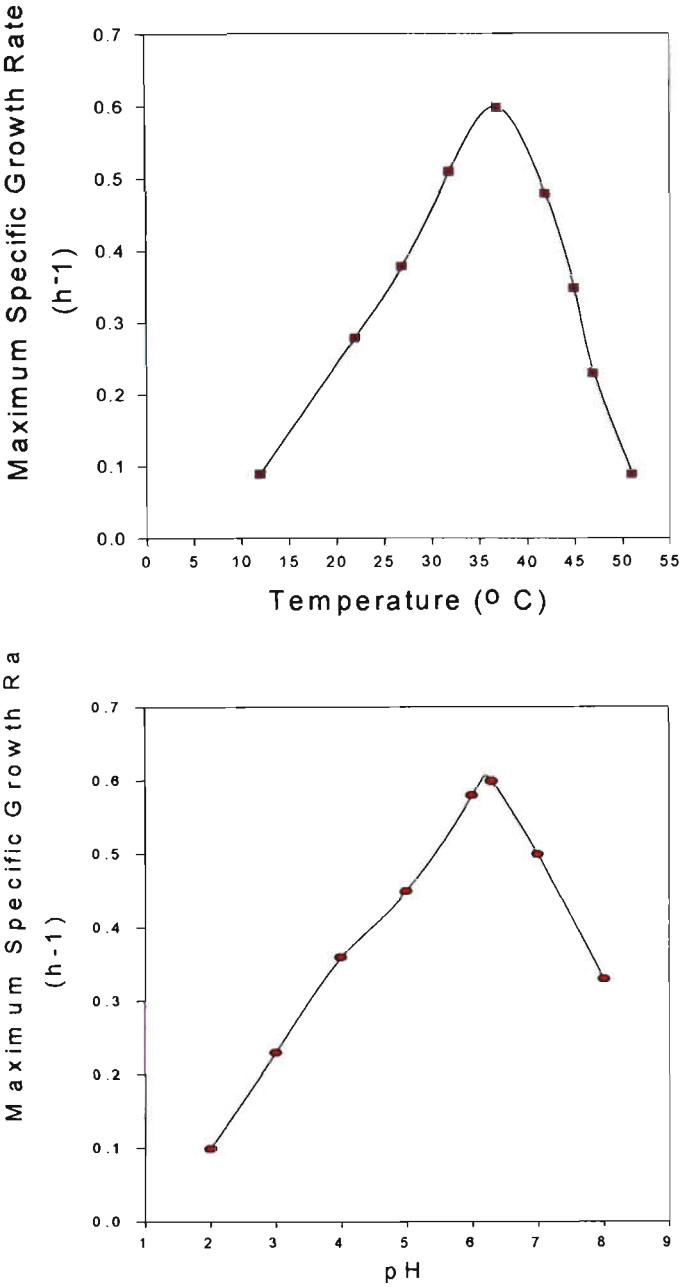


Figure 3.10 μ_{max} plotted against temperature (A) and pH (B)

Maximum specific growth rate (μ_{max}) was calculated at different temperatures and pHs. *Lactobacillus paracasei* strain VUP 12006 was grown in one litre of MRS broth in two-litre fermenters at temperatures between 12° and 51°C with the pH set at 6.3 (A) or at 37°C at pHs set between 2 and 8 (B). Nitrogen gas was sparged through the fermenter at 0.1 L/min.

Data from replicates was ± 7%. Strain VUP 12006 grew over the range of temperatures used (12-51°C) and showed a distinct optimum temperature for growth at 37°C ±0.5°C.

The results for determination of pH optimum for strain VUP 12006 are presented in Figure 3.10, which shows the maximum specific growth rate plotted against pH (pH range was 2 to 8). Growth was detected over the pH range tested and there was a sharp optimum pH at 6·3 ± 0·2.

The percentage change in the minimum to maximum specific growth rates (the slowest and fastest specific growth rates calculated from growth curves during growth at a specific temperature) was, in general, least in optimally grown cultures and highest at the extremes of growth (Table 3.7). The extent of growth after 24 hours was also greatly reduced at the higher temperatures. The optical density values dropped below the starting value after five hours and the calculated specific growth rate became negative after three hours for a culture grown at 51° C, indicating cell lysis. Specific growth rates were also affected by pH. The percentage difference in the minimum to maximum specific growth rates was lowest in optimally grown cultures and highest at the lowest pH, see Table 3.8. At pH 2.0 the calculated growth rate became zero after one hour.

3.3.2 Growth characteristics under optimum conditions (pH controlled at pH 6.3)

Once the optimum growth temperature and pH were determined, *Lb. paracasei* strain VUP 12006 was examined for basic growth characteristics at optimum temperature and pH. These characteristics included changes in optical density during the growth cycle, biomass production, glucose utilisation and the amount of NaOH required to neutralise metabolic acids produced during growth. Firstly biomass and optical density values (Figure 3.11) were compared using the method described in section 2.2.3.

Table 3.7 Percentage difference between minimum and maximum specific growth rates achieved during growth at each different temperature between 12 and 51°C and extent of growth.

T°C	12	22	27	32	37	42	45	47	51
% Difference	85	39	45	28	20	71	51	100	155
Extent of growth[a]	0.319	0.570	1.200	2.406	3.265	0.940	0.54	0.295	0.150

[a] Maximum OD_{600} reached after 6 hours in a typical experiment

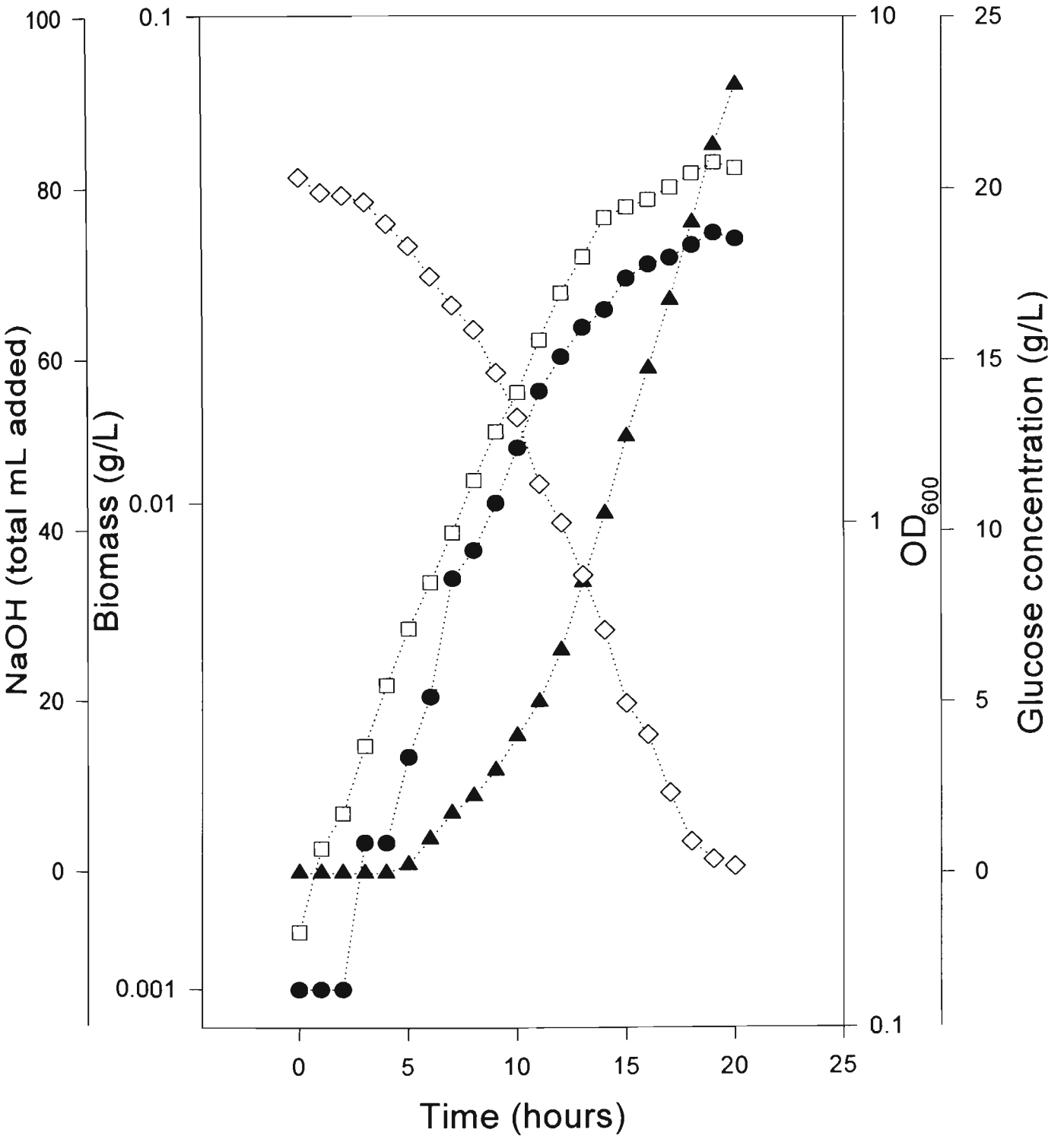
Table 3.8 Percentage difference between minimum and maximum specific growth rates achieved during growth at different pH values between 2 and 8 and extent of growth.

pH	2	3	4	5	6	6.3	7	8
% Difference	160	91	47	40	44	20	42	30
Extent of growth[a]	0.195	0.380	0.770	1.9565	2.500	3.265	2.550	0.690

[a] Maximum OD_{600} reached after 6 hours in a typical experiment

Figure 3.11 Growth characteristics of *Lb. paracasei* strain VUP 12006 under optimum conditions measured by changes in OD_{600}, biomass, glucose concentration and addition of sodium hydroxide.

Some basic characteristics of a culture growing in MRS broth at optimum temperature (37°) and pH (6.3) were determined by measuring changes in a number of parameters. These included: optical density measured at 600nm (□), glucose utilisation (◇), biomass production (●) and the amount of 2 M NaOH (mL) required to be added to the growing culture to control the pH at the predetermined optimum (▲). Cultures were grown in 1 L of MRS broth in a 2L fermenter. Nitrogen gas was sparged through the fermenter at 0.1 L/min. The inoculum was prepared from an overnight culture of strain VUP 12006 grown in MRS broth without pH control. The culture was centrifuged (10 min at 4000 rpm) and the cells collected and resuspended in MRS broth pre-warmed to 37°C. The fermenters were inoculated to give a starting OD of between 0.13 and 0.23 then growth was followed for 24 hours.


NaOH (total mL added)
Biomass (g/L)
OD600
Glucose concentration (g/L)
Time (hours)
100
80
60
40
20
0
0.1
0.01
0.001
10
1
0.1
25
20
15
10
5
0
0
5
10
15
20
25

The results indicate that biomass and optical density changed proportionally during growth and that optical density could be considered to reflect the extent of growth of the culture. It can also be seen that the culture reached exponential phase within one hour and that stationary phase was reached after about fifteen hours, using this particular inoculation procedure. Glucose utilisation was also examined (Figure 3.11). The rate of glucose utilisation was initially relatively slow and corresponded to the low biomass in the fermenter. As the biomass increased, the rate of glucose utilisation rose until the cells entered stationary phase and growth ceased when glucose was totally consumed. The sensitivity of the glucose assay was 0.2 g/L. Figure 3.11 shows the volume of NaOH added to the culture, noting that acids produced during growth were neutralised by 2 M NaOH in order for the culture to stay at pH 6.3. The utilisation of glucose is clearly mirrored by production of acids, as indicated by the total amount of NaOH added.

3.3.3 Growth characteristics for cultures commencing at optimum pH but without continued pH control

A control culture was grown at constant pH 6.3 while the second started at optimal pH (6.3) but the pH was not controlled. Figure 3.12 shows optical density values over 21 hours. The culture in which the pH was not controlled showed a slightly lower μ_{max} value (0.51) than the control (0.60), but the extent of growth as measured by optical density after twenty-one hours, was essentially the same. Figure 3.12 also shows the pH values decreasing during growth in the uncontrolled culture. The final pH reached by these cultures was generally around pH 3.0, which was consistent with the screening tests.

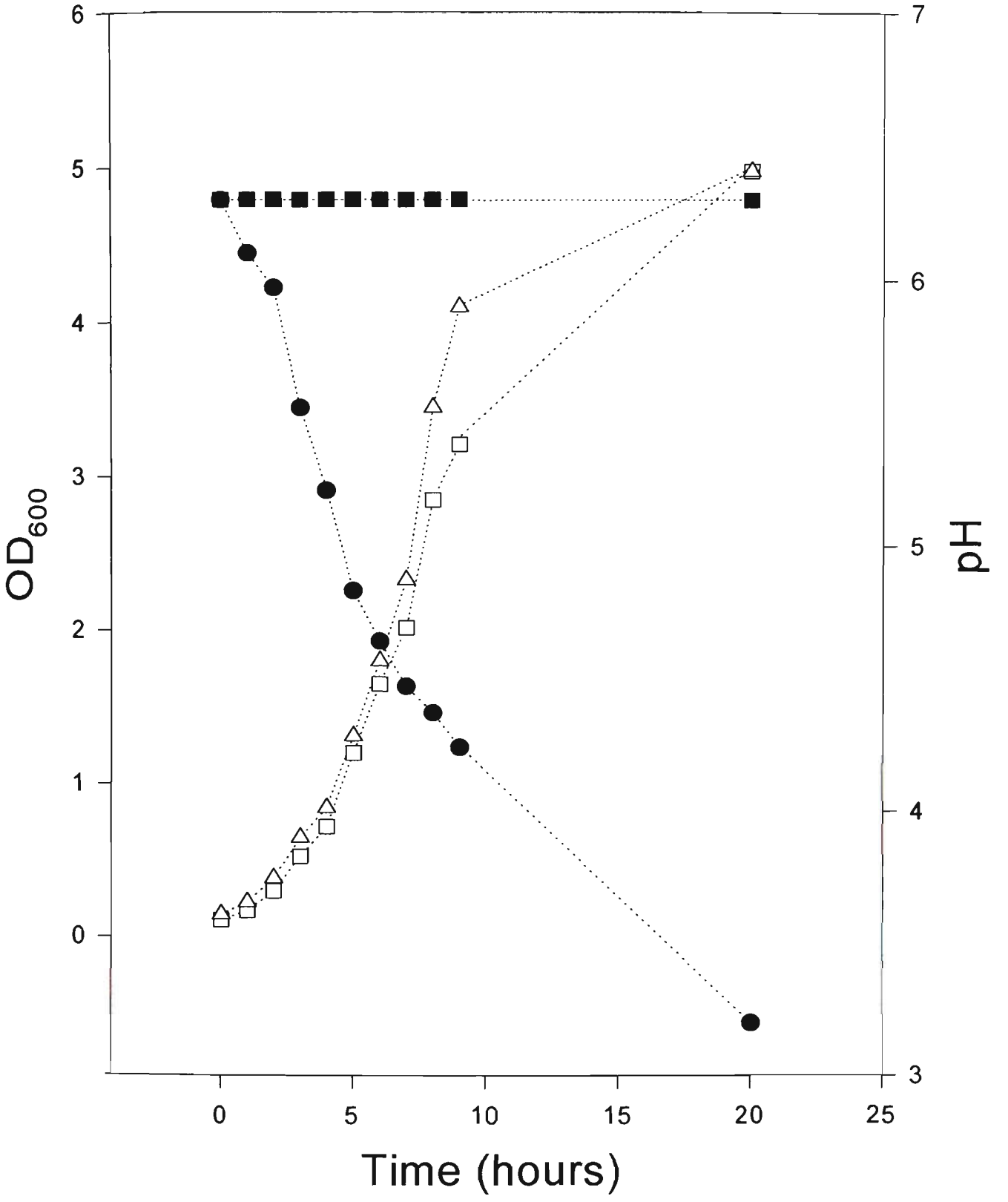
3.3.4 Effect of lactic acid concentration on growth

Potential inhibition by lactate on growth was examined by growing strain VUP 12006 in MRS broth containing, 5, 2.5, 1.25, 0.63, or 0.31 g/L of added lactate (Figure 3.13).

Figure 3.12 Growth characteristics of *Lb. paracasei* strain VUP 12006 grown without pH control, starting at optimum pH, measured by changes in OD_{600}.

A control culture of *Lb. paracasei* was grown under optimal conditions (37°C, pH 6.3 in 1 L of MRS broth) in a 2L fermenter and compared to an identical culture grown under the same conditions with the exception that the pH was not controlled at 6.3. Nitrogen gas was sparged through the fermenter at 0.1 L/min. The inoculum preparation and inoculation were as described in Figure 3.11.

Optical density (△) and pH (■) for growth controlled at pH 6.3/37°C and optical density (□) and pH (●) for growth at 37°C (pH not controlled).


6
5
4
3
2
1
0
7
6
5
4
3
OD_{600}
pH
0
5
10
15
20
25
Time (hours)

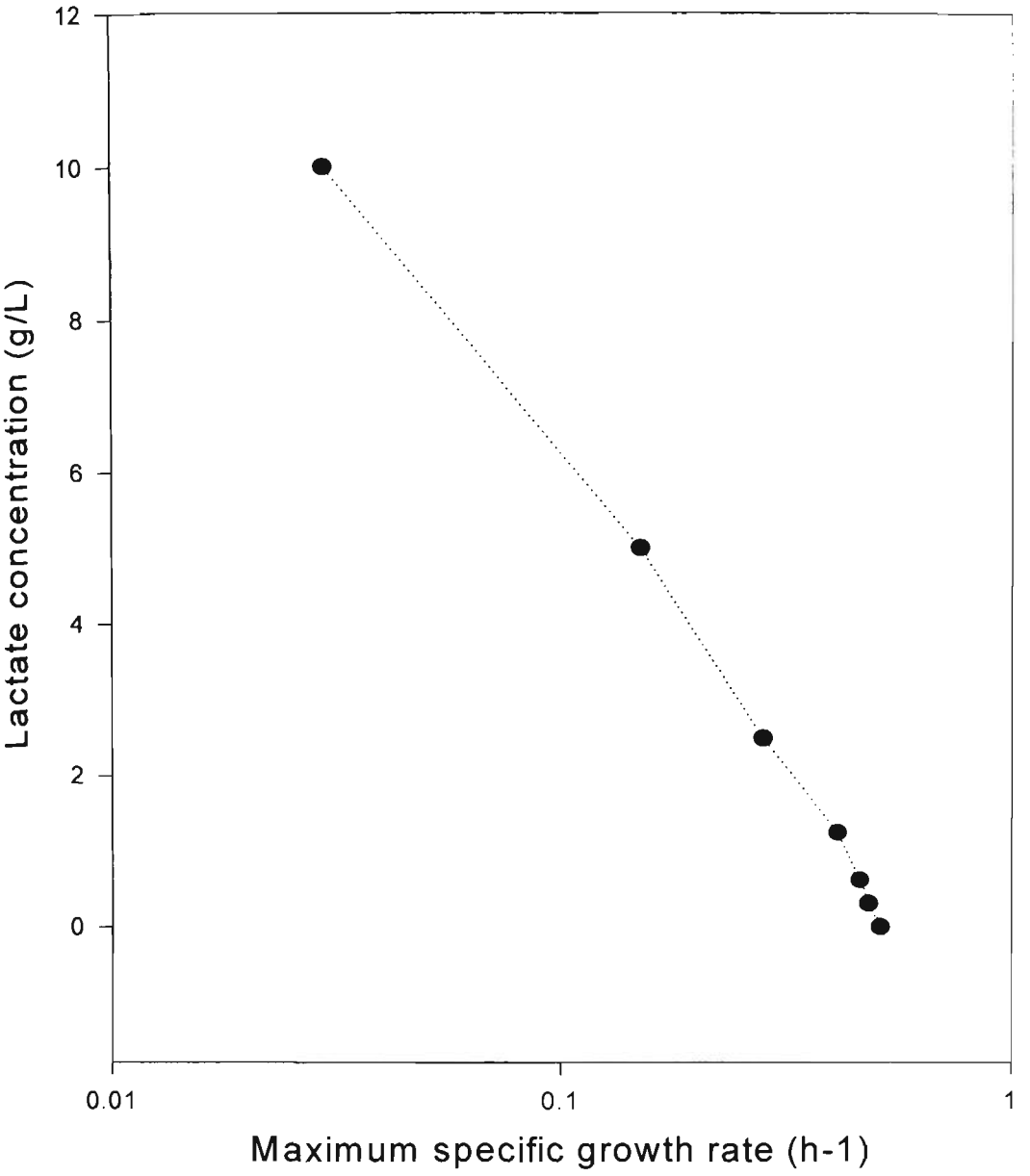


Figure 3.13 The effect of lactate concentration on the growth of *Lb. paracasei* strain VUP 12006.

Lactate concentrations between 0.31 and 10 g/L in MRS broth at 37°C were used to determine how well *Lb. paracasei* strain VUP 12006 could initiate growth in the presence of a metabolic end product. The maximum specific growth rate (μ_{max}) for each concentration was calculated over six hours of growth from samples collected hourly. Maximum specific growth rate (•) was then plotted against lactate concentration. A control culture of *Lb. paracasei* was grown under optimal conditions (37°C, pH 6.3 in MRS broth) in a 1L fermenter without added lactate and had a μ_{max} of 0.63. Nitrogen gas was sparged through all the fermenters at 0.1 L/min.

To differentiate between inhibition caused by altered pH and lactate ions, pH during growth at 37°C was maintained at a set point of 6.3. The inoculum was prepared by culturing strain VUP 12006 overnight in MRS broth.

Growth was severely affected by the presence of a high concentration of lactate in the growth medium despite the pH being held at 6.3. At a level of 10 g/L growth was essentially so slow it was equivalent to the growth rate observed in a culture growing at pH 3.0. At a lactate concentration of 2.5 g/L, the maximum specific growth rate of the challenged culture was slightly more than half that achieved by a culture that was grown in the absence of lactate. A control culture of *Lb. paracasei* was grown under optimal conditions (37°C, pH 6.3 in MRS broth) in a 1L fermenter without added lactate had a μ_{max} of 0.63. This can be compared to the level of lactate in the growth medium achieved after six hours of growth at optimum temperature and pH by the same strain.

3.3.5 Survival in extreme pH

Previous sections showed VUP 12006 grew poorly at very low pH. The following experiments were preformed to determine whether VUP 12006 could survive exposure to very low pH. Utilising the method of Gorden and Small (1993), *Lb. paracasei* strain VUP 12006 was subjected to extreme (lethal) acid conditions to determine the level of acid resistance. If greater than ten percent of challenged bacteria survived pH 2.5 for 2 hours they were considered acid resistant (section 2.3.9.2). This method was used to determine the ability of strain VUP 12006 to survive exposure to very low pH rather than being able to initiate growth in media at low pH.

3.3.5.1 Viability of strain VUP 12006 in MRS broth at pH 2 and 2.5

An ovemight culture of VUP 12006, grown in MRS broth at 37°C, was used to seed two fresh MRS broths at pH 2.0 and pH 2.5 respectively. The incubation temperature for this experiment was set at 37°C.

Samples were collected from these flasks every 30 minutes and viable cell counts performed (section 2.3.9.2). At pH 2.0, the percentage of colony forming units, as compared to the viable cell count at time zero, fell rapidly and only forty percent viability was detected after 30 minutes and after one hour it was not possible to detect any survivors (Figure 3.14). At pH 2.5 10% of the inoculum survived at 3.5 hours and 0.1% survived for 6 hours.

The rate of death was quicker for the first 3 hours and slowed during the second three hours. *Lb. paracasei* strain VUP 12000 was tested at the same time. This strain did not possess acid tolerance when tested according to the described protocol: at pH 2.0, after 2 hours, only 0.28% of starting numbers survived.

3.3.5.2 Relationship of growth phase to acid resistance

Strain VUP 12006 was challenged at different stages of the growth cycle as described in section 2.3.9.3. An overnight culture, grown in MRS broth at 37°C without pH control, was used to inoculate another flask run under the same conditions. Samples of the inoculum were removed from the fermenter every hour and inoculated into MRS broth at pH 2.5/37°C for 2 hours. The number of survivors was then determined (Figure 3.15). The results of this challenge indicate that the growth phase had a clear affect on the survival of this strain.

3.4 Effects of elevated temperature and low pH on growth

Strain VUP 12006 had clear optima with regard to temperature and pH and growth higher than 37°C or much below pH 6.3 may place the organism under stress. It was therefore proposed to observe changes that occurred in response to the strain growing outside optimum growth conditions.

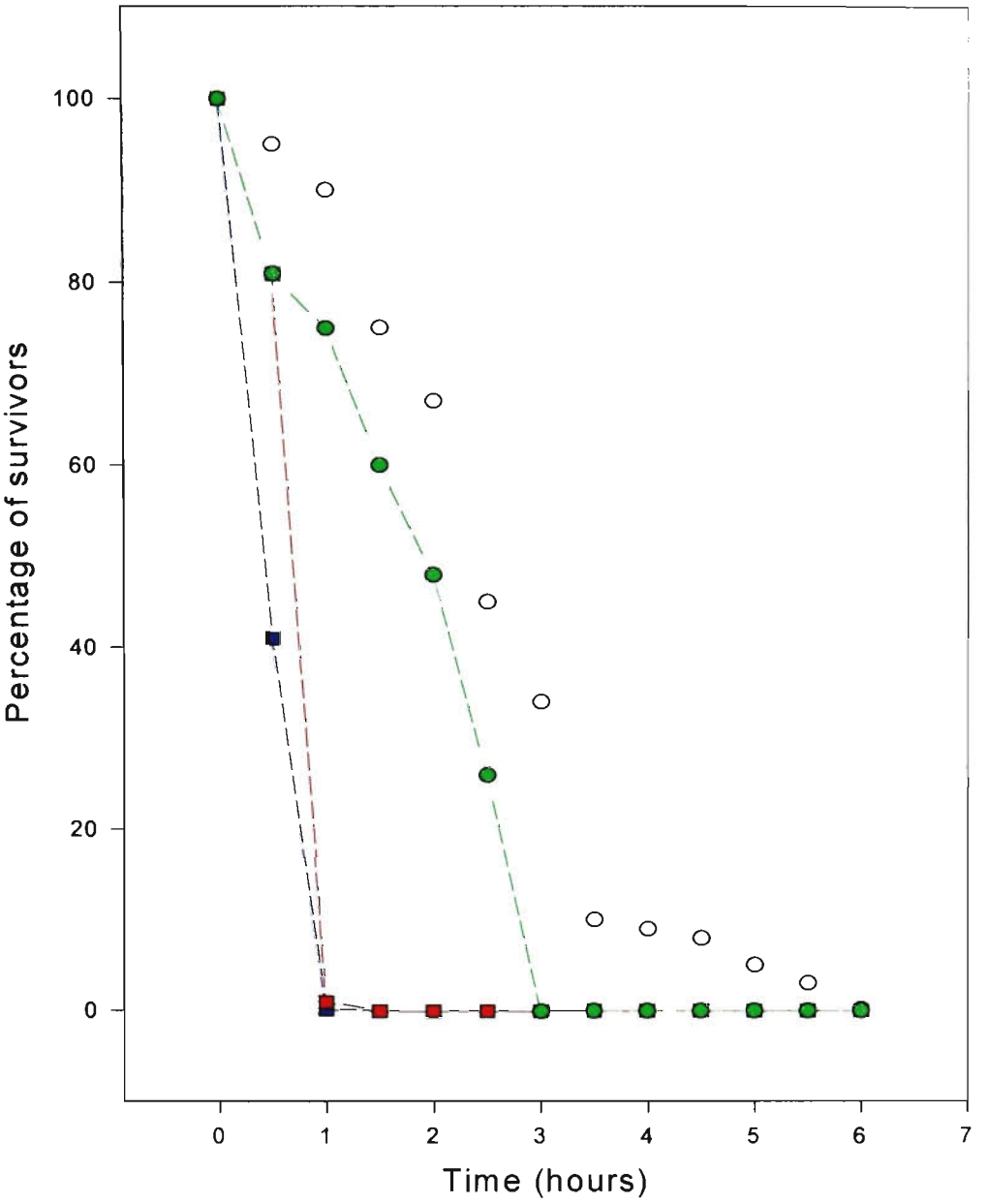


Figure 3.14 The effect of exposure to very low pH on the survival of *Lb. paracasei* strain VUP 12006.

An overnight culture of VUP 12006, grown in MRS broth at 37°C, was used to seed two fresh MRS broths at pH 2.0 (■) and pH 2.5 ( ) respectively, as described in section 2.3.9.2. VUP 12000 was used as a control {pH 2.0 (■) and pH 2.5 (●)}. The incubation temperature for this experiment was set at 37°C. Samples were collected from these flasks every 30 minutes and viable cell counts performed (section 2.3.9.4). The percentage of survivors was calculated from the viable cell counts relative to counts at time zero.

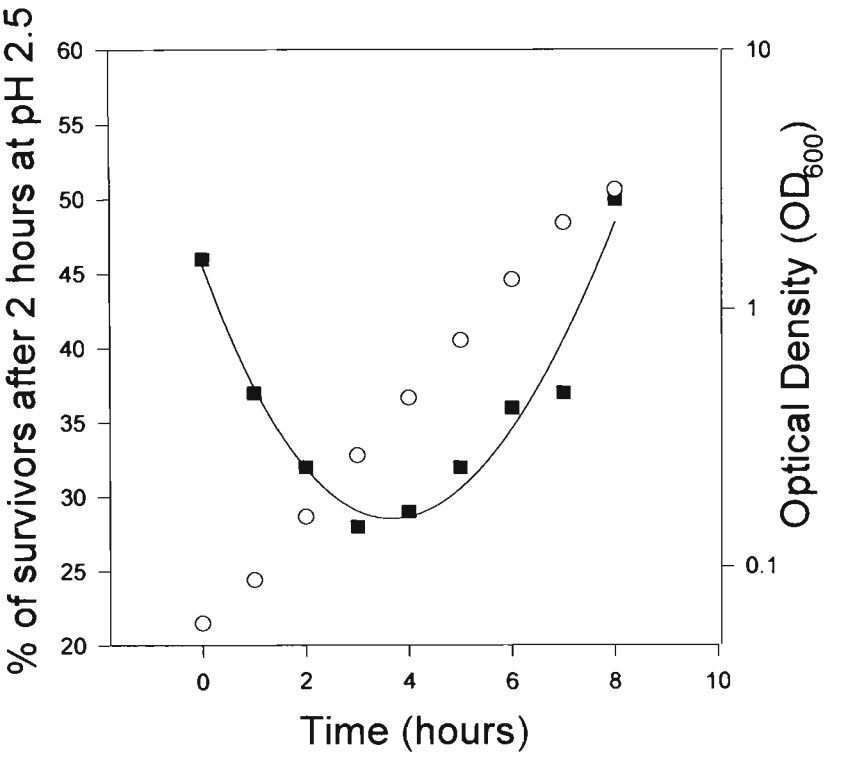


Figure 3.15 Relationship of growth phase to acid resistance for VUP 12006.

Lb. paracasei strain VUP 12006 was grown in a fermenter at 37°C in MRS broth without pH control. Every hour a sample of the culture was inoculated into MRS broth at pH 2.5. After 2 hours the percentage of remaining survivors was calculated. The graph shows % survivors (■) and optical density of the parent culture (o) plotted against time.

Data collected previously (Figure 3.10) showed μ_{max} values calculated at different temperatures and pHs. From this it was determined that VUP 12006 could be grown at pH 4.3/37°C and 45°C/pH6.3 to determine physiological changes. These conditions were chosen as they resulted in a decrease by 50% of the μ_{max} compared to optimum conditions and the conditions are similar to those encountered during yoghurt production.

Culture VUP 12006 was grown at 45°C/pH 6.3 or pH 4·0/37°C and the effect on growth rate (from OD_{600} changes), protein concentration, carbohydrate metabolism, protein profiles and FAME profiles determined. A control culture was grown at pH 6.3/37°C. These cultures were prepared from strain VUP 12006, which was sub-cultured from stationary phase after overnight growth in MRS broth at 37°C and in an initial pH of 6.3. The pH was not controlled during growth. The inoculum was further prepared by centrifugation (10 min at 4000 rpm) and the cells collected and resuspended in MRS broth pre-warmed to 37°C as described in section 2.3.7.1. This starter was inoculated to give a starting OD of 0.2 in three 1L fermenters (at 37°C/pH 6.3, 45°C/pH 6.3 or pH 4·0/37°C). The pH and temperature were maintained at the initial set point during growth.

3.4.1 Growth rate at 45°C or pH 4.0 of strain VUP 12006

The effect of elevated temperature and low pH on the extent of growth was measured from optical density (OD_{600}) readings (Figure 3.16). Elevated temperature and low pH had a marked effect on the extent and rate of growth. As determined from the optical density measured after eight hours, the control culture achieved approximately six times the extent of growth of the heat-stressed culture and four times the extent of growth of an acid-stressed culture.

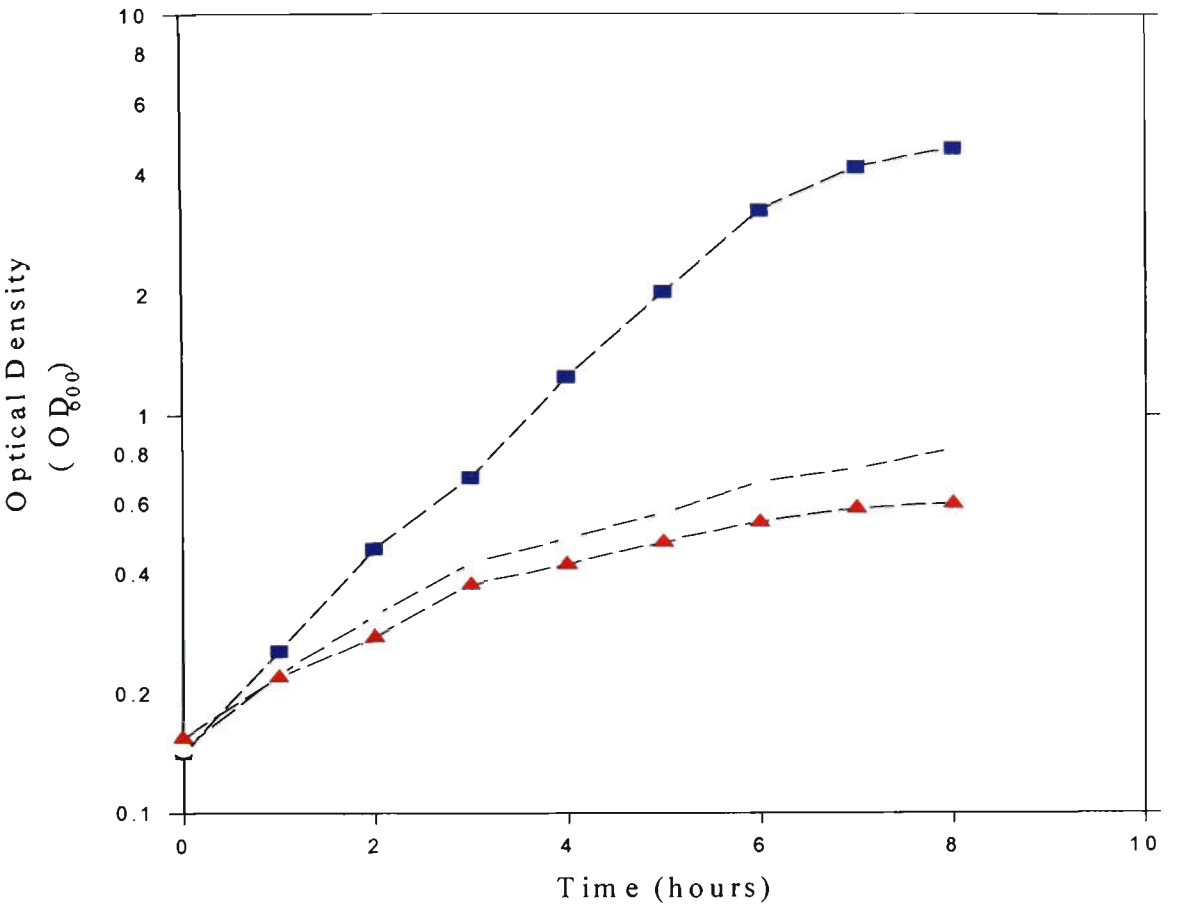


Figure 3.16 Effect of acid and high temperature on strain VUP 12006 measured by changes in optical density.

Cultures of strain VUP 12006 were grown in one litre of MRS broth in one-litre fermenters under controlled temperature and pH conditions. The inoculum was prepared from an overnight culture of strain VUP 12006 grown in MRS broth without pH control. The culture was centrifuged (10 min at 4000 rpm) and the cells collected and resuspended in MRS broth pre-warmed to 37°C, as described in section 2.2.6.1. These starter cells were used to inoculate 3 fermenters to an initial OD of 0.15 (pH 6.3 and 37°C) then growth was followed for eight hours. The conditions in two fermenters were immediately changed to 45°C and pH 6.3 ( ) and 37°C and pH 4.0 (▲) and the control was cultured at pH 6.3 and 37°C (■). Samples were taken hourly over eight hours and the optical density determined at 600nm. Nitrogen gas was sparged through the fermenters at 0.1 L/min.

Initially the heat-stressed culture grew at the same rate as the acid stressed culture but the growth rate had declined by the second hour.

3.4.2 Changes in protein concentration of cells grown at 45°C or at pH 4.0

Protein concentration of cells harvested periodically during growth at 45°C/pH 6.3, pH 4·0/37°C and pH 6.3/37°C was measured by the Lowry assay (section 2.4.3) (Figure 3.17). In the culture grown at 45°C the protein concentration fell below the starting value at the first hour and did not regain that value until after the fourth hour of culture, then tripled between hours five and six. The protein concentration of the culture grown at pH 4.0 remained stable for the first three hours and only began to rise at the fourth hour. This increase reflected the parallel rise in the control but was at a lesser rate. After six hours, protein levels in both test cultures were half that observed in the control. Measurement of protein was able to detect a lag period that was not obvious from OD_{600} readings but otherwise confirmed the lower growth rate and smaller biomass achieved at pH 4.0 or 45°C.

3.4.3 Changes in metabolite concentration of cells grown at 45°C or at pH 4.0

The glucose concentration started at 20 g/L in all cultures. After 6 hours growth, the glucose concentration in the control fell to 12.5 g/L (Figure 3.18). The culture grown at pH 4.0 mirrored the change in the glucose level until the end of the fifth hour. During the sixth hour in the culture grown at pH 4.0, the glucose level fell from 17.6 to 14.1 g/L while the control went from 18.0 to 12.5 g/L. The culture grown at 45°C showed a marked drop in glucose levels after the first hour. After five hours the glucose level in the control was still high at 18.0 g/L while the culture grown at 45°C had already dropped to 14.9 g/L. The culture grown at 45°C did not continue to demonstrate this high rate of glucose use and during the sixth hour the glucose level only decreased another 1.1 g/L compared to the control, which decreased 5.5 g/L.

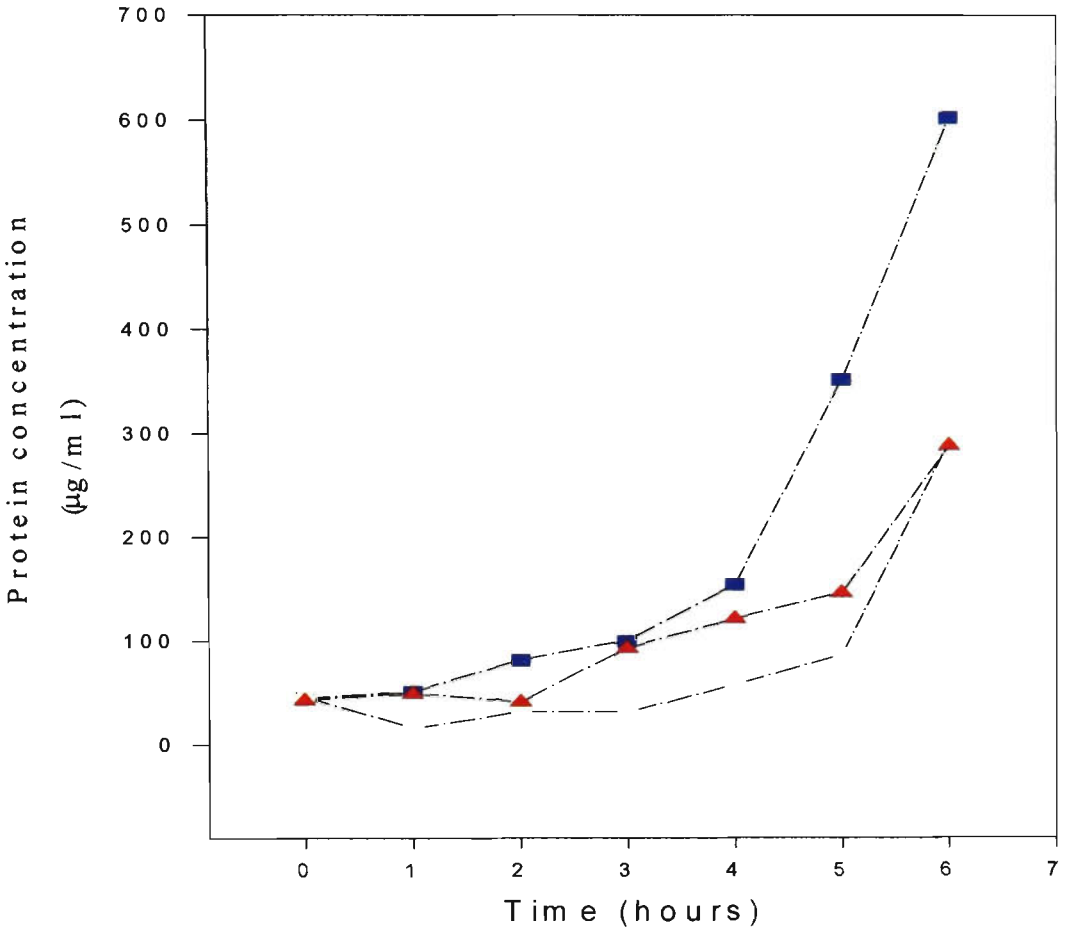


Figure 3.17 Effect of acid and high temperature on strain VUP 12006 measured by changes in protein concentration.

Cultures of strain VUP 12006 were grown in one litre of MRS broth in one-litre fermenters under controlled temperature and pH conditions. The inoculum was prepared from an overnight culture of strain VUP 12006 grown in MRS broth without pH control. The culture was centrifuged (10 min at 4000 rpm) and the cells collected and resuspended in MRS broth pre-warmed to 37°C, as described in section 2.2.6.1. These starter cells were used to inoculate 3 fermenters to an initial OD of 0.15 (pH 6.3 and 37°C) then growth was followed for six hours. The conditions in 2 fermenters were immediately changed to 45°C and pH 6.3 ( ) and 37°C and pH 4.0 (▲) and the control was cultured at pH 6.3 and 37°C (■). One mL samples were taken hourly over six hours and centrifuged to obtain a pellet which was resuspended in NaOH and boiled to disrupt the cells, (section 2.4.1.6). The protein concentration was determined from treated cells using the Lowry assay. Nitrogen gas was sparged through the fermenters at 0.1 L/min.

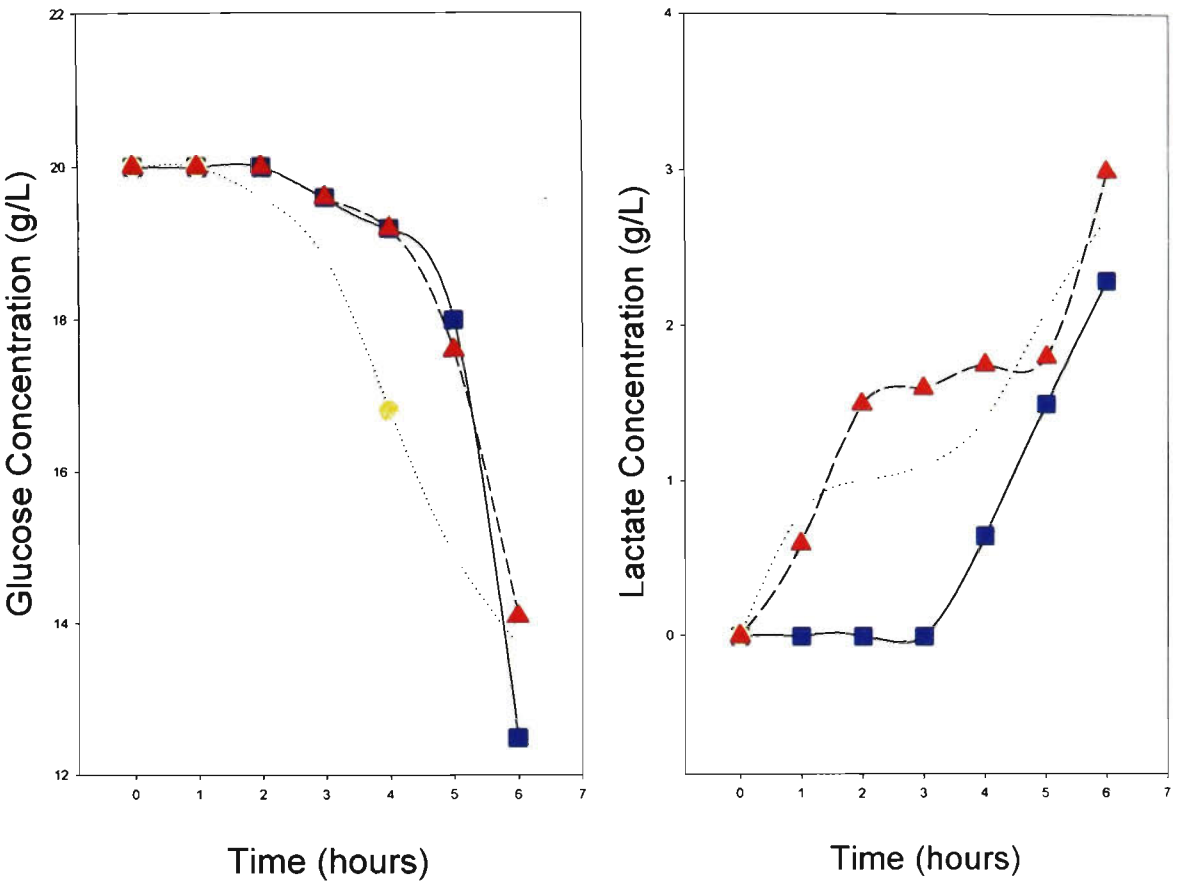


Figure 3.18 Effect of acid and high temperature on strain VUP 12006 measured by changes in glucose (A) and lactate concentration (B).

Cultures of strain VUP 12006 were grown as described in Figure 3.17. These starter cells were used to inoculate 3 fermenters to an initial OD of 0.15 (pH 6.3 and 37°C) then growth was followed for six hours. The conditions in 2 fermenters were immediately changed to 45°C and pH 6.3 ( ) and 37°C and pH 4.0 (▲) and the control was cultured at pH 6.3 and 37°C (■). Samples were taken hourly over six hours and the level of glucose and lactate determined by HPLC, (section 2.4.2.2). Nitrogen gas was sparged through the fermenters at 0.1 L/min.

Lactate levels were not detected in the control until the fourth hour but rose rapidly between hours four and six (Figure 3.18). The limit of lactate sensitivity was 0.1 g/L. However, in both the cultures grown at 45°C and pH 4.0, lactate levels increased rapidly for the first two hours in contrast to the relatively slow growth rate seen. Levels continued to rise, but at a decreased rate, between hours two and five then rose again rapidly in the sixth hour. A HPLC trace of a culture grown at pH 6.3 and 37°C taken two hours after inoculation is shown in Appendix 3.

3.4.4 Effect of growth at low pH and elevated temperature on protein profiles (1-D SDS-PAGE)

SDS-PAGE was run according to the method described in section 2.5.1. Estimation of molecular weight and quantification of the relative amount of protein present in each band was determined using an Ultrascan XL laser densitometer (Pharmacia) (section 2.5.3.3). In protein profiles of cultures grown under optimum conditions (pH 6.3, 37°C) approximately 30 major bands with molecular weights between 5 and 115 kDa were observed, (Figure 3.19). The banding pattern of proteins from the control culture appeared not to change significantly after the fifth hour of culture although the relative proportions of some proteins (for example, in the molecular weight range greater than 97.4 kDa) did vary at different stages of growth. The initial sample showed a banding pattern more similar to the overnight culture. However, growth at low pH and elevated temperature caused significant alteration to protein profiles compared to the control, see Figures 3.20 and 3.21. Growth at low pH in particular resulted in a significant change to the SDS-PAGE profile: late in the growth cycle the profile was made up of five major and several minor bands. Among the five major bands (25, 35, 45, 50 and 75 kDa), one band of approximately 42 kDa was particularly prominent. When the gel was scanned using laser densitometry, the density of this band corresponded to 24% of the total density of all proteins detected by silver staining in the twenty-four hour sample. This protein increased from being undetected in the first sample.

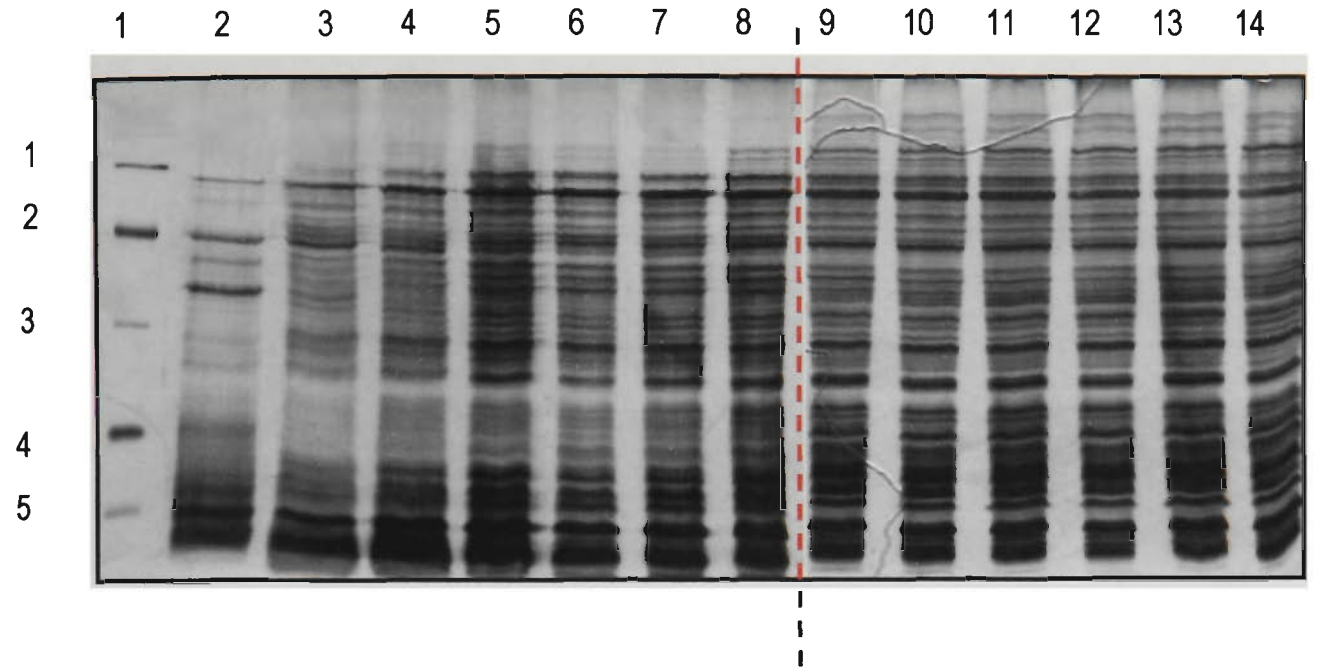


Figure 3.19 1-D SDS-PAGE of cellular proteins of *Lb. paracasei* strain VUP 12006 grown at pH 6.3 and 37°C.

Culture samples corresponding to lanes 2-13 were taken hourly after inoculation and cells were collected, concentrated, re-suspended in double strength loading buffer and boiled before analysis by 1-D SDS-PAGE (sections 2.4.1.1 and 2.5.1.1). The sample in lane 2 was collected immediately after inoculation and the sample in lane 14 was collected after 24 hours of culture. The dashed line indicates where two separately run gels have been joined. The gel was silver stained to visualise the protein bands.

Lane 1: Molecular Weight Standards
Lanes 2 to 13: Samples collected hourly for 12 hours
Lane 14: Sample collected after 24 hours growth

Molecular weight standards:

1. 97.4 kDa
2. 66.2 kDa
3. 45 kDa
4. 31 kDa
5. 21.5 kDa

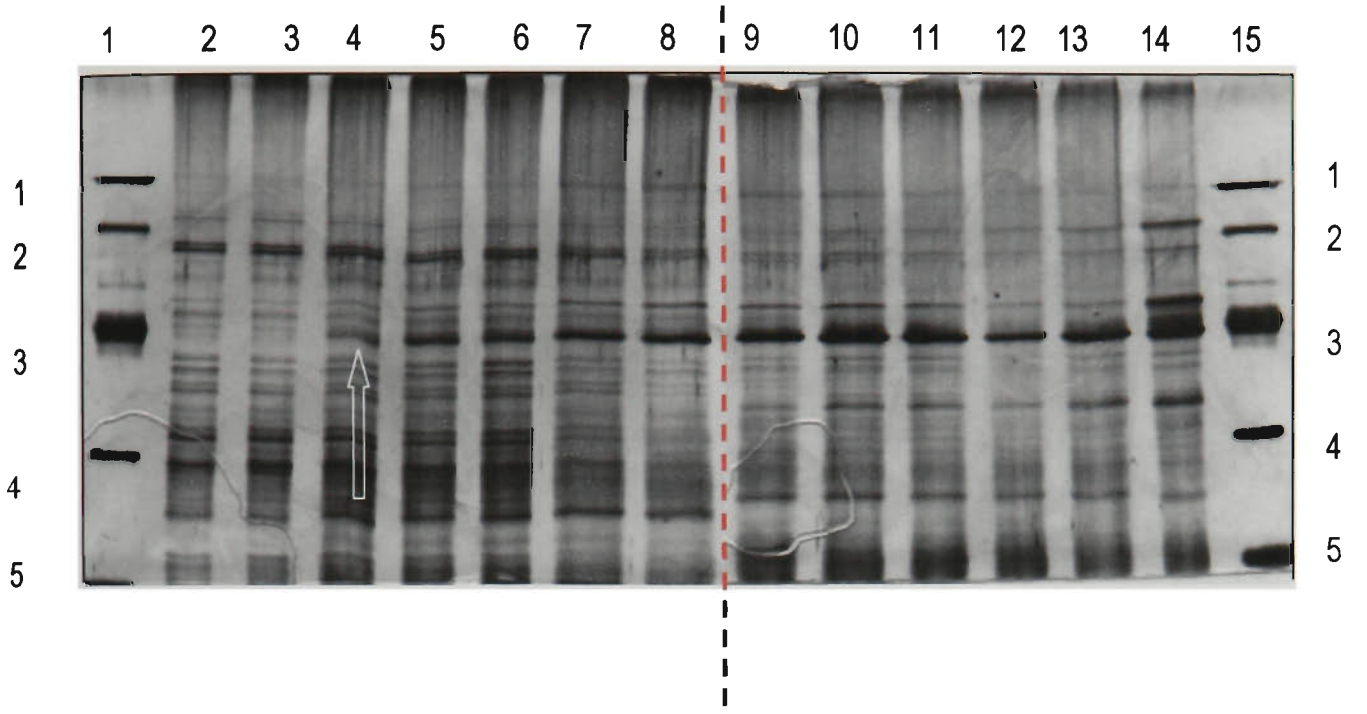


Figure 3.20 1-D SDS-PAGE of cellular proteins of *Lb. paracasei* strain VUP 12006 grown at pH 4.0 and 37°C.

Culture samples corresponding to lanes 2-14 were taken hourly after inoculation and cells were collected, concentrated, re-suspended in double strength loading buffer and boiled before analysis by 1-D SDS-PAGE (sections 2.4.1.1 and 2.5.1.1). The sample in lane 1 was collected immediately after inoculation and the sample in lane 14 was collected after 24 hours of culture. The dashed line indicates where two separately run gels have been joined. The gel was silver stained to visualise the protein bands. Protein banding pattern was produced during growth at low pH (pH 4.0 and 37°C). The arrow indicates protein band of approximately 42 kDa that was up-regulated during growth under acid stress.

Lanes 1and 15 MW Standards
Lanes 2 to 13 Samples collected hourly for 12 hours
Lane 14 Sample collected after 24 hours growth

Molecular Weight Standards

1. 97.4 kDa
2. 66.2 kDa
3. 45 kDa
4. 31 kDa
5. 21.5 kDa

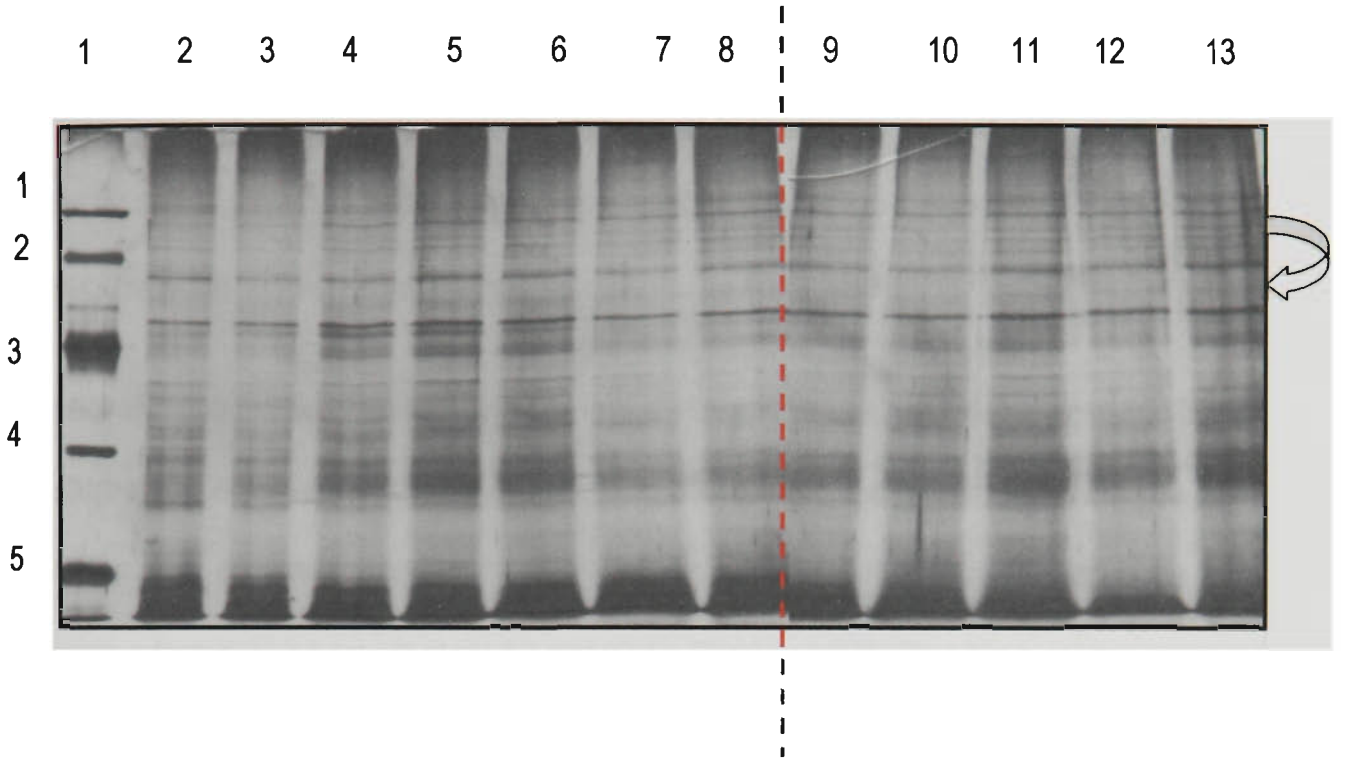


Figure 3.21 1-D SDS-PAGE of cellular proteins of *Lb. paracasei* strain VUP 12006 grown at pH 6.3 and 45°C.

Culture samples corresponding to lanes 2-13 were taken hourly after inoculation and cells were collected, concentrated, re-suspended in double strength loading buffer and boiled before analysis by 1-D SDS-PAGE (sections 2.4.1.1 and 2.5.1.1). The sample in lane 1 was collected immediately after inoculation and the sample in lane 14 was collected after 24 hours of culture. The dashed line indicates where two separately run gels have been joined. The gel was silver stained to visualise the protein bands. Protein banding pattern was produced during growth at pH 6.3 and 45°C. The arrow indicates the area between 50 and 90 kDa, where a number of minor bands that are up-regulated during growth at increased temperature, were detected by laser densitometry.

Lane 1 MW Standards
Lanes 2 to 13 Samples collected hourly for 12 hours

Molecular Weight Standards

1. 97.4 kDa
2. 66.2 kDa
3. 45 kDa
4. 31 kDa
5. 21.5 kDa

A protein of molecular weight around 42 kDa made up less than two percent of the proteins present in an optimally grown culture at any time throughout the growth cycle and in a culture grown at 45°C a protein of sirnilar size constituted orıly four to eight percent of the protein profile. During growth at 45°C only minor bands between around 50 to 90 kDa were up-regulated and they could only be detected by laser densitometry.

One of the other proteins that appeared during acid stress, a 50 kDa protein, also increased linearly throughout the culture time, as determined by laser densitometry and constituted between zero and ten percent of all proteins present. A protein of similar molecular weight was seen in the initial sample collected during growth at pH 6.3 and 37°C. The 35 and 75 kDa proteins were not present in significant amounts until the culture was exposed to acid stress for an extended time, greater than 12 hours, and may also be associated with stationary phase responses. The protein profile of heat stressed cultures displayed a general down-regulation of a number of proteins indicated by the decrease in the number of protein bands present in the profile. Increased expression of two proteins of about 60 and 70 kDa was detected by laser densitometry after a culture was grown at 45°C. An increase in expression of these proteins was not detected in control cultures.

3.4.5 Effect of growth at low pH and elevated temperature on immunoblot reactions

Following SDS-PAGE, proteins were transferred by electroblot to nitrocellulose membrane and detection of specific proteins was performed using a Western blot technique (section 2.5.4) (Figure 3.22). For convenience the immunoblot shown resulted from the combination of two antibodies in the reaction mix, however when only one antibody was used, only single bands of the appropriate size were observed. Bands visualised using this technique were quantified by laser densitiometry (section 2.5.3.3).

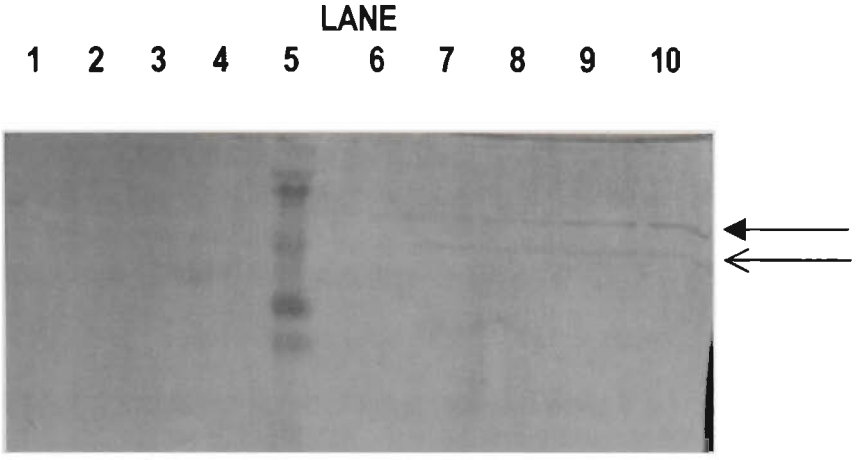


Figure 3.22 Immunoblot to detect regulation of HSP60 and HSP70 analogues during growth at 37°C and 45°C.

Culture samples grown at 37°C/pH 6.3, corresponding to lanes 1-4 and 45°C/pH 6.3, corresponding to lanes 6-9, were taken every two hours after inoculation and cells were collected, concentrated, re-suspended in double strength loading buffer and boiled before analysis by 1-D SDS-PAGE. The 1-D SDS-PAGE was electroblotted to nitrocellulose membrane and detection of specific proteins was performed using a Western Blot technique (2.5.4). Lane 10 is a positive control prepared from heat stressed *E. coli* cells grown at 42°C. Lane 5 is BIO-RAD pre-stained standards Myosin 202 kDa, β galactosidase 133kDa, Bovine serum albumin 71 kDa, Carbonic anhydrase 41.8 kDa, Soybean trypsin inhibitor 30.6 kDa and Lysozyme 17.8 kDa.

(approximately 70 kDa ◄—— , approximately 60 kDa <——)

Using this technique, the 60 and 70 kDa proteins up-regulated during growth at elevated temperature were shown to cross-react with antibodies made against *E. coli* hsp60 and *E. coli* hsp70. Positive controls prepared from heat stressed *E. coli* cells (grown at 45°C) were run on each gel. The production of the 60 kDa protein was found to increase four-fold during growth at 45°C but the same protein remained constant during growth at 37°C and pH 6.3.

Similarly, the 70kDa protein increased approximately four-fold while the level remained constant in the control. Cultures grown at low pH showed a similar response to the control culture in that protein bands at approximately 60 and 70 kDa cross-reacted with hsp60 and hsp70 antibodies but the levels remained unchanged during growth at low pH. It appears that growth at low pH did not cause up-regulation of analogues of HSP60 and HSP70.

3.4.6 Effect of acid and temperature shift on FAME profiles

FAME analysis was performed as described in section 2.2.6.3 except that samples for comparison were taken from the fermenters at the mid-exponential phase of growth. Compared to growth at pH 6.3, growth at pH 4.0 resulted in a reduction of the percentage of the $C_{18:1(9)}$ and $C_{18:3}$ combined peak from 26% to 11% and the $C_{19:0}$ was also reduced from 26% to 16%, (Table 3.9). A significant increase in relative abundance in the $C_{16:1}$ peak was observed at the same time, with an increase from 2% to 10% observed. Increasing the growth temperature resulted in the decrease of the relative abundance of $C_{19:0}$ from 26% to 12% while the $C_{18:0}$ peak increased from 5% to 14%. No other significant changes were seen.

3.4.7 The effect of change in pH during growth on the expression of the 45kDa protein

In previous sections it was shown that continuous growth at pH 4.0 resulted in the over-expression of a 42 kDa protein, which was not up-regulated during growth at elevated temperature (45°C) or during growth at optimal temperature and pH (37°C and pH 6.3).

Table 3.9 The effect of acid and temperature shift on FAME profile.

Long chain fatty acids present in *Lb. paracasei* strain VUP 12006 were converted into their corresponding methyl esters and and separated using a Varian gas chromatograph with a BPX-70 column. Twelve major peaks were found to be present. Data below is presented as a percentage of the specific fatty acids present during growth at low pH (pH 4.0 and 37 °C), high temperature (45°C and pH 6.3) and at 37°C and pH 6.3. Results were calculated from the area under a specific peak divided by the area under all peaks present in a specific strain and multiplied by 100. The data are means of two independent experiments.

[a] Methyl ester of fatty acids (%):											
FAME Retention time (min)	$C_{10:00}$	X1	X2	X3	$C_{14:00}$	X4	$C_{16:00}$	$C_{16:1}$	$C_{18:0}$	$C_{18:3}$ & $C_{18:1(9)}$ [b]	$C_{19:00}$
	2.05	3.5	3.8	4	4.2	5.9	6.3	6.8	9.6	10.2	12.3
Growth conditions											
Optimum	0	8	0	4	2	2	17	2	5	26	26
Low pH (pH 4)	1	0	1	6	0	7	15	10	11	11	16
High temperature (45° C)	0	0	2	2	0	9	17	2	14	21	12

[a] Relative proportion of fatty acid methyl ester based on percentage of total area for the peaks detected. [b] Peaks for fatty acid methyl esters 18:3 and 18:1(9) could not be separated completely at the base line during all runs using the method described in section 2.4.4 and are presented as a combined percentage.

To determine whether this protein was induced in response to acid stress, a series of fermentations were performed were the pH was shifted during growth from optimal to pH 4, then held at this pH. Alternatively the pH was shifted from pH 6.3 to 4.0 then returned to pH 6.3 to produce a spiked change in pH. The fermenters were set up, in duplicate, as follows:

Fermenter	**Growth conditions**
Fermenter one (F1)	Optimum growth conditions (37°C and pH 6.3) for 10 hours. Addition of NaOH was discontinued after 10 hours, pH was not controlled overnight.
Fermenter two (F2)	37°C/continuous growth at pH 4.0.
Fermenter three (F3)	37°C/pH shifted to pH 4 during exponential phase of growth (two hours after start of experiment). Low pH conditions were continued to the end of the experiment.
Fermenter four (F4)	37°C/pH not controlled. Growth commenced at pH 6.3. The pH dropped below 4 by the end of each experiment.
Fermenter five (F5)	37°C and pH 6.3, spiked to pH 4.0 then returned immediately to pH 6.3. The spike of acidic conditions was given at early exponential phase, two hours after start of experiment.

In F3 the pH took approximately ten minutes to reach pH 4.0 and in F5 the spike took nearly twenty minutes to cycle from pH 6.3 to 4.0 and back to 6.3. Cells were collected hourly and extracts prepared as described in sections 2.4.1.1. and 2.5.1.1. Protein profiles were determined by 1-D SDS-PAGE. Figure 3.23 shows the profiles seen for the five fermentation conditions, concentrating on the molecular weight range between approximately 30-70 kDa. Consistent with previous

experiments, the protein profiles of samples collected hourly from F1 (pH was controlled at 6.3), showed no apparent change in the regulation of the 42 kDa protein. When a spiked decrease in pH occurred during exponential growth at pH 6.3 (F5) there was no apparent change in the protein profile. However there was no attempt made to optimise the experimental protocol to detect induction of pH stress related proteins using this approach. When cells were cultured at pH 6.3 the pH decreased to pH 4.0 during exponential growth (F3) and there was a change in the protein profile so that it then resembled the one seen for continuous growth at pH 4.0 (F2). This showed the 42 kDa protein dominating during growth through to stationary phase. These data clearly show that for strain VUP 12006 the 42 kDa is linked to exposure to low pH.

3.5 Protein profiles of other *Lactobacillus* strains when grown at different pHs

Studies of 1-D SDS-PAGE profiles were performed on the other six strains of *Lactobacillus* described in section 3.2 to determine whether they showed similar responses to growth at low or high pH. Findings are presented in the following sections.

3.5.1 Growth at pH 4.0 or shift from pH 6.3 to 4.0 during growth

Lactobacillus strains, VUP 12001 and 12007, were grown either at pH 4.0, pH 6.3 or at pH 6.3 and shifted to pH 4.0 at early exponential phase (after 2 hours growth). These conditions are the same as those in which *Lb. paracasei* strain VUP 12006 was grown for the experiment described in section 3.4.13. SDS-PAGE was performed on protein samples taken hourly during growth in 1L fermenters in MRS broth at 37°C. The SDS-PAGE gels for strains VUP 12001 and 12007 are shown in Figure 3.24. These data show that during growth at pH 4.0 the 42 kDa protein is present at constant levels in VUP 12001 (gel B) and VUP 12007 (gel C) but not up-regulated as for strain VUP 12006 (gel A).

Figure 3.23 The effect of continuous acid shift or spike shift on the regulation of the 42 kDa acid stress protein as determined by 1-D SDS-PAGE for VUP 12006.

Lb. paracasei strain VUP 12006 was grown in a set of fermenters at 37°C in MRS broth. The pH in each of the fermenters varied according to the conditions described in section 3.4.8. Each gel is shown in close-up between approximately 30 and 70 kDa, as indicated on F1. An arrow beside each gel indicates the level of rnigration of a 45 kDa standard. Culture samples corresponding to lanes 1-11 were taken hourly after inoculation and cells were collected, concentrated, then re-suspended in double strength loading buffer and boiled before analysis by 1-D SDS-PAGE (sections 2.4.1.1 and 2.5.1.1). The sample in lane 1 was collected immediately after inoculation and the sample in lane 11 was collected after 24 hours of culture. The gel was silver stained to visualise the protein bands.

Key

Fermenter	Growth conditions (All cultures were grown at 37°C in MRS broth)
F1	Optimum growth conditions (pH 6.3) for 10 hours after which pH was not controlled.
F2	Continuous growth at pH 4.0
F3	pH shifted to pH 4 during exponential phase of growth (2 hours after start of experiment).
F4	pH not controlled. Growth commenced at pH 6.3.
F5	Commenced at pH 6.3, spiked briefly to pH 4.0 after 2 hours, then returned to pH 6.3.

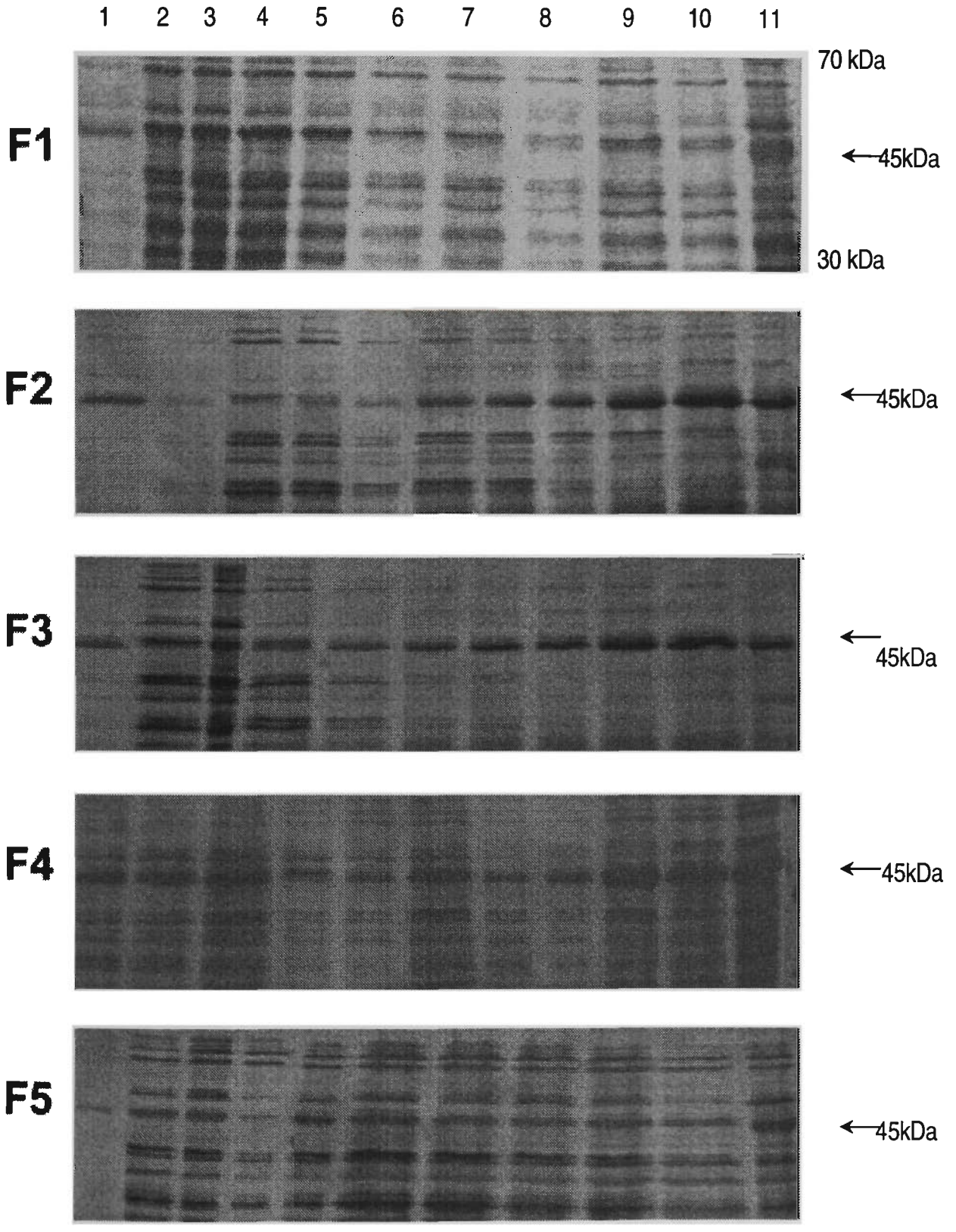

LANE
1 2 3 4 5 6 7 8 9 10 11
F1
70 kDa
45kDa
30 kDa
F2
45kDa
F3
45kDa
F4
45kDa
F5
45kDa

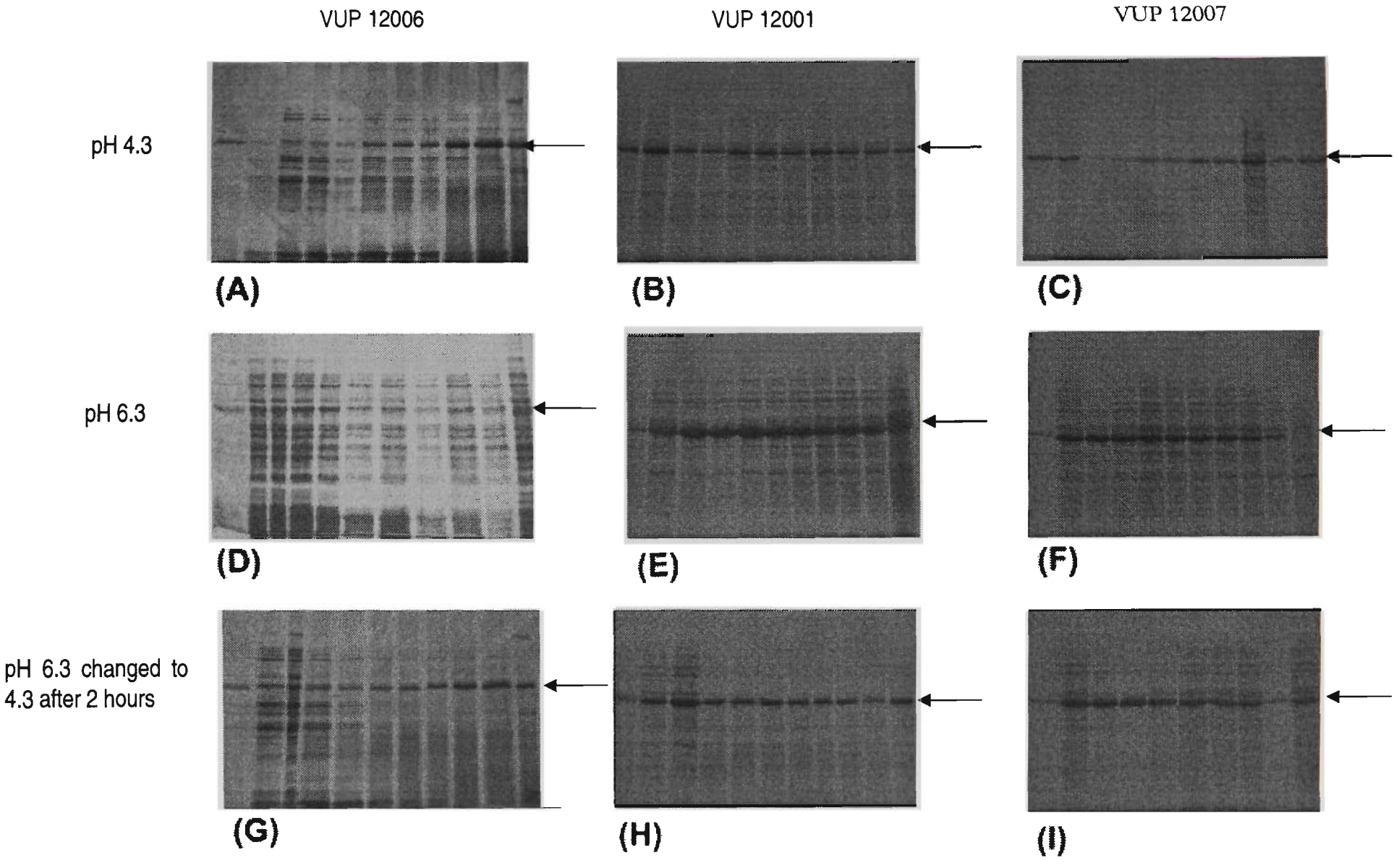
Figure 3.24 Comparison of 1-D SDS-PAGE protein profiles of *Lactobacillus* strains VUP 12006, 12001 and 12007 when grown at different pH values

Lactobacillus strains VUP 12006, 12001 and 12007 were grown in a set of one-litre fermenters at 37°C in MRS broth. The pH in each of the fermenters varied according to the conditions described below. Culture samples from each fermenter were taken hourly after inoculation and cells were collected, concentrated, re-suspended in double strength loading buffer and boiled before analysis by 1-D SDS-PAGE (sections 2.4.1.1 and 2.5.1.1).

Key

Gel	Strain	Growth pH
A	VUP 12006	4.0
B	VUP 12001	4.0
C	VUP 12007	4.0
D	VUP 12006	6.3
E	VUP 12001	6.3
F	VUP 12007	6.3
G	VUP 12006	6.3 changed to 4.3 after 2 hours
H	VUP 12001	6.3 changed to 4.3 after 2 hours
I	VUP 12007	6.3 changed to 4.3 after 2 hours

Arrow(◄———) indicates 42 kDa protein


VUP 12006
VUP 12001
VUP 12007
pH 4.3
pH 6.3
pH 6.3 changed to 4.3 after 2 hours
(A)
(B)
(C)
(D)
(E)
(F)
(G)
(H)
(I)

During growth at pH 6.3 in VUP 12006 (gel D) this protein is difficult to detect except by laser densitometry, but a protein of approximately 42 kDa is the most predominant protein produced during growth at pH 6.3 in *Lactobacillus* strains 12001 (gel E) and 12007 (gel F). Similar results were observed in the SDS-PAGE gels where the cultures were grown initially at pH 6.3 but shifted to pH 4.0 after one hour of incubation. The 42 kDa acid stress protein, which was observed to be up-regulated in strain VUP 12006 during growth at pH 4.0 was not seen to be up-regulated during growth in similar conditions in the other strains. A protein of identical molecular weight was seen in VUP 12001 and 12007 during growth at pH 6.3.

3.6 Partial purification and identification of acid stress protein of VUP 12006

Several attempts were made to partially purify the 42 kDa acid stress protein seen in SDS-PAGE gels that was up-regulated during growth at pH 4.0 in strain VUP 12006, with the aim of using the purified protein for N-terminal sequencing. The difficulties encountered during purification related to the method used to prepare samples used for 1-D SDS-PAGE. Initially protein samples were prepared by boiling whole cells in double strength loading buffer. When the same cells were homogenised as part of the preparation for 2-D SDS-PAGE, prepared as described in section 2.2.6.4, the banding patterns observed by 1-D SDS-PAGE were considerably different (Figure 3.25). It was observed that the protein that was markedly up-regulated during growth at pH 4.0 did not appear to be changed when time course samples were prepared by homogenisation and centrifugation, suggesting this protein was cell-membrane or surface-located.

Electrophoretic methods that used homogenised cells as a means of partially purifying the acid responsive protein were not found to be particularly useful. The methods used to partially purify the protein are described below.

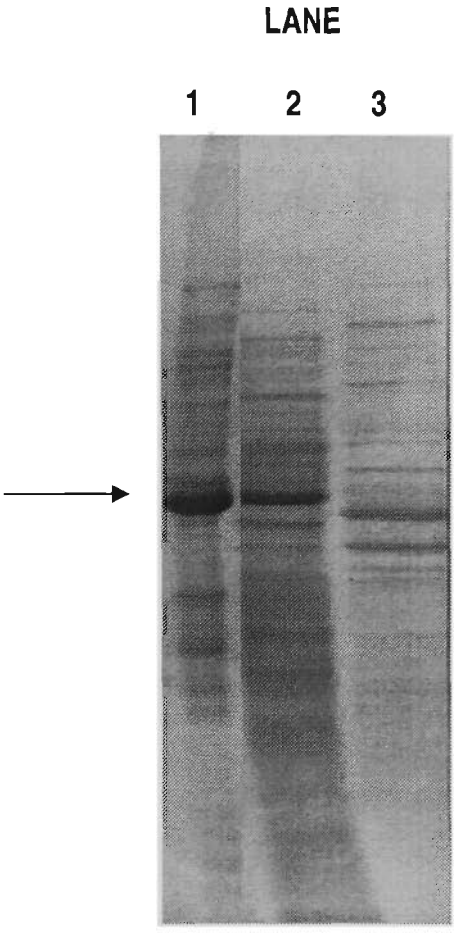


Figure 3.25 Comparison of protein banding patterns between cells prepared three ways.

Proteins were prepared from a single culture. Cells were resuspended in 40 mM Tris pH 7. After homogenisation the sample was centrifuged and both the clear supernatant and pellet were collected. A control sample of centrifuged whole cells was also collected. Approximately 200 µL of each sample was added to an equal volume of double strength loading buffer and heated to 100°C for 5 minutes. The samples were centrifuged and 20 µL was loaded onto a 1-D SDS-PAGE and visualised by Coomassie stain. Arrow marks level of 45 kDa standard.

Lane 1 Pellet of cellular debris after homogenisation

Lane 2 Whole cells

Lane 3 Supernatant removed from cellular debris

3.6.1 Partial purification using native PAGE and IEF (Prep and Rotofor cells)

Continuous elution electrophoresis-native PAGE was performed using a model 491 Prep Cell (BioRad). Approximately 0.5 g of crude homogenised protein was applied to the gel column, which was run according to the manufacturer's instructions (see section 2.4.3.4). The eluate from the column was collected as one mL fractions, which were subsequently run on 1-D SDS-PAGE to confirm which fraction(s) contained the protein of interest. Approximately 52 fractions, containing eluted proteins, were collected during each run. The protein of interest appeared between fractions 37 and 47. These fractions, however, were found to have low concentrations of the protein of interest and the fractions also contained several other protein bands. Using a similar approach, preparative isoelectric focussing using a Rotofor cell (BioRad) was performed according to the method described in section 2.4.3.5. Similar difficulties were experienced using this method, in that the fraction containing the protein of interest also contained several other proteins at much higher concentrations. It was decided not to persist with either method and to pursue partial purification through denaturing electrophoresis.

3.6.2 Purification by denaturing electrophoresis and N-terminal sequencing

1-D SDS-PAGE of a sample collected during the late exponential phase of growth at pH 4.0 (six hours after inoculation of the culture) was performed in a Protean II slab system (BioRad). The useable length of the resolving gel was 12 cm and using pre-stained protein standards as markers, the gel was allowed to run until the 31.6 kDa marker reached the end of the gel. The gel was electroblotted to PVDF membrane and the band visualised with Amido Black 10B. Along with the gel length, this allowed sufficient separation of protein bands for the 42 kDa protein seen in acid stressed cultures to be visualised and excised from the PVDF membrane without any apparent contamination from other bands (Figure 3.26).

The 42 kDa protein was sent for N-terminal sequencing at the Australian Proteome Analysis Facility (APAF) and the following result was obtained: Asp, Thr, Ser, Asp, Ser, Ile, Ala, Ser, Asn,

Lys, (Ser/Gln/Asp), Glu, Thr, Asn, Ala, Leu, Leu, Lys, Gln, Ile. Sequencing analysis was unable to determine the amino acid in position eleven. The initial search of protein databanks revealed no direct sequence match: however the closest match with 65% similarity was to a protein superfamily group of the cauliflower mosaic virus aphid transmission protein (PIR entry S01280). This search was performed through GeneFIND (http:www-nbrf.georgetown.edu/gf-cgi/genef) and ANGIS.

Subsequent searches through the same sites, after the searchable data base size had increased, yielded the following results, none were N-terminally located. The pattern match is shown below;

	Name of sequence match	Amino Acid Sequence
N-terminal sequence of acid stress protein submitted for pattern		D T S D S I A S N K S E T N A L L K Q I
Match 1	N-glycosylation site (PS00001)	D T S D S I A S N K S (Q D) E T N A L L K Q I
Match 2	Protein kinase C phosphorylation site (PS00005)	D T S D S I A S N K S (Q D) E T N A L L K Q I
Match 3	Casein kinase II phophorylation site (PS00006)	D T S D S I A S N K S (Q D) E T N A L L K Q I

The sequence was resubmitted for analysis using the tBLASTn program following the release of the draft genome of *Lb. casei* version '20Sep02'. The sequence was found to match the genome between positions 9501 and 9560 of scaffold 3, as shown below.

Query: 1 DTSDSIASNKSETNALLKQI 20

Sbjct: 9560 DTSDSIASNKSETNALLKQI 9501

Further studies were then performed to identify the gene sequence of the protein and its function during growth at low pH.

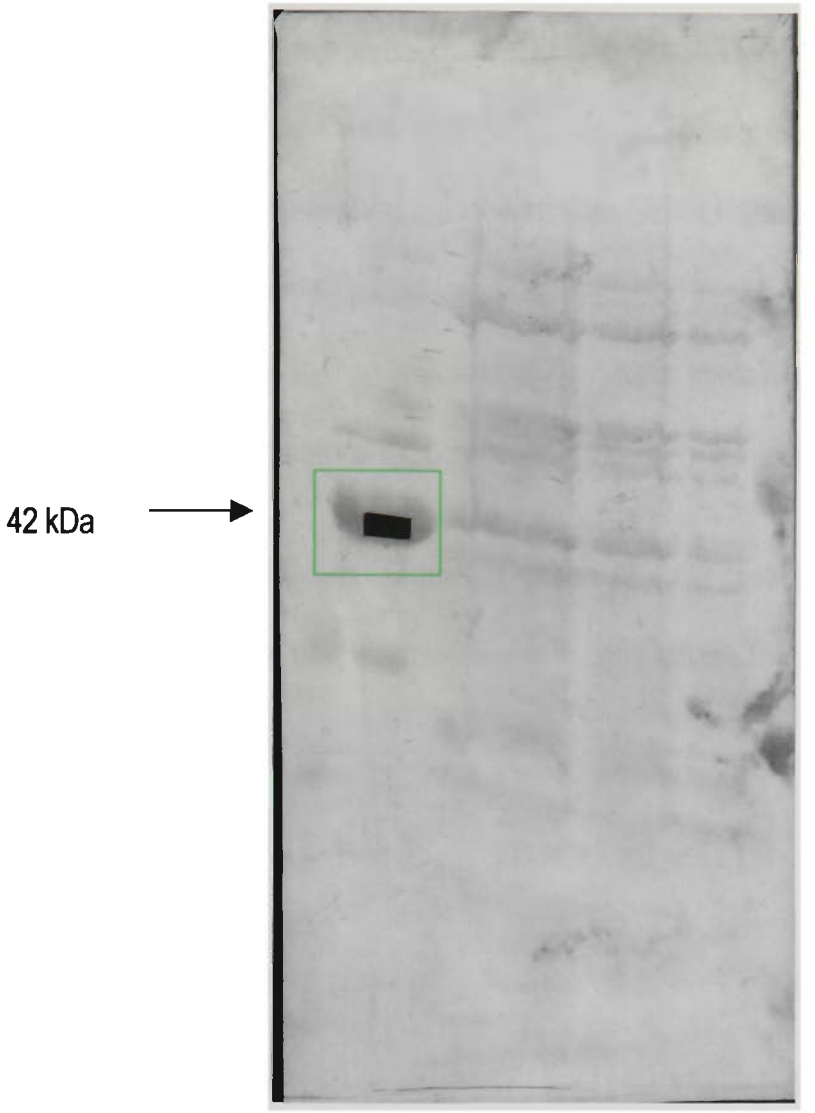


Figure 3.26 PVDF electro blotted membrane of 1-D SDS-PAGE showing excision of 42 kDa protein band for sequencing

Lb. paracasei strain VUP 12006 was grown at pH 4.0 and 37° for 6 hours. The cells were harvested, concentrated, re-suspended with double strength loading buffer and boiled before analysis by 1-D SDS-PAGE. The unstained gel was electroblotted to PVDF membrane and the protein bands were visualised with Amido black 10B (section 2.5.5). The 42 kDa band, marked by the green box, was excised from the gel and submitted for sequence analysis at The Australian Proteome Analysis Facility.

3.6.3 Construction of representative genomic library and screening for gene encoding the 42 kDa protein

Chromosomal DNA of *Lb. paracasei* strain VUP 12006 extracted according to the method described in section 2.6.7.1 was partially digested with *Sau*3A I (section 2.6.7.2). Aliquots taken at different times were run on a 0.5% agarose gel and samples showing maximum fluorescence in the 10-25 kb size range were used to clone into the *Bam*H I digested vector pBluescript SK+. See Figure 3.27. Ligation of vector and insert are described in section 2.6.7.3. *E. coli* TG2 cells were transformed with the ligation mix and colony blots containing 47 transformants and 1 position marker on each membrane were prepared as described in section 2.6.8.

Using results from the N-terminal sequence of the 42 kDa protein, a probe of 17-mer was constructed according to the method described in section 2.5.6. The probe was constructed with the following sequence:

5' G A A A C N A A (T or C) G C N (C or T) T N (C or T) C 3'

The melting temperature for the probe was calculated at 60.52°C using the following formula

Melting temperature = 81.5 + 16.6 (log10 [Na^+]) + 0.41 [(G + C)/n (100)] –600/n

Where n=17, [Na+]=0.5 and G+C =8.

The probe was labelled with (γ^{32} P) ATP and used to screen the representative genomic library by colony blot using the calculated melting temperature for the radio-labelled probe for hybridisation (section 2.6.11). Autoradiographic techniques were used to visualise positive reactions indicating which colonies contained transformed cells that hybridised with the probe.

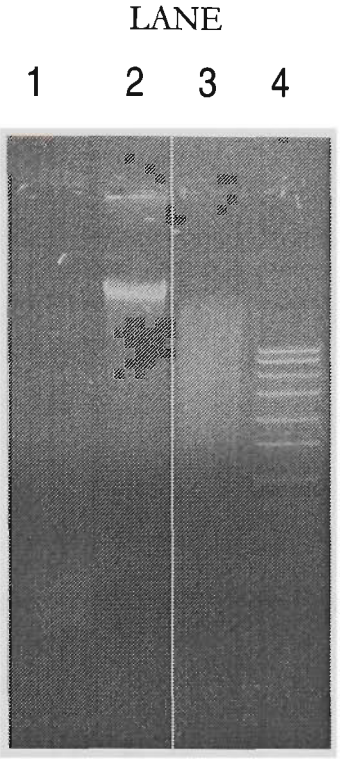


Figure 3.27 *Sau*3A I digestion of DNA from *Lb. paracasei* strain VUP 12006

Agarose electrophoresis gel (0.5% agarose) of *Lb. paracasei* DNA completely digested, undigested and partially digested with *Sau*3A I. The partially digested DNA was used to create a representative Genomic DNA library.

Key

Lane 1 Complete digest of *Lb. paracasei* DNA

Lane 2 Undigested *Lb. paracasei* DNA

Lane 3 *Lb. paracasei* DNA digested with 0.012 units of *Sau*3A I /µg DNA

Lane 4 SPP EcoRI marker (Progen)

SPP1/ EcoRI Fragment sizes in base pairs: 8,500, 7,350, 6,100, 4,840, 3,590, 2,810, 1,950

Over one thousand clones were screened and 19 positive *E. coli* transformants were detected. Preparations of plasmid DNA were made from these transformants as described in section 2.5.1. Size estimation of the plasmid by comparison with standards electrophoresed in 1% agarose indicated that only 5 clones had inserts over 300 bp. The inserts from the plasmids of these 5 clones were then sequenced, however, they failed to yield any significant matches when submitted to a BLASTn search. It was decided at that point to use PCR-based techniques to identify the gene associated with the sequenced amino acids, particularly as the draft genome of *Lb casei* strain ATCC 334 had recently been released to allow this approach.

3.6.4 Investigation of the *Lb. casei* ATCC 393 draft genome sequence for 42 kDa acid stress protein N-terminal sequence

The N-terminal amino acid sequence of the 42 kDa acid stress protein was submitted to the Oak Ridge National Laboratory (ORNL) internet site http://genome.ornl.gov/, see sections 2.6.15 and 2.6.16. A BLASTp search of the *Lactobacillus casei* draft genome (version '20sept02') was performed using that sequence data. As previously stated a sequence match with 100% homology was found between positions 9560 and 9501 in scaffold 3 of the draft genome. Scaffold 3 was one of 276 scaffolds in the draft genome and was 82,578 bp in length. Refer to Appendix 4 for a copy of the ORNL microbial BLAST results. At the same time, all known completed microbial genomes and the draft genomes of *Lb. bulgaricus*, *Lb. gasseri*, *Lc. lactis*, *Leuconostoc mesenteroides* and *B. longum* were searched for a match to the same N-terminal sequence but without further success.

The sequence information in Scaffold 3 was transferred to a file in ANGIS for further study. A six frame translation was performed to find and translate open reading frames (ORFs). The ORF containing the amino acid sequence of interest was found in Frame 4 (reverse) between positions 9644 and 8457. The predicted mass, length and pI of the protein were 40.802 kDa, 396 amino acids and 7.66 respectively. This information was obtained using the ANGIS Translate program.

An SPScan was used to scan the ORF for the presence of secretory signal peptides as the N-terminal sequence of the 42 kDa protein was not found at the start of the ORF. A cleavage site was predicated 28 amino acids from the start of the gene. This information is shown in Figure 3.28. The predicted mass of the cleavage product was 3.055 kDa. The predicted mass of the protein in *Lb. casei* ATCC 393 after cleavage was 37.979 kDa.

3.6.5 PCR based approach to identification of the 45 kDa protein

Based on the *Lb. casei* draft genome and using sequence information in the ORF containing the gene coding the 42 kDa protein of interest, three sets of PCR primers were designed to test for the presence of the gene in *Lb. paracasei* strain VUP 12006. The composition of the oligonucleotide primers used in this round of PCR is described in Table 3.10.

Table 3.10 PCR primers used in round one of gene identification.

Name	Sequence	Melting temperature
SP1S	AGATAGCCGAACACGATGCC	66.2
SP1AS	CACGACGACAGCAAAGAAAAC	64.6
SP2S	AACACAATGCTCTCACGAC	58.8
SP2AS	ACGACGACAGCAAAGAAAAC	61.8
SP3S	ATGGTGCGGGCAATTAAG	63.4
SP3AS	TGAAGATGCGAACACAGAAG	61.6

Figure 3.29 shows the location of these and subsequent primers based on sequence information from *Lb. casei* ATCC 334 between positions 7621 and 10381 of Scaffold 3 of the draft genome. The PCR reaction mixture (50μL) contained 30 pmol of each primer, 200μM each of dATP, dTTP, dCTP and dGTP, approximately 100ng of bacterial DNA, 2.5 U of *Taq* DNA polymerase (Sigma) and PCR buffer with 1.5 mM $MgCl_2$. DNA was amplified as follows: initial denaturation at 95°C for 2 minutes, followed by 30 cycles consisting of denaturation at 94°C for 30 seconds, annealing at 63°C for 30 seconds, extension at 68°C for 2 minutes and a 5 minute final extension step at 68°C.

```
SPScan of Scaffold_3.1

 Weight matrix: GenRunData:spgpos.dat

 Minimum score for SPs (threshold): 7.0

 Predicted cleavage sites indicated by '^'.

> sequence: Scaffold_3.

   name: Scaffold_3.  check: 7395  from: 1  to: 396

 1. 1 MKFNKVMITLVAAVTLAGSASAVTPVFA^DT 30

   Score: 11.5

   Probability: 3.137E-03

   SP length: 28

   McGeoch scan succeeded:

         Charged-region statistics:

               Length: 5   Charge: 2

               Hydrophobic-region statistics:

        Length: 8   Offset: 6   Total hydropathy: 57.4

               Maximum 8-residue hydropathy: 57.4, starting at 7
```

Figure 3.28 SPScan of Scaffold 3 of the *Lb. casei* draft genome.

Using sequence information obtained from Scaffold 3 of the *Lb. casei* draft genome an SPScan was performed. SPScan was used to determine the presence or absence of an amino acid signal peptide in the ORF containing the amino acid sequence of the 42 kDa acid stress protein. Information in this figure was copied from ANGIS Biomanager results file. The program detected a probable signal sequence of 28 amino acids.

7621 CGTCATTTCA TCGGTGACTG CTTCTACGTT CATGTCTGCT GGCTCCCGCG ATGGTTGCAA

7681 GCAGAAGCAG GACTGCCGCC GCCAAAGCAA ACCAAGTCCA ACCGGCAATG GGAACATGAG

7741 CATCTGCCAA ATAGCCAAAG CCATACATGA TAACAAGGTG GCCGCCAGCA AGGACTAGTG

7801 CAGTGAACGT CATGCTAAGG CGCTGCCAGA TTGTGACGGC GTTGGTGAAC CAAGCCGTGG

7861 CCATGACTAG TAGCTGTGCC AGCAGCAACA AAGCCATGAG TAGCTGTAAA ATTGTGTTGA

7921 GATACACCAT TTTATTGGGC TCCTTTAAAC GTGCTGTAAA GGACGAATCA ATTTTGCATT

7981 GTTGTACACG TATCATACCA ACAAAAATTC AGACTTGATA GTGTTATGCT GCGTCTCAGT

8041 T[illegible] AAGGTCG TTTGGTGAAA AGCCGATATC GATTCCGTGT

8101 TGGGTATCAT AACTGGTGAC AATTGCGATT CCTGTCATGT CGCGTTTGAT TGCTACCGGG

8161 GCGTGTCTCG AACAAGCGAA ATAGTTGCTG ATAAACCTCC TTAAGTGCCA AAAAAAGAAG

8221 CCACAAGGAA TGCGATCGAA CATTCTTTGT GACTTCTTTT AGATAACTCG GTAGTTTTTA

8281 AGAAAGAGCG TGAGCCAGAC CGGTTAGAAA CCGGAGTGTA AGTGGGCCTG AACGTGATGA

8341 CCGGGCTTTG GTCATTGCGT TCAGGGTCCT TACACGCAGG GTTCTGGTCT GGCGAACGCG

8401 TTTA[illegible] CCCAAG ACGTGATTAA GGGTAGGTAA AAATTACCGG

8461 TGGATATAAA CGTAGCTGCT TGCACC[illegible] CGGCC GGTTGGTGAG

8521 CTGAAGCCCA TGCCGCCTTG GCTGATCGTG ACACTGTCAC CGGAGACAGA TTGGACATAA

8581 GCAACGTGAC CATATGAACC ATCGGCTGTC CATTGACCGC CAACGGATTG ACCAGCGGCG

8641 AAGACGATGA TTGAGCCGGC TGCTGGCGTG TGGTTAACAG TGAAGCCAGC TGCTGCGGCT

8701 GAACCGCCCC ATTGTGCGCC GTTGCCCCAG CCATTACCAG CCCATGGTGC AACTGATTTG

8761 ACGTACCAGG TGCACTGACC CCAAGGATAG GTGTTACCTG AACCACTGAA GTCAGCGTGG

8821 CTGCCACTGC CTGAAG[illegible] ATGGTG GC GCTGCTTGAA

8881 CTGCTGGTAG TAGAAGAACT TGCGGTTGAA GCGGCAACGG TCTTCAAAGC GGCTTGGGTT

8941 GCAGCAGCTG CTTCAGACTG TTCTTGGGCA AATTGGCTTT GCAAAGCAAC CAACTCATCT

9001 TTATGATCAG CAATTTGTTT ATTCAAAGCA TCTTGTTTAT CGTTAGCATC TTTTTGTAGG

9061 GAAACCAACT GTGATTTCTG GGTTTCCAAT GAAGCCTTAG TTGAAACCAG CGTCTGACGG

9121 [illegible] CAAGCCAGCT AATTTATCCT TGGCAACTGT CACGGCATCA

9181 AGCGCATCTT TGCTGGCTTG GCTAAGTTTG CCAACCGTCA TGGTGCGGGC AATTAAGTCA

9241 GACAAGTTCT GGGAATTCAG AACAAAGTCG ATATAGACAT TGCCGCT[redacted]

9301 [illegible]CT GCAAGGAGAT CAACTGATCT TTCAAGTTGT TCTTGCGTGC TGTGACATTC

9361 TTTTGAGCGG CAACAATTTC ACCGCTGAGT GATGAAATCT TGGCATCAGT TGCGCTGATC

9421 TTAGCCGTTG CATCGCTGAT TTGACCATTC TTGGTATCGA TCTGCTTGTT CAAATTGATA

9481 A[illegible] ATTTGCTTC AACAGTGCAT TGGTTTCGCT TTTGTTGGAA

9541 GCGATACTGT CGCTTGTATC AGCGAAAACT GGTGTTACGG CGCTAGCAGA ACCTGCTAAG

9601 GTAACTGCAG CAACCAACGT GATCATGACT TTATTGAATT TCATTTTTGA CCTCACCCTT

9661 AATTTGATTA ACTAAAGGCT ACTTTACTGT GTCAATGTTA CGTCAGCGTT ACAGGACTGT

9721 TAGGAGTTGG TCTCAATCTT CATTTGCGCT TCAAAATTGC GATCATGAAC GTTTTTAATC

9781 CCAATTTTTG ATGAATTTTG TATGAAAAAA GCAGCGCCGT GACGACGCTG CCTGACAAAA

9841 TTGTAGGTTA TTCGTTTGAT TTCAGGGCTT CGACCATGTC CACCCGTTGG AGTCGACGAT

9901 GCGTGAGCCA GGTGACGACG CCGGTGAAGG CCAACATGAG TAACGTGGCC GTGAGGTAGC

9961 CGACAATGCT GATCGTGAGT GGAAAAACCA CGGCCTCAGT TTCGGCTTGA TACAAAATGT

10021 AGGCTGTCAG CAAATTGCCG AGCAGATAGC CGAACACGAT GCCGATCACT GTCAACACAA

10081 TGCTCTCACG ACTGATGTAC ATGGTGACCT CGCGATCGAA AAAGCCAAGC ACCTTGATTG

10141 TGGATAGTTC CCGAATGCGT TCCGAAACGT TGATGTTATT CAGATTGTAC AAGACCACGA

10201 ATGACAAAAC CGCACTGAGC AGAATAAAAA TCAAAACAAT AGGATCTAAC ATACCGCTCA

10261 TATTGCTTAA CGTTTTGTTT GCTCTGGTGG TGAAACTAAT CCCGACAATC GCGTGATGAT

10321 TGAGCAATTG CTTAGCGAGT CGATCATTTT GTTTGCTGCT TTGGGGCTGC AAGCGGACTA

Figure 3.29 *Lb. casei* strain ATCC 334 sequence data obtained from Scaffold 3 of draft genome version '20sept02' showing location of PCR primers.

Lb. casei strain ATCC 334 sequence data is shown between positions 7621 and 10381 of Scaffold 3 of the draft genome. PCR primers used to amplify DNA in this region are shown in this figure using colour coding. In general, the font is coloured for sense primers and antisense primers are highlighted. Primers were designed using PrimeGCG (ANGIS).

Name of primer	Identification	Name	Identification
SP1S	SP1S	2SP4S	
SP1AS	SP1AS	2SP4AS	2SP4AS
SP2S	SP2S	2SP5S	
SP2AS	SP2AS	2SP5AS	2SP5AS
SP3S	SP3S	3SP1S	3SP1S
SP3AS	SP3AS	3SP2S	3SP2S
2SP1S	2SP1S	3SP3S	3SP3S
2SP1AS		3SP1AS	3SP1AS
2SP2AS	2SP2AS	3SP4S	3SP4S
2SP3S	2SP3S	3SP2AS	3SP2AS

The results are shown in Figure 3.30. Genomic DNA from strain VUP 12006 amplified using SP1S/SP1AS, SP2S/SP2AS and SP3S/SP3AS were expected to yield PCR products of approximately 248, 217 and 281 bp respectively. As seen in Figure 3.30, the sizes of PCR fragments obtained were comparable to the sizes predicted from the *Lb. casei* genome. Sequence of the PCR products, in duplicate, was determined using a CEQ 2000 (Beckman Coulter) according to the manufacturer's instructions.

The sequence data for each fragment showed 100% homology to sequence found in the *Lb. casei* ATCC 334 ORF. Based on these results, further primer pairs were designed based on the sequence data available for *Lb. casei* ATCC 334 between positions 8401 and 10381 of scaffold 3. The composition of these oligonucleotide primers is described in Table 3.11.

Table 3.11 PCR primers used in round 2 of gene identification

Name	Sequence	Melting temperature
2SP1S	GTCCTTACACGCAGGGTTCT	63.0
2SP1AS	CGAACACCCAAACCATCTCT	63.8
2SP2AS	CGGCTCAATCATCGTCTTC	63.4
2SP3S	CCAGAGATGGTTTGGGTGTT	63.7
2SP4S	CGATGGTGCTGGTTGAAGT	63.8
2SP4AS	TGAAGACGGAACAGGAAAATG	63.8
2SP5S	GCATTTTCCTGTTCCGTCTT	62.9
2SP5AS	AAAAGGCTGGCAACTCTGTC	63.3

The PCR reaction mixture was identical to that used for round one of PCR. The expected sizes of PCR products and those determined from the agarose gel are shown in Table 3.12. The results of the second round of PCR are shown in Figure 3.30. A slight increase in the observed size of some product pairs compared with expected sizes was observed for some primer pairs.

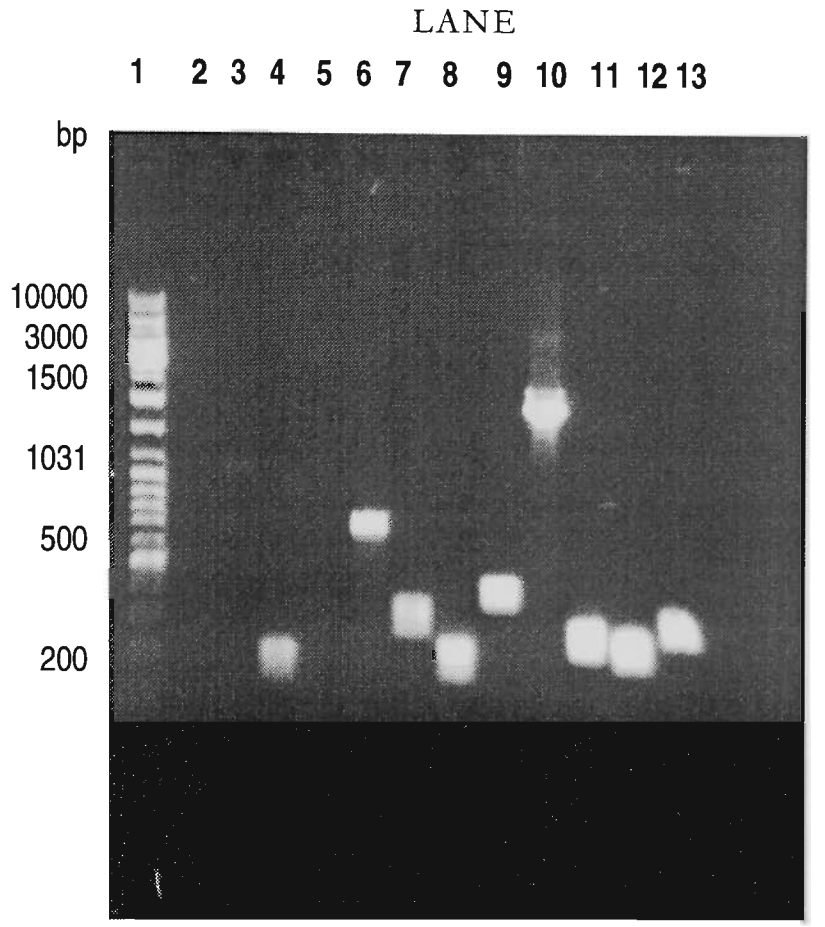


Figure 3.30 PCR amplification of DNA from *Lactobacillus* strain VUP 12006 using primers designed from *Lb. casei* ATCC 334 draft genome.

Lane 1 is marker (generuler, MBI Fermentas). Lanes 2-4 are PCR amplification products of DNA using primers 2SP1S/2SP1AS {141} (Lane 2), 2SP1S/2SP2AS {294} (Lane 3), 2SP3S/2SP2AS {172} (Lane 4), No sample (Lane 5), 2SP3S/2SP4AS {656} (Lane 6), 2SP4S/2SP4AS {294} (Lane 7), 2SP5S/2SP5AS {186} (Lane 8), 2SP5S/1SP3AS {331} (Lane 9), SP3S/SP2AS {1074} (Lane 10), SP1S/SP1AS {248} (Lane 11), SP2S/SP2AS {217} (Lane 12) and SP3S/SP3AS {281} (Lane 13). Expected product sizes, based on *Lb. casei* ATCC 334 draft genome, are shown in brackets {..} after each of the primer pairs.

Table 3.12 Expected and observed PCR product sizes from round two of gene identification.

Primer pair	PCR product size estimated from gel	Expected size based on *Lb. casei* draft genome
2SP1S/2SP1AS	No product	141 bp
2SP1S/2SP2AS	No product	292 bp
2SP3S/2SP2AS	≈170	172 bp
2SP3S/2SP4AS	≈700	656 bp
2SP4S/2SP4AS	≈300	294 bp
2SP5S/2SP5AS	≈190	186 bp
2SP5S/1SP3AS	≈350 bp	331 bp
2SP3S/1SP2AS	≈1200 bp	1074 bp

Genomic DNA from strain VUP 12006 amplified using combinations of primers pairs were expected to yield the following size fragments; it should be noted that some primers were used in more than one pair. It can be seen in Figure 3.30 that no products were obtained from either 2SP1S/2SP1AS or 2SP1S/2SP2AS. Sequence of the PCR products was performed at least in duplicate, from primer pairs 2SP3S/2SP2AS, 2SP3S/2SP4AS, 2SP4S/2SP4AS, 2SP3S/1SP2AS and 2SP5S/2SP5AS.

The sequence data, based on both forward and reverse primers, was aligned using the sequence consensus program in ANGIS. No stop codon was found in the sequence data obtained from *Lb. paracasei* strain VUP 12006, indicating that the gene had not yet been full sequenced: however, some differences were noted between the sequence data for two strains, notably an area of serine repeats (Figure 3.31), and further attempts were made to obtain the complete sequence of the gene. Initially anchored PCR was used in an attempt to extend known sequence data but this proved to be unsuccessful. A later trial of PCR, using primers designed from sequence data well down stream of the *Lb. casei* gene, proved more productive and the gene sequence was able to be determined. These results are presented in the following sections.

3.6.6 Anchored PCR

A variation of PCR, called anchored PCR, was used to obtain the remaining gene sequence. As sufficient sequence was only available to make a single primer, a known sequence was added to the end of the DNA by ligation of a known piece of DNA. The PCR was then performed with the gene-specific primer and the anchor primer. Briefly, DNA from *Lb. paracasei* strain VUP12006 was separately digested using the restriction enzymes EcoRI and PstI (Geneworks). EcoRI or PstI digested DNA (8ng) was subsequently ligated to pGEM-Teasy vector (50ng) using T4 DNA ligase. Using the primer combinations 2SP2AS/M13F and 2SP2AS/M13R several attempts were made to amplify DNA fragments. M13F and M13R were primers designed to amplify DNA from the pGEM-Teasy vector (Promega). The cornposition of the oligonucleotide primers used for anchored PCR is shown in Table 3.13.

Table 3.13 PCR primers used for anchored PCR

Name	Sequence	Melting temperature
pUC/M13-F	CGCCAGGGTTTTCCCAGTCACGAC	72.5
pUC/M13-R	TCACACAGGAAACAGCTATGAC	55.2

Although several fragments were obtained using this method, the size of the fragments was too small (<800 bp), given that the vector was included in the product. Due to the small size of the fragments, subsequent sequencing consistently yielded only sequence data that had previously been obtained and use of this method was discontinued.

3.6.7 Downstream PCR

Further primers were designed, based on the region 200-1500 bp downstream of the gene of interest in *Lb. casei* ATCC 334. The composition of these oligonucleotide primers is shown in Table 3.14.

Table 3.14 Composition of PCR primers used for third round of PCR

Name	Sequence	Melting temperature
3SP1S	AGACAGCAGCAGAGATAAACAAG	52.9
3SP2S	ACAGACAGCAGCAGAGATAAAC	52.6
3SP3S	TGCCAAAAAAAAGAAGCCACAAG	52.9
3SP4S	GCCAAATAGCCAAAGCCATAC	52.1
3SP1AS	CACCATCGCCAACAACACAG	54.4
3SP2AS	ATCACATCACGATCCCAACC	51.9

The PCR reaction mixture and amplification steps were identical to that previously described for the first two rounds of PCR. Genomic DNA from strain *Lb. casei* ATCC 334 amplified using combinations of primer pairs were expected to yield the size fragments described in Table 3.15.

Table 3.15 Expected size of PCR products

Primer pair	Expected size based on *Lb. casei* draft genome
3SP1S/3SP1AS	792 bp
3SP2S/3SP1AS	794 bp
3SP3S/3SP1AS	630 bp
3SP4S/3SP1AS	1090 bp
3SP1S/3SP2AS	360 bp
3SP2S/3SP2AS	362 bp
3SP3S/3SP2AS	198 bp
3SP4S/3SP2AS	658 bp

Results for *Lb. casei* ATCC 334 matched the expected product sizes, however the results for strain VUP 12006 showed that, of the eight primer pairs used, only four PCR amplification products were obtained from three primer pairs and these varied in size from the fragments obtained from *Lb. casei*. The results are shown in Figure 3.31. This data suggests that the area outside the gene varies between the species.

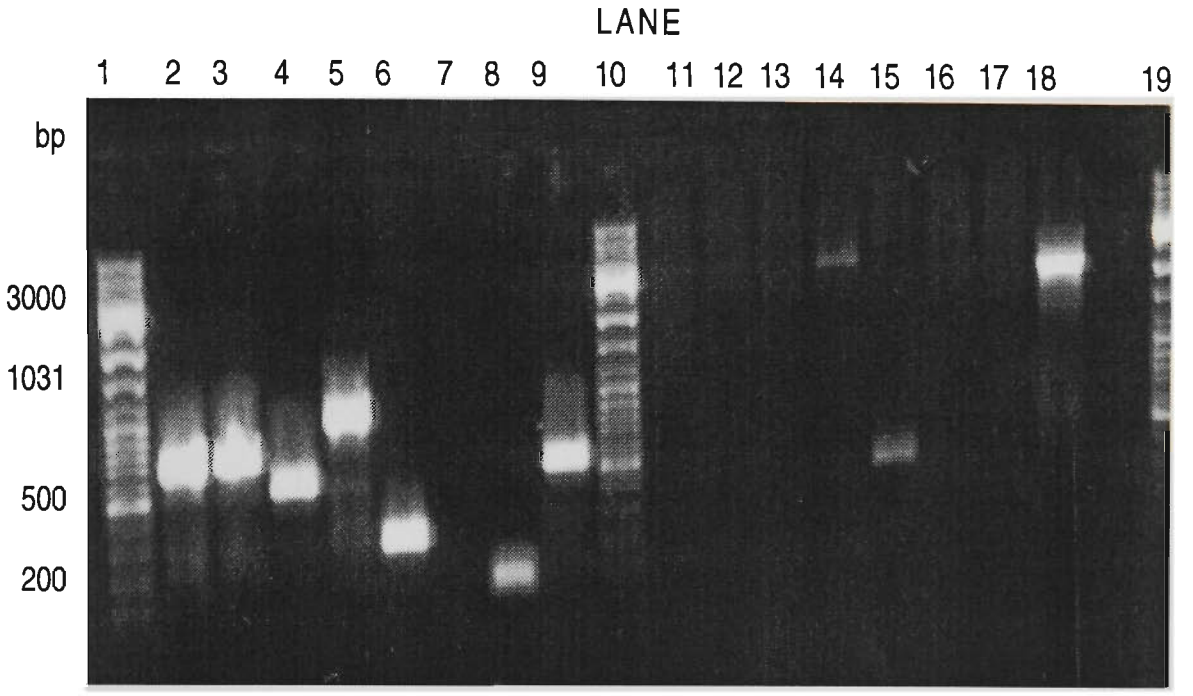


Figure 3.31 PCR amplification of DNA from *Lactobacillus* strain VUP 12006 using primers designed from Lb. casei ATCC 334 draft genome.

Lanes 1, 10 and 19 are marker (generuler, MBI Fermentas). Lanes 2-9 are PCR amplification products of *Lb. casei* strain GCRL-163 DNA, using primers 3SP1S/3SP1AS {792} (Lane 2), 3SP2S/3SP1AS {794} (Lane 3), 3SP3S/3SP1AS {630} (Lane 4), 3SP4S/3SP1AS {1090} (Lane 5), 3SP1S/3SP2AS {360} (Lane 6), 3SP2S/3SP2AS {362} (Lane 7), 3SP3S/3SP2AS {198} (Lane 8), 3SP4S/3SP2AS {658} (Lane 9). Expected product sizes, based on *Lb. casei* ATCC 334 draft genome, are shown in brackets {..} after the primer pairs. Lanes 11-18 are PCR amplification products of *Lb. paracasei* strain VUP 12006 using the same primer pairs.

Subsequently, the products obtained from strain VUP 12006 from those primers, were sequenced in duplicate. After a BLASTn search of the *Lb. casei* draft genome, sequence data from two PCR products, obtained from primer pairs 3SP4S/3SP1AS and 3SP4S/3SP2AS, were found to align with sequence from scaffold 3. The estimated size of these fragments, based on comparison to a generuler size marker (Fermentas), was approximately 3000 and 2500 bp.

A consensus sequence based on the alignment of the sequences obtained for these fragments and for the previous two rounds of PCR is shown in Figure 3.32. Consensus sequence data was obtained for each primer pair, individually for both foward and reverse primers, using the multiple alignment program ClustalW and CAP contig, a contig assembly program based on sensitive detection of fragment overlaps (BioEDIT). This process was repeated using the consensus sequence for each individual primer pair to build an overall consensus sequence.

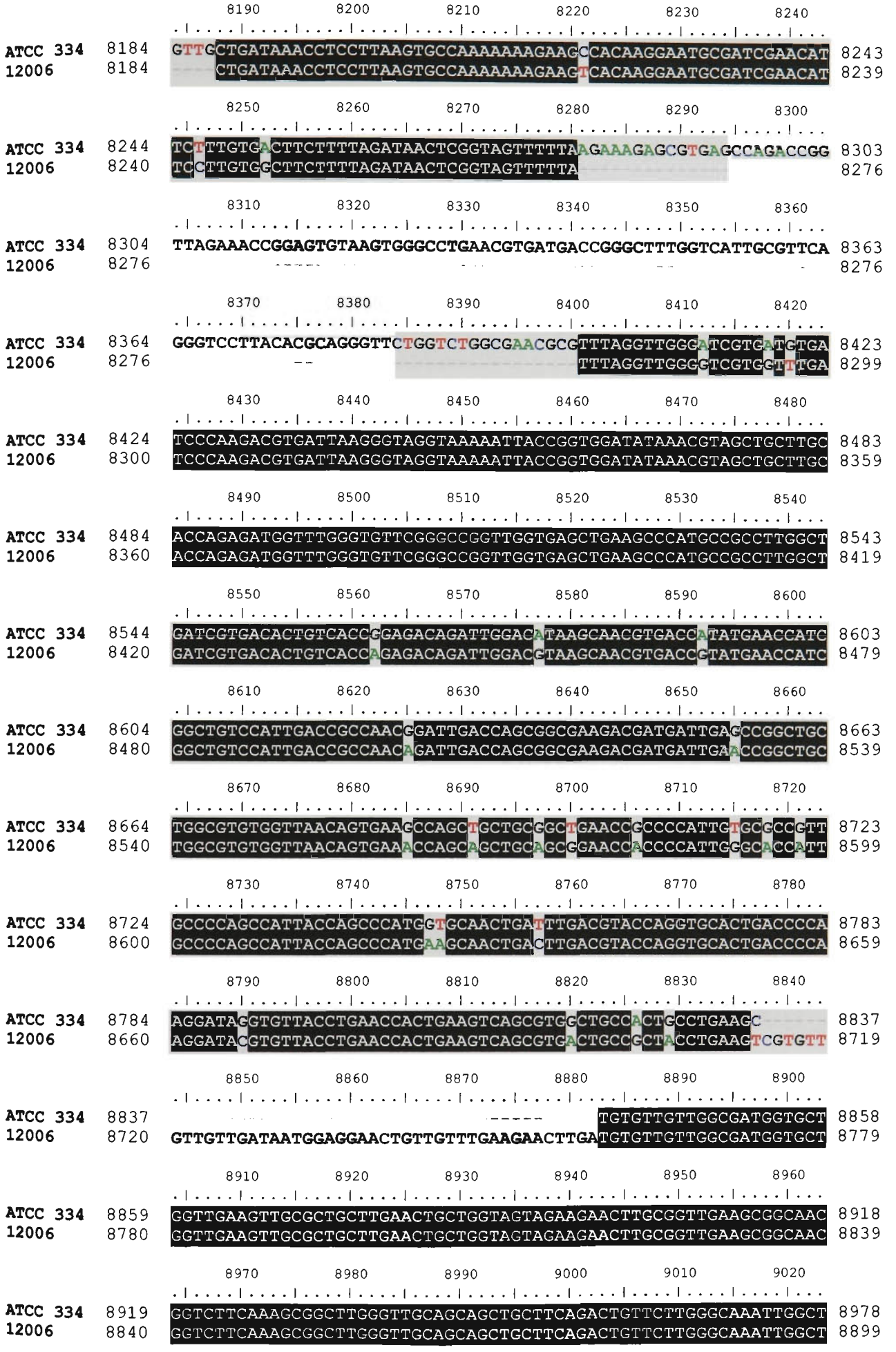
3.6.8 Submission of sequence data to ANGIS for analysis

The consensus sequence data obtained from the three rounds of PCR of interest was submitted to ANGIS to determine the presence of an ORF, to establish the amino acid translation sequence and make-up of the gene within the ORF and to detect the presence of signal peptides. Nucleotide and function similarities to known genes and a ClustalW alignment of the ORFs of *Lb. paracasei* VUP 12006 and *Lb. casei* 334 was also performed as well as an alignment to functionally similar proteins. Hydrophobicity data on the protein was also obtained using a Kyte and Doolittle plot of hydrophobicity .

Flip ORF, accessed through ANGIS, was used to find and translate in all six frames an open reading frame in the consensus nucleotide sequence of *Lb. paracasei* strain VUP 12006 shown in Figure 3.33. Nineteen ORFs were located in the submitted sequence but only one had a length greater than 300 bases and this was 1263 nucleotides in length and encoded a protein of 420 amino acid residues.

Figure 3.32 Consensus sequence of strain VUP 12006.

Sequence data based on PCR products derived from eleven primer pairs, was aligned against *Lb. casei* ATCC 334. Alignment is shown between positions 8184 and 10292 of scaffold 3 in the *Lb. casei* ATCC 334 draft genome. Two non-homologous regions can be seen between positions 8281 and 8404, and 8837 and 8884. Black boxes indicate areas of homology. Nucleotide bases are coloured either black (G), red (T), green (A) or blue (C).


8190 8200 8210 8220 8230 8240
ATCC 334 8184 GTTGCTGATAAACCTCCTTAAGTGCCAAAAAAAGAAGCCACAAGGAATGCGATCGAACAT 8243
12006 8184 CTGATAAACCTCCTTAAGTGCCAAAAAAAGAAGTCACAAGGAATGCGATCGAACAT 8239
8250 8260 8270 8280 8290 8300
ATCC 334 8244 TCTTTGTGACTTCTTTTAGATAACTCGGTAGTTTTTAAGAAAGAGCGTGAGCCAGACCGG 8303
12006 8240 TCCTTGTGGCTTCTTTTAGATAACTCGGTAGTTTTTA 8276
8310 8320 8330 8340 8350 8360
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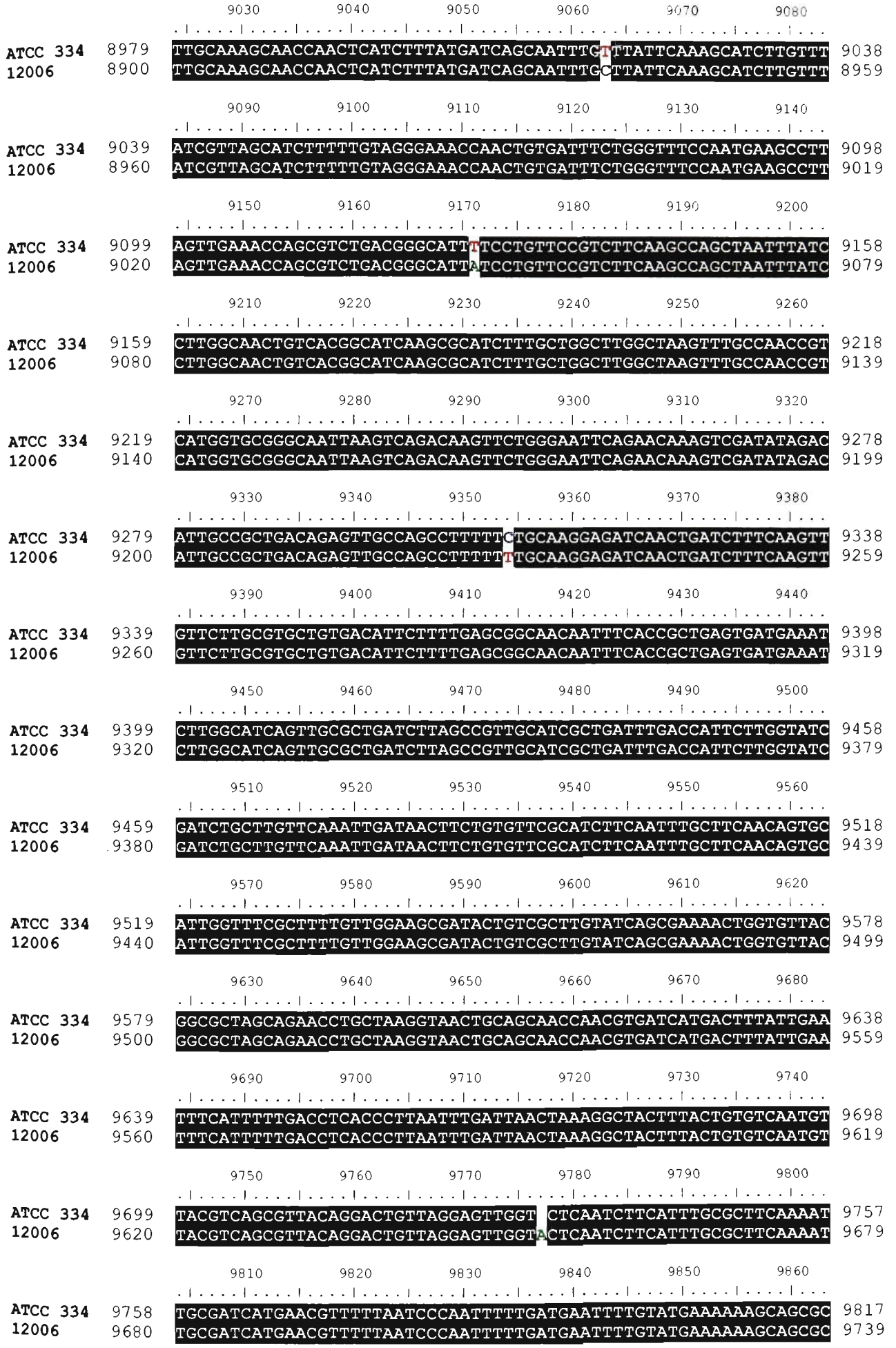
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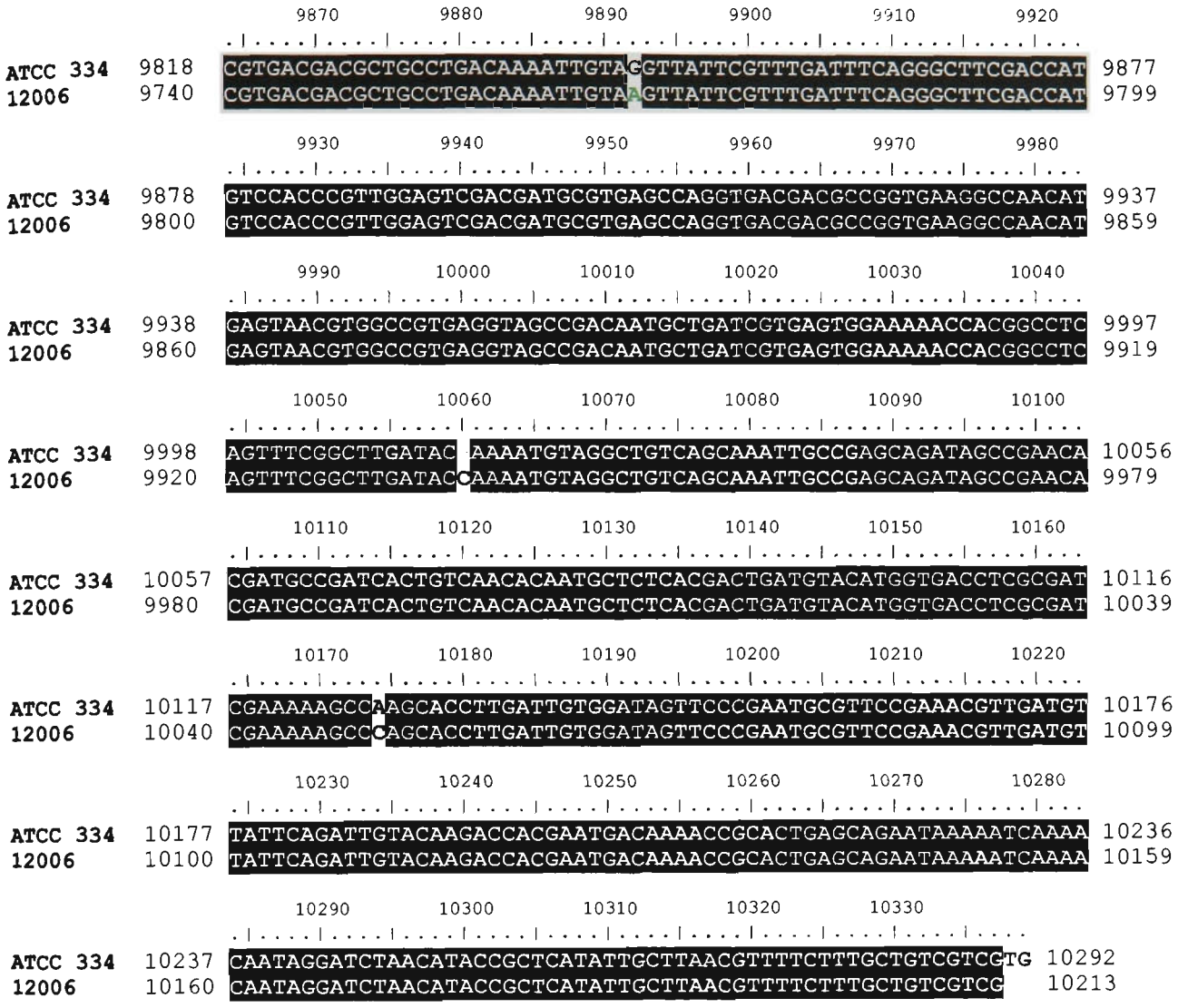
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 961  TCAGTTGCTTCATGGGCTGGTAATGGCTGGGGCAATGGTGCCCAATGGGGTAGTTCCGCT
1021  GCAGCTGCTGGTTTCACAGTCAACCACACGCCAGCAGCCGGTTCAATCATCGTCTTCGCC
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1141  GTCCAATCTGTCTCTGGTGACAGTGTCACGATCAGCCAAGGCGGCATGGGCTTCAGCTCA
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1202  TAA

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 421  *
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Figure 3.33 Nucleotide sequence (above) and amino acid sequence (below) from the ORF determined from the consensus nucleotide sequence of *Lb. paracasei* strain VUP 12006 coding for AphA.

Flip ORF (ANGIS) was used to find and translate an open reading frame in the consensus nucleotide sequence of *Lb. paracasei* strain VUP 12006. AphA is located between positions 9643 and 8437 relative to scaffold 3 of the *Lb. casei* ATCC 334 draft genome. A ribosome binding site, marked in red, was found 18 bases from the start of the ORF. The nucleotide sequence was translated into an amino acid sequence (420 aa's). The predicted secretory signal peptide is marked in blue (bold Methionine marks the start of gene) and the N-terminal amino acid sequence previously determined for this protein is marked in green. The start and stop codons are highlighted in yellow.

A ribosome binding site was found 18 bases from the start of the ORF. The first methionine residue following the ribosome binding site was determined to be the start of the gene, which will now be referred to as AphA. The amino acid sequence was translated from the nucleotide sequence of the ORF and found to be 420 amino acids in length with a mass of 43.49 kDa and pI of 9.61. This information is shown in Figure 3.33. When submitted to ANGIS for SPScan it was found that the gene had the same predicted secretory signal peptide as *Lb. casei* ATCC 334. This data was shown in Figure 3.28. The predicted cleavage site was confirmed by the N-terminal amino acid sequence obtained for VUP 12006. The mature form of the protein is about 39.34 kDa: this is slightly smaller than the size observed by 1-D SDS-PAGE. AphA was also examined for the presence of the C-terminal cell wall anchor motif LPTXTG but no such sequence could be found. The count and percentage of amino acid residues of the ORF is shown in Table 3.16. It can be seen from this data that the gene is relatively rich in serine (15.4%) and alanine (13.3%).

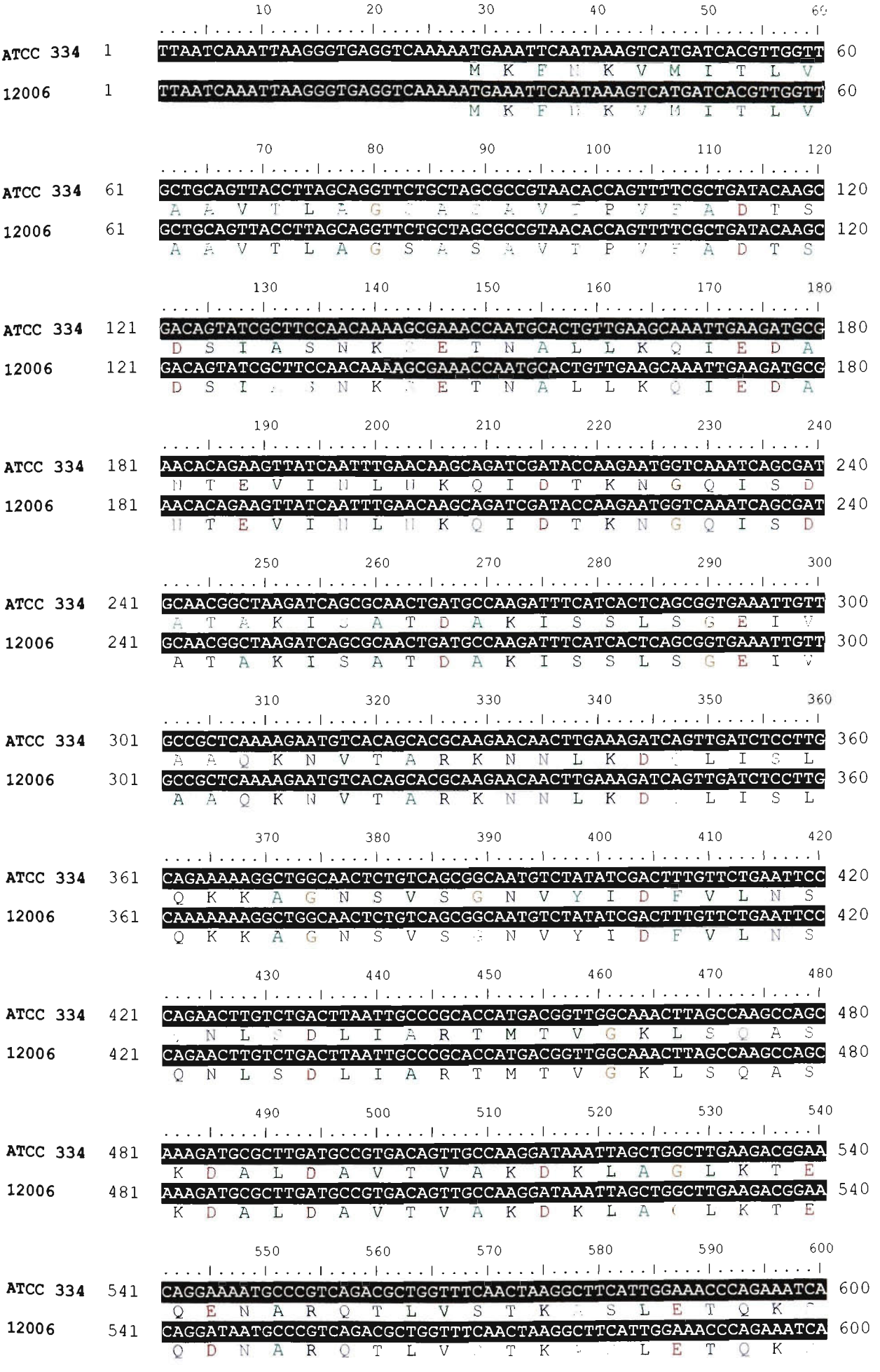
Table 3.16 Count and percentage of amino acids in ORF sequence.

residue	A	C	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y
count	56	1	21	9	7	29	5	22	28	26	4	29	5	27	6	65	37	29	6	8
%	13.3	0.23	4.98	2.13	1.66	6.88	1.18	5.22	6.65	6.17	0.95	6.88	1.18	6.41	1.42	15.43	8.78	6.88	1.42	1.9

A ClustalW alignment of the nucleotide and protein sequences comparing strains VUP 12006 and *Lb. casei* ATCC 334 is shown in Figure 3.34. A sequence difference, due to the presence of an additional 45 nucleotides (15 aa's) can be seen between positions 835-880 (positions 8884 and 8839 relative to Scaffold 3 of the *Lb. casei* ATCC 334 draft genome). This region contains two short serine repeats. There are also 29 other base variations between the two genes, of which four result in a change of amino acids and of these four, two changes result in coding for serine. To determine nucleotide and function similarities AphA was submitted to Genbank, Swissprot and spTrEMBL through ANGIS for BLASTn (nucleotide) and BLASTX (function) searches.

Figure 3.34 ClustalW alignment of the nucleotide and protein sequences comparing the AphA gene of strain VUP 12006 and sequence from scaffold 3 of *Lb. casei* ATCC 334.

Alignment of nucleotide sequence was performed using ClustalW. Shading denotes nucleotides that are identical in that position. A single letter amino acid translation is shown beneath the nucleotide sequence. A sequence difference, due to the presence of an additional 45 nucleotides (15 aa's), can be seen between positions 835-880 (positions 8884 and 8839 relative to Scaffold 3 of the *Lb. casei* ATCC 334 draft genome). This region contains two short serine repeats. An additional area of serine repeats can be seen between positions 773 and 800. The AphA gene is located between positions 9643 and 8437 relative to scaffold 3 of the *Lb. casei* ATCC 334.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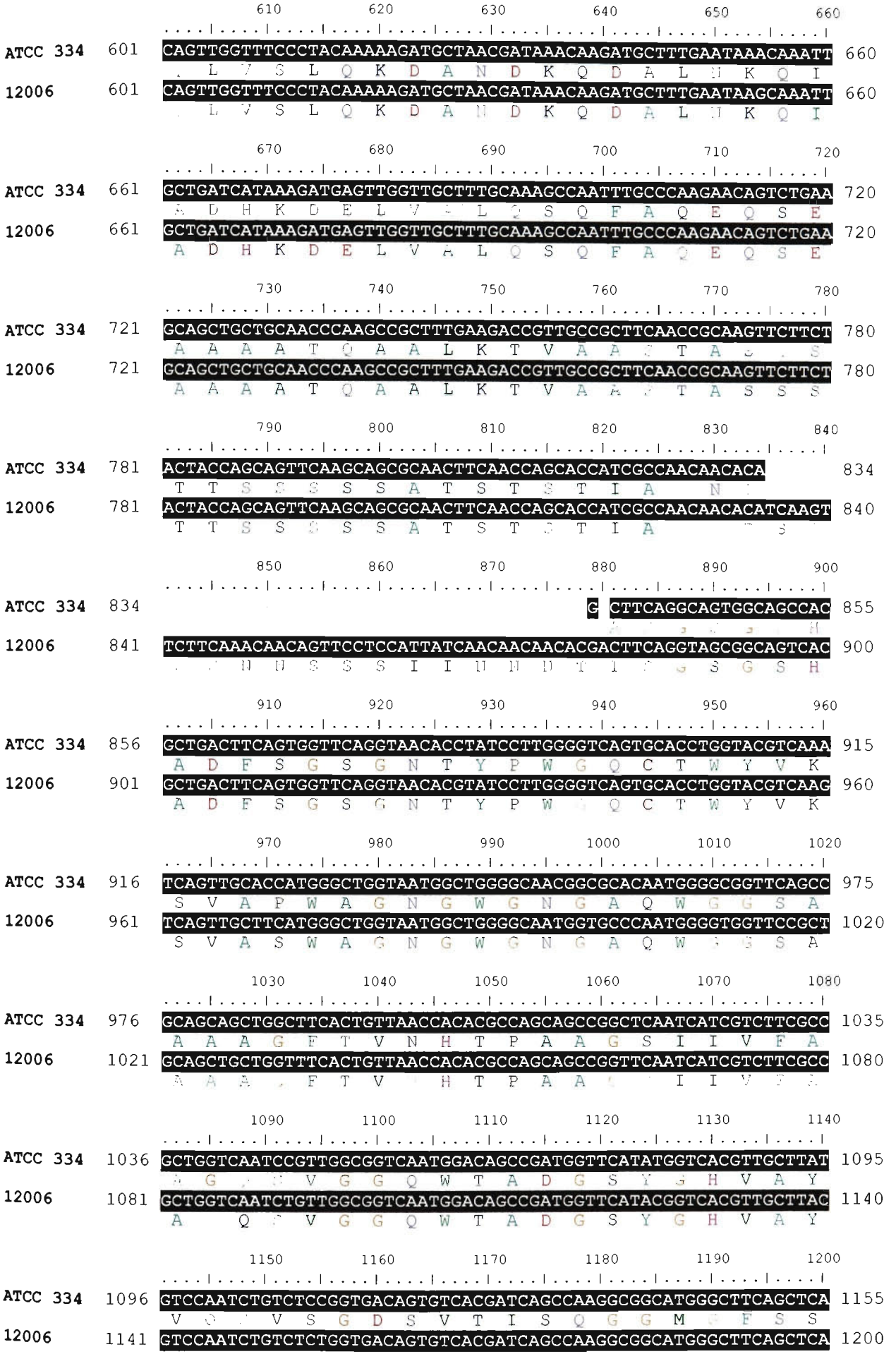
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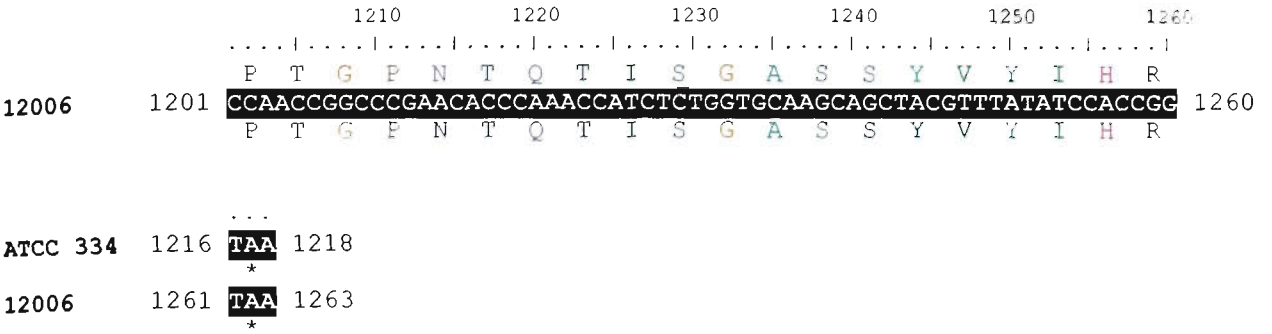
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ATCC 334 1216 TAA 1218
12006 1261 TAA 1263

The results can be seen in Appendix 5. The direct nucleotide search of completed databases was unsuccessful with only short insignificant matches of less than 30 bases. The protein search was more successful with several matching entries.

Proteins with similarity included: general stress protein GSP-781 (glucan-binding protein) from *Streptococcus mutans*, a secreted 45 kDa protein from *Streptococcus pneumoniae*, secreted antigen SagA from *Enterococcus faecium* and several other secreted proteins including an immunogenic secreted protein from *Streptococcus pyogenes*. It is interesting to note that the closest matching entries had the following lineage in common: Bacteria; Firmicutes; Lactobacillales of which the family Lactobacillales contains all members of the lactic acid bacteria.

A multiple alignment of the amino acid sequence of some of the functionally similar proteins is shown in Figures 3.35. Areas of close to 100 % homology can be seen in the multiple alignment particularly between positions 332 and 372. Some shared homology is also present in the N-terminal and C-terminal regions of the genes. Other similarities shared between these bacteria include the presence of secretory signal peptides and poly-serine domains. The signal sequence of each protein, its length and the size of the encoded proteins is shown in Table 3.17.

Table 3.17 Signal sequence for proteins determined to be functionally similar to AphA.

Name	Size of encoded protein (kDa)	Sequence	Length (in aa)
Lb. paracasei 12006	43.5	MKFNKVMITLVAAVTLAGSASAVTPVFADT	30
Lb. casei ATCC 334	40.8	MKFNKVMITLVAAVTLAGSASAVTPVFADT	30
Streptococcus mutans GSP-781	44.5	MKKRILSAVLVSGVTLSSATT	21
Strep. pyogenes	42.0	MKRILSAVLVSGVTLGAA	19
Strep. pneumonia	41.7	MKKKILASLLLSTVMVSQVAVLTTAHAET	29

Figure 3.35 Multiple alignment of the amino acid sequence of some of the functionally similar proteins to AphA gene in strain VUP 12006.

Black background denoted amino acids with 100 % homology. Similar residues are indicated by grey boxes. The hydrophobic terminus is indicated by (1). The poly-serine domain is indicated by (2). The peptide that was sequenced is indicated by (3).

Key:

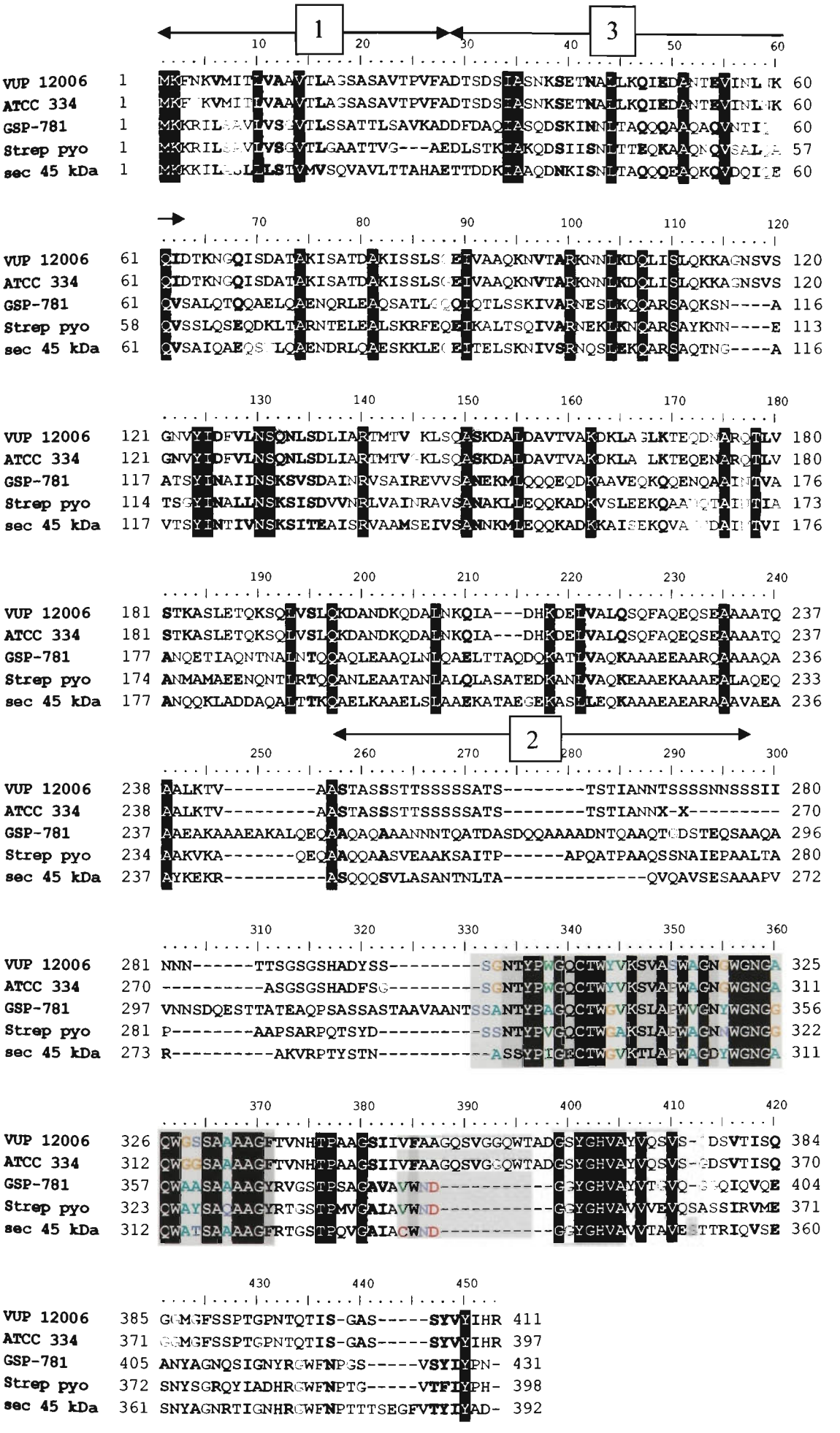
Lb. paracasei VUP12006 (VUP 12006)

Lb. casei ATCC 334 (ATCC334)

Strep. mutans (GSP-781) Swissprot Entry Q9AG98

Strep. pyogenes (Strep pyo) Swissprot Entry AE009955

Strep. pneumoniae (sec 45 kDa) Swissprot Entry AE007509


1
3
2
VUP 12006
ATCC 334
GSP-781
Strep pyo
sec 45 kDa

A Kyte and Doolittle plot of hydrophobicity is shown in Figure 3.36. A hydrophobic region, which can be seen at the start of AphA between positions 8 and 23, is consistent with the presence of a signal sequence. There is also another hydrophobic region around position 350, which may correspond to another membrane-spanning region.

3.7 Discussion

In this study, the response of *Lb. paracasei* strain VUP 12006 to environmental stress, principally heat and acid stress, has been examined. The response to acid stress has been particularly intriguing, with novel changes in protein profiles and fatty acid methyl ester profiles being observed. The results presented and their significance are discussed below.

3.7.1 Identification of test strains

This project initially planned to test the responses to an acid environment of strains of *Lb. acidophilus* principally because of the interest in this species as a probiotic supplement. Subsequently, twenty-five strains reported to be *Lb. acidophilus* were obtained from a variety of sources. However, only nineteen showed the basic colony and Gram stain morphology, which progressed these candidate strains through to the next stage of identification. Although this is a very small sample, it is interesting to note that 24% of strains from culture collections failed to match the expected colony and Gram stain morphology.

It is not known whether any of the strains had previously been tested using molecular biology techniques. However, it is known that the strains had been propagated from frozen stocks prior to receipt at the VUT laboratories and the possibility of strain contamination cannot be excluded. Such discrepancies have also been noted by Yeung (2002) who found a strain of *Streptococcus sanguis* in a commercial product labelled as containing *Lb. acidophilus*.

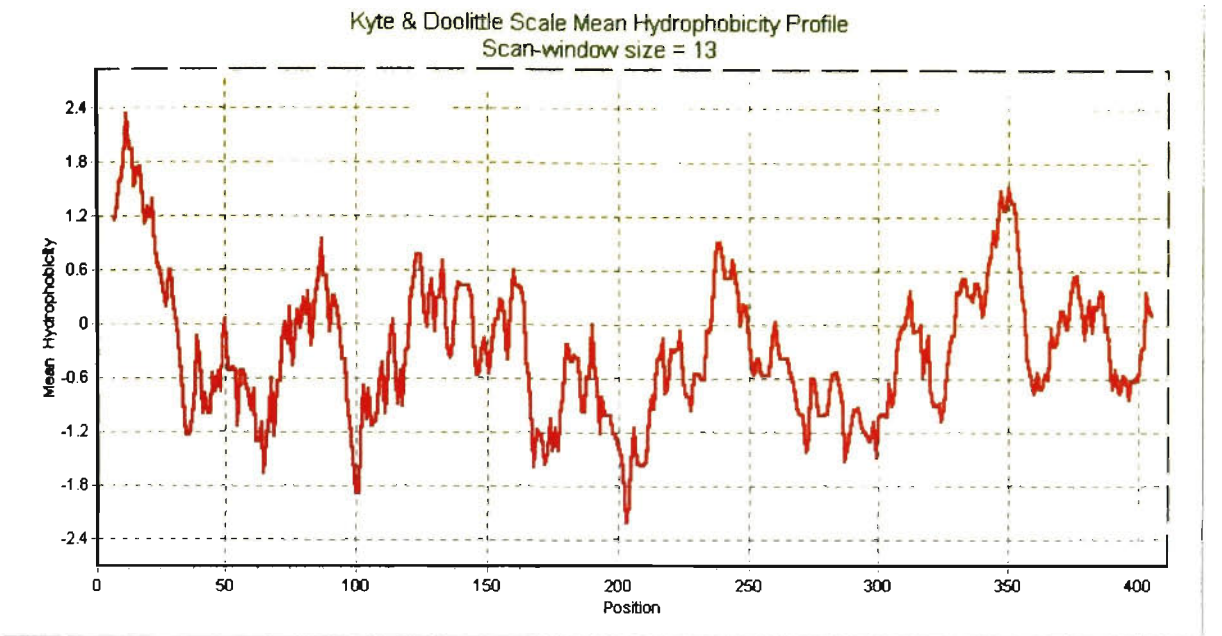


Figure 3.36 A Kyte and Doolittle plot of hydrophobicity.

A mean hydrophobicity profile was performed for AphA using the Kyte and Doolittle method using default parameters (BioEdit). Positive scores indicate a hydrophobic region. A hydrophobic region between positions 8 and 23 is consistent with the presence of a signal sequence. The poly serine area is located in a hydrophilic region between positions 249 and 279. The peak at position 350 may also correspond to possible membrane-spanning domain.

3.7.1.1 Traditional carbohydrate tests

Carbohydrate utilisation test kits are commercially available for the identification of LAB, however the cost per kit made the screening of multiple isolates prohibitive in this project. Identification of a small number of control strains was performed using lactobacilli fermentation broth, but difficulties were noted in interpretation of positive results and reproducibility was poor so it was then decided to develop a cheap and rapid method that could be the first step in identification of a variety of LAB to the species level. Three separate trials focussed on the use of minimal media using agar and liquid bases with 2% selected carbohydrate sources. In the first two trials different agar bases were tested but growth was generally very restricted in such minimal media and even when spectrophotometric assays were used to quantify changes in growth, interpretation of the results was very difficult. These trials were abandoned in favour of a method using a liquid based minimal media and an ELISA plate reader to measure changes in optical density. The advantages of this method are:

- results are reproducible and can easily be performed in duplicate on the same microtitre plate;
- the method works well on any air-tolerant bacteria;
- the volumes of broth used are very small (200µL per well), which keeps costs low;
- the plate set-up can be changed to suit the test protocol, for exarnple the plate may be set up to test fermentation of a single carbohydrate against 80 different tests or a single test against multiple carbohydrates, although control strains using this method take up a number of the test wells; and
- the results are obtained in one working day.

Traditional carbohydrate tests are no longer used as the full means of identification of LAB, but when compared with molecular biology based methods, they rnust be considered as the preferred starting point in a step-wise identification scheme because of the low cost. It has been stated by Du Plessis (1996) that differentiation and identification to species level based on biochemical and physiological tests is unreliable in *Lactobacillus* species. However, the experience of this study was that rapid carbohydrate tests in conjunction with Gram stain and growth characteristics, was the best option for first step identification when screening and comparing large numbers of isolates.

3.7.1.2 FAME fingerprints

It has been confirmed in this work that both SDS-PAGE and FAME fingerprinting require highly standardised methodology. Fingerprinting using FAME profiles has been successfully used as a way of differentiating both yeast and bacterial strains (Augustyn *et al.*, 1990, Johnsson *et al.*, 1995, Rementzis *et al.*, 1996). However, because the fatty acid composition of bacterial and yeast cells is dependent upon growth temperature, pH, growth medium and growth phase, comparison must be made using cultures grown under a rigid standard set of conditions. The standard deviation achieved in these results, <7% over 2 runs, is comparable to other studies, <5% by Blignaut, (1996), but the time and cost involved in this procedure suggest that, at least in this species, molecular based methods would be preferred as a means of strain identification, before using FAME profiles. The presence of lactobacillic acid in the profiles did, however, confirm species identification.

3.7.1.3 SDS-PAGE profiles

The same rigid culture conditions, as used in obtaining FAME profiles, are required when performing SDS-PAGE. Other studies using SDS-PAGE as a means of identification, have shown that the method is only reproducible when a standard set of whole cell proteins is used for normalisation between runs (Pot *et al.*, 1993) and these runs are required at least in triplicate.

The study by Dykes (1993) showed that triplicate readings of SDS-PAGE profiles performed on the same organism in independently run gels gave only 85-92% similarity; other authors have given figures of 85 to 93%. Furthermore, in order to handle complex identifications, such as comparisons of multiple species of LAB, computer-assisted comparison must be done using a databank of known LAB accessed using a software package such as GELCOMPAR (Pot *et al.*, 1993, Pot *et al.*, 1994). However, once a fully-standardised technique is used there appears to be good correlation with the results obtained by 16S rRNA gene analysis (Pot *et al.*, 1993).

While this work may have compared only a small number of cultures, it was found that over duplicate runs, visual comparison was sufficient to differentiate all but two of the test strains and of those two strains, one was a mutant of the other. With the exception of strain VUP 12006, that differentiation was based on changes in minor protein bands. In some regards this may suggest that, in this small sample, the major bands were good species markers. This appears to be supported with the observation of the marked differences in the major banding pattern of the strain VUP 12006. The principal difference of strain VUP 12006 to the other test strains was the presence of four highly-expressed protein bands, which although present in the other strains, were not expressed at such high levels. As over-expression of proteins may result from years of selection for flavour or functional properties in cultures used in manufacturing or industrial applications as well as species differences, this technique may not be as useful in identification of bacterial strains used by the manufacturing industry, at least when comparing profiles to known databanks. Apart from that observation, the method appears to be a reasonable second step for strain differentiation when screening large numbers of isolates in a situation similar to the type of identification process used in this project. The cost of performing these tests is not prohibitive and the time spent in extracting proteins from cultures is neither difficult nor arduous.

3.7.1.4 PCR (What's in a name?)

The identification of LAB has traditionally been performed using biochemical tests that are used to classify the organisms into one of three major fermentation groups: homofermentative lactobacilli, facultative heterofermentative lactobacilli and the obligate heterofermentative lactobacilli. These groups, however, do not reflect the phylogenetic relationship between species, either the three clusters proposed by Collins (1991) or more recently the five phylogenetic groups proposed by Schliefer and Ludwig (1995). This section will further discuss the difficulties of classification of unknown LAB and the use of molecular techniques to arrive at a valid identification.

Clearly, identification based on phylogenetic rather than phenotypic characteristics has now become a definitive means of identification, and the molecular biology based method used in this study was definitive in identifying the test strain. Nevertheless, the cost and time involved in obtaining those results make this method the third choice in the step-wise identification scheme. This method is probably best used in situations where tracking changes in the genetic make-up is important, for example strain substitutions in industrial applications. More recently, phylogenetic analysis based on partial HSP60 gene sequences has been developed (Jian *et al.*, 2001). The technique in which partial HSP60 genes are sequenced using degenerate PCR primers appears to have a higher resolving power than the 16S rRNA technique when determining differences between closely related strains. This technique may have a place in this type of study, where separation of closely related bacteria has proven difficult.

Molecular biology techniques were seen as the only way to confirm the identification of strain VUP 12006. The initial studies focussed on placing strain VUP 12006 in the correct genus. Dubernet *et al.* (2002) had recently published details of a genus-specific universal PCR primer pair, R16-1 and LbLMA1-rev. These authors had shown that PCR using the universal primers generated a single product of approximately 250 bp (exact size appeared to be related to species) and was specific for 21 *Lactobacillus* species including *Lb. acidophilus* and *Lb. casei*.

They also demonstrated that using DNA from *E. coli*, *Listeria*, *Staphylococcus* and LAB not on the list of 21 species, PCR using the same primers failed to generate any fragments. When strain VUP 12006 was tested using the universal primers a fragment of ≅250 bp was obtained, which was consistent with the strain being placed within the genus *Lactobacillus*. Interestingly, two control strains, *Lb. casei* GCRL-163 and *Lb. acidophilus* ASC 388 were run at the same time. *Lb. acidophilus* ASC 388 yielded a product of slightly larger than both strain VUP 12006 and *Lb. casei* GCRL-163. This variation in fragment size and slight differences in FAME data were the first indications that strain VUP 12006, was not an *acidophilus* strain.

In order to speciate strain VUP 12006 it was decided to perform a partial 16S rDNA sequence and compare the results to sequence data in Genbank. Although several potential PCR primer pairs have been published, it was decided to use PAF and 536R. PAF and 536R, which had been shown to identify a range of LAB, yielded products of 500 bp. These products were sequenced and the sequence data submitted for a BLASTn search.

It was found that the initial BLASTn search of sequence based on data from the forward primer identified strain VUP 12006 as *Lb. paracasei* while the data from the reverse primer most closely matched *Lb. casei* strain KH-1. Similar results were also obtained for *Lb. casei* ATCC 334 from the draft genome, in that the 16S sequence appeared to most closely match *Lb. paracasei.* The control strain of *Lb. casei* GCRL-163 sequenced at the same time yielded similarly unusual results while the *Lb. acidophilus* sequenced as expected and was most closely related to *Lb. acidophilus.*

This apparent contradiction in identification of the casei strains was made slightly clearer after examination of the alignment of strain VUP 12006 in the V1 region of the 16S rRNA gene with sequence data from type strains of both *Lb. casei* and *Lb. paracasei.* The sequence obtained from these variable regions had been shown by Ward and Timmins (1999) to differentiate

members of the *casei* complex. Examination of this region demonstrated that strain VUP 12006 was an exact match to *Lb. paracasei*. It should be noted that *Lb. casei* ATCC 334 and GCRL-163 showed similar sequence data in this region and it cannot easily be explained as to why they are not identified as *Lb. paracasei* as they differ in sequence to the type strain *Lb. casei* ATCC 393. Given these differences there is certainly further scope for study into the identification protocol for the *casei* group. Future work may well concentrate on strain classification based on other differences between the strains, particularly on genes other than the 16S rRNA gene and may well include the gene for HSP60 or similarly conserved genes.

3.7.1.5 Summary of identification techniques

In summary, as expected, carbohydrate utilisation studies were unable to speciate strain VUP 12006 and only minor differences were seen between the strains tested. Although sorbitol fermentation was negative, strain VUP 12006 had the typical fermentation pattern of *Lb. acidophilus*. It should also be pointed out that the range of carbohydrate tests was probably not extensive enough to differentiate the species being tested. FAME and SDS-PAGE profiles were equally unhelpful as an aid to identification, although strain differences were seen. A low level of discrimination between the strains is apparent and the fingerprints produced by both methods were easily influenced by the growth conditions and the growth phase prior to harvest. Molecular based identification, despite its cost and time-consuming methods, clearly produced the best results but these were not unambiguous either. Table 3.18 summarises the results relating to the usefulness of each technique used in this work.

3.7.2 Characterisation of *Lb. acidophilus* strain VUP 12006

3.7.2.1 Growth kinetics

Although it is hard to compare growth kinetics because of variation in culture conditions and species used, only one report has been published that would allow comparison of growth kinetics found in this study.

Table 3.18 Summary of the identification procedures used in this study.

Comparison was made on the basis of the apparent reliability of identification, cost, and time factors relating to the identification procedures used in this study. Cost per test decreases when performing more than 10 FAME profiles in a run, due to reduced set-up costs; approximately 56 samples were analysed to obtain FAME profile results. Costs for FAME profiles are marked Medium toHigh as each batch was done in runs of 10 or less: had the analysis been done in one or two batches, the cost would be Medium.

Test	Level of identification	Apparent reliability of result	Suitability for screening large numbers of isolates	[a]Cost	Minimum time taken to obtain results (does not include culture time)
Carbohydrate tests	Species	Good	No Yes	Single vial testing-medium Microtitre plate testing-low	24 hours 1 working day
FAME profiles	Species /strain	Good	Yes	Medium to High	24 hours
SDS-PAGE profiles	Strain	Very good	Yes	Medium	8 hours
PCR	Strain	Very good	Yes	High	>1 week

[a] cost of testing based on commercial rates. Low-< $10 per result. Medium-$10-$100 per result. High ->$100 per result

The growth kinetics for *Lb. paracasei* strain VUP 12006, determined during growth in fermenters, are similar to the values published in the literature for *Lb. acidophilus* (Kim *et al.*, 2001). In that study mid-log phase was reached in approximately 4 hours and stationary phase was established after around 14 hours. The work presented in section 3.4.2 demonstrated a slightly longer time until mid-log phase, around 6 hours, and stationary phase was reached after around 13 hours; but only an indirect comparison can be made when all parameters of the study were not included in the publication methods, particularly starter inocula and broth. A study by Gadgil (1997), working on growth modelling in batch cultures of *Lb. bulgaricus,* determined that the μ_{max} at pH 5.6 was around 0.45 $h(^{-1})$. Batch fermenter conditions were similar to those described in this work, including the use of MRS broth, however the flasks were agitated without aeration and no nitrogen sparging occurred. In this study, at pH 5.0, the μ_{max} was 0.50 $h(^{-1})$ and at pH 6.0 the μ_{max} was 0.59 $h(^{-1})$, which is slightly higher than that described for *Lb. bulgaricus*, but the difference may possibly be explained in strain variation and exposure of the flasks to room air.

3.7.2.2 Optimum growth conditions

The optimum growth conditions determined in this study, compared to published values of other *Lactobacillus* species, are shown below. While the value for temperature falls in the range of expected optimum values, the value for pH is slightly higher than that observed in some other species of *Lactobacillus*. As stated previously, it is critically difficult to compare these values, as the growth conditions were not fully elucidated in the publication.

	Values determined in this study for strain VUP 12006	Published values for the genus *Lactobacillus* (Gomez *et al.*, 1999)
Optimum temperature range	37 °C	35-40°C
Optimum pH	6.3 ± 0.2	5.5-6.0

3.7.2.3 Protein profiles and Western blot reactions

Stress, resulting from exposure to acid and heat, is commonly experienced by bacteria used in the food industry, particularly during the manufacture of fermented dairy products. One of the goals of this study was to detect proteins induced during acid and heat stress to identify the target areas within the cell responding to the stress. The results shown in this thesis, sections 3.4.9 and 3.4.10, indicate that growth at increased temperature (due to a 7°C rise in temperature above the optimum) resulted in a four-fold up-regulation of analogues of HSP60 and HSP70.

The presence of the *gro*ESL operon, encoding the GroES and GroEL chaperone proteins, has previously been localised on the chromosome of both *Lb. johnsonii* and *Lb. helveticus* (Broadbent *et al.*, 1998, Carey Walker *et al.*, 1999). The finding of the analogues is consistent with the presence of a similar operon in the tested strain of *Lb. paracasei.* Studies of the protein profiles showed that growth at low pH and high temperature, compared to growth at optimum pH and temperature, resulted in dissimilar changes to the banding pattern, suggesting that a universal stress response does not occur. The changes caused by decreasing pH during growth were rapid and appeared within one hour of the pH being lowered to pH 4.0, although a marked decrease in the generation time was observed at the same time.

3.7.2.4 FAMEs (Effect of acid and temperature shift on total cellular FAMEs)

The study of Decallone and others (1991) has clearly demonstrated that the age of a culture when sampled for comparison of FAME fingerprints is of fundamental importance. The profile clearly changes as a culture ages through exponential and growth phase. It was considered that this analysis of FAME profiles may also be a useful technique in understanding the mechanisms of adaptive tolerance to environmental change, so a study was made of the changes in FAME profiles taken at the same stage of growth but from cultures grown in different environments. The results were presented in section 3.4.6.

Bacterial cell membranes perform a vital function in protecting the cell from changes in the external environment such as increasing temperature or decreasing pH. Temperature dependent changes to fatty acid composition have been demonstrated in several bacteria including *Lb. plantarum*, *L. monocytogenes* and *C. botulinum* (Russell *et al.*, 1995, Evans *et al.*, 1998). In these organisms membrane fluidity at low temperatures was maintained by a decrease in chain length and an increase in the proportion of unsaturated fatty acid (UFA) chains as growth temperature decreased. In this study, it was determined that there was an increase in the presence of saturated $C_{18:0}$ (stearic acid) during heat stress from 6 to 14% and a decrease in $C_{18:1}$ (oleic acid) from 26 to 21%. The probable conversion, in part, of $C_{18:1}$ to $C_{18:0}$ by removal of one double bond would significantly increase the melting point and make the membranes less fluid at the higher temperature. Stearic acid has a high melting point of 69.6°C and oleic acid has a melting point of 13.4°C.

Following acid stress, marked changes were also noted in the pattern of fatty acids. Most changes were similar to those noted after the culture had undergone heat stress, particularly the decrease in peaks X1, $C_{14:0}$, $C_{18:0}$ and $C_{19:0}$ and increase in peaks X3, and $C_{18:1}$; however, $C_{16:1}$ increased from 2 to 10 % while no similar change occurred in a heat stressed culture. The overall similarity of changes suggests two possibilities. One possibility is of a general response to environmental stress rather than to a specific heat or acid stress response. The second is that heat and acid stress provoke similar responses to quite different stresses but not the responses that may be provoked by, for example, an oxidative stress. It would be expected that a different fatty acid profile would be seen after a decrease in temperature, so the idea of a single stress response seems unlikely and it must be questioned as to why the responses appear so similar. The answer may be found in the presence of cyclopropane fatty acids (CFAs) in the profile. The roles of cyclopropane fatty acids in the membranes of LAB are poorly understood, although it is known that in *E. coli* CFA synthesis occurs largely as the cells enter the stationary phase and

when starved for amino-acids (Eichel *et al.*, 1999). The function of CFAs is believed to be related to changes in the fluidity of the membrane in a similar manner to the way the presence of polyunsaturated fatty acids (PFAs) affect membrane fluidity, that is, they have a stabilizing effect on the membrane (Johnsson *et al.*, 1995, Grogan *et al.*, 1997). The presence of a CFA, $C_{19:0}$ cyc (lactobacillic acid) was observed in both control and stressed cultures. After heat and acid stress, the percentage composition of $C_{19:0}$cyc was found to decrease from 26% to 12% and 16% respectively, indicating lactobacillic acid plays only a limited role during heat and acid stress. Previously there have been only a few attempts at trying to explain how CFAs aid the cell in tolerating stress covering: growth over a limited temperature range, acid habituation and ethanol-induced changes to fatty acid composition (Khuller *et al.*, 1974, Couto *et al.*, 1996, Brown *et al.*, 1997, Evans *et al.*, 1998). When observing the changes in CFA composition of growth over a wide temperature range of two different strains of *Clostridium* sp., Khuller (1974) and Evans (1998) observed quite dissimilar effects. Khuller demonstrated an increase in CFA composition towards the cooler temperatures while in contrast Evans found the increase in warmer temperatures. Current information suggests that CFAs should be found in increased amounts in heat-affected cells, as they are expected to reduce the effect of temperature on membrane fluidity. However, this study and Khuller found otherwise and it may be considered that changes in other components which affect membrane fluidity that are perhaps less energetically expensive, may in part explain this difference. Following acid habituation, Brown *et al*, (1997) found increased levels of cyclopropane fatty acids ($C_{17:0}$cyc and $C_{19:0}$cyc) in *E. coli*. No similar changes were seen in this study. Acid habituation, compared to a sudden acid exposure may produce different FAME profiles and perhaps the two methods of acid exposure cannot be compared directly and more work needs to be performed in this area. In conclusion, in strain VUP 12006 it appears that CFAs do not play a significant role in the protection of the cell to heat and acid stress.

3.7.2.5 Lactic acid challenge

It has been shown previously that acidification of the cell is a principal factor in the cessation of cell division (Rallu *et al.*, 1996) and results shown in section 3.3.4 support these observations. In this experiment lactic acid was not used to provide a low pH environment. It can be seen that there is a logarithmic relationship between the μ_{max} value and lactate concentration indicating an inhibitory effect from the organic acid rather than the presence of $[H^+]$. Further studies of the affect of lactate were not pursued but may be followed up in future work.

3.7.3 Response of *Lb. acidophilus* strain VUP 12006 to challenge at lethal pH

3.7.3.1 Acid resistance is dependant on growth phase

The ability of *Lb. acidophilus* to survive at pH 2.5 for extended periods reflects an ability to control the internal pH of the cell by exclusion of hydrogen ions or by having the ability to pump protons from the cell. This study has shown that acid resistance is very dependent upon growth phase. In particular the ability to survive a lethal pH challenge was most ably demonstrated during stationary phase and lag phase where 50% and 45% of challenged bacteria, respectively, were able to survive for two hours. During exponential phase this value dropped to around 28%. This can be compared to the enteric pathogen *Shigella flexneri*, which was unable to demonstrate acid resistance until the late exponential phase (Gorden *et al.*, 1993). The results from the current study indicate that *Lb. paracasei* strain VUP 12006 is acid resistant according to the criteria of Gorden and Small (1993) and this was equivalent to many of the *Shigella* strains used in their study of acid resistance. Interestingly, it is noted that even after twenty-four hours the inoculum of *Lb. paracasei* continues to survive even if at very low numbers (<1%). An extensive study of acid resistance of the eight candidate LAB strains was not undertaken. However, when VUP 12000 (*Lb. acidophilus* type strain) was tested similarly, it was more rapidly killed by exposure to pH 2.5, demonstrating the relatively greater acid tolerance of VUP12006.

3.7.4 Comparison of test strains; the response to acid stress

The response to growth at low pH has been shown to vary between *Lactobacillus* strains. In the data shown in Figure 3.26 it can be seen that strain 12006, when compared with strains 12001 and 12007, was the only strain to up-regulate a 42 kDa protein during growth at pH 4.0. Although the optimum pH for growth is not known, strains 12001 and 12007 demonstrated an up-regulated protein of similar size (42 kDa) during growth at pH 6.3, but no analysis of that protein was undertaken to show whether the protein was the same as the protein produced by strain VUP 12006. These differences may be due to either species or strains variations, but clearly further work would need to be performed to determine if there was any link between the proteins produced by the strains. This could include N-terminal sequencing or antibody studies, using antibody prepared against the AphA protein of *Lb. paracasei*.

3.7.5 *Lb. paracasei* strain VUP 1200; structure and function of AphA

Several difficulties were experienced in attempts to identify as well as find the structure and function of the 42 kDa protein up-regulated in *Lb. paracasei* strain VUP12006 when exposed to low pH and these will be reported in this section.

3.7.5.1 identification of AphA

Initial investigations to establish the identification of AphA were only partially successful. The N-terminal sequence of 20 amino acids was obtained; however, the attempts at matching that sequence to a sequence in known protein databases were unproductive until the release of the *Lb. casei* ATCC 334 draft genome. Prior to the release of the draft genome several attempts were made to clone AphA into a representative genomic library, but the resulting transformants yielded only small inserts that showed no sequence homology to the N-terminal sequence. With the release of the draft genome this approach was changed to using PCR based techniques to obtain gene sequence.

The first round of PCR using 2 primer pairs designed around the upstream region and a third primer pair around the N-terminal region were successful in obtaining amplification products. These fragments showed 100% homology to sequence in *Lb. casei* 334, indicating that the N-terminal amino acid sequence obtained from *Lb. paracasei* VUP 12006 was coded in the region of scaffold three under investigation.

A second round of PCR was designed to find the complete sequence of the gene coding AphA. Using the known *Lb. casei* ATCC 334 sequence, primers were designed that would have covered the entire sequence if that sequence shared significant homology with the gene in *Lb. paracasei.* Although two primer pairs that used the same forward primer and had been designed to amplify DNA in the downstream region of the gene failed to produce products, six other primer pairs did produce products, which were sequenced and submitted to a BLASTn search of the draft *Lb. casei* genome. It was also found that the size of the expected PCR products was slightly lower for some primer pairs compared to the actual sizes obtained, suggesting differences between the genes in *Lb. casei* and *Lb. paracasei.* Analysis of the BLASTn results and subsequent alignment of the results with the draft genome of *Lb. casei* ATCC 334 revealed a region with 45 additional nucleotides compared to the draft genome (starting at position 8838) and this area was high in serine residues. This would appear to explain the size differences between some of the PCR products.

The data to this point provided over 90% of the AphA gene in *Lb. paracasei.* However, there was a gap between positions 1229 and 1263, so that the ORF was not defined. Consequently, anchored PCR was used in an attempt to walk down the gene from the region where the sequence was known. Although amplification products were obtained, the amplification products were all less than 800bp so that sequences beyond what was already known were not obtained. The reason small products were obtained from ligation of vector to *Lb. casei* VUP 12006 is not

immediately evident, but may be related to the selection of restriction endonuclease used or the vector.

PCR products obtained using these primers yielded expected results, in terms of fragment size, for *Lb. casei* ATCC 334, but only four amplification products from three primer pairs were observed when DNA from strain VUP 12006 was used. The size of the fragments was considerably larger than expected and they had to be sequenced to determine any relationship to AphA. Two of the amplification products were not from scaffold 3 and were not considered further, but the remaining two were found to share sequence homology in scaffold 3 of the draft genome of *Lb. casei* ATCC 334; the forward primer was the same for both these fragments and duplicate sequencing from the forward primer was identical for over 400 bases of sequence data obtained from *Lb. casei* ATCC 334.

It was found that the first ≈120 bases of the sequence for the forward primers were found to share 100% homology with sequence from *Lb. casei* ATCC 334 starting at position 7777 of scaffold 3. Beyond position 7897, little homology was observed between the remaining 290+ bases to sequence from *Lb. casei* ATCC 334. Given the size of the PCR fragments obtained from *Lb. paracasei*, an additional 2,000+ bases remain to be sequenced.

The sequence from the reverse primer 3SP1AS was used to obtain data that included a stop codon for AphA. The data obtained for this primer showed discontinuous homology. The sequence data obtained for *Lb. paracasei* matched *Lb. casei* between positions 8188 and 8281 and positions 8405 and 8851 with a non-matching region of 124 nucleotides between positions 8281 and 8405. Although this sequence data was continuous for strain VUP 12006 the gap was seen when a ClustalW alignment was performed; refer to Figure 3.33. The apparent difference between the two strains may account for the lack of amplification products when primer 2SP1S was used to amplify DNA from strain VUP 12006.

3.7.5.2 Structure and potential function of AphA of Lb. paracasei

The ORF encoded a protein of 420 amino acids with a predicted molecular mass of 43.49 kDa. A 28 amino acid signal peptide was predicted by SPScan and the cleavage site was confirmed by the N-terminal sequence. Thus the mature form of the protein is about 39.34 kDa, which is slightly smaller than the size observed by 1-D SDS-PAGE. From these results it can be concluded that AphA is translocated across the cytoplasmic membrane (due to the presence of the hydrophobic signal peptide) and is a secreted protein (the signal peptide has been removed as determined by the N-terminal sequence).

AphA did not posses the C-terminal cell wall anchor motif LPTXTG, which may be consistent with the protein being transitory in the cell wall before being excreted into the extracellular environment. No examination was made of the growth media, to look for the presence of AphA, in the current study so the presence or absence of the protein in the extracellular medium would have to be the subject of further study.

BLASTn (gene sequence) and BLASTx (protein sequence) were used to determine the relationship of AphA to known genes and proteins. The gene sequence was found not to have any significant matches in the known databases, although it was 94% homologous to an ORF found in the draft genome of *Lb. casei* ATCC 334. With the exception of that match, this gene was not conserved across unrelated or related genera. Protein relatedness was more apparent and there was significant homology to several members of the family *Lactobacillalles*. Many of the significant matches also showed the presence of secretory signal peptides and poly-serine domains and the proteins were of similar size, around 40-45 kDa.

The closest match was GSP-781, a glucan-binding protein from *Strep. mutans*. GSP-781 (general-stress protein) has been shown to be up-regulated significantly when *Strep. mutans* was exposed to high osmolarity and temperature, as well as low pH (Chia *et al.*, 2001). According to

Smith (1996) at least 6 proteins with the ability to bind α-1,6-glucan, glucan-binding proteins or Gbp have been identified in *Streptococcus* species. These include GpbA, GbpB and GbpC. One of these, GbpB, is thought to play a role in the molecular pathogenesis (dental caries) although the role has not been resolved. However, despite the presence of homologous areas there were no amino acid domains that suggested the biological function of the protein, although it has been suggested that GSP-781 may be associated with the maintenance of the stability of the cell wall or cell membrane (Chia *et al.*, 2001).

No study has been made of the putative proteins from *Strep. pneumoniae* or *Strep. pyogenes*. The Genbank references are related to whole genome sequences and not a study of a particular gene.

3.7.5.3 Further studies

The role of AphA in acid stress response or acid tolerance in *Lb. paracasei* is not known, nor is the physiological role defined. Further analysis may include genetic analysis through gene manipulation, which may correlate the role this protein plays in generalised stress response, acid tolerance or cell-surface maintenance. However, such studies must await the development of specific genetic tools to allow inactivation of the AphA gene (presuming that this would not be fatal). Further studies on the sub-cellular location of the protein need to be done, given the observation of the signal sequence and attempts at purification showed the loss of the AphA protein in the homogenates after centrifugation, suggesting the protein was located in the membrane-associated fraction.

Other areas of further study could also include the following:

- Obtain further up-stream and downstream sequence, which would help to determine the presence of an operon. As was noted in chapter 3, the downstream regions of the AphA

gene in *Lb. paracasei* strain VUP 12006 and the homologous region in *Lb. casei* ATCC 334 shared little homology, and over 2,000 bases of a PCR fragment remain to be sequenced.

- Preparation of antibodies against the 42 kDa protein identified during growth at low pH in *Lb. paracasei* strain VUP 12006. It would be useful to monitor the regulation of the gene through Western blot analysis. These antibodies could also be used to determine the presence of analogues of AphA in related strains, related species and non-related species as well as the quantification of AphA protein secreted into the growth medium. This protein has already been shown to have altered regulation in two related species compared to *Lb. paracasei* strain VUP 12006, see section 3.5.1.

3.8 Conclusion

- FAME, SDS-PAGE and carbohydrate utilisation profiles could not sufficiently differentiate the strains of LAB to enable them to be adequately identified to species level. Fingerprints produced by both methods were easily influenced by the growth conditions and the growth phase prior to harvest.

- 16S rRNA was able to differentiate to species level and strain VUP12006 was identified as *Lb. paracasei*. Molecular based identification, despite its cost and time-consuming methods, clearly produced the best results but these were not unambiguous either.

- *Lb. paracasei* VUP 12006 grew optimally at 37°C ± 0.5 and at pH 6.3 ± 0.2.

- When acid stressed, *Lb. paracasei* VUP 12006 produced a protein of approximately 42 kDa which was found to be cell membrane- or cell-surface-associated

- PCR based techniques were used to determine the nucleotide sequence of the gene known to encode the 42kDa AphA protein

- The genomic arrangement of the AphA gene was different in *Lb. paracasei* VUP 12006 compared to *Lb. casei* ATCC 334.

Chapter Four

STRESS RESPONSES IN *BIFIDOBACTERIUM* ANIMALIS (SYN. *LACTIS*) STRAIN VUP Bb 12

4.1 Introduction

At the time research for this thesis commenced, both *Lactobacillus* and *Bifidobacterium* strains were being investigated in the CBFT laboratories in terms of viability of probiotics in fermented dairy products. While investigations initially involving *Lb. paracasei* strain VUP 12006 were underway, as described in chapter 3, a strain of *Bifidobacterium* was also selected on which to undertake some preliminary studies on stress responses. Strain selection was based on known characteristics and use as a probiotic adjunct as described below:

1) At the time this project commenced, the bacterium was commonly supplied for use as a probiotic adjunct in yoghurt manufacture;

2) The strain had been previously shown to display functional characteristics (health benefits); and

3) Other features of the strain such as identification and survival characteristics of the strain had been described previously in the literature (Clark and Martin, 1994; Sgorbati *et al.*, 1995; Meile *et al.*, 1997; Kailaspathy and Chin, 2000; Shin *et al.*, 2000; Alander *et al.*, 2001; Crittenden *et al.*, 2001; O'Riordan *et al.*, 2001).

On this basis, *B. animalis (*syn. *lactis),* (stored as strain VUP Bb 12), was selected for characterisation of stress responses. *B. animalis (*syn. *lactis)* strain VUP Bb 12 has been used by the starter culture manufacturer, Christian Hansen, since 1986, and is included in several product

lines including ABT cultures, single cultures and anti-diarrhoea tablets. ABT cultures contain *Lb. acidophilus, S. thermophilus* and *B. animalis* syn. *lactis* strain Bb 12 (Wind, personal communication).

4.2 Identification of test strain

Genus and species designation of strain VUP Bb 12 was confirmed using the oligonucleotide primers described in Table 4.1. Lm3/Lm26 are genus specific primers, Ban2/23Si was used to confirm species and PAF/536R was used for sequencing the 16S rRNA gene.

Table 4.1 Primers used for identification of strain VUP 12006.

Name	Description	Tm
Lm3	CGGGTGCTNCCCACTTTCATG	63.4
Lm26	GATTCTGGCTCAGGATGAACG	54.6
Ban2	CATATTGGATCACGGTCC	50.5
23Si	CATTCGGACACCCTGGGATC	62.0
PAF	AGAGTTTGATCCTGGCTCAG	54.0
536R	GTATTACCGCGGCTGCTG	57.5

The PCR reaction mixture (50μL) for the first primer pair, Lm3/Lm26 contained 30 pmol of each primer, 200μM each of dATP, dTTP, dCTP and dGTP, approximately 100ng of bacterial DNA, 2.5 U of *Taq* DNA polymerase (Sigma) and PCR buffer with 50mM KCl pH 8.3, 10 mM Tris HCl and 1.5 mM $MgCl_2$.

DNA was amplified as follows: initial denaturation at 94°C for 4 minutes, followed by 30 cycles consisting of denaturation at 94°C for 30 seconds, annealing at 57°C for 30 seconds, extension at 72°C for 60 seconds and a 7 minute final extension step at 72°C. The products were stored at 4°C until required. Aliquots of the reaction mixture (10μL) were separated on a 0.8% agarose gel, stained with ethidium bromide and visualised under UV light. The results are shown in Figure 4.1.

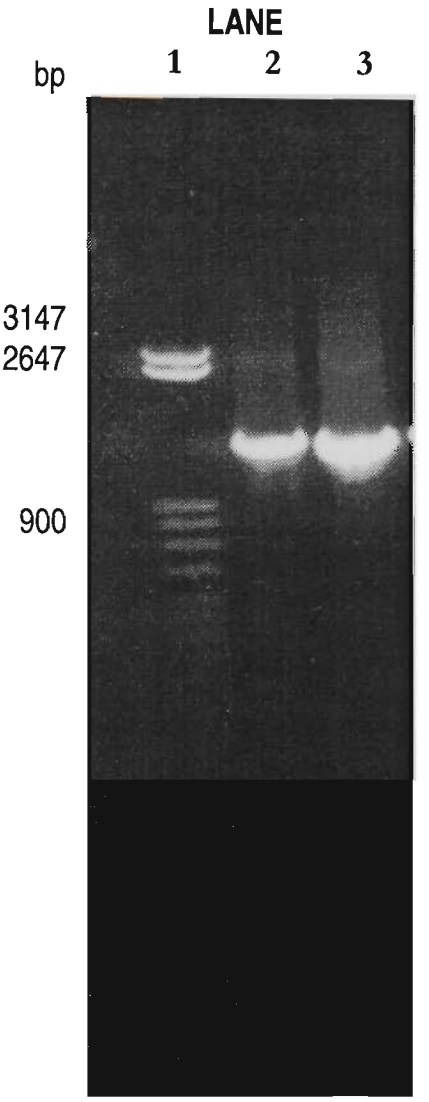


Figure 4.1 PCR amplification of DNA from *Bifidobacterium* strain VUP Bb 12 using primers Lm3 and Lm26.

Lane 1 is marker (EcoRI/Hind III digest, Progen). Lanes 2 and 3 are PCR amplification products of DNA using primers Lm3 and Lm26 (in duplicate). Products were obtained from the genomic DNA of strain VUP Bb 12. Products size was approximately 1770 bp.

Genomic DNA from strain VUP Bb 12 amplified using Lm3/Lm26 yielded a PCR product of approximately 1770 bp. Lm3/Lm26 have been shown to amplify all *Bifidobacterium* type strains and production strains using a 21 nucleotide sequence of the 16S gene that is highly conserved in *Bifidobacterium* (Kaufmann *et al.*, 1997). These results are therefore consistent with strain Bb 12 having a genus specific identification of *Bifidobacterium.*

Species level identification was confirmed using the primer pair PAF and 536R. The reaction mixture (50μL) for PAF/536R contained 30 pmol of each primer, 200μM each of dATP, dTTP, dCTP and dGTP, approximately 100ng of bacterial DNA, 2.5 U of *Taq* DNA polymerase (Sigma) and PCR buffer without $MgCl_2$. DNA was amplified as follows: initial denaturation at 94°C for 2 minutes, followed by 30 cycles consisting of denaturation at 94°C for 45 seconds, annealing at 55°C for 45 seconds, extension at 72°C for 60 seconds and a 7 minute final extension step at 72°C. The products were stored and electrophoresed as described above. The results are shown in Figure 4.2. Genomic DNA from strain Bb 12 amplified using PAF/536R yielded a PCR product of approximately 550 bp. Sequence of the PCR products was determined using a CEQ 2000 (Beckman Coulter) according to the manufacturer's instructions. The sequences in both the forward and reverse direction were edited in the software package 'bio-edit' to about the first 450 bases and submitted to ANGIS for a BLASTn search (2.6.14). The data for sequences showing significant alignment is shown in Figure 4.3. These results are consistent with strain Bb 12 having a species identification of *B. animalis.* As strain BB 12 was stated to be a junior synonym sub-species (*lactis*) of *B. animalis,* a third round of PCR, using the primer pair Ban 2/23Si, was used to confirm the final identification. The primer Ban 2 was designed by Ventura and Zink (2002) to anneal to a conserved region of *B. animalis* but not to *B. lactis.* Ventura and Zink feel *B. lactis* is not a junior synonym of *B. animalis* but rather a subspecies of *B. animalis* and they used these primers to investigate the taxonomic differentiation of the *B. lactis.*

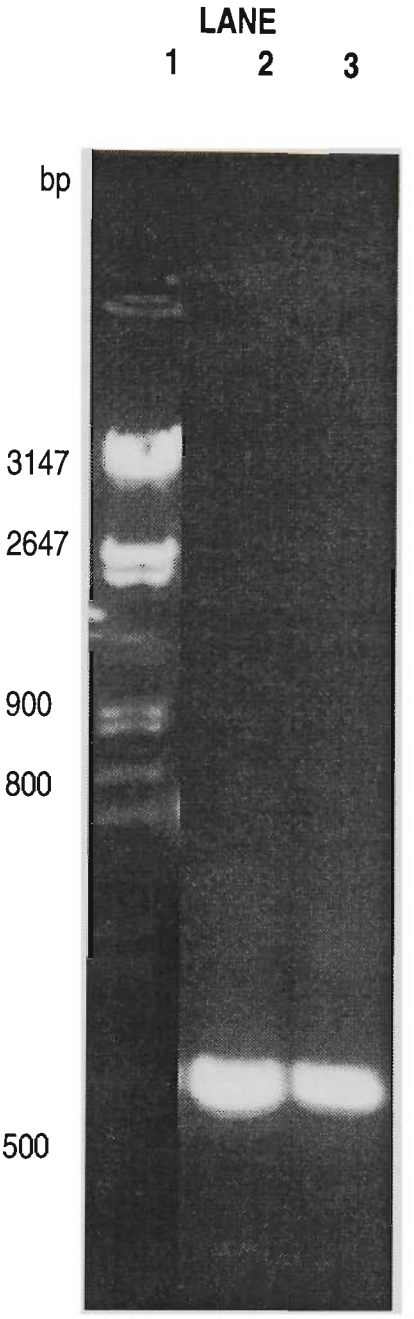


Figure 4.2 PCR amplification of DNA from *Bifidobacterium* strain VUP Bb 12 using primers PAF and 536R.

Lane 1 is marker (EcoRI/Hind III digest, Progen). Lanes 2 and 3 are PCR amplification products of DNA using primers PAF and 536R (in duplicate). Products were obtained from the genomic DNA of strain VUP Bb 12. Product size was approximately 550 bp.

Matching Entry (in GenBank Main)	Begin-End	Description	Score	E Value
AB050138	[1-460]	Bifidobacterium animalis gene for 16S rRNA, partial sequence, strain:YIT 4090.	842.0	0.0
AB050137	[1-460]	Bifidobacterium animalis gene for 16S rRNA, partial sequence, strain:YIT 4119.	842.0	0.0
AB050136	[1-460]	Bifidobacterium lactis gene for 16S rRNA, partial sequence, strain:YIT 4121.	842.0	0.0
AB050134	[1-460]	Bifidobacterium animalis gene for 16S rRNA, partial sequence, strain:Y 93050.	842.0	0.0
AB050133	[1-460]	Bifidobacterium animalis gene for 16S rRNA, partial sequence, strain:Y 96007.	842.0	0.0

```
AB050138  Bifidobacterium animalis gene for 16S rRNA, partial
sequence, strain:YIT 4090. Identities = 456/461 (99%)
     Strand = Plus / Minus

Query: 1        ggtgcttattcgaacaatccactcaacacggccgaaaccgtgccttgcccttgaacaaaa 60
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 477       ggtgcttattcgaacaatccactcaacacggccgaaaccgtgccttgcccttgaacaaaa 418

Query: 61       gcgggttacaacccgaaggcctccatcccgcacgcggcgtcgctgcatcaggcttgcgcc 120
                 |||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 417       gcggtttacaacccgaaggcctccatcccgcacgcggcgtcgctgcatcaggcttgcgcc 358

Query: 121      cattgtgcaatattccccactgctgcctcccgtaggagttctgggccgtatctcagtccc 180
                 |||||||||||||||||||||||||||||||||||| ||||||||||||||||||||
Sbjct: 357       cattgtgcaatattccccactgctgcctcccgtaggagt-ctgggccgtatctcagtccc 299

Query: 181      aatgtggccggtcaccctctcaggccggctacccgtcaacgccttggtgggccatcaccc 240
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 298       aatgtggccggtcaccctctcaggccggctacccgtcaacgccttggtgggccatcaccc 239

Query: 241      cgccaacaagctgataggacgcgaccccatcccatgccgcaaaagcatttcccaccccac 300
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 238       cgccaacaagctgataggacgcgaccccatcccatgccgcaaaagcatttcccaccccac 179

Query: 301      catgcgatggagcggagcatccggtattaccacccgtttccaggagctattccggtgcac 360
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 178       catgcgatggagcggagcatccggtattaccacccgtttccaggagctattccggtgcac 119

Query: 361      agggcaggttggtccacgcattactcacccgttcgccactctcaccccgacagcaagctg 420
                 |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 118       agggcaggttggt-cacgcattactcacccgttcgccactctcaccccgacagcaagctg 60

Query: 421      ccagggatcccgttcgacttgcaatgtg-taagcacgccgc 460
                 |||||||||||||||||||| ||||| ||||||||||||
Sbjct: 59        ccagggatcccgttcgacttgc-atgtgttaagcacgccgc 20
```

Figure 4.3 Result of BLASTn search of GenBank using sequence data from primer pair PAF/536R and DNA from strain VUP Bb 12.

16S rRNA gene sequence data from strain VUP Bb 12 and alignment of the closest matching GenBank entry is shown below the top 5 BLASTn results.

The conserved region was two insertions of eight nucleotides each. The PCR reaction mixture (50µL) for Ban 2/23Si contained 50 pmol of each primer, 200µM each of dATP, dTTP, dCTP

and dGTP, approximately 100ng of bacterial DNA, 2.5 U of *Taq* DNA polymerase (Sigma) and PCR buffer with 50mM KCl pH 8.3, 10 mM Tris HCl and 1.5 mM $MgCl_2$. DNA was amplified as follows: initial denaturation at 95°C for 5 minutes, followed by 30 cycles consisting of denaturation at 95°C for 30 seconds, annealing at 54°C for 60 seconds, extension at 72°C for 90 seconds and a 7 minute final extension step at 72°C. After running three trials no PCR products were obtained using the reaction mixture and conditions described above, although no positive control was available to confirm the result. According to Ventura and Zink, this negative result indicates a closer relationship of strain VUP Bb 12 to *B. lactis* rather than *B. animalis*. Based on currently accepted nomenclature and classification, these results are consistent with the identification remaining *B. animalis* syn. *lactis*. Evidence of the presence of the 'bifid shunt' has also been confirmed in this strain by use of the assay for the diagnostic enzyme fructose-6-phophate (Crittenden *et al.*, 2001, Sgorbati *et al.*, 1995). The phenotypic characterisation of strain VUP Bb 12 to genus level was checked at 3-monthly intervals using carbohydrate utilisation tests and the strain was monitored weekly for typical Gram stain and growth characteristics on MRS agar (section 2.2). Table 4.2 shows the parameters used for this characterisation.

4.3 Growth kinetics, protein profiling and effect of elevated temperature, low pH and aeration on growth curves of strain VUP Bb 12

The test strain was initially characterised by growth rate and protein profiles across a range of temperatures, pH levels and dissolved gases. Studies were conducted in one-litre fermenters according to the methods described in section 2.3.7. Cultures were grown in MRS broth with the addition of 0.05% cysteine-HCl and the fermenter was stirred at 150 rpm and sparged with nitrogen. Samples of fermenter contents were collected hourly for at least eight hours and again at twenty-fours hours after growth commenced.

Table 4.2 Characteristics of *B. animalis* (syn. *lactis*) strain VUP Bb 12.

Summary of phenotypic characteristics of *B. animalis* (syn. *lactis*) strain VUP Bb 12.

Characteristic	Description
Carbohydrate fermentation	Glucose (+), Galactose (+), Lactose (+)
Gram stain	Gram positive bacilli with many bifurcated forms and club shapes
Growth characteristics	Grew on MRS agar as small 1-2 mm, white-grey colonies after 48 hours anaerobic incubation at 42°C. Cultures failed to grow after 48 hours aerobic incubation on MRS agar.

4.3.1 Optimum temperature and pH for growth

4.3.1.1 Determination of optimum growth conditions

Specific growth rates for *B. animalis* strain VUP Bb 12 were calculated from absorbance values measured during growth over a range of temperatures (22 to 47°C) and pH values (4 to 7). The highest specific growth rate during the growth cycle (μ_{max}) was used to determine optimum growth conditions, as described in section 2.3.8. Figure 4.4 (A) presents the maximum specific growth rate plotted against temperature. The temperature values at the top of the peak formed by this plot were determined to be the optimum temperature range for growth. Optimum growth temperature was determined to be 42°C ± 0.5°C. Figure 4.4 (B) presents the maximum specific growth rate plotted against pH. The pH values at the top of the peak formed by this plot were determined to be the optimum pH range for growth. Optimum pH was determined to be 6.3 ± 0.3.

4.3.1.2 Growth kinetics

Strain VUP Bb 12 was cultivated in batch culture in a one-litre fermenter. The inoculum was prepared by harvesting stationary phase cells of an overnight culture that was not pH controlled during growth (section 2.3.7.1). The cells were centrifuged and made up in pre-warmed MRS broth. When grown in optimum conditions (42°C, pH 6.3 in MRS broth with nitrogen sparging and agitation at 150 rpm with a starting OD_{600} of 0.2.), mid-exponential phase was reached after approximately two hours and stationary phase was reached after approximately four hours. The maximum specific growth rate reached during growth in optimum conditions was 0.74 h^{-1}.

4.3.2 Observations of protein profiles of Bb 12 cultures grown between 22°C and 47°C

SDS-PAGE was used to study changes in protein profiles of strain VUP Bb 12 grown over a range of temperatures (22°C and 47°C).

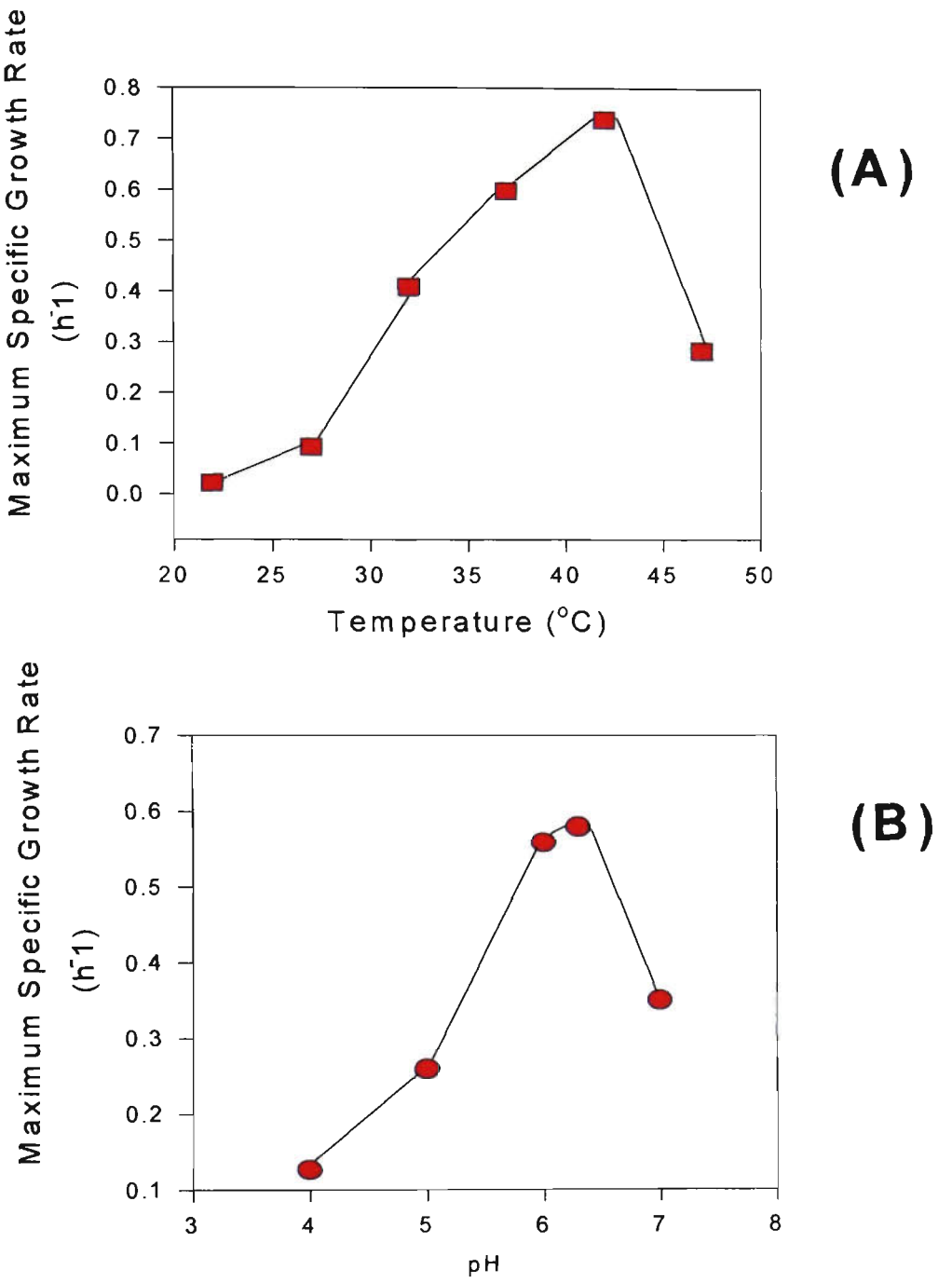


Figure 4.4 Optimum temperature and pH for growth of B. animalis (syn. lactis) Bb 12.

Maximum specific growth rate (μ_{max}) was calculated at different temperatures and pH levels. *B. animalis (*syn. *lactis)* strain VUP Bb 12 was grown in MRS broth with 0.05% cysteine-HCl in one-litre fermenters. Temperatures were set between 22 and 47°C with the pH fixed at 6.3 (A), or at 42°C at fixed pH levels between 4 and 7 (B). Nitrogen gas was sparged through the fermenter at 0.1 L/min. The peak of the resultant curve is the optimum growth range for temperature (A) and pH (B).

Cultures were grown in MRS broth with 0.05% cysteine-HCl in one-litre fermenters at constant pH (6.3) and the chosen temperature (Figure 4.5). The inoculum was prepared by harvesting stationary phase cells of an overnight culture that was not pH controlled during growth (section 2.3.7.1). The cells were centrifuged and made up in pre-warmed MRS broth. The protein profiles showed several areas of altered expression during the growth cycle across the range of temperatures shown and these areas are circled in Figure 4.5. Protein bands that were up-regulated during growth at 47°C, which could be detected by laser densitometry, included bands of approximately 19, 25, 40 and 65 kDa.

4.3.3 Observations of protein profiles of Bb 12 cultures grown between pH 4 and pH 7

SDS-PAGE was also used to study changes in protein profiles of strain VUP Bb 12 grown over a range of pH values (4.0 to 7.0). Cultures were grown in MRS broth with 0.05% cysteine-HCl in one-litre fermenters at constant temperature (42°C) and the chosen pH. The results are shown in Figure 4.6. The protein profiles for growth at neutral (pH 7.0) and mildly acid pH (pH 6.0) were essentially identical but at pH 5.0 an increase was noted in the density of a band at around 33 kDa. The same band was markedly increased during growth at pH 4.0.

Laser densitometry was performed on Coomassie stained SDS-PAGE gels prepared from samples collected from cultures grown at pH 4.0. It was determined that the band at 33 kDa made up around 4% of the amount of all bands present at the time growth commenced. This had increased to 21% after one hour, 33% after 3 hours, and 50% after 5 hours (Figure 4.7). The proportion of the up-regulated band peaked at 58% after overnight growth at pH 4.0. Laser densitometry also determined that the band is a doublet, but this can only be seen early in the growth cycle and after the second hour of growth at pH 4.0 the doublet merges and appears as a single band. A second band, of approximately 36 kDa, was also up-regulated during growth at pH 4.0, but not as strongly as the 33 kDa band.

Figure 4.5 SDS-PAGE profiles of *B. animalis (*syn. *lactis)* strain VUP Bb 12 grown over a range of temperatures.

Cultures of *B. animalis (*syn. *lactis)* strain VUP Bb 12 were grown at constant temperature and pH in one-litre fermenters containing MRS broth with 0.05% cysteine-HCl. The inoculum was prepared by harvesting stationary phase cells of an overnight culture that was not pH controlled during growth at 42°C (section 2.3.7.1). The cells were centrifuged and made up in pre-warmed MRS broth before inoculating to an initial OD_{600} of approximately 0.2. Temperatures were set at 32° (A), 37° (B), 42° (C) or 47°C (D) and the pH at 6.3. Samples of fermenter contents were taken hourly, concentrated by centrifugation and boiled with double strength loading buffer. The treated samples were centrifuged to remove cell debris and approximately 1μg of protein was applied to each lane. Protein bands were visualised by silver staining. Bands with altered expression during the growth cycle are circled in yellow.

Key

Gel (A) Lane 1 MW Standard, Lanes 2-9 samples collected hourly

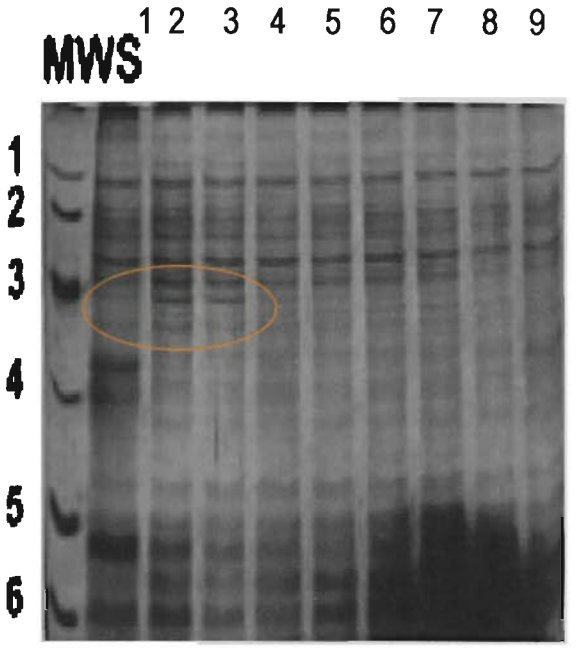
Gel (B) Lane 1 MW Standard, Lanes 2-8 samples collected hourly

Gel (C) Lane 9 MW Standard, Lanes 1-8 samples collected hourly

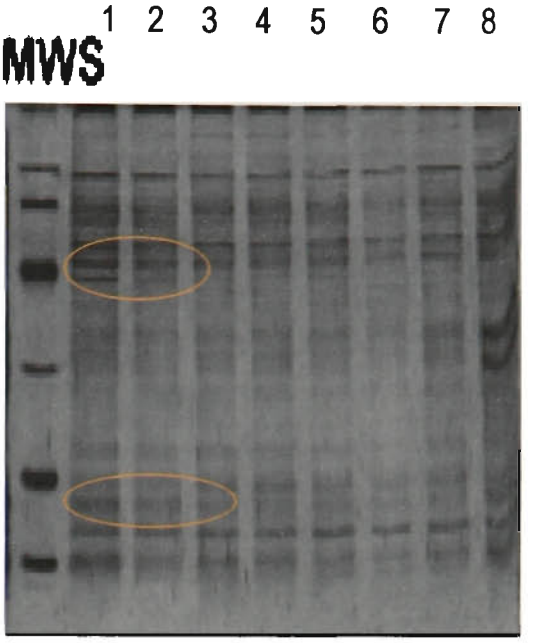
Gel (D) Lane 3 MW Standard, Lanes 1-2, 4-9 samples collected hourly

Molecular Weight Standards (MWS):

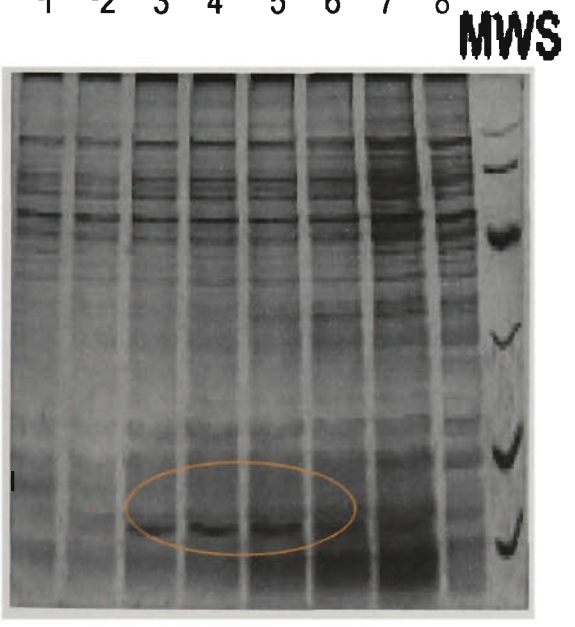
1. Phosphorylase B-97.4 kDa.
2. Bovine serum albumin-66.2 kDa
3. Ovalbumin-45 kDa
4. Carbonic anhydrase-31 kDa
5. Trypsin inhibitor-21.5 kDa
6. Lysozyme-14.4 kDa


MWS
1 2 3 4 5 6 7 8 9
1
2
3
4
5
6

(A)


MWS
1 2 3 4 5 6 7 8

(B)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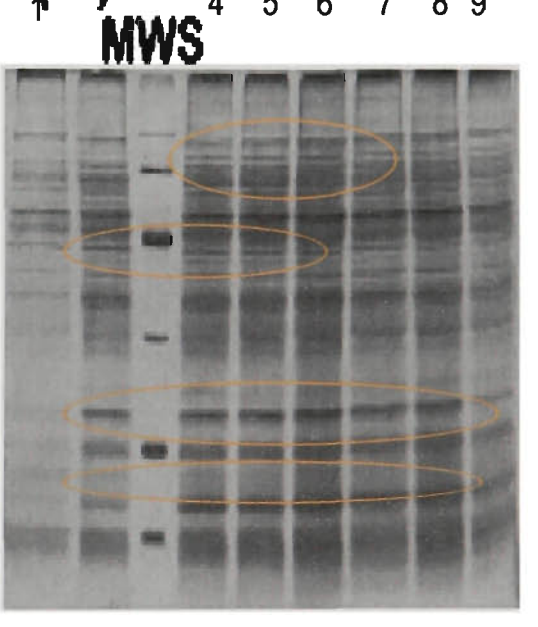
MWS

(C)


1 4 5 6 7 8 9
MWS

(D)

Figure 4.6 SDS-PAGE profiles of strain VUP Bb 12 grown over a range of pH levels.

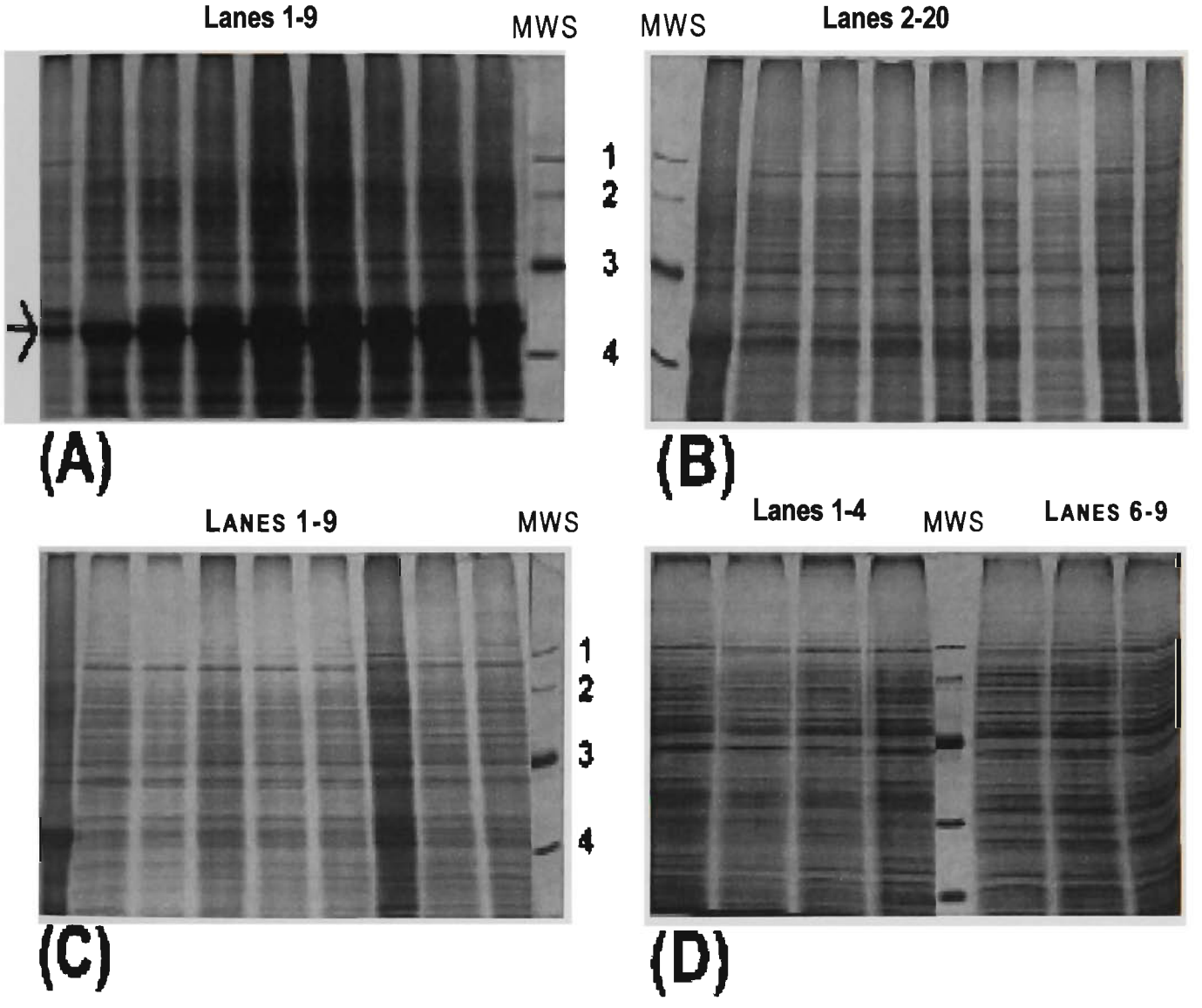
Cultures of *B. animalis* (syn. *lactis)* strain VUP Bb 12 were grown at constant temperature and pH in one-litre fermenters containing MRS broth with 0.05% cysteine-HCl. The inoculum was prepared by harvesting stationary phase cells of an overnight culture that was not pH controlled during growth at 42°C (section 2.3.7.1). The cells were centrifuged and made up in pre-warmed MRS broth before inoculating to an initial OD_{600} of approximately 0.2. Temperature was set at 42°C and the pH at 4.0 (A), pH 5.0 (B), pH 6.0 (C) or 7.0 (D). Samples of fermenter contents were taken hourly, concentrated by centrifugation and boiled with double strength loading buffer. The treated samples were centrifuged to remove cell debris and approximately 1μg of protein rich supernatant was applied to each lane. Protein bands were visualised by silver staining. Black arrow at left of gel (A) indicates the area of an up-regulated protein produced during growth at pH 4.0.

Key

Gel (A) Lane 10 MW Standard, Lanes 1-9 samples collected hourly during growth at pH 4.0

Gel (B) Lane 1 MW Standard, Lanes 2-10 samples collected hourly during growth at pH 5.0
Gel (C) Lane 10 MW Standard, Lanes 1-9 samples collected hourly during growth at pH 6.0
Gel (D) Lane 5 MW Standard, Lanes 1-4, 5-9 samples collected hourly during growth at pH 7.0

Molecular Weight Standards (MWS):
1. Phosphorylase B-97.4 kDa.
2. Bovine serum albumin-66.2 kDa
3. Ovalbumin-45 kDa
4. Carbonic anhydrase-31 kDa


Lanes 1-9
MWS
1
2
3
4
(A)
MWS
Lanes 2-20
(B)
LANES 1-9
MWS
1
2
3
4
(C)
Lanes 1-4
MWS
LANES 6-9
(D)

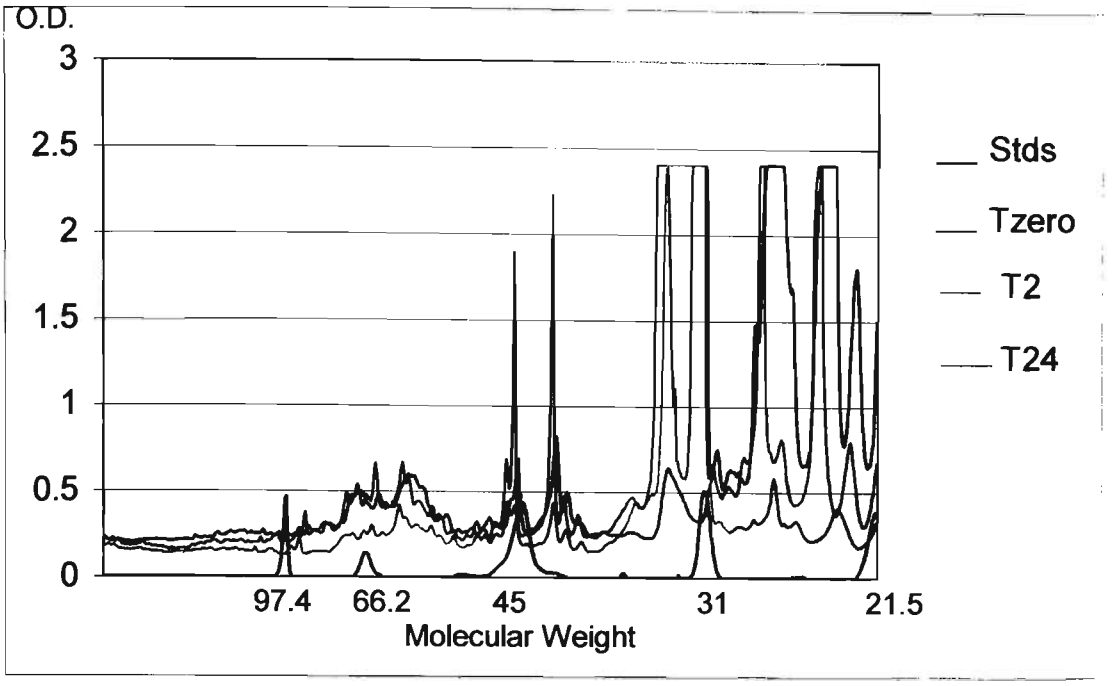


Figure 4.7 Laser densitometry of protein profiles of Strain VUP Bb 12 grown at pH 4.0 shown at selected time intervals.

A culture of *B. animalis* (syn. *lactis)* strain VUP Bb 12 was grown at constant temperature (42°C) and pH (4.0) in a one-litre fermenter containing MRS broth with 0.05% cysteine HCl. The inoculum was prepared by harvesting stationary phase cells of an overnight culture that was not pH controlled during growth at 42°C (section 2.3.7.1). The cells were centrifuged and made up in pre-warmed MRS broth before inoculating to an initial OD_{600} of approximately 0.2. Samples were collected hourly for SDS-PAGE protein profiles and the resulting profiles studied by laser densitometry. Selected Coomassie stained SDS-PAGE laser densitometry profiles are shown above. Samples for the profiles shown were collected at the start of growth (Tzero ----) and then after 2 (T2---) and 24 hours (T24---).

The molecular weight standards are shown in black and correspond to:

Molecular Weight Standards:
1. Phosphorylase B-97.4 kDa.
2. Bovine serum albumin-66.2 kDa
3. Ovalbumin-45 kDa
4. Carbonic anhydrase-31 kDa
5. Trypsin inhibitor 21.5 kDa

4.3.4 Observations of protein profiles of Bb 12 cultures without pH control

A culture of *B. animalis* (syn. *lactis*) strain VUP Bb 12 was grown in MRS broth with 0.05% cysteine-HCl in a one-litre fermenter at 42°C. The fermenter was sparged with nitrogen at 0.1 L/min. The starting pH was 6.3 but pH was not controlled and the pH of the fermenter reached approximately pH 5.2 after three hours and approximately 4.5 after eight hours. Over duplicate runs, samples were collected every two hours and prepared for SDS-PAGE as described in section 2.5.1.1 and run as described in section 2.5.1. The results are shown in Figure 4.8. An increase can be seen in the regulation of a protein of approximately 33 kDa. This protein has the same molecular weight as the protein up-regulated during growth at pH 4.0. A second protein of approximately 36 kDa was also up-regulated during growth without pH control.

4.3.5 Observations of protein profiles of VUP strain Bb 12 cultures grown in a fermenter sparged with instrument air

The effect of aeration of strain VUP Bb 12 was studied by exposing bacterial cells grown in a fermenter sparged with instrument air from the time of inoculation. Two fermenters were set up at the same time; one was continuously sparged with nitrogen (99.9% nitrogen with a maximum of 1 ppm oxygen) at 0.1 L/min (control), and the other continuously sparged with instrument air (20.9% oxygen, 78.1% nitrogen and 0.9% argon) at 0.1 L/min (potentially causing oxidative stress).

SDS-PAGE was used to observe protein profiles of strain VUP Bb 12 sampled while growing with aeration. Cultures were grown in MRS broth with 0.05% cysteine-HCl in one-litre fermenters at constant pH (6.3) and temperature (42°C). Samples were collected every two hours after inoculation and the results for SDS-PAGE analysis are shown in Figure 4.9. The protein profiles produced during aerated growth are very sirnilar to the control: no protein bands were obviously up-regulated during growth in instrument air but several minor bands of molecular weight greater than 97.4 kDa and around 25 kDa were down-regulated.

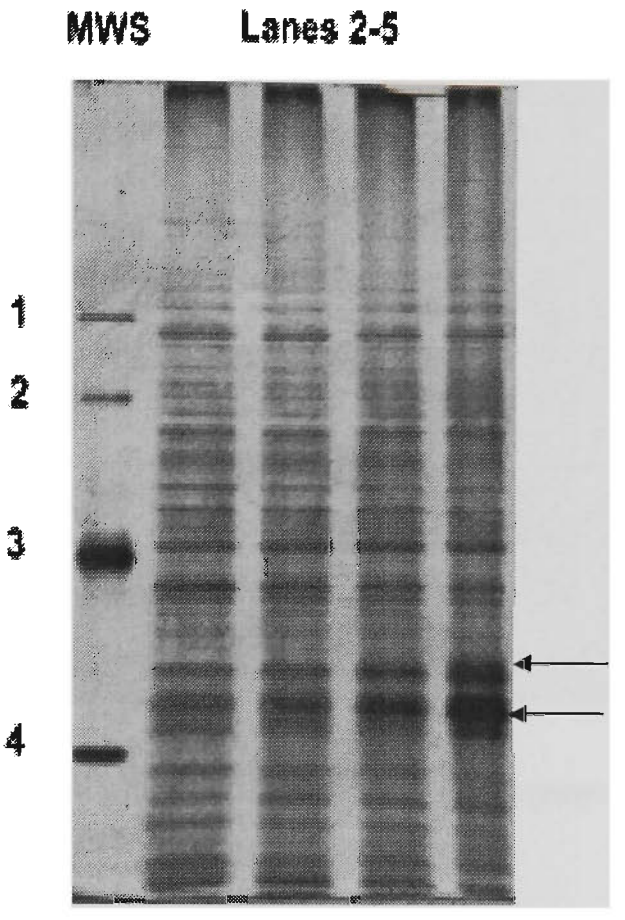


Figure 4.8 SDS-PAGE profile of strain VUP Bb 12 grown without pH control.

A culture of strain VUP Bb 12 was grown at constant temperature in a one-litre fermenter containing MRS broth with 0.05% cysteine-HCl. The inoculum was prepared by harvesting stationary phase cells of an overnight culture that was not pH controlled during growth at 42°C (section 2.3.7.1). The cells were centrifuged and made up in pre-warmed MRS broth before inoculating to an initial OD_{600} of approximately 0.2. Temperature was set at 42°C and the pH initially at pH 6.3. Samples of fermenter contents were taken every 2 hours (commencing two hours after inoculation), concentrated by centrifugation and boiled with double strength loading buffer. The treated samples were centrifuged to remove cell debris and approximately 1µg of protein was applied to each lane. Protein bands were visualised by silver staining. Black arrows at right of gel indicate area of up-regulated protein production during growth. The lower arrow indicates a protein band that has the same molecular weight as the protein band up-regulated during growth at pH 4.0.

Key
Lane 1 MW Standard, Lanes 2-5 samples collected 2 hourly

Molecular Weight Standards (MWS):
1. Phosphorylase B-97.4 kDa.
2. Bovine serum albumin-66.2 kDa
3. Ovalbumin-45 kDa
4. Carbonic anhydrase-31 kDa

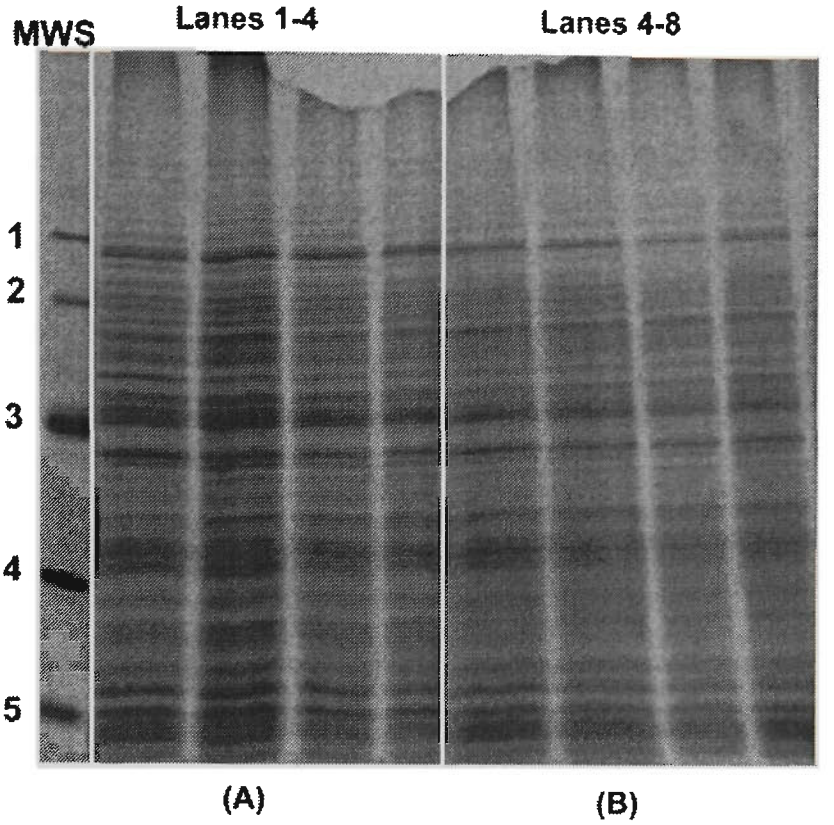


Figure 4.9 Comparison of *B. animalis* (syn. *lactis)* strain VUP Bb 12 grown in low and high oxygen environment.

Cultures of *B. animalis* (syn. *lactis)* strain VUP Bb 12 were grown at constant temperature (42°C) and pH (6.3) in one-litre fermenters containing MRS broth with 0.05% cysteine-HCl. The inoculum was prepared by harvesting stationary phase cells of an overnight culture that was not pH controlled during growth at 42°C (section 2.3.7.1). The cells were centrifuged and made up in pre-warmed MRS broth before inoculating to an initial OD_{600} of approximately 0.2. The culture in lanes 1-4 (A) was sparged with nitrogen during growth while the second culture (B) was grown in the same conditions except that instrument air (20.9% oxygen and 78.1% nitrogen) was being sparged through the system instead of nitrogen (99.9% nitrogen). Molecular weight standards (MWS) are shown on the left.

Key

Gel (A) Lanes 1-4, samples collected every two hours

Gel (B) Lanes 1-4, samples collected every two hours

Molecular Weight Standards (MWS):

1. Phosphorylase B-97.4 kDa.
2. Bovine serum albumin-66.2 kDa
3. Ovalbumin-45 kDa
4. Carbonic anhydrase-31 kDa
5. Trypsin inhibitor-21.5 kDa

4.3.6 N-terminal sequencing

The 33 kDa protein that was seen to be up-regulated during growth at pH 4.0 was submitted once for N-terminal sequencing to the Australian Proteome Analysis Facility. Protein samples were prepared from cultures grown at pH 4.0 and 37°C. Cells from the fermenter were harvested after six hours, concentrated by centrifugation and boiled with double strength loading buffer. The treated samples were centrifuged to remove cell debris and analysed by 1-D SDS-PAGE. After the presence of the 33 kDa protein was confirmed by Coomassie staining, the process was repeated except that the gel was electro-blotted to transfer the band of interest to PVDF membrane.

Amido Black 10B was used to visualise the band, which was excised from the membrane and submitted for sequencing (section 2.5.7). Results indicated that the N-terminus was blocked and sequencing data was not obtained.

4.3.7 Effect of heat, acid and oxidative stress on growth curves of strain VUP Bb 12

After determining the pH and temperature conditions for fastest growth (section 4.3.1.1) and performing preliminary SDS-PAGE gels on strain VUP Bb 12 cells, a separate set of experiments was performed to determine suitable conditions to study stress responses in detail. The temperature selected for heat stress was 45°. At 45° there was a significant effect on growth rate but an increase in biomass during growth was observed initially. The selected level of acid stress was initially pH 4.0, however this was later changed to pH 4.3 for studies into sudden decreases in pH or continuous exposure to low pH during growth. This level (pH 4.0) was determined from both prelirninary SDS-PAGE gels and growth kinetics data. Cultures grown at pH 4.0 had shown the development of a low molecular weight protein that was not seen at high pH values; however the maximum specific growth rate at pH 4.0 was very low (mean value of 0.111 over three

experiments). At pH 4.0 cultures produced insufficient levels of biomass for protein studies, but this was achieved at pH 4.3 while retaining SDS-PAGE gel characteristics.

The effect of elevated temperature, low pH and exposure to oxygen on the extent of growth was measured by changes in OD_{600} and compared to a control culture: typical results are shown in Figure 4.10. A starter culture of strain VUP Bb 12 was grown overnight as described in section 2.3.7.1 and used to inoculate the fermenters that contained MRS broth pre-heated to the selected temperature.

The relative changes in absorption for each of the fermenters varied with the different growth conditions. The biomass in the control cultures (42°C and pH 6.3) increased was approximately 11-fold after seven hours of incubation. Cultures grown at low pH increased biomass approximately 2.5-fold, reaching approximately one quarter the OD_{600} of the control. The culture grown at low pH grew slowly compared to the control strain, and failed to reach stationary phase during the first seven hours of growth at pH 4.0.

The four fermenters were set up as follows:

	Condition	**Temperature and pH**	**Gaseous environment**
(1)	Optimum growth conditions	42°C and pH 6.3	Nitrogen sparged, 0.1 L/min
(2)	Elevated temperature	45°C and pH 6.3	Nitrogen sparged, 0.1 L/min
(3)	Low pH	42°C and pH 4.0	Nitrogen sparged, 0.1 L/min
(4)	Aeration	42°C and pH 6.3	Instrument air sparged, 0.1 L/min

The biomass in culture grown at 45°C was essentially unchanged after seven hours, although the OD_{600} initially increased slightly to almost twice the the starting value after the first hour.

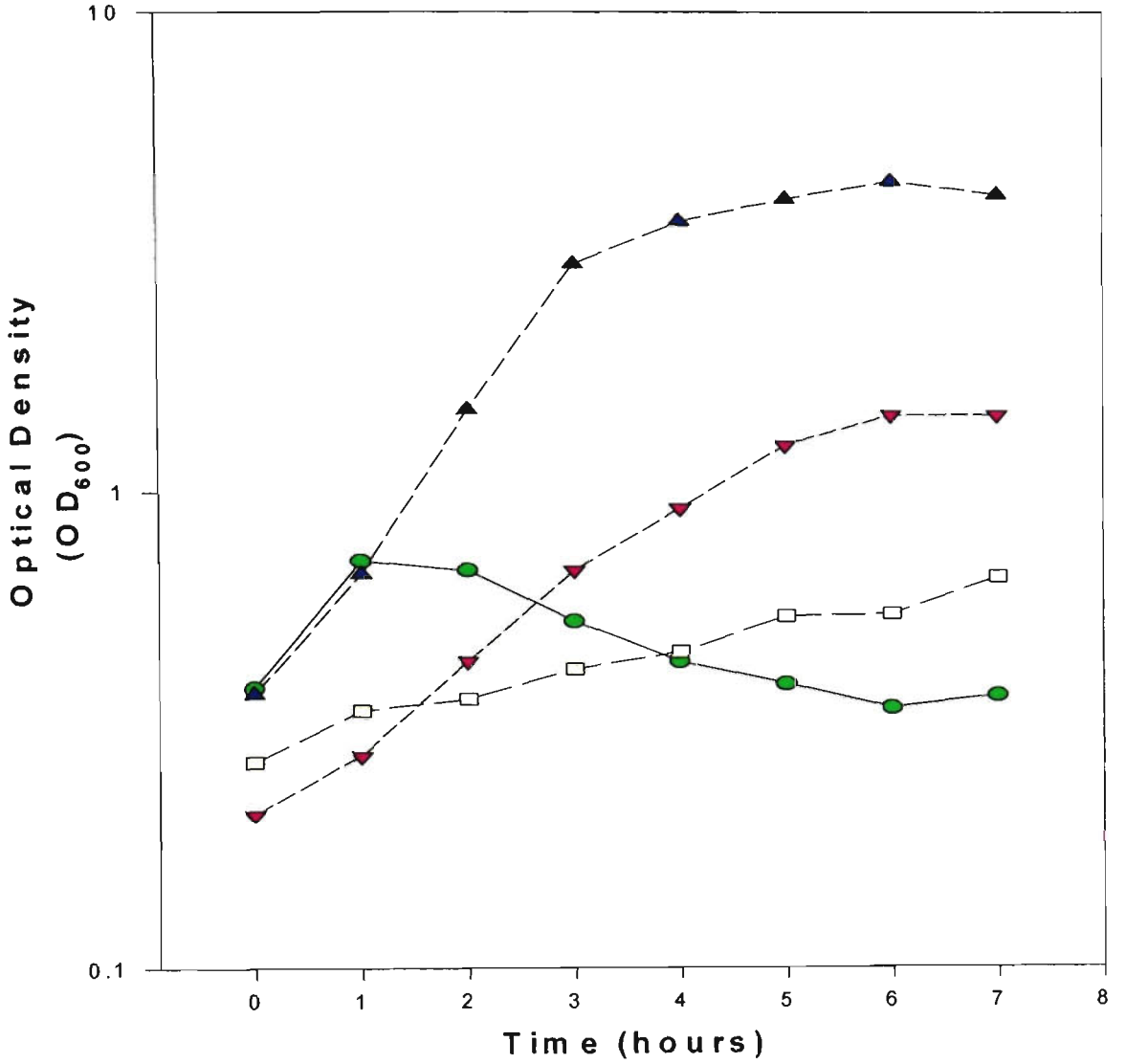


Figure 4.10 Effect of low pH and elevated temperature on the growth of strain VUP Bb 12 as measured by changes in optical density.

Cultures of strain VUP Bb 12 were grown in MRS broth in one-litre fermenters under controlled pH and temperature conditions. The inoculum was prepared by harvesting stationary phase cells of an overnight culture that was not pH controlled during growth at 42°C (section 2.3.7.1). The cells were centrifuged and made up in pre-warmed MRS broth before inoculating to an initial OD_{600} of approximately 0.2. The starter was used to inoculate four fermenters containing MRS broth preheated to the selected temperature. The set conditions of the fermenters were (1) 42°C and pH 6.3 (▲), (2) 45°C and pH 6.3 (●), (3) 42°C and pH 4.0 ( ) and (4) 42°C and pH 6.3 (▼) with aeration. Nitrogen was sparged through fermenters 1-3 at 0.1 L/min and instrument air was sparged through fermenter 4 at 0.1 L/min.

This transient increase in the OD of the culture grown at 45°C was slightly higher than that for the optimally-grown culture, but in the second hour the optical density remained stationary and in the following four hours the optical density decreased slowly. This data suggests that the temperature selected for studying heat stress may have been too high and the reduction in OD may be due to cell lysis. Cultures grown in MRS broth sparged with instrument air increased biomass around 6.5-fold. These cultures had a lower μ_{max} than the control strain and stationary phase was delayed by around two hours.

4.4 Analysis of physiological and biochemical changes in *B. animalis (*syn. *lactis)* strain VUP Bb 12 following growth in different pH conditions

Under the experimental conditions used, growth at elevated temperature and exposure to air during growth did not cause large changes in the cellular protein profile of strain VUP Bb 12 as detected by 1-D SDS-PAGE. In the case of growth at 47°C, this may have been due to cell death at the elevated temperature, shortening the period over which stress proteins could be expressed (see Figure 4.10). It was therefore decided that studies beyond the initial characterisation should focus on the effects of acid stress on strain VUP Bb 12, given that growth at low pH resulted in significant changes in cellular protein profile. The principle area of on-going examination then focussed on the responses of growing cultures to sudden decreases in pH and continuous exposure to low pH. The results of these experiments are described in the following sections.

4.4.1 Growth characteristics of VUP strain Bb 12 following sudden decrease in pH or continuous exposure to low pH

The effect of sudden decrease in pH or continuous exposure to low pH on strain VUP Bb 12 was determined by comparing changes in optical density during growth, viable cell counts, carbohydrate utilisation, 1 and 2-D SDS-PAGE profiles and FAME profiles.

This experiment was performed in duplicate, using seven 1L flasks, fitted with screw-capped lids and sampling ports, instead of fermenters; there were insufficient fermenters available to perform this experiment and at the same include all the selected growth conditions. This experimental set-up ensured that identical starter cultures and fixed growth conditions were applied to each flask. The flasks were rotated at 50 rpm on a rotating platform and were sparged through the sampling port with nitrogen immediately before inoculation and after each sampling. All flasks were incubated at 42°C. The pH was maintained using a citrate phosphate buffer system fixed at the desired pH (section 2.3.1.1). The ability of the citrate phosphate buffer to control pH at specific levels was confirmed during each experiment by the removal of an aliquot of the growth media and checking the pH was within 0.1 pH units of the set value. With the exception of Flask 7, the starter cultures were grown overnight at pH 6.3. The starter culture for Flask 7 was grown at pH 4.3. Seven flask fermentations were set up as follows:

Flask	**Growth conditions.**
Flask 1	Optimum pH 6.3
Flask 2	pH changed from 6.3 to 5.3 after 2 hours
Flask 3	pH changed from 6.3 to 4.3 after 2 hours
Flask 4	Constant pH 5.3
Flask 5	Constant pH 4.3
Flask 6	Changed from 6.3 to 5.3 after one hour then to 4.3 at the second hour
Flask 7	Inoculum grown at pH 4.3, constant pH of 4.3

4.4.1.1 Effect of acid stress on growth kinetics (as determined by optical density)

The effect of sudden decrease in pH, or continuous exposure to low pH on growth kinetics was determined by optical density of strain Bb 12 (Figure 4.11). In Figure 4.11 (A) the effect of a

sudden decrease in pH can be seen on cells grown at optimum pH (6.3) that were pH shifted to either pH 5.3 or 4.3. Clearly the most severe effect is on cells shifted to pH 4.3, where the optical density decreased significantly, almost back to the value at the start of the growth cycle. The cells also failed to recover from this sudden change in pH and the growth curve remained relatively flat after the exposure. A decrease in optical density was also noted in the cells shifted to pH 5.3, although the change was not as severe. A continuous increase in optical density was noted in the control cells, which was consistent with previous experiments.

In Figure 4.11 (B) the effect of continuous growth at pH 4.3 and 5.3 is shown compared to a control grown at pH 6.3. It can be seen that the growth rate from cells grown at pH 5.3 was very similar to that of cells grown at pH 6.3, but that rate decreased significantly late in the growth cycle. By comparison with results shown in Figure 4.11(A), it can be seen that the extent of growth of cells grown continuously at either 4.3 or 5.3 is higher than for cells shifted to that specific pH during exponential phase. This suggests that the ability of the strain to cope with low pH may be related to the growth phase.

To determine if a brief exposure to an intermediate pH was protective of exposure to lower pH, the pH of test flasks was shifted from pH 6.3 to 4.3 (flask 3) or from 6.3 to 5.3 then to 4.3 (flask 6). Figure 4.11 (C) shows that change in OD_{600} for flasks 3 and 6 were similar, which suggests that prior culture at pH 5.3 for 2 hours did not improve subsequent growth at pH 4.3. However, when the results are compared to viable cell counts (see Figure 4.12), this data suggested that a step down in pH to 5.3 prior to final transfer to pH 4.3 might have improved final cell viability.

Figure 4.11 (D) shows the comparison of two flasks grown continuously at pH 4.3. One flask was inoculated with a starter culture grown overnight at pH 4.3 (flask 7) and the other starter was grown at pH 6.3 then inoculated into broth at pH 4.3 (flask 5).

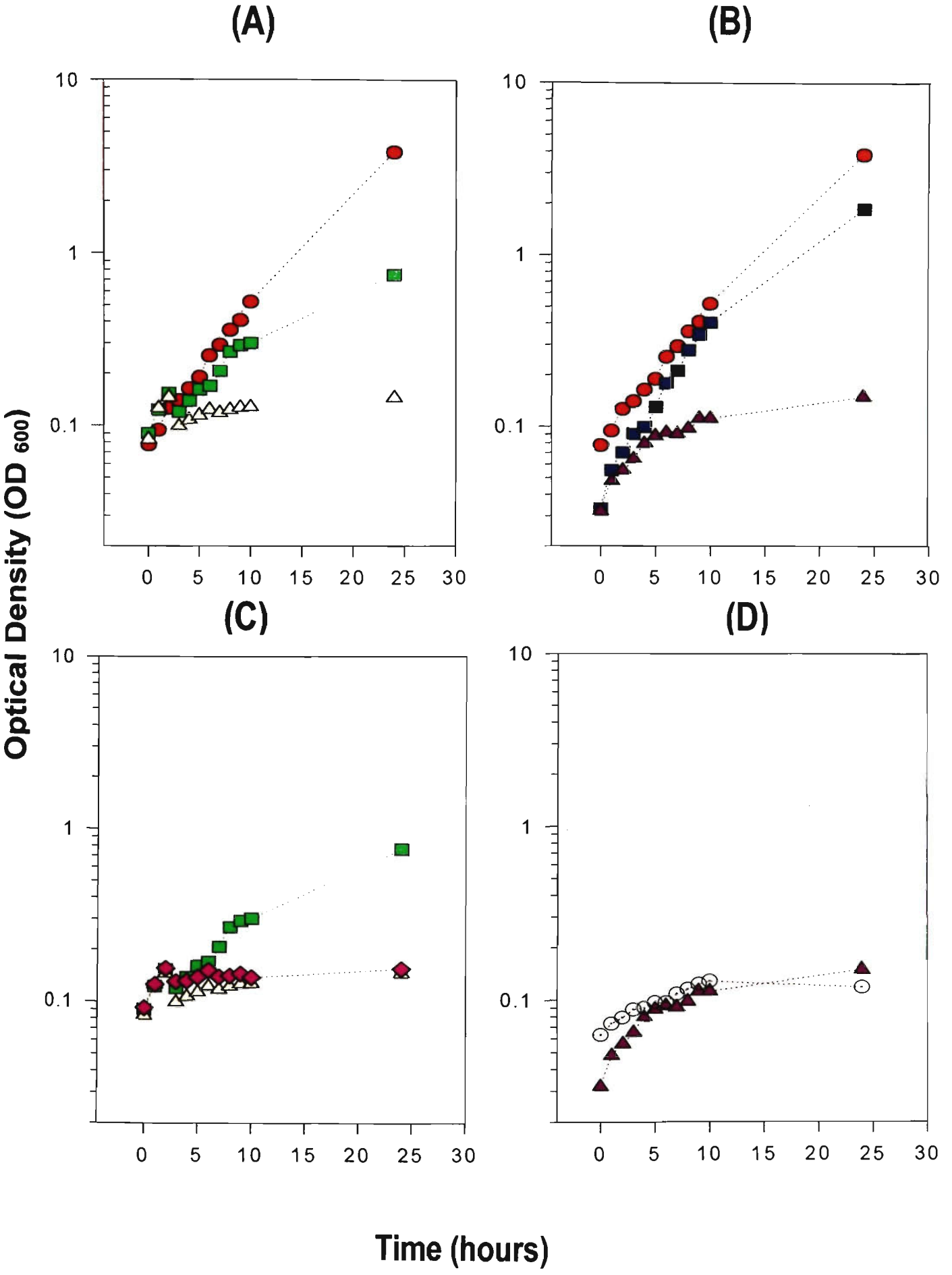
Figure 4.11 Optical density changes seen during culture of *B. animalis* (syn. *lactis*) strain VUP Bb 12 at different pH values and pH shift during growth.

Growth characteristics of *B. animalis* (syn. *lactis*) strain VUP Bb 12 during growth at low pH were determined by measuring changes in optical density. All flasks were held at constant temperature (42°C) and grown in MRS broth with 0.05% cysteine-HCl. The flasks were sparged with nitrogen, through a sampling port, every hour after sampling. Flasks were rotated at 50 rpm. Except for Flask 7, the inoculum was prepared by harvesting stationary phase cells of an overnight culture that was pH controlled at 6.3 during growth at 42°C (section 2.3.7.1). The cells were centrifuged and made up in pre-warmed MRS broth before inoculating to an initial OD_{600} of approximately 0.2, as described in section 2.3.7. The inoculum for Flask 7 was grown at pH 4.3 but otherwise was treated the same way.

The seven flasks were set up as follows:

Flask	Key	Growth conditions
Flask 1	●	Optimum pH (6.3)
Flask 2	■	pH changed from 6.3 to 5.3 after 2 hours
Flask 3		pH changed from 6.3 to 4.3 after 2 hours
Flask 4	■	Constant pH 5.3
Flask 5	▲	Constant pH 4.3
Flask 6	◆	Changed from pH 6.3 to 5.3 after 2 hours then to 4.3 after the third hour
Flask 7	⊙	Constant pH 4.3 (Inoculum grown at pH 4.3)

Graph (A) compares growth between the control (flask 1) and two flasks that started at pH 6.3 but the pH was changed after two hours to pH 5.3 (flask 2) and pH 4.3 (flask 3) respectively. Graph (B) compares growth between the control (flask 1) and two flasks grown continuously at pH 5.3 (flask 4) and pH 4.3 (flask 5). Graph (C) compares growth between three flasks. All flasks started at pH 6.3. The pH of two flasks was changed after two hours to pH 5.3 (flask 2) and pH 4.3 (flask 3). The third flask was changed to pH 5.3 after two hours and changed again after a further hour to pH 4.3. Graph (D) compares growth between two cultures grown at pH 4.3. One flask was pre-acid-stressed by growing the starter culture at pH 4.3 (flask 7) and the other starter was grown at pH 6.3 (flask 5).


(A)
(B)
(C)
(D)
10
1
0.1
0
5
10
15
20
25
30
Optical Density (OD 600)
Time (hours)

4.4.1.2 Effect of acid stress on viable cell counts

The effect of sudden decrease in pH, or continuous exposure to low pH, on viable cell counts was determined for strain VUP Bb 12 (Figure 4.12). Viable cell counts were performed by plating serial dilutions onto MRS agar plates and incubating the plates for 48 hours under anaerobic conditions (section 2.3.9.4). In Figure 4.12 (A) the effect of a sudden decrease in pH can be seen on cells growing at optimum pH then shifted to either pH 5.3 or 4.3. The viable cell count of the control culture grown at pH 6.3 increased over two orders of magnitude in the first eight hours and viability dropped only slightly in samples taken at 8 and 24 hours. Viable cell numbers in the culture grown at pH 5.3 increased almost two orders of magnitude in the first eight hours but viability decreased significantly, by over one order of magnitude, between the samples taken at 8 and 24 hours. After 24 hours growth at pH 4.3, no viable cells could be detected. Viable cell numbers increased only one order of magnitude in the first eight hours of growth at that pH. This data indicate that prolonged exposure to low pH had a significant impact on cell viability.

In Figure 4.12 (B) the effect of continuous growth at pH 4.3 and 5.3 is shown compared to a control grown at pH 6.3. At both pH 5.3 and 4.3, it can be seen that the viability of cells decreased significantly after eight hours growth. The decreases are similar to cultures that experience sudden decrease in pH during growth (flask 3). However, it must be noted that viable cells could still be detected in cultures grown continuously at pH 4.3 after 24 hours, whereas no viable cells could be detected in the culture experiencing the sudden pH decrease to pH 4.3.

To determine if a brief exposure to an intermediate pH decrease was protective of subsequent exposure to low pH, the pH of test flasks was shifted from pH 6.3 to 4.3 (flask 3) or from 6.3 to 5.3 then to 4.3 (flask 6). Figure 4.12 (C) shows the results of those changes. The findings showed that after twenty-four hours viable cells are still present in flask 6, while no viable cells could be detected in flask 3.

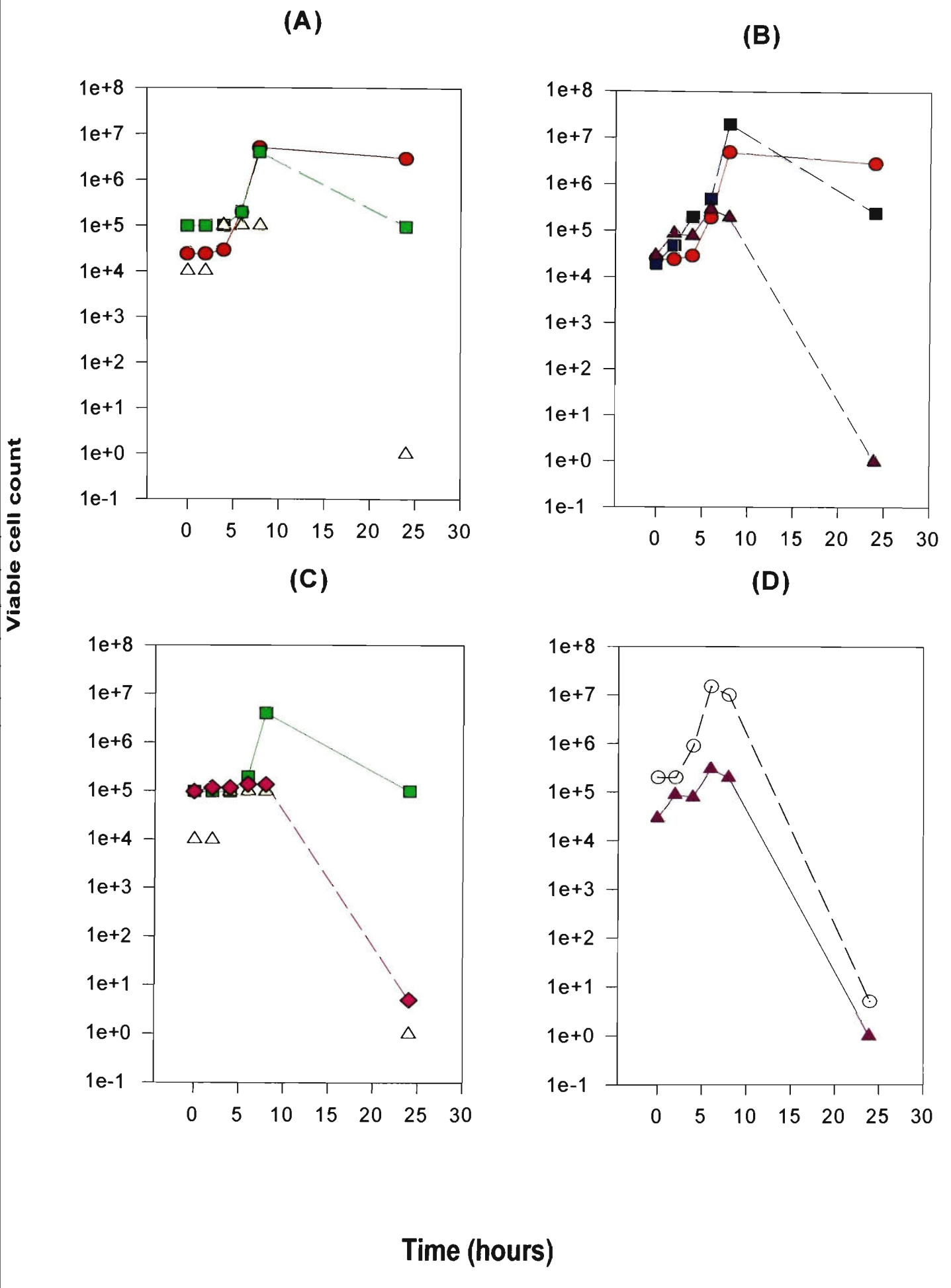
Figure 4.12 Viable cell count changes seen during culture of *B. animalis* (syn. *lactis)* strain VUP Bb 12 at different pH values and pH shift during growth.

Growth characteristics of *B. animalis* (syn. *lactis)* strain VUP Bb 12 during growth at low pH were determined by measuring changes in viable cell count. All flasks were held at constant temperature (42°C) and cultures grown in MRS broth with 0.05% cysteine-HCl. The flasks were sparged with nitrogen, through a sampling port, every hour after sampling. Flasks were rotated at 50 rpm. Except for Flask 7, the inoculum was prepared by harvesting stationary phase cells of an overnight culture that was pH controlled at 6.3 during growth at 42°C (section 2.3.7.1). The cells were centrifuged and made up in pre-warmed MRS broth before inoculating to an initial OD_{600} of approximately 0.2, as described in section 2.3.7. The inoculum for Flask 7 was grown at pH 4.3 but otherwise was treated the same way.

The seven flasks were set up as follows:

Flask	Key	Growth conditions
Flask 1	●	Optimum pH 6.3
Flask 2	■	pH changed from 6.3 to 5.3 after 2 hours
Flask 3		pH changed from 6.3 to 4.3 after 2 hours
Flask 4	■	Constant pH 5.3
Flask 5	▲	Constant pH 4.3
Flask 6	◆	Changed from pH 6.3 to 5.3 after 2 hours then to 4.3 after the third hour
Flask 7	⊙	Constant pH 4.3 (Inoculum grown at pH 4.3)

Graph (A) compares growth between the control (flask 1) and two flasks that started at pH 6.3 but the pH was changed after two hours to pH 5.3 (flask 2) and pH 4.3 (flask 3) respectively. Graph (B) compares growth between the control (flask 1) and two flasks grown continuously at pH 5.3 (flask 4) and pH 4.3 (flask 5). Graph (C) compares growth between three flasks. All flasks started at pH 6.3. The pH of two flasks was changed after two hours to pH 5.3 (flask 2) and pH 4.3 (flask 3). The third flask was changed to pH 5.3 after two hours and changed again after a further hour to pH 4.3. Graph (D) compares growth between two cultures grown at pH 4.3. One flask was pre-acid-stressed by growing the starter culture at pH 4.3 (flask 7), the other starter was grown at pH 6.3 (flask 5). When no viable cells were detected (<10/mL), this is shown as 1e+0 in all panels.

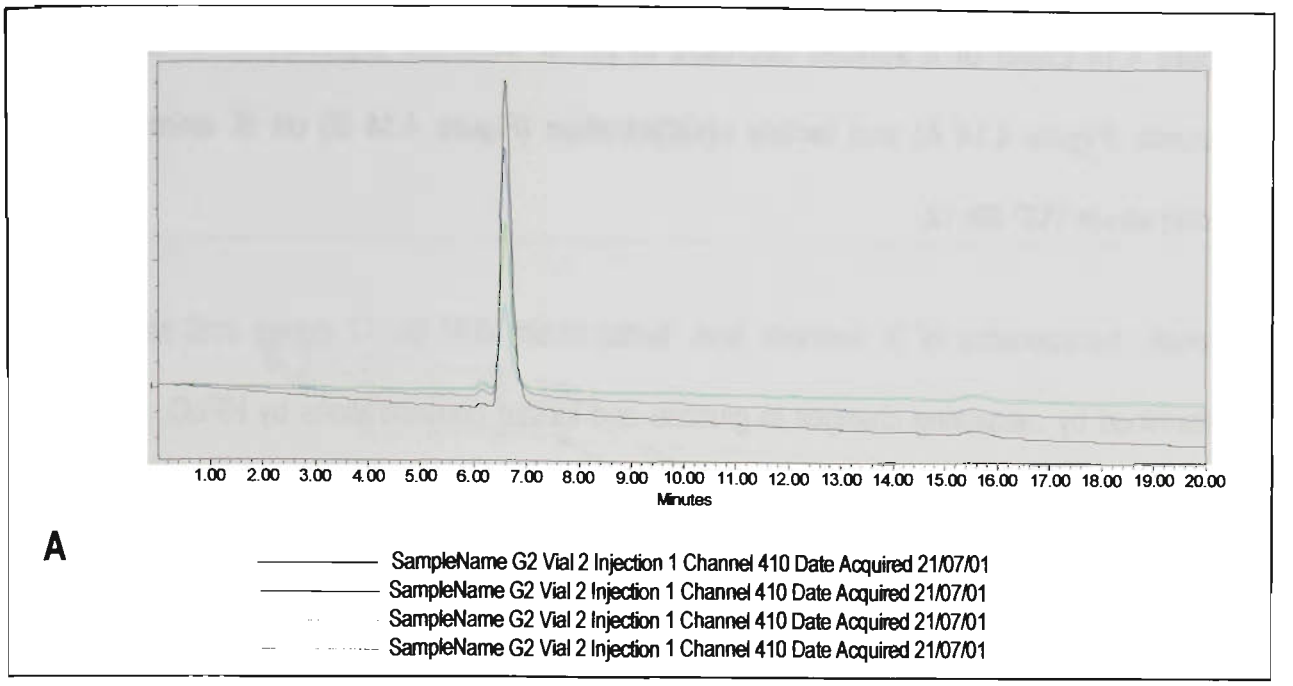

(A)
(B)
(C)
(D)
Viable cell count
1e+8
1e+7
1e+6
1e+5
1e+4
1e+3
1e+2
1e+1
1e+0
1e-1
0
5
10
15
20
25
30
Time (hours)

These results suggest some protection of cell viability may have occurred through prior exposure to pH 5.3 before shifting to pH 4.3, an observation that could not be seen from OD_{600} measurements. Figure 4.12(D) shows the comparison of two flasks grown continuously at pH 4.3. One flask was inoculated with a starter culture grown overnight at pH 4.3 and the other starter was grown at pH 6.3 then sub-cultured into broth at pH 4.3. Compared to the results observed for optical density, where pre-stressed cells demonstrated slightly poorer growth than the control, two minor differences were observed between the two cultures. Firstly, the increase in viable cells from the culture grown using an inoculum grown at pH 4.3 was over two orders of magnitude compared to an increase of less than half that in the non-stressed starter. Secondly, viable cells could be detected after twenty-four hours growth of the pre-stressed cultures. This data suggests that prior growth at low pH may assist subsequent growth and survival at low pH.

4.4.1.3 Effect of acid stress on carbohydrate utilisation

HPLC was used to measure changes in carbohydrate concentration and to determine what metabolic by-products were produced during growth. Examples of HPLC traces of standards and samples are shown in Figure 4.13. Comparison to the retention time of known standards was used to show utilisation of glucose and production of lactate and acetate. The limit of detection for glucose, acetate, citrate and formate was 0.2 g/l, and 0.1 g/L for lactate. Changes in citrate concentration reflected the concentration of the citrate-phosphate buffer used to control pH. Four other peaks were detected by HPLC, including formic acid, but the concentration of these analytes did not change either during growth under optimum conditions or at low pH (Figure 4.13).

The results for glucose utilisation and lactate production are shown in Figure 4.14. The glucose concentration was 20 g/L in all flasks at the start of growth. After 24 hours growth, the glucose level of both the control, growing at pH 6.3, and the flask growing constantly at pH 5.3 fell to around 14 g/L.



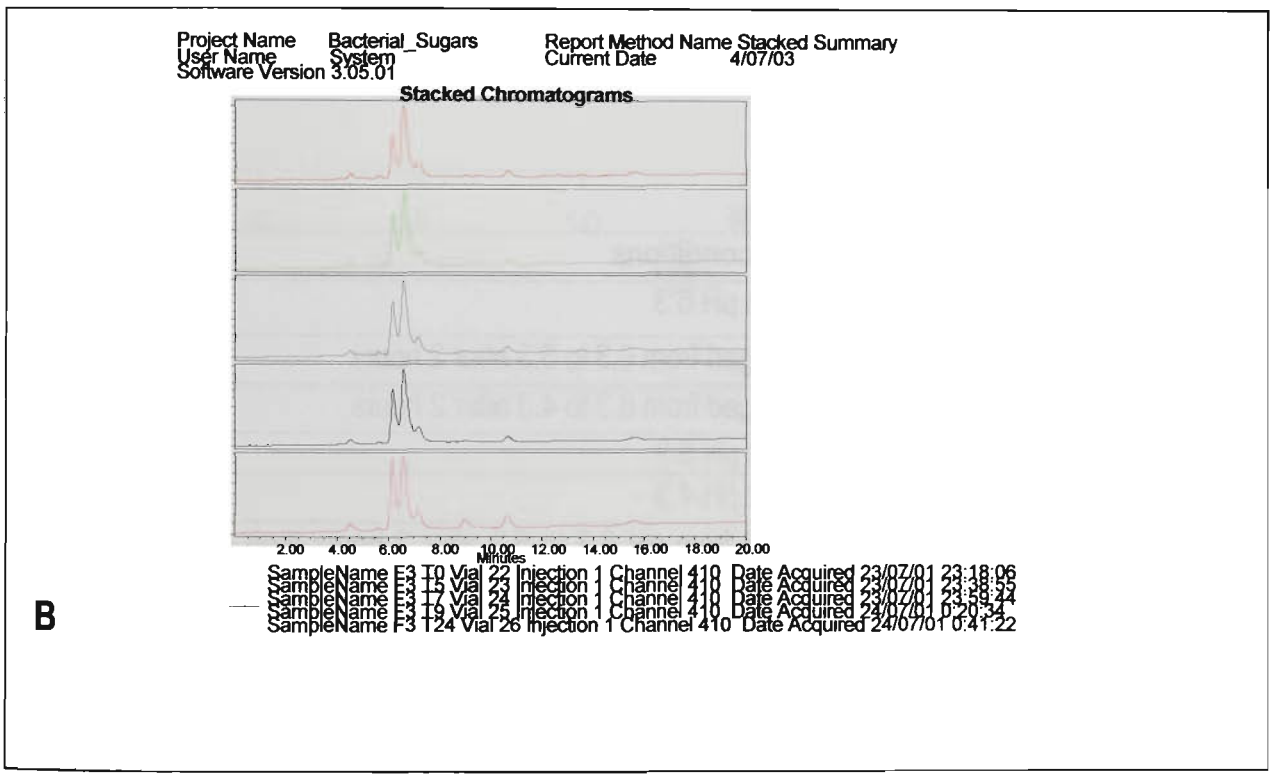


Figure 4.13 HPLC standards and samples.

Growth characteristics of *B. animalis* (syn. *lactis*) strain VUP Bb 12 were determined by measuring changes in glucose and lactate concentration by HPLC. Glucose standards (A) (0.2 to 1.0 g/L) were prepared from 1.0 g/L stock. Samples (examples were collected from a single fermenter at the time intervals indicated) are shown in (B).

Figure 4.14 Effect of a sudden decrease in pH or constant exposure to acidic pH on glucose (Figure 4.14 A) and lactate concentration (Figure 4.14 B) on *B. animalis* (syn. *lactis*) strain VUP Bb 12.

Growth characteristics of *B. animalis* (syn. *lactis*) strain VUP Bb 12 during acid stress were determined by measuring changes in glucose and lactate concentrations by HPLC. All flasks were held at constant temperature (42°C) and cultures grown in MRS broth with 0.05% cysteine-HCl. The flasks were sparged with nitrogen every hour after sampling. Flasks were rotated at 50 rpm. Starter cultures were prepared as described in section 2.3.7, one was grown at pH 6.3 and used to inoculate flasks1-6, the other was grown at pH 4.3 and was used to inoculate flask 7. Samples were taken every hour over the first nine hours of growth and again after 24 hours.

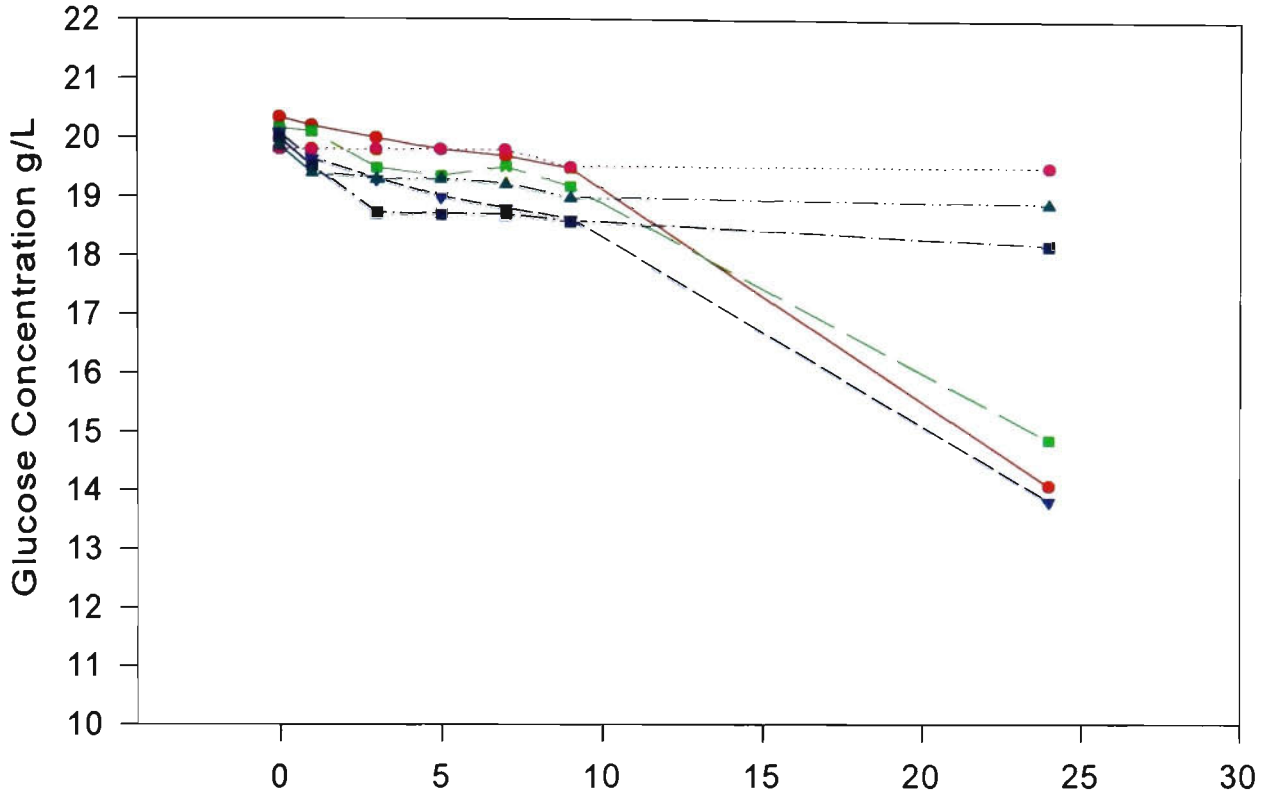
The seven flasks were set up as follows:

Flask	Key	Growth conditions
Flask 1	●	Optimum pH 6.3
Flask 2	■	pH changed from 6.3 to 5.3 after 2 hours
Flask 3	■	pH changed from 6.3 to 4.3 after 2 hours
Flask 4	▼	Constant pH 5.3
Flask 5	●	Constant pH 4.3
Flask 6	▲	Changed from pH 6.3 to 5.3 after 2 hours then to 4.3 after the third hour
Flask 7		Constant pH 4.3 (Inoculum grown at pH 4.3)

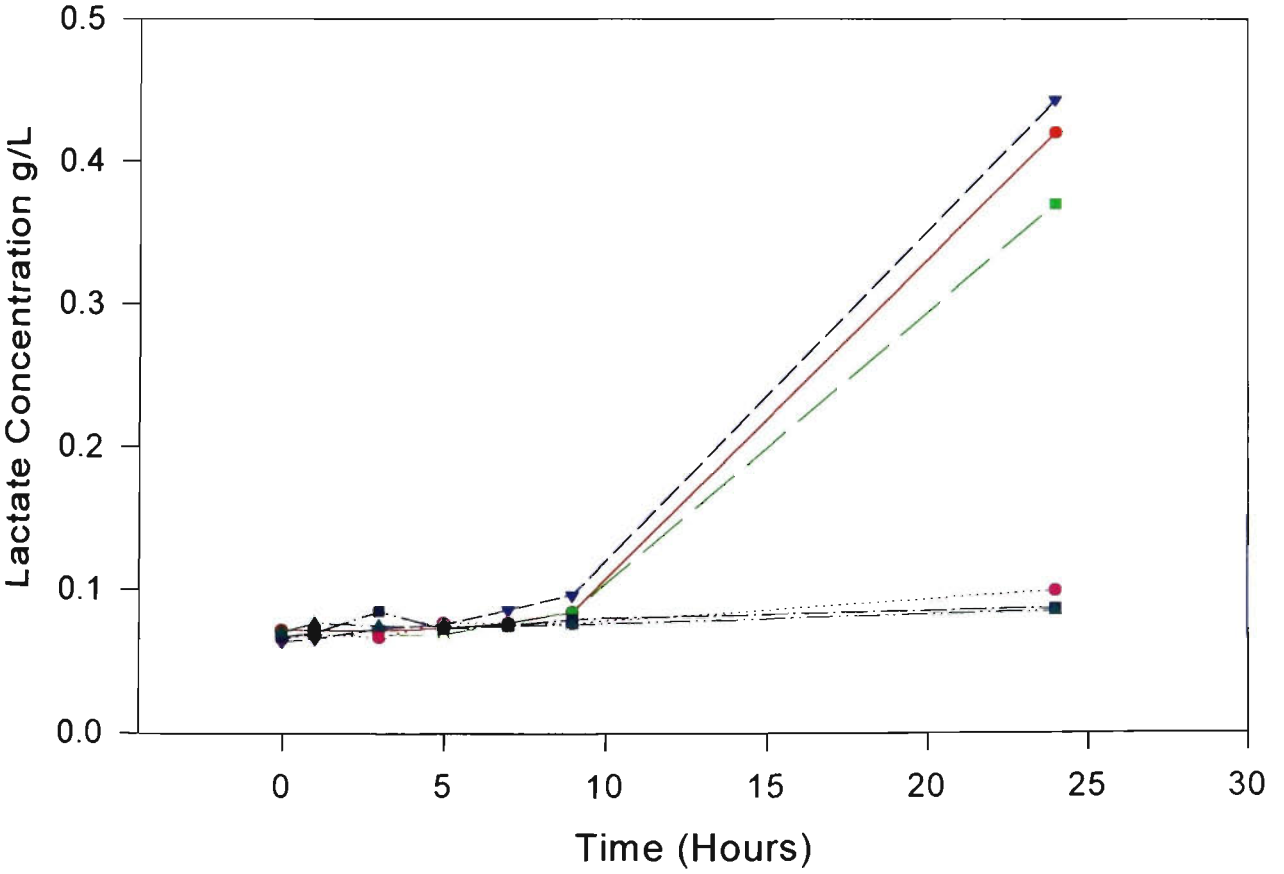
(A) Glucose utilisation

(B) Lactate production

(A)


Glucose Concentration g/L
22
21
20
19
18
17
16
15
14
13
12
11
10
0
5
10
15
20
25
30

(B)


Lactate Concentration g/L
0.5
0.4
0.3
0.2
0.1
0.0
0
5
10
15
20
25
30
Time (Hours)

The cultures in which the pH was changed to 5.3 after 2 hours reached around 15 g/l after the same time.

The cultures that were grown at pH 4.3 or shifted to pH 4.3 during the growth cycle, failed to utilise significant amounts of glucose once at pH 4.3, which is consistent with the relatively poor growth and subsequent viability of cells seen in these flasks (see Figure 4.12). In general, increased lactate levels were not detected in any flask during the first five hours of growth and levels mainly increased during the period between the ninth and twenty-fourth hours.

However, cultures grown at pH 4.3, including those that experienced sudden changes to pH 4.3 produced only very minor levels of lactate during the twenty-four hours over which samples were collected. The control, grown at pH 6.3, and the culture grown constantly at pH 5.3 produced the highest levels of lactate, around 0.42 g/L and 0.45 g/L respectively. The culture that was shifted to pH 5.3 after two hours produced around 0.37 g/L of lactate. This suggests that the ability to grow at pH 4.3 was very limited.

4.4.2 Effect of growth at low pH on protein profiles: 2-D SDS-PAGE Profiles

1-D SDS-PAGE profiles revealed little in terms of regulation of minor protein during sudden decrease in pH or continuous exposure to low pH, so it was decided to perform 2-D SDS-PAGE to better assess protein regulation during acid stress.

A method that was based on extraction of proteins in the presence of solvents was developed in an attempt to improve the extraction of hydrophobic proteins. This method development is shown in the following section. A more traditional method of separation of cytosolic proteins based on iso-electric focussing was also used to assess protein regulation during growth at low pH. This data is shown in section 4.4.2.3.

4.4.2.1 Methods development

As part of the preparation for 2-D SDS-PAGE, cell homogenates were prepared using a Bead beater-8 ® and 40mM Tris pH 7.0, as described in section 2.4.1.3. The protein profiles prepared using this technique had a major disadvantage, however, as the 33 kDa protein seen during acid stress was not detected in these cell-free extracts. This suggested that the protein may be a cell surface or membrane protein that would be retained in the cell debris after centrifugation of cell lysates. The cell debris, from broken cells, was therefore collected and prepared for SDS-PAGE in the same manner as whole cells. Analysis of protein profiles from this procedure revealed that the protein of interest was indeed retained with the cell debris and a method of extracting the protein would have to be found if the 33 kDa protein were to be tracked using 2-D SDS-PAGE. These results are shown in Figure 4.15. It should be noted that fine cellular material, which was not centrifuged from the samples, has stained as a strong dark background in the lane loaded with proteins prepared from cellular debris.

Cell stressors, by their nature, are often in close contact with cell membranes, and it was considered important to develop a method that preferentially extracted hydrophobic proteins so that changes in membrane proteins during cell stress could be observed. Given the difficulties associated with the use of 2-D SDS-PAGE as an analytical tool to study membrane proteins, in particular poor solubilization of membrane proteins, a literature search was performed to indicate suitable methodology. The literature suggested detergent partition and solvent extraction as two possible methods to extract hydrophobic proteins (Lopez *et al.*, 1991; Kobayashi *et al.*, 1998; Ferro *et al.*, 2000). In the end, two methods were examined: the first, using detergent extraction, was adapted from commercially available methods (Sigma, ProteoPrep Protein Precipitation Kit), and the second, extraction of protein using various solvents, was tested. In the first method, proteins were extracted in a bead beater cell in the presence of non-denaturing zwitterionic detergents made up in 40 mM Tris pH 7.

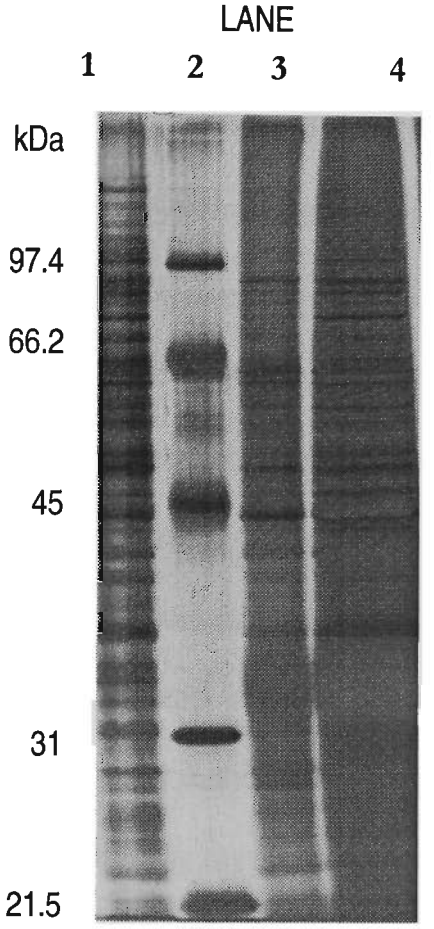


Figure 4.15 Comparison of protein banding between samples prepared three ways.

Proteins were prepared from a single culture of *B. animalis* (syn. *lactis*) in a bead beater. Cells were resuspended in 40mM Tris pH 7. After bead beating the sample was centrifuged and both the clear supernatant and pellet were collected. A control sample of centrifuged whole cells was also collected. Approximately 200 µL of each sample was added to an equal volume of double strength loading buffer and heated to 100°C for 5 minutes. The samples were centrifuged and 20µL was loaded onto a 1-D SDS-PAGE and visualised by silver staining.

Lane	Sample	Solvent solution
1	Supernatant removed from cellular debris	40mM Tris pH 7
2	MW Standard	
3	Whole cells	40mM Tris pH 7
4	Pellet of cellular debris left after bead beating	Double strength loading buffer

The detergents used were CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propane sulfonate) and Triton X-100 made up as described in Table 4.3. Cells were obtained from a culture grown at pH 4.3 and were harvested after 6 hours. These zwitterionic detergents do not denature proteins and they were also selected for compatibility with the reagents used in the first dimension separation of 2-D SDS-PAGE.

The extractions were performed as described in section 2.4.1.3. Protein profiles were compared using 1-D SDS-PAGE. The results are shown in Figure 4.16. Figure 4.16 shows that the single detergent extractions have an almost identical banding pattern to the samples prepared using TRIS alone. The banding pattern produced from the mixture of detergents (CHAPS and Triton X-100) showed only a slightly different banding pattern to that produced from the single detergent extractions. While this may appear to indicate that hydrophobic proteins might have a slightly higher representation in samples prepared using the detergent mixture, the banding patterns were not dissimilar enough to pursue this method as a means of extracting hydrophobic proteins. The second method used to preferentially extract hydrophobic proteins examined a number of common solvents, including octanol, acetone, chloroform, ethanol, methylated spirit, diethyl ether and n-hexane. The solvents used in this method varied in boiling point (34-195°C), density (0.60-1.492 g/mL) and miscibility with water, see Table 4.4.

Extractions were performed in a bead beater-8, as described in section 2.4.1.3. Extracts were prepared in duplicate from cells grown overnight in MRS broth at 42°C. The cells were centrifuged and divided equally (0.5 g wet weight) into bead beater tubes prior to solvent extraction. After treatment both the solvent and aqueous layers were removed separately and dried under nitrogen. After drying the samples were made up in 200 µL of 40 mM Tris pH 7.

Table 4.3. Solutions used for protein extraction from cell homogenates of strain VUP Bb 12.

	Extraction solution
Control 1	40mM Tris pH 7
Treatment 1	CHAPS 4% (w/v) in 40mM Tris pH 7
Treatment 2	Triton X-100 2% (w/v) in 40mM Tris pH 7
Treatment 3	CHAPS 4% (w/v) and Triton X-100 2% (w/v) in 40mM Tris pH 7
Control 2	Protein extract prepared by boiling the cells in double strength loading buffer

Table 4.4. Physical properties of solvents used to extract proteins from homogenates of strain VUP Bb 12.

	Boiling Point (Range)	Density d^{20}_4 (g/ml)	Miscibility with water
Octanol	193-195 °C	0.82	no
Acetone	55.5- 56.5 °C	0.790-0.792	yes
Chloroform	60.5-61.5 °C	1.492	no
Ethanol (95%)	80 °C	0.79	yes
Di-ethyl ether	34-35 °C	0.713-0.714	no
n-Hexane	69 °C	0.66	no
Methylated spirit	76-79 °C	0.806	yes

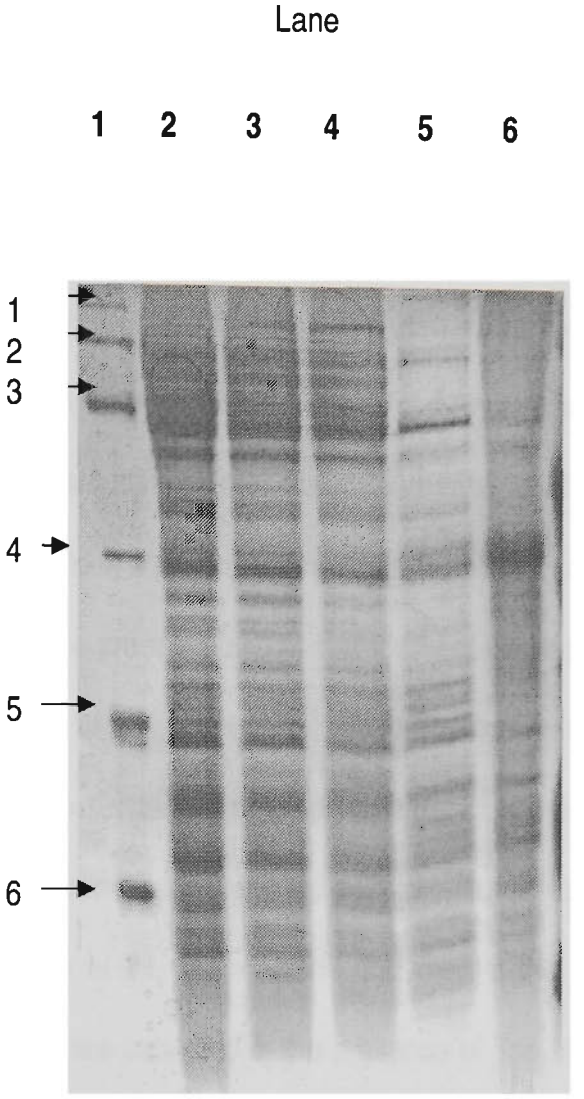


Figure 4.16 Comparison of detergent extraction methods.

Proteins were extracted from a single culture of *B. animalis* (syn. *lactis*) in a bead beater cell using non-denaturing zwitterionic detergents (CHAPS and Triton X-100). The extractions were performed as described in section 2.2.6.4. Protein profiles were compared using 1-D SDS-PAGE.

Lane	Sample	Extraction solution
1	Standards	1. 97.4 kDa 2. 66.2 kDa 3. 45 kDa 4. 31 kDa 5. 21.5 kDa 6. 14 kDa
2	Control 1	40mM Tris pH 7
3	Treatment 1	CHAPS 4% (w/v) in 40mM Tris pH 7
4	Treatment 2	Triton X-100 2% (w/v) in 40mM Tris pH 7
5	Treatment 3	CHAPS 4% (w/v) and Triton X-100 2% (w/v) in 40mM Tris pH 7
6	Control 2	Protein extract prepared by boiling the cells in double strength loading buffer

The aqueous layer in the octanol, chloroform and n-hexane treatments produced a gel rather than a liquid, which was difficult to handle and dry. 1-D SDS-PAGE gels were loaded with 20μL of protein-rich extract prepared by boiling 200μL of protein-rich extract with 200μL of double strength loading buffer. The results are shown in Figure 4.17. Several quite different banding patterns were obtained from a single culture. A total of 27 major bands could be identified from all of the extraction techniques combined; a major band constituted more than 5% of the sum of protein contained in all bands in a particular lane. Fifteen major bands were observed in protein samples for cells lysed in the presence of Tris buffer and glass beads alone, the highest of any treatment. Only one major band was observed in the octanol-treated samples. The banding patterns that most closely resembled the samples prepared in the presence of Tris buffer and glass beads alone, came from the water-miscible solvents, acetone, ethanol and methylated spirit. The size distribution of the major bands observed in the water-miscible solvents was almost identical to the samples extracted in the presence of Tris buffer and glass beads alone; however, some bands were present in higher amount compared to the Tris samples. In both the acetone and ethanol treated samples, bands at approximately 92, 68, 64, 61 and 51 kDa appeared to be preferentially extracted. Between 20 and 50% more of these bands was extracted by the addition of the solvents.

The water immiscible solvents produced markedly different banding patterns to the Tris-treated samples. Both octanol and chloroform produced a single major band of approximately 102 kDa. This band was seen in the aqueous and solvent layer of the octanol treated cells, although not as strongly in the aqueous layer. The aqueous layer of the chloroform treated cells appeared more like the Tris suspended cells but the clarity of the banding pattern was affected by the presence of strong background staining probably caused by cell debris caught up in the gel matrix from which the sample was prepared.

Key

Lane	Solvent used to assist extraction of proteins	Sample
1	-	Molecular Weight Standards 97.4 kDa 66.2 kDa 45 kDa 31 kDa
2	Octanol	aqueous layer
3	Acetone	solvent layer
4	Chloroform	solvent layer
5	Ethanol	aqueous layer
6	Ether	solvent layer
7	n-Hexane	solvent layer
8	Methylated spirit	aqueous layer
9	-	Tris-extracted cells
10	-	Tris-extracted cells
11	-	Biomass (whole cells boiled with equal volume of double strength loading buffer)
12	Octanol	solvent layer
13	Chloroform	aqueous layer
14	Ether	aqueous layer
15	n-Hexane	aqueous layer

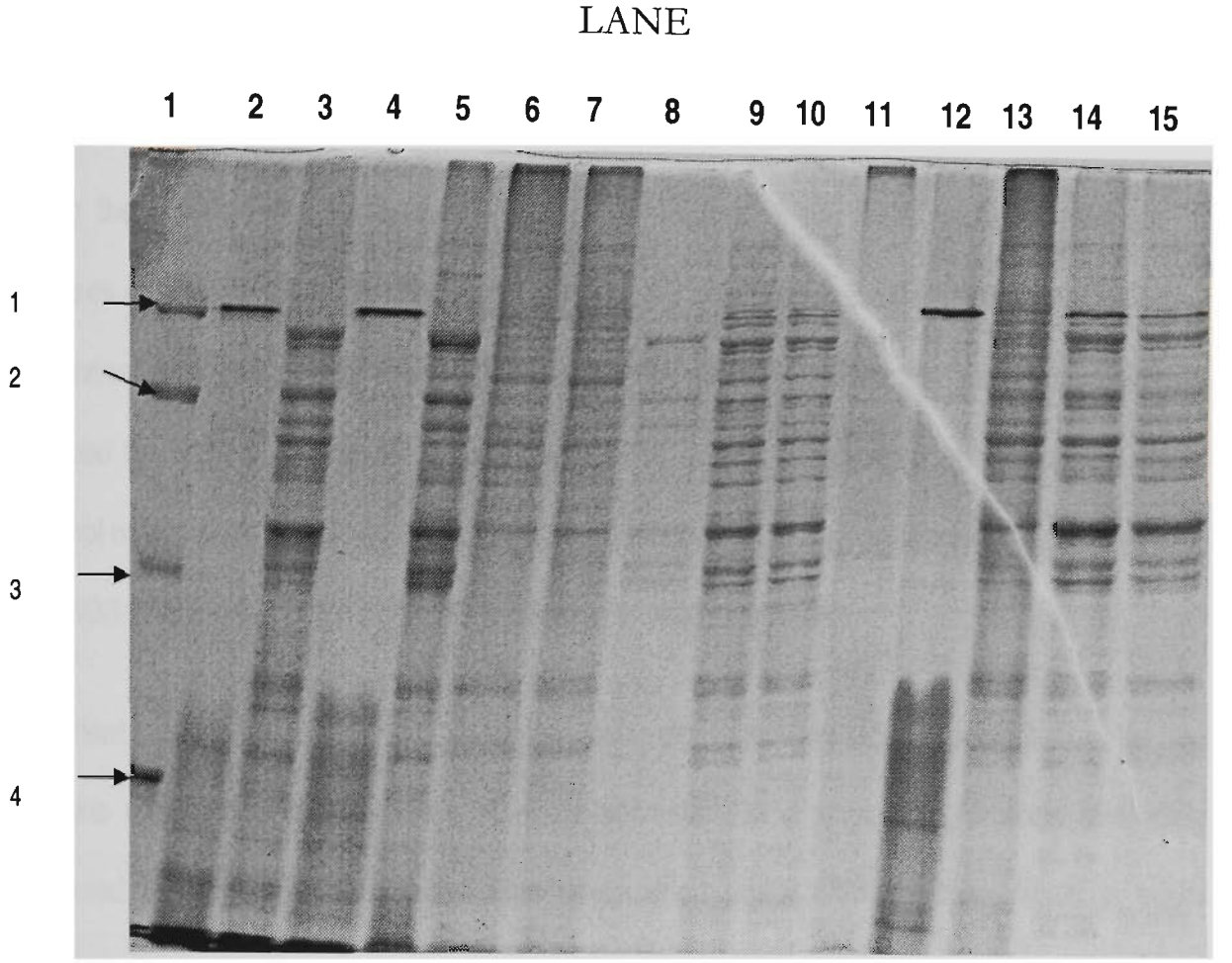


Figure 4.17 Solvent extraction of proteins from *B. animalis* (syn. *lactis)* strain VUP Bb 12.

B. animalis (syn. *lactis*) strain Bb 12 was grown overnight in MRS broth at 42°C. The culture was centrifuged and the cells divided into equal wet weights of 0.5 g. The cells were disrupted using the bead beater system, as described in section 2.2.6.4. A range of solvents was added to each sample to assist extraction of hydrophobic proteins. Where the solvent was immiscible in water, both the solvent and aqueous layers were collected then dried under nitrogen. A single sample was collected for water miscible solvents, which was also dried under nitrogen. These samples and whole cells were further prepared by boiling in double strength loading buffer. Equal loadings of 20 µL of the centrifuged supernatant were then loaded onto 1-D SDS-PAGE to compare the effectiveness of the extraction procedure.

The other water immiscible solvents, diethyl ether and n-hexane, produced very similar banding patterns to each other. Only six major bands were produced, which were all present in the Tris-suspended samples.

Using the results from the solvent extraction experiment, trials were made to determine if mixtures of solvents could be used to extract proteins from bacterial cells. These trials indicated that extracting proteins in the presence of a mixture of solvents of either 2 or 3 solvents drastically reduced the yield of protein for each sample. For example, a mixture of octanol, ether and ethanol or a mixture of hexane and chloroform produced insufficient protein to be visualised on a 1-D SDS PAGE gel. The cause of the reduction in yield is unclear.

Sufficient protein for testing was, however, obtained by mixing equal concentrations of the proteins extracted in the presence of single solvents. In a single trial, proteins separately extracted in chloroform and diethyl ether were dried under nitrogen, then mixed in equal concentrations after they had each been re-dissolved in Tris buffer. Comparison of proteins extracted in the presence of chloroform and diethyl ether and the mixture described above is shown in Figure 4.18.

The results indicated that a benefit (a reduction in the number of 2-D SDS PAGE gels that may be needed for comparison of profiles) could be obtained from preparing 2-D SDS PAGE gels from a 50:50 mixture of two water immiscible solvents with different banding patterns. Water miscible solvents had been shown to have banding patterns that were very similar to that of proteins extracted only in the presence of Tris buffer, and it would not have been advantageous to use one of those solvents in a mixture. In the following sections 'proteins extracted using solvents' refers to the combined chloroform and diethyl ether extracts mixed as described above.

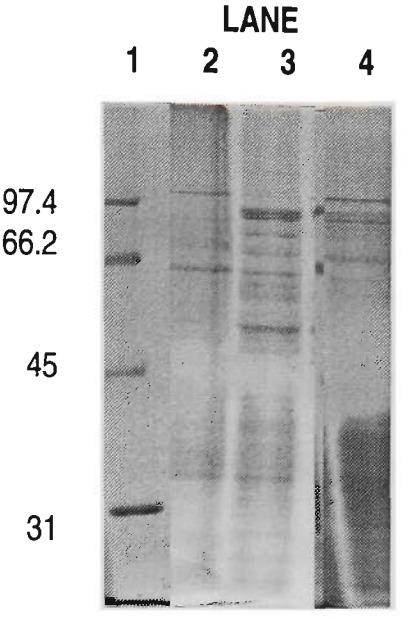


Figure 4.18 Comparison of proteins extracted in the presence of chloroform, diethyl ether and a mixture of chloroform and diethyl ether.

B. animalis (syn. *lactis*) strain VUP Bb 12 was grown overnight in MRS broth at 42°C and pH 4.3. The culture was centrifuged and the cells divided into equal wet weights of 0.5 g. The cells were disrupted using the bead beater system, as described in section 2.2.6.4. Either chloroform or diethyl ether was added to each sample to assist extraction of hydrophobic proteins. The solvent layer from each sample was collected, dried under nitrogen then re-dissolved in 200 µL of Tris buffer. 100 µL of each of these extracts were mixed to make a 50:50 mixture of each of the chloroform and diethyl ether extracts. All three samples were further prepared by boiling in an equal volume of double strength loading buffer. Equal loadings of 20 µL of the centrifuged supernatant were then loaded onto 1-D SDS-PAGE and compared after visualisation using a silver stain.

Lane	Sample	Lane	Sample
1	MW Standards	3	Diethyl ether treatment
2	Chloroform treatment	4	50:50 mixture of chloroform and diethyl ether extracts

4.4.2.2. 2-D SDS-PAGE for analysis of solvent-extracted proteins

2-D SDS-PAGE was performed on cultures grown at pH 6.3 and pH 4.3 (optimum pH and acid stressed conditions) in the conditions described for flask 2 and flask 5 in section 4.4.1. Up to 5 gels were run on the same samples using varying protein concentrations. Varying the loading allowed examination of all protein spots without problems of overloading masking proteins present in low abundance. The gels shown here are the ones that allowed proteins with altered regulation to be most easily observed. Proteins were extracted in the presence of either Tris or solvent, as described in sections 2.4.1.3 and 2.5.2. Samples were collected six hours after inoculation of the flasks. The results are shown in Figures 4.19, 4.20, 4.21 and 4.22. Figures 4.19 and 4.20 show 2-D SDS-PAGE profiles of cultures that were grown at pH 6.3. Figure 4.19 shows proteins that were present when cells were lysed in 40 mM Tris pH 7.0 and Figure 4.20 shows proteins extracted using solvent. Figures 4.21 and 4.22 show 2-D SDS-PAGE profiles of cultures that were grown at pH 4.3. Figure 4.21 shows proteins that were extracted using 40 mM Tris pH 7.0 and Figure 4.22 shows proteins extracted using solvent.

Over 150 protein spots could be detected in samples that were present when cells were lysed in Tris buffer in cultures grown at pH 6.3 and 4.3, and the spot profiles vary for each of the samples. In particular at least six proteins are up-regulated during growth at pH 4.3. These proteins are circled in red in Figure 4.21. Samples that were extracted in the presence of solvents demonstrated around half the number of protein spots that could be seen in Tris-extracted samples, but a number of up-regulated proteins could be seen and these are circled in Figure 4.22. Significantly, none of the proteins that were seen to be up-regulated in the Tris-extracted samples equated to the spots in the solvent-extracted samples in terms of pI and molecular weight. The 33 kDa protein could also be detected, but always appeared as a smear rather than a distinct spot. The best estimation at a pI was about 4.9. The 33 kDa protein is circled in blue in Figure 4.22.

Figure 4.19 Computer processed image of two dimensional SDS-PAGE of *B. animalis* (syn *lactis) strain VUP Bb 12* grown at pH 6.3 (cleared lysate in Tris)

2-D electrophoresis was performed on samples of proteins extracted from strain VUP Bb 12 grown in 1L fermenters in MRS broth with 0.05% cysteine hydrochloride. The temperature was controlled at 42°C and the pH at 6.3. Nitrogen was sparged through the fermenter at 0.1 L/min. Samples were collected six hours after inoculation of the fermenter and protein extracted according to the method described in section 2.4.1.3. The protein samples were desalted and the protein concentration measured. First dimension isoelectric focussing of a 20 μg protein sample was performed on a linear pH 4-7 immobilised pH gradient strip (Amrad Pharmacia Biotech) according to section 2.5.2. The second dimension SDS-PAGE was carried out on a homogeneous resolving gel (12%) and a 4% stacking gel. Protein detection was performed by silver staining according to section 2.5.3.2.

Key

Position (lane or strip)

1	Molecular weight standards 1. 97.4 kDa 2. 66.2 kDa 3. 45 kDa 4. 31 kDa 5. 21.5 kDa 6. 14 kDa	3	Molecular weight standards
2	IPG strip of strain VUP Bb 12 – culture grown at pH 6.3		

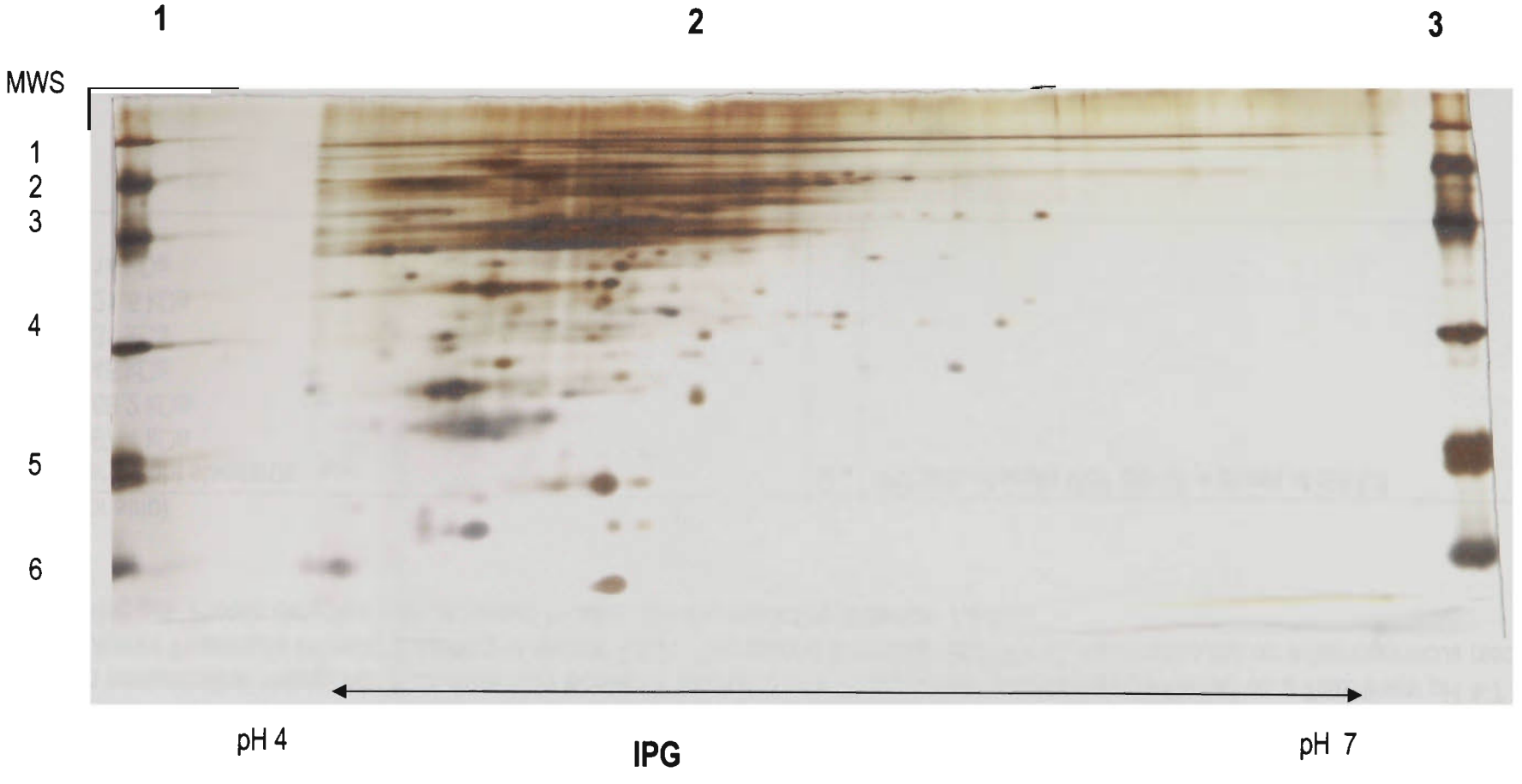

Position (lane or strip)
1
2
3
MWS
1
2
3
4
5
6
pH 4
IPG
pH 7

Figure 4.20 Computer processed image of two dimensional SDS-PAGE of *B. animalis* (syn *lactis) strain VUP Bb 12* grown at pH 6.3 (solvent extracted protein).

2-D electrophoresis was performed on samples of proteins extracted using chloroform and diethyl ether. Cultures were grown in 1L fermenters in MRS broth with 0.05% cysteine hydrochloride. The temperature was controlled at 42°C and the pH at 6.3. Nitrogen was sparged through the fermenter at 0.1 L/min. Samples were collected six hours after inoculation of the fermenter and protein extracted according to the method described in section 2.4.1.3. The protein samples were desalted and the protein concentration measured. First dimension isoelectric focussing of a 20 μg protein sample was performed on a 7cm, linear pH 4-7 immobilised pH gradient strip (Amrad Pharmacia Biotech) according to section 2.5.2. The second dimension SDS-PAGE was carried out on a homogeneous resolving gel (12%) and a 4% stacking gel. Protein detection was performed by silver staining according to section 2.5.3.2.

Key

Position (lane or strip)

1	Molecular weight standards 1. 97.4 kDa 2. 66.2 kDa 3. 45 kDa 4. 31 kDa 5. 21.5 kDa 6. 14 kDa	2	IPG strip of strain VUP Bb 12 – grown at pH 6.3

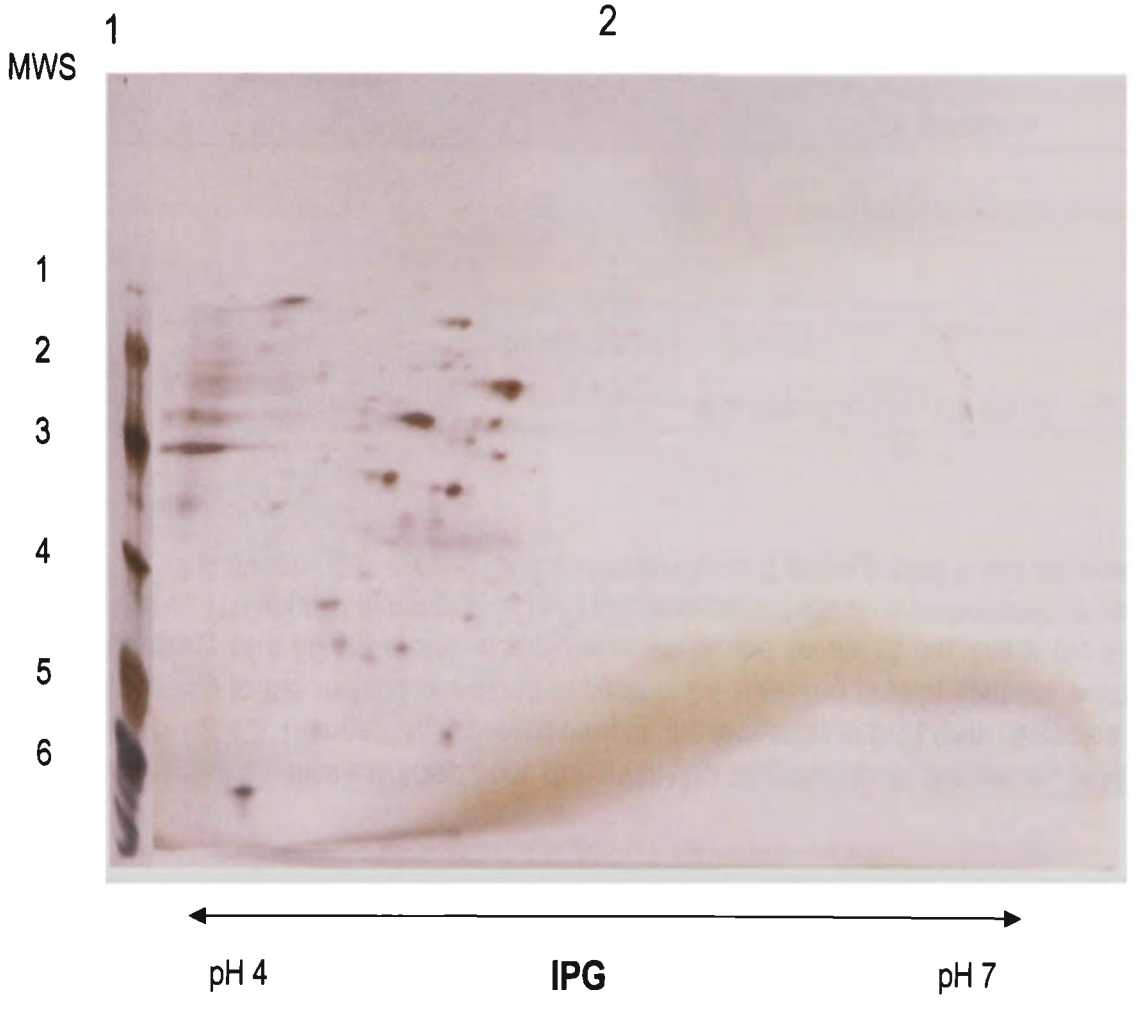

Position (lane or strip)
1
2
MWS
1
2
3
4
5
6
pH 4
IPG
pH 7

Figure 4.21 Computer processed image of two dimensional SDS-PAGE of *B. animalis* (syn *lactis) strain VUP Bb 12* grown at pH 4.3 (cleared lysate in Tris)

2-D electrophoresis was performed on samples of proteins extracted from strain VUP Bb 12 grown in 1L fermenters in MRS broth with 0.05% cysteine HCl. The temperature was controlled at 42°C and the pH at 4.3. Nitrogen was sparged through the fermenter at 0.1 L/min. Samples were collected six hours after inoculation of the fermenter and protein extracted according to the method described in section 2.4.1.3. The protein samples were desalted and the protein concentration measured. First dimension isoelectric focussing of a 50 μg protein sample was performed on an 18 cm, linear pH 4-7 immobilised pH gradient strip (Amrad Pharmacia Biotech) according to section 2.5.2. The second dimension SDS-PAGE was carried out on a homogeneous resolving gel (12%) and a 4% stacking gel. Protein detection was performed by silver staining according to section 2.5.3.2. Protein spots that are circled in red are up-regulated compared to pH 6.3.

Key

Position (lane or strip)

1	Molecular weight standards 1. 97.4 kDa 2. 66.2 kDa 3. 45 kDa 4. 31 kDa 5. 21.5 kDa	3	IPG strip of strain VUP Bb 12 – grown at pH 4.3
2	Molecular weight standards	4	Molecular weight standards

Position (lane or strip)

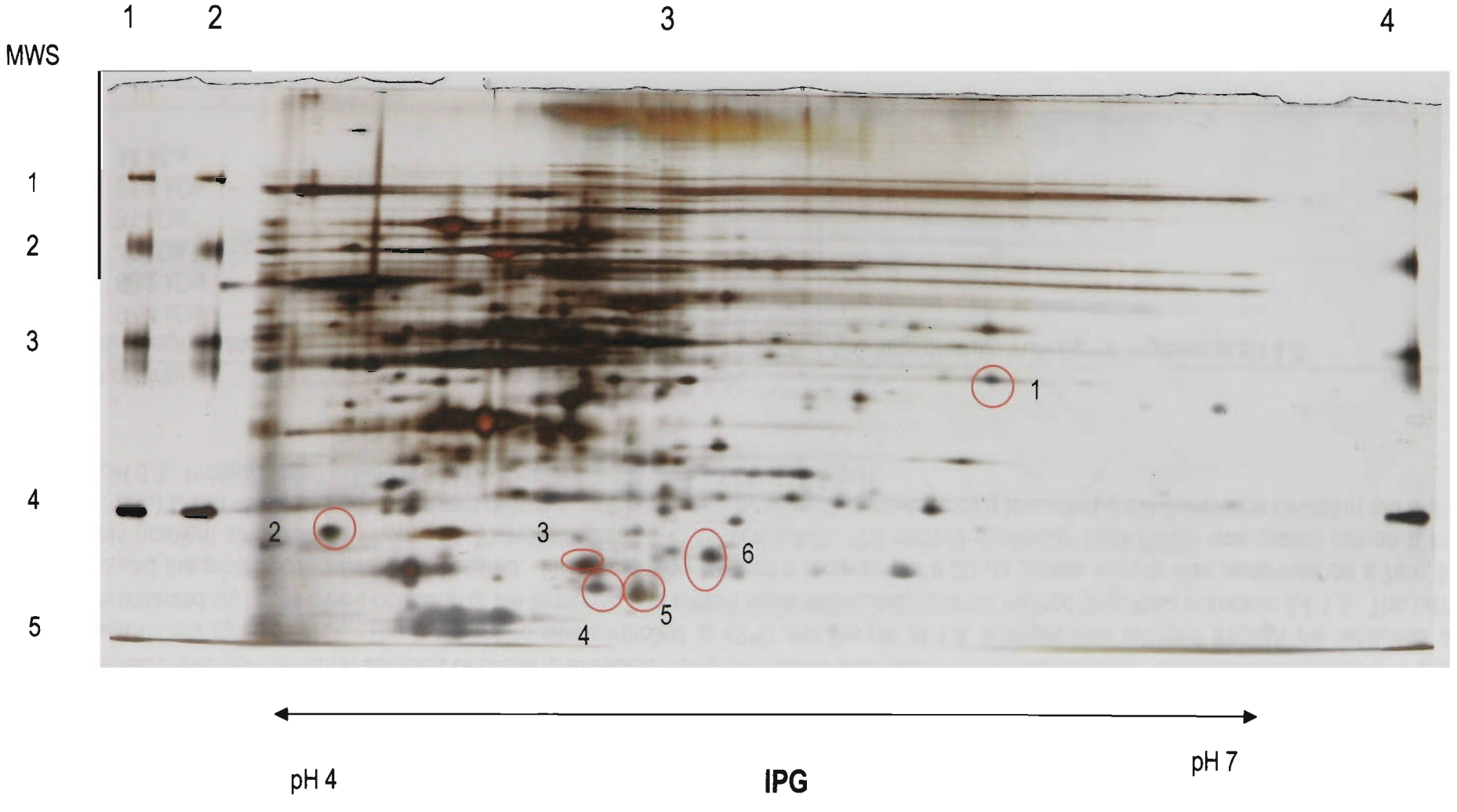


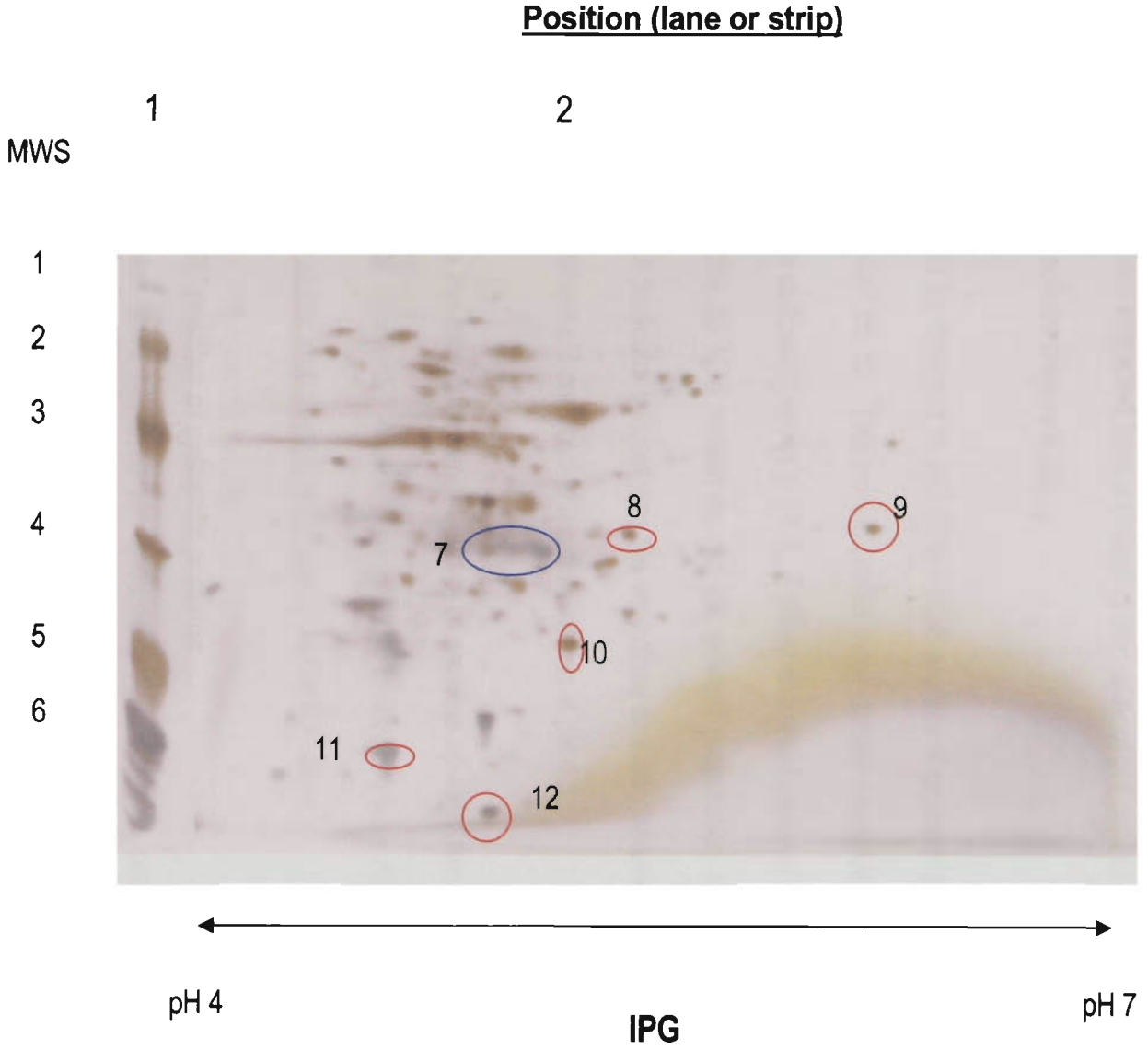
Figure 4.22 Computer processed image of two dimensional SDS-PAGE of *B. animalis* (syn *lactis) strain VUP Bb 12* grown at pH 4.3 (solvent extracted protein).

2-D electrophoresis was performed on samples of proteins extracted using chloroform and diethyl ether (see 4.4.2.1). Cultures were grown in 1 L fermenters in MRS broth with 0.05% cysteine HCl. The temperature was controlled at 42°C and the pH at 4.3. Nitrogen was sparged through the fermenter at 0.1 L/min. Samples were collected six hours after inoculation of the fermenter and protein extracted according to the method described in section 2.4.1.3. The protein samples were desalted and the protein concentration measured. First dimension isoelectric focussing of a 20 μg protein sample was performed on a 7cm, linear pH 4-7 immobilised pH gradient strip (Amrad Pharmacia Biotech) according to section 2.5.2. The second dimension SDS-PAGE was carried out on a homogeneous resolving gel (12%) and a 4% stacking gel. Protein detection was performed by silver staining according to section 2.5.3.2. Proteins circled in red are up-regulated compared to pH 6.3. Protein circled in blue is 33 kDa protein seen in earlier SDS-PAGE gels.

Key

Position (lane or strip)

1	Molecular weight standards 1. 97.4 kDa 2. 66.2 kDa 3. 45 kDa 4. 31 kDa 5. 21.5 kDa 6. 14 kDa	2	IPG strip of strain VUP Bb 12 – grown at pH 4.3


Position (lane or strip)
1
2
MWS
1
2
3
4
5
6
7
8
9
10
11
12
pH 4
pH 7
IPG

The same procedure was used to compare proteins extracted from *B. animalis* (syn. *lactis*) strain VUP Bb 12 obtained as freeze-dried powder (intended for use as a starter culture) to determine if the protein profiles were similar to either a culture grown at pH 6.3 or 4.3 and from a fermenter where the culture was grown without pH control. Figures 4.23 and 4.24 show 2-D SDS-PAGE profiles of protein extracted from the freeze-dried starter culture following suspension in 40 mM Tris pH 7.0 (Figure 4.23) and solvent (Figure 4.24). Figures 4.25 and 4.26 shows 2-D SDS-PAGE profiles of proteins extracted from a culture grown without pH control using 40 mM Tris pH 7.0 (Figure 4.25) and solvent (Figure 4.26).

Nearly 200 spots could be detected in 2-D SDS-PAGE profiles of proteins prepared from freeze-dried cultures strain VUP Bb 12. The protein profile most closely resembles Figure 4.22, which was prepared from the culture grown at pH 4.3. Solvent-extracted proteins from the freeze-dried cells also contained the 33 kDa protein as did the solvent preparation from a culture grown without pH control. Of particular interest was the very close similarity between samples prepared from cultures grown without pH control and a culture grown at pH 4.3.

Table 4.5 summarises the molecular weight and pI of proteins up-regulated during growth at pH 4.3. Known standards (Serva IEF markers, supplied by Invitrogen) were run on 2-D SDS-PAGE to confirm the linearity of the immobilised pH gradient on the IEF strips.

4.4.2.3 2-D SDS-PAGE profiles of cytosolic proteins

2-D SDS-PAGE profiles were obtained for cytosolic protein samples prepared by isoelectric focussing (IEF) using a Rotofor cell (BioRad), see section 2.5.5. Cell homogenates were prepared from cultures grown at pH 4.3 and 6.3 as described in section 4.4.1. Cell-free protein extracts were prepared using a Bead beater-8 ® and 40mM Tris pH 7.0, as described in section 2.4.1.3.

Figure 4.23 Computer processed image of two dimensional SDS-PAGE of *B. animalis* (syn. *lactis) strain VUP Bb 12* (Protein extracted from freeze dried cells).

2-D electrophoresis was performed on samples of proteins extracted from freeze dried cells into pH 7.0 Tris buffer as described in section 2.4.1.3. First dimension isoelectric focussing of a 50 μg protein sample was performed on an 18 cm, linear pH 4-7 immobilised pH gradient strip (Amrad Pharmacia Biotech) according to section 2.5.2. The second dimension SDS-PAGE was carried out on a homogeneous resolving gel (12%) and a 4% stacking gel. Protein detection was performed by silver staining according to section 2.5.3.2.

Key

Position (lane or strip)

1	Molecular weight standards 1. 97.4 kDa 2. 66.2 kDa 3. 45 kDa 4. 31 kDa 5. 21.5 kDa 6. 14 kDa	2	IEF sample of protein extracted into Tris buffer from strain VUP Bb 12 – freeze dried cells

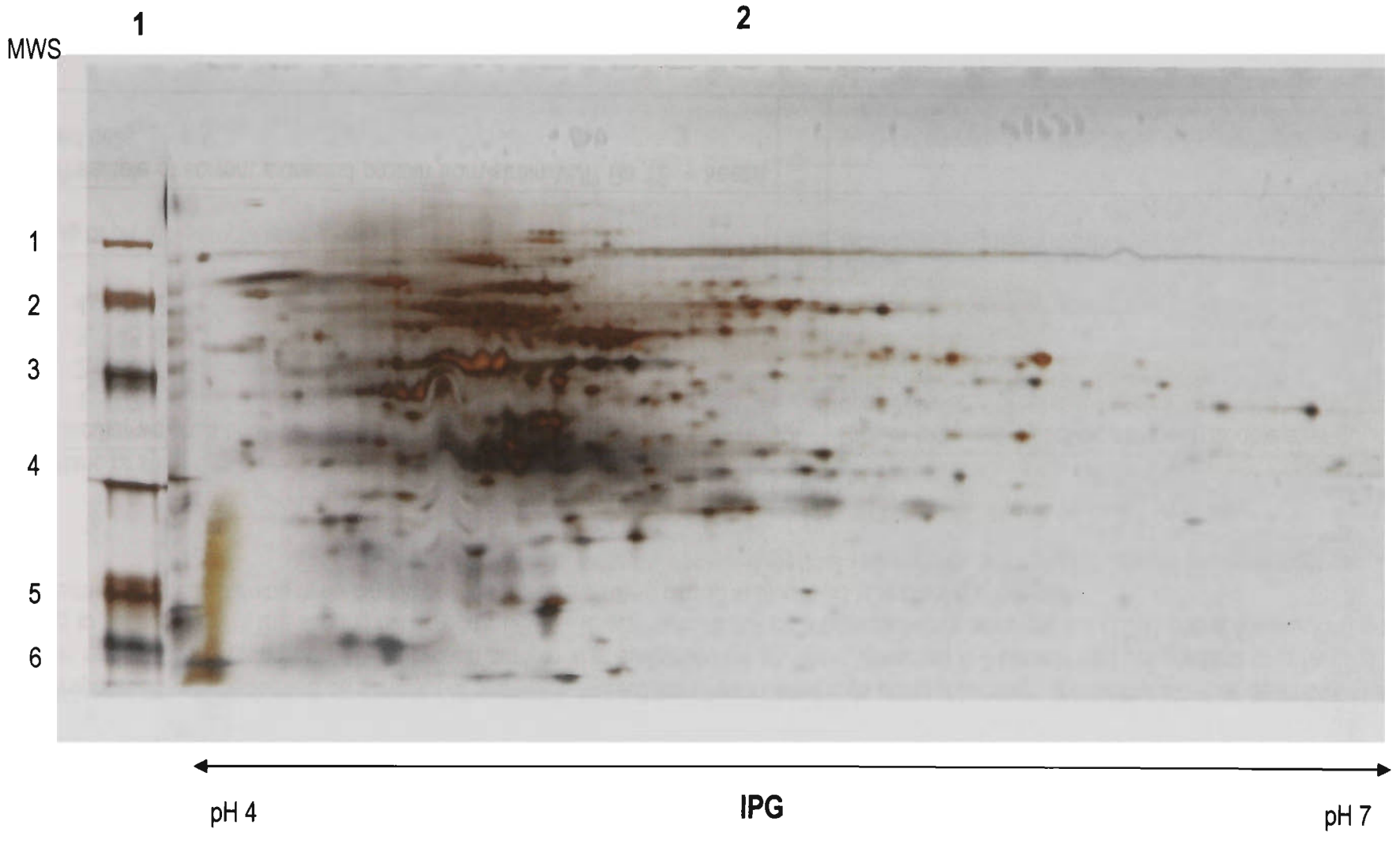

Position (lane or strip)
1
2
MWS
1
2
3
4
5
6
pH 4
IPG
pH 7

Figure 4.24 Computer processed image of two dimensional SDS-PAGE of *B. animalis* (syn *lactis) strain VUP Bb 12* (solvent extracted protein from freeze dried cells).

2-D electrophoresis was performed on samples of proteins extracted from freeze dried cells using chloroform and diethyl ether as described in section 2.4.1.3. First dimension isoelectric focussing of a 50 μg protein sample was performed on an 18cm, linear pH 4-7 immobilised pH gradient strip (Amrad Pharmacia Biotech) according to section 2.5.2. The second dimension SDS-PAGE was carried out on a homogeneous resolving gel (12%) and a 4% stacking gel. Protein detection was performed by silver staining according to section 2.5.3.2. Protein circled in blue is 33 kDa protein of interest.

Key

Position (lane or strip)

1	Molecular weight standards 1. 97.4 kDa 2. 66.2 kDa 3. 45 kDa 4. 31 kDa	4	Protein extracted by boiling cells with double strength loading buffer- freeze dried cells
2	1 μg of protein sample used to run IEF	5	Molecular weight standards
3	IEF sample of solvent extracted protein from strain VUP Bb 12 – freeze dried cells		

Position (lane or strip)

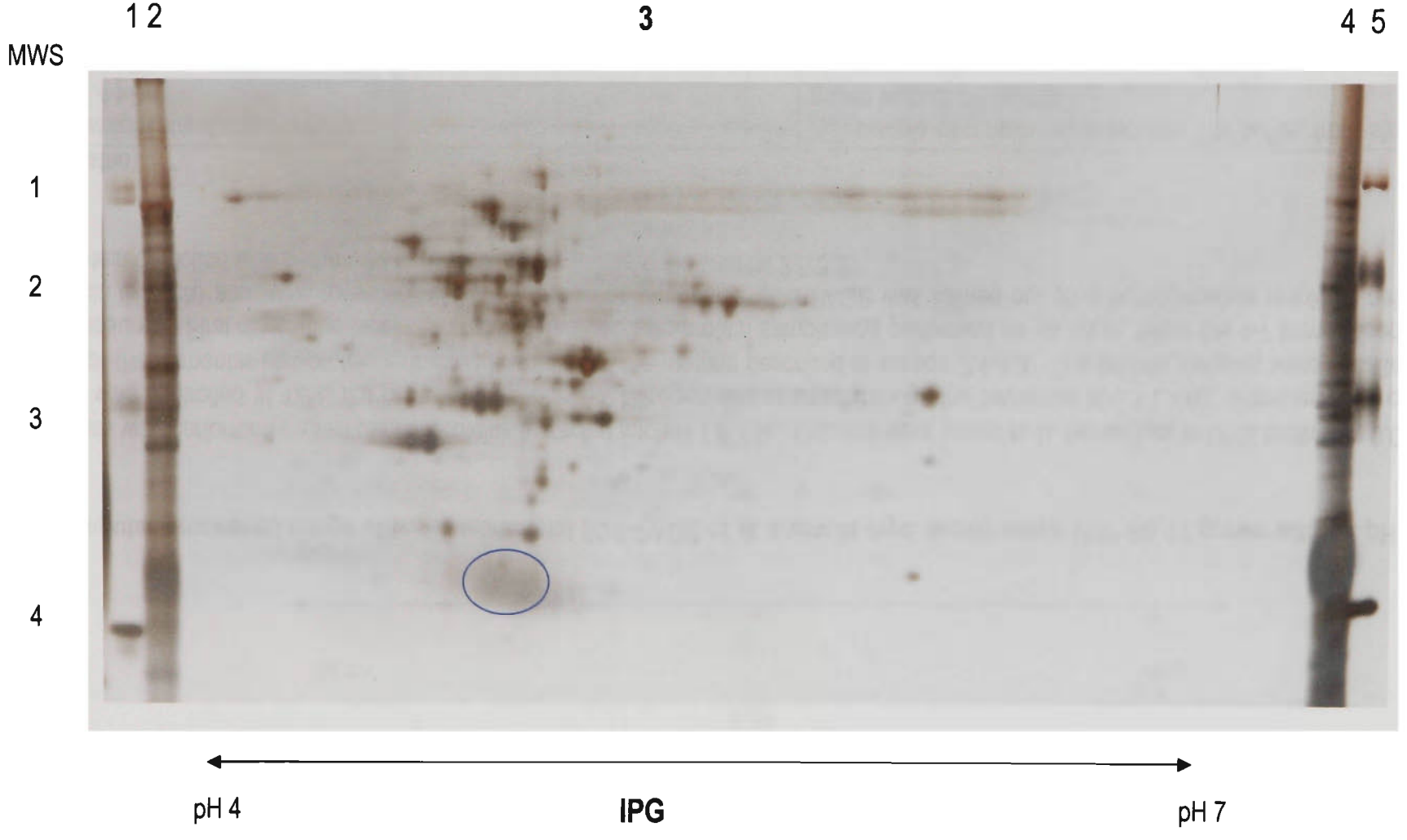


Figure 4.25 Computer processed image of two dimensional SDS-PAGE of *B. animalis* (syn. *lactis) strain VUP Bb 12* grown without pH control (cleared lysate in Tris)

2-D electrophoresis was performed on samples of proteins extracted into pH 7.0 Tris. Cultures were grown in 1L fermenters in MRS broth with 0.05% cysteine-HCl. The temperature was controlled at 42°C but pH was not controlled. Nitrogen was sparged through the fermenter at 0.1 L/min. Samples were collected six hours after inoculation of the fermenter and protein extracted according to the method described in section 2.4.1.3. The protein samples were desalted and the protein concentration measured. First dimension isoelectric focussing of a 50 μg protein sample was performed on an 18cm, linear pH 4-7 immobilised pH gradient strip (Amrad Pharmacia Biotech) according to section 2.5.2. The second dimension SDS-PAGE was carried out on a homogeneous resolving gel (12%) and a 4% stacking gel. Protein detection was performed by silver staining according to section 2.5.3.2.

Key

Position (lane or strip)

1	Molecular weight standards 1. 97.4 kDa 2. 66.2 kDa 3. 45 kDa 4. 31 kDa 5. 21.5 kDa	2	IEF sample of protein extracted into Tris buffer, from strain VUP Bb 12 – grown without pH control

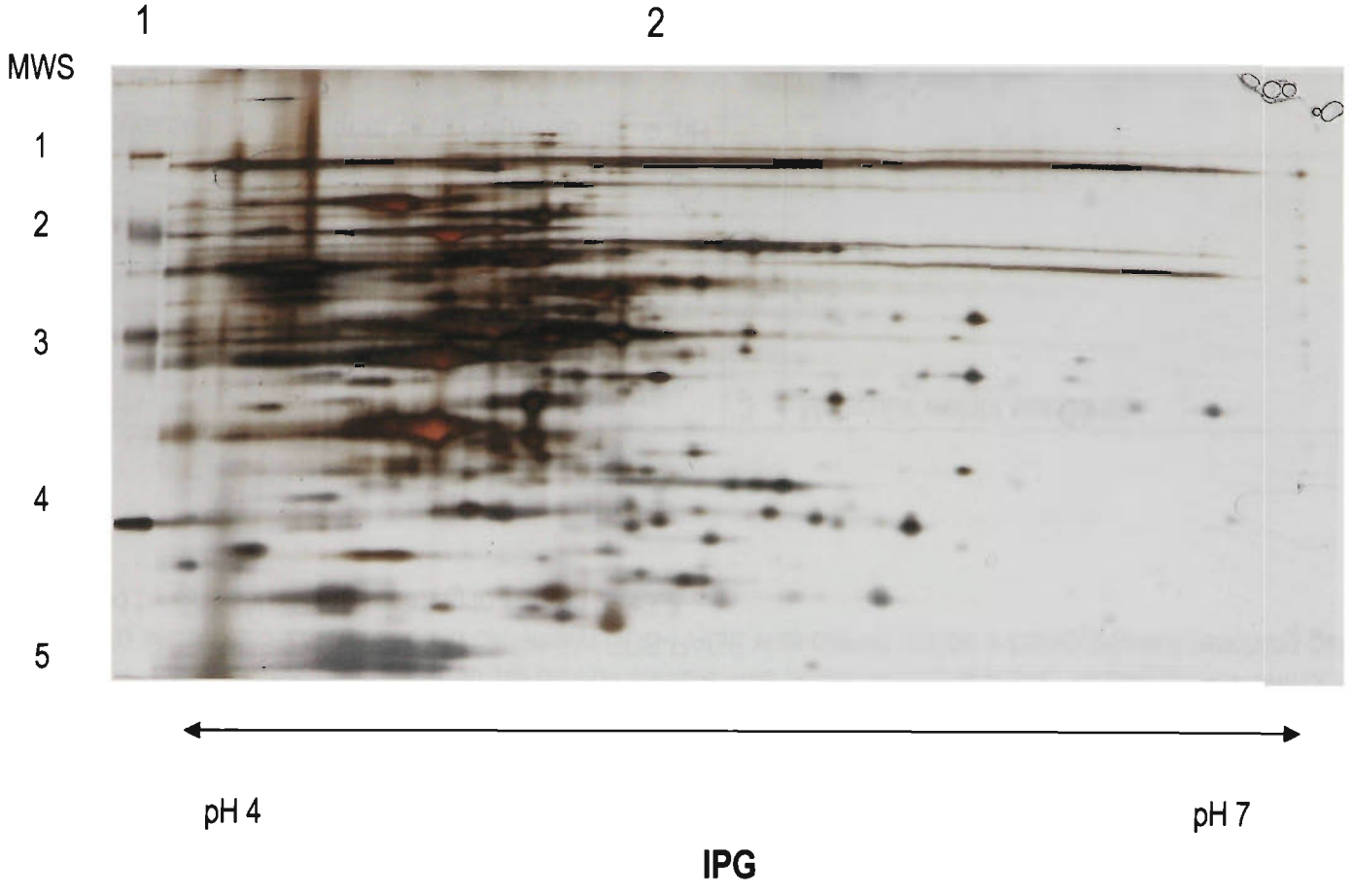

Position (lane or strip)
1
2
MWS
1
2
3
4
5
pH 4
pH 7
IPG

Figure 4.26 Computer processed image of two dimensional SDS-PAGE of *B. animalis* (syn. *lactis) strain VUP Bb 12* grown without pH control (solvent extracted protein).

2-D electrophoresis was performed on samples of proteins extracted from a culture grown without pH control, using chloroform and diethyl ether as described in section 2.4.1.3. First dimension isoelectric focussing of a 50 μg protein sample was performed on a linear, 18 cm, pH 4-7 immobilised pH gradient strip (Amrad Pharmacia Biotech) according to section 2.5.2. The second dimension SDS-PAGE was carried out on a homogeneous resolving gel (12%) and a 4% stacking gel. Protein detection was performed by silver staining according to section 2.5.3.2.

Key

Position (lane or strip)

1	Molecular weight standards 1. 97.4 kDa 2. 66.2 kDa 3. 45 kDa 4. 31 kDa 5. 21.5 kDa	3	Molecular weight standards
2	IEF sample of solvent extracted proteins from strain VUP Bb 12 – pH uncontrolled		

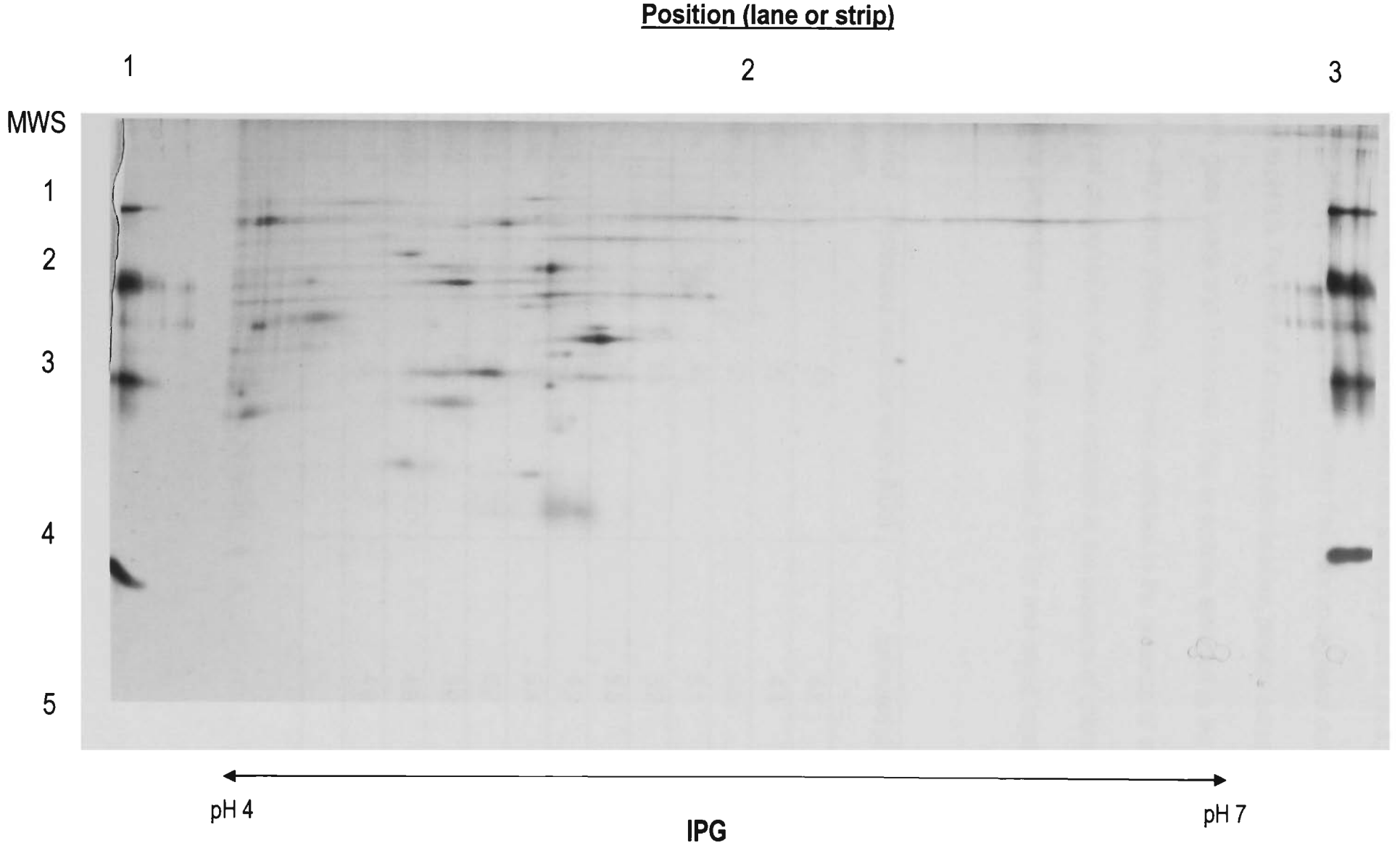

Position (lane or strip)
1
2
3
MWS
1
2
3
4
5
pH 4
pH 7
IPG

Table 4.5 Molecular weight and pI of protein up-regulated during acid stress.

2-D SDS-PAGE was performed on proteins extracted from samples grown at pH 4.3 and pH 6.3. Estimations of size and pI were performed on proteins that were up-regulated during growth at pH 4.3 compared to pH 6.3. The method of treatment refers to either, proteins extracted using the bead beater with glass beads and Tris buffer (Tris) or proteins extracted in the presence of chloroform and di-ethyl ether (Solvent). Proteins extracted in the presence of solvents were prepared from equal concentrations of protein extracted in the presence of chloroform and di-ethyl ether. These preparations were then re-dissolved in Tris and mixed together in equal volumes.

	Method of treatment	Estimated molecular weight (kDa)	Estimated pI
1	Tris	43	6.2
2	Tris	27	4.1
3	Tris	25	5.0
4	Tris	23	5.1
5	Tris	23	5.2
6	Tris	26	5.3
7	Solvent	33	4.9
8	Solvent	35	5.4
9	Solvent	36	6.2
10	Solvent	22	5.2
11	Solvent	13	4.6
12	Solvent	5	4.9

The crude protein mixture, prepared by bead-beating (section 2.4.1.3) was de-salted by dialysis against distilled water. The samples were then mixed with broad range carrier ampholytes pH 3-10 (2% v/v) to make a final volume of 55 mL. The protein-ampholyte solution was injected into the sample chamber and large air bubbles removed. Constant power (12 W) was applied to the Rotofor until the current had stabilised for one hour. Each run took approximately four hours. Twenty fractions were collected from each run and 100µL was mixed with an equal quantity of double strength loading buffer and heated to 100°C for 5 minutes. The samples were then centrifuged and loaded onto a 1-D SDS-PAGE gel.

Comparison of the results for cells grown at pH 6.3 and 4.3 are shown in Figures 4.27 and 4.28. No bands were observed in fractions 16-20 for samples prepared from cultures grown at either pH. Visual comparison of the fractions 1-15 indicated a number of differences. In particular where there were fewer bands, as in fractions 10, 11 and 12, a number of changes in banding patterns could be seen. Proteins between approximately 40-45 kDa and 10-20 kDa appeared to be up-regulated during growth at pH 4.3. Higher molecular weight proteins of approximately 90 and 70 kDa appear to be down-regulated at pH 4.3. Other differences could be seen in low molecular weight proteins in fractions 5-9 of samples prepared from cells grown at low pH, but they were primarily down-regulation changes.

Samples from fractions 10, 11 and 12 (both pH 6.3 and 4.3-derived samples) were selected for further separation by 2-D SDS-PAGE because they contained proteins that appeared to be up-regulated during growth at low pH and relatively low numbers of bands meant that spots would be easier to identify and handle during subsequent collection of protein for N-terminal sequencing. A pre-formed immobilised pH gradient on a 7 cm plastic support strip (Immobiline DryStrip pH 4-7, Amrad Pharmacia Biotech) was rehydrated overnight by placing the strip in a sterile screw-capped test tube containing 20 µg of protein in 40 µL of rehydration solution.

Figure 4.27 Computer processed image of one dimensional SDS-PAGE of *B. animalis* (syn. *lactis*) strain Bb12 grown with pH control at pH 6.3 (rotofor fraction 1-12) and 4.3 (rotofor fractions 1-13).

1-D electrophoresis was performed on samples of proteins extracted from *B. animalis* (syn. *lactis*) grown in 1L flasks in MRS broth. The temperature was controlled at 42°C but the pH of each flask was varied. Samples shown in lanes 2-14 were grown at pH 6.3 and samples shown in lanes 17-30 were grown at pH 4.3. Lanes at position 13, 16 and 28 are blank (contain no samples). Lanes 1, 15 and 31 contain molecular weight standards (MWS) 97.4 kDa, 66.2 kDa, 45 kDa, 31 kDa and 21.5 kDa. Nitrogen was sparged through the flasks prior to flask inoculation and after collection of each sample. Samples were collected six hours after inoculation of the flask and protein extracted according to the method described in section 2.4.1.3. The crude protein mixtures prepared by bead-beating were de-salted and the protein concentration measured. The protein extract was mixed with carrier ampholytes (2%) to a final volume of 51 mL and injected into the Rotofor (BioRad) chamber. Constant power (12W) was applied for approximately 4.5 hours until the proteins were separated according to their isoelectric point. The fractions, numbered from the acidic end of the rotofor, were applied to the lanes as listed below. Proteins up-regulated at pH 4.3 compared to pH 6.3 are circled in blue. Proteins down-regulated at pH 4.3 compared to pH 6.3 are circled in red.

Lane	pH	Rotofor Fraction	Lane	pH	Rotofor Fraction	Lane	pH	Rotofor Fraction
1	MWS		12	6.3	11	23	4.3	7
2	6.3	1	13	No sample		24	4.3	8
3	6.3	2	14	6.3	12	25	4.3	9
4	6.3	3	15	MWS		26	4.3	10
5	6.3	4	16	No sample		27	4.3	11
6	6.3	5	17	4.3	1	28	No sample	
7	6.3	6	18	4.3	2	29	4.3	12
8	6.3	7	19	4.3	3	30	4.3	13
9	6.3	8	20	4.3	4	31	MWS	
10	6.3	9	21	4.3	5			
11	6.3	10	22	4.3	6			

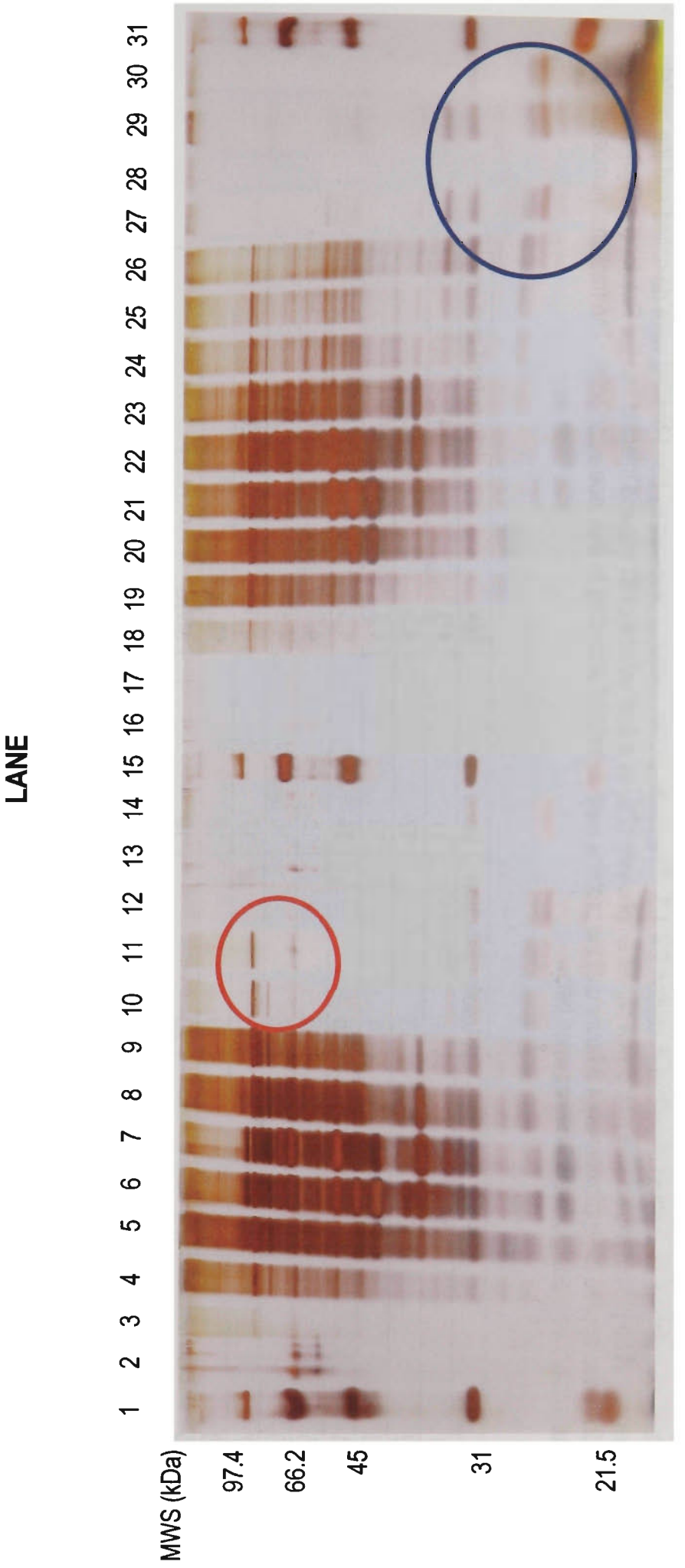
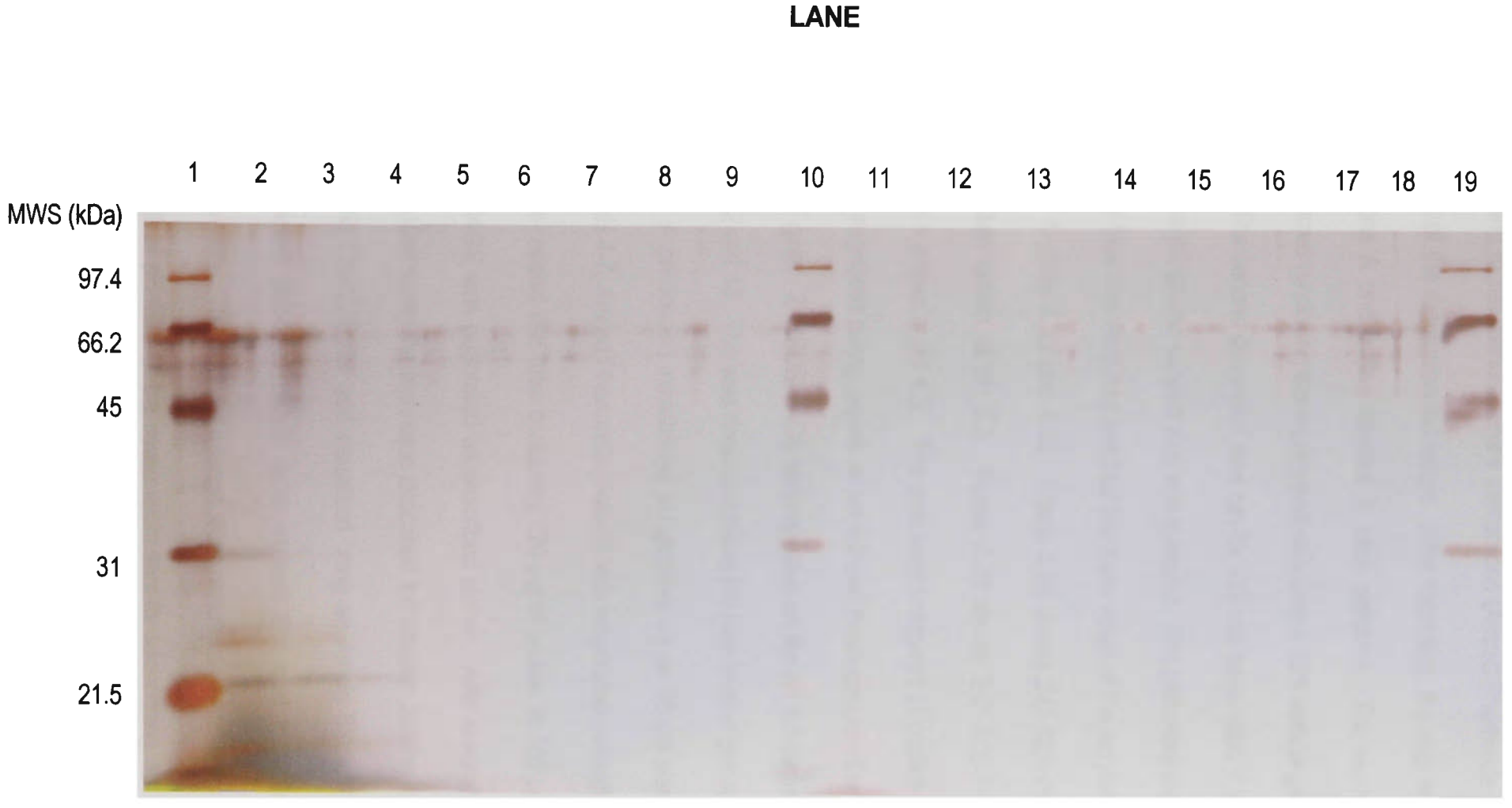

LANE
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31
MWS (kDa)
97.4
66.2
45
31
21.5

Figure 4.28 Computer processed image of one dimensional SDS-PAGE of *B. animalis* (syn. *lactis*) strain Bb12 grown with pH control at pH 6.3 (rotofor fraction 13-20) and 4.3 (rotofor fractions 14-20).

1-D electrophoresis was performed on samples of proteins extracted from *B. animalis* (syn. *lactis*) grown in 1L flasks in MRS broth. The temperature was controlled at 42°C but the pH of each flask was varied. Samples shown in lanes 2-9 were grown at pH 6.3 and samples shown in lanes 11-17 were grown at pH 4.3. Lane at position 18 is blank (contain no sample). Lanes 1,10 and 19 contain molecular weight standards (MWS) 97.4 kDa, 66.2 kDa, 45 kDa, 31 kDa and 21.5 kDa. Nitrogen was sparged through the flasks prior to flask inoculation and after collection of each sample. Samples were collected six hours after inoculation of the flask and protein extracted according to the method described in section 2.4.1.3. The crude protein mixtures prepared by bead-beating were de-salted and the protein concentration measured. The protein extract was mixed with carrier ampholytes (2%) to a final volume of 51 mL and injected into the Rotofor (BioRad) chamber. Constant power (12W) was applied for approximately 4.5 hours until the proteins were separated according to their isoelectric point. The fractions, numbered from the acidic end of the rotofor, were applied to the lanes as listed below.

Lane	pH	Rotofor Fraction	Lane	pH	Rotofor Fraction
1	MWS		11	4.3	14
2	6.3	13	12	4.3	15
3	6.3	14	13	4.3	16
4	6.3	15	14	4.3	17
5	6.3	16	15	4.3	18
6	6.3	17	16	4.3	19
7	6.3	18	17	4.3	20
8	6.3	19	18	No sample	
9	6.3	20	19	MWS	
10	MWS				


LANE
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
MWS (kDa)
97.4
66.2
45
31
21.5

The strip was focussed in three stages using a Multiphor II electrophoresis unit with 2-D application kit according to the manufacturer's instructions (Amrad Pharmacia Biotech). Times were at the high end of the recommended range. After focussing, the strip was removed and equilibrated in solutions A and B (ten minutes in each solution). The second dimension of separation was performed by placing the equilibrated strip onto a 12% vertical gel sandwich (CBS triple wide system). The second dimension was run for 120 volt hours (600 V, 40 mA, 10 W) at constant current before the plastic support strip was removed. The gels were then allowed to run until the bromophenol blue dye front had reached the lower edge of the acrylamide gel. These results are shown in Figures 4.29 and 4.30. Figure 4.29 shows 2-D SDS-PAGE of proteins extracted from a culture grown at pH 6.3. Figure 4.30 shows 2-D SDS-PAGE of proteins extracted from a culture grown at pH 4.3. The gels were compared to confirm the presence of proteins that were up-regulated during growth at pH 4.3 and these compared with earlier results using 1-D SDS PAGE gels. 2-D SDS-PAGE was repeated on the pH 4.3-derived sample using pooled fractions 11, 12 and 13. This was done to achieve the high level of protein required for N-terminal sequencing. A pre-formed immobilised pH gradient on an 18 cm plastic support strip (Immobiline DryStrip pH 4-7, Amrad Pharmacia Biotech) was rehydrated overnight by placing the strip in a sterile screw-capped test tube containing 120 μg of protein in 350 μL of rehydration solution. 2-D SDS PAGE was performed as described above. After electrophoresis, protein bands were electro-blotted using the technique described for Western blotting (section 2.5.4) to PVDF membrane using CAPS buffer and visualised using an Amido Black 10B stain. Seven spots of interest, labelled Bsb-2 to Bsb-8, were excised and sent to LaTrobe University, Department of Biochemistry. The spots of interest were proteins that had been seen to be up-regulated during growth at pH 4.3. Of the seven spots sent for sequencing, spots Bsb-2, Bsb-3 and Bsb-4 were found to have insufficient protein for sequencing and spots Bsb-5 and Bsb-6 had identical sequence patterns. Sequence data was obtained for spots Bsb-5, 6, 7 and 8.

Figure 4.29 Computer processed image of two dimensional SDS-PAGE of *B. animalis* (syn. *lactis*) strain Bb12 grown with pH control at pH 6.3.

2-D electrophoresis was performed on Rotofor fractions 11, 12 and 13. Samples of proteins were extracted from *B. animalis* (syn. *lactis*) grown in 1L flasks in MRS broth at pH 6.3 and at 42°C. Samples were collected six hours after inoculation of the flask and protein extracted according to the method described in section 2.4.1.3. The crude protein mixtures prepared by bead-beating were de-salted and the protein concentration measured. The protein extract was mixed with carrier ampholytes (2%) to a final volume of 51 mL and injected into the Rotofor (BioRad) chamber. Constant power (12W) was applied for approximately 4.5 hours until the proteins were separated according to their isoelectric point. First dimension isoelectric focussing of a 20µg protein sample was performed on a linear pH 4-7 immobilised pH gradient strip (Amrad Pharmacia Biotech) according to section 2.5.2. The second dimension SDS-PAGE was carried out on a homogenous resolving gel (15%) and a 4% stacking gel. Protein detection was performed by silver staining according to section 2.5.3.2

Key
Position (lane or strip)

1	Molecular weight standards (MWS) 97.4 kDa, 66.2 kDa, 45 kDa, 31 kDa, 21.5 kDa and 14 kDa
2	Rotofor fraction 11 (sample grown at pH 6.3)
3	MWS
4	Rotofor fraction 12 (sample grown at pH 6.3)
5	MWS
6	Rotofor fraction 13 (sample grown at pH 6.3)
7	MWS

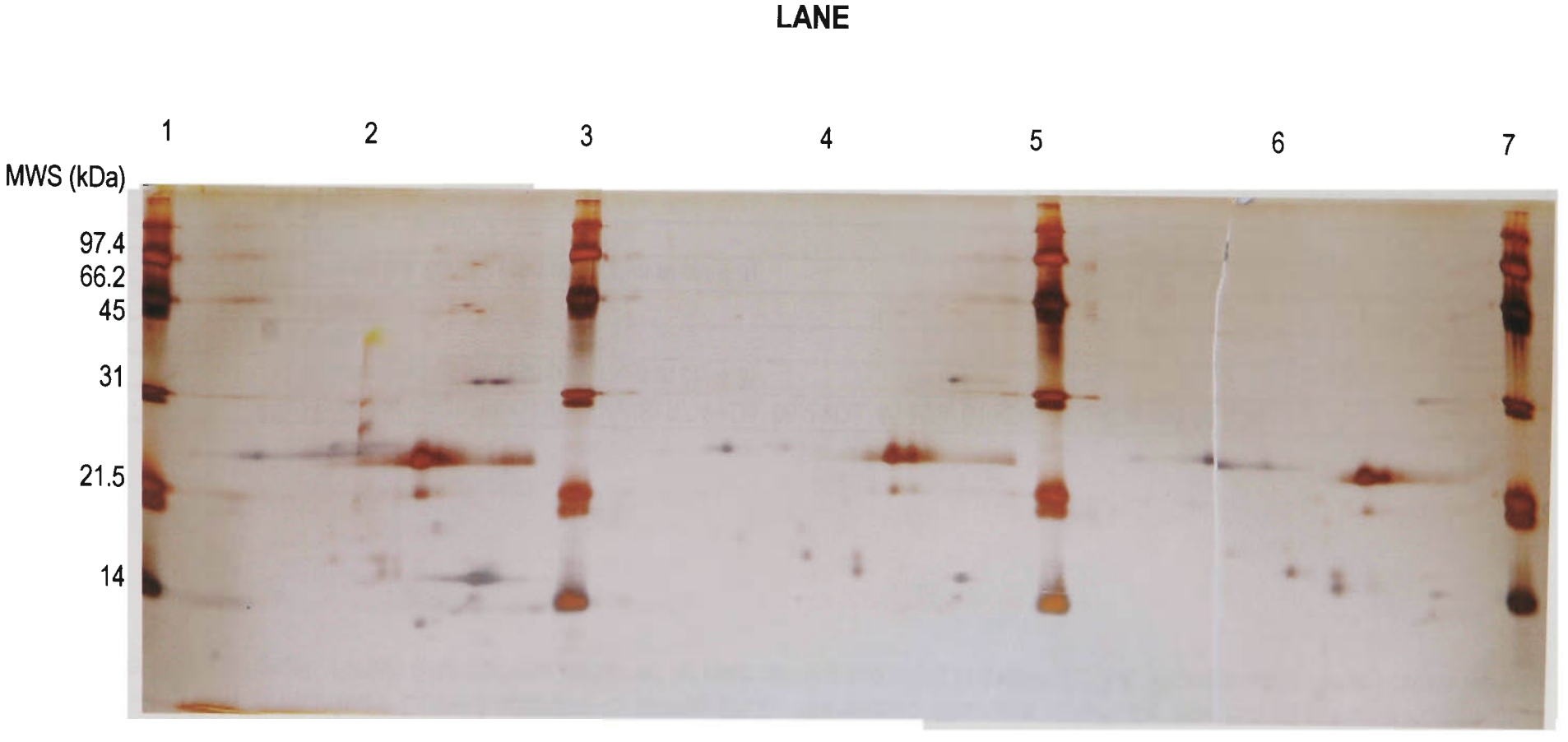

LANE
1
2
3
4
5
6
7
MWS (kDa)
97.4
66.2
45
31
21.5
14

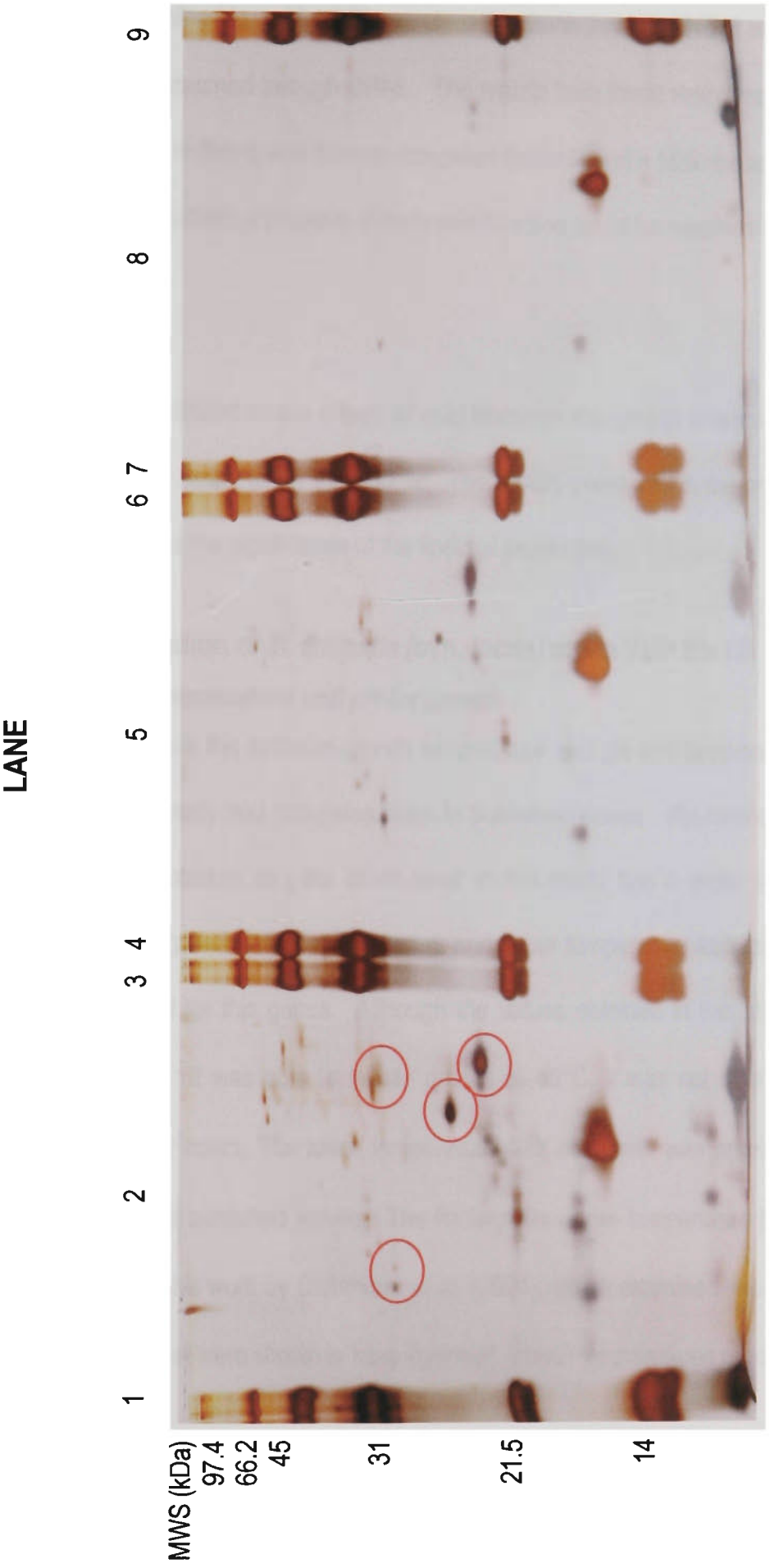
Figure 4.30 Computer processed image of two dimensional SDS-PAGE of *B. animalis* (syn. *lactis*) strain Bb12 grown with pH control at pH 4.3.

2-D electrophoresis was performed on Rotofor fractions 11, 12 and 13. Samples of proteins were extracted from *B. animalis* (syn. *lactis*) grown in 1L flasks in MRS broth at pH 4.3 and at 42°C. Samples were collected six hours after inoculation of the flask and protein extracted according to the method described in section 2.4.1.3. The crude protein mixtures prepared by bead-beating were de-salted and the protein concentration measured. The protein extract was mixed with carrier ampholytes (2%) to a final volume of 51 mL and injected into the Rotofor (BioRad) chamber. Constant power (12W) was applied for approximately 4.5 hours until the proteins were separated according to their isoelectric point. First dimension isoelectric focussing of a 20µg protein sample was performed on a linear pH 4-7 immobilised pH gradient strip (Amrad Pharmacia Biotech) according to section 2.5.2. The second dimension SDS-PAGE was carried out on a homogenous resolving gel (15%) and a 4% stacking gel. Protein detection was performed by silver staining according to section 2.5.3.2. Proteins within the red circles were N-terminal sequenced.

Key

Position (lane or strip)

1	Molecular weight standards (MWS) 97.4 kDa, 66.2 kDa, 45 kDa, 31 kDa, 21.5 kDa and 14 kDa
2	Rotofor fraction 11 (sample grown at pH 4.3)
3	MWS
4	MWS
5	Rotofor fraction 12 (sample grown at pH 4.3)
6	MWS
7	MWS
8	Rotofor fraction 13 (sample grown at pH 4.3)
9	MWS


LANE
1
2
3 4
5
6 7
8
9
MWS (kDa)
97.4
66.2
45
31
21.5
14

This data was submitted for a BLASTP search to GenBank through ANGIS and the draft genome of *B. longum* was searched through ORNL. The results from these searches are shown in Table 4.6. The matches to Bsb-6 and 8 were elongation factor-P and a 50S ribosomal protein L7/L12. Otherwise, only hypothetical proteins of no known function could be matched to Bsb-7.

4.5 Discussion

This chapter concentrated on the effects of acid stress on the growth kinetics and proteins made by *B. animalis (*syn. *lactis)* strain VUP Bb 12. The results presented in the previous sections are discussed below and the significance of the findings presented.

4.5.1 Characterisation of *B. animalis (*syn. *lactis)* strain VUP Bb 12

4.5.1.1 Optimum temperature and pH for growth

Table 4.7 summarises the optimum growth temperature and pH and temperature and pH range determined in this study and compares them to published values. By contrast to the published values for *Bifidobacterium* sp., the strain used in this study has a wider temperature growth range. The fastest growth rate for temperature and upper temperature limit for growth are similar to the values stated for this genus. Although the values obtained in this study (section 4.3.1) show that VUP Bb 12 was able to initiate growth at 45°C, it was not able to sustain growth beyond the first few hours. The lower temperature limit of growth was approximately 22°C, at least 3°C lower than published values. The findings for upper temperature limit are consistent with those found in the work by Crittenden *et al.* (2001), which examined two strains of *B. lactis* (B94 and DS 920) that were shown to have optimum growth temperatures around 40°C.

The pH determined to be the optimum for growth in this study, pH 6.3, falls within the expected range for this species, but the lower growth limit appears equal to (or possibly lower) than the previously published values for this genus.

Table 4.6 Results of N-Terminal sequencing of cytosolic proteins up-regulated during growth of *B. animalis* (syn. *lactis)* strain Bb 12 at pH 4.3.

Cytosolic proteins that were seen to be up-regulated during growth at pH 4.3 were N-terminally sequenced by The Department of Biochemistry at LaTrobe University. The data obtained from this sequencing was submitted to both Genbank and ORNL for BLASTP searches. Completed genomes were searched in Genbank and the draft genome of *B. longum* was searched in ORNL. The molecular weight, estimated pI and possible function of the closest protein matches are shown in columns 2, 3 and 6 respectively. The N-terminal sequence of protein matches in the *B. longum* draft genome is shown in column 5. The sequence of the closest protein match is shown in column 7.

Name	MW (kDa)	pI	N-terminal sequence	*B. longum* N-terminal sequence	Possible function	Sequence of related proteins
Bsb-5	20654	5.05	same as Bsb-6	same as Bsb-6		
Bsb-6	20654	5.05	A(Q)(T)(T)N D(I)K N G - - L N	MAQTTNDIKN GSVLN	"elongation factor P"	MAQTTNDIKNGSVLNLDGQLWTVMKFQHVKPGKGPAFVRT IKNVLSGKIVDKTFNAGMKMEFETVDNRTLQYSYEDGDN FVFMDMTTYDQIMVPKTLLGDKAKFLLEGTDCLVSFHDGTPLSVDLPGSV VLTITHTEPGLQGNRSNAGTKPATVETGAE IQVPLFINEG DRVKINTEDG SYTGRENN
Bsb-7	26973	4.64	SGHXKWAXXKHKXAAXDA	MSGHSKWATTKHKKAAIDA	??	MSGHSKWATTKHKKAAIDAKRGKLFAKLIKNIEIAARLGGGDPDGNPSLY DAIYKAKKASMPADNIARAVKRGAGAEDGAANYEDIVYEGYAPAGVGLII ECLTDNRNRAAAEVRSTLTKGNGSLATSGSVSFNFERKGQ IVVPSEGVDFDKLFETAAEAGAEDVTDDGEVFTVVTGPSD LFTVRKALQEAGFDYDSADQVMQPKNEVELSLEDARKVSK LIDNLDDLDDVQNIYSNWTASDEVMAQLDEE
Bsb-8	13247	4.56	A K F T Q D E L L E A F	MAKYTNDELLEAF	50S RIBOSOMAL PROTEIN L7/L12	MAKYTNDELLEAFGEMTLVELSEFVKAFEEKFDVEAAAPV AAVAAVAGAAAPAEEEKDEFDVILSAVGDKKIQVIKAVRAITNLGLAEAK ALVDGAPKAVLEKAKKEDAEKAKAQLEEAGASVELK

Table 4.7 Comparison of optimum growth temperature and pH to published values.

	Values determined in this study for strain VUP Bb 12	**Published values for *Bifidobacterium* spp. (Gomez and Malcata, 1999)**
Optimum temperature range	42°C	37-41°C
Optimum pH	6.3 ± 0.2	6-7
Growth (temperature) range low limit high limit	 ≈22°C Not determined in this study	 No growth below 25-28°C No growth above 43-45°C
Growth (pH) range low limit high limit	 ≈4.0 Not determined in this study	 No growth below 4.5-5.0 No growth above 8.0-8.5

Data shown in section 4.3.1 is problematical in its interpretation given the relatively few points used to determine growth kinetics. However, later data (Figure 4.10) clearly showed that strain VUP Bb 12 was able to both initiate growth and grow at pH 4.0. This capacity could offer significant advantage over other strains when considering the selection of bifidobacteria for use in fermented dairy products.

The observations of extended growth range, reflect the origin and development of the test strain. As a dairy starter culture and adjunct, the strain has undergone a stringent selection process over the last 20 years. Strain VUP Bb 12 was specifically selected through a series of mutations and propagations to make it suitable for use in dairy products (Mogensen, 2002).

The specific selection criteria were:

- the ability to withstand the high acidity and temperatures associated with dairy fermentation processes
- good survival characteristics (in terms of product shelf life) and
- contribution to good flavour in fermented products.

In terms of being able to survive the temperatures and pH values experienced during the manufacture of dairy products, the findings of this study support the suitability of strain VUP Bb 12 as a dairy adjunct.

4.5.1.2 Growth kinetics

This study showed that when strain VUP Bb 12 is grown in a complex synthetic medium, such as MRS broth, in a fermenter where fine control is maintained over temperature, pH, agitation and gaseous environment, μ_{max} values can reach 0.7 h^{-1}.

Few reports have been made of the growth kinetics of *Bifidobacterium* species and in particular *B. animalis (*syn. *lactis)*, as most research is targeted on survival in sub-optimum conditions rather than growth in optimum conditions. However, in a publication by Alander (2001) on the survival and persistence of *B. animalis* Bb-12 in the gastrointestinal tract, the growth kinetics of the strain was shown as a plot of OD versus time. In those growth curves, growth in TPY medium with 1% fructo-oligosaccharides or 1% gluco-oligosaccharides was compared to growth in TPY medium with 1% glucose (w/v). It was stated that cultures were grown in microtitre plates, at 37°C in anaerobic conditions and no other details were given. For cultures grown in 1% glucose, the growth curves showed a very long lag phase, extending nearly 12 hours. In this study lag phase lasted only one hour but this cannot be compared to the earlier study as the size of the inocula and prior growth conditions of the starter culture are unknown. Commencing at an OD of around 0.1 the final OD of the culture was 0.6 after 24 hours, which was not only much slower than the results achieved in this study but the extent of growth was also much less. These results are equally difficult to compare to the results from the study reported in this thesis, as the measurement of OD in microtitre plates cannot be directly compared to a measurement of OD in a spectrophotometer. The light path in a spectrophotometer is generally fixed at 1 cm while the volume of media in the microtitre plate well will determine the length of the light path and that will affect the OD values. Growth curves for the media containing oligosaccharides showed even longer lag phases and lower extents of growth compared to the glucose control (Alander *et al.*, 2001).

Other studies, using two commercial strains of *B. bifidum* (Bf-1 and Bf-6), have demonstrated mean doubling times of 237 ±10 minutes and 242 ±13 minutes respectively (Shin *et al.*, 2000).

In the present study, the calculated doubling time for *B. animalis (*syn. *lactis)* strain VUP Bb 12 grown in optimum conditions was 56 ± 1 minute. Apart from variation due to strain differences,

the marked differences between the results may be accounted for in the differences in culture methods. In the study by Shin (2000) several factors would have contributed to a slower growth rate, such as:

- the bacteria were cultured in 12% (w/w) reconstituted non-fat dry milk, not MRS with cysteine-HCl. Milk as a growth medium has been shown to provide essential nutrients but the lack of amino acids and small peptides does not to support 'extended growth' (Rasic and Kurmann, 1983);

- the cultures were grown in tubes (no volumes were stated) and the tubes were not mixed during culture by nitrogen gas sparging or stirred agitation. This alone may have significantly extended the doubling time and

- the tubes were incubated using GasPaks, an anaerobic pouch incubation system. Poor gas diffusion in liquid media may have resulted in high oxygen levels remaining in the growth medium for an extended period, which would also extend the doubling time.

4.5.1.3 Observations of protein profiles of strain Bb 12 grown between 32°C and 47°C

The only differences observed in the protein profiles prepared from cultures grown between 32°C and 47°C were noted in the cultures grown at the highest temperature (47°C), which was 5°C above optimum.

When bacterial cells are shifted to a temperature significantly higher than the optimum, the synthesis of heat shock proteins is rapidly induced (Lindquist and Craig, 1988; Yura *et al.*, 1993). The typical response expected to be seen in 1-D SDS-PAGE profiles in Gram-negative bacteria undergoing heat stress would be an increase in proteins of molecular weight corresponding to induction of some or all of the heat shock proteins, including HSP 60, HSP 70, HSP 90 and small HSPs. Strain VUP Bb 12 did demonstrate a heat stress response: in particular, up-regulation of

the higher molecular weight proteins corresponding to induction of HSP 70 and HSP 60 and some small molecular weight proteins was detected in SDS-PAGE gels.

The sizes of the bands that were induced (70, 65, 40, 25 and 19 kDa) also corresponded to the size of proteins induced in other lactic acid bacteria during heat shock. Labelling of heat shock proteins in *Leuconostoc oenos,* using the more sensitive ^{35}S technique, has demonstrated induction of proteins of approximately 75 kDa, 64 kDa, 24 kDa, 18 kDa and 14.5 kDa (Guzzo *et al.*, 1994).

Antibody or labelling techniques, using ^{35}S labelling for example, may have detected minor changes in regulation of proteins related to HSP 90 and HSP 70, but were not performed in the present study. Western blotting techniques were also not used to confirm that the 65 kDa protein reacted with antibodies produced against HSP 60.

Only a few reports have been made of heat shock in lactic acid bacteria including (Whitaker and Batt, 1991; Arnau *et al.*, 1996; Hartke *et al.*, 1997; Carey Walker *et al.*, 1999). In *Lb. johsonii* the groESL operon has been sequenced and transcription activity studied (Carey Walker *et al.*, 1999). Maximum transcription activity in this strain, VPI 11088, was found to occur at 55°C. The authors attributed the relatively high temperature of expression to the presence of two CIRCE elements, which they felt would contribute a stronger level of negative regulation. It may be possible that dual CIRCE elements are present in heat shock operons of other lactic acid bacteria, including strain VUP Bb 12. If that is so then the most suitable conditions for induction of the high molecular weight heat shock proteins may not have been tested, as the test range in this study was limited to 47°C, a temperature where growth was strongly inhibited. Future experiments would need to look at different temperatures or heat shock regimes and more sensitive techniques to observe any changes. Sequencing of the operons would also need to be performed to confirm the presence of dual CIRCE elements.

In *L. lactis* subsp. *lactis* LM0230 heat shock proteins were observed after a temperature shift from 30°C to 42°C (Whitaker and Batt, 1991). Two of the up-regulated proteins were homologues of HSP 60 and HSP 70. Other up-regulated proteins were found to have molecular weights of 93, 76, 67, 60, 51, 49, 42, 40, 28 and 24 kDa, some of which correspond to proteins detected in this study and the study by Guzzo (1994).

4.5.1.4 Observations of protein profiles of strain Bb 12 cultures grown between pH 4 and pH 7

Marked up-regulation of a protein of approximately 33 kDa was a feature of SDS-PAGE gels of whole cells of strain VUP Bb 12 grown under strongly acidic conditions. A 14-fold increase was observed in the relative amount of the protein present in cultures grown at pH 4.0. To my knowledge, the marked up-regulation of this protein has not previously been reported in the literature for this species. The same molecular weight protein was also noted to be faintly up-regulated during growth at pH 5.0 and strongly up-regulated during growth in a fermenter with no pH control. No increase in regulation was observed in cultures grown at pH 6.0 or 7.0.

A second protein of 36 kDa was also up-regulated during growth at low pH, although not as strongly as the 33 kDa protein; however, the relative amount of the two proteins appears linked as they appear to increase at the same rate. The 33 kDa protein was also observed in 2-D SDS-PAGE after solvent extraction using chloroform and diethyl ether. This is discussed in section 4.5.2.2.

4.5.1.5 Identification of 33 kDa protein up-regulated during acid stress

N-terminal sequencing was unable to provide data that could assist in the identification of the 33 kDa protein seen during growth at pH 4.3. Observations of 2-D SDS PAGE gels showed that the protein was not present as a discrete spot; it appeared as a large smear, and it was not possible to a have a second attempt at N-terminal sequencing because the protein concentration was not high enough in the smear. The unusual appearance of the protein in these gels may be due to

the presence of a carbohydrate or lipid bound to the protein that interferes with electrophoresis. The presence of carbohydrate or lipid could be detected through modified staining methods; for example glycoproteins can be detected using a modified Periodic Acid-Sciff method. However, these stains were not performed as part of this work and would be included in future studies.

4.5.1.6 Observations of protein profiles and growth kinetics of strain Bb 12 cultures grown in a fermenter sparged with instrument air

A study by Beerens (2000) suggested that *Bifidobacterium* spp. derived from animal sources were more likely to be tolerant of exposure to air than *Bifidobacterium* spp. derived from human sources. In this study, while no major changes were observed in protein profiles grown in the presence of instrument air compared to a control, several minor proteins were down-regulated, suggesting that aeration stress resulted in sub-lethal cellular damage only and strain VUP Bb 12 could be considered tolerant to exposures of this type.

While considered obligate anaerobes, *Bifidobacterium* species show considerable variation in the growth response and metabolism in the presence of oxygen (Shimamura *et al.*, 1992). Few studies have been made of oxidative stress in LAB, but these have included *Lactobacillus* and *Lactococcus* species (Duwat *et al.*, 1995; Marty-Teysset *et al.*, 2000; Guerzoni *et al.*, 2001). In a study of oxidative stress in *Lb. delbrueckii,* it was determined that aeration of cultures did not affect the μ_{max} values achieved during growth, but aerated cultures entered stationary phase much earlier than non-aerated cultures (Marty-Teysset *et al.*, 2000). The authors attributed this to an increase of H_2O_2 in the growth medium due to the presence of the enzyme NADH oxidase. This enzyme has also been reported in other lactobacilli and some streptococci, but not in bifidobacteria. In the present study entry into stationary phase was delayed by exposure to instrument air and the μ_{max} value was lower than for the control, which suggests that a similar mechanism was not present in strain VUP 12006.

4.5.2 Characterisation of *B. animalis* (syn. *lactis*) strain VUP Bb 12 during acid stress

The affect of a sudden decrease in pH or continuous exposure to low pH was determined for strain VUP Bb 12. The use of a citrate phosphate buffer was incorporated into the methodology so that up to seven different environmental conditions could be tested at the same time. Preliminary experiments indicated that the buffer did not affect the growth rate of strain VUP Bb 12. However, some decrease in growth rate did occur, compared to growth rates in fermenters, due probably to mixing on a rotating platform, which was not as effective as agitation achieved through gas sparging and the use of a propeller stirrer.

4.5.2.1 Effect of decrease in pH on growth kinetics, viable cell counts and carbohydrate utilisation

Several observations were made of the effect of a sudden decrease in pH or continuous exposure to low pH on growth kinetics, viable cell counts and carbohydrate utilisation and they are discussed in this section.

In terms of an increase in OD_{600}, cultures grown continuously at pH 5.3 performed nearly as well as cultures grown at pH 6.3 over the first 8 hours. This was reflected in the carbohydrate utilisation studies, where glucose use and lactate production, were very similar for the two cultures. It was also noted that entry into stationary phase did not occur due to lack of a carbon source, as well over half of the initial glucose supplied, was still present in the medium after twenty-four hours incubation. Growth at pH 5.3 did not appear to have any effect on the relative proportion of acetate and lactate produced, compared to growth at pH 6.3. However, at pH 4.3 production of lactate and acetate did not occur.

Literature reviews had suggested that changes in growth conditions would affect the relative amounts of acetate and lactate production (Sgorbati *et al.*, 1995). The theoretical ratio of acetate to lactate should be 1.5 to 1, based on known bifidobacterial glucose metabolism, and it has been

observed, in *B. lactis* strain UR1, that oxygen stress has resulted in a higher ratio of acetate to lactate with an associated decrease in formate (Meile *et al.*, 1997). Changes of this nature were not observed in this limited trial for either growth at low pH or with aeration.

Cultures grown at pH 4.3 demonstrated a comparatively strong growth rate in the first five hours and the viable cell count increased by nearly one order of magnitude. However, there was little or no growth for the next nineteen hours, as the culture appeared to go into stationary phase. It was not determined exactly at what point during the last nineteen hours of growth that viable cells could not be detected; however the viable cell count dropped over four orders of magnitude in that time. Significantly, during the 24 hours of growth, significant amounts of glucose were not utilised, nor were any significant amounts of lactate produced, despite the observation that viable cells could be detected. These results showed that growth at low pH severely affected the cells and growth was not significant. These cells also failed to survive at pH 4.3 overnight.

The major difference between cultures grown at pH 6.3, 5.3 and 4.3 was seen in the final viable cell counts. The twenty-four hour viable cell count for cultures grown at pH 5.3 and 4.3, showed two-orders and four-orders of magnitude loss of viability, respectively, over the previous sixteen hours, while the viable cell count of the control remained essentially unchanged. The exact mechanism to explain why cells grown at pH 5.3 and 4.3 lose viability so rapidly is not apparent from these studies but is possibly related to proton permeation of the cell membrane. The apparent shift into stationary phase also appears to be part of a protective mechanism for this strain when they are exposed to low pH environments with subsequent induction of the general stationary phase response, changes to fatty acid profiles and changes in cell morphology: all of which would contribute to protection of cells at low pH.

Cultures that were exposed to a sudden decrease in pH during growth did not achieve similar OD_{600} readings to those of cultures grown continuously at the same pH. Clearly, sudden

decreases in pH had a more detrimental effect on growth rates than continuous exposure to low pH, but the explanation may be that it was not the sudden shift that affected growth as much as the growth phase in which it occurred. The sudden change in pH occurred during the exponential phase of growth, the phase of growth where *Lb. acidophilus* strain 12006 was shown to be more acid-sensitive. Cultures exposed continuously to low pH were exposed from lag phase, and may well have adapted to these conditions, which is consistent with the production of the 33 kDa protein in cultures where pH was uncontrolled and in pH 4.3-grown cultures.

There appeared to be some degree of contradiction in the results observed when determining if a brief exposure to pH 5.3 would make a culture more acid-tolerant to pH 4.3. In terms of final OD_{600}, cultures exposed to a brief exposure at pH 5.3 prior to exposure to pH 4.3 did not appear to perform better than cultures shifted directly from pH 6.3 to 4.3. In contrast, after twenty-four hours continuous growth at pH 4.3, no viable cells could be detected; but low numbers of viable cells could be detected after twenty-four hours in cultures that were stepped down through pH 5.3. It can be seen from the results presented in the previous section that growth curves based on OD_{600} do not reflect viable cell counts and that viable cell counts better reflect the survival of cells at low pH.

A similar limitation of the experimental protocol was appreciated when trying to interpret the results that would indicate whether pre-acid-adapted starters performed better than control cultures prepared from starters grown at pH 6.3. While changes in OD indicate there was no difference between acid-adapted and non-adapted starter cultures, viable cell counts clearly indicated better growth and survival. This is strongly suggestive that using an acid-adapted culture is protective against subsequent growth at low pH. Clearly more work would be needed to confirm these observations. In the present study no attempt was made to optimise the growth conditions with regard to the growth phase when cultures were stepped down through pH 5.3 and

the cultures were permitted to grow less than one generation before further exposure to pH 4.3. Both of these conditions would have to be considered in future experimental design.

4.5.2.2 Effect of growth at low pH on protein profiles

Several difficulties were experienced when using SDS-PAGE profiles to study changes in protein regulation during growth at low pH. Changes in protein profile as determined by 1-D SDS-PAGE failed to show any further protein induction following sudden shift in pH so it was decided to switch to 2-D SDS-PAGE, where changes in lower-abundance proteins could be examined. As noted in the results section, the immediate disadvantage of the switch was that the 33 kDa protein could not be detected in samples prepared by standard preparation of cytosolic proteins by bead-beating and subsequent centrifugation of cellular debris. This suggested that the 33 kDa protein was a cell surface or membrane protein and was among the 30% of proteins that are calculated to be not detected by routine 2-D SDS-PAGE (Santoni *et al.*, 2000). Several attempts were made to improve the representation of the 33 kDa protein in 2-D gels. Detergent extraction, one of the methods commonly suggested in the literature, did solubilise more proteins in the presence of CHAPS and Triton X-100, but did not appear to be a candidate method for use in 2-D SDS-PAGE as the 1-D profile was not changed significantly. More success was obtained using solvents to extract hydrophobic proteins, although the 33 kDa protein was never detected as a discrete spot in 2-D gels and more often appeared as a smudge. However, further sequencing was not pursued because insufficient protein was transferred to PVDF membrane for detection by Amido Black 10B.

Methods for extracting cell-surface proteins were developed. In general, solvent extraction appears to have resulted in two profile types. Octanol and chloroform produced proteins of high molecular weight and profiles with few bands. The remaining solvents studied in this work produced profiles that contained many of the bands seen in the gel when proteins were extracted from whole cells into Tris buffer, but the relative abundance of the bands changed according to

the solvent type. For 2-D SDS-PAGE the best results were obtained from mixing equal volumes of chloroform- and di-ethyl ether-extracted proteins after the extracted proteins had been re-dissolved in Tris buffer. It was found that mixing the solvents together for use as an extraction solution resulted in very low yields of proteins and although the reason for this is unknown it is possible that mixing the solvents together caused the proteins to precipitate or complex with the cell debris. Whatever the cause, there was insufficient protein to run the sample by 1-D SDS-PAGE or in the first dimension of 2-D SDS-PAGE. This problem was solved by making separate extractions and making up the dried extracts in Tris buffer subsequently.

Results of 2-D SDS-PAGE using solvent-extracted proteins demonstrated the usefulness of the technique for showing changes in hydrophobic proteins during growth at pH 4.3 when compared to pH 6.3. The pI and estimated size of the solvent-extracted proteins up-regulated during growth at pH 4.3 were, with one exception, different to that obtained from cytosolic proteins. However, a protein concentration step would be required if these solvent extracted samples were to be used to determine the N-terminal sequence of up-regulated proteins.

4.5.2.3 Function of up-regulated cytosolic proteins

The function of the three proteins up-regulated during growth at pH can, in part, be deduced from the function of closely related proteins. A BLASTP search of Genbank revealed that the closest matches to all three proteins had very similar pI and molecular weight to that observed by 2-D SDS-PAGE.

Protein Bsb-6 was found to have sequence similarity to elongation factor P or EF-P. EF-P is associated with elongation of protein biosynthesis. In particular it serves as a catalyst in the formation of the very first peptide bond of bacterial proteins (Aoki *et al.*, 1997). In *E. coli,* EF-P is thought to affect the affinity of the ribosome for charged tRNA, thereby facilitating peptide synthesis. The protein Bsb-7 was found to have sequence homology to a number of hypothetical

proteins based on database matching to the completed genomes of *B. longum*, *Corynebacterium glutamicum* and *Mycobacterium leprae*. The function of these hypothetical proteins could not be determined.

Bsb-8 was found to have homology to the N-terminal sequence from the 50S ribosomal protein L7/L12. The 50S sub-unit function is related to formation of peptide bonds: in particular, it catalyses the peptidyl transferase reaction of protein synthesis. The ribosomal protein L7/L12 is an important component of this sub-unit and in *E. coli* is present in four copies organized as two dimers. The L7/L12 dimer is thought to interact with elongation factors as part of translation elongation indicating a possible relationship to Bsb-6 (a potential elongation factor).

The possible function of both Bsb-6 and 8 appear to be closely related to the protein synthesis, in particular ribosomal function. The presence of these proteins may be related to a number of factors, including production of novel proteins that are essential for growth during acid stress, or because the growth phases are out of step. In the first case, it is possible the cells are making proteins, like the 33 kDa protein seen during growth at pH 4.3, because they are essential for cell viability. In the second, the increase in ribosomal function could be related to the fact that cells grown at pH 6.3 have reached stationary phase, while those at pH 4.3 are potentially still in early exponential phase, refer to Figure 4.10.

4.5.2.4 Areas for further study

There are several areas that could be considered for further study. Firstly, in order to obtain the complete nucleotide sequence of the gene encoding the 33 kDa protein in *B. animalis* (syn. *lactis*), it would be of interest to obtain amino acid sequence data for that protein. While further attempts could be made at obtaining the N-terminal sequence using the traditional Edman degradation reaction, an alternative approach would be to obtain a peptide-mass fingerprint from a proteolytic digest of purified protein. Briefly, a trypsin digest would be performed on a spot cut

from an acrylamide gel (1-D) corresponding to the location of the 33 kDa protein. Then, using MALDI-TOF, an accurate mass of the peptides would be obtained and compared to data in protein databanks obtained from proteins digested by the same enzyme. Where this approach was insufficient further N-terminal or C-terminal data may be able to be obtained using PSD (post-source dissociation) or CID (collision-induced dissociation). Once sequence data had been obtained a similar approach to that used in Chapter 3 would be followed to obtain the sequence of the gene encoding this protein.

Other areas of further study could also include the following:

- Preparation of antibodies against the 33 kDa protein identified during growth at low pH in *B. animalis* (syn. *lactis*) strain VUP Bb 12. It would be useful to monitor the regulation of the gene through western blot analysis. These antibodies could also be used to determine the presence of analogues of the gene in related strains, related species and non-related species.

- Determination of the sub-cellular location of the 33 kDa protein. This work would assist in understanding the function of the protein.

- Further studies are also required to identify the proteins up-regulated during heat stress in *B. animalis* (syn. *lactis*) Bb 12. A different approach, using a lower challenge temperature, would be used to see if a stronger heat shock response could be initiated. Although heat shock studies could include western blot analysis, alternative approaches could involve N-terminal sequencing or peptide mass fingerprinting of 2-D SDS-PAGE spots.

4.6 Conclusion

- 16S rRNA was able to confirm strain Bb12 as *B. animalis* (syn.) *lactis*.

- *B. animalis* (syn.) *lactis* grew optimally at 42°C ± 0.2 and at pH 6.3 ± 0.2.

- When heat stressed at 47°C, *B. animalis* (syn.) *lactis* did not demonstrate a classic heat stress response.

- When acid stressed, *B. animalis* (syn.) *lactis* produced a protein of approximately 33 kDa which was found to be cell-membrane- or cell-surface-associated. The N-terminal sequence of this protein could not be determined.

- Cytosolic proteins Bsb-6 and Bsb-8 were N-terminally sequenced and identified as being functionally related to elongation factor-P and 50S ribosomal protein L7/L12 respectively.

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APPENDICES

Appendix 1

List of Laboratory Instrumentation, equipment and chemicals

Instrumentation and equipment

Anerobic jar and catalyst system (2L)
Supplied by Oxoid Australia Pty Ltd

Bioreactor (fermenter) 2L
Braun Biostat B
Supplied by B. Braun Australia

Bioreactor (fermenter) 1L 1012 motor controller and FC4 Data system
Applikon dependable instruments, Schiedam Holland
Supplied by Real Time Engineering

Cell Homogenizer.
Supplied by B. Braun, Australia

Cell Homogenizer. 50 mL Duran glass or stainless steel sample bottles
Supplied by B. Braun, Australia

Centrifuge J2-HS, refrigerated
Supplied by Beckman Industries Australia Ltd

Centrifuge, refrigerated
Sorvall Model RT7
Supplied by Amrad Pharmacia Biotech

Dry Block Heater
Supplied by Ratek Instruments Australia

Eppendorf tubes (1 mL)
Elkay Products Inc
Supplied by Pathtech Diagnostics

Fermenter (One litre Bio Reactor Under Computer Evaluation)
BRUCE system
Developed at CBFT, Vic University by Dr. Takashi Ueno

Gas Chromatograph model 3400, including Cx autosampler and Star chromatography software, version 4.0, Saturn 2000 GC/MS/MS and Gas chromatography sample vials
Supplied by Varian Australia Pty Ltd

Gas Chromatography column BPX-70
25 metre bonded phase, fused silica, very polar column
Supplied by SGE Australia

Gas supplies including high purity Nitrogen, Instrument air and gases for GC
Supplied by BOC Gases

GelAir Drying System
Protein gel dryer
Supplied by Bio-Rad Laboratories Pty Ltd

G100 Electrophoresis tank and fittings
Supplied by Amrad Pharmacia Biotech

HPLC column
Aminex HPX-87H
Supplied by Bio-Rad

Hybond N+ Nucleic acid transfer membrane
Supplied by Amersham Australia Pty Ltd

HPLC (including Varian 9100 Autosampler, Varian 9012 Pump,
Varian 9050 UV-VIS Detector and Varian Star 4.0 software
Supplied by Varian Australia Pty Ltd

Kimwipes Fine Grade Cat # 4103
Supplied by Kimberly Clark

Microfuge Eppendorf Centrifuge 54515C
Supplied by Crown Scientific Pty Ltd

Microfuge Microspin 24
Sorvall Instruments Dupont.
Supplied by Varian Australia Pty Ltd

Mini-Beadbeater-8
BioSpec products, Inc
Supplied by Daintree Scientific

Mini Prep Cell (preparative electrophoresis)
Supplied by Bio-Rad Laboratories Pty Ltd

Mini-Protean II cell
Supplied by Bio-Rad Laboratories Pty Ltd

Model 491 Prep Cell (preparative electrophoresis)
Supplied by Bio-Rad Laboratories Pty Ltd

Needles hypodermic 18G 1½ TW 1.00 x 38 mm
Terumo Corporation
Supplied by Pathtech Diagnostics

Nytran-Plus Nylon Membrane
Supplied by Progen Industries Ltd

Pasteur pipettes Cat# BILB 150
Supplied by Selby Scientific

pH meter model PHM220
Supplied by Radiometer Pacific

Photographics
Supplied by Prossper Photographics

Protean II cell
Supplied by Bio-Rad Laboratories Pty Ltd

Rotofor Cell
Bio-Rad Laboratories Pty Ltd

Screw top open hole vial caps 8 mm Alltech Cat # 16-000698-00
Screw cap vials 12x32 mm (amber) Alltech Cat # 95194
Supplied by Progen Industries Ltd

Syringes, Terumo Corporation 1 mL, 5 mL, 10 mL, 50mL
Supplied by Crown Scientific

Trans-Blot SD cell
Supplied by Bio-Rad Laboratories Pty Ltd

Triple wide vertical slab electrophoresis unit
Supplied by Silenus Labs Pty Ltd

Two-dimensional gel electrophoresis
Supplied by Pharmacia

Water baths
Supplied by Ratek Instruments Australia

Whatman Chromatography Paper 3MM Chr 46x57 cm Cat # 3030917
Supplied by Lab Supply

Chemicals

^{32}P Deoxycytidine 5'-Triphosphate
Supplied by Bresatec ACN 070 039 913

Acetic acid Glacial AnalaR Cat # 945040
Merck Pty Ltd ACN 005 063 791
Supplied by Crown Scientific

Acrylamide powder
Acrylamide 99.9%
Supplied by Bio-Rad Laboratories Pty Ltd

ATP Adenosine 5'-Triphosphate Disodium Salt Cat # A 6144
Supplied by Sigma Aldrich Pty Ltd

Agar Bacteriological (Agar No 1) Code 11, Cat # LP011F
Unipath Ltd, Basingstoke, Hampshire, England
Supplied by Oxoid Australia Pty Ltd

Ammonium Acetate Molecular Biology Grade MW=77.08 Cat # A 1542
Supplied by Sigma Aldrich Pty Ltd

Ammonium Persulphate MW=228.20
Supplied by Bio-Rad Laboratories Pty Ltd

Ampicillin D [-]- -Aminobenylpenicillin MW=371.4 Cat # A 6144
Supplied by Sigma Aldrich Pty Ltd

Antibodies (GroEL, Dna-K etc.)
Supplied by Sigma Immuno Chemicals, BioRad and DAKO

Biolog Lactic Acid Suspension Broth
Biolog Inc.
Supplied by SPL Diagnostics

Bis
(N,N'-methylene-bis-acrylamide)
Supplied by Bio-Rad Laboratories Pty Ltd

Bovine Serum Albumin Fraction V Laboratory Reagent Cat # 100 018
Supplied by Boehringer Mannheim Pty Ltd

Butan-2-ol
Supplied by Sigma Aldrich Pty Ltd

Coomassie Blue G-250 dye
Supplied by Bio-Rad Laboratories Pty Ltd

DEPC Diethyl Pyrocarbonate MW=162.1 Cat # D 5758
Supplied by Sigma Aldrich Pty Ltd

Dithiothreitol
Supplied by Sigma Aldrich Pty Ltd

DNA Grade Agarose (Molecular Biology Grade) Cat # 200-0011
Supplied by Progen Industries Ltd

EcoR1 Restriction Nuclease and Buffer Cat # 101S
Pst1 Restriction Nuclease and NEBuffer 3 Cat # 140S
Other restriction enzymes and buffers
Manufactured by New England Biolabs
Supplied by Genesearch Pty Ltd

Ethanol Absolute
Supplied by CSR Yarraville Distillery

Ethidium Bromide MW=394.3 Cat # E 8751
Supplied by Sigma Aldrich Pty Ltd

Ethylenediaminetetra acetic acid Disodium Salt: Dihydrate MW= 372.2 Cat # E 4884
Supplied by Sigma Aldrich Pty Ltd

ExpressHyb
Clontech
Supplied by Clontech Laboratories

Folin/Ciocalteau Reagent
Supplied by Sigma Aldrich Pty Ltd

Formaldehyde MW=30.03 37% Solution ACS Reagent Cat # F 1268
Supplied by Sigma Aldrich Pty Ltd

Formamide MW=45.04 Cat # F 7503
Supplied by Sigma Aldrich Pty Ltd

Gas Chromatography Lipid Standards
Product Numbers: 189-1, 189-3, 189-9, 189-4
Supplied by Sigma Aldrich Pty Ltd

D-Glucose BDH MW=180.16 Cat # 28450
Merck Pty Ltd ACN 005 063 791
Supplied by Crown Scientific

Glycerol BDH General Purpose Reagent Product # 28454
Merck Pty Ltd ACN 005 063 791
Supplied by Crown Scientific

Isoamyl Alcohol BDH AnalaR Product # 1003832
Merck Pty Ltd ACN 005 063 791
Supplied by Crown Scientific

Isopropanol (2-Propanol) MW=60.10 Cat # I 9516
Supplied by Sigma Aldrich Pty Ltd

IPTG Isopropyl- -D-Thiogalactopyranosid MW=238.3
Supplied by Boehringer Mannheim Pty Ltd

DL-Lactic acid (Sodium salt) FW=112.1
Supplied by Sigma Aldrich Pty Ltd

Lysozyme Cat # 1585657
Supplied by Boehringer Mannheim Pty Ltd

Magnesium Chloride BDH MW=246.47
Merck Pty Ltd ACN 005 063 791
Supplied by Crown Scientific

Magnesium Sulphate BDH AnalaR MW=246.47 Product # 10151
Merck Pty Ltd ACN 005 063 791
Supplied by Crown Scientific

2-Mercaptoethanol (2-Hydroxyethylmercaptan; -mercaptoethanol) MW=78.13 Cat # M 6250
Supplied by Sigma Aldrich Pty Ltd

Methanol HPLC Grade BDH
Merck Pty Ltd ACN 005 063 791
Supplied by Crown Scientific

Molecular weight standards
Supplied by Bio-Rad Laboratories Pty Ltd

Petroleum Ether BDH AnalaR, Product # 10179
Merck Pty Ltd ACN 005 0630791
Supplied by Crown Scientific

Phenol
Supplied by Novachem Pty Ltd

Polyvinylpirrolidine (Molecular Biology Reagent) MW=360,000
Supplied by Sigma Aldrich Pty Ltd

Potassium Acetate MW=98.24 Cat # P 1147
Supplied by Sigma Aldrich Pty Ltd

Potassium Hydroxide BDH MW=56.11 AnalaR, Product # 10210
Merck Pty Ltd ACN 005 0630791
Supplied by Crown Scientific

Potassium Chloride BDH MW=74.55, Merck (Laboratory Reagent) Product # 29594
Merck Pty Ltd ACN 005 0630791
Supplied by Crown Scientific

RNAse Ribonuclease I Bovine Pancreas
Supplied by Amrad Pharmacia Biotech

Rotolytes
Supplied by Bio-Rad Laboratories Pty Ltd

Sodium Acetate BDH AnalaR MW=82.03 Product # 10236
Merck Pty Ltd ACN 005 0630791
Supplied by Crown Scientific

Sodium Chloride BDH MW=58.44 AnalaR, Product #10241
Merck Pty Ltd ACN 005 0630791
Supplied by Crown Scientific

Sodium Hydroxide MW=40.00 Cat # 10236
Ajax Chemicals
Supplied by Crown Scientific

Sodium Lactate MW=112.07
Supplied by Crown Scientific

Sodium Lauryl Sulphate (SDS) MW=288.4 Cat # L4509
Supplied by Sigma Aldrich Pty Ltd

Sodium Citrate BDH MW=294.10 AnalaR, Product # 10242
Merck Pty Ltd ACN 005 0630791
Supplied by Crown Scientific

TEMED
(N,N,N',N'-tetramethyl-ethylenediamine)
MW=116.21
Supplied by Bio-Rad Laboratories Pty Ltd

Tris chloride MW=121.14 AnalaR, Product # 103157P
Merck Pty Ltd ACN 005 0630791
Supplied by Crown Scientific

Tris chloride MW=157.61 Cat # 103130.18
Supplied by ICN Biochemicals Australasia Pty Ltd

Triton X-100 t-octylphenoxypolyethoxyethanol Cat # X 100
Supplied by Sigma Aldrich Pty Ltd

Tryptone Code L42 Oxoid Australia, Cat # LP042B
Unipath Ltd, Basingstoke, Hapmshire, England
Supplied by Oxoid Australia Pty Ltd

Yeast Extract Code L21,
Unipath Ltd, Basingstoke, Hampshire, England
Supplied by Oxoid Australia Pty Ltd

X-ray Film X AR 5 (43x35) Cat # 1651512
Supplied by Integrated Sciences

Appendix 2

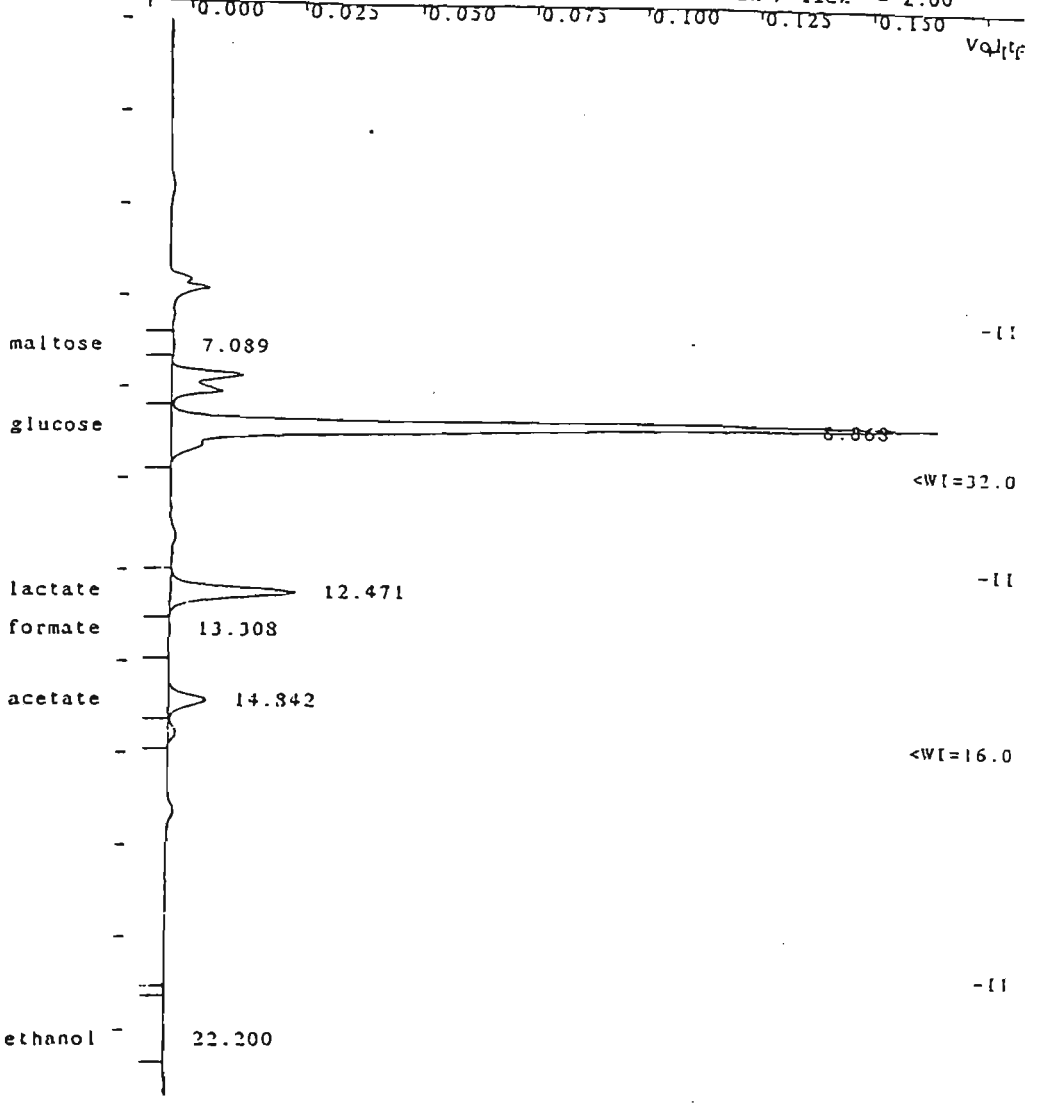
List of Suppliers

Amersham Australia Pty Ltd	PO Box 99 North Ryde NSW 2113
Amrad Pharmacia Biotech	34 Wadhurst Dve Boronia Vic 3155
B. Braun Australia	10 Arcade Rd. Box Hill North, Vic 3129
Beckman Industries Australia Ltd	Unit 16, 170 Forester Rd Mt Waverly Vic 3149
Bio-Rad Laboratories Pty Ltd	PO Box 210, Regents Park NSW 2143
BOC Gases	90 Bell St, PO Box 43 Preston Vic 3072
Boehringer Mannheim Pty Ltd	353 Whitehorse Rd Nunawading Vic 3131
Bresatec ACN 070 039 913	PO Box 11 Rundle Mall Adelaide SA 5000
Chr. Hansen Pty Ltd	49 Barry St. Bayswater, Vic 3153
Clontech Laboratories	4 Research Park Drive, Macquarie University Research Park North Ryde NSW 2113
Crown Scientific Pty Ltd	PO Box 101 Burwood Vic 3125
CSR Yarraville Distillery	PO Box 83 Yarraville Vic 3013
Daintree Scientific	20 Kiama Pde. St Helens Tasmania
DAKO Australia Pty Ltd	Lord St Botany New South Wales
Genesearch Pty Ltd	14 Technology Drive Arundel, Qld 4214
ICN Biochemicals Australasia Pty Ltd	PO Box 187, Unit 12 167 Prospect Highway Seven Hills NSW 2147
Integrated Sciences	1401 Burke Rd East Kew Vic 3102
Kimberly Clark	PO Box 343 Milsons Point NSW 2061
Lab Supply	PO Box 688 Mulgrave Nth Vic 3170
Novachem Pty Ltd	50 Garden St South Yarra Vic 3141
Oxoid Australia Pty Ltd	104 Northern Rd Western Heidelberg Vic 3081
Pathtech Diagnostics	PO Box 175 Balwyn Vic 3103

Progen Industries Ltd	2806 Ipswich Rd Darra Qld 4076
Prossper Photographics	149 Nelson Place Williamstown Vic 3016
Ratek Instruments Australia	PO Box 131 Mitcham Vic 3155
Real Time Engineering	Level 1 15-19 Boundary St. Rushcutters Bay NSW 2011
Selby Scientific	PO Box 276 Acacia Ridge Qld 4110
SGE Australia	7 Argent Place Ringwood Vic, 3134
Sigma Aldrich Pty Ltd and Sigma Immuno Chemicals	Unit 2, 14 Anella Ave Castle Hill NSW 2154
Silenus Labs Pty Ltd	34 Wadhurst Drive Boronia Vic 3155
SPL Diagnostics	1408 Centre Rd. Clayton, Vic 3168
Varian Australia Pty Ltd	679 Springvale Rd Mulgrave Vic 3170

Appendix 3

```
Title        : glumalt
Run File     : c:\star\maria\maria042.run
Method File  : C:\STAR\MARIA.MTH
Sample ID    : Sue 6b

Injection Date: 21-JAN-96 8:34 PM     Calculation Date: 21-JAN-96 8:50 PM

Operator     : Maria                  Detector Type: ADC8 (1 Volt)
Workstation: MS-DOS_6                 Bus Address  : 16
Instrument : Varian Star #1           Sample Rate  : 10.00 Hz
Channel    : A = RI-4 1 volts         Run Time     : 24.002 min

********** Star Chromatography Software ********** Version 4.01 **************

Chart Speed =    0.87 cm/min   Attenuation = 800          Zero Offset = 41
Start Time  =    0.000   min   End Time    = 24.000  min  Min / Tick  = 2.00
```



Appendix 4

Computational Biology at ORNL

Channel • Generation • Grail • GrailEXP • Pipeline • Parser • PROSPECT

ORNL Microbial Blast Results

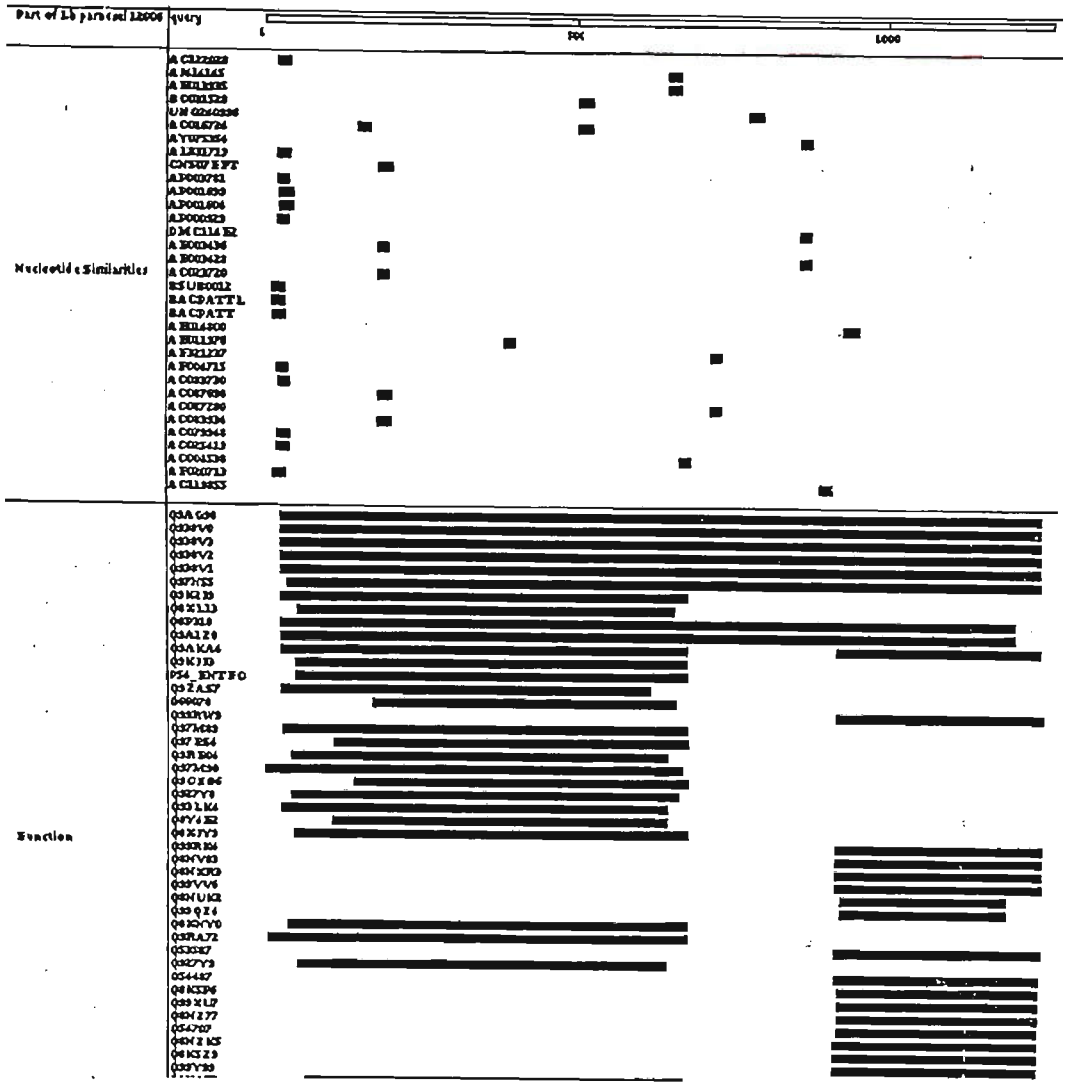
```
TBLASTN 2.2.3 [May-13-2002]

Reference: Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schaffer,
Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997),
"Gapped BLAST and PSI-BLAST: a new generation of protein database search
programs", Nucleic Acids Res. 25:3389-3402.

Query=
       (20 letters)

Database: /auto/microbe/lcas/chromosome/1/data/lcas.fna
           199 sequences; 2,618,854 total letters

Searching.done

                                                                    Score    E
Sequences producing significant alignments:                         (bits) Value

                                                                      41   1e-05
Scaffold3                                                             21   9.9
Scaffold7

>Scaffold3
          Length = 82578

 Score = 40.8 bits (94), Expect = 1e-05
 Identities = 20/20 (100%), Positives = 20/20 (100%)
 Frame = -2

Query: 1     DTSDSIASNKSETNALLKQI 20
              DTSDSIASNKSETNALLKQI
Sbjct: 9560 DTSDSIASNKSETNALLKQI 9501


>Scaffold7
          Length = 75568

 Score = 21.2 bits (43), Expect = 9.9
 Identities = 10/16 (62%), Positives = 12/16 (74%)
 Frame = -2

Query: 2     TSDSIASNKSETNALL 17
              +SD I +NKS T ALL
Sbjct: 24543 SSDMIRTNKSATYALL 24496


  Database: /auto/microbe/lcas/chromosome/1/data/lcas.fna
    Posted date:  Oct 7, 2002  5:30 PM
  Number of letters in database: 2,618,854
  Number of sequences in database:  199

Lambda     K      H
   0.293    0.110    0.252

Gapped
```

```
Matrix: BLOSUM62
Gap Penalties: Existence: 11, Extension: 1
Number of Hits to DB: 21,771
Number of Sequences: 199
Number of extensions: 27
Number of successful extensions: 2
Number of sequences better than 10.0: 4
Number of HSP's better than 10.0 without gapping: 2
Number of HSP's successfully gapped in prelim test: 0
Number of HSP's that attempted gapping in prelim test: 0
Number of HSP's gapped (non-prelim): 2
length of query: 20
length of database: 872,951
effective HSP length: 0
effective length of query: 24
effective length of database: 872,951
effective search space: 20950824
effective search space used: 20950824
frameshift window, decay const: 50,  0.1
T: 13
A: 40
X1: 17 ( 7.2 bits)
X2: 38 (14.6 bits)
X3: 64 (24.7 bits)
S1: 43 (21.3 bits)
S2: 43 (21.2 bits)
```

Analysis Summary

1. Nucleotide Similarities: blastn Result
2. Function: blastx Result
3. Gene Prediction: GenScan Result

Graphic Summary


Part of Lb paracsei 12006
query
1
500
1000
Nucleotide Similarities
Function

Details

blastn Result

Matching Entry	Begin-End	Description	Score	E Value
AC122028	[24-45]	Mus musculus chromosome 2 clone RP24-394D4, complete sequence.	44.1	0.47
AJ414145	[645-666]	Yersinia pestis strain CO92 complete genome; segment 5/20.	44.1	0.47
AE013935	[645-666]	Yersinia pestis KIM section 335 of 415 of the complete genome.	44.1	0.47
BC031528	[502-526]	Mus musculus, Similar to nuclear receptor coactivator 4, clone MGC:28270 IMAGE:4008986, mRNA, complete cds.	42.1	1.9
UHO240996	[774-798]	uncultured Holophaga/Acidobacterium Sva0777 16S rRNA gene, partial.	42.1	1.9
AC016724	[151-171]	Homo sapiens BAC clone RP11-339F22 from 2, complete sequence.	42.1	1.9
AC016724	[502-526]	Homo sapiens BAC clone RP11-339F22 from 2, complete sequence.	42.1	1.9
AY075354	[858-877]	Drosophila melanogaster GH28809 full length cDNA.	40.1	7.4
AL831719	[24-43]	Mouse DNA sequence from clone RP23-166I8 on chromosome 4, complete sequence.	40.1	7.4
CNS07EFT	[182-205]	Human DNA sequence chromosome 8 of Homo sapiens (human).	40.1	7.4
AP003781	[22-41]	Homo sapiens genomic DNA, chromosome 11q clone:RP11-49K4, complete sequences.	40.1	7.4
AP001699	[25-48]	Homo sapiens genomic DNA, chromosome 21q, section 43/105.	40.1	7.4
AP001604	[25-48]	Homo sapiens genomic DNA, chromosome 21, clone:KB1648B8, APP-D21S292 region, complete sequence.	40.1	7.4
AP000929	[22-41]	Homo sapiens genomic DNA, chromosome 11q, clone:RP11-778B17, complete sequence.	40.1	7.4
DMC114E2	[858-877]	Drosophila melanogaster cosmid 114E2.	40.1	7.4
DMC114E2	[858-877]	Drosophila melanogaster cosmid 114E2.	40.1	7.4
AE003436	[182-201]	Drosophila melanogaster chromosome X, section 20 of 74 of the complete sequence.	40.1	7.4
AE003428	[858-877]	Drosophila melanogaster chromosome X, section 12 of 74 of the complete sequence.	40.1	7.4
AC023720	[182-201]	Drosophila melanogaster X BAC RP98-7F15 (Roswell Park Cancer Institute Drosophila BAC Library) complete sequence.	40.1	7.4

BSUB0012	[16-35]	Bacillus subtilis complete genome (section 12 of 21): from 2195541 to 2409220.	40.1	7.4
BACPATTL	[16-35]	Bacillus subtilis phage SPB ATTL site.	40.1	7.4
BACPATT	[16-35]	B.subtilis phage SPB ATT site.	40.1	7.4
AE014800	[928-955]	Bifidobacterium longum NCC2705 section 187 of 202 of the complete genome.	40.1	7.4
AE011976	[384-403]	Xanthomonas axonopodis pv. citri str. 306, section 354 of 469 of the complete genome.	40.1	7.4
AF321237	[714-733]	Homo sapiens chromosome 11p15.4 clone RPC11-610i20 P2-containing olfactory receptor gene cluster, complete sequence.	40.1	7.4
AF004715	[22-41]	Homo sapiens jerky gene product homolog mRNA, complete cds.	40.1	7.4
AC093730	[25-44]	Homo sapiens BAC clone RP11-12K22 from 4, complete sequence.	40.1	7.4
AC087698	[182-205]	Homo sapiens chromosome 8, clone RP11-25K19, complete sequence.	40.1	7.4
AC087280	[714-733]	Homo sapiens chromosome 11, clone RP11-560B16, complete sequence.	40.1	7.4
AC083934	[182-205]	Homo sapiens chromosome 8, clone RP11-108L23, complete sequence.	40.1	7.4
AC079948	[24-43]	Homo sapiens 12 BAC RP11-316O1 (Roswell Park Cancer Institute Human BAC Library) complete sequence.	40.1	7.4
AC025419	[24-43]	Homo sapiens 12 BAC RP11-230G5 (Roswell Park Cancer Institute Human BAC Library) complete sequence.	40.1	7.4
AC004538	[665-684]	Homo sapiens PAC clone RP5-887P4 from 7p21-p22, complete sequence.	40.1	7.4
AF020713	[16-35]	Bacteriophage SPBc2 complete genome.	40.1	7.4
AC119855	[893-912]	Mus musculus, clone RP23-243H19, complete sequence.	40.1	7.4

blastx Result

Matching Entry	Begin-End	Description	Score	E Value
Q9AG98	[28-1251]	General stress protein GSP-781 (Glucan-binding protein B).	98.3	2e-19
Q938V0	[28-1251]	Glucan-binding protein B.	97.9	3e-19
Q938V3	[28-1251]	Glucan-binding protein B.	97.9	3e-19
Q938V2	[28-1251]	Glucan-binding protein B.	95.6	1e-18
Q938V1	[28-1251]	Glucan-binding protein B.	95.6	1e-18
Q97N55	[40-1251]	Secreted 45 kDa protein.	81.5	2e-14
Q9K2J9	[28-678]	Secreted antigen SagA.	81.5	2e-14

Q8XL13	[55-657]	Hypothetical protein CPE1229.	78.4	2e-13
Q8P318	[28-1209]	Putative secreted protein.	78.4	2e-13
Q9A1Z8	[28-1209]	Putative secreted protein (Secreted protein SibA precursor).	77.3	5e-13
Q9AKA4	[28-678]	PcsB protein precursor.	76.1	1e-12
Q9AKA4	[922-1251]	PcsB protein precursor.	52.7	1e-12
Q9KJJ3	[52-678]	Secreted antigen SagBb.	70.6	5e-11
P54_ENTFC	[52-678]	P54 protein precursor.	70.6	5e-11
Q9ZAS7	[28-618]	Putative secreted protein (Fragment).	69.9	8e-11
O66078	[175-660]	Putative extracellular protein Exp1 precursor (Fragment).	65.2	2e-09
Q99RW9	[922-1257]	Hypothetical protein SAV2304 (SsaA protein).	64.4	3e-09
Q97M89	[31-678]	Cell wall-associated hydrolase.	64.4	3e-09
Q97ES4	[112-681]	Uncharacterized protein containing conserved domain, related toYABE B.subtilis C-terminal domain.	63.6	6e-09
Q9RE04	[43-648]	P45 precursor (Peptidoglycan lytic protein P45).	62.8	1e-08
Q97M90	[1-672]	Cell wall-associated hydrolase.	62.1	2e-08
Q9CEB6	[142-681]	Hypothetical protein yuaE.	61.3	3e-08
Q927Y8	[43-666]	Peptidoglycan lytic protein P45.	60.9	4e-08
Q93LK4	[28-648]	SalA antigen.	60.9	4e-08
Q8Y4E2	[109-648]	Hypothetical protein lmo2504.	60.5	5e-08
Q8XJY9	[49-681]	Probable cell wall-binding protein.	56.6	8e-07
Q99RX4	[922-1257]	SsaA protein (Secretory antigen precursor SsaA homolog).	55.4	2e-06
Q8NV83	[922-1257]	MW2217 protein.	55.4	2e-06
Q8NXR3	[922-1257]	MW0627 protein.	53.9	5e-06
Q99VV6	[922-1257]	SA0620 protein (Secretory antigen SsaA homologue).	53.9	5e-06
Q8NUK2	[928-1200]	MW2565 protein.	53.5	7e-06
Q99QZ4	[928-1200]	Hypothetical protein SAV2644.	53.5	7e-06
Q8XNY0	[40-681]	Probable cell wall-binding protein.	53.1	9e-06
Q9RA72	[7-681]	M-like protein precursor.	51.5	3e-05
Q9RA72	[31-681]	M-like protein precursor.	36.7	3e-05