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**Surface characteristics of an adhesive  
thermophilic spore-forming *Bacillus*,  
isolated from milk powder**

A thesis presented in partial fulfilment of the requirements for the degree of

Doctor of Philosophy in Food Technology

At Massey University, Palmerston North, New Zealand

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**2008**

## ABSTRACT

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The growth of thermophiles during the manufacture of milk powder leads to a progressive increase in the number of thermophilic bacteria contaminating the final product. The limited residence time of the milk in the plant during milk powder manufacture and the concentration effect of converting milk into milk powder cannot explain the number of thermophiles found in the final product. This suggests that thermophiles are attaching to the large surface area of stainless steel found within a milk powder plant and then growing and developing into biofilms, with individual cells and/or biofilm fragments sloughing off into the product line and thus contaminating the final product.

The aim of the present study was to investigate the attachment mechanisms that enable the thermophile *Anoxybacillus flavithermus* (B12) to attach to stainless steel surfaces. Passing a B12 culture through a column of stainless steel chips, collecting the first cells to pass through, re-culturing and repeating the process six times, resulted in the isolation of a mutant, labelled X7, with 10-fold reduced ability to attach to stainless steel as well as a reduced ability to attach to plastic and glass.

A comparison of bacterial cell surface properties indicated that X7 was less hydrophobic than its parental strain B12. Cell surface charge measurements also suggest that X7 has less net negative surface charge. Disruption of extracellular polysaccharides and DNA appeared to have no effect on the attachment process. Removal of surface proteins caused a reduction in attachment of B12 and X7 as well as a reduction in surface hydrophobicity suggesting surface protein involvement in both.

Analysis by two-dimensional gel electrophoresis of lysozyme/mutanolysin extracted surface proteins revealed two proteins expressed at reduced levels in X7 compared with B12. One protein was identified by mass spectrometry as the cytoplasmic enzyme Formate acetyltransferase. The role of Formate acetyltransferase and the

second unidentified protein on the attachment process of *Anoxybacillus flavithermus* remains unclear.

## LIST OF PUBLICATIONS

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**Palmer J.S., Flint, S.H., Brooks, J. (2007)** Bacterial cell attachment, the beginning of a biofilm. *Journal of Industrial Microbiology and Biotechnology*. **34**:577-588.

## LIST OF PRESENTATIONS

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**Palmer J.S., Flint, S.H., Brooks, J., Schmid, J. (2005)** Surface proteins of an adhesive thermophilic sporeforming bacilli from milk powder. *New Zealand Microbiological Society Conference*, Dunedin, New Zealand, 2005.

**Palmer J.S., Flint, S.H., Brooks, J., Schmid, J. (2005)** Surface proteins of an adhesive thermophilic sporeforming bacilli from milk powder. ASM, Biofilms conference, Quebec, Canada. 2007

## ACKNOWLEDGEMENTS

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I would like to thank chief supervisor Prof John Brooks for this supervision throughout this project, even after his move from Massey University to Auckland Institute of Technology University. His ability to keep a balanced view of the overall project with metaphors such as “How will this make the boat go faster?” always kept the final goal of this project in mind.

Thanks must also go to my other supervisors Assoc/Prof Steve Flint for his never-ending encouragement and guidance. I would also like to thank Dr Jan Schmid for his helpful discussions and ideas throughout this project.

Thanks also to everyone in the Microbiology suite, in particular Ann-Marie Jackson, John Sykes, Mike Sahayam, Judy Collins and Susan Bassett as well as various post-graduate and visiting Dutch students working in the same laboratory.

I also gratefully acknowledge, Fonterra research centre staff members, Kylie Walker, Richard Ireland and Dr Skealt for their use of analytical equipment and technical advice.

Finally, I would like to thank family and friends who have encouraged and helped keep me sane the last few years. To Terri and my daughters (Bree and Sophie), no words can explain my gratitude to you for allowing me to pursue this dream.

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## List of Abbreviations

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g	acceleration due to gravity
AFM	atomic force microscope
BATH	bacterial adherence to hydrocarbons
bp	base pair
BLAST	basic local alignment search tool
BCA	bicinchoninic acid
BAP	biofilm associated protein
BSA	bovine serum albumin
°C	degrees Celsius
CHAPS	3-cholamidopropyl-dimethylammonio-1-propanesulfonate
CIP	cleaning in place
CID	collision induced spectra
CFU	colony forming units
Da	daltons
dNTP	deoxynucleotide triphosphate
DNA	deoxyribosenucleic acid
DLVO	Derjaguin, Landau, Verwey, Overbeek
DW	distilled water
DTT	Dithiothreitol
ESI	electrospray ionization
ESI-QUAD-TOF	electrospray ionization-quadrupole-time-of-flight
ESIC	electrostatic interactive chromatography
EB1	equilibration buffer 1
EB2	equilibration buffer 2
EDTA	ethylenediamine tetraacetic acid
eDNA	extracellular deoxyribosenucleic acid
EPS	extracellular polysaccharide
g	gram(s)
g/l	grams per litre
HPLC	high performance liquid chromatography
h	hour(s)
HCl	hydrochloric acid
HIC	hydrophobic interactive chromatography
IPS	immobilised pH gradient
IDT	impedence detection time
IEF	isoelectric electric focusing
pI	isoelectric point
kb	kilo base pairs
kDa	kilodalton
LPS	lipopolysaccharide
L	litre(s)
LB	Luria-Bertani
MgCl <sub>2</sub>	Magnesium chloride

MgSO <sub>4</sub>	Magnesium sulphate
m/z	mass charge ratio
MALDI	matrix-assisted laser desorption/ionization
MATH	microbial adherence to hydrocarbons
μg	microgram(s)
μl	microlitre(s)
μm	micrometre(s)
μM	micromolar
mA	milliampere
mg	milligram(s)
ml	millilitre(s)
mm	millimetre(s)
mM	millimolar
min	minutes
M	molar
KH <sub>2</sub> PO <sub>4</sub>	monobasic potassium phosphate
ng	nanogram
nm	nanometer
ONPG	o-nitrophenyl-β-D-galactosidase
TEMED	N-N-N-N-tetramethylene diamine
ORF	open reading frame
OD	optical density
%	percentage
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
KCl	potassium chloride
RAPD	random amplified polymorphic DNA
RI	refractive index
rpm	revolutions per minute
NaCl	sodium chloride
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SD(s)	standard deviation(s)
SSP1	Staphylococcus surface protein 1
SSP2	Staphylococcus surface protein 2
MS/MS	tandem mass spectroscopy
TOF	time-of-flight
TEM	transmission electron microscopy
TAE	tris-acetate-EDTA
Tris	tris(hydroxymethyl)aminomethane
TE	tris-HCl-EDTA
TSA	tryptic soya agar
TSB	tryptic soya broth
2D-PAGE	two dimensional-polyacrylamide gel electrophoresis
UV	ultraviolet
v/v	volume/volume

V	volts
VH	volt hours
w/v	weight/volume
XPS	x-ray photoelectric spectroscopy

#### Amino acids

A	alanine
E	glutamic acid
H	histidine
L	leucine
P	proline
S	serine
V	valine
C	cysteine
F	phenylalanine
I	isoleucine
M	methionine
Q	glutamine
T	threonine
Y	tyrosine
D	aspartic acid
G	glycine
K	lysine
N	asparagine
W	tryptophan

#### Nucleotides

A	adenosine
C	cytidine
G	guanosine
T	thymidine

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## Introduction and Project Objectives

### *1.1 Introduction*

In the manufacture of milk powder, plant run times are limited by contamination of the product by thermophilic bacteria, both vegetative and sporulated. During the process run, which is typically of 18 to 20 hours' duration, a gradual increase in thermophiles occurs in the final product, until a point is reached at which thermophile numbers have increased to  $10^5$  CFU/g, which is the cut-off point for acceptability to many customers. Levels higher than this result in downgrading of the product and consequent economic loss to the manufacturer.

Because of the limited residence time of the milk the plant (in the order of minutes) compared with thermophile growth rates, thermophilic growth in the liquid phase cannot explain the large numbers of thermophiles found in the final product. This leads to the conclusion that thermophiles are able to attach to a surface, grow and develop into a biofilm. Shedding of individual cells or sloughing of biofilm fragments into the product line results in contamination of the final product.

Bacterial cell attachment is thought to involve a complex interaction between a substrate and the bacteria and this is influenced by many potential extrinsic factors within the surrounding environment. The attachment mechanism of thermophiles isolated from milk powder has not been well studied and there are limited reports on what surface characteristics enable thermophiles to attach to stainless steel surfaces.

The focus of this study was to gain some fundamental understanding of the mechanism of attachment displayed by these bacteria and what aspects of the cell surface are responsible for the ability of these bacteria to attach to stainless steel.

## **1.2 Project Objectives**

The aim of the research presented in this thesis was to gain an understanding of what attachment mechanisms of the milk powder isolate *Anoxybacillus flavithermus* strain B12 are involved in the attachment of these bacteria to stainless steel surfaces.

In order to learn more about how bacteria attach to surfaces a literature review was carried out to establish what is already known about bacterial attachment in general, as well as bacterial attachment to stainless steel and is presented in chapter 2.

The first objective of the present study was to isolate by natural selection a strain of *A. flavithermus* lacking the ability or having reduced ability to attach to stainless steel, from a large population of *A. flavithermus* strain B12.

The second objective of the research presented in this thesis was to compare the surface characteristics of B12 and a strain with reduced attachment to stainless steel in an attempt to pinpoint surface characteristics important in the attachment process of B12. Surface characteristics previously reported to be involved in attachment include electrostatic interactions, surface hydrophobicity, cell surface proteins, extracellular DNA and cell surface polysaccharides.

From the results of these two objectives it was hoped enough information would be gained to identify what specific surface structures play an important role in the ability of strain B12 to attach to stainless steel surfaces. A greater understanding of the attachment process of B12 may help in future development of stainless steel surfaces that limit bacterial attachment in the dairy industry.

### Literature review

#### 2.1 *Thermophiles in foods*

##### 2.1.1 Thermophiles in the dairy industry

##### 2.1.2 Dairy biofilms

#### 2.2 *Initial bacterial attachment to surfaces*

##### 2.2.1 Conditioning of a surface

##### 2.2.2 Mass transport

##### 2.2.3 Initial attachment

##### 2.2.4 Surface charge

##### 2.2.5 Hydrophobicity

##### 2.2.6 DLVO theory

##### 2.2.7 Surface roughness and micro-topography

##### 2.2.8 Role of surface carbohydrates in attachment

##### 2.2.9 Role of surface proteins in attachment of *Staphylococcus* species

#### 2.3 *Conclusion*

## **2.1 Thermophiles in Foods**

Thermophiles can be found in a range of processed food products including canned food (Denny, 1981), sugar beet (Messner *et al.*, 1997), gelatin (De Clerck *et al.*, 2004) and cheese (Cosentino *et al.*, 1997). These thermophiles do not produce any known toxins and have not been associated with any health effects, but lipolytic and proteolytic enzyme activity resulting in the production of off-flavours is of concern (Denny, 1981; Chen *et al.*, 2004). In addition, large numbers of bacteria present in common food ingredients are often unacceptable to customers (Scott *et al.*, 2007).

In the canning industry, thermophilic spores can be difficult to kill because of their inherent heat resistance, and are generally more heat resistant than mesophilic spores (Denny, 1981). However, tolerance of thermophilic spores in canned foods allows the industry to produce foods of higher quality, because a less rigorous thermal process is required to kill only mesophilic spores. Thermophilic spores will remain dormant in canned foods as long as the cans are cooled properly and stored at room temperature (Denny, 1981).

### **2.1.1 Thermophiles in the Dairy industry**

The dairy industry produces a wide variety of perishable (e.g. yoghurt, butter) and semi-perishable (e.g. milk powder and casein) products, each product often with a specific population of contaminating bacteria. Fresh bovine milk is usually regarded as sterile, but during the milking process bacterial contamination can come from three sources: 1) infection of the udder, 2) exterior of the teats, 3) the milking and/or storage equipment (Chambers, 2002). Milk is stored on the farm at 7°C which limits growth to psychrotrophic organisms. During transport to and storage at the manufacturing site there is no refrigeration, with the industry relying upon temperature of the bulk milk to limit microbial growth. Muir *et al.* (1986) reported little to no increase in the number of thermophilic bacteria in the milk during this transport and storage period, with numbers

of thermophilic bacilli in raw milk generally in the range of <10 colony-forming units per ml (CFU/ml) (Flint *et al.*, 2001; Scott *et al.* 2007).

The dairy industry relies on pasteurisation (72°C for 15 seconds) as the main process to destroy heat sensitive and pathogenic bacteria present in raw milk. Nevertheless, the low numbers of thermophilic bacilli spores present in the raw milk are able to survive pasteurisation and potentially contaminate other areas of the plant. During milk powder manufacture, heat exchangers, regeneration and evaporator sections of a plant typically operate at between 45°C to 75°C, which is a range suitable for the growth of thermophiles. The milk is concentrated approximately 10-fold to form a powder, thus if no growth occurred during the manufacturing process, numbers of thermophiles in the milk powder should not exceed 100 CFU/ml. However, when evaporator run times exceed 16-20 hours, high numbers of thermophiles are commonly found in the final product (up to 10<sup>5</sup> CFU/g) (Murphy *et al.*, 1999; Stadhouders *et al.*, 1982). The residence time of the milk from storage tank to powder does not usually exceed 20-30 minutes, so there is insufficient time for thermophilic growth in the bulk milk during processing, even with a generation time of approximately 15-20 minutes for some thermophiles (Scott *et al.*, 2007). Consequently, the high contamination found after 16-20 hour run times are most probably due to biofilm growth and detachment on the large stainless steel surface areas found within a milk powder plant.

### **2.1.2 Dairy Biofilms**

The growth of thermophilic bacilli during milk powder manufacture is thought to occur as a biofilm (Flint *et al.*, 2001). A biofilm is generally described as an accumulation of microorganisms and their associated extracellular polymeric substances or polysaccharides actively attached to, growing and multiplying on a surface (Flint *et al.*, 1997). In sections of the milk powder plant where nutrient and temperature conditions are ideal for thermophilic growth, vegetative cells or spores may attach to stainless steel surfaces or milk fouled surfaces (Hinton *et al.*, 2002) and develop into a biofilm. Product contamination is most likely to occur when individual cells are released from the biofilm

or even when sloughing or breaking up of the biofilm occurs during processing, releasing large numbers of bacteria directly into product passing by. This release of bacteria into the passing product is sometimes referred to as the biotransfer potential (Wirtanen *et al.*, 1996).

The advantages accruing to a bacterial cell growing within a biofilm community are thought to include greater resistance to heat and chemicals (Carpentier *et al.*, 1998; Lee Wong and Cerf 1995; Austin and Bergeron, 1995). Hausner and Wuertz (1999) also reported higher rates of bacterial conjugation, suggesting that higher rates of genetic transfer are also taking place within biofilm structures. Bacterial cells growing in a biofilm also appear to undergo phenotypic changes, with some cells altering polysaccharide production (Davies and Geesey, 1995) or flagella expression (Belas *et al.*, 1986). Advances in proteomic profiling of bacteria suggest bacteria undergo a variety of phenotypic changes during biofilm growth and development and as much as 50% of the proteome can change compared with planktonic cells (Sauer *et al.*, 2002; Patrauchan *et al.*, 2007). However, the phenotypic changes occurring during biofilm growth do not appear to be homogeneous throughout, with concentration gradients of oxygen, nutrients and waste products resulting in multiple mini-environments within the biofilm structure. Consequently, the phenotypic make up of cells within a biofilm may vary in accordance with the local chemical and physical conditions, which may change as biofilm development proceeds (Stewart and Franklin, 2008). This phenotypic diversity of cells within a biofilm may be partially responsible for the observed resistance of biofilm cells to chemicals and heat.

Biofilm formation is thought to have detrimental effects on certain process characteristics, for instance, the reduction of heat transfer (Russell, 1993) and increased resistance to flow of fluids, resulting in increased energy costs to maintain given flow rates. (Criado *et al.*, 1994).

During correct Cleaning in Place (CIP) procedure, most thermophilic bacteria are eliminated (Parker *et al.*, 2003). However, high numbers of thermophile spores were

found to be associated with foulant in a milk powder plant (Scott *et al.*, 2007), which likely provide protection to embedded bacteria from cleaning chemicals (Hinton *et al.*, 2002). The exact nature of foulant and biofilm interaction remains elusive at present. Furthermore, Austin and Bergeron (1995) concluded that bacterial biofilms can develop on gaskets commonly used in the dairy industry. Even after regular CIP procedures extensive biofilms remained, potentially seeding the next manufacturing run.

Several groups (Norwood and Gilmour, 1999; Borucki *et al.*, 2003; Lundén *et al.*, 2000) have demonstrated that dominant or persistent strains of *L. monocytogenes* isolated from food processing plants have a higher rate of attachment to surfaces than sporadic or non-persistent strains. Amongst the large number of strains examined, significant variation was noted in the ability of some isolates to attach to stainless steel (Norwood and Gilmour, 1999; Lundén *et al.*, 2000). Borucki *et al.* (2003) concluded that persistent strains of *L. monocytogenes*, as well as demonstrating higher initial attachment rates, also show increased biofilm formation. The higher attachment and biofilm formation rates of persistent *L. monocytogenes* strains probably demonstrate the natural selection within food processing plants of strains of *L. monocytogenes* with increased ability to attach to inert surfaces, resist sanitizers and form extensive biofilms.

Work by Rückert *et al.* (2004) concluded that *Anoxybacillus flavithermus* is the dominant thermophilic bacterial contaminant of milk powders all over the world and appears to be a ubiquitous organism in milk powder plants. Perhaps *Anoxybacillus flavithermus* is an example of a persistent organism in milk powder manufacture. However, other thermophilic bacilli including *Bacillus licheniformis* and *Geobacillus stearothermophilus* species are commonly isolated from various milk powders (Rückert *et al.*, 2004).

The question remains “What major factors allow bacteria to attach to abiotic surfaces such as stainless steel?”, since the initial attachment process in biofilm formation is the most crucial stage for the future growth and development of the biofilm (Busscher *et al.*, 1995A).

## **2.2 Initial bacterial attachment to surfaces**

The dominating factor involved in the initial attachment of a bacterial cell to a surface has remained elusive and today it is thought that a multitude of factors are involved, including surface conditioning, mass transport, surface charge, hydrophobicity, surface roughness and surface micro-topography.

### **2.2.1 Conditioning of a surface**

During the first stage, molecules present in the bulk flow, both organic and inorganic are carried toward the surface either by diffusion or turbulent flow. This accumulation of molecules at the solid-liquid interface on surfaces found in many food industries is commonly called a conditioning film and leads to a higher concentration of nutrients at the surface compared with the liquid phase (Kumar & Anand, 1998).

The adsorption of organic molecules such as proteins to surfaces could play an important role in bacterial attachment, as this conditioning of the surface may alter the physical-chemical properties of the surface. Factors affected can include surface free energy, hydrophobicity and electrostatic charges (Dickson and Koochmaraie, 1989).

In the dairy industry, surface conditioning is often referred to as fouling and was suggested by Rosmaninho *et al.* (2007) to take place as soon as a dairy product is brought into contact with stainless steel. Hinton *et al.* (2002) concluded that fouled stainless steel allowed faster attachment and growth of dairy thermophiles. Conflicting opinions exist on the importance of a conditioning film in initial bacterial attachment, with Fletcher (1976) reporting that the presence of proteins such as albumin, gelatin and fibrinogen inhibited attachment of a marine *Pseudomonas* to poly-styrene. Parkar *et al.* (2001) demonstrated that the presence of skim milk on a surface of stainless steel, even at concentrations as low as 1%, reduced the attachment of spores and vegetative cells of thermophilic bacilli. Skim milk was also found to reduce the attachment of *S. aureus*,

*Listeria monocytogenes* and *Serratia marcescens* to stainless steel (Barnes *et al.*, 1999). Even individual milk components such as casein and  $\beta$ -lacto globulin were reported by Helke *et al.* (1993) to reduce attachment of *Listeria monocytogenes* and *Salmonella typhimurium* to stainless steel. One reason for the reduced attachment reported above may be that the proteins in the bulk fluid phase may act as competition for binding sites on the surface of the stainless steel, reducing the ability of bacteria to attach. However, Speer & Gilmour (1995) reported that stainless steel and rubber surfaces treated with either whey proteins or lactose demonstrated an *increase* in attachment of milk-associated micro-organisms. Flint *et al.* (2001) also reported higher attachment of *Bacillus stearothermophilus* to stainless coupons coated with skim milk foulant created by denaturing skim milk on to the surface by autoclaving. Holah & Gibson (2000) reported that Johal (1988) observed a reduction in the surface charge of stainless steel after conditioning of the stainless steel in meat juices and suggested that this was “enhancing the potential accumulation of bacteria on the surfaces”. Also Jeong and Frank (1994) suggested that the presence of proteins on a surface favours biofilm formation, as attached proteins could be a source of nutrients for bacteria. More recently, Verran and Whitehead (2006) reported that the presence of protein material such as Bovine Serum Albumin (BSA) on an inert surface retained bacterial cells at a higher rate, during a cleaning cycle, than cells on a clean surface. The shearing off of bacterial cells from a surface may result in bacterial footprints or bacterial surface polymers remaining behind on the surface and this may also play a role in further bacterial cell attachment (Neu 1992). These bacterial footprints are generally thought to consist of polysaccharides and/or glycoproteins and may modify the substratum physicochemistry (Bejarano & Schneider, 2004; Gómez-Suárez *et al.*, 2002). The conflicting observations reported on the importance of a conditioning film on bacterial attachment may be the result of different laboratory conditions, different bacterial strains, surfaces and differing organic molecules used to create a conditioning film.

The presence of primary colonisers that allow different species of oral bacteria to adhere to teeth has been observed for many years in the dental research and is commonly called co-aggregation (Whittaker *et al.*, 1996; Bos *et al.*, 1996; Yoshida *et al.*, 2005). Sasahara

and Zottola (1993) reported co-aggregation between two species of bacteria not usually associated with the oral cavity (*Listeria monocytogenes* and *Pseudomonas fragi*) on a glass surface. In pure culture form, *Listeria monocytogenes* showed sparse adherence to a glass surface; however when grown with *Pseudomonas fragi*, significant adherence of *Listeria monocytogenes* was observed. They concluded that *Pseudomonas fragi* was the primary coloniser and the exopolysaccharide produced by the *Pseudomonas fragi* was responsible for the observed increase in *Listeria monocytogenes* adherence. More recently Rickard *et al.* (2002) concluded co-aggregation between different freshwater bacteria is a common phenomenon in the aquatic environment. It seems logical to think that co-aggregation must also occur in many different environments outside the oral cavity of humans and may have an important role to play in the attachment of bacteria to surfaces in the food industry and in other medical or environmental areas. Interestingly, Trachoo and Brooks (2005) demonstrated that *Campylobacter* can co-aggregate with *Enterococcus* to form biofilms and these were found extensively in the poultry industry in Thailand.

### 2.2.2 Mass Transport

Mechanisms by which bacteria are transported to a surface can include Brownian motion, sedimentation due to differences in specific gravity between the bacteria and the bulk liquid, or convective mass transport, by which cells are physically transported towards the surface by the movement of the bulk fluid. Yang *et al.* (1999) demonstrated that the convective mass transport towards a substratum surface can occur in a stainless steel pipe. This convective mass transport produces a higher cell attachment rate in the presence of a T-junction than when the convective mass transport is parallel to a substratum or in this case a stainless steel surface. This suggests that convective mass transport towards a surface may help facilitate the close approach of bacteria to a surface and thus play a role in initial bacterial attachment. The convective mass transport results reported by Yang *et al.* (1999) also reinforce the importance of plant design in controlling microbial contamination, growth and ease of cleaning. In turbulent flow, the creation of multiple, small turbulent eddies may drive small particles including bacterial cells

towards surfaces, which may in turn override the Gibbs energy barrier required by bacteria to come into contact with a surface. The Gibbs energy barrier is the sum of the van der Waals interactions, commonly attractive and electrostatic interactions, usually repulsive, due to both bacteria and substratum surfaces being negatively charged (Vadillo-Rodríguez *et al.*, 2005). Balancing this increase in contact of bacteria with the substratum is the reduced thickness of the boundary layer, which would allow increased scouring and thus reduce the time available for bacteria to interact with the surface.

Davies (2000) reported that active transport, mediated by bacterial flagella activity and chemotaxis, has been considered an important mechanism enabling bacteria to interact with a surface. However, using *E. coli* and insertion mutagenesis to disrupt flagella operons and the *che* operon, responsible for chemotaxis, Pratt and Kolter (1998) demonstrated that motility is important for initial interaction with an abiotic surface, but chemotaxis played no part in bacterial attachment and was described by the authors as dispensable in initial biofilm formation by *E.coli*. Conversely, Klausen *et al.* (2003) reported that the flagella of *Pseudomonas aeruginosa* were not a necessary factor in the initial attachment of this organism to a solid surface, but played a role in biofilm development. Of interest also is a report by O'Toole and Kolter (1998) detailing that mutant non-flagellated cells of *Pseudomonas fluorescens* were defective in attachment to several abiotic surfaces, compared to non-mutant flagella producing cells. However, a change in the medium, with citrate instead of glucose as a carbon source, reinstated the cells attachment ability, suggesting the environmental conditions may have an effect on a cells mode of attachment. Recent research has tended to focus on characterization of important factors involved in all aspects of biofilm formation from initial attachment to biofilm maturation. A common theme reported in biofilm deficient mutants is the disruption of the genes involved in flagella synthesis (Montie *et al.*, 1982; Smit *et al.*, 1989; O'Toole and Kolter, 1998; Pratt and Kolter, 1998). However, the methods employed to isolate biofilm deficient mutants are often not very suitable for the isolation of mutants carrying mutations solely involved in attachment. The involvement of flagella in initial attachment of bacteria to abiotic surfaces is still not well understood, but this

may be a reflection on different laboratory conditions, different bacterial strains and surfaces employed.

### 2.2.3 Initial attachment

There are two generally accepted theories on the attachment of bacteria to solid surfaces. The first of these theories is a two-step process (Kumar & Anand, 1998; Marshall *et al.*, 1971). The first step involves the bacteria being transported close enough to allow initial attachment to take place, with the forces involved in this initial attachment being van der Waals forces, electrostatic forces and hydrophobic interactions (Gilbert *et al.*, 1991; van Loosdrecht *et al.*, 1987; Carpentier & Cerf, 1993). During this initial contact bacteria still show Brownian motion and can be easily removed by fluid shear forces, e.g. rinsing (Marshall *et al.*, 1971). The next crucial step in the attachment process is the irreversible attachment of cells to the surface, described by Dunne (2002) as bacteria locking on to the surface by the production of exo-polysaccharides and or specific ligands, such as pili or fimbriae that may form a complex with the surface. At the end of this stage much stronger physical or chemical forces are required to remove the bacteria from the surface, e.g. scraping, scrubbing or chemical cleaners.

In the transition from reversible attachment to irreversible attachment, various short range forces are involved, including covalent and hydrogen bonding as well as hydrophobic interactions (Kumar & Anand, 1998). Poortinga *et al.* (2001) expanded on the idea of covalent bonding in bacterial attachment and suggested that bacteria either donated electrons or accepted electrons from the substratum. Whatever the electron transfer, these results suggest that electron transfer between cell surface and the substratum plays an important role in bacterial attachment to inorganic and presumably to organic surfaces too.

The three step model proposed by Busscher and Weerkamp (1987) involves Lifshitz-van der Waals forces operating over several hundred nanometers (nm) as a first step. The second operates over distances of about 20 nm involving Lifshitz-van der Waals forces

and electrostatic interactions. The third step occurs at distances of around 5 nm, where specific adhesion receptors may facilitate strong adhesion.

The transition time from reversible to irreversible attachment has been an area of comparatively little work in the past. However, Meinders *et al.* (1995) demonstrated that over a time as short as ten minutes, attachment of thermophilic *Streptococcus* became 100 times less reversible. Flint *et al.* (1997A) found cultures of *Streptococcus thermophilus* and *Bacillus cereus* attached to stainless steel in less than 60 seconds and subsequent washing with distilled water proved ineffective in removing attached cells. Schwab *et al.* (2005) noted that numbers of *Listeria monocytogenes* attached to stainless steel were essentially the same either after 5 minutes or 24 hours incubation. Butler *et al.* (1979) reported similar findings with meat surfaces, noting considerable attachment of bacterial cells taking place in the first minute of contact, though small increases were also seen over time. Vadillo-Rodríguez *et al.* (2004) used atomic force microscopy (AFM) to measure the forces required to move away the AFM tip from the surface of *Streptococcus thermophilus* after the AFM tip had been in contact with the cell surface from anywhere between 1 to 200 seconds. An increasing amount of force was required to remove the AFM tip from the cell surface over time, which corroborates earlier work mentioned above, suggesting the bond strength increases between cell surface and substratum over a relatively short period of time.

#### **2.2.4 Surface charge**

Bacterial cells generally have a net negative charge on their cell wall at neutral pH (Rijnaarts *et al.*, 1999) However, the magnitude of the charge varies from species to species and is probably influenced by culture conditions (Gilbert *et al.*, 1991; Kim & Frank, 1994), age of the culture (Walker *et al.*, 2005), ionic strength (Dan, 2003), pH (Husmark & Ronner, 1990) and all wall structural and functional groups present. The charge on the cell surface is often determined as its zeta-potential, which is calculated from the mobility of the bacterial cell in the presence of an electrical field under defined salt concentration and pH. Most bacteria have a negative zeta potential at physiological

pH (pH 7) (Millsap *et al.*, 1997; Gilbert *et al.*, 1991; Lerebour *et al.*, 2004), however Jucker *et al.* (1996) isolated a bacterial strain of *Stenotrophomonas (Xanthomonas) maltophilia*, with a positive surface charge & positive zeta potential at physiological pH. This was compared with a strain of *Pseudomonas putida* with a negative surface charge and a negative surface potential at physiological pH. The *S.maltophilia* demonstrated high attachment efficiency to glass and Teflon, both of which have a negative surface charge. But as the ionic strength of the suspending medium was increased, a drop in attachment efficiency of *S. maltophilia* was noted as well as a change to a negative zeta potential, suggesting the importance of surface charge in attachment of *S.maltophilia* to glass and Teflon. Conversely, at high ionic strength the negatively charged *P. putida* demonstrated higher attachment efficiency and a decreasing (move towards zero) zeta potential, suggesting high ionic strength suppresses or overwhelms the natural surface charge of bacteria. Mafu *et al.* (1991) also concluded that high ionic strengths suppressed electrostatic interactions between *Listeria monocytogenes* and various inert surfaces. Furthermore, Giaouris *et al.* (2005) reported that higher sodium chloride concentrations (10.5%) inhibited the adherence of *Salmonella enterica* to stainless steel coupons. One explanation for the above observations mentioned by Jucker *et al.* (1996) and Van der Wal *et al.* (1997) is that the bacterial cell surface charge originates from the dissociation of acidic groups such as carboxyl, phosphate and amino groups as well as basic groups found on the cell surface. Consequently the zeta potential of the bacterial cell strongly depends upon the ionic strength of the suspending medium and the higher the ionic strength the more ions are available to shield and thus neutralise the charge of the cell surface. Electrostatic interaction chromatography (ESIC) has been used by several groups in the past to measure the overall surface charge of bacteria (Peng *et al.*, 2001; Ukuku and Fett, 2002; Flint *et al.*, 1997A; Dickson and Koohmaraie, 1989; Jones *et al.*, 1996; Narendran, 2003). The relative surface charge of cells is assessed by the affinity to either the anionic or cationic resins. The ESIC method usually involves passing a culture through an anionic or cationic column and comparing the relative retention to elution ratio of bacterial cells. The ESIC method assesses most bacteria as possessing a net negative charge, in line with most zeta potential measurements. Jones *et al.* (1996) noted that some isolates of *S. epidermidis* exhibited a high interaction with both anionic and

cationic exchange resins. They suggested there were localised regions of positively charged (cationic) molecules on the bacterial cells surface, but over the whole cell surface negatively charged (anionic) molecules outnumbered the cationic molecules, resulting in the cell having net negative charge.

Surface charge can also be influenced by the pH of the suspending medium as reported by Husmark and Ronner (1990). They demonstrated a maximum level of attachment of *Bacillus cereus* spores to hydrophilic and hydrophobic surfaces when the pH of the suspending medium was equal to the isoelectric point of the *Bacillus* spores, in this case pH 3. In the pH range above the isoelectric point (above pH 4) the observed decrease in spore attachment was thought to result from electrostatic repulsion between the spore surface and the substratum, because both surfaces had a negative charge.

Other groups to have reported positive correlations between cell surface charge and attachment include Ukuku & Fett (2002), Dickson & Koohmaraie (1989) and Van Loosdrecht *et al.* (1987). On the other hand, Flint *et al.* (1997A) compared 12 strains of thermophilic *Streptococci* and their attachment to stainless steel with respect to their surface charge, measured by separation through anionic and cationic exchange resins. They were unable to establish any relationship between numbers of cells attaching to stainless steel and cell surface charge. As commented by Flint *et al.* (1997A), at pH 7 all the thermophilic *Streptococci* cells displayed a negative surface charge and this is likely to repel the bacterial cell from surfaces such as stainless steel, owing to the inherent negative surface charge of this material. Gilbert *et al.* (1991) noted that increasing negative charge on the surface of *E. coli* resulted in reduced attachment, but no such correlation could be drawn for *S. aureus*, demonstrating that attachment cannot solely be explained by surface charge, but may be one of the contributing factors to bacterial attachment. Narendran (2003) also reported that bacterial attachment to meat surfaces could not be explained by the bacterial surface charge alone and suggested that bacterial attachment is very complex with many bacterial surface characteristics involved. The surface of the meat is also more complex than that of relatively inert surfaces, such as stainless steel.

The surface charge of inert surfaces to which bacteria can attach is also most likely to play an important role in bacterial attachment. Fukuzaki *et al.* (1995) reported that the zeta potential of stainless steel particles at pH 7 was weakly negative, with the stainless steel having an isoelectric point between pH 4.0 and 4.5. Bren *et al.* (2004) proposed that hydroxyl groups of surface oxides can interact with  $H^+$  and  $OH^-$  groups according to the following reaction,



(Me = metal)

The ratio of metals that are protonated (positively charged groups), neutral or dissociated (negatively charged groups) is obviously very dependent upon the pH of the overlying medium. Thus in low pH medium the dominant group would be  $MeOH_2^+$  but at neutral or higher pH values  $MeOH$  or  $MeO^-$  groups may dominate. Different metals may also have slightly different pKa and pKb values, so thus different ratios of metal oxides at the surface may produce metal surfaces with varying surface charges at the same pH value. A possible example of this may have been reported by Takehara and Fukuzaki (2002), when they observed that stainless steel exposed to  $HNO_3$ , ozone and  $300^\circ C$  heat treatment contained different ratios of Chromium and Iron oxides at the surface. The different treated stainless steel surfaces also demonstrated different relative adsorption curves for  $H^+$  and  $OH^-$  titrations, suggesting that the surface treatment may also play an important role in the surface charge of stainless steel and in turn have a role to play in the attachment of bacteria.

### 2.2.5 Hydrophobicity

Hydrophobic interactions have widely been suggested as being responsible for much of the adherence of cells to surfaces (Hood & Zottola 1995). Hydrophobicity is an interfacial phenomenon. It is very difficult to evaluate the results of most adhesion tests solely on the basis of hydrophobicity, since so many parameters are involved in most interfacial systems of interest (Doyle *et al.*, 1990). Although the hydrophobic effect has

been known for some time, it has been difficult to assign it a satisfying definition (Doyle, 2000). Put simply, a hydrophobic molecule would rather exist in another hydrophobic environment than in a hydrophilic environment, such as water. Husmark & Ronner 1992 demonstrated that bacterial spores generally attach at a higher rate than vegetative cells to surfaces. They attributed this observation to the higher hydrophobicity of spores and the hair like structures covering the surface of the spores. Zita and Hermansson (1997) correlated cell surface hydrophobicity of *E.coli* strains to the attachment to activated sludge flocs found in the treatment of waste water, suggesting that cell surface hydrophobicity may play an important role in the attachment of *E.coli*. Hydrophobicity of a cell surface has also been attributed to the adhesion of eukaryote microbes *Cryptosporidium parvum* and *Giardia lamblia* to solid surfaces (Dai *et al.*, 2004) and in the attachment of yeast to stainless steel surfaces in the apple processing industry (Brugnoni *et al.*, 2007). Davies (2000) concluded that differences in surface hydrophobicity of different bacterial cells result from the properties conferred upon the cell surface by molecules such as proteins and lipids. Evidence that hydrophobicity of cells may be related to protein structures on the cell surface does exist. Paul & Jeffrey (1985) noted that cells treated with proteolytic enzymes decreased the hydrophobicity of *Vibrio proteolytica* and this in turn reduced the adherence to hydrophobic surfaces such as polystyrene. Oakley *et al.* (1985) reported that *Streptococcus sanguis* cells treated with trypsin demonstrated reduced adhesion to hexadecane (a highly hydrophobic organic liquid), presumably because hydrophobic proteins were removed from the cell surface. X-ray photoelectron spectroscopy (XPS) analyses of bacterial surfaces by Reid *et al.* (1999) and Millsap *et al.* 1997 found that bacterial strains with high hydrophobicity ratings also tended to have a higher nitrogen/carbon ratio. Conversely cells with a higher hydrophilic rating tended to have higher oxygen/carbon ratio. These results tend to indicate that the presence of proteinaceous material at the cell surface increases the hydrophobicity of the cell surface. Walker *et al.* (2005) also suggested differences that they reported in the surface hydrophobicities of 3hr and 18hr cultures of *E. coli* are related to the surface proteins, specifically a decrease in hydrophilic (acidic) proteins present on the cell surface. The role of lipopolysaccharide (LPS) in attachment to solid surfaces remains unclear (Burks *et al.*, 2003; Razatos *et al.*, 1998 and Razatos *et al.*, 2000), but evidence is

mounting that the presence of LPS on cell surface tends to make a bacterial cell more hydrophilic in nature and that the loss of LPS results in the cell surface becoming more hydrophobic (Park and So, 2000; Al-Tahhan *et al.*, 2000). Interestingly, Kannenberg and Carlson (2001) reported that a reduction in oxygen levels induced structural modifications in the LPS of the bacterium *Rhizobium*, resulting in an increase in surface hydrophobicity of the cell. This tends to indicate that the bacterial cell is quite capable of sensing changes in its external environment and in turn change a major cell surface characteristic such as surface hydrophobicity.

There is an ongoing debate as to which is the best method to measure bacterial surface hydrophobicity. The three most popular methods include Bacterial Adherence To Hydrocarbons, commonly called the BATH test, as described by Rosenberg *et al.* (1980), which is now generally called the MATH test (Microbial Adherence To Hydrocarbons). The others are Hydrophobic Interaction Chromatography (HIC) (Smyth *et al.*, 1978) and Water Contact Angles measurements (Van der Mei *et al.*, 1998). In the MATH test, evidence exists that hydrophobicity is not the only interaction taking place between microbial cell and organic solvent (hydrophobic compound) such as hexadecane. (Indeed, both hexadecane and xylene have been found to disrupt cell walls of *S. thermophilus* and *Anoxybacillus* sp. respectively (Flint, pers. comm.). Ahimou *et al.* (2001), Busscher *et al.* (1995) and Van der Mei (1993) have all reported that the MATH test can be influenced by electrostatic interactions, with Busscher *et al.* (1995) reporting that hexadecane, the most commonly used hydrocarbon to measure hydrophobicity, is negatively charged in water, with a zeta potential of between -50 to -80 mV. Van der Mei *et al.* (1995) concluded the MATH test should be measured at pH values where the zeta potential of the test organism and/or hydrocarbon are near zero to reduce the potential interference of electrostatic interactions. Doyle (2000) suggested the MATH test should be performed under either high ionic strength or at the isoelectric point of the bacterial cells to minimise any possible electrostatic interactions, making any measurement of attachment to any hydrophobic hydrocarbon such as hexadecane valid.

Hydrophobic Interaction Chromatography (HIC) involves the interaction of hydrophobic cells with a hydrophobic column, such as Phenyl-Sepharose, with cells demonstrating high hydrophobicity being retained in the column and cells with low hydrophobicity being eluted. As early as 1978, Smyth *et al.* (1978) noted that increasing ionic concentration, in this case NaCl, affected cell attachment to a HIC column such as Phenyl-Sepharose. Wiencek *et al.* (1990) also reported that a high ionic strength was necessary to overcome electrostatic repulsion between bacterial spores and a hydrophobic column containing Phenyl-Sepharose. Wiencek *et al.* (1990) used both BATH and HIC methods to measure relative cell hydrophobicity on bacterial spores and noted that the relative hydrophobicities as determined by BATH and HIC generally agreed, even though a high concentration of NaCl was used to mask electrostatic repulsion between bacterial spores and Phenyl-Sepharose.

Evidence that hydrophobicity is a strong predictor of cell attachment to surfaces varies from group to group, with Peng *et al.* (2001), Gilbert *et al.* (1991), Iwabuchi *et al.* (2003), Liu *et al.* (2004) and Van Loosdrecht *et al.* (1987A) suggesting a strong correlation between hydrophobicity and cell attachment to surfaces. Van Loosdrecht *et al.* (1987A) went so far as to suggest that surface hydrophobicity is the key factor in determining bacterial attachment to solid surfaces and that surface charge can only become important when surface hydrophobicity is minimal. However, it must be noted that Van Loosdrecht *et al.* (1987A) used polystyrene discs, which are very hydrophobic, to measure cell adhesion, thus possibly favouring hydrophobic interactions. On the other hand Sorongan *et al.* (1991), Parment *et al.* (1992), Parkar *et al.* (2001) and Flint *et al.* (1997) concluded that hydrophobicity had little to no relationship in determining bacterial cell attachment. Nevertheless, bacterial cell surface hydrophobicity may be one of the many factors involved in initial attachment of micro-organisms to surfaces.

The hydrophobicity rating of stainless steel surfaces also appears to be a topic with limited published work, with surfaces often only described as simply hydrophilic or hydrophobic in nature (Carpentier and Cerf, 1993). Descriptions of the hydrophobic/hydrophilic nature of stainless steel vary from author to author, with

Brugnoni *et al.* (2007) and Teixeira *et al.* (2005) directly describing stainless steel as a hydrophobic surface and Li and Logan (2004) listing all metal oxides used in their study as hydrophobic. However, Lejeune (2003), Planchon *et al.* (2007A) and Lerebour *et al.* (2004) describe stainless steel surfaces as hydrophilic in nature, with Boulangé-Petermann (1996) commenting that all metal surfaces are hydrophilic surfaces compared with polymers. The hydrophilic/hydrophobic nature of stainless steel can be a relative term, with the hydrophobicity of stainless steel compared with glass (hydrophilic) most likely defining stainless steel as hydrophobic. On the other hand, stainless steel compared with Teflon (hydrophobic) may result in stainless steel being described as hydrophilic. Different grades of stainless steel may also play a role, with electro-polished stainless steel appearing more hydrophilic than standard 2B 316 stainless steel. This confusion and contradiction relating to the hydrophilic/hydrophobic nature of stainless steel will most likely remain until there is an agreement of the most appropriate method to measure surface hydrophobicity.

### 2.2.6 DLVO Theory

The DLVO (Derjaguin, Landau, Verwey, and Overbeek) theory of colloid stability has been used by several groups to try to explain attachment of micro-organisms to surfaces (Rijnaarts *et al.*, 1995 and Hermannsson, 1999). According to the DLVO theory, particle adhesion is driven by the sum of the Lifshitz-van der Waals interactions, usually described as attractive, and also electrostatic interactions, which may be repulsive or attractive, depending upon the charge of the two surfaces interacting. Rijnaarts *et al.* (1995A & 1999) concluded that electrostatic interaction is as a general rule repulsive between inert surfaces and bacterial surfaces at neutral pH, as most inert surfaces and bacterial surfaces are negatively charged at neutral pH. However, one strain of *Stenotrophomonas (Xanthomonas) maltophilia* was described by Jucker *et al.* (1996) as having a net positive surface charge at neutral pH. Bacterial cell surface charge originates from the presence of carboxyl, phosphate and amino groups in either dissociated or protonated form and the surface charge consequently depends upon the pH (Poortinga *et al.*, 2002) and/or ionic concentration (Van der Wal *et al.*, 1997) of the suspending

medium. Poortinga *et al.* (2002) suggested that at physiological pH values, i.e. between pH 5 and 7 bacterial cells are generally negatively charged due to the excess of negatively charged carboxyl and phosphate groups over positively charged amino groups.

One critical aspect to the DLVO theory is ionic strength of the solution. Rijnaarts *et al.* (1995 & 1999) described how at low ionic strength solution, for example <0.001 M, long range electrostatic repulsion dominates bacterial attachment, but at high ionic strength (>0.1 M) other factors such as steric interactions (hydrophobicity) dominate. Many workers have now realised that the DLVO theory does not take into account that the bacterial cell surface is not a model colloid particle but a highly dynamic surface that responds to changes in ionic strength, pH, the presence of macromolecules and even the presence of other surfaces (Poortinga *et al.*, 2002). These environmental changes may induce conformation changes to surface structures such as flagella and fimbriae that may have an important role in cell attachment. Pembrey *et al.* (1999) reported that the methods used to prepare cells for cell surface analysis can have an influence on the values of cell surface parameters, especially the use of high salt buffers vs. low salt buffers. Even centrifugation at 15,000g was suggested by Pembrey *et al.* (1999) to cause enough modification to cell wall structures to bring about differences in electrophoretic mobility and hydrophobicity compared with cells of the culture that were not centrifuged at 15,000g. Work presented by Pembrey *et al.* (1999) and Castellanos *et al.* (1997) tends to imply that the bacterial cell surface is not just an inert rigid structural component of the cell, but a delicate and complex array of proteins, carbohydrates and other components that the cell uses to sense its immediate environment, that can be easily damaged or altered by chemical and/or physical stress.

Methods for analysing electrostatic interaction such as zeta potential and electrophoretic mobility measurements tend to give results in terms of the cell surface overall charge, or the net surface charge at the macroscopic level. Dan (2003) suggested that the DLVO approach to bacterial adhesion tended to treat bacterial cells as traditional colloidal particles, characterised by having an even surface and a evenly distributed surface charge. The problem remains that cells contain many complicated surface structures such as

flagella, pili, fimbriae, glycoproteins, carbohydrates, teichoic acids and other biological materials composed of proteins (in *Bacillus* species up to 9% of total cell proteins are associated with the cell wall (Tjalsma *et al.*, 2000). These complicated surface structures may exert their own localised cell surface charge at a microscopic cell surface level that could possibility mediate attachment through local electrostatic attraction despite the cells having an overall electrostatic repulsion. Interestingly, Jones *et al.* (1996), measuring the cell surface charge of *S. epidermidis* strains, noted that some strains revealed a marked interaction with the cationic-exchange resin (negatively charged resin), though all strains exhibited as expected a highly negative cell surface, suggesting that different regions on the surface on the cell display different surface charges, even though the overall cell charge may be negative.

### **2.2.7 Surface Roughness and Micro-Topography**

Stainless steel is the most common food contact material in the food industry today, as it is easy to fabricate, durable, chemically and physiologically inert at a variety of food processing temperatures and pressures, generally corrosion resistant and usually easy to clean (Holah and Gibson, 2000). However, the micro-topography of stainless steel examined under SEM (Zoltai *et al.*, 1981) and more recently AFM (Arnold and Bailey, 2000) reveals cracks and crevices which could provide a greater area for cell attachment and possible protection from cleaning chemicals and fluid forces. Verran and Whitehead (2006) concluded that surfaces with scratches and pits of similar size to microbial cells retained higher numbers of cells than surfaces with surface features much larger than microbial cells. Various groups have observed greater cell attachment on surfaces with high surface roughness and thus concluded surface roughness is an important factor in bacterial attachment to inert surfaces (Pederson, 1990; Leclercq-Perlat and Lalande 1994). On the other hand, Mafu *et al.* (1990), Vanhaecke *et al.* (1990) and Flint *et al.* (2000) have reported no correlation between surface roughness and bacterial attachment to inert surfaces. Arnold and Bailey, (2000) reported that electro-polished stainless steel showed significantly fewer bacterial cells attaching and that biofilm formation on the electro-polished stainless steel was slower than untreated surfaces. Parkar *et al.* (2003)

commented that electro-polished stainless steel produced only a small reduction in initial attachment of thermophilic bacilli, but biofilm formation on this surface was patchy and less dense than on normal 316 stainless steel. Arnold and Bailey (2000) described electro-polishing as removing metal from an object's surface through an electrochemical process similar to, but the reverse of electroplating. Removal of metal ions, they suggested, reduces the chemical reactivity of the surface, changing the electrostatic interactions between metal and the surface of the micro-organism and thus rendering the surface less susceptible to bacterial attachment. Besides surface roughness, surface topography may also play a part in cell attachment to surfaces (Kumar and Anand, 1998). Several observations by SEM (Verran *et al.*, 2001, Zoltai and Zottola, 1981) have demonstrated bacteria attaching within the surface cavities of the steel surface. Verran *et al.* (2001) and Jullien *et al.* (2002) suggested that stainless steel topography had little effect on the total numbers of bacterial cells attaching, and that surface topography may affect biofilm development by protecting cells from removal and thus allowing biofilm re-growth to occur more rapidly. Flint *et al.* (2000) also commented that surface topography around the critical size close to the diameter of the bacterial cells may entrap bacteria on the stainless steel surface, thus providing cells with some degree of protection from cleaning agents.

### **2.2.8 Role of Surface Carbohydrates in Attachment**

The role of surface carbohydrates in initial attachment of bacteria to surfaces remains inconclusive. However, the role of surface carbohydrates in biofilm structure is now well documented, with Sutherland (2001) even describing exo-polysaccharides as the main cement holding a biofilm structure together.

Flint *et al.* (1997) reported that surface carbohydrate production by various *Streptococcus thermophilus* strains could not be related to the number of cells attaching to stainless steel. Parker *et al.* (2001) also found no correlation between the attachment to stainless steel of thermophilic bacilli and the amount of extracellular polysaccharide produced.

Allison and Sutherland (1987) compared the attachment of a polysaccharide-producing strain of bacteria with that of a non-producing strain and found no difference in the initial attachment of each strain. However, the polysaccharide-producing strain was able to form micro-colonies on a surface, while the non-polysaccharide producing strain was unable to form micro-colonies and remained as single attached cells.

Evidence supporting the role of surface carbohydrates in initial attachment was reported by Herald and Zottola (1989). They treated cells of *Pseudomonas fragi* with various compounds to disrupt proteins and carbohydrates and concluded that both surface polysaccharides and proteins play a role in attachment of *Pseudomonas fragi* to stainless steel.

The involvement of surface polysaccharides in attachment of bacteria to abiotic surfaces is still not well understood, but this may be a reflection of different laboratory conditions, different bacterial strains and surfaces employed.

### **2.2.9 Role of Surface Proteins in Attachment of *Staphylococcus* species**

The ability of coagulase-negative *Staphylococcus*, especially *S. epidermidis*, to colonise various biomaterials such as heart valves, catheters and artificial prostheses (Donlan, 2001; Knobloch *et al.*, 2001) is thought to be an important step in the pathogenesis of these organisms commonly seen in chronic infections. This relationship between *Staphylococcus* pathogenesis and biofilm formation has resulted in a considerable amount of effort being put into understanding *Staphylococcus* attachment and biofilm formation from a medical point of view. Key aspects of *Staphylococcus* initial attachment are thought to include surface hydrophobicity (Hogt *et al.*, 1986 and Hogt *et al.*, 1983) surface proteins (Veenstra *et al.*, 1996, Timmerman *et al.*, 1991, Heilmann *et al.*, 1997, Cucarella *et al.*, 2001) and teichoic acid structure (Gross *et al.*, 2001). Several surface proteins have been implicated in the ability of *Staphylococcus* strains to attach to inert surfaces. Cucarella *et al.* (2001) identified two mutants of *Staphylococcus aureus* by transposon mutagenesis, with a significant decrease in attachment to inert surfaces. Both

mutants had the Tn917 transposon inserted at the same chromosomal locus. This locus encoded a cell wall-associated protein of 2276 amino acids with a molecular weight of 254kDa (Arrizubieta *et al.*, 2004) called BAP (Biofilm Associated Protein) for short. All isolates of *S. aureus* harbouring the BAP gene were highly adherent to inert surfaces and were strong biofilm producers. The BAP gene was present in only 5% of the 350 *S. aureus* bovine mastitis isolates, suggesting the BAP gene is not a prerequisite for *S. aureus* strains to cause a mastitis infection in cows. Tormo *et al.* (2005) reported other strong biofilm producers from *S. epidermidis*, *S. chromogenes*, *S. xylosus* *S. simulans* *S. hyicus* all produced a BAP-like protein with an amino acid sequence similarity of greater than 80%, suggesting that the BAP surface protein is an important protein involved in attachment of *Staphylococcus* to surfaces.

Other groups have also described the isolation of mutants unable to attach to inert surfaces or unable to form a biofilm due to the loss of a surface protein. Heilmann *et al.* (1996) isolated a transposon-insertion mutant of *S. epidermidis* unable to attach to polystyrene. In comparison with the wild type, the mutant lacks five cell surface associated proteins with masses of 120, 60, 52, 45 and 38kDa. Restoration of the 60kDa band by complementation studies demonstrated that only the 60kDa band is required for initial attachment to polystyrene. Also noted was a decrease in the hydrophobicity of the mutant compared with the wild type strain and the more pronounced ability of the mutant to attach to a hydrophilic surface, in this case glass. Heilmann *et al.* (1996) suggested that the observed increase in attachment to glass by the mutant compared with the wild type may be a result of the mutant lacking the five surface proteins, allowing hydrophilic surface structures to become unmasked, thus making the cell surface more hydrophilic. This in turn increases the likelihood of hydrophilic/hydrophilic interaction between mutant bacterial cell surface and the glass surface, compared with hydrophobic/hydrophilic interaction between the wild type bacterial cell surface and the glass surface. Further analysis by Heilmann *et al.* (1997) showed that the 60kDa adhesion protein appeared to be a protein fragment of a much larger protein that bears sequence homology to an autolysin (AtlE) found in *S. aureus*. Heilmann *et al.* (1997) proposed that the 60kDa and 52kDa bands are the product of the cleaved 120kDa band, similar to the

At1E found in *S. aureus* which is composed of two lytic active domains of 60kDa and 52kDa in size. The ability of the 60kDa to bind to both polystyrene surfaces and plasma protein coated surfaces suggests that it is a multifunctional surface protein allowing cells to attach to inert surfaces and host cell surfaces. Veenstra *et al.* (1996) identified a 280kDa surface protein, subsequently named SSP1 (*Staphylococcus* Surface Protein) from *S. epidermidis* and with the use of immunogold labelling followed by electron microscopy suggested that SSP1 is located on fimbriae-like structures on the cell surface. Proteolytic cleavage of SSP1 by trypsin resulted in SSP2 of 250kDa as demonstrated by SDS-PAGE. The proteolytic cleavage of cells with SSP1 on the surface coincided with the loss of adhesive function and increased concentration of SSP2, suggesting the conversion of SSP1 to SSP2. Veenstra *et al.*, 1996 suggested that the bacterial cell may be able to control its own phenotype between a high adherent and a low adherent phenotype by the proteolytic cleavage of SSP1 to SSP2 until a more favourable environment is reached and adhesiveness can be restored.

No one has reported on the epidemiological distribution among isolates of the Genus *Staphylococcus* of the surface proteins associated with attachment, namely 250kDa reported by Veenstra *et al.* (1996), 60kDa reported by Heilmann *et al.* (1997) and 254kDa reported by Cucarella *et al.* (2001). The question still remains open on the distribution of the above mentioned surface proteins among *Staphylococcus* isolates: do some *Staphylococcus* isolates possess all three surface proteins or do some isolates only have one or even none? If some *Staphylococcus* isolates do possess all three surface proteins associated with attachment, then does each protein have a specific affinity with a particular surface e.g. hydrophobic or hydrophilic surfaces, or are they all generic in terms of overall surface affinity?

## **2.3 Conclusion**

The interaction between an environmental surface and bacterial cells appears to be mediated by a complex array of chemical and physical interactions, with each affected by the chemical and physical environment the bacterial cell and the surface it is being

exposed to, or has recently been exposed to. The multiple factors involved in cell attachment, such as surface conditioning, mass transport, surface charge, hydrophobicity, surface roughness and surface micro-topography can make it difficult to characterise the role and the overall importance each factor has in attachment. The understanding of bacterial attachment to abiotic surfaces such as stainless steel may help in the future development of surfaces with no or reduced attachment, or in developing an effective sanitation programme and thus reducing the potential contamination of processed products by spoilage or pathogenic bacteria.

### **Material and Methods**

#### **3.1 *Source of isolates***

#### **3.2 *Culture preparation***

#### **3.3 *Isolation of variant X7 from Anoxybacillus flavithermus Strain (B12) with Reduced Attachment to Stainless Steel***

#### **3.4 *Attachment Assay***

##### **3.4.1 *Epifluorescence microscopy***

##### **3.4.2 *Impedance detection***

##### **3.4.3 *Comparison of B12 and X7 attachment to stainless steel cuttings in a glass column and to stainless steel cuttings suspended in solution***

##### **3.4.4 *Polystyrene microtitre plate assay***

#### **3.5 *Comparison of B12 and X7 Bacterial Strains***

##### **3.5.1 *Random amplified polymorphic DNA (RAPD) analysis***

###### **3.5.1.2 *Agarose gel electrophoresis***

##### **3.5.2 *API biochemical profile***

##### **3.5.3 *Transmission electron microscopy (TEM)***

- 3.5.4 Supernatant exchange and effect on attachment**
- 3.5.5 Cell surface Hydrophobicity**
  - 3.5.5.1 Microbial adhesion to hydrocarbon test (MATH)*
  - 3.5.5.2 Hydrophobic interactive chromatography (HIC)*
  - 3.5.5.3 Attachment to Sigmacote coated Glass*
- 3.5.6 Cell surface charge**
  - 3.5.6.1 Zeta potential*
  - 3.5.6.2 Electrostatic interaction chromatography (EIC)*
  - 3.5.6.3 Effect of pH on attachment to stainless steel*
- 3.5.8 Quantitative analysis of protein concentration**
- 3.5.9 Treatment of cells with protein disrupting agents**
- 3.5.10 Quantitative analysis surface carbohydrates**
- 3.5.11 Treatment of cells with carbohydrate disrupting agent**
- 3.5.12 HPLC analysis of surface carbohydrates**
- 3.5.13 Treatment of cells with DNase I**
- 3.6 Comparison of Total and Surface extracted Proteins by polyacrylamide Gel Electrophoresis**
  - 3.6.1 One-dimensional polyacrylamide gel electrophoresis (SDS-PAGE) of cell proteins**
    - 3.6.1.1 Total cell protein extraction for one-dimensional polyacrylamide gel electrophoresis (SDS-PAGE)*

3.6.1.2 *Acid Glycine surface protein extraction for one-dimensional polyacrylamide gel electrophoresis (SDS-PAGE)*

3.6.1.3 *Lysozyme surface protein extraction for one-dimensional polyacrylamide gel electrophoresis (SDS-PAGE)*

3.6.1.4 *One-dimensional polyacrylamide gel electrophoresis (SDS-PAGE)*

3.6.1.4.1 *Gel staining and photography*

### **3.6.2 Two-dimensional polyacrylamide gel electrophoresis (2D PAGE) of cell proteins**

3.6.2.1 *Total cell protein extraction for two-dimensional polyacrylamide gel electrophoresis (2D PAGE)*

3.6.2.2 *Acid Glycine surface protein extraction for two-dimensional polyacrylamide gel electrophoresis (2D PAGE)*

3.6.2.3 *Lysozyme surface protein extraction for two-dimensional polyacrylamide gel electrophoresis (2D PAGE)*

3.6.2.4 *Two-dimensional polyacrylamide gel electrophoresis (2D PAGE) method*

3.6.2.4.1 *Isoelectric focusing*

3.6.2.4.2 *Second dimension and strip transfer*

3.6.2.4.3 *Gel Staining and Image acquisition*

3.6.2.4.4 *ImageMaster 2D software*

3.6.2.5 *Cytoplasmic enzyme assays*

## **3.7 Protein Identification**

**3.7.1 Electrospray ionisation–Quadrupole–Time-of Flight (ESI-QUAD-TOF)**

**3.7.2 Bioinformatics**

*3.7.2.1 BLAST searches*

*3.7.2.2 Sequence alignments and Phylogenetic comparison*

**3.7.3 Polymerase Chain reaction (PCR)**

**3.7.4 Extraction of PCR product from agarose**

**3.7.5 DNA cloning and Transformation for DNA sequencing**

*3.7.5.1 DNA cloning*

*3.7.5.2 Transformation*

**3.7.6 Plasmid extraction**

**3.7.7 DNA quantification**

**3.7.8 DNA sequencing**

### 3.1 Source of Isolates

The strains used in this study are either isolates from milk powder, variants of milk powder isolates or reference strains from culture collections. The strains are listed in Table 3.1.

**Table 3.1** List of isolates and their origin

Strain number	Culture type	Source
B12	<i>Anoxybacillus flavithermus</i>	Skim milk powder
X7	<i>Anoxybacillus flavithermus</i>	This Study
AF	Non-dairy <i>Anoxybacillus flavithermus</i>	Otago University
B13	<i>Geobacillus thermoleovorans</i>	Skim milk powder
GT	Non-dairy <i>Geobacillus thermoleovorans</i>	Fonterra Research Centre
GS	Non-dairy <i>Geobacillus stearothermophilus</i>	Reference culture ATCC 12980

## **3.2 Culture Preparation**

Cultures were maintained in long term storage by freezing at  $-75^{\circ}\text{C}$  using Microbank<sup>®</sup> beads (Pro-Lab Diagnostics, Austin, Texas, USA). For routine preparation of cultures, bacteria retrieved from frozen stocks were inoculated into Bacto Tryptic Soy broth and/or Bacto Tryptic Soy Agar (Difco, Becton Dickson & Co, USA). Cultures were grown aerobically for 8-12 hr at  $55^{\circ}\text{C}$ .

Quantitative estimates of viable cells were made by inoculating Bacto Tryptic Soy Agar plates with 0.1 ml of 10-fold serial dilutions prepared in sterile buffered peptone water (5 g per litre, Merck). The plates were incubated in plastic bags to prevent the plates from drying out, at  $55^{\circ}\text{C}$  for 24 hours and the number of colonies counted.

### **3.3 Isolation of variant X7 from *Anoxybacillus flavithermus* Strain (B12) with Reduced Attachment to Stainless Steel**

In order to isolate a strain of B12 with reduced attachment to stainless steel by natural selection, stainless steel cuttings (1-3 mm in size) were collected from an engineering lathe and soaked in three changes of 95% ethanol over 2 hours. The cleaned cuttings were air dried at room temperature and placed in a 1 cm diameter x 40 cm long glass column (Fig 3.1). The stainless steel filled glass column was autoclaved (121°C at 15 psi for 15 minutes), placed in a retort stand and allowed to stand overnight. Fifteen ml of a mid log phase culture of B12 (8hr) was added to the column and allowed to pass through. After passing through the column samples were collected into four fractions.

Fraction 1 – The first 0.5 ml collected was spread plated onto five plates (100 µl per plate) onto Tryptic Soy Agar (TSA) and incubated overnight at 55°C in sealed bags.

Fraction 2 – The second 0.5 ml eluted from the column was collected, and spread plated onto five plates TSA (100 µl per plate) and incubated as before.

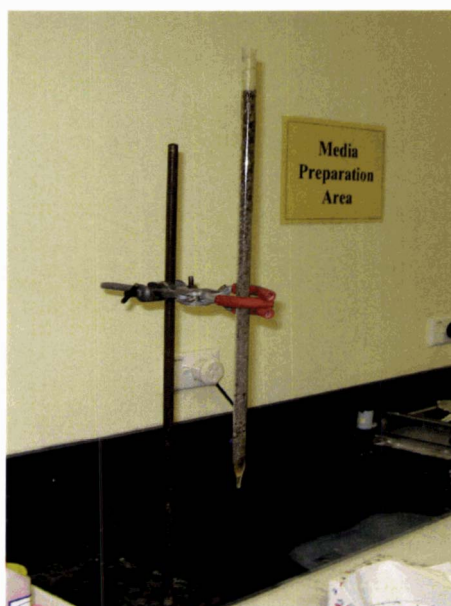
Fraction 3 – The next 2 ml was collected and spread plated onto twenty plates TSA (100 µl per plate) and incubated as before.

Fraction 4 – 5 ml was collected and 2 ml was spread plated onto twenty plates TSA (100 µl per plate) and incubated as before.

One of the first B12 colonies isolated after passage through the column was restreaked on to TSA for isolated colonies and incubated at 55°C overnight. After restreaking, one isolated colony was used to inoculate 15 ml Tryptic Soy Broth (TSB), incubated for 7 hours at 55°C and added to a cleaned glass column containing stainless steel chips and the above cycle repeated again.

To clean the column between runs, 40 ml of 95% ethanol was added to the column and allowed to stand for one hour. The column was then washed twice by the addition of 50 ml of 0.05 M of phosphate buffer (0.05 M  $\text{KH}_2\text{PO}_4$  (BDH); 0.05 M  $\text{Na}_2\text{HPO}_4$  (BDH)) at pH 7.0 and allowed to stand for one hour to remove ethanol. The column was then autoclaved, placed in a retort stand and allowed to stand overnight (Fig 3.1).

After repeating the process outlined above six times, bacterial colonies isolated from the first lot through the column in the seventh run were compared with the original B12 strain in their ability to attach to surfaces. The strain isolated following repeated selection through the column was eventually named X7.



**Fig 3.1:** Photograph of the glass column containing stainless steel chips used to select for attachment deficient strain X7.

## **3.4 Attachment Assays**

### **3.4.1 Epifluorescence microscopy**

To establish the number of bacteria attaching to a surface, Epifluorescence microscopy was used based on the method reported by Parkar *et al.* (2001). The fluorochrome stain Acridine Orange (BDH, 10 mg) was dissolved into 100 ml of 0.05 M phosphate buffer pH 7.0 to create a final working concentration of 0.01% solution. This was then filtered through a Sartorius filter (Medic Corporation, Lower Hutt, New Zealand) before use and stored at 4°C for up to 2 months.

Treated cells or control preparations of cells were diluted to 0.50 OD<sub>600nm</sub> in 0.05M phosphate buffer pH 7.0. Two ml of suspended cells were incubated with stainless steel pieces (laser cut, 1 cm<sup>2</sup>, 316 grade) in test tubes for 20 minutes. For the assessment of cell attachment to glass four ml of suspended cells were incubated with glass slides (2 cm<sup>2</sup>, Esco, Biolab, NZ) in test tubes for 20 minutes. Cells attached to stainless steel pieces or glass cover slides were washed five times with 4 ml deionised water then exposed to 4 ml Acridine Orange stain for 2 minutes at room temperature, washed five times in 4 ml sterile deionised water, air dried and mounted onto glass microscope slides using either Vaseline or epoxy resin. Bacterial cells were observed under ultraviolet (UV) illumination, using a Leitz Ortholux microscope with an H2 incident light excitation filter block (Ernst Leitz Wetzlar, GmbH, Wetzlar, Germany) and photographed using either a PJC 1600 film (Kodak, Rochester, New York, USA) or a digital camera (Nikon, CoolPix 990, Japan). The cells were counted in five separate fields and the average expressed as log<sub>10</sub> cells cm<sup>2</sup>. Each experiment was performed in duplicate.

### **3.4.2 Impedance detection**

In order to determine the counts of viable cell attached to stainless steel surfaces, stainless steel laser cut pieces (1 cm<sup>2</sup>) with attached cells were added to TSB in a MiniTrac 4000 impedance monitor (Sylab, MBH, Purkersdorf, Austria) with the temperature block set to

55°C. This method has been used by other groups studying thermophilic bacilli and demonstrated as a reliable method for the enumeration of attached cells (Flint & Brooks, 2001). In this study it provided a reliable method for the enumeration of *Anoxybacillus flavithermus*. The time taken to reach 5% of the electrode E value, termed the threshold level, was measured and called the impedance detection time (IDT). The larger the initial viable cell numbers present, the shorter the IDT.

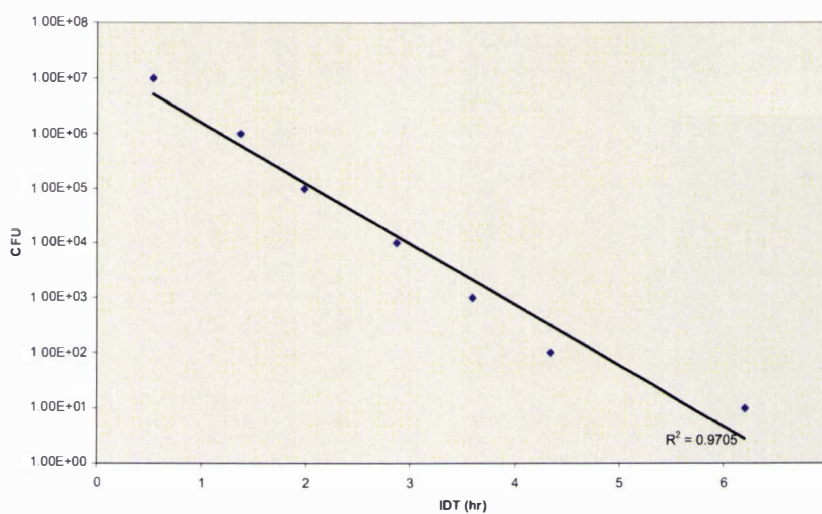
Individual Bactrac cells were prepared for inoculation by soaking in laboratory detergent (Cleanaid powder 2 g l<sup>-1</sup>) overnight, gently washing with a brush and rinsing with at least five changes of deionised water and sterilizing by autoclaving. Just before use, the deionised water was removed and replaced aseptically with 10 ml of sterile TSB medium. The number of viable cells present was determined from a calibration curve created by using serial 10-fold dilutions of planktonic cells incubated at 55°C in the Bactrac and plated onto TSA incubated at 55°C overnight in sealed plastic bags to prevent the plates drying out.

Calibration curves are given by the following equations

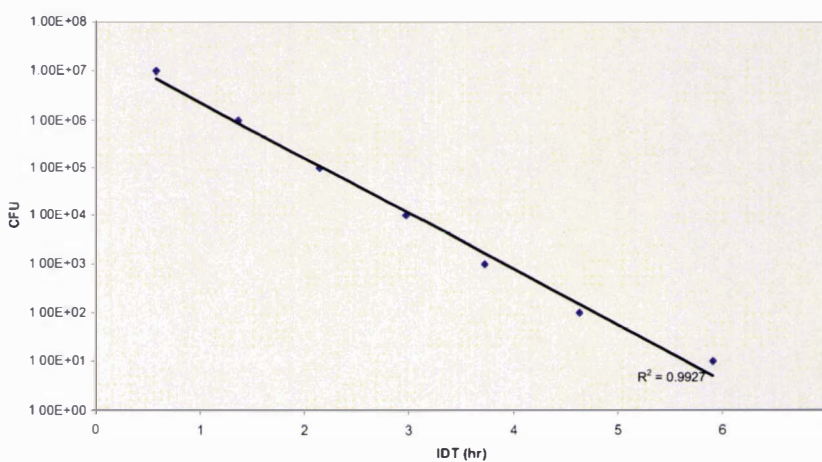
$$\text{B12} \quad \text{Log}_{10}(\text{CFU}) = -1.2558 * T + 7.6023 \quad r^2 = -0.9705$$

$$\text{X7} \quad \text{Log}_{10}(\text{CFU}) = -1.0235 * T + 7.7924 \quad r^2 = -0.9925$$

Where T = the time taken for the sample to reach impedance detection time



**Fig 3.2:** Calibration curve for planktonic *Anoxybacillus flavithermus* strain B12 in the MiniTrac 4000 impedance monitor using TSB as the growth medium. IDT = Impedance Detection Time.



**Fig 3.3:** Calibration curve for planktonic *Anoxybacillus flavithermus* strain X7 in the MiniTrac 4000 impedance monitor using TSB as the growth medium. IDT = Impedance Detection Time.

### **3.4.3 Comparison of B12 and X7 attachment to stainless steel cuttings in a glass column and to stainless steel cuttings suspended in solution**

Stainless steel cuttings were collected from a lathe and washed in ethanol for 2 hours, allowed to air dry at room temperature and placed in a 20 cm long x 1 cm diameter glass column as described in section 3.3. A 50 ml of mid-log phase culture of B12, (7-8 hr) was centrifuged at 1000 x g and resuspended in 0.05 M phosphate buffer pH 7.0 to OD<sub>600nm</sub> of 0.660. One ml of resuspended culture was added to the autoclaved column followed by the addition of 60 ml of 0.05 M phosphate buffer pH 7.0 and allowed to pass through the column. A total of 22 fractions each of 2 ml of eluted mixture of buffer and unattached cells were collected. Quantitative estimates of viable cells in each fraction were made by 10 -fold serial dilutions of each fraction to 10<sup>-4</sup> dilution, followed by plating of fractions as described in Section 3.2

To assess the ability of B12 and X7 to attach to stainless steel cuttings, the number of B12 or X7 cells removed from a solution containing suspended stainless cuttings was measured by assaying the number of cells remaining behind in suspension. Cultures of B12 and X7 were grown to mid-log phase (7-8 hr) in TSB at 55°C. Cells were collected by centrifugation (1000 x g for 20 minutes) and resuspended in the equal volume of 0.05 M phosphate buffer pH 7.0. Ten ml of the suspended cells were added to 2 g of stainless steel cuttings (collected and treated as above). After 5, 10 and 15 minutes, one ml samples of culture suspension were collected. Total cell numbers in each sample were estimated by serial dilutions plated out onto TSA and incubated overnight in plastic bags at 55°C.

### **3.4.4 Polystyrene microtitre plate assay**

To determine the ability of various strains to attach to surfaces, microtitre plate attachment assay was utilised based on the methods reported by Ziebuhr *et al.* (1999) and Loo *et al.* (2000). Cell cultures were centrifuged at 1000 x g for 20 minutes and

resuspended in 0.05 M phosphate buffer pH 7.0 to  $OD_{600nm}$  of 1.00. Resuspended cells (200  $\mu$ l) were incubated overnight in 96 well polystyrene microtitre plates (Falcon, nontreated polystyrene plates, Bacto Laboratories, Australia) at room temperature in a moistened closed container to prevent the sample from drying out. Wells were washed three times with 0.05 M phosphate buffer pH 7.0 and air dried at 55°C for 1 hr. 200  $\mu$ l 0.1% solution of crystal violet was added and held at room temperature for 2 minutes and then each well was washed five times with 0.05 M phosphate buffer pH 7.0. 200  $\mu$ l of 95% ethanol was added to each well and  $OD_{490nm}$  measured using a MicroELISA plate reader (Dynatech, Torrance, California)

### **3.5 Comparison of B12 and X7 Bacterial Strains**

#### **3.5.1 Random Amplified Polymorphic DNA (RAPD) analysis**

In order to determine parental relationship between the original B12 strain and the newly isolated strain X7 that demonstrates a lower affinity to stainless steel, Random Amplified Polymorphic DNA (RAPD) analysis was used (Williams *et al.*, 1990 and Ronimus *et al.*, 1997). RAPD uses Polymerase Chain Reaction (PCR) to detect polymorphisms by amplifying random portions of the cell DNA, with the use of short arbitrary DNA primers (9-10 bases in length).

In this study, the random primer OPR 13, 5'-GGACGACAAG-3' Ronimus *et al.* (1997) was utilized to identify any polymorphisms between the strains B12 and X7. Ronimus *et al.* (1997) and Flint *et al.* (2001) demonstrated that the primer OPR 13 produced the best profile based on the number of bands and their distribution when comparing and identifying different strains of thermophilic bacilli.

The PCR reaction used was based on the method reported by Ronimus *et al.* (1997) and Flint *et al.* (2001). A reaction mixture was prepared by combining the following: 460µl of H<sub>2</sub>O; 40 µl of 20X buffer (TFL buffer, Epicentre Technologies, Madison, Wisconsin, USA); 80 µl of 25mM MgCl<sub>2</sub>; 160 µl of dNTP (Promega, Madison, Wisconsin, USA); 1 µl of primer (Invitrogen, Carlsbad, California, USA); 10 µl of TFL polymerase (Epicentre Technologies). The reaction mixture was dispensed (75 µl per tube) into reaction tubes and 5 µl of culture (template) was added. The PCR reaction took place under the following conditions: 1 cycle of denaturation at 94°C for 3 minutes, 35 cycles of 94°C for 45 seconds, 55°C for 45 seconds and 72°C for 1 minute, followed by a final single step of 72°C for 5 minutes.

### 3.5.1.2 Agarose gel electrophoresis

Agarose electrophoresis was performed in a BRL, Horizon 11.14 (Life Technologies) apparatus for 2-3 hours. Agarose gels were made of Agarose UltraPure™ (Invitrogen) at 1.5% (w/v) in 1 X TBE buffer.

#### 1 X TBE buffer

89mM Tris (Bio-Rad)	10.9 g
89mM Boric acid (BDH)	5.56 g
2.5mM Na <sub>2</sub> EDTA (BDH)	0.93 g
Deionized water	1000 ml

Agarose was melted in a microwave and allowed to equilibrate to 50°C before the gel was poured. Sample loading buffer (1-2 µl) was mixed with DNA sample (10 µl) and loaded onto the gel. Either a 5 µl (0.2 µg/µl) sample of 1 kb DNA ladder (Invitrogen) or 5 µl (0.1 µg/µl) sample of EZ load 100 bp PCR molecular ruler (Bio-Rad) was included in the end wells. Electrophoresis was performed at 80 V for 2-3 hours.

#### Sample loading buffer

40% Sucrose (BDH) (w/v)	4 g
0.25% Bromophenol blue (BDH) (w/v)	25 mg
0.25% Xylene cyanol FF (BDH) (w/v)	25 mg
Double-distilled water	10 ml

Gels were stained in 0.005% Gelstar® (Cambrex Bioscience, Maine, USA) stain for 15 minutes, washed once in 1 X TBE and observed under UV light and photographed using Polaroid 667 film (Polaroid, St Aubens, Hertfordshire, UK).

### 3.5.2 API biochemical profile

The API 50 CH system (LaBalme LesGrottes, Montalieu, Vercieu, France) was used according to the manufactures instructions to provide information on any biochemical differences between strains B12 and X7.

### 3.5.3 Transmission electron microscopy (TEM)

To visualize surface features of both B12 and X7 strains, cells were examined under TEM. Sections of resin-embedded preparations were prepared as follows: an 8-10 hour culture was centrifuged at 1000 x g for 15 minutes and resuspended in deionised water. The cells were fixed at ambient temperature for 2-3 hr in a primary fixative solution consisting of 3% (w/v) glutaraldehyde (BDH), 2% formaldehyde solution (BDH) in 0.1 M phosphate buffer (0.1 M  $\text{KH}_2\text{PO}_4$  (BDH); 0.1 M  $\text{Na}_2\text{HPO}_4$  (BDH)) at pH 7.2. The cells were washed with three changes of phosphate buffer and exposed to a secondary fixative solution consisting of 1% osmium tetroxide (ProTech, Queensland, Australia) in 0.1 M phosphate buffer at ambient temperature for 30 minutes. After two washes in 0.1 M phosphate buffer, the cells were dehydrated using acetone (BDH) and embedded in Procure 812 epoxy resin (Pro Tech). Thin (90 nm) sections were cut using a microtome, stained with saturated uranyl acetate (BDH) in 50% ethanol followed by lead citrate (1.33 g lead nitrate (BDH) and 1.76 g sodium citrate (BDH) in 30 ml) and examined using a Philips 201c (Eindhoven, Netherlands) TEM.

In order to examine possible cell surface polysaccharide structures, both B12 and X7 were stained with the cationic inorganic stain Ruthenium red (Hood & Zottola, 1988), followed by examination under TEM. Embedded cells were prepared as follows: an 8-10 hour culture of cells was centrifuged at 1000 x g for 15 minutes and resuspended in deionised water. Cells were washed once in 0.1 M sodium cacodylate buffer (21.4 g/1000 ml of water) adjusted to pH 2 with HCl for 90 minutes at 37°C. The cells were fixed at ambient temperature in 2.5% (w/v) glutaraldehyde (BDH), 0.05% ruthenium red (Sigma)

in 0.1 M sodium cacodylate buffer for one hour. The cells were washed three times in 0.1 M sodium cacodylate buffer and exposed to a secondary fixative solution consisting of 2% osmium tetroxide (ProTech, Queensland, Australia) and 0.05% Ruthenium red in 0.1 M sodium cacodylate buffer for 30 minutes. The cells were dehydrated in acetone and embedded in Procure 812 epoxy resin (Pro Tech). Thin (90 nm) sections were prepared as described in section 3.5.3.

### **3.5.4 Supernatant exchange and effect on cell attachment**

An attempt was made to detect possible surface components produced by strain B12 but lacking in X7 that may accumulate in the supernatant of the culture and play an important part in attachment to stainless steel. X7 cells were added to B12 supernatant and B12 cells were added to X7 supernatant. Both were then assessed for their ability to attach to stainless steel. Cultures were centrifuged (1000 x g for 10 minutes) and the cells were washed once in 0.05 M phosphate buffer then resuspended using the supernatant from a culture ( $OD_{600nm}$  0.50) of the opposing strain and incubated for 20 minutes at 55°C. Attachment of the treated cells to stainless steel coupons was assessed by epifluorescence microscopy as described in section 3.4.1.

### **3.5.5 Hydrophobicity of the cell surface**

#### *3.5.5.1 Microbial adhesion to hydrocarbon test (MATH)*

In order to characterise the relative cell surface hydrophobicity of B12 and X7 cultures, a modified version of the microorganism adhesion to hydrocarbon (MATH) test (Rosenberg *et al.*, 1980) was employed. Ahimou *et al.* (2001), Busscher *et al.* (1995), Van der Mei (1993) and Doyle (2000) all suggested that electrostatic interactions can influence the MATH test because of the negative charge of hydrocarbons such as

hexadecane. Doyle (2000) proposed that the electrostatic interactions can be reduced by the presence of a high concentration of ions, usually NaCl.

In this trial, cultures of B12 and X7 were grown to mid-log phase, centrifuged at 1000 x g for 20 minutes and resuspended in 0.05 M phosphate buffer at pH 7.0 containing one of the following: sodium chloride concentrations 0 M, 1 M, 2 M, 3 M or 4 M. The final OD<sub>600nm</sub> was adjusted to 0.7-0.8 and 2.5 ml of the suspension was added to 2.5 ml of hexadecane, mixed for 30 seconds on a vortex mixer and incubated for 20 minutes at 30°C. The absorbance of the aqueous phase was measured at 600 nm using a UV/visible spectrophotometer (Hitachi, Japan) and the percentage hydrophobicity was determined using the formula below.

$$\% \text{ hydrophobicity} = (A_0 - A_1) / A_0 \times 100$$

A<sub>0</sub> = OD of the bacterial suspension before mixing with hexadecane.

A<sub>1</sub> = OD of the bacterial suspension after mixing with hexadecane.

### 3.5.5.2 Hydrophobic interactive chromatography (HIC)

In a further attempt to determine the cell surface hydrophobicity of B12 and X7 cultures, a modified version of HIC as reported by Smyth *et al.* (1978) and Peng *et al.* (2001) was employed. In HIC hydrophobic cells are separated from non-hydrophobic cells by passing a bacterial suspension through a hydrophobic resin (Phenyl Sepharose 6 fast flow, Amersham Biosciences).

Pasteur pipettes were plugged with glass wool and filled with one ml of suspension of hydrophobic resin as provided by the manufacturer. The resin was washed with 10 ml of 0.05 M phosphate buffer at pH 7.0 with the same concentration of NaCl added as the bacterial suspension to be tested i.e. 0 M, 1 M, 2 M, 3 M or 4 M NaCl. The bacterial cells were prepared by centrifuging a mid-log phase culture at 1000 g for 20 minutes and resuspended in 0.05 M phosphate buffer pH 7.0 with one of the concentrations of sodium

chloride to an OD<sub>600nm</sub> of 1.0. Each column was loaded with 0.3 ml of culture and eluted with 0.9 ml of 0.05 M phosphate buffer (pH 7.0) with appropriate NaCl concentration. The absorbance was measured at 600 nm using a UV/visible spectrophotometer (Hitachi, Japan) and the percentage of bacteria retained in the hydrophobic column was calculated from the absorbance of a ¼ dilution of the original bacterial suspension (OD<sub>600nm</sub> 0.7-0.8) and the absorbance of the sample eluted from the column.

$$\% \text{ retained by Column} = (A_0 - A_1) / A_0 \times 100$$

A<sub>0</sub> = OD of ¼ diluted bacterial suspension

A<sub>1</sub> = OD of the eluted bacterial suspension

### 3.5.5.3 Attachment to Sigmacote treated glass

In order to assess the role of surface hydrophobicity in the attachment of B12 and X7 to surfaces, glass slides were coated with Sigmacote<sup>®</sup> (Sigma) to increase surface hydrophobicity. Glass slides were soaked overnight in undiluted Sigmacote<sup>®</sup>. Mid-log phase cultures of B12 and X7 were collected by centrifugation (1000 g, 20 min). The cells were diluted in either 0 M, 1 M, 2 M, 3 M or 4 M NaCl concentrations dissolved in 0.05 M phosphate buffer (pH 7.0) to final OD<sub>600nm</sub> of 0.50. Coated or untreated glass slides were then exposed to the cell suspensions for 20 minutes and the number of attached cells was counted by epifluorescence microscopy as outlined in section 3.4.1, except that wash solutions also contained the same concentration of NaCl as the original cell suspension.

## 3.5.6 Cell surface charge

### 3.5.6.1 Estimation of Zeta potential

The surface charge of both B12 & X7 was expressed as the Zeta potential in a Malvern ZetaSizer IV (Malvern Instruments Ltd, UK) (Ahimou *et al.*, 2001). Cells were grown to

mid-log phase and washed twice by centrifugation at 1000 g for 20 minutes and resuspension in 5 mM NaCl solution. Cells were diluted to OD<sub>600nm</sub> 0.5-0.6 in solutions of defined pH, Table 3.2 (phosphate-citrate buffers) One ml of suspended cells were added to cuvettes for testing. Measurements were performed in triplicate.

**Table 3.2** Composition of citrate and phosphate buffers to create buffers in 2-8 pH range.

pH	X ml 0.01 M citric acid	Y ml 0.02 M Na <sub>2</sub> HPO <sub>4</sub>
2	89	11
3	79.5	20.5
4	61.4	36.6
5	48.5	51.5
6	36.8	63.2
7	17.6	82.4
8	6.4	93.6

### 3.5.6.2 Electrostatic interaction chromatography (EIC)

The relative surface charge of both B12 & X7 strains was assessed by separation through anionic (Dowex AG 1-8x 100-200 mesh) or cationic (Dowex AG 50W-X8 100-200 mesh) exchange resins (Bio-Rad). The method is based upon that of Pedersen (1980) and Flint *et al.* (1997). Pasteur pipettes were plugged with glass wool and filled with 0.5 g of ion exchange resin then eluted with 5 ml of 0.01 M phosphate buffer (0.01 M KH<sub>2</sub>PO<sub>4</sub> (BDH); 0.01 M Na<sub>2</sub>HPO<sub>4</sub> (BDH)) at pH 7.0. Bacterial suspensions were prepared by centrifuging a mid-log phase culture (8-10 hr) at 1000 g for 20 minutes and resuspended in phosphate buffer to OD<sub>600nm</sub> 0.7-0.8. Each column was loaded with 1 ml of bacterial suspension and then eluted with 3 ml of phosphate buffer. The absorbance of the eluted sample was measured at 600 nm using a UV/visible spectrophotometer (Hitachi, Japan) and the percentage of bacteria retained in the column was calculated from the absorbance of a ¼ dilution of the original bacterial suspension (OD<sub>600nm</sub> 0.7-0.8) and the absorbance of the sample eluted from the column.

$$\% \text{ retained by Column} = (A_0 - A_1) / A_0 \times 100$$

$A_0$  = OD of  $\frac{1}{4}$  diluted bacterial suspension

$A_1$  = OD of the eluted bacterial suspension

### 3.5.6.3 Effect of pH on cell attachment

To investigate the effect pH has on attachment of B12 and X7 to stainless steel, cells were suspended in buffer solutions within pH range of 3.0 – 7.2. The method was based upon that of Stanley (1983). Mid-log phase cultures of B12 and X7 were collected by centrifugation (1000 x g, 20 min). The cells were diluted in different pH buffers to  $OD_{600nm}$  of 0.50 for all pH values used. Stainless steel, 316 grade coupons (1 cm<sup>2</sup>) were then exposed to the cell suspensions for 20 minutes. The number of attached cells was counted by epifluorescence microscopy as outlined in section 3.4.1.

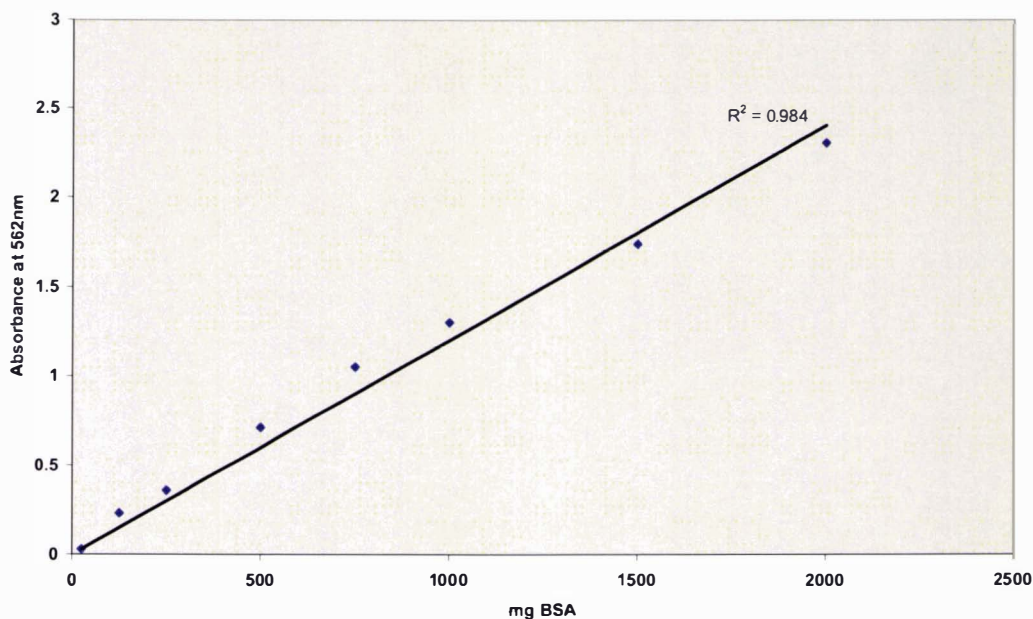
**Table 3.3:** Composition of citrate and phosphate buffers to create buffers in 3-7.2 pH range

Final pH	0.01 M Citric acid (x ml)	0.02 M Na <sub>2</sub> HPO <sub>4</sub> (y ml)
3	79.4 ml	20.5 ml
4.6	53.2 ml	46.7 ml
Final pH	0.01 M Na <sub>2</sub> HPO <sub>4</sub> (x ml)	0.02 M NaH <sub>2</sub> PO <sub>4</sub> (y ml)
5.8	4.0 ml	46.0 ml
6.6	18.7 ml	31.2 ml
7.2	36.0 ml	14.0 ml

The x and y volumes were mixed to give a final volume of 100 ml buffer at a required pH value and then used to dilute the cells to the appropriate OD .

### 3.5.8 Quantitative analysis of protein concentration

Protein concentration was determined by using the PIERCE BCA protein assay kit (Rockford, USA). The Pierce protein assay is a detergent-compatible colorimetric test for the detection and quantitation of total protein. The method combines the well known biuret reaction, where  $\text{Cu}^{2+}$  is reduced to  $\text{Cu}^{1+}$  by protein in an alkaline solution, with the sensitive colorimetric detection of  $\text{Cu}^{1+}$  cation by using a unique Bicinchoninic acid reagent. A 100  $\mu\text{l}$  aliquot of each standard or unknown sample was pipetted into test tubes. 2 ml of the working solution was added to each sample, well mixed and incubated at 37°C for 30 minutes. After incubation, the tubes were cooled to room temperature for 10 minutes. The absorbance of each tube was measured at 562 nm using a UV/visible spectrophotometer (Hitachi, Japan). Calibration curves were prepared against standard bovine serum albumin (BSA) solutions. The results were expressed as BSA equivalent mg/ml.



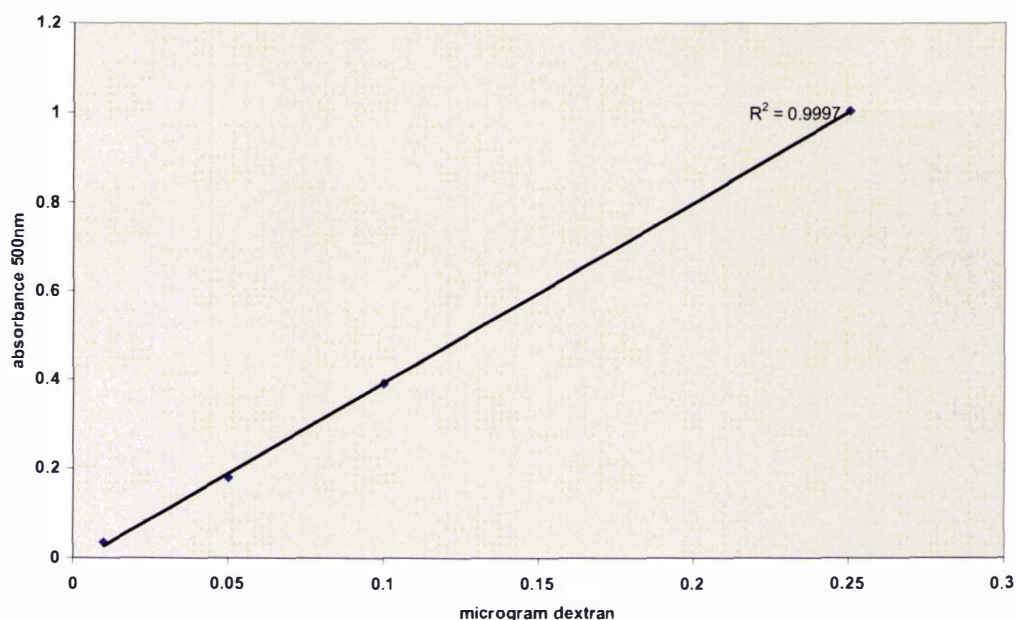
**Fig 3.4:** Calibration curve for protein estimation using PIERCE BCA protein assay kit.

### 3.5.9 Treatment of cells with protein disrupting agents

To investigate the effect of bacterial cell surface proteins on attachment, three different methods were used to remove surface proteins. Cultures of B12 and X7 were centrifuged (1000 x g for 10 minutes) and then the cells were resuspended to the original volume with either 1% trypsin (Sigma)(37°C for 24 hr) in 0.05M phosphate buffer pH 7.5 or 0.2 M glycine at pH 2.2 (20°C for 30 min) or 5 M LiCl<sub>2</sub> (20°C for 30 min). After treatment, cultures were washed twice and resuspended in 0.05 M phosphate buffer pH 7.0.

### 3.5.10 Quantitative analysis of surface carbohydrates

The amount of extracellular polysaccharides (EPS) produced by B12 and X7 was determined by the acid hydrolysis method of Dall and Herndon (1989) as described by Evans *et al.* (1994). Cultures of B12 and X7 were centrifuged (1000 x g for 10 minutes) and then the cells were resuspended to OD<sub>600nm</sub> = 1.1 (approximately equal to 1 x 10<sup>8</sup> cells ml<sup>-1</sup>) and sonicated at 60 W for 2 minutes in a sonicator water bath (Ultrasonics Pty, NSW, Australia). The samples were centrifuged (1000 x g for 10 minutes) to remove cells and 1 ml of the supernatant was added dropwise to 8 ml absolute alcohol to precipitate polysaccharides and stored at 4°C overnight. This was centrifuged at 10,000 x g for 20 minutes and the resulting pellet dissolved in 1 ml of water. To each sample, 7 ml of 77% Sulphuric acid (BDH) was added to digest the polysaccharide and the suspension was cooled on ice for 10 minutes. Cold 1% tryptophan (BDH) (1 ml) was added and each sample was heated in a boiling bath for 20 minutes then cooled to room temperature. Acid hydrolysis of EPS produces a furan which condenses with tryptophan to produce a coloured product that can be quantified at OD<sub>500nm</sub> using a UV/visible spectrophotometer. Calibration curves were prepared against standard dextran solutions (molecular weight 52,000; Sigma). The results were expressed as dextran units per 10<sup>8</sup> cells.



**Fig 3.5:** Calibration curve for the detection of EPS

### 3.5.11 Treatment of cells with carbohydrate disrupting agent

To investigate the effect cell surface polysaccharides have on cell attachment, sodium metaperiodate was used to oxidize carbohydrates as outlined by Hussain *et al.* (2001). Cultures of B12 and X7 were centrifuged (1000 x g for 10 minutes), resuspended in the original volume with 50 mM sodium metaperiodate (BDH) in 0.05 M sodium acetate at pH 4.2 for 30 minutes. After treatment, the cells were washed twice in 0.1 M phosphate buffer pH 7.5. Attachment of cells to stainless steel was assessed by epifluorescence microscopy (Section 3.4.1).

### 3.5.12 HPLC analysis of surface carbohydrates

To further analyse extracellular surface polysaccharides, surface polysaccharides of B12 and X7 cultures were extracted as described in section 3.5.10. The isolated surface polysaccharides were hydrolysed as described by Scherz and Günther (1998). To

approximately 10-15 mg of extracted polysaccharide, 5 ml of 1 M sulphuric acid was added and then heated under reflux at 100°C for 5 hours. After cooling, the sulphate ions were removed with the addition of 0.25 M barium hydroxide (BDH) solution to a pH of approximately 5.5-6.0. This solution was then filtered to remove the barium sulphate from suspension. The filtrate was frozen at -75°C and freeze dried (Telstar Crydos, Spain) until dry, followed by resuspension in 1 ml of water. HPLC was used to separate the hydrolysed polysaccharide samples. A DIONEX HPLC system with a Phenomenex RCM Monosaccharide (300mm x 7.8mm, L19) packing was used to separate the sugars. The mobile phase was water with a flow rate of 0.6 ml/min and the separated monosaccharides were detected by refractive index (RI). 1% solutions of Glucose, Arabinose, Galactose, Rhamnose, Mannose and Xylose were used as markers to identify hydrolysed polysaccharides.

### **3.5.13 Treatment of cells with DNase I**

To investigate the effect of bacterial extracellular DNA on attachment, cultures of B12 and X7 were centrifuged (1000 x g for 10 minutes). The cells were resuspended to the original volume in 0.1 M Tris-HCl, pH 7.5 containing 2 mM MgCl<sub>2</sub>, and 50 µg/ml of DNase I (Sigma) for one hour at 25°C. After treatment, cultures were washed twice and resuspended in 0.05 M phosphate buffer pH 7.0. Attachment of cells to stainless steel was assessed by epifluorescence microscopy (Section 3.4.1).

### **3.6 Comparison of Whole Cells and Extracted Surface Proteins by Polyacrylamide Gel Electrophoresis**

#### **3.6.1 One dimensional polyacrylamide gel electrophoresis (SDS-PAGE) of cell proteins**

##### *3.6.1.1 Total cell protein extraction for one-dimensional polyacrylamide gel electrophoresis (SDS-PAGE)*

To enable more detailed studies on the cell proteins of B12 and X7, SDS-PAGE electrophoresis was employed. Mid-log phase cultures of B12 and X7 strains grown in TSB were centrifuged at 1000 x g for 20 minutes and resuspended to an OD<sub>600nm</sub> of 1.2 in distilled water (DW). The cell suspension was then sonicated six times on ice for 30 seconds, at amplitude of 7 micrometres, with a 30 second break between each 30 second sonication. The cell suspension was centrifuged (10,000 x g for 20 minutes) to remove cellular debris. The supernatant was frozen to -80°C then freeze-dried (Telstar, Cryodas, Terrassa, Spain) in a 100 ml round bottom flask at -75°C until dry. Fractions were then frozen at -75°C for storage.

##### *3.6.1.2 Acid glycine surface protein extraction for one-dimensional polyacrylamide gel electrophoresis (SDS-PAGE)*

To enable studies of cell wall components and their involvement in cell attachment, a modified acid glycine extraction described by Dubreuil *et al.* (1988) was adopted. Cells of strain B12 and X7 were harvested by centrifugation (1000 x g for 20 minutes) and resuspended in 0.2 M glycine hydrochloride buffer pH 2.2 (3 g cells per 100 ml). This suspension was stirred at room temperature for 30 minutes. Cells were removed by centrifugation (1000 g for 20 minutes). The supernatant was neutralized to pH 7.0 by the addition of 2 M NaOH, frozen to -80°C then freeze-dried (Telstar, Cryodas, Terrassa, Spain) in a 100 ml round bottom flask at -75°C until dry. Fractions were then frozen at -75°C for storage.

### 3.6.1.3 Lysozyme surface protein extraction for one-dimensional polyacrylamide gel electrophoresis (SDS-PAGE)

The second method employed to extract surface proteins involved incubating the cells in lysozyme and mutanolysin (Coolbear *et al.*, 1992). Cells were harvested by centrifugation at 1000 x g for 20 minutes and suspended in an equal volume of 50mM Tris-HCl, pH 7.0 with 10 mM MgCl<sub>2</sub>, 24% sucrose, 50,000 units per ml of lysozyme (Sigma) and 90 units per ml of mutanolysin (Sigma) and incubated for 5 hrs at 37°C. The mixture was centrifuged (10,000 x g for 20 minutes). The supernatant was frozen to -80°C then freeze-dried (Telstar, Cryodas, Terrassa, Spain) in a 100 ml round bottom flask at -75°C until dry. Fractions were then frozen at -75°C for storage.

### 3.6.1.4 One dimensional polyacrylamide gel electrophoresis (SDS-PAGE)

To enable a more detailed study of the surface proteins of strains B12 and X7, samples from cell extracts (refer sections 3.6.1.2 and 3.6.1.3) and total cell proteins (3.6.1.1) were analysed using SDS-PAGE. Protein samples were separated by vertical electrophoresis according to the method by Laemmli (1970) in denaturing polyacrylamide gels containing SDS. The Protean® II xi electrophoresis cell (Bio-Rad), holding two 20cm x 20cm gels was used. Stock solutions were prepared as follows.

#### (1) Resolving Gel

##### (a) Running Acrylamide

Acrylamide (BDH)	30.0 g
Bis-Acrylamide (Sigma)	1.0 g
Double-distilled water	100 ml

##### (b) Resolving Gel Buffer (pH 8.8)

Tris(BDH)	18.10 g
Double-distilled water	100 ml

(c) 10% Solution of SDS (BDH) (prepared fresh)

(d) 10% Solution of Ammonium persulphate (BDH) (prepared fresh)

The 12% resolving gel was prepared as follows.

Resolving Gel Buffer (pH 8.8)	10.0 ml
Running Acrylamide	16.0 ml
Double-distilled water	14.9 ml
10% SDS solution	1.6 ml
Ammonium persulphate solution	0.2 ml
N,N,N, N'-Tetramethylethylenediamone (TEMED) (Sigma)	0.02 ml

The above solutions were mixed gently, trying not to create bubbles, and then autopipetted between vertical two glass plates separated with 1mm spacers in the casting unit (Bio-Rad). After pouring a small volume of deionised water (about 2 ml) was gently distributed on top of the resolving gel and left until polymerisation had taken place.

## (2) Stacking Gel

(a) Stacking Acrylamide

Acrylamide (BDH)	30.0 g
Bis-Acrylamide (Sigma)	1.6 g
Deionized water	100 ml

(b) Stacking Gel Buffer (pH 6.8)

Tris (BDH)	6.05 g
SDS (BDH)	0.04 g
Deionized water	100 ml

(c) 10% Solution of Ammonium persulphate (prepared fresh)

The stacking gel was prepared as follows.

Stacking Gel Buffer (pH 6.8)	2.5 ml
Stacking Acrylamide	1.3 ml
Deionized water	6.1 ml
Ammonium persulphate solution	0.05 ml
TEMED	0.01 ml

The above solutions were mixed gently, trying not to create any bubbles. Approximately 1 ml of the stacking gel solution was used to wash the surface of the resolving gel after the surface water had been decanted off. The mould was then filled with the stacking gel mixture to 5 mm from the top of the lower glass plate. A 15 tooth comb was inserted to a depth of 25 mm and the gel left until polymerized (40-70 minutes). After the stacking gel had polymerised the comb was removed and the wells washed out with deionised water.

Samples for SDS-PAGE separation were dissolved in sample buffer.

### (3) Sample Buffer

50mM Tris-HCl pH 6.8	1.0 ml
Glycerol (BDH)	0.8 ml
10% SDS	1.6 ml
Deionized water	3.8 ml

1 ml of the above solution was added to each tube of freeze dried protein and mixed well. Protein concentrations were measured by BCA protein assay kit (PIERCE), which is a detergent-compatible protein assay kit. The readings were compared with a standard curve prepared using serial dilutions of bovine serum albumin. Samples typically contained 300-700  $\mu\text{g}$  of protein per 1 ml of sample. Before boiling the sample for 2 minutes, 50  $\mu\text{l}$  of  $\beta$ -mercaptoethanol and 50  $\mu\text{l}$  of 0.01% Bromophenol blue were added to each 1 ml sample, to act as a tracking dye. After boiling, 50  $\mu\text{l}$  aliquots of the 1 ml

sample were placed in eppendorf tubes and either frozen at -75°C for future use, or used immediately.

Electrophoresis buffer was added to both top and bottom tanks prior to the addition of 5-20 µl samples to each well.

(4) Electrophoresis Buffer

Glycine (BDH)	28.8 g
Tris (BDH)	6.01 g
SDS	2.0 g
Deionized water	2000 ml

Electrophoresis was usually performed at a constant current of 30 mA at 4°C to avoid excessive heating, until the tracking dye was within 1-2 cm of the bottom of the gel. The molecular weight of proteins was determined by direct comparison with the migration distance of a set of molecular weight standards. The standards were Broad Range molecular weight standards (Bio-Rad) reconstituted according to the manufacturer's advice.

#### 3.6.1.4.1 Gel staining and photography

Once the tracking dye was within 2-3 cm of the bottom of the gel, the gel was removed and silver stained by a modified method described by Dunn (1993):

- (1) fixed in 50% ethanol and 10% acetic acid for 30 minutes
- (2) transferred to 5% ethanol, 1% acetic acid for 15 minutes
- (3) washed in deionised water for 20 minutes with at least 3 changes of water

- (4) soaked in a solution of 0.127 g/l of anhydrous sodium thiosulphate for 2 minutes
- (5) washed in deionised water for 20 minutes with at least 3 changes of water
- (6) soaked in 2 g/l of Silver nitrate for 20 minutes
- (7) washed in deionised water, twice for 30 seconds
- (8) placed in developer until the desired level of staining was attained  
Developer
  - 60 g/l sodium carbonate (BDH)
  - 500 µl/l of 37% formaldehyde (BDH)
  - 20 ml/l of 0.2 g/l of anhydrous sodium thiosulphate (BDH)
- (9) soaked in cold 5% acetic acid stop solution
- (10) stored in deionised water.

Gels were either photographed or scanned using a digital scanner.

### **3.6.2 Two-dimensional gel polyacrylamide electrophoresis (2D PAGE) of cell proteins**

#### *3.6.2.1 Total cell protein extraction for two-dimensional polyacrylamide gel electrophoresis (2D PAGE)*

Two-dimensional PAGE electrophoresis was used to compare the total proteins of B12 and X7 cells. Mid log phase cultures of B12 and X7 strains, grown in TSB, were centrifuged (1000 x g for 20 minutes) and resuspended to an OD<sub>600nm</sub> of 1.2 in distilled water. The cell suspension was then sonicated six times on ice for 30 seconds, with a 30 second break between each 30 second sonication, at amplitude of 7 micrometres. The cell

suspension was centrifuged (10,000 x g for 20 minutes) in order to remove cellular debris. The supernatant was mixed with cold acetone at a ratio of 1 ml supernatant per 3 ml of cold acetone and then left at -20°C for 18 hr to precipitate proteins. The supernatant/acetone mixture was then centrifuged (10000 x g for 20 minutes). The acetone was poured off and the pellet air dried to allow any residual acetone to evaporate. Fractions were then frozen at -75°C for storage.

### 3.6.2.2 *Acid glycine surface protein extraction for two dimensional polyacrylamide gel electrophoresis (2D PAGE)*

A modified acid glycine extraction described by Dubreuil *et al.* (1988) was adopted to study cell wall components and their involvement in cell attachment. Cells of strain B12 and X7 were harvested by centrifugation (1000 x g for 20 minutes) and resuspended in 0.2 M glycine hydrochloride buffer pH 2.2 (3 g cells per 100 ml). This suspension was stirred at room temperature for 30 minutes. Cells were removed by centrifugation (1000 g for 20 minutes). The supernatant was neutralized to pH 7.0 by the addition of 2 M NaOH and then dialyzed overnight, with a least five changes of DW to remove residual glycine, which could interfere with the isoelectric focusing step. The dialyzed supernatant was mixed with cold acetone at a ratio of 1 ml supernatant per 3 ml of cold acetone and the supernatant/acetone mixture left at -20°C for 18 hours to precipitate proteins in the supernatant and then centrifuged (10000 x g for 20 minutes). The acetone was poured off and the pellet air dried to allow any residual acetone to evaporate. Aliquots were then frozen at -75°C for storage.

### 3.6.2.3 *Lysozyme surface protein extraction for two dimensional polyacrylamide gel electrophoresis (2D PAGE)*

The second method employed to extract surface proteins involved incubating the cells in lysozyme and mutanolysin (Coolbear *et al.*, 1992). Cells were harvested by centrifugation (1000g for 20 minutes) and suspended in an equal volume of 50 mM Tris-HCl, pH 7.0 with 10 mM MgCl<sub>2</sub>, 24% sucrose, 50,000 units ml<sup>-1</sup> of lysozyme (Sigma) and 90 units

ml<sup>-1</sup> of mutanolysin (Sigma) and incubated for 5 hrs at 37°C. The mixture was centrifuged (10,000 x g for 20 minutes) and the supernatant mixed with cold acetone, at a ratio of 1 ml supernatant per 3 ml of cold acetone, and then stored at -20°C for 18 hours to precipitate proteins. The supernatant was then centrifuged at (10,000 x g for 20 minutes). The acetone was poured off and the pellet air dried to allow any residue acetone to evaporate off. Fractions were then frozen at -75°C for storage.

#### 3.6.2.4 *Two-dimensional polyacrylamide gel electrophoresis (2D PAGE) method*

##### 3.6.2.4.1 Isoelectric focusing

Samples for 2D PAGE were prepared by the addition of 1 ml of 6 M urea and 2% CHAPS (3-Cholamidopropyl-dimethylammonio-1-propanesulfonate) solution to each tube of acetone precipitated protein, mixed and allowed to stand for 1 hour at room temperature to allow the pellet to dissolve completely. Protein concentrations were measured by the BCA protein assay kit (Section 3.5.8). The readings were compared with a standard curve prepared using serial dilutions of BSA. Samples typically contained 600-1000 µg of protein per 1 ml of sample. Protein samples were diluted to approximately 600 µg/ml for whole cell samples and 900 µg/ml for lysozyme/mutanolysin and acid glycine samples. The remaining sample solution chemicals were then added.

Sample solution final concentration per ml of sample

2 M Thiourea (Sigma)	0.152 g
6 M Urea (BDH)	0.36 g
2.0% CHAPS (Sigma)	0.2 g
20 mM Dithiothreitol (DTT) (Sigma)	0.003 g
0.5% Biolytes pH 3-10 (Bio-Rad)	0.005 ml
Bacterial Protease Inhibitor Cocktail (Sigma)	50 µl

To rehydrate the 17 cm IPG ReadyStrip pH 3-10(IPG)(Bio-Rad), 320  $\mu$ l of sample containing 600-900  $\mu$ g/ml of dissolved protein was added to the focusing tray and the IPG strip carefully placed on top, ensuring no air bubbles were trapped beneath the IPG strip. 2 ml of mineral oil (Bio-Rad) was added over the IPG strip to prevent evaporation during the rehydration process. The IPG strip underwent passive rehydration overnight (12-16 hr) at room temperature, allowing the sample buffer and proteins to soak into the strip. After rehydration the strip was removed and excess mineral oil drained off. Paper wicks soaked in deionised water with the excess water drained off were placed at both ends of the channels covering the wire electrodes. The rehydrated IPG strip was replaced in the channels and fresh mineral oil placed over the strip. The focusing tray containing the strip was placed in a Protean IEF cell (Bio-Rad) ready for focusing. Three preset programs were executed in the overall focusing step,

- 1) Conditioning step 250 V for 15 minutes
- 2) Linear voltage ramping for 4 hr up to 10,000 V
- 3) Final focusing at 10,000 V until VH (Volt-Hours) reached 50,000-60,000 VH

The purpose of the conditioning step is to remove salt ions and charged contaminants. At each stage the current did not exceed 50  $\mu$ A per strip. Once isoelectric focusing (IEF) was completed, the strip was removed and excess mineral oil drained off, each strip was wrapped in plastic wrap, labelled and frozen at  $-75^{\circ}\text{C}$  until ready for equilibration and second dimension SDS-PAGE.

#### 3.6.2.4.2 Second dimension and strip transfer

The equilibration steps serve to saturate the IPG strip with SDS, which is required by second dimension electrophoresis to give all proteins a net negative charge. The reductant Dithiothreitol (DTT) ensures all disulphite bonds are broken. Iodoacetamide alkylates all thio groups, preventing their oxidation during electrophoresis, as well as reducing the

level of silver stain artifacts and point streaking. The strip was soaked in 10 ml of Equilibration Buffer One (EB1) for 10 minutes, with gentle agitation. After 15 minute in EB1, the strip was removed, excess liquid drained off on a paper towel and placed the in Equilibration Buffer Two (EB2) for 15 minutes with gentle agitation.

#### Equilibration Buffer One (EB1)

6 M Urea (BDH)	3.6 g
2% SDS (BDH)	0.2 g
20% Glycerol (BDH)	2.0 ml
2% w/v DTT (Sigma)	0.2 g
0.375 M Tris-HCl pH 8.8	8.0 ml
Bromophenol Blue 0.01% (Sigma)	0.02 ml

#### Equilibration Buffer Two (EB2)

6 M Urea (BDH)	3.6 g
2% SDS (BDH)	0.2 g
20% Glycerol (BDH)	2.0 ml
2.5% w/v Iodoacetamide (Sigma)	0.25 g
0.375 M Tris-HCl pH 8.8	8.0 ml
Bromophenol Blue 0.01% (Sigma)	0.02 ml

2D PAGE gels were prepared as described in section 3.6.1.4 with the following exceptions: no stacking gel was added and the resolving gel added until only 1cm of space was left at the top of the two glass plates; 2 ml of deionised water was added on top to assist in gel polymerisation. After polymerisation of the resolving gel, excess water was drained off and 0.5 ml of electrophoresis buffer added to the surface of the gel to assist in the placement of the strip onto the top of the gel. With the use of a spare gel

spacer, the strip was carefully placed on top of the gel, avoiding any air bubbles being trapped between the strip and the resolving gel surface. Molten agarose sealing solution (50°C) was laid above the strip to help fix it in place and left for 10 minutes to allow the agarose to set.

#### Agarose Sealing Solution

Electrophoresis buffer	50.0 ml
Agarose	0.5 g

Electrophoresis was carried out at 15 mA per gel for 30 minutes, before increasing the current to 20 mA per gel. Electrophoresis then continued until the tracking dye was within 1-2 cm of the bottom of the gel. During electrophoresis, the gel tank was connected to a water supply to remove excess heat.

#### 3.6.2.4.3 Gel Staining and Image acquisition

Gels were stained with SYPRO® Ruby (Bio-Rad) as per the manufacturer's instructions and scanned with a Typhoon 9400 laser scanner (Amersham, Biosciences). Scans were saved as TIFF files.

#### 3.6.2.4.4 ImageMaster 2D software

Image analysis was performed using ImageMaster 2D Platinum 6.0 software (Invitrogen). Gels were imported as TIFF files and gel images were aligned to match spots between gels. Gel alignment is achieved by identification of 3-4 landmark spots or easily identifiable protein spots on different gels to help the software with gel to gel comparisons. Spot detection was automatically performed by the software. Results were checked manually where necessary to ensure correct spot to spot matching.

### 3.6.2.5 Cytoplasmic enzyme assays

To ensure that B12 and X7 cells had not been ruptured during the cell wall proteins extraction protocol, the cytoplasmic enzymes Leucine Aminopeptidase and  $\beta$ -galactosidase were assayed. Leucine Aminopeptidase was assayed by a modified version of the method described by Schaumburg *et al.* (2004). This involved mixing 800  $\mu$ l of reaction buffer (20 mM Tris-HCl, pH 7.4) with 50  $\mu$ l of surface extract and 8  $\mu$ l L-leucine-*p*-nitroanilide (Sigma). Samples were assayed at 405 nm using a UV/visible spectrophotometer (Hitachi, Japan) after incubation for 10 minutes at room temperature, with a sonicated cell sample acting as a positive control.  $\beta$ -galactosidase was assayed by a modified method as described by Steinmoen *et al.* (2002). In the  $\beta$ -galactosidase assay, 240  $\mu$ l of reaction buffer (5 mM MgCl<sub>2</sub>, 250 mM  $\beta$ -mercaptoethanol, 50 mM KCl, 0.3 M Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O; 0.2 M NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O; 4 mg/ml *o*-nitrophenyl- $\beta$ -D-galactopyranoside (ONPG)(Sigma), pH 7.0) was added to 960  $\mu$ l of surface extract. After 20 minutes incubation at 37°C samples were assayed at 420 nm as described above, with a sonicated sample acting as a positive control.

## **3.7 Protein Identification**

### **3.7.1 Mass Spectroscopy**

To identify protein spots of interest, samples were digested with trypsin into peptides, followed by Electrospray Ionisation–Quadrupole–Time-of Flight (ESI-QUAD-TOF) analysis using the Thermo TSQ 7000 system. MS/MS data were searched against Mascot protein data base for identification using MS/MS Ion Search ([www.matrixscience.com](http://www.matrixscience.com)). This work was performed by Bioanalytical Mass Spectrometry Facility, University of New South Wales, Sydney, Australia.

### **3.7.2 Bioinformatics**

#### **3.7.2.1 BLAST searches**

The Basic Local Alignment Search Tool algorithm (BLAST), (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>) was used for searches to establish sequence identity. Nucleotide sequences were analysed for similarities to other nucleotide sequences by BlastN. BlastX was used to translate a nucleotide sequence into possible reading frames and then align these against a database of protein sequences.

#### **3.7.2.2 Sequence alignments and Phylogenetic comparison**

ClustalW2 (<http://www.ebi.ac.uk/Tools/clustalw2/index.htm>) software or CLC Sequence viewer 4.6 was used to align nucleotide or amino acid sequences for comparison. Phylogenetic analysis was performed with CLC Sequence 4.6 using Neighbour Joining with Bootstrapping analysis performed with 1000 repetitions.

### 3.7.3 Polymerase chain reaction (PCR)

The PCR reaction mixture was prepared by combining the following: 460  $\mu$ l of H<sub>2</sub>O; 40  $\mu$ l of 20X buffer (TFL buffer, Epicentre Technologies, Madison, Wisconsin, USA); 80  $\mu$ l of 25 mM MgCl<sub>2</sub>; 160  $\mu$ l of dNTP (Promega, Madison, Wisconsin, USA); 1  $\mu$ l of primer mix (Table 3.4) (Invitrogen, Carlsbad, California, USA); 10  $\mu$ l of TFL polymerase (Epicentre Technologies). The reaction mixture was dispensed (75  $\mu$ l per tube) into reaction tubes and 5  $\mu$ l of culture or gel extracted PCR product was added. The PCR reaction was conducted under the following conditions: 1 cycle of denaturation at 94°C for 3 minutes, 35 cycles of 94°C for 45 seconds, 55°C for 45 seconds and 72°C for 2 minutes (depending upon on the size of the fragment being amplified), followed by a final single step of 72°C for 5 minutes.

**Table 3.4:** Primers used in this study

Names	Sequences 5'-3"	Application
M13F	GCC AGG GTT TTC CCA GTC ACG A	Sequencing
M13R	AGC GGA TAA CAA TTT CAC ACA GCA	Sequencing
Primer 1	GGA GGC GTC CTT GAT ATG GAT AC	PCR amplification of PFL gene
Primer 1A	GGT GGC GTT CTT GAT ATG GAT AC	PCR amplification of PFL gene
Primer 2	GGA TAT TCC TCT GGA TGT TCC AT	PCR amplification of PFL gene
Primer 2A	GGA TAT TTC TCA GGA TGT TCC AT	PCR amplification of PFL gene
Primer B12 F1	TTA GAT ATT TAT ATC GAA CGC AC	Sequencing
Primer B12 R1	GCG CTC ATA ACA ATA TTT ATC GTC	Sequencing
Primer X7 F1	TTT AGC GGC GAC CCA ACG TGG	Sequencing
Primer B12 F2	CGG AGA CGA ATA ATG TCT TCG C	Sequencing
Primer B12 R2	GAA TTT GAG CCA ATC ACA TC	Sequencing
Primer X7 F2	GGC TTA GCG GTC GAC TTT GAA	Sequencing
Primer B12 R2	TGG TGT TCG CGC GAA TTT GAG G	Sequencing

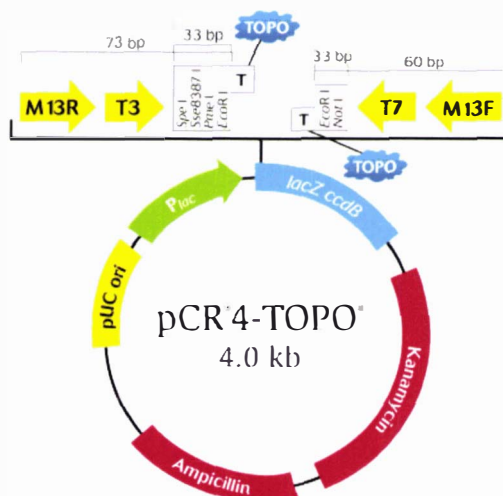
### 3.7.4 Extraction of PCR product from agarose

PCR products were separated by agarose gel electrophoresis followed by cutting the DNA bands out of the gel and extraction from agarose with the use of the QIAQuick™ gel extraction kit (Qiagen). Briefly, gels were viewed under long-wave UV lights and fragments of the appropriate size were excised with the use of a scalpel blade. Fragments were placed in a 1.5 ml Eppendorf tube and three volumes of QG buffer were added (where weight of agarose fragment in mg is equal to one volume in micro-litres). The mixture was incubated at 50°C for 10 minutes. One volume of isopropanol was added to the sample and mixed. To bind DNA, the sample was applied to a QIAQuick™ spin column and centrifuged at 12,000 x g for 1 minute. The column was washed with 750 µl of Qiagen PE buffer and centrifuged (12,000 x g for 1 minute). DNA was eluted using 50 µl of deionised water.

### 3.7.5 DNA Cloning and Transformation for DNA sequencing

#### 3.7.5.1 DNA Cloning

PCR products to be cloned were ligated into plasmid vector pCR®4-TOPO® (Invitrogen)(Fig 3.6). The TOPO TA cloning strategy allows for direct insertion of PCR amplified product containing deoxyadenosine (A) overhang to the 3' ends of PCR product. The TOPO component contains a Topoisomerase enzyme covalently bound to the plasmid vector, which is released upon the insertion of a PCR product, eliminating the need for DNA ligase. The pCR®4-TOPO® allows direct selection of recombinants via the disruption of the lethal *E.coli* gene, *ccdB*. The vector contains *ccbB* gene fused to the C-terminus of LacZα fragment. Insertion of a PCR product disrupts the LacZα- *ccbB* gene fusion permitting the growth of only positive recombinants upon transformation into TOP10 cells. Typically, 4 µl of fresh PCR product, 1 µl of salt solution (Invitrogen) were added to 1 µl of pCR®4-TOPO® plasmid. The reaction mixture was mixed gently and incubated at room temperature for 5 minutes, followed by either storage on ice if transformation were taking place the same day, or stored at -20°C for future use.



**Fig 3.6:** Plasmid vector pCR<sup>®</sup> 4-TOPO<sup>®</sup> (Invitrogen) used for cloning PCR products (taken from Invitrogen.com).

### 3.7.5.2 Transformation

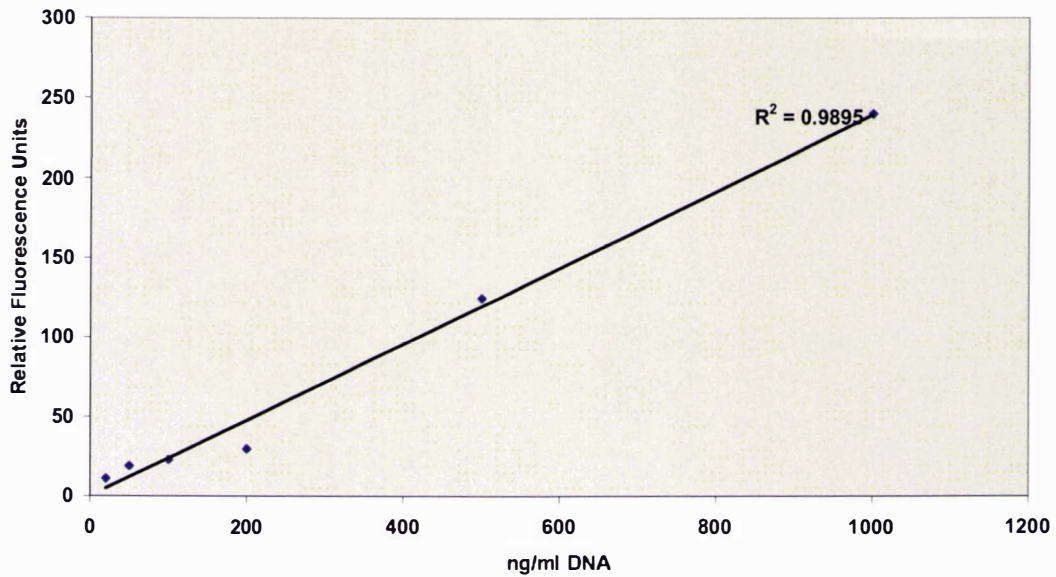
Transformation of *E.coli* One Shot<sup>®</sup> TOP 10 chemically competent cells (Invitrogen) was carried out using ligated PCR products and plasmid DNA (section 3.7.6). Vials of One Shot<sup>®</sup> TOP 10 *E.coli* cells were thawed on ice followed by the addition of 2  $\mu$ l of ligated plasmid and incubated on ice for 30 minutes. The reaction mixture was heat shocked for 30 seconds at 42°C, transferred to ice and 250  $\mu$ l of S.O.C medium (2% Tryptone, 0.5% Yeast Extract, 10 mM NaCl, 2.5mM KCl, 10 mM MgCl<sub>2</sub>, 10 mM MgSO<sub>4</sub> and 20 mM glucose)(Invitrogen) added then gently shaken for one hour at 37°C. 50  $\mu$ l was plated onto preheated (37°C) Luria-Bertani (LB) agarose plates (Merck) containing 50  $\mu$ g/ml of kanamycin (Merck) and incubated at 37°C overnight. Single colonies were transferred to 10 ml of LB broth containing 50  $\mu$ g/ml of kanamycin, grown overnight at 37°C then transferred to glycerol storage beads (Microbank , Pro-lab, Canada) for storage at -80°C.

### **3.7.6 Plasmid extraction**

Plasmids were extracted using PureLink™ Quick Plasmid Miniprep Kit (Invitrogen). Cells containing the plasmid of interest were grown overnight at 37°C in 10 ml of LB broth containing 50 µg/ml of kanamycin. Five to ten ml of cell suspension was pelleted by centrifugation (1000 x g for 20 minutes). The pelleted cells were resuspended in 250 µl of resuspension buffer (containing RNase). Cells were lysed by the addition of 350 µl lysis buffer, gently mixed and allow to stand for 5 minutes at room temperature. Cellular proteins were removed by the addition of 350 µl of precipitation buffer, gently mixed then centrifuged (12,000 x g for 1 minute). The supernatant was placed into a spin column and centrifuged (12,000 x g for 1 minute). The column was washed with 700 µl of wash buffer and centrifuged (12,000 x g for 1 minute). DNA was eluted from the column by the addition 70 µl of TE buffer followed by centrifugation (12,000 x g for 1 minute). DNA concentration was measured as described in Section 3.7.7.

### **3.7.7 DNA quantification**

DNA concentrations were measured using the DNA quantitation kit, Fluorescence assay (Sigma). The Fluorescence assay methods uses Bisbenzimidide H 33258 (Hoechst 33258), which binds to AT sequences in the minor groove of double-stranded DNA. When excited at 360 nm, the fluorescence emission at 460 nm of the dye increases at a linear rate over increasing DNA concentrations of 10 ng/ml to 10 µg/ml. A 2-10 µl aliquot of each standard or unknown sample was pipetted into 2000 µl of the dye solution. The emission at 460 nm of each sample was measured using Spectrofluorometer (RF-15-1, Shimadzu, Japan) Calibration curves were prepared against standard calf thymus DNA solutions.



**Fig 3.7:** Calibration curve for DNA estimation using Fluorescence assay kit.

### 3.7.8 DNA sequencing

DNA sequencing was performed using BigDye<sup>™</sup> fluorescent dye-labelled terminators based upon the dideoxy chain termination method (Sanger *et al.*, 1977) by the Allan Wilson Centre Genome Service at Massey University, Palmerston North. Primers used for sequencing were synthesised by Invitrogen (Table 3.4).

## CHAPTER 4.0

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### Isolation of isogenic strain X7 with reduced attachment to stainless steel from parental strain B12

#### 4.1 *Introduction*

#### 4.2 *Procedures*

##### 4.2.1 Isolation of mutant with reduced attachment to stainless steel

##### 4.2.2 Comparison of growth curves and Biochemical and RAPD profiles of B12 and X7

##### 4.2.3 Attachment of dairy and non-dairy isolates of *Anoxybacillus* and *Geobacillus* strains to stainless steel

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##### 4.3.1 Attachment of B12 and X7 to various surfaces

4.3.1.1 *Attachment of B12 and X7 to stainless steel measured by epifluorescence microscopy count*

4.3.1.2 *Attachment to stainless steel by cultures of B12 and X7 measured by impedance*

4.3.1.3 *Cumulative Percentage of Cells Collected Through Column*

4.3.1.4 *Attachment of B12 and X7 to suspended stainless steel chips*

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4.3.1.7 *Attachment of B12 and X7 cultures to plastic centrifuge tubes*

**4.3.2 Growth curves and attachment of B12 and X7**

**4.3.3 Biochemical and RAPD analysis of B12 and X7**

4.3.3.1 *RAPD comparison of B12 and X7*

4.3.3.2 *Morphology and biochemical test profiles of B12 and X7 using API 50CHB test strip*

**4.3.4 Attachment of dairy and non-dairy isolates of *Anoxybacillus* and *Geobacillus* strains to stainless steel**

**4.4 Discussion**

**4.5 Conclusion**

## 4.1 Introduction

The bacterium *Anoxybacillus flavithermus* is a common contaminant of milk powder products and is characterized as a thermophilic, spore-forming, nonpathogenic bacillus (Flint *et al.*, 2001; Parkar *et al.*, 2001). The bacteria are present naturally at low levels in raw milk (Murphy *et al.*, 1999) but can reach high levels in milk powder ( $>10^5$  CFU/g) in the final product. The growth of *A. flavithermus* in milk powder plants is believed to occur as a biofilm, where low numbers of spores or poorly cleaned contaminated surfaces result in seeding of the next manufacturing run, enabling rapid biofilm development. Bacteria can be released from the biofilm matrix, resulting in heavy contamination of the product passing by; this is sometimes referred to as the biotransfer potential of the biofilm (Wirtanen *et al.*, 1996).

The first and most essential stage in any biofilm development must be the initial attachment of bacteria to a surface, as all further biofilm development depends on the strength of the initial layer of cells. The initial attachment stage has been described as a two step process by Marshall *et al.* (1971). Van der Waals forces, electrostatic forces and hydrophobic interactions are thought to dominate the initial attachment. In the second step, production of exo-polysaccharides or specific ligands such as pili or fimbriae lock the bacteria onto the surface, requiring much greater physical or chemical energy to remove cells from the surface.

Many groups (Heilmann *et al.*, 1996; Cucarella *et al.*, 2001; Mosoni *et al.*, 2001) have created attachment-deficient mutants to gain an understanding of those aspects of the bacterial cell surface involved in initial attachment. Mutant and non-mutant strains have been compared, using physiological and/or biochemical methods. Isogenic stains are commonly defined as genetically identical or originating from the same parental strain. In analyzing phenotypic trials they have been used in medical microbiology to assign specific traits to important virulence factors in bacteria (Feng *et al.*, 2001; Rayner *et al.*, 1995). One common method successfully used in both Gram positive and Gram negative bacteria to create attachment deficient mutants is the use of transposon insertion

mutagenesis (Heilmann and Götz, 1998 and DeFlaun *et al.*, 1990). This requires a large amount of screening to identify mutants with limited ability to attach to surfaces. Other groups have recorded and studied bacteria that have undergone spontaneous changes (Iwabuchi *et al.*, 2003), or isolated bacteria from an environment where the ability of bacteria to attach to a surface may be an important survival mechanism (Jucker *et al.*, 1996). Mosoni *et al.* (2001) used natural selection within a large population of *Ruminococcus albus* to isolate strains lacking the ability to attach to cellulose.

The purpose of the present study was to isolate by natural selection a strain of *A. flavithermus* lacking the ability or having reduced ability to attach to stainless steel, from a large population of *A. flavithermus* strain B12 isolated from milk powder having a stronger affinity for stainless steel compared with non-dairy isolates of *A. flavithermus*.

## **4.2 Procedures**

### **4.2.1 Isolation of mutant with reduced attachment to stainless steel**

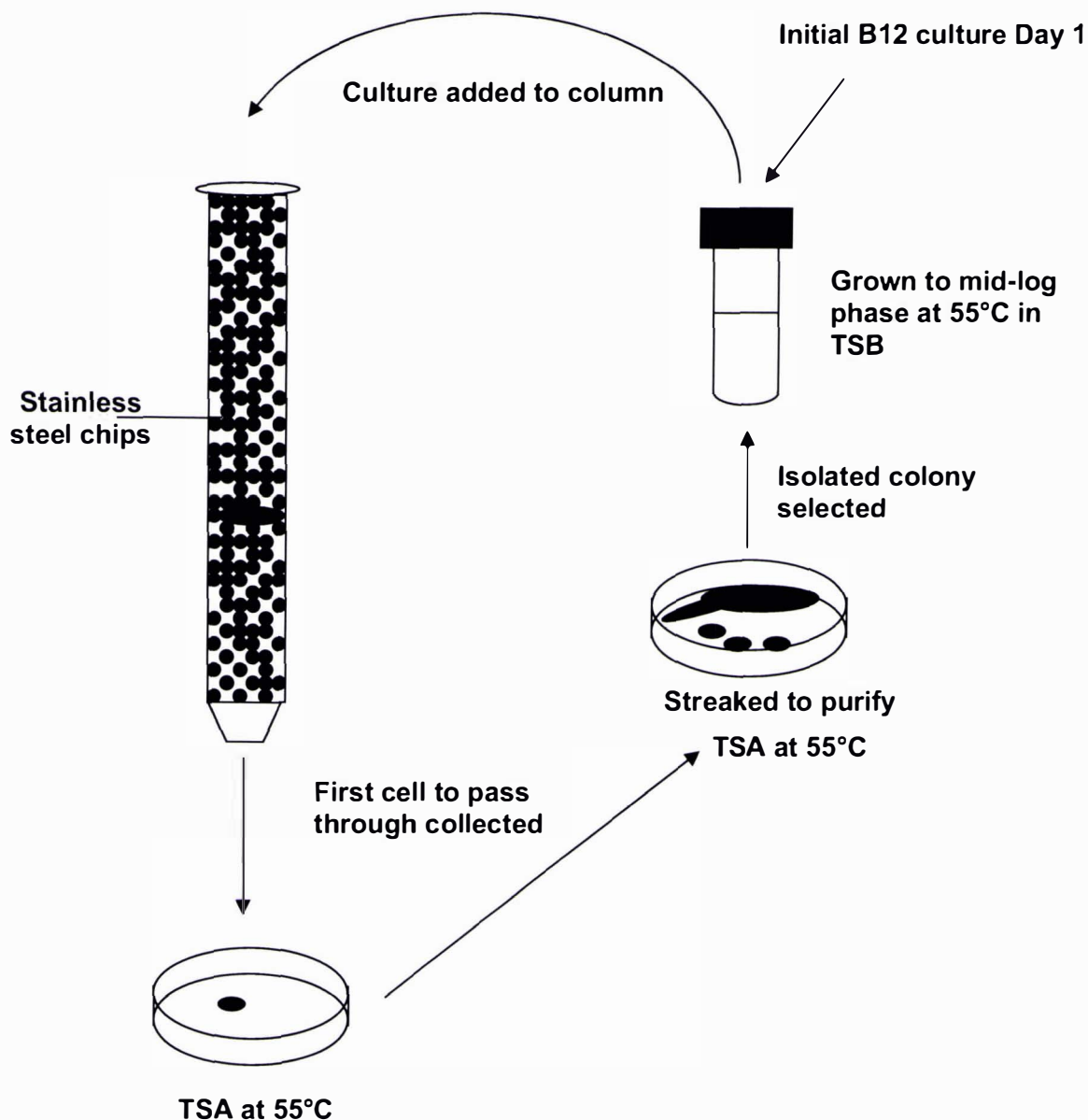
A culture of *A. flavithermus* strain B12 was added to a glass burette column filled with stainless steel chips and the first aliquot collected as described in Section 3.3. The culture was streaked to isolate a pure colony and then grown in a broth culture to mid-log phase, added to the stainless steel column and allowed to pass through again. This process was repeated six times (Fig 4.1). A culture identified as X7 in reference to the number of times the culture passed through the column, was isolated and compared with B12 on its ability to attach to stainless steel coupons as assessed by epifluorescence microscopy and impedance measurements. Attachment to glass and microtitre plates was also compared.

### **4.2.2 Comparison of growth curves and Biochemical and RAPD profiles of B12 and X7**

B12 and X7 cultures were compared by growth curves and ability to attach to stainless steel at the same time. Biochemical and RAPD profiles of B12 and X7 were compared.

### 4.2.3 Attachment of dairy and non-dairy isolates of *Anoxybacillus* and *Geobacillus* strains to stainless steel

Dairy and non-dairy isolates of *Anoxybacillus* and *Geobacillus* were compared on their ability to attach to stainless steel, using epifluorescence microscopy counts.



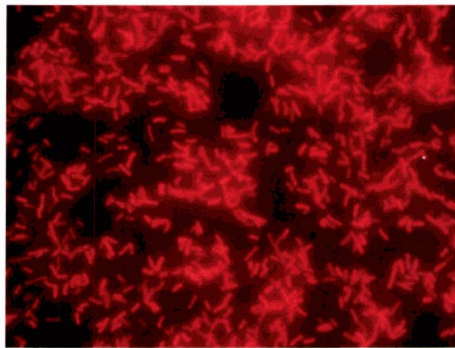
**Fig 4.1:** Summary of strategy used to isolate attachment deficient mutant from culture B12. This process was repeated six times to produce the isolate known as X7, derived by selection of natural variants of culture B12.

## 4.3 Results

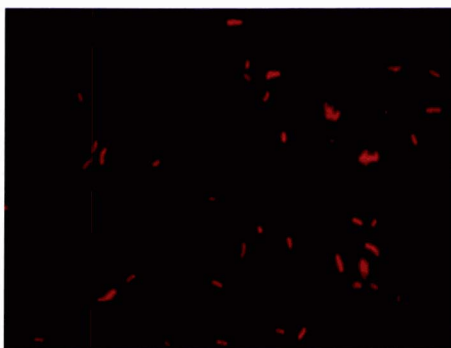
### 4.3.1 Attachment of B12 and X7 to various surfaces

#### 4.3.1.1 Attachment of B12 and X7 to stainless steel measured by epifluorescence microscopy count

The epifluorescence microscopy count method (Section 3.4.1) employed to analyse the number of bacteria attaching to stainless steel demonstrated that isolate X7 attaches to stainless steel at about  $1 \log_{10} \text{ cm}^2$  less than its parental strain B12 (Fig 4.2 and 4.3).



**Fig 4.2:** Epifluorescence microscopy count of strain B12 attached to stainless steel (x400 magnification,  $5.21 \pm 0.02 \log_{10} \text{ cells cm}^2$ )



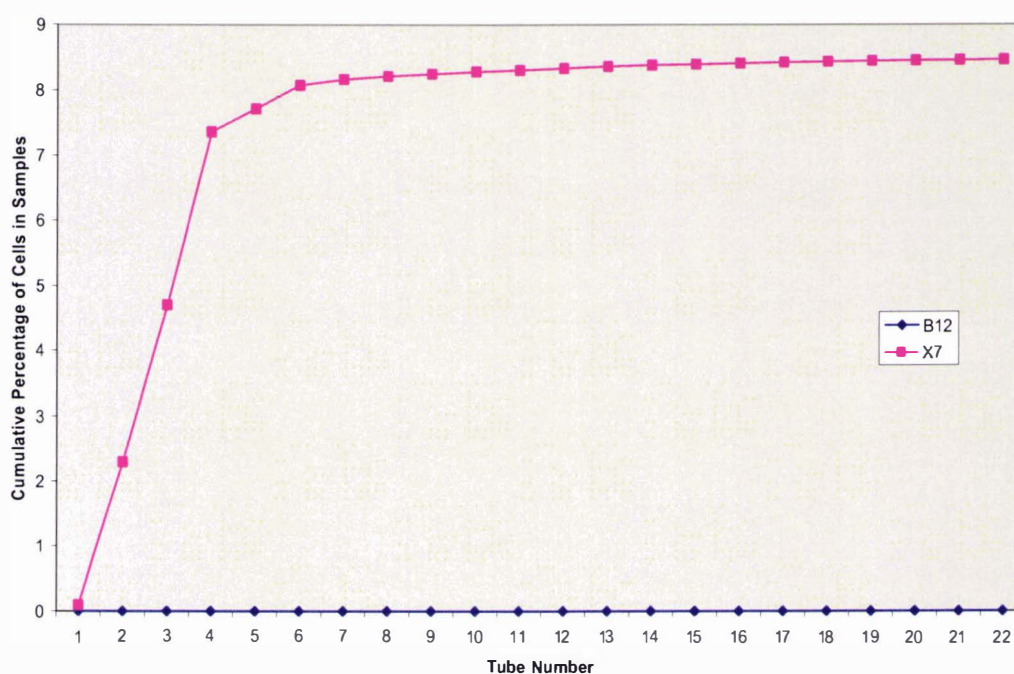
**Fig 4.3:** Epifluorescence microscopy count of strain X7 attached to stainless steel (x400 magnification,  $3.69 \pm 0.24 \log_{10} \text{ cells cm}^2$ )

#### *4.3.1.2 Attachment to stainless steel by cultures of B12 and X7 measured by impedance*

To compare the ability of B12 and X7 to attach to stainless steel coupons, impedance measurements were made using a MiniTrac 4000 (Sy-Lab, Austria). Impedance measures chemical changes that occur in a medium due to metabolic activity of the bacteria as they multiply, thus impedance measures only the live cells attached to a surface. The standard curve for impedance is shown in Section 3.4.2. Strain B12 attaches to stainless steel at levels about 10-fold higher than strain X7, with  $5.63 \pm 0.12 \log_{10}$  cells per coupon ( $2 \text{ cm}^2$ ) of B12 attaching, compared with X7 at  $4.53 \pm 0.12 \log_{10}$  cells per coupon. Errors represent the standard deviation from the mean of 3 replicates.

#### 4.3.1.3 Cumulative Percentage of Cells Collected Through Column

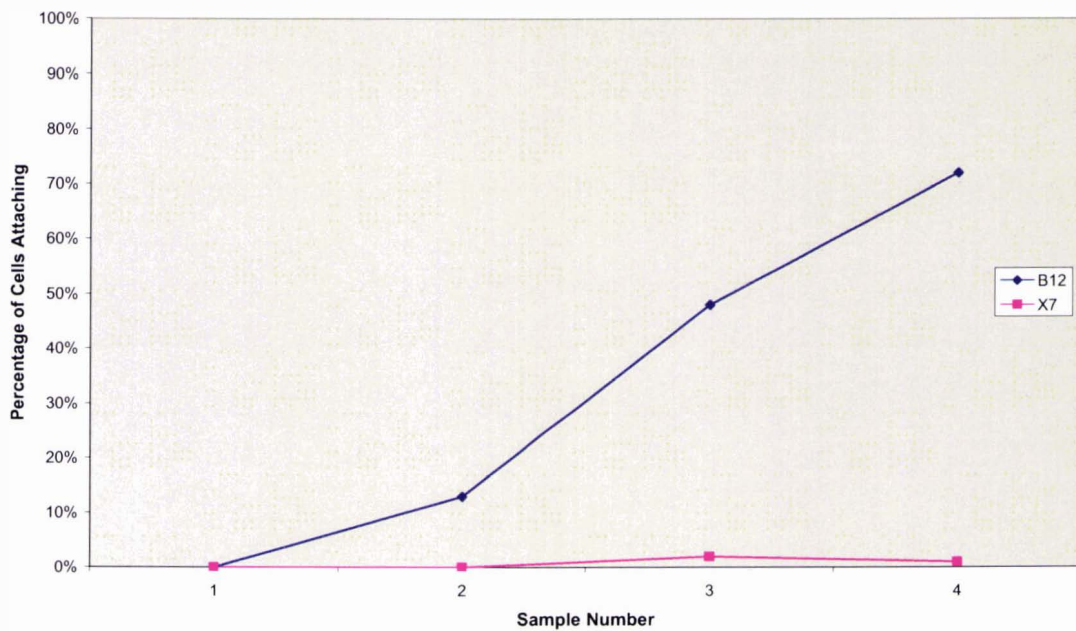
To further compare the ability of B12 and X7 to attach to stainless steel, cultures of B12 or X7 were added to a glass column containing stainless steel chips. Fractions containing eluted cells were then collected and plated out to measure the number of cells collected (Section 3.4.3). Almost 9% of the viable cells of X7 added to the column of stainless steel chips were collected after passing through the column. In contrast, less than 0.001% of the viable cells of B12 were collected after the culture was passed through the column (Fig 4.4).



**Fig 4.4:** Cumulative percentage of B12 and X7 cells collected after passing through a column filled with stainless steel chips.

#### 4.3.1.4 Attachment of B12 and X7 to suspended stainless steel chips

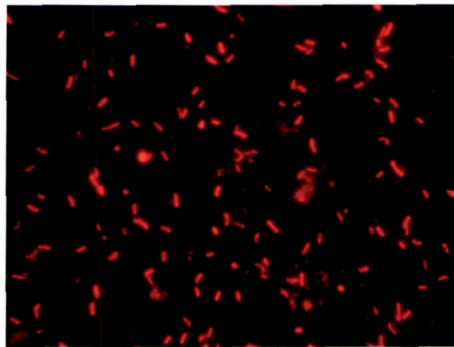
The mixing of washed stainless steel chips with planktonic mid-log phase cultures of B12 and X7 followed by plate counts of samples removed after 5, 10 and 15 minutes revealed a change in the number of cells present in the liquid phase with up to 70 % of B12 cells attached to the suspended stainless steel chips, compared with 1-2% of X7 cells attaching under the same conditions (Fig 4.5).



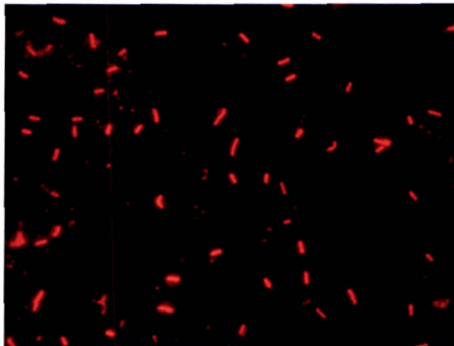
**Fig 4.5:** The attachment ability of B12 and X7 cells to suspended stainless steel chips by measuring the reduction of cells present in the suspended medium

#### 4.3.1.5 Attachment of B12 and X7 to Glass measured by epifluorescence microscopy count

The epifluorescence microscopy count method (Section 3.4.1) was employed to analyse the number of bacteria attaching to glass demonstrated that isolate X7 attaches to glass slightly less than its parental strain B12 (Fig 4.6 and 4.7).



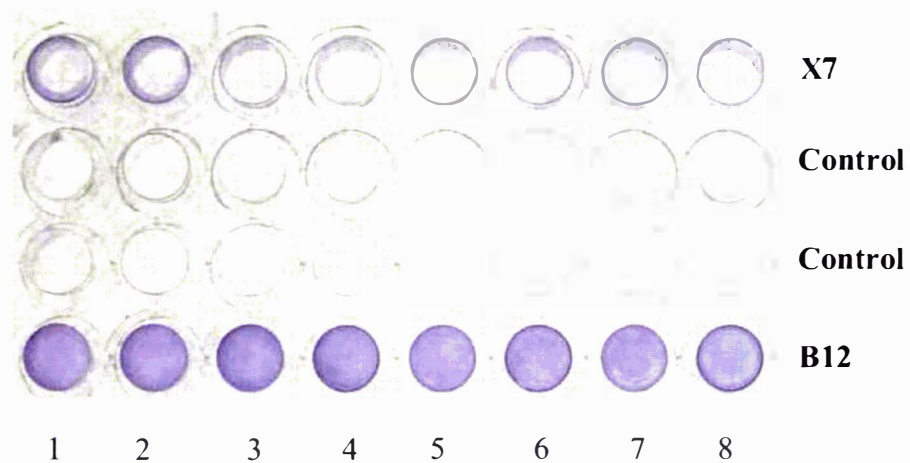
**Fig 4.6:** Epifluorescence microscopy count of strain B12 attached to glass (x400 magnification,  $4.13 \pm 0.08 \log_{10}$  cells per  $\text{cm}^2$ )



**Fig 4.7:** Epifluorescence microscopy count of strain X7 attached to glass (x400 magnification,  $3.78 \pm 0.28 \log_{10}$  cells per  $\text{cm}^2$ )

#### 4.3.1.6 Attachment of B12 and X7 cultures to polystyrene plates

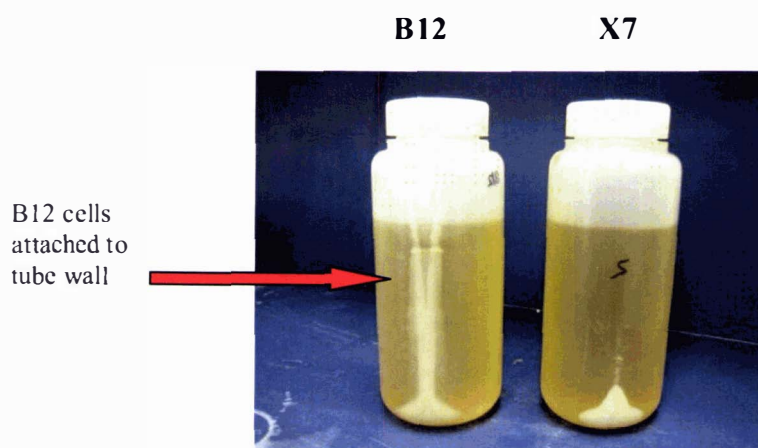
The isolate X7 demonstrated reduced attachment to a flat-bottomed polystyrene microtitre plate compared with the original B12 strain. Cultures of B12 added to flat-bottomed nontreated polystyrene microtitre plates, washed and stained with crystal violet gave OD<sub>490nm</sub> readings of between 0.25-0.29. X7 on the other hand produced optical density readings of 0.08-0.11. Figure 4.5 represents the crystal-violet stained polystyrene plates with the top row representing the X7 reduced attaching strain and the fourth row representing the original B12 strain. All wells of X7 and B12, labelled one to eight were loaded with equal volume and density of cells.



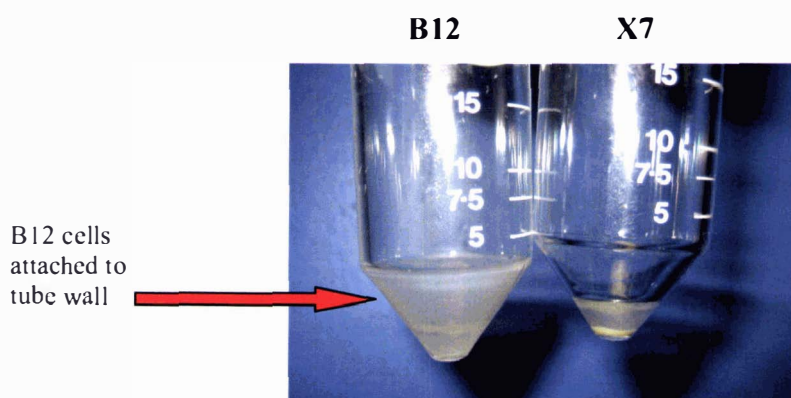
**Fig 4.8:** Attachment of B12 and X7 cultures to nontreated polystyrene microtitre plates stained with crystal violet. All wells of X7 and B12, labelled 1 to 8 were loaded with equal volume and density of cells. Control wells contained no cells.

#### 4.3.1.7 Attachment of B12 and X7 cultures to plastic centrifuge tubes

One observation made while working with cultures B12 and X7, was the centrifugation patterns produced by B12 and X7 cultures. Culture B12 appears to demonstrate a greater ability to attach to the plastic walls as indicated by the arrows in Figs 4.9 and 4.9A after centrifugation at 1000 x g for 20 minutes in 500 ml and 40 ml plastic centrifuge tubes respectively.



**Fig 4.9:** Two 500 ml plastic centrifuge tubes showing the ability of B12 to attach to the plastic wall of the centrifuge tube compared with X7, during centrifugation at 1000 x g for 20 minutes. Culture supernatant was left behind to provide contrast.



**Fig 4.9A:** Two 40 ml centrifuge tubes highlighting the ability of B12 to attach to the plastic wall of the centrifuge tube compared with X7, during centrifugation at 1000 x g for 20 minutes.

### **4.3.2 Growth curves and attachment of B12 and X7**

To compare the B12 and X7 strains the need arose to exclude any possible differences in growth curves that could be related to any metabolic differences between the two strains that could explain the observed disparity in attachment of cells to stainless steel. Consequently, growth curves of B12 and X7 (measured by optical density and viable counts) were compared over a 24 hr period, with samples being taken at 0, 2, 4, 5, 6, 7, 8, 10, 12 and 24 hr intervals. Little difference was noted between the optical density readings (Fig 4.10) of B12 and X7 over the 24 hr period, which peaked after 8 hr of growth. There was also little difference between the 2 strains in the total viable count per ml (Fig 4.11) over the same time period.

At the same time the ability of B12 and X7 to attach to stainless steel coupons was assessed using impedance measurement (MiniTrac 4000) to measure viable cell attachment rates and total cell attachment using epifluorescence microscopy. Both the impedance (Fig 4.12) and epifluorescence microscopy (Fig 4.13) demonstrated that the B12 strain had a higher rate of cell attachment to stainless steel than strain X7. It should be noted that the decreased attachment of viable B12 cells to stainless steel (Fig 4.12) after 10 hrs also coincided with a decrease in the optical density (Fig 4.10) over a similar time period.

Viable cell counts recorded low levels of viable cells after 10 hrs (Fig 4.11), but highest total numbers of cells viable and dead attaching to stainless steel was also at 10 hrs as determined by epifluorescence microscopy (Fig 4.13). Therefore, it appears that attachment of B12 and X7 to stainless steel does not require cells to be viable.

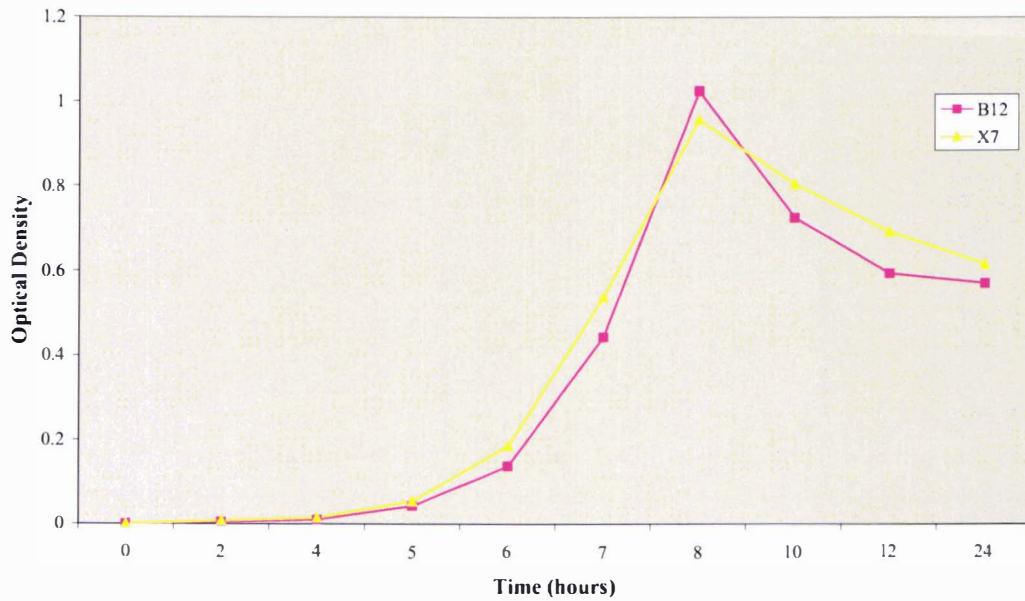


Fig 4.10: Optical density over 24 hour period of B12 and X7 cultures

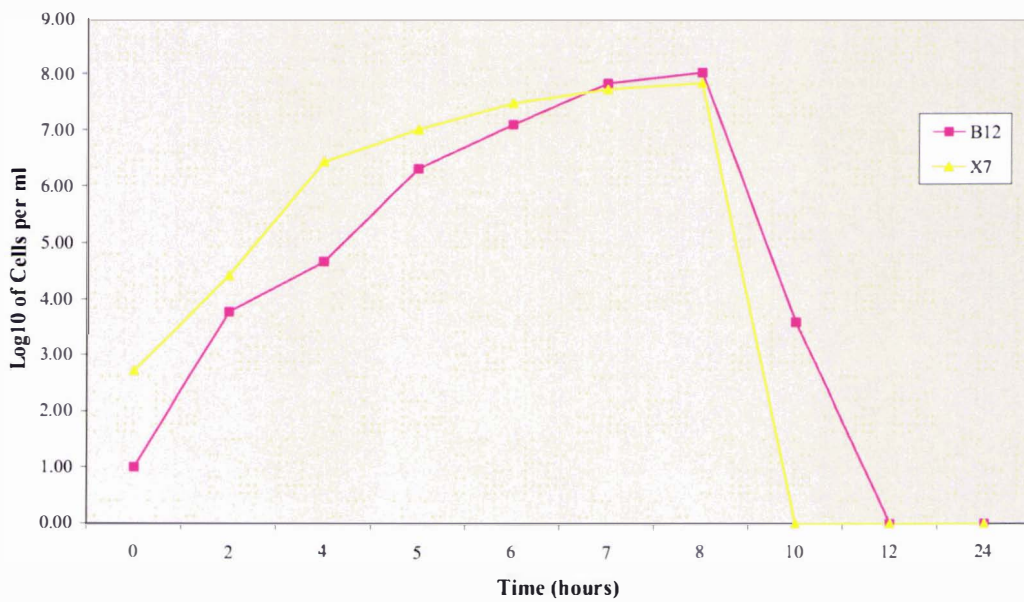
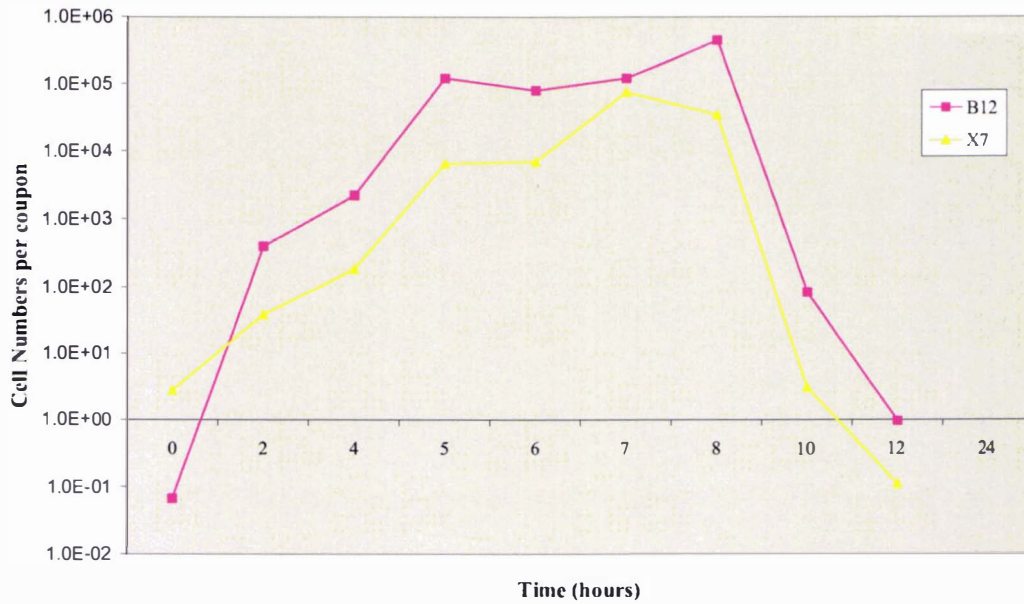
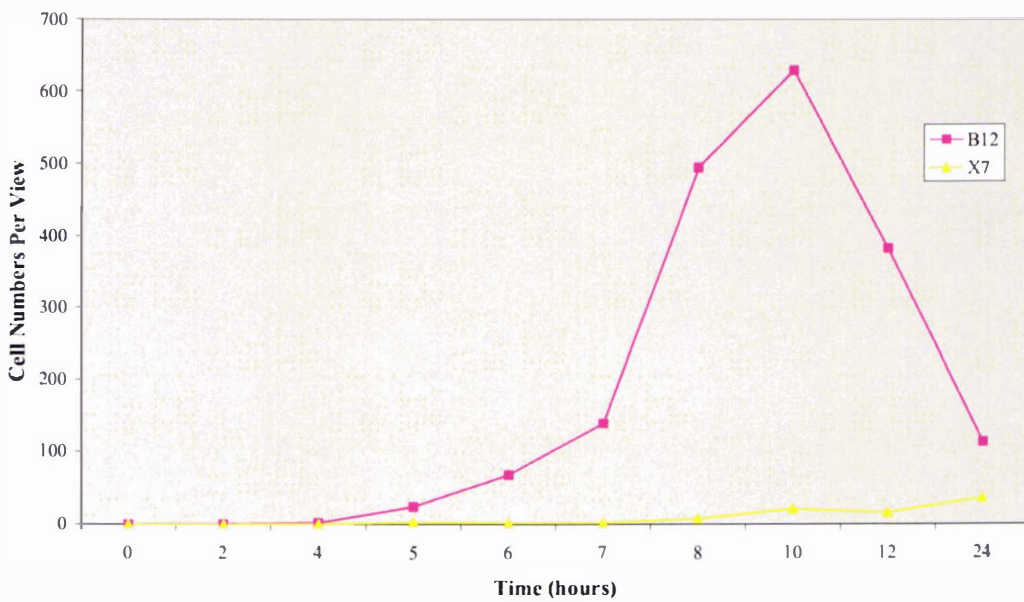


Fig 4.11: Total viable count (planktonic cells) of B12 and X7 cultures over 24 hour period



**Fig 4.12:** Impedance (MiniTrac 4000) count of B12 and X7 attached to stainless steel coupons.

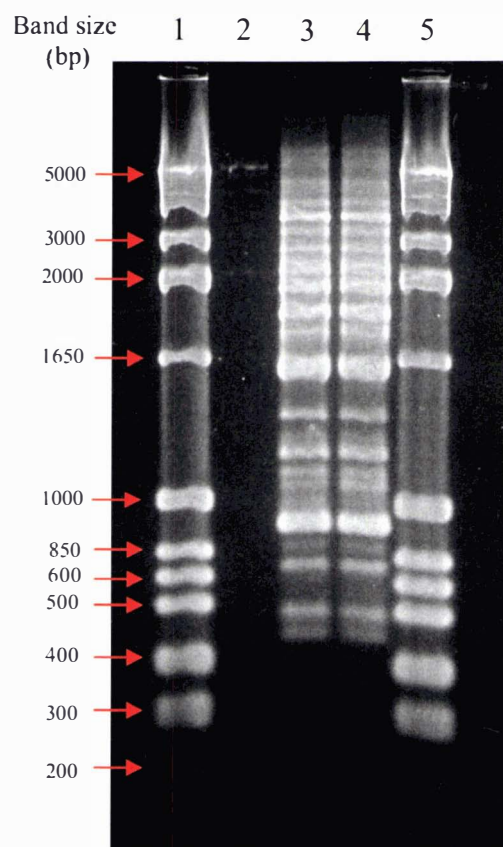


**Fig 4.13:** Epifluorescence microscopy count of B12 and X7 attached to stainless steel coupons

### 4.3.3 Biochemical and RAPD comparison of B12 and X7

#### 4.3.3.1 RAPD comparison of B12 and X7

Random Amplified Polymorphic DNA (RAPD) profiles of B12 and X7 using OPR-13 primers, as reported by Flint *et al.* (2001A), resulted in identical RAPD profiles (Fig 4.11) suggesting the very limited genetic variation between B12 and X7.



**Fig 4.14:** RAPD profiles of B12 and X7 cells. Lanes 3 and 4 contain DNA amplified from B12 and X7 respectively. Lanes 1 and 5 both contain 1 kb + ladder (Invitrogen). Lane 2 contains negative control.

#### 4.3.3.2 Morphology and biochemical test profiles of B12 and X7 using API 50CHB test strip

Biochemical profiles of B12 and X7 served two purposes in this study, firstly to confirm that X7 is not an environmental contaminant with a very different biochemical pattern. The second purpose was to identify any biochemical differences that could be related to the reduced attachment phenotype of X7. Cultures of X7 grown on TSA medium produced colonies that appeared to be identical to those of B12. Both strains observed under the microscope were Gram positive sporeforming rods similar in size, 2-5µm long at 55°C. The two isolates were tested using the API 50 CHB biochemical test kits (LaBalme, Les Grottes, France) and produced identical biochemical profiles (Table 4.1)

**Table 4.1** Biochemical test profiles of B12 and X7 cultures

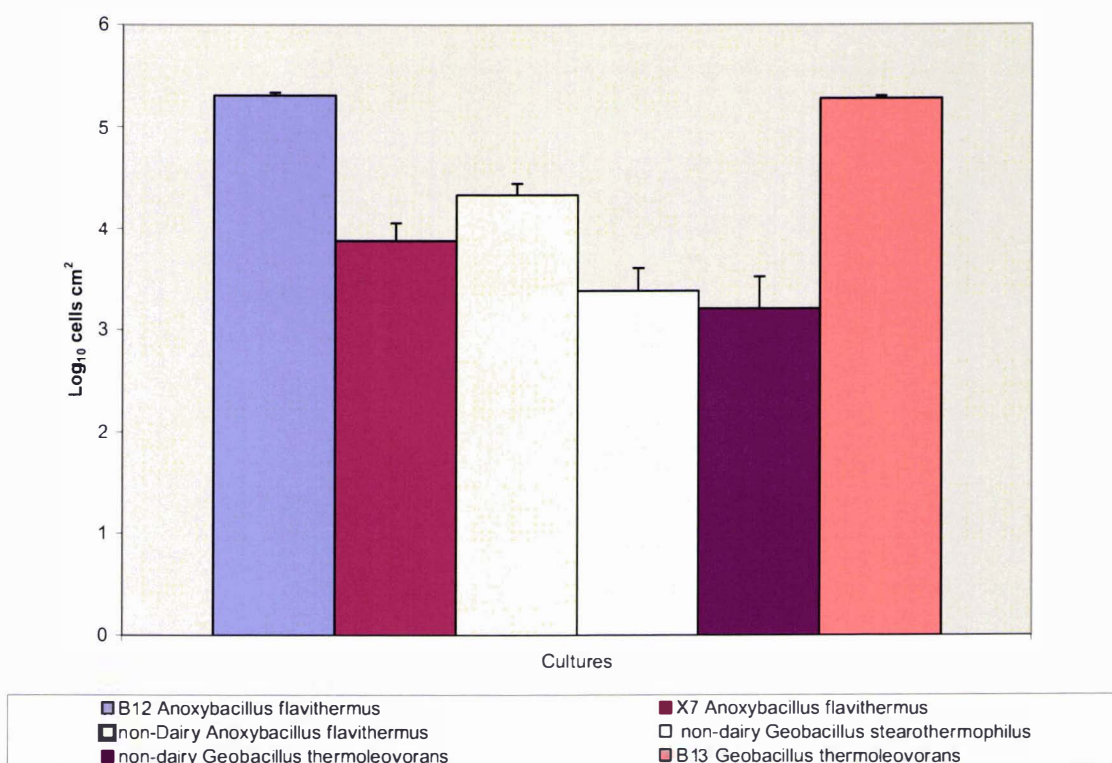
Test	Culture or isolate	
	B12	X7
Glycerol	-	-
Erythritol	-	-
D-Arabinose	-	-
L-Arabinose	-	-
Ribose	-	-
Amidon	-	-
β-Methyl-d-Xyloside	-	-
Galactose	+	+
Glucose	+	+
Fructose	+	+
Mannose	+	+
Sorbose	-	-
Rhamnose	-	-
Dulcitol	-	-
Inositol	-	-
Mannitol	-	-
Sorbitol	-	-
α-Methyl-D-Mannoside	-	-
α-Methyl-D-Glucoside	-	-
N-Acetyl-glucosamine	-	-
Amygdalin	-	-

**Table 4.1** Biochemical test profiles of B12 and X7 cultures (*continued...*)

Arbutin	-	-
Esculin	+	+
Salicin	-	-
Cellobiose	-	-
Maltose	+	+
Lactose	+	+
Melibiose	-	-
Sucrose	+	+
Trehalose	-	-
Inulin	-	-
Melezitose	-	-
Raffinose	-	-
Starch	+	+
Glycogen	-	-
Xylitol	-	-
Gentiobiose	-	-
D-Turanose	+	+
D-Lyxose	-	-
D-Tagatose	-	-
D-Fructose	-	-
L-Fructose	-	-
D-Arabitol	-	-
L-Arabitol	-	-
Gluconate	-	-
2-Keto-Gluconate	-	-
5-Keto-Gluconate	+	+

#### 4.3.4 Attachment of dairy and non-dairy isolates of *Anoxybacillus* and *Geobacillus* strains to stainless steel

To compare the ability of dairy and non-dairy isolates to attach to stainless steel, several dairy and environmental isolates of *Anoxybacillus* and *Geobacillus*, including B12 and X7 were assessed for their ability to attach to stainless steel as outlined in section 3.4.1. Both dairy strains of *A. flavithermus* (B12) and *Geobacillus thermoleovorans* (B13) attach at the rate of approximately 5-5.5 log<sub>10</sub> cells cm<sup>2</sup>, on the other hand the non-dairy isolates attach at one or more log values lower per cm<sup>2</sup>. The X7 variant of B12 *A. flavithermus* has attachment ability to stainless steel similar to that of non-dairy isolates.



**Fig 4.15:** Attachment of various *Anoxybacillus* and *Geobacillus* strains to stainless steel coupons. Error bars represent the standard deviation from the mean of 2 replicates.

#### 4.4 Discussion

In an attempt to gain greater understanding of the attachment process of milk powder isolate *A. flavithermus* (B12) to stainless steel, it was considered important to select for an isolate or mutant of B12 demonstrating reduced ability to attach. In the process of identifying what surface characteristics are important in bacterial attachment to surfaces, Hermansson (1999) considered comparisons of an isogenic strain with a parental strain may result in more consistent results and consequently limit surface heterogeneity common when comparing between different species or strains from different environments. A selection process of passing a culture through a column of stainless steel chips was used to isolate a spontaneous isogenic mutant or variant strain of culture B12 with reduced ability to attach to stainless steel. The process was repeated six times with the first fraction collected that contained a viable bacterium cultured and then the isolate was recultured and placed back into a cleaned and sterilized column for another round. The concept of isolating by natural selection a spontaneous mutant with reduced attachment to a substrate has been successfully reported before by Mosoni *et al.* (2001) studying *Ruminococcus albus* attachment to cellulose and by Kolenbrander (1982) investigating *Actinomyces* spp co-aggregation. A more common and powerful method reported by many groups to produce attachment or adhesion deficient mutants is to subject the bacteria to transposon insertion mutagenesis (Heilmann and Götz, 1998; Cucarella *et al.*, 2001; Espinosa-Urgel *et al.*, 2000). However, transposon insertion mutagenesis was not considered for this work due to the lack of a stable transposon suitable for thermophilic bacilli. The isolate produced after seven times through the column in this study was labelled X7, in reference to the number of times the culture passed through the column. Further work on X7 was undertaken to identify any possible phenotypic changes in relation to attachment compared with its parental strain B12.

Epifluorescence microscopy was initially used to determine the ability of X7 to attach compared with its parental strain B12 using stainless steel coupons as the substratum, as previously reported by Flint *et al.* (2000) and Parker *et al.* (2001). The X7 isolate consistently demonstrated on the order of  $1 \log_{10} \text{ cm}^2$  reduction in attachment to stainless

steel compared with the original B12 strain (Fig 4.2 and 4.3). Attachment of cells to stainless steel as measured by impedance, which measures only viable cell attachment, also demonstrated that X7 attaches about  $1 \log_{10} \text{ cm}^2$  less than its parental strain B12. One method to compare attachment rates of B12 and X7 populations utilized a glass column filled with stainless steel chips, measuring the number of cells that were able to pass through the column compared with the original number of cells added. This allows a comparison of the attachment ability of B12 and X7 as a population. Again the X7 isolate displayed its reduced ability to attach to stainless steel with about 9% of X7 population added being collected after passage through the column, compared with B12, where less than 0.001% of the added population was collected (Fig 4.4). Another method measured the percentage of cells attaching to suspended stainless steel chips. Samples of suspending medium were removed at 5, 10 and 15 minute periods and the number of cells remaining compared with the number of cells present before the addition to the stainless steel chips. In this case 72% of B12 cells attached to the stainless steel chips compared with less than 1% of X7 cells (Fig 4.5).

To compare the attachment rates of B12 and X7 to a variety of surfaces, the ability of bacterial cells to attach to glass and polystyrene microtitre plates was investigated. Results from the attachment of B12 and X7 to glass suggest X7 attaches slightly less than its parental strain B12. The microtitre plate method has been used by several groups (Ziebuhr *et al.*, 1999; Loo *et al.*, 2000 and Arrizubieta *et al.*, 2004) in the past to identify isolates that demonstrate a reduced ability to attach to surfaces. The 96 well microtitre plate method can accommodate simultaneous tests on multiple samples. The microtitre plate method also produced results supporting the confocal fluorescence microscopy evidence that the X7 isolate has a reduced ability to attach to surfaces compared with B12 (Fig 4.8). An interesting and unexpected observation was that centrifuged cultures of X7 formed a small pellet at the bottom of the tube, compared with culture B12 which appeared to attach to the side of the tube at a higher rate than X7 (Figs 4.9 and 4.9A).

The results comparing the attachment abilities of B12 and X7 to different substrates suggest the differences in the attachment ability of B12 and X7 is universal to multiple

surfaces and not specific to attachment to stainless steel. Iwabuchi *et al.* (2003) reported that the addition of extracellular polysaccharide (EPS) from *Rhodococcus* EPS producing strain to non-EPS producing strains of *Rhodococcus* resulted in reduced attachment to multiple surfaces. In addition, the more hydrophobic spores of *Bacillus cereus* demonstrated a greater ability to attach to multiple surfaces than the less hydrophobic spores from *Bacillus subtilis* (Faille *et al.*, 2002). Thus changes in the surface characteristics of a bacteria can modify attachment to multiple surfaces

In order to eliminate the possibility that differences in growth rate and/or total number of cells are responsible for the observed reduced attachment characteristic of X7, the growth characteristics of X7 and B12 were compared. No obvious differences between the Optical Density and plate counts over the different growth phases of B12 and X7 cultures were noted (Fig 4.10 & 4.11), but at the same time throughout the various growth phases of X7, a marked reduced ability of X7 to attach to stainless steel compared with culture B12 was noted. Of interest is the almost total non-viability of the cultures after 12 hrs of growth (Fig 4.11). This is probably due to the decrease in the pH of the medium, which was pH 5.6 in TSB after 24 hrs. Also, the maximum cell attachment measured by epifluorescence microscopy peaked at 10 hrs (Fig 4.13) suggesting that the cell attachment process is not influenced by cell viability, because by 10 hrs cell viability had reduced by several logs. The reduction in attachment of B12 shown in Fig 4.13 and 4.12 generally coincides with a reduction in the optical density of the culture shown in Fig 4.10 and is most probably associated with cell lysis as the culture enters the death phase of its growth curve.

Analysis using API 50CHB biochemical test kits (Table 4.1) and RAPD (Fig 4.14) for B12 and X7 were unable to distinguish between the cultures, suggesting that the only major difference between them is the reduced ability of X7 to attach to surfaces compared with its parent strain B12. API 50CHB biochemical analysis was utilised for two purposes, the first to make sure that the X7 isolate was not a contaminant unrelated to B12. The second purpose was to identify any biochemical changes demonstrated by X7 compared with its parental strain B12 that could be related to the lesser ability of X7 to

attach to surfaces. RAPD analysis of B12 and X7 cultures using OPR-13 primers as reported by Flint *et al.* (2001A) and Ronimus *et al.* (2003) resulted in identical RAPD profiles, providing evidence of the close genetic association between B12 and X7.

A comparison of the ability of dairy and non-dairy isolates of *A. flavithermus* and *Geobacillus thermoleovorans* to attach to stainless steel using epifluorescence microscopy resulted in both dairy isolates demonstrating a greater ability to attach to stainless steel compared with non-dairy isolates (Fig 4.15). This observation raises the suggestion that the dairy isolates may have evolved within the dairy factory to a phenotype with higher affinity to stainless steel. Bakker *et al.* (2004) concluded that bacteria isolated from a given niche may well have adapted to that particular niche over time through selective pressures, including environmental detachment forces such as cleaning regimes.

## **4.5 Conclusion**

The selection process to produce a mutant of B12 with lower ability to attach to stainless steel resulted in the successful isolation of a strain labelled X7. Strain X7 demonstrated a lower ability to attach to stainless steel coupons as measured by epifluorescence microscopy and impedance. X7 also displayed a lower capacity to attach to stainless steel chips and a lower ability to attach to polystyrene microtiter plates.

The growth curves of B12 and X7 were very similar and do not appear to explain the differences in attachment.

Biochemical and RAPD analysis could not distinguish between B12 and X7, strongly suggesting that the natural selection process successfully resulted in the isolation of a mutant strain of B12 that demonstrates a lower ability to attach to surfaces than the parental strain.

Lastly, one other key aspect of mutant X7 is that it is a stable phenotype that can be sub-cultured and duplicate the same phenotype.

### Cell Surface Properties of B12 and X7

#### 5.1 *Introduction*

#### 5.2 *Procedures*

#### 5.3 *Results*

##### 5.3.1 **Transmission Electron Microscopy of B12 and X7 cells**

##### 5.3.2 **Supernatant exchange of B12 and X7 and the effect on attachment of B12 and X7 to stainless steel coupons**

##### 5.3.3 **Surface Charge of B12 and X7**

###### 5.3.3.1 *Zeta potential*

###### 5.3.3.2 *Electrostatic Interaction Chromatography*

###### 5.3.3.3 *Effect of pH on the attachment of B12 and X7 to stainless steel coupons*

##### 5.3.4 **Hydrophobicity of B12 and X7**

###### 5.3.4.1 *Determination of cell surface hydrophobicity using MATH (Microbial Adhesion to Hydrocarbons).*

###### 5.3.4.2 *Determination of cell surface hydrophobicity using HIC (Hydrophobic Interactive Chromatography).*

###### 5.3.4.3 *Attachment of B12 and X7 to glass and Sigmacote coated glass.*

**5.3.5 Attachment of B12 and X7 cells to stainless steel coupons following disruption of surface proteins by various methods.**

**5.3.6 Surface Carbohydrates**

*5.3.6.1 The effect of Acidified Sodium metaperiodate treated cells and effect on attachment to stainless steel coupons.*

*5.3.6.2 Analysis of extracellular polysaccharide (EPS) from B12 and X7.*

**5.3.7 Effect of DNase I on attachment of B12 and X7 to stainless steel coupons.**

**5.3.8 Effect of various treatments on surface charge of B12 and X7 as measured by Electrostatic Interaction Chromatography.**

**5.3.9 Effect of various treatments on hydrophobicity of B12 and X7 as measured Microbial Adhesion to Hydrocarbons.**

**5.4 Discussion**

**5.5 Conclusion**

## 5.1 Introduction

The interaction between a bacterial surface and any substratum is mediated by multiple attachment mechanisms. Factors involved may include electrostatic interactions (Jucker *et al.*, 1996), cell surface hydrophobicity (Husmark & Ronner, 1992; van Loosdrecht *et al.*, 1987A), cell surface proteins (Flint *et al.*, 1997A; Cucarella *et al.*, 2001; Heilmann *et al.*, 1996) and surface polysaccharides (Fletcher & Floodgate, 1973; Lewis *et al.*, 1989). Specific surface structures, such as flagella, may also be involved in the attachment process (Klausen *et al.*, 2003). Other aspects not related to the genetic make up of the individual bacterial cell, but which can influence attachment rates, include convective mass transport (Yang *et al.*, 1999), nutritional conditions (O'Toole & Kolter, 1998), pH (Poortinga *et al.*, 2001), ionic strength (van der Wal *et al.*, 1997; Cowell *et al.*, 1998) and even cell preparation protocols (Pembrey *et al.*, 1999).

The physico-chemical characteristics of the substratum such as surface free energy, surface charge, hydrophobicity and roughness (Pereni *et al.*, 2006) as well as adsorption of organic molecules or conditioning film (Spicer & Gilmour, 1985) are also thought to play a role in bacterial attachment. Several, or all, of these mechanisms must act in some way to mediate attachment of bacterial cells; the problem remains the difficulty of isolating each individual factor that may be involved in attachment and then being able to manipulate each factor to understand its role in the attachment process.

In order to gain a greater understanding of attachment process, cell surface characteristics of the parent strain B12 were compared with the isogenic mutant strain X7 isolated from a B12 culture, having a phenotype demonstrating reduced ability to attach to surfaces. Hermansson (1999) suggested that isogenic mutant strains might be best employed in relating cell surface characteristics to cell attachment, thus avoiding species-to-species heterogeneity of the cell surface. Characteristics to be studied for their effects on attachment include surface charge; hydrophobicity; pH; surface proteins, carbohydrates and DNA.

## 5.2 Procedures

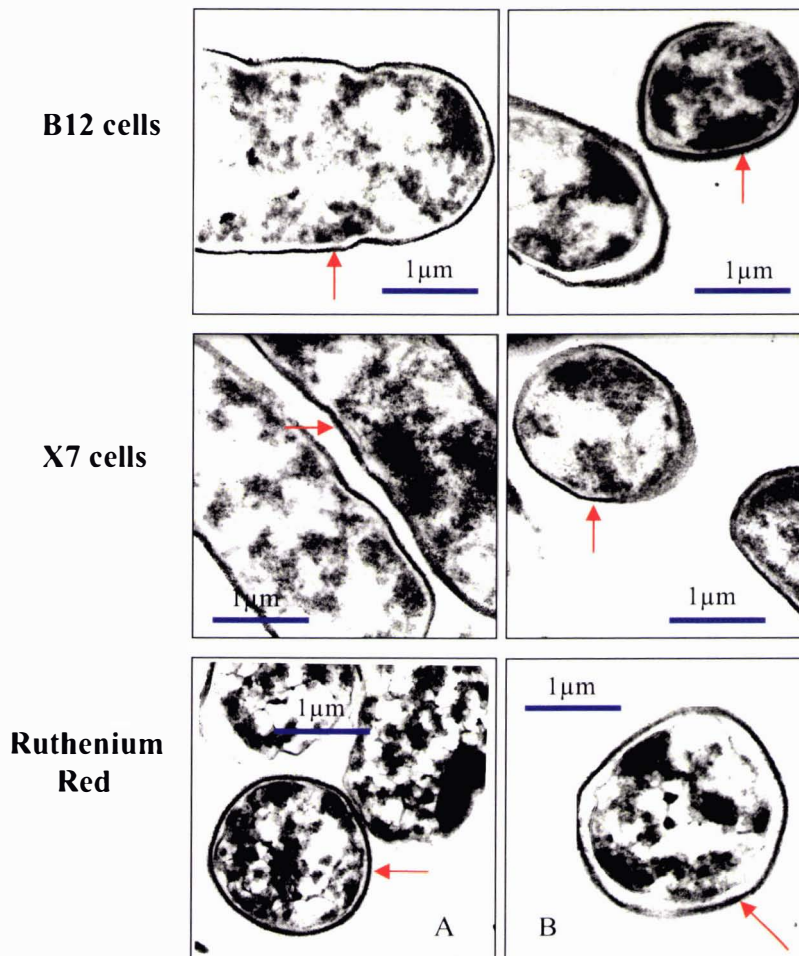
1. To conduct TEM investigations on B12 and X7 cells to observe any physical differences in surface between the two strains, unstained and stained with ruthenium red (refer section 3.5.3)
2. To identify any components present in B12 and X7 culture supernatant that may hinder or increase attachment by mixing B12 cells with X7 supernatant and X7 cells with B12 supernatant and then monitoring the ability of each culture to attach (refer section 3.5.4).
3. To measure the surface charge of B12 and X7 cells.
  - (a) Measure zeta potentials of B12 and X7 cells over pH range 2 to 8, using ZetaSizer electrophoretic mobility measurements (refer section 3.5.6.1).
  - (b) Measure surface charge of B12 and X7 cells by Electrostatic Interaction Chromatography (EIC) using anionic (Dowex AG 1-8x 100-200 mesh) or cationic (Dowex AG 50W-X8 100-200 mesh) exchange resins (refer section 3.5.6.2).
4. To observe the effect of pH on attachment of B12 and X7 to stainless steel coupons (refer section 3.5.6.3)
5. To determine cell surface hydrophobicity of B12 and X7 using MATH and HIC (refer sections 3.5.5.1 and 3.5.5.2).
6. To observe the effect of increased surface hydrophobicity on the attachment of B12 and X7 compared with uncoated glass by using Sigmacote coated glass (refer section 3.5.5.3).

7. To determine the influence of surface protein removal on the attachment of B12 and X7 to stainless steel coupons (refer section 3.5.9)
8. To determine the influence of polysaccharide removal on the attachment of B12 and X7 to stainless steel coupons and to determine the amount of EPS produced by each strain, in addition to comparing the monosaccharide composition of isolated EPS by HPLC (refer sections 3.5.10, 3.5.11 and 3.5.12).
9. To determine the influence of surface DNA removal on the attachment of B12 and X7 to stainless steel coupons (refer section 3.5.13).
10. To study the effect of removal of cell surface proteins and EPS on surface charge and hydrophobicity of B12 and X7.

## 5.3 Results

### 5.3.1 Transmission Electron Microscopy of B12 and X7 cells

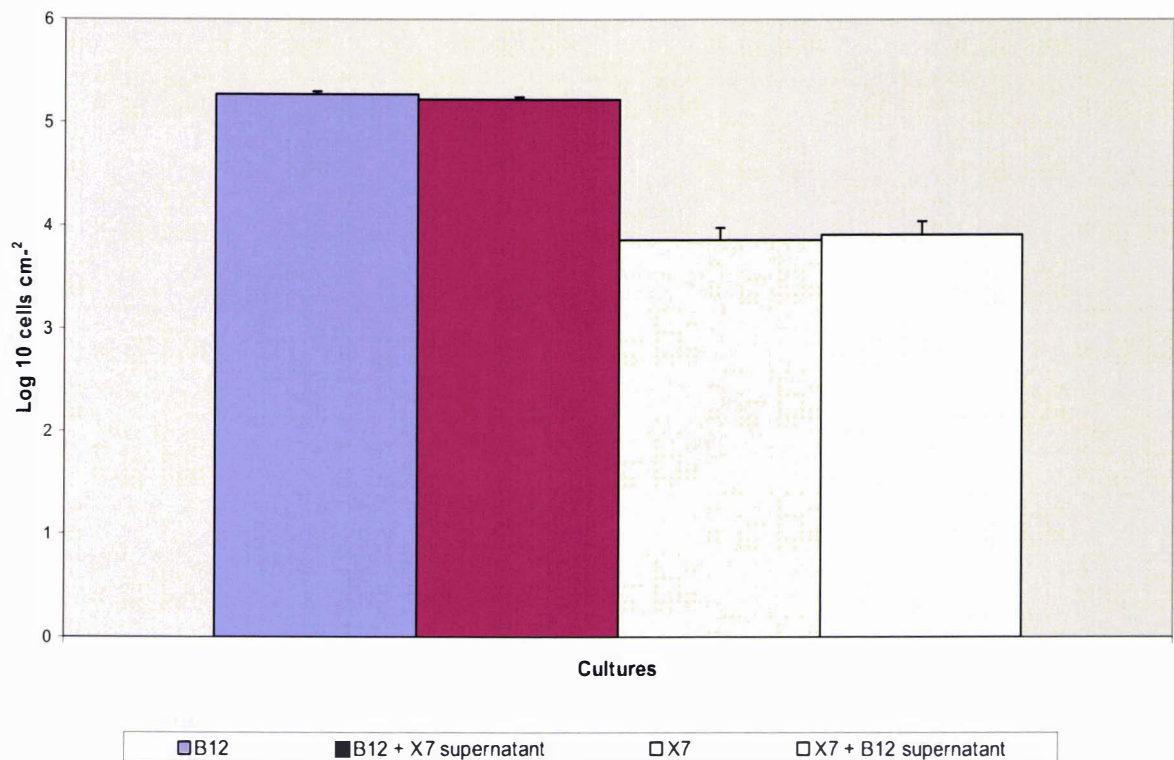
Transmission electron micrograph images of intact B12 and X7 cells and Ruthenium red (anionic polysaccharide stain) stained cells of B12 and X7 were similar with no obvious structural differences in the cell wall, as highlighted by the red arrows in Fig 5.1.



**Fig 5.1:** Transmission electron micrographs of intact B12 and X7 cells and Ruthenium red stained cells of B12 (labelled A) and X7 (labelled B). Red arrows point to outer layer of cell wall.

### 5.3.2 Supernatant exchange and the effect on attachment of B12 and X7 to stainless steel coupons.

The effect of treating centrifuged X7 cells with B12 culture supernatant (cells removed by centrifugation) and treating centrifuged B12 cells with X7 culture supernatant was investigated. After mixing the cells and supernatant fluids, the suspensions were incubated for 20 minutes at 55°C. The ability of each strain to attach to stainless steel was assessed by epifluorescence microscopy (Section 3.4.1). Incubation of X7 cells in B12 culture supernatant and B12 cells in X7 culture supernatant resulted in no obvious change in the number of the cells attached to stainless steel.

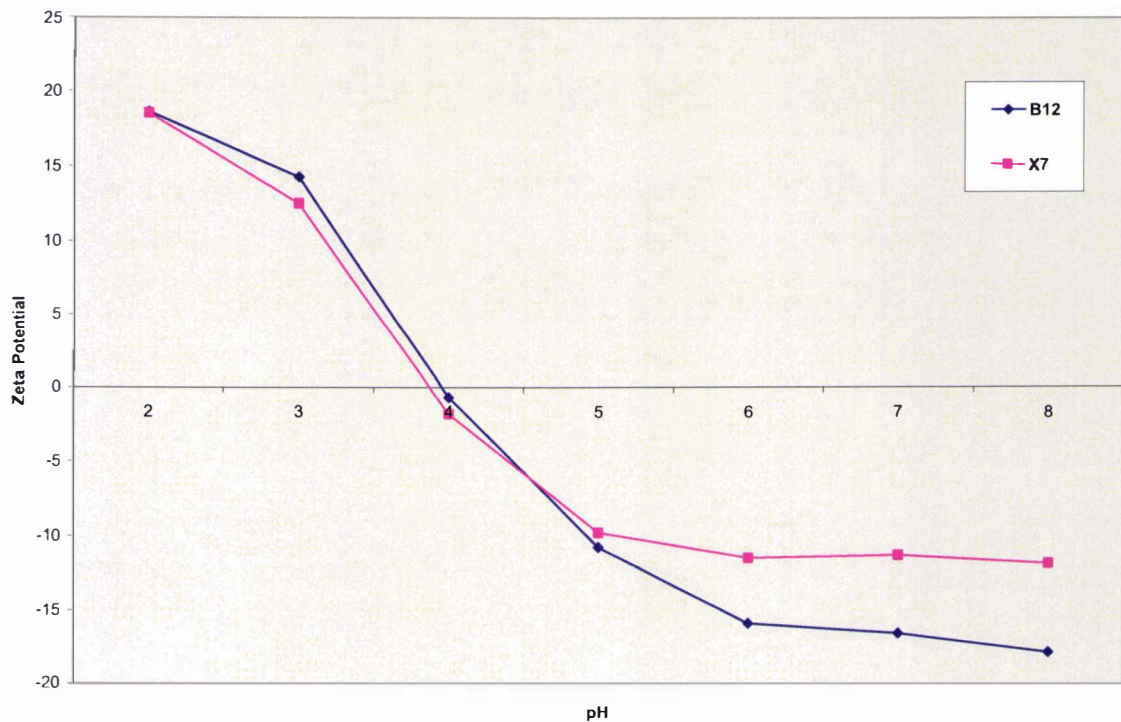


**Fig 5.2:** Supernatant exchange of B12 and X7 and the effect on attachment to stainless steel coupons. Error bars represent the standard deviation from the mean of 3 replicates.

### 5.3.3 Surface Charge of B12 and X7 cells

#### 5.3.3.1 Zeta potential

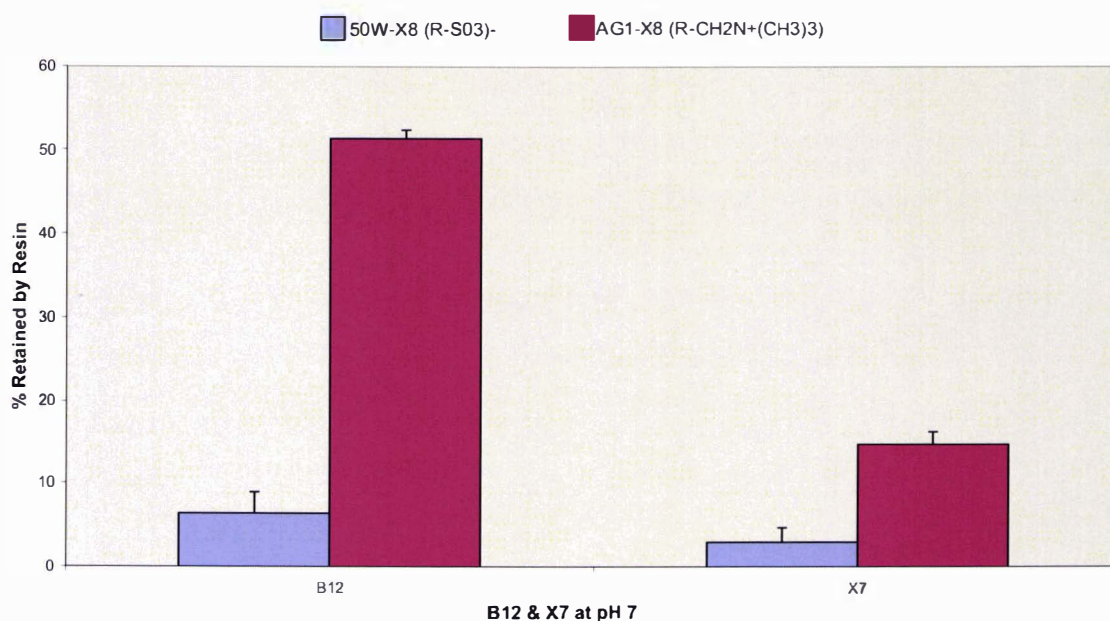
The electrophoretic mobility of strains B12 and X7, measured at pH values between 2 and 8 and expressed as zeta potentials, are shown in Fig 5.3. Both strains show a decrease in zeta potential with increasing pH, representative of the pH-dependent charge at the bacterial surface. Both strains have a similar isoelectric point of about pH 4, but strain B12 has a lower zeta potential (greater negative charge) than X7 over the pH range 6-8.



**Fig 5.3:** Zeta potentials of strains B12 and X7 as a function of pH. Error bars represent the standard deviation from the mean of 3 replicates.

### 5.3.3.2 Electrostatic Interaction Chromatography

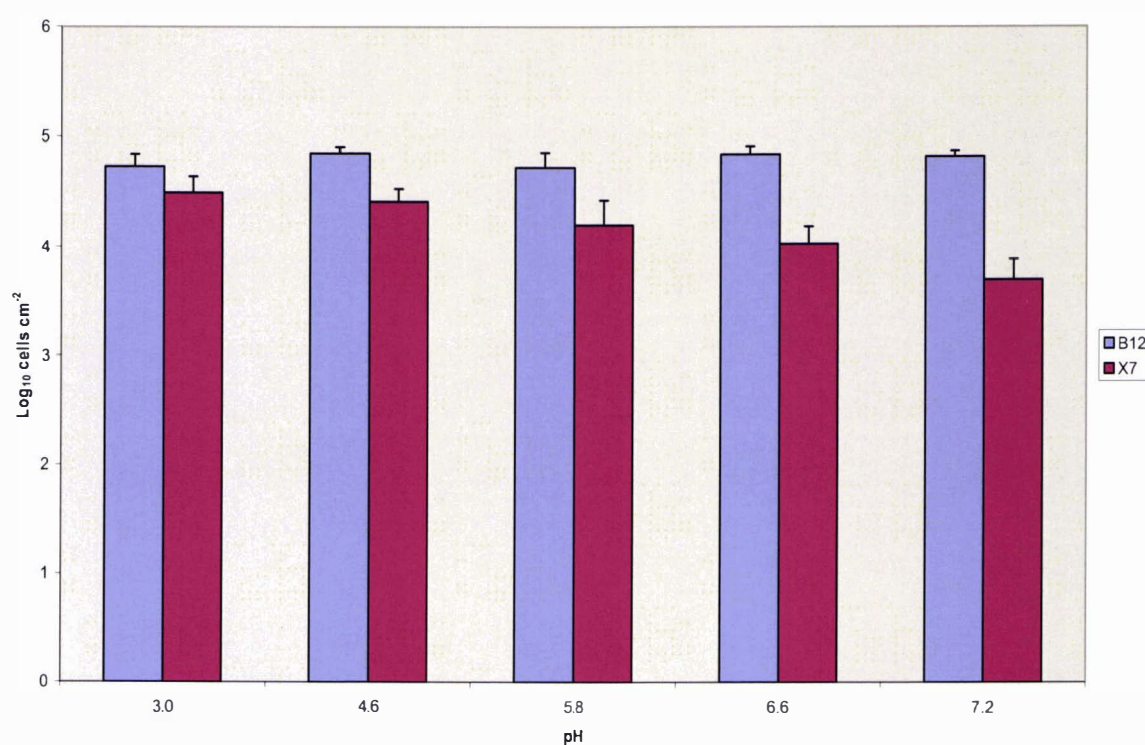
Both strains (B12 and X7) indicated a greater interaction and thus retention with the anionic exchange resin (functional group  $\text{R-CH}_2\text{N}^+(\text{CH}_3)_3$ ) than with the cationic column (functional group  $\text{R-SO}_3^-$ ). Figure 5.4 indicates that both strains have net negative charge at pH 7. Strain B12 demonstrated a higher retention rate to anionic resin at 51% compared with X7 at 14% retention.



**Fig 5.4:** Proportion of B12 and X7 cells retained on anionic (functional group  $\text{R-CH}_2\text{N}^+(\text{CH}_3)_3$  ■) and cationic (functional group  $\text{R-SO}_3^-$  □) exchange resins at pH 7. Error bars represent the standard deviation from the mean of 3 replicates.

### 5.3.3.3 Effect of pH on attachment of B12 and X7 to stainless steel coupons

The effect of pH on the ability of strains B12 and X7 to attach to stainless steel, as measured by epifluorescence microscopy, is shown in Fig 5.5. As the pH of the suspending medium decreased from pH 7.2 to 3.0 X7 showed a greater ability to attach. B12 showed neither a increase or decrease in attachment over the same pH range.

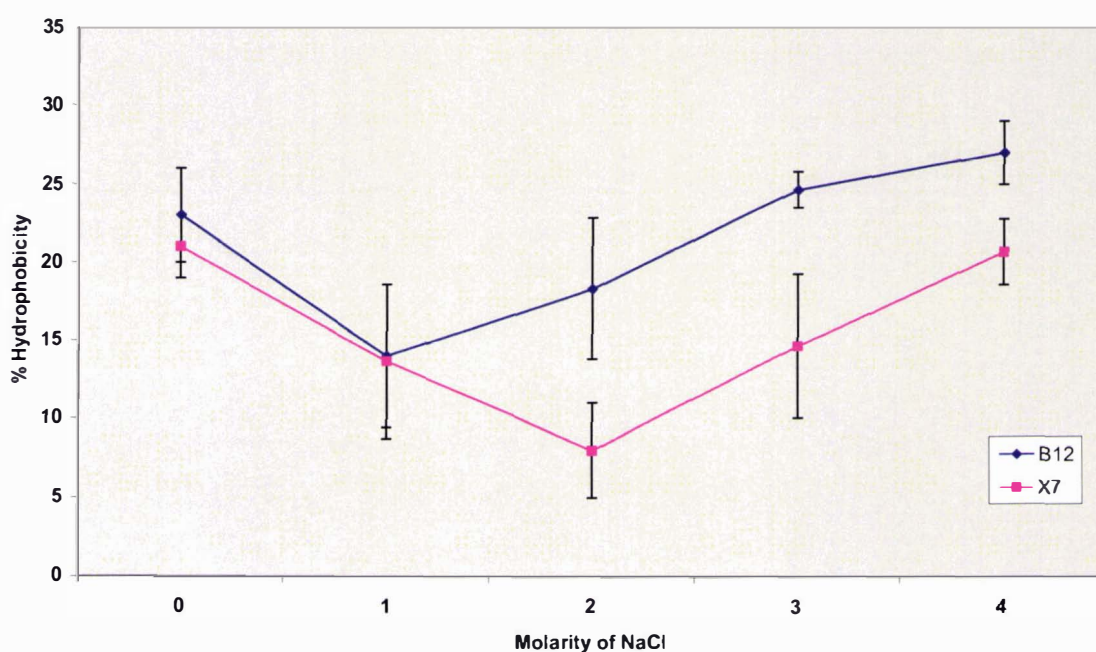


**Fig 5.5:** Effect of pH on attachment of B12 and X7 to stainless steel coupons as measured by epifluorescence microscopy. Error bars represent the standard deviation from the mean of 3 replicates.

### 5.3.4 Hydrophobicity of B12 and X7

#### 5.3.4.1 Determination of cell surface hydrophobicity using Microbial Adhesion to Hydrocarbons (MATH)

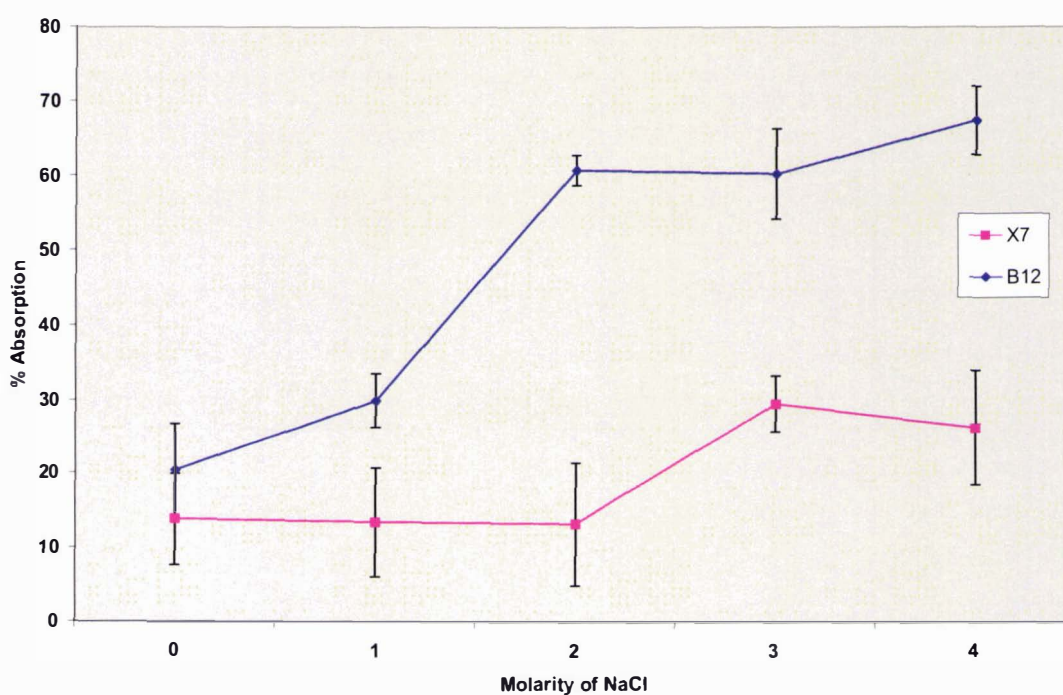
The percentage hydrophobicity of strains B12 and X7 increased at higher ionic strength, in this case NaCl. At lower ionic strengths (0 M and 1 M NaCl), the hydrophobicity of B12 and X7 are similar, but at higher ionic strengths (2 M-4 M NaCl), B12 is more hydrophobic than X7 (Fig 5.6). The hydrophobicity is expressed as % hydrophobicity =  $(A_0 - A_1) / A_0 \times 100$ . Where  $A_0$  = OD of the bacterial suspension before mixing with hexadecane and  $A_1$  = OD of the bacterial suspension after mixing with hexadecane.



**Fig 5.6:** Percentage hydrophobicity measurements of strains B12 and X7 using MATH test over increasing ionic strengths. Error bars represent the standard deviation from the mean of 3 replicates.

### 5.3.4.2 Determination of cell surface hydrophobicity using Hydrophobic Interaction Chromatography

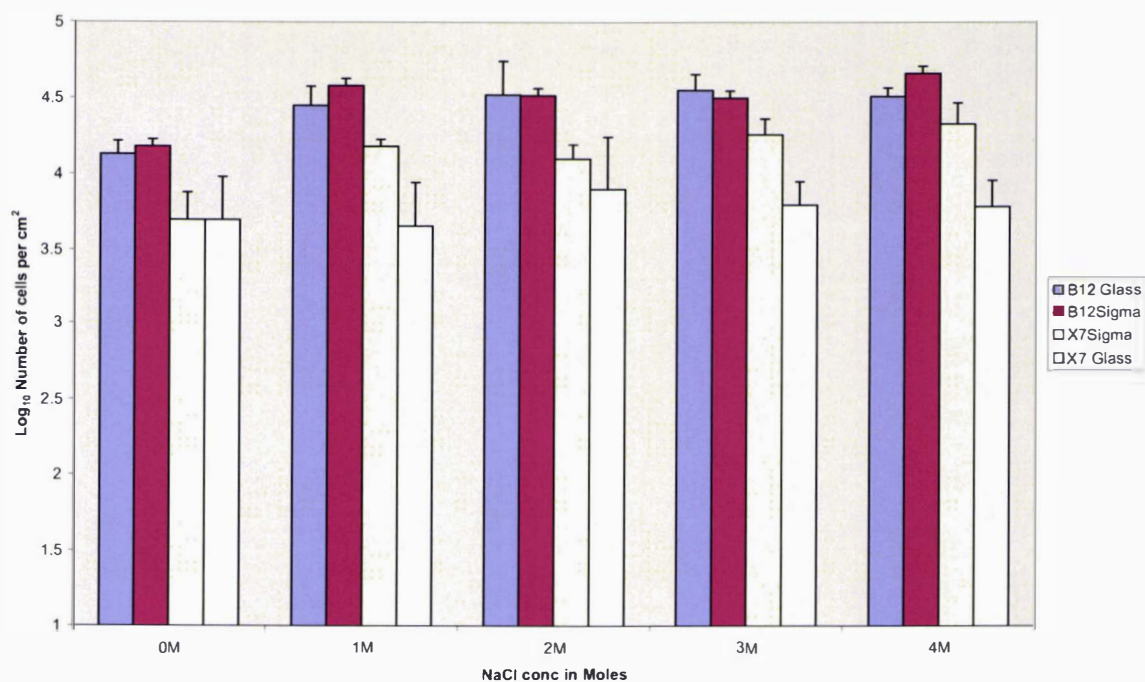
As the ionic strength of the suspending fluid increased, the hydrophobicity of strains B12 and X7 diverged; at higher ionic strengths strain B12 demonstrate a higher hydrophobicity than X7 (Fig 5.7). The hydrophobicity is expressed as % absorption =  $(A_0 - A_1)/A_0 \times 100$ . Where  $A_0$  = OD of a  $1/4$  dilution of the original bacterial suspension.  $A_1$  = OD of the bacterial suspension eluted from the column.



**Fig 5.7:** Percentage absorbance measurements of strains B12 and X7 using HIC test over increasing ionic strengths. Error bars represent the standard deviation from the mean of 3 replicates.

### 5.3.4.3 Attachment of B12 and X7 to glass and Sigmacote coated glass

The role played by hydrophobicity in the attachment of B12 and X7 was investigated by coating glass slides with Sigmacote<sup>®</sup> to increase surface hydrophobicity as previously reported by Krylov and Dovichi, (2000). The electrostatic interactions between surface and bacterial surface were modified by increasing NaCl concentration in the suspending medium. Overall, the more hydrophobic Sigmacote covered surface appeared to cause a slight increase in the attachment of both strains. X7 demonstrated an increased attachment to the more hydrophobic surface as the ionic strength of the suspending medium increased from 0 M NaCl to 4 M NaCl.



**Fig 5.8:** Attachment of B12 and X7 to Sigmacote coated and uncoated glass with increasing ionic strengths. Error bars represent the standard deviation from the mean of 3 replicates.

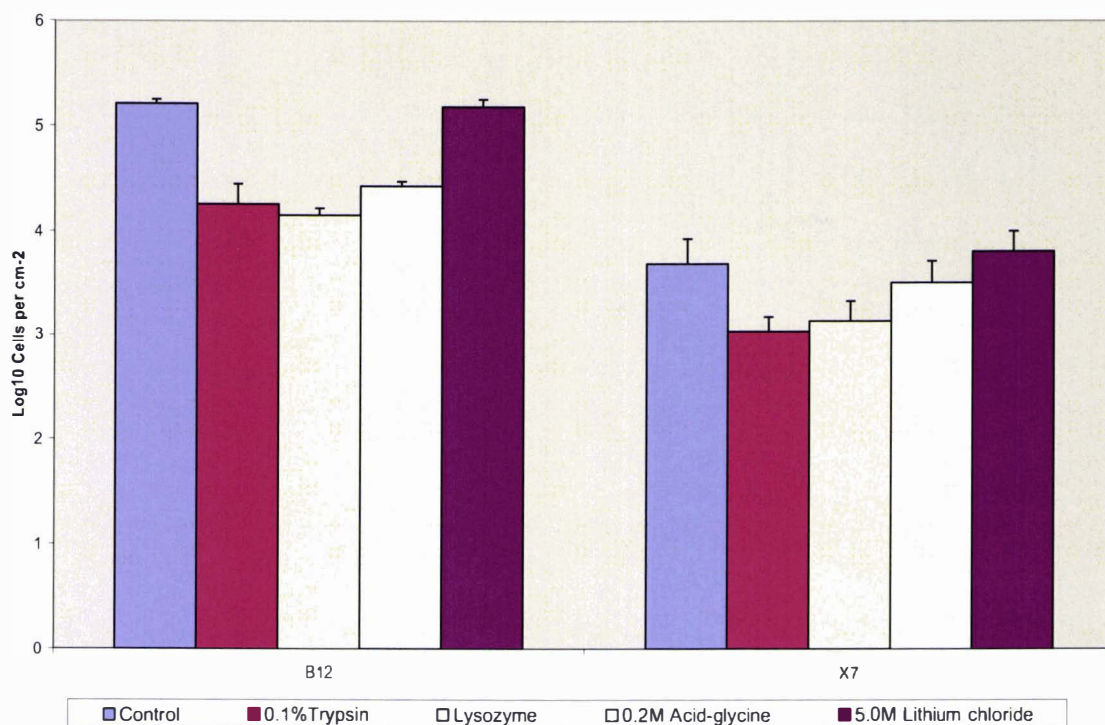
### **5.3.5 Attachment of B12 and X7 cells to stainless steel coupons following disruption of surface proteins by various methods.**

Cell suspensions of B12 treated with 0.1% of the protease trypsin attached to stainless steel at concentrations about  $1 \log_{10} \text{ cm}^2$  lower than untreated B12 cells. Treatment of X7 cells with 0.1% trypsin resulted in about a  $0.5 \log_{10} \text{ cm}^2$  reduction in the attachment of X7 to stainless steel, (Fig 5.9).

Acid-Glycine treatment of cell suspensions of B12 resulted in a  $0.8 \log_{10} \text{ cm}^2$  reduction in the ability of B12 to attach to stainless steel (Fig 5.9) while the same treatment caused only a slight reduction in the attachment of X7.

Lysozyme treatment of cell suspensions of B12 resulted in a  $1.1 \log_{10} \text{ cm}^2$  reduction in the attachment of B12 to stainless steel, but only a slight reduction in the attachment of X7 (Fig 5.9).

Treatment of cells with 5 M Lithium Chloride ( $\text{LiCl}_2$ ) to remove surface proteins caused no obvious reduction in the number of B12 or X7 cells attaching to stainless steel (Fig 5.9).

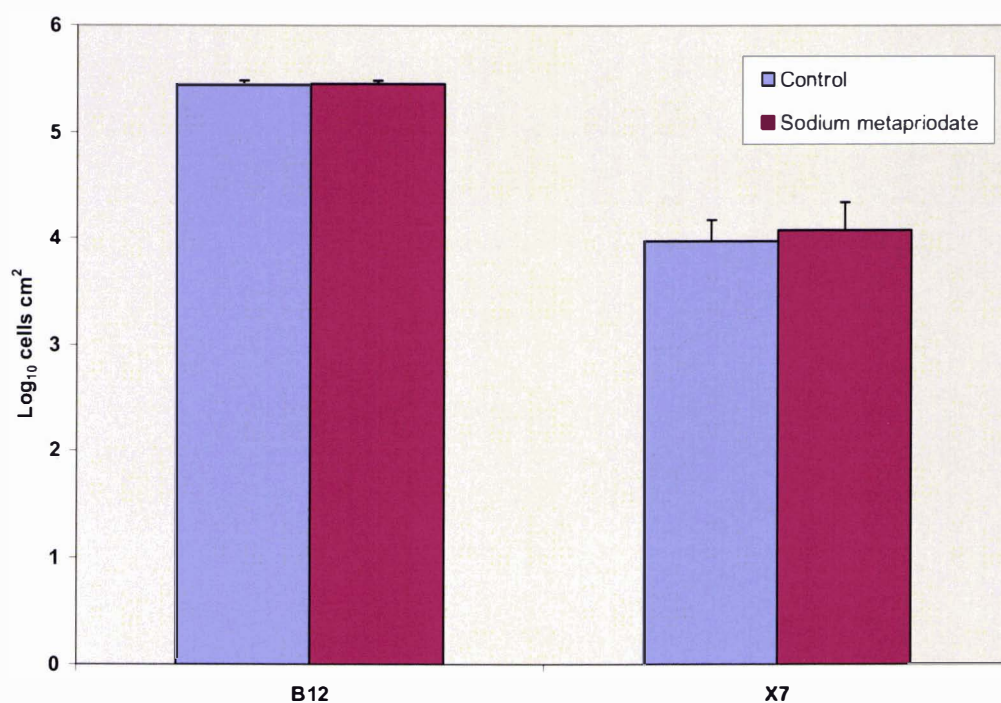


**Fig 5.9:** The attachment of B12 and X7 to stainless steel following disruption of surface proteins. Error bars represent the standard deviation from the mean of 3 replicates.

### 5.3.6 Surface Carbohydrates of B12 and X7

#### 5.3.6.1 The effect of acidified Sodium metaperiodate on attachment of B12 and X7 to stainless steel coupons.

Treatment of cell suspensions of B12 and X7 with acidified sodium metaperiodate, to disrupt polysaccharides appeared to have no effect on the ability of either B12 or X7 to attach to stainless steel (Fig 5.10). These results may be interpreted as showing that surface polysaccharides play a minimal role if any in the initial attachment of B12 and X7 strains to stainless steel.

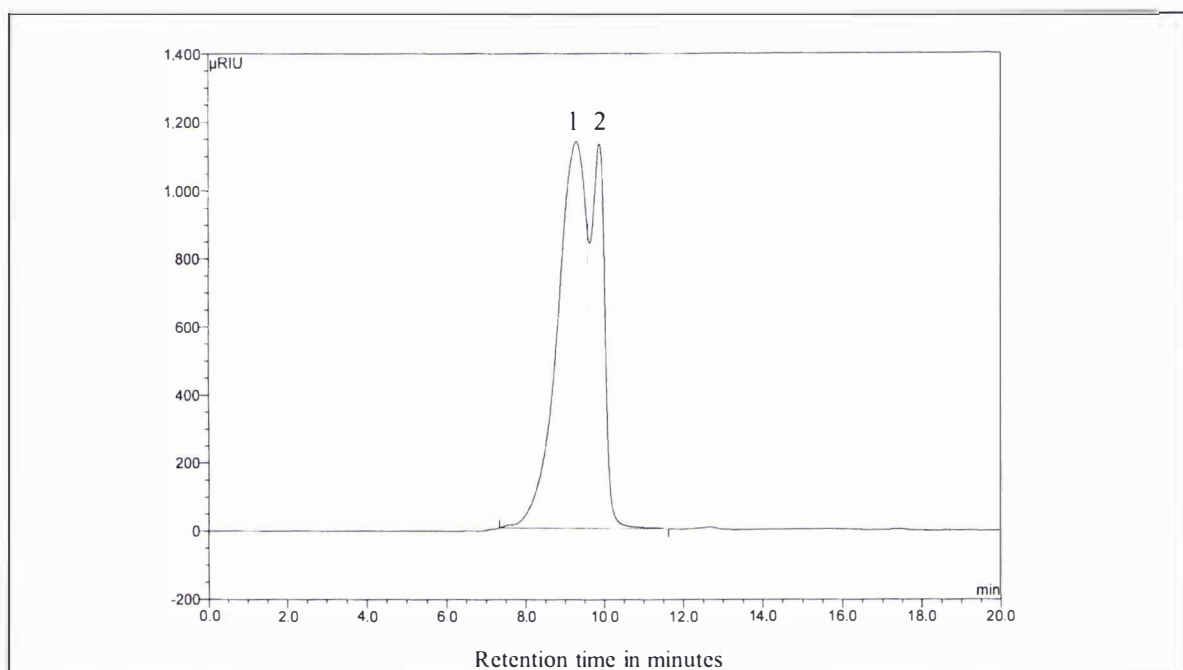


**Fig 5.10:** Attachment of B12 and X7 to stainless steel following treatment with acidified Sodium metaperiodate to remove surface carbohydrates. Error bars represent the standard deviation from the mean of 3 replicates.

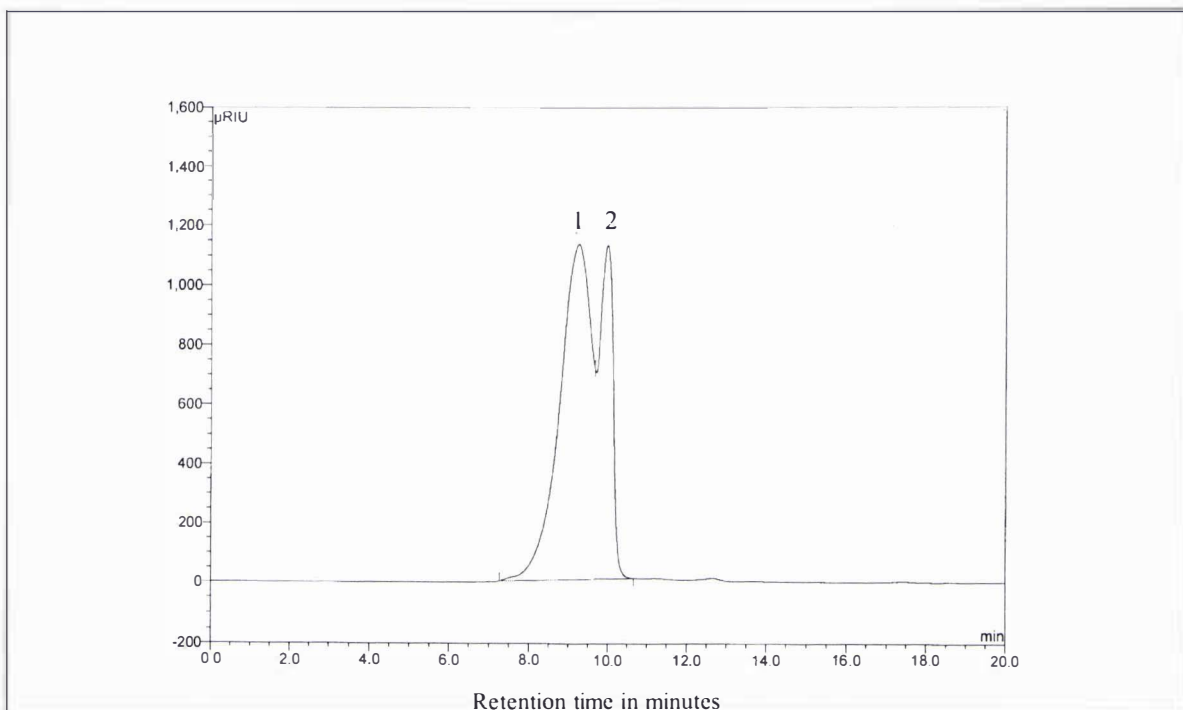
### 5.3.6.2 Analysis of extracellular polysaccharide (EPS) from B12 and X7

EPS recovered from standardized cells suspensions of B12 and X7 as described in section 3.5.9 was quantitated. Both strains were found to produce similar amounts of EPS with B12 cells producing 0.059  $\mu\text{g}$  dextran units per  $10^8$  cells per ml, S.D. of 0.0058 and X7 cells producing 0.057  $\mu\text{g}$  dextran units per  $10^8$  cells per ml, S.D. of 0.0024.

To determine the monosaccharide content of isolated EPS from B12 and X7 cultures, the isolated EPS was hydrolyzed with 1 M sulphuric acid and the resulting monosaccharides separated by HPLC. There appear to be no obvious differences in the monosaccharide composition of EPS from B12 and X7 cultures (Figs 5.11 and 5.12). In an attempt to identify the peaks labelled 1 and 2 present in Figs 5.11 and 5.12, 1% solutions of the following monosaccharides were used as markers: glucose, arabinose, galactose, rhamnose, mannose and Xylose (see Appendix A1 for monosaccharide marker chromatographs). Only peak 2 (9.81 minutes) matched the monosaccharide glucose (9.76 minutes) according to their relative retention times. However, peak 1 (9.21 minutes) could not be identified using the monosaccharide markers mentioned above.



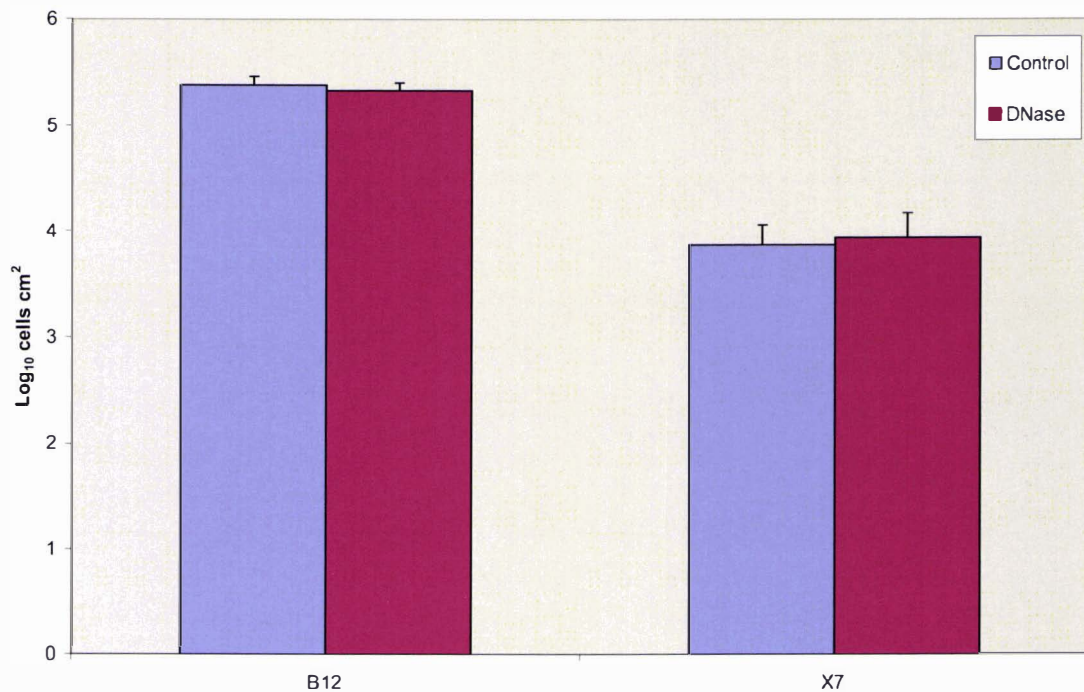
**Fig 5.11:** Monosaccharide composition of EPS isolated from strain B12 digested by Sulphuric acid and analysed by HPLC.



**Fig 5.12:** Monosaccharide composition of EPS isolated from strain X7 digested by Sulphuric acid and analysed by HPLC.

### 5.3.7 Effect of DNase I on attachment of B12 and X7 to stainless steel coupons

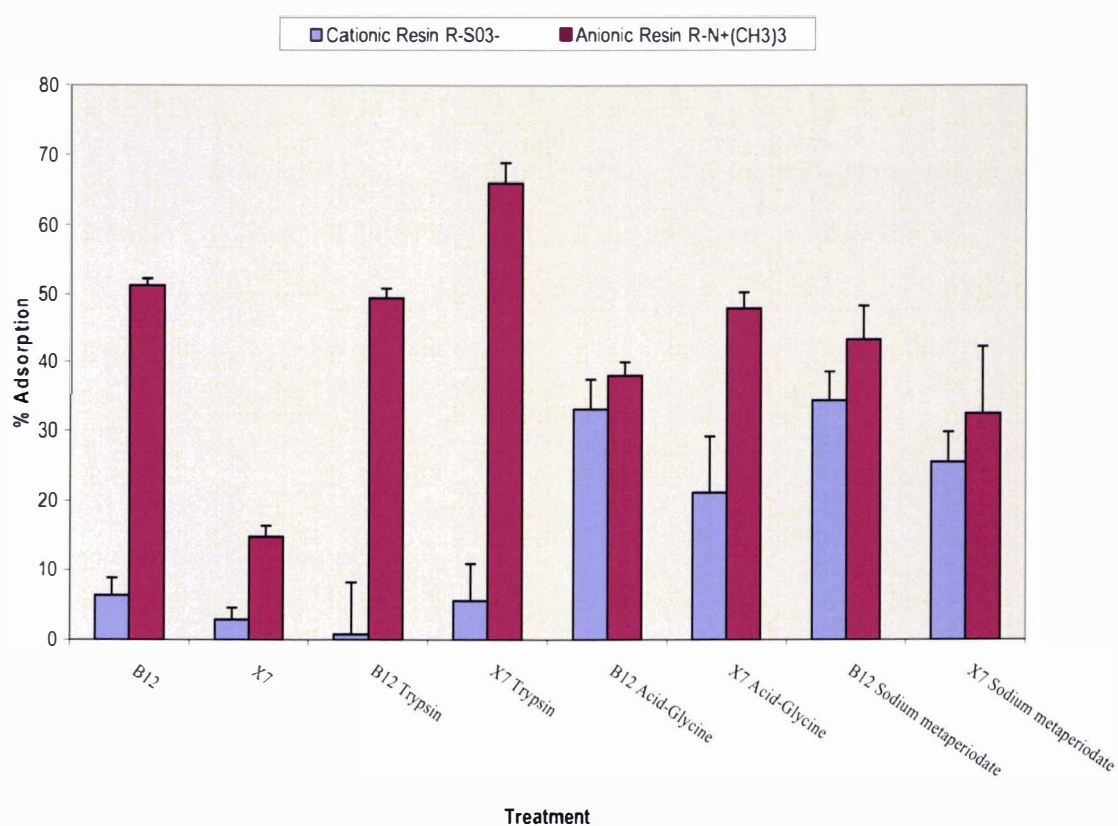
Treatment of B12 and X7 cells with DNase I caused no apparent reduction with respect to control untreated cells in their attachment to stainless steel (Fig. 5.13).



**Fig 5.13:** Attachment of B12 and X7 to stainless steel following treatment with DNase I to remove surface associated DNA. Error bars represent the standard deviation from the mean of 3 replicates.

### 5.3.8 Effect of various treatments on surface charge of B12 and X7 as measured by Electrostatic Interaction Chromatography.

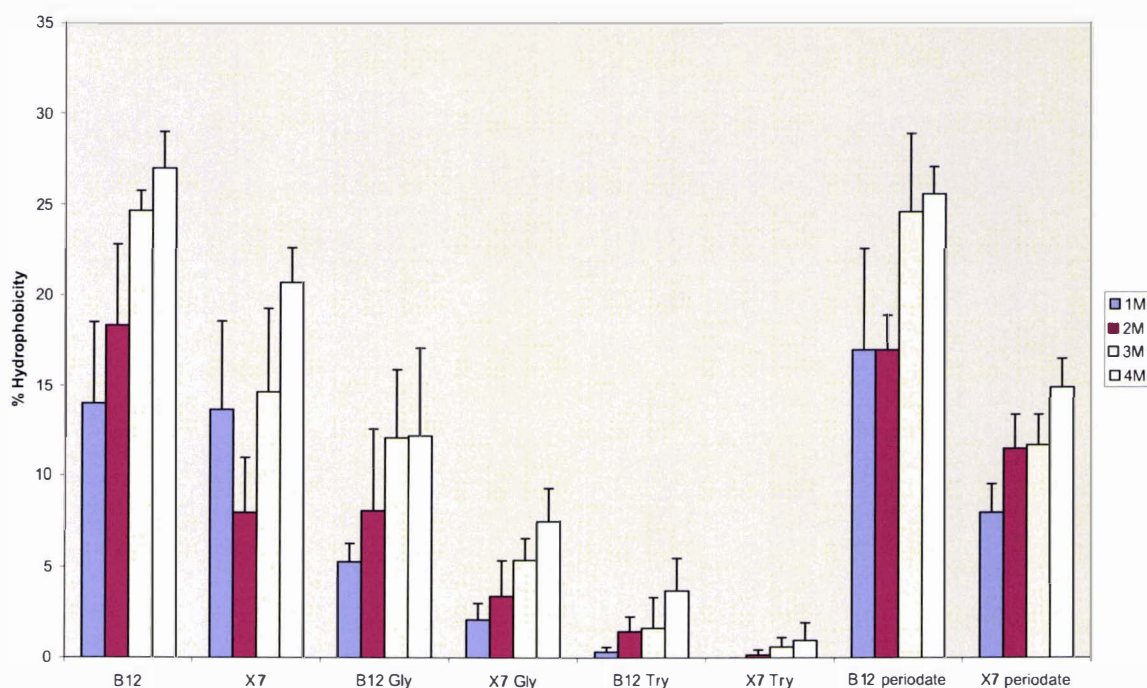
Removal of surface proteins by trypsin increased the negative charge (increased adsorption to anionic resin,  $R-N^+(CH_3)_3$ ) of stain X7, but had little effect on the positive charge of either B12 or X7 (adsorption to cationic resin,  $R-SO_3^-$ ). However, B12 and X7 cells treated with acid-glycine to remove surface proteins and sodium metaperiodate to disrupt surface carbohydrates demonstrated a greater positive charge (adsorption to cationic resin,  $R-SO_3^-$ ) (Fig 5.14).



**Fig 5.14:** Effect of Trypsin, Acid-glycine and Sodium metaperiodate treatment on surface charge of B12 and X7 cells as measured by Electrostatic Interaction Chromatography.

### 5.3.9 Effect of various treatments on hydrophobicity of B12 and X7 as measured by Microbial Adhesion to Hydrocarbons

The hydrophobicity of B12 and X7 cells appears to be influenced by surface proteins, as Trypsin and acid-glycine treatments of B12 and X7 cells reduced the hydrophobicity of both strains, with trypsin causing the greatest reduction in hydrophobicity compared with untreated cells. Measured hydrophobicity increased with increasing ionic strength of the suspending medium. In contrast, sodium metaperiodate treatment neither decreased nor increased hydrophobicity, suggesting that surface carbohydrates of B12 and X7 have no effect on surface hydrophobicity.



**Fig 5.15:** Effect of Trypsin, Acid-glycine and Sodium metaperiodate treatment on Hydrophobicity of B12 and X7 cells as measured by MATH (Microbial Adhesion to Hydrocarbons) in the presence 1, 2, 3 or 4 molar concentrations of NaCl.

## 5.4 Discussion

In this chapter, bacterial cell surface properties affecting attachment of strains B12 and X7 were studied in order to observe any differences between the two strains that could be associated with the greater propensity of B12 to attach to stainless steel compared with that of X7.

To compare and contrast the surface structures of B12 and X7, cells were viewed by transmission electron microscopy (TEM) with and without Ruthenium red staining. The anionic polysaccharide dye Ruthenium red has been reported in the past (Hood & Zottola, 1988; Gutierrez-Gonzalvez *et al.*, 1986) as a stain for surface anionic polysaccharides of bacteria that can be viewed under TEM conditions. No differences in cell wall structure were observed between the two strains. This suggests that the observed reduced attachment of X7 to stainless steel compared with the parental strain B12 cannot be associated with any major structural change to the cell wall or surface polysaccharides that can be seen using TEM.

Incubation of X7 cells in the supernatant of centrifuged B12 culture resulted in no increase in the ability of X7 to attach to stainless steel. The incubation of B12 cells in the supernatant of centrifuged X7 culture also resulted in no change of the ability of B12 to attach to stainless steel (Fig 5.2). This work was undertaken in an attempt to identify any components that may dissociate from the surface of B12 during the life of the culture and reside in the culture supernatant and play an important part in the ability of B12 to attach to stainless steel. What these surface components might be can be only speculated upon, but S-layer proteins are often non-covalently attached to the cell walls of Gram-positive bacteria and have the intrinsic ability to assemble into ordered two-dimensional crystalline structures. The functions of S-layer proteins still remain to be totally elucidated (Claus *et al.*, 2005), but they are thought to include adhesion sites for cell wall associated exoenzymes (Sára & Sleytr, 2000) and have been confirmed as mediating attachment of *Lactobacillus brevis* to human epithelial cells (Hynönen *et al.*, 2002). Extraction and analysis of S-layer proteins by SDS-PAGE may help identify the possible

involvement of S-layer proteins in the attachment of B12 and X7 to stainless steel. The potential for X7 to produce a surface component that inhibits attachment appears unlikely, as X7 culture supernatant had no effect on the ability of B12 to attach to stainless steel.

Surface charge is considered to be important in the ability of bacteria to attach to surfaces (Briandet *et al.*, 1999; Ukuku & Fett, 2002; Dickson & Koohmaraie, 1989; Van Loosdrecht, 1987). In this study, two methods were employed to analyse surface charge: Zeta potential and EIC. The Zeta potential of bacterial cells is calculated from the mobility of the bacterial cells in an electrical field under defined salt concentration and pH and has been used by many groups to define overall charge of the bacterial cell surface (Jucker *et al.*, 1996; Groenink *et al.*, 1998; Wilson *et al.*, 2001; Parkar *et al.*, 2001). Over various pH ranges, in this case pH 2-8, the Zeta potential of bacterial cells can be expected to change, as the pH of the suspending medium influences the dissociation of acidic groups such as carboxyl, phosphate and amino groups as well as basic groups found on a bacterial surface (Wal *et al.*, 1997). A comparison of the zeta potentials of B12 and X7 cells showed that at increasing pH values (pH 6-8), B12 cells are more electronegative than cells of X7 (Fig 5.3), but in the pH range of 2-5 B12 and X7 demonstrate very similar zeta potentials with an isoelectric point of about pH 4.

Several groups have used the EIC method in the past to measure charge on the surface of bacteria (Dickson & Koohmarare, 1989; Jones *et al.*, 1996; Peng *et al.*, 2001). The EIC values of B12 and X7 cells suggest that at pH 7, B12 cells have a greater negative charge than X7 cells (Fig 5.4). This result coincides with the zeta potential measurements that also suggested that B12 cells have a greater negative charge than X7 cells at pH 7. Although B12 and X7 cells showed a relatively low affinity for cationic resin (less than 10%), Jones *et al.* (1996) reported that some isolates of *S. epidermidis* exhibited a high retention for both anionic and cationic resins, suggesting there may be localized regions of positively charged and negatively charged molecules on the surface of the bacterial cell. Zeta potential measurements in comparison measure only net charge of bacterial cells and not the ratios of negative to positive charge regions on a cell surface as in EIC.

In general, most bacterial surfaces have a net negative charge at neutral pH (Rijnaarts *et al.*, 1999), but exceptions do exist (Jucker *et al.*, 1996). Several groups have reported that stainless steel also has a negative zeta potential over the pH 6-8 range (Fukuzaki *et al.*, 1995; Boulangé-Petermann *et al.*, 1995). Thus negative bacterial and stainless steel surface charges are likely to cause repulsion between the bacterial cell and stainless steel. On this basis, strain B12 might be expected to attach less than X7. That the opposite is observed suggests that bacterial surface charge is not an important factor in the ability of *Anoxybacillus flavithermus* to attach to stainless steel. Other groups have also reported no correlation in zeta potential and attachment to stainless steel (Parker *et al.*, 2001; Flint *et al.*, 1997A) demonstrating that attachment cannot be explained solely by surface charge. The electrostatic repulsion between stainless steel and bacterial cells may be overcome by non-electrostatic interactions, such as van der Waals forces, hydrophobic interactions and/or steric interactions. Boulangé-Petermann *et al.* (1995) noted that the zeta potential of stainless steel could vary with surface adsorption of ions such as chloride and phosphate at the solid/liquid interface resulting from different surface treatments. Further, they suggested that the zeta potential of stainless steel may not be an intrinsic surface parameter, but be dependent upon surface treatments and/or cleaning regimes, as well as the obvious pH and ionic strength of the suspending medium. Consequently, different pH values may affect the surface charge of stainless steel as well as the bacterial surface charge.

Changing the pH of the suspending medium resulted in no major change in the ability of B12 to attach to stainless steel over the pH range 3-7.2. However as the pH of the suspending medium lowered, strain X7 demonstrated a greater ability to attach to stainless steel (Fig 5.5). The observed increase may be due to the suspending medium moving toward the isoelectric point of strain X7; Husmark and Ronner (1990) reported *Bacillus cereus* spores showed maximum attachment to surfaces at their isoelectric point (pH 4). Lindsay *et al.* (2000) also reported dairy-associated *Bacillus* spp showing enhanced attachment to surfaces at pH 4.

Hydrophobic interactions are also thought to play a role in attachment (Peng *et al.*, 2001; Gilbert *et al.*, 1991; Liu *et al.*, 2004; Iwabuchi *et al.*, 2003). Two methods were employed to measure the hydrophobicity of strains B12 and X7: Hydrophobic interactive chromatography (HIC) and Microbial adhesion to hydrocarbon (MATH) (Mozes and Rouxhet, 1987). Overall the two methods indicated that B12 cells are generally more hydrophobic than X7 cells at high salt concentrations.

In the MATH test (Rosenberg *et al.*, 1980), the choice of organic phase is important, as some organic phases can destroy the surface of the bacteria. Flint *et al.* (1997A) used xylene as the organic phase in the MATH test, but in the present study, xylene was found to cause cell lysis, giving a false indicator of hydrophobicity, so hexadecane was used as an organic phase instead. One concern over the validity of the MATH test is the possible interplay between hydrophobic and charge properties involved in the MATH test. Ahimou *et al.* (2001); Busscher *et al.* (1995) and Van der Mei *et al.* (1993) have all expressed concern over the use of the MATH test without minimizing electrostatic interactions. Busscher *et al.* (1995) reported that hexadecane, the most commonly used hydrocarbon to measure hydrophobicity, has a zeta potential in water of between -50 and -80 mV at neutral pH. To overcome the potential interference of electrostatic interactions in the MATH test, Doyle (2000) suggested the use of high ionic strength conditions or cells suspended in medium at a pH equal to the isoelectric point of the bacterial cells.

In the study reported here, the MATH test was performed using cells suspended in solutions of 0 M, 1 M, 2 M, 3 M and 4 M NaCl as a modified version reported by Wiencek *et al.* (1990). The results imply that B12 has a higher surface hydrophobicity than X7 (Fig 5.6), but this difference occurred only at high levels of NaCl (2 M, 3 M and 4 M), apparently confirming the importance of reducing the electrostatic interaction when using the MATH as reported by Ahimou *et al.* (2001); Busscher *et al.* (1995) and Van der Mei *et al.* (1993).

HIC involves the interaction of bacterial cells with a hydrophobic column, in this case phenyl-sepharose, with cells demonstrating high hydrophobicity being retained in the

column and cells with low hydrophobicity being eluted. The percentage of retained cells gives an indication of the hydrophobicity of the bacteria.

HIC tests were performed on strains B12 and X7 using suspending media of increasing ionic strengths of NaCl as in the MATH test. The results again indicated that strain B12 has a greater surface hydrophobicity than strain X7 in the presence of higher concentrations of NaCl (Fig 5.7). The results of the present study are similar to those of Smyth *et al.* (1978), who demonstrated that, using HIC at higher ionic strengths of NaCl, the differences in hydrophobicity between *E. coli* K88 negative and *E. coli* K88 positive strains were more pronounced.

The use of NaCl concentrations in MATH and HIC will cause a reduction in the thickness of the double electric layer around a bacterial cell (Tomlinson *et al.*, 2007) reducing the effect of electrostatic interactions. However, the use of an isogenic mutant (X7) compared with the parental strain (B12) will limit heterogeneity of the cell surface that could be observed when comparing different species (Hermansson, 1999). Therefore, the results presented in this study suggest some differences in the cell surface characteristics of B12 and X7, resulting in a change in the hydrophobicity measurements between the two strains.

The role played by the hydrophobicity of the substratum surface in the attachment of strains B12 and X7 to surfaces was assessed by coating glass with Sigmacote. The addition of Sigmacote to the surface of glass is an example of a silanization surface modification, resulting in an increase of substratum surface hydrophobicity to equal that of the hydrophobic surface polystyrene (Krylov & Dovichi, 2000). The presence of Sigmacote on the glass surface resulted in no increase in attachment of strain B12. Strain B12 also demonstrated a greater attachment to glass (hydrophilic) surfaces than X7. Liu *et al.* (2004), however, reported that a microbial cell with high surface hydrophobicity could help cell attachment to both hydrophobic and hydrophilic surfaces. Increasing ionic concentration, from 0 M to 1 M NaCl resulted in a slight increase in the attachment of B12 to both glass and Sigmacote surfaces and X7 to Sigmacote glass, implying that once

surface charge is suppressed, hydrophobicity may play a role in attachment of both B12 and X7.

Many researchers have pointed to the role played by surface proteins in the attachment of bacteria to surfaces (Cucarella *et al.*, 2001; Paul and Jeffrey, 1985; Heilmann *et al.*, 1996). Flint *et al.* (1997A) reported that surface proteins of thermophilic streptococci are important in attachment to stainless steel, with trypsin or sodium dodecyl sulphate (SDS) treated cells demonstrating a 100-fold reduction in the number of bacteria attaching. Parkar *et al.* (2001), working with cells of thermophilic bacilli, also demonstrated that vegetative cells treated with trypsin or SDS exhibited decreased attachment to stainless steel.

Methods employed to disrupt or remove surface proteins from bacterial cells vary from group to group. Flint *et al.* (1997A) employed trypsin and SDS to remove surface proteins, resulting in the reduced ability of thermophilic streptococci to attach to stainless steel. SDS was not used in the present study because of the high rate of cell lysis observed when cells were exposed to 1-2% SDS. Trypsin treated cells of strains B12 and X7 both demonstrated a decrease in attachment to stainless steel, suggesting the importance of surface proteins in the attachment process.

Acid-glycine surface extraction involves exposing cells to 0.2 M glycine at pH 2.2 and has been used by Calabi *et al.* (2002) to extract surface layer proteins from *Clostridium difficile*. Acid-glycine has also been used by several groups to extract surface proteins from various *Campylobacter* species (Dubreuil *et al.*, 1988; Kervella *et al.*, 1993). Although the exact mechanism by which proteins were removed from the bacterial surface was not discussed, it is assumed it interferes with non-covalent bonds present on a cell surface, releasing non-covalently bonded surface proteins. Acid-glycine extraction of surface proteins from B12 and X7 strains resulted in a decrease in the attachment of both strains to stainless steel, but in general, trypsin treatment resulted in a greater reduction in attachment, highlighting the important role surface proteins play in the attachment process of both B12 and X7 to stainless steel.

Removal of cell wall proteins with lysozyme has been reported by Rojas *et al.* (2002) and Kelly *et al.* (2005). Lysozyme degrades the bacterial cell peptidoglycan layer by hydrolyzing the beta (1-4) linkages between N-acetyl-amino-2-deoxy-D-glucose residues. In the present study, lysozyme treatment of cells of B12 and X7 resulted in a similar reduction in attachment to stainless steel as trypsin treatment, again suggesting the potential importance of surface proteins in attachment. However, lysozyme treatment may remove also surface carbohydrates attached to peptidoglycan and has been reported in the past as a means of polysaccharide removal from bacterial cell walls (Parkar *et al.*, 2001).

Sun *et al.* (2007) reported the successful use of 5M lithium chloride as a method to extract surface proteins and gain greater understanding of the ability of *Lactobacillus plantarum* to attach to intestinal mucus layers. However, in the present study, the use of 5 M LiCl<sub>2</sub> did not appear to cause any reduction in the attachment of B12 or X7 to stainless steel.

Summarising, all but one of the methods used to extract surface proteins from bacterial surfaces resulted in a reduction in the attachment of both strains to stainless steel, suggesting that surface proteins of strains B12 and X7 play an important role in attachment.

Extracellular polysaccharides (EPS) of bacterial cells have been reported to aid the attachment of bacteria to glass surfaces (Tsuneda *et al.*, 2003), stainless steel (Lewis *et al.*, 1989) and mineral oxide surfaces (Jucker *et al.*, 1997). Conversely, the presence of extracellular polysaccharides has also been reported to inhibit bacterial attachment under certain circumstances (Ryu *et al.*, 2004; Gómez-Suárez *et al.*, 2002). In order to understand the potential role EPS may play in the attachment of B12 and X7 to stainless steel, acidified sodium metaperiodate was used to disrupt EPS, which results in the oxidation of carbohydrates into formic acid and formaldehyde (Scherz & Bonn, 1998).

Treatment of cells with acidified sodium metaperiodate caused no reduction in the attachment of either strain to stainless steel.

Extraction and quantification of EPS from B12 and X7 strains produced similar results, suggesting that the amount of EPS produced is not responsible for the difference in attachment rates. Parkar *et al.* (2001) also reported no correlation between EPS production and attachment of thermophilic bacilli to stainless steel and Flint *et al.* (1997A) concluded that EPS played no role in the attachment of thermophilic streptococci to stainless steel.

It was thought that acid hydrolysis of EPS into its monosaccharide components and analysis by HPLC might reveal subtle differences in the monosaccharide composition of the EPS between B12 and X7 that could be responsible for the different attachment rates. However, the monosaccharide composition of both strains was similar in terms of the number of monosaccharides identified, with 2 major peaks and total volume of the peaks. Only one of the two peaks (glucose) was identified, based on the relative retention time of a known solution of glucose (Fig 5.11 and Fig 5.12).

In summary, all three of these methods used to analyse EPS produced by B12 and X7 produced no conclusive results that could indicate EPS involvement in the ability of strain B12 to attach at a higher rate than strain X7.

Several researchers have pointed to the role played by extracellular DNA in biofilm growth and development and it has even been given the acronym eDNA (Steinberger & Holden, 2005; Allesen-Holm *et al.*, 2006). However, the role of eDNA on initial cell attachment is not well understood. Whitchurch *et al.* (2002) reported DNase I added to a medium flowing over a biofilm of *Pseudomonas aeruginosa* less than 82 hours old resulted in the biofilm being dissolved. But of interest is also the observation that surfaces exposed to a medium containing DNase I, after 3 days incubation contained very few attached cells, compared with medium without DNase I, which was extensively colonized. Cells of B12 and X7 exposed to DNase I produced very similar levels of

attachment compared with untreated cells, suggesting that eDNA is not involved in the attachment of B12 or X7 to stainless steel. To confirm that DNase I was not inactivated by proteolysis or other inhibitors during incubation with bacterial cells, samples of the suspending medium were taken after incubation with bacterial cells and DNase I activity was monitored by spectrophotometric means against pure DNA at 260 nm. The activity remained, confirming that B12 and X7 cells were incubated in the presence of active DNase I.

To obtain a better understanding of the role of proteins and carbohydrates in the distribution and magnitude of surface charge and surface hydrophobicity, cells modified by trypsin, acid-glycine and acidified sodium metaperiodate treatment underwent EIC and MATH tests to measure cell surface charge and cell surface hydrophobicity.

Surface modification of B12 and X7 with trypsin increased the negative charge of X7 (Fig 5.14). In addition, trypsin treatment of B12 and X7 caused a reduction in the hydrophobicity of both strains as measured by the MATH test (Fig 5.15). Acid-glycine treatment of B12 and X7 cells to remove surface proteins caused an increase in the positive charge of both strains). Nonetheless, acid-glycine treatment caused a reduction in the hydrophobicity of both strains, which is in agreement with the trypsin results, implying that cell surface hydrophobicity is linked with surface proteins.

Acidified sodium metaperiodate produced similar results to acid-glycine treatment in terms of surface charge, with both B12 and X7 demonstrating an increase in positive charge. On the other hand acidified sodium metaperiodate treatment had no effect on the cell surface hydrophobicity of either B12 or X7, implying that surface polysaccharides are not involved in surface hydrophobicity.

Protein and polysaccharide removal treatments tended to change the charge of B12 and X7 cells in general, this result is in agreement with observations by Castellanos *et al.* (1997), but no pattern relating changes in surface charge to attachment are obvious. However, in the present study cell surface proteins appear more important in determining

hydrophobicity, as also reported by Paul & Jeffrey (1985) and Smyth *et al.* (1978). When the effects of trypsin and acid-glycine treatment on reducing hydrophobicity and the effect of trypsin and acid-glycine treatment on attachment of cells to stainless steel are compared, there appears to be a relationship between the attachment of B12 and X7, surface proteins and hydrophobicity.

## **5.5 Conclusion**

The two main physicochemical differences between cell surfaces of B12 and X7 were

- 1) B12 has a greater negative surface charge than X7 as measured by zeta potential and EIC at pH 7.
- 2) B12 has a greater cell surface hydrophobicity than X7 as measured by MATH and HIC.

Surface proteins appear to be the most important surface characteristic associated with attachment of B12 and X7 to stainless steel, with surface proteins also the most important factor in determining surface hydrophobicity. EPS on the other hand appeared to play no role in the attachment or surface hydrophobicity of either B12 or X7.

The main difference between B12 and X7 appears to be a protein related characteristic that strain X7 lacks, resulting in a lower hydrophobicity that also appears to be involved in lowering the surface charge of B12. Based on this observation future work will involve the extraction and analysis of surface proteins from B12 and X7 cells

## CHAPTER 6.0

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# Comparison of total cell and surface extracted proteins from B12 and X7 cells using one-dimensional SDS-PAGE and two-dimensional PAGE gel electrophoresis

### 6.1 *Introduction*

### 6.2 *Procedures*

6.2.1 One-dimensional SDS-PAGE of total, acid-glycine and lysozyme/mutanolysin extracted proteins

6.2.2 Two-dimensional PAGE of total, acid-glycine and lysozyme/mutanolysin extracted proteins

### 6.3 *Results*

6.3.1 One-dimensional SDS-PAGE of total and surface extracted proteins

6.3.1.1 *One-dimensional SDS-PAGE of total cell proteins.*

6.3.1.2 *One-dimensional SDS-PAGE of acid-glycine surface extracted proteins.*

6.2.1.3 *One-dimensional SDS-PAGE of lysozyme/mutanolysin surface extracted proteins.*

6.2.1.4 *Summary of One-dimensional SDS-PAGE of total and surface extracted proteins*

6.3.2 Two-dimensional PAGE of total and surface extracted proteins

6.3.2.1 *Two-dimensional PAGE of total cell proteins.*

6.3.2.2 *Two-dimensional PAGE of acid-glycine surface extracted proteins.*

6.2.2.3 *Two-dimensional PAGE of lysozyme/mutanolysin surface extracted proteins.*

6.2.2.3.1 *Validation of lysozyme/mutanolysin extraction*

6.3.2.4 *Summary Two-dimensional PAGE of total & surface extracted proteins.*

## **6.4 Discussion**

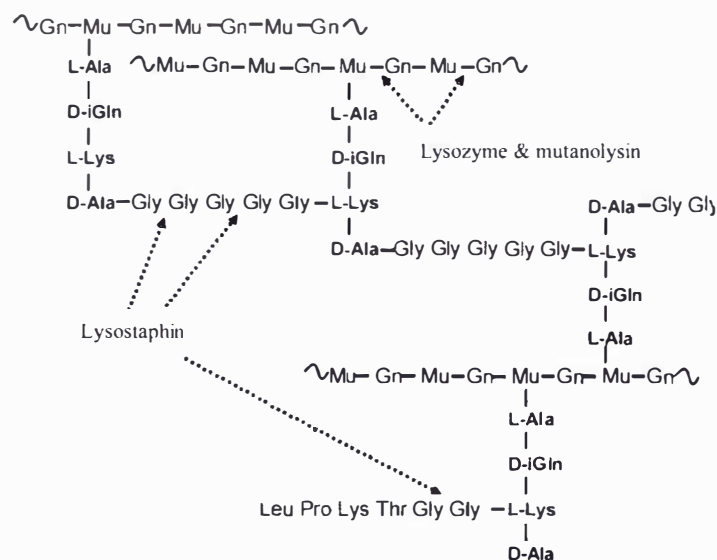
## **6.5 Conclusion**

## 6.1 Introduction

The interaction between a solid surface and the bacterial surface is mediated by numerous attachment mechanisms, but one important surface component commonly associated with bacterial attachment is surface proteins. Cucarella *et al.* (2001) reported a 254 kDa size surface protein termed BAP (Biofilm Associated protein) from *S. aureus* present in all isolates of *S. aureus* that were highly adherent to inert surfaces and consequentially all strong biofilm formers. Tormo *et al.* (2005) reported that other strong biofilm formers from *S. epidermidis*, *S. chromogenes*, *S. xylosus*, *S. simulans*, *S. hyicus* all produced a BAP-like protein with an amino acid sequence similarity of greater than 80% suggesting that the BAP surface protein is an important protein involved in the attachment of many *Staphylococcus* species to surfaces. Surface proteins from *S. epidermidis* associated with attachment to inert surfaces include a 280 kDa surface protein reported by Veenstra *et al.* (1996) and a 60 kDa surface protein (Heilmann *et al.*, 1997). The role of surface proteins in attachment is not confined to the Genus *Staphylococcus*; other research groups working with various different bacteria have also reported the important role surface proteins perform in the attachment of bacteria to solid surfaces. Examples include, *Shewanella oneidensis* (Thormann *et al.*, 2004), *Listeria monocytogenes* (Auvray *et al.*, 2007), *Stenotrophomonas maltophilia* (De Oliveira-Garcia *et al.*, 2003), *Azospirillum brasilense* (Dufrêne *et al.*, 1996), *Klebsiella pneumoniae* (Di Martino *et al.*, 2003) and thermophilic *Streptococci* (Flint *et al.*, 1997).

The use of two-dimensional gel electrophoresis in the analysis of surface proteins from bacteria, termed Surfaceome by Cullen *et al.* (2005), has received considerable interest from the medical area in recent times, especially in the identification of vaccine candidates from pathogenic bacteria. However, the various methods employed to extract surface proteins also vary from group to group, making comparisons difficult. The peptidoglycan degrading enzymes such as lysozyme dissolve bacterial cell wall peptidoglycan by hydrolyzing the  $\beta(1-4)$  linkages between N-acetyl-amino-2-deoxy-D-glucose residues, releasing surface related proteins into solution (Windholz, 1983). Lysozyme has been used to isolate surface proteins from Gram negative (Seyer *et al.*,

2005) and Gram positive (Chung *et al.*, 1998) bacteria. Nevertheless, *Streptococcus* species tend to be resistant to lysozyme, due to the presence of an O-acetyl in muramic residues (Hughes *et al.*, 2002). In addition some *Bacillus* species are also resistant to lysozyme due to the lack of an N-acetyl group in muramic residues. However, mutanolysin also categorised as a muramidase enzyme can attack the walls of *Streptococcus* and *Bacillus* species. Lysostaphin, a peptidase enzyme, which cleaves the pentaglycine cross-bridges in peptidoglycan, is also an alternative used against *Staphylococcus* species (Nandakumar *et al.*, 2005; Planchon *et al.*, 2007). These peptidoglycan degrading enzymes are usually used in the presence of an osmotic protective agent such as sucrose to limit cell lysis.



**Fig 6.1:** Lysozyme, mutanolysin and lysostaphin cleavage sites in the peptidoglycan layer of Gram positive bacteria (adapted from Gatlin *et al.*, 2006).

Other methods used to extract surface proteins have included acid-glycine (Wright *et al.*, 2005), lithium chloride (Nandakumar *et al.*, 2005), SDS (Flint *et al.*, 1997). More recently the use of biotinylation (Smither *et al.*, 2007) and trypsin digestion of cell surface proteins followed by analysis of the resulting peptides by matrix-assisted laser

desorption ionization time of flight mass spectrometry (MALDI-TOF) or tandem mass spectrometry (MS/MS) (Severin *et al.*, 2007) have also added to the repertoire of methods available to the researcher in the analysis of bacterial surface proteins.

The objective of work described in this chapter was to identify any specific proteins that may be involved in the attachment process of B12 and X7 to stainless steel, by comparing total proteins and surface extracted proteins using one-dimensional and two-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) & (2D PAGE).

## **6.2 Procedures**

### **6.2.1 One-dimensional SDS-PAGE of Total, Acid-glycine and Lysozyme/Mutanolysin extracted proteins**

In order to compare the total, acid-glycine and lysozyme/mutanolysin extracted proteins from B12 and X7 cells using one-dimensional SDS-PAGE, cultures of B12 and X7 were grown in TSB broth at 55°C till mid-log phase, collected by centrifugation and proteins extracted by either

- 1) Total proteins (section 3.6.1.1)
- 2) Acid-glycine (section 3.6.1.2)
- 3) Lysozyme/mutanolysin (section 3.6.1.3)

The resulting protein extracts were separated on a 12.5% SDS-PAGE according to the method of Laemmli and Favre (1973) using a Protean® II xi electrophoresis system (Bio-Rad) then silver stained as detailed in section 3.6.1.4.1.

## **6.2.2 Two-dimensional PAGE of Total, Acid-glycine and Lysozyme/Mutanolysin extracted proteins**

In order to compare the total, acid-glycine and lysozyme/mutanolysin extracted proteins from B12 and X7 cells using two-dimensional PAGE, cultures of B12 and X7 were grown in TSB broth at 55°C till mid-log phase, collected by centrifugation and proteins extracted by either

- 1) Total proteins (section 3.6.2.1)
- 2) Acid-glycine (section 3.6.2.2)
- 3) lysozyme/mutanolysin (section 3.6.2.3)

Samples were dissolved in 6 M urea and 2% CHAPS (3-Cholamidopropyl-dimethylammonio-1-propanesulfonate) to a protein concentration between 600-900 µg/ml, followed by the addition of other Rehydration/Sample chemicals (Section 3.6.4.4.1). 320 µl of sample was added to a focusing tray, followed by 17 cm IPG (immobilised pH gradient), pH 3-10 strips (Bio-Rad). 1.5 ml of mineral oil (Bio-Rad) was then overlaid on the strip and left overnight at room temperature to undergo passive rehydration. Isoelectric focusing was undertaken in a Protean isoelectric focusing IEF cell (Bio-Rad) using a three step ramping method as outlined in section 3.6.4.4.1. Once final focusing at 10,000 V reached 50,000-60,000 VH, strips were removed, drained of mineral oil and frozen at -75°C until ready for equilibration and second dimension SDS-PAGE.

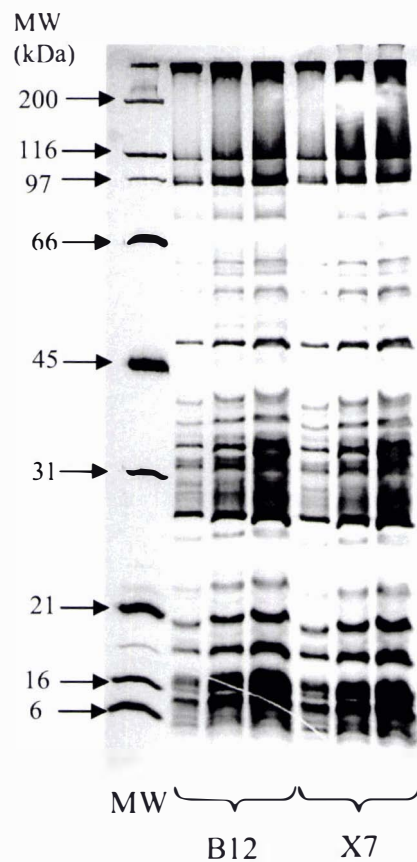
For the second dimension, strips were equilibrated as outlined in section 3.6.4.4.2, followed by separated on a 12.5% polyacrylamide gel using a Protean® II xi electrophoresis system (Bio-Rad). Gels were stained with SYPRO ruby (Bio-Rad) and laser scanned with a Typhoon 9400 laser scanner (Amersham, Biosciences) and saved as TIFF files. Imagemaster 2D software was used to compare gel images.

## 6.3 Results

### 6.3.1 One-dimensional SDS-PAGE of total & surface extracted proteins

#### 6.3.1.1 One-dimensional SDS-PAGE of total cell proteins

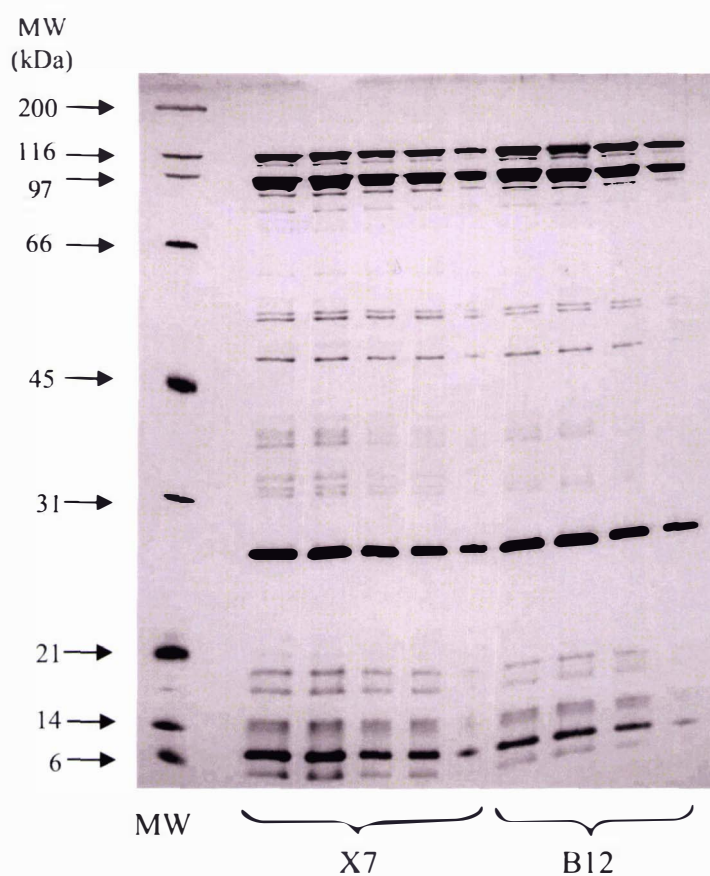
SDS-PAGE analysis of B12 and X7 total cell proteins, produced by sonication of B12 and X7 whole cells, revealed no differences in the banding patterns between the two strains (Fig 6.1).



**Fig 6.1:** Image of a typical SDS-PAGE of total cell proteins from B12 cells and X7 cells. B12 lanes contain 5, 10 and 15 µg of protein per well respectively. X7 lanes contain 5, 10 and 15 µg of protein per well respectively, with silver stain used to visualize protein bands.

6.3.1.2 One-dimensional SDS-PAGE of acid-glycine surface extracted proteins.

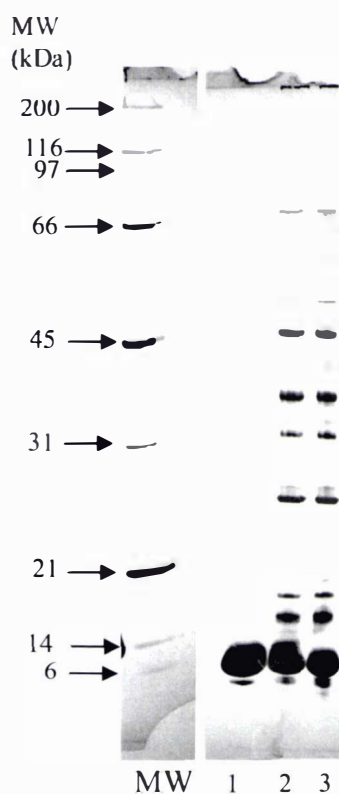
SDS-PAGE analysis of B12 and X7 acid –glycine surface extracted proteins revealed no differences in the banding patterns between the two strains (Fig 6.2).



**Fig 6.2:** Image of a typical SDS-PAGE of acid-glycine extracted surface proteins from X7 and B12. X7 lanes left to right contain 4.8, 4.8, 3.2, 3.2 and 1.6  $\mu\text{g}$  of protein per well respectively. B12 lanes left to right contain 4.8, 4.8, 3.2 and 1.6  $\mu\text{g}$  of protein per well respectively.

### 6.2.1.3 One-dimensional SDS-PAGE of lysozyme surface extracted proteins.

SDS-PAGE analysis of B12 and X7 lysozyme surface extracted proteins, demonstrated no differences in the banding patterns between the two strains (Fig 6.3).



**Fig 6.3:** Image of a typical SDS-PAGE of lysozyme extracted surface proteins from lysozyme control (lane 1), X7 (lane 2) and B12 (lane 3). Lane 2 & 3 contains approximately 10  $\mu\text{g}$  of protein. Lane 1 contains only lysozyme to act as a control and silver stain used to visualize protein bands.

#### *6.2.1.4 Summary of One-dimensional SDS-PAGE of total and surface extracted proteins*

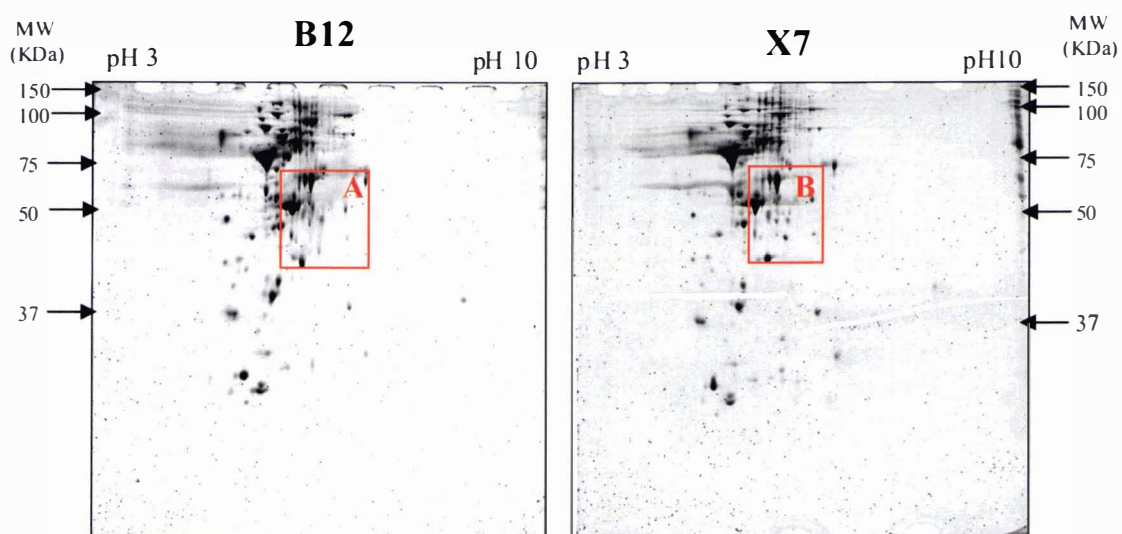
There was no obvious difference between the B12 and X7 total cell, acid-glycine and lysozyme extracted surface proteins, suggesting X7 is not lacking nor has gained any major surface proteins responsible for the different attachment rates of X7 and B12. However, one-dimensional SDS-PAGE can only differentiate between proteins with different molecular weights and has no ability to distinguish between proteins of similar size possessing a different charge or isoelectric point. With this in mind two-dimensional PAGE was undertaken in an attempt to separate proteins by size as well by charge giving greater resolution to complex protein mixtures.

### 6.3.2 Two-dimensional gel electrophoresis (2D-PAGE) of total and surface extracted proteins

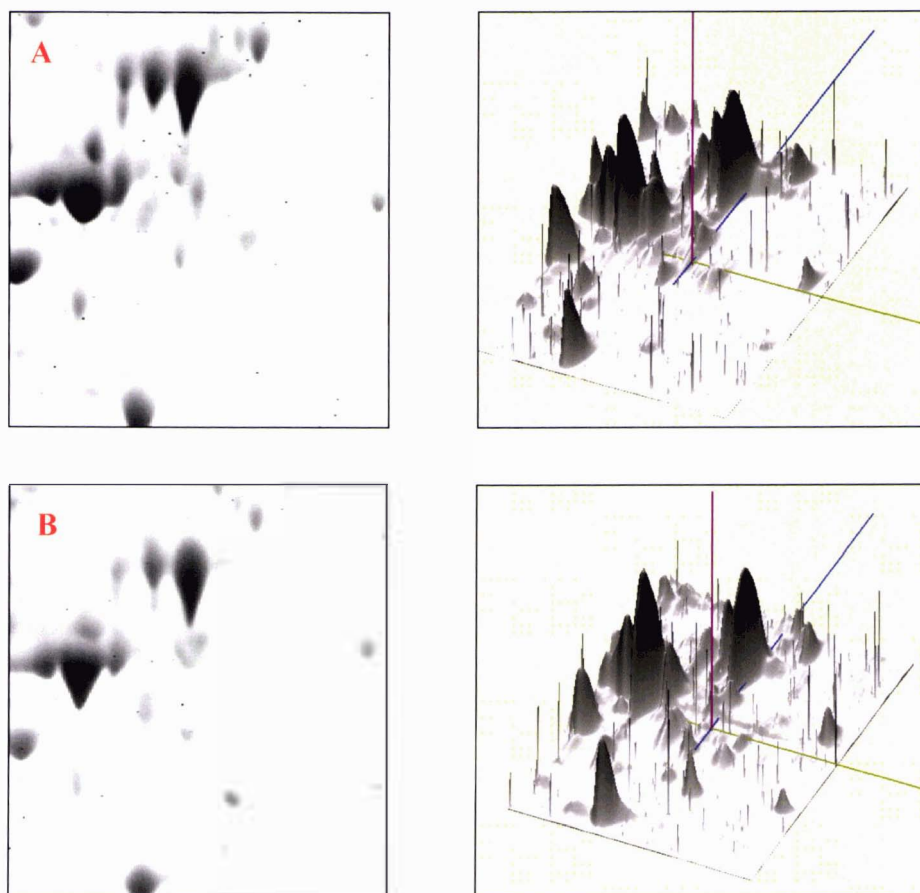
Two-dimensional gel electrophoresis remains one of the most popular techniques employed to study changes in protein expression. Protein samples are first separated in an immobilised pH gradient (IPG) gel strip until they reach a stationary position where their charge is zero. The pH at which the protein has zero net charge is called its isoelectric point (pI). The gel strip containing focused proteins is then transferred to an SDS-PAGE gel, the second dimension which separates proteins according to their molecular weight.

#### 6.3.1.1 Two-dimensional (2D-PAGE) of total cell proteins

2D-PAGE analysis of B12 and X7 total cell proteins, produced by sonication of B12 and X7 whole cells produced no major differences in protein expression (Fig 6.4). Red boxes labelled A and B outlined in Fig 6.4 were selected to demonstrate the use of SYPRO Ruby stain and Imagemaster software to analyse 2D-PAGE gels in Fig 6.5.



**Fig 6.4:** 2D-PAGE gels of whole cell protein extracts of B12 and X7 cells. 200  $\mu$ g of protein extract was run on a 17 cm, pH 3-10 strip, with the second dimension using a 12.5% SDS-PAGE gel, stained with Sypro Ruby and Laser scanned at 450 nm. Red squares (A and B) mark gel sections analysed in Fig 6.5 below.



**Fig 6.5:** Gel to gel comparisons of whole cell gel sections B12 (labelled A) and X7 (labelled B) and 3D images (representations of spot intensity) of the same gel sections using Imagemaster 2D software on the right column.

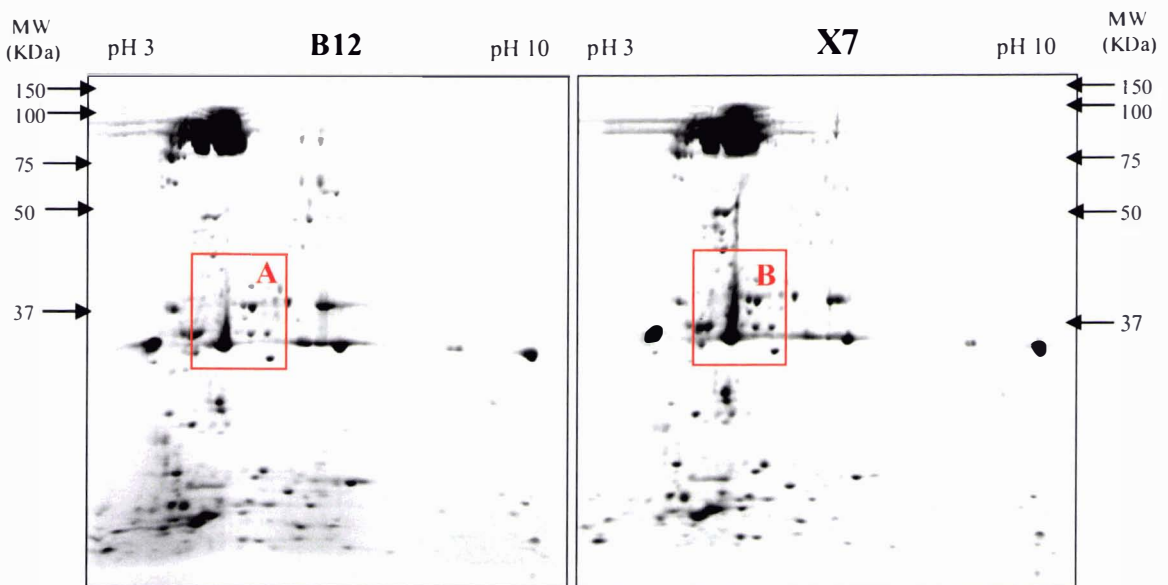
The use of Imagemaster 2D software enables the user to differentiate spot intensities on a gel and allows the user to view the spot intensities in three dimensions. The process involves selecting an area of interest on the gel, in this case the red region outlined in Fig 6.4. This area can then be observed (Fig 6.5 left column) and in a 3D diagram (Fig 6.5 right column). The height and width of the peaks in the 3D diagram is representative of the concentration and circumference of the gel spot respectively. The major benefits of using SYPRO Ruby stain are the sub-nanogram sensitivity equal to the best that silver staining techniques can provide (Tannu *et al.*, 2006). The other important benefit is that the stain is quantitative for a linear range that extends over three orders of magnitude in protein concentration (Nishihara & Champion, 2002). Silver stain in comparison has only

a limited quantitative range of about one order of magnitude. The large quantitative linear range offered by SYPRO Ruby makes it possible to compare protein expression levels accurately over a greater range of protein concentrations, allowing gel to gel comparisons with greater validity.

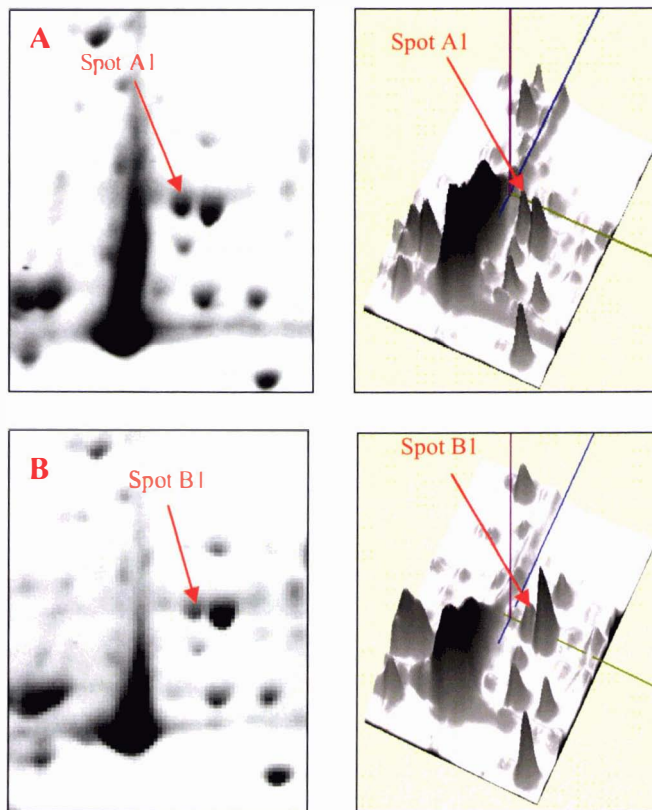
The analysis of whole cell extracts of B12 and X7 by 2D-PAGE gel electrophoresis produced no major differences in protein expression and repeatability between samples was good. 200  $\mu\text{g}$  of protein loaded on to each strip produced the best separation patterns, although strip manufacturers propose that protein loading of up to 300-400  $\mu\text{g}$  can be applied to each strip. The requirement for a lower loading in this work might be caused by the presence of interfering DNA or polysaccharides sometimes associated with whole cell extractions.

6.3.1.1 Two-dimensional (2D-PAGE) of acid-glycine surface extracted proteins.

2D-PAGE analysis of B12 and X7 acid-glycine surface extracted proteins produced no major differences in surface proteins (Fig 6.6). Red boxes labelled A and B outlined in Fig 6.6 demonstrate variation in spot intensity that can occur from sample to sample and highlights the importance that results were reproducible from different samples (Fig 6.7)



**Fig 6.6:** 2D-PAGE of acid-glycine surface protein extracts from B12 and X7 cells. 300µg of protein extract was run on a 17 cm, pH 3-10 strip, with the second dimension using a 12.5% SDS-PAGE gel, stained with SYPRO Ruby and laser scanned at 450 nm. Manually selected squares mark gel sections analysed in Fig 6.7 below.

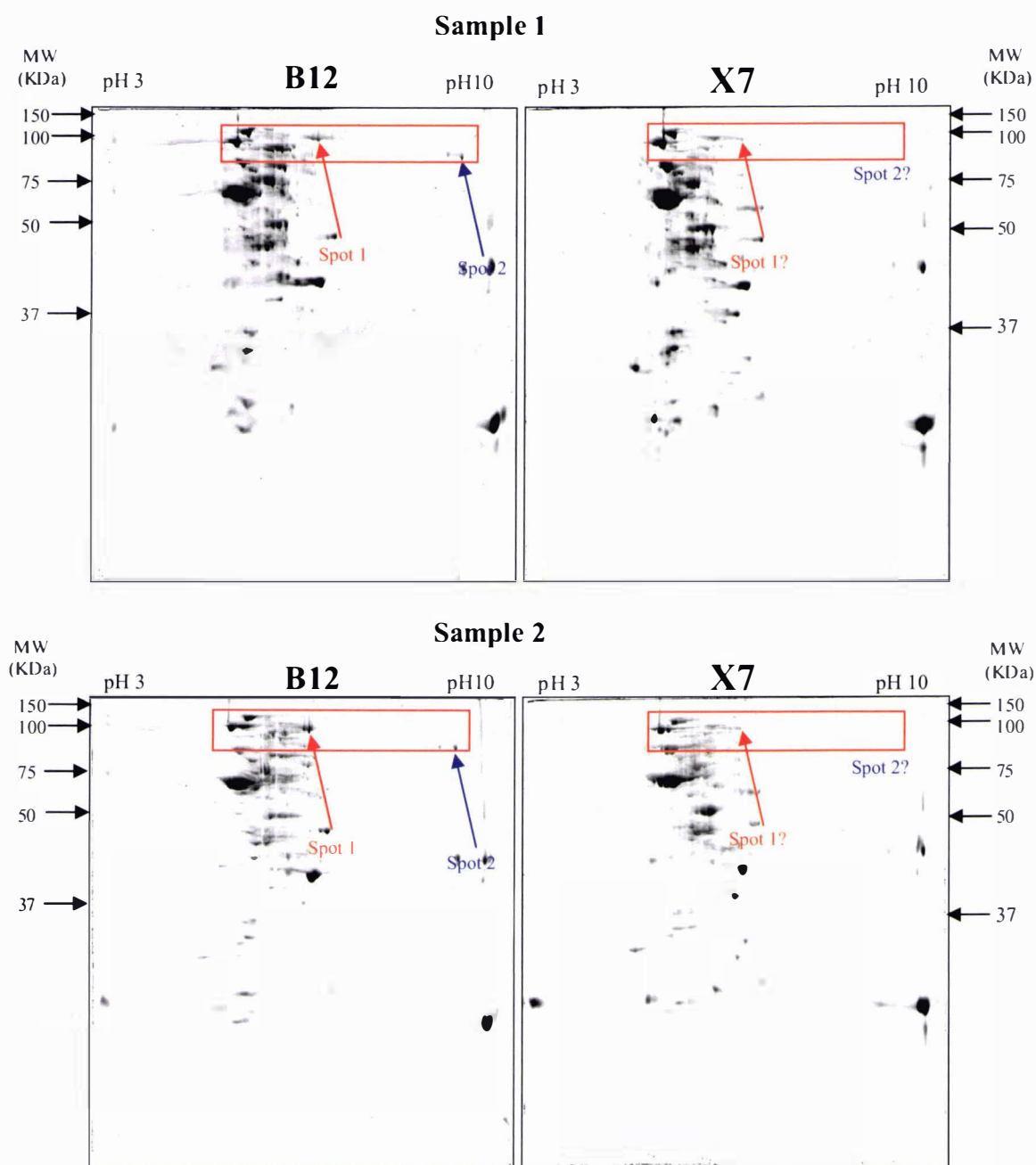


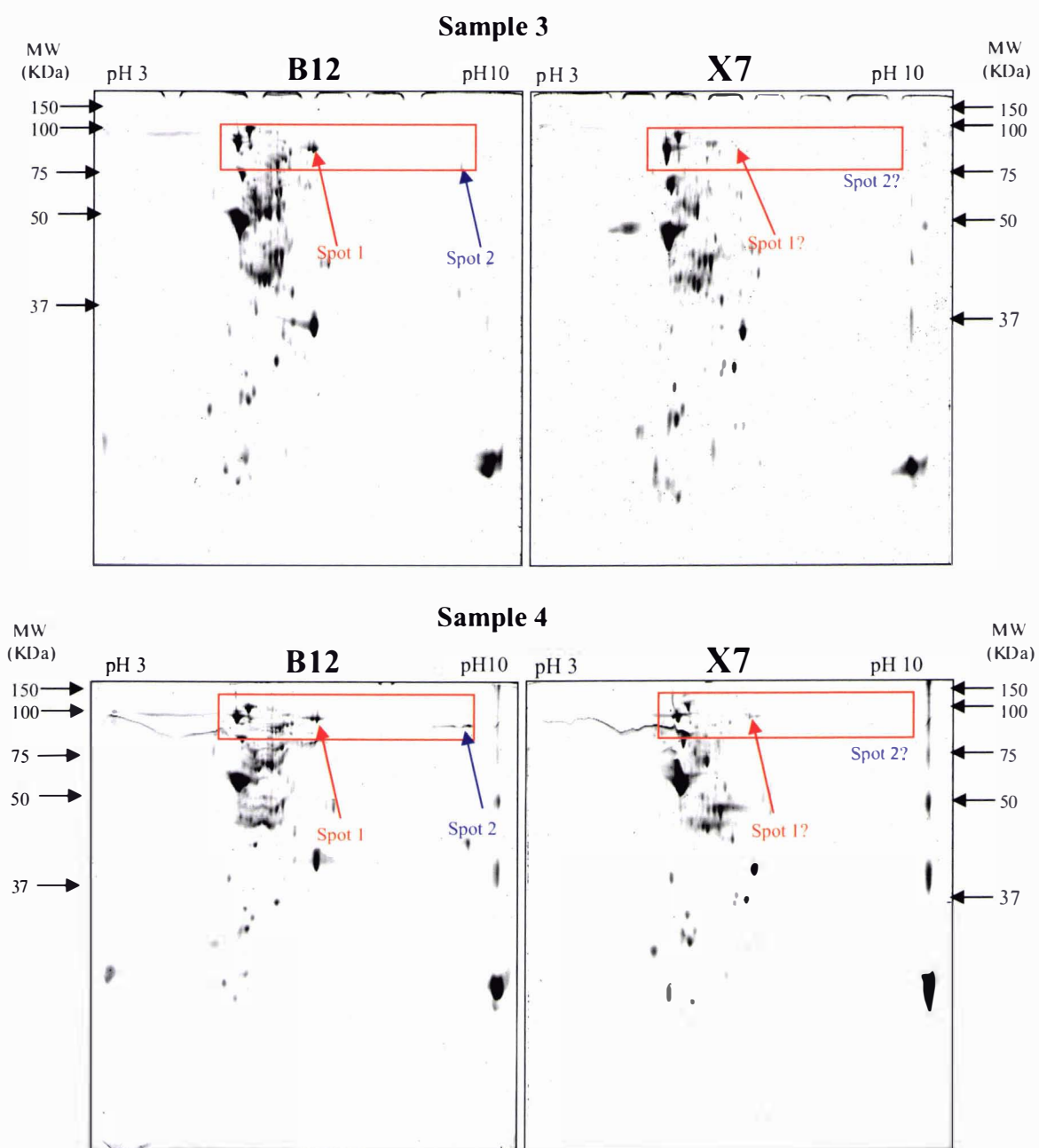
**Fig 6.7:** Comparisons of acid-glycine surface protein extracted gel sections B12 (labelled A) and X7 (labelled B) and 3D images of the same gel sections using Imagemaster 2D software on the right column. The acid-glycine sample strips were loaded with 300  $\mu\text{g}$  of protein, which is the maximum recommended by the strip manufactures.

Comparisons of acid-glycine surface extracted proteins from B12 and X7 cells revealed no major difference in the protein expression patterns (Fig 6.6). Although overall the level of reproducibility was high, a few spots did vary from sample to sample, but no spots were observed over the three separate samples that could suggest any major differences in protein expression between B12 and X7 cultures. One example of variation from sample to sample can be seen in Fig 6.7 with spot A1 from culture B12 appearing to be expressed at a higher level than the corresponding spot (B1) in culture X7. However, over the three samples tested, this minor difference was noted in only one sample, suggesting some sample to sample variability.

6.2.1.3 Two-dimensional SDS-PAGE of lysozyme/mutanolysin surface extracted proteins.

2D-PAGE analysis of B12 and X7 lysozyme/mutanolysin surface extracted proteins revealed two proteins present in higher concentration in the cell wall fraction of B12 compared with X7 (Red arrow for Spot 1 and Blue arrow Spot 2 Fig 6.8). Evaluation of Spot 1 and Spot 2 intensities between B12 and X7 (outlined in red boxes) are viewed in 3D images (Fig 6.9)

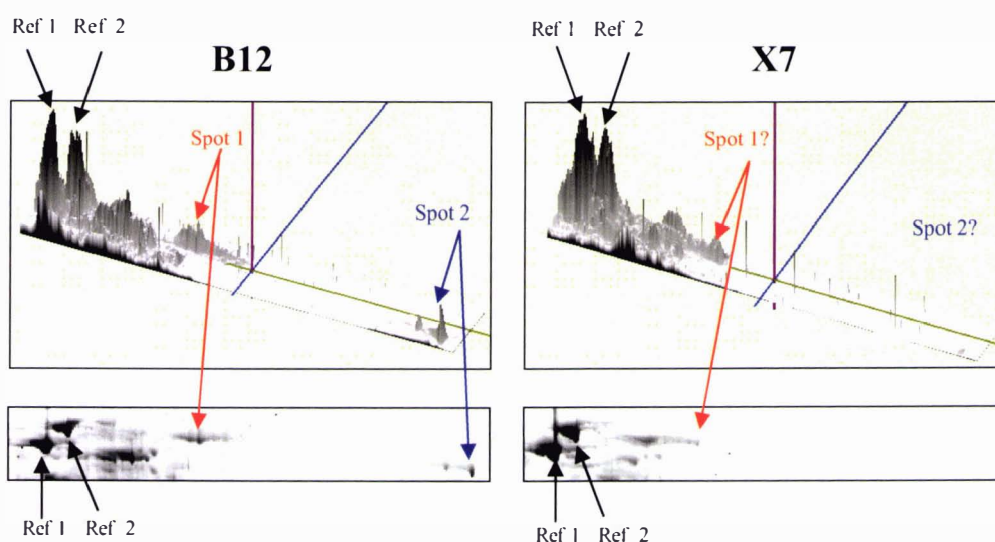




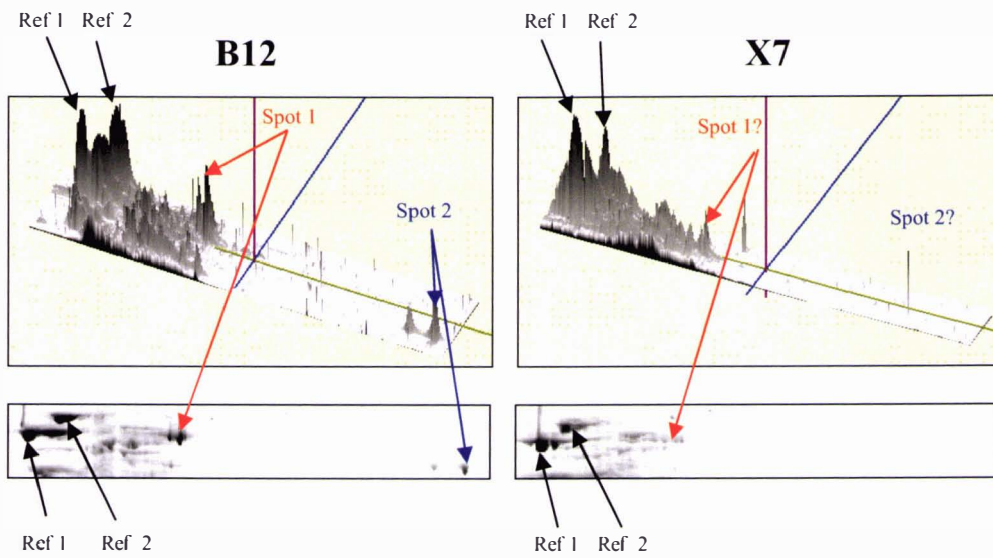
**Fig 6.8:** 2D-PAGE Comparisons of lysozyme/mutanolysin surface extracted protein gel sections from B12 and X7 from four separate samples. 300  $\mu$ g of protein extract was run on a 17 cm, pH 3-10 strip, with the second dimension using a 12.5% SDS-PAGE gel, stained with SYPRO Ruby and laser scanned at 450 nm. Arrows highlight differences between B12 and X7 that were reproducible in all four samples.

Comparisons of the lysozyme/mutanolysin extracts of surface proteins from strains B12 and X7 revealed two proteins that were consistently present at a higher concentration in strain B12, indicated by the square boxes in Fig 6.8. The gel regions containing these two proteins from four separate samples are highlighted in Fig 6.9 (gel Spot 1 and gel Spot 2). Reference Spots (Ref 1 & 2) serve as indicators of the relative protein loading between B12 and X7 samples, so changes in spot intensity, while surrounding spots remain unchanged, tends to indicate changes in protein levels. Upon closer analysis, gel Spot 1 appeared to consist of two components (Fig 6.9). The right hand component (labelled with red arrow) consistently showed a higher level intensity or concentration in strain B12, compared with strain X7 (Fig 6.9). From its position on the gels, the molecular weight and isoelectric point of Spot 1 is approximately 80,000 to 90,000Da and pH 6.5-7 respectively. Gel Spot 2 consistently showed a higher level of protein concentration in strain B12, compared with strain X7 (Fig 6.9). From its position on the gels, the molecular weight and isoelectric point of Spot 2 is approximately 75,000 to 85,000Da and pH 8.5-9 respectively.

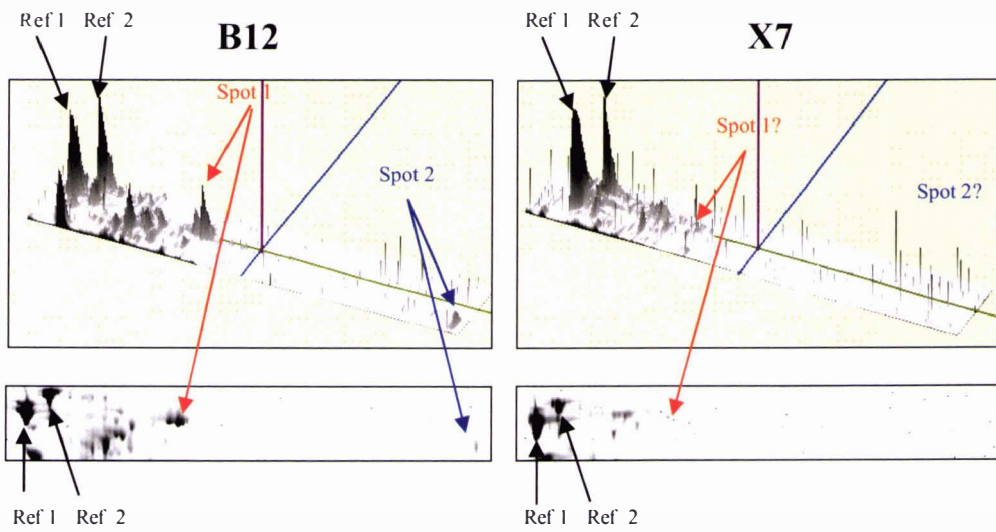
### Sample 1

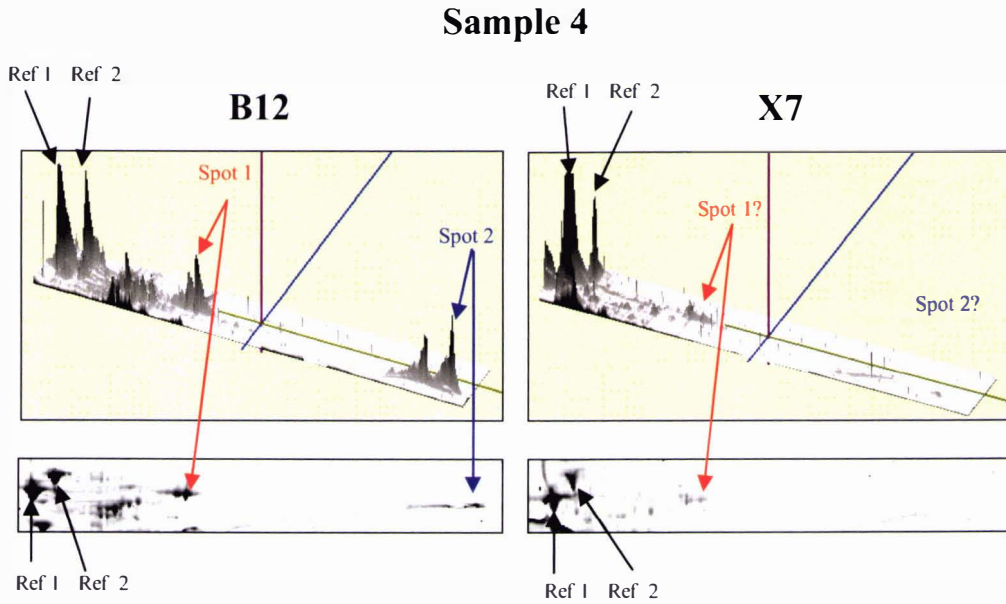


### Sample 2



### Sample 3





**Fig 6.9:** The increased concentration of Spot 1 (Red arrows) and Spot 2 (Blue arrows) in B12 cultures compared with X7 cultures over four separate samples. Black arrows point to reference Spots that indicate relative protein loading between B12 and X7 samples.

## 6.2.1.3.1 Validation of Lysozyme/Mutanolysin extraction

To ensure cells had remained intact during lysozyme/mutanolysin surface protein extraction, the cell supernatant was assayed for the cytoplasmic enzyme Aminopeptidase C (Schaumburg *et al.*, 2004) and  $\beta$ -Galactosidase (Steinmoen *et al.*, 2002) (Section 3.6.2.5). Results suggest that no major cytoplasmic contamination was taking place during treatment, with treated cells having similar levels of cytoplasmic contamination as non-treated cells over the five-hour treatment period and little difference in the rate of cell lysis between B12 and X7 (Table 6.0 and 6.1).

**Table 6.0:** Aminopeptidase C assay of lysozyme/mutanolysin surface extractions as a marker of cytoplasmic contamination over a 5 hr period of cell exposure to lysozyme/mutanolysin. Sonicated cells of B12 and X7 were used as positive controls for the assay. B12 and X7 controls contained no lysozyme/mutanolysin enzyme and were used to measure spontaneous cell lysis over the treatment period. Results were expressed as absorbance values at 405nm.

	B12 Lysozyme	X7 Lysozyme	B12 control	X7 control	B12 sonicated	X7 sonicated
10 minutes	0.022	0.021	0.008	0.007	0.38	0.37
3 hours	0.022	0.031	0.02	0.022	0.353	0.362
5 hours	0.025	0.036	0.019	0.023	0.341	0.345

**Table 6.1:**  $\beta$ -Galactosidase assay of lysozyme/mutanolysin surface extractions as a marker of cytoplasmic contamination over a 5 hr period. Sonicated cells of B12 and X7 were used as positive controls for the assay. B12 and X7 controls contained no lysozyme/mutanolysin enzyme and were used to measure spontaneous cell lysis over the treatment period. Results were expressed as absorbance values at 420nm.

	B12 Lysozyme	X7 Lysozyme	B12 Control	X7 Control	B12 Sonicated	X7 Sonicated
10 minutes	0.011	0.011	0.008	0.009	0.812	0.791
3 hours	0.017	0.018	0.009	0.011	0.929	0.954
5 hours	0.026	0.026	0.015	0.016	0.824	0.867

## 6.4 Discussion

Previous results from Chapter 5 suggested that surface proteins played an important role in the attachment of strains B12 and X7 to stainless steel surfaces, especially in the case of strain B12. Therefore, any differences in the total and/or surface protein composition could be responsible for the observed difference in attachment of the two strains. Consequently, the objective of this chapter was to identify by gel electrophoresis any differences in the total protein and/or surface protein composition of B12 and X7.

Initially, total cell proteins from both B12 and X7 were separated on a one-dimensional SDS-PAGE gel (Fig 6.1). The results produced no differences in the protein expression patterns between the two strains. One reason for this could be the relative abundance of proteins present in total protein samples, where 130 polypeptides can account for 70% of the protein mass of a typical cell (Herendeen *et al.*, 1979). Therefore, low abundance, but potentially important proteins may not be present in sufficient quantity to be detected by staining methods.

The extraction of bacterial surface proteins by treatment of cells with acid-glycine has been reported by several groups working with *Campylobacter* (Dubreuil *et al.*, 1988; Kervella *et al.*, 1993) and *Clostridium* (Wright *et al.*, 2005; Calabi *et al.*, 2002). The mechanism by which acid-glycine treatment removes surface proteins is generally not discussed by most authors, but is most probably due to the zwitterionic nature of the glycine molecule (Matubayasi *et al.*, 2003). Treatment of B12 and X7 cells with acid-glycine resulted in a reduction in the attachment of both strains to stainless steel (Fig 5.9), suggesting that acid-glycine treatment removed or modified some important surface proteins associated with the attachment of B12 and X7. However, in this study the acid-glycine proteins extracted and separated on a one-dimensional SDS-PAGE gel showed no variation in patterns between the two strains (Fig 6.2).

Surface protein extraction using lysozyme and/or mutanolysin, both of which are muramidases, dissolves bacterial cell wall mucopolysaccharides by hydrolyzing the  $\beta$ (1-

4) linkages between N-acetyl-amino-2-deoxy-D-glucose residues (Windholz, 1983), thus releasing proteins attached or embedded in this important bacterial surface layer. Mutanolysin enzyme will degrade the walls of *Streptococcus* and some *Bacillus* species that are resistant to egg white lysozyme (Heckels & Virji, 1988). Muramidase enzymes has been used by many groups in the past to degrade bacterial cell walls and to isolate surface proteins from *Lactobacillus* (Kelly *et al.*, 2005), *Bacillus* (Chung *et al.*, 1998) and *Pseudomonas* (Seyer *et al.*, 2005). Most research groups also add 15-20% sucrose to act as an osmotic protective agent during cell wall degradation. Furthermore, Coolbear *et al.* (1992) observed that the addition of 10 mM MgCl<sub>2</sub> resulted in decreased lysis of protoplasts formed as a result of lysozyme treatment of *Lactococcus* cells.

Results from total cell proteins and surface protein extraction methods, acid-glycine and lysozyme/mutanolysin analysis by one-dimensional SDS-PAGE gel of B12 and X7 cells, produced no obvious differences between the two strains. One limiting factor associated with the separation of proteins using one-dimensional SDS-PAGE is the case when one protein band could be composed of multiple proteins that by coincidence share the same or similar molecular weight, as one-dimensional SDS-PAGE resolves on the basis of size only, not on hydrophobicity or charge. However, previous results from Section 5.3.5 indicated that surface proteins are the most important factor in the attachment of strain B12 to stainless steel. Further analysis of surface proteins using more powerful protein analysis methods was therefore considered warranted.

In order to separate complex protein mixes with greater resolution, two-dimensional PAGE (2D-PAGE) was employed. 2D-PAGE is capable of separating proteins according to their charge as well as size. The high resolving power of the 2D-PAGE technique allows the analysis of complex mixtures of proteins. In addition, continuing refinements to 2D-PAGE protocols over the last decade, such as the now commonly used standard immobilized pH gradients, enable the user to resolve thousands of proteins on a single gel and when combined with mass spectrometry. This is why 2D PAGE was described by Görg *et al.* (2004) as the work horse of proteomics. However, the 2D-PAGE method can

under represent hydrophobic proteins present in a sample and may be unable to separate proteins with very acidic or alkaline isoelectric points (Westermeier and Naven, 2002).

Sample preparation of all three sample types (total protein, acid-glycine and lysozyme/mutanolysin surface extractions) for 2D-PAGE required some optimization, owing to the sensitivity of the 2D-PAGE technique to contaminating salts and polysaccharides in samples (Vandahl *et al.*, 2005). In this study, ice-cold acetone precipitation was employed to concentrate proteins and remove contaminating salts and polysaccharides. The use of trichloroacetic acid (TCA) combined with acetone is often recommended (Vandahl *et al.*, 2005). However, in this study, proteins precipitated with the TCA method proved difficult to solubilise into rehydration/sample solution.

Manual assessment of 2D PAGE gels of B12 and X7 total cell proteins and acid-glycine surface extracted proteins revealed no repeatable differences in the protein expression. SYPRO Ruby gel staining with Imagemaster software also found no differences in protein expression. Fig 6.7 provides a typical example of the sample to sample variation that can occur in 2D-PAGE systems. This emphasises the importance of multiple sample reproducibility in 2D-PAGE systems raised by Cahill and Nordheim, (2001).

Comparison of surface protein profiles of B12 and X7 by lysozyme/mutanolysin extraction showed two spots consistently present at a higher concentration in B12 compared with X7. The molecular weight and isoelectric point of Spot 1 (Fig 6.8) was approximately 80,000 to 90,000Da and pH 6.5-7 respectively. Concurrently, Spot 2 (Fig 6.8) was estimated to have a molecular weight of approximately 75,000 to 85,000Da and isoelectric point between pH 8.5-9. We believe that this is the first report of the 2D-PAGE technique being used to compare an isogenic mutant strain (X7), with reduced ability to attach to stainless steel against its parental strain (B12), in an attempt to identify surface proteins associated in the attachment of these bacterial to abiotic surfaces. However, Siciliano *et al.* (2008) recently used 2D-PAGE to undertake a comparative study of an aggregative *Lactobacillus crispatus* strain compared with a spontaneous isogenic mutant lacking the aggregative phenotype. Siciliano *et al.* (2008) noted that the

mutant strain expressed higher levels of enzymes involved in carbohydrate metabolism and transport and concluded that cells with a higher metabolic rate (i.e. mutant strain) are less likely to aggregate due to nutrient limitation.

Several authors have reported protein expression changes occurring during biofilm formation, using proteomics as a tool to measure these changes (Sauer *et al.*, 2002; Welin *et al.*, 2004; Trémoulet *et al.*, 2002). Sauer *et al.* (2002) concluded that over 50% of the *Pseudomonas aeruginosa* proteome demonstrated a change in expression levels when planktonic cells were compared with mature biofilms cells. O'Toole *et al.* (2000) suggested that biofilm formation is a process of microbial development not unlike bacterial spore formation. The question remains “Could B12 be permanently expressing a biofilm phenotype and X7 a planktonic phenotype?” The identification of proteins from lysozyme/mutanolysin surface extracts of B12 and X7 (labelled Spot one and Spot two, Fig 6.8) could help identify important surface proteins involved in the attachment process of these strains.

The link between surface proteins and their role in attachment of bacteria to solid surfaces has been well established and one of the most well studied bacteria in this area is *Staphylococcus aureus*, where multiple surface proteins have been implicated in the ability of this organism to attach to solid surfaces. Cucarella *et al.* (2001) reported a 254 kDa size surface protein termed BAP (Biofilm Associated protein) from *S. aureus* as a possible attachment protein. Furthermore, Tormo *et al.* (2005) disrupted the BAP gene of *S. aureus* and severely handicapped its capacity to form a biofilm. In addition, complementation studies with a biofilm negative strain of *S. aureus* with the BAP gene conferred the ability to form a biofilm, providing strong evidence for the importance of the BAP gene in biofilm formation. Other surface proteins from *S. epidermidis* associated with attachment to solid surfaces include a 280 kDa protein reported by Veenstra *et al.* (1996) and a 60 kDa protein described by Heilmann *et al.* (1997). Other bacterial groups with surface proteins involved in attachment to solid surfaces include *Listeria monocytogenes* (Auvray *et al.*, 2007), *Vibrio cholerae* (Watnick & Kolter, 1999),

*Enterococcus faecalis* (Tendolkar *et al.*, 2004), *Streptococcus thermophilus* (Flint *et al.*, 1997) and *Erysipelothrix rhusiopathiae* (Shimoji *et al.*, 2003).

One important aspect overlooked during extraction of cell surface proteins by research groups, is cytoplasmic contamination (Resch *et al.*, 2006; Hughes *et al.*, 2002). In the present study, Aminopeptidase C assay, as reported by Schaumburg *et al.* (2004), and  $\beta$ -galactosidase as described by Steinmoen *et al.* (2002) was used as a marker for cytoplasmic contamination during lysozyme/mutanolysin extraction of surface proteins from B12 and X7 cells. B12 cells treated with lysozyme/mutanolysin showed no increase in Aminopeptidase C or  $\beta$ -galactosidase activity in the supernatant compared with X7 cells, suggesting B12 is not showing a higher rate of cell lysis compared with X7. Thus it appears unlikely the higher concentrations of Spot 1 and 2 observed in B12 extracts are due to differences in the cell lysis rate between B12 and X7.

## **6.5 Conclusion**

Lysozyme/mutanolysin extracts of surface proteins from strains B12 and X7 revealed that two proteins were consistently present at a higher concentration on the surface of strain B12 compared with X7. It is possible that the higher concentration of the two proteins in the cell wall of B12 could be responsible for the observed difference in the attachment of B12 and X7. Further work was undertaken to identify Spot 1 and Spot 2 in an attempt to understand the role Spot 1 and Spot 2 may play in the attachment process of B12 and is reported in Chapter 7.

# Identification and Cloning of Proteins Found to be Present at Reduced Levels on the Surface of Mutant X7 Compared with its Parental Strain B12

## **7.1 Introduction**

## **7.2 Procedures**

## **7.3 Results**

**7.3.1 Identification of gel spots 1 & 2 by electrospray ionization quadrupole time-of-flight (ESI-QUAD-TOF)**

**7.3.2 PCR primer design by bioinformatic analysis of Formate acetyltransferase ORF from related bacteria**

**7.3.3 PCR amplification and cloning of the partial Formate acetyltransferase gene from B12 and X7**

**7.3.4 Bioinformatic analysis of Formate acetyltransferase DNA and predicted protein sequences from B12 and X7 sequences**

**7.3.5 Levels of Formate acetyltransferase from total cell proteins of B12 and X7.**

## **7.4 Discussion**

## 7.1 Introduction

Proteomics has been greatly advanced with development of mass spectrometry methods and is now regarded as indispensable technology in interpreting information encoded in genomes (Aebersold and Mann, 2003). Mass spectrometry is a technique that separates ions on the basis of mass and can provide both mass and internal sequence information on a molecule or protein, unlike N-terminal protein (Edman) sequencing that can provide only limited sequence information. Many proteins are “blocked” to Edman sequencing due to modification of the N-terminal amino group thus making the sequencing process technically difficult (Patterson *et al.*, 2001). On the other hand, protein sequencing by mass spectrometry provides internal sequence data from different regions of the protein, which can be used to design degenerate oligonucleotide primers to assist in gene cloning efforts. Mass spectrometry typically involves three steps: 1) ionisation of the molecule; 2) separation of the ions on the basis of their mass to charge ratio; and 3) detection of separated ions.

Electrospray ionisation (ESI) and matrix-assisted laser desorption/ionisations (MALDI) are two techniques most commonly used convert ions into a gaseous state for mass analysis. Following ionisation, the ions are separated according to their mass:charge ratio ( $m/z$ ) within a vacuum or electrical field (Westermeier and Naven, 2002). The most commonly used mass analysers include Time-Of-Flight (TOF) and Quadrupole mass analysers.

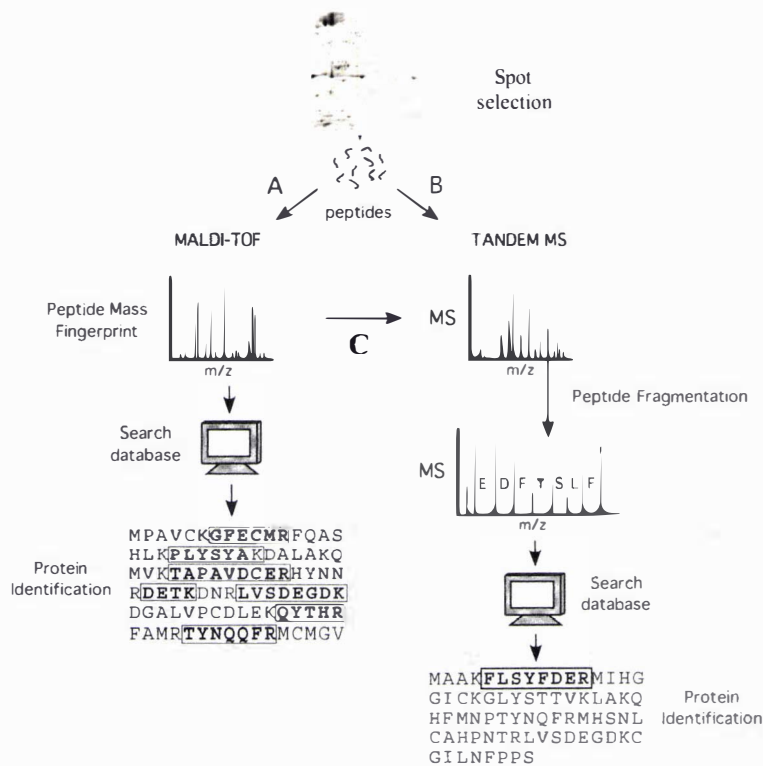
In TOF analysers, each ion is accelerated into a vacuum and maintains the constant velocity it acquired during acceleration. During this process, ions with a low mass-to-charge ratio travel more rapidly than ions with a high mass-to-charge ratio (Kinter, 2000). This allows the calculation of the mass-to-charge ratios based on their time of arrival at the detector.

In a Quadrupole mass analyser, electrical fields are used to separate ions according to their mass-to-charge ratio. By varying the strength and frequencies of the electrical fields, it is possible to induce a stable trajectory for an ion of given mass-to-charge ratio. This allows it to pass through the mass analyser to the detector, while all other ions are not detected. Small incremental changes to the electrical field allow all ions to be detected in a sample (Kinter, 2000).

Gel electrophoresis is one of the most common methods utilized in protein separation and when 2D-PAGE is employed, complex protein mixes containing thousands of proteins can be resolved on a single gel (Graves and Haystead, 2003). After gel staining, spots of interest can be cut out from the gel and then proteins within the excised gel can be digested with proteolytic enzymes such as trypsin to produce a multiple peptide fragment mix of an appropriate size for mass spectrometric analysis. These multiple peptide fragments are then analysed by mass spectrometry to obtain a peptide mass fingerprint, usually by MALDI-TOF analysis (labelled A in Fig 7.1). The peptide mass fingerprint is then searched against a database of peptide sequences based on the peptide profiles that would have been obtained following protein digestion by various proteolytic enzymes and the resulting peptide masses calculated. A protein is identified when a number of peptides from the digested sample match a number of theoretical peptides in the database (Fig 7.1). Most databases use algorithms that take the mass accuracy and the percentage of the protein sequence covered into account and attempt to calculate a level of confidence for a match (Berndt *et al.*, 1999; Eriksson and Fenyö, 2002).

A more accurate method of protein identification is the use of tandem mass spectrometry (MS/MS), sometimes referred to as collision induced spectra (CID). This results in not only peptide mass data, but also provides information about peptide sequence (Aebersold and Mann, 2003; Graves and Haystead, 2003). In this method, a specific peptide fragment can be selected to undergo further fragmentation in a collision chamber, resulting in a series of peptide ions, as most fragmentation occurs at the peptide bond. The fragmented peptide series then undergoes a second mass spectrometry analysis (labelled B in Fig 7.1). The mass of each fragment generated can be used to determine the amino acid sequence of the peptide.

A number of programs exist on websites that allow the user to search and fit a set of mass spectrometry peaks and search for peptides whose masses are identical or similar. MASCOT is a probability-based online search engine that allows the user to search the sequence database with peptide molecular weights obtained by enzymatic digestion of a protein, commonly called a peptide mass fingerprint. Sequence information can also be obtained from MS/MS data using the MASCOT search engine; the result is commonly called MS/MS Ion Search. Matches obtained with MS/MS data from a number of peptides from a single protein increase the confidence level for the protein identification (Aebersold and Mann, 2003; Graves and Haystead, 2003).



**Fig 7.1:** Protein identification by mass spectrometry. (A) Protein identification by matrix-assisted laser desorption/ionisation–time of flight (MALDI-TOF). Digestion of proteins with proteolytic enzymes into peptide fragments and then analysis by MALDI-TOF to measure the masses of the peptides is known as peptide mass fingerprinting. The peptide masses are then searched against the

peptide mass maps generated from the theoretical digest of proteins in a database. (B or C) In tandem mass spectrometry one peptide fragment is directed into a collision chamber for fragmentation. Mass analysis of the peptide fragments generated can be used to determine the amino acid sequence of the peptide (adapted from Graves and Haystead, 2003).

The identification of a single sequence or pieces of a single sequence itself is not particularly informative. However, finding similarities from sequences in existing data bases provides a powerful way to infer the function of the protein or nucleotide sequence. The Basic Local Alignment Search Tool (BLAST) can be used to determine sequence similarity. BLAST is a very powerful tool that allows the user to find sequences that share regions of homology, with the addition of statistical significance of each match, referred to as E-value. In general, the smaller the E-value, the better the alignment. For example, closely related alignments may have E-values of  $10^{-20}$  or less (Ma, 2008). The BLAST program allows the user to compare not only protein to protein and nucleotide to nucleotide similarities, but also allows the researcher to search with and against translated nucleotide queries in all three reading frames for one nucleotide strand and in all three reading frames for the opposite DNA strand. The BLAST options are outlined in Table 7.1

**Table 7.1:** Various BLAST search programs available

<b>blastp</b>	Amino acid query against a protein sequence database
<b>blastn</b>	Nucleotide query against a nucleotide database
<b>blastx</b>	Translated nucleotide query in all six reading frames, against a protein sequence database
<b>tblastn</b>	Amino acid query against a translated nucleotide database in all six reading frames
<b>tblastx</b>	Translated nucleotide query in all six reading frames against a translated nucleotide database in all six reading frames

Results from a BLAST search can allow the rapid prediction of functional and evolutionary relationships of sequences.

The ability to undertake multiple sequence alignments of DNA or proteins is an important tool in modern molecular biology and a cornerstone of bioinformatics. The ClustalW program is one of the most widely used multiple sequence alignment programs and is freely available for use. To use the ClustalW program, one must collect a set of protein or DNA sequences, commonly obtained from a database search. Applications include structural and functional predictions of genes and proteins, analysis of conserved regions within proteins to identify amino acids that are important for function or structural integrity of a protein, as well as assessing evolutionary relationships (Zvelebil and Baum, 2008).

The combination of mass spectrometry and DNA sequencing technology, mixed with the ever-expanding discipline of bioinformatics, now allows the modern researcher to identify most proteins of interest as well as clone and sequence the DNA encoding the protein sequence.

The objective of the work described in this chapter was to identify and sequence two protein spots from strain B12 that were noted in chapter 6 to occur at a lower concentration on the surface of strain X7. This would involve comparing the peptide sequences to the database in an attempt to find homologous proteins along with amplifying and cloning genes encoding spot 1 and spot 2.

## **7.2 Procedures**

In order to identify two protein spots singled out in two-dimensional PAGE of lysozyme/mutanolysin extracted proteins (chapter 6), the protein spots were removed and sent to Bioanalytical Mass Spectrometry in Australia. There the spots were subjected to trypsin digestion and mass spectrometry analysis (ESI-QUAD-TOF), followed by peptide identification using the MASCOT algorithm database search.

To clone and sequence the DNA encoding proteins identified by mass spectrometry, PCR primers were designed by comparing DNA sequences from five closely related bacterial species using ClustalW2. Conserved regions of DNA providing extensive coverage of the gene were chosen as PCR primers to amplify most of the Open Reading Frame (ORF) of Formate acetyltransferase from B12 and X7.

The PCR product containing most of the ORF was then cloned into the pCR<sup>®</sup>4-TOPO plasmid vector (Invitrogen), which was then transformed into *E.coli* One Shot<sup>®</sup> TOP 10 cells. Plasmids containing inserts were isolated using the PureLink<sup>™</sup> Quick Plasmid Miniprep Kit (Invitrogen) and sent for DNA sequencing.

DNA sequences of the ORF were compared with other DNA sequences and predicted amino acid sequences were compared back to peptides sequences identified by mass spectrometry (tandem MS/MS by ESI-QUAD-TOF).

In order to establish whether the expression levels of the protein were different in total cell proteins of B12 and X7, the levels of protein from two-dimensional SDS-PAGE of total cell proteins from B12 and X7 were compared using imagemaster 6.0 software.

## 7.3 Results

### 7.3.1 Identification of gel spots 1 & 2 by Electrospray ionization quadrupole time-of-flight (ESI-QUAD-TOF)

To identify the two surface proteins present at reduced levels in *Anoxybacillus flavithermus* strain X7 compared with B12, both spots were manually excised from the 2D PAGE gels and sent to the Bioanalytical Mass Spectrometry Facility (UNSW, Sydney, Australia) for mass spectrometry analysis (tandem MS/MS by ESI-QUAD-TOF), where proteins were digested by trypsin into peptides before analysis by mass spectrometry. Protein identification was performed using the MASCOT algorithm.

As a control, the lysozyme enzyme used to extract surface proteins was identified on the two-dimensional gels by its theoretical molecular weight and isoelectric point. This lysozyme spot was also manually excised and sent for mass spectrometry analysis and was successfully identified as lysozyme. A blank sample of the gel containing no visible spots was also sent for mass spectrometry to monitor background contamination and was found to contain only traces of common contaminant, human keratin.

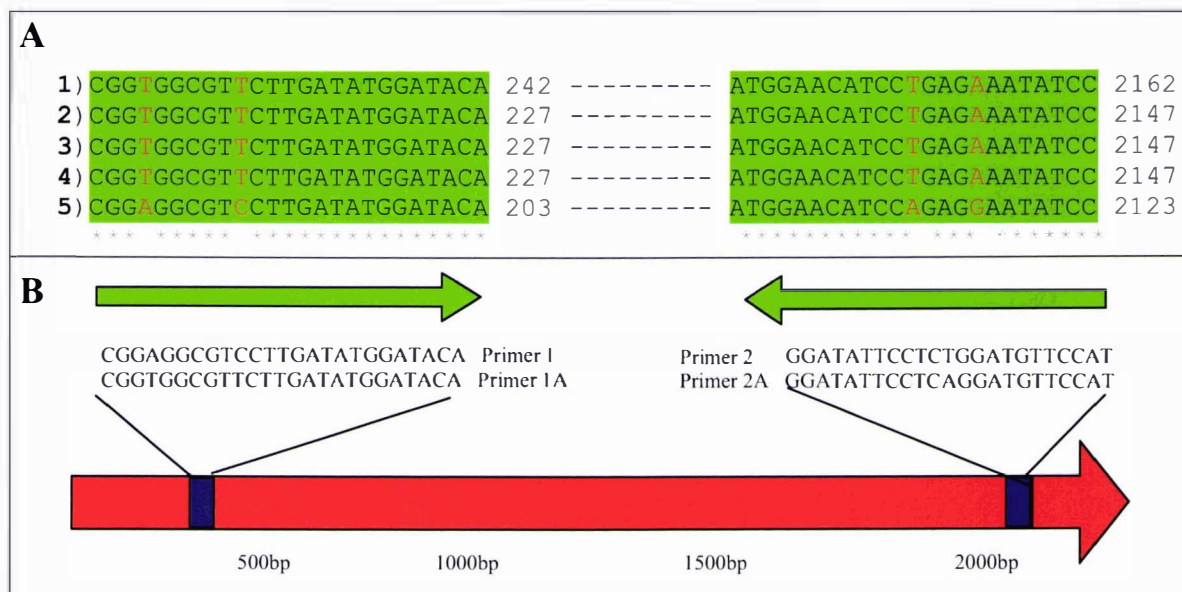
Mass spectrometry analysis of Spot 1 from B12 lysozyme/mutalysin extraction (Fig 6.8 and 6.9) provided eight hits against the bacterial enzyme Formate acetyltransferase, also known as Pyruvate formate lyase, with Probability Based MOWSE Score above 52. This indicates identity or extensive homology ( $p < 0.05$ ). The peptides showing homology to Formate acetyltransferase are listed in Appendix A2.1. The molecular weight of the eight Formate acetyltransferase enzymes containing peptides with similarity to Spot 1 ranged from 77,549 Da to 84,744 Da. Similarly, Spot 1 was estimated to have a molecular weight of 80,000 to 90,000 Da.

In contrast, mass spectrometry analysis of Spot 2 (Fig 6.8 and 6.9) from B12 lysozyme/mutalysin extraction gel was unable to identify this protein using the MASCOT algorithm when compared with all known protein sequences within the MASCOT database

and to sequences from only *Bacillus subtilis*. Peptides present in Spot 2 are listed in Appendix A2.2.

### **7.3.2 PCR primer design by bioinformatic analysis of Formate acetyltransferase ORF from related bacteria**

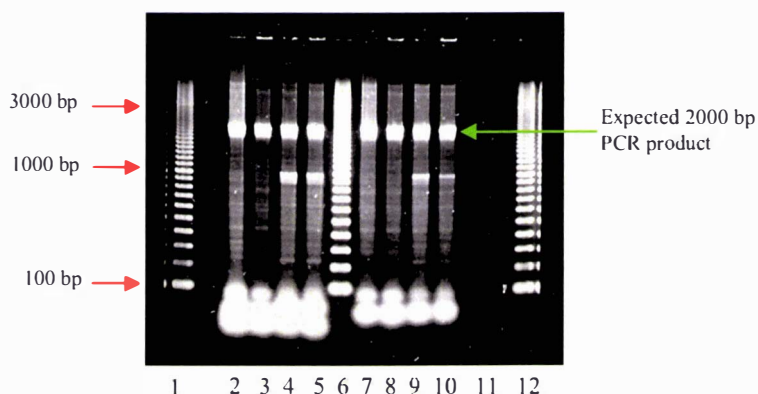
To sequence the gene encoding the ORF for Formate acetyltransferase from B12 and X7, PCR primers were designed by comparing DNA sequences of the Formate acetyltransferase gene from five strains of the Genus *Bacillus*. The downloaded sequences were compared using ClustalW2 (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>) (see Appendix A3 for complete sequence alignment). PCR primers were designed against areas showing extensive DNA homology as well as providing extensive coverage of the Formate acetyltransferase gene (Fig 7.2).



**Fig 7.2:** Amplification of Formate acetyltransferase ORF from B12 and X7. **(A)** DNA sequences of Formate acetyltransferase gene from five *Bacillus* species, showing extensive homology which were used to design PCR primers. 1) *Bacillus thuringiensis* str. Al Hakam, 2) *Bacillus anthracis* str. Sterne, 3) *Bacillus cereus* ATCC 10987, 4) *Bacillus thuringiensis* serovar konkukian, 5) *Bacillus licheniformis* ATCC 14580. **(B)** Direct PCR primers designed from the sequence comparison of five *Bacillus* species and the predicted position of the primers in the Formate acetyltransferase ORF from B12 and X7.

### 7.3.3 PCR amplification and cloning of the partial Formate acetyltransferase gene from B12 and X7

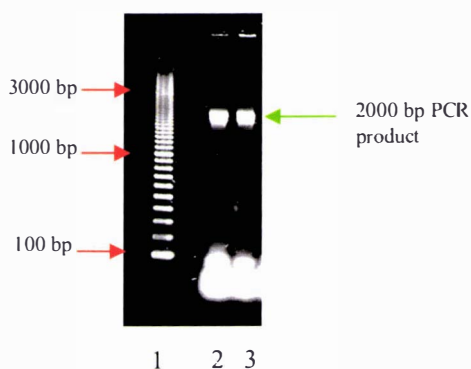
The PCR primers outlined in Fig 7.2 were used to amplify B12 and X7 genomic DNA at an annealing temperature of 55°C using all possible combinations of the four different primers. PCR products from all four combinations of primers (primers 1, 1A, 2 and 2A) and both B12 and X7 cultures were separated by gel electrophoresis. One large band with an estimated size of about 2000 base pairs (bp) (green arrow on Fig 7.3), which was the expected size of a PCR product derived from the Formate acetyltransferase ORF, was the dominant product. However, the use of primer 1A with either primer 2 or 2A also produced an additional product of around 1000 bp in size. As a consequence primer 1 and primer 2 were used in future PCR reactions to limit contamination of the PCR reaction (Fig 7.3).



**Fig 7.3:** Agarose gel electrophoresis of PCR products from the amplification of part of the Formate acetyltransferase gene. The green arrow represents a PCR product, which matches the expected size of the partial Formate acetyltransferase gene.

**Lane 1** - molecular weight standards, **Lane 2** – Primer 1 & 2 with B12, **Lane 3** - Primer 1 & 2 with X7, **Lane 4** – Primer 1A & 2 with B12, **Lane 5** – Primer 1A & 2 with X7, **Lane 6** - molecular weight standards **Lane 7** – Primer 1 & 2A with B12, **Lane 8** – Primer 1 & 2A with X7, **Lane 9** – Primer 1A & 2A with B12, **Lane 10** – Primer 1A & 2A with X7, **Lane 11** – Control, **Lane 12** - molecular weight standards.

The 2000 bp band produced from primers 1 and 2 was excised from the gel and extracted from the agarose with the use of QIAQuick gel extraction kit. The extracted 2000 bp band was then re-amplified to produce a cleaner PCR product (Fig 7.4).



**Fig 7.4:** Agarose gel electrophoresis of PCR products after second round of amplification. **Lane 1** - molecular weight standards, **Lane 2** – Primer 1 & 2 with B12, **Lane 3** - Primer 1 & 2 with X7

### **7.3.4 Bioinformatic analysis of Formate acetyltransferase DNA and predicted protein sequences from B12 and X7 sequences**

In order to sequence the PCR products, the PCR products of B12 and X7 were cloned into Plasmid vector pCR<sup>®</sup> 4-TOPO<sup>®</sup> (Invitrogen) and then transformed into *E.coli* One Shot<sup>®</sup> TOP 10 chemically competent cells (Invitrogen). Plasmids containing the Formate acetyltransferase gene fragment were then sent for sequencing, requiring three sets of forward and reverse primers to obtain the whole sequence (Section 3.7.8).

The DNA sequences (1946 bp) from the cloned PCR product from B12 and X7 were identical when translated in the same reading frame as the ORF from other closely related bacteria on which the primers were based (Appendix A4.1 and A4.2).

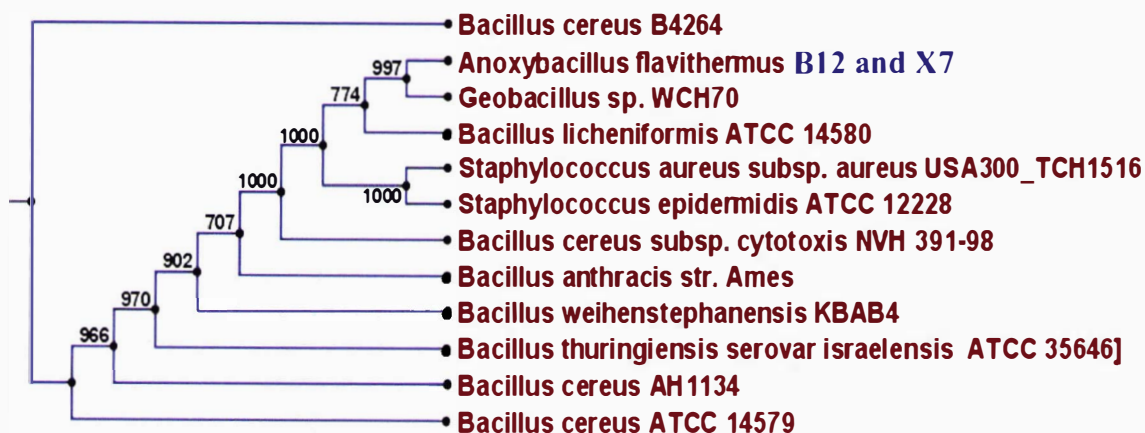
The 1946 pb DNA sequence from B12 and X7 encoded a 648 amino acid segment that was 91% identical, according to BLAST, to the Formate acetyltransferase enzyme from *Geobacillus* sp. WCH70. It showed 85%, 84% and 83% homology with Formate acetyltransferase enzyme from *Bacillus licheniformis* ATCC 14580, *Bacillus cereus* subsp. cytotoxis NVH (391-98) and *Bacillus weihenstephanensis* KBAB4, respectively (Appendix A5).

In addition, several of the peptides identified by mass spectrometry from Spot 1 matched peptides predicted from the *in-silico* translation of the B12 and X7 DNA sequence (Fig 7.5). Together these results provide strong evidence that Spot 1 is in fact the enzyme Formate acetyltransferase.



**Fig 7.5:** The predicted amino acid sequence from the cloned and sequenced DNA from the Formate acetyltransferase ORF amplified from B12 and X7. Underlined amino acids are sequences identified by mass spectrometry from 2D-PAGE gels spots as having homology with known Formate acetyltransferase sequences. Sequences underlined in green ( — ) are from B12 lysozyme/mutanolysin gels, sequences underlined in blue ( — ) are from B12 whole cell gels and sequences underlined in red ( — ) are from X7 whole cell gels.

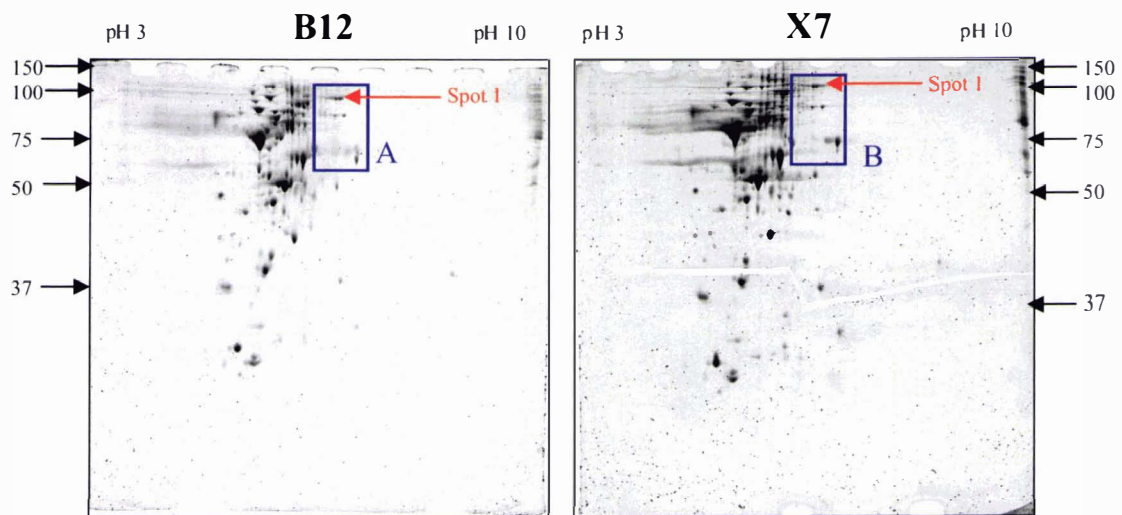
Phylogenetic analysis of the Formate acetyltransferase protein from *Anoxybacillus flavithermus* showed this protein to be grouped with the enzyme formate acetyltransferase from closely related bacterial species (Fig 7.6).



**Fig 7.6:** Phylogenetic relationship of Formate acetyltransferase from *Anoxybacillus flavithermus* with other related bacterial proteins. The phylogenetic tree in this figure was prepared in CLC sequence viewer 4.6 using Neighbour Joining and Bootstrapping (1000 replicates). For details of the sequences used in this alignment, see Appendix A5.2.

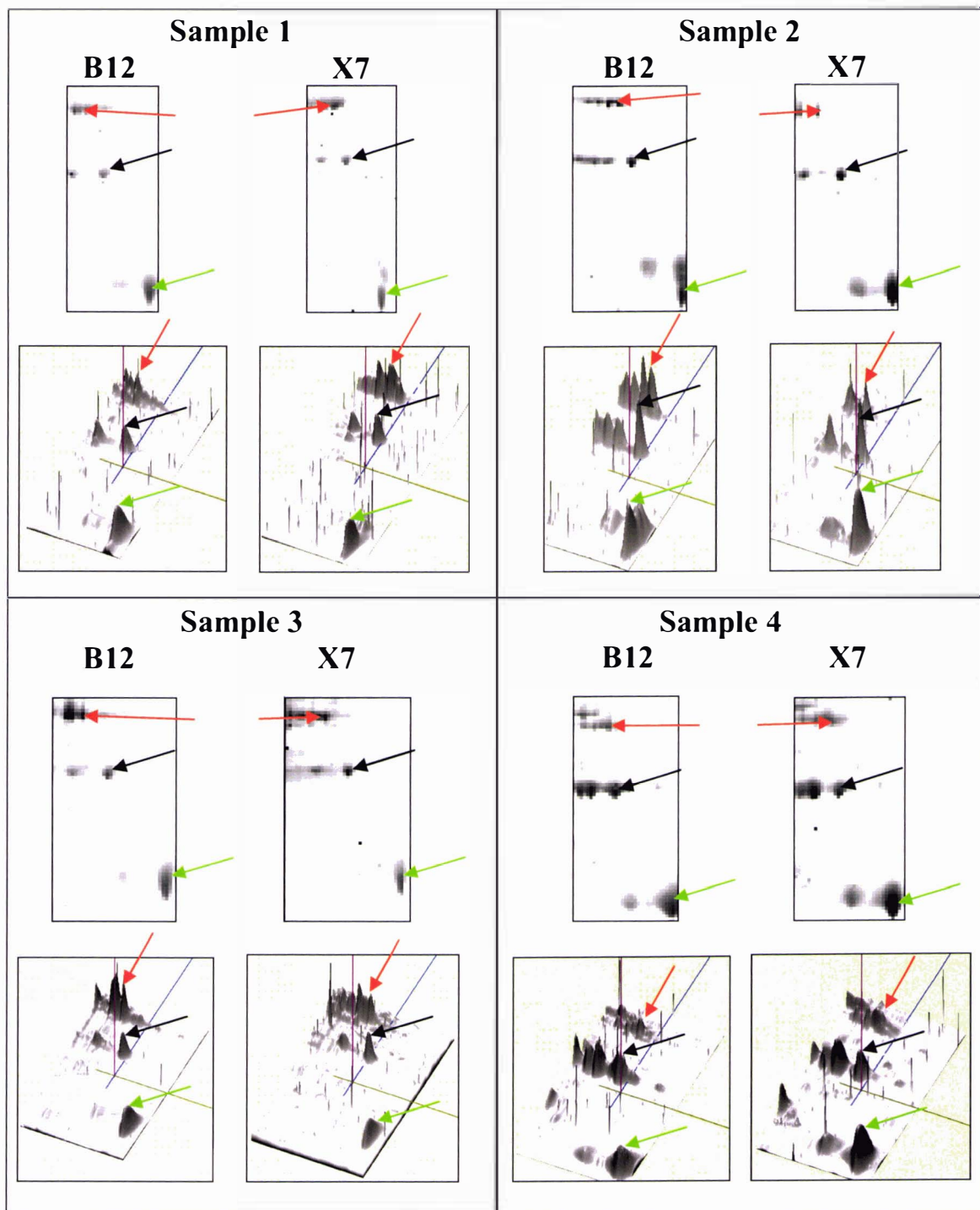
### 7.3.5 Levels of Formate acetyltransferase from total cell proteins of B12 and X7.

Comparisons of the lysozyme/mutanolysin extracts of surface proteins from strains B12 and X7 revealed that Formate acetyltransferase enzyme was consistently present at reduced levels in X7, compared with B12. In order to establish whether overall expression levels of Formate acetyltransferase enzyme were also different, gel spots thought to correspond to Formate acetyltransferase enzyme from total cell protein separations (Red arrows in Fig 7.7) were sent to Bioanalytical Mass Spectrometry in Australia. Confirmation of identity was by ESI-QUAD-TOF followed by MASCOT algorithm data base search. The spots were found to be Formate acetyltransferase enzyme (see Appendix A2.3 and A2.4). Once the Formate acetyltransferase enzyme was identified, with peptides linked back to the predicted amino sequence of the Formate acetyltransferase gene (Fig 7.5), the expression levels from total cell proteins were analysed from four separate samples (Fig 7.8).



**Fig 7.7:** Two-dimensional-PAGE (2D-PAGE) gels of whole cell protein extracts of B12 and X7 cells. 200  $\mu\text{g}$  of protein extract was run on a 17 cm, pH 3-10 strip, with the second dimension using a 12.5% SDS-PAGE gel, stained with Sypro Ruby and Laser scanned at 450 nm. Red arrows mark location of Formate acetyltransferase protein and Blue boxes mark sections analysed in Fig 7.8 below.

The gel regions containing the Formate acetyltransferase protein from total cell proteins from four separate samples are highlighted in Fig 7.8 (Blue squares labelled A and B in Fig 7.7). Black and green arrows point to reference Spots that indicate relative protein loading between B12 and X7 samples. Upon closer analysis, there appears to be no major difference in the expression of Formate acetyltransferase enzyme in B12 and X7 cultures.



**Fig 7.8:** The comparable expression levels of Formate acetyltransferase enzyme (red arrows) from B12 and X7 total cell proteins over four samples, with 3D images of the each gel underneath. Black and green arrows point to Reference Spots that indicate relative protein loading between B12 and X7 samples.

## 7.4 Discussion

Results from Chapter 6 identified two protein spots, extracted from the cell surface using lysozyme/mutanolysin treatment that were consistently present at a higher level in strain B12 compared with strain X7. Therefore, the objective of the work in this chapter was to identify the two proteins spots recognized in Chapter 6 as potentially responsible for the higher rate of attachment demonstrated by strain B12 over strain X7.

Mass spectrometry analysis by ESI-QUAD-TOF followed by MASCOT MS/MS Ion Search Query was able to identify protein Spot 1, with high confidence, as Formate acetyltransferase, also commonly known as Pyruvate formate lyase. Eight hits with a probability based Molecular Weight Search (MOWSE) ranging from 103 to 299 were described (Table 7.1). It should be noted that scores of over 52 indicate identity or extensive homology ( $p < 0.05$ ). In addition, all eight of the Formate acetyltransferase enzymes listed with extensive homology to Spot 1 come from Gram positive bacteria and four of the eight bacteria are also sporeformers, as is *Anoxybacillus flavithermus* used in this study.

However, mass spectrometry analysis of Spot 2, followed by a MASCOT MS/MS Ion Search Query was unable to identify any peptide homology in the MASCOT database. The inability to identify Spot 2 by mass spectrometry was not due to limited protein concentration or the lack of sample peaks in the mass spectrometry results (Russ Pickford, personal comm), but the lack of sequence homology present in the MASCOT database to match to protein Spot 2. The inability to identify protein spots is not uncommon in bacterial proteomics. Coquet *et al.* (2005) and Wei *et al.* (2006) reported several outer-membrane proteins from *Yersinia ruckeri* and *Shigella flexneri* respectively with no matching peptides in the database.

To help confirm the identity of Spot 1, five DNA sequences of the Formate acetyltransferase enzyme from the genus *Bacillus* were downloaded and compared, using the multiple sequence alignment program ClustalW2 to help identify DNA sequences highly conserved within the Formate acetyltransferase gene. When comparing the five

DNA sequences, the design of the PCR primers to amplify Formate acetyltransferase from B12 and X7 was based upon two important criteria:

- 1) Extensive DNA homology over an 18-25 bp region (Fig 7.2A).
- 2) The Amplified product would provide extensive nucleotide coverage of the Formate acetyltransferase gene (Fig 7.2B).

PCR primers were used to amplify a 1946 bp gene product that covered approximately 85% of the Formate acetyltransferase gene of B12 and X7, which was subsequently cloned and sequenced. Sequence alignments of B12 and X7 Formate acetyltransferase DNA were identical, suggesting that within the gene fragment sequenced, no minor changes in the amino acid sequence can explain the higher presence of Formate acetyltransferase in the cell wall of strain B12 compared with strain X7.

The *in-silico* translation of the cloned DNA sequence to a protein sequence produced a 648 amino acid sequence with 91%, 85%, 84% and 83% homology to the Formate acetyltransferase enzyme from *Geobacillus* sp. WCH70, *Bacillus licheniformis* ATCC 14580, *Bacillus cereus* subsp. cytotoxis NVH (391-98) and *Bacillus weihenstephanensis* KBAB4, respectively, according to BLAST analysis. This provides strong evidence that the cloned fragment from strains B12 and X7 does contain the majority of the coding sequence for Formate acetyltransferase present in B12 and X7.

Consequently, the *in-silico* translation of the cloned Formate acetyltransferase DNA from B12 and X7 should also encode the same peptides identified by mass spectrometry from Spot 1. In the present study, numerous peptide sequences identified by mass spectrometry from Spot 1 were also found to be encoded in the amplified Formate acetyltransferase gene fragment from B12 and X7 (Fig 7.5).

Two peptides commonly identified by mass spectrometry of Spot 1 (Appendix A2), but apparently not encoded within the 648 predicted amino acid sequence are (in standard International single letter code)

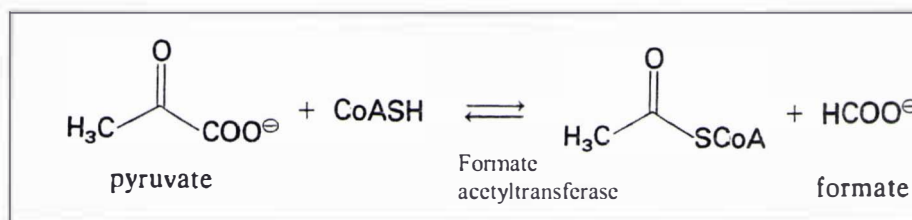
- 1) RVSGYAVNIFIKL
- 2) REQQIDVINRT

However, comparison of the entire amino acid sequence of Formate acetyltransferase from *Geobacillus* sp. WCH70, *Bacillus licheniformis* ATCC 14580, *Bacillus cereus* subsp. cytotoxis NVH (391-98) and *Bacillus weihenstephanensis* KBAB4, with the amplified region from strains B12 and X7, established that the two sequences above are positioned just outside the amplified region of DNA and thus are not encoded within the amplified region in Fig 7.5.

Overall, the ability to relate peptide sequences identified by mass spectrometry to predicted amino acid sequences from cloned and sequenced DNA provides a higher level of confidence that in this case, mass spectrometric analysis correctly identified protein Spot 1 as Formate acetyltransferase enzyme.

Phylogenetic analysis confirmed that the predicted amino acid sequence of Formate acetyltransferase from *Anoxybacillus flavithermus* was closely related to Formate acetyltransferase from closely related bacteria (Fig 7.6).

The Formate acetyltransferase enzyme, sometimes referred to as Pyruvate formate lyase is generally recognised as a cytoplasmic enzyme, whose function is to cleave pyruvate into formate and acetyl-CoA as outlined in Fig 7.8, and represents the initial step in the formation of mixed-acid end products in *Lactococcus lactis* and *Streptococcus mutans* (Melchiorsen *et al.*, 2002; Abbe *et al.*, 1982).



**Fig 7.9:** Formate acetyltransferase catalytic conversion of pyruvate to formate and acetyl-CoA (adapted from Walsh, 1979).

The Formate acetyltransferase enzyme appears to be the anaerobic counterpart to pyruvate-dehydrogenase enzyme, which cleaves pyruvate to acetyl-CoA and CO<sub>2</sub> during aerobic respiration. When *E.coli* cells are switched from aerobic conditions into anaerobic conditions, Formate acetyltransferase activity appears within minutes (Walsh, 1979). Peng and Shimizu (2003) reported a tenfold increase in the expression of Formate acetyltransferase genes when *E.coli* cells were switched from aerobic conditions into anaerobic conditions.

Factors reported to cause increased expression of Formate acetyltransferase include the presence of galactose (Melchiorsen *et al.*, 2002) in *Lactococcus lactis*, growth within a biofilm structure (Resch *et al.*, 2005; Zhu *et al.*, 2007) in *Staphylococcus aureus*, growth in anaerobic conditions (Zhu and Shimizu, 2004; Peng and Shimizu, 2003) in *E.coli* and bacterial growth in milk (Derzelle *et al.*, 2005) in *Streptococcus thermophilus*. Interestingly, three of the four factors mentioned above are known to be involved in milk powder production, from which *Anoxybacillus flavithermus* (B12) originates.

The enzyme Formate acetyltransferase from other bacteria is generally regarded as a cytoplasmic protein with no known cell surface localisation motifs such as LPXTG, a protein recognition sequence involved in attachment of surface proteins to the peptidoglycan layer in Gram positive bacteria, neither does it possess any membrane anchor domains or any signal sequence for translocation through the cell membrane. However, complete sequencing of the Formate acetyltransferase from B12 and X7 would be needed to confirm the lack of common cell surface targeting motifs such as LPXTG. Notwithstanding, the identification of intracellular proteins, particularly glycolytic

enzymes “moonlighting” (Schaumburg *et al.*, 2004) on a cell surface is not uncommon (Fischetti, 2000; Cole *et al.*, 2008; Cole *et al.*, 2005). Two glycolytic enzymes frequently found on the surface of *Streptococcus* include Enolase and Glyceraldehyde-3-phosphate dehydrogenase (Hughes *et al.*, 2002). Enolase has been implicated as a plasmin(ogen) binding protein from pathogenic streptococci (Bergmann *et al.*, 2001) and *Neisseria meningitidis* (Knaust *et al.*, 2007), which has been demonstrated to facilitate the cell invasion process (Bergmann *et al.*, 2001). The exact nature of Enolase secretion is still unknown; nevertheless, Bergmann *et al.* (2001) suggested the Enolase enzyme was secreted outside the cell wall then re-associated with the cell wall by interacting with receptors on the cell surface. On the other hand, Pancholi and Fischetti (1998) proposed that internal sequences within the Enolase enzyme, unknown to date, may target the enzymes to the cell surface. In addition, Boël *et al.* (2004), working with the glycolytic enzyme Enolase from *E.coli*, demonstrated that by changing the lysine amino acid at position 341 to glutamic acid, the catalytic activity of the enzyme was not reduced. However, the modified Enolase enzyme was no longer found on the cell surface, suggesting that the export of Enolase to the cell surface is by an unknown cellular process and not due to cytoplasmic contamination by cell lysis.

Recent work by Severin *et al.* (2007) identified Formate acetyltransferase enzyme on the surface of *Streptococcus pyogenes*. Severin *et al.* (2007) treated whole cells with the proteolytic enzyme trypsin to release surface proteins. The resulting mix of peptides generated were then analysed by tandem MS/MS, which identified 79 different proteins. In order to monitor potential cytoplasmic contamination, viable counts were taken after trypsin treatment and no reduction in cell numbers was recorded, suggesting little to no cytoplasmic contamination took place.

In order to establish if the higher expression of Formate acetyltransferase in B12 compared with X7 from lysozyme/mutanolysin surface extraction is present throughout the whole cell, the expression levels of Formate acetyltransferase were analysed from whole cell protein extractions. Initially, protein spots thought to represent Formate acetyltransferase from whole cell protein extractions were sent for mass spectrometry analysis to confirm spot identity. Overall, expression levels of Formate acetyltransferase

from whole cell samples of B12 and X7 are very similar; suggesting that the higher level of Formate acetyltransferase observed in surface extracts in strain B12 is not mirrored inside the cell.

## 7.5 Conclusion

Two lysozyme/mutanolysin extracted surface proteins were found to be expressed at a higher level in strain B12 than in X7 (Spot 1 and 2), but only one could be identified by mass spectrometry. This was found to be the enzyme Formate acetyltransferase. The Formate acetyltransferase enzyme is a well-known cytoplasmic enzyme involved in the conversion of pyruvate to formate and acetyl-CoA (Fig 7.8). Factors reported to cause higher expression of the Formate acetyltransferase enzyme in bacterial cells include: growth in milk (Derzelle *et al.*, 2005), anaerobic conditions (Zhu and Shimizu, 2004; Peng and Shimizu, 2003) and growth within a biofilm (Resch *et al.*, 2005; Zhu *et al.*, 2007). All three of these conditions would be encountered in the growth of *Anoxybacillus flavithermus* in milk powder plants. However, the mechanism of translocation and retention of the Formate acetyltransferase enzyme within the cell wall of *Anoxybacillus flavithermus* is not yet known. It's role in the cell wall that has lead to the phenotypic differences noted between B12 and X7, higher cell surface hydrophobicity and greater negative cell surface charge in B12 relative to X7 is not obvious at the current time. Asanuma and Hino (2002) reported that the presence of the Formate acetyltransferase does not guarantee the enzyme is active, as post-translational modification is required in *Lactococcus lactis* and *Streptococcus bovis* to produce active enzyme.

This work is not the first time the cytoplasmic enzyme Formate acetyltransferase has been identified on the surface of a bacterium (Severin *et al.*, 2007). Furthermore, the role that various cytoplasmic enzymes play in bacterial attachment and invasion by pathogenic *Streptococcus* has also been reported by several authors (Hughes *et al.*, 2002; Bergmann *et al.*, 2001; Pancholi and Fischetti, 1998 and 1993). In addition to Formate

acetyltransferase, Spot 2, which could not be identified by mass spectrometry, may be involved in the attachment process of B12 to surfaces.

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### Final Discussion, Future work and Conclusion

#### 8.1 Final Discussion

In the milk powder industry, high numbers of thermophilic bacteria may contaminate the product after long manufacturing runs. This contamination can cause customer rejection of the final product, resulting in reduced financial return. During milk powder manufacture, sections of the plant commonly operate at between 45° and 75°C, which is in the growth range of thermophiles. Raw milk usually contains <10 thermophiles per ml (Flint *et al.*, 2001; Scott *et al.*, 2007), but when run times exceed 18-20 hours, 10<sup>5</sup> thermophiles can be found per gram of milk powder (Murphy *et al.*, 1999; Stadhouders *et al.*, 1982). The 10-fold concentration effect of converting milk to powder cannot explain this increase and the residence time of the milk from storage tank to finished powder, usually 20-30 minutes, does not allow sufficient time for thermophilic growth in the bulk milk during processing. Therefore, the general assumption is that the thermophiles attach and grow, forming biofilms on the stainless steel surfaces within the plant. The resulting biofilm contaminates the product by individual cells or clumps of cells sloughing off.

The first critical stage in any biofilm formation is the initial attachment step, as future biofilm development depends upon the first layer of attached cells for anchorage to the surface. In this study, the isolation of a mutant with reduced ability to attach to surfaces was considered important in understanding what surface characteristics of *Anoxybacillus flavithermus* are involved in the attachment process. A strain of *A. flavithermus* (B12) from milk powder was selected as the parental strain because of its higher ability to attach to stainless steel compared with other non-dairy strains of *A. flavithermus* (Fig 4.15). A selection process of passing a culture through a glass column filled with stainless steel chips and collecting the first bacterial cells to be eluted from the column, re-culturing and repeating the process seven times, resulted in the isolation of a stable spontaneous mutant with, a 1 log<sub>10</sub> cells.cm<sup>2</sup> reduction in the ability to attach to stainless steel.

The resultant isogenic mutant or strain was labelled X7, in reference to the number of times the culture passed through the column. Strain X7 demonstrated a reduced ability to attach to stainless steel and a lower ability to attach to glass and polystyrene microtitre plates compared with its parental strain B12. Thus, the attachment deficiency demonstrated by X7 appears universal across multiple surface types and not specific to stainless steel. This observation raises the question, “what surface structures have changed in X7 that have resulted in its reduced ability to attach to multiple surfaces”? Furthermore, strain X7 was a stable phenotype that could be sub-cultured and present the same phenotype. Differences between B12 and X7 were even observable in the pellet formations following centrifugation. Strain B12 formed a smear on the sides and bottom of the centrifuge tube, while X7 formed a compact pellet on the bottom of the centrifuge tube, (Fig 4.4 and 4.4A).

To ensure that strain X7 was not a laboratory contaminant, Random Amplified Polymorphic DNA (RAPD) analysis, biochemical assays and observation of culture growth and colony morphology were used to compare B12 and X7. None of these techniques was able to distinguish between the two strains, strongly suggesting that the selection process resulted in the successful isolation of an isogenic mutant demonstrating a phenotype with reduced ability to attach to surfaces compared with its parental strain B12. Furthermore, the inability of standard techniques to distinguish between strains B12 and X7 shows limited heterogeneity of X7 compared with strain B12. With this in mind, any differences in the cell surface characteristics between the two strains may be responsible for the observed differences in their ability to attach to surfaces.

The first step in comparing the surface characteristics of the two strains involved the use of Transmission Electron Microscopy (TEM) in an attempt to observe any differences in the cell wall structure. Structural differences between a cellulose attachment deficient strain of *Ruminococcus albus* and its parental strain were observable when cells were viewed using TEM (Mosoni *et al.*, 2001). However, in this study, no differences were observed between B12 and X7. Consequently, no major structural modifications visible using TEM exist that could explain the different rates of attachment of B12 and X7.

Exchanging culture supernatants had no effect on the ability of each strain to attach to stainless steel. Thus, the supernatants from both B12 and X7 appear to be identical in terms of their effect on cell attachment. This result suggests that no secreted component is lacking in X7.

Cell surface charge is often associated with the adhesion of bacteria to surfaces (Briandet *et al.*, 1999; Ukuku and Fett, 2002). In this study, both Zeta potential measurements and Electrostatic Interactive Chromatography indicated that strain X7 has a lesser net negative charge compared with its parental strain B12 at neutral pH. Substrates such as stainless steel at neutral pH frequently possess a net negative charge, which naturally results in the repulsion of negatively charged cells from the surface (Hermansson, 1999). However, strain B12 has a greater ability to attach to stainless steel than X7, though B12 has a stronger negative charge than X7. One explanation for this observation may lie at a more microscopic scale. Bacterial cell surfaces may possess positively charged domains which may mediate attachment despite overall electrostatic repulsion. Cowan *et al.* (1994) hypothesised this for the interaction between *Treponema denticola* and human erythrocytes. Another explanation is that other physico-chemical properties such as hydrophobicity, van der Waals attractive forces and steric forces override electrostatic interactions in the attachment process of *A. flavithermus* to plastic, glass and stainless steel.

Hydrophobicity of the cell surface is also commonly considered to be associated with attachment of bacteria (Peng *et al.*, 2001; Iwabuchi *et al.*, 2003). The use of Microbial Adhesion To Hydrocarbons (MATH) and Hydrophobic Interactive Chromatography (HIC) methods to study cell surface hydrophobicity suggested that strain B12 had a higher surface hydrophobicity than strain X7. Increasing the surface hydrophobicity of the substratum by treatment with Sigmacote<sup>®</sup> resulted in the increased attachment of X7, implying that surface hydrophobicity interactions may play a role in the attachment of *A. flavithermus* to surfaces. In general strain B12 has a greater ability to attach to plastic (hydrophobic), glass (hydrophilic) and stainless steel than X7. The hydrophobicity rating of stainless steel is not well defined with Brugnoli *et al.* (2007) and Teixeira *et al.* (2005)

describing stainless steel as hydrophobic. However, Lejeune (2003), Planchon *et al.* (2007A) describe it as hydrophilic. Therefore, how can a cell with higher surface hydrophobicity attach at a higher rate to a hydrophilic surface? This discrepancy has been reported before, with Anand *et al.* (2007) noting that treatment of marine bacteria with 2, 4-dinitrophenol resulted in no change in surface charge, but reduced surface hydrophobicity, which in turn caused a reduction in attachment to glass and plastic surfaces. Liu *et al.* (2004) reported that a microbial cell with high surface hydrophobicity could help attachment to both hydrophobic and hydrophilic surfaces. The observation by Liu *et al.* (2004) could be a reflection on the relative hydrophobicity of the three phases i.e. aqueous phase, bacterial surface and substrate. Consequently, if the bacterial surface and solid surface are more hydrophobic than the aqueous phase this may cause an increase in the hydrophobic interaction between bacteria and substrate.

The role of extracellular polysaccharides, often associated with biofilm development (Sutherland, 2001), was investigated by measuring the amount of polysaccharide produced by B12 and X7, as well as the monosaccharide composition of the surface polysaccharides. However, results did not indicate any difference between the two strains and therefore argued against the involvement of polysaccharide in the initial attachment of B12 or X7 to surfaces. In addition, treatment of cells with polysaccharide degrading chemicals had no effect on attachment or surface hydrophobicity of the cells.

The role of surface proteins in attachment was investigated by treating cells with chemicals that disrupted or removed surface protein (trypsin, acid-glycine, lithium chloride and lysozyme). Except for lithium chloride, these treatments reduced the number of cells attaching by  $1 \log_{10}$  cells.cm<sup>2</sup> for both B12 and X7 strains. This suggests the important role surface proteins may play in attachment of B12 and X7 to stainless steel. Similar results were also reported by Parker *et al.* (2001) studying the surface properties of thermophilic bacilli. Removal of surface proteins also reduced the hydrophobicity of B12 and X7, suggesting surface proteins are an important factor in determining surface hydrophobicity.

One-dimensional SDS-PAGE of surface proteins extracted with lysozyme/mutanolysin, or acid-glycine and whole cell proteins did not reveal any differences between B12 and X7. Two-dimensional PAGE of acid-glycine extracted and whole cell proteins also revealed no differences between B12 and X7, signifying that no major disruption to the common cell surface protein display mechanisms such as the LPXTG motif (Fischetti, 2000) has taken place in X7. However, two-dimensional PAGE of lysozyme/mutanolysin extracted surface proteins from B12 showed two protein spots to be consistently present at a higher concentration compared with X7. The isoelectric point (pI) of Spot 1 was estimated to lie in the pH range 6.5-7.0, which probably will not affect the net charge of the cell. On the other hand, the pI of Spot 2 was estimated to lie between pH 8.5-9.0; this means that the Spot 2 protein would have a net positive net charge at neutral pH. This would most likely result in the cell surface of B12 having a minor increase in net positive charge compared with X7. However, measurements of zeta potential and electrostatic interaction demonstrate that B12 has a greater net negative charge on the cell surface compared with X7. From this result, it appears that the physical presence of Spot 1 and 2 proteins on the cell surface cannot account for the difference in surface charge observed between B12 and X7. Although the 2D-PAGE technique is expected to identify all proteins expressed by a cell, the reality is different (Huber, 2003). Hydrophobic proteins, high molecular weight and proteins present in small concentrations tend to be underrepresented in 2D-PAGE gels (Cash, 2004). Consequently, the possibility exists that proteins important in the attachment process of B12 could fall into this category.

One of the protein spots (Spot 1, Fig 6.8) was identified by mass spectrometry as the cytoplasmic enzyme Formate acetyltransferase, also known as Pyruvate formate lyase. PCR amplification of the DNA sequence (85% of the gene) followed by cloning and then sequencing of the Formate acetyltransferase gene fragment from both B12 and X7 resulted in identical DNA sequences. In addition, the predicted amino acid sequence from the cloned DNA encoded peptide sequences that were identified by mass spectrometry from the protein Spot 1 on the gel, providing greater confidence in the identification of Formate acetyltransferase.

The enzyme Formate acetyltransferase is generally regarded as a cytoplasmic enzyme involved in the conversion of pyruvate to acetyl-CoA and formate in anaerobic conditions. Factors reported to cause an increase in Formate acetyltransferase expression include growth in milk (Derzelle *et al.*, 2005), growth within a biofilm structure (Resch *et al.*, 2005; Zhu *et al.*, 2002) and obviously, anaerobic conditions (Zhu and Shimizu, 2004; Peng and Shimizu, 2003). All three of these conditions are known to be encountered in the growth of *A. flavithermus* in milk powder plants. Consequently, a higher level of Formate acetyltransferase expression in dairy isolates may be expected.

The identification of a cytoplasmic protein in a cell surface extraction protocol raises the question “Is cytoplasmic contamination via cell lysis responsible”? In this study two cytoplasmic enzymes (Leucine aminopeptidase and  $\beta$ -galactosidase) were monitored as indicators of any cytoplasmic contamination. Although a small amount of cytoplasmic contamination was present in the lysozyme/mutanolysin surface extracts, there was no difference in the level of contamination between B12 and X7 that could explain the higher level of Formate acetyltransferase present in the surface extracts from B12. Thus cytoplasmic contamination appears to be an unlikely source of the Formate acetyltransferase. A comparison of whole cell proteins by 2D PAGE suggested that the internal levels of Formate acetyltransferase in B12 and X7 were similar. This tends to indicate that the higher concentration of Formate acetyltransferase is limited to the cell wall of B12.

This is not the first report to identify Formate acetyltransferase on the surface of bacteria. Severin *et al.* (2007) identified Formate acetyltransferase on the surface of *Streptococcus pyogenes*. Furthermore, the identification of cytoplasmic enzymes on bacterial surfaces is not an uncommon occurrence (Fischetti, 2000; Cole *et al.*, 2008; Cole *et al.*, 2005). Some glycolytic enzymes such as Enolase found on a bacterial surface have been reported to enhance attachment to host tissue cells (Bergmann *et al.*, 2001; Knaust *et al.*, 2007) and even elicit protective immune responses in mice (Ling *et al.*, 2004). However, the nature of the transport mechanism to relocate a cytoplasmic enzyme such as Enolase, and in this study Formate acetyltransferase, to the cell surface remains elusive.

The results from this work raise three questions:

- i) What is actually different about X7 and how is Formate acetyltransferase involved in attachment?
- ii) How is Formate acetyltransferase relocated to the cell surface?
- iii) Does Formate acetyltransferase have an additional function when associated with the cell wall that is different from its function inside the bacterial cell i.e. is Formate acetyltransferase responsible for the observed increase in surface hydrophobicity and greater negative surface charge in B12? If so, is this difference due to the enzymatic activity of Formate acetyltransferase or is the physical presence of the enzyme in the cell wall responsible for differences in surface charge and hydrophobicity?

The inability to identify the second protein (Spot 2) from lysozyme/mutanolysin extracted proteins from strain B12 cannot be ignored, as this protein may play an important role in the attachment process. However, until identified, its role in attachment remains as speculation.

## **8.2 Future work**

Although the work presented in this thesis has yielded a lot of useful information, the results also uncovered many unanswered questions. Areas where more work is required include:

- 1) Further confirmation of the presence of Formate acetyltransferase on the cell surface. This could be accomplished by labelling surface protein with sulfo-NHS-LC-biotin, a technique sometimes termed surface biotinylation (Smither *et al.*, 2006). This biotin derivative cannot pass through cell membranes thus all surface proteins could be separated by 2D-PAGE and visualised by probing with Streptavidin. Protein spots of interest could then be sent for identification by mass spectrometry.

- 2) Full sequencing of the Formate acetyltransferase gene and adjacent DNA regions from B12 and X7 may identify any differences in control and/or transportation information that might be involved in the localisation of Formate acetyltransferase to the cell surface of B12.
- 3) Further work on the identification of the second protein present at a higher concentration in B12 (Spot 2) from lysozyme/mutanolysin extracted surface proteins. This might be achieved by MS/MS analysis to identify protein sequences, followed by PCR amplification, cloning and sequencing. Complete DNA sequencing of Spot 2 may provide enough information to identify the protein. Though identification of Spot 2 may not determine its role in attachment, it may suggest further studies to elucidate its role on the surface.
- 4) Disruption of the Formate acetyltransferase gene in strain B12, followed by attachment studies. The Formate acetyltransferase enzyme from *Streptococcus* is inactivated in the presence of oxygen (Asanuma and Hino, 2002), in spite of this *Streptococcus* is regarded as a Facultative anaerobic organism, as is *Anoxybacillus flavithermus*, which suggests this enzyme is not required for aerobic growth. Consequently, if disruption of the Formate acetyltransferase resulted in decreased attachment in aerobic conditions this would suggest a direct involvement of Formate acetyltransferase in attachment.
- 5) Measurement of any differences in gene expression between B12 and X7 by the use of DNA Microarray or Protein array technology. DNA or Protein array technology may be able to identify hydrophobic, or proteins with low or high isoelectric point (pI) values expressed by B12 or X7 that were not separated by the use of 2D PAGE technology in the present study. The presence of a hydrophobic surface protein with a low pI value on the surface of B12 involved in attachment, unable to be separated by 2D PAGE may explain the greater negative charge and higher hydrophobicity rating of B12 compared with X7.

- 6) Whole genome sequencing of B12 and X7 may be able to identify small differences in the DNA sequence of B12 and X7. Any differences between the DNA of B12 and X7 could then be annotated, as a result the identification of any ORF may help identify proteins important in the attachment process.

### **8.3 Final Conclusion**

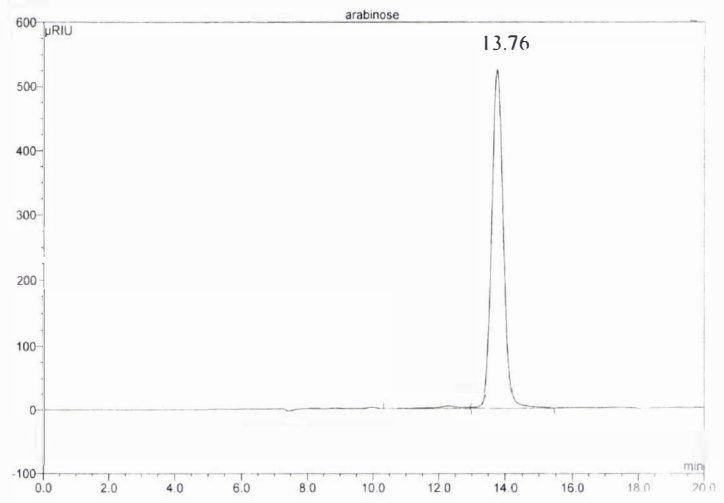
The objective of this research project was to gain an understanding of the attachment mechanisms of a thermophilic bacillus, *A. flavithermus* strain B12. The first step involved the isolation of an attachment deficient isogenic mutant of B12, which resulted in the isolation of X7. It was thought that by comparing the surfaces of B12 and X7 important cell surface attributes involved in cell attachment could be pinpointed. In general, strain B12 has a greater ability to attach to stainless steel, plastic and glass surfaces compared with X7, thus the attachment deficiency of X7 appears to be universal across multiple surface types. A comparison of the surface characteristics of B12 and X7 revealed that B12 has a greater negative cell surface charge and is more hydrophobic than X7. Disruption of surface proteins caused a reduction in hydrophobicity and attachment of both strains, indicating the possible importance of surface proteins in the attachment process. Two-dimensional PAGE analysis of lysozyme/mutanolysin extracted proteins highlighted two proteins that appeared to be present at a higher concentration on the cell surface of B12 compared with X7. One protein was identified as the cytoplasmic protein Formate acetyltransferase. However, the role of this enzyme in the attachment process of B12 remains unknown. The second protein was unable to be identified. When comparing the attachment of B12 and X7 with other dairy and non-dairy thermophilic bacilli, dairy isolates attach to stainless steel at a similar rate to B12. On the other hand, the ability of X7 to attach is comparable to non-dairy isolates. Perhaps the unique environment found within a milk powder plant, requiring a bacterial cell to tolerate growth in milk under anaerobic conditions and to have a high capacity to attach to stainless steel has possibly selected for a mutant strain with a phenotype adapted to survive the milk powder plant environment. Consequently, X7 may not be the true mutant, but a reversion to a phenotype more common outside a milk powder manufacturing plant.

# Appendix

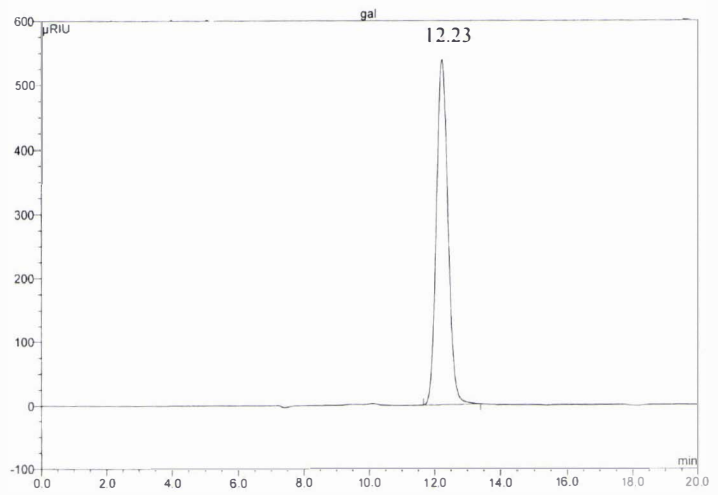
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## A1: Chromatographs of monosaccharide standards

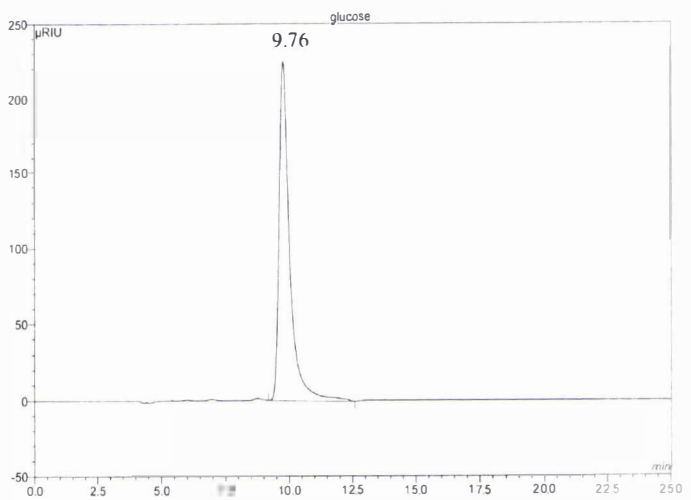
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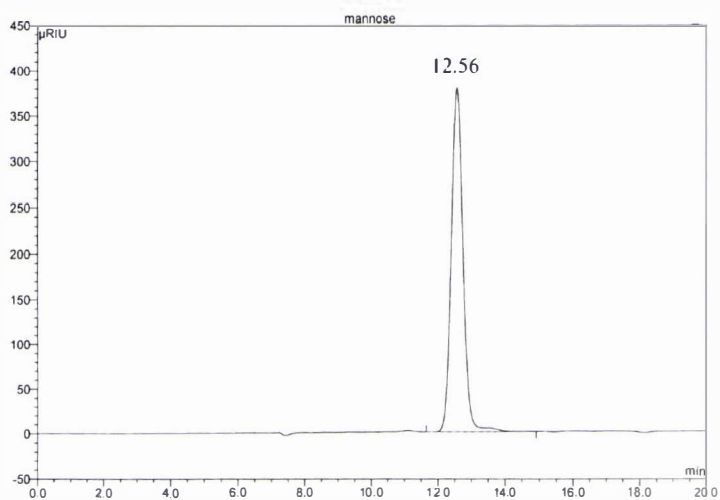
**1% Galactose**



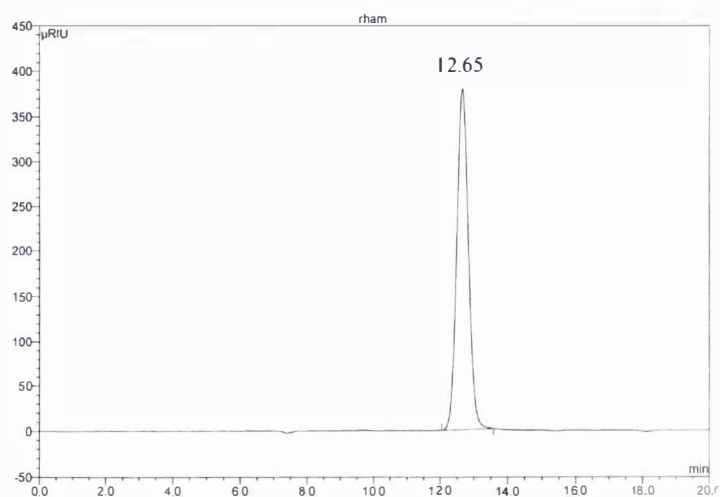
**1% Glucose**



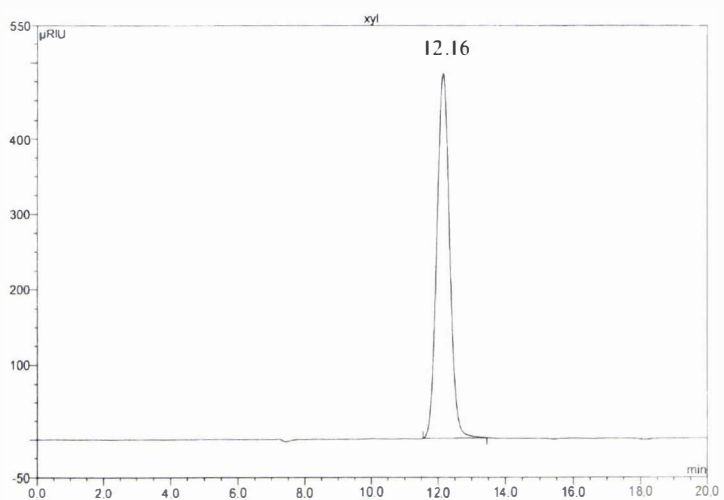
**1% Mannose**



**1% Rhamnose**



**1% Xylose**



# Appendix

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## A2: ESI-QUAD-TOF Mass spectrometry results

## Key for ESI-QUAD-TOF Mass spectrometry results

1. gi|68054704<sup>1</sup>      Mass: 84106<sup>2</sup>      Score: 299<sup>3</sup>      Queries matched: 6<sup>4</sup>

Formate acetyltransferase [*Exiguobacterium sibiricum* 255-15]

Observed <sup>5</sup>	Mr(expt) <sup>6</sup>	Mr(calc) <sup>7</sup>	Delta <sup>8</sup>	Miss <sup>9</sup>	Score <sup>10</sup>	Expect <sup>11</sup>	Rank <sup>12</sup>	Peptide <sup>13</sup>
549.2679	1096.5212	1096.5916	-0.0704	0	30	8.3	1	R.VSGYAVNFIK.L

1 – Accession link number

2 - Protein molecular weight

3 - Non-probabilistic protein score, derived from the ions scores

4 - Number of peptide matches

5 - Experimental m/z value

6 - Experimental m/z transformed to a relative molecular mass

7 - Calculated relative molecular mass of the matched peptide

8 - Difference (error) between the experimental and calculated masses.

9 - Number of missed enzyme cleavage sites

10 - Ions score

11 - Expectation value for the peptide match. (The number of times we would expect to obtain an equal or higher score, purely by chance. The lower this value, the more significant the result)

12 - Rank of the ions match, (1 to 10, where 1 is the best match).

13 - Sequence of the peptide in 1-letter code.

## A2.1 Spot 1 from B12 lysozyme/mutanolysin extraction

### Mascot Search Results

gi|68054704 Formate acetyltransferase [*Exiguobacterium sibiricum* 255-15]

gi|67917225 Formate acetyltransferase [*Clostridium thermocellum* ATCC 27405]

gi|89202507 Formate acetyltransferase [*Bacillus cereus* subsp. cytotoxis NVH 391-98]

gi|52080503 putative formate C-acetyltransferase [*Bacillus licheniformis* ATCC 14580]

gi|16800511 pyruvate formate-lyase [*Listeria innocua* Clip11262]

gi|106885378 formate acetyltransferase [*Clostridium phytofermentans* ISDg]

1. gi|68054704      Mass: 84106    Score: 299    Queries matched: 6

Formate acetyltransferase [*Exiguobacterium sibiricum* 255-15]

Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
549.2679	1096.5212	1096.5916	-0.0704	0	30	8.3	1	R.VSGYAVNFIK.L
557.7532	1113.4918	1113.5778	-0.0859	0	48	0.18	1	R.EQQIDVINR.T
575.2488	1148.4830	1148.5244	-0.0413	0	24	44	1	K.EQNGAAMSLGR.V + Oxidation (M)
652.3306	1302.6466	1302.6931	-0.0465	0	58	0.018	1	K.AGIFTGLPDAYGR.G
678.3424	1354.6702	1354.7132	-0.0430	0	57	0.021	1	R.VSTFLDIYIER.D
716.3745	1430.7344	1430.7881	-0.0536	1	83	4.8e-005	1	R.KAGIITGLPDAYGR.G

2. gi|67917225      Mass: 84351    Score: 275    Queries matched: 6

Formate acetyltransferase [*Clostridium thermocellum* ATCC 27405]

Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
549.2679	1096.5212	1096.5916	-0.0704	0	30	8.3	1	R.VSGYAVNFIK.L
557.7532	1113.4918	1113.5778	-0.0859	0	48	0.18	1	R.EQQIDVINR.T
575.2488	1148.4830	1148.5244	-0.0413	0	24	44	1	K.EQNGAAMSIGR.I + Oxidation (M)
652.3306	1302.6466	1302.6931	-0.0465	0	58	0.018	1	K.AGIFTGLPDAYGR.G
714.8156	1427.6166	1427.6602	-0.0436	0	33	4.9	1	K.ETLLDAMEHPEK.Y + Oxidation (M)
716.3745	1430.7344	1430.7881	-0.0536	1	83	4.8e-005	1	K.KAGIITGLPDAYGR.G

3. gi|89202507      Mass: 84405    Score: 167    Queries matched: 5

Formate acetyltransferase [*Bacillus cereus* subsp. cytotoxis NVH 391-98]

Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
549.2679	1096.5212	1096.5916	-0.0704	0	30	8.3	1	R.VSGYAVNFIK.L
557.7532	1113.4918	1113.5778	-0.0859	0	48	0.18	1	R.EQQIDVINR.T
575.2488	1148.4830	1148.5244	-0.0413	0	24	44	1	K.I.QNGAAMSLGR.T + Oxidation (M)
714.8156	1427.6166	1427.6602	-0.0436	0	33	4.9	1	R.ETLLDAMEHPEK.Y + Oxidation (M)
640.9893	1919.9461	1920.0237	-0.0776	0	34	4.3	1	R.TMATGIAGLSVVADSLSAIK.Y + Oxidation (M)

**4. gi|52080503      Mass: 83378    Score: 163    Queries matched: 4**putative formate C-acetyltransferase [*Bacillus licheniformis* ATCC 14580]

Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
549.2679	1096.5212	1096.5916	-0.0704	0	30	8.3	1	R.VSGYAVNFIK.L
575.2488	1148.4830	1148.5244	-0.0413	0	24	44	1	K.EQNGAAMSLGR.V + Oxidation (M)
678.3424	1354.6702	1354.7132	-0.0430	0	57	0.021	1	R.VSTFELDIYIIR.D
731.3300	1460.6454	1460.6969	-0.0515	0	53	0.055	1	R.EAQFLVDHFVMK.L + Oxidation (M)

**5. gi|16800511      Mass: 83744    Score: 103    Queries matched: 3**pyruvate formate-lyase [*Listeria innocua* Clip11262]

Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
502.2755	1002.5364	1002.5709	-0.0344	0	51	0.088	1	K.GALASLSSVAK.L
549.2679	1096.5212	1096.5916	-0.0704	0	30	8.3	1	R.VSGYAVNFIK.L
575.2488	1148.4830	1148.5244	-0.0413	0	24	44	1	K.EQNGAAMSLGR.T + Oxidation (M)

**Proteins matching the same set of peptides:****6. gi|16803446      Mass: 83772    Score: 103    Queries matched: 3**pyruvate formate-lyase [*Listeria monocytogenes* EGD-e]**7. gi|46907634      Mass: 83758    Score: 103    Queries matched: 3**formate acetyltransferase [*Listeria monocytogenes* str. 4b F2365]**8. gi|106885378      Mass: 77549    Score: 85      Queries matched: 2**formate acetyltransferase [*Clostridium phytofermentans* ISDg]

Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
575.2488	1148.4830	1148.5244	-0.0413	0	24	44	1	K.EQNGAAMSLGR.T + Oxidation (M)
628.8220	1255.6294	1255.6884	-0.0589	1	61	0.0079	1	R.LREELAEQIR.A

**Search Parameters**

Type of search : MS/MS Ion Search

Enzyme : Trypsin

Variable modifications : Carboxymethyl (C),Oxidation (M)

Mass values : Monoisotopic

Protein Mass : Unrestricted

Peptide Mass Tolerance :  $\pm 0.25$  DaFragment Mass Tolerance:  $\pm 0.2$  Da

Max Missed Cleavages : 3

Instrument type : ESI-QUAD-TOF

Number of queries : 123

Mascot: <http://www.matrixscience.com/>

## A2.2 Spot 2 from B12 lysozyme/mutanolysin extraction

## Mascot Search Results

Peptide matches not assigned to protein hits: (no details means no match)

Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
691.3072	1380.5998	1380.6408	-0.0410	0	46	0.26	1	ALEESNYELEGK
807.8999	1613.7852	1613.8961	-0.1108	3	37	2	1	QALQTRVETERRK
808.8765	1615.7384	1615.7940	-0.0556	0	35	3.3	1	LSSVEVSAAPAAEEEEK
536.8176	1071.6206	1071.6652	-0.0445	1	34	4	1	ILTTLKQQK
807.8972	1613.7798	1613.8637	-0.0839	2	30	11	1	K <sup>+</sup> LDVIGGYRTEHR
717.3294	1432.6442	1432.6802	-0.0359	0	30	11	1	VLCQAGGLLGI:GC <sup>+</sup> Carboxymethyl (C)
686.8774	1371.7402	1371.7907	-0.0505	1	29	12	1	IALLVLASACRIDK
526.7568	1051.4990	1051.4604	0.0386	0	29	11	1	QSSTEMPTR +Oxidation (M)
592.8483	1183.6820	1183.6785	0.0036	2	29	13	1	LLRLRNEDR
576.8105	1151.6064	1151.6774	-0.0710	1	29	13	1	LIRNIAAPER
649.3078	1296.6010	1296.6019	-0.0009	0	27	20	1	FTMDDGLANAVK+ Oxidation (M)
775.3928	1548.7710	1548.8120	-0.0409	2	27	22	1	AAAGVQKGSAPHRNK
525.2884	1048.5622	1048.5586	0.0036	0	26	27	1	SLLC <sup>+</sup> SIAGGK
595.3124	1188.6102	1188.6615	-0.0512	1	26	29	1	IIEEIFPAVR
616.3456	1230.6766	1230.6568	0.0199	0	25	34	1	IADVVDLGSNSIR
526.2590	1050.5034	1050.6073	-0.1038	1	23	39	1	KIATSIYGAK
523.2783	1044.5420	1044.5022	0.0399	0	23	61	1	AQSPCVTPSR
710.8326	1419.6506	1419.7146	-0.0640	0	22	59	1	YVNWIQQTIAAN
576.8043	1151.5940	1151.6774	-0.0834	1	21	66	1	IIGVDLNP <sup>+</sup> RR
569.7966	1137.5786	1137.5852	-0.0065	0	21	64	1	QEMSVLLFR +Oxidation (M)
633.3005	1264.5864	1264.6451	-0.0587	1	21	82	1	GQIQKFDTEPK
766.8905	1531.7664	1531.8622	-0.0958	1	21	88	1	RPIRPVVADGYKK
563.2538	1124.4930	1124.5349	-0.0419	0	21	75	1	IAD <sup>+</sup> EALYQSK
799.3870	1596.7594	1596.9926	-0.2331	3	20	92	1	KLERLVAIELKFGK
564.7487	1127.4828	1127.5683	-0.0854	1	20	97	1	DAARSDPGLAR
583.2805	1164.5464	1164.5775	-0.0310	0	20	95	1	LENEIQTYR
649.4286	1296.8426	1296.6785	0.1641	0	20	89	1	RPVDLGLNEER
675.3545	1348.6944	1348.6479	0.0466	2	20	1.1e+002	1	KGDLKNMMPDGK + Oxidation (M)
717.3375	1432.6604	1432.7310	-0.0705	1	19	1.2e+002	1	NDDIQVGIREFK
530.7681	1059.5216	1059.5672	-0.0456	1	19	1.3e+002	1	DVIGSAKQSR
528.2834	1054.5522	1054.5593	-0.0071	0	19	1.1e+002	1	HNITNCLK
558.7734	1115.5322	1115.5393	-0.0070	2	19	1.4e+002	1	CAHESAGKKK + Carboxymethyl (C)
513.5704	1537.6894	1537.8940	-0.2046	2	19	1.3e+002	1	VATDGTITV <sup>+</sup> KRHLK
563.7675	1125.5204	1125.6254	-0.1050	1	19	1.3e+002	1	KPQKQIGPSR
575.7795	1149.5444	1149.6142	-0.0697	2	18	1.4e+002	1	DVYDAKGGKVR
604.7966	1207.5786	1207.5105	0.0682	0	18	1.5e+002	1	DGEDAVDFANR
516.2875	1030.5604	1030.4903	0.0701	1	17	1.9e+002	1	ADGGGSRGGAAR
672.3223	1342.6300	1342.6881	-0.0580	1	17	2.1e+002	1	IYTFITIRENAK
649.3088	1296.6030	1296.6713	-0.0683	1	17	1.9e+002	1	TFDAFTKVVQA

A2: ESI-QUAD-TOF Mass Spec Results

634.3338	1266.6530	1266.4968	0.1562	0	17	2e+002	1	QSPGGGGCDMANK + Oxidation (M)
560.7911	1119.5676	1119.4913	0.0763	1	16	2.3e+002	1	C'SMSHRAVR + Carboxymethyl (C): Oxidation (M)
569.7986	1137.5826	1137.5600	0.0226	0	16	1.9e+002	1	NFLYVQMNR
533.2462	1064.4778	1064.5358	-0.0579	1	16	2.1e+002	1	KALMFMMAQK + Oxidation (M)
581.2682	1160.5218	1160.5937	-0.0719	1	16	2.6e+002	1	KGFGGLNSPER
569.2477	1136.4808	1136.5794	-0.0985	2	16	2.2e+002	1	AIRMAERMK + 2 Oxidation (M)
561.3039	1680.8899	1680.7885	0.1014	0	16	2.5e+002	1	VNMSDVENMALIMSK
678.4476	1354.8806	1354.7106	0.1701	0	16	2.1e+002	1	AALPDLQTHHPR
537.7552	1073.4958	1073.5790	-0.0832	0	16	2.7e+002	1	LVEGLAMTPK + Oxidation (M)
511.3313	1530.9721	1530.8626	0.1095	1	16	2.6e+002	1	IALKLMMQLLVDR
672.8144	1343.6142	1343.7019	-0.0877	2	16	2.8e+002	1	LRHMVDDKIFA
536.3083	1070.6020	1070.5291	0.0730	1	16	2.8e+002	1	CSRDDVVAGHK
583.3868	1164.7590	1164.6138	0.1452	0	15	2.6e+002	1	YTDAGGTLIVR
533.2401	1064.4656	1064.5138	-0.0481	0	15	2.8e+002	1	EVIFEEGSR
612.4062	1222.7978	1222.6631	0.1347	1	15	2.9e+002	1	LLEMDYLKAK
517.2468	1032.4790	1032.4835	-0.0045	0	15	3.8e+002	1	ADQSASGVGNK
692.3783	1382.7420	1382.7405	0.0016	0	14	3.6e+002	1	VVATAVVPDEVER
572.6213	1714.8421	1714.9227	-0.0806	2	14	3.8e+002	1	WALSRQRTATQIER
611.5472	2442.1597	2442.2100	-0.0503	2	14	4e+002	1	AQLKSESKCYDLAPAPEAAPAPK + Carboxymethyl (C)
503.1884	1004.3622	1004.4094	-0.0471	1	14	4.5e+002	1	HRSSCSDR + Carboxymethyl (C)
506.9355	1517.7847	1517.7759	0.0088	1	13	4.9e+002	1	LPSAGSLIEKNDMK + Oxidation (M)
534.2577	1066.5008	1066.5308	-0.0299	0	13	4.2e+002	1	GWAHLADAAR
505.2513	1008.4880	1008.6192	-0.1312	2	13	4.3e+002	1	APAITPRKR
570.4015	1138.7884	1138.5658	0.2226	0	12	4e+002	1	IHSSYYEK
713.3144	1424.6142	1424.7333	-0.1190	0	12	6.1e+002	1	MSYVALSGLAAAQK + Oxidation (M)
576.3119	1150.6092	1150.6206	-0.0114	1	11	6.6e+002	1	LLRGGGGPDGPR
654.2883	1306.5620	1306.5975	-0.0355	1	11	7.5e+002	1	TSSQKCEFIHQ
769.2954	1536.5762	1536.7453	-0.1690	2	11	5.8e+002	1	GTKKEETCIGIGETGK
567.2921	1698.8545	1698.9014	-0.0469	1	11	8.1e+002	1	ELLQYGLKGLCAYTK
625.9794	1874.9164	1874.9196	-0.0032	2	11	9.4e+002	1	RTKYVACGIFQSSTFK + Carboxymethyl (C)
523.2440	522.2367	522.2550	-0.0183	0	11	1.1e+002	1	FGGSR
649.6547	1945.9423	1946.1060	-0.1638	1	11	9.1e+002	1	APGIVRVLAGAAPEGHLAQR
546.0535	1635.1387	1634.9243	0.2144	1	10	2.2e+002	1	NLTILKETVQTIFK
564.2554	1126.4962	1126.5883	-0.0920	1	10	1e+003	1	GDWLRVPER
506.5790	1516.7152	1516.7746	-0.0594	1	10	1.1e+003	1	ITAAFGTAARATGHGK
512.2253	1022.4360	1022.4933	-0.0573	0	10	9.3e+002	1	QASATWFGR
634.4181	1266.8216	1266.6469	0.1748	0	9	9.5e+002	1	TYNNIRPAYR
590.3989	589.3916	589.2859	0.1057	0	9	1.2e+003	1	GA'PFGN
766.7120	2297.1142	2297.3153	-0.2012	3	9	1.4e+003	1	IKLYLARHRGDLVAATVMVR + Oxidation (M)
656.4337	1310.8528	1310.6367	0.2161	1	8	1.2e+003	1	SDPDWRHTAVK
671.9307	2012.7703	2012.9118	-0.1415	0	7	9.2e+002	1	CSIMMVGTSDHLYAATVR
561.5552	1681.6438	1681.8760	-0.2323	2	7	1.3e+003	1	DPQRHAPPPGVEARR
518.5782	2070.2837	2070.1374	0.1463	1	6	1.4e+003	1	QIGYVLQIQALPHKTAAR

## A2: ESI-QUAD-TOF Mass Spec Results

614.4155	1226.8164	1226.6771	0.1394	1	6	2.1e+003	1	IINNDVFHKK
680.3531	1358.6916	1358.5707	0.1210	0	5	3.2e+003	1	MDDAAAMAAAHER
627.1568	626.1495	626.2884	-0.1389	1	5	1.4e+003	1	HGRSGN
562.3827	561.3754	561.2758	0.0996	0	3	2.4e+003	1	AEAGSK
539.8650	1616.5732	1616.8092	-0.2360	1	2	2.9e+003	1	LNAAGRLEHAMAGYK + Oxidation (M)

### Search Parameters

Type of search : MS/MS Ion Search

Enzyme : Trypsin

Variable modifications : Carboxymethyl (C), Oxidation (M)

Mass values : Monoisotopic

Protein Mass : Unrestricted

Peptide Mass Tolerance :  $\pm$  0.25 Da

Fragment Mass Tolerance:  $\pm$  0.2 Da

Max Missed Cleavages : 3

Instrument type : ESI-QUAD-TOF

Number of queries : 101

Mascot: <http://www.matrixscience.com/>

## A2.3 Spot 1 from B12 total cell proteins

### Mascot Search Results

gi|125973025 formate acetyltransferase [*Clostridium thermocellum* ATCC 27405]

gi|86607744 formate acetyltransferase [*Synechococcus* sp. JA-2-3B'a(2-13)]

gi|52080503 putative formate C-acetyltransferase [*Bacillus licheniformis* ATCC 14580]

gi|116872838 formate acetyltransferase [*Listeria welshimeri* serovar 6b str. SLCC5334]

gi|150389979 formate acetyltransferase [*Alkaliphilus metalliredigens* QYMF]

gi|83594330 Formate acetyltransferase [*Rhodospirillum rubrum* ATCC 11170]

gi|106885378 formate acetyltransferase [*Clostridium phytofermentans* ISDg]

gi|119026010 formate acetyltransferase [*Bifidobacterium adolescentis* ATCC 15703]

#### 1. gi|125973025      Mass: 84351    Score: 540    Queries matched: 18

formate acetyltransferase [*Clostridium thermocellum* ATCC 27405]

Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
368.6835	735.3524	735.3915	-0.0391	0	51	0.093	1	R.IIGDYR.R
409.1825	816.3504	816.3726	-0.0222	0	55	0.043	1	K.TGNTPDGR.K
446.7154	891.4162	891.4814	-0.0652	0	40	1.3	1	R.VALYGVDL.L
473.2275	944.4404	944.4676	-0.0271	1	49	0.15	1	K.KTGNTPDGR.K
489.2474	976.4802	976.5164	-0.0362	0	25	3.5	2	R.AIMPFGGIR.M + Oxidation (M)
500.6995	999.3844	999.4596	-0.0752	0	31	8.2	1	K.QMQFFGAR.C + Oxidation (M)
524.7831	1047.5516	1047.5825	-0.0309	1	28	20	2	R.RVALYGVDL.L
549.2792	1096.5438	1096.5917	-0.0478	0	50	0.095	1	R.VSGYAVNFIK.L
549.3063	1096.5980	1096.5917	0.0064	0	(35)	3.2	1	R.VSGYAVNFIK.L
557.7695	1113.5244	1113.5778	-0.0534	0	52	0.067	1	R.EQQLDVINR.T
557.7970	1113.5794	1113.5778	0.0016	0	(40)	1.1	1	R.EQQLDVINR.T
575.2479	1148.4812	1148.5244	-0.0432	0	41	0.87	1	K.EQNGAAMSIGR.I + Oxidation (M)
575.2635	1148.5124	1148.5244	-0.0120	0	(25)	3.3	1	K.EQNGAAMSIGR.I + Oxidation (M)
652.3369	1302.6592	1302.6932	-0.0339	0	54	0.044	1	K.AGIITGLPDAYGR.G
652.3614	1302.7082	1302.6932	0.0151	0	(31)	7.8	1	K.AGIITGLPDAYGR.G
477.9219	1430.7439	1430.7881	-0.0443	1	(50)	0.1	1	K.KAGIITGLPDAYGR.G
716.3826	1430.7506	1430.7881	-0.0375	1	66	0.0024	1	K.KAGIITGLPDAYGR.G
716.4042	1430.7938	1430.7881	0.0057	1	(39)	1.4	1	K.KAGIITGLPDAYGR.G

#### 2. gi|86607744      Mass: 86312    Score: 515    Queries matched: 16

formate acetyltransferase [*Synechococcus* sp. JA-2-3B'a(2-13)]

Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
352.6837	703.3528	703.3765	-0.0237	1	30	14	1	K.HKTYR.N
352.6950	703.3754	703.3905	-0.0150	0	33	6.7	1	R.LPEAFK.R
368.6835	735.3524	735.3915	-0.0391	0	51	0.093	1	R.IIGDYR.R
409.1825	816.3504	816.3726	-0.0222	0	55	0.043	1	K.TGNTPDGR.R

446.7154	8914.162	8914.814	-0.0652	0	40	1.3	1	R.VALYGVDR.L
473.2275	944.4404	944.4676	-0.0271	1	49	0.15	1	K.KTGNTPDGR.R
487.2188	972.4230	972.4737	-0.0507	1	34	4.3	1	K.TGNTPDGR.R.A
489.2474	976.4802	976.5164	-0.0362	0	25	35	2	R.AIMPFGGIR.V + Oxidation (M)
500.6995	999.3844	999.4596	-0.0752	0	31	8.2	1	K.QMQFFGAR.V + Oxidation (M)
524.7831	1047.5516	1047.5825	-0.0309	1	28	20	2	R.VALYGVDR.L
549.2792	1096.5438	1096.5917	-0.0478	0	50	0.095	1	R.VSGYAVNFIK.L
549.3063	1096.5980	1096.5917	0.0064	0	(35)	3.2	1	R.VSGYAVNFIK.L
557.7695	1113.5244	1113.5778	-0.0534	0	52	0.067	1	R.EQQLDVINR.T
557.7970	1113.5794	1113.5778	0.0016	0	(40)	1.1	1	R.EQQLDVINR.T
575.2479	1148.4812	1148.5244	-0.0432	0	41	0.87	1	K.EQNGAAMSLGR.V + Oxidation (M)
575.2635	1148.5124	1148.5244	-0.0120	0	(25)	33	1	K.EQNGAAMSLGR.V + Oxidation (M)

### 3. gi|52080503 Mass: 83378 Score: 453 Queries matched: 13

putative formate C-acetyltransferase [*Bacillus licheniformis* ATCC 14580]

Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
352.6837	703.3528	703.3402	0.0127	0	30	14	1	K.HQTYR.Q
368.6835	735.3524	735.3915	-0.0391	0	51	0.093	1	R.IIGDYR.R
409.1825	816.3504	816.3726	-0.0222	0	55	0.043	1	K.TGNTPDGR.R
473.2275	944.4404	944.4676	-0.0271	1	49	0.15	1	K.KTGNTPDGR.R
487.2188	972.4230	972.4737	-0.0507	1	34	4.3	1	K.TGNTPDGR.R.A
500.6995	999.3844	999.4596	-0.0752	0	31	8.2	1	K.QMQFFGAR.A + Oxidation (M)
549.2792	1096.5438	1096.5917	-0.0478	0	50	0.095	1	R.VSGYAVNFIK.L
549.3063	1096.5980	1096.5917	0.0064	0	(35)	3.2	1	R.VSGYAVNFIK.L
575.2479	1148.4812	1148.5244	-0.0432	0	41	0.87	1	K.EQNGAAMSLGR.V + Oxidation (M)
575.2635	1148.5124	1148.5244	-0.0120	0	(25)	33	1	K.EQNGAAMSLGR.V + Oxidation (M)
730.3781	1458.7416	1458.8195	-0.0778	1	65	0.0033	1	R.KVGHITGLPDAYGR.G
487.8916	1460.6530	1460.6970	-0.0440	0	(26)	29	1	R.EAQELVDHFVVK.L + Oxidation (M)
731.3467	1460.6788	1460.6970	-0.0181	0	47	0.2	1	R.EAQELVDHFVVK.L + Oxidation (M)

### 4. gi|116872838 Mass: 83779 Score: 418 Queries matched:

formate acetyltransferase [*Listeria welshimeri* serovar 6b str. SLCC5334]

Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
352.6837	703.3528	703.3765	-0.0237	1	30	14	1	K.HKTYR.D
368.6835	735.3524	735.3915	-0.0391	0	51	0.093	1	R.IIGDYR.R
409.1825	816.3504	816.3726	-0.0222	0	55	0.043	1	K.TGNTPDGR.R
473.2275	944.4404	944.4676	-0.0271	1	49	0.15	1	K.KTGNTPDGR.R
487.2188	972.4230	972.4737	-0.0507	1	34	4.3	1	K.TGNTPDGR.R.A
500.6995	999.3844	999.4596	-0.0752	0	31	8.2	1	K.QMQFFGAR.A + Oxidation (M)
502.2757	1002.5368	1002.5709	-0.0341	0	77	0.00022	1	K.GALASLSSVAK.L
502.2807	1002.5468	1002.5709	0.0241	0	(51)	0.09	1	K.GALASLSSVAK.L
549.2792	1096.5438	1096.5917	-0.0478	0	50	0.095	1	R.VSGYAVNFIK.L
549.3063	1096.5980	1096.5917	0.0064	0	(35)	3.2	1	R.VSGYAVNFIK.L
575.2479	1148.4812	1148.5244	-0.0432	0	41	0.87	1	K.EQNGAAMSLGR.T + Oxidation (M)
575.2635	1148.5124	1148.5244	-0.0120	0	(25)	33	1	K.EQNGAAMSLGR.T + Oxidation (M)

**Proteins matching the same set of peptides:**

5. [gi|16800511](#)      **Mass:** 83744   **Score:** 417   **Queries matched:** 12

pyruvate formate-lyase [*Listeria innocua* Clip11262]

6. [gi|16803446](#)      **Mass:** 83772   **Score:** 417   **Queries matched:** 12

pyruvate formate-lyase [*Listeria monocytogenes* EGD-e]

7. [gi|46907634](#)      **Mass:** 83758   **Score:** 417   **Queries matched:** 12

formate acetyltransferase [*Listeria monocytogenes* str. 4b F2365]

8. [gi|127634467](#)      **Mass:** 83771   **Score:** 417   **Queries matched:** 12

formate acetyltransferase [*Listeria monocytogenes* 10403S]

9. [gi|150389979](#)      **Mass:** 84076   **Score:** 391   **Queries matched:** 15

formate acetyltransferase [*Alkaliphilus metalliredigens* QYMF]

Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
368.6835	735.3524	735.3915	-0.0391	0	51	0.093	1	R.IIGDYR.R
489.2474	976.4802	976.5164	-0.0362	0	25	35	2	R.AIMPEGGIR.T + Oxidation (M)
500.6995	999.3844	999.4596	-0.0752	0	31	8.2	1	K.QMQFFGAR.C + Oxidation (M)
549.2792	1096.5438	1096.5917	-0.0478	0	50	0.095	1	R.VSGYAVNFIK.L
549.3063	1096.5980	1096.5917	0.0064	0	(35)	3.2	1	R.VSGYAVNFIK.L
557.7695	1113.5244	1113.5778	-0.0534	0	52	0.067	1	R.EQQLDVINR.T
557.7970	1113.5794	1113.5778	0.0016	0	(40)	1.1	1	R.EQQLDVINR.T
575.2479	1148.4812	1148.5244	-0.0432	0	41	0.87	1	K.EQNGAAMSLGR.V + Oxidation (M)
575.2635	1148.5124	1148.5244	-0.0120	0	(25)	33	1	K.EQNGAAMSLGR.V + Oxidation (M)
652.3369	1302.6592	1302.6932	-0.0339	0	54	0.044	1	K.AGHTGLPDAYGR.G
652.3614	1302.7082	1302.6932	0.0151	0	(31)	7.8	1	K.AGHTGLPDAYGR.G
477.9219	1430.7439	1430.7881	-0.0443	1	(50)	0.1	1	R.KAGHTGLPDAYGR.G
716.3826	1430.7506	1430.7881	-0.0375	1	66	0.0024	1	R.KAGHTGLPDAYGR.G
716.4042	1430.7938	1430.7881	0.0057	1	(39)	1.4	1	R.KAGHTGLPDAYGR.G
502.2675	1503.7807	1503.8297	-0.0490	1	21	87	1	R.VALYGIDRLIEDK.N

10. [gi|83594330](#)      **Mass:** 83353   **Score:** 322   **Queries matched:** 9

Formate acetyltransferase [*Rhodospirillum rubrum* ATCC 11170]

Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
368.6835	735.3524	735.3915	-0.0391	0	51	0.093	1	R.IIGDYR.R
409.1825	816.3504	816.3726	-0.0222	0	55	0.043	1	K.IGNTPDGR.R
473.2275	944.4404	944.4676	-0.0271	1	49	0.15	1	K.KTGNTPDGR.R

487.2188	972.4230	972.4737	-0.0507	1	34	-1.3	1	K.TGNTPDGRR.A
500.6995	999.3844	999.4596	-0.0752	0	31	8.2	1	K.QMQFFGAR.A + Oxidation (M)
575.2479	1148.4812	1148.5244	-0.0432	0	41	0.87	1	K.EQNGAAMSLGR.V + Oxidation (M)
575.2635	1148.5124	1148.5244	-0.0120	0	(25)	33	1	K.EQNGAAMSLGR.V + Oxidation (M)
681.8401	1361.6656	1361.7191	-0.0534	0	62	0.0066	1	K.ALLYAINGGVDEK.S
681.8716	1361.7286	1361.7191	0.0096	0	(50)	0.1	1	K.ALLYAINGGVDEK.S

**11. gi|106885378 Mass: 77549 Score: 251 Queries matched: 6**

formate acetyltransferase [*Clostridium phytofermentans* ISDg]

Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
368.6835	735.3524	735.3915	-0.0391	0	51	0.093	1	R.HGDYR.R
409.1825	816.3504	816.3726	-0.0222	0	55	0.043	1	K.TGNTPDGR.K
473.2275	944.4404	944.4676	-0.0271	1	49	0.15	1	K.K.TGNTPDGR.K
575.2479	1148.4812	1148.5244	-0.0432	0	41	0.87	1	K.EQNGAAMSLGR.T + Oxidation (M)
575.2635	1148.5124	1148.5244	-0.0120	0	(25)	33	1	K.EQNGAAMSLGR.T + Oxidation (M)
628.8262	1255.6378	1255.6884	-0.0506	1	56	0.029	1	R.LREELAEQIR.A

**12. gi|119026010 Mass: 88896 Score: 81 Queries matched**

formate acetyltransferase [*Bifidobacterium adolescentis* ATCC 15703]

Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
368.6835	735.3524	735.3915	-0.0391	0	51	0.093	1	R.HGDYR.R
414.2079	826.4012	826.4701	-0.0689	1	31	7.7	1	K.IFTKYR.K

**Proteins matching the same set of peptides:**

**13. gi|23465524 Mass: 90431 Score: 80 Queries matched: 2**

formate acetyltransferase [*Bifidobacterium longum* NCC2705]

**14. gi|46190412 Mass: 88824 Score: 80 Queries matched: 2**

COG1882: Pyruvate-formate lyase [*Bifidobacterium longum* DJO10A]

**Search Parameters**

Type of search : MS/MS Ion Search

Enzyme : Trypsin

Variable modifications : Acrylamide (C), Carbamidomethyl (C), Oxidation (M)

Mass values : Monoisotopic

Protein Mass : Unrestricted

Peptide Mass Tolerance :  $\pm 0.25$  Da

Fragment Mass Tolerance:  $\pm 0.2$  Da

Max Missed Cleavages : 1

Instrument type : ESI-QUAD-TOF

Number of queries : 133

Mascot: <http://www.matrixscience.com/>

## A2.4 Spot 1 from X7 total cell proteins

### Mascot Search Results

gi|125973025 formate acetyltransferase [*Clostridium thermocellum* ATCC 27405]

gi|68054704 Formate acetyltransferase [*Exiguobacterium sibiricum* 255-15]

gi|86607744 formate acetyltransferase [*Synechococcus* sp. JA-2-3B'a(2-13)]

gi|52080503 putative formate C-acetyltransferase [*Bacillus licheniformis* ATCC 14580]

gi|116872838 formate acetyltransferase [*Listeria welshimeri* serovar 6b str. SLCC5334]

gi|83594330 Formate acetyltransferase [*Rhodospirillum rubrum* ATCC 11170]

gi|106885378 formate acetyltransferase [*Clostridium phytofermentans* ISDg]

gi|119026010 formate acetyltransferase [*Bifidobacterium adolescentis* ATCC 15703]

1. gi|125973025      **Mass:** 84351    **Score:** 698    **Queries matched:** 20

formate acetyltransferase [*Clostridium thermocellum* ATCC 27405]

Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
368.6815	735.3484	735.3915	-0.0431	0	50	0.096	1	R.HGDYR.R
409.1738	816.3330	816.3726	-0.0396	0	54	0.056	1	K.TGNTPDGR.K
445.7409	889.4672	889.5021	-0.0349	0	24	55	1	K.YPQLTIR.V
446.7126	891.4106	891.4814	-0.0708	0	36	3.2	1	R.VALYGVDR.L
473.2328	944.4510	944.4676	-0.0165	1	51	0.098	1	K.KTGNTPDGR.K
475.2475	948.4804	948.5141	-0.0337	1	31	10	1	R.GRIIGDYR.R
489.2388	976.4630	976.5164	-0.0534	0	34	4.8	1	R.AIMPFGGIR.M + Oxidation (M)
500.7031	999.3916	999.4596	-0.0680	0	36	2.8	1	K.QMQFFGAR.C + Oxidation (M)
524.7807	1047.5468	1047.5825	-0.0357	1	37	2.2	1	R.RVALYGVDR.L
549.2826	1096.5506	1096.5917	-0.0410	0	52	0.06	1	R.VSGYAVNFIK.L
549.2977	1096.5808	1096.5917	-0.0108	0	(24)	37	1	R.VSGYAVNFIK.L
557.7479	1113.4812	1113.5778	-0.0966	0	52	0.066	1	R.EQQLDVINR.T
575.2523	1148.4900	1148.5244	-0.0344	0	53	0.057	1	K.EQNGAAMSIGR.I + Oxidation (M)
575.2635	1148.5124	1148.5244	-0.0120	0	(41)	0.82	1	K.EQNGAAMSIGR.I + Oxidation (M)
652.3328	1302.6510	1302.6932	-0.0421	0	50	0.12	1	K.AGHITGLPDAYGR.G
652.3520	1302.6894	1302.6932	-0.0037	0	(40)	1	1	K.AGHITGLPDAYGR.G
477.9209	1430.7409	1430.7881	-0.0473	1	(47)	0.2	1	K.KAGHITGLPDAYGR.G
716.3788	1430.7430	1430.7881	-0.0451	1	83	4.6e-005	1	K.KAGHITGLPDAYGR.G
716.3944	1430.7742	1430.7881	-0.0139	1	(71)	0.00078	1	K.KAGHITGLPDAYGR.G
495.5940	1483.7602	1483.8107	-0.0505	1	58	0.014	1	K.LTREQQLDVINR.T

## 2. gi|68054704 Mass: 84106 Score: 605 Queries matched: 17

Formate acetyltransferase [*Exiguobacterium sibiricum* 255-15]

Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
368.6815	735.3484	735.3915	-0.0431	0	50	0.096	1	R.IIGDYR.R
409.1738	816.3330	816.3726	-0.0396	0	54	0.056	1	K.TGNTPDGR.R
473.2328	944.4510	944.4676	-0.0165	1	51	0.098	1	K.KTGNTPDGR.R
475.2475	948.4804	948.5141	-0.0337	1	31	10	1	R.GRIIGDYR.R
487.2216	972.4286	972.4737	-0.0451	1	36	2.7	1	K.TGNTPDGRR.A
500.7031	999.3916	999.4596	-0.0680	0	36	2.8	1	K.QMQFFGAR.A + Oxidation (M)
549.2826	1096.5506	1096.5917	-0.0410	0	52	0.06	1	R.VSGYAVNFIK.L
549.2977	1096.5808	1096.5917	-0.0108	0	(24)	37	1	R.VSGYAVNFIK.L
557.7479	1113.4812	1113.5778	-0.0966	0	52	0.066	1	R.EQQIDVINR.I
575.2523	1148.4900	1148.5244	-0.0344	0	53	0.057	1	K.EQNGAAMSLGR.V + Oxidation (M)
575.2635	1148.5124	1148.5244	-0.0120	0	(41)	0.82	1	K.EQNGAAMSLGR.V + Oxidation (M)
652.3328	1302.6510	1302.6932	-0.0421	0	50	0.12	1	K.AGHTGLPDAYGR.G
652.3520	1302.6894	1302.6932	-0.0037	0	(40)	1	1	K.AGHTGLPDAYGR.G
477.9209	1430.7409	1430.7881	-0.0473	1	(47)	0.2	1	R.KAGHTGLPDAYGR.G
716.3788	1430.7430	1430.7881	-0.0451	1	83	4.6e-005	1	R.KAGHTGLPDAYGR.G
716.3944	1430.7742	1430.7881	-0.0139	1	(71)	0.00078	1	R.KAGHTGLPDAYGR.G
495.5940	1483.7602	1483.8107	-0.0505	1	58	0.014	1	K.LTREQQIDVINR.I

## 3. gi|86607744 Mass: 86312 Score: 600 Queries matched: 16

formate acetyltransferase [*Synechococcus* sp. JA-2-3B'a(2-13)]

Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
368.6815	735.3484	735.3915	-0.0431	0	50	0.096	1	R.IIGDYR.R
395.6539	789.2932	789.3116	-0.0183	0	23	65	1	K.YCYER.L + Carbamidomethyl (C)
409.1738	816.3330	816.3726	-0.0396	0	54	0.056	1	K.TGNTPDGR.R
446.7126	891.4106	891.4814	-0.0708	0	36	3.2	1	R.VALYGVDR.L
473.2328	944.4510	944.4676	-0.0165	1	51	0.098	1	K.KTGNTPDGR.R
475.2475	948.4804	948.5141	-0.0337	1	31	10	1	R.GRIIGDYR.R
487.2216	972.4286	972.4737	-0.0451	1	36	2.7	1	K.TGNTPDGRR.A
489.2388	976.4630	976.5164	-0.0534	0	34	4.8	1	R.AIMPEGGIR.V + Oxidation (M)
500.7031	999.3916	999.4596	-0.0680	0	36	2.8	1	K.QMQFFGAR.V + Oxidation (M)
524.7807	1047.5468	1047.5825	-0.0357	1	37	2.2	1	R.VALYGVDR.L
549.2826	1096.5506	1096.5917	-0.0410	0	52	0.06	1	R.VSGYAVNFIK.L
549.2977	1096.5808	1096.5917	-0.0108	0	(24)	37	1	R.VSGYAVNFIK.L
557.7479	1113.4812	1113.5778	-0.0966	0	52	0.066	1	R.EQLDVINR.I
575.2523	1148.4900	1148.5244	-0.0344	0	53	0.057	1	K.EQNGAAMSLGR.V + Oxidation (M)
575.2635	1148.5124	1148.5244	-0.0120	0	(41)	0.82	1	K.EQNGAAMSLGR.V + Oxidation (M)
495.5940	1483.7602	1483.8107	-0.0505	1	58	0.014	1	K.LTREQQIDVINR.I

**4. gi|52080503      Mass: 83378    Score: 499    Queries matched: 14**putative formate C-acetyltransferase [*Bacillus licheniformis* ATCC 14580]

Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
368.6815	735.3484	735.3915	-0.0431	0	50	0.096	1	R.IIGDYR.R
395.6539	789.2932	789.3116	-0.0183	0	23	65	1	K.YCYER.I + Carbamidomethyl (C)
409.1738	816.3330	816.3726	-0.0396	0	54	0.056	1	K.TGNTPDGR.R
473.2328	944.4510	944.4676	-0.0165	1	51	0.098	1	K.KTGNTPDGR.R
475.2475	948.4804	948.5141	-0.0337	1	31	10	1	R.GRIIGDYR.R
487.2216	972.4286	972.4737	-0.0451	1	36	2.7	1	K.TGNTPDGRR.A
500.7031	999.3916	999.4596	-0.0680	0	36	2.8	1	K.QMQFFGAR.A + Oxidation (M)
549.2826	1096.5506	1096.5917	-0.0410	0	52	0.06	1	R.VSGYAVNFIK.L
549.2977	1096.5808	1096.5917	-0.0108	0	(24)	37	1	R.VSGYAVNFIK.L
575.2523	1148.4900	1148.5244	-0.0344	0	53	0.057	1	K.EQNGAAMSLGR.V + Oxidation (M)
575.2635	1148.5124	1148.5244	-0.0120	0	(41)	0.82	1	K.EQNGAAMSLGR.V + Oxidation (M)
730.3857	1458.7568	1458.8195	-0.0626	1	63	0.0055	1	R.KVGIITGLPDAYGR.G
731.3382	1460.6618	1460.6970	-0.0351	0	50	0.1	1	R.EAQELVDHFVVK.L + Oxidation (M)
487.9001	1460.6785	1460.6970	-0.0185	0	(24)	41	1	R.EAQELVDHFVVK.L + Oxidation (M)

**5. gi|16872838      Mass: 83779    Score: 435    Queries matched: 12**formate acetyltransferase [*Listeria welshimeri* serovar 6b str. SLCC5334]

Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
368.6815	735.3484	735.3915	-0.0431	0	50	0.096	1	R.IIGDYR.R
409.1738	816.3330	816.3726	-0.0396	0	54	0.056	1	K.TGNTPDGR.R
473.2328	944.4510	944.4676	-0.0165	1	51	0.098	1	K.KTGNTPDGR.R
475.2475	948.4804	948.5141	-0.0337	1	31	10	1	R.GRIIGDYR.R
487.2216	972.4286	972.4737	-0.0451	1	36	2.7	1	K.TGNTPDGRR.A
500.7031	999.3916	999.4596	-0.0680	0	36	2.8	1	K.QMQFFGAR.A + Oxidation (M)
502.2782	1002.5418	1002.5709	-0.0291	0	73	0.00064	1	K.GALASLSSVAK.L
502.2889	1002.5632	1002.5709	-0.0077	0	(47)	0.21	1	K.GALASLSSVAK.L
549.2826	1096.5506	1096.5917	-0.0410	0	52	0.06	1	R.VSGYAVNFIK.L
549.2977	1096.5808	1096.5917	-0.0108	0	(24)	37	1	R.VSGYAVNFIK.L
575.2523	1148.4900	1148.5244	-0.0344	0	53	0.057	1	K.EQNGAAMSLGR.I + Oxidation (M)
575.2635	1148.5124	1148.5244	-0.0120	0	(41)	0.82	1	K.EQNGAAMSLGR.I + Oxidation (M)

**Proteins matching the same set of peptides:****6. gi|16800511      Mass: 83744    Score: 434    Queries matched: 12**pyruvate formate-lyase [*Listeria innocua* Clp11262]**7. gi|16803446      Mass: 83772    Score: 434    Queries matched: 12**pyruvate formate-lyase [*Listeria monocytogenes* EGD-e]

8. gi|46907634 Mass: 83758 Score: 434 Queries matched: 12

formate acetyltransferase [*Listeria monocytogenes* str. 4b F2365]

9. gi|127634467 Mass: 83771 Score: 434 Queries matched: 12

formate acetyltransferase [*Listeria monocytogenes* 10403S]

10. gi|83594330 Mass: 83353 Score: 363 Queries matched: 10

Formate acetyltransferase [*Rhodospirillum rubrum* ATCC 11170]

Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
368.6815	735.3484	735.3915	-0.0431	0	50	0.096	1	R.IIGDYR.R
409.1738	816.3330	816.3726	-0.0396	0	54	0.056	1	K.TGNTPDGR.R
473.2328	944.4510	944.4676	-0.0165	1	51	0.098	1	K.K.TGNTPDGR.R
475.2475	948.4804	948.5141	-0.0337	1	31	10	1	R.GRIIGDYR.R
487.2216	972.4286	972.4737	-0.0451	1	36	2.7	1	K.TGNTPDGRR.A
500.7031	999.3916	999.4596	-0.0680	0	36	2.8	1	K.QMQFFGAR.A + Oxidation (M)
575.2523	1148.4900	1148.5244	-0.0344	0	53	0.057	1	K.EQNGAAMSLGR.V + Oxidation (M)
575.2635	1148.5124	1148.5244	-0.0120	0	(41)	0.82	1	K.EQNGAAMSLGR.V + Oxidation (M)
681.8506	1361.6866	1361.7191	-0.0324	0	54	0.046	1	K.ALIYAINGGVDEK.S
681.8812	1361.7478	1361.7191	0.0288	0	(53)	0.054	1	K.ALIYAINGGVDEK.S

11. gi|106885378 Mass: 77549 Score: 294 Queries matched: 7

formate acetyltransferase [*Clostridium phytofermentans* ISDg]

Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
368.6815	735.3484	735.3915	-0.0431	0	50	0.096	1	R.IIGDYR.R
409.1738	816.3330	816.3726	-0.0396	0	54	0.056	1	K.TGNTPDGR.K
473.2328	944.4510	944.4676	-0.0165	1	51	0.098	1	K.K.TGNTPDGR.K
475.2475	948.4804	948.5141	-0.0337	1	31	10	1	R.GRIIGDYR.R
575.2523	1148.4900	1148.5244	-0.0344	0	53	0.057	1	K.EQNGAAMSLGR.T + Oxidation (M)
575.2635	1148.5124	1148.5244	-0.0120	0	(41)	0.82	1	K.EQNGAAMSLGR.T + Oxidation (M)
628.8088	1255.6030	1255.6884	-0.0854	1	56	0.029	1	R.LREELAEQIR.A

12. gi|119026010 Mass: 88896 Score: 115 Queries matched: 3

formate acetyltransferase [*Bifidobacterium adolescentis* ATCC 15703]

Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
368.6815	735.3484	735.3915	-0.0431	0	50	0.096	1	R.IIGDYR.R
414.1922	826.3698	826.4701	-0.1003	1	35	3.1	1	K.IFIKYR.K
475.2475	948.4804	948.5141	-0.0337	1	31	10	1	R.GRIIGDYR.R

**Proteins matching the same set of peptides:**

**13. gi|23465524      Mass: 90431    Score: 114    Queries matched: 3**  
formate acetyltransferase [*Bifidobacterium longum* NCC2705]

**14. gi|46190412      Mass: 88824    Score: 114    Queries matched: 3**  
COG1882: Pyruvate-formate lyase [*Bifidobacterium longum* DJO10A]

**Search Parameters**

Type of search : MS/MS Ion Search

Enzyme : Trypsin

Variable modifications : Acrylamide (C),Carbamidomethyl (C),Oxidation (M)

Mass values : Monoisotopic

Protein Mass : Unrestricted

Peptide Mass Tolerance :  $\pm 0.25$  Da

Fragment Mass Tolerance:  $\pm 0.2$  Da

Max Missed Cleavages : 1

Instrument type : ESI-QUAD-TOF

Number of queries : 133

Mascot: <http://www.matrixscience.com/>

## A2.5 Lysozyme sample

### Mascot Search Results

1. gi|229157      Mass: 14305   Score: 373   Queries matched: 7

#### Lysozyme

Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
523.2618	1044.5090	1044.5352	-0.0262	0	47	0.24	1	K.GIDVQAWIR.G
714.7303	1427.4460	1427.6429	-0.1969	0	(63)	0.0014	1	K.FESNFNTQATNR.N
714.7966	1427.5786	1427.6429	-0.0643	0	(72)	0.00054	1	K.FESNFNTQATNR.N
714.7966	1427.5786	1427.6429	-0.0643	0	80	8.6e-005	1	K.FESNFNTQATNR.N
846.8757	1691.7368	1691.7725	-0.0357	0	99	1.4e-006	1	K.IVSDGDGMNAWVAWR.N +
Oxidation (M)								
877.3511	1752.6876	1752.8278	-0.1402	0	111	5.6e-008	1	R.NTDGSTDYGILQINSR.W
607.6168	1819.8286	1819.8675	-0.0389	1	36	2.5	1	K.IVSDGDGMNAWVAWR.N +
Oxidation (M)								

2. gi|126595      Mass: 14498   Score: 191   Queries matched: 2

#### Lysozyme C-3 (1,4-beta-N-acetylmuramidase)

Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
846.8757	1691.7368	1691.7725	-0.0357	0	99	1.4e-006	1	R.IVSDGDGMNAWVAWR.N +
Oxidation (M)								
877.8655	1753.7164	1753.8118	-0.0954	0	93	4.1e-006	1	R.NTDGSTDYGILEINSR.W

3. gi|47117006      Mass: 14467   Score: 122   Queries matched: 3

#### Lysozyme C (1,4-beta-N-acetylmuramidase C)

Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
722.8135	1443.6124	1443.6378	-0.0254	0	(26)	26	1	K.YESNFNTQATNR.N
722.8155	1443.6164	1443.6378	-0.0214	0	29	13	1	K.YESNFNTQATNR.N
877.8655	1753.7164	1753.8118	-0.0954	0	93	4.1e-006	1	R.NTDGSTDYGILEINSR.W

### **A3: DNA Sequence alignment of Formate acetyltransferase from five closely related *Bacillus* species.**

### A3: DNA Sequence alignment of Formate acetyltransferase from five closely related *Bacillus* species

The position where primers were designed are indicated by green shading with either orange (primer 1 and 1A) or blue (primer 2 and 2A) respectively

```
gi|118475778_505453-507717 Bacillus thuringiensis str. Al Hakam
gi|49183039_503644-505893 Bacillus anthracis str. Sterne
gi|42779081_575900-578149 Bacillus cereus ATCC 10987
gi|49476684_492607-494856 Bacillus thuringiensis serovar konkukian
gi|56182545_c2079245-2077020 Bacillus licheniformis ATCC 14580
```

```
ATGGAGGAGATTAGGATGACTCAAGTATTAGAAAATGTTAAAAACGCATG 50
-----ATGACTCAAGTATTAGAAAATGTTAAAAACGCATG 35
-----ATGACTCAAGTATTAGAAAATGTTAAAAACGCATG 35
-----ATGACTCAAGTATTAGAAAATGTTAAAAACGCATG 35
-----ATGGAACAATG 11
          * * * *
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GGAAAAC TTTAAAGGTGAAAAATGGAAAGCAGAGATTGATGTTCCGCGATT 100
GGAAAAC TTTAAAGGTGAAAAATGGAAAGCAGAGATTGATGTTCCGCGATT 85
GGAAAAC TTTAAAGGTGAAAAATGGAAAGCAGAGATTGATGTTCCGCGATT 85
GGAAAAC TTTAAAGGTGAAAAATGGAAAGCAGAGATTGATGTTCCGCGATT 85
GAAAGGTTTACCACAAACGTTTGGCAAAAAGAAGTCAATGTCCGCGATT 61
* * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
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TCATTTTAAATAATGTAAACGTTTACGAGGGAGACGAATCTTTCTTAGCG 150
TCATTTTAAATAATGTAAACGTTTACGAGGGAGATGAATCTTTCTTAGCG 135
TCATTTTAAATAATGTAAACGTTTAC AGGGAGATGAATCTTTCTTAGCG 135
TCATTTTAAATAATGTAAACGTTTACGAAGGGGACGAATCTTTCTTAGCG 135
TTATTTCTCTCGAAC TTTGAGCCATATCAAGGTGACGAATCGTTTCTCGAA 111
* * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
```

```
GGAGCAACTGAAGCAACGAAACAAC TTTGGGATCAAGTAATGGATTTAAC 200
GGAGCAACTGAAGCAACGAAACAAC TTTGGGATCAAGTAATGGATTTAAC 185
GGAGCAACTGAAGCAACGAAACAAC TTTGGGATCAAGTAATGGATTTAAC 185
GGAGCAACTGAAGCAACGAAACAAC TTTGGGATCAAGTAATGGATTTAAC 185
CCTCCGACGGGAAGCTACATCGGCATTATGGGATCATGTAATGGATTTGAC 161
* * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
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Primer 1 and 1A



```
AACGAAAGAACGTGAAAAACGGTGGCGTCTTGATATGGATACAAAAATTG 250
AACGAAAGAACGTGAAAAACGGTGGCGTCTTGATATGGATACAAAAATTG 235
AACGAAAGAACGTGAAAAACGGTGGCGTCTTGATATGGATACAAAAATTG 235
AACGAAAGAACGTGAAAAACGGTGGCGTCTTGATATGGATACAAAAATTG 235
AAAAAAGAGCGTGAAAAACGGAGGCGTCCTTGATATGGATACAGAGATCG 211
* * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
```

**A3: DNA Sequence Alignment of Formate acetyltransferase fro five Bacillus species**

```

TTTCTTCTATTACATCACATGAACCAGGATATTTAAATAAAGAGACTGAA 300
TTTCTTCTATTACATCACATGAACCAGGATATTTAAATAAAGAGACTGAA 285
TTTCTTCTATTACATCACATGAACCAGGATATTTAAATAAAGAGACTGAA 285
TTTCTTCTATTACATCACATGAACCAGGATATTTAAATAAAGAGACTGAA 285
TCTCAACGATCACCTCGCACGGTCCCGGATATTTGAACAAAGACCTGGAA 261
* * * * *
AAAGTAGTTGGTTTCCAAACTGATAAAACCA'TTTAAACGGTCTTTACAACC 350
AAAGTAGTCGGTTTCCAAACTGATAAAACCA'TTTAAACGGTCTTTACAGCC 335
AAAGTAGTCGGTTTCCAAACTGATAAAACCA'TTTAAACGGTCTTTACAACC 335
AAAGTAGTCGGTTTCCAAACTGATAAAACCA'TTTAAACGGTCTTTACAACC 335
AAAGTAGTCGGTTTCCAAACTGATAAAACCA'TTTAAACGGTCTTTACAACC 311
***** * * * *
ATATGGTGGTATTCGTATGGCGGAACAAGCTTGTGAATCTTATGGATATG 400
ATATGGTGGTATTCGTATGGCGGAACAAGCTTGTGAATCTTATGGATACG 385
ATATGGTGGTATTCGTATGGCGGAACAAGCTTGTGAATCTTATGGATACG 385
ATATGGTGGTATTCGTATGGCGGAACAAGCTTGTGAATCTTATGGATACG 385
TTTCGGCGGCATCCGAATGGCAAAGCAGGCATGCGAATCCTATGGT'TTTA 361
* * * * *
AAATGGATAAAGAGCTTAGTCGTATTTTTAGAGATTGGAGAAAAACTCAT 450
AAATGGATAAAGAACTTAGTCGTATTTTTAGAGATTGGAGAAAAACTCAT 435
AAATGGATAAAGAGCTTAGTCGTATTTTCAGAGATTGGAGAAAAACTCAT 435
AAATGGATAAAGAACTTAGTCGTATTTTTAGAGATTGGAGAAAAACTCAT 435
AACTGAATGAAGAAGTGGAAAGGATCTTTACCGATTACCGCAAAACTCAT 411
* * * * *
AACCAAGGTGTATTTGATGCTTATACACCAGAAATGAGAGCTGCTCGTAA 500
AACCAAGGTGTATTTGATGCTTATACACCAGAAATGAGAGCTGCTCGTAA 485
AACCAAGGCGTATTTGATGCTTATACACCAGAAATGAGAGCTGCTCGTAA 485
AACCAAGGTGTATTTGATGCTTATACACCAGAAATGAGAGCTGCTCGTAA 485
AACCAAGGCGTGT'TTGACGCATATACGGACGAAATGAAGCTCGCCCGAAA 461
***** * * * *
ATCAGGTGTTATTACGGGTCTTCCAGATGCATACGGACGTGGACGTATTA 550
ATCAGGTGTTATTACTGGTCTTCCAGATGCATACGGACGTGGACGTATTA 535
ATCAGGTGTTATTACTGGTCTTCCGGATGCATACGGACGTGGACGTATTA 535
ATCAGGTGTTATTACTGGTCTTCCAGATGCATACGGACGTGGACGTATTA 535
AGTCGGAATCATTACCGGACTGCCTGATGCTTACGGGCGCGGGCGCATCA 511
* * * * *
TCGGTGACTIONACCGCCGCGTAGCATTATATGGAGTAGATCATTTAATTGAA 600
TCGGTGACTIONACCGCCGCGTAGCATTATATGGAGTAGATCATTTAATTGAA 585
TCGGTGACTIONACCGCCGCGTAGCATTATATGGAGTAGATCATTTAATTGAA 585
TCGGTGACTIONACCGCCGCGTAGCATTATATGGAGTAGATCATTTAATTGAA 585
TCGGTGACTIONACCGGAGAGTGGCGCTTACGGCGTGGATTTCTTGATCGAT 561
***** * * * *

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A3: DNA Sequence Alignment of Formate acetyltransferase from five *Bacillus* species

```

GCGAAGAAAACAGATTTAAATTTAACTGGTGGTGTAAATGAGTGAAGATAC 650
GCGAAGAAAAGCAGATTTAAA'TTAACTGGCGGTGTAAATGAGTGAAGATAC 635
GCGAAGAAAAGT'AGATTTAAATTTAACTGGCGGTGTAAATGAGTGAAGATAT 635
GCGAAGAAAAGCAGATTTAAA'TTAACTGGCGGTGTAAATGAGTGAAGATAC 635
GAAAAGAAAAAAGATGCGGCCGGCACCTC'PCGGGTGATG'TCTGAAGAAAA 611
*   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *

AATGCGTTTACGCGAAGAGTTATCTGAGCAAATACGTGCACTTCAAGAGT 700
AATGCGTTTACGCGAAGAATTTATCTGAGCAAATACGTGCACTTCAAGAGT 685
AATGCGTTTACGCGAAGAGTTATCTGAGCAAATACGTGCACTTCAAGAGT 685
AATGCGTTTACGCGAAGAGTTATCTGAGCAAATACGTGCCCTTCAAGAGT 685
CATCCGCCCTTCGTGAAGAAGTGTCAAGAACAAATCCGAGCATTGAACGAAC 661
* * * * * * * * * * * * * * * * * * * * * * * * * * * *

TAAAACAAATGGCTGCTTCTCATGGCTTTGATATTTCTAAGCCAGCAACA 750
TAAAACAAATGGCTGCTTCTCATGGATTTGATATTTCTAAGCCAGCAACA 735
TAAAACAAATGGCTGCTTCTCATGGCTTTGATATTTCTAAGCCAGCAACA 735
TAAAACAAATGGCTGCTTCTCATGGCTTTGATATTTCTAAGCCAGCAACA 735
TTAAAGCGCTTGCAAAAAGCTATGGGTTTGACA'TTCCAAGCCTGCGGCG 711
*   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *

ACTGCTCAAGAAGCATTCCAATGGTTATACTTTGCATATCTTGCAGCAAT 800
AATGCTCAAGAAGCATTCCAATGGTTATACTTTGCATATCTTGCAGCAAT 785
ACTGCTCAAGAAGCATTCCAATGGCT'ATACTTCGCATATCTTGCAGCAAT 785
ACTGCTCAAGAAGCATTCCAATGGTTATACTTTGCATATCTTGCAGCAAT 785
AATGCAAGAGAAGCATTTCAATGGCTGTATTTTGCCTATTTGGCTGCCAT 761
*   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *

TAAAGAGCAAAACGGAGCTGCAATGAGTCTTGGACGTACTTCTACATTCT 850
TAAAGAGCAAAACGGAGCTGCAATGAGTCTTGGACGTACTTCTACATTCT 835
TAAAGAGCAAAACGGAGCTGCAATGAGTCTTGGCCGTACTTCTACATTCT 835
TAAAGAGCAAAACGGAGCTGCAATGAGTCTTGGACGTACTTCTACATTCT 835
TAAAGAGCAAAACGGAGCAGCAATGAGCCTTGGCCGCGTCCACGTTCC 811
***** * * * * * * * * * * * * * * * * * * * * * *

TAGATATTTATATTGAAAAGAGATTTAGCAAATGGTACTTTAACAGAAGAA 900
TAGATATTTATATTGAAAAGAGATTTAGCAAATGGTACTTTAACAGAAGAA 885
TAGATATTTATATTGAAAAGAGATTTAGCAAATGGTACTTTAACAGAAGAA 885
TAGATATTTATATTGAAAAGAGATTTAGCAAATGGCACTTTAACAGAAGAA 885
TTGATATTTACATCGAAAAGAGATTTGAAAACGGGCGTATTAACAGAGCGT 861
*   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *

GACGTACAAGAAATTTGTGGATCAC'TTCATTATGAAATTACGTCTTGTGAA 950
GACGTACAAGAAATTTGTGGATCACTTCATTATGAAATTACGTCTTGTGAA 935
GACGTACAAGAAATTTGTGGATCACTTCATTATGAAATTACGTCTTGTGAA 935
GACGTACAAGAAATTTGTGGATCACTTCATTATGAAATTACGTCTTGTAAA 935
GAAGCCCAAGAGCTTGTGCGACCATTTTCGTGATGAAGCTGCGTTTGGTCAA 911
* * * * * * * * * * * * * * * * * * * * * * * * * * * *

ATTTGCAAGAACACCTGATTATAATGAGTTATTCTCTGGTGACCCAACTT 1000
ATTTGCCAGAACACCTGATTATAATGAGTTATTCTCTGGTGACCCAACTT 985
ATTTGCAAGAACACCTGATTATAATGAATTTATTCTCTGGTGACCCAACTT 985
ATTTGCAAGAACACCTGATTATAATGAGTTATTCTCTGGCGACCCAACTT 985
ATTCGCGCGCACACCTGACTACAATGAAGTGTTCAGCGGCGATCCGACGT 961
* * * * * * * * * * * * * * * * * * * * * * * * * * * *

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A3: DNA Sequence Alignment of Formate acetyltransferase fro five Bacillus species

```

GGGTAAC TGAGTCTATCGGTGGTATGGCATTAGATGGTCGTCCATTAGTA 1050
GGGTAAC TGAGTCTATCGGTGGTATGGCATTAGATGGTCGTCCATTAGTA 1035
GGGTAAC TGAGTCTATCGGCGGTATGGCATTAGATGGCCGTCCATTAGTA 1035
GGGTAAC TGAGTCTATCGGTGGTATGGCATTGGATGGTCGTCCATTAGTA 1035
GGGTGACAGAATCAATCGGCGGAATGGCGCACGACGGACGCGCCCTGGTG 1011
**** * * * * * * * * * * * * * * * * * * * * * * * * * * * *

ACAAAGA AACTCATTCCGTTTCTTGCATACATTAGATAAATTTAGGACCAGC 1100
ACAAAGA AACTCATTCCGTTTCTTGCATACATTAGATAAATTTAGGACCAGC 1085
ACAAAGA AACTCATTCCGTTTCTTGCATACACTAGATAAATTTAGGACCAGC 1085
ACAAAGA AACTCATTCCGTTTCTTGCATACATTAGATAAATTTAGGACCAGC 1085
ACGAAAA AACTCGTTCCGTTTCTTGCATACGCTTGACAAATTTAGGCCCGGC 1061
* * * * * * * * * * * * * * * * * * * * * * * * * * * *

TCCAGA A CCAA AACTTAA CAGTTCTTTGGTCTAAACAATTA C C A C A G A A C T 1150
TCCAGA A CCAA AACTTAA CAGTTCTTTGGTCTAAACAATTA C C A C A G A A C T 1135
TCCAGA A CCAA AACTTAA CAGTTCTTTGGTCTAAACAATTA C C A C A G A A C T 1135
TCCAGA A CCAA AACTTAA CAGTTCTTTGGTCTAAACAATTA C C A C A G A A C T 1135
GCCTGA ACCGAATTTAA CCGTTCTTTGGTCTGT C A G A C T G C C G C A A A A G T 1111
* * * * * * * * * * * * * * * * * * * * * * * * * * * *

TTAAAA A C T A C T G T G C G A A A A T G T C T A T T A A A A C A T C A G C A A T T C A A T A T 1200
TTAAAA A C T A C T G T G C G A A A A T G T C T A T T A A A A C A T C A G C A A T T C A A T A T 1185
TTAAAA A C T A C T G T G C G A A A A T G T C T A T T A A A A C A T C A G C A A T T C A A T A T 1185
TTAAAA A C T A C T G T G C A A A A A T G T C T A T T A A A A C A T C A G C A A T T C A A T A T 1185
TTAAAA A C T A C T G T G C C A A A A T G T C G A T T A A A A C A A G C T C G A T C C A A T A C 1161
* * * * * * * * * * * * * * * * * * * * * * * * * * * *

GAAAA T G A T G A C A T T A T G C G T G C T G A C T A C G G C G A T G A C T A C G G A A T T G C 1250
GAAAA T G A T G A C A T T A T G C G T G C T G A C T A C G G C G A T G A C T A C G G A A T T G C 1235
GAAAA T G A T G A C A T T A T G C G T G C T G A C T A C G G C G A T G A T T A C G G A A T T G C 1235
GAAAA T G A T G A C A T T A T G C G T G C T G A C T A C G G T G A T G A C T A T G G A A T T G C 1235
GAAAA T G A C G A T A T C A T G C G T C C A G A A T A C G G T G A T G A C T A C G G A A T C G C 1211
* * * * * * * * * * * * * * * * * * * * * * * * * * * *

TTGTTG TGTATCTGCAATGAGAA TCGGTAAACAAATGCAAT TCTTTGGCG 1300
TTGTTG TGTATCTGCAATGAGAA TCGGTAAACAAATGCAAT TCTTTGGCG 1285
TTGTTG TGTATCTGCAATGAGAA TTGGTAAACAAATGCAGTTCTTTGGCG 1285
TTGTTG TGTATCTGCAATGAGAA TTGGTAAACAAATGCAGTTCTTTGGTG 1285
CTGCTG TGTATCGGCAATGGCAATCGGCAAA CAAATGCAGTTCTTTGGAG 1261
* * * * * * * * * * * * * * * * * * * * * * * * * * * *

CACGTG CAAACTTAGCAAAA GCATTA CTATATGCGATTAA CCGTGGTAAA 1350
CACGTG CAAACTTAGCAAAA GCATTA CTATATGCGATTAA CCGTGGTAAA 1335
CACGTG CAAACTTAGCAAAA GCATTA CTATATGCAATTAACCGTGGTAAA 1335
CACGTG CAAACTTAGCAAAA GCATTA TTATATGCGATTAA CCGTGGTAAA 1335
CACGCG CCAACTTGGCGAAA GCCTCTTTTATATGCGATTAA CCGCGGAAAA 1311
* * * * * * * * * * * * * * * * * * * * * * * * * * * *

GATGAAAA TCTAAAGCACAAGTTGGTCCTGAGTATGCACCGATTACTTC 1400
GATGAAAA TCTAAAGCACAAGTTGGTCCTGAGTATGCACCGATTACTTC 1385
GATGAAAA TCTAAAGCACAAGTTGGTCCTGAGTATGCACCGATTACTTC 1385
GATGAAAA TCTAAAGCACAAGTTGGCCCTGAGTACGCACCAATTA CTTC 1385
GACGAAA A GCATAAAA T GCAAGTCGGTCCGAAAA TGCCGCCGGTTGCTTC 1361
* * * * * * * * * * * * * * * * * * * * * * * * * * * *

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**A3: DNA Sequence Alignment of Formate acetyltransferase fro five Bacillus species**

ATGTC AATCTTAA CAATC ACATCTAACGTTGTATACGGTAAGAAA ACTGG 1850  
 ATGTC AATCTTAA CAAT CACATCTAACGTTGTATACGGTAAGAAA ACTGG 1835  
 ATGTC AATCTTAA CAATC ACATCTAACGTTGTATACGGTAAGAAA ACTGG 1835  
 ATGTC AATTTTAA CAATCA ATCTAACGTTGTATATGGTAAGAAA ACTGG 1835  
 ATGTC AATTTTAA CGATCACGTC AAACGTCGTTTACGGCAAGAAA CCGG 1811  
 \*\*\*\*\*

TAACACTCCAGATGGTCGCC TACTGGAGA ACCATTTGCACCAGGTGCGA 1900  
 TAACACTCCAGATGGTCGCCGCTACTGGAGA ACCATTTGCACCAGGTGCGA 1885  
 TAACACTCCAGATGGTCGCCGCTACTGGAGA ACCATTTGCACCAGGTGCGA 1885  
 TAACACTCCAGATGGTCGCCGCTACTGGAGA ACCATTTGCACCAGGTGCGA 1885  
 AAATACGCCGGATGGACGCCGCGGGAGAACCGTTTGCTCCAGGTGCGA 1861  
 \* \* \* \* \*

ACCCAATGCATGGACGTGATACAAAAGGTGCGTTAGCTTCATTATTATCT 1950  
 ACCCAATGCATGGACGTGATACAAAAGGTGCGCTAGCTTCATTATTATCT 1935  
 ACCCAATGCATGGACGTGATACAAAAGGTGCGTTAGCTTCATTATTATCT 1935  
 ACCCAATGCATGGACGTGATACAAAAGGTGCGTTAGCTTCATTATTATCT 1935  
 ATCCGATGCACGGCCGCGATACTAAAGGGACGCTTCGATCGCTGTCTTCA 1911  
 \* \* \* \* \*

GTAGCTAAATTACCAATGAAGATGCACAAGATGGTATTTCTAATACATT 2000  
 GTAGCTAAATTACCAATGAAGATGCACAAGATGGTATTTCTAATACATT 1985  
 GTAGCTAAATTACCAATGAAGATGCACAAGATGGCATTCTAATACATT 1985  
 GTAGCTAAATTACCAATGAAGATGCACAAGATGGTATTTCTAATACATT 1985  
 GTGGCAAAGCTGCCATTACAGCTATGCGCTCGACGGCATTTCCAACACCTT 1961  
 \* \* \* \* \*

CTCTATTATTC AAAAGCGCTTGGTAAAGAGGATGAAGTACAAGTACGTA 2050  
 CTCTATTATTC AAAAGCACTTGGTAAAGAGGATGAAGTACAAGTACGTA 2035  
 CTCTATTATTC AAAAGCACTTGGTAAAGAGGATGAAGTACAAGTACGTA 2035  
 CTCTATTATTC AAAAGCACTTGGTAAAGAGGATGAAGTACAAGTACGTA 2035  
 TTCAATCGTCCCGAAAAGCGCTTGGCAAAGACGAAGAGAGCCGCGCCGCCA 2011  
 \* \* \* \* \*

ACTTAGTATCAATGCTTGATGGTTATGCAGTAAAAGAAGGCCATCACTTA 2100  
 ACTTAGTATCAATGCTTGATGGTTATGCAGTAAAAGAAGGACATCACTTA 2085  
 ACTTAGTATCAATGCTTGATGGTTATGCAGTAAAAGAAGGACATCACTTA 2085  
 ACTTAGTATCAATGCTTGATGGTTATGCAGTAAAAGAAGGACATCACTTA 2085  
 ATTTGTCAAGCATCCTTGACGGATATGCCGAAAAACAGGACATCACTTA 2061  
 \* \* \* \* \*

AATATTAACGTA TTTAATCGTGAAACATT AATGGATGCAATGGAA CATCC 2150  
 AATATTAACGTA TTTAACCGTGAAACATT AATGGATGCAATGGAA CATCC 2135  
 AATATTAACGTA TTTAACCGTGAAACATT AATGGATGCAATGGAA CATCC 2135  
 AATATTAACGTA TTTAACCGTGAAACATT AATGGATGCAATGGAA CATCC 2135  
 AATGTAACGTA TTTAACAGAGAGACACTGCTCGACGCC ATGGAA CATCC 2111  
 \*\*\*\*\*



**Primer 2 and 2A**

██████████  
 TGA GAA TATCCACAATTAACAATTCGCGTATCTGGTTACGCTGTAAACT 2200  
 TGA GAA TATCCACAATTAACAATTCGCGTATCTGGTTACGCTGTAAACT 2185  
 TGA GAA TATCCACAATTAACAATTCGCGTATCTGGTTACGCTGTAAACT 2185  
 TGA GAA TATCCACAATTAACAATTCGCGTATCTGGTTACGCTGTAAACT 2185  
 A G A G G A A T A T C C G C A G T T A A C G A T T C G C G T C T C A G G C T A T G C G T C A A C T 2161  
 \*\*\*\*\*

### A3: DNA Sequence Alignment of Formate acetyltransferase fro five Bacillus species

```
TTATTAAATTAACTCGTGAACAACAAATCGATGTAATTAACCGTACAATG 2250
TTATTAAATTAACTCGTGAACAACAAATCGATGTAATTAACCGTACAATG 2235
TTATTAAATTAACTCGTGAACAACAAATCGATGTAATTAACCGTACAATG 2235
TTATTAAATTAACTCGTGAACAACAAATCGATGTAATTAACCGTACAATG 2235
TTATTAAGCTGACGAAAGAACAGCAGTTAGACGTCATCAGCAGAACCTTC 2211
```

```
***** * ** * * * * * * * * * * * * * * *
```

```
gi|118475778_505453-507717 CATGAAAGCATGTAA 2265
gi|49183039_503644-505893 CATGAAAGCATGTAA 2250
gi|42779081_575900-578149 CATGAAAGCATGTAA 2250
gi|49476684_492607-494856 CATGAAAGCATGTAA 2250
gi|56182545_c2079245-2077020 CATGAATCGATGTAG 2226
```

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***** *****
```

## Appendix

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**A4: Alignment of Formate acetyltransferase DNA sequences from B12 and X7 and *in-silico* translation of Formate acetyltransferase DNA sequence.**

### A4.1: Alignment of Formate acetyltransferase DNA sequences from B12 and X7



## A4.2: *in-silico* translation of Formate acetyltransferase DNA sequence consensus

gga ggc gtc ctt gat atg gat acg gaa atc gta tcg acc att aca tcg cac gga cct ggc tat tta aat aaa  
G G V L D M D T E I V S T I T S H G P G Y L N K

gaa ctt gaa aaa gtt gtc ggt ttt caa aca gac gaa cgg ttt aaa cgg gca ctt atg cca ttt ggt ggc att  
E L E K V V G F Q T D E P F K R A L M P F G G I

cgc atg gcg gag cag gcg tgc gaa gct tac gga tat gaa gtg agc gat gac gta aag aaa att ttt act  
R M A E Q A C E A Y G Y E V S D D V K K I F T

caa tat cgt aaa acg cat aac caa ggc gta ttt gac gtg tat acg gac gaa atg aaa gcg gcg cgc aaa  
Q Y R K T H N Q G V F D V Y T D E M K A A R K

gca ggc atc att aca ggg ctt cct gac gcc tat gga cgc ggc cgc att atc gga gac tat cgc cgc gtt  
A G I I T G L P D A Y G R G R I I G D Y R R V

gct tta tac ggc gtt gat cgt tta att gaa gaa aag aaa aaa gac tta cga aat aca ggt gcg cgt aca atg  
A L Y G V D R L I E E K K K D L R N T G A R T M

agc gaa gac att att cgt ctc cgc gaa gaa cta gca gag caa att cgc gcc ctc caa gaa cta aaa gaa  
S E D I I R L R E E L A E Q I R A L Q E L K E

atg gcg gca agc tac ggc tac gat att tca aag ccg gcg cgc aac gca cac gaa gcg ttc caa tgg tta  
M A A S Y G Y D I S K P A R N A H E A F Q W L

tat ttc gct tat ctt gcg gcg att aaa gaa caa aac ggt gca gca atg agt tta ggt cgc gtt tct acg ttt tta  
Y F A Y L A A I K E Q N G A A M S L G R V S T F L

gat att tat atc gaa cgc gac ttg caa gaa ggg aca tta aca gaa cgg gaa gca caa gag ctc gtt gac  
D I Y I E R D L Q E G T L T E R E A Q E L V D

cat ttc gtt atg aag tta cgc ctc gtc aaa ttc gcg cga aca cca gaa tat aat gag cta ttt agc ggc gac  
H F V M K L R L V K F A R T P E Y N E L F S G D

A4: Alignment of Formate acetyltransferase DNA Sequences from B12 and X7..

cca acg tgg gta acc gag tgc atc ggc ggt gtc gcg att gac ggc cgt ccg ctt gtg acg aaa aac tca  
P T W V T E S I G G V A I D G R P L V T K N S

ttc cgc ttc ttg cat acg ctt gat aac tta ggt cca gcg cca gaa cca aac tta acg gtt ctt tgg tgc aca  
F R F L H T L D N L G P A P E P N L T V L W S T

aaa tta cca gaa gcg ttc aaa aaa tat tgc gcg aaa atg tgc att caa aca agc tct atc caa tat gaa aat  
K L P E A F K K Y C A K M S I Q T S S I Q Y E N

gac gat tta atg cgc cct gag ttc ggc gat gac tac ggc att gcg tgc tgc gta tcc gcg atg cgc atc ggt  
D D L M R P E F G D D Y G I A C C V S A M R I G

aaa caa atg caa ttt ttc ggc gcg cgc gca aac ctt gcg aaa gca tta tta tat gcg att aat ggc ggc gta  
K Q M Q F F G A R A N L A K A L L Y A I N G G V

gat gaa aaa tta aaa gtg caa atc ggt cca gaa ttt gcg cca atc aca tct gaa tac tta gat tac gat gaa  
D E K L K V Q I G P E F A P I T S E Y L D Y D E

gtg atg cgc aaa ttt gac aac gtc atg gaa tgg cta gct gag ttg tac att aat acg tta aac gtc atc cac  
V M R K F D N V M E W L A E L Y I N T L N V I H

tac atg cac gat aaa tat tgt tat gag cgc att gaa atg gcg ctt cat gat aca aac gtc atg cgc acg atg  
Y M H D K Y C Y E R I E M A L H D T N V M R T M

gca acg gga att gcc ggt ctt tct gtc gtc gct gac tca ctt agc gca att aaa tat gca aaa gta aaa acg  
A T G I A G L S V V A D S L S A I K Y A K V K T

att cgc gac gaa aat ggc tta gcg gtc gac ttt gaa att gaa ggc gac ttc ccg aaa tat gga aac aac  
I R D E N G L A V D F E I E G D F P K Y G N N

gat gat cgc gtc gat tca att gca gtc gat att gtt gag cgc ttt atg aca aaa ttg cgc aaa cat aaa acg  
D D R V D S I A V D I V E R F M T K L R K H K T

tat cgc gat tca aaa cat aca aca tgc atc tta aca atc acg tca aac gtc gtc tat ggg aaa aag aca gga  
Y R D S K H T T S I L T I T S N V V Y G K K T G

A4: Alignment of Formate acetyltransferase DNA Sequences from B12 and X7..

aat aca cct gac ggc cgt cgc gca ggc gaa ccg ttt gca cct ggc gct aac ccg ttg cac ggc cgc gac  
N T P D G R R A G E P F A P G A N P L H G R D

aca aaa ggt gcg ctt gcg tcg tta agc tct gtc gca aaa tta ccg tat gaa tat gca tta gat ggc att tca  
T K G A L A S L S S V A K L P Y E Y A L D G I S

aac acg ttc tca atc gtg cca aaa gca ctc ggt aaa gat gat gca aca cgc gtt caa aac ctt gtg aca  
N T F S I V P K A L G K D D A T R V Q N L V T

att tta gac gga tat gct aaa aaa cgc ggt cac cat ttg aac atc aac gtc ttt aac cgc gaa acg ttg ctc  
I L D G Y A K K R G H H L N I N V F N R E T L L

gat gcg atg gaa cat cca gag gaa tat cc  
D A M E H P E E Y

## Appendix

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**A5: BLAST search of predicted amino acid sequence from B12 and X7 and Phylogenetic analysis Formate acetyltransferase sequences**

## A5:1 BLAST search of predicted amino acid sequence from B12 and X7.

[ref|ZP\\_02913786.1|](#) formate acetyltransferase [Geobacillus sp. WCH70]  
[gb|EDT35049.1|](#) formate acetyltransferase [Geobacillus sp. WCH70]  
 Length=749

Note B12 and X7 sequence in red

Score = 1257 bits (3252), Expect = 0.0, Method: Compositional matrix adjust.  
 Identities = 593/648 (91%), Positives = 632/648 (97%), Gaps = 0/648 (0%)

Query	1	GGVLDMDTIEIVSTITSHGPGYLNKELEKVVGFQTDPEPFKRALMPFGGIRMAEQACEAYGY	60
Sbjct	68	GGVLDMDTIVSTITSHRPGYLNKLEKIVGFQTDKPFKRALMPFGGIRMAQQSCEAYGY	127
Query	61	EVSDDVKKIFTQYRKHNQGVFDVYIDEMKAARKAGIITGLPDAYGRGRIIGDYRRVALY	120
Sbjct	128	KVSDVKKIFTQYRKHNQGVFDVYIDEMKARKAGIITGLPDAYGRGRIIGDYRRVALY	187
Query	121	GVDRLIEEKKKDLRNTGARTMSEDIIRLREELAEQIRALQELKEMAASYGYDISKPARNA	180
Sbjct	188	GVDRLIEEK+KDL+NTGARTM+EDIIRLREE+AEQIRAL ELK+MA SYGYDIS+PA+NA	247
Query	181	HEAFQWLYFAYLAAIKEQNGAAMSLGRVSTFLDIYIERDLQEGTLTEREAQELVDHFVMK	240
Sbjct	248	HEAFQWLYFAYLAAIKEQNGAAMSLGRVSTFLDIYIERDLQEGTLTEKEAQELVDHFVMK	307
Query	241	LRLVKFARTPEYNELFSGDPTWVTEISGGVAIDGRPLV KNSFRFLHTLDNLGPAPEPNL	300
Sbjct	308	LRLVKFARTPEYNELFSGDPTWVTEISGGMAIDGRPLVTKNSFRFLHTLDNLGPAPEPNL	367
Query	301	TVLWSTKLPEAFKKYCAKMSIQTSSIQYENDDLMRPEFGDDYGIACCVSAMRIGKQMQFF	360
Sbjct	368	TVLWS +LPEAFK+YCAKMSI+TSSIQYENDDLMR EFGDDYGIACCVS MRIGKQMQFF	427
Query	361	GARANLAKALLYAINGGVDEKLVQIGPEFAPITSEYLDYDEVMRKFDNVMEWLAELYIN	420
Sbjct	428	GARANLAKALLYAINGGVDEKLVQIGPEFAPITSEYLDYDEVMRKFDNVMEWLAELYIN	487
Query	421	TLNVIHYMHDKYCYERIEEMALHDSHILRTMATGIAGLSVVADSLSAIKYAKVKPIRDENG	480
Sbjct	488	TLNVIHYMHDKYCYERIEEMALHDSHILRTMATGIAGLSVVADSLSAIKYAKVKPIRDENG	547
Query	481	LAVDFEIEGDFPKYGNDDRVDIAVDIVERFMTKLRKHKTYRDSKHTLSILTITSNVVY	540
Sbjct	548	LAVDFEIEGDFPKYGNDDRVDIAVDLVERFMTKLRKHKTYRDSKHTLSILTITSNVVY	607
Query	541	GKKTGNTPDGRRAGEPFAPGANPLHGRDTKGALASLSSVAKLPYEYALDGISNTFSIVPK	600
Sbjct	608	GKKTGNTPDGRRAGEPFAPGANPLHGRDTKGALASLSSVAKLP+E+ALDGISNTFSIVPK	667
Query	601	ALGKDDATFVQNLVITILDGYAKKRGHHLNINVFNRETLLDAMEHPEEY	648
Sbjct	668	ALGKEEQTRVRNLVAILDGYMEKGGHHLNINVNLNRETLLDAMEHPEKY	715

A5: BLAST Search of predicted amino acid sequence.....

[gb|ABS20786.1](#) | formate acetyltransferase [Bacillus cereus subsp. cytotoxis NVH 391-98]  
Length=749

GENE ID: [5344916 Bcer98\\_0431](#) | formate acetyltransferase [Bacillus cereus subsp. cytotoxis NVH 391-98]

Score = 1170 bits (3027), Expect = 0.0, Method: Compositional matrix adjust.  
Identities = 549/648 (84%), Positives = 604/648 (93%), Gaps = 1/648 (0%)

Query 1 GGVLDMDTEIVSTITSHGPGYLNKELEKVVGFQTDPEPFKRALMPFGGIRMAEQACEAYGY 60  
Sbjct 69 GGVLDMDT+IVS+ITSHGPGYLNKELEKVVG QTD+PFKR+L PFGGIRMAEQ+CEAYG+  
GGVLDMDTKIVSSITSHGPGYLNKELEKVVGVQTDQPFKRSIQPFGGIRMAEQSCEAYGF 128

Query 61 EVSDDVKKIFTQYRKTHNQGVFDVYTDLMKAARKAGIITGLPDAYGRGRIIGDYRRVALY 120  
+V +++ +IF +RKTHNQGVFD YT EM+AARK+G+ITGLPDAYGRGRIIGDYRRVALY  
Sbjct 129 KVDEELSRIFRDWRKTHNQGVFDAYTPEMRAARKSGVITGLPDAYGRGRIIGDYRRVALY 188

Query 121 GVDRLIEEKKKDLRNTGARTMSEDIIRLREELAEQIRALQELKEMAASYGYDISKPARN 180  
GVDRLIE K++DL TG+ MSE++IRLREE++EQIRALQELKEMAAS+G+DISKPA NA  
Sbjct 189 GVDRLIEAKEEDLNLTGS-VMSEEVIRLREEVSEQIRALQELKEMAASHGFDISKPATNA 247

Query 181 HEAFQWLYFAYLAAIKEQNGAAMSLGRVSTFLDIYIERDLQEGTLTEREAQELVDHFV 240  
EAFQWLYFAYLAAIKEQNGAAMSLGR STFLDIY+ERDL+ GTLTE EAQE+VDHF+MK  
Sbjct 248 REAFQWLYFAYLAAIKEQNGAAMSLGRVSTFLDIYVERDLENGTLTEEEAQEIVDHFIMK 307

Query 241 LRLVKFARPEYNELFSGDPTWVTESIGGVAIDGRPLVTKNSFRFLHTLDNLGPAPEPNL 300  
LRLVKFARTP+YNELFSGDPTWVTESIGG+A+DGRPLVTKNSFRFLHTLDNLGPAPEPNL  
Sbjct 308 LRLVKFARTPDYNELFSGDPTWVTESIGGIALDGRPLVTKNSFRFLHTLDNLGPAPEPNL 367

Query 301 TVLWSTKLPEAFKKYCAKMSIQTSSIQYENDDMRPEFGDDYGIACCVSAMRIGKQMFFF 360  
TVLWS +LP+ FK YCAKMSI+TS+IQYENDDMRPE+GDDYGIACCVSAM+IGKQMFFF  
Sbjct 368 TVLWSKQLPQKFKNYCAKMSIKTSAIQYENDDMRPEYGGDDYGIACCVSAMKIGKQMFFF 427

Query 361 GARANLAKALLYAINGGVDEKLVQIGPEFAPITSEYLDYDEVMRKFDNVMEWLAELYIN 420  
GARANLAKALLYAINGG DEK KVQ+GPEFAPITS+ LDYDEV MFD MEWLA LY+N  
Sbjct 428 GARANLAKALLYAINGGDEKSKVQVGPPEFAPITSDVLDYDEVMHKFDMTMEWLAGLYLN 487

Query 421 TLNVIHYMHDKYCYERLEMALHDTNVMRTMATGIAGLSVVADSLSAIKYAKVKTRDENG 480  
TLNVIHYMHDKY YERIEMALHDT V+RTMATGIAGLSVVADSLSAIKYAKVK IRDENG  
Sbjct 488 TLNVIHYMHDKYSYERIEMALHDTVLRMTATGIAGLSVVADSLSAIKYAKVKPIRDENG 547

Query 481 LAVDFEIEGDFPKYGNDDRVDIAVDIVERFMTKLRKHKTYRDSKHTTISLTIITSNV 540  
+AVDFEIEGDFPKYGNDDRVD IAV++V+ FM KLRKHKTYR+S HT SILTIITSNV  
Sbjct 548 IAVDFEIEGDFPKYGNDDRVDIAVNLVKTFMNKLKHKTYRNSVHTMSLTIITSNV 607

Query 541 GKKTGNTPDGRRAGEPFAPGANPLHGRDTKGALASLSSVAKLPYEYALDGISNTFSIVPK 600  
GKKTGNTPDGRRAGEPFAPGANP+HGRDTKGALASL SVAKLPYE A DGISNTFSI+PK  
Sbjct 608 GKKTGNTPDGRRAGEPFAPGANPMHGRDTKGALASLSSVAKLPYEYALDQDGISNTFSIIPK 667

Query 601 ALGKDDATRVQNLVLTILDGYAKKRGHHLNINVFNRRETLLDAMEHPEEY 648  
ALGK++ +V NLV++LDGYA K GHHLNINVFNRRETLLDAMEHPE+Y  
Sbjct 668 ALGKEEEVQVSNLVSMLDGYAVKEGHHLNINVFNRRETLLDAMEHPEKY 715

[gb|AAU41019.1](#) | putative protein [Bacillus licheniformis DSM 13]  
Length=741

GENE ID: [3031414 BL01863](#) | putative formate C-acetyltransferase [Bacillus licheniformis ATCC 14580] (10 or fewer PubMed links)

Score = 1167 bits (3018), Expect = 0.0, Method: Compositional matrix adjust.  
Identities = 552/648 (85%), Positives = 595/648 (91%), Gaps = 1/648 (0%)

Query 1 GGVLDMDTEIVSTITSHGPGYLNKELEKVVGFQTDPEPFKRALMPFGGIRMAEQACEAYGY 60  
GGVLDMDTEIVSTITSHGPGYLNK+LEKVVG QTDPEPFKR+L PFGGIRMA+QACE+YG+  
Sbjct 61 GGVLDMDTEIVSTITSHGPGYLNKDLEKVVGVQTDPEPFKRSIQPFGGIRMAKQACESYGF 120

A5: BLAST Search of predicted amino acid sequence.....

Query	61	EVSDDDVKKIFTQYRKTHNQGVFDVYTD <del>EMKAARKAGIITGLP</del> DAYGRGRIIGDYRRVALY	120
		+++++V++IFT YRKTHNQGVFD YTEMK ARK GIITGLPDAYGRGRIIGDYRRVALY	
Sbjct	121	KLNEEVERIFTDYRKTHNQGVFDAYTDEM <del>KLARKVGIITGLP</del> DAYGRGRIIGDYRRVALY	180
Query	121	GVDRLIEEKKKDLRNTGARTMSEDIIRLREELAEQIRALQELKEMAASYGYDISKPARNA	180
		GVD LI+EKKKD T +R MSE+ IRLREEL+EQIRAL ELK +A SYG+DISKPA NA	
Sbjct	181	GVDFLIDEKKKDAAGT-SRV <del>MSEENIRLREELSEQIRALNELKALAKSYGFDISKPAANA</del>	239
Query	181	HEAFQWLYFAYLAAIKEQNGAAMSLGRVSTFLDIYIERDLQEGTLTEREAQELVDHFV <del>MK</del>	240
		EAFQWLYFAYLAAIKEQNGAAMSLGRVSTFLDIYIERDL+ G LTEREAQELVDHFV <del>MK</del>	
Sbjct	240	REAFQWLYFAYLAAIKEQNGAAMSLGRVSTFLDIYIERDLKTGVLTEREAQELVDHFV <del>MK</del>	299
Query	241	LRLVKFARTPEYNELFSGDPTWVTESIGGVAIDGRPLVTKNSFRFLHTLDNLGPAPEPNL	300
		LRLVKFARTP+YNELFSGDPTWVTESIGG+A DGR LVTKNSFRFLHTLDNLGPAPEPNL	
Sbjct	300	LRLVKFARTPDYNE <del>LFSGDPTWVTESIGGMAHDGRALVTKNSFRFLHTLDNLGPAPEPNL</del>	359
Query	301	TVLWSTKLPEAFPKYCAKMSIQTSSIQYENDDLMRPEFGDDYGIACCVSAMRIGKQM <del>QFF</del>	360
		TVLSW +LP+ PK YCAKMSI+TSSIQYENDD+MRPE+GDDYGIACCVSAM IGKQM <del>QFF</del>	
Sbjct	360	TVLWSVRLPQKFKNYCAKMSIKTSSIQYENDDIMRPEYGGDDYGIACCVSAMAIGKQM <del>QFF</del>	419
Query	361	GARANLAKALLYAINGGVDEKLVQIGPEFAPITSEYLDYDEV <del>MKFDNVMEWLAELYIN</del>	420
		GARANLAKALLYAINGG DEK K+Q+GPE P+ S+ LDYDEV <del>MKFDNVMEWLAELYIN</del>	
Sbjct	420	GARANLAKALLYAINGGKDEKHKMQVGPMPV <del>ASDVLDYDEVMHKFDQTMEWLAGLYIN</del>	479
Query	421	TLNVIHYMHDKYCYEREMALHDTNVMRTMATGIAGLSVVADSLSAIKYAKVKTIRDENG	480
		TLNVIHYMHDKYCYEREMALHDT ++RTMATGIAGLSVVADSLSA+KYAKV +RDENG	
Sbjct	480	TLNVIHYMHDKYCYEREMALHDT <del>EILRTMATGIAGLSVVADSLSAVKYAKVSVVRDENG</del>	539
Query	481	LA <del>DFEIEGDFPKYGNDDRVD</del> IAVDIVERFM <del>KLRKH</del> KTYRDSKHTTSILTITSNVVY	540
		+AVDFE EGDFPKYGNDDRVD+IAVDIV+REFM KLRKH+TYR S T SILTITSNVVY	
Sbjct	540	IAVDFEIEGDFPKYGNDDRVDIAVDIVKRF <del>MKKLRKHQTYRQSVQIMSILTITSNVVY</del>	599
Query	541	GKKTGNTPDGRRAGEPFAPGANPLHGRDTKGALASLSSVAKLPY <del>EYALDGISNTFSIVPK</del>	600
		GKKTGNTPDGRRAGEPFAPGANP+HGRDTKG LASLSSVAKLPY YALDGISNTFSIVPK	
Sbjct	600	GKKTGNTPDGRRAGEPFAPGANPMHGRDTKGT <del>LASLSSVAKLPYSYALDGISNTFSIVPK</del>	659
Query	601	ALGKDDATRVQNLVTLIDGYAKKRGHHLNINVENRETL <del>L</del> DAMEHP <del>EEY</del>	648
		ALGKD+ +R NL +ILDGYA K GHHLN+NVFNRETL <del>L</del> DAMEHP <del>EEY</del>	
Sbjct	660	ALGKDEESRAANLSS <del>ILDGYAAKTGHHLN</del> VN <del>VFNRETL</del> L <del>DAMEHP</del> EEY 707	

[ref|YP\\_001643320.1](#) formate acetyltransferase [Bacillus weihenstephanensis KBAB4]  
[gb|ABY41692.1](#) formate acetyltransferase [Bacillus weihenstephanensis KBAB4]  
 Length=749

[GENE ID: 5840596 BcerKBAB4\\_0426](#) | formate acetyltransferase  
 [Bacillus weihenstephanensis KBAB4]

Score = 1155 bits (2988), Expect = 0.0, Method: Compositional matrix adjust.  
 Identities = 542/648 (83%), Positives = 597/648 (92%), Gaps = 1/648 (0%)

Query	1	GGVLDMDTEIVSTITSHGPGYL <del>NKELEKVVGFQTD</del> EPFKRALMPFGGIRMAEQACEAYGY	60
		GGVLDMDT+IVS+ITSH PGYLNK++EKVVGFQTD+PFKR+L P+GGIRMAEQACEAYGY	
Sbjct	69	GGVLDMDTKIVSSIT <del>SHEPGYLNKDIEKVVGFQTDKPFKRS</del> LQPYGGIRMAEQACEAYGY	128
Query	61	EVSDDDVKKIFTQYRKTHNQGVFDVYTD <del>EMKAARKAGIITGLP</del> DAYGRGRIIGDYRRVALY	120
		E+ ++ KIF +RKTHNQGVFD YTEMKAARK+G+ITGLPDAYGRGRIIGDYRRVALY	
Sbjct	129	EMDKELSKI <del>FRDWRKTHNQGVFDAYTPEMKAARKSGVITGLP</del> DAYGRGRIIGDYRRVALY	188
Query	121	GVDRLIEEKKKDLRNTGARTMSEDIIRLREELAEQIRALQELKEMAASYGYDISKPARNA	180
		G+D LIE KK D TG MSED +RLREEL+EQ+RALQELK+MAAS+G+DISKPA NA	
Sbjct	189	GIDHLLIEAKKADYNLTGG-V <del>MSEDTMRLREELSEQMRALQELKQMAASHGFDISKPATNA</del>	247
Query	181	HEAFQWLYFAYLAAIKEQNGAAMSLGRVSTFLDIYIERDLQEGTLTEREAQELVDHFV <del>MK</del>	240
		EAFQWLYFAYLAAIKEQNGAAMSLGRVSTFLDIYIERDL GTLTEREAQELVDHFV <del>MK</del>	
Sbjct	248	QEAFQWLYFAYLAAIKEQNGAAMSLGRVSTFLDVYIERDLANGTLTEEKAQEIVDHF <del>IMK</del>	307
Query	241	LRLVKFARTPEYNELFSGDPTWVTESIGGVAIDGRPLVTKNSFRFLHTLDNLGPAPEPNL	300
		LRLVKFARTP+YNELFSGDPTWVTESIGG+A+DGRPLVTKNSFRFLHTLDNLGPAPEPNL	

A5: BLAST Search of predicted amino acid sequence.....

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Sbjct 308 LRLVKFARTPDYNELFSGDPTWVTESIGGMALDGRPLVTKNSFRFLHTLDNLGPAPENL 367
Query 301 TVLWSTKLPFAFKKYCAKMSIQTSSIQYENDDLMRPEFGDDYGIACCVSAMRIGKQMQFF 360
TVLWS +LPE FK YCAKMSI+TS+IQYENDD+MR ++GDDYGIACCVSAMRIGKQMQFF
Sbjct 368 TVLWSKQLPENFKNYCAKMSIKTSAIQYENDDIMRADYGDDYGIACCVSAMRIGKQMQFF 427
Query 361 GARANLAKALLYAINGGVDEKLVQIGPEFAPITSEYLDYDEVMRKFDNVMEWLAELYIN 420
GARANLAKALLYA+NGG DEK K Q+GPE+APITSE LDY+EVMRKFD MEWLA LY+N
Sbjct 428 GARANLAKALLYAVNGGKDEKSKAQVGPEYAPITSEVLDYEEVMRKFDMTMEWLAGLYLN 487
Query 421 TLNVIHYMHDKYCYERIEMALHDTNVMRTMA GIAGLSVVADSLSAIKYAKVKTIRDENG 480
TLNVIHYMHDKY YERIEMALHDTNV+RTMATGIAGLSVVADSLSAIKYA+VK IRDENG
Sbjct 488 TLNVIHYMHDKYSYERIEMALHDTNVLRTMATGIAGLSVVADSLSAIKYAQVKPIRDENG 547
Query 481 LAVDFEIEGDFPKYGNNDDRVDIAVDIVERFMTKLRKHKTYRDSKHHTSILTITSNVVY 540
+AVDFEIEGDFPKYGNNDDRVD IAV++V+ FM K+RKHKTYR+S HT SILTITSNVVY
Sbjct 548 IAVDFEIEGDFPKYGNNDDRVDIAVNLVKTFMNKIRKHKTYRNSVHTMSILTITSNVVY 607
Query 541 GKKTGNTPDGRRRAGEPFAPGANPLHGRDTK GALASLSSVAKLPYEYALDGISNTFSIVPK 600
GKKTGNTPDGRR GEPPFAPGANP+HGRDTK GALASL SVAKLPYE A DGISNTFSI+PK
Sbjct 608 GKKTGNTPDGRRTGEPPFAPGANPMHGRDTK GALASLSSVAKLPYEDAQDGISNTFSIIPK 667
Query 601 ALGKDDATRVNLVTLIDGYAKKRGHHLNINVFNRETL DAMEHPEEY 648
ALGK+D +V+NLV++LDGYA K GHHLNINVFNRETL+DAMEHPE+Y
Sbjct 668 ALGKEDDVQVRNLVSM LDGYAIKEGHHLNINVFNRETLMDAMEHPEKY 715

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## A5:2 Polypeptide sequences used in Formate acetyltransferase phylogenetic analysis

Polypeptide sequences used in Formate acetyltransferase phylogenetic analysis		
Species	Protein Name	Accession number
<i>Geobacillus</i> sp.WCH70	Formate acetyltransferase	ZP_02913786
<i>Bacillus cereus</i> subsp.cytotoxis NVH 391-98	Formate acetyltransferase	YP_001373781.1
<i>Bacillus licheniformis</i> ATCC 14580	Formate acetyltransferase	YP_079294.1
<i>Bacillus cereus</i> B4264	Formate acetyltransferase	ZP_02579581.1
<i>Bacillus cereus</i> AH1134	Formate acetyltransferase	ZP_02522437.1
<i>Bacillus cereus</i> ATCC 14579	Formate acetyltransferase	NP_830328.1
<i>Bacillus anthracis</i> str. Ames	Formate acetyltransferase	NP_843045.1
<i>Bacillus thuringiensis</i> serovar israelensis ATCC 35646	Formate acetyltransferase	ZP_00740300.1
<i>Staphylococcus epidermidis</i> ATCC 12228	Formate acetyltransferase	NP_763769.1
<i>Staphylococcus aureus</i> subsp. aureus USA300_TCH1516]	Formate acetyltransferase	YP_001574143.1

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