

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

**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

Jon Stuart Palmer

2008

ABSTRACT

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

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

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

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.

TABLE OF CONTENTS

Abstract	ii
List of Publications	iv
List of Presentations	iv
Acknowledgements	v
Table of Contents	vi
List of Figures	xix
List of Tables	xxviii
List of Abbreviations	xxix
Chapter 1	1
Introduction and project objectives	1
1.1 Introduction	1
1.2 Project objectives	2

Chapter 2

Bacterial cell attachment, the beginning of a biofilm – a review of the Literature	3
2.1 <i>Thermophiles in foods</i>	4
2.1.1 Thermophiles in the dairy industry	4
2.1.2 Dairy biofilms	5
2.2 <i>Initial bacterial attachment to surfaces</i>	8
2.2.1 Conditioning of a surface	8
2.2.2 Mass transport	10
2.2.3 Initial attachment	12
2.2.4 Surface charge	13
2.2.5 Hydrophobicity	16
2.2.6 DLVO theory	20
2.2.7 Surface roughness and micro-topography	22
2.2.8 Role of surface carbohydrates in attachment	23
2.2.9 Role of surface proteins in attachment of <i>Staphylococcus</i> species	24
2.3 <i>Conclusion</i>	26

Chapter 3

Methods and materials	28
3.1 <i>Source of isolates</i>	32
3.2 <i>Culture preparation</i>	33

3.3	<i>Isolation of variant X7 from Anoxybacillus flavithermus strain (B12) with reduced attachment to stainless steel</i>	34
3.4	<i>Attachment Assay</i>	36
3.4.1	<i>Epifluorescence microscopy</i>	36
3.4.2	<i>Impedance detection</i>	36
3.4.3	<i>Comparison of B12 and X7 attachment to stainless steel cuttings in a glass column and to stainless steel cuttings suspended in solution</i>	39
3.4.4	<i>Polystyrene microtiter plate assay</i>	39
3.5	<i>Comparison of B12 and X7 Bacterial Strains</i>	41
3.5.1	<i>Random amplified polymorphic DNA (RAPD) analysis</i>	41
3.5.1.2	<i>Agarose gel electrophoresis</i>	42
3.5.2	<i>API biochemical profile</i>	43
3.5.3	<i>Transmission electron microscopy (TEM)</i>	43
3.5.4	<i>Supernatant exchange and effect on attachment to stainless steel</i>	44
3.5.5	<i>Cell surface Hydrophobicity</i>	44
3.5.5.1	<i>Microbial adhesion to hydrocarbon test (MATH)</i>	44
3.5.5.2	<i>Hydrophobic interactive chromatography (HIC)</i>	45
3.5.5.3	<i>Attachment to Sigma coated Glass</i>	46

3.5.6	Cell surface charge	46
3.5.6.1	<i>Zeta potential</i>	46
3.5.6.2	<i>Electrostatic interaction chromatography (EIC)</i>	47
3.5.6.3	<i>Effect of pH on attachment to stainless steel</i>	48
3.5.8	Quantitative analysis of protein concentration	49
3.5.9	Treatment of cells with protein disrupting agents	50
3.5.10	Quantitative analysis surface carbohydrates	50
3.5.11	Treatment of cells with carbohydrate disrupting agent	51
3.5.12	HPLC analysis of surface carbohydrates	51
3.5.13	Treatment of cells with DNase I	52
3.6	<i>Comparison of Total and Surface extracted Proteins by Gel Electrophoresis</i>	53
3.6.1	One-dimensional gel electrophoresis (SDS-PAGE) of cell proteins	53
3.6.1.1	<i>Total cell protein extraction for one-dimensional gel electrophoresis (SDS-PAGE)</i>	53
3.6.1.2	<i>Acid Glycine surface protein extraction for one-dimensional gel electrophoresis (SDS-PAGE)</i>	53
3.6.1.3	<i>Lysozyme surface protein extraction for one-dimensional gel electrophoresis (SDS-PAGE)</i>	54
3.6.1.4	<i>One-dimensional gel electrophoresis (SDS-PAGE) method</i>	54

3.6.1.4.1	Gel staining and photography.....	57
3.6.2	Two-dimensional gel electrophoresis (2D PAGE) of cell proteins.....	58
3.6.2.1	<i>Total cell protein extraction for two-dimensional gel electrophoresis (2D PAGE)</i>	<i>58</i>
3.6.2.2	<i>Acid Glycine surface protein extraction for two-dimensional gel electrophoresis (2D PAGE)</i>	<i>59</i>
3.6.2.3	<i>Lysozyme surface protein extraction for two-dimensional gel electrophoresis (2D PAGE)</i>	<i>59</i>
3.6.2.4	<i>Two-dimensional gel electrophoresis (2D PAGE) method.....</i>	<i>58</i>
3.6.2.4.1	<i>Isoelectric focusing</i>	<i>60</i>
3.6.2.4.2	<i>Second dimension and strip transfer</i>	<i>61</i>
3.6.2.4.3	<i>Gel Staining and Image acquisition</i>	<i>63</i>
3.6.2.4.4	<i>ImageMaster 2D software.....</i>	<i>63</i>
3.6.2.5	<i>Cytoplasmic enzyme assay</i>	<i>64</i>
3.7	Protein Identification	65
3.7.1	Electrospray ionisation–Quadrupole–Time-of Flight (ESI-QUAD-TOF).....	65
3.7.2	Bioinformatics	65
3.7.2.1	<i>BLAST searches.....</i>	<i>65</i>
3.7.2.2	<i>Sequence alignments and Phylogenetic comparison .</i>	<i>65</i>
3.7.3	Polymerase Chain reaction (PCR)	66
3.7.4	Extraction of PCR product from agarose.....	67

3.7.5 DNA cloning and Transformation for DNA sequencing.....	67
3.7.5.1 <i>DNA cloning</i>	67
3.7.5.2 <i>Transformation</i>	68
3.7.6 Plasmid extraction	69
3.7.7 DNA quantification.....	69
3.7.8 DNA sequencing	70

Chapter 4

Isolation of isogenic strain X7 with reduced attachment to stainless steel from parental strain B12.....	70
4.1 Introduction.....	72
4.2 Procedures.....	73
4.2.1 Isolation of Strain X7	73
4.2.2 Comparison of growth rates and Biochemical and RAPD profiles of B12 and X7	73
4.2.3 Attachment of dairy and non-dairy isolates of <i>Anoxybacillus</i> and <i>Geobacillus</i> strains to stainless steel..	74
4.3 Results	75
4.3.1 Attachment of B12 and X7 to various surfaces.....	75

4.3.1.1	<i>Attachment of B12 and X7 to stainless steel measured by epifluorescence microscopy count</i>	75
4.3.1.2	<i>Attachment to stainless steel by cultures of B12 and X7 measured by impedance</i>	76
4.3.1.3	<i>Cumulative Percentage of Cells Collected Through Column</i>	77
4.3.1.4	<i>Attachment of B12 and X7 to suspended stainless steel chips</i>	78
4.3.1.5	<i>Attachment of B12 and X7 to Glass measured by epifluorescence microscopy count</i>	79
4.3.1.6	<i>Attachment of B12 and X7 cultures to polystyrene plates</i>	80
4.3.1.7	<i>Attachment of B12 and X7 cultures to plastic centrifuge tubes</i>	81
4.3.2	Growth rate and attachment of B12 and X7	82
4.3.3	Biochemical and RAPD analysis of B12 and X7	85
4.3.3.1	<i>RAPD comparison of B12 and X7</i>	85
4.3.3.2	<i>Morphology and biochemical test profiles of B12 and X7 using API 50CHB test strip</i>	86
4.3.4	Attachment of dairy and non-dairy isolates of <i>Anoxybacillus</i> and <i>Geobacillus</i> strains to stainless steel ..	88
4.4	Discussion	89
4.5	Conclusion	92

Chapter 5

Cell surface properties of B12 and X7	94
5.1 Introduction.....	96
5.2 Procedures	97
5.3 Results.....	99
5.3.1 Transmission Electron Microscopy of B12 and X7 cells	99
5.3.2 Supernatant exchange of B12 and X7 and the effect on attachment of B12 and X7 to stainless steel coupons.....	100
5.3.3 Surface Charge of B12 and X7	101
5.3.3.1 <i>Zeta potential.....</i>	101
5.3.3.2 <i>Electrostatic Interaction Chromatography.....</i>	102
5.3.3.3 <i>Effect of pH on the attachment of B12 and X7 to stainless steel coupons.....</i>	103
5.3.4 Hydrophobicity of B12 and X7	104
5.3.4.1 <i>Determination of cell surface hydrophobicity using MATH (Microbial Adhesion to Hydrocarbons).....</i>	104
5.3.4.2 <i>Determination of cell surface hydrophobicity using HIC (Hydrophobic Interactive Chromatography).....</i>	105
5.3.4.3 <i>Attachment of B12 and X7 to glass and Sigmacote coated glass</i>	106

5.3.5	Attachment of B12 and X7 cells to stainless steel coupons following disruption of surface proteins by various methods	107
5.3.6	Surface Carbohydrates.....	109
5.3.6.1	<i>The effect of Acidified Sodium metaperiodate treated cells and effect on attachment to stainless steel coupons.....</i>	<i>109</i>
5.3.6.2	<i>Analysis of extracellular polysaccharide (EPS) from B12 and X7.....</i>	<i>110</i>
5.3.7	Effect of DNase I on attachment of B12 and X7 to stainless steel coupons.....	112
5.3.8	Effect of various treatments on surface charge of B12 and X7 as measured by Electrostatic Interaction Chromatography	113
5.3.9	Effect of various treatments on hydrophobicity of B12 and X7 as measured Microbial Adhesion to Hydrocarbons.....	114
5.4	Discussion.....	115
5.5	Conclusion	124

Chapter 6

Comparison of total cell and surface extracted proteins from B12 and X7 cells using one-dimensional SDS-PAGE and two-dimensional PAGE gel electrophoresis.....	125
6.1 Introduction.....	127
6.2 Procedures.....	129
6.2.1 One-dimensional SDS-PAGE of total, acid-glycine and lysozyme/mutanolysin extracted proteins.....	129
6.2.2 Two-dimensional PAGE of total, acid-glycine and lysozyme/mutanolysin extracted proteins.....	130
6.3 Results.....	131
6.3.1 One-dimensional SDS-PAGE of total and surface extracted proteins.....	131
6.3.1.1 <i>One-dimensional SDS-PAGE of total cell proteins ...</i>	131
6.3.1.2 <i>One-dimensional SDS-PAGE of acid-glycine surface extracted proteins.....</i>	132
6.2.1.3 <i>One-dimensional SDS-PAGE of lysozyme/ mutanolysin surface extracted proteins.....</i>	133
6.2.1.4 <i>Summary of One-dimensional SDS-PAGE of total and surface extracted proteins.....</i>	134
6.3.2 Two-dimensional PAGE of total and surface extracted proteins.....	135

6.3.2.1	<i>Two-dimensional PAGE of total cell proteins</i>	136
6.3.2.2	<i>Two-dimensional PAGE of acid-glycine surface extracted proteins</i>	138
6.3.2.3	<i>Two-dimensional PAGE of lysozyme/mutanolysin surface extracted proteins</i>	140
6.3.2.3.1	Validation of lysozyme/mutanolysin extraction	145
6.4	Discussion	146
6.5	Conclusion	150

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	151
7.1	Introduction	152
7.2	Procedures	156
7.3	Results	158
7.3.1	Identification of gel Spots 1 and 2 by Electrospray ionization quadrupole time of flight (ESI-QUAD-TOF)	158
7.3.2	PCR primer design by bioinformatic analysis of Formate acetyltransferase ORF from related bacteria	159

7.3.3 PCR amplification and cloning of the Formate acetyltransferase gene from B12 and X7	160
7.3.4 Bioinformatic analysis of Formate acetyltransferase DNA and predicted protein sequences from B12 and X7 sequences	162
7.3.5 Levels of Formate acetyltransferase from total cell proteins of B12 and X7	165
7.4 <i>Discussion</i>	168
7.5 <i>Conclusion</i>	174
Chapter 8	
Discussion	175
8.1 <i>Final Discussion</i>	174
8.2 <i>Future work</i>	181
8.3 <i>Final Conclusion</i>	183
Appendix A1	184
Appendix A2	187
Appendix A3	204
Appendix A4	212

Appendix A5.....217

Bibliography.....223

LIST OF FIGURES

- Figure 3.1:** Photograph of the glass column containing stainless steel chips used to select for attachment deficient strain of B12.... 35
- Figure 3.2:** Calibration curve for planktonic *Anoxybacillus flavithermus* strain B12 on the MiniTrac 4000 impedance monitor using TSB as the growth medium. IDT = Impedance Detection Time 38
- Figure 3.3:** Calibration curve for planktonic *Anoxybacillus flavithermus* strain X7 on the MiniTrac 4000 impedance monitor using TSB as the growth medium. IDT = Impedance Detection Time 38
- Figure 3.4:** Calibration curve for protein estimation using PIERCE BCA protein assay kit 49
- Figure 3.5:** Calibration curve for the detection of EPS..... 51
- Figure 3.6:** Plasmid vector pCR[®] 4-TOPO[®] (Invitrogen) used for cloning PCR products..... 68
- Figure 3.7:** Calibration curve for DNA estimation using Fluorescence assay kit 70
- Figure 4.1:** Summary of strategy used to isolate attachment deficient mutant from culture B12. This process was repeated seven times to produce the isolate known as X7, derived by selection of natural variants of culture B12..... 75

Figure 4.2:	Epifluorescence microscopy count of strain B12 attached to stainless steel (x400 magnification, $5.21 \pm 0.02 \log_{10}$ cells per cm^2).....	76
Figure 4.3:	Epifluorescence microscopy count of strain X7 attached to stainless steel (x400 magnification, $3.69 \pm 0.24 \log_{10}$ cells per cm^2).....	76
Figure 4.4:	Cumulative percentage of B12 and X7 cells collected after passing through a column filled with stainless steel chips.....	78
Figure 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	79
Figure 4.6:	Epifluorescence microscopy count of strain B12 attached to glass (x400 magnification, $4.13 \pm 0.08 \log_{10}$ cells per cm^2).....	80
Figure 4.7:	Epifluorescence microscopy count of strain X7 attached to glass (x400 magnification, $3.78 \pm 0.28 \log_{10}$ cells per cm^2) ...	80
Figure 4.8:	Attachment of B12 and X7 cultures to polystyrene nontreated 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	81

Figure 4.9:	Two 500 ml plastic centrifuge tubes showing the ability of B12 to attach to the plastic wall of the centrifuge tube compared to X7, during centrifugation at 1000 x g for 20 minutes. Culture supernatant was left behind to provide contrast.....	82
Figure 4.9A:	Two 40 ml centrifuge tubes highlighting the ability of B12 to attach plastic wall of the centrifuge tube compared to X7, during centrifugation at 1000 x g for 20 minutes.....	82
Figure 4.10:	Optical density over 24 hour period of B12 and X7 cultures..	84
Figure 4.11:	Total viable count of B12 and X7 cultures over 24 hour period	84
Figure 4.12:	Impedance (MiniTrac 4000) count of B12 and X7 attached to stainless steel coupons	85
Figure 4.13:	Epifluorescence microscopy count of B12 and X7 attached to stainless steel coupons	85
Figure 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	86
Figure 4.15:	Attachment of various <i>Anoxybacillus</i> and <i>Geobacillus</i> strains to stainless steel coupons.....	89

- Figure 5.1:** Transmission electron micrographs of intact B12 & X7 cells and Ruthenium red stained cells of B12 (labelled A) and X7 (labelled B). Red arrows point to outer layer of cell wall 99
- Figure 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 100
- Figure 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 101
- Figure 5.4:** Proportion of B12 & X7 cells retained on anionic (functional group $R-CH_2N^+(CH_3)_3$) and cationic (functional group $R-SO_3^-$) exchange resins at pH 7. Error bars represent the standard deviation from the mean of 3 replicates 102
- Figure 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 103
- Figure 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 104
- Figure 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 105

- Figure 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 106
- Figure 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 108
- Figure 5.10:** Attachment of B12 & 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 109
- Figure 5.11** Monosaccharide composition of EPS isolated from strain B12 digested by Sulphuric acid and analysed by HPLC 111
- Figure 5.12:** Monosaccharide composition of EPS isolated from strain X7 digested by Sulphuric acid and analysed by HPLC 111
- Figure 5.13:** Attachment of B12 & X7 to stainless steel following treatment with DNAase I to remove surface associated DNA. Error bars represent the standard deviation from the mean of 3 replicates 112
- Figure 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 113

Figure 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... 114
Figure 6.1:	Lysozyme, mutanolysin and lysostaphin cleavage sites in the peptidoglycan layer of Gram positive bacteria (adapted from Gatlin <i>et al.</i> , 2006).....128
Figure 6.1:	Image of a typical SDS-PAGE of total cell proteins from B12 cells and X7 cells 131
Figure 6.2:	Image of a typical SDS-PAGE of acid-glycine extracted surface proteins from X7 and B12 132
Figure 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)..... 133
Figure 6.4:	2D-PAGE gels of whole cell protein extracts of B12 and X7 cells. 200µg of protein extract was run on a 17cm, pH 3-10 strip, with the second dimension using a 12.5% SDS-PAGE gel, stained with Sypro Ruby and Laser scanned at 450nm 135
Figure 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..... 136

- Figure 6.6:** 2D PAGE of acid-glycine surface protein extracts from B12 and X7 cells. 300µg of protein extract was run on a 17cm, pH 3-10 strip, with the second dimension using a 12.5% SDS-PAGE gel, stained with SYPRO Ruby and laser scanned at 450nm..... 138
- Figure 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 300µg of protein, which is the maximum recommended by the strip manufactures..... 139
- Figure 6.8:** 2D PAGE Comparisons of lysozyme/mutanolysin surface extracted protein gel sections from B12 and X7. 300µg of protein extract was run on a 17cm, pH 3-10 strip, with the second dimension using a 12.5% SDS-PAGE gel, stained with SYPRO Ruby and laser scanned at 450nm. Arrows highlight differences between B12 and X7 that were reproducible in all four samples..... 141
- Figure 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..... 144
- Figure 7.1:** Protein identification by mass spectrometry. (A) Protein identification by matrix-assisted laser desorption/ionisation – time of flight (MALDI-TOF). Proteins digested with proteolytic enzymes into peptide fragments and then

analysed 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) 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)..... 154

Figure 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 160

Figure 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 161

Figure 7.4: Agarose gel electrophoresis of PCR products after second round of amplification 161

- Figure 7.5:** The predicted amino acid sequence from the cloned and sequenced DNA from the Formate acetyltransferase gene fragment amplified from B12 and X7. Underlined amino acids are sequences identified by mass spectrometry from 2D PAGE gels Spots as having homology to known Formate acetyltransferase sequences..... 163
- Figure 7.6:** Phylogenetic relationship of Formate acetyltransferase from *Anoxybacillus flavithermus* with other related bacteria proteins. The phylogenetic tree in this figure was prepared using BLAST pairwise alignments, with Fast Minimum Evolution as the tree method. For details of the sequences used in this alignment, see Appendix A5.2..... 164
- Figure 7.7:** Two-dimensional-PAGE (2D-PAGE) gels of whole cell protein extracts of B12 and X7 cells. 200µg of protein extract was run on a 17cm, 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. 165
- Figure 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..... 167
- Figure 7.9:** Formate acetyltransferase catalytic conversion of pyruvate to formate and acetyl-CoA (adapted from Walsh, 1979)..... 171

LIST OF TABLES

Table 3.1:	List of isolates and their origin	32
Table 3.2:	Composition of citrate and phosphate buffers to create buffers in 2-8 pH range.....	47
Table 3.3:	Composition of citrate and phosphate buffers to create buffers in 3-7.2 pH range.....	48
Table 3.4:	Primers used in this study	66
Table 4.1:	Biochemical test profiles of B12 and X7 cultures.....	87
Table 6.0:	Aminopeptidase C assay of lysozyme/mutanolysin surface extractions as a marker of cytoplasmic contamination over a 5 hour 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.....	145
Table 6.1:	β -Galactosidase assay of lysozyme/mutanolysin surface extractions as a marker of cytoplasmic contamination over a 5 hour 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	145
Table 7.1:	Various BLAST search programs available	155

List of Abbreviations

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 ₂	Magnesium chloride

MgSO ₄	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 ₂ PO ₄	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