

The Role of Bacterial Extracellular Polymers in Cell Surface Chemistry, Metal Adsorption and Biomineralisation.

Janette Tourney

PhD
The University of Edinburgh
2008



Declaration

I, Janette Tourney declare that the work presented in this thesis, presented for the degree of Doctor of Philosophy, is all my own except where otherwise stated.

Acknowledgements

This project would not have been possible without help and support from a lot of people. Firstly, Bryne Ngwenya has been a fantastic supervisor throughout the duration of my PhD and I will always be extremely grateful to him. Without his encouragement during my final year as an undergraduate I would never have had the confidence to apply for a PhD in the first place.

I would also like to thank my co-supervisors Fred Mosselmans, especially for a lot of time spent conducting EXAFS analysis at Daresbury and on subsequent data analysis, Greg Cowie for access to and advice on organics analysis, and my advisor Simon Harley.

I have been able to make use of the expertise of many people during the course of this project. I would like to thank Gail Ferguson and Victoria Marlow (Microbiology, Edinburgh) for providing lab space and help with culturing bacteria during the early stages of the project. Juan Diaz-Mochon and Songsak Planonth (Chemistry, Edinburgh) provided access to and help with ATR-FTIR analysis. I am also grateful to Laurence Tetley (ICMB, Glasgow) for providing access to and help with TEM and SEM facilities, and Margaret Mullin and Eoin Robertson for assistance with sample preparation. Access to and training in the use of the zeta potentiometer was provided by Andrea Schaffer and Alan Simm (Engineering, Edinburgh). I would also like to thank Chris Daughney (GNS, New Zealand) for providing the FITMOD program and also a great deal of help while I was learning to use it. Within the department of Earth Sciences, I would like to thank Ann Mennim for metals analysis, Nic Odling for XRD analysis and Emma Passmore for SEM sample preparation and help with the analysis. Steve Mowbray conducted the DOC analysis and helped with numerous other tasks throughout the project. Marisa Magennis helped me in the lab and also during the EXAFS analysis in Daresbury. I also thank Ciaran Beggan for all his time spent helping me learn to use Matlab.

The other postgrads in the department have made the duration of my PhD a much more enjoyable experience. Sarah Appleby was a fantastic office-mate and kept me company during writing up. Vicki Parry, Clare Woulds, Marisa Magennis, Amber

Annett and Gillian McCay all deserve thanks for being generally supportive and supplying motivation when it was needed.

This work was funded by a School of Geosciences Teaching Studentship and I have been based at the School of Geosciences, The University of Edinburgh, throughout the duration of the project.

Abstract

Bacteria and other microorganisms can significantly influence the biogeochemical cycling of metal ions in many environmental settings. Recent studies have investigated the roles of bacterial cells in proton and metal adsorption, and in biologically-induced mineral precipitation. This study aimed to characterise the role of bacterial extracellular polymers in surface reactivity, metal adsorption and biomineralisation. This was undertaken using an EPS-producing, thermophilic, bacterial strain, *Bacillus licheniformis* S-86. Experimental work was undertaken comparing cells with the EPS layer intact (native cells) with cells from which the EPS layer had been extracted (EPS-free cells). The study incorporated surface characterisation by potentiometric titration, infrared analysis and electrophoretic mobility analysis. Investigation of the mechanisms of Zn adsorption to cell surfaces was undertaken by both macroscopic batch adsorption experiments, and spectroscopic (EXAFS) analysis. Mineralisation experiments were carried out in order to investigate the effect of EPS on CaCO_3 precipitation.

Surface complexation modelling of the potentiometric titration data indicated that the native and EPS-free cells contained four proton-active functional groups, with pK_a values of 3.3-3.4, 5.3-5.4, 7.4-7.5 and 9.9-10.1. These were tentatively identified as phosphodiester, carboxyl, phosphoryl and amine groups respectively, and ATR-FTIR analysis supported identification of the pK_a 5.3-5.4 site as carboxylic. The site concentrations of the pK_a 3.3-3.4 and 9.9-10.1 groups were significantly lower in the EPS-free cells than in the native cells. The macroscopic and EXAFS metal adsorption studies indicated that both the carboxyl and phosphoryl groups are involved in Zn complexation, and a lack of temperature-dependent adsorption provides evidence that Zn binds by an outer-sphere mechanism. Biomineralisation experiments indicated that the presence of EPS affects both CaCO_3 morphology and polymorphism. The presence of EPS appears to favour precipitation of the stable polymorph calcite over the metastable polymorph vaterite. The results of this study have shown that EPS, and potentially the associated dissolved organic carbon, can significantly affect the surface reactivity of bacterial cells.

Table of Contents

Chapter 1: Introduction	1
1.1. Microbe-Metal Interactions	1
1.2. Research Rationale	2
1.3. Review of Previous Research Findings	3
1.3.1. Bacterial cell wall structure	3
1.3.2. Cell surface charge and chemistry	5
1.3.2.1. Potentiometric titrations	5
1.3.2.2. Electrophoretic mobility studies	7
1.3.3. Surface complexation modelling	8
1.3.3.1. Determining deprotonation constants and site concentrations	9
1.3.3.2. Electrostatic modelling	12
1.3.4. Macroscopic adsorption studies	17
1.3.4.1. Langmuir adsorption isotherms	17
1.3.4.2. Discrete-site SCM approach to metal adsorption	18
1.3.4.2.1. Adsorbing metal	19
1.3.4.2.2. Bacterial species	21
1.3.4.2.3. Growth conditions and growth phase	24
1.3.4.2.4. Adsorption temperature	25
1.3.4.2.5. Bacterial extracellular polymeric substances (EPS)	26
1.3.5. Spectroscopic studies	28
1.3.5.1. Infrared adsorption studies (FTIR and ATR-FTIR)	28
1.3.5.2. X-ray absorption fine structure spectroscopy (EXAFS) studies	30
1.3.6. Predicting metal adsorption behaviour in complex systems	32
1.3.7. Biomineralisation studies	34
1.3.7.1. Carbonate minerals	36
1.3.7.2. Iron oxides	39
1.3.7.3. Silicate minerals.	43
1.3.8. Summary	45
1.4. Project Objectives and Research Approach	47
1.5. References	51
 Chapter 2: The effect of extracellular polymers (EPS) on the proton adsorption characteristics of the thermophile <i>Bacillus licheniformis</i> S-86.	 69
Results published in <i>Chemical Geology</i> (2008), Vol. 247, p1-15.	
Abstract	69
2.1. Introduction	70
2.2. Materials and Methods	72
2.2.1. Cell culture and preparation	72
2.2.2. EPS extraction	73
2.2.3. Cell characterisation by SEM imaging	73
2.2.4. Potentiometric titrations	74
2.2.5. Data modelling	75
2.2.6. Attenuated total reflectance Fourier transform infrared spectroscopy	76

<i>(ATR-FTIR)</i>	
2.2.7. <i>Fourier transform infrared spectroscopy (FTIR)</i>	76
2.2.8. <i>Dissolved organic carbon (DOC) analysis</i>	77
2.3. Results	77
2.3.1. <i>Cell characterisation by SEM imaging</i>	77
2.3.2. <i>Potentiometric titrations</i>	78
2.3.2.1. <i>Native cells</i>	78
2.3.2.2. <i>EPS-free cells</i>	78
2.3.3. <i>Titration modelling</i>	81
2.3.3.1. <i>Native cells</i>	81
2.3.3.2. <i>EPS-free cells</i>	81
2.3.4. <i>Infrared analysis</i>	84
2.3.5. <i>Dissolved organic carbon analysis</i>	87
2.4. Discussion	88
2.4.1. <i>EPS extraction protocol</i>	88
2.4.2. <i>Surface functional groups</i>	89
2.4.3. <i>Infrared analysis</i>	92
2.4.4. <i>Correlation between titration modelling and infrared analysis</i>	102
2.4.5. <i>Implications for metal adsorption and modelling</i>	104
2.5. Conclusions	105
2.6. References	107

Chapter 3: Investigation the effect of an EPS layer on the electrostatic properties of a bacterial strain.

Abstract	113
3.1. Introduction	114
3.2. Materials and Methods	116
3.2.1. <i>Cell culture, preparation and EPS extraction</i>	116
3.2.2. <i>Cell characterisation by SEM imaging</i>	116
3.2.3. <i>Cell characterisation by TEM imaging</i>	116
3.2.5. <i>Potentiometric Titrations</i>	117
3.2.6. <i>Data modelling</i>	118
3.2.7. <i>Electrophoretic mobility measurements</i>	120
3.3. Results	120
3.3.1. <i>Cell characterisation by SEM imaging</i>	120
3.3.2. <i>Cell characterisation by TEM imaging</i>	121
3.3. <i>Potentiometric titrations</i>	122
3.3.1. <i>Native cells</i>	122
3.3.2. <i>EPS-free cells</i>	122
3.3.4. <i>Electrophoretic mobility measurements</i>	124
3.3.4.1. <i>Native cells</i>	124
3.3.4.2. <i>EPS-free cells</i>	124
3.4. Discussion	126
3.4.1. <i>Potentiometric titrations and data modelling</i>	126
3.4.2. <i>Electrophoretic mobility measurements</i>	135
3.5. Conclusions	138
3.6. References	140

Chapter 4: A macroscopic and EXAFS study of Zn sorption to the surface of the EPS-producing <i>Bacillus licheniformis</i> S-86.	145
Abstract	145
4.1. Introduction	146
4.2. Materials and Methods	149
4.2.1. Cell culture and preparation	149
4.2.2. EPS extraction	149
4.2.3. Metal adsorption experiments	150
4.2.4. Data modelling	151
4.2.5. EXAFS spectroscopy	152
4.3. Results	154
4.3.1. Macroscopic Zn adsorption	154
4.3.1.1. Native cells	154
4.3.1.2. EPS-free cells	154
4.3.3. Zn EXAFS analysis	157
4.3.3.1. Standards	157
4.3.3.2. Native cells	163
4.3.3.3. EPS-free cells	164
4.4. Discussion	164
4.4.1. Zn adsorption	164
4.4.2. The effect of EPS on metal adsorption	175
4.5. Conclusions	176
4.6. References	178
 Chapter 5: A variable temperature study of Zn adsorption by the EPS producing thermophile, <i>Bacillus licheniformis</i> S-86.	 183
Abstract	183
5.1. Introduction	184
5.2. Materials and Methods	187
5.2.1. Cell culture and preparation	187
5.2.2. Zn adsorption experiments	188
5.2.3. Data modelling	188
5.3. Results and Discussion	190
5.3.1. Zn adsorption curves	190
5.3.2. Data modelling	193
5.3.3. The effect of elevated temperature of Zn adsorption	195
5.3.4. Zn complexation mechanism	198
5.3.5. Implications for a universal bacterial-metal surface complexation model	199
5.4. Conclusions	199
5.5. References	201
 Chapter 6: CaCO₃ precipitation in the presence of an EPS-producing bacterium.	 206
Abstract	206
6.1. Introduction	207
6.2. Materials and Methods	209
6.2.1. Cell culture and preparation	209
6.2.2. EPS extraction	209

6.2.3. Dissolved organic carbon (DOC) analysis	210
6.2.4. Mineralisation experiments	210
6.2.5. Sample imaging by scanning electron microscopy (SEM)	211
6.2.6. Mineral characterisation by X-ray diffraction (XRD) analysis	211
6.3. Results	211
6.3.1. Dissolved organic carbon (DOC) analysis	211
6.3.2. Sample imaging by scanning electron microscopy (SEM)	212
6.3.3. Mineral characterisation by X-ray diffraction (XRD) analysis	217
6.4. Discussion	220
6.4.1. Calcium carbonate morphology	221
6.4.2. Calcium carbonate polymorphism	223
6.4.3. Heterogeneous nucleation on cell surfaces	225
6.4.4. The effect of organic molecules on mineralisation	226
6.5. Conclusions	229
6.6. References	231
6.7. Appendix: XRD Spectra	236

Chapter 7: Conclusions and general synthesis.	252
7.1. Summary of Project Aims and Objectives	252
7.2. Summary of Major Findings	253
7.2.1. The effect of EPS on cell surface chemistry and electrostatic properties (Chapters 2 and 3)	253
7.2.2. The effect of EPS on metal adsorption (Chapter 4)	256
7.2.3. Metal adsorption at elevated temperatures (Chapter 5)	257
7.2.4. The effect of EPS on CaCO ₃ mineralisation (Chapter 6)	258
7.3. The Environmental Role of EPS in Bacterial Cell Surface Reactivity	259
7.4. Further Work	262
7.5 References	263

List of Figures

Figure 1.1: Generalised representation of bacterial cell wall structures.	4
Figure 1.2: Examples of chemical functional groups present in bacterial cell walls and extracellular polymers.	
Figure 1.3: Example acid- base titration curves conducted as a function of biomass concentration and pH.	7
Figure 1.4: Acid- base titration data and model fitting curves obtained using FITEQL.	11
Figure 1.5: Equilibrium adsorption models applied to Cu adsorption data.	17
Figure 1.6: Metal adsorption onto <i>Bacillus subtilis</i> (165mg/l) as a function of ionic strength.	20
Figure 1.7: Cd adsorption onto natural bacterial consortia.	22
Figure 1.8: Bacterial 'fingerprint' spectral region obtained by FTIR analysis.	28
Figure 1.9: Experimental setup for EXAFS analysis.	30
Figure 1.10: EXAFS spectrum of Fe ₂ O ₃ .	30
Figure 1.11: FESEM image of calcified <i>Myxococcus xanthus</i> cells and vaterite spherulites.	38
Figure 1.12: TEM micrograph of lepidocrocite mineralisation on the cell wall of <i>Bacillus subtilis</i> .	40
Figure 1.13: TEM image of chains of magnetosomes within a magnetotactic bacterium.	41

Figure 1.14: TEM image of a silica crust forming on the sheath of a cyanobacterial filament.	44
Figure 2.1a: SEM image of native <i>B. licheniformis</i> cells.	78
Figure 2.1b: SEM image of EPS-free <i>B. licheniformis</i> cells.	78
Figure 2.2a: Potentiometric titration curves for native <i>B. licheniformis</i> cells in 0.01 M NaNO ₃ .	80
Figure 2.2b: Potentiometric titration curves for EPS-free <i>B. licheniformis</i> cells in 0.01 M NaNO ₃ .	80
Figure 2.3a: ATR- FTIR spectra for native <i>B. licheniformis</i> cells.	85
Figure 2.3b: ATR- FTIR spectra for EPS-free <i>B. licheniformis</i> cells.	85
Figure 2.4a: FTIR spectra for native <i>B. licheniformis</i> cells (baseline-corrected).	86
Figure 2.4b: FTIR spectra for EPS-free <i>B. licheniformis</i> cells (baseline-corrected).	86
Figure 2.5: Dissolved organic carbon (DOC) results for supernatants from native and EPS-free <i>B. licheniformis</i> samples as a function of pH.	87
Figure 2.6a: ATR- FTIR spectra for native <i>B. licheniformis</i> cells (normalised to the amide II peak).	94
Figure 2.6b: ATR- FTIR spectra for EPS-free <i>B. licheniformis</i> cells (normalised to the amide II peak).	94
Figure 2.7a: FTIR spectra for native <i>B. licheniformis</i> cells (baseline-corrected and normalised to the amide II peak).	95
Figure 2.7b: FTIR spectra for EPS-free <i>B. licheniformis</i> cells (baseline-corrected and normalised to the amide II peak).	95
Figure 2.8: Peak absorbance ratios for peaks 1 and 5, as a function of pH (ATR-FTIR spectra).	103
Figure 3.1a: SEM image of native <i>B. licheniformis</i> cells.	121
Figure 3.1b: SEM image of EPS-free <i>B. licheniformis</i> cells.	121
Figure 3.2a: TEM image of a native <i>B. licheniformis</i> cell.	121
Figure 3.2b: TEM image of an EPS-free <i>B. licheniformis</i> cell.	121
Figure 3.3a: Potentiometric titration curves for native <i>B. licheniformis</i> cells.	123
Figure 3.3b: Potentiometric titration curves for EPS-free <i>B. licheniformis</i> cells.	123
Figure 3.4a: Electrophoretic mobility measurements for native <i>B. licheniformis</i> cells.	125
Figure 3.4b: Electrophoretic mobility measurements for EPS-free <i>B. licheniformis</i> cells.	125
Figure 3.5: Light microscope images of <i>B. licheniformis</i> suspensions.	127
Figure 4.1a: SEM image of native <i>B. licheniformis</i> cells.	150
Figure 4.1b: SEM image of EPS-free <i>B. licheniformis</i> cells.	150
Figure 4.2a: Zn adsorption edges for native cells.	156
Figure 4.2b: Zn adsorption edges for EPS-free cells.	156
Figure 4.3: i) EXAFS spectra and ii) Fourier transforms for: A) Zn standards and native cells B) standards and EPS-free cells; C) Native and EPS-free cells at pH 3; D) Native and EPS-free cells at pH 5; E) Native and EPS-free cells at pH 7.	159
Figure 4.4: Light microscope images of <i>B. licheniformis</i> suspensions.	166
Figure 4.5a: Histogram of Log K values for Zn adsorption.	170
Figure 4.5b: Example model fits to adsorption data for Zn.	170
Figure 5.1: Zn adsorption isotherms for <i>B. licheniformis</i> S-86.	190

<i>Figure 5.2: Light microscope images of B. licheniformis suspensions.</i>	192
<i>Figure 5.3: Histogram of Zn-site stability constants obtained at 25 °C, 40 °C and 50 °C.</i>	196
<i>Figure 5.4: Model fits to data.</i>	197
<i>Figure 6.1: Dissolved organic carbon released into solution as a function of pH by native an EPS-free B. licheniformis S-86 cells.</i>	212
<i>Figure 6.2: SEM images for the control experiment at a) 48 h, and b) 7 days.</i>	213
<i>Figure 6.3: SEM images for the native cell experiment at a) 48 h, and b) 7 days.</i>	214
<i>Figure 6.4: SEM images for the EPS-free cell experiment at a) 48 h, and b) 7 days.</i>	216
<i>Figure 6.5: SEM images for the EPS-solution experiment at a) 48 h, and b) 7 days.</i>	217
<i>Figure 6.6: XRD spectrum for the EPS-free cell experiment at 48 h.</i>	219
<i>Figure 6.7: XRD spectrum for the EPS solution experiment at 48 h.</i>	219
<i>Figure 6.8: Free energy of clusters in solution as a function of the number of ions in the cluster.</i>	224
<i>Figure 7.1: The environmental roles of EPS in metal-microbe interactions and biomineralisation.</i>	260

List of Tables

<i>Table 2.1: FITEQL results for native B. licheniformis cells.</i>	83
<i>Table 2.2: FITEQL results for EPS-free B. licheniformis cells.</i>	83
<i>Table 2.3: ATR-FTIR peaks.</i>	96
<i>Table 2.4: FTIR peaks.</i>	97
<i>Table 3.1: FITMOD modelling results for EPS-free cells.</i>	131
<i>Table 3.2: FITMOD modelling results for native cells.</i>	131
<i>Table 4.1: EXAFS results for the Zn standards and biomass samples.</i>	158
<i>Table 4.2: Mean pK_a values, site concentrations and associated 1x standard deviations for B. licheniformis S-86.</i>	168
<i>Table 4.3a: FITEQL modelling results for Zn adsorption to native cells.</i>	168
<i>Table 4.3b: FITEQL modelling results for Zn adsorption to EPS-free cells.</i>	169
<i>Table 5.1: Site concentrations and deprotonation constants for B. licheniformis S-86.</i>	193
<i>Table 5.2: FITEQL modelling results for experiments conducted at 25 °C, 40 °C and 50 °C.</i>	194
<i>Table 7.1: Possible effects of EPS production on metal adsorption relative to non-EPS producing strains.</i>	259

1. Introduction

1.1. Microbe-Metal Interactions

The ability to develop a process-based understanding of the interactions between the many organic and inorganic surfaces and dissolved species is a pre-requisite to developing a predictive framework for the behaviour of dissolved species present in environmental settings. Alongside the study of components such as minerals and dissolved organic matter, investigations focussing on metal adsorption and mineralisation at microbial surfaces are increasingly recognised as an essential contribution towards reaching a full understanding of highly complex natural systems.

Biogeochemically, bacterial cells and other microbes may exert influence over metal ions by four principle processes:

- cell surface adsorption, the non-metabolic adsorption of metal ions and other solutes to the cell walls of bacteria and other microscopic organisms such as fungi, archaea and algae;
- biomineralisation, the precipitation of minerals within a microbial cell, on the cell surface or in close association with the cell. Biomineralisation may be categorised as either metabolic or non-metabolic, depending upon the mechanism of mineral precipitation;
- microbial oxidation and reduction of metal species, which may occur as a consequence of cell metabolic processes. Metals are often utilised as terminal electron acceptors by bacteria living in anoxic conditions. The consequent change in oxidation state of the metal may result in precipitation if the resulting species is insoluble (Abdelouas et al., 2005, Francis et al., 2000). In most environments, microbes have a significant or even controlling influence over the cycling of redox-sensitive metals;

- metabolic uptake, which may lead to the sequestration of metals within the cell interior. Bioessential metals are required for cell metabolic processes, particularly the manufacture of enzymes.

The results of this study are presented as five chapters (Chapters 2-6) written in the form of stand-alone manuscripts intended for peer-reviewed publication. The introduction will therefore outline the main research issues and project objectives and provide an in-depth review of the corresponding literature. The final section of the introduction explains the research approaches undertaken during this study and outlines the thesis structure. A concluding chapter (Chapter 7) brings together the findings of Chapters 2-6 and outlines a model for the role of extracellular polymers in the surface reactivity of bacteria.

1.2. Research Rationale

Research into the processes of metal adsorption and biomineralisation at cell surfaces has traditionally focused on addressing the following major issues:

1. The mechanisms and controls on the surface adsorption process are not well understood. Specifically, the nature and types of surface sites involved in metal binding, and the type of bonding to these sites have not been fully constrained.
2. The mechanisms controlling different types of biomineralisation are not fully understood, particularly the relationship between cell surface adsorption and any subsequent mineral precipitation. This lack of knowledge inhibits attempts to establish characteristic properties of biogenic minerals by which they may be identified in either modern environments or the geological record.
3. Research into metal adsorption and biomineralisation would facilitate a quantitative description of the contribution made by biosorption and biomineralisation to the biogeochemical cycling of major and trace elements.

4. The predictive capability in bioremediation through biosorption and biomineralisation is presently limited by an incomplete mechanistic description of surface adsorption and biomineralisation under field scale conditions.

To provide a framework for this study, Section 1.3 describes previous studies which have attempted to address these issues.

1.3. Review of Previous Research Findings

1.3.1. Bacterial cell wall structure

Bacteria are prokaryotes, meaning they have no nucleus within the cell and their nuclear components are therefore unconfined within the cytoplasm. The cytoplasm is contained within the plasma membrane, and is enclosed by the cell wall. The bacterial cell wall is structurally and biochemically well-characterised (Madigan et al., 2003).

Bacteria may be classified as either Gram-positive or Gram-negative, depending upon their reaction with the Gram stain. The ability of Gram-positive strains to retain the Gram stain is a consequence of important differences in the cell wall structure between Gram-positive and Gram-negative species. Most significantly, Gram-positive strains have a much larger amount of peptidoglycan compared to Gram-negative strains, which have only a thin layer of peptidoglycan between the cytoplasmic membrane and their additional outer membrane (van der Wal et al., 1997). Simplified structures of these cell types are shown in Figure 1.1.

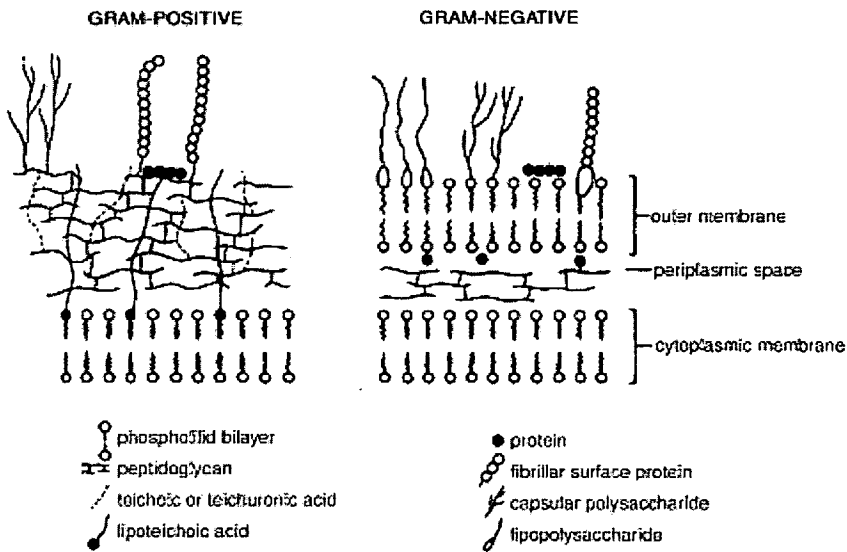


Figure 1.1: Generalised representation of bacterial cell wall structures (Poortinga et al., 2002).

Biochemically, cell walls are complex structures and are rich in polymers such as peptidoglycan, teichuronic acids, teichoic acids and enzymes (van der Wal et al., 1997). These polymers contain a range of functional groups including phosphoryl, carboxyl, hydroxyl, amine and phosphodiester groups (Figure 1.2). The outer membrane of the Gram-negative cell consists of proteins, phospholipids and lipopolysaccharides, and therefore also contains abundant functional groups.

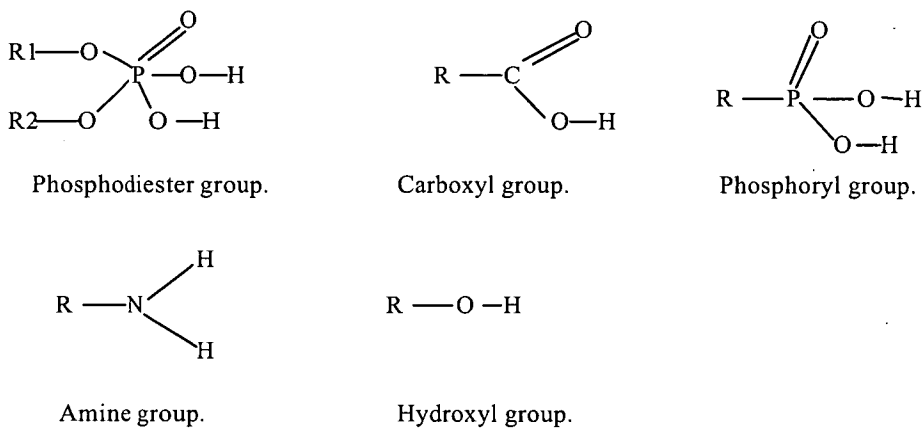


Figure 1.2: Examples of chemical functional groups present in bacterial cell walls and extracellular polymers. R represents rest of molecule.

The presence of these organic functional groups within the bacterial cell wall is of central importance to studies of cell surface reactivity, electrostatic interactions and metal adsorption. These groups may either lose or gain protons depending on the ambient pH conditions, thereby imparting either a positive or negative charge to the cell wall (van der Wal et al., 1997). At circumneutral pH most cell walls are likely to have a negative surface charge. Due to this negative charge, cell walls are able to complex cationic metal ions from solution (Beveridge et al., 1982). In addition, many bacterial species produce extracellular polymeric capsules. These may be produced to aid adhesion, to assist motility or as protection from dehydration or toxicity (Merroun et al., 2003). The extracellular polymers consist largely of polysaccharides, proteins, nucleic acids and lipids (Guibaud et al., 2004), but the composition varies according to growth conditions and environmental factors (Omoike and Chorover, 2004).

1.3.2. Cell surface charge and chemistry

The initial objective in many metal adsorption studies is to establish the total concentration of cell wall functional groups present, the number of different functional groups, and to try and assign identities to these groups based on their deprotonation constants. As the reactivity of these cell wall functional groups is based on their tendency to deprotonate as a function of pH, quantifying these groups is based on measurement of the charge variation in the system as pH is varied. This may be undertaken by means of potentiometric titrations of bacterial cell suspensions (Section 1.3.2.1). A second method of quantifying cell surface charge is measurement of electrophoretic mobility, which is discussed in Section 1.3.2.2 below.

1.3.2.1. Potentiometric titrations

Potentiometric titrations are carried out using bacterial biomass of known concentration suspended in an electrolyte, which comprises of ions that will not form complexes with the cell wall functional groups (Daughney and Fein, 1998; Ngwenya

et al., 2003; Haas, 2004; Borrok and Fein, 2005; Burnett et al., 2006a). The suspension is then pH-adjusted to either low or high pH and titrated upwards or downwards with a strong acid or base. It is important that the titration procedure does not cause irreversible damage or conformational changes to the cells. The reversibility of bacterial potentiometric titrations over the range of approximately pH 3-11 was established by Daughney and Fein (1998). Titrations are generally not undertaken outwith this pH range as the likelihood of cell damage is high. This constraint limits investigation of the full spectrum of functional groups that may be present within bacterial cell walls. Nevertheless, the range covered by most titrations is sufficient to cover potential pH-charge relationships across most environments of interest.

The data obtained from potentiometric titrations is most commonly plotted as net H^+ exchanged vs. pH. The concentration of H^+ exchanged is equal to the difference between the total acid or base added during the titration and the equilibrium (measured) H^+ and OH^- concentrations, and may be calculated as follows:

$$[H^+]_{exchanged} = C_a - C_b + [OH^-] - [H^+] \quad (1.1)$$

Where C_a = concentration of acid added, C_b = concentration of base added, $[OH^-]$ = equilibrium concentration of OH^- , $[H^+]$ = equilibrium concentration of H^+ (Haas et al., 2001).

The resulting titration curves indicate the overall buffering capacity of the bacterial cell wall. Figure 1.3 shows example titration curves and demonstrates increasing buffering capacity as biomass concentration increases. The characteristic weak inflection points are due to the presence of different organic functional groups that deprotonate across overlapping pH ranges. Subsequent surface complexation modelling can be undertaken using the titration data in order to establish functional group concentrations and stability constants (discussed in Section 4.2.2).

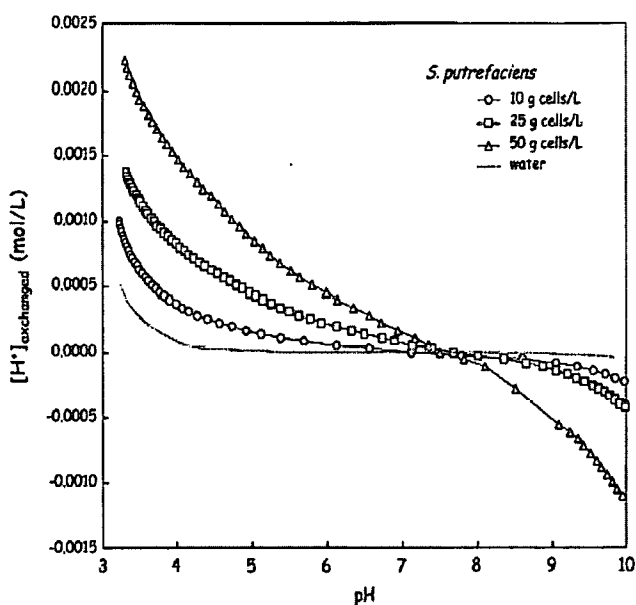


Figure 1.3: Example acid- base titration curves conducted as a function of biomass concentration and pH (Haas et al., 2001).

Potentiometric titrations and surface complexation modelling provide useful quantitative data about the cell surface chemistry, but cannot directly establish the identities of the proton-active functional groups. Assignment of identities is often based on comparison with previous literature and with deprotonation constants established for comparable organic ligands. However, the deprotonation constants for groups within the bacterial cell wall may not be identical to those established for the same group in isolation, so identification of groups by such means is necessarily tentative. A number of recent studies have undertaken spectroscopic analysis as a means of directly confirming functional group identities (Jiang et al., 2004; Dittrich and Sibler, 2006; Leone et al., 2007). Spectroscopic studies are discussed in detail in Section 4.3.

1.3.2.2. Electrophoretic mobility studies

As bacterial cells have charged surfaces, they are mobile when placed in an electric field. The magnitude of this electrophoretic mobility provides a measure of the surface charge density (Poortinga et al., 2002). The magnitude of the electrophoretic

mobilities displayed by bacterial cells is governed by three main factors: i) electrophoretic mobilities decrease with increasing ionic strength due to electrostatic interaction of the electrolyte ions with the ionised cell surface groups and consequent reduction of the cell wall electrical potential (Yee et al., 2004a); ii) as electrophoretic mobility is determined by the cell surface charge density, it increases with increasing pH owing to progressive deprotonation of the cell wall organic functional groups (Poortinga et al., 2002); iii) swelling of the cell wall leads to a decrease in surface charge density and hence decreased electrophoretic mobility. Cell wall swelling occurs both as a response to decreasing ionic strength and to increased pH (Poortinga et al., 2002; Gaboriaud et al., 2006). These effects may counteract each other, and the observed electrophoretic mobility results from the balance of these contributory factors.

A number of studies have undertaken electrophoretic mobility measurements of bacterial cells (van der Wal et al., 1997; Phoenix et al., 2002; MacLean et al., 2004; Yee et al., 2004a; Claessens et al., 2005; Fein et al. 2005; Burnett et al., 2006a; Leone et al., 2007; Heinrich et al., 2007). These studies have found that bacterial cells are negatively charged at most pH values. Generally, cells remain negatively charged as low as pH 3, indicating that the isoelectric point, at which all the surface functional groups are fully protonated, occurs at lower pH. As for potentiometric titrations, analysis conducted below pH 3 is liable to damage the cells.

1.3.3. Surface complexation modelling

Early studies investigating metal uptake by bacteria and other microbes looked at bulk adsorption, often at constant pH, and took the approach of fitting the data to Langmuir or Freundlich isotherms (Plette et al., 1996; Pagnanelli et al., 2000; Esposito et al., 2001). These empirical models provide useful constraints on adsorption capacity and charging behaviour under the given experimental conditions, but the results cannot be used to predict behaviour under different conditions. As more detailed constraints on the adsorption mechanism are sought, most studies have adopted the surface complexation modelling approach, in which proton and metal complexation is viewed as taking place to discrete surface functional groups.

Surface complexation modelling provides site concentrations and stability constants that can be applied to conditions other than those under which the experiments were conducted.

An accurate model of adsorption behaviour must address two principle considerations; firstly, quantifying and characterising the reactive functional groups in the bacterial cell wall, and secondly, accounting for the effects of electrostatic interactions on surface adsorption properties. Each of these issues will be discussed below.

1.3.3.1. Determining deprotonation constants and site concentrations

Most studies aiming to characterise bacterial proton and metal adsorption behaviour begin with the objective of determining the concentrations of the proton-active functional groups, and their respective deprotonation constants (K_a values). The deprotonation constants are usually quoted as $-\log K_a$, or pK_a . Each different functional group deprotonates across a characteristic pH range (typically $pK_a \pm 2$).

The deprotonation of a functional group can be represented by the following generic reaction (Borrok and Fein, 2004):



Where R is the bacterium and A represents a functional group on the bacterial surface or volume (in the case of EPS). The deprotonation or acidity constant, K_a , for this reaction is expressed as:

$$K_a = \frac{[R - A^{-}]a_{H^{+}}}{[R - AH^{\circ}]} \quad (1.3)$$

where $[R-A^-]$ and $[R-AH^0]$ represent the concentration of deprotonated and protonated sites and a_{H^+} represents the activity of protons in the solution.

Use of Equation (1.1) assumes the cell wall to be fully protonated at the start of the titration. However, as explained by Westall et al. (1995) and Borrok et al. (2005), the presence of very low pK_a functional groups lead to development of negative charge on the cell wall even at the low starting pH values for potentiometric titrations. In order to account for this charge imbalance, the total proton concentration (T_H) may be calculated as follows;

$$T_H = T_H^0 + [C_a - C_b] \quad (1.4)$$

Where C_a is the concentration of acid added and C_b is the concentration of base added. T_H^0 represents the charge imbalance due to the initial protonation state of the cell wall and is treated as an adjustable parameter during data optimisation modelling (Westall et al., 1995; Fein et al., 2005).

Having quantified the system charge excess as a function of pH using either Equation (1.1) or Equation (1.4), the next step is to constrain the number of different functional groups present, and their respective pK_a values and concentrations. This requires some form of data optimization approach. Many studies (e.g. Fein et al., 1997; Haas et al., 2001; Yee and Fein, 2001; Gorman-Lewis et al., 2005; Wightman and Fein, 2005; Johnson et al., 2007) have opted to use versions of the optimization program FITEQL (Herbelin and Westall, 1999), which finds the minimum number of surface sites required to fit the data. FITEQL calculates the variance, $V(Y)$, between the experimental data and the model:

$$V(Y) = \frac{\sum \left(\frac{Y_{calc} - Y_{obs}}{S_{obs}} \right)^2}{n_p n_{II} - n_u} \quad (1.5)$$

where Y_{calc} is the calculated value, Y_{obs} is the experimental value, S_{obs} is the error associated with the experimental data, n_p is the number of data points, n_u is the number of adjustable parameters in the model, and n_{II} is the number of 'Group II' components, for which both the total and free (dissolved) concentrations are known (Ngwenya et al., 2003).

A variance of less than 20 is considered a good fit to the experimental data (Herbelin and Westall, 1999). Examples of model fits to titration data are shown in Figure 1.4, which shows in this case that a three-site model was required to optimally fit the data.

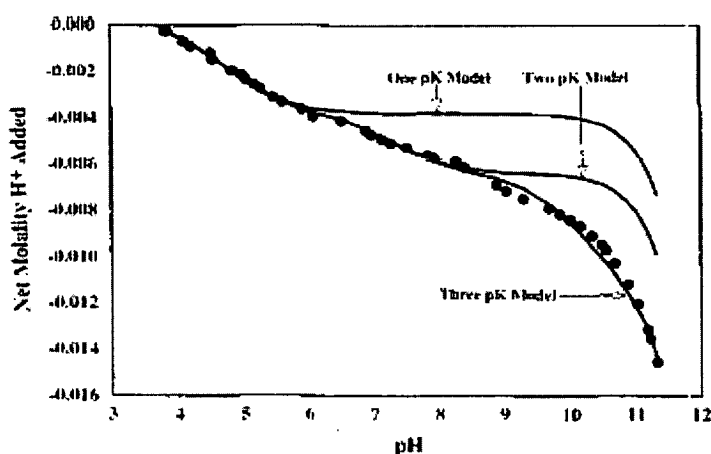


Figure 1.4: Acid- base titration data and model fitting curves obtained using FITEQL (Daughney and Fein 1998).

The other commonly used method of obtaining pK_a values and site concentrations is to employ a pK_a -spectrum approach, using the linear programming method (LPM) (Brassard et al., 1990; Cox et al., 1999; Sokolov et al., 2001; Dittrich and Sibling, 2006). A variation on this approach is the Fully Optimized Continuous (FOCUS) pK_a affinity distribution method developed by Martinez et al. (2002). The programs used for the LPM optimise the site concentrations to a spectrum of regularly-spaced 'fixed' pK_a values. The charge balance equation is set out as follows:

$$\text{Charge excess} = C_{Bj} - C_{Aj} + [H^+]_j - [OH^-]_j = \Sigma \left(\frac{K_{ai} L_{Ti}}{K_{ai}} \right) + [H^+]_j + S \quad (1.6)$$

Where C_{Bj} and C_{Aj} are the concentrations of acid and base for the j th addition of titrant, $[H^+]_j$ and $[OH^-]_j$ are measured, S is a constant accounting for the initial protonation state of the surface, and the surface sites are considered as the sum of n surface site $[L^-]$ with dissociation constants K_{ai} and total concentrations L_{Ti} (Dittrich and Sibling 2006).

The advantage to this approach over surface complexation modelling programs such as FITEQL is greater flexibility in the number of sites required, as FITEQL involves optimising the data around a predetermined number of sites. In addition, the 'pK_a spectrum' is believed to be a more accurate representation of the variable and overlapping pK_a ranges found in organic macromolecules than the small number of discrete pK_a values assigned by FITEQL (Cox et al., 1999).

Surface complexation modelling does not enable identification of the functional groups. These must be inferred by comparison with previously published work, and deprotonation constants for comparable organic ligands. As yet, there is no firm consensus on the identities of the proton-active functional groups, an issue which is discussed further in Chapter 2.

1.3.3.2. *Electrostatic modelling*

As mentioned previously, bacterial cell surfaces contain many organic functional groups that deprotonate over discrete pH ranges. Consequently, the bacterial surface is negatively charged at most pH values. Because of the cell surface charge that develops as a consequence of functional group deprotonation, there are potentially electrostatic interactions between the cell surface and the surrounding electrolyte that may affect the calculated site concentrations, pK_a values and metal complexation behaviour. Such electrostatic effects are manifested by variations in complexation parameters between experiments conducted at different ionic strengths. It seems that adsorption of some species is strongly affected by variation in ionic strength,

whereas others are relatively unaffected (discussed in Section 1.3.4.2.1).

Quantifying the electrostatic component requires assignation of a surface potential value. However, because the bacterial cell surface is a highly complex, heterogeneous structure, determining surface potential is extremely difficult. As the assigned values are often arbitrary and unrealistic, many recent studies have opted to neglect the electrostatic component (Chatellier and Fortin, 2004; Gorman-Lewis et al., 2005; Wightman and Fein, 2005; Claessens and van Capellen, 2007; Johnson et al., 2007; Tourney et al., 2008). Using a nonelectrostatic approach produces surface complexation parameters that are only applicable to the ionic strength conditions used for that particular study.

Many of the earlier surface adsorption studies used a Constant Capacitance approach (Fein et al., 1997; Daughney and Fein, 1998; Haas et al., 2001; Yee and Fein, 2001; Ngwenya et al., 2003). This approach assumes that all adsorption takes place within a plane immediately adjacent to the bacterial surface, and uses the following relationship between surface charge (σ), surface potential (Ψ_s) and capacitance (C);

$$C = \frac{\sigma}{\Psi_s} \quad (1.7)$$

The Constant Capacitance approach also produces ionic-strength-dependent complexation constants. However, the Double Layer Model (DLM) and Triple Layer Model (TLM) both incorporate ionic strength in the calculations of surface potential. The apparent (ionic strength-dependent) equilibrium constants obtained (K_a) may then be corrected for electrostatic effects to obtain intrinsic equilibrium constants (K_{int}) that are invariant with ionic strength, using the Boltzmann equation:

$$K_{int} = K_a e^{\left(\frac{-\Delta Z F \Psi_s}{RT}\right)} \quad (1.8)$$

Where: F = Faraday's constant, R = Gas constant, T = temperature, K_{int} = equilibrium constant referenced to zero surface charge, Ψ_s = electric potential of the bacterial cell surface, ΔZ = change in charge of the surface species in the reaction (Yee *et al* 2004).

A value must be assigned for the capacitance of the bacterial surface, but this cannot be directly measured so must be optimised. However, because surface charge is related to surface potential (Equation 1.7), and surface potential is affected by the surface site concentration, this results in a model with parameters that cannot be resolved independently (Daughney and Fein 1998). The Constant Capacitance approach was originally developed for use with mineral surfaces, which are far more homogeneous and structurally simple than bacterial cell walls and therefore have more easily measurable capacitance values. A capacitance of 8 F m^{-2} was used in many of the aforementioned studies, but is now considered unrealistically high. Owing to these limitations, the Constant Capacitance approach is generally no longer used for bacterial adsorption studies. Borrok and Fein (2005) compared proton, Pb, Cd and Sr adsorption data modelled using a nonelectrostatic approach, a Diffuse Layer model (DLM) and a Triple Layer model (TLM). It was found that the DLM and TLM could not accurately reproduce the experimental data as they overpredicted the effects of the electrostatic interactions. As with the Constant Capacitance approach, the DLM and TLM are intended for application to mineral adsorption studies and cannot provide a realistic representation of the bacterial cell surface environment.

Fein *et al.* (2005) undertook modelling of potentiometric titration data using a Langmuir-Freundlich model, a Constant Capacitance model and a nonelectrostatic model. They found that each approach provided an excellent fit to the data, thus highlighting the problem associated with use of modelling approaches that do not provide a realistic physical representation of the system under investigation.

Some recent studies have adopted the Donnan core-shell model, as this provides a more realistic representation of the cell wall-electrolyte interface (Plette *et al.*, 1995; Martinez *et al.*, 2002; Yee *et al.*, 2004; Burnett *et al.*, 2006a; 2006b; Heinrich *et al.*,

2007). The Donnan model views the cell wall as a 3-dimensional semi-permeable membrane throughout which the binding sites are distributed (Wonders et al., 1997; Wasserman and Felmy, 1998), in contrast to the Constant Capacitance model, which represents all the surface charge as concentrated in a single plane at the cell surface. This provides a means of calculating the surface potential (Ψ_s), for correction of apparent stability constants (Equation 1.7). The potential within the cell wall volume (Donnan volume), the Donnan potential (Ψ_D), is assumed to be equal to the surface potential at the interface between the Donnan volume and the bulk solution. This is an approximation however, as the Donnan potential is not the same as the surface potential.

The Donnan potential is calculated as follows:

$$\Psi_D = \left(\frac{RT}{zF} \right) \operatorname{arcsinh} \left(\frac{\rho}{2zFc} \right) \quad (1.9)$$

Where ρ is the cell wall charge per unit volume, z is the valency of the symmetrical electrolyte, R is the Gas Constant, T is temperature and c is the concentration of all ions (mol/unit volume) (Wonders et al., 1997).

The cell wall charge ρ (C m^{-3}) is calculated as follows:

$$\rho = \frac{[L^-]_T F}{nv} \quad (1.10)$$

Where F is Faraday's constant, n is the cell density (cells g^{-1}) and v is the cell wall volume (m^3/cell). $[L^-]_T$ is the charge excess (equivalent to H^+ exchanged as defined in Equation (1.1)) (Yee et al., 2004).

The Donnan potential calculated by this method may then be substituted for Ψ_s in Equation (1.7), and intrinsic stability constants obtained. The data optimisation program FITMOD, a modification of FITEQL 2.0 (Westall, 1982), provides ionic-strength-independent stability constants by this approach (Burnett et al., 2006a: 2006b; Hetzer et al., 2006; Heinrich et al., 2007).

The plots of charge excess vs. pH obtained by using Equation (1.1) are ionic-strength dependent. However, the Donnan model may be used to construct ‘master curves’ from the experimental data, which are invariant with ionic strength (Plette et al., 1995; Wonders et al., 1997; Martinez et al., 2002). The master curve can then in turn be used to calculate intrinsic site concentrations and deprotonation constants.

Master curves are plotted as charge excess vs. pH_s , where pH_s is calculated as follows:

$$pH_s = pH_{bulk} - \left(\frac{F\Psi_s}{2.303RT} \right) \quad (1.11)$$

Where pH_{bulk} is the measured solution pH and Ψ_s , the surface potential, is considered equal to the Donnan potential, Ψ_D (calculated as in Equation 1.8) (Martinez et al. 2002).

The ionic strength dependent titration curves will be seen to collapse into a single master curve, usually closest to the curve for the highest ionic strength experiment, as electrostatic effects are suppressed with increasing ionic strength (Martinez et al. 2002).

1.3.4. Macroscopic adsorption studies

The data obtained from macroscopic metal adsorption experiments may be modelled either by using adsorption isotherms or by a discrete site surface complexation approach. Both of these methods are discussed below.

1.3.4.1. Langmuir adsorption isotherms

Macroscopic metal adsorption studies generally consist of batch adsorption experiments conducted as a function of pH. Many early studies conducted experiments as a function of metal concentration, at constant pH, and fitted the results to Langmuir or Freundlich-type isotherms (Sag and Kutsal, 1995; Chang et al., 1997; Veglio et al., 1997; Sar et al., 1999; Pagnanelli et al., 2000; Esposito et al., 2001). The example in Figure 1.5 shows the equilibrium amount of Cu adsorbed by 1 g l^{-1} biomass at pH 5.

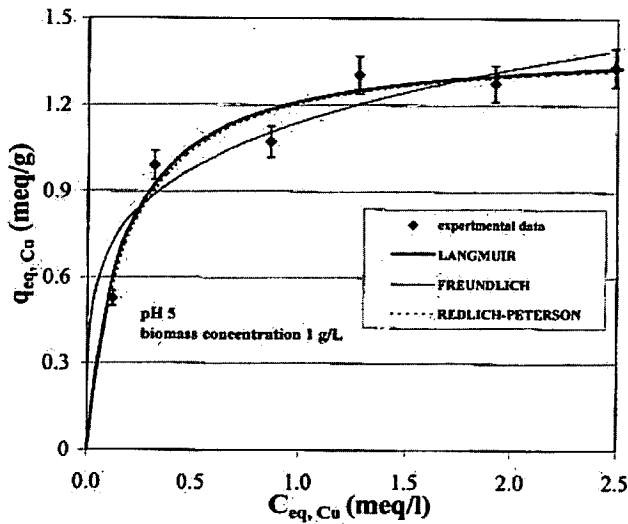


Figure 1.5: Equilibrium adsorption models applied to Cu adsorption data (Esposito et al., 2001).

These provide useful information relating to the bulk adsorption capacities of bacterial cells under the given experimental condition. Furthermore, such studies have incorporated investigations of adsorption kinetics, establishing that metal

complexation to bacterial cells is rapid (Chang et al., 1997; Sar et al., 1999), and reversible (Fowle and Fein, 2000). However, this approach cannot provide the thermodynamic parameters necessary to extrapolate to other systems. Many recent studies have undertaken discrete-site surface complexation modelling as a more mechanistically realistic means of characterising the adsorption mechanism. This approach views adsorption as occurring to a number of different sites with individual complexation constants and site concentrations that can be resolved by this data modelling approach (discussed in Section 1.3.4.2).

1.3.4.2. Discrete-site SCM approach to metal adsorption

The overall objective of such proton and metal adsorption studies is to gain an understanding of the adsorption mechanism. However, in order to apply experimental adsorption parameters to complex systems potentially containing millions of different bacterial strains, it is also necessary to develop and test the ‘universality’ of a generalised surface complexation model for bacterial metal adsorption (Fein et al., 1997; Fowle and Fein, 1999; Yee and Fein, 2001; Borrok et al., 2004a; 2004b; Fein, 2006). Developing a set of adsorption constants that are applicable to a wide range of bacterial species and environmental conditions would greatly facilitate prediction of metal-microbe interactions in a given setting, and also enable better understanding of the extent to which microbes influence biogeochemical cycling of metals under different conditions. However, this cannot be done until the effects of a range of experimental variables can be understood and accurately quantified. As the effects of these experimental parameters on metal adsorption are often a direct consequence of the effects on cell surface chemistry, studies of both proton and metal adsorption will be discussed.

Macroscopic adsorption studies have been used in order to investigate the contributions to proton and metal adsorption of variables such as other metal species (Macaskie and Basnakova, 1998; Chatellier and Fortin, 2004; Takahashi et al., 2005; Claessens and van Capellen, 2007; Tsuruta, 2006), ionic strength (Daughney and Fein, 1998; Small et al., 2001; Borrok and Fein, 2005; Kenward et al., 2006), bacterial strain (Haas et al., 2001; Yee and Fein, 2001; Ngwenya et al., 2003; Borrok

et al., 2004a; 2004b; Burnett et al., 2006a; 2006b; 2007; Hetzer et al., 2006; Heinrich et al., 2006), growth conditions (Daughney et al., 2001; Borrok et al., 2004c; Haas, 2004; Hong and Brown, 2006; Guine et al., 2007) and adsorption temperature (Wightman et al., 2001; Aksu et al., 2002; Takahashi et al., 2005; Gorman-Lewis et al., 2006). Each of these variables will be discussed below.

1.3.4.2.1. Adsorbing metal

Most environmentally significant groups of metals have been investigated in adsorption studies at some point, including radionucleides (Macaskie and Basnakova, 1998), redox-sensitive species (Chatellier and Fortin, 2004; Wightman and Fein, 2005), rare earth elements (REE) (Texier et al., 1999; Takahashi et al., 2005; Tsuruta, 2006), metal anions such as selenate (Kenward et al., 2006) and mercury (Daughney et al., 2002). Some metals adsorb as hydrated (outer-sphere) complexes whereas others adsorb as inner-sphere complexes. Inner-sphere complexes involve strong covalent bonds, whereas outer-sphere complexes are held in the diffuse layer by electrostatic interactions (Stumm and Morgan, 1996). This difference in adsorption mechanism explains why outer-sphere metals adsorb to lesser extents as ionic strength is increased and the diffuse layer collapses, whilst inner-sphere metals are unaffected (Kenward et al., 2005).

Yee et al. (2004) found that adsorption of Ca(II), Sr(II) and Ba(II) to *Bacillus subtilis* was strongly dependent on ionic strength (Figure 1.6), as was neptunyl adsorption to the same species (Gorman-Lewis et al., 2005). Adsorption of Cd^{2+} , Pb^{2+} and Cu^{2+} was found to exhibit strong ionic strength dependence by Daughney and Fein (1998). Ionic strength dependence was also observed by Small et al. (2001) for Sr adsorption to *Shewanella alga*, by Burnett et al. (2006a) for proton adsorption to *Anoxybacillus flavithermus* and by Kenward et al. (2006) for adsorption of selenate to *Shewanella putrefaciens*. A study by Borrok and Fein (2005) concluded that proton, Cd and Pb adsorption to two *Pseudomonas species* was only slightly ionic strength dependant, whereas adsorption on Sr was strongly affected by ionic strength. Haas et al. (2001) found that U(VI) adsorption to *Shewanella putrefaciens* was not strongly ionic strength dependent. These examples indicate that there is contradictory evidence in

the literature regarding the types of complexes formed by Cd and Pb. However, this may be resolvable by spectroscopic analysis (Stumm and Morgan, 1996).

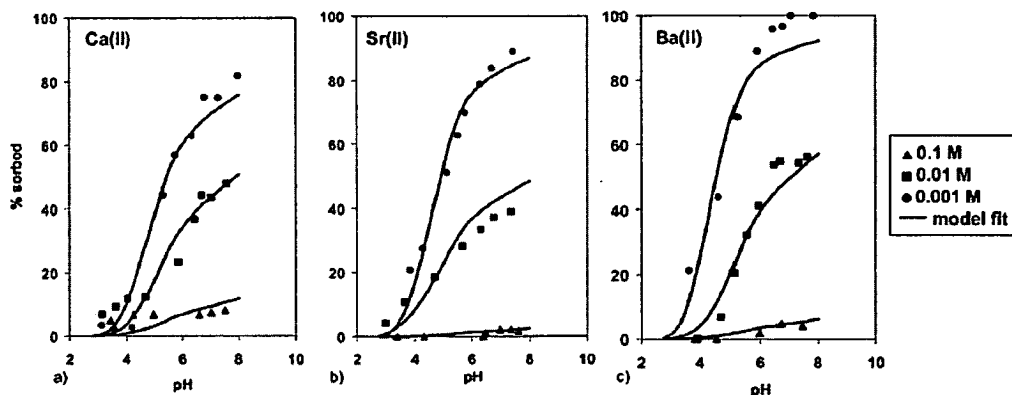


Figure 1.6: Metal adsorption onto *Bacillus subtilis* (165mg/L) as a function of ionic strength (Yee et al., 2004)

The type of complex formed also affects the response to temperature variation (Takahashi et al., 2005). Many studies have established that some metals are more strongly bound than others and there will be an order of preference if a mixture of different metals is used in the adsorption study. The studies by Takahashi et al. (2005) and Tsuruta (2006) indicate that some rare earth elements are preferentially adsorbed over others. Takahashi et al. (2005) found that *B. subtilis* and *E. coli* preferentially complexed heavy REE, particularly Tm, Yb and Lu. As this pattern was also found to be present in natural microbial mats, it was suggested that REE enrichment patterns could act as an indicator of bacterial activity in the geological record. Texier et al. (1999) found that Eu and Yb were preferentially adsorbed to *P. aeruginosa* compared to La, which is in agreement with the findings of Takahashi et al. (2005). However, the strong inhibition of lanthanide adsorption by Al (Texier et al., 1999) suggests that REE enrichment may be difficult to detect in most environmental settings, given the prevalence of Al. The variation in the stability constants (log K) values for metal-site complexes indicate varying binding affinities for different metals. For example, the presence of Cu will inhibit Zn complexation (Claessens and van Capellen, 2007). Studies of mixed-metal systems by Fowle and

Fein (1999) and Burnett et al. (2007) found that the binding of the different metals present in a mixed system could be predicted by the stability constants established for single-metal systems. In addition, the stability constants obtained from multi-metal systems were comparable to those from the single metal systems. This suggests that these stability constants can be used to predict metal binding to bacteria in natural or industrial settings.

It is well known that many bacterial species are able to induce redox transformations in metal ions, either as a consequence of changes to their immediate surroundings caused by metabolic effects, or more directly when the metal species is used as an electron donor by chemolithotrophic bacterial strains. This potentially has implications for the mechanism of metal adsorption to cell surfaces. For example, it was found by Kenward et al. (2006) that selenate adsorption to *S. putrefaciens* was partly irreversible due to reduction of selenate to elemental Se, which was subsequently precipitated to the cell surface. Cell surface precipitation due to changing redox state may also be a contributory factor to the mechanisms controlling surface adsorption of iron. Chatellier and Fortin (2004) conducted Fe(II) adsorption experiments under anoxic conditions and observed that adsorption was fully reversible. However, it was pointed out that in an oxic system iron oxidation would occur, potentially leading to precipitation of Fe(III) on the cell surfaces. Wightman and Fein (2005) found that, although Fe(III) adsorption to *B. subtilis* under oxic conditions could successfully be explained by an adsorption model, the irreversibility of the process under certain conditions indicated that precipitation may occur.

1.3.4.2.2. Bacterial species

As there are countless millions of different bacterial species, many of which cannot be cultured under laboratory conditions, a widely-applicable metal adsorption model relies upon similar adsorption characteristics regardless of the types of bacteria present. A number of studies have therefore sought to investigate potentially different adsorption behaviour displayed by different groups of bacteria.

An early study by Daughney et al. (1998) compared the thermodynamics of metal adsorption onto two bacterial species, *Bacillus subtilis* and *Bacillus licheniformis*.

Although the two species were found to have slightly different proton and metal adsorption characteristics, the large number of studies conducted since then have generally indicated that the site concentrations and stability constants for a wide range of different bacterial species fall within a relatively narrow range. Yee and Fein (2001) undertook proton and Cd adsorption studies using 7 different bacterial strains and found very similar adsorption behaviour. Proton and metal adsorption studies by Borrok et al. (2004a) and Johnson et al. (2007) using natural consortia from various environments found that, although the consortia generally adsorbed less metal than laboratory-cultured strains, there was little variation in adsorption between the different consortia. Figure 1.7 indicates the close correspondence between adsorption by a range of different bacterial consortia and the adsorption predicted using an averaged set of complexation parameters. However, a similar study conducted using bacterial consortia from contaminated environments (Borrok et al. 2004b) found a wide range of adsorption behaviour, indicating that adaptation may be an important factor controlling surface chemistry and metal adsorption characteristics.

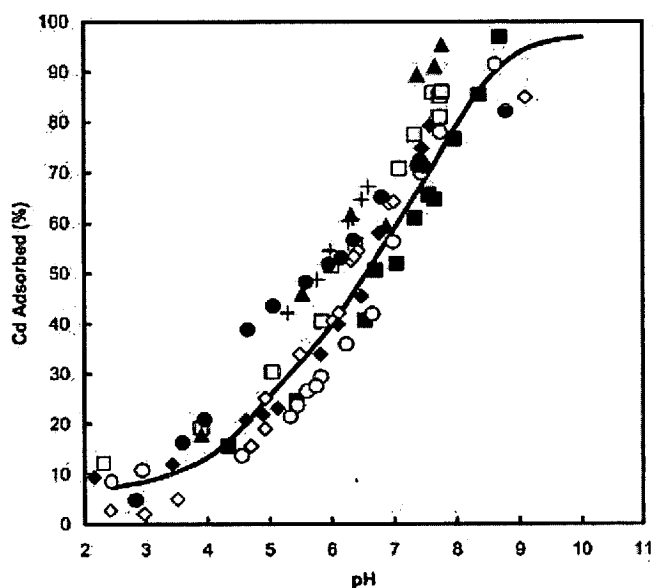


Figure 1.7: Cd adsorption onto natural bacterial consortia. Symbols indicate consortia from different sites, the solid line indicates predicted adsorption using 'averaged' complexation parameters (Borrok et al., 2004a).

Bacteria may be categorised as either gram-positive or gram-negative according to their cell wall structure (Section 1.3.1). Many proton and metal adsorption studies have focussed on gram-positive species, such as the well-characterised *Bacillus subtilis* (Daughney et al., 2001; Fein et al., 2001; Chatellier and Fortin, 2004; Gorman-Lewis et al., 2005; Wightman and Fein, 2005). Haas et al. (2004) investigated U(VI) adsorption to a gram-negative facultative anaerobe, *Shewanella putrefaciens*, and found that adsorption behaviour was similar to that of gram-positive strains. Ngwenya et al. (2003) undertook a study of Zn, Pb and Cu adsorption using a gram-negative *Enterobacter* species, and concluded that the adsorption parameters were not significantly different to those established for gram-positive strains.

Thermophilic bacterial strains are adapted to live in high-temperature environments such as hot springs or hydrothermal systems. As such environments are often metal-rich, studies have been undertaken to investigate the possibility that thermophiles may display significantly different proton and metal adsorption characteristics to mesophilic species. The recent studies by Burnett et al. (2006a; 2006b; 2007), and Heinrich et al. (2007), all conducted using the thermophile *Anoxybacillus flavithermus*, have found that the proton-active surface site concentration is slightly lower than comparable mesophiles (Burnett et al., 2006a), but metal adsorption characteristics are similar to those of non-thermophilic species (Burnett et al., 2006b). A study of Cd adsorption by Hetzer et al. (2006), conducted using the thermophiles *Geobacillus stearothermophilus* and *Geobacillus thermocatenulatus*, indicated that the majority of the Cd adsorbed was likely complexed by a different functional group to those most commonly indicated by surface complexation modelling of metal adsorption to mesophiles. However, generalisations cannot be made until further studies have been carried out with a wider range of thermophilic bacterial species.

1.3.4.2.3. Growth conditions and growth phase

A number of studies have carried out proton and/or metal adsorption investigations as a function of bacterial growth conditions. The approaches undertaken include varying nutrient availability (Borrok et al., 2004c; Haas, 2004; Guine et al., 2007), varying oxygen supply (Borrok et al., 2004c; Haas, 2004), and varying the nitrogen source and C:N ratio of the growth medium (Hong and Brown, 2006). Borrok et al. (2004c) found that Co adsorption by *Pseudomonas fluorescens* and *Shewanella oneidensis* was unaffected by growth conditions or oxygen concentration. However, whilst Haas (2004) found that the surface chemistry of *Shewanella putrefaciens* was unaffected by growth conditions, there were significantly different deprotonation constants and lower site concentrations when the cells were cultured anaerobically. The findings of Hong and Brown (2006) indicated that cell surface chemistry was sensitive to changes in growth medium composition. Specific surface sites on each of the two species studied were found to be affected by either nitrogen source or C:N ratio. The study by Guine et al. (2007) incorporated varying phosphate availability, and this was found to have a slight effect on Zn complexation. The 15 % decrease in Zn adsorption observed under P-limited conditions indicates at least some involvement of phosphate groups in Zn complexation. Eboigbodin et al. (2007) investigated the effect of glucose concentration on the surface properties of *E. coli*, concluding that the presence of glucose in the growth medium lead to significantly increased concentrations of amine and hydroxyl surface groups. This had the consequence of reducing aggregation caused by cell-to-cell interactions. Sharma et al. (2003) found that varying the energy source available to the chemolithotroph *Acidithiobacillus ferrooxidans* lead to variations in the isoelectric point, and therefore surface charge, of the cells. Jiang et al. (2004) found that ATR-FTIR analysis did not show any difference in surface properties of bacterial cells grown in two different growth media. However, this may be because both media compositions were nutrient rich and equally able to satisfy the cell requirements.

The effect of cell growth phase has also been investigated by various studies. Jiang et al. (2004) found no significant difference in bacterial infrared absorbance spectra as a function of growth phase and Haas (2004) found that growth phase had no significant effect on the surface chemistry of *S. putrefaciens*. However, other recent

studies have indicated that growth phase is a significant factor (Daughney et al., 2001; Guine et al., 2007). A study by Chang et al. (1997) found that Pb and Cd were optimally adsorbed by *Pseudomonas aeruginosa* at different growth stages, whereas Cu adsorption was unaffected by growth phase.

The issue of microbial adaptation to metal-contaminated environments has been addressed by Borrok et al. (2004b), who found that bacterial consortia from both natural and industrial contaminated environments displayed a wide range of proton and Cd adsorption characteristics, strongly suggesting that their reactivity is affected according to the environment they are adapted to live in.

1.3.4.2.4. Adsorption temperature

Establishing the effect of temperature variation on proton and metal adsorption is important to quantifying these processes in extreme environments, but is of particular significance to the development of bioremediation technologies. As yet, few studies have investigated this aspect of bacterial adsorption behaviour. Bulk Ni adsorption experiments conducted using an algal species as a function of temperature (Aksu et al., 2002) indicated increasing adsorption up to 45 °C. However, a study by Wightman et al. (2001) found that the protonation characteristics of *Bacillus subtilis* and the anaerobic thermophile TOR-39 did not change significantly over the temperature range 30 °C – 75 °C. These findings are supported by the conclusions of Gorman-Lewis et al. (2005), who conducted a titration calorimetry study using *Bacillus subtilis* and established reaction enthalpies that suggest differences in proton and Cd adsorption parameters over the temperature range 25 °C to 75 °C would be too small to detect using a batch experiment approach. The temperature-dependence of metal adsorption reactions may however depend upon whether the metal in question adsorbs to the cell surface as an inner- or outer-sphere complex. Takahashi et al. (2005) conducted experiments investigating the adsorption of rare earth elements onto the cell walls of *B. subtilis* and *E. coli* at temperatures of 4 °C, 24 °C and 37 °C. The observed temperature-dependent adsorption behaviour was thought to indicate the formation of inner-sphere complexes. As the release of the hydration shell of water molecules upon formation of an inner-sphere complex leads to an increase in

entropy, which is consistent with the increase in metal complexation at higher temperatures.

1.3.4.2.5. Bacterial extracellular polymeric substances (EPS)

Bacterial extracellular polymers are complex, heterogeneous substances commonly produced by cells for functions thought to include biofilm formation, protection from predation and stress resistance (Parikh and Madamwar, 2006). Kinzler et al. (2003) and Sand and Gehrke (2006) found that the EPS produced by the chemolithotrophic acidophiles *Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* served to enhance sulphide dissolution by facilitating cellular attachment to the sulphide surfaces, and by concentrating Fe(III) ions at the sulphide surface, thus increasing the oxidation rate. The production of EPS was also found to be important in bacterial adhesion to goethite (Omoike et al., 2004; Omoike and Chorover, 2006).

The production and composition of bacterial EPS is known to vary depending on the species of bacteria and factors such as growth medium and temperature (Omoike and Chorover, 2004; Noghabi et al., 2007). However, the composition is predominantly carbohydrates and proteins with lesser amounts of uronic and nucleic acids (Frolund et al., 1996; Comte et al., 2006a). Infrared analysis of bacterial EPS by ATR-FTIR reveals a 'fingerprint' spectral region qualitatively similar to that obtained for EPS-free bacterial cells (Omoike and Chorover, 2004). EPS may be described as either 'soluble' or 'bound'; generally, soluble EPS is easily removed by washing, whereas the bound fraction requires a stronger chemical or physical extraction method such as treatment with EDTA, formaldehyde, ion-exchange resin or sonication (Comte et al., 2006b). It was found by Comte et al. (2006a) that soluble EPS had a higher polysaccharide content than bound EPS. Also, Kives et al. (2006) found that there were differences in the polysaccharide compositions between EPS from planktonic and biofilm-associated *Pseudomonas fluorescens* cells. As polysaccharides are rich in organic functional groups, such variations in composition may potentially lead to differences in cell surface reactivity. A number of studies have investigated the proton and metal binding properties of EPS isolated from bacterial cells, biofilms or sludge flocs (McLean et al., 1990; Loaec et al., 1997; Salehizadeh and Shojaosadati,

2003; Lamelas et al., 2006; Quiroz et al., 2006; Noghabi et al., 2007). Guibaud et al., (2004) determined complexation constants by a polarographic method for Cu, Ni and Zn adsorption on EPS, and found that Zn complexation was minimal, whilst Cu and Ni were likely adsorbed by carboxylic functional groups present in the EPS. It was found by Guibaud et al. (2005b) that the same functional groups were present in EPS isolated from activated sludge as were present in EPS from pure cultures. However, the EPS from pure cultures had lower proportions of polysaccharides and uronic acids than those from activated sludge and were less efficient at metal sequestration. This difference in composition and metal-complexation behaviour may be an indication that adaptation due to prior metal exposure is an important factor controlling surface properties of bacterial EPS. The study by Comte et al. (2006c) established two pK_a values for EPS extracted from activated sludge by a number of different extraction protocols, and found that the EPS always had a greater affinity for Pb^{2+} than Cd^{2+} . However, the pK_a values and metal complexation capacity could be significantly affected by the extraction protocol used, particularly if chemical methods of EPS extraction were employed. This highlights an inherent difficulty associated with studying the properties of EPS. Lamelas et al., (2006) studied proton and Cd binding by isolated EPS, finding that the EPS had more Cd binding sites than the bacterial cell walls, but the stability constant for one of the two types of site was lower than those for bacterial cells.

Generally, the studies mentioned above have indicated that bacterial EPS has a similar reactivity towards protons and metals as the bacterial cell wall, and as EPS may be produced in large quantities depending on the conditions, the contribution to metal complexation is likely to be significant. As yet, no investigation into the cell surface reactivity of an EPS-producing bacterial strain has been undertaken.

1.3.5. Spectroscopic studies

1.3.5.1. Infrared adsorption studies (FTIR and ATR-FTIR)

Fourier transform infrared analysis (FTIR) and attenuated total reflectance Fourier transform infrared analysis (ATR-FTIR) have been used in a small number of recent studies as a means of identifying the functional groups involved in proton and metal binding at cell surfaces (Omoike and Chorover, 2004; Jiang et al., 2004; Dittrich and Sibler, 2006; Burnett et al., 2006b; Chen and Yang, 2006). The choice of ATR-FTIR over FTIR is advantageous for studies of biological samples as they may be analysed in their native state. The process of freeze-drying, grinding with KBr and pressing into a disc that is required for FTIR analysis may result in some important speciation changes within the bacterial samples. Jiang et al. (2004) conducted an ATR-FTIR study of bacterial cell surface chemistry as a function of pH, growth phase and growth medium. The bacterial ‘fingerprint’ spectral region between approximately 1750 cm^{-1} and 950 cm^{-1} (Figure 1.8) reveals peaks characteristic of amide, carboxyl, phosphoryl and carbohydrate groups, and as the protonated and anionic forms of a given group absorb at different positions within the electromagnetic spectrum, infrared analysis as a function of pH may be correlated with the deprotonation constants obtained from potentiometric titration data.

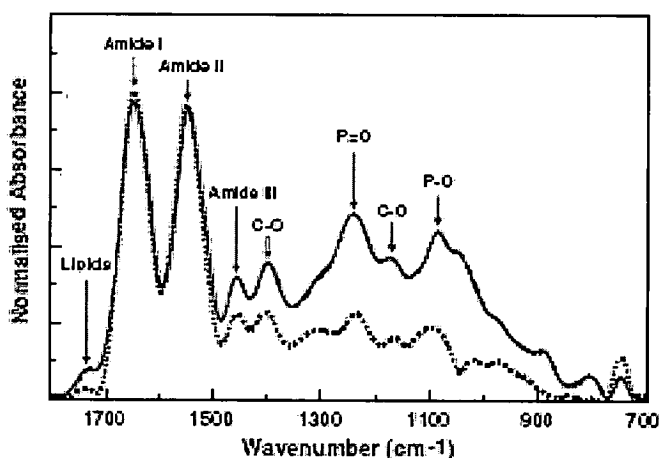


Figure 1.8: Bacterial ‘fingerprint’ spectral region obtained by FTIR analysis, with peak identities indicated. The dotted line represents the spectra obtained after Cd adsorption to the cells (Burnett et al., 2006b).

Jiang et al. (2004) observed changes in peak intensity as pH increased, indicating deprotonation of the corresponding functional groups. They found that IR spectra were not affected by varying either growth phase or growth medium, suggesting that neither of these variables significantly affects surface chemistry. Yee et al. (2004b) also used synchrotron radiation FTIR analysis to investigate the deprotonation of a cyanobacterial species, and observed that the peak corresponding to the carboxyl anion increased in intensity over the range pH 3.2- 6.5. Dittrich and Sibling (2006) found correspondence between the identities assigned to the functional groups identified by surface complexation modelling of titration data obtained from cyanobacterial suspensions, and the carboxyl, amide, phosphate, hydroxyl and carbohydrate peaks present in ATR-FTIR spectra. The study by Heinrich et al. (2007) compared surface complexation modelling of potentiometric titration data, obtained using the thermophilic bacterium *Anoxybacillus flavithermus*, with ATR-FTIR data. They were able to identify carboxyl and phosphodiester groups, but overlapping peaks in the bacterial spectra prevented identification of any other groups potentially active in proton binding. Burnett et al. (2006b) compared ATR-FTIR spectra of *A. flavithermus* both before and after exposure to Cd solution, but were unable to conclusively identify changes in the peaks due to Cd binding. However, Chen and Yang (2006) were able to infer Cu binding to both carboxyl and amine groups on the surface of an algal, rather than bacterial, species using FTIR spectroscopy. The results of these studies suggest that useful information can be obtained from IR analysis, but the large number of overlapping peaks within some regions of the bacterial spectrum, together with interference from potential conformational changes (Heinrich et al., 2007), and the possibility that the IR wave may penetrate cell interiors (Jiang et al., 2004), limits the resolution of this method. Specifically, it is often difficult to extract quantitative information regarding concentrations of reactive functional groups using this technique.

1.3.5.2. X-ray absorption fine structure spectroscopy (EXAFS) studies

Many recent attempts to accurately characterise the mechanisms involved in surface adsorption of metal ions to bacterial cells, specifically the identities of the functional groups involved, have focussed on the use of EXAFS. The typical experimental instrumentation for EXAFS analysis is shown in Figure 1.9.

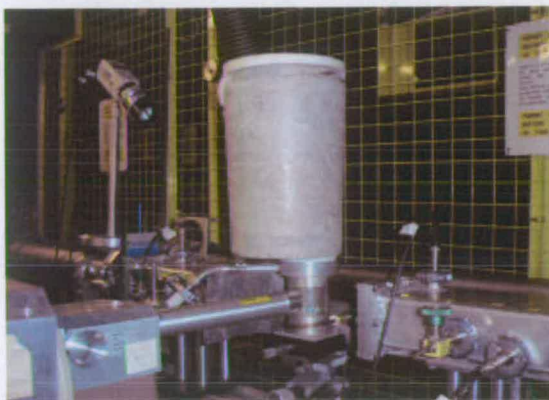


Figure 1.9: Experimental instrumentation for EXAFS analysis.

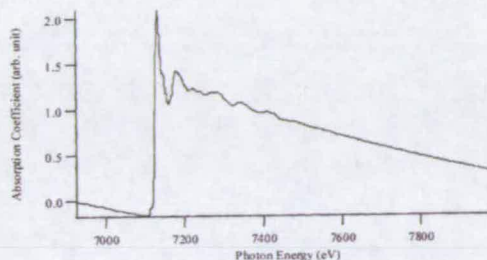


Figure 1.10: EXAFS spectrum of Fe₂O₃ (from Jiang, 2002).

During EXAFS analysis, a beam of high energy x-rays is directed at the sample. The energy of this incoming x-ray may be entirely transferred to the production of a photoelectron (a resonant absorption), for the incoming x-rays that are carrying the same energy as a bound core electron (Jiang 2002). The binding energies at which these core photoelectrons are ejected are characteristic of each element. The ejected photoelectron propagates as a wave which is then scattered by the neighbouring atoms, producing EXAFS oscillations. Figure 1.10 shows an example EXAFS spectrum for Fe₂O₃. The sharp jump in energy at approximately 7100 eV is known as the edge step, which results from attenuation of the incoming x-ray photon's energy and photoelectron production. The position of the edge step is unique to each element, as the core electron binding energies are different for each element. The region within approximately 50 eV of the edge step is known as the X-ray near edge structure (XANES), and the oscillations following this are the EXAFS part of the spectrum, produced by backscattering of emitted photoelectrons from surrounding atoms (Jiang 2002). Interpretation of EXAFS spectra enables identification of the

local binding environment, through constraining the interatomic bond distances and angles. In addition XANES analysis provides information regarding valence states.

A number of EXAFS studies have investigated binding of uranium to bacterial cell walls. Hennig et al. (2001) conducted a study of U(VI) complexation at the surfaces of *Bacillus cereus* and *Bacillus sphaericus*, concluding that binding was primarily to phosphoryl functional groups in the cell walls. Kelly et al. (2002) investigated UO_2^{2+} binding to *Bacillus subtilis* and found that binding was to phosphoryl groups at pH 1.67, but that carboxyl groups became increasingly important as pH increased to 4.80. Merroun et al. (2005) concluded that U(VI) binding to *Bacillus sphaericus* S-layer proteins and cell walls was to both carboxyl and phosphate groups at pH 4.5.

A study by Boyanov et al. (2003), investigating Cd binding to *Bacillus subtilis*, found that complexation was primarily to phosphoryl groups below pH 4.4, but increasingly to carboxyl groups at higher pH. In addition, a second phosphoryl-type site was thought to be involved at pH 7.8. However, Burnett et al. (2006b) found that Cd was bound only to carboxyl groups on the surfaces of *A. flavithermus*, at low metal:biomass ratios. The same conclusion was reached regarding Cu and Pb complexation. Sarret et al. (1998) and Toner et al. (2005) both conducted studies of Zn complexation, to fungal cell walls and a bacterial biofilm respectively. Both studies found that phosphoryl complexation was predominant, with a minor contribution from carboxyl ligands. Guine et al. (2006) identified phosphoester, carboxyl and sulfhydryl ligands in a study of Zn complexation by three different bacterial species.

Although the number of EXAFS studies conducted to date is small, the conclusions outlined above indicate that there is some contradiction between results of macroscopic studies and the spectroscopic studies. Specifically, macroscopic studies generally indicate that carboxyl-type ligands are predominantly involved in metal binding, even at low pH. However, the spectroscopic studies generally indicate that complexation is primarily to phosphoryl groups at low pH, with carboxyl complexation only becoming significant at circumneutral pH. Integrated studies employing both techniques are required in order to resolve this issue, especially as

the small number of studies conducted so far may not be ideally comparable in terms of biomass:metal ratios and the pH ranges chosen for analysis.

1.3.6. Predicting metal adsorption behaviour in complex systems

Establishing the applicability of metal adsorption characteristics obtained for laboratory cultures of bacteria to complex geochemical environments presents a real challenge, but this must be addressed in order to accurately predict and quantify microbial contributions to geochemical cycles (Fein, 2000; 2006).

The similarity of stability constants obtained from multi-metal systems to constants obtained for the same metals in single-metal systems is encouraging (Burnett et al., 2007), and studies conducted using bacterial consortia also indicate that the range of adsorption behaviour displayed by different species is relatively small (Borrok et al., 2004a; 2004b; Johnson et al., 2007). Both of these findings suggest that laboratory-derived stability constants may be applicable to more complex settings. The linear-free-energy approach developed by Fein et al. (2001) is a means of predicting metal adsorption behaviour based on the correlation between the metal-bacteria stability constants and metal-organic ligand stability constants. Whilst this approach works well in some cases (Johnson et al., 2007), the study by Gorman-Lewis et al. (2005) using Np suggests that the linear-free-energy approach is not applicable to all metal species.

Natural systems comprise many components other than metal ions and bacterial cells. A small number of studies have investigated the adsorption of organic contaminants to bacterial cell walls. Ju et al. (1997) found that lindane adsorption to a selection of gram-positive and gram-negative strains increased with increasing ionic strength and decreased with increasing pH, due to electrostatic repulsion between the negatively-charged cells and the lindane. MacLean et al. (2004) concluded that iodide was adsorbed to a single positively-charged site on the *B. subtilis* cell wall. The extent of adsorption decreased with increasing pH, but decreased with increasing ionic strength. This was taken as an indication that iodide is adsorbed as an outer-sphere complex. As concluded by MacLean et al. (2004), although the direct adsorption of such compounds may be limited under

environmental pH, significant complexation may occur by means of bridging mechanisms involving metal cations. Thus, Xiao et al. (2007) observed that adsorption of phenanthrene and tetrachlorobenzene to *E. coli* was significantly increased in the presence of metal cations, supporting the bridging mechanism hypothesis. This is an example of the formation of Type A ternary surface complexes, in which a metal ion forms a bridge between two negatively charged ligands or surfaces. Type B ternary surface complexes involve an organic ligand acting as a bridge between two neutral or positively charged surfaces or ligands (Ngwenya, 2007). The formation of these cell-metal-ligand ternary complexes may be significant in many environmental situations (Wightman and Fein, 2001; Borrok et al., 2007).

Most natural environments will contain substantial amounts of heterogeneous organic matter such as humic and fulvic acids. As these are known to be rich in functional groups (particularly carboxylic and phenolic) and active in proton and metal complexation, it is important to understand how they interact with and compete with bacterial cell surfaces. Fein et al. (1999) found that humic acid adsorbed strongly to *B. subtilis* cell surfaces at low pH, by a hydrophobic mechanism that could be accurately predicted using a surface complexation modelling approach. Because of these interactions, the formation of ternary complexes between bacteria, metals and organic acids must also be accounted for in natural systems. It was found by Wightman and Fein (2001) that there was no competition between Cd and humic substances for adsorption sites on *B. subtilis*, but that a soluble fraction of the humic acid may act as a competing ligand for Cd, forming solution complexes. Borrok and Fein (2004) used a chemical equilibrium approach to predict proton and Cd partitioning between bacteria and humic substances. They found that although humic substances have a higher concentration of binding sites and a greater affinity for Cd than cell surfaces, both types of complex are likely to be environmentally significant. An investigation by Borrok et al. (2007) found that bacteria-metal-organic matter complexes are stable at circumneutral pH, suggesting that such complexes are common in many environments. Furthermore, this type of complexation was found to increase Cu and Ni adsorption by 35-40 % at

circumneutral pH, which could have important implications for predictions of metal cycling in natural settings.

The bacteria themselves may act as a source of additional organic matter, either by producing EPS, or through the dissolved organic carbon (DOC) released upon cell lysis (Ngwenya, 2007). Bacterial extracellular polymers may complex metal ions in the subsurface, which may significantly enhance metal transport if the EPS is of the soluble fraction (Chen et al., 1995; Czajka et al., 1997; Jensen-Spaulding et al., 2004). However, bound, particularly biofilm-associated EPS, may have the effect of removing and sequestering metal ions from solution. Zn complexation by a bacterial biofilm has been observed by Toner et al. (2005; 2006) and the longer-term fate of such complexed metals is as yet unknown and merits further study.

Investigation of a model multi-component system, which contained bacteria, fungi, mineral phases and peat (Ledin et al., 1999), indicated that the bacteria were responsible for complexation of up to 38 % of the available metal, despite comprising a relatively small proportion of the system. Few studies as yet have attempted to apply the adsorption constants obtained by laboratory studies to real environmental settings. However, Daughney et al. (2002) were able to use the results of Hg-bacteria surface complexation modelling to predict the distribution of Hg in a number of acidic lakes and wetlands in Canada. The laboratory-derived parameters provided reasonably good fits to the field data, provided that the model was expanded to include Hg complexation by organic matter.

1.3.7. Biomineralisation studies

As mentioned in Section 1.1, the term ‘biomineralisation’ encompasses a number of different processes. Broadly, they may be categorised as ‘biologically controlled mineralisation’ (Bazylinski and Frankel, 2003) or ‘biologically induced mineralisation’ (Frankel and Bazylinski, 2003). The first category encompasses intracellular mineralisation, which generally occurs at specific locations and under certain conditions. The formation of magnetosomes by magnetotactic bacteria is an example of biologically controlled mineralisation, and occurs as a means for the bacteria to orientate themselves within the Earth’s magnetic field (Bazylinski and

Frankel, 2003). Examples of extracellular biologically controlled mineralisation include the formation of coccolith plates, mineral shells and tests by organisms such as foraminifera and molluscs (De Yoreo and Vekilov, 2003). Organisms exert thermodynamic control over crystal nucleation and growth during biologically controlled mineralisation (Bazylinski and Frankel, 2003).

Biologically induced mineralisation covers all the extracellular, less well defined mineralisation processes, including precipitation on the cell wall, precipitation due to microbial redox and other metabolically-related mineralisation. For example, the S-layers formed on some bacterial surfaces may act as a 'template' for mineralisation. Schultze-Lam et al. (1992) observed the formation of fine-grained gypsum and calcite on the S-layer of a cyanobacterial strain, *Synechococcus* GL24. This is a passive process, over which the cell has no control, and it may or may not provide any advantage to the microbe. It has been suggested that promotion of mineralisation helps prevent cell death by entombment, that precipitation removes toxic metals from the environment, or that chemotropic bacteria may promote mineral precipitation as a means of facilitating energy generation (Ehrlich, 1996; Fortin 2003). A full understanding of the mechanisms involved in microbial mineralisation will help to illuminate whether or not microbes gain any advantage from the process.

This discussion of recent research into biomineralisation will focus on those studies concerned with microbially-induced mineral precipitation, i.e. precipitation on or in close proximity to the bacterial cell wall. Studies have investigated biologically induced precipitation of a range of minerals, including ores such as gold (Watterson, 1991; Karthikeyan and Beveridge, 2002; Reith et al., 2006), and uraninite (Min et al., 2005). However, the majority of studies have looked at the abundant, and therefore geologically important, minerals that can be categorised as either carbonates, iron oxides or silicates. Each of these groups will be discussed below.

1.3.7.1. Carbonate minerals

Biogenic carbonate minerals are found in a range of natural forms and in many different environments. Microbialites (microbial carbonate deposits) have been described from both alkaline lakes (Arp et al., 1999; Dupraz et al., 2004) and Hawaiian sea caves (Leveille et al., 2000). Dupraz et al. (2004) observed that the micrite precipitation in microbialites from the Bahamas initiated within the extracellular-polymer biofilm surrounding the microbial cells. Leveille et al. (2000) also concluded that metal ions complexed from solution by the EPS provided the nucleation sites for microbialite mineralisation in Hawaiian sea caves. However, Arp et al., (1999) investigated microbialite precipitation in the alkaline Pyramid Lake, Nevada, and concluded that the biofilm EPS inhibited precipitation by acting as a Ca^{2+} buffer, and that mineralisation consequently initiated in EPS-free regions.

Carbonate precipitation is closely tied to the microbial metabolism, as the balance of photosynthesis and respiration influences the pH and carbonate saturation of the surrounding environment. Dupraz and Visscher (2005) describe how this, together with the effects of EPS production, are the predominant factors controlling microbialite precipitation. The resulting microbial carbonates are widespread and constitute an important component of the global carbon cycle. They also preserve valuable information concerning geochemical conditions. For example, Andres et al., (2006) describe carbon isotope fractionation in Bahamian stromatolites, which acts as a record of dissolved organic carbon (DIC) in the surrounding environment. Atmospheric carbon dioxide levels were inferred from mesoproterozoic stromatolites (Kah and Riding, 2007). Microbes are known to thrive in conditions inhospitable to other forms of life, and the biominerals formed in association with the cells can act as a record of past life. For example, Chafetz and Guidry (1999) describe bacterial shrubs from hot-water travertine deposits, and Tazaki et al., (2006) observed the formation of carbonate pisoliths around filamentous microbes living in hot springs. Hydrozincite was observed to precipitate in a contaminated stream, the Rio Naracauli, Italy, as a consequence of a microbially-induced pH increase and bicarbonate concentration decrease (Podda et al., 2000; Zuddas and Podda, 2005). This mineralisation proves environmentally beneficial as a wide range of heavy metals are removed from the stream by incorporation into the hydrozincites.

Whilst these studies illustrate the geological and environmental significance of microbial carbonate deposits, it is also important to establish the mechanisms underlying precipitation of these minerals. A number of studies have conducted laboratory investigations into carbonate precipitation in the presence of bacterial cells or microbial extracellular polymers. Kawaguchi and Decho (2002) investigated the effect of cyanobacterial EPS extracted from Bahamian stromatolites on CaCO_3 polymorphism. They found that EPS from the unlithified layers of the stromatolites had higher uronic acid and carbohydrate contents than the EPS from lithified layers, but similar protein contents. The EPS from the lithified layer was found to induce aragonite precipitation whereas EPS from the unlithified layer induced calcite formation, an effect believed to be due to the differences in biochemical composition of the two types of EPS. Rivadeneyra et al. (1998) investigated carbonate precipitation by strains of the halophile *Halomonas eurihalina*, as function of medium type and incubation temperature. Precipitation was observed by at least some strains under all the experimental conditions, with crystal quantity and size decreasing as the salt content of the medium increased. Crystallisation was observed to take place in a sequence beginning with filaments, then discs were observed, then eventually spherulites were formed. Aragonite mineralisation was favoured over calcite in liquid media. It was suggested that the precipitation mechanism involved adsorption of Ca or Mg ions to the cell wall, and mineralisation owing to the locally increased concentration of these ions. Rivadeneyra et al. (2006) studied precipitation of carbonate and phosphate by the halophile *Chromohalobacter marismortui*, as a function of medium type. Most of the precipitates formed had small crystals and general low crystallinity, and the mineralogy was found to vary according to medium composition. At high salt concentrations in liquid media vaterite and monohydrate were produced in addition to aragonite and struvite, whereas in solid media Mg-calcite and a phase similar to kutnahorite were formed. The composition and mineralogy of the precipitates produced by this strain were found to be different to those of biominerals produced by other halophiles in the same media. No precipitation was observed in cultures inoculated with heat-killed cells. Rodriguez-Navarro et al. (2007) conducted a study into the precipitation of vaterite by the soil bacterium *Myxococcus xanthus*. It was concluded that precipitation of vaterite, the

least stable CaCO_3 phase, occurs because the organic molecules present in the culture medium or produced by the bacterial cells (EPS for example) inhibit the transformation to more stable aragonite or calcite. Incorporation of organics into the vaterite precipitates stabilises them. Figure 1.11 (Rodriguez-Navarro et al., 2007) shows examples of vaterite spherules precipitated in association with *Myxococcus xanthus* cells, which are themselves calcified.

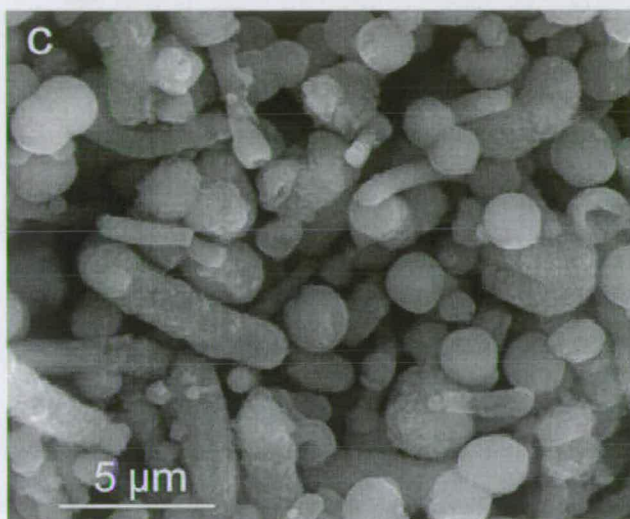


Figure 1.11: FESEM image of calcified *Myxococcus xanthus* cells and vaterite spherulites (Rodriguez-Navarro et al., 2007).

Warren et al. (2001) found that microbial CaCO_3 precipitation had the effect of removing Sr by solid phase capture, as the Sr was incorporated into the carbonate structures. However, UO_2 capture was lower and reversible, and capture of Cu was very low. Bacterial degradation of urea was found to induce rapid biomineralisation, and it was hypothesised that the cell surfaces act as nucleation templates for crystal growth. Mitchell and Ferris (2006) observed that urea hydrolysis by *Bacillus pasteurii* resulted in the production of ammonium, which increased the pH of the experimental system. This led to precipitation of rhombohedral calcite crystals in both the biotic and abiotic sectors of the microcosm, indicating that cell surfaces are not controlling mineralogy or morphology. However, the crystals in the biotic section were larger and had a wider size range than those in the bacteria-free section.

Dick et al. (2006) also investigated ureolysis-induced CaCO_3 precipitation on limestone by a range of *Bacillus* species. It was found that the ability to deposit CaCO_3 varied considerably between strains, with the most effective strains being characterised by high ureolytic efficiency and very negative zeta potentials. This was a consequence of easier adhesion due to the positive zeta potential associated with the limestone substrate. Stocks-Fischer et al. (1999) found that *Bacillus pasteurii* acted as a nucleation site for CaCO_3 precipitation, as cells were found to be encapsulated by CaCO_3 , and that precipitation in the presence of cells was considerably faster than abiotic precipitation. CaCO_3 precipitation was induced by the production of ammonia and consequent pH increase, due to the use of urea as a nitrogen source by *B. pasteurii*. The study by Lian et al. (2006) investigated carbonate mineralisation induced by *Bacillus megaterium*. It was found that the cells induced precipitation of calcite, but that when the supernatant centrifuged from the growth suspensions was added instead of cells, vaterite precipitation occurred. Braissant et al. (2003) investigated CaCO_3 precipitation in the presence of xanthan (purified EPS) and amino acids. It was found that sphere formation in both calcite and vaterite was enhanced by increasing concentrations of amino acids and xanthan. The relative proportions of calcite and vaterite produced differed according to the bacterial strain present, and in abiotic experiments was related to the amounts of EPS and amino acids added.

1.3.7.2. Iron oxides

Biogenic iron oxides can form as a consequence of the microbial metabolism, when bacteria oxidise Fe^{2+} to Fe^{3+} under oxic conditions. This leads to mineralisation in close proximity to the cells. Such biologically-induced precipitates, which are generally amorphous and nanocrystalline, may also form under anoxic conditions due to bacteria using Fe^{2+} as an electron donor and consequently forming Fe^{3+} .

A number of studies have been conducted with the aim of characterising the structures and mineralogy of biogenic iron oxides. The bacterial species *Gallionella* has been found to precipitate hematite on the stalk surfaces, which subsequently accretes secondary ferrihydrite and goethite (Hallberg and Ferris, 2004). Chatellier

et al. (2004) investigated the iron oxides formed by Fe^{2+} oxidation, as a function of bacterial species and various inorganic ligands. It was found that the bacterial cells did not affect the mineralogy of the lepidocrocite precipitates, but that the particle size was reduced and the morphology altered. Figure 1.12 (Fortin and Langley, 2005) shows an example of lepidocrocite nanocrystals, formed during Fe^{2+} oxidation, on the cell wall of *Bacillus subtilis*. Chan et al. (2004) found that mine waters contained iron oxide precipitates at the centre of which were elongated akagenite pseudo-crystals. It was shown that the akagenite forms around exopolysaccharide fibres extruded by the microbes. Additionally, the negatively-charged functional groups present in bacterial cell walls can encourage accumulation of pre-formed mineral precipitates, which tend to have a positive surface charge, from their surroundings (Glasauer et al., 2001).

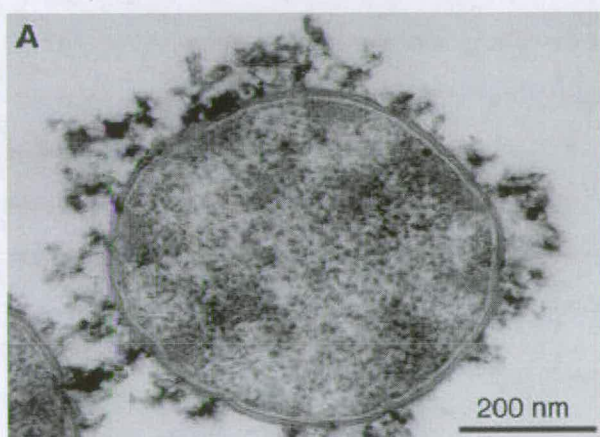


Figure 1.12: TEM micrograph of lepidocrocite mineralisation on the cell wall of *Bacillus subtilis* (Fortin and Langley, 2005).

Warren and Ferris (1998) investigated the mechanism of iron precipitation on bacterial surfaces, concluding that Fe surface adsorption, nucleation and precipitation occur as a continuum. However, Rancourt et al. (2005) found that Fe adsorbed to bacterial cell wall functional groups could not be incorporated into Fe^{3+} -oxide precipitates, owing to reduction by the cell wall to Fe^{2+} and an incompatible

coordination environment. The exact mechanisms by which iron oxide minerals form on bacterial cell surfaces therefore remain a subject of debate.

Microbial cells may become completely encapsulated and 'fossilised' by iron oxide precipitates (Fortin and Langley, 2005), thus leading to their preservation on geological timescales. Such bacterial microfossils are an important biomarker indicating the presence of life in ancient or inhospitable environments, as are bacterial intracellular magnetosomes, consisting of crystalline magnetite or goethite (example, Figure 1.13). Extracellular, or biologically-induced, magnetite may also form as a by-product of Fe^{3+} oxidation by dissimilatory iron-reducing bacteria under anoxic conditions. The Fe^{2+} produced by the reduction reaction subsequently reacts with Fe^{3+} to form magnetite. These bacterially induced magnetite precipitates are also recognised in ancient sediments (Konhauser, 1997, 1998). The geological importance of biogenic iron precipitation is still uncertain, but it is thought that there may have been a significant biological contribution to the mineralisation of the Precambrian banded iron formations (Konhauser and Ferris, 1996; Brown et al., 1999; Konhauser et al., 2002; Fortin and Langley 2005).



Figure 1.13: TEM image of chains of magnetosomes within a magnetotactic bacterium (Bazylinski and Frankel, 2003).

The use of biogenic iron precipitates as indicators of palaeoenvironmental conditions has also been investigated. Mortimer et al. (1997) found that the composition of biogenic siderite precipitated in the presence of *Geobacter metallireducens*, did not

reflect the composition of the solution from which it precipitated, but that Mn and Ca concentrations were inversely proportional to the rate of microbial activity. This indicates that iron oxides precipitated under the influence of microbial populations do not directly reflect the chemical conditions under which they were formed. Jones and Renaut (2007) observed that mineralised microbes could be found in the goethite precipitates from an acidic hot spring, but not in the lepidocrocite or jarosite. This was thought to be a consequence of differing environmental conditions under which the three minerals precipitate.

Evidence of a biological contribution to iron oxide minerals in a range of different environments have been found by a number of recent field studies. Little et al. (2004) studied iron oxide deposits from deep-sea vents and found filamentous textures similar to those precipitated by iron-oxidising bacteria. Some deposits appeared to have formed around bacterial cells. These structures were also observed in jaspers dating back 490 ma and could therefore potentially be used as a biomarker. Kennedy et al. (2003) also studied biogenic iron oxides from oceanic hydrothermal vents. The oxides were found to contain abundant bacterial organic matter, and it was hypothesised that the bacteria influence iron oxide precipitation by increasing the rate of Fe^{2+} oxidation, and by providing nucleation sites, hence lowering the concentration of Fe^{3+} required for precipitation to occur. Kasama and Murakami (2001) found that Fe-stalactites precipitated approximately 4 times faster in the presence of microorganisms, compared to abiotic precipitation rates. It was thought that this was due to microbial exopolysaccharides and surface properties rather than the microbial metabolism.

The amorphous, poorly-crystalline nature of biogenic iron oxides, together with the large proportion of organic material they often contain, enables such deposits to sequester large concentrations of other metal cations from their surrounding environment. Ferris et al. (1999; 2000) studied biogenic iron oxides forming in underground caves, and found that they were composites of iron oxides and bacterial organic matter. These iron oxides were shown to incorporate large quantities of other metals in their structures, possibly due to adsorption of metals to the negatively charged surface groups of the organic fraction. A study by Leblanc et al. (1996) found that bacterial iron-oxide stromatolites in a polluted stream were able to

sequester large amounts of arsenic in their structures, leading to a 2-3 order of magnitude reduction of As concentrations downstream. Iron oxides from hydrothermal systems may contain high trace metal concentrations within their structures (Kennedy et al., 2003).

1.3.7.3. Silicate minerals

Silicate mineralisation by microbes is well-known to occur in geothermal environments such as hot springs, and can result in the geological preservation of cells that have become completely encrusted by mineral precipitates. Konhauser and Ferris (1996) examined microbial mats from Icelandic hot springs, and found that the cyanobacterial cells were completely encapsulated by silica and/or iron precipitates, thus greatly enhancing their preservation potential. Figure 1.14 (Phoenix et al., 2000) shows an amorphous silica crust forming on the surface of the outer sheath surrounding the cyanobacterium *Calothrix*, an isolate from an Icelandic hot spring. This is of particular interest as some of the earliest examples of fossilised microbes are believed to have lived in conditions similar to those found in hot spring environments (Konhauser and Ferris, 1996; Phoenix et al., 2001). Studies investigating the mechanism of silicate precipitation on cell surfaces have generally concluded that metal cations adsorbed to cell wall functional groups are instrumental in silicate biomineral formation, as aqueous Si is subsequently bound to the metal ions rather than directly to the cell walls. These biogenic minerals are often iron-silicate composites that nucleate on the cell wall after Fe is adsorbed to surface functional groups (Konhauser, 1998). Urrutia and Beveridge (1994), Fein et al. (2002) and Yee et al. (2003) carried out laboratory studies that indicated that silicate minerals on cell surfaces form only in the presence of metal ions. Aqueous Si showed little affinity for the cell walls in the absence of metal cations. Yee et al. (2003) proposed that the cells may play an important role in facilitating aggregation of Si precipitates, but that they are not essential as nucleation surfaces. The binding of metal ions to the cell wall may contribute to cell preservation by mechanisms other than facilitating mineralisation. A study by Ferris et al. (1988) showed that encapsulation by silica alone did not inhibit cell lysis, but that the presence of metal

ions in the precipitates did, presumably because the autolytic enzymes are inhibited by metal ions.



Figure 1.14: TEM image of a silica crust (indicated by large arrow) forming on the sheath (indicated by small arrow) of a cyanobacterial filament. Scale bar = $2\mu\text{m}$ (Phoenix et al., 2000).

It is unclear whether or not encapsulation in silica provides any advantage to the cells, but it was found by Phoenix et al. (2001) that iron-silica precipitates confer significant UV resistance, and it was proposed that this may have been an important factor enabling colonisation of shallow-water environments by bacteria during the Precambrian. An earlier study (Phoenix et al., 2000) had established that mineralised cyanobacteria were able to photosynthesise at comparable rates to unmineralised populations.

Silicate biomineralisation is not limited to geothermal environments. Silicate-rich clay minerals have also been observed to form in close association with bacterial cell

surfaces. Kawano and Tomita (2001) observed amorphous Fe-containing silica and smectite forming on the surfaces of bacterial cells isolated from weathered volcanic ash. Clay-like phases were also found to occur on the surfaces of bacteria from Brazilian river water (Konhauser et al. 1993). Although these minerals may have nucleated on the cell surfaces, the final mineral compositions were controlled by the composition of the river water, and did not differ between bacterial species. Silicate precipitates are also known to form on the surfaces of the acidophile *Acidithiobacillus ferrooxidans*, an iron-oxidising bacterial species common to mine drainage waters. Fortin and Beveridge (1997) found that *A. ferrooxidans* promoted silicate precipitation at pH values as low as 2.2, whereas abiotic precipitation under the same conditions did not occur.

1.3.8. Summary

The results of numerous proton and metal adsorption studies have greatly increased understanding of the adsorption mechanism, and of the effects that different experimental parameters have on adsorption characteristics. However, macroscopic and spectroscopic studies have not as yet provided a definitive description of the adsorption mechanism. Specifically, uncertainty remains as to the identities of the functional groups involved in metal complexation (Sarret et al., 1998; Kelly et al., 2002; Boyanov et al., 2003; Chatellier et al., 2004; Guine et al., 2006). In addition, the effects of cell surface electrostatic interactions on proton and metal complexation are not adequately accounted for by surface complexation models (Borrok and Fein, 2005; Fein et al., 2005).

The available evidence generally shows that variations in bacterial species do not significantly affect adsorption behaviour (Yee and Fein, 2001; Ngwenya et al., 2003; Borrok et al., 2004a). However, it seems that varying growth conditions, or bacterial adaptation to extreme or perturbed environments, may lead to observable differences in cell surface chemistry and hence adsorption characteristics (Daughney et al., 2001; Borrok et al., 2004b; 2004c; Hong and Brown, 2006; Guine et al., 2007). The influence of extracellular polymeric substances (EPS) on surface reactivity is an aspect of this question, as EPS may be produced in response to variations in growth

conditions. The production of EPS by bacterial cells is commonplace, particularly in biofilms, which are comprised largely of EPS. These polymers may also be produced by planktonic cells, and may be soluble, contributing to the dissolved organic carbon component in a system. Soluble organic matter contains fractions that may be highly significant complexing agents of dissolved species.

Consequently, the production of EPS by bacterial cells may affect the transportation and bioavailability of metals in aqueous environments (Czajka et al., 1997; Jensen-Spaulding et al., 2004). A number of recent studies have attempted to characterise the proton and metal binding properties of isolated EPS (Loaec et al., 1997; Guibaud et al., 2004; 2005a; Comte et al., 2006a; 2006c). However, the effects of an EPS layer on the surface properties of bacterial cells is a relatively unexplored area of research.

As extremophilic species such as acidophiles or halophiles are adapted to extreme growth conditions, they may have significantly different surface chemistry and metal adsorption behaviour compared to mesophiles. Study of such bacterial strains has been limited as they are often difficult to culture under laboratory conditions, but further investigation is necessary in order to understand the contribution made by microbes to metal cycling in extreme environments.

Predictive metal adsorption models should also consider the competing effects of other subsurface and aqueous components, particularly organic matter such as humic acids and the dissolved organic carbon potentially produced by the bacterial cells themselves. It is also important to consider ternary interactions, particularly when attempting to predict the transportation behaviour of organic contaminants.

The mechanism of mineral precipitation on cell surfaces is not well understood, and it has not yet been established whether or not metal adsorption and precipitation is a continuous process (Warren and Ferris, 1998; Glasauer et al., 2001; Rancourt et al., 2005).

1.4. Project Objectives and Research Approach

This project was undertaken with the intention of investigating the surface chemistry and metal adsorption characteristics of an extremophilic bacterial species, as extremophiles may have significantly different surface chemistry to mesophiles. The thermophile *Bacillus licheniformis* S-86, which has an optimum growth temperature of 55 °C, was chosen. In addition, this species produces extracellular polymers, thus enabling investigation of the effect of EPS on surface chemistry, metal adsorption and calcium carbonate precipitation. As the presence of an EPS layer significantly alters the cell surface environment, this enables investigation of the electrostatic interactions at the cell surface when an EPS layer is present. The use of this species therefore enabled investigations of several poorly-understood aspects of this research area, as outlined in Section 1.2 and Section 1.3.8.

The study incorporated a number of specific objectives aimed at investigating the contribution made by EPS to cell surface reactivity, metal adsorption characteristics and microbially-induced mineralisation;

1. *To characterise and quantify the contribution made by extracellular polymers (EPS) to bacterial cell surface chemistry and electrostatic properties;*
2. *To conduct an integrated macroscopic and spectroscopic study of metal adsorption to an EPS-producing, thermophilic bacterial strain;*
3. *To investigate the effect of elevated adsorption temperature on metal adsorption by a thermophilic EPS-producing bacterial strain;*
4. *To investigate if carbonate mineral precipitation on the surfaces of cells is affected by the presence of EPS, and whether bacterial cells affect the morphology and crystal structure of the carbonates.*

Many recent studies in this area have used combinations of the different approaches outlined above in order to attain a consistent mechanistic explanation of proton and metal binding to bacterial cell surfaces. During this study, the approaches undertaken in order to attain the research objectives incorporated emerging experimental methods in this field of research as well as established techniques as

part of an integrated approach towards obtaining consistent mechanistic models of cell surface adsorption and mineralisation.

Cell surface characterisation incorporated use of potentiometric titrations, infrared spectroscopy (FTIR and ATR-FTIR) and electrophoretic mobility analysis. In addition, the cells were characterised by SEM and TEM. Investigation of the metal adsorption mechanism was undertaken by both macroscopic batch experiments and EXAFS analysis. The final aspect of the project involved biomineralisation experiments, and the morphologies and mineralogies were characterised by SEM and XRD respectively. The thesis reports the findings of this project as follows:

Chapter 2: The effect of extracellular polymers (EPS) on the proton adsorption characteristics of the thermophile Bacillus licheniformis S-86.

This chapter describes the results of analysis undertaken to characterise the surface chemistry of the gram-positive, EPS-producing, thermophilic bacterial strain *Bacillus licheniformis* S-86. In order to determine the effect of the EPS layer on surface chemistry, all analysis was undertaken comparing EPS-covered (native) cells with EPS-free cells that had been treated with cationic exchange resin in order to remove the EPS layer. Discrete-site surface complexation modelling of potentiometric titration data was undertaken using a nonelectrostatic approach. Infrared analysis of native and EPS-free cells, as a function of pH, was undertaken using both FTIR with KBr pellets, and ATR-FTIR, in which the cells are analysed as wet pastes. In addition, solution dissolved organic carbon concentrations were determined in order to compare DOC release by native cells with that by EPS-free cells. The results in this chapter formed the basis of Tourney et al. (2008).

Chapter 3: Investigating the electrostatic interactions at cell surfaces of an extracellular polymer (EPS)-producing bacterial strain.

The production of EPS significantly changes the electrolyte-cell surface environment, therefore it was decided to investigate the effect of an EPS layer on the electrophoretic mobility of the bacterial cells. In addition, electrostatic modelling of

data from potentiometric titrations conducted at 0.01M and 0.1 M ionic strength was undertaken using FITMOD, which incorporates the Donnan electrostatic correction. This approach was used to test the ability of the Donnan core-shell model to account for the effects of EPS on the cell surface environment. As the EPS layer thickness and hence Donnan thickness could not be measured by electron microscopy, an approach was adopted whereby the Donnan thickness was manually varied in order to investigate whether the deprotonation constants and site concentrations for the native cells would converge to optimal values.

Chapter 4: A macroscopic and spectroscopic study of Zn adsorption by an extracellular polymer (EPS)-producing bacterial strain, Bacillus licheniformis S-86.

The effects of EPS production on the mechanisms of Zn complexation by *B. licheniformis* S-86 were investigated by means of an integrated approach combining both macroscopic batch experiments and spectroscopic analysis. Batch adsorption experiments were undertaken in order to compare the metal adsorption capacity of the native cells with that of the EPS-free cells, thus indicating whether or not the production of EPS confers any additional metal adsorption ability. Modelling of the metal adsorption data using FITEQL 4.0 then enabled identification of the surface functional groups involved in Zn binding, and of the complexation constants for each functional group. Spectroscopic (EXAFS) analysis was carried out in order to constrain the mechanism of Zn adsorption to both native and EPS-free cells.

Chapter 5: The effect of temperature variation on Zn adsorption by an extracellular polymer (EPS)-producing thermophilic strain.

The effect of varying adsorption temperature on metal complexation by bacterial cells was investigated, as this aspect of metal adsorption has not received extensive study. The experiments were undertaken using an EPS-producing bacterial strain, *Bacillus licheniformis* S-86, which may show temperature-dependent adaptations to its surface chemistry compared to non EPS-producing species. Batch adsorption experiments were undertaken using Zn, at temperatures of 40 °C and 50 °C. The

data obtained from these macroscopic adsorption experiments was used to determine whether Zn sorption capacity varies with temperature, and whether subsequent surface complexation modelling of the adsorption data would show any temperature-dependent variation in complexation constants. This approach provided further constraints on the adsorption mechanism as temperature-dependent adsorption indicates inner-sphere complexation, whereas a lack of temperature dependence is indicative of outer-sphere complexation.

Chapter 6: The effect of bacterial cells and extracellular polymers on CaCO₃ mineralogy and morphology.

The results of an investigation into the effect of extracellular polymers on bacterially-influenced precipitation of calcium carbonate are reported in Chapter 6. Mineralisation experiments were undertaken using the ammonium free-drift method, and solutions were prepared containing native cells, EPS-free cells and the EPS solution extracted from the bacterial cells using cationic exchange resin. The control experiment contained no biological additives. Sampling was undertaken at intervals throughout the 7-day experimental duration. Crystal morphology was examined using scanning electron microscopy (SEM) and mineralogy was determined by X-ray diffraction (XRD). This approach enables identification of any differences in calcium carbonate morphology or mineralogy related to the presence of bacterial cells, cells with an EPS layer or extracted EPS in the experimental solution.

Chapter 7: General conclusions and synthesis.

This chapter summarises the main conclusions of this study, and explains the importance of these findings to the broader aim of characterising the role of EPS in complex environmental systems. Potential objectives for future research are also outlined.

1.5. References

- Abdelouas, A., Grambow, B., Fattahi, M., Andres, Y., Leclerc-Cessac, E. (2005) Microbial reduction of ^{99}Tc in organic matter-rich soils. *Science of the Total Environment*, 336, 255-268
- Aksu, Z. (2002) Determination of the equilibrium, kinetic and thermodynamic parameters of the batch biosorption of nickel(II) ions onto *Chlorella vulgaris*. *Process Biochemistry*, 38, 89-99
- Andres, M.S., Sumner, D.Y., Reid, R.P., Swart, P.K. (2006) Isotopic fingerprints of microbial respiration in aragonite from Bahamian stromatolites. *Geology*, 34, 973-976
- Arp, G., Thiel, V., Reimer, A., Michaelis, W., Reitner, J. (1999) Biofilm exopolymers control microbialite formation at thermal springs discharging into the alkaline Pyramid Lake, Nevada, USA. *Sedimentary Geology*, 126, 159-176
- Bazylinski, D.A., Frankel, R.B. (2003) Biologically controlled mineralisation in prokaryotes. *Reviews of Mineralogy and Geochemistry*, 54, 217-247
- Beveridge, T.J., Forsberg, C.W., Doyle, R.J. (1982) Major sites of metal binding in *Bacillus licheniformis* walls. *Journal of Bacteriology*, 150, 1438-1448
- Borrok, D., Fein, J.B. (2004) Distribution of protons and Cd between bacterial surfaces and dissolved humic substances determined through chemical equilibrium modelling. *Geochimica et Cosmochimica Acta*, 68, 3043-3052
- Borrok, D.M., Fein, J.B. (2005) The impact of ionic strength on the adsorption of protons, Pb, Cd, and Sr onto the surfaces of Gram negative bacteria: testing non-electrostatic, diffuse, and triple-layer models. *Journal of Colloid and Interface Science*, 286, 110-126
- Borrok, D., Fein, J.B., Kulpa, C.F. (2004a) Proton and Cd adsorption onto natural bacterial consortia: testing universal adsorption behaviour. *Geochimica et Cosmochimica Acta*, 68, 3231-3238



- Borrok, D.M., Fein, J.B., Kulpa Jr. C.F. (2004b) Cd and proton adsorption onto bacterial consortia grown from industrial wastes and contaminated geologic settings. *Environmental Science and Technology*, 38, 5656-5664
- Borrok, D., Turner, B.F., Fein, J.B. (2005) A universal surface complexation framework for modelling proton binding onto bacterial surfaces in geologic settings. *American Journal of Science*, 305, 826-853
- Borrok, D., Aumend, K., Fein, J. (2007) Significance of ternary bacteria-metal-natural organic matter complexes determined through experimentation and chemical equilibrium modelling. *Chemical Geology*, 238, 44-62.
- Borrok, D., Fein, J.B., Tischler, M., O'Loughlin, E., Meyer, H., Liss, M., Kemner, K.M. (2004c) The effect of acidic solutions and growth conditions on the adsorptive properties of bacterial surfaces. *Chemical Geology*, 209, 107-119
- Boyanov, M.I., Kelly, S.D., Kemner, K.M., Bunker, B.A., Fein, J.B., Fowle, D.A. (2003) Adsorption of cadmium to *Bacillus subtilis* bacterial cell walls: A pH-dependent X-ray adsorption fine structure spectroscopy study. *Geochimica et Cosmochimica Acta*, 67, 3299-3311
- Braissant, O., Cailleau, G., Dupraz, C., Verrecchia, E.P. (2003) Bacterially induced mineralisation of calcium carbonate in terrestrial environments: The role of exopolysaccharides and amino acids. *Journal of Sedimentary Research*, 73, 485-490
- Brassard, P., Kramer, J.R., Collins, P.V. (1990) Binding site analysis using linear programming. *Environmental Science and Technology*, 24, 195-201
- Brown, D.A., Sherrieff, B., Sawicki, J.A., Sparling, R. (1999) Precipitation of iron minerals by a natural microbial consortium. *Geochimica et Cosmochimica Acta*, 63, 2163-2169
- Burnett, P-G., Heinrich, H., Peak, D., Bremer, P.J., McQuillan, A.J., Daughney, C.J. (2006a) The effect of pH and ionic strength on proton adsorption by the thermophilic bacterium *Anoxybacillus flavithermus*. *Geochimica et Cosmochimica Acta*, 70, 1914-1927

- Burnett, P.-G.G., Daughney, C.J., Peak, D. (2006b) Cd adsorption onto *Anoxybacillus flavithermus*: Surface complexation modelling and spectroscopic investigations. *Geochimica et Cosmochimica Acta*, 70, 5253-5269
- Burnett, P.-G.G., Handley, K., Peak, D., Daughney, C.J. (2007) Divalent metal adsorption by the thermophile *Anoxybacillus flavithermus* in single and multi-metal systems. *Chemical Geology*, 244, 493-506
- Chafetz, H.S., Guidry, S.A. (1999) Bacterial shrubs, crystal shrubs, and ray-crystal shrubs: bacterial vs. abiotic precipitation. *Sedimentary Geology*, 126, 57-74
- Chan, C.S., De Stasio, G., Welch, S.A., Girasole, M., Frazer, B.H., Nesterova, M.V., Fakra, S., Banfield, J. (2004) Microbial polysaccharides template assembly of nanocrystal fibers. *Science*, 303, 1656-1658
- Chang, J.-S., Law, R., Chang, C.-C. (1997) Biosorption of lead, copper and cadmium by biomass of *Pseudomonas aeruginosa* PU21. *Water Research*, 31, 1651-1658
- Chatellier, X., Fortin, D. (2004) Adsorption of ferrous ions onto *Bacillus subtilis* cells. *Chemical Geology*, 212, 209-228
- Chatellier, X., West, M.M., Rose, J., Fortin, D., Leppard, G.G., Ferris, F.G. (2004) Characterisation of iron-oxides formed by oxidation of ferrous ions in the presence of various bacterial species and inorganic ligands. *Geomicrobiology Journal*, 21, 99-112
- Chen, J.-H., Lion, L.W., Ghiorse, W.C., Shuler, M.L. (1995) Mobilization of adsorbed cadmium and lead in aquifer material by bacterial extracellular polymers. *Water Research*, 29, 421-430.
- Chen, J.P., Yang, L. (2006) Study of a heavy metal biosorption onto raw and chemically modified *Sargassum* sp. via spectroscopic and modelling analysis. *Langmuir*, 22, 8906-8914
- Claessens, J., Van Capellen, P. (2007) Competitive binding of Cu^{2+} and Zn^{2+} to live cells of *Shewanella putrefaciens*. *Environmental Science and Technology*, 41, 909-914

- Claessens, J., van Lith, Y., Laverman, A.M., Van Capellen, P. (2005) Acid-base activity of live bacteria: Implications for quantifying cell wall charge. *Geochimica et Cosmochimica Acta*, 70, 267-276
- Comte, S., Guibaud, G., Baudu, M. (2006a) Biosorption properties of extracellular polymeric substances (EPS) resulting from activated sludge according to their type: Soluble or bound. *Process Biochemistry*, 41, 815-823
- Comte, S., Guibaud, G., Baudu, M. (2006b) Relations between extraction protocols for activated sludge extracellular polymeric substances (EPS) and EPS complexation properties Part 1. Comparison of the efficiency of eight EPS extraction methods. *Enzyme and Microbial Technology*, 38, 237-245
- Comte, S., Guibaud, G., Baudu, M. (2006c) Relations between extraction protocols for activated sludge extracellular polymeric substances (EPS) and complexation properties of Pb and Cd with EPS Part II: Consequences of EPS extraction methods on Pb²⁺ and Cd²⁺ complexation. *Enzyme and Microbial Technology*, 38, 246-252
- Cox, J.S., Smith, D.S., Warren, L.A., Ferris, F.G. (1999) Characterising heterogeneous bacterial surface functional groups using discrete affinity spectra for proton binding. *Environmental Science and Technology*, 33, 4514-4521
- Czajka, D.R., Lion, L.W., Schuler, M.L., Ghiorse, W.C. (1997) Evaluation of the utility of bacterial extracellular polymers for the treatment of metal-contaminated soils: Polymer persistence, mobility and the influence of lead. *Water Research*, 31, 2827-2839
- Daughney, C.J., Fein, J.B. (1998) The effect of ionic strength on the adsorption of H⁺, Cd²⁺, Pb²⁺, and Cu²⁺ by *Bacillus subtilis* and *Bacillus licheniformis*: A surface complexation model. *Journal of Colloid and Interface Science*, 198, 53-77
- Daughney, C.J., Fein, J.B., Yee, N. (1998) A comparison of the thermodynamics of metal adsorption onto two common bacteria. *Chemical Geology*, 144, 161-176
- Daughney, C.J., Fowle, D.A., Fortin, D. (2001) The effect of growth phase on proton and metal adsorption by *Bacillus subtilis*. *Geochimica et Cosmochimica Acta*, 65, 1025-1035

- Daughney, C.J., Siciliano, S.D., Rencz, A.N., Lean, D., Fortin, D. (2002) Hg(II) adsorption by bacteria: A surface complexation model and its application to shallow acidic lakes and wetlands in Kejimikujik National Park, Canada. *Environmental Science and Technology*, 36, 1546-1553
- De Yoreo, J.J., Vekilov, P.G. (2003) Principles of crystal nucleation and growth. *Reviews of Mineralogy and Geochemistry*, 54, 57-93
- Dick, J., De Windt, W., De Graef, B., Saveyn, H., Van der Merren, P., De Belie, N., Verstraete, W. (2006) Bio-deposition of a calcium carbonate layer on degraded limestone by *Bacillus* species. *Biodegradation*, 17, 357-367
- Dittrich, M., Sibling, S. (2006) Influence of H^+ and calcium ions on surface functional groups of *Synechococcus* PCC 7942 cells. *Langmuir*, 22, 5435-5442
- Dupraz, C., Visscher, P.T. (2005) Microbial lithification in marine stromatolites and hypersaline mats. *Trends in Microbiology*, 13, 429-438
- Dupraz, C., Visscher, P.T., Baumgartner, L.K., Reid, R.P. (2004) Microbe-mineral interactions: early carbonate precipitation in a hypersaline lake (Eleuthera Island, Bahamas). *Sedimentology*, 51, 745-765
- Eboigbodin, K.E., Ojeda, J.J., Biggs, C.A. (2007) Investigating the surface properties of *Escherichia coli* under glucose controlled conditions and its effect on aggregation. *Langmuir*, 23, 6691-6697
- Ehrlich, H.L. (1996) How microbes influence mineral growth and dissolution. *Chemical Geology*, 132, 5-9
- Esposito, A., Pagnanelli, F., Lodi, A., Solisio, C., Veglio, F. (2001) Biosorption of heavy metals by *Sphaerotilus natans*: an equilibrium study at different pH and biomass concentrations. *Hydrometallurgy*, 60, 129-141
- Fein, J.B. (2000) Quantifying the effects of bacteria on adsorption reactions in water-rock systems. *Chemical Geology*, 169, 165-280
- Fein, J.B. (2006) Thermodynamic modelling of metal adsorption onto bacterial cell walls: Current challenges. *Advances in Agronomy*, 90, 179-202

- Fein, J.B., Martin, A.M., Wightman, P.G. (2001) Metal adsorption onto bacterial surfaces: Development of a predictive approach. *Geochimica et Cosmochimica Acta*, 65, 4267-4273
- Fein, J.B., Scott, S., Rivera, N. (2002) The effect of Fe on Si adsorption by *Bacillus subtilis* cell walls: insights into non-metabolic bacterial precipitation of silicate minerals. *Chemical Geology*, 182, 265-273
- Fein, J.B., Daughney, C.J., Yee, N., Davis, T.A. (1997) A chemical equilibrium model for metal adsorption onto bacterial surfaces. *Geochimica et Cosmochimica Acta*, 61, 3319-3328
- Fein, J.B., Boily, J-F., Guclu, K., Kaulbach, E. (1999) Experimental study of humic acid adsorption onto bacteria and Al-oxide mineral surfaces. *Chemical Geology*, 162, 33-45
- Fein, J.B., Boily, J-F., Yee, N., Gorman-Lewis, D., Turner, B. F. (2005) Potentiometric titrations of *Bacillus subtilis* cells to low pH and a comparison of modelling approaches. *Geochimica et Cosmochimica Acta*, 69, 1123-1132
- Ferris, F.G., Fyfe, W.S., Beveridge, T.J. (1988) Metallic ion binding by *Bacillus subtilis*: Implications for the fossilization of microorganisms. *Geology*, 16, 149-152
- Ferris, F.G., Konhauser, K.O., Lyven, B., Pedersen, K. (1999) Accumulation of metals by bacteriogenic iron oxides in a subterranean environment. *Geomicrobiology Journal*, 16, 181-192
- Ferris, F.G., Hallberg, R.O., Lyven, B., Pedersen, K. (2000) Retention of strontium, caesium, lead and uranium by bacterial iron oxides from a subterranean environment. *Applied Geochemistry*, 15, 1035-1042
- Fortin, D. (2004) What biogenic minerals tell us. *Science*, 303, 1618-1819
- Fortin, D., Beveridge, T.J. (1997) Role of the bacterium *Thiobacillus* in the formation of silicates in acidic mine tailings. *Chemical Geology*, 141, 235-250
- Fortin, D., Langley, S. (2005) Formation and occurrence of biogenic iron-rich minerals. *Earth-Science Reviews*, 72, 1-19

- Fowle, D.A., Fein, J.B. (1999) Competitive adsorption of metal cations onto two gram positive bacteria: Testing the chemical equilibrium model. *Geochimica et Cosmochimica Acta*, 63, 3059-3067
- Fowle, D.A., Fein, J.B. (2000) Experimental measurements of the reversibility of metal-bacteria adsorption reactions. *Chemical Geology*, 168, 27-36
- Francis, A.J., Dodge, C.J., Gillow, J.B., Papenguth, H.W. (2000) Biotransformation of uranium compounds in high ionic strength brine by a halophilic bacterium under denitrifying conditions. *Environmental Science and Technology*, 34, 2311-2317
- Frankel, R.B., Bazylinski, D.A. (2003) Biologically induced mineralisation by bacteria. *Reviews of Mineralogy and Geochemistry*, 54, 95-114
- Frolund, B., Palmgren, R., Keiding, K., Nielsen, P.H. (1996) Extraction of extracellular polymers from activated sludge using a cation exchange resin. *Water Research*, 30, 1749-1758
- Gaboriaud, F., Daugue, E., Bailet, S., Jorand, F., Duval, J., Thomas, F. (2006) Multiscale dynamics of the cell envelope of *Shewanella putrefaciens* as a response to pH change. *Colloids and Surfaces B: Biointerfaces*, 50, 123-131
- Glasauer, S., Langley, S., Beveridge, T.J. (2001) Sorption of Fe(hydr)oxides to the surface of *Shewanella putrefaciens*: Cell-bound fine-grained minerals are not always formed de novo. *Applied and Environmental Mineralogy*, 67, 5544-5550
- Gorman-Lewis, D., Fein, J., Jensen, M.P. (2006) Enthalpies and entropies of proton and cadmium adsorption onto *Bacillus subtilis* cells from calorimetric measurements. *Geochimica et Cosmochimica Acta*, 70, 4862-4873
- Gorman-Lewis, D., Fein, J.B., Soderholm, L., Jensen, M.P., Chiang, M.-H. (2005) Experimental study of neptunyl adsorption onto *Bacillus subtilis*. *Geochimica et Cosmochimica Acta*, 69, 4837-4844
- Guibaud, G., Tixier, N., Bouju, A., Baudu, M. (2004) Use of a polarographic method to determine copper, nickel and zinc constants of complexation by extracellular polymers extracted from activated sludge. *Process Biochemistry*, 39, 833-839

- Guibaud, G., Comte, S., Bordas, F., Baudu, M. (2005a) Metal removal from single and multimetallic equimolar systems by extracellular polymers extracted from activated sludges as evaluated by SMDE polarography. *Process Biochemistry*, 40, 661-668
- Guibaud, G., Comte, S., Bordas, F., Dupuy, S., Baudu, M. (2005b) Comparison of the complexation potential of extracellular polymeric substances (EPS), extracted from activated sludges and produced by pure bacteria strains, for cadmium, lead and nickel. *Chemosphere*, 59, 629-638
- Guine, V., Martins, J.M.F., Causse, B., Durand, A., Gaudet, J-P., Spadini, L. (2007) Effect of cultivation and experimental conditions on the surface reactivity of the metal-resistant bacteria *Cupriavidus metallidurans* CH34 to protons, cadmium and zinc. *Chemical Geology*, 236, 266-280
- Guine, V., Spadini, L., Sarret, G., Muris, M., Delolme, C., Gaudet, J.-P., Martins, J.M.F. (2006) Zinc sorption to three gram-negative bacteria: Combined titration, modelling, and EXAFS study. *Environmental Science and Technology*, 40, 1806-1813
- Haas, J.R. (2004) Effects of cultivation conditions on acid-base titration properties of *Shewanella putrefaciens*. *Chemical Geology*, 209, 67-81
- Haas, J.R., Dichristina, T.J., Wade Jr, R. (2001) Thermodynamics of U(VI) sorption onto *Shewanella putrefaciens*. *Chemical Geology*, 180, 33-54
- Hallberg, R., Ferris, F.G. (2004) Biomineralisation by Gallionella. *Geomicrobiology Journal*, 21, 325-330
- Heinrich, H.T.M., Bremer, P.J., Daughney, C.J., McQuillan, A.J. (2007) Acid-base titrations of functional groups on the surface of the thermophilic bacterium *Anoxybacillus flavithermus*: Comparing a chemical equilibrium model with ATR-IR spectroscopic data. *Langmuir*, 23, 2731-2740
- Hennig, C., Panak, P.J., Reich, T., Rossberg, A., Raff, J., Selenska-Pobell, S., Matz, W., Bucher, J.J., Bernhard, G., Nitsche, H. (2001) EXAFS investigation of uranium(VI) complexes formed at *Bacillus cereus* and *Bacillus sphaericus* surfaces. *Radiochimica Acta*, 89, 625-631

- Herbelin, A.L., Westall, J.C. (1999) FITEQL 4.0: a Computer Program for Determination of Chemical Equilibrium Constants from Experimental Data; Report 99-01. Department of Chemistry Oregon State University, Corvallis
- Hetzer, A., Daughney, C.J., Morgan, H.W. (2006) Cadmium ion biosorption by the thermophilic bacteria *Geobacillus stearothermophilus* and *G. thermocatenulatus*. *Applied and Environmental Microbiology*, 72, 4020-4027
- Hong, Y., Brown, D.G. (2006) Cell surface acid-base properties of *Escherichia coli* and *Bacillus brevis* and variation as a function of growth phase, nitrogen source and C:N ratio. *Colloids and Surfaces B: Biointerfaces*, 50, 132-139
- Jensen-Spaulding, A., Cabral, K., Schuler, M.L., Lion, L.W. (2004) Predicting the rate and extent of cadmium and copper desorption from soils in the presence of bacterial extracellular polymer. *Water Research*, 38, 2231-2240
- Jiang, D.T. (2002) X-ray absorption fine structure spectroscopy. *Synchrotron Radiation: Earth, Environmental and Material Sciences Applications*. G.S. Henderson and D.R. Baker (Eds). Short Course Series, 30, 65-98. Mineralogical Society of Canada
- Jiang, W., Saxena, A., Song, B., Ward, B.B., Beveridge, T.J., Myeni, S.C.B. (2004) Elucidation of functional groups on gram-positive and gram-negative bacterial surfaces using infrared spectroscopy. *Langmuir*, 20, 11433-11442
- Johnson, K.J., Szymanowski, J.E.S., Borrok, D., Huynh, T.Q., Fein, J.B. (2007) Proton and metal adsorption onto bacterial consortia: Stability constants for metal-bacterial surface complexes. *Chemical Geology*, 239, 13-26
- Jones, B., Renaut, R.W. (2007) Selective mineralisation of microbes in Fe-rich precipitates (jarosite, hydrous ferric oxides) from acid hot springs in the Waiotapu geothermal area, North Island, New Zealand. *Sedimentary Geology*, 194, 77-98
- Ju, Y-H, Chen, T-C., Liu, J.C. (1997) A study on the biosorption of lindane. *Colloids and Surface B: Biointerfaces*, 9, 187-196
- Kah, L.C., Riding, R. (2007) Mesoproterozoic carbon dioxide levels inferred from calcified cyanobacteria. *Geology*, 35, 799-802

- Karthikeyan, S., Beveridge, T.J. (2002) *Pseudomonas aeruginosa* biofilms react with and precipitate toxic soluble gold. *Environmental Microbiology*, 4, 667-675
- Kasama, T., Murakami, T. (2001) The effect of microorganisms on Fe precipitation rates at neutral pH. *Chemical Geology*, 180, 117-128
- Kawaguchi, T., Decho, A.W. (2002) A laboratory investigation of cyanobacterial extracellular polymeric secretions (EPS) in influencing CaCO₃ polymorphism. *Journal of Crystal Growth*, 240, 230-235
- Kawano, M., Tomita, K. (2001) Microbial biomineralisation in weathered volcanic ash deposit and formation of biogenic minerals by experimental incubation. *American Mineralogist*, 86, 400-410
- Kelly, S.D., Kemner, K.M., Fein, J.B., Fowle, D.A., Boyanov, M.I., Bunker, B.A., Yee, N. (2002) X-ray absorption fine structure determination of pH-dependent U-bacterial cell wall interactions. *Geochimica et Cosmochimica Acta*, 66, 3855-3871
- Kennedy, C.B., Scott, S.D., Ferris, F.G. (2003) Characterisation of bacteriogenic iron oxide deposits from Axial Volcano, Juan de Fuca Ridge, Northeast Pacific Ocean. *Geomicrobiology Journal*, 20, 199-214
- Kenward, P.A., Fowle, D.A., Yee, N. (2006) Microbial selenate sorption and reduction in nutrient limited systems. *Environmental Science and Technology*, 40, 3782-3786
- Kinzler, K., Gehrke, T., Telegdi, J., Sand, W. (2003) Bioleaching-a result of interfacial processes caused by extracellular polymeric substances (EPS). *Hydrometallurgy*, 71, 83-88
- Kives, J., Orgaz, B., SanJose, C. (2006) Polysaccharide differences between planktonic and biofilm-associated EPS from *Pseudomonas fluorescens* B52. *Colloids and Surfaces B: Biointerfaces*, 50, 104-108
- Konhauser, K.O. (1997) Bacterial iron mineralisation in nature. *FEMS Microbiology Reviews*, 20, 315-326
- Konhauser, K.O. (1998) Diversity of bacterial iron mineralisation. *Earth-Science Reviews*, 43, 91-121

- Konhauser, K.O., Ferris, F.G. (1996) Diversity of iron and silica precipitation by microbial mats in hydrothermal waters, Iceland: Implications for Precambrian iron formations. *Geology*, 24, 323-326
- Konhauser, K.O., Fyfe, W.S., Ferris, F.G., Beveridge, T.J. (1993) Metal sorption and mineral precipitation by bacteria in two Amazonian river systems: Rio Solimoes and Rio Negro, Brazil. *Geology*, 21, 1103-1106
- Konhauser, K.O., Hamade, T., Raiswell, R., Morris, R.C., Ferris, F.G., Southam, G., Canfield, D.E. (2002) Could bacteria have formed the Precambrian banded iron formations. *Geology*, 30, 1079-1082
- Lamelas, C., Benedetti, M., Wilkinson, K.J., Slaveykova, V.I. (2006) Characterization of H^+ and Cd^{2+} binding properties of the bacterial exopolysaccharides. *Chemosphere*, 65, 1362-1370
- Leblanc, M., Achard, B., Ben Othman, D., Luck, J.M. (1996) Accumulation of arsenic from acidic mine waters by ferruginous bacterial accretions (stromatolites). *Applied Geochemistry*, 11, 541-554
- Ledin, M., Krantz-Ruckler, C., Allard, B. (1999) Microorganisms as metal sorbents: comparison with other soil constituents in multi-compartment systems. *Soil Biology and Biochemistry*, 31, 1639-1648
- Leone, L., Ferri, D., Manfredi, C., Persson, P., Shchukarev, A., Sjöberg, S., Loring, J. (2007) Modeling the acid-base properties of bacterial surfaces: A combined spectroscopic and potentiometric study of the gram-positive bacterium *Bacillus subtilis*. *Environmental Science and Technology*, 41, 6465-6471
- Leveille, R.J., Fyfe, W.S., Longstaffe, F.J. (2000) Geomicrobiology of carbonate-silicate microbialites from Hawaiian basaltic sea caves. *Chemical Geology*, 169, 339-355
- Lian, B., Hu, Q., Chen, J., Ji, J., Teng, H.H. (2006) Carbonate biomineralisation induced by soil bacterium *Bacillus megaterium*. *Geochimica et Cosmochimica Acta*, 70, 5522-5535

- Little, C.T.S., Glynn, S.E.J., Mills, R.A. (2004) Four-hundred-and-ninety-million-year record of bacteriogenic iron oxide precipitation at sea-floor hydrothermal vents. *Geomicrobiology Journal*, 21, 415-429
- Loaec, M., Olier, R., Guezennec, J. (1997) Uptake of lead, cadmium and zinc by a novel bacterial exopolysaccharide. *Water Research*, 31, 1171-1179
- Macaskie, L.E., Basnakova, G. (1998) Microbially-enhanced chemisorption of heavy metals: A method for the bioremediation of solutions containing long-lived isotopes of neptunium and plutonium. *Environmental Science and Technology*, 32, 184-187
- MacLean, L.C.W., Martinez, R.E., Fowle, D.A. (2004) Experimental studies of bacteria-iodide adsorption interactions. *Chemical Geology*, 212, 229-238
- Madigan, M.T., Martinko, J.M., Parker, J. (2003) *Biology of Microorganisms* (10th Edition). Prentice Hall
- Martinez, R.E., Smith, D.S., Kulczyzcki, E., Ferris, F.G. (2002) Determination of intrinsic bacterial surface acidity constants using a Donnan shell model and a continuous pK_a distribution method. *Journal of Colloid and Interface Science*, 253, 130-139
- McLean, R.J.C., Beauchemin, D., Clapham, L., Beveridge, T.J. (1990) Metal-binding characteristics of the gamma-glutamyl capsular polymer of *Bacillus licheniformis* ATCC 9945. *Applied and Environmental Microbiology*, 56, 3671-3677
- Merroun, M.L., Ben Chekroun, K., Arias, J.M., Gonzalez-Munoz, M.T. (2003) Lanthanum fixation by *Myxococcus xanthus*: cellular location and extracellular polysaccharide observation. *Chemosphere*, 52, 113-120
- Merroun, M.L., Raff, J., Rossberg, A., Hennig, C., Reich, T., Selenska-Pobell, S. (2005) Complexation of uranium by cells and S-layer sheets of *Bacillus sphaericus* JG-A12. *Applied and Environmental Microbiology*, 71, 5532-5543
- Min, M., Xu, H., Chen, J., Fayek, M. (2005) Evidence of uranium biomineralisation in sandstone-hosted roll-front uranium deposits, northwestern China. *Ore Geology Reviews*, 26, 198-206

- Mitchell, A.C., Ferris, F.G. (2006) The influence of *Bacillus pasteurii* on the nucleation and growth of calcium carbonate. *Geomicrobiology Journal*, 23, 213-226
- Mortimer, R.J.G., Coleman, M.L., Rae, J.E. (1997) Effect of bacteria on the elemental composition of early diagenetic siderite: implications for palaeoenvironmental interpretations. *Sedimentology*, 44, 759-765
- Ngwenya, B.T. (2007) Enhanced adsorption of zinc is associated with aging and lysis of bacterial cells in batch incubations. *Chemosphere*, 67, 1982-1992
- Ngwenya, B.T., Sutherland, I.W., Kennedy, L. (2003) Comparison of the acid-base behaviour and metal adsorption characteristics of a gram-negative bacterium with other strains. *Applied Geochemistry*, 18, 527-538
- Noghabi, K.A., Zahiri, H.S., Yoon, S.C. (2007) The production of a cold-induced extracellular biopolymer by *Pseudomonas fluorescens* BM07 under various growth conditions and its role for heavy metals absorption. *Process Biochemistry*, 42, 847-855
- Omoike, A., Chorover, J. (2004) Spectroscopic study of extracellular polymeric substances from *Bacillus subtilis*: Aqueous chemistry and adsorption effects. *Biomacromolecules*, 5, 1219-1230
- Omoike, A., Chorover, J. (2006) Adsorption to goethite of extracellular polymeric substances from *Bacillus subtilis*. *Geochimica et Cosmochimica Acta*, 70, 827-828
- Omoike, A., Chorover, J., Kwon, K.D., Kubicki, J.D. (2004) Adhesion of bacterial exopolymers to a α -FeOOH: Inner-sphere complexation of phosphodiester groups. *Langmuir*, 20, 11108-11114
- Pagnanelli, F., Petrangeli Papini, M., Toro, L., Trofoni, M., Veglio, F. (2000) Biosorption of metal ions on *Arthrobacter* sp.: Biomass characterisation and biosorption modelling. *Environmental Science and Technology*, 34, 2773-2778
- Parikh, A., Madamwar, D. (2006) Partial characterisation of extracellular polysaccharides from cyanobacteria. *Bioresource Technology*, 97, 1822-1827
- Phoenix, V.R., Adams, D.G., Konhauser, K.O. (2000) Cyanobacterial viability during hydrothermal biomineralisation. *Chemical Geology*, 169, 329-338

- Phoenix, V.R., Konhauser, K.O., Adams, D.G., Bottrell, S.H. (2001) Role of biomineralisation as an ultraviolet shield: Implications for Archean life. *Geology*, 29 (9), 823-826
- Phoenix, V.P., Martinez, R. E., Konhauser, K.O., Ferris, F.G. (2002) Characterisation and implications of the cell surface reactivity of *Calothrix* sp. Strain KC97. *Applied and Environmental Microbiology*, 68, 4827-4834
- Plette, A.C.C., Benedetti, M.F., Van Riemsdijk, W.H. (1996) Competitive binding of protons, calcium, cadmium, and zinc to isolated cell walls of a gram-positive soil bacterium. *Environmental Science and Technology*, 30, 1902-1910
- Plette, A.C.C., van Riemsdijk, W.H., Benedetti, M.F., van der Waal, A. (1995) pH dependent charging behaviour of isolated cell walls of a gram-positive soil bacterium. *Journal of Colloid and Interface Science*, 173, 354-363
- Podda, F., Zuddas, P., Minacci, A., Pepi, M., Baldi, F. (2000) Heavy metal coprecipitation with hydrozincite $[Zn_5(CO_3)_2(OH)_6]$ from mine waters caused by photosynthetic microorganisms. *Applied and Environmental Microbiology*, 66, 5092-5098
- Poortinga, A.T., Bos, R., Norde, W., Busscher, H.J. (2002) Electric double layer interactions in bacterial adhesion to surfaces. *Surface Science Reports*, 47, 1-32
- Quiroz, N.G.A., Hung, C-C., Santschi, P.H. (2006) Binding of thorium(IV) to carboxylate, phosphate and sulphate functional groups from marine exopolymeric substances (EPS). *Marine Chemistry*, 100, 337-353
- Rancourt, D.G., Thibault, P-J., Mavrocordatos, D., Lamarche, G. (2005) Hydrated ferric oxide precipitation in the presence of nonmetabolising bacteria: Constraints on the mechanism of a biotic effect. *Geochimica et Cosmochimica Acta*, 69, 553-577
- Reith, F., Rogers, S.L., McPhail, D.C., Webb, D. (2006) Biomineralization of gold: Biofilms on bacterioform gold. *Science*, 313, 233-236
- Rivadeneyra, M.A., Delgado, G., Ramos-Cormenzana, A., Delgado, R. (1998) Biomineralisation of carbonates by *Halomonas eurihalina* in solid and liquid media with different salinities: crystal formation sequence. *Research in Microbiology*, 149, 277-287

- Rivadeneira, M.A., Martin-Algarra, A., Sanchez-Navas, A., Martin-Ramos, D. (2006) Carbonate and phosphate precipitation by *Chromohalobacter marismortui*. *Geomicrobiology Journal*, 23, 1-13
- Rodriguez-Navarro, C., Jimenez-Lopez, C., Rodriguez-Navarro, A., Gonzalez-Munoz, M.T., Rodriguez-Gallego, M. (2007) Bacterially mediated mineralisation of vaterite. *Geochimica et Cosmochimica Acta*, 71, 1197-1213
- Sag, Y., Kutsal, T. (1995) Copper(II) and nickel(II) adsorption by *Rhizopus arrhizus* in batch stirred reactors in series. *The Chemical Engineering Journal*, 58, 265-273
- Salehizadeh, H., Shojasadati, S.A. (2003) Removal of metal ions from aqueous solution by polysaccharide produced from *Bacillus firmus*. *Water Research*, 37, 4231-4235
- Sand, W., Gehrke, T. (2006) Extracellular polymeric substances mediate bioleaching/biocorrosion via interfacial processes involving iron(III) ions and acidophilic bacteria. *Research in Microbiology*, 157, 49-56
- Sar, P., Kazy, S.K., Asthana, R.K., Singh, S.P. (1999) Metal adsorption and desorption by lyophilised *Pseudomonas aeruginosa*. *International Biodeterioration and Biodegradation*, 44, 101-110
- Sarret, G., Manceau, A., Spadini, L., Roux, J-C., Hazemann, J-L., Soldo, Y., Eyeberry-Berard, L., Menthonnex, J-J. (1998) Structural determination of Zn and Pb binding sites in *Penicillium chrysogenum* cell walls by EXAFS spectroscopy. *Environmental Science and Technology*, 32, 1648-1655
- Schultze-Lam, S., Harauz, G., Beveridge, T.J. (1992) Participation of a cyanobacterial S layer in fine-grain mineral formation. *Journal of Bacteriology*, 174 (24), 7971-7981
- Sharma, P.K., Das, A., Rao, H., Forssberg K.S.E. (2003) Surface characterisation of *Acidithiobacillus ferrooxidans* cells grown under different conditions. *Hydrometallurgy*, 71, 285-292
- Small, T.D., Warren, L.A., Ferris, F.G. (2001) Influence of ionic strength on strontium sorption to bacteria, Fe(III) oxide and composite bacteria-Fe(III) oxide surfaces. *Applied Geochemistry*, 16, 939-946

- Sokolov, I., Smith, D.S., Henderson, G.S., Gorby, Y.A., Ferris, F.G. (2001) Cell surface electrochemical heterogeneity of the Fe(III)-reducing bacteria *Shewanella putrefaciens*. *Environmental Science and Technology*, 35, 341-347
- Stocks-Fischer, S., Galinat, J.K., Bang, S.S. (1999) Microbiological precipitation of CaCO₃. *Soil Biology and Biochemistry*, 31, 1563-1571
- Stumm, W., Morgan, J.J. (1996) *Aquatic Chemistry, Chemical Equilibria and Rates in Natural Waters*, 3rd Edition. Wiley, New York
- Takahashi, Y., Chatellier, X., Hattori, K.H., Kato, K., Fortin, D. (2005) Adsorption of rare earth elements onto bacterial cell walls and its implication for REE sorption onto natural microbial mats. *Chemical Geology*, 219, 53-67
- Tazaki, K., Okuno, M., Furumoto, M., Watanabe, H. (2006) Biomineralisation of pisoliths in hot springs. *Materials Science and Engineering C*, 26, 617-623
- Texier, A-C., Andres, Y., Le Cloirec, P. (1999) Selective biosorption of lanthanide (La, Eu, Yb) ions by *Pseudomonas aeruginosa*. *Environmental Science and Technology*, 33, 489-495
- Toner, B., Manceau, A., Marcus, A.M., Millet, D.B., Sposito, G. (2005) Zinc sorption by a bacterial biofilm. *Environmental Science and Technology*, 39, 8288-8294
- Toner, B., Manceau, A., Webb, S.M., Sposito, G. (2006) Zinc sorption to biogenic hexagonal-birnessite particles within a hydrated bacterial biofilm. *Geochimica et Cosmochimica Acta*, 70, 27-43
- Tourney, J., Ngwenya, B.T., Mosselmans, J.W.F., Tetley, L., Cowie, G.L. (2007) The effect of extracellular polymers on the proton adsorption characteristics of the thermophile *Bacillus licheniformis* S-86. *Chemical Geology*, 247, 1-15
- Tsuruta, T. (2006) Selective accumulation of light or heavy rare earth elements using gram-positive bacteria. *Colloids and Surfaces B: Biointerfaces*, 52, 117-122
- Urrutia, M.M., Beveridge, T.J. (1994) Formation of fine-grained metal and silicate precipitates on a bacterial surface (*Bacillus subtilis*). *Chemical Geology*, 116, 261-280

- van der Wal, A., Norde, W., Zehnder, A.J.B., Lyklema, J. (1997) Determination of the total charge in the cell walls of Gram-positive bacteria. *Colloids and Surfaces B: Biointerfaces*, 9, 81-100
- Veglio, F., Beolchini, F., Gasbarro, A. (1997) Biosorption of toxic metals: an equilibrium study using free cells of *Arthrobacter* sp. *Process Biochemistry*, 32, 99-105
- Warren, L.A., Ferris, F.G. (1998) Continuum between sorption and precipitation of Fe(III) on microbial surfaces. *Environmental Science and Technology*, 32, 2331-2337
- Warren, L.A., Maurice, P.A., Parmar, N., Ferris, F.G. (2001) Microbially mediated calcium carbonate precipitation: Implications for interpreting calcite precipitation and for solid-phase capture of inorganic contaminants. *Geomicrobiology Journal*, 18, 93-115
- Wasserman, E., Felmy, A.R. (1998) Computation of the electrical double layer properties of semipermeable membranes in multicomponent electrolytes. *Applied and Environmental Microbiology*, 64, 2295-2300
- Watterson, J.R. (1991) Preliminary evidence for the involvement of budding bacteria in the origin of Alaskan placer gold. *Geology*, 20, 315-318
- Westall, J.C., Jones, J.D., Turner, G.D., Zachara, J.M. (1995) Models for association of metal ions with heterogeneous environmental sorbents. 1. Complexation of Co(II) by leonardite humic acid as a function of pH and NaClO₄ concentration. *Environmental Science and Technology*, 29, 951-959
- Wightman, P.G., Fein, J.B. (2001) Ternary interactions in a humic acid-Cd-bacteria system. *Chemical Geology*, 180, 55-65
- Wightman, P.G., Fein, J.B. (2005) Iron adsorption by *Bacillus subtilis* bacterial cell walls. *Chemical Geology*, 216, 177-189
- Wightman, P.G., Fein, J.B., Wesolowski, D.J., Phelps, T.J., Benezeth, P. Palmer, D.A. (2001) Measurement of bacterial surface protonation constants for two species at elevated temperatures. *Geochimica et Cosmochimica Acta*, 65, 3657-3669

- Wonders, J.H.A.M., Van Leeuwen, H.P., Lyklema, J. (1997) Metal- and proton-binding properties of a core-shell latex: interpretation in terms of colloid surface models. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 120, 221-233
- Xiao, L., Qu, X., Zhu, D. (2007) Biosorption of nonpolar hydrophobic organic compounds to *Escherichia coli* facilitated by metal and proton surface binding. *Environmental Science and Technology*, 41, 2750-2755
- Yee, N., Fein, J. (2001) Cd adsorption onto bacterial surfaces: A universal adsorption edge? *Geochimica et Cosmochimica Acta*, 65, 2037-2042
- Yee, N., Fowle, D.A., Ferris, F.G. (2004a) A Donnan potential model for metal sorption onto *Bacillus subtilis*. *Geochimica et Cosmochimica Acta*, 68, 3657-3664
- Yee, N., Benning, L.G., Phoenix, V.R., Ferris, F.G. (2004b) Characterisation of metal-cyanobacteria sorption reactions: A combined macroscopic and infrared spectroscopic investigation. *Environmental Science and Technology*, 38, 775-782
- Yee, N., Phoenix, V.R., Konhauser, K.O., Benning, L.G., Ferris, F.G. (2003) The effect of cyanobacteria on silica precipitation at neutral pH: implications for bacterial silicification in geothermal hot springs. *Chemical Geology*, 199, 83-90
- Zuddas, P., Podda, F. (2005) Variations in physico-chemical properties of water associated with bio-precipitation of hydrozincite $[Zn_5(CO_3)_2(OH)_6]$ in the waters of Rio Naracauli, Sardinia (Italy). *Applied Geochemistry*, 20, 507-517

2. The effect of extracellular polymers (EPS) on the proton adsorption characteristics of the thermophile *Bacillus licheniformis* S-86.

Abstract

This study investigated proton adsorption to an extracellular polymeric substance (EPS) producing bacterial strain, *Bacillus licheniformis* S-86, in order to characterise and quantify the contribution made by EPS to cell surface reactivity. Potentiometric titrations were conducted using both native cells and cells from which the EPS layer had been extracted. Discrete-site surface complexation modelling indicated the presence of four different functional groups in both native and EPS-free cells. These sites are assigned to phosphodiester, (pK_a 3.3-3.4), carboxylic (pK_a 5.3-5.4), phosphoryl (pK_a 7.4-7.5) and amine (pK_a 9.9-10.1) type groups. The pK_a values for the four groups were very similar for native and EPS-free cells, indicating no qualitative difference in composition, but site concentrations in the native cells were statistically found to be significantly higher than those in the EPS-free cells for the pK_a 3.3-3.4 and pK_a 9.9-10.1 sites. Infrared analysis provided supporting evidence that site 2 is carboxylic in nature but did not reveal any difference in IR absorption between the native and EPS-free cells. Dissolved organic carbon (DOC) analysis conducted during this study indicated that DOC release by cells is significant, and that the EPS layer is the major contributor.

2.1. Introduction

Investigating the acid-base properties of the bacterial cell wall is the first step towards fully understanding the roles of cell surfaces in metal adsorption and mineralisation. Owing to their abundance and high relative surface area, bacteria and other microscopic organisms are recognised as important components of many geochemical processes, particularly in the cycling of trace metals (Ledin et al., 1999; Fein, 2000). Furthermore, the potential industrial applications of bacteria as biosorbents for heavy metals and other environmental contaminants (Wong et al., 1993; Volesky, 2001) merit further development.

The objective of the present study was to directly quantify and characterise the contribution made by extracellular polymeric substances (EPS) to the proton adsorption capacity of an EPS-producing bacterial strain, *Bacillus licheniformis* S-86, by use of acid-base titrations and surface-complexation modelling. The differences observed were further investigated by infrared analysis of the bacterial cells using attenuated total-reflectance Fourier-transform infrared spectroscopy (ATR-FTIR) and Fourier-transform infrared spectroscopy (FTIR), which enable identification of IR peaks corresponding to cell surface functional groups. When conducted at a range of pH values, these techniques also indicate speciation changes in particular groups (Jiang et al., 2004). In addition, dissolved organic carbon (DOC) analysis of cell suspension supernatants was undertaken in order to assess the impact of EPS on solution DOC concentration, as this may potentially affect metal complexation behaviour within the system. This approach enables important insights into the role of EPS as a reactive surface, and provides further constraints on the conditions under which proton adsorption by bacterial species can be modelled with a single set of stability constants.

Recent studies have concluded that the acid-base characteristics of differing bacterial strains from varying environments are sufficiently similar to be described by a generalised 'universal' model (Borrok et al., 2005). The proton- and metal-adsorption behaviour of cell surfaces were found not to vary significantly with bacterial strain (Yee and Fein 2001; Ngwenya et al., 2003; Borrok et al., 2004a), growth phase or growth conditions (Haas, 2004). However, slight differences and

inconsistencies have been noted but not satisfactorily explained. For example, Daughney et al. (2001) investigated the effect of growth phase on proton and metal adsorption by *B. subtilis* and found that there were variations in surface site concentrations, deprotonation constants and metal complexation constants between the exponential, stationary and sporulated growth phases of *Bacillus subtilis*. Burnett et al. (2006a) found significantly lower surface site densities for the thermophile *Anoxybacillus flavithermus* compared to published values for mesophilic species in a study of proton adsorption characteristics. Hong and Brown (2006) investigated the acid-base properties of *Escherichia coli* and *Bacillus brevis* as a function of varying growth phase, nitrogen source and C:N ratio. Modelling of potentiometric titration data indicated the presence of four types of surface site on each species, and it was found that for *E. coli*, the fourth site concentration varied as a function of C:N ratio, whereas for *B. brevis* the concentration of the first site varied as a function of nitrogen source. Ngwenya et al. (2003) investigated proton and metal adsorption by a gram-negative strain, using a constant-capacitance approach, and concluded that although site densities for proton adsorption were of the same order of magnitude as published values for gram positive strains, there was an overall tendency for gram negative strains to display slightly lower site densities. In each case, the potential reasons for the observed differences in adsorption behaviour, for example the smaller quantity of peptidoglycan in gram-negative cells, merit further investigation.

Recently, the important role of bacterial EPS has become more widely recognised. These polymers potentially have a significant effect on the acid-base properties, and hence metal-adsorption characteristics, of bacterial cells. In addition, EPS is the major component of biofilms, and is an important control on cell adhesion to surfaces. Guine et al. (2006) found that the theoretical cell wall site densities for three bacterial strains were much lower than those necessary to explain the observed Zn loading, and concluded that the excess loading may be explained by the contribution made by EPS. Toner et al. (2005) investigated the sorption of Zn by a bacterial biofilm and concluded that metal complexation by the EPS component was likely to be significant, but difficult to quantify owing to the current lack of understanding of bacterial EPS in terms of compositional variability as well as

complexation behaviour. Merroun et al. (2003) observed complexation of large amounts of lanthanum in the EPS of *Myxococcus xanthus*, using TEM preparations.

A number of recent studies have attempted to characterise and quantify proton and metal adsorption by isolated bacterial EPS (Guibaud et al., 2003; Comte et al., 2006a; Lamelas et al., 2006). However, no study as yet has undertaken a direct comparison of the acid-base behaviour of intact cells from an EPS-producing strain with cells that have had the EPS layer removed. Such a comparison is critical to understanding the role of EPS in proton and metal adsorption by intact cells, since recent studies appear to indicate that the presence of EPS does not significantly affect cellular electrophoretic mobility and hence surface charge (e.g. Liu et al., 2007).

2.2. Materials and Methods

2.2.1. Cell culture and preparation

The thermotolerant, gram-positive strain *Bacillus licheniformis* S-86 was obtained from Professor M. Baigori, PRIOIMI Biotechnology, Argentina. Experimental batches were grown from the primary culture by inoculating 2-L Pyrex flasks containing 1 litre of Luria Broth growth medium (10 g L⁻¹ bacto-tryptone, 5 g L⁻¹ bacto-yeast extract, 5 g L⁻¹ NaCl). The medium was sterilised prior to inoculation by autoclaving at 121 °C for 30 minutes. Cultures were then incubated on a shaker for 24 hours at 30 °C. The optimum growth temperature for this strain is 55 °C but preliminary analysis indicates that growth temperature does not affect the cell surface properties. Small batches of cells grown at 55 °C were observed to produce large quantities of EPS, and titration results were similar to those obtained using cells grown at 30 °C. Cells were harvested by centrifugation at 23,420 x g for 15 minutes at 4 °C. The harvested cells were then washed three times in 18 MO ultrapure water and either used immediately or frozen and freeze-dried (lyophilised) for 3-5 days, for use in subsequent experiments. Comparison of fresh and lyophilised cells by SEM/TEM showed that there was no difference in visible cell integrity as a result of the lyophilisation process, indicating that lyophilisation does not lead to cell damage and consequent effects on surface properties.

2.2.2. EPS extraction

A range of different approaches have been used in order to remove EPS from cell surfaces. Some studies have used genetic mutation as a means of obtaining EPS-free cells (Breeveld et al., 1998; Liu et al., 2007). However, the majority have utilised either chemical methods such as treatment with EDTA or formaldehyde, or physical methods such as sonication or cation exchange resin (Guibaud et al., 2003; Comte et al., 2006b). In this study, it was found that ultrasonication did not remove the EPS layer (SEM images, not shown), and chemical extraction methods may introduce contamination (see Discussion, Section 2.4.1), so treatment by cation exchange resin was undertaken.

Following harvesting, cells were washed three times in 18 MO ultrapure water in order to remove residual growth medium. The cell pellet was then resuspended in ultrapure water and added to approximately 30 g cationic exchange resin (CER, Dowex Marathon C, 30-40 mesh), which was washed several times in 18 MO deionised water prior to use (Frolund et al., 1996). The suspension was left stirring at 4 °C overnight, then allowed to settle for five minutes in order to remove ion exchange resin. The cells were washed a further two times in deionised water, settling between washes in order to separate any remaining ion exchange resin.

2.2.3. Cell characterisation by SEM imaging

Specimens were prepared for SEM analysis using the cationic dye ruthenium red, based on the method of Erlandsen et al. (2004). Samples were prepared in duplicate for both native and EPS-free cells. Cell suspensions were deposited on polylysine-coated glass coverslips and fixed for 1 hr in 2.5 % glutaraldehyde (EM grade 1, Sigma) in 0.1 M cacodylate (sodium cacodylate, Agar Scientific) buffer (pH 7.4, 20 °C) containing 0.15 % ruthenium red (Sigma), 5 mM CaCl_2 and 5 % sucrose for 1 hr, then rinsed in cacodylate buffer 3 times over 15 mins before treatment with buffered 1 % osmium tetroxide for 1 hour. Glutaraldehyde and OsO_4 act to stabilise the cells osmotically and mechanically by binding to proteins and lipids respectively (Pentilla et al. 1974). Treatment with ruthenium red further stabilises extracellular polymers (discussed in Section 2.3.1). Unbound OsO_4 was then removed by repeated rinses

with deionised water and the specimens dehydrated for 5 minutes in each of 70 %, 90 %, 100 % (twice) and anhydrous ethanol. The coverslips were then immersed in hexamethyldisilazane (HMDS) (TAAB Laboratories Equipment Ltd.) twice before being allowed to air dry. The coverslips were mounted on SEM stubs and gold/palladium sputter-coated. Samples were imaged using a JEOL 6400 SEM at 6 kV.

2.2.4. Potentiometric titrations

Lyophilised cells were rehydrated in 18 MO ultrapure water for 1 hour, then washed a further two times in ultrapure water. The cells were subsequently rinsed three times in the NaNO_3 electrolyte. Titrations were carried out at 25 °C on 50 ml suspensions of varying cell concentration, in 0.01 M NaNO_3 . Cell concentrations were determined by sub-sampling 5 ml and evaporating to dryness until a constant weight had been achieved and corrected for electrolyte contribution (Ngwenya, 2007). The suspensions were initially acidified to approximately pH 3 using 1 M HNO_3 , then titrated to pH 11 with 0.5 M NaOH. The freshly prepared NaOH was transferred to a bottle fitted with a drying tube containing anhydrous NaOH and standardised in triplicate using potassium hydrogen phthalate (KHP). Titrations were carried out using an automated DL53 Mettler-Toledo burette assembly programmed in dynamic mode, so that successive titrant additions were made only when a stability of 0.1 mV s^{-1} had been attained.

Titration curves were plotted in terms of total proton concentration (T_H) vs. pH. The total proton concentration was obtained from the experimental data according to the method of Westall et al. (1995). This approach incorporates determination of the initial charge imbalance (T_H^0), which represents the concentration of H^+ required to fully protonate the bacterial cell walls at the starting point of the titration (Borrok et al., 2005). At the starting pH for the potentiometric titrations conducted during this study (approximately pH 3), the bacterial cells are still negatively charged and only become fully protonated below pH 2.5 (Chapter 3). Starting titrations at pH values this low will likely cause cell lysis and/or potential displacement of structural cations

(Borrok et al., 2004c), so T_H^0 must be estimated. In this study, T_H^0 was treated as an adjustable parameter and optimised during the data modelling (see below).

T_H is calculated as follows:

$$T_H = T_H^0 + [C_a - C_b] \quad (2.1)$$

Where C_a is the concentration of acid added and C_b is the concentration of base added (Westall et al., 1995).

2.2.5. Data Modelling

The deprotonation of a functional group can be represented by the following generic reaction (Borrok and Fein, 2004):



Where R is the bacterium and A represents a functional group on the bacterial surface or volume (in the case of EPS). The deprotonation or acidity constant, K_a , for this reaction may be expressed as:

$$K_a = \frac{[R - A^{-}]a_{H^{+}}}{[R - AH^{\circ}]} \quad (2.3)$$

where $[R - A^{-}]$ and $[R - AH^{\circ}]$ represent the concentration of deprotonated and protonated sites and $a_{H^{+}}$ represents the activity of protons in the solution.

Titration data were modelled using the data optimization program FITEQL 4.0 (Herbelin and Westall, 1999) in order to obtain site concentrations and acidity constants for the proton-active functional groups present in the bacterial EPS and cell

wall. The non-electrostatic model was chosen, neglecting the effects of surface charge. FITEQL calculates the variance, $V(Y)$, between the experimental data and the model: a variance of less than 20 is considered a good fit to the experimental data.

2.2.6. Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR)

Lyophilised cells (both native and EPS-free) were rehydrated in 18 MO ultrapure water, and then centrifuged for 10 minutes at 12,000 rpm. The cell pellets were then resuspended in 0.01 M NaClO_4 and four samples were prepared each for the native and EPS-free cells, with pH values adjusted to approximately 3, 5, 8 and 10. Sodium perchlorate was chosen as the electrolyte in preference to NaNO_3 , as a large nitrate peak obscures the carboxyl signal at approximately 1400 cm^{-1} , creating difficulties in spectrum interpretation. The suspensions were centrifuged again for 10 minutes at 12,000 rpm to remove excess electrolyte. The wet cell pastes were then loaded directly onto the ATR cell. Analysis was conducted using a Bruker Tensor 27 ATR-FTIR spectrometer equipped with a 45 degree germanium ATR crystal. Background scans using reserved supernatant from the cell suspensions were run prior to each sample, and 500 scans were collected both for the background and for each sample, at a resolution of 2 cm^{-1} . Background subtraction and baseline correction were undertaken using the IR software package OPUS 5.5.

2.2.7. Fourier transform infrared spectroscopy (FTIR)

Cells were suspended in 18 MO ultrapure water and three samples each were prepared for both native and EPS-free cells. Suspensions were adjusted to pH values of approximately 3, 5 and 8, then centrifuged for 10 minutes at 12,000 rpm in order to remove excess water. The cell pellets were then freeze dried prior to FTIR analysis. The lyophilised cells were ground with FTIR-grade KBr at a concentration of 1 % cells. Infrared pellets (1 g) were prepared by pressurising the KBr/cell mixture at 10 tonnes for approximately 90 seconds. FTIR analysis was conducted

using a JASCO FT/IR-410 spectrometer. Background scans were conducted using a KBr pellet, and 500 scans were collected for both the background and the samples.

2.2.8. Dissolved organic carbon (DOC) analysis

Batches of both native and EPS-free cells were pH-adjusted across a range of approximately pH 3 to 7 and equilibrated on a rotating rack for a period of 1 hr (corresponding to the equilibration time used for subsequent metal adsorption experiments, see Chapters 4 and 5). The samples were centrifuged for 10 minutes at 12,000 rpm and the supernatant decanted. The supernatant was then filtered (0.2 μm cellulose acetate membrane syringe filters) and stored in ashed glass vials with lids containing Teflon septa. Samples were stored frozen for subsequent DOC analysis, which was carried out with a Shimadzu 6000 TOC/TDN analyser, which incorporates high-temperature catalytic oxidation and a non-dispersive infrared detector. In each case, blank determinations were undertaken and sample DOC concentrations were corrected accordingly. External calibration standards were prepared from a stock solution of 1 g L⁻¹ potassium hydrogen phthalate. Dry weights were calculated for these cell suspensions, thus enabling a comparison of the DOC concentration produced for native and EPS-free cells.

2.3. Results

2.3.1. Cell characterisation by SEM imaging

Extracellular polymers are polysaccharide-rich and are not stabilised by the fixation protocols commonly used for biological SEM sample preparation (Erlandsen et al., 2004). These polymers are liable to collapse during standard SEM dehydration processes as they are generally bound to each other only by weak electrostatic forces (Merroun et al., 2003). Pretreatment with ruthenium red enhances imaging by stabilising the EPS via cross-links formed between the polymers and by facilitating osmium deposition (Merroun et al., 2003; Erlandsen et al., 2004). SEM analysis of the samples showed a clear difference in appearance between the native and EPS-

free cells. Figure 2.1a is an image of native *B. licheniformis* cells. A fibrous substance can clearly be seen coating the cell surfaces and draped between cells. This covering of extracellular polymers is absent or minimal on cells that were treated with cationic exchange resin (Figure 2.1b).

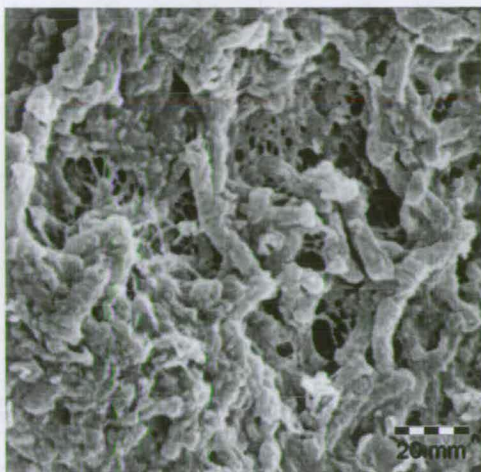


Figure 2.1a: SEM image of native *B. licheniformis* cells. (20 μm = 20 μm).

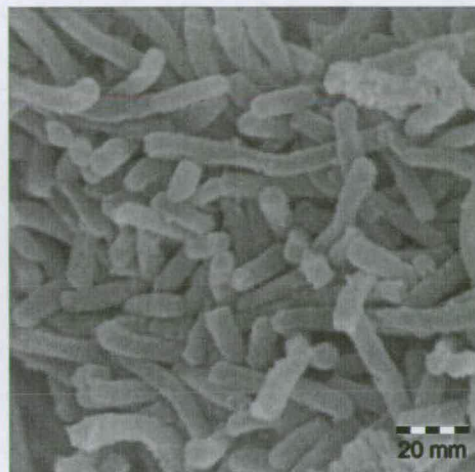


Figure 2.1b: SEM image of EPS-free *B. licheniformis* cells. (20 μm = 20 μm).

2.3.2. Potentiometric titrations

2.3.2.1. Native cells

Figure 2.2a shows representative titration curves at different bacterial concentrations plotted as T_H vs. pH (Equation 2.1). The bacterial buffering capacity derives from the deprotonation of functional groups on the cell surface and in the EPS layer. Weak inflection points on the titration curves for both the native and EPS-free cells indicate the deprotonation of a number of different functional groups, across overlapping pH ranges. It can be seen that buffering capacity increased with cell concentrations, as the number of available surface sites increased.

2.3.2.2. EPS-free cells

Three representative titration curves for EPS-free *B. licheniformis* cells at varying cell concentrations are shown in Figure 2.2b. Again, buffering capacity increases with increasing cell concentration. Although cell concentrations are not exactly

duplicated between the treatments, a quick comparison between Figures 2.2a and 2.2b indicates that the native cells have a higher buffering capacity relative to the EPS-free cells (for example, the 15.53 g L⁻¹ EPS-free experiment shows a lower buffering capacity than the 12.43 g L⁻¹ untreated experiment), suggesting that the extraction of EPS removed some of the proton-active sites. By using surface-complexation modelling, it should be possible to quantify site concentrations for the two experiments, which will allow us to establish whether (i) all of the sites are affected equally, and, if not, (ii) which sites are predominantly associated with EPS.

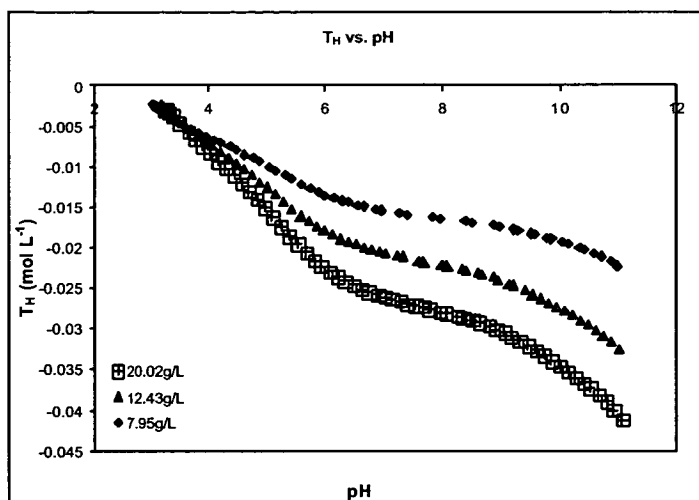


Figure 2.2a: Potentiometric titration curves for native *B. licheniformis* cells in 0.01 M NaNO₃.

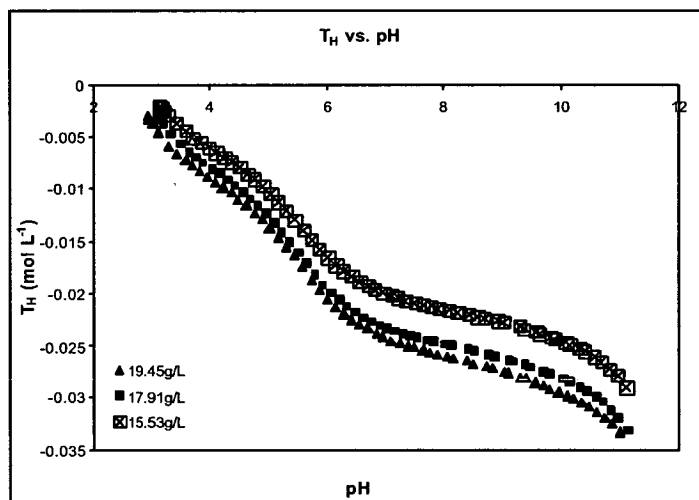


Figure 2.2b: Potentiometric titration curves for EPS-free *B. licheniformis* cells in 0.01 M NaNO₃.

2.3.3. Titration modelling

2.3.3.1. Native cells

The FITEQL 4.0 results, based on a discrete-site non-electrostatic surface complexation model, for the native *B. licheniformis* cell titrations are shown in Table 2.1. Average values and standard deviations for the eight titrations are also shown in Table 2.1. The modelling indicated that the data was optimally described by a 4-site model. A 3-site model could not adequately account for the entire buffering capacity, and the models did not converge at 5 sites. The pK_a values obtained, with corresponding 1 x standard deviations are 3.4 ± 0.2 , 5.3 ± 0.1 , 7.4 ± 0.2 and 9.9 ± 0.1 . The site concentrations and corresponding 1 x standard deviations (normalised to the dry mass of bacteria in each titration) were $8.53 \pm 2.45 \times 10^{-4} \text{ mol g}^{-1}$, $9.06 \pm 2.14 \times 10^{-4} \text{ mol g}^{-1}$, $2.87 \pm 0.59 \times 10^{-4} \text{ mol g}^{-1}$ and $6.50 \pm 1.44 \times 10^{-4} \text{ mol g}^{-1}$. T^0_H for the untreated cells was determined as $1.79 \pm 0.61 \times 10^{-3} \text{ mol g}^{-1}$.

2.3.3.2. EPS-free cells

Table 2.2 shows the FITEQL results for the individual titrations undertaken using EPS-free *B. licheniformis* cells and averaged pK_a , site concentration and $V(Y)$ values for the 4 titrations. As for the native cells, the FITEQL results indicated that the data was optimally fit by a 4-site model, with pK_a values and 1 x standard deviations of approximately 3.3 ± 0.1 , 5.4 ± 0.2 , 7.5 ± 0.4 and 10.1 ± 0.2 . The site concentrations and their corresponding 1 x standard deviations were $5.47 \pm 1.13 \times 10^{-4} \text{ mol g}^{-1}$, $6.82 \pm 1.09 \times 10^{-4} \text{ mol g}^{-1}$, $2.22 \pm 0.62 \times 10^{-4} \text{ mol g}^{-1}$ and $3.26 \pm 0.68 \times 10^{-4} \text{ mol g}^{-1}$. T^0_H for the EPS-free cells was $4.54 \pm 2.43 \times 10^{-4} \text{ mol g}^{-1}$.

Comparison between the two datasets using the student's t-test (for $n_1 = 8$ and $n_2 = 4$, $df = n_1 + n_2 - 2 = 10$, $t_{\text{critical}} = 2.22$ ($p = 0.05$)) shows that pK_a values for the first 3 sites may be considered identical, as t_s for each site is below the t_{critical} value of 2.22. The t_s value of 3.35 obtained for the fourth site indicates a statistical difference between the pK_a values for the two cell treatments. The site concentrations are all lower for the EPS-free cells compared to the native cells. Comparison of the datasets by t-test indicates that the first and fourth sites have significantly different concentrations.

Because surface complexation modelling does not provide reliable functional group identity, IR analysis was used to investigate whether it was possible to (i) correlate the titration/modelling data with changes in specific peak intensities and (ii) identify the chemical nature of the functional groups affected by EPS removal.

Table 2.1: FITEQL results for native *B. licheniformis* cells.

C (g L ⁻¹)	pK _a 1	C1 (mol g ⁻¹)	pK _a 2	C2 (mol g ⁻¹)	pK _a 3	C3 (mol g ⁻¹)	pK _a 4	C4 (mol g ⁻¹)	TH ₀ (mol g ⁻¹)	V(Y)
7.95	3.20	8.76 x 10 ⁻⁴	5.21	9.16 x 10 ⁻⁴	7.03	9.03 x 10 ⁻⁴	9.81	5.17 x 10 ⁻⁴	-2.00 x 10 ⁻³	0.73
8.85	3.09	9.99 x 10 ⁻⁴	5.19	8.44 x 10 ⁻⁴	7.18	2.66 x 10 ⁻⁴	9.91	5.35 x 10 ⁻⁴	-1.80 x 10 ⁻³	0.78
9.41	3.34	1.20 x 10 ⁻³	5.27	1.21 x 10 ⁻³	7.44	3.75 x 10 ⁻⁴	9.98	8.84 x 10 ⁻⁴	-2.64 x 10 ⁻³	0.92
9.63	3.35	1.16 x 10 ⁻³	5.26	1.17 x 10 ⁻³	7.33	3.62 x 10 ⁻⁴	9.94	8.34 x 10 ⁻⁴	-2.57 x 10 ⁻³	0.82
10.37	3.59	7.28 x 10 ⁻⁴	5.32	9.40 x 10 ⁻⁴	7.49	2.83 x 10 ⁻⁴	9.96	6.95 x 10 ⁻⁴	-1.70 x 10 ⁻³	0.85
12.43	3.53	7.00 x 10 ⁻⁴	5.29	8.83 x 10 ⁻⁴	7.43	2.60 x 10 ⁻⁴	9.92	6.45 x 10 ⁻⁴	-1.45 x 10 ⁻³	0.81
14.20	3.16	6.25 x 10 ⁻⁴	5.11	5.67 x 10 ⁻⁴	7.63	2.47 x 10 ⁻⁴	9.97	5.85 x 10 ⁻⁴	-8.73 x 10 ⁻⁴	1.69
20.02	3.65	5.42 x 10 ⁻⁴	5.33	7.12 x 10 ⁻⁴	7.57	2.00 x 10 ⁻⁴	9.97	5.09 x 10 ⁻⁴	-1.32 x 10 ⁻³	0.78
Mean	3.37	8.53 x 10 ⁻⁴	5.25	9.06 x 10 ⁻⁴	7.39	2.87 x 10 ⁻⁴	9.93	6.50 x 10 ⁻⁴	-1.13 x 10 ⁻³	
1x SD	0.21	2.45 x 10 ⁻⁴	0.075	2.14 x 10 ⁻⁴	0.20	5.86 x 10 ⁻⁵	0.06	1.44 x 10 ⁻⁴	1.60 x 10 ⁻³	

Table 2.2: FITEQL results for EPS-free *B. licheniformis* cells.

C (g L ⁻¹)	pK _a 1	C1 (mol g ⁻¹)	pK _a 2	C2 (mol g ⁻¹)	pK _a 3	C3 (mol g ⁻¹)	pK _a 4	C4 (mol g ⁻¹)	TH ₀ (mol g ⁻¹)	V(Y)
10.23	3.31	7.41 x 10 ⁻⁴	5.02	5.31 x 10 ⁻⁴	8.03	3.14 x 10 ⁻⁴	10.34	4.23 x 10 ⁻⁴	-8.09 x 10 ⁻⁴	1.31
15.53	3.42	4.67 x 10 ⁻⁴	5.51	7.78 x 10 ⁻⁴	7.43	1.92 x 10 ⁻⁴	10.14	3.16 x 10 ⁻⁴	-2.59 x 10 ⁻⁴	0.79
17.91	3.32	5.08 x 10 ⁻⁴	5.49	7.45 x 10 ⁻⁴	7.41	1.87 x 10 ⁻⁴	10.16	2.99 x 10 ⁻⁴	-3.90 x 10 ⁻⁴	0.91
19.45	3.13	4.99 x 10 ⁻⁴	5.43	6.75 x 10 ⁻⁴	7.17	1.94 x 10 ⁻⁴	9.88	2.65 x 10 ⁻⁴	-3.60 x 10 ⁻⁴	0.82
Mean	3.29	5.47 x 10 ⁻⁴	5.36	6.82 x 10 ⁻⁴	7.51	2.22 x 10 ⁻⁴	10.13	3.26 x 10 ⁻⁴	-5.54 x 10 ⁻⁴	
1x SD	0.12	1.13 x 10 ⁻⁴	0.23	1.09 x 10 ⁻⁴	0.37	6.17 x 10 ⁻⁵	0.19	6.79 x 10 ⁻⁵	2.43 x 10 ⁻⁴	

2.3.4. Infrared analysis

Figures 2.3a and 2.3b show spectra obtained by ATR-FTIR for native and EPS-free cells respectively, each with pH values adjusted to approximately 3, 5, 8 and 10.

There are a number of peaks in the characteristic microbial 'fingerprint' region between 2000 cm^{-1} and 750 cm^{-1} , appearing at approximately $1708\text{--}1717\text{ cm}^{-1}$ (Peak 1), $1632\text{--}1635\text{ cm}^{-1}$ (Peak 2), $1543\text{--}1548\text{ cm}^{-1}$ (Peak 3), $1454\text{--}1457\text{ cm}^{-1}$ (Peak 4), $1394\text{--}1401\text{ cm}^{-1}$ (Peak 5), $1218\text{--}1224\text{ cm}^{-1}$ (Peak 6), $1080\text{--}1084\text{ cm}^{-1}$ (Peak 7), 1056 cm^{-1} (Peak 7a), $968\text{--}974\text{ cm}^{-1}$ (Peak 8) and 917 cm^{-1} (Peak 9) (Table 2.3). The infrared spectra obtained by analysis with FTIR are shown in Figures 2.4a and 2.4b.

Spectra are shown for both the native and EPS-free samples at pH values of approximately 3, 5 and 8, focussing on the region between approximately 2000 cm^{-1} and 500 cm^{-1} . The major peaks within this region are listed in Table 2.4 and are at approximately 1652 cm^{-1} (Peak 1), 1538 cm^{-1} (Peak 2), 1455 cm^{-1} (Peak 3), $1385\text{--}1394\text{ cm}^{-1}$ (Peak 4), $1231\text{--}1236\text{ cm}^{-1}$ (Peak 5), $1162\text{--}1172\text{ cm}^{-1}$ (Peak 6), 1066 cm^{-1} (peak 7), 963 cm^{-1} (Peak 8) and 915 cm^{-1} (Peak 9).

Variations in the baseline absorbance and the higher absorbance by EPS-free samples for the ATR-FTIR samples indicate that sample absorbance was significantly affected by variations in the cell densities of the wet pastes used. The FTIR spectra may also show changes in absorbance that are due to inconsistencies in sample preparation rather than changes in cell surface chemistry. Changes in absorbance as a function of pH will therefore be discussed below with reference to normalised spectra.

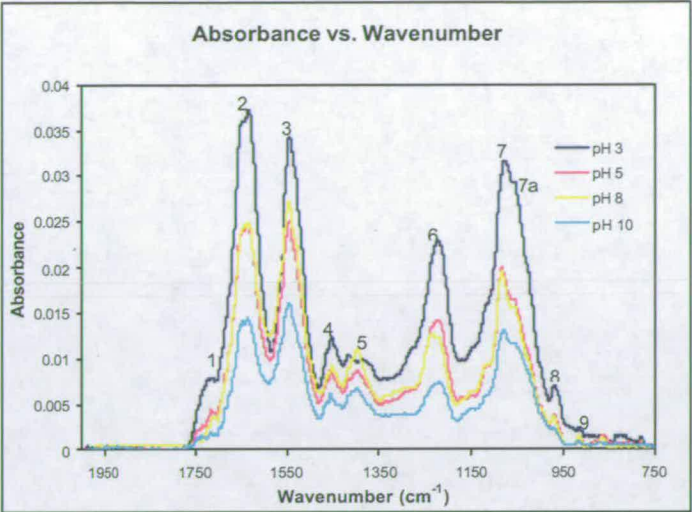


Figure 2.3a: ATR- FTIR spectra for native *B. licheniformis* cells.

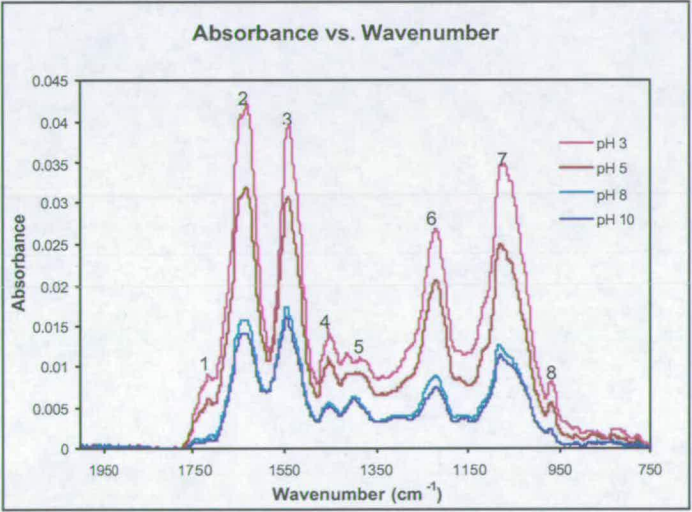


Figure 2.3b: ATR- FTIR spectra for EPS-free *B. licheniformis* cells.

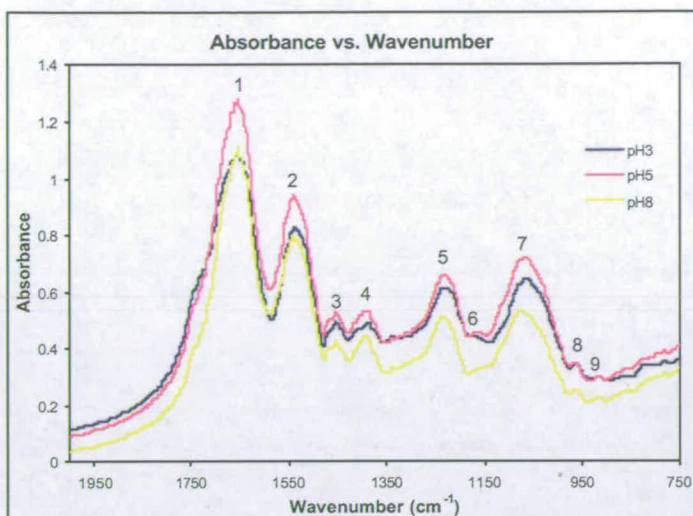


Figure 2.4a: FTIR spectra for native *B. licheniformis* cells (baseline-corrected).

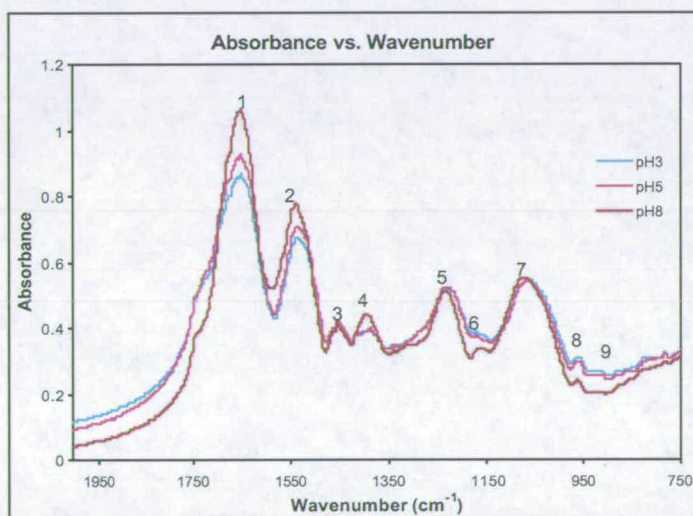


Figure 2.4b: FTIR spectra for EPS-free *B. licheniformis* cells (baseline-corrected).

2.3.5. Dissolved organic carbon analysis

Figure 2.5 shows DOC production as a function of pH for known concentrations of native (0.93 g L^{-1}) and EPS-free (1.24 g L^{-1}) *B. licheniformis* cells. The DOC concentration for the native cell samples increases from approximately 27 mg g^{-1} at pH 3 to 40 mg g^{-1} at pH 6.5. For the EPS-free cells, the concentration is approximately 5 mg g^{-1} at pH 3, and rises to 12 mg g^{-1} at pH 6.5. It can be seen that the DOC concentration in supernatant from the native cells was approximately four times higher than that for the EPS-free cells. This is consistent with DOC results for a range of different suspension concentrations (results not shown).

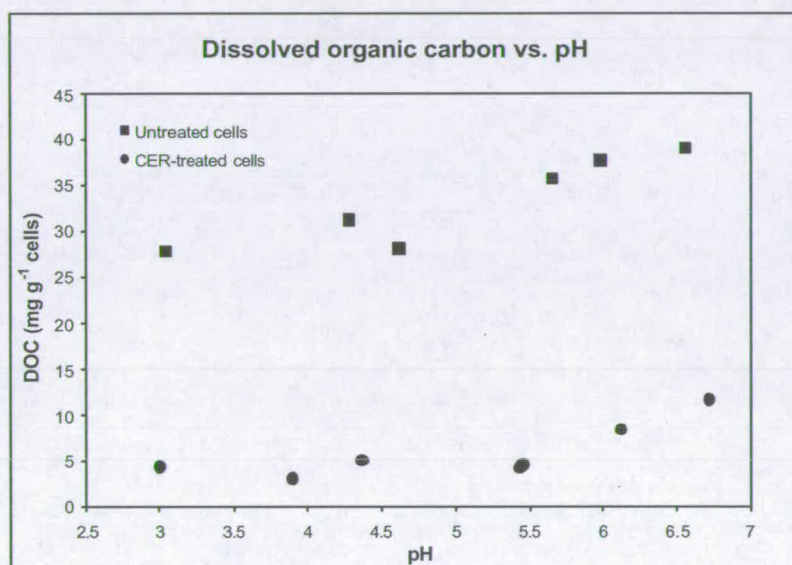


Figure 2.5: Dissolved organic carbon (DOC) results for supernatants from native (untreated) and CER-treated (EPS-free) *B. licheniformis* samples as a function of pH.

2.4. Discussion

2.4.1. EPS extraction protocol

Bacterial EPS may be defined as either 'soluble' or 'bound' (Comte et al., 2006a), the soluble fraction being extractable by centrifugation and the bound fraction requiring a chemical or physical extraction method. As a consequence of this rather flexible definition, EPS extraction protocols are numerous and vary in the level of treatment to which the bacterial cells are subjected. Comte et al. (2006b) investigated the efficiency of a number of different chemical and physical EPS extraction methods and found that the bound EPS composition and quantity varied significantly depending on the method chosen. Chemical treatment methods, though more effective at EPS removal, were liable to introduce contamination into the EPS which was undesirable as the extracted EPS was to be preserved for future DOC analysis. In order to minimise any risk of chemical alteration to the residual cell walls, it was decided to use a physical extraction method, treatment by cationic exchange resin, to remove the EPS layer from the *B. licheniformis* cells. This method enables EPS separation by removing cations from, and hence weakening, the EPS structure (Frolund et al., 1996). The SEM images obtained (Figures 2.1a and 2.1b) indicate that this method successfully removed most EPS from the cells and more intrusive chemical extraction methods were not necessary in this case. Figure 2.1a shows the substantial EPS coating on the untreated bacteria, supporting the finding of Toner et al. (2005) that repeated electrolyte rinses did not remove the EPS coating from cell aggregates. Any EPS removed during the washing process belongs to the 'soluble' fraction. The adsorption properties of soluble EPS also merit further investigation, as transportation of any metals bound to this fraction is likely to be significantly enhanced in aqueous environments (Chen et al., 1995, Czajka et al., 1997). The 'bound' EPS that is the subject of this study will remain associated with the bacterial cells under most conditions, as part of a biofilm in many environmental settings, so is likely to have an inhibitory effect upon the transportation of any bound ions (Warren and Haack, 2001). There is no visible evidence of any structural alteration in the EPS-free cells compared to the native cells, indicating that this method of EPS extraction does not cause any significant damage.

As the cationic exchange resin is acidic, there is a possibility that this treatment method may affect the buffering capacity of the cell wall. However, it has been observed that acid-washing of bacterial cells, a preparation step routinely carried out in early adsorption studies, increases the concentration of available surface sites, possibly by displacing structural Mg and Ca cations (Borrok et al., 2004c). As the EPS-free cells showed a decrease in available sites compared to the native cells (Tables 2.1 and 2.2), it may be concluded that the cationic exchange resin probably did not cause displacement of structural cations. If this did occur, data obtained would lead to an underestimate of the contribution made by the EPS fraction to proton adsorption by *B. licheniformis* S-86.

2.4.2. Surface functional groups

The titration curves in Figures 2.2a and 2.2b are similar in shape, showing the weak inflection points indicative of buffering by bacterial cell surfaces. Comparison of the experimental data curves indicates that the EPS-free cells have lower buffering capacities than the native cells across the titration range of approximately pH 3–pH 11.

It was decided to optimise the data with FITEQL 4.0, using a non-electrostatic model, as the electrostatic effects of bacterial cell surfaces are as yet poorly understood or quantified (Fein et al., 2005; Borrok et al., 2005). In particular, the presence of EPS on cell surfaces makes models based on surface capacitive effects meaningless. This is in contrast to the majority of previous studies, which have used a range of modelling approaches, including the constant capacitance model (Daughney and Fein, 1998; Ngwenya et al., 2003) and the diffuse and triple layer models (Borrok and Fein, 2005). Generally, electrostatic models such as the constant capacitance approach converge to three sites (Daughney and Fein, 1998, Haas et al., 2001; Ngwenya et al., 2003), whereas non-electrostatic models tend to indicate four surface sites (Borrok et al., 2004a, 2004b). Other approaches, such as the Linear Programming Method (LPM), have produced 5-site models (Cox et al., 1999; Sokolov et al., 2001; Dittrich and Sibbler, 2006). Martinez et al. (2002) used a fully

optimised continuous pK_a distribution method (FOCUS) to identify four surface sites on *B. subtilis* and *E. coli*.

The similar findings of a wide range of studies, employing a number of different data modelling approaches, highlights the issue of non-uniqueness in optimization of these data sets. The study by Borrok and Fein (2005) involved modelling a data set using non-electrostatic, diffuse and triple-layer models. Despite these approaches being conceptually different, they are mechanistically similar and very similar results were obtained. Fein et al. (2005) conducted potentiometric titrations and optimized the data with non-electrostatic, constant capacitance and Langmuir-Freundlich models. Again, each approach provided an excellent fit to the data. Developing a robust data modelling strategy will require more detailed investigation of the bacterial cell surface and its immediate surroundings, perhaps by spectroscopic means, in order to place more rigorous physical and chemical constraints on the proton adsorption environment.

The deprotonation constants and site concentrations obtained here (Tables 2.1 and 2.2) are comparable to those obtained by similar studies. Burnett et al. (2006a) suggested a 3-site model for *Anoxybacillus flavithermus*, with pK_a 1 = 4.94, pK_a 2 = 6.85, pK_a 3 = 7.85 and site concentrations of $5.33 \times 10^{-4} \text{ mol g}^{-1}$, $1.79 \times 10^{-4} \text{ mol g}^{-1}$ and $1.42 \times 10^{-4} \text{ mol g}^{-1}$. Borrok et al. (2004a) investigated natural bacterial consortia and obtained a 4-site model with average pK_a values of 3.12, 4.70, 6.57 and 8.99, with corresponding site densities (based on wet weights) of $6.65 \times 10^{-5} \text{ mol g}^{-1}$, $6.78 \times 10^{-5} \text{ mol g}^{-1}$, $3.68 \times 10^{-5} \text{ mol g}^{-1}$ and $4.47 \times 10^{-5} \text{ mol g}^{-1}$. The study by Chatellier and Fortin (2004) using *B. subtilis* converged to a 3-site model with pK_a values of 4.45, 6.74 and 9.08 and site concentrations of $5.6 \times 10^{-4} \text{ mol g}^{-1}$, $5.3 \times 10^{-4} \text{ mol g}^{-1}$ and $6.1 \times 10^{-4} \text{ mol g}^{-1}$ respectively. The total site densities for this study are 2.70×10^{-3} and $1.78 \times 10^{-3} \text{ mol g}^{-1}$ dry bacteria for the native and EPS-free cells respectively. These values are therefore comparable to those found for other gram-positive bacteria such as *B. subtilis*, and only slightly higher than comparable thermophiles such as *Anoxybacillus flavithermus*, which was found to have a total site density of $8.54 \times 10^{-4} \text{ mol g}^{-1}$ (Burnett et al., 2006a). Borrok et al. (2005) carried out nonelectrostatic modelling for 160 bacterial potentiometric titration data sets and obtained an average site density of $3.2 \times 10^{-4} \text{ mol wet g}^{-1}$. Based on a mean wet:dry

weight ratio of 8:1 for bacterial cells (Borrok et al., 2005), this is equivalent to 2.6×10^{-3} mol dry g⁻¹, which is comparable with the results of this study. As the cells are not fully protonated at pH 3 and the cells cannot be adjusted to sufficiently low pH to achieve a 'zero proton' condition without causing cellular damage, the charge imbalance (T^0_H) was treated as an adjustable parameter in FITEQL (Westall et al., 1995; Borrok et al., 2005; Fein et al., 2005). The value of $1.79 \pm 0.61 \times 10^{-3}$ mol g⁻¹ obtained for the native cells is significantly larger ($p = 0.05$) than the value obtained for the EPS-free cells ($4.54 \pm 2.43 \times 10^{-4}$ mol g⁻¹), which indicates that the native cells have a greater buffering capacity than the EPS-free cells. The standard deviations for the site concentrations are fairly high for both native and EPS-free cells. This may reflect variation in the amount of soluble EPS removed during washing of the native cells, and in the case of the EPS-free cells may indicate some inconsistency in the efficiency of the EPS removal.

The pK_a values obtained for the native and EPS-free *B. licheniformis* cell titrations are very similar, indicating that the same proton-active functional groups are present in the EPS fraction as in the bulk cell wall. As yet, there is no general agreement on the identities of the major bacterial cell-wall functional groups. Biochemically, cell walls are complex structures. The peptidoglycan layer is rich in teichoic and teichuronic acids, which contain phosphoryl and carboxyl groups. Hydroxyl, amine and phosphodiester groups are also present in the peptidoglycan (Beveridge, 1989; Cox et al., 1999; Ngwenya et al., 2003). The composition of EPS is highly variable, but consists mostly of polysaccharides, with substantial amounts of protein (Merroun et al., 2003). In addition, the EPS may host a range of bacterial exudates, such as nucleic acids and lipids (Guine et al., 2006).

As deprotonation constants are known for a wide range of aqueous organic acids, experimental values have in the past been compared with known pK_a values in order to identify the functional groups on the bacterial cell wall. This procedure relies on the assumption that organic acids in the cell wall deprotonate across the same pH ranges as when in aqueous solution. Generally, pK_a values between 2 and 6 are ascribed to carboxyl groups, pK_a values of 5.6-7.2 to phosphoryl groups and pK_a values of approximately 10 to amine or hydroxyl groups (Ngwenya et al., 2003). Some studies have ascribed sites with pK_a values around 4 to phosphodiester groups

(Sokolov et al., 2001; Martinez et al., 2002; Guine et al., 2006). Based on comparison of our data with published functional group pK_a data, Site 1 is tentatively assigned to a phosphodiester, Site 2 is either carboxyl or phosphoryl, Site 3 is likely a phosphoryl group and Site 4 is most likely to correspond to an amine group.

Although the t-test results indicated a significant difference between the native and EPS-free pK_a values for site 4, both pK_a values fall within the range that may be ascribed either to amine groups. As yet, spectroscopic evidence has not permitted confirmation of the identities of these principle functional groups, although recent studies suggest that metal adsorption at low pH ($pH < 4$) occurs primarily to phosphoryl groups rather than carboxyl sites (Hennig et al., 2001; Kelly et al., 2002; Boyanov et al., 2003).

The lower site concentrations observed in the EPS-free cell suspensions, particularly for the first and fourth groups, indicate that EPS contributes a relatively large proportion of these functional groups to the overall proton adsorption capacity of *B. licheniformis* S-86.

2.4.3. Infrared analysis

As macroscopic adsorption modelling does not provide definitive identification of surface functional groups, infrared analysis was undertaken in order to further probe the origin of qualitative differences and/or similarities between native and EPS-free bacterial cells. In addition, variations in peak absorption intensity between samples can indicate the relative concentrations of the corresponding functional groups (Jiang et al., 2004). These variations may therefore indicate quantitative changes in sample composition depending upon whether the cell samples contain EPS or not.

As shown in Figures 2.3a and 2.3b, direct comparison between ATR-FTIR samples cannot be made in this case because the absorption intensity varies between native and EPS-free cells, with higher intensities recorded for EPS-free cells. Analysis of the wet to dry weight scaling indicates that this arises because of the higher cell density (approximately 5:1) in EPS-free cells, and consequent deposition of relatively more cells on the surface of the ATR crystal. Because ATR-FTIR analysis involves the use of samples in the form of wet cell pastes, their consistencies are

difficult to precisely control by centrifugation alone. Additionally, such small samples are subject to changes in moisture content over the duration of the analysis, and this was observed when consecutive scans were conducted on a given sample. To permit direct comparison between treatments, the absorption data was normalised to the amide II peak at approximately 1545 cm^{-1} in order to isolate pH-dependent spectral variations from those due to variations in cell density (Figures 2.6a and 2.6b). This peak has been used in other studies as its intensity does not vary as a function of pH (Heinrich et al., 2007). In the present case, the use of this peak provides an internal scaling mechanism such that the amide II peak has an absorbance of unity for all samples. The FTIR spectra were also normalised to the amide II peak in order to remove the effects of varying sample concentration. The normalised spectra are shown in Figures 2.7a and 2.7b.

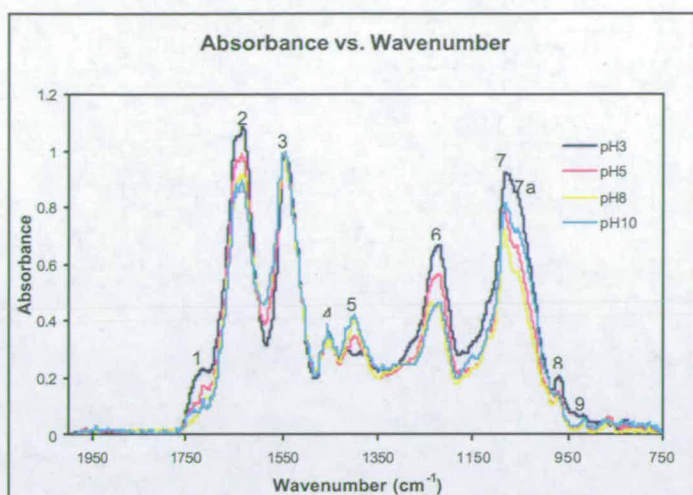


Figure 2.6a: ATR-FTIR spectra for native *B. licheniformis* cells (normalised to the amide II peak).

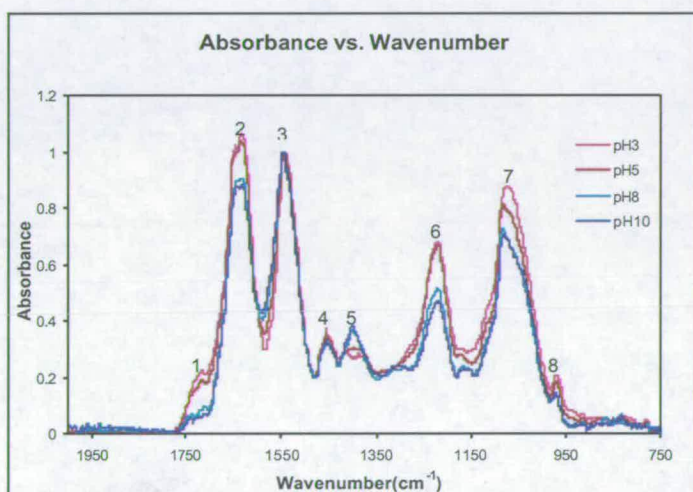


Figure 2.6b: ATR-FTIR spectra for EPS-free *B. licheniformis* cells (normalised to the amide II peak).

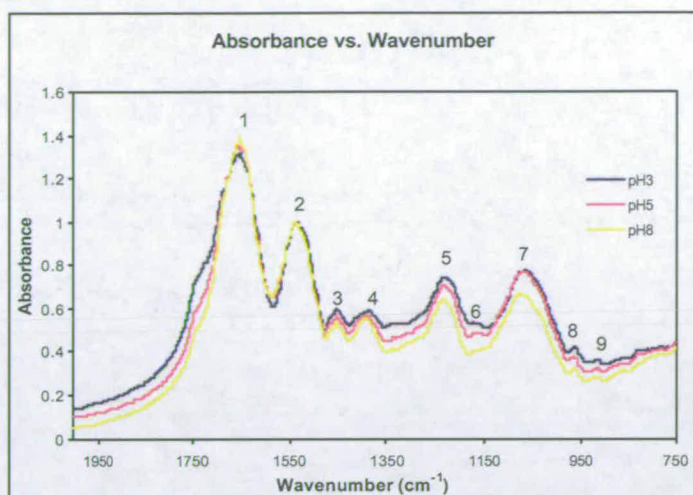


Figure 2.7a: FTIR spectra for native *B. licheniformis* cells (baseline-corrected and normalised to the amide II peak).

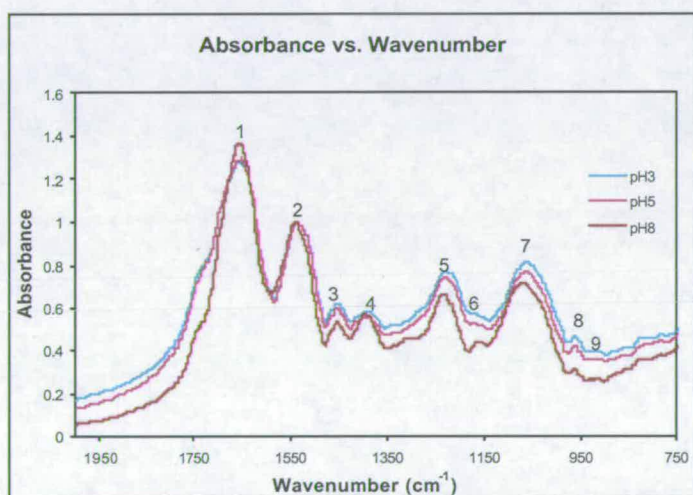


Figure 2.7b: FTIR spectra for EPS-free *B. licheniformis* cells (baseline-corrected and normalised to the amide II peak).

Table 2.3: ATR-FTIR peaks. Peaks identified according to Pagnanelli et al. (2000), Benning et al. (2004), Jiang et al. (2004), Guibaud et al. (2005), Burnett et al. (2006b), Dittrich and Sibling (2006) and Heinrich et al. (2007. *1: N= native cells, *2: C= EPS-free cells.

Peak No.	N ^{*1} pH 3	Abs	N pH 5	Abs	N pH 8	Abs	N pH 10	Abs	C ^{*2} pH 3	Abs	C pH 5	Abs	C pH 8	Abs	C pH 10	Abs	N3/C3	N5/C5	N8/C8	N10/C10	Peak Identity
1	1716	0.231	1714	0.174	1708	0.134	1717	0.127	1717	0.227	1717	0.194	1711	0.095	1712	0.071	1.017	0.897	1.410	1.789	COOH, Carboxylic acids
2	1633	1.084	1634	0.987	1633	0.915	1635	0.897	1634	1.062	1634	1.033	1632	0.899	1633	0.879	1.021	0.955	1.018	1.020	C=O, Amide I
3	1544	1.00	1544	1.00	1548	1.00	1544	1.00	1543	1.00	1544	1.00	1547	1.00	1547	1.00	*	*	*	*	N-H and C-N, Amide II
4	1455	0.363	1456	0.354	1455	0.337	1457	0.386	1456	0.370	1456	0.363	1454	0.328	1455	0.330	0.981	0.975	1.027	1.169	CH ₂ and CH ₃ , Amide III
5			1398	0.349	1398	0.404	1399	0.417	1394	0.269	1398	0.305	1400	0.367	1401	0.381	*	1.144	1.101	1.094	COO-
6	1223	0.666	1223	0.566	1222	0.455	1224	0.464	1222	0.677	1218	0.663	1222	0.512	1220	0.469	0.836	0.854	0.889	0.989	COOH and P=O, Carboxyls and phosphodiester
7	1080	0.922	1083	0.799	1084	0.724	1082	0.815	*	*	1082	0.810	1083	0.726	1081	0.705	1.060	0.986	0.997	1.156	P(OH) ₂ , Phosphodiester
7a			1056	0.665	1056	0.578	1055	0.725	*	*	*	*	*	*	*	*	*	*	*	*	C-OH, PO ₂ - and P(OH) ₂ contributions
8	969	0.207	970	0.149	971	0.134	973	0.147	968	0.209	969	0.184	971	0.135	972	0.139	0.990	0.810	0.992	1.058	
9	917	0.068	916	0.055	916	0.055	917	0.056	*	*	*	*	*	*	*	*	*	*	*	*	C-O, Polysaccharides

Table 2.4: FTIR peaks. Peaks identified according to Pagnanelli et al (2000), Jiang et al (2004), Guibaud et al (2005) and Burnett et al (2006b). *1: N= native cells, *2: C= EPS-free cells.

Peak No.	N ^{*1} pH 3	Abs	N pH 5	Abs	N pH 8	Abs	C ^{*2} pH 3	Abs	C pH 5	Abs	C pH 8	Abs	N3/C3	N5/C5	N8/C8	Peak Identity
1	1651	1.091	1651	1.283	1652	1.111	1651	0.867	1652	0.930	1651	1.065	1.26	1.38	1.04	Amide I (C=O/ C-N stretch)
2	1537	0.830	1537	0.940	1538	0.798	1537	0.676	1538	0.713	1538	0.779	1.23	1.32	1.02	Amide II (N-H bend/ C-N stretch)
3	1455	0.493	1455	0.531	1455	0.427	1455	0.417	1455	0.426	1455	0.416	1.18	1.25	1.02	Amide III (CH2/ C-OH bend)
4	1385	0.488	1393	0.529	1394	0.444	1386	0.393	1387	0.399	1394	0.445	1.24	1.33	1.00	Carboxyl (COO- stretch)
5	1231	0.614	1232	0.659	1236	0.510	1231	0.519	1232	0.527	1234	0.513	1.18	1.25	0.99	Phosphodiester (P=O stretch) or Carboxyl (C=O bend)
6	1171	0.442	1169	0.453	NP	NA	1171	0.387	1172	0.375	1162	0.337	1.14	1.21	NA	Phosphate (P=O stretch)
7	1065	0.644	1066	0.720	1078	0.529	1065	0.549	1065	0.547	1066	0.552	1.17	1.32	0.96	Phosphodiester (P-O stretch)
8	963	0.346	964	0.351	964	0.254	963	0.312	962	0.299	963	0.241	1.11	1.17	1.05	Phosphate (P-OH stretch or P-O-C stretch)
9	915	0.297	915	0.300	916	0.226	916	0.267	914	0.257	915	0.207	1.11	1.17	1.09	

Table 2.3 details the peak positions and relative absorbances of the major peaks observed in the region between approximately 2000 cm^{-1} and 750 cm^{-1} for the ATR-FTIR samples. It can be seen that there were small variations in wavenumber for each peak as pH increased, but these variations generally did not show any consistent trend, with the exception of Peak 8, which shifted to higher wavenumbers as pH increased. Peak 7a is a poorly-defined shoulder on the larger Peak 7, and was observed only in the native pH 5, 8 and 10 samples. Peak 9 was not observed at all in the EPS-free samples. Absorbance generally decreased with increasing pH. However, for several peaks in spectra from both native and EPS-free samples, a slight increase in absorbance was observed at pH 10. Peak 5 showed increasing absorbance with pH for both native and EPS-free samples.

The major peaks observed in the FTIR spectra are listed in Table 2.4. The positions of Peaks 1, 2 and 3 are quite consistent across all 6 samples. Peak 7 is shifted to 1078 cm^{-1} in the native pH 8 sample, and Peak 4 can be seen to shift towards higher wavenumbers as pH increases. Peak 5 also shows shifts to slightly higher wavenumbers with increasing pH. Peak 6 does not appear in the native pH 8 sample. Figures 2.7a and 2.7b show variations in peak height (extent of absorption) as a function of sample pH for native and EPS-free cells respectively. For the native cells, the pH 5 sample shows the greatest absorbance and the pH 8 sample shows the least absorbance, with the exception of Peak 1, where the pH 3 and pH 8 samples absorb to a similar extent. The pH 8 sample shows significantly less absorption for Peaks 8 and 9. For the EPS-free cells (Figure 2.7b), the pH 8 sample shows the greatest absorbance for Peaks 1, 2 and 4, with the pH 3 sample showing the least absorbance. The 3 samples absorb to similar extents at Peaks 3, 5 and 7. Significantly less absorption is seen in the pH 8 sample for Peaks 8 and 9.

It can be seen that both methods of infrared analysis produce very similar spectra, with the same functional groups identifiable from both ATR-FTIR and FTIR. In this case, the FTIR method has the advantage of producing more defined peaks. However, the results obtained by ATR FTIR are more consistent with respect to the spectra obtained as a function of pH, as described below.

The spectra obtained are consistent with those obtained by other studies using bacterial biomass (Pagnanelli et al., 2000; Omoike and Chorover, 2004; Jiang et al., 2004; Guibaud et al., 2005; Burnett et al., 2006b; Dittrich and Sibbler, 2006; Heinrich et al., 2007). Tables 2.3 and 2.4 list the identities assigned to the major peaks based on comparison with previous studies. Several of the peaks can be assigned to particular functional groups with confidence. However, there is difficulty in assigning identities to those peaks found between approximately 950 cm^{-1} and 1250 cm^{-1} . In this region, overlapping absorbance ranges of both carboxylic and phosphoryl groups lead to more than one functional group contributing to the position and intensity of any given peak and there is consequently no consensus in the literature as to the identities of these peaks.

Both the ATR-FTIR and FTIR data shows some variation in peak position with changing pH (Figures 2.6a, 2.6b, 2.7a and 2.7b). This is due to the effects of surface group speciation changes, which lead directly to major changes in corresponding peak absorbances, and potentially also affect the positions and absorbances of neighbouring peaks. The amide peaks are affected by the speciation changes of overlapping carboxyl peaks, and are also subject to variations caused by changes in protein conformations (Heinrich et al., 2007; Dittrich and Sibler, 2006; Jiang et al., 2004). A further potential cause of observed non-systematic changes is that spectra may be affected by the composition of the cell interior as well as the cell wall (Jiang et al., 2004).

Although infrared analysis is not a fully quantitative technique, previous studies have observed changes in absorption intensity with pH that have been ascribed to changes in protonation state of the functional groups present. The ATR-FTIR results detailed in Figures 2.6a and 2.6b and Table 2.3 indicate decreasing peak intensity with increasing pH for the protonated carboxyl group (Peak 1) that appears as a shoulder on the amide I peak ($1632\text{--}1635\text{ cm}^{-1}$). The spectral range at which this peak occurs (approximately $1708\text{--}1717\text{ cm}^{-1}$) is towards the lower end of the range of positions at which this peak has been observed in other studies. Jiang et al. (2004), Dittrich and Sibbler (2006) and Heinrich et al. (2007), in studies of intact bacterial and cyanobacterial cells and isolated bacterial cell walls, observed this peak at approximately 1720 cm^{-1} . However, Omoike and Chorover (2004) analysed EPS

isolated from *B. subtilis* and observed this peak at 1714 cm^{-1} . The lower spectral range observed in the present study may therefore be a consequence of the EPS-producing nature of *B. licheniformis* S-86. The corresponding deprotonated carboxyl group (at approximately 1398 cm^{-1}) is indistinct or absent at pH 3 and becomes increasingly intense as pH increases (Figures 2.6a and 2.6b). These characteristic changes in carboxyl group speciation were also observed in cell and EPS infrared analysis by Jiang et al. (2004), Omoike and Chorover (2004), Dittrich and Siblir (2006) and Heinrich et al. (2007). It can be seen from Figures 2.6a and 2.6b that there was a large increase in absorption for the carboxyl anion between pH 5 and pH 8 and that there was little difference between the pH 8 and pH 10 peaks. This deprotonation range supports the assignment of site 2, with pK_a 5.3-5.4, to a carboxyl group, since functional groups generally deprotonate over a pH range that is equivalent to the $\text{pK}_a \pm 2$. For FTIR analysis the results do not show clear trends. The --COO^- peak at approximately 1400 cm^{-1} is apparent in all of the spectra obtained, and the absorbance intensity of this peak increases with pH in the FTIR samples, with the exception of that for the native pH 8 sample, which is similar to the absorbance for the pH 3 sample. The protonated carboxyl group (peak 1 in the ATR-FTIR spectra) is not observed in any of the FTIR spectra. The inconsistent speciation behaviour observed in the FTIR spectra compared to the ATR-FTIR spectra may be because samples analysed by ATR-FTIR in their original state (as a wet paste in this case) are better able to retain their protonation state than those lyophilised and mixed with KBr for FTIR analysis. In addition, the analysis may have been affected by the thickness of the FTIR discs. Generally, these results indicate that ATR-FTIR provides more consistent results for samples of this kind, and subsequent discussion will therefore focus on interpretation of the ATR-FTIR spectra.

Speciation changes in the phosphoryl groups in bacterial cell walls were also observed by Heinrich et al. (2007), who identified a phosphodiester group at 1086 cm^{-1} . This peak also appeared in the ATR-FTIR spectra in the present study, and showed a tendency towards lower absorbance as pH increased, with the exception of the native pH 10 sample, which showed slightly increased absorbance. However, this peak overlapped with another peak at approximately 1056 cm^{-1} , so changes in

the phosphoryl group speciation cannot be resolved. None of the other peaks can be confidently identified as phosphoryl, as they are influenced by contributions from overlapping carboxylic groups. The amide I and amide III peaks also showed declining absorbances with increased pH, again with slight increases in absorbance at pH 10. As for the phosphodiester group, the overall trend towards lower absorption is in accordance with deprotonation of these groups. However, peaks indicating the appearance of corresponding anions cannot be identified in the spectra and are likely obscured by larger overlapping peaks. Generally, infrared analysis is of only limited use for identification of surface functional groups, due to the many overlapping peaks in the important spectral region. This is consistent with the findings of Heinrich et al. (2007).

The slight increase in absorbance observed for a number of peaks at pH 10 may be explained by changes in residual cell surface chemistry as a consequence of DOC release by the cells (Figure 2.5). The background scans were conducted using supernatant reserved from the cell suspensions (Jiang et al., 2004) and this background was subsequently subtracted from the sample spectra, which should remove any effects due to DOC in solution. It was shown by Ngwenya (2007) that DOC production increased with pH for both live and heat-killed cell suspensions of an *Enterobacter* species. Although much of the DOC in that study was believed to originate from cell lysis, the greater concentration released by live cells suggested a contribution from cellular exudates. In this study, only live cells were used, thus the substantially greater concentration of DOC released by native cells (Figure 2.5) indicates that the EPS layer was a major contributor to DOC released into solution. DOC release from the EPS-free cells may indicate other sources, such as DOC release by cells as response to the electrolyte environment, or cell lysis, although it is likely that residual traces of EPS also contribute. The observed increase in DOC in solution with pH may also reflect desorption of hydrophobic fractions from the cell surface (Borrok et al., 2007). Analysis of total dissolved nitrogen (TDN) may help to identify the DOC source as C:N ratios are likely to be very different for intracellular material compared to that for DOC derived from EPS. If DOC release affects the residual cell surface chemistry, this may have small effects on peak absorbances and

positions, and consequently may have masked some pH-dependent speciation changes.

2.4.4. Correlation between titration modelling and infrared analysis

The FITEQL data modelling results (Tables 2.1 and 2.2) indicated significant differences in functional group concentrations between the native and EPS-free cells, and that this decrease was particularly marked (approximately 2- fold) in the pK_a 1 (from $8.53 \pm 2.45 \times 10^{-4} \text{ mol g}^{-1}$ to $5.47 \pm 1.13 \times 10^{-4} \text{ mol g}^{-1}$) and pK_a 4 sites (from $6.50 \pm 1.44 \times 10^{-4} \text{ mol g}^{-1}$ to $3.26 \pm 0.68 \times 10^{-4} \text{ mol g}^{-1}$), which have been tentatively identified as phosphodiester and amine, respectively.

In order to quantify the difference in the extent of absorption observed after EPS had been removed from cells, ratios of the absorbance intensity (using amide II peak normalised values) of native cells to EPS-free cells were calculated (Tables 2.3 and 2.4). Plotting difference spectra (Benning et al., 2004; Heinrich et al., 2007) was not considered appropriate in this case, as there were small shifts in peak position between samples. The titration data modelling results suggest that highest ratios should be seen for groups corresponding to the pK_a 1 and pK_a 4 sites, as the native cells are known to have contained significantly higher concentrations of these sites than the EPS-free cells. Conversely, absorbance ratios for the pK_a 2 site should be closest to 1. As both data modelling and IR results suggest that site 2 is carboxylic, it may be expected that the protonated carboxyl peak (Peak 1 in the ATR-FTIR spectra) and the carboxyl anion (Peak 5 in the ATR-FTIR spectra) would display peak absorbance ratios close to 1. However, the ratios for Peak 1 range from 0.897 at pH 5 to 1.789 at pH 10 for the ATR-FTIR spectra. For Peak 5, the ratios are more consistent, ranging between 1.094 at pH 10 to 1.144 at pH 5 (Peak 1 and Peak 5 absorbance ratios are shown graphically in Figure 2.8). This disparity may be a consequence of the large amide I peak overlapping with Peak 1 and affecting the observed absorbances. Peak 5 is relatively unaffected by overlapping peaks and this may explain the more consistent absorbance ratios observed. The absorbance ratios calculated for the FTIR spectra generally increase slightly from pH 3 to pH 5, then decrease to values close to 1.0 for the pH 8 spectra.

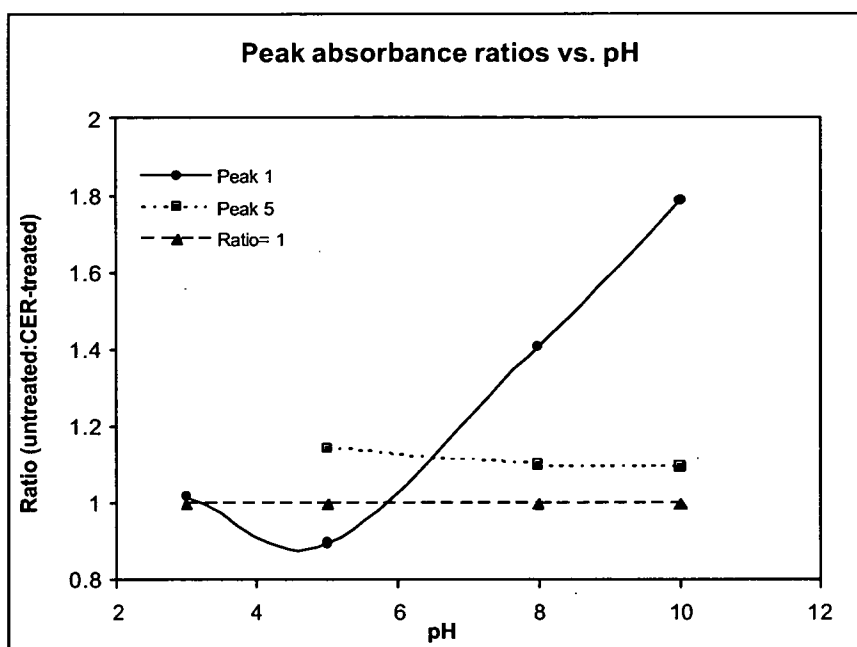


Figure 2.8: Peak absorbance ratios for peaks 1 and 5, as a function of pH (ATR-FTIR spectra). Native: EPS-free absorbance ratio of 1 is shown for reference.

The similarity in composition between the native and EPS-free cells suggests that changes in functional group speciation, and corresponding peak absorbance ratios, should remain consistent as a function of pH. However, Figure 2.5 suggests that a significant proportion of the EPS layer is solubilised as DOC, to a slightly greater extent as pH increases. The IR spectra should therefore show increasingly similar spectra for native and EPS-free cells as pH increases and the EPS layer is lost from the native cells as DOC, and absorbance ratios should approach 1 at high pH. Table 2.3 shows that, with the exception of Peak 1, absorbance ratios remain fairly constant with changing pH for each peak, and there is no consistent trend towards absorbance ratios of 1 at high pH. The variations observed here may be explained by the effects of overlapping peaks, which are particularly pronounced for Peak 1, as this appears as a shoulder on the much larger Peak 2.

This interpretation is necessarily cautious, owing firstly to the difficulties in assigning identities to the four surface groups indicated by the FITEQL modelling, and secondly to the ambiguities involved in relating some IR peaks to particular functional groups. Furthermore, the effect of DOC release on concentrations of residual cell surface and EPS functional groups is unknown, and is likely to have had

a significant effect upon the IR spectra observed. A further complication may stem from conformational changes that cause pH-dependent absorption in the UV, and possibly the IR, regions (Ngwenya et al., 2007; Heinrich et al., 2007). The loosely-bound EPS layer may be more susceptible to such conformational changes than the EPS-free cell wall. Considering these limitations, it is perhaps unsurprising that the calculated ratios for the ATR-FTIR spectra do not show any consistent trends, either for particular peaks or as a function of pH. The absorbance ratios for the FTIR spectra do show an overall decrease to values close to 1 as pH increases, which suggests that aspects of the solution chemistry, avoided by the use of lyophilised cells, may be obscuring the expected changes in the ATR-FTIR absorbance ratios.

2.4.5. Implications for metal adsorption and modelling

Recent studies have indicated that metal adsorption to bacterial cell surfaces occurs preferentially to certain functional groups as a function of pH (Sarret et al., 1998; Burnett et al., 2006b). The presence of EPS could therefore have a significant effect on the amount of metal adsorption observed, especially under conditions in which adsorption is likely to occur primarily to the relatively abundant phosphodiester or amine groups. A small number of studies have assessed the metal-complexation characteristics of isolated EPS (Guibaud et al., 2003; 2004; Comte et al., 2006c; Lamelas et al., 2006), but the contribution to metal adsorption made by EPS in situ merits further investigation. Future studies should aim to assess the likelihood that cells will produce significant quantities of EPS under the experimental conditions. This assessment should also be made when attempting to apply experimental results to environmental settings.

In order to confidently predict the proton and metal adsorption behaviour by bacterial cells in the natural environment, it is necessary to attain a consensus on the identities of the principle functional groups involved, and on the peak identities in the bacterial IR spectrum. Furthermore, the issue of non-uniqueness in data optimisation modelling for biological surfaces such as bacterial cells should be addressed. In order to attain a consistent model, a more thorough understanding of the three-

dimensional cell wall environment and the associated electrostatic interactions must be attained.

Finally, it has been suggested that the production of EPS by many bacterial species is a response to varying environmental conditions, and EPS composition is known to vary according to growth conditions (Lamelas et al., 2006). It is also believed that EPS production can occur as a response to metal exposure, probably in order to protect the cell from toxic metal concentrations by means of sequestration within the EPS (Toner et al., 2005). This may be a contributing factor to the increased metal complexation capacities of bacteria isolated from contaminated environments compared to those from uncontaminated environments (Borrok et al., 2004b).

The results of this part of the project indicate that metal adsorption studies should consider the potential involvement of cellular EPS in proton and metal adsorption studies, particularly those aimed at investigating the effect of variables such as growth temperature and nutrient availability, and also studies investigating differences in adsorption properties between species. The effect of EPS on metal adsorption to bacterial cell walls was investigated as part of this project by means of an integrated macroscopic and spectroscopic study. The results are discussed in Chapter 4.

2.5. Conclusions

This study has shown that the extracellular polymers produced by *B. licheniformis* S-86 contribute significantly to the proton absorption capacity of the bacterial cells. Chemical equilibrium modelling of the data using FITEQL 4.0 indicated that the EPS contains the same proton-active surface functional groups as does the bulk cell wall, but these groups are present in slightly different proportions. Specifically, removal of EPS causes the concentrations of the pK_a 3.3-3.4 (phosphodiester) and pK_a 9.9-10.1 (amine) functional groups to decrease by up to 50 %. At present, assigning identities to the functional groups indicated by the data optimisation program can only be tentative, owing to the lack of consensus on these group identities between recent macroscopic and spectroscopic studies.

Infrared analysis qualitatively supports the titration data. The EPS-free samples showed very similar spectra to the native samples, indicating that the identities of the dominant functional groups present do not differ between the EPS and the bulk cell wall, and these groups are not altered by the EPS-extraction procedure. The ATR-FTIR spectra clearly show the changing speciation of the carboxyl group as a function of pH, and the pH range over which this occurs (pH 3-pH 8) supports the identification of site 2 as carboxylic. Peaks corresponding to the phosphoryl surface sites are less easily identified, owing to the presence of numerous overlapping peaks in the relevant spectral regions. The IR analysis undertaken here is not sufficiently diagnostic to resolve differences in functional group concentrations between native and EPS-free cells. Whilst FTIR provides a stronger signal, the ATR-FTIR results for samples analysed as a function of pH are more consistent. This suggests that ATR-FTIR, using samples in their native state, is probably more appropriate when investigating samples for which preservation of the initial protonation state is important.

This study has also indicated that substantial DOC is released from cells, increasing slightly with raised pH, with the untreated cells producing approximately four times as much DOC as the EPS-free cells. This may have a significant effect upon cell surface chemistry, and hence IR spectra, particularly at high pH. The release of DOC into solution may also affect the observed metal adsorption characteristics, particularly for EPS-producing strains. Further investigation of the solution chemistry, incorporating TDN and carbohydrate analysis, will further distinguish intra- and extra-cellular sources of the DOC released.

The findings of this study have highlighted the significance of EPS as a contributor to both bacterial cell surface chemistry and, owing to DOC release, solution chemistry. The environmental prevalence of EPS-producing bacterial strains ensures that studies aiming to characterise and quantify the bacterial contributions to metal binding and cycling should aim to incorporate EPS and DOC as important system variables.

2.6. References

- Benning, L.G., Phoenix, V.R., Yee, N., Konhauser, K.O. (2004) The dynamics of cyanobacterial silicification: An infrared micro- spectroscopic investigation. *Geochimica et Cosmochimica Acta* 68, 743-757
- Beveridge, T.J. (1989) Metal ions and bacteria. In: Beveridge, T.J., Doyle, R.J. (Eds.), *Metal Ions and Bacteria*, pp 1-29
- Borrok, D., Fein, J.B. (2004) Distribution of protons and Cd between bacterial surfaces and dissolved humic substances determined through chemical equilibrium modelling. *Geochimica et Cosmochimica Acta* 68, 3043-3052
- Borrok, D.M, Fein, J.B. (2005) The impact of ionic strength on the adsorption of protons, Pb, Cd, and Sr onto the surfaces of Gram negative bacteria: testing non-electrostatic, diffuse and triple-layer models. *Journal of Colloid and Interface Science* 286, 110-126
- Borrok, D., Fein, J.B., Kulpa, C.F. (2004a) Proton and metal adsorption onto natural bacterial consortia: testing universal adsorption behaviour. *Geochimica et Cosmochimica Acta* 68, 3231-3238
- Borrok, D.M., Fein, J.B., Kulpa, C.F. (2004b) Cd and Proton Adsorption onto Bacterial Consortia Grown from Industrial Wastes and Contaminated Geologic Settings. *Environmental Science and Technology* 38, 5656-5664
- Borrok, D., Turner, B.F., Fein, J.B. (2005) A Universal Surface Complexation Framework For Modelling Proton Binding Onto Bacterial Surfaces In Geologic Settings. *American Journal of Science* 305, 826-853
- Borrok, D., Aumend, K., Fein, J.B. (2007) Significance of ternary bacteria-metal-natural organic matter complexes determined through experimentation and chemical equilibrium modelling. *Chemical Geology* 238, 44-62
- Borrok, D., Fein, J.B., Tischler, M., O'Loughlin, E., Meyer, H., Liss, M., Kemner, K.M. (2004c) The effect of acidic solutions and growth conditions on the adsorptive properties of bacterial surfaces. *Chemical Geology* 209, 107-119

- Boyanov, M.I., Kelly, S.D., Kemner, K.M., Bunker, B.A., Fein, J.B., Fowle, D.A. (2003) Adsorption of cadmium to *Bacillus subtilis* bacteria cell walls: A pH-dependent X-ray absorption fine structure spectroscopy study. *Geochimica et Cosmochimica Acta* 67, 3299-3311
- Breedveld, M., Bonting, K., Dijkhuizen, L. (1998) Mutational analysis of exopolysaccharide biosynthesis by *Lactobacillus sakei* 0-1. *FEMS Microbiology Letters* 169, 241-249
- Burnett, P-G., Heinrich, H., Peak, D., Bremer, P.J., McQuillan, A.J., Daughney, C.J. (2006a) The effect of pH and ionic strength on proton adsorption by the thermophilic bacterium *Anoxybacillus flavithermus*. *Geochimica et Cosmochimica Acta* 70, 1914-1927
- Burnett, P-G., Daughney, C.J., Peak, D. (2006b) Cd adsorption onto *Anoxybacillus flavithermus*: Surface complexation modelling and spectroscopic investigations. *Geochimica et Cosmochimica Acta* 70, 5253-5269
- Chatellier, X., Fortin, D. (2004). Adsorption of ferrous ions onto *Bacillus subtilis* cells. *Chemical Geology* 212, 209-228
- Chen, J-H., Lion, L.W., Ghiorse, W.C., Schuler, M.L. (1995) Mobilization of adsorbed calcium and lead in aquifer material by bacterial extracellular polymers. *Water Research* 29, 421-430
- Comte, S., Guibaud, G., Baudu, M. (2006a) Biosorption properties of extracellular polymeric substances (EPS) resulting from activated sludge according to their type: Soluble or bound. *Process Biochemistry* 41, 815-823
- Comte, S., Guibaud, G., Baudu, M. (2006b) Relations between extraction protocols for activated sludge extracellular polymeric substances (EPS) and complexation properties of Pb and Cd with EPS. Part I. Comparison of the efficiency of eight EPS extraction methods. *Enzyme and Microbial Technology* 38, 246-252
- Comte, S., Guibaud, G., Baudu, M. (2006c) Relations between extraction protocols for activated sludge extracellular polymeric substances (EPS) and complexation properties of Pb and Cd with EPS. Part II. Consequences of EPS extraction methods on Pb²⁺ and Cd²⁺ complexation. *Enzyme and Microbial Technology* 38, 246-252

- Cox, J.S., Smith, D.S., Warren, L.A., Ferris, F.G. (1999) Characterising Heterogeneous Bacterial Surface Functional Groups Using Discrete Affinity Spectra for Proton Binding. *Environmental Science and Technology* 33, 4514-4521
- Czajka, D.R., Lion, L.W., Shuler, M.L., Ghiorse, W.C. (1997) Evaluation of the utility of bacterial extracellular polymers for treatment of metal-contaminated soils: Polymer persistence, mobility, and the influence of lead. *Water Research* 31, 2827-2839
- Daughney, C.J., Fein, J.B. (1998) The Effect of Ionic Strength on the Adsorption of H^+ , Cd^{2+} , Pb^{2+} , and Cu^{2+} by *Bacillus subtilis* and *Bacillus licheniformis*: A Surface Complexion Model. *Journal of Colloid and Interface Science* 198, 53-77
- Daughney, C.J., Fowle, D.A., Fortin, D. (2001) The effect of growth phase on proton and metal adsorption by *Bacillus subtilis*. *Geochimica et Cosmochimica* 65, 1025-1035
- Dittrich, M. and Sibling, S. (2006) Influence of H^+ and calcium ions on the surface functional groups of *Synechococcus* PCC 7942 cells. *Langmuir* 22, 5435-5442
- Erlandsen, S.L., Kristich, C.J., Dunny, G.M, Wells, C.L. (2004) High-resolution Visualisation of the Microbial Glycocalyx with Low-voltage Scanning Electron Microscopy: Dependence on Cationic Dyes. *Journal of Histochemistry and Cytochemistry* 52, 1427-1435
- Fein, J.B. (2000) Quantifying the effects of bacteria on adsorption reactions in water-rock systems. *Chemical Geology* 169, 265-280
- Fein, J.B., Boily, J-F., Yee, N., Gorman-Lewis, D., Turner, B. (2005) Potentiometric titrations of *Bacillus subtilis* cells to low pH and a comparison of modelling approaches. *Geochimica et Cosmochimica Acta* 69, 1123-1132
- Frolund, B., Palmgren, R., Keiding, K., Nielsen, P.H. (1996) Extraction of extracellular polymers from activated sludge using a cation exchange resin. *Water Research* 30, 1749-1758
- Guibaud, G., Tixier, N., Bouju, A., Baudu, M. (2003) Relation between extracellular polymers' composition and its ability to complex Cd, Cu and Pb. *Chemosphere*, 52, 1701-1710

- Guibaud, G., Tixier, N., Bouju, A., Baudu, M. (2004) Use of a polarographic method to determine copper, nickel and zinc constants of complexation by extracellular polymers extracted from activated sludge. *Process Biochemistry* 39, 833-839
- Guibaud, G., Comte, S., Bordas, F., Dupuy, S., Baudu, M. (2005) Comparison of the complexation potential of extracellular polymeric substances (EPS), extracted from activated sludges and produced by pure bacterial strains, for cadmium, lead and nickel. *Chemosphere* 59, 629-638
- Guine, V., Spadini, L., Sarret, G., Muris, M., Delolme, C., Gaudet, J.-P., Martins, M.F. (2006) Zinc Sorption to Three Gram- Negative Bacteria: Combined Titration, Modeling, and EXAFS Study. *Environmental Science and Technology* 40, 1806-1813
- Haas, J.R. (2004) Effects of cultivation conditions on acid- base titration properties of *Shewanella putrefaciens*. *Chemical Geology* 209, 67-81
- Haas, J.R., Dichristina, T.J., Wade, R. (2001) Thermodynamics of U(VI) sorption onto *Shewanella putrefaciens*. *Chemical Geology* 180, 33-54
- Heinrich, H.T.M., Bremer, P.J., Daughney, C.J., McQuillan, A.J. (2007) Acid- base titrations of functional groups on the surface of the thermophilic bacterium *Anoxybacillus flavithermus*: Comparing a chemical equilibrium model with ATR-IR spectroscopic data. *Langmuir* 23, 2731-2740
- Hennig, C., Panak, P.J., Reich, T., Rossberg, A., Raff, J., Selenska- Pobell, S., Matz, W., Bucher, J.J., Bernhard, B., Nitsch, H. (2001) EXAFS investigation of uranium (VI) complexes formed at *Bacillus cereus* and *Bacillus sphaericus* surfaces. *Radiochimica Acta*, 89, 625-631
- Herbelin, A.L., Westall, J.C. (1999) FITEQL 4.0: a Computer Program for Determination of Chemical Equilibrium Constants from Experimental Data; Report 99-01. Department of Chemistry Oregon State University, Corvallis
- Hong, Y., Brown, D.G. (2006) Cell surface acid- base properties of *Escherichia coli* and *Bacillus brevis* and variation as a function of growth phase, nitrogen source and C:N ratio. *Colloids and Surfaces B: Biointerfaces* 50, 132-139

- Jiang, W., Saxena, A., Song, B., Ward, B.B., Beveridge, T.J., Myneni, S.C.B. (2004) Elucidation of Functional Groups on Gram- Positive and Gram- Negative Bacterial Surfaces Using Infrared Spectroscopy. *Langmuir* 20, 11433-11442
- Kelly, S.D., Kemner, K.M., Fein, J.B., Fowle, D.A., Boyanov, M.I., Bunker, B.A., Yee, N. (2002) X-ray absorption fine structure determination of pH-dependent U-bacterial cell wall interactions. *Geochimica et Cosmochimica Acta* 66, 3855-3871
- Lamelas, C., Benedetti, M., Wilkinson, K.J., Slaveykova, V.I. (2006) Characterization of H^+ and Cd^{2+} binding properties of the bacterial exopolysaccharides. *Chemosphere* 65, 1362-1370
- Ledin, M., Krantz- Rulcker, C., Allard, B. (1999) Microorganisms as metal sorbents: comparison with other soil constituents in multi- component systems. *Soil Biology and Biochemistry* 31, 1639-1648
- Liu, Y.; Yang, C-H and Li, J. (2007) Influence of extracellular polymeric substances on *Pseudomonas aeruginosa* transport and deposition profiles in porous media. *Environmental Science and Technology* 41, 206-213
- Martinez, R.E., Smith, D.S., Kulczycki, E., Ferris, F.G. (2002) Determination of Intrinsic Bacterial Surface Acidity Constants using a Donnan Shell Model and a Continuous pK_a Distribution Method. *Journal of Colloid and Interface Science* 253, 130-139
- Merroun, M.L., Ben Chekrou, K., Arias, J.M., Gonzalez- Munoz, M.T. (2003) Lanthanum fixation by *Myxococcus xanthus*: cellular location and extracellular polysaccharide observation. *Chemosphere* 52, 113-120
- Ngwenya, B.T. (2007) Enhanced adsorption of zinc is associated with aging and lysis of bacterial cells in batch incubations. *Chemosphere*, 67, 1982-1992
- Ngwenya, B.T., Sutherland, I.W., Kennedy, L. (2003) Comparison of the acid-base behaviour and metal adsorption characteristics of a gram-negative bacterium with other strains. *Applied Geochemistry* 18, 527-538
- Omoike, A., Chorover, J. (2004) Spectroscopic Study of Extracellular Polymeric Substances from *Bacillus subtilis*: Aqueous Chemistry and Adsorption Effects. *Biomacromolecules* 5, 1219-1230

- Pagnanelli, F., Petrangeli Papini, M., Toro, L., Trifoni, M., Veglio, F. (2000) Biosorption of Metal Ions on *Arthrobacter* sp.: Biomass Characterisation and Biosorption Modelling. *Environmental Science and Technology* 34, 2773-2778
- Penttilä, A., Kalimo, H., Trump, B.F. (1974) Influence of glutaraldehyde and/or osmium tetroxide on cell volume, ion content, mechanical stability and membrane permeability of Ehrlich ascites tumor cells. *The Journal of Cell Biology*, 63, 197-214
- Sarret, G., Manceau, A., Spadini, L., Roux, J-C., Hazemann, J-L., Soldo, Y., Eyebert-Berard, L., Menthonnex, J-J. (1998) Structural determination of Zn and Pb binding sites in *Penicillium chrysogenum* cell walls by EXAFS spectroscopy. *Environmental Science and Technology* 32, 1648-1655
- Sokolov, I., Smith, D.S., Henderson, G.S., Gorby, Y.A., Ferris, F.G. (2001) Cell Surface Electrochemical Heterogeneity of the Fe(III)- Reducing Bacteria *Shewanella putrefaciens*. *Environmental Science and Technology* 35, 341-347
- Toner, B., Manceau, A., Marcus, M.A., Millet, D.B., Sposito, G. (2005) Zinc sorption by a bacterial biofilm. *Environmental Science and Technology* 39, 8288-8294
- Volesky, B. (2001) Detoxification of metal- bearing effluents: biosorption for the next century. *Hydrometallurgy* 59, 203-216
- Warren, L.A., Haack, E.A. (2001) Biogeochemical controls on metal behaviour in freshwater environments. *Earth-Science Reviews* 54, 261-320
- Westall, J.C., Jones, J.D., Turner, G.D., Zachara, J.M. (1995) Models for association of metal ions with heterogeneous environmental sorbents. 1. Complexation of Co(II) by leonardite humic acid as a function of pH and NaClO₄ concentration. *Environmental Science and Technology* 29, 951-959
- Wong, P.K., Lam, K.C., So, C.M. (1993) Removal and recovery of Cu(II) from industrial effluent by immobilized cells of *Pseudomonas putida* II-11. *Applied Microbiology and Biotechnology* 39, 127-131
- Yee, N., Fein, J. (2001) Cd adsorption onto bacteria surfaces: A universal adsorption edge? *Geochimica et Cosmochimica Acta* 65, 2037-2042

3. Investigating the effect of an EPS layer on the electrostatic properties of a bacterial strain.

Abstract

The production of extracellular polymeric substances (EPS) by bacteria significantly alters the physical structure of the cell wall-solution interface. This study investigated the effect of the EPS layer on the electrostatic properties of the cell surface by conducting potentiometric titration data modelling and electrophoretic mobility (EM) analysis using both native cells, with the EPS-layer intact, and EPS-free cells of the gram-positive thermophile, *Bacillus licheniformis* S-86. Modelling of potentiometric titration data was undertaken using the data optimisation program FITMOD, which incorporates electrostatic correction using the Donnan core-shell model. The modelling showed that both native and EPS-free cells have 4 proton-active surface sites. However, no differences were found with regard to the effect of the EPS layer on the Donnan volume. Nevertheless, the modelling indicated that the native cells had a higher total site concentration than the EPS-free cells, in agreement with previous non-electrostatic modelling.

Electrophoretic mobility measurements indicated that the isoelectric point for both cell types was below pH 2. At low ionic strength (0.001 M), expansion of the EPS layer causes the native cells to display lower EM than the EPS-free cells. As ionic strength increases, this effect decreases and at 0.1 M the native cells have a higher EM than the EPS-free cells, in agreement with the FITMOD modelling and also previous nonelectrostatic modelling of potentiometric titration results which indicated that there is a relatively high charge density in the EPS layer.

3.1. Introduction

A large number of studies have investigated proton and metal adsorption to bacterial cell surfaces in recent years. Much of the recent work has focussed on accurately constraining the adsorption mechanisms, by means of both macroscopic batch experiments and spectroscopic analysis (Fein et al., 1997; Haas et al., 2001; Boyanov et al., 2003; Ngwenya et al., 2003; Chatellier and Fortin, 2004; Toner et al., 2005; Guine et al., 2006). Since bacteria develop surface charge, it is intuitive to assume that surface complexation modelling of macroscopic adsorption data would require characterisation of the electrostatic interactions between the cell surface and the electrolyte in which the adsorption reaction is taking place. However, this has proven difficult owing to the complex nature of the bacterial cell surface—specifically, the difficulty in determining the bacterial surface potential, which cannot be directly measured.

Most approaches to modelling the electrostatic component of surface adsorption reactions were originally formulated for use in modelling adsorption to well-characterised, two-dimensional mineral surfaces and are not well suited for application to the complex bacterial surface environment. Early studies made use of the constant capacitance model (Fein et al., 1997; Daughney et al., 1998; Ngwenya et al., 2003), which relates cell surface charge to surface potential by the capacitance. This approach is of limited relevance to bacterial surfaces owing to the difficulty in assigning a realistic capacitance to the bacterial surface, and the value of 8 F m^2 used in the above-mentioned studies is now generally considered unrealistically high. Owing to these difficulties, many studies have opted for a non-electrostatic approach, which undertakes surface complexation modelling without inclusion of an electrostatic component (Chatellier and Fortin, 2004; Gorman-Lewis et al., 2005; Wightman and Fein, 2005; Claessens and Van Capellen, 2007; Johnson et al., 2007). Neither the constant capacitance approach nor the non-electrostatic approach accurately represents the reality of the cell surface environment.

Recently, a number of studies have adopted the Donnan core-shell model as a more mechanistically realistic means of accounting for electrostatic interactions at the bacterial cell surface. The Donnan model represents the cell wall as a 3-dimensional,

ion-permeable, volume rather than a 2-dimensional, ion-impermeable surface, with a specific potential known as the Donnan potential. The Donnan model approach has been used successfully to describe bacterial proton adsorption by Plette et al. (1995), Martinez et al. (2002) and Yee et al. (2004). In addition, Hetzer et al. (2006), Burnett et al. (2006a; 2006b) and Heinrich et al. (2007) have applied the Donnan model to thermophilic bacterial species. The studies by Yee et al. (2004), Hetzer et al. (2006) and Burnett et al. (2006b) also used a Donnan model approach to explain metal adsorption to bacterial cells.

The important contribution made by bacterial EPS to proton and metal adsorption is increasingly recognized. Recent work has investigated the contribution made by the EPS layer to proton and metal adsorption by an EPS-producing bacterial strain, *Bacillus licheniformis* S-86. As the presence of an EPS layer on bacterial cells significantly alters the character of the cell-electrolyte interface region compared to cells with no EPS layer, it is important to investigate the effect this has on the electrostatic interactions at the cell-electrolyte interface. However, no study as yet has applied this (or any other) form of electrostatic model to proton or metal adsorption by an extracellular polymeric substance (EPS)-producing bacterial strain. This is potentially an interesting application, as the presence of an EPS-layer can affect the magnitude of the “softness” parameter (Tsuneda et al., 2003) due to an increased shell volume in the general core-shell model.

The aims of this study were to; i) investigate the effectiveness of the Donnan electrostatic model as a means of obtaining intrinsic site deprotonation constants and concentrations for an EPS-producing strain, and (ii) characterise the effect of an EPS layer on the electrophoretic mobility of the cells.

3.2. Materials and Methods

3.2.1. Cell culture, preparation and EPS extraction

Full details of the procedures undertaken for growth and preparation of *Bacillus licheniformis* S-86 cultures are included in Chapter 2. Experimental batches were grown in the Luria Broth growth medium and incubated on a shaker for 24 hours at 30 °C. EPS extraction was undertaken using cationic exchange resin, as described in Chapter 2.

3.2.2. Cell characterisation by SEM imaging

Specimens were prepared for SEM analysis using the cationic dye ruthenium red, based on the method of Erlandsen et al. (2004). Samples were prepared in duplicate of both native and EPS-free cells. Cell suspensions were deposited on polylysine-coated glass coverslips and fixed for 1 hr in 2.5 % glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4, 20 °C) containing 0.15 % ruthenium red for 1 hr, then rinsed in cacodylate buffer 3 times over 15 mins before treatment with buffered 1 % osmium tetroxide for 1 hour (further details in Sections 2.2.3 and 2.3.1). Unbound OsO₄ was then removed by repeated rinses with deionised water and the specimens dehydrated for 5 minutes in each of 70 %, 90 %, 100 % (twice) and anhydrous ethanol. The coverslips were then immersed in hexamethyldisilazane (HMDS) twice before being allowed to air dry. The coverslips were mounted on SEM stubs and gold/palladium sputter-coated. Samples were imaged using a JEOL 6400 SEM at 6 kV.

3.2.3. Cell characterisation by TEM imaging

As SEM images indicated that the EPS does not form a coherent capsule around the bacterial cells (Section 3.3.1), TEM imaging was used to determine the cell wall thickness of EPS-free cells only. Samples were prepared for TEM analysis according to the method of Zufferey et al. (2003), as follows. Cell pellets were fixed for 1 hour at 4 °C in 2.5 % glutaraldehyde (EM grade 1, Sigma) in 0.1 M cacodylate (sodium cacodylate, Agar Scientific) buffer containing 0.15 % ruthenium red

(Sigma), 5 mM CaCl_2 and 5 % sucrose. The samples were then washed 3 times in cacodylate buffer, and then fixed in a solution of 1 % osmium tetroxide, 2 mM CaCl_2 and 0.15 % ruthenium red for 1 hr at room temperature. In order to improve contrast during the electron microscopy, the samples were stained for 30 minutes with 1 % aqueous uranyl acetate (TAAB Laboratories Equipment Ltd.) prior to a graded ethanol dehydration (70 %, 90 %, 100 % and anhydrous ethanol), propylene oxide rinses (2 x 5 mins) and embedding in Epon-Araldite resin (Araldite/Embed 812 kit, Science Services Ltd.). Ultrathin sections were prepared on 200 mesh grids and stained in Reynold's lead citrate (an electron-opaque stain) for 5 mins. Samples were viewed on a Zeiss 912 Omega transmission electron microscope, recording zero-loss images on a 2 K Proscan digital camera system.

3.2.5. Potentiometric titrations

Lyophilised cells were rehydrated in 18 MO ultrapure water for 1 hour, then washed a further two times in ultrapure water. The cells were subsequently rinsed three times in the NaNO_3 electrolyte. Titrations were carried out at 25 °C on 50 ml suspensions of varying cell concentration, in 0.01 M and 0.1 M NaNO_3 . Cell concentrations were determined by sub-sampling 5 ml and evaporating to dryness until a constant weight had been achieved and corrected for electrolyte contribution (Ngwenya, 2007). Biomass concentrations were also calculated in terms of wet weight as this was required for the subsequent data modelling. The suspensions were initially acidified to approximately pH 3 using 1 M HNO_3 , then titrated up to pH 11 with 0.5 M NaOH. The freshly prepared NaOH was transferred to a bottle fitted with a drying tube containing anhydrous NaOH and standardised in triplicate using potassium hydrogen phthalate (KHP). Titrations were carried out using an automated DL53 Mettler-Toledo burette assembly programmed in dynamic mode, so that successive titrant additions were made only when a stability of 0.1 mV s^{-1} had been attained.

3.2.6. Data modelling

Data modelling was undertaken using the data optimisation program FITMOD (supplied by Dr C. Daughney, GNS, New Zealand), a modified version of FITEQL 2.0 (Westall, 1982). FITMOD incorporates the Donnan core-shell electrostatic model and therefore provides intrinsic surface site stability constants and concentrations.

The deprotonation of a functional group can be represented by the following generic reaction (Borrok and Fein, 2004):



Where R is the bacterium and A represents a functional group within the bacterial cell wall (or EPS) volume. The deprotonation or acidity constant, K_a , for this reaction may be expressed as:

$$K_a = \frac{[R - A^{-}]a_{H^{+}}}{[R - AH^{\circ}]} \quad (3.2)$$

where $[R - A^{-}]$ and $[R - AH^{\circ}]$ represent the concentration of deprotonated and protonated sites and $a_{H^{+}}$ represents the activity of protons in the solution.

The intrinsic deprotonation constants (K_a^{int}) are calculated by means of the following equation (Stumm and Morgan, 1996):

$$K_a^{\text{int}} = K_a^{\text{app}} e^{\left(\frac{-ZF\Psi_D}{RT} \right)} \quad (3.3)$$

where K_a^{app} = the deprotonation constant referenced to zero surface charge, Z = charge on the adsorbing ion, F = Faraday's constant, Ψ_D = Donnan potential, R = Gas constant and T = temperature (K).

The Donnan potential is calculated according to soft-particle theory (Ohshima, 1995);

$$\Psi_D = \frac{RT}{zF} \left[\frac{Z\rho_D}{2zc} + \left\{ \left(\frac{Z\rho_D}{2zc} \right)^2 + 1 \right\}^{1/2} \right] \quad (3.4)$$

Where z = electrolyte valency, ρ_D = charge density within the Donnan volume (C m^{-3}), c = electrolyte concentration (mol m^{-3}). The charge density is optimised by FITMOD (Burnett et al., 2006a).

Use of the Donnan model in FITMOD requires input of the following parameters in addition to the potentiometric titration data; i) cell wall thickness (or the cell wall plus EPS-layer thickness for native cells); ii) specific surface area ($\text{m}^2 \text{g}^{-1}$ wet weight); iii) biomass concentration (g L^{-1} wet weight). These parameters then enable the model to calculate the specific cell wall volume, which is taken to be equal to the Donnan volume. The cell wall (and EPS layer) thicknesses and cell dimensions were estimated from TEM and SEM images (Figures 3.1-3.2). The specific surface area (A) could then be calculated according to Hetzer et al. (2006);

$$A = \frac{2}{\rho} \left(\frac{r+1}{r \times l} \right) \quad (3.5)$$

where ρ = density (kg L^{-1}), r = radius, l = length.

The wet biomass concentration was recorded prior to titrations and the wet:dry mass ratio for *B. licheniformis* was obtained as described in Section 2.5 (Chapter 2).

FITMOD calculates the variance, $V(Y)$, between the experimental data and the model: a variance of less than 20 is considered a good fit to the experimental data.

3.2.7. Electrophoretic mobility measurements

Electrophoretic mobility measurements were conducted using a Brookhaven Instruments Corporation Zeta PALS Zeta Potential Analyser. This method utilises Phase Analysis Light Scattering (PALS), which involves measurement of the phase shift of a particle rather than the frequency shift (as in laser Doppler electrophoresis). Dilute (slightly turbid) suspensions of native and EPS-free *B. licheniformis* cells were prepared in 0.001 M, 0.01 M and 0.1 M NaNO₃. In order to conduct mobility determinations as a function of pH, the suspensions were pH-adjusted using HNO₃ and NaOH of ionic strength equal to that of the electrolyte. Approximately 1.6 ml of the cell suspension was transferred to a Perspex cuvette, into which the electrode was inserted. The electric field strength varied between 3 V cm⁻¹ and 8 V cm⁻¹, and electrophoretic mobility values were calculated by the ZetaPALS software according to the field strength at the time of measurement. An average electrophoretic mobility value was obtained for each sample based on 10 replicate measurements (conducted under alternating field directions). The pH of each cell suspension was recorded immediately after electrophoretic mobility analysis was undertaken.

3.3. Results

3.3.1. Cell characterisation by SEM imaging

SEM analysis of the samples showed a clear difference in appearance between the native (untreated) and EPS-free (CER-treated) cells. Figure 3.1a is an image of untreated *B. licheniformis* cells. A fibrous substance can clearly be seen coating the cell surfaces and draped between cells. This covering of extracellular polymers is absent or minimal on cells that were treated with cationic exchange resin (Figure 3.1b). The average cell dimensions obtained from the SEM images of *B. licheniformis* were length= 1.64 ± 0.38 μm , and width= 0.44 ± 0.08 μm .

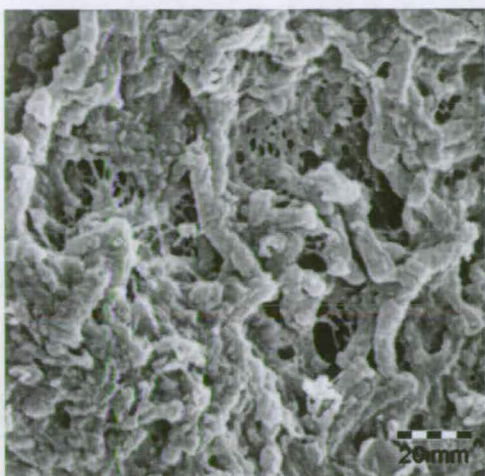


Figure 3.1a: SEM image of untreated *B. licheniformis* cells (20 μm = 2 μm).

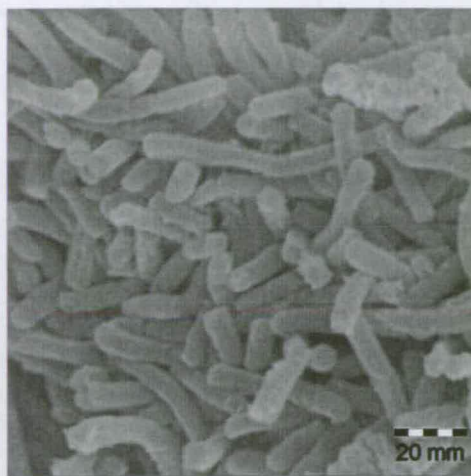


Figure 3.1b: SEM image of CER-treated *B. licheniformis* cells (20 μm = 2 μm).

3.3.2. Cell characterisation by TEM imaging

Example TEM images obtained for native and EPS-free cells are shown in Figures 3.2a and 3.2b respectively. The cell wall appears more defined in the image of the EPS-free cell, whereas the native cell has a distinct outer layer of EPS. This layer of EPS varies in thickness around the circumference of the cell and there is no distinct interface with the area surrounding the cell. Because of this, it was not considered possible to accurately measure the thickness of the EPS layer. The average cell wall thickness obtained using measurements from a number of EPS-free cell images was 22.0 ± 5.2 nm.

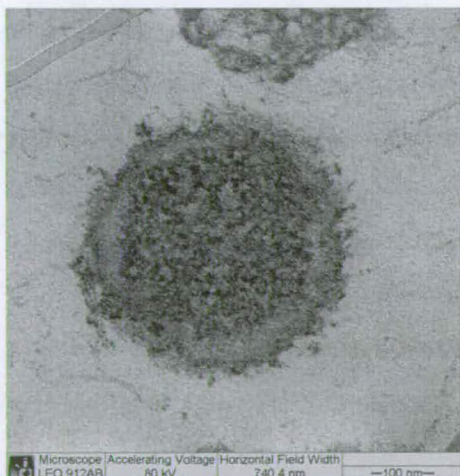


Figure 3.2a: TEM image of a native *B. licheniformis* cell.

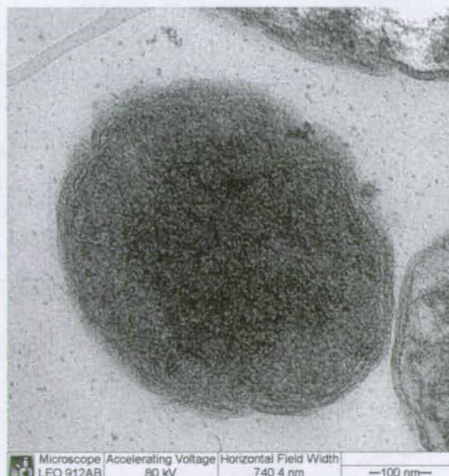


Figure 3.2b: TEM image of an EPS-free *B. licheniformis* cell.

3.3. Potentiometric titrations

3.3.1. Native cells

Representative potentiometric titration curves for the native cells are shown in Figure 3.3a. The curves are normalised to the dry mass of bacteria used, so variations in buffering capacity should reflect ionic strength dependent behaviour. Because the wet to dry weight ratio is much higher for the native cells than for the EPS-free cells (due to water held within the EPS layer), titration curves plotted in terms of wet weight are not directly comparable. Titration curves are plotted as $-C_b$ vs. pH, the form in which the data was input into FITMOD. The reproducibility between the titrations conducted at different biomass concentrations is good for the 0.01 M titrations and reasonable for the 0.1 M titrations, as the mass-normalised titration curves are in close agreement. There is little difference in buffering capacity between the curves at 0.01 M and 0.1 M.

3.3.2. EPS-free cells

Representative potentiometric titration curves for the EPS-free cell suspensions are shown in Figure 3.3b. The dry-mass-normalised curves at each ionic strength show good agreement between the two different biomass concentrations used. The titration curves do not show any systematic variability in buffering capacity as a function of ionic strength.

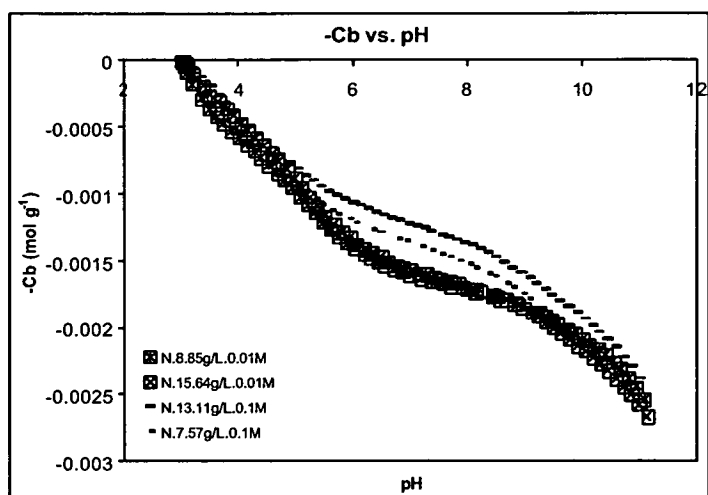


Figure 3.3a: Potentiometric titration curves for native *B. licheniformis* cells.

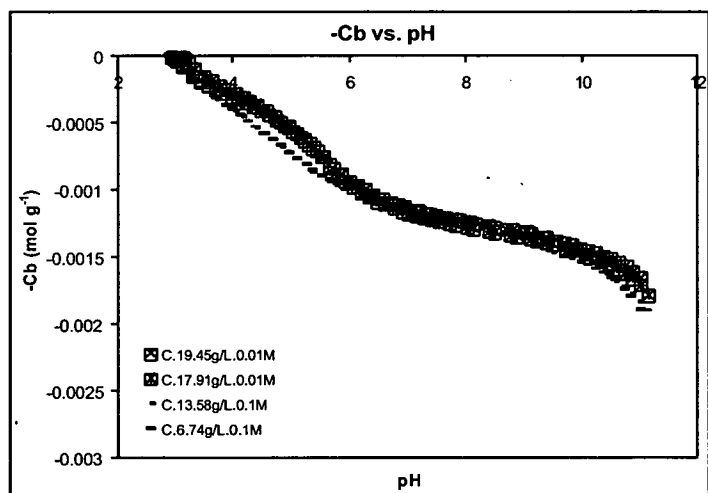


Figure 3.3b: Potentiometric titration curves for EPS-free *B. licheniformis* cells.

3.3.4. Electrophoretic mobility measurements

3.3.4.1. Native cells

The electrophoretic mobility results for native cells, as a function of pH and at 3 different ionic strengths, are shown in Figure 3.4a. The electrophoretic mobility remains negative at all suspension pH values, and increases in magnitude with increasing pH, from approximately -1.3 at pH 2.5 to approximately -3.3 at pH 6.5. The native cells show little variation in electrophoretic mobility with increasing ionic strength. The 0.01 M and 0.1 M suspensions show decreasing magnitude of electrophoretic mobility above approximately pH 6.5, as the electrophoretic mobility for both of these ionic strength experiments rises to between -2.5 and -2 at pH 7.5. The cell suspensions at 0.001 M NaNO₃ show consistently increasing electrophoretic mobility with increasing pH. The electrophoretic mobility reaches a maximum of approximately -4.0 at pH 6.5.

3.3.4.2. EPS-free cells

The experiments conducted using EPS-free cells (Figure 3.4b) show distinct increases in electrophoretic mobility with as ionic strength decreases from 0.1 M to 0.001 M NaNO₃, but little change in EM as pH increases. The electrophoretic mobility for the 0.1 M cell suspensions remains at approximately -1.5 across the experimental pH range. The 0.01 M suspensions increase slightly in electrophoretic mobility from -2.4 at pH 3, to -3.1 at pH 4.7, then there is a slight decrease to -2.5 at pH 7. The 0.001 M suspensions show a more defined trend towards increasingly negative electrophoretic mobilities, from approximately -3.8 at pH 3.7, to approximately -4.9 at pH 6.6.

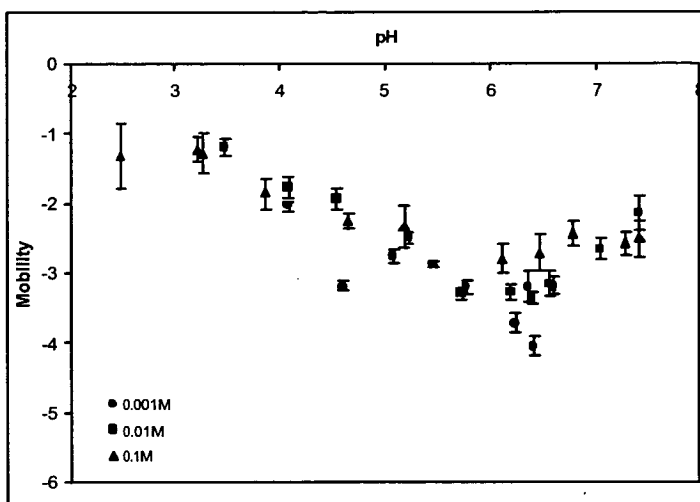


Figure 3.4a: Electrophoretic mobility measurements for native *B. licheniformis* cells. Error bars represent 1 standard deviation, based on 10 replicate measurements.

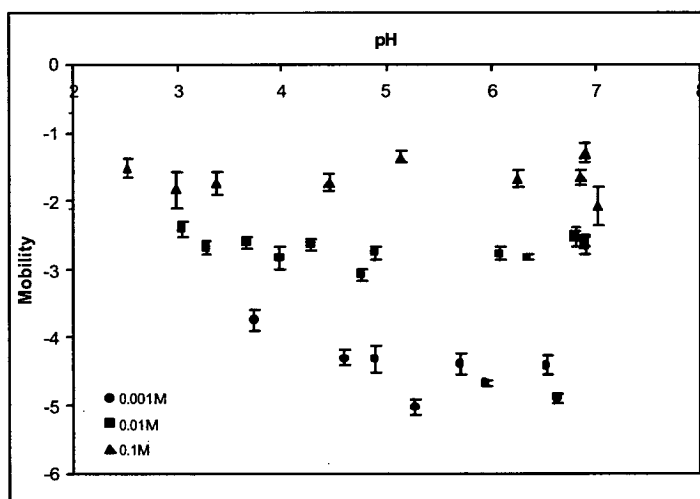


Figure 3.4b: Electrophoretic mobility measurements for EPS-free *B. licheniformis* cells. Error bars represent 1 standard deviation, based on 10 replicate measurements.

3.4. Discussion

3.4.1. Potentiometric titrations and data modelling

The potentiometric titration curves shown in Figures 3.3a and 3.3b demonstrate that there is little ionic-strength dependent variability in the buffering capacity of *B. licheniformis*. Titrations conducted at the same ionic strength and different biomass concentrations should plot on top of one another when normalised to the biomass concentration. The native cell titration curves show some variation as a function of biomass concentration, indicating that the relationship between the experimentally determined biomass concentration and the availability of reactive surface sites in solution is not straightforward. In this case, it is thought that the production of EPS by the cells is encouraging aggregation in solution, particularly at high biomass concentrations. Light microscope images were taken of *B. licheniformis* cell suspensions at 0.2 g L^{-1} , 1 g L^{-1} and 2 g L^{-1} , and pH values of 3, 5 and 7 (Figure 3.5 shows examples of the images taken at pH 5). Aggregation of cells at low pH due to neutralisation of the negative cell surface charge is commonly observed in bacterial cell suspensions. However, in the *B. licheniformis* cell suspensions it was found that aggregation was considerable even pH 7, and that the effect increased as biomass concentration increased. Figure 3.5 shows that the native cell suspensions are considerably less homogeneous than the EPS-free cells, explaining why the mass-normalised titration curves for the EPS-free cells are consistent whereas those for the native cells are not. It can be seen that some aggregation does occur in the EPS-free cell suspensions, possibly due to some residual EPS left after the extraction treatment, or possibly because the cells continue to produce EPS during the experimental procedure. However, this appears to be minor and the high stirring rate used during titrations will help to minimise the effects.

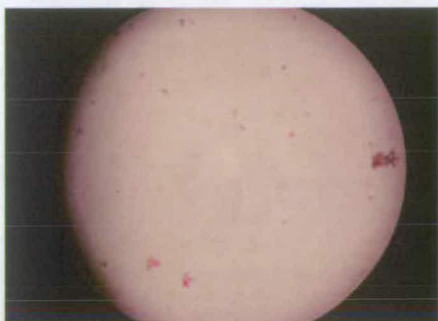


Figure 3.5ai.

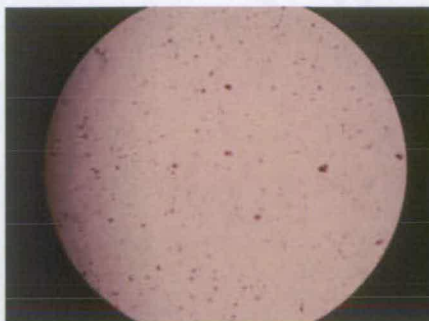


Figure 3.5aii.

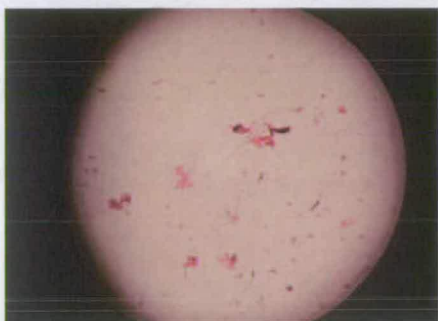


Figure 3.5bi.

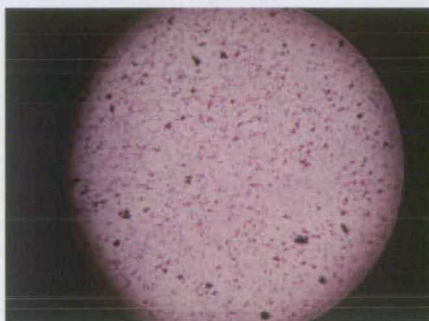


Figure 3.5bii.



Figure 3.5ci.

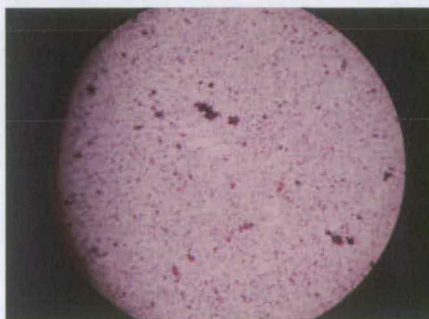


Figure 3.5cii.

Figure 3.5: Light microscope images of *B. licheniformis* suspensions. a: Biomass concentration of 0.2 g L^{-1} , pH 5 (Image diameter approx 1 mm): i) Native cells; ii) EPS-free cells. b: Biomass concentration of 1 g L^{-1} , pH 5 (Image diameter approx 1 mm): i) Native cells; ii) EPS-free cells. c: Biomass concentration of 2 g L^{-1} , pH 5 (Image diameter approx 1 mm): i) Native cells; ii) EPS-free cells.

A lack of ionic strength dependence indicates that the protons are bound strongly and the complexation mechanism is not easily affected by electrostatic interactions. However, previous studies have not reached a consensus on the effect of ionic strength on bacterial surface protonation behaviour. Titrations by Haas et al. (2001) at 0.05 M and 0.1 M did not show any difference in buffering capacity for *Shewanella putrefaciens*, and Yee et al. (2004) also found that ionic strength dependence of proton adsorption to *Bacillus subtilis* was insignificant. Borrok and Fein (2005) found that ionic strength (0.001 M – 0.6 M) had little effect on proton binding to *Pseudomonas putida* or *Pseudomonas mendocina*, and Fein et al. (2005) found that the buffering capacity of *Bacillus subtilis* did not vary significantly with ionic strength (0.01 M – 0.3 M). However, Burnett et al. (2006a) observed increasing buffering capacity with increasing ionic strength over the range 0.001 M - 0.1 M for *Anoxybacillus flavithermus*. Daughney and Fein (1998) also observed a trend of increasing buffering capacity with increasing ionic strength, for *B. subtilis* and *B. licheniformis*.

Previous nonelectrostatic modelling of potentiometric titration data obtained at an ionic strength of 0.01 M (Chapter 2) indicated that the native cells, and hence the EPS-layer, contained a higher concentration of proton-active functional groups than the EPS-free cells. In order to obtain intrinsic (non ionic-strength-dependent) stability constants and site concentrations, data modelling using the optimisation program FITMOD, which incorporates the Donnan core-shell model, was undertaken during this study. The Donnan core-shell model provides a more mechanistically realistic approximation to the cell wall-electrolyte interface, because it interprets the cell wall as a 3-dimensional ion-penetrable volume (Hetzer et al., 2006).

Electrostatic models such as the constant capacitance and Stern double-layer view the cell surface as an ion-impenetrable planar surface (Stumm and Morgan, 1996). The correction for electrostatic and ionic strength dependent adsorption applied by FITMOD is based on the soft-particle theory developed by Ohshima (1995).

This approach involves a number of assumptions that may not be realistic when applied to bacterial cells. For example, use of the Donnan model assumes uniform distribution of charge within the cell wall volume (Plette et al., 1995), but it has been

suggested that charge may be concentrated towards the poles of Bacilli species (Hetzler et al., 2006). The effectiveness of the Donnan electrostatic model as a means of correcting for ionic strength dependence is highly dependent upon accurate measurements for the cell wall thickness and cell dimensions, as these parameters are required to obtain the Donnan volume. Accurate measurements can be obtained for EPS-free cells using SEM and TEM images (Figures 3.1b and 3.2b); however, the presence of an EPS layer significantly complicates the estimation of cell wall thickness. As shown in Figures 3.1a and 3.2a, the EPS does not constitute a consistent capsule around each cell, but forms a 'matrix' within which the cells are embedded, and the quantity and thickness of EPS present on each cell surface is variable. In order to undertake data modelling for the native cells, the cell wall thickness measured for the EPS-free cells was taken as the starting point (or minimum thickness) and then manually increased in order to find an optimal soft shell width corresponding to the effective Donnan thickness that reproduces the pK_a values of EPS-free cells. This approach is justified because non-electrostatic modelling had already shown that pK_a values are statistically unaffected by the presence of EPS.

The results of the FITMOD modelling are shown in Table 3.1 (EPS-free cells) and Table 3.2 (native cells). Sites concentrations are reported as moles per dry gram of bacteria in order to facilitate comparison with the nonelectrostatic modelling results (Chapter 2). Generally, four different surface sites were required to fit the data for both native and EPS-free cells. Models also converged to 4 sites when data optimisation was undertaken using a nonelectrostatic approach. As FITMOD corrects the complexation parameters for ionic strength-dependent behaviour, the deprotonation constants and site concentrations should be similar for titrations conducted at different ionic strengths. Modelling results are presented for the 0.01 M and 0.1 M titrations, as well as average pK_a values and site concentrations calculated by combining the results obtained at both ionic strengths.

For the EPS-free cells, the mean pK_a values with associated 1x standard deviations are 3.4 ± 0.5 (site 1), 4.9 ± 1.0 (site 2), 7.3 ± 0.6 (site 3) and 9.0 ± 0.6 (site 4). The site concentrations and 1x standards deviations are $8.06 \pm 2.53 \times 10^{-4} \text{ mol g}^{-1}$ (site 1), $4.38 \pm 2.03 \times 10^{-4} \text{ mol g}^{-1}$ (site 2), $2.69 \pm 1.14 \times 10^{-4} \text{ mol g}^{-1}$ (site 3) and 3.85 ± 0.74

$\times 10^{-4} \text{ mol g}^{-1}$. The deprotonation constants obtained using a nonelectrostatic model (Chapter 2) were 3.3 ± 0.12 , 5.4 ± 0.2 , 7.5 ± 0.4 and 10.1 ± 0.2 , which are fairly similar to the intrinsic deprotonation constants obtained using the Donnan model. However, given the large standard deviations it is not possible to interpret the two sets of constants as significantly different. The site concentrations for the EPS-free cells obtained using a nonelectrostatic model were $5.47 \pm 1.13 \times 10^{-4} \text{ mol g}^{-1}$ (site 1), $6.82 \pm 1.09 \times 10^{-4} \text{ mol g}^{-1}$ (site 2), $2.22 \pm 0.62 \times 10^{-4} \text{ mol g}^{-1}$ (site 3) and $3.26 \pm 0.68 \times 10^{-4} \text{ mol g}^{-1}$ (site 4). These values are also similar to those obtained using FITMOD. The intrinsic concentration of site 1 is higher than ionic-strength-dependent concentration and that of site 2 is lower, but the concentrations for sites 3 and 4 are very close to those obtained using the nonelectrostatic approach. The nonelectrostatic model indicated that site 2 had the highest concentration and site 3 the lowest, but by using FITMOD the highest concentration is obtained for site 1. Site 3 remains the surface site with the lowest concentration. The total site concentration for EPS-free cells using electrostatic modelling ($1.90 \times 10^{-3} \text{ mol g}^{-1}$) is in close agreement with that obtained using nonelectrostatic modelling ($1.78 \times 10^{-3} \text{ mol g}^{-1}$).

The nonelectrostatic data modelling indicated that there were no significant differences between the pK_a values obtained for native and EPS-free cells. However, the presence of EPS modifies the cell surface environment and may significantly affect the electrostatic interactions at the cell-electrolyte interface. It was therefore hypothesised that the pK_a values and site concentrations for the native cells should converge towards the values obtained for the EPS-free cells, as the Donnan thickness entered into the model input file approached the true thickness of the cell wall and EPS layer.

Table 3.1: FITMOD modelling results for EPS-free cells. Donnan thickness 22nm.

0.01M	pK1	pK2	pK3	pK4	[Site1] (mol g⁻¹)	[Site2] (mol g⁻¹)	[Site3] (mol g⁻¹)	[Site4] (mol g⁻¹)	V(Y)
mean	3.03	4.20	6.64	8.42	6.68E-04	5.41E-04	1.60E-04	3.23E-04	31.17
s.d	0.26	0.09	0.03	0.04	5.01E-05	1.26E-04	6.19E-06	1.16E-05	
0.1M	pK1	pK2	pK3	pK4	[Site1] (mol g⁻¹)	[Site2] (mol g⁻¹)	[Site3] (mol g⁻¹)	[Site4] (mol g⁻¹)	V(Y)
mean	3.61	5.31	7.72	9.45	8.98E-04	3.69E-04	3.41E-04	4.26E-04	8.53
s.d	0.54	1.03	0.36	0.40	2.78E-04	2.39E-04	7.84E-05	6.77E-05	
All	pK1	pK2	pK3	pK4	[Site1] (mol g⁻¹)	[Site2] (mol g⁻¹)	[Site3] (mol g⁻¹)	[Site4] (mol g⁻¹)	V(Y)
mean	3.38	4.86	7.29	9.04	8.06E-04	4.38E-04	2.69E-04	3.85E-04	17.58
s.d	0.51	0.95	0.64	0.63	2.35E-04	2.03E-04	1.14E-04	7.44E-05	

Table 3.2a. FITMOD modelling results for native cells. Donnan thickness 22nm.

0.01M	pK1	pK2	pK3	pK4	[Site1] (mol g⁻¹)	[Site2] (mol g⁻¹)	[Site3] (mol g⁻¹)	[Site4] (mol g⁻¹)	V(Y)
mean	2.43	4.26	6.94	8.78	1.33E-03	6.02E-04	2.81E-04	5.91E-04	16.37
s.d	0.69	0.32	0.23	0.22	4.26E-04	1.78E-04	4.29E-05	8.26E-05	
0.1M	pK1	pK2	pK3	pK4	[Site1] (mol g⁻¹)	[Site2] (mol g⁻¹)	[Site3] (mol g⁻¹)	[Site4] (mol g⁻¹)	V(Y)
mean	4.13	6.07	8.19	9.75	1.61E-03	3.70E-04	6.51E-04	8.42E-04	12.52
s.d	0.10	0.35	0.28	0.25	6.92E-04	8.39E-05	2.63E-04	1.52E-04	
All	pK1	pK2	pK3	pK4	[Site1] (mol g⁻¹)	[Site2] (mol g⁻¹)	[Site3] (mol g⁻¹)	[Site4] (mol g⁻¹)	V(Y)
mean	3.28	5.16	7.56	9.27	1.47E-03	4.86E-04	4.66E-04	7.17E-04	14.44
s.d	1.02	1.01	0.71	0.56	5.53E-04	1.79E-04	2.64E-04	1.75E-04	

Table 3.2b. FITMOD modelling results for native cells. Donnan thickness 40nm.

0.01M	pK1	pK2	pK3	pK4	[Site1] (mol g⁻¹)	[Site2] (mol g⁻¹)	[Site3] (mol g⁻¹)	[Site4] (mol g⁻¹)	V(Y)
mean	2.67	4.49	7.17	9.03	1.31E-03	6.17E-04	2.79E-04	5.92E-04	14.75
s.d	0.67	0.30	0.21	0.22	4.37E-04	1.70E-04	4.06E-05	8.16E-05	
0.1M	pK1	pK2	pK3	pK4	[Site1] (mol g⁻¹)	[Site2] (mol g⁻¹)	[Site3] (mol g⁻¹)	[Site4] (mol g⁻¹)	V(Y)
mean	4.16	5.85	8.21	9.85	1.47E-03	4.60E-04	6.06E-04	8.87E-04	8.48
s.d	0.11	0.28	0.29	0.22	6.47E-04	9.14E-05	2.31E-04	1.91E-04	
All	pK1	pK2	pK3	pK4	[Site1] (mol g⁻¹)	[Site2] (mol g⁻¹)	[Site3] (mol g⁻¹)	[Site4] (mol g⁻¹)	V(Y)
mean	3.41	5.17	7.69	9.44	1.39E-03	5.38E-04	4.43E-04	7.39E-04	11.61
s.d	0.91	0.77	0.60	0.48	5.15E-04	1.53E-04	2.33E-04	2.08E-04	

Table 3.2c. FITMOD modelling results for native cells. Donnan thickness 60nm.

0.01M	pK1	pK2	pK3	pK4	[Site1] (mol g ⁻¹)	[Site2] (mol g ⁻¹)	[Site3] (mol g ⁻¹)	[Site4] (mol g ⁻¹)	V(Y)
mean	2.81	4.63	7.27	9.23	1.29E-03	6.39E-04	2.76E-04	5.93E-04	12.98
s.d	0.65	0.26	0.14	0.22	4.51E-04	1.61E-04	3.71E-05	7.99E-05	
0.1M	pK1	pK2	pK3	pK4	[Site1] (mol g ⁻¹)	[Site2] (mol g ⁻¹)	[Site3] (mol g ⁻¹)	[Site4] (mol g ⁻¹)	V(Y)
mean	4.14	5.88	8.25	9.90	1.46E-03	4.79E-04	6.25E-04	8.64E-04	9.51
s.d	0.11	0.37	0.29	0.24	7.21E-04	7.81E-05	2.69E-04	1.43E-04	
All	pK1	pK2	pK3	pK4	[Site1] (mol g ⁻¹)	[Site2] (mol g ⁻¹)	[Site3] (mol g ⁻¹)	[Site4] (mol g ⁻¹)	V(Y)
mean	3.47	5.25	7.76	9.56	1.37E-03	5.59E-04	4.50E-04	7.28E-04	11.24
s.d	0.83	0.73	0.57	0.42	5.65E-04	1.45E-04	2.58E-04	1.80E-04	

Table 3.2d. FITMOD modelling results for native cells. Donnan thickness 80nm.

0.01M	pK1	pK2	pK3	pK4	[Site1] (mol g ⁻¹)	[Site2] (mol g ⁻¹)	[Site3] (mol g ⁻¹)	[Site4] (mol g ⁻¹)	V(Y)
mean	2.89	4.71	7.40	9.29	1.26E-03	6.61E-04	2.73E-04	5.94E-04	11.53
s.d	0.62	0.23	0.15	0.23	4.67E-04	1.57E-04	3.40E-05	7.98E-05	
0.1M	pK1	pK2	pK3	pK4	[Site1] (mol g ⁻¹)	[Site2] (mol g ⁻¹)	[Site3] (mol g ⁻¹)	[Site4] (mol g ⁻¹)	V(Y)
mean	4.15	5.75	8.25	9.97	1.21E-03	5.58E-04	5.65E-04	9.24E-04	6.84
s.d	0.14	0.20	0.29	0.23	5.26E-04	1.43E-04	1.94E-04	2.45E-04	
All	pK1	pK2	pK3	pK4	[Site1] (mol g ⁻¹)	[Site2] (mol g ⁻¹)	[Site3] (mol g ⁻¹)	[Site4] (mol g ⁻¹)	V(Y)
mean	3.53	5.23	7.82	9.63	1.30E-03	6.10E-04	4.19E-04	7.59E-04	9.19
s.d	0.79	0.59	0.50	0.42	4.79E-04	1.49E-04	2.02E-04	2.44E-04	

Table 3.2e. FITMOD modelling results for native cells. Donnan thickness 100nm.

0.01M	pK1	pK2	pK3	pK4	[Site1] (mol g ⁻¹)	[Site2] (mol g ⁻¹)	[Site3] (mol g ⁻¹)	[Site4] (mol g ⁻¹)	V(Y)
mean	2.95	4.78	7.46	9.37	1.24E-03	6.81E-04	2.71E-04	5.95E-04	10.39
s.d	0.59	0.20	0.13	0.23	4.81E-04	1.57E-04	3.12E-05	7.96E-05	
0.1M	pK1	pK2	pK3	pK4	[Site1] (mol g ⁻¹)	[Site2] (mol g ⁻¹)	[Site3] (mol g ⁻¹)	[Site4] (mol g ⁻¹)	V(Y)
mean	4.15	5.74	8.26	10.01	1.31E-03	5.80E-04	5.57E-04	9.30E-04	6.59
s.d	0.11	0.19	0.29	0.23	5.39E-04	7.61E-06	1.88E-04	2.55E-04	
All	pK1	pK2	pK3	pK4	[Site1] (mol g ⁻¹)	[Site2] (mol g ⁻¹)	[Site3] (mol g ⁻¹)	[Site4] (mol g ⁻¹)	V(Y)
mean	3.55	5.26	7.86	9.69	1.27E-03	6.30E-04	4.14E-04	7.63E-04	8.49
s.d	0.75	0.55	0.48	0.40	4.74E-04	1.55E-04	1.97E-04	2.50E-04	

The results of the FITMOD modelling for the native cell titrations are shown in Table 3.2. The modelling was initially undertaken using a cell wall thickness of 22 nm, which produced pK_a values and 1x standard deviations of 3.28 ± 1.02 (site 1), 5.16 ± 1.01 (site 3), 7.56 ± 0.71 (site 3) and 9.27 ± 0.56 (site 4). Site concentrations

were $1.47 \pm 0.55 \times 10^{-3} \text{ mol g}^{-1}$ (site 1), $4.86 \pm 1.79 \times 10^{-4} \text{ mol g}^{-1}$ (site 2), $4.66 \pm 2.64 \times 10^{-4} \text{ mol g}^{-1}$ (site 3) and $7.17 \pm 1.75 \times 10^{-4} \text{ mol g}^{-1}$ (site 4). Except for site 1, the pK_a values are slightly higher than those for the EPS-free cells, but the standard deviations for both data sets are large. The intrinsic concentrations are all higher than those obtained with a nonelectrostatic model, although the difference is small for site 2. As seen for the EPS-free cells, the highest site concentration is for site 1 and the lowest for site 3. The nonelectrostatic modelling indicated that the highest site concentration for the native cells was for site 2 and the lowest for site 3.

The titration data for the native cells was subsequently remodelled using cell wall widths of 40, 60, 80 and 100 nm in order to investigate whether an optimal cell wall width and hence Donnan thickness could be found. The results in Table 3.2 show that the average $V(Y)$ values decrease as cell wall width is increased from 22 nm to 100 nm. The pK_a values all increase with cell wall width, the concentrations of sites 1 and 3 decrease and the concentrations of sites 2 and 4 increase. The pK_a values obtained using a cell wall thickness of 100 nm (Table 3.2e) are close to the values obtained using for native cells using a nonelectrostatic model, which were 3.4 ± 0.2 (site 1), 5.3 ± 0.1 (site 2), 7.4 ± 0.2 (site 3) and 9.9 ± 0.1 (site 4). The total site concentration decreases only slightly as cell wall width is increased from 22 nm to 100 nm, from $3.14 \times 10^{-3} \text{ mol g}^{-1}$ to $3.08 \times 10^{-3} \text{ mol g}^{-1}$. This is approximately 1.5 times as large as the total site concentration for the EPS-free cells and close to the total site concentration for native cells obtained using a nonelectrostatic model ($2.70 \times 10^{-3} \text{ mol g}^{-1}$).

Other studies have been undertaken using the Donnan model to account for electrostatic interactions, for non-EPS-producing bacterial strains (Martinez et al., 2002; Burnett et al., 2006a; Hetzer et al., 2006; Heinrich et al., 2007). Martinez et al. (2002) used the Donnan model in combination with a fully optimised continuous pK_a distribution method and a master curve approach. This provided intrinsic deprotonation constants and site concentrations for four surface sites on both *B. subtilis* and *E. coli*. Burnett et al. (2006a) and Heinrich et al. (2007) both used FITMOD to characterise the surface chemistry of *Anoxybacillus flavithermus*, a thermophilic strain. Burnett et al. (2006a) identified 3 surface sites from potentiometric titration data across an ionic strength range of 0.001 M - 0.1 M

NaNO₃. Heinrich et al. (2007) obtained a 2-site model that was able to account for both titration data and electrophoretic mobility measurements as a function of pH and ionic strength. Hetzer et al. (2006) also investigated thermophilic bacterial species using FITMOD and found that *Geobacillus thermocatenulatus* had 3 surface sites whereas *G. stearothermophilus* had 2 surface sites. Generally, the other studies using FITMOD have found a lower number of surface sites than the 4 sites found for *B. licheniformis* in the present study. However, the number of investigations that have used the Donnan model approach is small to date, and the results of this investigation are in agreement with earlier nonelectrostatic modelling (Chapter 2), as well as with the findings of Martinez et al. (2002).

Although lower standard deviations and $V(Y)$ values indicate that the models provide a better fit to the experimental data as cell wall width is increased, and the pK_a values appear to be stabilising, it was considered unrealistic to increase the Donnan thickness above 100 nm. Taylor and Lower (2008) measured the thickness of EPS on the surfaces of *Acidithiobacillus ferrooxidans* cells, and found that the average thickness was 29 nm. Assuming a similar EPS layer thickness for *B. licheniformis* would result in a total Donnan thickness (EPS plus cell wall) of approximately 50 nm. This approach was therefore unable to fully account for the presence of EPS on the cell surfaces. This may be due to the heterogeneous distribution of EPS across the cell surfaces, or the EPS structure may lead to behaviour that cannot be adequately described by the Donnan model. For example, swelling of the cell wall and EPS layer at low ionic strength may lead to significant variations in the specific surface area. Plette et al. (1995) found that swelling of the cell walls of *Rhodococcus erythropolis* A177 was significant below ionic strengths of 0.1 M. For both cell treatments, a lower $V(Y)$ value is obtained for the experiments conducted at the higher ionic strength (0.1 M), suggesting that EPS-layer expansion at low ionic strength may be a source of error. This effect should be less apparent in the EPS-free cells, but standard deviations are high for both EPS-free and native cells, suggesting that expansion of the cell wall is also a significant source of error.

The site concentrations obtained using FITMOD are generally close to those obtained using a nonelectrostatic model. The Donnan model approach also suggested that the native cells have a higher total site concentration than the EPS-free

cells. The most significant difference between the two modelling approaches is in the relative abundances of sites 1 and 2. Using FITMOD, site 1 is the most abundant site, whereas nonelectrostatic modelling indicated that site 2 was most abundant. Both modelling approaches found that site 3 was the least abundant. The pK_a values obtained using FITMOD are lower than those obtained using a nonelectrostatic approach, and there is greater divergence between the pK_a values for the native cells and EPS-free cells. However, the standard deviations for the FITMOD values are large and it is not possible to ascertain whether the two types of cells have significantly different pK_a values as a consequence of the presence of EPS.

3.4.2. Electrophoretic mobility measurements

Figures 3.4a and 3.4b show that, for both cell types and under all experimental conditions, the electrophoretic mobility at approximately pH 2.5, the lowest pH measured, remains negative. This suggests that the isoelectric point (IEP) for these cells (the point at which the overall surface charge is zero) is well below pH 2, which implies that the cell wall functional groups are not fully protonated even at very low pH. This finding is in agreement with that of Leone et al. (2007), who conducted EM measurements using *Bacillus subtilis* and found that the cell surfaces were negatively charged even at pH 1.4. Fein et al. (2005) measured the electrophoretic mobility of *Bacillus subtilis* in 0.1 M NaClO_4 and found that although the EM decreased in magnitude with decreasing pH, it remained negative even at pH 2. Other studies have found slightly higher values for the IEP of bacterial cells. van der Wal et al. (1997) conducted electrophoresis on a variety of bacterial species and found IEP values ranging from below pH 2 for *Rhodococcus erythropolis* to pH 4.6 for *Bacillus brevis*. Phoenix et al. (2002) conducted electrophoretic mobility and potentiometric titration analysis of a sheath-forming cyanobacterial species, and were able to correlate low electrophoretic mobilities with a lower charge density in the sheath, established by titration. Yee et al. (2004) found that the isoelectric point for *Bacillus subtilis* was at pH 2.4. MacLean et al. (2004) obtained an IEP of pH 2.1-2.2, also for *Bacillus subtilis*. Claessens et al. (2005) found an IEP at pH 3.5- 4.0 for *Shewanella putrefaciens*. Burnett et al. (2006a) and Heinrich et al. (2007) obtained

an IEP at pH 2.8-3.0 for *Anoxybacillus flavithermus*. The wide range of IEP values reported here for *Bacillus subtilis* confirm that the pH-dependence electrophoretic mobility is not characteristic of a particular strain (Poortinga et al., 2002). This may indicate that the bacterial cell surface is sensitive to variations in growth conditions.

The generally low isoelectric points obtained during this and other studies indicate the presence of very low pK_a functional groups within the bacterial cell walls.

However, the likelihood of inducing structural cell damage at such low pH values is an obstacle to validating the presence of these groups by potentiometric titration.

The magnitude of the electrophoretic mobilities displayed by bacterial cells is governed by three main factors: i) electrophoretic mobilities decrease with increasing ionic strength due to electrostatic interaction of the electrolyte ions with the ionised cell surface groups and consequent reduction of the cell wall electrical potential (Yee et al. 2004); ii) as electrophoretic mobility is determined by the cell surface charge density, it increases with increasing pH owing to progressive deprotonation of the cell wall organic functional groups (Poortinga et al., 2002); iii) swelling of the cell wall leads to a decrease in surface charge density and hence decreased electrophoretic mobility. This occurs both as a response to decreasing ionic strength and to increased pH (Poortinga et al., 2002; Gaboriaud et al., 2006). Obviously, these effects may counteract each other, and the observed EM results from the balance of these contributory factors.

A distinct increase in EM with decreasing ionic strength is observed for the EPS-free cells, but this effect is minimal for the native cells (Figures 3.4a and 3.4b). This behaviour was also observed in the studies by van der Wal et al. (1997), Yee et al. (2004), Claessens et al. (2005), Burnett et al (2006a) and Heinrich et al. (2007), all of which were conducted across the same ionic strength range as was used in the present study. Lectin-staining carried out by Burnett et al. (2006a) indicated the presence of minimal extracellular polysaccharides on *A. flavithermus*, and in other studies it is assumed that the strains used are non-EPS-producing.

The presence of a substantial EPS layer (Figures 3.1a and 3.2a) suggests that the native cells may be considerably more susceptible to fluctuating cell wall (including the EPS layer) volume than the EPS-free cells. The comparatively small increase in

EM for the native cells as ionic strength decreases is likely due to a significant decrease in the charge density of the native cells, owing to swelling of the cell wall and EPS layer. The effect of increasing pH on the cell wall volume can also be observed in the electrophoretic mobility results. For the native cells (Figure 3.4a), this effect is clearly demonstrated above approximately pH 6 for the experiments conducted at 0.001 M and 0.01 M NaNO₃. This trend is less obvious for the EPS-free cells (Figure 3.4b), but it can be seen that there is little increase in the magnitude of the electrophoretic mobility above approximately pH 4. As this is an effect controlled by the changing volume of the cell wall, the presence of an EPS layer and consequently larger effective cell wall volume explains the enhanced response of the native cells compared to the EPS-free cells. The experiments conducted at the highest ionic strength for both types of cells show consistently increasing EM as pH increases.

In order to compare the electrophoretic mobilities of native vs. EPS-free cells, it is necessary to consider all of the competing factors mentioned above. Comparison of Figures 3.4a and 3.4b shows that at 0.001 M, the EPS-free cells have greater EM than the native cells. At 0.01 M the EPS-free cells still have a slightly greater magnitude of EM, although above pH 6 there is little difference between the two cell types. At 0.1 M, when the effects of cell wall swelling are minimised, the native cells have a greater magnitude of EM than do the EPS-free cells. This is in agreement with the results of both the FITMOD modelling and earlier non-electrostatic potentiometric titration data modelling (Chapter 2), which indicated that native cells (and hence the EPS layer) have a higher charge density than EPS-free cells.

3.5. Conclusions

Potentiometric titrations undertaken at 0.01 M and 0.1 M ionic strength using both native and EPS-free cells indicated that the effect of ionic strength on the titration curves is minimal. However, variable reproducibility in titrations conducted at different biomass concentrations indicates that cell aggregation in suspension is affecting the titrations conducted using native cells. This is an aspect of the behaviour of EPS-producing cells that merits further investigation as it will significantly affect metal adsorption behaviour. This is further discussed in Chapters 4 and 5. In the context of this study, cell aggregation in suspension will probably lead to underestimates of the site concentrations for native cells. Electrostatic modelling of the potentiometric titration data using FITMOD, which corrects protonation constants using the Donnan approach, indicated that both native and EPS-free cells contain 4 reactive surface sites. Manually varying the cell wall thickness (and hence Donnan thickness) for the native cells did not lead to convergence of protonation constants of $V(Y)$ values within a reasonable range of 22 nm – 100 nm, indicating that this approach could not fully account for the effect of EPS production on the effective Donnan volume. The site concentrations obtained using FITMOD were in general agreement with those obtained using nonelectrostatic modelling (Chapter 2), and it was confirmed that the native cells contain a higher concentration of surface sites than do the EPS-free cells. Data optimisation using FITMOD indicated that site 1 was the site with the highest concentration, whereas site 2 had the highest concentration using the nonelectrostatic model. Sites 1 and 2 were tentatively identified as phosphodiester and carboxyl sites respectively (Chapter 2) and the significance of their relative concentrations will be further discussed with respect to metal adsorption in Chapters 4, 5 and 7. The pK_a values obtained using FITMOD showed a greater divergence between the values obtained for native and EPS-free cells than those obtained using a nonelectrostatic approach. However, it is not possible to ascertain whether or not there is a significant difference between the two datasets owing to the large standard deviations. This aspect of the protonation behaviour is important as the pK_a values are all considerably lower than those obtained using a nonelectrostatic model. If the differences are significant, this would suggest that electrostatic interactions do have an effect on the protonation behaviour

of this bacterial species, and that there is potentially some difference between the pK_a values for native and EPS-free cells. However, the functional group identities based on the pK_a values obtained using FITMOD would probably not differ from those assigned to the four groups identified using nonelectrostatic modelling (phosphodiester, carboxyl, phosphoryl and amine respectively).

The electrophoretic mobility analysis showed that swelling of the EPS layer is the dominant influence on the magnitude of the electrophoretic mobility at low ionic strength. At high ionic strength (0.1 M NaNO_3), the native cells have a greater magnitude of electrophoretic mobility than do the EPS-free cells, indicating that there is a relatively high charge density on the surfaces of the native cells and the EPS layer. This is in agreement with the results of the FITMOD modelling and earlier potentiometric titration analysis, which indicated a higher concentration of functional groups within the cell walls of native cells compared to EPS-free cells.

3.6. References

- Borrok, D., Fein, J.B. (2004) Distribution of protons and Cd between bacterial surfaces and dissolved humic substances determined through chemical equilibrium modelling. *Geochimica et Cosmochimica Acta*, 68, 3043-3052
- Borrok, D.M., Fein, J.B. (2005) The impact of ionic strength on the adsorption of protons, Pb, Cd, and Sr onto the surfaces of Gram negative bacteria: testing non-electrostatic, diffuse, and triple-layer models. *Journal of Colloid and Interface Science*, 286, 110-126
- Boyanov, M.I., Kelly, S.D., Kemner, K.M., Bunker, B.A., Fein, J.B., Fowle, D.A. (2003) Adsorption of cadmium to *Bacillus subtilis* bacterial cell walls: A pH-dependent X-ray adsorption fine structure spectroscopy study. *Geochimica et Cosmochimica Acta*, 67, 3299-3311
- Burnett, P-G., Heinrich, H., Peak, D., Bremer, P.J., McQuillan, A.J., Daughney, C.J. (2006a) The effect of pH and ionic strength on proton adsorption by the thermophilic bacterium *Anoxybacillus flavithermus*. *Geochimica et Cosmochimica Acta*, 70, 1914-1927
- Burnett, P-G.G., Daughney, C.J., Peak, D. (2006b) Cd adsorption onto *Anoxybacillus flavithermus*: Surface complexation modelling and spectroscopic investigations. *Geochimica et Cosmochimica Acta*, 70, 5253-5269
- Chatellier, X., Fortin, D. (2004) Adsorption of ferrous ions onto *Bacillus subtilis* cells. *Chemical Geology*, 212, 209-228
- Claessens, J., Van Capellen, P. (2007) Competitive binding of Cu^{2+} and Zn^{2+} to live cells of *Shewanella putrefaciens*. *Environmental Science and Technology*, 41, 909-914
- Claessens, J., van Lith, Y., Laverman, A.M., Van Capellen, P. (2005) Acid-base activity of live bacteria: Implications for quantifying cell wall charge. *Geochimica et Cosmochimica Acta*, 70, 267-276

- Daughney, C.J., Fein, J.B. (1998) The effect of ionic strength on the adsorption of H^+ , Cd^{2+} , Pb^{2+} and Cu^{2+} by *Bacillus subtilis* and *Bacillus licheniformis*: A surface complexation model. *Journal of Colloid and Interface Science*, 198, 53-77
- Daughney, C.J., Fein, J.B., Yee, N. (1998) A comparison of the thermodynamics of metal adsorption onto two common bacteria. *Chemical Geology*, 144, 161-176
- Erlandsen, S.L., Kristich, C.J., Dunny, G.M., Wells, C.L., 2004. High-resolution Visualisation of the Microbial Glycocalyx with Low-voltage Scanning Electron Microscopy: Dependence on Cationic Dyes. *Journal of Histochemistry and Cytochemistry* 52, 1427-1435
- Fein, J.B., Boily, J-F., Yee, N., Gorman-Lewis, D., Turner, B. F. (2005) Potentiometric titrations of *Bacillus subtilis* cells to low pH and a comparison of modelling approaches. *Geochimica et Cosmochimica Acta*, 69, 1123-1132
- Fein, J.B., Daughney, C.J., Yee, N., Davis, T.A. (1997) A chemical equilibrium model for metal adsorption onto bacterial surfaces. *Geochimica et Cosmochimica Acta*, 61, 3319-3328
- Gaboriaud, F., Dauge, E., Bailet, S., Jorand, F., Duval, J., Thomas, F. (2006) Multiscale dynamics of the cell envelope of *Shewanella putrefaciens* as a response to pH change. *Colloids and Surfaces B: Biointerfaces*, 50, 123-131
- Gorman-Lewis, D., Fein, J.B., Soderholm, L., Jensen, M.P., Chiang, M.-H. (2005) Experimental study of neptunyl adsorption onto *Bacillus subtilis*. *Geochimica et Cosmochimica Acta*, 69, 4837-4844
- Guine, V., Spadini, L., Sarret, G., Muris, M., Delolme, C., Gaudet, J.-P., Martins, J.M.F. (2006) Zinc sorption to three gram-negative bacteria: Combined titration, modelling, and EXAFS study. *Environmental Science and Technology*, 40, 1806-1813
- Haas, J.R., Dichristina, T.J., Wade Jr, R. (2001) Thermodynamics of U(VI) sorption onto *Shewanella putrefaciens*. *Chemical Geology*, 180, 33-54
- Heinrich, H.T.M., Bremer, P.J., Daughney, C.J., McQuillan, A.J. (2007) Acid-base titrations of functional groups on the surface of the thermophilic bacterium

- Anoxybacillus flavithermus*: Comparing a chemical equilibrium model with ATR-IR spectroscopic data. *Langmuir*, 23, 2731-2740
- Hetzer, A., Daughney, C.J., Morgan, H.W. (2006) Cadmium ion biosorption by the thermophilic bacteria *Geobacillus stearothermophilus* and *G. thermocatenulatus*. *Applied and Environmental Microbiology*, 72, 4020-4027
- Johnson, K.J., Szymanowski, J.E.S., Borrok, D., Huynh, T.Q., Fein, J.B. (2007) Proton and metal adsorption onto bacterial consortia: Stability constants for metal-bacterial surface complexes. *Chemical Geology*, 239, 13-26
- Leone, L., Ferri, D., Manfredi, C., Persson, P., Shchukarev, A., Sjöberg, S., Loring, J. (2007) Modeling the acid-base properties of bacterial surfaces: A combined spectroscopic and potentiometric study of the gram-positive bacterium *Bacillus subtilis*. *Environmental Science and Technology*, 41, 6465-6471
- MacLean, L.C.W., Martinez, R.E., Fowle, D.A. (2004) Experimental studies of bacteria-iodide adsorption interactions. *Chemical Geology*, 212, 229-238
- Martinez, R.E., Smith, D.S., Kulczyzcki, E., Ferris, F.G. (2002) Determination of intrinsic bacterial surface acidity constants using a Donnan shell model and a continuous pK_a distribution method. *Journal of Colloid and Interface Science*, 253, 130-139
- Ngwenya, B.T. (2007) Enhanced adsorption of zinc is associated with aging and lysis of bacterial cells in batch incubations. *Chemosphere*, 67, 1982-1992
- Ngwenya, B.T., Sutherland, I.W., Kennedy, L. (2003) Comparison of the acid-base behaviour and metal adsorption characteristics of a gram-negative bacterium with other strains. *Applied Geochemistry*, 18, 527-538
- Ohshima, H. (1995) Electrophoretic mobility of soft particles. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 103, 249-255
- Phoenix, V.P., Martinez, R.E., Konhauser, K.O., Ferris, F.G. (2002) Characterisation and implications of the cell surface reactivity of *Calothrix* sp. Strain KC97. *Applied and Environmental Microbiology*, 68, 4827-4834

- Plette, A.C.C., van Riemsdijk, W.H., Benedetti, M.F., van der Wal, A. (1995) pH dependent charging behaviour of isolated cell walls of a gram-positive soil bacterium. *Journal of Colloid and Interface Science*, 173, 354-363
- Poortinga, A.T., Bos, R., Norde, W., Busscher, H.J. (2002) Electric double layer interactions in bacterial adhesion to surfaces. *Surface Science Reports*, 47, 1-32
- Stumm, W., Morgan, J.J. (1996) *Aquatic Chemistry, Chemical Equilibria and Rates in Natural Waters*, 3rd Edition. Wiley, New York
- Taylor, E.S., Lower, S.K. (2008) Thickness and surface density of extracellular polymers on *Acidithiobacillus ferrooxidans*. *Applied and Environmental Microbiology*, 74, 309-311
- Toner, B., Manceau, A., Marcus, A.M., Millet, D.B., Sposito, G. (2005) Zinc sorption by a bacterial biofilm. *Environmental Science and Technology*, 39, 8288-8294
- Tsuneda, S., Jung, J., Hayashi, H., Aikawa, H., Hirata, A., Sasaki, H. (2003) Influence of extracellular polymers on electrokinetic properties of heterotrophic bacterial cells examined by soft particle electrophoresis theory. *Colloids and Surfaces B: Biointerfaces*, 29, 181-188
- van der Wal, A., Norde, W., Zehnder, A.J.B., Lyklema, J. (1997) Determination of the total charge in the cell walls of Gram-positive bacteria. *Colloids and Surfaces B: Biointerfaces*, 9, 81-100
- Westall, J.C. (1982) FITEQL: A computer program for the determination of chemical equilibrium constants from experimental data: Version 1.2, Report 82-01, Department of Chemistry, Oregon State University
- Wightman, P.G., Fein, J.B. (2005) Iron adsorption by *Bacillus subtilis* bacterial cell walls. *Chemical Geology*, 216, 177-189
- Yee, N., Fowle, D.A., Ferris, F.G. (2004) A Donnan potential model for metal sorption onto *Bacillus subtilis*. *Geochimica et Cosmochimica Acta*, 68, 3657-3664
- Zufferey, R., Allen, S., Barron, T., Sullivan, D.R., Denny, P.W., Almeida, I.C., Smith, D.F., Turco, S.J., Ferguson, M.A., Beverley, S.M. (2003) Ether phospholipids

and glycosylinositol phospholipids are not required for amastigote virulence or for inhibition of macrophage activation by *Leishmania major*. *Journal of Biological Chemistry*, 278, 44708-44718

4. A macroscopic and EXAFS study of Zn sorption to the surface of the EPS-producing *Bacillus licheniformis* S-86.

Abstract

This study investigated Zn adsorption to an extracellular polymeric substance (EPS)-producing bacterial strain, *Bacillus licheniformis* S-86. The contribution made by the EPS layer to metal adsorption was investigated by conducting batch metal adsorption experiments and EXAFS analysis using both native and EPS-free cells. Results show that EPS removal does not affect the metal adsorption capacity at the metal:biomass ratios used here. Macroscopic adsorption experiments indicate Zn complexation is predominantly to site 2 (pK_a 5.3-5.4) and site 3 (pK_a 7.4-7.5) but with adsorption to site 1 (pK_a 3.3-3.4) also evident. Spectroscopic (EXAFS) analysis shows Zn-carboxyl complexation, but possibly with a significant contribution from a second functional group. As the concentration of sites 2 and 3 are only slightly affected by EPS extraction, complexation to these functional groups explains why EPS removal does not reduce the amount of Zn adsorbed by the cells. However, the EPS layer is relatively enriched in site 1 compared to the EPS-free cell wall, so the production of EPS by cells in heavily metal-contaminated environments may significantly increase complexation capacity compared to non-EPS-producing strains.

4.1. Introduction

Interactions between metals and microbes such as bacteria, fungi and archaea are increasingly recognised to contribute importantly to the geochemical cycling of trace metals, radionuclides and other contaminant species (Fein, 1997; Fein 2006). The adsorption of aqueous metal ions to cell surfaces is recognised as a significant process in many settings, and is potentially the precursor to biomineralisation, but despite many recent studies investigating bacterial surface complexation, the mechanisms are not yet satisfactorily explained. In order to achieve this, it is first necessary to ascertain that adsorption is not affected by parameters such as bacterial species, growth conditions and adaptation.

Early studies investigating mechanisms of metal adsorption to bacterial cell surfaces are based on macroscopic adsorption experiments and use of data optimisation models in order to quantify surface site concentrations and binding constants (Pagnanelli et al., 2000; Haas et al., 2001; Ngwenya et al., 2003). However, this approach does not provide the identities of the sites involved, and site identification has been based on comparison with previously published studies and data for comparable organic ligands. These studies have been complemented in recent years by spectroscopic investigations using techniques such as extended x-ray absorption spectroscopy (EXAFS) (Sarret et al., 1998; Hennig et al., 2001; Kelly et al., 2002; Boyanov et al., 2003; Toner et al., 2005; Guine et al., 2006). Markai et al. (2003) and Merroun et al. (2003) conducted metal adsorption studies using time-resolved laser-induced fluorescence spectroscopy (TRLFS). Such spectroscopic methods enable the characterisation of the metal binding environment, as nearest-neighbour bond distances and angles may be ascertained by comparison with reference spectra and standards.

Comparison of recent metal adsorption investigations reveals contradictions between the results obtained for macroscopic and spectroscopic studies. Generally, macroscopic adsorption studies have concluded that the principal group involved in metal binding is carboxylic in nature, with phosphoryl groups becoming involved at lower cell:metal ratios. Fein et al. (1997) observed this for Cd, Cu and Al complexation by *Bacillus subtilis*. Haas et al. (2001) studied U(VI) adsorption by

Shewanella putrefaciens and found that adsorption occurred to both carboxylic and phosphoryl surface groups. Ngwenya et al. (2003) also concluded that Pb, Cu and Zn were complexed by carboxyl and phosphoryl groups. Gorman-Lewis et al. (2005) concluded that Np(V) ion adsorption occurs to phosphoryl and carboxyl groups on the cell wall. Burnett et al. (2006) found that Cd adsorption occurred to carboxyl groups, and that an additional carboxyl or phosphoryl site may be involved at low cell:metal ratios.

Spectroscopic studies, by contrast, have generally found that phosphoryl groups are more important metal-binding sites than carboxylic groups. Hennig et al. (2001) found that U(VI) was primarily bound to phosphoryl groups on *Bacillus cereus* and *Bacillus sphaericus* surfaces. Kelly et al. (2002) investigated U(V) ion binding to *Bacillus subtilis*, and found that binding at very low pH (pH 1.67) occurs to protonated phosphoryl groups, with carboxylic-type groups becoming involved in binding as pH increases. Boyanov et al. (2003) concluded that Cd binds to phosphoryl groups below pH 4.4, and increasingly to carboxylic groups at higher pH. Sarret et al. (1998), Toner et al. (2005) and Guine et al. (2006) all conducted EXAFS investigations into Zn binding environments. Guine et al. (2006) identified Zn binding by both phosphodiester and carboxylic groups. Toner et al. (2005) also concluded that both types of groups were involved, but that Zn binding was predominantly to phosphoryl groups. Sarret et al. (1998) reached a similar conclusion during a study conducted using a species of fungus, finding that Zn and Pb have reversed affinities for phosphoryl and carboxyl surface groups.

In addition to identifying the cell surface groups involved in proton and metal adsorption, many studies have worked towards understanding the effects of factors such as growth conditions (Borrok et al., 2004c; Guine et al., 2007), temperature (Wightman et al., 2001), and bacterial strain (Yee and Fein 2001; Ngwenya et al., 2003; Borrok et al., 2004a; 2004b), that may alter cell surface chemistry or metal adsorption behaviour. With the exception of bacterial adaptation, which may affect metal complexation parameters under some circumstances (Borrok et al., 2004b), it is generally accepted that these parameters do not affect proton and metal adsorption significantly. Protonation and metal adsorption behaviour can therefore be modelled using a single 'average' set of parameters.

A further factor, the effects of which have not yet been fully established, is the presence of extracellular polymeric substances (EPS) on cell surfaces. In many natural settings bacterial cells are present within a biofilm matrix, much of which is composed of biochemically-complex EPS secreted by the cells. These polymers may also be produced by planktonic cells, and consist of fractions with varying degrees of solubility (Comte et al., 2006a). The role of EPS in metal adsorption must therefore be fully understood in order to accurately characterise the effect of bacterial cells on metal transport, under the wide range of applicable environmental conditions. A small number of studies have investigated the proton and metal binding behaviour of isolated EPS (Guibaud et al., 2003; 2004; Comte et al., 2006c; Lamelas et al., 2006). These studies have found that metal complexation characteristics of EPS are similar to those established for bacterial cells, but that there may be variations in site concentrations and stability constants. A study by Toner et al. (2005) investigated Zn sorption to a bacterial biofilm, a significant proportion of which comprises EPS and Merroun et al. (2003) observed that substantial quantities of lanthanum were sequestered in the extracellular polymers produced by the bacterium *Myxococcus xanthus*.

The present study has undertaken macroscopic adsorption experiments and EXAFS analysis of Zn adsorption to the surfaces of *Bacillus licheniformis* S-86, an EPS-producing bacterial strain. The objectives of this approach were to characterise the metal binding environments and to investigate the effect of the EPS layer on metal adsorption capacity, binding environments and stability constants.

Previously undertaken potentiometric titrations and surface complexation modelling (Tourney et al., 2008) using both native and EPS-free cells have established the presence of four proton-adsorbing surface sites on both EPS-containing cells and the residual cell walls of cells from which the EPS layer had been extracted. Data optimisation using FITEQL 4.0 indicated similar pK_a values for the four sites in both cell treatments, indicating that there is no significant difference in the types of surface functional groups involved in proton binding. However, the native cells contained approximately double the concentrations of site types 1 and 4, indicating that the proportions of the four functional groups present vary between the EPS and the residual cell wall. The four sites were tentatively assigned to phosphodiester (site

1), carboxyl (site 2), phosphoryl (site 3) and amine (site 4) type groups. Given the differences in total site concentration between the two cell types, it was hypothesised that native cells should adsorb more metal than EPS-free cells.

4.2. Materials and Methods

4.2.1. Cell culture and preparation

The thermotolerant gram-positive strain *Bacillus licheniformis* S-86 was obtained from Professor M. Baigori, PRIOIMI Biotechnology, Argentina. Experimental batches were grown from the primary culture by inoculating 2 L Pyrex flasks containing the Luria Broth growth medium (10 g L⁻¹ bacto-tryptone, 5 g L⁻¹ bacto-yeast extract, 5 g L⁻¹ NaCl). The medium was sterilised prior to inoculation by autoclaving at 121 °C for 30 minutes. Cultures were then incubated on a shaker for 24 hours at 30 °C. Comparison with cultures grown at 55 °C indicated that EPS production was not affected by the lower growth temperature. Cells were harvested by centrifugation at 23,420 x g for 15 minutes at 4 °C. The harvested cells were then washed 3 times in 18 MO ultrapure water and either used immediately or freeze-dried for use in subsequent experiments. There were no differences between fresh and freeze-dried cells, both in terms of EPS characteristics (SEM) and metal adsorption behaviour.

4.2.2. EPS extraction

EPS extraction was carried out as described in Chapter 2. SEM images of native (untreated) and EPS-free (cation-exchange resin treated) cells are shown in Figures 4.1a and 4.1b.

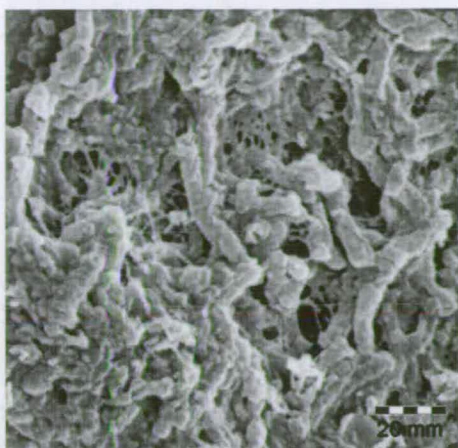


Figure 4.1a: SEM image of native *B. licheniformis* cells. (20 μm = 2 μm).

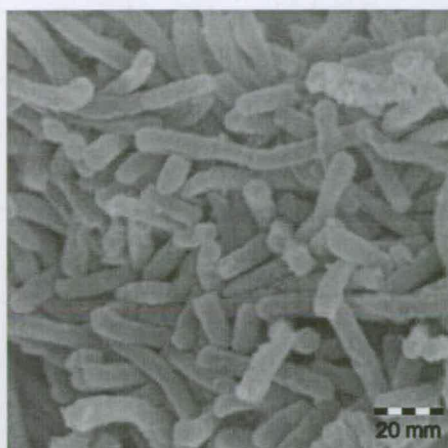


Figure 4.1b: SEM image of EPS-free *B. licheniformis* cells. (20 μm = 2 μm).

4.2.3. Metal adsorption experiments

Suspensions of native cells and EPS-free cells at varying cell concentration were prepared in 0.01 M NaNO₃ electrolyte (experimental parameters are shown in Tables 4.3a and 4.3b). 5 ml subsamples were removed and dried to constant weight in order to calculate the cell concentration for each experiment. Aqueous Zn (1000 ppm atomic absorption standard in HNO₃, Fisher Scientific) was added to the suspensions to obtain final metal concentrations of approximately 10 ppm. 20 ml volumes of suspension were transferred into 30 ml polycarbonate centrifuge tubes that had been acid washed prior to use. The samples were pH adjusted using NaOH and HNO₃ across an initial pH range of approximately pH 3 to pH 7.5. The suspensions were allowed to equilibrate on a rotating rack for 1 hour, and subsequently centrifuged at 17,210 x g for 10 minutes. A 10 ml volume of the supernatant was filtered using a 0.2 μm cellulose nitrate filter into an acid washed bottle. This solution was acidified to 1 % v/v HNO₃ (12.5 M HNO₃, Merck, Analar grade) and refrigerated prior to metal analysis by flame atomic absorption spectroscopy. The final pH of the remaining supernatant was recorded as pH was free to drift during the experiment. Metal concentration was determined using a Varian Spectra AA300 atomic absorption spectrophotometer. Analysis was conducted using an air/acetylene flame at a wavelength of 213 nm. The standards were prepared by serial dilution of a 1000 ppm Zn atomic absorption standard (Fisher Scientific), to concentrations chosen to

bracket sample concentrations (0- 10 ppm Zn). The standards were prepared in 0.01 M NaNO₃, acidified to 1 % HNO₃. Analytical precision was within 2 %.

4.2.4. Data modelling

The data was modelled assuming a 1:1 stoichiometry between the metal ion and a deprotonated surface site. The deprotonation of a functional group can be represented by the following generic reaction (Borrok and Fein 2004):



Where R is the bacterium and A represents a functional group on the bacterial cell wall volume. For native cells, this volume also includes the EPS layer present on the cell surface. The deprotonation or acidity constant, K_a, for this reaction may be expressed as:

$$K_a = \frac{[R - A^{-}]a_{H^{+}}}{[R - AH^{\circ}]} \quad (4.2)$$

where [R-A⁻] and [R-AH⁰] represent the concentration of deprotonated and protonated sites and a_{H⁺} represents the activity of protons in the solution

Subsequent metal adsorption to deprotonated surface functional groups is assumed to occur as follows (where M^{m+} is the metal ion):



Data modelling undertaken based on metal adsorption to protonated surface sites was tested during this study, but this resulted in models that either did not converge, or

converged with very poor fits to the experimental data. Stability constants are calculated according to the following mass-action equation:

$$K = \frac{[R - SM^{(m-1)}]}{[R - S^-][M^{m+}]} \quad (4.4)$$

Modelling was undertaken using previously determined pK_a values (Table 4.2). Stability constants for Zn complexation were then determined using FITEQL 4.0 (Herbelin and Westall, 1999). Initial metal concentrations were obtained by running blank (biomass-free) adsorption experiments. The non-electrostatic model was chosen, neglecting the effects of surface charge as these are not well quantified at present.

FITEQL calculates the variance, $V(Y)$, between the experimental data and the model:

$$V(Y) = \frac{\sum \left(\frac{Y_{calc} - Y_{obs}}{S_{obs}} \right)^2}{n_p n_{II} - n_u} \quad (4.5)$$

where Y_{calc} is the calculated value, Y_{obs} is the experimental value, S_{obs} is the error associated with the experimental data, n_p is the number of data points, n_u is the number of adjustable parameters in the model, and n_{II} is the number of 'Group II' components, for which both the total and free (dissolved) concentrations are known (Ngwenya et al., 2003). A variance of less than 20 is considered a good fit to the experimental data (Herbelin and Westall, 1999).

4.2.5. EXAFS spectroscopy

Suspensions of native (untreated) cells and EPS-free cells were prepared in 0.01 M $NaNO_3$ electrolyte. 5 ml subsamples were removed and dried to constant weight in order to obtain cell concentrations for the suspensions. Solutions of Zn were added to the suspensions to obtain final metal concentrations of 20 ppm, then the suspensions were divided in 3 and pH-adjusted to approximately pH 3, 5 and 7.

Experiments were carried out using 50 ml polycarbonate centrifuge tubes that had been acid washed prior to use. The suspensions were allowed to equilibrate on a rotating rack for 1 hour, following which they were centrifuged at 12,000 rpm for 10 minutes and the supernatants decanted. The cell pellets were then briefly rinsed with pH-adjusted electrolyte and centrifuged for a further 10 minutes at 12,000 rpm. The supernatant was discarded and the cell pastes were loaded into aluminium slots and covered with Kapton tape. The samples were stored frozen prior to EXAFS analysis.

Standards were prepared in order to enable comparison with the presumed carboxyl or phosphoryl binding sites on the biomass. Zn acetate (Merck, Analar grade) and Zn phosphate (Merck, Analar grade) were ground to a fine powder with boron nitride (ratio of 1:5) and then spread evenly onto sellotape, which was layered 6-8 times to form the solid standard. These reagents were also used to prepare 0.05 M Zn acetate and 0.01 M Zn phosphate solution standards. Solutions were adjusted to pH 7 using 0.1 M HNO₃ and NaOH.

Experiments were conducted at the synchrotron radiation source (SRS), station 16.5, at CCLRC Daresbury Laboratory, UK. The storage ring operated at 2 GeV with currents between 100 and 250 mA. Zn K-edge spectra were collected for the samples and standards using a sagittally focusing Si (220) crystal monochromator. Prior to data collection, monochromator energy calibration was carried out using Zn foil. Solution standard spectra were collected in transmission mode, solid standards and sample spectra were analysed in fluorescence mode using a 30-element Ge solid state detector. Samples were analysed using a cryostat. Between three and eight scans were collected and averaged for each standard and sample. Background subtraction, calibration and normalisation to an edge-step of unity were undertaken using the Daresbury Laboratory programs Excalib and Exspline. Spectra were k^3 -weighted and Fourier-transformed in the k -range of 3 Å⁻¹ - 13 Å⁻¹. EXAFS data fitting and refinement was undertaken using the Daresbury Laboratory program Excurv98. Shell-by-shell fitting was carried out and the quality of fit was evaluated using the R factor:

$$R_{exafs} = \sum_i^N \frac{1}{\sigma_i} \left(\left| \chi_i^{\text{exp}}(k) - \chi_i^{\text{th}}(k) \right| \right) \times 100\% \quad (4.6)$$

where $\chi_i^{\text{exp}}(k)$ and $\chi_i^{\text{th}}(k)$ are the experimental and theoretical EXAFS respectively and σ_i is defined as:

$$\frac{1}{\sigma_i} = k_i^n / \sum_j^n k_j^n \left| \chi_j^{\text{exp}}(k_j) \right| \quad (4.7)$$

An R-value of around 20 % is generally considered a good fit to the data.

4.3. Results

4.3.1. Macroscopic Zn adsorption

4.3.1.1. Native cells

The Zn adsorption edges shown in Figure 4.2a indicate that adsorption is close to zero at pH 3 and increases to a maximum of approximately 90 % at pH 6.5, for the experiments conducted at cell concentrations of 1.47 g L⁻¹ and 2.19 g L⁻¹. Adsorption edges for experiments conducted at lower cell concentrations show less Zn adsorption, with a maximum of approximately 50 % Zn adsorbed at 0.44 g L⁻¹ cells, and approximately 25 % of the available Zn adsorbed at 0.24 g L⁻¹ cells. This dependence on mass is well known and indicates that site concentration controls total metal adsorbed. For both native and EPS-free cells, standard deviations are calculated based on the precision of the AAS analysis (analytical precision).

4.3.1.2. EPS-free cells

The Zn adsorption edges for experiments conducted using EPS-free cells are shown in Figure 4.2b. Adsorption is minimal at pH 3 and increases to a maximum of approximately 90 % Zn adsorption, at pH greater than 6, for the experiments

conducted at concentrations of 1.09 g L^{-1} cells and greater. The isotherm for the experiment conducted at 0.57 g L^{-1} cells indicates a maximum Zn sorption capacity of approximately 80 %, and at 0.33 g L^{-1} cells the maximum adsorption capacity is approximately 55 %.

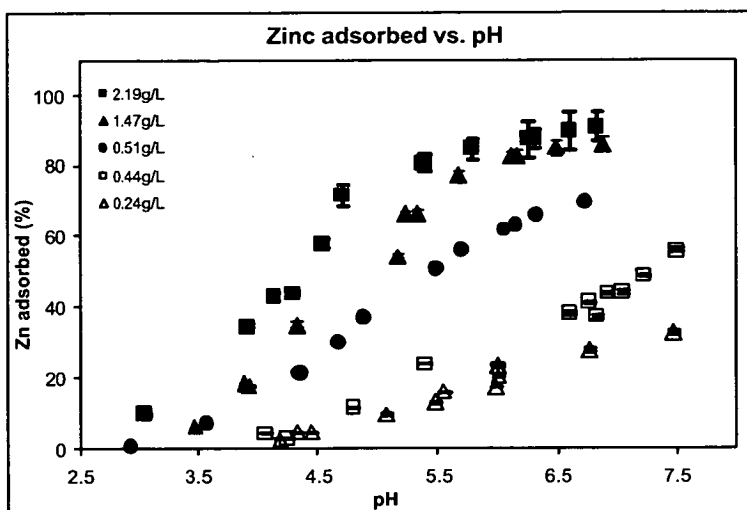


Figure 4.2a: Zinc adsorption edges for native cells (error bars indicate 2 standard deviations, based on analytical precision).

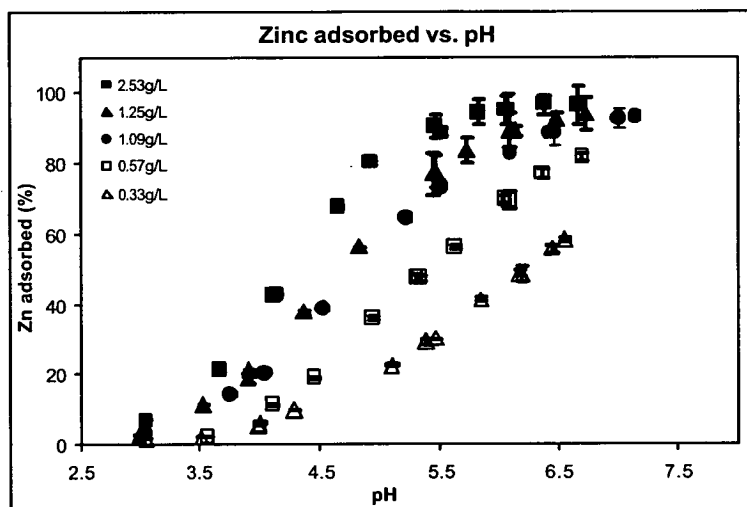


Figure 4.2b: Zinc adsorption edges for EPS-free cells (error bars indicate 2 standard deviations, based on analytical precision).

4.3.3. Zn EXAFS analysis

4.3.3.1. Standards

Zn acetate and Zn phosphate standards were analysed as both solids and solutions, and in each case better signals were obtained from the solution standards (Table 4.1 shows data for the solution standards). For all EXAFS data modelling, the coordination numbers were fixed. The EXAFS data analysis indicates that the Zn-acetate binding environment consists of a carbon shell at 2.83 Å, between two oxygen shells at 2.08 Å and 3.85 Å. This falls within the range of Zn-C interatomic distances for anhydrous Zn acetate of approximately 2.70 Å – 3.45 Å (Sarret et al., 1998). The Zn-phosphate standard indicated a phosphorus shell at 3.10 Å and oxygen shells at 1.93 Å and 3.66 Å. This Zn-P distance is close to the value obtained by Sarret et al. (2002) of 3.08 Å, for a Zn phytate standard. However, the R-value of 26.72 indicates that the fit of the model spectrum to the experimental spectrum for Zn-phosphate is sub-optimal, suggesting that this may not be a suitable standard to use for the purposes of this study. Whilst the R-factors for the attempted Zn-phosphate binding for the biomass samples apparently indicate better fits than for the Zn-carboxyl models (Table 4.1), it should be noted that the Zn-P bond distances are unrealistically small and do not correspond to the Zn-P bond distance for the Zn phosphate standard. EXAFS spectra, Fourier transforms and model fits are shown in Figure 4.3.

Table 4.1: EXAFS results for the zinc standards and biomass samples. CN = coordination number of the scattering atom (Zn), R = interatomic distance, σ^2 = Debye-Waller factor (low values indicate improvement in fit as the number of shells increases).

Sample	pH	Concentration	Atom	CN	R (Å)	σ^2 (Å ²)	R-factor
Zinc acetate		0.05 M	O	6	2.08	0.02	20.37
			C	2	2.83	0.04	
			O	4	3.85	0.03	
Zinc phosphate		0.01 M	O	6	1.93	0.03	26.72
			P	2	3.10	0.04	
			O	4	3.66	0.02	
Native	3.04	0.67 g L ⁻¹	O	6	2.06	0.01	36.85
			C	2	2.84	0.03	
			O	4	3.77	0.01	
			O	6	2.06	0.01	34.31
			P	2	2.57	1.88	
			O	4	3.78	0.01	
Native	4.97	0.67 g L ⁻¹	O	6	2.06	0.02	44.23
			C	2	2.84	0.03	
			O	4	3.79	0.03	
			O	6	2.05	0.02	43.33
			P	2	2.37	0.05	
			O	4	3.78	0.03	
Native	7.00	0.67 g L ⁻¹	O	6	2.05	0.02	33.24
			C	2	2.84	0.04	
			O	4	3.81	0.03	
			O	6	2.06	0.02	34.24
			P	2	3.22	0.40	
			O	4	3.81	0.02	
EPS-free	2.97	0.57 g L ⁻¹	O	6	2.09	0.01	42.43
			C	2	2.97	0.04	
			O	4	3.88	0.03	
			O	6	2.07	0.01	40.41
			P	2	2.37	0.02	
			O	4	3.81	0.03	
EPS-free	4.97	0.57 g L ⁻¹	O	6	2.08	0.01	25.17
			C	2	2.89	0.04	
			O	4	3.86	0.04	
			O	6	2.08	0.01	25.15
			P	2	2.40	0.05	
			O	4	3.84	0.04	
EPS-free	6.95	0.57 g L ⁻¹	O	6	2.05	0.02	27.46
			C	2	2.83	0.04	
			O	4	3.80	0.02	
			O	6	2.05	0.02	27.64
			P	2	2.33	0.06	
			O	4	3.79	0.02	

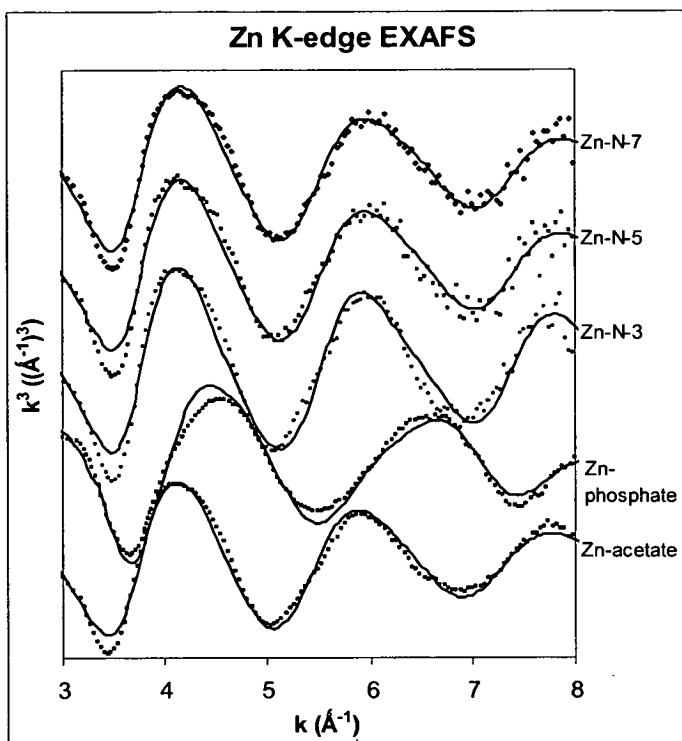


Figure 4.3ai. EXAFS spectrum for Zn standards and native cells. Points = data; Lines = best-fit models.

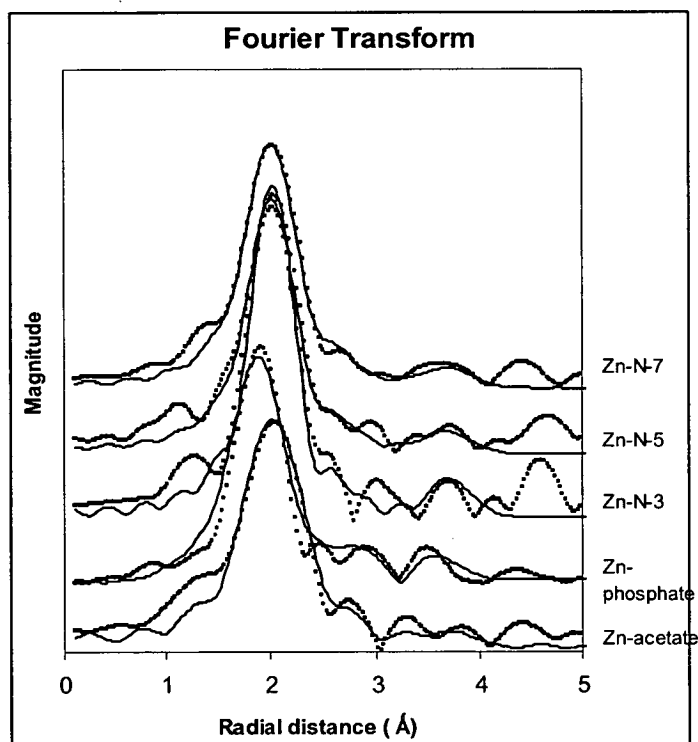


Figure 4.3aii. Fourier transform for Zn standards and native cells. Points = data; Lines = best-fit models.

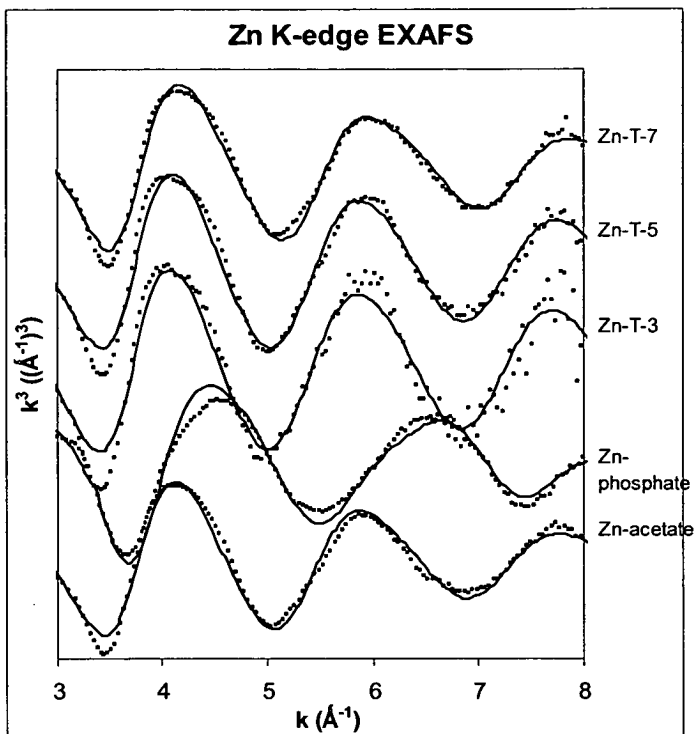


Figure 4.3bi. EXAFS spectra for Zn standards and EPS-free cells. Points = data; Lines = best-fit models.

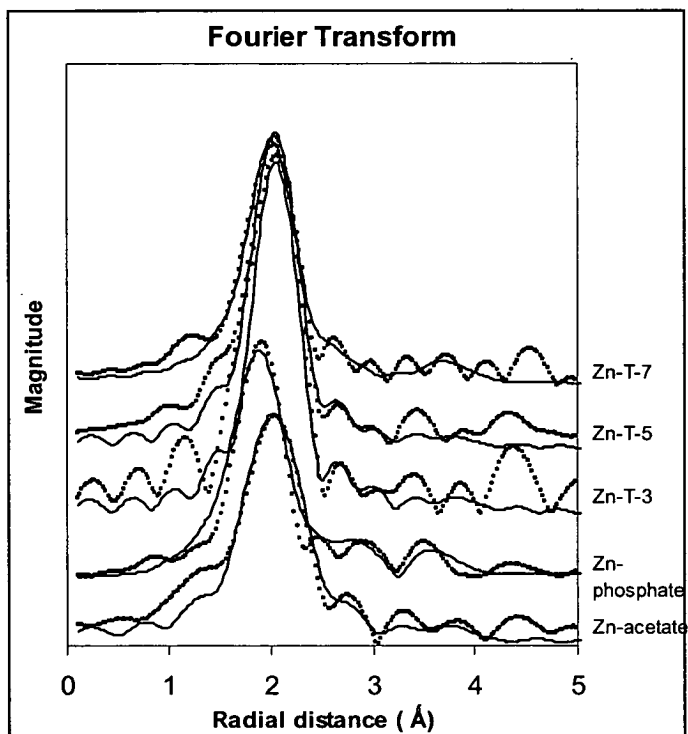


Figure 4.3bii. Fourier transform for Zn standards and EPS-free cells. Points = data; Lines = best-fit models.

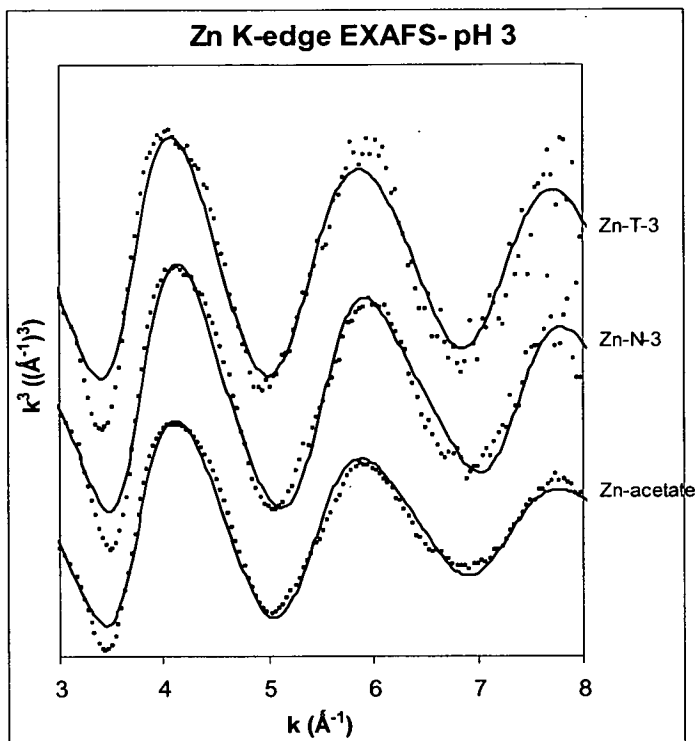


Figure 4.3ci. EXAFS spectrum for native and EPS-free cells at pH 3. Points = data; Lines = best-fit models.

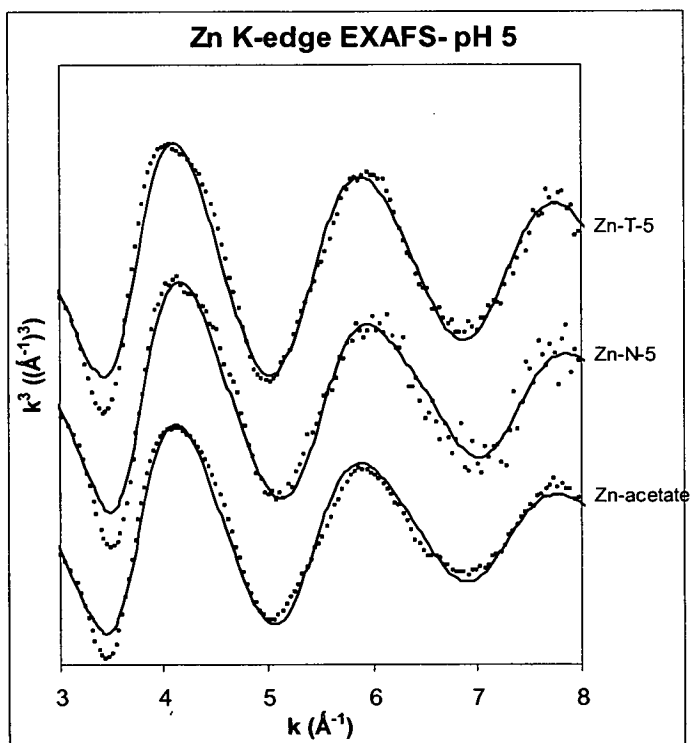


Figure 4.3di. EXAFS spectrum for native and EPS-free cells at pH 5. Points = data; Lines = best-fit models.

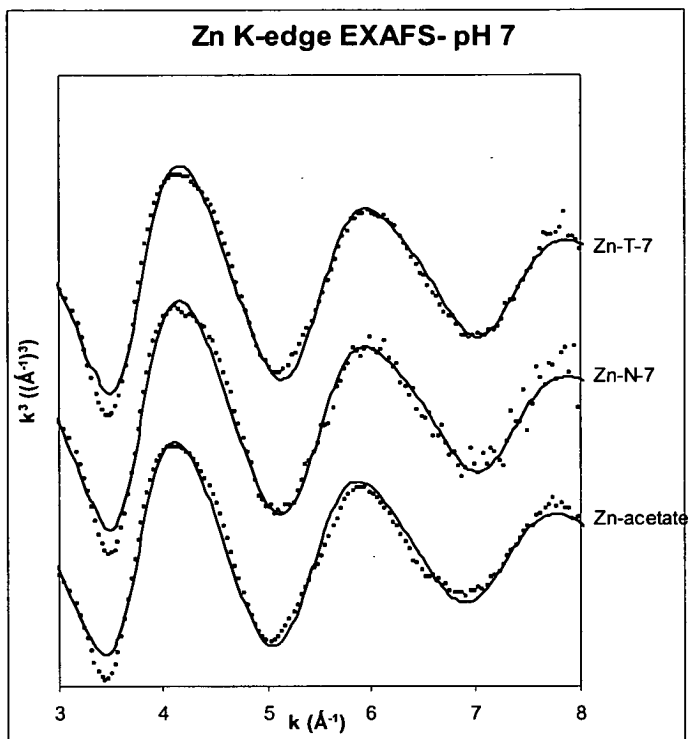


Figure 4.3ei. EXAFS spectrum for native and EPS-free cells at pH 7. Points = data; Lines = best-fit models.

4.3.3.2. Native cells

The k^3 -weighted EXAFS spectra for the native cells are shown with those for the standards in Figure 4.3. It can be seen that the spectra for the biomass samples are clearly out of phase with that of the Zn phosphate standard, but in phase with that for the Zn acetate standard. The spectra for the biomass samples all have greater amplitudes than the Zn acetate standard, and the amplitudes of the biomass spectra decrease slightly with increasing pH. Decreasing amplitude of the EXAFS spectrum indicates an increased range of interatomic (Zn-C or Zn-P) bond distances (Sarret et al., 1998). The Fourier transforms show peaks at approximately 2.0 Å and 2.8 Å that are in phase with the peaks shown in the Fourier transform for the acetate standard. The pH 3 and pH 5 samples also show a peak at approximately 3.0 Å. This peak appears at approximately the same position as a peak seen in the Fourier transforms either for phosphate standard.

The EXAFS parameters and data modelling results are shown in Table 4.1. In each case, lower Debye-Waller factors were obtained for models incorporating carbon shells, and Zn-ligand bond distances were closer to those obtained for the Zn-acetate standard than those for the Zn-phosphate standard. The Zn-P distances for the biomass samples are generally unrealistically low. The Zn-C bond distances for the native cells were slightly higher than those obtained for the Zn-acetate standard, at 2.84 Å (pH 3.04), 2.84 Å (pH 4.97) and 2.84 Å (pH 7.00).

4.3.3.3. EPS-free cells

The biomass and standard k^3 weighted EXAFS spectra shown in Figure 4.3 indicate that the biomass samples are in phase with the Zn acetate standard rather than the Zn phosphate standard. There is a decrease in amplitude with increasing pH of the biomass samples, all of which have spectra with greater amplitudes than that of the Zn acetate standard. There is also a slight shift to higher k -values in the spectrum for the pH 7 sample, relative to the other spectra. The Fourier transforms for the EPS-free cells show peaks at approximately 2.0 Å and 2.8 Å, in phase with peaks in the Zn-acetate Fourier transform. The Fourier transforms for the EPS-free cells at each

pH show a small peak at approximately 3.0 Å, in phase with a peak in the Zn-phosphate Fourier transform.

As for the native cells, the best-fit models (based on the Debye-Waller factor, Fit Index and bond distances) to the EXAFS data (Table 4.1) indicated Zn binding to a carboxyl-type ligand. The Zn-C bond distances were less consistent than those obtained for the native cells, at 2.90 Å (pH 2.97), 2.89 Å (pH 4.97) and 2.83 Å (pH 6.95).

4.4. Discussion

4.4.1. Zn adsorption

The initial observation drawn from Figures 4.2a and 4.2b is that mass-dependent adsorption is less apparent than may be expected. The curves for native cells in particular lack a consistent trend as a function of biomass concentration.

Specifically, there is little difference in maximum adsorption between the 1.47 g L⁻¹ and 2.19 g L⁻¹ experiments, and a disproportionately large difference between the 0.57 g L⁻¹ and 0.44 g L⁻¹ experiments.

As this behaviour was not been observed during similar experiments carried out using non EPS-producing strains, it was hypothesised that the presence of bound EPS may induce aggregation of cells in suspension. Aggregation of cells will lead to a reduction in the cell surface area available for adsorption, and a lack of correspondence between the experimentally determined biomass concentration and the amount of metal adsorbed. In order to test this hypothesis, gram-stained suspensions of native and EPS-free cells at different biomass concentrations of 0.2 g L⁻¹, 1 g L⁻¹ and 2 g L⁻¹, and pH values of approximately 3, 5 and 7 were examined. As shown in Figure 4.4, considerable aggregation occurs when the cells are in suspension. Aggregation was observed to increase with increasing biomass concentration, and also with decreasing pH due to protonation of surface functional groups and consequent reduction of the electrostatic repulsion between cells. Cell aggregation at low pH is a generally observed occurrence because of these

electrostatic interactions; however, considerable aggregation was also observed at pH 5 and 7 and this is most likely caused by the presence of EPS. Some degree of aggregation was observed even at the lowest biomass concentration, indicating that this problem cannot be avoided by conducting experiments at low concentration. Comparison of the native and EPS-free cell suspensions shows that aggregation is considerably less in the EPS-free suspensions, and that the large quantities of EPS surrounding the clusters of native cells are not visible. However, some aggregation does still occur and this may either be caused by small quantities of residual EPS that was not removed by treatment with cationic exchange resin, or it is possible that the cells continue to produce some EPS after the harvesting and washing procedure is carried out. This may explain why the EPS-free experimental curves also show less mass-dependent trend than may be expected.

A further possible reason for loss of adsorption curve resolution, particularly at high pH, is competition for metal complexation by dissolved organic carbon (DOC) released from the EPS layer. As discussed in Chapter 2, the EPS layer is the major source of DOC from the *B. licheniformis* cells and the quantity in solution increases with pH, possibly due to the desorption of hydrophobic fractions (Borrok et al., 2007). It is thought likely that fractions of the DOC are active in metal adsorption, forming metal-ligand complexes in solution (Ngwenya, 2007) and thus decreasing the amount of Zn adsorbed to the cell surfaces.

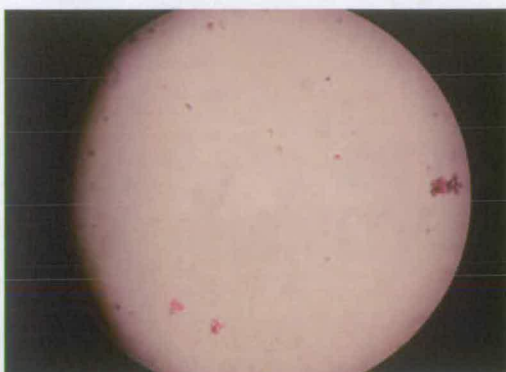


Figure 4.4ai.

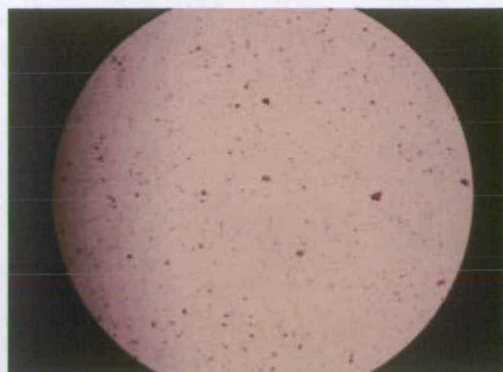


Figure 4.4aii.

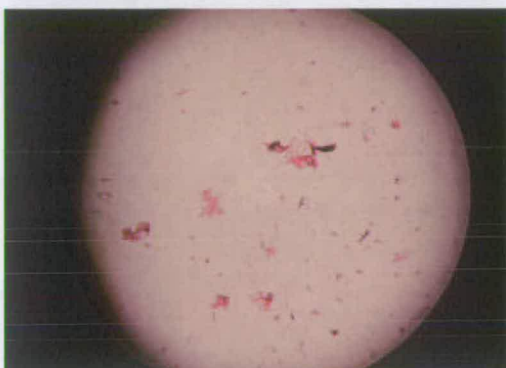


Figure 4.4bi.

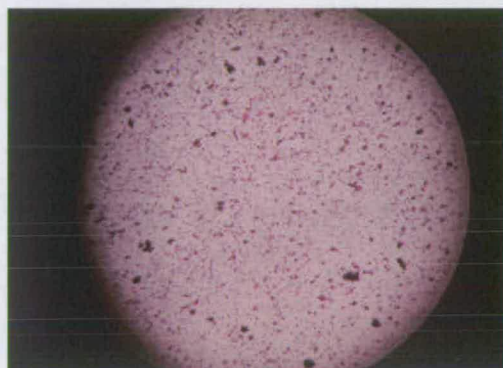


Figure 4.4bii.



Figure 4.4ci.



Figure 4.4cii.

Figure 4.4: Light microscope images of *B. licheniformis* suspensions. a: Biomass concentration of 0.2 g L^{-1} , pH 5 (Image diameter approx 1 mm): i) Native cells; ii) EPS-free cells. b: Biomass concentration of 1 g L^{-1} , pH 5 (Image diameter approx 1 mm): i) Native cells; ii) EPS-free cells. c: Biomass concentration of 2 g L^{-1} , pH 5 (Image diameter approx 1 mm): i) Native cells; ii) EPS-free cells.

Comparison of Figures 4.2a and 4.2b suggests that the EPS-free cells are able to complex a larger quantity of Zn than the native cells. For example, the EPS-free experiment at 1.25 g L^{-1} shows a greater maximum adsorption of Zn than the native cell experiment at 1.47 g L^{-1} , and the EPS-free experiment at 0.57 g L^{-1} shows a considerably larger maximum adsorption than the native cell experiment at 0.51 g L^{-1} , despite the relatively small difference in biomass concentration. This is probably a reflection of the greater extent to which native cells aggregate in solution compared to EPS-free cells rather than a real difference in adsorption capacity.

The FITEQL data modelling results, calculated using previously determined site concentrations and deprotonation constants (Table 4.2) show that, for most of the native and EPS-free experiments, the optimal model fit involves Zn complexation by both site 2 and site 3 (Tables 4.3a and 4.3b). In many cases all of sites 1, 2 and 3 are required to optimally fit the experimental data. The Zn-site 2 log K values are quite consistent for both the native and EPS-free cells, but the stability constants for the other two sites show considerable variability. This may be because their involvement in Zn complexation is less consistent, or it may suggest that there is some heterogeneity within a functional group in terms of Zn complexation behaviour, possibly due to interactions with neighbouring organic ligands. The number of sites required to fit the data does not decrease with increasing biomass concentration as is normally observed in such studies. As discussed above, this is most likely due to the considerable degree of aggregation that occurs in the cell suspensions due to the presence of EPS. This scenario will lead to a reduced available surface area and the requirement for additional surface sites to complex the available metal. This may explain why, for example, 2 sites are required to explain Zn adsorption for the two lowest-biomass native cell experiments, but 3 sites are required for many of the experiments conducted at higher biomass concentrations. Such behaviour has not been observed with other, non-EPS producing strains in our laboratory, suggesting that this is a feature of EPS-driven aggregation. Although the Zn-site stability constants for sites 1 and 3 are less consistent than those for site 2, the histogram of native and EPS-free stability constants (Figure 4.5a) shows that the differences between the mean stability constants for each site are within the bounds of experimental error. Comparison of the log K values by the student's t-test ($p=$

0.05) indicates that the differences between native and EPS-free cell log K values are not significant for sites 1 and 3. However, the log K values for site 2 are more consistent for both cell treatments, and the t-test results show a significant difference between the two. As surface sites within bacterial cell walls may represent a range of closely-related functional groups, it is possible that these results reflect some slight differences in composition between the EPS and the bulk cell wall. Alternatively, the difference in structure may lead to differing interactions with neighbouring molecules and hence slightly differing metal complexation characteristics.

Table 4.2: Mean pK_a values, site concentrations and associated 1x standard deviations for *B. licheniformis* S-86.

	Native cells		EPS-free cells		Type
	pK _a	[Site] (mol g ⁻¹)	pK _a	[Site] (mol g ⁻¹)	
Site 1	3.36 ± 0.21	8.53 ± 2.45 × 10 ⁻⁴	3.29 ± 0.12	5.47 ± 1.13 × 10 ⁻⁴	Phosphodiester
Site 2	5.25 ± 0.08	9.06 ± 2.14 × 10 ⁻⁴	5.36 ± 0.23	6.82 ± 1.09 × 10 ⁻⁴	Carboxyl
Site 3	7.39 ± 0.20	2.87 ± 0.59 × 10 ⁻⁴	7.51 ± 0.37	2.22 ± 0.62 × 10 ⁻⁴	Phosphoryl
Site 4	9.93 ± 0.06	6.50 ± 1.44 × 10 ⁻⁴	10.13 ± 0.19	3.26 ± 0.68 × 10 ⁻⁴	Hydroxyl/amine

Table 4.3a: FITEQL modelling results for Zn adsorption to native cells.

[Biomass] (g L ⁻¹)	Log K 1	Log K 2	Log K 3d	V(Y)
0.24		3.29	4.20	5.26
0.44		3.20	4.24	5.08
0.51	2.15	3.73	4.69	1.56
0.57	3.32	3.99	5.64	1.51
0.76	3.00	3.78	5.88	4.81
0.91	1.97	3.64	5.57	3.36
0.93		3.60	5.05	5.42
1.02	2.78	3.60	5.25	8.03
1.47	1.76	3.53	4.86	9.75
2.19	2.27	3.50		5.17
Mean	2.46	3.58	5.04	
St dev.	0.58	0.23	0.60	

Table 4.3b: FITEQL modelling results for Zn adsorption to EPS-free cells.

[Biomass] (g L ⁻¹)	Log K 1	Log K 2	Log K 3d	V(Y)
0.33		3.67	5.42	5.20
0.57			6.50	1.30
0.69	3.02		6.47	20.18
1.09	2.11	3.82	5.80	0.44
1.25	3.02	3.95		5.92
1.25	2.00	3.87	5.80	0.93
1.83		3.89		3.04
2.53	1.06	3.87	5.97	0.39
Mean	2.24	3.85	5.99	
St dev.	0.82	0.10	0.42	

Although the results are not always consistent with regard to the number of sites required to fit the experimental data, it was decided to calculate average stability constants for all three sites (using combined native and EPS-free results), and to obtain predictive adsorption curves using a 3-site model. The calculated average stability constants were 2.37 ± 0.66 (site 1), 3.68 ± 0.23 (site 2) and 5.42 ± 0.71 (site 3). Representative experimental and model curves are shown in Figure 4.5b. The model curves for both the native cell experiments considerably overpredict the amount of Zn adsorbed at pH 6.5, whereas the model curves for the EPS-free experiments show slight underprediction of maximum sorption by the 1.25 g L⁻¹ experiment and a close fit to the experimental data for the 0.57 g L⁻¹ experiment. These observations are probably primarily driven by the effects on mass-dependent adsorption caused by the cell aggregation discussed earlier. The closer fits to the data for the EPS-free cells, particularly at lower biomass concentration, are probably a reflection of the closer correlation of the calculated site concentrations with the experimentally-determined biomass concentration, due to less cell aggregation.

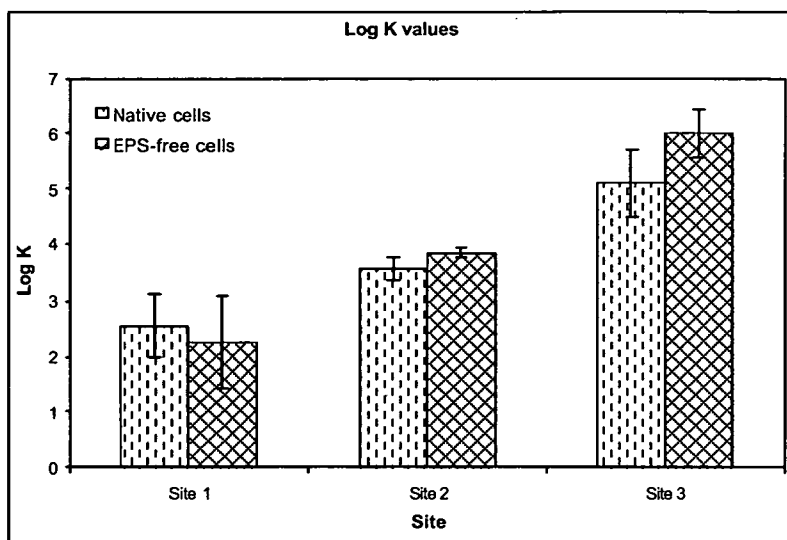


Figure 4.5a: Histogram of Log K values for Zn adsorption (error bars indicate 1 standard deviation).

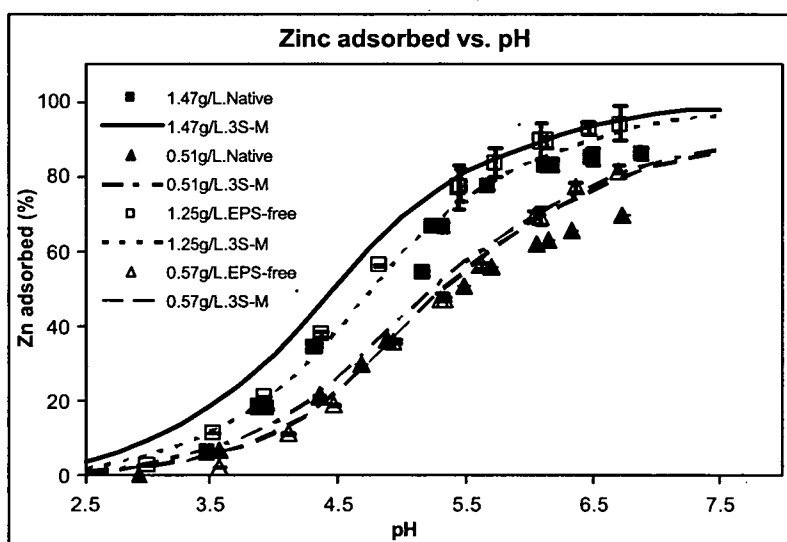


Figure 4.5b: Example model fits to adsorption data for zinc (error bars indicate 2 standard deviations, based on analytical precision).

The results of the macroscopic adsorption experiments lead to the conclusion that Zn binding, for both native and EPS-free cells occurs principally to the carboxylic and phosphoryl groups, but that contributions from the phosphodiester group may also be significant. As the carboxyl group will begin to deprotonate at a pH of approximately 3.3 ($\text{pK}_a \pm 2$), it is likely that Zn binding occurs to this group across the whole pH range at which these experiments were conducted. The phosphodiester groups will also be in the deprotonated form across the whole experimental pH range, and the phosphoryl groups will start to deprotonate at approximately pH 5.4. Most macroscopic studies of Zn adsorption have concluded that Zn is bound primarily to carboxyl-type sites; however, the pK_a values and assignments of the proton-active surface sites do not necessarily correspond to those used in this study. Generally, previous studies have assigned sites with pK_a values of approximately 4-5 to carboxyl groups, and sites with pK_a values between 6 and 8 to phosphoryl groups (Fein et al., 1997; Plette et al., 1996; Ngwenya et al., 2003; Claessens and van Capellen 2007). The pK_a 7.4-7.5 site therefore corresponds to the phosphoryl sites identified in these other studies, but the pK_a 5.3-5.4 site falls slightly outwith the usual range assigned to carboxyl groups. Daughney et al. (1998) established pK_a values for *B. licheniformis* of 5.2, 7.5 and 10.2, assigned to carboxyl, phosphoryl and hydroxyl type groups respectively, and these are in very close agreement with sites 2, 3 and 4 defined here. Chatellier and Fortin (2004) highlighted the problem of ambiguous identifications of proton-active surface sites, as their study of ferrous iron adsorption to *B. subtilis* suggested that adsorption occurred to a site with pK_a of approximately 6.7, and that the pK_a 4.5 site usually assumed to be carboxylic only made a minor contribution to Fe adsorption.

The Zn-site stability constants (log K values) found here are broadly comparable with those of other studies. Plette et al. (1996) investigated metal binding to isolated cell walls of a gram-positive bacterium and described metal binding to both carboxylic (pK_a 4.6) and phosphatic (pK_a 7.8) sites, with Zn-site log K values of 1.9 and 4.1 respectively. Ngwenya et al. (2003) investigated proton adsorption to a gram-negative bacterium and found three sites, with pK_a values of 4.3 (carboxyl), 6.9 (phosphoryl) and 8.9 (hydroxyl/amine). The carboxyl and phosphoryl sites were found to be involved in Zn adsorption, with log K values of 3.3 and 5.1. Claessens

and van Capellen (2007) explained Zn binding to *S. putrefaciens* with a 2-site model, with log K values of 4.3 for the acidic group and 5.5 for the neutral group, defined as carboxylate and phosphoryl in this study. Guine et al. (2006) conducted Zn adsorption experiments using 3 different bacterial species, and found log K values of between 2.0 and 3.3 for Zn-carboxyl, between 4.0 and 5.3 for Zn-phosphoryl and between 5.2 and 5.6 for Zn-amine complexes. The wide range of log K values reported by this study for each site highlight considerable variation in stability constants between species. The log K values reported in the literature are fairly similar to the mean log K values established by the present study. There is considerable overlap between the log K values established for the 'acidic' site in other studies and both the Zn-site 1 and Zn-site 2 log K values reported here, which reflects variations in the pH range constrained, and in the number of proton-active sites identified by different studies.

The results of this study indicate that Zn does have a high affinity for the phosphoryl site (pK_a 7.4-7.5) compared to the carboxyl site, as the Zn-phosphoryl stability constants (log K values) of 5.04 and 5.99 for native and EPS-free cells respectively are substantially higher than those for the pK_a 5.3-5.4 (carboxyl) site, which are 3.58 and 3.85. However, previous potentiometric titrations indicated that the pK_a 7.4-7.5 site is only 30 % as abundant as site 2, in both native and EPS-free cells.

Furthermore, the adsorption experiments were undertaken to a maximum pH of 7, at which pH less than 50 % of the site 3 functional groups would be deprotonated.

These factors may explain why the pK_a 5.3-5.4 site is more significant in complexation, despite the lower affinity for Zn. A high affinity of Zn for phosphoryl sites was also indicated in the macroscopic adsorption studies by Plette et al. (1996), Ngwenya et al. (2003), Claessens and van Capellen (2007) and Guine et al. (2007). Fein et al. (2001) found that Co, Nd, Ni and Sr adsorption could all be explained by carboxyl binding alone, but that Zn required a phosphoryl site in addition.

The results of the EXAFS analysis are supportive of the macroscopic adsorption data. The dominance of the carboxylic group (pK_a 5.3-5.4) indicated by the FITEQL modelling results is confirmed by the EXAFS spectra (Figure 4.3), which show a clear correspondence between the biomass samples and the Zn-acetate standard, but not with the Zn-phosphate standard. The data analysis (Table 4.1) indicates that the

Zn binding environment in the biomass samples consists of 6 oxygen atoms in an octahedral configuration around the central Zn. This coordination is known to occur in Zn acetate (van Niekerk et al., 1953), accounting for the good correspondence between the Zn acetate standard and the biomass samples. The model fits show the second shell to consist of 2 carbon atoms in each case. The slight deviations in Zn-C bond distances from that obtained for the Zn-acetate standard, together with the moderately high R-factors, imply that the binding environment is non-uniform. This is likely due to the significant degree of binding to an additional site, either the phosphoryl (pK_a 7.4-7.5) site identified as significant in the macroscopic adsorption experiments, or the pK_a 3.3-3.4 (phosphodiester) site which would be fully deprotonated across the experimental pH range. Inspection of the Fourier transforms (Figure 4.3) for both the native and EPS-free cells reveals the presence of peaks at approximately 3.0 Å that appear to correspond to the phosphorus shell in the Zn-phosphate standard. However, the best-fit models did not incorporate this peak. A decrease of amplitude in the EXAFS spectra, as observed here with increasing pH, was observed by Sarret et al. (1998) as Zn concentration increased and interpreted as indicating an increasing distribution of bond lengths (Zn-C or Zn-P) as different types of sites became involved in Zn binding. As Zn adsorption increased with pH, the decreased amplitude of the EXAFS signals in this study very probably does reflect increasing involvement of the phosphate site. However, a highly heterogeneous binding environment will not produce a resolvable EXAFS signal, so these results indicate that the complexation to carboxyl groups is the predominant form of Zn binding in these samples. As mentioned earlier, the model spectrum fit to the experimental EXAFS spectrum for the Zn phosphate standard is not ideal. It is likely that use of a wider range of Zn phosphate and phosphodiester standards would enable a more detailed characterisation of the Zn binding environment. Linear combination fitting can be undertaken to constrain the contribution made by minor functional groups, provided that suitable standard spectra are available.

These results are interesting because the majority of previous studies investigating Zn complexation by microbial cell surfaces have concluded that Zn is bound predominantly by phosphoryl-type ligands (Sarret et al., 1998; Toner et al., 2005; Guine et al., 2006). In each of these studies, it was concluded that the contribution of

the carboxyl groups to Zn sorption was very minor compared to that of the phosphoryl groups. However, despite the high affinity of Zn for phosphoryl groups indicated by the log K values obtained here, the macroscopic and spectroscopic data both show that carboxylic groups are more significant. This contrast with previous work may be explained in part by differences in the microorganisms used. Sarret et al. (1998) investigated adsorption to *Penicillium chrysogenum*, a fungus with cell walls containing predominantly phosphoryl type groups (95 %) and minor carboxylic groups (5 %)- it is therefore unsurprising that the Zn was predominantly bound to phosphoryl groups. Toner et al. (2005) were investigating Zn adsorption to a biofilm, the composition of which may differ significantly from that of isolated bacterial cells. This raises the issue of biochemical composition of bacterial extracellular polymers, which are generally considered to consist mostly of carbohydrates and proteins and relatively little phosphate-rich nucleic acids (Comte et al., 2006a). However, Matsukawa et al. (2004) reported a nucleic acid content of up to 80 % in the EPS of a *P. aeruginosa* biofilm. The findings of Toner et al. (2005) suggest that either the biofilm composition was considerably more P-rich than is typical of EPS, or that Zn had an overwhelming preference for relatively minor phosphate groups. The study by Guine et al. (2006), investigating Zn sorption to 3 gram-negative bacterial species, found that Zn had a higher affinity for the phosphoryl sites than the carboxyl, but the binding observed could not account for the full extent of observed Zn adsorption, and it was suggested that other mechanisms play an important part, particularly adsorption to EPS components. EXAFS studies conducted using other metals have found that at low pH, phosphoryl bonding is dominant but that carboxyl groups become more important as pH increases. Boyanov et al. (2003) found that Cd binding was mainly to phosphoryl groups below approximately pH 4, but that carboxyl-type binding increased with pH. Kelly et al. (2002) conducted an EXAFS investigation using the uranyl ion, concluding that binding at very low pH (pH 1.67) was exclusively to phosphoryl groups, but that carboxylic groups became progressively more dominant in uranyl complexation at higher pH values (pH 3.22 and pH 4.80). A study conducted using cyanobacteria (Yee et al., 2004) concluded that Cu, Cd and Pb are predominantly bound to carboxylic groups at circumneutral pH. Burnett et al. (2006) also used

EXAFS to confirm Cd-carboxyl binding on the cell walls of *Anoxybacillus flavithermus*. The findings of this study differ from other spectroscopic studies using Zn, but previous investigations have shown that other metals form carboxyl-complexes on cell walls. Despite the high affinity of Zn for phosphoryl groups, Zn-carboxyl binding is dominant in the macroscopic modelling and EXAFS results owing to the relatively high concentration of carboxyl groups in the cell walls and EPS.

4.4.2. The effect of EPS on metal adsorption

The results obtained here for Zn binding to native and EPS-free *B. licheniformis* S-86 cells consistently indicate that the carboxylic pK_a 5.3-5.4 site is the surface functional group predominantly involved in metal complexation. The results of previous potentiometric titrations and data modelling show that the concentration of this site is only slightly reduced by removal of the EPS layer from cell surfaces (Table 4.1), but the site concentration remains relatively high compared to those of the other surface sites. Therefore, as metal binding occurs predominantly to the carboxyl site, the amount of metal adsorbed by the cells is not substantially reduced by removal of the EPS layer. The concentration of site 3 changes little after EPS-removal, which also explains the lack of difference between adsorption to native and EPS-free cells. As site 1 is also involved in Zn binding and is significantly reduced in concentration after EPS removal, it may be that production of EPS can significantly enhance metal complexation when the metal concentration is particularly high, for example in a contaminated environment or in a bioreactor or at low pH, when site 1 will be the predominant available ligand.

A small number of studies have investigated the complexation characteristics of isolated EPS and have attempted to link the complexation of individual metals to the EPS composition. Guibaud et al. (2004) found that Ni and Cu adsorption to EPS extracted from activated sludge was related to the amount of proteins, humic acids, polysaccharides, and uronic acid in the case of Ni, suggesting that carboxylic groups control adsorption of these metals. The 2005 study by Guibaud et al. found that EPS extracted from pure bacterial strains is more protein rich and relatively depleted in

polysaccharides and uronic acids than EPS from sludges. The pure EPS was less able to complex metals than EPS from sludges, but there was a relationship between phosphorus content and the ability to bind Pb and Ni for all types of EPS studied. The analysis carried out to date is not sufficiently detailed or conclusive to link metal adsorption capacity to EPS composition, particularly in comparison to that of the bacterial cell wall. A particular difficulty in conducting such analysis is that the composition of the extracted EPS is highly variable depending upon the extraction method used (Comte et al., 2006b; 2006c). However, this issue would merit further investigation.

4.5. Conclusions

This study has investigated the binding environments of Zn on the surfaces of *Bacillus licheniformis* S-86, an EPS-producing bacterial strain. The results of both the macroscopic adsorption experiments and EXAFS analysis indicate that Zn is complexed by both carboxyl and phosphoryl-type groups, although the contribution from the pK_a 3.3-3.4 group (believed to represent phosphodiester groups) is also significant. The Zn EXAFS spectra and modelling results indicate that Zn is predominantly bound to a carboxylic surface site, which supports the macroscopic adsorption data from this study and many previous macroscopic studies. Although other EXAFS investigations of bacterial Zn adsorption have indicated that complexation is primarily to phosphoryl-type surface sites, and this study appears to contradict that, spectroscopic investigations using other metals have observed phosphoryl bonding only at very low pH, and increasing dominance of carboxyl complexation as pH increases. The conclusions of this study are in line with these findings.

The dominant involvement of the carboxyl group, which is only slightly reduced in concentration by EPS removal, explains why there is little difference in metal adsorption capacity between native and EPS-free cells at the experimental metal:biomass ratios used here. However, at high metal concentrations the adsorption capacity of the carboxyl site will be exceeded, and sites 1 and 3 will

become increasingly important. Under these conditions, it is likely that native cells will have a considerably higher metal adsorption capacity compared to EPS-free cells, as the EPS layer is relatively rich in the phosphodiester sites. This conclusion suggests that EPS production will significantly enhance metal adsorption under metal contaminated conditions. This supports the hypothesis that EPS production is a defensive mechanism intended to protect the cells from toxic metal concentrations (Comte et al., 2006a), as under such conditions the EPS layer will have the ability to sequester a relatively large quantity of metal, preventing it from reaching the cell wall.

4.6. References

- Borrok, D., Aumend, K., Fein, J.B., 2007. Significance of ternary bacteria-metal-natural organic matter complexes determined through experimentation and chemical equilibrium modelling. *Chemical Geology* 238, 44-62
- Borrok, D., Fein, J.B. (2004) Distribution of protons and Cd between bacterial surfaces and dissolved humic substances determined through chemical equilibrium modelling. *Geochimica et Cosmochimica Acta*, 68, 3043-3052
- Borrok, D., Fein, J.B., Kulpa, C.F. (2004a) Proton and Cd adsorption onto natural bacteria consortia: testing universal adsorption behaviour. *Geochimica et Cosmochimica Acta*, 68, 3231-3238
- Borrok, D., Fein, J.B., Kulpa, C.F. (2004b) Cd and proton adsorption onto bacterial consortia grown from industrial wastes and contaminated geological settings. *Environmental Science and Technology*, 38, 5656-5664
- Borrok, D., Fein, J.B., Tischler, M., O'Loughlin, E., Meyer, H., Liss, M., Kemner, K.M. (2004c) The effect of acidic solutions and growth conditions on the adsorptive properties of bacterial surfaces. *Chemical Geology*, 209, 107-119
- Boyanov, M.I., Kelly, S.D., Kemner, K.M., Bunker, B.A., Fein, J.B., Fowle, D.A. (2003) Adsorption of cadmium to *Bacillus subtilis* bacterial cell walls: A pH-dependent X-ray adsorption fine structure study. *Geochimica et Cosmochimica Acta*, 67, 3299-3311
- Burnett, P-G.G., Daughney, C.J., Peak, D. (2006) Cd adsorption onto *Anoxybacillus flavithermus*: Surface complexation modelling and spectroscopic investigations. *Geochimica et Cosmochimica Acta*, 70, 5253-5269
- Chatellier, X., Fortin, D. (2004) Adsorption of ferrous ions onto *Bacillus subtilis* cells. *Chemical Geology*. 212, 209-228
- Claessens, J., van Capellen, P. (2007) Competitive binding of Cu^{2+} and Zn^{2+} to live cells of *Shewanella putrefaciens*. *Environmental Science and Technology*, 41, 909-914

- Comte, S., Guibaud, G., Baudu, M. (2006a) Biosorption properties of extracellular polymeric substances (EPS) resulting from activated sludge according to their type: soluble or bound. *Process Biochemistry*, 41, 815-823
- Comte, S., Guibaud, G., Baudu, M. (2006b) Relations between extraction protocols for activated sludge extracellular polymeric substances (EPS) and complexation properties of Pb and Cd with EPS. Part I. Comparison of the efficiency of eight EPS extraction methods. *Enzyme and Microbial Technology*, 38, 246-252
- Comte, S., Guibaud, G., Baudu, M. (2006c) Relations between extraction protocols for activated sludge extracellular polymeric substances (EPS) and complexation properties of Pb and Cd with EPS. Part II. Consequences of EPS extraction methods on Pb^{2+} and Cd^{2+} complexation. *Enzyme and Microbial Technology*, 38, 246-252
- Daughney, C.J., Fein, J.B., Yee, N. (1998) A comparison of the thermodynamics of metal adsorption onto two common bacteria. *Chemical Geology*, 144, 161-176
- Fein, J.B. (2000) Quantifying the effects of bacteria on adsorption reactions in water-rock systems. *Chemical Geology*, 169, 265-280
- Fein, J.B. (2006) Thermodynamic modelling of metal adsorption onto bacterial cell walls: Current challenges. *Advances in Agronomy*, 90, 179-202
- Fein, J.B., Martin, A.M., Wightman, P.G. (2001) Metal adsorption onto bacterial surfaces: Development of a predictive approach. *Geochimica et Cosmochimica Acta*, 65, 4267-4273
- Fein, J.B., Daughney, C.J., Yee, N., Davis, T.A. (1997) A chemical equilibrium model for metal adsorption onto bacterial surface. *Geochimica et Cosmochimica Acta*, 61, 3319-3328
- Gorman-Lewis, D., Fein, J.B., Soderholm, L., Jensen, M.P., Chiang, M-H. (2005) Experimental study of neptunyl adsorption onto *Bacillus subtilis*. *Geochimica et Cosmochimica Acta*, 69, 4837-4844
- Guibaud, G., Tixier, N., Bouju, A., Baudu, M. (2003) Relation between extracellular polymers' composition and its ability to complex Cd, Cu and Pb. *Chemosphere*, 52, 1701-1710

- Guibaud, G., Tixier, N., Bouju, A., Baudu, M. (2004) Use of a polarographic method to determine copper, nickel and zinc constants of complexation by extracellular polymers extracted from activated sludge. *Process Biochemistry*, 39, 833-839
- Guibaud, G., Comte, S., Bordas, F., Dupuy, S., Baudu, M. (2005) Comparison of the complexation potential of extracellular polymeric substances (EPS) extracted from activated sludges and produced by pure bacterial strains, for cadmium, lead and nickel. *Chemosphere*, 59, 629-638
- Guine, V., Martins, J.M.F., Causse, B., Durand, A., Gaudet, J-P., Spadini, L. (2007) Effect of cultivation and experimental conditions on the surface reactivity of the metal-resistant bacteria *Cupravidus metallidurans* CH34 to protons, cadmium and zinc. *Chemical Geology*, 236, 266-280
- Guine, V., Spadini, L., Sarret, G., Muris, M., Delolme, C., Gaudet, J-P., Martins, J.M.F. (2006) Zinc sorption to three gram-negative bacteria: Combined titration, modelling and EXAFS study. *Environmental Science and Technology*, 40, 1806-1813
- Haas, J.R., Dichristina, T.J., Wade Jr. R. (2001) Thermodynamics of U(VI) sorption onto *Shewanella putrefaciens*. *Chemical Geology*, 180, 33-54
- Hennig, C., Panak, P.J., Reich, T., Rossberg, A., Raff, J., Selenska-Pobell, S., Matz, W., Bucher, J.J., Bernhard, G., Nitsche, H. (2001) EXAFS investigation of uranium(VI) complexes found at *Bacillus cereus* and *Bacillus sphaericus* surfaces. *Radiochimica Acta*, 89, 625-631
- Herbelin, A.L., Westall, J.C (1999) FITEQL 4.0: a Computer Program for Determination of Chemical Equilibrium Constants from Experimental Data; Report 99-01. Department of Chemistry Oregon State University, Corvallis
- Kelly, S.D., Kemner, K.M., Fein, J.B., Fowle, D.A., Boyanov, M.I., Bunker, B.A., Yee, N. (2002) X-ray adsorption fine structure determination of pH-dependent U-bacterial cell wall interactions. *Geochimica et Cosmochimica Acta*, 66, 3855-3871
- Lamelas, C., Benedetti, M., Wilkinson, K.J., Slaveykova, V.I. (2006) Characterization of H⁺ and Cd²⁺ binding properties of the bacterial exopolysaccharides. *Chemosphere*, 65, 1362-1370

- Markai, S., Andres, Y., Montavon, G., Grambow, B. (2003) Study of the interaction between europium (III) and *Bacillus subtilis*: fixation sites, biosorption modelling and reversibility. *Journal of Colloid and Interface Science*, 262, 351-361
- Matsukawa, M., Greenberg, E.P. (2004) Putative exopolysaccharides synthesis genes influence *Pseudomonas aeruginosa* biofilm development. *Journal of Bacteriology*, 2004, 4449-4456
- Merroun, M.L., Ben Chekroun, K., Arias, J.M., Gonzalez-Munoz, M.T. (2003) Lanthanum fixation by *Myxococcus xanthus*: cellular location and extracellular polysaccharide observation. *Chemosphere*, 52, 113-120
- Ngwenya, B.T. (2007) Enhanced adsorption of zinc is associated with aging and lysis of bacterial cells in batch incubations. *Chemosphere*, 67, 1982-1992
- Ngwenya, B.T., Sutherland, I.W., Kennedy, L. (2003) Comparison of the acid-base behaviour and metal adsorption characteristics of a gram-negative bacterium with other strains. *Applied Geochemistry*, 18, 527-538
- Pagnanelli, F., Petrangeli Papini, M., Toro, L., Trifoni, M., Veglio, F. (2000) Biosorption of metal ions on *Arthrobacter* sp.: Biomass characterisation and biosorption modelling. *Environmental Science and Technology*, 34, 2773-2778
- Plette, A.C.C., Benedetti, M.F., van Riemsdijk, W.H. (1996) Competitive binding of protons, calcium, cadmium, and zinc to isolated cell walls of a gram-positive soil bacterium. *Environmental Science and Technology*, 30, 1902-1910
- Sarret, G., Manceau, A., Spadini, L., Roux, J-C., Hazemann, J-L., Soldo, Y., Eyebert-Berard, L., Menthonnex, J-J. (1998) Structural determination of Zn and Pb binding sites in *Penicillium chrysogenum* cell walls by EXAFS spectroscopy. *Environmental Science and Technology*, 32, 1648-1655
- Sarret, G., Saumitou-Laprade, P., Bert, V., Proux, O., Hazemann, J-L., Traverse, A., Marcus, M.A., Manceau, A. (2002) Forms of zinc accumulated in the hyperaccumulator *Arabidopsis halleri*. *Plant Physiology*, 130, 1815-1826
- Toner, B., Manceau, A., Marcus, M.A., Millet, D.B., Sposito, G. (2005) Zinc sorption by a bacterial biofilm. *Environmental Science and Technology*, 39, 8288-8294

- Tourney, J., Ngwenya, B.T., Mosselmans, J.W.F., Tetley, L., Cowie, G.L. (2008) The effect of extracellular polymers on the proton adsorption characteristics of the thermophile *Bacillus licheniformis* S-86. *Chemical Geology*, 247, 1-15
- van Niekerk, J.N., Schoening, F.R.L., Talbot, J.H. (1953) The crystal structure of zinc acetate dehydrate, $\text{Zn}(\text{CH}_3\text{COO})_2 \cdot 2\text{H}_2\text{O}$. *Acta Crystallographia*, 6, 720-723
- Wightman, P.G., Fein, J.B., Wesolowski, D.J., Phelps, T.J., Benezeth, P., Palmer, D.A. (2001) Measurement of bacterial surface protonation constants for two species at elevated temperatures. *Geochimica et Cosmochimica Acta*, 65 3657-3669
- Yee, N., Fein, J. (2001) Cd adsorption onto bacterial surfaces: A universal adsorption edge? *Geochimica et Cosmochimica Acta*, 65, 2037-2042
- Yee, N., Benning, L.G., Phoenix, R., Ferris, F.G. (2004) Characterization of metal-cyanobacterial sorption reactions: A combined macroscopic and infrared spectroscopic investigation. *Environmental Science and Technology*, 38, 775-782

5. A variable-temperature study of Zn adsorption by the EPS-producing thermophile, *Bacillus licheniformis* S-86.

Abstract

This study investigated the effect of varying temperature on Zn adsorption to the surface of a gram-positive bacterium, *B. licheniformis* S-86, an extracellular polymer (EPS)-producing thermophile. Batch adsorption experiments were undertaken at 40 and 50 °C and the results analysed using the data optimisation program FITEQL 4.0. The best-fit models indicated that Zn complexes are formed principally with surface sites likely to represent carboxylic and phosphoryl groups, with additional contribution from a phosphodiester type group. These results are very similar to those obtained for Zn adsorption experiments previously conducted at room temperature. Stability constants show minimal, non-systematic, variations with temperature, thus indicating that adsorption temperature does not have an observable effect on the Zn adsorption characteristics. These results suggest that metal adsorption at temperatures up to 50 °C by EPS-producing bacterial species may be predicted using the same 'universal' set of complexation constants developed using non EPS-producing strains at room temperature. They suggest that we should be able to predict with confidence, mass distributions of metals in a wide range of environments using thermodynamic values determined for non EPS-producing bacterial strains.

5.1. Introduction

A large number of recent studies have investigated proton and metal adsorption to bacterial cell surfaces (Fein et al., 1997; Pagnanelli et al., 2000; Yee and Fein 2001; Ngwenya et al., 2003; Burnett et al., 2006a; 2006b; Ngwenya 2007). The overall objective is establishing a 'universal' thermodynamic model that enables prediction of the metal adsorption behaviour of bacterial species and consortia from a wide range of natural and industrial environments. Such a model is critical to allow realistic representation of microbial influences in contaminant transport models. Achieving this will also contribute greatly to the development of bioremediation technologies, and also aid understanding of the role of microbes in biogeochemical cycling of metals.

To this end, studies have attempted to identify the various parameters that may have an effect upon the surface chemistry and metal adsorption characteristics of bacterial cells. These include, among others, competition between metals ions in solution (Claessens & van Cappellen, 2007), bacterial species (Yee & Fein, 2001; Borrok et al., 2004a), environmental adaptation (Borrok et al., 2004b), growth phase (Daughney et al., 2001; Haas, 2004), growth medium (Hong & Brown, 2006), the presence of other organics (e.g. Fein and Delea, 1999; Borrok et al., 2007), the role of EPS (Lamelas et al., 2006; Tourney et al., 2008) and temperature (Takahashi et al., 2004, Gorman-Lewis et al., 2006).

Claessens and van Cappellen (2007) investigated competitive binding of Cu^{2+} and Zn^{2+} to *Shewanella putrefaciens* cell surfaces, concluding that the higher affinity of Cu^{2+} for the cell wall sites leads to reduced Zn^{2+} binding in the presence of Cu^{2+} . The observed adsorption behaviour is therefore likely to change depending on the identities of the particular metal species present.

A number of studies have attempted to ascertain whether adsorption varies according to the bacterial strains present. Metal adsorption studies by Yee and Fein (2001) and Borrok et al. (2004a; 2004b), conducted using ranges of bacterial species and consortia from both natural and contaminated environments, have established that metal adsorption parameters are sufficiently similar to be represented by a single model. However, the larger range in Cd adsorption parameters observed for

consortia from contaminated environments compared to consortia from natural environments (Borrok et al., 2004b) suggests that adaptation may be an important factor. Ngwenya et al. (2003) compared Pb, Cu and Zn adsorption by a gram-negative *Enterobacteriaceae* with adsorption parameters obtained for a range of gram-positive strains, concluding that the deprotonation constants, site densities and metal stability constants did not differ significantly depending on whether gram-positive or gram-negative cells were involved.

Wightman and Fein (2001) and Borrok and Fein (2004) investigated the distribution of Cd in systems containing both bacterial cells and humic substances. Wightman and Fein (2001) found that the presence of humic acid decreased Cd adsorption to the cell surfaces and proposed that this was because a fraction of the humic acid was acting as a competing ligand for Cd. Borrok and Fein (2004) determined through chemical equilibrium modelling that dissolved humic substances have both a greater number of binding sites than bacterial surfaces, and a greater affinity for Cd. It was demonstrated by Borrok et al. (2007) that bacteria-metal-natural organic matter complexes form readily, and are likely to be significant in most natural waters. The ability of humic substances to compete with the cells for metal adsorption, and the potential formation of ternary complexes call for the presence of other organic substances in a microbial system to be considered in any modelling approach.

The effect of cell growth phase on Cd^{2+} and Fe^{3+} adsorption was investigated by Daughney et al. (2001), who found that site concentrations, stability constants and the metal sorption capacity vary significantly depending on whether the cells are in the exponential, stationary or sporulated phases. Borrok et al. (2004c) and Guine et al. (2007) both found that varying the type of nutrient medium during cell cultivation had no significant effect upon metal adsorption.

Surface characterisation of EPS-producing bacterial cells (Tourney et al., 2008) and isolated EPS (Omoike and Chorover, 2004) has shown that the chemical functional groups present in the EPS are similar to those found in the cell wall of EPS-free cells. Studies investigating the metal adsorption properties of EPS have generally focussed on isolated EPS extracted either from bacterial cells or from sludges, and

have concluded that EPS has similar metal-binding properties to bacterial cells (Guibaud et al., 2003; 2005; Lamelas et al., 2006; Noghabi et al., 2007). TEM observation of lanthanum fixation by *Myxococcus xanthus* (Merroun et al., 2003) showed that La fixation occurred both in the cell wall and in the EPS layer secreted by this bacterium. The results of metal adsorption studies conducted as part of this project (Chapter 4) are summarised below.

As yet, few studies have investigated the effect of varying temperature upon bacterial metal adsorption. An investigation by Wightman et al. (2001) assessed the effect of temperature variation on proton adsorption by a mesophilic bacterial strain, with experiments conducted at 20, 50 and 75 °C. In addition, acidity constants and site concentrations were determined at 50 °C for a thermophilic bacterium, TOR-39 (an anaerobic Fe-reducing strain). It was found that bacterial surface protonation was not significantly temperature dependent within this temperature range. These findings are supported by Gorman-Lewis et al. (2006) who conducted calorimetric studies of proton and Cd adsorption by *Bacillus subtilis*, and concluded that the increase in Cd adsorption between 25 °C and 75 °C would probably be too small to detect using bulk adsorption experiments.

The objective of this study was to investigate whether metal adsorption to an EPS-producing strain of *Bacillus licheniformis* S-86 is temperature-dependent. The study was motivated by two related observations: (i) Bacteria from contaminated environments have been shown to have a slightly different chemistry to other neutrophilic species (Borrok et al., 2004b), suggesting that these bacteria have developed adaptive mechanisms to cope with the contamination; (ii) EPS is known to sequester metals in biofilms and can also be an adaptive response to stress (such as temperature perturbation) and/or contamination (Loaec et al., 1997; Guibaud et al., 2005; Toner et al., 2005; Comte et al., 2006a). It was therefore posited that an EPS-producing strain of bacteria may adsorb more metal at higher temperature.

Previous experimental investigations have established that both EPS-free cells and EPS-covered (native) cells of *Bacillus licheniformis* S-86 contain 4 proton-active functional groups, believed to correspond to phosphodiester, carboxyl, phosphoryl and amine groups (Tourney et al., 2008). The deprotonation constants (pK_a values)

do not differ significantly between the two types of cells, but removal of the EPS layer results in decreased concentrations of the four functional groups, particularly sites 1 and 4, each of which reduces in concentration by up to 50 %. Subsequent macroscopic and EXAFS Zn adsorption experiments established that Zn was bound primarily to site 2, but with additional involvement of site 3 and possibly site 1, at all cell:metal ratios and for both native and EPS-free cells. Because the concentrations of sites 2 and 3 are only slightly reduced by EPS removal, the overall Zn complexation capacity of the EPS-free cells is no different from the native cells.

This study was undertaken using native cells only (with the EPS layer intact), thereby ensuring that the cells retained any adaptive characteristics in case EPS production was also an adaptive response to their thermophilic lifestyle. Zn adsorption experiments were conducted at 40 and 50 °C, and the data compared with experiments conducted at room temperature (25 °C).

5.2. Materials and Methods

5.2.1. Cell culture and preparation

The thermotolerant, gram-positive strain *Bacillus licheniformis* S-86 was obtained from Professor M. Baigori, PRIOIMI Biotechnology, Argentina. Experimental batches were grown from the primary culture by inoculating 2-L Pyrex flasks containing 1 L of the Luria Broth growth medium (10 g L⁻¹ bacto-tryptone, 5 g L⁻¹ bacto-yeast extract, 5 g L⁻¹ NaCl). The medium was sterilised prior to inoculation by autoclaving at 121 °C for 30 minutes. Cultures were then incubated on a shaker for 24 hours at 30 °C. The optimum growth temperature for this strain is 55 °C but preliminary analysis indicated that growth temperature does not affect the production of EPS. Cells were harvested by centrifugation at 23,420 x g for 15 minutes at 4 °C. The harvested cells were then washed 3 times in 18 MO ultrapure water and either used immediately or freeze-dried for use in subsequent experiments. The use of freeze dried cells has no apparent effect on surface properties.

5.2.2. Zn adsorption experiments

Suspensions of native cells at varying cell concentration were prepared in 0.01 M NaNO₃ electrolyte (experimental parameters are shown in Table 5.2). 5 ml subsamples were removed and dried to constant weight in order to obtain the cell concentration for each experiment. Aqueous Zn (1000 ppm atomic absorption standard in HNO₃; Fisher Scientific) was added to the suspensions to obtain final metal concentrations of approximately 10 ppm. 20 ml volumes of suspension were divided into 50 ml polycarbonate centrifuge tubes that had been acid washed and rinsed 3 times in 18 MO ultrapure water prior to use. The samples were pH adjusted using NaOH and HNO₃ across an initial pH range of approximately pH 3 to pH 7.5. The suspensions were allowed to equilibrate in a temperature-controlled oven or water bath for 1 hour, and subsequently centrifuged at 17,210 x g for 10 minutes. A 10 ml volume of the supernatant was filtered using a 0.2 µm cellulose nitrate filter into an acid washed bottle. This solution was acidified to 1 % v/v HNO₃ (15.5 M HNO₃, Merck, Analar grade) and refrigerated prior to analysis by flame atomic adsorption spectroscopy. The final pH of the remaining supernatant was recorded as pH was free to drift during the experiment. AAS analysis was conducted using an air/acetylene flame at a wavelength of 213 nm. The standards were prepared by serial dilution of a 1000 ppm Zn atomic absorption standard (Fisher Scientific), to concentrations chosen to bracket sample concentrations (0- 10 ppm Zn). The standards were prepared in 0.01 M NaNO₃, acidified to 1 % HNO₃. Analytical precision was within 2 %.

5.2.3. Data Modelling

The deprotonation of a functional group can be represented by the following generic reaction (Borrok and Fein 2004):



Where R is the bacterium and A represents a functional group on the bacterial surface or volume (in the case of EPS). The deprotonation or acidity constant, K_a , for this reaction may be expressed as:

$$K_a = \frac{[R - A^-]a_{H^+}}{[R - AH^0]} \quad (5.2)$$

where $[R - A^-]$ and $[R - AH^0]$ represent the concentration of deprotonated and protonated sites and a_{H^+} represents the activity of protons in the solution.

Subsequent metal adsorption to deprotonated surface functional groups is assumed to occur as follows (where M^{m+} is the metal ion):



Stability constants are calculated according to the following mass-action equation:

$$K = \frac{[R - SM^{(m-1)}]}{[R - S^-][M^{m+}]} \quad (5.4)$$

Titration data obtained previously for *Bacillus licheniformis* S-86 were modelled using the data optimisation program FITEQL 4.0 (Herbelin and Westall, 1999) in order to obtain site concentrations and acidity constants for the proton-active functional groups present in the bacterial EPS and cell wall (Table 5.1). The study undertaken by Wightman et al. (2001) found that deprotonation constants for *Bacillus subtilis* did not vary significantly with temperature in the range 30- 75 °C. This supports the use in this study of deprotonation constants determined a room temperature to model metal adsorption at temperatures up to 50 °C. Log K values for OH^- , $ZnOH^+$ and $ZnOH^0_2$ were adjusted for temperature (Baes and Mesmer,

1981; Stumm and Morgan, 1996). Stability constants for Zn complexation were then determined using these values, and total Zn concentrations obtained by running blank (cell-free) adsorption experiments. The non-electrostatic model was chosen, neglecting the effects of surface charge. FITEQL calculates the variance, $V(Y)$, between the experimental data and the model: a variance of less than 20 is considered a good fit to the experimental data (Herbelin and Westall, 1999).

5.3. Results and Discussion

5.3.1. Zn adsorption curves

Example Zn adsorption curves for experiments conducted at room temperature (25 °C), 40 °C and 50 °C are shown in Figure 5.1. Adsorption is minimal at pH 3 and increases to a maximum of approximately 90 % of the available Zn, at approximately pH 6.5 for the experiments conducted at biomass concentrations of 0.82 g L⁻¹ and greater. The maximum Zn adsorption for the 0.59 g L⁻¹ and 0.62 g L⁻¹ experiments is approximately 75 % at pH 6.5.

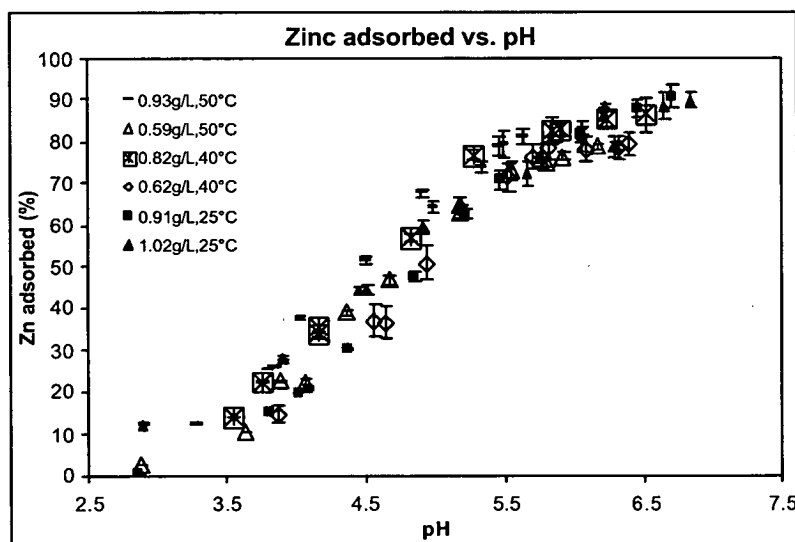


Figure 5.1: Zinc adsorption isotherms for *B. licheniformis* S-86. Error bars indicate 2 standard deviations, based on analytical precision.

The distribution of the experimental Zn adsorption curves in Figure 5.1 clearly indicates that varying the reaction temperature between 25 °C and 50 °C does not affect the Zn adsorption capacity of the bacterial cells. For example, comparing similar biomass concentrations (e.g. 0.91 g L⁻¹ at 25 °C and 0.93 g L⁻¹ at 50 °C) shows little difference in adsorption density, the differences being limited to between pH 3.5 and 5. The variation in amount of Zn adsorbed is a function of biomass concentration, as has been demonstrated previously for various different metals by numerous adsorption studies (Fein et al., 1997; Esposito et al., 2001; Ngwenya et al., 2003). The adsorption curves in Figure 5.1 show less mass-dependent variation in the amount of Zn adsorbed than may be expected. This is likely because cells producing copious amounts of EPS have a tendency to aggregate in suspension. As a consequence, the experimentally determined biomass concentration will not correlate with the cell surface area available for adsorption, resulting in loss of resolution of adsorption curves based on biomass concentration. Figures 5.2a-5.2d show light microscope images of gram-stained *B. licheniformis* cell suspensions (in 0.01 M NaNO₃ electrolyte) at different biomass concentrations and pH values. Clusters of EPS-embedded cells can clearly be seen within the suspension, and the large quantity of EPS produced by this species is apparent in the images. Cell aggregation is commonly observed for most bacterial species at low pH (approximately pH 3) due to protonation of the organic functional groups and consequent decrease in electrostatic repulsion between cells. However, *B. licheniformis* cells were also observed to aggregate at higher pH values and to an increasing extent as biomass concentration increased. Some degree of cell aggregation was observed even at low (0.2 g L⁻¹) biomass concentrations and circumneutral pH, suggesting that this effect is responsible for the lack of clear mass-dependent adsorption.

As discussed in Chapter 4 (Section 4.4.1), the formation of Zn-ligand complexes in solution is likely to increase with increasing pH due to increased release of dissolved organic carbon (DOC) into solution from the EPS layer (Borrok et al., 2007; Ngwenya, 2007). The metal-complexing fraction of the DOC acts in competition to the cell wall, possibly leading to further loss of resolution in the adsorption curves as pH increases.



Figure 5.2a.

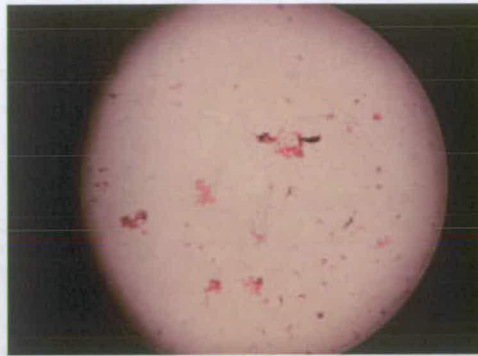


Figure 5.2b.



Figure 5.2c.

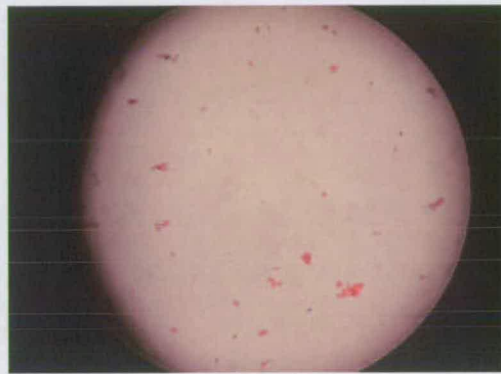


Figure 5.2d.

Figure 5.2: Light microscope images of *B. licheniformis* suspensions. a: Biomass concentration of 0.2 g L^{-1} , pH 5 (Image diameter approx 1 mm). b: Biomass concentration of 1 g L^{-1} , pH 5 (Image diameter approx 1 mm). c: Biomass concentration of 0.2 g L^{-1} , pH 7 (Image diameter approx 1 mm). d: Biomass concentration of 1 g L^{-1} , pH 7 (Image diameter approx 1 mm).

5.3.2. Data modelling

The FITEQL modelling for the variable-temperature experiments (Table 5.2) provided best-fit models for Zn adsorption onto the proton-active surface sites established by previous potentiometric titrations (Table 5.1). Adsorption to the protonated pK_a 7.4 site (phosphoryl) was tested but these models either did not converge or converged with very high $V(Y)$ values. Data modelling for each experiment indicated that the pK_a 5.3 and pK_a 7.4 sites (carboxyl and phosphoryl) are of principle importance in Zn complexation to the cell surfaces (Table 5.2). At the metal:cell ratios used here, the pK_a 3.4 (phosphodiester) site was also required to account for the observed Zn complexation in all of the 40 °C experiments and 3 of the 50 °C experiments. The results of previous Zn adsorption experiments conducted at room temperature also indicated that adsorption is primarily due to the carboxyl and phosphoryl groups, with some contribution from the pK_a 3.4 phosphodiester group.

Table 5.1: Site concentrations and deprotonation constants for *B. licheniformis* S-86.

	Native cells		Type
	pK_a	[Site] (mol/g)	
Site 1	3.36 ± 0.21	$8.53 \pm 2.45 \times 10^{-4}$	Phosphodiester
Site 2	5.25 ± 0.08	$9.06 \pm 2.14 \times 10^{-4}$	Carboxyl
Site 3	7.39 ± 0.20	$2.87 \pm 0.59 \times 10^{-4}$	Phosphoryl
Site 4	9.93 ± 0.06	$6.50 \pm 1.44 \times 10^{-4}$	Amine

Table 5.2: FITEQL modelling results for experiments conducted at 25 °C, 40 °C and 50 °C.

[Biomass] (g L ⁻¹)	log K 1	log K 2	log K 3	V(Y)
0.24, 25°C		3.29	4.20	5.26
0.44, 25°C		3.20	4.24	5.08
0.51, 25°C	2.15	3.73	4.69	1.56
0.57, 25°C	3.32	3.99	5.64	1.51
0.76, 25°C	3.00	3.78	5.88	4.81
0.91, 25°C	1.97	3.64	5.57	3.36
0.93, 25°C		3.60	5.05	5.42
1.02, 25°C	2.78	3.60	5.25	8.03
1.47, 25°C	1.76	3.53	4.86	9.75
2.19, 25°C	2.27	3.50		5.17
Mean 25°C	2.46	3.58	5.04	
St.dev 25°C	0.58	0.23	0.60	
0.82, 40°C	2.33	3.98	5.15	3.44
0.74, 40°C	2.63	4.13	4.93	1.24
0.62, 40°C	3.37	3.80	3.70	3.16
0.46, 40 °C	2.46	3.92	4.53	2.34
0.76, 40°C	1.92	3.38	5.17	1.70
0.82, 40°C	2.07	3.48	4.97	2.53
Mean 40°C	2.46	3.78	4.74	
St. dev 40°C	0.51	0.29	0.56	
0.93, 50°C	2.47	3.99		3.02
0.59, 50°C	2.57	3.54	4.78	5.18
0.81, 50°C	2.21	3.45	4.74	1.01
Mean 50°C	2.42	3.66	4.76	
St. dev 50°C	0.19	0.29	0.03	
Mean-All	2.46	3.66	4.90	
St. dev-All	0.48	0.26	0.55	

It is immediately apparent from the results shown in Table 5.2 that the FITEQL modelling does not show the expected mass-dependent trend in results. The number of sites required to account for the amount of Zn adsorbed should decrease as biomass concentration increases but this is not consistently observed in the results of this study. As discussed above, this is most likely a consequence of cell aggregation due to the EPS-producing nature of this strain. The modelling results indicate the unpredictable nature of this effect. Nevertheless, the importance of the carboxylic pK_a 5.3 site in Zn complexation emerges as a consistent trend from the data modelling results.

Generally, metal adsorption studies conducted using a macroscopic batch experiment approach have found that adsorption at circumneutral pH is to carboxylic functional groups, although the pK_a assigned to this group is often slightly lower than the pK_a value of 5.3 described here (Fein et al., 1997; Ngwenya et al., 2003; Claessens and

van Capellen 2007). However, Haas et al. (2001) identified a pK_a 5.2 group on *Shewanella putrefaciens* as carboxylic. Interestingly, Plette et al. (1996) and Ngwenya et al. (2003) both found that phosphoryl-type groups are more influential in Zn binding than is indicated by the results of this study. However, there is no consensus on this as yet. Previous spectroscopic work is consistent with a predominantly Zn-carboxyl binding environment (Chapter 4), but modelling of macroscopic adsorption data at both room temperature and elevated temperature suggests a significant contribution from a surface site with pK_a of approximately 7.4, likely to correspond to a phosphoryl group. The pK_a 3.4 site, likely to correspond to a phosphodiester group also appears to have a significant role in Zn complexation at each experimental temperature.

5.3.3. *The effect of elevated temperature on Zn adsorption*

The Zn-site stability constants (log K values) for the experiments conducted at elevated temperature (Table 5.2) may be compared with the mean log K values established for Zn adsorption experiments conducted at 25 °C. The room temperature experiments produced results indicating that site 2 is consistently involved in Zn complexation and that site 3 is also required to explain the observed adsorption in most cases. Generally a 3-site model is required although the stability constants obtained for sites 1 and 3 show considerable variability, ranging over approximately 1 order of magnitude. At 40 °C, the modelling results show that 3 sites are required for each experimental dataset and at 50 °C, 2 of the 4 experiments were optimally fit by 3-site models. The log K values for the Zn-site complexes show little variation as a function of temperature, and these small changes in do not show any systematic trends with increasing temperature. The histogram of log K values as a function of temperature (Figure 5.3) shows that the log K values for the 3 different temperatures are not distinguishable from experimental error. The student's t-test confirmed that there were no significant differences between the log K values at the three different temperatures at the 0.05 significance level.

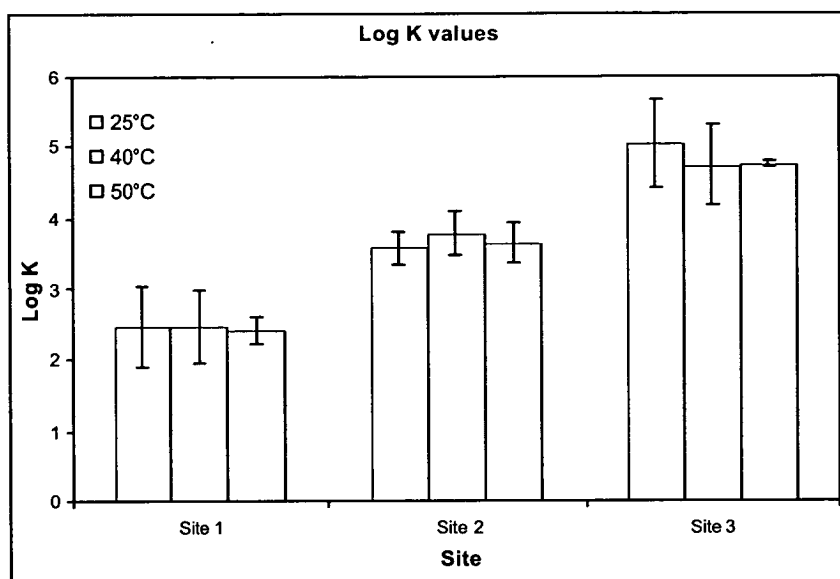


Figure 5.3: Histogram of Zn-site stability constants obtained at 25 °C, 40 °C and 50 °C. Error bars indicate 1 standard deviation.

Averaged stability constants were calculated for all three sites by combining data obtained at all three experimental temperatures. These averaged stability constants were used to obtain predictive Zn adsorption curves, examples of which are shown in Figure 5.4 together with the corresponding experimental adsorption curves. It can be seen that the model curves slightly underpredict the extent of adsorption, particularly at higher pH. This underprediction applies to experimental adsorption curves conducted at all three temperatures, providing further evidence that the reaction mechanism is not affected by temperature variation within the experimental range.

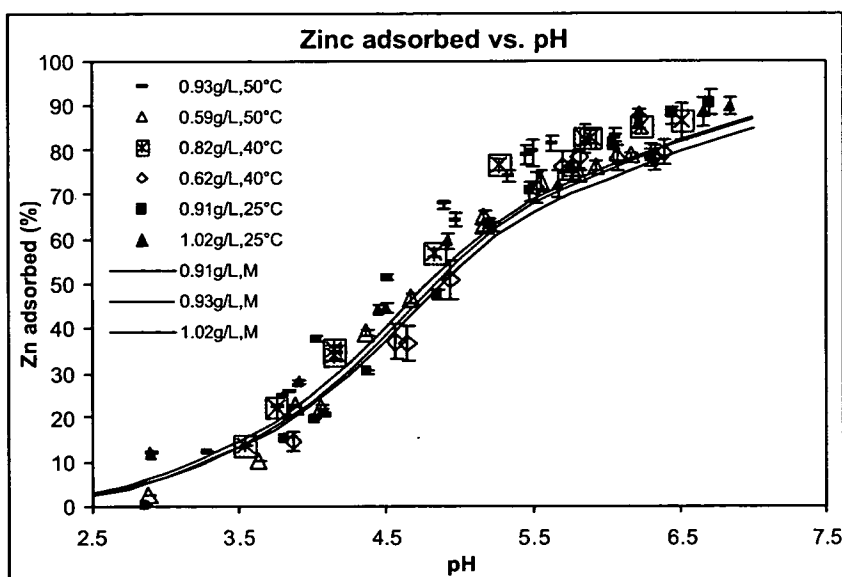


Figure 5.4: Model fits to data. Averaged stability constants used to obtain model curves are calculated using data from all 3 temperatures. M= 3-site model curve. Error bars indicate 2 standard deviations, based on analytical precision.

Few other studies have investigated adsorption to bacterial cells as a function of experimental temperature. Aksu (2002) conducted batch biosorption experiments and found that the quantity of Ni adsorbed to an algal species, *Chlorella vulgaris*, showed a significant increase as adsorption temperature increased from 15 °C to 45 °C. Takahashi et al. (2005) also observed increasing adsorption with temperature, in this case for lanthanide adsorption to *B. subtilis*, over the temperature range 5 °C to 37 °C. Wightman et al. (2001) conducted experiments at temperatures up to 75 °C, using two bacterial species, in order to assess the effect on protonation constants. It was found that neither protonation constants nor site concentrations varied significantly as a function of temperature in this range, for either species of bacteria. One of the two bacterial species used by Wightman et al. (2001), TOR-39, was a thermophile isolated from a deep sedimentary basin. Comparison of protonation characteristics for TOR-39 at 50 °C with those determined at the same temperature for the mesophilic *B. subtilis* indicated that the total site concentration for the thermophile was slightly lower than that of *B. subtilis*, but that the protonation constants for the two species were very similar.

Reaction enthalpies for Cd adsorption onto *Bacillus subtilis* (Gorman-Lewis et al., 2006) were used to estimate the changes in Cd stability constants observable over a temperature range of 25-75 °C, based on the protonation constant data of Wightman et al. (2001). It was concluded that the changes in log K would be approximately 0.005 log units for the carboxylic site, which would have little effect on metal adsorption, and approximately 0.4 log units for the phosphoryl site. This would lead to a slight increase in Cd adsorption, but this was thought likely to be undetectable using batch experiments. The results of the present study suggest that changes in the stability constants on this magnitude would not be distinguishable from experimental uncertainty.

5.3.4. *Zn complexation mechanism*

Because the formation of inner-sphere complexes involves the release of hydration water molecules, this leads to an increase in entropy (Langmuir, 1997). Conversely, outer-sphere complex formation leads to decreased entropy because hydration water molecules are retained and the disorder of the system is decreased (Gorman-Lewis et al., 2006). A positive reaction entropy leads to an increased magnitude of the free energy, and hence an increased log K value for the complexation reaction. This implies that an increase in adsorption with increasing temperature is indicative of inner-sphere complexation. The increase in lanthanide adsorption with increasing temperature observed by Takahashi et al. (2005) was considered to be consistent with an increase in entropy, and hence the formation of inner-sphere complexes on the surfaces of *B. subtilis*. Gorman-Lewis et al. (2006) used a calorimetric method to measure reaction enthalpies for Cd adsorption to *B. subtilis*, and used these to infer positive reaction entropies, indicating inner-sphere complexation. Temperature-dependent log K values for Cd adsorption were then calculated, based on their measured reaction entropies. The changes in log K were sufficiently small that increased Cd adsorption with increasing temperature would not be observable by macroscopic experimental methods. A lack of temperature dependent variation in log K values, as is observed here for Zn adsorption, indicates outer-sphere complexation.

5.3.5. Implications for a 'universal' bacteria-metal surface complexation model

An important objective of the many proton and metal adsorption studies undertaken in recent years has been to develop a 'universal' surface complexation model that enables microbial adsorption in any environmental or industrial setting to be easily predicted, without the need to consider every species present separately. In order to achieve this, it must be firmly established that adsorption to bacterial cells does not vary significantly depending on species or growth conditions. Studies by Yee and Fein (2001) and Ngwenya et al. (2003) compared different species of bacteria and concluded that there was little variation in adsorption properties between strains. Studies by Borrok et al. (2004a) and Johnson et al. (2007) investigated metal adsorption to natural bacterial consortia and concluded that surface complexation models could successfully predict adsorption using averaged stability constants and site concentrations. The results of the present study suggest that such averaged values may also be applicable to EPS-producing strains at temperatures up to 50 °C. This is an important conclusion as a large proportion of bacterial species in many environments may produce EPS.

5.4. Conclusions

This study has provided evidence that complexation of Zn to the cell surfaces of *B. licheniformis* is not significantly affected by adsorption temperature, up to 50 °C. In addition, the Zn-site stability constants show no significant variation as a function of temperature. The lack of temperature dependence observed here is in accordance with the findings of the small number of other studies investigating the temperature dependence of microbial proton and metal adsorption. The results of this study also provide evidence that the EPS-producing nature of this thermophilic strain does not affect its metal adsorption characteristics as a function of temperature. These findings suggest that a 'universal' surface complexation model remains applicable to thermophilic and EPS-producing bacterial strains within the temperature range investigated here. They suggest that we should be able to predict with confidence,

mass distributions of metals in a wide range of environments using thermodynamic values determined for non EPS-producing bacterial species.

5.5. References

- Aksu, Z. (2002) Determination of the equilibrium, kinetic and thermodynamic parameters of the batch biosorption of nickel(II) ions onto *Chlorella vulgaris*. *Process Biochemistry*, 38, 89-99
- Baes, C.F., Mesmer, R.E. (1981) The thermodynamics of cation hydrolysis. *American Journal of Science*, 281, 935-962
- Borrok, D., Fein, J.B. (2004) Distribution of protons and Cd between bacterial surfaces and dissolved humic substances determined through chemical equilibrium modelling. *Geochimica et Cosmochimica Acta*, 68, 3043-3052
- Borrok, D., Fein, J.B., Kulpa, C.F. (2004a) Proton and Cd adsorption onto natural bacteria consortia: testing universal adsorption behaviour. *Geochimica et Cosmochimica Acta*, 68, 3231-3238
- Borrok, D., Fein, J.B., Kulpa, C.F. (2004b) Cd and proton adsorption onto bacterial consortia grown from industrial wastes and contaminated geological settings. *Environmental Science and Technology*, 38, 5656-5664
- Borrok, D., Aumend, K., Fein, J. (2007) Significance of ternary bacteria-metal-natural organic matter complexes determined through experimentation and chemical equilibrium modelling. *Chemical Geology*, 238, 44-62
- Borrok, D., Fein, J.B., Tischler, M., O'Loughlin, E., Meyer, H., Liss, M., Kemner, K.M. (2004c) The effect of acidic solutions and growth conditions on the adsorptive properties of bacterial surfaces. *Chemical Geology*, 209, 107-119
- Burnett, P-G., Heinrich, H., Peak, D., Bremer, P.J., McQuillan, A.J., Daughney, C.J. (2006a) The effect of pH and ionic strength on proton adsorption by the thermophilic bacterium *Anoxybacillus flavithermus*. *Geochimica et Cosmochimica Acta*, 70, 1914-1927
- Burnett, P-G.G., Daughney, C.J., Peak, D. (2006b). Cd adsorption onto *Anoxybacillus flavithermus*: Surface complexation modelling and spectroscopic investigations. *Geochimica et Cosmochimica Acta*, 70, 5253-5269

- Claessens, J., van Capellen, P. (2007) Competitive binding of Cu^{2+} and Zn^{2+} to live cells of *Shewanella putrefaciens*. Environmental Science and Technology, 41, 909-914
- Comte, S., Guibaud, G., Baudu, M. (2006a) Biosorption properties of extracellular polymeric substances (EPS) resulting from activated sludge according to their type: Soluble or bound. Process Biochemistry, 41, 815-823
- Daughney, C.J., Fowle, D.A., Fortin, D. (2001) The effect of growth phase on proton and metal adsorption by *Bacillus subtilis*. Geochimica et Cosmochimica Acta, 65, 1025-1035
- Esposito, A., Pagnanelli, F., Lodi, A., Solisio, C., Veglio, F. (2001) Biosorption of heavy metals by *Sphaerotilus natans*: an equilibrium study at different pH and biomass concentrations. Hydrometallurgy, 60, 129-141
- Fein, J.B., Delea, D. (1999) Experimental study of the effect of EDTA on Cd adsorption by *Bacillus subtilis*: a test of the chemical equilibrium approach. Chemical Geology, 161, 375-383
- Fein, J.B., Daughney, C.J., Yee, N., Davis, T.A. (1997) A chemical equilibrium model for metal adsorption onto bacterial surface. Geochimica et Cosmochimica Acta, 61, 3319-3328
- Gorman-Lewis, D., Fein, J.B., Jensen, M.P. (2006) Enthalpies and entropies of proton and cadmium adsorption onto *Bacillus subtilis* bacterial cells from calorimetric measurements. Geochimica et Cosmochimica Acta, 70, 4862-4873
- Guibaud, G., Tixier, N., Bouju, A., Baudu, M. (2003) Relation between extracellular polymers' composition and its ability to complex Cd, Cu and Pb. Chemosphere, 52, 1701-1710
- Guibaud, G., Comte, S., Bordas, F., Dupuy, S., Baudu, M. (2005) Comparison of the complexation potential of extracellular polymeric substances (EPS), extracted from activated sludges and produced by pure bacteria strains, for cadmium, lead and nickel. Chemosphere, 59, 629-638
- Guine, V., Martins, J.M.F., Causse, B., Durand, A., Gaudet, J-P., Spadini, L. (2007) Effect of cultivation and experimental conditions on the surface reactivity of the

- metal-resistant bacteria *Cupravidus metallidurans* CH34 to protons, cadmium and zinc. *Chemical Geology*, 236, 266-280
- Haas, J.R. (2004) Effects of cultivation conditions on acid-base titration properties of *Shewanella putrefaciens*. *Chemical Geology*, 209, 67-81
- Haas, J.R., Dichristina, T.J., Wade Jr, R. (2001) Thermodynamics of U(VI) sorption onto *Shewanella putrefaciens*. *Chemical Geology*, 180, 33-54
- Herbelin, A.L., Westall, J.C (1999) FITEQL 4.0: a Computer Program for Determination of Chemical Equilibrium Constants from Experimental Data; Report 99-01. Department of Chemistry Oregon State University, Corvallis
- Hong, Y., Brown, D.G. (2006) Cell surface acid-base properties of *Escherichia coli* and *Bacillus brevis* and variation as a function of growth phase, nitrogen source and C:N ratio. *Colloids and Surfaces B: Biointerfaces*, 50, 132-139
- Johnson, K.J., Szymanowski, J.E.S., Borrok, D., Huynh, T.Q., Fein, J.B. (2007) Proton and metal adsorption onto bacterial consortia: Stability constants for metal-bacterial surface complexes. *Chemical Geology*, 239, 13-26
- Lamelas, C., Benedetti, M., Wilkinson, K.J., Slaveykova, V.I. (2006) Characterization of H⁺ and Cd²⁺ binding properties of the bacterial exopolysaccharides. *Chemosphere*, 65, 1362-1370
- Langmuir, D. (1997) *Aqueous Environmental Geochemistry*, Prentice Hall, Upper Saddle River, New Jersey
- Loaec, M., Olier, R., Guezennec, J. (1997) Uptake of lead, cadmium and zinc by a novel bacterial exopolysaccharide. *Water Research*, 31, 1171-1179
- Merroun, M.L., Ben Chekroun, K., Arias, J.M., Gonzalez-Munoz, M.T. (2003) Lanthanum fixation by *Myxococcus xanthus*: cellular location and extracellular polysaccharide observation. *Chemosphere*, 52, 113-120
- Ngwenya, B.T. (2007) Enhanced adsorption of zinc is associated with aging and lysis of bacterial cells in batch incubations. *Chemosphere*, 67, 1982-1992

- Ngwenya, B.T., Sutherland, I.W., Kennedy, L. (2003) Comparison of the acid-base behaviour and metal adsorption characteristics of a gram-negative bacterium with other strains. *Applied Geochemistry*, 18, 527-538
- Noghabi, K.A., Zahiri, H.S., Yoon, S.C. (2007) The production of a cold-induced extracellular biopolymer by *Pseudomonas fluorescens* BM07 under various growth conditions and its role for heavy metals absorption. *Process Biochemistry*, 42, 847-855
- Omoike, A., Chorover, J. (2004) Spectroscopic study of extracellular polymeric substances from *Bacillus subtilis*: Aqueous chemistry and adsorption effects. *Biomacromolecules*, 5, 1219-1230
- Pagnanelli, F., Petrangeli Papini, M., Toro, L., Trifoni, M., Veglio, F. (2000) Biosorption of metal ions on *Arthrobacter* sp.: Biomass characterisation and biosorption modelling. *Environmental Science and Technology*, 34, 2773-2778
- Plette, A.C.C., Benedetti, M.F., Van Riemsdijk, W.H. (1996) Competitive binding of protons, calcium, cadmium, and zinc to isolated cell walls of a gram-positive soil bacterium. *Environmental Science and Technology*, 30, 1902-1910
- Stumm, W., Morgan, J.J. (1996) *Aquatic Chemistry: Chemical equilibria and rates in natural waters*, 3rd Edition, Wiley, New York
- Takahashi, Y., Chatellier, X., Hattori, K.H., Kato, K., Fortin, D. (2005) Adsorption of rare earth elements onto bacterial cell walls and its implication for REE sorption onto natural microbial mats. *Chemical Geology*, 219, 53-67
- Toner, B., Manceau, A., Marcus, A.M., Millet, D.B., Sposito, G. (2005) Zinc sorption by a bacterial biofilm. *Environmental Science and Technology*, 39, 8288-8294
- Tourney, J., Ngwenya, B.T., Mosselmans, J.W.F., Tetley, L., Cowie, G.L. (2008) The effect of extracellular polymers on the proton adsorption characteristics of the thermophile *Bacillus licheniformis* S-86. *Chemical Geology*, 247, 1-15
- Wightman, P.G., Fein, J.B. (2001) Ternary interactions in a humic acid-Cd-bacteria system. *Chemical Geology*, 180, 55-65

Wightman, P.G., Fein, J.B., Wesolowski, D.J., Phelps, T.J., Benezeth, P., Palmer, D.A. (2001) Measurement of bacterial surface protonation constants for two species at elevated temperatures. *Geochimica et Cosmochimica Acta*, 65 3657-3669

Yee, N., Fein, J. (2001) Cd adsorption onto bacterial surfaces: A universal adsorption edge? *Geochimica et Cosmochimica Acta*, 65, 2037-2042

6. CaCO₃ precipitation in the presence of an EPS-producing bacterium.

Abstract

The effect of bacterial extracellular polymers on calcium carbonate morphology and polymorphism has been investigated for this study. Free-drift mineralisation experiments were conducted using cells of the EPS-producing thermophile *Bacillus licheniformis* S-86 that either had the EPS layer intact (native cells) or had the EPS layer removed (EPS-free cells). In addition, experiments were undertaken in the presence of the extracted EPS-solution. It was found that the abiotic control and EPS-free cell experiments contained vaterite and calcite when sampled after 12 h and 48 h, whereas the native cell and EPS-solution experiments contained only calcite. The results suggest that dissolved organic carbon (DOC) released from the EPS complexes Ca²⁺ ions in solution, reducing the calcium carbonate saturation and favouring calcite precipitation over vaterite. After 7 days, all experiments contained only calcite, indicating that the metastable polymorph vaterite is not stabilised by the presence of organic matter over this time period. However, distinct morphological differences between the calcite crystals precipitated in the four experiments were preserved after a period of 7 days, suggesting that crystal morphology may be a better indicator of biologically induced carbonate mineralisation than polymorphism.

6.1. Introduction

Bacteria and other microorganisms have a number of roles in natural environments that influence the biogeochemical cycling of metals. The metabolic activities of microbes induce redox transformations in metal species that often have controlling influences over their bioavailability (Abdelouas et al., 2005; Francis et al., 2000). In addition, metal adsorption to cell surfaces and biofilms may significantly affect transportation in many environmental and industrial settings (Fein, 2000; Borrok et al., 2004a; 2004b; Fein, 2006). It is also well known that bacterial cells can influence the precipitation of minerals in their immediate environment (Ehrlich, 1996; Douglas and Beveridge, 1998; Frankel and Bazylinski, 2003). Microbially induced precipitation of carbonates is particularly important in both modern and ancient aqueous, marine and terrestrial settings (Krumbein and Giele, 1979; Chafetz et al., 1991; Chafetz and Buczynski, 1992; Castanier et al., 1999; Bosak and Newman, 2003; Cailleau et al., 2005), and numerous studies have investigated the precipitation mechanisms (Stocks-Fischer et al., 1999; Warren et al., 2001; Lian et al., 2006; Mitchell and Ferris, 2006; Rodriguez-Navarro et al., 2007).

Such biologically-induced mineralisation is a passive process that occurs either as a consequence of changes in the solution chemistry or pH caused by the microbial metabolism, or because mineral nucleation on the cell surfaces enables energy barriers to be overcome. Recent studies have observed that the bacterial cells may influence the morphology and polymorphism of mineral phases precipitated, thus potentially providing a way of distinguishing microbially-induced minerals from abiotic precipitates (Ben Chekroun et al., 2004; Bosak and Newman, 2005; Lian et al., 2006; Rodriguez-Navarro et al., 2007).

The effects of various different organic molecules have been investigated recently (Sondi and Matijevic, 2001; Becker et al., 2003; Jimenez-Lopez et al., 2003; Lakshminarayanan et al., 2003; Sondi and Salopek-Sondi, 2005), and a small number of studies have raised the issue of extracellular polymeric substances (EPS) as a potentially important influence on biogenic mineral precipitation (Kawaguchi and Decho, 2002; Braissant et al., 2003; 2007). Kawaguchi and Decho (2002) and Braissant et al. (2003) conducted investigations into the effects of isolated EPS on

calcite precipitation, both studies concluding that the biochemical composition of the EPS, particularly the amino acid content, is an important factor controlling calcite morphology and polymorphism. Braissant et al. (2007) investigated carbonate precipitation by an EPS-producing sulphate-reducing bacterium, and concluded that Ca-binding by the EPS was responsible for controlling carbonate precipitation within the EPS layer. Generally, although it is apparent that organic molecules, including EPS, have an important influence over bacterially-induced carbonate precipitation, there is as yet no consensus as to the mechanisms by which organic molecules influence mineralisation.

Production of EPS significantly alters the surface environment of bacterial cells, as well as providing an additional source of organic ligands and dissolved organic carbon (DOC). As EPS is very common in natural environments, particularly as a major component of biofilms, it is important to establish how EPS influences biomineralisation kinetics and mechanisms. Although previous studies have shown that the presence of an EPS layer does not significantly alter the proton or metal adsorption characteristics of bacterial cells (Tourney et al., 2008), it is possible that the EPS-layer interacts significantly with ions in solution to alter the kinetic pathways of mineral nucleation and crystal growth. The aims of this study were to investigate the effect of EPS on calcium carbonate morphology and polymorphism, using an EPS-producing bacterial strain, *Bacillus licheniformis* S-86. In order to assess whether the EPS layer changes the effects on precipitation compared to the EPS-free cell surface, experiments were conducted using both cells with the EPS layer intact (native cells) and cells from which the EPS layer had been extracted. In addition, experiments were conducted using the extracted EPS-solution, without the presence of cells. These were compared with abiotic control experiments and the precipitation products assessed for morphology and mineralogy by SEM and XRD.

6.2. Materials and Methods

6.2.1. Cell culture and preparation

The thermotolerant, gram-positive strain *Bacillus licheniformis* S-86 was obtained from Professor M. Baigori, PRIOIMI Biotechnology, Argentina. Experimental batches were grown from the primary culture by inoculating 2-L Pyrex flasks containing the Luria Broth growth medium (10 g L⁻¹ bacto-tryptone, 5 g L⁻¹ bacto-yeast extract, 5 g L⁻¹ NaCl). The medium was sterilised prior to inoculation by autoclaving at 121 °C for 30 minutes. Cultures were then incubated on a shaker for 24 hours at 30 °C. The optimum growth temperature for this strain is 55 °C but preliminary analysis indicates that growth temperature does not affect the cell surface properties. Cells were harvested by centrifugation at 23,420 x g for 15 minutes at 4 °C. The harvested cells were then washed three times in 18 MO ultrapure water and either used immediately or freeze-dried for use in subsequent experiments. Examinations of cells by SEM/TEM, as well as surface titrations studies have shown that the freeze-drying process does not affect cell viability and surface properties.

6.2.2. EPS extraction

Following harvesting, cells were washed three times in 18 MO ultrapure water in order to remove residual growth medium. The cell pellet was then resuspended in ultrapure water and added to approximately 30 g cationic exchange resin (CER, Dowex Marathon C, 30-40 mesh), which was washed several times in 18 MO deionised water prior to use (Frolund et al., 1996). The suspension was left stirring at 4 °C overnight, then allowed to settle for five minutes in order to remove ion exchange resin. The cells were washed a further two times in deionised water, settling between washes in order to separate any remaining ion exchange resin. The supernatant from the extraction was preserved for DOC analysis and for use in mineral precipitation experiments.

6.2.3. Dissolved organic carbon (DOC) analysis

Supernatant was reserved from the cell suspension treated with cation exchange resin to remove the EPS layer. The supernatant was then filtered (0.2 μm cellulose acetate membrane syringe filters) and stored in ashed glass vials with lids containing Teflon septa. Samples were stored frozen for subsequent DOC analysis, which was carried out with a Shimadzu 6000 TOC/TDN analyser, which incorporates high-temperature catalytic oxidation and a non-dispersive infrared detector. In each case, blank determinations were undertaken and sample DOC concentrations were corrected accordingly. External calibration standards were prepared from a stock solution of 1 g L^{-1} potassium hydrogen phthalate.

6.2.4. Mineralisation experiments

Mineralisation experiments were conducted according to the ammonia free-drift method (Lian et al., 2006). The experiments were conducted in petri dishes enclosed within a large dessicator. The Petri dishes contained 25 ml volumes of a 10 mM CaHCO_3 solution (prepared by combining equal volumes of 20 mM $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ (Sigma, ACS grade) and NaHCO_3 (Merck, Analar grade) solutions). The pH was monitored during mixing of the two solutions and adjusted to below pH 6 using concentrated HCl (11.3 M, Merck Analar grade) in order to ensure precipitation did not occur. The initial pH of each experimental solution was between 5.0 and 5.5, and was allowed to drift freely during the course of the experiment. The pH was measured only at the end of the 7 day experimental period in order to avoid introducing contamination into the solutions. For each of the four experiments, the final pH was approximately 9.1. The Petri dishes were inoculated with either i) native cells; ii) EPS-free cells; or iii) the extracted EPS solution (the supernatant from the CER treatment described in Section 2.2). The concentrations of cells used for the native and EPS-free experiments were comparable. A blank experiment contained only the $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ and NaHCO_3 solution. The ammonium carbonate ($(\text{NH}_4)_2\text{CO}_3$) (Fisher Scientific, laboratory reagent grade) was placed in an open dish at the base of the dessicator. All experimental equipment and solutions were UV-sterilised prior to the experiments, and all preparation and sampling was conducted

within a microbiological safety cabinet in order to ensure that no microbial contamination occurred. Sampling was undertaken by removal of glass cover slips placed on the bases of the Petri dishes, at time intervals of 12 h, 24 h, 48 h, 72 h, 96 h and 7 days. The cover slips were rinsed thoroughly with ultrapure water then allowed to air-dry prior to analysis.

6.2.5. Sample imaging by scanning electron microscopy (SEM)

Glass coverslips removed from the bases of the Petri dishes during the mineralisation experiments were mounted directly onto the SEM stubs and gold sputter-coated to a thickness of approximately 20 nm. A Philips XL30CP scanning electron microscope with tungsten filament was used. Analysis was undertaken using a secondary electron detector with accelerating voltage of 20 kV, at a working distance of 10 mm.

6.2.6. Mineral characterisation by X-Ray diffraction analysis (XRD)

X-ray diffraction analysis of the samples was carried out on a Bruker D8 Advance Diffractometer using CuK α primary radiation generated at an accelerating voltage of 40KV. The diffracted x-rays were recorded by a SoLx energy dispersive detector with the source and detector in a 2 θ configuration. The samples were scanned in a range of 4-60° 2 θ with a dwell time of 1s/0.01 2 θ . The resulting intensity-2 θ plots were electronically filtered to remove CuK α II peaks. The amounts of phases present were determined by Rietveld analysis using the TOPAZ analysis package.

6.3. Results

6.3.1. Dissolved organic carbon (DOC) analysis

The supernatant from the EPS extraction procedure was found to have a dissolved organic carbon content of 38.7 ± 3.9 ppm. This supernatant was added to the experimental solution at a ratio of 1:4, resulting in an experimental DOC

concentration of 7.7 ± 0.8 ppm. The results of previous DOC analysis on suspensions of native and EPS-free cells (Chapter 2) are shown in Figure 6.1.

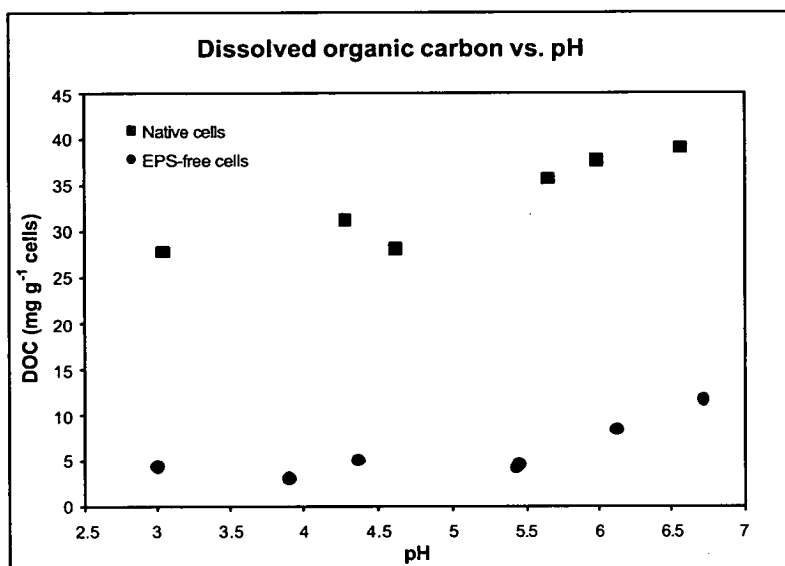


Figure 6.1: Dissolved organic carbon released into solution as a function of pH by native and EPS-free *B. licheniformis* S-86 cells.

6.3.2. Sample imaging by scanning electron microscopy (SEM)

Preliminary light microscope analysis was used to identify the most significant samples to analyse by SEM. For each of the four experimental compositions, the 12 h, 24 h and 48 h samples showed similar characteristics, with the crystal size increasing slightly between 12 h and 48 h. After 48 h, the morphologies of the crystals appeared to change, reaching a consistent appearance between 96 h and 7 days. It was therefore decided to analyse the 48 h and 7 day samples by SEM, the results of which are shown in Figures 6.2- 6.5 and are described below.

The blank sample at 48 h (Figure 6.2a) show both rhomb-shaped crystals and spherical crystals. Figure 6.2a(ii) shows that the two morphologies could occur in close association. The spherical crystals appear to be aggregates of thin plates. These plate-like structures also appear to grow outwards from the surfaces of the spheres (Figure 6.2a(iii)). The 7 day blank sample (Figure 6.2b) is much more

homogeneous in composition, consisting of angular crystals. These appear to be twins and aggregates of rhombic structures (Figure 6.2bii).

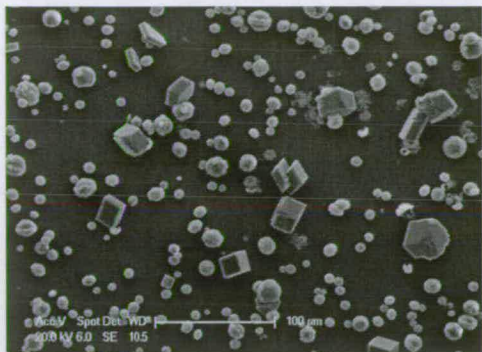


Figure 6.2ai. SEM image of control experiment at 48 hrs (showing distribution of crystal types).

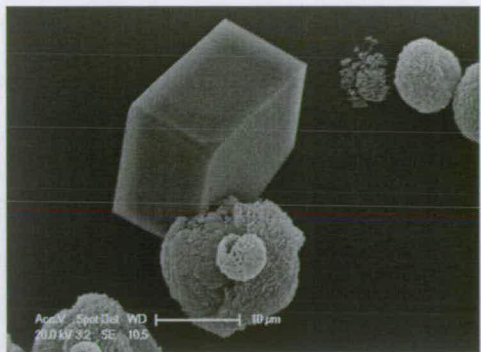


Figure 6.2aii. SEM image of control experiment at 48 hrs (showing crystal detail).

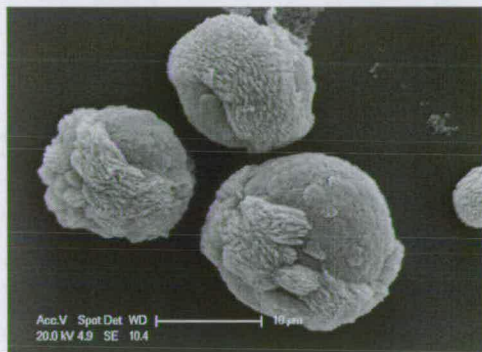


Figure 6.2aiii. SEM image of control experiment at 48 hrs (showing detail of spherical precipitates).

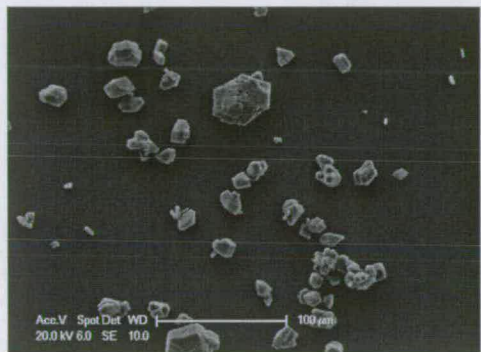


Figure 6.2bi. SEM image of control experiment at 7 days (showing distribution of crystals).

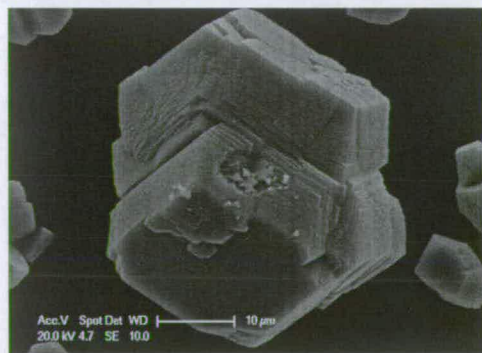


Figure 6.2bii. SEM image of control experiment at 7 days (showing crystal detail).

The sample from the native cell-containing experiment at 48 h (Figure 6.3a) shows both angular crystalline structures and spherical structures. As shown in Figure 6.3aii, the surfaces of the spheres show layers made up of small aggregates. The surfaces are quite smooth, but punctuated by spherical and oval depressions. An example of the rhombic structures seen in this sample (Figure 6.3aiii) shows multiple faces, with distinct edge-step features. The sample taken at 7 days from this experiment (Figure 6.3b) comprises fewer and larger crystals than that at 48 h. At 7 days, all of the crystals are rhombic in structure, most of these displaying the multiple faces seen in Figure 6.3bii.

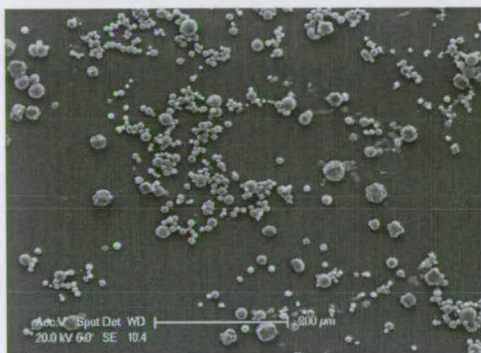


Figure 6.3ai. SEM image of the native cell experiment at 48 hrs (showing crystal distribution).

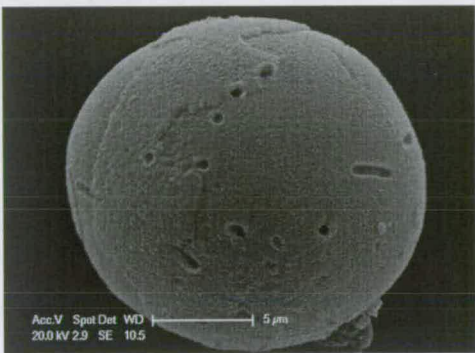


Figure 6.3aii. SEM image of the native cell experiment at 48 hrs (showing spherulitic precipitate).

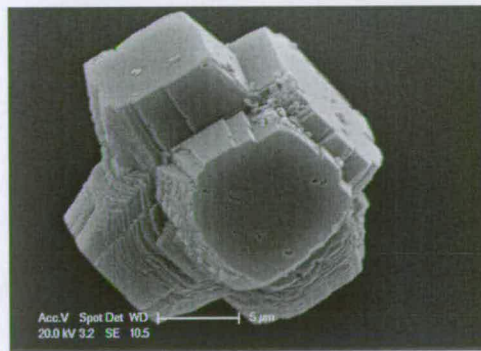


Figure 6.3aiii. SEM image of native cell experiment at 48 hrs (showing detail of crystal).

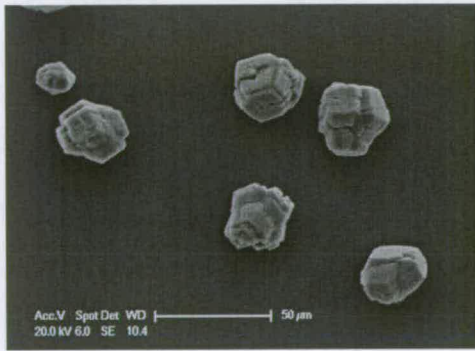


Figure 6.3bi. SEM image of native cell experiment at 7 days (showing crystal distribution).

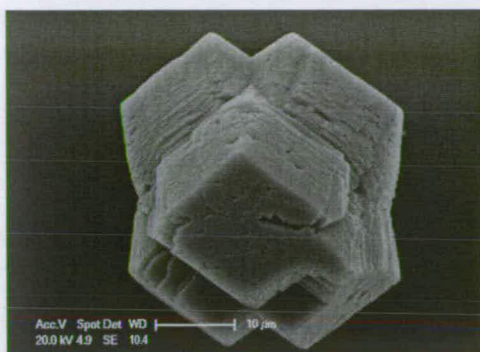


Figure 6.3bii. SEM image of native cell experiment at 7 days (showing detail of crystal).

Figure 6.4a shows the images taken of the 48 h samples from the EPS-free cell-containing experiment. This sample also contains both rhombic and spherical crystals. The rhombic crystals generally have well-defined faces and edges, although the faces are often heavily pitted by round and oval-shaped depressions (Figure 6.4aiii). The spherical crystal shown in Figure 6.4ai appears to comprise plate-like structures, both parallel to and perpendicular to the surface of the sphere. The spherical structure shown in Figure 6.4aii appears to have a more granular composition. The surfaces of the structures shown in both Figure 6.4aii and Figure 6.4aiii appear to be colonised by bacterial cells. The 7 day samples from the EPS-free cell experiments exclusively contain rhombic crystals, such as the example shown in Figure 6.4b in which the crystals appear intergrown. This crystal also shows evidence of surface colonisation by bacterial cells.

The SEM images from the experiments containing the EPS solution are shown in Figure 6.5a and 6.5b. At 48 h (Figure 6.5a), the sample consists exclusively of angular crystals. These are generally either aggregates of planar, elongate crystals, or cubic-shaped crystals. The crystals often have large holes in the centres of the faces, as shown in Figure 6.5aii. The images of the sample taken at 7 days (Figure 6.5b) show rhombic crystals with multiple faces.



Figure 6.4ai. SEM image of EPS-free cell experiment at 48 hrs (showing detail of spherical precipitate).



Figure 6.4aii. SEM image of EPS-free cell experiment at 48 hrs (showing surface detail of precipitate).

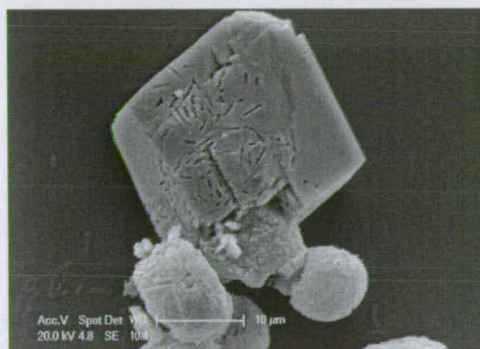


Figure 6.4aiii. SEM image of EPS-free cell experiment at 48 hrs (showing crystal detail).

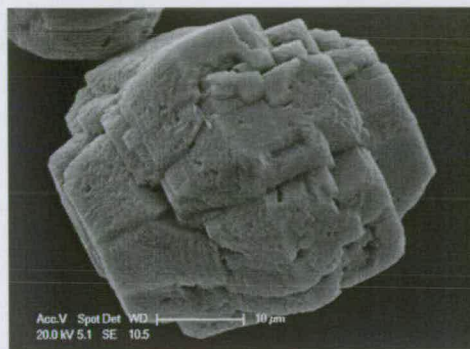


Figure 6.4b. SEM image of EPS-free cell experiment at 7 days (showing crystal detail).

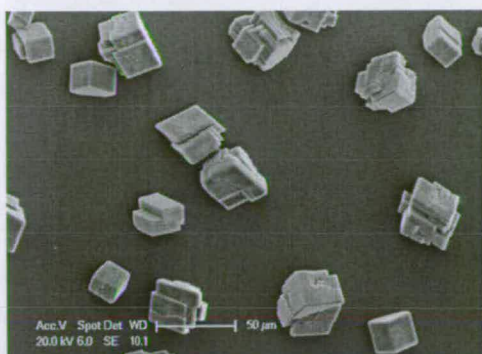


Figure 6.5ai. SEM image of EPS-solution experiment at 8 hrs (showing crystal distribution).



Figure 6.5aii. SEM image of EPS-solution experiment at 48 hrs (showing surface structure).

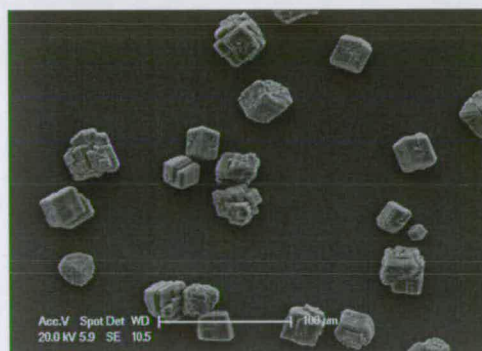


Figure 6.5bi. SEM image of EPS-solution experiment at 7 days (showing crystal distribution).



Figure 6.5bii. SEM image of EPS-solution experiment at 7 days (showing surface structure).

6.3.3. Mineral characterisation by X-Ray diffraction analysis (XRD)

Samples collected at 12 h, 48 h and 7 days were analysed by XRD. Duplicates were analysed for the samples collected at 48 h. The XRD spectra obtained indicate that both calcite and vaterite were present in the control and EPS-free cell samples taken at 12 h. At 48 h, one each of the two control and EPS-free cell samples contained vaterite and calcite, whereas the other contained only calcite. The 12 h and 48 h native cell and EPS solution samples contained only calcite. The samples collected from all four experiments after 7 days contained only calcite.

Determination of calcite to vaterite ratios in the control (12h) and EPS-free (12h) samples showed 70 % calcite: 30 % vaterite and 58.6 % calcite: 41.4 % vaterite respectively. The control (48 h) and EPS-free (48 h) samples contained 41 % calcite:

59 % vaterite and 40 % calcite: 60 % vaterite respectively, indicating that the proportion of vaterite increases until 48 h, after which it is replaced by calcite. These ratios should be taken as approximations due to errors potentially introduced by preferred-orientation of crystals. Figure 6.6 shows an example XRD spectrum from a vaterite and calcite-containing sample (EPS-free cell experiment, 48 h) and Figure 6.7 shows an example spectrum containing only calcite (EPS-solution, 48 h). All XRD spectra are included in the appendix. Each figure shows spikes marking the positions at which peaks would occur for calcite (marked by black squares) and vaterite (marked by red circles).

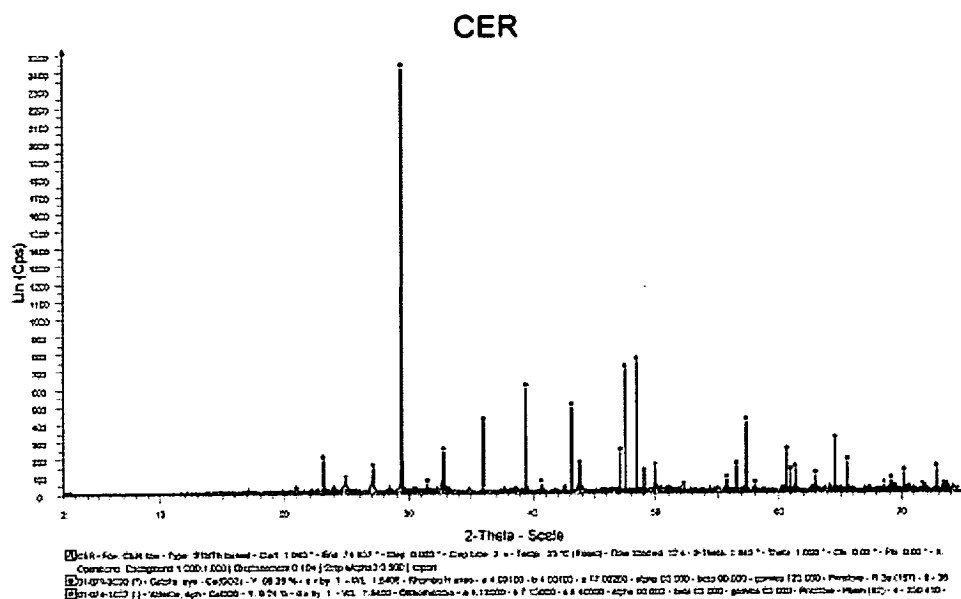


Figure 6.6: XRD spectrum for the EPS-free cell experiment at 48 h.

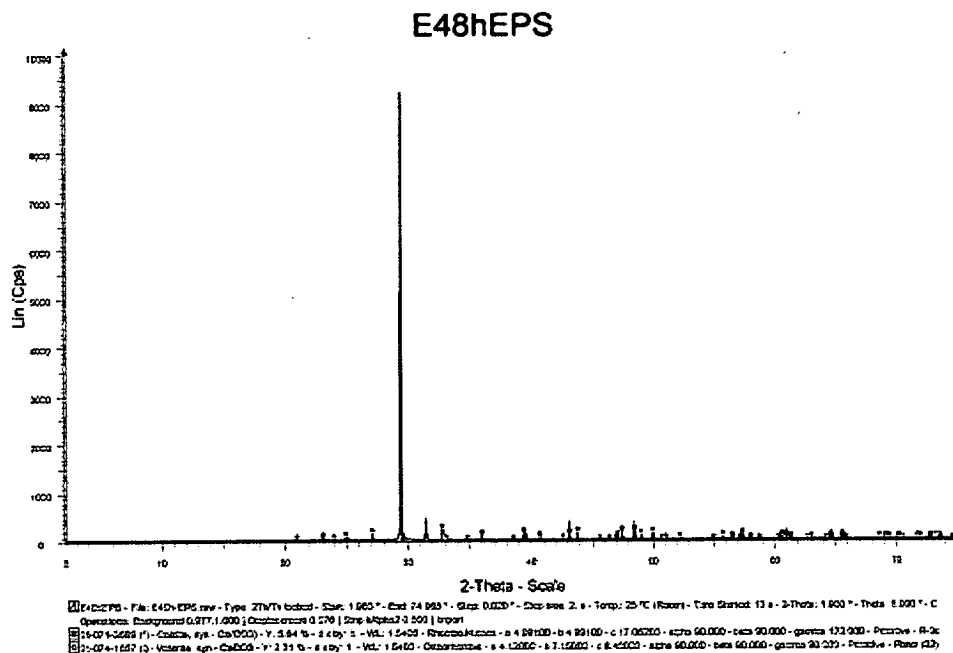


Figure 6.7: XRD spectrum for the EPS-solution experiment at 48 h.

6.4. Discussion

We can estimate the saturation state of the calcium carbonate mixture at the beginning of the experiment based on the initial pH and the Ca^{2+} and HCO_3^- concentrations using the following reaction for calcite dissolution (Langmuir 1997):



The solubility product constant can be formulated as:

$$K_{sp} = \frac{[\text{Ca}^{2+}][\text{HCO}_3^-]}{[\text{H}^+]} = \frac{K_{so}}{K_2} = \frac{10^{-8.4}}{10^{-10.3}} \approx 79.4$$

where

$$K_{so} = [\text{Ca}^{2+}][\text{HCO}_3^-] = 10^{-8.4}$$

and

$$K_2 = \frac{[\text{CO}_3^{2-}][\text{H}^+]}{[\text{HCO}_3^-]} = 10^{-10.3}$$

At the starting pH of these experiments (pH 5.5), the ion activity product (IAP), with Ca^{2+} and HCO_3^- concentrations equal at 0.01M is:

$$IAP = \frac{0.01 * 0.01}{10^{-5.5}} \approx 31.6$$

Hence, the saturation index (SI) of the solution at the start is:

$$SI = \log\left(\frac{31.6}{79.4}\right) \approx -0.4$$

As the SEM and XRD results from the control experiment show, bacterial cells are not required to induce precipitation either by modifying the solution chemistry or by

providing a nucleation surface. However, the results described in Section 3 indicate that the presence of cells and/or EPS affects both the polymorphism and morphology of calcium carbonate precipitation compared to the control experiment. The presence of vaterite in the EPS-free cell experiments but not the native cell or EPS-solution experiments suggests that the EPS is significantly affecting polymorphism. The morphologies differ in each of the four experiments after a period of 48 h; however, both the morphologies and CaCO_3 polymorphism evolve as a function of time.

Throughout this study, the EPS present must be considered as two components- the solid EPS present on the surfaces of the native cells, but also as a dissolved component added to the solution chemistry owing to the release of dissolved organic carbon from the EPS layer. In the case of the EPS-solution experiment there is no solid component, thus removing one variable from the system. These aspects of EPS will be discussed below with respect to their potential effects on the precipitation of crystals on the cell surfaces (heterogeneous nucleation) and also the organic-inorganic interactions that may occur both in solution and at the surfaces of growing crystals.

6.4.1. Calcium carbonate morphology

A number of distinctly different morphologies were observed in the present study. Well-developed calcite rhombs are seen in the control experiments at 48 h (Figure 6.2a_{ii}), in accordance with the findings of Lian et al. (2006). The rhombs observed in the EPS-free cell (48 h) experiment (Figure 6.4a_{iii}) are pitted by bacteria-shaped holes that presumably formed as the crystal grew around the cells, which subsequently lysed. The EPS-solution (48 h and 7 d) experiments also contained rhombic crystals. These are characterised by rough, pitted surfaces and rough, 'stretched' edges (Figure 6.5a_{ii}). These roughened edges were seen in the 'supernatant' experiments conducted by Lian et al. (2006), but in that study, large cavities in the faces were only observed in the cell-containing experiments. The uneven crystal growth seen in the EPS-solution experiments in the present study cannot be due to the influence of bacterial colonisation on the crystal surfaces.

Instead, it is postulated that they are caused by adsorption of organic molecules to the active growth sites on the crystal and consequent crystal growth inhibition (Stumm and Morgan, 1996). The cell-containing experiments of the present study were found, at 48 h (e.g. Figure 6.3aiii), to contain distinct crystals with a shape similar to the '3-dimensional cross' described by Lian et al. (2006). The development of this morphology was explained by Lian et al. (2006) as a consequence of bacterial colonisation of the cell surfaces. The bacterial colonies on the crystal faces were thought to adsorb ions from solution, leading to a higher local supersaturation state and more rapid crystal growth in the centres of the faces. The SEM images obtained during this study clearly show bacterial colonisation on the crystals, and the absence of this morphology from the control and EPS-solution experiments suggests that the controlling factor does involve the presence of cells. The control, EPS-free and native cell experiments all contain spherical precipitates. However, two distinct morphologies can be identified. The examples shown in Figures 6.2aiii and 6.4ai, from the control (48 h) and EPS-free cell (48 h) experiments have surfaces composed of layers of plate-like structures. In addition, parallel rows of these thin plates circumnavigate the spheres, standing perpendicular to the surface. By contrast, the examples from the native-cell experiment (Figure 6.3aii) have a much smoother surface that appears to be comprised of layers of many tiny nanospheres. Some of these 'smooth' spheres were also found in the EPS-free cell experiment at 48 h. Based on the XRD results discussed above, it seems apparent that these two spherical morphologies actually represent different CaCO_3 polymorphs. The 'platy' spheres are found only in the vaterite-containing experiments and are therefore likely to be vaterite. No vaterite was found by XRD in the native cell experiments, suggesting that the smooth spheres may not be vaterite, or that the amount of vaterite present in the native cell experiments is too small to detect by XRD analysis. Calcite is not known to precipitate with this spherical morphology, but the spheres may represent an amorphous precursor, or possibly monohydrocalcite (Rivadeneyra et al., 1998; 2004; Jimenez-Lopez et al., 2001).

The final morphology identified in the present study is the aggregates of crystals seen in all of the experiments after a period of 7 days, but also in the EPS-solution and native-cell experiments at 48 h. The crystals seen in the 7 day samples retain some

of the distinguishing features seen at 48 h. The crystals from the control experiment are more defined and angular than those from the other experiments, and the aggregates often consist of a number of smaller rhombic crystals. The crystals from the 7 day EPS-solution samples, although basically rhombic in shape, retain the roughened edges and faces seen at 48 h. The 7 day samples from both the EPS-free cell and native cell experiments contain examples of the '3-d cross' morphology discussed earlier. Evidence of bacterial colonisation on the crystal surfaces can still be seen in the 7 day samples from these experiments. This contrasts with the findings of Mitchell and Ferris (2006), whose SEM images showed that crystals precipitated in the absence of *Bacillus pasteurii* had rougher faces and a less ordered appearance than those precipitated in the presence of bacteria, after a period of 7 days. It was concluded that the presence of bacteria has a stabilising effect on the formation of calcite rhombohedra; however, the results of the present study suggest the opposite effect.

6.4.2. Calcium carbonate polymorphism

Morphology and polymorph selection during biologically controlled carbonate precipitation for the production of shells and tests by higher organisms is controlled by biological 'templates' that regulate the interfacial surface energies during nucleation and crystal growth (Stumm and Morgan, 1996, Rodriguez-Navarro et al., 2007). However, the mechanisms controlling precipitation in the presence of bacterial cells are less specific. The effects on the kinetics of crystallisation result from a balance of contributory factors, including alterations in surface chemistry, nucleation on cell surfaces and complexation of dissolved species by organic ligands both in solution and within the cell walls. The presence of vaterite in the control experiments shows that the vaterite was simply a transitional phase in this study. This is in accordance with the Ostwald Step Rule (Figure 6.8), which states that phases will precipitate in order of increasing stability, as the least stable phase has a lower solid-solution interfacial tension to overcome (Stumm and Morgan, 1996). The results of this study indicate that the presence of EPS has an effect on the

kinetics of crystallisation that affects CaCO_3 polymorph formation during the earlier stages of the experimental duration.

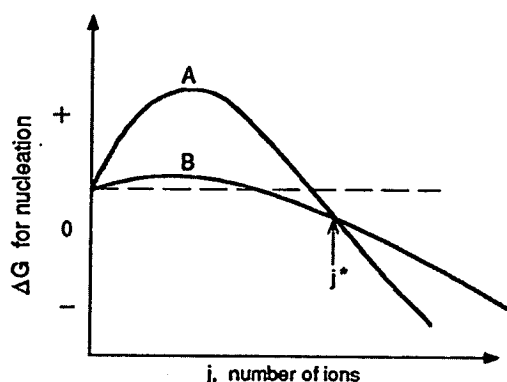


Figure 6.8: Free energy of clusters in solution as a function of the number of ions in the cluster. Curve A corresponds to the thermodynamically stable phase, curve B to the precursor phase (from Stumm and Morgan 1996).

Vaterite is a metastable calcium carbonate polymorph with a hexagonal crystal structure, usually observed to precipitate as spherules (Grasby, 2003). Homogeneous precipitation of calcium carbonate from inorganic solutions at room temperature usually results in the formation of both calcite and vaterite, although the vaterite usually transforms rapidly to calcite (Han et al., 2006). Vaterite is very rarely found to precipitate under natural conditions, except in association with organic matter. A rare example of abiotic vaterite precipitation in a modern natural environment are spherules found in supraglacial sulphur springs in the Canadian high Arctic, precipitating under unusually high-pH and low temperature conditions that are thought to stabilise vaterite (Grasby, 2003).

Precipitation of vaterite has been observed in a number of laboratory studies, generally in association with bacterial cells or some other organic additive (Braissant et al., 2003; Jimenez-Lopez et al., 2003; Ben Chekroun et al., 2004; Lian et al., 2006; Rodriguez-Navarro et al., 2007). Braissant et al. (2003) found that vaterite precipitation was enhanced when high concentrations of xanthan (purified EPS) and amino acids were added to the solution. Lian et al. (2006) found that vaterite was not

precipitated in the control experiments, but was precipitated in experiments containing supernatant from the cell centrifugation step. These supernatant experiments contained large spherulitic vaterite grains with a 6-fold symmetry, that comprised up to 60 % of the crystals formed. It was proposed that vaterite precipitation may have been favoured in the supernatant solutions because there was a lack of substrate for calcite precipitation, whereas the organic functional groups present in EPS (which is likely to comprise a large fraction of the supernatant solution) facilitated the precipitation of vaterite.

Rodriguez-Navarro et al. (2007) conducted a study of vaterite precipitation by the soil bacterium *Myxococcus xanthus*. Precipitation was initiated by the increased pH and carbonate alkalinity as a consequence of microbial transformation of NH_3 to NH_4^+ and OH^- . This study found that vaterite precipitation did not occur in the abiotic controls, but did occur in biotic experiments, including those containing the biological additive Bacto-Casitone rather than bacterial cells. These vaterite precipitates transformed to calcite over the course of the experiments (1 month).

Comparing the results of the present study with previously published literature indicates that the presence of vaterite in the control experiments is unusual.

However, under the high calcium carbonate saturation conditions used throughout this study, the formation of the metastable polymorph is favoured (Han et al., 2006; Mitchell and Ferris, 2006). The spherical vaterite morphology observed here is in agreement with the findings of previous studies. However, the presence of a second spherical morphology in the native cell experiments merits further investigation as this may represent minor vaterite precipitation.

6.4.3. Heterogeneous nucleation on cell surfaces

A number of studies have investigated the precipitation of minerals directly onto bacterial cell surfaces. Mineralisation under these circumstances is largely controlled by the changes in surface energy caused by the interactions between the cell surface organic molecules and the surfaces of the growing crystal. Generally, mineral phases become increasingly metastable as surface energy decreases (Navrotsky, 2004). A nucleation surface may interact with the initial nanocrystals in such a way that

surface energy is decreased, and consequently the precipitation of an otherwise metastable phase may be enhanced. It is thought that bacterial surfaces may also facilitate precipitation in undersaturated solutions by lowering the activation energy barrier and hence critical saturation state for precipitation to take place (Mitchell and Ferris, 2006).

The presence of an EPS layer on the surface of the native cells may lead to differences in carbonate precipitation compared to the EPS-free cells due to differences in the electrostatic interactions, mineral adhesion properties and geometric matching. However, EPS can affect the biomineralisation system in two different ways; as an in-situ solid phase on the native cell surfaces, but also by altering the solution chemistry due to release of dissolved organic carbon. It is important to distinguish these two factors, as in-situ EPS could affect heterogeneous nucleation on the cell surfaces, whereas release of DOC could affect precipitation directly from solution.

6.4.4. The effect of organic molecules on mineralisation

The presence of organic molecules can affect mineralisation by influencing the kinetics of crystal nucleation and growth. Many previous studies (discussed in Section 6.4.2) have found that the metastable CaCO_3 polymorph vaterite is precipitated in addition to calcite when organic additives or bacterial cells are present in the solution. As described by Rodriguez-Navarro et al. (2007), organic polymers are thought to stabilise vaterite, thus explaining why it is often observed in biological systems but rarely in abiotic systems. Most studies of carbonate precipitation in the presence of organic molecules have proposed that vaterite is stabilised by incorporation of organic matter into the mineral structure.

Jimenez-Lopez et al. (2003) investigated the effect of lysozyme (a globular protein) on calcium carbonate precipitation, and found that spherulites formed at an early stage of the experiment, but these were transformed to calcite rhombs. It was found that the rate of transformation of spherulites to rhombs increased with increasing lysozyme concentration. Differences in morphology depending on lysozyme concentration were thought to be a consequence of electrostatic interactions between

the protein molecules and the calcite surfaces, as this molecule is too large to participate in geometric matching with the carbonate ions at the mineral surfaces.

The disappearance of vaterite from the control experiments after a period of 7 days shows that the phase is transitional in the absence of organic interactions. This is unsurprising as vaterite is unstable under most conditions due to its high solubility and low density relative to aragonite and calcite (Rodriguez-Navarro et al., 2007). However, none of the experiments contained vaterite after 7 days, indicating that polymorph stabilisation by organic molecules was not significant over this time period. As vaterite was present in only one of the duplicate control and EPS-free cell experiments sampled at 48 h the phase must transform to calcite relatively early in the experimental duration.

A small number of studies have focussed on the role of bacterial EPS in the precipitation of CaCO_3 polymorphs (Kawaguchi and Decho, 2002; Braissant et al., 2003; Bosak and Newman, 2005). It was found by Kawaguchi and Decho (2002) that the effect on CaCO_3 polymorph selection depended on which part of the stromatolite the EPS had been extracted from. EPS from the lithified layer encouraged aragonite precipitation, whereas EPS from the unlithified layer encouraged calcite formation. This was thought to be controlled by differences in the biochemical composition of the EPS from the two layers. Braissant et al. (2003) and Bosak and Newman (2005) both found that extracted EPS encouraged the precipitation of vaterite spherules. This effect was ascribed to the presence of amino acids (Braissant et al., 2003), or potential binding of Ca^{2+} or incorporation of the EPS into the growing crystal (Bosak and Newman, 2005). It should be noted that comparing results of studies on EPS is difficult, as definitions of the EPS fraction (for examples, 'soluble' vs. 'bound' EPS (Comte et al., 2006a)) and extraction protocols vary greatly (Comte et al., 2006b; 2006c).

Based on previous work undertaken using *Bacillus licheniformis* S-86, the reactivity of the bacterial extracellular polymers is very similar to that of the cell wall.

Previous studies have shown that the protonation characteristics and metal adsorption behaviour are not significantly affected by the presence of EPS on the cell surfaces (Chapters 2, 4, 5). As discussed in Section 6.4.2, the presence of vaterite in both the

abiotic control experiment and the EPS-free cell experiment, and the absence of vaterite in the EPS-solution experiment, indicate that the nucleation surface is not controlling the polymorph selection. The use of the EPS-solution is an effective means of establishing that the effect of EPS is not due to the effect on the cell surface and hence potential mineral nucleation environment.

The results of the present study suggest that the presence of EPS favours the precipitation of calcite rather than vaterite. Jimenez-Lopez et al. (2003) found that lysozyme favoured both calcite and the metastable phase, so it seems that EPS does not affect the mineralisation process by the same mechanisms as lysozyme. On the other hand, Hernandez-Hernandez et al. (2008) conducted a study of the effect of purified eggshell proteins on calcium carbonate precipitation, and found that all fractions favoured calcite precipitation over other polymorphs. It was thought that this was due to Ca^{2+} complexation by the organic molecules and consequent lowering of the carbonate saturation state.

The differences in both morphology and polymorphism between the control experiment and the EPS-solution experiment, neither of which contain a solid organic phase, suggest that the effect of EPS on solution chemistry is the primary factor controlling the effects on CaCO_3 precipitation observed during this study. It has been established that EPS-containing cells release substantially more dissolved organic carbon (DOC) to solution than do EPS-free cells (Figure 6.1). As stated by Stumm and Morgan (1996), the presence of DOC can interfere with crystal growth and morphology by absorbing to active growth sites on the crystals. In addition, whilst organic ligands in the solid phase act to stabilise mineral precipitates, organic ligands in solution can inhibit crystallisation (Rodriguez-Navarro et al., 2007). As Figure 6.1 suggests, the native cells used during this study are likely to release considerably more DOC into the experimental solutions than the EPS-free cells, and DOC analysis of the EPS-solution indicates a significant DOC concentration (7.7 ± 0.8 ppm). Although DOC is a complex mixture of organic compounds, it is known that fractions are liable to have significant metal-complexation capabilities (Christensen et al., 1999; Guthrie et al., 2005). Complexation of Ca^{2+} by DOC would lead to decreased calcium carbonate saturation in the bulk solution which would favour precipitation of the less soluble polymorph calcite over vaterite

(Mitchell and Ferris, 2006; Hernandez-Hernandez et al., 2008). Whilst the in-situ EPS layer may also affect heterogeneous nucleation on cell surfaces, it seems likely that the release of DOC into the experimental solutions is the factor controlling CaCO₃ polymorphism in this study.

6.5. Conclusions

This study was undertaken in order to investigate the effect of bacterial extracellular polymers on calcium carbonate morphology and polymorphism. In the free-drift experiments conducted, vaterite precipitation was observed as a precursor to the more stable calcite phase. However, the presence of EPS appeared to inhibit vaterite formation.

Crystal morphology was observed to vary significantly depending on whether the experimental solution contained bacterial cells or EPS. The '3-dimensional cross' described by Lian et al. (2006) was observed in cell-containing experiments during this study, and the association with bacterial cells helps to confirm that this effect is a consequence of bacterial colonisation on the cell surfaces and subsequent increased CaCO₃ supersaturation in the region of the bacterial colonies. Spherical precipitates were also observed during this study, although it seems that the 'platy' spheres were composed of vaterite whereas the 'smooth' spheres were not.

It was seen that, after a period of 12 h, the abiotic control and EPS-free cell experiments contained vaterite, whereas the native cell and EPS-solution experiments did not. The results of this study suggest that the effect of EPS on polymorphism is related to the substantial release of DOC from the EPS layer. High concentrations of DOC in solution appear to favour calcite precipitation. It is proposed that the mechanism responsible for this effect may be complexation of Ca²⁺ by fractions of the DOC, thus decreasing the carbonate supersaturation. This will favour precipitation of the less soluble polymorph calcite over vaterite.

During the 7-day experimental duration, it was found that the vaterite present in the EPS-free experiments at 12 h and 48 h was converted to calcite by 7 days. This

suggests that the presence of bacterial surfaces and associated organic molecules may not have a sufficiently stabilising effect on vaterite that it is able to persist over long time periods and act as an indicator of biologically-induced carbonate mineralisation. However, the distinct morphologies exhibited in samples from the four different experiments were retained in the day-7 samples, and therefore the morphologies of carbonate crystals may be a better indication of bacterial activity than polymorphism. The long-term persistence of these morphologies under different environmental conditions therefore merits further investigation.

6.6. References

- Abdelouas, A., Grambow, B., Fattahi, M., Andres, Y., Leclerc-Cessac, E. (2005) Microbial reduction of ^{99}Tc in organic matter-rich soils. *Science of the Total Environment*, 336, 255-268
- Becker, A., Becker, W., Marxen, J.C., Epple, M. (2003) In-vitro crystallization of calcium carbonate in the presence of biological additives- comparison of the ammonium carbonate method with double-diffusion techniques. *Z. Anorg. Allg. Chem.*, 629, 2305-2311
- Ben Chekroun, K., Rodriguez-Navarro, C., Gonzalez-Munoz, M.T., Arias, J.M., Cultrone, G., Rodriguez-Gallego, M. (2004) Precipitation and growth morphology of calcium carbonate induced by *Myxococcus xanthus*: Implications for recognition of bacterial carbonates. *Journal of Sedimentary Research*, 74, 868-876
- Borrok, D., Fein, J.B., Kulpa, C.F. (2004a) Proton and Cd adsorption onto natural bacterial consortia: testing universal adsorption behaviour. *Geochimica et Cosmochimica Acta*, 68, 3231-3238
- Borrok, D.M., Fein, J.B., Kulpa Jr. C.F. (2004b) Cd and proton adsorption onto bacterial consortia grown from industrial wastes and contaminated geologic settings. *Environmental Science and Technology*, 38, 5656-5664
- Bosak, T., Newman, D.K. (2003) Microbial nucleation of calcium carbonate in the Precambrian. *Geology*, 31, 577-580
- Bosak, T., Newman, D.K. (2005) Microbial kinetic controls on calcite morphology in supersaturated solutions. *Journal of Sedimentary Research*, 75, 190-199
- Braissant, O., Cailleau, G., Dupraz, C., Verrecchia, E.P. (2003) Bacterially induced mineralization of calcium carbonate in terrestrial environments: The role of exopolysaccharides and amino acids. *Journal of Sedimentary Research*, 73, 485-490
- Braissant, O., Decho, A.W., Dupraz, C., Glunk, C., Prezekop, K.M., Visscher, P.T. (2007) Exopolymeric substances of sulphate-reducing bacteria: Interactions with calcium at alkaline pH and implication for formation of carbonate minerals. *Geobiology*, 5, 401-411

- Cailleau, G., Braissant, O., Dupraz, C., Aragno, M., Verrecchia, E.P. (2005) Biologically induced accumulations of CaCO_3 in orthox soils of Biga, Ivory Coast. *Catena*, 59, 1-17
- Castanier, S., Le Metayer-Levrel, G., Perthuisot, J-P. (1999) Ca-carbonates precipitation and limestone genesis- the microbiological point of view. *Sedimentary Geology*, 126, 9-23
- Chafetz, H.S., Buczynski, C. (1992) Bacterially induced lithification of microbial mats. *Palaios*, 7, 277-293
- Chafetz, H.S., Rush, P.F., Utech, N.M. (1991) Microenvironmental controls on mineralogy and habit of CaCO_3 precipitates: an example from an active travertine system. *Sedimentology*, 38, 107-126
- Christensen, J.B., Botma, J.J., Christensen, T.H. (1999) Complexation of Cu and Pb by DOC in polluted groundwater: A comparison of experimental data and predictions by computer speciation models (WHAM and MINTEQA2). *Water Research*, 33, 3231-3238
- Comte, S., Guibaud, G., Baudu, M. (2006a) Biosorption properties of extracellular polymeric substances (EPS) resulting from activated sludge according to their type: Soluble or bound. *Process Biochemistry*, 41, 815-823
- Comte, S., Guibaud, G., Baudu, M., 2006b. Relations between extraction protocols for activated sludge extracellular polymeric substances (EPS) and complexation properties of Pb and Cd with EPS. Part I. Comparison of the efficiency of eight EPS extraction methods. *Enzyme and Microbial Technology* 38, 246-252
- Comte, S., Guibaud, G., Baudu, M (2006c) Relations between extraction protocols for activated sludge extracellular polymeric substances (EPS) and complexation properties of Pb and Cd with EPS. Part II: Consequences of EPS extraction methods on Pb^{2+} and Cd^{2+} complexation. *Enzyme and Microbial Technology*, 38, 246-252
- Douglas, S., Beveridge, T.J. (1998) Mineral formation by bacteria in natural microbial communities. *FEMS Microbiology Ecology*, 26, 79-88
- Ehrlich, H.L. (1996) How microbes influence mineral growth and dissolution. *Chemical Geology*, 132, 5-9

- Fein, J.B. (2000) Quantifying the effects of bacteria on adsorption reactions in water-rock systems. *Chemical Geology*, 169, 165-280
- Fein, J.B. (2006) Thermodynamic modelling of metal adsorption onto bacterial cell walls: Current challenges. *Advances in Agronomy*, 90, 179-202
- Francis, A.J., Dodge, C.J., Gillow, J.B., Papenguth, H.W. (2000) Biotransformation of uranium compounds in high ionic strength brine by a halophilic bacterium under denitrifying conditions. *Environmental Science and Technology*, 34, 2311-2317
- Frankel, R.B., Bazylinski, D.A. (2003) Biologically induced mineralisation by bacteria. *Reviews of Mineralogy and Geochemistry*, 54, 95-114
- Frolund, B., Palmgren, R., Keiding, K., Nielsen, P.H., 1996. Extraction of extracellular polymers from activated sludge using a cation exchange resin. *Water Research* 30, 1749-1758
- Grasby, S.E. (2003) Naturally precipitating vaterite ($\mu\text{-CaCO}_3$) spheres: Unusual carbonates formed in an extreme environment. *Geochimica et Cosmochimica Acta*, 67, 1659-1666
- Guthrie, J.W., Hassan, N.M., Salam, M.S.A., Fafous, I.I., Murimboh, C.A., Murimboh, J., Chakrabarti, C.L., Gregoire, D.C. (2005) Complexation of Ni, Cu, Zn, and Cd by DOC in some metal-impacted freshwater lakes: a comparison of approaches using electrochemical determination of free-metal-ion and labile complexes and a computer speciation model, WHAM V and VI. *Analytica Chimica Acta*, 528, 205-218
- Han, Y.S., Hadiko, G., Fuji, M., Takahashi, M. (2006) Crystallization and transformation of vaterite at controlled pH. *Journal of Crystal Growth*, 289, 269-274
- Hernandez-Hernandez, A., Vidal, M.L., Gomez-Morales, J., Rodriguez-Navarro, A.B., Lebas, V., Gautron, Y., Nys, J.M., Garcia Ruiz, J.M. (2008) Influence of eggshell matrix proteins on the precipitation of calcium carbonate (CaCO_3). *Journal of Crystal Growth*, 310, 1754-1759
- Jimenez-Lopez, C., Caballero, E., Huertas, F.J., Romanek, C.S. (2001) Chemical, mineralogical and isotope behaviour, and phase transformation during the

precipitation of calcium carbonate minerals from intermediate ionic solution at 25 °C. *Geochimica et Cosmochimica Acta*, 65, 3219-3231

Jimenez-Lopez, C., Rodriguez-Navarro, A., Dominguez-Vera, J.M., Garcia-Ruiz, J.M. (2003) Influence of lysozyme on the precipitation of calcium carbonate: A kinetic and morphologic study. *Geochimica et Cosmochimica Acta*, 67, 1667-1676

Kawaguchi, T., Decho, A.W. (2002) A laboratory investigation of cyanobacterial extracellular polymeric secretions (EPS) in influencing CaCO₃ polymorphism. *Journal of Crystal Growth*, 240, 230-235

Krumbein, W.E., Giele, C. (1979) Calcification in a coccoid cyanobacterium associated with the formation of desert stromatolites. *Sedimentology*, 26, 593-604

Lakshminarayanan, R., Valiyaveetil, S., Loy, G.L. (2003) Selective nucleation of calcium carbonate polymorphs: Role of surface functionalization and poly(vinyl alcohol) additive. *Crystal Growth and Design*, 3, 953-958

Langmuir, D. (1997) *Aqueous Environmental Geochemistry*, Prentice Hall, Upper Saddle River, New Jersey

Lian, B., Chen, J., Ji, J., Teng, H.H. (2006) Carbonate biomineralisation induced by soil bacterium *Bacillus megaterium*. *Geochimica et Cosmochimica Acta*, 70, 5522-5535

Mitchell, A.C., Ferris, F.G. (2006) The influence of *Bacillus pasteurii* on the nucleation and growth of calcium carbonate. *Geomicrobiology Journal*, 23, 213-226

Navrotsky, A. (2004) Energetic clues to pathways to biomineralisation: Precursors, clusters, and nanoparticles. *PNAS*, 101 (33), 12096-12101

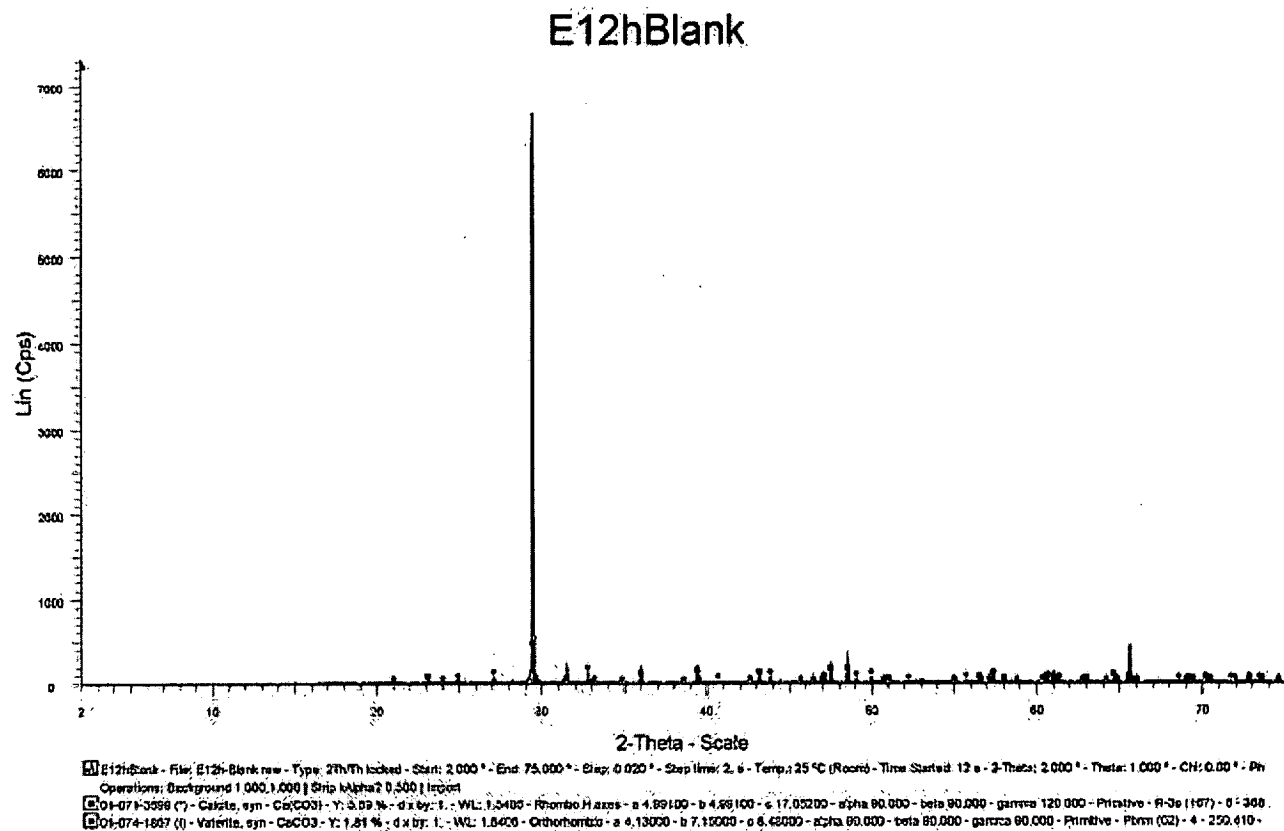
Rivadeneyra, M.A., Delgado, G., Ramos-Cormenzana, A., Delgado, R. (1998) Biomineralization of carbonates by *Halomonas eurihalina* in solid and liquid media with different salinities: crystal formation sequence. *Research in Microbiology*, 149, 277-287

Rivadeneyra, M.A., Parraga, J., Delgado, R., Ramos-Cormenzana, A., Delgado, G. (2004) Biomineralization of carbonates by *Halobacillus trueperi* in solid and liquid media with different salinities. *FEMS Microbiology Ecology*, 48, 39-46

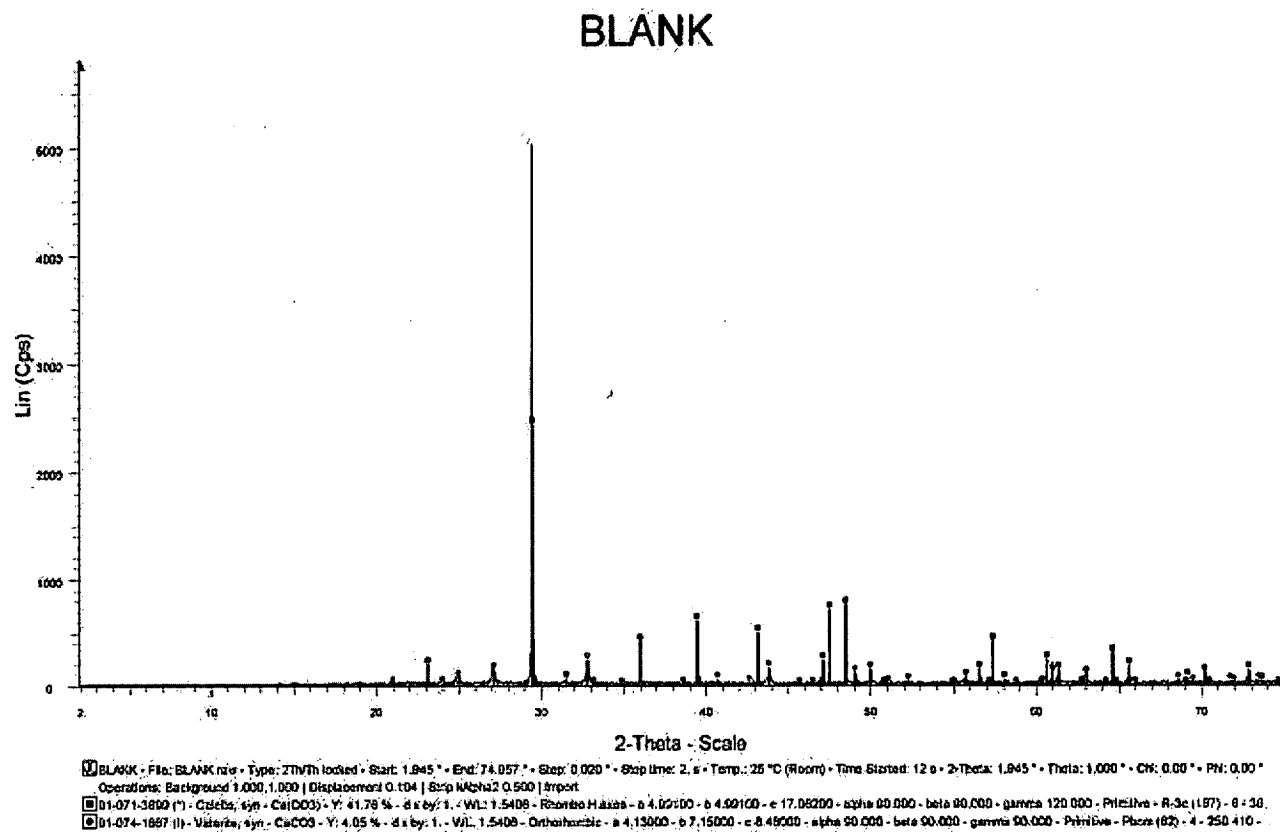
- Rodriguez-Navarro, C., Jimenez-Lopez, C., Rodriguez-Navarro, A., Gonzalez-Munoz, M.T., Rodriguez-Gallego, M. (2007) Bacterially mediated mineralization of vaterite. *Geochimica et Cosmochimica Acta*, 71, 1197-1213
- Sondi, I., Matijevic, E. (2001) Homogeneous precipitation of calcium carbonates by enzyme catalyzed reaction. *Journal of Colloid and Interface Science*, 238, 208-214
- Sondi, I., Salopek-Sondi, B. (2005) Influence of the primary structure of enzymes on the formation of CaCO_3 polymorphs: A comparison of plant (*Canavalia ensiformis*) and bacterial (*Bacillus pasteurii*) ureases. *Langmuir*, 21, 8876-8882
- Stocks-Fischer, S., Galinat, J.K., Bang, S.S. (1999) Microbiological precipitation of CaCO_3 . *Soil Biology and Biochemistry*, 31, 1563-1571
- Stumm, W., Morgan, J.J. (1996) *Aquatic Chemistry, Chemical Equilibria and Rates in Natural Waters*, 3rd Edition. Wiley, New York
- Tourney, J., Ngwenya, B.T., Mosselmans, J.W.F., Tetley, L., Cowie, G.L. (2008) The effect of extracellular polymers on the proton adsorption characteristics of the thermophile *Bacillus licheniformis* S-86. *Chemical Geology*, 247, 1-15
- Warren, L.A., Maurice, P.A., Parmar, N., Ferris, F.G. (2001) Microbially mediated calcium carbonate precipitation: Implications for interpreting calcite precipitation and for solid-phase capture of inorganic contaminants. *Geomicrobiology Journal*, 18, 93-115

6.7. Appendix: XRD Spectra

1a: Control experiment, 12 h.

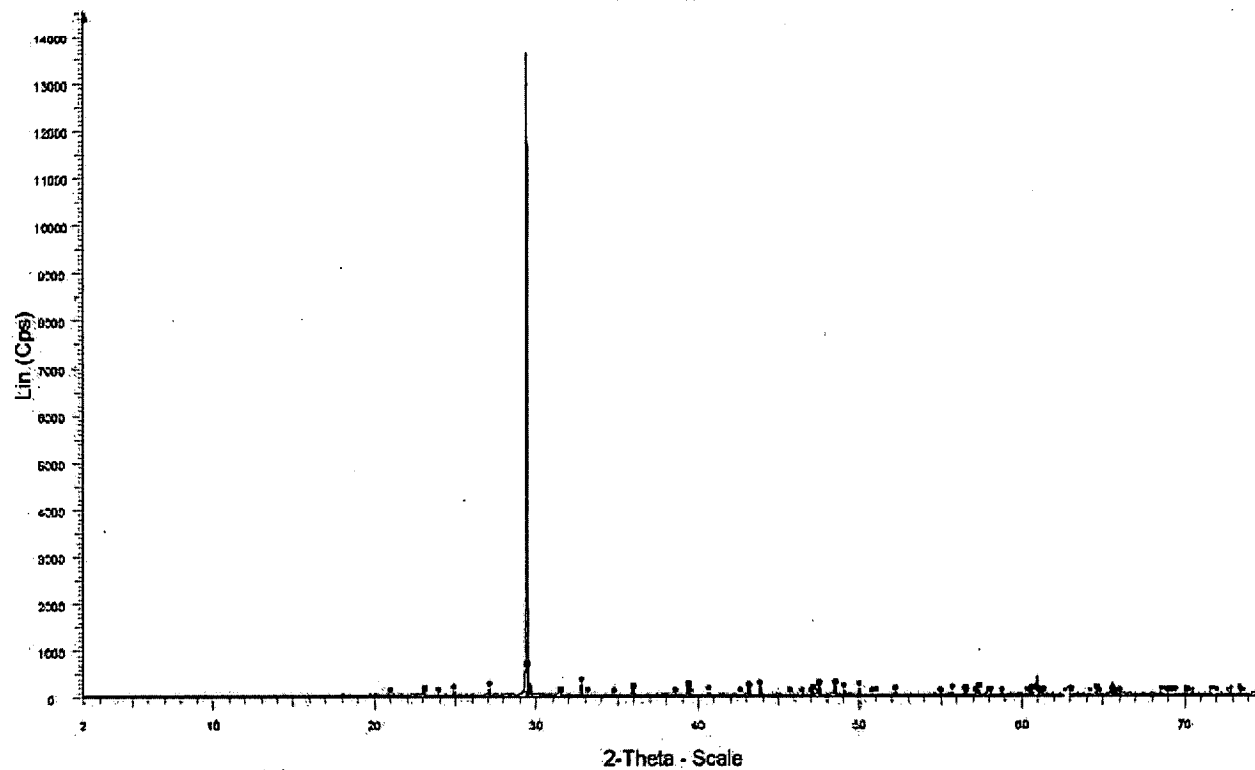


1b1: Control experiment, 48 h.

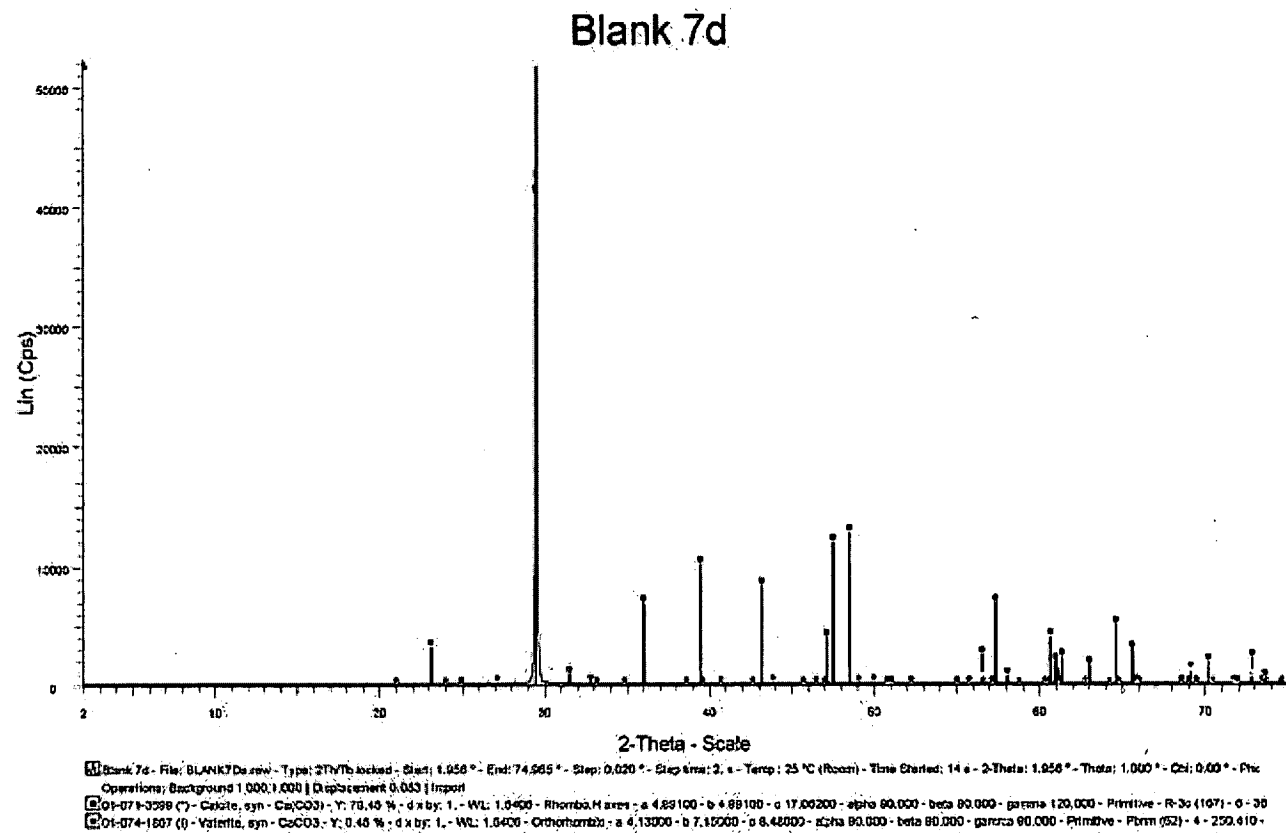


Ibii: Control experiment, 48 h.

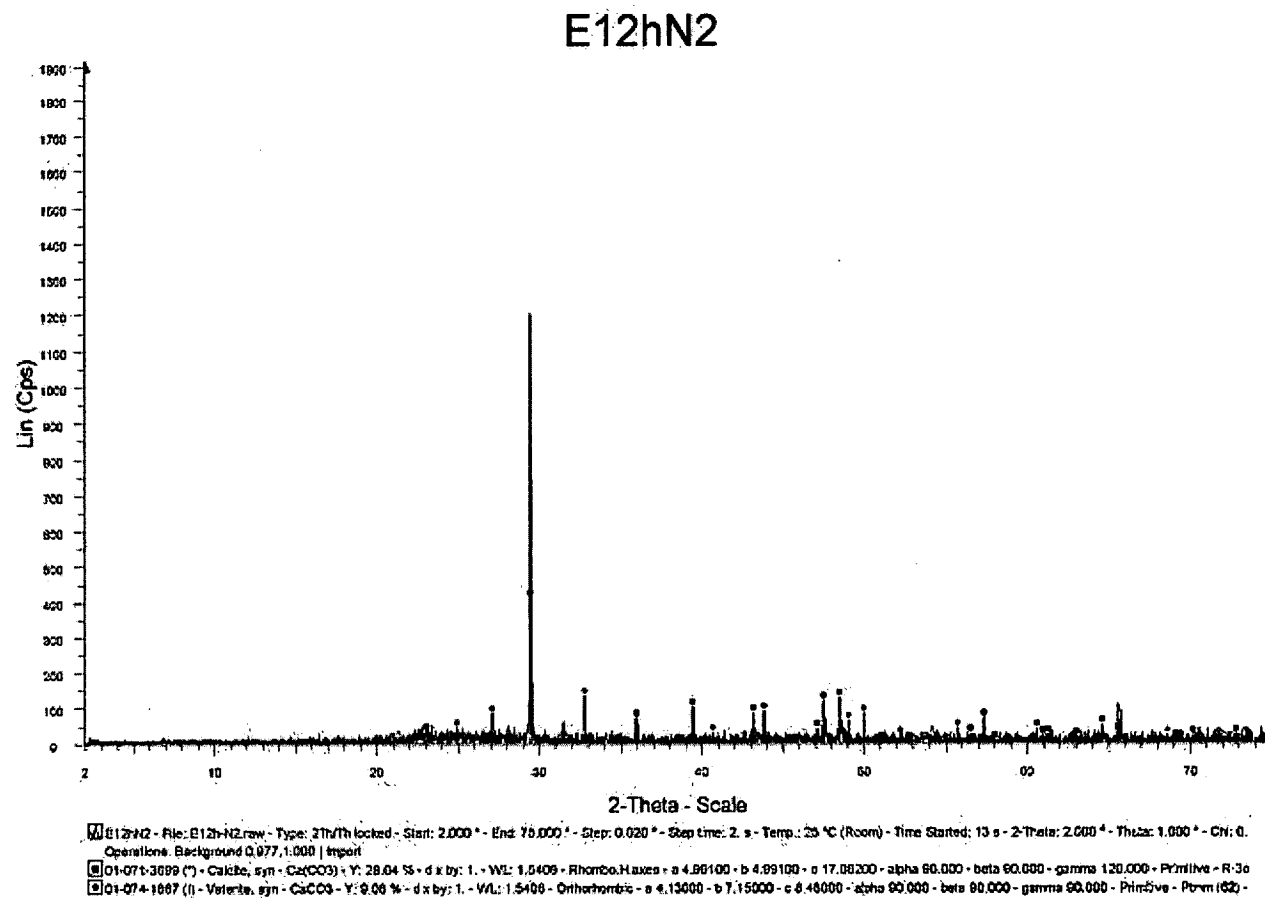
E48hBlank



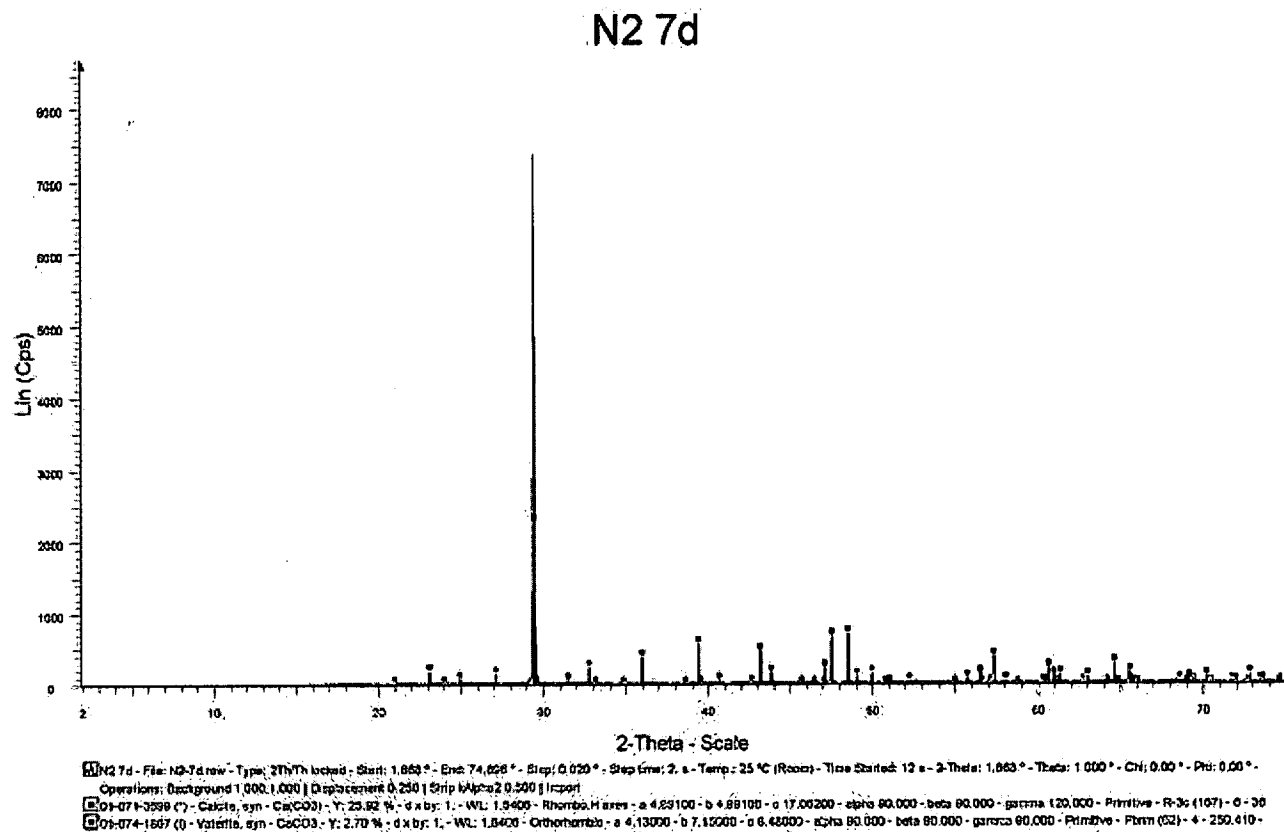
☒ E48hBlank - File: E48h-Blank3.raw - Type: 2Th/Th locked - Start: 1.928° - End: 74.941° - Step: 0.020° - Step time: 2.4 - Temp: 25 °C (Room) - Time Started: 14 s - 2-Theta: 1.928° - Theta: 1.000° -
 Operations: Background 0.877, 1.000 | Displacement 0.141 | Strip kAlpha2 0.500 | Import
☒ 01-071-3559 (*) - Calcite, syn - $\text{Ca}(\text{CO}_3)$ - Y: 3.85 % - d x by: 1. - WL: 1.5406 - Rhombo.Haxes - a 4.99100 - b 4.99100 - c 17.06200 - alpha 90.000 - beta 90.000 - gamma 120.000 - Primitive - R-3c
☒ 01-074-1857 (f) - Vaterite, syn - CaCO_3 - Y: 1.82 % - d x by: 1. - VL: 1.5406 - Orthorhombic - a 4.13000 - b 7.15000 - c 8.45000 - alpha 90.000 - beta 90.000 - gamma 90.000 - Primitive - Pmm (62)



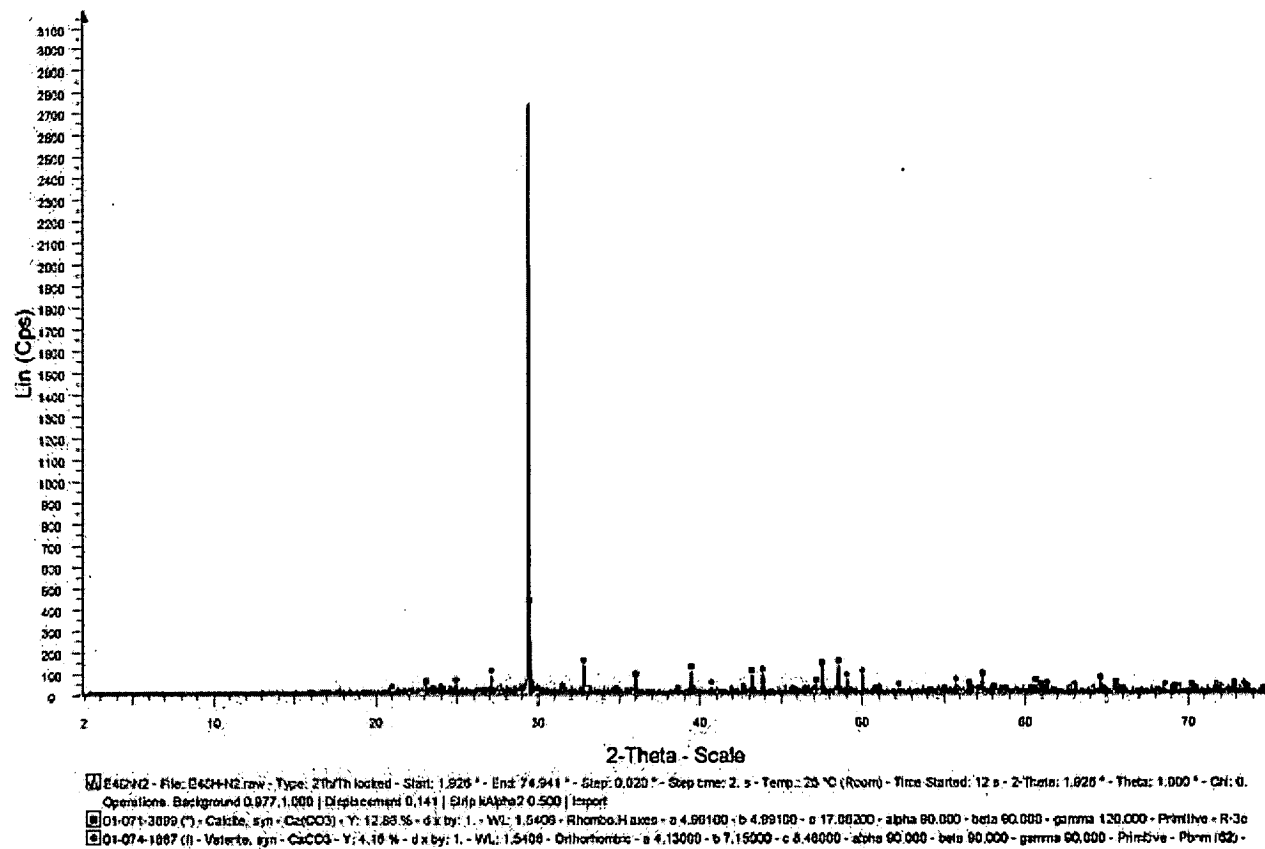
2a: Native cell experiment, 12 h.



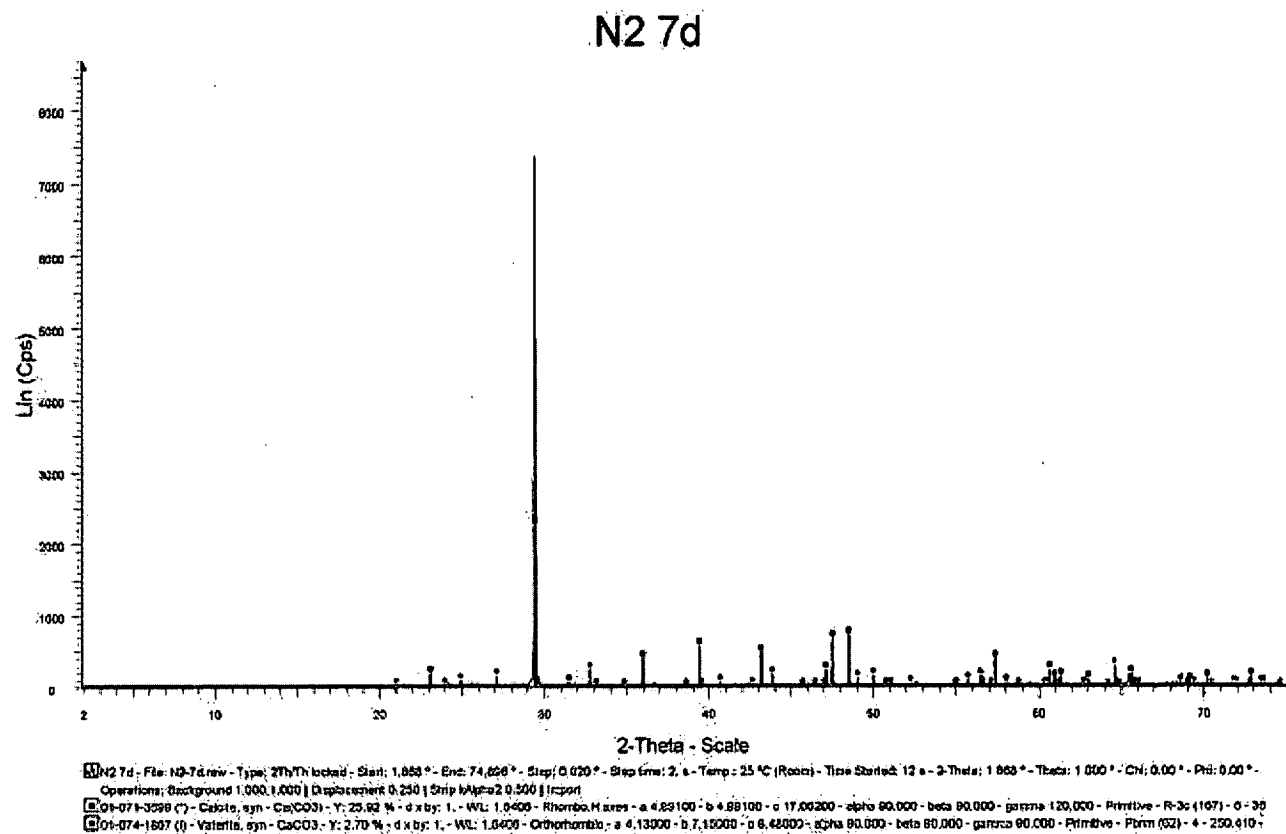
2bi: Native cell experiment, 48 h.

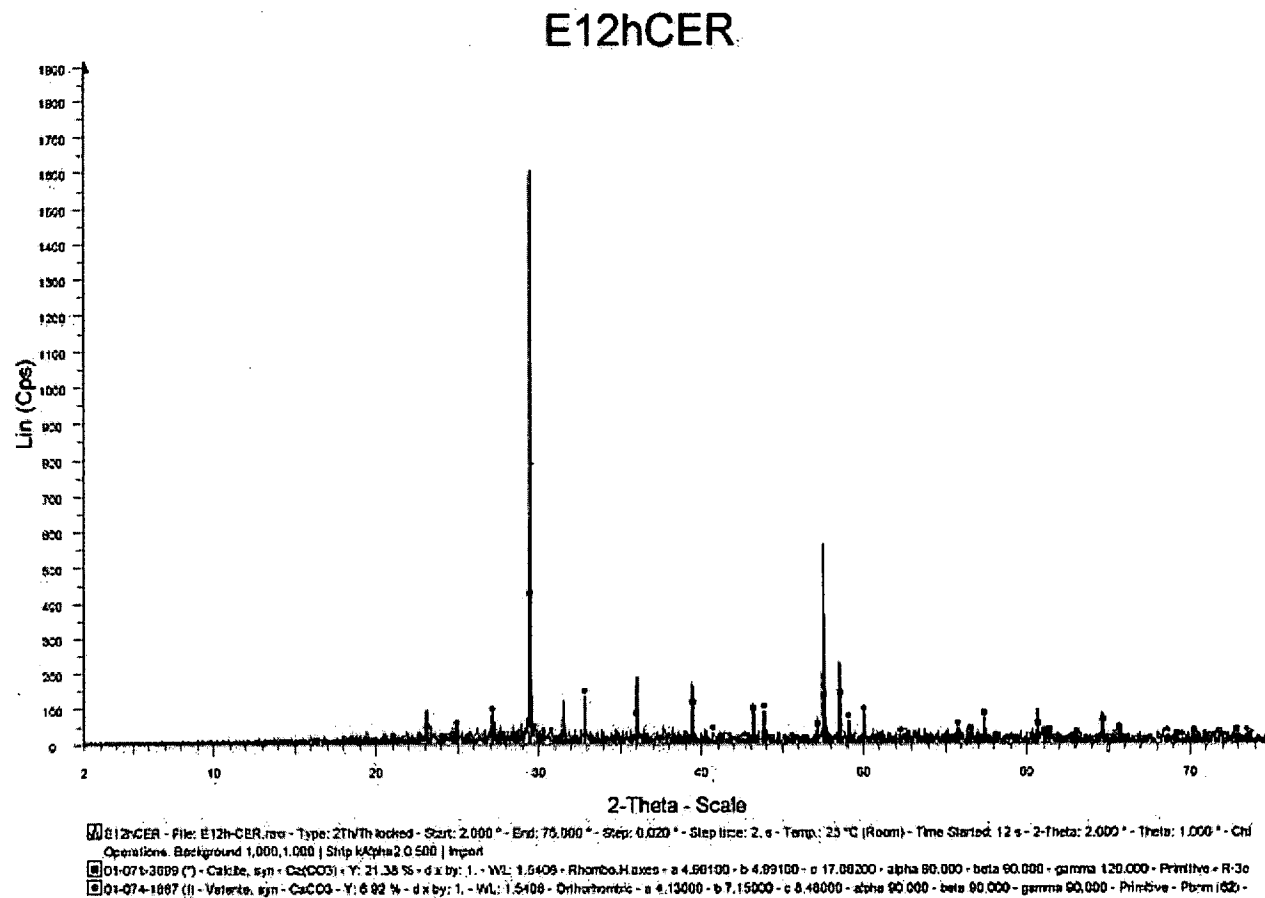


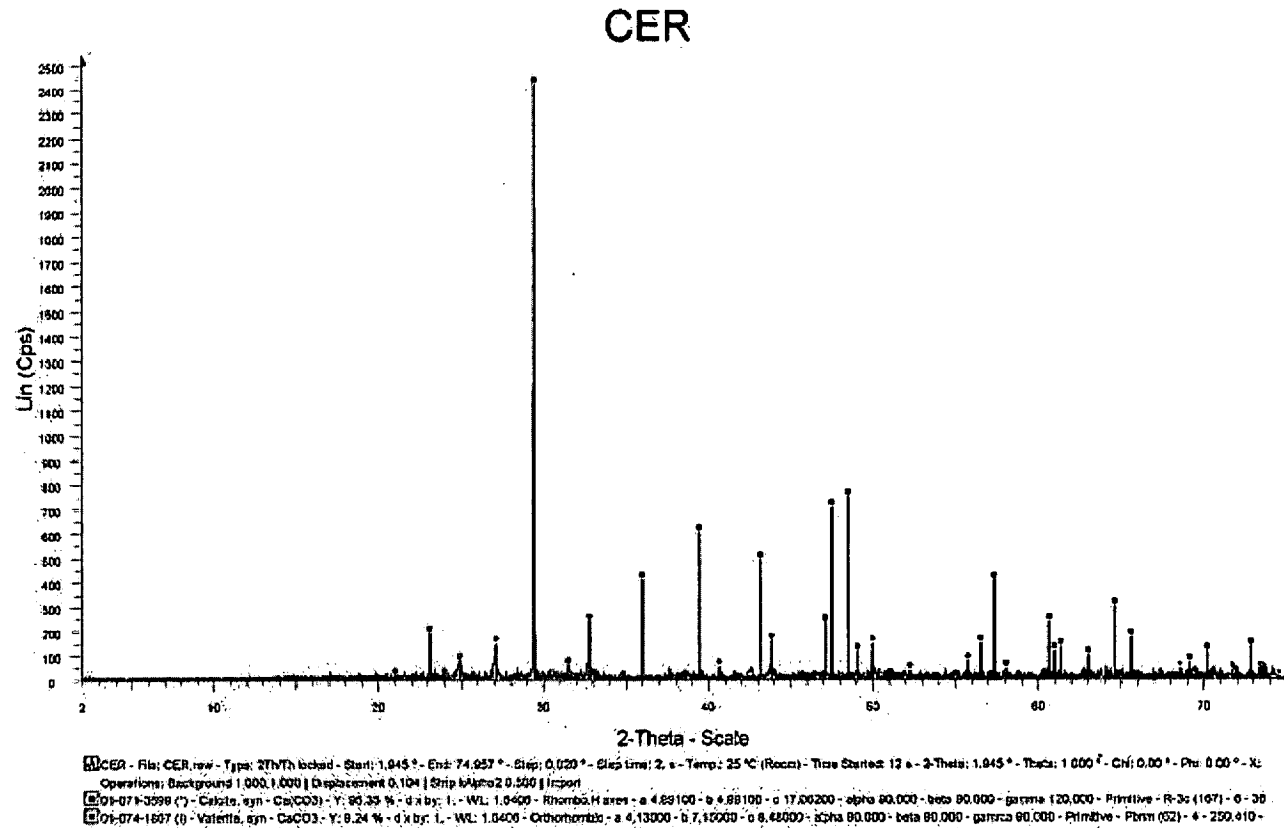
E48hN2



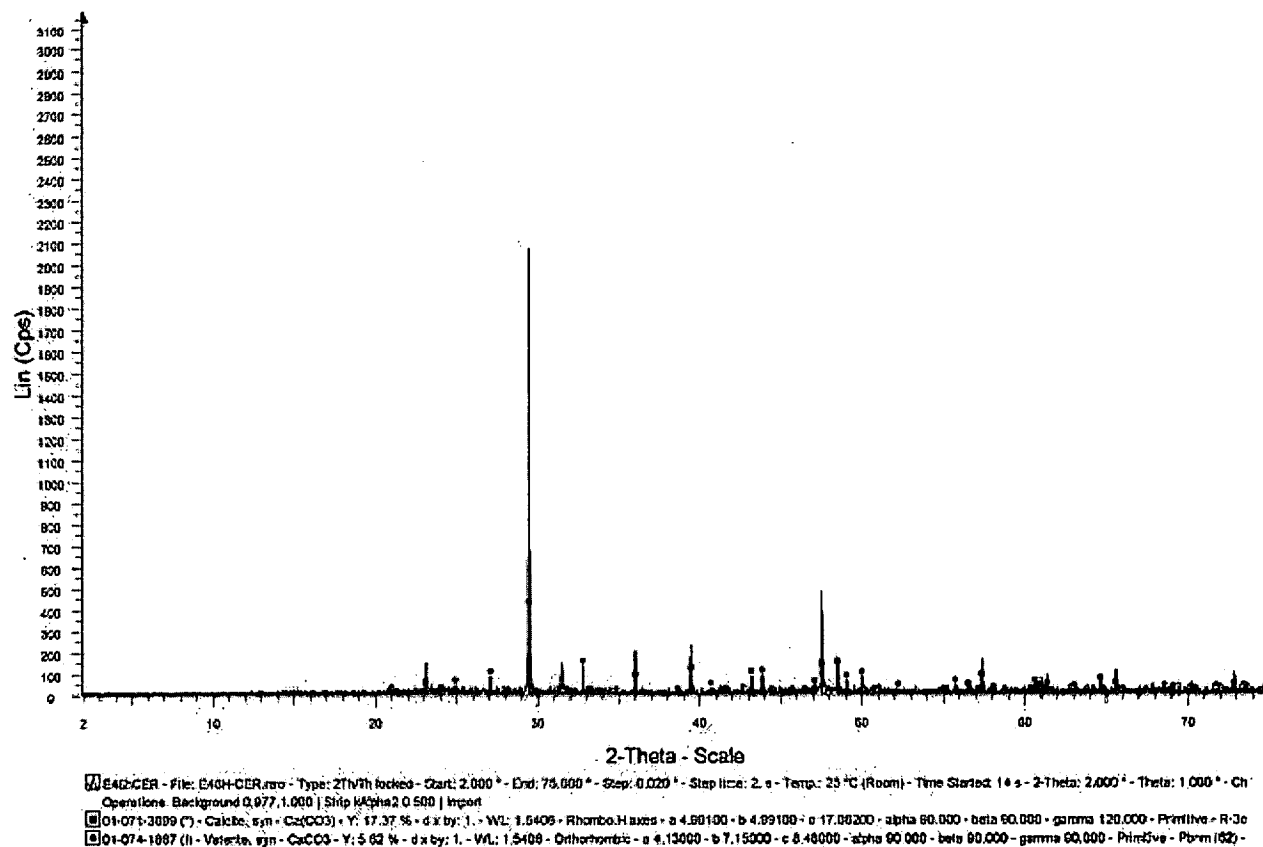
2c: Native cell experiment, 7 days.

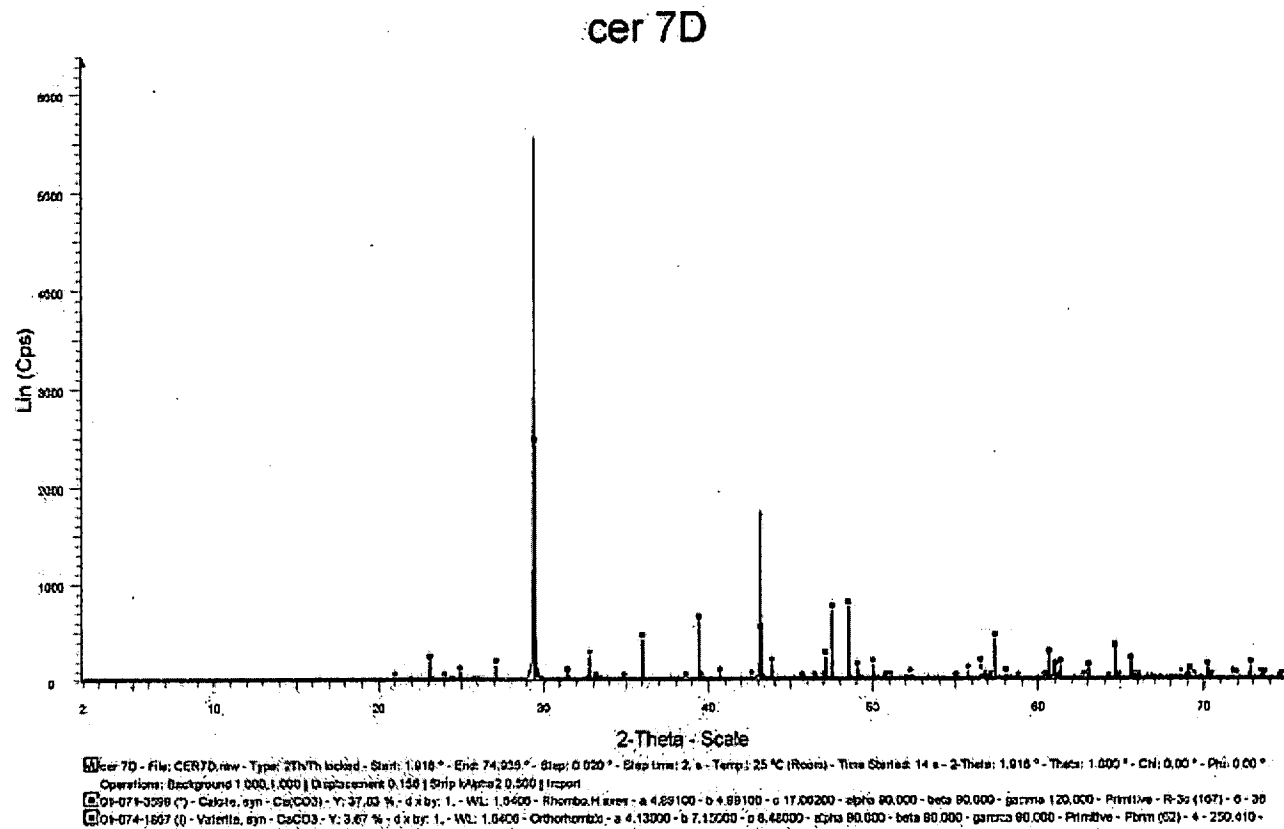


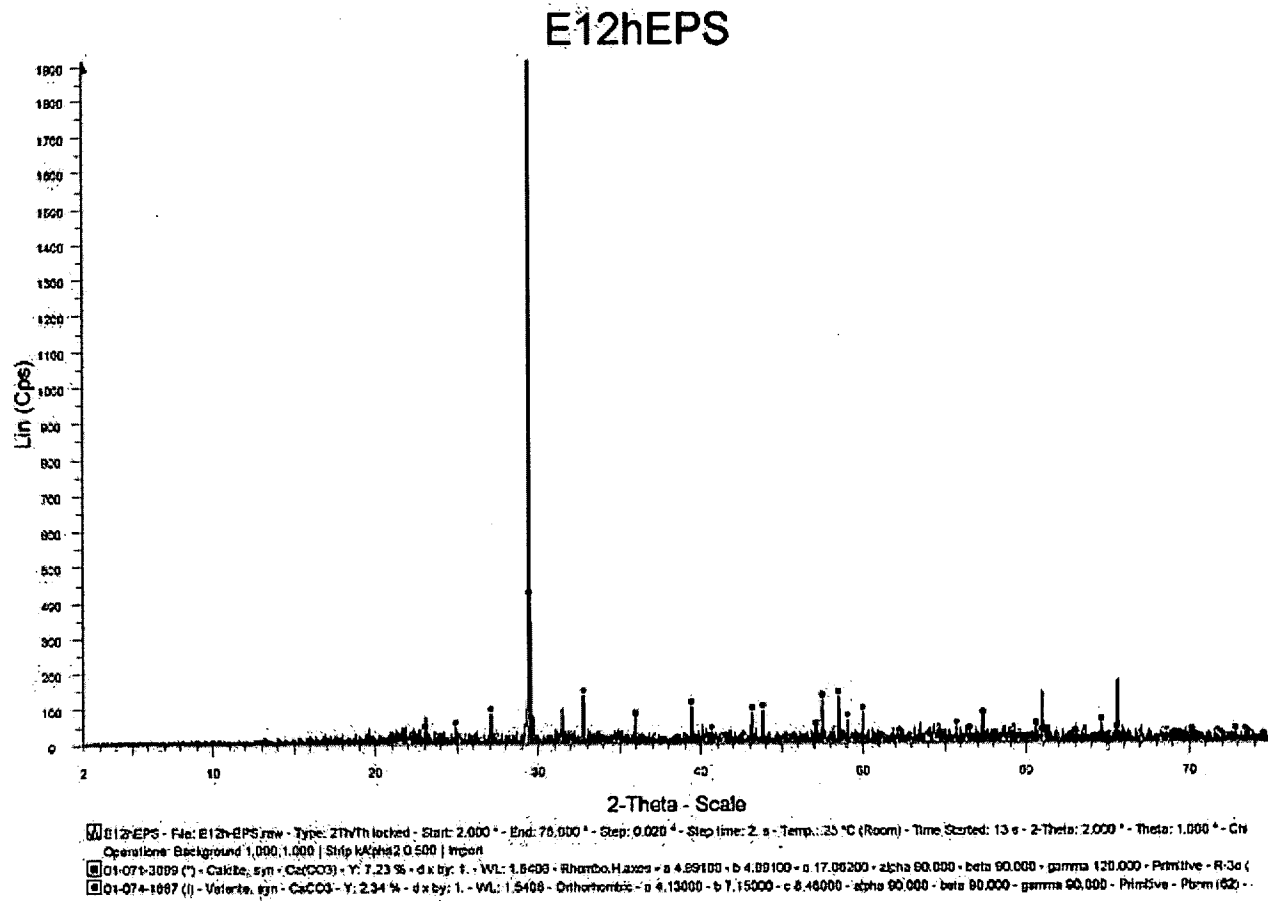




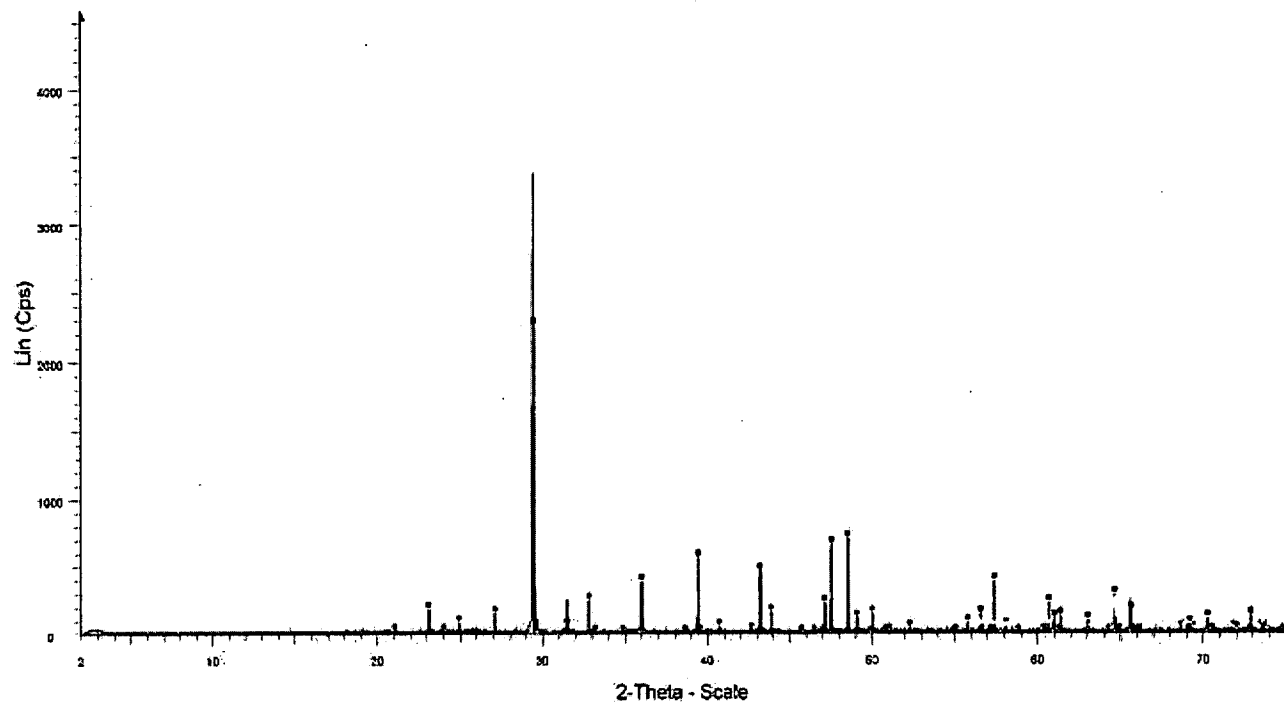
E48hCER



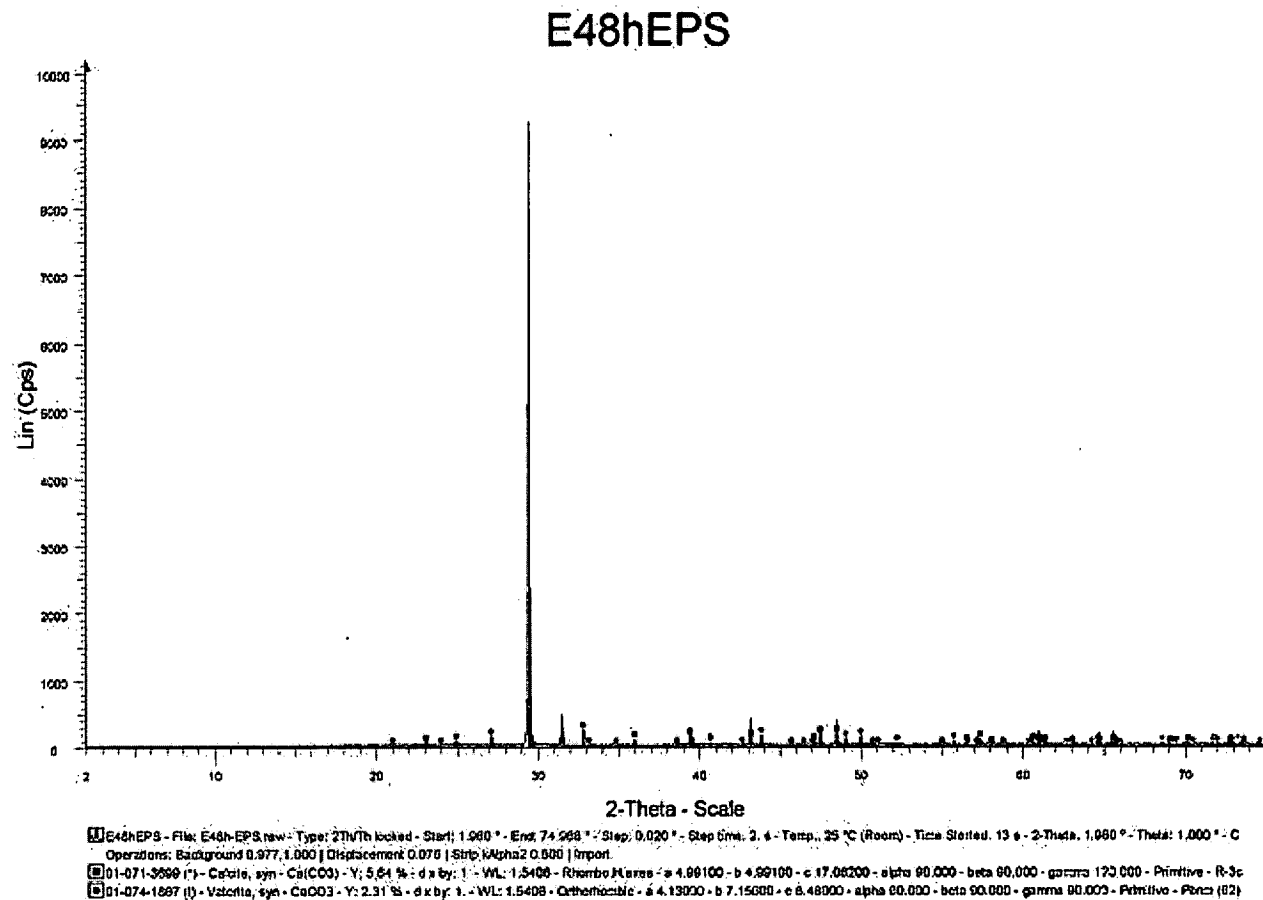


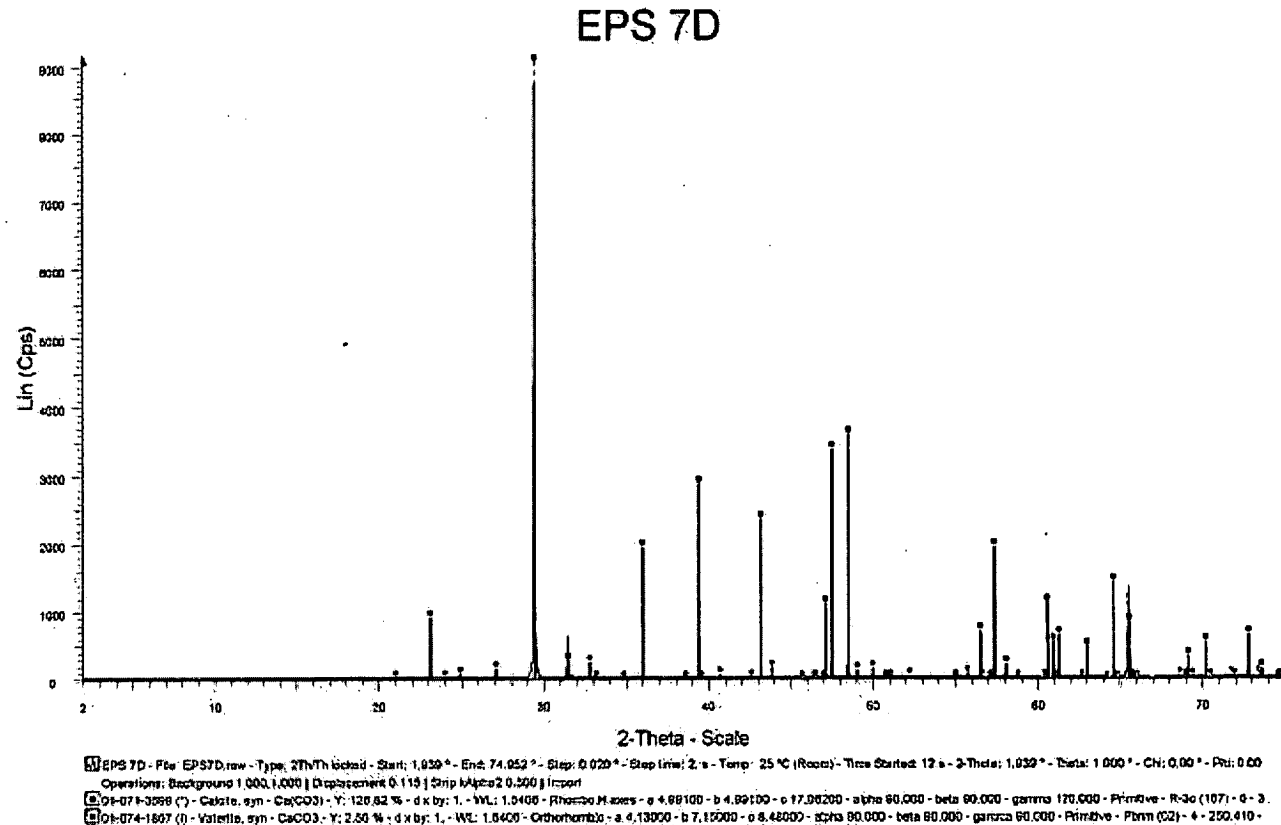


F-EPS



F-EPS - File: F-EPS.new - Type: 2Th/Th locked - Start: 1.045 ° - End: 74.057 ° - Step: 0.020 ° - Step time: 2. s - Temp: 25 °C (Room) - Time Started: 14 s - 2-Theta: 1.045 ° - Theta: 1.000 ° - Chi: 0.00 ° - Phi: 0.00 ° -
 Operations: Background 1.000, 1.000 | Output: 0.104 | Strip Alpha 2.0.500 | Import
01-074-3598 (*) - Calcite, syn - CaCO₃ - Y: 49.29 % - d x by: 1 - WL: 1.9400 - Rhombo. H axes - a 4.89100 - b 4.89100 - c 17.00200 - alpha 90.000 - beta 90.000 - gamma 120.000 - Primitive - R-3c (197) - O - 36
01-074-1607 (f) - Vaterite, syn - CaCO₃ - Y: 0.13 % - d x by: 1 - WL: 1.9400 - Orthorhombic - a 4.13000 - b 7.15000 - c 6.48000 - alpha 90.000 - beta 90.000 - gamma 90.000 - Primitive - P6mm (52) - 6 - 250.610





7. Conclusions and general synthesis.

7.1. Summary of Project Aims and Objectives

The basis of this project was an investigation of the effects of extracellular polymeric substances (EPS) on the surface chemistry and metal adsorption characteristics of bacterial cells. In addition, the effect of EPS on mineral precipitation was also investigated. This was undertaken using a thermophilic bacterial strain, *Bacillus licheniformis* S-86, and experiments were conducted using both cells with the EPS layer present (native cells) and cells from which the EPS had been extracted by treatment with cationic exchange resin.

The specific project objectives were as follows:

1. *To characterise and quantify the contribution made by extracellular polymers (EPS) to bacterial cell surface chemistry and electrostatic properties;*
2. *To conduct an integrated macroscopic and spectroscopic study of metal adsorption to an EPS-producing, thermophilic bacterial strain;*
3. *To investigate the effect of elevated adsorption temperature on metal adsorption by a thermophilic EPS-producing bacterial strain;*
4. *To investigate if carbonate mineral precipitation on the surfaces of cells is affected by the presence of EPS, and whether bacterial cells affect the morphology and crystal structure of the carbonates.*

7.2. Summary of Major Findings

7.2.1. The effect of EPS on cell surface chemistry and electrostatic properties (Chapters 2 and 3)

Surface characterisation of the native and EPS-free *B. licheniformis* cells incorporated potentiometric titrations, infrared analysis and electrophoretic mobility analysis. Data optimisation modelling was undertaken using both a nonelectrostatic approach and the Donnan electrostatic correction.

Nonelectrostatic modelling of the potentiometric titration data, using FITEQL 4.0, revealed that both native and EPS-free *B. licheniformis* cell walls contain 4 proton-active functional groups. The pK_a values obtained did not differ significantly between the two cell treatments and may be approximated as 3.3-3.4 (site 1), 5.3-5.4 (site 2), 7.4-7.5 (site 3) and 9.9-10.1 (site 4). Data optimisation modelling indicated that there are some differences in site concentrations between the native and EPS-free cells. The site concentrations obtained for the native cells were $8.53 \pm 2.45 \times 10^{-4} \text{ mol g}^{-1}$, $9.06 \pm 2.14 \times 10^{-4} \text{ mol g}^{-1}$, $2.87 \pm 0.59 \times 10^{-4} \text{ mol g}^{-1}$ and $6.50 \pm 1.44 \times 10^{-4} \text{ mol g}^{-1}$. For the EPS-free cells, the site concentrations were $5.47 \pm 1.13 \times 10^{-4} \text{ mol g}^{-1}$, $6.82 \pm 1.09 \times 10^{-4} \text{ mol g}^{-1}$, $2.22 \pm 0.62 \times 10^{-4} \text{ mol g}^{-1}$ and $3.26 \pm 0.68 \times 10^{-4} \text{ mol g}^{-1}$. These results indicate that the EPS layer is enriched in sites 1 and sites 4 relative to the EPS-free cell wall. The differences in site concentrations between sites 2 and 3 in the two cell treatments are smaller, particularly for site 3. Assigning identities to the four cell wall/EPS functional groups was undertaken based on comparison of the pK_a values with those from published literature. It was thought that the sites are most likely to correspond to phosphodiester (pK_a 3.3- 3.4), carboxyl (pK_a 5.3- 5.4), phosphoryl (pK_a 7.4- 7.5) and amine groups (pK_a 9.9- 10.1).

Analysis of the samples was undertaken using both FTIR and ATR-FTIR in order to observe any changes in the bacterial spectra either as a function of pH or according to whether or not an EPS layer is present. It was found that ATR-FTIR provided more consistent results, possibly because this technique allows the biomass to be analysed in its native state, as a wet paste in this case. This suggests that the drying and mixing with KBr necessary to prepare FTIR pellets may not be suitable for samples of this nature. The infrared spectra obtained did not indicate any changes in

functional groups between the native and EPS-free cells, but clearly showed the changing speciation of the carboxyl group at $1708\text{--}1717\text{ cm}^{-1}$ (protonated carboxyl) and approximately 1400 cm^{-1} (carboxyl anion). The pH range over which this occurred (pH 3– 8) supports identification of the pK_a 5.4 group as carboxylic, as deprotonation typically occurs across the pH range of $\text{pK}_a \pm 2$.

Further data modelling was undertaken using the Donnan core-shell model (FITMOD) in order to correct for electrostatic interactions between the cell wall and the electrolyte. Potentiometric titrations were carried out at ionic strengths of 0.01 M and 0.1 M and data modelling undertaken to obtain intrinsic (non ionic-strength-dependent) deprotonation constants and site concentrations. This approach was also found to produce four surface functional groups, although reproducibility was poorer than that using the nonelectrostatic approach. The cell wall thickness measured by TEM for the EPS-free cells was assumed to represent the Donnan thickness required by FITMOD to calculate the Donnan volume. The thickness of the EPS layer on the native cells could not be resolved by electron microscopy, therefore the Donnan thickness was manually increased in increments starting from the 22 nm measured for the cell wall. It was hypothesised that the $V(Y)$ values should decrease as the input Donnan thickness approached the true value, and that the deprotonation characteristics should approach the values obtained for the EPS-free cells. Based on the 0.01 M and 0.1 M titration data, the average pK_a values and corresponding 1x standard deviations for the EPS-free cells were 3.4 ± 0.5 (site 1), 4.9 ± 1.0 (site 2), 7.3 ± 0.6 (site 3) and 9.0 ± 0.6 (site 4). The site concentrations and 1x standard deviations are $8.06 \pm 2.53 \times 10^{-4}\text{ mol g}^{-1}$ (site 1), $4.38 \pm 2.03 \times 10^{-4}\text{ mol g}^{-1}$ (site 2), $2.69 \pm 1.14 \times 10^{-4}\text{ mol g}^{-1}$ (site 3) and $3.85 \pm 0.74 \times 10^{-4}\text{ mol g}^{-1}$. For the native cells, the average pK_a values at a Donnan thickness of 100 nm were 3.6 ± 0.8 (site 1), 5.3 ± 0.6 (site 2), 7.9 ± 0.5 (site 3) and 9.7 ± 0.4 (site 4). The site concentrations and 1x standard deviations are $1.27 \pm 0.47 \times 10^{-3}\text{ mol g}^{-1}$ (site 1), $6.30 \pm 1.55 \times 10^{-4}\text{ mol g}^{-1}$ (site 2), $4.14 \pm 1.97 \times 10^{-4}\text{ mol g}^{-1}$ (site 3) and $7.63 \pm 2.50 \times 10^{-4}\text{ mol g}^{-1}$. The complexation characteristics for the native cells did not show a tendency to approach those for the EPS-free cells, and it was considered unrealistic to further increase the Donnan thickness. Although the pK_a values obtained using the electrostatic approach deviate somewhat from those obtained using nonelectrostatic modelling,

they are within a close enough agreement to correspond to the same functional groups identified using the initial nonelectrostatic modelling approach. The most significant difference in the site concentrations obtained using the Donnan model compared to the nonelectrostatic results is the higher concentration of site 1. The total site concentrations are similar, and the electrostatic modelling also indicates a substantially higher concentration of functional groups in the native cells compared to the EPS-free cells. The disparity between the intrinsic pK_a values for the EPS-free and native cells indicates that the Donnan model was not fully accounting for the effect of the EPS layer on the cell surface electrostatic properties. It is thought that errors may be introduced due to substantial expansion of the cell walls and EPS layer, particularly at low ionic strength.

Electrophoretic mobility analysis was undertaken, comparing native and EPS-free cells. It was found that the effects of ionic strength variation on the cell wall volume were much more pronounced for the native cells. As ionic strength decreased from 0.1 M to 0.001 M, swelling of the cell wall (and EPS layer for the native cells) caused an apparent decrease in the cell surface charge. At low ionic strength, native cells have lower electrophoretic mobilities than the EPS-free cells, indicating a lower surface charge density. However, at 0.1 M when the effects of cell wall swelling are minimised, the native cells have a higher electrophoretic mobility and hence higher surface charge density than the EPS-free cells, in agreement with the findings based on potentiometric titration data.

Measurement of dissolved organic carbon concentrations released into solution by the cells revealed that native cells produce considerably more DOC than EPS-free cells. This shows that the EPS layer is a significant source of DOC to solution, and EPS-producing bacterial strains may consequently influence solution chemistry in their surrounding environment to a greater extent than non EPS-producing bacterial strains.

Another significant outcome of this part of the study was the finding that the presence of EPS encourages cell aggregation in solution. Cell aggregation is commonly observed at low pH, due to neutralisation of cell surface charge and reduction in electrostatic repulsion. However, light microscope images show that

native *B. licheniformis* S-86 cells undergo considerable aggregation even at circumneutral pH. This leads to loss of mass-dependent resolution in potentiometric titration curves. The large standard deviations associated with the site concentrations reflect this loss of resolution, but it is likely that the site concentrations obtained for the native cells are minimum values. Consequently, the differences in the site concentrations between the native and EPS-free cells may be greater than reported here.

7.2.2. The effect of EPS on metal adsorption (Chapter 4)

The effect of EPS on Zn adsorption was investigated by means of an experimental approach that incorporated both macroscopic batch experiments and spectroscopic (EXAFS) analysis. Modelling of adsorption data from the macroscopic adsorption experiments using FITEQL 4.0 indicated that both site 2 (carboxylic) and site 3 (phosphoryl) were involved in Zn binding. Generally, all of sites 1, 2 and 3 were required to explain the observed Zn uptake from solution but stability constants for site 1 were found to vary greatly. The data modelling showed a lack of mass-dependent resolution, as the expected increase in the number of sites required for metal binding with decreasing biomass concentration was not consistently observed. This is thought to be a consequence of cell aggregation in solution, which is particularly significant for native cells, but was observed to occur to a lesser extent in EPS-free cells, either due to residual EPS following the extraction treatment, or continued EPS production by the cells throughout the experimental duration. The extent of metal adsorption was similar for both native and EPS-free cells. The mean stability constants obtained for Zn binding to native cells were 2.46 ± 0.58 (site 1), 3.58 ± 0.23 (site 2) and 5.04 ± 0.60 (site 3). For EPS-free cells the mean log K values were 2.24 ± 0.82 (site 1), 3.85 ± 0.10 (site 2) and 5.99 ± 0.42 (site 3). There was found to be no significant difference (t-test) between the stability constants obtained for native and EPS-free cells.

EXAFS analysis of Zn binding to *B. licheniformis* S-86 was consistent with Zn complexation by a carboxylic functional group. However, fairly high R-values are an indication that the fit of the model spectra to the experimental data is sub-optimal.

This may suggest that there is also Zn complexation by site 3, a phosphoryl-type group, which would be in agreement with the macroscopic experimental results. However, the Zn-phosphate standard used for these experiments did not provide a sufficiently good EXAFS spectrum to enable linear combination fitting to be undertaken. Application of this method can provide an indication of the extent of metal binding accounted for by minor functional groups.

The dominance of the carboxylic group (site 2), and likely significance of site 3 (phosphoryl group) in Zn complexation explains why there is no observable difference in metal complexation characteristic between native and EPS-free cells. The site concentrations calculated for the two cell treatments show that the concentrations of sites 2 and 3 are affected relatively little by removal of the EPS layer. As the concentrations of the principle binding sites in the EPS layer are similar to those in the EPS-free cell wall, metal complexation is not significantly affected by EPS production.

7.2.3. Metal adsorption at elevated temperatures (Chapter 5)

Zn adsorption experiments were conducted at 40 °C and 50 °C using native cells of the EPS-producing *B.licheniformis* S-86, in order to investigate whether this bacterial species shows any adaptation enabling increased metal uptake at elevated temperature. As for the room-temperature experiments, it was found that sites 2 and 3 were consistently involved in Zn complexation, with the additional involvement of site 1 in most cases. The mean stability constants obtained for 25 °C, 40 °C and 50 °C were 2.46 ± 0.48 (site 1), 3.66 ± 0.26 (site 2) and 4.90 ± 0.55 (site 3). No significant differences (t-test) were found between the log K values at different temperatures. This lack of temperature-dependent adsorption behaviour is consistent with an outer-sphere complexation mechanism for Zn-binding to the bacterial cells.

7.2.4. The effect of EPS on CaCO_3 mineralisation (Chapter 6)

Calcium carbonate precipitation experiments were undertaken using the ammonium free-drift method, in the presence of native *B. licheniformis* cells, EPS-free cells and a solution of the EPS extracted from cells by treatment with cation exchange resin. A blank experiment contained only the calcium and carbonate solutions. The experiments were run over the course of one week, and samples removed for analysis at 24 h, 48 h and 7 days. SEM analysis was used to observe the morphologies of the CaCO_3 precipitated. It was found that each of the four experiments contained different characteristic crystal morphologies in the 48 h samples. Some of these morphological features were retained in the 7 day samples, although the precipitates had generally recrystallised to large, irregular crystalline structures. Mineralogical analysis by XRD revealed that the 12 h and 48 h samples from the blank and EPS-free cells contained varying proportions of vaterite, in addition to calcite. The native cell and EPS-solution experiments contained only calcite at 12 h and 48 h, and by 7d all of the experiments contained only calcite. These results indicated that the presence of EPS inhibits precipitation of vaterite, which is a metastable precursor to calcite. It is thought that this effect was due to release of DOC into solution by the EPS present on the native cells. The EPS-solution also contains a high concentration of DOC. Fractions of the released DOC are likely to complex Ca ions in solution, thus lowering the Ca saturation. As precipitation of the more soluble and less stable polymorph (vaterite) is favoured by high saturation conditions, lowering the concentration of available Ca in solution would favour calcite precipitation.

The observation that all vaterite is replaced by calcite after a period of 7 days indicates that stabilisation of vaterite by organic matter is not significant on this timescale under the experimental conditions used here. However, lysis of the cells after several days would release large quantities of DOC, further encouraging the precipitation of calcite over vaterite.

7.3. The Environmental Role of EPS in Bacterial Cell Surface Reactivity

The results described above have shown that the bacterial extracellular polymers produced by *B. licheniformis* S-86 have a similar reactivity towards protons and Zn as the EPS-free cell wall. This suggests that the production of EPS by bacterial cells does not significantly enhance their metal complexation abilities under normal environmental conditions. Further to this, the results of the variable-temperature adsorption experiments have shown that the thermophilic bacterial species used during this study does not appear to have enhanced metal-complexation characteristics under elevated temperature conditions. These results provide evidence to support the theory that a ‘universal’ surface complexation model can be applied to bacterial adsorption of protons and metals in a wide variety of environmental settings. Under some circumstances, the presence of an EPS layer may enable enhanced metal adsorption (summarised in Table 7.1), and future studies should aim to consider the possibility that such conditions may occur.

Table 7.1: Possible effects of EPS production on metal adsorption relative to non-EPS producing strains.

Condition	Effect of EPS	Outcome
Low pH.	Higher concentration of low pK_a site 1.	Enhanced metal adsorption.
High pH.	Increase in DOC released-competes for metal complexation.	Reduced metal adsorption.
High cell concentration in solution (e.g. bioreactor).	Aggregation and reduced available surface area.	Reduced metal adsorption.
Contaminated environment.	EPS provides protection.	Enhanced metal adsorption.
Occurrence of surface precipitation.	EPS provides larger volume for precipitation to occur within.	Enhanced adsorption/precipitation.

The results of the calcium carbonate mineralisation studies indicate that EPS may have a very significant role as a source of dissolved organic carbon, and hence as a factor altering the solution chemistry of the surrounding environment. It was established that significantly more DOC is released into the surrounding medium if EPS is present on the cells, and this is particularly important when investigating the characteristics of biofilms, a large proportion of which consists of EPS. This study found that DOC release by EPS can alter the mineralogy and morphology of minerals

precipitated in close proximity or on the surfaces of the cells, and it is possible that this effect may be observed on or in the vicinity of microbial biofilms.

There are a number of other potential environmental roles of EPS in addition to those directly investigated by this study. Figure 7.1 summarises a range of possible interactions between EPS and metal ions, and the effects of EPS production on biomineralisation.

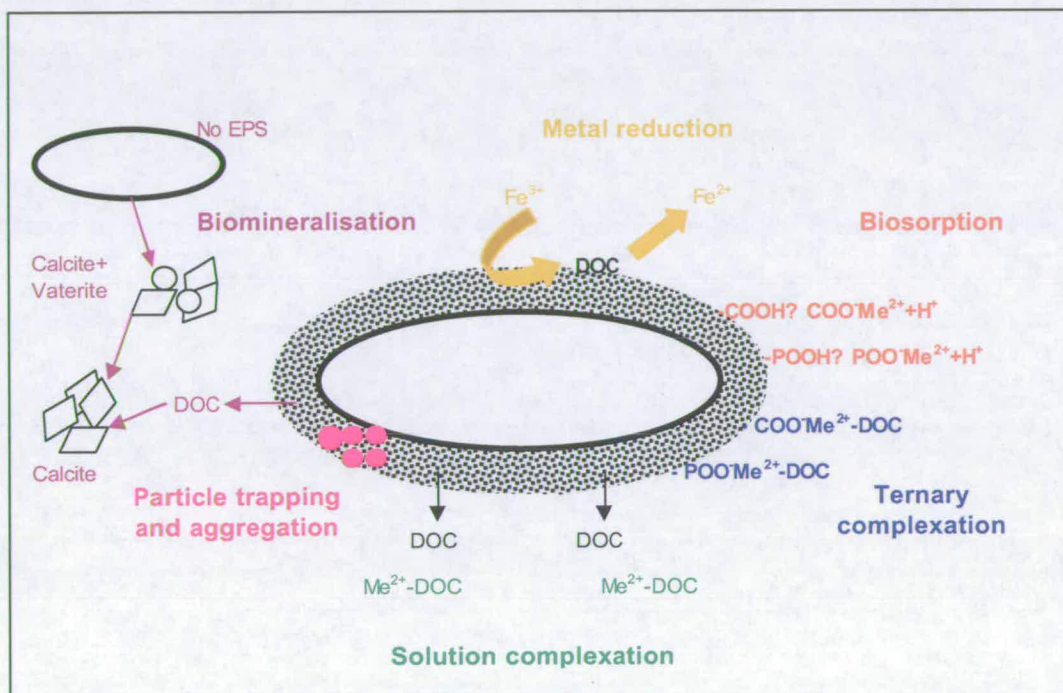


Figure 7.1: The environmental roles of EPS in metal-microbe interactions and biomineralisation.

This study did not address the issue of particle trapping and aggregation within EPS, but recent other recent studies have shown that this is likely a significant factor influencing biogeochemical cycling of metals. For example, Moreau et al. (2007) studied biofilms of sulphate-reducing bacteria and found that they contained spheroidal biogenic ZnS nanocrystals. It was proposed that entrapment within the extracellular polymers of the biofilm could significantly limit dispersion of the nanoparticles. This has important implications for transport of the products of bioremediation processes and biominerals (Moreau et al., 2007). For example, *Geobacter sulfurreducens* reduces U(VI) to uraninite, which subsequently forms

nanoparticles outside the cell (Lloyd, 2003) and it is likely that these particles would form aggregates within any EPS present. Merroun et al. (2003) used transmission electron microscopy to observe significant quantities of lanthanum adsorbed within the EPS layer of *Myxococcus xanthus*, as well as on the cell wall.

Characterising the role of bacterial cells in transportation of metals, radionuclides and other contaminants is a principle aim of metal adsorption studies. Attempts to predict contaminant transport in aquifers or other subsurface environments should consider the potential for bacteria both to enhance transportation, in the case of sorption to planktonic cells, or inhibit transportation if sorption occurs to biofilm-associated cells. Studies by Chen et al. (1995), Czajka et al. (1997) and Jensen-Spaulding et al. (2004) have shown that EPS in solution can complex metal ions and increase transportation rates. On the other hand, Liu et al. (2007) demonstrated that the presence of EPS increased bacterial adhesion to glass beads in column experiments. Limiting the transportation of cells through adhesion would presumably also limit the transportation of any adsorbed contaminants. Furthermore, Hand et al. (in press) describe clogging of pores within porous media by bacterial extracellular polymers, which would also decrease the rate of contaminant transport within the system.

The role of bacteria and other microbes in redox cycling is well established. Bacteria are known to be instrumental in the redox transformations of numerous metals and metalloids including iron, manganese and chromium (Lloyd, 2003). Redox cycling of metals within biofilms can be complex, as biofilms may be stratified in terms of redox potential. Metal reduction requires the presence of labile organic matter as an electron donor, and it is possible that EPS could act as a significant source of such organic matter. It has been established by this study that significant quantities of DOC are released from the EPS layer, and it is therefore possible that EPS production may confer an enhanced metal reduction ability compared to non-EPS-producing bacterial strains.

Ternary complexation involving both metals and organic ligands is a potentially significant process at cell surfaces. The interactions between bacterial cell, metals and other organic ligands have been investigated by studies including Wightman and

Fein (2001), Borrok and Fein (2004) and Borrok et al. (2007). It was found by Borrok et al. (2007) that all fractions of the natural organic matter studied were capable of forming ternary complexes with bacterial cells and metals, and that the formation of ternary complexes increased as pH decreased. However, the incorporation of metals was found to be complex and varied according to the identity of the metal species. It was thought that ternary complexation could significantly increase binding of metals with lower affinities for the bacterial surface, such as Ni. In addition to the processes discussed above, EPS is believed to have important roles in bioleaching (Kinzler et al., 2003; Sand and Gehrke, 2006) and adhesion to surfaces (Omoike et al., 2004). The interactions and competing effects between these different processes are likely to be complex and variable according to the environmental conditions. Understanding each of these processes individually is the first step towards fully characterising the environmental role of EPS.

7.4. Further Work

The results of this study, together with the other possible environmental roles of EPS discussed in Section 7.3, have raised some issues that merit further investigation. Specifically, future work could aim to address the following objectives:

- The production of EPS induces significant cell aggregation in solution, which results in poor resolution of both proton and metal adsorption curves as a function of biomass concentration. This impedes discrete-site surface complexation modelling, as the number of sites required to optimally fit the data does not increase with decreasing biomass concentration as expected. Further work should aim to assess how significantly cell aggregation affects the stability constants and site concentrations obtained through surface complexation modelling. This would contribute to the wider research area investigating the effect of EPS on contaminant transport.

- A systematic investigation of the variation in EPS composition with growth conditions and temperature would be of value, as some studies have identified potentially important differences in EPS composition according to varying conditions. Guibaud et al. (2005) found that EPS extracted from pure bacterial cultures was less effective as a metal adsorbent than EPS from activated sludge owing to differences in the biochemical composition. Noghabi et al. (2007) investigated EPS production by a psychrotrophic bacterium and found that polymer production was significantly affected by growth conditions. It has been shown by Eboigbodin et al. (2007) that the tendency of *E. coli* cells to aggregate varies according to the concentration of surface functional groups, which in turn is affected by the growth medium. Furthermore, Kives et al. (2006) found that the polysaccharide content of EPS produced by planktonic *Pseudomonas fluorescens* B52 differed from that of EPS produced by the same strain living in a biofilm. The potentially variable composition of EPS should therefore be considered when attempting to apply generalised surface complexation models to environmental settings.
- The release of substantial quantities of dissolved organic carbon into solution from the EPS layer has been raised as a potentially important factor affecting several aspects of this project. As discussed in Section 7.3, future studies investigating metal adsorption and mineralisation on bacterial cells or biofilms should aim to include DOC as an important variable within the system. Characterising the reactivity of this DOC towards protons and metals would be a valuable contribution to this area of research and would perhaps enable a modelling approach that includes DOC as a component. Establishing which fractions of the EPS layer are released into solution, under different environmental conditions, is important when attempting to apply laboratory-obtained complexation constants to more complex systems. This study would be of importance to determining the potential role of EPS as an organic matter source in redox cycling of metals, and in the formation of ternary surface complexes.

7.5. References

- Borrok, D., Fein, J.B. (2004) Distribution of protons and Cd between bacterial surfaces and dissolved humic substances determined through chemical equilibrium modelling. *Geochimica et Cosmochimica Acta*, 68, 3043-3052
- Borrok, D., Aumend, K., Fein, J. (2007) Significance of ternary bacteria-metal-natural organic matter complexes determined through experimentation and chemical equilibrium modelling. *Chemical Geology*, 238 (1-2), 44-62
- Chen, J-H., Lion, L.W., Ghiorse, W.C., Shuler, M.L. (1995) Mobilization of adsorbed cadmium and lead in aquifer material by bacterial extracellular polymers. *Water Research*, 29, 421-430
- Czajka, D.R., Lion, L.W., Schuler, M.L., Ghiorse, W.C. (1997) Evaluation of the utility of bacterial extracellular polymers for the treatment of metal-contaminated soils: Polymer persistence, mobility and the influence of lead. *Water Research*, 31, 2827-2839
- Eboigbodin, K.E., Ojeda, J.J., Biggs, C.A. (2007) Investigating the surface properties of *Escherichia coli* under glucose controlled conditions and its effect on aggregation. *Langmuir*, 23, 6691-6697
- Guibaud, G., Comte, S., Bordas, F., Dupuy, S., Baudu, M. (2005) Comparison of the complexation potential of extracellular polymeric substances (EPS), extracted from activated sludges and produced by pure bacteria strains, for cadmium, lead and nickel. *Chemosphere*, 59, 629-638
- Hand, V., Lloyd, J.R., Vaughan, D.J., Wilkins, M.J., Boulton, S. (2008) Experimental studies of the influence of grain size, oxygen availability and organic carbon availability on bioclogging in porous media. *Environmental Science and Technology*, In Press
- Jensen-Spaulding, A., Cabral, K., Schuler, M.L., Lion, L.W. (2004) Predicting the rate and extent of cadmium and copper desorption from soils in the presence of bacterial extracellular polymer. *Water Research*, 38, 2231-2240

- Kinzler, K., Gehrke, T., Telegdi, J., Sand, W. (2003) Bioleaching-a result of interfacial processes caused by extracellular polymeric substances (EPS). *Hydrometallurgy*, 71, 83-88
- Kives, J., Orgaz, B., SanJose, C. (2006) Polysaccharide differences between planktonic and biofilm-associated EPS from *Pseudomonas fluorescens* B52. *Colloids and Surfaces B: Biointerfaces*, 50, 104-108
- Liu, Y., Yang, C-H., Li, J. (2007) Influence of extracellular polymeric substances on *Pseudomonas aeruginosa* transport and deposition profiles in porous media. *Environmental Science and Technology*, 41, 198-205
- Lloyd, J.R. (2003) Microbial reduction of metals and radionucleides. *FEMS Microbiology Reviews*, 27, 411-425
- Merroun, M.L., Ben Chekroun, K., Arias, J.M., Gonzalez-Munoz, M.T. (2003) Lanthanum fixation by *Myxococcus xanthus*: cellular location and extracellular polysaccharide observation. *Chemosphere*, 52, 113-120
- Moreau, J.W., Weber, P.K., Martin, M.C., Gilbert, B., Hutcheon, I.D., Banfield, J.F. Extracellular proteins limit the dispersal of biogenic nanoparticles. *Science*, 316, 1600-1603
- Noghabi, K.A., Zahiri, H.S., Yoon, S.C. (2007) The production of a cold-induced extracellular biopolymer by *Pseudomonas fluorescens* BM07 under various growth conditions and its role for heavy metals absorption. *Process Biochemistry*, 42 (5), 847-855
- Omoike, A., Chorover, J., Kwon, K.D., Kubicki, J.D. (2004) Adhesion of bacterial exopolymers to an α -FeOOH: Inner-sphere complexation of phosphodiester groups. *Langmuir*, 20, 11108-11114
- Sand, W., Gehrke, T. (2006) Extracellular polymeric substances mediate bioleaching/biocorrosion via interfacial processes involving iron(III) ions and acidophilic bacteria. *Research in Microbiology*, 157, 49-56
- Wightman, P.G., Fein, J.B. (2001) Ternary interactions in a humic acid-Cd-bacteria system. *Chemical Geology*, 180, 55-65