PHYSICOCHEMICAL IMPACTS OF SOLUBLE METALS ON BACTERIAL LIPID CHEMISTRY AND FUNCTION

A Thesis

Presented to

The Faculty of Graduate Studies

of

The University of Guelph

by

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In partial fulfillment of requirements

for the degree of

Doctor of Philosophy

August 2011

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ABSTRACT

PHYSICOCHEMICAL IMPACTS OF SOLUBLE METALS ON BACTERIAL LIPID CHEMISTRY AND FUNCTION

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Bacterial membranes are dynamic structures, and contain lipid components that are individually simple, but complex as a whole system. The presence of charged functional groups makes them capable of interaction with ubiquitous environmental metals. Physiological responses of bacteria to metals, in preservation of membrane functions and integrity, are unclear. In this study, membrane lipid profiles were characterized for *Shewanella putrefaciens* CN32. Both fatty acid chemistry and hydrophilic headgroup chemistry were assayed, after growing the cells in a chemically defined medium spiked with Mn, V, or U. Cultures were grown in both aerobic and anaerobic conditions, to examine the effects of O$_2$ and CO$_2$ gases, as well as the combined effects of these gases with metals. The results were compared to scanning transmission X-ray microscopy (STXM) elemental maps and near-edge X-ray absorption fine structure (NEXAFS) spectra of isolated and purified *S. putrefaciens* CN32 envelopes at V, Mn, Ca, C, N, and O edges. It was found that there were strong correlations between membrane fluidity and fatty acid composition of strain CN32 membranes. The acyl chain chemistry was minimally affected by metal presence in the growth medium, however these subtle changes correlated with significant alterations in the fluid states of the membranes. Uranium seemed to fall outside this relationship, strongly stabilizing cell membranes. Metals in all treatments adsorbed to cell membranes,
determined using either NEXAFS or electron microscopy, with the exception of V in aerobic conditions.

Permeability effects of metal exposure to Ca(II), Cu(II), Mn(II), U(VI), V(IV), and Zn(II) were also assessed. Bacterial strains for these studies included *S. putrefaciens* CN32, *Escherichia coli* AB264 (wildtype K-12), *Pseudomonas aeruginosa* PAO1 wildtype, and *Bacillus subtilis* 168, in order to compare published data from the membrane chemistry of those organisms to *S. putrefaciens* CN32 membranes. Each metal had the same overall impact on each bacterial strain, regardless of variations in cell membrane and surface sugar chemistry, however the strengths of these effects were different for each organism. All metals with the exception of U permeabilized cell walls, while U rendered the membrane much less permeable. These impacts on permeability were concentration dependent from 0.001 mM to 1 mM concentrations. The research demonstrated that growth environment has a significant impact on the physicochemical state of bacterial membranes. Metals in those environments have varying complexation chemistry according to pH and redox conditions, and impact membrane attributes and dynamics depending on cell wall chemical composition.
DEDICATION

For family and friends, past and present.

I could not have done it without you.
ACKNOWLEDGEMENTS

This research would not have been possible without the support of many people. I would like to thank my advisor Dr. Susan Glasauer, for her patience and support over the years. There have been ups and downs along the entirety of this research, and her advice, understanding, and guidance on both professional and personal levels has been invaluable.

Thanks also go out to my current advisory committee; Dr. Jack Trevors, Dr. Susan Koval, and Dr. John Dutcher. Your thoughts and suggestions regarding my research have been very insightful, and your support has been very much appreciated. Special thanks to the late Dr. Terry Beveridge, whose work, and early suggestions, helped trigger my interest in bacterial cell walls.

I also wish to extend thanks to my colleagues, Al Mattes, Dan Puddephatt, Kamini Khosla, Deanna Nemeth, David Boyle, Peter Smith, and Sarah Barabash for their thoughts and suggestions. Special thanks to Dan Puddephatt, whose knowledge of chemistry as it pertains to bacterial surfaces has helped tremendously over the course of this study. Special thanks are also necessary for Dianne Moyles, who electron microscopy expertise has saved the day time and time again.

Finally, I would like to thank my family and friends. My parents have stuck by my curious life decision of enrolling in a Ph.D program, and offered relentless support when I’ve needed it the most over the years. Lastly, but certainly not least, I would like to thank Sarah Glover. You always know the right things to say to keep me going, in times when I’m ready to give up. With all my heart, thank you.
“If you would be a real seeker after truth, it is necessary that at least once in your life you doubt, as far as possible, all things.”

- René Descartes (1596-1650)
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XAFS  X-ray absorption fine structure
XRD  X-ray diffraction
INTRODUCTION

Research objectives

The research presented in this dissertation is comprised of three separate studies. The objective of the first experiment was to identify whether Shewanella putrefaciens CN32 responded to metals in the growth environment by altering membrane phospholipid chemistry, and to identify these alterations in membrane chemistry during growth in the presence of Mn, V, or U. The first experiment assayed the fluid state of S. putrefaciens CN32 membranes after cultivation in the presence of Mn(II), V(IV), or U(VI). Experiments characterized fluorescent probe rotation in membrane hydrophobic regions, fatty acid saturation, and thermal resistance of whole cell membranes.

The objective of the second experiment was to characterize surface functional groups, to determine potential metal-binding sites within the cell wall. The second study assessed the metal reactive groups within strain CN32 cell envelopes and phospholipids when cultured in the same conditions as the first experiment. Functional groups were characterized using X-ray spectroscopy, and column and thin layer chromatography.

The objective of the third experiment was to identify the impacts of Ca, Cu, Mn, U, V, and Zn on the cell wall permeability of a range of bacteria common in metal-membrane studies. The third experiment examined the impacts of soluble Ca(II), Cu(II), Mn(II), U(VI), V(IV), and Zn(II) on the membrane permeability of S. putrefaciens CN32, Escherichia coli AB264, Pseudomonas aeruginosa PAO1, and Bacillus subtilis 168.
Hypotheses

Upon review of pertinent literature, the following research hypotheses were investigated:

1. Mn(II), V(IV), and U(VI) will reduce membrane fluidity, therefore having a stabilizing effect on cell membrane fluidity.
2. These metals will impact membrane fatty acid chemistry, in particular saturation.
3. Culturing in aerobic conditions will yield differences in phospholipid functional group chemistry in comparison to anaerobic culturing. These differences will impact surface-metal associations.
4. Metal associations with cell wall functional groups will increase membrane permeability, as a function of metal concentration.
5. Calcium(II), Cu(II), Mn(II), U(VI) (as UO$_2^{2+}$), V(IV) (as VO$_2^{2+}$), and Zn(II) will have similar permeabilizing effects on the bacterial cell walls. Saturated fatty acids will form tighter packing associations with adjacent lipids and decrease permeability.
6. Based on compiled literature, chemical variations in Gram-positive and Gram-negative bacterial envelopes are not expected to affect cell permeability.
CHAPTER 1

THE DYNAMIC NATURE OF THE BACTERIAL SURFACE: IMPLICATIONS FOR METAL-MEMBRANE INTERACTION

Submitted to FEMS Microbiology Reviews

CHAPTER ONE

The dynamic nature of the bacterial surface: Implications for metal-membrane interaction

1.1 Abstract

Bacterial surfaces are chemically complex and diverse structures. Lipids, proteins, and carbohydrates conform dynamically to the cellular microenvironment, where conditions can shift over extremely short time scales. Bacteria must rapidly compensate for changes in their environment to survive. Cell envelopes carry a plethora of ionogenic reactive groups on proteins, lipids, and carbohydrates. This influences electrochemical interactions and adhesion to surfaces, and assists in the organization of components within the cell envelope. In this literature review, I explore the chemistry of Gram-positive and Gram-negative bacterial surfaces in a context of metal-reactivity. I define surfaces here as the area of the cell envelope exposed to the aqueous environment, and interacting with soluble ions. Metal-binding sites on bacterial envelope components are identified from the outer membrane inward to the cytoplasmic membrane, and I discuss the physicochemical processes of metal binding to these surface ligands. This literature review also addresses some of the practical approaches to quantifying the thermodynamic values of the protolytic and adsorptive reactions within the cell wall. The practical approaches are supported by a review of the underlying theoretical molecular interactions of membrane reactive groups with exogenous ions. The metal binding capacity and sources of ligand variability are also discussed in detail.
1.2 Introduction

Bacterial cell envelopes are chemically diverse structures, which serve functions that are essential for cell survival. Membranes are semi-permeable and contain transport proteins to enable the passing of molecules and ions, including metals (Shabala et al. 2009; Reyes et al. 2009; Ruiz et al. 2009). They are also the sites for chemical reception (Weber and Silverman 1988; Gunsalus and Park 1994; Celani and Vergassola 2010; Hazelbauer and Lai), DNA replication and cell division (Mott and Berger 2007; Kaguni 2006; Cooper 1991), motility (Rajagopala et al. 2007; Kaiser 2000), and respiration (Richardson 2000; Shi et al. 2007; Myers and Myers 2001). At the most basic level, membranes sequester intracellular components from highly variable environments that include toxic metal species. On the other hand, the ionogenic constituents of bacterial envelopes provide reactive groups that readily interact with metals spanning the periodic table (Beveridge 1989; Beveridge 2005; Beveridge and Koval 1981). The extent of metal binding depends not only on the metal chemical species, but also on the environmental metal concentration, redox conditions, and pH. Cellular chemistry also strongly affects metal associations: cell wall polyelectrolyte composition, cell respiration (Urrutia Mera et al. 1992; Daughney et al. 2001), the excretion of extracellular material (Ferris et al. 1989a; Lamelas et al. 2006; Ueshima et al. 2008), or the synthesis of proteinaceous motility or respiratory appendages (Gorby et al. 2006; Ferris and Beveridge 1985; Lin et al. 2002). In the case of the latter, these external structures are not always observed in the laboratory. This can be due to equipment limitations, harsh physical or chemical treatments, or culture conditions. They are, however, often common in situ. As such, it is necessary to approach the characterization of metal-membrane interactions in a manner that accounts for the physiological complexity of the cell surface and its associated biological structures.
Bacteria must necessarily interact with metals. These cell-metal associations occur wherever bacteria are found: in higher organisms, in plant rhizospheres, soils, and in toxic waste sites. The ability of bacteria to respire metals, metalloids, or even radionuclides (Liu et al. 2002; Nealson and Saffarini 1994; Lloyd 2003; Lovely 1993; Myers and Nealson 1988b) may be exploited in the bioremediation of contaminated sites (Lloyd and Renshaw 2005; Lovley 1995; Lovley and Phillips 1992; Wang and Xiao 1995). This is achieved through selecting for bacterial processes that lead to metal immobilization. Metal reduction by bacteria does not always immobilize metals, however, and reduction can solubilize metals such as V(V) (Carpentier et al. 2003), Mn(IV) (DiChristina et al. 2002; Lovely 1991), and Fe(III) (Arnold et al. 1990; Jones et al. 1984). This is often undesirable, and can lead to costly corrosion damages (Little and Lee 2000; Little et al. 1998; Rao et al. 2000), and groundwater toxification (Boice et al. 2009a; Bouchard et al. 2010; Baker and Banfield 2003). Conversely, bacterial solubilization of metal can be exploited in ore extraction. In order to predict or manipulate these outcomes, it is essential to understand both the metal chemistry and microbiological processes thoroughly.

This literature review explores the metal-reactive groups at bacterial surfaces, and the processes involved in metal binding to these sites. Titration and spectroscopic experimental approaches that identify metal-reactive groups within bacterial cell walls are also critically reviewed. Bacteria respond to subtle changes in their environments. Understanding the structural variability of bacterial surfaces is, therefore, necessary to minimize artefact reporting in cell surface studies, and to predict more accurately how bacteria impact the dynamics of metals in natural systems.
1.3  **Cell membrane components of geomicrobiological importance**

Most cell membranes carry a net negative charge. The charge contributions of individual components, and the spatial configurations of these charged groups, play dominant roles in electrostatic interaction with metals. Bacterial cell wall chemistry varies across species, yet their membrane compositions at the base level consist of similar molecules. Both Gram-positive and Gram-negative cell wall structures have been reviewed in the past (Schleifer and Kandler 1972, 1973; Beveridge and Murray 1980; Beveridge and Matias 2000; Beveridge 1999; Graham et al. 1991). In this section individual biochemical structures are described, to highlight the metal-reactive sites and suggest geomicrobiological implications.

1.3.1  **Proteins**

Cell membranes contain variable amounts of protein. This depends not only on species, but also on the Gram classification. There are striking differences in total content (Hancock and Nikaido 1978) and functionality between those proteins in cytoplasmic compared to outer membranes of Gram-negative bacteria, as reviewed in past literature (DiRienzo et al. 1978; Nikaido 1996; Saier 2006). Outer membrane proteins are lower in quantity than cytoplasmic membrane proteins (about 35-60%) and are mainly porin channels and secretory proteins. Protein-rich (about 80% protein by weight) cytoplasmic membranes contain proteins necessary for functions that include signal transduction, transport, and respiration. This review defines membrane proteins as integral, peripheral, and transmembrane, and excludes more external motility or respiratory appendages, such as flagella, pili, or nanowires.
There are many membrane proteins known to have active sites specific for certain metals. Usually these are redox- or catalytically-active metals such as Fe (Mason and Cammack 1992), Mn (Wieghardt 1989), Cu (Buse et al. 1999), or Zn (Vallee and Auld 1990). Among these proteins are those involved in respiration and signal transduction. Metal specificities of protein ligands often follow the Irving-Williams series of $\text{Mn}^{2+} < \text{Fe}^{2+} < \text{Co}^{2+} < \text{Ni}^{2+} < \text{Cu}^{2+} > \text{Zn}^{2+}$ (1948); outlined and put into the context of metalloproteins in a thorough review by Waldron and Robinson (2009). It is important to note that metal binding to proteins is not limited to active sites. While histidine, methionine, cysteine, aspartic and glutamic acid residues often provide metal ligands in the active sites of metalloproteins (Fig. 1.1a), membrane proteins almost always display net anionic charges across their surfaces in circumneutral pH, with the MntR protein of *B. subtilis* as an example (Fig. 1.1b). Kotrba et al. (1999) inserted short metal binding peptides into integral *E. coli* outer membrane LamB proteins to assess the metal binding capacity, and found that surface display of putative metal binding peptides rich in anionic amino acids increased Cd$^{2+}$ binding substantially in vitro.

Cationic peptides have important roles in maintaining bacterial membrane integrity. Protein-lipid interactions may be limited by the presence of counterions or electrolytes in solution, which can dissociate acidic lipid clusters that are sequestered by cationic membrane proteins (Mbamala et al. 2005). Membrane lipids diffuse within the bilayer to minimize the amount of energy needed to maintain the overall structure. This lipid de-mixing impacts membrane stability by limiting reorganization; migrating lipids offset hydrophobic mismatch of bilayer thickness with hydrophobic domains of transmembrane proteins (Andersen and Koepe 2007). In addition, limiting electrostatic interactions with counterions or electrolytes in solution increases the
influence of van der Waals (induced dipole-induced dipole) and entropic (including hydrophobic and hydrogen bonding) forces in protein-lipid interaction (Israelachvili 2011).

Figure 1.1. Image of MntR protein 2F5D from the Protein Data Bank (Kliegman et al. 2006) from *Bacillus subtilis* rendered in PyMOL (Version 1.3, Schrödinger, LLC) displaying (a) ribbon structure with enlarged Mn$^{2+}$ ions, as well as amino acid ligands for Mn 1150 from chain A, also enlarged for clarity; and (b) MntR electrostatic potential map calculated using DelPhi (Nicholls and Honig 1991), which calculates based on a finite difference solution to the Poisson-Boltzmann equation, applied to a molecular surface rendered in GRASP2 (Petrey and Honig 2003). MntR represses the expression of *mntABCD* in the *mnt* operon, when Mn$^{2+}$ ions are bound within the active sites. The *mntABCD* genes encode MntABCD Mn(II) transport proteins.
1.3.2 Phospholipids

Phospholipids are major components of bacterial membranes, and possess phosphoryl and occasionally carboxyl groups in their hydrophilic regions that are available for metal cation binding at circumneutral pH. Bacterial phospholipid head group composition varies within a membrane, as well as across species. Typical bacterial membranes consist predominantly of phosphatidylethanolamine (PE), phosphatidylglycerol (PG), diphosphatidylglycerol (DPG; cardiolipin), and to lesser extents phosphatidylserine (PS) or lyso-variations of PE or PG (Seltmann and Holst 2002). Depending on the pH of the solution, the spatial orientation of the head groups may be altered. For example, in PS at low pH the protonated head group lies parallel with the membrane bilayer, while in circumneutral pH environments, the headgroup is deprotonated and resides perpendicular to the membrane (Sanson et al. 2002). It is not unreasonable to predict a similar effect in other lipids that are anionic at circumneutral pH, such as PG.

Inward headgroup folding may also be observed in higher ionic strength environments, subsequently masking charged groups. The C-C bonds of the head group freely rotate, determined using NMR and molecular dynamics simulations, and can alter the proximity of phosphate groups to adjacent molecules (Dufourc et al. 1992; Robinson et al. 1994). Cations often occupy the space between negatively charged phosphate groups; the space between the ligands is then the sum of \(2r_C + 4r_{H_2O}\) where \(r_C\) and \(r_{H_2O}\) are the radii of the cation and water molecules respectively (van Leeuwen and Buffle 2009). Each ion of Mg\(^{2+}\) and Ca\(^{2+}\), for example, is surrounded by a shell of six water molecules (Magini 1988). The uranyl ion (MW 270.03 g \cdot mol\(^{-1}\)) is composed of two axial oxygen atoms and coordinates with 5 equatorial waters.
(Tsushima and Suzuki 2000), 1 - 2 of which are replaced by phosphoryl, or carboxyl oxygen atoms. It is notable that uranyl adsorption to a phospholipid such as PS (MW ~385.30 g · mol⁻¹ depending on acyl chain chemistry) can drastically increase the overall weight of the lipid-metal complex. The molecular weight of UO₂²⁺ is 70% of the total weight of a PS molecule, while the binding of transition metals such as Mn²⁺, Fe²⁺, or VO²⁺ (MW 54.94, 55.85, 66.94 g · mol⁻¹ respectively) would increase PS weight by 14-17%. Conservation of momentum law dictates that lateral lipid diffusion rates are reduced under these metal-binding scenarios, especially in the case of a bidentate lipid-metal-lipid complex, and would also likely affect the permeability of the membrane. Lateral lipid diffusion occurs at a rate that is dependent on acyl chain saturation chemistry, however it typically ranges from 1.1-2.2 μm² · s⁻¹ (Nomura et al. 2008). In addition, if counterions are present on phosphoryl or carboxyl phospholipid metal-reactive sites, diffusion will be more heavily influenced by hydrophobic and van der Waals attractions between lipid hydrocarbon chains (Israelachvili 2011).
Figure 1.2. Phospholipid bilayer rendered in (a) gel, (b) fluid, and (c) disordered phase states. Depending on the phase of the bilayer, the metal-reactive functional groups within the hydrophilic region change proximity, and impact the metal binding capacity of the membrane. Coordinates for these phase states were obtained courtesy of Dr. Helmut Heller (Leibniz-Rechenzentrum, LRZ).
Rather than examining effects of metal binding on lipid structure and dynamics, an alternate approach is to identify what effect lipid fatty acid chemistry has on metal binding. Analogous to the head groups, there is constant rotation, albeit governed by intermolecular forces, at the C-C bonds of the hydrophobic fatty acid tails. Acyl chain hydrophobic interactions tend to keep the lipids packed tight (Seltmann and Holst 2002; Israelachvili 2011). Unsaturated or cyclopropyl fatty acids may, however, physically limit close proximity. The increased distances between adjacent phosphate groups in membranes with higher concentrations of these lipids (Fig. 1.2) may reduce metal-ligand (1:2) binding in metal complexes that would permit bidentate associations, and promote metal-ligand (1:1) binding. Fatty acids are known to become less saturated at lower temperatures, and assume more saturated compositions at higher temperatures, as reviewed in detail by Zhang and Rock (2008). It was reported by Huster et al. (2000) that an increase in unsaturated fatty acids results in a twofold decrease in Ca\(^{2+}\) binding to lipid membranes. This suggests that in terms of phospholipid-metal associations, higher temperatures may induce slightly higher quantities of metal binding due to higher saturated fatty acid content of membranes. Alternatively, the opposite may occur in colder environments, with membranes consisting of more unsaturated fatty acids reducing the degree of metal binding. It is important to note that while phospholipid acyl chain saturation chemistry may alter metal binding, most metal is bound within the brush of surface sugars.

1.3.3 Peptidoglycan and teichoic acids

Peptidoglycan structure and synthesis has been thoroughly reviewed (Vollmer et al. 2008; Vollmer and Bertsche 2008; Meroueh et al. 2006; Schleifer and Kandler 1972, 1973). Its ability to bind metals (Matthews et al. 1979; Hoyle and Beveridge 1984; Falla and Block 1993;
Beveridge and Murray (1980) is often overlooked in Gram-negative cells, amidst other negatively charged surface components. Amino acid chains cross-linking \( \beta-(1\rightarrow4) \)-linked \( N \)-acetylglucosamine (GlcNAc) and \( N \)-acetylmuramic acid (MurNAc) disaccharide repeats also have free carboxyl groups at the terminal residues. Bacterial species with peptidoglycan having \textit{meso}-diaminopimelic acid (m-DAP) linkages, such as \textit{E. coli} and most bacilli, often have D-glutamic acid residues amidst the peptide chains linking the disaccharides. Both glutamic acid and \textit{m}-DAP offer additional carboxyl groups, which are able to bind metals, such as demonstrated with \( \text{UO}_2^{2+} \) (Barkleit et al. 2009) and \( \text{Na}^+ \) and \( \text{Mn}^{2+} \) (Matthews et al. 1979) for \textit{Bacillus subtilis}, and \( \text{Ni}^{2+} \) and \( \text{Cu}^{2+} \) for \textit{Pseudomonas fluorescens} (Falla and Block 1993). This is consistent with the findings of Kelly et al. (2002) in which XAFS was used to investigate Gram-positive cells exposed to \( 83 \mu\text{M of } \text{UO}_2^{2+} \) at pH values of 1.67, 3.22, and 4.8. This study reported monodentate binding by phosphate groups and bidentate binding by carboxyl metal reactive groups (MRGs). In Gram-negative cells, metal-peptidoglycan interactions are reserved for metals that are capable of passing through outer membrane porin channels, as mentioned previously. This also limits the passage of metal precipitates into the periplasm. Chemotype I peptidoglycan structures are similar for both \textit{E. coli} and many \textit{Bacillus} species (Schleifer and Kandler 1972). Gram-positive peptidoglycan binds much more metal, presumably due to the higher amount of material present at the surface, including the covalently attached teichoic or teichuronic acids (Collins and Stotzky 1989).

Teichoic acids, with the exception of lipoteichoic acids, are covalently bound to the MurNAc acid molecules in Gram-positive peptidoglycan, and the structure has been well defined (Ward 1981; Archibald 1974). D-alanine residues offer \( \text{NH}_3^+ \) groups in addition to the glycerol or
riboitol phosphate groups, and hydroxyl groups, contributing to charge heterogeneity for the teichoic acids. Aside from D-alanine, compounds such as glucose or N-acetylamino sugars can be substituted on the same free hydroxyl group (Seltmann and Holst 2002). Past work by Beveridge et al. (1982) has demonstrated teichoic acids binding Na\(^+\), K\(^+\), Mg\(^{2+}\), Ca\(^{2+}\), Mn\(^{2+}\), Fe\(^{3+}\), and Ni\(^{2+}\) in *Bacillus licheniformis* cell walls. In addition, Doyle et al. (1980) observed Na\(^+\), Ca\(^{2+}\), Mn\(^{2+}\), Ni\(^{2+}\), Sr\(^{2+}\), Zn\(^{2+}\), and Mg\(^{2+}\) binding to *B. subtilis* 168 peptidoglycan and teichoic acids, and noted that the binding involved negative cooperativity.

Poly-(polyol phosphate) and poly-(glycosylpolyol phosphate) teichoic acids are the most well known, including both glycerol and ribitol as polyol components. Other teichoic acid classes present in Gram-positive bacteria are teichuronic acids and lipoteichoic acids. Teichuronic acids lack the phosphate found in the polyphosphate teichoic acids, but contain uronic acid. This has been speculated to sequester cations. The exact reason for the need to sequester metals, or for teichoic acids in general for that matter, is currently not well understood, but it is known to affect the structural integrity and morphology of cells (Bhavsar et al. 2004). In environments with high concentrations of soluble and potentially toxic metals, additional metal-reactive groups (MRGs) within the cell wall may serve to further detoxify the bacterial microenvironment (Azenha et al. 1995).

### 1.3.4 Lipopolysaccharides

Lipopolysaccharides are often the first line of defense for Gram-negative bacteria in protection against antagonisms in their microenvironments. Lipopolysaccharide chemistries vary across species (Heinrichs et al. 1998; Darveau 1998; Seltmann and Holst 2002). They can be generally
classified as rough or smooth depending on their O-side chain presence or absence; S-type and R-type respectively. LPS molecules consist of glucosamine pairs joined via $\beta(1\rightarrow6)$ linkages, with multiple fatty acid tails in amide and ester linkages to each sugar (comprising the lipid A molecule), as well as a core oligosaccharide region. Lipid A molecules are flanked by phosphate groups at the terminal hydrophilic ends of the disaccharide structure, and are known to be stabilized by cations, typically believed to be $\text{Ca}^{2+}$ or $\text{Mg}^{2+}$ (Hancock 1984; Seltmann and Holst 2002). Outer-leaflets of Gram-negative outer membranes consist mainly of lipid A, a 2-keto-3-deoxyoctonate (KDO)-containing core region and O-side chains. The phosphate MRGs of this region contain two free oxygen atoms (Huijbregts et al. 2000) available to interact with solute ions; the third oxygen forms a double bond with the phosphorous atom and is neutrally charged. Depending on ligand availability in polyvalent metal-bridging scenarios, association with nearby carboxyl or hydroxyl groups on sugars such as KDO in the LPS core region is possible. It has been suggested that internal cross-linking of carboxyl groups and amino groups in core molecules may occur, effectively limiting the available anionic metal-reactive groups in the core region (Ferris 1989; Ferris and Beveridge 1986b). Gram-negative LPS will adsorb uranyl ions to two phosphoryl groups per metal at pH values above 6 (Barkleit et al. 2008). This shifts to a 1:1 ratio at pH values less than 4 because the ion competes with protons for MRG sites, as determined using time-resolved laser-induced fluorescence spectroscopy (Barkleit et al. 2008).

S-type LPS consists of the same basic core structure as R-type LPS, but possesses O-specific polysaccharide (O-antigen) repeats. O-specific polysaccharides consist of 20-40 repeating units of 2-8 monosaccharides, and when present, comprise the largest section of the LPS molecule. The geometry of the glycan polymer is rarely fully perpendicular to the membrane plane in a
linear chain. Despite proximity to one another, however, it has been noted that solution metal concentrations of about 5 mM (depending on the metal) may be necessary for cross-linking O-specific side chains (Nguyen et al. 2003). Spatial occupancies of O-polysaccharide chains tend to vary based on glycosidic bond rotations and interactions with adjacent molecules and the microenvironment. Recent experimental and molecular dynamics simulation studies suggest that glycan repeats fold back and collapse toward the LPS core region when 50 mM Ca\(^{2+}\) is present (Schneck et al. 2009), and further suggest that this serves to decrease the permeability of cationic molecules. Langley and Beveridge (1999) observed consistent Cu\(^{2+}\) binding to core regions across varying O-side chain compositions in *Pseudomonas aeruginosa* PAO1 wildtype and PAO1 mutants, with differences in O-sidechain chemistries correlated with the additional binding and precipitation of metals such as iron and lanthanum.

### 1.4 Metal reactions in the region between the envelope and the bulk solution

The formation of covalent complexes between soluble ions and membrane MRGs occur according to the rules proposed by Pauling (1929). Ligand oxygen and sulfur atoms will orient about the cations as much as sterically possible in a geometric arrangement. The coordination number and the radius ratio predict this arrangement. There are rarely sufficient oxygen and sulfur atoms from proximal biogenic functional groups to occupy more than two positions in the coordinated arrangement, which will be discussed further. The remaining volume around the cation is filled with water molecules. This section will discuss the diffusion of soluble ions from bulk solution to inner-sphere coordination with a MRG in a bacterial envelope, and review techniques that characterize the functional groups on a bacterial surface. Surfaces are defined in this thesis as the area of a bacterial envelope where charged ligands are exposed to the aqueous
milieu. This conflicts with past definitions of the bacterial surface, but soluble ions will bind periplasm-exposed MRGs, and as such these reactive layers must be considered part of the surface area within an envelope.

1.4.1 The process of metal binding to metal-reactive groups

Metals bind to MRGs in a two-step reaction where a hydrated ion approaches the MRG, and one water molecule is shed and replaced with the oxygen of the binding ligand to form a specific bond (Sposito 2004). The latter step in this conception is the rate limiting step (Eigen 1963), resulting in the rate of inner-sphere adsorption being approximately equal to the rate of desolvation (Zhang and Buffle 2009). As a metal ion diffuses through the bulk solution, it may enter the cell wall, or it may be excluded and remain in the bulk solution (Levitz et al. 2006). Within the polyelectrolyte brush of the cell, the rate of ion accumulation from the bulk solution into outer-sphere coordination with MRGs is characterized by the differential equation:

$$\frac{d[(ML)]}{dt} = k_d^{in}[M][L] + k_d^{os}[(ML)] - k_d^{in}[(M.L)] - k_d^{in}[(ML)]$$ (1)

The rate that ions are bound into specific coordination with the membrane MRGs is described by the differential equation

$$\frac{d[(ML)]}{dt} = k_d^{in}[(M.L)] - k_d^{in}[ML]$$ (2)

where the $k$ values are the rate constants with subscripts $d$ indicating a desorption, $a$ indicating an adsorption, and superscripts $in$ referring to inner-sphere complexes and $os$ referring to outer-sphere complexes (Fig. 1.3)(Buffle et al. 2007). Formulae 1 and 2 include terms for the bulk ion molar concentration of the metal [M], the concentration of the MRG [L], non-specific
coordination of the fully hydrated ion with the MRG [(M.L)], and inner-sphere complexes composed of the metal bound to the MRG [ML].

Figure 1.3. Diagramatic representation of equation (2) in which the rate of outer-sphere coordination is the rate at which the fully hydrated metal diffuses into outer-sphere coordination with an arbitrary Gram-negative MRG, plus the rate that metals desorb from inner-sphere coordination with the MRG, minus the rate that outer-sphere coordinated metals diffuse back out to the bulk solution, and minus the rate that outer-sphere coordinated metals adsorb to the MRG.

1.4.2 Macroscopic experiments determining the protonation state of MRGs

The effects of pH and proton competition for MRGs are necessarily considered when evaluating metal-membrane interaction. The unitless interaction energy between an ion with charge $z_M$ and a number of MRGs with charge $z_i$ is a function of the electron charge ($e$) in C, the permittivity of the solution ($\varepsilon\varepsilon_0$) in J$^{-1}$Cm$^{-1}$, the Boltzmann constant ($k$) in JK$^{-1}$, the temperature ($T$) in K, the
center to center point of closest approach between the charged ion and the MRG ($a_i$) in m, and
the inverse Debye length ($\kappa$) in m$^{-1}$

$$U^\infty = \frac{Z_M e^2}{4\pi \varepsilon_0 kT} \sum_i \frac{Z_i}{a_i} \left(1 - \frac{\kappa a_i}{1 + \kappa a_i}\right)$$

(3)

In this, it can be seen that if the charge of the MRG ($z_i$) is neutralized by the binding of a proton
in the lower pH range, the interaction energy becomes zero (van Leeuwen et al. 2007). With
zero interaction energy, the rates of adsorption and desorption into and out of electrostatic
coordination are also zero (van Leeuwen and Buffle 2009). This can occur on the MRGs in
direct contact with the external environment at low pH, but this is also an important
consideration at the cytoplasmic membrane where proton flux occurs during cellular respiration
(Urrutia Mera et al. 1992).

Potentiometric titrations of viable whole cells or cell constituents have been used to determine
the values for the deprotonation constants of the range of MRGs (Table 1.1). The titrations
involve a series of preparatory steps that can include an EDTA rinse to remove membrane bound
cations (Cox et al. 1999; Fein et al. 1997; Fowle and Fein 2000). This procedure has, however,
been shown to strip exchangeable cations from the membrane surface (Haas 2004), release LPS
(Leive 1974), and promote autolysis (Leduc et al. 1982). As an alternate method, bacterial cells
have been prepared by rinsing cells with an acidic solution to remove cations present in the
growth medium from MRGs (Daughney et al. 2001; Fein et al. 2001). Using the LIVE/DEAD®
Bacterial Viability Kit (Invitrogen) cells have been shown to remain viable at the low pH of the
rinse (~pH 1 – pH 2) (Sokolov et al. 2001). On the other hand, Heinrich et al. (2007) have
shown low pH to produce previously undetected proton reactive sites that may have originated
from lysates.
Table 1. Compilation of protonation constants for whole Gram-positive and Gram-negative bacteria, with proposed functional groups and site densities, based on published literature.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Reference</th>
<th>pH Range</th>
<th>a</th>
<th>b</th>
<th>℃</th>
<th>pK1</th>
<th>pK2</th>
<th>pK3</th>
<th>pK4</th>
<th>pK5</th>
<th>pK6</th>
<th>pK7</th>
<th>pK8</th>
<th>pK9</th>
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</thead>
<tbody>
<tr>
<td>Streptococcus faecalis</td>
<td>Yee and Fein 2001</td>
<td>2.51 (0.3)</td>
<td>10.23 (0.40)</td>
<td>10.0 (0.01)</td>
<td>0.21 (0.01)</td>
<td>0.0317 (0.00074)</td>
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<tr>
<td>Staphylococcus aureus</td>
<td>Haas et al. 2001</td>
<td>2.51 (0.3)</td>
<td>10.23 (0.40)</td>
<td>10.0 (0.01)</td>
<td>0.21 (0.01)</td>
<td>0.0317 (0.00074)</td>
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<tr>
<td>Sporosarcina ureae</td>
<td>Haas et al. 2001</td>
<td>2.51 (0.3)</td>
<td>10.23 (0.40)</td>
<td>10.0 (0.01)</td>
<td>0.21 (0.01)</td>
<td>0.0317 (0.00074)</td>
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<tr>
<td>Bacillus subtilis</td>
<td>Cox et al. 1999</td>
<td>2.51 (0.3)</td>
<td>10.23 (0.40)</td>
<td>10.0 (0.01)</td>
<td>0.21 (0.01)</td>
<td>0.0317 (0.00074)</td>
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<tr>
<td>Bacillus megaterium (sporulated + stationary)</td>
<td>Cox et al. 1999</td>
<td>2.51 (0.3)</td>
<td>10.23 (0.40)</td>
<td>10.0 (0.01)</td>
<td>0.21 (0.01)</td>
<td>0.0317 (0.00074)</td>
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1.4.3 Determination of the proton binding constants from titration data

Stability constants for proton binding within bacterial cell walls are often identified using potentiometric titrations. This process has been reviewed, and well critiqued, by Borrok et al. (2005). Cell suspensions are either titrated from an alkaline to acidic pH, or are back titrated from acidic to basic. This requires an initial addition of acid or base. The (de)protonation constants are calculated by balancing the electroneutrality equation:

\[
[B^+] + [H^+] + [\equiv \text{NH}_3^+] = [\equiv \text{PO}_4^2^-] + [\equiv \text{COO}^-] + [A^-]
\]

(4)

where \([B^+]\) is the cationic component of the added base and \([A^-]\) is the anionic component of the added acid; the two components of the inert electrolyte cancel out (Stumm and Morgan 1996). The symbol \([\equiv X]\) represents membrane bound MRGs. Other functional groups have also been proposed including phosphodiester \([\equiv \text{PO}_4^2\equiv]\) (Sokolov et al. 2001; Cox et al. 1999; Borrok et al. 2005; Ueshima et al. 2008) and hydroxyl \([\equiv \text{OH}]\) (Yee and Fein 2001; Cox et al. 1999; Sokolov et al. 2001; Ngwenya et al. 2003; Guine et al. 2006; Barkleit et al. 2008). The net neutrality can be related to the protonation or deprotonation constants in several forms. Sokolov et al. (2001) and Cox et al. (1999) have used a linear programming approach by iterating the \(K_a\) values in the form:

\[
C_{b_i} - C_{a_i} + [H^+]_{i} - [OH^-]_{i} = \sum_{i=1}^{n} \left( \frac{K_{a_i} L_{T_i}}{K_{a_i} + [H^+]} \right) + S
\]

(5)

where \(S\) is the original protonation state of the cell, \(L_T\) is the total concentration of the \(i^{th}\) MRG, and \(C_{a_i}\) and \(C_{b_i}\) are concentrations of acid and base for the \(i^{th}\) titrant addition respectively. In the electroneutrality equation (4), the negatively charged MRGs can be represented by:

\[
[\equiv X^-] = S_d Q_{\text{max}} X (1 - \theta_X)
\]

(6)
and amines by:

\[
[\equiv \text{NH}_3^+] = S_d Q_{\text{max}N} (\theta_N)
\]  \hspace{1cm} (7)

where \(S_d\) represents the suspension density of the cells in g \cdot L^{-1} of electrolyte, \(Q_{\text{max}X}\) is the total number of protonated and deprotonated MRGs, and the term \(\theta_X\) is a value between and including 0 and 1. For reactions involving only protons, \(\theta_X\) is described with a Langmuir-Freundlich equation (Plette et al. 1995):

\[
\theta_X = \frac{(K_X[H^+])^m_X}{1 + (K_X[H^+])^m_X}
\]  \hspace{1cm} (8)

In all but the linear-programming methods, non-linear regression is required to determine the critical parameters including the \(K_X\) values. Like the linear-programming technique, non-linear regression is an iterative tool that minimizes the sums of squared errors (Ritz and Streibig 2008).

A closer study of these values across the literature indicates that there are three predominant peaks in the distribution of values (Fig. 1.4). Two of these peaks are within the chemically and biologically relevant range of pH 4 – pH 9.

![Figure 1.4. Density plot of published log K values, relating the protonation of Gram-positive and Gram-negative MRGs with the actual values circled along the bottom.](image)
1.4.4 The metal binding capacity of MRGs

Several models are available for evaluating metal adsorption as a function of the electrostatic potential, including the constant capacitance model (CCM), the diffuse layer model (DLM), the triple layer model (TLM), and the NICA-Donnan model. Readers interested in a thorough review of the CCM, DLM, and TLM are directed to the review by Goldberg and Criscenti (2008). Linear programming does not include an electrostatic parameter. It has been used to determine binding capacities of an anion (I⁻) by bacterial cells (MacLean et al. 2004), but will not be further discussed in this review. On the other hand, the CCM has been used extensively. For the purpose of fitting the CCM to titration data, Haas and Northup (2004) used a capacitance (C) of 1.0 Fm⁻² for the Gram-negative bacterium S. putrefaciens based on the relationship relating capacitance to the Born solvation coefficient of the electrolyte (ω_ML) in cal · mol⁻¹ and the aqueous effective radius (r_e,ML) in Å (Sahai and Sverjensky 1997):

\[
C = 5.929 - 32.928 \left( \frac{1}{\omega_{ML} r_{e,ML}} \right)
\]  

(9)

Using curve-fitting software, Fein et al. (1997) determined that the capacitance (C) relating membrane charge of B. subtilis to surface potential was 8.0 Fm⁻², whereas that value calculated from (9) should be 1.2 Fm⁻². Because bacterial membranes behave as polyelectrolyte brushes (Gaboriaud et al. 2005), it is preferable to assess the electrostatic potential between membranes and the bulk ions using a Donnan model (Yee et al. 2004). This model suggests that at low ionic strengths, there is a strong potential between the charges in the membrane and the bulk solution, attracting counterions from the bulk solution and shifting the effective binding coefficients (Lyklema et al. 2005). The inverse is also true in which high electrolyte salt concentrations screen effective charge potential between the cell and the bulk solution. This creates a plateau in the magnitude of counterion attraction, despite increasing charge density (Dague et al. 2006).
Metal binding capacities of bacterial cell wall MRGs are determined by treating suspensions of cells with metal solutions across a sweep of pH values (Daughney et al. 2001; Fowle and Fein 1999; Fowle et al. 2000; Kulczycki et al. 2005; MacLean et al. 2004). Spectroscopy studies may be used to support batch experiments. In studying the adsorption of uranyl ions (UO$_2^{2+}$) to LPS from *Pseudomonas aeruginosa* S10, Barkleit et al. (2008) showed that the metal adsorbs to the phosphate MRGs in mono and bidentate formations. On the other hand, Plette et al. (1996) determined coefficients for carboxylic groups of Gram-positive *Rhodococcus erythropolis* A177 cell wall extractions to be 0.55, 1, and 0.9 for Ca$^{2+}$, Cd$^{2+}$, and Zn$^{2+}$ respectively, and 1, 1.2, and 1 for phosphatic MRGs. These values refer to polydentate binding when less than 1, monodentate binding when equal to 1, and binding of several ions to a single MRG when greater than 1 (Koopal et al. 2005). These titration and spectroscopic techniques offer an excellent means of characterizing total-surface functional groups. This stands in sharp contrast to extraction and characterization of individual components using methods that extract, separate, and chemically characterize structures such as lipids, proteins, or carbohydrates.

1.5 **Sources of metal-reactive group variability in laboratory experiments**

The dynamic nature of the bacterial surface is due, in part, to constant fluctuation in the biochemical composition of the membrane. This also includes synthesis of extracellular polysaccharides and proteins. Alterations in membrane protein, LPS, capsular or extracellular polymeric substance (EPS) synthesis, motility or respiration appendages, or biofilm lifestyles all alter available MRGs. In the case of EPS, the environment typically regulates biosynthesis indirectly. This section deals with the occasionally subtle cellular responses to changes in
environment, and seeks to identify somewhat unpredictable or unforeseen sources of surface MRG variability in research.

1.5.1 Culture conditions

Cell walls are one of the first lines of defence that bacteria have against their environment. As such, the versatile nature of the bacterial envelope can adapt to numerous environmental stimuli, for protective as well as metabolic purposes. In laboratory flask cultures, the age of the culture plays a dominant role in defining membrane lipid chemistry, as reviewed by Denich et al. (2003). It is inevitable that culture age impacts metal binding, given the high MRG content of bacterial cell envelopes. Guiné et al. (2007) reported that culture growth phase, phosphate concentrations, as well as viability, affect the acid-base properties and Cd and Zn reactivity of Gram-negative Cupriavidus metallidurans CH34 cells. Growth stage-dependent changes in Cd-binding were also observed in Gram-positive Bacillus subtilis cells (Daughney et al. 2001). This is likely a function of exhausted phosphate in the growth medium over time. It has been observed that less phosphate in medium results in decreased phosphoryl-dense teichoic acids, and increases in carboxyl-sparse teichuronic acids, in the B. subtilis cell wall (Lang et al. 1982). In contrast, Jiang et al. (2004) examined several pseudomonads and bacilli at different growth stages using infrared spectroscopy, and overall metal sorption and metal-binding functional groups were not greatly affected by the age of the culture. In this study, only two medium types (trypticase soy broth and Luria-Bertani broth) were used, and both are high in nutrients and not chemically defined. The duration and storage conditions of the cells while adhered to attenuated total reflectance (ATR) crystal surfaces may have had an impact, as cellular response to new
environments such as osmotic stress or anaerobiosis can occur in a matter of minutes (Golding et al. 2005; Weber et al. 2006).

Erlenmeyer flasks typically used in culturing bacteria limit the oxygen available for respiration in a manner that allows greater O$_2$ availability at higher shaking revolutions and lower culture volume-flask volume ratios (Efthymiou and Vieit 1979; Wise 1951). In addition, higher ionic strength media solutions can affect mass transfer of oxygen in aerobic media (Zieminski and Whittemore 1971). This suggests that additional considerations have to be taken when culturing facultative anaerobes. The presence of electron acceptors such as nitrate in growth medium can force anaerobic growth if cultures are not shaken sufficiently, or oxygen is limited from decreased solution-air surface area. Anaerobic respiration pathways, such as the nitrate reduction pathway, require synthesis of specific surface and periplasmic proteins (González et al. 2006; Moreno-Vivian et al. 1999; Stewart 1988).

1.5.2 Cell preparations and sample damage

Potentiometric titration methods requiring an inert background electrolyte commonly utilize salts such as NaNO$_3$ (Cox et al. 1999), KNO$_3$ (Sokolov et al. 2001), NaClO$_4$ (Ueshima et al. 2008), and NaCl (Haas et al. 2001). The ability of many anaerobes and facultative anaerobes to respire NO$_3^-$ can change the ionic strength of the electrolyte. In the case of titration studies, nitrate respiration, or respiration protein biosynthesis, may occur throughout the duration of the experiment. In titration vessels, cells are weighed and added to starting pH-adjusted electrolyte in order to estimate ligand concentrations per unit weight. Functional group concentrations estimated by Haas et al. (2001) appear to be an order of magnitude smaller than those determined.
by other groups. The values determined by Haas are, however, in terms of wet mass whereas other groups use dry cell conversions for their densities (Table 1.1). In selecting an electrolyte, it is necessary to consider that microbes may respire solution components; the electrolyte may also impact cell surface integrity. Peripheral proteins, in particular, can be removed readily with simple washing using low salt buffers (Zhao et al. 2004; Archer et al. 1978). Counterions present in electrolyte solutions reduce the overall negative charge of the cell wall, which lessens the electrostatic interactions between surface-displayed ionic amino acid residues in proteins, and phospholipid head groups. This contributes to the release of peripheral proteins.

Bacteria have a multitude of systems to adapt to acidic environmental conditions, which act to change the electrochemical nature of the membrane. Cells produce amino-acid decarboxylases at acidic pH resulting in production of terminal carboxyl MRGs and a buffering of environmental cations (Bearson et al. 1997; Gale and Epps 1942). Other mechanisms of pH tolerance in bacteria have been well reviewed (Booth 1985; Cotter and Hill 2003), and include H\(^+\) efflux pumps and monovalent cation/H\(^+\) antiporters (Padan et al. 2001; Hunte et al. 2005), and lipid fatty acid modifications that are dependent on species (Mykytczuk et al. 2010; Brown et al. 1997; Chang and Cronan 1999). In an examination of twenty-one acid tolerant bacteria, Svensäter et al. (1997) characterized a median killing pH of 3.0 (pH 2.3 – 4.5) for both Gram-positive and Gram-negative cells. Cell lysis in these situations releases highly charged nucleic acids and ribosomes, and exposes the inner leaflet of the CM, adding many additional anionic reactive groups. This illustrates the importance of determining structural integrity of cells at low-pH starting points in titration experiments (Fig. 1.5), as well as in metal removal washes of cell pellets.
Exposure to oxygen is often inevitable, even in anaerobic bacteriology. Working under anoxia in a glovebox is useful for culturing or preparations, but maintaining that anoxic state prior to analysis is not always possible. Bacteria are able to respond to oxygen presence rapidly, exporting quantifiable membrane proteins within 10 minutes (Weber et al. 2006). Cell surface analyses such as potentiometric titrations, or membrane characterizations such as protein profiling, may report artefacts if anoxia is not fully maintained.

1.5.3 Extracellular structures

Bacteria in natural environments commonly adopt a biofilm, rather than planktonic, lifestyle. Biofilm polysaccharide matrices typically consist of up to 97% water, 1-2% polysaccharides, 2-
5% cellular biomass, and trace amounts of intracellular material arising from lysates (Sutherland 2001b). Polysaccharides are the major structural component of biofilms, and due to their often polyanionic nature, may be rendered more rigid by multivalent cations within the biofilm matrix (Sutherland 2001a). Major anionic metal-binding sites on these sugars include carboxyl and hydroxyl groups on uronic acids (D-glucuronic, D-galacturonic, D-mannuronic); at circumneutral pH hydroxyl groups are, however, protonated. Baker et al. (2010) have proposed phosphoryl contributions to the EPS, but ethanol precipitation of EPS also precipitates DNA, RNA, and proteins that remain in the supernatant from culture lysates. These can appear as artefacts in potentiometric titrations, and in the case of nucleic acids, can give false positives for phosphoryl groups. Toner et al. (2005b) examined the effect of Zn sorption to *Pseudomonas putida* biofilms using EXAFS spectroscopy, and observed that while 20% of the adsorbed metal was bound to carboxyl groups and 80% was bound to phosphoryl ligands, the latter was likely due to cell surface MRGs. Tourney et al. (2009) characterized Zn adsorption to EPS-producing *Bacillus licheniformis* S-86 suspension MRGs, and found that adsorption occurred mainly at carboxyl and phosphoryl functional groups for both the planktonic cells and EPS-cell clusters, with the carboxyl functional groups dominating. The presence of EPS had little effect on the total zinc bound, and the phosphoryl and phosphodiester contributions emphasize the contributions of surface lipids and teichoic acids on metal binding in Gram-positive bacteria. In laboratory culture experiments, biofilms can form at the liquid-air interface on the sides of flasks, or on the bottoms of stationary anaerobic serum bottles, even with shaking. While the chemical composition of the growth medium will affect the physical biofilm structure (Stoodley et al. 1998), it is important to note that EPS biosynthesis can occur when the stationary growth phase is reached (Davies et al. 1993; Petry et al. 2000).
Figure 1.6. Protein surfaces computed in the same manner as Figure 1.1, showing (a) PDB protein 2ZBI flagellin (Maruyama et al. 2008); and (b) PDB protein 3PHS pilin (Krishnan et al. 2007) electrostatic surface maps. Flagellin is strongly anionic across its surface, and repeats of this protein suggest that flagella will serve as effect metal-binding templates. Pilin is much more zwitterionic, and is less likely to associate with metal cations.
Late exponential phase and early stationary phase also tend to induce the synthesis and assembly of flagella; biosynthesis occurs as growth medium becomes nutrient deficient, with *flhB* operon expression (Amsler et al. 1993). Flagella have been shown to associate with mineral clusters in *Shewanella putrefaciens* CN32 cultures (Glasauer et al. 2001), but dedicated studies of the ability of unmodified flagella or pili to bind metals, to my knowledge, have yet to be performed. Crystal structures of flagellin and pilin have been identified, and calculating the electrostatic surface of these proteins (Fig. 1.6) demonstrates that highly anionic flagellin repeats should bind metals, while more zwitterionic pilin repeats are likely to have relatively lower metal-binding capacity. This suggests that exposed anionic residues on flagellin polymers may provide additional metal reactive sites in nutrient- or electron acceptor-limited situations.

### 1.6 Environmental implications

The ubiquity of metals, both required and toxic, in the environment presents obstacles that microorganisms are able to overcome in order to survive. In areas with high concentrations of respirable metals, soluble products of dissimilatory metal reduction (DMR) may become a toxicological concern; the roles that microorganisms play in environmental metal cycling inevitably have human impacts, and have been well documented (Nordberg et al. 2007). Bacteria are known to reduce metals such as Mn (Myers and Nealson 1988a; Myers and Myers 2001; Nealson et al. 1989) and V (Carpentier et al. 2003; Myers et al. 2004; Ortiz-Benrad et al. 2004) to more soluble forms over physiological pH ranges, increasing their mobility in aqueous systems. The fates of soluble products of DMR between the time of reduction and diffusion into the bulk solution are not well defined. It is, however, assumed that they follow the same binding constraints outlined previously, and saturate cell wall MRGs. Studies probing the
physicochemical effects of soluble metal adsorption to cell walls will shed light on how cells are stressed by metals in their environments, and offer insight on how to exploit these changes as a means of biocontrol. This also holds true for bacteria used to bioremediate metals. Metals such as U(VI) are stable as relatively soluble UO$_2^{2+}$ complexes under many environmental conditions. While several reports indicate that U-contaminated drinking water of around 0.7 Bq/L may not induce organ cancer in humans (Kurttio et al. 2006b; Boice et al. 2009b; Auvinen et al. 2005; Kurttio et al. 2006a; Boice et al. 2009a), there have been conflicting reports from animal studies (Brugge et al. 2005; Domingo 2001). Bacterial uranium respiration reduces U(VI) to U(IV), which is rendered less mobile due to its relative insolubility; this process may be used to remediate toxic U in subsurface environments by DMR bacteria (Anderson et al. 2003; Lloyd 2003; Lloyd and Renshaw 2005; Lovley 1995; Lovley and Phillips 1992). While DMR bacteria respire U(VI), growth, division, and intracellular functions likely occur unhindered. When metals bind bacterial surface functional groups, this does not affect the export of newly synthesized molecules to the membrane, or intracellular protein activity. In fact, bacteria such as *S. putrefaciens* CN32 that are coated with metals or mineral precipitates (Fig. 1.7) are still able to grow and proliferate. This underlies the need to understand how membrane dynamics and MRG turnover affect metal-membrane interactions *in situ.*
Figure 1.7. Unstained representative TEM micrograph of a dividing *Shewanella putrefaciens* CN32 cell, harvested from trace element growth medium that was amended with V(IV). Despite being coated with soluble metal, and precipitates, cell division still occurs.

Although metal toxicity to bacterial cells varies with species, a useful compilation has been recorded mainly for *E. coli* by Nies (2007). It is important to note that while many metals are known to have inhibitory effects on cell functions, toxicity concentrations compiled by Nies do not differentiate between interferences with intracellular functions, and threats to membrane integrity causing lysis. Counterions and electrolytes are known to alter membrane organization in bacteria (Israelachvili 2011). Reducing electrostatic interactions between membrane
components causes the environmental composition to play a much stronger role in determining cell survival. In this way, metal counterions may induce lysis in a similar manner as H\(^+\) ion excess. In natural environments, however, planktonic cells would be much more at risk than those existing within a biofilm. Biofilm growth is preferred in many environments that are sub-optimal for bacterial survival, with the biofilm matrix providing an effective buffer from predators, toxic materials, and pH stresses (Costerton et al. 1987). While biofilm matrices are mainly composed of water as previously mentioned, the rigidity of the polysaccharide structure is influenced by available multivalent cations (Decho 1999; Hernández et al. 2010; Sutherland 2001b). Metal cross-linking of matrix MRGs can turn the biofilm into a more rigid gel and increase its chemical resistance, which poses an obstacle in areas where biofilms can cause structural damages, such as pipelines or sewer drains.

1.7 Summary

The variability of MRGs across genera, species, and even strains of bacteria, can be extremely high, and strongly dependent on growth phase and growth environment. This makes general cross-species comparisons of surface chemistry extremely challenging. Despite the complexity of bacterial surfaces, the underpinning systems must obey the rules postulated by Pauling (1929) for crystal coordination, where sterically possible. Bacteria have high surface area-to-volume ratios, and an abundance of reactive ligands available to bind metals. Cells do not, however, simply behave as templates for metal binding. Cell membranes respond to environmental stimuli by redistributing lipid components and optimizing the interbilayer forces. This usually occurs through lateral diffusion, \textit{de novo} biosynthesis, or enzymatic activity. Metal counterion presence on these MRGs alters chemical structures and dynamics of membrane components, and may
force the membrane into energetically unfavourable configurations. Compromising the integrity of bacterial membranes in this manner implies that metals could mediate antibiotic activity, or be used in chemotherapeutic research.

Bacteria are living organisms, but all regulation and response is based on physicochemical organization. Within a bacterium, physical influences impact biogenic structures and organelles the same as they would impact any other chemical structure in the universe. The possibility exists for underlying quantum control at the nanoscale and sub-nanoscale level, as reviewed by Trevors and Masson (2011). Cellular responses to stimuli can be, therefore, considered Boolean; there are very few exceptions where activations or repressions of cellular controls are not controlled by yes or no, true or false, on or off logic. As such, the cascades of organized events working simultaneously and leading up to processes such as cell division or chemotaxis are astounding. Predicting bacterial reactions to metals in situ relies on understanding that unconscious responses of bacteria to stimuli occur simultaneously, across the entire organism, within molecular timeframes (Trevors 2010). Overall responses represent the end product of a multitude of interactions, which optimize survival based on billions of years of evolutionary trial and error.
CHAPTER 2

Changes in *Shewanella putrefaciens* CN32 membrane lipid chemistry and fluidity in the presence of soluble Mn(II), V(IV), and U(VI)

Submitted to the Geomicrobiology Journal

French, S., J.T. Trevors, S. Glasauer. 2011. Changes in *Shewanella putrefaciens* CN32 membrane lipid chemistry and fluidity in the presence of soluble Mn(II), V(IV), and U(VI). Geomicrobiol. J. *Accepted.*
CHAPTER TWO
Changes in *Shewanella putrefaciens* CN32 membrane lipid chemistry and fluidity in the presence of soluble Mn(II), V(IV), and U(VI)

2.1 Abstract
In natural reducing environments, such as anoxic sediments and soils, bacteria that respire metals may be exposed to high concentrations of soluble transition metals. The aim of this study was to identify physiological and biochemical adaptations of *Shewanella putrefaciens* CN32 membranes to soluble Mn(II), V(IV), and U(VI). Responses of CN32 to these metals were assessed, in aerobic and anaerobic cultures, by means of membrane fluidity and fatty acid composition assays. During aerobic growth, all metals had a stabilizing effect on fluidity, while under anoxic conditions this was only observed for bacteria treated with U(VI). Membrane gel-to-fluid phase transition temperatures were higher under anaerobic conditions and were not affected by the metal treatments. Fatty acid desaturation demonstrated linear correlation with significant increases in membrane fluidity, despite metal treatments that did not significantly alter fatty acid chemistry. Scanning transmission X-ray microscopy and near-edge X-ray absorption fine structure spectroscopy at Mn 2p- and V 2p-edges revealed that both Mn(II) and V(IV) were associated non-specifically with CN32 membranes, with V(IV) associating as VO$^{2+}$ under anoxic conditions only.
2.2 Introduction

*Shewanella putrefaciens* CN32 is a Gram-negative bacterium that can chemically reduce a range of transition metals, metalloids, and radionuclides (Liu et al. 2002; Nealson and Saffarini 1994). This may have application to remediating metal contaminated subsurface environments (Borch et al. 2010; Anderson et al. 2003; Lovley and Phillips 1992). Metal sorption to bacteria is hypothesized to have critical consequences for survival due to the strong associations with reactive functional groups within the cell wall (Beveridge 1989). Past studies of CN32 have included genetic and protein analyses (Beliaev and Saffarini 1998; DiChristina and DeLong 1994; Wade and DiChristina 2000), surface and ultrastructure characterization (Korenevsky and Beveridge 2007; Gorby et al. 2008; Gorby et al. 2006; Fredrickson et al. 2003), and respiration (Glasauer et al. 2007; Pinchuk et al. 2008; Fredrickson et al. 2004). An outstanding knowledge gap is, however, the impact of soluble metals on cell envelope physicochemistry and lipid dynamics.

Bacterial membranes are complex structures that are the primary sites for cell respiration (Richardson 2000), and reception of environmental stimuli (Weber and Silverman 1988; Okusu et al. 1996; Gunsalus and Park 1994). Membrane components such as lipids and proteins interact through a variety of intermolecular forces (Grasso et al. 2002; Israelachvili 2011; Hermansson 1999), allowing lipid components to spatially reconfigure and diffuse laterally; average diffusion rates are around 2 µm²s⁻¹ (Nomura et al. 2008; Chan et al. 1991) as a result of van der Waals, electrostatic and entropic intermembrane forces. This allows for a necessarily fluid membrane, permitting lipids to diffuse and adapt to environmental changes, and also to conform to membrane proteins and lipids biosynthesized *de novo*. Prokaryotes alter membrane
protein profiles (Beliaev et al. 2005; Moore et al. 2005) and lipid chemistry in response to stress, including temperature, pressure, and organic solvents (Mykytczuk et al. 2007; Lopez et al. 2006; Cronan 2006; Denich et al. 2003). Mykytczuk et al. (2011) reported minor variations in Acidithiobacillus ferrooxidans strain lipid acyl chain chemistry when exposed to Cu$^{2+}$ and Ni$^{2+}$, and variable changes in membrane fluidity. There are no reports, to my knowledge, that correlate variations in membrane fluidity with metal cation stimuli in the dissimilatory metal reducing bacterium S. putrefaciens CN32.

For this study, manganese, vanadium and uranium were chosen as the target metals based on environmental relevance (Borch et al. 2010; Adriano 2001). These metals are also redox-active, and span a range of coordination chemistry. Manganese is ubiquitous in marine and terrestrial sediments. Its chemical lability under environmentally relevant redox conditions makes it of interest for the bioremediation of some toxic metals, including uranium (Fredrickson et al. 2002). Manganese has a minimum inhibitory concentration of around 20 mM for Escherichia coli growth (Nies 2007), although this can be expected to vary across bacterial species. Furthermore, Mn(II) is interchangeable with iron in oxygen tolerance systems such as metalloprotein catalysis of superoxide dismutation (Martin et al. 1986), and its necessity for cell growth and division is well documented (Jakubovics and Jenkinson 2001). It has been associated with gasoline containing the additive methylcyclopentadienyl manganese tricarbonyl (Zayed et al. 1999; Bolté et al. 2004), and is common in industrial waste or landfill sites (Šarić and Lucchini 2007). The USEPA does not consider Mn an element of concern, and does not stipulate a maximum primary contaminant level (USEPA 2003). The World Health Organization recommended level is 500 µg/L (WHO 2008), however, and a study by Bouchard et al (2010) found a significant decrease
in IQ for children in Quebec with chronic exposure to levels as low as around 200 µg/L. The biogeochemistry of this abundant element is, therefore, of interest.

Vanadium is a relatively abundant transition metal (similar to Zn in global concentration) with complex redox chemistry. It exists as species of V(III), V(IV) and V(V) over a range of pH-Eh conditions common in surface and subsurface environments (Evans and Barabash 2010). Bacteria typically tolerate concentrations of V(IV) in excess of 3 mM (Bell et al. 2004), despite the potential interference of V with enzymes such as protein kinases (Stankiewicz and Tracey 1995; Mukherjee et al. 2004). Vanadium is present in localized areas in high concentration, such as in groundwater associated with mining or smelting operations (Boice et al. 2009a), or near gas power plants (Juichang et al. 1995). It is a common element in coal and crude oil, and can be a contaminant in oil-impacted soils (Bell et al. 2004).

Uranium(IV) and U(VI) are the common chemical valences for U in surface and subsurface environments. Uranium(IV) is considered relatively insoluble compared to U(VI), although the solubility may be increased when complexed with particular organic and inorganic ligands (Langmuir 1978; Haas and Northup 2004). Bacteria can tolerate U(VI) concentrations of up to around 2 mM (Nies 2007); however, this value will depend on U speciation, solubility, and chemical complexation. Uranium(VI) complexes readily adsorb to bacterial surfaces (Barkleit et al. 2008, 2009; Haas et al. 2001). The mechanism of toxicity for bacteria exposed to U is not known, and may be due to compromised structural integrity or interference with membrane functions. Subsurface bioremediation of soluble U(VI) has been proposed as a long-term cleanup strategy at contaminated sites (Lovley and Phillips 1992; Wall and Krumholz 2006;
Anderson et al. 2003; Lloyd and Renshaw 2005). Understanding how U impacts subsurface bacteria is, therefore, important, as their ubiquity and metabolic versatility can determine U immobilization in these environments.

In environments with high concentrations of soluble metals, disruption of intermolecular attractive forces within the bacterial membrane may induce fluidization. To test this hypothesis, changes in cell membrane fluidity were characterized. Changes in phospholipid acyl chain chemistry induced by exposure to soluble Mn(II), V(IV) and U(VI), in the presence and in the absence of oxygen, were also characterized. The results provide insight into the stability of cell membranes under relevant environmental conditions, and have implications for biogeochemical phenomena that are postulated to occur at cell membranes, such as biomineralization. The research combines conventional spectrofluorometry and chromatography, as well as soft X-ray spectromicroscopy, to assess metal effects on the bacterial envelope.

2.3 Materials and Methods

2.3.1 Media and cell culture conditions

Stock cultures of *S. putrefaciens* CN32 were stored at -80°C, and grown from frozen stock on trypticase soy agar (TSA). Isolated colonies were grown in trypticase soy broth (TSB) to exponential phase (8 hours). Cells were harvested through centrifugation for 10 min at 6300 x g, washed in 5 mM sterile HEPES buffer (pH 6.8), pelleted and resuspended in buffer. The suspension was used to inoculate defined medium (DM) (Fredrickson et al. 1998), which was modified to incorporate 1 mM NaH$_2$PO$_4$ rather than 3.9 mM, and 20 mM lactate was used as the electron donor. Amino acid supplements were added as 20 µg/mL each of L-arginine, L-
glutamic acid, and L-serine, which were deoxygenated, filter-sterilized and incorporated after autoclaving the medium. The initial concentration of the DM was $\sim 5.0 \times 10^8$ cells $\cdot$ L$^{-1}$, determined using a standard curve comparing optical density (OD; 600 nm) of the cells in DM and spread plate dilution counts of colony-forming units. Bacteria were grown in DM until they reached the exponential growth phase (12 hours) before they were harvested and transferred to DM supplemented with either 1 mM VCl$_4$, 1 mM MnCl$_2$, or 1 mM UO$_2$(CH$_3$COO)$_2$ $\cdot$ 2H$_2$O, and again grown to mid-exponential growth phase (12, 12, 14, 15 hours for control, Mn(II), V(IV), and U(VI) respectively). Control cultures were prepared by growing the bacteria in the same DM devoid of metal supplementation.

Anaerobic cell cultures were prepared in an anaerobic chamber (Coy Laboratory Products, Grass Lake, Michigan, USA) with an average hydrogen content of 2.3%, and a balance of argon. The cultures were generated from aerobic DM cultures that were harvested at exponential phase, and then used to inoculate anaerobic DM with a final concentration of $\sim 5.0 \times 10^8$ cells/L of concentrated CN32 cells using the same procedure as for the aerobic treatments, but prepared using deoxygenated buffer in the glove box under H$_2$/Ar. Sodium fumarate (13 mM) was added as the terminal electron acceptor for anaerobic cultures. The media was deoxygenated with oxygen-scrubbed N$_2$ gas prior to inoculation. The same metal treatments were used for the oxic and anoxic media as well (exponential growth times of 26, 26, 30, 30 hours for control, Mn-, V-, and U-treated respectively). Cells that were cultured in the presence of U were examined as whole mounts using a Philips CM-10 transmission electron microscope (TEM; FEI, Hillsboro OR, USA) to check for potential U precipitates. All chemicals were of reagent grade or higher,
from Sigma-Aldrich (Oakville ON, CAN), and all glassware was acid washed overnight in 0.1 M HCl, then rinsed three times with Milli-Q nanopure water and oven-dried prior to use.

2.3.2 Membrane fluidity analysis

Membrane fluidity was examined through quantification of the fluorescence polarization of the membrane probe 1,6-diphenyl-1,3,5-hexatriene (DPH; excitation 358 nm, emission 428 nm), which was solubilized in tetrahydrofuran. DPH has absorption and emission transition dipoles aligning parallel to the probe hydrocarbon chains, which assume parallel alignment with FA chains when stabilized in membranes (Fig. 2.1). Higher saturated or trans-acting FA induce more probe stabilization and higher fluorescence polarization values; branched, cyclopropyl, unsaturated, and cis-acting FA result in less probe stabilization.

Bacteria were resuspended to an OD<sub>600</sub> of 0.20 in 5 mM HEPES (pH 6.8), and 3 mL of the suspension was placed in a methacrylate cuvette. One microlitre of DPH solution was added in the dark, to a final concentration of 4.3 µM, and then allowed to incorporate for 10-15 minutes. Anaerobic samples were prepared in the glove box; a small square of parafilm was placed over the mouth of the cuvette, with a cuvette stopper inserted on the top. The capped cuvette was placed into a spectrofluorometer (Photon Technology International, Birmingham, NJ, USA) equipped with a temperature controller and electronic stirrer. The sterile temperature probe was inserted through a small hole in the cuvette cap, piercing the parafilm. A small amount of silicon seal was placed around the hole to minimize sample oxygenation before the temperature probe
penetrated the parafilm, and after it was pushed into the cuvette cap. The temperature probe that was in contact with the culture inside the cuvette did not contact the silicon gel.

An initial emission scan was run in order to ensure an emission peak at 428 nm after excitation at 358 nm. After obtaining this peak, fluorescence counts were collected through time-based scans of 15 seconds at 25°C, with data collection every 1 second and perpendicular excitation. The same scan was then repeated with parallel excitation. The data gathered over 15 seconds were averaged and used in the fluorescence polarization equation of Shinitzky and Barenholz (Shinitzky and Barenholz 1978). The results were background-subtracted, taking into account both cell-free probe emissions, and probe-free cell emissions. The final calculated results were subjected to an ANOVA, followed by Dunnett and Bonferroni post tests (P < 0.05 was considered statistically significant).
Figure 2.1. DPH molecule embedded between two saturated phospholipid molecules. DPH fluoresces strongly when embedded in this orientation, but fluorescence is negligible when in solution.

2.3.3 Membrane phase transition temperature determination

After membrane fluidity analyses were completed, the temperature of the same bacterial suspensions was rapidly lowered to 15°C and a temperature ramp was run to a maximum temperature of ~65°C. The excitation polarizer was set to the vertical direction and the emission detector to horizontal, using the same calculated correction (G) factor as used in the membrane fluidity experiments. The melt curve was run at a rate of 5°C/min. This rate was chosen to minimize cellular modifications of the membrane. Data acquisition took place every second for
the duration of the ramp. The emission counts were used to calculate the fluorescence polarization in the same manner as for the membrane fluidity experiments. Rather than taking the average over 15 seconds, however, the value was calculated at each data point and plotted against temperature. The resulting data points for each sample were fit to 4-parameter logistic curves:

\[
y = \min + \frac{(\max - \min)}{1 + \left(\frac{x}{\frac{T_m}{\text{Hillslope}}}\right)^{-\text{Hillslope}}}
\]

with a maximum of 200 iterations using SigmaPlot (Systat Software Inc.). The phase transition temperature (T\text{m}) was calculated from the midpoint of the linear slope (Fig. 2.2). Maximum and minimum fluorescence polarization values, and the magnitude of difference between the maximum and minimum (ΔFP) are recorded for each curve.

### 2.3.4 Fatty acid compositional analyses

Fatty acid (FA) extraction was performed following a previously established method (Sasser 2001), with minor modifications. Bacteria grown under conditions identical to those for the membrane fluidity analyses were used to examine the chemical structure of the FAs in CN32. After the bacteria were treated with metals and harvested, wire loops of bacteria were transferred aseptically from cell pellets to sterile screw-top test tubes corresponding to each treatment, in triplicate. The bacteria were saponified with 1 mL of 30% NaOH in methanol-water (1:1 v/v), vortexed, and placed in a heating block for 5 mins at 100°C. The vortexing and heating cycle was repeated, and the samples were cooled for 30 minutes. Two millilitres of 6.0 N HCl-methanol (13:11 v/v) was added to the tubes, followed by heating for 10 minutes at 80°C in a heating block, then cooling in a cold water bath.
Figure 2.2. Typical membrane melting curve, comparing fluorescence polarization of DPH in CN32 membranes to temperature, as the membrane enters a more disordered state. The curve corresponds to a four-parameter logistic curve, where $T_m$ is the temperature where the membrane is more than halfway in a fluid state than a gel state. The pre- and post-transition values are relatively linear. Conclusions regarding thermal stability can be derived from both the difference between these values ($\Delta FP$), as well as the more disordered post-transition fluorescence polarization values.

FAs were extracted into the upper organic phase with the addition of hexane-methyl tert-butyl ether (1:1 v/v), and were gently agitated for 10 minutes. The lower phase was discarded, and the samples washed with 3 mL of 0.3 M NaOH in preparation for GC. Previously established GC-FID conditions were used (Sasser 2001). Sherlock software (Microbial Identification Inc., Newark DE, USA) was utilized to identify peaks and to test that FA profiles demonstrated homology towards *S. putrefaciens*.

### 2.3.5 Cell envelope preparation and purification

After being cultured in the presence of metals, CN32 cells were harvested at their respective exponential phases for each metal and resuspended in 5 mM HEPES buffer (pH 6.8), then passed
twice through a French pressure cell at an average of 1-2 drops per second, at a pressure of approximately 15 000 PSI. To the cell lysates, 50 µg · mL⁻¹ of DNase I (Sigma) and 100 µg · mL⁻¹ of RNase A (Sigma) were added, followed by incubation on ice for 20 mins. The samples were then centrifuged at 6300 x g for 10 minutes to remove cell debris, the lysates were transferred into fresh centrifuge tubes, and the process repeated. The suspensions were subsequently centrifuged at 48 000 x g for 20 minutes to harvest the envelopes. The pellets were washed and resuspended in 5 mM HEPES (pH 6.8) and this process was repeated 5 times. After the last wash, the pellets were resuspended in Milli-Q water and spun down at 6300 x g for 10 mins again to ensure that there was no more debris left in the solution. Samples of the envelope extracts were stained with 2% uranyl acetate, mounted on electron microscopy grids, and examined using TEM to ensure purity. The envelopes were then lyophilized and stored at -80°C.

2.3.6 Scanning transmission X-ray microscopy

Scanning transmission X-ray microscopy (STXM) measurements were performed at beamlines 11.0.2 and 5.3.2 of the Advanced Light Source at Lawrence Berkeley National Lab, Berkeley, CA (Kilcoyne et al. 2003). A Fresnel zone plate lens (25 nm outer zones) is used to focus a monochromatic X-ray beam onto the sample. The sample is scanned through the fixed beam and transmitted photons are detected via a phosphor scintillator -photomultiplier tube assembly to provide a 2D image of the sample volume probed. On both beamlines, the theoretical spatial and spectral resolutions during our measurements were 30 nm and ± 0.1 eV respectively.

Vanadium standard materials were purchased from Alfa Aesar (Ward Hill MA, USA), and Mn standard materials were obtained from B. Toner (2005a); all standards were XRD confirmed.
Standard materials and CN32 membranes were deposited onto a 100 nm thick silicon nitride window (Silson Ltd, Northampton, UK) and analyzed at room temperature under He at pressure < 1 atm. X-ray images recorded at energies just below and at the relevant absorption edges were converted into optical density (OD = ln(Io/I)) images and used to derive elemental maps. NEXAFS spectra from regions of interest were obtained from image sequences (stacks) collected at energies spanning the relevant absorption edge. Potential beam-induced damage to the samples was carefully checked at V and Mn 2p-edges by recording fast stacks at few energy points spanning the relevant absorption edge and then a full finer stack on a similar region in close proximity. At least two different sample regions were analyzed for each element and experiments were performed on different time replicates. The photon energy was calibrated at the V 2p-edges using the O 1s → 3s transition at 538.9 eV of gaseous CO₂ and at Mn 2p-edges using the Mn2p₃/₂ of MnCl₂ set at 640 eV. All STXM data processing was done using the aXis2000 software (Hitchcock 2008).

Figure 2.3. TEM whole mount of *S. putrefaciens* CN32 harvested after 36 hours of anaerobic growth at 25°C in DM supplemented with 1 mM UO₂(CH₃COO)₂ · 2H₂O (as UO₂²⁺), demonstrating no surface precipitates typically associated with U(VI) reduction.
2.4 Results

2.4.1 Membrane fluidity

*Shewanella putrefaciens* CN32 cells grown in the presence of U(VI) were examined under TEM to identify surface precipitates (Fig. 2.3). No surface precipitates commonly associated with U(VI) reduction were observed in the culture. Membrane fluidity for each treatment was evaluated by calculating fluorescence polarization of the DPH probe (Table 2.1). DPH approximates a cylindrical shape and is embedded parallel to saturated or trans-acting fatty acids, with absorption and emission transition dipoles parallel to the long axis (Lentz 1993). This causes the fluorescence polarization to increase when the probe is stabilized in the membrane, and decrease when the probe freely rotates in a more fluid membrane. As such, membrane fluidity is inversely proportional to fluorescence polarization.

Oxygen presence fluidized the membranes of bacteria cultured without treatment metal, and the membranes of CN32 cultured with U. Under aerobic conditions, Mn, V, and U stabilized membranes, and exhibited similar values for fluidity. There was no significant change in fluidity for anaerobic bacteria treated with Mn or V; bacteria cultured with added U were the only anaerobic metal treatment that had significant membrane stabilization.
Table 2.1. Data summary of fluorescence polarization (FP) data for single temperature and melt-curve experiments. Data are presented as means (± SD; n=3), and temperature gradient values were obtained from 4-parameter logistic curve fitting in SigmaPlot 11. Pre- and post-transition fluorescence polarization values were obtained by averaging maximum and minimum values at segments of linearity prior to, and after, phase transition.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>FP (25°C)</th>
<th>Tm</th>
<th>FP Pre-Transition</th>
<th>FP Post-Transition</th>
<th>ΔFP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aerobic</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.239(0.006)a</td>
<td>33.5(0.389)</td>
<td>0.361(0.031)</td>
<td>0.156(0.020)a</td>
<td>0.205(0.037)</td>
</tr>
<tr>
<td>Mn(II)</td>
<td>0.288(0.012)b</td>
<td>35.1(1.340)</td>
<td>0.295(0.129)</td>
<td>0.233(0.012)b</td>
<td>0.063(0.129)</td>
</tr>
<tr>
<td>V(IV)</td>
<td>0.277(0.012)b</td>
<td>27.3(0.839)</td>
<td>0.304(0.025)</td>
<td>0.216(0.017)b</td>
<td>0.088(0.030)</td>
</tr>
<tr>
<td>U(VI)</td>
<td>0.299(0.014)b</td>
<td>27.7(0.954)</td>
<td>0.295(0.034)</td>
<td>0.287(0.035)b</td>
<td>0.008(0.049)b</td>
</tr>
<tr>
<td><strong>Anaerobic</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.314(0.007)a</td>
<td>32.1(3.550)</td>
<td>0.346(0.031)</td>
<td>0.251(0.070)a</td>
<td>0.095(0.076)</td>
</tr>
<tr>
<td>Mn(II)</td>
<td>0.298(0.004)</td>
<td>37.4(1.610)</td>
<td>0.336(0.022)</td>
<td>0.231(0.016)</td>
<td>0.106(0.027)</td>
</tr>
<tr>
<td>V(IV)</td>
<td>0.299(0.020)</td>
<td>36.3(4.480)a</td>
<td>0.309(0.065)</td>
<td>0.265(0.035)</td>
<td>0.044(0.074)</td>
</tr>
<tr>
<td>U(VI)</td>
<td>0.387(0.039)ab</td>
<td>33.0(2.470)</td>
<td>0.356(0.045)</td>
<td>0.280(0.027)</td>
<td>0.076(0.052)</td>
</tr>
</tbody>
</table>

*aSignificantly different from identical metal treatment in opposite atmospheric growth conditions
*bSignificantly different from control under identical atmospheric growth conditions

### 2.4.2 Membrane melt curves

Four-parameter logistic curves were utilized to determine phase-transition characteristics for aerobic and anaerobic treatments (Table 2.1). Anaerobic cultures treated with V(IV) showed significant membrane stabilization compared to aerobic cultures treated with the same metal. Anaerobic cultures not treated with metal also demonstrated significantly greater thermal stability when membranes were in a more disordered fluid state (post phase transition).

Under oxic conditions, both V(IV) and U(VI) significantly stabilized the membranes against heat-induced fluidization compared to the aerobic control. In addition, aerobic cultures treated
with U(VI) demonstrated only minute fluidization of the membrane when bacteria were heated. Gel-phase fluidity was similar in all treatments; when heated above 55°C, however, membranes in all aerobic metal treatments were more stable than the control.

2.4.3 Fatty acid composition

Fatty acid profiles are shown with the sums of [unsaturated, cyclopropyl, branched], and saturated hydrocarbon chains (Table 2.2). Fatty acid composition did not show unequivocal trends with metal treatment. Fatty acid ratios of [unsaturated, cyclopropyl, branched]:saturated derivation were used to approximate overall FA chemistry in membrane phospholipids. Ratios of unsaturated:saturated FA saturation chemistry have been previously used to estimate overall membrane lipid biochemistry (Trevors 2003; Denich et al. 2003; Quivey et al. 2000; Kim et al. 2001). These studies do not, however, account for branched or cyclopropyl fatty acids, despite their fluidizing effects on cell membranes (Russell 1984; Eze and McElhaney 1981). Ratios in the present study were used to approximate phospholipid shape, representing the relationship between more conical:cylindrical phospholipids. The ratios varied significantly between the aerobic and anaerobic control treatments. Exposure to oxygen was correlated with a decreased proportion of saturated FA, and an increased proportion of unsaturated FA. Bacteria cultured with U(VI) under oxic conditions had a significantly higher proportion of saturated FA than the aerobic control. Vanadium(IV)-treated cultures had a significantly lower proportion of saturated FA under anoxic conditions. A linear relationship was observed between the FA ratios and the fluorescence polarization values for all metal treatments, with the exception of the anaerobic U(VI) cultures (Fig. 2.4), with no pattern to the residuals.
Table 2.2. FA profile for extracted *S. putrefaciens* CN32 membrane phospholipids, displaying all FA present > 1% in total content with the exception of the rare cyclopropyl moieties. Values are displayed in mean percentages (± SD; n=3).

<table>
<thead>
<tr>
<th>FA</th>
<th>Control</th>
<th>Aerobically Grown (% ± SD)</th>
<th>Anaerobically Grown (% ± SD)</th>
<th>Control</th>
<th>Mn(II)</th>
<th>V(IV)</th>
<th>U(VI)</th>
<th>Control</th>
<th>Mn(II)</th>
<th>V(IV)</th>
<th>U(VI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saturated</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12:0</td>
<td>5.11(0.37)</td>
<td>4.48(0.35)</td>
<td>5.83(0.60)</td>
<td>4.58(0.11)</td>
<td>4.62(0.12)</td>
<td>5.23(0.60)</td>
<td>4.75(0.15)</td>
<td>5.06(0.96)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14:0</td>
<td>1.42(0.07)</td>
<td>1.31(0.02)</td>
<td>2.50(0.21)</td>
<td>1.24(0.04)</td>
<td>1.58(0.14)</td>
<td>1.76(0.17)</td>
<td>1.26(0.12)</td>
<td>1.30(0.06)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>15:0</td>
<td>0.64(0.02)</td>
<td>0.72(0.02)</td>
<td>0.37(0.02)</td>
<td>0.58(0.02)</td>
<td>1.14(0.04)</td>
<td>1.17(0.10)</td>
<td>1.02(0.01)</td>
<td>1.11(0.02)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16:0</td>
<td>17.66(0.51)*</td>
<td>18.45(0.01)</td>
<td>19.67(0.69)</td>
<td>21.50(0.09)*</td>
<td>22.89(0.22)*</td>
<td>18.56(0.95)*</td>
<td>18.52(0.09)*</td>
<td>18.38(1.22)*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17:0</td>
<td>0.34(0.02)*</td>
<td>1.11(1.02)</td>
<td>0.18(0.02)</td>
<td>1.14(0.44)</td>
<td>1.60(0.44)*</td>
<td>2.01(0.50)</td>
<td>0.83(0.02)</td>
<td>1.31(0.84)</td>
<td></td>
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</tr>
<tr>
<td>18:0</td>
<td>1.22(0.13)*</td>
<td>1.98(0.48)</td>
<td>1.16(0.23)</td>
<td>2.79(0.40)*</td>
<td>3.40(0.63)*</td>
<td>2.33(0.33)</td>
<td>2.01(0.09)</td>
<td>1.75(0.42)*</td>
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<tr>
<td>Branched</td>
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<td></td>
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</tr>
<tr>
<td>12:0 3OH</td>
<td>3.22(0.08)</td>
<td>2.96(0.25)</td>
<td>3.61(0.32)</td>
<td>3.11(0.06)</td>
<td>2.56(2.22)</td>
<td>3.43(0.21)</td>
<td>3.00(0.04)</td>
<td>3.42(0.54)</td>
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</tr>
<tr>
<td>13:0 iso</td>
<td>4.12(0.28)*</td>
<td>3.40(0.25)</td>
<td>3.90(0.40)</td>
<td>3.16(0.10)*</td>
<td>2.84(1.00)*</td>
<td>3.82(0.46)</td>
<td>3.56(0.10)</td>
<td>3.65(0.58)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13:0 3OH</td>
<td>1.05(0.02)</td>
<td>0.94(0.08)</td>
<td>1.01(0.08)</td>
<td>0.95(0.02)</td>
<td>1.22(0.03)</td>
<td>0.98(0.07)</td>
<td>0.91(0.02)*</td>
<td>1.03(0.20)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>15:0 iso</td>
<td>10.75(0.38)*</td>
<td>9.84(0.37)</td>
<td>8.08(0.46)</td>
<td>8.93(0.28)</td>
<td>8.20(0.33)*</td>
<td>11.23(1.09)*</td>
<td>10.07(0.12)*</td>
<td>9.89(0.26)*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17:0 iso</td>
<td>1.76(0.10)*</td>
<td>2.00(0.13)</td>
<td>1.08(0.11)</td>
<td>2.44(0.06)*</td>
<td>2.08(0.05)*</td>
<td>1.94(0.02)</td>
<td>1.93(0.04)*</td>
<td>1.91(0.26)*</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Cyclopropyl</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>17:0 cyclo o8c</td>
<td>0.00(0.00)</td>
<td>0.00(0.00)</td>
<td>1.77(0.02)*</td>
<td>0.00(0.00)</td>
<td>0.00(0.00)</td>
<td>0.00(0.00)</td>
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<td></td>
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</tr>
<tr>
<td>19:0 cyclo o8c</td>
<td>0.00(0.00)</td>
<td>0.00(0.00)</td>
<td>0.98(0.16)*</td>
<td>0.00(0.00)</td>
<td>0.00(0.00)</td>
<td>0.00(0.00)</td>
<td>0.00(0.00)</td>
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</tr>
<tr>
<td>17:1 o8c</td>
<td>1.83(0.04)*</td>
<td>2.59(0.21)*</td>
<td>0.93(0.01)*</td>
<td>1.80(0.01)*</td>
<td>3.36(0.08)*</td>
<td>3.46(0.21)*</td>
<td>3.42(0.00)*</td>
<td>3.73(0.10)*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:1 o9c</td>
<td>5.50(0.28)</td>
<td>6.02(0.25)</td>
<td>3.99(0.32)*</td>
<td>6.82(0.14)*</td>
<td>6.09(0.08)</td>
<td>5.78(0.14)</td>
<td>5.82(0.14)*</td>
<td>6.25(0.72)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>18:1 o7c</td>
<td>7.21(0.36)</td>
<td>8.07(0.35)*</td>
<td>8.79(0.71)*</td>
<td>9.18(0.20)*</td>
<td>8.20(0.29)</td>
<td>6.93(0.17)*</td>
<td>8.38(0.15)</td>
<td>7.97(0.90)*</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>72.79(2.79)</td>
<td>70.74(4.85)</td>
<td>69.88(3.78)</td>
<td>67.26(1.99)</td>
<td>63.64(7.04)</td>
<td>67.65(4.57)</td>
<td>70.41(1.79)</td>
<td>69.80(6.59)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total saturated</td>
<td>26.61(1.14)*</td>
<td>28.29(1.98)</td>
<td>29.89(1.79)</td>
<td>32.07(1.13)*</td>
<td>35.67(1.63)*</td>
<td>31.48(2.70)</td>
<td>28.68(0.51)*</td>
<td>29.40(3.88)*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ratio (unsat/sat)</td>
<td>2.74(0.16)*</td>
<td>2.50(0.24)</td>
<td>2.34(0.19)</td>
<td>2.10(0.10)*</td>
<td>1.78(0.21)*</td>
<td>2.15(0.23)</td>
<td>2.45(0.08)*</td>
<td>2.37(0.39)*</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

aSignificantly different from identical metal treatment in opposite atmospheric growth conditions
bSignificantly different from control under identical atmospheric growth conditions
Figure 2.4. Scatter plot comparison and linear regression of fluorescence polarization values and FA ratios, demonstrating a relationship between the two factors. Anaerobically grown cells grown in the presence of U(VI) displayed values that fell outside the relationship trend visible within the other treatments.

2.4.4 STXM analyses of bacterial envelopes

Bacterial envelopes harvested from *S. putrefaciens* CN32 cultures were examined under TEM to ensure that the samples were free of whole cells (Fig. 2.5). STXM (Kirz et al. 1995) and NEXAFS spectroscopy (Stöhr 1992) were performed on envelopes at V 2p and Mn 2p edges. Elemental mapping and NEXAFS at Mn 2p edges (Fig. 2.6) on aerobic and anaerobic Mn-treated bacterial envelopes revealed diffuse manganese consistent with Mn$^{2+}$ (Toner et al. 2005a; Pecher et al. 2003; Grush et al. 1996; Gilbert et al. 2003). Elemental mapping and NEXAFS at V 2p edges (Fig. 2.7) confirmed that V(IV) as VO$^{2+}$ was sparsely distributed over the envelopes of anaerobic V-treated cells. Vanadium was not detected (detection limit of ~0.1 mM) in
association with aerobic V-treated cell envelopes. Vanadium 2p spectra exhibit a core electronic $V \ 2p_{3/2} \rightarrow V \ 3d$ transition (515-520 eV) and $V \ 2p_{1/2} \rightarrow V \ 3d$ transition (522-527 eV) (Abbate et al. 1993). No precipitates were observed in the purified envelope extracts in either of these metal treatments using TEM or STXM. NEXAFS spectra of 3d transition metals such as V and Mn exhibit distinct L$_2$ and L$_3$ bands due to the spin-orbit splitting of the 2p-orbitals (de Groot et al. 1990). The fine structure of these bands has been attributed to the crystal field splitting of 3d orbitals (Brik et al. 2006; de Groot et al. 1990; van der Laan and Kirkman 1992). From more-oxidized to more-reduced 3d metals (V and Mn), the occupancy of electrons in the 3d state increases, which leads to a lower absorption energy. Uranium edges were not examined using STXM due to the unavailability of the 11.0.2 beamline, so uranyl ion association was examined by electron density using TEM; no precipitates were observed in association with the bacteria or in the extracellular region.

![Figure 2.4. TEM micrograph of purified S. putrefaciens CN32 envelopes from anaerobically grown cells, treated with 2% (w/v) uranyl acetate to enhance contrast. Scale bar is 500 nm.](image)
Figure 2.6. STXM analyses of aerobically grown CN32 cell envelopes (A) STXM image recorded at 640 eV; (B) Mn distribution map and (C) Mn 2p NEXAFS spectra of manganese-treated CN32 membrane extracts along with Mn(II) standard MnCl$_2$, Mn(III) standard γ-MnOOH and Mn(IV) standard δ-MnO$_2$ shown for comparison. Stacks were recorded in multiple regions of the sample shown in (A), and spectra representative of the sample are depicted in (C). Indicated in (C) are L$_3$ and L$_2$ edges (2p$_{3/2}$ and 2p$_{1/2}$ respectively). Scale bars are 10 µm.
Figure 2.7. STXM analyses of anaerobically grown CN32 cell envelopes; (A) STXM image recorded at 517.5 eV; (B) V distribution map and (C) V 2p NEXAFS spectra of vanadium-treated CN32 membrane extracts along with V(V) standard $\alpha$-V$_2$O$_5$ and V(IV) standards VO$_2$ and VCl$_4$ $\cdot$ H$_2$O (labeled VO$^{2+}$) shown for comparison. Multiple stacks from the sample in (A) were assayed, and shown in (C) are spectra that represent sample X-ray absorbance. Indicated in (C) are the transition energies at L$_3$ and L$_2$ edges. Scale bars are 10 µm.
2.5 Discussion

In this study, the ability of Mn, V, and U to induce chemical changes in *S. putrefaciens* CN32 membranes, as well as to affect the fluid state of CN32 bilayers, was assessed. Bacterial membranes are able to respond to environmental stressors such as temperature, pH, and salinity, largely through desaturation of membrane FA (Mykytczuk et al. 2007; Denich et al. 2003; Los and Murata 2004; Zhang and Rock 2008). Mykytczuk et al. (2011) observed similar subtle variations in FA chemistry across a very broad range of 1-200 mM Cu$^{2+}$ and Ni$^{2+}$ metals in *A. ferrooxidans*, and similar variability in T$_{m}$ and fluidity using the same DPH probe. *A. ferrooxidans* and *S. putrefaciens* CN32 have dissimilar envelope chemistries, and are typically grown in different growth medium, so actual values cannot be compared, but trends in variability are similar in my experiment.

Desaturation of FA affects the shape of phospholipid molecules, which is defined here as geometrical approximations of spatial occupation. Modes of FA modification in *Shewanella* have yet to be documented, and it is unclear if desaturation is due to *de novo* biosynthesis as previously reviewed (Cronan 2003; Zhang and Rock 2008), or through oxidative desaturation documented in other species of bacteria (Weber et al. 2001; Aguilar et al. 2001; Zhang et al. 2007). Unsaturated FA assume a conical shape, resulting in a reduced packing density and an increase in membrane fluidity. Cyclopropyl FA, and some branched FA also tend to have a conical lipid shape, contributing to membrane fluidization. The linear relationship between FA shape (presented as a ratio of unsaturated:saturated) and fluidity is supported by our results.
Anaerobic U-treated bacteria fell outside the plot of unsaturated:saturated ratio, which suggests that the strong stabilization of cell membranes in this treatment is due to additional factors. Although U(VI) was associated with the cell wall, as seen by TEM, no characteristic uranium precipitates (Lloyd 2003; Merroun and Selenska-Pobell 2008; Ferris et al. 1989b) formed in the cell wall or in the medium. The growth medium was deoxygenated with N2 to eliminate carbonates that can complex with U above pH 6.5 (Fig. 2.8). One explanation is that these uranyl associations stabilized the membrane lipids, promoting DPH association. Since the uranyl ion is capable of bidentate bonding to LPS (Barkleit et al. 2008), and, presumably, to phospholipids, it may behave differently than the other metals examined in this study by forming lipid-uranyl-lipid interactions. This would limit lateral diffusion, potentially enhancing DPH binding and fluorescence. Given the linear geometry of the uranyl ion, this manner of stabilization of membrane lipids seems possible.

Figure 2.8. Summary of major U complex proportions (0.01 M) across a pH 3-9 gradient under atmospheric conditions. Omitted are complexes present in relatively trace proportions, consistently below 0.1% across the pH range. Curves were calculated using the MICROQL algorithm of Westall (1986), using parameters based from CHEAQS (Verweij 2010).
In contrast, aerobic U(VI)-treated cells stayed within the correlation of FA ratio and fluidity, but membranes were much more thermally stable than for all other treatments. It is unclear, however, whether this is mainly due to sugar crosslinking within the LPS or because the phospholipid head groups are stabilized by U; in the latter case, it must be in a manner that does not affect DPH fluorescence. It has been postulated that metal concentrations greater than 5 mM are necessary to cross-link anionic components found in the lipopolysaccharide regions (Nguyen et al. 2003; Langley and Beveridge 1999). Barkleit et al. (2008) observed, however, that 0.1 mM UO$_2^{2+}$ was bound by two phosphoryl ligands at circumneutral pH. This suggests that bidentate sorption and consequent membrane stabilization are possible at sub-millimolar concentrations at the outer membrane. In our aerobic medium, aqueous uranyl-phosphate complexes dominate U speciation (Table 3). The speciation calculations do not consider contributions of cell surface chemistry or of soluble complexation agents from the bacteria, but they do provide some insight into the metal chemistry of the growth medium. The dominant uranyl phosphate complexes are uncharged, and will not associate with the cell wall as strongly as cationic uranyl species. Given the 1 mM concentrations used in our research, however, and assuming even 20% of the U(VI) complexes in the growth medium are cationic, it suggests that there are excess cationic uranyl complexes available for binding even at cell concentrations of about $5 \times 10^8$ cells/mL. The only two uranyl complexes that differed substantially between aerobic and anaerobic treatments were (UO$_2$)$_2$(CO$_3$)(OH)$_3$ and (UO$_2$)$_3$(OH)$_5^+$. These molecules are large, and may not breach the OM or substantially impact fluidity. Changes in membrane protein content, however, may account for some differences seen in the thermal stability of the aerobic U(VI) treatment.
Table 2.3. Uranium complexes present in aerobic U-treated cell medium, calculated without bacterial presence. Complexes present < 1% were omitted. Data was generated using CHEAQS (Verweij 2010).

<table>
<thead>
<tr>
<th>Aqueous uranium complex</th>
<th>Aerobic DM content (%)</th>
<th>Anaerobic DM content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(UO$_2$)$_2$(PO$_4$)</td>
<td>53.38</td>
<td>57.47</td>
</tr>
<tr>
<td>(UO$_2$)$_2$(CO$_3$)(OH)$_3$</td>
<td>13.82</td>
<td>-</td>
</tr>
<tr>
<td>(UO$_2$)$_2$(OH)$_5$</td>
<td>13.54</td>
<td>24.13</td>
</tr>
<tr>
<td>(UO$_2$)(PO$_4$)$^-$</td>
<td>5.47</td>
<td>5.89</td>
</tr>
<tr>
<td>a(UO$_2$)(NTA)$^-$</td>
<td>4.11</td>
<td>4.33</td>
</tr>
<tr>
<td>(UO$_2$)(CO$_3$)$^-$</td>
<td>3.34</td>
<td>-</td>
</tr>
<tr>
<td>(UO$_2$)$_2$(OH)$_2^{2+}$</td>
<td>2.64</td>
<td>3.89</td>
</tr>
<tr>
<td>(UO$_2$)$^{2+}$</td>
<td>1.64</td>
<td>1.99</td>
</tr>
</tbody>
</table>

aNTA – nitrilotriacetic acid

Since the DPH probe associates within the hydrophobic region of the bilayer, proteins simply exclude the probe from their space and can be expected to have less of an effect on fluorescence polarization readings than unsaturated FA. Their influence would be more apparent in thermal stability studies due to the fact that overall bilayer stability is assayed, rather than only lipid fluidity. Some CN32 cells likely produced U(VI) respiratory proteins, as even with rapid shaking flask cultures may not be fully aerobic (Wise 1951). Due to frequent albeit limited oxygen exposure, however, the respiratory proteins would not be extensively utilized. Charged membrane proteins sequester lipids by electrostatic interaction within the membrane, and form stabilizing protein-lipid domains (Epand 2006). Previous studies have shown that proteins do increase the ordering of lipid membranes (Jähnig 1979), but further study is required to identify or confirm potential protein effects in our experiment.

We observed variations between gel-to-fluid phase transition temperatures in anaerobic bacteria treated with Mn. Under anoxic conditions, Mn(II) and V(IV) adsorbed to, and stabilized, CN32 cell walls. Predicting metal speciation in complex growth media is challenging. This applied
especially to V, for which the interpretation of even simple solution chemistry is complicated by inconsistencies in published stability data (Evans and Garrels 1958; Wanty and Goldhaber 1992; Takeno 2005). In general, V(IV) behaves as an anion under oxic conditions, and as a cation under reducing conditions. Difficulties in determining V complexation are amplified in chemically complex growth media in the presence of actively dividing and respiring cells. Most anionic vanadium complexes are likely removed after five washes, or at least beneath STXM detection limits. This is supported by our NEXAFS studies, in which V(IV) was only detected in association with the bacteria under anoxic conditions. In addition, the anaerobic V-treated cells had significantly higher T_m than their aerobic counterparts, further supporting that only cell wall-associated V(IV) exhibits stabilizing effects on CN32 membranes. Beveridge and Koval (1981) did not observe V binding to aerobically-prepared Escherichia coli AB264 envelopes after 10 minutes of exposure, but did detect Mn and U. E. coli AB264 possesses rough LPS that may be comparable to S. putrefaciens CN32; however, phospholipid compositions likely differ between these species.

The fatty acid ratios calculated in this experiment provided an estimate of overall membrane lipid chemistry. Lipids that were summed in the [unsaturated, cyclopropyl, branched] totals are assumed to all have the same degree of chemical deviation from saturated FA, and thus make the same contribution to membrane phase state. This is not the case; although branched FA will contribute to a more fluid membrane, the cyclo and unsaturated FA should play a much stronger role in membrane fluidization than branched FA. The associations of DPH with membrane lipids are, however, likely to be more equally affected by branched, cyclo, and unsaturated FA. For this reason, the linear relationship between the FA ratio and fluorescence polarization is still
a useful means of detecting non-lipid contributors to changes in membrane fluidity. This is the first study of fatty acid chemistry and membrane fluidity of *S. putrefaciens* CN32, and comparative data are therefore not available.

Metal-membrane interactions are key to surface-associated biomineralization, in which the bacterial surface is thought to serve as a template for heterogeneous mineral nucleation (Ferris et al. 1989b; Beveridge 1989; Fortin et al. 1997; Schultze-Lam et al. 1996; Ferris et al. 1986). Our results suggest that the association of metals with bacterial surfaces will be a function not only of the metal, but also of the redox conditions. Both cell wall chemistry and metal chemistry are functions of oxygen presence (Hunter et al. 2010; Korenevsky and Beveridge 2007; Haas 2004), and are highly complex with substantial gaps in understanding of their combined impacts on biogeochemical processes.

### 2.6 Conclusion

The ubiquity of soluble metals in natural environments, and the persistence of the microorganisms living amongst them, suggests that bacterial cell wall structure and function is maintained. Our results demonstrate that metals induce subtle biochemical changes in FA saturation chemistry, that are sufficient to significantly affect overall membrane fluidity. These impacts are dependent not only on the metal, but also on the chemical environment in which cells are growing. In addition, adsorption of metals to cell walls can enhance the thermal stability in a manner that is independent of overall fluidity. This is the first study, to our knowledge, that examines purified cell envelopes using STXM rather than whole cells to observe the association of metals with cell surface components. The use of STXM-NEXAFS to examine
purified bacterial envelopes offers a means to eliminate intracellular contamination, when speciating and characterizing surface-bound metals, without harsh chemical treatment that may alter metal chemistry. Metal-induced changes in bacterial cell membrane have extensive implications in understanding biomineralization, predicting bioremediation or cell survival in environments contaminated with metals, and interpreting the effects of metals on bacteria in laboratory scale studies.
CHAPTER 3

CHARACTERIZING *Shewanella putrefaciens* CN32 PHOSPHOLIPID FUNCTIONAL GROUP CHEMISTRY AFTER CULTURING IN THE PRESENCE OF SOLUBLE Mn(II), V(IV), AND U(VI)

Submitted to the Journal of Microbiological Methods

CHAPTER THREE

Characterizing *Shewanella putrefaciens* CN32 phospholipid functional group chemistry after culturing in the presence of soluble Mn(II), V(IV), and U(VI)

3.1 Abstract

The proportions of phospholipid functional groups in bacteria are known to vary across growth conditions, suggesting that growth environments will impact the surface reactivity of bacteria to soluble metal species. This research identified variations in phospholipid functional groups in *Shewanella putrefaciens* CN32 during aerobic and anaerobic growth, and with Mn(II)-, V(IV)-, or U(VI)-amendment in chemically defined medium. Phospholipids were separated using thin-layer chromatography (TLC), and cell wall functional groups were characterized using near-edge X-ray absorption fine structure (NEXAFS) spectroscopy. TLC results indicated that anaerobic growth conditions induced higher phosphatidylglycerol and lower diphosphatidylglycerol proportions in cell membranes. In addition, metal-amendment to the culture media increased phosphatidylethanolamine in anaerobic growth environments. NEXAFS CNO 1s were very similar across treatment, despite variations in phospholipid chemistry observed in TLC separations. Carbon 1s→π* transitions at 298-299 eV were indicative of envelope protein content, which was confirmed using a traditional colorimetric protein assay. The results of this study indicate that anaerobic growth has a strong impact on cell membrane phospholipid proportions, especially when cells are grown in the presence of soluble metal cations. NEXAFS observations suggest that CNO-K edge spectroscopy may not be specific enough to identify the contributions of individual components to cell envelope chemical compositions, but may complement other methods of surface characterization.
3.2 Introduction

Membrane-metal associations are ubiquitous in nature. The large surface area-to-volume ratios of bacterial surfaces, and the multitude of metal-reactive sites within the surface components (Beveridge 1989; Beveridge 2005; Hoyle and Beveridge 1984), offer bacterial biomass as adsorption surfaces in environments with high concentrations of soluble metals. This reduces the mobility of soluble metal complexes and has been proposed to be a means of metal immobilization, and remediation in aqueous environments (Valls and de Lorenzo 2002; Chang and Huang 1998; Veglio and Beolchini 1997; Gavriescu 2004). The effectiveness of this immobilization is dependent not only on the environmental redox and pH conditions, but also on the metal-reactive group chemistry within the polyelectrolyte brush of surface sugars, proteins, and lipids.

Metal-reactive components in Gram-negative bacterial envelopes are summarized as peptidoglycan, lipopolysaccharides (LPS), proteins, and phospholipids. The structural diversity of bacterial envelopes presents a challenge in determining contributions of functional groups on specific structural components, towards metal binding. Metal-reactive sites on peptidoglycan and LPS are not expected to change substantially across environmental metal stimuli; proteins and phospholipids, however, are known to undergo selective biosynthesis depending on the cellular environment (Zhang and Rock 2008; Romantsov et al. 2009; Cronan 2006; Silver and Phung 2005; Dowhan 1997). Synthase, phosphatase, and decarboxylase enzymes required for biosynthesis of diphosphatidylglycerol (DPG), phosphatidylserine (PS), phosphatidyglycerol (PG), or phosphatidylethanolamine (PE) are embedded in the cytoplasmic membrane. These proteins are regulated by mechanisms that require anionic or zwitterionic lipids to activate or de-
repress, maintaining a balance of zwitterionic and acidic functional groups within the membrane. Comprehensive reviews by Zhang and Rock (2008), and Cronan (2003) outline this regulation of headgroup, as well as acyl chain, biochemistry. Charged functional groups of phospholipids influence electrostatic interactions with adjacent proteins or lipids. Selective biosynthesis due to charge-masking metal cation stresses may be a major tolerance mechanism in these types of environments.

Electrostatic interactions between membrane lipids, proteins, and sugars play a major role in maintaining bacterial compartmentalization. Metal counterions, as well as increasing ionic strength, are known to lessen electrostatic influences and emphasize contributions from intermolecular entropic (including hydrophobic effect) and van der Waals forces (Israelachvili 2011; Hermansson 1999; Grasso et al. 2002). Lipid de-mixing can be a direct result of this, sequestering acidic phospholipids (Tokutomi et al. 1981; Coorssen and Rand 1995; Silvius and Gagne 1984), and impacting protein-lipid interactions (Epand 2006). This type of sequestration, however, may not be applicable to membranes containing high proportions of unsaturated fatty acids (Tilcock et al. 1988). Metals in solution, as examined in chapters 2 and 4, also impact membrane attributes such as fluidity and permeability. To our knowledge, no studies have, however, been performed that address alterations in phospholipid functional group chemistry that may occur in response to high concentrations of soluble metal cations.

In this experiment, I characterized phospholipid headgroups after exposure to soluble Mn$^{2+}$, VO$^{2+}$, and UO$_2$$^{2+}$ ions. Conventional lipid purifications and separations were compared to NEXAFS CNO-1s spectra of purified membranes in order to assess the ability of STXM and
NEXAFS to identify changes in membrane lipid composition at these edges. Changes in lipid diffusion after cation exposure was also assayed in this study. Reorganization of synthetic membranes with defined chemical composition can be calculated or predictable; in vivo membrane responses to soluble cations are much more complex, but reorganize due to the same forces despite the chemical heterogeneity. As such, observing the impacts of metals on lipid diffusion, in membranes comprised of lipids extracted from whole cells, offers insight into how those lipids laterally migrate within the cell envelope in the presence of those same metals. It was hypothesized that soluble metal ions would induce changes in phospholipid functional group chemistry, due to a need to maintain both membrane fluidity and cellular integrity when electrostatic interactions within the membrane are masked. It was also expected that the molecular diversity in the cell envelope would complicate spectral analyses. There may exist, however, signature transition energies that act as indicators for changes in individual components such as proteins or amine-containing phospholipid functional groups.

3.3 Materials and methods

3.3.1 Reagents and cell culture conditions

Stock cultures of Shewanella putrefaciens CN32 were grown from frozen stocks, as stated in chapter 2.3.1, and cultured on TSA as previously described. Isolated colonies were transferred to 250 mL of TSB, and grown aerobically on a 25°C shaker table (300 RPM) for 8 hours to mid-exponential growth phase. Cells were spun down at 6300 x g for 10 minutes, and resuspended in 5 mM HEPES buffer (pH 6.8). This suspension was used to inoculate 500 mL of chemically defined medium (DM) (Fredrickson et al. 1998) that had been pH adjusted to 6.8. Medium was modified to include 1 mM NaH₂PO₄, 20 mM lactate was used at the electron donor, and
additional supplements of L-arginine, L-glutamic acid, and L-serine were included at 20 µg/mL concentrations. Phosphate was sterilized and added after autoclaving the medium, and amino acid supplements were filter sterilized through a 0.22 µm filter and added after the medium had cooled. *S. putrefaciens* CN32 was cultured aerobically in identical treatment conditions as in Chapter 2.

Anaerobic cultures were grown in a similar manner as for aerobic growth, but the final metal-treatment medium was deoxygenated for 1 hour, and the headspace of the large serum bottle for 15 minutes, with N₂ gas bubbling prior to autoclaving. The terminal electron acceptor in the anaerobic cultures was 13 mM fumarate. Phosphate and amino acid supplements were also deoxygenated prior to sterilization, and added afterward to the medium in a glovebox (Coy Laboratory Products) with average hydrogen content of 2.3%, and a balance of Ar. Anaerobic culture treatments were prepared in the same manner as in Chapter 2. All glassware was bathed overnight in 0.1 M HCl, triple-rinsed in Millipore nanopure water, and oven-dried prior to use.

### 3.3.2 Total lipid extraction

Total lipids were obtained using a modified Bligh and Dyer extraction (1959). Cell pellets (~5g wet weight) from harvested cells were resuspended in 10 mL methanol and 5 mL chloroform, and placed in a 4°C shaker running at 300 RPM for 4 hours in Oak Ridge PTFE tubes. After 4 hours, an additional 5 mL of chloroform and 5 mL of sterile water was added, and samples were shaken for an additional 30 mins. Aqueous and organic phases were separated by centrifuging at 4355 x g for 20 minutes. After centrifugation, there were clear phase separations. The upper phase, and the debris between the phases, were both carefully removed. The lower organic phase
was collected, and filtered through sterilized Whatman #1 paper to ensure precipitates were removed. The crude lipid samples were transferred into sterile screw-top test tubes, while maintaining anoxia using the Hungate technique. Under the same gentle stream of N₂ gas, the lipids were dried in the tubes, weighed, resuspended in deoxygenated chloroform, and stored under anoxia at -20°C in the dark.

### 3.3.3 Phospholipid purification

Phospholipids were separated from crude total lipids using the method of Daniels et al. (2007) with some minor modifications. Silica resin (100-200 mesh, 75-150 µm particle size, 60 Å pore size) was activated at 125°C for a minimum of 4 hours. Fresh chloroform was mixed with 12 g of the activated resin to create a slurry with a final volume of 40 mL. This slurry was flushed with N₂ gas in a fume hood, and then was poured into a 1 cm x 30 cm chromatography column, with a fritted glass disc and greaseless Teflon stopcock, to a 1:10 column width-resin height ratio. The column was tapped gently to remove air bubbles, and chloroform was drained until it just entered the resin.

The crude lipids in chloroform were added to the top of the resin bed. The column was opened to allow entry of the sample, at an average drip rate of 1 mL/min. After the sample had entered the resin, an additional 5 mL of chloroform was added to the top, and drained as above. A separatory funnel was used to regulate the flow of the solvent into the column, and also acted as a reservoir. A gentle stream of N₂ gas was flushed through the collection vessels while the column was running, and vessels had headspace flushed with the gas for 5 minutes before immediate capping and storage at -20°C.
Initially, 50 mL of chloroform was passed through the column, the collection vessel changed, followed by 60 mL of chloroform-acetone (1:1 v/v). A volume of 60 mL of acetone was then passed though and collected in the same vessel. The collection vessel was changed after this, and 60 mL of methanol was passed though. The first two vessels contained neutral, glyco, and sulfolipids; the last (methanol elution) vessel containing phospholipids had the methanol removed in a 40°C water bath under a stream of N₂ gas. The resulting residue was resuspended in 1 mL of freshly prepared and deoxygenated chloroform-methanol (1:1 v/v), and stored in the dark at -20°C until required.

3.3.4 Thin layer chromatography and phospholipid spot analysis

Whatman K6 thin-layer chromatography (TLC) silica gel plates were prerun with chloroform-methanol (1:1 v/v), placed in a 110°C oven for one hour, then cooled in a desiccator. The TLC chamber was lined with solvent-saturated Whatman 3MM paper; a solvent system of chloroform-methanol-water (65:25:4 v/v/v) was used for the first dimension, and chloroform-methanol-7 N ammonium hydroxide (60:35:5 v/v/v) was used for the second dimension. Vapors were allowed to equilibrate in the tank for each dimension after adding filter paper saturated with the respective solvent. A spray reagent of 1% iodine in anhydrous ethanol was used to visualize TLC spots, and plates were air-dried in a fume hood. After drying, plates were scanned using a flatbed scanner, and saved as high-resolution image files for software densiometry analysis. Positive control plates were also run, to compare to lipids extracted from each metal-treated culture. Positive controls were PE, PG, DPG, PS, phosphatidic acid (PA), and phosphatidylcholine (PC). Lyso-PE or lyso-PG standards were not run, but there was no
apparent indication of either lipid (characterized by an $R_f \leq 0.2$ with the second dimension solvent) on stained plates.

The open-source public domain software package ImageJ (Abramoff et al. 2004; Burger and Burge 2008) was used as a densiometry application to obtain relative proportions of lipids on the TLC plates (Fig. 3.1). Images were converted to 8-bit greyscale, and black and white tones were inverted for ease of visualization. There was no substantial difference in background noise after a 50-pixel rolling ball radius background subtraction, so the original image was used. The image was calibrated with the uncalibrated optical density function. The scale was set to pixels, and spots were traced and quantified as values of integrated density, which is a sum of the grey value intensity of each pixel in the selected spot. To ensure that each lipid type gave the same spot intensity with identical concentrations, a plate was spotted with 5 $\mu$g of lipid, and detected with our iodine spray. No particular headgroup chemistry elicited a substantially more intense spot from the indicator reagent when equal concentrations and volumes (to emulate larger or smaller spot sizes) were spotted adjacently on a TLC plate. Integrated density values were converted to percent of the total lipids providing contrast on the TLC plate, and treatments were compared in InStat (Graphpad Software Inc) using an ANOVA and Bonferroni tests. Variation in lipid content was considered significant if $P < 0.01$.

3.3.5 Liposome preparations and lipid diffusion assays

Phospholipids from section 3.3.3 were used to generate liposome suspensions, which were used to estimate the direct effects soluble metals have on phospholipid diffusion within strain CN32 membranes. The organic solvent from the previously extracted phospholipids was removed by
running a gentle stream of argon gas over the lipids in a 30ºC heating block, to offset the cooling from the compressed gas. About 20 mg of phospholipid from the aerobic control treatment were treated with 2.5 mL of 5 mM HEPES (pH 6.8) and allowed to sit for 1 hour at 25ºC in the dark. After this incubation, the samples were vortexed at maximum speed for 2 minutes, and had a cloudy appearance. Transmission electron microscopy revealed that these suspensions consisted of large, non-uniform vesicles. The lipid suspensions were, therefore, sonicated in a bath sonicator for 5 minutes to make the liposomes smaller and more uniform in size. Liposomes were diluted with 5 mM HEPES (pH 6.8), to optical densities (at 600 nm wavelength) of 0.10. Subsamples of these suspensions were stained with 2% uranyl acetate, and examined under transmission electron microscopy to ensure consistent liposome sizes, and to also ensure that the sizes were appropriate of liposomes and not micelles. Micelles have a maximum diameter of twice the length of the longest acyl chain and headgroup combination (Israelachvili 2011), while liposomes are substantially larger. Phospholipids from anaerobically cultured \textit{S. putrefaciens} CN32 cells were not observed to form liposomes under the same preparation conditions, and could therefore not be assayed in this manner.

Relative membrane lipid diffusion was assayed using the fluorescent dye 1-pyrenebutyric acid (PBA; excitation: 342 nm, main monomer emission: 375 nm, excimer emission: 475 nm). The PBA molecule aligns its hydrocarbon rings parallel to acyl chains within the hydrophobic region of the bilayer, while maintaining its carboxylic acid group at the hydrophilic region (Fig. 3.1). Pyrene monomers have differing fluorescence emissions than pyrene excimer complexes (Galla and Sackmann 1974). Excimers form when monomers collide and interact when embedded in the same plane within a bilayer. Correlating the ratio of excimers:monomers to the concentration
of pyrene within the sample gives a relative diffusion rate from the slope of the linear fit. Two-millilitre liposome solutions were incorporated with 1 µL of 4, 7, 10, and 20 µM PBA in deoxygenated ethanol (final concentrations), from concentrated stock solutions. Stock solutions of PBA were diluted in anhydrous deoxygenated ethanol, to ensure that all liposome solutions had 1 µL of ethanol added, regardless of concentration. Probe was allowed to incorporate into the liposomes for 15 minutes, after which excimer:monomer ratios were stabilized, and fluorescence lifetime reported to be stable (Rharass et al. 2006). Manganese(II), V(IV), or U(VI) were then added to the cuvette to final concentrations of 0.01, 0.1, and 1 mM, and allowed to incorporate for 15 minutes. After 15 minutes, the excimer:monomer ratios were again observed to stabilize. Fluorescence emission scans were performed at 342 nm excitation, capturing 375 nm and 475 nm emissions simultaneously using a dual emission detection spectrofluorometer (Photon Technology International, Birmingham NJ, USA). All treatments were prepared in triplicate, and the excimer:monomer ratios were plotted against PBA concentration and fitted to linear functions. Variations of slope within metal treatments, in comparison to the control, are indicative of relative changes in PBA diffusion (Galla and Sackmann 1974).
Figure 3.1. Pyrenebutyric acid monomer aligned between two phosphatidylethanolamine molecules. The monomer fluoresces at a different wavelength than an excimer complex, which allows for estimations of phospholipid diffusion based on the ratio of excimer:monomer fluorescence.

3.3.6 Bacterial envelope extraction

After harvesting *S. putrefaciens* CN32 from metal-treated media, total cell envelopes were extracted using the method of Beveridge and Koval (1981), as outlined in chapter 2. Cell envelopes in this study are defined as containing LPS, peptidoglycan, and both outer membrane and cytoplasmic membranes. A subsample of the extracted envelopes were visualized using transmission electron microscopy (TEM) with 2% uranyl acetate stain to ensure purity. The pure
envelope samples were loaded onto 100 nm thick silicon nitride windows (Silson Ltd, Northampton UK) in preparation for scanning transmission X-ray microscopy (STXM).

The protein quantity in the envelopes was assayed using a BioRad DC protein kit (BioRad Laboratories), which uses a modified Lowry assay. Washed envelopes were weighed and solubilized in 1 mL of buffer that was non-reactive with kit reagents (0.1 M Tris-HCl, 4 M urea, 1% Triton X-100, 0.025 M EDTA). Dilutions were accounted for, and absorbance at 750 nm was compared to a standard curve generated from absorbances of known bovine serum albumin (BSA) concentrations in the same solubilization buffer. Protein content of washes from initial whole cell harvesting was assayed as well to identify potential loss of peripheral proteins, and protein quantity was < 0.5 mg/mL of supernatant.

3.3.7 Scanning transmission X-ray microscopy

STXM analyses were performed at the Advanced Light Source (Lawrence Berkeley National Lab, Berkeley, CA, USA) on the molecular environmental sciences beamline 5.3.2 (250-600 eV) (Kilcoyne et al. 2003). Uranium K, L, M, and N_{1.5} edges (about 730 – 115000 eV) were not appropriate to examine on the 5.3.2 beamline, which is tuned for a lower energy range. This microscope uses a Fresnel zone plate lens to focus a monochromatic x-ray beam onto a sample. The sample is scanned through the fixed beam and transmitted photons are detected via a phosphor scintillator -photomultiplier tube assembly to provide a 2D image of the sample volume probed. On both beamlines, the theoretical spectral resolution during our measurements was ± 0.1 eV. Silica nitride windows with S. putrefaciens CN32 envelopes were analyzed under He at pressure < 1 atm.
Transmission images at single energies below and at the C-1s, N-1s, or O-1s absorption edge energies were used to derive chemical maps. Elemental distribution maps were obtained by subtracting optical density images recorded below and at the elemental absorption edge. Image sequence stacks acquired at multiple energies spanning the relevant absorption edge were used to create near-edge X-ray absorption fine structure (NEXAFS) spectra. The stack images were aligned via a spatial cross-correlation analysis and NEXAFS spectra were extracted from groups of pixels from relevant spatial areas. Potential beam-induced damage to the samples was carefully checked at the C-1s edge by recording fast stacks at few energy points spanning the C-1s energy range, and then a full finer stack on a similar region close by. The energy calibration was performed at C-1s using the 3p Rydberg peaks of gaseous CO$_2$ at 292.74 eV and 294.76 eV. Bacterial envelopes for all treatments were analyzed, as well as PE, PG, DPG, PS, and PC phospholipid standards (Sigma Aldrich, Oakville CAN). Lipopolysaccharide, bovine serum albumin protein (BSA), and DNA were also examined using NEXAFS at the CNO-1s edges. All STXM data processing was done using the IDL-based software package aXis2000 (Hitchcock 2008).

NEXAFS spectra were fit to Gaussian peaks and an arctangent step (Fig. 3.2) using the X-ray spectroscopy software package ATHENA (Ravel and Newville 2005), then optimized in Igor Pro (Wavemetrics Inc, Lake Oswego OR, USA) to minimize the residuals.
3.4 Results

3.4.1 TLC lipid separations

Thin layer chromatography plates were quantified using the ImageJ software package (Fig. 3.2). Membrane phospholipid compositions were similar for all aerobic treatments, however anoxic environments induced variation in phospholipid headgroup composition (Table 3.1). In anoxic conditions, V-treatment induced significantly higher PE proportions, and lower PG in S. putrefaciens CN32 cells. In addition, Mn and V treatments had a significantly lower DPG content than the control after culturing under anaerobic conditions. Anaerobic bacteria exhibited less DPG, and anaerobic control bacteria had significantly more PG within their envelopes than aerobic control counterparts. In addition to the major three lipids, there was also indication of an unknown lipid, which was present in varying amounts depending on treatment. Overall, in anoxic conditions V amendment to the growth medium induced a change from the control lipid composition, and the ratio of PE to PG varied between aerobic and anaerobic control treatments.

Table 3.1. Phospholipid compositions of S. putrefaciens CN32 cells grown in both aerobic and anaerobic conditions, with 1 mM concentrations of Mn(II), V(IV), or U(VI) amended into the medium. Values are presented as mean percent content (n=3, ± SD) of total lipids separated using 2D TLC. The zwitterionic:anionic ratio is also presented, as PE:[PG+DPG].

<table>
<thead>
<tr>
<th></th>
<th>PE</th>
<th>PG</th>
<th>DPG</th>
<th>Unknown</th>
<th>PE:[PG+DPG]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aerobic</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>66.82 (4.05)</td>
<td>12.98 (5.07)</td>
<td>14.34 (7.03)</td>
<td>5.86 (4.47)</td>
<td>2.45</td>
</tr>
<tr>
<td>Mn-treated</td>
<td>66.50 (0.53)</td>
<td>12.56 (0.62)</td>
<td>15.12 (1.01)</td>
<td>5.82 (1.33)</td>
<td>2.40</td>
</tr>
<tr>
<td>V-treated</td>
<td>60.90 (0.63)</td>
<td>16.37 (1.06)</td>
<td>14.87 (1.12)</td>
<td>7.86 (0.97)</td>
<td>1.95</td>
</tr>
<tr>
<td>U-treated</td>
<td>63.12 (0.28)</td>
<td>13.86 (0.37)</td>
<td>16.97 (0.46)</td>
<td>6.05 (0.42)</td>
<td>2.05</td>
</tr>
<tr>
<td><strong>Anaerobic</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>55.18 (1.79)</td>
<td>27.05 (2.23)</td>
<td>9.57 (1.44)</td>
<td>8.20 (1.65)</td>
<td>1.51</td>
</tr>
<tr>
<td>Mn-treated</td>
<td>69.28 (9.80)</td>
<td>20.49 (6.00)</td>
<td>4.41 (1.50)</td>
<td>5.82 (3.10)</td>
<td>2.78</td>
</tr>
<tr>
<td>V-treated</td>
<td>77.23 (1.80)</td>
<td>15.08 (0.80)</td>
<td>4.78 (0.50)</td>
<td>2.91 (2.20)</td>
<td>3.89</td>
</tr>
<tr>
<td>U-treated</td>
<td>66.11 (2.90)</td>
<td>16.65 (1.30)</td>
<td>10.53 (0.40)</td>
<td>6.71 (1.10)</td>
<td>2.43</td>
</tr>
</tbody>
</table>

*Indicates significant deviation from anaerobic treatment under identical metal treatment conditions

*b Indicates significant deviation from respective control value
Figure 3.2. Representative portion of TLC plate showing separation of aerobic control phospholipids. (A) Integrated density profiles generated in ImageJ demonstrate the need for 2D TLC, due to the overlap of some of the spots in the x or y plane; (B) 3D surface plot showing full separation of the lipids when run in two dimensions. These separate spots were traced around in ImageJ to obtain relative lipid quantities.
3.4.2 Lipid diffusion assays

Phospholipid liposomes were checked for purity using TEM (Fig. 3.3). Excimer:monomer ratios of PBA fluorescence were plotted against PBA concentration, and fit to linear functions (Fig. 3.4). Soluble Mn(II), V(IV), and U(VI) had varying effects on phospholipid diffusion in liposome suspensions. The relative effects of each metal on diffusion were estimated from the slopes of the linear fits (Table 3.2). Manganese(II) significantly increased the diffusion of lipids in *S. putrefaciens* CN32 phospholipid liposomes at 1 mM concentrations, but decreased the diffusion at lower concentrations. Vanadium(IV) induced significant increases in diffusion, based on higher excimer formations, at 1 mM concentrations only. Lower concentrations of V(IV) did not significantly impact lipid diffusion. Uranium(VI) had significant concentration-dependent inhibitory effects on diffusion, with the strength of the inhibition proportional to the concentration of the metal. At 0.1 and 1 mM concentrations, the uranyl ion significantly inhibited diffusion, and at the higher concentration impeded the formation of PBA excimers in the liposomes. There was low correlation between excimer:monomer ratio and pyrene concentration in the 1 mM U(VI) treatment, but the mean excimer:monomer ratio values for each of the plotted points were 0.360 ± 0.013. This indicates that the slope of the fit was about 0, based on the low standard deviation.

3.4.3 Envelope protein quantification

Envelope protein content was assayed and tabulated (Table 3.3). Control treatments had significantly less (P < 0.01) protein than Mn- or V-treated cell envelopes. This was observed to be independent of phospholipid composition, but was judged to be an important preliminary assay in analyzing π* transitions in NEXAFS spectra.
Table 3.2. Slopes of linear correlations of pyrene excimer:monomer ratio-to-pyrene concentration. Values are presented as mean slope (n=3, ± SD).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Metal concentration (mM)</th>
<th>Mean slope (± SD)</th>
<th>Percent of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>3.12 x 10^{-2} (1.65 x 10^{-3})</td>
<td>-</td>
</tr>
<tr>
<td>Mn(II)</td>
<td>0.01</td>
<td>2.07 x 10^{-2} (2.05 x 10^{-3})</td>
<td>6.63</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>2.12 x 10^{-2} (1.05 x 10^{-3})</td>
<td>67.95</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>4.84 x 10^{-2} (1.19 x 10^{-3})</td>
<td>155.13</td>
</tr>
<tr>
<td>V(IV)</td>
<td>0.01</td>
<td>2.63 x 10^{-2} (5.28 x 10^{-3})</td>
<td>84.29</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>3.16 x 10^{-2} (5.31 x 10^{-3})</td>
<td>101.28</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1.29 x 10^{-1} (7.04 x 10^{-3})</td>
<td>413.46</td>
</tr>
<tr>
<td>U(VI)</td>
<td>0.01</td>
<td>1.87 x 10^{-2} (1.87 x 10^{-3})</td>
<td>59.94</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>4.62 x 10^{-3} (3.70 x 10^{-4})</td>
<td>14.81</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>-9.10 x 10^{-4} (1.07 x 10^{-3})</td>
<td>-2.92</td>
</tr>
</tbody>
</table>

*a Indicates statistically significant deviation from control value (P < 0.01)

Figure 3.3. Transmission electron micrographs showing liposomes generated from *S. putrefaciens* CN32 phospholipid extracts, stained with 2% uranyl acetate for contrast. Presented are (A) liposomes before sonication; and (B) liposomes after sonication, which have a consistent diameter of about 140 nm.
Figure 3.4. Linear fits of mean pyrene excimer:monomer ratio (SD < 1%) correlated with pyrene concentration for (A) Mn(II)-treated liposomes; (B) V(IV)-treated liposomes; and (C) U(VI)-treated liposomes. Treatments were offset in the y-axis for clarity, and metal treatment concentrations follow the legend of: No metal addition □, 0.01 mM metal □, 0.1 mM metal △, 1 mM metal ▽.
Table 3.3. *Shewanella putrefaciens* CN32 envelope protein content after aerobic and anaerobic growth in control, Mn-treated, and V-treated growth media. Values are presented as mean protein weight (n=3, ± SD) per unit envelope weight.

<table>
<thead>
<tr>
<th></th>
<th>Envelope Protein (mg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aerobic</strong></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.256 (0.003)</td>
</tr>
<tr>
<td>Mn-treated</td>
<td>0.359 (0.008)</td>
</tr>
<tr>
<td>V-treated</td>
<td>0.315 (0.006)</td>
</tr>
<tr>
<td><strong>Anaerobic</strong></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.190 (0.001)</td>
</tr>
<tr>
<td>Mn-treated</td>
<td>0.383 (0.008)</td>
</tr>
<tr>
<td>V-treated</td>
<td>0.344 (0.015)</td>
</tr>
</tbody>
</table>

### 3.4.4 CNO NEXAFS spectroscopy

CNO 1s NEXAFS spectra demonstrate subtle differences across metal treatments, as well as between aerobic and anaerobic growth. At the C-K edge (Fig. 3.5, 3.6), peaks at 285 and 285.3 eV were similar in combined intensity across treatments, typically corresponding to $1s \rightarrow \pi^*(C=C)$ transitions. This likely indicates histidine and aromatic amino acid residues in proteins, and unsaturated fatty acids. Prominent peaks at 287.3 eV were consistent for all aerobic treatments. Anaerobic treatments exhibited lower absorbance at this energy, and typically correspond to aliphatic $1s \rightarrow \pi^*(C-H)$ transitions. The dual peaks at 288 and 289 eV were relatively equal in intensity across treatments, however the 289 eV peak had a stronger contribution towards overall spectral shape. These peak energies correspond to $1s \rightarrow \pi^*(C=O)$ carboxylic and carbonate transitions respectively. In the overall treatment spectra, the aerobic and anaerobic control treatments displayed less prominent absorbance at these energies; which had high absorbance in the BSA protein standard. This corresponded to the protein content trends in the envelopes. Past the ionization threshold of 290 eV, $1s \rightarrow \sigma^*(C-C)$ transitions at 292.6 and 300 eV were present in high amounts for both aerobic and anaerobic Mn-treated envelopes compared to the other
treatments. The complicated carbon structure of the cell envelope, and the energetic proximity of many $\pi^*$ transitions (Kaznacheyev et al. 2002; Plekan et al. 2008), make it challenging to differentiate contributions of individual carbonaceous components towards the overall C spectra in any of our samples. Aligning the standards and treatment spectra demonstrates, however, that the major peak at 288 eV in treatments is likely due to protein, with the broadening at 288.5 eV from phospholipid, and 289.5 eV from LPS.

Nitrogen spectra at the K edge (Fig. 3.7) showed minimal absorption at 399.5 eV, mainly in aerobic control and Mn-treated envelopes, which coincided with the DNA standard, and suggests incomplete DNase digestion of cell lysate DNA. There was distinctly higher absorption observed at the $1s \rightarrow \pi^*_{(N-H)}$ 401.3 eV transition for all aerobic envelopes, especially for aerobic control and Mn-treated envelopes. Absorbance peaks around 405.6 eV likely correspond to $1s \rightarrow \sigma^*_{(N-C)}$ transitions, and were much higher in aerobic and anaerobic control treatments than any metal treatment. In addition, these absorbance peaks were all higher in anaerobic treatments compared to their anaerobic counterparts. It is notable that in the aerobic Mn-treated envelopes, a strong secondary peak at 407.3 eV was also present. This gave a similar overall shape to the spectrum rather than a prominent peak at 405.6 eV, but explains the broadening of the peak after 406 eV. Peaks at 412 eV were very broad, and difficult to compare, but were similar across all treatments.

O-K edge spectra (Fig. 3.8) exhibited distinct $1s \rightarrow \pi^*_{(C=O)}$ transitions at 532.3 eV, suggestive of ester, ketone, and aldehyde groups. The overall intensities varied, however, across treatment, as aerobic control treatments displayed the highest absorbance at this energy while anaerobic
controls had the lowest absorbance. Peaks at about 536.4 eV likely correspond to $1s \rightarrow \sigma^*_\text{(O-H)}$ transitions, and were relatively consistent across treatments. A large, broad absorbance peak at 538.5 eV corresponding to the $1s \rightarrow \sigma^*_\text{(C-O)}$ transition was observed for all treatments. This peak was highest for the aerobic control, but stayed relatively consistent across treatments; statistical comparison was challenging, given that spectra were representative of various regions of the sample, rather than means of $n$ recorded stacks. A final $\sigma^*$ transition was observed at about 543 eV that was consistent across all treatments.

Figure 3.5. Representative NEXAFS fit after recording stacks at various portions of a sample at the C-K edge. This figure represents the NEXAFS spectrum for a C-1s aerobic control treatment. The residuals (A), fit spectrum (B), and Gaussian fits with arctangent step (C) are presented, with major transition energies discussed in section 3.4.3.
Figure 3.6. C-K edge NEXAFS spectra of envelopes from Mn(II) and V(IV) treated CN32 cells grown aerobically and anaerobically. These normalized spectra are presented alongside protein, DNA, phospholipid, and LPS standards. Envelope spectra are representative from stacks obtained from spatially different regions on the STXM window.
Figure 3.7. N-K edge NEXAFS spectra comparing metal-treated CN32 envelopes to phospholipid, protein, LPS, and DNA standards. BSA was used as a representative protein, but it should be noted that N spectra across many types of protein do not differ substantially (Zubavichus et al. 2008). Only phospholipids containing N were assayed at the N-K edge.
Figure 3.8. O-K edge NEXAFS spectra comparing metal-treated CN32 cell envelopes with protein, phospholipid, LPS, and DNA standards.
3.5 Discussion

Exposure to atmospheric oxygen and CO₂, as well as Mn-, V-, and U-amendment to the defined medium, altered both the lipid composition and protein content of the CN32 cell walls. Metal-amendment, and atmospheric exposure were the only direct variations in the growth media and environment in this study. The impacts of these two factors will, therefore, be discussed.

Functional group variations in CN32 envelopes across aerobic and anaerobic cultures were expected. In aerobic flask cultures, it is challenging to maintain an oxic environment throughout the flask, even with intense shaking (Wise 1951). Facultative anaerobes such as CN32 may contain more envelope protein growing aerobically than anaerobically, due to synthesis of anaerobic respiration proteins embedded alongside those necessary for aerobic growth. Myers and Myers (1992) observed striking differences in cytochromes and overall protein content between aerobic and anaerobically grown *Shewanella oneidensis* MR-1 (formerly *Shewanella putrefaciens* MR-1) cells, but a weight percentage of total envelope protein content was not given. Interestingly, Zubavichus et al. (2008) determined that protein type does not seem to substantially change CNO-K edge NEXAFS spectra, despite variation in protein size and structure. This is a strong indicator that the subtle variations observed in our spectra may be due to phospholipid chemistry. Although the protein contribution to the overall spectra may not change in shape, it will still have an impact on the spectral intensity. As such, between 288-289 eV in the C 1s spectra, the aerobic and anaerobic controls have substantially lower absorbance. This corresponds to a peak in the BSA protein standard, and, consistent with our protein quantifications, these treatments had the lowest protein content.
The metal-treated bacteria in both aerobic and anaerobic culture conditions had higher envelope protein content than cells grown without metal amendment. In chapter 2, under identical culture conditions Mn was found to bind to envelopes as Mn$^{2+}$, and V complexes were identified as VO$^{2+}$, using NEXAFS spectroscopy. There are known transport proteins for both Mn and V ions, but the transcriptional regulators of their biosynthesis are currently not well understood.

Manganese(II) transporters are known to be inhibited at 1 mM concentrations in *Synechocystis* sp., the organism in which the mntCAB ATP binding cassette (ABC) transporter system was originally identified (Yamaguchi et al. 2002; Bartsevich and Pakrasi 1995). In addition, Mn(II)-sensing proteins controlling the synthesis of general cation Nramp (natural resistance-associated macrophage protein) transporter are also known to be repressed at available metal concentrations greater than 1 µM in *Bacillus subtilis* (Que and Helmann 2000). Vanadium-uptake in bacteria is much less understood in comparison to Mn and other metals. Research by Bellenger et al. (2008a; 2008b) demonstrated vanadate-uptake in *Azotobacter* controlled by siderophore-style chelation, but this may be more a function of V-interaction with iron chelators (Baysse et al. 2000). Due to the high concentrations of metals used in our research, it is likely that the increased quantity of protein in envelopes harvested from metal-amended CN32 cultures in comparison to control cultures was not due to transport machineries. Hu et al. (2005) have shown that when *Caulobacter crescentus* was grown in the presence of 1 mM U(VI), genes encoding signal transduction and receptor proteins were substantially up-regulated. In the same study they also observed up-regulation of efflux-pump and oxidative stress genes when grown in Cd(II). These observations suggest that protein content of U(VI)-treated *S. putrefaciens* CN32 cell envelopes likely exceeded that of the control, but further investigation is necessary to
confirm this notion. This increased protein content in metal-treated cell envelopes was reflected in the fluidity and thermal stability results in chapter 2, and will be further discussed in chapter 5.

Phospholipid functional group chemistry can determine metal binding in a manner dependent on environmental redox and pH conditions. Phospholipid compositions of *S. putrefaciens* CN32 membranes derived from this experiment are similar to those published for *Shewanella fidelis*, *Shewanella affinis*, *Shewanella frigidimarina* and *Shewanella pacifica* (Frolova et al. 2005). This study also reported an unknown phospholipid present at about 2-8% of total lipid, but the R_f values for this lipid were not mentioned in the report and could not be compared to the ones on our plates. Most bacteria predominately exhibit PE, PG, and DPG (Seltmann and Holst 2002; Cronan 2003; Zhang and Rock 2008). Regulation of the pathways synthesizing phospholipids have been linked to osmotic stresses and the ionic strength of the environment (Romantsov et al. 2009), temperature (Heimburg 2007; Russel 1984a), and organic solvents (Weber and De Bont 1996; Segura et al. 1999). Lipids PE, PG, and DPG will be individually discussed within the context of our research.

The dominant lipid in many bacteria is PE, and the significant decrease in anaerobic control PE content is accompanied by a significant rise in PG in comparison to the aerobic control. Zhang and Rock (2008) have indicated that the ratio of zwitterionic-to-anionic phospholipids is regulated by the tight regulation of PE, with PG being synthesized continuously, and DPG being synthesized reversibly through the Cls enzyme from the PG precursor. As a result, zwitterionic:anionic ratios in bacteria are usually maintained consistently. In *S. putrefaciens* CN32 they were between 2.0-2.5 for all treatments with the exceptions of the anaerobic control
(1.5), and anaerobic V-treated (3.9). This is the first study to investigate the effects of metal ions or anoxia on regulation of phospholipid biosynthesis, and comparative data are, therefore, not available. The low DPG content observed in *S. putrefaciens* CN32 is common in other species of *Shewanella*, and *E. coli* cells totally lacking the ability to synthesize this lipid are still able to grow and divide normally (Nishijima et al. 1988). In *S. putrefaciens* CN32, DPG was lowest under anaerobic conditions, primarily in Mn- and V-treated cells. Due to its dimeric nature, with four acyl chains and a relatively small headgroup surface area, DPG is flexible about its C-C bonds and serves to reduce the exposure of membrane hydrophobic regions to the aqueous milieu. This is especially true at the cell poles and when adjacent to proteins. Romantsov et al. (2009) have compiled an excellent review of literature suggesting that DPG content increases as a function of osmotic stress. Ionic strengths of media in our study were calculated in CHEAQS, and were found to be 0.0172 (± 0.00023) M, with the exception of the Mn(II)-amended media, which were 0.0211 and 0.192 M for oxic and anoxic media respectively. There were no significant differences in DPG content in Mn-treated bacteria, so this was likely not a factor in our study. In U(VI)-treated cultures, cells did not have a significantly different DPG content than in the respective aerobic or anaerobic control treatments.

In our treatments, PG was higher in the anaerobic control, but did not change substantially across the other treatments. Compared to the variations in PE and DPG content, the steady levels of PG despite soluble cation presence suggest it may be less involved in biochemical adaptation to environmental metal stimuli. In their comprehensive review, Zhang and Rock (2008) suggest that PG is synthesized continuously in bacteria, and that turnover rate is high. This helps to explain why PG levels remain relatively steady. It is important to note that since our lipid
amounts are listed as percentages of total lipid, if one lipid is in lower quantity, the other lipids will be consequently higher. In cases of high PG, due to the continuous synthesis of this lipid it is more likely that PE or DPG syntheses are impacted. The higher PG content in the anaerobic control treatment may have also influenced liposome formation from phospholipids extracted from *S. putrefaciens* CN32 cells under those conditions. Why this would be the case is unclear, as pure PG liposomes can form, but it is important to note that liposome formation is also strongly influenced by the acyl chain branching and saturation chemistry. In chapter 2 it was shown that significantly more saturated fatty acids were present in anaerobically cultured *S. putrefaciens* CN32 bacteria, which may have impacted liposome formation from phospholipids extracted from the anaerobic control treatment.

The various metal treatments had strong impacts on the phospholipid diffusion within liposomes formed from the aerobic control bacteria. Manganese(II) concentrations less than 1 mM inhibited diffusion, yet beyond this increased the diffusion substantially. A similar trend was observed in V(IV)-treated liposomes, but the increase in diffusion was much higher. These observations likely coincide with charge reversal of the phospholipid liposomes under these conditions. Unlike cell membranes, liposomes do not include charged LPS, peptidoglycan, or protein within their membranes. This decreases the amount of negative charge that would need to be addressed before charge reversal could occur. Charge reversal is an important concept in lipid diffusion, as electrostatic repulsion is a major contributor to a dynamic membrane. As the charge of the membrane approaches 0 due to cation binding, the electrostatic repulsion is reduced, and hydrophobic and van der Waals attractions reduce the mobility of lipids. In scenarios where charge reversal occurs, the electrostatic repulsion is reintroduced, potentially
with stronger effects if bound metals increase the magnitude of the charge beyond that of the initial anionic charge of the liposome. In the U(VI)-treated liposomes, the concentration-dependent inhibition of diffusion may be due to bidentate lipid-metal-lipid complexation. This was observed to be possible in LPS (Barkleit et al. 2008), and based on the proximity of the anionic ligands, is a possibility in liposomes as well. While this study examined lateral diffusion, it is probable that the binding of the uranyl ion (binding confirmed as an efficient TEM stain for liposomes; Fig. 3.1) will inhibit transverse diffusion as well.

The diffusion assay examined membranes from a natural source, but the liposomes were still generated synthetically. The liposomes combined lipids from the outer membrane, as well as both leaflets of the cytoplasmic membrane into phospholipid combinations that may not be applicable to whole cell scenarios. The absence of proteins is another issue that should be acknowledged, as protein sequestration of phospholipids will affect their diffusion. Results from the diffusion assay do, however, still stand as valuable data due to the sheer number of phospholipid molecules within bacterial envelopes.

The spectra from CNO-K edges of lipid standards and LPS offer insight into the overall shapes of spectra from sample treatments. Standards did not seem to correspond to variations in lipid type in bacteria assayed through TLC. Given the chemically similar nature of lipid headgroups, and immense variation in fatty acid chemistry (Table 2.2), this is not surprising. Phospholipid headgroups typically differ by a single functional group. As such, NEXAFS is a powerful tool in determining the chemical forms of metals bound to cell envelopes (Fig. 2.5, 2.6), however CNO spectra of elaborate cellular organelle structures such as envelopes are difficult to interpret using
this technique. This was seen in the C 1s transitions presented in our results that were ubiquitous across both standards and cell envelope treatments. Individual components such as phospholipids presented alongside our spectra (Fig. 3.6) will deviate slightly across acyl chain chemistry, as length of hydrocarbon chains will alter the absorbance at 1s→σ^*(C−C) and 1s→π^*(C−H) transitions. These variations occur within a single pure culture (Table 2.2), and restrict the use of NEXAFS as a means of identifying specific chemical variations in lipids within cell envelopes. There are no indicator transitions for a particular lipid functional group available that are not shared by another lipid, LPS, or protein molecule. Envelope structures such as peptidoglycan and LPS are not known to chemically vary in response to environmental metal stimuli. LPS can be variable on cell surfaces, as observed for the A- and B-band variability in Pseudomonas aeruginosa (Rivera et al. 1988; Hancock and Nikaido 1978), but this is believed to maintain endotoxin variability in pathogen virulence systems (Gunn 2001; Guo et al. 1997; Raetz et al. 2007). This is not the case in S. putrefaciens CN32 cells, which maintain stable core chemistry, with acyl chain modification presumably typical of Gram-negative bacteria (Raetz et al. 2007).

In this experiment, 2D TLC was used instead of high-performance liquid chromatography (HPLC) to separate phospholipids. Given the possibility of lysophospholipids in CN32 (Frolova et al. 2005), TLC was required due to the difficulty of quantifying these lipids using HPLC (Helmerich and Koehler 2003). Two-dimensional TLC and HPLC offer comparable means of separation efficiency with complex lipid mixtures, with detection limits being the drawback in TLC. ImageJ is capable of resolving even minute changes in lipid spots when visualized using a non-fluorescent stain, but iodine detection limits for phospholipids are still only 0.1-0.5 µg (Fried and Sherma 1999). If a lipid is present on the TLC plate, but exists below the detection
limits for the stain, the spot will not be quantified and the entire percentage composition of the other lipids will change accordingly. While this is not expected to have occurred in our experiment, it is a possibility and must be considered.

**Conclusion**

The results of this research indicate that *Shewanella putrefaciens* CN32 cells growing anaerobically alter their phospholipid headgroup proportions in favor of PG, at the cost of DPG. Anaerobic growth conditions and metal-amendment to the growth medium also induces higher PE content than in control, or aerobically cultured cells. Manganese(II), V(IV), and U(VI) have similar impacts on CN32 headgroup proportions in anaerobic conditions, but do not induce significant changes when cultured aerobically. Manganese(II)- and V(IV)-treatments increase the protein content of CN32 cell walls, and this impact is observed across both aerobic and anaerobic treatments. Manganese(II) and V(IV) also increase phospholipid diffusion in membrane liposomes at 1 mM concentrations, while U(VI) strongly inhibits diffusion proportionally to metal concentration. CNO 1s NEXAFS spectra do reveal cell wall functional groups, but attributing peaks to individual components is challenging due to the chemical complexity of bacterial cell walls. Protein spectra do not vary in spectral shape, only in intensity. As such, the 1s→π* resonance at 288-289 eV in C 1s spectra may be helpful in estimating protein quantity within a cell wall, but using NEXAFS as a sole means of relative protein quantification would be inappropriate.

Our findings complement research performed in chapter 2, and elaborate on the idea that bacterial growth environments can greatly affect membrane lipid chemistry. Bacterial envelopes
are rich in functional groups that extend far beyond the cytoplasmic membrane. Incidentally, the overall quantities or proportions of functional groups within the envelope may not be substantially altered across growth conditions, but within phospholipids this is not the case.
CHAPTER 4

IMPACTS OF SOLUBLE METALS ON THE PERMEABILITY OF GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIA

Submitted to Antonie van Leeuwenhoek

CHAPTER FOUR

Impacts of soluble metals on the permeability of Gram-positive and Gram-negative bacteria

4.1 Abstract

The impacts that soluble Ca(II), Cu(II), Mn(II), U(VI), V(IV), and Zn(II) have on *Escherichia coli* AB264, *Shewanella putrefaciens* CN32, *Pseudomonas aeruginosa* PAO1, and *Bacillus subtilis* 168 cell permeability were investigated in this study. The nucleic acid-binding fluorescent probe 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) was used as an indicator of membrane permeability. Variations in DAPI fluorescence intensity after cells had been exposed to 0.001, 0.01, 0.1 and 1 mM metal concentrations indicated permeability effects of the soluble metal cations. Ca, Cu, Mn, V, and Zn permeabilized cell walls; U, in contrast, appeared to impede penetration of the probe. With the exception of V, metal effects were consistent across all strains of bacteria. These effects differed in strength. Cell wall-bound metals were quantified using inductively coupled optical emission spectrometry, and corresponded to the permeability variations seen within the range of concentrations used (0.001 to 1 mM). This suggested that metal counterions bound to the cell wall have a stronger effect on permeability, rather than only as contributors to background electrolyte effects. Fatty acid profiles of lipids extracted from whole cells of each species were obtained using gas chromatography and flame ionization detection. Lipid acyl chain chemistry suggested that unsaturated fatty acid content had a weak positive correlation with metal-induced permeability, likely due to hydrophobic attraction within the bilayer.
4.2 Introduction

Soluble metals are ubiquitous in environments where bacteria are found. Diffusion of these metals towards, and adsorption to, bacterial surfaces occurs readily and can be explained by the availability of metal-reactive groups within bacterial cell envelopes (Beveridge 1989; Beveridge 2005; Beveridge and Koval 1981; Beveridge and Murray 1976). There have been few reports that identify effects of soluble metals on membrane permeability, although the impacts of various alkaline Earth and transition metals on lipopolysaccharide (LPS) dynamics (Schneck et al. 2009), antimicrobial peptide effectiveness (Pink et al. 2003), and phospholipid chemistry (Popova et al. 2008) have been investigated. Cell permeability itself is a function of the physicochemical properties of the cell envelope as well as the substances passing through it. The necessarily semi-permeable cytoplasmic membrane may be adversely affected by metal adsorption, with implications for cell survival in bioremediation environments, as well as for development of antimicrobial agents. Essential divalent cations such as Ca$^{2+}$ and Mg$^{2+}$ effectively cross-link and stabilize outer membrane (OM) lipopolysaccharide (LPS) molecules in Gram-negative bacteria (Ferris and Beveridge 1986a; Ferris 1989). Conversely, these same metals have been used to increase the permeability of membranes for plasmid introduction (Mandel and Higa 1970; Norgard et al. 1978; Chung et al. 1989), which is a common technique in molecular microbiology. Little is known, however, about the permeabilizing effects that other metals may have within cell envelopes.

Bacterial membranes host a variety of carboxyl, phosphoryl, hydroxyl, and amide groups that are necessary to maintain cell structure and function. These sites are present on proteins, LPS or teichoic acids, peptidoglycan, and lipids. Metal binding to the metal reactive groups poses an
obstacle in maintaining the necessarily fluid cytoplasmic membrane (CM), and for functions such as respiration or selective permeability. Phospholipids in particular tend to regulate the overall fluidity of cell membranes (Russel 1984b; Cronan 2006; Denich et al. 2003), primarily through chemical changes in fatty acid (FA) chemistry. Fatty acid desaturation can occur due to desaturase activity within the membrane, which has been identified in *Bacillus subtilis* and *Pseudomonas aeruginosa* (Aguilar et al. 2001; Weber et al. 2001; Zhang et al. 2007), or through *de novo* biosynthesis (Cronan 2003; Zhang and Rock 2008). Cation binding decreases the homogeneity of phospholipids within the membrane, inducing de-mixing (Mbamala et al. 2005; Gelbart and Bruinsma 1997; Leckband et al. 1993; Träuble et al. 1976), and may also dehydrate the phospholipid headgroups. This effectively shrinks their surface area and exposes more of the hydrophilic regions (Leckband et al. 1993; Israelachvili 2011). Hydrophobic effects and FA stereochemistry dictate that FA saturation will play a pivotal role in determining the permeability of the membrane in such a scenario.

Metals appear to impact cell walls differently across bacterial species. Bacteria used in past studies of metal-membrane interaction include various *Bacillus, Pseudomonas, E. coli,* and *Shewanella* species. Each of these organisms shares similar chemistry at the CM; the peptidoglycan and surface sugars are, however, very different. From these chemical differences arise variations in charged sites within the polyelectrolyte brush of the cell wall, which subsequently impact metal binding capacity and surface charge. Comparisons of metal-induced permeability effects on cell walls across species with different LPS or teichoic acid chemistries will supplement current understanding of total cell permeability. The effects Ca$^{2+}$ and Mg$^{2+}$ on membrane spatial organization have been studied extensively in lab and theoretical studies.
(Leckband et al. 1993; Pink et al. 2003; Schneck et al. 2009; Bashford et al. 1988). The impacts of other environmentally ubiquitous metals such as Zn and Mn, and redox-active contaminant metals such as V or U, have not been considered in spite of widespread presence in the environment and proposed bioremediation strategies (Mattes et al. 2011). The intermolecular forces responsible for membrane organization and permeability are becoming increasingly well understood (Mbamala et al. 2005; Israelachvili 2011; Träuble et al. 1976), but a great deal of this understanding arises from synthetic membrane studies in highly controlled environments. Experimental permeability data that include a range of metals, and use whole cells, would help to apply this theoretical understanding to bacteria growing in environments abundant in metals.

Fluorescent probes used in the past to study permeability include 1-N-phenylnaphthylamine (NPN)(Loh et al. 1984; Hancock and Wong 1984), 3,3’-dipropylthiadicarbocyanine iodide (DiSC3-5) for membrane potential and CM permeability (Wu and Hancock 1999), and propidium iodide (Wu et al. 2003) such as in the BacLight™ LIVE/DEAD stain (Invitrogen). Both NPN and DiSC3-5 are pH and redox sensitive, and not suited for permeability studies when metal addition changes the sample pH, or when 3 mL of suspension is used with a potential redox gradient in the cuvette. Propidium iodide is used to assess membrane-compromised cells only; in the cases where membranes are rendered less permeable, this probe will not be suitable. The fluorescent probe 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) has not been used in the past as a probe in bacterial cell wall permeability research. Its negligible fluorescence in solution, high intracellular fluorescence, and cationic nature, however, suggest it is ideal for metal-membrane permeability studies. DAPI is also water soluble, which allows for interesting
comparisons to soluble metals. It is for these reasons that the DAPI probe is used in this research as a metal-insensitive probe of total cell permeability.

The purpose of this experiment is to identify cell wall permeability effects associated with soluble species of the metals Ca(II), Cu(II), Mn(II), U(VI), V(IV), and Zn(II), in *E. coli*, *B. subtilis*, *P. aeruginosa*, and *S. putrefaciens*. How lipid FA chemistry and metals bound within these diverse cell walls impact permeability will also be examined. I hypothesize that metals will permeabilize the bacterial cell walls, with the strength of the permeabilization dependent on metal binding. It is also expected that saturated acyl chain content within the membrane will decrease permeability, as electrostatic effects within the membrane are lessened by metal cations in solution.

4.3 Materials and methods

4.3.1 Cell culture conditions

Cultures of *Bacillus subtilis* 168 and *E. coli* AB264 (K-12 with only core regions present within its LPS) were provided by Dr. Susan Koval (University of Western Ontario, London CAN). *Pseudomonas aeruginosa* PAO1 cultures were provided by Dr. Joseph Lam (University of Guelph, Guelph CAN). *Shewanella putrefaciens* CN32 was grown from stock cultures stored at -80°C in trypticase soy broth (TSB)-sterile glycerol (1:1 v/v). All cell cultures were grown initially on trypticase soy agar (TSA) for 30 hours at 37°C, and at 25°C for CN32. Isolated colonies were asceptically transferred into 10% TSB, and shaken at 300 RPM until growth reached the exponential phase: 8 hours for CN32, and 5 hours for the other species, at the previously described temperatures. Cultures were harvested at 4300 x g for 10 minutes, in
preparation for subsequent analyses, and all cultures were prepared in triplicate. All culture glassware was acid washed overnight in 0.1 M HCl, then rinsed three times in Millipore nanopure water and oven dried prior to autoclaving with growth medium, in order to eliminate residual metals.

4.3.2 Metal content of trypticase soy broth

The metal profiles of TSB were examined using a Varian Vista Pro inductively coupled plasma-optical emission spectrometer (ICP-OES; Agilent Technologies) with axially viewed plasma. Digestion methods were modified from EPA Method 3050B (USEPA 1996). TSB powder was transferred into a high-pressure Teflon bomb, to which 9.0 mL of HNO₃ was added, followed by gentle swirling and 3 mL of HCl. The sample was pre-digested for two hours, then tightly sealed and placed into an oven for three hours at 110°C. The vessel was allowed to cool at 25°C for two hours, after which the solution was filtered through Whatman #42 filter paper and brought to 25 mL final volume using Millipore 18.3 MΩ-cm water. Stock standards (SCP Science, Quebec CAN) of 1000 mg/L were diluted with the same acid concentrations and water as the sample digestions to make final concentrations of 0.50, 5.00, and 10.00 mg/L as calibration standards. A reverse aqua regia (3:1 HNO₃:HCl v/v) blank mixture was also put through the digestion procedure, and a separate multi-element 1.0 ppm check standard was analyzed. The detection limits for these analyses are around 0.1 mg/L for most elements. ICP-OES glassware was acid washed in 1 N HNO₃ overnight, rinsed three times in Millipore nanopure water, and oven dried prior to use.
4.3.3 Membrane permeability assays

Metal salts used in the permeability experiment were CaCl₂, CuCl₂, MnCl₂, UO₂(CH₃COOH)₂, VCl₄, and ZnCl₂. These metals were prepared individually as concentrated stock solutions, which were deoxygenated by bubbling oxygen-scrubbed N₂ gas through the solution for 30 mins/100 mL, followed by 10 minutes of headspace degassing. This step lowers the redox potential, and purges carbon dioxide from the headspace to eliminate carbonate complexation with metals, in the stock solutions. The solutions were filter sterilized (0.22 µm filter) into acid-washed autoclaved serum bottles in an anaerobic chamber. No precipitation or discoloration was observed in any of the anoxic vials after this preparation. Metal treatments of 0.0010, 0.010, 0.10, and 1.0 mM concentrations were used for each metal, for each analysis step. All metals in this study were the highest grade available, and purchased from Sigma-Aldrich (Oakville, CAN).

Figure 4.1. The chemical structure of 4,6-diamidino-2-phenylindole dihydrochloride (DAPI), which binds in the minor grooves of DNA molecules, and through intercalation to RNA molecules (Tanious et al. 1992).

Permeability was measured using the fluorescent probe DAPI (Fig. 4.1; excitation 345 nm, emission 455 nm). While typically used as a nucleic acid stain, it was found that pre-treating bacteria with metals altered the permeability of the CM to DAPI entering the cell. This offered an indication of the degree of permeability through quantification of DAPI fluorescence. The
assay was accomplished by preparing washed culture pellets to an OD\textsubscript{600} of 0.20 ($\sim 2 \times 10^8$ cells/mL) in 5 mM HEPES (pH 6.8), followed by aliquoting 3 mL of suspension into a methacrylate cuvette (path length 1 cm) with a clean magnetic stirrer. Afterwards, metal solution was added to the desired concentration, maintaining pH at 6.8, and the solution was left to equilibrate for 15 minutes. To this, 1 $\mu$L of 0.9 mM DAPI probe in ethanol was added, to a final concentration of 0.3 $\mu$M. The solution was left to equilibrate in the dark for 15 minutes. Time-based emission scans of DAPI fluorescence were performed in an earlier experiment, and fluorescence intensity was found to plateau within 15 minutes for each metal treatment. Fluorescence emission spectra were obtained by collecting fluorescence intensity between 400-575 nm using a spectrofluorometer equipped with a temperature controller and electronic stirrer, and slit width of 4 nm (Photon Technologies International, London ON, CAN). This was repeated three times for each culture at 25ºC, for each metal, as well as controls for metal-free cells, background fluorescence of probe, and any probe-free background emission from the solution.

### 4.3.4 Metal content of bacterial cell walls

Quantification of cell wall-associated metals in the permeability experiment was performed using ICP-OES. The accurate concentrations of the metal stock solutions used for the permeability assay were obtained using the same ICP parameters as in the TSB metal quantification, but without the acid digestion. Bacterial cultures were prepared according to the methods in the permeability assay (section 4.3.3), brought to an OD\textsubscript{600} of 0.20, and incubated with the same metals as in the permeability assay under the same conditions. Cells were pelleted at 4300 x g for 20 minutes, and the supernatant was carefully assessed for metal content using
the same ICP conditions as with the stock solution metal quantifications. These values were subtracted from the concentrations of the stock solutions to estimate the metal associated with the cell surfaces in our permeability assay conditions.

4.3.5 Fatty acid extraction and lipid profiling

Membrane FA were extracted and characterized according to the methods in Chapter 2, with minor changes. Small subsamples of TSB cultures used in the permeability assay were washed five times with sterile water to remove potential artifact lipids from the soy broth. Lipids were subsequently extracted from cells in a wire loop of this pellet. The sums of saturated and unsaturated FA were calculated from the data outputs as previously described in Chapter 2. The ratios of unsaturated:saturated FA offer a means to generalize membrane structure by estimating the overall shape of total phospholipids (Kim et al. 2001; Trevors 2003). Saturated and trans-acting lipids tend to have a cylindrical shape with respect to occupied space, while unsaturated, branched, cyclopropyl, and cis-acting lipids have a more conical shape. This shape is dependent on the surface area of the headgroup as well. This includes dehydration effects, which arise from water-binding limitations after cation-binding within a lipid headgroup (Tatulian et al. 1991). Phospholipid shapes regulate packing density and order within the membrane, and will greatly affect membrane permeability.

4.4 Results

4.4.1 Metal composition of TSB medium

Metal content of TSB medium was analyzed using ICP-OES, in order to determine which metals were present in the common growth medium prior to exposure to the various metal treatments.
(Table 4.1). This was necessary for the permeability experiment, to ensure that the metals of interest were not present in comparable concentrations in the TSB medium prior to exposure in the permeability assay. Calcium, at a concentration of 0.02 mM, was the only element present in 10% TSB that may have been available to cells at a concentration comparable to ones in the permeability assay. This does not necessarily mean that surfaces are calcium-rich prior to metal treatment, but may affect the extent of Ca$^{2+}$ binding to the cell wall upon metal treatment during permeability assays. The TSB medium has a high concentration of Na and K, with other elements present in lower concentrations. Aluminum and Mo were present in concentrations just above the minimum detection limits, and this suggested that other elements might be present, but exist below the detection limits of ICP-OES.

Table 4.1. Metal composition of TSB broth medium, quantified using ICP-OES. Values are presented as means (n=3, ± SD), with ICP-OES detection limits of about 0.1 mg/L. Elements As, Ba, Cd, Co, Cr, Cu, Hg, Mn, Ni, Pb, Sb, Se, and Sr were also analyzed, but were absent or below the detection limits.

<table>
<thead>
<tr>
<th></th>
<th>Powder (mg/g)</th>
<th>100% TSB (mg/L)</th>
<th>10% TSB (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Al</td>
<td>0.006 (0.002)</td>
<td>0.192 (0.045)</td>
<td>0.019 (0.005)</td>
</tr>
<tr>
<td>Ca</td>
<td>0.257 (0.012)</td>
<td>7.702 (0.367)</td>
<td>0.770 (0.037)</td>
</tr>
<tr>
<td>Fe</td>
<td>0.018 (0.001)</td>
<td>0.541 (0.028)</td>
<td>0.054 (0.003)</td>
</tr>
<tr>
<td>K</td>
<td>37.001 (1.300)</td>
<td>1110.027 (39.012)</td>
<td>111.003 (3.901)</td>
</tr>
<tr>
<td>Mg</td>
<td>0.252 (0.001)</td>
<td>7.573 (0.022)</td>
<td>0.757 (0.002)</td>
</tr>
<tr>
<td>Mo</td>
<td>0.005 (0.001)</td>
<td>0.155 (0.015)</td>
<td>0.015 (0.002)</td>
</tr>
<tr>
<td>Na</td>
<td>79.153 (1.463)</td>
<td>2374.585 (43.892)</td>
<td>237.458 (4.389)</td>
</tr>
<tr>
<td>Si</td>
<td>0.104 (0.002)</td>
<td>3.120 (0.073)</td>
<td>0.312 (0.007)</td>
</tr>
<tr>
<td>Zn</td>
<td>0.014 (0.001)</td>
<td>0.422 (0.016)</td>
<td>0.042 (0.002)</td>
</tr>
</tbody>
</table>

4.4.2 Membrane permeability assays

Based on the DAPI assay, cell permeability varied in response to metals, as functions of the metal and the concentrations (Fig. 4.2). Uranium consistently rendered cells less permeable to the DAPI probe in all strains of bacteria used in this research. All other metals had consistent
permeabilizing effects on the cell wall, with the exception of V in PAO1 suspensions. At concentrations greater than 0.10 mM, Ca, Cu, and Zn had lower permeability than at 0.10 mM concentrations in *B. subtilis* 168 and *P. aeruginosa* PAO1. This was observed in *E. coli* AB264 at 1 mM concentrations of Cu only. Metals in solution had a minimal impact on permeability of *B. subtilis* 168 cells, while other strains demonstrated similar levels of permeabilization to the DAPI probe.

Permeability changes in membranes due to metal exposure tended to follow a log-linear regression that resulted in plateaus in permeability at the highest metal concentrations. Metal presence for all metals except V and U caused a metal-induced blue-shift of DAPI emission spectra towards a peak of 460 nm (data not shown); control treatments had an emission peak at about 470 nm. This observation required full emission spectra to be obtained from 400-560 nm each time, incorporating the peak fluorescence intensity into the presented mean (Fig. 4.2). Fluorescence emission at 460 nm corresponds to minor groove DAPI-DNA complexes. An emission wavelength closer to 470 nm corresponds to DAPI-RNA intercalation complexes (Tanious et al. 1992). The metal-induced blue-shifts suggest that DAPI may preferentially bind to RNA in bacteria, with DAPI-DNA complexes forming when intracellular probe concentrations are higher. This is in contrast to published findings (Tanious et al. 1992; Kapuscinski 1995), but it is important to note that these studies were performed using pure polyA-T or polyA-U solutions rather than bacterial suspensions.

To ensure that fluorescence plateaus were not due to DAPI saturation of nucleic acids in the cell suspensions, several cuvettes containing only cells and buffer were heated to 90ºC for 10 minutes
to disrupt and lyse cells, and release DNA into solution. Incorporating DAPI into these cuvettes after they were rapidly returned to 25°C using the water-cooling stage in the spectrofluorometer resulted in a large increase in fluorescence. This indicated that plateaus in permeability were not due to DAPI saturation.

DAPI-metal associations, if occurring, did not seem to interfere with DAPI-DNA complexation. Even at the lowest metal concentration, metals were present in substantially higher solution concentrations than the DAPI probe, and changes in fluorescence were still linked with metal concentration. It was, therefore, assumed that changes in DAPI fluorescence were due to the impacts of metals on cell wall permeability rather than concentration-dependent DAPI-metal complexation inhibiting DAPI-nucleic acid associations.

4.4.3 Envelope-associated metals

The quantity of metals associated with bacteria in suspension was found to increase according to treatment concentration (Fig. 4.3). The Zn treatments were an exception (Fig. 4.3d); in this case, cells seemed to become saturated with Zn at concentrations ≤ 0.10 mM. For U at all concentrations, nearly all U was associated with *S. putrefaciens* and *P. aeruginosa* cells. Such extensive binding was only observed for U-treatments. Uranium was also the only metal that rendered the bacteria less permeable to the DAPI probe. Uranium- and Cu-treated cells both had positive log-linear relationships between quantity of metal associated with the cell wall, and the treatment concentration. The other metals were below the ICP detection limits at lower concentrations, and could not be plotted logarithmically because they had zero values for mean. Calcium and Mn either did not bind, or concentrations were below the detection limits of ICP-
OES. *S. putrefaciens* CN32 and *P. aeruginosa* PAO1 bound the highest amount of metal for each of the Cu, U, V, and Zn treatments. *B. subtilis* 168 and *E. coli* AB264 bound the least amount of these metals. Observations coincide somewhat with the permeability data, where *Shewanella* and *Pseudomonas* had the highest DAPI fluorescence in metal treatments, and *Bacillus* was least affected. The observation that Ca was present at levels below the ICP-OES detection limits may be due to its presence in the TSB medium, and probable persistence within the cell envelope after transfer to the buffer solution, reducing its ability to bind more Ca during the metal-association experiment. Removal of Mn from solution by the bacteria will be discussed further on.
Figure 4.2. Total cell permeability of DAPI probe presented as mean peak fluorescence intensity of $\sim 2 \times 10^8$ cells $\cdot$ mL$^{-1}$ ($n=3$, ± SD) after exposing cells to (a) Ca, (b) Cu, (c) Mn, (d) U, (e) V, and (f) Zn for 15 minutes at 25°C. Species presented are *E. coli* AB264 (○), *S. putrefaciens* CN32 (□), *P. aeruginosa* PAO1 (△), and *B. subtilis* 168 (▽). Plots are offset in the y-axis for clarity.
Figure 4.3. Molar concentrations of metals bound to various bacterial species in suspension; cell concentrations are \( \sim 2.0 \times 10^8 \) cells \( \cdot \) mL, the same concentrations used in the permeability assay. All metal concentrations are displayed as their mean (\( n=3, \pm SD \)). Bound Ca and Mn were quantified as well, but were either not present, or below the detection limits of ICP-OES (\( \sim 0.1 \) mg/L). Bacterial species presented are *E. coli* AB264 (○), *S. putrefaciens* CN32 (□), *P. aeruginosa* PAO1 (Δ), and *B. subtilis* 168 (▽).
4.4.4 Fatty acid profiles

Fatty acids extracted from *S. putrefaciens* CN32, *B. subtilis* 168, *P. aeruginosa* PAO1, and *E. coli* AB264, and their corresponding percentages were determined along with unsaturated:saturated ratios (Table 4.2). Fatty acid ratios varied across species, but standard deviations were high. *S. putrefaciens* CN32 possessed a large percentage of 16:1 w7c FA, indicative of fluid membranes. In contrast, *E. coli* AB264 was found to have more [saturated, branched, cyclo] FA than saturated chains, in particular palmitic acid, which is reflected in the FA ratio. The high energetic cost of cyclopropyl FA formation indicates that *E. coli* AB264 may be fermenting sugars within the TSB medium, as these FA are typically found in acid-stressed cell membranes (Brown et al. 1997; Chang and Cronan 1999), and flask cultures are never fully aerobic (Wise 1951). *P. aeruginosa* PAO1 membrane acyl chains were predominantly comprised of 16:0 and 18:1 w7c FA. *B. subtilis* 168 had an extremely low percentage of saturated FA and high standard deviations. When FA ratios were plotted against peak relative DAPI fluorescence values for each metal, a general linear relationship was observed between the permeability effect of the metal and quantity of unsaturated FA in the membrane (Fig. 4.4). Correlations between FA ratio and DAPI permeability varied with the metal treatment, with Ca and Cu treatments having the highest correlations ($R^2 = 0.97$ and 0.90 respectively). Manganese-, U-, and Zn-treated cells had weaker correlations ($R^2 = 0.82, 0.68,$ and 0.62), and there was no correlation between permeability and FA ratio in V-treated cells ($R^2 = 0.21$).
<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>E. coli AB264</th>
<th>S. putrefaciens CN32</th>
<th>P. aeruginosa PAO1</th>
<th>B. subtilis 168</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saturated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12:0</td>
<td>4.77 (0.74)</td>
<td>3.52 (0.67)</td>
<td>4.00 (0.20)</td>
<td>0.51 (0.88)</td>
</tr>
<tr>
<td>14:0</td>
<td>9.47 (0.39)</td>
<td>2.31 (0.60)</td>
<td>0.82 (0.17)</td>
<td>0.51 (0.89)</td>
</tr>
<tr>
<td>15:0</td>
<td>1.81 (0.13)</td>
<td>3.74 (0.18)</td>
<td>0.53 (0.05)</td>
<td>0.00 (0.00)</td>
</tr>
<tr>
<td>16:0</td>
<td>39.76 (0.98)</td>
<td>11.00 (0.39)</td>
<td>28.00 (0.03)</td>
<td>3.57 (1.21)</td>
</tr>
<tr>
<td>17:0</td>
<td>0.84 (0.11)</td>
<td>1.18 (0.05)</td>
<td>0.00 (0.00)</td>
<td>0.00 (0.00)</td>
</tr>
<tr>
<td>18:0</td>
<td>0.52 (0.90)</td>
<td>0.50 (0.48)</td>
<td>1.09 (0.45)</td>
<td>0.85 (1.47)</td>
</tr>
<tr>
<td>Branched</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10:0 3OH</td>
<td>0.00 (0.00)</td>
<td>0.14 (0.24)</td>
<td>3.34 (0.25)</td>
<td>0.00 (0.00)</td>
</tr>
<tr>
<td>13:0 iso</td>
<td>0.00 (0.00)</td>
<td>6.13 (0.34)</td>
<td>0.00 (0.00)</td>
<td>0.00 (0.00)</td>
</tr>
<tr>
<td>12:0 2OH</td>
<td>0.00 (0.00)</td>
<td>0.00 (0.00)</td>
<td>5.01 (0.28)</td>
<td>0.00 (0.00)</td>
</tr>
<tr>
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<td>2.97 (0.14)</td>
<td>5.34 (0.22)</td>
<td>0.00 (0.00)</td>
</tr>
<tr>
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<td>0.00 (0.00)</td>
<td>1.12 (0.22)</td>
<td>0.00 (0.00)</td>
<td>1.05 (0.91)</td>
</tr>
<tr>
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<td>0.00 (0.00)</td>
<td>1.96 (0.05)</td>
<td>0.00 (0.00)</td>
<td>0.00 (0.00)</td>
</tr>
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<td>14.03 (0.19)</td>
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<td>21.85 (1.26)</td>
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<td>0.00 (0.00)</td>
<td>39.22 (2.17)</td>
</tr>
<tr>
<td>16:0 iso</td>
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<td>0.00 (0.00)</td>
<td>3.54 (0.35)</td>
<td></td>
</tr>
<tr>
<td>17:0 cyclo</td>
<td>16.67 (4.52)</td>
<td>0.00 (0.00)</td>
<td>2.31 (0.47)</td>
<td>0.00 (0.00)</td>
</tr>
<tr>
<td>17:0 iso</td>
<td>0.00 (0.00)</td>
<td>0.81 (0.06)</td>
<td>0.00 (0.00)</td>
<td>10.98 (0.56)</td>
</tr>
<tr>
<td>17:0 anteiso</td>
<td>0.00 (0.00)</td>
<td>0.00 (0.00)</td>
<td>0.00 (0.00)</td>
<td>11.86 (0.82)</td>
</tr>
<tr>
<td>13:0 3OH</td>
<td>0.00 (0.00)</td>
<td>1.41 (0.08)</td>
<td>0.00 (0.00)</td>
<td>0.00 (0.00)</td>
</tr>
<tr>
<td>15:1 iso</td>
<td>8.51 (0.35)</td>
<td>1.27 (0.27)</td>
<td>0.00 (0.00)</td>
<td>0.00 (0.00)</td>
</tr>
<tr>
<td>14:0 3OH</td>
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<td>0.00 (0.00)</td>
<td>0.00 (0.00)</td>
<td>2.05 (0.28)</td>
</tr>
<tr>
<td>16:1 iso</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17:1 iso</td>
<td>0.20 (0.35)</td>
<td>0.15 (0.26)</td>
<td>0.54 (0.93)</td>
<td>0.00 (0.00)</td>
</tr>
<tr>
<td>17:1 anteiso</td>
<td>0.00 (0.00)</td>
<td>0.00 (0.00)</td>
<td>0.00 (0.00)</td>
<td>1.79 (1.55)</td>
</tr>
<tr>
<td>17:1 iso w10c</td>
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<td>9.11 (1.03)</td>
<td>0.00 (0.00)</td>
<td>0.00 (0.00)</td>
</tr>
<tr>
<td>17:1 w8c</td>
<td>0.00 (0.00)</td>
<td>2.37 (0.08)</td>
<td>0.00 (0.00)</td>
<td>0.00 (0.00)</td>
</tr>
<tr>
<td>18:1 w8c</td>
<td>4.14 (1.18)</td>
<td>3.87 (0.12)</td>
<td>31.58 (2.22)</td>
<td>0.00 (0.00)</td>
</tr>
<tr>
<td>19:0 cyclo</td>
<td>3.02 (1.25)</td>
<td>0.00 (0.00)</td>
<td>5.27 (1.25)</td>
<td>0.00 (0.00)</td>
</tr>
<tr>
<td>16:1 w6c</td>
<td>4.81 (1.53)</td>
<td>28.77 (0.16)</td>
<td>10.15 (1.11)</td>
<td>0.00 (0.00)</td>
</tr>
<tr>
<td>16:1 w7c</td>
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<td>0.00 (0.00)</td>
<td>0.00 (0.00)</td>
<td>0.00 (0.00)</td>
</tr>
<tr>
<td>18:0 anteiso</td>
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<td></td>
<td></td>
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<tr>
<td>Unsaturated</td>
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<td></td>
</tr>
<tr>
<td>17:1 anteiso</td>
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<td>51.13 (3.89)</td>
<td>50.72 (7.99)</td>
<td>7.82 (4.30)</td>
</tr>
<tr>
<td>w9c</td>
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<td></td>
</tr>
<tr>
<td>17:1 iso w10c</td>
<td>76.36 (10.93)</td>
<td>52.48 (4.19)</td>
<td>51.84 (4.52)</td>
<td>94.99 (11.44)</td>
</tr>
<tr>
<td>17:1 w8c</td>
<td>0.33 (0.00)</td>
<td>0.97 (0.00)</td>
<td>0.98 (0.00)</td>
<td>0.08 (0.00)</td>
</tr>
<tr>
<td>Ratio (unsat/sat)</td>
<td>0.00 (0.00)</td>
<td>0.00 (0.00)</td>
<td>0.00 (0.00)</td>
<td>0.00 (0.00)</td>
</tr>
</tbody>
</table>

Table 4.2. Mean FA percentage compositions (n=3, ± SD) for E. coli AB264, S. putrefaciens CN32, P. aeruginosa PAO1, and B. subtilis 168, after growth to exponential phase in 10% TSB. Full FA profiles are presented along with ratios of unsaturated:[saturated, branched, cyclopropyl] lipid chemistries.
Figure 4.4. Correlation between unsaturated: [saturated, cyclo, branched] FA and mean peak relative DAPI fluorescence (n=3, ± SD) for each metal used in this study. General trends demonstrate an increase in permeability as a function of increased [saturated, cyclo, branched] FA content. Uranium values are presented not as a peak relative fluorescence, but rather as the minimum relative fluorescence, due to the membrane being rendered more impermeable by this metal.
4.5 Discussion

This research examined the impacts of selected soluble metals on cell wall permeability. It is important to note that molecules diffusing through cell walls must first pass through the surface polysaccharides before reaching the CM. The bacterial surface is defined in this study as the area within the cell wall that is exposed to the aqueous milieu, and includes the phospholipids flanking the periplasm and peptidoglycan in Gram-negative cells. The surface polysaccharides of bacteria used in this study varied with the strain. *P. aeruginosa* PAO1 exhibits smooth LPS with chemically distinct shorter A-band and longer B-band LPS within its surface under most conditions (Rivera et al. 1988; Hancock and Nikaido 1978). Alternatively, *E. coli* AB264 (Beveridge and Graham 1991; Beveridge 1993) and *S. putrefaciens* CN32 (Korenevsky et al. 2002; Vinogradov et al. 2002) possess chemically similar rough LPS on their surfaces. *B. subtilis* 168 possesses thick carboxyl-containing peptidoglycan, and teichoic acids containing high densities of phosphodiester groups (Beveridge and Murray 1976; Seltmann and Holst 2002; Graham and Beveridge 1994).

Rough LPS has fewer metal reactive sites available within its core structure than the surfaces of cells with smooth-type LPS that contain core regions as well as O-antigen repeats. Smooth LPS offers higher metal buffering capacity from anionic ligands on the glycan repeats. Barkleit et al. (2008) observed that excess metal was required to fully saturate LPS carboxyl sites before inner phosphoryl sites played a role in binding uranyl ions on purified LPS from *Pseudomonas aeruginosa* S10. Given the abundance of metal-reactive sites on surface sugars surrounding bacteria, it is not unreasonable to predict similar effects in whole cells during our permeability studies. Smooth LPS also provides spatial hindrance in cation-rich solution, for materials
approaching the CM. Schneck et al (2009) identified a Ca$^{2+}$-dependent collapse of LPS in *P. aeruginosa* dps 89, a PAO1 mutant containing only B-band LPS (Lightfoot and Lam 1991). It is plausible that this could be occurring for PAO1 at 1 mM metal concentration in our relatively dilute bacterial suspensions, but this would mainly affect permeability for larger molecules rather than soluble metals. In fact, despite smooth LPS (as B-bands) on PAO1 cells, overall permeability effects in our study were very similar to cells expressing only rough LPS.

The contributions of phospholipids to overall permeability can be summarized according to their spatial occupation within the bilayer plane, which encompasses the hydrophobic FA region, and the hydrophilic head group region. Phospholipid head groups have different proportions of phosphoryl, carboxyl, hydroxyl, or amine groups depending on the lipid, which affect protein-lipid interaction as well as electrostatic repulsion (Israelachvili 2011). Binding of free cations to surface phosphoryl or carboxyl ligands at circumneutral pH will contribute to neutralization of the surface charge, and lessen electrostatic effects. As metals adsorb to these ligands, it is possible for charge reversal of the membrane to occur. In the case of the cationic DAPI, this may be indicated by results showing decreased membrane permeability to DAPI for metal concentrations greater than 0.1 mM. In the case of Ca$^{2+}$, charge reversal can occur between 0.1-1.0 mM concentrations (McLaughlin et al. 1981; Eisenberg et al. 1979). Collins and Stotzky (1992) report charge reversals for several bacterial species, including *P. aeruginosa* and *B. subtilis*, at circumneutral pH and 0.1 mM concentrations of Cu$^{2+}$. In that study, charge reversal was also observed for bacterial suspensions containing 0.1 mM Zn$^{2+}$, but higher pH was necessary for the charge reversal to occur. This indicates increased positive charge of the surface in the presence of Zn$^{2+}$, relative to Cu$^{2+}$. The affinity of cell walls for the uranyl ion observed in
our metal association assay may induce this reversal much sooner; charge reversal would close
the membrane to the DAPI probe (Fig. 1). Uranium(VI) as $\text{UO}_2^{2+}$ greatly lowered the surface
charge of $E. \text{coli}$ membranes in a concentration dependent manner, from 0.0010 – 1.0 mM
concentrations (McQuillen 1951). The same concentrations of U have also been shown to
induce a positive surface potential in synthetic PE membranes, although other divalent cations
had a negligible impact (McLaughlin et al. 1971).

The chemistry of the FA region of lipid molecules strongly impacts spacing between lipids and
adjacent molecules. Cell wall permeability trends in our research suggest that reduced cell wall
permeability to DAPI corresponds to higher amounts of unsaturated FA. Huster et al. (2000)
reported that unsaturated FA presence in artificial membranes decreased the amount of $\text{Ca}^{2+}$
binding twofold. This observation may help to explain the relationship between the FA ratio and
the strength of the metal permeability effects observed in our research. Exposure of hydrophilic
head group regions to polyvalent cations causes de-mixing, clustering acidic phospholipids into
domains (Israelachvili 2011; Hui et al. 1983). This tight lipid clustering causes neutral and basic
phospholipids to be more loosely packed and exposed to the aqueous milieu, and their
hydrophobic regions ‘expand’ to occupy the newly-available space (Israelachvili 2011). More
prevalemt hydrophobic attraction in these conditions, combined with the tight geometric packing
of saturated FA, helps to explain the correlations between cell wall permeability and FA
saturation in our research.
Table 4.3. Dominant metal species (≥ 1%) in solution of 5 mM HEPES buffer, under atmospheric gas conditions, and pH adjustment to 6.8. Data was obtained using CHEAQS (Verweij 2010).

<table>
<thead>
<tr>
<th>Metal</th>
<th>0.001 mM</th>
<th>0.01 mM</th>
<th>0.1 mM</th>
<th>1 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca</td>
<td>93% Ca^{2+}</td>
<td>93% Ca^{2+}</td>
<td>93% Ca^{2+}</td>
<td>93% Ca^{2+}</td>
</tr>
<tr>
<td></td>
<td>6% Ca(HCO_3)^+</td>
<td>6% Ca(HCO_3)^+</td>
<td>6% Ca(HCO_3)^+</td>
<td>6% Ca(HCO_3)^+</td>
</tr>
<tr>
<td>Cu</td>
<td>86% CuCO_3</td>
<td>86% CuCO_3</td>
<td>86% CuCO_3</td>
<td>83% CuCO_3</td>
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<tr>
<td></td>
<td>8% Cu^{2+}</td>
<td>8% Cu^{2+}</td>
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<td>8% Cu^{2+}</td>
</tr>
<tr>
<td></td>
<td>2% Cu(OH)^+</td>
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</tr>
<tr>
<td></td>
<td>2% Cu(HCO_3)^+</td>
<td>2% Cu(HCO_3)^+</td>
<td>2% Cu(HCO_3)^+</td>
<td>2% Cu(HCO_3)^+</td>
</tr>
<tr>
<td></td>
<td>1% CuCO_3</td>
<td>1% CuCO_3</td>
<td>1% CuCO_3</td>
<td>1% CuCO_3</td>
</tr>
<tr>
<td>Mn</td>
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<td>85% Mn^{2+}</td>
<td>85% Mn^{2+}</td>
<td>86% Mn^{2+}</td>
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</tr>
<tr>
<td></td>
<td>7% Mn(HCO_3)^+</td>
<td>7% Mn(HCO_3)^+</td>
<td>7% Mn(HCO_3)^+</td>
<td>7% Mn(HCO_3)^+</td>
</tr>
<tr>
<td>U</td>
<td>63% (UO_2)(CO_3)_3^{2-}</td>
<td>63% (UO_2)(CO_3)_3^{2-}</td>
<td>61% (UO_2)(CO_3)_3^{2-}</td>
<td>48% (UO_2)(CO_3)_3^{2-}</td>
</tr>
<tr>
<td></td>
<td>36% (UO_2)(CO_3)_3^{4-}</td>
<td>36% (UO_2)(CO_3)_3^{4-}</td>
<td>35% (UO_2)(CO_3)_3^{4-}</td>
<td>32% (UO_2)(CO_3)_3^{4-}</td>
</tr>
<tr>
<td></td>
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<td>3% (UO_2)_2(CO_3)(OH)_3</td>
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</tr>
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<td></td>
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</tr>
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<td>81% Zn^{2+}</td>
<td>81% Zn^{2+}</td>
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<td>10% Zn(HCO_3)^+</td>
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<td>8% ZnCO_3</td>
<td>8% ZnCO_3</td>
<td>8% ZnCO_3</td>
<td>8% ZnCO_3</td>
</tr>
</tbody>
</table>

Calculations of metal speciation (Table 4.3) indicated that Ca(II), Cu(II), Mn(II), and Zn(II) had similar complexation chemistry at all concentrations in HEPES buffer at pH 6.8. The charges of these metal complexes were relatively independent of metal-association with the cell wall.

Copper carbonate complexes are neutrally charged, but they still associate with cell membranes and have strong permeabilization effects. Alternatively, Zn^{2+} ions were the dominant form of Zn, but found to associate only weakly even at 1 mM concentrations across all examined species, and yet had permeabilizing effects in all species except *B. subtilis*. Uranium complexes with carbonates to form anionic complexes in solution according to Table 4.3, but high concentrations still bound to cells in treatment suspensions. Calcium and Mn were not detected on the cell surface to compare the predicted chemical complexes in Table 4.3 to bound metal, which also suggests that permeability effects in the context of those metals may be due to their presence as electrolytes in the assay solutions. Stability data for V is conflicting (Evans and Garrels 1958;
Wanty and Goldhaber 1992; Takeno 2005), which made deriving V complexes in solution unreliable. From these published reports, it can be generalized that V behaves as an anion in aerobic environments at circumneutral pH. Data from the metal-binding assay indicated that V(IV) associated with the cell wall in similar amounts as Cu(II) for all species, with very different end results in DAPI permeability. It is unclear why this would be the case.

Observed cell wall penetration by the relatively large DAPI probe brings into question the ability of the soluble metal cations used in this study to enter the cytoplasm as well. Metal toxicities for \textit{E. coli} have been compiled by Nies (2007), and found to be \( \geq 50 \text{ mM}, 1 \text{ mM}, 20 \text{ mM}, 2 \text{ mM}, 5 \text{ mM}, \) and \( 1 \text{ mM} \) for Ca(II), Cu(II), Mn(II), U(VI), V(V), and Zn(II) respectively. These toxicities are likely a function of cell permeability, in a manner dependent on the ability of the metal itself to interfere with cell processes once in the cytoplasm. Metals such as Ca(II) and Mn(II) are lower in binding preference than other elements in biological scenarios (Williams 2001), and Mn(II) is the lowest element in the Irving-Williams series (Irving and Williams 1948; Irving and Williams 1953). This lower binding affinity to biological ligands helps to explain how Ca and Mn toxicity may be less than other metals, due to less interference with cell functions. The high affinities of ligands for metals Cu(II) and Zn(II) are reflected in their higher toxicities. Experiments that examine the hierarchy of metal affinity to bacterial components usually leave out elements such as U or V, with more attention paid to other, more common, first row transition metals. In fungi and algae, U(VI) has been found to bind much more strongly than even Cu(II) in biosorption efficiency ordering (Gadd 1990).
In environments with relatively high concentrations of soluble metals, the permeability of bacterial membranes is dependent not only on metal binding, but also on FA saturation chemistry at the CM. As was expected, higher saturated FA content resulted in weaker permeability effects after exposure to metal cations. In toxic metal bioremediation, or in environments where bacteria must survive high concentrations of toxic metals, cells with typically high proportions of saturated FA content may prove to be less permeable to toxic metals and compounds. Our findings may have promising implications for medical microbiology or chemotherapeutic research. The observation that even micromolar concentrations of common metals can permeabilize cell walls suggests that less toxic metals such as Ca(II) or Mn(II) may be able to increase permeability of CMs to antimicrobial agents.

### 4.6 Conclusion

This research examined the effects that soluble metal ions had on the total cell wall permeability of *E. coli* AB264, *S. putrefaciens* CN32, *P. aeruginosa* PAO1, and *B. subtilis* 168, to a cationic probe. Fatty acid saturation in phospholipid molecules was shown to play an important role in metal-induced permeabilization effects, with unsaturated FA content positively correlated with increases in membrane permeability. This study also indicates that cell wall adsorption of metal species helps buffer CM permeability to cationic compounds. The buffering effect depends on both the overall charge of the cell wall after metal binding, as well as the surface ligand saturation.
CHAPTER 5

CONCLUDING DISCUSSION
CHAPTER FIVE

Concluding discussion

The mobility of toxic soluble metals in the environment can be a serious health risk for humans. Toxic metals may be present in leachate from mining sites, refineries, or mineral formations, as previously discussed. Contaminated environments notwithstanding, soluble metals are ubiquitous on Earth, and are necessary for growth and survival of most organisms. Bacterial cell walls are chemically complex, highly charged structures, and serve a multitude of functions that have been outlined in previous chapters. Despite metal adsorption to cell walls, bacteria still maintain their structure and membrane function. The major objective of this research was to identify whether Shewanella putrefaciens CN32 responded to metals in its growth environment by modulating its membrane phospholipid chemistry. The objective also included correlating those changes in lipid chemistry to variations in membrane attributes such as fluidity and diffusion, as well as whole cell permeability. The objectives guided a strategy that included characterizing both hydrophilic functional groups and FA chemistry within the hydrophobic region, as well as identifying changes in lipid dynamics within the membrane. Individual data from each respective chapter has been discussed with respect to the overall objective, and will not be further addressed. The results of the three combined studies reveal trends in the data, and will be discussed in this chapter along with potential in situ and in vitro applications of those results.

Electrostatic interactions between lipids within phospholipid bilayers are dependent on functional groups within the hydrophilic headgroup. Zwitterionic lipids such as PE will exhibit positive charges if phosphoryl groups have adsorbed metal cations. Alternatively, the charge on
PG will become more neutral when binding a cation, and lessen the electrostatic repulsion between membrane lipids (Israelachvili 2011). The attractive hydrophobic and van der Waals interactions then become more prevalent within the membrane, increasing its thermal resistance. In chapter 2, it was predicted that the proportions of phospholipid functional groups have a strong impact on thermal stability. Metal-treated CN32 cells exhibited increased anionic PG, which coincided with increased membrane thermal stability. There was higher protein content in the metal-treated CN32 envelopes in anaerobic conditions as well (Table 3.2), which was positively correlated with higher thermal stability; this is a notion that was also suggested in chapter 2. Thermal stability of bacterial membranes can be viewed as an indicator of overall membrane strength. From the research conducted in chapters 2 and 3, it is apparent that increased membrane strength is required when high concentrations of soluble metals are present under anaerobic growth conditions.

In light of the impacts on thermal stability, metal association with phospholipid headgroups had minimal impacts on membrane fluidity. Fluidity was instead dominated by fatty acid saturation chemistry. This was predicted in chapter 2, and confirmed when comparing fluidity data to functional group characterizations. In aerobic conditions, metal treated cell cultures were more fluid than the control (Table 2.1), and did not change significantly when treated with metals but were cultured anaerobically. Incidentally, when grown under anaerobic conditions, cultures had similar phospholipid functional group proportions but demonstrated increased PG and decreased DPG when treated with metals under anoxia. This demonstrates that membrane fluidity is not a function of headgroup chemistry, especially since increases in fluidity had a strong positive correlation with unsaturated FA proportions. The hypothesis that Mn(II), V(IV), and U(VI)
would reduce the fluidity of CN32 membranes was partially confirmed, as this only occurred in aerobically cultured bacteria. Under anaerobic growth conditions this was not the case, and the hypothesis was rejected. Because fluidity is related to FA saturation, the hypothesis that Mn(II), V(IV), and U(VI) would impact FA saturation was validated as well. These observations indicate that in the hydrophobic region of the bilayer, the hydrocarbon chains are not affected by adsorption of metals to surface functional groups. Intracellular activities are largely independent of metal binding to the cell surface. This allows bacteria to control membrane lipid packing and function within their cytoplasm, in environments where external stresses may threaten cell survival. This holds true in environments where physical or chemical factors such as temperature (Abboud et al. 2005), pH (Mykytczuk et al. 2010; Chang and Cronan 1999), high concentrations of metals (chapter 2), or salinity (Oliver and Colwell 1973; Wood 1999) may compromise membrane integrity.

Aerobic and anaerobic growth conditions were hypothesized to impact metal-membrane interactions. This was observed in Chapter 2, where V-binding differed in aerobic and anaerobic conditions (Fig. 2.7). The charge of V complexes at high and low redox potential was expected to differ as well, but further study is necessary to elaborate on differences in V-binding under aerobic compared to anaerobic growth. The speciation of V is challenging to determine in vitro, and chemical treatments and chromatographic separations of V species may not be representative of its solution complexation chemistry. As such, V species in growth medium can be identified at the V-L$_{2,3}$ edges (Fig. 2.7). NEXAFS can be used to characterize V 2p spectra using STXM to target the background growth medium to examine dominant solution complexes, as well as possible V-binding to any extracellular materials. Chemical modeling using custom algorithms
or software such as CHEAQS (Verweij 2010), or the Geochemist’s Workbench® (Rockware Inc, University of Illinois), can characterize chemical complexes in solution. When organic ligands are introduced, the calculations become more complex (Cabaniss and Shuman 1988a, b; Tipping and Hurley 1992; Tipping 1994), and are not necessarily representative of the solution chemistry in the presence of bacteria. This is observed in the case of U(VI). Its solution chemistry in growth medium (pH 6.8; Table 2.3), or 5 mM HEPES buffer (pH 6.8; Table 4.3), indicates that it commonly behaves as an anion in solutions exposed to both atmosphere and inert gas. Despite this, it behaves as a high-affinity electron microscopy stain (Fig. 2.1, 2.4), binding to cell walls as the uranyl ion (Barkleit et al. 2008, 2009; Fowle et al. 2000; Koban and Bernhard 2007).

Additional study is required to determine the affinity of individual bacterial surface ligands for a broad range of elements, and to identify the ability of cell wall ligands to disrupt solution complexes in favor of surface ligand binding.

Future studies stemming from this research will serve to elaborate on, and possibly exploit, the trends observed for bacteria cultured with and exposed to soluble metals. An important future direction would be to identify if these observations hold true in environments with high concentrations of soluble metals; examining the FA compositions and membrane strengths of the indigenous bacterial consortia. Alternatively, the abilities of metals Ca, Mn, and Zn to increase the permeability of bacterial membranes may be exploited for bacteriocidal ends. Even sub-millimolar concentrations of soluble metals can impact membrane physicochemistry, and this knowledge can be applied to whichever ends would benefit from altering the integrity of bacterial membranes. This may include increasing the efficiency of various antibiotics by compromising the bacterial envelope, or maximizing the antibacterial effects of detergents.
Controlled \textit{in vitro} experiments allow careful manipulation of a single aspect of the environment, but \textit{in situ} this will rarely be the case. Bacteria have adapted to changing environments for billions of years. Intricacies of each cell, in each population, in each community, within an ecosystem, simply cannot be anticipated. By understanding the responses of cells to soluble metals in controlled environments, it is possible, however, to refine predictions of cell responses \textit{in situ}.
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SUPPLEMENTARY DATA

Figure S1. Lactate dehydrogenase (LDH) enzyme activity in the presence of soluble metals. LDH was incubated with NAD in 5 mM HEPES buffer (pH 6.8) containing both glycine and hydrazine, in the dark for the amount of time shown. NADH was assayed at 340 nm, with the pyruvate irreversibly complexing with hydrazine. This prevented the reaction from proceeding in reverse. Uranium formed precipitates that made it challenging to identify LDH activity, but based on growth curves soluble metals did not substantially impact growth.
Figure S2. Scanning transmission X-ray microscopy images of CN32 envelopes (Fig. 2.4) mounted on Si₃N₄ slides. Images shown represent (A) envelopes at 300 eV; (B) C map; (C) Ca map; (D) O map. The optical densities are listed below the STXM images, with C being the densest, followed by O, and Ca. Mg was not detected.
Figure S3. C 1s spectral fits for CN32 envelopes extracted from cells grown aerobically, in the presence of 1 mM Mn$^{2+}$. These fits complement the spectra presented (Fig. 3.3). Shown here are (A) residuals, minimized in Igor Pro (Wavemetrics Inc); (B) experimental and fit spectra; (C) Gaussian fits and tangential step function.
Figure S4. C 1s spectral fits for CN32 envelopes extracted from cells grown aerobically, in the presence of 1 mM VO$^{2+}$. These fits complement the spectra presented (Fig. 3.3). Shown here are (A) residuals, minimized in Igor Pro (Wavemetrics Inc); (B) experimental and fit spectra; (C) Gaussian fits and tangential step function.
Figure S5. C 1s spectral fits for CN32 envelopes extracted from cells grown anaerobically in the absence of metal-amendment. These fits complement the spectra presented (Fig. 3.3). Shown here are (A) residuals, minimized in Igor Pro (Wavemetrics Inc); (B) experimental and fit spectra; (C) Gaussian fits and tangential step function.
Figure S6. C 1s spectral fits for CN32 envelopes extracted from cells grown anaerobically, in the presence of 1 mM Mn$^{2+}$. These fits complement the spectra presented (Fig. 3.3). Shown here are (A) residuals, minimized in Igor Pro (Wavemetrics Inc); (B) experimental and fit spectra; (C) Gaussian fits and tangential step function.
Figure S7. C 1s spectral fits for CN32 envelopes extracted from cells grown anaerobically, in the presence of 1 mM VO$^{2+}$. These fits complement the spectra presented (Fig. 3.3). Shown here are (A) residuals, minimized in Igor Pro (Wavemetrics Inc); (B) experimental and fit spectra; (C) Gaussian fits and tangential step function.
Figure S8. N 1s spectral fits for CN32 envelopes extracted from cells grown aerobically, in the absence of metal-amendment. These fits complement the spectra presented (Fig. 3.4). Shown here are (A) residuals, minimized in Igor Pro (Wavemetrics Inc); (B) experimental and fit spectra; (C) Gaussian fits and tangential step function.
Figure S9. N 1s spectral fits for CN32 envelopes extracted from cells grown aerobically, in the presence of 1 mM Mn$^{2+}$. These fits complement the spectra presented (Fig. 3.4). Shown here are (A) residuals, minimized in Igor Pro (Wavemetrics Inc); (B) experimental and fit spectra; (C) Gaussian fits and tangential step function.
Figure S10. N 1s spectral fits for CN32 envelopes extracted from cells grown aerobically, in the presence of 1 mM VO$^{2+}$. These fits complement the spectra presented (Fig. 3.4). Shown here are (A) residuals, minimized in Igor Pro (Wavemetrics Inc); (B) experimental and fit spectra; (C) Gaussian fits and tangential step function.
Figure S11. N 1s spectral fits for CN32 envelopes extracted from cells grown anaerobically, in the absence of metal-amendment. These fits complement the spectra presented (Fig. 3.4). Shown here are (A) residuals, minimized in Igor Pro (Wavemetrics Inc); (B) experimental and fit spectra; (C) Gaussian fits and tangential step function.
Figure S12. N 1s spectral fits for CN32 envelopes extracted from cells grown anaerobically, in the presence of 1 mM Mn$^{2+}$. These fits complement the spectra presented (Fig. 3.4). Shown here are (A) residuals, minimized in Igor Pro (Wavemetrics Inc); (B) experimental and fit spectra; (C) Gaussian fits and tangential step function.
Figure S13. N 1s spectral fits for CN32 envelopes extracted from cells grown anaerobically, in the presence of 1 mM VO$^{2+}$. These fits complement the spectra presented (Fig. 3.4). Shown here are (A) residuals, minimized in Igor Pro (Wavemetrics Inc); (B) experimental and fit spectra; (C) Gaussian fits and tangential step function.
Figure S14. O 1s spectral fits for CN32 envelopes extracted from cells grown aerobically, in the absence of metal-amendment. These fits complement the spectra presented (Fig. 3.5). Shown here are (A) residuals, minimized in Igor Pro (Wavemetrics Inc); (B) experimental and fit spectra; (C) Gaussian fits and tangential step function.
Figure S15. O 1s spectral fits for CN32 envelopes extracted from cells grown aerobically, in the presence of 1 mM Mn$^{2+}$. These fits complement the spectra presented (Fig. 3.5). Shown here are (A) residuals, minimized in Igor Pro (Wavemetrics Inc); (B) experimental and fit spectra; (C) Gaussian fits and tangential step function.
Figure S16. O 1s spectral fits for CN32 envelopes extracted from cells grown aerobically, in the presence of 1 mM VO\(^{2+}\). These fits complement the spectra presented (Fig. 3.5). Shown here are (A) residuals, minimized in Igor Pro (Wavemetrics Inc); (B) experimental and fit spectra; (C) Gaussian fits and tangential step function.
Figure S17. O 1s spectral fits for CN32 envelopes extracted from cells grown anaerobically, in the absence of metal-amendment. These fits complement the spectra presented (Fig. 3.5). Shown here are (A) residuals, minimized in Igor Pro (Wavemetrics Inc); (B) experimental and fit spectra; (C) Gaussian fits and tangential step function.
Figure S18. O 1s spectral fits for CN32 envelopes extracted from cells grown anaerobically, in the presence of 1 mM Mn$^{2+}$. These fits complement the spectra presented (Fig. 3.5). Shown here are (A) residuals, minimized in Igor Pro (Wavemetrics Inc); (B) experimental and fit spectra; (C) Gaussian fits and tangential step function.
Figure S19. O 1s spectral fits for CN32 envelopes extracted from cells grown anaerobically, in the presence of 1 mM VO$^{2+}$. These fits complement the spectra presented (Fig. 3.5). Shown here are (A) residuals, minimized in Igor Pro (Wavemetrics Inc); (B) experimental and fit spectra; (C) Gaussian fits and tangential step function.