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Resolving biogeochemical phenomena at high spatial resolution through electron microscopy

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Our understanding of microbe-metal interactions has advanced dramatically since the mid-1970s when little was known about the reactivity of bacterial cell wall components toward metal ions in the extracellular milieu. Although certain metals such as UO_2^{++} and Pb^+ were known to react with components of bacterial cell walls and used to visualize their structure by electron microscopy (Garland *et al.*, 1975), little physicochemical data were available on the specificity and sites of interactions (Humphrey & Vincent, 1966; Heptinstall *et al.*, 1970; Irvin *et al.*, 1975; Lambert *et al.*, 1975; Raymond & MacLeod, 1975). Furthermore, there were no model systems to explore the mechanisms of these interactions. This began to change when Beveridge and Murray used isolated cell walls of *Bacillus subtilis* to quantify metal ion binding to wall components. Beveridge demonstrated that cell walls concentrated cations such as Mg^{++}, Na^+, K^+, Cu^{++} and Fe^{+++}, but not Ba^{++}, Li^+ or Al^{+++} (Beveridge & Murray, 1976). Since these initial studies, Beveridge and his students and collaborators have contributed greatly to our understanding of the complex interactions between microbial cell surface polymers and metals in the environment. As fellow scientists working in this research area, we have developed a deep admiration of Beveridge's scientific insight, technical skills and collegial demeanor. Not surprisingly, Beveridge's research has had a significant impact on our research, as well as on the research of our collaborators and colleagues, and will likely influence the work of future generations of scientists working in the field of geobiology. Some examples are cited below.

Beveridge's research from the very beginning provided the type of detailed description of the interaction between bacterial cell wall components and various metal ions that offered unique perspectives on function. For example, through a quantitative comparison of metal bound to chemically modified cell walls of *B. subtilis*, Beveridge determined that cell wall carboxyl groups more than other functional groups played an important role in metal uptake (Beveridge & Murray, 1980). These results revealed for the first time the importance of cell wall biopolymer chemistry on metal ion uptake by bacterial cells. The interplay of metal concentrations and other environmental variables undoubtedly influence cell wall chemistry and structure. Future researchers investigating metal interactions with bacteria in their natural habitats will likely find that as cell wall biopolymer chemistry changes in response to the conditions of the surrounding environment, other functional groups may assume a greater role in binding metals.

A potentially useful approach to characterize at the micrometer- and nanometer-scale functional groups associated with microbial cell walls and extracellular polymers that bind metals is synchrotron radiation-based scanning transmission X-ray microscopy (STXM). Distinct from other high-resolution techniques, STXM allows samples to remain hydrated at ambient atmospheric pressure, thus minimizing introduction of artifacts. One of us (TB) has used this approach to generate carbon K-edge and Fe L_{III}-edge maps and near-edge X-ray absorption fine structure (NEXAFS) spectra that correlate the abundance of Fe with carbonate and carboxyl concentrations at specific sites on a single cell of the bacterium *Shewanella putrefaciens* using ferric-citrate as terminal electron acceptor and lactate as the electron donor (Fig. 1A–C). Since this approach can also distinguish different species of a metal and their relative abundance at different sites on a single cell (Fig. 1D,E), it has the potential to correlate functional moieties of cell wall and extracellular polymers such as amide, phenolic or ketone, aromatic groups (carbon K-edge), and phosphate groups (P K-edge) with a specific species of a metal (i.e. Fe(II) and Fe(III) using Fe L_{III}-edge spectra).

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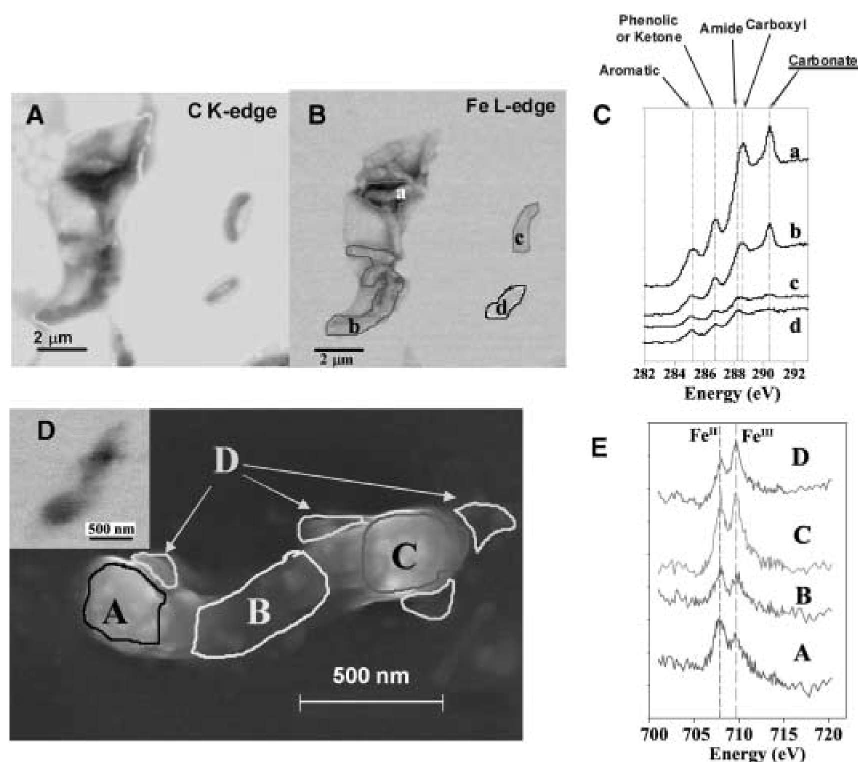

Fig. 1 Scanning transmission X-ray microscopy maps and corresponding spectra of cells of *Shewanella putrefaciens* respiring on ferric-citrate. (A) Carboxylic carbon K-edge map; dark regions delineate high concentration of carboxylic carbon from cells; (B) Fe(II) L-edge map; dark regions delineate extracellular areas where Fe(II) is concentrated; (C) Carbon K-edge spectra of different areas within 1b; (D) Scanning electron microscopy image and Fe L-edge map (inset; showing the same cell) of different Fe species across a single cell of *S. putrefaciens*; (E) Fe L-edge spectra showing relative abundance of Fe(II) and Fe(III) at different sites on cell in 1D.

Beveridge and collaborators have also contributed greatly to our current understanding of those components of bacterial cell walls that bind various metals. Using the cell envelope of the Gram-negative bacterium *Escherichia coli* as a model system, and protocols to isolate envelope components without disrupting their structure, Beveridge obtained the first high-resolution transmission electron microscopic images of the sites of metal interaction. The images revealed that most metal deposition occurred at or near polar head groups of the membrane fraction or along the peptidoglycan layer (Beveridge & Koval, 1981; Hoyle & Beveridge, 1983). This research ultimately led to an understanding of how cations such as Mg^{2+} and Ca^{2+} maintain membrane structure in Gram-negative bacteria. Later, Beveridge extended his investigation of metal interactions with bacterial envelopes to other Gram-positive bacteria such as *Bacillis cereus* and Gram-negative bacteria such as *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* (Mullen *et al.*, 1989; Krueger *et al.*, 1993). This and other research of the Beveridge laboratory have revealed that bacterial cell walls, independent of their structural differences, are capable of binding large quantities of different metals. These findings have prompted other researchers to explore the use of bacteria and their cell wall components for the recovery of metals from various waste streams and other metal-contaminated environments (Kuyucak & Volesky, 1988; Wang *et al.*, 2000; Kim *et al.*, 2002). More recently, Fein and coworkers have employed surface complexation models to describe metal adsorption to bacterial cell walls (Yee & Fein, 2003; Gorman-Lewis *et al.*, 2005; Wightman & Fein, 2005). Future technologies to remove cations from various aqueous environments could very well be based on the types of interactions that bacteria utilize to scavenge metal ions from their environment.

Having detected carboxyl group-containing uronic acids in isolated fractions of extracellular polymeric substances (EPS) excreted by a freshwater sediment bacterium, one of us (GGG) became interested in their potential to bind Cu^{2+} ions that typically adsorb to the organic fraction of sediments. Geesey's laboratory adopted Beveridge's quantitative approach to describe the interactions of the metal ion with various EPS fractions. The strongest interaction occurred with the uronic acid-containing EPS fraction, consistent with the earlier findings of Beveridge and Murray that carboxyl groups of bacterial biopolymers participate in metal binding (Beveridge & Murray, 1980). Larry Jang, a collaborating chemical engineer, recognized the value of Beveridge's quantitative descriptions of biopolymer-metal interactions, and for nearly a decade evaluated various numerical models to describe and parameterize the binding of metal ions by biopolymers (Jang *et al.*, 1990; Jang *et al.*, 1999).

Demonstration by Beveridge and colleagues of the strong interaction of metal ions with microbial cell wall structures also prompted Geesey's research group to investigate the role of microbial biofilm EPS in microbially influenced corrosion of metals. The formation of high-affinity, stable complexes between Cu^{2+} ions concentrated on metallic copper surfaces submerged in aqueous media and carboxyl groups in EPS of

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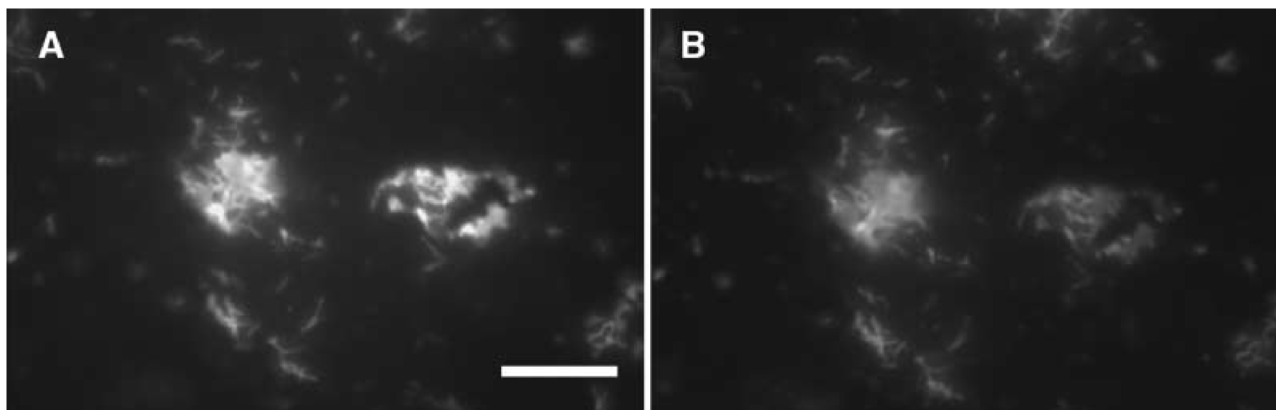

Fig. 2 Epifluorescence microscopy images of sulfate-respiring *Desulfovibrio desulfuricans* Essex 6 growing on a hematite surface. Hematite-associated cells were fixed with 4% paraformaldehyde, washed with phosphate-buffered saline (pH 7.2), permeabilized with 0.5 mg mL^{-1} lysozyme, treated with DNase, subjected to *in situ* reverse transcription polymerase chain reaction (IS RT-PCR) with 1 μM IRIS-Fuchsia labeled dUTP and primers Hyd1F and Hyd5R which are specific to the [NiFe] hydrogenase, and poststained with the nucleic acid stain 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) before viewing under an Olympus BX60 epifluorescence microscope. (A) Total DAPI-stained cells on hematite surface viewed using the MWU filter cube. (B) IS RT-PCR labeled cells expressing the [NiFe] hydrogenase gene imaged in the same field of view as panel A using MWIG filter cube. Bar represents 10 μm.

microbial biofilms growing on the surface was proposed as a mechanism of microbially influenced corrosion of copper tubing used in potable water distribution systems and industrial heat exchangers (Geesey *et al.*, 1986). This was later supported by experimental data showing dissolution and surface pitting of metallic Cu in the presence of bacterial biofilms and purified EPS (Jolley *et al.*, 1989; Bremer & Geesey, 1991). Beveridge's work further suggests the likelihood that metal-microbe interactions may be responsible for other mechanisms of microbially influenced corrosion of alloys containing other metals such as iron, chromium, and nickel. These mechanisms, however, have yet to be elucidated.

In the future, a combined microscopic, genetic and surface spectroscopic approach may be useful for revealing new mechanisms of microbially influenced corrosion of metals. *In situ* reverse transcription polymerase chain reaction (IS RT-PCR) can resolve individual cells and microcolonies of cells that express specific genes at a surface (Magnuson *et al.*, 2004). One of us (CLR) has used this approach to evaluate hydrogenase gene expression in sulfate-reducing bacteria. Figure 2 shows individual fluorescent cells in a microcolony of *Desulfovibrio desulfuricans* Essex 6 expressing the gene encoding the [Ni-Fe] hydrogenase, an enzyme that promotes depolarization of the cathode of a corrosion reaction by catalyzing the reversible reaction $H_2 \leftrightarrow 2H^+ + 2e^-$. *In situ* reverse transcription polymerase chain reaction should reveal targeted genes that are expressed by cells in the vicinity of a corrosion product or pit on a corroding metal surface since it can be performed without disrupting the physical association between the cells and these substrata. A corrosion product or pit formed on a metal surface can be spatially correlated with fluorescent cells expressing a particular gene of interest in the same field of view under a microscope by switching from reflected fluorescence to reflected differential interference contrast (DIC) optics (Magnuson *et al.*, 2004). Degenerate primers can be substituted for specific primers to probe for genes whose products have not previously been considered in corrosion reactions. This approach, when combined with confocal scanning laser microscopy, quantitative PCR and STXM offer novel strategies to resolve not only mechanisms of microbially influenced corrosion but also the genes and metabolic pathways involved in microbial transformations of minerals. As discussed below, Beveridge typically employed multiple analytical approaches to resolve structure-function relationships between components of the bacterial cell surface.

Iron-reducing bacteria such as *Geobacter* and *Shewanella* spp. transfer electrons from respiratory cytochromes at the cell surface to solid phase electron acceptors such as iron oxides. These bacteria are thought to utilize a variety of mechanisms including direct contact (Arnold *et al.*, 1998), soluble shuttles (Lovley *et al.*, 1996; Marsili *et al.*, 2008) and EPS such as polysaccharides (Marshall *et al.*, 2006), nanowires (Gorby *et al.*, 2006) and geopili (Reguera *et al.*, 2005) to facilitate this process. Using TEM to examine thin sections of samples of cells of *Shewanella oneidensis* MR-1 respiring anaerobically on ferrihydrite, Beveridge and collaborators at the Pacific Northwest National Laboratory recently observed EPS extending between the surface of the cell and the iron oxide surface (Fig. 3). Future studies are planned to use TEM in combination with ferritin-conjugated antibodies that target products of genes that are expressed during respiration on this substrate to resolve at the ultrastructural level their physical locations during the electron transfer reaction.

Beveridge's success in describing cell wall-metal interactions stems from the wide network of collaborations he has established over the past 30 years with scientists whose expertise

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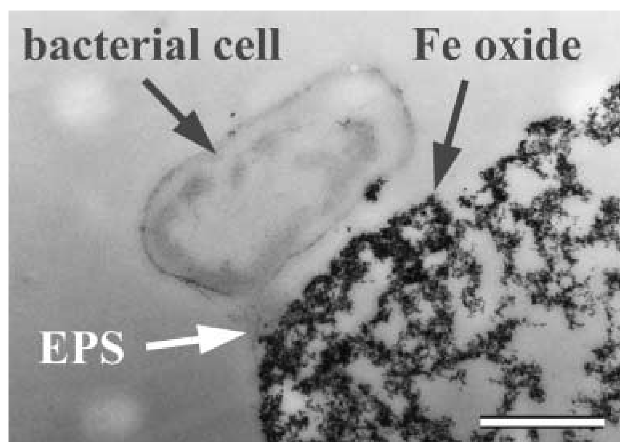

Fig. 3 Transmission electron micrograph of a thin section of a cell of *Shewanella oneidensis* MR-1 associated with an iron oxide surface via extracellular polymeric substances after incubation for 11 days in anaerobic liquid medium containing 30 mM HEPES buffer, 18 mM lactate as electron donor and 5 mM Si-stabilized ferrihydrite as electron acceptor. Bar denotes 0.5 µm.

complements his own in electron microscopy. Collaborative research was a trademark of Beveridge's approach to science long before it became a necessity in today's research climate. Terry's easy-going manner facilitated collaborative interactions, while his passion for science made the collaborations highly productive. We cannot think of many other scientists who have used the collaborative research approach as successfully as Beveridge. For example, through collaboration, he combined transmission electron microscopy (TEM) with small angle X-ray scattering, atomic absorption spectroscopy, and energy-dispersive X-ray spectroscopy (EDX) to describe the interactions of Ga, U, and Pt with *P. fluorescens* (Krueger *et al.*, 1993). Through other collaborations, he combined TEM with EDX, and inductively coupled plasma atomic-emission spectroscopy to investigate the influence of cell physiology on the binding and uptake of metals by cells. Using this suite of analytical techniques Beveridge and coworkers showed that protons pumped into the cell envelope of *B. subtilis* during respiration to establish a proton motive force compete with metal cations for binding sites in the envelope (Urrutia Mera *et al.*, 1992). Actively respiring cells bound less metal on their surface than did moribund cells. Terry always gave the impression, however, that he valued the personal interactions with his colleagues as much or more than the resulting scientific discoveries.

Among Beveridge's most significant contributions to science is his work on the involvement of microorganisms in mineral diagenesis and the role of metal cations in the preservation of microbial cells in the fossil record (Ferris *et al.*, 1986; Ferris *et al.*, 1988). In the early 1980s, he and his collaborators employed high-resolution electron microscopy to follow the mineralization of bacteria and their component parts (Beveridge *et al.*, 1983). They demonstrated that the loading of cell walls of *B. subtilis* with metal ions in an artificial sediment environment leads to the formation of authigenic mineral phases such as crystalline metal sulfides, and the preservation of bacterial cells in sedimentary rock. Beveridge, in collaboration with Ron Oremland at the US Geological Survey, using a suite of complementary analytical techniques that included UV-VIS spectroscopy, Raman spectroscopy, and transmission and scanning electron microscopy, showed that nanospheres of elemental Se formed through anaerobic respiration of Se oxyanion had different properties than nanospheres formed abiotically (Oremland *et al.*, 2004). They were also successful in resolving differences in the properties of nanospheres of Se formed by different Se-respiring organisms. These findings will likely lead to the use of microorganisms in the fabrication of new materials requiring the novel properties of microbiologically derived Se. More recently, Beveridge and collaborators demonstrated the formation of intracellular granules of ferric and ferrous iron formed by *S. putrefaciens* CN32 during anaerobic respiration on solid phase ferric oxide (Glasauer *et al.*, 2007). This was the first time mixed-valence metal particles had been detected *in situ* at high resolution.

It is important to note that Beveridge and collaborators have also shown that fine-grained precipitates associated with the membranes of many Gram-negative bacteria are not necessarily formed *de novo* at these sites (Glasauer *et al.*, 2001). Large quantities of nanoparticles of ferrihydrite, goethite and hematite, formed through abiotic reactions elsewhere, can be transported to and become bound to cell walls of *Shewanella putrefaciens* CN32. They further demonstrated that nanocrystals of these minerals can penetrate the outer membrane and peptidoglycan layers of the cell envelope. Given these results, one gains a heightened appreciation of the rigor of the earlier studies performed by Beveridge that enabled him to resolve *de novo* mineral formation on bacterial cell walls. As the field of biogeochemistry matures, the 'Beveridge paradigm' that microbial cells are important sites of metal cation binding and mineral formation will only gain significance.

The observations made by Beveridge and colleagues during their early investigations of metal interactions with natural microbial biofilms in aquatic environments further heightened our appreciation for his scientific insight. In work carried out with Konhauser and others, Beveridge recognized that biofilm structures are important sites of mineral precipitation in lotic (flowing water) environments (Konhauser *et al.*, 1994). They provided evidence that epilithic microbial biofilms dominate the reactivity of the rock-water interface and appear to control mineral formation in these dynamic systems. Although we had similar evidence from electron micrographs prepared from samples of microbial biofilms that formed in mountain streams nearly two decades earlier, unlike Beveridge, we failed to recognize at the time a role for microbial biofilms in this biogeochemical context. Following Beveridge's lead, we now pay closer attention to the mineral precipitates that accumulate in

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biofilms during their development on various substrata (Neal *et al.*, 2001).

Beveridge and collaborators also demonstrated that cell walls and EPS associated with microbial biofilms formed in subsurface environments were capable of scavenging mobile aqueous phase ions present in very low concentrations, leading to the formation of ferrihydrite or hematite under aerobic conditions and siderite under anaerobic conditions (Brown *et al.*, 1994). Based on these observations, they proposed that mineral development through such a mechanism could influence the transport of material in radioactive waste disposal sites. It has since been reported that iron oxides such as those formed in the presence of microbial biofilms have significant sorption capacity for radionuclides such as uranyl ions (Payne *et al.*, 1994; Bruno *et al.*, 1995; Sato *et al.*, 1997; Duff *et al.*, 2002). The importance of Beveridge's discoveries in the area of microbially mediated mineral diagenesis can be traced to recent US Department of Energy-sponsored research to explore the feasibility of stimulating Fe- and U-precipitating bacteria to immobilize uranyl ions in subsurface aquifers contaminated with nuclear waste (Anderson *et al.*, 2003; Wu *et al.*, 2006). New evidence of microbiologically mediated formation of nanocrystals of uraninite as part of a strategy to bioremediate uranium-contaminated subsurface environments (Suzuki *et al.*, 2002; Sani *et al.*, 2004) support Beveridge's earlier proposal for this practical application of microbe-metal interactions (Brown *et al.*, 1994).

Beveridge's successful collaborations and insightful interpretations of experimental data resulted largely from his electron microscopy skills. Few scientists can take credit for as many high-resolution images of microbial structures in the published peer-reviewed literature as Beveridge. Furthermore, few electron microscopists have worked harder than Beveridge to minimize or eliminate artifacts introduced to samples during dehydration in preparation for examination by electron microscopy. He was quick to evaluate a new approach to preserve bacterial cell structures for high-resolution electron microscopic examination and extract any new information it offered on cell structure, organization, and function. In a recent article, based on a presentation at the symposium, 'Bacterial Sculpture: Peptidoglycan Metabolism and Cell Shape', convened during the ASM General Meeting in Atlanta, 5–9 June 2005, Beveridge shared his first-hand experiences with cryotechniques, which in his view, are the best available means to eliminate or minimize deformation of biopolymer structure that typically occurs when dehydrating samples for electron microscopic examination (Beveridge, 2006). In this paper, he revealed how never before seen features of bacterial cell walls preserved as frozen hydrated thin sections provide new insight into maintenance of peptidoglycan structure. He also credited this preservation method for enabling the acquisition of new structural information on LPS and EPS in Gram-negative bacterial biofilms. His enthusiasm was only slightly tempered by evidence that the density of EPS in his samples appears to be too low to avoid some condensation of polymers during water removal by this approach. Resolution of the structure of bacterial biofilm EPS in their native state may thus have to await future developments in sample preparation and/or instrumentation and the interest and skills of a new generation of microbiologists. In view of the strong foundation established by Beveridge in structural microbiology during his 30-year tenure at the University of Guelph, one anticipates that his legacy will ensure the institution's leadership in this area of research into the foreseeable future.

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