Journal of Nuclear and Radiochemical Sciences, Vol. 6, No.1, pp. 7-10, 2005

Bacterial Cell Wall Structure and Implications for Interactions with Metal Ions and Minerals

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Received: November 15, 2004; In Final Form: November 15, 2004

New techniques in the rapid freezing of cells, so that they are vitrified, and cryo-transmission electron microscopy (cryoTEM) of the *frozen hydrated thin-sections* from the vitrified cells are showing their true native structure. Unlike other forms of TEM, frozen hydrated thin-sections cannot be contrasted by heavy-metal stains (such as U, Pb, and Os) and their contrast is via the inherent density of the constituent molecules within the cells. Therefore, these frozen sections show the true mass distribution within the biomatter. Another cryoTEM technique, *freeze-substitution*, also produces thin sections for viewing by TEM, but these are plastic sections of heavy-metal stained cells. The heavy-metal ions of the stain complex to the available reactive sites of the biomatter. When such images are compared to those from the frozen hydrated sections, a clear interpretation of native structure and its metal reactivity can be made. These types of observations will be invaluable for the study of microbe-metal/radionuclide interactions.

1. Introduction

Prokaryotes are the Earth's smallest life form and, yet, have the largest surface area-to-volume ratio of all cells.[1, 2] They are also the most ancient form of life and have persisted on Earth for at least 3.6 Ga, even (we think) in some of the most extreme environments imaginable, such as the deep subsurface. Most of these early primitive (and today's modern) natural environments possessed reasonably high amounts of metal ions that were capable of precipitation under suitable pH or redox conditions. Deep-seated in such geochemical situations is the likelihood of suitable interfaces that lower the local free-energy so that interfacial metal precipitation is promoted. Bacteria, being minute and having highly reactive surfaces (interfaces), are exquisitely efficient environmental particles for metal ion adsorption and mineral nucleation. Metal ions interact with available surface reactive groups (or ligands) available on the bacterial surface and precipitates grow as environmental counter-ions interact with more and more metal at the site.[3–7] Once formed these precipitates are under the influence of natural geochemical and additional microbially-mediated conditions[8] that instigate the development of fine-grain minerals, usually via dehydration so that crystalline phases are eventually developed.[9] These minerals commence as so-called 'nano-mineral phases' and grow with time to become larger and larger. This bacterially-induced mineralization is likely the natural phenomenon that so encases some cells in fine-grain minerals that they die and become *bona fide* 'microfossils'.[10] In ancient times, these mineral-encased prokaryotes, enduring low-temperature metamorphic geological conditions, survived as microfossils still existent in such very old Precambrian formations as the ~2.0 Ga Gun Flint Chert, north of Lake Superior in Canada.

It is certain that bacterial surfaces interact with environmental metals ions and can provide nucleation sites for mineral precipitation but it has been extremely difficult to study such systems with high precision on a cell to cell basis, even though a wide base of techniques exists.[11] This is because the cells are extremely small and the interactive structures even smaller, and because the reactive sites responsible for adsorbing metals retain their reactivity only over a certain range of pH and Eh, which is difficult to monitor over micro-scale distances. This article will outline our new advances in elucidating the structure of bacterial surfaces.

2. Use of Cryo-Transmission Electron Microscopy

Using conventional fixation, embedding and thin-section techniques, the transmission electron microscopic observation of bacteria has been a most powerful method for elucidating the internal cytoplasmic organization and the juxtaposition of encompassing envelope layers of cells.[12, 13] Yet, there are many drawbacks to such conventional techniques since the cells are chemically fixed, using harsh fixatives (such as glutaraldehyde and osmium tetroxide), and dehydrated before embedding in a plastic resin for thin sectioning.[14] Essential lipids are extracted, proteins are denatured, and nucleic acids are artificially condensed. The cells are a specter of their former selves. Clearly, the images of such cells have been beneficial to our initial perception of the structural organization of prokaryotic cells[12] but hydration (which these embedded cells no longer have) is a necessary prerequisite for the maintenance of native structure. With proper expertise, care and equipment, it is now possible through the use of cryo-transmission electron microscopy (cryoTEM) to obtain a better and more natural view of bacteria.[15–18]

Freeze-substitution. One cryoTEM technique that became popular during the 1980–90s was freeze-substitution.[18–21] Here, cells are rapidly frozen at ~–196 ˚C so as to vitrify them in amorphous ice, which is not crystalline and is a kind of glass.[13, 14] Hence the cells are *physically* 'fixed' since there is no time during freezing for structure to degrade; in fact, freezing occurs within milli- to micro-seconds and all molecular motion is stopped. If the bacteria are thawed, they come back to the living state and continue to grow and divide. This is a clear-cut measure of how well the cells are preserved. Once vitrification is accomplished and cellular structure preserved, the temperature is raised from –196 ˚C to –80 ˚C and the cells put into a freeze-substitution mixture. This consists of a cryogenic fluid (such as acetone) containing a chemical fixative (osmium tetroxide), a heavy metal stain

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Published on Web 07/31/2005

(uranyl acetate), and a molecular sieve (for trapping water).[20, 21] At this low temperature, the cells do not melt and remain vitrified, and structure is preserved. The cellular and surrounding ice (water) sublimes and is trapped in the molecular sieve. Eventually, the specimen is thoroughly dehydrated and fixed in this freeze-substitution mixture with the structure maintaining many of its native features. Now, it can be embedded in plastic resin, cured and thin sectioned for viewing by TEM.

Freeze-substitution and Gram-positive (*Bacillus subtilis*) cell walls. The results of freeze-substitution are breath taking, especially when examining the surface structures (Figure 1). The fabric of Gram-positive cell walls is no longer featureless (as seen in conventional embeddings),[22] but is a tripartite structure (Figure 1). The region associated with the plasma membrane (immediately above the bilayer) is highly contrasted due to the acquisition of large quantities of heavy metal stain. The middle region is lightly contrasted since the wall polymers (mainly peptidoglycan) are stretched almost to the breaking point so that the mass-to-volume ratio is much reduced compared with other wall regions. The outermost region consists of thin fibres that extend into the external milieu. This tripartite cell wall structure is compatible with current models of cell wall turnover.[21, 22]

More importantly for this chapter is the fact that the polymeric structure of the wall has been preserved by freeze-substitution and the available reactive sites within the wall have been 'decorated' with the heavy metal stain so that we obtain a clear picture of *where the reactive sites reside*. Many sites are in the region immediately apposed to the membrane since, here, new wall polymers are being extruded and compacted via penicillin-binding proteins in the membrane. As an area of *de novo* wall assembly, where new polymers are being cross-linked into the pre-existing wall fabric, many reactive groups are available for decoration in this region since the mass is great and the availability of reactive groups is high. The middle region is different though. It is the area in the cell wall that resists turgor pressure and maintains the integrity of the cell. It has to be highly cross-linked to hold the cell together under such a pressure load and is considerably stretched. This region, then, has few reactive groups left available for interaction with the heavy metal stain since most have been used to cross-link the network together for strength. The outermost region is an area where the cell wall is being broken down by the wall's constituent autolysins. Covalent bonds are being broken and new reactive groups being made. This region probably has little mass (since it is being shed during cell wall turnover) but an excess of reactive sites that are readily decorated by the stain.

Frozen hydrated Gram-positive (*B. subtilis*) cell walls. A more difficult and therefore less used cryoTEM technique is the use of frozen hydrated sections.[16, 23] This technique requires skill and perseverance. As in freeze-substitution, cells are vitrified but now, instead of processing the cells so that conventional plastic thin sections are obtained, this frozen material is immediately put into a *cryo*-ultramicrotome and thin sectioned. The cells are sectioned while vitrified and the frozen sections immediately mounted into a *cryo*-specimen holder and inserted into the *cryo*-chamber of a *cryo*TEM. We emphasize the word '*cryo*' because the temperature must be maintained at –196 to –140 ˚C during all manipulations, otherwise the amorphous ice embedding the cells will become crystalline and ruin the native structure to be observed. No chemical fixatives or heavy metal stains are used during the entire process and, since the sections remain vitrified, all cellular macromolecules and polymers remain in a hydrous state.

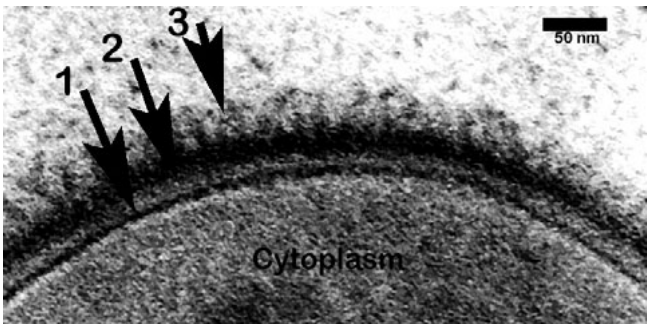

Figure 1. Thin-section image of a freeze-substituted of a Gram-positive *Bacillus subtilis* cell showing three distinct regions in the cell wall, the inner (#1), middle (#2) and outer (#3) regions that correspond to cell wall turnover and to the available reactive groups within the cell wall network. Bar = 50 nm.

One of the advantages of the cryo-sectioning technique is that no artificial chemical fixatives or heavy metal stains need to be used. However, implicit in the use of TEM is that the specimen must possess enough density to efficiently scatter high voltage electrons from the electron gun of the microscope (i.e., the electron potential is typically ~100,000 – 200,000 electron volts). Biomaterials, once thin sectioned, rarely have enough density to effectively scatter such high powered electrons since the thin sections are only ~50 nm thick and the biomatter possesses only low atomic number elements (such as H, C, O, N, etc.). This is the primary reason why conventional and freeze-substitution thin sections use stained material; the heavy metal stains increase the density (or the mass-to-volume ratio) of the specimen so that the contrast becomes great enough for the cells to be easily visualized.[14] Frozen hydrated thin sections of unstained bacteria do not have this luxury since they cannot be stained once sectioned. (The staining fluids would immediately freeze over the sample and obliterate the structure of the cells.)

Clearly, then, these frozen hydrated sections of bacteria are difficult to see since their contrast is close to that of the surrounding vitrified ice. For this reason, we rely on the inherent phase function of the lenses of the cryoTEM and use phase contrast to help imaging by over focusing to see the bacteria. Certain microscopes (e.g. those with energy filters) can also derive more additional contrast for the specimen. But even then there are additional problems in visualizing frozen sections. The energy of the electron beam is often high enough to locally increase the specimen's temperature, resulting in the formation of crystalline ice (from amorphous ice) and, eventually, in ice sublimation. Since bacteria are excellent nucleation particles (remember how efficient they are for forming fine-grain minerals!), the amorphous to crystalline phase transition of ice frequently occurs on the bacteria and their structure is obscured. Furthermore, since the specimen is kept so cold, the frozen sections act as 'cold traps' for the condensation of extraneous molecules within the high vacuum of the microscope column, thereby often contaminating the structure of the specimen. With all these associated problems, it is a wonder that frozen hydrated sections of bacteria can be imaged, but they can and they are extraordinary (Figure 2).

These images differ from what we see in freeze-substitution images. Remember, now we have no heavy metal stains to assist contrast and must rely on the inherent density imparted on the cell by the constituent atoms within its molecules. Proteins will be more readily discerned from the surrounding ice than, say, carbohydrates because they are usually larger, contain nitrogen and (sometimes) sulfur, and tend to fold tighter. And, most important, all cellular constituents remain hydrated and therefore not artificially condensed because of a dehydration regimen. The ribosomes are larger and more robust and can be seen because of their large concentration of protein and rRNA (here the phosphorus adds additional contrast) (Figure 2). Even the bilayer of the membrane can be seen because of the inherent contrast of the phosphorus in the phos-

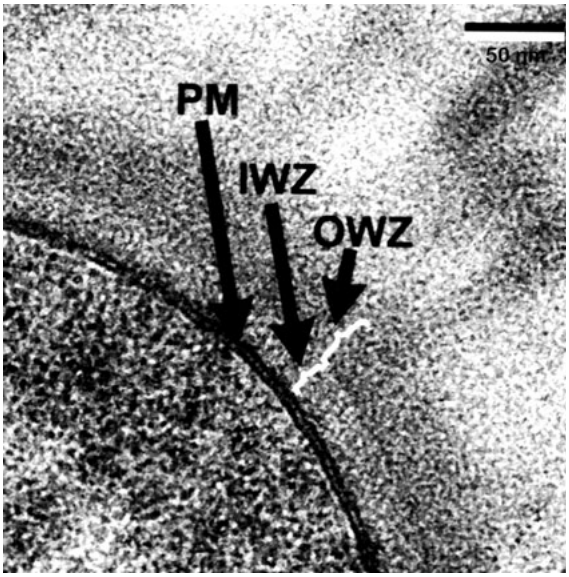

Figure 2. Frozen hydrated thin section of the *B. subtilis* cell wall showing the plasma membrane (PM) the inner wall zone (IWZ) and the outer wall zone (OWZ). Here the IWZ corresponds to region #1 and the OWZ to region #2 of the freeze-substituted wall in Figure 1. Region #3 is not seen. These IWZ and OWZ have different dimensions than the regions in Figure 1 and are visualized entirely due to the density of the constituent macromolecules. Bar = 50 nm.

pholipids. Most important for this chapter, though, is the cell wall. Here, we get a clear idea of the mass distribution within the Gram-positive wall of *B. subtilis* (Figure 2). Immediately above the bilayered membrane is a low-density space with little contrast. Experiments have shown that this is a periplasmic space.[24] Above this is a more densely contrasted region that represents the peptidoglycan-teichoic acid network of the wall. Unlike freeze-substitutions, there is not an outermost fibrous region (cf., Figures 1 and 2).

3. Correlation of Freeze-Substitution and Frozen Hydrated Images

How can we reconcile the differences seen in Figures 1 and 2 remembering that the cell in Figure 1 has been dehydrated and decorated with a heavy metal stain? Since it is dehydrated, we would expect there would be a certain amount of contraction of the wall regions in freeze-substitution images (Figure 1) because the structures are no longer hydrated. We would also expect reactive groups to be labeled. On the other hand, frozen hydrated structure would not be condensed and this is why the thickness of each wall region is greater in Figure 2 as compared to Figure 1. This same image shows a periplasmic space whereas Figure 1 does not. The periplasm has contracted and condensed in Figure 1, but it has been preserved in its natural state in Figure 2. Accordingly, the periplasm in Figure 1 is more concentrated and (it seems) more reactive since it stains strongly. In Figure 2, the periplasm has not condensed and it has low density. The conclusion, then, is that the natural state of the periplasm in these cells is as a relatively low-density matrix of highly reactive biomatter occupying a definite periplasmic space defined by the membrane and the middle region. Presumably the periplasm in this space consists of new wall polymers, secreted proteins (and their associated chaperones), and both periplasmic enzymes and oligosaccharides.[24]

The region above this periplasmic space is wider in frozen sections than in freeze-substitutions (cf., Figures 1 and 2) and, since this is the hydrated structure, the increased width shows its natural state. Both figures reveal it to be of relatively low contrast; freeze-substitutions suggest there are few available reactive groups and frozen sections suggest that there is little

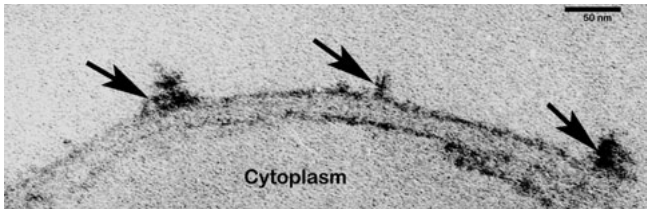

Figure 3. Conventional thin section of a *B. subtilis* that has been subjected to 50 mM $FeCl_3$ treatment for 15 min at 22°C. The iron has begun to precipitate from solution onto the cell wall (arrows). Notice that most iron is associated with the wall surface and with the periplasm. No stains other than the iron have been used on this cell.

density.[25] Therefore this region, as the stress-bearing region of the wall, has been stretched taunt (thereby reducing its mass), and most reactive groups have been utilized to ensure that the network is cemented firmly together. This forms a strong but elastic fabric of wall polymers, mainly of peptidoglycan.[25, 26]

The outermost fibrous region, which is highly decorated with stain in Figure 1, is not seen in Figure 2. Accordingly, this outermost region has so little mass it cannot be seen but is highly reactive. This is in accordance with cell wall turnover since this is a region where autolysins are breaking down old peptidoglycan making it soluble. This would reduce its mass while at the same time would generate many new reactive sites due to hydrolysis.[24]

4. How Does This New Interpretation of Wall Structure Correlate with Metal Ion Interaction and Mineralization?

It is undeniable that metal ions interact strongly with bacterial surfaces and mineralize them.[3, 7, 9, 27–29] Cell walls adsorb metal ions and minerals are nucleated in Gram-positive cell walls because of metal ion interaction with the peptidoglycan and secondary polymers (Figure 3).[4, 5] Our new structural observations on Gram-positive walls now show the quantity of hydrated biomatter that resides in the wall for metal interaction (Figure 2). They also reveal where are the most reactive and likely regions for metal ion interaction and mineral growth (Figure 1). The outermost fibrous region would be the most accessible reactive region and, here, there would be little problem of metal ion access and of mineral particle growth. Since many wall polymers are in the act of being solubilized, these polymers and their precipitates would be sloughed from the cells but could continue to grow and mature into *bona fide* mineral phases in the external milieu. The middle region has less reactivity since it is highly cross-linked. Because turgor pressure stretches this region almost to the breaking point, the peptidoglycan would be in a relatively 'open' configuration[25] so that most metal ions could penetrate through. The inner region (or periplasmic space) is a highly reactive loose gel of polymers and proteins that would both interact with and precipitate metal ions. Here, though, since the periplasm resides within a confined space between the plasma membrane and the middle wall region, mineral growth would be restricted to the accessible space.

These correlations and interpretations allow certain predictions to be made as to where environmental metal ions should interact with Gram-positive bacterial surfaces. Gram-negative surfaces are structurally more complicated[12, 13, 15, 19, 21] but they have also been imaged via freeze-substitution and frozen hydrated sections[16, 19–21, 30] and are, therefore, also able to be correlated. These Gram-negative surfaces also interact strongly with metal ions.[27] It is therefore undeniable that bacterial surfaces make excellent nucleation sites for the development of fine-grained minerals. Hopefully these new electron microscopy observations on bacterial walls help shed some light on these metal ion-cell surface interactions.

Acknowledgments. The author thanks his student V. Matias for supplying the frozen hydrated section used in Figure 2. A much more extended version of this paper will be published in an up-coming Society for General Microbiology book in late 2005 entitled *'Micro-organisms and Earth Systems–Advances in Geomicrobiology'*. The research presented in this article was made possible through funding provided by a Canadian National Science and Engineering Research Council (NSERC) Discovery grant and a United States Department of Energy Natural and Accelerated Bioremediation Research Program (US-DOE-NABIR) grant to TJB. The electron microscopy was performed in the Guelph Regional Integrated Imaging Facility (GRIIF), which is partially funded by an NSERC- Major Facility Access grant to TJB.

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