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Bacterial biomineralization: Where to from here?

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It is widely accepted that bacteria bind metals and form minerals, but where did this understanding come from? The answer is simple, Terry Beveridge. His pioneering research in the 1970s, as a PhD student at the University of Western Ontario under the supervision of Drs Robert Murray and William Fyfe, demonstrated that the outer surfaces of the common soil bacterium, *Bacillus subtilis*, could bind so much metal that they formed dense aggregates (e.g. Beveridge & Murray, 1976). Transition metals, in particular, imparted such strong electron-scattering power into the cell wall that some of them have subsequently been used as contrasting agents for electron microscopy (Beveridge, 1978). Indeed, many of the advances made on bacterial ultrastructure over the past three decades were made possible by metal staining and visualization of thin sections under the transmission electron microscope (TEM). From this work, another pivotal paper followed. In it, Terry used a variety of chemical treatments to modify or remove electronegative and/or electropositive groups within the cell wall of *B. subtilis*, and in doing so, showed that it was the anionic carboxyl groups in Gram-positive cells that contributed most to metal binding (Beveridge & Murray, 1980). By contrast, subsequent research with Gram-negative cells, such as *Escherichia coli*, showed that phosphate groups in the outer membrane were the main metal-binding groups (Ferris & Beveridge, 1986).

The innovation did not end there. Irrespective of what bacteria Terry studied, what he repeatedly observed was that considerable variations in metal immobilization could be displayed by a single species. In some instances, cell walls were diffusely stained, while at other times so much metal was fixed to the cell surface that it formed a distinct mineral phase. This then led Terry to propose a two-step mechanism for bacterial biomineralization; the first step in time is an electrostatic interaction between the metal cations and the anionic sites in the cell wall. Those metals then act as nucleation sites for the deposition of more solutes from solution, potentially leading to mineral precipitation (e.g. Beveridge & Fyfe, 1985). Crucially, this model has stood the test of time, and it now has been used to explain the formation of a wide array of biominerals, ranging from iron minerals (Konhauser, 1998) to carbonates (Thompson & Ferris, 1990) to silicates (Konhauser *et al.*, 2004).

It is in this regard that I first met Terry during the second year of my PhD at the University of Western Ontario, back in 1991. I was studying the factors influencing river chemistry in the Amazon and came across some papers of his describing how bacteria bind metals. During my fieldwork I had noticed that a biofilm covered all of the sediment I collected, and it occurred to me that given the large surface area of the river bed, the volume of water that fell into direct contact with the biomass must have been substantial. Accordingly, I wanted to analyse that material to see what metals were bound and if any authigenic minerals had formed – and the right place to do that work was at the University of Guelph, in Terry's laboratory. I subsequently spent many months analysing material under the TEM and became familiarized with experimental techniques used to study microbe–metal interactions. Without question, my interests in geomicrobiology directly stem from that time spent in Terry's laboratory.

As I moved on to my Postdoctoral Fellowship with Grant Ferris at the University of Toronto, and then my first faculty position at the University of Leeds, my interests continued to center on biomineralization processes. But, the more I studied, the more I began to think about the relative importance of bacteria to natural mineral-forming processes, and whether microbes actively enhance mineralization reactions or just play the cursory role of a solid surface on which nucleation reactions take place. In other words, how important are bacteria to many of the widely described biomineralization processes? One obvious mineral to focus on was amorphous silica because of its widespread formation in association with hot-spring deposits, the diversity of microorganisms present in some silicifying environments (Blank *et al.*, 2002), and the numerous studies reporting microbial silicification leading to cellular preservation (e.g. Oehler, 1976; Francis *et al.*, 1978).

What made silica particularly interesting to me was the fact that it had been suggested many years ago that the role of

microorganisms in silica precipitation was largely a passive process (e.g. Walter *et al.*, 1972). In geothermal systems, waters originating from deep, hot reservoirs, at equilibrium with quartz, commonly contain dissolved silica concentrations significantly higher than the solubility of amorphous silica at 100 °C. Therefore, when these fluids are discharged at the surface, degassing, rapid cooling to ambient temperatures, and evaporation all conspire together to cause the solution to suddenly become supersaturated with respect to amorphous silica (Fournier, 1985). In such supersaturated solutions, the discharged monomeric silica, $Si(OH)_4$, polymerizes, initially to oligomers (e.g. dimers, trimers, and tetramers) and eventually to polymeric species with spherical diameters of 1–5 nm, as the silanol groups (-Si-OH-) of each oligomer condense and dehydrate to produce the siloxane (-Si-O-Si-) cores of larger polymers. The polymers grow in size through Ostwald ripening such that a bimodal composition of monomers and particles of colloidal dimensions (>5 nm) are generated. These either remain in suspension due to the external silanol groups exhibiting a residual negative surface charge, they coagulate via cation bridging and nucleate homogenously, or they precipitate heterogeneously on a solid substratum (Iler, 1979). As microorganisms are present in these polymerizing solutions, they will inevitably become silicified, much the same as other submerged solids, e.g. pollen, wood, leaves, and sinter.

Experimental evidence now exists that appears to corroborate the view that the microbial role in silicification is predominantly incidental and not limited to any particular taxa. In particular, bacteria have little affinity for monomeric silica, even at high bacterial densities and low pH conditions, where most organic functional groups are fully protonated (Fein *et al.*, 2002). Similarly, under highly supersaturated conditions, the rates of silica polymerization are independent of the presence of bacterial biomass, and the amount of silica removed from solution in abiotic and biotic experiments is virtually indistinguishable (e.g. Yee *et al.*, 2003; Benning *et al.*, 2004; Lalonde *et al.*, 2005). Presumably, in concentrated silica solutions there is such a strong chemical driving force for silica polymerization, homogeneous nucleation, and ultimately silica precipitation that there is no obvious need for microbial catalysis. It has also been observed that silicification occurs on dead cells, and continues autocatalytically and abiogenically for some time after their death due to the high reactivity of the newly formed silica. Consequently, silica precipitated in the porous spaces between bacterial filaments has the same basic size and morphology as the silica precipitated on the original filaments (e.g. Jones *et al.*, 1998). These findings certainly support the notion that microbial silicification at thermal springs occurs simply because microorganisms grow in a polymerizing solution where silicification is inevitable. This observation has recently been supported by Amores & Warren (2007), who found no evidence of biologically enhanced rates of silicification at circum-neutral pH in Yellowstone biofilms compared to abiotic systems. Interestingly, however, enhanced rates were observed at acidic (pH 3) conditions, suggesting that under low pH conditions, a biotic impact on precipitation rates may be observed.

Whether abiotic or biologically enhanced, silicification is a commonplace process and microorganisms are certainly capable of adapting to, and surviving, this dramatic entombment. A study by a former PhD student of mine, Vernon Phoenix, showed that silicification of the cyanobacterium *Calothrix* sp. took place exclusively on the outer surface of the sheath (an extracellular polysaccharide layer that surrounds the cell). In fact, when *Calothrix* was grown in silica supersaturated conditions, its sheath doubled to tripled in thickness (Phoenix *et al.*, 2000). Concomitantly, the filaments developed extensive mineral crusts up to 5 μm thick upon the sheath surface, yet the cells' pigments still autofluoresced, they continued to generate oxygen, and the mineralized colonies exhibited comparable rates of photosynthesis to the nonmineralized colonies. Furthermore, the cell walls and cytoplasm remained free from silica precipitate. In a more recent study with the thermophilic chemolithoautotroph *Sulfurihydrogenibium azorense*, a current PhD student, Stefan Lalonde, showed that the bacterium, when grown as a H_2 oxidizer, reacted to increasing silica concentrations by producing excess protein that appears linked to biofilm production (Lalonde *et al.*, 2005). Since silicification was observed to be restricted to the biofilm, and not the cell surface, we proposed that *S. azorense* may prevent cellular silicification to some degree by providing abundant reactive sites in the surrounding matrix and regulating biofilm production appropriately, with potential contributions from metabolic effects. Thus, it appears that some microorganisms can thrive in silica-rich environments because they form protective layers that isolate the cells from the potentially damaging effects of silicification, while others may employ yet-undiscovered means of ensuring their survival under these extreme conditions.

Given the tremendous diversity of microorganisms and their habitats, these observations must just scrape the surface of a very diverse array of complex microbe–silica interactions. Evidently, future biomineralization research must (i) examine further the microbial response to silicification, asking just what organisms can survive this process, how this is achieved, and ultimately what are the molecular-scale responses, and (ii) if some microorganisms can survive silicification, can they use this mineral suit-of-armour to their advantage? This second question is the subject of an accompanying paper and is thus not discussed here (see Phoenix & Konhauser, 2008). To elucidate the first question, we have begun tackling this issue by looking at the effects of silica on a filamentous heterocyst-forming cyanobacterium, *Anabaena* sp. PCC 7120. This organism, also known as *Nostoc muscorum*, has served as a model cyanobacterium for the study of heterocyst differentiation and nitrogen fixation, and its genome has recently been sequenced (Kaneko *et al.*, 2001). As it is typically found in freshwater systems such as lakes and rivers, and not typically

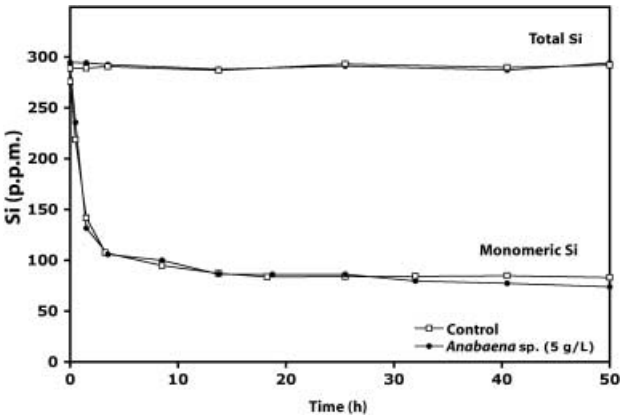

Fig. 1 Experimental silicification of a concentrated (5 g L^{-1} wet mass) suspension of *Anabaena* sp. Supersaturation with respect to amorphous silica was achieved by neutralization to pH 8 such that silica polymerization occurred spontaneously. Relative to the control, the presence of a high concentration of *Anabaena* sp. had no detectable influence on the polymerization process (monomeric Si), and did not promote precipitation in any way (total Si).

associated with geothermal environments, we were interested in discovering whether such species would survive exposure to highly siliceous solutions, and if they did, what type of physiological response would they exhibit? It turns out that batch cultures of *Anabaena* sp. grew readily even with high concentrations of silica (300 p.p.m. Si) in their growth media. This is not surprising given that interaction between cell surfaces and silica monomers, polymers and colloids is generally weak; the negative surface charge exhibited by nearly all microbes at circumneutral pH inhibits the adsorption of the similarly charged silica species, and freshly formed silica polymers and colloids are highly hydroxylated (silanol-rich) and thus more prone to forming hydrogen bonds. Indeed, when we exposed concentrated suspensions of freshly harvested, exponential phase *Anabaena* cells to silica supersaturated solutions, they had no effect on the rate or extent of silica polymerization, nor did they facilitate mineral nucleation or sequester the newly formed polymers and colloids from solution (Fig. 1). This does not necessarily mean, however, that the cells cannot become entombed with amorphous silica after prolonged exposure to highly supersaturated solutions. Figure 2 shows TEM micrographs of *Anabaena* sp. filaments before and after 30 days of repeated exposure to supersaturated silica solution, where the solution was refreshed every time that spontaneous polymerization was complete (~2 days). After 30 days, cells had become encased in an amorphous silica coating, but despite this, close-up images of the cell–silica interface (Fig. 1C) did not reveal any evidence that the *Anabaena* sp. cell wall played a role in mineral nucleation. Instead, it appears that *Anabaena* sp. was merely an innocent bystander, simply occupying space in which amorphous silica could not accumulate.

The apparently minimal interaction between *Anabaena* sp. and amorphous silica species made this system an interesting starting point for evaluating the opposite of most silicification studies to date, that being the effect of silica on the microbe. While some disruption in cellular homeostatic functions may be expected in the case of microbes that are readily encrusted with amorphous silica, it is our aim to identify whether the cells respond to the mere presence of silica. In other words, the fact that *Anabaena* sp. does not physically interact with amorphous silica to any large extent means that we can focus on cellular response that is independent of any stress specifically associated with physical encrustation.

So, why are potential silica stress responses interesting? It is likely that the Earth's oceans were at or near saturation with respect to amorphous silica throughout the Precambrian (Maliva *et al.*, 2005). If much of bacterial life on Earth evolved in silica-rich waters, did they evolve some mechanism to recognize, or even cope with, a mineralizing agent such as amorphous silica? If so, did this occur early or late? Or are the above-mentioned biological responses to the silicification

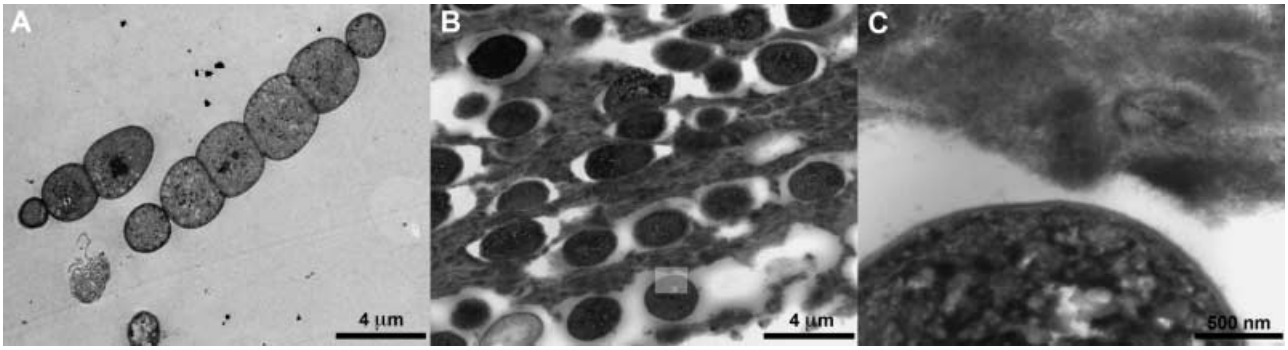

Fig. 2 Transmission electron microscope micrographs of (A) intact and unsilicified filaments of *Anabaena* sp. and (B) filaments that were grown for 30 days in a supersaturated silica solution that was replaced every 2 days. Despite becoming entombed in a matrix of amorphous silica, high magnification ((C), inset of (B)) reveals minimal interaction between amorphous silica and the cell wall.

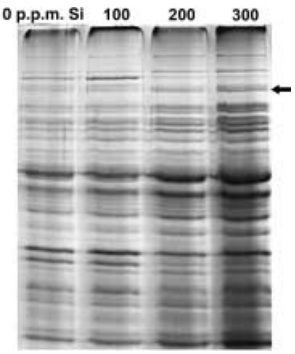

Fig. 3 SDS-PAGE gel of soluble protein extracts obtained from cultures of *Anabaena* sp. grown in BG-11 with the addition of 0, 100, 200 or 300 p.p.m. Si. Several changes in protein expression are evident despite the limited window afforded by the gel. The band highlighted by the arrow was excised for sequencing by mass spectrometry and identified as a 116 000 Da hypothetical membrane transporter.

process merely manifestations of other stress-response systems, where cells are coping with something perhaps more familiar, e.g. osmotic stress? To begin addressing these questions, we evaluated protein expression on a gross scale using polyacrylamide gel electrophoresis. From our preliminary results, two things are clear (Fig. 3). First, it is apparent that the presence of silica induces changes in protein expression. A SDS-PAGE gel of soluble protein extracts from *Anabaena* sp., grown with different concentrations of silica added to BG-11 media, reveal several changes in protein expression. To date we have focused on a high molecular weight (116 kDa) band that was expressed at high silica concentrations (200–300 p.p.m. Si) but not at low concentrations. Sequencing by mass spectrometry provided a strong match to a hypothetical protein (NCBI accession BAB74740) whose function is only vaguely known by homology; this particular protein possesses a conserved domain that represents a predicted membrane transporter and putative toxin regulator.

Our second and perhaps more profound finding is that changes in protein expression are evident in our gels despite the fact that less than 100 proteins are visualized on the gel out of the 5368 potential proteins in the genome. Clearly, response to the presence of silica is not limited to a single gene product. In this case, the microbe-solute interaction is a two way street, where the microbe not only affects mineralization, but conversely, mineralization impacts on the microbe. It is likely that many microbes will exhibit some form of response to mineralization, and in this regard, on-going investigation with *Calothrix* sp. has also revealed numerous changes in protein expression, including one of similar molecular weight to that mentioned above for *Anabaena* sp. (data not shown). Unfortunately, the *Calothrix* genome is uncharacterized to date, and protein identification by peptide fragment sequencing has proven unsuccessful to date.

From this preliminary research, it is obvious that we need to expand our limited window into protein expression by combining traditional techniques that investigate changes in physiology (i.e. bulk amino acid composition, chlorophyll content) and metabolic activity (i.e. rates of N_2 fixation and photosynthesis) with the more cutting edge tools available to microbiologists today. As we do not specifically know how a genetic response to the silicification process might manifest itself, we are pushed in the direction of expression profiling, or comparing snapshots of gene or protein expression obtained under different conditions. In recent years, DNA microarray technology, where some or all genes of a single organism may be individually spread across a single glass slide and individually evaluated for changes in gene expression, has become poised to reveal the true depth of microbe–environmental interactions. For example, Singh *et al.* (2003) evaluated transcription of 3165 genes of cyanobacterium *Synechocystis* sp. PCC 6803 grown on iron-deficient media, and found that 85 genes showed statistically significant changes in transcription level, while a further 731 genes changed by a factor of >1.25, relative to iron-sufficient controls. It appears that in order to fully understand the environment from the microbe's point of view, hundreds to thousands of highly interrelated processes must be considered.

While our lessons learned from the protein gels may not be unexpected in the eyes of a professional microbiologist, it brings us geomicrobiologists full circle. That is full circle in the realization that interactions between microbes and their physical environment are more profound than we often imagine, or can imagine. My students have newfound respect for the complexity and intricacy of microbes as they interact with their environment, much as I did upon entering Terry's laboratory. I have no doubt that the geomicrobiological studies that began in an innocuous laboratory in Guelph, Ontario, will inspire students and professors alike for the generations to come.

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