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# From iron oxides to infections

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I was fortunate to have met Terry Beveridge early during my time in graduate school, when my advisor, Francois Morel (a geochemist), recognized the importance of my having a microbiologist on my PhD advising committee. At the time, I was studying microbial precipitation of arsenic trisulfide ($As_2S_3$) by *Desulfotomaculum auripigmentum* and needed the help of a geomicrobiologist to take electron micrographs of my samples (Newman *et al.*, 1997). What better person to ask than Terry Beveridge? Not knowing much about what I was doing, I naively went off to Guelph to spend a week working in Terry's laboratory during the fall of 1994. Little did I know that this would be the start of one of the most inspirational scientific and personal relationships of my career. From that week forward, I turned to Terry whenever I had a question about microbiology, and looked forward to his responses: Terry had a way of making me feel like I had hit upon something profound (when in fact my questions were pretty trivial), and his answers not only were thoughtful and informed, but had the effect of getting me to think about other problems that were much more interesting.

As I moved on from graduate school, and started to consider the world of microbiology more broadly, I sought advice from Terry regarding where to go for a postdoc. Although I was still quite naïve, I'd read enough by then to realize that Terry was not just an accomplished geomicrobiologist, but had also done pioneering work in more medically related fields. Had I not been committed to learning bacterial genetics, I would have happily joined his laboratory to study membrane vesicles and their contents, which Terry was very excited about at the time (Beveridge, 1999). Over the years, I continued to see Terry on a regular basis at meetings, and exchanged e-mail with him about science. The more I learned about microbiology, the more I came to appreciate that his studies of cellular ultrastructure were not only technically outstanding, but covered a vast intellectual territory, ranging from iron oxides to infections. It is Terry's depth, intellectual versatility, optimism and generosity that I would like to pay homage here, as the less naïve I have become, the more I have come to appreciate how very special this combination of qualities is.

Something that characterizes Terry's ocuvre is its fundamental nature. Whether studying *Bacillus subtilis, Staphylococcus aureus, Eschericbia coli, Pseudomonas aeruginosa* or mixed cultures from the environment, he had the ability to observe a cell and articulate general principles about how it holds itself together. His way of seeing was both rigorous and highly creative (Beveridge 2006). For example, in a recent study on the native cell wall organization of *S. aureus*, Terry and Valério Matias inferred specific mechanisms of cell wall growth in *S. aureus* based on densitometry plots of its outer wall zone (Matias & Beveridge, 2006). A casual observer would easily miss such insights; yet, upon taking a closer look and thinking harder, they are difficult to escape: this is but one example of how Terry turned a 'simple' image into something rich in mechanistic content, creating something of an epiphany for the reader. His visual acuity reminds me of that of one of my other heroes in science – Barbara McClintock, the renegade cytogenetieist who found evidence for transposition in maize decades before it

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was rediscovered in bacteria. Like Terry, she was able to infer fundamental mechanisms of biological organization by staring down the microscope with a keen eye and mind, possessing that elusive scientific gift – a 'feeling for the organism' (Keller, 1983).

In the spirit of writing something forward-looking in honor of Terry, I thought I would focus on my version of the microbiological continuum from iron oxides to infections. Moving back and forth between the world of geomicrobiology and the world of medical microbiology is something Terry did incredibly successfully throughout his career, and his work has served as an inspiration to me of how one can focus on fundamental biological principles (in his case, the physical organization of bacterial cell walls) and apply them broadly. The fundamental biological principles my laboratory seeks to understand primarily revolve around how cellular electron transfer processes work under diverse anaerobic conditions. In some cases, these processes affect the geochemistry of the environment (such as respiratory arsenate reduction or phototrophic iron oxidation); in others, they are more germane to the survival of microorganisms in clinical contexts. How we tie these fields together as geobiologists is what I would like to focus on here.

For many geobiologists, the rock record provides an incentive to study the function of certain biomolecules by demonstrating their evolutionary importance, but it can also affect our thinking about biological processes in other ways. For example, many bacteria live together in biofilms, communities of cells attached to surfaces. Despite their ubiquity – from the lungs of cystic fibrosis patients, to medical implants, to the surfaces of rocks in sediments (Costerton *et al.*, 1995; Costerton *et al.*, 1999) – we know very little about the rules of metabolism that sustain life in these environments. Indeed, if we penetrate only a few microns below the surfaces of most biofilms, we encounter an anaerobic world (Stewart, 2003), similar in some important respects to conditions on Earth billions of years ago. Bacteria living in these environments face the challenge of sustaining their metabolism under conditions where oxidants for cellular reducing power are limited. Because the effectiveness of antibiotic treatment depends significantly on the physiological state of biofilm cells (Brown *et al.*, 1988; Davies, 2003), what factors control their metabolic state and its dynamics are very important to understand. Can we gain insights into how biofilm communities survive today by considering the evolutionary origins of their metabolism?

Our entry into this problem came from considering how bacteria respire iron (III) (hydro) oxides, probably the most abundant and important terminal electron acceptors for ancient cellular respiration (Vargas *et al.*, 1998). Working first with the merabolically versatile bacterium, *Shewanella oneidensis*, we demonstrated that it excretes small organic molecules (e.g. quinones) and hypothesized that these molecules might mediate electron transfer from the cell to mineral surfaces (Newman & Kolter, 2000). Although we and others later realized that excreted quinones were *not* responsible for catalyzing mineral reduction (Hernandez & Newman, 2004; Myers & Myers, 2004; Lies *et al.*, 2005), our results suggested that self-produced electron shuttles might be an important mechanism for mineral transformation by different types of bacteria. By looking at their chemical structures, we inferred that certain redox-active antibiotics (e.g. phenazines and some glycopeptides) produced by common soil bacteria (e.g. *Pseudomonas chlororaphis* and *Streptomyces coclicolor*) and clinical isolates (e.g. *Pscudomonas aeruginosa*, an opportunistic pathogen of individuals with the disease cystic fibrosis) could function as extracellular electron shuttles (Hernandez & Newman, 2001; Shyu *et al*, 2002). We went on to show that this was indeed the case, and that they could be exchanged between diverse bacterial species (Hernandez *et al.*, 2004). Coincidental, two recent independent studies have shown that flavins can mediate extracellular electron transfer for different *Shewanella* species (Marsili *et al.*, 2008; von Canstein *et al.*, 2008). This makes sense, given that phenazines and flavins are structurally related (Fig. 1).

Due to the rich history of *Pseudomonas* research, we decided to focus on the phenazine molecules it produces (Turner & Messenger, 1986). Most current literature emphasizes the role of phenazines as virulence factors, which generate toxic-by-products (e.g. $O_2$ and $H_2O_2$) when oxidized in an aerobic environment (Hassan & Fridovich, 1980; Thomashow, 1996; Kerr, 2000). For this reason, phenazines are conventionally thought to be toxic to other organisms and are believed to provide the producer with a competitive advantage (Thomashow, 1996; Pierson & Pierson, 1996; Kerr, 2000). However, because phenazines may have evolved in a world without $O_2$ (acylated versions of these compounds are important electron carriers in the membranes of some methanogenic archaea (Abken *et al.*, 1998)) and are often produced at concentrations below their toxic threshold under anaerobic conditions (Hernandez *et al.*, 2004; Price-Whelan & Newman, 2008), we hypothesized that their 'antibiotic' activity might be a consequence of the geochemical conditions prevalent on Earth today, but not a reflection of their original function (Price-Whelan *et al.*, 2006).

In the past two years, we have tested this hypothesis in several ways using *P. acruginosa* strain PA14. We have shown that: (i) phenazines function effectively as electron shuttles to Fe(III) minerals (Hernandez *et al.*, 2004; Wang & Newman, 2008a), which may aid in Fe(II) acquisition (Dietrich *et al.*, 2006); (ii) phenazines help modulate intracellular redox homeostasis as oxidants for NADH and/or by affecting carbon flux through central metabolic pathways (Price-Whelan *et al.*, 2007); (iii) phenazines are signaling molecules, influencing the expression of a limited set of genes during the transition from exponential growth into stationary phase (Dietrich *et al.*, 2006); (iv) phenazines are produced in biofilms (Lies *et al.*, 2008), as expected – given that phenazine biosynthesis had previously been shown to be up-regulated by quorum sensing and low oxygen tension (Whiteley *et al.*, 1999; Chin-A-Woeng *et al.*, 2001); and (v) phenazines play an important role in biofilm development, dramatically affecting the morphology of multicellular communities (Fig. 2) (Dietrich *et al.*, 2008). We are beginning to work out the molecular pathways that underpin these phenomena by identifying and characterizing the proteins that respond to phenazines as well as those required for phenazine trafficking within and between cells (e.g. transcription factors that directly sense phenazines, oxidoreductases that reduce them and membrane complexes that transport them). We have also begun to develop specific analytical tools – both electrochemical (Buchler *et al.*, 2003; Lies *et al.*, 2008) and spectroscopic (Wang & Newman, 2008b) – to localize and quantify phenazine distribution in multicellular communities and at the single-cell level. Ultimately, we seek to understand the trafficking of the various phenazines that *P. aeruginosa* produces, as we have preliminary evidence that they have different functions (Dietrich & Newman, 2008; Wang & Newman, 2008a). Regardless of which phenazine docs what, our results demonstrate that phenazines are much more than 'antibiotics', profoundly affecting the producing organism metabolically and developmentally.

In the next few years, we would like to extend these findings. One of the exciting implications of our work is that the functions we are finding for phenazines may apply to a broad class of redox-active natural products. For example, in studying one of the transcription factors that senses phenazines in *Pseudomonas aeruginosa* (SoxR), we found bioinformatic evidence that SoxR might sense similar compounds in other organisms (Teal, 2007). Ironically, SoxR was first discovered as a transcription factor that senses oxidative stress in *E. coli*, where it indirectly controls gene expression of products necessary for responding to superoxide and nitric oxide (Demple *et al.*, 2002). Not only did the biosynthesis of redox-active natural products likely predate the evolution of $O_2$, but this pattern of gene regulation appears to be limited to *E. coli* and *Salmonella;* in most other bacterial genomes, the SoxR rcgulon appears quite small, affecting only a handful of genes that are involved in the transport and transformation of small molecules (Teal, 2007). Choosing *S. coelicolor* as a test case, we found that the pigmented polykeride, actinorhodin (structurally similar to phenazines, see Fig. 1), appears to activate SoxR (Dietrich *et al.*, 2008). Moreover, *S. coelicolor* mutants defective in actinorhodin

biosynthesis share similar developmental phenotypes as *P. aeruginosa* mutants defective in phenazine biosynthesis (Fig. 2). While much remains to be done, we are encouraged that many 'secondary' metabolites may be of primary importance for the development and maintenance of diverse microbial communities, especially when they are $O_2$-limited.

In addition to determining the generality our findings, we hope to pursue the potential clinical relevance of our work by focusing on the pathology of *P. aeruginosa.* We would like to test the hypothesis that phenazine production and cycling [or that of another redox-active compound, such as melanin (Nosanchuk & Casadevall, 2003)] is important for the survival of *P. aeruginosa* in the CF lung, where *P. aeruginosa* is thought to exist in a biofilm-like state (Singh *et al* 2000). Encouragingly, the phenazine pyocyanin appears critical for lung infection in mice (Lau *et al.*, 2004). If our hypothesis proves true that redox-active small molecules help diverse biofilms survive and/or develop *in vivo,* it would suggest a new class of targets for rational drug design to control infection. Future research would then be aimed at developing specific methodologies to control their trafficking in this context.

I imagine Terry would have gotten a kick out of the electron shuttle connection between *S. oncidensis* and *P. aeruginosa*, as he spent much of his career studying these two organisms. With a twinkle in his eye, he probably would have smiled and asked 'But have you considered … ?' or 'What do you know about …?', helping me focus on all that remained to be learned. Going forward in my career, I will miss being able to chat with Terry about science, not only missing his great insight and stimulating questions, but his warmth and encouragement. Among the many things I learned from Terry was the importance of not taking oneself too seriously. Once I wrote to him to ask for words of wisdom to impart to my student about using electron microscopy to study bacterial cell biology, and he responded with: 'Sure! A bottle of scotch while imaging cells is always helpful.' This makes me laugh every time I think of it, and is a poignant and powerful reminder to me of how science is often at its best when mixed with a sense of humor. In so many ways, I feel privileged to be able to count Terry Beveridge as one of my mentors, and I will be forever grateful for the example he set for me both as a scientist and a human being.

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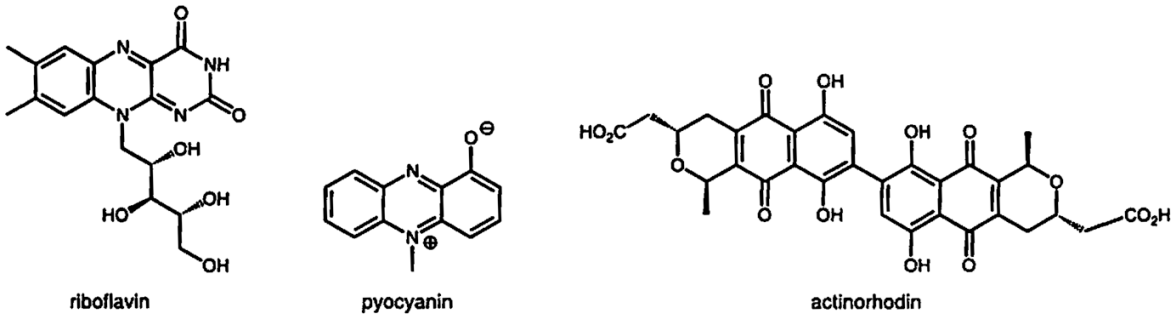


**Fig. 1.**

Structures of different electron shuttles: riboflavin made by *Shewanella oneldensis*, pyocyanin, a phenazine made by *Pseudomonis aeruginosa*, and actinorhodin made by *Streptomyces coellcolor*.

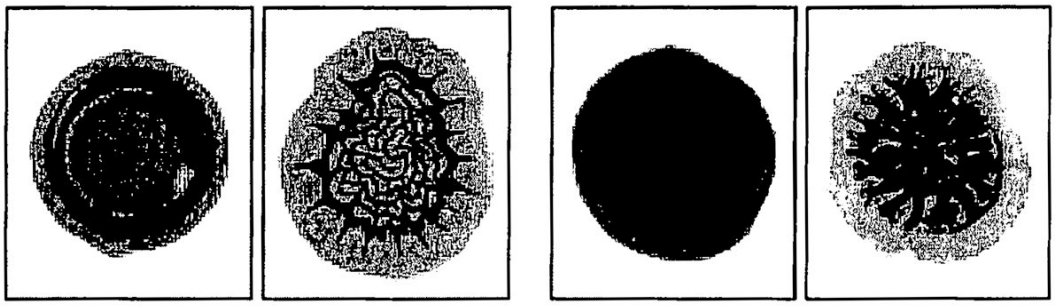

**Fig. 2.**

Colony morphologies as a function of small molecule production. Left pair: WT and mutant that cannot make phenazine (*Pseudomonas aeruginosa.* diameter ~8 mm). Right pair: WT and mutant that cannot make actinorhodin (*Streptomyces coellcolor.* diameter −4 mm). For each pair, the WT is on the left and the mutant is on the right.