Discovery of Zetaproteobacteria.
by David Emerson

This is a story that provides some insight into how science actually works. It starts at Loihi Seamount, what is often referred to as the next Hawaiian Island. Loihi is an undersea volcano located about 25 miles southeast of the Big Island, putting it at eastern edge of the Hawaii hotspot, a thin spot in the Earth's crust that has given rise to all the Hawaiian Islands, and by most estimates the entire Emperor Seamount chain, which extends WNW past Midway Island and then doglegs NNW for another 1500 miles nearly to the Aleutian Islands. Presumably as the Pacific plate continues to sail west over the Hawaiian hotspot, Loihi will keep growing until it breaks the surface and then, perhaps in half a million years or so, it could become the largest mountain on Earth, just as Mauna Kea is today.

It was in the 1970s that it became appreciated by oceanographers that Loihi was an active undersea volcano, which meant that it might have undersea hot water venting going on. In the mid 1980's Dave Karl and some colleagues at the University of Hawaii did the first deep-sea submersible dives at Loihi and discovered several diffuse flow, low temperature vent fields covered in rust at Loihi's summit. They scooped up some of the floculant rust deposits, and with one look in the microscope it was immediately obvious that biology was at work. The rust had a high abundance of stalk-like structures reminiscent of the long-known Fe-oxidizing bacterium *Gallionella ferruginea*. Dave got in touch with Bill Ghiorse, my Ph.D advisor at the time at Cornell University, who was the leading expert in the U.S. on *Gallionella*, and iron bacteria in general.

I was close to finishing my Ph.D in Bill's lab at the time, and Bill graciously invited me to come along to do what grad students do best, most of the work. So Bill and I packed up some culture media for growing *Gallionella* and went on a vent cruise to Loihi in 1988 along with Dave Karl. However, the discovery of Zetaproteobacteria then was not to be. At that time the University of Hawaii's deep sea submersible, used to dive over 3,000 ft to the vents, had its own barge and tender, while the science crew was on a larger oceanographic ship; dive teams were shuttled back and forth. Through a rather embarrassing episode that had nothing to do with me, the submersible was damaged on its voyage to the site. The upshot was that I got to spend two extraordinarily boring weeks in the tropics, pounding around on an uncomfortable research ship that spent its entire time parked in the same spot in the brisk SE trade winds; the monotony broken occasionally by the excitement of the geologists on board exclaiming over a few rock fragments they were able to dredge up from the bottom. These all looked amazingly alike to me. We did eventually get one dive in at the very end of the cruise. The graduate student union being what it was, Dave and Bill were the ones to go down in the submersible Pisces V, and collected a few samples from the vents. Upon their re-surfacing, I immediately started some cultures, which soon began producing nice iron oxide filaments, an encouraging sign. I was able to nurse these along until we crossed the six timezones back to Cornell, where with one look in the microscope I knew I had a marine fungus whose hyphae became coated in Fe-oxides. Since my shipboard lab bench (a 3 foot long sheet of plywood) was directly underneath the rather ancient duct

for the research vessel's air conditioning system, which, this being the tropics, was black from the mold growing around it, we decided it prudent not to call Nature and tell them to hold the presses, but chalk the whole thing up to experience.

After this experience, I could never forget Loihi, but probably shouldn't be blamed for deciding that freshwater, terrestrial iron springs, preferably within a few minutes drive or bicycle from the lab, would be a much better place to study the iron bacteria. Indeed, it was studying these freshwater communities that allowed me to develop some better methods for culturing and isolating obligate Fe-oxidizers. In the meantime, Dave Karl took on Craig Moyer as a graduate student, and set him on the Loihi project, wisely using cultivation-independent techniques (at that time still quite new) to study the communities associated with the vents. Craig was quite successful, and isolated a number of DNA clones representative of the bacteria living in the iron mats. Fortuitously, Craig decided to do a postdoc with Jim Tiedje in the Center for Microbial Ecology at Michigan State University where I was a postdoc with John Breznak. So, through Craig I got reconnected to Loihi.

Craig sent me some fresh Loihi Fe mat samples he collected on a dive series in 1996, by then I had moved on to the ATCC and George Mason University. Using the techniques I had developed for freshwater Fe-oxidizers, I was able to isolate strain PV-1, which we later described as *Mariprofundus ferrooxydans*, the first cultivated Zetaproteobacteria. Of course the whole story is not nearly so simple. It took me about a year of dilution series and transfers in iron gradient medium using different, inert gel-stabilizing agents, before I was reasonably convinced I had a pure culture. Interestingly, I only periodically noted stalk formation by PV-1 during this time. I am still not sure whether stalk formation varied some with the media I was using, or perhaps I really had a mixed culture of stalk and non-stalk forming cultures. Or maybe I just wasn't paying close enough attention. I am still continually surprised by how easy it is to miss the obvious, until it becomes obvious. In any event, with time, the culture medium and the organism both stabilized to a consistent stalk-forming culture.

Of course, this entire time I was under the impression that what I had isolated was a marine *Gallionella*. It was a small bean shaped cell that formed a helical stalk composed of iron oxides. It required microaerobic conditions and preferred opposing gradients of oxygen and Fe(II) and was an obligate Fe-oxidizer, thus morphologically and physiologically it had all the attributes of *Gallionella.* One of our primary goals was to determine the 16S sequence of PV-1 to determine who it was related to. I collaborated with Craig on this.

This, as turns out, was another cautionary tale. Prior to isolating PV-1, I had also isolated several freshwater Fe-oxidizing bacteria, and we also wanted to determine the 16S sequence of these bacteria. In the meantime, my graduate student Johanna Weiss isolated several more strains from the rhizosphere of freshwater wetland plants. At the time we were using small gel-stabilized gradient tubes for all our culture work. While these worked well for dilution, isolation, and regular growth maintenance, it was a challenge to scale up the growth to get enough material for reliable DNA harvests. We also tried many different DNA extraction methods, where our continuum of success ran from total failure to 'there might be something there'. With a lot of work, and on a good

day, we were able to get nanogram quantities of DNA. We would ship these off to Craig, who, on a good day, was able to get a PCR amplification of the 16S gene to work. Eventually we got 16S amplicons, and then DNA sequences, for PV-1 and our freshwater strains and found they were all closely related and belonged to the Gammaproteobacteria, or so we thought. This was interesting. We were not surprised they were all related, they were all lithotrophic Fe-oxidizers after all. It was a little surprising that they were all nearly identical , especially since cell morphologies varied quite a bit, and they came from different habitats. What was really puzzling however, was that they clustered in among the Xanthomonadales a group of heterotrophic plant associated organisms, and they were not related to the single sequence available for *Gallionella ferruginea*, which had been done earlier by Lotta Hallbeck in Sweden. It was relatively easy to come up with arguments to convince ourselves that we must have the correct phylogenies, furthermore, we were able to independently do at least one 16S sequence in my lab, which gave the same result that Craig had gotten. Still, there was some niggling doubt, which was one reason I held off on publishing the detailed phylogeny of these organisms.

During this same time, Johanna developed better growth techniques for Fe-oxidizers that allowed us to get much higher growth yields of the pure cultures, and we went to using the MoBio soil DNA extraction kits which seemed to work better than most of the techniques we had tried. So together Johanna, and Jeremy Rentz, a postdoc in my lab, were able to get much better DNA yields for all the Fe-oxidizers, in the microgram range. Jeremy undertook some 16S analysis with this DNA, and wouldn't tell me the results until he had done it at least three times. The phylogenies were completely different! The freshwater strains were quite diverse, but, with one exception, all clustered with *Gallionella ferruginea* and formed what will probably turn out to be a new order in the Betaproteobacteria. This confirmed Lotta Hallbeck's earlier work. PV-1, however, was really different and did not cluster with any of the known classes of Proteobacteria. With the new DNA, Craig also confirmed Jeremy's results and soon developed some robust phylogenetic trees, showing the uniqueness of PV-1 and some of his early clones from Loihi. We have never been able to track down the precise cause of the earlier 'false gamma' results we got, but it almost had to have been some consistent trace contaminant in the PCR reagents.

Subsequently, we obtained a draft genome sequence for PV-1 (as well as complete genomes of freshwater Fe-oxidizers) which allowed us to look at other phylogenetically conserved genes. This confirmed PV-1's placement as a deep-branching member of the Proteobacteria, likely worthy of being a unique class. This really was quite a surprise. *Mariprofundus* and *Gallionella* have such similar physiologies, morphologies, and ecotypes, yet at the genetic level share only about 25 highly homologous genes in common (< 1% of the genome). Clearly the most likely explanation is that the requirements to grow on Fe(II) place a strong selection on bacteria to develop common modalities, even if they are unrelated.

So who really discovered Zetaproteobacteria? Craig Moyer developed his clone libraries at about the same time I was figuring out how to grow freshwater Fe-oxidizing bacteria. His clone PVB OTU4 from a vent at Loihi is the first example in genbank of

what would come to be known as the Zetaproteobacteria. However it was not until I obtained the pure culture of *M. ferrooxydans*, and we later showed it was related to OTU4, that the context for the Zetaproteobacteria was established. So its a nice tale about how different approaches can support each other to build a stronger scientific story. It also illustrates how the tension between ‘getting a paper out’ and ‘getting a paper out that is correct’ is an unending dynamic that every scientist faces.