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A Novel Lineage of Proteobacteria Involved in Formation of Marine Fe-Oxidizing Microbial Mat Communities

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**Background.** For decades it has been recognized that neutrophilic Fe-oxidizing bacteria (FeOB) are associated with hydrothermal venting of Fe(II)-rich fluids associated with seamounts in the world’s oceans. The evidence was based almost entirely on the mineralogical remains of the microbes, which themselves had neither been brought into culture or been assigned to a specific phylogenetic clade. We have used both cultivation and cultivation-independent techniques to study Fe-rich microbial mats associated with hydrothermal venting at Loihi Seamount, a submarine volcano. Methodology/Principle Findings. Using gradient enrichment techniques, two iron-oxidizing bacteria, strains PV-1 and JV-1, were isolated. Chemolithotrophic growth was observed under microaerobic conditions; Fe(II) and Fe⁵⁺ were the only energy sources that supported growth. Both strains produced filamentous stalk-like structures composed of multiple nanometer sized fibrils of Fe-oxyhydroxide. These were consistent with mineralogical structures found in the iron mats. Phylogenetic analysis of the small subunit (SSU) rRNA gene demonstrated that strains PV-1 and JV-1 were identical and formed a monophyletic group deeply rooted within the Proteobacteria. The most similar sequence (85.3% similarity) from a cultivated isolate came from *Methylophaga marina*. Phylogenetic analysis of the RecA and GyrB protein sequences confirmed that these strains are distantly related to other members of the Proteobacteria. A cultivation-independent analysis of the SSU rRNA gene by terminal-restriction fragment (T-RF) profiling showed that this phylotype was most common in a variety of microbial mats collected at different times and locations at Loihi. Conclusions. On the basis of phylogenetic and physiological data, it is proposed that isolate PV-1T (= ATCC BAA-1019: JCM 14766) represents the type strain of a novel species in a new genus, *Mariprofundus ferrooxydans* gen. nov., sp. nov. Furthermore, the strain is the first cultured representative of a new candidatus class of the Proteobacteria that is widely distributed in deep-sea environments, Candidatus *ζ* (zeta)-Proteobacteria cl. nov.

**INTRODUCTION.** Bacteria are known for the wide variety of reduced inorganic substrates they are able to oxidize and utilize as energy sources for growth. These include sulfur, ammonia, methane, hydrogen, and iron. The oxidation of ferrous (Fe (II)) to ferric (Fe(III)) iron is especially enigmatic because thermodynamically it yields a minimal amount of energy for growth, approximately $ΔG°' = -109 \text{ kJ} \cdot \text{mol}^{-1}$, and at neutral pH chemical oxidation of Fe(II) to Fe(III), or iron oxyhydroxides, i.e. rust, occurs rapidly in the presence of oxygen [1]. To circumvent these problems, bacteria that oxidize Fe(II) to obtain energy at circumneutral pH have two key requirements for growth, low O₂ partial pressures and sustained Fe(II) concentrations. This kind of metabolism has received scant attention in marine systems, first, because practically nothing is known about marine iron-oxidizing bacteria, and second, because the oceans are generally considered to be oxygenated and depleted in Fe(II). In addition, marine sediments tend to be rich in sulfide, which reacts rapidly with Fe(II) to precipitate FeS.

An important exception to these oceanic iron depleted conditions occurs in areas of hydrothermal venting, either at seamounts or at crustal spreading centers. Here, anoxic vent fluids charged with Fe(II) come in contact with the cold, oxygenated ocean water. It is estimated that the present-day flux of Fe(II) from hydrothermal venting is approximately $3 \times 10^{13} \text{ mol yr}^{-1}$ [2]. As a result, there are often substantial deposits of Fe-oxides at these vents, and in nearly every case that has been examined, the morphology of Fe-oxides provide abundant evidence for microbial activity [3,4,5,6,7]. The tell-tale signs of this activity are the tubular sheath casts of filamentous Fe-oxidizing bacteria (FeOB), or the twisted stalks of *Gallionella*-like organisms. Another potential habitat for marine FeOB is freshly formed oceanic basalts that result from volcanic and/or tectonic activity. In this case, leaching of Fe(II) directly from the mineral or glass surfaces may occur allowing for growth of biofilms of FeOB on the weathered rocks [8,9].

These types of habitats are more common than is often appreciated, since crustal spreading centers and subduction zones, as well as seamounts with either active volcanism or cold seepage of mineral rich fluid flow are common throughout the world’s oceans [10]. Because iron is the fourth most abundant element in the Earth’s crust, it is often the most abundant potential energy source at the boundaries of the oxygenated ocean and the ✿To whom correspondence should be addressed. E-mail: demerson@gmu.edu

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reducing subsurface [11]. However, we know very little about the population biology of prokaryotic lithotrophic communities that could potentially live off the Fe(II)-rich waters and vent fluids associated with these kinds of boundary habitats [1,12].

We previously reported on the role that lithotrophic FeOB appear to play in the formation of extensive microbial mats that form at the Loihi Seamount [13]. During that study two novel FeOB were isolated, strains JV-1 and PV-1. These organisms were striking because as they grew, they formed filamentous stalk-like structures that appeared to consist primarily of an amorphous iron oxyhydroxide. These filaments bore a strong morphological resemblance to the types of oxides seen in the mats at Loihi[13]. We report here a more complete characterization of these two isolates and show that their metabolism is limited to Fe-oxidation. A cultivation-independent approach confirmed that they are common in iron mats located at Loihi. They are phylogenetically novel and represent the first cultured isolates of a new class of Proteobacteria that until now has only been represented by sequences in environmental SSU rRNA gene libraries associated with the floor of the deep ocean.

RESULTS

Growth characteristics of PV-1 and JV-1

Both strains grew microaerobically with Fe$^{2+}$ as the sole energy source and CO$_2$ as the only available carbon source indicating the doubling time at 23°C and was not observed at 35°C. Growth appeared to be optimal between 5.5–7.2 and appeared optimal between 6.2 and 6.5. A spot test for catalase activity was negative. Growth characteristics of PV-1 and JV-1, including FeS or on FeCO$_3$ (siderite) in gradient culture and on FeCl$_2$, Fe(NH$_4$)$_2$(SO$_4$)$_2$, and FeSO$_4$ in liquid bottle cultures. They were also able to grow on zero valent iron, Fe(0). A growth curve for strain PV-1 comparing growth on Fe(0) and FeS is shown in Figure 1. Final growth yields tended to be higher on FeS, but for strain PV-1 comparing growth on Fe(0) and FeS is shown in Figure 1. Another dominant set of fatty acids was 16:1 w7c/15 iso 2OH, however these two fatty acids could not be discriminated from one another and are reported as summed in Feature 3 in Table 1. The abundance of the branched chain fatty acid 11:0 iso 3OH in PV-1 is unusual. This is generally considered a rare fatty acid in bacteria [14]. It has been shown to be diagnostic of the family Xanthomonadaceae and normally comprises <10% of the total fatty acids in these organisms [15,16].

Morphology

Cells of strains PV-1 and JV-1 were curved rods (approximately 0.5 x 2–5 μm). The most distinguishing characteristic of these strains was the filamentous stalk-like structures of iron oxyhydroxides that they formed during cell growth, Figure 2 and [13]. The cells had Gram negative type cell walls when viewed by TEM, Figure 2c, and the fibrillar Fe-oxyhydroxides were observed as well as lesser amounts of fine particulate oxides sometimes associated with the cells. HRTEM showed that the stalks were themselves composed of a set of discrete fibers aligned parallel to the length of the stalk, Figure 2f. The stalk shown in Figure 2f is between 0.6 μm and 70 nm in diameter. The sample was not stained; thus contrast is due to iron minerals on or within the stalk. It was confirmed by energy dispersive X-ray analysis that the major element in these structures was the filamentous stalk-like structures of iron oxyhydroxides that they formed during cell growth, Figure 2 and [13]. The cells had Gram negative type cell walls when viewed by TEM, Figure 2c, and the fibrillar Fe-oxyhydroxides were observed as well as lesser amounts of fine particulate oxides sometimes associated with the cells. HRTEM showed that the stalks were themselves composed of a set of discrete fibers aligned parallel to the length of the stalk, Figure 2f. The stalk shown in Figure 2f is between 0.6 and 0.7 microns wide, with individual fibers that are each ~70 nm in diameter. The sample was not stained; thus contrast is due to iron minerals on or within the stalk. It was confirmed by energy dispersive X-ray analysis that the major element in these structures was the filamentous stalk-like structures of iron oxyhydroxides that they formed during cell growth, Figure 2 and [13].

FAME analysis

The MIDI system was only able to identify approximately 45% of the fatty acids in lipid extracts from late-log phase PV-1 cells. The dominant fatty acid was 11:0 iso 3OH, followed in relative abundance by 17:0 iso 3OH, 16:0, and 18:1 iso H (Table 1). Another dominant set of fatty acids was 16:1 w7c/15 iso 2OH, however these two fatty acids could not be discriminated from one another and are reported as summed in Feature 3 in Table 1. The

Table 1. FAME Analysis, showing fatty acids ≥2% of total.

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Percent (%)</th>
<th>Average</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Run 1</td>
<td>Run 2</td>
<td></td>
</tr>
<tr>
<td>12:0 iso</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>11:0 iso 3OH</td>
<td>27</td>
<td>28</td>
<td>27.5</td>
</tr>
<tr>
<td>15:1 w8c</td>
<td>1</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>16:0</td>
<td>14</td>
<td>9</td>
<td>11.5</td>
</tr>
<tr>
<td>18:1 iso H</td>
<td>5</td>
<td>6</td>
<td>5.5</td>
</tr>
<tr>
<td>17:0 iso 3OH</td>
<td>13</td>
<td>14</td>
<td>13.5</td>
</tr>
<tr>
<td>Summed in Feature 3$^1$</td>
<td>26</td>
<td>23</td>
<td>24.5</td>
</tr>
<tr>
<td>Summed in Feature 7$^2$</td>
<td>6</td>
<td>7</td>
<td>6.5</td>
</tr>
</tbody>
</table>

$^1$16:1 w7c/15 iso 2OH
$^2$19:0 CYCLO w10c/19w6

doi:10.1371/journal.pone.0000667.t001
was Fe (data not shown). Treatment of the stalks with 0.3 M oxalic acid caused a rapid and nearly complete dissolution, suggesting that they are composed primarily of poorly crystalline iron oxyhydroxides (Supplemental Figure S1). When glass surfaces (microscope slides) were placed in a culture of PV-1 growing in liquid culture, the cells attached to the glass surface and formed stalks, Figure 2a–d. This confirmed that the cells grew at the apical ends of the stalks; presumably these cells are extruding the Fe oxyhydroxide filaments as they grow.

Phylogeny
PV-1 and JV-1 SSU rDNA sequences were identical (1488 bases). Their genotypic similarity was confirmed with rep-PCR, where both strains gave nearly identical banding patterns (Supplemental Figure S2), that were distinct from freshwater FeOB. A phylogenetic analysis based on comparison of the SSU rRNA gene with other bacteria revealed that PV-1 and JV-1 were unusual members of the phylum Proteobacteria in that they did not cluster with any of the known classes of $\alpha$, $\beta$, $\gamma$, $\varepsilon$, or $\delta$-Proteobacteria (Figure 3) [17,18]. Instead, the strains branched deeply within the Proteobacteria as shown in a phylogenetic tree created by maximum likelihood [19], Figure 3. Very similar tree topologies were found by neighbor-joining, maximum parsimony or minimum evolution methods using MEGA3 [20](data not shown). The novelty of the strain PV-1 within the Proteobacteria was confirmed by phylogenetic analysis of two other highly conserved proteins, gycrase beta-subunit (GyrB), and the recombination protein A (RecA). Phylogenetic trees for the GyrB and RecA proteins translated from the gene sequences are shown in Figure 4a and 4b, respectively. In both cases, PV-1 clustered within the Proteobacteria, but formed a distinct lineage from the other described classes of Proteobacteria.

A BLAST search of GenBank using the SSU rRNA gene sequence from PV-1 revealed a group of environmental clones that clustered within the PV-1/JV-1 lineage, Figure 3. All these clones were from deep-sea marine habitats, primarily associated with hydrothermal activity. A T-RFLP (terminal restriction fragment length polymorphism) analysis of Fe-rich microbial mats collected at several different vent sites around the summit of Loihi in 2004 found that the PV-1 phylotype was consistently present at all the sampled sites, Figure 5. The vent water temperatures at these sites ranged from 10° to 65°C, and there were two main T-RF clusters, Loihi Group I that was associated with cooler vents <40°C and Loihi Group 2 that was associated with vents >40°C. The PV-1/JV-1 phylotype appeared most strongly associated with the Group 1 populations, Figure 5. Light microscopy indicated the presence of filamentous Fe oxyhydroxides similar to those formed by PV-1 at all these sites as well (data not shown).

DISCUSSION
A systematic analysis of these two microbes from microbial iron mats at Loihi Seamount indicates that they appear to be the first cultured isolates of a candidatus class of Proteobacteria, the “$\zeta$-Proteobacteria”. The most conspicuous trait of these two strains is their obligate dependence upon Fe(II) as an energy source and microaerobic conditions. They are incapable of heterotrophic growth; neither can they grow on other inorganic energy sources such as reduced S-compounds or H2. These strains are also obligate aerobes, they do not oxidize Fe(II) when grown anaerobically with nitrate, nor can they reduce Fe(III) in the presence of acetate. The ability of these strains to grow on Fe0 metal is of interest, as we are not aware of other reports of Fe-oxidizing bacteria growing on metallic iron, although there is evidence that there can be direct electron flow from Fe2+ to support the growth of methanogens and obligately anaerobic sulfate-reducing bacteria [21]. Under aerobic conditions the reaction of iron metal is

$$2\text{Fe}^0 + \text{O}_2 + 2\text{H}_2\text{O} \rightarrow 2\text{Fe}^{2+} + 4\text{OH}^- \quad (1)$$

Presumably the actual substrate for the FeOB is Fe(II) that is released upon chemical oxidation of the Fe0; this process would be speeded up due to the ionic strength of seawater. Because these bacteria are growing under suboxic gradient conditions, O2 may not always be present, in which case the oxidant of of Fe5+ may be water which results in hydrogen production:

$$\text{Fe}^0 + 2\text{H}_2\text{O} \rightarrow \text{Fe}^{2+} + \text{H}_2 + 2\text{OH}^- \quad (2)$$

However, since these bacteria have been shown not to use H2 as an energy source, their growth substrate will still be Fe(II). We have not yet demonstrated CO2 fixation by either PV-1 or JV-1, however both grow well without any added organic C source. The draft genome sequence of PV-1 (NZ_AATS00000000) reveals it has a gene for the small subunit ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) protein, as well as genes for both form I and form II of the large subunit Rubisco, indicating that PV-1 has the genetic complement necessary for autotrophic growth.

As was noted earlier, these marine Fe-oxidizers produce a unique filamentous stalk-like structure composed primarily of Fe-oxyhydroxides [13]. More detailed electron micrographic analysis presented here indicates that the Fe-containing stalks are themselves composed of bundles of iron-rich fibrils. The processes of how these fibrils are excreted and their subcellular localization are currently under investigation. While it is presumed that an organic matrix is excreted by the cells that may serve as a ‘scaffold’ for the formation of Fe-oxyhydroxides, the composition of the matrix is unknown. Presumably it contains acidic polysaccharides that play a role in metal binding and coordination [22,23]. At a gross morphological level there appears to be a remarkable congruence between the filamentous Fe oxyhydroxides produced by strains PV-1 and JV-1 and the ferricydrite-rich stalks produced by Gallionella ferruginea [24]. G. ferruginea cells share a similar morphology to PV-1 and JV-1 cells, since they are also curved rods. The ferricydrite-containing stalk of G. ferruginea is noted for its regular helical structure. Both PV-1 and JV-1 can produce twisted stalks, and at times these can appear helical; however as
Figure 3. Maximum-likelihood phylogenetic tree showing the evolutionary placement of *Mariprofundus ferrooxydans* (strains PV-1 and JV-1) belonging to the novel class of zeta-Proteobacteria along with representatives from other previously described classes of Proteobacteria. Scale bar represents 5 nucleotide substitutions per 100 positions.

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viewed by light microscopy, and shown in Fig 2 (a–d) they do not appear to have the regular helicity that is often observed with G. ferruginea. The overall morphological similarity between G. ferruginea and the PV-1 and JV-1 strains suggests that reports of Gallionella spp. from deep-sea marine habitats may need to be re-evaluated.

It is also interesting to note that certain lithoautotrophic S-oxidizing bacteria that have been found at hydrothermal vents also may produce copious filamentous sulfur structures [25]. The morphology of these sulfur filaments bears a notable resemblance to the iron stalks formed by strains PV-1 and JV-1; however, the organism identified with filamentous sulfur formation is an epsilon-Proteobacteria, Candidatus Arcobacter sulfidicus [26], and, as far as is known, does not oxidize iron. It will be interesting to determine if the mechanisms for deposition of iron-rich filaments and sulfur-rich filaments share any commonalities.

Comparison to other neutrophilic FeOB

Edwards et al. [27] described two groups of marine FeOB that were most closely related to Hyphomonas jannaschiana and Marinobacter aquaeolei, which are z- and γ-Proteobacteria, respectively. These organisms can both grow heterotrophically using organic substrates, as well as lithoautotrophically, on Fe(II), although their doubling times are appreciably slower than either PV-1 or JV-1 on Fe(II). Neither of these organisms forms a filamentous iron oxyhydroxide. Freshwater FeOB isolates described to date include G. ferruginea, as discussed above [28], FeOB TW2 [29], and two strains isolated from groundwater, ES-1, and ES-2 [30]; The latter two organisms have recently been described as ‘Sideroxydans lithotrophicus’ and ‘Gallionella ferricapsiformans’, novel members of the β-Proteobacteria [31]. Another group of obligately lithotrophic FeOB has been isolated from the rhizosphere of wetland plants. These organisms are also members of the β-Proteobacteria and include a new species, ‘Sideroxydans paludicola’ and a novel genus ‘Ferrotrophicum radicicola’ [32]. Like other freshwater isolates they do not grow with elevated salt concentrations, nor do they form morphologically unique Fe-oxides. Another differentiating feature of these marine FeOB is that the dominant fatty acid that was identified was the branched chain hydroxy 11:0 iso 3OH; this fatty acid was not found in the freshwater strains, instead the saturated straight chain fatty acids 14:0 and 16:0 were more prevalent in these isolates.
Environmental Relevance
Several clones from deep-sea environmental samples were the closest relatives to PV-1 based upon SSU rRNA gene sequences retrieved through a BLASTN search (Figure 3). For near full-length sequences (>1200 bases), a clone from the Kebrit Deep (depth 1468m), in the Red Sea [33] was 95.4% similar (1373 bases) to PV-1 and a Loihi Seamount hydrothermal vent clone, Loihi Seamount PV’B OTU4 [34,35] was 93.4% similar (1455 bases). In addition to the Loihi clone, other related SSU rRNA gene signatures have been observed from deep-sea hydrothermal systems, including the Guaymas Basin [36], the Cleft Segment hydrothermal system off the coast of Oregon [37], and the Mariana Trench in the western Pacific. With the exception of the Moyer et al. [35] study that predated the current work at Loihi, none of the reports yielding related sequences were specifically investigating FeOB. However, the observation of sequences related to PV-1 from numerous hydrothermal systems suggests FeOB may be more widespread than is currently recognized [38,39,40].

Observation of iron-oxide filaments similar to those made by PV-1 in deep-sea sediments have also been made at the Axial Volcano of the Juan de Fuca Ridge [41] and Vai’ulu’u Volcano near Samoa [7]. Representatives of the Mariprofundus group have also been detected by T-RFLP and clone library analyses of microbial mats from N.W. Eifuku Volcano along the Mariana Island Arc, as well as the Cleft Segment of the Juan de Fuca Ridge and have been shown to co-occur with characteristic Fe-rich filaments [37]. Numerous samples of microbial mats collected at different times from different vent sites from Loihi also share predominant populations that correspond with those expected from the PV-1/ JV-1 phylotype (data not shown). These findings corroborate the T-RFLP analysis presented here (Figure 5), that show the Mariprofundus phylotype exists at numerous vent sites at Loihi.

Description of Mariprofundus gen. nov.
Mariprofundus gen. nov. (Mar.is.pro.fund’u.s. L. masc. n. maris the sea; L. adj. profundus deep; L. masc. n. Mariprofundus a deep-sea organism).
Cells are motile curved rods that appear Gram-negative by transmission electron microscopy. Growth is obligately lithotrophic and requires Fe$^{2+}$ as the energy source. Growth is oxygen dependent and requires marine salts.

Description of Mariprofundus ferrooxydans sp. nov.
Mariprofundus ferrooxydans (ferr.ox’y.dans. L. n. ferrum iron; Gr. adj. oxys sour; N.L. v. oxydo to sour, oxidize; N.L. part. adj. ferrooxydans iron-oxidizing).
Displays the following properties in addition to those given by the genus description. Growth requires microaerobic conditions. The cells do not grow on other reduced inorganic energy sources besides iron or on organic compounds. As a result of growth filamentous stalk-like structures containing poorly crystalline iron oxyhydroxide are produced. The optimum growth temperature is 30°C and the optimum pH range is 6.0–6.5. The G+C content of the DNA of PV-1 is 54%. The cells are catalase negative. The dominant fatty acids are 11:0 iso 3OH, 17:0 iso 3OH, 16:0, and 18:1 iso H The type strain is PV-1T, ATCC® BAA-1019 (=JCM 14766) isolated from iron-rich microbial mats associated with regions of hydrothermal venting at Loihi Seamount in the Pacific Ocean. The class, order, and family description for this species are provided in the supplemental information (Supplemental Text S1).

MATERIALS AND METHODS
Source and growth conditions
Both isolates were enriched from Fe-rich mats associated with hydrothermal venting at Loihi Seamount as described previously.
K2HPO4 according to the following formulation (per L deionized-H2O):

water (ASW) amended with nitrogen and phosphate sources

same techniques were used for the isolation of PV-1 [13].

isolation of JV-1 have been published previously and much the

published elsewhere [42]. They all share in common the provision

plates, or bottle cultures. Details of these techniques have been

CaCl2 Fe0 particles as an iron source instead of the agarose stabilized FeS

after the mineral salts medium was autoclaved at 121°C for 20 min.

Gaspak jars as previously described [13].

were inoculated into the liquid and the plates were incubated in

medium in a standard Petri plate, and the plate was gently swirled

layer. Approximately 100 mg of Fe 0 was added to 15 ml of ASW

epifluorescence microscopy.

after staining with Syto 13 (Molecular Probes) and viewing by

Gradient tubes were considered positive or negative for growth

pH range for growth of each strain was tested by varying

The pH range for growth of each strain was tested by varying

Fe-oxides were vortexed for 30 seconds and aliquots were

the buffers used in the top layer of the gradient tubes. Sodium

Bacterial strain frustic acid estimation (FAME) were identified using the procedure

For standard transmission electron microscopy (TEM), PV-1 cells

For high resolution transmission electron microscopy (HRTEM), a late log phase culture of PV-1 was grown in gradient plates and preserved with 2% glutaraldehyde. A few microliters of preserved sample was deposited onto a formvar-coated copper grid, rinsed with deionized water, and air-dried. The sample was coated with carbon and examined on a Philips CM-200 transmission electron microscope, operated at an accelerating voltage of 200 kV.

Fatty acids analysis

Fatty acid methyl esters (FAME) were identified using the procedure recommended by Microbial Identification System (MIDI, Sherlock Microbial Identification System Version 4.0, MIS Operating Manual, March 2001, Newark, DE). The cells were grown in gradient plates (100 ml total) until the late log phase of growth and then harvested by centrifugation. Excess Fe(III) was removed from the cell pellet by treating the sample with 0.33 M oxalic acid for one hour at 37°C, and then washing it three times by centrifugation with de-ionized water. The extraction of FAMEs and their analysis by gas chromatography was done as previously described [44].

Phylogenetic Analysis

DNA was extracted from phosphate buffered (50 mM) saline (pH 8.0) washed pellets containing cells and iron oxides using the MoBio PowerSoil DNA Isolation kit. This minimal procedure produced DNA of high quantity and quality, which was not matched in previous studies [13,30] due to low cell counts produced DNA of high quantity and quality, which was not matched in previous studies [13,30] due to low cell counts.
org); details will be published elsewhere. The GyrB sequence was aligned with 53 homologous sequences from species in the Proteobacteria, using the L-INS-i option of the MAFFT program [48,49]. A tree was inferred from 725 homologous positions using the TREEFINDER algorithm (www.treefinder.de). The Dayhoff model of amino acid substitution was used and the discrete gamma distribution model, with four rate categories and an estimated alpha parameter, was implemented. Branch support was estimated using the LRS approximations [50] in TREEFINDER. The RecA sequence was aligned to 60 homologous sequences from the Proteobacteria and 317 positions were used to build the tree. The same software and parameters were used for aligning and inferring the tree. A list of the taxa used to construct both RecA and GyrB trees is provided in Supplementary Table S1.

Repetitive element PCR (Rep-PCR) was carried out using the DiversiLab system from Bacterial Barcodes (www.bacterialbarcodes.com). PV-1 and JV-1 cells were grown in gradient plates, harvested, and subjected to DNA extraction as described above. The DNA was amplified using the Stenotrophomonas kit provided by Bacterial Barcodes, which contains a proprietary set of primers for repetitive elements common among Proteobacteria. The PCR products were separated on an Agilent 2100 Bioanalyzer, and a dendrogram comparing the new profiles to profiles from a database of samples was generated using the DiversiLab software from Bacterial Barcodes.

**T-RFLP analysis**

Microbial mat samples were collected in 2004 using a suction sampling apparatus (Emerson and Moyer, 2002) either within the caldera or near the summit of Loihi Seamount from hydrothermal vent sites ranging in depth from 1150 to 1325 mbsl. Metagenomic DNA was extracted from these mat samples using the FastDNA Spin Kit following the manufacturer's protocol (Qbiogene, Irvine, CA). Extracted DNA was pooled, cleaned, and concentrated using Montage PCR Centrifugal Filter Devices (Millipore, Bedford, MA). The DNA was then quantified using a NanoDrop spectrophotometer (Nanodrop Technologies, Wilmington, DE) and diluted to 10 ng·µl⁻¹ using filter sterilized 10 mM Tris, pH 8.0. Three replicate SSU rDNA amplifications were performed, each using 50 ng of metagenomic DNA and bacterial Domain specific primers 68F and 1492R with PCR conditions as previously described [13]. The forward primer was labeled with 6-FAM (6-carboxyfluorescein) on the 5' end. The amplicons were visually assayed for size by 1% agarose gel electrophoresis against a 1-kb ladder DNA size standard. Only reactions where corresponding negative controls yielded no amplification products were used. The remaining fluorescently-labeled PCR products were desalted and treated with eight tetrameric restriction enzymes as previously described in Chao et al. [51]. The fragments were separated by capillary electrophoresis using an ABI 3100 genetic analyzer using POP-6 with a 50 cm capillary array (Applied Biosystems, Foster City, CA). The fluorescently labeled 5' terminal-restriction fragments were sized against the Genescan ROX-500 internal size standard using GeneMapper v3.7 (Applied Biosystems). Only fragments between 50 and 500 nucleotides were included in the analysis as this size range has been shown to have the highest degree of precision [52]. Resulting electropherograms were then imported into BioNumerics v4.61 (Applied Maths, Austin, TX). Community fingerprints were compared in BioNumerics using the Pearson product-moment correlation [53] and unweighted pair group method with arithmetic mean (UPGMA) cluster analysis. The cophenetic correlation coefficient was calculated to assess the robustness of the assigned clusters.

**SUPPORTING INFORMATION**

**Table S1**

| Found at: | doi:10.1371/journal.pone.0000667.s001 (0.05 MB DOC) |

**Text S1**

| Found at: | doi:10.1371/journal.pone.0000667.s002 (0.02 MB DOC) |

**Figure S1** A time series of images showing the effect of treating a stalk with oxalic acid. The oxalate reduces the iron oxides in the stalk causing shrinkage and substantial reduction in size of the stalk; however a remnant of material remains. In this stalk there was no visual change after 10 minutes. No cell was present on this stalk. For this experiment, stalks from a fresh culture of PV-1 were viewed at 1,000× by phase contrast microscopy and a drop of 0.3 M oxalic acid was placed at the edge of the coverslip allowing the oxalate to diffuse under the coverslip and reduce the Fe-oxides. Photomicrographs were captured at the indicated times. The bar = 5 µm.

| Found at: | doi:10.1371/journal.pone.0000667.s003 (0.88 MB TIF) |

**Figure S2** Rep-PCR comparison of strains PV-1 and JV-1 with freshwater FeOB strains ES-2 ('Gallionella capsiferriformans'), ES-1 ('Sideroxydans lithotrophicus'), and BrT ('Sideroxydans paludicola'). Stenotrophomonas maltophilia is included as a control. In duplicated runs PV-1 and JV-1 shared 99% similarity in their rep-PCR profiles. Rep-PCR profiles with % similarities <95% usually indicate unrelated strains.

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**Author Contributions**

Conceived and designed the experiments: DE CM. Performed the experiments: DE JR RD HA CC. Analyzed the data: DE JR TL RD CM. Wrote the paper: DE JR TL CM.

**REFERENCES**


