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ORIGINAL ARTICLE

Physiological and ecological implications of an iron- or hydrogen-oxidizing member of the Zetaproteobacteria, *Ghiorsea bivora*, gen. nov., sp. nov.

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Chemosynthetic Fe-oxidizing communities are common at diffuse-flow hydrothermal vents throughout the world's oceans. The foundational members of these communities are the Zetaproteobacteria, a class of Proteobacteria that is primarily associated with ecosystems fueled by ferrous iron, Fe(II). We report here the discovery of two new isolates of Zetaproteobacteria isolated from the Mid-Atlantic Ridge (TAG-1), and the Mariana back-arc (SV-108), that are unique in that they can utilize either Fe(II) or molecular hydrogen (H₂) as sole electron donor and oxygen as terminal electron acceptor for growth. Both strains precipitated Fe-oxyhydroxides as amorphous particulates. The cell doubling time on H₂ vs Fe(II) for TAG-1 was 14.1 vs 21.8 h, and for SV-108 it was 16.3 vs 20 h, and it appeared both strains could use either H₂ or Fe(II) simultaneously. The strains were close relatives, based on genomic analysis, and both possessed genes for the uptake NiFe-hydrogenase required for growth on H₂. These two strains belong to Zetaproteobacteria operational taxonomic unit 9 (ZetaOTU9). A meta-analysis of public databases found ZetaOTU9 was only associated with Fe(II)-rich habitats, and not in other environments where known H₂-oxidizers exist. These results expand the metabolic repertoire of the Zetaproteobacteria, yet confirm that Fe(II) metabolism is the primary driver of their physiology and ecology.

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Introduction

Ferrous iron is an important driver of chemosynthetic or chemolithoautotrophic microbial ecosystems at both deep and shallow marine hydrothermal vents, and it is now well documented that specialized communities adapted for microaerobic growth on Fe(II) thrive at these vents (Kato *et al.*, 2012; Hoshino *et al.*, 2016). A number of Fe-oxidizing bacteria (FeOB) produce organo-metallic filamentous Fe-oxides to construct a woven fabric mat that provides enough structural integrity for colonization by other microbes (Chan *et al.*, 2016). The population structure of active marine iron mats is dominated by members of the Zetaproteobacteria, one of the seven officially recognized classes of Proteobacteria (Makita *et al.*, 2016). In general, the

Proteobacteria are well known for the remarkable breadth of their metabolic diversity, and the different classes of Proteobacteria occupy a wide variety of both oxic and anoxic niches, where they are often conspicuous for their high relative abundances. The Zetaproteobacteria are unique in this sense, as they are only found associated with marine systems, or ancient marine sediments, where Fe(II) is a prevalent electron donor (McAllister *et al.*, 2011; Scott *et al.*, 2015). Thus, the Zetaproteobacteria are more metabolically specialized than the other classes of Proteobacteria, with the exception of the class Acidithiobacillia, which are obligate iron or sulfur utilizers, but restricted to acidic environments (Williams and Kelly, 2013). Consistent with the Zetaproteobacteria being specialists for Fe(II)-based lithotrophy, thus far all the known isolates are obligate Fe-oxidizers that prefer microaerobic conditions. This makes the Zetaproteobacteria a compelling group of organisms for understanding how aspects of phylogenetic diversity contribute to functional unity.

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The Fe(II)-rich ecosystems of these FeOB inhabit are almost exclusively associated with lower temperature (<100 °C) diffuse vents. The cumulative contribution of diffuse hydrothermalism to the biogeochemical cycling of elements at the seafloor may be as great, or greater than at high-temperature vents (Wankel *et al.*, 2011; Resing *et al.*, 2015). Nonetheless, the microbiology and geochemistry of diffuse-flow systems is not as well studied as high temperature, focused flow vents most typified by black smokers. In theory, lower temperature vents offer greater niche-space for colonization by diverse microbial communities. The thermal gradients in diffuse-flow systems are less extreme; therefore, they encompass a spatially larger habitable zone that can include the subsurface (Orcutt *et al.*, 2011). As a result, they may offer heterogeneous gradients of electron donors and acceptors to support diverse microbial ecosystems. Previous studies have shown that the diversity of Zetaproteobacteria phylotypes is as great, or greater, within a particular vent site than it is between vent sites separated by large geographical distances (Davis and Moyer, 2008; McAllister *et al.*, 2011; Scott *et al.*, 2015). Other recent studies showed that physicochemical differences in temperature, oxygen and Fe(II) concentrations, as well as hydrodynamics played an important role in shaping the community structure of microbial iron mats (Fullerton *et al.*, 2017; Scott *et al.*, 2017). What is not known is how metabolic differences—specifically the ability to utilize alternative electron donors to iron or for that matter alternative electron-acceptors—drives community diversity, and whether Zetaproteobacteria are all obligate Fe oxidizers. Here, we describe two new isolates of Zetaproteobacteria that come from quite different hydrothermal systems at nearly opposite sides of the Earth. These two strains are close phylogenetically and share many phenotypic attributes including the ability to use H₂ in addition to Fe(II) as their sole electron donor, thus expanding the metabolic repertoire of the Zetaproteobacteria.

Materials and methods

Source and isolation of strains

TAG-1 and SV-108 were isolated from marine hydrothermal vents on the Mid-Atlantic Ridge (MAR) in the Atlantic Ocean and the Southern Mariana Trough in the Pacific Ocean, respectively. At the MAR an iron mat sample was collected from the Trans-Atlantic Geotraverse (TAG) vent site (3626 mbsl; 26°9′45.9″N, 044°46′38.5″W) using a microbial mat sampler (described in Breier *et al.*, 2012) capable of collecting samples with sub-centimeter vertical resolution. The sample was associated with a small (approximately 10 cm²), fluffy yellow mat at a diffuse vent site with a temperature of 5 °C at the vent orifice. Upon return to the ship a subsample of this material was used to initiate an enrichment culture

for FeOB using an artificial seawater medium (ASW) amended with zero valent iron powder (ASW/ZVI) as the Fe(II) source (Emerson and Floyd, 2005; Emerson *et al.*, 2007). An initial set of serial dilutions from 10⁻² to 10⁻⁷ was done in Petri plates each containing 15 ml of ASW/ZVI and incubated in an anaerobic jar under a microoxic atmosphere created using a Campypak (Becton Dickinson, Franklin Lakes, NJ, USA) as described previously (Emerson and Floyd, 2005). After 10 days there was significant growth at the 10⁻² dilution, as well as some cells present at 10⁻³ and 10⁻⁴ dilutions. A subsequent dilution series (10⁻²–10⁻⁷) was carried out in ASW/ZVI and within 6 days, the original 10⁻² dilution had growth at 10⁻⁶, while there was no growth from the original 10⁻⁴ dilution. This second 10⁻⁶ dilution culture was placed in a serum bottle under a N₂ atmosphere and returned to Bigelow Laboratory where three subsequent dilutions to extinction (10⁻³ to 10⁻⁸) were carried out, again under micro-aerobic growth conditions. In each case the highest dilution that yielded growth after 1 week was either 10⁻⁶ or 10⁻⁷, in no case was there growth at 10⁻⁸. Observations by phase contrast and epifluorescence microscopy showed a uniform, rod-shaped cell morphology, but no distinctive morphology for the iron oxides.

At the Mariana, an iron mat sample was collected at the Snail Vents (also known as the Fryer Site), near Marker 108 (2850 mbsl, 12°57′166″N, 143°37′142″W). Snail Vents are on the Malaguana–Gadao Ridge axis of the back-arc spreading ridge in the Southern Mariana Trough. The sample was from flocculant yellow iron mat associated with a diffuse vent with an orifice temperature of 27 °C. The same procedure was followed in the isolation as used for TAG-1, including initial growth in a 10⁻² dilution aboard the ship followed by four series of dilution to extinction. The final culture also yielded a rod-shaped cell that precipitated Fe-oxides, but did not produce a distinctive iron oxide morphology. Neither of these strains grew on marine R2A medium, indicating the absence of heterotrophic bacteria. Both strains were grown in batch culture on ASW/ZVI for DNA extraction and sequencing of the small subunit (SSU) rRNA gene. The SSU rRNA sequences of both strains indicated a single phylotype was present that was most closely related to the Zetaproteobacteria. The MAR isolate was designed TAG-1, and the Mariana isolate was designed SV-108.

Both bacterial strains were deposited in the culture collections as following number: strain TAG-1, DSMZ 103937, JCM 31637, NCMA B5; strain SV-108, NCMA B6.

Cultivation of TAG-1 and SV-108.

Both strains were cultivated in ASW that contained either 0.5 g l⁻¹ (9.35 mM) ammonium chloride or 0.8 g l⁻¹ (9.41 mM) sodium nitrate as the N-source. The pH was adjusted and buffered to 6.5 with 20 mM

sodium bicarbonate and bubbling with carbon dioxide. Cultivation was conducted in 20 ml ASW medium in 30-ml sealed glass bottles under N₂/CO₂ headspace with regular feeding of 200 µl sterilized air every 48 h to maintain microoxic conditions and 1 mM ferrous chloride (FeCl₂) or 700 µl H₂ (approximately 4% v/v of headspace) as the electron donor, or in a 15 ml of ASW/ZVI in a Petri plate under a microoxic atmosphere created using a Campypak. Bacterial growth on FeCl₂ and hydrogen, and on ammonia and nitrate in bottles, was compared respectively through microscopic cell-counting using SYTO13 staining (Thermo Scientific, Waltham, MA, USA) at different time points of cultivation and establishing the growth curves as has been described previously (Emerson *et al.*, 2007). Concentrations of Fe(II) in the culture were monitored using the phenanthroline method (Tamura *et al.*, 1974). The temperature and pH optima for growth was determined at different temperatures (5–37 °C) and pH values (5.5–7.5, buffered by 20 mM MES or HEPES).

Monitoring of hydrogen oxidation by TAG-1 and SV-108

Bacterial cells of strain TAG-1 and SV-108 were inoculated into 20 ml of ASW medium in a 30-ml bottle, with a headspace filled with N₂/CO₂ and supplied with additional 700 µl hydrogen (final approx. 4% v/v) and 300 µl sterilized air. The H₂ concentration was measured each day by taking a 100 µl of headspace gas with a sterile syringe. Gas pressure in the culture vials was measured using a digital pressure meter (GMH 3111, Greisinger Electronic, Regenstauf, Germany) to calculate the amount of H₂ consumed. To replenish the supply of oxygen, 200 µl sterilized air was supplied to the culture every 2 days. An aliquot of the medium (approximately 0.1 ml) was removed from the culture bottle each day for cell counts. All incubations were prepared as biological triplicates, and included a cell-free control. The H₂ concentration in the headspace of the TAG-1 and SV-108 incubations was measured with a Hewlett-Packard Co. 5980 series II gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA) coupled to a thermal conductivity detector (TCD-GC) equipped with a molecular sieve 13X column (3 m × 1/8"; Alltech, Unterhaching, Germany). The conditions for analysis were as follows: oven temperature, 40 °C; injection temperature, 150 °C; detection temperature, 175 °C; carrier gas (argon) flow rate, 30 ml min⁻¹ (27 psi). The concentration of H₂ was monitored and quantified on 32 Karat software (Beckmann Coulter, Krefeld, Germany). A standard curve for H₂ was prepared for every measurement using 0.04–3.74% (v/v) H₂ standards prepared in serum bottles.

Whole-genome sequencing of TAG-1 and SV-108

For genome sequencing both TAG-1 and SV-108 were grown in approximately 100 ml of a

H₂-containing medium. After 5 days of growth, cells were concentrated by centrifugation (16 000 *g* for 10 min), and DNA was extracted using a PowerMax Soil kit (Mo Bio Laboratories, Solana Beach, CA, USA). The genome of TAG-1 was sequenced at the Department of Energy's, Joint Genome Institute (JGI) as part of their microbial sequencing program, using Illumina technology as previously described (Emerson *et al.*, 2013). The genome of SV-108 was sequenced and assembled by the sequencing facility at the Bigelow Laboratory Single Cell Genomics Center using Illumina (Nextseq) technology. The assembled genomes of both organisms were uploaded to JGI's Integrated Microbial Genome and Microbiome (IMG/MER) for annotation. Subsequent analysis of both genomes was done using tools provided on the IMG/MER website (<https://img.jgi.doe.gov/cgi-bin/mer/main.cgi>; Markowitz *et al.*, 2009, 2014). The IMG Taxon ID for the genome of TAG-1 is 2852580733 and for SV-108 it is 2617270712.

Phylogenetic analyses

To understand the phylogeny of TAG-1 and SV-108, phylogenetic trees based on maximum-likelihood using the program MEGA (ver. 7.0.21; Kumar *et al.*, 2016) were created with SSU rRNA gene sequences, concatenated sequences of housekeeping genes, and some key functional gene sequences, respectively. The housekeeping genes used for building concatenated trees were as follows: DNA gyrase subunit B (*gyrB*), isoleucyl-tRNA synthase (*ileS*), leucyl-tRNA synthase (*leuS*), CTP synthase (*pyrG*), leader peptidase A (*lepA*), DNA recombination and repair protein (*recA*), elongation factor P (*efp*), RNA polymerase α -subunit (*rpoA*), RNA polymerase major σ factor (*rpoD*), Hsp70 cochaperone (*dnaJ*) and tmRNA-binding protein (*smpB*) (Gil *et al.*, 2004; Santos and Ochman, 2004). For all analyses, the relevant nucleic acid sequences were aligned using ClustalW in MEGA and the trees were created using Tamura-Nei model with 1000 bootstrap iterations.

Whole-genome comparisons

Similarities among whole genomes of TAG-1 and SV-108 with other Zetaproteobacteria isolates (*Mariprofundus ferrooxydans* PV-1, JV-1 and M34, *Mariprofundus* spp. EKF-M39, and DIS-1) were investigated by calculating average amino-acid identities (AAIs) and nucleic acid identities (ANIs). AAIs were calculated by CompareM (ver. 0.0.21; <https://github.com/dparks1134/CompareM>). ANIs were calculated using JSpeciesWS Online Service (<http://jspecies.ribohost.com/jspeciesws/>; Richter and Rosselló-Móra, 2009; Richter *et al.*, 2015). The synteny between the genomes of TAG-1 and SV-108 was conducted using Mauve (ver. 2.4.0; Darling *et al.*, 2010).

Biogeography and ecological analysis

Initial phylogenetic and classification analysis using ZetaHunter (<https://github.com/mooreryan/ZetaHunter>, last accessed 01.01.2017) to assess SSU rRNA gene sequences showed that TAG-1 and SV-108 belong to Zetaproteobacteria ZetaOTU9 (McAllister *et al.*, 2011; Figure 4). To better understand the global distribution of these isolates and ZetaOTU9, we used the Integrated Microbial Next Generation Sequencing (IMNGS, <https://www.imngs.org>, last accessed 04.01.2017; Lagkouvardos *et al.*, 2016) server. IMNGS is an online platform that allows users to conduct comprehensive searches of SSU rRNA gene sequences against raw sequence read archives retrieved from the International Nucleotide Sequence Database Collaboration. The current build (accessed January 2017) has roughly 88 500 samples, 32 890 of which are from human-associated studies and the remainder from a wide array of environmental and host-associated habitats. IMNGS offers two types of queries—a taxonomy query and a similarity query. To gain a general sense of Zetaproteobacteria distribution, we first ran the taxonomy query. Because there are no lower taxonomic ranks for Zetaproteobacteria, we screened the entire class. We then conducted a similarity search of all near full-length SSU rRNA single amplified genome (SAG) and pure culture sequences classified at ZetaOTU9 (by ZetaHunter) against the IMNGS database using a similarity threshold of 99% and a minimum length of 200 bp.

Results

Growth studies of TAG-1 and SV-108

TAG-1 and SV-108 are both rod-shaped cells with dimensions of 0.3×1.5 – $2 \mu\text{m}$ (Figure 1). Motility was often, but not always, observed, and both strains contained a complement of flagellar synthesis genes and chemotaxis genes (Supplementary Tables S1 and S2). When cultivated with Fe(II), Fe-oxhydroxides precipitated that had no discernible morphology, although the cells were often associated with the oxides. Both strains grew on either FeCl₂ or H₂ with ammonium chloride as a N source (Figure 2a). Growth on H₂ was faster than growth on FeCl₂ especially in TAG-1, with calculated doubling times of 14.1 and 21.8 h (TAG-1) and 16.3 and 20.0 h (SV-108) for H₂ and Fe(II), respectively. These doubling times were calculated based on the rapid growth phases of the growth curves in Figure 2, thus represent an optimal doubling time. For both strains consumption of H₂ supplied in the headspace corresponded with cell growth, and the addition of H₂ to week-old cultures led to rapid H₂ consumption (Figure 2b). There was no consumption of H₂ in the cell-free controls, indicating no abiotic H₂ consumption. TAG-1 consumed H₂ in the headspace with a rate of $4.83 \mu\text{mol day}^{-1}$ during the first 6 days of incubation, and $12.27 \mu\text{mol day}^{-1}$ following a

second addition of H₂ (Supplementary Figure S1). Cell yield of TAG-1 was 2.36×10^7 cells per $\mu\text{mol H}_2$ consumed, which is a comparable value to the calculated cell biomass yield of *Cupriavidus necator*, a model H₂-oxidizing chemolithoautotrophic bacterium (Bongers, 1970). When TAG-1 was grown with both Fe(II) and H₂, or either substrate alone, the consumption of Fe(II) and H₂ co-occurred (Figure 3). SV-108, on the other hand, showed slower H₂ oxidation in the presence of Fe(II) than cultures without Fe(II) during first 5–6 days, while Fe(II) disappeared more quickly in the presence of H₂ (Figure 3), suggesting this organism may have a growth preference for Fe(II) over H₂.

Growth studies revealed that nitrate had an inhibitory or bacteriostatic effect on the growth of SV-108, since there was a pronounced lag phase of 3–6 days for cultures grown on either Fe(II) or H₂ in the presence of 9.41 mM sodium nitrate, but not on 9.35 mM ammonium chloride (Figure 2a). Nitrate-grown cells of SV-108 that were sub-cultured into new nitrate-containing medium exhibited a similar lag phase (data not shown), indicating this lag was not strictly due to an adaptation to nitrate. TAG-1 did not show any differential response in growth based on nitrogen source.

The temperature (20 °C) and pH (6.5–7.0) optima of both strains are summarized in Supplementary Table S3. Neither strain grew on heterotrophic R2A medium under microoxic conditions. It was discovered that organic compounds could inhibit the growth of both strains. Both strains can be cryo-preserved in 10% glycerol (w/v final concentration); however, upon recovery, the glycerol stocks had to be diluted 1:100 or 1:1000 in order to initiate growth. This phenomenon was tested further by adding either glycerol or glucose to either TAG-1 or SV-108 grown on ZVI; neither strain grew in the presence of 0.5% glycerol or 1% glucose; however, at lower concentrations (0.1% glycerol or 0.5% glucose) the cells were able to grow so long as H₂ was present.

Phylogenetic analysis of TAG-1 and SV-108

The respective genome sizes of TAG-1 and SV-108 were 2.16 and 2.14 Mb; other pertinent genome information is shown in Supplementary Table S4. Phylogenetic analysis confirmed the morphological and physiological observations that these two strains are close relatives, sharing 99% homology between their respective SSU rRNA genes. The SSU rRNA gene phylogeny showed the two strains clustered closely together within the Zetaproteobacteria (Figure 4). More detailed phylogenetic trees that used concatenated functional genes *gyrB-ileS-leuS-efp-lepA-pyrG-recA-rpoA-rpoD-dnaJ-smpB* confirmed this relationship (Supplementary Figure S2). TAG-1 and SV-108 clustered most closely with three SAGs that originated from the MAR, and formed a small phylogenetic clade (Figure 4). The ANIs and AAls between isolates of Zetaproteobacteria with sequenced genomes

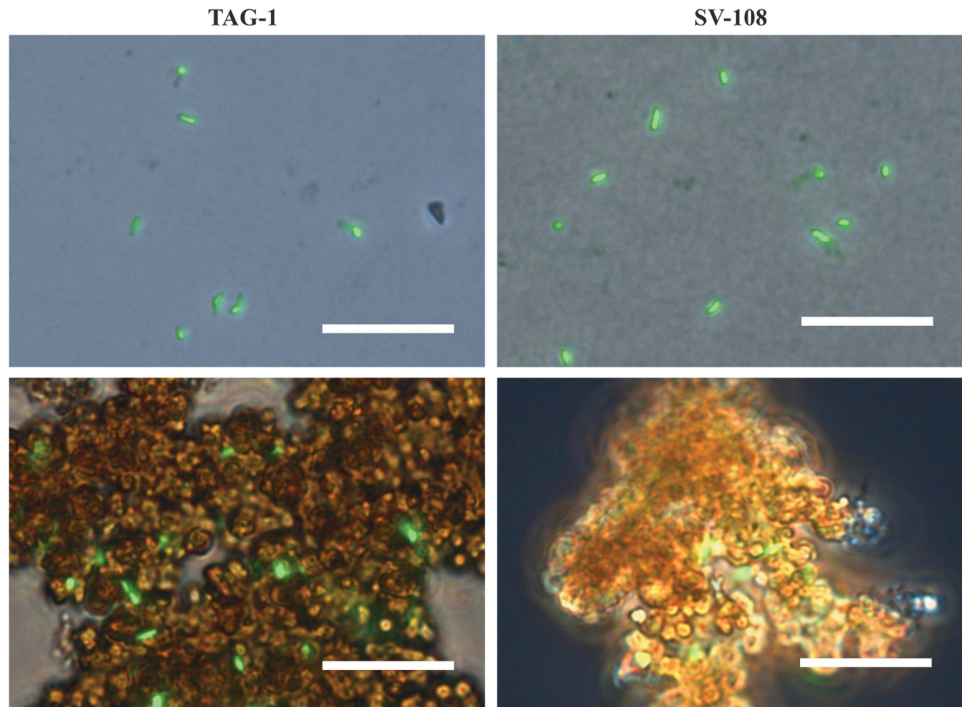


Figure 1 Microscopic images of TAG-1 (left) and SV-108 (right) cells grown on H₂ (above) and FeCl₂ (below). Cells were stained with SYTO13. Scale bars indicate 10 μm.

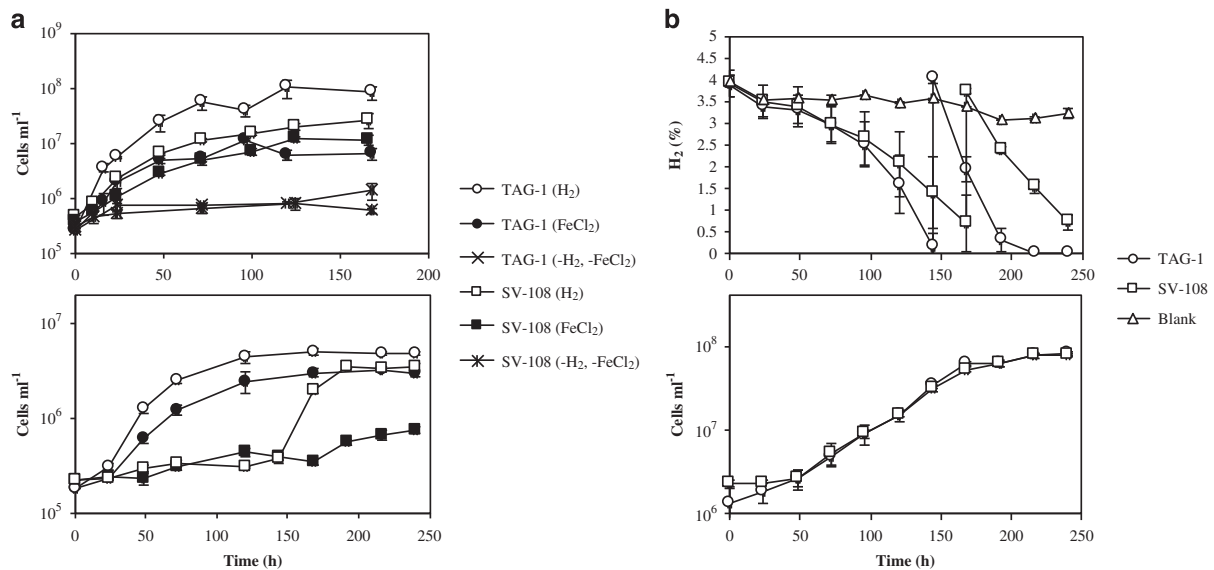


Figure 2 (a) Growth curves of TAG-1 (○, ●) and SV-108 (□, ■) on H₂ (○, □) or FeCl₂ (●, ■) with ammonium chloride (upper, *n* = 3) or sodium nitrate (lower, *n* = 2). Hydrogen and Fe(II) sources were regularly fed. Growth curves with neither H₂ nor Fe(II) source (with ammonium chloride) were also shown. (b) Monitoring of hydrogen concentrations (upper) and cell numbers (lower) in strain TAG-1 (○) and SV-108 (□) cultures (*n* = 3). Additional H₂ was fed after 144 h (TAG-1) and 168 h (SV-108) of incubation. Error bars indicate standard deviations.

(PV-1, JV-1, M34, EKF-M39, DIS-1, TAG-1, and SV-108) are shown in Supplementary Figure S3, and provided further confirmation of the relatedness of TAG-1 and SV-108 to one another, and distance from the other Zetaproteobacteria isolates. As shown in Supplementary Figure S4 there was considerable

synteny between the genomes of TAG-1 and SV-108. Based on their relatedness to one another, but genetic, physiological, and morphological differences from other Zetaproteobacteria, it is reasonable to propose TAG-1 and SV-108 are strains of a novel genus and species.

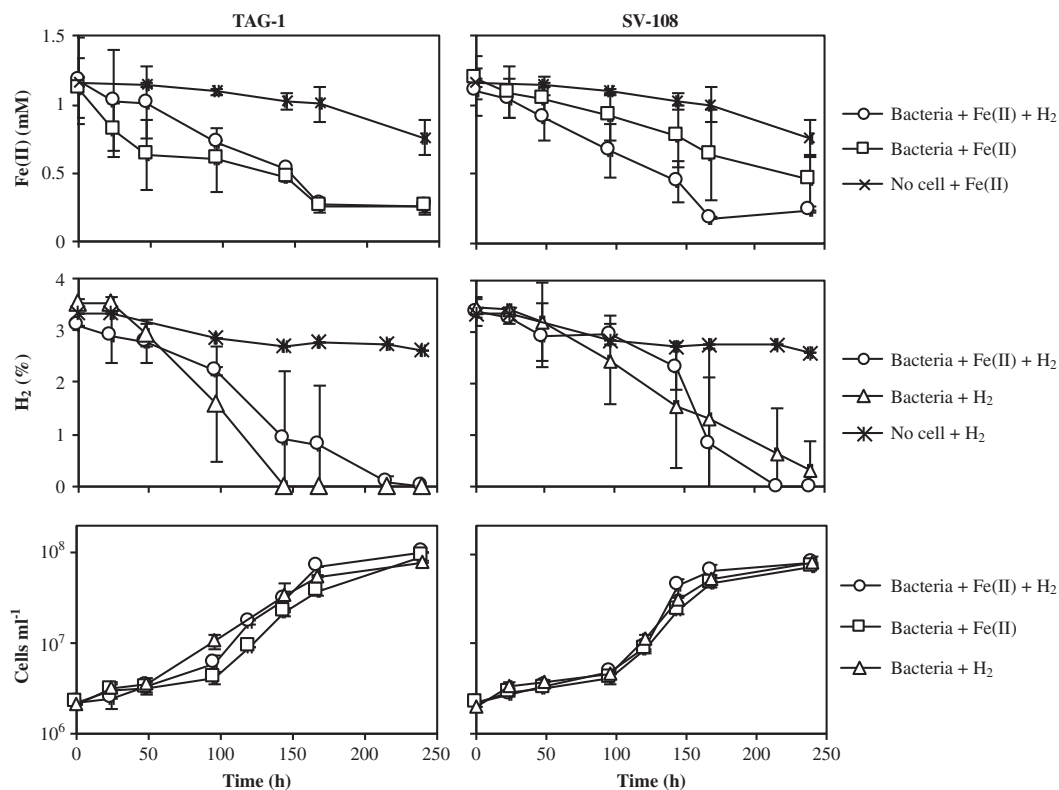


Figure 3 Monitoring of Fe(II) (upper) and hydrogen (middle) concentrations and cell numbers (lower) in strain TAG-1 (left column) and SV-108 (right) cultures which were supplied with simultaneous use of Fe(II) and hydrogen (○) or only Fe(II) (□) or hydrogen (Δ). Error bars indicate SD ($n=3$).

Genome analysis of TAG-1 and SV-108

The mechanism of iron oxidation in the Zetaproteobacteria remains largely unknown; however, several candidate genes or gene clusters have been identified either through comparative genomics or proteomics (Singer *et al.*, 2011; Emerson *et al.*, 2013; Barco *et al.*, 2015; Fullerton *et al.*, 2017). Thus far, a gene in *M. ferrooxydans* identified as a distant homolog of Cyc2, an outer membrane protein in *Acidithiobacillus* that has iron oxidation activity, and designated Cyc2_{PV-1} has been found in the genomes of all marine and freshwater lithotrophic FeOB (Barco *et al.*, 2015). TAG-1 (IMG locus tag DM09DRAFT_0629) and SV-108 (Ga0073143_10672) both encode Cyc2_{PV-1} homologs according to BLAST analysis. The alternative respiratory complex III (AC-III) including the *actB* gene that encodes for a molybdopterin oxidoreductase is another important component in the genomes of a number of marine and freshwater FeOB. TAG-1 has this complex, but also contains a second gene encoding a molybdopterin oxidoreductase. This second gene is in a cluster that includes the large and small subunits of the assimilatory nitrate reductase. This same gene cluster is conserved in the genomes of several Zetaproteobacteria (Field *et al.*, 2014; Fullerton *et al.*, 2017). Interestingly, SV-108 did not have the AC-III complex, but does encode nitrite and nitrate assimilatory reductases (Supplementary Figure S5).

Consistent with their ability to grow on H₂, both TAG-1 and SV-108 possessed nearly identical gene clusters that consisted of the subunits and assembly proteins of the H₂-uptake NiFe-hydrogenase (Figure 5; Greening *et al.*, 2016). The assembly protein genes (*hypA*, *B*, *C*, *D*, *E* and *F*) were also detected in genomes of other isolates of Zetaproteobacteria; however, these strains do not have subunit genes of the uptake NiFe-hydrogenase, but instead had the genes for a NAD-reducing hydrogenase.

In terms of autotrophic potential, TAG-1 and SV-108 had gene clusters consistent with CO₂ fixation via the Calvin–Benson–Bassham pathway, including the large subunit *cbbM* gene of the Form II ribulose-1,5-bisphosphate carboxylase (RubisCO) and the phosphoribulokinase gene (Berg, 2011). A phylogenetic comparison of the *cbbM* gene of TAG-1 and SV-108 showed a 92% similarity of nucleic acid sequences between these two genes, and lower (66–79%) similarities with other Zetaproteobacteria isolates and SAGs (Supplementary Figure S6). Neither TAG-1 or SV-108 encoded Form I RubisCO, implying they may have a preference for high CO₂ and low oxygen conditions (Tabita *et al.*, 2008).

For nitrogen acquisition, both TAG-1 and SV-108 have a suite of genes involved in nitrogen metabolism, as listed in Supplementary Table S5. These include an assimilatory nitrate reductase, and nitrite reductase that, as noted above, contain a

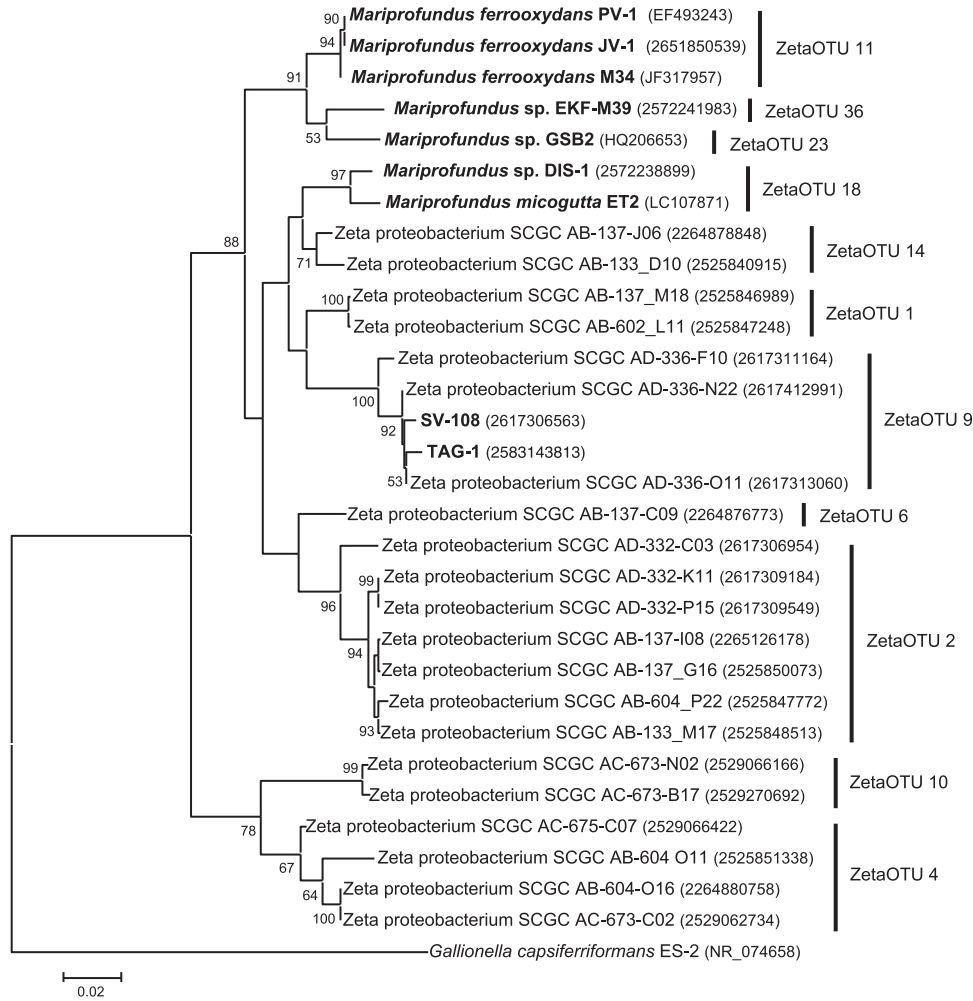


Figure 4 Maximum-likelihood phylogenetic tree of SSU rRNA gene of TAG-1, SV-108 and other Zetaproteobacteria isolates and SAGs. Isolated strains of Zetaproteobacteria are in bold, and GenBank accession no. or Gene IDs on IMG are given in parentheses. The tree was rooted with SSU rRNA gene of *G. capsiferiformans* ES-2. The tree was created with 1000 bootstrap iteration and the values below 50 are not reported.

molybdopterin oxidoreductase. They also have a nitric oxide reductase as well as ammonium transporters. TAG-1 and SV-108 share all nitrogen-related genes checked in this study. The reason for nitrate-sensitivity of SV-108 is still unclear.

Ecological analysis

A previous study found that the Zetaproteobacteria are primarily associated with high iron environments in the ocean (Scott *et al.*, 2015). ZetaOTU9, the clade containing TAG-1 and SV-108, was present at diffuse-flow Fe(II)-rich vents at Rainbow and Snakepit hydrothermal vent fields, in addition to TAG, located on the MAR, although a previous study, based on an earlier phylogenetic analysis, identified ZetaOTU9 as OTU3 at TAG, Rainbow and Snakepit (Scott *et al.*, 2015). Somewhat surprisingly, at the Snail Vent site in the Southern Mariana Trough, analysis of community abundance by SSU rRNA amplicon sequencing found that although the

relative abundance of Zetaproteobacteria was 10–12% of community, ZetaOTU9 only accounted for 0.05% of the Zetaproteobacteria reads (Hager *et al.*, 2017). This may help explain why only a low dilution (10^{-2}) enrichment was successful. A comprehensive taxonomic search of SSU rRNA raw sequencing read archives using IMNGS found Zetaproteobacteria were present (mostly at abundances of $<0.01\%$) in only 700 of over 55 500 available non-human samples. A similarity search of representative sequences classified as ZetaOTU9 against IMNGS found hits in only 17 of these samples (Table 1). The two samples in the IMNGS where ZetaOTU9 had substantial abundance, accounting for 22–37% of the total community, was from freshly synthesized basalt chips incubated on abyssal plain of the Atlantic Ocean (Henri *et al.*, 2016). These were colonized by Zetaproteobacteria that were presumed to be growing on Fe(II) released from the basalt. Remarkably ZetaOTU9 accounted for $>90\%$ of the Zetaproteobacteria reads from these samples.

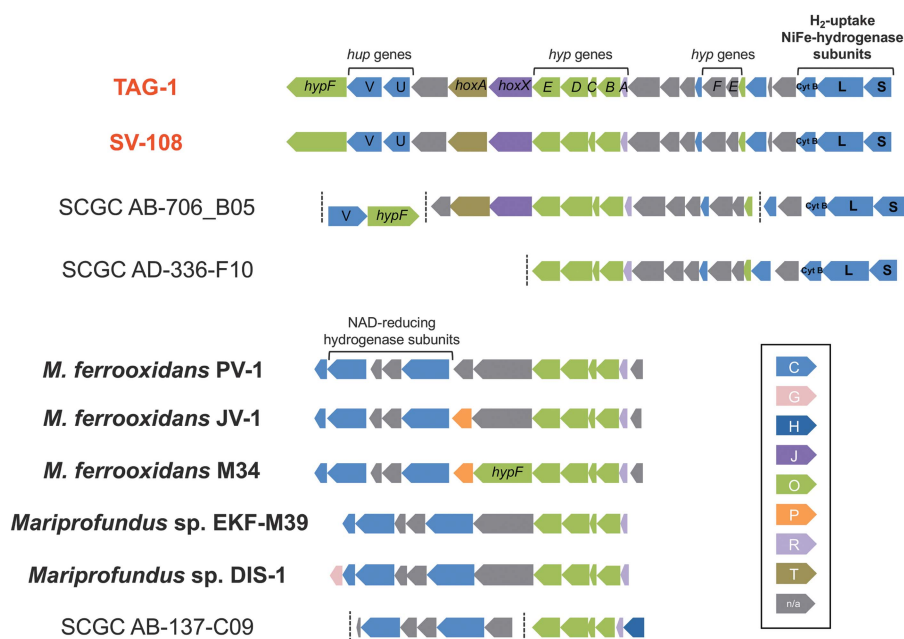


Figure 5 Gene clusters of NiFe-hydrogenase encoded by TAG-1, SV-108 and other Zetaproteobacteria isolates and SAGs. Genes are shown in different colors according to COG functional category as listed in a box. Genes annotated as subunits of H₂-uptake NiFe-hydrogenase are labeled with bold letters (L: large subunit, S: small subunit, and Cyt B: cytochrome *b* subunit). Dash lines indicate end of the contig.

Table 1 Distribution of SSU rRNA gene of ZetaOTU9 SAGs and pure culture isolates

SRA IDs	Size	Zeta reads	ZetaOTU9 reads
ERR313278 ^a	12769	11	1
ERR313285 ^a	12765	11	1
ERR313301 ^a	159368	127	19
SRR1573430 ^b	31256	3417	3403
SRR1573432 ^b	16973	3330	3112
SRR2130144 ^c	37846	4	4
SRR2130147 ^c	36968	3	3
SRR2130148 ^c	53226	1	1
SRR2130149 ^c	45262	1	1
SRR2130151 ^c	30321	6	6
SRR2130152 ^c	49326	18	9
SRR2130153 ^c	32058	11	9
SRR2130155 ^c	20423	7	7
SRR592604 ^d	5902	1	1
SRR592631 ^d	4566	1	1

Size refers to the total reads in each library, and ZetaOTU9 reads are a subset of the Zeta reads.

^aMarine sediments of the Barents Sea.

^bMAR Basaltic glass.

^cPo river Prodelta and Mar Piccolo of Taranto surface sediments bacterial communities.

^dLogatchev field in the MAR.

Discussion

Previous isolates of marine FeOB have all been obligate Fe-oxidizers, thus TAG-1 and SV-108 are the first Zetaproteobacteria shown to grow microaerobically on H₂ as an alternative substrate. Phylogenetic analysis of conserved genes as well as comparative genomics clearly shows these two strains are not

closely related to other isolates belonging to the Zetaproteobacteria. Furthermore, they do not produce any identifiable extracellular organo-metallic structures such as stalks, sheaths or organized filaments that are characteristic of other known marine FeOB. Consistent with a lack of stalk production, TAG-1 and SV-108 do not possess the putative genes for stalk-formation (*xag* operon), although these are conserved in the genomes of several *Mariprofundus* species (Kato *et al.*, 2015). Presumably these two strains produce a different type of exopolymer that prevents them from becoming encrusted in Fe-oxides, as has been proposed for several, non-stalk forming freshwater FeOB (Emerson *et al.*, 2013). Despite coming from hydrothermal vents on opposite sides of the world, the two isolates share most of their genes in common and their genomes have substantial regions of synteny. Together these results demonstrate TAG-1 and SV-108 represent a novel genus within the Zetaproteobacteria, and the name ‘*Ghiorsea bivora*’ is proposed.

Metabolism

Thermodynamically H₂ is a better energy source than Fe(II) with a moderately higher free energy, $\Delta G^\circ = -237 \text{ kJ mol}^{-1}$ ($-474 \text{ kJ per mol O}_2 \text{ reduced}$) for H₂ vs $\Delta G^\circ = -90 \text{ kJ mol}^{-1}$ for Fe(II) ($-360 \text{ kJ per mol O}_2 \text{ reduced}$) (Emerson *et al.*, 2010). The doubling times of TAG-1 and SV-108 were 55.0% and 23.2% more rapid for H₂-grown cells compared to Fe(II)-grown cells, respectively, and cell yields were marginally

higher on H₂ compared to Fe(II) (Figure 2a). While we could not totally account for auto-oxidation of Fe(II) in the substrate utilization experiments, it appeared both substrates were consumed simultaneously, indicating these organisms are not adapted to preferentially use either Fe(II) or H₂, despite H₂ being the more energetically favorable electron donor.

Both strains share nearly identical suites of genes involved in H₂ metabolism that include the canonical genes for the oxygen-tolerant, membrane-bound H₂-uptake NiFe-hydrogenase (Greening *et al.*, 2016). These proteins contain signal sequences and are presumably located in the periplasm where they can initiate H₂ oxidation coupled to energy conservation via the electron transport chain. The phylogenetic analysis of the large subunit of the hydrogenase revealed that two SAG genomes belonging to ZetaOTU9 (SCGC AB-706_B05 and SCGC AD-336-F10) also contain uptake NiFe-hydrogenase (Figure 5). Together these genes from the Zetaproteobacteria fall into a diverse cluster of related genes in the Gammaproteobacteria, with the closest relatives coming from lithotrophic sulfur-oxidizing bacteria (Figure 6). This raises the possibility that H₂ oxidation was acquired through different horizontal gene transfers.

Regarding iron oxidation, we still lack definitive evidence as to how FeOB conserve energy from Fe(II) oxidation (White *et al.*, 2016); however, these two strains illustrate the diversity of specific pathways that are likely involved in this process. Both strains have a gene homolog to Cyc2_{PV-1} that a recent proteomic analysis indicated could be an important outer membrane cytochrome that may initiate iron oxidation. The Cyc2 homologs encoded by TAG-1, SV-108 and an SAG belonging to ZetaOTU9 (SCGC AD-336-F10) show high gene homologies (Supplementary Figure S7). Neither strain however, possesses a gene homologous to the Cyc1_{PV-1}, a soluble cytochrome that has been suggested to play a role in shuttling electrons across the periplasm in *M. ferrooxydans*. Both strains have the capacity to produce the different complexes of the electron transport chain; however, TAG-1 does not have a cytochrome bc₁ complex (complex III), although it does have the AC-III, while SV-108 does not have AC-III but does have the cytochrome bc₁ complex III. Unlike most of the other FeOB genomes, neither strain has the cytochrome bd complex that encodes a terminal oxidase with high oxygen affinity that can couple directly with the quinone pool. In both strains, complex IV, the terminal oxidase of the electron transport chain, is encoded by a single gene cluster *ccb₃*-type cytochrome oxidase. This is in contrast to *M. ferrooxydans* that has this same gene cluster, but also has a second cluster of genes that encode a *ccb₃*-type cytochrome oxidase. Proteomic analysis showed that this second cytochrome oxidase was the most highly expressed in *M. ferrooxydans* (Barco *et al.*, 2015). Somewhat surprisingly,

TAG-1, but not SV-108, also has a gene cluster that includes the *coxA* (DM09DRAFT_0178) and *coxB* (DM09DRAFT_0179) genes encoding an aa3-type terminal cytochrome oxidase with lower affinity for oxygen. Thus far, TAG-1 is only genome from an FeOB isolate that has this cytochrome oxidase, although it is present in SAGs and metagenomes of Zetaproteobacteria (Fullerton *et al.*, 2017).

Based on this genomic analysis, we hypothesize that TAG-1 may exhibit more flexibility regarding its ability to grow and survive under higher or more dynamic oxygen concentrations. A related aspect is the capacity for these organisms to defend against reactive oxygen species such as hydrogen peroxide (H₂O₂) and super-oxides. This is especially relevant given that Fenton chemistry involves the reaction of H₂O₂ with Fe(II) to produce hydroxyl radicals that are extremely damaging to cellular organic matter (Imlay, 2008). Somewhat surprising then is that in the genome of SV-108, neither catalase or superoxide dismutase was found, while TAG-1 had a catalase-peroxidase gene (DM09DRAFT_0584) but also lacked superoxide dismutase (Supplementary Table S6). Superoxide dismutase is a ubiquitous gene in aerobic organisms, and is even found in many anaerobes (Hewitt and Morris, 1975; Imlay, 2008). Thus far, the sequenced genomes of all other FeOB have contained superoxide dismutase (Mumford *et al.*, 2016). For defense against H₂O₂ production, both TAG-1 and SV-108 contain two copies of a cytochrome *c*-peroxidase gene. Cytochrome *c*-peroxidase is excreted to the periplasm, and may act as a defense mechanism against exogenously produced H₂O₂. The most abundant genes involved in reactive oxygen species protection for both strains were peroxiredoxins (TAG-1 = 4 copies; SV-108 = 5 copies; Supplementary Table S6) that utilize a thiol-based mechanism to react with H₂O₂ in the cytoplasm. Presumably it is this mechanism that is the primary defense for reactive oxygen species in TAG-1 and SV-108. It should be noted, however, that our results cannot eliminate the possibility of either mis-annotation of functional genes, or that a gene is missing from these near complete, but not finished genomes. Some physiological capabilities of TAG-1 and SV-108, such as oxygen tolerance and utilization of nitrogen-species, still have to be evaluated via further cultivation-based tests.

Ecological implications

All previous isolates of Zetaproteobacteria have been obligate Fe-oxidizers, so the discovery of these two H₂-utilizing strains adds a new dimension to the physiology of this group. Because the Zetaproteobacteria are phylogenetically differentiated from other Proteobacteria at the class level, we are able to effectively track individual OTUs within them (McAllister *et al.*, 2011). TAG-1 and SV-108, as well as the two SAGs that also had uptake hydrogenase genes (Figure 5) belong to ZetaOTU9; however, none

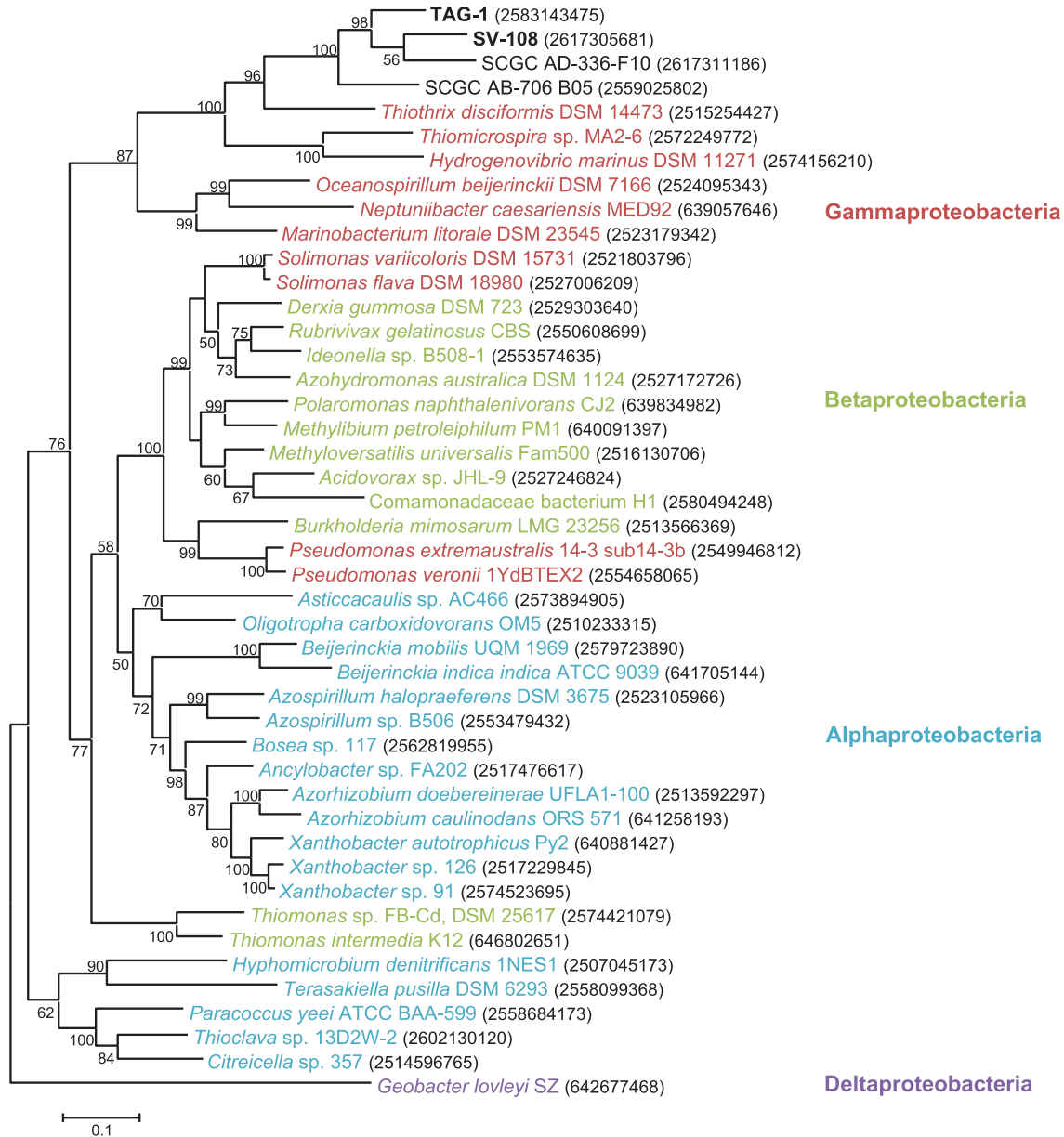


Figure 6 Maximum-likelihood phylogenetic trees of H₂-uptake NiFe-hydrogenase large subunit gene of TAG-1, SV-108, Zetaproteobacteria SAGs and related bacterial species. Gene IDs on IMG are given in parentheses, and bacterial classes (Alpha-, Beta-, Gamma- and Deltaproteobacteria) are shown in different colors. The tree was rooted with the gene of *G. lovleyi* (Deltaproteobacteria). The tree was created with 1000 bootstrap iteration and the values below 50 are not reported.

of the other genomes of Zetaproteobacteria, either from pure cultures, SAGs, or metagenomes have these hydrogenase genes, suggesting that growth on H₂ may be a trait only found in ZetaOTU9. When analyzed relative to the dominant ZetaOTUs in a variety of chemosynthetic iron communities, the overall abundance of ZetaOTU9 is often at background levels of <0.1% of the community. However, in samples where its abundance is >1%, it is often a dominant phylotype of the Zetaproteobacteria. For example, among vent sites at the MAR, ZetaOTU9 was prevalent in five samples (two from TAG, two from Snakepit, one from Rainbow), accounting for

60–95% of Zetaproteobacteria reads, but made-up less than 5% of the reads in three other samples (one each from TAG, Snakepit and Rainbow; Scott *et al.*, 2015). At the Mariana, as mentioned in the results, ZetaOTU9 was in low abundance at the Snail Vents where SV-108 was isolated. However, in a broader survey of chemosynthetic iron mats along the Mariana Arc and back-arc, ZetaOTU9 accounted for >5% of the Zetaproteobacteria reads in 6 of 21 discrete samples from 5 different sites. At one of these sites ZetaOTU9 accounted for 75% of the total Zetaproteobacteria reads (Hager *et al.*, 2017). An extensive survey of iron mats at Lō‘ihi Seamount,

found ZetaOTU9 was only present in veil-like mats that are structurally dominated by sheath-forming Zetaproteobacteria (Scott *et al.*, 2017).

In addition to being abundant on fresh basalts (Henri *et al.*, 2016) as noted above, other sites where ZetaOTU9 is abundant are mild steel coupons incubated in coastal seawater (McBeth and Emerson, 2016), and in the subsurface of the ocean crust (Kato *et al.*, 2009). These latter habitats are locales where H₂ may be present. On steel, ZVI, given the right conditions, can react with seawater to produce H₂, and in the subsurface water–rock reactions may generate H₂. The capacity to use H₂ as a sole electron donor would certainly give strains like TAG-1 and SV-108 a competitive advantage over obligate Fe(II)-oxidizers. Unfortunately, it has not been possible to obtain H₂ measurements from the iron mats where ZetaOTU9 was abundant. It is worth pointing out, however, that samples from the MAR, Lōihi, and the Mariana were all collected at high (sub-centimeter) spatial resolution, thus the corresponding presence and absence of ZetaOTU9 with specific samples that in some cases were only a few centimeters apart, indicates that there may be chemical (e.g., the presence of H₂), mineralogical and/or physical drivers that may be controlling their population dynamics. This underscores the importance of being able to collect relevant physicochemical data at spatial scales that can be co-registered with community analysis, so fundamental drivers of microbial diversity are revealed.

The IMNGS meta-analysis included a number of sites where H₂ is likely present as a potential energy source, such as sediments and hydrothermal vent communities, but where the abundance of Fe(II) is insufficient to support Fe(II)-fueled chemosynthesis. This indicates that despite having the metabolic flexibility of being able to use H₂ in addition to Fe(II), TAG-1 and SV-108, and by extension ZetaOTU9, are still limited to Fe(II)-rich habitats. Thus the adaptations required to grow on Fe(II) likely play a stronger role in selection than the capacity to grow on H₂. There can be appreciable fluxes of H₂-associated diffuse venting systems, and the dynamics of these fluxes indicate there is significant microbial metabolism of H₂ (Wankel *et al.*, 2011). The discovery that this particular clade of FeOB that grow as well or better on H₂ than Fe(II) supports this finding, and indicates the Zetaproteobacteria contribute to chemosynthesis in these systems via H₂ utilization in addition to iron oxidation. A similar role has been suggested for chemolithoautotrophic sulfur-oxidizing bacteria like *Thiomicrospira* (Hansen and Perner, 2015).

Taxonomy

TAG-1 and SV-108 represent the most unique isolates within the Zetaproteobacteria discovered to date. We propose they form a novel genus and species, thus representing the second genus and third species within this class.

Description of *Ghiorsea* gen. nov.

Ghiorsea gen. nov. (Ghi.or.se.a. N. L. fem. n. *Ghiorsea*, named after contemporary American microbiologist William C Ghiorse, in recognition of his important contributions to the field of geomicrobiology.

Gram-negative rod-shaped cells. Growth is obligately lithotrophic, oxygen-dependent, and requires marine salts; habitat, marine microbial iron mats. The type species is: *Ghiorsea bivora* gen. nov. sp. nov.

Description of *Ghiorsea bivora* sp. nov.

G. bivora (bi.vor.a, L. prefix *bi* two; L. comb. *-vora*, ones that eat; N.L. n. *bivora* one that eats two substrates).

Displays the following properties in addition to those given by the genus description. The cells are rods, approximately 0.3 × 1.5–2 μm. Motility is often observed. Lithotrophic growth on either ferrous iron or on hydrogen gas under microaerobic conditions. Result of growth on ferrous iron is the precipitation of iron-oxyhydroxides with no determinant structure. No observable growth on organic compounds; elevated concentrations of organics may be bacteriostatic. The optimum growth temperature is 20 °C. The pH range for growth is 6.0–7.0. The G+C content of the DNA is 43.7%. The type strain is TAG-1^T (=DSMZ 103937; =JCM 31637; =NCMA B5; IMG genome identification number 2582580733) isolated from an iron-rich microbial mat associated with diffuse hydrothermal venting at the TAG hydrothermal vent site on the MAR.

Conflict of Interest

The authors declare no conflict of interest.

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