Genomic Insights into Two Novel Fe(II)-Oxidizing *Zetaproteobacteria* Isolates Reveal Lifestyle Adaption to Coastal Marine Sediments

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ABSTRACT The discovery of the novel *Zetaproteobacteria* class greatly expanded our understanding of neutrophilic, microaerophilic microbial Fe(II) oxidation in marine environments. Despite molecular techniques demonstrating their global distribution, relatively few isolates exist, especially from low-Fe(II) environments. Furthermore, the Fe(II) oxidation pathways used by *Zetaproteobacteria* remain poorly understood. Here, we present the genomes (>99% genome completeness) of two *Zetaproteobacteria*, which are the only cultivated isolates originating from typical low-Fe [porewater Fe(II), 70 to 100 μM] coastal marine sediments. The two strains share <90% average nucleotide identity (ANI) with each other and <80% ANI with any other *Zetaproteobacteria* genome. The closest relatives were *Mariprofundus aestuarium* strain CP-5 and *Mariprofundus ferrinatatus* strain CP-8 (96 to 98% 16S rRNA gene sequence similarity). Fe(II) oxidation of strains KV and NF is most likely mediated by the putative Fe(II) oxidase Cyc2. Interestingly, the genome of strain KV also encodes a putative multicopper oxidase, PcoAB, which could play a role in Fe(II) oxidation, a pathway found only in two other *Zetaproteobacteria* genomes (*Ghiorsea bivora* TAG-1 and SCGC AB-602-C20). The strains show potential adaptations to fluctuating O_2 concentrations, indicated by the presence of both cbb_3- and aa_3-type cytochrome c oxidases, which are adapted to low and high O_2 concentrations, respectively. This is further supported by the presence of several oxidative-stress-related genes. In summary, our results reveal the potential Fe(II) oxidation pathways employed by these two novel chemolithoautotrophic Fe(II)-oxidizing species and the lifestyle adaptations which enable the *Zetaproteobacteria* to survive in coastal environments with low Fe(II) and regular redox fluctuations.

IMPORTANCE Until recently, the importance and relevance of *Zetaproteobacteria* were mainly thought to be restricted to high-Fe(II) environments, such as deep-sea hydrothermal vents. The two novel *Mariprofundus* isolates presented here originate from typical low-Fe(II) coastal marine sediments. As well as being low in Fe(II), these environments are often subjected to fluctuating O_2 concentrations and regular mixing by wave action and bioturbation. The discovery of two novel isolates highlights the importance of these organisms in such environments, as Fe(II) oxidation has been shown to impact nutrients and trace metals. Genome analysis of these two strains further supported their lifestyle adaptation and therefore their potential preference for coastal marine sediments, as genes necessary for surviving dynamic O_2 concentrations and oxidative stress were identified. Furthermore, our analyses also expand our understanding of the poorly understood Fe(II) oxidation pathways used by neutrophilic, microaerophilic Fe(II) oxidizers.

KEYWORDS coastal marine sediments, Fe(II) oxidation, Fe(II)-oxidizing bacteria, *Zetaproteobacteria*

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Microaerophilic Fe(II)-oxidizing bacteria originating from marine habitats belong almost exclusively to the relatively novel *Zetaproteobacteria* class. Originally, the biogenic Fe(III) (oxyhydr)oxides found in Fe(II)-rich marine habitats were thought to be produced by microaerophilic Fe(II)-oxidizing *Betaproteobacteria* such as *Gallionella* and *Leptothrix*, as the biomineral structures they formed were reminiscent of the sheaths and twisted stalks found in terrestrial environments (1–3). However, subsequent 16S rRNA gene sequencing surveys failed to confirm the presence of these *Betaproteobacteria* and instead provided the first sequence of the *Zetaproteobacteria* class (4). The first isolates of *Zetaproteobacteria* (strains PV-1 and JV-1) were obtained from the Lōʻihi Seamount, Hawaii, and were shown to be obligate microaerophilic Fe(II)-oxidizing bacteria (5, 6). Subsequent isolation efforts and gene surveys from Fe(II)-rich marine habitats revealed deep-branching 16S rRNA gene sequences, which were used to establish the *Zetaproteobacteria* as a novel class within the *Proteobacteria* (see reference 7 for a comprehensive review).

Zetaproteobacteria typically inhabit circumneutral and saline environments where opposing gradients of Fe(II) and O_2 exist, thus occupying a niche with low O_2 concentrations yet sufficient concentration of the electron donor, Fe(II). In doing so, they are able to outcompete the abiotic oxidation of Fe(II) by molecular oxygen, which is slow at low O_2 concentrations (8), a strategy employed by all microaerophilic Fe(II)-oxidizing bacteria regardless of habitat type (9–11). Though *Zetaproteobacteria* were first identified at an Fe(II)-rich hydrothermal vent (4, 5), molecular methods, including 16S rRNA gene surveys and metagenomic studies, have shown the global distribution of *Zetaproteobacteria* in various Fe(II)-rich, and also Fe(II)-poor, habitats. These include Fe-rich microbial mats at hydrothermal vents (12, 13) and deep-sea sites (14, 15), shallow and deep marine subsurface sediments (16–19), a redox-stratified estuarine water column (20, 21), biofilms formed on mild steel incubated in coastal waters (22, 23), worm burrows in marine sediments and saline environments (24), near-shore coastal environments (25, 26), a tidal river (27), and, surprisingly, in terrestrial CO_2-rich springs (28–31). Further investigation of the tidal river (27) revealed that *Zetaproteobacteria* were only present in those environments with 5 ppt salinity or higher, while the occurrence of *Zetaproteobacteria* in the terrestrial CO_2-rich springs could be explained by the relatively high salinity at the sites (ranging between 9 and 14 ppt salinity) (28, 30, 31). Analysis of the various habitat types revealed the common characteristics shared among environments where *Zetaproteobacteria* have been found, which include brackish to hypersaline waters, microoxic conditions, and a supply of Fe(II) (7). The concentrations of dissolved Fe(II) in these habitats vary over several orders of magnitude, from high Fe(II) at hydrothermal vents (up to 934 μM) (7) to low Fe(II) in coastal sediments (70 to 100 μM) (25) and redox-stratified water columns ($<$1 μM) (20, 21). However, these low dissolved Fe(II) concentrations of $<$100 μM, which can be found in many habitats worldwide such as coastal marine sediments, are sufficient for maintaining a chemolithoautotrophic lifestyle and highlight the physiological flexibility within this *Proteobacteria* class.

The majority of information regarding the distribution of the *Zetaproteobacteria* has been obtained through sequencing of the 16S rRNA gene, which was then further used to define the diversity within this class. Analysis of full-length 16S rRNA genes using the specifically designed classification pipeline ZetaHunter (32) has identified at least 60 *Zetaproteobacteria* operational taxonomic units (ZOTUs) based on 97% sequence similarity (7). Further analysis of the ZOTUs indicated that dominant ZOTUs varied between different habitat types, which suggests that predominant geochemical conditions at individual sites favor the growth of specific ZOTUs (7). To date, there are relatively few isolates of *Zetaproteobacteria*, considering their widespread distribution, owing to the difficulty in maintaining the specific O_2 and Fe(II) concentrations required for growth in the laboratory and increasing autocatalytic Fe(II) oxidation over time (33). In total, there are currently 17 isolates representing 9 ZOTUs with almost all belonging to the genus *Mariprofundus* (7, 34). Members of the *Mariprofundus* genus are obligate neutrophilic, microaerophilic, and autotrophic Fe(II) oxidizers (7), of which the *Mariprofundus fer-*

TABLE 1 Overview of genomic features in strains KV and NF

Organism	Genome size (Mbp)	Genome completeness (%)	Genome contamination (%)	No. of contigs	GC (%)	No. of total genes	Protein coding gene count	No. of RNA genes	No. of 16S rRNA genes	tRNA	IMG genome ID
Strain KV	2.53	>99	<1	28	51.9	2,506	2,441	53	1	46	2757320575
Strain NF	2.52	>99	<1	22	50.9	2,499	2,453	58	2	47	2757320574

roxydans strain PV-1 is the type strain (35). Recently two *Zetaproteobacteria* isolates belonging to a novel second genus, *Ghiorsea*, were isolated and found to have physiological characteristics similar to those of *Mariprofundus*. However, they were shown to be facultative Fe(II) oxidizers which were also able to grow via H₂ oxidation (36). The majority of *Zetaproteobacteria* isolates originate from Fe(II)-rich environments; exceptions include two isolates from a redox-stratified estuarine water column, *Mariprofundus ferrinatatus* strain CP-8 and *Mariprofundus aestuarium* strain CP-5 (20, 21), as well as two isolates from coastal marine sediments, *Zetaproteobacteria* sp. strain S2.5 and *Zetaproteobacteria* sp. strain S1OctC (25), hereafter named strains KV and NF after Kalø Vig and Norsminde Fjord, respectively. Strains KV and NF are currently the only cultured representatives of ZOTU3 (7) and were isolated from two coastal marine sediments in Denmark (25, 37). These sediments are considered to represent typical coastal marine sediments due to their comparatively low dissolved Fe(II) porewater concentrations (70 to 100 μM) (25). Both strains are oxygen dependent, neutrophilic, autotrophic, and obligate Fe(II) oxidizers that produce slightly curved rod-shaped cells approximately 1 μm long and 0.4 μm wide. They require marine salts for growth (salinity range, 6.9 to 23 ppt) and show no observable growth on organic substrates. The length of the twisted stalks produced by both strains range from 15 to 78 μm, and the ferric iron minerals produced have been identified as lepidocrocite and potentially ferrihydrite (25). Though the physiology (25) and stalk formation (38) of strains KV and NF have already been investigated, the phylogenetic properties and genomic potential have yet to be explored. As such, the mechanism for Fe(II) oxidation and the genomic potential of these isolates are currently unknown.

Therefore, the aims of this study were to (i) determine the potential Fe(II) oxidation mechanism of the isolates, (ii) understand their genomic potential, and (iii) compare their genomes with those of other microaerophilic Fe(II)-oxidizing bacteria to identify similar and dissimilar genomic traits.

RESULTS AND DISCUSSION

Phylogenetic diversity of strains KV and NF. The genome sizes of strains KV and NF are 2.53 and 2.52 Mbp, respectively. Strain KV has a total of 2,441 protein-coding genes, while strain NF has 2,453 (Table 1). Analysis of the predicted genes revealed no clear differences in metabolic potential between strains KV and NF (data not shown). This is supported by physiological experiments conducted by Laufer et al. (25) where both strains had similar cell and twisted-stalk morphologies and were only able to grow using the same combinations of electron donors and electron acceptors [Fe(II) and O₂]. Phylogenetic analysis of the two isolates revealed that strains KV and NF are closely related, sharing 98.8% gene sequence similarity based on full-length 16S rRNA genes extracted from the genomes (Fig. S1 in the supplemental material). Comparison with other *Zetaproteobacteria* isolates and single amplified genomes (SAGs), as well as microaerophilic Fe(II)-oxidizing bacteria from freshwater environments, showed that the strains clustered closely together within the *Zetaproteobacteria* (Fig. 1; Fig. S1). Based on the 16S rRNA genes, strains KV and NF were most closely related to two isolates from a redox-stratified water column in the Chesapeake Bay (20, 21), sharing between 96 and 98% 16S rRNA gene sequence identity, respectively (Fig. 1; Fig. S1). The Chesapeake Bay isolates, *M. ferrinatatus* strain CP-8 and *M. aestuarium* strain CP-5, were classified into ZOTU groups 37 and 18, respectively, whereas strains KV and NF belong to ZOTU3 (7). Comparisons with other selected Fe(II)-oxidizing bacteria and *Zetapro-*
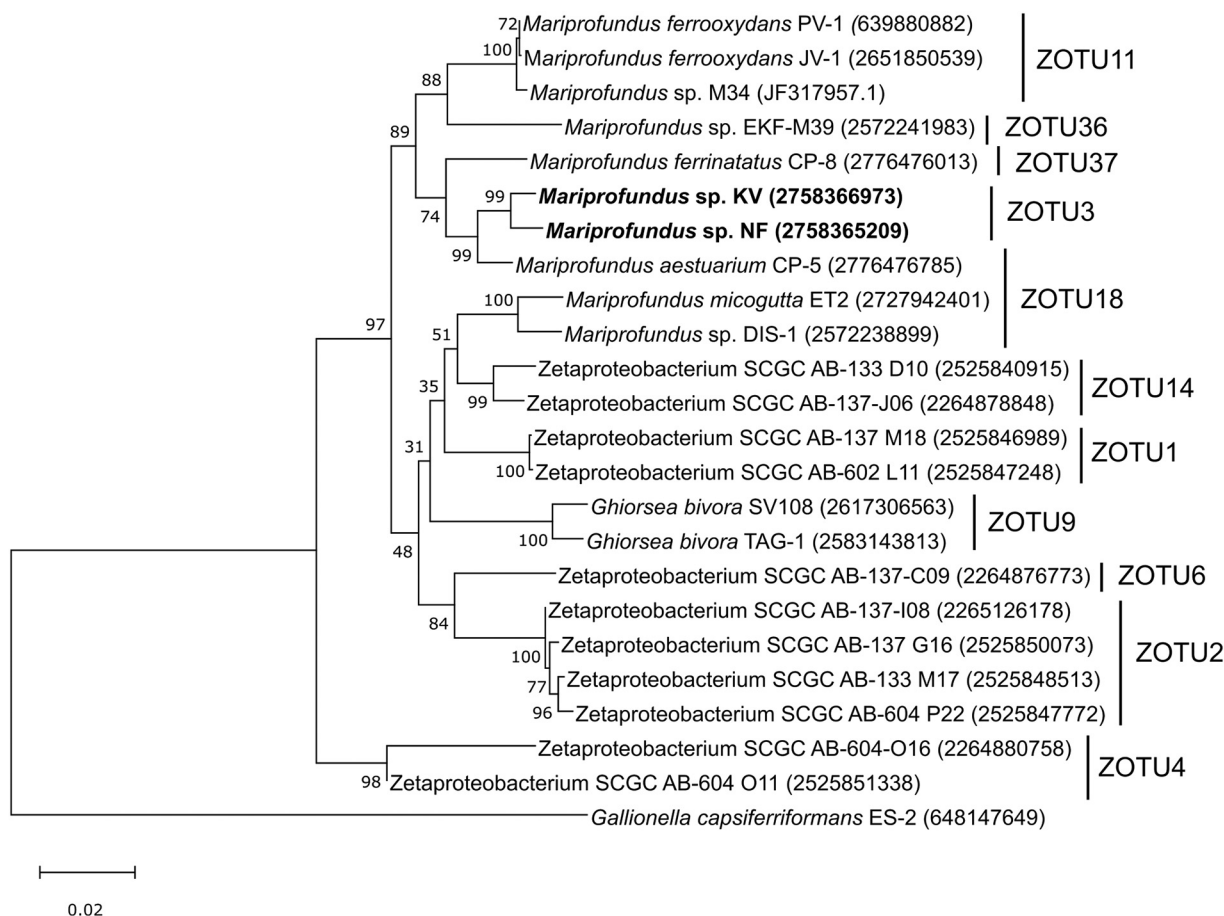
FIG 1 Neighbor-joining phylogenetic tree of full-length 16S rRNA genes of strains KV, NF, and selected *Zetaproteobacteria* isolates and single amplified genomes (SAGs) with the corresponding ZOTU classification shown to the right of the figure, according to McAllister et al. (7). The investigated strains of this study, strains KV and NF, are in bold, and the GenBank accession number or gene IDs from IMG are shown in parentheses. The tree was rooted with the 16S rRNA gene of *Gallionella capsiferriformans* strain ES-2, and bootstrap values were calculated based on 1,000 replicates. The scale bar represents the number of base substitutions per site.

teobacteria SAGs showed lower 16S rRNA gene homology ($<$95%; Fig. S1). Whole-genome comparisons using average nucleotide identity (ANI) with sequenced *Zetaproteobacteria* genomes and SAGs as well as other Fe(II)-oxidizing bacteria revealed that strains KV and NF share only 87% similarity with each other and are less than 80% similar to any of the other genomes investigated (Fig. S1). A similar trend was observed based on average amino acid identity (AAI) comparisons with strains KV and NF, exhibiting 90% identity to each other and less than 80% to other isolates (Fig. 2; Fig. S1).

Carbon utilization. Strains KV and NF are maintained in the laboratory under microoxic conditions with Fe(II) as the electron donor and CO_2 as a sole carbon source. Analysis of the genome revealed that both strains, KV and NF, encode key genes involved in the Calvin-Benson-Bassham (CBB) cycle (Table S1). Both genomes contain the gene for form II ribulose 1,5-bisphosphate carboxylase (RuBisCo) large subunit (*cbbM*), closely related to other *Mariprofundus* species *cbbM* genes (Fig. S2); *cbbM* is an essential gene for inorganic carbon fixation and is consistent with our growth observations in the laboratory (37). Form II RuBisCo has a low affinity for CO_2 and is adapted to low-O_2 and high-CO_2 environments (39). Though the genomes contain the gene for form II RuBisCo, they do not contain the gene for form I RuBisCo (*cbbL*). This observation is consistent with findings in other *Zetaproteobacteria*, for example, strains CP-5, CP-8, EKF-M39, TAG-1, and SV-108 (21, 36, 40). This is somewhat surprising, as form I RuBisCo gene is better adapted to higher O_2 concentrations and provides more efficient CO_2 fixation under these conditions (21). It has been hypothesized that the
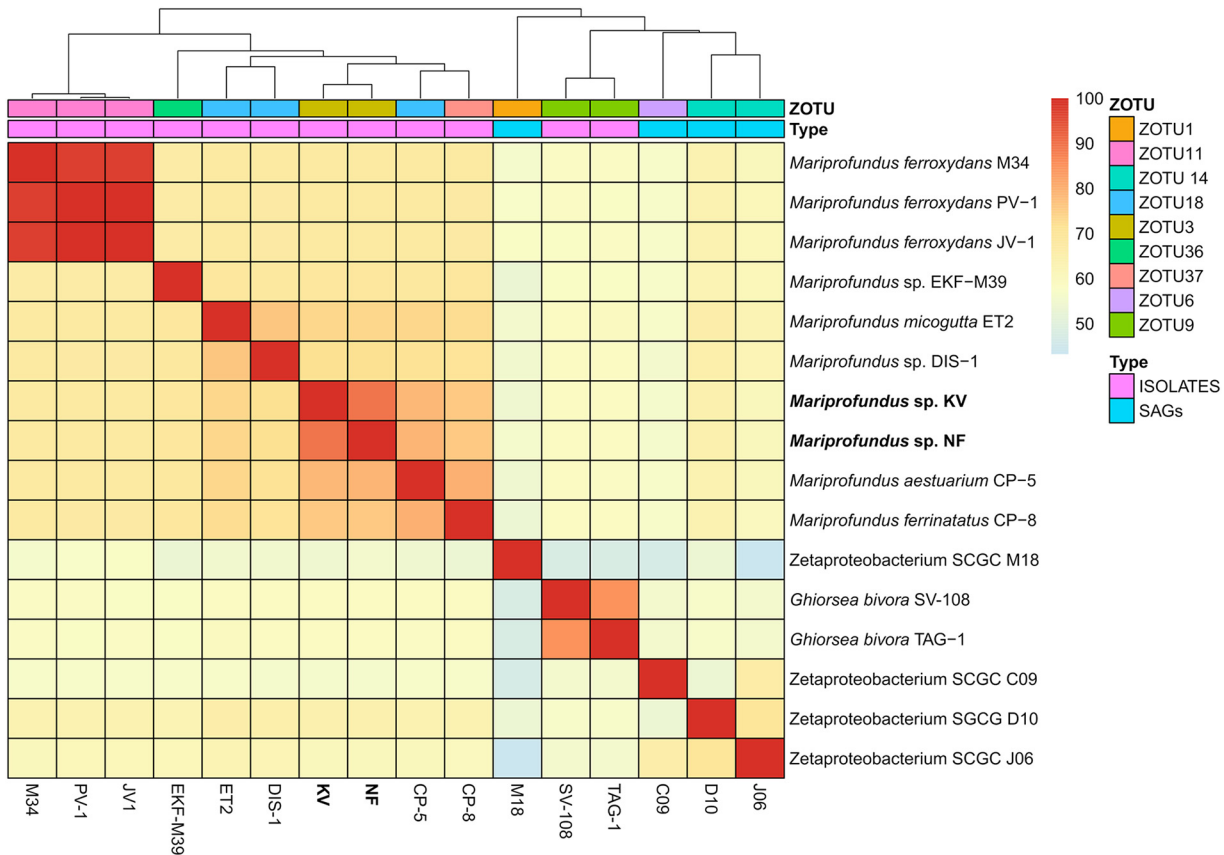
FIG 2 Heatmap and dendrogram based on average AAI between strains KV and NF as well as selected *Zetaproteobacteria* isolates and SAGs.

presence of both the form I and form II RuBisCo genes helps with adaptation, enabling these *Zetaproteobacteria* to maintain a chemolithoautotrophic lifestyle in habitats where fluctuations of O_2 occur, for example, in strain DIS-1, which is capable of tolerating fully oxygenated environments (23). Nevertheless, neither the genome of strain KV nor the genome of strain NF contains the gene for form I RuBisCo, suggesting they have a preference for high-CO_2 and low O_2-conditions, even though seasonal- and tidal-induced fluctuations in the O_2 concentration in the sediments from which they were isolated have been observed (37). It is important to note that while these results reveal the genomic potential of the isolates, further physiological experiments to determine gene function and expression are needed to validate these hypotheses.

Also present in the genomes are gene clusters containing the two RuBisCo gene activation proteins CbbO and CbbQ. Furthermore, the genomes of strains KV and NF each contain two carbonic anhydrase-encoding genes, which are predicted to function by rapidly converting CO_2 to bicarbonate in preparation for cellular uptake (35). The main product from the CBB cycle is glycerate 3-P, and the genomes of strains KV and NF possess the genes necessary to convert this into pyruvate via the encoded Embden-Meyerhof-Parnas pathway (Table S1); pyruvate can be further metabolized in the tricarboxylic acid (TCA) cycle for energy generation. The TCA cycle is present and almost complete in both genomes (see present and missing genes in Table S1).

Electron transport in the outer and inner membranes. The mechanisms for Fe(II) oxidation in neutrophilic Fe(II)-oxidizing bacteria, including the *Zetaproteobacteria*, remain largely unknown. To avoid intracellular mineral encrustation under neutrophilic and microaerophilic conditions, enzymatic Fe(II) oxidation is likely to occur in the outer membrane via extracellular electron transfer (EET) (41). The genomes of many microaerophilic Fe(II)-oxidizing bacteria, originating from both freshwater and marine habitats, contain homologs of an outer membrane cytochrome protein (Cyc2) (42),
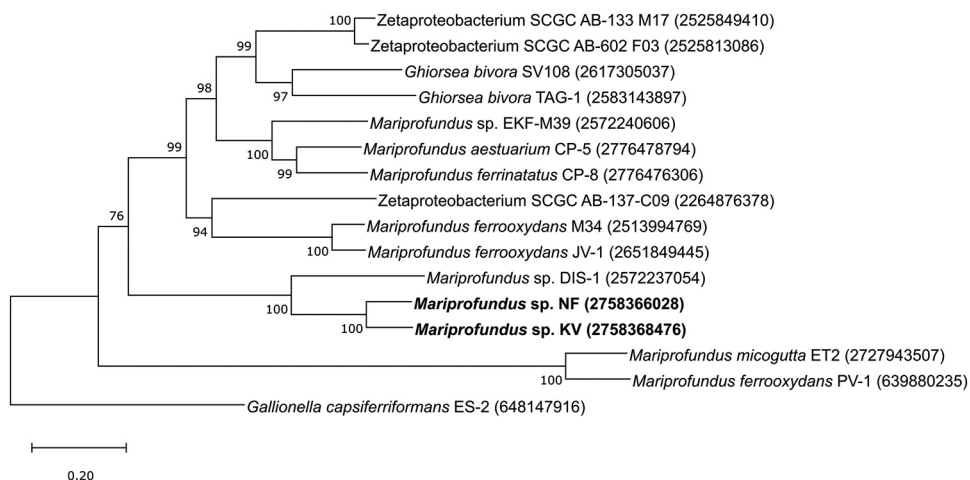
FIG 3 Neighbor-joining phylogenetic tree of the Cyc2 proteins based on amino acid sequences of strains KV and NF, as well as selected *Zetaproteobacteria* isolates and SAGs. The strains investigated in this study, strains KV and NF, are in bold, and the gene IDs from IMG are shown in parentheses. The tree was rooted with the Cyc2 protein of *G. capsiferriformans* strain ES-2, and bootstrap values were calculated based on 1,000 replicates. The scale bar represents the number of base substitutions per site.

which has been shown to oxidize Fe(II) in the acidophilic *Acidithiobacillus ferrooxidans* (43). The genomes of strains KV and NF contain homologs of the putative EET gene *cyc2* found in the *Mariprofundus* type strain PV-1 (44) according to BLAST analysis (Fig. 3; Table S2). Furthermore, the genes also encode a predicted protein sequence for a CXXCH heme-binding motif at the N terminus, a common feature in *cyc2* gene sequences (21). A recent study demonstrated that the *cyc2* gene was highly expressed by diverse *Zetaproteobacteria in situ* in biogenic Fe(III) (oxyhydr)oxide mats at several hydrothermal vents. In addition, in laboratory incubations amended with Fe(II), the *cyc2* gene showed increased expression, substantiating the role of Cyc2 in Fe(II) oxidation in neutrophilic Fe(II) oxidizers (45). No homologs of the $cyc1_{PV-1}$ gene, a periplasmic cytochrome *c* shown to be present in some other *Zetaproteobacteria* and highly expressed in the proteome of strain PV-1 during Fe(II) oxidation (44), were present in the genomes of strains KV and NF. In addition, no homologs of other known genes involved in Fe(II) oxidation were detected using the blastp function in the Joint Genome Institute's Integrated Microbial Genome (IMG) with an E value cutoff of 10^{-5}. These genes included *mtoAB* (46), *pioAB* (47), and the putative EET gene *ompB* (homolog *mofA*), which was found in a Fe(II)-oxidizing *Gallionellaceae* sp. in culture KS (41). This was further supported by the results generated using the FeGenie software (48) to search for protein families involved in Fe(II) oxidation (Cyc1, Cyc2, FoxABC, FoxEYZ, sulfocyanin, PioABC, and MtoAB), which only identified Cyc2 in the genomes of strains KV and NF. More recently, several new putative models for enzymatic Fe(II) oxidation were proposed, which include the outer membrane porin-cytochrome *c* complex (PCC) and the outer membrane multicopper oxidase (MCO; Fig. 4A) (42). Though there were no gene homologs corresponding to the tentatively named PCC3 and PCC4 systems (42) in strain KV or NF, interestingly, genes encoding an MCO belonging to the PcoA and PcoB protein families were identified in the genome of strain KV and contained the expected number of transmembrane regions for this gene (typically 10 to 14) (Fig. 4B and C). No homologs of *pcoA* or *pcoB* were identified in strain NF. Comparison of the genomes showed a strong synteny of surrounding genes between the strains, further supporting the case that *pcoAB* was not present in strain NF (Fig. S3). However, further analysis of genomes of the closest relatives revealed that a homolog of the *pcoB* gene was also present in a *Zetaproteobacteria* SAG (SCGC AB-602-C20) as well as *Ghiorsea bivora* strain TAG-1, though these were more closely related to each other than to *pcoB* in the genome of strain KV (Fig. 4B). The *pcoAB* genes are homologous to the *copAB* genes found in *Pseudomonas syringae* and are involved in copper resistance (49). It has
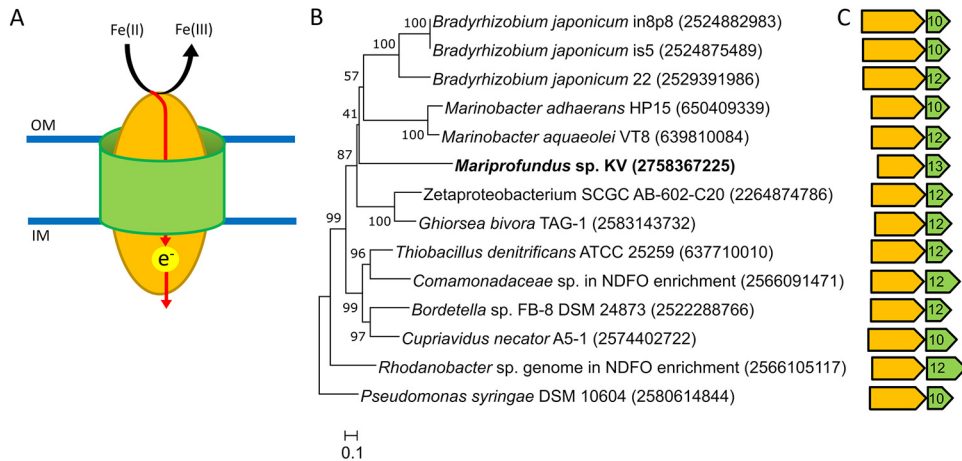
FIG 4 (A) PcoAB multicopper oxidase system (adapted from reference 42) showing the PcoA cytochrome (orange), PcoB porin (green), and the electron flow (red) between the outer membrane (OM) and inner membrane (IM). (B) Neighbor-joining phylogenetic tree of PcoB proteins based on amino acid sequence in strain KV (bold), strain TAG-1, a *Zetaproteobacteria* SAG SCGC AB-602-C20, and freshwater bacteria (IMG gene IDs are shown in parentheses, and the scale bar represents the number of base substitutions per site). (C) PcoAB gene clusters showing only PcoA (orange) and PcoB (green) genes; numbers in the porin-encoding genes show the predicted number of transmembrane regions.

been hypothesized that PcoA may have a broad substrate range and may have a role in Fe(II) oxidation for many Fe(II)-oxidizing *Betaproteobacteria* in which the gene has been identified (42). It was also shown that PcoA in *Pseudomonas aeruginosa* was able to oxidize Fe(II) to Fe(III) as part of Fe acquisition (50). In summary, identification of *cyc2* homologs in the genomes of strains KV and NF, and *pcoAB* in the genome of strain KV, suggest that these are the main mechanisms for outer membrane Fe(II) oxidation. While the presence of *pcoB* in strain NF is certainly an exciting discovery, further experimental work is needed to confirm its hypothesized role in Fe(II) oxidation.

The presence of the alternative respiratory complex III (AC-III) is a common feature in many *Zetaproteobacteria* and other Fe(II)-oxidizing bacteria (36), and it has been hypothesized that they are able to use the AC-III, which is thought to be analogous to the *bc1* complex, as well as a molybdopterin oxidoreductase (encoded by the *actB* gene) to facilitate Fe(II) oxidation (35). Neither the *actB* nor AC-III genes were present in genomes of strains KV and NF. However, both genomes carry genes for the *bc1* complex (also known as complex III), which is a key component of the electron transport chain (Table S2). A novel *bc* complex was identified in the closest relatives of strains KV and NF, *M. ferrinatatus* strain CP-8 and *M. aestuarium* strain CP-5, and suggested to function as a quinone reductase in the electron transport chain (21). However, homologs of the novel *bc* complex were not observed in the genomes of strains KV and NF, suggesting *bc1* is important in these two strains. Recently, an EET transport system was proposed for strain PV-1 which included the outer membrane Cyc2, periplasmic Cyc1, and the inner membrane AC-III (44). However, the lack of homologs of Cyc1 and AC-III in the genomes of strains KV and NF suggests that a different pathway is functional in these two strains. In summary, based on the Fe(II) oxidation gene analysis, it can be hypothesized that strains KV and NF employ the outer membrane Cyc2 (or PcoAB in KV), a currently unidentified periplasmic protein, and the *bc1* complex for EET during Fe(II) oxidation (Fig. 5).

Within the *Zetaproteobacteria*, an operon of ~20 genes was reported to be highly conserved, and this large cluster was originally shown to be conserved in strain PV-1 and *Sideroxydans lithotrophicus* strain ES-1 (51). While the function of these genes remains hypothetical, annotated genes present in certain *Zetaproteobacteria* SAGs suggest that they are involved in electron transport and possibly have a role in Fe(II) oxidation (40). However, no homologs were identified in the genomes of strains KV and NF. It is important to note that this cluster was only identified in *Zetaproteobacteria*
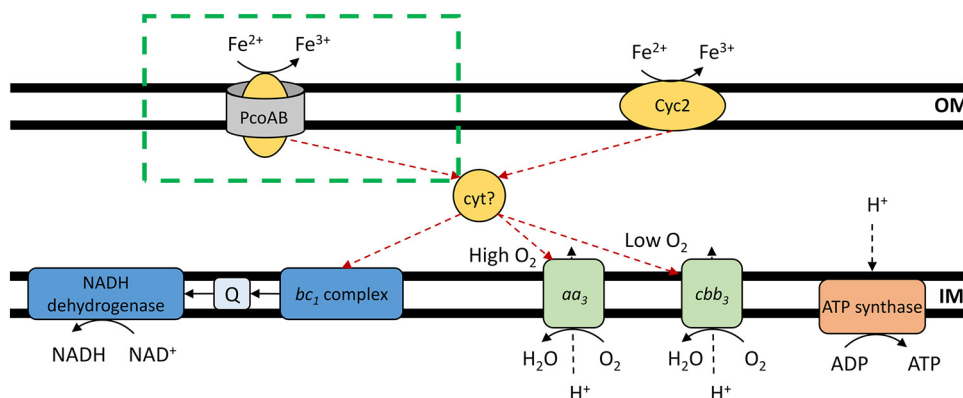
FIG 5 Proposed electron transport chains for the investigated isolates strain KV and strain NF. OM, outer membrane; IM, inner membrane; cbb_3, cytochrome cbb_3 oxidase; aa_3, cytochrome aa_3 oxidase; Q, quinone pool. The electron flow is shown in red. The dashed green box signifies the presence of genes encoding PcoAB found only in strain KV. Cyc2 is likely to be the Fe(II) oxidase in both strains, although strain KV may also oxidize Fe(II) using the PcoAB porin-cytochrome complex. Two terminal oxidases are present in both strains (shown in green) with the high-O_2-affinity cbb_3 probably utilized under low-oxygen conditions and the low-affinity aa_3 probably utilized under higher-oxygen conditions. The pathway for production of reducing equivalents for CO_2 fixation in both strains is shown in blue.

SAGs belonging to specific ZOTU groups (1, 2, and 6) (40), and it is possible that the occurrence of this gene cluster is a genomic trait limited to specific *Zetaproteobacteria* OTUs and may not be present in ZOTU3.

The genomes of strains KV and NF encode the inner membrane cytochrome *c* oxidase cbb_3-type subunit (Table S2). The cbb_3-type cytochromes have the highest affinity to O_2 out of all cytochrome oxidases involved in microaerophilic respiration (35) and may also be used to inhibit O_2 poisoning under microoxic conditions (52, 53). Homologs of cbb_3-type oxidases have been found in almost all *Zetaproteobacteria* genomes, aside from strains CP-5 and CP-8 (21). Therefore, cbb_3-type cytochromes are considered to be adapted for low-O_2 conditions due to their high affinity to O_2 (54). Furthermore, the genomes of strains KV and NF also contain an aa_3-type cytochrome oxidase, which has a lower affinity to O_2 and is therefore suited to higher-oxygen conditions (55). Typically, neutrophilic Fe(II) oxidizers are not expected to be adapted for higher-O_2 conditions, as they are unable to outcompete abiotic Fe(II) oxidation under such high-O_2 conditions (9). However, aa_3-type cytochromes are present in other *Zetaproteobacteria,* such as strain TAG-1 (36), *Mariprofundus micogutta* (56), and several SAGs (40), which generally also contain a cbb_3-type oxidase. As an exception, the most closely related strains to KV and NF, strains CP-5 and CP-8, only contain the aa_3-type oxidase, which is likely due to the higher-oxygen conditions to which these strains are exposed in the water column (21). The occurrence of genes encoding both cbb_3- and aa_3-type oxidases in the genomes of strains KV and NF could potentially indicate that they are able to modify their electron transport chain in response to variable oxygen concentrations. However, the range of oxygen concentrations under which the strains can grow is unknown.

Previous cultivation experiments showed that the strains did not grow under either full atmospheric oxygen conditions or at around 2% O_2 when supplied with organic substrates, including yeast extract and acetate, lactate, and pyruvate (see reference 25 for a full list of organic substrates tested), which suggested that growth is limited to microoxic conditions with Fe(II) as an electron donor. This is supported in part by a previous experiment using strain KV, which was grown in gradient tubes and appeared to have optimal growth at O_2 concentrations of 20 to 40 μM (33); however, the full range of microoxic conditions under which strain KV and especially strain NF are able to grow with Fe(II) as electron donor is unknown. Furthermore, it is not known which cytochrome is expressed by the isolates. Additional experiments determining growth

under different O_2 concentrations in combination with transcriptomics to determine which cytochrome *c* oxidase is expressed may answer this question.

Based on the genomic evidence presented above, an electron transport model for the Fe(II) oxidation pathways in strains KV and NF is proposed (Fig. 5) whereby Fe(II) oxidation and initial electron transfer occurs in the outer membrane using either Cyc2 (KV and NF) or PcoAB (KV) before being transferred to the electron transport chain in the inner membrane.

Oxidative stress. Under high Fe(II) concentrations and fluctuating O_2 conditions, the production of reactive oxygen species such as superoxide, hydrogen peroxide, and hydroxyl radicals can occur. Several stress-related genes have been found in the genomes of strains KV and NF (Table S3). Both genomes encode a Fe-Mn-type super-oxide dismutase, which is common among other *Zetaproteobacteria* isolates except strain TAG-1 (23) and is also present in SAGs from ZOTU10 (40). Two copies of the cytochrome *c* peroxidase were found in the strain KV and strain NF genomes, which is thought to assist in the protection against external sources of hydrogen peroxide (57). This is consistent with other *Zetaproteobacteria* isolates, which typically contain at least two copies of the cytochrome *c* peroxidase gene (23). Interestingly, the genomes of strains KV and NF both encode a catalase-peroxidase enzyme, which is mostly absent from other known *Zetaproteobacteria* isolates. The exceptions to this are strain DIS-1, which is capable of tolerating fully oxygenated waters (23), and strain CP-5, which was found in the oxygen-influenced water column and is likely to be adapted to higher O_2 concentrations based on the occurrence of only an aa_3-type cytochrome (21). Taken together, strains KV and NF possess several potential stress-related genes. As these isolates originate from low-Fe(II) habitats, it is probable that such adaptations are due to the fluctuating O_2 concentrations to which they are expected to be frequently exposed in the environment. The factors leading to dynamic redox conditions include day-night cycles, during which microbial O_2 production rates vary, as well as biotur-bation and wave action, which can all introduce O_2 into the sediment (37).

Additional features within the genomes. Analysis of nitrogen-cycling genes revealed that the genomes of strains KV and NF are missing most of the genes for denitrification and nitrogen fixation. They do, however, carry a nitrilase gene, similar to observations in *Zetaproteobacteria* SAGs (40), which have been shown to produce ammonia from the hydrolysis of organic nitrogen compounds (58). Two copies of ammonium transporters are also present in each genome, suggesting that strains KV and NF may use ammonium as the primary nitrogen source (Table S4). This is consistent with growth observations in the laboratory where ammonium is the only nitrogen source supplied (37). It has recently been discovered that two *Zetaproteobacteria* strains are also able to metabolize H_2 (36); however, based on genomic investigations, this is likely not a possibility for strains KV and NF, which is consistent with previous physi-ology experiments (25). While both genomes carry the assembly protein genes *hypAB-CDEF* (*hypA* gene ID 2758368043 and 2758366628 for strains KV and NF, respectively), they do not have the necessary H_2-uptake NiFe hydrogenase. Furthermore, there is little genomic evidence for any other lithotrophic metabolism employed by these strains, including methanotrophy, sulfur oxidation, or ammonia oxidation, suggesting that Fe(II) oxidation is the primary energy acquisition process for these strains.

Genes related to pyruvate metabolism were present in the genomes of strains KV and NF (Table S1). Recently, it was shown that the genomes of several *Zetaproteobac-teria* isolates contain genes related to anaerobic pathways, including genes encoding pyruvate formate lyase (Pfl), pyruvate ferredoxin oxidoreductase (Pfor), phosphotrans-acetylase (Pta), and acetate kinase (AckA) (59). However, as growth has not been linked to anaerobic metabolism in *Zetaproteobacteria*, the authors proposed that the microbes are able to generate sufficient energy for persistence and/or maintenance using these genes, in a process referred to as "auxiliary anaerobic metabolism," which relies on endogenous glycogen as a carbon source (59). This metabolism could be used as a potential survival strategy to allow the microbes to survive under oxygen-limiting

conditions. In our genomes, genes encoding Pfl, Pfor, Pta, and AckA were detected. However, it is unlikely that Pta and AckA are used for acetate assimilation under oxic conditions, although they might be employed as part of the proposed model for auxiliary anaerobic metabolism (59). This is consistent with observations from previous physiological studies on strains KV and NF that showed no growth on acetate with O_2 as the electron acceptor; in addition, no growth was observed under anoxic conditions with either a lactate and acetate mixture (5 mM each) or yeast extract (2 mg/ml) as the electron donor and nitrate as the electron acceptor. In support of strains KV and NF potentially employing the anaerobic auxiliary metabolism, other genes detailed within the auxiliary anaerobic metabolism model are also present within the genomes of strains KV and NF, including genes encoding NADH-reducing hydrogenase, the Rnf complex, glycogen phosphorylase, and phosphoglucomutase.

In conclusion, analysis of the genomes of two novel *Zetaproteobacteria* isolates, strains KV and NF, has greatly expanded our understanding of neutrophilic and microaerophilic microbial Fe(II) oxidation. These isolates are the only cultivated representatives of ZOTU3 and are unique in the fact that they were isolated from typical coastal marine sediments, containing comparatively low concentrations of dissolved Fe(II) and total Fe. The genomes of strains KV and NF contain homologs of the putative Fe(II) oxidase *cyc2* gene, while the genome of strain KV also includes homologs of the novel MCO system PcoAB, a trait found in only two other *Zetaproteobacteria* so far. The genomes appear to confirm Fe(II) oxidation as the primary energy acquisition process, and both isolates possess the necessary genes to maintain a chemolithoautotrophic lifestyle. Furthermore, evidence for strain adaptations to fluctuating O_2 concentrations, including oxidative-stress-related genes and high- and low-O_2-affinity terminal oxidases, is found in the genomes. These results expand our understanding of *Zetaproteobacteria* in coastal marine sediments and can be further used to gain insights into how prevailing environmental conditions drive genomic diversity.

This study provides the basis for further investigations—specifically, determining the predicted function and physiological impacts of the putative genes involved in Fe(II) oxidation discussed here, which could be achieved by conducting further experiments, for example, by combining growth experiments with transcriptomics and proteomics, and determining predicted gene function using knockout mutants. Furthermore, the hypotheses presented here regarding the selective use of the terminal oxidases cbb_3 and aa_3 during extracellular electron transfer should be tested, for example, by investigating gene expression (e.g., transcriptomics) under fluctuating oxygen conditions.

MATERIALS AND METHODS

Cultivation and DNA extraction. The isolation and preliminary characterization of strains KV and NF are described in detail in Laufer et al. (25). Following isolation, both strains were maintained in petri dishes containing 10 ml of modified artificial seawater medium (ASW; salinity, 23 ppt) and approximately 0.5 g of zero-valent iron (ZVI) (37), here referred to as ZVI plates. In order to grow sufficient biomass for DNA extractions, an aliquot of each stock culture was transferred into multiple ZVI plates (10 ml each), which were incubated in gas-tight acrylic jars (Merck, Germany) containing an oxygen-scrubbing gas pack that produced a microoxic atmosphere with 6 to 10% atmospheric O_2 (BD GasPack EZ Campy; Becton, Dickinson and Co., NJ). After 2 weeks, the aqueous phase from parallel ZVI plates containing cells and Fe(III) (oxyhydr)oxide minerals was collected while the ZVI powder was held at the bottom of the petri dish using a strong magnet. In total, 500 ml was harvested for each strain. Cells and Fe(III) (oxyhydr)oxide minerals were harvested by centrifugation (16,000 × *g* for 6 min), and oxalate solution (28 g ammonium oxalate and 15 g oxalic acid per liter; adjusted to pH 3) was added to the cell/mineral pellet, with subsequent incubation for 10 min to dissolve Fe(III) (oxyhydr)oxide minerals. Following this, cells were centrifuged, the supernatant was removed, and the cell pellet was washed in Tris-EDTA buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA). A number of DNA extraction methods were tested prior to the large-scale harvesting of biomass with the Mo Bio DNA PowerSoil DNA extraction kit (Mo Bio Laboratories Inc., CA, USA), which consistently delivered the best results for DNA yield and quality. Therefore, samples were extracted using this kit according to the manufacturer's instructions in preparation for shotgun sequencing.

Genome sequencing and analysis. Sequencing with the Illumina MiSeq v2 kit was done by IMGM Laboratories GmbH (Munich, Germany) and produced 3,390,779 and 4,056,803 read pairs with 250-bp read lengths for strains KV and NF, respectively. Reads were trimmed and adapters removed using Trimmomatic v0.36 (60). Afterward, more than 97% of both sets of paired-end reads remained. Success

of Trimmomatic was controlled with FastQC v0.11.5 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). PhiX contamination was tested with Bowtie v2.3.3 by aligning trimmed paired-end reads with the PhiX genome (https://support.illumina.com/sequencing/sequencing_software/igenome.html). No more than three read pairs mapped to PhiX for each sample. Around 65% of NF and 55% of KV trimmed paired-end reads were merged with FLASH v1.2.11 (61). Merged and unmerged paired-end reads were assembled with SPAdes v3.11.0 (62) using "– careful" and multiple k-mer sizes (-k 21, 33, 55, 77, 99, and 127). Final assembly was evaluated with QUAST v4.5 (63), which counted 22 (N_{50} = 313,772) and 28 (N_{50} = 186,387) contigs ≥500 bp with around 250× and 290× coverage for genomes of strains NF and KV, respectively. Genome completeness was above 99% and contamination below 1%, estimated by CheckM version 1.0.11 (64) with Prodigal v2.6.2 (65), HMMER v3.1b2 (http://hmmer.org/), and pplacer v1.1.alpha19 (66) based on lineage-specific marker sets (*Proteobacteria*) and the reduced reference genome tree using the current CheckM database (database created 16 January 2015).

The assembled genomes of both strains were uploaded to the Joint Genome Institute's Integrated Microbial Genome and Microbiome Expert Review (IMG/MER) pipeline for annotation, and subsequent analysis was performed using tools available through the pipeline (https://img.jgi.doe.gov/cgi-bin/mer/main.cgi; 67). Putative Fe(II) oxidation genes were identified using the blast function in IMG (E value cutoff, 10^{-5}). In addition, the assembled genomes were analyzed using the FeGenie tool (48) to search for genes related to iron acquisition, storage, and reduction/oxidation.

Phylogenetic analysis and whole-genome comparisons. To determine the phylogeny of strains KV and NF, neighbor-joining phylogenetic trees were produced using MEGA X (68) for the 16S rRNA gene sequences annotated using the IMG/MER pipeline and the translated protein sequences of specific genes of interest (*cyc2*, *cbbM*, and *pcoB*) in comparison to other *Zetaproteobacteria* isolates, single amplified genomes (SAGs), and closely related freshwater Fe(II)-oxidizing bacteria. For these analyses, gene and protein sequences were aligned using ClustalW in MEGA X to produce neighbor-joining trees with 1,000 bootstrap replicates. To calculate the pairwise nucleotide identity of the 16S rRNA gene, the BLAST pairwise alignment was used (69). Whole-genome comparisons between the genomes of strains KV and NF were achieved by calculating the average nucleotide identities (ANIs) and the average amino acid identities (AAIs). ANIs were calculated using IMG/MER, and the AAIs were calculated using an online AAI calculator tool (70).

Data availability. The IMG taxon IDs for genomes of strains KV and NF are 2757320575 and 2757320574, respectively. Raw sequencing data and genome assemblies have been deposited at DDBJ/ENA/GenBank under BioProject accession number PRJNA563526.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.9 MB.

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