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 aestuariun strain CP-5 and Mariprofundus ferrinatus 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 O2 concentrations, indicated by the presence of both cbb3- and aa3-type cytochrome c oxidases, which are adapted to low and high O2 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 O2 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 O2 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 biominal 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 Lo‘ihi 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 O2 exist, thus occupying a niche with low O2 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 O2 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 CO2-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 CO2-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 O2 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-
roxodans 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 *Zetaproteobacteria* Adaptation to Coastal Sediments Applied and Environmental Microbiology September 2020 Volume 86 Issue 17 e01160-20 aem.asm.org

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<tr>
<th>Organism</th>
<th>Genome size (Mbp)</th>
<th>Genome completeness (%)</th>
<th>Genome contamination (%)</th>
<th>No. of contigs</th>
<th>GC (%)</th>
<th>No. of total genes</th>
<th>Protein coding gene count</th>
<th>No. of RNA genes</th>
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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 CO₂ 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 CO₂ and is adapted to low-O₂ and high-CO₂ 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 O₂ concentrations and provides more efficient CO₂ fixation under these conditions (21). It has been hypothesized that the
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 O₂ 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-CO₂ and low O₂-conditions, even though seasonal- and tidal-induced fluctuations in the O₂ 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 CO₂ 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),
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 CXCH 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. 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.

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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.
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 $\mu$M (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 O2 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 O2 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 superoxide 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 O2 concentrations based on the occurrence of only an aa3-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 O2 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 O2 production rates vary, as well as bioturbation and wave action, which can all introduce O2 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 H2 (36); however, based on genomic investigations, this is likely not a possibility for strains KV and NF, which is consistent with previous physiology 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 H2-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 Zetaproteobacteria isolates contain genes related to anaerobic pathways, including genes encoding pyruvate formate lyase (Pfl), pyruvate ferredoxin oxidoreductase (Pfor), phosphotransacetylase (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 Pfi, 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 O₂ 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 O₂ concentrations, including oxidative-stress-related genes and high- and low-O₂-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 cbb₃ and aa₃ 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 Lauffer 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 O₂ (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 IMG/M 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/genome.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 $\geq 500$ bp with around 250$\times$ and 290$\times$ 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 calculator tool (70). 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 and 28 ($N$) of interest (September 2020 Volume 86 Issue 17 e01160-20 aem.asm.org). Whole-genome comparisons between the genomes of strains KV and NF, 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 calculator tool (70). Additional 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/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.

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.
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