Evidence for auxiliary anaerobic metabolism in obligately aerobic *Zetaproteobacteria*

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Abstract

Zetaproteobacteria are obligate chemolithoautotrophs that oxidize Fe(II) as an electron and energy source, and play significant roles in nutrient cycling and primary production in the marine biosphere. *Zetaproteobacteria* thrive under microoxic conditions near oxic–anoxic interfaces, where they catalyze Fe(II) oxidation faster than the abiotic reaction with oxygen. Neutrophilic Fe(II) oxidizing bacteria produce copious amounts of insoluble iron oxyhydroxides as a by-product of their metabolism. Oxygen consumption by aerobic respiration and formation of iron oxyhydroxides at oxic–anoxic interfaces can result in periods of oxygen limitation for bacterial cells. Under laboratory conditions, all *Zetaproteobacteria* isolates have been shown to strictly require oxygen as an electron acceptor for growth, and anaerobic metabolism has not been observed. However, genomic analyses indicate a range of potential anaerobic pathways present in *Zetaproteobacteria*. Heterologous expression of proteins from *Mariprofundus ferrooxydans* PV-1, including pyruvate formate lyase and acetate kinase, further support a capacity for anaerobic metabolism. Here we define auxiliary anaerobic metabolism as a mechanism to provide maintenance energy to cells and suggest that it provides a survival advantage to *Zetaproteobacteria* in environments with fluctuating oxygen availability.

Introduction

Fe(II) is an important electron and energy source in deep ocean biosphere. *Zetaproteobacteria* are the major Fe(II) oxidizing bacteria (FeOB) in marine environments and shown to be widely distributed in deep ocean microbial communities [1–5]. Fe(III) oxyhydroxides produced as a by-product from neutrophilic FeOB may bind phosphate and other metals, thus affecting the biogeochemical cycling of multiple nutrients in addition to iron and carbon [6]. Therefore, understanding the metabolic strategies used by *Zetaproteobacteria* will help us understand key sources and flux of metabolic energy in the marine biosphere.

Zetaproteobacteria are obligate chemolithoautotrophic FeOB, relying on electrons generated from Fe(II) oxidation to generate reducing equivalents and ATP for carbon dioxide fixation. *Mariprofundus ferrooxydans* PV-1, the first cultivated member of the *Zetaproteobacteria*, was isolated from the Loihi seamount near Hawaii [1]. Very little is known about the metabolic strategies used by *Zetaproteobacteria* to survive in the environment, owing to the difficulty of laboratory cultivation and the lack of genetic tools. Cultivated isolates of *Zetaproteobacteria* have been shown to be strictly microaerophilic [1, 7–9]. Coupling oxygen reduction to Fe(II) oxidation is a challenge for FeOB because oxygen rapidly oxidizes Fe(II) abiotically at atmospheric concentrations. At micromolar and submicromolar oxygen concentrations the kinetics of abiotic Fe(II) oxidation by oxygen is slow enough to allow FeOB growth [10], and thus, *Zetaproteobacteria* are thought to thrive at oxic–anoxic interfaces where the oxygen concentrations are low [1, 5, 7, 9, 10].

The depletion of oxygen by biotic and abiotic reactions at the oxic–anoxic interfaces where the oxygen concentrations are already very low can limit the continuous availability of oxygen. Under oxygen limiting conditions, anaerobic metabolism may provide a survival advantage to

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Zetaproteobacteria by providing maintenance energy in an oxygen independent manner. Because growth linked to anaerobic metabolism has not been shown in *Zetaproteobacteria*, we refer to this as “auxiliary anaerobic metabolism” where energy is generated for persistence and/or maintenance rather than growth. We analyzed the genomes of nine different *Zetaproteobacteria* isolates to search for genes known to be associated with anaerobic metabolism in central carbon metabolism, anaerobic respiration, and fermentation. We also validated activity of several genes via heterologous expression by complementing mutants in *Shewanella oneidensis*. Methods used for analyzing zeta-proteobacterial genomes, *S. oneidensis* strain construction, and growth curves are described in the Supplementary information.

Results and discussion

Zetaproteobacteria genomes contain genes for anaerobic metabolism

All *Zetaproteobacteria* genomes analyzed contain multiple genes encoding for proteins known to be involved in anaerobic metabolism in other bacteria (Table 1). Pyruvate formate lyase (Pfl) and pyruvate ferredoxin oxidoreductase (Pfor) decarboxylate pyruvate to acetyl CoA under anoxic conditions to link glycolysis with the Krebs cycle, whereas pyruvate dehydrogenase is known to perform the same function under oxic conditions. The presence of Pfl encoding genes is strongly suggestive of anaerobic metabolism. Pfl from *Escherichia coli* has been reported to be extremely oxygen sensitive where exposure to oxygen leads to cleavage of the enzyme [11]. Pfl has been found to be essential for different anaerobic metabolisms in diverse bacteria [12–14]. Phosphotransacetylase (Pta) and acetate kinase (AckA) convert acetyl CoA to acetate and produce ATP by substrate level phosphorylation. *Zetaproteobacteria* are unable to grow heterotrophically on sugars and therefore are unlikely to use Pta and AckA for acetogenesis under oxic conditions as an overflow metabolism which occurs when sugars are metabolized at high growth rate in *E. coli* [15]. Moreover, the inability of *Zetaproteobacteria* to grow on acetate ([1], Table S2) limits the possible use of Pta and AckA for acetate assimilation under oxic conditions, but is consistent for a role of these proteins in auxiliary anaerobic metabolism. The Rnf complex, an anaerobic ferredoxin-dependent respiratory enzyme reported to be involved in ATP production and nitrogen fixation [16, 17], was also found to be encoded in all *Zetaproteobacteria* genomes analyzed. Under anaerobic conditions, the Rnf complex catalyzes the oxidation of reduced ferredoxin coupled to reduction of NAD⁺ to form NADH and generation of

Table 1 List of *Zetaproteobacteria* isolates analyzed in this study.

Protein homologs for auxiliary anaerobic metabolism in <i>Zetaproteobacteria</i>													
Strain	PflA and PflB	Pfor	Rnf complex	HoxY and HoxH	HycG and HycE	Pta	AckA	LdhA	AdhI	AdhB	NirK	NapA and NapB	OTR
<i>Mariprofundus ferrooxydans</i> PV-1	+	+	+	+	+	+	+	−	+	+	−	−	+
<i>Mariprofundus ferrooxydans</i> JV-1	+	+	+	+	+	+	+	−	+	+	−	−	+
<i>Mariprofundus ferrooxydans</i> M34	+	+	+	+	−	+	+	−	+	+	−	−	+
<i>Mariprofundus micogutta</i> ET2	−	+	+	+	−	+	+	−	+	+	−	−	−
<i>Mariprofundus</i> sp. DIS-1	+	+	+	+	−	+	+	−	+	+	−	+	−
<i>Mariprofundus aestuarium</i> CP-5	+	+	+	+	−	+	+	−	+	+	−	−	−
<i>Mariprofundus ferrinatatus</i> CP-8	+	+	+	+	−	+	+	−	+	+	−	−	−
<i>Mariprofundus</i> sp. EKF-M39	−	+	+	+	+	−	−	+	+	+	+	−	−
<i>Ghiorsea bivora</i> TAG-1	−	−	+	+	−	−	−	−	+	+	−	−	+
List of homologs for proteins associated with anaerobic metabolism present on genomes of different <i>Zetaproteobacteria</i> isolates													
<i>PflA</i> pyruvate formate lyase, <i>PflB</i> pyruvate formate lyase activating protein, <i>Pfor</i> pyruvate ferredoxin oxidoreductase, <i>Rnf</i> complex Rnf complex consisting of six proteins RnfABCDGE, <i>HoxY</i> and dehydrogenase, <i>AdhI</i> and <i>AdhB</i> alcohol dehydrogenase, <i>NirK</i> nitrite reductase, <i>NapA</i> and <i>NapB</i> nitrate reductase, <i>OTR</i> octaheme containing tetraethionate reductase													

List of homologs for proteins associated with anaerobic metabolism present on genomes of different *Zetaproteobacteria* isolates

PflA pyruvate formate lyase, PflB pyruvate formate lyase activating protein, Pfor pyruvate ferredoxin oxidoreductase, Rnf complex Rnf complex consisting of six proteins RnfABCDGE, HoxY and HoxH Type 3 NAD-reducing Ni-Fe hydrogenases, HycG and HycE type 4 hydrogen evolving Ni-Fe hydrogenases, Pta Phosphotransacetylase, AckA acetate kinase, LdhA fermentative lactate dehydrogenase, AdhI and AdhB alcohol dehydrogenase, NirK nitrite reductase, NapA and NapB nitrate reductase, OTR octaheme containing tetrahaem reductase

proton motive force (PMF). Fe(II) dependent oxygen reduction has been postulated as the only mechanism to produce PMF and ATP in *Zetaproteobacteria* [18]. Identification of genes encoding AckA and Rnf complex proteins suggest the presence of additional ATP and PMF generation mechanisms in *Zetaproteobacteria*.

Zetaproteobacteria have not been shown to grow heterotrophically, which limits the possibility of using exogenous organic carbon sources as substrate for anaerobic metabolism. One potential substrate for auxiliary anaerobic metabolism could be endogenous storage compounds. *Zetaproteobacteria* genomes were found to encode genes for synthesis and hydrolysis of glycogen (Table S1), which can serve as carbon and electron source under anoxic conditions. Anaerobic metabolism solely reliant on hydrolysis of endogenous storage compounds coupled to homolactic, heterolactic, and mixed acid fermentations has been described in other lithoautotrophic and photoautotrophic bacteria [19–21]. In situ gene expression studies from environmental iron mat samples showed that genes predicted to be involved in glycogen synthesis in *Zetaproteobacteria* were upregulated within two minutes of Fe(II) addition [22]. A proteomics study of *M. ferrooxydans* PV-1 reported the presence of proteins involved in glycogen hydrolysis ([18], data set S1). These results are consistent with the hypothesis that glycogen can be both produced and utilized by *Zetaproteobacteria*.

Redox homeostasis under anoxic conditions can be maintained either by reduction of electron acceptors other than oxygen or by fermentation. Based on the presence of genes encoding for NirS and NirK type nitrite reductases in *zetaproteobacterial* metagenomes from iron mats at Loihi Seamount, Singer et al. [23] speculated that *Zetaproteobacteria* may be able to reduce nitrite. None of the genomes analyzed in our study were found to encode a NirS homolog; and only the *Mariprofundus* sp. EKF-M39 genome was found to encode a homolog of NirK, indicating the potential for nitrite reduction in this strain. Three of the analyzed *Zetaproteobacteria* genomes were found to contain a gene encoding a putative octaheme tetrathionate reductase (OTR, Table S2). In vitro studies on OTRs from *S. oneidensis* and *Beggiatoa* spp. showed its ability to reduce tetrathionate, nitrite, and hydroxylamine, though the physiological function of this protein remains unclear [24, 25]. The presence of genes encoding putative OTRs therefore raises the possibility of nitrite, tetrathionate, and/or hydroxylamine reduction by *Zetaproteobacteria*. In addition, *Mariprofundus* sp. DIS-1 contains homologs of NapA and NapB, which suggests the possibility of nitrate reduction by this strain.

All the *Zetaproteobacteria* genomes analyzed in this study were found to contain both zinc-containing and iron-containing alcohol dehydrogenases, suggesting the

possibility of fermentative production of alcohol to maintain redox homeostasis. The *M. ferrooxydans* EKF-M39 genome encodes a putative fermentative lactate dehydrogenase, which converts pyruvate to lactate while reoxidizing NADH to NAD⁺. Most of the *Zetaproteobacteria* genomes analyzed were found to encode for group 3b NAD-reducing Ni-Fe hydrogenases [26]. These hydrogenases are known to be reversible, and can play a role in reoxidizing NADH/NADPH. Three of the genomes also encode for group 4 Ni-Fe hydrogen evolving hydrogenases [26], which can act as components of a formate hydrogen lyase complex linking formate oxidation by formate dehydrogenase (FdhH) to hydrogen production. Genes predicted to encode components of FdhH were not found in any of the *Zetaproteobacteria* genomes analyzed.

Model for auxiliary anaerobic metabolism in *M. ferrooxydans* PV-1

M. ferrooxydans PV-1 is the most studied *Zetaproteobacteria* isolate, and therefore we chose it as a model to characterize auxiliary anaerobic metabolism in *Zetaproteobacteria*. We tested the functionality of homologs for PflB, PflA, Pta, and AckA from *M. ferrooxydans* PV-1 (Table 1) using heterologous expression studies. Deletion mutants of *S. oneidensis* were chosen as the hosts for heterologous expression because *S. oneidensis* requires Pfl, Pta, and AckA for growth under anaerobic conditions [14, 27]. Heterologous expression of *pflA* and *pflB* from *M. ferrooxydans* PV-1 was able to complement growth of a *S. oneidensis* mutant lacking *pflB* (Fig. 1a). Expression of both genes was required for growth, suggesting PflB from *M. ferrooxydans* was unable to interact with PflA from *S. oneidensis* (data not shown). Heterologous expression of *pta* and *ackA* from *M. ferrooxydans* PV-1 was also able to complement the anaerobic growth of a *S. oneidensis* mutant strain lacking *pta* and *ackA* (Fig. 1b). Consistent with previous observations, we were unable to detect evidence for heterotrophic growth of *M. ferrooxydans* PV-1 or utilization of other electron acceptors (Table S2), based on direct cell counts as described in Supplementary information.

An overview of auxiliary anaerobic metabolism in *M. ferrooxydans* PV-1 is presented in Fig. 1c. We propose that this metabolism is driven by endogenous glycogen produced during optimal aerobic growth conditions. *M. ferrooxydans* PV-1 is unlikely to utilize extracellular glycogen for either growth or survival. Utilization of extracellular glycogen requires hydrolysis of glycogen to glycans or glucose, which are transported inside the cell by specific transporter proteins for further metabolism. The *M. ferrooxydans* PV-1 genome does not encode for any known transporter proteins for either glycans or glucose. When oxygen is limiting, we propose that intracellular glycogen is

ferredoxin to generate PMF and reduce NAD to NADH. NADH could then be reoxidized by NAD-reducing hydrogenases, while using PMF, to produce hydrogen to maintain the redox homeostasis under anoxic conditions.

Auxiliary anaerobic metabolism may differ in other neutrophilic FeOB. Four genomes of Fe(II) oxidizing *Betaproteobacteria* (*Sideroxydans lithotrophicus* ES-1 [29], *Gallionella capsiferriformans* ES-2 [29], *Ferriphaselus* sp. R-1 [30], and *Ferriphaselus amnicola* OYT1 [31]) were found to have some, but not all of the components described in Fig. 1c and none found to encode a Pta homolog. From this preliminary analysis, Fe(II) oxidizing *Betaproteobacteria* appear to have a more limited capacity for auxiliary anaerobic metabolism, though additional genomes and more work with cultured isolates are warranted.

Although growth of *Zetaproteobacteria* requires Fe(II) oxidation coupled to aerobic respiration, our analysis is consistent with a role for auxiliary anaerobic metabolism in these bacteria. The presence of alternative PMF and ATP generating mechanisms could support survival strategies when Fe(II) oxidation is unavailable for *Zetaproteobacteria*. The mechanisms proposed can help direct future research to understand the ecology of *Zetaproteobacteria* and suggest that anaerobic metabolism can benefit even strict aerobes living near the oxic–anoxic interfaces.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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