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ORIGINAL ARTICLE

Deep-sea hydrothermal vent *Epsilonproteobacteria* encode a conserved and widespread nitrate reduction pathway (Nap)

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Despite the frequent isolation of nitrate-respiring *Epsilonproteobacteria* from deep-sea hydrothermal vents, the genes coding for the nitrate reduction pathway in these organisms have not been investigated in depth. In this study we have shown that the gene cluster coding for the periplasmic nitrate reductase complex (*nap*) is highly conserved in chemolithoautotrophic, nitrate-reducing *Epsilonproteobacteria* from deep-sea hydrothermal vents. Furthermore, we have shown that the *napA* gene is expressed in pure cultures of vent *Epsilonproteobacteria* and it is highly conserved in microbial communities collected from deep-sea vents characterized by different temperature and redox regimes. The diversity of nitrate-reducing *Epsilonproteobacteria* was found to be higher in moderate temperature, diffuse flow vents than in high temperature black smokers or in low temperatures, substrate-associated communities. As NapA has a high affinity for nitrate compared with the membrane-bound enzyme, its occurrence in vent *Epsilonproteobacteria* may represent an adaptation of these organisms to the low nitrate concentrations typically found in vent fluids. Taken together, our findings indicate that nitrate reduction is widespread in vent *Epsilonproteobacteria* and provide insight on alternative energy metabolism in vent microorganisms. The occurrence of the *nap* cluster in vent, commensal and pathogenic *Epsilonproteobacteria* suggests that the ability of these bacteria to respire nitrate is important in habitats as different as the deep-sea vents and the human body.

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Introduction

The contribution of deep-sea vent microorganisms to the sulfur cycle has been extensively investigated (Jannasch, 1995; Karl, 1995; Reysenbach *et al.*, 2002). However, the isolation and characterization of a number of novel bacteria and archaea from deep-sea hydrothermal vents revealed that most of them can use nitrate in addition to sulfur as their terminal electron acceptor. During growth, these microorganisms reduce nitrate to dinitrogen (Takai *et al.*, 2004; Nakagawa *et al.*, 2005; Takai *et al.*, 2006) or ammonium (Blochl *et al.*, 1997; Vetriani *et al.*, 2004; Voordeckers *et al.*, 2008; Perez-Rodriguez *et al.*, 2010, 2012). In most cases, nitrate reduction is coupled to the oxidation of molecular hydrogen (and less frequently of formate or thiosulfate), and these organisms have a shorter doubling time when nitrate, rather than sulfur, is supplemented as the terminal electron acceptor (Vetriani *et al.*, 2004; Voordeckers *et al.*, 2005; Perez-Rodriguez *et al.*, 2010, 2012). In particular, most of the *Epsilonproteobacteria* isolated from deep-sea hydrothermal vents conserve energy by reducing nitrate (reviewed in Campbell *et al.*, 2006). *Epsilonproteobacteria* have been found to be abundant in geographically distant deep-sea vent sites, and colonization experiments that were carried out in the vicinity of active deep-sea vents revealed that between 66 and 98% of the microorganisms associated with these substrates belonged to this group of bacteria (Lopez-Garcia *et al.*, 2003; Takai *et al.*, 2003; Alain *et al.*, 2004). Overall, these observations indicate that *Epsilonproteobacteria* represent a large fraction of the primary producers at deep-sea hydrothermal vents

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at temperatures between 25 and 60 °C, and imply that the use of nitrate as a terminal electron acceptor in these environments may be more common than previously thought.

Data on the nitrogen chemistry of hydrothermal fluids indicate a depletion of both nitrate and nitrite in vent fluids. Nitrate, however, is present in bottom seawater (Millero, 1996) and it is therefore available to be used by vent bacteria both as an electron acceptor and as a nitrogen source. More recently, Bourbonnais *et al.* (2012a, b) found that the rates for nitrate reduction to dinitrogen gas and ammonium in fluids from diffuse flow vents on the Juan de Fuca Ridge were up to ~1000 and 150 nmol N l^{-1} per day, respectively, and that the isotopic composition of nitrate and ammonium suggested a role for vent microorganisms in nitrogen cycling.

Prokaryotes are able to synthesize three distinct types of nitrate reductases. All three enzymes reduce nitrate to nitrite, but they are involved in different physiological processes (reviewed in Lin and Stewart, 1998; Moreno-Vivian *et al.*, 1999; Potter *et al.*, 2001; Richardson *et al.*, 2001). The Nas enzyme is located in the cytoplasm and participates in nitrogen assimilation, whereas the other two nitrate reductases (Nar and Nap) are both linked to respiratory electron transport systems. The Nar enzyme is a three-subunit complex attached to the cytoplasmic side of the membrane. The Nap enzyme is a two-subunit complex located in the periplasmic space of Gram-negative bacteria. All three types of nitrate reductases contain molybdenum bound to the bis-molybdopterin guanine dinucleotide cofactor at the active site. However, although the assimilatory enzyme (Nas) uses NAD(P)H or ferredoxin as reductant, the respiratory Nar and Nap enzymes ultimately take electrons from the membranous quinone pool.

The periplasmic nitrate reductase (NapA, encoded by the *napA* gene) has been described in several different organisms, and its physiological functions may vary in different bacteria (Moreno-Vivian *et al.*, 1999; Potter *et al.*, 2001; Richardson *et al.*, 2001). Although in some bacteria NapA has a dissimilatory function to maintain redox balance during growth on highly reduced carbon sources (for example, in *Rhodobacter* and *Paracoccus* spp.; Richardson *et al.*, 2001), in some enteric and rumen bacteria, such as *Escherichia coli* and *Wolinella succinogenes*, NapA is expressed during anaerobic growth and catalyzes the first step in the respiratory reduction of nitrate to ammonium (Grove *et al.*, 1996; Simon *et al.*, 2003). Within the *Epsilonproteobacteria*, the *napA* gene has been detected in the genomes of the rumen bacterium *W. succinogenes* (Simon *et al.*, 2003); of the pathogens *Helicobacter hepaticus* (Suerbaum *et al.*, 2003), *Campylobacter jejuni* (Parkhill *et al.*, 2000) and several other *Campylobacter* spp. (Kern and Simon, 2009a); and of *Arcobacter butzleri* (Miller *et al.*, 2007), *Sulfurimonas denitrificans* (Sievert *et al.*, 2008) and several *Epsilonproteobacteria* isolated from marine geothermal environments (Inagaki *et al.*, 2003; Voordeckers *et al.*, 2005; Nakagawa *et al.*, 2007; Campbell *et al.*, 2009; Sikorski *et al.*, 2010; Giovannelli *et al.*, 2011). The genome sequence of these organisms showed that *Epsilonproteobacteria* encode the periplasmic nitrate reductase, NapA, and not for the membrane-bound enzyme, NarG (in contrast to, for example, *E. coli*, which encodes both enzymes).

The *napA* gene represents the core entity of the periplasmic nitrate reductase gene cluster. The *napDAB* genes encode the molybdopterin-containing reductase (NapA), the diheme component of the periplasmic electron transfer system (NapB, the redox partner of NapA) and a putative chaperone involved in the maturation of NapA (NapD). In *Epsilonproteobacteria,* the role of individual products of the *nap* gene cluster has been investigated in *W. succinogenes* (Kern *et al.*, 2007). Consistent with the lack of the NapC (Simon *et al.*, 2003), the electron transfer to NapAB in *W. succinogenes* is mediated by the NapGH membrane-bound iron–sulfur periplasmic quinone dehydrogenase complex (Kern and Simon, 2008). NapL is an iron–sulfur periplasmic protein present in all epsilonproteobacterial genomes. Although the function of NapL and NapF is currently unclear, recent data indicate that in *W. succinogenes* the NapGHF complex is involved in menaquinol oxidation, electron transfer to periplasmic NapA and maturation of the cytoplasmic NapA precursor (Kern *et al.*, 2007; Kern and Simon, 2009b).

Although surveys of genes involved in nitrate reduction have been carried out in soils and estuarine environments (Bru *et al.*, 2007; Smith *et al.*, 2007; Dong *et al.*, 2009), the distribution and diversity of the nitrate reductase in natural microbial communities from deep-sea geothermal environments are scarce. Recently, Perez-Rodriguez *et al.* (2013) identified *Gamma*- and *Alphaproteobacteria*-related, membrane-bound nitrate reductases, NarG, in vent microbial communities. However, there is no information on the distribution and diversity of the periplasmic nitrate reductase, NapA, in deep-sea environments. In this study we have reconstructed the structure of the *nap* gene cluster in *Epsilonproteobacteria* isolated from deep-sea hydrothermal vents whose genomes have been sequenced; we have established that reference strains of vent *Epsilonproteobacteria* encode and express the periplasmic nitrate reductase (NapA); and we have reconstructed the phylogeny of this enzyme. Furthermore, we have assessed the diversity, distribution and phylogeny of the NapA enzyme in natural microbial communities from different vent microhabitats. Our findings show that the periplasmic nitrate reductase is conserved and widespread in deep-sea vent microbial communities, and provide insight on alternative energy metabolism in vent microorganisms.

Materials and methods

Reference strains and genome-based reconstruction of the nitrate reduction pathway

The *Epsilonproteobacteria* used as reference strains in this study were cultured according to the conditions described in the original references (Supplementary Table S1 and Supplementary Information). The genomes of reference strains of *Epsilonproteobacteria* (Supplementary Table S2) were used to reconstruct the *nap* gene clusters. Both genes and predicted proteins were searched using BLAST against the public database. The Kyoto Encyclopedia of Genes and Genomes (http://www.genome.jp/kegg/) database, the Gene Orthology systems and BLAST algorithm were used to reconstruct the pathway of nitrate reduction in *Caminibacter mediatlanticus*.

RNA extraction and qualitative detection of napA transcripts

Total RNA was extracted from 20 ml of cultures of *C. mediatlanticus* and *Caminibacter profundus* grown in modified SME medium supplemented with either potassium nitrate or elemental sulfur (Voordeckers *et al.*, 2005), using the UltraClean Microbial RNA Isolation Kit according to the protocol supplied with the kit (MoBio Laboratories, Solana Beach, CA, USA). Following DNase I treatment, total RNA was used as a template to detect *napA* transcripts by reverse transcriptase-PCR, using the SuperScript III One-Step RT-PCR System with primers NapV16F and NapV17R (Supplementary Table S4) according to the manufacturer's specifications (Invitrogen, Inc., Carlsbad, CA, USA). The identity of the transcripts was confirmed by sequencing.

Sample collection

Four types of samples were used in this study: (1) an active, high-temperature black smoker sulfide chimney was collected from the 'Rainbow' vent field on the Mid-Atlantic Ridge (MAR); (2) hydrothermal fluids were collected in titanium samplers from two diffuse flow vents located at *Alvinella* Pillar (AP) and at East Wall (EW), respectively, on the East Pacific Rise (EPR) and were filtered shipboard on 0.2 μm Supor Gelman filters (Ann Arbor, MI, USA); (3) bacterial filaments were collected from an exclusion cage that had been deployed about 1 m above a diffuse flow vent located at Marker 89 on the EPR for 1 year. All samples were collected using the deep-submergence vehicle *Alvin*. Characteristics of samples and sampling locations are summarized in Supplementary Table S3.

DNA extraction

Individual replicate samples of the Rainbow chimney, the EW and AP hydrothermal fluids, and Mk 89 bacterial filaments (Supplementary Table S3) were collected and pooled together. Genomic DNA was extracted from pooled samples of each site and from cultures of each reference strain as previously described (Vetriani *et al.*, 2003; Voordeckers *et al.*, 2005, 2008).

DNA amplification by PCR

A fragment of about 1150 bp of the *napA* gene, coding for the periplasmic nitrate reductase, was amplified from the genomic DNA of reference strains (or was downloaded from GenBank, if available) and from the genomic DNA extracted from the four vent samples (Supplementary Table S3). PCR was carried out using primers NapV16F (5′-GCNCCNTGYMGNTTYTGYGG-3′) and NapV17R (5′-RTGYTGRTTRAANCCCATNGTCCA-3′), which were designed based on the *napA* sequence of *Paracoccus pantotrophus*, *Ralstonia eutropha* and *E. coli* (Flanagan *et al.*, 1999), but which also target the epsiloproteobacterial *napA* gene (Supplementary Table S4). PCR conditions for amplification reactions were as follows: 30 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min and extension at 72 °C for 2 min, with a final extension time of 10 min during the last cycle. In order to reduce PCR bias, at least four independent PCR reactions per sample were carried out in parallel and pooled.

Library construction, restriction fragment length polymorphism screening and sequencing

The amplified *napA* gene fragments were cloned into the pCR4-TOPO plasmid vector and the ligation products were transformed into competent *E. coli* Oneshot cells (Invitrogen, Inc.). Four *napA* environmental libraries were constructed (Supplementary Table S3), and 189 *napA* clones were screened by restriction fragment length polymorphism analysis using the *Mnl*I restriction endonuclease. Representative clones for each library showing unique restriction fragment length polymorphism patterns were selected and their sequences (about 1000 nucleotides) were determined for both strands.

Phylogenetic analyses

Translated amino acid sequences (http://www.ebi.ac.uk/emboss/transeq/) were aligned with ClustalX v 1.8 (Thompson *et al.*, 1997) and manually adjusted using Seaview (Galtier *et al.*, 1996). Phylogenetic distances were calculated using the Observed Divergence matrix and the neighbor-joining method was used to evaluate tree topologies. Phylo_win was used to plot tree topologies (Galtier *et al.*, 1996) and their robustness was tested by bootstrap analysis with 1000 resamplings. 16S rRNA gene-based phylogenetic analyses were carried out as previously described (Perez-Rodriguez *et al.*, 2013).

Cluster analysis of clone libraries
Cluster analysis of the four clone libraries was carried out using the R-software (R-Cran project, http://cran.r-project.org/). Briefly, the NapA sequences obtained from each library were sorted according to their phylogenetic grouping and fed to R for the determination of the distance matrix based on Jaccard dissimilarity. Cluster analysis was done using the average linkage method.

Nucleotide sequence accession numbers
The sequences obtained in this study are available through GenBank under the following accession numbers: EF644686–EF644758, EF644772–EF644780, EF644815–EF644826 and EF683089–EF683090. The accession numbers of the NapA sequences of the reference strains are indicated in Figure 2a.

Results

Reconstruction of the nap gene cluster and of the nitrate reduction pathway in vent Epsilonproteobacteria
We reconstructed the *nap* gene clusters from the genomes of deep-sea vent *Epsilonproteobacteria* and compared them with those of the pathogen *C. jejuni* and the rumen commensal *W. succinogenes* (Figure 1a). The organization of the *nap* gene cluster, *napAGHBFLD*, was conserved in all vent *Epsilonproteobacteria*, whereas *C. jejuni* lacked the *napF* gene (Figure 1a). Furthermore, a number of open reading frames (ORFs) were found in the *nap* gene clusters of *Sulfurimonas autotrophica*, *S. denitrificans*, *Sulfurovum* sp. NBC37, *Nitratiruptor* sp. SB155 and *Nitratiruptur salsuginis* (Figure 1a).

The nitrate reduction pathway in *C. mediatlanticus* was reconstructed from its genome sequence and a model based on the gene functions assigned to *W. succinogenes* (Kern *et al.*, 2007) was proposed (Figure 1b; see Discussion for further information).

Detection and phylogenetic analysis of the NapA periplasmic nitrate reductase in reference strains of vent Epsilonproteobacteria
Phylogenetic analysis of the amino acid sequence deduced from the *napA* genes placed the enzymes of all the *Epsilonproteobacteria* in a group distinct from the gammaproteobacterial NapA (Figure 2a). Three major clusters were identified: one of these clusters comprised sequences from the *Nautiliales*, which includes the genera *Caminibacter* and *Nautilia*, all isolated from deep-sea hydrothermal vents (Figure 2a). Within the *Nautiliales*, the enzymes from *C. mediatlanticus* and *Caminibacter* sp. strain TB-1 (98% sequence identity) formed a subgroup separated from those of *Caminibacter hydrogeniphilus* and *C. profundus* (93% sequence identity; Figure 2a). The sequence identity between the enzymes from the two subgroups ranged between 84 and 87%. The NapA from *Nautilia profundicola* and *Nautilia nitratireducens* formed a subgroup closely related to the enzymes from *C. hydrogeniphilus* and *C. profundus* (87–88% sequence identity), whereas the NapA from *Nautilia* spp. MT3, MT4 and MT5 formed a unique cluster (Figure 2a). In contrast with the NapA phylogeny, the 16S rRNA gene-based phylogenetic analysis placed all the *Nautilia* spp. in a unique group, closely related but separated from the *Caminibacter* spp. (compare Figures 2a and b). Taken together, these observations suggest an event of horizontal gene transfer of the *napA* gene.

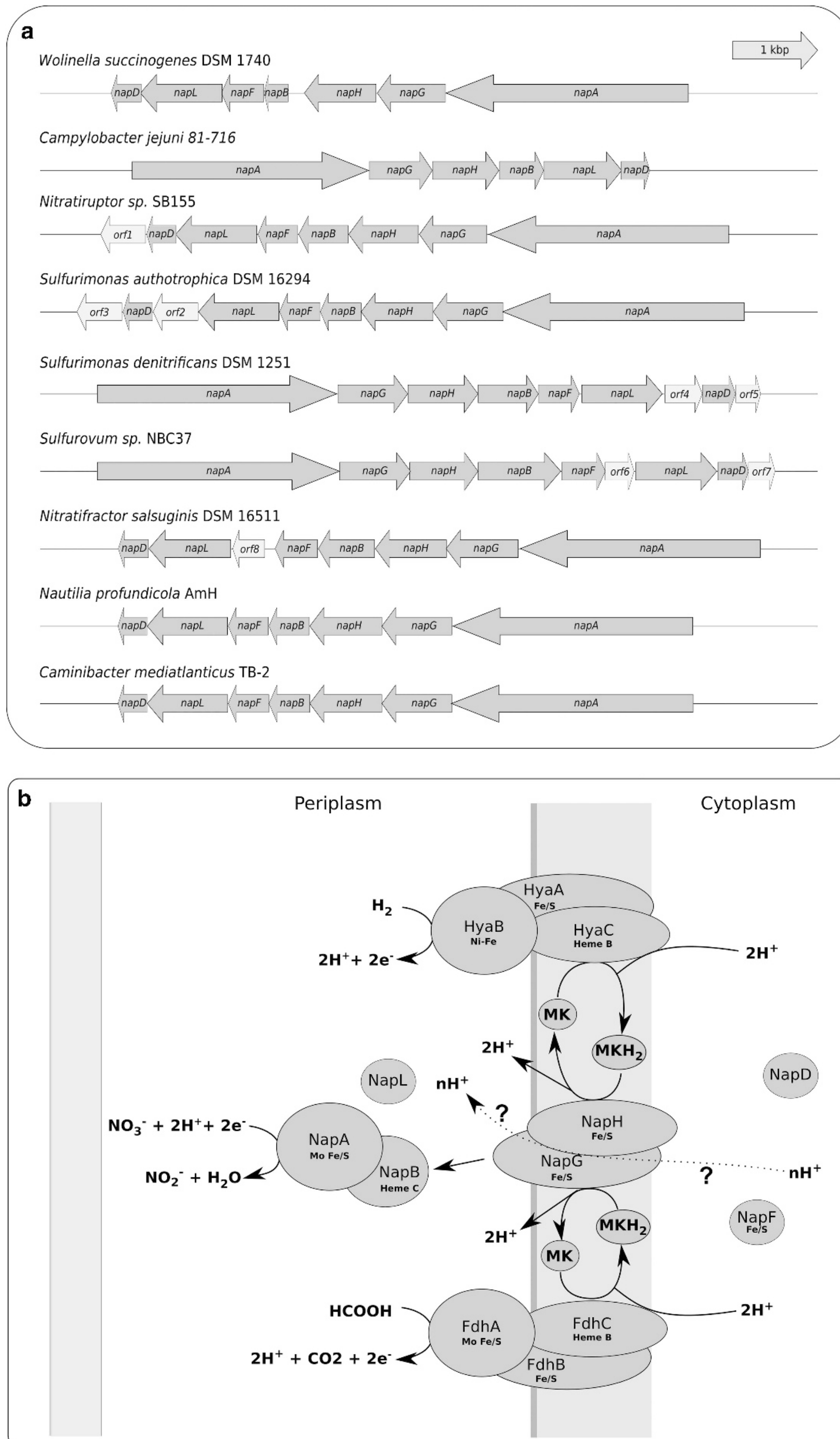
The other two clusters contained NapA sequences from members of the *Campylobacterales* (Figure 2a). One of these two clusters included sequences from pathogenic and commensal *Epsilonproteobacteria* (*Campylobacter* spp., *Helicobacter* spp. and *W. succinogenes*; Figure 2a). The second cluster of NapA from the *Campylobacterales* included sequences from organisms isolated from geothermal and reducing environments (for example, *Sulfurovum* spp., *Sulfurimonas* spp. and *Sulfurospirillum* spp.) and from the pathogen *A. butzleri* (Figure 2a). The NapA from *Nitratifractor salsuginis* clustered with the enzyme of *Sulfurovum* sp. NBC37, whereas the 16S rRNA gene-based phylogeny placed this taxon in a unique lineage (compare Figures 2a and b).

The NapA from *Thioreductor micantisoli* was placed in an independent lineage related to the *Nautiliales* (77% identity to the NapA from *C. mediatlanticus*), whereas the sequence from *Hydrogenimonas thermophila* formed a discrete lineage related to one of the two clusters of *Campylobacterales* (68% identity to the NapA of *Sulfurospirillum barnesii)*. Finally, the NapA from *Nitratiruptor* sp. SB155 formed a unique lineage outside of the three main clusters (Figure 2a).

Detection of napA *transcripts in* Caminibacter *spp.*
Total RNA was extracted from cultures of *C. mediatlanticus* and *C. profundus*, which were grown anaerobically at their optimal growth temperatures (Supplementary Table S1) under a CO_2/H_2 atmosphere (Supplementary Figure S1A, lanes 1 and 2). When the RNA extracted from cultures of the two species grown in the presence of nitrate as their sole terminal electron acceptor was subjected to reverse transcriptase-PCR with *napA*-specific primers, *napA* transcripts were detected in both cases (Supplementary Figure S1B, lanes 3 and 4). When the two cultures were grown in the presence of elemental sulfur as their sole terminal electron acceptor, faint signals for the *napA* transcript were also detected, indicating that the periplasmic nitrate reductase may be constitutively expressed at a basal level (data not shown).

Diversity and phylogenetic analysis of NapA nitrate reductase in vent microbial communities
Genomic DNA was successfully extracted from four samples, representing vent microhabitats

characterized by different temperature and redox regimes: an active, high-temperature sulfide chimney from the Rainbow vent site on the MAR sulfide (fluid emission at the orifice: 158 °C), hydrothermal fluids from two sites on the EPR AP fluid (40 °C) and EPR EW fluid (25 °C; maximum free sulfide



concentration of 175 μM), and bacterial filaments attached to an artificial substrate (EPR white filaments; 2.5 °C; H_2S concentration range: 0.2–32 μM; Supplementary Table S3). All the *napA* gene fragments retrieved from the vent natural microbial communities investigated in this study were phylogenetically related to the *Epsilonproteobacteria*, and a total of eight NapA groups were identified based on phylogenetic clustering (Figures 3 and 4). Out of these eight groups, six included sequences related to reference strains (Figure 3).

The MAR sulfide showed the lowest diversity of NapA sequences (Supplementary Figure S2), 86% of which were related to the enzymes of *Caminibacter* and *Nautilia* spp. (the amino acid sequence identity of these clones to the NapA of *C. mediatlanticus* ranged from 81 to 100%). The remaining 14% of the sequences from this site was related to the enzyme of *N. salsuginis* (83–88% identity to the NapA of *N. salsuginis*; Figures 3 and 4).

The EPR AP fluid showed the highest diversity of NapA sequences, which were grouped into five different clusters (Figures 3 and 4). The majority (76%) of the sequences retrieved from this sample were related to the NapA of *Sulfurovum* sp. NBC37 (84–90% identity), whereas 12% were related to *N. salsuginis* (82% identity) and 3% each to *Nitratiruptur* sp. SB155 (71% identity), and to *Caminibacter* and *Nautilia* spp. (81% and 79% identity to the enzymes of *C. mediatlanticus* and *N. profundicola*, respectively). Phylogenetic analysis placed the remaining 6% of the EPR AP fluid sequences in a unique cluster, designated as Group I, unrelated to the NapA of any cultured *Epsilonproteobacteria* (Figure 3). Within Group I, the amino acid sequence of the NapA fragment designated as

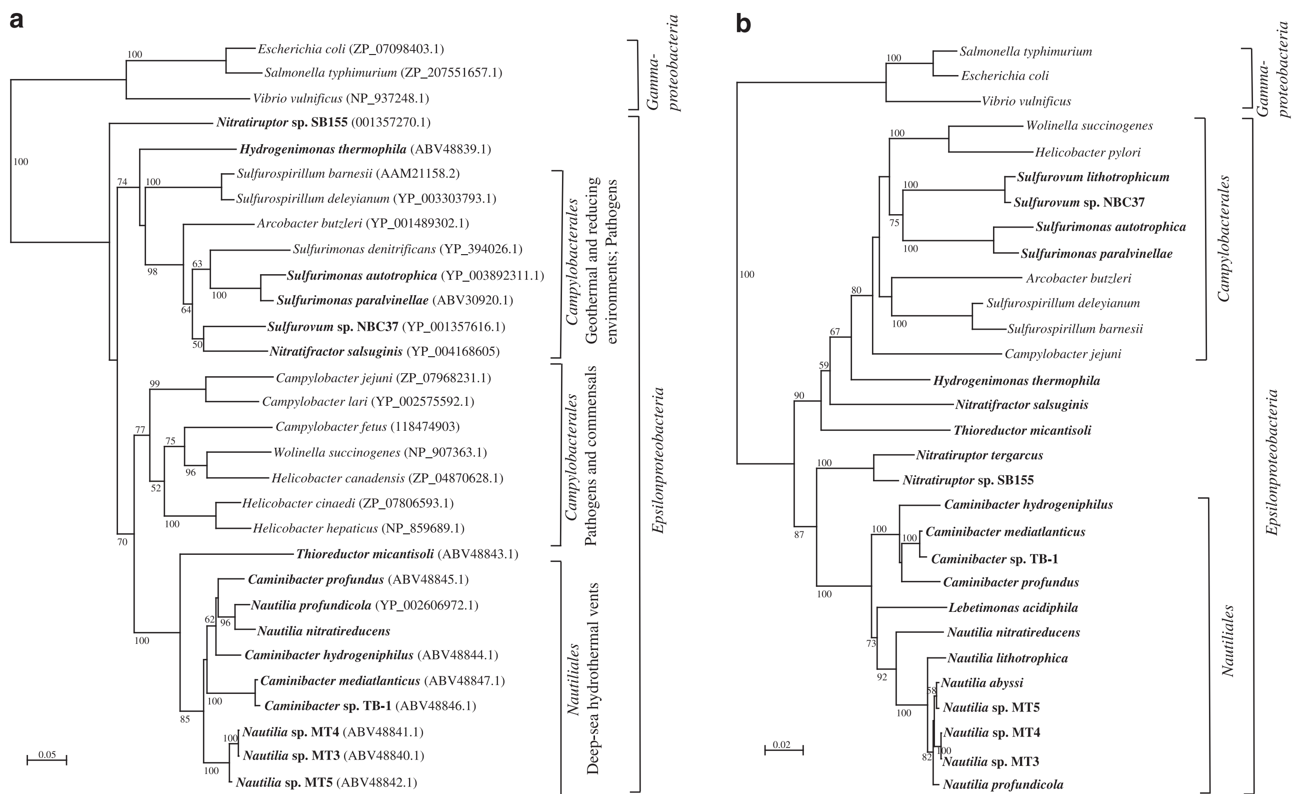


Figure 2 (**a**) Neighbor-joining phylogenetic tree showing the position of the periplasmic nitrate reductase, NapA, of reference strains of *Epsilonproteobacteria*. Bootstrap values based on 1000 replications are shown at branch nodes. Bar, 5% estimated substitutions. (**b**) Neighbor-joining phylogenetic tree inferred from 16S rRNA gene sequences showing the position of reference strains of *Epsilonproteobacteria*. Bootstrap values based on 1000 replications are shown at branch nodes. Bar, 2% substitutions. Sequences from *Epsilonproteobacteria* isolated from deep-sea hydrothermal vents are in bold.

Figure 1 (**a**) Organization of the *nap* gene cluster in different *Epsilonproteobacteria*. Individual genes are drawn to scale. (**b**) Proposed model of the Nap respiratory nitrate reduction pathway in *Caminibacter mediatlanticus*. Question marks denote speculative processes and the dotted line indicates the proposed contribution of the Nap complex to the generation of the proton motive force. NapAB, periplasmic nitrate reductase complex; NapHG, membrane-bound iron–sulfur complex; NapD, putative chaperone; NapL, periplasmic iron–sulfur protein; NapF, putative iron–sulfur adaptor protein; HyaA, nickel–iron-dependent hydrogenase, large subunit; HyaB, nickel–iron-dependent hydrogenanse, small subunit; HyaC, nickel–iron-dependent hydrogenase, heme-b-containing subunit; FdhA, formate dehydrogenase, molibdenum-containing large subunit; FdhB, formate dehydrogenase, small subunit; FdhC, formate dehydrogenase, heme-b-containing subunit; MK, menaquinone.

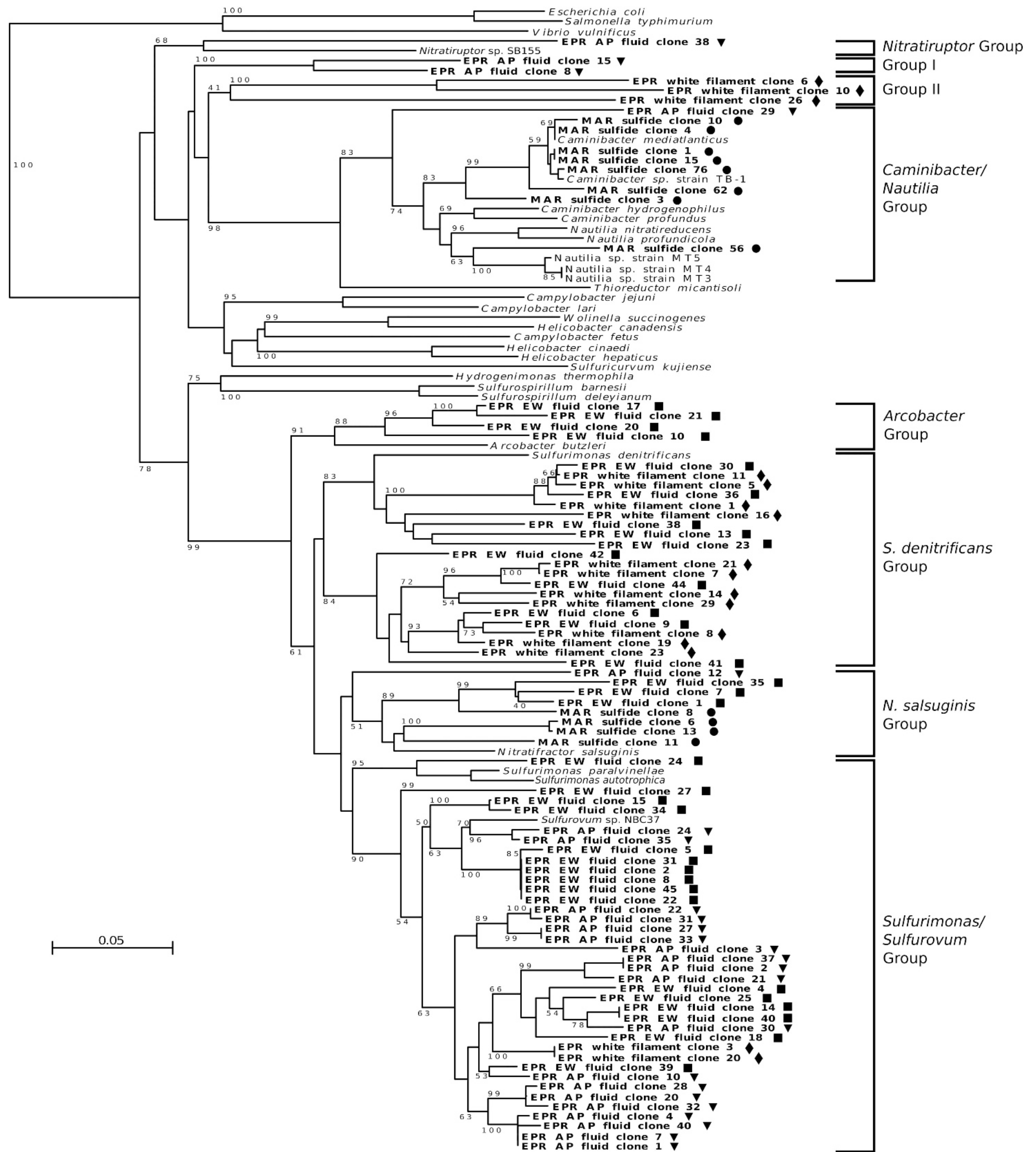


Figure 3 Neighbor-joining phylogenetic tree of the amino acid sequence deduced from a fragment of the gene encoding for the periplasmic nitrate reductase (*napA*) from the Rainbow chimney (MAR sulfide; circles), the East Wall (EPR EW fluid; squares) and *Alvinella* Pillar (EPR AP fluid; triangles) diffuse flow vents, and from substrate-attached bacterial filaments (EPR white filament; diamonds). Representative sequences obtained in this study are in bold. Percentages >50% of 1000 bootstrap resampling that support each topological element are shown at the branch nodes. Bar, 5% estimated substitutions.

EPR AP fluid clone 15 (Figure 3) was 69%, 70%, 71% and 72% identical to the enzymes of *C. mediatlanticus*, *N. profundicola*, *W. succinogenes* and *Campylobacter lari*, respectively.

The NapA sequences retrieved from the EPR EW fluid were grouped into four clusters (Figures 3 and 4). Forty-four percent of the sequences from this sample were related to *Sulfurovum* and *Sulfurimonas* spp.,

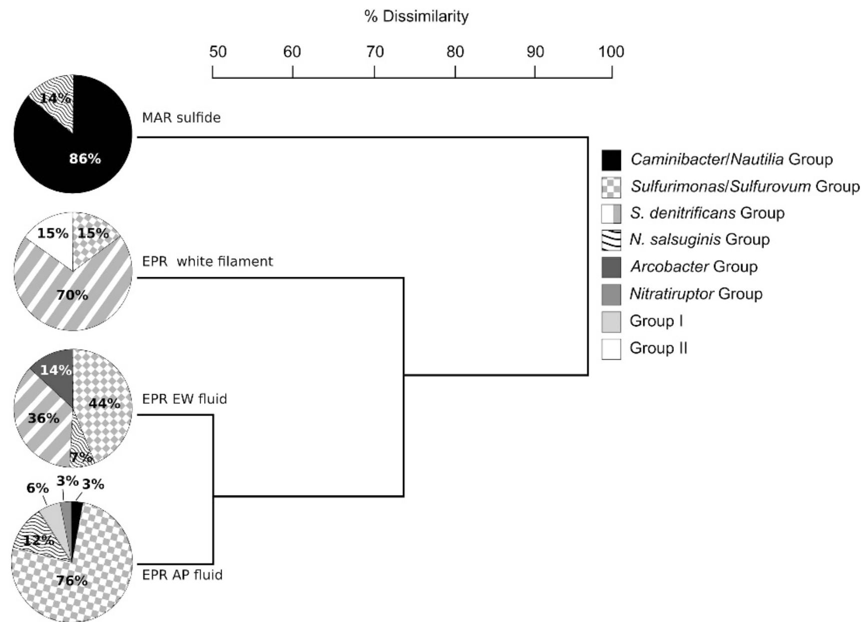


Figure 4 Cluster analysis of the four vent microbial communities based on the phylogenetic affiliation of the NapA sequences. The frequency of the eight groups of sequences detected is shown (see text for details).

with 85–93% identity to the enzyme of *Sulfurovum* sp. NBC37 and 78–85% identity to the enzyme of *S. autotrophica* (Figure 3). The amino acid sequence of the NapA fragment designated as EPR EW fluid clone 24 was the most closely related to enzymes of *Sulfurimonas* spp. (87% and 85% identity to the enzymes of *Sulfurimonas paralvinellae* and *S. autotrophica*, respectively). The second major group (36%) of NapA sequences retrieved from the EPR EW fluid sample was related to *S. denitrificans* (~ 80% identity). The remaining sequences from the EPR EW fluid clustered with *A. butzleri* (14% of the sequences; 80% identity) and with *N. salsuginis* (7% of the sequences; 83% identity).

The NapA sequences from the EPR white filament community were distributed into three groups: 70% of the sequences were related to *S. denitrificans* (80–81% identity), whereas 15% clustered with *Sulfurovum* and *Sulfurimonas* spp. (90% and 82% identity to the enzyme of *Sulfurovum* sp. NBC37 and *S. autotrophica*, respectively; Figures 3 and 4). The remaining 15% of the sequences from the EPR white filament community formed a unique cluster, designated as Group II, unrelated to the NapA of any cultured *Epsilonproteobacteria* (Figure 3). The amino acid sequence of EPR white filament clone 6, a NapA clone representative of Group II, was 66% or less identical to the enzymes of *C. mediatlanticus*, *C. hydrogeniphilus*, *Nautila* sp. MT3, *W. succinogenes*, *Helicobacter cinaedi* and *Helicobacter canadensis*.

Cluster analysis of the periplasmic nitrate reductase-encoding microbial communities indicated that EPR AP and EPR EW fluids were the most similar (51.1% Jaccard dissimilarity), whereas the MAR sulfide community shared the least number of *napA* genes with the other three communities (100%, 97.4% and 95.2% dissimilarity with the EPR white filaments, EPR EW fluid and EPR AP fluid, respectively; Figure 4). The EPR white filament community was more similar to the EPR EW fluid (56.4% dissimilarity) than to the EPR AP fluid communities (93.4% dissimilarity; Figure 4). This is consistent with the finding that *Caminibacter* and *Nautilia*-related periplasmic nitrate reductases were detected only in the MAR sulfide and in the EPR AP fluid libraries, whereas *Sulfurovum*-related enzymes were detected in the other three communities. *S. denitrificans*-related sequences were detected in both the EPR EW fluid and EPR white filament communities (Figure 4).

Discussion

The nitrate reduction pathway of vent Epsilonproteobacteria

Comparative analysis of the *nap* gene clusters showed that the *napAGHBFLD* structure is conserved in all vent *Epsilonproteobacteria* (Figure 1a). However, some differences were found. The *nap* gene clusters of *Nitratiruptor* sp. SB155, *Sulfurovum* sp. NBC31, *N. salsuginis*, *S. denitrificans* and *S. autotrophica* contained some predicted ORFs, which were not present in the gene clusters of the strict anaerobes, *C. mediatlanticus* and *N. profundicola* (Figure 1a). These ORFs are hypothetical proteins with no predicted conserved domain, except for *orf1* (locus tag NIS_1801) in the operon of *Nitratiruptor* sp. SB155, *orf2* (Saut_1245) in *S. autotrophica* and *orf4* (Suden_1520) in *S. denitrificans*, which have a putative active site similar to the histidine kinase

signal transduction sensor for oxygen. The identities of the deduced amino acid sequences of these ORFs are 66% between *orf2* and *orf4*, 61% between *orf1* and *orf2*, and 63% between *orf1* and *orf4*, whereas all the other ORFs are unrelated. Although *Nitratiruptor* sp. SB155 and *S. denitrificans* are facultative microaerophiles, *S. autotrophica* is an obligate aerobe. Hence, in these bacteria the putative histidine kinase might mediate signal transduction in response to oxygen sensing.

It is worth noting that most of the mesophilic *Epsilonproteobacteria* isolated from deep-sea hydrothermal vents reduce nitrate to dinitrogen gas, whereas their thermophilic relatives produce ammonium as the end product of nitrate respiration (Supplementary Table S1 and references therein). This is consistent with data showing that ammonification is more prevalent than denitrification in nitrate-reducing thermophiles (Blochl *et al*., 1997; Vetriani *et al*., 2004; Perez-Rodriguez *et al*., 2012), and may be related to a possible decrease in nitrate concentrations as the hydrothermal fluid temperature increases. In line with this hypothesis, an increase in the significance of nitrate ammonification relative to denitrification as the concentration of nitrate decreased was observed in estuarine sediments (Dong *et al*., 2009). Phylogenetic analyses of the denitrification or ammonification pathways in these bacteria should provide further clues on their respective evolutionary histories.

The proposed model for the nitrate reduction pathway in *C. mediatlanticus* shows the NapAB reductase complex, containing the molybdenum-containing active site, which receives electrons from the membrane-bound NapGH heterodimer and carries out the reduction of nitrate to nitrite (Figure 1b). NapGH is a transmembrane Fe/S cluster that functions as a quinone oxidoreductase and mediates the transfer of electrons from the membrane quinone pool. In *C. mediatlanticus* and other thermophilic *Epsilonproteobacteria*, the reduction of nitrate is generally coupled with oxidation of hydrogen gas. The enzyme complex responsible for the oxidation of hydrogen (Hya complex, also known as Hyn, Hyd, Hup or Hox) includes three subunits that couple hydrogen oxidation with the transfer of electrons to the quinone pool and the generation of the proton gradient. In the proposed model, HyaAB (locus tags CMTB2_07241, CMTB2_07236) is responsible for the oxidation of hydrogen mediated by the Ni-Fe active site, whereas HyaC (CMTB2_07251) is a heme-b-containing protein responsible for the electron transfer to the quinone pool (Figure 1b). The Nap cluster in the genome of *C. mediatlanticus* is functionally linked to the formate dehydrogenase complex (genes *fdh*ABC; CMTB2_03503, CMTB2_03508 and CMTB2_03513, respectively), which may provide reducing equivalents through a menaquinol–menaquinone transport chain (Figure 1b). However, at this point the role of the formate dehydrogenase in *C. mediatlanticus* is unclear, as this bacterium does not seem to use formate as an energy source (Voordeckers *et al*., 2005). Interestingly, the close relatives *N. nitratireducens*, *Nautilia lithotrophica* and *N. profundicola* use formate as an alternative energy source to hydrogen (Miroshnichenko *et al*., 2002; Smith *et al*., 2008; Perez-Rodriguez *et al*., 2010), and the *fdh*ABC gene clusters of *C. mediatlanticus* and *N. profundicola* are highly conserved (86%, 83% and 65% amino acid identity for FdhA, B and C, respectively).

Community structure of nitrate-reducing Epsilonproteobacteria *in deep-sea hydrothermal vents*

The community structure of nitrate-reducing microorganisms was investigated by surveying the distribution of the periplasmic nitrate reductase in four vent microhabitats representing different temperature and redox regimes. Three independent lines of evidence gathered in this study strongly suggest that at deep-sea hydrothermal vents, chemolithoautotrophic *Epsilonproteobacteria* mediate NapA-catalyzed respiratory nitrate reduction to either dinitrogen gas or to ammonium: (1) nitrate-reducing *Epsilonproteobacteria* encode the periplasmic nitrate reductase, NapA (Figure 1a), and not for the membrane-bound enzyme, NarG; (2) the *napA* gene is expressed in vent *Epsilonproteobacteria* during growth with nitrate as the sole electron acceptor (Supplementary Figures S1 and S3); and (3) all the *napA* genes retrieved from the vent samples were related to epsilonproteobacterial enzymes (Figure 3). Furthermore, epsilonproteobacterial *nap* gene transcripts were recently detected in a chimney-associated microbial biofilm (Dahle *et al*., 2013). As *Epsilonproteobacteria* are widespread and, in some cases, abundant members in deep-sea hydrothermal vent microbial communities, nitrate reduction in these environments appears to be more relevant than previously recognized.

The observation that the NapA recovered from the MAR sulfide community were predominantly related to enzymes from *Caminibacter* and *Nautila* spp. (Figures 3 and 4) is in line with data obtained from 16S rRNA gene and ATP citrate lyase (an enzyme involved in CO_2 fixation via the reductive tricarboxylic acid cycle) libraries constructed from the same chimney sample, which revealed a prevalence of *Epsilonproteobacteria* of the genus *Caminibacter* associated with this vent (Voordeckers *et al*., 2008). The isolation of *C. mediatlanticus*, *Caminibacter* sp. strain TB-1 (Voordeckers *et al*., 2005) and *C. profundus* (Miroshnichenko *et al*., 2004) from the Rainbow hydrothermal vent further confirmed the occurrence of these organisms at this site.

Analyses of the periplasmic nitrate reductase from the EPR EW and AP fluids suggest that the moderate temperatures (25–40 °C) and mildly reducing conditions (due to the advection of oxygenated seawater) usually associated with diffuse flow vents are conducive to the establishment of a diverse

community of *Epsilonproteobacteria*. In general, a correlation exists between temperature and redox state of hydrothermal fluids in that higher-temperature fluids tend to be more reduced than lower-temperature ones (Luther *et al.*, 2001; Le Bris *et al.*, 2006). To some extent, this may explain the presence of clones related to the mesophilic, microaerobic, thiosulfate-oxidizing *Sulfurovum* and *Sulfurimonas* spp. in the EPR AP and EW fluid, and in the substrate-associated white filament communities (Figures 3 and 4). The distribution of *napA* genes related to mesophilic and thermophilic *Epsilonproteobacteria* appears to reflect the adaptation of these organisms to different temperature and redox regimes. For instance, the *Caminibacter*/*Nautilia* group dominated the high-temperature MAR sulfide (86%), which is in line with the anaerobic and thermophilic metabolism of these organisms (Figure 4).

Implications of napA expression in nitrate depleted environments

Although several microorganisms, including *E. coli*, encode for both the periplasmic and membrane-bound nitrate reductase, it is not clear why *Epsilonproteobacteria* encode the periplasmic type, NapA. In *E. coli*, the expression of the *nap* genes is induced at low concentrations of nitrate (μM range), whereas the cytoplasmic nitrate reductase (Nar) is expressed at nitrate concentrations in the mM range (Cole, 1996; Potter *et al.*, 1999; Wang *et al.*, 1999). Competition experiments, where mutant strains of *E. coli* lacking either Nar or Nap were carried out using continuous-culture techniques that maintained the concentration of nitrate in the μM range, showed that the Nap^{+} strain outcompeted the strain expressing only the Nar enzyme during nitrate-limited growth (Potter *et al.*, 1999). The saturation constants (K_s) for nitrate for the two strains were estimated to be 15 and 50 μM for Nap and Nar, respectively (Potter *et al.*, 1999). These observations, also supported by a study where NapA-mediated nitrate reduction in estuarine sediments remained important at low nitrate concentrations (Dong *et al.*, 2009), imply that between the two enzymes NapA is better adapted to function in nitrate-depleted environments. *Epsilonproteobacteria* include very different organisms, such as deep-sea vent chemolithoautotrophs (for example, *Caminibacter* and *Sulfurimonas* spp.) and human pathogens (for example, *Campylobacter* and *Helicobacter* spp.). However, the Nap nitrate reductase system appears to be conserved within these different bacteria. Nitrate is depleted in hydrothermal fluids but it is present at μM concentrations in deep seawater (Millero, 1996), and vent microorganisms are exposed to it along redox gradients that form at the interface between fluids and seawater. As nitrate concentrations in the μM range are common to both deep seawater and fluids in the human body (Lentner, 1984; Millero, 1996), we hypothesize that the nitrate-deficient nature of these environments is a factor selecting for the high nitrate affinity NapA enzyme in both pathogenic and vent *Epsilonproteobacteria*. Future comparative analyses of the genes and enzymes comprising the *nap* cluster in pathogenic and vent *Epsilonproteobacteria* may provide insights into the evolution of the periplasmic nitrate reductase complex and nitrate scavenging strategies.

Conflict of Interest

The authors declare no conflict of interest.

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