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Genome of the Epsilonproteobacterial Chemolithoautotroph *Sulfurimonas denitrificans*▽†

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Sulfur-oxidizing epsilonproteobacteria are common in a variety of sulfidogenic environments. These autotrophic and mixotrophic sulfur-oxidizing bacteria are believed to contribute substantially to the oxidative portion of the global sulfur cycle. In order to better understand the ecology and roles of sulfur-oxidizing epsilonproteobacteria, in particular those of the widespread genus *Sulfurimonas*, in biogeochemical cycles, the genome of *Sulfurimonas denitrificans* DSM1251 was sequenced. This genome has many features, including a larger size (2.2 Mbp), that suggest a greater degree of metabolic versatility or responsiveness to the environment than seen for most of the other sequenced epsilonproteobacteria. A branched electron transport chain is apparent, with genes encoding complexes for the oxidation of hydrogen, reduced sulfur compounds, and formate and the reduction of nitrate and oxygen. Genes are present for a complete, autotrophic reductive citric acid cycle. Many genes are present that could facilitate growth in the spatially and temporally heterogeneous sediment habitat from where *Sulfurimonas denitrificans* was originally isolated. Many resistance-nodulation-development family transporter genes (10 total) are present; of these, several are predicted to encode heavy metal efflux transporters. An elaborate arsenal of sensory and regulatory protein-encoding genes is in place, as are genes necessary to prevent and respond to oxidative stress.

Only recently have epsilonproteobacteria been recognized as an environmentally relevant group of bacteria, as 16S rRNA-based sequencing surveys have identified them in a vast array of habitats, including brackish, marine, and subsurface (see, e.g., references 3, 11, 25, 39, 45, 71, and 74; reviewed in reference 6). Over the last years, quite a few cultured representatives of this group have been obtained from these environments, and currently most cultured members of the free-living epsilonproteobacteria are chemolithoautotrophs or mixotrophs, capable of either oxidizing reduced sulfur compounds and hydrogen with oxygen and/or nitrate or oxidizing hydrogen with elemental sulfur coupled to the fixation of inorganic carbon (reviewed in reference 6). These organisms use the reductive citric acid cycle for carbon fixation (24, 67). Given their abundance, sulfur-oxidizing epsilonproteobacteria, in particular members of the genus *Sulfurimonas*, are believed to be relevant to the function of the global sulfur cycle (6).

Genome data from these organisms would be key to metagenomic sequencing efforts in habitats where they are abundant and would also, by comparison to other epsilonproteobacteria, be helpful for determining the traits unique to a free-living, autotrophic lifestyle versus a host-associated, heterotrophic lifestyle. Recently, the genome sequences of *Sulfurovum* sp. strain NBC37-1 and *Nitratiruptor* sp. strain SB155-2, two sulfur-oxidizing epsilonproteobacteria from deep-sea hydrothermal vents, were published; these sequences revealed that these organisms share many features with their pathogenic (e.g., *Campylobacter* and *Helicobacter* spp.) epsilonproteobacterial relatives (41). Given the remarkable variety of habitats where sulfur-oxidizing epsilonproteobacteria are found, it was of great interest to also conduct these analyses on nonvent epsilonproteobacteria. To represent the abundant sulfur-oxidizing epsilonproteobacteria present in coastal marine sediments, we chose to sequence and analyze the genome of the sulfur-oxidizing chemolithoautotroph *Sulfurimo-*

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TABLE 1. Comparative genome features of epsilonproteobacteria[a]

Species	Size (Mbp)	% Coding	% GC	rRNA operons	No. of CDS
Sulfurimonas denitrificans DSM1251	2.20	93.8	34.5	4	2,104
Sulfurovum sp. strain NBC37-1	2.56	90.1	43.8	3	2,466
Nitratiruptor sp. strain SB155-2	1.88	95.1	39.7	3	1,857
Campylobacter fetus 82-40	1.80	90.0	33.3	3	1,719
Campylobacter jejuni NCTC 11168	1.64	95.4	30.6	3	1,629
Campylobacter jejuni RM1221	1.78	91.8	30.3	3	1,838
Helicobacter hepaticus ATCC 51449	1.80	93.4	35.9	1	1,875
Helicobacter acinonychis Sheeba	1.55	89.0	38.2	2	1,618
Helicobacter pylori[b] 26695	1.67	90.0	38.9	2	1,576
Helicobacter pylori J99	1.64	90.7	39.2	2	1,491
Helicobacter pylori HPAG1	1.59	91.0	39.1	2	1,544
Wolinella succinogenes DSM1740	2.11	94.5	48.5	3	2,043

[a] Data for all taxa, except for *Sulfurivom* sp. strain NBC37-1 and *Nitratiruptor* sp. strain SB155-2, were collated from the Integrated Microbial Genomes webpage and had been generated using consistent methodology. For *Sulfurivom* sp. strain NBC37-1 and *Nitratiruptor* sp. strain SB155-2, data were collected from reference 41, for which slightly different methodologies were used to identify coding sequences (CDS).

[b] For *H. pylori*, the 16S gene is not collocated with the 23S and 5S genes in an operon. Additionally, an orphan 5S sequence is found in strain 26695.

nas denitrificans DSM1251. Based on its phenotype, *S. denitrificans* was originally named *Thiomicrospira denitrificans* (70). Subsequent sequencing revealed the polyphyletic nature of *Thiomicrospira*, with members from both the gammaproteobacteria and epsilonproteobacteria (40). As a result, *Thiomicrospira denitrificans* was eventually removed from the genus *Thiomicrospira* and placed within the genus *Sulfurimonas* (68). In addition to marine sediments, bacteria belonging to this genus have been isolated or detected in a variety of sulfidogenic environments, including deep-sea hydrothermal vents, the oxic-anoxic interface of marine anoxic basins, and oil fields (6, 18, 25, 32), making organisms of this genus globally significant.

MATERIALS AND METHODS

Genome sequencing, annotation, and analysis. DNA libraries were created and sequenced to an approximately 13× depth of coverage at the Production Genomics Facility of the Joint Genome Institute (JGI) using the whole-genome shotgun method as previously described (7, 57). Gaps were closed and base quality problems were addressed by sequencing finishing reads, and PHRED/PHRAP/CONSED were used for assembly (12, 13, 20). Automated and manual annotations were conducted by ORNL in a manner similar to that described previously (7, 57). Results were collated and presented via GenDB (37) for manual verification. The prediction of membrane transporters was based on a transporter annotation pipeline that uses several predictive approaches such as BLAST, COG, PFAM, and TIGRFAM HMM searches, transmembrane topology prediction algorithms, and takes advantage of a curated database of transporters. Details of this pipeline and database have been published in references 50, 51, and 57. The main limitation of this approach is the ability to accurately predict precise transporter specificities. Based on both internal and external testing, this methodology is highly successful at identifying putative transporters and predicting approximate substrate specificity. However, making precise substrate predictions, e.g., serine transport rather than transport of another amino acid, is more problematic. The other related limitation is that the approach is dependent on comparison with known experimentally characterized transporters, and so completely novel transporters, which have never had homologs experimentally characterized, will not be predicted by this methodology.

To uncover genes involved in oxidative stress, the *S. denitrificans* genome was examined with a series of BLAST queries, using genes known to be involved in oxidative stress response in *Helicobacter pylori* (73).

Identification of genes encoding signal transduction and regulatory proteins. The complements of genes that encode signal transduction and regulatory proteins were compared among *S. denitrificans* DSM1251, *Thiomicrospira crunogena* XCL-2, and *Nitrosococcus oceani* ATCC 19707. To compare signal transduction and regulatory protein genes among these obligate autotroph genomes, genes were identified by querying the predicted gene products to the InterPro (PRINTS, PFAM, TIGRFAM, PRODOM and SMART) and COG databases (via HMM search for InterPro; via RPSblast for COGs) to identify domains indicative of a role in these processes (e.g., EAL, GGDEF, PAS/PAC). Genes with predicted domains above the trusted cutoff score (InterPro) or an *e* value of less than e^{-5} (COGs) were assigned a product description and classified using a set of rules based on the domain architecture of the protein. The final results were manually verified.

Nucleotide sequence accession number. The complete sequence of the *S. denitrificans* genome is available from GenBank (accession number NC_007575).

RESULTS AND DISCUSSION

Genome structure. The *S. denitrificans* DSM1251 genome is one of the largest epsilonproteobacterial genomes yet sequenced, consisting of a single 2.2-Mbp chromosome (Table 1). The coding density and G+C content are similar to those of the other epsilonproteobacteria (Table 1). Four rRNA operons are present, which, due to their elevated G+C content (~50%) relative to the genome average (34.5%), are visible as positive G+C content anomalies on the genome map (Fig. 1). Three of these operons (the 16S-tRNA^{Ala}-23S-5S operons) are 100% identical and are oriented in the same direction, while the fourth (the 16S-tRNA^{Ile}-23S-5S operon) is in the opposite orientation, and its 5S and 23S genes each have a single nucleotide substitution compared to the others. The free-living *S. denitrificans*, *Sulfurovum* sp. strain NBC37-1, and *Nitratiruptor* sp. strain SB155-2 have more rRNA operons than those epsilonproteobacteria that are known to be exclusively host related (Table 1), which is likely a reflection of an adaptation to fluctuating environmental conditions and the necessity for versatility (30, 41, 65).

Two large (17,627-bp) identical transposons are apparent as negative-G+C-content anomalies (30.0%) (Fig. 1). Flanked by identical 12-bp inverted repeats, these transposons (Suden_0690 to Suden_0702 and Suden_1587 to Suden_1599) include genes encoding transposases as well as proteins similar to the TniB (46%) and TniQ (47%) transposase accessory proteins found in mercury resistance transposons in *Xanthomonas* sp. strain W17 and other systems (29). These transposons also include genes encoding a type I restriction-modification methyltransferase and restriction enzyme (see the supplemental material). Interestingly, one of the copies of this transposon interrupts a flagellin biosynthetic operon, which may explain why, unlike close relatives (25,

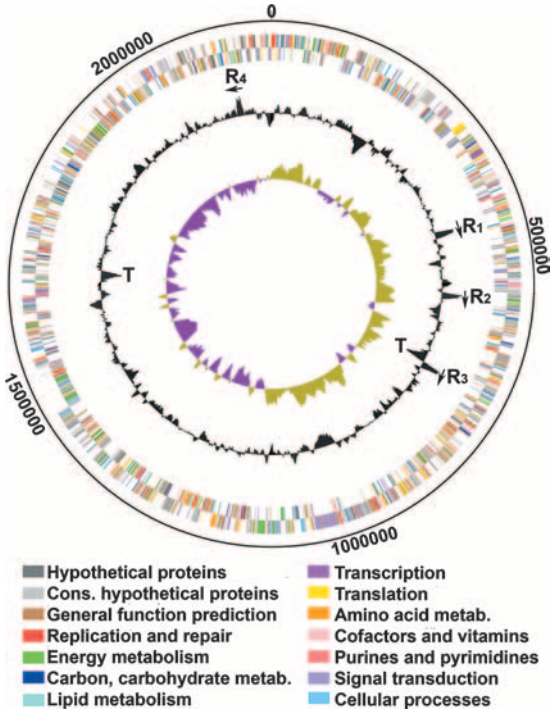

FIG. 1. Map of the *Sulfurimonas denitrificans* DSM1251 genome. The two outer rings include protein-encoding genes, which are color coded based on their membership in COG categories. Ring 3 depicts the deviation from the average G+C content level (%), while the innermost ring is the GC skew (= [G−C]/[G+C]). R1, R2, R3, and R4 are rRNA operons (with their orientations indicated with arrows), and the two regions marked T are identical large transposons. The G+C and GC skew rings were calculated with a sliding window of 10,000 bp with a window step of 100. Cons., conserved.

68), this strain of *S. denitrificans* is nonmotile (70). Other transposase and integrase genes are described in the supplemental material.

Transporters. *S. denitrificans* has a modest complement of genes (97 total) predicted to encode transporters. This number is similar to those of other sequenced heterotrophic epsilonproteobacteria (75 to 124 genes), which is surprising given that nutrient requirements for *S. denitrificans*, believed to be an obligate autotroph, are simple compared to those of the others. This similarity in transporter numbers is due in part to the expansion of a few transporter families in this species compared to other epsilonproteobacteria. Amt family transporters are encoded in the *S. denitrificans* (Suden_0641 and Suden_0643), *Sulfurovum* sp. strain NBC37-1 (two copies), *Nitratiruptor* sp. strain SB155-2 (one copy), and *Wolinella succinogenes* (one copy) genomes, but not in *Helicobacter pylori*, *Helicobacter hepaticus*, or *Campylobacter jejuni*, and are likely to facilitate ammonium uptake. Perhaps their absence in *Helicobacter* and *Campylobacter* spp. is due to nitrogen requirements for these species being met primarily from exogenous urea and/or amino acids (35, 54, 69). A formate-nitrite transporter (FNT) gene is present in *S. denitrificans* (Suden_0716) and absent from other sequenced epsilonproteobacteria. Neither gene context nor sequence comparison clarifies the substrate for this transporter. Also notable is the presence of an abundance of resistance-nodulation-cell division (RND) superfamily genes (10 genes [Suden_0270, Suden_0536, Suden_0799, Suden_0876, Suden_0877, Suden_0883, Suden_1281, Suden_1440, Suden_1499, and Suden_2011]) compared to what is seen for other epsilonproteobacteria, including the two hydrothermal vent species (2 to 6 genes). Many of these are predicted to encode transporters for metal efflux in *S. denitrificans*. As in *W. succinogenes* and the two hydrothermal vent epsilonproteobacteria, an apparent operon that encodes a cytoplasmic arsenate reductase (Suden_0314), arsenite permease (Suden_0313), and regulatory protein ArsR (Suden_0315) is present (41, 61). Apparently, the sediment ecosystem inhabited by *S. denitrificans* requires a level of resistance to metals and other toxins similar to or perhaps enhanced over that of the digestive tract habitats and hydrothermal vents favored by the other sequenced species.

Electron donors. *S. denitrificans* was originally isolated in a chemostat with thiosulfate as the electron donor and nitrate as the electron acceptor (70). However, prior to this study, the pathways and complexes involved were not identified. Neutrophilic sulfur-oxidizing bacteria use two types of sulfur oxidation pathways: one involving a multienzyme complex catalyzing the complete oxidation of reduced sulfur compounds to sulfate (Sox pathway) (15, 28), and another implementing sulfite and elemental sulfur as important intermediates (27, 47, 59). The genome of *S. denitrificans* reveals that the oxidation of reduced sulfur compounds proceeds via the Sox pathway (Fig. 2). Homologs for genes encoding all components that are required for a fully functional complex in vitro, i.e., SoxB, SoxXA, SoxYZ, and SoxCD (15), could be identified. As for other obligate sequenced autotrophs (41, 57), the *sox* genes in *S. denitrificans* do not occur in one cluster, as in the model organism *Paracoccus pantotrophus* GB17 (15), but in different parts of the genome. *S. denitrificans* has basically two clusters, one containing *soxXYZAB* (Suden_0260 to Suden_0264) and another one containing *soxZYCD* (Suden_2057 to Suden_2060). SoxZY are known to interact with both SoxAB and SoxCD, and their duplication could possibly indicate differential regulation of these two loci. SoxCD has homologies to sulfite dehydrogenase (SorAB) but has been shown to act as a sulfur dehydrogenase (15). In addition, it has recently been shown that organisms that lack *soxCD* but do have *soxB*, *soxXA*, and *soxYZ* use the Sox system to oxidize thiosulfate to sulfur, which is either stored inside the cell or excreted (21). However, elemental sulfur formation by *S. denitrificans* has not been reported. Recently, sulfur oxidation enzymes were also measured in the closely related bacteria *Sulfurimonas autotrophica* and *Sulfurimonas paralvinellae* (67). In this case, sulfite dehydrogenase was detected using an assay that would not be expected to measure such activity were these organisms to use the Sox system (C. G. Friedrich, personal communication), indicating that other *Sulfurimonas* spp. might either not use the Sox system or use a modified version of it. In this regard, it is interesting that the SoxC sequence identities of *S. denitrificans* to sequences of those organisms that have a contiguous *sox* gene set are significantly lower (44%) than when SoxC sequences from organisms in which *sox* genes occur in one cluster are compared among themselves (>63%). Both *soxB* and *soxC* genes exhibit the highest similarities with genes from *Sulfurovum* sp. strain NBC37-1 (41), which suggests that both clusters of *sox* genes are not recent additions to this epsilonproteobacterial lineage. In fact, a phylogenetic analysis based on a large number of SoxB sequences from a variety of sulfur-oxidizing bacteria is even

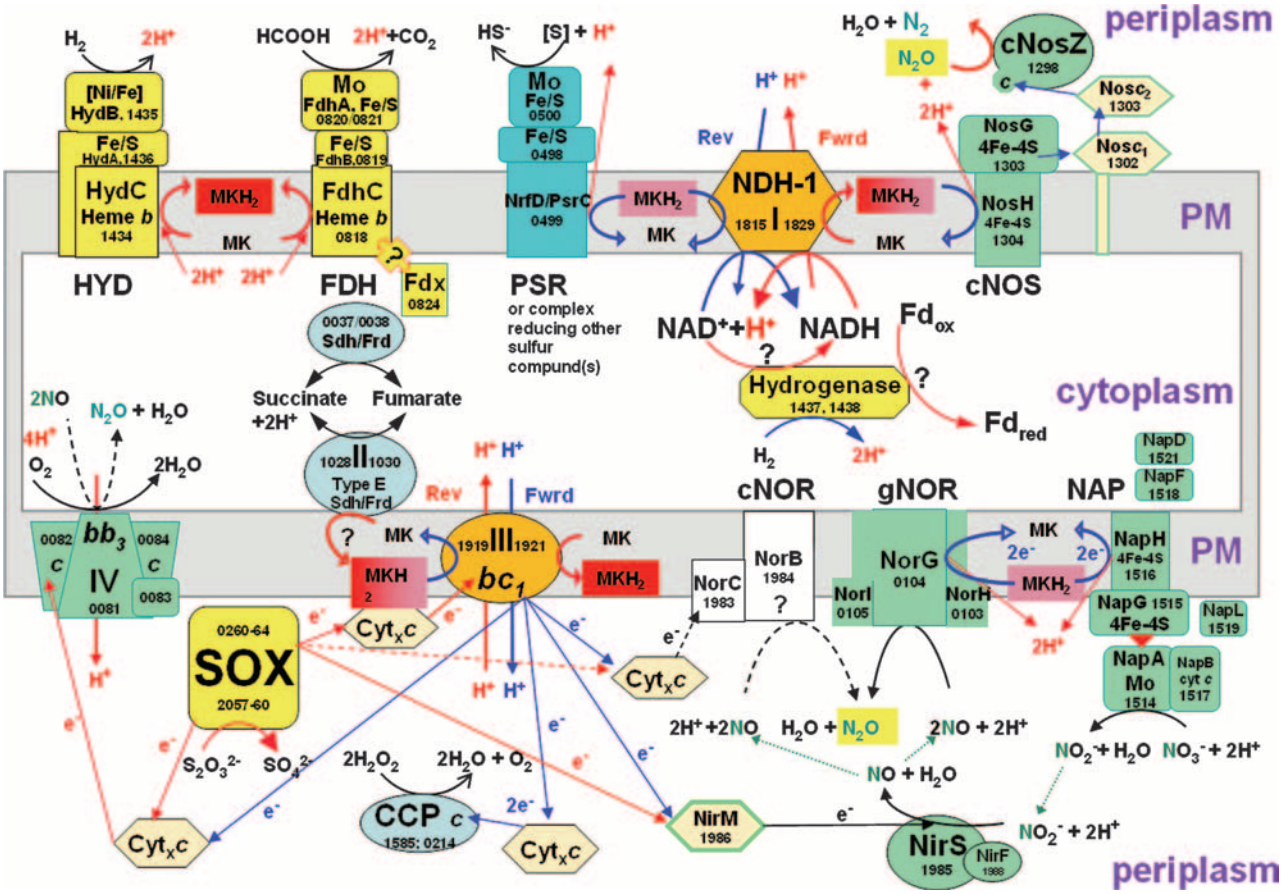

FIG. 2. Model for electron transport in *Sulfurimonas denitrificans*. Abbreviations: I, NADH dehydrogenase; II, succinate dehydrogenase/fumarate reductase; III, bc_1 complex; IV, cbb3 cytochrome *c* oxidase; Cyt_n c, cytochrome *c*; CCP *c*, cytochrome *c* peroxidase; cNOS, cytochrome *c* nitrous oxide reductase; FDH, formate dehydrogenase; HYD, hydrogenase; MK, menaquinone; MKH_2, menaquinol; Mo, molybdenum-containing cofactor; NAP, periplasmic nitrate reductase; NIR, nitrite reductase; NOR, nitric oxide reductase; NOS, nitrous oxide reductase; PM, plasma membrane; PSR, polysulfide reductase; SOX, sulfur oxidation system. Candidate monoheme cytochromes *c* listed as "Cyt_xc" are Suden_0904, Suden_0741, and Suden_0578 (all COG2863) as well as Suden_0865, Suden_1329, and Suden_1112 (no COG assignment).

suggestive of an origin of the Sox system in epsilonproteobacteria (36).

Besides those for the Sox system, *S. denitrificans* also has a gene encoding a sulfide:quinone oxidoreductase (Suden_0619). Sulfide:quinone oxidoreductase catalyzes the oxidation of sulfide to elemental sulfur in *Rhodobacter capsulatus* (56), leading to the deposition of sulfur outside the cells. At present, its role in *S. denitrificans* is unclear, as this species has not been shown to deposit elemental sulfur, though this possibility has not been exhaustively explored with differing cultivation conditions.

The genome also provided evidence for the ability to use H_2 and formate as electron donors (Fig. 2). Based on this information, *S. denitrificans* was successfully cultivated with H_2 as its electron donor and nitrate as the electron acceptor (S. Sievert and S. Molyneaux, unpublished data). The *S. denitrificans* genome encodes two Ni-Fe hydrogenase systems: one cytoplasmic enzyme and one membrane-bound hydrogenase complex. The genes encoding the two subunits of the cytoplasmic enzyme (which lack TAT motifs) (Suden_1437 and Suden_1438) are adjacent to genes encoding the periplasmic hydrogenase (its small subunit has a TAT motif, and a *b*-type cytochrome subunit would function to anchor it to the membrane and shunt electrons to the quinone pool) (Suden_1434 to Suden_1436). The small subunit of the cytoplasmic hydrogenase of *S. denitrificans* forms a cluster with sequences from the two deep-sea hydrothermal vent epsilonproteobacteria and *Aquifex aeolicus* and is distantly related to H_2-sensing hydrogenases of alphaproteobacteria and cyanobacteria (41). Nakagawa et al. (41) suggest that the cytoplasmic hydrogenase acts as an H_2-sensing hydrogenase in *Sulfurovum* sp. strain NBC37-1 and *Nitratiruptor* sp. strain SB155-2. However, an alternative, and in our view more likely, function for the cytoplasmic enzyme as a catalytically active hydrogenase is suggested by the sequence similarity of both subunits to the enzyme from *A. aeolicus*. In *A. aeolicus*, the cytoplasmic hydrogenase can reduce electron acceptors with very negative redox midpoint potentials and therefore has been suggested to provide low-potential electrons to the reductive citric acid cycle (4). This would circumvent the necessity for reverse electron transport and thus increase its growth efficiency, similar to what has been found for certain Knallgas bacteria using the Calvin cycle for CO_2 fixation. Further experiments are needed to confirm the actual

role of the cytoplasmic hydrogenase. Following these hydrogenase genes are several genes encoding hydrogenase-assembly related functions (Suden_1424 to Suden_1433).

A formate dehydrogenase complex is encoded by an operon similar in gene order to one found in *W. succinogenes* (Suden_0816 to Suden_0824). Formate dehydrogenase α subunits contain a selenocysteine residue (26) which is encoded by a stop codon. A putative selenocysteine codon (TGA) followed by a palindromic region was found between two open reading frames that are homologous to the amino and carboxy ends of formate dehydrogenase; accordingly, these open reading frames have been combined into a single coding sequence for the α subunit of this enzyme, Suden_0820, which includes the molybdopterin-binding and iron-sulfur cluster domains typically found in this subunit, as well as the TAT pathway signal sequence which would shunt this subunit to the periplasm. The β and γ subunits are encoded by Suden_0819 and Suden_0818, respectively, with the latter having an unprecedented N-terminal addition with two more predicted transmembrane segments (making six in total). Suden_0824 encodes a ferredoxin, which may shuttle the electrons from formate oxidation to cellular processes. Formate dehydrogenase maturation is likely facilitated by the products of Suden_0823, which encodes a TorD family protein that functions in molybdoprotein formation, and Suden_0817, which encodes an FdhD/NarQ family maturation protein. Suden_0816 encodes a protein belonging to the aminotransferase class V PFAM, as does the SelA protein, which catalyzes a step in selenocysteinyl-tRNA synthesis. Other proteins likely to be involved in selenocysteine synthesis are encoded nearby (Suden_0830 encodes selenophosphate synthase, Suden_0831 encodes L-seryl-tRNA selenium transferase, and Suden_0832 encodes selenocysteine-specific translation elongation factor SelB). Interestingly, the hydrothermal vent epsilonproteobacteria do not appear to have this complex. Though a homolog to the α subunit of formate dehydrogenase was present in both *Nitratiruptor* sp. strain SB155-2 and *Sulfurovum* sp. strain NBC37-1, genes encoding the other subunits were not apparent from BLASTp searches of their genomes. In addition, *S. denitrificans* has a gene encoding the large subunit of a formate dehydrogenase H (*fdhF*, Suden_1902), which is most similar to the one in *W. succinogenes* (WS0126). However, its function and substrate are not apparent based on its sequence or genomic context.

Electron acceptors. All genes required for the complete reduction of nitrate to N_2 are present (Fig. 2). However, *S. denitrificans* has some notable modifications compared to the canonical denitrification pathway. Similar to *Bradyrhizobium japonicum*, *S. denitrificans* appears to have only a periplasmic nitrate reductase (Nap) and not a cytoplasmic membrane-bound nitrate reductase (Nar) (2), which is present in most organisms producing N_2 from nitrate (52). The *nap* gene cluster (*napABHGFLD*; Suden_1514 to Suden_1519, Suden_1521) has the same arrangement as the one identified in *W. succinogenes*, which is unusual in that it lacks a gene encoding the NapC subunit (64). Possibly, *S. denitrificans* is able to denitrify under microaerobic conditions, as the Nap enzyme has been implicated in aerobic denitrification (38). Nitrite reduction to nitric oxide is likely catalyzed by a cytochrome cd_1-dependent nitrite reductase (*nirS*, *nirF*; Suden_1985, Suden_1988), whose genes are present in a gene cluster (Suden_1976 to Suden_1989) that also includes siroheme synthesis genes and two genes annotated as *norCB* (Suden_1983 and Suden_1984).

The *norCB* genes, which encode the small and large subunits of nitrous oxide-forming nitric oxide reductase (cNOR), a member of the heme-copper oxidase (HCO) superfamily (16), are usually clustered together with additional genes required for enzyme assembly and activation (76). These latter genes, *norD* and *norQ*, are missing from the *S. denitrificans* genome. While there is evidence for functional cNOR in bacteria that lack the *norQ* (*cbbQ*) gene, there are presently no experimental reports that demonstrate the functionality of cNOR in bacteria that also lack the *norD* gene. Attempts to test this for *Hydrogenobacter thermophilus* with *Pseudomonas aeruginosa* or *Escherichia coli* as expression hosts were inconclusive (66). The *S. denitrificans norCB* genes are most closely related by sequence similarity to the *norCB* genes in the genomes of *H. thermophilus* and *Methylococcus capsulatus* as well as *Sulfurovum* sp. strain NBC37-1 and *Nitratiruptor* sp. strain SB155-2, the latter of which are two newly sequenced marine epsilonproteobacteria (41). All of these genomes also lack the *norD* gene.

Even though the functionality of cNOR is questionable, *S. denitrificans* is a complete denitrifier and must be able to reduce NO. Attempts to find other inventory implicated in NO reduction were successful and yielded additional candidate systems. One of them, NADH:flavorubredoxin-NOR, also known as the NorVW complex (17, 19), was also not complete and thus likely nonfunctional because a NorW-encoding gene was not identified. Interestingly, the genome encodes NorV in the unusual form of two genes: one encoding a rubredoxin (Suden_1582) is succeeded by a flavodoxin gene (Suden_1581). Although both cNOR and NADH:flavorubredoxin-NOR may not have catalytic activity, it is possible that their NO-binding capacity has a function in NO sequestration and detoxification.

Interestingly, the *S. denitrificans* genome also encodes a previously unidentified member of the HCO superfamily that is also a candidate for catalyzing nitric oxide reduction. These HCO genes follow a set of pseudogenes normally involved in nitrate reduction (Suden_0100 to Suden_0102). Based on structural modeling and genome analysis, it is expected that this new HCO family is a novel nonelectrogenic quinone-oxidizing nitric oxide reductase, gNOR (J. Hemp, M. G. Klotz, L. Y. Stein, and R. B. Gennis, unpublished data). The gNOR family, encoded by the *norGHJ* genes (Suden_0103 to Suden_0105), is unique within the HCO superfamily in that it exhibits a novel active-site metal ligation, with one of the three conserved histidine ligands being replaced with an aspartate. This ligation pattern strongly suggests that the active-site metal is an iron. Structural modeling of members of the gNOR family has identified three conserved acidic residues which form a charged pocket within the active site, a feature shared with the cNOR family (49). Besides in *S. denitrificans*, gNOR also appears to be present in *Sulfurovum* sp. strain NBC37-1 and *Persephonella marina* strain EX-H1, whereas it is missing from *Nitratiruptor* sp. strain SB155-2. Since phylogenetic analysis demonstrates that nitric oxide reductase activity has evolved multiple times independently within the heme-copper superfamily, these shared features between the distantly related gNOR and cNOR families are interesting examples of convergent evolution (Hemp et al., unpublished).

Nitrous oxide reduction is carried out by nitrous oxide reductase encoded by an unusual *nos* gene cluster (Suden_1298 to Suden_1308) similar to one previously identified in *W. succinogenes* (63). As in *Wolinella*, the NosZ in *S. denitrificans* (Suden_1298) contains a C-terminal extension of about 200 residues that carries a monoheme cytochrome *c* binding motif (CXGCH), suggesting that it too functions as a cytochrome *c* nitrous oxide reductase (Fig. 2). This feature is also shared by NosZ of *Sulfurovum* sp. strain NBC37-1 and *Nitratiruptor* sp. strain SB155-2, which form a cluster with NosZ from *S. denitrificans* (see Fig. S3 in the supplemental material), possibly allowing the design of primers to screen for the presence of denitrifying epsilonproteobacteria in the environment. It has been hypothesized that the *nos* gene cluster in *W. succinogenes* codes for proteins involved in an electron transport chain from menaquinol to cytochrome *c* nitrous oxide reductase (63), and it is likely that the same holds true for *S. denitrificans*, as well as *Sulfurovum* sp. strain NBC37-1 and *Nitratiruptor* sp. strain SB155-2. In addition, *S. denitrificans* has an almost identical copy of *nosZ* next to a c553-type monoheme cytochrome *c* (Suden_1770, Suden_1769), but its function is at this point unknown. Interestingly, *S. denitrificans* also has a gene coding for a large subunit of a ferredoxin-nitrite reductase (*nirB*; Suden_1241), which could be involved in nitrite assimilation or detoxification (8). However, no gene coding for the small subunit was identified, raising questions about its function.

Additional electron acceptors are suggested by this organism's gene complement (Fig. 2). Like most alpha-, beta-, gamma-, and epsilonproteobacteria but unlike deltaproteobacteria, the *S. denitrificans* genome contains a cluster of four genes that encode the FixNOQP proteins, which constitute a proton-pumping cbb3-type cytochrome *c* oxidase, suggesting an ability to use oxygen as a terminal electron acceptor. This is somewhat unexpected, since *S. denitrificans* was originally described as an obligate denitrifier and is quite sensitive to oxygen (S. Sievert, unpublished data). cbb3-type cytochrome *c* oxidase complexes have extremely high affinities for oxygen (48), which might allow this organism to use oxygen as an electron acceptor under extremely low oxygen tensions, or alternatively, to scavenge oxygen to prevent poisoning. Another possibility might be the involvement of cbb3-type cytochrome *c* oxidase in the catalytic reduction of NO (14). It is interesting that, in line with other epsilonproteobacteria, the genome does not contain genes encoding FixGHIS, which are present in all other bacteria that express a cbb3-type cytochrome *c* oxidase and are involved in the assembly and maturation of the cbb3-type cytochrome *c* oxidase complex (33, 44). The reasons for the absence of *fixGHIS* in epsilonproteobacteria remain unknown, and it needs to be tested experimentally what the actual role of cbb3-type cytochrome *c* oxidase in *S. denitrificans* is.

Additionally, *S. denitrificans*, *Nitratiruptor* sp. strain SB155-2, and *Sulfurovum* sp. strain NBC37-1 may be able to use sulfur compounds as terminal electron acceptors. Genes Suden_0498 to Suden_0500 encode an Fe/S protein, an NrfD-related membrane anchor (eight helices), and an unusually long molybdopterin-containing oxidoreductase that contains a twin-arginine translocation pathway signal, respectively; homologs of these three genes are also encoded adjacent to each other in the two hydrothermal vent epsilonproteobacteria. Together, these proteins could form a periplasm-facing membrane-bound complex that is most likely involved in the reduction of sulfur compounds like elemental sulfur, polysulfide, thiosulfate, or tetrathionate. However, only a limited number of these molybdopterin-containing oxidoreductases have been characterized and it is presently not possible to infer substrate specificity from the primary structure. Further experiments are needed to confirm the substrate used by this complex.

Carbon fixation and central carbon metabolism. Genes encoding the enzymes of the reductive citric acid cycle are apparent (Fig. 3), which is consistent with prior biochemical and genetic analyses of this organism (24). Of particular note are genes encoding the enzymes necessary for the cycle to operate in the reductive direction: pyruvate:acceptor oxidoreductase (Suden_0096 to Suden_0099, based on similarity to biochemically characterized orthologs in *H. pylori* [23]), pyruvate carboxylase (Suden_0622, Suden_1259, based on biochemically characterized orthologs from *C. jejuni* [72]; however, see below), 2-oxoglutarate:ferredoxin oxidoreductase (Suden_1052 to Suden_1055, as for pyruvate:acceptor oxidoreductase, based on reference 23), and ATP-dependent citrate lyase (Suden_0570, Suden_0571 [24]). Also noteworthy is the presence of two copies of succinate dehydrogenase/fumarate reductase, one of which has a subunit that would anchor it to the membrane (Suden_1028 to Suden_1030), while the other lacks this subunit and may be cytoplasmic (Suden_0037, Suden_0038). The membrane-bound form is unusual in that it has a cysteine-rich type-E membrane anchor. It is similar to SdhABE from *W. succinogenes*, which has been characterized as a membrane-bound fumarate-reducing complex with subunits SdhAB facing the periplasm. In contrast to *W. succinogenes*, the *S. denitrificans* gene encoding subunit A is about 43 residues shorter and lacks the TAT signal peptide present in its *W. succinogenes* homolog, something that appears to be shared with *Sulfurovum* sp. strain NBC37-1 and *Nitratiruptor* sp. strain SB155-2, both of which are also chemolithoautotrophic epsilonproteobacteria using the reductive citric acid cycle for carbon fixation. Thus, it appears that in these organisms the membrane-bound fumarate-reducing complex faces into the cytoplasm. At present, the exact function of the two fumarate reductases is unknown, although it is likely that the membrane-bound one, due to its potential for additional energy generation, might be involved in the reductive citric acid cycle for autotrophic carbon fixation. The intriguing possibility that *S. denitrificans* might also be able to carry out fumarate respiration has to await further experimentation. However, *S. denitrificans* does not contain an *frdCAB* operon typical for menaquinol:fumarate reductase sustaining fumarate respiration in other epsilonproteobacteria.

The acetyl coenzyme A (acetyl-CoA) and oxaloacetate produced by the reductive citric acid cycle could be funneled to central carbon metabolism: acetyl-CoA could be converted to pyruvate via pyruvate:acceptor oxidoreductase (see above) and oxaloacetate could be used to form phosphoenolpyruvate via phosphoenolpyruvate carboxykinase (Suden_1696). Acetyl-CoA could also be directed to fatty acid synthesis (acetyl-CoA carboxylase [Suden_1174, Suden_1608]). Genes that suggest an ability to supplement autotrophic growth with acetate assimilation are present in all three sulfur-oxidizing epsilonproteobacteria. Two possible systems for converting acetate to acetyl-CoA are present: acetyl-CoA ligase (Suden_1451), as

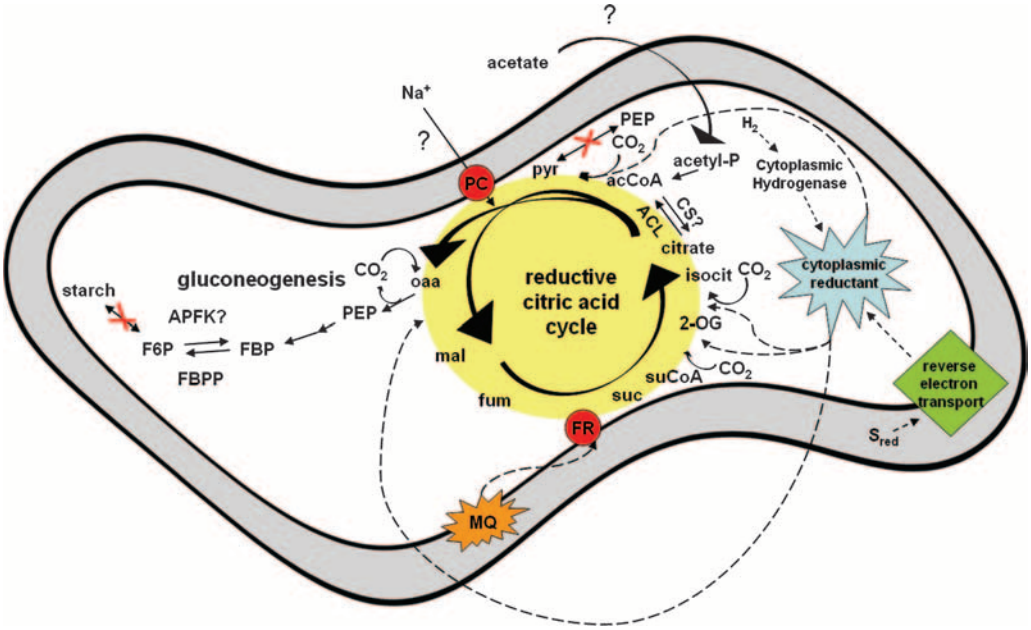

FIG. 3. Central carbon metabolism in *Sulfurimonas denitrificans*. Abbreviations: 2-OG, 2-oxoglutarate; acCoA, acetyl-CoA; ACL, ATP-citrate lyase; APFK, ATP-dependent phosphofructokinase; CS, citrate synthase; F6P, fructose 6-phosphate; FBP, fructose 1,6-bisphosphate; FBPP, fructose 1,6 bisphosphate phosphatase; FR, fumarate reductase; fum, fumarate; isocit, isocitrate; mal, malate; MQ, menaquinone; OAA, oxaloacetate; PC, pyruvate carboxylase; PEP, phosphoenolpyruvate; pyr, pyruvate; suc, succinate; suCoA, succinyl-CoA.

well as phosphate acetyltransferase (Suden_0055) and acetate kinase (Suden_0056). These are also present in *Sulfurovum* sp. strain NBC37-1, while acetate kinase is absent from *Nitratiruptor* sp. strain SB155-2. Perhaps the two systems have different affinities for acetate, as has been demonstrated for methanogens (60), and are differentially expressed depending on the environmental concentrations of this organic acid.

In order for *S. denitrificans* to grow autotrophically using the reductive citric acid cycle, there must be a means of carboxylating pyruvate to form oxaloacetate. In some organisms, this is accomplished by the tandem activities of phosphoenolpyruvate synthetase and phosphoenolpyruvate carboxylase, while others use pyruvate carboxylase (55). In contrast to the two autotrophic epsilonproteobacteria *Sulfurovum* sp. strain NBC37-1 and *Nitratiruptor* sp. strain SB155-2, which have genes encoding phosphoenolpyruvate synthase as well as pyruvate kinase (41), the latter of which is usually involved in ATP synthesis during glycolysis, *S. denitrificans* does not appear to have any genes that might encode an enzyme that could interconvert phosphoenolpyruvate and pyruvate. Instead, it may use pyruvate carboxylase, as genes encoding both the biotin carboxylase subunit (Suden_0622) and biotin carboxyl carrier subunit (Suden_1259) of this enzyme are present. The biotin carboxyl carrier subunit gene (Suden_1259) occurs in an apparent operon with other genes homologous to the subunits of sodium-transporting oxaloacetate decarboxylase (Suden_1258 to Suden_1260). Suden_1259, which encodes the α subunit of this complex, has a high level of sequence similarity with pyruvate carboxylase genes from various *Campylobacter* species (including one from *C. jejuni* which has been biochemically characterized) (72), while the β and γ subunits (Suden_1258 and Suden_1260), which are absent from the heterotrophic epsilonproteobacteria, are similar to those found from *Sulfurovum* sp. strain NBC37-1, *Nitratiruptor* sp. strain SB155-2, many gammaproteobacteria, many *Chlorobia* spp., and *Desulfotalea psychrophila*, a deltaproteobacterial sulfate reducer. Heterotrophic organisms that have this complex ferment citrate. After cleaving citrate to acetate and oxaloacetate, they use the oxaloacetate decarboxylase complex to couple the exothermic decarboxylation of this organic acid to the extrusion of sodium ions. For these organisms, the other genes necessary for citrate fermentation (e.g., the gene for the citrate transporter) are nearby (9). This is not the case for *S. denitrificans*. An alternative function for Suden_1259 is suggested by phylogenetic analysis, which places it within a clade with the biochemically characterized pyruvate carboxylase from *C. jejuni* (Fig. 4) and separate from biochemically characterized oxaloacetate decarboxylase genes from *Klebsiella pneumoniae* and *Vibrio cholerae* (5). Other members of this clade include the genes from *Chlorobia* spp., *Sulfurovum* sp. strain NBC37-1, and *Nitratiruptor* sp. strain SB155-2, which also use the reductive citric acid cycle for carbon fixation and are not known to ferment citrate. It is possible to operate the oxaloacetate decarboxylase complex as a pyruvate carboxylase by imposing a sodium gradient across the membrane (10). It is tempting to speculate that in the autotrophic epsilonproteobacteria, *Chlorobia* spp., and possibly *D. psychrophila*, this complex functions as a pyruvate carboxylase. Interestingly, the sequenced autotrophic epsilonproteobacteria and *Desulfotalea psychrophila* are all marine organisms, and *Chlorobia* spp. evolved in the marine environment (1). Only 5 of the 10 sequenced *Chlorobia* spp. have the sodium-transporting oxaloacetate decarboxylase/pyruvate carboxylase complex, while the other 5, including *C. tepidum*, have the α subunit (on which Fig. 4 is based) but not the three-

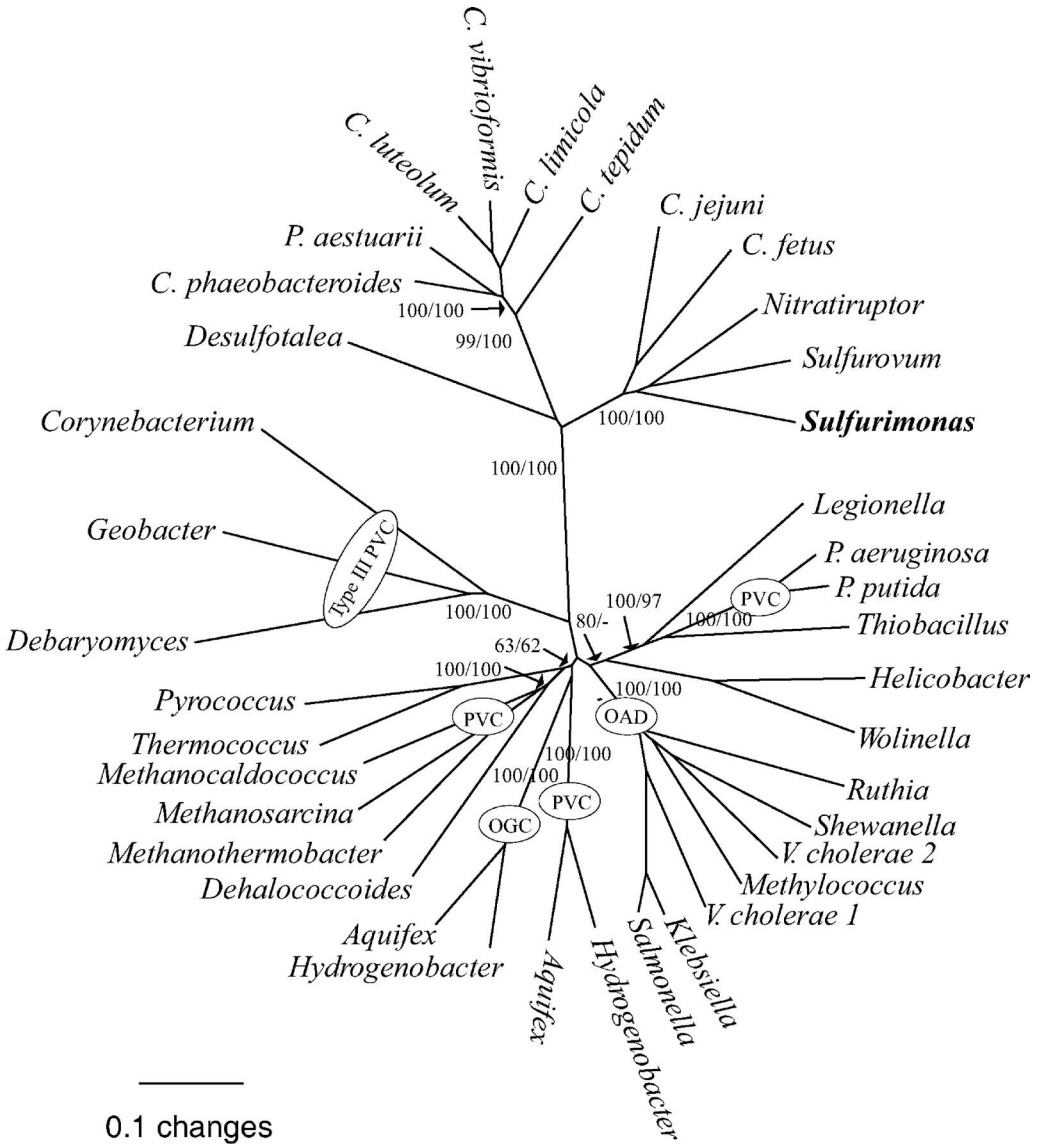

FIG. 4. Phylogenetic relationships of α subunits of oxaloacetate decarboxylase (OAD), pyruvate carboxylase (PVC), oxoglutarate carboxylase (OGC), and type III pyruvate carboxylase to the product of Suden_1259 of *Sulfurimonas denitrificans*. Sequences were aligned using the program package MacVector. Neighbor-joining and parsimony trees based on the predicted amino acid sequences were calculated using PAUP 4.0b10. Bootstrap values (1,000 replicates) for the major nodes are given for the neighbor-joining (first value) and parsimony (second value) analyses. *P. aestuarii*, *Prosthecochloris aestuarii*.

subunit pump. With the exception of *C. limicola*, which was isolated from a mineral hot spring, all *Chlorobia* spp. containing the sodium pump have a requirement for sodium. Thus, it is likely that these organisms have found a way to couple pyruvate carboxylation, which is energetically unfavorable, to a sodium gradient, something that was previously proposed but never shown for an organism (10). Whether the complex encoded by Suden_1258 to Suden_1260 functions as a pyruvate carboxylase or an oxaloacetate decarboxylase is a key point begging clarification, which will be nontrivial, given that a genetic system has not been developed for this organism.

Carbon fixed by the reductive citric acid cycle can be shunted through gluconeogenesis, as all genes necessary for this pathway are present. The presence of genes encoding citrate synthase (Suden_2100) and ATP-dependent (irreversible) phosphofructokinase (Suden_0549) are enigmatic, as their roles in this obligate autotroph are unclear.

Genes are apparent whose products could utilize the carbon skeletons synthesized by central carbon metabolism for ammonia assimilation (see below) and amino acid, nucleotide, fatty acid, and phospholipid synthesis. Cysteine biosynthesis is notable in that the reduction of sulfate proceeds via adenosine 5′-phosphosulfate rather than 3′-phosphoadenylylsulfate in a pathway that was until recently known only for plants (42). Genes encoding assimilatory sulfate reduction cooccur in an apparent operon (Suden_0154 to Suden_0160). Most likely, this operon-like structure is turned on or off depending on whether *S. denitrificans* is inhabiting an environment with a

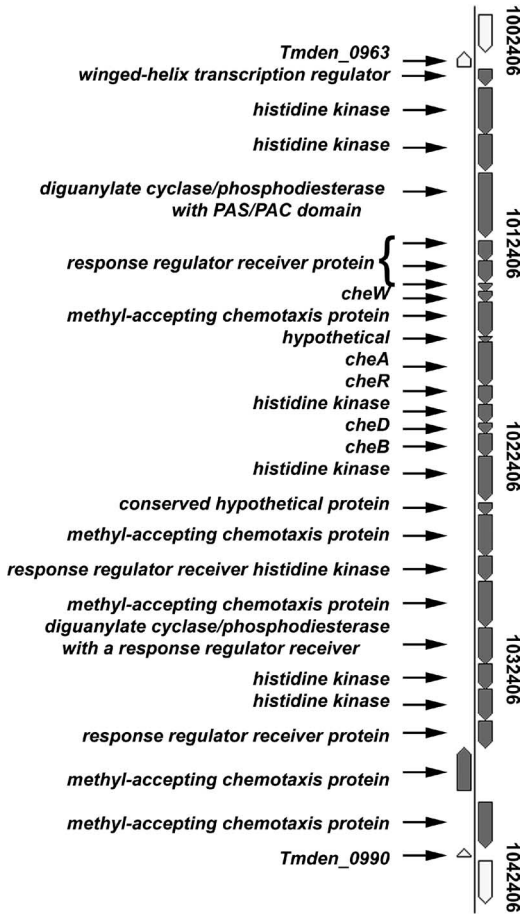

FIG. 5. A large gene cluster from the *Sulfurimonas denitrificans* genome that includes many of the genes for chemotaxis signal transduction.

high concentration of reduced inorganic sulfur compounds. Interestingly, sulfate assimilation in *Sulfurovum* sp. strain NBC37-1 and *Nitratiruptor* sp. strain SB155-2 appears to proceed via 3′-phosphoadenylylsulfate.

Nitrogen assimilation. The *S. denitrificans* genome contains *nirC* (*focA*) and *nirB* genes, encoding the formate-nitrite transporter (Suden_0716) and the large subunit of NAD(P)H-dependent ammonia-forming siroheme nitrite reductase (Suden_1241), respectively, along with the inventory for siroheme synthesis (Suden_1977, *cysG*, siroheme synthase; Suden_1989, *cobA-cysG*, uroporphyrinogen III methylase); however, it lacks the *nirD* gene, which encodes the small subunit of siroheme nitrite reductase. Because the genome also lacks *nrfHA* genes, which encode respiratory nitrite ammonification capacity in many delta- and epsilonproteobacteria (62), it appears that *Sulfurimonas* is solely dependent on ammonia uptake from the environment. The genome indeed contains two genes encoding different ammonia permeases (noted above) (22, 43), one AmtB-like (Suden_0641) and one rhesus factor-like (Suden_0643) permease, which are clustered together with the gene encoding nitrogen regulatory protein PII (*glnK*, Suden_0642). Whereas AmtB proteins function as ammonia gas uptake channels, the substrate for Rh-like protein channels is still debated and includes ammonia as well as CO_2 (43). The genome contains also all the additional genes needed for 2-oxoglutarate sensing and the regulation of nitrogen assimilation (reviewed in reference 34).

Chemotaxis and other regulatory and signaling proteins. Close relatives of *S. denitrificans* are motile, while this particular strain is nonmotile, probably due to the interruption of a flagellar biosynthetic operon by a transposon (see "Genome structure" above). Based on the presence of all of the genes necessary to encode the flagellar apparatus, none of which display any evidence of degeneration, an abundant sensory apparatus necessary to detect the presence of chemoattractants or repellants and communicate this information to the flagellar motor, as well as the sequence identity of this transposon with a duplicate in the genome (see above), it is likely that nonmotility is a recently acquired property. Interestingly, many of the genes encoding the chemotaxis components are in a large cluster with multiple kinases and response regulators (Fig. 5), as in *Nitratiruptor* sp. strain SB155-2 (41), suggesting interconnectivity between the chemotaxis and other signal transduction systems. Perhaps the original enrichment and isolation procedure for this strain (in a chemostat) might have selected for a nonmotile strain.

The *S. denitrificans* genome encodes a relative abundance of signaling proteins. Particularly well represented in these genomes are genes encoding proteins with EAL and GGDEF domains (based on PFAM hits, 16 and 38 genes, respectively), which likely function in the synthesis and hydrolysis of the intracellular signaling compound cyclic diguanylate (53). Further, six proteins with PAS/PAC domains which may function as redox sensors are encoded (75). The genomic repertoire of signaling and regulatory genes was compared with those of two other free-living, obligate chemolithoautotrophs for which these data are available (Table 2). Some features are similar to *Thiomicrospira crunogena*: both of these species have a relative abundance of signal transduction proteins compared to *Ni-*

TABLE 2. Regulatory and signaling proteins of *Sulfurimonas denitrificans* and other obligate chemolithoautotrophs

Functional description of proteins	No. of proteins of indicated description in[a]:		
	S. denitrificans	*T. crunogena*	*N. oceani*
Transcription/elongation/ termination factors	56	72	104
Two-component transcriptional regulator, winged-helix family	19	4	2
Signal transduction	146	128	75
Chemotaxis signal transduction	28	27	8
Methyl-accepting chemotaxis	13	14	1
Nonchemotaxis signal transduction	118	101	67
Signal transduction histidine kinase	36	17	18
Cyclic nucleotide signal transduction	42	49	16
Total	202	200	179

[a] *Sulfurimonas denitrificans* DSM1251 is compared to gammaproteobacteria *Thiomicrospira crunogena* XCL-2 and *Nitrosococcus oceani* ATCC 19707.

trosococcus oceani (31), which may be a response to habitats spatially (sediments; *S. denitrificans*) or temporally (hydrothermal vents; *T. crunogena*) more heterogeneous than the open ocean (*N. oceani*). Both have a large number of genes encoding methyl-accepting chemotaxis proteins (Table 2). Unlike what is seen for *T. crunogena*, none of the methyl-accepting chemotaxis proteins from *S. denitrificans* are predicted to have PAS/PAC domains that could bind redox-sensitive cofactors (57), but a gene encoding a protein with a PAS/PAC domain is present in the large cluster of chemotaxis genes described above (Fig. 5), suggesting potential communication between sensing cellular or environmental redox conditions and the chemotactic apparatus. Another similarity between *T. crunogena* and *S. denitrificans* is an abundance of genes predicted to be involved with cyclic nucleotide signal transduction, and many of these are predicted to have EAL and/or GGDEF domains, indicating a role for cyclic diguanylate in intracellular signaling in this organism. Many of these predicted proteins also have PAS/PAC domains, as in *T. crunogena* (57).

Unique among the three species compared here, *S. denitrificans* has a relative abundance of signal transduction histidine kinases and an expanded complement of winged-helix family two-component transcriptional regulators (Table 2). Both *T. crunogena* and *N. oceani* can use a rather limited variety of electron donors and acceptors compared to *S. denitrificans*. Perhaps this expansion in histidine kinases and transcriptional regulators coordinates the expression of the complexes necessary for the oxidation of multiple electron donors (e.g., H_2, reduced sulfur compounds, formate) and the reduction of multiple electron acceptors (O_2 and NO_x).

Oxidative stress. *S. denitrificans* has reasonably elaborate defenses against oxidative stress, on par with what has been observed for the pathogenic epsilonproteobacterium *H. pylori* (73). Initially, this was considered surprising to us, because this nonpathogenic species does not have to elude the oxidative arsenal of a host immune system, nor, as a microaerophile capable of growth via denitrification, does it grow in the presence of high concentrations of oxygen. However, given the presence of enzymes with labile iron-sulfur clusters with irreplaceable roles in central carbon metabolism in this organism (e.g., pyruvate:acceptor oxidoreductase [Suden_0096 to Suden_0099], 2-oxoglutarate:acceptor oxidoreductase [Suden_1053 to Suden_1055]), perhaps added defenses are a necessary part of survival.

Several genes whose products could prevent the buildup of intracellular Fe^{2+} that can spur hydroxyl radical generation via the Fenton reaction are present. A gene that encodes the Fur protein (Suden_1272), which regulates iron uptake (14), is present. A gene encoding iron-binding ferretin (Suden_1760) is also present.

Many enzymes to detoxify reactive oxygen and nitrogen species are encoded in this genome. An iron/manganese superoxide dismutase (Suden_1129) is present in this species but not in the other sulfur-oxidizing epsilonproteobacteria (41). This enzyme and ruberythrin (Suden_0739) could convert superoxide to hydrogen peroxide, which in turn could be dispatched by catalase (Suden_1323), peroxiredoxins (Suden_0132, Suden_0630, Suden_1778, Suden_1803), or cytochrome *c* peroxidase (Suden_0214, Suden_1585). Peroxiredoxins, particularly alkylhydroperoxide reductases, might be specifically targeted toward low levels of H_2O_2 or organic peroxides (58, 73). To dispense with nitric oxide that escapes the periplasmic and membrane-associated respiratory nitrogen reduction complexes, a truncated hemoglobin gene (Suden_0993) which may convert nitric oxide to nitrate is present (46). Thioredoxins (Suden_0342, Suden_0501, Suden_1867, Suden_2099) and thioredoxin reductase (Suden_1869) could funnel electrons to these oxidative stress proteins, as glutathione does not appear to play this role in this organism, since genes encoding glutathione synthetase or gamma-glutamate-cysteine ligase are absent.

Genes are apparent whose products could enable a cell to cope with damage inflicted by any reactive oxygen or nitrogen species that escape cellular defenses. Endonucleases III (Suden_0516) and IV (Suden_1835) and MutS (Suden_0755) could repair oxidative DNA damage. Methionine sulfoxide reductase (Suden_0012) and alkylhydroxide reductase (Suden_1778) could contend with any methionine residues or lipids that had been oxidized by interaction with reactive oxygen or nitrogen species, while nitroreductases (Suden_0519, Suden_1158) could prevent oxidized cellular nitrogenous and other compounds from generating peroxide.

Conclusions. *S. denitrificans* has several unique features which differentiate it from the other epsilonproteobacteria that have been sequenced to date. It has a larger genome than most of the others, which likely provides the sensory, regulatory, and metabolic versatility necessary for survival in a habitat more heterogeneous than found in a metazoan host. For example, the numerous genes whose products have redox sensory domains likely function to position these cells in the redoxcline to enable them to obtain the electron donors and acceptors needed for growth. Furthermore, these cells are quite versatile with respect to electron donors and acceptors, as the genome data suggest a capability of using donors and acceptors beyond those based on cultivation studies. Although *S. denitrificans* has been isolated from coastal marine sediments, its genome shares many features with two recently described autotrophic deep-sea hydrothermal vent epsilonproteobacteria, including the potential to utilize a variety of redox substrates (hydrogen gas, reduced sulfur compounds, oxygen, and nitrate), its responses to oxidative stress and high metal content, and a genome size intermediate between the two. This suggests that while these habitats at first appear strikingly different, they require similar adaptations on the scale of the microbes. Several additional features, which are absent from their hydrothermal vent relatives, are present in *S. denitrificans*, and these additional features may be particularly valuable in the sediment habitat. Their formate dehydrogenase complex would enable *S. denitrificans* to utilize a major by-product of fermentation that would cooccur with it should sediment organic carbon loads be high. The presence of the additional oxidative stress protein (superoxide dismutase) may enable *S. denitrificans* to cope with diurnal shifts in sediment oxygen concentration, and several additional resistance-nodulation-cell division family efflux pumps relative to what is seen for hydrothermal vent epsilonproteobacteria suggest that survival in marine sediments requires a degree of versatility and defense against environmental insult beyond what is necessary at moderate temperatures at hydrothermal vents.

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