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The genome of the epsilonproteobacterial chemolithoautotroph *Sulfurimonas denitrificans*

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(abstract)

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 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 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 (11 total) are present, several of which are predicted to encode heavy metal efflux transporters. An elaborate arsenal of sensory and regulatory protein-encoding genes is in place, as well as genes necessary to prevent and respond to oxidative stress.

61 Epsilonproteobacteria are represented as molecular isolates from a vast array of
62 habitats, including brackish, marine, and subsurface (e.g., (3, 11, 25, 39, 45, 71, 74);
63 reviewed in (6)). Over the last years, quite a few cultured representatives of this group
64 have been obtained from these environments, and currently all cultured members are
65 chemolithoautotrophs or mixotrophs, capable of using sulfur and/or hydrogen as electron
66 donors, while denitrifying and/or growing as microaerophiles (reviewed in (6)). These
67 organisms use the reductive citric acid cycle for carbon fixation (24, 67). Given their
68 abundance, sulfur-oxidizing epsilonproteobacteria, in particular members of the genus
69 *Sulfurimonas*, are believed to be relevant to the function of the global sulfur cycle (6).
70 Genome data from these organisms would be key to metagenome sequencing
71 efforts in habitats where they are abundant, and would also, by comparison to other
72 epsilonproteobacteria, be helpful for determining the traits unique to a free-living,
73 autotrophic lifestyle versus a host-associated, heterotrophic lifestyle. Recently, the
74 genome sequences of *Sulfurovum* sp. NBC37-1 and *Nitratiruptor* sp. SB155-2, two
75 sulfur-oxidizing epsilonproteobacteria from deep-sea hydrothermal vents were published,
76 which revealed that these organisms share many features with their pathogenic (e.g.,
77 *Campylobacter* and *Helicobacter* spp.) epsilonproteobacterial relatives (41). Given the
78 remarkable variety of habitats where sulfur-oxidizing epsilonproteobacteria are found, it
79 was of great interest to also conduct these analyses on non-vent epsilonproteobacteria. To
80 represent the abundant sulfur-oxidizing epsilonproteobacteria present in coastal marine
81 sediments, we chose to sequence and analyze the genome of the sulfur-oxizing
82 chemolithoautotroph *Sulfurimonas denitrificans* DSM1251. Based on its phenotype, *S.*
83 *denitrificans* was originally named *Thiomicrospira denitrificans* (70). Subsequent

84 sequencing revealed the polyphyletic nature of the *Thiomicrospiras,* with members from
85 both the gammaproteobacteria and epsilonproteobacteria (40). As a result,
86 *Thiomicrospira denitrificans* was eventually removed from the genus *Thiomicrospira* and
87 placed within the genus *Sulfurimonas* (68). In addition to marine sediments, bacteria
88 belonging to this genus have been isolated or detected in a variety of sulfidogenic
89 environments, including deep-sea hydrothermal vents, the oxic-anoxic interface of
90 marine anoxic basins, and oilfield (6, 18, 25, 32), making organisms of this genus
91 globally significant.
92

93 **MATERIALS AND METHODS**

94

95 **Genome sequencing, annotation, and analysis.** DNA libraries were created and
96 sequenced to approximately 13X depth of coverage at the Production Genomics Facility
97 of the Joint Genome Institute (JGI) using the whole-genome shotgun method as
98 previously described (7, 57). Gaps were closed and base quality problems were
99 addressed by sequencing finishing reads, and PHRED/PHRAP/CONSED were used for
100 assembly (12, 13, 20). Automated and manual annotation were conducted by ORNL
101 similarly to (7, 57). Results were collated and presented via GenDB (37) for manual
102 verification. The prediction of membrane transporters was based on a transporter
103 annotation pipeline that uses several predictive approaches such as BLAST, COG, PFAM
104 and TIGRFAM HMM searches, transmembrane topology prediction algorithms, and
105 takes advantage of a curated database of transporters. Details of this pipeline and
106 database have been published in (50, 51, 57). The main limitation of this approach is the

107 ability to accurately predict precise transporter specificities. Based on both internal and
108 external testing, this methodology is highly successful at identifying putative transporters
109 and predicting approximate substrate specificity. However, making precise substrate
110 predictions, eg., serine transport rather than amino acid transport, is more problematic.
111 The other related limitation is that the approach is dependent on comparison with known
112 experimentally characterized transporters, so completely novel transporters which have
113 never had homologues experimentally characterized will not be predicted by this
114 methodology.

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

118 **Identification of genes encoding signal transduction and regulatory proteins.**
119 The complement of genes that encode signal transduction and regulatory proteins were
120 compared among *S. denitrificans* DSM1251, *Thiomicrospira crunogena* XCL-2, and
121 *Nitrosococcus oceani* ATCC 19707. To compare signal transduction and regulatory
122 protein genes among these obligate autotroph genomes, genes were identified by
123 querying the predicted gene products to the InterPro (PRINTS, PFAM, TIGRFAM,
124 PRODOM and SMART) and COGs databases (via HMM search – InterPro, or RPSblast
125 – COGs) to identify domains indicative of a role in these processes (e.g., EAL, GGDEF,
126 PAS/PAC). Genes with predicted domains above the trusted cutoff score (InterPro) or an
127 e value less than e^{-5} (COGs) were assigned a product description and classified using a

128 set of rules based on the domain architecture of the protein. The final results were
129 manually verified.

130 **Nucleotide sequence accession number.** The complete sequence of the *S.*
131 *denitrificans* genome is available from GenBank (accession number NC_007575).
132
133

134 **RESULTS AND DISCUSSION**

135

136 **Genome structure.** The *S. denitrificans* DSM1251 genome is one of the largest
137 epsilonproteobacterial genomes yet sequenced, consisting of a single 2.2 Mbp
138 chromosome (Table 1). The coding density and G+C content are similar to the other
139 epsilonproteobacteria (Table 1). Four rRNA operons are present, which, due to their
140 elevated G+C content (~50%) relative to the genome average (34.5%), are visible as
141 positive G+C content anomalies on the genome map (Fig. 1). Three of these operons
142 (*16S-tRNA*^{Ala}*-23S-5S*) are 100% identical, and are oriented in the same direction, while
143 the fourth (*16S-tRNA*^{Ile}*-23S-5S*) is in the opposite orientation, and its 5S and 23S genes
144 each have a single nucleotide substitution compared to the others. The free-living *S.*
145 *denitrificans, Sulfurovum* sp. NBC37-1, and *Nitratiruptor* sp. SB155-2 have more rRNA
146 operons than those epsilonproteobacteria that are known to be exclusively host-related
147 (Table 1), which is likely a reflection of an adaptation to fluctuating environmental
148 conditions and the necessity for versatility (30, 41, 65).
149 Two large (17,627 bp), identical transposons are apparent as negative G+C
150 content anomalies (30.0%; Fig. 1). Flanked by identical 12 bp inverted repeats, these

151 transposons (*Suden_0690 – 0702; Suden_1587 - 1599*) include genes encoding
152 transposases, as well as proteins similar to the TniB (46%) and TniQ (47%) transposase
153 accessory proteins found in mercury resistance-transposons in *Xanthomonas* sp. W17 and
154 other systems (29). These transposons also include genes encoding a type I restriction-
155 modification methyltransferase and restriction enzyme (see Supplemental Materials).
156 Interestingly, one of the copies of this transposon interrupts a flagellin biosynthetic
157 operon, which may explain why, unlike close relatives (25, 68), this strain of *S.*
158 *denitrificans* is nonmotile (70). Other transposase and integrase genes are described in
159 Supplemental Materials.
160
161 **Transporters.** *S. denitrificans* has a modest complement of genes (97 total)
162 predicted to encode transporters. This number is similar to other sequenced heterotrophic
163 epsilonproteobacteria (75-124 genes), which is surprising given that nutrient
164 requirements for *S. denitrificans,* believed to be an obligate autotroph, are simple,
165 compared to the others. This similarity in transporter numbers is due in part to the
166 expansion of a few transporter families in this species compared to other
167 epsilonproteobacteria. Amt-family transporters, are encoded in the *S. denitrificans*
168 (*Suden_0641* and *0643*), *Sulfurovum* sp. NBC37-1 (2 copies), *Nitratiruptor* sp. SB155-2
169 (1 copy) and *Wolinella succinogenes* (1 copy) genome, but not in *Helicobacter pylori,*
170 *Helicobacter hepaticus,* or *Campylobacter jejuni,* and are likely to facilitate ammonium
171 uptake. Perhaps their absence in *Helicobacter* and *Campylobacter* spp. is due to nitrogen
172 requirements for these species being met primarily from exogenous urea and/or amino
173 acids (35, 54, 69). A FNT (formate-nitrite transporter) gene is present in *S. denitrificans*

174 (*Suden_0716),* and absent from other sequenced epsilonproteobacteria. Neither gene
175 context nor sequence comparison clarifies the substrate for this transporter. Also notable
176 is the presence of an abundance of Resistance-Nodulation-Cell Division (RND)
177 Superfamily genes (11 genes; *Suden_0270; Suden_0536; Suden_0799; Suden_0876;*
178 *Suden_0877; Suden_0883; Suden_1281; Suden_1440; Suden_1499; Suden_1574;*
179 *Suden_1575; Suden_2011*), compared to other epsilonproteobacteria, including the two
180 hydrothermal vent species (2-6 genes). Many of these are predicted to encode
181 transporters for metal efflux in *S. denitrificans*. As in *W. succinogenes* and the two
182 hydrothermal vent epsilonproteobacteria, an apparent operon is present that encodes a
183 cytoplasmic arsenate reductase (*Suden_0314)*, arsenite permease (*Suden_0313)*, and
184 regulatory protein ArsR (*Suden_0315;* (41, 61). Apparently the sediment ecosystem
185 inhabited by *S. denitrificans* requires a similar or perhaps enhanced level of resistance to
186 metals and other toxins, than the digestive tract habitats and hydrothermal vents favored
187 by the other sequenced species.
188
189 **Electron donors.** *S. denitrificans* was originally isolated in a chemostat with thiosulfate
190 as electron-donor and nitrate as electron-acceptor (70). However, prior to this study, the
191 pathways and complexes involved were not identified. Neutrophilic sulfur-oxidizing
192 bacteria use two types of sulfur oxidation pathways: one involving a multienzyme
193 complex catalyzing the complete oxidation of reduced sulfur compounds to sulfate (Sox
194 pathway) (15, 28), and another implementing sulfite and elemental sulfur as important
195 intermediates (27, 47, 59). The genome of *S. denitrificans* reveals that the oxidation of
196 reduced sulfur compounds proceeds via the Sox pathway (Fig. 2). Homologs for genes

197 encoding all components that are required for a fully functional complex in vitro, i.e.,
198 SoxB, SoxXA, SoxYZ, and SoxCD (15), could be identified. As in other obligate
199 sequenced autotrophs (41, 57), the *sox* genes in *S. denitrificans* do not occur in one
200 cluster, as in the model organism *Paracoccus pantotrophus* GB17 (15), but in different
201 parts of the genome. *S. denitrificans* has basically two clusters, one containing *soxXYZAB*
202 (*Suden_260-264*) and another one containing *soxZYCD* (*Suden_2057-2060*). SoxZY are
203 known to interact with both SoxAB and SoxCD and their duplication could possibly
204 indicate differential regulation of these two loci. SoxCD has homologies to sulfite
205 dehydrogenase (SorAB), but has been shown to act as a sulfur dehydrogenase (15). In
206 addition, it has recently been shown that organisms that lack *soxCD*, but do have *soxB*,
207 *soxXA*, and *soxYZ* use the Sox system to oxidize thiosulfate to sulfur, which is stored
208 either inside the cell or excreted (21). However, elemental sulfur formation by *S.*
209 *denitrificans* has not been reported. Recently, sulfur oxidation enzymes were also
210 measured in the closely related bacteria *Sulfurimonas autotrophica* and *Sulfurimonas*
211 *paralvinellae* (67). In this case, sulfite dehydrogenase was detected using an assay that
212 would not be expected to measure such activity were these organisms to use the Sox
213 system (C. G. Friedrich, pers. comm.), indicating that other *Sulfurimonas spp.* might
214 either not use the Sox system or use a modified version of it. In this regard it is
215 interesting to note that the SoxC sequence identities of *S. denitrificans* to sequences of
216 those organisms that have a contiguous *sox* gene set are significantly lower (44%) than
217 when SoxC sequences from organisms in which *sox* genes occur in one cluster are
218 compared among themselves (>63%). Both *soxB* and *soxC* genes exhibit highest
219 similarities with genes from *Sulfurovum* sp. NBC37-1 (41), which suggests that both

220 clusters of *sox* genes are not recent additions to this epsilonproteobacterial lineage. In
221 fact, a phylogenetic analysis based on a large number of SoxB sequences from a variety
222 of sulfur-oxidizing bacteria is even suggestive of an origin of the sox system in
223 epsilonproteobacteria (36).

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

230 The genome also provided evidence for the ability to also use H_2 and formate as
231 electron donors (Fig. 2). Based on this information, *S. denitrificans* was successfully
232 cultivated with H_2 as its electron donor and nitrate as the electron acceptor (Sievert and
233 Molyneaux, unpublished data). The *S. denitrificans* genome encodes two Ni-Fe
234 hydrogenase systems: one cytoplasmic, and one membrane-bound hydrogenase complex.
235 The genes encoding the two subunits of the cytoplasmic enzyme (which lack TAT
236 motifs; *Suden_1437-8*) are adjacent to genes encoding the periplasmic hydrogenase (its
237 small subunit has a TAT motif, and a b-type cytochrome subunit would function to
238 anchor it to the membrane and shunt electrons to the quinone pool; *Suden_1434-6*). The
239 small subunit of the cytoplasmic hydrogenase of *S. denitrificans* forms a cluster with
240 sequences from the two deep-sea hydrothermal vent epsilonproteobacteria and *Aquifex*
241 *aeolicus* and is distantly related to H_2-sensing hydrogenases of alphaproteobacteria and
242 cyanobacteria (41). Nakagawa et al. (41) suggest that the cytoplasmic hydrogenase acts

243 as a H_2-sensing hydrogenase in *Sulfurovum* NBC37-1 and *Nitratiruptor* SB155-2.
244 However, an alternative, and in our view more likely, function for the cytoplasmic
245 enzyme as a catalytically active hydrogenase is suggested by the sequence similarity of
246 both subunits to the enzyme from *A. aeolicus*. In *A. aeolicus,* the cytoplasmic
247 hydrogenase can reduce electron acceptors with very negative redox midpoint potentials,
248 and therefore has been suggested to provide electrons with low potential electrons to the
249 reductive citric acid cycle (4), which would circumvent the necessity for reverse electron
250 transport and thus increase its growth efficiency, similar to what has been found in
251 certain Knallgas bacteria using the Calvin cycle for CO_2-fixation. Further experiments are
252 needed to confirm the actual role of the cytoplasmic hydrogenase. Following these
253 hydrogenase genes are several genes encoding hydrogenase-assembly related functions
254 (*Suden_1433-24).*
255 A formate dehydrogenase complex is encoded in an operon similar in gene order to
256 one found in *W. succinogenes* (*Suden_0816-24).* Formate dehydrogenase α subunits
257 contain a selenocysteine residue (26) which is encoded by a stop codon. A putative
258 selenocysteine codon (TGA) followed by a palindromic region was found between two
259 open reading frames (ORFS) that are homologous to the amino and carboxy ends of
260 formate dehydrogenase; accordingly, these ORFS have been combined into a single
261 coding sequence for the α subunit of this enzyme, *Suden_0820*, which includes the
262 molybdopterin-binding and iron-sulfur cluster domains typically found in this subunit, as
263 well as the TAT-pathway signal sequence which would shunt this subunit to the
264 periplasm. The β and γ subunits are encoded by *Suden_0819* and *Suden_0818,*
265 respectively, with the latter having an unprecedented N-terminal addition with two more

266 predicted transmembrane segments (making it six in total). *Suden_0824* encodes a
267 ferredoxin, which may shuttle the electrons from formate oxidation to cellular processes.
268 Formate dehydrogenase maturation is likely facilitated by the products of *Suden_0823,*
269 which encodes a TorD family protein that functions in molybdoprotein formation, and
270 *Suden_0817*, which encodes a FdhD/NarQ family maturation protein. *Suden_0816*
271 encodes a protein belonging to the aminotransferase class V PFAM, as does the SelA
272 protein, which catalyzes a step in selenocysteinyl-tRNA synthesis. Other genes likely to
273 be involved in selenocysteine synthesis are encoded nearby (*Suden_0830:*
274 selenophosphate synthase; *Suden_0831:* L-seryl-tRNA selenium transferase;
275 *Suden_0832:* selenocysteine-specific translation elongation factor SelB). Interestingly,
276 the hydrothermal vent epsilonproteobacteria do not appear to have this complex. Though
277 a homolog to the α subunit of formate dehydrogenase was present in both *Nitratiruptor*
278 SB155-2 and *Sulfurovum* sp. NBC37-1, genes encoding the other subunits were not
279 apparent from BLASTp searches of their genomes. In addition, *S. denitrificans* has a gene
280 encoding the large subunit of a formate dehydrogenase H (*fdhF*, *Suden_1902*), which is
281 most similar to the one in *W. succinogenes* (WS0126). However, its function and
282 substrate are not apparent based on its sequence or genomic context.
283
284 **Electron acceptors.** All genes required for the complete reduction of nitrate to N_2
285 are present (Fig. 2). However, *S. denitrificans* has some notable modifications compared
286 to the canonical denitrification pathway. Similar to *Bradyrhizobium japonicum*, *S.*
287 *denitrificans* appears to have only a periplasmic nitrate reductase (Nap) and not a
288 cytoplasmic membrane-bound nitrate reductase (Nar) (2), which is present in most

289 organisms producing N_2 from nitrate (52). The *nap* gene cluster (*NapABHGFLD;*
290 *Suden_1514-1519, 1521*) has the same arrangement as the one identified in *W.*
291 *succinogenes*, which is unusual in that it lacks a gene encoding the NapC subunit (64).
292 Possibly, *S. denitrificans* is able to denitrify under microaerobic conditions, as the Nap
293 enzyme has been implicated in aerobic denitrification (38). Nitrite reduction to nitric
294 oxide is likely catalyzed by a cytochrome cd_1-dependent nitrite reductase (*nirS, nirF*;
295 *Suden_1985, 1988*), whose genes are present in a gene cluster (*Suden_1976-1989*) that
296 also includes siroheme synthesis genes and two genes annotated as *norCB* (*Suden_1983-*
297 *1984*).
298 The *norCB* genes, which encode the small and large subunits of nitrous oxide-
299 forming nitric oxide reductase (cNOR), a member of the heme-copper oxidase (HCO)
300 superfamily (16), are usually clustered together with additional genes required for
301 enzyme assembly and activation (76). These latter genes, *norD* and *norQ*, are missing
302 from the *S. denitrificans* genome. While there is evidence for functional cNOR in
303 bacteria that lack the *norQ/cbbQ* gene, there are presently no experimental reports that
304 demonstrate functionality of cNOR in bacteria that also lack the *norD* gene. Attempts to
305 test this for *Hydrogenobacter thermophilus* using *Pseudomonas aeruginosa* or
306 *Escherichia coli* as an expression hosts were inconclusive (66). The *S. denitrificans*
307 *norCB* genes are closest related by sequence similarity to the *norCB* genes in the
308 genomes of *H. thermophilus, Methylococcus capsulatus* as well as *Sulfurovum* sp.
309 NBC37-1 and *Nitratiruptor* sp. SB155-2, the latter of which are two newly sequenced
310 marine epsilonproteobacteria (41). All of these genomes also lack the *norD* gene.
311 Even though the functionality of cNOR is questionable, *S. denitrificans* is a

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

321 Interestingly, the *S. denitrificans* genome also encodes a previously unidentified
322 member of the HCO superfamily that is also a candidate for catalyzing nitric oxide
323 reduction. These HCO genes follow a set of pseudogenes normally involved in nitrate
324 reduction (*Suden_0100-0102*). Based on structural modeling and genome analysis it is
325 expected that this new HCO family is a novel non-electrogenic quinone-oxidizing nitric
326 oxide reductase, gNOR (J. Hemp, M. G. Klotz, L. Y. Stein, R. B. Gennis, unpubl. data).
327 The gNOR family, encoded by the *norGHJ* genes (*Suden_0103-0105*), is unique within
328 the HCO superfamily in that it exhibits a novel active-site metal ligation, with one of the
329 three conserved histidine ligands being replaced with an aspartate. This ligation pattern
330 strongly suggests that the active-site metal is an iron. Structural modeling of members of
331 the gNOR family has identified three conserved acidic residues, which form a charged
332 pocket within the active site, a feature shared with the cNOR family (49). Besides *S.*
333 *denitrificans,* gNOR also appears to be present in *Sulfurovum* sp. NBC37-1 and
334 *Persephonella marina* strain EX-H1, whereas it is missing in *Nitratiruptor* sp. SB155-2.

335 Since phylogenetic analysis demonstrates that nitric oxide reductase activity has evolved
336 multiple times independently within the heme-copper superfamily, these shared features
337 between the distantly related gNOR and cNOR families are interesting examples of
338 convergent evolution (J. Hemp, M. G. Klotz, L. Y. Stein, R. B. Gennis, unpubl. data).
339 Nitrous oxide reduction is carried out by nitrous oxide reductase encoded by an
340 unusual *nos* gene cluster (*Suden_1298-1308)* similar to one previously identified in *W.*
341 *succinogenes* (63). As in *Wolinella*, the NosZ in *S. denitrificans* (*Suden_1298)* contains a
342 C-terminal extension of about 200 residues that carries a monoheme cytochrome c
343 binding motif (CXGCH), suggesting it, too, functions as a cytochrome c nitrous oxide
344 reductase (cNOS; Fig. 2). This feature is also shared by NosZ of *Sulfurovum* sp. NBC37-
345 1 and *Nitratiruptor* sp. SB155-2, which form a cluster with NosZ from *S. denitrificans*
346 (Fig. S3)*,* possibly allowing the design of primers to screen for the presence of
347 denitrifying epsilonproteobacteria in the environment. It has been hypothesized that the
348 *nos* gene cluster in *W. succinogenes* codes for proteins involved in an electron transport
349 chain from menaquinol to cytochrome c nitrous oxide reductase (63), and it is likely that
350 the same holds true for *S. denitrificans,* as well as *Sulfurovum* sp. NBC37-1 and
351 *Nitratiruptor* sp. SB155-2. In addition, *S. denitrificans* has an almost identical copy of
352 *nosZ* next to a c553-type monoheme cytochrome c (*Suden_1770, 1769*), but its function
353 is at this point unknown. Interestingly, *S. denitrificans* also has a gene coding for a large
354 subunit of a ferredoxin-nitrite reductase (*nirB*; *Suden_1241*), which could be involved in
355 nitrite assimilation or detoxification (8). However, no gene coding for the small subunit
356 was identified, raising questions about its function.
357 Additional electron acceptors are suggested by this organism's gene complement

358 (Fig. 2). Like some other delta- and epsilonprotreobacteria, the *S. denitrificans* genome
359 contains a cluster of four genes (*Suden_0081-0084*) that encode the FixNOQP proteins,
360 which constitute a proton-pumping cbb_3- type cytochrome c oxidase., suggesting an
361 ability to use oxygen as a terminal electron acceptor. This is somewhat unexpected, since
362 *S. denitrificans* was originally described as an obligate denitrifier and is quite sensitive to
363 oxygen (S. Sievert, unpubl. data). *Cbb3*-type cytochrome c oxidase complexes have
364 extremely high affinities for oxygen (48), which might allow this organism to use oxygen
365 as an electron acceptor under extremely low oxygen tensions, or alternatively, to
366 scavenge oxygen to prevent poisoning. Another possibility might be the involvement of
367 *cbb3*-type cytochrome c oxidase in the catalytic reduction of NO (14). It is interesting,
368 that, in line with other epsilonproteobacteria, the genome does not contain genes
369 encoding FixGHIJ, which are present in all other bacteria that express a cbb_3-type
370 cytochrome c oxidase and are involved in assembly and maturation of the cbb_3-type
371 cytochrome c oxidase complex (33, 44). The reasons for the absence of *fixGHIJ* in
372 epsilonproteobacteria remain unkown, and it needs to be tested experimentally what the
373 actual role of cbb_3-type cytochrome c oxidase in *S. denitrificans* is.
374 Additionally, *S. denitrificans, Nitratiruptor* sp. SB155-2, and *Sulfurovum* sp.
375 NBC37-1 may be able to use sulfur compounds as terminal electron acceptors. Genes
376 *Suden_0498-0500* encode an Fe/S-protein, a NrfD-related membrane anchor (8 helices),
377 and an unusually long molybdopterin-containing oxidoreductase that contains a twin-
378 arginine translocation pathway signal, respectively; homologs of these three genes are
379 also encoded adjacent to each other in the two hydrothermal vent epsilonproteobacteria.
380 Together these proteins could form a periplasm-facing membrane-bound complex that is

381 most likely involved in the reduction of sulfur compounds like elemental sulfur,
382 polysulfide, thiosulfate or tetrathionate. However, only a limited number of these
383 molybdopterin-containing oxidoreductases have been characterized and it is presently not
384 possible to infer substrate specificity from the primary structure. Further experiments are
385 needed to confirm the substrate used by this complex.
386
387 **Carbon fixation and central carbon metabolism.** Genes encoding the enzymes
388 of the reductive citric acid cycle are apparent (Fig. 3), which is consistent with prior
389 biochemical and genetic analyses of this organism (24). Of particular note are genes
390 encoding the enzymes necessary for the cycle to operate in the reductive direction:
391 pyruvate:acceptor oxidoreductase (*Suden_0096 – 0099,* based on similarity to
392 biochemically characterized orthologs in *H. pylori* (23)), pyruvate carboxylase
393 (*Suden_0622, Suden_1259*, based on biochemically characterized orthologs from *C.*
394 *jejuni* (72), but see below), 2-oxoglutarate:ferredoxin oxidoreductase (*Suden_1052 – 55*,
395 as for pyruvate:acceptor oxidoreductase, based on (23)), and ATP-dependent citrate lyase
396 (*Suden_0570, Suden_0571*)(24). Also noteworthy is the presence of two copies of
397 succinate dehydrogenase/fumarate reductase, one of which has a subunit that would
398 anchor it to the membrane (*Suden_1028 - 1030*), while the other lacks this subunit and
399 may be cytoplasmic (*Suden_0037, Suden_ 0038*). The membrane-bound form is unusual
400 in that it has a cysteine-rich, type-E membrane anchor. It is similar to SdhABE from *W.*
401 *succinogenes* which has been characterized as a membrane-bound fumarate-reducing
402 complex with subunits SdhAB facing the periplasm. In contrast to *W. succinogenes*, the
403 *S. denitrificans* gene encoding subunit A is about 43 residues shorter and lacks the TAT

404 signal peptide present in its *W. succinogenes* homolog, something that appears to be
405 shared with *Sulfurovum* sp. NBC37-1 and *Nitratiruptor* sp. SB155-2, both of which are
406 also chemolithoautotrophic epsilonproteobacteria using the reductive citric acid cycle for
407 carbon fixation. Thus, it appears that in these organisms the membrane-bound fumarate-
408 reducing complex faces into the cytoplasm. At present the exact function of the two
409 fumarate reductases is unknown, although it is likely that the membrane-bound one, due
410 to its potential for additional energy generation, might be involved in the reductive citric
411 acid cycle for autotrophic carbon fixation. The intriguing possibility that *S. denitrificans*
412 might also be able to carry out fumarate respiration has to await further experimentation.
413 However, *S. denitrificans* does not contain a *frdCAB* operon typical for
414 menaquinol:fumarate reductase sustaining fumarate respiration in other
415 epsilonproteobacteria.

416 The acetyl-CoA and oxaloacetate produced by the reductive citric acid cycle
417 could be funneled to central carbon metabolism: acetyl-CoA could be converted to
418 pyruvate via pyruvate:acceptor oxidoreductase (see above) and oxaloacetate could be
419 used to form phosphoenolpyruvate via phosphoenolpyruvate carboxykinase
420 (*Suden_1696*). Acetyl-CoA could also be directed to fatty acid synthesis (acetyl-CoA
421 carboxylase; *Suden_1174; Suden_1608*). Genes are present that suggest an ability to
422 supplement autotrophic growth with acetate assimilation in all three sulfur-oxidizing
423 epsilonproteobacteria. Two possible systems for converting acetate to acetyl-CoA are
424 present: acetyl-CoA ligase (*Suden_1451*), as well as phosphate acetyltransferase
425 (*Suden_0055*) and acetate kinase (*Suden_0056*), and are also present in *Sulfurovum* sp.
426 NBC37-1, while acetate kinase is absent in *Nitratiruptor* sp. SB155-2. Perhaps the two

427 systems have different affinities for acetate, as has been demonstrated in methanogens
428 (60), and are differentially expressed depending on environmental concentrations of this
429 organic acid.

430 In order for *S. denitrificans* to grow autotrophically using the reductive citric acid
431 cycle, there must be a means of carboxylating pyruvate to form oxaloacetate. In some
432 organisms this is accomplished by the tandem activities of phosphoenolpyruvate
433 synthetase and phosphoenolpyruvate carboxylase, while others use pyruvate carboxylase
434 (55). In contrast to the two autotrophic epsilonproteobacteria *Sulfurovum* NBC37-1 and
435 *Nitratiruptor* SB155-2*,* which have genes encoding phosphoenolpyruvate synthase as
436 well as pyruvate kinase (41), the latter of which is usually involved in ATP synthesis
437 during glycolysis, *S. denitrificans* does not appear to have any genes that might encode an
438 enzyme that could interconvert phosphoenolpyruvate and pyruvate. Instead, it may use
439 pyruvate carboxylase, as genes encoding both the biotin carboxylase subunit
440 (*Suden_0622*) and biotin carboxyl carrier subunit (*Suden_1259)* of this enzyme are
441 present. The biotin carboxyl carrier subunit gene (*Suden_1259*) occurs in an apparent
442 operon with other genes homologous to the subunits of sodium-transporting oxaloacetate
443 decarboxylase (*Suden_1258 – 60*). *Suden_1259*, which encodes the α subunit of this
444 complex, has a high level of sequence similarity with pyruvate carboxylase genes from
445 various *Campylobacter* species (including one from *C. jejuni* which has been
446 biochemically characterized) (72), while the β and γ subunits (*Suden_1258* and *1260*),
447 which are absent from the heterotrophic epsilonproteobacteria, are similar to those found
448 from *Sulfurovum* sp. NBC37-1, *Nitratiruptor* sp. SB155-2, many gammaproteobacteria,
449 many *Chlorobia*, and *Desulfotalea psychrophila*, a deltaproteobacterial sulfate reducer.

450 Heterotrophic organisms that have this complex ferment citrate. After cleaving citrate to
451 acetate and oxaloacetate, they use the oxaloacetate decarboxylase complex to couple the
452 exothermic decarboxylation of this organic acid to the extrusion of sodium ions. For these
453 organisms, the other genes necessary for citrate fermentation (e.g., citrate transporter) are
454 encoded nearby (9). This is not the case in *S. denitrificans*. An alternative function for
455 *Suden_1259* is suggested by phylogenetic analysis, which places it within a clade with
456 the biochemically characterized pyruvate carboxylase from *C. jejuni* (Fig. 4) and separate
457 from biochemically characterized oxaloacetate decarboxylase genes from *Klebsiella*
458 *pneumoniae* and *Vibrio cholerae* (5). Other members of this clade include the genes from
459 the *Chlorobia, Sulfurovum* sp. NBC37-1, and *Nitratiruptor* sp. SB155-2, which also use
460 the reductive citric acid cycle for carbon fixation and are not known to ferment citrate. It
461 is possible to operate the oxaloacetate decarboxylase complex as a pyruvate carboxylase
462 by imposing a sodium gradient across the membrane (10). It is tempting to speculate that
463 in the autotrophic epsilonproteobacteria, the *Chlorobia*, and possibly *D. psychrophila,*
464 this complex functions as a pyruvate carboxylase. Interestingly, the sequenced
465 autotrophic epsilonproteobacteria and *Desulfotalea psychrophila* are all marine
466 organisms, and the *Chlorobia* evolved in the marine environment (1). Only five of the ten
467 sequenced *Chlorobia* have the sodium-transporting oxaloacetate decarboxylase/pyruvate
468 carboxylase complex, while the other five, including *C. tepidum,* have the alpha subunit
469 (on which Fig. 4 is based), but not the three-subunit pump. With the exception of *C.*
470 *limicola*, which was isolated from a mineral hot spring, all *Chlorobia* containing the
471 sodium pump have a requirement for sodium. Thus, it is likely that these organisms have
472 found a way to couple pyruvate carboxylation, which is energetically unfavorable, to a

473 sodium gradient, something that was previously proposed, but never shown in an
474 organism (10). Whether the complex encoded by *Suden_1258-1260* functions as a
475 pyruvate carboxylase or an oxaloacetate decarboxylase is a key point begging
476 clarification, which will be nontrivial, given that a genetic system has not been developed
477 in this organism.

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

483 Genes are apparent whose products could utilize the carbon skeletons synthesized
484 by central carbon metabolism for ammonia assimilation (see below) and amino acid,
485 nucleotide, fatty acid, and phospholipids synthesis. Cysteine biosynthesis is notable, in
486 that the reduction of sulfate proceeds via adenosine 5'-phosphosulfate (APS) rather than
487 3′-phosphoadenylylsulfate (PAPS) in a pathway that was until recently only known from
488 plants (42). Genes encoding assimilatory sulfate reduction co-occur in an apparent operon
489 (*Suden_0154-0160)*. Most likely this operon-like structure is turned on or off depending
490 on whether *S. denitrificans* is inhabiting an environment with a high concentration of
491 reduced inorganic sulfur compounds. Interestingly, sulfate assimilation in *Sulfurovum* sp.
492 NBC37-1 and *Nitratiruptor* SB155-2 appears to proceed via PAPS.

493

494 **Nitrogen assimilation.** The *S. denitrificans* genome contains *nirC* (*focA*) and
495 *nirB* genes encoding the formate-nitrite transporter (*Suden_0716*) and the large subunit of

496 NAD(P)H-dependent ammonia-forming siroheme nitrite reductase (*Suden_1241*),
497 respectively, along with the inventory for siroheme synthesis (*Suden_1977*, *cysG*,
498 siroheme synthase; *Suden_1988*, *cobA-cysG*, uroporphyrinogen III methylase); however,
499 it lacks the *nirD* gene, which encodes the small subunit of siroheme nitrite
500 reductase. Because the genome also lacks *nrfHA* genes, which encode respiratory nitrite
501 ammonification capacity in many delta- and epsilonproteobacteria (62), it appears that
502 *Sulfurimonas* is solely dependent on ammonia uptake from the environment. The genome
503 contains, indeed, two genes encoding different ammonia permeases (noted above) (22,
504 43), one AmtB-like (*Suden_0641*) and one Rhesus factor-like (*Suden_0643*) permease,
505 which are clustered together with the gene encoding nitrogen regulatory protein PII
506 (*glnK*, *Suden_0642*). Whereas AmtB proteins function as ammonia gas uptake channels,
507 the substrate for Rh-like protein channels is still debated and includes ammonia as well as
508 CO_2 (43). The genome contains also all the additional genes needed for 2-oxoglutarate-
509 sensing and regulation of nitrogen assimilation (reviewed in (34)).
510
511 **Chemotaxis and other regulatory and signaling proteins.** Close relatives of *S.*
512 *denitrificans* are motile , while this particular strain is nonmotile, probably due to the
513 interruption of a flagellar biosynthetic operon by a transposon (see Genome Structure,
514 above). Based on the presence of all of the genes necessary to encode the flagellar
515 apparatus, none of which display any evidence of degeneration, an abundant sensory
516 apparatus necessary to detect the presence of chemoattractants or repellants, and
517 communicate this information to the flagellar motor, as well as the sequence identity of
518 this transposon with a duplicate in the genome (see above), it is likely that nonmotility is

519 a recently acquired property. Interestingly, many of the genes encoding the chemotaxis
520 components are in a large cluster with multiple kinases and response regulators (Fig. 5),
521 as in *Nitratiruptor* sp. SB155-2 (41), suggesting interconnectivity between the
522 chemotaxis and other signal transduction systems. Perhaps the original enrichment and
523 isolation procedure for this strain (in a chemostat) might have selected for a non-motile
524 strain.

525 The *S. denitrificans* genome encodes a relative abundance of signaling proteins.
526 Particularly well-represented in these genomes are genes encoding proteins with EAL
527 and GGDEF domains (based on hits to PFAMS, 16 and 38 genes, respectively), which
528 likely function in the synthesis and hydrolysis of the intracellular signaling compound
529 cyclic diguanylate (53). Further, six proteins with PAS/PAC-domains are encoded, which
530 may function as redox sensors (75). The genomic repertoire of signaling and regulatory
531 genes was compared with two other free-living, obligate chemolithoautotrophs for which
532 these data are available (Table 2). Some features are similar to *Thiomicrospira*
533 *crunogena*: both of these species have a relative abundance of signal transduction
534 proteins compared to *Nitrosococcus oceani* (31), which may be a response to more
535 spatially (sediments; *S. denitrificans)* or temporally (hydrothermal vents; *T. crunogena*)
536 heterogeneous habitats, compared to the open ocean (*N. oceani*). Both have a large
537 number of genes encoding methyl-accepting chemotaxis proteins (MCPs; Table 2).
538 Unlike *T. crunogena,* none of the MCPs from *S. denitrificans* are predicted to have
539 PAS/PAC domains that could bind redox-sensitive cofactors (57), but a gene encoding a
540 protein with a PAS/PAC domain is present in the large cluster of chemotaxis genes
541 described above (Fig. 5), suggesting potential communication between sensing cellular or

542 environmental redox conditions and the chemotactic apparatus. Another similarity
543 between *T. crunogena* and *S. denitrificans* is an abundance of genes predicted to be
544 involved with cyclic nucleotide signal transduction, and many of these are predicted to
545 have EAL and/or GGDEF domains, indicating a role for cyclic diguanylate in
546 intracellular signaling in this organism. Many of these predicted proteins also have
547 PAS/PAC domains, as in *T. crunogena* (57).

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

556

557 **Oxidative stress.** *S. denitrificans* has reasonably elaborate defenses against
558 oxidative stress, on par with what has been observed for pathogenic
559 epsilonproteobacterium *H. pylori* (73). Initially this was considered surprising to us,
560 because this nonpathogenic species does not have to elude the oxidative arsenal of a host
561 immune system, nor, as a microaerophile capable of growth via denitrification, does it
562 grow in the presence of high concentrations of oxygen. However, given the presence of
563 enzymes with labile iron-sulfur clusters with irreplaceable roles in central carbon
564 metabolism in this organism (e.g., pyruvate:acceptor oxidoreductase; *Suden_0096* –

565 *0099;* 2-oxoglutarate:acceptor oxidoreductase; *Suden_1053 - 1055*), perhaps added
566 defenses are a necessary part of survival.
567 Several genes are present whose products could prevent the buildup of
568 intracellular Fe^{2+} that can spur hydroxyl radical generation via the Fenton reaction. A
569 gene is present that encodes the Fur protein (*Suden_1272*), which regulates iron uptake
570 (14). A gene encoding iron-binding ferretin (*Suden_1760*) is also present.
571 Many enzymes to detoxify reactive oxygen and nitrogen species are encoded in
572 this genome. An iron/manganese superoxide dismutase (*Suden_1129)* is present in this
573 species, but not the other sulfur-oxidizing epsilonproteobacteria (41). This enzyme and
574 ruberythrin (*Suden_0739)* could convert superoxide to hydrogen peroxide, and this
575 superoxide could in turn be dispatched by catalase (*Suden_1323)*, peroxiredoxins
576 *(Suden_0132, Suden_0630, Suden_1778, Suden_1803)*, or cytochrome c peroxidase
577 *(Suden_0214; Suden_1585).* Peroxiredoxins, particularly alkylhydroperoxide reductases,
578 might be specifically targeted towards low levels of H_2O_2 or organic peroxides (58, 73).
579 To dispense with nitric oxide that escapes the periplasmic and membrane-associated
580 respiratory nitrogen reduction complexes, a truncated hemoglobin gene is present
581 *(Suden_0993)*, which may convert nitric oxide to nitrate (46). Thioredoxins
582 (*Suden_0342; Suden_0501; Suden_1867; Suden_2099)* and thioredoxin reductase
583 *(Suden_1869)* could funnel electrons to these oxidative stress proteins, as glutathione
584 does not appear to play this role in this organism, since genes encoding glutathione
585 synthetase or gamma-glutamate-cysteine ligase are absent.
586 Genes are apparent whose products could enable a cell to cope with damage
587 inflicted by any reactive oxygen or nitrogen species (ROS and RNS) that escape cellular

588 defenses. Endonuclease III (*Suden_0516)* and IV *(Suden_1835)* and MutS (*Suden_0755)*
589 could repair oxidative DNA damage. Methionine sulphoxide reductase *(Suden_0012)* and
590 alkylhydroxide reductase *(Suden_1778)* could contend with any methionine residues or
591 lipids that had been oxidized by interaction with ROS or RNS, while nitroreductases
592 (*Suden_0519; Suden_1158)* could prevent oxidized cellular nitrogenous and other
593 compounds from generating peroxide.
594
595 **Conclusions**. *S. denitrificans* has several unique features which differentiate it
596 from the other epsilonproteobacteria that have been sequenced to date. It has a larger
597 genome than most of the others, which likely provides the sensory, regulatory, and
598 metabolic versatility necessary for survival in a habitat more heterogeneous than found in
599 a metazoan host. For example, the numerous genes whose products have redox sensory
600 domains likely function to position these cells in the redoxcline to enable them to obtain
601 the electron donors and acceptors needed for growth. Furthermore, these cells are quite
602 versatile with respect to electron donors and acceptors, as the genome data suggest a
603 capability of using donors and acceptors beyond those based on cultivation studies.
604 Although *S. denitrificans* has been isolated from coastal marine sediments, its genome
605 shares many features with two recently described autotrophic deep-sea hydrothermal vent
606 epsilonproteobacteria, including the potential to utilize a variety of redox substrates
607 (hydrogen gas, reduced sulfur compounds, oxygen, and nitrate), its responses to oxidative
608 stress and high metal content, and a genome size intermediate between the two. This
609 suggests that while these habitats appear at first strikingly different, they require similar
610 adaptations on the scale of the microbes. Several additional features, which are absent in

611 their hydrothermal vent relatives, are present in *S. denitrificans* that may be particularly
612 valuable in the sediment habitat. Their formate dehydrogenase complex would enable *S.*
613 *denitrificans* to utilize a major by-product of fermentation that would co-occur with it
614 should sediment organic carbon loads be high. The presence of the additional oxidative
615 stress protein (superoxide dismutase) may enable *S. denitrificans* to cope with diurnal
616 shifts in sediment oxygen concentration, and several additional RND-family efflux
617 pumps relative to hydrothermal vent epsilonproteobacteria suggest that survival in marine
618 sediments requires a degree of versatility and defense against environmental insult
619 beyond what is necessary at moderate temperatures at hydrothermal vents.
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622
623
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625
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879

880

881 **FIGURE LEGENDS**

882

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

890

891

892 FIG. 2. Model for electron transport in *Sulfurimonas denitrificans.* Abbreviations:
893 I—NADH dehydrogenase; II—succinate dehydrogenase/fumarate reductase; III—bc_1
894 complex; IV—cbb_3 cytochrome c oxidase; Cyt_n c—cytochrome c; CCP—cytochrome c
895 peroxidase; FDH—formate dehydrogenase; HYD—hydrogenase; MK—menaquinone;
896 MKH_2—menaquinol; Mo—molybdodenum containing cofactor; NAP—periplasmic
897 nitrate reductase; NIR—nitrite reductase; NOR—nitric oxide reductase; NOS—nitrous
898 oxide reductase; PM—plasma membrane; PSR—polysulfide reductase; SOX—sulfur
899 oxidation system. Candidate monoheme cytochromes *c* listed as "Cyt_x *c*" are
900 *Suden_0904*, *Suden_0741*, *Suden_0578* (all COG2863) as well as *Suden_0865*,
901 *Suden_1329* and *Suden_1112* (no COG assignment).

902

903

904 FIG. 3. Central carbon metabolism in *Sulfurimonas denitrificans.* Abbreviations:
905 2-OG—2-oxoglutarate; acCoA—acetyl-Coenzyme A; ACL—ATP-citrate lyase;
906 APFK—ATP-dependent phosphofructokinase; CS—citrate synthase; F6P—fructose 6-
907 phosphate; FBP—fructose 1,6-bisphosphate; FBPP—fructose 1,6 bisphosphate
908 phosphatase; FR—fumarate reductase; fum—fumarate; isocit—isocitrate; mal—malate;
909 MQ—menaquinone; OAA—oxaloacetate; PC—pyruvate carboxylase; PEP—
910 phosphoenolpyruvate; pyr—pyruvate; suc—succinate; suCoA—succinyl-Coenzyme A.
911

912 FIG. 4. Phylogenetic relationships of alpha-subunits of oxaloacetate
913 decarboxylase (OAD), pyruvate carboxylase (PVC), oxoglutarate carboxylase (OGC),
914 and type III pyruvate carboxylase to the product of *Suden_1259* of *Sulfurimonas*
915 *denitrificans*. Sequences were aligned using the program package MacVector. Neighbor-
916 joining and Parsimony trees based on the predicted amino acid sequences were calculated
917 using PAUP 4.0b10. Bootstrap values (1,000 replicates) for the major nodes are given for
918 the neighbor-joining (first value) and parsimony analyses (second value).
919

920 FIG. 5. A large gene cluster from the *Sulfurimonas denitrificans* genome that
921 includes many of the genes for chemotaxis signal transduction.
922
923
924
925

926

927

928 TABLE 1. Comparative genome features for epsilonproteobacteria[‡]

929

Species	Size (Mbp)	% coding	%GC	rRNA operons	# CDS
Sulfurimonas denitrificans DSM1251	2.20	93.8	34.5	4	2104
Sulfurovum sp. NBC37-1	2.56	90.1	43.8	3	2466
Nitratiruptor sp. SB155-2	1.88	95.1	39.7	3	1857
Campylobacter fetus 82-40	1.80	90.0	33.3	3	1719
Campylobacter jejuni NCTC 11168	1.64	95.4	30.6	3	1629
Campylobacter jejuni RM1221	1.78	91.8	30.3	3	1838
Helicobacter hepaticus ATCC 51449	1.80	93.4	35.9	1	1875
Helicobacter acinonychis Sheeba	1.55	89.0	38.2	2	1618
*Helicobacter pylori*26695	1.67	90.0	38.9	2*	1576
Helicobacter pylori J99	1.64	90.7	39.2	2*	1491
Helicobacter pylori HPAG1	1.59	91.0	39.1	2*	1544
Wolinella succinogenes DSM1740	2.11	94.5	48.5	3	2043

930

931 [‡]Data for all taxa, except for *Sulfurivom* sp. NBC37-1 and *Nitratiruptor* sp.

932 SB155-2 were collated from the Integrated Microbial Genomes webpage and had been

933 generated using consistent methodology. For *Sulfurivom* sp. NBC37-1 and *Nitratiruptor*

934 sp. SB155-2, data were collected from (41), for which slightly different methodologies

935 were used to identify coding sequences (CDS).

936 *In *H. pylori*, the 16S gene is not collocated with the 23S and 5S genes in an

937 operon. Additionally, an orphan 5S sequence is found in strain 26695

938

939

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

941 obligate chemolithoautotrophs*

942

Number:			
S. denitrificans	*T. crunogena*	*N. oceani*	Functional Description
56	72	104	Transcription/Elongation/Termination Factors
19	4	2	Two component transcriptional regulator, winged helix family
146	128	75	Signal Transduction proteins
28	27	8	Chemotaxis Signal Transduction proteins
13	14	1	Methyl-accepting chemotaxis proteins
118	101	67	Non-Chemotaxis Signal Transduction
36	17	18	Signal Transduction Histidine Kinase
42	49	16	Cyclic nucleotide signal transduction
202	200	179	Total

943 **Sulfurimonas denitrificans* DSM1251 is compared to gammaproteobacteria

944 *Thiomicrospira crunogena* XCL-2 and *Nitrosococcus oceani* ATCC 19707.

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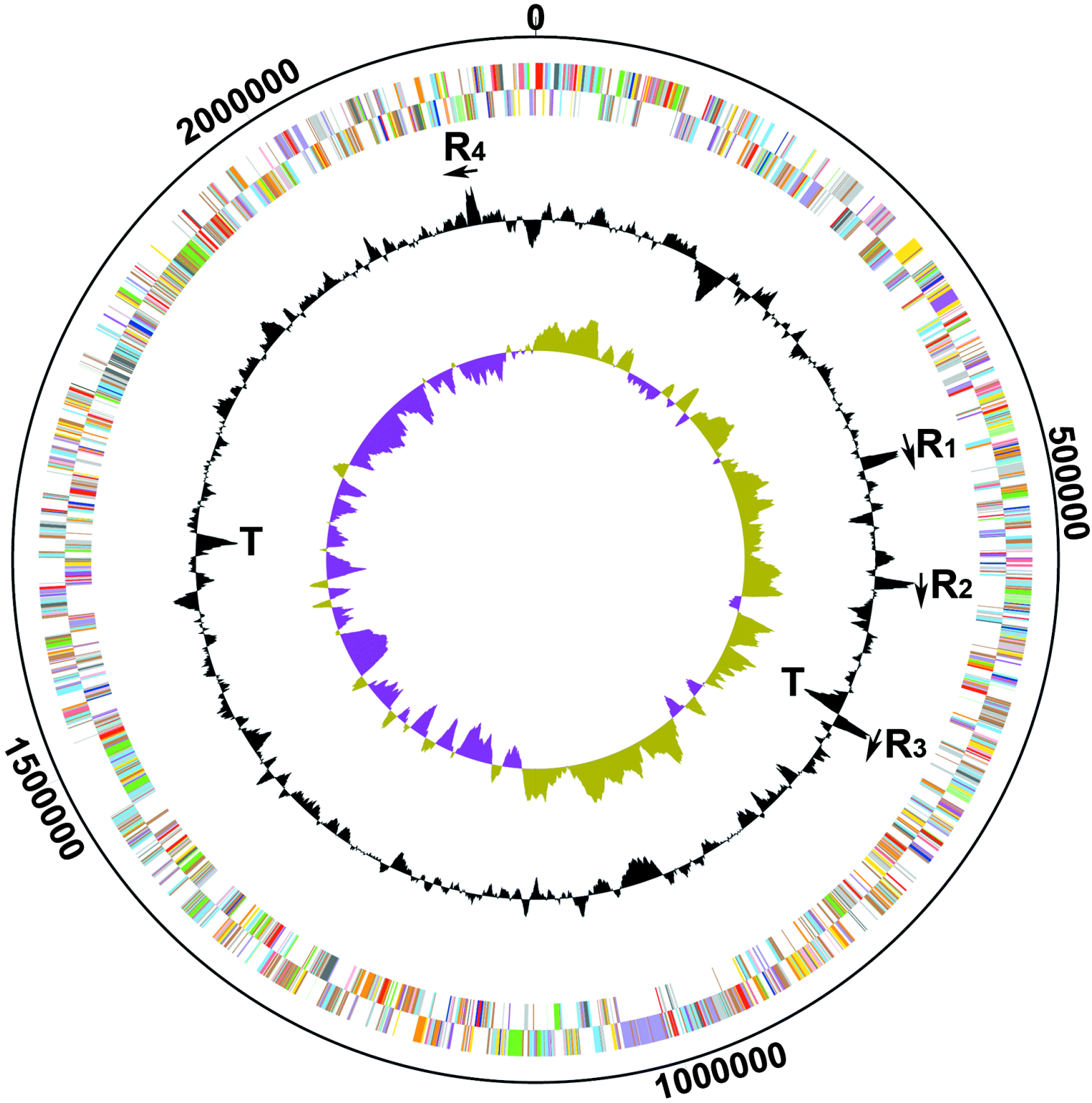
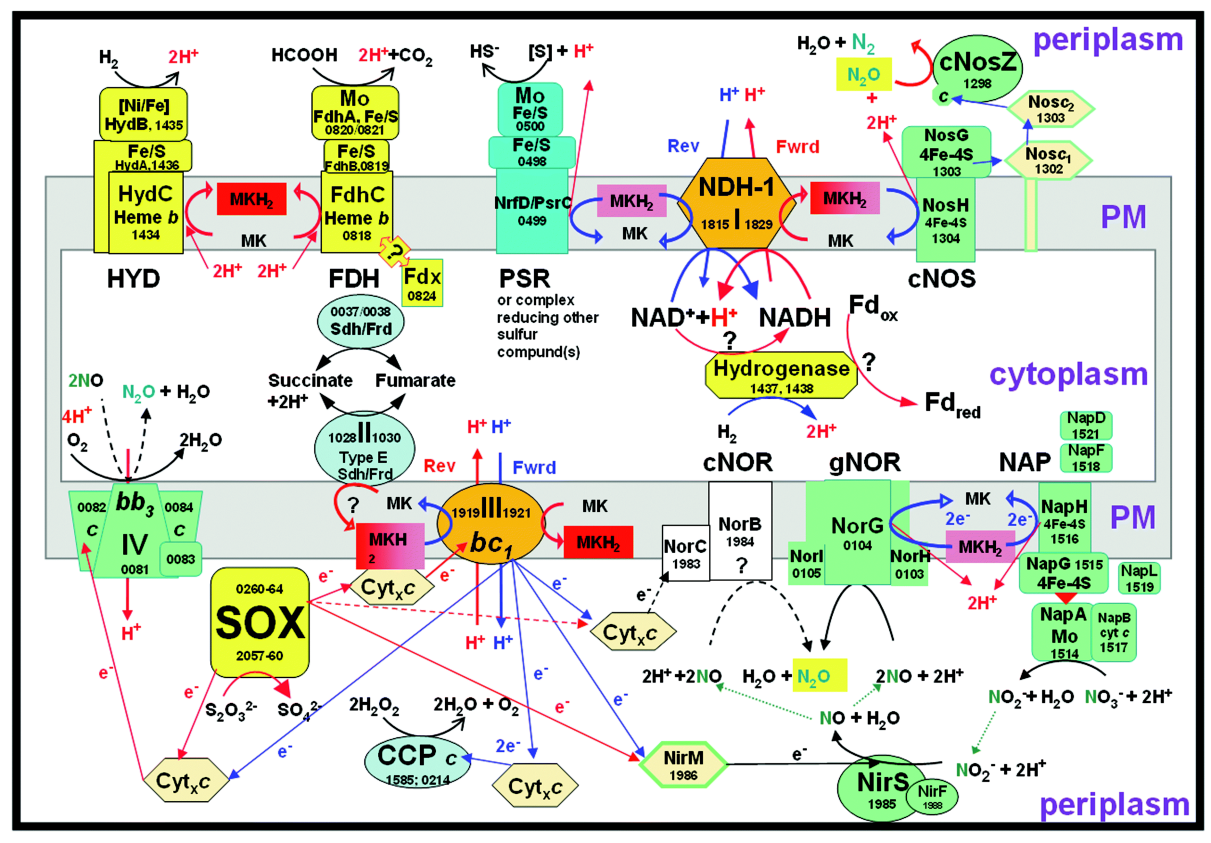


Hypothetical proteins
Cons. hypothetical proteins
General function prediction
Replication and repair
Energy metabolism
Carbon, carbohydrate metab.
Lipid metabolism
Transcription
Translation
Amino acid metab.
Cofactors and vitamins
Purines and pyrimidines
Signal transduction
Cellular processes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cytoplasm
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HydC
Heme b
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MKH2
MK
2H+
2H+
HYD
HCOOH
2H+ +CO2
Mo
FdhA, Fe/S
0820/0821
Fe/S
FdhB, 0819
FdhC
Heme b
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Fdx
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FDH
0037/0038
Sdh/Frd
Succinate
+2H+
Fumarate
HS-
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NrfD/PsrC
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or complex
reducing other
sulfur
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Fwrd
NDH-1
1815 | 1829
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NAD+ +H+
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NADH
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Hydrogenase
1437, 1438
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Fdred
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2H+
H2O + N2
N2O
+
2H+
cNosZ
1298
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Nosc2
1303
Nosc1
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NosG
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1303
NosH
4Fe-4S
1304
cNOS
2NO
N2O + H2O
4H+
O2
2H2O
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Type E
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NAP
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4Fe-4S
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4Fe-4S
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NapA
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NapB
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SOX
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S2O3 2-
SO4 2-
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2H2O + O2
CCP c
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H2O + N2O
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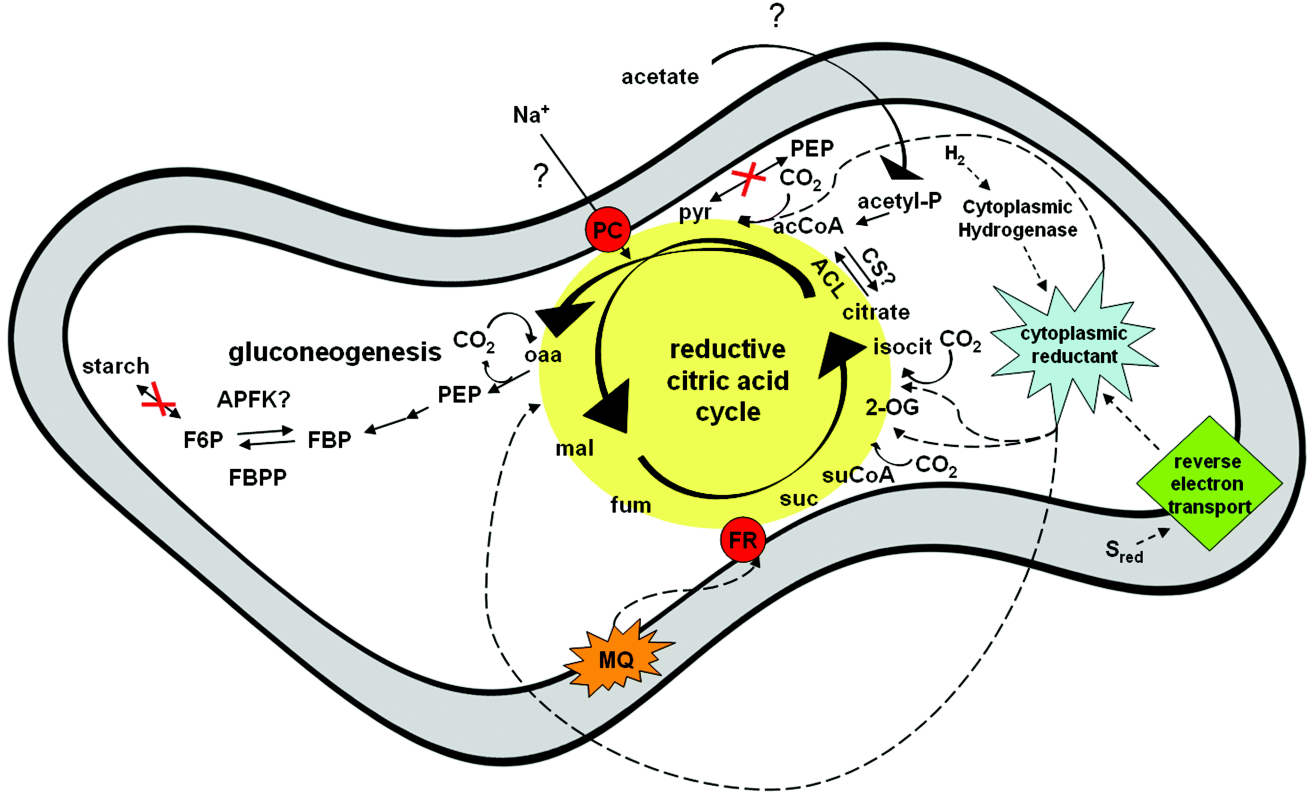
NirF
1988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PEP
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Hydrogenase
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CS?
citrate
cytoplasmic
reductant
gluconeogenesis
CO2
oaa
reductive
citric acid
cycle
isocit
CO2
starch
APFK?
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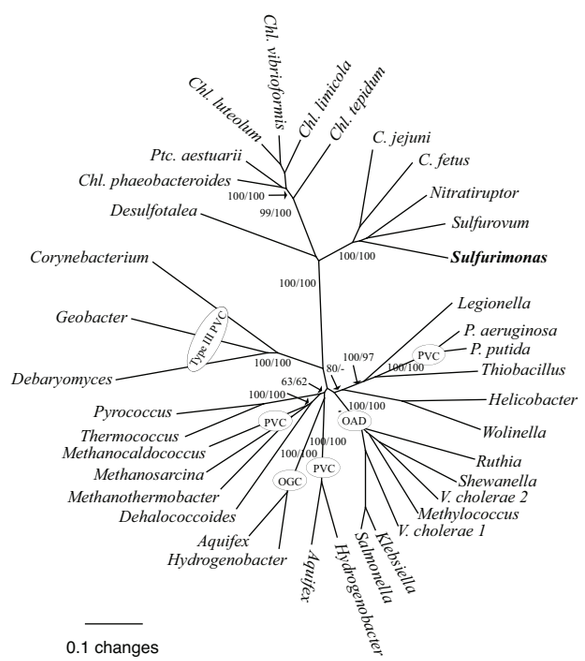
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Ptc. aestuarii
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Nitratiruptor
Desulfotalea
Sulfurovum
Sulfurimonas
Corynebacterium
Legionella
Geobacter
P. aeruginosa
P. putida
Type III PVC
PVC
Thiobacillus
Debaryomyces
Helicobacter
Pyrococcus
OAD
Wolinella
Thermococcus
Methanocaldococcus
Ruthia
Methanosarcina
Shewanella
Methanothermobacter
V. cholerae 2
Methylococcus
Dehalococcoides
V. cholerae 1
OGC
Aquifex
Hydrogenobacter
Aquifex
Hydrogenobacter
Salmonella
Klebsiella
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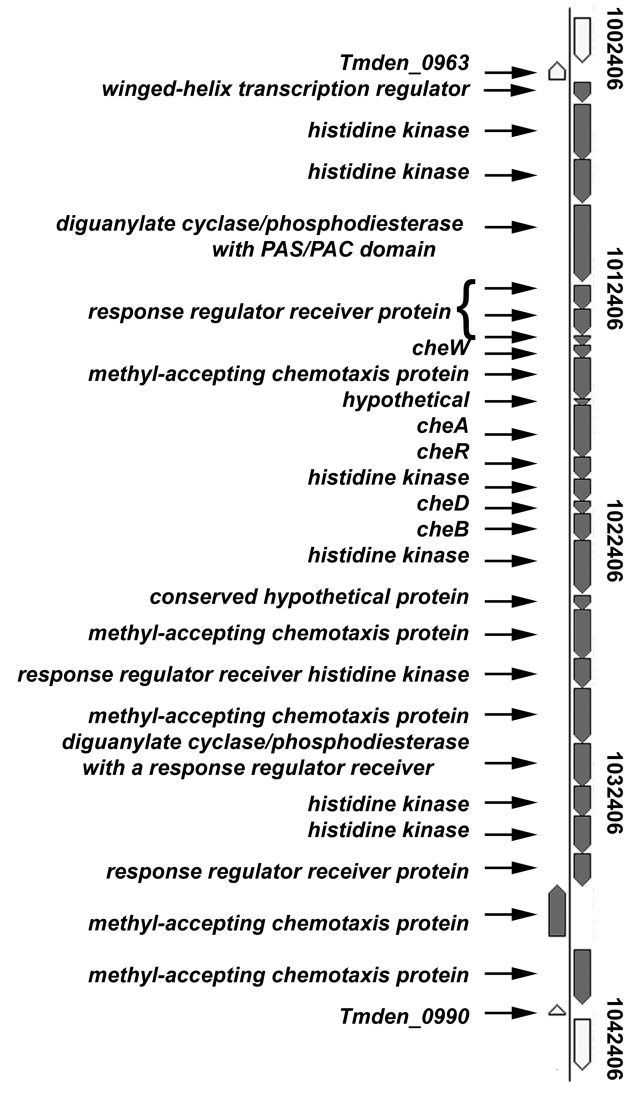
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Tmden_0963
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histidine kinase
histidine kinase
diguanylate cyclase/phosphodiesterase
with PAS/PAC domain
response regulator receiver protein
cheW
methyl-accepting chemotaxis protein
hypothetical
cheA
cheR
histidine kinase
cheD
cheB
histidine kinase
conserved hypothetical protein
methyl-accepting chemotaxis protein
response regulator receiver histidine kinase
methyl-accepting chemotaxis protein
diguanylate cyclase/phosphodiesterase
with a response regulator receiver
histidine kinase
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response regulator receiver protein
methyl-accepting chemotaxis protein
methyl-accepting chemotaxis protein
Tmden_0990

1 **SUPPLEMENTAL MATERIAL**

2 **Further details of genome structure.** In addition to the large transposon
3 interrupting one of the flagellar biosynthetic operons (Fig. S1), another transposase gene
4 (*Tmden_1713*) is located near a $tRNA^{Thr}$ gene, adjacent to a hypothetical protein gene
5 (*Tmden_1712)* whose 3'-end is 82% identical, at the nucleotide level, with two
6 transposase genes located downstream (*Tmden_1724* and *Tmden_1725*). These two
7 genes, and regions 5' and 3' of each (totaling 1302 bp apiece), are 100% identical to each
8 other. This region also includes genes encoding a recombinase and phage integrase
9 (*Tmden_1723;* Fig. S2); the presence of the phage integrase gene, identical repeats, and
10 juxtaposition to a tRNA gene suggest that this portion of the genome may be a remnant of
11 a degraded or partially excised prophage. Other potential tranposase genes are present
12 (*Tmden_0961; Tmden_1698; Tmden_1708)*, but have insufficient sequence similarity to
13 known proteins for deducing their function convincingly.

14 Twelve phage integrase genes are present. As expected for phage genes, six of
15 these are near tRNA genes (*Tmden_0248, Tmden_0779, Tmden_0800, Tmden_1618,*
16 *Tmden_1723; Tmden_1743)* which are common insertion sites for lysogenic phages (1),
17 and three are flanked by clusters of genes encoding hypothetical proteins *(Tmden_1247;*
18 *Tmden_1618; Tmden_1633)*, which is consistent with the observation that many phage
19 genes are unique and uncharacterized (2). Two phage integrase genes *(Tmden_0938;*
20 *Tmden_0959)* flank genes encoding a type I restriction modification system gene cluster
21 and are part of a larger region (bp 977850 – 1002764; 5 o'clock, Fig. 1), that have a
22 negative G+C anomaly (31.1%). The remaining two are included in the large transposons
23 described above (*Tmden_0693* and *Tmden_1590)*.

24 Another negative G+C anomaly, which also has a GC skew anomaly, is visible on
25 the genome map at approximately 1 o'clock (bps 192095 – 210856; 30.5% G+C; Fig. 1).
26 Although this region contains several genes encoding hypothetical proteins, as one might
27 expect were it derived from a phage, it does not appear to include any transposase or
28 integrase genes, nor does it include any repeated sequences that might suggest recent
29 gene rearrangement in this region.

30 **Restriction-modification systems.** *S. denitrificans* has numerous restriction-
31 modification (RM) systems encoded in its genome. Eleven DNA methyltransferase genes
32 are present, and encode methyltransferases similar to those found in Type I
33 (*Tmden_0697*, *Tmden_0942, Tmden_1594*), Type II (*Tmden_0121; Tmden_0129;*
34 *Tmden_0130; Tmden_0478; Tmden_0537; Tmden_1565; Tmden_1839; Tmden_1855*)
35 and Type III (*Tmden_1355*) restriction-modification systems. For 6 of these
36 methyltransferases, genes encoding restriction enzymes are nearby (Type 1:
37 *Tmden_0700; Tmden_0948; Tmden_1597;* Type II: *Tmden_128; Tmden_1854;* Type III:
38 *Tmden_1350)* and for two of them the genes appear to encode fused methylase/restriction
39 enzymes (*Tmden_0478, Tmden_0537*)(5). Based on genome sequence data, such large
40 numbers of RM systems are not unusual for epsilonproteobacteria: *Helicobacter pylori*
41 has 24 RM systems (3), *C, jejuni* has 10, and *W. succinogenes* has 5 (4, 5). If active,
42 perhaps in *S. denitrificans* these systems provide a robust defense against the introduction
43 of phage and other 'non-native' DNA into the genome.
44

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59 **FIGURE LEGENDS.**
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61 FIG. S1. Two large identical transposons from the *Sulfurimonas denitrificans*
62 genome. Numbers indicate the position of the regions, in nucleotides, with respect to the
63 origin of replication, and the arrows indicate the presence of the inverted repeat
64 sequences at each end: > = TGTCATTTACAA; < = TTGTAAATGACA.
65
66 FIG. S2. Map of a region from the *Sulfurimonas denitrificans* genome that
67 includes a small repeated region. The duplicate copies of this repeat include the two
68 adjacent transposase genes (shaded grey), while a third region with a high level of
69 identity (82%) is included within a hypothetical gene upstream (also shaded grey).
70 Numbers indicate the position of the regions, in nucleotides, with respect to the origin of
71 replication.
72
73
74 FIG. S3. Phylogenetic relationships of NosZ from different bacteria and the
75 archaeon *Pyrobaculum calidifontis*. Suden_1298 is part of a novel nos cluster previously
76 identified in *Wolinella succinogenes* (63). All epsilonproteobacterial sequences have a C-
77 terminal extension and contain a heme *c*-binding motif. The sequences from
78 *Dechloromonas aromatica* and *Magentospirillum magnetotacticum* also have a
79 (somewhat shorter) C-terminal extension, but are lacking a heme *c*-binding motif (63)
80 Sequences were aligned using the program package MacVector. Neighbor-joining and
81 Parsimony trees based on the predicted amino acid sequences were calculated using

82 PAUP 4.0b10. Bootstrap values (1,000 replicates) for the major nodes are given for the

83 neighbor-joining (first value) and parsimony analyses (second value).

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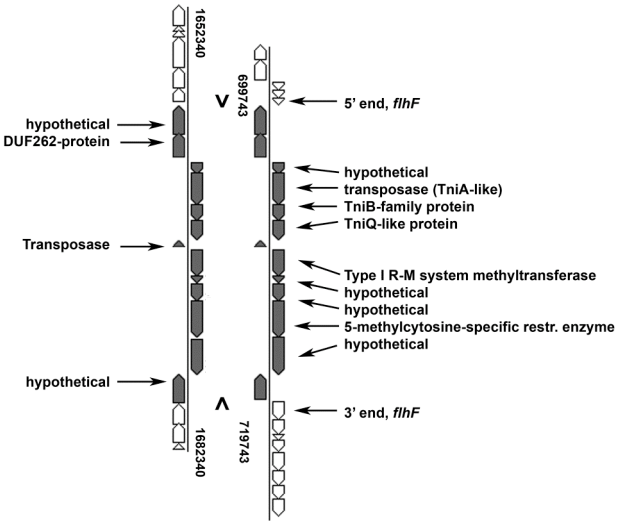

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94 FIG. S1

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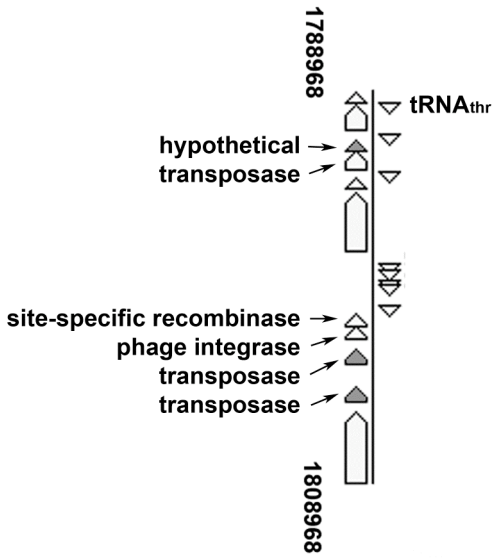
1788968
tRNAthr
hypothetical
transposase
site-specific recombinase
phage integrase
transposase
transposase
1808968

FIG. S2

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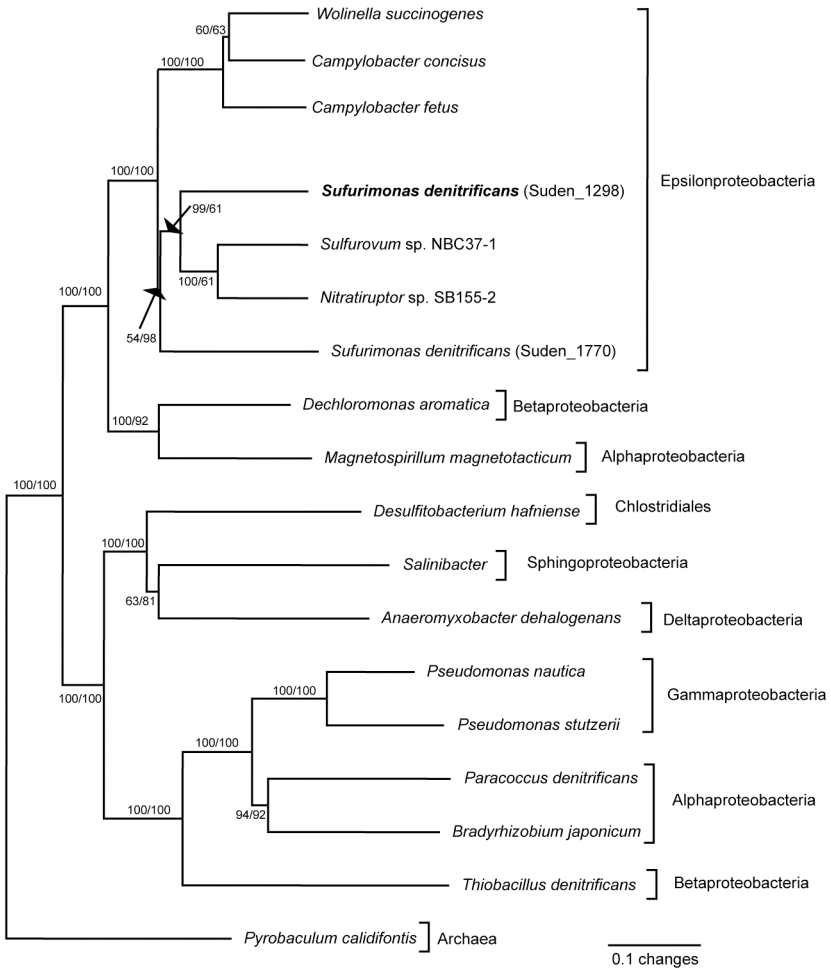

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113 FIG. S3