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Clustered Genes Related to Sulfate Respiration in Uncultured Prokaryotes Support the Theory of Their Concomitant Horizontal Transfer†

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The dissimilatory reduction of sulfate is an ancient metabolic process central to today's biogeochemical cycling of sulfur and carbon in marine sediments. Until now its polyphyletic distribution was most parsimoniously explained by multiple horizontal transfers of single genes rather than by a not-yet-identified "metabolic island." Here we provide evidence that the horizontal transfer of a gene cluster may indeed be responsible for the patchy distribution of sulfate-reducing prokaryotes (SRP) in the phylogenetic tree. We isolated three DNA fragments (32 to 41 kb) from uncultured, closely related SRP from DNA directly extracted from two distinct marine sediments. Fosmid ws39f7, and partially also fosmids ws7f8 and hr42c9, harbored a core set of essential genes for the dissimilatory reduction of sulfate, including enzymes for the reduction of sulfur intermediates and synthesis of the prosthetic group of the dissimilatory sulfite reductase. Genome comparisons suggest that encoded membrane proteins universally present among SRP are critical for electron transfer to cytoplasmic enzymes. In addition, novel, conserved hypothetical proteins that are likely involved in dissimilatory sulfate reduction were identified. Based on comparative genomics and previously published experimental evidence, a more comprehensive model of dissimilatory sulfate reduction is presented. The observed clustering of genes involved in dissimilatory sulfate reduction has not been previously found. These findings strongly support the hypothesis that genes responsible for dissimilatory sulfate reduction were concomitantly transferred in a single event among prokaryotes. The acquisition of an optimized gene set would enormously facilitate a successful implementation of a novel pathway.

Dissimilatory sulfate reduction or sulfate respiration is a key process in the mineralization of organic matter in marine sediments. Up to 50% of organic carbon in coastal sediments is mineralized anaerobically by sulfate-reducing prokaryotes (SRP) (20). This process is one of the oldest types of biological energy conservation. Evidence from geological sulfur isotope records suggests that it arose for the first time approximately 3.5 billion years ago (47). The early origin and appearance of dissimilatory sulfate reduction (DSR) should be reflected in a widespread distribution among prokaryotes and a paralleled phylogeny of the 16S rRNA gene and functional genes. However, this metabolic pathway is patchily scattered and occurs solely within four bacterial and two archaeal lineages (43, 53, 57). Comparative phylogenetic studies on the 16S rRNA gene and the two key enzymes, dissimilatory sulfite reductase (DsrAB) and adenosine-5′-phosphosulfate reductase (AprAB), suggested multiple, independent events of horizontal gene transfer (HGT) of the respective functional genes (22). For instance, the DsrAB sequence of *Archaeoglobus* spp. is more closely related to bacterial sequences than would be expected from their 16S rRNA phylogeny. Thus, a bacterial origin of *dsrAB* of *Archaeoglobus* spp. is conceivable (12, 22, 53). The tree topology of AprA partially differs from both the 16S rRNA and DsrAB trees, indicating independent horizontal transfers of both *aprAB* and *dsrAB* (12). These studies suggested that *Firmicutes* and *Deltaproteobacteria*, respectively, might have served as donor lineages.

The respiration of sulfate requires a set of several enzymes (39). Three cytoplasmic proteins were identified. Besides DsrAB and AprAB, an ATP sulfurylase is involved to activate sulfate. It is still unclear which membrane proteins universal for SRP are essential to mediate the transfer of electrons to cytoplasmic DsrAB and AprAB. Evidence is accumulating that membrane complexes containing periplasmic triheme cytochrome *c* and heterodisulfide reductase-resembling proteins are involved (5, 15, 32, 33, 41).

In general, HGT is regarded as a major force in the evolution of prokaryotes (2, 25). The HGT of genes of metabolic pathways, such as carbon dioxide fixation (7) and photosynthesis (19), has been documented between distantly related groups. For DSR, the putative HGT of a cluster of essential genes arranged as a mobilizable "metabolic" or "genomic island" (GEI) was discussed, since a stepwise acquisition of single genes seems unlikely (12, 22). However, the available data of the finished or ongoing sequencing of the genomes of

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four sulfate-reducing prokaryotes illustrate that the relevant genes for DSR are dispersed throughout the genomes (16, 23, 44) and *Desulfovibrio desulfuricans* (DOE Joint Genome Institute, Walnut Creek [http://www.jgi.doe.gov]). This observation and the diverging phylogenies of key enzymes ostensibly contradict the hypothesis of the HGT of a genomic island (12, 22, 53). Nevertheless, the horizontal transfer of GEIs is an important mechanism for the evolution and adaptation of both pathogenic and nonpathogenic prokaryotes (9). It is increasingly recognized that gene clusters similar to horizontally transferable pathogenicity islands are also constituents of nonpathogenic bacteria. Such genetic structures can translocate gene functions required for catabolic activities and symbiosis. However, no evidence for a GEI linked to ecologically highly relevant processes, such as dissimilatory sulfate reduction, has been obtained.

The evolution of dissimilatory sulfate reduction cannot be easily inferred from a few cultured organisms, since the vast majority of SRP remain unexplored, and is mainly known by their 16S rRNA clone sequences (8). The initial aim of our study was to investigate the genomic context of key enzymes of DSR of uncultured SRP from marine sediments. The gene neighborhood was expected to reveal novel genes functionally involved in sulfate respiration. Therefore, we established environmental DNA libraries containing large inserts (35 to 45 kb) from two sites highly active in sulfate reduction: (i) sediment of an intertidal sand flat in the German Wadden Sea and (ii) sediment from a marine methane hydrate area, the southern Hydrate Ridge off the coast of Oregon. The libraries were screened for either *aprA* or *dsrAB*, and selected clones were completely sequenced.

MATERIALS AND METHODS

Study sites and sampling. Two marine sediments were investigated. (i) The upper 25 cm of surface sediment of an intertidal sand flat ("Janssand") in the German Wadden Sea was sampled (53°43′N, 07°41′E). Sediment cores were collected at low tide on 23 March 2002 with polyacryl tubes, closed at both ends with airtight rubber stoppers, and transported on ice for further processing in the lab. The cores were sectioned and immediately frozen at −20°C. DNA was extracted from sediment of the 5- to 12-cm horizon.

(ii) Hydrate Ridge sediment samples were obtained during the RV Sonne cruise SO148-1 on 28 July 2000 at the crest of the southern Hydrate Ridge at the Cascadia convergent margin off the coast of Oregon (Northeast Pacific, 44°34′N, 125°09′W, 780-m water depth).

Sediment cores were taken above the hydrate at areas of active gas seeping at a water depth of 777 m using a video-guided multiple corer. Cores were sectioned, and surface sediments were anoxically stored at 5°C in stoppered bottles (250 ml) without headspace. In the laboratory, the sediment was overlayered with artificial seawater medium, gassed with methane, and incubated at 12°C.

Fosmid library construction. Sediment from 5- to 12-cm depth from the intertidal sand flat of the Wadden Sea was taken for library construction. DNA was extracted as previously described (56) by overnight incubation with proteinase K, gel purified to get rid of humic substances, and additionally purified using the GeneClean Turbo kit (Qbiogene, Carlsbad, CA). A fosmid library was constructed using the EpiFOS fosmid library production kit (Epicenter, Madison, WI) according to the manufacturer's instructions with the following modifications: the concentrated DNA was blunt ended, purified, and concentrated using MICROCON YM-100 columns (Promega, Mannheim, Germany). DNA fragments of appropriate length for cloning were obtained after separation by pulsed-field gel electrophoresis (PFGE). The gel (1.3% low-melting-point agarose) was run on a field contour-clamped homogeneous electric field–PFGE mapper (Bio-Rad, Munich, Germany) at 14°C, 6 V cm^{-1}, for 18 h with 1- to 21-s pulses in 1× Tris-acetate-EDTA buffer. Gel bands containing 40- to 50-kb-long DNA were excised, digested with gelase (Epicenter), purified, and concentrated by application of MICROCON YM-100 columns. Packaging into phage heads and transduction were performed as indicated in the manufacturer's instructions. Three libraries containing approximately 34,000 clones with insert sizes ranging from 32 to 44 kilobases in total were prepared.

The fosmid library from Hydrate Ridge sediments was established as follows. DNA from 1- to 3-cm sediment depth was extracted as described above. The DNA was further purified by anion-exchange chromatography using Genomic-tip 20/G (QIAGEN, Hilden, Germany), embedded in 1% low-melting-point agarose, and dialyzed against Tris-EDTA buffer. The fosmid library was constructed as indicated above with the following modifications. After prior equilibration for 0.5 h at 40°C with end repair mix devoid of enzymes, the end repair reaction was performed in the intact agarose plug. The end repair reaction was performed with doubled nucleotide concentration, and size selection was carried out on a 1% SeaPlaque GTG agarose (FMC BioProducts) PFGE gel (0.5× Tris-borate-EDTA, 1 to 10 s, 14°C, 120°, 11 h). DNA was excised, equilibrated with Tris-EDTA buffer, solubilized with gelase (Epicenter), and concentrated. Ligation, packaging, and transduction were conducted as described above.

Screening for *aprA* and *dsrAB*. In total, 11,000 clones from the intertidal sand flat library were screened for *aprA* genes. For amplification of the alpha subunit of the adenosine-5′-phosphosulfate reductase gene (*aprA*), primers APS-1-F and APS-4-RV were used to amplify a fragment of approximately 400 bp. The primer sequences and the PCR protocols will be published elsewhere (B. Meyer and J. Kuever, unpublished data). Screening for *dsrAB* genes in the fosmid library from Hydrate Ridge sediments was performed similarly. The full-length *dsrAB* genes were amplified from the fosmid extracts and cells using the previously described primers and PCR conditions (53).

Sequencing, ORF finding, and sequence annotation. Fosmids were sequenced by a shotgun approach based on plasmid libraries with 1.5- and 3.5-kb inserts. Sequences of small inserts were determined by using Big Dye chemistry (ABI), M13 primers, and ABI3730XL capillary sequencers (ABI) up to a 10-fold coverage. Resulting reads were assembled by Phrap44 and finished in Consed (13). Open reading frames (ORFs) were predicted by the gene prediction program GLIMMER (6), which is integrated into the open source program package GENDB (36). Annotation of the identified ORFs was accomplished on the basis of similarity searches against different databases, such as Pfam, Swissprot, Uniprot, and Interpro. Signal peptides and transmembrane helices were also predicted. All results were evaluated manually. Potential transcription termination sites were predicted using the program TransTerm (11).

Phylogenetic analysis. Full-length *dsrAB* sequences of the three fosmid clones were translated into proteins and phylogenetically analyzed using the ARB program package (31). Maximum likelihood trees were constructed using JTT amino acid substitution matrix for evolutionary distance along with the ProML program of the Phylip program package integrated in ARB. Distance matrix trees were calculated using the neighbor-joining function of ARB. Deletions and insertions were not considered in the DsrAB treeing methods. The amino acid sequences of QmoB and Sat were aligned using the ClustalW program package. For phylogeny analysis, calculations were performed without and with a 50% positional conservation filter. Subsequently, consensus trees were generated from the results of all treeing methods. Protein length heterogeneities were considered, and terminal sequence stretches were excluded from calculations. For QmoB 899 amino acid positions and for Sat 433 amino acid positions were included in the calculations.

Nucleotide sequence accession numbers. The nucleotide sequence data are available at GenBank under accession numbers CT025835 (fosmid ws39f7), CT025836 (fosmid ws7f8), and CT025834 (fosmid hr42c9).

RESULTS

Sequencing of fosmids and annotation. Screening of the Wadden Sea library revealed fosmids ws39f7 and ws7f8 containing an adenosine-5′-phosphosulfate reductase gene (*aprAB*). From Hydrate Ridge sediment, fosmid hr42c9 with a gene locus of the dissimilatory sulfite reductase (*dsrAB*) was retrieved. The full sequences of all three fosmids were determined.

For the 38.5-kb insert of fosmid ws39f7, 35 ORFs were predicted. The deduced amino acid sequences of 30 ORFs showed significant similarities to functional proteins in databases (Table 1). The second fosmid, ws7f8, contained an insert of 32 kb with 30 predicted ORFs (see Table S2 in the supplemental material), of which 27 showed homologs in either pub-

TABLE 1. ORFs of ws39f7

ws39f7 ORF no. (aa)[a]	Predicted protein	Closest homolog (BLASTx, E value) in public databases and Pfam hits (E value)
1 (431)	Quinone-interacting membrane-bound oxidoreductase complex subunit A (QmoA)	*Chlorobium tepidum* (e-133)
2 (748)	QmoB	*C. tepidum* (e-0.0)
3 (405)	Dissimilatory sulfite reductase subunit A (DsrA)	Clone AY197455, Guaymas basin (e-151)
4 (362)	Dissimilatory sulfite reductase subunit B (DsrB)	Clone AY197455, Guaymas basin (e-151)
5 (81)	Probable dissimilatory sulfite reductase subunit D (DsrD)	Fosmid ws7f8 ORF13
6 (414)	Siroheme a-amid synthase (DsrN)	*Desulfobacula toluolica* (e-84)
7 (558)	Iron-sulfur-binding protein, glutamate synthase subunit (DsrL/GltD)	*C. tepidum* (e-102)
8 (105)	Dissimilatory sulfite reductase subunit C (DsrC)	Pfam (e-52), *Desulfitobacterium hafniense* (e-34)
9 (177)	Conserved hypothetical protein	*D. desulfuricans* (e-27)
10 (335)	Hdr-like menaquinol-oxidizing enzyme, subunit C (HmeC/DsrM)	*Archaeoglobus fulgidus* (e-57)
11 (538)	HmeD/DsrK	*A. fulgidus* (e-159)
12 (110)	HmeE/DsrJ	*D. desulfuricans* (e-32)
13 (257)	HmeA/DsrO	*A. fulgidus* (e-48)
14 (385)	HmeB/DsrP	*Moorella thermoacetica* (e-119)
15 (567)	Pyridine nucleotide-disulfide oxidoreductase	*A. fulgidus* (e-90), Pfam (e-50)
16 (214)	Siroheme synthase (CysG)	*Bacillus halodurans* (e-23)
17 (518)	Uroporphyrinogen III synthase/methyltransferase (CysG/HemD)	*D. desulfuricans* (e-92)
18 (392)	QmoC	*C. tepidum* (e-73)
19 (407)	Sulfate adenylyltransferase (Sat)	Pfam (e-147), *C. tepidum* (e-145)
20 (146)	Adenosine-5′-phosphosulfate reductase subunit B (AprB)	*A. fulgidus* (e-45)
21 (641)	Adenosine-5′-phosphosulfate reductase subunit A (AprA)	*A. fulgidus* (e-0.0)
22 (92)	Conserved hypothetical protein	*C. tepidum* (e-9)
23 (285)	Conserved hypothetical protein containing unknown protein family UPF0153	*C. tepidum* (e-56), Pfam 0.0002, Pfam (e-34), *A. fulgidus* (e-24)
24 (315)	Nitrate reductase, gamma subunit	
25 (538)	Reductase, iron-sulfur-binding subunit	*A. fulgidus* (e-83)
26 (129)	Response regulator receiver domain	Pfam (e-16)
27 (182)	Transcriptional regulator	Pfam (e-23), *Clostridium acetobutylicum* (e-16)
28 (227)	Conserved hypothetical protein	*D. desulfuricans (e-04)*
29 (590)	Reductase, iron-sulfur-binding subunit containing response regulator receiver domain	*Desulfovibrio vulgaris* (e-61), Pfam (e-18)
30 (515)	Two-component regulator sensor, histidine kinase	*D. desulfuricans* (e-116), Pfam (e-35)
31 (210)	Conserved hypothetical protein	*D. desulfuricans* (e-19)
32 (510)	Di- and tricarboxylate transporter	*D. desulfuricans* (e-0.0)
33 (174)	Conserved hypothetical protein containing a cystathione-β-synthase (CBS) domain	Pfam (e-07)
34 (203)	Response regulator receiver domain	Pfam (e-27), *Geobacter metallireducens* (e-18)
35 (198)	Protein related to phosphoenolpyruvate synthase	*D. vulgaris* (e-20)

[a] Number of amino acids is in parentheses.

lic databases or fosmids ws39f7 and hr42c9. Fosmid hr42c9 harbored a 40.6-kb insert displaying 38 predicted ORFs (see Table S3 in the supplemental material). The majority of ORFs in all three fosmids mutually showed the highest similarities, indicating a close phylogenetic relationship of the corresponding organisms.

Sulfur energy metabolism. All three fosmids encoded several proteins implicated in dissimilatory sulfate reduction. The majority significantly resembled proteins from sulfate/sulfite-reducing prokaryotes and sulfur-oxidizing bacteria (Table 1 and Tables S2 and S3 in the supplemental material). The following analysis is focused on ORFs found in fosmid ws39f7, since it provided the largest gene set involved in DSR (Table 1). To improve clarity, "ORF" refers to the predicted gene whereas "Orf" refers to the deduced protein. The arrangement of the genes in the fosmids and of selected homologs in other organisms is depicted in Fig. 1.

As noted above, fosmids ws39f7 and ws7f8 included the gene for adenosine-5′-phosphosulfate reductase (*aprAB*, ORF20 and ORF21). Besides, a gene coding for the ATP sulfurylase (*sat*, ORF19) was identified directly upstream of *aprAB* (Fig. 1). These enzymes catalyze the activation of sulfate and the subsequent reduction of adenosine-5′-phosphosulfate (APS) to sulfite. In contrast, the *sat-aprAB* cluster was not found on fosmid hr42c9.

Fosmids ws39f7 and ws7f8 carried genes encoding four pro-

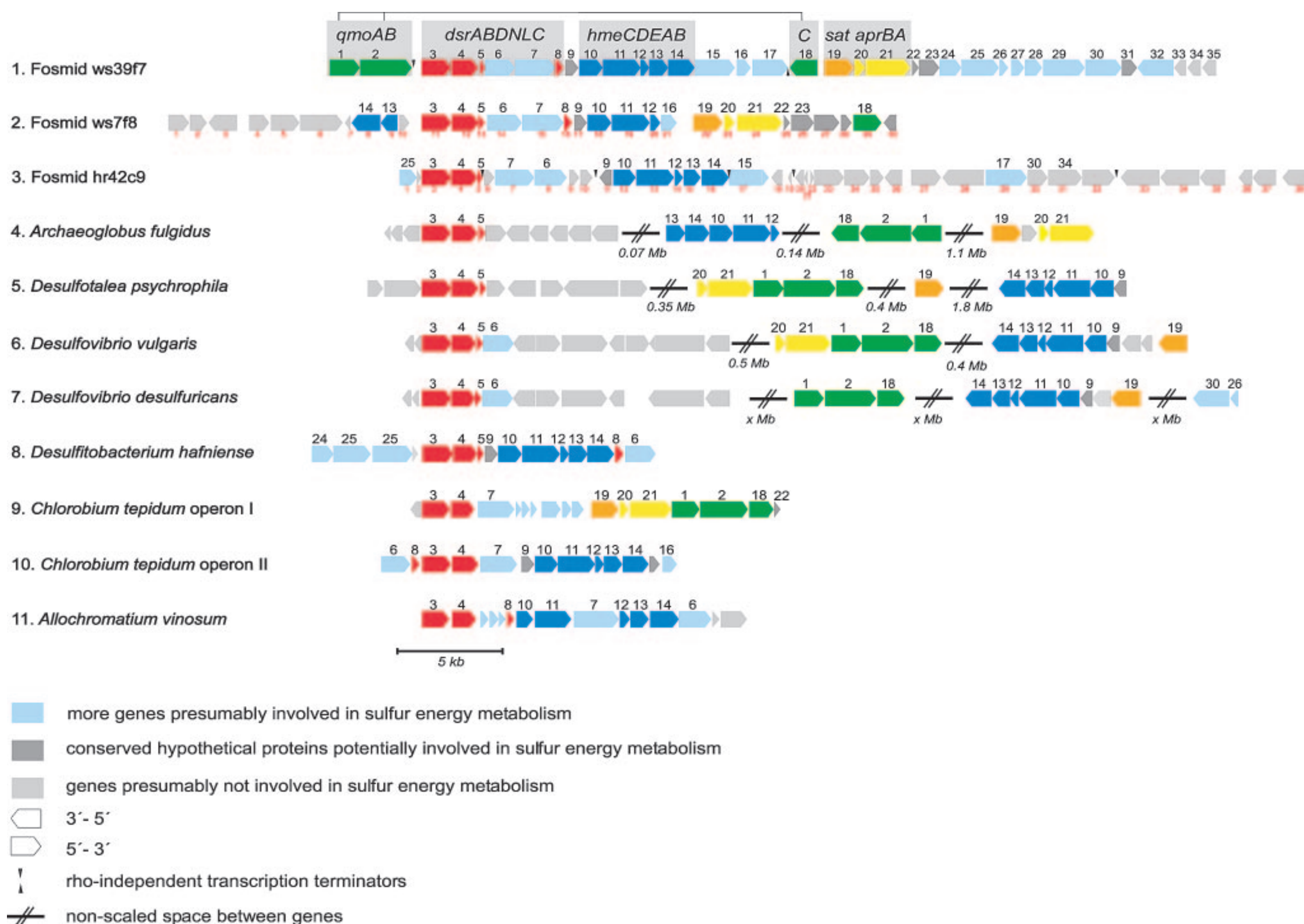

FIG. 1. Genomic organization of the *dsrAB* locus in fosmids ws39f7, ws7f8, and hr42c9, sequenced SRP genomes, *Desulfitobacterium hafniense*, *Chlorobium tepidum*, and *Allochromatium vinosum*. ORF numbers match those in Table 1. Numbers above ORFs refer to the homolog ORF in fosmid ws39f7.

posed subunits of the cytoplasmic dissimilatory sulfite reductase (DsrABDC, ORFs 3, 4, 5, and 8), accounting for the terminal steps in the reduction of sulfite to hydrogen sulfide. The putative dissimilatory sulfite reductase protein (ORF3 and 4) contains the characteristic siroheme binding sites and is phylogenetically closely related to DsrAB from known SRP (see the end of Results) (Fig. 2). ORF5 is predicted to be functionally similar to DsrD despite the absence of significant database hits. The predicted protein size of approximately 9 kDa and the position downstream of *dsrB* are in good accordance to DsrD in other SRP (16, 23, 44) and the sulfite-reducing bacterium *Desulfitobacterium hafniense* (Fig. 1). The DsrD amino acid sequence is highly variable. Nevertheless, in all homologs DNA-binding motifs are conserved. Its function is still not fully resolved (18), but it has been postulated that DsrD might play a role in DNA binding (37) or interact with DsrAB (28) to bind sulfite. It has never been identified in sulfur-oxidizing organisms; thus, it appears to be specifically involved in the dissimilatory reduction of sulfite (5, 23). The gene encoding DsrC (ORF8) was displayed in fosmids ws39f7 and ws7f8. DsrC was proposed to further reduce oxidized sulfur intermediates, such as thiosulfate (48, 50). Both DsrC and DsrD are not essentially tightly associated with DsrAB (18, 40). Thus, they also may act as independent proteins rather than as subunits of the Dsr complex.

Fosmid ws39f7 displayed two genes that are likely responsible for the synthesis of siroheme, the presumed prosthetic group of DsrAB. ORF16 encodes a subunit of siroheme synthase (CysGA). It is followed by a gene (ORF17) coding for a bifunctional protein that represents a fusion of siroheme synthase (CysGB) and uroporphyrinogen III synthase (HmeD), an arrangement very similar to the *dsr* locus in *Chlorobium tepidum* (Fig. 1). Downstream of *dsrD*, a gene (ORF6), which is related to *dsrN* in SRP (16, 23, 26, 27, 44), *D. hafniense*, and sulfur-oxidizing bacteria, was predicted (5, 10). The derived protein resembled cobyrinic acid *a*,*c*-diamide synthase, which is part of the vitamin B_{12} biosynthetic pathway and catalyzes the amidation of cobyrinic acid. However, a distinct function of this protein in DSR and also in sulfur oxidation was proposed (5, 26). Generally, siroheme is considered to be the prosthetic group of DsrAB. In contrast, in *Desulfovibrio* spp. an amidated siroheme was identified, and a DsrN-like protein was accounted for the amidation step (26, 35). In *D. desulfuricans*, *Desulfovibrio vulgaris*, *D. hafniense*, *Thermodesulforhabdus norvegica* (27), *Desulfobacter vibrioformis* (26), and our fosmids, *dsrN* homologs are found in the direct neighborhood of *dsrAB*

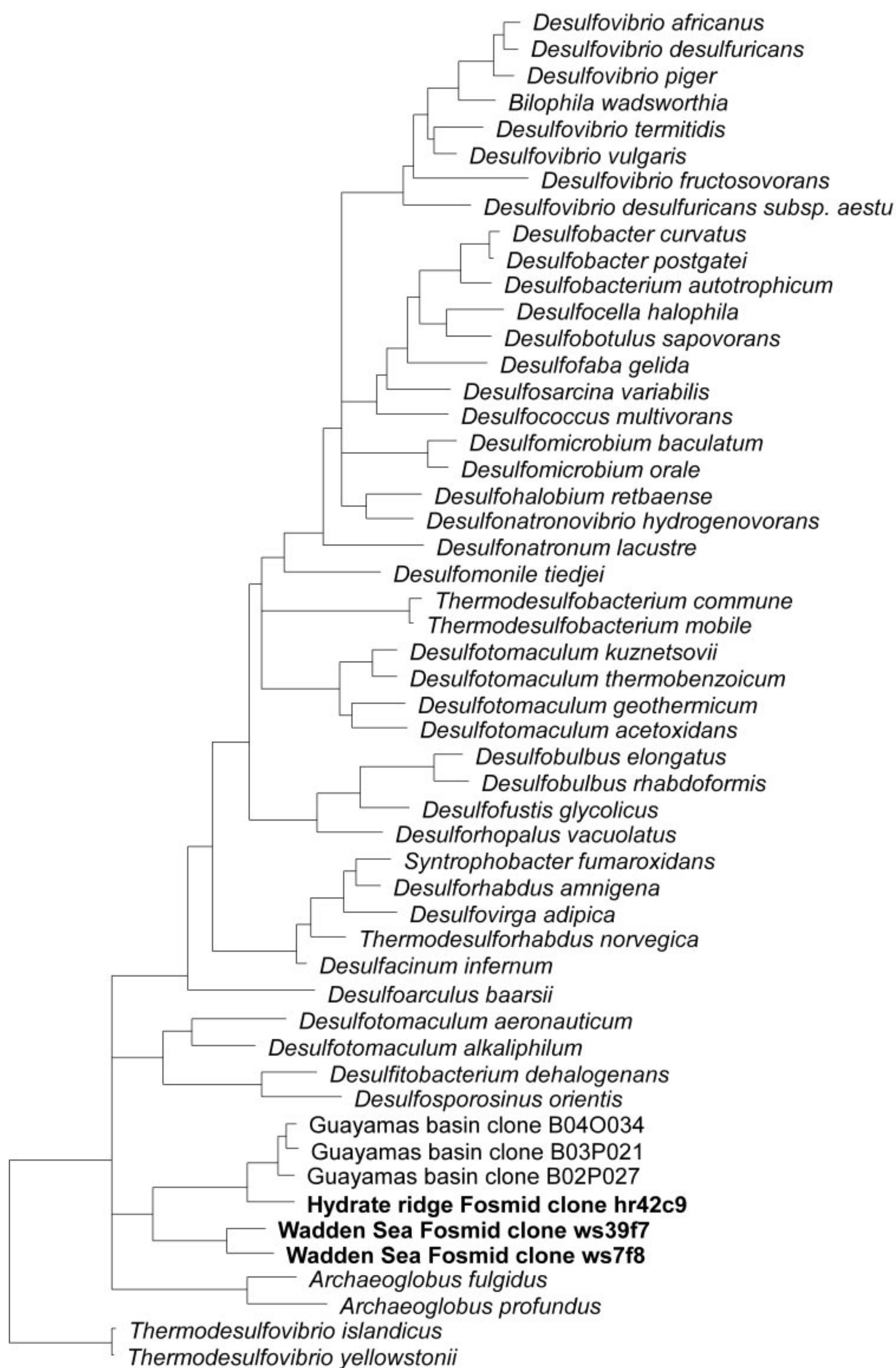

FIG. 2. DsrAB-based phylogenetic reconstruction. The scale bar corresponds to 10% estimated sequence divergence. The tree was inferred using the maximum likelihood and neighbor-joining methods. Multifurcations were introduced when branching orders were not supported by all phylogenetic methods used.

(Fig. 1), suggesting siroheme amide as the actual prosthetic group of DsrAB. Interestingly, in all fosmids *dsrN* is juxtaposed to a *dsrL*-like gene (ORF7). The amino acid sequence of Orf7 showed high similarities to DsrL in phototrophic sulfur-oxidizing bacteria in both N- and C-terminal sequences and to a subunit of glutamate synthase (GltD). Like in DsrL of *Allochromatium vinosum*, Orf7 has two flavin adenine dinucleotide (FAD)- and two Fe_4S_4-binding sites as well as one NAD(P)H-binding motif. For the reverse pathway of sulfur oxidation, Dahl et al. (5) proposed a function in electron transfer from NAD(P)H or FAD to an acceptor protein to other proteins of the *dsr* locus rather than in glutamate synthesis. Related proteins are also present in known genomes of bacterial SRP and sulfite-respiring *D. hafniense* and *Moorella thermoacetica*.

Membrane proteins of electron transport. On three fosmids investigated in this study, genes were found in proximity to *dsrAB* that are most probably involved in the electron transfer to AprAB and DsrAB (Fig. 1). Orf10 to Orf14 of fosmid ws39f7 showed high similarity to the heterodisulfide reductase-like menaquinol-oxidizing enzyme complex (HmeABCDE) in *Archaeoglobus fulgidus* and to the DsrMKJOP complex in *A. vinosum*. The identified complex is distinct to other respiratory complexes, such as the high-molecular-weight cytochrome (Hmc) complex in *D. vulgaris* (45), with respect to gene arrangement and gene composition. The amino acid sequences of Orf10 to Orf14 revealed specific characteristics supporting a function similar to that predicted for the Hme complex of *A. fulgidus* (32). Heme *b*- and heme *c*-binding sequence motifs were identified in ORF10 and 12. Cysteine motifs for Fe-S binding were found in ORF11 and 13. ORF10 encodes a transmembrane heme *b*-type protein and likely is the homolog of HmeC/DsrM. Orf11 is an Fe-S protein related to a DsrK/HmeD. The deduced protein from ORF12 is predicted as a periplasmic triheme with a typical sec-signal peptide that may serve as a membrane anchor on the periplasmic side, similar to DsrJ and HmeE. Just as DsrO/HmeA, Orf13 contains an Fe-S binding site and an arginine leader sequence indicative for the Tat transport system across membranes (1). Orf14 is related to DsrP/HmeB and concordantly showed 10 membrane-spanning helices and similarities to polysulfide reductase motifs. This protein family participates in the electron transfer from quinones to terminal acceptors.

The four purified proteins HmeACDE in *A. fulgidus* were proposed to play a critical role in electron transfer from the menaquinol to sulfur intermediates, facilitating the reduction of sulfate to sulfide (32, 33). In *Archaeoglobus profundus*, HmeC and HmeD homologs are involved in the oxidation of H_2, the only known electron source for this organism (33). In *A. vinosum*, DsrMKJOP also copurified with DsrAB and were shown to be indispensable for the oxidation of sulfur intermediates (5, 42). The proximity of *hme* to *dsrAB* in all of our investigated fosmids, in *D. hafniense*, and in sulfur-oxidizing *C. tepidum* and *A. vinosum* supports an interaction of the gene products (Fig. 1). Genome comparison revealed that genes of Hme-related membrane complexes consistently show a conserved structure, which contains at least five genes. In contrast to *A. fulgidus*, the other investigated genomes contain an additional homolog gene with unknown function linked to this operon (ORF9) (Fig. 1). We repeatedly found homologs to ORF9 that are linked to genes encoding the *b*-type cytochromes in the DsrMKJOP/Hme operon of SRB genomes, in *D. hafniense*, and in *C. tepidum* but not in *A. fulgidus* (Fig. 1). The predicted protein has a calculated mass of 21 kDa and displays highest sequence similarity to a conserved hypothetical protein in *D. desulfuricans*.

On fosmid ws39f7, the two genes upstream of *dsrAB* (ORF1 and 2) probably form an interactive unit with ORF18. These three genes encode a membrane complex homolog to the quinone-interacting membrane-bound oxidoreductase (QmoAB) complex in *D. desulfuricans* (ORF1, 2, and 18), a novel membrane-bound respiratory complex (41). It also showed high similarity to heterodisulfide reductase in methanogenic archaea. The deduced proteins of ORF1 and ORF2 code for soluble proteins each containing Fe-S and FAD for electron and proton transport according to QmoA and QmoB in *D. desulfuricans*. No signal peptides or transmembrane helices were identified, indicating a cytoplasmic location. The third gene, ORF18, encoded six membrane-spanning helices and thus likely is a transmembrane protein as QmoC. It harbored both a hydrophobic domain with homology to the heme *b* protein HdrE and a hydrophilic domain with homology to the Fe-S protein HdrC in *A. fulgidus*. Based on comparison of our data with the detailed analysis of sequences and proteins in *D. desulfuricans*, ORF1, 2, and 18 proteins likely are functionally similar to the Qmo complex. The derived proteins are predicted to interact physically and to catalyze the transfer of electrons to AprAB for the reduction of APS. The *qmo* operon is juxtaposed next to *aprAB* in *Desulfotalea psychrophila*, *D. vulgaris*, and *C. tepidum* and partially in fosmid ws39f7, which also confirms a direct functional linkage to AprAB (Fig. 1). This is supported by the absence of *qmoABC*, *sat*, and *aprAB* in the sulfite-reducing *D. hafniense* and *M. thermoacetica*.

On all three fosmids, additional genes that are potentially involved in sulfur-based energy metabolism were identified (Table 1 and Tables S2 and S3 in the supplemental material). The derived protein of ORF28 (ws39f7) showed a weak similarity to high-molecular-weight cytochrome complex subunit E (HmcE) of *D. vulgaris* (45). It is probably physically associated with an Fe-S protein encoded by ORF29 that in turn resembles the HmcF subunit. The latter seems to form a fused transcriptional unit with a response regulator receiver domain (ORF29) and may be functionally linked with the adjacent two-component regulator sensor (ORF30). Similarly, Orf25 displayed the best hit to an Fe-S protein of *A. fulgidus*. In *D. hafniense*, the homologs are in close proximity to *dsrAB*, which supports a functional linkage to DsrAB (Fig. 1). Similarly to ORF29, a close link of ORF25 to regulatory elements (ORF27 and 28) was found. A dedicated function could not be assigned to both two-protein units; however, the close association with regulatory elements suggests a role in transcriptional control. We further found a predicted NADH oxidase (Orf15) that was homolog to NoxA-3 of *A. fulgidus* and showed highest similarity to a predicted gene product in *D. hafniense*. Related homologs were also found in known genomes of SRP and in *C. tepidum*, indicating a specific relation to sulfur-based energy metabolism. NADH oxidases might also translocate electrons directly from NADH to APS, as was proposed for *D. vulgaris* (4). More generally, NADH oxidases play a role in protection of proteins under oxygen stress (34).

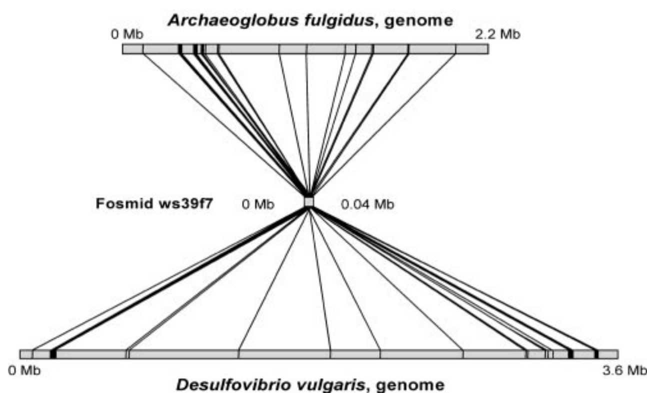

FIG. 3. Comparison of positions of ortholog genes on fosmid ws39f7 and complete genomes of *Archaeoglobus fulgidus* and *Desulfovibrio vulgaris*. Ortholog genes are connected by black lines. Mb, megabases.

Novel genes probably involved in DSR. Besides ORF9 linked to the Hme complex, several other ORFs could not be assigned to functionally characterized proteins. Homologs to Orf23 were identified in all SRP genomes, in *Desulfobacula toluolica*, in *C. tepidum*, and in *Thiobacillus denitrificans* but not in the draft sequences of sulfite-respiring *D. hafniense* and *M. thermoacetica*, indicating a role in electron transfer to AprAB. Orf23 displayed high motif similarities to a protein family of unknown function (UPF0153). These proteins contain eight conserved cysteine residues that may form a metal-binding site. Their function is still unknown but might be a part of an oxidoreductase complex. The deduced protein has a calculated mass of 34 kDa. In both fosmid ws39f7 and fosmid ws7f8, the respective gene was coupled to a gene encoding a conserved hypothetical protein (ORF22) and to *aprAB* (Fig. 1). The hypothetical protein derived from ORF22 is related to a gene in *C. tepidum*, which is also adjacent to *aprAB* (Fig. 1). Another probable protein was encoded by ORF31, which showed weak but significant hits to hypothetical proteins in known SRP genomes and *D. hafniense*. The gene context does not allow designation of a function, but it is apparently linked to regulatory elements on fosmid ws39f7, in *D. desulfuricans*, and in *D. hafniense*. Furthermore, a gene encoding a putative transmembrane transport protein (ORF33) that is associated with regulatory elements was identified. The deduced protein belongs to a family of di- and tricarboxylate transporters and to sodium/sulfate symporter proteins. It showed similarity to an uncharacterized transport protein in *D. desulfuricans* and might be involved in sulfate transport into the cytoplasm.

Genome comparison of gene arrangement. When the gene arrangement on fosmid ws39f7 is compared to that in the genomes of *A. fulgidus*, *D. psychrophila*, *D. vulgaris* (16, 23, 44), and *Desulfobacterium autotrophicum* (unpublished data), it is shown that the homolog genes are dispersed similarly throughout the genomes (Fig. 3). Clustering of genes involved in DSR was also observed to a lower extent in fosmids ws7f8 and hr42c9 (Fig. 1). In sulfite-respiring *D. hafniense*, 11 homolog genes were found to cluster with *dsrAB*. Phototrophic sulfur-oxidizing *A. vinosum* and in particular *C. tepidum* also show a high degree of gene clustering similar to fosmid ws39f7. In *C. tepidum*, 17 homolog genes are located in the neighborhood of two physically separated *dsrAB* copies (Fig. 1), with a gene order partially identical to that of fosmid ws39f7.

Phylogenetic analysis. The phylogenetic reconstruction of DsrAB (Fig. 2) and AprAB (data not shown) clearly indicated an affiliation of the respective organisms of fosmids ws39f7, ws7f8, and hr42c9 with sulfate-respiring and not with sulfur-oxidizing prokaryotes. However, the distinct treeing approaches did not allow a clear assignment to any characterized group of SRP (Fig. 2). DsrAB of the fosmids branched deeply and affiliated with a novel group of uncultured SRP from the Guaymas basin (78% sequence similarity) (Fig. 2) and from an acidic bog fen (30) (data not shown in tree). The most similar cultured relative was the firmicute *Desulfotomaculum ruminis* (75% sequence similarity). Typical deletions for "authentic" *Desulfotomaculum* spp., *Thermodesulfovibrio* spp., and *Archaeoglobus* spp. were found (22). In accordance with Loy et al. (30), the novel *dsrAB* sequences of our fosmid are likely not pseudogenes. A tetranucleotide analysis (51) also did not allow affiliation of the fosmid sequences with a phylogenetic group.

Additionally, phylogenetic analyses of the QmoB subunit and the ATP sulfurylase were performed. In both cases, an affiliation with either sulfate-reducing or sulfur-oxidizing prokaryotes was not well supported by any method, resulting in branching between both functional groups (Fig. 4A and 5A). In both proteins, the similar insertion/deletion patterns of sulfur-oxidizing bacteria and fosmid clones suggest a common ancestor (Fig. 4B and 5B). Moreover, the QmoB phylogeny and the alignment pattern suggest an affiliation of fosmid ws39f7 and *C. tepidum* with *A. fulgidus* (Fig. 4B). These findings are consistent with phylogenetic analyses and alignment patterns in QmoA and QmoC (not shown). Besides, the E values after BLAST search indicated that several genes of the investigated fragments show highest similarity to *C. tepidum* rather than to SRP (Table 1 and Tables S2 and S3 in the supplemental material).

DISCUSSION

The vicinity of the *dsr* locus in so far uncultured SRP from marine sediments was investigated to gain a more comprehensive image of the genetics and evolution of DSR. To study genes involved, three DNA fragments were isolated from fosmid libraries.

Comprehensive model of dissimilatory sulfate reduction. Based on our gene context analyses, a more comprehensive model for dissimilatory sulfate reduction in prokaryotes can be proposed (Fig. 6). To indicate the level of gene clustering, we refer to ORFs in fosmid ws39f7. According to this model, sulfate is transported into the cell either by a hypothetical sodium/sulfate symporter protein (Orf32) or by general sulfate transporters not encoded on the fosmid. The cytoplasmic steps of DSR are already well known (39). In the cytoplasm, sulfate is activated by an ATP sulfurylase (Orf19, EC 2.7.7.4) to form APS. An electron translocation to APS is catalyzed by AprAB (Orf20 and 21, EC 1.8.99.2), resulting in sulfite and adenosine monophosphate. DsrABDC subunits (Orf3, 4, 5, and 8, EC 1.8.99.3) reduce sulfite to intermediates and finally to hydrogen sulfide. Furthermore, a complete gene set required for the synthesis of the prosthetic group of DsrAB, which is generally regarded to be siroheme (24), was identified. Recent experi-

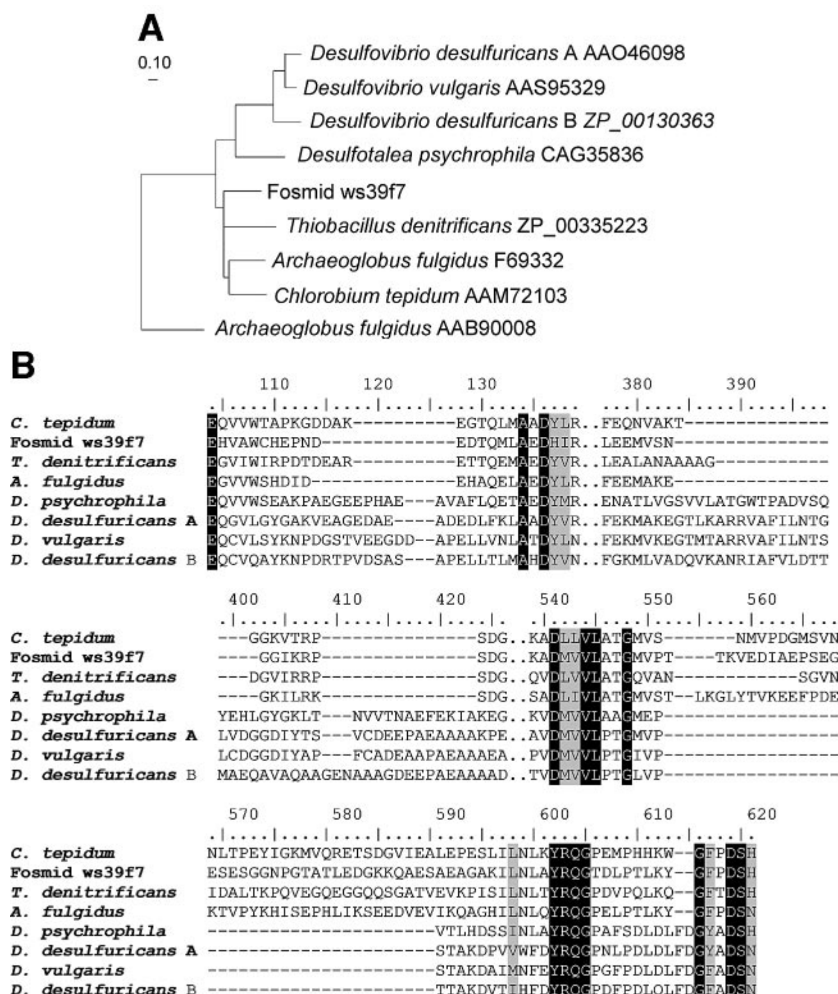

FIG. 4. (A) QmoB-based phylogenetic reconstruction. The scale bar corresponds to 10% estimated sequence divergence. The tree was inferred using the maximum likelihood, maximum parsimony, and neighbor-joining methods. Multifurcations were introduced when branching orders were not supported by all phylogenetic methods used. (B) Partial amino acid sequence alignment of the QmoB protein showing insertions/deletions supporting an affiliation of QmoB with sulfur-oxidizing bacteria. Similar residues are highlighted according to the BLOSUM62 matrix for evolutionary substitution. The alignment is numbered according to the *C. tepidum* sequence.

mental results and the analysis of the genetic context of the *dsr* locus provide evidence that siroheme-amide may serve as the actual prosthetic group rather than siroheme (26, 35) (Table 1 and Fig. 1). The physical proximity of *dsrN* and *dsrAB* in our fosmids, *D. hafniense*, and phototrophic sulfur oxidizers (5) strongly supports this hypothesis. The juxtaposition of *dsrL* to *dsrN* points at the DsrL protein to provide glutamine for amidation of siroheme. In contrast, Dahl et al. (5) proposed that DsrL functions as a NADPH:acceptor oxidoreductase.

It is still unclear which proteins universally present in SRP transfer electrons to the cytoplasmic enzymes. A few membrane complexes have been described for *Desulfovibrio* spp. and *Archaeoglobus* spp., such as the DvH Hmc (45), Dd27k 9Hc (46), DvH TpIIc3 (52), Hme (32, 33), and Qmo (41) complexes. These complexes are related, and all but Qmo contain a periplasmic cytochrome *c* subunit. However, they differ substantially in their gene order, protein structure, and number of heme-binding sites. Here it is shown that the HmeA-E/DsrMKJOP complex to date appears to be the only cytochrome *c*-transmembrane complex of which all conserved homologs are universally present among known SRP and also in phototrophic sulfur-oxidizing bacteria (5, 23).

Similarly, the *qmoABC* homologs along with the gene arrangement appear to be unique in published genomes of sulfate-respiring prokaryotes, *D. autotrophicum* (data not shown), *C. tepidum*, and *T. denitrificans*. This complex does not show periplasmic components; therefore, it was speculated to account for the electron transfer directly to AprAB for the reduction of adenosine-5′-phosphosulfate (41). This observation is also confirmed by the capability of *Desulfotomaculum aeronauticum* to utilize sulfate only by amendment of the quinone precursor menadione. Apparently here electrons are transferred from the menaquinone pool to APS rather than from periplasmic electron sources (17).

Haveman et al. (15) found a downregulation of *dsrMKJOP*/*hme*, *qmoABC*, and ATP synthase genes in *D. vulgaris* upon inhibition of DSR by nitrite amendment. They concluded that both membrane complexes donate electrons to AprAB and

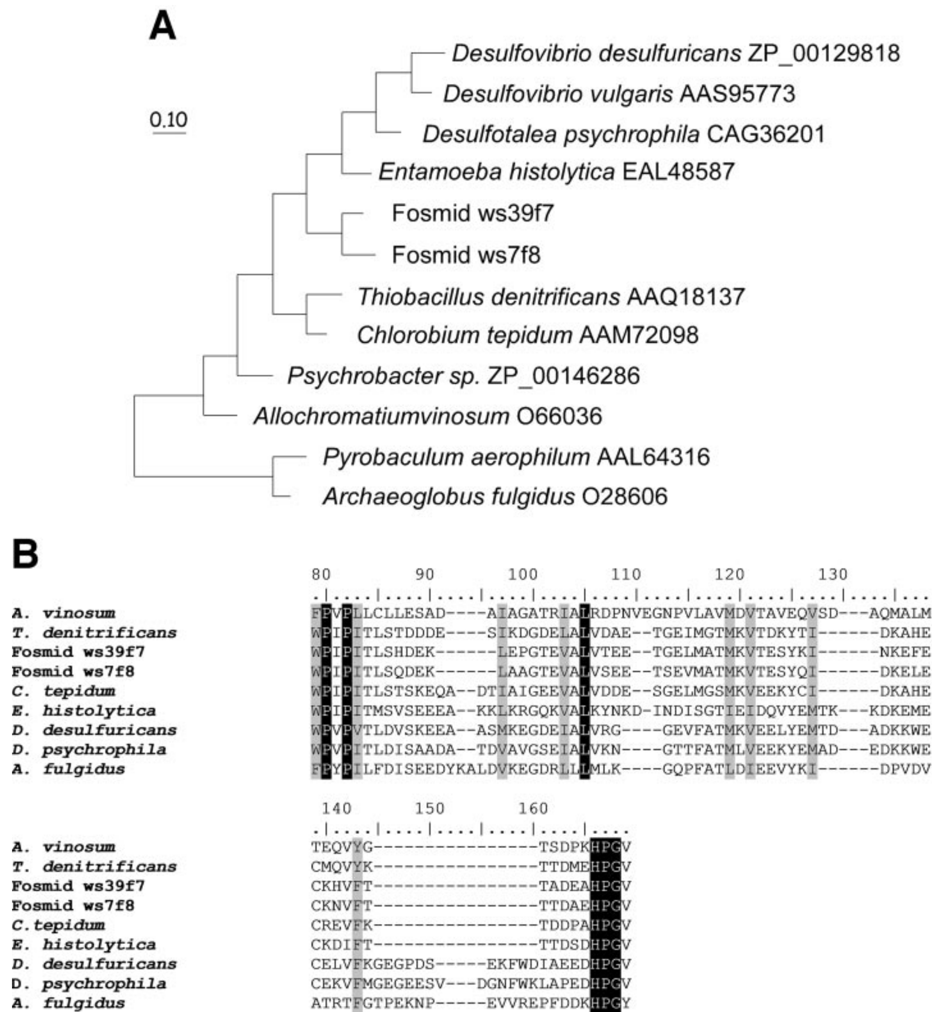

FIG. 5. (A) Sat-based phylogenetic reconstruction. The scale bar corresponds to 10% estimated sequence divergence. The tree was inferred using the maximum likelihood, maximum parsimony, and neighbor-joining methods. Multifurcations were introduced when branching orders were not supported by all phylogenetic methods used. (B) Partial amino acid sequence alignment of the Sat protein showing insertions/deletions supporting an affiliation of Sat with sulfur-oxidizing bacteria. Similar residues are highlighted according to the BLOSUM62 matrix for evolutionary substitution. The alignment is numbered according to the *A. vinosum* sequence.

DsrAB. Here it is shown that these genes are more universally distributed among SRP and most likely are of general importance in electron transfer of DSR. The proteins homologous to HmeA-E/DsrMKJOP and QmoABC (32, 41) may function as universal menaquinone oxidoreductases and transmembrane electron/proton transfer proteins in SRP. Such an obligate interaction would provide for the missing link between the menaquinone pool/periplasm and the cytoplasmic enzymes. The ubiquitous presence of the *dsrMKJOP*/*hme* and *qmoABC* genes in sulfate-reducing prokaryotes and their presence in some sulfite-reducing and phototrophic sulfur-oxidizing bacteria emphasize their relevance for general sulfur-based energy metabolism.

In this model, the previously supposed cytoplasmic electron shuttles (39, 43) from the membrane donors to cytoplasmic DsrAB and AprAB are not illustrated. In contrast, there is evidence for a direct interaction of DsrABDC with the membrane components. In *Desulfovibrio* spp. (49) and also in *A. vinosum* (5), a membrane-bound fraction of DsrAB was observed.

Several conserved hypothetical proteins that were often closely linked to known genes, such as ORF9 and ORF23, were identified. Thus, they might play a so far unknown role in sulfur-based energy metabolism. Further experiments should elucidate their role in these pathways.

Not all components important for sulfate respiration may necessarily be encoded on this subgenomic fragment. Pyrophosphatase, additional sulfate transporters, quinones, ferredoxins, flavodoxins, and oxidoreductases are widespread and could be easily supplied by general metabolism.

Horizontal gene transfer of the DSR pathway. Such a tight clustering of essential genes involved in DSR as in fosmid ws39f7 has not been previously observed. The results support previous speculations of a metabolic island of DSR (12, 22). Accordingly, fosmid ws39f7 might be the remaining part of an ancient, putative metabolic island or GEI of DSR. In any case,

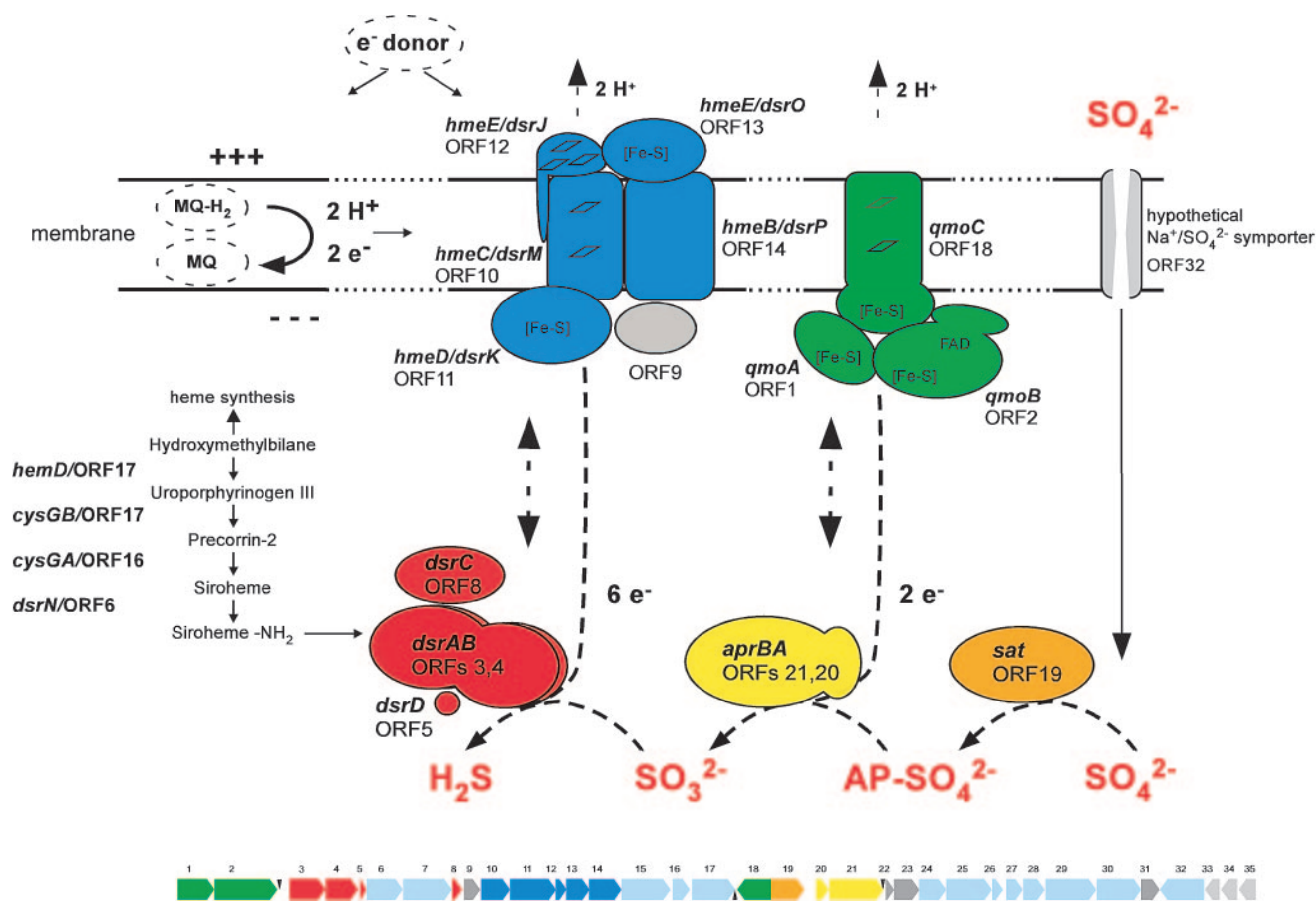

FIG. 6. Tentative schematic model of dissimilatory sulfate reduction based on the deduced proteins potentially encoded on fosmid ws39f7. The illustrations of Hme and Qmo complexes were modified after Mander et al. (32) and Pires et al. (41). ▱ indicates heme.

there are implications of the tight clustering of DSR-related genes for horizontal gene transfer and for the evolution of SRP.

The "selfish operon hypothesis" (29) is currently the most parsimonious explanation for clustering of genes that are not necessarily cotranscribed or do not permanently interact physically. It states that gene clusters are created and maintained by selection for transferability. Organisms with clustered and unclustered genes are assumed to be equally fit. Our data support evolution of SRP via the transfer of a complex gene cluster rather than individual genes. Since multiple genes, such as *dsrAB*, *sat*, and *aprAB*, and specific membrane proteins (*hme*/*qmo*) are required for a function, only the acquisition of all these genes is beneficial to the recipient. Thus, only organisms with close linkage of all genes can serve as donors. It is difficult to imagine that essential genes for such a complex metabolic pathway were acquired stepwise. In this case, one has to assume that these single, not-yet-functional genes were maintained in the genome until the core set of enzymes was completed. The transfer of a more complete set of genes in a single HGT event would enormously increase the chances for a successful implementation of DSR into energy metabolism. The close functional linkages of DsrAB to Hme and AprAB to QmoA-C suggest a paralleled evolution of cytoplasmic and membranous proteins. Additional sequence data from SRP should reveal whether *hme* and *qmo* were also involved in HGT.

The existence of a DSR gene cluster was previously doubted due to two observations. First, comparative phylogenetic studies on the 16S rRNA gene, DsrAB, and AprAB suggested multiple and independent horizontal gene transfers (12, 22). Second, the observed gene dispersal in SRP genomes provided evidence against the existence of a metabolic island of sulfate reduction. Apparently the dispersal of genes does not remarkably affect the capability to respire sulfate.

These results do not necessarily contradict the HGT of a gene cluster of DSR. Once such a fragment has been integrated into the genome and the DSR pathway has been implemented, single genes may have been exchanged by in situ ortholog gene displacement (38), explaining extant phylogenetic inconsistencies. Boucher et al. (2) demonstrated an unparalleled phylogeny of Sat and AprAB in *A. fulgidus* despite the fact that these genes are physically and functionally closely linked (Fig. 1). Moreover, the maintenance of a horizontally received gene cluster in the recipient could have been superimposed by high genome plasticity, as gene order generally evolves faster than the protein sequence (55). Genome plasticity is strongly influenced by mobile elements such as transposons (21, 54). Genes can be shuffled concomitant with a loss of mobile elements adjacent to a previous GEI. However,

mobilizable elements generally are not a prerequisite to prove ancient HGT events.

Due to limited sequence data, mobility elements, which are indicative for GEIs (9, 14), were not yet identified. Thus, fosmid ws39f7 does not meet this strict prerequisite of a classical GEI. Furthermore, the sequence data are still too scarce to compare GC content and codon usage in the flanking regions. Attempts have started to sequence regions adjacent to the presented fosmids to further investigate the existence of a GEI. A heterolog expression of recombinant genes was not successful yet. First experiments did not reveal a sulfide production from sulfite by *Escherichia coli* hosting fosmid ws39f7.

Generally, archaea extensively share genes with *Firmicutes* (3). Sulfate-respiring *Firmicutes* were involved in HGT affecting *dsrAB* and *aprA* (12), whereas a close phylogenetic relation of DsrAB from archaea and *Firmicutes* has also been discussed (53) but not proven. The pattern of insertions and deletions supports a common ancestor of DsrAB of *Firmicutes*, *Nitrospirae*, and *Archaeoglobus* spp. Finally, one might speculate that a genomic island of DSR was once transferred from a putative common ancestor of the GEI-bearing organism and sulfate-respiring *Firmicutes* to ancestral *Archaeoglobus* spp. Moreover, the mosaic phylogenetic affiliation of investigated genes also indicates a close interaction among sulfate-reducing and sulfur-oxidizing prokaryotes during evolution. It is surprising that the operon structure in ws39f7, gram-positive *D. hafniense*, and sulfur-oxidizing *C. tepidum* is partially conserved. Eisen et al. (10) reported that many genes from *C. tepidum* resemble those from *Archaeoglobus* spp., both being thermophilic organisms. In particular, sulfur oxidation genes are more similar to genes in *A. fulgidus* than in aerobic *Sulfolobales*. Thus, one could hypothesize that the investigated fosmids could represent a genetic link between both functional groups. Consequently, the DSR gene cluster reported here might represent a conserved progenitor of the scattered genes found in most modern SRP and might be regarded as "a living molecular fossil." Under this assumption, future HGT events to organisms incapable of DSR and successful implementation of this trait are imaginable.

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