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Phylogeny of Dissimilatory Sulfite Reductases Supports an Early Origin of Sulfate Respiration

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Microorganisms that use sulfate as a terminal electron acceptor for anaerobic respiration play a central role in the global sulfur cycle. Here, we report the results of comparative sequence analysis of dissimilatory sulfite reductase (DSR) genes from closely and distantly related sulfate-reducing organisms to infer the evolutionary history of DSR. A 1.9-kb DNA region encoding most of the α and β subunits of DSR could be recovered only from organisms capable of dissimilatory sulfate reduction with a PCR primer set targeting highly conserved regions in these genes. All DNA sequences obtained were highly similar to one another (49 to 89% identity), and their inferred evolutionary relationships were nearly identical to those inferred on the basis of 16S rRNA. We conclude that the high similarity of bacterial and archaeal DSRs reflects their common origin from a conserved DSR. This ancestral DSR was either present before the split between the domains *Bacteria*, *Archaea*, and *Eucarya* or laterally transferred between *Bacteria* and *Archaea* soon after domain divergence. Thus, if the physiological role of the DSR was constant over time, then early ancestors of *Bacteria* and *Archaea* already possessed a key enzyme of sulfate and sulfite respiration.

The ability to use sulfate as a terminal electron acceptor is characteristic of several bacterial lineages and one thermophilic genus of *Archaea*. In these prokaryotes, the enzyme dissimilatory sulfite reductase (DSR) catalyzes the six-electron reduction of (bi)sulfite to sulfide, which is the central energy-conserving step of sulfate respiration (25). One archaeal (*Archaeoglobus fulgidus*) and four bacterial DSRs have so far been characterized by enzyme properties (8, 14, 21, 22, 36). Characterized bacterial enzymes and typical sources include desulfoviridin (e.g., *Desulfovibrio vulgaris*), desulforubidin (e.g., *Desulfovibrio desulfuricans* Norway), P582 (e.g., *Desulfotomaculum ruminis*), and desulfofuscidin (e.g., *Thermodesulfovibrio yellowstonii*). Although they differ in absorption spectra, electrophoretic mobilities, and redox properties, all characterized bacterial enzymes have an $\alpha_2\beta_2$ structure or an $\alpha_2\beta_2\gamma_2$ structure (3, 8, 27) and possess iron-sulfur clusters and siroheme prosthetic groups. Gene sequences were previously determined for the DSR genes of *A. fulgidus* and *Desulfovibrio vulgaris* (8, 20) and were used to assign them to a redox enzyme superfamily characterized by a repeat structure common to sulfite and nitrite reductases (7). This superfamily also encompasses gene sequences of assimilatory nitrite and sulfite reductases from higher plants, fungi, algae, and bacteria (used biosynthetically) and the small, monomeric sulfite reductase from *Desulfovibrio vulgaris* (35). The physiological role of the monomeric reductase is unresolved, but the *Desulfovibrio vulgaris* enzyme resembles spectroscopically the low-molecular-weight sulfite reductases isolated from *Methanosarcina barkeri* and *Desulfuromonas acetoxidans* (24).

Members of the redox enzyme superfamily share enzyme properties or gene sequence motifs with the anaerobically expressed sulfite reductase from *Salmonella typhimurium* (17), the inducible sulfite reductase from *Clostridium pasteurianum* (13), and the "reverse sulfite reductases" detectable in the phototrophic sulfur bacterium *Chromatium vinosum* and in the sulfur-oxidizing chemolithotroph *Thiobacillus denitrificans* (31, 32). Thus, all characterized enzymes that catalyze either the oxidative or reductive (dissimilatory or assimilatory) transformation between sulfite and sulfide appear to be related. This study addresses the question of archetype. Was there a common progenitor, and if so, what was its physiological function? The recent observation of high sequence similarity between the DSRs of *A. fulgidus* and *Desulfovibrio vulgaris* (20), representatives of the *Archaea* and *Bacteria* domains, respectively, suggested either a horizontal gene transfer or a common origin of a highly conserved reductase. To distinguish between these alternatives, we determined the gene histories of the α and β subunits for representative sulfate reducers. Both were consistent with similar analysis of the 16S rRNA genes from these organisms, suggesting a single ancestral progenitor.

MATERIALS AND METHODS

Isolation of nucleic acids, gene amplification procedures, and Southern hybridization. Genomic DNA was isolated from the reference organisms as previously described (4). The primers DSR1F (5′-AC[C/G]CACTGGAAGCACG-3′), DSR2F (5′-CTGGAAGGA[C/T]GACATCAA-3′, modified from reference 20), DSR3F (5′-GAAGAA[C/G]ATG[A/T]ACGGGTT-3′), and DSR4R (5′-GTGTAGCAGTTACCGCA-3′, modified from reference 20) were dissolved to a concentration of 10 pmol/μl. For PCR amplification, 1 μl of each primer solution, 10 to 100 ng of DNA, 5 μl of 10× PCR buffer (500 mM Tris [pH 8.3], 20 mM $MgCl_2$, 5 to 10% Ficoll, 10 mM Tartrazine), 5 μl of 10× bovine serum albumin (2.5 mg/ml), 5 μl of 10× deoxynucleoside triphosphates (2 mM [each] dATP, dCTP, dGTP, and dTTP), and 2 U of *Taq* DNA polymerase were combined in a final reaction volume of 50 μl and loaded and sealed in a capillary tube. After initial denaturation for 15 s at 94°C, amplification was carried out in a 1650 Air Thermo-Cycler (Idaho Technology) for 30 cycles with each cycle consisting of 15 s at 94°C, 20 s at 54°C, and 54 s at 72°C. The reaction was completed by a final extension at 72°C for 1 min. PCR products were loaded together with a 1-kb DNA ladder molecular size marker on a 0.8% agarose gel to evaluate the PCR. Southern transfers were performed by treating the gel with

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250 mM HCl for 10 min (DNA depurination) and blotting the DNA onto a MagnaCharge Nylon membrane (MSI) following instructions published by Boehringer Mannheim Corporation (3a). A 243-bp double-stranded DNA probe labeled with digoxigenin-11-dUTP was prepared by PCR (as described above) with the primers DSR1F and DSR5R (5′-TGCCGAGGAGAACGATGTC-3′) and *Desulfovibrio vulgaris* template DNA. This probe targets a conserved region of the analyzed DSR α subunits. The blots were hybridized with the probe at 60°C overnight and washed at 65°C at intermediate stringency following the Boehringer Mannheim protocol. The digoxigenin-labeled probe and molecular weight markers were detected colorimetrically with the nitroblue tetrazolium salt and 5-bromo-chloro-3-indolylphosphate system (Boehringer Mannheim) according to the manufacturer's instructions.

DSR gene cloning, sequencing, and phylogeny inference. Untreated and *Eco*RI-digested PCR products were ligated into pCR™II plasmids and transformed into ONE SHOT competent *Escherichia coli* cells following the manufacturer's directions (TA Cloning System; Invitrogen). DNA sequences were obtained from double-stranded insert templates with M13 forward and reverse, infrared dye-labeled primers and a 4000L automated sequencer according to the manufacturer's instructions (LI-COR). Deduced amino acid sequences were aligned manually by pooling the amino acids into six groups (9) with the GDE 2.2 sequence editor (33a). Nucleic acid sequences of the gene fragments were then aligned according to the amino acid alignment.

Protein phylogeny. To construct phylogenetic trees based on the amino acid alignments, we prepared three data sets: one contained the DSR α-subunit sequences, a second contained β-subunit sequences, and a third contained a concatenated α- and β-subunit data set. For distance and parsimony analysis, gaps and missing sequence information were coded as missing data, yielding 186, 191, and 377 positions for α-subunit, β-subunit, and α- and β-subunit data sets, respectively. For protein maximum-likelihood methods, all positions where two or more sequences had missing data were deleted, while at those positions where only a single sequence was missing information, missing data were coded as a 21st amino acid. Final data sets consisted of 180, 184, and 363 positions for α-subunit, β-subunit, and α- and β-subunit data sets, respectively. Protein distances were inferred by using a maximum-likelihood method implemented in the PROTDIST program, with the Dayhoff PAM 001 matrix as the amino acid replacement model. Trees were inferred from the distances by using FITCH with global rearrangements (11). Unweighted amino acid parsimony analysis was completed with test versions 4.0.0d59 and 4.0.0d60 of the PAUP* program written by D. L. Swofford. Maximum-parsimony trees were determined by the branch-and-bound algorithm. Protein maximum-likelihood trees were calculated in two ways. Exhaustive searches were performed by using the PROTML 2.2 (2) program with the JTT-f amino acid replacement model to select the tree which conferred the greatest likelihood on the data. To account for rate heterogeneity among sites, protein maximum-likelihood trees were also estimated with the PUZZLE 3.1 program employing the JTT-f model with a mixed eight-category discrete gamma-plus-invariant-site model with default parameter estimation methods (34).

Nucleotide phylogeny. All nucleotide-level analyses were based on first and second codon positions of alignments that corresponded to the amino acid alignments described above for the DSR sequences. α-Subunit, β-subunit, and α- and β-subunit data sets consisted of 384, 466, and 850 aligned positions, respectively. A total of 1,041 aligned positions were utilized for the 16S rRNA data set. In all cases, missing data or alignment gaps were treated as missing information. The PAUP* program (and references therein) was used to perform nucleotide parsimony, distance, and maximum-likelihood analysis. Distance matrices were estimated by the maximum-likelihood method with the Hasegawa-Kishino and Yano (HKY) model with a discrete gamma-invariant-site model with trees selected under the minimum evolution criterion. The transition/transversion ratio and rate heterogeneity parameters were estimated via maximum likelihood on an HKY maximum-likelihood-distance–minimum-evolution topology. Maximum-likelihood analysis was performed with the same model. Heuristic tree-searching procedures for distance and maximum-likelihood methods involved simple stepwise addition with tree bisection reconnection rearrangements. Unweighted parsimony analysis was performed as described above.

Bootstrap analysis. Bootstrap analysis for protein distance methods utilized programs in the PHYLIP package. Bootstrap estimates for the protein maximum-likelihood trees utilized the resampling estimated-log likelihood method implemented in the PROTML 2.2 program. All other bootstrapping was performed with the PAUP* program. For protein distance, protein parsimony, nucleotide distance, and nucleotide parsimony analyses, 500 bootstrap resamplings were analyzed. Due to time constraints, nucleotide maximum-likelihood bootstrap analysis was based on 250 resamplings.

Nucleotide sequence accession number. The sequences for the DSR α and β subunits have been deposited in GenBank (accession no. U58114 to U58129).

RESULTS AND DISCUSSION

We first evaluated four primers, designed on the basis of sequence conservation between the DSR genes of *A. fulgidus* and *Desulfovibrio vulgaris*, in different combinations for PCR amplification of genomic DNA from a wide variety of reference organisms (Table 1). One primer pair (DSR1F-DSR4R) amplified the expected ~1.9-kb fragment from all 22 sulfate-reducing bacteria tested. No amplification could be observed with DNA from bacterial and archaeal species that do not derive energy from sulfate reduction (Fig. 1), demonstrating that this primer pair does not amplify (i) genes encoding assimilatory sulfite reductase, (ii) genes encoding the sulfite reductase from an organism having the capacity to respire sulfite but not sulfate (*Shewanella putrefaciens*), or (iii) the genes for the reverse sulfite reductases, which show some similarity to DSR with respect to catalytic parameters and subunit composition (31, 32). This is also consistent with recent sequence analysis of this enzyme (siroheme sulfite reductase) from the sulfur-oxidizing phototrophic bacterium, *Chromatium vinosum* (16). Comparative analysis revealed that the evolutionary distance between the enzymes from *Chromatium vinosum* and *Desulfovibrio vulgaris* is greater than that separating the enzymes from *A. fulgidus* and *Desulfovibrio vulgaris*. Although these data suggest a yet-earlier divergence between oxidative and reductive modes of dissimilatory sulfur metabolism, in the absence of additional sequences for the oxidative type of DSR, we do not consider it further.

We sequenced the 1.9-kb amplification products from *Desulfobotulus sapovorans*, *Desulfonema limicola*, *Desulfococcus multivorans*, *Desulfobacter latus*, *Desulfovibrio* sp. strain PT-2, *Desulfovibrio* sp. strain PIB2, *Desulfotomaculum ruminis*, and *Thermodesulfovibrio yellowstonii*. However, the product from *Thermodesulfobacterium commune* was not stable in *E. coli* and sequence information is not yet available for this organism. We are evaluating alternative cloning strategies and hope to include this sequence in future analyses. All sequences showed high similarity to each other and to those previously determined for *Desulfovibrio vulgaris* and *A. fulgidus* (Tables 2 and 3), and much less similarity to other members of the siroheme-containing redox enzyme superfamily (7). With the exception of reverse sulfite reductase from *Chromatium vinosum*, no extensive alignment was possible.

Phylogenetic trees for the DSR α and β subunits and combined α and β subunits were estimated from the amino acid and nucleotide data sets by distance, parsimony, and maximum-likelihood methods (Fig. 2). Trees were also inferred from a 16S rRNA data set containing a wider array of *Bacteria* and *Archaea* (Fig. 3). Overall, highly similar orderings of taxa were found between 16S rRNA and DSR trees.

The archaeon *A. fulgidus* branches closest to *Thermodesulfovibrio yellowstonii* and *Desulfotomaculum ruminis* in α-subunit, β-subunit, and α- and β-subunit DSR data sets (Fig. 2). However, which of these two bacteria is closest to *A. fulgidus* depends on both the DSR subunit and the phylogenetic methods. α-Subunit and α- and β-subunit amino acid-based analyses recover a *Desulfotomaculum ruminis-A. fulgidus* clade in the majority of bootstrap replicates, whereas nucleotide-level analyses of these data sets and both nucleotide and amino acid analyses of the β-subunit data set support a *T. yellowstonii-A. fulgidus* grouping. Trees inferred from the 16S rRNA data set also show the *Archaea* (represented by *A. fulgidus* and *Methanococcus jannaschii*) joining the bacterial subtree close to these two taxa with *Thermodesulfobacterium commune* usually forming the deepest bacterial branch (26). Distance and maximum-likelihood methods are congruent in finding *Thermodesulfovibrio yellowstonii* the next taxon to diverge. Bootstrap support for the clustering of *Thermodesulfovibrio yellowstonii*, *Thermodesulfobacterium commune*, and *A. fulgidus* is strong only for distance methods (Fig. 3). Furthermore, the clustering of *Desulfotomaculum ruminis* with these taxa is only moderately supported by parsimony and likelihood methods. Similar

TABLE 1. PCR amplification of genomic DNA from reference organisms

Species or isolate[a]	Source or strain	PCR products obtained with primer pair[b]				Hybridization with DSR probe
		I	II	III	IV	
SRs of the δ-*Proteobacteria*						
Desulfovibrio vulgaris	ATCC 29579	+	+	+	+	+
Desulfovibrio desulfuricans	ATCC 27784	+	+	+[2]	+	+
Desulfovibrio africanus	ATCC 19996	(+)	−	+	−	+
Desulfovibrio sp. strain PT-2	D. A. Stahl	+	+	+	+	+
Desulfovibrio oxyclinae	Y. Cohen	+	+	+	−	+
Desulfovibrio sp.	D. Gevertz	+	+	+	−	+
Desulfovibrio sp. strain G11	M. J. McInerney	+	+	+	+	+
Desulfoarculum baarsii	M. J. McInerney	−	−	+	−	+
Desulfobacterium niacini	DSM 2650	−	+	+	−	+
Desulfobacterium vacuolatum	DSM 3385	−	+	+	−	ND
Desulfococcus multivorans	ATCC 33890	−	+	+	−	+
Desulfonema ishimotoi Jade 02	F. Widdel	(+)	+	+	−	+
Desulfonema ishimotoi Tokyo 01	F. Widdel	−	+	+	(+)	+
Desulfonema limicola	ATCC 33961	−	−	+	−	+
Desulfobotulus sapovorans	ATCC 33892	+	−	+	−	+
Desulfomonas pigra	ATCC 29098	+	+	+	+	+
Desulfobacter latus	ATCC 43918	+	−	+	−	+
Desulfomicrobium baculatus	DSM 1743	+	−	+	−	+
Desulfobulbus propionicus	ATCC 33891	−	−	+	−	+
Nitrospira division SR						
Thermodesulfovibrio yellowstonii	R. Devereux	−	−	+	−	+
Thermodesulfobacterium division SR						
Thermodesulfobacterium commune	ATCC 33708	−	−	+	−	+
Gram-positive division SR						
Desulfotomaculum ruminis DL	ATCC 23193	−	+	+	−	+
δ-*Proteobacteria* with "reverse" SR						
Beggiatoa sp. strain MS-81-1c	D. Nelson	−	−	−	−	−
Beggiatoa sp. strain OH-75-2a	D. Nelson	−	−	−	−	−
Beggiatoa sp. strain 81-6	D. Nelson	−	−	−	−	−
Chromatium vinosum	ATCC 17899	−	−	−	−	−
Thiobacillus denitrificans	ATCC 25259	−	−	−	−	−
δ-*Proteobacteria* sulfite-respiring bacterium						
Shewanella putrefaciens	ATCC 8071	ND	ND	−	ND	−

[a] SR, sulfate reducer.

[b] +, PCR product of the expected size; −, no PCR product; (+), low yield of PCR product; +[2], two similar-sized PCR products; ND, not determined. PCR amplification of genomic DNA from 22 sulfate-reducing bacteria, 5 bacteria considered to possess a reverse-type sulfite reductase, and 1 bacterium having the capacity to respire sulfite with the DSR primers. The primer pair DSR1F-DSR4R (III) amplified the expected ~1.9-kb fragment for all sulfate reducers tested. Primer pairs DSR1F-DSR3R (I), DSR2F-DSR4R (II), and DSR2F-DSR3R (IV) generated the expected ~1.1-kb, ~1.4-kb, and ~0.5-kb fragments for only some of the sulfate-reducing bacteria analyzed. Sufficient quality of each genomic DNA for successful PCR amplification was demonstrated using conserved 16S rDNA-targeted primers (data not shown). Amplification products of all sulfate reducers with primer pair III hybridized specifically with a DNA probe complementary to a conserved region of the α subunit of the DSR.

results were obtained with a taxonomically reduced 16S rRNA data set that included only those taxa used in the DSR analyses (data not shown).

For all methods with both DNA and amino acid data sets of all the DSR data sets, the δ-subclass of the *Proteobacteria* (δ-*Proteobacteria*) forms a clade that receives highly significant bootstrap support (all bootstrap values were >97%). The monophyly of the δ-*Proteobacteria* receives much poorer support from 16S rRNA analysis. This is largely due to a weak tendency for *Desulfotomaculum ruminis* to cluster with the *Desulfovibrio* group.

Although the branching order within the δ-*Proteobacteria* is very similar between DSR and 16S rRNA data sets, where taxa overlap, a few minor differences are apparent. First, *Desulfovibrio vulgaris* and *Desulfovibrio* sp. strain PT2 strongly form a grouping to the exclusion of all other sequences in the 16S rRNA tree (bootstrap values were ≥99% for all three phylogenetic methods). These two also form a grouping for β-subunit and α- and β-subunit data sets but do not form a clade in optimal protein maximum-likelihood or nucleotide maximum-likelihood trees of the α-subunit data set. However, for this data set, protein distance and nucleotide distance methods do recover the *Desulfovibrio vulgaris-Desulfovibrio* sp. strain PT2 grouping with 87 and 55% bootstrap support, respectively (data not shown). Several factors are probably responsible for the failure of the likelihood methods to recover this relationship. First, since the α-subunit data set contains relatively few aligned positions, inferences based on this data set will be subject to large random error. Furthermore, the relatively short branches leading to the *Desulfovibrio vulgaris* and *Desulfovibrio* sp. strain PT2 sequences in the α-subunit trees compared to the β-subunit tree (Fig. 2) suggest that the α subunit of these two organisms may have diverged so little from the common ancestral sequence of the δ-*Proteobacteria* that the

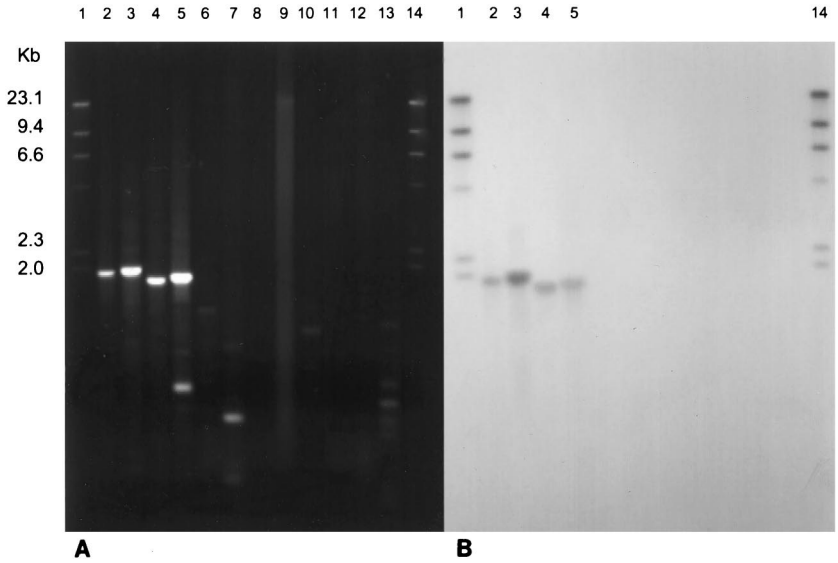

FIG. 1. PCR specificity determinations using the DSR primer pair DSR1F-DSR4R with genomic DNA from *Desulfovibrio vulgaris* ATCC 29579 (lane 2), *Desulfomicrobium baculatus* DSM 1743 (lane 3), *Desulfotomaculum ruminis* ATCC 23193 (lane 4), *Thermodesulfovibrio yellowstonii* (provided by R. Devereux) (lane 5), *E. coli* (provided by the University of Illinois [UI]) (lane 6), *Shewanella putrefaciens* ATCC 8071 (lane 7), *Nitrosomonas* sp. strain C56 (provided by J. Waterbury) (lane 8), *Thiobacillus denitrificans* ATCC 25259 (lane 9), *Arthrobacter globiformis* ATCC 8010 (lane 10), *Beggiatoa* sp. strain MS 81-1-C (provided by D. Nelson) (lane 11), *Chromatium vinosum* ATCC 17899 (lane 12), and *Methanosarcina acetivorans* (UI) (lane 13) Lanes 1 and 14 contain molecular weight markers. (A). In addition, genomic DNA obtained from the following bacteria was used for further specificity evaluation of the DSR primer set (data not shown): Fe reducer TT4B (provided by L. Krumholtz), *Nitrospira briensis* C128 (provided by Waterbury), *Nitrobacter hamburgensis* 14X (provided by J. Waterbury), *Nitrosovibrio tenuis* C141 (provided by J. Waterbury), *Oxalobacter formigenes* ATCC 35274, *Zoogloea ramigera* ATCC 19623, *Fibrobacter succinogenes* ATCC 19169, *Bacillus subtilis* ATCC 6051, a *Streptomyces* sp. (UI), *Streptococcus pyogenes* ATCC 12344, *Pseudomonas aeruginosa* (UI), *Beggiatoa* sp. strain OH-75-2a (provided by D. Nelson), and *Methanobacterium thermoautotrophicum* (UI). A fragment of the expected length was exclusively obtained with DNA from the sulfate reducers (*Desulfovibrio vulgaris*, *Desulfovibrio baculatus*, *Desulfotomaculum ruminis*, and *Thermodesulfovibrio yellowstonii*). Sufficient quality of each genomic DNA for successful PCR amplification was demonstrated in control reactions with conserved 16S rDNA-targeted primers (data not shown). The identity of the amplified products was confirmed by Southern hybridization with a DNA probe targeting a conserved region in the α subunit of DSR (B).

branch joining them is extremely short and cannot be resolved with the number of data available. Inclusion of more sequences in phylogenetic analysis often increases the efficiency of the methods for finding the correct topology (15). Consistent with this, our preliminary analyses of larger DSR data sets containing more δ-*Proteobacteria* with each of the phylogenetic methods recover a relationship between *Desulfovibrio vulgaris* and *Desulfovibrio* sp. strain PT2 in trees of α-subunit, β-subunit, and α- and β-subunit amino acid and nucleotide data sets. Furthermore, analyses of data sets including a partial α-subunit and complete β-subunit DSR sequence from *Desulfovibrio gigas* reveals that the latter organism is an immediate sister to a strongly supported *Desulfovibrio vulgaris-Desulfovibrio* sp. strain PT2 clade.

A second anomaly is the positioning of *Desulfovibrio oxyclinae*. In 16S rRNA trees, *Desulfovibrio oxyclinae* robustly groups with *Desulfovibrio africanus*, with these two taxa appearing as sisters to a *Desulfovibrio vulgaris-Desulfovibrio* sp. strain PT2 clade (Fig. 3). However, all of the DSR data sets show it as a

TABLE 2. Sequence similarities of 16S rRNA in sulfate-reducing prokaryotes[a]

Fragment	Percent similarity with fragment						
	Aful	Dvul	DPT2	Dlat	Dsap	Dmul	Tyel
Aful							
Dvul	66						
DPT2	66	95					
Dlat	66	81	81				
Dsap	67	83	82	85			
Dmul	65	82	82	86	89		
Drum	64	80	80	79	80	79	
Tyel	71	80	81	79	81	81	80

[a] Sequence data were extracted from available data bases. Only alignment positions represented in all aligned sequences for which nucleotides were determined unambiguously were included in the pairwise calculations. Fragments: Aful, *Archaeoglobus fulgidus*; Dvul, *Desulfovibrio vulgaris*; DPT2, *Desulfovibrio* sp. strain PT-2; Dlat, *Desulfobacter latus*; Dsap, *Desulfobotulus sapovarans*; Dmul, *Desulfococcus multivorans*; Tyel, *Thermodesulfovibrio yellowstonii.*

TABLE 3. Sequence similarities of DSR α- and β-subunit gene fragments in sulfate-reducing prokaryotes[a]

Fragment	Percent similarity (α subunit/β subunit) with fragment								
	Aful	Dvul	DPT2	DP1B	Dlat	Dsap	Dlim	Dmul	Drum
Aful									
Dvul	62/57								
DPT2	58/56	83/89							
Doxy	60/56	79/72	75/72						
Dlat	59/55	70/65	67/71	67/68					
Dsap	57/60	72/70	71/72	73/71	70/77				
Dlim	58/59	66/65	65/64	68/64	70/71	71/73			
Dmul	57/58	65/73	67/74	67/69	63/67	72/77	71/75		
Drum	61/61	61/62	59/62	58/59	60/59	60/64	57/63	51/61	
Tyel	56/62	50/54	51/53	49/53	52/55	53/57	58/59	53/53	53/61

[a] Only alignment positions represented in all aligned sequences for which nucleotides were determined unambiguously were included for the pairwise calculations. Fragments (GenBank accession numbers for each DSR sequence [α/β] are given in parentheses.): Aful, *Archaeoglobus fulgidus*: Dvul, *Desulfovibrio vulgaris*; DPT2, *Desulfovibrio* sp. strain PT-2 (U58114/U58115); DP1B, *Desulfovibrio oxyclinae* P1B2 (U58116/U58117); Dlat, *Desulfobacter latus* (U58124/U58125), Dsap, *Desulfobotulus sapovorans* (U58120/U58121); Dlim, *Desulfonema limicola* (U58128/U58129); Dmul, *Desulfococcus multivorans* (U58126/U58127); Drum, *Desulfotomaculum ruminis* (U58118/U58119); Tyel, *Thermodesulfovibrio yellowstonii* (U58122/U58123).

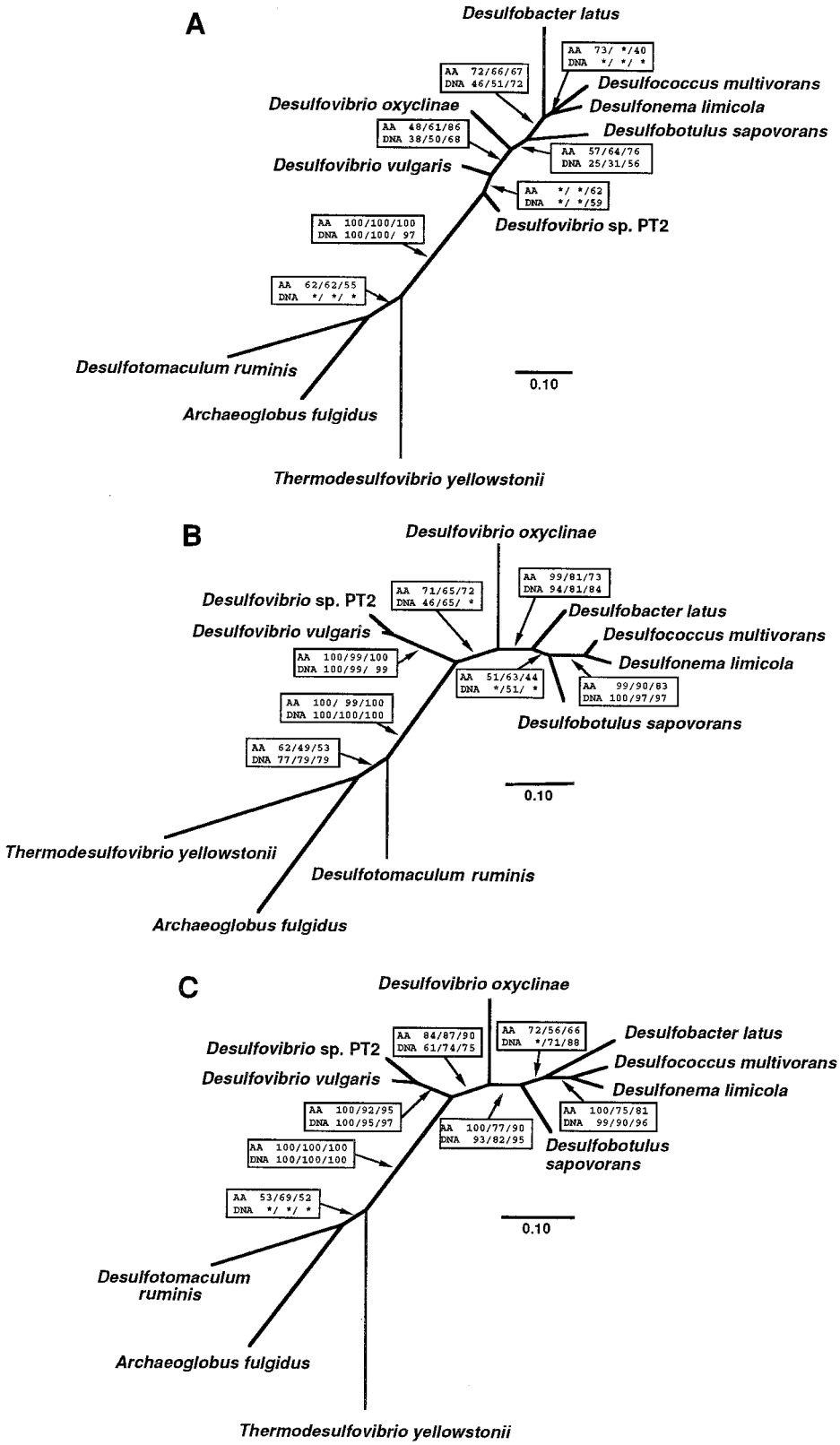

FIG. 2. Phylogenetic trees reflecting the relationships of the analyzed sulfate-reducing prokaryotes based on DSR sequences. Tree topologies and branch lengths were obtained from protein maximum-likelihood analysis of the DSR α-subunit (log likelihood = −2046.54) (A), β-subunit (log likelihood = −2116.25) (B), and α- and β-subunit data sets (log likelihood = −4188.60) (C) with the JTT-f amino acid substitution model. Bootstrap values for branches are reported in boxes with arrows pointing to the relevant branch. Bootstrap values are reported in the order distance/parsimony/likelihood for both amino acid (AA) and DNA data sets. Asterisks indicate that the branch in question was not recovered in the majority of bootstrap replicates by the phylogenetic method. The scale bar indicates the number of expected amino acid substitutions per site per unit of branch length.

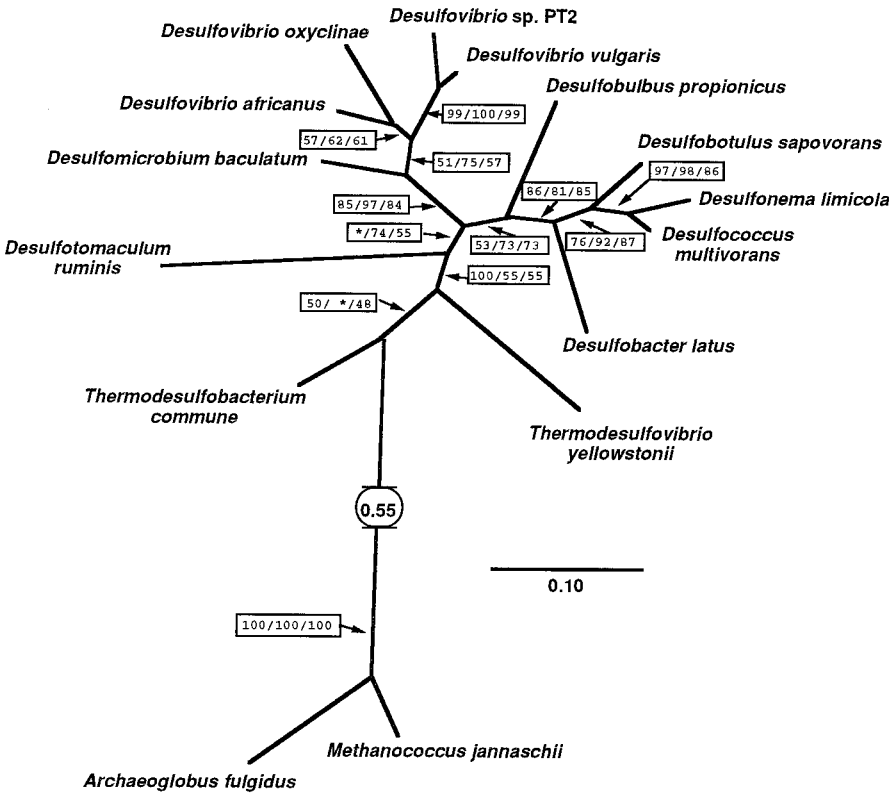

FIG. 3. Phylogenetic relationships between *Archaea* and *Bacteria* inferred from comparisons of 16S rRNA genes. The tree topology and branch lengths (log likelihood = −6573.00) were obtained by the maximum-likelihood method with the HKY model with a discrete gamma-invariant-site model with an alpha shape parameter (α) of 0.48, a proportion of invariant sites of 0.20, and a transition/transversion ratio of 1.54 (parameters were obtained by maximum-likelihood optimization). Bootstrap values are shown in boxes with arrows indicating the relevant branch. Bootstrap values are reported in the order distance/parsimony/likelihood, and asterisks indicate that the branch in question was not recovered in the majority of bootstrap replicates with the phylogenetic method used. The scale bar indicates expected nucleotide substitutions per site per unit of branch length. A section of the branch connecting the *Archaea* and *Bacteria* has been removed for ease of presentation. The length of this section is reported in an ellipse on the branch.

separate branch most closely related to the *Desulfobacter latus-Desulfococcus multivorans-Desulfonema limicola-Desulfobotulus sapovorans* clade, with moderate bootstrap support. Once again, however, inclusion of further δ-*Proteobacteria* DSR sequences (36a) suggests that this conflict may be an artifact of limited taxonomic representation. When phylogenetically broader data sets are considered, the bootstrap support for the *Desulfobacter latus-Desulfococcus multivorans-Desulfonema limicola-Desulfobotulus sapovorans-Desulfovibrio oxyclinae* clade decreases, indicating that the branching order among these groups is poorly resolved.

A final point of conflict between 16S rRNA and α- and β-subunit DSR topologies is the relative branching order of *Desulfotobacter latus* and *Desulfotobotulus sapovorans*. In this case, the branches in question in the DSR trees do not receive strong bootstrap support, and conflicts between phylogenetic methods are apparent (Fig. 2), indicating once again that the branching order among these taxa is not well resolved.

Topology aside, there are several notable differences in branch lengths between the DSR and 16S rRNA trees. For instance, in the 16S rRNA tree, a long branch connects the *Archaea*, *A. fulgidus* and *Methanococcus jannaschii*, to the *Bacteria* (Fig. 3). In contrast, archaeal and bacterial DSR sequences are not particularly distant; for α-subunit, β-subunit, and α- and β-subunit data sets, the branch leading to *A. fulgidus* is approximately the same length as branches leading to *Desulfotomaculum ruminis* and *Thermodesulfovibrio yellowstonii*. In contrast, the branch connecting the δ-*Proteobacteria* to the rest of the tree is relatively long in the DSR data set and quite short for 16S rRNA.

There are several explanations for these differences in branch length. First, it is possible that both sets of genes are tracing the same evolutionary history but have suffered periodic increases and/or decreases in their rate of substitution at different times. For instance, a periodic increase in the rate of substitution in the 16S rRNA gene may have occurred along the branch between *Archaea* and *Bacteria*, leading to the long branch length observed relative to that in DSR trees. Similarly, an increase in substitution rate in both subunits of DSR may have occurred on the branch connecting δ-*Proteobacteria* to all other taxa. However, it is also possible that the disparity in branch lengths may betray different evolutionary histories for the 16S rRNA and DSR data sets. For instance, the high degree of similarity between *A. fulgidus* DSR and homologs from *Desulfotomaculum ruminis* and *Thermodesulfovibrio yellowstonii* could indicate that the former organism acquired its DSR genes from one of the latter lineages by lateral gene transfer. Lateral transfer between gram-positive eubacteria and *Archaea* is not without precedent; for instance, the hsp70 and glutamine synthetase genes of *Archaea* may have been acquired by lateral transfer from gram-positive eubacteria (5, 29). Moreover, lateral transfer would explain why sulfate respiration is not widespread among known *Archaea* but is instead phylogenetically restricted to *A. fulgidus* and close relatives (37). The resolution of these alternatives will depend upon the availability of additional DSR sequences. In this regard, we note the recent pub-

lication of α- and β-subunit DSR sequences of the sulfite reductase of the crenarchaeote *Pyrobaculum islandicum* (23). Preliminary phylogenetic analyses by us show these sequences to be highly divergent but to share a most recent common ancestor with the *Archaeoglobus* sequences, suggesting that the *Archaea* are monophyletic in DSR trees. However, *P. islandicum* lacks the capacity for sulfate respiration, and its DSR may be under different functional constraints. Molitor and associates have suggested that the protein from *P. islandicum* and the sulfite reductases from sulfate reducers and from sulfur oxidizers represent three independent lineages that originated prior to the divergence of *Archaea* and *Bacteria* (23).

However, in the absence of additional data, the near congruence of 16S rRNA and DSR for sulfate-reducing *Bacteria* and *Archaea* suggests that the gene histories of the DSR subunits represent the phylogeny of the organisms. Because members of the domain *Archaea* are considered to be more related to *Eucarya* than to *Bacteria* (10, 18, 38), one plausible implication is that DSR was already present in the progenitor of the three recognized domains of life. Although we cannot exclude the possibility that ancestral DSR evolved within either *Bacteria* or *Archaea* soon after the split of the domains and was then transferred to the other domain by an early lateral gene transfer event, the capacity for sulfate or sulfite respiration appears to have a very early origin in either case.

This conclusion is justified only if these genes are orthologous and retain their ancestral physiological role. There are at least two supporting lines of evidence that the progenitor genes coded for an enzyme similar in function to the modern DSR. First, in contrast to assimilatory sulfite and nitrite reductases, both subunits of all sequenced DSRs contain a conserved ferredoxin-like domain. The identical position of this domain in the DSRs of *Bacteria* and *Archaea* indicates that this unique feature of DSRs was present before the divergence of the two domains (8). Second, as life may have originated in hot environments (1, 19, 38), the occurrences of sulfate-reducing prokaryotes among hyperthermophilic *Archaea* (*Archaeoglobus profundus* and *A. fulgidus*) and deep-branching thermophilic bacteria (*Thermodesulfovibrio yellowstonii* and *Thermodesulfobacterium commune*) are consistent with an early origin. Third, isotopic data suggest that dissimilatory sulfate reduction began 2.8 to 3.1 billion years ago (28, 33) but acquired global significance only after sulfate concentrations had significantly increased in the Precambrian oceans approximately 2.35 billion years ago (6). The isotopic data are reasonably consistent with a recent estimate of the time of domain divergence, ca. 3.1 to 3.6 billion years ago, based on sequence comparisons of a large number of different proteins (12). Since our data indicate that the progenitor genes of DSR evolved before or soon after the divergence of the three domains, organisms able to reduce sulfate, or at least sulfite, may have given rise to all known forms of bacterial and archaeal life. This view is consistent with recent speculations that respiratory electron transport systems evolved prior to oxygenic photosynthesis (30).

If our inference is correct, it is difficult to understand why the capacity for this lifestyle has such restricted phylogenetic distribution among *Bacteria* and *Archaea*. Dissimilatory sulfate reduction is found in only three primary bacterial lineages and is restricted to a single archaeal genus, *Archaeoglobus*. One possible explanation for the apparently limited phylogenetic distribution of sulfate-reducing microorganisms is that most bacterial and archaeal lineages have lost the appropriate genes during evolution. On the other hand, we might simply have failed to isolate representatives of the natural diversity of sulfate-reducing prokaryotes. We have started to systematically evaluate the second possibility by using the PCR primers described in this study to amplify DSR genes directly from total DNA isolated from a variety of habitats (sulfidogenic aquifers, gastrointestinal sites, microbial mats, lake sediments, and biofilm reactors). Our initial phylogenetic analyses of "environmental" DSR sequences have revealed novel sequences that are distinct from described sulfate-reducing assemblages (36a). This suggests great undescribed natural diversity of sulfate-reducing prokaryotes and is also consistent with the early origin of this possibly archtypical phenotype.

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