

Molecular Characterization of Sulfate-Reducing Bacteria in the Guaymas Basin†

Ashita Dhillon,¹ Andreas Teske,^{2,‡} Jesse Dillon,³ David A. Stahl,³ and Mitchell L. Sogin^{1*}

Marine Biological Laboratory, The Josephine Bay Paul Center for Comparative Molecular Biology and Evolution,¹ and Biology Department, Woods Hole Oceanographic Institution,² Woods Hole, Massachusetts, and Department of Civil and Environmental Engineering, University of Washington, Seattle, Washington³

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The Guaymas Basin (Gulf of California) is a hydrothermal vent site where thermal alteration of deposited planktonic and terrestrial organic matter forms petroliferous material which supports diverse sulfate-reducing bacteria. We explored the phylogenetic and functional diversity of the sulfate-reducing bacteria by characterizing PCR-amplified dissimilatory sulfite reductase (*dsrAB*) and 16S rRNA genes from the upper 4 cm of the Guaymas sediment. The *dsrAB* sequences revealed that there was a major clade closely related to the acetate-oxidizing delta-proteobacterial genus *Desulfobacter* and a clade of novel, deeply branching *dsr* sequences related to environmental *dsr* sequences from marine sediments in Aarhus Bay and Kysing Fjord (Denmark). Other *dsr* clones were affiliated with gram-positive thermophilic sulfate reducers (genus *Desulfotomaculum*) and the delta-proteobacterial species *Desulforhabdus amnigena* and *Thermodesulforhabdus norvegica*. Phylogenetic analysis of 16S rRNAs from the same environmental samples resulted in identification of four clones affiliated with *Desulfobacterium niacini*, a member of the acetate-oxidizing, nutritionally versatile genus *Desulfobacterium*, and one clone related to *Desulfobacula toluolica* and *Desulfotignum balticum*. Other bacterial 16S rRNA bacterial phylotypes were represented by non-sulfate reducers and uncultured lineages with unknown physiology, like OP9, OP8, as well as a group with no clear affiliation. In summary, analyses of both 16S rRNA and *dsrAB* clone libraries resulted in identification of members of the *Desulfobacteriales* in the Guaymas sediments. In addition, the *dsrAB* sequencing approach revealed a novel group of sulfate-reducing prokaryotes that could not be identified by 16S rRNA sequencing.

The Guaymas Basin is one of the series of deep semiclosed basins in the Gulf of California (5) where tectonic activity of the site generates a high heat flow (19). The highly productive surface waters and terrigenous input from Baja California and the Mexican mainland lead to rapid sediment accumulation and have created sediment layers up to 400 m deep (36). Hydrothermal fluids are discharged both through chimneys at 270 to 325°C and through the porous sediment (21). Compared to other known hydrothermal vent sites, the Guaymas Basin is unique in that the organic matter in the accumulating sediment is pyrolyzed under high-temperature conditions to petroleum-like products, such as gasoline-range aliphatic and aromatic hydrocarbons and residual polar asphaltic material (2, 35, 36).

Petroleum is thought to undergo microbial degradation at precipitated mineral mound surfaces or unconsolidated sediments, as well as the sediment-water interface (2, 35). In addition to petroleum hydrocarbons, the chemical milieu of the Guaymas vents includes short-chain organic acids and ammonia, which are released via pyrolysis of organic material in the sediments (23, 44). Many bacterial populations in Guaymas

Basin sediments have adapted to the petroleum hydrocarbon-rich environment, e.g., the anaerobic sulfate-reducing bacteria that oxidize alkanes, aromatic compounds, and fatty acids (30, 34) and aerobic aromatic hydrocarbon-degrading bacteria with a preference for aromatic carboxylic acids (11). Thus, the microbial communities in the Guaymas Basin may depend more on the utilization of hydrocarbon constituents derived primarily from photosynthetic biomass than on chemoautotrophic carbon assimilation with sulfide oxidation as an energy source.

The sulfate-reducing bacteria (SRB) are a large and extremely diverse physiological group of anaerobic microorganisms capable of degrading a wide range of organic substrates, including petroleum-based products like alkanes (34, 37), toluene (4, 32), benzene (30), and polyaromatic hydrocarbons (10, 51). The strictly anaerobic life style and slow growth rate of SRBs make it very difficult to isolate and identify representatives that grow on hydrocarbons. A culture-independent alternative approach based on the 16S rRNA gene sequence has been used to characterize bacterial communities, including novel biodegraders (1, 29, 37, 51). Sequence analyses of PCR-amplified 16S rRNA coding regions provide the most general framework for studies of natural microbial diversity and abundance (8, 15, 29, 33). However, 16S rRNA-based analysis does not provide an unambiguous link to the physiology or metabolic capabilities of a bacterium, particularly in newly discovered phylogenetic lineages without cultured isolates and known phenotypes (6, 8, 15). Hence, the functional gene approach has been used to identify bacteria responsible for biogeochemical processes in the environment (7, 16, 25).

* Corresponding author. Mailing address: Marine Biological Laboratory, Josephine Bay Paul Center for Comparative Molecular Biology and Evolution, NASA Astrobiology Institute, 7 MBL St., Woods Hole, MA 02543. Phone: (508) 289-7246. Fax: (508) 457-4727. E-mail: sogin@mbl.edu.

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‡ Present address: Dept. of Marine Sciences, University of North Carolina, Chapel Hill, Chapel Hill, N.C.

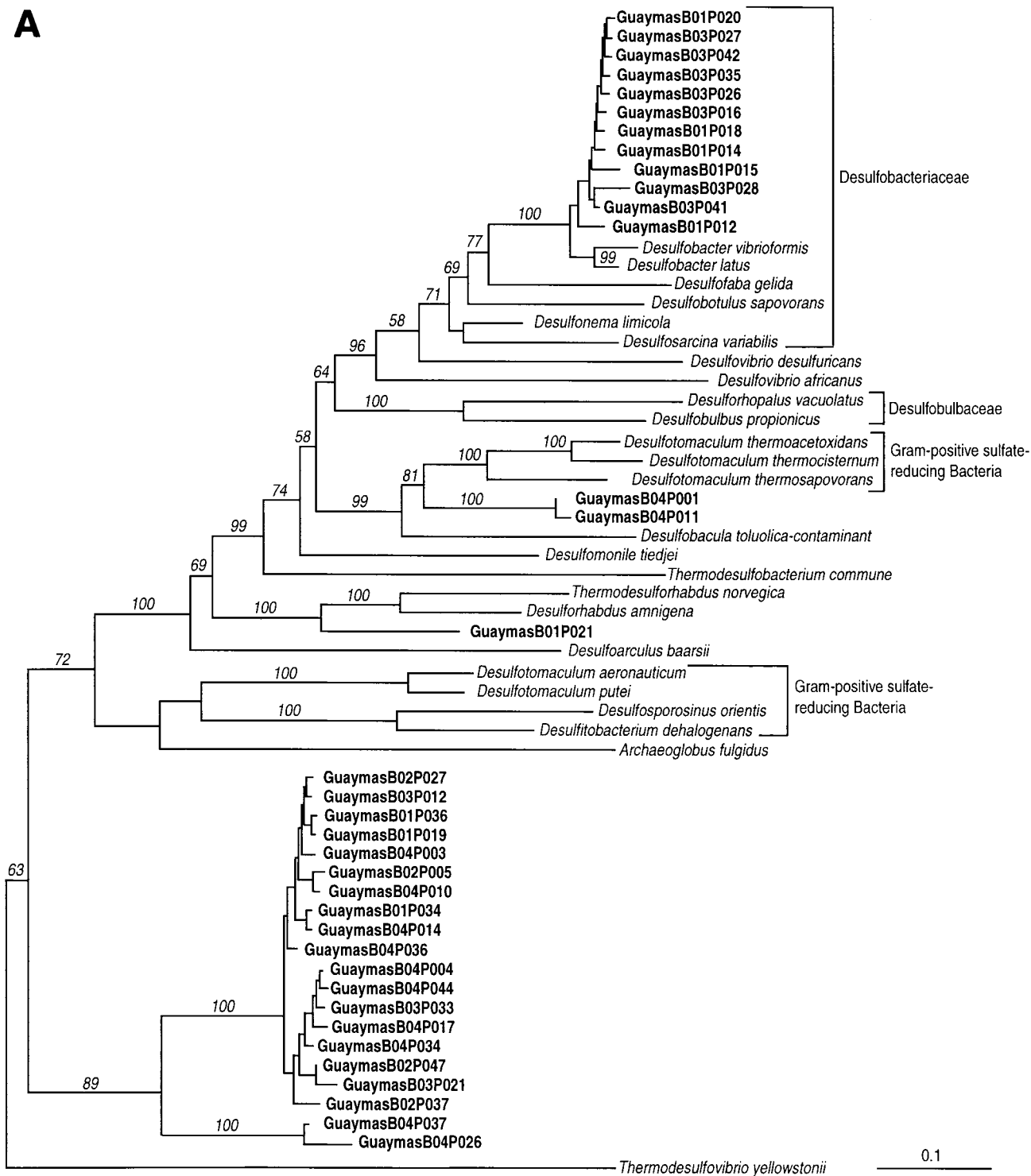


FIG. 1. (A) Phylogenetic tree based on the translated amino acid sequences of PCR-amplified *dsrAB* genes from sulfate-reducing prokaryotes. Sequences were retrieved from Guaymas core B sediment layers (1 to 4 cm). The tree was constructed by using the maximum-likelihood method in PUZZLE. The bootstrap analysis was done by using puzzleboot and 100 replicates. Bootstrap values are shown for branches with more than 50% bootstrap support. (B) Phylogenetic tree based on the translated, partial amino acid sequences of *dsrAB* genes from sulfate-reducing prokaryotes. Sequences were retrieved from Guaymas core B sediment layers (1 to 4 cm). The tree was constructed by using the maximum-likelihood method in PUZZLE. The bootstrap analysis was done by using puzzleboot and 100 replicates. Bootstrap values are shown for branches with more than 50% bootstrap support. Group I, group II, and group III sequence clusters were defined as described in reference 43; group IV was based on this data set.

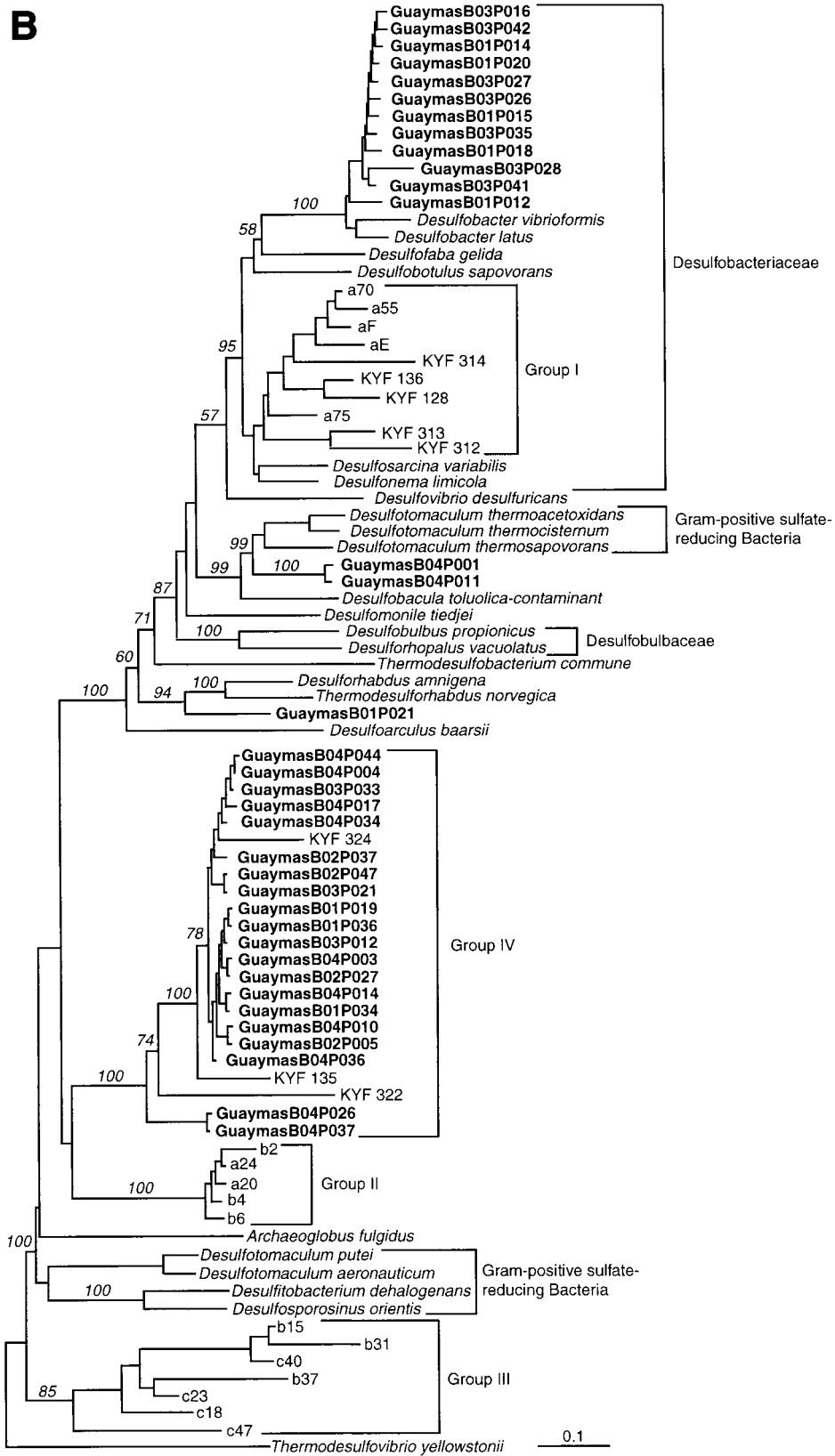


FIG. 1—Continued.

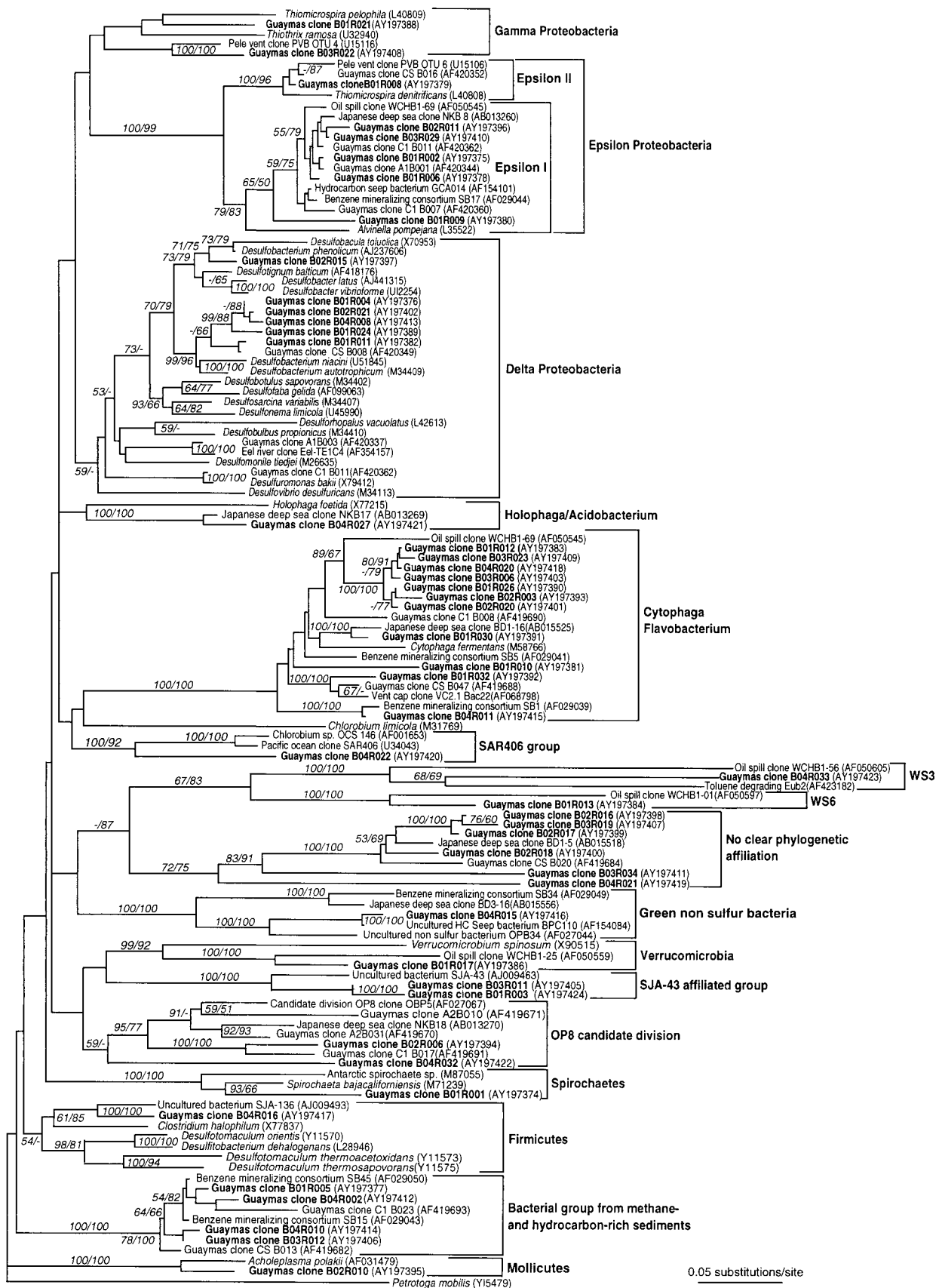


FIG. 2. Phylogenetic tree based on 16S rRNA sequences of bacterial clones from Guaymas core B sediment layers (1 to 4 cm). The tree was constructed with PAUP*. Bootstrap values based on 500 replicates each (for distance and parsimony) are shown for branches with more than 50% bootstrap support.

A key enzyme of dissimilatory sulfate reduction, dissimilatory sulfite reductase (EC 1.8.99.3), catalyzes the reduction of sulfite to sulfide, an essential step in the anaerobic sulfate respiration pathway. The key gene coding for this essential enzyme has been found in all SRBs that have been tested so far (17, 45). The 1.9-kb DNA fragment encoding most of the α and β subunits of dissimilatory sulfite reductase can be amplified by PCR from all recognized lineages of SRBs (17, 45). Comparative amino acid sequence analysis of the dissimilatory sulfite reductase genes (*dsrAB*) has recently been used to investigate the evolutionary history of anaerobic sulfate (sulfite) respiration (13, 17, 45) and the environmental diversity of uncultured SRB populations (7, 16, 25, 43). In this study we investigated *dsr* diversity in the Guaymas Basin using full-length sequences of PCR-amplified *dsrAB* gene fragments. This is the first report of PCR-amplified *dsrAB* sequences from environmental DNA that include nearly full-length alpha- and beta-subunit sequences.

MATERIALS AND METHODS

Sampling sites and characteristics. Sediment cores were retrieved during dives with the research submersible *Alvin* (Woods Hole Oceanographic Institution) on a cruise to the Guaymas Basin in April 1998. Core B (*Alvin* dive 3205; 28 April 1998; diameter, 6 in.; length, 25.3 cm) was obtained from the Everest Mound area in the Southern Guaymas vent field (27°00.888'N, 111°24.734'W). The designations of various clones obtained from core B from the Guaymas Basin begin with B, while the designations of clones obtained from core A (dive 3203; Everest Mound; 27°00.762'N, 111°24.656'W) and core C (dive 3207; Orpheus site; 27°00.435'N, 111°24.612'W) begin with A and C, respectively, as described in a previous study (40). Core B had no *Beggiatoa* mat, and the upper 6 cm consisted of a black layer of liquid petroleum mud. Gas bubbles appeared in the sediment at a depth of 7 cm. The in situ temperature profile of the sediment obtained by using *Alvin*'s thermoprobe was determined prior to coring. The uppermost 5 cm of the core had a temperature range of 3 to 16°C, while the temperature range for the segment from 5 to 23 cm was 16 to 140°C. The cores were brought to the surface within 6 h of sampling and were sliced at 1-cm intervals within 6 h after retrieval. Multiple 2-ml portions of the sediments were taken from each 1-cm layer and immediately frozen at -80°C for nucleic acid extraction.

Nucleic acid extraction, 16S rRNA and *dsr* gene amplification, cloning, and sequencing. Total genomic DNA was extracted from 30 mg of sediment from each layer by using a MOBIO cloning kit (MO BIO Labs Inc., Solana Beach, Calif.). Bacterial oligonucleotides 8F and 1492R (40) were used to prime the 16S rRNA PCRs. We employed primers *dsr*1F and *dsr*4R (45) to amplify the *dsr* genes from environmental samples. Each PCR mixture (final volume, 50 μ l) contained 5 μ l of each primer solution (10 pmol/ μ l), 1 μ l of DNA, 5 μ l of 10 \times PCR buffer, 1.25 μ l of 10 \times bovine serum albumin (10 mg/ml), 5 μ l of a 10 \times deoxynucleoside triphosphate mixture (2 mM dATP, 2 mM dCTP, 2 mM dGTP, 2 mM dTTP), and 2.5 μ l of *Taq* DNA (2 U/ μ l). Amplification was carried out with an Eppendorf Mastercycler gradient PCR machine. After initial denaturation for 1 min at 94°C, 30 cycles were performed, with each cycle consisting of 1 min at 94°C, 1 min at 54°C, and 3 min at 72°C. The reaction was completed by a final extension step at 72°C for 1 min. PCR products were purified on a 0.8% agarose gel and then poly(A) tailed and cloned by using a TOPOXL PCR cloning kit before they were transformed into *Escherichia coli* by using the manufacturer's protocols (Invitrogen, San Diego, Calif.).

The *dsrAB* sequences were determined with an ABI 3700 by using the M13 universal primers SP010 and SP030 to initiate DNA synthesis in cycle sequencing reactions. The 16S rRNA sequences were determined with a LICOR 4200 sequencer by using the same sequencing primers. The 16S rRNA sequences were assembled by using Phrap (12) and were aligned with sequences from the GenBank database by using the ARB software (www.arb-home.de) fast aligner utility. To obtain full-length *dsr* sequences, the internal degenerate sequencing primers 1FI (5'-CAGGAYGARTKACCG-3') and 1RI (5'-CCCTGGGTRT GRAYRAT-3') were designed on the basis of sequence conservation around positions 500 and 1500 in the *dsrAB* amplicons. The *dsrAB* sequences were assembled by using the software package Sequencher (<http://www.genecode.com>).

Phylogenetic analysis. The *dsrAB* sequences were translated by using MacClade (<http://macclade.org>) and were aligned by using ClustalX (42). We used the quartet puzzling algorithm in PUZZLE, version 5.0, to estimate a tree with maximum-likelihood branch lengths. A bootstrap analysis was done by using puzzleboot v 1.03 script. For searches under distance criteria we used 100 replicates per data set. The analysis included the full-length translated region of the *dsrAB* genes, excluding the region between the stop codons. In the case of the partial *dsrAB* tree, partial regions corresponding the Aarhus Bay and Kysing Fjord (Denmark) forward and reverse sequences were used in the analysis. The 16S rRNA sequence alignment for phylogenetic inference was minimized by using a mask in ARB (1,296 positions were used for analysis). The dendrogram was constructed with the ARB database evolutionary distance (neighbor-joining algorithm with likelihood settings). Distances were calculated by using the best-fit model TrN+I+G, as selected by Model Test, version 3.06 (31). The robustness of inferred topologies was tested by bootstrap resampling with minimum evolution and parsimony methods implemented in PAUP*, version 4.08b (39), by using the same distance model. The 16S rRNA analyses were checked for chimeras by using the CHIMERA-CHECK online analysis program of the RDP-II database (22). Three chimeras that were identified were removed from the phylogenetic analysis. For the *dsrAB* sequences, complete and partial *dsrAB* gene trees gave the same phylogenetic structure, ruling out the possibility of chimeras. The amino acid alignment was also checked for ambiguities visually. Blast searches were done for complete *dsrAB* fragment nucleotide sequences; the deeply branching group did not show any statistically significant similarity to other *dsrAB* sequences in the database.

Nucleotide sequence accession numbers. The Guaymas Basin 16S rRNA and *dsrAB* gene sequences have been deposited in the GenBank database under the following accession numbers: 16S rRNA, AY197374 to AY197384, AY197386, AY197388 to AY197403, AY197405 to AY197424; and *dsrAB*, AY197425 to AY197459.

RESULTS

Diversity of *dsrAB* genes. Five different phylogenetic groups of *dsrAB* genes were found in the Guaymas sediment (Fig. 1A). Nearly 34% of the sequences were closely related to *dsrAB* genes of the gram-negative delta-proteobacteria *Desulfobacter latus* and *Desulfobacter vibrioformis*, which are members of the acetate-oxidizing genus *Desulfobacter* (Fig. 1A). A second *dsrAB* cluster that accounted for 57% of the sequences amplified from Guaymas sediments branched deeply in the *dsrAB* tree and was phylogenetically distinct from sequences of cultured strains (Fig. 1A). This cluster contained two distinct lineages; two novel *dsrAB* sequences (B04P026 and B04P037) formed a sister group to a tightly clustered assemblage of 18 *dsrAB* sequences whose phylogenetic affinity was uncertain (Fig. 1A). Only small numbers of other *dsrAB* sequences were found. A single *dsrAB* sequence (B01P021) from the Guaymas sediments formed a monophyletic lineage with *Thermodesulfobacter norvegica* and *Desulfobacter amnigena*. Two *dsrAB* sequences grouped with a *dsr* clade of thermophilic, gram-positive SRB, represented by *Desulfotomaculum thermocisternum*, *Desulfotomaculum thermoacetoxidans*, and *Desulfotomaculum thermosapovorans*.

Comparisons with partial *dsrAB* sequences from environmental samples that included the alpha and beta subunits provided additional resolution while confirming the mutually exclusive, well-supported major branches of the *dsrAB* trees based on full-length *dsrAB* fragments (Fig. 1B). The deeply branching Guaymas sequences lacking cultured relatives were related but were not identical to sequences from coastal marine sediments, including clones KYF 324 and KYF 135 from Kysing Fjord and related *dsrAB* lineages from Aarhus Bay, Denmark (43) (Fig. 1B). None of the *dsrAB* sequences from the *Desulfobacter*-related delta-proteobacterial group from the

Guaymas Basin or from cultured isolates were related to *Desulfobacter*-related environmental sequences (group I) isolated from Kysing Fjord and Aarhus Bay (Fig. 1B) (43).

Bacterial 16S rRNA gene sequences. The bacterial clone libraries from the Guaymas sediments were very diverse and included relatives of numerous cultured and uncultured lineages in the *Proteobacteria*, as well as numerous other bacterial phyla (Fig. 2), and some were related to clones from a previous study (40). Most 16S rRNA gene sequences of delta-proteobacterial SRB (B02R021, B04R008, B01R024, B01R011, B01R004) and the previously sequenced Guaymas clone CSB008 (40) clustered with *Desulfobacterium niacini* and *Desulfobacterium autotrophicum* (>90% bootstrap support). Member of the acetate-oxidizing, nutritionally versatile genus *Desulfobacterium* also degrade short-chain fatty acids, ethanol, lactate, and tricarboxylic acid (TCA) cycle intermediates (48, 49). One Guaymas clone (B02R015) was related to the acetate and aromatic hydrocarbon oxidizers *Desulfobacula toluolica* (32) and *Desulfobacterium phenolicum* (48). In brief, the 16S rRNA sequences were most closely related to the sequences of cultured SRB that oxidize acetate and a wide range of substrates completely to CO₂. The most common bacterial phylogenetic types in core B with significant numbers of clones were members of the epsilon subclass of the *Proteobacteria*, the *Cytophaga-Flavobacterium-Bacteroides* phylum, the uncultured bacterial group associated with methane- and hydrocarbon-rich sediments, and an uncultured lineage with no clear phylogenetic affiliation (Fig. 2). Other phylogenetic lineages that contained only one or two Guaymas clones included the gamma subclass of the *Proteobacteria*, the *Spirochaetes*, the *Verrucomicrobia*, the *Holophaga-Acidobacterium* lineage, the green nonsulfur bacterial phylum, the *Firmicutes*, and the uncultured WS3 and WS6 lineages obtained previously from a jet fuel-contaminated aquifer (8). Also found were the uncultured OP8 candidate subdivision from deep-sea sediments and geothermal springs (15), sequences related to clone SJA43 from an anaerobic trichlorobenzene-degrading bioreactor (50), and a sequence related to SAR406.

DISCUSSION

Diversity of *dsrAB* genes. The *dsrAB* sequences within the *Desulfobacteriales*, close to *Desulfobacter latus* and *Desulfobacter vibrioformis*, could represent true *Desulfobacter* species or *dsrAB* genes of the gram-negative, delta-proteobacterial species *Desulfobacula toluolica* (32; M. Wagner, unpublished results). The latter possibility is supported by the sequence of a 16S rRNA clone related to *Desulfobacula toluolica* in the 16S rRNA library (B02R015). Furthermore, the current *dsrAB* database does not include representatives of the closely related genus *Desulfobacterium* (17, 45), a genus of nutritionally versatile acetate oxidizers that is well represented in the 16S rRNA clone library of the Guaymas Basin (Fig. 2). Therefore, the *Desulfobacter*-related *dsrAB* sequences retrieved from the Guaymas sediments could be derived from SRB belonging to the genus *Desulfobacterium*, whose 16S rRNA genes were found frequently in the Guaymas samples. Acetate-oxidizing and short-chain fatty acid-degrading, mesophilic SRB of the genera *Desulfobacter*, *Desulfobacula*, and *Desulfobacterium* should find favorable growth conditions in the relatively cool,

acetate- and short-chain fatty acid-rich surface layers of the Guaymas sediments (21).

Two nearly identical *dsrAB* sequences (B04P001 and B04P011) grouped with *dsrAB* genes of thermophilic representatives of the gram-positive genus *Desulfotomaculum*, including *Desulfotomaculum thermocisternum*, *Desulfotomaculum thermoacetoxidans*, and *Desulfotomaculum thermosapovorans*. The phylogenetic positions of thermophilic *Desulfotomaculum* species in *dsrAB* and rRNA gene trees are discordant. Alternative explanations for this include possible lateral gene transfer (17) or ancestral gene duplication followed by differential loss in the genus *Desulfotomaculum*. The nutritional demands of these sulfate reducers match the abundance of acetate and short-chain fatty acids in the Guaymas sediments. *Desulfotomaculum* species degrade a wide spectrum of substrates, including acetate, short-chain fatty acids, ethanol, and lactate (47), while in the absence of sulfate they can grow acetogenically on benzoate, propionate, butyrate, and ethanol (41).

The *dsrAB* clone related to *Desulforhabdus amnigena* and *T. norvegica* (Fig. 1A) indicated that SRB that were terminal oxidizers of acetate with the potential for aromatic hydrocarbon degradation were present. *Desulforhabdus amnigena* has been isolated from anaerobic granular sludge and uses acetate as a sole carbon and energy source (28). It does not metabolize benzoate or other aromatic compounds, but its close relative, the sulfate-reducing strain PROT1, is known to be a toluene degrader (4). The thermophilic gram-negative species *T. norvegica* oxidizes acetate, lactate, TCA cycle intermediates, and short-chain fatty acids; it was isolated from hot (70 to 75°C) oil field water samples, an environmental setting similar to the Guaymas Basin (3). At present, it is not possible to identify the SRB carrying the new clade of *dsrAB* sequences that are related only to environmental *dsrAB* sequences from Kysing Fjord; no cultured isolate is available that can connect *dsrAB* and 16S rRNA phylotypes. Although in a previous study the workers demonstrated that there are anaerobic methane-oxidizing microbial communities in Guaymas sediments (40), the *dsrAB* data set does not allow unambiguous identification of syntrophic SRB or their substrates that are involved in this process (14, 38). The sulfate-reducing members of anaerobic methanotrophic consortia in Guaymas sediments so far have not been identified by fluorescence in situ hybridization. 16S rRNA probes for members of the genus *Desulfosarcina*, the common sulfate-reducing syntrophs in methane-oxidizing consortia, have not hybridized with the bacterial cells in consortia from Guaymas Basin and several other locations (18, 27). *Desulfosarcina* sequences were not found in this survey and were not found in previous 16S rRNA-based studies (40). The *dsrAB* sequences without any cultured relatives that are predominant in this data set were most closely related to *dsrAB* clones from sulfate-reducing sediment layers and not to phylogenetically distinct *dsrAB* clones from the methane-oxidizing zone (43).

Bacterial diversity in the Guaymas sediments. Most of the rich bacterial diversity in Guaymas core B was affiliated with sequences isolated from hydrocarbon-rich environments, cold seep environments, or hydrocarbon-rich enrichments (20, 26, 30). The diverse proteobacterial and nonproteobacterial lineages in this study represent a mixture of phylotypes similar to that found in a previous study (40), related to presumably sulfur-oxidizing lineages (gamma and epsilon subclasses of the

Proteobacteria) and to phylotypes frequently found in geothermal springs, hydrocarbon-rich cold seeps, and anthropogenic oil spills (8, 15).

The 16S rRNA clones of SRB belonging to the phylotype *Desulfobacteriales* found in this study represent a group of organisms capable of complete oxidation of acetate and other electron donors, such as short-chain fatty acids, TCA cycle intermediates, and aromatic compounds. These substrates are end products of hydrolysis and fermentation of complex polymeric compounds by the diverse, heterotrophic microbial populations in the Guaymas Basin (11, 40) and, in addition, the products of thermogenic petroleum generation in the Guaymas sediments (35, 36).

Candidates for novel SRB are not necessarily limited to the classical delta-proteobacterial SRB. The highly diverse members of the uncultured phylum candidate divisions OP8 and phylogenetic groups with no clear affiliation could include previously unrecognized SRB that fill the niche. For example, members of the lineage associated with hydrocarbon- and methane-rich sediments were found repeatedly in sulfate-reducing enrichments from Guaymas sediment (30) and in freshly harvested Guaymas sediment characterized by high sulfate reduction rates (40). Although their environmental distribution suggests anaerobic metabolism and involvement in the degradation of aromatic hydrocarbon and petroleum compounds, the physiology and environmental activity of these diverse bacterial groups remain to be determined by pure-culture studies.

The two-pronged approach of using 16S rRNA gene and *dsrAB* clone library sequencing provided a more detailed picture of the SRB community in the Guaymas Basin than either of these techniques alone. Similar mutually compatible results were obtained in microbial ecology studies of hypersaline cyanobacterial mats that targeted the same system with 16S rRNA gene probing and *dsrAB* gene sequencing (24, 25). For the Guaymas Basin, the 16S rRNA gene and *dsrAB* data sets indicate the significance of members of the *Desulfobacteriaceae*, most likely the genera *Desulfobacterium* and *Desulfobacula*, and of the thermophilic gram-positive SRB in the sediment. The problem of congruence between the *dsrAB* and 16S rRNA data sets presents a continuing challenge, particularly in the interpretation of *dsrAB* sequences related to the thermophilic *Desulfotomaculum* species with discordant 16S rRNA and *dsrAB* phylogenies (17). Beyond the reach of 16S rRNA gene sequences, the *dsrAB* data set demonstrated the presence of uncultured and unknown major clades of sulfate-reducing prokaryotes in the Guaymas sediments that could not be identified by 16S rRNA gene sequencing.

Finally, this PCR-based survey was not necessarily complete. Sulfate reduction rate measurements in Guaymas sediments have consistently revealed high activities for mesophilic, thermophilic, and hyperthermophilic sulfate reducers (9). By using sediment slurries from the site where core B was taken, temperature gradient sulfate reduction measurements were obtained, which demonstrated that there were active, moderately or extremely thermophilic, sulfate-reducing populations (46). In the near-surface sediment layer (0 to 6 cm), the highest sulfate reduction rates were measured over a broad temperature range, ca. 40 and 70°C (maximum rate, 300 nmol of SO₄²⁻ ml⁻¹ day⁻¹), which matches the grow temperatures of the

thermophilic *Desulfotomaculum* species and of *T. norvegica*; an additional peak occurred between 80 and 90°C, which matched the temperature optimum of archaeal sulfate reducers of the genus *Archaeoglobus*. With increasing sediment depth, the temperature profile of sulfate-reducing activity moved towards the range of *Archaeoglobus*, and the overall sulfate reduction rates decreased (46). Apparently, the PCR-based survey did not detect *Archaeoglobus*-related *dsrAB* genes, although the activity of these hyperthermophilic sulfate reducers could be measured. These results emphasize the need for quantitative molecular approaches in microbial community analysis that circumvent PCR and cloning biases.

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