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Seasonal Changes in the Relative Abundance of Uncultivated Sulfate-Reducing Bacteria in a Salt Marsh Sediment and in the Rhizosphere of *Spartina alterniflora*

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Phylogenetic diversity and community composition of sulfate-reducing bacteria in a salt marsh sediment and in the rhizosphere of *Spartina alterniflora* were investigated. Uncultivated *Desulfobacteriaceae* family-related phylotypes were studied by selectively amplifying 16S rRNA gene fragments from DNA extracted from salt marsh rhizosphere samples. Two novel phylotypes were retrieved from rhizosphere samples, with A01 having 89.1% sequence similarity with *Desulfococcus multivorans* and 4D19 having 96.3% sequence similarity with *Desulfosarcina variabilis*. Additionally, six sequences that were extremely closely related to *Desulfococcus multivorans* (99% sequence similarity) were found. Reference RNAs containing sequences identical to corresponding cloned regions of A01 or 4D19 16S rRNA were synthesized via in vitro transcription and were used in subsequent quantitative membrane hybridization experiments. Oligonucleotide probes A01-183 and 4D19-189 were designed to specifically target these two novel phylotypes and were tested for target specificity against synthesized RNA and reference RNAs extracted from pure cultures. The newly designed probes were then used, together with eubacterial probes, to determine the relative abundances of the novel phylotypes in the salt marsh sediment and the rhizosphere. Mean relative abundances of A01-183 and 4D19-189 targets were 7.5 and 3.4%, respectively, suggesting that the target organisms of A01-183 and, to a lesser extent, of 4D19-189 play an important role in the salt marsh sediment and the *Spartina* rhizosphere. A seasonal trend of increased A01 relative abundance during the period of vegetative plant growth was evident, suggesting a close interaction between A01 and *S. alterniflora*.

The advent of molecular microbial ecology has provided a glimpse of the extensive genetic diversity of natural microbial communities and, at the same time, has underscored the fact that microbial community structure and dynamics are largely unknown. Approaches to describe the genetic diversity of natural soil or sediment communities have included DNA reassociation experiments (39), 16S ribosomal DNA (rDNA) retrieval and analysis (3), fractionation of total bacterial DNA by G+C content (20), and cross-hybridization of bacterial DNA from two communities (32), all of which have pointed to highly complex assemblages of bacterial populations. For example, Torsvik et al. (39) estimated that 10^3 to 10^4 different genomic equivalents were present in 1 g of soil, while Borneman et al. (3) recovered 124 previously undescribed 16S rRNA gene sequences from an agricultural soil. These studies of genetic diversity have provided valuable but qualitative insights into microbial community composition. Several investigations of the quantitative significance of various phylogenetic groups in soils and sediments have been conducted, but these have generally included culturing (e.g., reference 4) or enrichment (5, 35, 38) steps or have been limited to extremely broad phylogenetic groups, such as domains (e.g., reference 23). As a result, very little is known about community structure and how it is influenced by different environmental conditions or different microhabitats.

In plant-inhabited soils and sediments, a particularly important microhabitat is the rhizosphere, or region immediately surrounding and influenced by the plant roots. Key biogeochemical processes such as organic matter decomposition, pollutant degradation (2), and nonsymbiotic nitrogen fixation (37) occur at accelerated rates in the rhizosphere and greatly influence ecosystem functions. Despite its importance, very little is known about this subset of the total soil microbial community or how it differs from its nonrhizosphere counterpart.

In the current study, we investigated a natural sediment and rhizosphere community by combining a survey of microbial phylogenetic diversity with a study of the environmental significance of novel phylotypes. We chose the community of sulfate-reducing bacteria (SRB) inhabiting a salt marsh sediment because both the biogeochemical dynamics of this community and the physiology and 16S rRNA phylogeny of SRB have been well studied. Sulfate reduction is the dominant terminal electron-accepting process and accounts for more than half of the total decomposition of organic matter in salt marshes (21). In addition, the salt marsh cordgrass, *Spartina alterniflora*, is the most thoroughly studied marine wetland plant, and considerable information is available on *Spartina*-sediment interactions, production of organic compounds by its roots, and aspects of its decomposition in sediments (6, 16, 19, 22, 26). Sulfate-reducing activity has been shown to be closely tied to plant growth stages, suggesting that plant-SRB interactions in the *S. alterniflora* rhizosphere play an important role in salt marsh biogeochemical cycles (16, 19). To date, the 16S rRNA phylogeny of SRB is one of the most complete, and

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hybridization probes are available for each of the major groups (7, 8, 10). The phylogenetic groups are also defined by distinct physiological features, in particular, the ability to use specific electron donors. Therefore, comparative rRNA methods may also provide information on the types of substrates used by rhizosphere bacteria.

Using membrane hybridization with RNA extracted from marsh sediment samples and various probes for gram-negative mesophilic SRB (10), Devereux et al. (9) found that the family *Desulfobacteriaceae* (41) (targeted by probe 804 [10]) accounted for up to 20% of total bacterial rRNA and appeared to be the most important group of SRB in the salt marsh sediment. However, the relative abundances of probed genera within the *Desulfobacteriaceae* accounted for only a small fraction of the relative abundance of the family as a whole, suggesting that other undescribed *Desulfobacteriaceae* species played a significant role in the salt marsh microbial community. Therefore, the objectives of the current study were to (i) qualitatively investigate the diversity of the *Desulfobacteriaceae* and search for novel phylotypes by retrieving and analyzing 16S rDNA directly from rhizosphere samples; (ii) design and optimize 16S rRNA-targeted oligonucleotide probes that specifically target novel sequences, if found; and (iii) apply the newly designed and currently available probes to quantitatively investigate the population dynamics of the targeted phylotypes in the marsh sediment and the rhizosphere.

MATERIALS AND METHODS

Study site and sample collection. Samples were collected from a tall-form, creekside stand of *S. alterniflora* in Chapman's Marsh in southeastern New Hampshire. Iron and sulfur biogeochemistry (16, 19), production and emission of biogenic sulfur gases (30), acetate cycling in the rhizosphere of *S. alterniflora* (17), and the effect of plant growth stage on sulfate reduction (19) have been studied previously at this site. Boardwalks were used to access sampling sites. Sediment cores (5-cm diameter) were collected with a handheld corner (Wildco Wildlife Supply Co., Saginaw, Mich.) equipped with a separate plastic liner for each sample and were held anoxically on ice (19) for transport to the laboratory. Sediment cores were either processed within 1 to 2 h of sample collection or stored at −80°C until used. Cores for 16S rDNA sequence retrieval were collected on 22 August 1994 and 8 September 1994, and cores for RNA probing were collected biweekly or monthly from 3 November 1993 to 5 October 1994.

DNA extraction and purification. The upper 2.5 cm of each core was used for DNA extractions, as sulfate reduction rates (SRR) have been found to be highest in this depth zone (19). Bulk sediment was removed from roots in the upper 2.5 cm of each core by briefly rinsing roots with phosphate buffer (8.7 mM $Na_2HPO_4 \cdot H_2O$) that was adjusted with NaCl to the same salinity as sediment pore water (about 26 ppt). While most of the sediment was removed from roots through rinsing, microscopic observation of root hairs stained with DAPI (4′,6-diamidino-2-phenylindole) (15) showed that root hairs remained densely covered with rhizosphere bacteria. A total of 10 g (wet weight) rinsed roots, 10 g of sterilized 0.1-mm-diameter zirconia-silica beads (BioSpec Products, Inc., Bartlesville, Okla.), and 10 ml of extraction buffer (150 mM NaCl, 10 mM Tris-HCl [pH 8.0], 100 mM EDTA, 4% sodium dodecyl sulfate) were combined in a bead mill homogenizer cup (BioSpec Products, Inc.) that was packed in ice. The mixture was homogenized five times for 15 s and cooled on ice between homogenizations. The sample was then subjected to three freeze-thaw cycles at −80 and 65°C, transferred to a centrifuge tube, and centrifuged for 8 min at 8,000 × *g*. The supernatant was transferred to a clean tube, the pellet was washed with 3 ml of 10 mM Tris-HCl (pH 8.0) and centrifuged, and the resulting supernatant was combined with the previous fraction. Two grams of acid-washed polyvinylpolypyrrolidone was added to the supernatant, which was then incubated on ice for 30 min and centrifuged at 8,000 × *g* for 8 min. The resulting pellet was washed with 3 ml of 10 mM Tris-HCl (pH 8.0), which was combined with the previous supernatant after centrifugation. The supernatant was then extracted with Tris-buffered phenol (pH 8.0), phenol-chloroform-isoamyl alcohol (25:24:1), and chloroform-isoamyl alcohol (24:1). DNA was precipitated with ethanol at −20°C overnight, washed with 80% ethanol, dried briefly, and resuspended in approximately 400 μl of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The extracted DNA was further purified with SpinBind cartridges (FMC BioProducts, Rockland, Maine) and by low-melting-point gel electrophoresis as described by Moré et al. (29), except that electrophoresis was carried out for 1 h and EDTA was not added to the electrophoresis gel or running buffers. DNA (6 to 23 kb) was recovered from agarose gels by SpinBind cartridge purification as described by the manufacturer, except that EDTA was omitted from the wash buffer.

Amplification and cloning of *Desulfobacteriaceae* 16S rDNA. Primers fD1 (5′-gggaattcgtcgacAGAGTTTGATCCTGGCTCA-3′ [40]) and r804 (5′-ggaag cttggatccCAACGTTTACTGCGTGGA-3′) were used to amplify an 830-bp region of 16S rDNA from members of the *Desulfobacteriaceae* (annealing sites are in uppercase letters). Primer r804 was derived from the *Desulfobacteriaceae*-specific probe designed by Devereux et al. (10). PCR conditions were as described by Weisburg et al. (40), except that PCR mixtures contained 2 mM $MgCl_2$ and 40 temperature cycles were used. In order to ensure specific amplification of *Desulfobacteriaceae* 16S rDNA, the optimal annealing temperature (65°C) was determined by using DNA extracted from *Desulfovibrio vulgaris* and *Escherichia coli* as negative controls (each contains two mismatches with primer r804) and DNA extracted from *Desulfococcus multivorans* as a positive control (1). PCR products were analyzed by electrophoresis in 0.8% agarose gels (33).

Amplified 16S rDNA fragments were purified from the PCR mixture, ligated bidirectionally into plasmid vector pNoTA/T7 (Five Prime Three Prime, Inc., Boulder, Colo.), and transformed into competent *E. coli* cells with the Prime PCR Cloning Kit (Five Prime Three Prime, Inc.). White colonies were screened for inserts by using PCR to selectively amplify cloned inserts. For this purpose, PCR conditions described above were used, except that the template consisted of 1 to 2 μl of clone cells.

Clones that were found to contain the 830-bp insert were placed into categories by restriction fragment length polymorphism (RFLP) analysis. Cloned inserts were amplified by using whole cells as templates in PCR, as described above. The PCR products were then concentrated and desalted by ultrafiltration with 10,000 NMWL Ultrafree-MC filter units (Millipore Corp., Bedford, Mass.). Each PCR product was digested separately with the tetrameric endonucleases *Msp*I, *Hha*I, and *Hin*fI (33), and restriction fragments were resolved by gel electrophoresis in 4% MetaPhor agarose (FMC Bioproducts).

Sequencing and phylogenetic analyses. Plasmid DNA of at least one clone from each RFLP category was purified with the Perfect Prep system (Five Prime Three Prime, Inc.) and sequenced with a PRISM Ready Reaction Dye Deoxy Terminator Cycle Sequencing Kit (Perkin-Elmer Cetus) and an ABI 373A automated sequencer (Applied Biosystems, Foster City, Calif.). Primers M13 -20, M13 reverse (Stratagene, Inc., La Jolla, Calif.), and R536 (5′-ACCGCGGC KGCTGGC-3′) were used in sequencing reactions.

Retrieved 16S rDNA sequences were checked for potential chimeras with the CHECK_CHIMERA program of the Ribosomal Database Project (RDP) (25) and were aligned with 16S rRNA sequences of other members of the *Desulfobacteriaceae*, *Myxococcus xanthus*, and *E. coli*. Unambiguously aligned base positions were used to calculate Jukes-Cantor distances and construct phylogenetic trees with maximum parsimony, neighbor-joining, and least-squares methods available in the phylogenetic analysis package PHYLIP 3.57 (13). For maximum parsimony and neighbor-joining trees, 100 bootstrapped data sets were analyzed.

Generation of RNA standards by in vitro transcription. Reference RNAs for the uncultivated phylotypes were generated by in vitro transcription of cloned rDNAs A01, 2B14, and 4D19. In order to generate sense 16S rRNA transcript, it was necessary to subclone inserts into a plasmid vector containing an RNA polymerase promoter upstream from the insert. Cloned inserts were cleaved from pNoTA with *Sal*I and *Bam*HI (endonucleases whose recognition sequences had been incorporated into the 5′ ends of primers fD1 and r804, respectively). The insert was then separated from pNoTA DNA by gel electrophoresis in 2.5% NuSieve agarose (FMC Bioproducts) and cloned into pBluescript II KS^+ (pBS; Stratagene, Inc.) that had been previously digested with *Bam*HI and *Sal*I (33). pBS was transformed into competent *E. coli* cells, and transformants were screened for inserts.

Plasmid DNA was isolated from transformants and linearized by digestion with *Xba*I, which cleaved pBS downstream of insert sequences. *Xba*I and RNases were then inactivated by treatment with 50 μg of proteinase K per ml for 30 min at 37°C. The mixture was extracted twice with Tris-buffered phenol–chloroform–isoamyl alcohol (25:24:1), and the linearized plasmid was precipitated with ethanol and resuspended in sterile distilled H_2O. The in vitro transcription reaction mixtures contained transcription buffer (40 mM Tris-HCl [pH 8.0], 8 mM $MgCl_2$, 50 mM NaCl, 2 mM spermidine, 30 mM dithiothreitol), 1 μg of linearized pBS DNA, 400 μM (each) ribonucleotide, 30 mM dithiothreitol, 1 μl of RNase inhibitor (Boehringer Mannheim Corp., Indianapolis, Ind.), and 10 U of T7 RNA polymerase (Stratagene, Inc.) in a volume of 25 μl. After incubation of the reaction mixture at 37°C for 1.5 h, an equal volume of sterile distilled H_2O was added and the template DNA was degraded by adding 1 μl of RNase-free DNase I (Stratagene, Inc.) and incubating the mixture at 37°C for 15 min. T7 RNA polymerase was inactivated by heating the mixture to 75°C for 10 min, and RNA products were purified with NuClean R50 Sephadex spin columns (VWR Scientific, Inc., Bridgeport, N.J.). Purified RNAs were analyzed by spectrophotometry and denaturing polyacrylamide gel electrophoresis (33).

Probe design and optimization. Oligonucleotide probes for novel *Desulfobacteriaceae* 16S rRNA sequences were designed by examining an alignment of the cloned inserts (this study) and all previously described *Desulfobacteriaceae* 16S rRNA sequences available from the RDP (25). Regions that contained sequences unique to the novel phylotypes were considered as potential probe target sites. Probe specificity was checked with the RDP's CHECK_PROBE utility (25). Other oligonucleotide probes used in this study included 814 (complementary to *Desulfococcus multivorans*, *Desulfosarcina variabilis*, and *Desulfobotulus sapovorans* 16S rRNA) (10); EUB338 (complementary to almost all

TABLE 1. 16S rRNA oligonucleotide probes and target groups

Target(s)	Probe	Probe sequence	Target site[a]	Wash temp (°C)
A01	A01-183	CCCCTAAGAAAATACGAT	183–201	40
A01	A01-267	CTAACCATCGCGGCCTTG	267–285	53
4D19	4D19-189	CCCTTGATCCAACATTCC	189–207	46
Most bacteria	EUB338[b]	GCTGCCTCCCGTAGGAGT	338–356	48
Most bacteria and A01	A01-338	GCTGCCTCCCGTAGGMGT[c]	338–356	48
Desulfococcus multivorans, *Desulfosarcina variabilis*, and *Desulfobotulus sapovorans*	814[d]	ACCTAGTGATCAACGTTT	814–831	45

[a] *E. coli* numbering.
[b] Reference 36
[c] M refers to A or C.
[d] Reference 10.

known *Bacteria* 16S rRNAs) (36); and a modified bacterial probe, A01-338, described below (Table 1).

Optimal wash temperatures (i.e., T_ds) for the newly designed probes were determined following hybridization of ^{32}P-labeled probes (10) with the reference RNAs generated by in vitro transcription. To determine approximate T_ds, wash temperatures of 35 to 65°C were tested in 5°C increments on replicate membranes. These approximate T_ds were then refined, and probes were tested for specificity against nontarget RNAs by testing wash temperatures that bracketed the approximate T_ds. Nontarget RNAs consisted of rRNA extracted from *Desulfococcus multivorans*, *Desulfosarcina variabilis*, and *Desulfoarculus baarsii* (50 ng/blot), as well as in vitro-transcribed RNAs A01, 2B14, and 4D19 (12.5 ng/blot). It should be noted that it was necessary to omit poly(A) from hybridization buffers for hybridizations with probe A01-183 because the probe target contained a U-rich region (Fig. 1; Table 1) that appeared to be blocked by poly(A). However, other blocking agents present in the hybridization solutions were sufficient for reducing background signal.

Application of probes to environmental RNA. RNA was extracted from sediment and rhizosphere samples as previously described (10), except that the extraction buffer consisted of 7.5 ml of 50 mM sodium acetate, 10 mM EDTA, and 1.3 ml of phenol equilibrated with 50 mM sodium acetate–10 mM EDTA. For rhizosphere samples, excess bulk sediment was briefly rinsed from roots as described above. For bulk sediment samples, RNA was extracted directly from sectioned cores containing both roots and sediment. After resuspension in 100 to 500 μl of sterile distilled H_2O, nucleic acids were further purified with Sephadex G-25 spin columns (28) and were analyzed by spectrophotometry.

Probes were hybridized with membranes containing serial dilutions of environmental RNAs and reference RNAs. Reference RNAs consisted of RNA generated by in vitro transcription (above) for probes A01-183 and 4D19-189 or rRNA extracted from *Desulfobotulus sapovorans* for probe 814. The relative abundances of specific probe targets were calculated as percentages of total bacterial 16S rRNA as described by Stahl et al. (36). Samples for which the slope of probe signal per unit of RNA was not linear (i.e., $R^2 < 0.90$) were omitted from analyses.

Nucleotide sequence accession numbers. The cloned sequences were submitted to GenBank under accession no. U85478 to U85480.

Species/Sequence	A01-183
Target	CAUCGUAUUUUCUUAGGGG
4D19	UAUCUAAUAUUCCUUGGAA
Desulfococcus multivorans	AGUCAUUUAGGCUGUGGUU
Desulfosarcina variabilis	UAUCCAAAUAUCUUCGGAU
Desulfoarculus baarsii	GACCACGACAACUGCGGUU
Desulfobotulus sapovorans	UGUUGURUUUUCUUCGGGG
Desulfonema limicola	AGUCAUUA-AUACCCCGGU
Desulfonema magnum	UAUC-UUGAGAACUUCGGU

Species/Sequence	4D19-189
Target	GGAAUGUUGGAUCAAGGG
A01	GGGGGAUGCGGUCAAGGU
Desulfococcus multivorans	GGUUUAGAUGAUGAAAGG
Desulfosarcina variabilis	GGAUUUUUGGAUCAAGGG
Desulfoarculus baarsii	CGGUUGUUGCGGNNAAG
Desulfobotulus sapovorans	GGGAAUGC-AACCAAAGA
Desulfonema limicola	GGUAUUAAUGAUGAAAGA
Desulfonema magnum	GGUUUUUAAGAUCAAAGG

FIG. 1. Comparison of probes A01-183 and 4D19-189 with aligned sequences from nontarget SRB and other closely related sequences. Mismatches with probe target sequence are shown in boldface and underlined.

RESULTS

Amplification and restriction analysis. A total of 65 of 100 clones screened contained an insert of the expected size (approximately 830 bp) and were further analyzed for RFLPs. From RFLP analyses, eight unique operational taxonomic units were found, based on a combination of five unique *Hha*I fragment patterns, four unique *Hin*fI patterns, and three unique *Msp*I patterns.

Sequence analysis and consideration of potential chimeras. Although the CHECK_CHIMERA program of the RDP (25) suggested that one of the eight sequences, A01, may be chimeric, further analysis of this sequence indicated otherwise. Using A01's predicted secondary structure, we found complementary base pairing for all 74 bp where the two potential chimera fragments formed helices. In addition, both potential fragments had higher identity with A01's closest relative, *Desulfococcus multivorans*, than with its second closest relative, *Desulfosarcina variabilis*. We therefore were confident that A01 was not chimeric.

We found that the EUB338 target in clone A01 contained a G340 residue, resulting in a G-A mismatch with the published EUB338 probe. This mismatch may be due to an error in nucleotide incorporation by *Taq* polymerase, although it would also be tolerated by predicted secondary structure models as it results in a noncanonical base pair, G-A, that is common in 16S rRNA (42). In order to account for this mismatch, a modified EUB338 probe, A01-338 (5′-GCTGCCTCCCGTAGGMGT-3′) was used for hybridizations in which A01 RNA was used as a standard. The EUB338 target site in the remaining seven 16S rDNA fragments that were sequenced contained the expected EUB338 probe target.

Phylogeny of retrieved 16S rDNA sequences. Phylogenetic analysis revealed that all of the cloned sequences clustered within the family *Desulfobacteriaceae* near *Desulfococcus multivorans* and *Desulfosarcina variabilis* (Fig. 2). Topologies of maximum parsimony, neighbor-joining, and least-squares phylogenetic trees were very similar (data not shown). Two se-

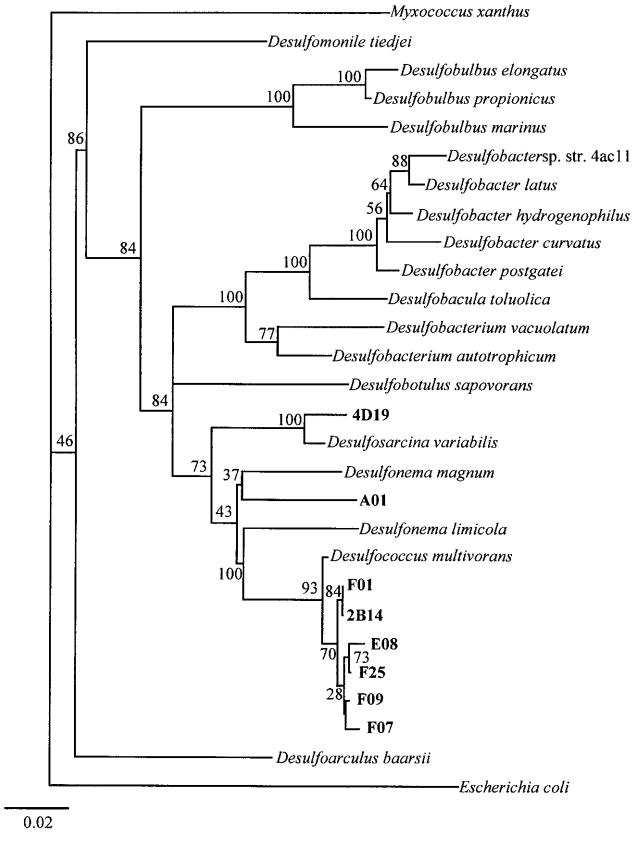

FIG. 2. Phylogenetic tree of cloned 16S rRNA gene fragments and 16S rRNA sequences from members of the *Desulfobacteriaceae* family constructed with a neighbor-joining algorithm. Jukes-Cantor evolutionary distances were calculated from 688 base positions. Bootstrap values (out of 100 trees) are shown adjacent to nodes. The scale bar is in fixed nucleotide substitutions per sequence position.

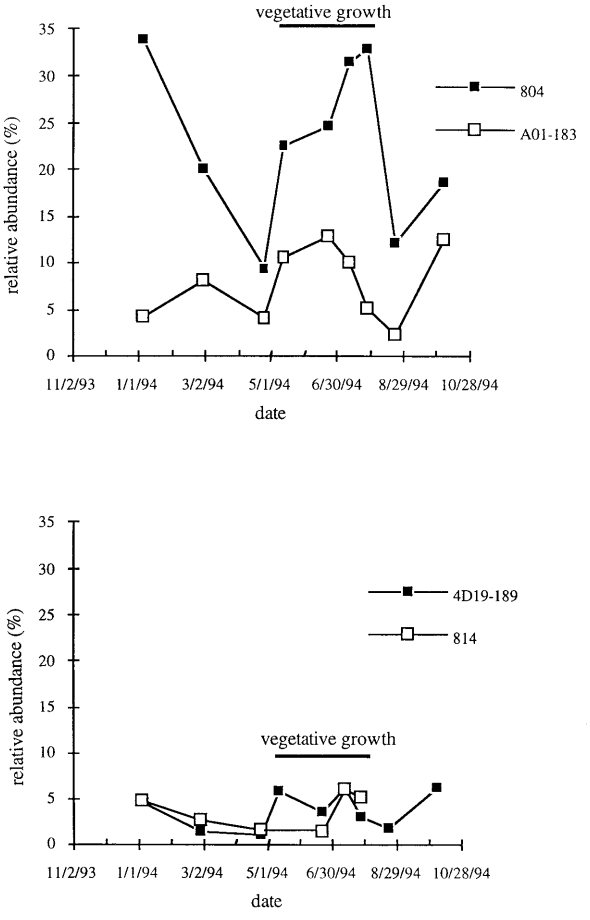

FIG. 3. Seasonal trends in rhizosphere relative abundances for 804 and A01-183 (top) and 4D19-189 and 814 (bottom) target rRNAs in the salt marsh. The period of vegetative growth for *S. alterniflora* is indicated. *S. alterniflora* begins flowering immediately after the vegetative growth period.

quences were unique. A01 had 89.1% identity with *Desulfococcus multivorans*, and 4D19 had 96.1% identity with *Desulfosarcina variabilis*. The remaining six sequences (2B14, E08, F01, F07, F09, and F25) were very closely related to *Desulfococcus multivorans* (having 99.0 to 99.7% identity), suggesting that they represented strains of this species.

Design and optimization of oligonucleotide probes. Oligonucleotide probes that targeted unique sequences of the two novel phylotypes described above were designed (Fig. 1; Table 1). Both probe targets are within a region that is highly variable in sequence and also is somewhat variable in length (42). The number and position of mismatches of each probe with currently available 16S rRNA sequences (Fig. 1) are indicative of high probe specificity, especially for probe A01-183, which has four mismatches with its closest known nontarget relatives. The high identity of the remaining six sequences with *Desulfococcus multivorans* made it impossible to design a specific probe(s) for them with more than one mismatch with known nontarget sequences. However, it is likely that these extremely close relatives of *Desulfococcus multivorans* were targeted by probe 814, which was designed to target *Desulfococcus multivorans*, *Desulfosarcina variabilis*, and *Desulfobotulus botulus* (10).

The empirically determined probe T_ds were 40°C for A01-183 and 46°C for 4D19-189 (Table 1). Specificity tests of both probes showed that no detectable probe remained bound to nontarget reference RNAs after membranes were washed at their respective T_ds.

Relative abundances of A01, 4D19, and 814-targeted organisms. We wished to determine the contribution of the uncultivated SRB, represented by the cloned sequences, to the SRB community. For this purpose, we synthesized RNA transcripts from the cloned rDNAs to use as reference rRNAs in the generation of standard curves. The RNA transcript thus produced contained a sense RNA sequence identical to positions 9 to 822 (*E. coli* numbering) of the uncultivated organisms' 16S rRNA and included the target sequences of both the EUB338 probe and the newly designed probes.

Over all depths and sampling dates, the mean relative abundance detected by probe A01-183 was (7.5 ± 3.5)% bacterial rRNA. The corresponding mean relative abundance detected by probe 4D19-189 was (3.4 ± 2.1)%. Relative abundances of 814 target rRNA were quite low (mean, 3.1%) and were lower than the sum of A01 and 4D19 relative abundances for all sampling dates and treatments for which data points from all three probes were available.

Seasonal trends in the relative abundances detected by probes A01-183, 4D19-189, and 814 for rhizosphere and bulk sediment RNA samples are shown in Fig. 3 and 4. These trends indicated that the relative abundance of A01 in the rhizosphere increased during the period of vegetative plant growth and

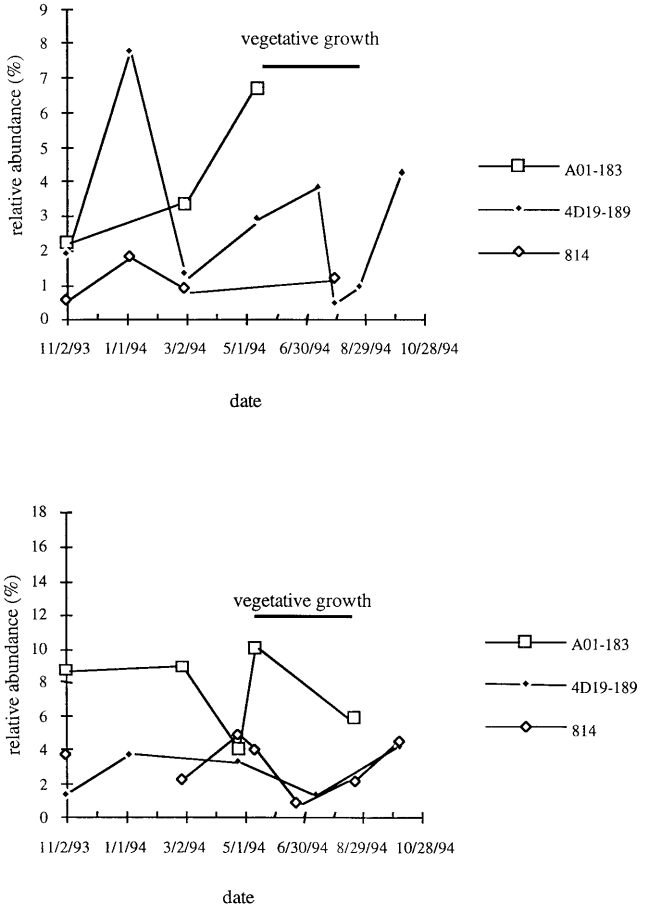

FIG. 4. Seasonal trends in the relative abundances of A01-183, 4D19-189, and 814 probe target rRNAs in 0- to 2-cm-depth (top) and 2- to 4-cm-depth (bottom) bulk sediment. The period of vegetative growth for *S. alterniflora* is indicated. *S. alterniflora* begins flowering immediately after the vegetative growth period.

then declined after the onset of reproductive plant growth, immediately following vegetative growth. Depth profiles in A01 relative abundances and bacterial rRNA absolute abundances were measured in samples from three depths (0 to 2, 2 to 4, and 6 to 8 cm), taken on 12 May 1994, at the beginning of the plant growing season (see Table 3). Relative abundance of A01 increased with depth, while absolute abundance of bacterial rRNA, expressed as picomoles of 338 target per gram of sediment, was highest in the upper sediment. Although absolute abundance values should be interpreted with caution due to sample-to-sample variability in RNA extraction efficiency, this trend of higher amounts of bacterial rRNA in the upper sediment is indicative of a more active community compared to the lower sediment.

DISCUSSION

Selective amplification of *Desulfobacteriaceae* 16S rRNA genes yielded two novel phylotypes, both members of the nutritionally versatile *Desulfococcus-Desulfosarcina-Desulfonema* assemblage. Oligonucleotide probes that specifically targeted the novel phylotypes were used in combination with reference RNAs synthesized from cloned rDNAs to determine the quantitative significance of the novel phylotypes. These quantitative probing experiments suggested that phylotype A01 played a dominant role in the salt marsh sediment and *S. alterniflora* rhizosphere microbial communities and that seasonal population dynamics of A01 were correlated with the phenology, or growth stages, of *S. alterniflora.*

Relative abundances and potential ecological roles of phylotypes A01 and 4D19. While amplification and cloning of 16S rDNAs are often used to examine microbial diversity, much more needs to be known of the representation, and ecology, of uncultivated bacteria in the natural community. Yet, hybridizations to determine the relative abundance of an rRNA require a source of RNA to generate the standard curves needed for quantitation. Synthetic rRNA, obtained in a manner similarly described and shown by Polz and Cavanaugh (31) to generate useful standard hybridization curves, was used to observe the dynamics of two novel, uncultivated phylotypes in marsh sediments.

If probes A01-183 and 4D19-189 are specific for A01 and 4D19, respectively (i.e., if they do not target other unknown phylotypes), then the rather high relative abundances of A01 and, to a lesser extent, 4D19 found here may provide an important insight into sediment and soil microbial communities. As discussed above, while molecular studies of soil-sediment microbial communities have suggested extremely high complexity, with up to 10^4 species present in a gram of soil (39), the community structure or quantitative distribution of individual phylotypes remains poorly understood. Here, the rather high relative abundances of A01 and 4D19 suggest that while the overall sediment community may be highly diverse, there were a small number of well-adapted species in the sediment habitat that play a significant role in microbial community dynamics.

The physiological characteristics of the novel phylotypes' closest relatives suggest that the versatility of this group of SRB may contribute to their success in the salt marsh sediment. The closest relative of A01, *Desulfococcus multivorans*, is capable of utilizing many electron donors including formate, lactate, ethanol, acetate, C_3 to C_{16} fatty acids (41), secondary alcohols such as 2-propanol and 2-butanol, isobutyrate, and propionate (14). Other electron donors utilized by members of the *Desulfococcus-Desulfosarcina-Desulfonema* assemblage include H_2, fumarate, malate, and benzoate (41). All members of this group are capable of complete oxidation of organic carbon to CO_2 (41). Such nutritional versatility could be advantageous in a complex environment such as the salt marsh sediment and the rhizosphere. In this habitat, potential electron donors for SRB include compounds directly released from roots, such as products of fermentative metabolism in roots during periods of hypoxia or anoxia (i.e., ethanol, malate, and probably acetate) (19), as well as low-molecular-weight compounds such as fatty acids and amino acids released from areas of root necrosis or from sloughed root cells. Electron donors may also be indirectly supplied to SRB by fermentative and acetogenic bacteria that incompletely oxidize dissolved organic carbon (DOC) released from roots and detritus. Acetate, for example, is thought to be an important intermediate produced by fermenters and subsequently utilized by SRB (34). Other compounds that have been found to stimulate sulfate reduction in salt marsh sediments include lactate, ethanol, butanol, and formate (34).

Another physiological trait that may be advantageous in the salt marsh rhizosphere is the ability to tolerate and/or utilize oxygen, which is transported to the roots and rhizosphere of *S. alterniflora* via lacunae (27). Although there are no reports of oxygen-supported SRB growth, *Desulfococcus multivorans* is one of several SRB that respire oxygen (12), a trait that it may share with its close relatives, A01 and 4D19. This trait would result in both tolerance and removal of oxygen and would

TABLE 2. Mean relative abundances of probe targets in bulk sediment and rhizosphere samples

Treatment	Probe	Mean relative abundance	SD	*n*
Sed[b] (0–2 cm)	A01-183	4.1	2.3	3
	4D19-189	2.9	2.4	8
	814	1.1	0.5	4
	804[a]	4.9	2.1	10
Sed[b] (2–4 cm)	A01-183	7.5	2.5	5
	4D19-189	2.8	1.3	5
	814	3.2	1.5	7
	804[a]	13.6	2.7	9
Sed[b] (6–8 cm)	A01-183	11.4	1.1	2
	4D19-189	4.1	2.6	4
	814	3.8	4.3	4
	804[a]	19.2	3.1	4
Rhiz[c]	A01-183	7.8	3.9	9
	4D19-189	3.8	2.0	9
	814	3.7	2.0	6
	804[a]	22.8	8.8	9
Total	A01-183	7.5	3.5	19
	4D19-189	3.4	2.1	26
	814	3.1	2.3	21
	804[a]	14.2	8.8	32

[a] 804 relative abundances measured by Hines et al. (18).
[b] Bulk sediment samples taken from depths indicated in parentheses.
[c] Rhizosphere samples taken from a depth of 0 to 4 cm.

thereby allow for continued metabolism in its presence, giving these SRB a competitive advantage in a rapidly changing redox environment.

The low relative abundances of targets of probe 814 (Table 2; Fig. 3 and 4), which Devereux et al. (10) designed to target the *Desulfococcus-Desulfosarcina-Desulfobotulus* group, suggested that 814 did not target the novel phylotypes A01 and 4D19, which are also members of this group. It was impossible to directly determine whether the novel phylotypes contained the 814 target (*E. coli* positions 814 to 831) because the cloned 16S rDNAs consisted of base positions 9 to 822. However, recently published 16S rRNA sequences from other organisms that fall within the *Desulfococcus-Desulfosarcina-Desulfobotulus* group provide evidence that the 814 probe does not target all group members. For example, 16S rRNA sequences from members of the genus *Desulfonema*, which is a close relative of *Desulfosarcina* and *Desulfococcus*, contain a mismatch with 814 in the center of the probe-target hybrid. Similarly, environmental clones A34 and A52 (11), also closely related to *Desulfococcus* and *Desulfosarcina*, contained the 804 target sequence but had a mismatch with the 814 probe. Thus, it is quite possible that while the novel phylotypes were targeted by 804 and were monophyletic with 814-targeted organisms, they did not contain the 814 target.

Seasonal and spatial trends in relative abundances. Although it is impossible to assess the statistical significance of seasonal patterns in relative abundances given the limited size of our data set, several interesting trends related to plant growth stage were apparent. In the rhizosphere, the relative abundance of A01 and, to a lesser extent, 4D19 exhibited a seasonal trend that was similar to the trend of relative abundances detected by probe 804, which targets members of the *Desulfobacteriaceae* family (Fig. 3). Rhizosphere relative abundances increased immediately after the onset of vegetative plant growth and then decreased when the plants began to flower, immediately after vegetative growth (Fig. 3). A similar trend in SRR has also been observed by Hines and coworkers at the same study site as that in the current study (19).

It is likely that the trends of increased relative abundances of *Desulfobacteriaceae* members and SRR during the period of vegetative plant growth were a direct result of DOC released from roots and rhizomes during this period. First, this seasonal pattern was observed only in the rhizosphere and was not found in the bulk sediment (Fig. 4), suggesting a direct influence of roots. Second, the physiological changes in *S. alterniflora* during the vegetative growth period provide evidence for its role in influencing rhizosphere bacterial dynamics. In tall-form stands of *S. alterniflora*, such as the current study site, almost all plants flower and flowering occurs simultaneously, resulting in a clear distinction between vegetative and reproductive growth stages. As *S. alterniflora* enters the vegetative growth period, it remobilizes nonstructural carbohydrates from rhizome stores and translocates new photosynthate (24) to rapidly growing roots and rhizomes. This increased supply of soluble carbohydrates, along with lysates from sloughed-off root cap cells from rapidly growing young roots, results in increasing amounts of DOC leaking from plant roots and rhizomes (19). When plants reach the reproductive growth stage, carbon is reallocated to flowering structures, carbohydrates are immobilized in rhizomes (24), and therefore, release of DOC into the rhizosphere decreases rapidly (19). Here, decreases in A01 and 804 (Fig. 3) relative abundances in the rhizosphere correspond with the onset of *S. alterniflora* flowering and decreases in SRR (19).

Although not coincident with general seasonal ecological patterns in the salt marsh, increased relative abundances of 804 (19), A01, and 4D19 (Fig. 3) probe targets observed in the rhizosphere on 5 October 1994 did coincide with increased SRR on the same date (19). This apparent stimulation of the SRB community may be due to either fortuitous availability of organic substrates at the sampling location or perhaps initial degradation of senescing root hairs.

The lack of any clear seasonal patterns in relative abundances in the bulk sediment (Fig. 4) was somewhat surprising given the strong seasonal variation in SRR (19). However, these data are relative and not absolute abundances and therefore reflect relative increases in target rRNA compared with total bacterial rRNA. Thus, a lack of clear seasonal trends suggests that varying abundances of other bacteria masked the seasonal trends in abundances of target SRB. Environmental factors that may affect fermentative, acetogenic, and sulfate-reducing bacteria in similar manners include temperature and general availability of organic carbon (as opposed to availability of specific substrates that may disproportionately affect one group). It should be noted that several bulk sediment data points were omitted from analyses due to nonlinear relationships between the probe signal and amount of RNA probed (see Materials and Methods). Although it is possible that a seasonal trend would have been evident if more data points were available for bulk sediment samples, Hines et al. (18) found that seasonal trends in relative abundances of other SRB probes were either not evident or less pronounced in the bulk sediment than in the rhizosphere at the same site.

Analysis of A01 rRNA relative abundances, absolute abundance of bacterial rRNA, and biogeochemical parameters measured at different sediment depths on 12 May 1994 indicated that SRR and absolute abundances or bacterial rRNA decreased with depth, while the relative abundance of A01 and total reduced sulfur increased with depth (Table 3). Taken together, these data suggest that (i) while the upper sediment

TABLE 3. A01-183 relative abundances, absolute abundance of bacterial probe target, SRR, and total reduced sulfur measured on 12 May 1994

Depth (cm)	A01-183 relative abundance (%)	EUB338 target (pmol g of sediment^{-1})	SRR (nmol ml^{-1} day^{-1})[a]	Total reduced sulfur (μmol ml^{-1})[a]
0–2	6.6	9.5	2,078	86
2–4	10.1	4.7	1,339	144
6–8	12.1	5.1	749	167

[a] Measured by Hines et al. (18).

harbors a more active SRB community, other bacterial groups are also active in the upper sediment and (ii) A01 is relatively better adapted to the lower sediments than the upper sediments.

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