

## Diversity of Sulfate-Reducing Bacteria in Oxic and Anoxic Regions of a Microbial Mat Characterized by Comparative Analysis of Dissimilatory Sulfite Reductase Genes

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**Sequence analysis of genes encoding dissimilatory sulfite reductase (DSR) was used to identify sulfate-reducing bacteria in a hypersaline microbial mat and to evaluate their distribution in relation to levels of oxygen. The most highly diverse DSR sequences, most related to those of the *Desulfonema*-like organisms within the  $\delta$ -proteobacteria, were recovered from oxic regions of the mat. This observation extends those of previous studies by us and others associating *Desulfonema*-like organisms with oxic habitats.**

The relationship of sulfate-reducing bacteria (SRB) to oxygen has been of particular interest since the publication of earlier reports of exceptionally high rates of sulfate reduction in the oxygenated surface regions of some microbial mats (4, 6, 16, 44). These observations are inconsistent with the generally accepted paradigm that environmentally available electron acceptors are depleted sequentially according to the thermodynamically predicted order of preference. Although there is no apparent restriction on the use of sulfate in the presence of a thermodynamically preferred electron acceptor, such as oxygen and nitrate, it is generally assumed that the use of sulfate under these conditions places an organism at a selective disadvantage. However, selective advantage or disadvantage can be evaluated only with a full understanding of the environmental context, from considering such factors as syntrophy, microhabitat, and varying physical and chemical environments. In turn, this understanding cannot be achieved without knowledge of population structure. In this study we use comparative sequencing of genes encoding a key enzyme in sulfate respiration, dissimilatory sulfite reductase (DSR), to directly identify SRB populations within the oxic and anoxic regions of a microbial mat community.

Although pure culture remains fundamental to microbiology, the biases associated with culture-based descriptions of community structure are now generally acknowledged. Thus, this well-established approach is increasingly complemented by the use of a variety of molecular tools. In particular, comparative sequencing of the 16S rRNAs now provides the most general framework for studies of natural microbial diversity and abundance (21, 35, 38, 39; for reviews, see references 2, 14, and 32). One limitation of the rRNA-based analysis is that it does not provide a direct link to physiology. To some extent, populations identified by rRNA sequence are expected to

share metabolic features with close relatives characterized by results of pure culture, but little inference can be made for more distant relatives. Thus, novel lineages of SRB—which may contribute to sulfate reduction in oxic habitats—cannot be identified by rRNA sequence alone.

A direct molecular identification of novel SRB must consider the presence of enzymes required for sulfate respiration or the genes encoding them. To this end we earlier demonstrated that a 1.9-kb DNA fragment encoding most of the alpha and beta subunits of the DSR could be amplified by PCR from all recognized lineages of SRB with a single primer set (46). DSR catalyzes the six-electron reduction of sulfite to sulfide and hence is required by all SRB. Development of a general PCR primer set was possible because of the remarkable conservation of the DSR sequence. This conservation was first suggested by the combined studies led by Voordouw and Trüper showing that the bacterial (*Desulfovibrio*) and archaeal (*Archaeoglobus*) genes have approximately 60% nucleotide similarity (11, 20, 25, 45).

Our previous studies demonstrated that this PCR primer set would amplify the appropriate DNA fragment only from sulfate-respiring microorganisms and that the phylogenies of DSRs of hitherto analyzed reference strains are consistent with that inferred from the 16S rRNA (46). The homologous enzyme from the sulfide-oxidizing organism *Chromatium vinosum* is phylogenetically well separated from those of sulfate respirers (20). Thus, environmental studies based on DSR sequence analyses should provide a more direct measure of SRB diversity and distribution. We here describe the use of this approach to directly evaluate the diversity of SRB populations in a hypersaline cyanobacterial mat from Solar Lake (Sinai, Egypt). In particular, we addressed the issue of the relationship of the presence of SRB to the availability of oxygen by determining the distribution of DSR sequence types within previously described oxic and anoxic depth intervals within this mat (7, 12, 31, 34).

**Mat maintenance and analysis.** Mats maintained in aquaria were characterized after 5 h of exposure to light as described in the accompanying paper (31) and as previously established (16, 24, 34).

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TABLE 1. Distribution of characterized DSR clones by depth and phylogenetic affiliation

Clade or lineage	Clone(s) (GenBank accession no.) found at indicated depth (mm) in indicated mat zone							
	0.05	0.3	0.4	0.6	Fluctuating oxic-anoxic	4.0	9.0	
Clade A	237 (AF179340, AF179320), 244 (AF179341, AF179321)	78 (AF179332, AF179312), 74 (AF179333, AF179313)	934 (AF179345, AF179325), 91 (AF179331, AF179311)	22 (AF179347, AF179327), B5 (AF179348, AF179328)				
		713 (AF179343, AF179323), 716 (AF179344, AF179324), 72 <sup>a</sup>						
					9 (AF179346, AF179326)			
Lineage C								52 (AF179329, AF179310)
Lineage D						8037 (AF179336, AF179316)		
Clade E							9065 <sup>a</sup>	
Lineage F								50 (AF179329, AF179309)
								917 (AF179334, AF179314), 920 (AF179339, AF179319)

<sup>a</sup> Not shown in the phylogenetic DSR tree (Fig. 2) due to limited sequence information. Phylogenetic affiliation of these cloned DSR sequences was determined by analyzing a reduced number of positions by the different tracing methods (data not shown).

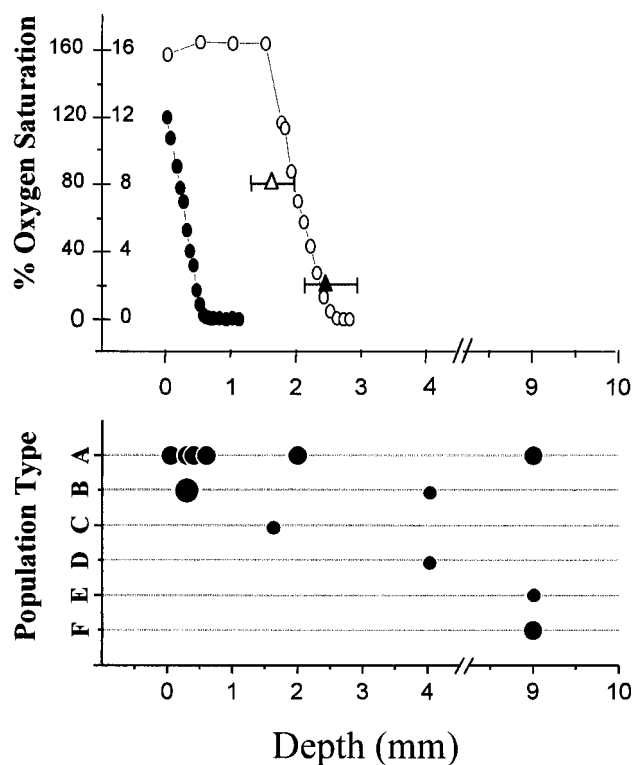


FIG. 1. Distribution of DSR sequence type by sample depth in the mat in relation to oxygen concentration. The number of clones recovered from each sample depth affiliated with each sequence type (A to F) is represented by the size of the corresponding circle (one to three clones). The medians and ranges for the one-half maximum-oxygen (open triangle) and minimum-oxygen (filled triangle) values were calculated from six independent microelectrode measurements (31).

**Nucleic acid analyses.** Total DNAs were extracted from mat depths of 0.05, 0.3, 0.4, 0.6, 1.6, 2.0, 4.0, and 9.0 mm, and a longitudinal sample was taken from a complete mat core by a modification of the method described by Tsai and Olson (42). Briefly, mat sections were washed with 1 ml of TE (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) and incubated in 200  $\mu$ l of lysozyme solution (0.15 M NaCl, 0.3 M Na<sub>2</sub>-EDTA, 15 mg of lysozyme per ml) for 3 h at 37°C. Then, 200  $\mu$ l of lysis buffer (0.1 M NaCl, 0.5 M Tris-HCl [pH 8.0], 10% sodium dodecyl sulfate) was added and the suspension was subjected to three cycles of freezing (ethanol and dry ice for 5 min) and thawing (65°C water bath for 10 min). Finally, proteinase K was added to a final concentration of 50  $\mu$ g/ml and the tubes were incubated at 30°C for 30 min. DNA was purified by phenol extraction, precipitated with isopropanol, washed with 80% (vol/vol) ethanol, and resuspended in water.

**PCR amplification, cloning, and sequencing.** PCR amplification was carried out in a 1650 Air Thermo-Cycler (Idaho Technology, Idaho Falls, Idaho) under the reaction conditions and with the DSR1F and DSR4R primers previously described (46). PCR products (ca. 1.9 kb) were ligated either directly with the TA Cloning System into pCRII plasmids and transformed into ONE SHOT competent *Escherichia coli* cells according to the directions of the manufacturer (Invitrogen, San Diego, Calif.) or following recovery from an agarose gel with an agarose gel DNA extraction kit (Boehringer Mannheim GmbH, Mannheim, Germany). Partial sequences were obtained from the 3' and 5' ends of each insert with a LI-COR 4000L automated sequencer and infrared dye-labeled M13 for-

ward and reverse primers (LI-COR Corp., Lincoln, Nebr.). Clones and GenBank accession numbers are listed in Table 1.

PCR amplification of DNAs obtained from the indicated depths (Fig. 1) yielded amplification products of the expected 1.9-kb size. A total of 24 DSR clones were sequenced (Table 1). Deduced DSR amino acid sequences were aligned with the Genetic Data Environment (GDE) version 2.2 sequence editor (37a) implemented in the ARB software environment (40). Distance matrix {FITCH and KITCH (PHYLIP version 3.5 [15a]) and neighbor-joining (ARB)}, parsimony (PROTPARS, PHYLIP version 3.5), and maximum-likelihood (PROTML, PHYLIP version 3.5) analyses were performed on a concatenated alpha- and beta-subunit amino acid data set. Missing sequence information was coded as missing data, yielding 291 total positions for the combined alpha-subunit (128 positions) and beta-subunit (163 positions) data set. Bootstrap analysis (1,000 resamplings) was performed for the parsimony method with a program in the PHYLIP package.

No two sequences were identical, and all were affiliated with the bacterial domain. Comparison of phylogenetic trees obtained with the different methods revealed, in general, consistent topologies for both alpha and beta DSR subunits. For consideration of maximum sequence information, we present results of a phylogenetic analysis of a concatenated alpha- and beta-subunit amino acid data set (Fig. 2). A more complete development of the basis for this analysis is presented in our previous study of DSR phylogeny (46).

Six well-resolved lineages of DSR sequences are represented by the cloned sequences (A to F). Many of these lineages are not associated with established SRB groups. Although some may be related to cultured SRB not yet characterized by DSR sequencing, others almost certainly represent undescribed SRB. In overview, lineages A to E are within or closely related to the  $\delta$  subgroup of the class *Proteobacteria*. The F lineage is well resolved from the others and can be classified only as a member of the bacterial domain. The more deeply diverging sequences (E and F) were recovered either from the deep, permanently anoxic, regions of the mat or from longitudinal slices consisting primarily of the permanently anoxic region (2.5 to 9 mm). The depth distribution of sequence types in relation to oxygen is shown in Fig. 1.

**Clade A.** Clade A includes the greatest number of cloned sequences; 8 of 11 clones originated from the oxic zone (0 to 1.5 mm), and 2 of 3 clones originated from the chemocline. All 12 sequences affiliated with clade A are unique and closely related to described *Desulfonema* and *Desulfococcus* species within the  $\delta$ -proteobacteria.

**Clade B.** Clade B is comprised of five unique sequences, including those of the three remaining clones from the oxic zone of the mat. This clade is affiliated with the *Desulfococcus-Desulfonema* lineage within the  $\delta$ -proteobacteria.

**Lineage C.** Lineage C contains two cloned sequences, distantly related to *Desulfobacter* spp. These sequences were recovered from a depth of 1.6 mm (chemocline during the day and anoxic zone at night) and from a longitudinal section. However, the phylogenetic position is not fully resolved; neighbor-joining and FITCH analyses indicated a relatively close relationship with clade B sequences, which was not supported by maximum-likelihood and parsimony methods.

**Lineage D.** Lineage D is defined by one clone recovered from a depth of 4 mm, a region that is permanently anoxic. Although not related to any described SRB, it shows high sequence similarity to a DSR sequence recovered from an aromatic-hydrocarbon-degrading, sulfate-reducing enrichment (17a).

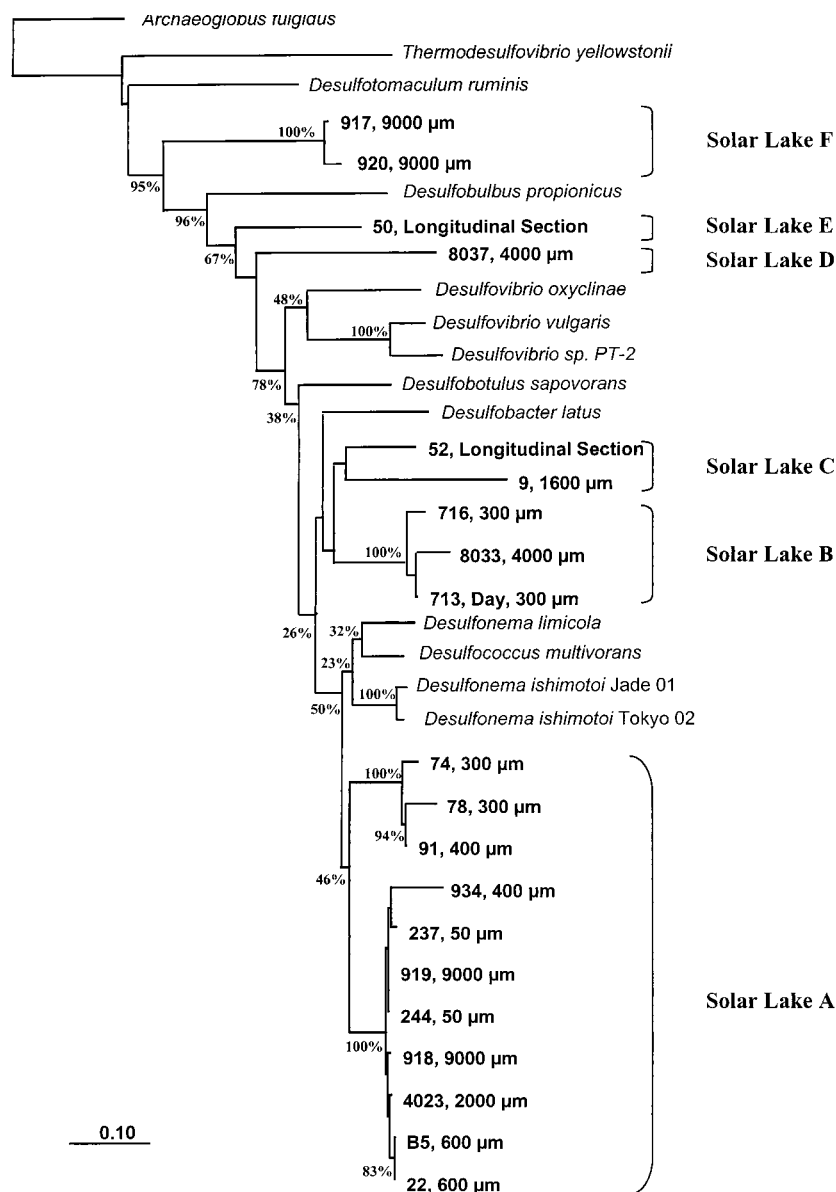


FIG. 2. Phylogenetic tree reflecting the relationships of the analyzed DSR clones retrieved from the mat with the DSRs from characterized sulfate-reducing prokaryotes (46). The DSR sequences of *Desulfobulbus propionicus* and *Desulfonema ishimotoi* (47) were added to the data set so that we could more accurately define the phylogenetic depth of the  $\delta$ -subclass SRB in the DSR tree and provide additional reference. Tree topology was obtained from FITCH distance matrix analysis of the DSR alpha- and beta-subunit amino acid data set. Bootstrap values were determined from parsimony analysis with an identical data set. Branches for which no bootstrap value is indicated were not recovered in the majority of bootstrap replicates by the parsimony method. The scale bar indicates the number of expected amino acid substitutions per site per unit of branch length.

**Clade E.** Clade E is represented in the phylogenetic tree by one clone recovered from a longitudinal section of the mat and another clone isolated from the 9-mm depth. It is not related to any available pure culture sequence and only peripherally related to *Desulfobulbus* spp.

**Lineage F.** Lineage F is deeply diverging and defined by two highly similar DSR sequences not closely related to any available pure culture sequence. It demonstrates a weak specific association with *Desulfotomaculum ruminis* by KITCH analysis but forms an independent lineage by all other applied treeing methods.

There is a general need in microbial ecology to more directly relate community structure to community functions. Within the analytical framework of comparative gene sequencing, the

most direct linkages are provided by genes encoding key physiological attributes. Several genes have been used in this way, including those for nitrogenase (3, 52–54), [NiFe] hydrogenase (48), ribulose biphosphate carboxylase/oxygenase (33), methane monooxygenase (30), and ammonia monooxygenase (36, 37). However, with the possible exception of ammonia monooxygenase (restricted to two well-defined lineages within the proteobacteria), none of these genes provide fully comprehensive or consistent coverage. The DSR gene appears to be the first example of a gene encoding a widely distributed metabolic trait of sufficiently high sequence conservation to be recoverable from all recognized archaeal and bacterial lineages with a single PCR primer set and to also display phylogenetic relationships generally consistent with the 16S rRNA.

Of particular interest was the relationship of SRB to oxygen. During the diurnal cycle, these mats are exposed to changing chemical gradients, most notably of O<sub>2</sub>, H<sub>2</sub>S, and pH. With reference to O<sub>2</sub> exposure, this and previous studies have defined three general depth intervals (8, 9, 18, 23, 24, 34): (i) permanently oxic (0 to 0.5 mm), (ii) fluctuating oxic and anoxic (0.5 to 2.5 mm), and (iii) permanently anoxic (deeper than 2.5 mm). The most striking observation was that all DSR sequences derived from the permanently oxic zone are affiliated with clades A and B (Fig. 1 and Table 1), whose members are closely related to the *Desulfonema-Desulfococcus* group of metabolically versatile, completely oxidizing SRB (17, 50). At this depth dissolved oxygen concentrations varied from approximately 160% saturation during the day to below 10% saturation during the night.

It has been recognized for some time that many SRB are oxygen tolerant and that some may have a limited capacity to respire oxygen. Many *Desulfovibrio* spp. have these characteristics (1, 10, 13, 15, 22, 29, 43). Several *Desulfovibrio* species isolated from oxic regions of microbial mats (e.g., *Desulfovibrio oxyclinae* and *Desulfovibrio halophytica*) have been shown to have a high affinity for oxygen but a limited capacity to respire it for growth (26, 27). However, no DSR sequences related to *Desulfovibrio* were recovered in this study. In addition, our previous studies of a similar mat in Guerrero Negro (Baja California Sur, Mexico) with group-specific rRNA probes revealed a minor presence of *Desulfovibrio* species in the near surface (upper 2 mm). A common feature of both mat systems is the near-surface abundance of *Desulfonema*-like populations, as revealed by the DSR sequences recovered in this study and with group-specific probes to characterize the population structure of the Guerrero Negro mat (35). Since DNAs recovered from environmental samples may be derived in part from dead or inactive cells, the DSR sequence alone does not provide direct evidence for an active sulfate-respiring population. However, rRNA-based analyses provide additional support for the presence of an active SRB microbiota in the oxic regions of this mat community. The general patterns of DSR distribution are also consistent with the results of the high-resolution study of rRNA abundance in this mat community presented in our accompanying paper (31).

Although pure culture isolates of *Desulfonema* have not been examined for their relationship to oxygen, other members of this family have been demonstrated to either reduce oxygen or be oxygen tolerant, including *Desulfobacterium autotrophicum* strains (13, 15, 29), several *Desulfobacter* species (10, 13), and a *Desulfococcus multivorans* strain (13, 15). More recently, *Desulfonema* spp. were identified by in situ hybridization in sea sediments (28), and high numbers of *Desulfonema* spp. have been identified by most-probable-number counts in the upper 2 mm of a field sample of the Solar Lake cyanobacterial mat and identified by sequencing of 16S rRNA gene fragments amplified from DNAs isolated near the chemocline (41). Thus, molecular and microbiological data derived from two independent field sites and from the aquarium systems consistently show the *Desulfonema*-like SRB to be dominant in the permanently oxic region of hypersaline cyanobacterial mat communities. However, no identical DSR sequences were recovered in our initial analysis and we anticipate that continued studies will reveal much greater sequence diversity, pointing to a very complex ecology of SRB in the near surface of the mat.

We anticipate that comparative DSR sequence analyses will provide a useful complement to the microbiological and molecular tools now used to study the ecology of sulfate-respiring microorganisms. For example, DSR sequence type could be used to assist in monitoring successful enrichment of previ-

ously unknown SRB. Or, if a close phylogenetic relationship implies phenotypic similarity, this information could assist in the design of specific enrichment strategies. Since this sequence is an explicit indicator of the capacity for sulfate respiration, DSR-based analyses should also foster a better understanding of the more general environmental roles of these organisms (9a). The metabolic diversity of this group has been revised repeatedly in recent years, suggesting a more general participation in the flow of carbon and electrons in anoxic habitats than earlier thought. For example, their carbon sources, long thought to be limited to simple organic acids and alcohols, now include a wide variety of aliphatic and aromatic compounds (5, 49, 51; for a review, see reference 19). Also, although sulfate respiration unites them, SRB are not restricted to this mode of existence. As a group, they have the capacity to use a broad variety of electron acceptors, including sulfite, thiosulfate, sulfur, nitrite, and nitrate (10, 15, 19, 22, 29, 43). Some members derive energy from disproportionation of sulfur, thiosulfate, and sulfide and incomplete sulfate reduction to thiosulfate and sulfur (for a review, see reference 50). It is conceivable that within certain habitats, sulfate respiration may be a minor metabolic mode for some members of this functionally defined assemblage.

**Nucleotide sequence accession numbers.** See Table 1 for nucleotide sequence accession numbers.

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