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DIVERSITY OF SULFATE-REDUCING GENES (*dsr*AB) IN SEDIMENTS FROM PUGET SOUND

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ABSTRACT

The aims of this study were to characterize the population structure and diversity of sulfate-reducing bacteria (SRB) from three distinct sites at Puget Sound, and relate the biogeochemical properties of the sediments to the sulfate-reducer communities. The population composition and diversity of sulfate-reducing bacteria carrying *dsr*AB genes from surface Puget Sound sediments was investigated using a polymerase chain reaction-based cloning approach. Sediment cores were collected from three different locations: Carr Inlet (C1A), Shallow Bud Inlet (S1A), and Turning Basin (T1A). A total of 498 *dsr*AB clones were sequenced from the three sites. Ecological indices indicated that T1A had the highest diversity and evenness values and C1A had the lowest. Correlations were also found between diversity indices and geochemical parameters. The diversity of the SRB decreased with decreasing carbon concentrations and sulfate reduction rates, and increasing levels of oxygen. A phylogenetic comparison revealed that the majority of the *dsr*AB sequences were associated with the delta-proteobacterial phylotypes *Desulfonema*, *Desulfococcus* and *Desulfosarcina*, suggesting that complete oxidizers with high substrate versatility dominate in the sediments. The environmental conditions and energy sources available in the sediments may have dictated microbial community structure and diversity of SRBs. Distinctive community structures of SRBs in Puget Sound sediments were found to vary at different sites with different redox profiles. The dominance of the *Desulfobacteraceae*-like sequences may be due to the presence of a diverse spectrum of substrates in the sediments. This study represents one of the first efforts to characterize the population of sulfate-reducing microbes in the oxygenated regions of Puget Sound sediments. The phylogenetic identification of *dsr*AB genes in the sediment samples allows the composition of sulfate-reducing prokaryotic communities to be inferred, and working hypotheses about their likely carbon substrates to be formed.

Keywords: Carbon bioavailability, dissimilatory sulfite reductase genes, microbial community, microbial diversity

INTRODUCTION

Puget Sound is an inland sea of 6118 km^2 with deepwater ports, saltwater beaches and sheltered inlets along interior waterways. It is located at the northwestern corner of the State of Washington and is the major corridor for interstate and international marine transportation. A wide variety of chemical compounds have been discharged in Puget Sound, many of which are sequestered into the sediments [1]. The sequestration of these compounds into the sediments decreases their bioavailability for transformation, thus anaerobic processes such as sulfate reduction become the main route to remediation [2, 3]. Sulfate-reducing bacteria (SRB) are the key players in this process. They live in sediments and collectively degrade even the most recalcitrant substrates such as aliphatic and aromatic compounds [4]. SRBs are a large and extremely diverse physiological group of anaerobic microorganisms that use sulfate as a terminal electron acceptor for respiration and organic carbon as growth substrates, including petroleum hydrocarbon components [5–8]. Some SRBs are not completely dependent on sulfate; they can also use alternative electron acceptors such as Fe (III) [9, 10] and nitrate [11], can disproportionate inorganic sulfur compounds [12], or can grow under fermentative conditions [11]. Although some SRBs have been shown to survive in the presence of oxygen, no growth has been observed under this condition [13, 14].

Sulfate reduction is the dominant anaerobic process in marine sediments [15–17], accounting for the oxidation of more than 50% of the total organic carbon in some systems [15, 17, 18]. The sulfate-reducing microbiota are expected to be strongly influenced by organic carbon dynamics in the continental margin sediments. The relative contribution of sulfate reduction to overall carbon oxidation in the sediments is significant, but varies with increasing water/sediment depth and distance offshore [17, 19]. Sulfate reduction rates are generally highest in shallow sediments and decrease with increasing water depth [19]. Ultimately, sulfate reduction

depends on organic carbon flux into the sediment [20, 21]. The difference in the organic carbon dynamics between sites may affect the selection and diversity of sulfate-reducer communities. In this study, we sequenced the functional gene for dissimilatory sulfite reductase (*dsr*AB) in the sediments to characterize the SRB involved in the anaerobic carbon oxidation pathway in the sediments. The *dsr*AB gene encodes the α- and β-subunits of the protein, which catalyses the final step on sulfate respiration—the reduction of sulfite to sulfide [22–24]. Three sites were selected which vary in terms of water depth, concentrations of carbon and oxygen, and nutrient availability. Such differences may have an impact on the structure of the sulfate-reducer communities in the sediments.

The relationship of SRBs to oxygen has been of particular importance since the publication of earlier reports of exceptionally high rates of sulfate reduction in oxygenated regions of some microbial mats [25, 26]. These observations were contrary to the accepted paradigm that the relative importance of SRB would occur in anoxic environments. The oxygen-tolerant SRB populations were mostly members of the genus *Desulfonema*, acetate-oxidizing, nutritionally versatile, filamentous, and motile organisms that can migrate and aggregate [27, 28]. Full understanding of the interacting biological, chemical, and physical forces that affect sulfate reduction rates would reveal the importance of SRBs present in oxic sediment layers. In turn, this understanding cannot be achieved without knowledge of the population structure in the oxygenated zone of the sediments. In the present study, samples were collected from the upper 0.5 cm aerobic horizon of the sediments. To date, microbiological studies of Puget Sound sediments have focused on isolation of novel polycyclic aromatic-hydrocarbon degrading bacteria [5] and characterization of denitrifier microbial communities [29]. The work described here represents one of the first studies to describe the population of sulfate-reducing microbes in the oxygenated regions of Puget Sound sediments. In particular, we addressed the distribution of SRB sequence types to the availability of oxygen, carbon, and nutrients in the sediments. Ultimately, the SRB population described here may help us in defining the resiliency of the ecosystem to anthropogenic impact and characterize novel biodegraders, understand their potential roles in carbon and sulfur dynamics, and provide a means for management strategies and the assessment of restoration potential in Puget Sound sediments.

EXPERIMENTAL PROCEDURES

Sampling Sites and Geochemical Parameters

We analysed sediment cores from three locations in Puget Sound (Figure 1). Carr Inlet (C1A) is a site with a high concentration of nitrate. Shallow Bud Inlet (S1A) presents an area with a low concentration of nitrate due to its consumption by algae. Turning Basin (T1A) has a high concentration of H_2S in the sediments [1]. At the C1A site, silt (50%) dominated the surface layer of the sediments followed by sand (25%) and clay (25%). The sediment is olive green in colour and the surface porosity is about 0.90% [1]. The T1A sediment is about 18% sand, 56% silt and 26% clay, and is brownish green and has a surface porosity of about 0.95%. At the S1A site, the sediments were mainly dominated by a mixture of sand and mud, are brownish green and have a surface porosity similar to that of T1A sediments [1].

The overlying waters and porewaters were sampled with a whole core squeezing apparatus and analysed for oxygen concentration with a microelectrode (Microelectrodes, Inc., Bedford, N.H.), and for nitrate concentration by the cadmium reduction method [30, 31]. Cores were obtained from each site using a Soutar box corer. Sediment samples for molecular analysis were stored −80°C until used. Sediment samples for chemical analyses were stored at 4°C and were analysed as soon as the samples were shipped in the laboratory.

The percentage weight of organic carbon was determined on freeze dried, ground sediment samples by the method of Hedges and Stern [32] using either a Carlo-Erba model 1106 CHN elemental analyser or a Leeman Laboratories CHNS elemental analyser. The NH_4^+ and NO_3^- contents of the sediments were measured by the methods of Strickland and

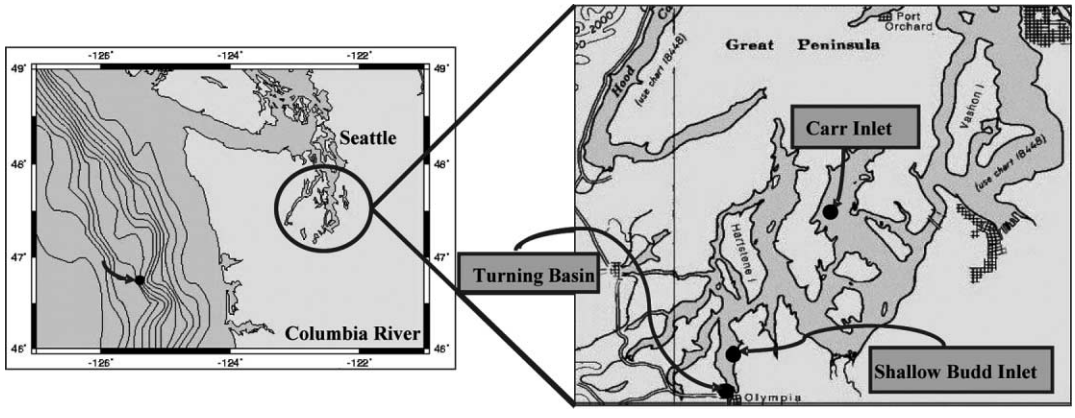

Figure 1. Map showing the study area (Puget Sound) and indicating the locations of the collection sites.

Parsons [33]. Sulfate reduction rates were determined by measuring the reduction of $^{35}SO_4$ following methods described previously [34–36]. The Fe^{2+} and Mn^{2+} ions in the sediments were measured using colorimetric methods [37, 38].

Community DNA Extraction and Purification

For all three sites (C1A, S1A, and T1A), DNA was extracted from the top sediment layer (0.5 cm). Triplicate sediment samples (0.5 g) from each site were ground in liquid nitrogen as described previously [39], prior to DNA extraction. The total community DNA from each replicate sediment sample was extracted and purified using UltraClean Soil DNA Isolation Kit (MoBio Laboratories, Solana Beach, CA). DNA quantity was determined spectrophotometrically (Nanodrop ND 1000, Nanodrop Technologies, Inc., Delaware, USA). The replicate nucleic acid extracts were pooled for polymerase chain reaction (PCR). All nucleic acids were stored at –20°C until used.

PCR Amplification and Cloning of *dsr*AB Genes

The dissimilatory (bi-)sulfite reductase (*dsr*) primers used in this study were those of Wagner *et al.* [22]: (dsr-IF: 5′ AC [C/G] CAC TGG AAG CAC G 3′ and dsr-4R: 5′ GTG TAG CAG TTA CCG CA 3′). PCR reactions (20 µl total volume) consisted of 1 × PCR buffer (50 mM KCl, 10 mM Tris, 0.1% Triton X-100, pH 9.0), 1mM dNTPs, 1.5 mM $MgCl_2$, 1 µM of each primer, 4 µg BSA, 2.5U *Taq* polymerase (Roche Molecular Biochemicals, Indiana, USA), and 50 ng DNA template. The thermal cycling protocol used included initial denaturation at 94°C for 2 minutes, followed by 25 cycles of 94°C for 30 seconds, 58°C for 1 minute, and 72°C for 1 minute. A final extension step at 72°C for 7 minutes was also used. The primer pair dsr-1F and dsr-4R amplified a 1942 bp (base pairs) length, corresponding to the target DNA segment. Amplicons of the expected size were excised from 0.8% low melting agarose gels and purified using the QIAquick gel extraction kit (Qiagen, Inc., Valencia, CA, USA) following the manufacturer's instructions. Amplicons were cloned into the pCR™ II vector from a TA-cloning kit (Invitrogen, San Diego, CA, USA). Competent *Escherichia coli* cells were transformed according to the provided protocol (Invitrogen). Approximately 200 white colonies from each sediment sample were randomly selected and screened for *dsr*AB inserts, which was detected with primers specific to the polylinker of the vector pCR™ II (see [40]). Each single colony was picked, resuspended in 60 µl 0.1 × TE buffer (1 mM Tris, 0.1 mM EDTA) boiled for 10 minutes at 100°C and then stored at –20°C. The inserts were amplified (20 µl reactions) with the TA specific primers for pCR™ II vector [40]. The inserts were visualized on a 1.5% TAE agarose gel stained with ethidium bromide and clones with the expected size were used for further analysis. PCR products (30 µl) amplified with vector-specific primers were purified with PCR_{96} Cleanup plates (Millipore Corporation, Bedford, MA, USA).

Sequencing and Phylogenetic Analysis

DNA sequencing was performed with ABI PRISM BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster, CA, USA), and the ABI PRISM 3700 DNA Analyzer (PE Applied Biosystems). Nucleotide sequences were assembled and edited using the ChromasPro™ program version 1.31 (Technelysium Pty Ltd, Queensland, Australia). Unique *dsr*AB clones (≥97% sequence identity) from each site were identified by direct sequence comparison, and were designed as operational taxonomic units (OTUs). Altogether, 498 representative *dsr*AB clones were sequenced: 164 from C1A; 172 from S1A; and 162 from T1A (Table 1). The *dsr*AB partial sequences were aligned using ClustalW multiple sequence alignment [41] in the sequence analysis package MegAlign (Lasergene DNASTAR, Inc, Madison, WI, USA). The aligned sequences were analysed by distance matrix methods using DNADIST program. Comparative sequence analyses were conducted on both nucleotide sequences and translated sequences. BLASTp similarity searches were also carried out to compare translated *dsr*AB sequences with those from GenBank, to be certain that the *dsr*AB sequences recovered from this study indeed represent the right protein. Phylogenetic analyses of *dsr*AB genes were performed using MEGA version 3 [42], and phylogenetic trees were constructed with distance matrices and maximum likelihood methods. For sequences with at least 97% identical nucleotides in a given clone library, only one was used for phylogenetic tree. The nucleic acid tree phylogenies have similar topology compared with translated amino acids.

Data Analyses

The diversity (Shannon–Weiner and Simpson index) and evenness (equitability) indices were based upon the

Table 1. Characteristics and diversity estimates of *dsr*AB gene clones from sediments samples collected at three different sites in Puget Sound.

Sites	Number of clones obtained[a]	Number of OTUs identified[b]	H[c]	$1/D$[d]	Evenness[e]
C1A	164	49	2.84	3.01	0.73
S1A	172	50	3.18	3.43	0.77
T1A	162	64	3.84	4.19	0.86

[a] Number of clones analysed from each library.

[b] Operational taxonomic units based on unique partial *dsr*AB gene sequences (≥97%).

[c] Shannon–Weaver index, higher number represents more diversity.

[d] Reciprocal of Simpson's index, higher number represents more diversity.

[e] As evenness approaches 1, the population is more evenly distributed.

distribution of OTUs obtained from clone libraries using the equations from Krebs [43] (see also [44]). In this study, OTUs were estimated based on clones with sequence similarities of at least 97%. The evenness index (E) measures the equitability of species abundance for a sample. As E approaches a value of 1, the population is more evenly distributed [43, 45].

LIBSHUFF analysis [46] was used to compute homologous and heterologous coverage within and between *dsr*AB clone libraries from different depths. In this analysis, the predicted coverage of a sampled library was denoted by the homologous coverage, whereas the heterologous coverage was represented by the observance of a similar sequence in a separate library. The values were reported over a sequence similarity range or evolutionary distance (D) based upon a distance matrix. For LIBSHUFF and diversity analyses, 468 *dsr*AB sequences (134 from C1A; 172 from S1A; and 162 from T1A) were considered. Sequences from the three libraries were also compared with each other to check for overlapping sequences. The diversity captured from each clone library was estimated by rarefaction analysis using the analytical approximation algorithm of Hughes *et al*. [47] and 95% confidence intervals estimated as described by Hecke *et al*. [48]. Calculations were performed with the free software program Analytical Rarefaction 1.3 (available at http://www.uga.edu/~strata/software/). The *dsr*AB sequences described in this study have been submitted to GenBank under the accession numbers DQ996682 to DQ996752 (C1A gene sequences); DQ996753- DQ996841 (S1A gene sequences); and DQ996842- DQ996911 (T1A gene sequences).

RESULTS

Geochemical Properties Across the Study Sites

As shown in Table 2, the geochemical properties of the surface sediments collected from C1A, S1A, and T1A were different from each other. The T1A (the shallowest site; 3 m water depth) sediment had the highest concentrations of carbon, NH_4^+-N (ammonium), and sulfate reduction rate, and lowest concentrations of NO_3^--N (nitrate), Fe^{2+}, and Mn^{2+}. The C1A (the deepest site; 125 m water depth) sediment had the highest concentration of NO_3^--N and Mn^{2+} and lowest concentrations of carbon, SO_4^{-2}, and sulfate reduction rate. The S1A had the highest oxygen concentration but the lowest NH_4^+-N.

Statistical Analysis of Clone Libraries

The 498 clones retrieved from this study produced 163 OTUs (≥97% nucleotide sequence similarity) across all sites. Sediment sample from T1A generated the highest number of OTUs, while sediment samples collected from C1A and S1A had similar numbers of OTUs (Table 1). Sequence analysis of the 498 *dsr*AB clones revealed that the sediments harbour a diverse community of sulfate reducers. On the basis of both Shannon–Weaver index values and reciprocal Simpson's index values, sediments retrieved from C1A and S1A depths were less diverse than the T1A sediments, supporting the conclusion that the T1A sediments had the highest diversity of all sites studied (Table 1). In general, diversity indices results showed that sediments from S1A were more similar to C1A than T1A. Rarefaction analysis indicated that majority of the recovered diversity was sampled within 98 analysed clones for C1A, 105 clones for S1A, and 123 clones for T1A. Ecological indices indicated that T1A had the highest diversity and evenness values. Sixty four OTUs were estimated at this site based on at least 97% nucleic acid similarity of *dsr*AB sequences. The *dsr*AB gene clone libraries were also compared between the sites in terms of differences between coverage curves (LIBSHUFF analysis, version 1.2), as described previously [46]. Clone libraries from S1A and T1A had the lowest ΔC_{xy} compared with each other, indicating that these two libraries were most similar. It appears that clone libraries from C1A and T1A were the most dissimilar (Table 3).

Distinctive community structures of SRBs in Puget Sound sediments were found to vary at different sites with different redox profiles. Overlapping sequences found between sites indicate a change in community structure with increasing water depth, carbon content, and sulfate reduction rate, and decreasing concentrations of oxygen and nitrate. The most obvious difference was generally observed between sites C1A (deepest water depth, lowest carbon concentration, and highest oxygen and nitrate concentrations) and T1A (shallowest water depth, greatest carbon concentration, and lowest oxygen and nitrate concentrations). This difference was reflected not only by LIBSHUFF analysis of clone libraries, but also by examining the percentage of overlapping sequences of *dsr*AB clones. Sequences in site T1A showed more overlap with S1A than with C1A. Overall, the sequences from each library had little overlap, except for the predominant *dsr*AB clones.

Table 2. Biogeochemical properties of at different sediment samples collected at 0.5 cm depths from Carr inlet, Shallow bud inlet and Turning basin at Puget Sound.

Sites	Water depth (m)	Carbon (%)	O_2 (μM)	Sulfate reduction rate (mmol m^2 day^{-1})	NH_4^+ (μM)	NO_3^- (μM)	SO_4^{-2} (μM)	Fe^{2+} (μM)	Mn^{2+} (μM)
C1A	125	1.80	178.57	7.5	23.69	13.95	20.80	23.40	227.8
S1A	10	2.50	249.17	12.0	6.62	0.83	23.20	35.25	14.93
T1A	3	3.20	84.94	25.0	31.21	0.53	23.40	3.89	2.71

Table 3. LIBSHUFF analyses of the marine sediment communities at different sites based on *dsr*AB clone libraries.

Sample comparison [a]	Clones (n_x)	Clones (n_y)	ΔC_{xy} [b]	*P* value
C1A versus S1A	164	172	0.81	0.001
C1A versus T1A	164	162	1.01	0.001
S1A versus T1A	172	162	0.54	0.001

[a] The respective clone number for each site is given by n_x and n_y, and ΔC_{xy} represents the difference in coverage of the two clone libraries. The software for the analysis was used according to specified directions at http://www.arches.uga.edu/~whitman/libshuff.html.

[b] An increase in ΔC_{xy} values represents greater dissimilarity between the given *dsr*AB communities.

Sequences were used to construct phylogenetic trees including other marine *dsr*AB clones and known SRBs from the database such as that from the Pacific Coast of Mexico and Washington margins. Three *dsr*AB clone libraries were constructed (one from each site; see Figures 2–4), and the clones were sequenced with the primer *dsr*-4R. The resulting phylogenies were largely congruent, differing only in the exact placement of individual sequences within low-order branch clusters. Assuming that our sampling of clones was random (no biases due to sampling, sample handling, DNA extraction, and cloning), our results suggest that the majority of the *dsr*AB sequences relate to δ *Proteobacteria* (*Desulfacinum*, *Desulfonema*, *Desulforhopalus*, *Desulfosarcina*, *Desulfococcus*, *Desulfovibrio*, *Syntrophobacter* and *Desulfoarculus*) and *Firmicutes* (*Desulfotomaculum*). When *dsr* sequences from representatives of δ-*Proteobacteria* and *Firmicutes* were compared with unique *dsr* clones, many of the Puget Sound clones were more closely related to one another than previously reported SRBs (Figures 2–4) although a wide range of sequence divergence was observed among *dsr*AB clones from three different sediment samples (40–98% nucleic acid similarity). All of the *dsr*AB clones were also considerably divergent from the *dsr*AB clones retrieved from marine sediments of the Pacific coast of Mexico (clones M300238, M300226, M306064, M300079 and M300278) and Washington margins (clones W301122, W306461, W307206, W307149, and W3001182) by Liu *et al*. [49]. None of the Washington and Mexico *dsr*AB clones grouped with the *dsr*AB clones from C1A, S1A and T1A, although the sediments from two these sites were also collected from 0.5 cm water–sediment layer. Similarities among these clones vary between 30 and 40% (Figures 2–4).

Comparisons of partial *dsr*AB sequences between clone libraries in this study and other *dsr*AB sequences recovered from other oxygenated environments such as the aerobic layers of the microbial mats [28] and surface sediment layers of the Guaymas basin [50] surveys provided additional resolution (Figure 5). The Guaymas surface sediments are almost certainly anaerobic and warm or hot. The sulfidic vent fluid is upwelling through the sediment column right to the sediment and water interface, where thick mass of sulfide-oxidizing bacteria (Beggiatoa) intercept the sulfide and oxidize it with nitrate of seawater origin. The OTUs from each clone library showed overlap, except for the dominant *dsr*AB clones. For example, C1A (n = 38 clones) overlapped with T1A (n = 40 clones); T1A (n = 34 clones) overlapped with S1A (n = 61 clones); and S1A (n = 61 clones) overlapped with C1A (n = 37 clones). Dominant sequences in this study are wedges in the phylogenetic tree (Figure 5) and are listed in the captions are dominant. Sediment S1A shared 20% (35 clones) and 17% (39 clones) of the *dsr*AB clones with T1A and C1A, respectively, while C1A (lowest carbon concentration and sulfate reduction rate) had little overlap (11 clones; 6%) with T1A (highest carbon concentration and sulfate reduction rate).

Puget Sound sequences lacking cultured relatives were related but not identical to sequences from Guaymas basin survey [50], including clones B01P021, B04P004, B04P026, and B03P021 (Figure 5). None of the *dsr*AB sequences from Solar Lake mat [28] were related to any of the *dsr*AB sequences in the present study. Although the *dsr*AB sequences recovered were related to *dsr*AB communities that demonstrated capabilities for anaerobic metabolism and degradation of aromatic hydrocarbon and petroleum compounds [5–8], the physiology and activities of these diverse bacterial groups remain to be determined by pure culture, to understand their potential for bioremediation.

DISCUSSION

Carbon cycling in marine sediments is coupled to a variety of different electron acceptors, including oxygen, nitrate, sulfate, manganese, and iron [15, 17–18]. In natural environments, SRBs are important in sulfur and carbon cycling, being responsible for 50% of the mineralization of organic matter in marine sediments [51]. The ultimate fate of carbon and sulfur in marine environments is largely determined by the activities of the native microbiota. In the sediments, the relative contribution of sulfate reduction is significant, but varies with increasing water/sediment depth, distance offshore, and carbon availability [17]. The present study demonstrated that the composition and diversity of sulfate reducer assemblages at different water depths changed, with greatest diversity occurring at shallowest water depth (3 m) sample (T1A). These results were consistent with those found by Liu *et al.* [49]. Direct comparison of the entire sequence libraries between sediment depths (LIBSHUFF analysis) indicated that the sediment collected from the deepest water depth (125 m; C1A) was significantly different to the sediments retrieved from the shallowest water depth (3 m; T1A). The diversity of the SRB decreased with decreasing carbon concentrations and sulfate reduction rates, and increasing levels of oxygen.

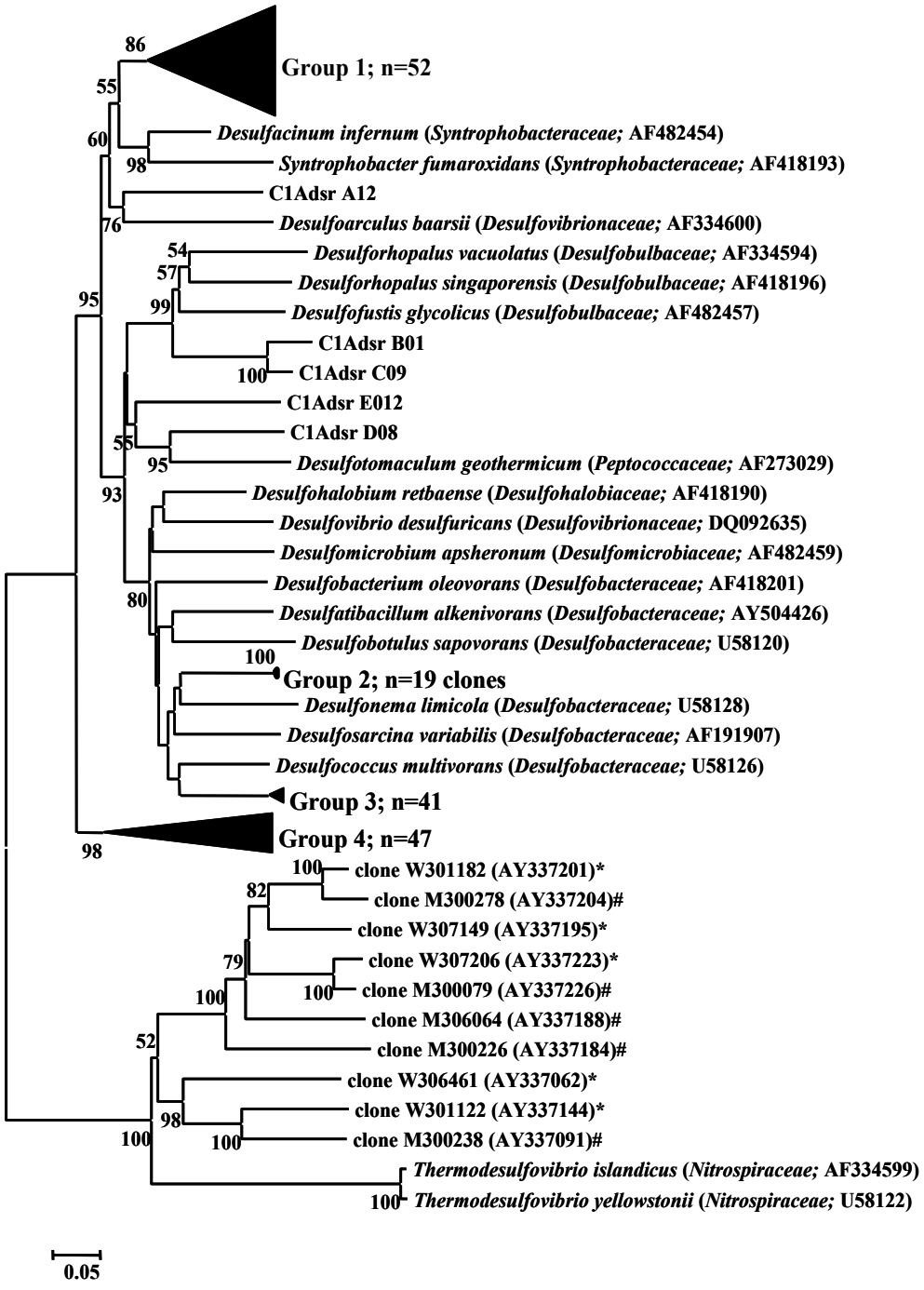

Figure 2. Phylogenetic tree reflecting the relationships of the analysed *dsr*AB clones retrieved from Carr Inlet (C1A). The *dsr* sequences of 20 known SRBs and 10 marine clones, were added to the data set so we could more accurately define the phylogenetic depth of the δ-subclass SRB in the *dsr*AB tree and provide additional reference. Numbers before branch points represent percentages of 500 bootstrap samples that supported each branch. Bootstrap values under 50% are not shown. The scale bar represents 0.05 substitutions per nucleotide position. Reference sequences denoted with # and * were marine clones retrieved by Liu *et al.* [49] from the Pacific coast of Mexico and Washington margins, respectively. Analysis was performed at the nucleotide level for reasons of higher resolution.

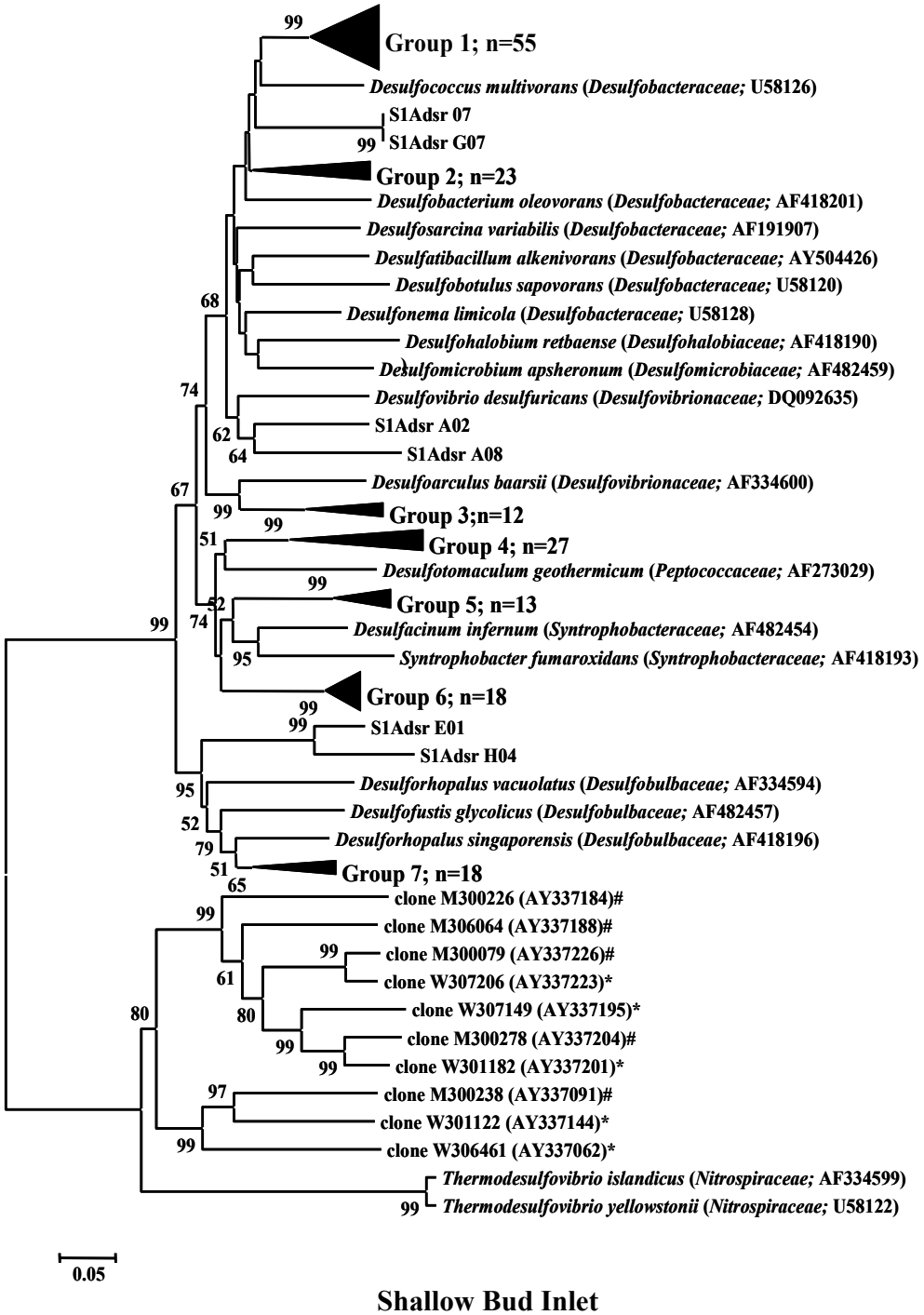

Figure 3. Phylogenetic tree reflecting the relationships of the analysed *dsr*AB clones retrieved from Shallow Budd Inlet (S1A). The *dsr* sequences of 20 known SRBs and 10 marine clones, were added to the data set so we could more accurately define the phylogenetic depth of the δ-subclass SRB in the *dsr*AB tree and provide additional reference. Numbers before branch points represent percentages of 500 bootstrap samples that supported each branch. Bootstrap values under 50% are not shown. The scale bar represents 0.05 substitutions per nucleotide position. Reference sequences denoted with # and * were marine clones retrieved by Liu *et al.* [49] from the Pacific coast of Mexico and Washington margins, respectively. Analysis was performed at the nucleotide level for reasons of higher resolution.

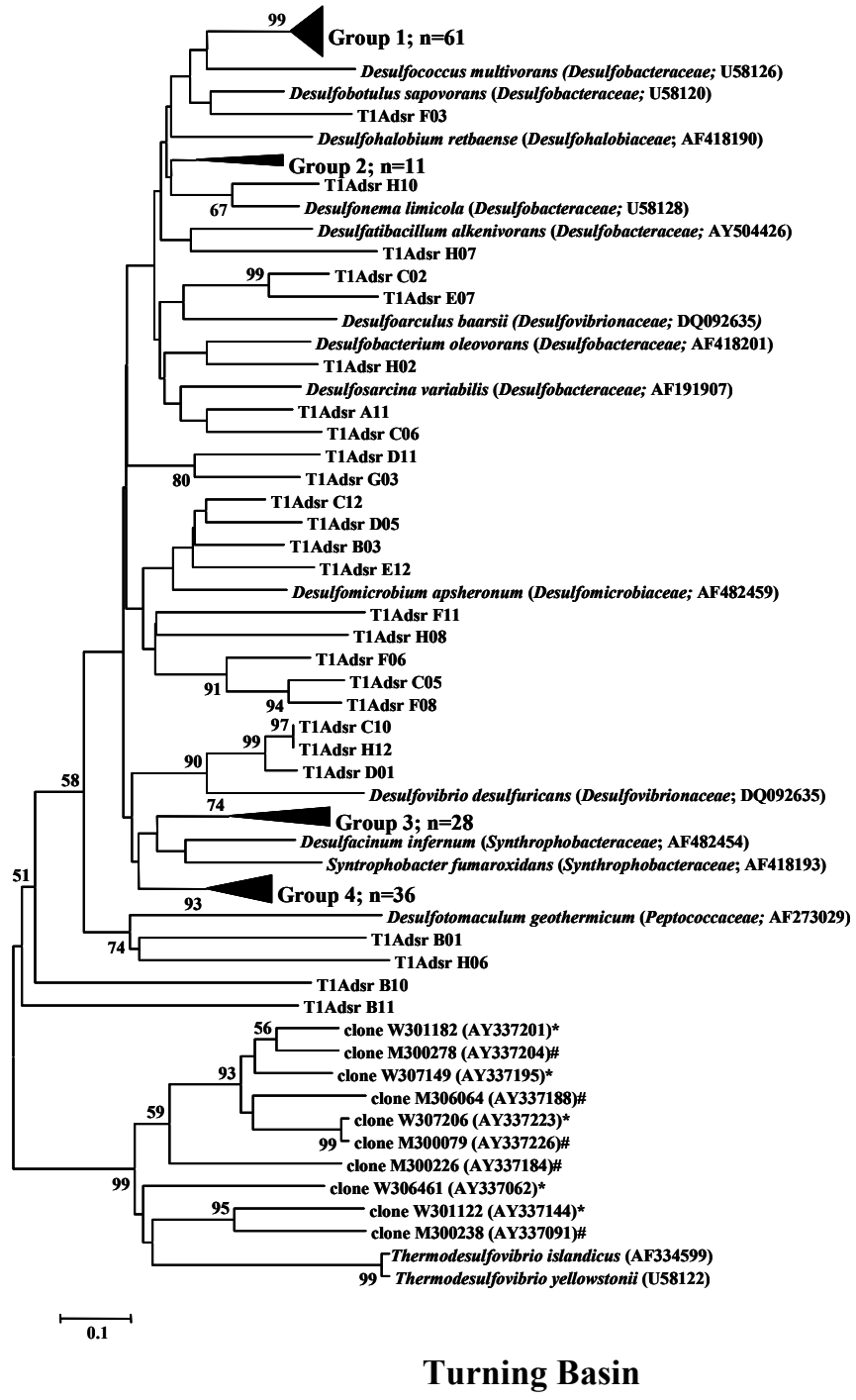

Figure 4. Phylogenetic tree reflecting the relationships of the analysed *dsr*AB clones retrieved from Turning Basin (T1A). The *dsr* sequences of 20 known SRBs and 10 marine clones, were added to the data set so we could more accurately define the phylogenetic depth of the δ-subclass SRB in the *dsr*AB tree and provide additional reference. Numbers before branch points represent percentages of 500 bootstrap samples that supported each branch. Bootstrap values under 50% are not shown. The scale bar represents 0.05 substitutions per nucleotide position. Reference sequences denoted with # and * were marine clones retrieved by Liu *et al.* [49] from the Pacific coast of Mexico and Washington margins, respectively. Analysis was performed at the nucleotide level for reasons of higher resolution.

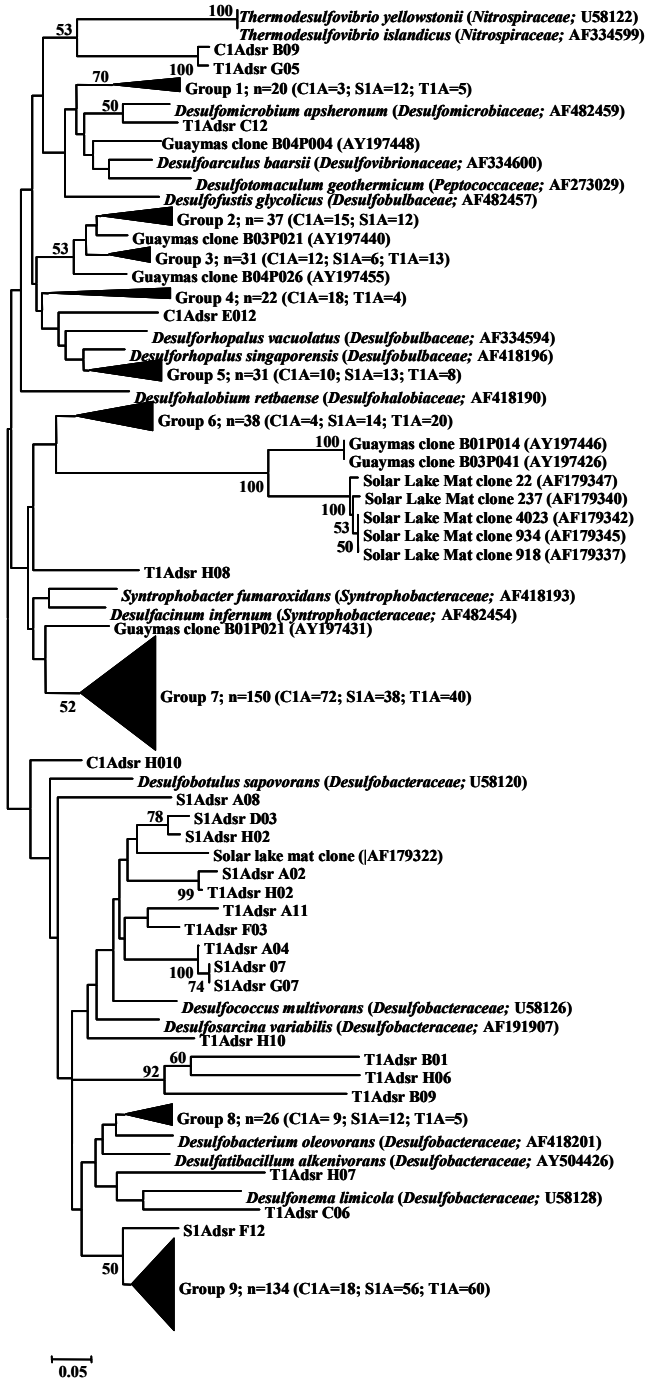

Figure 5. Phylogenetic tree reflecting the relationships of the analysed *dsr*AB clones retrieved from all three sites. The *dsr* sequences of 20 known SRBs and other environmental clones retrieved from the Solar Lake mat and Guaymas basin were added to the data set so we could more accurately define the phylogenetic depth of the δ-subclass SRB in the *dsr*AB tree and provide additional reference. Numbers before branch points represent percentages of 500 bootstrap samples that supported each branch. Bootstrap values under 50% are not shown. The scale bar represents 0.05 substitutions per nucleotide position. The eight clone groups represent closely related sequences. Groups 1, 2, 3, 4, 5, 6, 7, and 8 represent 49, 12, 10, 48, 5, 5, and 8 closely related sequences, respectively. Analysis was performed at the nucleotide level for reasons of higher resolution.

The phylogenetic composition of the SRB community in all three locations demonstrated differences and commonalities among three different sites (Table 4). *Desulfobacteaceae*-like sequences including *Desulfococcus*, *Desulfosarcina*, and *Desulfonema* dominated the SRB communities in all three sites, suggesting that complete-oxidizing SRBs with high substrate versatility dominate the Puget Sound sediments. For example, *Desulfococcus multivorans*, which was associated with the largest clone group (Figures 3 and 4), has metabolic capabilities that are well matched to carbon substrates such as low molecular weight organic acids, alkanes, and aromatic hydrocarbons found in Puget Sound sediments [5]. *Desulfosarcina variabilis* and *Desulfonema limicola*, on the other hand, are capable of oxidizing acetate and other organic compounds (short chain fatty acids, alcohols) completely to CO_2 (see [11, 52]). The nutritional versatility of the genera *Desulfococcus*, *Desulfosarcina*, and *Desulfonema* may allow them to thrive in habitats with diverse substrate spectra such as that found in Puget Sound sediments. Several *dsr*AB sequences from Puget Sound were related to cultured members of the delta-proteobacterial group *Syntrophobacteraceae*. Suprisingly, the *Desulfovibrio*, *Desulfomicrobium*, and *Desulfohalobium*-like sequences, which were found at high abundance from the clone libraries, were not detected in the C1A site (Table 4). These incomplete-oxidizing sulfate reducers may be at competitive disadvantage in the Puget Sound sediments where diverse carbon substrates predominate. *Desulfovibrio*, *Desulfomicrobium*, and *Desulfohalobium* are Gram-negative sulfate reducers, most of which oxidize their substrates incompletely to acetate [11]. Previous studies have demonstrated the dominance of *Desulfovibrio* species in lake sediments [53–55]. Other *dsr*Ab sequences related to the incompletely oxidizing genus *Desulforhopalus*, which specializes in the incomplete (acetate-producing) oxidation of

Table 4. Phylogenetic affiliation of *dsr*AB clone libraries from three different sites.

Phylogenetic affiliation	Number of clones		
	C1A	S1A	T1A
Class *δ-Proteobacteria*			
Desulfobacteraceae			
Desulfobacterium oleovorans			1
Desulfatibacillum alkenivorans			1
Desulfobotulus sapovorans			1
Desulfonema limicola	19		12
Desulfosarcina variabilis			4
Desulfococcus multivorans	41	80	61
Desulfobulbaceae			
Desulfofustis glycolicus	2	18	
Desulforhopalus singaporensis			
Desulforhopalus vacuolatus		2	
Desulfohalobiaceae			
Desulfohalobium retbaense			
Desulfomicrobiaceae			
Desulfomicrobium apsheronum			9
Desulfovibrionaceae			
Desulfoarculus baarsii	1	12	2
Desulfovibrio desulfuricans		2	3
Syntrophobacteraceae			
Desulfacinum infernum	52	13	28
Syntrophobacter fumaroxidans		18	36
Other δ-Proteobacteria	47		8
Class *Nitrospira*			
Nitrospiraceae			
Thermodesulfovibrio islandicus			
Thermodesulfovibrio yellowstonii			
Class *Clostridia*			
Peptococcaceae			
Desulfotomaculum geothermicum	2	27	2

propionate, propanol, lactate, lactate, and ethanol; the completely oxidizing *Desulfofustis*, which specializes in the complete oxidation of glycolate, was not detected in the T1A site. This result suggests that propionate, propanol, or ethanol, which are commonly used substrates by this genus, are insignificant components in the sulfate-reducing carbon pathway in site T1A.

The higher level of diversity in the shallowest sediment may arise from active decomposition and transformation of deposited organic matter, which is encouraged by faunal bioturbation and current mixing of sediment, replenishing nutrients and energy sources [56]. Many studies have shown that as organic carbon descends through the water column, labile compounds are preferentially oxidized, leaving behind recalcitrant organic compounds that are less susceptible to enzymatic degradation [17, 19, 57]. Thus, both the absolute amount of carbon reaching the sediment floor and the overall bioavailability decrease as the water depth increases [17, 58, 59]. Hence, it is assumed that the overall carbon bioavailability increases with decreasing slope depth. The sediments from the shallowest water depth (T1A) are also expected to have a ready supply of labile carbon and energy sources [17], thus increasing the diversity [19, 60]. The reduced diversity observed at the deepest water depth (C1A) may be due to inadequate carbon and energy sources for sulfate-reducer metabolism. This reduces species richness and limits the density of its taxa [61, 62].

Majority of the clones were seemingly affiliated with delta subdivision of the class *Proteobacteria*. Few sequences showed presumptive membership to *Firmicutes*. Nearly 80% of the clone sequences were members of the *Desulfobacteraceae*. The predominance of phylotypes of *Desulfobacteraceae* is consistent with other high-throughput sequencing surveys of surfacial marine sediments [3, 49, 63–67]. The persistent and notable pattern of *Desulfobacteraceae* in sediments could be due to their nutritional diversity, growth characteristics, and ecophysiological flexibility [6, 8, 11, 13, 68]. Members of the family *Desulfobacteraceae* are also morphologically diverse. Cells are either spherical, ovoid, spherical, spiral, or viroid in shape; they occur in pairs, in aggregates, and in multicellular filaments as characteristics for the genus *Desulfonema* [52]. Many are motile and their motility is mainly due to flagella and filamentous forms that exhibit gliding motility. The flagellar or filamentous morphology and gliding movement are features that are not decisive in the study of phylogenetic and metabolic relationships, but they are highly relevant for competition with other SRBs in their natural environment. Motility can offer a competitive advantage especially in migrating to niches where nutrients are available. Potential benefits of motility may include increased efficiency of nutrient acquisition, and avoidance of toxic substances and damaging oxygen species [69]. The capacity for gliding movement, as in *Desulfonema* spp., provide an explanation for the assumed selection and resulting abundance of this group in natural habitats [52].

The *dsr*AB clones retrieved from this study were distantly related to *dsr*AB clones retrieved from other marine sediment surveys such as those from the continental margins of Washington (carbon = 1.5–3.0%; sulfate reduction rate = 1.19 mmol m^2 day^{-1}; water depth = 119–997 m) and the Pacific Coast of Mexico (carbon = 7.0–9.0%; sulfate reduction rate = 0.04–5.96 mmol m^2 day^{-1}; water depth = 340–387 m) [49]. Most of the clones from the Washington and Mexico sediments seem to be a sister group of *Thermodesulfovibrio*. The physical and geochemical variations in the various microhabitats such as temperature, pH, oxidation reduction potential and concentrations of the inorganic and organic substrates would have a great impact on the selection of a community or founding populations that may be endemic rather than cosmopolitan or both [60, 70]. Interestingly, several Guaymas clones showed presumptive membership to *Desulfotomaculum*, *Desulfosarcina*, and *Desulfobacter* spp., respectively. These genera clustered with many Puget Sound clones. Molecular investigations have reported *Desulfotomaculum* spp. thriving under a variety of harsh conditions, including mine tailings [71] and heavy-metal contaminated estuarine sediments [72]. The extensive physiological capabilities, namely spore production and utilization of many different electron donors and acceptors, of *Desulfotomaculum* spp. seemingly permit adaptation to anthropogenically impacted or otherwise challenging environmental conditions. *Desulfosarcina*- and *Desulfobacter*-like sequences, which were recovered from the Guaymas basin, corresponded with many *dsr*AB sequences retrieved from this study. Strains within these genera are capable of oxidizing a great variety of electron donors completely to CO_2 (see [73]), which would clearly provide a competitive advantage in environments where a broad range of carbon compounds are readily available.

This study disclosed the structure and diversity of sulfate-reducer communities at different sites in Puget Sound. By examining sediments from different sites, we were able to determine the community structure in relation to varying levels of environmental gradients. These varying environmental gradients selected for members of *Desulfobacteraceae*, a metabolically versatile group. Assuming physiological likeness to the closest phylogenetic relatives, metabolic versatility may permit ample oxidation of refractory carbon compounds to support the growth of these organisms in Puget Sound sediments. However, further efforts are needed to isolate these diverse sulfate reducers as revealed by *dsr*AB sequences, to establish their physiological and ecological functions, and to understand their potential roles in carbon and sulfur dynamics in sediments. Further study with the development tools for the detection of physical and geochemical variations, and isotope characterization are also needed to provide a better comprehensive understanding of the ecological significance of the SRBs in marine sediments. Nonetheless, the sequence data analysed in this study should provide a basis to design probes and primers to quantitatively assess the diverse range of sulfate reducers present in the environment.

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