Linkage of High Rates of Sulfate Reduction in Yellowstone Hot Springs to Unique Sequence Types in the Dissimilatory Sulfate Respiration Pathway

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Diversity, habitat range, and activities of sulfate-reducing prokaryotes within hot springs in Yellowstone National Park were characterized using endogenous activity measurements, molecular characterization, and enrichment. Five major phylogenetic groups were identified using PCR amplification of the dissimilatory sulfite reductase genes (dsrAB) from springs demonstrating significant sulfate reduction rates, including a warm, acidic (pH 2.5) stream and several nearly neutral hot springs with temperatures reaching 89°C. Three of these sequence groups were unrelated to named lineages, suggesting that the diversity and habitat range of sulfate-reducing prokaryotes exceeds that now represented in culture.

Sulfate-reducing prokaryotes (SRP) are widely distributed in the environment. Their habitat range includes freshwater, marine, and hypersaline aquatic systems, cold oceanic sediments, the deep subsurface, hydrothermal vents, and hot springs (7, 13, 14, 21, 22, 24). Although cultivated SRP are phylogenetically and physiologically diverse, they are restricted to four divisions within the Bacteria and one within the Archaea (25). Over 90% of described species are affiliated with the delta subdivision of the Proteobacteria (ca. 14 genera and 50 species) or the Firmicutes (Desulfothermus spp.). Those few that do not associate with these two divisions are affiliated with the thermophilic bacterial genera Thermodesulfobacterium and Thermodesulfovibrio or a single archael genus, Archaeoglobus.

We now know their natural diversity is much greater than represented in culture, as revealed by endogenous activity measurements and more recent use of explicit molecular criteria (4–6, 8, 9, 17, 19). The report of sulfate respiration at 110°C in hydrothermal marine sediments is well beyond the maximum growth temperature of any isolate (16). Direct environmental surveys based on comparative sequencing of genes encoding the dissimilatory sulfite reductase (dsrAB) enzyme have identified novel clades in different environments, including a hypersaline microbial mat community, a uranium tailing site, a coastal fjord, and a hydrothermal vent worm (5, 6, 17, 19).

A more complete census of the environmental diversity of SRP has considerable importance for resolving their varied environmental roles, not necessarily restricted to sulfate reduction, and developing a better understanding of their origins and evolution. Since the genes in the pathway for sulfate respiration are sufficiently conserved to be recovered by PCR amplification, and since virtually all recognized genera have been characterized by comparative sequencing of genes encoding the 16S rRNA and the dissimilatory sulfite reductase (dsrAB) (18), these sequences now provide a framework to explicitly identify environmental populations. In the present study, we used a threefold approach, incorporating endogenous activity measurements, molecular characterization, and enrichment to characterize the diversity and activity of SRP in various thermal springs in Yellowstone National Park, Wyo.

Site descriptions and analytical methods. The sediments of different geothermal pools and microbial mats in shallow runoff streams presented a wide range of temperatures (ca. 40 to 90°C), pH (ca. 2.5 to 6.6), and sulfate concentrations (ca. 0.5 to 90 mM) (Table 1). Using a combined temperature-pH probe (WTW, Ft. Myers, Fla.) (pH 330), temperature and pH were measured on site. Sulfate concentrations were determined by capillary electrophoresis following the return to the lab (Quanta 2000 capillary ion analyzer; Waters Corp., Milford, Mass.). Using replicate mat (3 cm deep) or sediment (≤10 cm deep) samples collected in 5-ml syringe corers, endogenous measurements of sulfate-reduction rates (SRR) were initiated on site. A 25-μl Hamilton syringe was used to evenly distribute 10 μl (185 kBq) of carrier-free Na₂[³⁵SO₄] immediately before sealing each sample with a butyl rubber stopper and incubating in site water for 30 min, a time during which we have shown uptake of sulfate to be linear at one of the most active sites (New Pit Spring; data not shown). However, since linearity was not determined for all sites, reported values should be treated as conservative rates. Reactions were terminated (and sulfide trapped) by expelling syringe contents into 10 ml of a 20% zinc acetate solution. Sodium molybdate (6.6 mM) was added to a control sample to inhibit microbially mediated sulfate reduction. An additional control was immediately quenched with zinc acetate following Na₂[³⁵SO₄] addition. Samples were processed following return to the laboratory using the single-step chromium reduction method of Fossing and Jørgensen (12) and rates calculated by the method of Jørgensen (15).

Triplicate ~0.5-ml samples from selected sites were frozen on dry ice and stored at −80°C for later determination (using...
the Bligh and Dryer technique [2] as modified by Findlay et al. [10, 11]) of total phospholipid phosphate levels.

Enrichment cultures. Using 20% filtered source water and either 3 mM acetate or H2 (5 lb/in2) plus 1.5 mM acetate (Table 1) as described by Boone et al. (3), dilution series enrichment cultures were developed from selected sites under a N2/CO2 atmosphere. Cultures were incubated at 55°C and transferred three to four times over a 4-month period prior to molecular characterization.

Molecular characterization. Site material was frozen immediately following collection by placement on dry ice. Using a high-salt-sodium dodecyl sulfate-heat method (27), total DNA was later extracted from approximately 2 to 5 g of sediment or high salt-sodium dodecyl sulfate-heat method (27), total DNA di-

<TABLE>
<table>
<thead>
<tr>
<th>Study site, and specific location</th>
<th>Sample type</th>
<th>pH</th>
<th>Temp (°C)</th>
<th>SO4 concn (mM)</th>
<th>SRR (nmol of SO4 cm⁻³ day⁻¹)</th>
<th>Phospholipid content (nmol of P g⁻¹)</th>
<th>Enrichments obtained</th>
<th>No. of clades found with DSR PCR amplification of samples from²:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mud Volcano area, Obsidian Pool</td>
<td>Sediment</td>
<td>6.3</td>
<td>89</td>
<td>0.6</td>
<td>104 (144)¹</td>
<td>9.7 (3.7)</td>
<td>–</td>
<td>1</td>
</tr>
<tr>
<td>Nymph Creek</td>
<td>Scraped Mat</td>
<td>2.5</td>
<td>38–47</td>
<td>4.7</td>
<td>212 (110)</td>
<td>n.a.²</td>
<td>–</td>
<td>2</td>
</tr>
<tr>
<td>Black Sediment Pool</td>
<td>Sediment</td>
<td>6.6</td>
<td>69</td>
<td>0.6</td>
<td>87 (97)</td>
<td>n.a.</td>
<td>–</td>
<td>3</td>
</tr>
<tr>
<td>Mammoth Springs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>New Pit Spring</td>
<td>Mat</td>
<td>6.1</td>
<td>56</td>
<td>31.6</td>
<td>11,111 (1,497)</td>
<td>n.a.</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>Bath Lake Vista</td>
<td>Mat</td>
<td>6.5</td>
<td>54</td>
<td>6.3</td>
<td>n.a.</td>
<td>n.a.</td>
<td>+</td>
<td>2</td>
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<tr>
<td>Roland’s Well</td>
<td>Mat</td>
<td>6.5</td>
<td>52</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>+</td>
<td>1</td>
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<tr>
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<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Site A</td>
<td>Sediment</td>
<td>2.3</td>
<td>88</td>
<td>87.5</td>
<td>704¹</td>
<td>n.a.</td>
<td>–</td>
<td>0</td>
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<tr>
<td>Site B</td>
<td>Sediment</td>
<td>2.2</td>
<td>42</td>
<td>6.5</td>
<td>1.6 (1.4)</td>
<td>n.a.</td>
<td>–</td>
<td>0</td>
</tr>
<tr>
<td>Site E</td>
<td>Sediment</td>
<td>4.1</td>
<td>91</td>
<td>0.3</td>
<td>3.9 (3.1)</td>
<td>n.a.</td>
<td>–</td>
<td>0</td>
</tr>
<tr>
<td>Cinder Pool</td>
<td>Sediment</td>
<td>2.5</td>
<td>91</td>
<td>0.5</td>
<td>2²</td>
<td>2.4 (1.2)</td>
<td>–</td>
<td>0</td>
</tr>
<tr>
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<td>Sediment</td>
<td>3.0</td>
<td>80</td>
<td>2.6</td>
<td>5.6¹</td>
<td>1.8 (0.6)</td>
<td>–</td>
<td>0</td>
</tr>
<tr>
<td>Mt. Washburn area</td>
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<td></td>
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<td></td>
<td></td>
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<td></td>
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<tr>
<td>Site A</td>
<td>Sediment</td>
<td>2.2</td>
<td>46</td>
<td>54.0</td>
<td>b.d.¹</td>
<td>25.8 (1.8)</td>
<td>–</td>
<td>0</td>
</tr>
<tr>
<td>Site B</td>
<td>Sediment</td>
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<td>81</td>
<td>39.1</td>
<td>b.d.¹</td>
<td>n.a.</td>
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<td>0</td>
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<td>77</td>
<td>38.2</td>
<td>b.d.¹</td>
<td>6.9 (5.1)</td>
<td>–</td>
<td>0</td>
</tr>
<tr>
<td>Inkpot Spring</td>
<td>Sediment</td>
<td>6.2</td>
<td>86</td>
<td>24.1</td>
<td>b.d.¹</td>
<td>1.3 (0.1)</td>
<td>–</td>
<td>0</td>
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</tbody>
</table>

¹ n.a, data not available.
² Values represent means (standard deviations). n = 3 for all samples except those from New Pit, for which n = 2.
³ The rate was determined from one core; the other two cores gave results below the detection limit.
⁴ b.d., below detection limit.
⁵ –, enrichment was attempted with no success.
⁶ Values represent the number of clades present. Zeros represent unsuccessful attempts at amplification. DSR, dissimilatory sulfite reductase.
FIG. 1. Unrooted dendrogram depicting relationships among *dsrAB* gene sequences. A mask of 483 amino acid positions (gaps and ambiguous residues were excluded) was created with ARB software. The backbone tree was constructed using the neighbor-joining algorithm in PAUP*. Previously published (18) sequences from SRP grown in cultures are shown in italic characters; sequences from environmental (env) and enrichment culture (enrich) clone libraries are shown in boldface. For groups for which no member grown in culture is known, the closest sequences from the GenBank database that are representative of strains not grown in culture were included in plain characters for reference. Subtrees were constructed similarly using partial sequences (162 to 273 residues) (shown with dashed lines) and added manually to the backbone tree by using a fully sequenced member as an anchor lineage. Values at nodes are bootstrap confidence values expressed as percentages of 1,000 replications. Nodes without values indicate those unsupported by a majority of tree reconstructions. *Archaeoglobus profundus* was used as an outgroup in all trees. Accession numbers for all sequences are in parentheses.
sequenced members as reference lineages. In cases in which sequences were not closely related to sequences from cultivated SRP, the nearest relative sequences from BLAST (1) searches of the GenBank dsrAB database were added to the tree for reference. Bootstrap resampling of the neighbor-joining and parsimony trees was performed using 1,000 and 100 replicates, respectively. For likelihood analysis using quartet puzzling, at least 1,000 replicates were performed. Phylogenetic trees and subtrees inferred from neighbor-joining, parsimony, and maximum-likelihood analyses of aligned dsrAB sequences all showed similar topologies, with the only major differences observed in the deeply branching nodes not resolved by bootstrap analysis. These trees were in general agreement with previously published phylogenies of strains grown in cultures (18).

Mean SRR varied from below detectable levels to over 11,000 nmol cm$^{-3}$ day$^{-1}$ among the sites investigated. SRR values for controls were less than 10% of measured rates at the most active sites (>10 nmol of SO$_4$ cm$^{-3}$ day$^{-1}$) and also significantly less than mean values for lower activity sites (<50%). Values shown in Table 1 have been corrected by subtracting the control values. The high rates observed at Nymph Creek (212 nmol of SO$_4$ cm$^{-3}$ day$^{-1}$) and New Pit Spring (11,111 nmol of SO$_4$ cm$^{-3}$ day$^{-1}$) likely reflect input of endogenous electron donors from associated photosynthetic microbial mat communities. Nymph Creek, an acidic 38- to 47°C stream, contains a mat dominated by the alga Cyanidium caldarium, while New Pit Spring, a more alkaline 47°C spring, contains a mat composed primarily of photosynthetic bacteria. However, significant SRR were also observed in a nonphotosynthetic high-temperature (89°C) near-neutral spring (Obsidian Pool [OP]) of relatively low biomass (Table 1). The SRR in such low-biomass springs may be using non-biologically derived electron donors (such as H$_2$ or CO) such as are present in many geothermal environments.

The low-pH (2.2 to 3.0) springs in Norris Geyser Basin exhibited a range of temperatures (42 to 91°C) and SRR values. With the exception of one replicate in Norris (site C), rates were usually low (<10 nmol cm$^{-3}$ day$^{-1}$) or below detection. Rates at all sites near Mt. Washburn, including both low-pH (ca. 2) and near-neutral-pH (ca. 6) springs, were below detection. However, the high sulfate concentrations in this region (>21 mM SO$_4$; Table 1) significantly reduce the sensitivity of our analysis. Thus, longer incubation times, or higher isotope concentrations, may be needed for detection. Although Zeikus et al. (26) reported detectable SRR at Inpocket Spring in the Mt. Washburn area, measurable rates were only observed for samples incubated at the cooler margin of the spring. Reduction rates in samples incubated in higher-temperature water, as was done in this study, were close to the lower limit of detection.

We attempted to amplify dsrAB sequences from all sites demonstrating appreciable sulfate reduction (>10 nmol of SO$_4$ cm$^{-3}$ day$^{-1}$) and from stable enrichments. With the exception of New Pit Spring and Site C in Norris Geyser Basin, successful amplification generally corresponded with higher activity sites. Environmental sequences affiliated with five major clades (Fig. 1); three were novel and two were represented by named species. For the latter, sequences from three near-neutral springs grouped with Desulfobacula toluolica, while those from Nymph Creek were most closely related to gram-positive Desulfitobaculum spp. Another Nymph Creek dsrAB sequence belonged to a novel, deeply branching clade (Fig. 1) with strong nucleotide identity (85%) to another environmental clone recovered from a hot spring in Japan (K. Mori and S. Hanada, unpublished data). The Nymph Creek data suggest that SRP can inhabit highly acidic environments, although no sulfate-reducing microorganism has been reported that is capable of growth at such low pH. Like this Nymph Creek sequence, sequences from the two other novel clades were not closely related to any SRP grown in cultures and, in these cases, the sequences were only modestly related (<80% nucleotide identity) to environmental clones from the database (4, 20) (Fig. 1). Most of the Black Sediment Pool (BSP) sequences and all of the OP sequences form a closely related assemblage within a novel clade. The three BSP sequences in this clade are representative of 12 closely related sequences differing by <1% at the nucleotide level. The close relationship between sequence types recovered from BSP and OP suggests some similarity of habitat. Although the temperatures of these springs differ by 20°C, they share similar chemistry and their SRR are statistically indistinguishable (t = 0.16; P = 0.8772) (Table 1). Thus, their affiliation may be more closely linked to chemical parameters than to temperature. Also, apparent sequence diversity in the lower-temperature spring was greater than for OP. Two additional sequence types recovered from BSP were related to sequences from lower temperature (ca. 50 to 55°C) sites in the Mammoth Springs area (Bath Lake Vista and Roland’s Well), possibly suggesting that SRP diversity is restricted at higher temperatures.

Our limited inspection of sites by enrichment was intended primarily to provide some perspective on the types of SRP recoverable (using standard enrichment methods) from these sites. As has been commonly observed for other such comparisons, there was no congruence between environmental and enrichment sequences. All dsrAB sequences derived from enrichments were closely affiliated with representatives grown in cultures (Fig. 1).

In an early publication of investigations using comparative 16S rRNA sequencing to define relationships among SRP, the patchy distribution of sulfate-respiring lineages within the bacterial tree was also noted (8). At that time, we offered the suggestion that diversity was not fully represented in the available culture collection. The results of this study provide more direct evidence that the environmental diversity of SRP greatly exceeds that represented by organisms grown in cultures and includes deeply diverging lineages not closely related to described sulfate-reducing groups. These findings underscore the importance of further investigation of SRP in these and other environments. A more complete understanding of the diversity and habitat distribution of SRP is an essential precursor to determining the contribution of members of this important functional group to biogeochemical cycling and of understanding their origins and evolution.

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REFERENCES