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Microbially mediated sulphide production in a thermal, acidic algal mat community in Yellowstone National Park

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Summary

Our objective in this study was to characterize prokaryotic sulphide production within the oxygenic, predominantly eukaryotic algal mat in an acidic stream, Nymph Creek, in Yellowstone National Park (YNP). We used microsensors to examine fluctuations in H_2S and O_2 concentrations over time through the vertical aspect of the ~3 mm mat in a 46–48°C region of the creek. We also used analyses of PCR-amplified 16S rRNA gene sequences obtained from denaturing gradient gels, and PCR-amplified sequences of a functional gene associated with microbial sulphate respiration (*dsrA*) to characterize the bacterial community in the same region of the mat. During midday, photosynthesis rates were high within the first 500 µm interval of the mat and high oxygen concentrations (600% air saturation) penetrated deeply (>1800 µm) into the mat. During early evening and night, oxygen concentrations within the first 1100 µm of the mat decreased over time from 60% air saturation (a.s) to 12% a.s. A precipitous decline in oxygen concentration occurred at a depth of 1100 µm in all night measurements and anoxic conditions were present below 1200 µm. Within this anoxic region, sulphide concentrations increased from nearly 0 µM at 1200 µm depth to 100 µM at 2400 µm depth. Enrichment cultures inoculated with Nymph Creek mat organisms also produced H_2S. Sequence analyses of 16S rRNA and *dsrA* genes indicated the presence of at least five bacterial genera including species involved in dissimilative sulphate or sulphur reduction.

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Introduction

Concentrations of various oxidized sulphur species, including elemental sulphur (S°), sulphite (SO_3^{2-}), thiosulphate ($S_2O_3^{2-}$), and sulphate (SO_4^{2-}), are typically high in environments such as acidic geothermal springs, acid mine drainages and volcanic lakes; consequently, there is potential for biogeochemical sulphur oxidoreduction in these environments (Brock, 1978; Herlihy and Mills, 1985; Ward *et al*., 1989; Gyure *et al*., 1990; Satake, 1997; Chang *et al*., 2001). For example, sulphate-reducing bacteria (SRB) have been isolated from various acidic niches in mine waste (Fortin *et al*., 2000), and SRB, as well as biogenic H_2S, are readily detected and/or cultured from alkaline hot springs and thermal vents (Hugenholtz *et al*., 1998; Miroshnichenko *et al*., 1998; Sievert and Kuever, 2000). In contrast, neither SRB nor biogenic sulphide production has been observed in acidic thermal springs, in part because anaerobic SRB were thought not to tolerate O_2 evolved by the photosynthetic algae in these ecosystems (reviewed in Widdel and Bak, 1992). However, it is now known that many SRB tolerate O_2 for short times; indeed, some continue to reduce sulphate in the presence of O_2 (Canfield and Des Marais, 1991; Sigalevich *et al*., 2000), or even eliminate it by using it as an electron acceptor (Sigalevich and Cohen, 2000). Thus, anaerobic SRB could occur in an oxygenic, acidic algal mat, such as that of Nymph Creek.

Sulphide microsensors are used to monitor H_2S concentrations over µm-scale intervals in microbial biofilms (Kühl and Steuckart, 2000) and sediments in acidic environments (Kühl *et al*., 1998). Such concentration profiles provide a more direct view of processes occurring *in situ*. This prompted us to investigate bacterial H_2S production and potential sulphate and/or sulphur reduction in the algal mat in Nymph Creek.

Results and discussion

Nymph Creek is located near Norris Geyser Basin in Yellowstone National Park (YNP), Wyoming. It is a 1–2 m wide, 1–10 cm deep geothermally heated, acidic stream

originating from multiple springs. Its most prominent feature is a bright green, ~2–3 mm-thick predominately algal mat composed of a unicellular *Cyanidium caldarium*-like alga that covers much of the stream bed (Doemel and Brock, 1971; M. J. Ferris, K. B. Sheehan and J. M. Henson, unpubl. data). The pH of the bulk water is ~2.7 and the temperature at the source is ~57°C year-round (M. J. Ferris, K. B. Sheehan and J. M. Henson, unpubl. data). Elemental sulphur was present in mat samples at 40 μg g^{-1} mat dry weight in a previous study (Ward *et al*., 1989), and the water chemistry of recent samples demonstrated high sulphur and iron concentrations in Nymph Creek's bulk water and mat (Sheehan *et al*., 2003). All analyses in this study were conducted on a sunny region of the mat near the creek's source where temperatures were between 46 and 48°C. All samples were collected during August, 1999 and 2000.

Microsensor analyses

We used microelectrodes to measure variations in sulphide and O_2 concentrations over time through the vertical aspect of the mat (Fig. 1). Midday profiles of photosynthesis rates showed a peak at a depth of 200 μm and oxygen concentrations approaching nearly 600% air saturation (a.s.) penetrating deeply (1800 μm) into the mat (Fig. 1A and B). These microelectrode measurements agreed well with previous microelectrode analyses of oxygen in the Nymph Creek mat during midday that were conducted over a decade ago (Revsbech and Ward, 1983). However, in contrast to the study of Revsbech and Ward (1983), our measurements show fluctuating concentrations and zones with constant oxygen levels in the upper mm of the mat in both light and darkness indicating some advective transport of spring water through part of the mat. Perco-

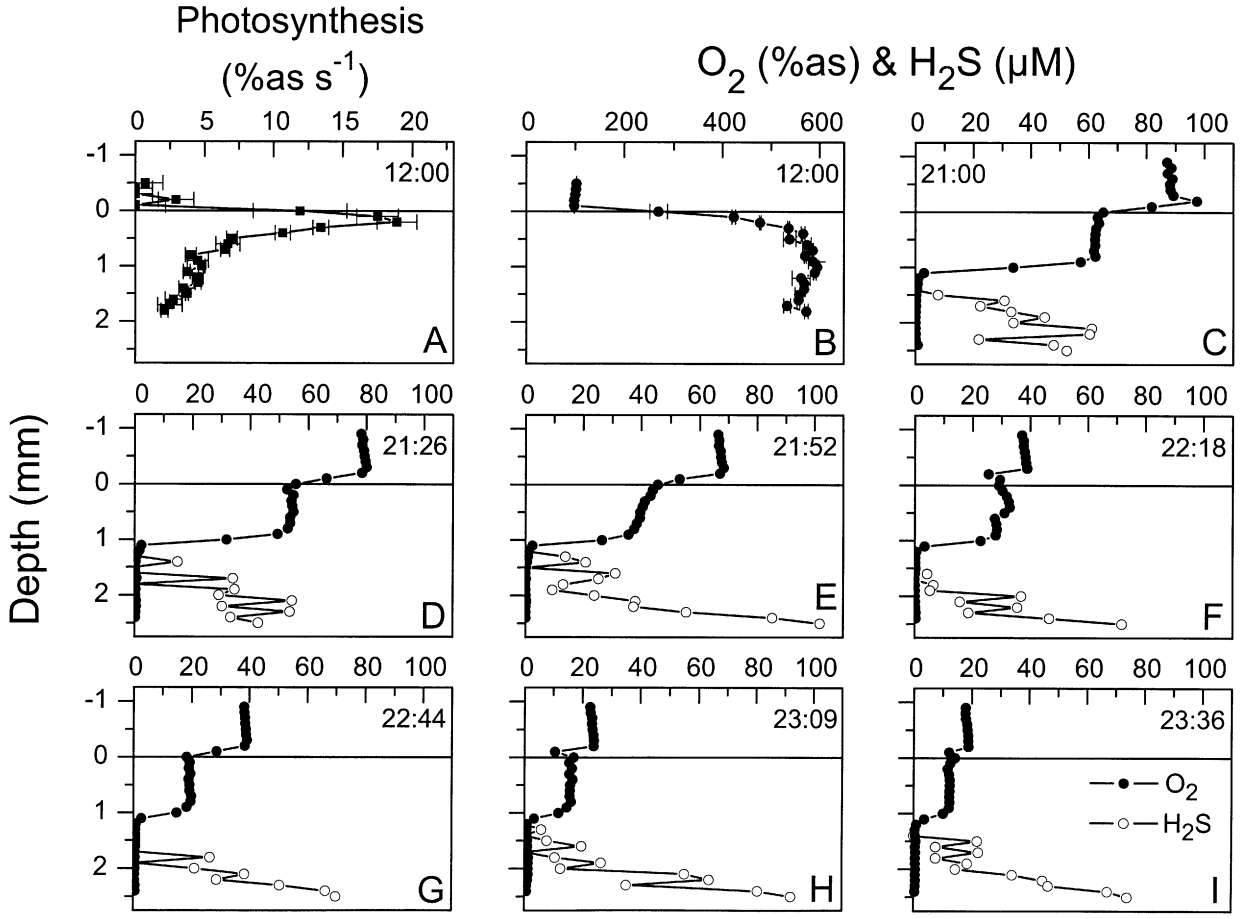

Fig. 1. *In situ* sulphide and oxygen production throughout the Nymph Creek mat as measured by sulphide-specific (Kühl and Steuckart, 2000) and Clark-type O_2 (Revsbech and Ward, 1983) microsensors. The O_2 microsensor had a <10 μm diameter tip, a stirring sensitivity of <2%, and a t_{90} response time of <0.5 s. The H_2S microsensor had a <20 μm tip painted with black enamel to avoid light interference. Microelectrodes were calibrated, mat profiles measured, and conversions and photosynthesis rates calculated as previously described (Wieland and Kühl, 2000).
A. Midday photosynthesis rates.
B. Midday oxygen concentrations; sulphide was not detected in the mat during mid day.
C–I. Sulphide and oxygen concentrations throughout the mat from early to late-evening.
Error bars represent standard deviation of three replicate measurements.

lation of water through the mat may also explain the fluctuating H_2S concentrations in the mat. Entrapped air bubbles in the mat fabric could also in part explain our profiles, but we did not see any air bubbles in our sample. Quantitative calculations of reaction rates from our microsensor data are problematic and await further detailed studies of the microenvironment and transport mechanisms in the mat. However, zones of net O_2 and H_2S production and consumption could still be clearly identified.

Between 21:00 and 23:30 h, seven O_2 and H_2S concentration profiles were recorded at approximately 30 min intervals. O_2 concentrations in the mat, and in the water overlying the mat, declined over time. By 21:00, O_2 concentration in the upper 1100 μm interval of the mat decreased from a midday high of 600% a.s. to 60% a.s. By 23:30, oxygen concentration over the same depth interval decreased to 12% a.s. A precipitous drop in O_2 concentration occurred in each profile between the depths of 1100 and 1200 μm (Fig. 1D–I). Below ~1200 μm depth, O_2 was not detected and the mat was anoxic. Sulphide concentrations were undetectable during midday, but increased to ~100 μM in the lower mat depths during the evening (Fig. 1D–I). The concentrations of H_2S in Nymph Creek source water, measured annually from 1999 to 2001, were 4, 6 and 2 μM respectively; thus, it is unlikely that the source water itself accounts for the high concentrations (>60 μM) measured in the mat (Nordstrom, pers. comm.; Ball *et al*., 2002). These O_2 and H_2S concentrations are consistent with a microbial mat community where photosynthesis provides organic carbon for heterotrophic microbial growth, depleting oxygen and providing an anoxic environment favourable for H_2S production.

Enrichment cultures

As *in situ* microsensor analyses suggested that H_2S was present, we confirmed the presence of H_2S-production by inoculating mat samples into Lactate C medium with pH adjusted to a range of 2–7 (Widdel and Bak, 1992) to enrich for sulphate-reducers. All enrichment cultures with pH 5–7 had black FeS precipitate after 24–48 h at 37°C; Lactate C medium below pH 5 did not develop precipitate, even after prolonged incubation. Enrichment cultures plated onto Lactate C agar medium failed to produce SRB colonies. Extinction dilution series from five enrichment cultures demonstrated that sulphate-reducing organisms were always 7–8 orders of magnitude less numerous than other anaerobes, as only 1:10 dilutions produced FeS, whereas other anaerobes grew in 10^{-9} dilutions. Our attempts to amplify *dsrA* sequences from enrichment cultures failed, perhaps because of the low ratio of sulphate-reducers/total anaerobes, and/or because of PCR inhibition. An alternative explanation is that sulphur-reducing bacteria were responsible for H_2S and that dilution of elemental sulphur, which is not present in Lactate C medium, restricted the production of sulphide and growth of sulphur reducers in the less diluted enrichments.

No enrichments showed H_2S production below pH 5. This is consistent with the physiologies of known mesophilic SRB, where H_2S is not produced at pH < 5 in laboratory cultures even when the isolates are cultured from acidic environments (Herlihy and Mills, 1985; Gyure *et al*., 1990; Satake, 1997; Fortin *et al*., 2000; Chang *et al*., 2001). Microelectrode pH analyses conducted by Revsbech and Ward (1983) demonstrated that the pH was 2.7 through the Nymph Creek mat. However, the mat could harbour microniches of higher pH. Sulphate reduction reactions can consume protons and/or produce proton-scavenging HS^- or HCO_3^- ions (reviewed in Widdel and Bak, 1992).

An alternative explanation for apparent H_2S production at pH 2.7 in Nymph Creek is that the pH of the sediment below the mat was appreciably higher than that of Nymph Creek bulk water and that sulphide produced at pH > 5 diffused upward into the mat. To test this, a pH probe with a 4-mm tip diameter was inserted 2 cm into the stream bed. The probe remained in position overnight to allow the surrounding mat and sediments to stabilize. The pH before sunrise the following morning was 2.7. Thus, the overall pH just below the mat was low during hours when H_2S concentrations in the mat were high, making the diffusion explanation unlikely.

Finally, sulphate and/or sulphur reduction may actually occur at low pH, but we and others are thus far unable to reproduce the correct culture conditions to observe this in the laboratory.

DGGE analyses

Vertical 2–3 mm mat samples for genetic analyses were gently removed, transferred into 2 ml plastic screw-capped microcentrifuge tubes, and stored frozen. Bacterial populations were examined by DGGE analysis of PCR-amplified bacterial 16S rRNA gene segments with primers expected to amplify all eubacteria (Ferris *et al*., 1996). This approach revealed the presence of species related to cultivated and uncultivated bacteria from low pH and/or high temperature environments (Table 1). Lanes 1 and 8 in Fig. 2 represent the DGGE profile of a 46–48°C region of the mat. This profile was reproducible and the species composition revealed by sequencing individual DGGE bands was similar to a bacterial profile from a neighbouring acidic hot spring, where the same PCR-DGGE approach was used to analyse potential arsenite-oxidizing bacterial populations (Jackson *et al*., 2001).

Table 1. Sequences and organisms detected, their nearest neighbours in GenBank, and potential electron donors and/or acceptors of their nearest cultivated neighbours.

Method detected	GenBank accession number	Nearest GenBank relative and Phylogenetic affiliation	GenBank nearest relative	% Similarity	Potential electron donors and/or acceptors
16S rRNA DGGE-fragment 1	(AY305278)	*Hydrogenobacter acidophilus* (*Aquificales*) from a solfataric Japanese field (Shima *et al*., 1994);	D16296	96	Requires S° (Shima *et al*., 1994) oxidizes H_2, reduces S°, $S_2O_3^{2-}$ (Stohr *et al.,* 2001)
		Hydrogenobaculum sp. NOR3L3B (*Aquificales*) from acidic Norris Geyser Basin, YNP (Eder and Huber, 2002);	AJ320225	99	
		Uncultured bacterium from acidic spring, YNP (Jackson *et al.,* 2001)	AF325179	98	
16S rRNA DGGE-fragment 2	(AY305279)	*Desulphurella multipotens* (δ proteobacteria) from thermal spring in Kamchatka (Miroshnichenko *et al*., 1998);	Y16943	98	Anaerobe; reduces S°
		Desulphurella kamchatkensis from thermal spring in Kamchatka (Miroshnichenko *et al*., 1998);	Y16941	98	
		Desulphurella acetivorans;	X72768	98	
		uncultured bacterium from acidic spring, YNP (Jackson *et al.,* 2001);	AF325180	98	
		uncultured bacterium from acidic Montserrat springs (Burton and Norris, 2000)	AF232926	97	
16S rRNA DGGE-fragment 3	(AY305280)	Uncultured proteobacterium from acidic, saline water of Vulcano;	AF339745	96	Unknown
		Desulfitobacterium metallireducens (*Firmicutes*) (Finneran *et al.,* 2002);	AF297871	84	Anaerobe; reduces SO_3^{2-} and Fe^{3+} and other metals
		Desulfosporosinus sp. (*Firmicutes*)	AF295659	84	Anaerobe; reduces SO_4^-
16S rRNA DGGE-fragment 4	(AY305281)	*Desulfotomaculum thermobenzoicum* (*Firmicutes*) from sludge of a methane fermentation reactor (Tasaki *et al*., 1991)	L15628	96	Anaerobe; reduces SO_4^{2-}, $S_2O_3^{2-}$ SO_3^{2-}, and S° (Redburn and Patel, 1993)
16S rRNA DGGE-fragment 5	(AY305282)	*Acidimicrobium ferroxidans* (*Firmicutes*);	U75647	97	Facultative anaerobe; oxidizes H_2 and reduces Fe^{3+} or oxidizes organics and reduces O_2 (Bridge and Johnson, 1998)
		Uncultured *Acidimicrobium* sp. from YNP	AY191899	97	
16S rRNA DGGE-fragment 6	(AY305283)	Uncultured bacterium clone BG6, from acidic spring, YNP	AY191889	99	Unknown
Cloned *dsrA* gene sequences					
Clone 171–1	AY321359	*Pyrobaculum islandicum* (*Crenarchaeota*) from geothermal power plant (Dahl *et al*., 2001)	U75249		Reduces S°, $S_2O_3^{2-}$, SO_3^{2-}
Clone 229–1	AY321360				
Clone 230–1	AY321361				
		Chromatium vinosum (γ proteobacteria)	U84760		Oxidizes S°

Six prominent DGGE bands (lanes 2–7) were isolated and ~300 bp sequences of these segments were compared with those in GenBank as summarized in Table 1. Four of the bands were >96% similar to their closest related GenBank relative, a finding that suggests that they could be the same species, or at least have similar metabolism to their nearest GenBank neighbour (Stackebrandt *et al*., 1997).

The sequence of band 1 was 96% similar to a hydrogen-oxidizing *Hydrogenobaculatum acidophilum*, previously named, *Hydrogenobacter acidophilus* (Stohr *et al*., 2001). The sequence was 98% similar to an uncultured bacterium identified in a neighbouring thermal, acidic spring in YNP (Jackson *et al*., 2001), and most (99%) similar to another *Hydrogenobaculum* sequence from Norris Basin in YNP (Eder and Huber, 2002; see Table 1 for accession numbers). Similar sequences were detected in acid mine drainages (Bond *et al*., 2000) and in a Japanese solfataric field (Shima *et al*., 1994).

Band 2 was 98% similar to three sulphur-reducing *Desulphurella* species: *D. multipotens*, *D. acetivorans*, and *D. kamchatkensis. Desulphurella kamchatkensis* was iso-

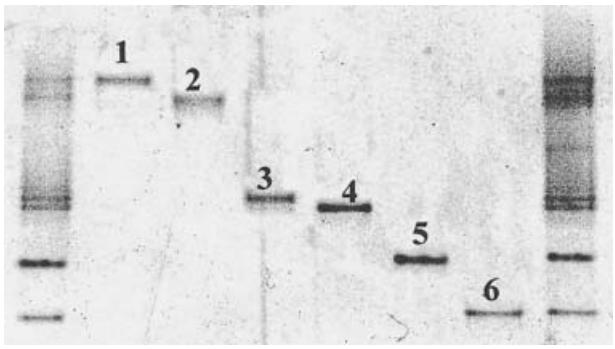

Fig. 2. DGGE analysis of products obtained from PCR amplification of 16S rRNA gene sequences in mat samples from Nymph Creek. Nucleic acids were extracted from samples using a modification of a method described by Moré *and* co-workers (Moré *et al*., 1994; Sheehan *et al.,* 2003). DGGE analyses of bacterial populations was as described previously (Ferris *et al*., 1996) except that the gels were electrophoresed at 60 V for 17 h. The PCR primer set should amplify a ~323 bp region of eubacterial 16S rRNA genes from nucleotides 1071–1391 (*E. coli* numbering; Ferris *et al*., 1996). Multiple successive rounds of band re-picking and PCR re-amplification were necessary in some cases to enrich for a single targeted band. Each PCR product exhibiting an individual band upon DGGE re-analyses was cleaned with a PCR product cleaning kit (QIAGEN, Hilden, Germany), sequenced, and compared to sequences in GenBank (Altschul *et al*., 1997). All sequences were submitted to GenBank (see Table 1). Lanes 1 and 8: DGGE profile of 16S rRNA gene segments amplified directly from DNA extracted from mat samples.) Lanes 2–7: DGGE profile of bands re-amplified from the initial algal mat profiles (Lanes 1 and 8), confirming correspondence with those in the community profile and suitability for genetic sequencing. Scale bar = 0.1 changes.

lated previously from a thermal vent at neutral pH in Kamchatka (Miroshnichenko *et al*., 1998). Fragment 2 also was 98% and 97% similar to two uncultured bacterial sequences from a neighbouring acidic spring in YNP (Jackson *et al*., 2001) and a thermal, acidic pool on the Caribbean island of Montserrat respectively (Burton and Norris, 2000). *Desulphurella* species are anaerobes and reduce elemental sulphur to sulphide; *D. kamchatkensis* also requires sulphur for growth (Miroshnichenko *et al*., 1998).

Band 3 was most similar (96%) to a recently described isolate of *Desulfitobacterium metallireducens*, an anaerobic iron- and elemental sulphur-reducing Gram-positive eubacterium (Finneran *et al*., 2002).

Band 4 was most similar (96%) to the cultivated species *Desulfotomaculum thermobenzoicum* (Redburn and Patel, 1993), a thermophilic, Gram-positive SRB originally isolated from a methane fermentation reactor. The genus *Desulfotomaculum* includes low-GC, Gram-positive, *Bacillus*/*Clostridia*-like organisms (Stackebrandt *et al*., 1997).

Band 5 was most similar (97%) to the iron-oxidizing *Acidimicrobium ferrooxidans.* In aerobic conditions, this acidophile uses O_2 as an electron acceptor and Fe(II) as its donor. In the absence of oxygen, it can utilize organic compounds as electron donors and reduce Fe(III) as its electron acceptor (Bridge and Johnson, 1998).

Band 6 was 89% similar to the thermophile, *Meiothermus rosaceus*, cultured from a Chinese hot spring and related to the *Thermus/Deinococcus* group of eubacteria (Chen *et al*., 2002) and 99% similar to an uncultured organism from a nearby YNP spring.

The inferred physiologies of bacteria represented by DGGE bands 2, 3 and 4 were consistent with H_2S production, as their closest GenBank neighbours were microbes that participate in geomicrobiological sulphur cycling. Cultivated species within these genera are anaerobic and can reduce sulphate, sulphite, thiosulphite and/or elemental sulphur to H_2S.

*Functional (*dsrA*) gene analyses*

Identifications based on 16S rRNA gene sequence similarities are not necessarily indicative of physiology. Therefore, a functional gene associated with dissimilatory sulphur metabolism was PCR-amplified, cloned and sequenced directly from mat samples. The gene *dsrA* encodes a subunit of the bisulphate reductase enzyme complex that is found in organisms that respire on sulphate and sulphite, as well as some organisms that oxidize sulphur via the reverse reaction. Ten cloned sequences yielded three different *dsrA* sequences that were phylogenetically within a single clade (Fig. 3). The presence of *dsrA* sequences suggested that the community had the genetic capacity for sulphate/sulphite respiration and/or sulphur oxidation, as illustrated by the relationship of Nymph Creek *dsrA* sequences with both sulphur-oxidizing (*Chromatium*) and sulphate/sulphite-respiring (*Pyrobaculatum, Thermodesulfovibrio*) organisms. The three related Nymph Creek *dsrA* sequences could represent either archaeal or eubacterial species since related *dsrA* sequences are found in both domains because of lateral transfer events involving *dsrA* genes (Klein *et al*., 2001).

In conclusion, we used microsensor data to demonstrate that the predominantly eukaryotic mat on the Nymph Creek streambed became anoxic and produced sulphide at its lower depths at night. Culture enrichments, as well as *dsrA* and 16S rRNA gene sequences, strongly suggested that sulphide production was biologically produced by prokarya either through sulphate reduction by SRB, or by sulphur respiration, perhaps by the *Desulphurella* species detected by DGGE. Neither our enrichment cultures from Nymph Creek, nor known, cultured SRB, even those isolated from acidic environments (Herlihy and Mills, 1985; Satake, 1997; Chang *et al*., 2000; Fortin *et al*., 2000; Gyure *et al*., 1990), produced FeS at pH < 4. Perhaps sulphate or sulphur reduction does occur at low pH in Nymph Creek, but our culture conditions, thus far, are not ideal to observe this in the laboratory.

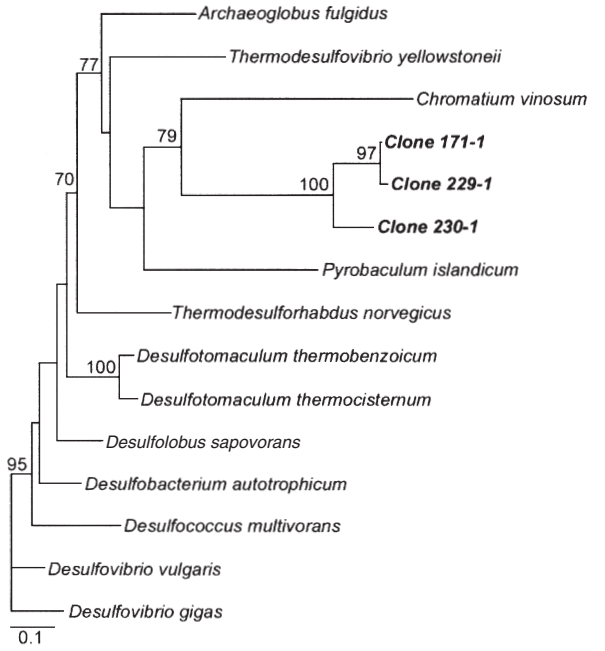

Fig. 3. Phylogenetic tree showing the relationship between *dsrA* clones (in bold) amplified directly from Nymph Creek mat samples and *dsrA* genes from known sulphite- and sulphate-reducing bacteria. PCR primers *DSR*420F (5′-ACCCACTGGAAGCACG-3′) and 1140R (5′-AGTGCATGCAACGGACG-3′) (Karkhoff-Schweizer *et al*., 1995; T. S. Magnuson, unpubl.), specific for *dsrA* (a subunit of the bisulphite reductase gene), amplified a 700 bp region of archaeal and eubacterial genes. DNA of a known sulphate-reducing bacterium, *Desulfovibrio vulgaris,* was used as a positive control. PCR conditions were as described (Karkhoff-Schweizer *et al*., 1995), and products were purified, cloned, and sequenced (Sheehan *et al.,* 2003). Sequences were translated to amino acids (Hall, 2001) and aligned (Thompson *et al*., 1994) with the following parameters: Gap opening 10, Gap Extension 0.2. Two different criteria (distance matrix, maximum parsimony) were used, and both resulted in the same hypothetical phylogenetic relationships. The tree was drawn using the PAUP* program (Swofford, 2002), using a maximum parsimony criterion, with a bootstrap value of 1000. Bootstrap values equal or greater than 70 are shown.

Alternatively, Nymph Creek and other SRB or sulphur-reducing prokaryotes in acidic environments could be sequestered in undetected microniches of low pH.

Acknowledgements

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Note added in proof

Fishbain *et al.* recently reported high rates of sulphate reduction and the presence of sulphite reductase genes in Nymph Creek microbial mats [Fishbain, S., Dillon, J.G., Gough, H.L., and Stahl, D.A. (2003) *Appl Environ Microbiol* **69:** 3663–3667.].

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