Environmental Microbiology (2001) **3**(8), 532–542

Molecular analysis of microbial community structure in an arsenite-oxidizing acidic thermal spring

Colin R. Jackson, Heiko W. Langner, Jessica Donahoe-Christiansen, William P. Inskeep and Timothy R. McDermott*

Thermal Biology Institute and Department of Land Resources and Environmental Sciences, Montana State University, Bozeman, MT 59717, USA.

Summary

Electron microscopy (EM), denaturing gradient gel electrophoresis (DGGE) and 16S rDNA sequencing were used to examine the structure and diversity of microbial mats present in an acid-sulphate–chloride (pH 3.1) thermal (58–62°C) spring in Norris Basin, Yellowstone National Park, WY, USA, exhibiting rapid rates of arsenite oxidation. Initial visual assessments, scanning EM and geochemical measurements revealed the presence of three distinct mat types. Analysis of 16S rDNA fragments with DGGE confirmed the presence of different bacterial and archaeal communities within these zones. Changes in the microbial community appeared to coincide with arsenite oxidation activity. Phylogenetic analysis of 1400 bp 16S rDNA sequences revealed that clone libraries prepared from both arsenic redox active and inactive bacterial communities were dominated by sequences phylogenetically related to *Hydrogenobacter acidophilus* and *Desulphurella* sp. The appearance of archaeal 16S rDNA sequences coincided with the start of arsenite oxidation, and sequences were obtained showing affiliation with both *Crenarchaeota* and *Euryarchaeota*. The majority of archaeal sequences were most similar to sequences obtained from marine hydrothermal vents and other acidic hot springs, although the level of similarity was typically just 90%. Arsenite oxidation in this system may result from the activities of these unknown archaeal taxa and/or the previously unreported arsenic redox activity of *H. acidophilus*- or *Desulphurella*-like organisms. If the latter, arsenite oxidation must be inhibited in the initial high-sulphide zone of the spring, where no change in the distribution of arsenite versus arsenate was observed.

*For correspondence. E-mail timmcder@montana.edu; Tel. (+1) 406 994 2190; Fax (+1) 406 994 3933.
Received 5 June, 2001; revised 12 June, 2001; accepted 26 June, 2001.

Introduction

Traditional microbiological techniques may underestimate the diversity of microorganisms in environmental samples and could potentially provide unrealistic descriptions of microbial community structure. Molecular methods based on the analysis of 16S rDNA sequences can provide information about the complexity of prokaryotic communities in natural environments and have revealed entirely new phylogenetic lineages (Pace, 1997). Although a number of pioneering studies have used these techniques to describe novel and diverse 16S rDNA sequences present in hot spring environments (e.g. Ward *et al*., 1990; Barns *et al*., 1994; Hugenholtz *et al*., 1998), few studies have attempted comprehensive surveys of the microbial communities present in these systems or tried to link structural patterns to biogeochemical processes.

Hot spring ecosystems are often characterized by chemical conditions that facilitate a diversity of biogeochemical and metabolic processes. For example, organisms capable of oxidizing hydrogen (Kawasumi *et al*., 1984; Shima and Suzuki, 1993), hydrogen sulphide (Caldwell *et al*., 1976), sulphur and thiosulphate (Kawasumi *et al*., 1984; Shima and Suzuki, 1993) or reducing sulphur (Bonch-Osmolovskaya *et al*., 1990; Miroshnichenko *et al*., 1998), nitrate and nitrite (Caldwell *et al*., 1976) or iron-dependent photosynthesis (Pierson *et al*., 1999) have all been identified in, or isolated from, thermal environments. Despite the occurrence of such microorganisms in these systems, the contribution(s) of these and other organisms to the entire microbial community present at a particular site is generally unknown.

As well as often having abundant concentrations of hydrogen and sulphur, hydrothermal springs often contain high concentrations of arsenic (Stauffer and Thompson, 1984; Tanaka, 1990; Koch *et al*., 1999). In such environments, arsenic concentrations average 1–3 mg l^{-1}, but can be as high as 150 mg l^{-1} in some hot springs in Yellowstone National Park (YNP), WY, USA (Stauffer and Thompson, 1984). Arsenic in thermal systems exists predominantly as two inorganic redox species, arsenate (As_V) and arsenite (As_{III}) (Koch *et al*., 1999). Arsenite is believed to be the most likely species in emerging geothermal waters (Nicholson, 1993), although arsenate is the thermodynamically favourable species in most aerobic systems (Ferguson and Gavis, 1972; Cullen

and Reimer, 1989). The oxidation of arsenite to arsenate can occur through both abiotic and biotic processes, although biological oxidation is typically much faster (Cullen and Reimer, 1989).

In prokaryotes, arsenate reduction can occur via a dissimilatory route, in which arsenate is used as an electron acceptor under anaerobic or microaerobic conditions (Newman *et al.*, 1998). Arsenate reduction may also occur via a detoxification pathway, whereby arsenate is taken up via a phosphate transporter, reduced by an arsenate reductase and then extruded via an arsenite efflux pump (Ji *et al.*, 1993). The detoxification pathway can occur either aerobically or anaerobically and is not necessarily governed by prevailing redox conditions. Oxidation of arsenite can also be important as a detoxification process (Anderson *et al.*, 1992; Ji *et al.*, 1993) or can serve as an energy source for chemolithoautotrophic metabolism (Ilyaletdinov and Abdrashitova, 1981; Santini *et al.*, 2000). Despite the potential importance of arsenite oxidation, few studies have examined the organisms or communities that carry out this process. Wilkie and Hering (1998) showed that the microbial community associated with submerged macrophytes was capable of arsenite oxidation in a stream receiving geothermal inputs, but they did not attempt to characterize this community.

One of our motivations for studying arsenic redox activity in prokaryotes is our interest in evolutionary aspects of microbe–arsenic interactions; presumably, arsenic toxicity was an issue at a very early stage in microbial evolution. In the current study, we describe the structure of the microbial communities present in an arsenite-oxidizing spring, as determined by a variety of molecular techniques. Samples were initially characterized using denaturing gradient gel electrophoresis (DGGE) and described further by cloning, restriction fragment length polymorphism (RFLP) analysis and subsequent sequencing and phylogenetic analysis of 16S rRNA genes. The work reported here was conducted in collaboration with Langner *et al.* (2001), in whose work the rates of *in situ* arsenite oxidation, aqueous geochemistry and mat solid phase chemistry in this acid-sulphate thermal spring are discussed in greater detail.

Results

Arsenic speciation

Chemical analysis showed that pH remained fairly constant along the spring channel and that temperature decreased with distance from the source (Table 1). Repeated analysis of arsenic species along the course of the spring showed that arsenite represented roughly 90–95% of total soluble arsenic until a point corresponding to the beginning of a brown-coloured zone (sites 5 and 6), where the percentage of total soluble arsenic as arsenate increased to roughly 25%. The percentage of arsenate continued to increase to over 40% above the ensuing green mat (Table 1). Across the same distance, there was no measurable change in iron chemistry, with all iron being present as Fe(II) at a constant concentration of 60 μM at all sites (Langner *et al.*, 2001). Similarly sulphate levels remained constant (1.2 mM) along the course of the spring, whereas sulphide levels declined from 65 μM in the source water to non-detectable levels by site 8 (Langner *et al.*, 2001). A diel study revealed no differences in spring chemistry between day and night samples (Langner *et al.*, 2001). Rates of arsenite oxidation determined in *ex situ* reaction vessels for mat samples obtained from site 6 followed pseudo first-order kinetics; rate constants were determined to be $k = 0.045\ \text{min}^{-1}$ for live samples compared with $k = 0.0054\ \text{min}^{-1}$ for formaldehyde-killed controls (slope was not statistically different from zero). Spring water incubated without mat samples showed no arsenite oxidation whether live or formaldehyde killed, whereas mat samples taken from site 4 (just before the brown mat) showed arsenite oxidation rates of $0.023\ \text{min}^{-1}$ for live samples and no arsenite oxidation for formaldehyde-killed controls. Live mat samples taken from the green mat (site 8) showed arsenite oxidation rates similar to those at site 4 ($k = 0.028\ \text{min}^{-1}$), suggesting that the highest rates of arsenite oxidation were occurring in the brown mat (Langner *et al.*, 2001). As the focus of this study was to initiate characterization of the arsenite oxidizers present in this spring, subsequent studies pertained mainly to comparing the microbial communities present in these initial spring zones (sites 3 and 6) that contrasted in arsenite oxidation.

Table 1. Characteristics of sampling sites examined in the present study.

Site	Distance (m) from source	Temperature (°C)	pH	As_V/As_T	Visual description
1	0.0	62.1	3.18	0.05	Spring source
2	1.7	60.3	3.16	0.07	Yellow mat
3	2.7	59.2	3.15	0.06	End of yellow mat
4	3.7	58.3	3.14	0.09	No visible mat
5	4.5	56.9	3.12	0.15	Start of brown mat
6	5.0	52.8	3.12	0.25	Middle of brown mat
7	5.3	51.0	3.11	0.38	End of brown mat
8	5.6	47.3	3.07	0.40	Green mat
9	5.9	43.6	3.02	0.43	End of green mat

As_V/As_T is the proportion of total arsenic as arsenate (As_V) (sampling date:16 November 1999; data are from an average of two separate determinations). Total arsenic was ≈ 33 μM at each site.

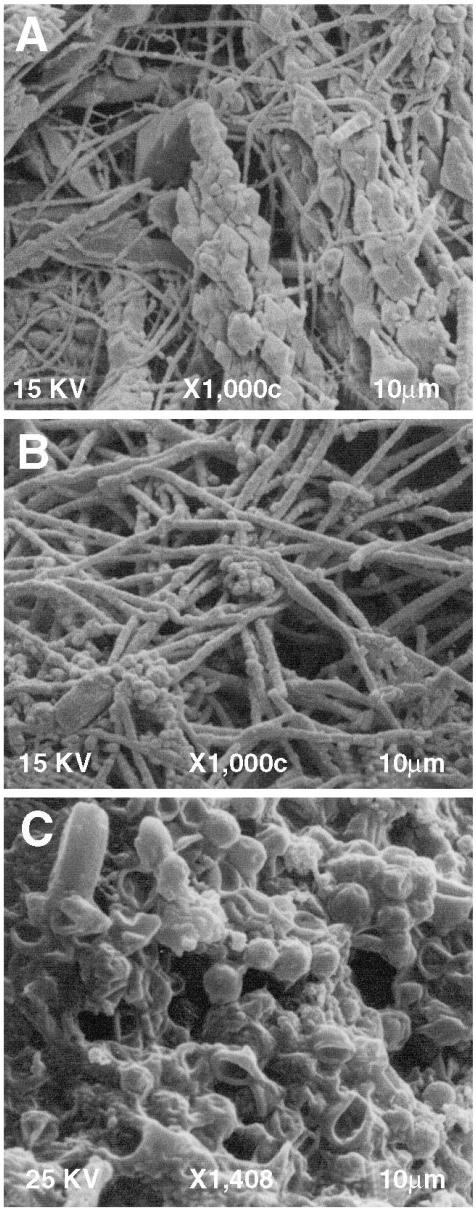

Fig. 1. Scanning electron micrographs of the three microbial communities observed in this acidic hot spring: (A) yellow filamentous (site 3); (B) brown (site 6); and (C) green (site 8).

Electron microscopy

Scanning electron microscopy (SEM) was used to examine the ultrastructure of the communities present at different locations within the spring. Visual assessment suggested three dominant zones of yellow, brown and green mats. SEM of representatives of these zones (sites 3, 6 and 8) showed that they also differed based on cell morphology and crystalline deposits associated with the cells (Fig. 1). Mat communities at sites 3 and 6 were similar, in that filamentous growth was the predominant cellular morphology (Fig. 1A and B), although they differed significantly in the chemical precipitates associated with the mat. Elemental S was the predominant solid phase within site 3 (Langner *et al*., 2001), which is consistent with the yellow colour, whereas an arsenic-rich, Fe oxyhydroxide phase dominated site 6 (Langner *et al*., 2001), which is consistent with the brown colour of this zone. Site 8 was green and appeared to be dominated by algal cells, probably *Cyanidium* sp. (personal communication; D. Castenholz, unpublished observations).

Molecular characterization

DNA could be extracted from all sampling sites except site 1, which was located at the spring source. Bacterial 16S rDNA could be amplified from sites 2–9 using bacterial-specific DGGE primers, but archaeal-specific DGGE primers only amplified DNA obtained from sites 5–9. DGGE analysis of these amplification products revealed changes in the microbial communities at each site for both Bacteria (Fig. 2A) and Archaea (Fig. 2B). DGGE banding patterns were relatively simple, with no more than 15 distinguishable bands being present in the amplified DNA from a particular site. DGGE profile complexity tended to increase with distance from the source and concomitant with a reduction in temperature (Table 1). DGGE patterns were reproducible (data not shown).

Based on the DGGE findings, sites 3 and 6 were selected as typical representatives of the yellow and brown mat communities, respectively, and also showed contrasting arsenite oxidation activity. The members of these microbial communities were examined in greater detail by phylogenetic analysis of near full-length 16S rRNA genes. Bacterial 16S rDNA was successfully amplified from both sites but, as with the primers used for DGGE analysis, archaeal 16S rDNA amplification was only successful with DNA from site 6. Spiking of site 3 DNA preparations with *Sulfolobus* DNA yielded a polymerase chain reaction (PCR) product, so it was concluded that the absence of archaeal PCR product was not an artifact. These three PCR products (bacterial 16S rDNA from sites 3 and 6, archaeal 16S rDNA from site 6) were cloned, and at least 90 clones from each PCR product were analysed by restriction fragment length polymorphism (RFLP) analysis. Restriction digests with *Eco*RI, *Rsa*I and *Hae*III defined a number of operational taxonomic units (OTUs) at each site (Fig. 3). The tetrameric enzymes, *Rsa*I and *Hae*III, were most useful in separating bacterial 16S rDNA types, whereas all three enzymes were useful in identifying different archaeal 16S rDNA fragments. With the bacterial primers, 20 and 22 OTUs were observed in the PCR products from the yellow and brown zones respectively. A total of 40 OTUs was observed in the archaeal PCR products from the brown zone.

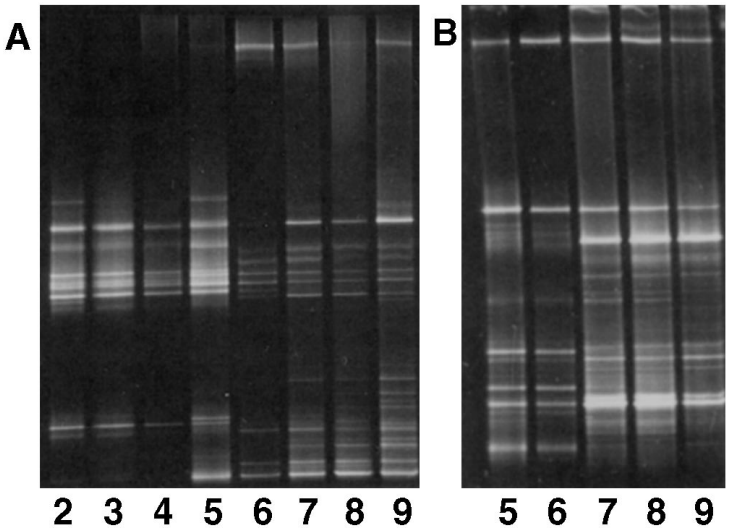

Fig. 2. DGGE analysis of 16S rDNA fragments amplified from the arsenite-oxidizing thermal spring.
A. DGGE profiles obtained using Bacteria-specific primers from DNA obtained from different locations within the spring.
B. DGGE profiles obtained using Archaea-specific primers at the same sites. DNA from sites 2–4 could not be amplified with archaeal primers. Numbers correspond to the sample sites described in Table 1 and increase moving away from the spring source (site 1, at which no DNA was detected).

Sequencing of the 3′ and 5′ ends (total 750–800 bp) of each major RFLP-defined OTU resulted in the pooling together of some OTUs. Many of the OTUs had very similar sequences for these regions, particularly for the bacterial 16S rDNA samples (Table 2). Based on the 97% similarity criterion used in this study, the dominant bacterial OTUs (those represented by two or more clones) derived from both yellow and brown mats contained only two distinct sequence types, represented by clones B5 and B45. The archaeal clones were more diverse, with six major sequence types being recognized: clones A1, A6, A9, A10, A13 and A14 (Table 2). Thus, eight clones (six archaeal and two bacterial) were used for near full-length 16S rDNA sequence analysis.

When analysed using BLAST, the two bacterial 16S rDNA sequences were found to correspond closely with sequences listed in GenBank. The most frequently obtained sequence type (sequences similar to clone B5) accounted for 84% of the bacterial clones obtained from the yellow mat and 79% of the bacterial clones obtained from the brown mat (Table 2, Fig. 3). This sequence type was 97% similar to the sequence listed for *Hydrogenobacter acidophilus* (accession number D16296). The other major bacterial sequence type (sequences similar to clone B45) showed 97% similarity to the four *Desulphurella* species (*D. kamchatkensis*, *D. multipotens*, *D. acetivorans* and *D. propionica*; accession numbers Y16941, Y16943, X72768and Y16942respectively). This sequence type accounted for 3% of bacterial clones obtained from the yellow mat and 10% of bacterial clones obtained from the brown mat. Taxonomic assignment for both sequence types was not altered when they were aligned and their phylogeny determined using ARB (data not shown).

Both RFLP and DGGE analysis suggested a greater diversity of Bacteria at sites 3 and 6 than was obtained from sequence analysis. In an attempt to reconcile this difference, full-length clones from various OTUs were used as PCR templates using the DGGE primers. DGGE analysis of these PCR products showed that OTUs with the same phylogeny could account for separate bands in the site 6 community DGGE profile (Fig. 4). Examination of the sequences of these OTUs in the 1070–1392 region of 16S rDNA used in DGGE identified minor variations that could account for different migration in DGGE gels. Further, sequencing of the individual site 6 community DGGE bands (PCR purified from the DGGE gel) showed that the sequences of each clone matched exactly with that of the similarly migrating band in the community DGGE profile. Thus, we concluded that *H. acidophilus*-like sequences accounted for four of the DGGE bands in the site 6 community profile and *Desulphurella*-like sequences accounted for two. Other bands in the bacterial DGGE profile (particularly those in the lower part of the gel; Fig. 2A) could not be accounted for by sequenced OTUs and may represent bacterial sequences that were not detected by the cloning/sequencing approach. Representatives of these lower bands were also PCR purified and sequenced. Based on $\approx$300 bp, one band showed phylogenetic affiliation to both an uncultured sequence obtained from an acid mine drainage site (clone BA46 from Bond *et al.*, 2000) and *Acidimicrobium ferrooxidans*, an iron-oxidizing thermoacidophile (Clark and Norris, 1996). Two other bands showed affiliation with *Meiothermus* sp., a genus of moderate thermophiles (Nobre *et al.*, 1996). Thus, although our clone libraries were dominated by *H. acidophilus*- and *Desulphurella*-like sequences, other bacterial sequence types may also be present in the spring mat community.

When analysed using BLAST, none of the archaeal 16S rDNA sequences obtained showed $>$95% homology to

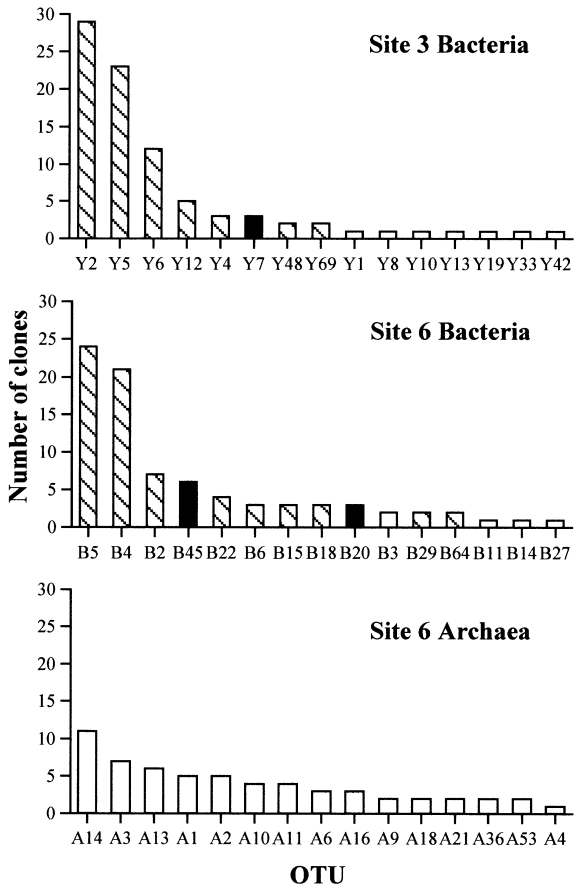

Fig. 3. Frequency plots of different operational taxonomic units (OTUs) as defined by RFLP screening of cloned 16S rDNA sequences. OTUs are identified by the clone library from which they were obtained (Y, bacterial DNA from site 3; B, bacterial DNA from site 6; A, archaeal DNA from site 6), followed by a number designating the first clone in that sample that showed those restriction patterns. Clone libraries contained near full-length 16S rDNA sequences amplified from site 3 bacterial DNA, site 6 bacterial DNA and site 6 archaeal DNA. A total of 20, 22 and 40 OTUs were obtained from each clone library, respectively, but, to simplify presentation, only the 15 most numerous OTUs are shown for each sample. For bacterial DNA libraries, hatched bars represent OTUs containing *Hydrogenobacter*-like sequences, and shaded bars represent OTUs containing *Desulphurella*-like sequences.

sequences listed in GenBank, and the mean similarity to any previously reported sequence was 89%. Phylogenetic analysis revealed that five of the six sequences grouped with 16S rDNA sequences in the *Crenarchaeota*, *and* one sequence (clone A10) grouped with the *Thermoplasmales* in the *Euryarchaeota*. Although nearest-neighbour, maximum-parsimony and maximum-likelihood methods yielded slightly different trees, similar phylogenies for both our sequences and major archaeal lineages were obtained using all three methods, so that only the maximum-likelihood analysis is reported (Fig. 5).

Table 2. Grouping of RFLP-defined OTUs by 16S rDNA sequence similarity.

Sequenced clone	RFLP-defined OTUs with similar 16S rDNA sequences	Percentage similarity
Clone A1	A1, A18	98.5
Clone A6	A6	–
Clone A9	A9	–
Clone A10	A2, A10, A11	97.8
Clone A13	A13, A16	99.9
Clone A14	A14, A3	99.4
Clone B5	Y2, Y5, Y6, Y12, Y4, Y48, Y69, B5, B4, B2, B22, B6,B15, B18, B29, B64	98.2
Clone B45	Y7, B45, B20	98.3

Sequence similarity is for regions corresponding to ≈ 8–450 and 1040–1490 according to the *Escherichia coli* 16S rRNA gene numbering system and represents the sequence similarity between the two least similar OTUs within a particular sequence type.

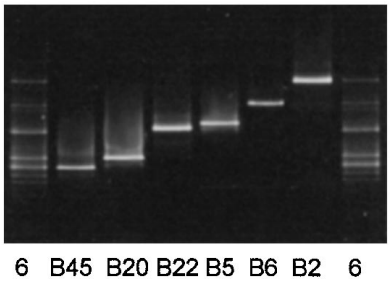

Fig. 4. DGGE analysis of 16S rDNA fragments amplified from RFLP-defined OTUs obtained from site 6 that correspond to DGGE bands in the whole bacterial community DNA obtained from this site. 16S rRNA gene sequence analysis showed B45 and B20 to be very similar to *Desulphurella* sp., and B22, B5, B6 and B2 to be very similar to *Hydrogenobacter acidophilus*. Only the portion of the site 6 profile (end lanes) that compares with the isolated OTUs is shown.

Discussion

Moderate to high levels of arsenic are common in geothermal waters, although no definitive work has been reported that characterizes the microbial communities associated with As transformations in such environments. Further, although significant progress has been made towards understanding the biochemistry and physiology of arsenate reduction in other environmental systems (both detoxification and arsenate respiration), very little is known about biological arsenite oxidation. The primary goal of this study was to determine which microorganisms inhabit an arsenite-oxidizing thermal spring as an initial step towards identifying those organisms involved in arsenite oxidation. Visual assessments and chemical measurements showed that arsenite oxidation coincided with a transition from the yellow to brown microbial communities with rapid net rates of arsenite oxidation associated with the brown mat. SEM suggested that the yellow and brown zones were composed of filamentous microbial communities (Fig. 1), but associated with

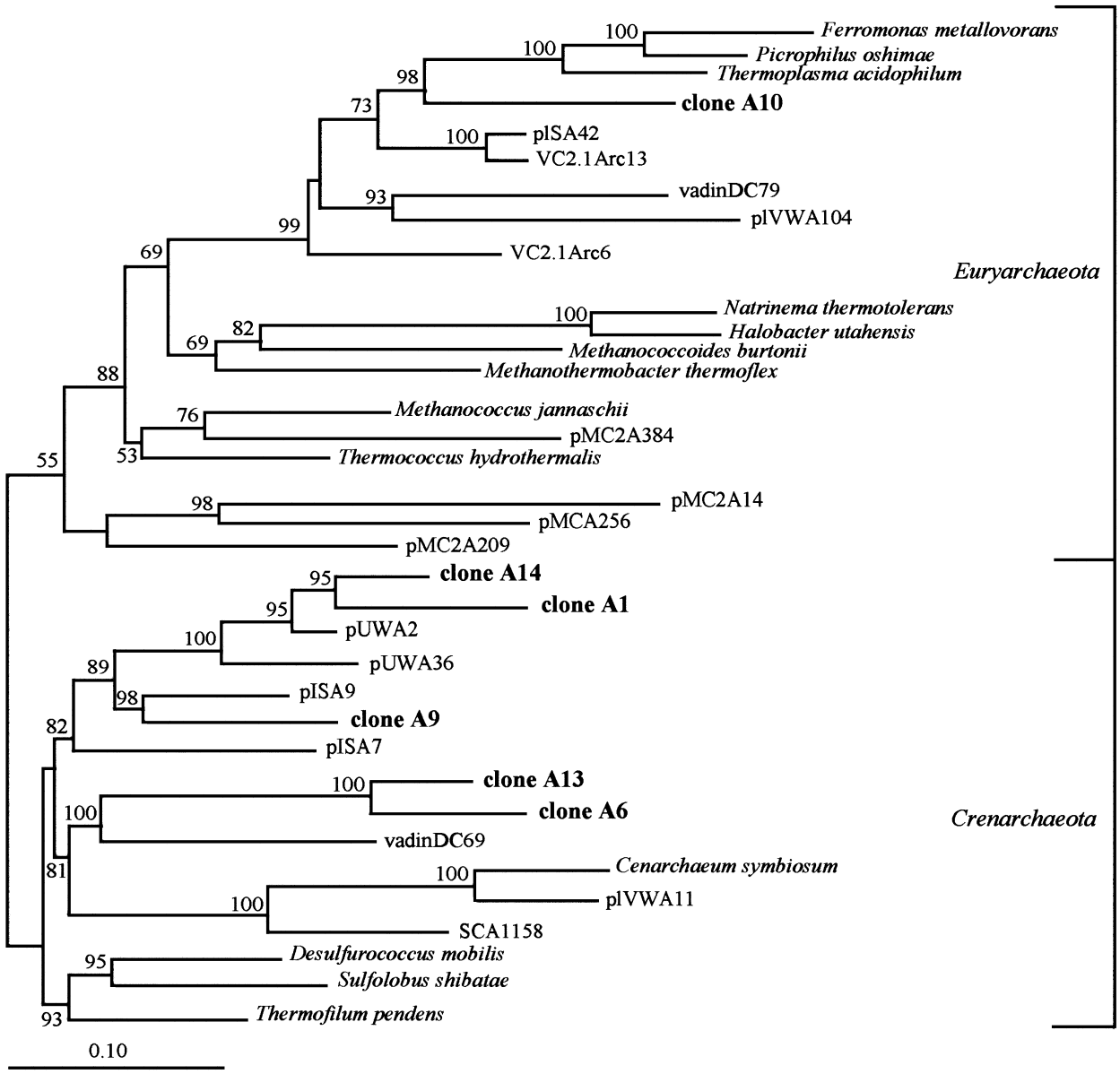

Fig. 5. Phylogenetic tree for the archaeal 16S rDNA sequences obtained from hot spring microbial mats in this study. The tree was obtained using maximum-likelihood analysis with *Aquifex pyrophilus* as the outgroup. Only sequences obtained in this study (bold type), nearest relatives and major archaeal lineages are shown. Bootstrap values are based on 100 runs and are only shown where > 50. Similar trees were obtained using neighbour-joining and maximum-parsimony analyses.

different chemical precipitates (Langner *et al.*, 2001). DGGE of PCR-amplified bacterial and archaeal 16S rDNA fragments also revealed differences in these locations. Changes in both bacterial and archaeal DGGE profiles between sites 3 and 6 (Fig. 2) appeared to coincide with the onset of arsenite oxidation and suggested that the identity of the arsenite oxidizers could potentially be inferred from differences in DGGE banding patterns. Cloning and sequencing of full-length bacterial 16S rDNA sequences suggested that the yellow and brown mat communities were very similar in terms of the bacterial community, with clone libraries obtained from both regions being dominated by sequences similar to those of *H. acidophilus* and *Desulphurella* sp. In contrast, Archaea were apparently absent from site 3, whereas several novel archaeal sequences were cloned from site 6.

RFLP-defined OTUs (or phylotypes) have been used in previous measurements of microbial diversity (Nüsslein and Tiedje, 1998; 1999), and it has been suggested that RFLP analysis [or amplified rDNA restriction analysis (ARDRA)] has less taxonomic resolution than sequencing

and phylogenetic analysis (Nüsslein and Tiedje, 1999). In this study, RFLP analysis of full-length 16S rDNA clones was used to define OTUs in order to focus the sequencing effort. OTU groupings suggested significant bacterial diversity (Fig. 3) and were in general agreement with diversity suggested by DGGE profiles (Fig. 2). However, many of the OTUs proved to have near-identical sequences, at least for the partial (total 750 bp for both ends of the molecule) sequences examined in the initial screening, suggesting that the number of RFLP-defined OTUs may not be an accurate reflection of phylogenetic diversity in this system.

Additional studies helped to clarify the apparent discrepancy between OTU/DGGE bacterial diversity estimates and the results from 16S rDNA sequence analysis. Near full-length 16S rDNA clones representative of different OTUs were used as templates for DGGE analysis and compared with the original bacterial community profile from site 6 (Fig. 4). Six distinguishable bands in the site 6 profile could be accounted for by *H. acidophilus* and *Desulphurella* sp. related sequences, based on migration in the DGGE gel and sequence, confirming that OTUs showing the same phylogeny had minor sequence variations that could be detected in DGGE gels. Some of the apparent diversity suggested by DGGE probably represented minor variation in these sequence types rather than entirely novel taxa. Thus, like the RFLP-defined OTU approach, the number of bands appearing in DGGE gels would appear to be an overestimate of species diversity in this system. However, not all bands in DGGE profiles could be accounted for by sequenced OTUs from our clone libraries, and the additional bands may represent sequences that were not detected in our cloning and sequencing approach.

The two bacterial taxa identified have physiological and ecological traits consistent with this hydrothermal system. *H. acidophilus* has been isolated previously from acidic mud samples taken from solfataras in Japan (Shima and Suzuki, 1993) and shows growth at pH 3.0–4.0 and temperatures of 65°C, the prevailing conditions in the first few metres of this spring. In addition to oxidizing hydrogen for its obligately chemolithoautotrophic lifestyle, *H. acidophilus* also requires elemental sulphur or thiosulphate (Shima and Suzuki, 1993). The presence of elemental sulphur (rhomboid crystals) at site 3 was shown by SEM (Fig. 1A), confirmed by SEM-EDX analysis (Langner *et al.*, 2001) and is consistent with the physiology of *H. acidophilus*. The presence of elemental sulphur is also consistent with the presence of *Desulphurella* sp., as judged from the physiology of all four previously characterized *Desulphurella* species: *D. acetivorans* (Bonch-Osmolovskaya *et al.*, 1990), *D. multipotens* (Miroshnichenko *et al.*, 1994), *D. kamchatkensis* and *D. propionica* (Miroshnichenko *et al.*, 1998). However, the 16S rDNA sequence similarity of these four species is $>99\%$ (Miroshnichenko *et al.*, 1998), whereas the cloned sequence obtained in this study was only 97% similar to these sequences. In addition, although all four *Desulphurella* sp. show optimum growth at temperatures found in this system (55–60°C) and are obligate anaerobic sulphur reducers, none of the four characterized species is reported to be capable of growth at pH values <4.5. Thus, based on phylogeny and environmentally inferred physiology, it is likely that the *Desulphurella*-like organisms present in this acidic spring may represent either a new acidophilic species of *Desulphurella* or a closely related acidophilic taxa. In any case, a metabolism based on anaerobic sulphur reduction is consistent with environmental conditions in this system.

Both DGGE and a cloning–sequencing approach failed to show the presence of Archaea in the initial part of the spring. Although this does not prove their absence, it seems unlikely that two different sets of primers would consistently fail to amplify Archaea in some samples while amplifying those in others. Furthermore, when the yellow mat sample (site 3) was spiked with archaeal DNA (from *Sulfolobus* sp.), we were able to amplify this spiked DNA with both sets of primers (data not shown). Therefore, it is reasonable to conclude cautiously that the first few metres of the spring were not colonized by Archaea during the course of this study.

The six archaeal 16S rDNA sequences obtained from site 6 appeared to be novel sequences that had not been reported previously for either isolates or environmental clones. Five appeared to be members of the kingdom *Crenarchaeota*, and one (clone A10) grouped with the order *Thermoplasmales* in the kingdom *Euryarchaeota*. Others have cloned 16S rDNA sequences from marine hydrothermal vents that group with the *Thermoplasmales*, including the sequences pISA42 and pIVWA104 (Takai and Horikoshi, 1999) and VC2.1Arc13 and VC2.1Arc6 (Reysenbach *et al.* 2000) shown in Fig. 5. The known members of the *Thermoplasmales* are all acidophilic, and the presence of a sequence belonging to a thermoacidophile is consistent with this system. Although little is known of the biogeochemistry associated with many Archaea, some taxa within the *Thermoplasmales* are capable of iron oxidation (Edwards *et al.*, 2000), although any role these organisms may have in arsenic cycling is unknown.

Three of the Crenarchaeotal sequences (clones A1, A9 and A14) grouped with sequences obtained from marine hydrothermal vents (e.g. pISA9; Takai and Horikoshi, 1999) and an acidic hot spring (e.g. pUWA2 and pUWA36; Takai and Sako, 1999). These three clones appear to be members of the terrestrial hot spring Crenarchaeotic group 1 suggested by Takai and Horikoshi (1999), and our phylogenetic analysis supports

the existence of this group. The other sequences within this group were obtained from much hotter environments (90–100°C) than the spring in this study (53°C), so that the data acquired in the present study suggest that members of this group may also be found under less extreme thermal conditions. The remaining archaeal sequences obtained from this spring (clones A6 and A13) grouped with each other, but were $>10\%$ dissimilar to previously reported archaeal sequences. Along with a sequence obtained from biofilms in an anaerobic fluidized-bed digester (vadinDC69; Godon *et al*., 1997), they formed a fairly deep lineage within the *Crenarchaeota*.

The oxidation of arsenite in this thermal spring did not occur in formaldehyde-killed controls, providing evidence that the rapid oxidation rates are probably linked to one (or more) of the microbial populations identified above. It is possible that the microbes were only involved indirectly by producing oxidants capable of rapid arsenite oxidation (Cherry *et al*., 1979; Cullen and Reimer, 1989). However, as discussed more fully by Langner *et al.* (2001), our data show that abiotic oxidation rates caused by either O_2 or Fe(III) could not account for the rapid oxidation observed *in situ* or in *ex situ* reaction vessels. For example, solid phase Fe(III) hydroxides, which were observed in the mats from site 6, were present in all *ex situ* reactions, including the formaldehyde-killed controls in which arsenite oxidation rates were minor relative to unkilled vessels. Furthermore, in experiments in which we compared arsenite oxidation in paired serum bottles containing $FeCl_3$ and As_2O_3 (15 μM each; simulates levels of each in the mat material) and with or without mat inoculum, arsenite oxidation was complete in inoculated samples within 2 days, whereas $<5\%$ was oxidized in abiotic controls, consistent with the results from formaldehyde-killed samples in *ex situ* reaction vessels. The co-occurrence of Archaea and arsenite oxidation may not be coincidental. The majority of archaeal sequences obtained did not group with cultured representatives of the Archaea, so it is impossible to infer any physiological traits. But, given the paucity of information regarding arsenite oxidation in prokaryotes in general, inferring arsenite oxidation activity solely from phylogeny would be difficult at this time. The phylogenetic information obtained in this study was, however, used to design enrichment methods for the isolation of arsenite-oxidizing micro-organisms from this system. Initial enrichment experiments suggest that arsenite oxidation only occurs in microaerobic conditions under an 85% H_2, 10% CO_2, 5% air atmosphere (unpublished observations). Molecular analyses of these enrichments suggests the presence of *H. acidophilus* and *Desulphurella* sp. and the absence of Archaea (unpublished observations), implying that *H. acidophilus* and/or *Desulphurella* sp. are involved in arsenite oxidation in this system. However, the absence of arsenite oxidation in the early part of the spring (yellow mat community, sites 2–4) that is also colonized by *H. acidophilus* and *Desulphurella* sp. suggests that this activity may be inhibited by the prevailing chemical conditions in this part of the spring. The most noticeable chemical difference between sites 3 and 6 is the disappearance of aqueous sulphide and the accumulation of elemental sulphur in the spring sediment (Langner *et al*., 2001). We are currently focusing on studies to understand the relationships between microbial growth, sulphide chemistry and arsenic biogeochemistry in this thermal environment.

Experimental procedures

Site description, sampling and determination of arsenite oxidation rates

Samples were obtained from a small hot spring (NHSP106 in the YNP thermal inventory, co-ordinates 44°43′54.8″N 110°42′39.9″W) located in Norris Geyser Basin, Yellowstone National Park, WY, USA. The spring is shallow (1–2 cm deep) and narrow (0.5 m across) and is fed by source water of ≈63°C and pH 3.1. Total arsenic concentration is relatively constant along the region of the channel studied and is ≈33 μM (Langner *et al*., 2001). A transect of nine sampling sites was established from the spring source to ≈6 m downstream. The proportion of arsenic as arsenate changed dramatically at different locations along the transect, beginning to increase at ≈4.5 m downstream, and coincided with a visual change in the microbial mats from yellow (sites 2–4) to brown (sites 5–7). A summary of the physical and chemical characteristics of each sampling site is provided in Tables 1, and a more thorough description of the site chemistry is provided by Langner *et al.* (2001).

In order to verify that arsenite oxidation was associated with the brown mat, cores (2.2 cm diameter, 0.5 cm thickness) were taken from the surface material at site 6 and transferred to polyethylene bottles. Killed controls received formaldehyde (4% w/v), and both live and killed samples were immediately covered with 30 ml of spring water taken from site 5. Sample bottles were incubated for 9 min in a neighbouring spring at a similar temperature. Changes in arsenic speciation were determined by taking 1 ml samples after 0, 1, 3 and 9 min and filtering 0.2 ml into each of two sample bottles (one for the determination of total arsenic and one for arsenate) containing 5 ml of distilled water. Arsenate bottles received 1 ml of 2 M Tris buffer (pH 6), and both bottles were sparged with nitrogen gas. While sparging, samples received 1 ml of 0.8 M $NaBH_4$ solution in 0.25 M NaOH, added in 0.2 ml increments over 4 min to reduce arsenite to arsine gas. The samples were sparged for a further 3 min to drive off all arsine and preserved in 0.1 M HCl. Upon return to the laboratory, arsenic concentrations in arsenate and total arsenic samples were determined by hydride generation atomic absorption spectrometry (Jones *et al*., 2000), and rates of arsenite oxidation were determined. Similar rates of arsenite oxidation were determined for samples taken from before the brown mat (site 4) and the

green mat (site 8) and for spring water without a mat sample present (Langner *et al.*, 2001).

Sediment samples for molecular analyses were collected from each sample point by aseptically scooping mat material directly into a 2 ml microcentrifuge tube and flash frozen on site using a dry ice–ethanol mixture. Samples were maintained at −80°C before DNA extraction. Additional samples from sites located in the three dominant mat types (site 3, yellow; site 6, brown; site 8, green) were taken for SEM and processed and analysed using standard methods (Goldstein *et al.*, 1992). These samples were examined using a JSM-6160 scanning electron microscope (Jeol USA) at the Image and Chemical Analysis Laboratory at Montana State University.

Nucleic acid extraction

Nucleic acids were extracted from each sample using three cycles of freeze–thaw, followed by the high-salt, sodium dodecyl sulphate (SDS)-based, extended heating method of Zhou *et al.* (1996). The solution obtained was extracted with chloroform, precipitated in isopropanol and resuspended in Tris–EDTA (TE) buffer. After visual inspection in agarose gels, the DNA was purified using Sepharose 4B columns as described previously (Jackson *et al.*, 1997).

Partial 16S rDNA amplification and DGGE analysis

DGGE was used to obtain an initial description of the prokaryotic community present at each site. A region of the 16S rDNA in each sample was PCR amplified using two sets of primers, one specific for domain *Bacteria*, the other specific for domain *Archaea*. The bacterial primers were the Bac1070f (5′-ATGGCTGTCGTCAGCT-3′) and universal Univ1392r (5′-ACGGGCGGTGTGT-AC-3′) primers described by Ferris *et al.* (1996), which amplify a 323 bp section of the 16S rRNA gene. The Univ1392r primer was attached to a 40 bp GC clamp to facilitate analysis with DGGE; these primers have been used previously to examine bacterial communities present in hot springs (Ferris *et al.*, 1996; 1997). Final reaction mixtures contained 0.4 μM each primer, 0.2 mM each deoxyribonucleotide triphosphate, 2.5 mM $MgCl_2$, 1.0 U of *Taq* polymerase (Promega) and a buffer composed of 10 mM Tris-HCl, 50 mM KCl and 0.1% Triton X-100. Reactions were performed in 50 μl tubes in a GeneAmp PCR System 9700 (PE Applied Biosystems), using the following programme: 2 min at 95°C, followed by 26 cycles of 95°C (45 s), 43°C (45 s), 72°C (45 s) and a final 7 min at 72°C. The other primer set consisted of Arc931f (5′-AGGAATTGGCGGGGGAGCA-3′), derived from the taxon-specific probes described by Amann *et al.* (1995), and the Univ1392r primer with the GC clamp. Together, these primers amplify a 461 bp section of the 16S rRNA gene of members of the domain *Archaea*. Amplification conditions were as for the bacterial primers, except for the annealing temperature, which was 56°C. Negative control amplifications (no template) were performed routinely.

Amplification products were viewed in agarose gels and analysed using DGGE. The amplified products were electrophoresed through a 40–70% urea–formamide gradient in 8% acrylamide gels at 60 V and 60°C for 16 h. Separate gels were run for the bacterial and archaeal PCR products; both sets of amplification products separated well under these conditions. After electrophoresis, gels were stained in SYBR green (Bio-Rad), and the banding patterns were examined under ultraviolet light.

Full-length 16S rDNA amplification and cloning

Based upon the different banding patterns observed in DGGE gels and the patterns of arsenite oxidation observed in the hot spring, two locations, sites 3 and 6, were selected for a more thorough characterization of the microbial community. DNA from each of these sites was amplified using primer sets that amplify almost the entire 16S rRNA gene. The bacterial primer set consisted of the Bacteria-specific primer Bac8f (5′-AGAGTTTGATCCTGGCTCAG-3′) and the universal primer Univ1492r (5′-GGTTACCTTGT TACGACTT-3′). The archaeal primers were the Archaea-specific primer Arc2f (5′-TTCCGGTTGATCCYGCCGGA-3′) and Univ1492r. All primers were designed based on the probes described by Amann *et al.* (1995). PCR concentrations were as for the DGGE primer sets, and amplification was performed using the programme: 2 min at 95°C, followed by 30 cycles of 95°C (1 min), 45°C (1 min), 72°C (3 min) and a final 7 min at 72°C. The same conditions were suitable for both Archaea- and Bacteria-specific primers.

PCR products obtained from each reaction were visualized on agarose gels to confirm that the correct sized band was present, and this band was cut from the gel and purified using UltraFree-DA gel extraction kits (Millipore). The purified PCR fragments were cloned using a TOPO TA cloning kit (Invitrogen). Approximately 90 positive clones from each reaction were transferred to a clone library for further analysis.

RFLP analysis and sequencing

The inserts from the selected clones were amplified using the M13f and M13r primers provided by the manufacturer (Invitrogen). These amplify the entire cloned PCR product plus an ≈250 bp region on either side of the insert. The same amplification conditions used for the full-length 16S rDNA amplification were suitable, and the template was added by touching each clone with a sterile toothpick and then touching this toothpick into the reaction mixture. The PCR products (length ≈1900 bp) were subjected to separate restriction enzyme digests for 4 h at 37°C, with the enzymes *Eco*RI, *Rsa*I and *Hae*III. After the digest, the products were electrophoresed in 2.2% agarose gels for 2 h at 110 V. Clones from each 16S rDNA sample (as defined by site and taxon) that showed the same restriction patterns with all three enzymes were grouped as discrete operational taxonomic units (OTUs).

In order to focus our sequencing effort on the dominant members of the prokaryote community, only those OTUs that were represented by two or more clones (i.e. at least 2% of the total amplified 16S rDNA) were chosen for sequencing. DNA was amplified from each representative clone using the M13 primers described above, and the product was purified

using Microcon PCR centrifugal filter devices (Millipore). The ends of the 16S rDNA insert were sequenced using the Univ1492r and Bac2f or Arc8f (depending upon whether the clone was from an archaeal or bacterial sample) primers, using an ABI Prism 310 genetic analyser (Applied Biosystems). These partial sequences (containing a total of $\approx$ 750 bp of usable information) were used in a second screening step to determine whether any of the OTUs defined by RFLP analysis were essentially the same or very similar sequences. We chose 97% similarity as a criterion for refined grouping within an OTU as, at sequence homology below this value, it is unlikely that organisms are related at the species level (Stackebrandt and Goebel, 1994). Thus, if the partial sequences for different RFLP-defined OTUs differed by $<$ 3%, they were assumed to be sufficiently similar that full-length sequence analysis for each was not merited, and sequence microheterogeneity may have arisen from allelic variation within a population or potential PCR and/or sequencing error (Barns *et al*., 1994). In addition, the partial sequence data for each OTU was checked against sequences in GenBank (Benson *et al*., 2000) using BLAST (Altschul *et al*., 1990) to confirm that, whenever two or more OTUs were grouped together, all representatives of this group matched the same GenBank sequences (i.e. to ensure that the 97% similarity criterion only grouped OTU sequences together if they were more similar to each other than to separate GenBank entries). Sequencing and BLAST searching each end of the molecule also allowed for screening of chimeric sequences.

For clones that were sufficiently different, the entire insert was sequenced using the primers Bac8f, Bac338f (5′-ACTCCTACGGGAGGCAGC-3′), Univ522f (5′-CAGCMGCCGCGG-TAAT-3′, where M is A or C), Bac785f (5′-GGATTAGATACCCTGGTAG-3′), Bac1070f, Univ1492r, Bac1086r (5′-AGCTGACGACAGCCAT-3′), Bac804r (5′-CTACCAGGGTATCTAATCC-3′), Univ538r (5′-ATTACCGCGGCKGCTG-3′, where K is G or T) and Bac356r (5′-GCTGCCTCCCGTAGGAGT-3′) for bacterial 16S rDNA clones. For the archaeal clones, Arc2f, Univ522f, Arc931f, Arc1206f (5′-RCACGCGGGCTRCAA-3′, where R is A or G), Univ1492r, Arc1231r (5′-TTGYAGCCCGCGTGY-3′, where Y is C or T), Arc950r (5′-TGCTCCCCCGCCAATTCCT-3′) and Univ522r were used. Sequences were assembled with SEQUENCHER 3.1.1 (Gene Codes Corporation) and checked against sequences in GenBank using BLAST to find the closest relatives. Sequences were subsequently aligned manually, and the phylogeny was determined using neighbour-joining, maximum-parsimony (PHYLIP) and maximum-likelihood (FASTDNAML) methods contained within the ARB software package (distributed by W. Ludwig and O. Strunk, Technical University of Munich, Germany), using sequences included in version 8.0 of the Ribosomal Database Project (Maidak *et al*., 2000). *Aquifex pyrophilus* was used as the outgroup in each analysis, and 100 samplings were used to generate bootstrap data. Phylogenetic analyses were restricted to nucleotide positions that were unambiguous for all sequences.

Nucleotide sequence accession numbers

The $\approx$ 1400 bp sequences for bacterial clones B5 and B45 and archaeal clones A1, A6, A9, A10, A13 and A14 have been deposited in GenBank under accession numbers AF325179, AF325180, AF325181, AF325182, AF325183, Af325184, AF325185and AF325186 respectively.

Acknowledgements

This work was supported by funding from the US Environmental Protection Agency (R827457-01-0), NASA (project no. NAG5-8807) and the Montana Agricultural Experiment Station (projects 911310 and 914398).

References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990) Basic local alignment search tool. *J Mol Biol* **215**: 403–410.

Amann, R.I., Ludwig, W., and Schleifer, K.-H. (1995) Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol Rev* **59**: 143–169.

Anderson, G., Williams, J., and Hille, R. (1992) The purification and characterization of arsenite oxidase from *Alcaligenes faecalis*, a molybdenum-containing hydroxylase. *J Biol Chem* **267**: 23674–23682.

Barns, S.M., Fundyga, R.E., Jeffries, M.W., and Pace, N.R. (1994) Remarkable archaeal diversity detected in a Yellowstone National Park hot spring environment. *Proc Natl Acad Sci USA* **91**: 1609–1613.

Benson, D.A., Karsch-Mizrachi, I., Lipman, D.J., Ostell, J., Rapp, B.A., and Wheeler, D.L. (2000) GenBank. *Nucleic Acids Res* **28**: 15–18.

Bonch-Osmolovskaya, E.A., Sokolova, T.G., Kostrikina, N.A., and Zavarzin, G.A. (1990) *Desulfurella acetivorans* gen. nov. and sp. nov. – a new thermophilic sulfur-reducing eubacterium. *Arch Microbiol* **153**: 151–155.

Bond, P.L., Smriga, S.P., and Banfield, J.F. (2000) Phylogeny of microorganisms populating a thick, subaerial, predominantly lithotrophic biofilm at an extreme acid mine drainage site. *Appl Environ Microbiol* **66**: 3842–3849.

Caldwell, D.E., Caldwell, S.J., and Laycock, J.P. (1976) *Thermothrix thioparus* gen. and sp. nov., a facultatively anaerobic facultative chemolithotroph living at neutral pH and high temperature. *Can J Microbiol* **22**: 1509–1517.

Cherry, J.A., Shaikh, A.U., Tallman, D.E., and Nicholson, R.V. (1979) Arsenic species as an indicator of redox conditions in groundwater. *J Hydrol* **43**: 373–392.

Clark, D.A., and Norris, P.R. (1996) *Acidimicrobium ferrooxidans* gen. nov., sp. nov. mixed-culture ferrous iron oxidation with *Sulfobacillus* species. *Microbiology* **142**: 785–790.

Cullen, W.R., and Reimer, K.J. (1989) Arsenic speciation in the environment. *Chem Rev* **89**: 713–764.

Edwards, K.J., Bond, P.L., Gihring, T.M., and Banfield, J.F. (2000) An archaeal iron-oxidizing extreme thermophile important in acid mine drainage. *Science* **287**: 1796–1799.

Ferguson, J.F., and Gavis, J. (1972) A review of the arsenic cycle in natural waters. *Water Res* **6**: 1259–1274.

Ferris, M.J., Muyzer, G., and Ward, D.M. (1996) Denaturing gradient gel electrophoresis profiles of 16S rRNA-defined populations inhabiting a hot spring microbial mat community. *Appl Environ Microbiol* **62**: 340–346.

Ferris, M.J., Nold, S.C., Revsbech, N.P., and Ward, D.M. (1997) Population structure and physiological changes within a hot

spring microbial mat community following disturbance. *Appl Environ Microbiol* **63**: 1367–1374.

Godon, J.-J., Zumstein, E., Dabert, P., Habouzit, F., and Moletta, R. (1997) Molecular microbial diversity of an anaerobic digestor as determined by small-subunit rDNA sequence analysis. *Appl Environ Microbiol* **63**: 2802–2813.

Goldstein, J.I., Newbury, D.E., Echlin, P., Joy, D.C., Romig, A.D., Lyman, C.E., *et al.* (1992) *Scanning Electron Microscopy and X-Ray Microanalysis.* New York: Plenum Press.

Hugenholtz, P., Pitulle, C., Hershberger, K.L., and Pace, N.R. (1998) Novel division level bacterial diversity in a Yellowstone hot spring. *J Bacteriol* **180**: 366–376.

Ilyaletdinov, A.N., and Abdrashitova, S.A. (1981) Autotrophic oxidation of arsenic by a culture of *Pseudomonas arsenitoxidans*. *Mikrobiologiya* **50**: 197–204.

Jackson, C.R., Harper, J.P., Willoughby, D., Roden, E.E., and Churchill, P.F. (1997) A simple, efficient method for the separation of humic substances and DNA from environmental samples. *Appl Environ Microbiol* **63**: 4993–4995.

Ji, G., Silver, S., Garber, E.A.E., Ohtake, H., Cervantes, C., and Corbisier, P. (1993) Bacterial molecular genetics and enzymatic transformations of arsenate, arsenite, and chromate. In *Biohydrometallurgical Techniques.* Torma, A.E., Apel, M.L., and Brierly, C.L. (eds). Warrendale, PA: The Minerals, Metals and Materials Society, pp. 529–539.

Jones, C.A., Langner, H.W., Anderson, K., McDermott, T.R., and Inskeep, W.P. (2000) Rates of microbially mediated arsenate reduction and solubilization. *Soil Sci Soc Am J* **64**: 600–608.

Kawasumi, T., Igarashi, Y., Kodama, T., and Monoda, Y. (1984) *Hydrogenobacter thermophilus* gen. nov., sp. nov., an extremely thermophilic, aerobic, hydrogen-oxidizing bacterium. *Int J Syst Bacteriol* **34**: 5–10.

Koch, I., Feldmann, J., Wang, L., Andrews, P., Reimer, K.J., and Cullen, W.R. (1999) Arsenic in the Meager Creek hot springs environment, British Colombia, Canada. *Sci Total Environ* **236**: 101–117.

Langner, H.W., Jackson, C.R., McDermott, T.R., and Inskeep, W.P. (2001) Rapid oxidation of arsenite in a hot spring ecosystem in Yellowstone National Park. *Environ Sci Technol* **35**: 3302–3309.

Maidak, B.L., Cole, J.R., Lilburn, T.G., Parker, C.T., Jr, Saxman, P.R., Stredwick, J.M., *et al.* (2000) The RDP (Ribosomal Database Project) continues. *Nucleic Acids Res* **28**: 173–174.

Miroshnichenko, M.L., Gongadze, G.A., Lysenko, A.M., and Bonch-Osmolovskaya, E.A. (1994) *Desulfurella multipotens* sp. nov., a new sulfur-respiring thermophilic eubacterium from Raoul Island (Kermadec archipelago, New Zealand). *Arch Microbiol* **161**: 88–93.

Miroshnichenko, M.L., Rainey, F.A., Hippe, H., Chernyh, N.A., Kostrikina, N.A., and Bonch-Osmolovskaya, E.A. (1998) *Desulfurella kamchatkensis* sp. nov. and *Desulfurella propionica* sp. nov., new sulfur-respiring thermophilic bacteria from Kamchatka thermal environments. *Int J Syst Bacteriol* **48**: 475–479.

Newman, D.K., Ahmann, D., and Morel, F.M.M. (1998) A brief review of dissimilatory arsenate reduction. *Geomicrobiol J* **15**: 255–268.

Nicholson, K. (1993) *Geothermal Fluids: Chemistry and Exploration Techniques.* Berlin: Springer Verlag.

Nobre, M.F., Trüüper, H.G., da Costa, M.S. (1996) Transfer of *Thermus ruber* (Loginova *et al.* 1984), *Thermus silvanus* (Tenreiro et al. 1995), and *Thermus chliarophilus* (Tenreiro et al. 1995) to *Meiothermus* gen. nov. as *Meiothermus ruber* comb. nov., *Meiothermus silvanus* comb. nov. and *Meiothermus chliarophilus* comb. nov., respectively, and emendation of the genus *Thermus*. *Int J Syst Bacteriol* **46**: 604–606.

Nüsslein, K., and Tiedje, J.M. (1998) Characterization of the dominant and rare members of a young Hawaiian soil bacterial community with small-subunit ribosomal DNA amplified from DNA fractionated on the basis of its guanine and cytosine composition. *Appl Environ Microbiol* **64**: 1283–1289.

Nüsslein, K., and Tiedje, J.M. (1999) Soil bacterial community shift correlated with change from forest to pasture vegetation in a tropical soil. *Appl Environ Microbiol* **65**: 3622–3626.

Pace, N.R. (1997) A molecular view of microbial diversity and the biosphere. *Science* **276**: 734–740.

Pierson, B.K., Parenteau, M.N., and Griffin, B.M. (1999) Phototrophs in high-iron-concentration microbial mats: physiological ecology of phototrophs in an iron-depositing hot spring. *Appl Environ Microbiol* **65**: 5474–5483.

Reysenbach, A.-L., Longnecker, K., and Kirshtein, J. (2000) Novel bacterial and archaeal lineages from an in situ growth chamber deployed at a mid-Atlantic ridge hydrothermal vent. *Appl Environ Microbiol* **66**: 3798–3806.

Santini, J.M., Sly, L.I., Schnagl, R.D., and Macy, J.M. (2000) A new chemolithoautotrophic arsenite-oxidizing bacterium isolated from a gold mine: phylogenetic, physiological, and preliminary biochemical studies. *Appl Environ Microbiol* **66**: 92–97.

Shima, S., and Suzuki, K.-I. (1993) *Hydrogenobacter acidophilus* sp. nov., a thermoacidophilic, aerobic, hydrogen-oxidizing bacterium requiring elemental sulfur for growth. *Int J Syst Bacteriol* **43**: 703–708.

Stackebrandt, E., and Goebel, B.M. (1994) Taxonomic note: a place for DNA–DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**: 846–849.

Stauffer, R.E., and Thompson, J.M. (1984) Arsenic and antimony in geothermal waters of Yellowstone National Park, Wyoming, USA. *Geochim Cosmochim Acta* **48**: 2547–2561.

Takai, K., and Horikoshi, K. (1999) Genetic diversity of Archaea in deep-sea hydrothermal vent environments. *Genetics* **152**: 1285–1297.

Takai, K., and Sako, Y. (1999) A molecular view of archaeal diversity in marine and terrestrial hot water environments. *FEMS Microbiol Ecol* **28**: 177–188.

Tanaka, T. (1990) Arsenic in the natural environment. Part II: arsenic concentrations in thermal waters from Japan. *Appl Organomet Chem* **4**: 197–203.

Ward, D.M., Weller, R., and Bateson, M.M. (1990) 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. *Nature* **345**: 63–65.

Wilkie, J.A., and Hering, J.G. (1998) Rapid oxidation of geothermal arsenic(III) in streamwaters of the Eastern Sierra Nevada. *Environ Sci Technol* **32**: 657–662.

Zhou, J., Bruns, M.A., and Tiedje, J.M. (1996) DNA recovery from soils of diverse compositions. *Appl Environ Microbiol* **62**: 316–322.