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Archaeal and bacterial communities in geochemically diverse hot springs of Yellowstone National Park, USA

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ABSTRACT

Microbiological and geochemical surveys were conducted at three hot springs (Obsidian Pool, Sylvan Spring, and 'Bison Pool') in Yellowstone National Park (Wyoming, USA). Microbial community structure was investigated by polymerase chain reaction (PCR) amplification of 16S rRNA gene sequences from DNA extracted from sediments of each hot spring, followed by molecular cloning. Both bacterial and archaeal DNA was retrieved from all samples. No Euryarchaea were found, but diverse Crenarchaea exist in all three pools, particularly affiliating with deep-branching, but uncultivated organisms. In addition, cloned DNA affiliating with the Desulphurococcales and Thermoproteales was identified, but the distribution of taxa differs in each hot spring. The bacterial community at all three locations is dominated by members of the Aquificales and Thermodesulfobacteriales, indicating that the 'knallgas' reaction (aerobic hydrogen oxidation) may be a central metabolism in these ecosystems. To provide geochemical context for the microbial community structures, energy-yields for a number of chemolithoautotrophic reactions are provided for >80 sampling sites in Yellowstone, including Obsidian Pool, Sylvan Spring, and 'Bison Pool'. This energy profile shows that the knallgas reaction is just one of many exergonic reactions in the Yellowstone hot springs, that energy-yields for certain reactions can vary substantially from one site to the next, and that few of the demonstrated exergonic reactions are known to support microbial metabolism.

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INTRODUCTION

Yellowstone's diverse geochemical environments host a variety of chemotrophic and phototrophic thermophilic communities (Brock, 1978; Barns *et al.*, 1994, 1996; Hugenholtz *et al.*, 1998; Pierson *et al.*, 1999; Langner & Inskeep, 2000; Reysenbach *et al.*, 2000; Jackson *et al.*, 2001; Johnson *et al.*, 2001; Langner *et al.*, 2001; Blank *et al.*, 2002; Boomer *et al.*, 2002; Fouke *et al.*, 2003; Papke *et al.*, 2003; Inskeep *et al.*, 2004; Jahnke *et al.*, 2004; Macur *et al.*, 2004; Spear *et al.*, 2005). Since the early work of Thomas Brock, numerous thermophiles have been isolated from these springs, including members of *Thermus*, *Thermocrinis*, *Sulfolobus*, *Thermomicrobium*, *Thermoanaerobium*, *Thermodesulfovibrio*, *Thermodesulfobacterium*, *Thermoanaerobacter*, *Anaerobranca*, *Thermobacteroides*, *Acidianus*, and *Sulphurococcus* (Brock & Freeze, 1969; Brock *et al.*, 1972; Jackson *et al.*, 1973; Zeikus *et al.*, 1979, 1983; Ben-Bassat & Zeikus, 1981; Wiegel & Ljungdahl, 1981; Henry *et al.*, 1994; Engle *et al.*, 1995). However, many of these organisms appear only infrequently in 16S rRNA libraries, which feature primarily sequences from novel, as yet uncultured, strains (Barns *et al.*, 1994, 1996; Hugenholtz *et al.*, 1998; Reysenbach *et al.*, 2000). As an example, 16S rRNA signatures of 54 novel Bacteria and 36 novel Archaea, including members of the deeply branching Korarchaea, were identified in Obsidian Pool (Mud Volcano area) (Barns *et al.*, 1994, 1996; Hugenholtz *et al.*, 1998). Based on these molecular surveys, this pool appears to be one of the most genetically diverse sites on Earth, yet the vast majority of these organisms have not been cultured.

The environmental factors that most directly influence hot spring microbial diversity remain unresolved. Temperature has perhaps received the most attention, but other constraining factors may include pH, redox disequilibria, trace element

levels, organic matter composition, solar radiation budgets and trophic relationships. It has been argued previously that geochemical energy-yields may be a key determinant of microbial community structure and diversity in thermal environments (Baross, 1995; Amend & Shock, 2001). Accordingly, geochemical energy-yields from dozens of redox reactions were evaluated in deep-sea hydrothermal systems (McCollom & Shock, 1997; Bach & Edwards, 2003), shallow submarine hydrothermal systems (Amend *et al.*, 2003, 2004) and continental hot springs (Shock *et al.*, 2005a,b; Spear *et al.*, 2005). It was shown that *in situ* energy-yields depend much more on chemical composition (such as redox sensitive compounds) than on temperature, and the energy available to thermophiles from any given metabolism can vary substantially from one site to the next. In illustration, in >80 Yellowstone locations investigated by Shock *et al.* (2005a,b), the free energy from ferric iron reduction, represented by

$$\text{goethite} + 0.5H_{2,g} + 2H^+ \rightarrow Fe^{2+} + 2H_2O, \quad (1)$$

(catalysed by the Obsidian Pool bacterium *Geothermobacterium ferrireducens* (Kashefi *et al.*, 2002)) is strongly pH dependent and highly variable [−8 to −55 kJ (mol e^-)$^{-1}$]. In contrast, the knallgas reaction,

$$H_{2,g} + 0.5O_{2,g} \rightarrow H_2O, \quad (2)$$

(mediated by many species of Aquificales found in Yellowstone (Huber *et al.*, 1998; Spear *et al.*, 2005), yields ~100 kJ (mol e^-)$^{-1}$ on average at all locations studied.

To more directly investigate the effects of temperature, pH and other geochemical parameters on thermophile diversity, the microbial community structures at one location in each of three Yellowstone hot springs were determined and interpreted in light of energy-yields. Extensive geochemical analyses were performed over a period of several years at >80 sample locations to provide environmental context, and three individual springs were chosen for a focused microbiological survey. The three springs[1] – Obsidian Pool, Sylvan Spring, and 'Bison Pool' (Fig. 1) – are all ~80 °C, range in pH from 5.5 to 8.1, and differ substantially in their overall geochemical characteristics.

CHARACTERIZATION OF SAMPLING AREAS

Hot spring features, including those investigated in this study, are generally categorized in two classes – vapor-dominated (acid-sulphate) systems and water-dominated (alkaline-chloride and carbonate) systems (Fournier, 1989). At Yellowstone, vapor-dominated systems are fed via fractures by reduced gases (e.g. H_2 and H_2S) from the 3–10 km deep magma body (Fournier & Pitt, 1985; Kennedy *et al.*, 1985; Fournier *et al.*, 1994). Approaching the surface, the sulphides are oxidized in accord with, for example,

$$H_2S + 2O_2 \rightarrow SO_4^{-2} + 2H^+, \quad (3)$$

resulting in acidic fluids of elevated sulphate concentrations capable of considerable chemical weathering, which releases cations into solution (White *et al.*, 1971). Acid-sulphate systems at Yellowstone include the Mud Volcano area (encompassing Obsidian Pool) and Fountain Paint Pots. In contrast, water-dominated systems (alkaline-chloride and carbonate) are characterized by substantial water–rock interaction and subsurface boiling in deep-heated reservoirs, but little interaction with reduced mantle-derived gases (White *et al.*, 1971; Fournier, 1989). Many of the large geyser basins at Yellowstone, including the Lower Geyser Basin (encompassing 'Bison Pool'), host alkaline-chloride and carbonate thermal features. Owing to the differing geochemical consequences of these processes, chloride and sulphate concentrations (as well as pH) can be used to chemically categorize thermal waters (Ellis & Mahon, 1964, 1967; Krauskopf, 1964), an example of which is depicted in Fig. 2.

'Bison Pool'

'Bison Pool' (Fig. 1A) is the unofficial name for a small hot spring in the Sentinel Meadow (Lower Geyser Basin) area

[1] The thermal feature referred to here as 'Sylvan Spring' (Fig. 1C) of the northern Sylvan Springs group (Gibbon Geyser Basin) has been given multiple names in the literature, causing some confusion. Early observations (Allen & Day, 1935) described the region much as it appears today, with extensive chemical weathering, acidic pools, and sulphur depositing fumaroles, but these did not mention the pool shown in Fig. 1C. Researchers from the US Geological Survey (USGS) made note that this pool, the largest in the group, was not obvious on an aerial photograph taken prior to 1959 (J. Ball, pers. comm. September, 2004), indicating that the feature may have developed more recently. Whittlesey (1988) names this feature 'Dante's Inferno' (Whittlesey 1998), placing a different 'Sylvan Spring' ~300 feet to the north. A sketch map in Brock (1978) misidentified Dante's Inferno as Sylvan Spring, and as a result, many refer to the location by this name. This naming is reinforced because the feature is the largest hot spring in the group (thus lending its name to the whole region). We have decided to adhere to the naming convention used by the majority of workers in this field, calling the feature in Fig. 1C 'Sylvan Spring'. The USGS team did not name this feature in their geochemical surveys of the area (Ball *et al.*, 1998, 2001), and this decision should not confuse others when referencing these works. Shock *et al.* (2005b) consistently refer to the feature as 'Sylvan Spring', labelling it as such on detailed maps of the area.

In addition, it should be noted that 'Bison Pool' is an unofficial name for the feature whose GPS coordinates are given in the Methods section. While some researchers refer to this location as Rosette Geyser, an official National Park designation of a hot spring in the area, our efforts to compare older maps and data (Allen & Day, 1935) with our maps and field observations suggest that these features are different. Indeed, there are several dormant and extinct features in the general area, which may be Rosette Geyser. For our purpose, we will leave this matter unresolved, and provide GPS coordinates.

Fig. 1 Photographs of (A) 'Bison Pool' (pH, 8.1), (B) Obsidian Pool (pH, 6.5), and (C) Sylvan Spring (pH, 5.5), with arrows indicating the sampling locations. At the arrow in (B), the pool is ~2 m across.

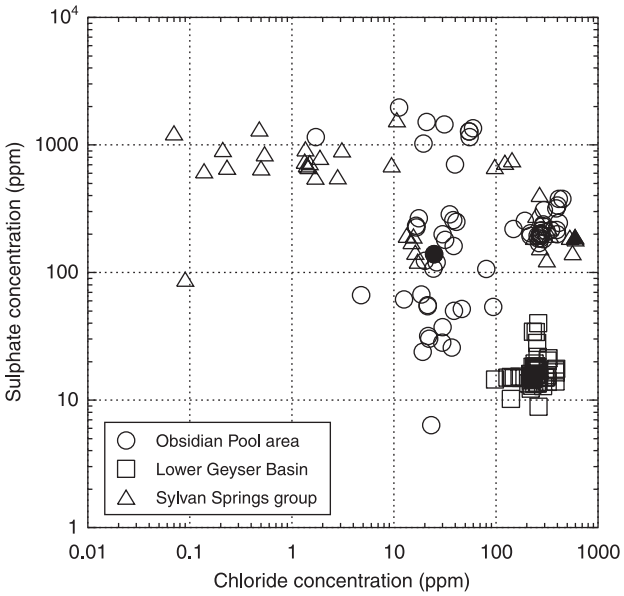

Fig. 2 Sulphate and chloride concentrations (in ppm) measured in 1999–2003 from the Lower Geyser Basin (open squares), the greater Obsidian Pool area (open circles), and the Sylvan Spring area (open triangles). Corresponding filled symbols indicate the three key 2001 locations used in the focused microbiological survey ('Bison Pool', square; Obsidian Pool, circle; Sylvan Spring, triangle).

that features mound-forming siliceous sinter deposits. It can be seen in Fig. 2 that samples from the Lower Geyser Basin (open squares), including 'Bison Pool' (filled square), exhibit high chloride and low sulphate concentrations. In addition, the Sentinel Meadow hot springs are alkaline (pH 7–9.5, where 'Bison Pool' ≈ 8) and generally feature certain elevated trace element concentrations (e.g. B, Al, Rb, W) relative to the other two study areas. These geochemical signatures suggest that the Lower Geyser Basin thermal fluids have undergone substantial water–rock interaction. The sampling location used for the microbiological survey reported here for 'Bison Pool' (arrow, Fig. 1A) was 82.6 °C at a pH of 8.1.

Obsidian Pool

The Mud Volcano region, home of the greater Obsidian Pool area (~1 km^2), has been defined as a vapor-dominated system (White *et al.*, 1971; Zohdy *et al.*, 1973). Gas-delivering faults associated with the Sour Creek resurgent dome intersect the area (Kennedy *et al.*, 1985). However, the greater Obsidian Pool area also contains thermal features characteristic of water-dominated systems (open circles in Fig. 2). With few exceptions, the thermal waters contain intermediate to high levels of chloride (10–500 ppm), but sulphate concentrations range from the lowest (~5 ppm) to the highest (>1000 ppm) values we observed. Our Obsidian Pool sample (arrow, Fig. 1B and filled circle in Fig. 2), 79.9 °C, is characterized by moderate levels of chloride (~20 ppm) and sulphate (~100 ppm) with a near neutral pH of 6.5 (similar to hot water-dominated systems), but elevated cation concentrations (e.g. NH_4^+, Fe^{+2}, Ca^{+2}, Mg^{+2}), characteristic of vapor-dominated systems. This sample can be interpreted as a diluted acid-sulphate fluid.

Sylvan Spring

Like the greater Obsidian Pool area, the Sylvan Springs area (Gibbon Geyser Basin) is characteristic of both vapor-dominated and water-dominated systems. However, Sylvan Spring fluids (open triangles in Fig. 2) are all high in sulphate (>100 ppm), but range in chloride levels from the lowest (<0.1 ppm) to the highest (~600 ppm) measured. Numerous springs in a ravine formed by a NE–SW trending fault that appears to follow an anticlinal fold hinge are highly acidic (pH 1.9–3.2), and the surrounding rock is extensively weathered. Sylvan Spring proper (Fig. 1C and filled triangle in Fig. 2) and other features on the flanks of the ravine exhibit both acid-sulphate and alkaline-chloride signatures. They range in pH from 4.1 to 7.5 (Sylvan Spring ~5.5), and, compared to most acidic features in the area, their sulphate concentrations are lower and their chloride concentrations are higher (Fig. 2). In addition, the Sylvan Spring sample used in the microbiological survey (81 °C) contains ~550 ppm SiO_2(aq), ~300 ppm Na^+, and elevated trace elements (data not shown) typical of an alkaline-chloride system.

METHODS

Sample locations/collection

Sediment and hydrothermal fluid samples for a focused, three-pool survey of geochemistry and microbiology were collected in 2001 from Obsidian Pool (Mud Volcano Area), Sylvan Spring (North Sylvan group, Gibbon Geyser Basin), and 'Bison Pool' (an unnamed feature in the Sentinel Meadow, Lower Geyser Basin). Specifically, samples were taken at one location in each pool (arrows, Fig. 1) at the following coordinates: Obsidian Pool (44°36′10.9″N, 110°51′54.7″E), Sylvan Spring (44°41′55.9″N, 110°46′5.8″E), 'Bison Pool' (44°34′10.9″N, 110°51′54.7″E). Samples for DNA extraction were retrieved at these locations from just below the sediment/water interface (top 5 cm). Sediment samples were collected with a sterile spatula, placed in 50 mL Falcon tubes, kept on ice in the field, and stored (within 6 h) at –20 °C. Sediments for total cell counts using DAPI (4′,6′diamidino-2-phenylindole; Sigma, St. Louis, MO, USA) were stored in a 4% paraformaldehyde:phosphate-buffered-solution (PFM:PBS). The sediment was allowed to fix for 3–6 h on ice, the fixative was decanted, PBS: 96% ethanol (EtOH, 1 : 1 v/v) was added and samples were stored at –20 °C. The depth to the interface between thermal fluid and sediment was 5–10 cm in each case. Hydrothermal fluid and gas at these three locations were collected concurrently with the microbiological samples (for details see Shock *et al.*, 2005a). Due to the volume of sample needed for the extensive geochemical analysis, hydrothermal fluid was collected from the 5–10 cm above the sediment interface. Ideally, these analyses would be done using pore fluid from the upper 5 cm of sediment. This is, however, beyond the scope of this investigation: it is probable that there is a rapid mixing of fluids in the sediment–surface interface due to the very active nature of these features. In addition, to provide environmental context, a broad geochemical survey was conducted between 1999 and 2003 at various other features in the Obsidian Pool, Sylvan Spring, and Sentinel Meadow geothermal systems.

DNA extraction from sediment samples

Because different DNA extraction methods have different recovery efficiencies (Hugenholtz *et al.*, 1998; Miller *et al.*, 1999), five methods were applied to each of the frozen samples: bead-beating using the Bio101 extraction kit (Q-BIOgene's FastDNA Spin Kit for Soil; Carlsbad, CA, USA), bead-beating (adapted from Bond *et al.* (2000)), freeze/thaw (adapted from Barns *et al.* (1994) and pers. comm. with C. Blank), freeze/thaw/grind (adapted from Hurt *et al.* (2001)), and chemical extraction (adapted from Miller *et al.* (1999)). To prevent damage to extracted nucleic acids, the extraction protocols were adjusted to suit the pH at each study area. For specific protocols, see Meyer-Dombard (2004).

PCR of extracted community DNA

DNA was amplified with primer pairs 21F–1391R (targeting archaea) and 27F-1492R (targeting bacteria) (Lane, 1991) using a Hybaid PCR Express thermalcycler. Cycling conditions for 21F–1391R: preheat at 95 °C for 5 min; 30 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1.5 min; extension at 72 °C for 15 min. Cycling conditions for 27F–1492R: preheat at 95 °C for 5 min; 35 cycles at 95 °C for 1 min, 52 °C for 1 min, 72 °C for 1 min; and extension at 72 °C for 5 min. The reaction mixtures included (per 20 μL reaction) 1.8 μL 25 mM $MgCl_2$, 0.2 μL dNTP mix (12.5 μmol of each dATP, dGTP, dTTP and dCTP; Bioline, Randolph, CA, USA), 0.25 μL 5 U μL^{-1} Ampli*Taq* Gold (Applied Biosystems, Foster City, CA, USA), 2.0 μL 10× Ampli*Taq* Gold reaction buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 0.25 pmol μL^{-1} primer (IDT), and 13.75 μL ddH_2O.

Molecular cloning of amplified community DNA

Polymerase chain reaction (PCR) products from the five extractions were combined and cloned using the Invitrogen TOPO TA cloning kit (cat. no. K4500-01; Carlsbad, CA, USA). This resulted in six libraries, one archaeal and one bacterial for each of the three samples. The efficiency of the five extraction methods was not evaluated in this study. Between 192 and 384 white colonies were picked from each library, and the plasmids were purified using the QIAGEN Miniprep Kit (cat. no. 27106; Valencia, CA, USA). The insert was amplified using M13F and M13R primers. The reaction mix included: 1.2 μL 25 mM $MgCl_2$, 0.2 μL dNTP mix, 0.25 μL 5 U μL^{-1} Ampli*Taq*

Gold, 2.0 μL 10× Ampli*Taq* Gold reaction buffer, 2 pmol μL^{-1} primer (forward and reverse), and 14.95 μL ddH_2O. Reactions were cycled as follows: preheat at 95 °C for 5 min; followed by 25 cycles at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min; followed by a 15-min extension at 72 °C. Products were initially screened using restriction fragment length polymorphism (RFLP) by *HhaI* (New England BioLabs, Ipswich, MA, USA) digestion. Digests were run on a 4% MetaPhor gel (Cambrex, Baltimore, MD, USA) to identify unique banding patterns. Following maximization of the rarefaction curves (data not shown), screening was done by short segment (~700 bp) sequencing.

Sequencing and phylogenetic inference

At least one clone representing each unique RFLP banding pattern in each library was chosen for sequencing. A ~700 bp fragment of the 16S rRNA gene was sequenced for 35 bacterial and 51 archaeal clones from Sylvan Spring, 49 bacterial and 67 archaeal clones from 'Bison Pool', and 50 bacterial and 77 archaeal clones from Obsidian Pool. Following this initial screening, the full 16S rRNA gene was sequenced for unique clones from the 'Bison Pool' (22) Obsidian Pool (33), and Sylvan Spring (14) libraries. Plasmids with DNA inserts were directly sequenced using the primers 21F/958R/1391R and 27F/907R/1492R. Cycling conditions for sequencing reactions were: preheating step at 96 °C for 3 min; 35 cycles at 96 °C for 30 s, annealing temperature (T_a) for 15 s (where T_a = 55 °C (21F), 52 °C (27F, 958R), 45 °C (907R, M13R), 57.3 °C (1392R), 50 °C (1492R), 48.3 °C (M13F)), and 60 °C for 4 min. Sequencing reactions contained 37.5 pmol μL^{-1} primer, 1.0 μL BigDye Terminator premix version 3.1 (Applied Biosystems), and ddH_2O to bring the final volume to 5 μL. Products were sequenced on an Aurora Genetic Analysis System (SpectruMedix, State College, PA, USA).

Contiguous sequences were assembled using SEQUENCHER (version 4.1.4; Gene Codes Corporation, Ann Arbor, MI, USA), submitted to GenBank (accession numbers DQ243725–DQ243783), and compared to the NCBI (National Center for Biotechnology Information) database using BLAST (Altschul *et al.*, 1997) to find closest relatives. Approximately 1200–1400 nucleotide bases were aligned using the software BIOEDIT (version 5.0.9; http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and then manually adjusted using the predicted secondary structure of the molecule as a template. Chimeric analyses were largely performed by careful alignment comparisons, but the CHIMERACHECK (Cole *et al.*, 2003) and BELLEROPHON (Huber *et al.*, 2004) software were also used as guidelines. Phylogenetic inference of homologous positions was performed using neighbour joining (NJ), maximum parsimony (MP), and maximum likelihood (ML) with the software PAUP (version 4b.10; Sinhauer Associates, Sutherland, MA, USA). MP analyses were performed with random addition of taxa (1000 replicates). 'Long-branch attraction' issues were addressed by considering branch lengths of >10% of the total number of nucleotides aligned as long branches, and the analysis was adjusted appropriately. In addition, the placement of taxa within monophyletic groups was tested by addition–subtraction–replacement experiments. For ML tree construction, evolutionary models were evaluated with a likelihood ratio test to find the simplest model appropriate for the data (Huelsenbeck & Rannala, 1997), which was then used to construct the trees. This process was iterated three times with 1000 replicates in the final iteration. All trees were evaluated using only unambiguously aligned nucleotides and bootstrapped (100–1000 replicates) (Felsenstein, 1985). Taxa not uniquely affiliated with a phylogenetic position are noted in the results.

Geochemistry

Analyses of water and gas samples collected between 1999 and 2001 are reported in Shock *et al.* (2005a,b). Temperature, pH, conductivity, and alkalinity were measured in the field with hand-held meters (YSI 30, Orion 290 A plus). Portable spectrophotometers (Hach, Loveland, CO, USA) were used in the field to measure dissolved oxygen, nitrate, nitrite, total ammonia, ferrous iron, total sulphide, and dissolved silica. Major ions and trace elements were analysed in the laboratory using ion chromatography (Dionex, Sunnyvale, CA, USA) and inductively coupled plasma mass spectrometry (Finnigan), respectively. Gases were collected in evacuated Giggenbach bottles containing known amounts of NaOH (Giggenbach, 1975; Giggenbach & Goguel, 1989; Giggenbach *et al.*, 2001) and analysed by gas chromatography and established wet chemical methods (Giggenbach & Goguel, 1989) in the laboratory of Tobias Fischer, University of New Mexico. Results of gas analyses are not reported directly here, but these data were used in the energetic calculations.

The compositional data were converted to chemical activities using the computer code EQ3 and used to compute values of the overall Gibbs free energy (ΔG_r) as described in Amend & Shock (2001) and Shock *et al.* (2005a). Briefly, values of ΔG_r for >180 inorganic reactions were computed using the relation:

$$\Delta G_r = \Delta G_r^\circ + RT \ln Q_r, \quad (4)$$

where ΔG_r° stands for the standard state Gibbs free energy of reaction, R and T represent the gas constant and temperature (in Kelvin), respectively, and Q_r denotes the activity product, which is expressed as:

$$Q_r = \Pi a_i^{\nu_{i,r}} \quad (5)$$

where a_i stands for the activity of the i^{th} component in the reaction, and $\nu_{i,r}$ denotes the stoichiometric reaction coefficient. By convention, a negative value of ΔG_r indicates an exergonic, or energy-yielding, reaction.

RESULTS AND DISCUSSION

The archaeal and bacterial community structures in sediments from three hot springs in Yellowstone were determined and then interpreted in the context of *in situ* chemical composition and reaction energetics. The three sites (Obsidian Pool, Sylvan Spring, and 'Bison Pool') chosen for this comparative geobiological study of springs are of similar temperature (79.9–82.6 °C), but variable chemistry (e.g. pH: 5.5–8.1; conductivity: 650–5050 μS cm^{-1}). Whole cell counts in the three sediment samples are as follows: Obsidian Pool, 2.3×10^6 cells cm^{-3}; Sylvan Spring, 3.2×10^6 cells cm^{-3}; and 'Bison Pool', 2.1×10^6 cells cm^{-3}.

Community structure analysis

Archaeal and bacterial DNA was successfully amplified from all three sediment samples. PCR products from all extractions were pooled, and two libraries (archaeal and bacterial) were constructed for each sample. The assembled sequences of the 16S rRNA gene for each clone were submitted to GenBank and assigned accession numbers DQ243725–DQ243783. All sequenced clones, their phylogenetic affiliations, the closest GenBank match, percentage similarity to the GenBank data, and the numbers of clones screened by RFLP patterns and partial gene sequencing are recorded in Table 1.

Following RFLP screening, rarefaction curves (data not shown) for five of six libraries maximized at <40 plasmids; the exception was the Obsidian Pool archaeal library, which had not maximized even after screening 66 clones. The remaining clones analysed were screened using short segment sequencing. Analysis of RFLP banding patterns revealed 13 bacterial and 35 archaeal patterns at Obsidian Pool, 8 bacterial and 26 archaeal patterns at Sylvan Spring, and 16 bacterial and 32 archaeal patterns at 'Bison Pool'. The clonal distribution differed between the RFLP and sequencing screening methods in some cases. For example, 12 of 67 clones screened using sequencing, but only 5 of 75 clones screened using RFLP were identified as YNP BP A23. The differences are attributed to difficulties in identifying unique banding patterns using RFLP, and the consistency of conserved restriction sites.

The distribution and diversity of clones representing unique taxa are shown in Fig. 3 as percentages of the total number of clones screened by partial (~700 bp) 16S rRNA sequencing. The six pie charts represent the archaeal (Fig. 3A,C,E) and bacterial (Fig. 3B,D,F) libraries from 'Bison Pool', Obsidian Pool, and Sylvan Spring; each pie wedge is colour-coordinated such that taxa within each domain can be readily compared among the three sample locations. These results reflect the distribution of clones within each library, but do not necessarily quantify the diversity in the natural environment. Figure 3 reveals that, although certain taxa are similar between all three sediment samples, the distribution of taxa between the hot springs varies greatly.

Phylogenetic analysis of archaeal clones

Phylogenetic evaluation of archaeal sequences is shown in Fig. 4A; a subset of the 'uncultured' crenarchaeal clones are highlighted in Fig. 4B. Except where noted below, neighbour-joining and maximum-likelihood analyses were in close agreement with the maximum parsimony tree depicted here. Samples from the current study are shown in bold and coded according to sample location. All three archaeal communities were dominated by Crenarchaeota, containing members of the Desulphurococcales, Thermoproteales, and 'uncultured' Crenarchaeota. Sulfolobales were not observed in this study despite their reported presence at the Sylvan Springs (Brock *et al.*, 1972; Brock, 1978). No Euryarchaeota and only one Korarchaeon (from Obsidian Pool) were found in the three samples. It can be seen in Figs 3 and 4 that the archaeal communities from the three pools are similar on the order level, but vary significantly on the genus level, examples of which are discussed separately below.

Clones affiliating with the Desulphurococcaceae were found in all three pools, but no members of the Pyrodictiaceae were identified. The Desulphurococcaceae clones fell into five groups, four of which (D-I to D-IV) may be novel clades, forming unassociated monophyletic groups that are not affiliated with the other known genera of Desulphurococcaceae (*Aeropyrum*, *Thermodiscus*, *Ignicoccus*, *Staphylothermus*, *Stetteria*, and *Sulfophobococcus*). The remaining clones (SSp A84 and BP A49) fall within clades containing *Desulphurococcus mobilis* (96.2% similarity) and *Thermosphaera aggregans* (99.1% similarity). The novel phylotypes are generally well supported by the bootstrap analysis. However, the placement of group D-II may be affected by long-branch attraction artefacts; neighbour-joining and maximum-likelihood analyses (not shown) placed BP A73 as a separate branch within group D-IV. Further, group D-I may represent a separate monophyletic group related to *Aeropyrum*, *Thermodiscus*, and *Ignicoccus*, and group D-III may affiliate most closely with *Pyrodictium* and *Pyrolobus*. Regardless of the exact placement of each clade, significant diversity in the Desulphurococcaceae is evident in all three sediments.

Differences in the distribution of D-I, D-II, D-III, and D-IV among the three sites are noteworthy. It can be seen in Fig. 3A,C,E, and Fig. 4 that group D-I is comprised entirely of 'Bison Pool' clones, and group D-IV contains only samples from Obsidian Pool and Sylvan Spring. However, groups D-II and D-III are represented in all three pools. Note that these novel Desulphurococcales represent >65% of the 'Bison Pool' library, compared to 11% of the Obsidian Pool and 23% of the Sylvan Spring libraries (Fig. 3).

Within the Thermoproteales, close relatives of members of the Thermoproteaceae and Thermofilaceae were found in this study. Clones from all three samples (ObP A130, ObP A166, SSp A81, and BP A6) form a clade with the previously identified Yellowstone clone 'pBA2' (Reysenbach *et al.*, 2000). While closely related (99.5% similarity), short-branch attraction

Table 1 Inventory of clones analysed, the associated figure number, affiliated phylogenetic group, closest GenBank match and % similarity (based on BLAST search), and screening results (RFLP and short-segment sequencing)

Clone* (type representative)	Fig	Phylogenetic Group†	Closest GenBank match‡ (% similarity)	Clonal distribution§
YNP BP A23	4a	D-I (unknown Desulfurococcales)	*Aeropyrum pernix* (AB078022) (94%)	12/67 : 5/75
YNP BP A10			*A. camini* (AB109559) (94%)	12/67 : 4/75
YNP BP A78			*Staphylothermus marinus* (X99560) (92%)	12/67 : 1/75
YNP BP A73	4a	D-II (unknown Desulfurococcales)	*Stetteria hydrogenophilia* (Y07784) (94%)	1/67 : 3/75
YNP SSp A61			*S. marinus* (92%)	1/51 : 1/88
YNP ObP A84			*S. marinus* (92%)	2/77 : NA
YNP BP A60			*S. marinus* (93%)	6/67 : 4/75
YNP BP A32			*S. marinus* (95%)	2/67 : 1/75
YNP BP A9	4a	D-III (unknown	*Sulfophobococcus zilligii* X98064 (91%)	1/67 : 1/75
YNP ObP A110		Desulfurococcales)	(AB075802) (91%)	1/77 : 1/89
YNP BP A22			*Pyrodictium occultum* (M21087) (93%)	5/67 : 1/75
YNP SSp A24			*Caldococcus noboribetus* (D85038) (94%)	1/51 : 1/88
YNP SSp A51	4a	D-IV (unknown	*Desulfurococcus mobilis* (M36474) (89%)	1/51 : 1/88
YNP ObP A25		Desulfurococcales)	*D. mobilis* (89%)	3/77 : 2/89
YNP BP A49		Desulfurococcales/	*Thermosphaera aggregans* (X99556)	2/67 : 2/75
	4a	*Thermosphaera*	(94%)	
YNP SSp A84		Desulfurococcales/	*D. mobilis* (90%)	4/51 : 16/88
	4a	*Desulfurococcus*		
YNP ObP A130		Thermoproteales/	*Pyrobaculum organotrophicum*	1/77 : 2/89
	4a	*Thermoproteus*	(AB063647) (92%)	
YNP BP A6			*Thermoproteus neutrophils* (AB009618) (98%)	7/67 : NA
YNP ObP A166			*P. organotrophicum* (93%)	5/77 : 3/89
YNP SSp A81			*P. organotrophicum* (92%)	2/51 : 4/88
YNP SSp A50		Thermoproteales/	*Vulcanisaeta distributa* (AB063630)	2/51 : 3/88
	4a	*Vulcanisaeta*	(97%)	
YNP SSp A10	4a	Thermoproteales/	*Thermofilum pendens* (X14835) (96%)	3/51 : 1/88
YNP BP A17		*Thermofilum*	*T. pendens* (94%)	2/67 : 1/75
YNP BP A81	4b	UC-I (Uncultured	Clone pJP41 (L25301) (93%)	1/67 : 1/75
YNP ObP A97		Crenarchaeota)	Clone pJP41 (87%)	4/77 : NA
YNP ObP A136			Clone pJP41 (87%)	4/77 : 2/89
YNP ObP A150			Clone pJP33 (L25300) (99%)	2/77 : 8/89
YNP BP A89			Clone pJP33 (99%)	1/67 : 14/75
YNP ObP A31			Clone pJP96 (U63338) (99%)	7/77 : 5/89
YNP SSp A70	4a	UC-II (Uncultured Crenarchaeota)	Clone pISA9 (AB019732) (86%)	2/51 : 8/88
YNP ObP A62			Clone HAuD-LA19 (AB113630) (97%)	1/77 : NA
YNP ObP A68			Clone SUBT-13 (AF361212) (98%)	1/77 : NA
YNP ObP A5	4a	UC-IV (Uncultured Crenarchaeota)	Clone 19b-34 (UAR294865) (91%)	1/77 : 2/89
YNP ObP A23			Clone pJP89 (L25305) (99%)	4/77 : 3/89
YNP ObP A16	4a	UC-V (Uncultured Crenarchaeota)	Clone pISA7 (AB019733) (85%)	7/77 : 8/89
YNP ObP A153			Isolate S10TFL (X99564) (89%)	7/77 : 8/89
YNP ObP A17			Clone pISA7 (87%)	1/77 : 2/89
YNP BP A103			Clone pUWA2 (AB007307) (86%)	1/67 : NA
YNP ObP A10			Isolate S10TFL (X99564) (84%)	1/77 : 1/89
YNP ObP A42	4a	Korarchaeota	Clone pJP78 (U63344) (98%)	2/77 : 5/89
YNP ObP B89	5	Aquificales/	*Thermocrinis ruber* (L09672) (97%)	4/50 : 1/66
YNP ObP B32		*Thermocrinis*	*Thermocrinis ruber* (L09672) (97%)	10/50 : NA
YNP BP B97			*Thermocrinis ruber* (L09672) (97%)	11/49 : 6/83
YNP BP B71			*Thermocrinis ruber* (L09672) (97%)	11/49 : 23/83
YNP BP B86			*Thermocrinis ruber* (L09672) (98%)	6/49 : 14/83
YNP SSp B90	5	Aquificales/	Isolate YNP-SS1 (AF507961) (99%)	22/35 : 1/86
YNP SSp B60		*Hydrogenothermus*	Isolate YNP-SS1 (AF507961) (99%)	22/35 : 3/86
YNP ObP B24			Isolate YNP-SS1 (AF507961) (99%)	1/50 : 3/66
YNP BP B68	5	Unknown	Clone SHA-2 (AJ306740) (83%)	4/49 : 2/83
YNP BP B72		Aquificales	*Thermodesulfobacterium hveragerdicum* (X96725) (89%)	4/49 : 4/83
YNP SSp B24	5	Thermodesulfobacteriales	Clone SRI-27 (AF25595) (99%)	1/35 : 4/86
YNP ObP B80		*Geothermobacterium*	*Geothermobacterium ferrireducens* (AF411013) (98%)	3/50 : 3/66
YNP BP B73			*G. ferrireducens* (99%)	2/49 : NA
YNP ObP B48	5	Thermodesulfobacteriales	Clone SRI-93 (AF255596) (98%)	1/50 : 3/66
YNP ObP B45			Clone SRI-27 (98%)	5/50 : 3/66
YNP ObP B34	5	Actinobacteriales	*Nesterenkonia* sp. (AY574575) (96%)	2/50 : 1/66

Table 1 *Continued*

Clone* (type representative)	Fig	Phylogenetic Group†	Closest GenBank match‡ (% similarity)	Clonal distribution§
YNP ObP B94	5	Clostridiales	*C. thermocellum* (L09173) (91%)	1/50 : 1/66
YNP ObP B118	5	Proteobacteria	*S. xenophaga* (AY611716) (99%)	1/50 : NA
YNP ObP B70	5	beta-Proteobacteria	*Tepidimonas* sp. (AY324139) (96%)	1/50 : 3/66
YNP BP B75	5	gamma-Proteobacteria	*Stenotrophomonas* sp. (AJ293468) (99%)	1/49 : 1/83
YNP SSp B57			*Stenotrophomonas* sp. (AJ293468) (99%)	3/35 : 1/86
YNP ObP B67			*Stenotrophomonas* sp. (AJ293468) (99%)	1/50 : 1/66

*YNP, Yellowstone National Park; BP, 'Bison Pool'; ObP, Obsidian Pool; SSp, Sylvan Spring; A no., archaeal clone no.; B no., bacterial clone no.

†As seen in Figs. 4 and 5.

‡Based on BLAST search. Percentage similarity determined by BLAST search and distance matrices (PAUP).

§Number of clones like sample/number of clones screened by sequencing : number of clones like sample/number of clones screened by RFLP.

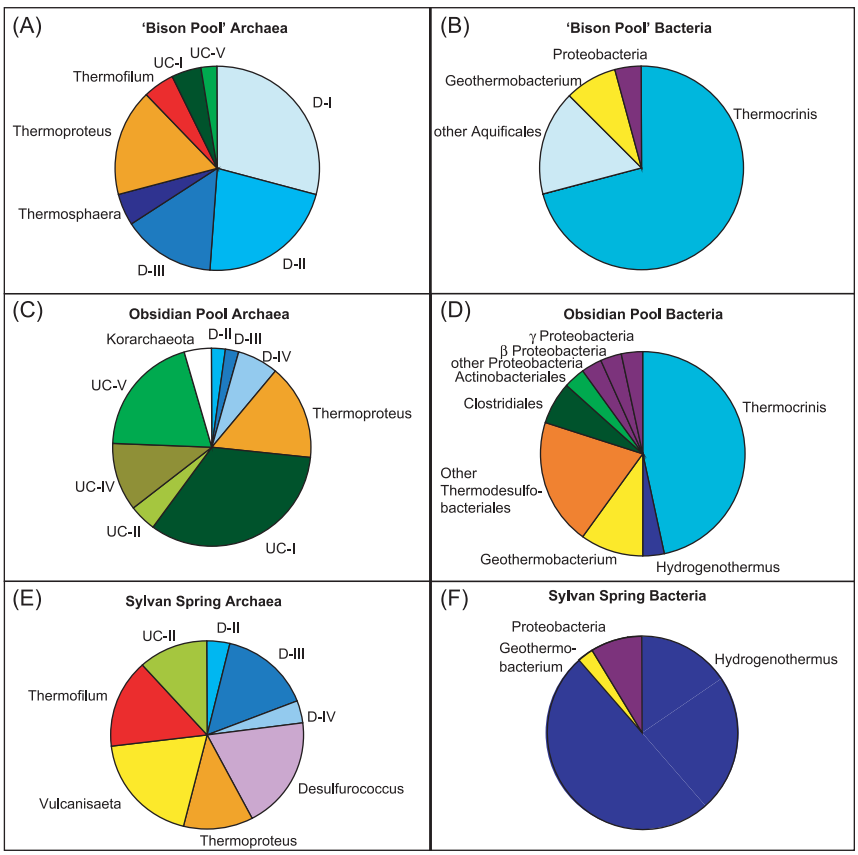

Fig. 3 Diversity and distribution of plasmids within archaeal and bacterial clone libraries for 'Bison Pool' (A, B), Obsidian Pool (C, D), and Sylvan Spring (E, F). Pie wedges indicate the percentages of each taxon in the total library, as determined by screens of partial (~700 bp) 16S rRNA sequences (see Table 1). Colours are coordinated for all archaeal libraries (A, C, E) and all bacterial libraries (B, D, F), but not across domain boundaries.

artefacts prevented confident placement of each individual clone. These *Thermoproteus*-like organisms are ubiquitous in all three hot springs, representing 12–17% of the archaeal clone libraries (Fig. 3A,C,E). Within the Thermofilaceae, clones related to *Thermofilum pendens* (94.4% similarity) are only represented in Sylvan Spring and 'Bison Pool'. However, an earlier study found evidence of this family at Obsidian Pool (Barns *et al.*, 1994, 1996), suggesting a temporally dependent community structure.

Many of the archaeal clones recovered from the three sediments cluster within the 'uncultured' Crenarchaeota – a group of organisms known only by their gene sequences. No naming system has been unanimously adopted for these phylotypes, and the clades represented in Fig. 4 are simply labelled 'UC' (uncultured Crenarchaeota) I–V, which also incorporates the terrestrial hot spring crenarchaeal group (THSC) (Takai & Sako, 1999; Takai *et al.*, 2001). The uncultured Crenarchaeota feature prominently in the Obsidian Pool library (69%), but less so in the 'Bison Pool' (7%) and Sylvan Spring (12%) libraries (Fig. 3A,C,E).

A phylogenetic analysis of group UC-I is depicted in Fig. 4, utilizing clones from groups UC-II, III, and IV as an

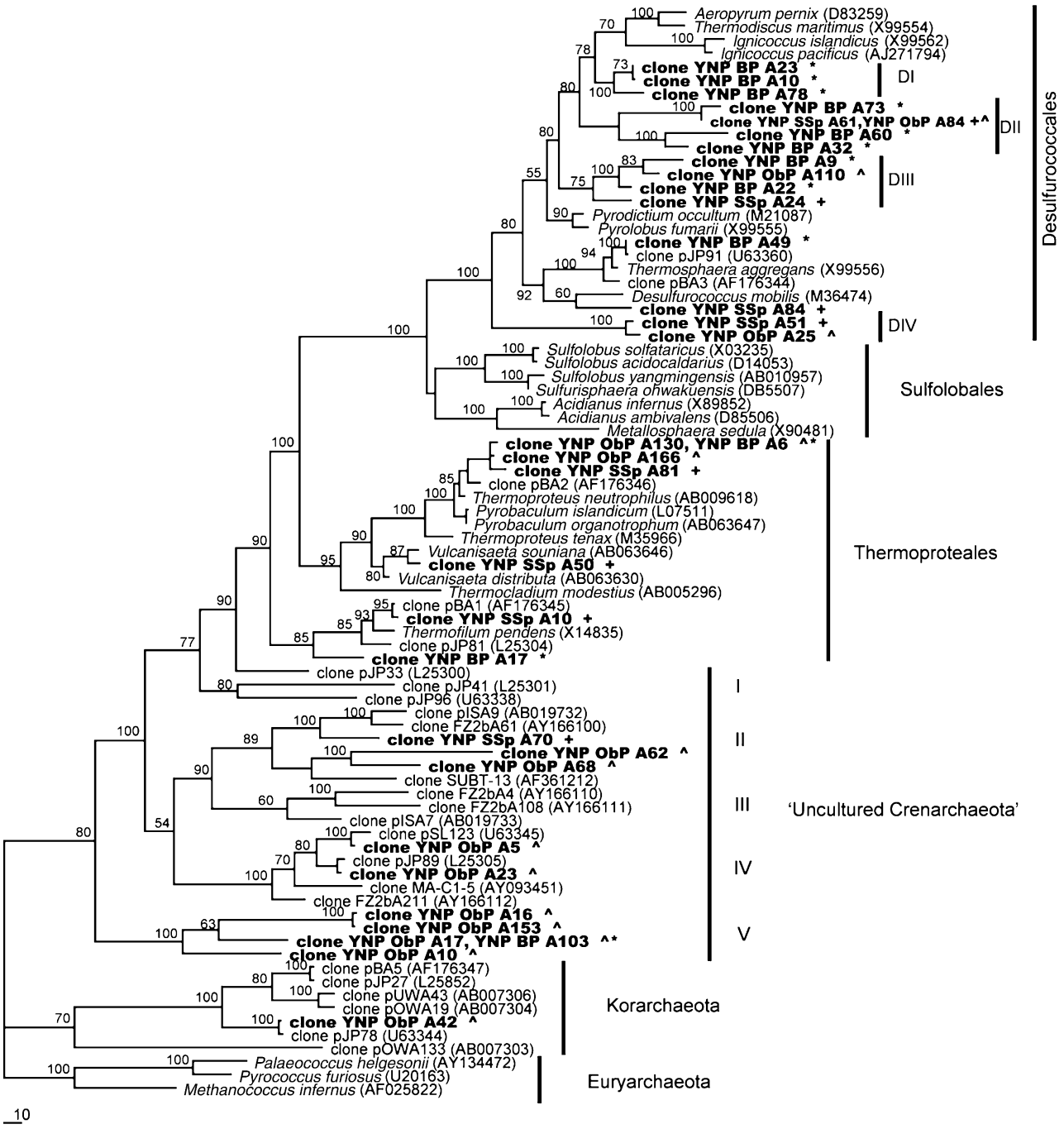

Fig. 4 Maximum parsimony phylogenetic inference from 16S rRNA gene sequences (1000 random addition replicates) of the Crenarchaeota (A) and a subgroup (UC-1) of the 'uncultured' Crenarchaeota (B). Bootstrap values indicate 100 parsimony replicates. Organisms from this study in bold, with * for 'Bison Pool', + for Sylvan Spring, and ^ for Obsidian Pool. Bar indicates 10 changes.

outgroup. Group UC-I is comprised of sequences similar to previously identified clones pJP33, pJP96, and pJP41 from Obsidian Pool (Barns *et al.*, 1994, 1996). The placement of the root of this clade relative to the other UC groups is problematic due to long branches, the lack of informative characters in the 16S rRNA, and the paucity of sequences from similar organisms to support the topology. When isolated in this monophyletic analysis, Obsidian Pool and 'Bison Pool' clones maintain a topology with high confidence numbers overall, although one node is not well supported. UC-I features clones from both the Obsidian Pool (33%) and 'Bison Pool' (10%) libraries, but not from Sylvan Spring.

Groups UC-II through V are shown on the phylogenetic tree in Fig. 4, together with representatives from a submarine

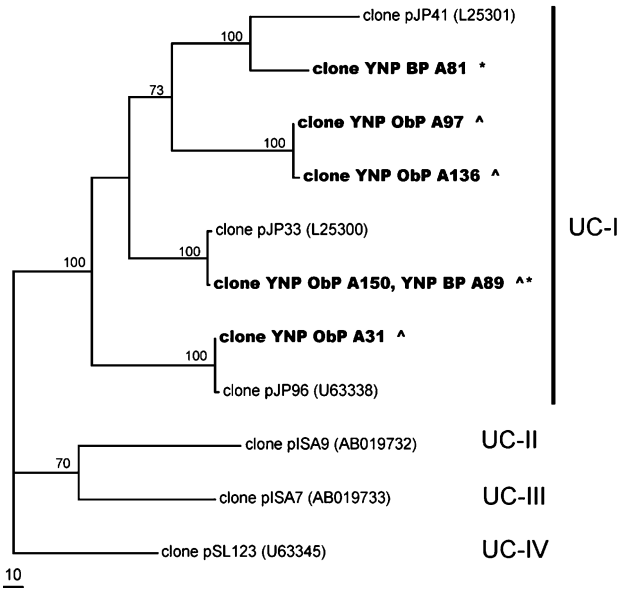

Fig. 4 *Continued*

sulphide chimney (Finn) on the Juan de Fuca Ridge (Schrenk *et al.*, 2003), subterranean hot springs in Iceland (Marteinsson *et al.*, 2001), hydrate-bearing deep marine sediments (Reed *et al.*, 2002), sediments from the Okinawa Trough (Takai & Horikoshi, 1999), and other clones from Obsidian Pool (Barns *et al.*, 1994, 1996). Phylotype UC-II was found in Obsidian Pool and Sylvan Spring, phylotype UC-III was absent from all three samples, phylotype UC-IV was present only in Obsidian Pool, and phylotype UC-V was found in Obsidian Pool and 'Bison Pool'. Groups UC-II through IV are supported by high bootstrap values. However, the placement of UC-V was difficult to constrain due to long-branch affects; in analyses with the five UC groups plus the Korarchaeota, each UC-V clone pulled away from the groupings shown in Fig. 4, but without the Korarchaeota, the clones enjoyed high bootstrap values in their shown topology in all analyses. Despite the consequent lower bootstrap value, this phylotype may represent a new, deeply branching clade within the 'uncultured' Crenarchaeota, which is thus far unique to Yellowstone at Obsidian Pool and 'Bison Pool'.

Obsidian Pool is also home to the deepest-branching archaeal kingdom – the Korarchaeota (Barns *et al.*, 1994, 1996). In this study, the Korarchaeota are represented only by the Obsidian Pool clone ObP A42, which is 93.3% similar to clone pJP78 (Barns *et al.*, 1994, 1996). Notably, clone pJP27-like organisms – the previously identified Korarchaeota at Obsidian Pool (Barns *et al.*, 1994, 1996) – were not identified in this study. Independent verification of the presence and identity of Korarchaeota from a hydrothermal environment has not been previously shown.

Only a few previous studies at Yellowstone have targeted the Archaea in systems geochemically similar to those studied here. Barns *et al.* (1994, 1996) identified 36 archaeal sequences in Obsidian Pool sediments, representing 12 major crenarchaeal clades, and one group of Korarchaeota. However, independent attempts to amplify DNA from another Obsidian Pool sample and from Queen's Laundry (Sentinel Meadow in the Lower Geyser Basin) using archaeal-specific primers were unsuccessful (Graber *et al.*, 2001; Blank *et al.*, 2002). Figure 6A shows the distribution of clones (relative to the total library size) for each major group of Crenarchaea found in Obsidian Pool in this study and in Barns *et al.* (1994). The 12 crenarchaeal groups found by Barns and colleagues were also identified here. However, only eight were retrieved from our Obsidian Pool sample, and four of those were also found in the Sylvan Spring or 'Bison Pool' samples. The remaining four were present exclusively in the Sylvan Spring and/or 'Bison Pool' samples. In addition, four clades (D-II, D-IV, UC-II, UC-V) from this study were not identified by Barns *et al.* (1994, 1996).

Phylogenetic analysis of bacterial clones

A phylogenetic inference from bacterial sequences is shown in Fig. 5. It can be seen that most of the bacterial sequences affiliate with the Aquificales, but a number of clones also fall within the Thermodesulfobacteriales. The Aquificales clones are numerically well-represented in all three libraries (Fig. 3B,D,F). Members of the Aquificaceae were only identified in 'Bison Pool' and Obsidian Pool libraries. Within the Aquificaceae, several clones, including ObP B89 and seven others that were not included in the phylogenetic tree (due to short-branch artefacts), represent apparent subspecies of *Thermocrinis ruber*, the pink filament-forming hyperthermophiles identified in a number of Yellowstone locations (Reysenbach *et al.*, 2000; Jackson *et al.*, 2001; Jahnke *et al.*, 2001; Blank *et al.*, 2002; Eder & Huber, 2002; Kato *et al.*, 2004; Zhang *et al.*, 2004; Spear *et al.*, 2005). Organisms belonging to the family Hydrogenothermaceae within the Aquificales were found at Sylvan Spring (>85% of the library) and Obsidian Pool, but not at 'Bison Pool' (Fig. 3B,D,F). Two clones from 'Bison Pool' (BP B68 and BP B72) branch well outside the established Aquificaceae and Hydrogenothermaceae clades and may represent a new phylotype within the Aquificales. Alternatively, these clones may represent novel members of the genus *Desulphurobacterium*; a more definitive placement of the root of this clade will depend on new cultured representatives from these groups. Clones from Sylvan Spring (SSp B60 and SSp B90) and Obsidian Pool (ObP B24) are similar to clones found by other studies (pBB and OPB13) that form a group in the genus *Sulphurihydrogenibium*. Cultured organisms in this group are found in other terrestrial environments and have been shown to oxidize sulphur, hydrogen, arsenite, and selenite with a variety of electron acceptors (Nakagawa *et al.*, 2005).

Clones from all three sample libraries fall within the order Thermodesulfobacteriales (Fig. 5), but feature most prominently in the Obsidian Pool sample (Fig. 3D). Clones SSp B24, ObP B80, and BP B73 are ~98% similar to *Geothermobacterium*

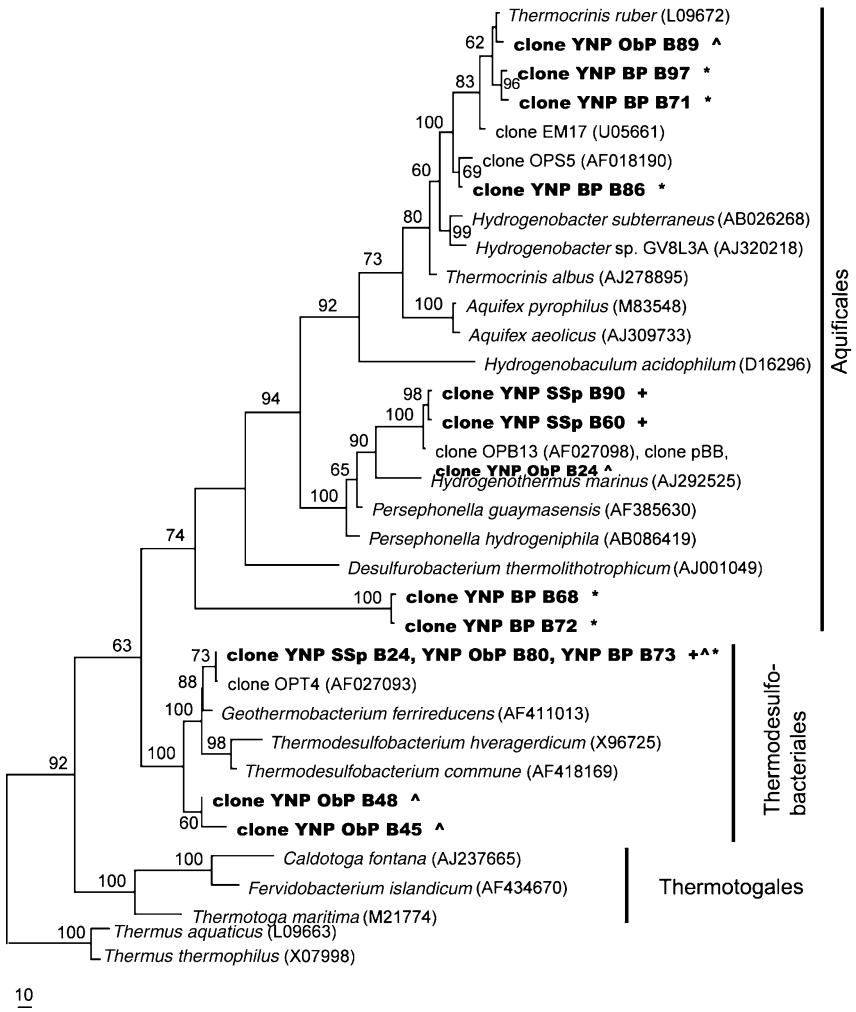

Fig. 5 Maximum parsimony phylogenetic inference from 16S rRNA sequences (1000 random addition replicates) of the bacteria. Bootstrap values indicate 100 parsimony replicates. Organisms from this study in bold, with * for 'Bison Pool', + for Sylvan Spring, and ^ for Obsidian Pool. Bar indicates 10 changes.

ferrireducens, a thermophilic Fe(III) reducing organism recently isolated from Obsidian Pool (Kashefi *et al.*, 2002). In addition, clones ObP B48 and ObP B45 from Obsidian Pool may represent a new phylotype within the Thermodesulfobacteriales. Besides the strictly thermophilic Aquificales and Thermodesulfobacteriales, *Stenotrophomonas*-like clones were found at all three locations, and clones clustering with the Actinobacteriales, Clostridiales, and subdivisions of the Proteobacteria were retrieved from Obsidian Pool sediments. These clones are included in Table 1, but not in the phylogenetic tree in Fig. 5.

While few archaeal gene surveys have been carried out in these three hydrothermal areas, several investigations have targeted bacterial diversity there. For example, at Queen's Laundry, also in the Sentinel Meadow area, Blank *et al.* (2002) found almost exclusively *Thermocrinis ruber*-like organisms and low percentages of clones that affiliated with *Thermodesulfobacterium* and the Thermotogales. These findings are in close agreement with our results at 'Bison Pool' (see Fig. 6B). In addition, *Fervidobacterium* strain 'BP1' (belonging to the Thermotogales) was isolated from 'Bison Pool' (Meyer-Dombard, 2004), although sequences belonging to the Thermotogales were not found in our 'Bison Pool' bacterial library. It should be noted that Queen's Laundry and 'Bison Pool' are only ~0.5 km apart in Sentinel Meadow, and that they feature very similar chemical compositions. A key difference in the samples is that the DNA from Queen's Laundry was extracted from siliceous sinter at the edge of the pool, and the DNA from 'Bison Pool' was extracted from water saturated sediment. This geochemical difference may explain the subtle community variations.

One of the best studied sites in Yellowstone is Obsidian Pool. In addition to the archaeal libraries of Barns *et al.* (1994, 1996), Hugenholtz *et al.* (1998) identified 54 novel bacterial sequences from an Obsidian Pool sediment sample, including 12 novel clades. Surprisingly, only 4 of the 54 sequences – and none of the novel groups – were similar to clones from this study. A further study at Obsidian Pool was conducted by Graber *et al.* (2001), in an investigation of bacterial diversity down a high temperature gradient in an outflow channel. In a 76 °C sample, the Aquificales dominated, with minor representation by the Thermotogales. At 70 °C, Planctomycetales,

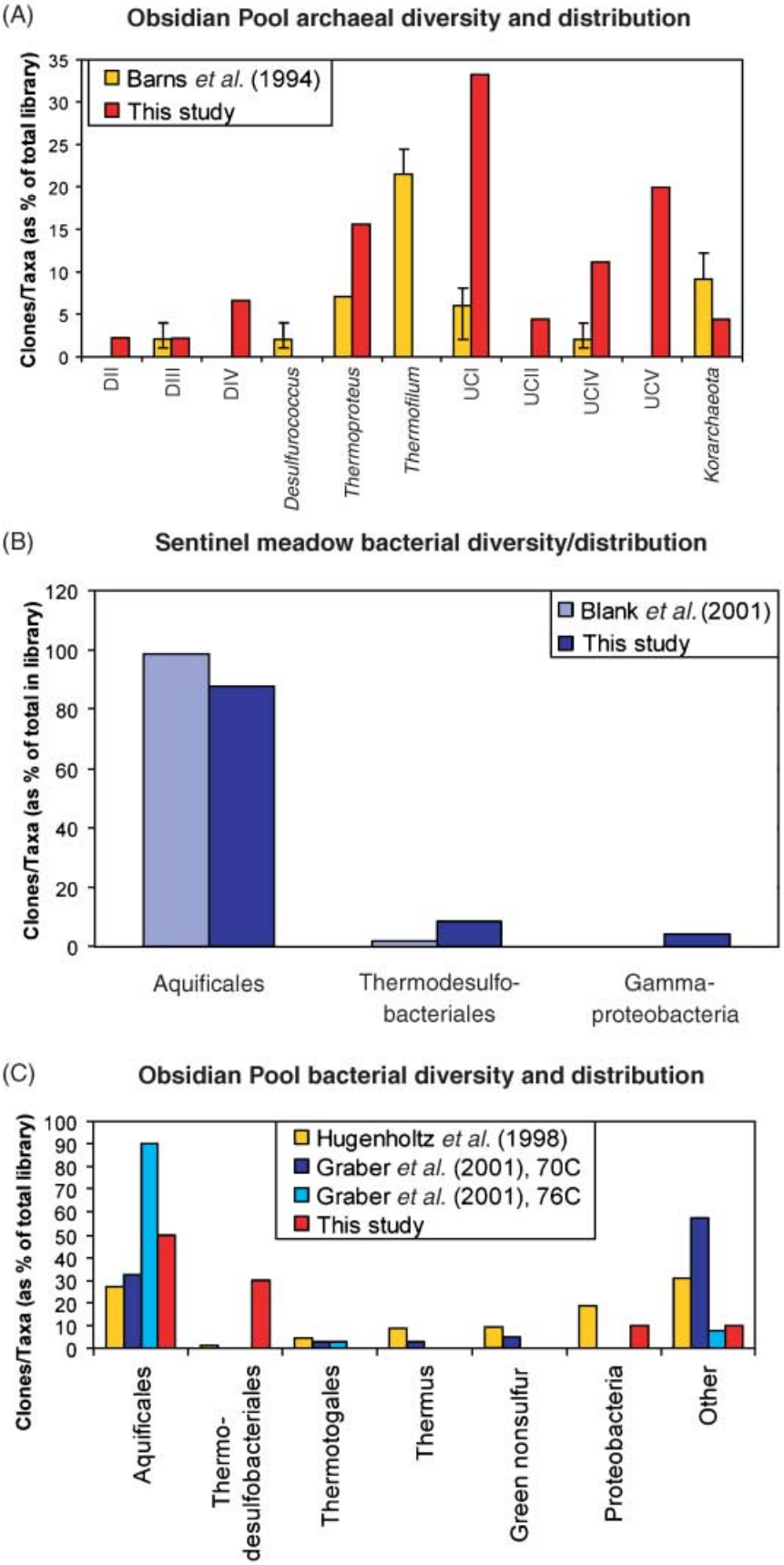

Fig. 6 Comparison of diversity and distribution in archaeal clone libraries at Obsidian Pool (A), bacterial clone libraries at Sentinel Meadow (B), and bacterial clone libraries at Obsidian Pool (C) from this investigation and several studies reported in the literature. Bars depict percentages of the total library represented by each taxon, as determined by 16S rRNA sequencing. Error bars in (1) result from ambiguous reporting in source.

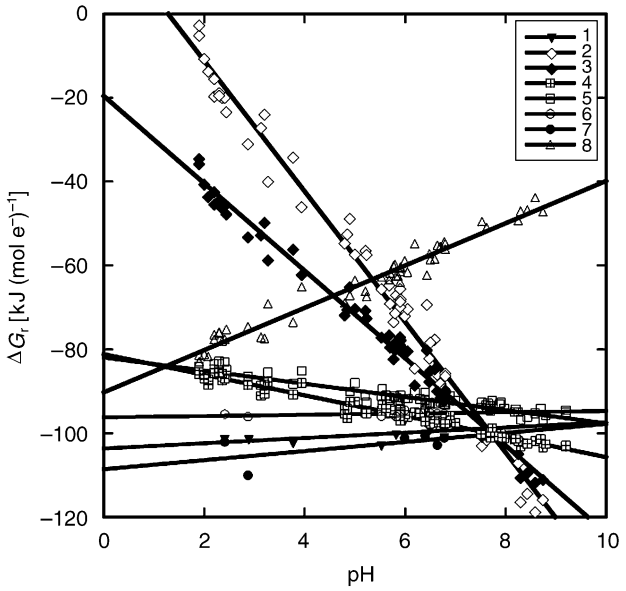

Fig. 7 Values of overall Gibbs free energy [ΔG_r, in kJ (mol e$^-$)$^{-1}$] as a function of pH for eight aerobic oxidation reactions at the Lower Geyser Basin, the greater Obsidian Pool area, and the Sylvan Spring area. Geochemical data to compute values of ΔG_r taken from Shock *et al*. (2005a,b). Numbers in legend refer to reactions given in Table 2. ('Bison Pool', pH 8.1; Obsidian Pool, pH 6.5; Sylvan Spring, pH 5.5.)

Table 2 Chemolithoautotrophic reactions considered in Figs 7 and 8

Reaction Number	Reactions	Electrons transferred
1	$H_2(g) + 0.5O_2(aq) \rightarrow H_2O$	2
2	$3Fe^{+2} + 0.5O_2(aq) + 3H_2O \rightarrow$ Magnetite $+ 6H^+$	2
3	$3Fe^{+2} + 0.25O_2(aq) + 1.5H_2O \rightarrow$ Goethite $+ 2H^+$	1
4	$S^0 + 1.5O_2(aq) + H_2O \rightarrow SO_4^{-2} + 2H^+$	6
5	$H_2S(aq) + 2O_2(aq) \rightarrow SO_4^{-2} + 2H^+$	8
6	$CH_4(g) + 2O_2(aq) \rightarrow CO_2(g) + 2H_2O$	8
7	$CO(g) + 0.5O_2(aq) \rightarrow CO_2(g)$	2
8	Pyrite $+ 2H^+ + 0.5O_2(aq) \rightarrow 2S^0 + Fe^{+2} + H_2O$	2
9	$S^0 + H_2(g) \rightarrow H_2S(aq)$	2
10	$4H_2(g) + SO_4^{-2} + 2H^+ \rightarrow H_2S(aq) + 4H_2O$	8
11	Goethite $+ 0.5H_2(g) + 2H^+ \rightarrow Fe^{+2} + 2H_2O$	1

Rhodothermus, and several phototrophic orders (including the green nonsulphur bacterium *Chloroflexus*) were recovered; neither high-temperature *Rhodothermus*, nor *Chloroflexus* was found in the present study (Fig. 6C). Further, the Aquificales clones from these three studies exhibited significant sequence variability. Note that samples in the three studies were retrieved from different locations in Obsidian Pool, and that the collections spanned an 8-year period (1993–2001). The differences in the bacterial communities at Obsidian Pool may be due to temporal variations in geochemistry, compositional variations in microenvironments, or the resolution afforded by the applied molecular techniques.

This is the first molecular study to examine microbial diversity in the Sylvan Springs area. However, Bridge & Johnson (1998) and Johnson *et al.* (2001, 2003) cultured novel species of *Sulfobacillus*, *Actinobacterium*, and *Methylobacterium* from the same geothermal basin. The metabolic capabilities of these organisms include dissimilatory oxidation of ferrous iron and tetrathionate, as well as reduction of ferric iron. These Bacteria were isolated from undisclosed low temperature locations more acidic than our Sylvan Spring sample. However, these species add to the overall known microbial diversity of the region, which also includes members of the archaeal order Sulfolobales (Brock *et al.*, 1972).

Geochemical energy and microbial diversity

The three hot spring sediment samples targeted in this study are similar in temperature (~80 °C), but significantly different in chemical composition. We showed above that Obsidian Pool, Sylvan Spring, and 'Bison Pool' share certain archaeal and bacterial taxa, but also that they feature variations in community composition. It is beyond the reach of this communication to definitively assert which of the numerous geochemical factors present in these extreme ecosystems most directly control hot spring community structure. The diverse geochemistry in Yellowstone hydrothermal systems results in a complex structure of energy regimes, and a first order investigation of our gene surveys in the context of *in situ* energy-yields for chemolithoautotrophic reactions can help interpret thermophilic ecosystem dynamics (Shock *et al.*, 2005a).

Values of ΔG_r of >180 reactions were calculated for >80 thermal features at Yellowstone (Shock *et al.*, 2005a,b) using data collected between 1999 and 2001; here, we highlight only some of the most exergonic examples in the Obsidian Pool area, Sylvan Springs area, and Sentinel Meadow (Fig. 7), with particular attention paid to the three key springs. Values of ΔG_r (normalized per electron transferred) of eight aerobic oxidation reactions in tens of sample locations are plotted as functions of pH in Fig. 7. The reactions, listed in Table 2, represent the oxidation of methane, hydrogen, carbon monoxide, ferrous iron, and reduced sulphur. It can be seen in this figure that ΔG_r values of some of the reactions are relatively insensitive to changes in pH, yielding large but nearly invariable amounts of energy [80–120 kJ (mol e$^-$)$^{-1}$] over the pH range 2–9. Only the energy-yields for Fe^{2+} and pyrite oxidation (reactions 2, 3 and 8) vary substantially with changing pH, increasing from 0 to 40 kJ (mol e$^-$)$^{-1}$ to >100 kJ (mol e$^-$)$^{-1}$, and decreasing from ~80 kJ (mol e$^-$)$^{-1}$ to ~50 kJ (mol e$^-$)$^{-1}$, respectively, over the pH range of 2 to >8. One of the most exergonic reactions is the knallgas reaction (reaction 1 in Table 2), yielding ~100 kJ (mol e$^-$)$^{-1}$ at all Yellowstone springs investigated here. This reaction represents the central catabolic process of many of the Aquificales, and it was recently proposed as the dominant source of energy for primary productivity in Yellowstone hot springs (Spear *et al.*, 2005). This high energy-yield for reaction (1) may be responsible for the

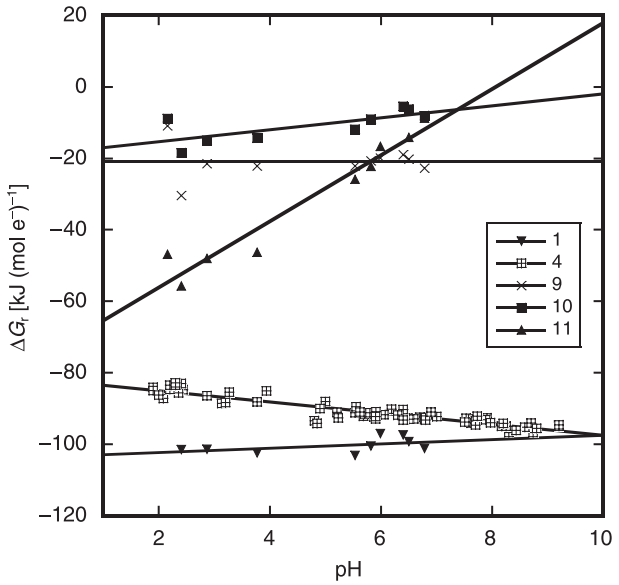

Fig. 8 Values of overall Gibbs free energy [ΔG_r, in kJ (mol e$^-$)$^{-1}$] as a function of pH for five reactions that represent metabolisms mediated by known organisms closely affiliated with clones from this study. Data to calculate values of ΔG_r taken from Shock *et al*. (2005a,b). Numbers in legend refer to reactions given in Table 2. ('Bison Pool', pH 8.1; Obsidian Pool, pH 6.5; Sylvan Spring, pH 5.5.)

apparent ubiquity of the Aquificales at Yellowstone, including at our three key sites (Reysenbach *et al*., 2000; Jackson *et al*., 2001; Jahnke *et al*., 2001; Blank *et al*., 2002; Eder & Huber, 2002; Kato *et al*., 2004; Zhang *et al*., 2004; Spear *et al*., 2005). However, it can also be seen in Fig. 7 that aerobic respiration reactions with other electron donors are equally, if not more, exergonic. Interestingly, of the eight reactions featured in Fig. 7, only two (reactions 1 and 4) are known to be used by phylotypes (the Aquificales and *Sulphurihydrogenibium*) identified in this study, and most exergonic reactions analysed in Shock *et al*. (2005a,b) are not known to support microbial metabolism at Yellowstone.

Values of ΔG_r for five reactions that are known metabolic strategies of taxa similar to the clones identified in this study are shown in Fig. 8 (energy-yields for reactions (1) and (4) catalysed by certain Aquificales are replotted from Fig. 7). For example, some members of the archaeal orders Desulphurococcales and Thermoproteales can obtain energy by the reduction (with H_2) of elemental sulphur (reaction 9) and sulphate (reaction 10). Further, *Geothermobacterium ferrireducens* isolated from Obsidian Pool by Kashefi *et al*. (2002) (close relatives of which were found in all three springs) makes a living by reducing Fe(III); this metabolism is represented here by the reduction of ferric iron in the hydroxide mineral goethite (FeOOH) (reaction 11). Note that the reduction of sulphur species (reactions 9, 10) yields significantly less energy [~5–35 kJ (mol e$^-$)$^{-1}$] than the aerobic reactions (1, 4), which yield >80 kJ (mol e$^-$)$^{-1}$, and both sets of reactions are only weakly dependent on pH. Reaction (11), on the other hand, yields significantly more energy at low pH [~50 kJ (mol e$^-$)$^{-1}$] than at circumneutral pH [~15 kJ (mol e$^-$)$^{-1}$]. At the *in situ* pH (~6.5) of Obsidian Pool fluids, *Geothermobacterium ferrireducens* and other organisms mediating reaction (11) obtain only ~15 kJ (mol e$^-$)$^{-1}$. These findings suggest that 10–20 kJ (mol e$^-$)$^{-1}$ may be a sufficient amount of energy to support *Geothermobacterium* and chemolithoautotrophic strains of Desulphurococcales and Thermoproteales. This is consistent with minimum energy requirements observed for chemolithoautotrophs in Cape Lookout Bight, North Carolina (Hoehler *et al*., 2001). It is also plausible, however, that organisms relying on reactions (9–11) thrive at greater depths in Yellowstone thermal sediments, where conditions are likely more reducing and, hence, these reactions more exergonic. Further studies are necessary that explicitly target metabolic activity and rate measurements of certain redox reactions, especially along redox gradients with depth in thermal sediments.

Many of the organisms in Yellowstone hot springs are known only through clone libraries in this and other investigations (Barns *et al*., 1994, 1996; Takai & Horikoshi, 1999; Marteinsson *et al*., 2001; Reed *et al*., 2002; Schrenk *et al*., 2003). These organisms, identified here as groups UC-I to UC-V and D-I to D-IV, represent 30–90% of the phylotypes found in this study at Obsidian Pool, Sylvan Spring, and 'Bison Pool', but their metabolisms and energy sources are completely unknown. It is therefore difficult to use their phylogenetic positions to help interpret the microbial communities in a geochemical context. However, our abilities to culture the 'unculturables', and thereby elucidate their metabolisms, may be aided by designing growth media that target the most exergonic reactions. Recall that several of the reactions represented in Fig. 7 (and most of the >180 reactions considered by Shock *et al*. (2005a,b)) are not currently known to support any thermophiles. It seems unlikely that these large and numerous energy sources are left untapped by the usually resilient and metabolically versatile organisms in extreme environments.

CONCLUSIONS

The archaeal and bacterial communities in three Yellowstone sediment samples were investigated within the context of geochemical signatures of their greater geothermal regions. Variability in the thermophilic community structures of these features is argued to be a reflection of geochemical conditions and the resulting differences in available energy. Examining the geochemistry of hydrothermal fluids in tandem with the biological community reveals the complexity of the metabolic framework and thermophilic community structure as a function of geochemistry. Even relatively minor differences in pH and other geochemical factors, which translate directly to varying energy-yields from chemolithotrophic reactions, may be responsible for the observed differences in community structure in the thermal environments at Obsidian Pool, Sylvan Spring, and 'Bison Pool'. Within the Bacteria, clones

similar to the alkaline-loving *Thermocrinis* are found only in the circum-neutral Obsidian Pool and alkaline 'Bison Pool'. The Sylvan Spring Aquificales are most similar to the genera *Sulphurihydrogenibium* (made up of terrestrial organisms) (Nakagawa *et al.*, 2005) and *Hydrogenothermus*, *Persephonella*, and *Desulphurobacterium* (primarily marine genera), some of which tolerate mildly acidic environs. It is possible, though not established (Jahnke *et al.*, 2001; Spear *et al.*, 2005), that the apparent ubiquity of the Aquificales within these Yellowstone environments is due to the abundance of energy available via aerobic hydrogen oxidation. Conversely, all three pools feature relatives of *Geothermobacterium ferrireducens* and other members of the Thermodesulfobacteriales, despite relatively modest energy-yields from the reduction of Fe(III). This raises questions about the *in situ* activities of these taxa. In the archaeal domain, the Desulphurococcales are well represented in Sylvan Spring, 'Bison Pool', and Obsidian Pool; the Thermoproteales have a larger presence in Sylvan Spring and Obsidian Pool than in 'Bison Pool'; and the greatest diversity of 'uncultured' Crenarchaeota is found in Obsidian Pool. Because the majority of clones representing the archaeal communities in these features does not affiliate with any cultured organisms, one can only speculate about their modes of metabolism, though geochemical context is essential in developing a model of community dynamics. Future studies focusing on culturing representatives of these groups and metagenomic studies targeting metabolic activity will further link microbial presence and geochemical variation.

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