Arch Microbiol (2003) 180 : 60–68
DOI 10.1007/s00203-003-0562-3

ORIGINAL PAPER

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Novel thermo-acidophilic bacteria isolated from geothermal sites in Yellowstone National Park: physiological and phylogenetic characteristics

Received: 7 January 2003 / Revised: 15 April 2003 / Accepted: 8 May 2003 / Published online: 7 June 2003

Abstract Moderately thermophilic acidophilic bacteria were isolated from geothermal (30–83 °C) acidic (pH 2.7–3.7) sites in Yellowstone National Park. The temperature maxima and pH minima of the isolates ranged from 50 to 65 °C, and pH 1.0–1.9. Eight of the bacteria were able to catalyze the dissimilatory oxidation of ferrous iron, and eleven could reduce ferric iron to ferrous iron in anaerobic cultures. Several of the isolates could also oxidize tetrathionate. Six of the iron-oxidizing isolates, and one obligate heterotroph, were low G+C gram-positive bacteria (*Firmicutes*). The former included three *Sulfobacillus*-like isolates (two closely related to a previously isolated Yellowstone strain, and the third to a mesophilic bacterium isolated from Montserrat), while the other three appeared to belong to a different genus. The other two iron-oxidizers were an *Actinobacterium* (related to *Acidimicrobium ferrooxidans*) and a *Methylobacterium*-like isolate (a genus within the α-Proteobacteria that has not previously been found to contain either iron-oxidizers or acidophiles). The other three (heterotrophic) isolates were also α-Proteobacteria and appeared be a novel thermophilic *Acidisphaera* sp. An ARDREA protocol was developed to discriminate between the iron-oxidizing isolates. Digestion of amplified rRNA genes with two restriction enzymes (*Sna*BI and *Bsa*AI) separated these bacteria into five distinct groups; this result was confirmed by analysis of sequenced rRNA genes.

Keywords Acidophiles · Biodiversity · Iron oxidation · Iron reduction · Moderate thermophiles · Yellowstone National Park

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Abbreviations *ARDREA* Amplified ribosomal DNA restriction enzyme analysis · *Fe*$\underline{O}$ Ferrous sulfate overlay medium · *FeS*$\underline{O}$ Ferrous sulfate/potassium tetrathionate overlay medium · *TSB* Tryptone soya broth

Introduction

Terrestrial geothermal areas are located in various regions of our planet, most particularly around the borders of tectonic plates and in areas where the Earth's crust is relatively thin. In these sites, thermal springs, fumaroles and geysers may be commonplace. Solfatara fields may also be present; these are acidic hot springs, mud pots and soils whose low pH derives from the oxidation of sulfur, formed by the condensation of volcanic gases (H_2S and SO_2). The elevated temperatures and extreme acidity in solfatara fields accelerate the dissolution of minerals, so that these environments may also contain elevated concentrations of soluble metals and silica.

Probably the most famous and well-studied geothermal area is Yellowstone National Park (Wyoming, USA). Much of the pioneering microbiological work in Yellowstone was carried out by Thomas Brock and co-workers (e.g. Brock 1978, 2001). Solfatara fields within Yellowstone, such as Sylvan Springs and the Norris Geyser area, are sites from which moderately thermophilic and acidophilic bacteria (and the eukaryotic alga *Cyanidium caldarium*) and extremely thermophilic and acidophilic archaea (e.g. *Sulfolobus* spp.) have been isolated. In addition, Johnson et al. (2001a) isolated a number of iron-oxidizing *Sulfobacillus*-like moderate thermophiles and *Alicyclobacillus*-like bacteria from sites (40-81 °C; pH 2.1–2.9) within Sylvan Springs, and from the Frying Pan Hot Spring in the Norris Geyser area. Many of these isolates were found to be able to reduce ferric iron to ferrous. Similar gram-positive thermo-acidophilic bacteria were also isolated from the Caribbean island of Montserrat, just prior to the catastrophic volcanic events of 1996 (Atkinson et al. 2000).

The known biodiversity of moderately thermophilic prokaryotes has expanded greatly during the past 20 years

(Hallberg and Johnson 2001). In addition to *Sulfobacillus* sp., there are two other recognized species of moderately thermophilic iron-oxidizing bacteria, *Leptospirillum thermoferrooxidans* (although the single isolate of this gram-negative bacterium has been lost) and *Acidimicrobium (Am.) ferrooxidans*. The archaeon *Ferroplasma acidiphilum* can also oxidize ferrous iron, although its upper growth temperature is about 45 °C (Golyshina et al. 2000). Sulfur-oxidizing moderate thermophiles include *Acidithiobacillus (At.) caldus* and *Hydrogenobacter acidophilus* (Shima and Suzuki 1993); the latter is the most thermophilic of all known acidophilic bacteria (temperature maximum of about 70 °C) although it is less acidophilic than *At. caldus*. *Sulfobacillus* sp. can also oxidize reduced sulfur and, like *Am. ferrooxidans*, metabolize organic carbon compounds as well as fix carbon dioxide (Clark and Norris 1996). Currently, the only classified strictly heterotrophic thermo-acidophilic bacteria are *Alicyclobacillus (Alb.)* sp.

In this paper, we describe the isolation and physiological characterization of moderately thermophilic iron-metabolizing acidophiles, including novel species of bacteria, from two areas in Yellowstone National Park.

Materials and methods

Sampling sites and analyses

Samples (designated YS1–YS6) were taken from six sites within Yellowstone National Park, in October 2000. Three of these sites (YS1–3) were located close to the Gibbon river, about 200 m east of Beryl Spring, and three (YS4–6) from Frying Pan Hot Spring, at the outer fringe of the Norris Geyser area. Measurements of pH and temperature were recorded on site using an Orion model 1230 field meter and combination pH/temperature probe. Water and slurry samples were placed into sterile 25-ml polypropylene tubes, and kept cool (4 °C) until processed in the Bangor laboratory.

Isolation of acidophiles from field samples and enrichment cultures

Dispersed samples were streak-inoculated onto overlaid solid media containing either ferrous sulfate (Feo) or ferrous sulfate plus potassium tetrathionate (FeSo); both media (pH~2.5) also contained a small amount (0.025%, w/v) of tryptone soya broth (TSB). Details of the formulation and preparation of these media are given elsewhere (Johnson 1995). Plates were incubated at 45 °C for up to 20 days. Enrichment cultures (50 ml in 100-ml conical flasks) were set up in 10 mM ferrous sulfate/0.02% (w/v) yeast extract, and in 1% (w/v) pyrite media, both adjusted to pH 2.0 (with sulfuric acid). These were also incubated, with intermittent shaking, at 45 °C. After 6 days (iron/yeast extract enrichments) or 35 days (pyrite enrichments), samples from these cultures were again streak-inoculated onto Feo and FeSo solid media.

Plates were examined using a binocular microscope (at 10–40× magnification) and colonies differentiated based on their color (e.g., resulting from oxidation of ferrous iron and deposition of orange/brown ferric iron) and morphological characteristics. Representative colonies were viewed under a phase-contrast microscope (at 400× magnification) to record cell sizes, morphologies, and the presence or absence of endospores.

Isolates were purified by repeated single-colony isolation on Feo or FeSo solid media and maintained in ferrous iron/yeast extract liquid media (the majority of isolates), or 10 mM ferrous sulfate/0.025% TSB (pH 1.5) for one isolate (Y0018).

Physiological characterization of isolates

pH and temperature limits

The moderate thermophiles were grown in 10 mM ferrous sulfate/0.02% (w/v) yeast extract (or TSB, for isolate Y0018) liquid medium and incubated at 45 °C. The lowest pH value at which isolates could grow was determined by adjusting media pH to 1.90, 1.50, 1.30, 1.00 and 0.80 (with sulfuric acid). Their upper temperature limits were determined by growing isolates in liquid medium (pH 2.0) at 45, 50, 55, 60, 65° and 68 °C.

Oxidation of iron and tetrathionate

To test for iron oxidation, isolates were inoculated into 10 mM ferrous sulfate/0.02% yeast extract (TSB for Y0018) medium (pH 2.0) and incubated at 45 °C. Changes in ferrous iron concentrations were measured using the ferrozine colorimetric method (Lovley and Phillips 1987). Oxidation of tetrathionate was examined by growing isolates in a liquid medium containing 5 mM potassium tetrathionate/0.02% yeast extract (or TSB; pH 2.5) and monitoring changes in sulfate concentrations (turbidometrically: Hydrocheck system, WPA, Cambridge, UK) and culture pH.

Utilization of glucose

Cultures were grown in liquid medium containing 10 mM glucose/0.02% yeast extract (or TSB)/1 mM ferrous sulfate (pH 2.0), and the same medium excluding glucose. The optical densities (at 600 nm) of ±glucose cultures were compared after incubation (at 45 °C) for 1 week. Fermentative growth on glucose was also tested using 10 mM glucose/0.02% yeast extract (or TSB)/1 mM ferrous sulfate (pH 2.0) liquid medium in cultures incubated anaerobically (described below).

Reduction of ferric iron

Isolates were inoculated into 20 ml of liquid medium containing 10 mM ferric sulfate/10 mM glucose/0.02% yeast extract (or TSB), pH 2.0, in 28-ml universal bottles. Uninoculated control cultures were also prepared. The bottles were stoppered with cotton wool inserts, and put into an AnaeroJar, within which an anoxic atmosphere was generated using the AnaeroGen system (Oxoid, UK). Ferric iron reduction was monitored by measuring changes in ferrous iron concentrations using the ferrozine assay.

Growth rates

Iron-oxidizing isolates were grown in liquid medium containing 25 mM ferrous sulfate/0.02% yeast extract (TSB for Y0018), pH 1.8, and incubated, shaken (150 rpm) at 45 °C. Culture doubling times (t_d) were evaluated from semi-logarithmic plots of iron oxidized against time. For the heterotrophic isolates (Y004/8/12/13), the liquid medium contained 10 mM glucose/0.02% yeast extract/1 mM ferrous sulfate (pH 2.0), and growth curves were prepared using optical density (at 600 nm) measurements; these cultures were incubated at both 45 and 55 °C.

Oxidation of pyrite

Iron-oxidizing isolates (excluding Y0018) were inoculated into a liquid medium (100 ml in 250-ml conical flasks) containing 1% ground rock pyrite (Yahya and Johnson 2002) and 0.02% yeast extract (pH 2.0) and incubated, shaken (150 rpm), at 45 °C. Samples were withdrawn at regular intervals to measure concentrations of soluble iron using atomic absorption spectrometry.

Phylogenetic characterization of isolates

Differentiation of isolates using amplified ribosomal DNA restriction enzyme analysis

Iron-oxidizing isolates were grown in 10 mM ferrous sulfate/0.02% yeast extract (or TSB) liquid medium, harvested and lysed (by resuspending in lysis solution containing 0.05 M NaOH+ 0.25% SDS). Their 16S rRNA genes were amplified using Touchdown PCR (Don et al. 1991) in the presence of 2% DMSO. Gene amplification used forward (5′-AGAGTTTGATCMTGGCTCAG-3′) and reverse (5′-TACGGYTACCTTGTTACGA-CTT-3′) primers complimenting positions 8–27 and 1,510–1,492 of the *Escherichia coli* 16S rRNA gene, respectively (Lane 1991). The PCR reaction was carried out in a Progene Techne (Cambridge, UK) thermocycler as follows: an initial denaturation at 95 °C for 5 min, followed by 20 cycles (95 °C for 30 s, 57 °C (-0.5 °C per cycle) for 30 s and 72 °C for 1.5 min) and by 15 cycles (95 °C for 30 s, 47 °C for 30 s and 72 °C for 1.5 min) and a final 10-min incubation at 72 °C. Restriction enzyme digests of the purified products were carried out using *Bsa*AI (New England Biolabs, Beverly, Mass., USA) and *Sna*BI (Promega, Madison, Wis., USA), using manufacturers' protocols. These particular restriction enzymes had previously been shown to be useful in differentiating moderately thermophilic iron-oxidizing bacteria (N. Okibe and D. B. Johnson, unpublished data). Digested DNA was mixed with DNA loading buffer (0.25% (w/v) bromophenol blue in 30% (v/v) glycerol), placed into wells in a 2% agarose gel prepared using high-resolution blend agarose (type 3:1; Amresco), and DNA fragments separated by applying a 50 V current. The gel was stained in an ethidium bromide bath (~10 min) prior to analyzing the DNA pattern under UV light.

Analysis of 16S rRNA gene sequences

The 16S rRNA genes of all 12 isolates were amplified by Touchdown PCR (as above) and purified using purification kits (Qiagen Genomics; Washington, D.C., USA) using the manufacturer's instructions.

The amplified 16S ribosomal gene was sequenced on an ABI Prism Model 3700 (Applied Biosystems, Foster City, Calif., USA). Two hundred nanograms of purified PCR product were incubated with 2 µl Big Dye version 3 Ready Reaction Mix (containing *Taq* FS polymerase, buffer, deoxynucleotides, and the four Big Dye fluorescent dideoxynucleotide terminators), 2 µl 2.5× dilution buffer, 1 µl of sequencing primer (to span the near full-length gene, primers included 27F, 1492R, 515F, 907R, 1100R; 1.6 pmol) and water to a final volume of 10 µl. A consensus sequence was generated for each portion of the 16S ribosomal RNA gene based on a minimum of five independent sequence determinations with each primer. Overlaps between primer reads were used to assemble the final sequence used for subsequent analyses.

The sequence data were compared with 16S rRNA sequences deposited in public databases using the BLAST search program (Altschul et al. 1997). The 16S rRNA gene sequences of various bacteria (including those closely related to the unknown sequence as indicated from the BLAST search) obtained from the GenBank database were aligned with those of YTF3 using ClustalW (Thompson et al. 1994). These alignments were then used to construct a distance matrix (Jukes and Cantor 1969), followed by phylogenetic tree construction by neighbor-joining (Saitou and Nei 1987). These algorithms were provided in PHYLIP version 3.5c (Felsenstein 1993). A phylogenetic tree was constructed using Treeview software (Page 1996).

Results

All of the six sites sampled were acidic (pH range 2.7–3.7) but showed wide variation in temperature (range 30–83 °C). Microscopy indicated that prokaryotes were present in all six samples, with different-sized rods and refractile endospores being readily observed. Sample YS6 was teeming with microorganisms with diverse morphologies, including *Cyanidium*-like eukaryotes (which were also found in YS2), filamentous algae, and diatom-like structures. Fewer microorganisms were evident in YS3 than in the other samples.

Isolation of acidophiles by direct plating

Samples from the four hottest (>70 °C) sites all failed to produce colonies when streaked directly onto overlay plates incubated at 45 °C, with the exception of water from site YS3 plated onto Feo medium, where one colony with a "fried egg" morphology (which is typical of *Sulfobacillus* spp.) developed. This isolate (designated Y006) was selected for more detailed characterization.

At least three different colony types were apparent on Feo medium inoculated with water from site YS2. These were: (1) flat, rhizoidal, ferric-iron-stained colonies (iden-

Table 1 Diversity and putative identities of isolates obtained on ferrous iron and ferrous iron/tetrathionate overlay media, inoculated with enrichment cultures grown in either ferrous iron/yeast extract or pyrite liquid media and incubated at 45 °C. Isolates indicated in **bold** are those subjected subsequently to physiological and phylogenetic analyses. One isolate (Y006; a *Sulfobacillus*-like bacterium) was obtained by direct plating of YS3 water onto a ferrous sulfate overlay plate

Enrichment culture		
	Ferrous sulfate/yeast extract	Pyrite
YS1	*Sulfobacillus*-like (**Y002**)	*Sulfobacillus*-like
YS2	Novel iron-oxidizers (**Y005**) *Alicyclobacillus*-like (**Y004**) *At. caldus*-like	Novel iron-oxidizers (as Y005) *Alicyclobacillus*-like *At. caldus*-like
YS3	No isolates obtained	*Sulfobacillus*-like Gram-negative heterotrophs (**Y0013**)
YS4	*Alicyclobacillus*-like *Sulfobacillus*-like Gram-negative heterotrophs (**Y008**)	Novel iron-oxidizers (as Y005) *Sulfobacillus*-like Gram-negative heterotrophs (as Y008) *At. caldus*-like
YS5	Novel iron-oxidizer (as Y005) *Sulfobacillus*-like	Novel iron-oxidizers (as Y005) *Sulfobacillus*-like (**Y0015**, **Y0016**, and **Y0017**)
YS6	*Alicyclobacillus*-like	Novel iron-oxidizers (as Y005) Gram-negative heterotrophs (**Y0012**) *Sulfobacillus*-like *Acidimicrobium*-like (**Y0018**)

tical to isolate Y005, described below); (2) larger, gelatinous off-white colonies, composed of cells with physiological features (spore-forming heterotrophs that did not oxidize ferrous iron) that were very similar to those of previously-isolated *Alicyclobacillus*-like bacteria (Johnson et al. 2001a); (3) very small, unstained colonies, which were the most numerous – about 20-fold greater than those in (1) and (2). The latter were similar to colonies (e.g. heterotrophs Y008, Y0012, Y0013) that were also isolated from enrichment cultures (Table 1). Type (3) colonies were also the dominant form observed on FeSo plates, although *At. caldus*-like isolates (with characteristic bright-white central zones, caused by the deposition of elemental sulfur) also grew on this medium. Besides confirming that the *At. caldus*-like isolates grew autotrophically on tetrathionate (at 45 °C), forming sulfate, no further characterization was carried out on these bacteria.

Isolates with morphological and physiological traits similar to those of iron-oxidizing *Sulfobacillus* sp., heterotrophic *Alicyclobacillus*, and "Y008-like" bacteria also grew on the Feo plate streak-inoculated with water from site YS6. In addition, there were also numerous small green colonies on this plate; these were identified as *Cyanidium caldarium*-like eukaryotic algae. Very similar colonies were also found on the FeSo plate inoculated with YS6 water, although in smaller numbers, and *At. caldus*-like colonies were the most abundant on this overlay medium.

Isolation of moderate thermophiles from enrichment cultures

In contrast to the results obtained from direct plating of Yellowstone samples, moderately thermophilic isolates were obtained from 45 °C enrichment cultures from all six sample sites (Table 1). In general, a greater diversity of isolates was obtained from the cooler sites (YS2 and YS6) than from those that were >70 °C (YS1, YS3, YS4, and YS5). For example, only endospore-forming *Sulfobacillus*-like isolates were obtained from enrichment cultures of the 78 °C site YS1. Iron-oxidizing colonies that had the same distinctive morphology as those seen on *Feo* medium inoculated directly with YS2 water (Y005-type) were obtained from enrichment cultures of all three samples (YS4, YS5 and YS6) taken at the Frying Pan Hot Spring, as well as, again, site YS2. Gram-positive, sporulating iron-oxidizers (*Sulfobacillus*-like) and non-iron-oxidizers (*Alicyclobacillus*-like) were widely distributed in plated enrichment cultures, as were gram-negative, non-spore-forming heterotrophic isolates (Y008, Y0012, and Y0013). In contrast, *At. caldus*-like isolates were recovered only from enrichment cultures of the two cooler sites (YS2 and YS6).

Physiological characteristics of isolates

Physiological characteristics of the Yellowstone isolates (excluding *At. caldus*-like bacteria and eukaryotes) are listed in Table 2. Eight of the isolates that were studied in detail were iron-oxidizers, and six of these were spore-forming bacteria that could also oxidize tetrathionate, suggesting that they were *Sulfobacillus* sp. The two iron-oxidizing isolates that did not form spores were Y0018, which grew as thin rods and occasionally formed chains, and Y005, which was a filamentous bacterium, forming flocs (1–3 mm diameter) in liquid medium. All of the iron-oxidizers grew in ferrous iron/yeast extract liquid medium, except isolate Y0018 (although TSB successfully substituted for yeast extract). While the four other isolates (Y004, Y008, Y0012, and Y0013) were grown routinely in iron/yeast extract medium, they did not oxidize ferrous iron. Interestingly, isolates Y008, Y0012, and Y0013 (which were phylogenetically closely related to each other) were able to oxidize tetrathionate, but only in media containing organic carbon.

There was considerable variation in tolerance to high temperatures and low pH amongst the 12 isolates. The most thermo-tolerant isolates were the non-iron-oxidizing het-

Table 2 Physiological characteristics of Yellowstone isolates. *ND* Not determined

Isolate	Cell morphology	Endo-spores	$T_{maximum}$ (°C)	$pH_{minimum}$	$S_4O_6^{2-}$ oxidation	Fe^{2+} oxidation	Fe^{3+} reduction	Utilization of glucose	t_d (h)[a]
Y002	3–4 µm rods	+	55	1.3	+	+	+	+	3.1
Y004	2–3 µm rods	+	60	1.0	–	–	(+)	+	8.8
Y005	Long (>20 µm) filaments	–	55	1.9	–	+	–	+	19
Y006	3–4 µm rods	+	55	1.3	+	+	+	+	2.2
Y008	Small 1–2 µm rods	–	65	1.9	+	–	+	+	ND
Y0010	3–4 µm rods	+	50	1.3	+	+	+	+	4.2
Y0012	Small 1–2 µm rods	–	65	1.9	(+)	–	+	+	7.8/(2.8)
Y0013	Small 1–2 µm rods	–	65	1.9	(+)	–	+	+	7.8/(2.8)
Y0015	Single/paired 3 µm rods	+	60	1.3	+	+	(+)	(+)	3.4
Y0016	Single/paired 3 µm rods	+	60	1.3	+	+	+	+	9.7
Y0017	Broad 4 µm rods	+	50	1.3	+	+	+	+	5.6
Y0018	Slender 3–4 µm rods	–	55	1.3	–	+	+	+	6.6

[a]Determined at 45 °C, except for numbers in parentheses (55 °C)

Table 3 Relatedness of Yellowstone isolates to known acidophilic bacteria, and accession numbers of isolate sequences deposited in GenBank. *ND* Sequence not deposited in GenBank

Isolate	Length of 16S rRNA gene determined: Accession number	Closest sequence identity[a] (%) Reference
Y002	1423 bp: ND	SLC66 (95.3%): Johnson et al. 2001b
Y004	1405 bp: AY140236	Bacterium K1 (97.6%): Karavaiko et al. 2000
Y005	1404 bp: AY140237	*Methylobacterium fujisawaense* (96.3%): Green et al. 1988
Y006	1425 bp: ND	SLC66 (95.3%) (99.9% identity with Y002)
Y008	1340 bp: AY140238	*Acidisphaera (As.) rubrifaciens* (93.3%): Hiraishi et al. 2000
Y0010	1333 bp: AY140235	SLC66 (95.3%) (99.9% identity with Y002)
Y0012	1384 bp: ND	*As. rubrifaciens* (93.4%) (99.9% identity with Y008)
Y0013	1290 bp: ND	*As. rubrifaciens* (93.5%) (98.8% identity with Y008)
Y0015	1397 bp: ND	"*Sulfobacillus yellowstonensis*" YTF1 (99.0%): Johnson et al. 2001a
Y0016	1394 bp: ND	"*Sb. yellowstonensis*" YTF1 (98.9%) (99.2% identity with Y0015)
Y0017	1295 bp: ND	"*Sulfobacillus montserratensis*" L15 (98.0%): Yahya et al. 1999
Y0018	1262 bp: ND	*Acidimicrobium ferrooxidans* TH3 (97.6%): Clark and Norris 1996

[a]Based on sequence similarities of 16S rRNA genes

erotrophs, particularly the gram-negative isolates Y008, Y0012, and Y0013, which grew at temperatures up to 65 °C. In contrast, the most heat-sensitive isolate (Y0017) did not grow above 50 °C. The Y008/12/13 group was, however, amongst the least acid-tolerant of the isolates, and did not grow below pH 1.9, whereas *Alicyclobacillus*-like Y004 was the most acid-tolerant isolate and grew at pH 1.0.

Growth of all 12 isolates was superior in media containing organic carbon, and many of the isolates failed to grow in "inorganic" medium. However, autotrophic growth in tetrathionate medium was observed for all of the *Sulfobacillus*-like isolates (Y002/6/10/15/16 and 17). The optical densities of liquid cultures of *Sulfobacillus*-like isolates were also much greater in glucose-supplemented than in glucose-free (ferrous iron/yeast extract) liquid medium, as were those of Y0018 and the heterotrophic isolates that did not oxidize iron (Table 2). Most isolates were also found to reduce ferric iron in glucose-containing medium incubated under anoxic conditions. All of the 10 mM ferric iron was reduced within 10 days, except in cultures of *Alicyclobacillus*-like isolate Y004 (~12% of the Fe^{3+} present) and *Sulfobacillus*-like isolate Y0015 (~20%); limited ferric iron reduction (mean 2.5%) was found in abiotic control cultures. Greater numbers of cells at the end of incubation indicated that many of the isolates could grow via anaerobic respiration, using ferric iron as terminal electron acceptor, although no quantitative data were obtained. However, no growth in glucose medium occurred in anaerobic cultures that did not contain ferric iron, indicating that none of the isolates was able to ferment glucose.

Culture doubling times varied between isolates, although these were determined at fixed temperatures that were not necessarily the temperature optima of the different bacteria. Growth rates determined at 50 °C for the iron-oxidizing isolates were all slower (or very similar) than those determined at 45 °C (data not shown). In contrast, the gram-negative heterotrophs (Y0012 and Y0013), which had both previously found to be more thermo-tolerant than the other isolates, grew much more rapidly at 55 °C than at 45 °C (Table 3).

Leaching of pyrite at 45 °C by the six *Sulfobacillus*-like bacteria and isolate Y005 is shown in Fig. 1. The most successful pyrite-oxidizing isolate was Y0017, followed by Y0015 and Y0016, which displayed virtually identical leaching trends. Mean values are shown, and there was generally <5% variation between replicates. Two of the *Sulfobacillus*-like isolates (Y002 and Y006) did not oxidize pyrite during the 20-day incubation period whereas, following a lag period of about 12 days, the rate of mineral oxidation by isolate Y005 was similar to that of the most efficient *Sulfobacillus* isolates.

Phylogenetic characteristics of isolates

ARDREA proved to be a very useful method for discriminating between the eight iron-oxidizing Yellowstone iso-

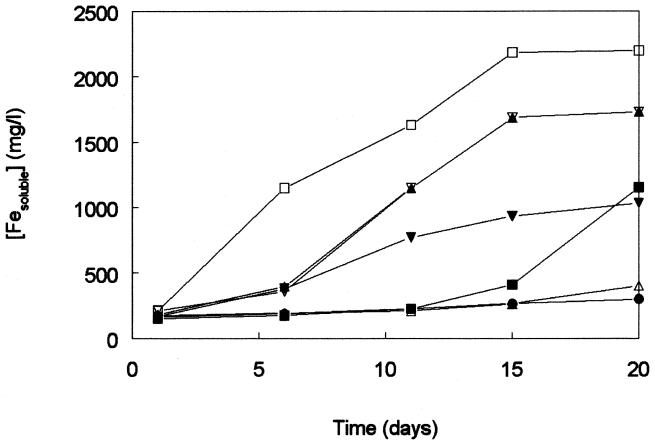

Fig. 1 Oxidation of pyrite by Yellowstone isolates. Y002 (△), Y005 (■), Y006 (●), Y0010 (▼), Y0015 (▲), Y0016 (▽), Y0017 (□)

lates. Digestion of amplified 16S rRNA genes with *Sna*BI produced two distinct fragment profiles (Fig. 2a), one from isolates Y002, Y006, Y0010, and Y0017, and the second from isolates Y005, Y0015, Y0016, and Y0018. When the amplified genes were cut with *Bsa*AI, isolates Y002, Y006, and Y0010 again produced identical patterns, as did isolates Y0015 and Y0016. However, the digested DNA fragments of isolates Y005, Y0017, and Y0018 were different from each other and from other isolates (Fig. 2b).

PCR amplification of the 16S rRNA genes from the 12 isolates gave products varying from 1,262 to 1,425 bp. Comparison of these sequences confirmed results from ARDREA, namely that isolates Y002, Y006, and Y0010 were closely related to each other (99.9% sequence identity) as were isolates Y0015 and Y0016 (99.2% sequence identity). The other four iron-oxidizing isolates were not related to each other or to these two groups (the highest sequence identity (84.3%) being Y0017 with the Y002/6/10 cluster). The 16S rRNA gene sequence analysis of the heterotrophic isolates also correlated well with physiological data, confirming that the gram-negative isolates (Y008, Y0012, and Y0013) were highly related to each other and were distinct from the gram-positive spore-forming heterotroph Y004. The nearest known relatives of each of the Yellowstone isolates, determined by reference to GenBank data, are shown in Table 3, together with the accession numbers of the 16S rRNA gene sequences that were deposited in GenBank. The phylogenetic relationships of the 12 Yellowstone isolates to each other and to other moderately thermophilic bacteria are shown in Fig. 3.

All of the isolates have been deposited in the Acidophile Culture Collection held at the University of Wales, Bangor (http://biology.bangor.ac/research/bart/).

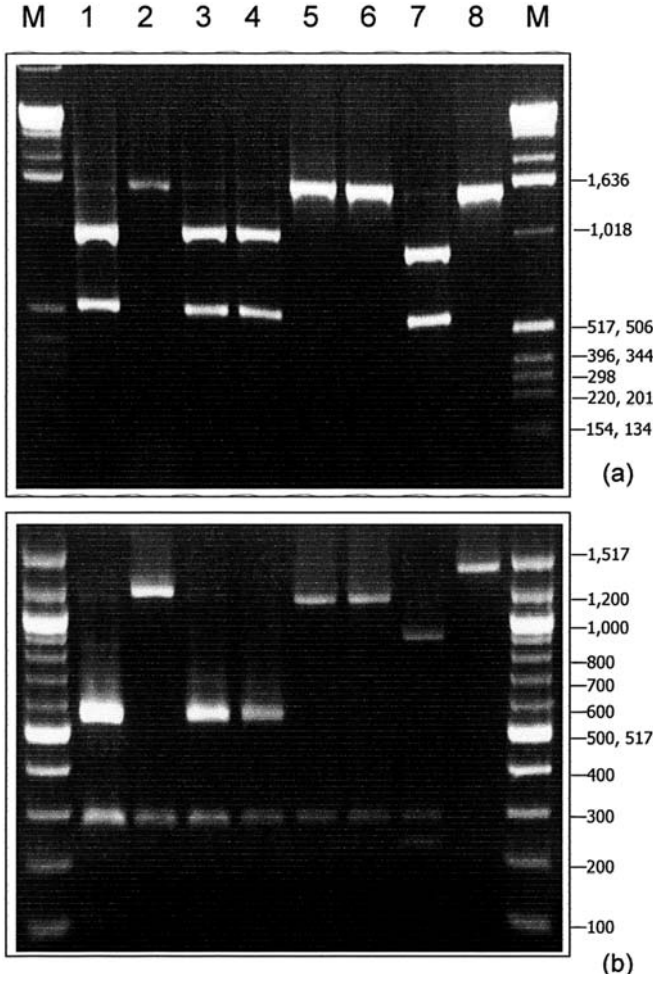

Fig. 2 Electrophoretic analysis of 16S rRNA genes from iron-oxidizing Yellowstone isolates digested with **a** *Sna*BI and **b** *Bsa*AI. *Lanes*: *M* 100-bp DNA ladder (New England Biolabs), *1* Y002, *2* Y005, *3* Y006, *4* Y0010, *5* Y0015, *6* Y0016, *7* Y0017, *8* Y0018

Discussion

This work has focused on the isolation and characterization of moderately thermophilic acidophiles from two geothermal areas in Yellowstone. At least four of the sites were seemingly too hot (>70 °C) for active growth of moderate thermo-acidophiles, which generally have temperature maxima of about 55–60 °C (Hallberg and Johnson 2001). Indeed, direct isolation of moderate thermophiles on solid media was only successful with the two cooler sites, YS2 and YS6 (with the exception of the single spore-forming isolate Y006 isolated from the 83 °C site YS3). However, enrichment in various liquid media followed by plating onto highly efficient and selective solid media resulted in the successful isolation of moderate thermophiles from all six sites. Many of the isolates were spore-formers and might therefore be present as inactive endospores in the >70 °C sites. However, a number of the isolates (iron-oxidizers Y005 and Y0018, and heterotrophs Y008, Y0012, and Y0013) do not form spores

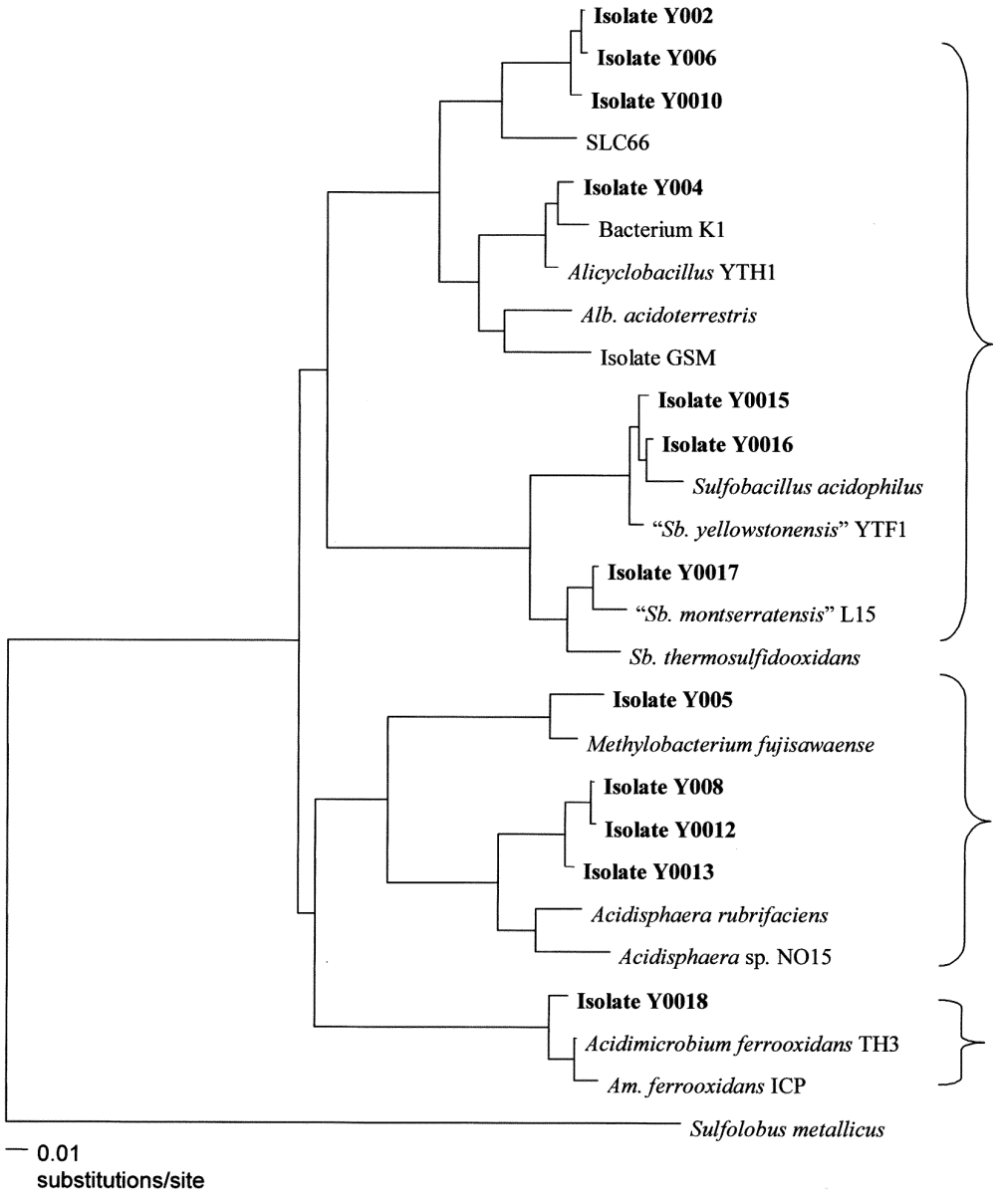

Fig. 3 Phylogenetic relationships of the Yellowstone isolates (*bold*) to known acidophilic prokaryotes. The phylogenetic tree was rooted with *Sulfolobus metallicus*. *Bar* 0.1 nucleotides substitution per 100 for the horizontal branch lengths. Alb. *Alicyclobacillus*, Am. *Acidimicrobium*, Sb. *Sulfobacillus*

and were presumably present in these hot sites as vegetative cells. It is interesting that *At. caldus*-like bacteria (gram-negative sulfur-oxidizers) were only isolated from enrichment cultures from the two cooler sites (YS2 and YS6) whereas gram-negative *Acidisphaera (As.)*-like isolates (Y008/12/13) were also obtained from sites YS3 (83 °C) and YS4 (72 °C). This may be related to differences in temperature maxima of these bacteria, which is >10 °C greater for the *Acidisphaera*-like isolates, making them amongst the most thermo-tolerant acidophilic bacteria to have yet been described.

Many of the isolates that were characterized were able to catalyze the dissimilatory oxido-reduction of iron and the oxidation of sulfur. The co-existence of bacteria that either oxidize or reduce iron (and some that can do both, depending on oxygen concentration) has been reported for lower temperature acidic environments (e.g., Johnson 2001). Both ferrous and ferric iron are soluble in low pH (<~2.3) liquors and, whereas the oxidation of ferrous iron is potentially a source of energy for prokaryotes, ferric iron is an attractive alternative electron acceptor to oxygen in acidic environments, particularly as the redox potential of the ferrous/ferric couple (+770 mV at pH 2.0) is quite similar to that of the O_2/H_2O couple (+820 mV). Since the solubility of oxygen is lower in thermal than in cooler waters, oxygen-depleted and anoxic conditions are more likely to be widespread in geothermal pools and streams, such as those sampled in the current work. The ability to utilize alternative electron acceptors to oxygen would, therefore, appear to be advantageous to indigenous microflora, and it is not surprising that 11 of the 12 isolates were able to reduce ferric iron (some more effectively than others) in anoxic media. Whether the isolates could grow via anaerobic respiration on ferric iron was not as-

certained, although this has been demonstrated for other strains of *Sulfobacillus* sp. and *Am. ferrooxidans* (Bridge and Johnson 1998).

The ARDREA protocol was useful in differentiating the moderately thermophilic iron-oxidizing isolates. The two restriction enzymes used gave identical digestion patterns for isolates Y002, Y006, and Y0010, and for isolates Y0015 and Y0016, and bacteria within these two groups were found subsequently, from analyses of their 16S rRNA genes, to be closely related to each other. The other three iron-oxidizers were all distinct from each other and from the other two groups of bacteria. The ARDREA technique has been used by other research groups to differentiate and identify acidophiles, although this has been applied mostly to mesophiles, such as *At. ferrooxidans* (e.g. Selenska-Pobell et al. 1998) and *Leptospirillum* spp. (Coram and Rawlings 2002). Rawlings (1995) has also described a method for discriminating between *At. thiooxidans* (a mesophilic sulfur-oxidizer) from moderately thermophilic *At. caldus*. This is the first reported use of ARDREA for discriminating between and identifying species of iron-oxidizing moderate thermophiles.

Comparison of the sequences of the 16S rRNA genes of the Yellowstone isolates with those available in GenBank produced some interesting results. Isolates Y0015 and Y0016 were very closely related to a *Sulfobacillus* isolate (*"Sb. yellowstonensis"* YTF1) that had previously been isolated from Frying Pan Hot Spring in Yellowstone (Johnson et al. 2001a); these bacteria all share a number of physiological traits, such as temperature maxima of about 60 °C. In contrast, the *Alicyclobacillus*-like isolate (Y004) shared only 82.3% identity with an earlier Frying Pan isolate (*Alicyclobacillus* YTH1), and it was more closely related (97.6%) to bacterium K1 (Karavaiko et al. 2000). In common with all currently classified species of *Alicyclobacillus*, isolate Y004 does not oxidize ferrous iron, while strain K1 has been reported to do so.

Isolate Y0017 was also identified as a *Sulfobacillus* sp., although its nearest known relative is a mesophile ("*Sb. montserratensis"*) isolated from the Caribbean island of Montserrat (Yahya et al. 1999). Interestingly, isolate Y0017 was the least thermophilic of all of the current Yellowstone isolates and, like "*Sb. montserratensis*" (Yahya and Johnson 2002), it was able to accelerate the oxidative dissolution of pyrite very effectively. The other three spore-forming iron-oxidizing isolates (Y002, Y006, and Y0010) were found to be most closely related to an unclassified low G+C gram-positive mesophilic bacterium (SLC66) that had been oxidized from sulfidic regolith subjected to accelerated bio-oxidation (Johnson et al. 2001b); however, the sequence identity was relatively low (95.3–95.4%) indicating that these are different species. The Y002/6/10 cluster has some physiological traits in common with SLC66, e.g., in being poor pyrite-oxidizers. The phylogeny of this group of bacteria needs to be resolved, although it would appear that they are not members of the *Sulfobacillus* genus, since the 16S rRNA sequence identity(of isolate Y002) with *Sb. thermosulfidooxidans* is only 81.1% and 83.9% with *Sb. acidophilus*.

Isolate Y0018 is most closely related to *Am. ferrooxidans* TH3, with 97.6% 16S rRNA gene sequence identity. Both bacteria have the same temperature maximum (55 °C), and can reduce ferric iron but cannot oxidize reduced sulfur (Bridge and Johnson 1998; Clark and Norris 1996). The type strain of *Am. ferrooxidans* is ICP, which was isolated from a geothermal pool in Iceland, while TH3 (the original strain) was isolated from a copper leach dump in New Mexico (Clark and Norris 1996). Isolate Y0018 is only the third bacterium of the genus *Acidimicrobium* to have been documented, and its relatively low sequence identitiy with *Am. ferrooxidans* TH3 and ICP suggests that it may be a novel species, but more data are required to confirm this.

Phylogenetic analysis of the Yellowstone isolates did, however, reveal two distinctly novel groups of thermoacidophiles. The first of these were isolates Y008, Y0012, and Y0013, whose closest known relative is *Acidisphaera rubrifaciens* (93–94% 16S rRNA gene identity). Currently, *As. rubrifaciens* (isolated from acidic hot springs and mine drainage in Japan) is the single designated species of the genus *Acidisphaera* (Hiraishi et al. 2000), although a bacterium (NO-15) sharing 94.5% rRNA gene identity with the Japanese isolate was isolated from acid mine drainage in Norway (Johnson et al. 2001c). The Yellowstone isolates were even more distantly related to NO-15 (91.9% gene identity) than to the original strain. However, both the original strain and the Norwegian isolate are mesophilic (temperature optimum 30–35 °C) whereas the Yellowstone isolates were found to grow at temperatures up to 65 °C. In view of this, and the relatively low 16S rRNA gene identity, it is clear that the Yellowstone isolates represent a novel species, or possibly genus, of acidophilic heterotrophic bacteria.

The other interesting discovery was the isolation of an iron-oxidizing bacterium (Y005) that is very distantly related to all known acidophiles or iron-oxidizers, and is most closely related to the methylotroph *Methylobacterium fujisawaense* (a mesophilic non iron-oxidizing neutrophile; Green et al.1988). The filamentous, floc-forming nature of Y005 (and other isolates, such as Y003, that were not characterized) readily distinguished it from the other iron-oxidizers in this study. Although it was maintained routinely in ferrous iron yeast extract medium, preliminary experiments have shown that Y005 can also be subcultured in ferrous iron/methanol (10–20 mM) medium but not in "inorganic" ferrous sulfate medium, suggesting that, as indicated by phylogenetic analysis, it is a methylotrophic bacterium. Again, further research is required to clarify the detailed phylogeny of isolate Y005. It is interesting to note, however, that this organism was found in both the Gibbon River and Frying Pan samples, and that it could accelerate the oxidative dissolution of pyrite, suggesting that it may have potential application in commercial mineral oxidation processes that operate at moderately thermophilic (40–55 °C) temperatures.

This study of moderately thermo-acidophilic bacteria in geothermal sites in Yellowstone National Park has yielded new insights into the biodiversity of this group of bacte-

ria. The discovery of novel species and strains of α-Proteobacteria, *Actinobacteria,* and iron-oxidizing *Firmicutes*, in the relatively small number of sites that were examined, indicates that there is still much to be learned about the diversity of these extremophilic bacteria.

Acknowledgements Naoko Okibe is grateful for financial assistance provided by The Institution of Mining and Metallurgy (UK), the Gen Foundation and Glaxo Ltd. We also appreciate the help of Zhe-Xue Quan (Korean Advanced Institute of Science and Technology, Taejon) with the bacterial growth experiments, and thank Lynn Petzke for her assistance in re-analyzing the phlyogenetic tree.

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