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Microbiology of acidic, geothermal springs of Montserrat: environmental rDNA analysis

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Abstract DNA was extracted from water and sediment samples taken from acidic, geothermal pools on the Caribbean island of Montserrat. 16*S* rRNA genes were amplified by PCR, cloned, sequenced, and examined to indicate some of the organisms that might be significant components of the in situ microbiota. A clone bank representing the lowest temperature pool that was sampled (33°C) was dominated by genes corresponding to two types of acidophiles: *Acidiphilium*-like mesophilic heterotrophs and thermotolerant *Acidithiobacillus caldus*. Three clone types with origins in low- and moderate- (48°C) temperature pools corresponded to bacteria that could be involved in metabolism of sulfur compounds: the aerobic *A. caldus* and putative anaerobic, moderately thermophilic, sulfur-reducing bacteria (from an undescribed genus and from the *Desulfurella* group). A higher-temperature sample indicated the presence of a *Ferroplasma*-like organism, distinct from the other strains of these recently recognized acidophilic, iron-oxidizing members of the Euryarchaeota. Acidophilic Archaea from undescribed genera related to *Sulfolobus* and *Acidianus* were predicted to dominate the indigenous acidophilic archaeal population at the highest temperatures.

Key words Acidophiles · Thermophiles · Hot springs · 16*S* rDNA · Phylotypes

Introduction

A great diversity of Bacteria and Archaea in hot spring samples from Yellowstone National Park (USA) was revealed by analysis of rRNA genes that were amplified by the polymerase chain reaction (PCR) from environmental DNA (Barns et al. 1994; Hugenholtz et al. 1998). Many sequences were not sufficiently related to those in databases to indicate affiliations of source organisms to described genera or families. A lower microbial diversity might be expected in strongly acidic hot springs. Previous molecular ecological analyses of acidic environments have concerned industrial sites and relatively low temperatures. Acidic water samples from a mineral sulfide mine in Australia provided sequences from acidophiles that were already familiar from previous work with laboratory cultures (Goebel and Stackebrandt 1995). A similar analysis of acidic samples from another mine site (Iron Mountain, CA, USA) found that all sequences shared greater than 90% identity (and many greater than 97%) with bacterial sequences in databases (Edwards et al. 1999). This article describes analysis of 16*S* rDNA clone libraries to predict the putative principal constituents of the in situ microbiota at various temperatures in acidic geothermal springs on the Caribbean island of Montserrat. A microbial survey of the same sites based on enrichment culture procedures has been described (Atkinson et al. 2000).

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Materials and methods

Sample collection and DNA extraction

Samples of water and sediment were taken from four sites (covering a wide temperature range) at Galway's and Gages Upper Soufrières, Montserrat, in 1996 (Atkinson et al. 2000). Samples were examined by phase-contrast microscopy, but only several days after their collection and maintenance at about 20°C. Samples for DNA extraction were frozen within 2 h of collection, transported frozen to the laboratory, stored at −70°C, and thawed just before analysis. The DNA extraction procedure was that of Barns et al. (1994). In summary, 10 ml of each water–sediment

sample was mixed with 10 ml extraction buffer before a sequence of treatments with lysozyme, proteinase K, lysis buffer, and freeze–thaw cycles. Extractions with phenol and with phenol–chloroform–isoamyl alcohol were followed by treatment of the aqueous phase with polyvinylpolypyrrolidone before precipitation and washing of DNA.

16*S* rDNA cloning, sequencing, and analysis

Amplifications of 16*S* rDNA sequences were performed with a Perkin-Elmer DNA Thermal Cycler 480, using 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1.5 min. Reaction mixtures contained 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, 150 μM each of dATP, dCTP, dGTP, and dTTP, a universal prokaryotic reverse primer (R1492, 5′-TACGGYTACCTTGTTACGACTT-3′), and either a eubacterial (F27, 5′-AGAGTTTGATC MTGGCTCAG-3′) or archaeal (F25, TCYGGTTGA-TCCYGCCRG-3′) forward primer (each primer at 100 pmol). PCR products were cloned using *Taq* polymerase and the Original TA Cloning Kit (Version D) vector (pCR2.1) and host *Escherichia coli* strain (Invitrogen). Groups of clones derived from each sample site were subdivided on the basis of restriction fragment-length polymorphism (RFLP) comparisons following *Sau*3AI and *Rsa*I digests of inserts excised with *Eco*RI. Cloned rRNA genes of subgroup representatives were sequenced using an Applied Biosystems Model 373A Automatic Sequencer. Cloned sequences were checked using the CHIMERA_CHECK version 2.7 of the Ribosomal Database Project (RDP) (Maidak et al. 1999). Databases were searched for similar sequences using BLAST programmes (Altschul et al. 1997). Percentage identities of selected sequences to those of clones were determined following GCG BESTFIT alignments (Genetics Computer Group, University of Wisconsin, USA) and minor, manual modifications where appropriate. Phylogeny analysis used the PILEUP programme of GCG and DNAdist, DNApars, and FITCH programmes of PHYLIP version 3.57 (Felsenstein 1995). GenBank accession numbers for rDNA sequences determined in this work are given in the Results (Fig. 1).

Results and discussion

Site characteristics and preliminary observations on samples

Water temperatures and pH values at sample sites were recorded (Table 1). The conditions at two of the sites that provided samples from which DNA was extracted (near sites N and 11; Table 1) were different from those reported in an account of the site locations (Atkinson et al. 2000). The differences reflected sampling of adjacent but different pools within these designated sample sites. The Galway's Soufrière samples were from small, shallow pools and contained some solid taken from the surface of sediments to a depth of a few centimeters. The Gages Upper Soufrière site was a small pool (with very little sediment) about a meter from its source spring at the base of a bare rock wall where the temperature was 93°C.

Samples that had not been frozen were examined by microscopy several days after they were collected. The numbers of organisms in sediment-rich samples were difficult to estimate, but there was a clear range (Table 1) from numerous rods of various sizes (Galway's Soufrière site L) to only one or two *Sulfolobus*-like organisms visible per field of view (Gages Upper Soufrière site 11). Very little DNA was extracted from samples with few visible cells (Table 1). The highest concentration obtained was still generally at least an order of magnitude less than that obtained using the same procedure with equally acidic material (pH 2) from coal spoil in the UK where quantities ranged from 0.3 μg to several micrograms of DNA per 10 ml of the slurry samples (Burton and Norris, unpublished work).

Environmental 16*S* rDNA analysis

Initial sequencing of more than 300 nucleotides (encoding in most cases the 5′-rRNA domain termini) of clones with specific RFLPs revealed some with insert sequences that were identical to or differed in only one or two positions from those of other more common RFLP types. A regrouping of clone types to recognize the sequence identities

Table 1. Characteristics of hot spring samples and the sample sites at Galway's and Gages Upper Soufrières, Montserrat

	Galway's (near N[a])	Galway's (L[a])	Gages Upper (near 11[a])	Galway's (G[a])
Temperature (°C)	33	48	73	98
pH	3.2	3.0	1.6	3.0
Microscopy[b]	+++ Rods	++++++ Rods	+ *Sulfolobus*-like	++ *Sulfolobus*-like; very few rods
DNA extracted (ng/10 ml sample)	<10	40	<5	20

[a] Site designations refer to Atkinson et al. (2000)
[b] Estimated relative cell numbers and predominant morphologies

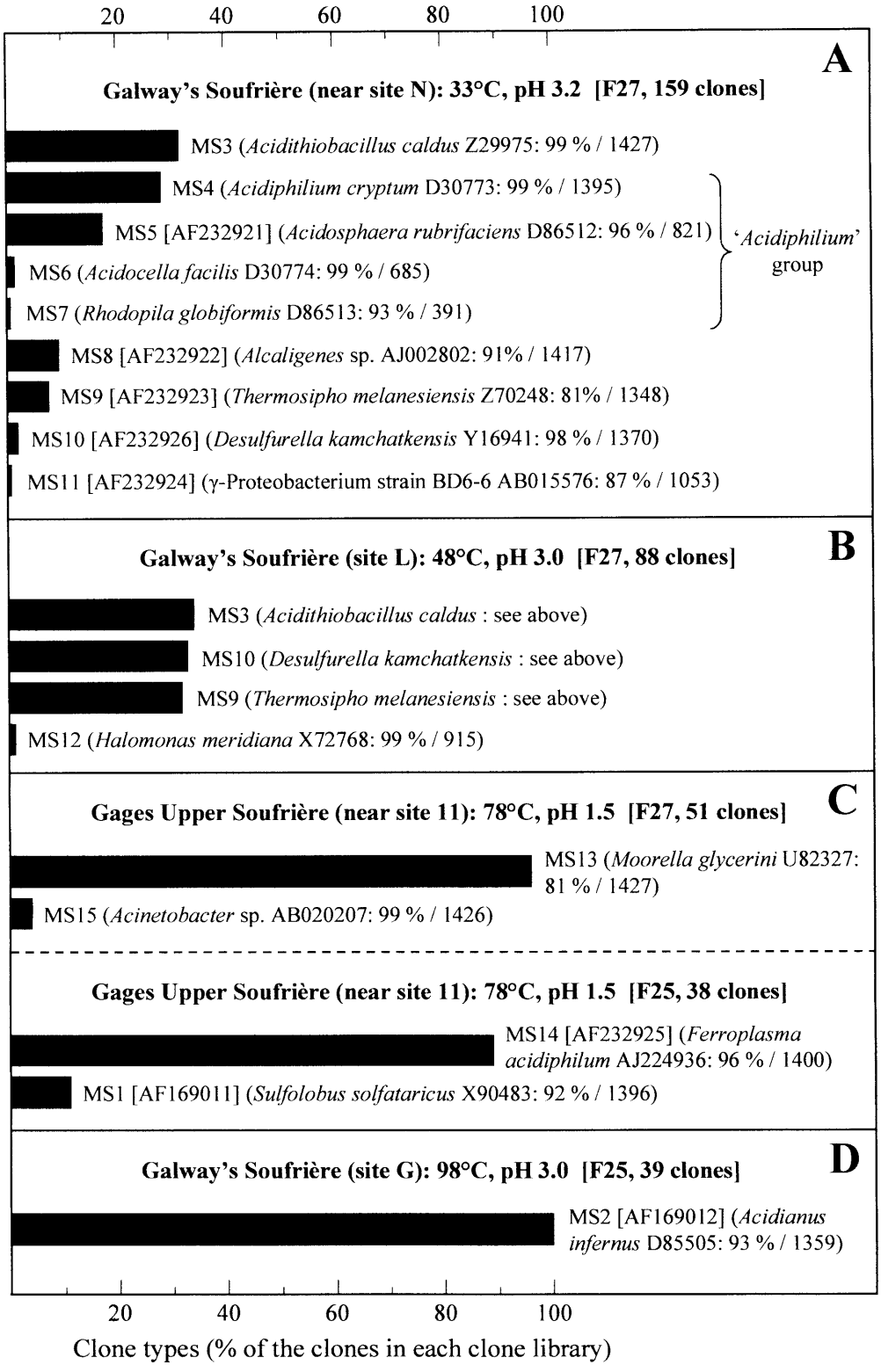

Fig. 1A–D. Analysis of 16*S* rDNA clone libraries. Clone types (*MS numbers*) from the indicated sample sites (**A–D**) are shown as a percentage of the total clones in each of five clone libraries. The forward primers used in the PCR and the number of clones with approximately 1500-bp inserts for which RFLPs were examined are given. GenBank accession numbers of MS clone types are given (in *square brackets*). MS clone type sequences were not deposited in the cases of a short sequence (*MS7*), a sequence with several gaps (*MS12*), and those with greater than 99% identity to established database sequences. Database sequences with the highest percentage identities to MS clone sequences are given (in *parentheses*) with source organism names, GenBank accession numbers, and the numbers of nucleotides over which the comparisons were made

before further sequencing of representative clone types added 5 clones to 77 subsequently found to represent *Acidithiobacillus* (formerly *Thiobacillus*) *caldus* (clone MS3, from two different samples), 5 clones to 40 found to represent *Acidiphilium cryptum* (clone MS4), 3 clones to 27 in the case of clone MS10, and one or two transfers in some other cases. Very closely related species, such as some *Acidiphilium* species that share greater than 99% rRNA gene sequence identity, might not therefore have been distinguished in this sequence analysis. However, almost identical sequences seen in environmental gene analysis could arise from PCR artifacts and multiple gene copy

variation as well as from true strain variation (Field et al. 1997; Wintzingerode et al. 1997).

Many factors preclude relating frequencies of rDNA sequences in clone libraries to relative abundance of organisms in the environment (Felske et al. 1997; Wintzingerode et al. 1997). These factors include potential biases associated with DNA extraction and the PCR and cloning procedures. Some organisms from the Montserrat samples were probably indicated in the clone libraries to the exclusion of other strains that were present in equally low numbers in mixtures because of the random selection of low-abundance genes. However, multiple detection of identical sequences could have indicated source organisms that were likely to be significant members of the communities (see Fig. 1).

All but one of the clone types that comprised more than 10% of clones in each library corresponded to acidophiles in cases in which greater than 90% identity to database sequences was found (Fig. 1). The exception was clone type MS10, with about 98% identity to several *Desulfurella* species (see later). Two clone types that were recovered frequently (MS9 and MS13) did not correspond to database sequences (<82% identity), precluding speculation of the likelihood of acidophily of the source organisms. Two clone types from warm samples had predicted sources that were nonacidophilic and nonthermophilic (clones MS12 and MS15, corresponding to *Halomonas* and *Acinetobacter* species, respectively), but these were single clones and contamination at the sample site or later by nonindigenous strains cannot be ruled out. Many nonacidophilic closest relatives (principally *Alcaligenes* species and other β-Proteobacteria) were also indicated for the source of the fourth most common clone type (MS8) from the 33°C sample (Fig. 1A). However, a maximum of 91% identity to known strains in this case did not preclude a novel acidophile as the sequence source.

Some of the same sequences (>99.5% identity) were recovered from 33°C and 48°C samples. These pairs of sequences were given a single code designation (Fig. 1A,B). The presence of moderate thermophiles in lower-temperature pools might be expected because many of these contained hot spots with higher temperatures than those recorded as representative of the bulk water volume. Clones MS9 and MS10 constituted greater proportions of the clone library from the warmer site, and clone MS10 most closely corresponded to moderately thermophilic *Desulfurella* species (see later). However, sequence identities (98%) were not sufficient to discount a novel strain with a different temperature range for growth. Clone MS3 corresponded to *Acidithiobacillus caldus*, which is known to grow well at both these temperatures (Norris et al. 1986; Hallberg and Lindström 1994) so its presence in both pools would not be surprising.

One cloned sequence in this study appeared obviously chimeric. The sequence (from the 78°C sample) encoded a 16*S* rRNA 5′-domain (the first 550 nucleotides) with highest identity to *Brevibacillus thermorubrum* (but only 84% identity) and central and 3′-domains (positions 550–1171) that corresponded to *Pseudomonas* species (e.g., 99.5% identity to *Pseudomonas rhodesiae*).

Low-temperature site: predicted community types

Approximately a third of the clone types from 33°C and 48°C samples corresponded to *Acidithiobacillus caldus* (Fig. 1A,B). As noted, sulfur-oxidizing *A. caldus* grows over a wide temperature range. Its widespread distribution is already known to include hot springs in the United States, Iceland, and New Zealand and acidic mine sites and coal spoils in England and Australia.

Acidophilic, heterotrophic α-Proteobacteria were indicated as likely source organisms of 48% of the rRNA genes amplified from the 33°C sample (Fig. 1A). The predominant cloned sequence (MS4) corresponded to *Acidiphilium*, placing it in a cluster of sequences from *Acidiphilium cryptum*, *Acidiphilium organovorum*, and *Acidiphilium multivorum*, which share 99% 16*S* rRNA gene sequence identities. The second common sequence from the *Acidiphilium*-like group (clone MS5; 18% of the clones) did not appear to represent a previously described species; its greatest identity was to *Acidosphaera rubrifaciens*, 96%, but this was only marginally more than that to *Acidiphilium cryptum*, 95%. Limited sequencing of two clone types that were obtained only once or twice indicated further variety in the *Acidiphilium* cluster. Clone MS6 corresponded to *Acidocella* (formerly *Acidiphilium*) *facilis*. The likely source of clone MS7 remained unidentified, with similar percentage sequence identities between 92% and 93% to *Rhodopila globiformis*, *Acidiphilium cryptum*, *Acidosphaera rubrifaciens*, and clone MS5 (Fig. 1A). *Acidiphilium* and related species have been found in acidic mine drainage worldwide but have been little studied in natural acidic environments where the emphasis has generally been on thermophiles.

Two other clone types representing unidentified bacteria came only from the low-temperature sample (Fig. 1A). Clone type MS8, as noted earlier, showed just over 90% sequence identity to rRNA genes of several β-Proteobacteria of the *Alcaligenes* group, and a single clone of type MS11 showed 87% sequence identity to the gene from an unidentified γ-Proteobacterium from a deep-sea sediment.

Moderate temperature site: predicted community types

As noted earlier for *Acidithiobacillus caldus*, significant clone types from the 48°C sample were also obtained from the lower-temperature sample. Clone MS10 corresponded to several species of *Desulfurella* with similar percentage identities of rRNA gene sequences (*Desulfurella kamchatkensis* Y16941, 98.2% identity; *D. multipotens* Y16943, 98.1%; *D. acetivorans* X72768, 97.8%; *D. propionica* Y16942, 97.8%). The described species have optimum temperatures between 50° and 60°C and an optimum pH for growth of 6.5–7.0 (Bonch-Osmolovskaya et al. 1990; Miroshnichenko et al. 1994). They are anaerobic, sulfur-

respiring organisms, which suggests that the related sequence from Montserrat samples could reflect sampling of the pool sediments where there might have been a sharp pH gradient between niches appropriate for acidophilic, sulfur-oxidizing *A. caldus* in the water and neutrophilic, sulfur-reducing *Desulfurella* in the sediment. Alternatively, clone MS10 could represent a new species of acid-tolerant *Desulfurella*. Acid-tolerant sulfate-reducing bacteria were isolated from Montserrat samples but remain to be characterized (Sen and Johnson 1999; Atkinson et al. 2000). Clone type MS9 did not correspond to a described species. A database search indicated that the most closely related rRNA genes were from thermophilic sulfur-reducing anaerobes, but none with greater than about 81% identity to MS9 (e.g., *Thermosipho melanesiensis*; Fig. 1). However, a high sequence identity of MS9 (more than 97%) was found to rRNA genes cloned from DNA extracted from an acidic, geothermal site on the Aeolian Island of Vulcano, Italy (S. Simmons and P. Norris, unpublished data).

Heterotrophic, acidophilic bacteria were not represented among rRNA genes amplified from the 48°C sample DNA, even though moderately thermophilic *Alicyclobacillus* and *Sulfobacillus* species were readily isolated in enrichment cultures established with samples from low and moderate-temperature pools (Atkinson et al. 2000). Their absence from clone libraries could reflect the small size of the clone banks, in situ numerical dominance of the bacteria that did provide the cloned genes, or a major contribution from germinating spores to their establishment in enrichment cultures. A bias within the experimental protocol against these spore-forming gram-positive bacilli is considered unlikely because alicyclobacilli and sulfobacilli were more prominently represented than gram-negative thiobacilli in rDNA clone libraries of acidic coal spoil samples from the UK, which were examined at the same time as the Montserrat samples using the same procedures (data not shown).

High-temperature sites: predicted community types

PCR products were obtained from the 98°C sample only when archaeal primers were used (Fig. 1D), whereas archaeal and eubacterial primers provided two groups of clones from the 78°C sample (Fig. 1C). The predominant eubacterial rRNA gene that was cloned (MS13) did not correspond to a described species. A database search showed most sequence similarity to genes from various thermophilic species of the *Bacillus–Clostridium* group, such as *Moorella glycerini*, a neutrophilic, fermentative anaerobe isolated from Yellowstone National Park, USA (Slobodkin et al. 1997), but the sequence identity of just over 80% with MS13 could place the source organism of the cloned sequence in a separate bacterial division. There was little indication from microscopy, ATP analysis, or DNA extraction (see Table 1) of an extensive autochthonous microbiota at the 78°C sample site, but the cloned archaeal sequence did share significant identity with known thermoacidophiles.

At the time of sampling and analysis, clone MS14 indicated a novel group of the Euryarchaeota but now probably confirms the widespread distribution in industrial and natural acidic environments of iron-oxidizing species of recently described *Ferroplasma* species. Clone MS14 shared 96% sequence identity with *Ferroplasma acidiphilum* (Golyshina et al. 2000), an iron-oxidizing relative of heterotrophic euryarchaeal acidophiles of the *Thermoplasma* and *Picrophilus* group. *Ferroplasma acidiphilum* was isolated from a mineral sulfide-processing bioreactor (Golyshina et al. 2000). Similar isolates have been found in acidic mine drainage (Edwards et al. 2000) and appeared to inhabit a copper bioleaching heap from which a rDNA sequence with high similarity to that of *F. acidiphilum* was amplified (Vásquez et al. 1999). However, greater sequence identity of clone MS14 (>98% identity over 1383 nucleotides) was found to rDNA from another iron- and pyrite-oxidizing strain that was isolated from a commercial mineral-processing bioreactor (Norris and Burton, unpublished data) and to an organism isolated from New Zealand hot springs (Norris and Burton, unpublished data).

The optimum growth temperatures of these strains were just above 50°C with growth at 62°C but not at 65°C (Norris and Burton unpublished data), which indicated a temperature range closer to that of *Picrophilus* species than that of the mesophilic *F. acidiphilum*. In view of the 78°C temperature recorded at the Montserrat sample site and the apparently extremely low biomass in situ, it is possible that the strain represented by clone MS14 might have been active only on the fringe of the pool, where a gradient of decreasing temperatures occurred. Archaeal-specific primers were not used with the PCR of DNA from the 48°C sample, so the likely presence of *Ferroplasma*-like organisms at more moderate temperature sites cannot be confirmed.

A temperature gradient within the sample site might also explain the recovery of a sequence (clone MS2) related to those of *Sulfolobus*-like organisms from the hottest site, even though the temperature at the measurement point was higher than the maximum growth temperature of known thermoacidophiles. Two different rDNA sequences (MS1 and MS2) were obtained from the 78° and 98°C sample sites with similar percentage identities (about 91%–93%) to several *Sulfolobus*-like organisms, only one of which is indicated for each clone (Fig. 1C,D). A phylogenetic placement of these sequences among those of *Sulfolobus*-like thermoacidophiles indicated an uncertain relationship to *Acidianus infernus* and *Sulfolobus metallicus* with low bootstrap values (Norris et al. 2000). The finding of only novel sequences of *Sulfolobus*-like rRNA genes from sites that gave rise to enrichment cultures dominated by *S. metallicus* and previously cultured higher temperature strains (Atkinson et al. 2000; Norris et al. 2000) requires further investigation.

This initial survey has provided sequences that could be used to target the previously uncultured organisms in laboratory enrichment cultures under various conditions to facilitate their isolation, as described for the isolation of a previously uncultured thermophile from a Yellowstone

National Park hot spring (Huber et al. 1995). The phylogenetic analysis has indicated potentially suitable culture conditions for some of the uncultured organisms that were the sources of the rRNA genes.

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References

Altschul SF, Madden TL, Schäffer AA, Jinghui Z, Zheng Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402

Atkinson T, Cairns S, Cowan DA, Danson MJ, Hough DW, Johnson DB, Norris PR, Raven N, Robinson C, Robson R, Sharp RJ (2000) A microbiological survey of Montserrat Island hydrothermal biotopes. Extremophiles 4:305–313

Barns SM, Fundyga RE, Jeffries MW, Pace NR (1994) Remarkable archaeal diversity detected in a Yellowstone National Park hot spring environment. Proc Natl Acad Sci USA 91:1609–1613

Bonch-Osmolovskaya EA, Sokolova TG, Kostrikina NA, Zavarzin GA (1990) *Desulfurella acetivorans* gen. nov. and sp. nov. – a new thermophilic sulfur-reducing eubacterium. Arch Microbiol 153:151–155

Edwards KJ, Goebel BM, Rodgers TM, Schrenk MO, Gihring TM, Cardona MM, Hu B, McGuire MM, Hamers RJ, Pace NR (1999) Geomicrobiology of pyrite (FeS_2) dissolution: case study at Iron Mountain, California. Geomicrobiol J 16:155–179

Edwards KJ, Bond PL, Gihring TM, Banfield JF (2000) An archaeal iron-oxidizing extreme acidophile important in acid mine drainage. Science 287:1796–1799

Felsenstein J (1995) PHYLIP (Phylogeny Inference Package), version 3.57c. Department of Genetics, University of Washington, Seattle

Felske A, Rheims H, Wolternik A, Stackebrandt E, Akkermans ADL (1997) Ribosome analysis reveals prominent activity of an uncultured member of the class Actinobacteria in grassland soils. Microbiology 143:2983–2989

Field KG, Gordon D, Wright T, Rappé M, Urbach E, Vergin K, Giovannoni SJ (1997) Diversity and depth-specific distribution of SAR11 cluster rRNA genes from marine plankton. Appl Environ Microbiol 63:63–70

Goebel BM, Stackebrandt E (1995) Molecular analysis of the microbial biodiversity in a natural acidic environment. In: Jerez CA, Vargas H, Toledo H, Wiertz JV (eds) Biohydrometallurgical processing, vol 2. University of Chile, Santiago, pp. 43–52

Golyshina OV, Pivovarova TA, Karavaiko GI, Kondrat'eva TF, Moore ERB, Abraham W-R, Lünsdorf H, Timmis KN, Yakimov MM, Golyshin PN (2000) *Ferroplasma acidiphilum* gen. nov., sp. nov., an acidophilic, autotrophic, ferrous-iron-oxidizing, cell wall-lacking, mesophilic member of the *Ferroplasmaceae* fam. nov., comprising a distinct lineage of the Archaea. Int J Syst Evol Microbiol 50:997–1006

Hallberg KB, Lindström EB (1994) Characterization of *Thiobacillus caldus* sp. nov., a moderately thermophilic acidophile. Microbiology 140:3451–3456

Huber R, Burgraff S, Mayer T, Barns SM, Rossnagel P, Stetter KO (1995) Isolation of a hyperthermophilic archaeum predicted by in situ RNA analysis. Nature (Lond) 376:57–58

Hugenholtz P, Pitulle C, Hershberger KL, Pace NR (1998) Novel division level bacterial diversity in a Yellowstone hot spring. J Bacteriol 180:366–376

Maidak BL, Cole JR, Parker CT Jr, Garrity GM, Larsen N, Li B, Lilburn TG, McCaughey MJ, Olsen GJ, Overbeek R, Pramanik S, Schmidt TM, Tiedje JM, Woese CR (1999) A new version of the RDP (Ribosomal Database Project). Nucleic Acids Res 27:171–173

Miroshnichenko ML, Gongadze GA, Lysenko AM, Bonch-Osmolovskaya EA (1994) *Desulfurella multipotens* sp. nov., a new sulfur-respiring thermophilic eubacterium from Raoul Island (Kermadec archipelago, New Zealand). Arch Microbiol 161:88–93

Norris PR, Marsh RM, Lindström EB (1986) Growth of mesophilic and thermophilic acidophilic bacteria on sulfur and tetrathionate. Biotechnol Appl Biochem 8:318–329

Norris PR, Burton NP, Foulis NAM (2000) Acidophiles in bioreactor mineral-processing. Extremophiles 4:71–76

Sen AM, Johnson DB (1999) Acidophilic sulphate-reducing bacteria: candidates for bioremediation of acid mine drainage. In: Amils R, Ballester A (eds) Biohydrometallurgy and the environment toward the mining of the 21st century. Elsevier, Amsterdam, pp. 709–718

Slobodkin A, Reysenbach A-L, Mayer F, Wiegel J (1997) Isolation and characterization of the homoacetogenic thermophilic bacterium *Moorella glycerinii* sp. nov. Int J Syst Bacteriol 47:969–974

Vásquez M, Moore ERB, Espejo RT (1999) Detection by polymerase chain reaction-amplification and sequencing of an archaeon in a commercial-scale copper bioleaching plant. FEMS Microbiol Lett 173:183–187

Wintzingerode FV, Göbel UB, Stackebrandt E (1997) Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiol Rev 21:213–229