Extremophiles (2006) 10:525–530
DOI 10.1007/s00792-006-0527-y

ORIGINAL PAPER

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Ferroplasma cupricumulans sp. nov., a novel moderately thermophilic, acidophilic archaeon isolated from an industrial-scale chalcocite bioleach heap

Received: 27 March 2006 / Accepted: 4 April 2006 / Published online: 24 May 2006

Abstract A new species of *Archaea* was isolated from an industrial mineral sulphide bioleach heap. Strain BH2, a non-motile pleomorphic coccus, was capable of chemomixotrophic growth on ferrous sulphate and yeast extract. Growth was not supported in the absence of yeast extract. Phylogenetic analysis based on the 16S rRNA gene showed that strain BH2 was most closely related to the species *Ferroplasma acidiphilum*; however, it showed only 95% sequence similarity with this species. Strain BH2 had a temperature optimum of 53.6°C and a temperature range for growth between 22 and 63°C. Thus, it is the first moderately thermophilic member of the genus *Ferroplasma*. The optimum pH for the growth of the strain occurred between pH 1.0 and 1.2 and the lowest pH at which growth was observed was 0.4. Based on 16S rRNA gene sequence analysis and other physiological characteristics, strain BH2 constitutes a new species within the genus *Ferroplasma*. The name *Ferroplasma cupricumulans* is proposed for the new species and strain BH2 (DSM 16651) is proposed as the type strain.

Keywords Acidophiles · Heap bioleaching · Ferroplasma · Moderate thermophile · Archaea · Ferroplasma cupricumulans sp. nov.

Communicated by G. Antranikian

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Introduction

For many years it was thought that *Acidithiobacillus ferrooxidans* (Temple and Colmer 1951) was the most significant microorganism in the leaching of metal sulphides and a major contributor to acid mine drainage (Rawlings 2002; Goebel et al. 2000). In recent years, advances in knowledge of the bioleaching of mineral sulphide ores have compelled researchers to explore extreme mineral leaching environments for microorganisms that have potential commercial applications (Rawlings 2002). This research, together with the development of rapid molecular techniques for the identification of cells by culture-independent methods, has resulted in a greater understanding of the microbial diversity of these extreme sites and has led to the revision and expansion of the acidophilic microbial taxa.

One group of microorganisms, which has been revised because of such studies, is the order *Thermoplasmatales*. Previously this order contained two genera of heterotrophic *Archaea*: *Thermoplasma* and *Picrophilus* (Segerer et al. 1988; Schleper et al. 1995). More recently the order was expanded to include the family *Ferroplasmaceae*, which was first described in 2000 by Golyshina et al. in their description of the chemolithotrophic acidophilic archaeon *Ferroplasma acidiphilum* strain Y^T isolated from a pyrite/arsenopyrite-oxidising bioreactor in Kazakhstan. A second species of the same genus, *Ferroplasma acidarmanus* (Fer1), was also isolated later from an extremely acidic mine drainage site at Iron Mountain (Edwards et al. 2000). Unlike *F. acidiphilum*, *F. acidarmanus* (Fer1) is described as a mixotrophic microorganism capable of growth on ferrous iron and heterotrophic growth on yeast extract alone. Both strains are mesophilic iron-oxidising acidophiles (Dopson et al. 2004).

A number of strains of both of these species of *Ferroplasma* have since been isolated or identified from commercial bioleaching operations (Okibe et al. 2003; Kinnunen et al. 2004) and from naturally occurring

acidic sites (Gonzalez-Toril et al. 2003; Burton and Norris 2000).

Here we describe the isolation and characterisation of a novel moderately thermophilic strain within the genus *Ferroplasma*, for which we propose the name *Ferroplasma cupricumulans*. The strain was isolated from industrial-scale chalcocite bioleach heaps in Monywa, Myanmar (formerly Burma).

Materials and methods

Heap solids and leachate solutions were collected from several of the Myanmar Ivanhoe Copper company (MICCL) heaps for enrichment. The heaps were primarily chalcocite heaps (0.4% copper) that contained up to 5% pyrite. The Myanmar heaps were not 'forced aerated' like many industrial bioleach heaps, although temperatures as high as 46°C were measured in rapidly cooling, freshly excavated material collected from the interior of the heaps.

Enrichment and isolation

Pregnant leachate solutions (PLS) were inoculated into basal nutrient medium containing (g L^{-1}): $(NH_4)_2SO_4$, 1.5; $MgSO_4.7H_2O$, 0.25; KH_2PO_4, 0.25; yeast extract, 0.1. The medium was adjusted to pH 1.8 with concentrated H_2SO_4 (Plumb et al. 2002) with 10 g L^{-1} ferrous sulphate and 2 g L^{-1} sodium tetrathionate, and incubated statically at 50°C. It yielded cells with morphology typical of cells without rigid cell walls. Cells were enumerated using the most probable number (MPN) technique. Strain BH2 was later isolated by serial decimal dilution to extinction on a low pH basal medium (pH 0.8) with 20 g L^{-1} ferrous sulphate and 2 g L^{-1} potassium tetrathionate. Additional samples including heap solids were obtained at a later date and enriched as in the earlier case.

Phylogenetic analysis

Genomic DNA was obtained from isolates by phenol-chloroform extraction and precipitated with sodium acetate and isopropanol (Plumb et al. 2001). DNA extracts were purified before amplification using the Ultra-Clean PCR Clean-up Kit (MO BIO Laboratories Inc.) The 16S rRNA gene was amplified using the HotstarTaq Master Mix (Qiagen) with the primer 25F arch (5′-TCY GGT TGA TCC YGC CRG-3′) (GeneWorks Pty Ltd.) and a modified 1492R primer (5′-ACG GIT ACC TTG TTA CGA CTT-3′) Amplification products were purified using the Ultra-Clean PCR Clean-up Kit (MO BIO Laboratories Inc.).

Sequencing of the strain was conducted using the ABI protocol for sequencing using a Big Dye Terminator v3.1 Cycle Sequencing Kit as recommended by the manufacturer (Applied Biosystems) PCR and sequencing reactions were performed using a Peltier Thermal Cycler (MJ Research). Electrophoresis of the purified sequence was conducted by the Western Australian Genome Resource Centre. Sequences were assembled and edited using ChromasPro software (Technelysium Pty Ltd.). Assembled sequences were aligned with sequences from the GenBank database using BLAST (Basic Local Alignment Search Tool) (Altschul et al. 1997). Phylogenetic analysis was conducted using the ARB program (Ludwig et al. 2004). A phylogenetic tree was constructed using the distance matrix and neighbour-joining method with the Jukes and Cantor single parameter correction method (Jukes and Cantor 1969) Purified genomic DNA was analysed for its mol% G + C content by Dr Peter Schumann of the DSMZ.

Phenotypic characterisation

The temperature range for growth of the strain was determined in a temperature gradient incubator (Terratec). Cultures were prepared in custom-made 20 mL L-shaped tubes with 18 mL of basal medium (pH 0.8) supplemented with 20 g L^{-1} of ferrous sulphate and 2 g L^{-1} of potassium tetrathionate. Each tube was inoculated with 2 mL from an actively growing culture on the same medium. Twenty-four tubes were placed into the temperature gradient incubator and incubated at temperatures between 35 and 65°C. A temperature gradient of less than 40°C is advised by the manufacturer to ensure accurate temperature control within the heating block. The activity of the culture at each of the 24 temperatures was determined from the ferrous iron oxidation rate. Ferrous iron in solution was measured using the method of Wilson (1960).

For strain BH2, ferrous iron oxidation was not exponential with time, rather the rate of ferrous iron oxidation followed zero order kinetics. The square root of the reciprocal of the time taken for ferric iron concentration to increase by 2 g L^{-1} in each tube was plotted for each temperature and the Ratkowsky equation (Ratkowsky et al. 1983) was fitted to the data to determine the theoretical maximum, minimum and optimum temperatures for activity of the strain. The pH optimum for the strain was determined using the ferric iron production rate. Cultures were prepared in 250-mL Erlenmeyer shake flasks over a pH range. After inoculation of each flask with a standardised inoculum, the change in the concentration of ferrous iron in solution was measured over time.

Strain BH2 was tested for growth on a number of substrates. Cultures were prepared in 50 mL sterile tubes with 1% w/v of each of the test substrates in basal medium. All substrates except elemental sulphur were added from filter-sterilised stock solutions. Elemental sulphur was sterilised by heating at 100°C for 1 h repeated twice on consecutive days. The effects of each substrate on growth were tested with and without yeast

extract (0.01%). Growth of the strain on each substrate was monitored using a phase contrast microscope after three successive subcultures. The substrates tested included D-glucose, D-cellobiose, yeast extract, meat extract, tryptone, sodium acetate, elemental sulphur, potassium tetrathionate, sodium thiosulphate and manganese sulphate.

Strain BH2 was also tested for growth under anaerobic conditions on basal medium (pH 1.2) with the addition of ferric sulphate as an electron acceptor and potassium tetrathionate as an electron donor. Cultures (50 mL) were prepared under nitrogen gas in 100-mL serum bottles. Following inoculation, the headspace was replaced with CO_2. Ferrous iron concentrations in solution and cell numbers were monitored over time.

Results and discussion

Two strains were isolated from enrichment cultures inoculated with heap solids. Both strains showed variable morphology typical of wall-less cells. Early in the growth of the culture they appeared as large irregular-shaped cocci, often with budding cells typical of other species of wall-less *Archaea* (Segerer et al. 1988). Older cultures were dominated by very small, more regular-shaped cocci (approx. 0.2–0.5 μm). Results of the MPN cultures indicated that these morphotypes were dominant within the heap samples (Hawkes et al. 2005).

16S rRNA gene sequencing (GenBank AY907888) of the two isolates indicated that both were most closely related to the archaeon *F. acidiphilum*. A BLAST search of the GenBank database showed that the 16S rRNA gene of both strains shared 99% sequence similarity with the uncultured archaeon MS14, a clone sequence identified from samples obtained from geothermally heated pools on the island of Montserrat (Burton and Norris 2000). The most closely related cultured microorganism was *F. acidiphilum*Y^T with both strains sharing 95% sequence similarity.

Although strains BH1 and BH2 were isolated under different conditions (pH 1.2 and 0.8, respectively) both strains shared 100% similarity in their 16S rRNA gene sequence. Phylogenetic analysis of the 16S rRNA gene of strain BH2 showed that the strain constituted a new moderately thermophilic species within the genus *Ferroplasma* (Fig. 1). The G + C content of genomic DNA was determined to be 34.0%, which is lower than the G + C contents obtained for *F. acidiphilum* (36.5%) and *F. acidarmanus* (36.8%).

Temperature and pH optimum

The temperature optimum for BH2 was determined using the rate of ferric iron generation as a measure of microbial activity rather than using a direct measure for growth. The optimal temperature for ferrous iron oxidation, determined after fitting the Ratkowsky equation,

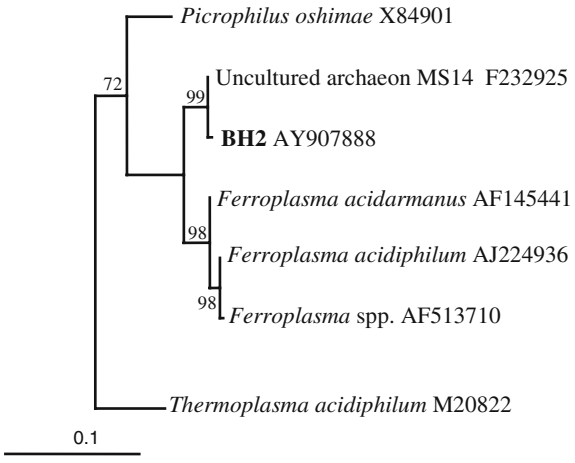

Fig. 1 Phylogenetic analysis based on the 16S rRNA gene sequences of species within the order of *Thermoplasmatales*.. The archaeon *Sulfolobus acidocaldarius* was used as the out-group for tree construction (not shown). The scale shows 0.1 nucleotide substitutions per position in the gene. Bootstrap probabilities (percentage) are based on 10,000 iterations

was 53.6 ± 0.46°C and the theoretical temperature maximum and minimum were 63 ± 0.21 and 22.6 ± 0.84°C, respectively (Fig. 2). Subsequent experiments in which turbidity was measured in addition to ferrous iron oxidation confirmed that the oxidation rate was related to growth, with the theoretical temperature optimum determined using the turbidity data 52.9 ± 0.76°C (Fig. 3). The zero-order reaction kinetics was held for both biomass growth and ferrous iron oxidation. This zero-order reaction rate for ferrous iron oxidation has also been observed in *F. acidiphilum* (Franzmann et al. 2005). The growth kinetics of these strains is yet to be explained, however it is possible that the constant ferrous iron oxidation rate is the result of substrate limitation within the medium. The temperature

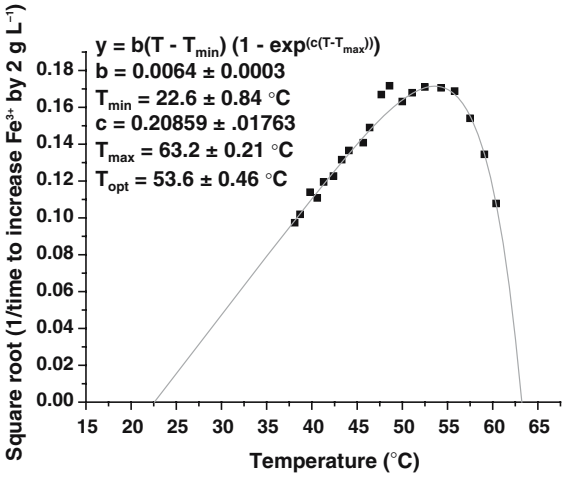

Fig. 2 The response activity of strain BH2 to changing temperature. The Ratkowsky equation was fitted to the data from which the theoretical T_{min}, T_{opt} and T_{max} were determined

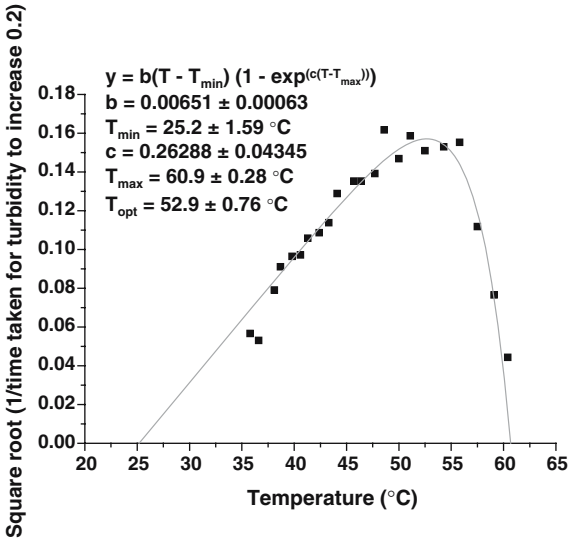

Fig. 3 The growth response of strain BH2 (based on turbidity) to changing temperature. The Ratkowsky equation was fitted to the data from which the theoretical T_{min}, T_{opt} and T_{max} were determined

optimum of BH2 distinguishes it from the two previously described species of *Ferroplasma*. Unlike strains of *F. acidiphilum* and *F. acidarmanus*, strain BH2 is a moderately thermophilic species with a temperature optimum well above *F. acidiphilum* and *F. acidarmanus*.

The pH optimum was also determined using the rate of ferrous iron oxidation. The optimum pH range for microbial activity for strain BH2 was between 1 and 1.2. Some microbial activity occurred at the upper and lower limits of the pH test range, however activity at these extremes was low (Fig. 4).

Ferric iron reduction under anaerobic conditions

Strain BH2 showed the ability to reduce ferric iron in the presence of 0.02% yeast extract when grown under an atmosphere of CO_2. The ability to reduce ferric iron under anaerobic conditions did not form part of the initial characterization of *F. acidiphilum*, which was described as an obligate aerobe. A latter comparative study of four strains of *F. acidiphilum* and *F. acidarmanus* (Dopson et al. 2004) showed that ferric iron reduction occurred in all strains tested when incubated with a suitable electron donor under anaerobic conditions. Given that the heaps from which BH2 was isolated were not 'forced aerated', it is likely that oxygen transfer was limited within the heaps and that microaerophilic or anoxic areas existed. It is possible that this ability to reduce ferric iron under anaerobic conditions led to the dominance of strain BH2 in the MICCL bioleach heaps. The high ferric iron concentration and low oxygen concentration within the heap environment would favour microorganisms capable of this type of metabolism (Bridge and Johnson 1998; Johnson and McGinness 1991).

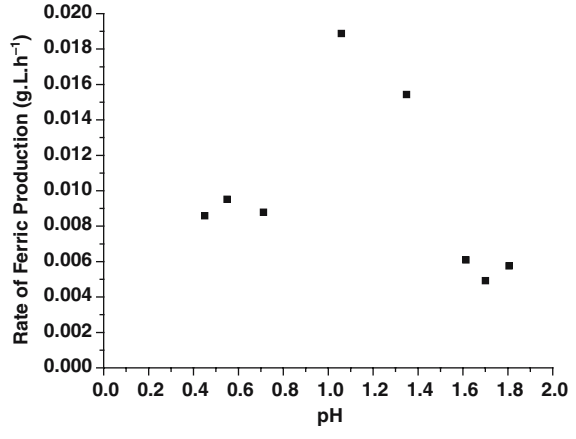

Fig. 4 Response of the activity of strain BH2 to changing pH

Growth on organic and inorganic substrates

Significant growth was observed only in those cultures that contained ferrous iron and yeast extract. Cell numbers persisted in the culture with yeast extract only; however, cell numbers remained low after successive subcultures. No growth was obtained on any other substrate when tested in the absence of ferrous iron and yeast extract. No growth was obtained from aerobic cultures containing elemental sulphur and other reduced sulphur compounds including potassium tetrathionate and sodium thiosulphate.

Some debate surrounds the potential for autotrophic growth by the previously described species of *Ferroplasma*, and indeed the requirement for organic carbon is different for members of the two described species. *F. acidarmanus* Fer1 was shown to have an obligate requirement for organic carbon and may grow organotrophically on yeast extract alone (Dopson et al. 2004). *F. acidiphilum* however, was described as a strict autotroph that required a small amount of yeast extract as a micronutrient source (Golyshina et al. 2000). Like *F. acidiphilum*, growth of strain BH2 could not be sustained on yeast extract alone and growth could not be sustained without the addition of yeast extract or ferrous iron. These results suggest that strain BH2 grows chemomixotrophically.

Conclusions

Some key phenotypic differences exist between the two previously described species of *Ferroplasma* and strain BH2 isolated from the Myanmar heap bioleach operation. Based on 16S rRNA gene sequence analysis, strain BH2 appears most closely related to *F. acidiphilum*; however, it differs from *F. acidiphilum* in that it appears to have an obligate growth requirement for organic carbon. Interestingly, strain BH2 appears to have metabolic capabilities similar to those of *F. acidarmanus*; however, in this study it could not be grown organo-

trophically on yeast extract alone. It is difficult to ascertain the growth requirements of the strain using a complex substrate, such as yeast extract. Further study of the essential growth requirements provided in the yeast extract is required. This may also elucidate the nature of the proposed limiting growth factor indicated by the zero-order growth kinetics observed in this study. Strain BH2 was moderately thermophilic, a novel characteristic for a member of the genus *Ferroplasma* that clearly differentiates this strain from previously described species of *Ferroplasma*.

Based on phylogenetic analysis of the 16S rRNA gene and physiological differences, strain BH2 constitutes a new species of the *Archaea* within the genus *Ferroplasma* for which we propose the name *F. cupricumulans*.

Emended description of the genus Ferroplasma (Golyshina et al. 2000; Dopson et al. 2004) In addition to the characteristics of the genus described by Golyshina *et al.* (2000) and Dopson *et al.* (2004), the genus includes facultative anaerobic species capable of chemomixotrophic and chemo-organotrophic growth. The G + C content is 34–37 mol%. The type species of the genus is *F. acidophilum* strain Y^T.

Description of F. cupricumulans sp. nov. Hawkes, Franzmann (O'Hara and Plumb 2005) F. cupricumulans (cu.pri.cu' mu.lans. N.L. net. n. cuprum copper, L. part. pres. cumulans heaping up) Isolated from a commercial chalcocite heap leaching operation in Myanmar. *F. cupricumulans* is a moderately thermophilic archaeon, which grows optimally at 55°C and which has a maximum temperature of 63°C for growth. Morphology was described for this genus. It has an optimum pH of 1.0–1.2. The strain grows chemomixotrophically, oxidising ferrous iron in the presence of yeast extract. A facultative aerobe, the stain reduces ferric iron under anaerobic conditions. The mol% G + C content of genomic DNA is 34.0%.

The strain has been deposited within the German Collection of Microorganisms and Cell Cultures (DSMZ) (DSM 16651) and the Japan Collection of Microorganisms (JCM) (JCM 13668).

Acknowledgments The authors would like to acknowledge Paul Monaghan and the Myanmar Ivanhoe Copper company (MICCL) for their assistance with sampling. We also would like to thank Prof. Hans G. Trüper for the Latin name of *Ferroplasma cupricumulans*. Dr Peter Schumann of Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) determined the mol% G + C content of strain BH2.

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