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ScienceDirect
Trans. Nonferrous Met. Soc. China 23(2013) 1774−1782
Transactions of Nonferrous Metals Society of China
www.tnmsc.cn

Isolation, characterization of *Acidiphilium* sp. DX1-1 and ore bioleaching by this acidophilic mixotrophic organism

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Received 11 January 2013; accepted 5 June 2013

Abstract: The isolation and characterization of a subspecies of *Acidiphilium* that not only acts as an enhancer of other autotrophic acidophiles in bioleaching, but also has significant leaching capacity towards marmatite was described. *Acidiphilium* sp. DX1-1, a Gram-negative, motile, short rod-shaped bacterium that accumulates intracellular polyhydroxybutyrate, was isolated from the Dexing copper mine area in China. It is mesophilic and acidophilic with an optimum growth at 30 °C and pH 3.5. Phylogenetic analyses identify that it is a member of genus *Acidiphilium* and closely related to *Acidiphilium cryptum* and *Acidiphilium multivorum*. It is mixotrophic, utilizing organic substrates and a range of inorganic substrates, such as sulfur, ferric iron and a variety of sulfide minerals. *Acidiphilium* sp. DX1-1 is able to bioleach 40% of the zinc content of marmatite with the initial pH 3.5 within a month, which is even higher than that of *A. ferrooxidans* or the mixed culture with *A. ferrooxidans* at even lower pH.
Key words: *Acidiphilium* sp.; bioleaching; marmatite; chalcopyrite; polyhydroxybutyrate; 16S rRNA

1 Introduction

Bioleaching, also known as biooxidation or biomining, is the use of microorganisms to extract metals from ores and tailings. The microorganisms used for bioleaching are mainly chemoautotrophs, such as *Acidithiobacillus ferrooxidans*, *Acidithiobacillus thiooxidans* and *Leptospirillum* spp., that gain energy by oxidization of ferrous iron (Fe^{2+}), sulfur (S^0) and reduced inorganic sulfur compounds [1]. The consequential ferric iron (Fe^{3+}) and low pH of the leaching effluent facilitates subsequent metal oxidation and extraction [2]. These chemoautotrophs are highly sensitive to organic compounds released from themselves and by cell lysis. The dissolved organic carbon is metabolized by heterotrophic and mixotrophic microorganisms, such as *Acidimicrobium*, *Ferroplasma, Sulfobacillus* and *Acidiphilium* spp., that also play significant roles in the bioleaching process [3]. It would therefore be of interest to identify organisms that able to bioleach metal ores in the presence of both organic and inorganic substrates.

Acidiphilium spp. are Gram-negative, mesophilic, aerobic, acidophilic, heterotrophic, rod-shaped bacteria. The genus *Acidiphilium* currently contains six species: *Acidiphilium acidophilum* (formerly *Thiobacillus acidophilus*) [4−6], *Acidiphilium angustum* [7], *Acidiphilium cryptum* [8], *Acidiphilium multivorum* [9], *Acidiphilium organovorum* [10] and *Acidiphilium rubrum* [7]. The *Acidiphilium* spp. bacteria can grow on a wide range of organic substrates, and some species are able to reduce ferric iron (Fe^{3+}) and/or oxidize elemental sulfur [8,11−13]. These species are often found in close association with the acidophilic chemoautotrophs in acidic mining environments. The type species *A. cryptum* was isolated from *Acidithiobacillus ferrooxidans* cultures [8].

In this work, we describe the isolation, characterization of *Acidiphilium* sp. DX1-1 and ore bioleaching by this acidophilic mixotrophic organism, which not only acts as an enhancer of other autotrophic acidophiles in bioleaching, but also has a significant leaching capacity by itself towards marmatite.

Foundation item: Projects (2004CB619204, 2010CB630901) supported by the National Basic Research Program of China; Project (NCET-07-0869) supported by Program of New Century Excellent Talents in Ministry of Education of China; Projects (50321402, 50374075) supported by the National Natural Science Foundation of China
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DOI: 10.1016/S1003-6326(13)62660-6

2 Experimental

2.1 Sample origin

Liquid samples were collected from the Yangtao Wu reservoir in the Dexing copper mine of Jiangxi province in China, which was mainly composed of the acid mine drainage, infiltration water and rain water. The pH of collected samples was 3.0, and the water temperature was 27 °C when sampling. Chemical analysis of water sample was carried out by inductively coupled plasma-atomic emission spectroscopy (ICP-AES). Besides iron and sulfur, the most abundant metals present were magnesium and aluminium (Table 1).

Table 1 Concentrations of selected elements and ions of water sample from Yangtao Wu reservoir

Element and ion	Content/ ($mg \cdot mL^{-1}$)	Element	Content/ ($mg \cdot mL^{-1}$)	Element	Content/ ($mg \cdot mL^{-1}$)
S	4401	Si	56.26	W	1.81
Mg	1102	P	6.52	Sn	1.1
Fe	981.83	Co	4.09	Hg	1.07
Fe^{2+}	125	Ni	4.02	Mo	0.59
Al	944	Zn	3.18	Cr	0.35
Ca	336.35	Sb	3.14	Cd	0.15
Cu	100	As	1.95	Ti	0.04
Mn	64.38	Pb	1.89	Ag	0.01

2.2 Culture conditions

9K basic medium ($(NH_4)_2SO_4$ 3.0 g, KCl 0.1 g, KH_2PO_4 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $Ca(NO_3)_2$ 0.01 g, distilled water 1000 mL) with different proportions of $FeSO_4 \cdot 7H_2O$ and glucose were used for flasks enrichment of the organisms [14]. 9K-glucose solid medium (9K basic medium with 1% glucose and 1.5% agar powder, pH 3.5) was used for isolation and purification. 9K-glucose liquid medium (9K basic medium with 1% glucose) was used for growing cells and determining the physiochemical characterization. 9K basic liquid medium, adjusted the pH to 3−3.5 with H_2SO_4, was used for energy utilization determination. All the 9K basic medium described above was sterilized by autoclaving at 121 °C for 20 min, the $FeSO_4 \cdot 7H_2O$ and glucose solutions were filter sterilized with a 0.22 μm cutoff filter. Inoculum was incubated under aerobic shacking conditions of 30 °C and 200 r/min.

2.3 Enrichment and isolation

Acidiphilium spp. in the water sample were enriched in the 9K basic medium supplemented with gradually increasing concentration of glucose from 0.01% to 1% and decreasing concentration of $FeSO_4$ from 4.5% to 0 in four serial subculturing steps [6].

Isolation of glucose-grown *Acidiphilium* spp. colonies was performed on 9K-glucose solid medium. Eleven single colonies were picked, of which one isolate named DX1-1 was chosen for further investigation. DX1-1 was deposited in China Center for Type Culture Collection (CCTCC) under the number as CCTCC CSU 208092.

2.4 Morphology and subcellular structure observation

Morphological characteristics of the isolate were observed with an optical microscope (Olympus CX−31) after Gram staining. Surface and inner substructures of the cells harvested from the logarithmic growth phase and fixed by 4% glutaraldehyde were examined with a scanning electron microscope (SEM, JEOL JSM−6360 LV) and transmission electron microscope (TEM, JEOL JEM−1230) after cytochemical treatment and staining.

2.5 Physiochemical characterization

1 mL of log phase culture (about 1×10^8 cell) was inoculated into 100 mL of 9K-glucose liquid medium to determine the optimal temperature and pH for growth. Effects of the temperature and the pH on the growth of the strain were determined by direct cells counting under a microscope. Growth curves of the strain were obtained under optimal conditions and by counting the cells.

To test inorganic substrate utilization, the strain DX1-1 inoculum was inoculated in 9K basic medium supplemented with 1% (w/v) alternative inorganic substrate—$FeSO_4 \cdot 7H_2O$, S^0, $Na_2S_2O_3$, $Fe_2(SO_4)_3$, $FeCl_3$, CuS, pyrrhotite, chalcopyrite, pyrite, marmatite and jarosite. The characteristics of the organic substrates were investigated by BIOLOG method [15]

2.6 G+C content, 16S rRNA gene sequencing and phylogenetic analysis

Cells were harvested by centrifugation and washed with TE buffer (pH 8.0). Genomic DNA was extracted by UNIQ-10 Spin Column Genomic DNA Minipreps Kit (Sangon) according to the protocol of the Kit. The G + C content of DNA was determined by China Center for Type Culture Collection (CCTCC), using HPLC as described by MESBAH et al [16].

Amplification of 16S rRNA gene of the isolate was carried out in PCR cycler (T-Gradient Thermoblock, Biometra) using universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) [17]. Amplification reactions were performed in a total volume of 50 μL. The reaction mixture contained 5 μL of 10×PCR buffer, 5 μL of 20 mmol/L $MgCl_2$, 1 μL of 10 mmol/L dNTPs mixture, 1 μL of primers 27F (5 pmol/μL) and 1492R (5 pmol/μL), respectively, 0.5 μL of *Taq* polymerase

(NEB, 5 U/μL) and 1 μL of template DNA (about 100 ng/μL). The following thermo cycling procedure was used: an initial holding at 94 °C for 3 min, followed by 30 cycles at 94 °C for 45 s，55 °C for 1 min, and 72 °C for 1 min, then followed by an extension to the last cycle at 72 °C for 7 min. The PCR product was purified using E.Z.N.A^{TM} Gel Extraction Kit (OMEGA). The product was integrated into pBS-T vector using the pBS-T PCR Products Clone Kit (Tiangen Biotech, Beijing). 3 μL of ligation mixture was used to transform into *Escherichia coli* DH5α competent cells and plated on Luria-Bertani (LB) medium containing ampicillin (100 μg/mL), 5-bromo-4-chloro-3-indolyl-*β*-*D*-galactopyranoside (X-Gal, 200 μg/mL) and isopropyl *β*-*D*-thiogalactopyranoside (IPTG, 40 μg/mL). The white recombinant colonies were screened and sequenced on an ABI 3730 sequencer (Sunbiotech Co., Ltd, China).

The 16S rRNA gene sequence of strain DX1-1 was comparatively analyzed with the nucleic acid database of GenBank. Phylogenetic relationships of strain DX1-1 and other *Acidiphilium* spp. bacteria were derived by aligning sequences by Clustal X.

2.7 Extraction and identification of intracellular polymers

Intracellular polymers were extracted and recovered by SDS-EDTA method [18]. Sodium dodecyl sulfate (SDS) and EDTA were added into the cell suspension to the final concentration of 7 g/L and 10 g/L, respectively. The mixture (pH 7.0) was stirred at 50 °C for 15 min and followed by a centrifugation at 4000 r/min for 15 min. The precipitate was washed with distilled water and further purified with chloroform. The Fourier transform infrared (FTIR) spectroscopy was used to identify the purified intracellular polymers. The IR spectra of the extracted polymers and the standard PHB (Sigma-Aldrich) were recorded on a Nicolet FTIR 740 spectrometer in the range of 4000−400 cm^{-1} using KBr pellets.

2.8 Leaching experiment

Leaching experiments were carried out with the isolate DX1-1 and the mixture of the isolate DX1-1 and *Acidithiobacillus ferrooxidans* QSX-1 (accession number: CCTCC AB 206200). QXS-1 was isolated by our lab and deposited in China Center for Type Culture Collection (CCTCC). Marmatite and chalcopyrite were used for the leaching experiment separately.

2.9 Marmatite leaching experiments

The pure isolated strain DX1-1, *A. ferrooxidans* QSX-1 which had been propagated by cultivation on marmatite for one generation and their mixed cultures were used to leach marmatite collected from Dachang mining area of Guangxi province in China. The chemical components of the marmatite were 47.4% Zn, 16.99% Fe, 30.85% S and 4.76% others. Experiments were carried out in 250 mL flasks containing 100 mL of 9K basic medium plus 5% (w/v) of marmatite powder (below 200 mesh, <74 μm). Diluted sulfuric acid (2%) was used to adjust the pH value of the leaching solutions. Marmatite leaching by sole strain DX1-1 (initial pH 3.5), *A. ferrooxidans* QSX-1 (initial pH 2.0) and the mixture of the two strains (initial pH 2.0 and 3.5) during 30 d was observed and compared with control group. Approximately identical number of cells (about 1×10^7 cell) were used as inoculum and the experiments were conducted at 30 °C and under 200 r/min of shaking condition. 1 mL of samples were removed every 2 or 3 d for determining the concentrations of soluble Zn(II) ions by an atomic absorption spectrophotometer (Hitachi Z-8000). The lost water (sampling and evaporation) in the medium was supplemented with sterilized 9K basic medium after sampling.

2.10 Chalcopyrite leaching experiments

The chalcopyrite leaching experiments were performed with the same protocol, except the concentrations of soluble Cu(II) ions were determined every 4 d. The chalcopyrite used in the leaching experiment was collected from Daye mining area of Hubei province in China. The mineralogical composition of the chalcopyrite sample was 60.8% chalcopyrite ($CuFeS_2$), 20.7% pyrite (FeS_2), 8.4% $CaCO_3$, 4.6% SiO_2 and 5.5% others.

2.11 Nucleotide sequence accession number

The nucleotide sequences of the 16S rRNA gene reported in this study was deposited in the GenBank nucleotide sequence databases under the accession number EF556220.

3 Results

3.1 Cell morphology and ultrastructure

The isolate DX1-1 was adaptively enriched in 9K-$FeSO_4$-glucose liquid culture, isolated and further purified from 9K-glucose solid medium. Colonies on solid medium appearing were round, ivory-white, translucent, convex, smooth and 2–3 mm in diameter after incubation at 30 °C for 3 d (Fig. 1(a)).

DX1-1 is Gram-negative, motile and short rod-shaped. Cells are (1.7±0.2) μm long and (0.7±0.05) μm wide and can form short chains (Fig. 1(b)). Transmission electron microscopy (TEM) (Fig. 1(c)) revealed a large number of granules inside the cells, accounting for approximately 90% of the cell volume. These were extracted and identified as the high

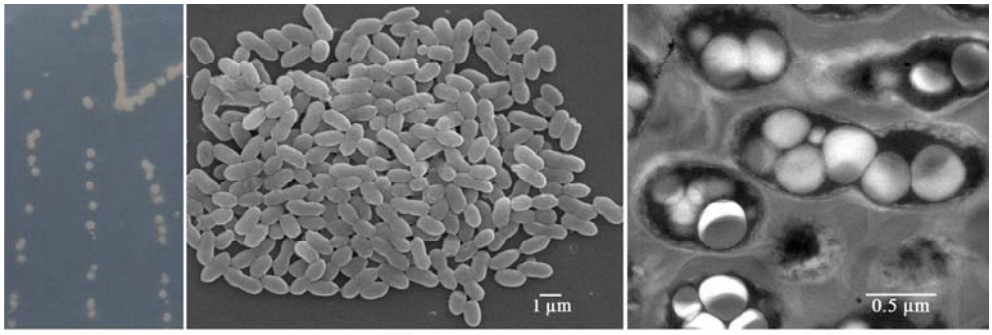

Fig. 1 Colony morphology and subcellular microstructures of strain DX1-1: (a) Colony on solid medium; (b) Scanning electron microscope (SEM); (c) Transmission electron microscope (TEM)

molecular mass polyester polyhydroxybutyrate (PHB), which is utilized as a stored carbon source in many species of bacteria and can be harvested for using as being biodegradable thermoplastic. The FTIR spectrum of the polymer obtained from the strain DX1-1 is essentially identical to that of the standard PHB from Sigma (Fig. 2). The FTIR spectra of both PHB samples show typical ester (C═O) absorption at 1724 cm^{-1} and a series of intense bands located at 1000–1300 cm^{-1} corresponding to the stretching of the C—O bond of the ester group. The other absorption bands at 1380, 1457 and 2976 cm^{-1} region correspond to —CH_3, —CH_2 and —CH groups.

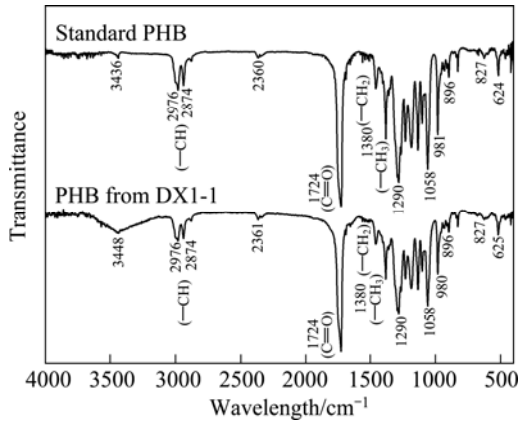

Fig. 2 FTIR spectra of polymer from strain DX1-1 and standard PHB from Sigma

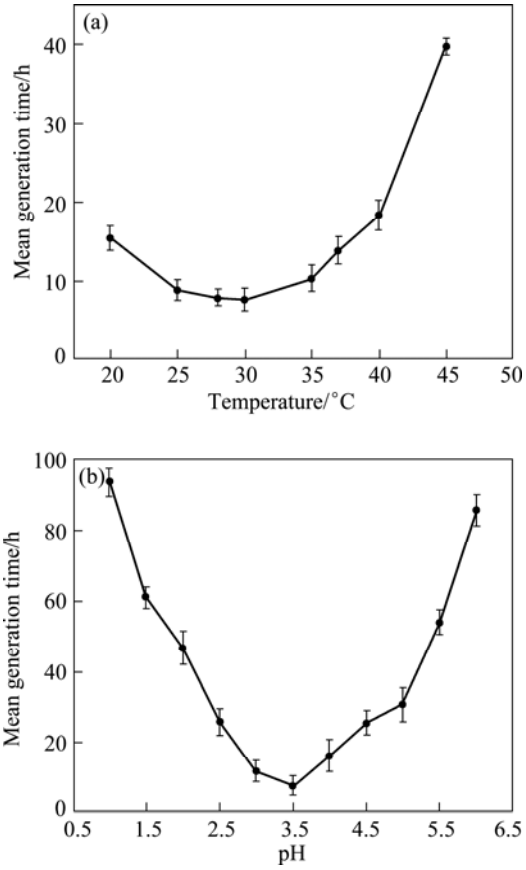

Fig. 3 Effect of temperature (a) and pH (b) on growth of strain DX1-1

3.2 Optimal growth conditions

The strain DX1-1 is mesophilic and able to grow between 20 and 45 °C, with a temperature optimum of 30 °C (Fig. 3(a)). The pH range for growth is between 1.0 and 6.0 with an optimum at pH 3.5 (Fig. 3(b)). To study the growth curve of strain DX1-1, cells in the logarithmic growth phase were inoculated into flasks containing 100 mL of 9K-glucose liquid medium (pH 3.5, initial concentration 1.0×10^6 cell/mL) and incubated on an orbital shaker at 30 °C and 200 r/min. The growth curve indicated that the logarithmic growth phase of DX1-1 starts at about 30 h, and the stationary phase begins at about 128 h with the cell concentration reaching 1.21×10^9 cell/mL (Fig. 4(a)). The specific growth rate constant (μ) was calculated to be 0.09 h^{-1} with a mean generation time of 7.67 h.

3.3 Utilization of substrates

Strain DX1-1 can grow on a wide range of inorganic substrates, including autotrophical growth by oxidizing elemental sulfur, reducing ferric iron (Fe^{3+}) to

ferrous iron (Fe^{2+}), and utilizing jarosite and a variety of sulfide minerals (Table 2). DX1-1 grows well in the 9K basic medium supplemented with 1% elemental sulfur (Fig. 4(b)). The growth rate constant (μ) was 0.024 h^{-1} and the mean generation time was 28.90 h. Elemental sulfur was oxidized to the final metabolite sulfate, reducing the pH value of the medium from 3.5 to 1.0 in 17 d. DX1-1, like other sulfur oxidizing bacteria, can play an important role in bioleaching of metal sulfide ores during a process that accumulates elemental sulfur as a critical intermediate [19]. We also evaluated growth on organic substrates using the BIOLOG technique, demonstrating that DX1-1 can grow heterotrophically using a wide range of organic substrates (Table 3).

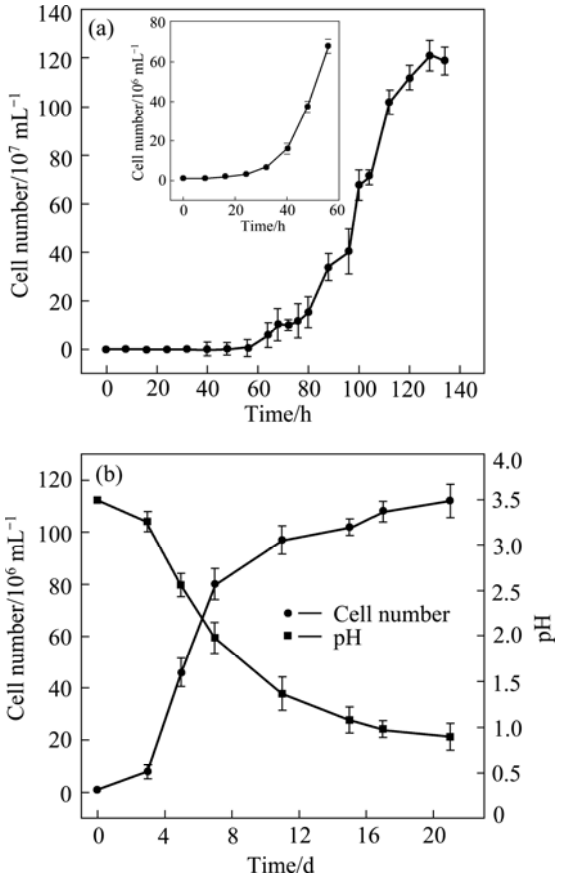

Fig. 4 Growth curves of strain DX1-1 cultured in 9K-glucose (a) and 9K-S (b) and pH change during growth in 9K-S medium (b) (Insert in (a) is the growth curve during the first 60 h)

Table 2 Inorganic substrate utilization of strain DX1-1

Substrate	Utilization	Substrate	Utilization
$FeSO_4·7H_2O$	+/−	Jarosite	+
Elemental sulfur	+	CuS	−
$Na_2S_2O_3$	−	Pyrrhotite	−
$Fe_2(SO_4)_3$	+	Chalcopyrite	+
$FeCl_3$	+	Pyrite	+
$Fe(OH)_3$	+	Marmatite	+

+ means that it can be utilized; − means that it cannot be utilized; +/− means uncertain.

Table 3 Organic substrate utilization of strain DX1-1

Substrate	Utilization	Substrate	Utilization
Control	−	*P*-hydroxy acid	−
α-cyclodextrine	+	Itaconic acid	+
Dextrin	+	*α*-ketobutyric acid	+
Glycogen	+/−	*α*-ketoglutarate	+
Tween 40	+	*α*-aminolevulinicaid	+
Tween 80	+	*D*-*L*-lactic acid	+
N-acetyl-*D*-galactosamine	+	Malonate	+
N-acetyl-*D*-glucosamine	−	Propionic acid	+
Adon sugar alcohols	+	Quinate	+
L-arabinose	−	*D*- glucarate acid	+
D- arabinose	+	Decanedioic acid	+/−
D-cellobiose	+	Succinate	+
i- erythritol	+	Bromosuccinic acid	+
D- fructose	+/−	Daminozide	+
L-fucose	+	Glucuronic amide	+
D-galactose	+	*L*-propanamide	+
Gentiobiose	+	*D*-alanine	+
α-*D*-glucose	+	*L*-alanine	+
m- inose	+	*L*-propylamine acyl-gly	+
α-*D*-lactose	+	*L*- asparagine	+
Lactulose	+	*L*-aspartic acid	+
Maltose	+	*L*-glutamate	+
D-mannitol	+	Glycyl-*L*-aspartic acid	+
D-mannose	+/−	Glycyl-*L*-glutamic acid	+/−
D-melibiose	+	*L*-histidine	−
β-methyl-*D*-glucoside	+	Hydroxy-*L*-proline	+
Alor ketose	+	*L*-leucine	+
D-raffinose	+	*L*- ornaline	+
L-rhamnose	+	*L*-phenylalanine	+
D-sorbierite	+	*L*-proline	+
Sucrose	−	*L*-pyroglutamate	−
D-trehalose	+	*D*-serine	−
Turanose	+	*L*-serine	+
Xylitol	+	*L*-threonine	+/−
Methyl acetone	+	*D*,*L*-carnitine	+
Monomethyl succinate	+	*γ*- aminobutyric acid	+
Aceticacid	+	Urocanic acid	+/−
Cis-aconitic acid	+	Inosine	+
Citric acid	+	Uridine	+
Methanoic acid	+	Thymidine	+
D-galactonolactone	+	Phenethylamine	+
D-galacturonic acid	+	Putrescine	+
D-gluconic acid	+	2-aminoethanol	+
D-glucosamine	+	2,3-butanediol	+
D-glucuronate	+	Glycerol	+
α-hydroxybutyrate	+/−	*D*,*L*-*α*-glycerol phosphate	+
β- hydroxybutyrate	+/−	1-phosphate glucose	+
γ- hydroxybutyrate	+	*β*- phosphate glucose	+

+ means that it can be utilized; − means that it can not be utilized; +/− means uncertain.

3.4 Phylogenetic analysis of DX1-1

Phylogenetic analysis of the 16S ribosomal RNA (16S rRNA) gene (comprising 1450 bp, between positions 27 and 1492 in *E. coli* 16S rRNA gene sequence numbering) indicated that DX1-1 is a member of the alpha subclass of the proteobacteria and belongs to the genus *Acidiphilium* (Fig. 5). The 16S rRNA gene sequence of DX1-1 (GenBank: EF556220) shares identity of 99.72% and 99.66% to that of *Acidiphilium cryptum* ATCC33463 and *Acidiphilium multivorum* AIU301, respectively (Table 4). The G+C content of genomic DNA of strain DX1-1 is 62.3% (mole fraction), which is lower than that of all the other *Acidiphilium* spp. strains (Table 4).

3.5 Bioleaching studies of DX1-1

Bioleaching of marmatite and chalcopyrite by DX1-1, *A. ferrooxidans* QXS-1 and a mixed culture of these two strains were investigated at initial pH of 2.0 and 3.5 (Fig. 6).

In the marmatite leaching study, *Acidiphilium* sp. DX1-1 worked very efficiently on marmatite (Fig. 6(a)). The total extraction amount of zinc in 30 d was 9.30 g/L with the initial pH 3.5 in the leaching solution. This was about 34 times higher than the extracted amount (0.27 g/L) in the acidic media without bacteria under the same conditions. Due to the large initial inoculum, the amount of zinc extracted increased rapidly to 8.78 g/L during the first 18 d. However, the leaching rate slowed down with little contribution to the total extraction amount during the following 12 d, suggesting that the higher concentrations of the metal ions accumulated during the leaching procedure may inhibit growth. In contrast, the typical bioleaching bacterium *A. ferrooxidans* worked less efficiently than DX1-1 on marmatite. The total zinc extraction amount was 7.21 g/L in 30 d with the initial pH 2.0 of the leaching solution.

The zinc extraction efficiency of the mixed culture consisting of both strains (1:1) was investigated with different initial pH of the leaching solution: pH 2.0 (the

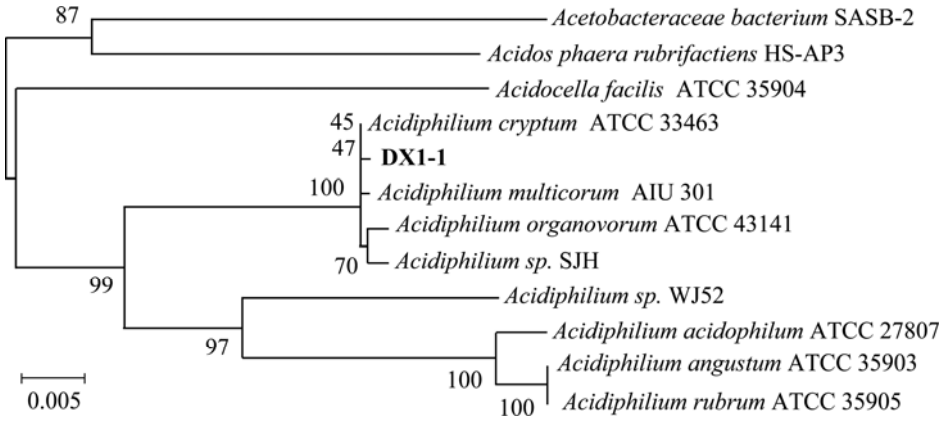

Fig. 5 Phylogenetic tree derived from 16S rRNA gene sequence of strain DX1-1 (Numbers at nodes indicate bootstrap values of neighbor-joining analysis (>50%) from 1000 replicates. The scale bar indicates 0.005 substitutions per nucleotide position)

Table 4 DNA base composition of 16S rRNA homology among *Acidiphilium* species [8,20]

Strain	Mole fraction of G+C/%	16S rRNA sequence similarity to DX1-1/%	16S rRNA gene sequence accession number
DX1-1	62.3	100	EF556220
Acidiphilium cryptum ATCC 33463	67.3	99.72	D30773
Acidiphilium cryptum JF-5	69.8	99.24	Y18446
Acidiphilium multivorum AIU 301	67.6	99.66	AB006711
Acidiphilium organovorum ATCC43141	67.4	99.50	D30775
Acidiphilium sp. SJH	n/a	99.43	AY040740
Acidiphilium angustum ATCC 35903	63.4	94.85	D30772
Acidiphilium rubrum ATCC 35905	63.2	94.85	D30776
Acidiphilium sp. WJ52	n/a	94.79	AY495956
Acidiphilium acidophilum ATCC 27807	63.5	94.23	D86511
Acidocella facilis ATCC35904	64.2	93.14	D30774
Acidosphaera rubrifaciens HS-AP3	n/a	93.11	D86512

n/a is not available.

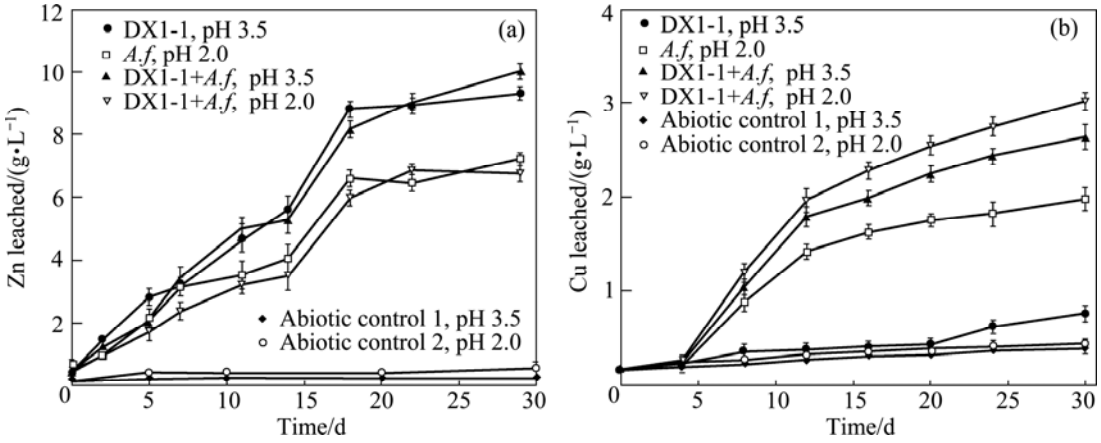

Fig. 6 Comparison of marmatite leaching (a) and chalcopyrite leaching (b) by sole or mixture of *Acidiphilium* sp. DX1-1 and *A. ferrooxidans* during 30 d flasks shaking

optimal pH of *A. ferrooxidans*) and pH 3.5 (the optimal pH of *Acidiphilium* sp. DX1-1), respectively. The mixed culture revealed similar marmatite leaching ability to the pure culture with the same initial pH. It was deduced that *Acidiphilium* sp. DX1-1 was the dominant bacterium in the mixed-culture facilitated marmatite bioleaching system at its optimal pH 3.5, while *A. ferrooxidans* was the dominant species at pH 2.0. The mixed culture did not enhance the leaching rate of marmatite. However, the leaching capacity in the mixed culture group with the initial pH 3.5 was not inhibited after 22 d. Interactions between the two species might enhance the metal ion resistance ability of the bacteria. However, this advantage was not very obvious in the mixed culture group with an initial pH of 2.0.

In the case of chalcopyrite leaching, *Acidiphilium* sp. DX1-1 was not particularly effective in monoculture at pH 3.5 (Fig. 6(b)). The copper extraction amount was 0.77 g/L in 30 d, which is only slightly higher than that of the control without bacteria. However, the mixed culture of *Acidiphilium* sp. DX1-1 and *A. ferrooxidans* QXS-1 revealed a significant increase in the leaching rate of copper. The extraction amount of copper was 3.01 g/L in 30 d in the presence of mixed culture with the initial pH 2.0, which was about 1.5 times higher than that of a culture containing only *A. ferrooxidans* QXS-1. The leaching rate of the mixed culture with the initial pH of 3.5 was slower than that of a mixed culture with the initial pH of 2.0, but still faster than that of culture containing only *A. ferrooxidans* QXS-1.

4 Discussion

Our morphological and phylogenetic analyses demonstrated that strain DX1-1 is a member of genus *Acidiphilium* and closely related to the species *A. cryptum* and *A. multivorum*. The *Acidiphilium* species showed the phylogenetic heterogeneity and a relatively broad range of 16S rRNA gene sequence similarity levels which varied between 94% and 100% (Table 4) [21]. JOHNSON et al [11,22] classified all the *Acidiphilium* species into two groups, based on 16S rRNA gene sequence, and suggested that the bacteria in the same group are different strains of a single species rather than different species. The isolate DX1-1 belongs to the first group which includes *A. cryptum*, *A. multivorum*, *A. organovorum* and *Acidiphilium* sp. SJH. The second group includes *A. acidophilum, A. organovorum* and *A. rubrum*. The bacteria in the same group share >99% (*A. organovorum* and *A. rubrum* share 100%) 16S rRNA gene homology and have similar physiological characteristics.

Acidiphilium sp. DX1-1 can grow either heterotrophically or autotrophically and shows a relatively wide adaptability of substrate utilization. Like other *Acidiphilium* spp. members, DX1-1 can utilize a variety of organic substrates as carbon source. This advantage renders culturing of this bacterium easy in both liquid and solid media. This bacterium also can be incorporated into the under layer of the double-layer gel for isolating autotrophic species, such as *Leptospirillum* sp., because of its ability to metabolize organic compounds [23].

The intracellular PHB accumulation was also observed in other *Acidiphilium* species (e.g. *A. angusturn* ATCC 35903 [7], *A. multivorum* [9] and *A. cryptum* JF-5 [13]). PHB is the intracellular energy and carbon store that is produced by a large variety of microorganisms apparently in response to conditions of metabolic stress. It is accumulated under conditions of nitrogen or phosphorus limitation when there is an abundant carbon source in the medium, and is catabolized when other common energy or carbon sources are not available. This trait is a great advantage for DX1-1 as it allows it to be co-cultured in inorganic medium with other

chemoautotrophs (such as *A. ferrooxidans* and *Leptospirillum* spp.) that are sensitive to organic compounds.

The utilization of inorganic substrates has been observed in some *Acidiphilium* species and strains, which renders these bacteria more likely to be considered mixotrophic. *A.organovorum* ATCC43141 and *Acidiphilium* SJH showed ferric iron reduction ability; *A. rubrum* is able to oxidize sulfur; *A. cryptum* JF-5 and *A. acidophilum* ATCC27807 can reduce ferric iron and oxidize sulfur. *Acidiphilium* sp. DX1-1 demonstrates broader inorganic substrate utilization and is able to oxidize sulfur and reduce ferric iron, as well as utilizing some sulfide minerals such as marmatite.

Prior to this work, the role of *Acidiphilium* spp. in bioleaching was believed to be as an enhancer of other autotrophic iron-oxidizing acidophiles by metabolizing organic compounds that inhibit growth of the iron oxidizers. However, we demonstrate here that *Acidiphilium* sp. DX1-1 has a significant leaching capacity towards marmatite with the initial pH 3.5, which is even higher than that of *A. ferrooxidans* and the mixed culture with *A. ferrooxidans* at even lower pH. *Acidiphilium* sp. DX1-1 was also able to leach chalcopyrite but to a less extent than mixed cultures containing *A. ferrooxidans*. Thus, *Acidiphilium* sp. DX1-1 acted as the enhancer of *A. ferrooxidans* in mixed culture during the chalcopyrite leaching process and increased the leaching capacity of *A. ferrooxidans*.

Overall, our work demonstrates that *Acidiphilium* sp. DX1-1 has properties that may render it use in metal bioleaching on an industrial scale, with the added potential utility of biopolymer synthesis during growth.

5 Conclusions

1) An acidophilic mixotrophic bioleaching bacterium *Acidiphilium* sp. DX1-1 was isolated. DX1-1 can grow either heterotrophically or autotrophically and shows a relatively wide adaptability of substrate utilization.

2) 9K-glucose cultured DX1-1 accumulates intracellular PHB. This trait is a great advantage for DX1-1, as it allows it to be co-cultured in inorganic medium with other chemoautotrophs that are sensitive to organic compounds.

3) DX1-1 exhibits enhanced bioleaching capacity. It not only acts as an enhancer of other autotrophic iron-oxidizing acidophiles by metabolizing organic compounds which inhibit growth of the iron oxidizers, but also has a significant leaching capacity towards marmatite.

Acknowledgements

The authors thank Dr. Joel H. WEINER and Dr. Richard A. ROTHERY for reviewing this manuscript and helpful discussion.

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嗜酸兼性异养菌 *Acidiphilium* sp. DX1-1 的分离、特征及其浸矿行为

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摘 要：研究了一株源自江西德兴铜矿矿区的中温嗜酸兼性异养菌 *Acidiphilium* sp. DX1-1 的分离、鉴定、特征及其浸矿行为。菌株 *Acidiphilium* sp. DX1-1 为短杆状革兰氏阴性菌，最适合的生长温度为 30 °C，最适合的生长 pH 约为 3.5。该菌株具有广泛的底物利用特性，可以利用有机物进行异养生长并在细胞内积累聚羟基丁酸酯，也可以利用单质硫、三价铁等无机物进行自养生长。系统发育分析表明 DX1-1 属于 *Acidiphilium* 属，与 *Acidiphilium cryptum* and *Acidiphilium multivorum* 的同源性大于 99%。在铁闪锌矿生物浸出过程中，*Acidiphilium* sp. DX1-1 表现出极强的浸矿能力，其作用不仅仅是之前报道的作为其他自养嗜酸浸矿细菌的辅助者。在初始 pH3.5 时，DX1-1 能够在一个月内单独地浸出铁闪锌矿中 40%的锌。该浸出率高于它与 *A. ferrooxidans* 混合以及 *A. ferrooxidans* 单独浸出铁闪锌矿(初始 pH 均为 2.0)的浸出率。

关键词：*Acidiphilium* sp.；生物浸矿；铁闪锌矿；黄铜矿；聚羟基丁酸酯；16S rRNA

(Edited by Hua YANG)