16S rDNA and 16S–23S internal transcribed spacer sequence analyses reveal inter- and intraspecific *Acidithiobacillus* phylogeny

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In order to contribute to our understanding of *Acidithiobacillus* taxonomy, we determined 16S rDNA sequences and the 16S–23S internally transcribed spacer (ITS) sequence of 35 Chinese *Acidithiobacillus* isolates and three reference strains representing three validly described species and used them to construct phylogenetic trees. The two phylogenetic trees were roughly similar topologically, and *Acidithiobacillus* strains were assigned to eight phylogenetic groups. In addition, the results of phylogenetic analysis were consistent with those obtained by randomly amplified polymorphic DNA (RAPD) cluster analysis. Compared with a phylogenetic tree based on the 16S rRNA sequences, the ITS tree showed more clearly the inter- and intraspecific genealogical relationships of the genus *Acidithiobacillus*. Similarity values of the ITSs varied from 60.5 % to 84.7 % between representative strains of different species, and the maximum level of ITS divergence between strains belonging to the same species was 13 %. Coupling phylogenetic analysis and phenotypic characteristics, we concluded that at least each of the three *Acidithiobacillus ferrooxidans* phylogenetic groups should be considered a separate subspecies, and that five sulfur-oxidizing Chinese *Acidithiobacillus*-like isolates represent one or two new species of the genus *Acidithiobacillus*. The ITS may be a potential target for the development of fluorescent *in situ* hybridization probes for more accurately detecting distinct ecotypes of *Acidithiobacillus* strains and other closely related sulfur-oxidizing bacteria.

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INTRODUCTION

The genus *Acidithiobacillus* accommodates a group of Gram-negative, rod-shaped bacteria that derive energy from the oxidation of reduced sulfur compounds (Kelly & Wood, 2005). These bacteria appear to be present in a wide range of environments, especially in sites where pH is low, such as mining areas, hot sulfur springs and bioleaching operations. Many attempts have been made to describe a large number of *Acidithiobacillus* strains (Harrison, 1982; Karavaiko *et al.*, 2003; Bergamo *et al.*, 2004). More recently, bacteria of the genus *Acidithiobacillus* have been widely studied for bioleaching, biodegradation and desulfurization of coal and natural gas (Acharya *et al.*, 2004; Rawlings & Johnson, 2007). However, since their natural

habitats are ecologically extremely diverse, different *Acidithiobacillus* strains of the same species developing in various ecological niches are characterized by differences in growth rate, tolerance to heavy metal ions and activities of ferrous iron and/or sulfide mineral oxidation (Leduc & Ferroni, 1994; Kondratyeva *et al.*, 1999; Ageeva *et al.*, 2001). Moreover, strains belonging to the same *Acidithiobacillus* species exhibit a high degree of interstrain genetic variability with respect to the DNA G+C content, level of DNA–DNA homology of total genomes, the number and sizes of plasmids and chromosomal DNA structure (Harrison, 1982; Amils *et al.*, 1998; Karavaiko *et al.*, 2003; Rawlings, 2005; Valenzuela *et al.*, 2006).

To date, the revised genus *Acidithiobacillus* consists of four validly described species: *Acidithiobacillus thiooxidans*, *Acidithiobacillus ferrooxidans*, *Acidithiobacillus albertensis* and *Acidithiobacillus caldus* (Kelly & Wood, 2000). However, it has been found that some *Acidithiobacillus*-like isolates could not be assigned to any of the known *Acidithiobacillus* species (Harrison, 1982; Lane *et al.*, 1992; Karavaiko *et al.*, 2003), and some other sulfur-oxidizing strains were proposed to represent a novel species of *Acidithiobacillus* (Norris, 2007). Therefore, large-scale

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Abbreviations: RAPD, randomly amplified polymorphic DNA; ITS, internal transcribed spacer; UPGMA, unweighted pair group method with arithmetic means.

The GenBank/EMBL/DDBJ accession numbers for the 16S rDNA nucleotide sequence and 16S–23S rDNA intergenic spacers data reported in this study are DQ676505–DQ676511, EF059755–EF059762 and EU084695–EU084714.

investigations of the genotypic features of indigenous *Acidithiobacillus* strains in relation to their physiological properties might give a better understanding of the inter- and intraspecific genealogical relationships of this genus, and could be used for clarifying the taxonomic status of controversial strains and the rapid screening of strains important in industrial environments, such as bioleaching operations.

While the 16S rDNA sequence is a good tool for inferring inter- and intragenetic relationships, sequencing of the 16S–23S rDNA internal transcribed spacer (ITS) has been suggested to be well suited for typing and identification of bacteria at both the species and the strain level (Barry *et al.*, 1991; Gurtler & Stanisich, 1996), because of marked variation of the ITSs in both sequence and size between strains and species. Based on diversities between ITS sequences, it will be possible to construct species- and strain-specific oligonucleotides that can be used to detect bacteria in their natural environments.

In this work, in order to contribute to our understanding of *Acidithiobacillus* taxonomy, 35 Chinese *Acidithiobacillus* isolates and four reference strains were examined by randomly amplified polymorphic DNA (RAPD) and sequencing of the 16S rDNA and the 16S–23S rDNA ITS. Phylogenetic data obtained from the ITS sequences were compared with corresponding data derived from the 16S rDNA analysis, considering both the resolution and branching of the phylogenetic trees. To clarify the taxonomic status of some controversial strains, we analysed levels of ITS sequence divergence between the Chinese *Acidithiobacillus* isolates and representative strains from various parts of the world. In addition, molecular data on the grouping of the *Acidithiobacillus* isolates are discussed relating to phenotypic characteristics.

METHODS

Bacterial strains and isolation. Samples of water and sediment were collected from different mining sites in China (Table 1). Samples were serially diluted and spread onto a variety of solid media specifically designed for isolating acidophilic sulfur-oxidizing and iron-oxidizing bacteria, or subjected to enrichment in liquid medium and then streaked onto solid medium (Hallberg & Lindstrom, 1994; Johnson, 1995). Each isolate was purified by repeated single-colony isolation, and preliminary identification of isolates was carried out on the basis of colony morphologies and cell characteristics (Johnson, 1995). The identities of the isolates were confirmed by analyses of their 16S rRNA genes, and some key physiological traits (e.g. oxidation of iron and/or sulfur and autotrophy or heterotrophy) were examined (Kelly & Wood, 2000, 2005). Of the 35 isolates, 20 were identified as representing *A. ferrooxidans*, seven as representing *A. thiooxidans* and three as representing *A. caldus*; the remaining five were not identified to species level and were termed *Acidithiobacillus*-like strains. The strains used in this study and their sources are listed in Table 1.

DNA preparation. Bacterial cells were harvested by centrifugation at 15 000 g for 10 min and washed with acid water (pH 1.2) to remove ferric iron precipitate. Genomic DNA was isolated from

the tested strains as described by Novo *et al.* (1996) and Bergamo *et al.* (2004), with modifications. After treatment with 10 µl RNase (20 mg ml⁻¹), DNA concentrations and purity were determined by spectrophotometric readings. The DNA was stored at -70 °C until needed.

RAPD-PCR. Ten 10-mer primers (OPA-02, OPA-07, OPA-13, OPC-02, OPC-08, OPC-09, OPC-11, OPC-15, OPC-19, OPC-20) from Operon Technologies and 20 10-mer primers (S-21 to S-40, Sangon Biological Engineering Technology) were chosen randomly for the PCR. In addition, five longer primers (17–22-mer) were tested for the ability to differentiate *Acidithiobacillus* isolates: PJ108, PJ118 (Lado & Yousef, 2003); M13 (Jonas *et al.*, 2000); ERIC2 (Versalovic *et al.*, 1991); and BG2 (van Belkum *et al.*, 1993).

PCR was carried out in 25 µl containing 20 ng genomic DNA, 2.5 mM MgCl₂, 0.4 µM primer, 2 U *Taq* DNA polymerase (TaKaRa Biotechnology), 200 µM of each dNTP in 10 mM Tris/HCl, pH 8.3, 50 mM KCl and 0.001 % gelatin, 0.1 % Triton X-100, under a drop of mineral oil. A Bio-Rad iCycler Thermal Cycler was used for amplification. The cycling programme when using 10 nt primers was essentially as described by Welsh & McClelland (1990). The cycling programme when using longer (>17 nt) primers was as described by Williams *et al.* (1990), with minor modifications. Each amplification was done in triplicate and results were reproducible when DNA obtained by different extraction procedures of the same strain was used (Novo *et al.*, 1996; Bergamo *et al.*, 2004). The amplification products (10 µl) were separated by electrophoresis on 1.5 % agarose 0.5 × TBE gels. The gels were stained with ethidium bromide and photographed under UV. The 200 bp DNA ladder (TaKaRa Biotechnology) was used as a size marker in all gels.

Data from the RAPD-PCR were scored in binary form (presence/absence) and similarity matrices were generated using the Jaccard coefficient (Sneath & Sokal, 1973). A dendrogram was constructed by using the UPGMA (unweighted pair group method with arithmetic means) with the software package NTSYS-pc 2.01 (Rohlf, 1997). Cluster analyses were performed on the combined datasets generated by RAPD-PCR fingerprinting.

Sequencing and phylogenetic analysis of 16S rDNA and 16S–23S rDNA intergenic spacers. The 16S rRNA genes were amplified using the primers 27F and 1525R (Lane 1991). The primers 5'-GACTGGGGTGAAGTCGTAAC-3' and 5'-TGGCTGGGTGCC-CCATTCGG-3' were used to amplify the 16S–23S rDNA intergenic spacers (Sagredo *et al.*, 1992). PCRs (50 µl) contained 30–50 ng DNA, 0.4 µM each primer, 200 µM each dNTP, 2 mM MgCl₂ and 2 U *Taq* DNA polymerase (TaKaRa Biotechnology), in the buffer supplied by the manufacturer. Amplifications were performed using a Bio-Rad iCycler Thermal Cycler and included an initial denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min and final extension at 72 °C for 7 min. Direct sequencing of the PCR products was performed with an ABI PRISM BigDye terminator v3.1 sequencing Ready Reaction kit (PE Applied Biosystems) and an ABI PRISM 3730 genetic analyser (PE Applied Biosystems), using both forward and reverse PCR amplification primers. All sequencing procedures were repeated at least twice for each strain.

The 16S rRNA gene sequences and 16S–23S rDNA intergenic spacers were compared to sequences in the GenBank database by using BLAST (Altschul *et al.*, 1990). The sequences were aligned by using the CLUSTAL_X program (Thompson *et al.*, 1997). Phylogenetic trees were constructed using the MEGA v. 4.0 (Tamura *et al.*, 2007) packages with p-distances and the neighbour-joining method (Saitou & Nei, 1987). Bootstrap values were calculated from 1000 replications using the MEGA program.

Table 1. Reference strains and isolates used in this study

<i>Acidithiobacillus</i> strain	Description and original of isolation		
	Sampling location	Mine type	Type of sample
<i>A. ferrooxidans</i>			
ATCC 23270 ^T	Type strain from ATCC, USA	Coal mine	Bituminous effluent
ATCC 19859	Strain from ATCC, USA	Copper ore	Acid drainage
BY-3	Baiyin city, Gansu, China	Copper mine	Soil sample, pH 3.5
H4	Jingyuan county, Gansu, China	Coal mine	Sediment from effluent, pH 6.0
H3	Jingyuan county, Gansu, China	Coal mine	Acid drainage, pH 5.5
LY-2	Lueyang county, Shaanxi, China	Copper mine	Soil sample, pH 3.5
S-1	Shizuishan city, Ningxia, China	Coal mine	Effluent from wastes, pH 5.5
S-2	Shizuishan city, Ningxia, China	Coal mine	Sediment from effluent, pH 5.8
FY-3	Fuyun county, Xinjiang, China	Nickel–copper mine	Effluent, pH 4.0
JDC	Jinduicheng, Shaanxi, China	Molybdenum mine	Soil sample, pH 3.1
HJY	Yuanqu county, Shanxi, China	Copper mine	Acid drainage, pH 3.5
TKY-2	Zhongtiao Mountain, Shanxi, China	Copper mine	Acid drainage, pH 3.5
YP-3	Qianshan county, Jiangxi, China	Copper mine	Acid drainage, pH 2.1
DX-1	Dexing county, Jiangxi, China	Copper mine	Effluent from wastes, pH 1.8
DX-2	Dexing county, Jiangxi, China	Copper mine	Acid effluent from heap leaching
TSK	Anqing city, Anhui, China	Copper mine	Acid effluent, pH 2.2
DBS	Qujiang county, Guangdong, China	Copper mine	Effluent, pH 3.8
HNQ	Liuyang county, Hunan, China	Copper–zinc mine	Effluent, pH 2.5
TGS	Tongling city, Anhui, China	Copper mine	Acid drainage from wastes, pH 3.2
DGS	Tongling city, Anhui, China	Copper mine	Soil sample, pH 3.2
CHMS	Jiujiang city, Jiangxi, China	Copper mine	Effluent, pH 3.5
DXH	Dexin county, Jiangxi, China	Copper mine	Acid drainage from wastes, pH 3.0
<i>A. thiooxidans</i>			
ATCC 19377 ^T	Type strain from ATCC, USA	Clay	Soil sample
BY-s	Baiyin city, Gansu, China	Copper mine	Acid drainage from wastes, pH 3.5
JY	Jingyuan county, Gansu, China	Coal mine	Effluent, pH 5.5
LYS	Lueyang county, Shaanxi, China	Copper mine	Soil sample, pH 3.5
SZS	Shizuishan city, Ningxia, China	Coal mine	Sediment from effluent, pH 5.5
TKY-t	Zhongtiao Mountain, Shanxi, China	Copper mine	Acid drainage, pH 3.8
YP-2	Qianshan county, Jiangxi, China	Copper mine	Acid drainage, pH 2.0
DX-3	Dexing city, Jiangxi, China	Copper mine	Acid effluent from heap leaching
<i>A. caldus</i>			
DSM8584 ^T	Type strain from DSM	Coal spoils	Soil sample
DBS-5	Qujiang county, Guangdong, China	Copper mine	Soil sample, pH 4.5
YP-6	Qianshan county, Jiangxi, China	Copper mine	Acid drainage, pH 3.0
DX-4	Dexing city, Jiangxi, China	Copper mine	Acid effluent of heap leaching
<i>Acidithiobacillus</i> sp.			
DBS-2	Qujiang county, Guangdong, China	Copper mine	Effluent from wastes, pH 3.5
TSK-3	Anqing city, Anhui, China	Copper mine	Acid effluent, pH 2.5
DBS-4	Qujiang county, Guangdong, China	Copper mine	Soil sample, pH 4.5
FY-2	Fuyun county, Xinjiang, China	Copper–nickel mine	Effluent, pH 4.0
YP-5	Qianshan county, Jiangxi, China	Copper mine	Acid drainage, pH 2.5

RESULTS AND DISCUSSION

Cluster analysis based on RAPD-PCR data

The genomic DNA of each strain used in this work was amplified using 35 different random primers. Twenty-five of the primers tested produced a large number of specific

and reproducible banding patterns revealing polymorphisms, whereas the other 10 primers (S-21, S-22, S-27, S-28, S-32, S-33, S-34, S-35, S-36, OPC-09) were excluded from the data analysis due to the production of non-reproducible banding pattern. The number of bands for each primer varied from 4 to 32 with an average of about 18 bands per primer. The size of the amplified product ranged

from approximately 200 to 4000 bp. In addition, primers of 10 bases produced fewer amplified products than longer primers (data not shown).

Amplified DNA fragments that were reproducible were scored as 0 (absence) and 1 (presence) in a matrix dataset and the relationships between the strains were estimated using Jaccard's coefficient of similarity (data not shown). The cluster analysis, performed by distance data matrix from RAPD-PCR profiles, divided the 35 *Acidithiobacillus* strains into two major clusters (Fig. 1). Cluster I comprised all the *A. ferrooxidans* strains, whereas cluster II was composed exclusively of sulfur-oxidizing strains, and contained all these strains. Within cluster I, the 22 strains of *A. ferrooxidans* could be divided into four distinct groups at the 34 % similarity level. Group Ia included most of the *A. ferrooxidans* isolates from different regions in China and isolates TKY-2 and JDC were the most divergent strains (35 % average similarity, Fig. 1); group Ib comprised isolates DBS, YP-3, DX-1, TSK-1, BY-3, type strain ATCC 23270^T, and reference strain ATCC 19859; group Ic consisted of isolates FY-3 and TGS. Isolate DX-2 was the most divergent organism in cluster I and was assigned to single-member group Id. In comparison with cluster I, cluster II, formed by the 13 sulfur-oxidizing strains, included two groups: group IIa consisted of isolates BY-s, JY, YP-2, LYS, DX-3, TKY-t and the *A. thiooxidans* type strain ATCC 19377^T; group IIb comprised five sulfur-oxidizing *Acidithiobacillus*-like isolates YP-5, FY-2, DBS-2, TSK-3,

DBS-4 and *A. thiooxidans* strain SZS. Similarity between strains from the two clusters ranged from 5.7 % to 19 %. Among strains from cluster I (strains of *A. ferrooxidans*), the similarities ranged from 16.2 % (ATCC 23270^T and JDC) to 71.6 % (ATCC 23270^T and ATCC 19859), and among strains of cluster II (only sulfur-oxidizing strains) was 18.3 % (LYS and TSK-3) to 66 % (LYS and YP-2). Among the strains within *A. ferrooxidans* group Ib, the similarities ranged from 33.3 % to 71.6 %. The similarity between group Ic strains TGS and FY-3 was 45 %.

Thus, RAPD-PCR profiles revealed considerable genomic diversity within *A. ferrooxidans*. These data are in agreement with those of Novo *et al.* (1996), who concluded that various *A. ferrooxidans* strains have a very wide similarity coefficient range (almost 0 % to over 98 %). There is a good correlation between the relationships of the Chinese *Acidithiobacillus* strains determined by RAPD analysis and corresponding data derived from repetitive element (rep)-PCR, with the exception of *A. ferrooxidans* isolates TGS and FY-3 belonging to the same group with most of the Chinese *A. ferrooxidans* isolates in the rep-PCR analysis (Ni *et al.*, 2008). In addition, as shown in Table 1 and in Fig. 1, no clear correlation was found between the genotypic groups of the *Acidithiobacillus* sp. strains and either the geographical location or type of habitat/sample from which the strains were isolated; this is consistent with results obtained by other workers (Novo *et al.*, 1996; Karavaiko *et al.*, 2003).

Fig. 1. Dendrogram representing the relationships between 35 *Acidithiobacillus* strains based on UPGMA cluster analysis of the RAPD profiles derived from 25 primers using Jaccard's similarity coefficient.

Comparative analysis of 16S rDNA sequence and 16S–23S rDNA intergenic spacers

Sequences of the 16S rDNA and the 16S–23S rDNA ITS were determined for 35 *Acidithiobacillus* isolates recovered from wastes or sediments in China. Although it is known that the genome of *A. ferrooxidans* has two *rrn* operons (Salazar *et al.*, 1989), in each case only one ITS-PCR product was obtained, suggesting that the two ITS copies in the genome of each of the *Acidithiobacillus* isolates did not differ in size. According to our sequencing results, the structure and organization of the 16S–23S intergenic spacers of the Chinese *Acidithiobacillus* isolates were consistent with the data obtained by other researchers (Venegas *et al.*, 1988; Sagredo *et al.*, 1992). The 16S–23S intergenic spacers of all Chinese *Acidithiobacillus* isolates contain two highly conserved genes for tRNA^{Ile} and tRNA^{Ala}, which split the ITS into three regions (ITS1, ITS2 and ITS3). The tRNA^{Ile} and tRNA^{Ala} genes of *Acidithiobacillus* spp. are 87.3 % and 86.8 % homologous, respectively, to the corresponding tRNA genes of *E. coli*. The other feature of the ITS of all the *Acidithiobacillus* strains is the presence of a 9 bp antitermination consensus sequence (TGTTCTTTTG) resembling box A, which is found in all the 16S–23S intergenic spacers of *E. coli*, as well as in a number of other bacterial species (Berg *et al.*, 1989). A detailed comparison of the sequence revealed variations in the length of the ITS among the *Acidithiobacillus* strains examined (Table 2). The divergence between the ITS sequences arises from insertions and deletions of short sequences located in the ITS3 region (see Fig. 4). These sequences could be interesting candidates for the development of species- and strain-specific probes. The size of the ITS of the *A. ferrooxidans* strains varied from 434 to 456 bp. The *A. thiooxidans* strains possessed different ITS in sizes ranging from 451 to 490 bp. The *Acidithiobacillus*-like strains YP-5, FY-2 and DBS-4 possessed an identical ITS of 421 bp, whereas the ITS of the other two sulfur-oxidizing strains DBS-2 and TSK-3 was shorter: only 413 and 415 bp. *A. caldus* strains presented the smallest ITSs, of 379–382 bp. A *cfol* site was found in the ITS of *A. ferrooxidans* and *A. thiooxidans*, while no *cfol* site was present in the ITS of *A. caldus* and five *Acidithiobacillus*-like strains.

Table 2. ITS Length heterogeneity of *Acidithiobacillus* spp.

Group	Spacer length (bp)					
	ITS1	ITS2	ITS3	tRNA ^{Ile}	tRNA ^{Ala}	Total
1	44–45	3	233–240	77	76	434–441
2	44–45	3	249–255	77	76	449–456
3	45	3	240	77	76	454
4	45	3	256	77	76	457–490
5	45–50	3	256–289	77–78	76–77	451–465
6	46–47	3	177–179	77	76	379–382
7	50	3	207–210	77	76	413–416
8	48	3	217	77	76	421

Phylogenetic trees were constructed based on the 16S rRNA gene and 16S–23S rDNA spacer sequences of the Chinese *Acidithiobacillus* isolates and the additional *Acidithiobacillus* strains available from GenBank (Figs 2 and 3). The phylogenetic tree constructed on the basis of the ITS sequences was topologically roughly similar to that generated by 16S rDNA sequences. The *Acidithiobacillus* strains were separated into eight phylogenetic groups. The strains assigned to a species showed higher phylogenetic coherence on the topology tree, suggesting the identities of all tested strains to be correct. An analysis of the 16S rRNA gene sequence indicated that all the *A. thiooxidans* and *A. ferrooxidans* strains constituted a monophyletic cluster with an 89 % bootstrap value and that the species *A. caldus* was phylogenetically close to *A. thiooxidans* and *A. ferrooxidans*. As shown in Fig. 2, the *A. caldus* strains and *A. thiooxidans* DSM 612 took an intermediate position between *A. ferrooxidans*, *A. thiooxidans* and the five sulfur-oxidizing *Acidithiobacillus*-like isolates. In contrast, the 16S–23S rDNA spacer sequences revealed that *A. caldus* strains and five sulfur-oxidizing *Acidithiobacillus*-like isolates showed a higher degree of relatedness, and constituted a monophyletic cluster (C) with a bootstrap value of 100 %, whereas *A. thiooxidans* strains also constituted another monophyletic cluster (B) with a bootstrap value of 99 %. The ITS nucleotide sequence divergence between representative strains belonging to different phylogenetic groups is shown in Fig. 4. Moreover, results of phylogeny analysis, especially the phylogenetic tree constructed on 16S–23S rDNA spacer sequence, were in line with the dendrogram obtained by RAPD cluster analysis based on the whole genome. In the RAPD clustering analysis, the most divergent *A. ferrooxidans* strain DX-2 fell into none of the phylogenetic major groups; the Chinese *A. thiooxidans* isolate SZS most distantly related to *A. thiooxidans* ATCC 19377^T was assigned to the independent phylogenetic group 5 (Fig. 3). There was an exception, in that the representative strains of RAPD groups Ib and Ic were combined into the phylogenetic group 1.

Studies of acidithiobacilli by our laboratory and other workers (Goebel & Stackebrandt, 1994; Paulino *et al.*, 2001) have demonstrated that the 16S rRNA gene sequences of strains belonging to different *Acidithiobacillus* species are less than 5 % divergent, except for presumptively identified *Acidithiobacillus* strains, and that most strains from the same *Acidithiobacillus* species show greater than 98 % identity in their 16S rDNA sequences. The 16S rRNA gene sequence divergence between *A. thiooxidans* and *A. ferrooxidans* strains is less than 3 %, whereas the 16S rRNA gene sequence of *A. thiooxidans* has more than 99 % identity to that of the currently presumed *A. albertensis* strains. Consequently, the variations in the 16S rRNA gene sequences are not sufficient to provide useful information to allow clear differentiation of closely related *Acidithiobacillus* strains (Fox *et al.*, 1992). Compared with the 16S rRNA gene sequence, the 16S–23S rDNA ITS exhibited considerable variation in structure and is more

Fig. 2. Unrooted phylogenetic tree derived from the analysis of 16S rDNA sequences of Chinese *Acidithiobacillus* isolates and related reference strains. The tree was constructed by using the neighbour-joining method; bootstrap values (1000 replicates) lower than 70% are not shown. The numbers in parentheses correspond to the GenBank accession numbers.

appropriate for studies at the intraspecies level. The ITS-based phylogeny markedly improved the resolution and complemented the phylogeny of the genus *Acidithiobacillus* based on 16S rRNA gene sequences. Our analysis results showed that the levels of ITS sequence similarity within members of the same species were markedly greater than the levels of similarity between members of different species. Overall similarity values of the ITSs varied from

60.5 to 84.7% between representative strains of different species of the genus *Acidithiobacillus* (data not shown). Sequences from the spacer of the *A. thiooxidans* and *A. ferrooxidans* strains ranged from 86.7 to 99.6% and 92.4 to 100% similarity, respectively. The *Acidithiobacillus* strains DBS-2, TSK-3 and the erroneously identified *A. thiooxidans* KCTC8928P, which were assigned to phylogenetic group 7, showed about 80% and 69–74%

Fig. 3. Neighbour-joining tree based on 16S–23S ITS sequences, showing the inter- and intraspecific relationships of members of the genus *Acidithiobacillus*. Bootstrap values (1000 replicates) lower than 70% are not shown. The numbers in parentheses correspond to the GenBank accession numbers.

ITS identity with *A. caldus* DSM 8584^T and *A. thiooxidans* ATCC 19377^T, respectively. Phylogenetic group 8 consisted of *Acidithiobacillus* strains YP-5, FY-2 and DBS-4, which showed about 80% and 72.5% ITS identity with *A. caldus* DSM 8584^T and *A. thiooxidans* ATCC 19377^T, respectively. The ITS and the 16S rRNA gene sequence similarity between the two groups was 85.6–87.7% and 96–97%, respectively. In addition, although *A. albertensis* was tentatively assigned to the new genus *Acidithiobacillus*, there have up to now been no research reports about the ITS of *A. albertensis*. As its 16S rDNA sequence and phenotypic traits are highly similar to most *A. thiooxidans* strains (more than 99% 16S rDNA sequence identity) (Kelly &

Wood, 2005), the information on ITS is expected to contribute to improvement of the taxonomy and phylogeny of *A. albertensis*.

From the alignment, we could also verify the existence of intraspecific polymorphism in the ITSs of *Acidithiobacillus* spp. Most of the *A. ferrooxidans* strains, including the type strain ATCC 23270^T, fell into three phylogenetic groups. Each of the three *A. ferrooxidans* phylogenetic groups possessed a distinct ITS, ranging in size from 440 to 441 bp (group 1), 448 to 456 bp (group 2) and 452 to 454 bp (group 3), respectively. *A. thiooxidans* strains were assigned to phylogenetic groups 4 and 5 (Fig. 3). The majority of

19377	-AAAGAAATGGGTC-CTAGACCCACACACGCCACTCGGTAAA--GAATCGGGCCTATAGCTCAGCTGGCTAGAGCACACGACTGATAATCGTGAGGTCA	96
SZS	-AAAGAAACGGGTC--TAGACCCACACACGCCACTCGGTAAA--GAATCGGGCCTATAGCTCAGCTGGCTAGAGCACACGACTGATAATCGTGAGGTCA	95
SSP	-AAAGAAAAGGGTC--CAGACCCACACACGCCACTCGGTAAA--GAATTGG-CTATAGCTCAGCTGGCTAGAGCACACGACTGATAATCGTGAGGTCA	94
33020	-AAAGAAAAGGGTC--TAGACCCACACACGCCACTCGGTAAA--GAATTGGGCTATAGCTCAGCTGGCTAGAGCACACGACTGATAATCGTGAGGTCA	95
23270	-AAAGAAAAGGGTC--TAGACCCACACACGCCACTCGGTAAA--GAATTGGGCTATAGCTCAGCTGGCTAGAGCACACGACTGATAATCGTGAGGTCA	95
TSK-3	-AGAGAAATAGGGTATCTAGACCCACACACGCCACTCGGTAGAAAGACTTGGGCTATAGCTCAGCTGGCTAGAGCACACGACTGATAATCGTGAGGTCA	99
YP-5	-AAAGAGACGGGTA-CTAGACCCACACACGCCACTCGGTAGAGAGACTTGGGCTATAGCTCAGCTGGCTAGAGCACACGACTGATAATCGTGAGGTCA	98
DSM8584	AAAGAGAACGGGTA-CTAGACCCACACACGCCACTCGGTAGC--GACTTGGGCCATAGCTCAGCTGGCTAGAGCACACGACTGATAATCGTGAGGTCA	97
ITS1		
19377	GTGGTTCGAGTCCACTTGGGCCACCAATGGGGCTGTAGCTCAGCTGGGAGAGCACTGCTTTGCAAGCAGGGGGTCAACCGGTCGATCCCGGTCA	196
SZS	GTGGTTCGAGTCCACTTGGGCCACCAATGGGGCTGTAGCTCAGCTGGGAGAGCACTGCTTTGCAAGCAGGGGGTCAACCGGTCGATCCCGGTCA	195
SSP	GTGGTTCGAGTCCACTTGGGCCACCAATGGGGCTGTAGCTCAGTGGGAGAGCACTGCTTTGCAAGCAGGGGGTCAACCGGTCGATCCCGGTCA	194
33020	GTGGTTCGAGTCCACTTGGGCCACCAATGGGGCTGTAGCTCAGTGGGAGAGCACTGCTTTGCAAGCAGGGGGTCAACCGGTCGATCCCGGTCA	195
23270	GTGGTTCGAGTCCACTTGGGCCACCAATGGGGCTGTAGCTCAGTGGGAGAGCACTGCTTTGCAAGCAGGGGGTCAACCGGTCGATCCCGGTCA	195
TSK-3	GTGGTTCGAGTCCACTTGGGCCACCAATGGGGCTGTAGCTCAGCTGGGAGAGCACTGCTTTGCAAGCAGGGGGTCAACCGGTCGATCCCGGTCA	199
YP-5	GTGGTTCGAGTCCACTTGGGCCACCAATGGGGCTGTAGCTCAGCTGGGAGAGCACTGCTTTGCAAGCAGGGGGTCAACCGGTCGATCCCGGTCA	198
DSM8584	GTGGTTCGAGTCCACTTGGGCCACCAATGGGGCTGTAGCTCAGTGGGAGAGCACTGCTTTGCAAGCAGGGGGTCAACCGGTCGATCCCGGTCA	197
ITS2		
19377	CCACCAAGGGGAA--AGGCCCGCG--CGCAGCGGGTCGACGCTGGGGTGGAGAAAGTAGGCGGCA--GGAAGCGGGTAACAGGTAA--CAG-GAAGTAGA	288
SZS	CCACCAAGGGGAA--AGGCCCGCG--CGCAGCGGGTCGACGCTGGGGTGGAGAAAGTAGGCGGCA--GGAAGCGGGTAACAGGTAA--CTGTAAAGCGCA	289
SSP	CCACCAAGGGGATGGAAAGGCCCGCAAGAGCGCGGGCTGGTCTGCTGGG-----AAGCGGTATGGAAAGTGATTTGGGATTTGGATTTTGTGTGTA	285
33020	CCACCAAGGGGAAAGAGGCCCGCAAGAGCGCGGGCTGGTCTGCTGGG-----AAGCGGTATAGACAGGGATGTAGGGTTGAGGATGTAGTGCCTA	286
23270	CCACCAAGGGGATAGAAAGGCCCGCAAGAGCGCGGGCTGGTCTGCTGGG-----AAGCAGTAGAGAAAGGGTTTAGGGTT-----TAGTGTGTA	279
TSK-3	CCACCAAGATG-----TTCTGGTG-----GAAAGGCGAGCTGCCCAAG-----GCTGGGGCCAGTGGTAGGAGAGGTTGG-----	264
YP-5	CCACCAAGAT-----TGCTGGTG-----GGGAGAT-----CTATCCCGAG-----AGGGGTAGGTAGGAGTAGGGAAGGGTAGA-----GGAA	265
DSM8584	CCACCAAGAC-----GGTG-----GGGTGG-----GGGAGCCGCGGGTCC-----	234
ITS3		
19377	GAATAGGAAAGTAATTA--GAGTCCGTAGTAGTGAGTTTCTGAATAAAGAGCTGAGTACTACCGATTTCG--AGTATGGAAGCGGTA--TTGTTCTTT	383
SZS	GAATAGGAAAGCTAGTAAAGAGCTGTAGTAGTGAGTTTCTGATC--AGAAAGCTGAGTACTACCGATTTCAG--GGTAACGAAGCGGTA--TTGTTCTTT	383
SSP	GTGCTGAATGACGCTTTTAAGGAT-----GTAGGAAGTTAAAGG-----GCTATTGATCACTGCCACTCGGAAAGGTTTGTAGTGCCTTAGTTTGTCTTT	377
33020	GTGCTGAATAACGCTTTAGGGAT-----ATAGGAAGTTAAAGTAAAGTGCTATTGATCACTGCCACTCGGAAAGGT--CGAGTGTGTAGTTTGTCTTT	379
23270	GT-CTGAATAACGCTTTAGGGAT-----GTAGGAAGT--AAGGT--GCTATTGATCACTGCCACTCGGAAAGAT--TGAGTGCCTTAGTTTGTCTTT	365
TSK-3	-----GTAGGTTAG--TGGAAGA-----AAGGGATGGAGA--GTCCTTGTGCACTACGTAC--CGGAAGGT--GCAGTAGTA--TTGTTCTTT	338
YP-5	GAGAAAGTAGGTAGGTAGTGGAG-----AAGGGATGAATT--GTCCTTGTGCACTGCGTAC--CGGAAGGT--GCAG--AGTA--TTGTTCTTT	345
DSM8584	-----GTGGTGTGGGCACTGGG-----CAGTGCAGAGCT--TTGGGTTGTGCACTGTACAT--TGGAAGAT--GTGC--GGTA--T-GTTCTTT	307
19377	GACAGTTGAGGAAAGGGAAGGCCATGTTTGTCTATCTTGCCGATGGCAGAC--CTCCAGGTGGATGCTTGGGGATATAT	458
SZS	GACAGTTGAGGAAAGGGAAGGCCATGTTTGTCTATCTTGCCGATGGCAGAC--CTCCAGGTGGATGCTTGGGGATATAT	458
SSP	GACAGTTGAGGAAAGGGAAGGCCATGTTTGTCTATCTTGCCGATGGCAGAC--CTCCAGGTGGATGCTTGGGGATATAT	452
33020	GACAGTTGAGGAAAGGGAAGGCCATGTTTGTCTATCTTGCCGATGGCAGAC--CTCCAGGTGGATGCTTGGGGATATAT	454
23270	GACAGTTGAGGAAAGGGAAGGCCATGTTTGTCTATCTTGCCGATGGCAGAC--CTCCAGGTGGATGCTTGGGGATATAT	440
TSK-3	GACAAATGGAAGAAAGGGAAGGCCGTTGCCAGTTCTGGGACTTGGTAGCGATCTCGCGAAGGTATTGCGGGATATAT	415
YP-5	TACAAAGAAAGGGAAGGCCGTTGCCAGTTCTGGGACTTGGTAGCGATCTCGC--AAGGTATTGCGGGATATAT	421
DSM8584	GACAAAGTGAAGGGAAGGCCAGATGACCGGTCCTGGGACGGGTCATC--TCCCCGAGGGTATTGGGGATATAT	382

Fig. 4. Alignment of 16S–23S rDNA ITS sequences of representative strains belonging to eight *Acidithiobacillus* phylogenetic groups. The length of the ITS is indicated at the end of the sequences in nucleotides. Asterisks indicate identity, and hyphens represent alignment gaps. The box A antiterminator sequence is outlined.

A. thiooxidans strains, including the type strain ATCC 19377^T, were recovered in a very tight group (group 4) with more than 97 % ITS sequence similarity. The Chinese *A. thiooxidans* isolate SZS was assigned to a separate group 5, which contained a unique *A. thiooxidans* strain STAM61 with the largest ITS of 490 bp. While ITS sequence similarity levels between members of the two *A. thiooxidans* groups were only 88–90 %, the two groups showed more than 98 % identity in their 16S rRNA gene sequence. *A. thiooxidans* strain CASQ22, described by Bergamo *et al.* (2004), which possessed an ITS with a smallest size of 409 bp, and fell into none of groups, showed only ITS sequence similarity on average of less than 87 % to other *A. thiooxidans* strains, suggesting that this strain might correspond to a different genospecies or genomovar from the remaining *A. thiooxidans* strains. Consequently, the ITS could also be used as a tool to clarify controversial strains.

Determination of cutoff points of genomic data

As seen in Fig. 3, the tree constructed by using 16S–23S ITS sequences showed clearly the inter- and intraspecific genealogical relationships of the *Acidithiobacillus* species. Thus, levels of ITS sequence divergence may also be considered as a genomic criterion for distinguishing closely related *Acidithiobacillus* strains and species definitions. In this study, *Acidithiobacillus* strains that share less than 87 % similarity in the 16S–23S rDNA spacer sequence are nearly always members of different species, which is consistent with the data obtained by Leblond-Bourget *et al.* (1996) with the genus *Bifidobacterium*. The maximum levels of the 16S–23S rDNA spacer sequence divergence for the species *A. thiooxidans* were about 13 %. For *A. ferrooxidans* and *A. caldus*, the maximum levels of the 16S–23S rDNA spacer sequence divergence were about 8 %.

Although levels of ITS sequence divergence between strains belonging to the same species may help determine relationships of closely related strains, there is up to now no precise boundary limit to clarify species definitions and controversial strains. In this study, the levels of the 16S–23S rDNA spacer sequence divergence between five sulfur-oxidizing *Acidithiobacillus*-like isolates and two closely related species *A. caldus* and *A. thiooxidans* were more than 13 %. This result was also consistent with those derived from 16S rDNA sequences analysis (level of similarity <95 %). Preliminary phenotypic experiments revealed that *Acidithiobacillus*-like isolates YP-5, FY-2 and DBS-4 were distinguished from *A. thiooxidans* ATCC 19377^T not only in that they were moderate acidophiles (optimum growth pH between 4.0 and 4.5) when growing between 30 and 35 °C, but also in that they were also able to oxidize elemental sulfur at temperatures ranging from 40 °C to 45 °C. In addition, isolates YP-5, FY-2 and DBS-4 grew faster in the media supplied with 0.01 % yeast extract than without yeast extract on the basis of their specific growth rate (Ni *et al.*, 2008), indicating that the three isolates are physiologically similar to *A. caldus* DSM^T 8584, and should be mixotrophic. In contrast, *Acidithiobacillus* strains DBS-2 and TSK-3 are unable to grow above 40 °C (Ni *et al.*, 2008). Following the recommendation of Stackebrandt *et al.* (2002) that phenotype continues to play a salient role in the decision about cutoff points of genomic data, these novel isolates should represent one or two new species of the genus *Acidithiobacillus* or even indicate a new genus. Further studies on these sulfur-oxidizing isolates are in progress in our laboratory.

Previous work has shown that *A. ferrooxidans* strains could be divided into several genomic groups (genomovars) on the basis of DNA–DNA hybridization similarity, and that strains from the different *A. ferrooxidans* genomovars had genome similarity of only 10–50 % (Harrison, 1982; Karavaiko *et al.*, 2003). However, genomovars are commonly distinguished at a genome similarity level of 70 %. Even if the genomic similarity value for circumscribing the species could be lowered to 50 % according to the suggestion of Rossello-Mora & Amann (2001), the current situation, that strains from the different genomovars were assigned to a single species *A. ferrooxidans*, should be controversial. On the other hand, in most cases only small variations in 16S rRNA gene sequence were observed with strains belonging to *A. ferrooxidans* (less than 2 % sequence divergence).

In this study, the majority of *A. ferrooxidans* strains isolated from various acid habitats were assigned to three phylogenetic groups by 16S rDNA sequences and the 16S–23S rDNA spacer sequence analysis. ITS sequence similarity levels within and between the phylogenetic groups of *A. ferrooxidans* were about 97.5–100 % and 92–97 %, respectively (data not shown). Strains ATCC 23270^T, SSP and ATCC 33020 were used as representative strains of the three *A. ferrooxidans* phylogenetic groups. The Chinese *A. ferrooxidans* isolates were assigned to phylogenetic

groups 1 and 2, as shown in Figs 1 and 2. *A. ferrooxidans* isolates related to strain ATCC 33020 were not recovered in this study. Previous studies have demonstrated that *A. ferrooxidans* ATCC 33020 (representing the phylogenetic group 3) possesses distinctive phenotypic characteristics, including more sensitivity to uranium and an ability to accumulate a large amount of uranium in comparison with other ecotypes of *A. ferrooxidans* strains (Merroun & Selenska-Pobell, 2001). Our phenotypic analysis (Ni *et al.*, 2008) also revealed that *A. ferrooxidans* strains from phylogenetic group 1 were able to grow at 40 °C or pH 1.3, and able to oxidize elemental sulfur rapidly in iron-free 9k medium (pH 3.5) (pH values of media decreased to 1.6–1.8 after 1 week). In contrast, *A. ferrooxidans* strains belonging to phylogenetic group 2 failed to grow or grew very poorly under the corresponding conditions and grew slowly in iron-free 9k medium (pH 3.5) supplied with 1 % sulfur powder (pH values of media decreased only to 2.0–2.5 after 1 week). Thus each of the three *A. ferrooxidans* phylogenetic groups corresponds to a different physiological group. As was the case with *A. ferrooxidans* ATCC 33020, each of the three phylogenetic groups has been proposed to be considered as a distinct species or a separate subspecies (Selenska-Pobell *et al.*, 1998; Johnson *et al.*, 2005). At least, each phylogenetic group should be represented by a subspecies, a concept currently being defined (Brenner *et al.*, 2004). This problem needs further elucidation by other phenotypic characteristics or genomic information.

In a wide range of sulfur-containing environments, diverse members of the genus *Acidithiobacillus* and other closely related sulfur-oxidizing bacteria are abundant and their metabolism versatile (Friedrich, 1998). However, pure cultures of these sulfur-oxidizing bacteria are difficult to isolate because of their slow growth and synergistic interactions with heterotrophic organisms (Baker & Banfield, 2003). It is generally believed that there is a selection for certain sulfur-oxidizing bacteria during the process of purification and that purified cultures reflect the conditions used for isolation, rather than the actual dominance of the particular strains in the environment (Schrenk *et al.*, 1998). Therefore, pure cultures of acidithiobacilli available today are considered unrepresentative of the true diversity of the genus *Acidithiobacillus* in the environment. In fact, a bacterial clone from acidic volcano samples was proposed to represent a novel species of *Acidithiobacillus* (Simmons & Norris, 2002). Recently, there have been several attempts to construct specific oligonucleotides based on 16S rRNA gene sequences (Peccia *et al.*, 2000; Bond & Banfield, 2001; Bouchez *et al.*, 2006; Okabe *et al.*, 2007), with the aim of detecting and identifying a variety of the sulfur-oxidizing bacteria at the species level or above *in situ*. As described in this study, however, *Acidithiobacillus* strains from various niches possess only small variations in 16S rRNA gene sequences. *Acidithiobacillus* strains sharing identical 16S rRNA gene sequences show only much less than 50 % genome DNA similarity (Goebel & Stackebrandt, 1994). In

contrast, *Acidithiobacillus* strains show higher ITS polymorphisms, including either species-, genospecies- and/or even strain-specific nucleotide changes. Thus, as was the case with the iron-oxidizing species *Leptospirillum ferroplasma* (Tyson *et al.*, 2005), ITS may be a potential target for the development of fluorescent *in situ* hybridization probes for more accurately detecting distinct ecotypes of strains within the *Acidithiobacillus* species and other closely related sulfur-oxidizing bacteria, pure cultures of which are difficult to obtain from environmental samples.

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