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16S/23S Intergenic Spacer Region as a Genetic Marker for *Thiobacillus thiooxidans* and *T. ferrooxidans*

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Abstract Bioleaching is the process in which insoluble metal sulfide is oxidized by specialized iron- and/or sulfur-oxidizing lithotrophic bacteria in acidic, metal-rich environments. Most of these processes are carried out by the genus *Thiobacillus*. Three novel *Thiobacillus* strains (*Thiobacillus thiooxidans* AZ11, *Thiobacillus thiooxidans* MET, and *Thiobacillus thiooxidans* TAS) associated with bioleaching have been isolated from soil and sludge (Korean patent No. 1999-0073060 for *T. thiooxidans* AZ11, Korean patent No. 1999-0005798 for *T. thiooxidans* MET, and Korean patent No. 1999-0073059 for *T. thiooxidans* TAS). A partial sequence of 16S ribosomal RNA gene (16S rDNA) and the entire sequence of 16S/23S intergenic spacer region (ISR) were determined in the three above novel strains and in *Thiobacillus ferrooxidans* ATCC19859 as a reference strain. When phylogenetic analysis was performed based on G+C contents and sequence alignments, *T. ferrooxidans* ATCC19859 was found to be closely related to previously registered *T. ferrooxidans* strains in a monophyletic manner, while the three novel *T. thiooxidans* strains were classified in a paraphyletic manner. Close examination on the base composition of 16S/23S ISR revealed that the 5' part (nucleotide residues 21-200) was specific for the genus *Thiobacillus*. On the other end, the 3' part (nucleotide residues 201-520) showed specificity in *T. ferrooxidans* strains, but not in *T. thiooxidans* strains. These results suggest that the proximal and distal halves of 16S/23S ISR could be used as a genetic marker for the identification of the genus *Thiobacillus* and the species *T. ferrooxidans*, respectively.

Key words: Bioleaching, *Thiobacilli*, 16S rDNA, 16S/23S ISR, phylogenetic tree, phylogenetic marker

The current drain of natural resources has increased the importance and attractiveness of leaching in the industrial mineral process. Generally, chemical and biological methods have been used. The latter, however, is preferred due to growing need to use lower-grade ores, relative ease of implementation, low start-up costs required, and less environmental contamination [2, 3]. Even small amounts of lead, zinc, and copper in ore can be refined by bioleaching to a higher grade with good yields [24]. The major warrior in bioleaching is *Thiobacillus* species [22].

Thiobacillus has been spotlighted for its ability to leach copper from the copper sulfides after oxidation [26]. Mixtures of highly acidophilic, chemolithotrophic bacteria including *Thiobacillus ferrooxidans*, *Thiobacillus thiooxidans*, *Leptospirillum ferrooxidans*, and genus *Acidiphilium* have been applied in the industrial bioleaching process [12, 15, 27]. The roles of bacteria in bioleaching and biooxidation of pyrite and other metal sulfides to soluble metal sulfates and sulfuric acid have also been well established [23, 28]. *T. ferrooxidans* oxidizes reduced sulfur compounds and iron(II) to iron(III) ions. While *T. thiooxidans* is able to oxidize only reduced sulfur compounds, *L. ferrooxidans* can oxidize only iron(II) ions [11].

Since the mixture of chemolithotrophic and heterotrophic microorganisms are found in acidic, sulfur-, and metal-rich environments, the identification and evaluation of individual species are important in the industrial bioleaching process [25]. Nevertheless, special growth conditions of these microorganisms make their detection with classical cultivation-dependent methods very difficult, and can often lead to the omission of some constituent organisms [25]. In addition, multiple serotypes are present in different isolates of a single species [13]. To detect genomic diversity and to examine phylogenetic relationship among subspecies of sulfur- and iron-oxidizing bacteria, molecular methods such as G+C content, DNA-DNA hybridization, and analysis of ribosomal RNA gene have been used [14, 21]. Recently, PCR-mediated analysis of the 16S/23S intergenic spacer region (ISR) was introduced to differentiate among bacteria found in acidic mining environments [18]. The well-

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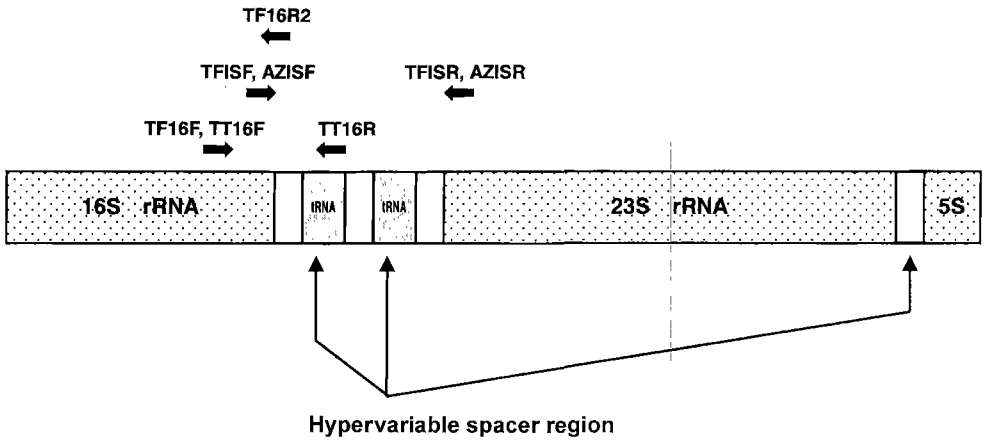

Fig. 1. Schematic representation of the prokaryotic rRNA gene.
The primers used in this study are indicated above the bar.

conserved ribosomal RNA gene (termed rDNA hereafter) has also been found useful for organizing evolutional relationships even among physiologically related species [4].

In prokaryotes, the rDNA loci contain the genes for all three rRNA species, 16S, 23S, and 5S genes (Fig. 1). These genes are separated by a spacer region which exhibits a high degree of variation at the level of genus and species. Therefore, the rDNA sequence is recognized as a powerful taxonomic tool in bacteria [18]. In particular, the 16S rDNA sequence contains more variable regions that have been useful in the differentiation of genus and species [12].

Three strains were recently isolated and identified as *T. thiooxidans* AZ11, MET, and TAS [5, 6, 7]. Here, we report the phylogenetic affiliation and sequence alignments of these with iron and sulfur oxidizers, *T. ferrooxidans* and *T. thiooxidans*, based on the sequencing analysis of 16S rDNA and 16S/23S ISR. This is preliminary data for the determination of genetic marker which could be used for the primary screening of bioleaching strains from nature. The phylogenetic framework and sequence alignments established in this study may offer a more directed approach towards the comparative biochemistry of those organisms and promote the development of a phylogenetic marker for the detection of useful microorganisms involved in bioleaching.

MATERIALS AND METHODS

Strains and Cultures

Bacteria strains used in this study were *T. ferrooxidans* ATCC19859 as a reference strain, *T. thiooxidans* AZ11 [Korean Culture Type Collection (KCTC) 8929P], *T. thiooxidans* MET (KCTC8930P), and *T. thiooxidans* TAS (KCTC8928P). These three strains were originally isolated from soil, activated sludge, and digested sludge, respectively, and their functions in bioleaching were tested [5, 6, 7].

T. ferrooxidans was cultivated in M16 medium (3 g $MgSO_4 \cdot 7H_2O$, 0.2 g $(NH_4)_2 HPO_4$, 45 g $FeSO_4 \cdot 7H_2O$, and 0.1 g KCl per liter, pH 2.0, 28°C) with continuous aeration for 4 days. *T. thiooxidans* was cultured in thiosulfate medium (8 g $Na_2S_2O_3 \cdot 5H_2O$, 2 g K_2HPO_4, 0.2 g $MgCl_2 \cdot 7H_2O$, 0.4 g NH_4Cl, 2 g KH_2PO_4, and 0.01 g $FeSO_4 \cdot 7H_2O$ per liter at pH 5–6, 30°C) with aeration for 10 days. The cultures were filtered through Whatman No. 1 filter paper to remove precipitated ferric iron and sulfate precipitates. The supernatant was centrifuged at 8,000 rpm for 20 min to obtain cell pellet. *Escherichia coli* DH5α (*sup*E44Δ *lac*U169 (FΦ80lcZΔ M15) *hsd*R17 *rec*A*end*A1*gyr*A96*thi*-1*rel*A1) was grown in Luria Bertani medium (1% Bacto-tryptone, 0.5% Bacto-yeast extract and 1% NaCl) and used as a recipient for recombinant DNA.

The cells of the three strains were short rods (0.5 μm× 1 μm), motile, and Gram-negative. The colonies on the thiosulfate agar plate were small, with a diameter of 0.5–1 mm and whitish yellow by extracellular deposition of sulfur (1 to 2 mm diameters). The intracellular sulfur was nonvisible, which was confirmed under phase contrast microscopy. They could chemolithotrophically grow by utilizing energy from the oxidation of reduced sulfur compounds such as thiosulfate, elemental sulfur, and sulfide. However, they could not grow heterotrophically, nor oxidize ferrous ion to ferric ion. They had ubiquinone-8 as coenzyme in the respiration chain. Their main cellular fatty acids were hexadecanoic acid (16:0) as nonpolar fatty acids, and hydroxytetradecanoic acid (3-OH 14:0) as hydroxyl fatty acids. On the basis of the results of the ubiquinone system and cellular fatty acid composition, these three strains were identified as *T. thiooxidans*.

Table 1. Primers used for the amplification of *T. ferrooxidans* and *T. thiooxidans* 16S rDNA and 16S/23S ISR.

Primers	Sequence	Strain	Source (GenBank/EMBL Accession number)
TF16F	5'-GGCGGCATGCCTAACACA-3'	*T. ferrooxidans* ATCC19859	ABO39820
TF16R2	5'-ACGTATTCACCGCGGCATGCTGAT-3'		This study
TFISF	5'-GTAATCGCGGATCAGCAT-3'		ABO39820
TFISR	5'-TTAAACAGTTGCAGCCAC-3'		U18089
TT16F	5'-ACGCTGGCGGCATGCCTAACA-3'	*T. thiooxidans* AZ11	Y11596
TF16R	5'-ACGTATTCACCGCGGCATGCTGAT-3'		This study
AZISF	5'-TTCCTACGCATTACTCACCCGTCCGC-3'		This study
AZISR	5'-ACTACGGCCAACCTTCCCAGGTCGT-3'		Y11596
TT16F	5'-ACGCTGGCGGCATGCCTAACA-3'	*T. thiooxidans* MET	Y11596
TF16R2	5'-ACGTATTCACCGCGGCATGCTGAT-3'		This study
TFISF	5'-GTAATCGCGGATCAGCAT-3'		ABO39820
TFIS	5'-TTAAACAGTTGCAGCCAC-3'		U18089
TT16F	5'-ACGCTGGCGGCATGCCTAACA-3'	*T. thiooxidans* TAS	Y11596
TT16R	5'-AACGTATTCACCGCGGCATGC-3'		Y11596
TFISF	5'-GTAATCGCGGATCAGCAT-3'		ABO39820
TFISR	5'-TTAAACAGTTGCAGCCAC-3'		U18089

DNA Preparation

Genomic DNA was extracted as follows: Each strain was harvested by centrifugation at 14,000 rpm. Residual jarosites were removed by low-speed centrifugation, and sulfate precipitates were removed by repeated washing with distilled water. Cells were washed with 100 µl of TEN buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 10 mM NaCl) with vigorous vortexing. Cells were suspended with 50 µl SET buffer (20% sucrose, 50 mM Tris-HCl, pH 7.6, 50 mM EDTA) and lysed with lysozyme (5 mg/ml in TEN buffer) and 10% SDS. DNA was purified by treatment with phenol followed by precipitation with ethanol.

Table 2. Sequence information of 16S rDNA and 16S/23S ISR used in alignments.

rDNA	GenBank/EMBL Accession number	Strain	Ref.
16S rDNA	Y11596	*T. thiooxidans* ATCC19377	De Wulf-Duran, P.
	X72851	*T. thiooxidans*	Goebel, B. M.
	M79401	*T. thiooxidans* DSM612	Lane, D. J.
	AB039820	*T. ferrooxidans* NASF-1	Kamimura, K.
	X75267	*T. ferrooxidans* N-Fe4	Goebel, B. M.
	X75268	*T. ferrooxidans* N-Fe3	Goebel, B. M.
	AF359940	*T. thiooxidans* AZ11 KCTC 8929P	Cho *et al.*
	AF359941	*T. thiooxidans* MET KCTC 8928P	Cho *et al.*
	AF362021	*T. thiooxidans* TAS KCTC 8930P	Cho *et al.*
	AF362022	*T. ferrooxidans* ATCC19859	This work
	X91080	*S. thermosulfidooxidans*	Durand, P.
	AF124350	*S. orisratti* ATCC700640	Zhu, H.
16S/23S ISR	X07395	*T. ferrooxidans*	Venegas, A.
	X98209	*T. thiooxidans*	Vasquez, M.
	U51430	*T. cuprinus*	Moreira, D.
	X98211	*Thiobacillus* sp.	Vasquez, M.
	AJ237906	*Leptospirillum* sp. DSM8468	Espejo, R. T.
	U55311	*E. coli* K-12	Garcia-Martinez, J.
	AF362022	*T. ferrooxidans* ATCC19859	This work
	AJ278723	*T. ferrooxidans*	Seleska-Pobell, S.
	AJ278722	*T. ferrooxidans*	Seleska-Pobell, S.
	U18089	*T. ferrooxidans*	Flores-Rozas, H.
	AF359942	*T. thiooxidans* AZ11 KCTC 8929P	Cho *et al.*
	AF359943	*T. thiooxidans* MET KCTC 8928P	Cho *et al.*
	AF362021	*T. thiooxidans* TAS KCTC 8930P	Cho *et al.*

Amplification of rDNA Genes

Bacterial rDNAs were amplified from purified genomic DNA by PCR [19], using the primers shown in Table 1. The primer sequences were designed based on strictly conserved sequences specific for 16S and 23S rDNA from various bioleaching-associated bacteria (Table 2). TF16F, TT16F, TF16R, and TT16R were selected from the highly conserved region adjacent to the 16S rDNA sequence. TFISF and AZISF were selected from the sequence adjacent to 16S/23S ISR. TFISR and AZISR were derived from the highly conserved 23S rDNA sequence. TF16R2 originated from the sequence of 16S rDNA of *T. ferrooxidans* ATCC19859 revealed in this study (Fig. 1, Table 1).

PCR was carried out in 50 μl volumes containing 10 ng of each genomic DNA and 25 μl of ExTaq premix (TaKaRa, Japan), and 10 pmole of primers excluding TT16F and TF16R2 or 20 pmole (TT16F and TF16R2). The amplification cycle profile consisted of denaturation at 95°C for 1 min, annealing for 1 min at 54°C (for TFISF/TFISR and TF16F/TF16R2) or at 53°C (for TT16F/TT16R and TT16F/TF16R2) or at 55°C (for AZISF/AZISR), and extension for 3 min at 72°C.

Sequence Alignment and Phylogenetic Analysis

Using the computing program, Clustal [29], rDNA sequences obtained in this work were aligned to homologous rDNA sequences [16, 17, 20] of sulfur- and iron-oxidizing bacteria available from the GenBank database (Table 2). Regions showing ambiguity were excluded from the subsequent phylogenetic analyses based on 16S/23S ISR. The DNA base compositions in 16S rDNA and 16S/23S ISR were investigated by using DNAsis program. To investigate phylogenetic relationships of iron- and sulfur-oxidizing bacteria, Parsimony analysis was carried out by PAUP program accompanied by 1000 bootstrap. Maximum Likelihood analysis [9] was also performed by DNAml.

Nucleotide Sequences Accession Numbers

Sequences used in phylogenetic analyses were available from the GenBank or EMBL database. The new sequences are available under the following GenBank accession numbers: 16S rDNA of AZ11 (AF359940), 16S/23S ISR DNA of AZ11 (AF359942), 16S rDNA of MET (AF359941), 16S/23S ISR DNA of MET (AF359943), 16S rDNA and 16S/23S ISR DNA of TAS (AF362021), 16S rDNA and 16S/23S ISR DNA of ATCC19859 (AF362022).

RESULTS

Cloning and Sequencing of 16S rDNA and ISR

In this study, we examined the sequences of 16S/23S in an attempt to identify the phylogenetic position of the three strains of *T. thiooxidans* (AZ11, MET, and TAS), which had been previously isolated by screening the ability of sulfur oxidation [5-7]. The sequences of 16S rDNA and 16S/23S ISR of *T. ferrooxidans* ATCC19859, which were used as a control during the isolation of those three strains, were not known at the time our study was conducted. Therefore, we used this strain as a reference for amplification and phylogenetic analysis. Using primers TFISF and TFISR common to *T. ferrooxidans* NASF-1 and *T. ferrooxidans* (U18089), the fragment of 2.3 kb covering the 3' end of 16S rDNA, entire 16S/23S ISR, and 5' part of 23S rDNA of *T. ferrooxidans* ATCC19859 was successfully amplified. The 16S/23S ISR sequence exhibited significant homology with those of several strains of *T. ferrooxidans* and *T. thiooxidans* registered at the GenBank.

When the primers TF16F and TF16R derived from the 16S rDNA sequence of *T. ferrooxidans* NASF-1 were used to amplify the partial 16S rDNA sequence, no amplified fragment was obtained in spite of repeated trials with slight modifications. This failure led us to substitute TF16R with TF16R2 from the 2.3 kb fragment sequence of *T. ferrooxidans* ATCC19859. With primers TF16F and TF16R2, the fragment of 1.3 kb representing partial 16S rDNA sequence of *T. ferrooxidans* ATCC19859 was amplified. Thus, the sequences of partial 16S rDNA and entire 16S/23S ISR of *T. ferrooxidans* ATCC19859 was completely determined.

When the primers TF16F and TF16R2 were used for amplification of 16S rDNA of *T. thiooxidans* AZ11, MET, and TAS, no amplified fragments were produced, probably due to primer incompatibility. To search for proper primers, two more primers (TT16F and TT16R) derived from the sequence of *T. thiooxidans* ATCC19377 (Y11596) were synthesized. Among combinations of forward primers (TF16F and TT16F) with reverse primers (TF16R2 and TT16R), TT16F and TF16R2 turned out to be adequate for amplification of 16S rDNA sequences of *T. thiooxidans* AZ11 and *T. thiooxidans* MET, while TT16F and TT16R were adequate for *T. thiooxidans* TAS. Meanwhile, TFISF and TFISR were surprisingly effective for amplification of 16S/23S ISR of *T. thiooxidans* MET and *T. thiooxidans* TAS, but not for *T. thiooxidans* AZ11. To amplify 16S/23S ISR of *T. thiooxidans* AZ11, we used forward (AZISF) and reverse (AZISR) primers derived from its 16S rDNA sequence and from *T. thiooxidans* ATCC19377 (Y11596), respectively. Thus, approximately 1.3 kb fragments of 16S rDNA and 0.6 kb fragments of 16S/23S ISR for the three above strains were obtained and sequenced for analysis. Schematic representation of primers is shown in Fig. 1 and their sequences were listed in Table 1.

G+C Contents

The G+C contents of partial sequences of 16S rDNA and complete 16S/23S ISR sequences of *T. ferrooxidans* ATCC19859 and of the three novel *T. thiooxidans* AZ11,

Table 3. Nucleotide composition of 16S rDNA sequences of species used in this study.

Species	A	T	G	C	G+C	Total
1. *T. ferrooxidans*ATCC19859 (AF362022)	24.0	19.80	33.10	23.10	56.20	1499
2. *T. ferrooxidans* NASF-1 (AB039820)	24.5	19.10	33.10	23.30	56.40	1490
3. *T. ferrooxidans* N-Fe4 (X75267)	24.3	19.10	33.60	22.80	56.40	1333
4. *T. ferrooxidans* N-Fe3 (X75268)	24.4	19.20	33.60	22.90	56.50	1326
5. *T. thiooxidans* AZ11 KCTC 8929P (AF359940)	24.1	19.60	33.40	23.00	56.40	1340
6. *T. thiooxidans* ATCC 19377 (Y11596)	24.25	19.29	33.35	23.09	56.44	1472
7. *T. thiooxidans* TAS KCTC 8930P (AF362021)	20.66	21.40	34.04	22.87	56.91	1495
8. *T. thiooxidans* DSM612 (M79401)	24.03	18.20	32.93	22.85	55.78	1269
9. *T. thiooxidans* (X72851)	23.46	18.60	34.52	23.89	58.41	1338
10. *T. thiooxidans* MET KCTC 8928P (AF359941)	25.62	21.18	31.73	21.45	53.18	1346
11. *S. orisratti* ATCC 700640 (AF124350)	26.50	22.00	29.50	22.00	51.50	1337
12. *S. thermosulfidooxidans* (X91080)	21.30	15.80	36.20	26.80	63.00	1438

MET, and TAS strains were examined along with those of reference strains (Tables 3 and 4). In the case of 16S rDNA, *T. ferrooxidans* rarely varied, whereas *T. thiooxidans* varied to some degree within the range of 5%. Even though 16S rDNA sequences were partial, the G+C contents of *Thiobacillus* species remarkably contrasted those of a nonlithotrophic bacterium, *Streptococcus orisratti* (51.5%) and a chemolithotrophic bacterium, *Sulfobacillus thermosulfidooxidans* (63.0%) (Table 3). In the case of 16S/23S ISR, the G+C contents in *T. ferrooxidans* and *T. thiooxidans* were relatively constant with values of 53% and 55–56%, respectively (Table 4). Interestingly, *T. thiooxidans* TAS was close to *T. ferrooxidans* in terms of G+C contents.

Phylogenetic Relationship

When 16S rDNA sequences of 4 strains of *T. ferrooxidans*, 6 strains of *T. thiooxidans* including 3 KCTC strains, and *S. orisratti* as an outgroup control were analyzed by using PAUP, it was found that *T. thiooxidans* ATCC19377 was closer to the *T. ferrooxidans* strains rather than to the rest of the *T. thiooxidans* strains examined (Fig. 2). Meanwhile, all four strains of *T. ferrooxidans* were relatively close to each other. It should be noted that *T. ferrooxidans* ATCC19859, which has been classified based on biochemical characteristics, was also close to the three other *T. ferrooxidans* strains at the molecular level. In contrast, the *T. thiooxidans* strains appeared to be a paraphyletic group. Compared with other *T. thiooxidans* strains, the three novel strains (AZ11, MET, TAS) were more distant from *T. ferrooxidans*. ISR analysis by Maximum Likelihood showed that *T. ferrooxidans* strains constituted a monophyletic group, when *E. coli*, *Leptospirillum species*, and *Thiobacillus cuprinus* were involved in the outgroup (Fig. 3). However, *T. thiooxidans* strains formed a paraphyletic group as in 16S rDNA analysis and were divided into two groups, KCTC strains and the rest. The latter was found to be closer to *T. ferrooxidans* than to the former. In addition, *Leptospirillum* species interrupted the phylogenetic relationship of the two groups.

Homology of 16S rDNA and 16S/23S ISR Sequences

We compared the sequences of 16S rDNA and 16S/23S ISR of *T. ferrooxidans* ATCC 19859 and the three novel *T. thiooxidans* strains (AS11, MET, and TAS) available with those of several other *T. ferrooxidans* and *T. thiooxidans*

Table 4. Nucleotide composition of 16S/23S ISR nucleotide sequences of species used in this study.

Species	A	T	G	C	G+C	Total
1. *T. ferrooxidans* (U18089)	23.85	22.93	32.56	20.64	53.20	436
2. *T. ferrooxidans* ATCC 19859 (AF362022)	23.80	22.70	32.90	20.60	53.50	441
3. *T. ferrooxidans* (AJ278723)	23.00	23.23	32.74	20.79	53.53	452
4. *T. ferrooxidans* (X07395)	24.80	21.50	32.20	21.50	53.70	572
5. *T. ferrooxidans* (AJ278722)	24.55	21.65	32.81	20.98	53.79	436
6. *T. thiooxidans* (X98209)	25.00	19.60	33.60	21.50	55.10	545
7. *T. thiooxidans* MET KCTC 8928P (AF359943)	23.78	20.27	20.66	35.08	55.74	513
8. *T. thiooxidans* AZ11 KCTC 8929P (AF359942)	23.37	19.71	34.95	21.74	56.69	492
9. *T. thiooxidans* TAS KCTC 8930P (AF362021)	26.89	19.17	32.14	21.32	53.46	555
10. *Thiobacillus* sp. (X98211)	26.50	21.40	30.20	22.00	52.20	48.7
11. *T. cuprinus* (U51430)	21.80	27.20	27.50	23.50	51.00	600
12. *L. ferrooxidans* DSM8468 (AJ237906)	26.00	14.90	35.50	23.60	59.10	496
13. *E. coli* K-12 (U55311)	28.50	26.30	23.40	21.80	45.20	354

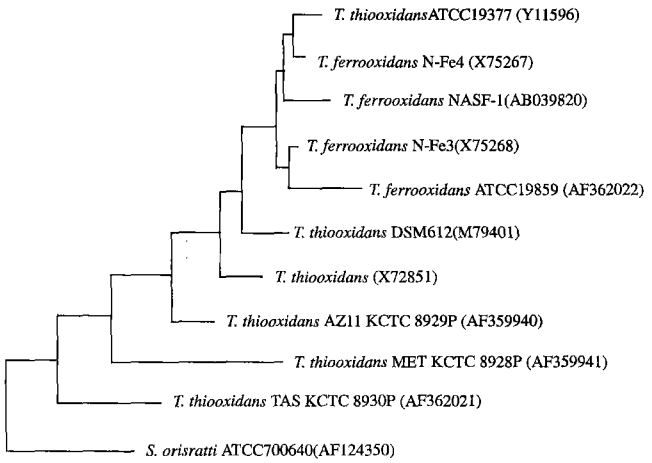

Fig. 2. Philogenetic tree based on the partial 16S rDNA sequences of Acidophilic bacteria and *Streptococcus orisratti* as an outgroup.
The Genbank accession numbers are shown in parentheses.

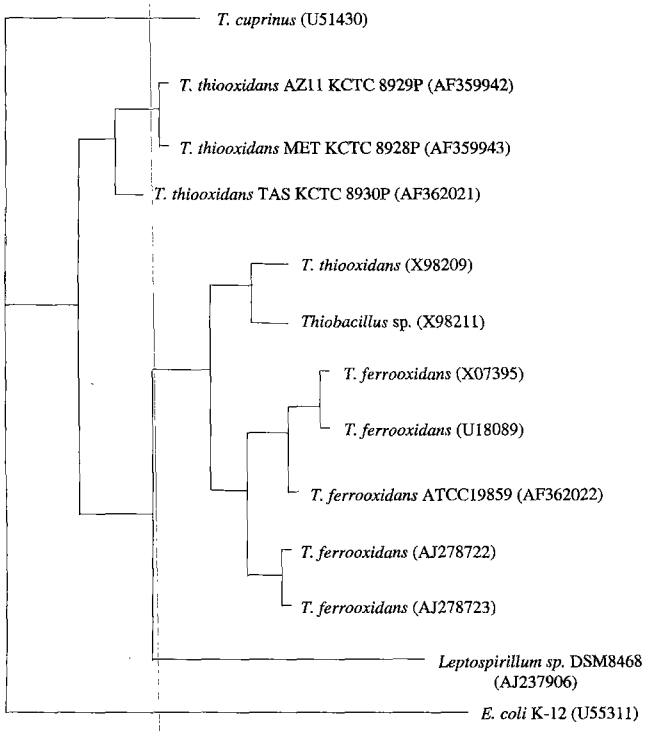

Fig. 3. Phylogenetic tree based on the 16S/23S ISR sequences of acidophilic bacteria and *E. coli* K-12 and *Leptospirillum* species as outgroup.
The GenBank accession numbers are shown in parentheses.

strains and outgroup species such as *Streptococcus* registered at the GenBank. When aligned with each other, high homology was found among the three novel strains (data not shown). The homology was higher in 16S/23S ISR than in 16S rDNA of all strains examined.

Clustral [29] program was used to determine the degree of homology among particular sequences by counting different bases from major bases in every 20 base-block. We found that the value became higher as more unrelated sequences were included. When the sequences of 16S rDNA were examined in *Thiobacillus* species only (Fig. 4A) and *Thiobacillus* plus *S. orisratti* (Fig. 4B), it was found that 16S rDNA sequences were not well conserved over the region examined except for several conserved domains indicated by arrows. When the sequences of 16S/23S ISR were examined, blocks 2–10 (nucleotide residues 21–200) were found to be conserved in *Thiobacilli* species only, whereas the remaining region was somewhat divergent (Fig. 5A). Meanwhile, when the 16S/23S ISR sequences of *Leptospirillum* species, *T. cuprinus* and *E. coli* were included, blocks 2–10 were found to be divergent as was the remaining blocks (Fig. 5B). These findings indicated that blocks 2–10 might be a genus-specific sequence, which could be used as a genetic marker to identify *Thiobacillus* species.

Next, we were interested in determining if the blocks numbered 11–25 (nucleotide residues 221–520) contained a species-specific sequence. As shown in Fig. 5C, that region was very well conserved among the five *T. ferrooxidans* strains. This result was in contrast to that of Fig. 5A, in which *T. ferrooxidans* and *T. thiooxidans* strains were combined, strongly indicating that the nucleotide residues 221-520 of ISR seemed to be specific to *T. ferrooxidans*. In the case of *T. thiooxidans*, however, high homology in that region among the three novel strains (AZ11, MET, and TAS) (Fig. 5D) was significantly disturbed even by including the ISR sequence of *T. thiooxidans* (X98209) (Fig. 5E). Thus, in contrast *T. ferrooxidans*, inconsistency in homology among *T. thiooxidans* strains indicated that the ISR sequence of bps 220–520 could not be used as *T. thiooxidans*-specific genetic marker.

DISCUSSION

Identification of individual organisms in the mixture of bioleaching bacteria is of importance for isolation of more functional bacterium in bioleaching as well as for phylogenetic expansion. Since *Thiobacillus* species have been identified only based on their biochemical ability to oxidize sulfur and iron, discrimination of species from nature remains obscure. As an alternative way for identification, molecular biological tools have been developed. For example, the sequence of 16S rDNA which is well conserved among species has been used for taxonomy. Recently, the sequence of 16S/23S ISR has sometimes provided a better criterion for phylogenetic classification [1, 30].

In the present study, we focused on determining the nucleotide sequences of 16S rDNA and 16S/23S ISR for classification of three *T. thiooxidans* strains recently

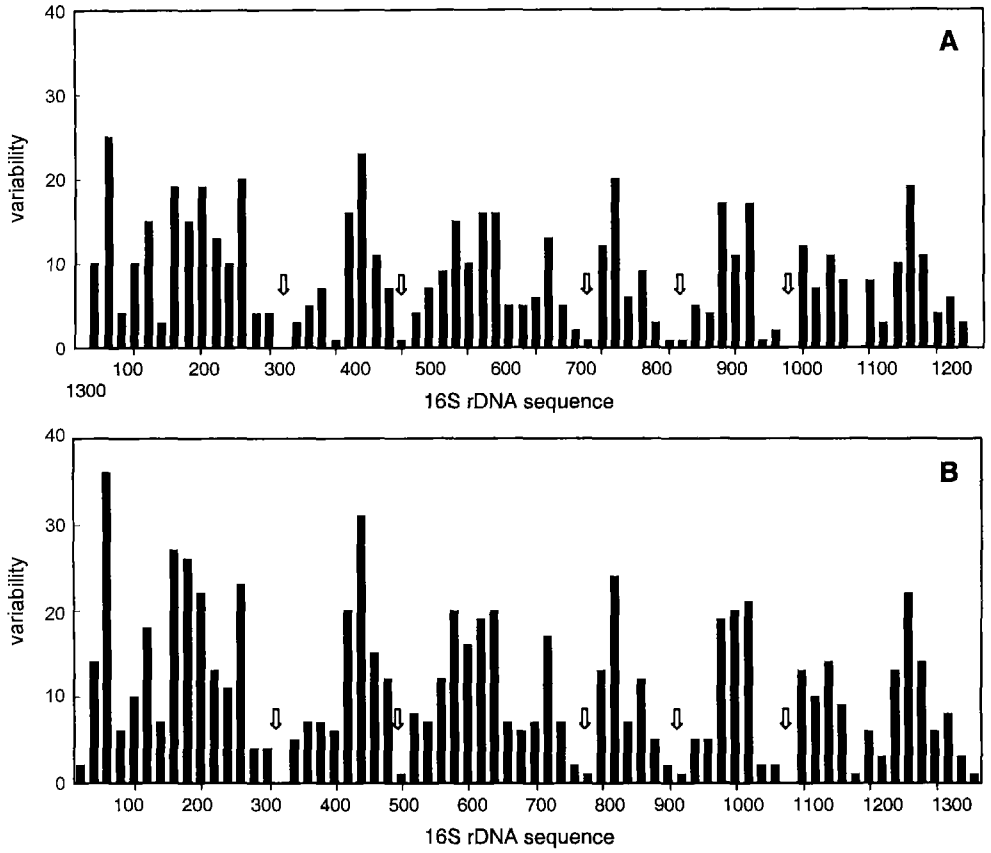

Fig. 4. Divergence of 16S rDNA sequence in ingroup strains only (A) and ingroup strains along with *S. orisratti* as an outgroup (B). The numbers of different bases from major bases in a 20 base-block are shown in the Y-axis. Arrows indicate conserved regions among tested strains.

isolated and one strain of *T. ferrooxidans* ATCC19859. They were compared with corresponding sequences of some other *T. thiooxidans* and *T. ferrooxidans* strains available from the GenBank or the EMBL database. In both cases, *T. ferrooxidans* strains were faithfully grouped, whereas *T. thiooxidans* strains were grouped in a rather promiscuous way (Figs. 2 and 3). As shown in Fig. 3, the three novel *T. thiooxidans* strains excluding *T. thiooxidans* (X98209) were more distant phylogenetically from *T. ferrooxidans* than *Leptospirillum* species. This may correlate with the information that G+C contents of 16S rDNA and 16S/23S ISR were constant among *T. ferrooxidans* strains in contrast to fluctuations among *T. thiooxidans*. These results may suggest either that there is a need for either more detailed criteria for classification of *T. thiooxidans*, reclassification of the three *T. thiooxidans* strains, or reconsideration of phylogenetic characterization of *Leptospirillum* species.

Isolation and evaluation of strains is important for identification of new functional strains from the natural environment. Two different methods have been commonly used for that purpose: *in situ* hybridization using specific probes and PCR-mediated detection using specific primers [21]. Acquisition of proper probe for the former and primers for the latter are essential for the success of these approaches. When variation occurs in the sequence of interest, PCR-mediated detection, per se, cannot frequently be used as shown in this study. Accordingly, hybridization seems to be more efficient for detection and/or identification of closely related sequences. If the hybridization probe possesses genus- or species-specificity, it can be a useful genetic marker especially for the classification of organisms in a mixed culture of bioleaching-associated bacteria. Therefore, we tried to search for genetic markers, if any. As shown in Fig. 4, the partial sequence of 16S rDNA in several *Thiobacilli* examined in this study was not found to contain any genus- or species-specific regions. In contrast, the 5' region of 16S/23S ISR sequence (nucleotide residues 21–200) was found to be hypothetically specific for the genus of *Thiobacillus*, even though the number of sequences examined was limited. Expansion of available sequences will help determine whether that region can be used as a genetic marker.

The 3' region of 16S/23S ISR sequence (nucleotide residues 201–520) exhibited low degree of homology among several *Thiobacilli* species (Fig. 5A). However, significantly high homology was regained when only *T. ferrooxidans* strains were compared (Fig. 5C), suggesting that 3' region

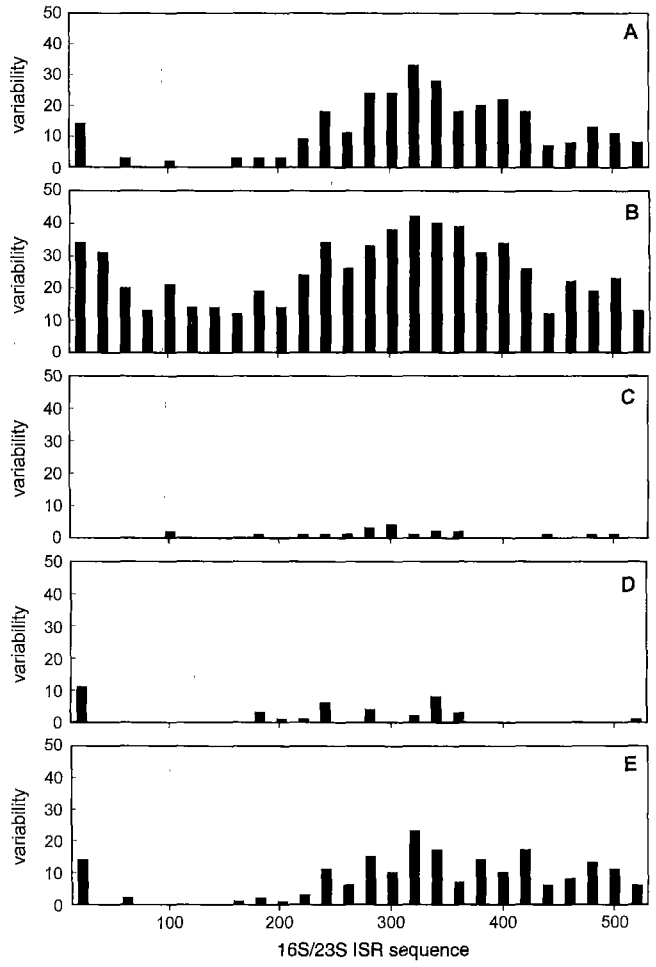

Fig. 5. Divergence of 16S/23S ISR sequence.
Ingroup strains only (A); ingroup strains along with *Leptospirillum* species, *E. coli* K-12 and *T. cuprinus* as an outgroup (B); *T. ferrooxidans* strains only (C); three *T. thiooxidans*, AZ11, MET, and TAS (D); three *T. thiooxidans*, AZ11, MET, and TAS with *T. thiooxidans* (X98209) (E). The numbers of different bases from major bases in a 20 base-block are shown in the Y-axis.

of 16S/23S ISR might be used as a genetic marker specific for *T. ferrooxidans*. Meanwhile, significantly high homology was also regained when only the three novel strains of *T. thiooxidans* were compared (Fig. 5D), but lost when including the sequences of two other *T. thiooxidans* strains. This was not surprising since those two strains were found to be close to *T. ferrooxidans* rather than to *T. thiooxidans* as shown in a phylogenetic tree based on ISR sequence (Fig. 3).

In conclusion, the 5' part of 16S/23S ISR of either *T. ferrooxidans* and *T. thiooxidans* can be used as a genetic marker for the identification of *Thiobacilli* genus from a mixed population. Additionally, the 3' part of 16S/23S ISR of *T. ferrooxidans* can be used only for *T. ferrooxidans* species, whereas the 3' part of 16S/23S ISR of *T. thiooxidans* species is not limited to its own species.

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