Discrimination among the Three *Acidithiobacillus* Species, *A. ferrooxidans, A. thiooxidans* and *A. caldus*, Based on Restriction Fragment Length Polymorphism Analysis of the 16S–23S rDNA Intergenic Spacer Region

Satoshi Wakai, Kentaro Yamamoto, Tadayoshi Kanao Tsuyoshi Sugio and Kazuo Kamimura

(Department of Biological Function)

The PCR-amplified 16S-23S rDNA intergenic spacer regions (ISRs) of *Acidithiobacillus ferrooxidans, A. thiooxidans,* and *A. caldus* strains were sequenced and evaluated for differentiation and identification of these bacteria. The total length of the 16S-23S ISRs of *A. ferrooxidans* and *A. thiooxidans strains* and *A. caldus* GO-1 were 441, 456, and 379 bp, respectively. Two genes, encoding $tRNA^{Ile}$ and $tRNA^{Ala}$, and the box A-like sequences were highly conserved in the ISRs of all *Acidithiobacillus* species. The restriction fragment length polymorphism (RFLP) profiles of the PCR-amplified 16S-23S rDNA ISRs digested by *Hae*III and *Alu*I could clearly discriminate *A. ferrooxidans* from *A. thiooxidans* and *A. caldus*. The results indicated that RFLP analysis of the 16S-23S ISRs is an easy and rapid method for discrimination and identification of *Acidithiobacillus* species.

Key words : *Acidithiobacillus*, 16S-23S rDNA, RFLP analysis, tRNA

Introduction

A. ferrooxidans, A. thiooxidans, and *A. caldus* are especially found in sites where sulfur compounds are abundant, such as acid mine drainages, sewage treatments and marine habitats. They are Gram-negative, non-spore forming bacteria that derive energy from the oxidation of reduced inorganic sulfur compounds and fix CO_2 as the carbon source [1]. *A. ferrooxidans* is characterized by its ability to oxidize both ferrous iron and reduced inorganic sulfur compounds. Since growth rates of *Acidithiobacillus* species are very low and the growth is chemoautotrophic, ecological studies of *Acidithiobacillus* species are hampered by difficulties in isolating and identifying strains using culture-based methods. Therefore, the development of a rapid and selective method is required to identify *Acidithiobacillus* species in natural environments.

In a previous paper, we reported that a 16S rDNA fingerprinting technique could differentiate *A. ferrooxidans* from *A. caldus*, but failed to differentiate *A. ferrooxidans* from *A. thiooxidans* [2]. In most eubacteria, the genes for rRNA are organized in operons with the genes encoding the 16S, 23S, and 5S rRNAs separated by intergenic spacer regions. Since ISRs exhibit a great deal of length and sequence variation, polymorphism analysis of the 16S–23S rDNA spacer fragments has been successfully used to identify and discriminate bacteria in environmental samples[3–7]. In this report, we present additional data on the sequences and RFLP profiles of the PCR-amplified 16S–23S rDNA ISRs from *A. ferrooxidans, A. thiooxidans*, and *A. caldus*.

Materials and Methods

Bacterial strains and culture condition

Bacterial strains used in this investigation were as follows: iron-oxidizing bacteria *Acidithiobacillus ferrooxidans* ATCC 13598, ATCC 13661, ATCC 14119, ATCC 19859, ATCC 21834, ATCC 23270, and ATCC 33020 from a type culture collection center; *A. ferrooxidans* NASF-1[8] and Funis 2-1[9] isolated from natural environments; sulfur-oxidizing bacteria *A. thiooxidans* IFO 13701, IFO 13724, and JCM 3867 from type culture collection centers; a moderately thermophilic sulfur-oxidizing bacterium *A. caldus* GO-1[10] from a natural environment. These bacteria were cultured as described by Kamimura et al.[2].

Amplification of 16S–23S rDNA ISRs

DNA was isolated and purified as described previ-

Received October 1, 2005

ously[2]. An amplification of the 16S–23S rDNA ISR was performed using a primer pair P16SF (GCCTTGTACACACCGC) and P23SR (CTTAGATGTTTCAGTTC), which anneal to positions 1387 to 1402 of the 16S rDNA and positions 203 to 187 of the 23S rDNA, *Escherichia coli* numbering (GenBank accession no. V00331), respectively. Amplifications were performed with chromosomal DNA prepared from 13 strains of *Acidithiobacillus* species. PCR reactions were carried out in the following mixture; 10 μl 10 × PCR buffer, 100 ng purified chromosomal DNA, 0.5 μM each primer, 200 μM dNTP and 2 U Taq polymerase (Takara Biochemicals, Shiga) in a total volume of 100 μl. Thermal cycling parameters were as follows; the first denaturation, 94 °C for 2 min; denaturation, 94 °C for 30 s; annealing, 43 °C for 30 s; extention, 72 °C for 60 s; cycle number, 35; the final extension, 72 °C for 2 min. The amplification products were analyzed by electrophoresis on 1 % agarose gel.

Sequencing and sequence analysis of 16S–23S rDNA ISRs

The 16S–23S rDNA ISRs amplified by PCR and purified with a Geneclean Kit (Funakoshi Co., Tokyo) were directly sequenced as described previously[2]. The nucleotide sequence data of intergenic spacers from *A. ferrooxidans* ATCC 19859, ATCC 13598, ATCC 14119, ATCC 21834, ATCC 23270, ATCC 33020, NASF–1, and Funis 2–1; *A. thiooxidans* IFO 13701, IFO 13724 and JCM 3857; and *A. caldus* GO–1 have been submitted to the DDBJ/EMBL/GenBank nucleotide sequence databases under accession nos. AB 189131, AB 189132, AB 189133, AB 198134, AB 189135, AB 189136, AB 189137, AB 189138, AB 189139, AB 189140, AB 189141, and AB 189142, respectively. The DDBJ CLUSTALW System was used for an alignment of sequences[11].

RFLP analysis of 16S–23S rDNA ISRs

Restriction fragment length polymorphism (RFLP) analysis was conducted to discriminate 16S–23S rDNA ISRs from 13 strains of *Acidithiobacillus* species. PCR products were digested in two reactions separately using restriction endonucleases, *Alu*I and *Hae*III (Takara Biomedicals), at 37 °C overnight. The digested fragments were separated by electrophoresis on 4 % NuSieve 3:1 agarose gels (FMC Bioproducts) in 1 × Tris-acetate-EDTA buffer (pH 8.0). After electrophoresis, gels were stained with ethidium bromide, and then visualized on a UV transilluminator.

Results and Discussion

PCR amplification using primers, P16SF and P23SR, designed from the flanking terminal sequences of the 16S and 23S rRNA genes, was performed with chromosomal DNA isolated from 13 strains of *Acidithiobacillus* species. Amplifications of the 16S–23S rDNA ISRs from *A. ferrooxidans* and *A. thiooxidans* strains yielded products having a nearly similar size of approximately 770 bp (Fig. 1). An amplification of the region from *A. caldus* GO–1 yielded a slightly shorter band with approximately 690 bp in size (Fig. 1).

A. ferrooxidans has been known to have two rRNA operons and two tRNAs, $tRNA^{Ile}$ and $tRNA^{Ala}$, in the spacer between the 16S and 23S rDNA[12,13]. The size of the 16S–23S rDNA ISRs from 8 strains of *A. ferrooxidans* was 441 bp, that from 3 strains of *A. thiooxidans* was 456 bp, and that from *A. caldus* GO–1 were 379 bp. Sequences from the 16S–23S rDNA ISR of *A. ferrooxidans* or *A. thiooxidans* strains ranged from 99.1 to 100 %, or from 99.8 to 100 % similarities, respectively. The 16S–23S rDNA sequence similarity between *A. ferrooxidans* strains and *A. thiooxidans* strains or *A. caldus* GO–1 was from 79.0 to 79.4 %, or from 77.9 to 78.6 %, respectively. The sequence similarity between *A. thiooxidans* strains and *A. caldus* GO–1 was 78.1 %. Nucleotide sequences of *A. ferrooxidans* NASF–1, *A. thiooxidans* IFO 13701 and *A. caldus* GO–1 were aligned and is shown in Fig. 2. Two tRNA genes, $tRNA^{Ile}$ (77 bp) and $tRNA^{Ala}$ (76 bp), were involved in the spacer region of three *Acidithiobacillus* species. The antitermination element, box A–like sequences (TGTTCTTTGACA), was identical among three *Acidithiobacillus* species, and had two mismatches with the corresponding sequence of *E. coli*

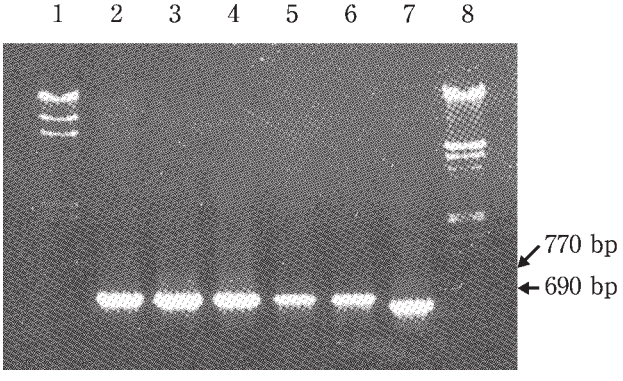

Fig. 1 Agarose gel electrophoresis of PCR-amplified fragments of the 16S–23S rDNA intergenic spacer regions from the strains of *Acidithiobacillus*.
Lanes: 1, λDNA *Hind*III restriction fragments; 2; *A. ferrooxidans* ATCC 23270; 3, *A. ferrooxidans* NASF–1; 4, *A. thiooxidans* IFO 13701; 5, *A. thiooxidans* IFO 13724; 6, *A. thiooxidans* JCM 3867; 7, *A. caldus* GO–1; 8, λDNA *Hind*III/*Eco*RI restriction fragments.

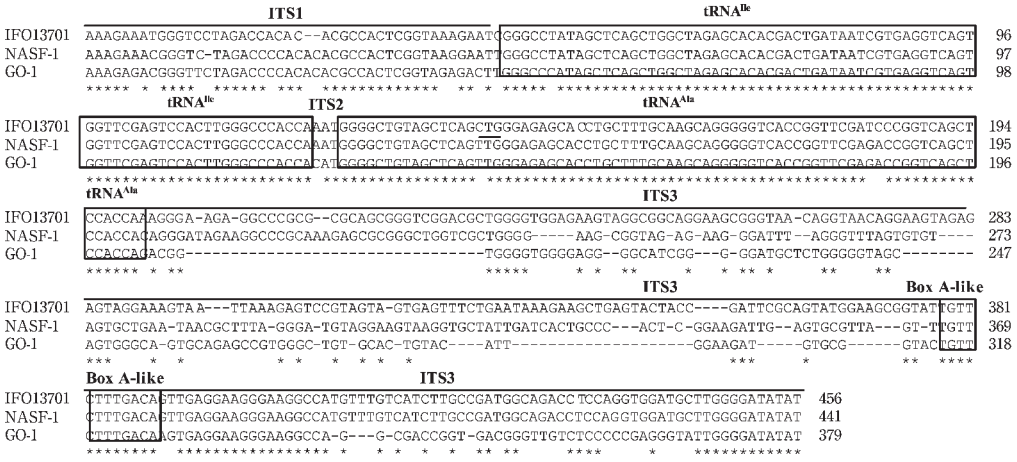

Fig. 2 Alignment of the 16S-23S rDNA intergenic spacer sequences from *A. thiooxidans* IFO 13701, *A. ferrooxidans* NASF-1, and *A. caldus* GO-1. Identical bases are indicated by asterisks and sequence gap are indicated by dashes.

(TGCTCTTTAACA) (Fig. 2). Another highly conserved region of 17 nucleotides, following the box A element, was observed in intergenic spacer regions of three *Acidithiobacillus* species. The tRNA genes of all *A. ferrooxidans* and *A. thiooxidans* strains were separated by an AAT intergenic spacer (ITS 2) (Fig. 2), the first base of which was C in *A. caldus* GO-1 (Fig. 2). Although nucleotide sequences of 5'-terminal region of intergenic spacer 1 (ITS 1) and 3'-terminal region of ITS 3 were relatively conserved among *Acidithiobacillus* species, heterogeneity was observed in sequences of 5'-terminal region of ITS 3. Our blastn searches on databases with heterogeneous sequences in the ITS 3 regions of three *Acidithiobacillus* species revealed that each region was specific to species, supporting a previous suggestion that ITS 3 region is a potential target for the development of molecular probe for differentiating Acidithiobacilli[7].

As shown in our previous report, a phylogenic tree constructed based on 16S rDNA sequences from *Acidithiobacillus* strains indicates high sequence similarity between *A. ferrooxidans* and *A. thiooxidans*[2]. Karavaiko *et al*. have also reported a comparative phylogenetic analysis of the 16S rDNA genes of *Acidithiobacillus* species, and indicated that *A. thiooxidans* comprised a monophyletic cluster with *A. ferrooxidans* in the phylogenetic tree[14]. Since *A. ferrooxidans* was not clearly differentiated from *A. thiooxidans* by RFLP profiles of the 16S rDNA fragments, RFLP analysis of the PCR-amplified 16S-23S rDNA intergenic spacer fragments from *Acidithiobacillus* strains was carried out to examine whether the analysis is used to identify *Acidithiobacillus* species from natural environments. The 16S-23S rDNA ISRs from 13 strains of *Acidithiobacillus* were digested with two

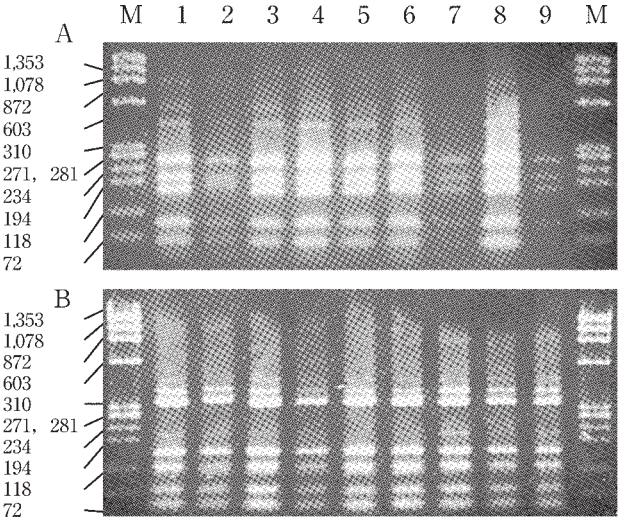

Fig. 3 Restriction fragment length profiles of the 16S-23S rDNA intergenic spacer regions amplified from *A. ferrooxidans* strains. Amplified products were digested with the restriction endonucleases, *Hae*III (A) and *Alu*I (B).
Lanes: 1, ATCC 13598; 2, ATCC 13661; 3, ATCC 14119; 4, ATCC 19859; 5, ATCC 21834; 6, ATCC 23270; 7, ATCC 33020; 8, NASF-1; 9,Funis 2-1. Lane M, molecular marker (ϕX 174 DNA *Hae*III restriction fragments).

restriction endonucleases, *Alu*I and *Hae*III, and analyzed by electrophoresis. With *Hae*III and *Alu*I, *A. ferrooxidans* strains except for strain ATCC 13661 displayed the identical RFLP profiles (Fig. 3A and 3B). Although strain ATCC 13661 was discriminated from the other *A. ferrooxidans* strains by RFLP profiles with *Hae*III, all the strains showed identical RFLP profiles with *Alu*I (Fig. 3B). Three strains of *A. thiooxidans* displayed identical RFLP profiles with *Hae*III and *Alu*I (Fig. 4A and 4B, lanes 3-5). RFLP

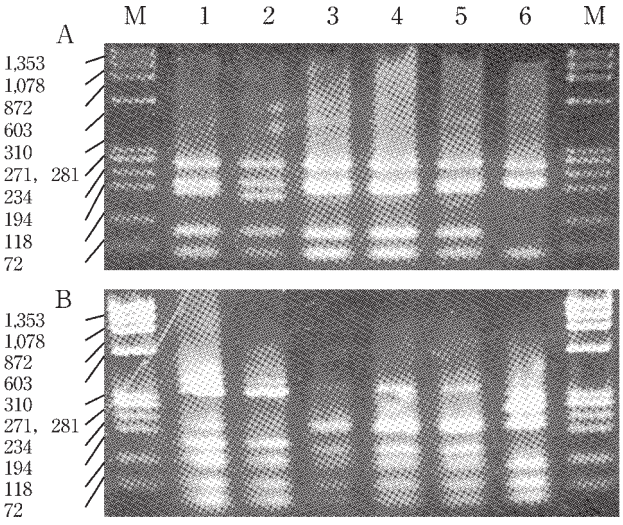

Fig. 4 Restriction fragment length profiles of the 16S–23S rDNA intergenic spacer regions amplified from *Acidithiobacillus* strains. Amplified products were digested with restriction endonucleases, *Hae*III (A) and *Alu*I (B). Lanes: 1, *A. ferrooxidans* ATCC 13661; 2, *A. ferrooxidans* NASF-1; 3, *A. thiooxidans* IFO 13701; 4, *A. thiooxidans* IFO 13724; 5, *A. thiooxidans* JCM 3867; 6, *A. caldus* GO-1. Lane M, molecular marker (ϕX 174 DNA *Hae*III restriction fragments).

profiles of *A. caldus* GO-1 were different from those of *A. ferrooxidans* and *A. thiooxidans*. Thus, *A. ferrooxidans* could be discriminated from *A. thiooxidans* strains and *A. caldus* GO-1 by RFLP profiles with *Hae*III and *Alu*I (Fig. 4).

Since a database for 16S–23S rDNA ISR sequences was established enabling a comparison of the sequences from different bacteria (http: //ulises.umh.es/RISSC)[15], the RFLP analysis of the 16S–23S rDNA ISRs may be successfully used to study the diversity of bacterial population in environmental samples from acid mine drainages, sewage treatments, and marine habitats.

References

1) Vishniac, W. V. : The genus *Thiobacillus*, p. 456–461, *In* Buchanan, R. E. and Gibbons, N. E. (ed.), Bergey's Manual of Determinative Bacteriology. 8th ed., Williams and Wilkins, Baltimore (1974)
2) Kamimura, K., S. Wakai and T. Sugio : Identification of *Thiobacillus ferrooxidans* strains based on restriction fragment length polymorphism analysis of 16S rDNA. Microbios, **105**, 141–152 (2001)
3) Jensen, M. A., J. A. Webster and N. Straus : Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms. Appl. Environ. Microbiol., **59**, 945–952 (1993)
4) Grütler, V. and V. A. Stanisich : New approaches to typing and identification of bacteria using the 16S–23S rDNA spacer region. Microbiology., **142**, 3–16 (1996)
5) Garcia-Martinez, S. G. Acinas, A. I. Anton and F. Rodriquez-Valera : Use of the 16S–23S ribosomal genes spacer region in studies of prokaryotic diversity. J. Microbiol. Methods, **36**, 55–64 (1999)
6) Pizarro, J., E. Jedlicki, O. Orellana, J. Romero and R. T. Espejo : Bacterial population in samples of bioleached copper ore as revealed by analysis of DNA obtained before and after cultivation. Appl. Environ. Microbiol., **62**, 1323–1328 (1996)
7) Bergamo, R. F., M. T. M. Novo, R. V. Verissimo, L. C. Paulino, N. C. Stoppe, M. I. Z. Sato, G. P. Manfio, P. I. Prado, O. Garcia, Jr. and L. M. M. Ottoboni : Differentiation of *Acidithiobacillus ferrooxidans* and *A. thiooxidans* strains based on 16S–23S rDNA spacer polymorphism analysis. Res. Microbiol., **155**, 559–567 (2004)
8) Sugio, T., A. Fujioka, M. Tsuchiya, N. Shibusawa, K. Iwahori and K. Kamimura : Isolation and some properties of a strain of the iron-oxidizing bacterium *Thiobacillus ferrooxidans* resistant to 2, 4-dinitrophenol. J. Ferment. Bioeng., **86**, 134–137 (1998)
9) Sugio, T., L. J. White, D. Shute, D. Choate and R. C. Blake II : Existence of a hydrogen sulfide: ferric iron oxidoreductase in iron-oxidizing bacteria. Appl. Environ. Microbiol., **58**, 431–433 (1992)
10) Kamimura, K., T. Okayama, K. Murakami and T. Sugio : Isolation and characterization of a moderately thermophilic sulphur-oxidizing bacterium. Microbios, **99**, 7–18 (1999)
11) Thompson, J. D., T. J. Gibson, F. Plewniak, D. Jeanmougin and G. Higgins : The CLUSTAL_W windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res., **25**, 4876–4882 (1997)
12) Salazar, O., M. Takamiya and O. Orellana : Characterization of the two tRNA gene operons present in *Thiobacillus ferrooxidans*. FEBS Lett., **242**, 436–443 (1989)
13) Vengas, A., E. Hevia and H. Sanchez : Sequence of two tRNA genes from a *Thiobacillus ferrooxidans* ribosomal operon. Nuc. Acids Res., **16**, 8179 (1988)
14) Karavaiko, G. I., T. P. Turova, T. F. Kondrat'eva, A. M. Lysenko, T. V. Kolganova, S. N. Ageeva, L. N. Muntyan and T. A. Pivovarova : Phylogenetic heterogeneity of the species Acidithiobacillus ferrooxidans. Int. J. Syst. Evol. Microbiol., **53**, 113–119 (2003)
15) Garcia-Martinez, J., I. Bescos, J. J. Rodriguez-Sala and F. Rodriguez-Valera : RISSC : a novel database for ribosomal 16S–23S RNA genes spacer regions. Nuc. Acids Res., **29**, 178–180 (2001)

16S-23S rDNA スペーサー領域の制限断片長多型解析による *Acidithiobacillus* 属3種，*A. ferrooxidans*，*A. thiooxidans* および *A. caldus* の識別

若井　暁・山本健太郎・金尾　忠芳・杉尾　剛
上村　一雄
（生物機能開発学講座）

Acidithiobacillus ferrooxidans，*A. thiooxidans* および *A. caldus* の16S-23S rDNA スペーサー領域の塩基配列を決定し，これらのバクテリアの識別・同定への有効性を評価した．*A. ferrooxidans*，*A. thiooxidans* および *A. caldus* のスペーサー領域の長さは，それぞれ441，456および379bp であった．3種の *Acidithiobacillus* スペーサー領域では，$tRNA^{Ile}$，$tRNA^{Ala}$をコードする遺伝子の塩基配列が高度に保存されていた．PCR 増幅した16S-23S rDNA スペーサー領域を *Hae*Ⅲおよび *Alu*Ⅰで酵素処理することによって得られた断片の解析によって，*A. ferrooxidans* を *A. thiooxidans* と *A. caldus* から識別できた．*Acidithiobacillus* 種の16S-23S rDNA スペーサー領域の制限断片長多型解析は，*Acidithiobacillus* 属の種の同定および *A. ferrooxidans* に属する株の同定のための迅速で，技術的に簡便な方法であることが明らかとなった．