Characterization of a putative chromosome segregation and condensation protein (ScpB) in an acidophilic iron–oxidizing bacterium
Acidithiobacillus ferrooxidans

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Acidithiobacillus ferrooxidans is one of the most widely used microorganisms in bioleaching operations to recover copper from low-grade copper sulfide. This bacterium uses ferrous iron and reduced inorganic sulfur compounds (RISCs) as energy sources. Transcriptions of genes thought to be involved in the oxidation of RISCs have been known to be highly activated in *A. ferrooxidans* cells grown on RISCs, while transcriptions of genes involved in the iron oxidation were repressed in the cells grown on RISCs. A gene encoding a putative chromosome segregation and condensation protein (ScpB) with a helix-turn-helix motif was found in the upstream region of sulfide : quinone oxidoreductase gene, whose expression was up-regulated in cells grown in sulfur and tetrathionate. A semi-quantitative PCR analysis using cDNA prepared from iron-, sulfur-, or tetrathionate-grown cells revealed that the transcription of *scpB* was up-regulated in cells grown on sulfur or tetrathionate as the energy source. Electrophoretic mobility shift assays were employed to examine whether the ScpB functions as a transcription factor. The result indicated that the recombinant His-tagged ScpB protein was able to nonspecifically bind *in vitro* to DNA. This is the first report on a direct association of ScpB with DNA.

Key words : *Acidithiobacillus ferrooxidans*, Acidophile, ScpB, transcription factor

Introduction

Ferric irons generated by iron-oxidizing bacteria are essential for the bioleaching from sulfide ores, because they chemically catalyze the solubilization of metal ions from the ores. However, a sulfur layer formed on the surface of sulfide ores prevents ferric ions from attacking the sulfide-metal bond, resulting in a decrease in the bioleaching efficiency. Therefore, in addition to iron-oxidizing activity, one of the important microbial activities in the bioleaching operation is sulfur-oxidizing activity to prevent the formation of the elemental sulfur layer on the surface of ores[14]. An iron-oxidizing bacterium *Acidithiobacillus ferrooxidans* is a chemolithoautotrophic acidophile that has been studied for industrial bioleaching application[12,14,16]. This bacterium obtains its energy for growth and cell maintenance from the oxidation of ferrous iron and/or reduced inorganic sulfur compounds (RISCs) under acidic conditions. In the iron oxidation, components involved in the electron transfer from ferrous iron to oxygen are thought to be two cytochromes c (Cyc 2 and Cyc 1), a rusticyanin (Rus), and an aa_3–type cytochrome c oxidase[16]. These proteins are encoded in the *rus* operon. A higher expression of this operon has been reported in ferrous iron-grown cells than in sulfur-grown ones[19].

Although some enzyme activities thought to be involved in the aerobic oxidation of RISCs by *A. ferrooxidans* were detected[1,4,13], the oxidation pathway of RISCs is still unclear. We have proposed that sulfur oxidation in sulfur-grown *A. ferrooxidans* cells proceeds *via* sulfide as an intermediate and involves a sulfide : quinone oxidoreductase (Sqr) and a *bd*–type ubiquinol oxidase[7,17,18]. We have also reported the involvement of tetrathionate hydrolase (Tth) in tetrathionate and sulfur oxidations in *A. ferrooxidans*[8]. Although both the iron-oxidizing and sulfur-oxidizing activities are indispensable for the bioleaching, the iron-oxidizing activity was decreased in *A. ferrooxidans* cells grown on sulfur[18,19]. We have reported that the expressions of *sqr* and *tth* genes are up–regulated in cells grown on sulfur or tetrathionate[8,18]. By expression proteomics an exported 21–kDa putative thiosulfate sulfur transferase protein and a 33–kDa putative thiosulfate/sulfate binding protein (ModA 1) were found to be synthesized in much higher amounts in cells grown in sulfur or thiosulfate as com-

pared with those grown in ferrous iron[11]. Proteomic and bioinformatic analysis using immobilized pH gradients and mass spectrometry has also revealed that a sulfate/molybdate-binding protein (probably ModA1) was synthesized in high abundance during sulfur growth condition[2]. Although these results suggested the presence of a specific mechanism for the transcriptional regulation of genes involved in iron or sulfur oxidation in *A. ferrooxidans*, the regulation mechanisms have been poorly understood. The clarification of regulatory mechanism for the expression of genes involved in the oxidations of iron and RISCs is expected to provide us with a new technique to improve the leaching efficiency. In the present study we examined the transcription of a gene encoding a putative chromosome segregation and condensation protein (ScpB) having a helix-turn-helix (HTH) motif and evaluated its binding activity to putative promoter regions of genes involved in iron or sulfur oxidation in *A. ferrooxidans*.

Materials and methods

Microorganisms, media, and culture conditions

A. ferrooxidans ATCC 23270 was used in this study. Iron-, sulfur-, or tetrathionate-grown cells were prepared as described previously[17,18]. *E. coli* strains were grown in Luria-Bertani(LB)medium at 37℃. Ampicillin was added at the final concentration of 50 μg mL^{-1}.

Total RNA isolation, reverse transcription polymerase chain reaction (RT-PCR)

The transcriptional level of the *scpB* gene was semiquantitatively determined by RT-PCR. The 16S RNA gene was used as the endogenous control. The sequences of oligonucleotide primers used in the experiments were designed based on nucleotide sequences found in the available finished DNA genome sequence of *A. ferrooxidans* ATCC 23270 (http://cmr.jcvi.org/cgi-bin/CMR/GenomePage.cgi?org=gtf) (Table 1). The specificity of the primers was confirmed by PCR using genomic DNA from *A. ferrooxidans* ATCC 23270. Cells grown at 30℃ with shaking in 1 liter of iron-medium for 4 days and sulfur medium or tetrathionate medium for 7 days were harvested by centrifugation and suspended in 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA. Total RNA was immediately isolated using a PureLink™ Micro-to-Midi Total RNA Purification System (Invitrogen Japan KK, Tokyo, Japan) with a RNase-free DNase (Invitrogen) treatment. RNA concentrations were determined using a Quant-iT™ RNA Assay kit (Invitrogen) with a Qubit™ fluorometer (Invitrogen). Equal amounts of total RNA (1 μg) were used to prepare cDNA using a PrimeScript 1st strand Synthesis kit according to the manufacturer's protocol (Takara-Bio, Shiga, Japan). The reaction mixture for cDNA synthesis contained 0.5 mM dNTP mixture, 1 μL of 10 μM reverse primer (PscpB-R), 1 μg of total RNA,

Table 1 Primers used to amplify *scpB* gene, 16S rRNA gene, and promoter regions of genes involved in the sulfur or iron oxidation

Primers	Sequence (5'> 3')	Genes or DNA regions
ScpB-F-Nhe	TTTGGCGAGCTAGCCGTGAAGCGCTCCTGG	*scpB* gene
ScpB-R-Hind	GGAATATTATAGAAGCTTCCCAATT	*scpB* gene
ScpB-F	AAGCGCTCCTGGCATTGTTTTTGTCCAG	Partial *scpB* gene
ScpB-R	TGAAGCTGCTGCATGATCTGCACGCTGA	Partial *scpB* gene
Sqr-P-F	GGCAGGCATAACGGACTGAAACCTTA	*sqr* promoter
Sqr-P-R	GGGTTGGACGGAACGAACTGGAAATA	*sqr* promoter
ScpB-P-F	GTATCTCCATGAGCTTCTGCCGCTT	*scpB* promoter
ScpB-P-R	AAACAATGCCAGGAGCGCTTCACGCAT	*scpB* promoter
Tth-P-R	GGCCAGCAATAATGCCGATA	*tth* promoter
Tth-P-F	GGGATGCAGCCATAAATATA	*tth* promoter
Cyc 2-P-R	TTCCACCAACTGCTGCTAAT	*cyc 2* promoter
Cyc 2-P-F	AAATAGAACGTGTGGGTGCT	*cyc 2* promoter
341 F	CCTACGGGAGGCAGCAG	16S rRNA gene
518 R	ATTACCGCGGCTGCTGG	16S rRNA gene

4 μL of Prime Script buffer, 0.5 μL of RNase inhibitor, 0.5 μL of Prime Script TRase, in a total volume of 20 μL. The PCR reaction for *scpB* gene was performed in a reaction mixture containing 1 μL of 10 μM forward (ScpB-F) and reverse (ScpB-R) primers, 5 μL of cDNA aliquot, 2 μL of 10 mM dNTPs mixture, 5 μL PCR buffer and TaKaRa Ex taq (Takara-Bio). Amplifications were carried out in a TaKaRa PCR Thermal Cycler (TaKaRa-Bio) under the following conditions: denaturation at 94℃ for 1 min followed by 30 cycles at 94℃ for 30 sec, annealing at 55℃ for 30 sec, extension at 72℃ for 1 min and a final polymerization step at 72℃ for 7 min. Samples (15 μL) of each PCR reaction were analyzed by 3 % agarose gel electrophoresis. Before RT-PCR, all samples were proven to be free of contaminating DNA by performing control PCRs without a preceding RT step. Buffers, cycling conditions, and the amount of template were the same as for RT-PCRs.

Cloning of *scpB* gene in an expression vector

The *scpB* gene was amplified from *A. ferrooxidans* genome DNA using an Amplitaq Gold PCR Master Mix (Applied Biosystems, Tokyo, Japan) and ScpB primers containing a NheI site in the forward primer (ScpB-F-Nhe) and a HindIII site in the reverse primer (ScpB-R-Hind) (Table 1). The amplified product was purified using a GFX ™ PCR DNA and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, UK) and cloned in pETcoco-2 (Novagen, Madison, WI, USA) to yield a construct (pETcoco-scpB) in which the entire coding region of the *scpB* gene is fused to a 6 ×His tag at the N-terminal. The insertion of the gene in the expression vector was verified by PCR using specific primers and flanking primers (T7 promoter and terminator). The positive clones were sequenced using the flanking primers.

Production and purification of His-tagged recombinant ScpB protein

The pETcoco-scpB was transformed into *E. coli* BL 21 (DE 3) expression strain. A positive clone was incubated overnight in 10 mL of LB medium with 50 μg mL^{-1} of ampicillin at 37℃. The culture was then transferred to 1 liter of LB medium with the same antibiotic concentration and 0.1% of arabinose. When the culture reached an optical density of 0.6 at 600 nm, the synthesis of His-tagged ScpB was induced by adding 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to the culture. Cells were further incubated at 22℃ for 12 h and harvested by centrifugation (15,000× *g*, 10 min). The His-tagged ScpB was purified using a Ni-charged Chelating Sepharose Fast Flow resin (Amersham Biosciences, Buckinghamshire, UK) according to the manufacturer's recommendations. After washing the resin with phosphate/NaCl (PN) buffer (20 mM phosphate, 0.5 M NaCl, pH 7.4) containing 100 mM imidazole, the bound protein (His-tagged ScpB) was eluted with PN buffer containing 400 mM imidazole. The His-tagged ScpB protein was loaded on gel filtration column (G 3000 SW, Tosoh, Tokyo, Japan) with eluting buffer containing 10 mM Tris-HCl (pH 7.0), 80 mM KCl, 0.2 mM EDTA, 0.2 mM dithiothreitol, and 10% glycerol. Fractions containing the His-tagged ScpB were kept at −70°C until subsequent analyses. The concentration of the purified His-tagged ScpB was determined by the Bradford method and the purity was verified by SDS-PAGE analysis as described previously[17].

Electrophoretic mobility shift assay

For the DNA-protein interaction analysis, the promoter regions were amplified using specific sets of primers as shown in Table 1. Primer pairs, Sqr-P-F and Sqr-P-R, ScpB-P-F and ScpB-P-R, Tth-P-F and Tth-P-R, Cyc 2-P-F and Cyc 2-P-R, were used to amplify the putative promoter regions of the *sqr*, *scpB*, *tth*, and *cyc 2* genes, respectively. Primers, 341 F and 518 R, were used to amplify a partial 16 S rRNA gene of *A. ferrooxidans*. This partial 16 S rDNA was used for a comparative assay to determine the specificity of the His-tagged ScpB protein. These amplified fragments were purified with a GF ™ PCR DNA and Gel Band Purification Kit (GE Healthcare). The different concentrations of purified His-tagged ScpB in a buffer consisting of 10 mM Tris-Cl (pH 7.0), 80 mM KCl, 0.2 mM EDTA, 0.2 mM dithiothreitol, 10% glycerol, and 0.75 μM bovine serum albumin was mixed with 0.1 pmol of the DNA for 30 min on ice in a total volume 20 μL as described by Qin et al[10]. The DNA/His-tagged ScpB mixture was separated by polyacrylamide gel electrophoresis on a 7 % nondenaturating gel at 4 ℃ for 60 min at 100 V. The gel was stained with a SYBR Green I Nucleic Gel Stain (Cambrex Bio Science Rockland, Inc.USA). The DNA bands were visualized by a fluorescence image analyzer, FMBIO II (Hitachi Solutions, Ltd. Tokyo, Japan).

Database analysis

Nucleotide sequences were obtained through The Comprehensive Microbial Resource web site at http://cmr.jcvi.org/cgi-bin/CMR/GenomePage.cgi?org=gtf. The sequence data were used to search the DNA and

protein databases for homologues through the web site at the National Center for Biotechnology Information (NCBI ; http://www.ncbi.nlm.nih.gov/BLAST/).

Results and discussion

Analysis of open reading frame around sqr gene

We have already reported that expressions of *sqr* and *tth* genes were up-regulated in *A. ferrooxidans* grown on sulfur or tetrathionate and repressed in cells grown on ferrous iron[8,18]. The results suggested the presence of proteins which regulate the transcription of genes involved in the oxidation of RISCs. When regions surrounding *sqr* gene on locus AFE_1792 (NCBI numbering) in the genomic sequence of *A. ferrooxidans* ATCC 23270 were analyzed, a gene on locus AFE_1791 annotated to be a chromosome segregation and condensation protein B (ScpB) was found in the upstream region of *sqr* gene (Fig. 1A). The gene encoded a polypeptide of 177 amino acids, with a predicted molecular mass of 19,389 Da (Fig. 1B). Our BLAST analysis of the database at NCBI Web site revealed the presence of a helix-turn-helix (HTH) motif signature common to bacterial regulatory proteins in the N-terminal region of ScpB. The BLAST search also revealed that ScpB showed a relatively high percentage of identity (45%) to a putative transcriptional regulator (Ypuh-like) in *Pseudomonas stutzeri* and to chromosome segregation and condensation proteins (ScpBs) in

several prokaryotes, such as the putative ScpBs of *Acidithiobacillus ferrivorans* SS 3 (74%), *Acidithiobacillus thiooxidans* (68%), *Acidithiobacillus caldus* (49%), *Methylococcus capsulatus* (47%), *Cellvibrio japonicas* (41%), and *Legionella pneumophila* (39%). ScpB has been initially discovered in *Bacillus subtilis*[15]. Usually, *scpB* gene is located in an operon with *scpA* in prokaryotes. The *scpB* gene of *A. ferrooxidans* was also found in an operon with *scpA* gene (Fig. 1A). ScpA, as well as ScpB, has been proposed to interact with prokaryotic structural maintenance of chromosomes (SMC) protein[15]. A complex composed of SMC, ScpA, and ScpB is thought to be required for chromosome segregation and condensation in *B. subtilis*. Although the *B. subtilis* ScpB has a helix-turn-helix (HTH) motif in the N-terminal region, it did not bind to dsDNA or ssDNA regardless of the presence or absence of ATP[5]. Three distinct MSC complexes were found in bacteria[9]. The first complex, MukBEF, is found in enterobacteria and certain other γ-subdivision proteobacteria. The second complex, SMC-ScpAB, has been found in many other bacteria and archaebacteria. The third complex, MksBEF, has been recently identified and is found to be broadly present in diverse bacteria. Although a *smc* gene has been found in *A. ferrooxidans* genome[3], there is no available information on the interaction between the SMC and the ScpB in *A. ferrooxidans*. Our BLAST search analysis revealed that the *A. ferrooxidans* ScpB

(A)

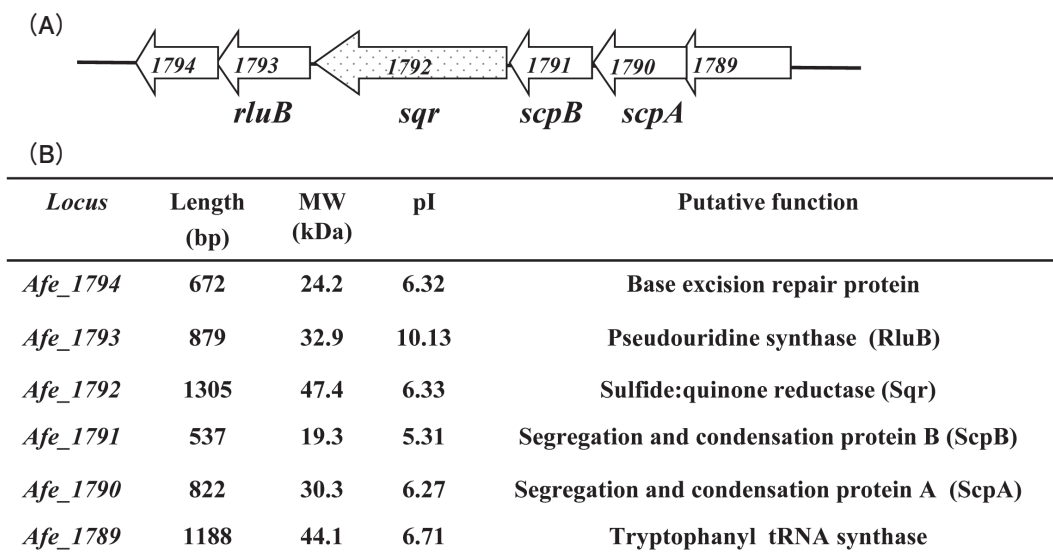| Locus	Length (bp)	MW (kDa)	pI	Putative function
Afe_1794	672	24.2	6.32	Base excision repair protein
Afe_1793	879	32.9	10.13	Pseudouridine synthase (RluB)
Afe_1792	1305	47.4	6.33	Sulfide:quinone reductase (Sqr)
Afe_1791	537	19.3	5.31	Segregation and condensation protein B (ScpB)
Afe_1790	822	30.3	6.27	Segregation and condensation protein A (ScpA)
Afe_1789	1188	44.1	6.71	Tryptophanyl tRNA synthase

Fig. 1 (A) Schematic map of the contig region containing the putative gene cluster context around the *sqr* gene of *A. ferrooxidans* 23270. (B) Putative functions of proteins encoded in genes surrounding *sgr* (loci were numbered according to NCBI numbering).

showed a low level of identity to the *B. subtilis* ScpB （30%）. The BLAST search analysis using the amino acid sequence of the *A. ferrooxidans* ScpA also revealed a low level of identity to the *B. subtilis* ScpA （28%）. The *A. ferrooxidans* ScpB is located in the upstream region of *sqr* gene, which is up-regulated in cells grown on RISCs, and has an HTH motif signature common to regulatory proteins. Although the *B. subtilis* ScpB is known to be involved in chromosome segregation and condensation, we think that the ScpB of *A. ferrooxidans* may act as a transcription factor rather than as a chromosome segregation and condensation protein.

Transcription of the *scpB* Gene in *A. ferrooxidans*

To study the expression pattern of the *scpB* gene at the transcriptional level, total RNA was isolated from *A. ferrooxidans* cells grown on ferrous iron, sulfur or tetrathionate at exponential growth phase. Partial *scpB* gene having the expected length was amplified using cDNA prepared from sulfur-or tetrathionate-grown cells, as well as PCR product with genomic DNA （Fig. 2B）, indicating that *scpB* gene was actually transcribed in *A. ferrooxidans* cells. The transcription of *scpB* gene
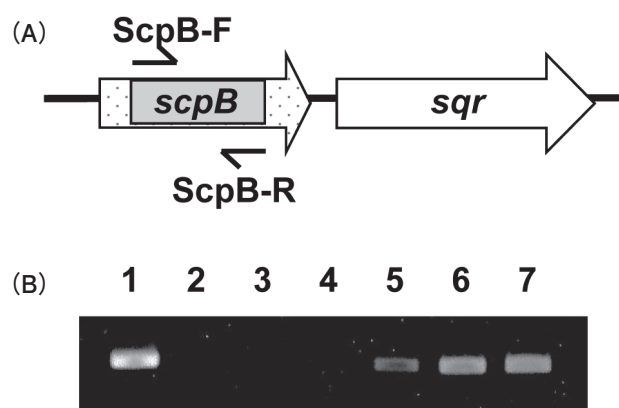
seemed to be slightly up-regulated in cells grown in sulfur and tetrathionate

ScpB binding to promoter regions of genes involved in iron or sulfur oxidation.

Although the *A. ferrooxidans* ScpB was annotated as a chromosome segregation and condensation protein, an HTH motif was found in the N-terminal region and the transcription of *scpB* gene was slightly up-regulated in cells grown in sulfur and tetrathionate. These results implied that the *A. ferrooxidans* ScpB plays a role in the transcriptional regulation and associates with promoter regions of genes involved in the iron or sulfur oxidation. EMSA assays with promoter regions and purified regulatory proteins are a well-recognized method to reveal the DNA-associating ability of regulatory proteins. The *scpB* gene from *A. ferrooxidans* ATCC 23270 was expressed in *E. coli* with the pETcoco-scpB. A protein with the expected molecular mass of His-tagged ScpB was synthesized in *E. coli* with the pETcoco-scpB （Fig. 3）. The protein was purified and used for EMSA assays. To evaluate functions of the recombinant ScpB protein, we initially carried out the EMSA analysis using the ScpB protein and the promoter region for *sqr* gene. As shown in Fig. 4, the ScpB was able to shift
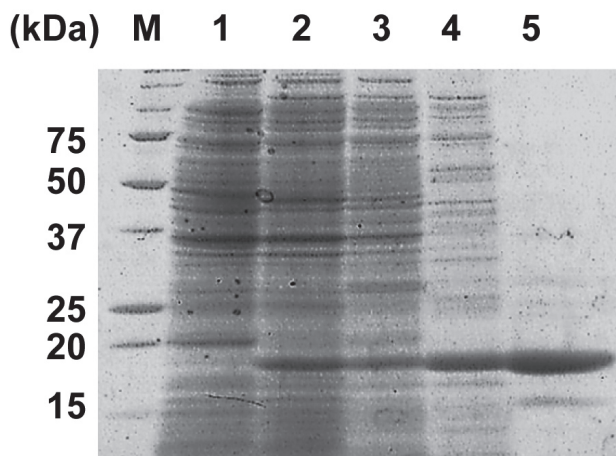

Fig. 2　RT-PCR analysis of the *scpB* gene in *A. ferrooxidans* cells grown in ferrous iron, sulfur, or tetrathionate.
（A）Schematic map of the genetic context of *scpB* and *sqr*. Arrows indicate locations of primers used for the RT-PCR analysis. （B）Agarose gel electrophoresis of RT-PCR products. The RT reaction was carried out on 1 μg of total RNA obtained from *A. ferrooxidant* ATCC 23270 cells grown in ferrous iron, sulfur, or tetrathion-ate. RT-PCR products amplified using cDNA from RNA prepared from cells grown in ferrous iron （lane 5）, sulfur （lane 6）, or tetrathionate （lane 7） were analyzed. PCR reactions with RNA prepared from cells grown in ferrous iron （lane 2）, sulfur （lane 3）, or tetrathionate （lane 4） were carried out in order to exclude amplifica-tion due to genomic DNA contamination. PCR products amplified using genomic DNA were also analyzed （lane 1）.

Fig. 3　SDS-PAGE analysis of the recombinant ScpB synthe-sized in *E. coli*.
Lane 1, cell-free extract from *E. coli* BL 21 （DE 3） with pETcoco; lane 2, cell-free extract from *E. coli* BL 21 （DE 3） with pETcoco-scpB; lane 3, supernatant obtained by centrifugation of cell-free extract from *E. coli* BL 21 （DE 3） with pETcoco-scpB at 100,000×g for 1 h; lane 4, sample obtained by affinity chromatog-raphy using a Ni-charged Chelating Sepharose Fast Flow resin, lane 5, ScpB obtained by gel filtration. Proteins were stained with Coomassie Blue. Molecular masses in kDa are indicated on the left. Lane M, marker proteins.
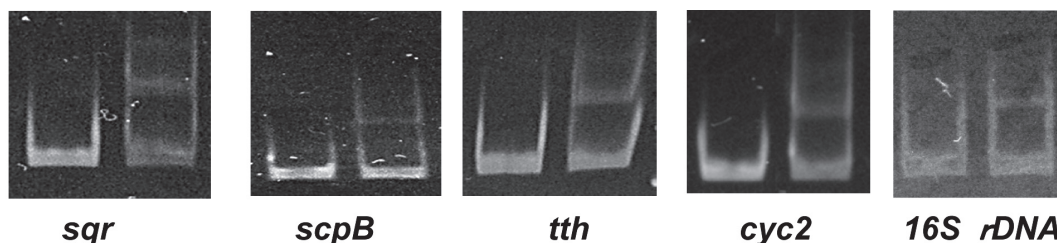
Fig. 4 Electrophoretic mobility shift assays using recombinant *A. ferrooxidans* ScpB protein and DNAs of promoter regions (*sqr*, *scpB*, *tth*, and *cyc 2* promoter regions) or a 16 S rDNA. DNAs were incubated with (right lane) and without (left lane) recombinant ScpB in each EMSA analysis.

the mobility of the promoter DNA of *sqr* gene (Fig. 4, lane *sqr*). When ScpB protein boiled at 100℃ was used in the assay under the same condition, a shifted DNA band was not detected (data not shown). Therefore, the recombinant ScpB protein was demonstrated to have a capacity for forming a protein-DNA complex. To examine whether the ScpB protein is able to associate to other promoter regions of genes involved in the iron or sulfur oxidation, the ScpB protein was incubated with DNA fragments of promoter regions for *tth or cyc 2*. The migrations of the promoter DNAs (*tth* and *cyc 2*) were retarded by incubation with the ScpB protein (Fig. 4 lanes *tth* and *cyc 2*). An association of the ScpB protein with its own promoter region was also examined, resulting in a shifted DNA band (Fig. 4 lane *scpB*). An incubation with 16 S rRNA gene also resulted in a shifted DNA band (Fig. 4 lane *16 S rDNA*).

Conclusion

In the present study we found that the transcription of *scpB* gene in *A. ferrooxidans* was slightly up-regulated in cells grown in RISCs, and the recombinant ScpB protein showed the capability of associating with promoter regions. One of the questions was whether the ScpB protein is involved in the regulation of the expression of *sqr* or *tth* gene. The results obtained in this study did not give us a clear answer because the recombinant ScpB protein nonspecifically bound to DNAs including promoter regions of genes thought to be involved in iron or sulfur oxidation and to the 16 S rRNA gene. Genome-wide annotation procedure has been recently applied to identify *A. ferrooxidans* transcriptional factors[6]. Although the ScpB has an HTH motif, it was not recognized as a transcriptional factor in the report. Therefore, at this point it is not possible to assign a definitive role to the ScpB protein in *A. ferrooxidans*. However, since the *scpB* gene of *A. ferrooxidans* is found in an operon with the *scpA*

gene like other prokaryotes, it strongly suggests that it functions as a chromosome segregation and condensation protein. Although the binding activity of ScpB protein from *B. subtilis* to dsDNA or ssDNA has not been reported[5], this is the first report on the ScpB protein having the capability of associating with DNA.

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好酸性鉄酸化細菌 *Acidithiobacillus ferrooxidans* の染色体凝集・分配タンパク質（ScpB）の性質

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　Acidithiobacillus ferrooxidans は，低品位の銅鉱石から銅を回収するバイオリーチングにおいて使用される微生物の一つである．この細菌は，エネルギー源として二価鉄イオンや還元型無機硫黄化合物（RISC）を使用する．鉄の酸化に関与する遺伝子の転写は，*A. ferrooxidans* が RISC で生育したときには抑制されるが，RISC の酸化に関与すると考えられている遺伝子の転写は活性化されることが知られている．硫黄やテトラチオン酸で生育したときにその発現が上方制御される硫化水素：キノン酸化還元酵素のすぐ上流に，ヘリックスターンヘリックスモティーフを持つ，ScpB と推定されるタンパク質をコードする遺伝子が存在していた．鉄，硫黄，テトラチオン酸生育細胞から調製した cDNA を用いた半定量的 PCR 分析の結果，硫黄やテトラチオン酸で生育した細胞内の *scpB* 遺伝子の転写は，鉄生育細胞と比較すると上方制御されていた．組換え ScpB タンパク質を用いたゲルシフトアッセイ法で，ScpB が転写制御因子として機能するかどうかを調べた．その結果，ScpB は DNA に結合したが，結合の特異性はなかった．ScpB が直接 DNA と相互作用する報告はこれまでになかった．