Genetic and Immunochemical Characterization of Thiocyanate-Degrading Bacteria in Lake Water

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Natural aquatic and soil samples were screened for the presence of thiocyanate-degrading bacteria. Using thiocyanate supplementation, we established an enrichment culture containing such bacteria from lake water. The dominant bacteria had the *scnC*-LS5 gene encoding thiocyanate hydrolase, which was closely related to the enzyme found previously in *Thiobacillus thioparus* THI115 isolated from activated sludge.

Carbonyl sulfide (COS) is a major sulfur compound in the troposphere contributing to sulfate aerosol, which accumulates in the stratosphere and influences the climate (3). About one-half of the COS originates from marine and soil environments (11). Biological processes have been considered to be responsible for the unique sulfur flow (14). However, little is known about the diversity and composition of the bacterial communities that are involved in the production and degradation of COS in the environment. It has been observed that production of COS in soil is stimulated by addition of thiocyanate (2, 14, 15). Katayama et al. (6, 7) isolated *Thiobacillus thioparus* THI112 and THI115 from activated sludge and showed that these bacteria are obligate chemolithotrophs that utilize thiocyanate as a sole energy source and produce ammonia and COS as the reaction products (8, 12). A unique enzyme responsible for degradation of thiocyanate was purified from this bacterium and designated thiocyanate hydrolase (7). The *scnA*, *scnB*, and *scnC* genes encoding the three subunits (α , β , and γ) of this enzyme have been cloned and sequenced. The deduced amino acid sequences exhibit significant homology to the sequences of nitrile hydratases from various bacteria (10). Furthermore, thiocyanate-degrading activity has been found in the facultative chemolithotroph *Paracoccus thiocyanatus* (9), in which a thiocyanate hydrolase-like enzyme has been detected (K. Hatayama and Y. Katayama, unpublished data), indicating that microbes carrying this enzyme may be distributed widely in water and soil environments.

In the present study, we detected thiocyanate-degrading and COS-producing bacteria in lake water by using an enrichment culture supplemented with thiocyanate. To quantify such bacteria in a natural aquatic environment, we used a fluorescent immunostaining technique with thiocyanate hydrolase-specific antibodies. Furthermore, *scnC*-specific primers were used to characterize the microbes harboring the related genes. To determine phylogenetic relationships among thiocyanate-degrading bacteria, we analyzed 16S ribosomal DNA (rDNA) frag-

ments by a PCR-denaturing gradient gel electrophoresis (DGGE) method.

Thiocyanate degradation and COS emission in environmental bacteria. Water and soil samples were collected from various locations in Japan (Table 1). The microorganisms in the samples were grown at 30°C in TC medium (7) containing 0.1 g of potassium thiocyanate per liter (TC1 medium). Consumption of thiocyanate in the medium was measured spectrophotometrically (7). To measure the amount of COS, a gas sample was collected from the headspace of a flask equipped with a rubber stopper and analyzed by gas chromatography (7). As shown in Table 1, degradation of thiocyanate and COS emission in the cultures were evident in almost all samples examined. These results indicate that thiocyanate-degrading microbes are distributed widely in various natural and man-made environments. Compared to soil and activated sludge samples, aquatic samples required a longer lag time before maximum degradation activity was observed.

To enrich thiocyanate-degrading bacteria, 100 ml of surface water from Lake Sagami was supplemented with 0.1 g of potassium thiocyanate per liter and incubated in a 500-ml flask at 30°C. When the thiocyanate was degraded, the culture was supplemented with additional potassium thiocyanate. The supplementation procedure was repeated three times. Then 10 ml of the resultant culture was inoculated into 90 ml of TC medium containing 0.5 g of potassium thiocyanate per liter (TC5 medium), and the procedure was repeated three times. In the resultant enrichment culture, designated culture LS5, the 0.45 mmol of thiocyanate initially present in the medium was degraded in 38 h (Fig. 1). The concentration of COS increased in the early phase of thiocyanate degradation but decreased later. As observed with *T. thioparus* THI115 (7), COS molecules exported by the bacterial cells were subsequently ingested by the same bacteria and utilized as an energy source.

The production of thiocyanate hydrolase was analyzed by Western blotting. As shown in Fig. 2, analysis of a crude extract of the microorganisms from culture LS5 grown in TC5 medium produced three bands that were indistinguishable from the α -, β -, and γ -subunit bands of the thiocyanate hydrolase of *T. thioparus* THI115. These protein bands were not obtained with a control sample prepared from culture LS5 grown in 0.01×

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TABLE 1. Thiocyanate degradation and COS emission in various environmental samples

Location (prefecture)	Date	Source	Thiocyanate degradation ^a	COS emission ^b
Lake Abashiri (Hokkaido)	August 1995	Lake water	—	—
Lake Kussharo (Hokkaido)	August 1995	Lake water	—	—
River Toyohira (Hokkaido)	August 1995	River water	+	+
Lake Onuma (Hokkaido)	August 1995	Swamp water	+	+
Tokyo University of Agriculture and Technology (Tokyo)	May 1995	Farm soil	+	+
Minamitama sewage works (Tokyo)	November 1994	Activated sludge ^c	+	+
Lake Sagami (Kanagawa)	January 1998	Lake water	+	+
Lake Suwa (Nagano)	September 1994	Lake water	+	+
Coast Kono (Fukui)	March 1994	Soil	+	+
Lake Mikata (Fukui) ^d	March 1994	Soil in shore	+	+
Lake Mikata (Fukui) ^d	December 1994	Lake water	+	+
Lake Hiruga (Fukui) ^d	December 1994	Lake water	+	+
Lake Kugushi (Fukui) ^d	December 1994	Lake water	+	+
Lake Suga (Fukui) ^d	December 1994	Lake water	±	+
Lake Suigetsu (Fukui) ^d	December 1994	Brackish water	±	+
Lake Yogo (Shiga)	March 1994	Lake water	+	+
Lake Yogo (Shiga)	March 1994	Soil in shore	+	+
Lake Biwa (Shiga) ^e	March 1994	Lake water	+	+
Lake Biwa (Shiga) ^e	March 1994	Soil in shore	+	+
Niihama (Ehime)	July 1994	Brackish water	+	+

^a +, complete degradation of thiocyanate in the medium; ±, incomplete degradation of thiocyanate in the medium; —, no degradation of thiocyanate in the medium.

^b +, significant emission of COS from the culture; —, no emission of COS from the culture.

^c Activated sludge was obtained from an aeration tank at facilities used to treat municipal wastewater.

^d Lake Mikata, Lake Hiruga, Lake Kugushi, Lake Suga, and Lake Suigetsu are connected and are collectively referred to as the Mikata-Goko lakes.

^e Samples were obtained from the north basin of Lake Biwa.

NBY medium (pH 7.0) containing 0.1 g of meat extract (Kyokuto, Tokyo, Japan) per liter, 0.05 g of NaCl per liter, 0.1 g of Bacto Peptone (Difco) per liter, and 0.05 g of Bacto Yeast Extract (Difco) per liter.

The size of the bacterial population producing thiocyanate hydrolase was estimated by fluorescent immunostaining (24) by using antisera raised against the α and γ subunits of the enzyme and fluorescein isothiocyanate-labeled secondary antibody. To stain the cytoplasmic enzyme, the cell wall was permeabilized by treatment for 30 to 60 min at 37°C with a lysozyme solution containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM MgCl₂, lysozyme (40 μ g/ml), DNase I (1 mg/ml), and gelatin (10 μ g/ml). The total cell number was

determined by 4',6'-diamidino-2-phenylindole (DAPI) staining. As shown in Table 2, 93% of the cells in culture LS5 were stained with the antibodies, while only 0.02% of the cells in the fresh surface water of Lake Sagami were stained. Moreover,

FIG. 1. Thiocyanate degradation (●) and COS emission (○) in enrichment culture LS5. The microorganisms in culture LS5 (10 ml) were inoculated into 90 ml of TC5 medium containing 5 mM potassium thiocyanate in a 500-ml flask. The ordinates indicate the residual amount of thiocyanate in the medium and the total amount of COS in the flask. The error bars indicate standard deviations based on duplicate experiments.

FIG. 2. Western blot analysis of the thiocyanate hydrolase. Crude extracts were prepared from culture LS5 grown in TC5 medium (LS5/TC5) or in 0.01× NBY medium (LS5/0.01× NBY). Thiocyanate hydrolase purified from *T. thioparus* THI115 was used as a standard.

TABLE 2. Occurrence of thiocyanate-degrading bacteria in lake water and an enrichment culture

Sample	Density of thiocyanate-degrading bacteria (cells ml ⁻¹)	Density of total bacteria (cells ml ⁻¹)	% of thiocyanate-degrading bacteria
Freshwater from Lake Sagami ^a	2.7×10^{2b}	1.3×10^{6c}	0.02
Enrichment culture LS5	8.4×10^7	9×10^7	93

^a The sample was collected on 14 January 1998.

^b The standard deviation was $\pm 1.0 \times 10^2$ cells ml⁻¹.

^c The standard deviation was $\pm 1.0 \times 10^6$ cells ml⁻¹.

the fluorescence intensities of the latter cells were quite low, indicating that enzyme synthesis may be inducible by addition of thiocyanate. Since the thiocyanate level in the lake surface water was below the detection limit (data not shown), enzyme synthesis may be induced to only a limited extent in the bacteria present in such an environment. There may be some localized habitat in the lake where certain populations of bacteria take up the free form of thiocyanate liberated from thioglucoside (23) or cyanide (22) and survive as a reservoir.

Characterization of *scnC*-related genes. For PCR analysis of the bacteria in culture LS5, we designed the following degenerate primers: 5'-GTNGCNMRNGCNTGGBTNGAYCC-3' and 5'-GGICKIWSIGGIADIACNADRTA-3' (where B is C, G, or T; D is A, G, or T; K is G or T; M is A or C; N is A, C, G, or T; R is A or G; S is C or G; W is A or T; and Y is C or T). This primer set could be used to amplify part of the *scnC* gene encoding Val⁶⁸ to Pro¹⁹⁴ of the γ subunit of thiocyanate

hydrolase, which was highly homologous to the corresponding parts of nitrile hydratases of various bacteria (10). Using this primer set, we amplified a DNA fragment of the same size (~380 bp) from the genomic DNA of the bacteria in culture LS5. Direct sequencing of the PCR product revealed a highly homologous gene, which we designated *scnC*-LS5. The nucleotide and amino acid sequences of *scnC*-LS5 were 85% (284 of 333 residues) and 96% (107 of 111 residues) identical to those of *scnC*, respectively. The amplified *scnC*-LS5 gene was cloned into the pCR2.1 vector (Invitrogen). We also observed non-specific PCR bands at ~350 and ~290 bp whose sequences were not related to the *scnC* gene.

For Southern blot analysis, genomic DNA digested with restriction enzymes were blotted onto Immobilon-Ny+ (Millipore) and incubated with the [α -³²P]dCTP-labeled *scnC*-LS5 probe. After hybridization, the filter was washed with 0.2× SSC-0.1% sodium dodecyl sulfate for 30 min at 42°C (1× SSC

FIG. 3. Genomic Southern blot analysis of *scnC* and *scnC*-related genes. Genomic DNA (1 μ g) extracted from *T. thioparus* THI115 or culture LS5 was digested with *EcoRI* or *HindIII* and separated on a 1.0% agarose gel. The positions of DNA size markers are indicated on the right.

FIG. 4. DGGE patterns of the PCR products amplified with 16S rDNA primers. The template DNA was extracted from enriched bacteria from culture LS5 grown in TC5 medium (LS5/TC5) or from the control (culture LS5 grown in 0.01× NBY medium [LS5/0.01× NBY]). The denaturant gradient was formed by assuming that 7 M urea and 40% formamide provided 100% denaturing conditions at 60°C. The patterns of DNA stained with ethidium bromide are shown on the right.

FIG. 5. Phylogenetic identities of the bacteria represented by the 16S rDNA bands obtained by DGGE (bands A, B, C, D, and E). The phylogenetic tree was constructed by using the CLUSTAL X search (20) and TreeView (<http://taxonomy.zoology.gla.ac.uk/rod/treeview.html>) programs. The 16S rDNA sequences of bacterial strains were obtained from GenBank; the accession numbers are indicated after the names of the organisms. The numbers at the nodes are bootstrap values (based on 1,000 trials). Scale bar = 0.1 change per nucleotide.

is 0.15 M NaCl plus 0.015 M sodium citrate). The hybridization signals were detected with BAS-1500 (Fujifilm, Tokyo, Japan). *Eco*RI and *Hind*III digests of the genomic DNA of *T. thioparus* THI115 contained 5.0- and 9.0-kb single bands, respectively, while *Eco*RI and *Hind*III digests of culture LS5 DNA contained 7.0- and 7.2-kb single bands, respectively (Fig. 3). The results indicated that *scnC* and *scnC-LS5* were single-copy genes and were distinct from each other.

Phylogenetic analysis of thiocyanate-degrading bacteria. Using the bacterial genomic DNA samples, we performed a touchdown PCR (4) to amplify 16S rDNA. The forward primer (5'-CGCCCGCCGCGCGCGGGCGGGGCGGGGGC ACGGGGGGCCTACGGGAGGCAGCAG-3') contained a part of the *Escherichia coli* 16S rDNA sequence (nucleotide positions 341 to 357) with a 5' extension of a 40-bp GC clamp that was necessary for subsequent DGGE (17). The reverse primer (5'-CCCCGTCAATTCCTTTGAGTTT-3') was complementary to nucleotide positions 907 to 928. DGGE analysis of the PCR products (16, 19) was performed with a DCode universal mutation detection system (Bio-Rad) according to

the protocols provided by the manufacturer. Samples were subjected to electrophoresis for 4 h at 200 V through a 6% polyacrylamide gel with a denaturant gradient. As shown in Fig. 4, the PCR products obtained from culture LS5 grown in TC5 medium and from culture LS5 grown in 0.01× NBY medium showed different migration patterns after DGGE. The DNA fragments were purified from the gel and subjected to direct sequencing. The 16S rDNA sequence of the major DNA band from culture LS5 grown in TC5 medium, band B, indicated that the dominant bacteria in culture LS5 grown in TC5 medium, which should have had the *scnC-LS5* gene, were closely related to a strain of *T. thioparus* (13) for which a 16S rDNA sequence (accession no. M79426) has been reported. Minor DNA bands A, C, and D were phylogenetically related to the 16S rDNA of *Cytophaga johnsonae*, *Aquaspirillum delicatum* (21), and *Rhodanobacter lindaniclasticus* (18), respectively (Fig. 5), although the participation of these bacteria in thiocyanate degradation has not been analyzed. The position of major band E from culture LS5 grown in 0.01× NBY medium was close to the position of 16S rDNA of *Burkholderia*

pyrocinia (1), and this band was not obtained with culture LS5 grown in TC5 medium. At least some of the accompanying heterotrophic population might have utilized excretion products of the autotrophs.

An enrichment procedure may enrich only some members of desired bacterial groups (5). Alternative enrichment procedures might more effectively amplify other thiocyanate-degrading bacteria that have not been found yet. Currently, we are collecting more *scnC*-related genes, including those derived from members of other genera, such as *P. thiocyanatus*. This approach should be helpful for designing versatile and specific degenerate primers for analysis of the diverse thiocyanate-degrading bacteria that may be distributed widely in natural and man-made environments.

Nucleotide sequence accession number. The nucleotide sequence of *scnC*-*LS5* has been deposited in the DDBJ database under accession number AB0074989.

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