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Isolation and identification of thiocyanate utilizing chemolithotrophs from gold mine soils

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Abstract

A mixed bacterial culture capable of growing in potassium-thiocyanate containing medium (200 mg KSCN) has been isolated from bacterial suspensions of soil samples collected near gold mines in Kumjung (Korea). The isolates were initially characterized by metabolic profile analysis and were identified as *Bacillus thermoglucosidasius*, *Bacillus cereus*, *Bacillus licheniformis*, *Bacillus mycoides*, *Brevibacterium epidermidis*, *Brevibacterium otitidis*, and *Corynebacterium nitrilophilus*. One of the seven isolates was initially characterized as *Brevibacterium epidermidis*, which is not known to degrade thiocyanate. However, using 16S rDNA sequencing, this strain was identified as a member of *Klebsiella*. The strain shows high similarity values (95.8 to 96.4%) with *Klebsiella* species, and the closest known relative was found to be *K. ornithinolytica* ATCC 31898. The result indicates that species of the genus *Klebsiella* were the closest phylogenetic relatives of the investigated strain. This is the first known report of a member of *Klebsiella* that is capable of utilizing thiocyanate as sole source of carbon and nitrogen.

Introduction

Thiocyanate (SCN-) is a simple inorganic compound which arises from a diverse range of natural and industrial sources (Sorokin et al. 2001). Thiocyanate is toxic to many higher organisms at relatively low concentrations (1–2 mM) because it has strong tendencies to bind to proteins and acts as a non-competitive inhibitor (Wood et al. 1998). The toxic effects of SCN^- include inhibition of halide transport to the thyroid gland, stomach, cornea and gills as well as the inhibition of a variety of enzymes. SCN^- induced central nervous system effects on humans include irritability, nervousness, hallucination, psychosis, mania, delirium and convulsions (Lewis 1992).

A number of chemolithotrophic and chemoheterotrophic bacteria can utilize thiocyanate as a source of energy and nutrients. These include *Arthrobacter*, *Escherichia*, *Methylobacterium*, and *Pseudomonas* species, which are mostly isolated from activated sludge processes (Boucabeille et al. 1994; Hung & Pavlostathis 1998, 1999; Wood et al. 1998; Kim & Katayama 2000). The aerobic biodegradation of thiocyanate proceeds as follows. First, thiocyanate is hydrolysed to cyanate (OCN^-) and sulfide, followed by hydrolysis of cyanate to ammonia and bicarbonate, and oxidation of sulfide to sulfate (Equation (1)) (Hung & Pavlostathis 1999).

$$SCN^- + 3H_2O + 2O_2 \rightarrow HCO_3^- + NH_4^+ + SO_4^{2-} + H^+. \quad (1)$$

In contrast to the biodegradation of thiocyanate by the activated sludge process, less is known about the biotransformation of thiocyanate performed by soil microorganisms. The soil ecosystem includes a variety of different microbial groups which are generally adapted to the inorganic and organic constituents of a given site. For example, it has been discovered that indigenous soil microorganisms found near gold mines

are capable of degrading a variety of refractory compounds including thiocyanate that has contaminated the soil (Grigor'eva et al. 1999). Prior to our study, minor attention has been paid to finding soil microorganisms capable of utilizing thiocyanate as sole carbon and nitrogen source. Therefore, the objectives of our research were to isolate and to identify soil microorganisms capable of utilizing thiocyanate as sole carbon and nitrogen source.

Materials and methods

Enrichment, treatability, and isolation of microorganisms

Luria-Bertani (LB) medium supplemented with 5 g of yeast extract and 150 mg of potassium thiocyanate (KSCN) l^{-1} was used as an enrichment medium. The following mineral salts (M9) medium was used to isolate and acclimate thiocyanate utilizing microbes: 0.2 g KSCN, 6 g Na_2HPO_4, 3 g KH_2PO_4, 1 g NaCl, 0.01 g $CaCl_2$, 0.5 g $MgSO_4$, 0.04 g $FeSO_4 \cdot 7H_2O$, 0.0015 g $MnSO_4 \cdot H_2O$ in 1 l distilled water. All reagents were of analytical grade and the pH of the media was adjusted to 7.0 with 3 N NaOH.

Twenty five soil samples were taken as described by Fulthorpe & Schofield (1999) at 5 to 20 cm deep from topsoil around gold mines in Kumjung (Korea). Five grams of each soil sample was suspended in 45 ml of distilled water. Ten ml of each soil suspension was well mixed, and then an aliquot (2 ml) of the mixed suspension was plated on the enrichment medium. After 7 days incubation, the colonies that formed were inoculated and incubated in the isolation medium. Turbid cultures of 25 ml were subcultured at 7 days intervals into fresh isolation media for acclimation. After five successive subculturings as outlined above, aliquots of the turbid culture were streaked onto isolation agar plates. Distinct microbial colonies were repeatedly streaked until pure colonies were obtained for identification.

All batch cultures were carried out in 2 l Erlenmeyer flasks, with working volumes of 250 ml, at pH 7 and 28 °C. Samples were taken periodically to analyze residual thiocyanate. All experiments were performed in triplicate. Concentration of thiocyanate was determined according to the procedure in Standard Methods (APHA 1998).

Characterizing the microorganisms by Biolog analysis

The isolates were initially characterized by their fermentation pattern using an automated identification system with microplates of 95 different carbon sources (BioLog microstation system, BIOLOG Inc.). After the completion of the Biolog array, the 16S rDNA sequence of one isolate, characterized as *Brevibacterium epidermidis*, was analyzed to further investigate the natural relationship of the microorganism because the strain has not been reported to utilize thiocyanate.

16S rDNA analysis of the selected strain

Genomic DNA of the microbe was extracted by phenol-chloroform solvents and ethanol precipitation (Sambrook & Russell 2001). The dried DNA was then dissolved in TE buffer (pH 7.5) and used as a DNA template. Two custom-made oligonucleotide primers, EBP5A (5′-CGTGCTTAACACATGCAAG; *B. epidermidis* positions 3 to 22) and EBP5B (5′-CTGATCTGCGATTACTAGC; *B. epidermidis* positions 1302 to 1274), were used to amplify the 16S rDNA of the selected isolate. Each PCR mixture of 50 μl contained 100 mM Tris-HCl (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$, 200 μM of each deoxynucleotide triphosphate (dNTP), 10 pmol of each primer, 5U of *Taq* DNA polymerase (Takara *Taq*, Takara), and 0.5 μg of the DNA extracted. PCR amplification was carried out in a thermal cycler (PTC-100, MJ research) with the following program: an initial denaturation at 94 °C for 4 min, followed by 35 cycles with each cycle consisting of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min; and a final extension at 72 °C for 7 min.

Purified PCR fragments from a commercial kit (QIAquick, Qiagen) were used as templates for the DNA sequencing with an automated sequencer (ABI PRISM 3100, Perkin-Elmer). The 16S rDNA sequencing was initially performed with a sense primer, EBP5A, and an anti-sense primer, EBP5B. An internal primer of EBP5C (5′-AATCGGAATTACTGGGC), based on the 16S rDNA sequencing result of the template, was additionally used in the sequencing of the internal region of the template to determine the full sequence of the 16S rDNA, which was compared to the reference sequences in the GenBank database. The Vector NTI Suite program (version 5.5.1, InforMax) was also used to calculate similarity (S_{ab}) values and estimate phylogenetic relationship.

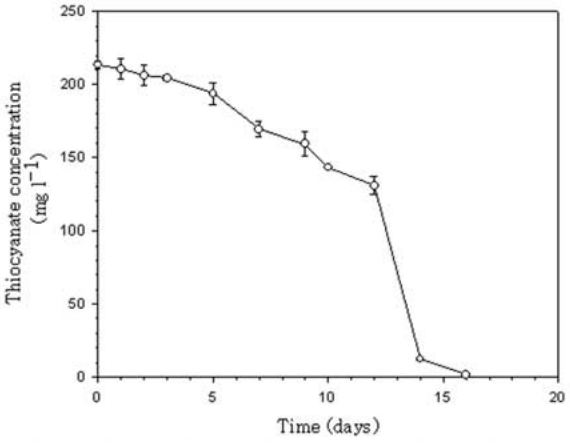

Figure 1. Degradation of thiocyanate in the medium after five successive subculturings. Turbid cultures of 25 ml were subcultured at 7 day intervals into fresh isolation media to ensure that only thiocyanate utilizing microbes were enriched. The experiments were done in triplicate and carried out in batch modes using 2 l of Erlenmeyer flasks with working volume of 250 ml at 28 °C.

Results and discussion

Figure 1 shows the mineralization of thiocyanate in the isolation medium. After 16 days of incubation, the thiocyanate had been totally degraded. This indicated that the mixed cultures isolated from the soil were capable of using thiocyanate as a growth nutrient because the substance represented the sole source of carbon and nitrogen in the medium. The concentration of thiocyanate gradually decreased at a rate of 9.9 mg SCN l^{-1} d^{-1} for the first 12 days of incubation, and then sharply decreased at a rate of 32.6 mg SCN l^{-1} d^{-1} after that. The initial low rate of thiocyanate degradation was probably due to lag phase of the microbial growth.

Seven different colonies were obtained from the isolation steps as described earlier. All the microorganisms were characterized as gram-positive bacteria and were preliminarily identified using a BioLog assay. They were *Bacillus thermoglucosidasius*, *Bacillus cereus*, *Bacillus licheniformis*, *Bacillus mycoides*, *Brevibacterium epidermidis*, *Brevibacterium otitidis*, and *Corynebacterium nitrilophilus*.

Almost the complete 16S rDNA sequence (ca. 1300 bp) was determined to establish the phylogenetic position of the selected strain, *B. epidermidis*. The phylogenetic tree by sequence searches of GenBank database shows the high similarity of the strain with members of *Klebsiella* species (Figure 2) and the sequence homology values with various *Klebsiella* species ranged 95.8 to 96.4%. The investigated strain showed the highest sequence homology, 96.4%, with the strain of *Klebsiella ornithinolytica* ATCC 31898 (accession number AF129441). The sequence of the investigated strain was separately compared to the sequence of *Brevibacterium epidermidis* NCDO 2286 (accession number X76565). There were 408 mismatches between the two strains, which was a similarity of 72.3%. Therefore, it could be concluded that species of the genus *Klebsiella* were the closest phylogenetic relatives of the selected strain.

Klebsiella spp. are chemoorganotrophs that can grow in a minimal medium with an ammonium or nitrate ion as the sole nitrogen source (Balows et al. 1992; Madigan et al. 1997). Because thiocyanate was the only source of carbon and nitrogen for microbial growth in the isolation medium, the isolate, believed to be a *Klebsiella* strain, is likely to be a chemolithotrophic microorganism which utilizes thiocyanate autotrophically.

It is interesting to note that the sequences of the sense and anti-sense primers (Rochelle 2001) (i.e., EBP5A and EBP5B, respectively) used in this study are very similar to the corresponding regions in *Brevibacterium epidermidis* and *Klebsiella ornithinolytica* (Figure 3). This may account for the high sequence similarity between the investigated strain and *K. ornithinolytica*. Complete identity of the internal primers (i.e., EBP5C) of the two strains provides another confirmation that the investigated strain, originally identified as *B. epidermidis*, is a member of the genus *Klebsiella* in the family Enterobacteriaceae.

Conclusions

Mixed cultures capable of utilizing thiocyanate as sole source of carbon and nitrogen were isolated from soil samples near gold mines. From these cultures, seven distinct colonies were isolated that were able to degrade thiocyanate in the isolation medium containing thiocyanate autotrophically. One of the seven isolates was initially characterized as *Brevibacterium epidermidis*, but this strain was identified as a member of *Klebsiella* by 16S rDNA sequencing. This is the first known report of a member of *Klebsiella* that is capable of utilizing thiocyanate as sole source of carbon and nitrogen, which indicates that some strains of *Klebsiella* sp. can utilize thiocyanate chemoautolithotrophically.

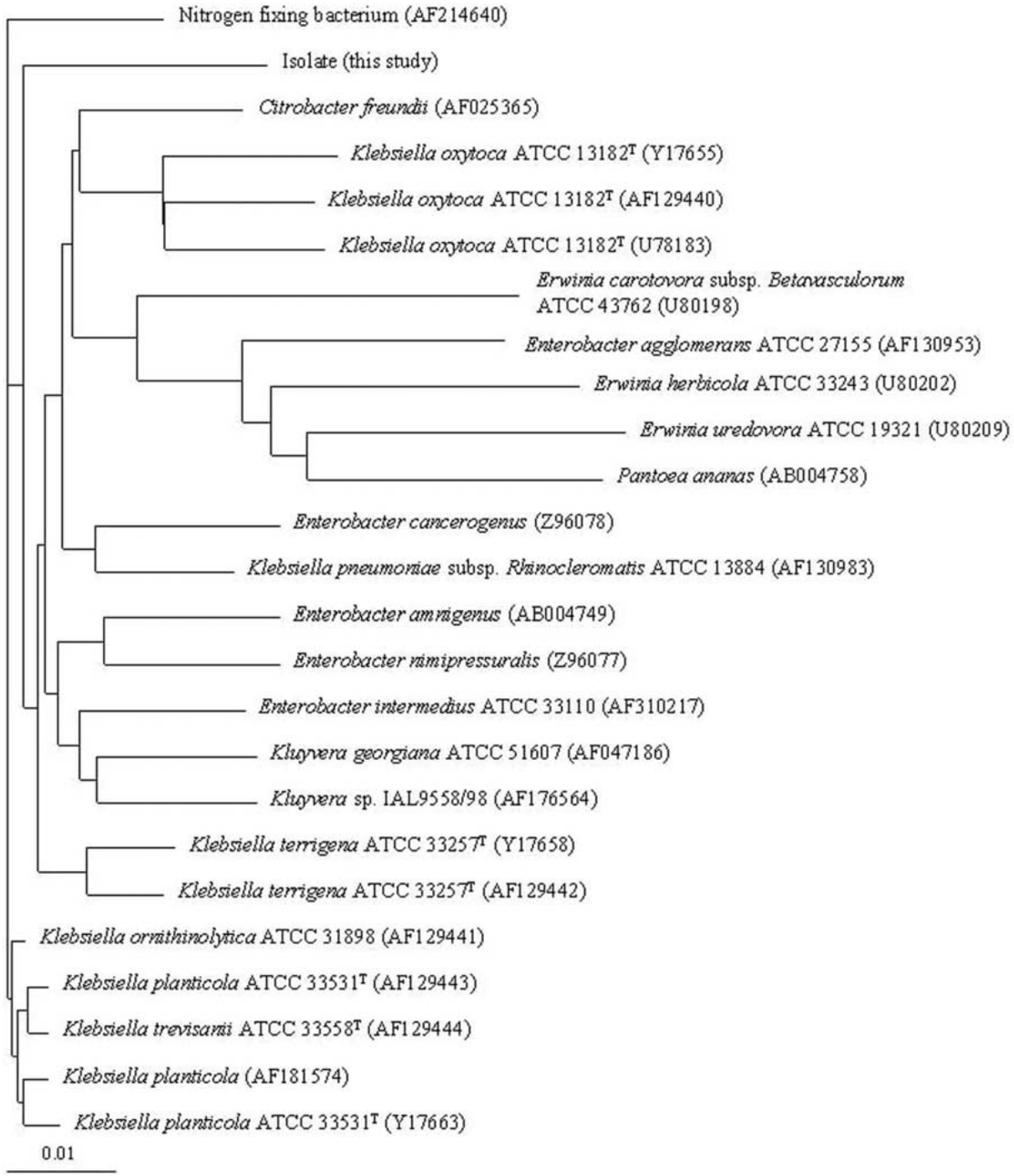

Figure 2. Phylogenetic tree showing the affiliation of 16S rDNA sequence of the selected isolate to the sequences of different *Klebsiella* species. Genomic DNA of the microbe was extracted by phenol-chloroform solvents and ethanol precipitation. Purified PCR fragments using a commercial kit were used as templates for the DNA sequencing with an automated sequencer. The Vector NTI Suite program (version 5.5.1, InforMax) was also used to calculate similarity values and to build phylogenetic relationship.

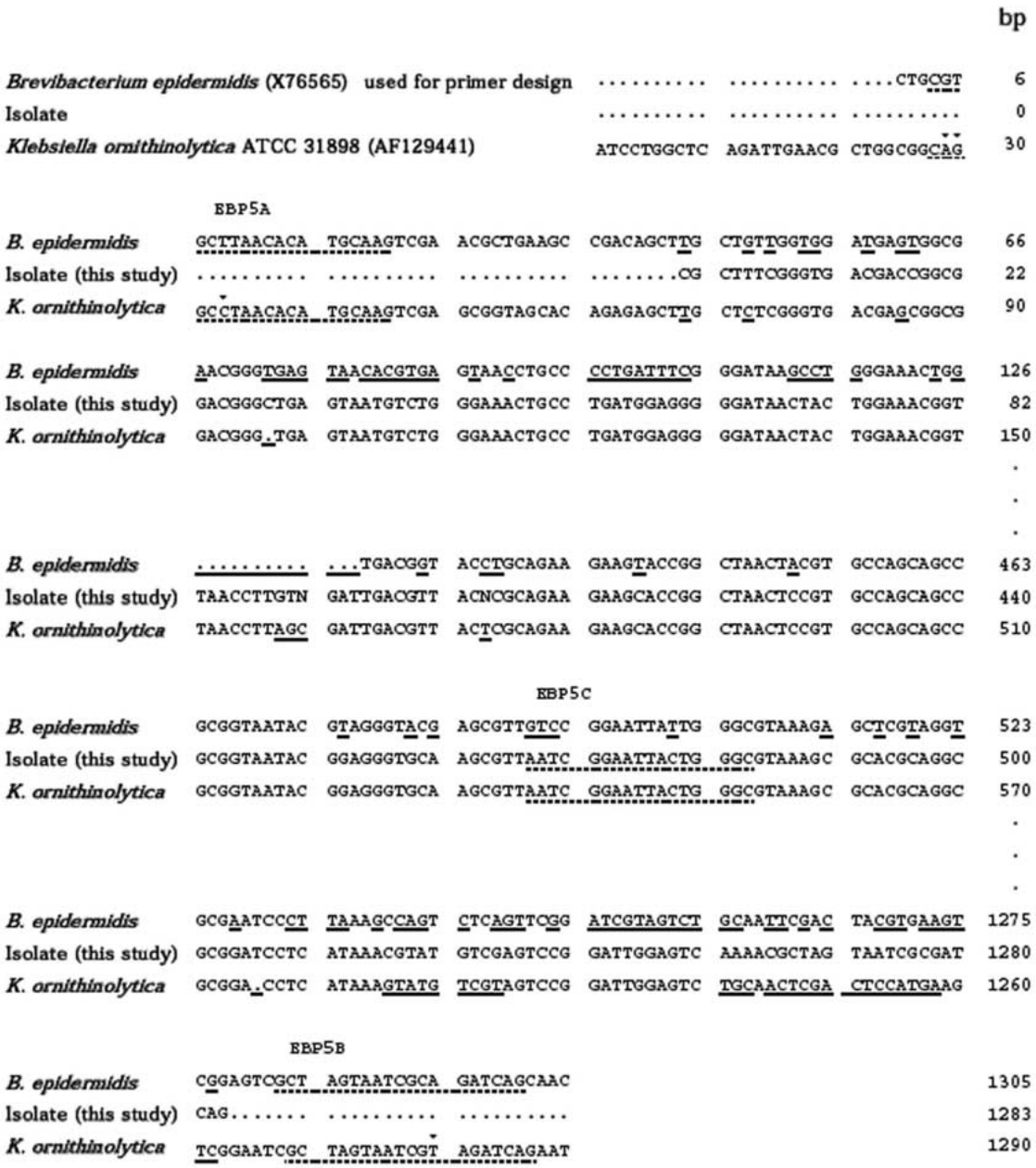

Figure 3. Comparison of homology in 16S rDNA sequences among *B. epidermidis* NCDO 2286, *K. ornithinolytica* ATCC 31898, and the investigated strain. The underlined letters indicate the nucleotide differences among strains. The 16S rDNA sequencing was initially performed with a sense primer and an anti-sense primer. An internal primer was designed based on the sequencing result of the template and was additionally used for sequencing an internal region of the template to determine full sequence of the 16S rDNA. The dotted underline letters indicate the target sequences of the primers used for sequencing. The differences in primer sequences of *B. epidermidis* NCDO 2286 and *K. ornithinolytica* ATCC 31898 are indicated by ▼.

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