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Isolation, Characterization, and Phylogenetic Position of a New Sulfur-Oxidizing Bacterium

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A sulfur-oxidizing bacterium was isolated from mine wastewater and characterized. The isolate was gram-negative, rod (0.2×1.2~1.5 μm), nonmotile, catalase positive, and oxidase positive. The optimal pH and temperature for growth were 7.0 and 30°C, respectively. The optimum thiosulfate concentration was 70 mM and the maximum growth rate was 0.081 hr^{-1}. The major ubiquinone contained in the isolate was Q-8. The cellular fatty acid composition was $C_{16:0}$, $C_{16:1}$, $C_{18:1}$, C_{17cyc}, and C_{19cyc} as nonpolar fatty acids, and 3-OH C10:0 and 3-OH $C_{12:0}$ as hydroxylated fatty acids. The isolate was a facultative chemolithoautotroph which can grow autotrophically on sodium thiosulfate and sodium sulfide and which can grow heterotrophically on yeast extract. It can also grow mixotrophically on sodium thiosulfate and yeast extract. Comparison of the 16S rRNA gene sequence of the isolate with that of *Thiobacillus* species and *Paracoccus thiocyanatus* revealed that it is closely related to *T. caldus* which belongs to the β-subclass of the class *Proteobacteria*. However, the isolate could not grow at extremely low pH (pH 1~3.5). On the basis of the phenotypic, chemotaxonomic and phylogenetic data, the isolate was tentatively named *Thiobacillus* sp. strain C.

Key words: Facultative chemolithoautotroph, sulfur-oxidizing bacterium, *Thiobacillus*

Environmental pollution has become a major source of concern for society. Some environmental problems are associated with the emission of notorious sulfur-containing compounds such as sulfur dioxide (SO_2) and hydrogen sulfide (H_2S). Sulfur dioxide is largely produced from the burning of sulfur-containing fossil fuels and is a major source of acid rain. Hydrogen sulfide is emitted from industrial wastes such as petrochemical plants, pulp plants, methanogenic waste treatment plants, and etc. Due to its toxicity, corrosiove guality, and putrid smell even in low concentration, the removal of hydrogen sulfide has been a major issue in the world. The biological oxidation of hydrogen sulfide may be an effective method to remove it (4, 14, 15, 20)

Thiobacillus species are known to be involved in the oxidation of hydrogen sulfide in wastewater treatment systems. Based on the Bergey's Manual, *Thiobacillus* species are gram-negative, rod, motile by polar flagella, and can oxidize reduced sulfur compounds (7). They are divided into three subclasses. The obligate chemolithoautotrophs obtain energy from the oxidation of reduced sulfur compounds and use carbon dioxide as a carbon source (e.g. *T. ferrooxidans, T. thiooxidans, T. neapolitanus, T. denitrificans*, etc). The facultative chemolitho-autotrophs can grow autotrophically on reduced sulfur compounds and carbon dioxide, but can grow heterotrophically on organic compounds (e.g. *T. intermedius, T. novellus, T. delicatus, T. versutus*, etc.). Chemolithoheterotrophs, on the other hand, cannot grow autotrophically as they cannot fix carbon dioxide, but they can use reduced sulfur compounds as energy sources (e.g. *Thiobacillus* Q). On the basis of ubiquinone and fatty acid composition, *Thiobacillus* can be subclassified into Group I-1 (*T. novellus, T. versutus*), Group I-2 (*T. acidophilus*), Group II (*T. intermedius, T. delicatus*), Group III-1 (*T. denitrificans, T. thioparus*), Group III-2 (*T. neapolitanus*), and Group III-3 (*T. ferrooxidans, T. thiooxidans*) (6). In recent years, several thermophilic bacteria such as *T. caldus, T. thermosulfatus*, and *T. hydrothermalis* were identified (1,

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2, 11). From the phylogenetic study of sulfur- and iron-oxidizing eubacteria, members of the genus *Thiobacillus* were assigned to three of the five subclasses of the class *Proteobacteria* (8, 12). However, updated taxonomic reclassification clarified the phylogenetic tree of the genus *Thiobacillus* and placed nearly all members in the beta subclass of *Proteobacteria* (5, 11, 19).

In this paper, the isolation and the characterization of a new strain of autotrophic sulfur-oxidizing bacterium from a mine wastewater are described.

Materials and Methods

Isolation and growth conditions

From 60 samples collected from soils, mine wastewaters, industrial wastewaters, and sea waters, several sulfur-oxidizing bacteria were isolated. The enrichment culture medium was composed of basal salts (g/l) K_2HPO_4 (6.0), KH_2PO_4 (2.0), NH_4Cl (0.5), $MgSO_4 \cdot 7H_2O$ (0.8), Na_2EDTA (0.5) and $ZnSO_4 \cdot 7H_2O$ (0.22), and the following trace elements (mg/l) $CaCl_2 \cdot 2H_2O$ (50), $MnSO_4 \cdot 5H_2O$ (10), $FeSO_4 \cdot 7H_2O$ (50), $(NH4)_6\ Mo_7O_{24} \cdot 4H_2O$ (10), $CuSO_4 \cdot 5H_2O$ (10) and $CoCl_2 \cdot 6H_2O$ (10). Furthermore, the medium contained 30 mM sodium thiosulfate as an energy source. The pH of the medium was adjusted to 7.0. To avoid precipitation during autoclaving, $MgSO_4 \cdot 7H_2O$, $Na_2S_2O_3$, and basal salts were sterilized separately. Cells were grown in a 300 ml flask and agitated at 120 rpm at 30°C for 7 days. A 1 ml aliquot of the turbid suspension was then transferred to a fresh medium and incubated as before. After serial transfers, 0.1 ml of the suspension were spread onto solid thiosulfate (30 mM)-basal agar plates and the plates were incubated at 30°C for 7 days. From the plates, several fast-growing colonies were isolated and characterized.

Physiological and biochemical properties

Optimum growth conditions such as temperature, thiosulfate concentration, and pH were tested. The effects of nitrogen sources and growth stimulators were also determined. To determine the growth rates, cells were grown in 15 l fermentor (New Brunswick Scientific, Bioflo 3000), and the optical density at 600 nm was measured.

The isolates were tested for their ability to utilize the following sulfur-containing substrates: $Na_2S_2O_3$, $Na_2S_2O_4$, Na_2SO_4, $PbSO_4$, $ZnSO_4$, $FeSO_4$, Na_2S, CuS, FeS, KSCN, $K_2Al_2(SO_4)_4 \cdot 24H_2O$ (each 0.5%), and elemental sulfur (0.05%). Heterotrophic growth was also tested in basal salts supplemented with the following sunstances: 0.05% complex organic substrates (yeast extract, peptone, nutrient broth); 0.2% sugars (glucose, galactose, lactose, xylulose, arabinose, fructose, ribose, mannose, cellobiose); 0.2% aliphatic alcohols (methanol, ethanol, propanol); 0.2% organic acids (oxalic acid, acetic acid, succinic acid, citric acid).

Chemotaxonomy

Ubiquinone system was determined by thin layer chromatography as described by Katayama-Fujimura *et al.* using the reverse phase silica gel plate (HPTLC, RP-18, 10X10 cm, Merck) (6). A mixture of acetone and water (80:20) was used as a developing solution.

Fatty acid composition was determined by the method of Katayama-Fujimura *et al.* using a gas chromatograph (Shimadzu GC-14A) equipped with a coated fused silica capillary column (DURA bond-1, 0.25 mm × 30 m) (6). Temperatures for column, injector, and detector were 180, 250, and 250°C, respectively. Elongation coefficient A16 was calculated by the ratio between concentration of cis-vaccenic and palmitoleic acids.

16S rRNA genes sequencing and phylogenetic analysis

Cells in the late-exponential phase of growth were harvested, washed with sterile 1% (w/v) saline, and resuspended in 100 µl sterile distilled water. Crude lysates were prepared by protease digestion, heat treatment, and centrifugation (3). 16S rDNA fragments that corresponded to position 8-1510 of the *Escherichia coli* numbering system were amplified by polymerase chain reaction (PCR) from the crude extract (3) and sequenced following the method of Shin *et al.* (10). The 16S rDNA sequence of the isolate was aligned with the representative sequences of *Thiobacillus* species and *Paracoccus thiocyanatus* using CLUSTAL W software (13). Alignment gaps and undetermined or ambiguous base positions were not taken into consideration for the calculations. The bootstrap option in this program package was used for statistical analysis of branching patterns on the phylogenetic tree with 1,000 bootstrapped trials (13). The 16S rDNA sequence has been deposited in the GenBank database under accession number AF 023264. The accession numbers for the other nucleotide sequences used to construct phylogenetic trees are as follows: *T. acidophilus*, D86511; *T. baregensis*, Y09280; *T. caldus*, Z29975; *T. ferrooxidans*, Y11595; *T. hydrothermalis*, M90662; *T. novellus*, D32247; *T. thermosulfatus*, U27839; *T. thiooxidans*, Y11596; *T. thioparus*, M79426; and

Table 1. Comparision of morphological and biochemical characteristics of the isolate with *Thiobacillus* type strains

Characteristics	*Thiobacillus* sp. *strains C*	*T. caldus*	*T. thiooxidans*	*T. ferrooxidans*	*T. delicatus*
Cell morphology	rod	short Rod	short rod	rod	rod
Cell size	0.2×1.2~1.5 μm	1.2~-0.7×1.8~0.8 μm	0.6×1.0~2.0 μm	0.5×1.0 μm	0.4~0.6×0.7~1.6 μm
Colony color	white	transparent	transparent or whitish yellow	white with sulfur	whitish yellow
Growth on thioulfate agar plate	positive	positive	positive	positive	positive
Motility	negative	positive	positive	positive	negative
Gram reaction	negative	negative	negative	negative	negative
Denitrification					
NO_2	positive	nd[a]	negative	negative	positive
N_2	positive	nd	negative	negative	negative
Nitrogen source with Urea	positive	nd	nd	nd	nd
Ammonium chloride	positive	positive	nd	positive	positive
Potasium nitrate	positive	positive	nd	positive	positive
Temperature for optimum growth	37°C	45°C	28~30°C	30~35°C	30~35°C
growth range	25~42°C	32~52°C	10~37°C	10~37°C	15~42°C
pH for optimum growth	7.0	2.0~2.5	2.0~3.0	2.5	5.0
growth range	4.5~8.5	1.0~3.5	0.5~5.5	1.3~4.5	5.0~7.0
Ubiquinone system	Q-8	Q-8	Q-8	Q-8	Q-10
Major fatty acids[b,c] Non-hydroxylated fatty acid	$C_{16:0}$, $C_{16:1}$ +17cyc, $C_{18:1+19cyc}$	nd	$C_{16:0}$, $C_{16:1}$ +17cyc, $C_{18:1+19cyc}$	$C_{16:0}$, $C_{16:1}$ +17cyc, $C_{18:1+19cyc}$	$C_{16:1}$, $C_{16:1}$ +17cyc, $C_{18:1+19cyc}$
Hydroxylated fatty acid	3-OH $C_{10:0}$ 3-OH $Cl_{2:0}$	nd	3-OH $C_{14:00}$	3-OH $C_{14:00}$	3-OH $C_{10:0}$ 3-OH $C_{12:0}$

[a] nd, not determined
[b] $C_{16:1+17cyc}$ (hexadecenoic acid plus cyclopropane of C_{17}), $C_{16:0}$ (hexadecanoic acid),
$C_{18:1+19cyc}$ (octadenoicacid plus cyclopropane of C_{19})
[c] 3-OH $C_{10:0}$ (3-hydroxydodecanoic acid)

Paracoccus thiocyanatus, D32242.

Results

Morphology

The isolated sulfur-oxidizing bacterium was gram-negative, nonmotile, and rod-shape with dimensions of 0.2×1.2~1.5 μm. Colonies were whitish and small (1~2 mm in diameter) (Table 1).

Biochemical and cultural characteristics

Catalase and oxidase were present in the isolate. Nitrate was reduced to nitrite, and nitrite was also reduced to N_2 (Table 1). The ranges of pH and temperature at which growth was observed were 4.5~8.5 and 25~42°C, respectively. The optimum pH and temperature for growth were 7.0 and 37°C, respectively (Fig. 1 and Fig. 2). The isolate could grow on thiosulfate in the concentration range of 30~210 mM, and the maximum growth rate occurred at 70 mM (Fig. 3). The generation time was found to be 8.5 hr and drying cell yield was 0.11 g cell/g thiosulfate.

Substrate utilization

The isolate was able to grow only on $Na_2S_2O_3$ or Na_2S among the sulfur-containing substrates tested. It was also found that heterotrophic growth occurs

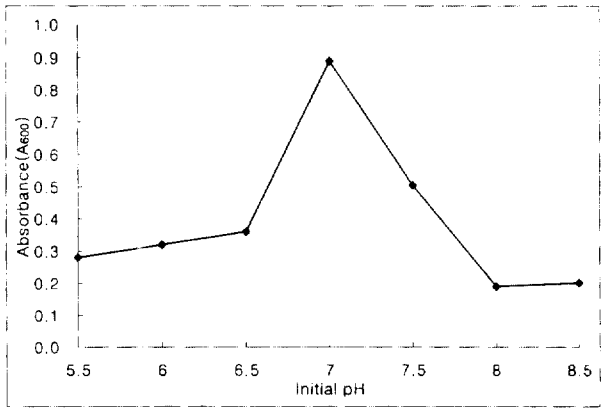

Fig. 1. Effect of initial pH on the growth of *Thiobacillus* sp. strain C.

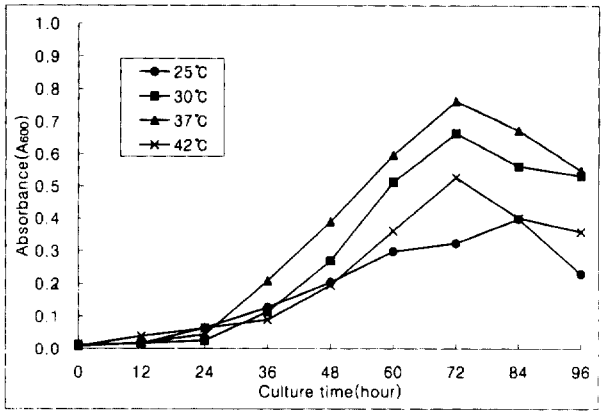

Fig. 2. Effect of temperature on the growth of *Thiobacillus* sp. strain C.

only with 0.05% yeast extract (Table 2). During mixotrophic growth with thiosulfate and yeast extract, however, growth was stimulated and the growth rate was about two times higher than that of thiosulfate-grown cultures.

Chemotaxonomic markers

The isolate contained ubiquinone Q-8 as a major component. The cellular fatty acid composition was $C_{16:0}$, $C_{16:1}$, $C_{18:1}$, C_{17cyc}, and C_{19cyc} as nonhydroxylated fatty acids, and 3-OH $C_{10:0}$, and 3-OH $C_{12:0}$ as β-hydroxy acids.

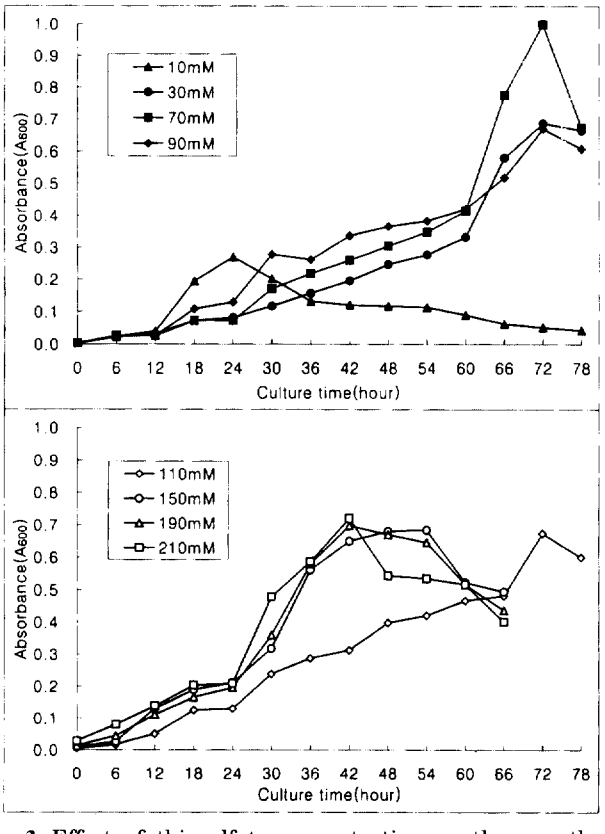

Fig. 3. Effect of thiosulfate concentration on the growth of *Thiobacillus* sp. strain C.

Table 2. Test of various substrates as a source of carbon and/or energy

Substrates	Growth	Substrates	Growth
$Na_2S_2O_3$	+[a]	Yeast extract	+
$Na_2S_2O_4$	−[b]	Peptone	−
Na_2S	+	Oxalic acid	−
Na_2SO_4	−	Citric acid	−
CuS	−	Acetic acid	−
FeS	−	Succinic acid	−
$FeSO_4$	−	Glucose	−
$PbSO_4$	−	Fructose	−
Element Sulfur	−	Lactose	−
$K_2Al_2(SO)_4 \cdot 4H_2O$	−	Arabinose	−
KSCN	−	Galactose	−
$K_3Fe(CN)_6$	−	Ribose	−
Methanol	−	Mannose	−
Ethanol	−	Methylamine	−
Propanol	−	Xylose	−
Phenol	−	Cellobiose	−
Glycerol	−	Nutrient	−

[a]+ positive ultilization, [b]- negative utilization

Phylogenetic analysis

TTGAACGCTGGCGGCATGCCTAACACATGCAAGTCGAACGGCAGCAGGT
CCTTCGGGATGCTGGCGAGTGGCGGACGGGTGAGTAACGCGTAGGAATC
TGTCCTCGAGTGGGGGATAACCCAGGGAAACTTGGGCTAATACCGCATA
CGCCCTGAGGGGGAAAGCGGGGGATCTTCGGACCTCGCGCTGGAGGAGG
AGCCTGCGTCCGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGA
CGATCGGTAGCTGGTCTGAGAGGACGATCAGCCACACTGGGACTGAGAC
ACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTTCGCAATGG
GGGCAACCCTGACGAAGCAATGCCGCGTGTGTGAAGAAGGCCTTCGGGT
TGTAAAGCACTTTCAGCGGGGACGAAAAGGTACGGGCGAACAGTCCGTG
CTGTTGACGTGAACCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAG
CCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAA
AGGGCGCGTAGGCGGTCATCTCAGTCTGCTGTGAAATCCCCGGGCTCAA
CCTGGGAATGGCAGTGGATACTGGATGGCTGGAGTCTGGGAGAGGGTCG
TGGAATTCCACGTGTAGCGGTGAAATGCGTAGATATGTGGAGGAACACC
AATGGCGAAGGCAGCGACCTGGCCCGAGACTGACGCTGAAGTGCGAAAG
CGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACG
ATGGATACTAGCGTGTTGGCAGTTTAACNNNNNNGTGCCGCACGTAACG
CATTAAGTATCCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAG
GAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAT
GCAACGCGCAGAACCTTACCTGGGCTTGACATGTGAGGAATCCTGCAGA
GATGTGGGAGTGCCTTCGGGGAGCCGCAACACAGGTGCTGCATGGCTGT
CGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAA
CCCTTGCCCTTAGTTGCCAGCAGTTCGGCTGGGCACTCTAAGGGGACTG
CCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCC
TTTATGTCCAGGGCTACACACGTGCTACAATGGCGCATACAGAGGGATG
CCAACTCGCGAGAGGGAGCCGACCCCAGAAAGTGCGCCGTAGTTCGGAT
TGCAGTCTGCAACTCGACTGCATGAAGTCGGAATCGCTAGTAATCGCGG
ATCAGCACGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCG
TCACACCATGGGAGTGGGCTGTACCAGAAGCCGGTAGCCTAACCGCAAG
GAGGGCGCCGACCACGGTATGGTTCATGACTGGGGTGAAGTCGTAACAA
GGTAGCCGTAGGGGAACCTGC

Fig. 4. The 16S rDNA nucleotide sequence of *Thiobacillus* sp. strain C.

Table 3. Matrix showing relationships between spesies of *Thiobacillus* and *Paracoccus thiocyanatus* based on the levels of similarity of 16S rDNA sequences[a]

Organism	Level(%) of similarity with									
	1	2	3	4	5	6	7	8	9	10
1. *Thiobacillus* sp. C										
2. *T. thiooxidans*	91.5									
3. *T. ferrooxidans*	90.8	97.9								
4. *T. baregensis*	84.3	82.1	82.6							
5. *T. acidophilus*	83.2	83.0	82.7	78.5						
6. *T. caldus*	91.7	95.2	95.4	81.6	82.8					
7. *T. novellus*	82.8	80.7	80.9	79.0	83.3	80.0				
8. *T. thermosulfatus*	83.3	82.3	82.5	81.7	80.1	82.6	80.1			
9. *Paracoccus thiocyanatus*	82.9	80.7	80.3	79.2	82.5	80.9	87.1	81.4		
10. *T. hydrothermalis*	84.4	85.2	85.5	86.4	82.7	84.6	81.6	83.9	80.4	
11. *T. thioparus*	85.6	84.2	84.0	84.0	80.9	83.8	79.8	87.8	81.7	84.4

[a] Sites with gaps and sites where nucleotides were not determined, were not included in the comparision.

The 16S rDNA fragments of the isolate were amplified by PCR and sequenced. The determined sequences consisted of 1491 residues (Fig. 4). The sequence was compared with a data set consisting of 10 reference sequences derived from the databases of GenBank. Comparison of the 16S rDNA sequences revealed that the isolated strain belongs to the beta subdivision of *Proteobacteria* and clusters together with members of the *Thiobacillus* group. This is in agreement with chemotaxonomic studies. Table 3 shows the level of overall percent similarity for each pair of sequences which could be aligned. On the basis of evolutionary distance values obtained, a neighbor-joining phylogenetic tree was constructed (Fig. 5). The tree demonstrates that the isolate falls into the *Thiobacillus* cluster (1000% support of bootstrapping) and is most closely related to *T. caldus*.

Discussion

An aerobic sulfur-oxidizing bacterium isolated from mine wastewater is gram negative, rod-shaped, and can grow autotrophically by utilizing limited reduced sulfur compounds, such as sodium thiosulfate and sodium sulfide as energy sources. Based on these characteristics, we concluded that the isolate belongs to the genus *Thiobacillus* among the colorless sulfur bacteria (7).

The principle quinone in the isolate is ubiquinone Q-8 which is a marker of obligately chemolithotrophic *Thiobacilli* (6). Moreover, the cellular fatty acid compositions are very similar to

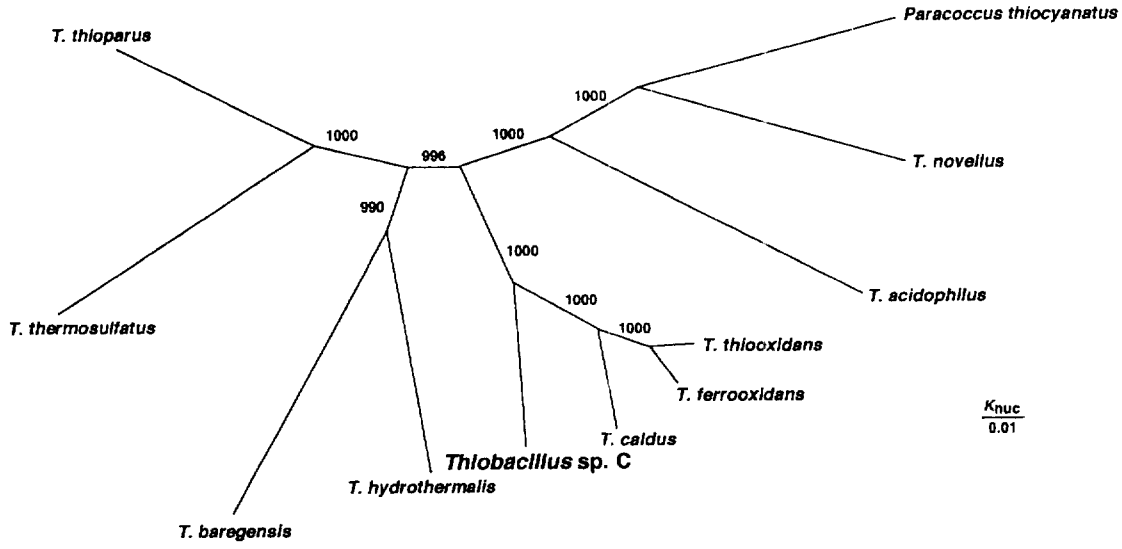

Fig. 5. Phylogenetic relationship of *Thiobacillus* sp. strain C to other selected proteobacteria based on 16S rRNA sequences. Numbers are percent probabilities obtained with 1,000 bootstrapped runs for individual nodes. *K*nuc, nucleotide substitution rate.

those of *T. ferrooxidans* and *T. thiooxidans* involved in Group-III-3 of the genus *Thiobacillus* (6).

T. caldus is gram negative, rod, and contains ubiquinone Q-8. However, unlike other acidophilic members of *Thiobacillus*, it has an optimal growth temperature of 45°C representing the first moderately thermophilic, acidophilic *Thiobacillus* species (2). Two thermophilic bacteria, *T. tepidarius* (17) and *T. aquaesulis* (18), are known as neutrophiles. Thus *T. caldus* is assigned to the Group-III-3 which includes acidophilic bacteria (2). Comparing the 16S rRNA nucleotide sequences of the isolate with those of other sulfur-oxidizing bacteria, it was noted that the isolate has 91.5% homology with *T. caldus*.

As shown in Table 1, most of the chemotaxonomic characteristics of the isolate are closely related to those of the strains of Group-III-3. However, there are several differences as follows. First, the strains of Group-III-3 are typical acidophiles which can grow under acidic conditions below pH 4, whereas the isolate can grow only in the neutral pH range 4.5 to 8.5. Second, unlike *T. ferrooxidans* or *T. thiooxidans*, the isolate cannot use elemental sulfur or metal sulfide (Table 2). However, it can grow heterotrophically only on yeast extract among the organic compounds tested. Of course, the isolate can grow without any additional growth factors, but the growth was stimulated by the addition of yeast extract. The stimulation of growth of chemolithobacteria by exogenous organic substance has been described by Rittenberg (9). We thus conclude that the isolate is a facultative chemolithoautotroph.

Although some of their physiological and cultural characteristics are different from each other, the phylogenetic tree shows that the isolate, *T. caldus*, *T. ferrooxidans*, and *T. thiooxidans* fall into a monophyletic cluster which may correspond to the thiobacillus group. Thus we conclude that the isolate is a novel facultative bacterium which is closely related to the obligate acidophilic sulfur-oxidizing bacteria. Further studies are required to identify its characteristics more accurately, and the isolate was tentatively named *Thiobacillus* sp. strain C.

Acknowledgments

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