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ORIGINAL PAPER

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Characterization of *Symbiobacterium toebii*, an obligate commensal thermophile isolated from compost

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Abstract A symbiotic thermophilic bacterium, strain SC-1, was isolated from hay compost (toebi) in Korea. The new isolate exhibited an obligate commensal interaction with a thermophilic *Geobacillus* strain and required crude extracts and/or culture supernatant from *Geobacillus* sp. SK-1 for axenic growth. The growth factors from *Geobacillus* sp. SK-1 were irreversibly inactivated by phenol or protease treatment, suggesting that they might be proteins. The cells of strain SC-1 were non-spore forming, nonmotile rods that were stained Gram-negatively. The isolate was a microaerophilic heterotroph. Growth was observed between 45° and 70°C (optimum: 60°C; 2.4-h doubling time) and pH 6.0 and 9.0 (optimum: pH 7.5). The G+C content of the genomic DNA was 65 mol%, and the major quinones were MK-6 and MK-7. A phylogenetic analysis of its 16S rDNA sequence indicated that strain SC-1 is closely related to *Symbiobacterium thermophilum* and so was named *Symbiobacterium toebii* on the basis of its physiological and molecular properties.

Key words *Symbiobacterium toebii* · Thermophile · Symbiotic bacterium · Compost · Commensalism · Microaerophilic

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Introduction

There are large numbers of unidentified microorganisms in natural environments that can be seen only under a microscope or detected only by molecular ecological methods. Although large numbers of new thermophilic bacteria have been isolated in recent years, only a small fraction of environmental bacterial communities can be cultivated by current techniques because of our inability to understand and reproduce the microenvironmental habitats found in nature (Lee et al. 1996b; Ward et al. 1998). It has been assumed that biological interactions (e.g., symbiotic relationships) are essential for the growth of some microorganisms. For example, the growth of the bacterial parasite *Bdellovibrio* requires a Gram-negative bacterial host (Ruby 1992). Syntrophic bacteria can grow only when methanogens remove hydrogen, the metabolic product of the syntrophic bacteria.

A new thermophilic bacterium, strain SC-1, detected from compost during the screening of thermostable tyrosine phenol-lyase-producing bacteria, exhibited an obligate commensal interaction with a thermophilic *Geobacillus* strain (Lee et al. 1996a, 1997). The growth of strain SC-1 has never been observed in enrichment cultures in the absence of *Geobacillus* sp. strain SK-1. Strain SC-1 has been isolated using a medium containing crude extracts and the culture supernatant of strain SK-1 (Rhee et al. 2000). This type of bacterial interaction has also been reported previously (Suzuki et al. 1988). Recently, with the use of a dialyzing culture vessel, the independent growth of *Symbiobacterium thermophilum* has been confirmed, and *S. thermophilum* has been validly described for the first time (Ohno et al. 1999, 2000). However, more research on the evolutionary phylogeny of these microorganisms is required to gain understanding of their microbial interactions in the ecosystem.

The aim of this work was to characterize the isolate SC-1 from compost in Korea, which exhibits a symbiotic interaction with *Geobacillus* sp. strain SK-1. Accordingly, in this paper the morphological and physiological characteristics of the isolate SC-1 are described along with its phylogenetic position on the basis of 16S rDNA sequences.

Materials and methods

Strains and culture media

A symbiotic thermophilic bacterium, strain SC-1, was isolated from hay compost (toebi) in Korea. The enrichment culture conditions and isolation of strain SC-1 (= KCTC 0307BP; KCTC, Korean Collection for Type Cultures) have been previously described by Rhee et al. (2000). The enrichment cultures were initiated by inoculating a basal medium (BM) with 1% (w/v) hay compost. The BM contained 5 g polypeptone, 1 g yeast extract, 6 g K_2HPO_4, 2 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, and 0.5 g of L-tyrosine per liter of deionized water. After enrichment, two kinds of cells, designated strains SK-1 (= KCTC 0306BP) and SC-1 (= KCTC 0307BP), were always observed microscopically.

To cultivate strain SC-1, a modified BM (MBM) containing cell extracts and culture supernatant from the *Geobacillus* strain SK-1 was used. *Geobacillus* strain SK-1 was cultivated in BM at 60°C for 48 h. The culture supernatant of strain SK-1 was filter-sterilized with a sterilizing filter (0.2 µm pore size; Sartorius, Goettingen, Germany) and added to a 1 : 1 ratio by volume with BM. For the cell extract preparation, a culture broth of strain SK-1 was centrifuged at 5,000 *g* for 15 min, and then the cell pellets were washed with a 20 mM phosphate buffer (pH 7.6) and broken with an ultrasonicator (Branson Ultrasonics, Danbury, CT, USA). The crude extract was centrifuged at 10,000 *g* for 30 min, and the supernatant was used as the cell extract. The protein in the cell extract was determined by the Bradford method (Bradford 1976).

The cultures were incubated under a microaerobic or anoxic atmosphere because cell growth was inhibited under aerobic conditions. For cultivation under microaerobic conditions ($CO_2/N_2/O_2$, 10 : 85 : 5), the *Campylobacter* microaerophilic system (Difco, Detroit, MI, USA), and palladium catalysts (Difco) were used at 60°C in an anaerobic jar (Difco). For cultivation under anoxic conditions (CO_2/N_2, 10 : 90), MBM containing 10 mM nitrate as an electron acceptor was used with an anaerobic system (Difco). The anaerobic condition was confirmed by the reduction of a resazurin indicator strip (Difco). For liquid cultures under anoxic conditions, the cultures were grown in 2-l bottles with butyl rubber stoppers. After the bottles were sealed, the headspace of the bottles was replaced with sterile N_2 gas. The bottles were then incubated at 60°C with intermittent shaking.

The effect of pH on growth was determined in MBM with four different buffers at a final concentration of 50 mM: citrate-Na_2HPO_4 buffer, pH range 5.0–7.0; phosphate buffer, pH range 6.0–8.0; Tris-HCl buffer, pH range 7.0–9.0; and glycine-NaOH buffer, pH range 8.5–10.5.

Growth determination of strain SC-1

The cell growth of strain SC-1 was monitored using the following three methods. First, the growth of strain SC-1 was determined by direct cell counts using a haemacytometer (Superior, Marienfeld, Germany) under light microscopy. Second, the amount of indole accumulated during growth of strain SC-1 was determined with Ehrlich's reagent (Yokota et al. 1989). Third, under nitrate-reducing conditions, the growth of strain SC-1 was monitored by determining the amount of nitrite accumulated. Nitrite was determined with a colorimetric method described by Hanson and Phillips (1981).

Morphology

The shapes and sizes of living and stained cells were determined by light microscope. The Gram reaction was determined using a Difco Gram stain kit according to the manufacturer's recommended protocol. The spore-forming ability of the new isolate was examined using Schaeffer's sporulation agar (Schaeffer et al. 1965) with the addition of the cell extract and culture supernatant of strain SK-1. The search for flagella was performed with a model H7000 transmission electron microscope (Hitachi, Tokyo, Japan) after staining with 0.5% to 2% (w/v) phosphotungstic acid. The thin sections were stained with uranyl acetate and lead citrate. The scanning electron microscopy of the new isolate was carried out as previously described by Padilla et al. (1997).

Determination of G+C content and chemotaxonomic characterizations

The chromosomal DNA was isolated and purified by the method described previously (Yoon et al. 1996). The guanosine + cytosine (G + C) content was then determined using the high-performance liquid chromatography (HPLC) method of Tamaoka and Komagata (1984).

Menaquinones were extracted and purified as described by Komagata and Suzuki (1987). The purified menaquinones were then dissolved in acetone and separated by an isocratic reverse phase HPLC by using methanol–isopropyl alcohol (50:50, v/v) as the eluent. Fatty acids were extracted and analyzed based on the instructions of the Microbial Identification System (MIDI, Newark, DE, USA) as described previously (Sasser 1990). The diamino acid of the peptidoglycan was determined by the method described previously (Komagata and Suzuki 1987).

Sequencing of 16S rDNA gene and data analysis

The 16S rDNA was amplified by polymerase chain reaction (PCR) analysis using two universal primers, designated F1 (5′-AGAGTTTGATCCTGGCTCAG-3′), corresponding to positions 8 to 27 of *E. coli* 16S rDNA, and R13 (5′-AGAAAGGAGGTGATCCAGCC-3′), corresponding to positions 1,544 to 1,525 of *E. coli* 16S rDNA, described previously (Yoon et al. 1998). The PCR product was purified by using a QIAquick PCR purification kit (Qiagen, Valencia, CA, USA). The purified 16S rDNA was sequenced using an ABI PRISM BigDye Terminator Cycle Sequencing Ready

Reaction kit (Applied Biosystems, Foster City, CA, USA) as recommended by the manufacturer. The primers used for sequencing were F340 (5′-ACTCCTACGGGAGGCAGC AG-3′), R8 (5′-AGGGTTGCGCTCGTTG-3′), and the two amplification primers, F1 and R13, described above. The purified sequencing reaction mixtures were automatically electrophoresed using an Applied Biosystems model 310 automatic DNA sequencer. The 16S rDNA sequence of the new isolate was repeatedly determined to check the reproducibility of the PCR-mediated rDNA sequence.

The 16S rDNA sequence of strain SC-1 was aligned with the 16S rDNA gene sequences of representatives of related taxa using Clustal W software (Thompson et al. 1994). The reference sequences were obtained from the Ribosomal Database Project (RDP) database. The multiple sequence alignment was then corrected as necessary. The phylogenetic analyses were restricted to nucleotide positions (1,213 homologous positions) that were unambiguously alignable in all sequences. The G+C content of the rDNA genes was calculated and a transversion analysis performed, as described by Woese et al. (1991). A maximum likelihood (ML) analysis was carried out using the program package MOLPHY ver. 2.3b3 (Adachi and Hasegawa 1996). A ML distance matrix was calculated using NucML, and a neighbor-joining (NJ) topology as the starting tree for the ML method was reconstructed by NJdist in MOLPHY. A ML tree was obtained using NucML with the R (local rearrangement search) option based on the HKY model (Hasegawa et al. 1985). The local bootstrap probabilities were estimated using the RELL (resampling of estimated log-likelihood) method (Kishino et al. 1990; Hasegawa and Kishino 1994).

Nucleotide sequence accession number

The 16S rDNA sequence of strain SC-1 was deposited in the GenBank data library under accession number AF190460. The accession numbers of the sequences used as references were as follows: *Geobacillus stearothermophilus*, X60640; *Geobacillus thermoleovorans*, Z26923; *Geobacillus thermoglucosidasius*, X60641; *Bacillus subtilis*, X60646; *Paenibacillus alvei*, X57304; *Paenibacillus azotofixans*, X60648; *Alicyclobacillus acidocaldarius*, X60742; *Alicyclobacillus cycloheptanicus*, X51928; *Symbiobacterium* sp. SC-1, AF190460; *Desulfotomaculum ruminis*, M34418; *Desulfotomaculum nigrificans*, X62175; *Heliobacterium chlorum*, M11212; *Selenomonas sputigena*, RDP TreeNum 1510; *Phascolarctobacterium faecium*, X72865; *Peptococcus niger*, X55797; *Moorella thermoautotrophica*, X77849; *Moorella thermoacetica*, M59121; *Thermaerobacter marianensis*, AB011495; *Desulfotomaculum australicum*, M96665; *Ammonifex degensii*, U34975; *Caldicellulosiruptor saccharolyticus*, l09178; *Dictyoglomus thermophilum*, X69194; *Thermoanaerobacter ethanolicus*, L09162; *Thermoanaerobacter thermocopriae*, L09169; *Syntrophospora bryantii*, M26491; *Syntrophomonas wolfei*, AF022248; *Thermoanaerobacter saccharolyticum*, L09169; *Thermoanaerobacter thermosaccharolyticum*, M59119; *Clostridium thermocellum*, L09173; *Clostridium butyricum*, M59085; *Atopobium minutum*, M59059; *Escherichia coli*, A14565.

DNA-DNA hybridization

Symbiobacterium thermophilum IAM 14863^T, donated by T. Beppu, was used as the reference strain for DNA-DNA hybridization studies. Chromosomal DNA of strain SC-1 was prepared by the procedures of Yoon et al. (1996). DNA-DNA hybridization was carried out according to the modified quantitative bacterial dot method of Tjernberg et al. (1989). DNA extracted from each strain was fixed on a nitrocellulose filter using slot-blot device (Minifold II, Schleicher and Schuell, Dassel, Germany) and labeled with ^{32}P with a random primed labeling kit (Roche, Palo Alto, CA, USA). The hybridization temperature was 50°C in 2×standard saline citrate (SSC, 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), in the presence of 50% formamide.

Results

Enrichment and isolation

Using the enrichment techniques described above, a mixed culture containing strain SC-1 and *Geobacillus* sp. strain SK-1 was obtained. Although the growth of strain SC-1 was never observed in the absence of *Geobacillus* sp. strain SK-1, the growth of strain SC-1 did not require live *Geobacillus* strain SK-1 when extract or culture supernatant of strain SK-1 were present in the medium (Fig. 1a). Therefore, strain SC-1 was isolated using an MBM agar containing the crude extract and/or culture supernatants of strain SK-1 under an anoxic atmosphere. After incubation for 3 days under microaerobic or anoxic conditions, the colonies observed on the MBM agar plates were circular, transparent, and less than 0.1 mm in diameter (Fig. 1b).

Morphological and physiological characteristics

The cells of strain SC-1 were found to be nonmotile, slightly curved rods, 1–5 μm long and 0.2–0.3 μm wide (Fig. 2a). No flagella were observed by transmission electron microscope of negatively stained cells. The cells of strain SC-1 stained Gram-negative and no spore formation on the sporulation agar medium or MBM was observed. A thin-section electron micrograph is shown in Fig. 2b.

The growth of strain SC-1 was detected under both microaerobic and anoxic conditions, because a pure culture of strain SC-1 grew optimally under a reduced oxygen tension. Under anoxic conditions, strain SC-1 grew anaerobically, reducing nitrate to nitrite stoichiometrically. Therefore, determination of accumulated nitrite was used as an alternative method for growth measurement of strain SC-1. The organism grew at temperatures between 45° and 70°C, with an optimum of 60°C, and the generation time at

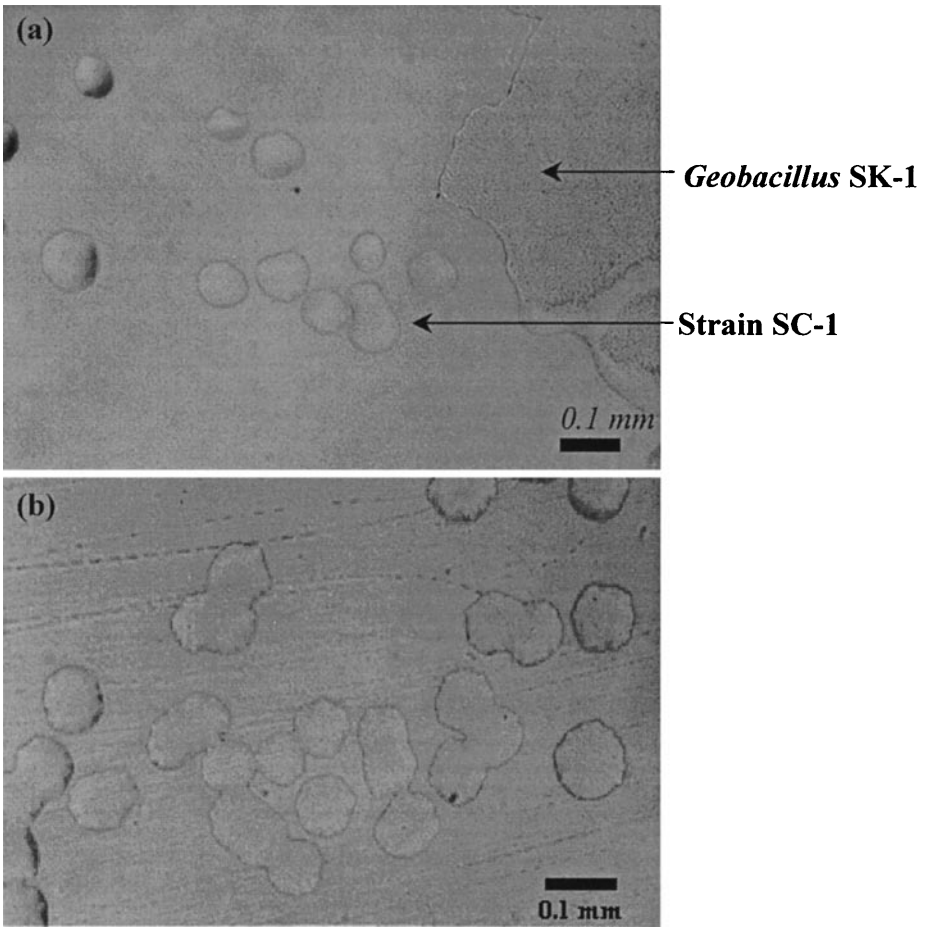

Fig. 1a,b. Colony configurations of strains *SC-1* and *SK-1* on modified basal medium (MBM) agar containing crude extracts and culture supernatants of strain *SK-1* under an anatomy microscope. **a** Mixed culture of strains *SC-1* and *SK-1*. **b** Pure culture of strain *SC-1*

this temperature was about 2.4 h at pH 7.5 (Fig. 3a). No growth was observed at 80°C. The growth of the new isolate at 60°C occurred at pH values of 6.0–9.0, with an optimum about 7.5 (Fig. 3b). No growth was detected at pH values below about 6.0 or above 9.0.

Biochemical characteristics

It was reported previously that heat-stable and unstable factors could support the growth of SC-1 (Rhee et al. 2000); therefore, in this study, we attempted to purify and identify the symbiotic factors from its symbiotic partner, *Geobacillus* sp. SK-1. It was shown that heat-stable (low molecular weight) factors were not always essential for the growth of strain SC-1. The heat unstable factors were aggregated and precipitated at pH 4.0 and renatured by returning the precipitate to pH 7.0. The resulting factor was irreversibly inactivated by phenol or protease treatment, thereby suggesting that the heat-unstable factors may be proteins. Because the membrane fraction of *Geobacillus* showed low growth activity (less than 5% of the cytoplasmic fraction), the factors may be in the cytoplasm. More research on the function of these factors is required to understand the unknown microbial interactions in the ecosystem.

The growth yield of strain SC-1 observed in pure cultures was about one-fifth that observed in mixed cultures. In addition, because of its obligate dependency on the extract and culture supernatant of strain SK-1 and its low growth yield in pure culture, it was difficult to determine conventional phenotypic characteristics, such as nutrient utilization, growth in different media, and acid production from sugars. Tryptophan indole-lyase (0.34 U/mg protein) and tyrosine phenol-lyase (0.01 U/mg protein) were induced by tryptophan and tyrosine, respectively. However, tyrosine and tryptophan had no effect on the growth of strain SC-1.

Chemotaxonomic characteristics and DNA base composition

The major quinones found in strain SC-1 were 61% menaquinone 6 (MK-6) and 39% menaquinone 7 (MK-7). The fatty acid profile of strain SC-1 was characterized mainly by 39% iso-$C_{15:0}$, 28% iso-$C_{17:0}$, 10% iso-$C_{16:0}$, 7% $C_{16:0}$, 7% anteiso-$C_{17:0}$, 2% anteiso-$C_{15:0}$, and 2% $C_{18:0}$. Strain SC-1 did not contain diaminopimelic acid as the diamino acid in its cell wall. The G+C content of strain SC-1 was 65 mol%.

16S rDNA sequence analysis

An almost complete 16S rDNA sequence of strain SC-1, comprising 1,521 nucleotides (>96% of *E. coli* 16 rDNA

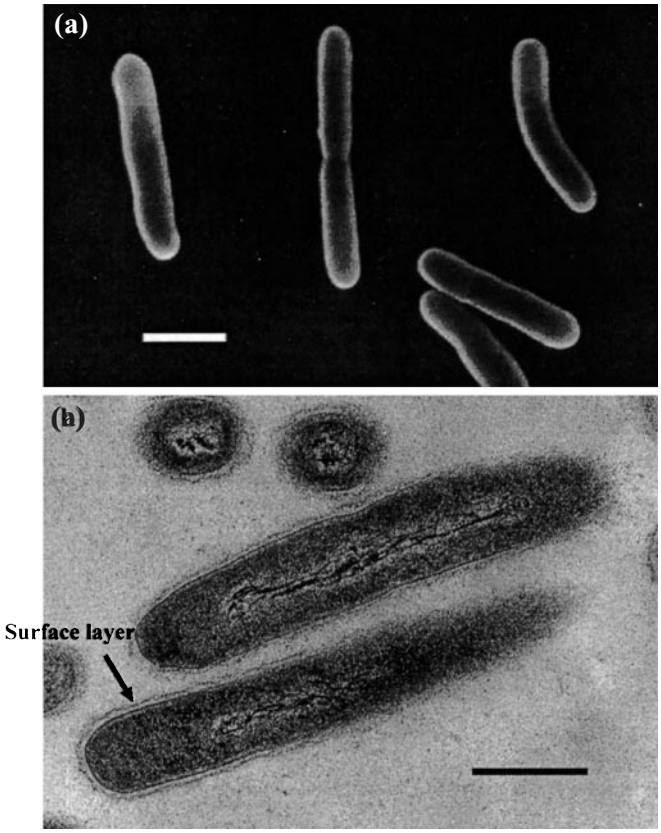

Fig. 2a,b. Electron micrographs of strain SC-1. **a** Scanning electron micrograph of exponentially growing cells occurring singly or in pairs; bar, 0.5 μm. **b** Transmission electron micrograph of a thin section of an exponentially growing cells; bar, 0.3 μm

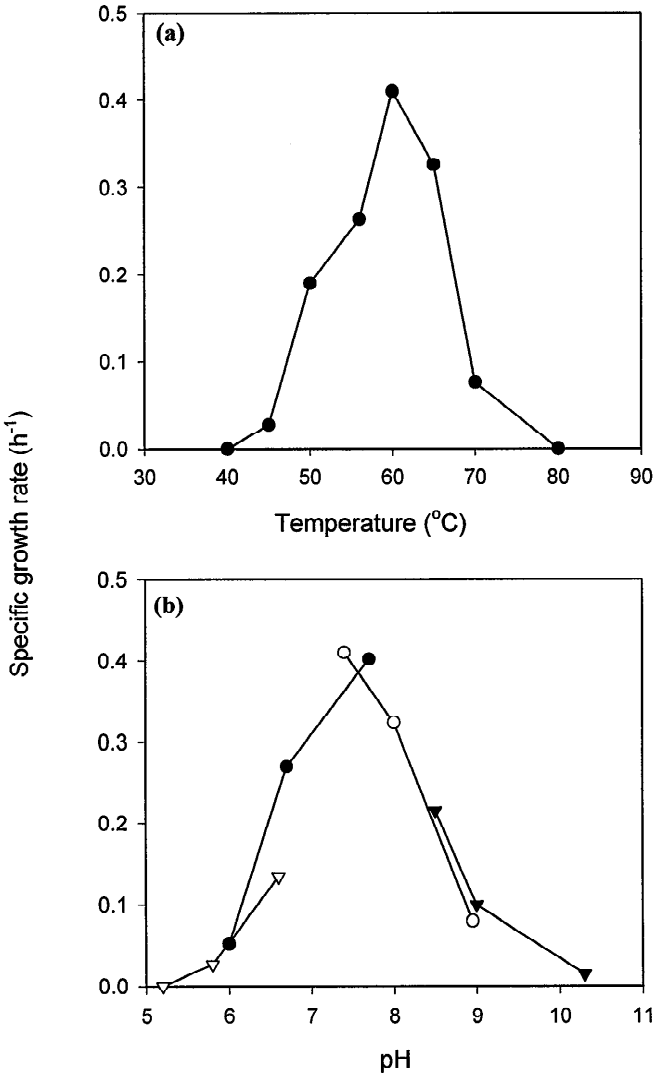

Fig. 3a,b. Effects of temperature **(a)** and pH **(b)** on growth of strain SC-1. **a** Growth determined in MBM at pH 7.5. No growth occurred below 45°C or above 70°C. **b** Growth determined in MBM with various buffers at 60°C. The pH of the medium containing appropriate buffer systems was adjusted with HCl and NaOH at room temperature. *Open triangles*, citrate-Na_2HPO_4 buffer; *solid circles*, phosphate buffer; *open circles*, Tris-HCl buffer; *solid triangles*, glycine-NaOH buffer

sequence), was determined in this study. An inspection of the predicted secondary structures and evaluation by the CHECK-CHIMERA program of the RDP (Larsen et al. 1993) indicated that the rDNA sequence of SC-1 was free of artifacts. A BLAST search was conducted to obtain those sequences that were potentially related to that of strain SC-1. The 16S rDNA sequence of strain SC-1 showed about 98% sequence similarity with that of *Symbiobacterium thermophilum* (AB004913), which was submitted only once prior to this study. These results indicate that strain SC-1 is a member of the *Symbiobacterium* subphylum. However, on the basis of a sequence similarity analysis, the 16S rDNA sequence of the isolate exhibited less than 87% similarity with all other known sequences. The most similar 16S rDNA sequences were those of *Geobacillus thermoleovorans* (Rainey et al. 1994), *Alicyclobacillus acidocaldarius* (Wisotzkey et al. 1992), *Moorella thermoautotrophica* (Wiegel et al. 1981), *Moorella thermoacetica* (Slobodkin et al. 1997), *Thermaerobacter marianensis* (Takai et al. 1999), *Desulfotomaculum australicum* (Stackebrandt et al. 1997), *Thermoanaerobacter ethanolicus*, and *Thermoanaerobacter thermocopriae* (Collins et al. 1994) with 85%–87% similarity except for *S. thermophilum*. Thus, the 16S rDNA sequence analysis indicates that *Symbiobacterium* is closely related to the *Bacillus-Clostridium* subphylum (Collins et al. 1994) of the Gram-positive bacteria, even though the isolate appears to be phylogenetically distant from these bacteria at the genus level (Fig. 4).

A phylogenetic analysis by the NJ and ML methods suggested a similar topology for the affiliation of the new isolate, indicating a distinct branch denoting an early divergence from the genera of the *Bacillus-Clostridium* subphylum (Collins et al. 1994). Although the base composition disparities among the genomic DNAs of low-G + C-content Gram-positives range from 31.0% to 72.5%, the base composition disparities among the 16S rDNA sequences were relatively small (from 51.7 mol% G + C content in *Paenibacillus alvei* to 63.0 mol% G + C content in *Thermaerobacter marianensis*). The 16S rDNA of strain SC-1 had a 59.0 mol% G+C content, which is similar to that of thermophilic *Geobacillus* species (from 54.1 mol%

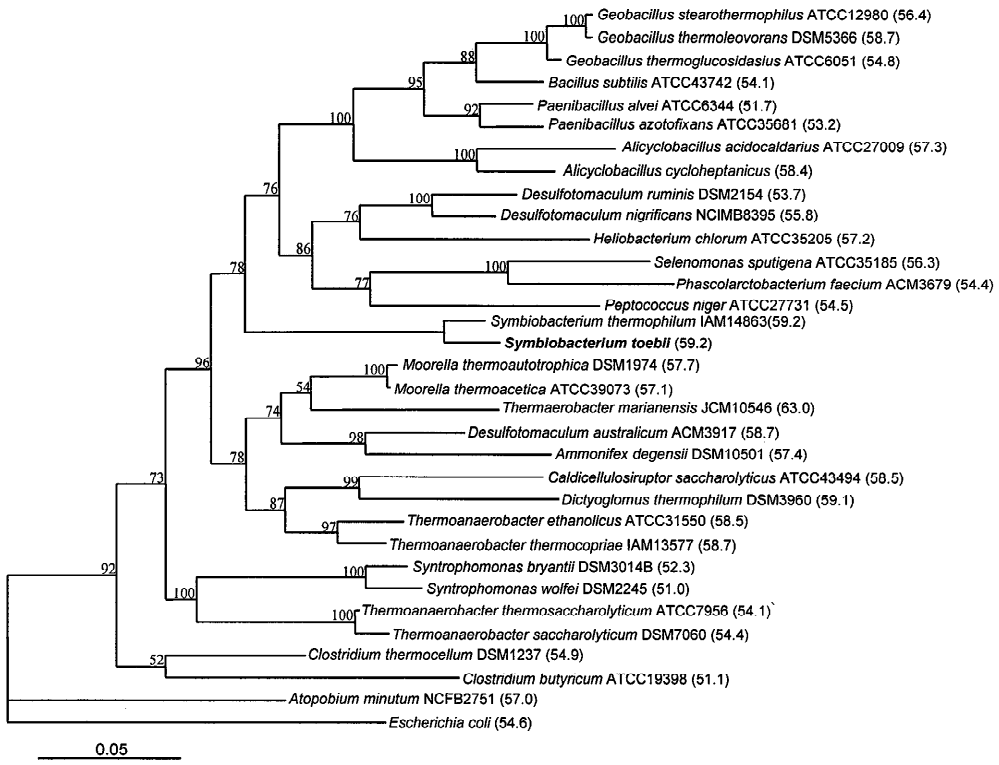

Fig. 4. Phylogenetic dendrogram of representative low-G+C-content, spore-forming bacteria within the *Bacillus-Clostridium* subphylum based on 16S rDNA sequences by using the maximum likelihood method on 1,213 homologous sequence positions. *Numbers on the tree* represent the bootstrap values (expressed as a percentage of 1,000 replications). *Numbers after the name* are the G+C content in mol% of homologous sequence positions. Scale bar indicates 5 substitutions per 100 nucleotides. The *E. coli* 16S rDNA sequence served as an outgroup

in *Geobacillus thermoglucosidasius* to 58.7 mol% in *Geobacillus thermoleovorans*). Furthermore, when using a transversion analysis for the generation of a phylogenetic tree, it was confirmed that the base composition disparities had little influence on the tree topology.

DNA-DNA hybridization

For the elucidation of the novelty of the new isolate strain SC-1, DNA-DNA hybridization experiments were carried out. This test showed that the chromosomal homology between *Symbiobacterium thermophilum* and *Symbiobacterium* sp. SC-1 was approximately 30%, indicating that the new isolate was a novel species of the *Symbiobacterium* genus.

Discussion

Most thermophilic bacteria have been isolated from geothermal environments associated with volcanic activity (Zeikus 1979; Kristjansson and Stetter 1992). However, other thermally heated environments, including man-made environments and naturally solar-heated environments, have also been reported to harbor thermophilic bacteria (Mathrani and Ahring 1991; Huang et al. 1998). Strain SC-1 was isolated from hay compost (toebi) from farmland in Korea. Because the isolate SC-1 exhibited an obligate symbiotic (commensal) interaction with a thermophilic *Geobacillus* sp. SK-1, crude extracts and culture supernatants of strain SK-1 were required for the pure growth of the isolate (Rhee et al. 2000). Bacterial commensal interaction with partner microorganisms is rare, whereas other types of interactions among bacteria are widely observed (Shimao et al. 1984; Ruby 1992).

Because the growth of strain SC-1 exhibits an essential requirement for symbiotic factors from *Geobacillus* sp. SK-1, an attempt was made to purify and identify these symbiotic factors. As a result, it is suggested that the symbiotic factors might be proteins because they were irreversibly inactivated by phenol or protease treatment. However, the complete identification of the symbiotic factors has been thus far unsuccessful. Consequently, further research is required to identify these symbiotic factors, which may contribute to a better understanding of microbial interactions in ecological environments and to the discovery of other microbial groups ubiquitous yet still unknown.

Suzuki et al. (1988) described an obligate symbiotic bacterium *Symbiobacterium thermophilum* with physiological similarities to strain SC-1 with respect to growth temperature, optimal pH, and tyrosine phenol-lyase and tryptophan indole-lyase activity. Recently, Ohno et al. (1999, 2000) established a pure culture of *S. thermophilum* from its supporting *Geobacillus* strain validly described as *S. thermophilum*. Since strain SC-1 exhibits a high similarity to *S. thermophilum* in its 16S rDNA and physiological characteristics, it was tentatively identified as *Symbiobacterium* sp. strain SC-1. However, *S. thermophilum* does not have

round-end rod morphology, and its cell-wall structure is also slightly different, and the genome size determined by pulsed-field gel electrophoresis was different from that of *S. thermophilum* (Hong et al. 2000). A thin-section electron micrograph of strain SC-1 showed that the cell wall was relatively thin and loose, causing its Gram negativity and flexible (curved) rod shape. A cell-wall analysis showed that strain SC-1 contained menaquinone and a branched-chain fatty acid, which are widespread in Gram-positive bacteria. Thus, these results support the position of strain SC-1 close to the Gram-positive bacteria, even though it stained Gram negatively. Strain SC-1 is a microaerophilic thermophile of the domain Bacteria. Therefore, on the basis of its physiological and molecular properties, strain SC-1is named *Symbiobacterium toebii*.

Most thermophilic strains isolated from compost are members of the genus *Bacillus* (Storm 1985). However, Ohno et al (2000) and we have discovered these novel thermophilic bacteria, *S. thermophilum* and *S. toebii*, and have confirmed the wide distribution of strain SC-1 in compost (unpublished data). Accordingly, more studies are needed to elucidate the diversity of bacteria affected by microbial interactions because *Symbiobacterium* sp. is surely not the only example in the ecosystem.

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