

Symbiobacterium toebii sp. nov., Commensal Thermophile Isolated from Korean Compost

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Abstract A thermophilic nonspore-forming rod isolated from hay compost in Korea was subjected to a taxonomic study. The microorganism, designated as SC-1^T, was identified as a nitrate-reducing and nonmotile bacterium. Although the strain was negatively Gram-stained, a KOH test showed that the strain SC-1^T belonged to a Gram-positive species. Growth was observed between 45 and 70°C. The optimal growth temperature and pH were 60°C and pH 7.5, respectively. The G+C content of the genomic DNA was 65 mol% and the major quinone types were MK-6 and MK-7. A phylogenetic analysis based on 16S rDNA sequences revealed that the strain SC-1^T was most closely related to *Symbiobacterium thermophilum*. However, the level of DNA-DNA relatedness between strain SC-1^T and the type strain for *Symbiobacterium thermophilum* was approximately 30%. Accordingly, on the basis of the phenotypic traits and molecular systematic data, the strain SC-1^T would appear to represent a new species within the genus *Symbiobacterium*. The type strain for the new species is named SC-1^T (=KCTC 0307BP^T; DSM 15906^T).

Key words: *Symbiobacterium toebii* sp. nov., thermophilic, commensal bacterium, symbiosis

While screening thermostable tyrosine phenol-lyase-producing bacteria [7, 8], a novel thermophilic eubacterium was detected in compost. The strain exhibited a commensal interaction with *Geobacillus toebii* SK-1^T and was isolated using cell extracts of the partner SK-1^T [12, 13, 14, 16].

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Therefore, we subjected strain SC-1^T to polyphasic characterization to investigate whether it represents a new species within the genus *Symbiobacterium* [10, 17].

For the cultivation of strain SC-1^T, a basal medium (BM) was used containing 5 g of polypeptone, 10 g of yeast extract, 6 g of K₂HPO₄, 2 g of KH₂PO₄, and 0.025 g of MgSO₄·7H₂O per liter of deionized water. Since strain SC-1^T is a commensal bacterium, cell-free crude extracts of *Geobacillus toebii* SK-1^T were consistently added to the BM. The cell extracts were prepared by sonicating a centrifuged pellet of fully-grown SK-1^T, followed by addition to the BM after filter-sterilization. The strain SC-1^T were cultured at 60°C under anaerobic conditions. For cultivation under anaerobic conditions (CO₂/N₂, 10:90), the BM containing 30 mM nitrate as an electron acceptor was incubated in an anaerobic jar (Difco) using an anaerobic system and palladium catalysts (Difco). For cultivation under microaerophilic conditions (CO₂/N₂/O₂, 10:85:5), a campylobacter microaerophilic system (Difco) was used instead of an anaerobic system. The cell growth of the strain SC-1^T was monitored based on the nitrite accumulated, which was determined by the colorimetric method described by Hanson & Phillips [3].

Since the growth of the strain SC-1^T is known to depend on cell-to-cell communication with *Geobacillus toebii* strain SK-1^T [12, 13], the strain SC-1^T was isolated using a modified BM (MBM) containing crude extracts of mixed cultures under an anoxic atmosphere. Strain SK-1^T was found to provide higher stimulation for the growth of the strain SC-1^T than any other *Bacillus* or *Geobacillus* species tested [16]. The colonies of the strain SC-1^T observed on MBM agar plates were circular, transparent, and less than 0.1 mm in diameter. The Gram reaction was determined

using a Difco Gram Stain Kit according to the manufacturer's recommended protocol (Difco). To distinguish false-negative Gram staining, a KOH test was performed in parallel with the Gram stain reaction. The KOH test was performed by mixing a visible amount of cells from a colony in a loopful of 3% aqueous KOH on a glass slide [11]. Although SC-1^T indicated Gram-negative staining, the KOH test revealed that the strain SC-1^T was a Gram-positive species. False-negative Gram staining with this strain appeared to occur with a long incubation time [11]. The shapes and sizes of the living and stained cells were determined by light microscopy. The individual cells of the strain SC-1^T were nonmotile rods, 1 to 5 µm in length and 0.2 to 0.3 µm wide. As observed by light microscopy at 60°C, the cells were nonmotile, while transmission electron microscopy of negatively stained cells indicated that the cells had no flagella. The search for flagella was also performed using a model H7000 transmission electron microscope (Hitachi) after the preparations were stained with 0.5 to 2% (w/v) phosphotungstic acid. A thin section was stained with uranyl acetate and lead citrate. No spore formation by physiological induction was observed under any culture conditions.

Strain SC-1^T grew anaerobically with nitrate reduction under anoxic conditions. Nitrite was the final product of nitrate reduction and accumulated during anoxic growth. Although the strain SC-1^T was previously isolated with 10 mM nitrate [7, 8], when 30 mM initial nitrate was added to the media, this produced the highest amount of nitrite, approximately 28 mM, and best growth of the strain SC-1^T (Fig. 1). Under microaerophilic conditions, growth was enhanced in an atmosphere that contained a lower O₂ concentration. The organism grew at temperatures between 45 and 70°C, exhibiting optimum growth at 60°C, and the generation time at these temperatures was about 2.4 h at pH 7.5. No growth was observed at 80°C. Growth of the new isolate at 60°C occurred at pH between 6.0 and 9.0, with optimum growth at about 7.5. No growth was

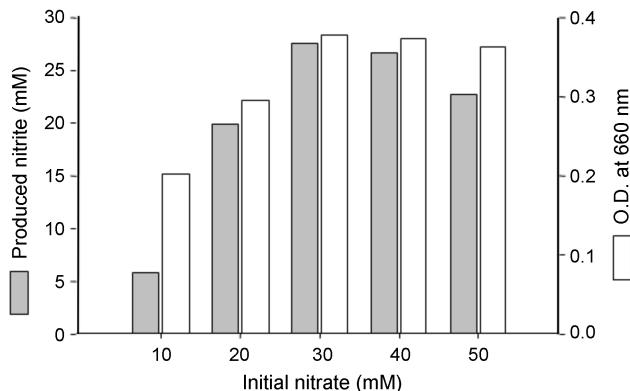


Fig. 1. Effect of initial nitrate concentration on growth of strain SC-1^T.

detected below pH 6.0 or above pH 9.0. The isolate was unable to grow in an MBM that did not contain yeast extract and polypeptone.

The menaquinones were extracted and purified as described by Komagata and Suzuki [6]. The purified menaquinones were then dissolved in acetone and separated by isocratic reverse phase HPLC using methanol-isopropyl alcohol (50:50, v/v) as the eluting solvent. The major quinones found in the strain SC-1^T were 61% of menaquinone 6 and 39% of menaquinone 7. The fatty acids were extracted and analyzed according to the instructions of the Microbial Identification System (MIDI; Microbial ID), as described previously [15]. The fatty acid profile of the strain SC-1^T was characterized as mainly 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}. The chromosomal DNA was isolated and purified by a previously described method [9, 21]. The G+C content was determined by the method of Tamaoka and Komagata [18]. The DNA was hydrolyzed and the resultant nucleotides were analyzed by isocratic-reverse high-performance liquid chromatography (HPLC). The result showed that the G+C content of the strain SC-1^T was 65 mol%. The diamino acid of the peptidoglycan was determined by a previously described method [6], and the result showed that strain SC-1^T did not contain any form of diaminopimelic acid as the diamino acid in its cell wall.

The susceptibility of the strain SC-1^T to antibiotics was determined by transferring an exponentially growing culture into fresh media containing filter-sterilized antibiotics at a concentration of 0–100 µg/ml [4]. The growth of the

Table 1. Enzymatic profile of strain SC-1^T determined using API-ZYM kit.

Enzyme assayed for	Extacellular	Intracellular
Alkaline phosphatase	++	+++
Esterase (C4)	++	+
Esterase lipase (C8)	+	-
Lipase (C14)	-	-
Leucine arylamidase	++	++
Valine arylamidase	-	-
Cysteine arylamidase	-	-
Trypsin	-	-
α-chymotrypsin	+++	+
Acid phosphatase	+++	+++
Naphthol-AS-BI-phosphohydrolase	+++	+++
α-galactosidase	+++	+++
β-galactosidase	-	-
β-glucuronidase	-	-
α-glucosidase	+++	-
β-glucosidase	++	-
N-acetyl-β-glucosaminidase	-	-
α-mannosidase	-	-
α-fucosidase	-	-

strain SC-1^T was inhibited by the addition of ampicillin, chloramphenicol, and tetracycline to the media before incubation at a concentration of 2 µg/ml, yet not by the addition of 200 µg/ml of kanamycin. The general enzymatic profile of the strain SC-1^T was determined using an API-ZYM kit (BioMerieux, France), which scores 19 different enzyme activities, including β-glucosidase [1, 5], as presented in Table 1. The strain produced alkaline phosphatase, esterase (C4), leucine arylamidase, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, and α and β-glucosidase. Since the current paper is the first ever to report enzymatic profile of a *Symbiobacterium*, the API-ZYM system was able to provide new and helpful characteristics for the rapid identification of the genus *Symbiobacterium*.

The 16S rDNA was amplified by a PCR using two universal primers, as described previously [22]. The PCR product was then purified using a QIAquick PCR purification kit (Qiagen) and sequenced using an ABI PRISM BigDye Terminator Cycle sequencing Ready Reaction kit (Applied Biosystems) according to the manufacturer's instructions. The purified sequencing reaction mixtures were automatically

electrophoresed using an Applied Biosystems model 310 automatic DNA sequencer. The 16S rDNA sequence of strain SC-1^T was aligned with representative 16S rDNA sequences of related taxa using CLUSTAL W software [19]. Any gaps at the 5' and 3' ends of the alignment were omitted from further analyses. As a result, an almost complete 16S rDNA sequence of strain SC-1^T comprising 1521 nucleotides (>96% of *E. coli* 16 rDNA sequence) was determined in the current study. A 16S rDNA sequence analysis indicated that strain SC-1^T was a member of the genus *Symbiobacterium* (Fig. 2). Based on sequence similarity analysis, the 16S rDNA sequence of the new isolate exhibited 98.5% similarity with *Symbiobacterium thermophilum* IAM14863^T. DNA-DNA hybridization was performed to determine the entire genomic relatedness between strain SC-1^T and the type strain for *Symbiobacterium thermophilum*^T. Since the DNA-DNA relatedness was approximately 30%, this indicated distinct genomic species [20]. In conclusion, therefore, all the results described in this report support the proposal that a new species, *Symbiobacterium toebeii* sp. nov. is created for strain SC-1^T. The properties of the new species are summarized below.

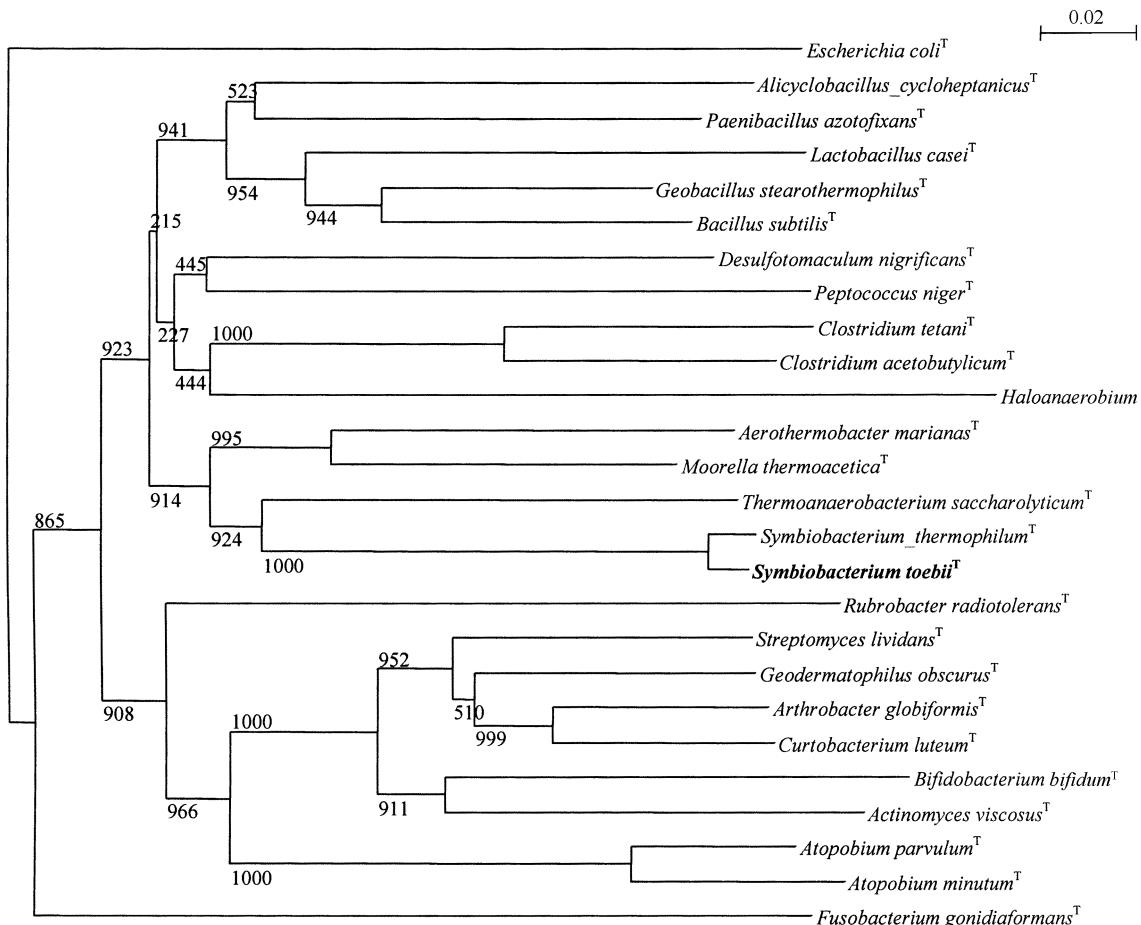


Fig. 2. Phylogenetic tree showing position of strain SC-1^T within genus *Symbiobacterium* and related taxa.

Description of *Symbiobacterium toebii* sp. nov.

Symbiobacterium toebii (to.e.bi.i. M. L. neut. n. *toebii* referring to *toebi*, the farmland compost in Korea, the original source of the organism). Straight to slightly curved rods, 0.2–0.3×1.0–5.0 µm, with rounded ends. Gram stain reaction is negative. However, a KOH test revealed that strain SC-1^T belongs to a Gram-positive species. Cells occur singly or in pairs and are nonmotile. No spores were observed. The cells are microaerophilic or anaerobic with denitrification. The temperature range for growth is 45–70°C, with an optimum temperature of 60°C. The pH range for growth is 6.0–9.0, with optimum growth occurring at 7.5. Growth requires crude extracts of *Geobacillus*. The G+C content in the genomic DNA is about 65 mol%, as determined by HPLC. The fatty acids are 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}. Isolated from farmland compost in Gongju, Korea. The type strain is strain SC-1^T, which has been deposited in the Korean Collection for Type Cultures as KCTC 0307BP^T and DSM 15906^T.

The GenBank accession number for the 16S rDNA sequence of strain SC-1^T is AF190460.

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