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NOTE

# *Symbiobacterium thermophilum* gen. nov., sp. nov., a symbiotic thermophile that depends on co-culture with a *Bacillus* strain for growth

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**A Gram-negative and tryptophanase-positive thermophile, whose growth is dependent on co-culture with an associating *Bacillus* strain, had been reported and tentatively named *Symbiobacterium thermophilum* strain T$^T$. Axenic culture of strain T$^T$ was recently established by dialysing cultures with the supporting bacterial strains or adding their culture broth. Phylogenetic analysis of strain T$^T$, based on the 16S rDNA sequence, was conducted for the validation of *S. thermophilum*. The sequence of strain T$^T$ was located at the outermost position in the high-G+C Gram-positive group distinctly isolated from any other branches hitherto known. Ten sequences identical to that of strain T$^T$, and one sequence closely related to it, were identified for the first time from soil and compost samples. The outer membrane of strain T$^T$ had a three-layered structure, outside the cytoplasmic membrane, which is similar to the S-layer in the cells of members of the *Bacillaceae*. Chemical analysis of the cells revealed that menaquinone-6 is a major component of the quinone system. According to these results, along with several previous observations (i.e. a G+C DNA content of 65 mol% and the identification of iso-$C_{15:0}$ and iso-$C_{17:0}$ acids as major cellular fatty acids), the new taxon *Symbiobacterium thermophilum* gen. nov., sp. nov. is proposed. The type strain is *S. thermophilum* strain T$^T$ (= IAM 14863$^T$).**

**Keywords:** *Symbiobacterium thermophilum*, 16S rDNA, tryptophanase, high-G+C Gram-positive bacteria

A thermophilic bacterium (strain T$^T$) producing tryptophanase and tyrosine-phenyl lyase ($\beta$-tyrosinase) was obtained from compost at 60 °C in mixed culture with a thermophilic *Bacillus* strain (Suzuki *et al*., 1988) and was used as a source of these distinctly heat-stable enzymes (Suzuki *et al*., 1991, 1992; Hirahara *et al*., 1992, 1993). We originally isolated these organisms from a sample collected in Japan; isolation of similar organisms in Korea was also reported by Lee *et al*. (1997). Strain T$^T$ is a Gram-negative small rod with a DNA G+C content of 65·1 mol% and it contains branched fatty acids as major cellular components (Suzuki *et al*., 1988). In addition, we observed that strain T$^T$ was able neither to grow independently in any artificial medium nor to produce pure colonies; instead, it grew only in co-culture with the *Bacillus* strain in liquid media. Although the bacterium was tentatively designated as *Symbiobacterium thermophilum* strain T$^T$, the unique properties have prevented us from establishing its axenic culture (essential for its validation as a novel taxon). However, our recent study (Ohno *et al*., 1999) revealed that this bacterium grows in the pure state in dialysing culture, separated from the *Bacillus* strain by a cellulose membrane. A quantitative PCR with specific DNA sequences as primers enabled us to quantify the growth of strain T$^T$ in the culture. Furthermore, the method allowed us to detect limited growth of strain T$^T$ in the conditioned medium supplemented with the cultured broth not only of the *Bacillus* strain but also of various bacterial

The DDBJ accession numbers for the 16S rRNA gene sequences of strains T$^T$ and YK67 are AB004913 and AB004915.

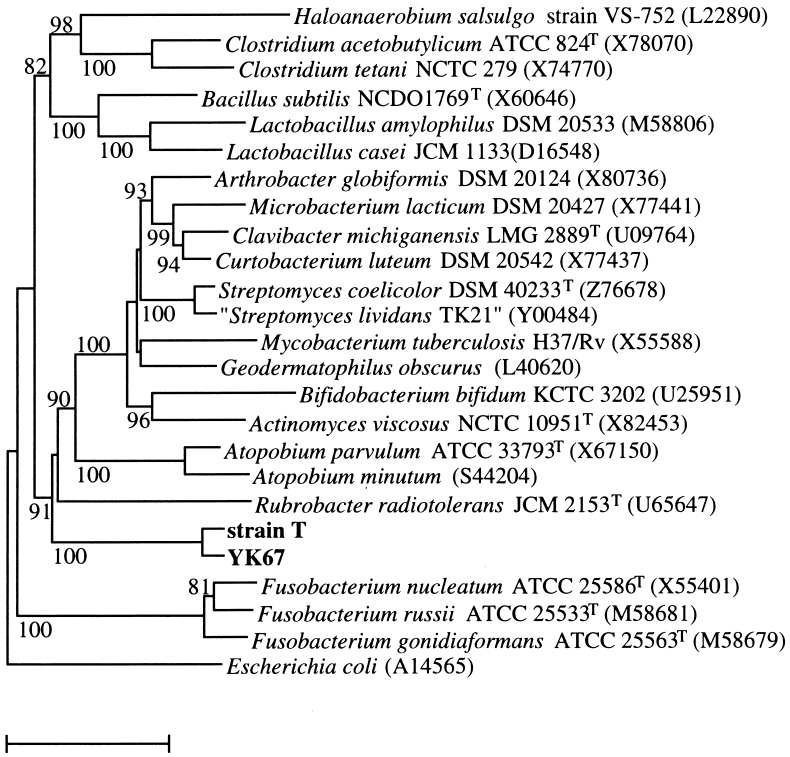


***Fig. 1.*** Unrooted tree showing the position of a novel phylogenetic branch of *Symbiobacterium thermophilum* strains $T^T$ and YK67 with representative sequences from other Gram-positive bacteria and *Escherichia coli*. The tree, constructed using the neighbour-joining method, was based on a comparison of aligned positions of 1005 nucleotides (excluding deleted and ambiguously aligned sites). Each bootstrap value is expressed as a percentage of 1000 replications. Values above 80 % are given at branching points. Bar, 10 % sequence divergence.

species, even including *Escherichia coli*. We also found that the conditioned agar medium supported the formation of minute colonies (less than 0·2 mm in diameter) of strain $T^T$ with low colony-forming efficiencies (0·5–1·0 %) even under microaerobic conditions. Thus, axenic culture of strain $T^T$ has been established, although its nutritional requirements remain to be chemically identified. Here, we report the unique phylogenetic location of strain $T^T$ by means of 16S rDNA typing and provide a description for the taxonomic validation of *S. thermophilum*.

Cells from the axenic culture of strain $T^T$ were subjected to a PCR to amplify its 16S rDNA with universal primers (5′-CGCGGATCCAGAGTTTGATCMTGGCTCAG-3′ and 5′-CGCGGATCCTACCTTGTTACGACTTCACCCCAG-3′ corresponding to positions 7–26 and 1484–1507, according to *E. coli* numbering, respectively). The amplified 1·5 kb fragment was digested with *Bam*HI and cloned onto M13 mp19 at the *Bam*HI cleavage site. Its nucleotide sequence was determined using an automatic sequencer (model 4000L; Li-cor). A database search in the Ribosomal Data Project (RDP) web site (http://www.cme.msu.edu/RDP/) did not reveal any sequences closely related to that of strain $T^T$. The construction of a phylogenetic tree with various sequences from Gram-negative and -positive bacteria always resulted in the exclusion of the strain $T^T$ branch from the Gram-negative group. It was included in the Gram-positive group as the outermost branch in the high-G+C Gram-positive cluster. A representative tree constructed using the neighbour-joining method is shown in Fig. 1. Calculation by the maximum-likelihood method (Felsenstein, 1982) resulted in similar isolation of the branch from other groups (data not shown). These results indicate that strain $T^T$ is a most isolated member of the Gram-positive bacteria, positioned close to the branches of the *Fusobacterium* group and the Gram-negative bacteria.

We attempted to detect the presence of *S. thermophilum* and its relatives from environmental samples. Among 89 compost or soil samples collected at several districts in Japan, 43 gave tryptophanase-positive mixed cultures when cultured stationary in liquid Luria–Bertani medium at 60 °C. The specific primers of 16S rRNA gene of *S. thermophilum* (5′-CGCGGATCCTCTGCTCTGGGATAACAGGC-3′ and 5′-CGCGGATCCGAACTGAGACCGCCTTTTGC-3′, corresponding to positions 108–127 and 1278–1297 of strain $T^T$ numbering, respectively) were used to amplify the *S. thermophilum*-specific sequence in these tryptophanase-positive mixed cultures; we obtained

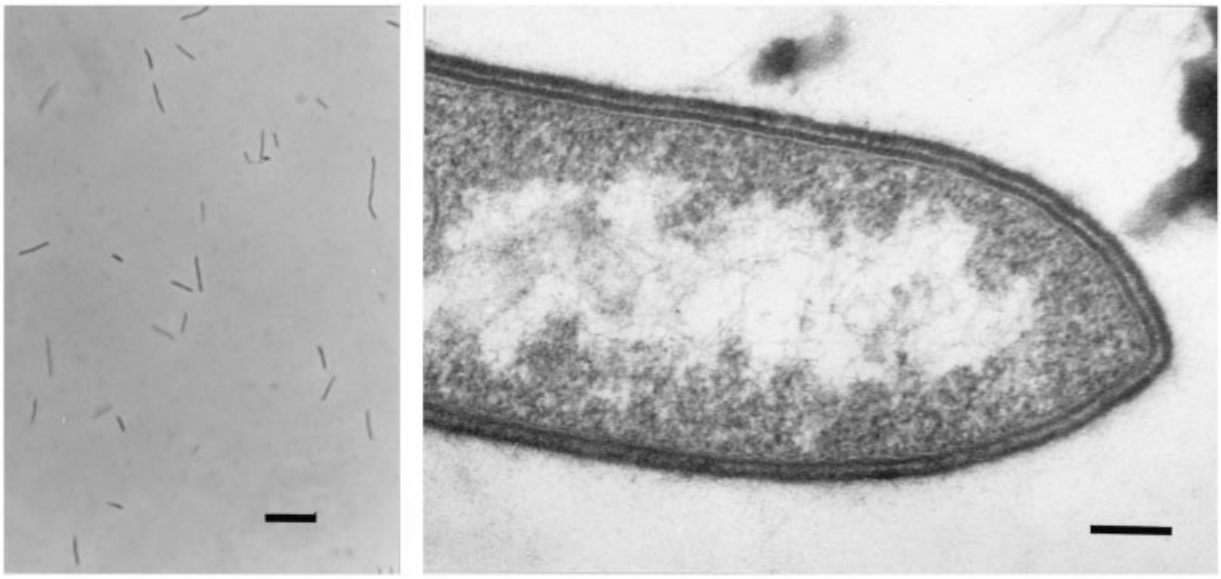

***Fig. 2.*** *S. thermophilum* strain $T^T$. (left) Phase-contrast micrograph; bar, 10 μm. (right) Thin-sectioned electron micrograph showing the outer membrane structure; bar, 100 nm.

11 fragments showing similarity to the sequence of strain $T^T$. Nucleotide sequencing elucidated that 10 out of the 11 fragments were almost identical to that from strain $T^T$ (99·8–100 % identity in 1150 nucleotides) and one sequence (YK67) was closely related (96·9 % identity in 1155 nucleotides) (Fig. 1). This result implies the presence of species diversity within the *Symbiobacterium* branch as well as wide distribution of *S. thermophilum* and its related strains in the environment.

We also conducted 16S rRNA sequence analysis of the associating *Bacillus* strain. Cells from its pure culture were subjected to a universal PCR reaction to amplify an approximately 1·5 kb 16S rDNA fragment and the nucleotide sequence was determined by using the same strategy as that used for strain $T^T$. The phylogenetic position of the strain was located in the cluster of thermophilic members of the *Bacillaceae* (data not shown), which is in accordance with its general taxonomical properties (Suzuki *et al*., 1988). As we reported recently (Ohno *et al*., 1999), the ability to support the growth of *S. thermophilum* is not limited to the *Bacillus* strain but is distributed among other species of the *Bacillaceae* and even among a wider variety of bacterial species.

We previously reported that the surface structure of strain $T^T$ consisted of three layers, apparently being similar to the surface of the outer membranes of Gram-negative bacteria; however, the observation was based on an electron micrograph of rather lower resolution (Suzuki *et al*., 1988). The phylogenetic position indicated by the 16S rDNA sequence prompted us to re-examine the surface structure. A thin-sectioned preparation of *S. thermophilum* showed the existence of a three-layered structure (approximate total thickness 27 nm), outside the cytoplasmic membrane, which consisted of an innermost electron-dense layer, a middle electron-transparent layer and an outer electron-dense layer (Fig. 2). This unit structure is different from the surface structure of Gram-negative bacteria but rather resembles the S-layer structure observed with the cells of the *Bacillaceae* (Tsukagoshi *et al*., 1982). Thus, we conclude that the cell-surface structure of strain $T^T$ is different from that of typical Gram-negative bacteria, though its Gram-staining is negative.

We carried out a chemical analysis of the strain $T^T$ cells in order to identify its respiratory quinone. The cells were extracted with chloroform/methanol (2:1, v/v) and the extract was analysed by two-dimensional TLC and HPLC according to standard methods (Collins *et al*., 1980; Collins & Jones, 1981). The results indicated that menaquinone-6 is the major component of the quinone system in strain $T^T$. Our previous analysis revealed the presence of the iso-branched chain $C_{15:0}$ and $C_{17:0}$ acids as the major cellular fatty acids of strain $T^T$ (Suzuki *et al*., 1988). Menaquinones and the branched fatty acids are known to exist mostly in Gram-positive, but not Gram-negative, bacteria. In addition, Grundy & Henkin (1999) recently reported the presence of a T-box sequence in front of the leucyl-tRNA synthase gene in our registered sequence of the tryptophanase gene cluster of *S. thermophilum* strain $T^T$ (DDBJ accession no. AB010832). T-box is a consensus sequence distributed widely and specifically among Gram-positive bacteria. All these results indicate that *S. thermophilum* strain $T^T$ and its related sequences constitute a characteristic new branch belonging to the Gram-positive group.

### Description of *Symbiobacterium* gen. nov.

*Symbiobacterium* (sym.bi.o.bac.te′ri.um. Gr. adj. *symbiotikos* symbiotic; Gr. n. *bakterion* a small rod; *Symbiobacterium* symbiotic small rods).

Cells are Gram-negative, straight rods with a multilayered cell surface structure. Non-motile and non-sporulating. Iso-branched chain $C_{15:0}$ and $C_{17:0}$ acids are the major components of the cellular fatty acids. Menaquinone-6 is the major component of the quinone system. It is a symbiont requiring a diffusible metabolite(s) of the associating bacterial species for independent growth. The optimum temperature for growth is approximately 60 °C. Microaerophilic. The type species is *Symbiobacterium thermophilum*.

### Description of *Symbiobacterium thermophilum* sp. nov.

*Symbiobacterium thermophilum* (ther.mo′phil.um. Gr. n. *therme* heat: Gr. adj. *philos* friend, loving; *thermophilum* heat-loving).

Cells are straight rods (0·25–0·35 × 1·5–7 mm), occurring singly or in pairs. The Gram-reaction is negative, but molecular taxonomy indicates that this bacterium belongs to the Gram-positive group, at the outermost phylogenetic branch. Non-motile. Microaerophilic, requiring a diffusible bacterial metabolite(s) for its independent growth. Catalase-positive. Positive for indole production. Produces inducible tryptophanase and tyrosine-phenyl lyase activity. The temperature range for growth is 45–65 °C. The optimum pH for growth is approximately 7·5. Isolated in mixed culture with a *Bacillus* strain from compost in Hiroshima Prefecture, Japan. Established as axenic cultures by adding the culture filtrates of the *Bacillus* strain or other bacterial species. The DNA G + C content is 65·1 mol %. The type strain is strain $T^T$ (= IAM14863$^T$).

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