**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, has been reported and tentatively named *Symbiobacterium thermophilum* strain T. Axenic culture of strain T was recently established by dialysing cultures with the supporting bacterial strains or adding their culture broth. Phylogenetic analysis of strain T, based on the 16S rDNA sequence, was conducted for the validation of *S. thermophilum*. The sequence of strain 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, and one sequence closely related to it, were identified for the first time from soil and compost samples. The outer membrane of strain 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 (= IAM 14863T).

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

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A thermophilic bacterium (strain T) producing tryptophanase and tyrosine-phenyl lyase (*β*-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 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 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, 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 in the culture. Furthermore, the method allowed us to detect limited growth of strain T in the conditioned medium supplemented with the cultured broth not only of the *Bacillus* strain but also of various bacterial...
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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.

Cells from the axenic culture of strain T\(^T\) were subjected to a PCR to amplify its 16S rDNA with universal primers (5'-CGCGGATCAGAAGTTGATCMTGGCTCAG-3' and 5'-CGCGGATCCTACC-TTGTTACGACTTCACCCCAG-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'-CGCGGATCCCAGAAGTTGATCMTGGCTCAG-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

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*.

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11 fragments showing similarity to the sequence of strain T<sup>T</sup>. Nucleotide sequencing elucidated that 10 out of the 11 fragments were almost identical to that from strain T<sup>T</sup> (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<sup>T</sup>. 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<sup>T</sup> 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<sup>T</sup> 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<sup>T</sup> 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<sup>T</sup>. Our previous analysis revealed the presence of the iso-branched chain C<sub>15</sub>:0 and C<sub>17</sub>:0 acids as the major cellular fatty acids of strain T<sup>T</sup> (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<sup>T</sup> (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<sup>T</sup> 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. symbioktos symbiotic; Gr. n. bakterion a small rod; *Symbiobacterium* symbiotic small rods).

Cells are Gram-negative, straight rods with a multi-layered cell surface structure. Non-motile and non-sporulating. Iso-branched chain C<sub>15,10</sub> and C<sub>17,0</sub> 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; *thermo-philum* heat-loving).

Cells are straight rods (0.25–0.35 x 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 supporting *B. brevis* strain or *Bacillus* with a growth is approximately 7 mol%. The type strain is strain T (= IAM148635).

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**References**


