Growth of a Tryptophanase-producing Thermophile, *Symbiobacterium thermophilum* gen. nov., sp. nov., Is Dependent on Co-culture with a *Bacillus* sp.

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An obligately symbiotic bacterium, strain T, associating with a specific strain S of a thermophilic *Bacillus* sp. was isolated during screening for micro-organisms that produce heat-stable tryptophanase and β-tyrosinase. A colony containing both strains was finally isolated by successive selection with bacitracin. In mixed culture with *Bacillus* strain S under low aeration conditions at 60 °C, growth of strain T was initiated following lysis of the *Bacillus* cells. No independent growth of strain T was observed in any of the media tested, even in the presence of other thermophilic *Bacillus* strains or cell-free extracts of strain S or its killed cells. No physical adhesion between cells of strains S and T was observed microscopically. Tryptophanase and β-tyrosinase production by strain T were induced by L-tryptophan and L-tyrosine, respectively. The name *Symbiobacterium thermophilum* gen. nov., sp. nov. is proposed for strain T which is a Gram-negative rod (0·25–0·35 μm diameter) with a multilayer surface structure containing *meso*-diaminopimelic acid. The G+C content of the DNA is 65·1 mol%; iso-$C_{15:0}$, iso-$C_{17:0}$ and anteiso-$C_{17:0}$ acids are the major cellular fatty acids. The type strain is strain IAM 13621.

INTRODUCTION

Association and co-operation between two different micro-organisms leading to growth and expression of some physiological functions have frequently been observed. For example, a *Methanobacterium* sp. associating with another bacterium to produce methane from ethanol (Bryant *et al.*, 1967), and oxidative degradation of polyvinyl alcohol resulting from co-operation between two *Pseudomonas* strains (Shimao *et al.*, 1984). In such associations, each partner can grow independently if appropriate media and conditions are provided, although the association is essential for expression of the metabolic activities stated. On the other hand, in parasitic symbiosis, growth of the symbiont requires obligate association with its partner. Bdellovibrios kill and lyse the Gram-negative bacteria which are essential for their growth (Starr & Huang, 1972) and the bacterium YLM-1 lyses living cells of *Saccharomyces cerevisiae* to acquire catalase, which is essential for its growth (Yamamoto *et al.*, 1987).

We have done screening tests to find thermophilic micro-organisms that produce heat-stable tryptophanase and β-tyrosinase, as possible replacements for the mesophilic bacteria used in enzymic synthesis of L-tryptophan (Nakazawa *et al.*, 1972) and aromatic amino acids (Enei *et al.*, 1973), respectively. Distribution of these two enzymes was thought to be limited almost exclusively to the Enterobacteriaceae (DeMoss & Moser, 1969), but we have found that a mixed culture of thermophiles from compost samples produces these enzymes. Interestingly, this new tryptophanase- and β-tyrosinase-producing bacterium, strain T, only grew in co-culture with a specific thermophilic *Bacillus*, strain S. This paper describes the isolation and properties of this thermophilic strain T, which is named *Symbiobacterium thermophilum* gen. nov., sp. nov., together with the characteristics of its symbiosis with the specific *Bacillus* strain.

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METHODS

Media. Trp-PEP medium, which contains 0·2% L-tryptophan, 0·5% polypeptone (Daigo Chemicals), 0·1% Bacto yeast extract (Difco), 0·3% K_2HPO_4, 0·1% KH_2PO_4, 0·05% $MgSO_4.7H_2O$ and 0·05% pyridoxal 5′-phosphate (Sigma) was used for enrichment and mixed cultures. Tyr-PEP medium, containing 0·2% L-tyrosine instead of L-tryptophan, was used for induction of β-tyrosinase. Trp-PEP agar medium was prepared by addition of 1·5% (w/v) agar.

Independent growth of *S. thermophilum* was tested in Trp-PEP medium supplemented with 0·1% each of malt extract (Difco), tomato-juice (Kagome tomato paste, Kagome Co.) or a vitamin mixture solution which contained biotin (20 μg ml^{-1}), folic acid (20 μg ml^{-1}), riboflavin (5 μg ml^{-1}), thiamin (5 μg ml^{-1}), pantothenic acid, nicotinic acid and thioctic acid (each at 500 μg ml^{-1}), vitamin B_{12} (1 μg ml^{-1}) and pyrroloquinoline quinone (1 μg ml^{-1}; Sigma). Trp-PEP medium, supplemented with 0·1% each of potato or oat meal extracts prepared according to Booth (1971), nutrient broth (Difco), Bacto-tryptone (Difco) and heart infusion broth (Difco) were also examined for their ability to support the growth of *S. thermophilum*. Sucrose (10%, w/v) was added to Trp-PEP medium to test the effect of osmotic stabilization.

Isolation and cultivation of thermophilic bacteria. Approximately 100 mg each of soil, compost or 0·5 ml hot spring water from various locations in Japan was added to 10 ml Trp-PEP medium or Tyr-PEP medium in an L-shaped tube and incubated at 60–70 °C for 24–30 h with gentle shaking at 30 strokes min^{-1}. The presence of tryptophanase-producing organisms in the culture was determined by detecting indole formation with Kovacs' reagent (Smibert & Krieg, 1981). Phenol formation in Tyr-PEP medium was detected with 4-aminoantipirine (Lacoste *et al.*, 1959). A culture derived from compost from Hiroshima Prefecture, which had been shown to produce indole by Kovacs' test, was diluted 10^5-fold and 0·1 ml samples were inoculated into 10 L-shaped tubes containing 10 ml Trp-PEP medium. After 24 h cultivation at 60 °C, three of the 10 subsidiary cultures gave positive indole reactions. A sample (0·1 ml) of the culture with the highest activity was inoculated into 10 ml Trp-PEP medium containing 1 mg bacitracin ml^{-1} (Sigma). After cultivation at 60 °C for 24 h, a sample containing 10^2–10^3 total cells was spread on a nitrocellulose membrane (Toyo TM-2, Toyo Roshi) placed on Trp-PEP agar medium and incubated at 60 °C for 24 h. Colonies were preserved by replica plating and the master plate was sprayed with Kovacs' reagent. Red-stained colonies were transferred into 10 ml Trp-PEP medium and cultured at 60 °C for 24 h. The culture was preserved by lyophilization.

Lyophilized cells of the tryptophanase-producing culture were inoculated into 200 ml Trp-PEP medium in a 500 ml Erlenmeyer flask and cultured at 60 °C for 2 d without shaking. The seed culture was transferred into 1 litre of fresh medium in a 1·5 l jar fermenter (Iwashiya-Sawada Co., model MB-C) and cultured at 60 °C under three different aeration conditions corresponding to mass transfer coefficients (K_{La}) of 67·8, 8·6 and 2·1.

Estimation and direct counting of cell numbers. Since *S. thermophilum* strain T did not form colonies independently, the most probable number of its viable cells in the mixed culture was estimated by the multiple-tube cultivation technique (Taylor, 1962). Five L-shaped tubes, each containing 10 ml Trp-PEP medium, were inoculated with 0·1 ml of appropriately diluted enrichment culture and indole formation was examined after 24 h cultivation at 60 °C. The number of positive tubes was recorded and the most probable number index of tryptophanase-positive cells calculated by reference to statistical tables.

In order to count the number of colony forming units, 0·1 ml of appropriately diluted culture was spread on a membrane filter placed on the surface of Trp-PEP agar medium and incubated at 60 °C for 24 h. The total cell numbers of *S. thermophilum* strain T and *Bacillus* strain S in the mixed culture were determined in a Petroff-Hausser counting chamber by phase contrast microscopy (× 1000 magnification).

Separation of S. thermophilum cells from Bacillus strain S cells. This was done by two-step centrifugation. The mixed culture (10 ml) was first centrifuged at 500 ***g*** for 15 min to sediment the *Bacillus* cells selectively and then centrifuged at 10000 ***g*** for 15 min to pellet the precipitate of *S. thermophilum* cells.

Selective lysis of the *Bacillus* cells by lysozyme was also used for isolation of *S. thermophilum* cells as follows. Cells collected from 10 ml of the mixed culture by centrifugation at 10000 ***g*** for 15 min were suspended in 10 ml 100 mM-sodium phosphate buffer (pH 7·0) containing 0·54 mM-EDTA and 100 mM-trisodium citrate. Lysozyme (Sigma) was added to give a final concentration of 300 μg ml^{-1}. The *Bacillus* cells were selectively lysed during incubation for 15 min at 35 °C and the residual *S. thermophilum* cells were collected by centrifugation at 10000 ***g*** for 15 min.

Nutritional requirements of S. thermophilum. Approximately 1 × 10^8 *S. thermophilum* cells were added to 10 ml of various media in L-shaped tubes, and incubated at 60 °C with gentle shaking. To examine the effect of catalase, bovine catalase (1000 units ml^{-1}; Sigma) was added to Trp-PEP medium at 2–3 h intervals to compensate for heat inactivation. To prepare a cell-free extract of *Bacillus* strain S, cells were collected from 100 ml of an exponentially growing culture, suspended in 10 ml 100 mM-sodium phosphate buffer (pH 7·0), and sonicated. The resulting extract was sterilized by filtration (Corning; 0·22 μm filter) and added aseptically to the medium at a concentration of 10% (v/v). The strain S cells, killed by adding streptomycin (Meiji Seika Kaisha) (100 μg ml^{-1}) to the exponentially growing culture, were collected and washed by successive centrifugation, and added aseptically

to a final concentration of 3×10^8 cells ml^{-1}. Co-cultivation with strains of *Bacillus stearothermophilus*, ATCC 8005 and ATCC 12980, or *Bacillus coagulans*, ATCC 7050 and ATCC 1194, was also attempted; approximately 1×10^8 exponentially growing cells of each of the *Bacillus* strains together with 1×10^8 cells of *S. thermophilum* were inoculated into 10 ml Trp-PEP medium and incubated at 60 °C with gentle shaking. Total counts were used to monitor growth of *S. thermophilum*.

A U-shaped cultivation tube, equipped with a central diaphragm consisting of a nitrocellulose membrane (Millipore; 0·22 μm) was used to determine whether *Bacillus* strain S produced a diffusible growth factor for *S. thermophilum*. Trp-PEP medium (10 ml) was added to both sides of the tube separated by the membrane and *Bacillus* strain S inoculated in one side. After cultivation at 60 °C for 3 h to obtain exponential growth of strain S, approximately 1×10^8 cells of the isolated *S. thermophilum* cells were inoculated in the other side and the tube was further incubated at 60 °C for 24 h with shaking at 30 strokes min^{-1}.

Indole assay. Indole accumulated in the culture media was determined by the method of Kupfer & Atkinson (1964).

Determination of mol% G+C. DNA from strains T and S was isolated by the method of Saito & Miura (1963) and the mol% G+C contents determined by the T_m method (Marmur & Doty, 1962) or by liquid chromatographic analysis of acid hydrolysates (Ko *et al.*, 1977).

Analysis of diaminopimelic acid. The isomeric types and contents of diaminopimelic acid in peptidoglycans of *S. thermophilum* strain T were determined in a whole cell lysate by thin-layer chromatography, according to the method of Staneck & Roberts (1979). The relative content of diaminopimelic acid was determined by comparing the density of the spot in the chromatogram with that from the same amount of cells of *Bacillus subtilis* Marburg strain IFO 13719, using a scanning densitometer.

Analysis of fatty acid contents. Cellular fatty acids were analysed by gas chromatography according to the method of Suzuki *et al.* (1981).

Electron microscopy. Cells of strains S and T were fixed with glutaraldehyde and stained using the method of Ryter & Kellenberg (1958). Ultrathin sections were observed with a transmission electron microscope (JEM JEOL 200CX).

Biochemical tests. Nitrate reducing activity and other biochemical properties (items listed in Table 2) of the pure culture of strain S and the mixed culture of strains T and S were examined according to the methods described by Holding & Collee (1971).

Tryptophanase and β-tyrosinase assays. About 10 g wet wt cells of *S. thermophilum* strain T, obtained from the mixed culture with *Bacillus* strain S in Trp-PEP medium or Tyr-PEP medium in a jar fermenter with aeration of $K_{La} = 8{\cdot}6$, were disrupted by grinding with 20 g alumina and extracted with 15 ml 50 mM-potassium phosphate buffer (pH 6·5) containing 100 μM-pyridoxal 5′-phosphate (Sigma), 1 mM-2-mercaptoethanol and 250 μM-phenylmethylsulphonyl fluoride. Tryptophanase activity in the crude extract was assayed by measuring indole formation from L-tryptophan at 65 °C (McEvoy-Bowe, 1963). One unit of activity was defined as the amount of the enzyme that produced 1 μmol indole min^{-1}. β-Tyrosinase activity was assayed by determining the amount of pyruvate formed from L-tyrosine at 65 °C (Friedmann & Haugen, 1943). One unit of activity was defined as the amount of the enzyme that produced 1 μmol pyruvate min^{-1}.

RESULTS

Enrichment culture of a tryptophanase-producing thermophile

Distinct accumulation of indole and phenol was observed in several cultures from compost samples. Inoculation of the cultures into fresh Trp-PEP medium reproducibly gave indole-forming cultures, which suggested the presence of viable tryptophanase-positive organisms. However, no single tryptophanase-positive colony was detected by plating these cultures on Trp-PEP agar. Therefore, the number of tryptophanase-positive organisms in the culture was estimated by the multiple-tube cultivation technique with diluted samples. The most probable number (MPN) of tryptophanase-positive organisms in one of the original cultures obtained from Hiroshima Prefecture was estimated to be $1{\cdot}6 \times 10^6$ cells ml^{-1}, while the total number of colony-forming units (c.f.u.) was approximately 5×10^8 ml^{-1}. To enrich the tryptophanase-positive organisms, the original culture was diluted and transferred to multiple tubes containing Trp-PEP medium. One of the resulting test-tube cultures was estimated to contain $2{\cdot}4 \times 10^7$ tryptophanase-positive cells ml^{-1} in a total of $1{\cdot}04 \times 10^9$ bacterial cells ml^{-1} (2·3% enrichment).

To achieve further enrichment, selection by various antibacterial drugs was examined: selection by bacitracin was most successful. The 2·3% enrichment culture described above was inoculated into Trp-PEP medium containing 1 mg bacitracin ml^{-1} and cultured at 60 °C for

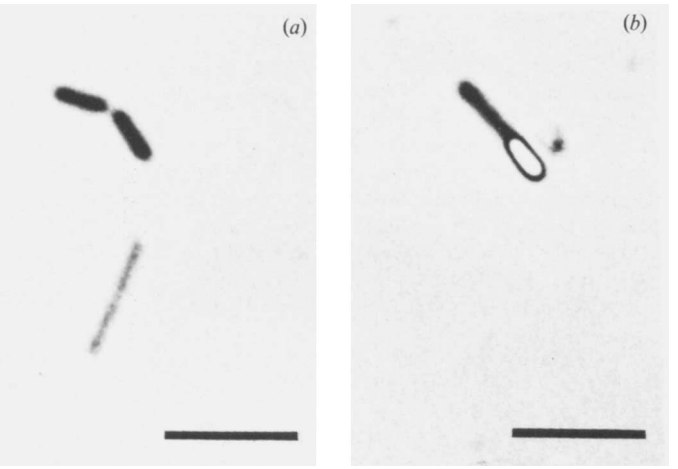

Fig. 1. Phase-contrast photomicrographs of *S. thermophilum* strain T and *Bacillus* strain S. (*a*) Thin cell of *S. thermophilum* with a dividing *Bacillus* strain S cell. (*b*) *Bacillus* strain S forming a prespore and a mature spore. Bar, 10 μm

24 h. The culture was estimated to contain $1{\cdot}8 \times 10^9$ tryptophanase-positive cells ml^{-1} in a total of $3{\cdot}3 \times 10^9$ cells ml^{-1} (55% enrichment). Approximately 1% of the colonies subsequently obtained on membrane cultures on agar gave a distinct positive reaction when tested with Kovacs' reagent. Microscopic observation of the tryptophanase-positive colonies revealed that each was a mixture of two different cell morphologies: a small rod-shaped cell, tentatively designated strain T, and a large rod-shaped cell, designated strain S (Fig. 1*a*). All the tryptophanase-negative colonies were composed of only one cell type, corresponding to strain S. When a mixed colony containing both strains T and S was inoculated in Trp-PEP medium and incubated at 60 °C for 24 h, the liquid culture gave a distinct positive reaction for the presence of indole. Production of phenol from L-tyrosine, indicative of β-tyrosinase activity, was also observed with the mixed colony grown in Tyr-PEP medium. The culture inoculated with a pure colony of strain T was negative for both tryptophanase and β-tyrosinase activities. Lyophilized samples of the mixed culture could be stored for at least 1 year at 4 °C without significant loss of viability.

Growth profiles in mixed culture containing strains T and S

Growth and indole formation were determined in mixed cultures in Trp-PEP medium grown under different aeration conditions. With relatively low aeration ($K_{La} = 8{\cdot}6$), strain S began to grow exponentially and then lysed with formation of visible aggregates of cell debris at the late exponential phase (Fig. 2*a*). Growth of strain T was initiated simultaneously with lysis of strain S, and then a gradual increase in cell number and accumulation of indole in the medium were observed. Most of the residual cells of strain S showed a terminal bulge indicating the presporulation stage or contained refractile endospores at the polar position (Fig. 1*b*). After 24 h cultivation, the number of strain S cells decreased markedly, while the number of strain T cells reached approximately $1{\cdot}1 \times 10^{10}$ ml^{-1}; at this point an almost pure culture of strain T, containing less than 0·1% of strain S, was obtained. In contrast, no lysis of strain S was observed in pure culture under the same conditions (Fig. 2*a*) and only 2–3% of total cells formed spores, even after 24 h cultivation. The cell yield in the pure culture of strain S was distinctly higher than in the mixed culture. No indole accumulation was detected in the pure culture of strain S.

Mixed cultures with lower aeration ($K_{La} = 2{\cdot}1$) at 60 °C gave an essentially similar profile (Fig. 2*b*) to that described above, while no distinct lysis of strain S along with slower growth of strain T was observed at the higher aeration value ($K_{La} = 67{\cdot}8$) (Fig. 2*c*). In cultures incubated

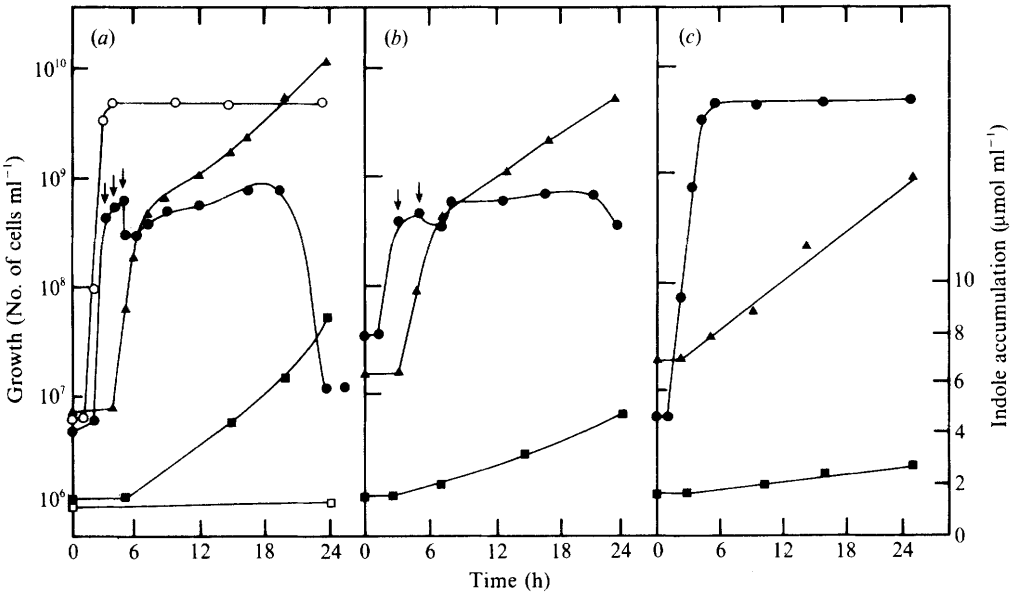

Fig. 2. Growth profile of *S. thermophilum* strain T in mixed culture with *Bacillus* strain S under different aeration conditions. The mixed population was inoculated into Trp-PEP medium and incubated at 60 °C with K_{La} = 8·6 (*a*), 2·1 (*b*) and 67·8 (*c*). Cell numbers of strain T (▲) and strain S (●) and indole formation (■) were measured in the mixed culture. *Bacillus* strain S was also grown in pure culture with aeration (K_{La}) = 8·6 (*a*), and cell numbers (○) and indole formation (□) were measured. Arrows indicate the points where visible aggregation of cell debris was observed.

at 50 °C under these three different aeration conditions, strain S became dominant and almost no growth of strain T occurred (data not shown). These different patterns of growth in the mixed cultures corresponding to changes in aeration and temperature were reproducibly obtained using seed cultures from a mixed colony of strains T and S.

Separation of strain T cells from the mixed culture, using the marked difference in cell size, was possible by a two-step process of centrifugation. In addition, strain S cells were selectively lysed by treatment with lysozyme in the presence of EDTA. These procedures allowed the isolation of strain T cells from the mixed culture.

Cell-free extracts of strain T exhibited strong tryptophanase activity [0·4–0·5 units (mg protein)$^{-1}$], but no β-tyrosinase activity was detected in the extract of cells grown in Trp-PEP medium. In contrast, distinct β-tyrosinase activity [approximately 0·02 units (mg protein)$^{-1}$], but little tryptophanase activity, was observed with the extract of cells grown in Tyr-PEP medium.

Description of strain T

The most remarkable characteristic of strain T is its obligate requirement for the presence of growing strain S cells. No independent growth was observed in any of the media tested. Furthermore, the addition of a vitamin mixture containing pyrroloquinoline quinone, catalase and osmotic stabilizer had no effect. Addition of cell-free extracts of strain S and streptomycin-killed strain S cells could not support independent growth of strain T. When isolated living cells of strain T were inoculated into Trp-PEP medium separated with a nitrocellulose membrane in a U-shaped tube from growing cells of strain S, no independent growth of strain T was observed. These results seemed to exclude the possibility that diffusible factors or nutrients provided by strain S were supporting growth of strain T. Nutritional feeding by direct contact between strain T and S cells can also be excluded, since no such contact was observed microscopically.

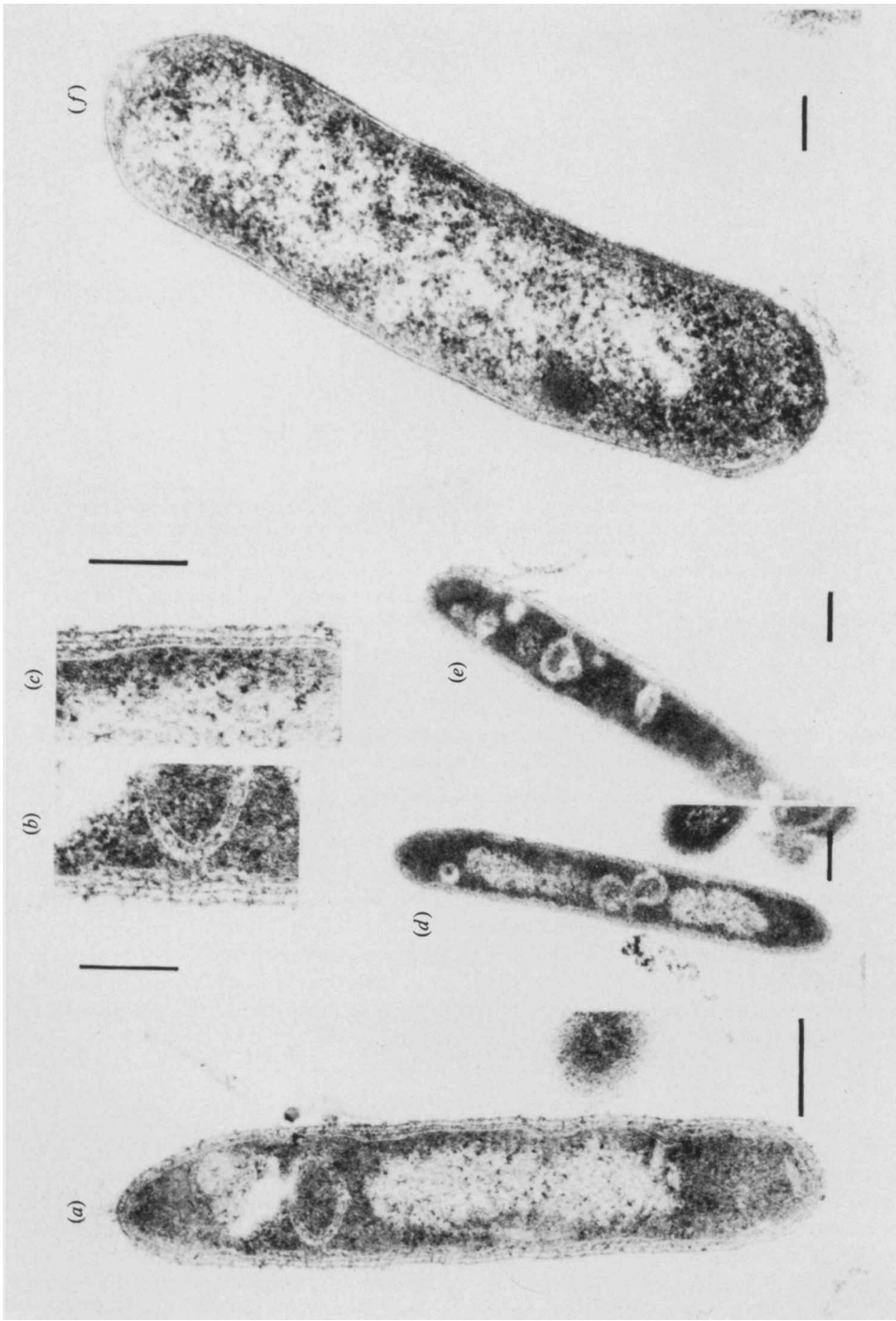

Fig. 3. Electron micrographs of ultrathin sections of *S. thermophilum* strain T and *Bacillus* strain S. (*a*, *d* and *e*) Cells of strain T with various forms of inner membrane invagination. (*b* and *c*) Enlarged profiles of surface structures from (*a*). (*f*) Cell of *Bacillus* strain S. Bars in (*a*), (*d*), (*e*) and (*f*), 0·2 μm; bars in (*b*) and (*c*), 0·1 μm.

Table 1. *Fatty acid composition of S. thermophilum strain T and Bacillus strain S*

ND, Not detected.

Fatty acids	Percentage composition	
	S. thermophilum strain T	*Bacillus* strain S
n-$C_{15:0}$	ND	8·0
n-$C_{16:0}$	2·0	1·6
iso-$C_{15:0}$	39·5	36·3
iso-$C_{16:0}$	3·7	23·0
iso-$C_{17:0}$	30·5	20·6
anteiso-$C_{15:0}$	5·9	2·3
antieso-$C_{17:0}$	18·4	8·2

Table 2. *Properties of Bacillus strain S and related Bacillus strains*

Data for *B. coagulans* and *B. stearothermophilus* are from Gordon *et al.* (1973): d, reactions differ.

	Bacillus strain S	*B. coagulans*	*B. stearothermophilus*
Cell size (μm)	0·6–0·9 × 2·5–6·0	0·6–1·0 × 2·5–5·0	0·6–1·0 × 2–3·5
Spore	Cylindrical, subterminal	Ellipsoidal or cylindrical, subterminal	Ellipsoidal, subterminal
Gram-reaction	+	+	d
Motility	+	+	d
Catalase	+	+	d
Anaerobic growth	+	+	−
Voges–Proskauer reaction	−	+	−
Methyl red reaction in Voges–Proskauer broth	+	d	−
Growth temperature:			
Maximum (°C)	66	55–60	65–70
Minimum (°C)	40	15–25	30–45
Growth:			
At pH 5·7	+	+	d
In 0·02% azide	+	+	−
Acid from:			
Glucose	+	+	+
Arabinose	−	d	d
Xylose	−	d	d
Mannitol	−	d	d
Hydrolysis of starch	+	+	+
NO_2^- from NO_3^-	−	d	d
G + C (mol%)	53·3	45·4–56·0	46·0–52·0

On the basis of these observations, we suggest that strain T is a novel strictly symbiotic bacterium which cannot be identified as a member of any of the genera described in *Bergey's Manual of Determinative Bacteriology* (Buchanan & Gibbons, 1974) or *Bergey's Manual of Systematic Bacteriology* (Krieg, 1984; Sneath, 1986). Therefore, we propose the name *Symbiobacterium thermophilum* gen. nov., sp. nov. Although axenic culture of this bacterium has thus far proved impossible, reproducible isolation of the living cells in an almost pure state has been achieved and this fulfils the requirements for description of a type strain according to the International Code of Nomenclature of Bacteria (Lapage *et al.*, 1975). Electron-micrographs of ultrathin sections show that the cell surface structure is composed of multiple layers (Fig. 3*a*, *b*, *c*) and is distinctly different from the Gram-positive type structure of strain S (Fig. 3*f*). The innermost layer of strain T frequently forms invaginations (Fig. 3*a*, *d*, *e*). Analysis of cellular fatty acids shows that the main components are iso-C_{15}, iso-C_{17} and anteiso-C_{17} saturated acids (Table 1).

Properties of strain S

Strain S is an aerobic spore-former which can grow independently in nutrient broth up to 66 °C. Cylindrical endospores are formed at the polar position in swollen sporangia (Fig. 1*b*). Its physiological properties (Table 2) are intermediate between *B. stearothermophilus* and *B. coagulans* (Gordon *et al.*, 1973). Several type culture strains of these species, i.e. *B. stearothermophilus* ATCC 8005 and ATCC 12980, and *B. coagulans* ATCC 7050 and ATCC 1194, failed to support growth of strain T in place of strain S in mixed culture. Iso-C_{15}, iso-C_{16} and iso-C_{17} saturated fatty acids are the major components of cellular fatty acids while anteiso-C_{15} acid is a minor component (Table 1). The DNA base composition is 53·3 mol% G+C as determined by liquid chromatographic assay of the acid hydrolysate. The type strain is strain IAM 13622 (culture collection of the Institute of Applied Microbiology, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan).

DISCUSSION

Growth of *S. thermophilum* obligately requires co-culture with *Bacillus* strain S. Such an obligate requirement is also observed in the parasitic symbiosis of bdellovibrios with Gram-negative bacteria; however, *S. thermophilum* cells do not exhibit direct contact with the host cells, as observed with bdellovibrios. Ectosymbiosis without direct contact of the partner cells suggests feeding of diffusible nutrients or effectors required by one or both partners. Recently, co-operation between two different pseudomonads in oxidative degradation of polyvinyl alcohol has been explained in terms of the supply of pyrroloquinoline quinone by one cell to the other (Shimao *et al.*, 1984). The obligate requirement of living yeast cells for growth of the bacterium YLM-1 can be replaced by adding catalase to the medium (Yamamoto *et al.*, 1987). In mixed culture of *S. thermophilum* with *Bacillus* strain S under low aeration conditions, lysis of strain S occurs concomitantly with initiation of growth of *S. thermophilum*. In the following stage, growth of strain S appeared to be in balance with lysis, which could support further growth of *S. thermophilum*. No distinct lysis of strain S was observed in mixed culture with high aeration, although partial lysis of strain S could also occur to support slower growth of *S. thermophilum*. It seems probable that *S. thermophilum* requires some diffusible substances derived from lysis of *Bacillus* strain S cells. However, none of the nutrients or additives examined supported independent growth of *S. thermophilum*. All attempts to detect diffusible factors in the cells or culture media of *Bacillus* strain S have thus far failed. A possible substance responsible for the ectosymbiosis of *S. thermophilum* with *Bacillus* strain S clearly requires future investigation. The formal proposal and description of *S. thermophilum* are as follows.

Symbiobacterium gen. nov.

Sym.bio.bac.te'.ri.um; Gr. adj. *symbiotikos* symbiotic; Gr. n. *bakterion* a small rod; L. n. *Symbiobacterium* a symbiotic bacterium.

Obligate symbiont requiring co-culture with a specific *Bacillus* strain under conditions of relatively low aeration and at around 60 °C. Cells are small rods with a multi-layered cell surface structure. Non-motile and non-sporulating. The G+C content of the DNA is 65·1 mol% by the T_m method. The type species is *Symbiobacterium thermophilum*.

Symbiobacterium thermophilum sp. nov.

Ther.mo.phil'.um; Gr. n. *therme* heat; L. adj. *philum* loving; L. n. *thermophilum* heat-loving. Cells are small rods, 0·25 to 0·35 μm in diameter and 1·5 to 7 μm in length, occurring singly or in pairs. Gram-staining is negative, and analysis of diaminopimelic acid shows the presence of the *meso*-isomer, the relative content of which is one-sixth that in *B. subtilis*.

Aerobic, but does not grow independently in any medium tested, obligately requiring co-culture with a specific thermophilic *Bacillus* strain S. Growth temperature range in mixed culture is 55 to 65 °C. Indole production positive, inducible tryptophanase and β-tyrosinase activity. Almost no other physiological activities can be identified due to the absence of independent growth, but nitrate reduction in the mixed culture appears to be due to *S.*

thermophilum, since pure culture of the supporting *Bacillus* strain S lacks nitrate reductase activity.

Isolated in mixed culture with a *Bacillus* strain S from compost in Hiroshima Prefecture, Japan, and separated from the latter by fractional centrifugation or treatment with lysozyme and EDTA.

The DNA base composition is 65·1 mol% G+C.

Holo Type strain: the separated cells of *Symbiobacterium thermophilum* strain T (= IAM 13621).

Bacillus strain S is a thermophilic *Bacillus* species which possesses physiological properties intermediate between *B. stearothermophilus* and *B. coagulans*. The composition of cellular fatty acids in strain S is similar to that of *B. stearothermophilus*, but different from that of *Bacillus subtilis* (Kaneda, 1977). The taxonomic position of strain S in the genus *Bacillus* also requires future investigation.

In general, isolation of a specific pair of obligate symbionts from nature is extremely difficult. During this work, accidental discovery of the effectiveness of bacitracin for selection of such a pair enabled us to overcome this difficulty. In addition, plating on a membrane filter on the surface of agar medium facilitated colony formation at high temperatures and a mixed colony thus emerged from cells of the two strains in contact with each other on the membrane. Although no established microbiological technique for isolating such obligate symbionts is yet available, a wide distribution of this type of symbiosis in nature seems highly probable.

Tryptophanase and β-tyrosinase from Enterobacteriaceae can be used for enzymic synthesis of L-tryptophan and some aromatic amino acids, respectively, through reverse reactions (Nakazawa *et al.*, 1972; Enei *et al.*, 1973). The enzymes of *S. thermophilum* are potentially useful due to their higher heat-stability, which we will report elsewhere.

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