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A novel microbial interaction: obligate commensalism between a new gram-negative thermophile and a thermophilic *Bacillus* strain

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Abstract Obligately commensal interaction between a new gram-negative thermophile and a thermophilic *Bacillus* strain was investigated. From compost samples, a mixed culture showing tyrosine phenol-lyase activity was enriched at 60°C. The mixed culture consisted of a thermophilic gram-negative strain, SC-1, and a gram-positive spore-forming strain, SK-1. In mixed cultures, strain SC-1 started to grow only when strain SK-1 entered the stationary phase. Although strain SC-1 showed tyrosine phenol lyase activity, we could not isolate a colony with any nutrient medium. For the isolation and cultivation of strain SC-1, we added culture supernatant and cell extract of the mixed culture to the basal medium. The supernatant and cell extract of the mixed culture contained heat-stable and heat-labile factors, respectively, that are essential to the growth of strain SC-1. During pure cultures of strain SK-1, the heat-stable growth factors were released during the growth phase and the heat-labile growth factors were produced intracellularly at the early stationary phase. Strain SC-1 was gram-negative and microaerophilic, and grows optimally at 60°C. Based on these results, we propose a novel commensal interaction between a new gram-negative thermophile, strain SC-1, and *Bacillus* sp. strain SK-1.

Key words Gram-negative thermophile · Tyrosine phenol-lyase · Commensalism · Growth factors

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Introduction

The extremophilic nature of many bacteria has stimulated intense efforts to probe the potential biotechnological applications of their stable enzymes. Large numbers of new thermophilic bacteria have been isolated in recent years (Stetter 1996). However, because of our inability to understand and reproduce real microenvironmental niches, only a small fraction of the bacterial communities can be cultivated by current techniques (Lee et al. 1996; Ward et al. 1998).

It is difficult to isolate some microorganisms because of their interaction with other organisms (e.g., in symbiotic relationships) that influence naturally occurring bacteria. For example, growth of the bacterial parasite *Bdellovibrio* requires a gram-negative bacteria host (Ruby 1992). Syntrophic bacteria can grow only when other bacteria remove hydrogen, the metabolic product of syntrophic bacteria (Mcinerney 1992).

In our laboratory, we have detected a new thermophilic gram-negative bacterium, strain SC-1, during the screening of thermostable tyrosine phenol-lyase-producing thermophiles. The thermostable biocatalyst of strain SC-1 has been characterized and reported previously (Lee et al. 1997, 1999). However, because strain SC-1 obligatorily requires essential growth factors from *Bacillus* sp. strain SK-1, we could not separate strain SC-1 from strain SK-1 and obtain a single colony. This type of bacterial association was reported previously by Suzuki et al. (1988). Recently, by using a dialyzing culture vessel, independent growth of *Symbiobacterium thermophilum* was confirmed (Ohno et al. 1999).

We have isolated a single colony of strain SC-1 from the mixed culture of strain SC-1 and SK-1 and cultivated strain SC-1 independently. For the isolation and cultivation of strain SC-1, we used the crude extract and culture supernatant of its partner (*Bacillus* sp. strain SK-1) as growth factors. In this article, we describe the isolation of strain SC-1 from the mixed culture and the characterization of the interaction between the two microorganisms.

Materials and methods

Collection of samples and enrichment

To initiate enrichment cultures of tyrosine phenol lyase-producing microorganisms, a basal medium (BM) was used. The BM contained 5 g polypeptone, 1 g yeast extract, 6 g K_2HPO_4, 2 g KH_2PO_4, 0.5 g $MgSO_27{\cdot}H_2O$, and 0.5 g of L-tyrosine per liter of deionized water. Enrichment cultures were initiated by inoculating BM with 1% compost samples. The samples were collected from compost yards in Kongjoo, Korea. The cultures were performed in 500-ml Erlenmeyer flasks with 200 ml BM at 60°C in a gyratory shaker. After 2 weeks of incubation, phenol, as a product of tyrosine phenol-lyase, was detected in the culture broth with Ehrlich's reagent (Yokota et al. 1989). Two milliliters of the culture showing phenol-positive reaction were transferred to 200 ml fresh BM three times. The phenol was fully accumulated after each transfer.

We tried to isolate the tyrosine phenol-lyase-producing microorganisms by using an endpoint dilution technique. The most diluted tubes showing phenol-positive reaction were transferred three times using the process of endpoint dilution. After three transfers, we always observed two kinds of cells in the phenol-positive cultures.

Isolation of strains from the enrichment culture

A single colony of strain SK-1 was isolated by spreading the enrichment cultures onto BM agar plates. No colony of the other strain, SC-1, was detected on the BM agar plates. To isolate a single colony of strain SC-1, we used modified BM (MBM) containing the cell extract and culture supernatant of the mixed cultures and 10 mM nitrate. The culture supernatant was filter-sterilized and added in a 1:1 ratio by volume with BM. Cell extracts of centrifuged pellets were added after filter-sterilization. Endpoint diluted cultures spread onto the MBM agar plates and were incubated under an anoxic atmosphere (see Section on cultivation). The colonies that appeared were transferred to new MBM plates. A single colony was transferred into liquid MBM to check purity and confirm its ability to produce tyrosine phenol-lyase. For maintenance, the pure culture was stored in a glycerol-MBM medium (50:50 v/v) mixture at −20°C.

Light microscopy and electron microscopy

The shapes and sizes of living and stained cells were determined by light microscopy. The Gram reaction was determined by light microscopy after cells were treated according to the method described by Bartholomew (1962). Physical contact between strains SC-1 and SK-1 was monitored by light microscopy during cultures. For transmission electron microscopy, samples were fixed with glutaraldehyde and platinum-shadowed. Thin sections were obtained according to the method described by Miroshnichenko et al. (1989).

Determination of growth-inhibitory activity of strain SC-1 on strain SK-1

Growth-inhibiting activity of culture broth of the strain SC-1 on strain SK-1 was tested according to the paper disc method described by Satoh et al. (1996). Culture supernatants of mixed cultures and pure cultures of strain SC-1 were tested. Filter papers that absorbed 200 µl culture supernatants were overlaid onto the BM agar plate spread with strain SK-1. The plates were incubated overnight at 60°C, and a clear zone was observed around the filter paper.

Cultivation

Growth of strain SK-1 was monitored with a spectrophotometer and viable cell counting. Cell growth of strain SC-1 was monitored by the following two methods. First, growth of strain SC-1 was determined by direct cell counts using a hemacytometer (Superior, Marienfeld, Germany) and light microscopy. Second, in nitrate-reducing conditions, the growth of strain SC-1 was monitored by determining the nitrite accumulated. Nitrite was determined with the colorimetric method described by Hanson and Phillips (1981).

For cultivation under anoxic atmosphere conditions (CO_2/N_2, 10:90 v/v), MBM plates were incubated in an anaerobic jar (Difco, Detroit, MI, USA) with the anaerobic system and palladium catalysts (Difco). The Anaerobic jar was incubated at 60°C. For cultivation under microaerobic conditions ($CO_2/N_2/O_2$, 10:85:5 v/v), the *Campylobacter* microaerophilic system (Difco) was used instead of the Anaerobic system. For liquid cultures under anoxic conditions, the cultures were grown in serum bottles with a butyl rubber stopper and aluminum crimp seals. After the bottles were sealed, the headspace of the serum bottles was filled with sterile N_2 gas. The bottles were incubated at 60°C with intermittent shaking.

Growth factors

The supernatants and cell extracts of *Bacillus* sp. SK-1 contained essential growth factors for strain SC-1. To determine the growth-stimulating activity of cell extract and culture supernatant of strain SK-1 during batch cultures, the culture broth samples were collected as time courses. The collected samples were centrifuged at 5000 × *g* for 15 min and divided into culture supernatant and cell pellets. The cell pellets were washed with 20 mM phosphate buffer (pH 7.6) and broken with an ultrasonicator (Branson Ultrasonics, Danbury, CT, USA). The crude extracts were centrifuged at 10000 × *g* for 30 min and the supernatant was used as cell extract. To determine the activity of the culture supernatant, the culture supernatants prepared as time courses were autoclaved and added to the BM containing active cell extract. Conversely, for determination of the activity of cell extract, various cell extracts were filter-sterilized and added to the BM containing active culture

supernatant. For the bioassay of growth factors, strain SC-1 was inoculated into the prepared assay medium and incubated under an anoxic atmosphere. Growth was determined by measuring nitrite accumulated during nitrate respiration.

The production of growth factors in other cells was tested. Culture supernatants and cell extracts of the following cells were tested as a source of growth-stimulating factors: *Escherichia coli* BL21, *Bacillus subtilis* ATCC6051, *Bacillus thermoglucosidasius* DSM 2542, *Bacillus* sp. strain KLS01 (growth optimum, 55°C), *Thermus thermophilus* HB8, *Saccharomyces cerevisiae*, and CHO (Chinese hamster ovary) cells. All the cells were harvested at the early stationary phase, and cell extracts and culture supernatants were prepared as already described.

Results

Enrichment of thermostable tyrosine phenol lyase-producing mixed cultures

The enrichment of tyrosine phenol lyase-producing thermophiles was attempted with composts at 60°C. Initially, a culture showing a phenol-positive reaction was detected after 1 week of incubation with various composts. After successive transfers, the activity was maintained. To isolate a pure culture, the enrichment broth was serially diluted in BM and incubated. The endpoint diluted culture showing tyrosine phenol lyase activity always had two types of bacterial strains, strain SC-1 and strain SK-1. However, only strain SK-1 appeared on the BM plates, and it had no tyrosine phenol lyase activity. We could not isolate strain SC-1 from the mixed cultures.

The mixed culture of strain SC-1 and SK-1 and production of phenol were monitored in a batch culture (Fig. 1). When strain SK-1 entered the stationary phase, strain SC-1 started to grow with the production of phenol. Under our cultivation conditions, when *Bacillus* sp. strain SK-1 entered the stationary phase, we could not observe endospores, and cells of *Bacillus* were lysed significantly during the stationary phase.

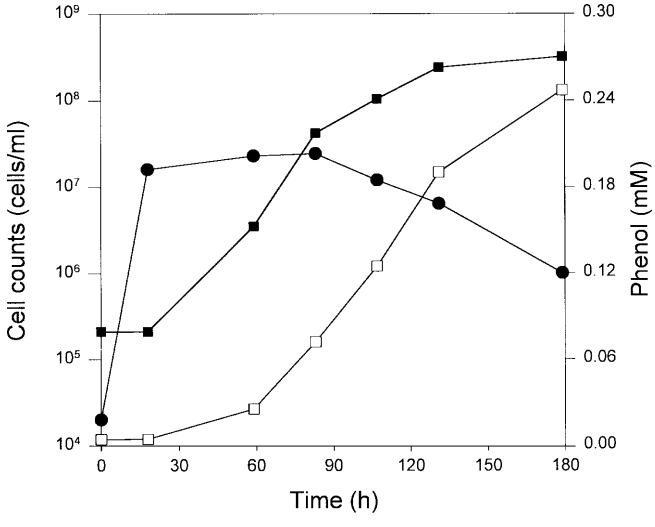

Fig. 1. Mixed culture of strains SC-1 and SK-1. *Solid squares*, strain SC-1; *solid circles*, strain SK-1; *open squares*, phenol concentration

Pure culture of strain SC-1

No colony of strain SC-1 was detected on the plates of any nutrient media used. When supernatants and cell extracts of pellets from mixed cultures were supplemented to BM (called MBM), microcolonies (<0.1 mm in diameter) were detected on the MBM agar after 3 days of incubation under anoxic conditions. However, under aerobic conditions, detection of the microcolony was difficult even by microscopic observation (with 40× magnification). The growth yield in nitrate-reducing conditions was more than 10 times higher than that in aerobic conditions. However, the growth yield of pure cultures was significantly lower than that of mixed cultures (<25%).

Morphological and physiological characteristics

Strain SK-1 had typical characteristics of *Bacillus*. Strain SK-1 was aerobic, motile, spore-forming, gram-positive, 0.5 μm in diameter, and 2–5 μm long. Growth was observed from 35° to 65°C.

The colony of strain SC-1 was translucent. Strain SC-1 was rod shaped, gram negative, 0.2–0.3 μm in diameter, and 1–5 μm long. Optimal temperature and pH for the growth were about 60°C and 7.5, respectively. We could detect colonies of strain SC-1 on the MBM plates incubated under microaerobic conditions and nitrate-reducing conditions. We confirmed that strain SC-1 could grow in nitrate-reducing conditions with the accumulation of stoichiometric amounts of nitrite, and no N_2 gas was produced. Electron photomicrographs of ultrathin sections showed that strain SC-1 and SK-1 had no special morphology or surface structures such as multilayers or invagination (Fig. 2).

The association between strain SC-1 and SK-1 could be maintained under anoxic conditions because they were able to grow in nitrate-reducing conditions. When a minimal medium containing glucose as the sole carbon source was used, only strain SK-1 grew. Because strain SC-1 showed a low growth yield at MBM and was unable to grow in minimal media, we were not able to determine the general physiological characteristics.

Growth factors

Although we used both culture supernatant and cell extract of mixed cultures for the isolation of strain SC-1, we could cultivate strain SC-1 with only culture supernatant. When the culture supernatant was autoclaved, the growth-stimulating activity disappeared. The growth-stimulating activity recovered if we supplemented cell extract from the mixed culture. However, the cell extract alone has no growth-stimulating activity on strain SC-1. Therefore, at

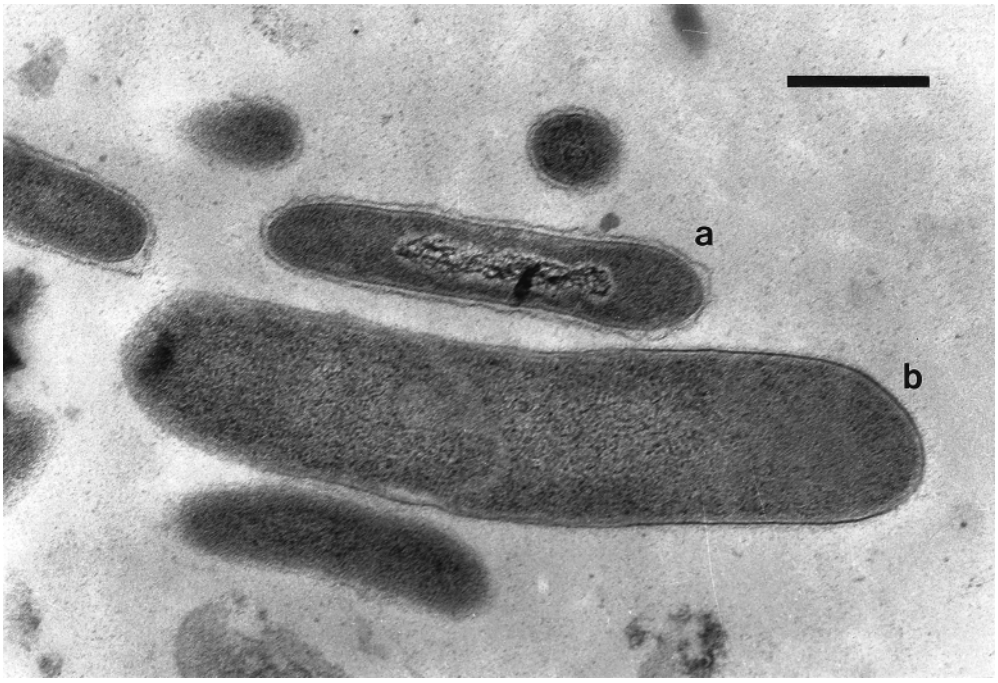

Fig. 2. Transmission electron photomicrographs of cells from mixed culture show strain SC-1 (*a*) and strain SK-1 (*b*). *Bar* 0.5 μm

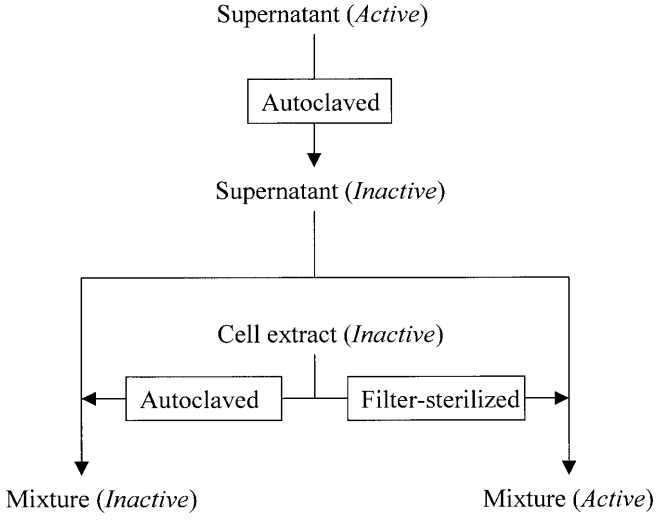

Fig. 3. Identification scheme of proposed growth factors. *Active*, growth stimulation on strain SC-1; *Inactive*, no activity of growth stimulation on strain SC-1

least two factors in the supernatant were necessary for the growth of strain SC-1 as described in Fig. 3. The growth factors present in the supernatant and cell extract were designated as heat-labile and heat-stable growth factors, respectively.

The factors were also produced in the pure culture of strain SK-1. Cell growth of strain SK-1 in the pure culture and mixed culture is shown in Fig. 4a. As shown in Fig. 4b, the extracellular heat-stable factors were released during the growth phase of SK-1 and the intracellular heat-labile factors were produced in the early stationary phase. Therefore, the growth of strain SC-1 was stimulated by the samples prepared only at the overlapping period of production of heat-stable and heat-labile factors (during about 30 h) (Fig. 4b). In the mixed cultures, growth-stimulating activity of samples on strain SC-1 could be sustained for about 100 h during the stationary phase of strain SK-1 (Fig. 4c).

We tested the production of growth factors in other cells. All the eubacterial cells tested produced growth factors. When the growth factors of thermophiles were used, strain SC-1 had typical rod-shaped morphology. However, when growth factors of mesophiles such as *E. coli* and *B. subtilits* were used, filamentous growth of strain SC-1 was observed. Eukaryotic cells such as yeast and animal cells could not produce factors for growth of strain SC-1. All other additives such as vitamins and trace elements had no significant growth-stimulating activities on strain SC-1.

Influence of strain SC-1 on the growth of strain SK-1

Growth of strain SK-1 was not significantly affected by strain SC-1 during the mixed cultures (see Fig. 4a). When paper disks containing the culture supernatant of strain SC-1 and mixed culture were added to strain SK-1-spread agar plates, no clear zones were observed around the disks. Furthermore, we did not observe any physical contact between strain SC-1 and SK-1 during batch cultures by microscopic observation.

Discussion

A novel gram-negative thermophile, strain SC-1, was isolated from compost. Growth of strain SC-1 was obligatorily

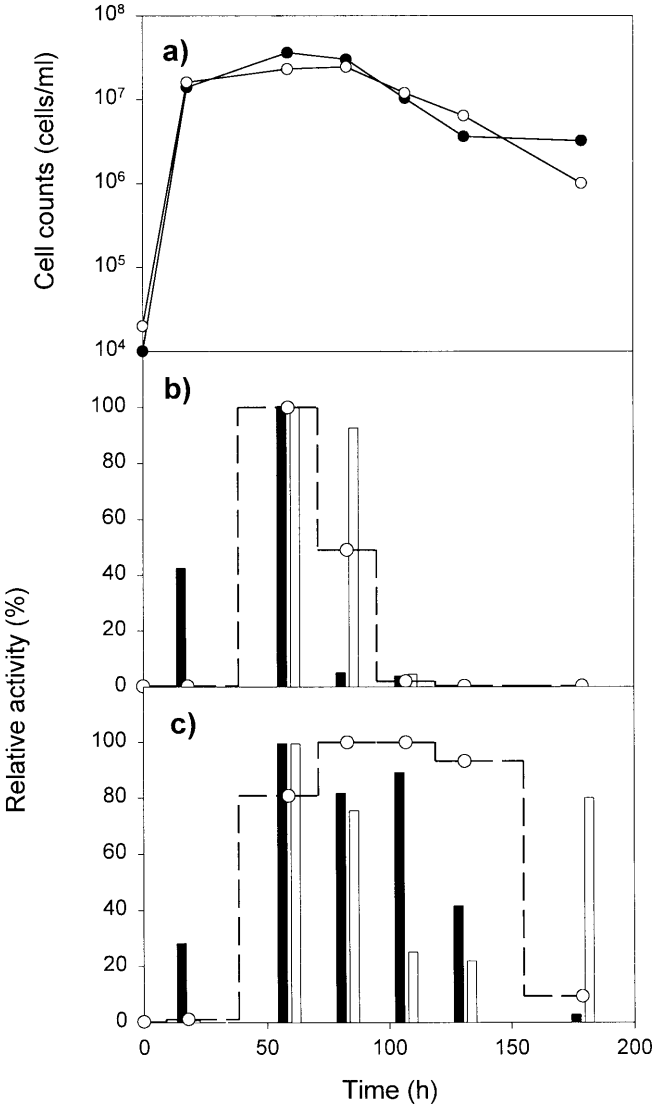

Fig. 4a–d. Production profile of growth factors during the batch culture of strain SK-1 and mixed culture. **a** Growth curve of strain SK-1 in pure (*solid circles*) and mixed cultures (*open circles*). **b,c** Activity of growth factors in pure culture and mixed cultures of strain SK-1, respectively. *Black bars*, activity of heat-stable factors; *white bars*, activity of heat-labile factors

dependent on the growth of *Bacillus* sp. strain SK-1. Considering that strain SC-1 has industrially important thermostable enzymes such as tyrosine phenol-lyase (Lee et al. 1997, 1999), strain SC-1 has potential for use in biotechnology. Description of the isolation and characterization of strain SC-1 and the interaction between the two bacterial strains follows.

During enrichment for the isolation of thermophiles producing tyrosine phenol-lyase at 60°C, we found mixed cultures of strain SC-1 and SK-1. Growth of strain SC-1 was strictly dependent on the growth of strain SK-1, and we did not observe any colonies of strain SC-1 with various culture media available. These results imply that when SK-1 enters the stationary phase, the conditions of the media might be changed to permit the growth of strain SC-1.

According to the characteristics of SK-1, we identified it as *Bacillus* sp. strain SK-1. We could not detect any special characteristics related to associations with strain SC-1. Identification of strain SC-1 by conventional physiological tests was difficult because of its dependence on strain SK-1 and its low growth in pure cultures. Considering the characteristics of strain SC-1, especially the requirement for *Bacillus* sp., strain SC-1 has a high similarity to *Symbiobacterium thermophilum* strain T, as previously suggested by Suzuki et al. (1988). Morphologically, strain SC-1 had different types of cell-surface structures compared with those of *S. thermophilum* because strain SC-1 had no special surface structures. Considering its dependency on stability of the growth factors, growth conditions (optimum oxygen tension, pH, and temperature) must be reconfirmed after characterization of the growth factors. According to these novel interaction and physiological data, strain SC-1 had no strong affiliation with any bacterial groups previously reported. Taxonomic study of strain SC-1 by phylogenetic and chemotaxonomic methods will be reported elsewhere.

Although enrichment cultures were carried out under aerobic conditions, no colony was observed under aerobic conditions. Under the microaerobic conditions or anoxic conditions, we detected microcolonies of strain SC-1. After the growth of strain SK-1, oxygen tension decreased to permit growth of strain SC-1, and this might be one of the association factors for the growth of strain SC-1.

We did not observe any physical interaction between strain SC-1 and SK-1 by light microscopy. This result means that the association is mediated by diffusion of growth factors released from the cells. This result corresponds with the result of Ohno et al. (1999) that showed growth of strain SC-1 without direct contact between two strains. They confirmed pure cultures of *Symbiobacterium thermophilum* by separating *Bacillus* with a dialysis membrane.

Apparent aggregation by lysis of *Bacillus* and significant growth repression of *Bacillus* in the mixed culture as described by Suzuki et al. (1988) were not observed. The culture supernatant of strain SC-1 had no growth-inhibitory activity on SK-1. Considering these results, strain SC-1 has no significant inhibitory activity on the growth of strain SK-1 (Fig. 4a).

The nature of the growth factors was characterized. According to the results, we suggest that at least two kinds of factors from SK-1 might be necessary for the growth of strain SC-1 (Fig. 3). As shown in Fig. 4, the heat-stable factors of the culture supernatant were released as metabolites during the growth phase of SK-1 and disappeared in the stationary phase. The intracellular heat-labile factors were produced in the early stationary phase. These heat-labile factors might be released by the lysis of SK-1 during the stationary phase of the mixed culture. Lysis of strain SK-1 in the stationary phase is observed also in the pure cultures (Fig. 4a). Although strain SK-1 produced these growth factors without induction of strain SC-1, the production pattern of growth factors in the mixed cultures was different from that in the pure culture. The factors were sustained a long time in the stationary phase. This result implies that strain SC-1 might be involved in regulating the

production of heat-labile factors of strain SK-1, although the growth profile of *Bacillus* was not significantly changed by the presence of strain SC-1. Taken together, because we did not detect any advantages or disadvantages of this interaction on the growth of *Bacillus* partner, we propose this interaction as an obligate commensalism.

Ohno et al. (1999) reported that the factors diffusible through the dialysis membrane (molecular weight cutoff, 3500 Da) stimulated the growth of *Symbiobacterium thermophilum*. However, the essential growth factors of strain SC-1 could not cross the ultrafiltration membrane (molecular weight cutoff, 10000 Da). When the heat-labile factors were analyzed with gel filtration chromatography (FPLC) with a Superose 12HR column (Pharmacia LKB, Uppsala, Sweden), growth-stimulating activity was observed around the 30-kDa fraction (data not shown).

The production of growth factors is widespread in the microbial world, considering that all the bacterial strains tested showed growth-stimulation activity on strain SC-1. Therefore, thermophilic strain SK-1 might not be a specific partner of strain SC-1 in the ecosystem. However, growth factors of mesophiles such as *E. coli* and *B. subtilis* do not permit optimal growth of strain SC-1, considering the filamentous growth of strain SC-1 that occurs with growth factors of these bacteria.

Only a limited number of genera and species of gram-negative thermophilic eubacteria have been isolated (Storm 1985). The sources of these gram-negative thermophiles are mainly volcanic marine and terrestrial hot springs. In composts, most of the thermophilic strains isolated are in the genus *Bacillus* (Storm 1985; Beffa et al. 1996). Our findings indicate that there might be a new group of gram-negative thermophiles in compost that could have obligately commensal associations with other microorganisms.

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