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Characterization of Growth-supporting Factors Produced by *Geobacillus toebii* for the Commensal Thermophile *Symbiobacterium toebii*

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***Symbiobacterium toebii* is a commensal symbiotic thermophile that cannot grow without support from a partner bacterium. We investigated the properties of *Symbiobacterium* growth-supporting factors (SGSFs) produced by the partner bacterium *Geobacillus toebii*. SGSFs occurred in both the cell-free extract (CFE) and culture supernatant of *G. toebii* and might comprise multifarious materials because of their different biological properties. The heavy SGSF contained in the cytosolic component exhibited heat- and proteinase-sensitive proteinaceous properties and had a molecular mass of >50 kDa. In contrast, the light SGSF contained in the extracellular component exhibited heat-stable, proteinase-resistant, nonprotein properties and had a molecular mass of <10 kDa. Under morphological examination using light microscopy, *S. toebii* cultured with the culture supernatant of *G. toebii* was filamentous, whereas *S. toebii* cultured with the CFE of *G. toebii* was rod-shaped. These results strongly suggest that the SGSFs produced by *G. toebii* comprise two or more types that differ in their growth-supporting mechanisms, although all support the growth of *S. toebii*. Upon the examination of the distribution of SGSFs in other bacteria, both cytosolic and extracellular components of *Geobacillus kaustophilus*, *Escherichia coli*, and *Bacillus subtilis* had detectable growth-supporting effects for *S. toebii*, indicating that common SGSF materials are widely present in various bacterial strains.**

Keywords: *Symbiobacterium toebii*, *Geobacillus toebii*, symbiobacterium growth-supporting factor (SGSF), commensalism, symbiosis

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Approximately 99% of microorganisms in nature cannot be successfully cultivated under artificial culture conditions, even in the presence of appropriate nutrients [3]. Recently, several improved cultivation techniques have been reported, and many previously uncultivable bacteria were identified from compost, sludge, marine sediment, and other materials [1, 4–6]. Notwithstanding these efforts, most microorganisms remain uncultivable because of the complexity of microbial communities and the inability to create similar microenvironments with characteristics such as extremely high substrate concentrations or the presence of specific ingredients required for microbial growth [8, 13, 19, 22].

Several bacteria that require growth factors or specific diffusible components have been isolated in the laboratory [1, 6, 9, 12, 16]. A common feature of these bacteria is that they require a specific compound(s) for their growth that is supplied by a neighbor or partner bacterium that occurs in the same environment. For example, *Catellibacterium nectariphilum* requires the partner bacterium *Sphingomonas* sp. for its growth [16]. It cannot grow on artificial media alone and requires diffusible compounds from the partner bacterium. These compounds are heat-stable nonpeptides <1,000 Da in molecular mass, but they have not yet been identified. In addition, Kaeberlein *et al.* [6] developed a novel cultivation method for uncultivable microorganisms using a diffusion chamber method with 0.03-μm pore-size polycarbonate membranes and successfully isolated two diffusible-compound-requiring bacteria; one bacterium showed 93% 16S RNA gene sequence similarity with *Lewinella persica* and the other was *Arcobacter nitrofigilis*. These bacteria required diffusible compounds for their growth that were able to pass through 0.03-μm pores and seemed to be signal compounds that originated from their neighbors; however, these compounds have not yet been characterized.

Previously, we reported that *Symbiobacterium toebii* requires some growth-supporting factors (GSFs) from its partner bacterium *Geobacillus toebii* and does not show sustainable growth in artificial culture in the absence of *G. toebii* [11, 12]. However, pure *S. toebii* can be cultured by adding the culture supernatant and/or cell-free extract (CFE) of *G. toebii* directly to the culture medium [12]. Additionally, many novel, previously uncultivated anaerobic bacteria were isolated from compost and enriched using CFE of *G. toebii* [1]. The GSFs that occur in the CFE of *G. toebii* have also been examined; some of these have proteinaceous properties and low and high molecular weights, but they have not yet been characterized [1].

Symbiobacterium thermophilum, a congener of *S. toebii*, is a growth factor-requiring bacterium that exhibits no growth under artificial culture conditions unless co-cultivated with its partner bacterium *Bacillus* strain S [9, 10, 18]; GSFs occur in the culture supernatant but not in the CFE of the partner bacterium, and *Escherichia coli*, *Bacillus subtilis*, or *Thermus thermophilus* may substitute for *Bacillus* strain S as the helper bacterium for the growth of *S. thermophilum*. The GSFs produced by *Bacillus* strain S are of low molecular weight and can pass through molecular weight cut-off (MWCO) 3,500 cellulose membrane [10]. Watsuji *et al.* [20] reported CO_2 and bicarbonate as candidate GSFs and suggested a syntropic relationship between *S. thermophilum* and its partner bacteria based on cultivation results. We previously discussed the *Symbiobacterium* GSF (SGSF) produced by *G. toebii* as a proteinaceous material with a high molecular mass of approximately 30 kDa [12]. Recently, Bae *et al.* [1] successfully isolated several novel groups of microorganisms by supplying the CFE of *G. toebii*; they also discussed the characteristics of the GSFs. However, despite the extensive research regarding the GSFs produced by several bacteria, no clear result has yet been reported. Considering these previous reports, the characteristics of the GSFs produced by *G. toebii* appear to differ from those produced by *S. thermophilum* and other bacteria, although it is possible that the differences could have occurred because of differences in cultivation techniques.

We investigated and summarized the characteristics of the SGSFs produced by *G. toebii*. Our results provide important clues to allow the characterization of the SGSFs produced by *G. toebii* and to understand the commensal symbiotic relationship between two bacteria that belong to different genera.

MATERIALS AND METHODS

Bacterial Strains, Media, and Culture Conditions

Symbiobacterium toebii KCTC 0307BP^T (DSM 15906^T) and *Geobacillus toebii* KCTC 0306BP^T (DSM 14590^T) were isolated from hay compost in Korea [14, 15]. *Escherichia coli* JM83, *Bacillus subtilis* 168, and *Geobacillus kaustophilus* JCM 20319^T were used to investigate the growth-supporting effects of the symbiotic relationship. *Escherichia coli* was cultured at 37°C, *B. subtilis* at 30°C, and *G. toebii* and *G. kaustophilus* at 55°C in Luria-Bertani broth. The PETN medium contained 6 g of K_2HPO_4, 2 g of KH_2PO_4, 5 g of bacto tryptone, 10 g of yeast extract, and 30 mM $NaNO_3$ per liter of deionized water. After autoclaving, 100 μg/ml CFE of *G. toebii* was added to the PETN broth as a SGSF supplement, and 50 μg/ml kanamycin was used to prevent contamination by *G. toebii*. *Symbiobacterium toebii* was cultivated at 60°C under anaerobic conditions (CO_2/H_2, 10:90) using BD BBL GasPak plus anaerobic system envelopes (Becton Dickinson Microbiology Systems, Cockeysville, MD, U.S.A.). The anaerobic conditions were monitored using BD GasPak Dry anaerobic indicator strips. Yeast extract and bacto tryptone were purchased from BD (Becton Dickinson and Company, Sparks, MD, U.S.A.). Agar and other chemical components were purchased from Nacalai Tesque (Kyoto, Japan).

Preparation of the CFE and Culture Supernatant of the Partner Strains

To prepare CFE for each of the various bacterial partner strains, cells were harvested at the late exponential phase by centrifugation at 7,000 ×*g* for 10 min at 4°C. The pellet was suspended with 50 mM potassium phosphate buffer, pH 7.2, after washing twice with the same buffer, and then disrupted using an ultrasonic disruptor (UD-201; TOMY, Tokyo, Japan). The crude extract was centrifuged at 9,000 ×*g* for 30 min at 4°C, and the resulting supernatant, so-called cell-free extract (CFE), was filtered through a 0.22-μm pore-size membrane (Minisart-plus, 0.22-μm CA-membrane+GF-prefilter; Sartorius AG, Germany). The membrane fraction was prepared by ultracentrifugation of the prepared CFE at 105,000 ×*g* for 60 min at 4°C. After washing twice with potassium phosphate buffer, pH 7.2, the pellet was suspended in the same potassium phosphate buffer.

To prepare culture supernatants for each of the bacterial partner strains, cells were removed from the culture at the mid-logarithmic phase, and the culture supernatant was filter-sterilized by passing it through a 0.22-μm pore-size membrane. The sterilized bacterial culture supernatant was mixed with 2× PETN culture broth (1:1, v/v) for the cultivation of *S. toebii*. The protein concentrations of the CFE, membrane fraction, and culture supernatant were determined using the Bio-Rad protein quantification kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.).

Determination of *S. toebii* Growth

The growth of *S. toebii* was monitored using three different methods. The number of cells was counted directly using a Bacteria Counting Chamber (depth 0.02 mm; Erma, Tokyo, Japan) under a light microscope (Olympus BX40F; Olympus Optical Co. Ltd., Tokyo, Japan). The optical density was measured using a spectrophotometer (Hitachi U-3000; Tokyo, Japan) at 600 nm. Growth was monitored under nitrate-reducing conditions based on the nitrite produced, which was determined using the colorimetric method of Gerhardt *et al.* [2]. The cell shapes were examined under a light microscope after the cells were stained with 0.3% crystal violet (BD, Sparks, MD, U.S.A.).

Investigation of the Biological Properties of SGSFs Contained in the CFE and Culture Supernatant of *G. toebii*

To estimate the sizes of the SGSFs, the CFE and culture supernatant of the various helper bacterial strains were fractionated by molecular

weight using 20 ml of MWCO 10,000 or 50,000 cellulose membrane concentrators (Vivascience AG, Hannover, Germany). For heat treatment, PETN medium containing the CFE or culture supernatant was autoclaved at 105°C for 20 min. To prepare proteinase-digested CFE or culture supernatant, the PETN medium containing the CFE or culture supernatant was treated with 50 μg/ml Proteinase K (Merck, KGaA, Darmstadt, Germany) at 60°C for 5 h.

Results

Growth-supporting Activity of Cellular Components from *G. toebii*

To investigate the cellular location of SGSFs, we examined the growth-supporting activity of three components derived from the culture broth of *G. toebii*: CFE, a cytosolic component; culture supernatant, an extracellular component; and the membrane fraction, a membrane component. The cytosolic and extracellular components showed positive activity, and the best growth-supporting activity was observed using the cytosolic component (Fig. 1). Interestingly, the extracellular component showed approximately 70% of the growth-supporting activity of the cytosolic component. In contrast, the membrane component did not show growth-supporting activity for *S. toebii* (Fig. 1). This indicates that SGSFs occur in both the cytosolic and extracellular components of *G. toebii*.

S. toebii Morphology

We compared the morphology of *S. toebii* cultured with CFE or with culture supernatant of *G. toebii* to determine the physiological effects of the SGSFs on *S. toebii*. *Symbiobacterium toebii* was cultured for 30 h under anaerobic conditions, and the cell shape was examined under a light microscope (Fig. 2). Normal rod-shaped cells were detected in CFE-supplemented PETN medium (Fig. 2B), whereas *S. toebii* was filamentous when cultured in culture supernatant-supplemented PETN medium (Fig. 2A). These results strongly indicate that the SGSFs contained in the cytosolic and extracellular components of *G. toebii* differed, and their growth-supporting mechanisms might also differ. Therefore, we investigated the biological properties of the SGSFs contained in the cytosolic and extracellular components of *G. toebii*.

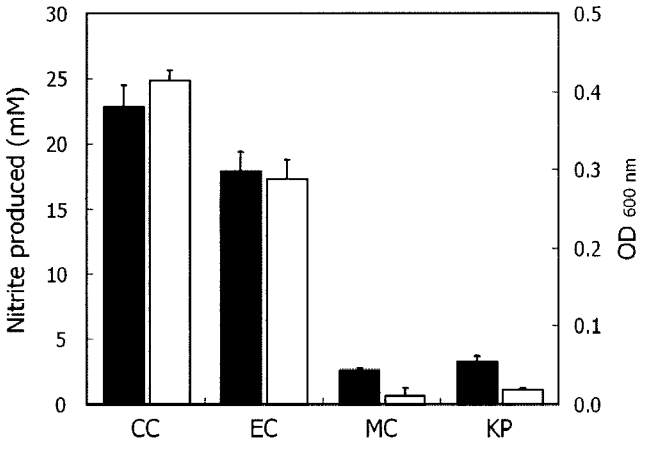

Fig. 1. Growth-supporting effects of the cytosolic, extracellular, and membrane components of *G. toebii*.
The growth of *S. toebii* was determined by quantifying the nitrite produced (■) and optical density at 600 nm (□) after 48 h in culture at 60°C under anaerobic conditions. The results represent the mean (± standard deviation) of three independent cultures (the membrane fraction was tested using two independent cultures). CC, cytosolic component; EC, extracellular component; MC, membrane component; KP, 50 mM potassium phosphate buffer, pH 7.2.

Characterization of the SGSFs in the Cytosolic Component of *G. toebii*

To investigate the biological properties of the SGSFs, their growth-supporting activity was examined after dividing the cytosolic component using a MWCO 50,000 cellulose membrane concentrator. The biochemical properties were also characterized by Proteinase K treatment and autoclaving

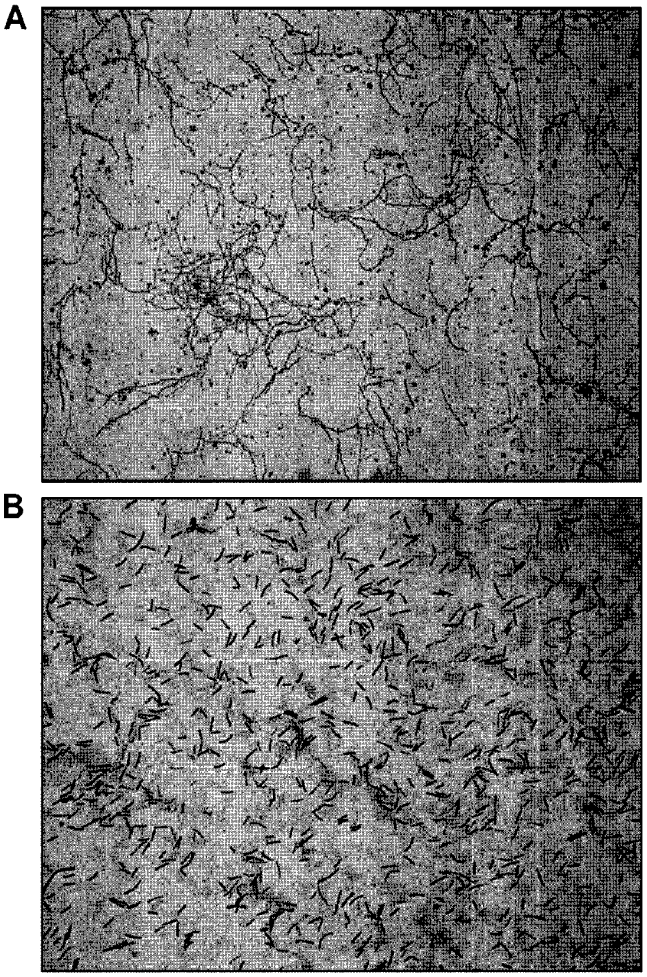

Fig. 2. Morphology of *S. toebii* cultivated with the (**A**) culture supernatant and (**B**) cell-free extract of *G. toebii*, as examined under a light microscope.
S. toebii was incubated at 60°C under anaerobic conditions for 30 h. Cells were stained using 0.3% crystal violet.

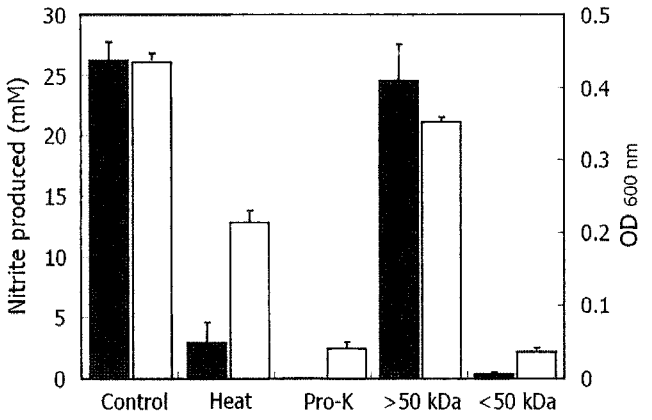

Fig. 3. Biological features of the *Symbiobacterium* growth-supporting factor (SGSF) contained in the cytosolic component of *G. toebii*.

The growth of *S. toebii* was determined by quantifying the nitrite produced (■) and optical density at 600 nm (□) after 48 h in culture at 60°C under anaerobic conditions. The results represent the mean (± standard deviation) of three independent cultures. Control, no treatment (positive control); Heat, heat-treated cytosolic component; Pro-K, Proteinase-K-treated cytosolic component; >50 kDa, molecular mass fraction of cytosolic component >50 kDa; <50 kDa, molecular mass fraction of cytosolic component <50 kDa. Unpredicted turbidity of the heat-treated cytosolic component was detected by heat-denaturation of high concentration of proteins contained in the cytosolic component of *G. toebii*.

at 105°C for 20 min. For the cytosolic components of *G. toebii*, growth-supporting activity occurred for substances with molecular mass >50 kDa, whereas no growth-supporting activity was detected for substances with molecular mass <50 kDa (Fig. 3). Moreover, the significant growth-supporting effect was not detected after Proteinase K and heat treatment of the cytosolic component at 60°C for 5 h and 105°C for 20 min, respectively (Fig. 3). This indicates that the SGSF contained in the cytosolic component of *G. toebii* is proteinaceous material and has a molecular mass >50 kDa.

Although *G. toebii* was cultured at 55°C, it was possible that the proteins extracted from the cells were unstable in the culture broth during the cultivation of *S. toebii* at 60°C. To investigate the stability of the SGSF contained in the cytosolic component in culture medium at 60°C, we examined and compared the growth curves of *S. toebii* that were cultured in culture medium that contained the cytosolic component of *G. toebii* and were pre-incubated for 0, 1, 3, 17, and 24 h at 60°C. Growth was similar for pre-incubations of 0, 1, 3, and 17 h, but the growth lag time increased significantly, by approximately 30 h, for the 24-h pre-incubation (Fig. 4). This demonstrates that the SGSF of the cytosolic component has strong stability and maintained its original growth-supporting activity in culture broth for 24 h, even though it has proteinaceous properties. Additionally, we noted a similar doubling time at the exponential phase of 24-h pre-incubations; below, we discuss different factors that may be related to this adaptation at the initial growth stage of *S. toebii*.

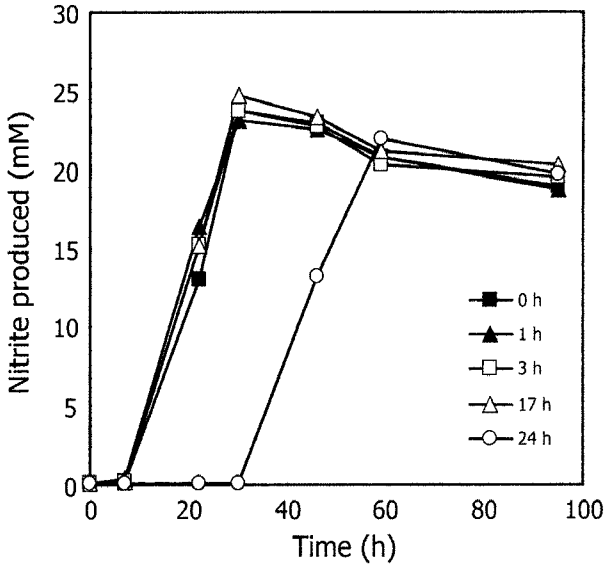

Fig. 4. Heat resistance and growth-supporting effect of the cytosolic component of *G. toebii*.

Cell-free extract (20 mg/ml) was added to 100 ml of PETN broth and incubated without shaking at 60°C; 1.0% *S. toebii* was inoculated after 0, 1, 3, 17, or 24 h. The growth of *S. toebii* was determined by quantifying the nitrite produced.

Characterization of the SGSFs in the Extracellular Component of *G. toebii*

The extracellular component of *G. toebii* was characterized using the same approach as for the cytosolic component (Fig. 5). The fraction with low molecular mass (*i.e.*, <10 kDa) showed growth-supporting activity for *S. toebii*,

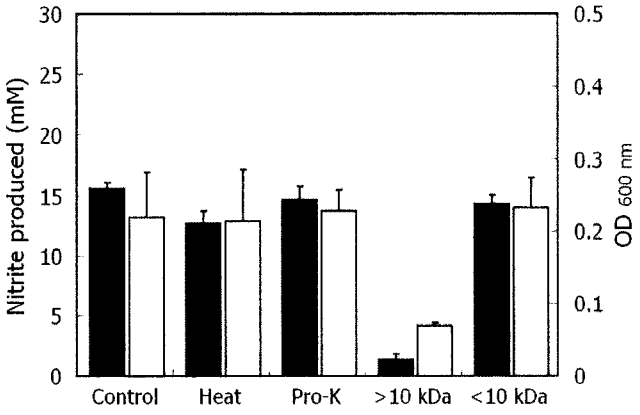

Fig. 5. Biological features of the *Symbiobacterium* growth-supporting factor (SGSF) contained in the extracellular component of *G. toebii*.

The growth of *S. toebii* was determined by quantifying the nitrite produced (■) and optical density at 600 nm (□) after 48 h of culture at 60°C in anaerobic conditions. The results represent the mean (± standard deviation) of three independent cultures (the fractionation results were determined for two independent cultures). Control, no treatment (positive control); Heat, heat-treated extracellular component; Pro-K, Proteinase-K-treated extracellular component; >10 kDa, molecular mass fraction of extracellular component >10 kDa; <10 kDa, molecular mass fraction of cytosolic component <10 kDa.

but the fraction with high molecular mass (>10 kDa) did not. In addition, the growth-supporting activity of this SGSF was sustained after Proteinase K treatment at 60°C for 5 h and heat treatment at 105°C for 20 min. This indicates that the SGSF contained in the extracellular component of *G. toebii* is smaller than the SGSF in the cytosolic component and is composed of non-proteinaceous material. Thus, it strongly suggests that the extracellular and cytosolic SGSFs comprise different materials.

Growth-supporting Activities of Other Microorganisms

The growth-supporting activities of *E. coli* JM83, *B. subtilis* 168, and *G. kaustophilus* JCM 20319 for *S. toebii* were examined using both the CFEs and culture supernatants. The CFEs and culture supernatants prepared from these strains showed strong growth-supporting activities, similar to the results with *G. toebii* added to the culture as a positive control (Fig. 6). Interestingly, although these mesophilic bacteria were not closely related to *G. toebii* phylogenetically and differed from *S. toebii* and *G. toebii* in optimal growth temperature, they showed similar growth-supporting effects for *S. toebii* as the novel partner bacterium *G. toebii*. Thus, the SGSFs contained in the CFEs or culture supernatants of various bacteria might be common materials that are widely distributed in microorganisms in nature.

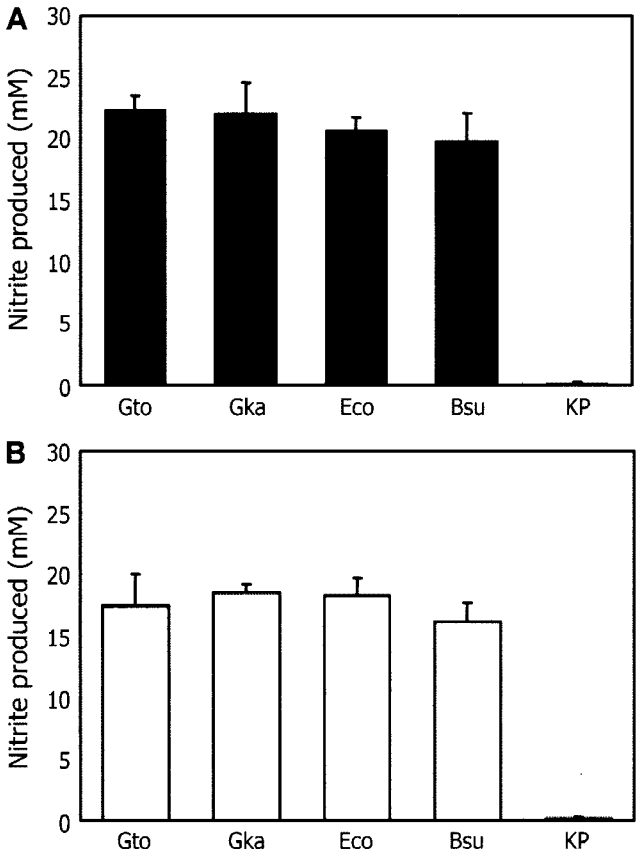

Fig. 6. Growth-supporting effects for *S. toebii* of the (**A**) cell-free extract and (**B**) culture supernatant of *G. toebii*, *G. kaustophilus*, *E. coli*, and *B. subtilis*.

The growth of *S. toebii* was determined by quantifying the nitrite produced after 48 h of culture at 60°C in anaerobic conditions. The results represent the mean (± standard deviation) of three independent cultures. Gto, *G. toebii*; Gka, *G. kaustophilus*; Eco, *E. coli*; Bsu, *B. subtilis*; KP, 50 mM potassium phosphate buffer, pH 7.2 (negative control).

Discussion

This is the first investigation of the properties of light and heavy SGSFs in the culture supernatant and CFE, respectively, of *G. toebii* for the commensal bacterium *S. toebii*. Growth-supporting activity for *S. toebii* was found in both the CFE and culture supernatant, but not in the membrane fraction of *G. toebii*. The light SGSF in the extracellular component of *G. toebii* had nonproteinaceous properties and a molecular mass <10 kDa. These properties are similar to those of the GSFs for *C. nectariphilum* and *S. thermophilum* that were detected in the culture supernatants of the partner strains *Sphingomonas* sp. and *Bacillus* strain S, respectively [10, 17]. Because of these similar properties, we propose that the light SGSF for *S. toebii* may be a compound similar to those for *C. nectariphilum* and *S. thermophilum*. The heavy SGSF contained in the cytosolic component had proteinaceous properties and a molecular mass >50 kDa. This was similar to a previous report, except that the molecular mass was approximately 30 kDa [12]. However, we found no growth-supporting activity in the cytosolic component fraction that was <50 kDa in molecular mass. Bae *et al.* [1] reported five groups of microorganisms that require the cell extract of *G. toebii* for growth, and the GSFs contained in the cell extract exhibited different properties according to their molecular weights and the isolated microorganism. Even though the GSFs for groups IV and V exhibited nonproteinaceous properties despite their high molecular weights, those for groups I and II exhibited properties similar to the heavy SGSF for *S. toebii*, which exhibited a high sensitivity to Proteinase K and high molecular mass of >10 kDa. Thus, we suspect that the heavy SGSF for *S. toebii* maybe be composed of materials similar to those of the high-molecular-weight GSFs for groups IV and V.

It was previously suggested that growth-supporting activity was only found in the CFE of *G. toebii* and that the growth of *S. toebii* is stimulated by the lysis of *G. toebii* at the stationary phase, because the growth of *S. toebii* was initiated during the stationary phase of *G. toebii* in mixed cultivation of these bacteria [12]. However, we found that the light SGSF in the culture supernatant had 60–70% of the growth-supporting activity of the heavy SGSF, as well as different properties and composition. In addition, all CFEs and culture supernatants prepared from the late lag to the stationary growth stages of *G. toebii* exhibited similar

Table 1. Characteristics of several growth-supporting factors (GSFs) required by GSF-dependent bacterial strains.

Characteristic	*Symbiobacterium toebii*		Unclassified clostridiales	*Symbiobacterium thermophilum*	*Catellibacterium nectariphilum*
General features of GSF-dependent bacterium					
Partner strain	*Geobacillus toebii*		*Geobacillus toebii*	*Bacillus* sp.	*Sphingomonas* sp.
Growth temperature	60°C		60°C	60°C	30°C
Oxygen tension	Anaerobic & microaerobic		Anaerobic	Anaerobic	Aerobic
Denitrification	+		+	+	-
Biological properties of GSFs					
Cellular location	Extract[a]	Sup[b]	Extract	Sup	Sup
Estimated Mr[c]	>50 kDa	<10 kDa	>10 kDa for groups 1–5 <10 kDa for groups 4 & 5	<3.5 kDa	<1 kDa
Heat stability	S[d]	R[e]	S	R	R
Proteinase K stability	S	R	R (groups 3–5), S (groups 1 & 2)	ND[f]	R
Effect of other microbes					
Escherichia coli	+	+	ND	+ (Sup)	50%
Bacillus subtilis	+	+	ND	+ (Co-culture)	-
Geobacillus kaustophilus	+	+	ND	ND	ND
Thermus thermophilus	+	ND	ND	+ (Co-culture)	ND
Saccharomyces cerevisiae	-	ND	ND	- (Sup)	ND
References	This study, [9, 10]		[1]	[7, 15]	[13, 14]

[a]Cell-free extract. [b]Culture supernatant. [c]Molecular mass. [d]Sensitive. [e]Resistant. [f]Not determined.
+, Positive reaction or results; -, negative reaction or results.

growth-supporting activities (unpublished data). Thus, the heavy and light SGSFs are produced constantly throughout all growth stages of *G. toebii*, and the light SGSF may be excreted from the cells to the culture medium, but not released by cell lysis. The membrane fraction did not show growth-supporting activity for *S. toebii*, indicating that the SGSFs and the growth-supporting mechanisms are not related to compounds located on or within the membrane of *G. toebii*, and the growth support may not involve cell-to-cell interaction between *S. toebii* and *G. toebii*.

To verify the biological properties of the light and heavy SGSFs produced from *G. toebii*, we focused on fundamental properties such as molecular weight and heat and proteinase stability. We summarized the characteristics of GSFs reported here and previously (Table 1). Although the heavy SGSF in the cytosolic component is unstable at 105°C for 20 min, it showed lengthy heat stability at 60°C for 24 h (Fig. 4). The optimal growth and doubling times at the exponential phase of *S. toebii* were similar when the CFE of *G. toebii* was pre-incubated for 0, 1, 3, 17, and 24 h, and only the 24-h pre-incubation resulted in a significantly extended lag time. This indicates that the heavy SGSF contained in the CFE of *G. toebii* can survive for at least 24 h in the culture broth, even though it has proteinaceous properties. We also suspect that other factors in addition to SGSFs may be related to the adaptations at the initial growth stage of *S. toebii* to develop the delicate environment that is required for the initial growth of *S. toebii*. Indeed, we have investigated the commensal relationship between *S. toebii* and *G. toebii* to protect against the toxicity of reactive oxygen species in the culture medium (unpublished data).

Because there are reports that various microorganisms support the growth of commensal thermophiles [1, 9, 17], we also examined the growth-supporting effects of several microorganisms for *S. toebii*. We investigated the growth-supporting effects of both the cytosolic and extracellular components separately, whereas most previous studies examined either the culture supernatant or CFE as a supplement for growth-factor-requiring bacteria (Fig. 6). Considering the abundant distribution of SGSFs in both the cytosolic and extracellular components in various microorganisms, we suspect that SGSFs are common materials that are essential for the growth of a variety of bacteria.

Recently, Watsuji *et al.* [20] reported that the supplementation of CO_2 induces the growth of *S. thermophilum*, even in the absence of a partner bacterial strain. They focused on carbonic anhydrase, a ubiquitous enzyme that catalyzes the conversion between CO_2 and bicarbonate. However, *S. toebii* did not grow in culture medium supplemented with 10 mM ammonium, sodium, and potassium bicarbonate, or with 10–200 mM potassium bicarbonate instead of the CFE or culture supernatant of *G. toebii* (data not shown). In addition, carbonic-anhydrase-encoding genes or homologous genes were not detected in the published genome sequence of *Geobacillus kaustophilus* HTA426,

even though it shows a growth-supporting effect similar to that of *G. toebii* (Fig. 6).

The molecular weight of the heavy SGSF in the cytosolic component was greater than that reported previously. Possibly, the low reproducibility of the cultivation and unclear threshold for the determination of growth resulted in an obscure definition of growth in previous studies of *S. toebii*. However, we had clear cultivation results from three biological tests and obtained a high concentration of nitrite produced in each experiment using a stable cultivation technique. Our data will provide fundamental information with which to understand microbial uncultivability in nature.

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