
Minoru TAKEDA, Toshiharu NISHIYAMA, Shogo NOMOTO, Sachie SHINMARU, Ichiro SUZUKI, and Jun-ichi KOIZUMI

Division of Materials Science and Chemical Engineering, Faculty of Engineering, Yokohama National University, Tokiwadai 79-5, Hodogaya, Yokohama 240-8501, Japan

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**Paenibacillus** sp. strain TB is capable of degrading the sheath prepared from a sheathed bacterium, *Sphaerotilus natans*. *S. natans* was able to grow alone on casamino acids but strain TB was not. Cocultivation of strain TB and *S. natans* was examined in a medium supplemented with casamino acids as a growth substrate. The growth of strain TB was observed when the sheath was supplied to the medium or in cocultivation with *S. natans*. The phospholipid amount reached a maximum after 24 h of cocultivation and subsequently kept almost the same level for 96 h. The sheath amount also reached a maximum after 24 h and then gradually declined. The cell concentration of strain TB increased throughout the cocultivation. By competitive PCR targeted for amplification of a part of 16S rDNA, the abundance ratio (*S. natans*/strain TB) of 6.7 was obtained at 72 h. Almost no growth of strain TB was detected in a coculture with a sheath-less mutant of *S. natans*. The evidence allows the conclusion that strain TB grew by utilizing the intact sheath in coculture with *S. natans*.

**Key words:** cocultivation; *Sphaerotilus natans*; *Paenibacillus* sp.; sheath; commensalism

*Sphaerotilus natans* is one of the sheathed filamentous bacteria often found in streams with abundant organic compounds and in activated sludge. Romano and Peloquin reported that the sheath is composed of polysaccharide, protein, and lipid. We extended their work and demonstrated that the sheath is constructed from polysaccharide, which is composed of glucose and (N-acetyl)galactosamine, and a peptide rich in glycine and cysteine. Such characteristics are similar to those of the sheath prepared from *Leptothrix discophora*, which is constructed from a heteropolysaccharide composed of N-acetylgalactosamine and uronic acids and a protein rich in cysteine. *L. discophora* is a close relative of *S. natans* in terms of physiological and phylogenetic properties. *Leptothrix* spp. and *S. natans* have ability to extracellularly produce iron and manganese oxides (*S. natans* oxidizes only iron); metal oxidizing activity is associated with the sheath. *S. natans* is known to be one of the filamentous bacteria causing bulking problems of activated sludge. Biodegradation of the sheath responsible for the filamentation of *S. natans* was first demonstrated by discovery of two *Paenibacillus* strains (strains TB and TK). Strain TB is able to grow on purified sheath or the polysaccharide moiety of sheath as a sole carbon source. In this study, we attempted to clarify whether strain TB can use intact sheath in coculture with *S. natans* or not. The results will make a significant contribution to the knowledge of the ecophysiology of the sheath-forming and sheath-degrading bacteria.

The sheath-forming bacterial strain used in this study was *Sphaerotilus natans* IFO13543. It was cultured and its sheath was prepared in the manner previously described. A sheath-degrading bacterium, *Paenibacillus* sp. strain TB (JCM 11186 = IAM 14962 = KCTC 13912), was grown in sheath medium. Due to filamentation and entanglement of the filaments of *S. natans*, its growth could not be estimated by colony or direct cell counting. Consequently, total bacterial growth in pure or mixed cultures was monitored by the increase in the amount of phospholipid. Phospholipid was extracted from the cells based on the method of Bligh and Dyer with modifications as described below. Cells were washed with distilled water and suspended in 1 ml of distilled water. Chloroform (1.25 ml) and methanol (2.5 ml) were added and vortexed. After low-speed centrifugation, the chloroform phase was recovered and vapo-

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† To whom correspondence should be addressed. Tel: +81-45-339-4266; Fax: +81-45-339-4267; E-mail: mtake@ynu.ac.jp
estimated based on the amount of glucose incorporated into the sheath according the method previously described. S. natans is able to accumulate a large amount of polyhydroxyalkanoates (PHA) when the organism is grown on sugars and organic acids. Granules of PHA cannot be removed during sheath preparation and remain within the sheath up to 38% (w/w). The contaminating granules may influence the measurement of sheath amount. Therefore, pure cultivations of both bacteria were examined at 30°C with shaking in a medium composed of proteose peptone No. 3 (Difco), tryptone peptone, yeast extract, or casamino acids (0.2%), K2HPO4 (0.01%), CaCO3 (0.05%), and MgSO4·7H2O (0.02%). Both bacteria were able to grow individually in media supplemented with proteose peptone No. 3, tryptone peptone, or yeast extract as a source of energy. While in a medium supplemented with casamino acids, S. natans grew forming a sheath coincident with cell growth, but the growth of strain TB was not detected. Sheath-dependent growth of strain TB was tested at 30°C with shaking in the casamino acids medium supplemented with 0.01% sheath. Pure cultivation of strain TB was possible when purified sheath was added to the casamino acid medium (Fig. 1). In this case, the sheath was completely broken down in 96 h and cell growth was observed in reverse proportion to the amount of residual sheath. By microscopic observation, 9.2×10⁶ cells/ml of strain TB cells were detected in a 96-h culture. Based on these results, we decided to use casamino acid medium for further analysis and cocultivation.

S. natans extracellularly produces acidic exopolysaccharide (EPS) together with sheath. EPS of S. natans was prepared by the procedure described below. S. natans was cultured at 30°C for 48 h in the medium used to obtain the sheath-less mutant. Cells from 31 of culture were removed by centrifugation and the supernatant recovered was passed through a 0.45-μm membrane filter. CTAB solution (1%) was dropped into the filtrate, which was being stirred. The precipitate was harvested by centrifugation and dissolved in 100 ml of 1 M NaCl solution. Ethanol (200 ml) was added, mixed, and centrifuged, and the precipitate was recovered. After it was rinsed with cold 70% ethanol, the precipitate was dialyzed against distilled water. The dialysate was passed through a column (1.5×10 cm) packed with Amberlite 50W-X8 (H+), it was lyophilized; usually 50–90 mg of purified EPS was obtained per 1 l of culture. To investigate whether EPS was produced by S. natans in the casamino acids medium or not, the bacterium was grown in the medium for 4 days with shaking. Cells and sheath were removed by centrifugation and then the supernatant was passed through a glass filter (GA100, Advantec). To the filtrate, ethanol was added and stirred. EPS was harvested by centrifugation and dried at 105°C. The amount of partially purified EPS recovered from pure culture of S. natans grown on casamino acid was 8–10 mg per 1 l.

The effects of EPS on the growth of strain TB were examined by the following procedure. Strain TB was inoculated into the casamino acids medium supplemented with 0.1% purified EPS and incubated at 30°C for 7 days. Growth of strain TB was judged by microscopic observation at intervals of 24 h. No growth was observed in the medium supplemented with purified EPS, indicating that EPS cannot be assimilated by strain TB. Growth of strain TB was also attempted in the presence of 0.1% sheath and 0.1% purified EPS using the casamino acid medium as a basal medium. It was revealed that EPS does not inhibit sheath-dependent growth of strain TB because almost the same concentration of cells was microscopically detected in the presence or absence of EPS. The following experiment was further performed. S. natans was cultured on the casamino acids medium for 48 h and cells were removed by centrifugation. The supernatant was passed through a sterilized membrane filter (0.22 μm). The filtrate (50 ml) or fresh casamino acids medium (50 ml, as a control) was aseptically poured into a 500-ml flask containing 50 ml of the casamino acids medium supplemented with 0.01% sheath (final concentration). Strain TB was inoculated and shaken for 72 h at 30°C. After cultivation, the cell concentration was microscopically measured and the amount of residual sheath was colorimetrically measured. Almost the same amount of cells (8–9×10⁶ cells/ml) was observed and sheath was completely broken down whether the culture filtrate of S. natans was added or not. It was confirmed that no growth inhibitors against strain TB were secreted by S. natans, at least in pure culture.
Cocultivation of strain TB and \( S. \text{natans} \) was done using the casamino acids medium at 30°C with shaking. The phospholipid amount reached a steady level (2.6–3.1 mg/l) after 24 h of cocultivation of strain TB and \( S. \text{natans} \). In pure culture of \( S. \text{natans} \), almost the same time-dependent change in the amount of phospholipid was observed and 1.7–2.3 mg/l of phospholipid was found at the steady state. The amount of sheath reached a maximum of 3.1 mg/l after 24 h and then it gradually decreased to 1.8 mg/l in the next 72 h. Decline of the sheath amount at stationary phase was not observed in the pure culture of \( S. \text{natans} \), suggesting that the decline is ascribed to degradation by strain TB. In pure culture of \( S. \text{natans} \), most cells were enclosed in sheath (Fig. 2a). Whereas, in the mixed culture the sheath was completely broken down and some fibrillar materials, probably shreds of sheath, can be seen instead (Fig. 2b). Cells of \( S. \text{natans} \) and strain TB in coculture were distinguishable by microscopic observation as shown in Fig. 2b. The growth of strain TB in the coculture was microscopically measured with respect to culture time (Fig. 3). The cell concentration of strain TB gradually increased throughout cocultivation and reached \( 4.5 \times 10^6 \) cells/ml after 96 h. Cocultivation of strain TB and a sheath-less mutant of \( S. \text{natans} \) was also examined. Since \( S. \text{natans} \) often loses its sheath-forming capability under eutrophic conditions,1) the bacterium was repeatedly cultured statically in a medium composed of 0.4% glucose, 0.2% proteose-peptone No. 3, 0.02% yeast extract, 0.05% CaCO\(_3\), and 0.02% MgSO\(_4\)・7H\(_2\)O to prompt the appearance of sheath-less mutants. A smooth colony indicating disappearance of the sheath was selected on an agar plate of 1% tryptone. Only \( 2 \times 10^5 \) cells were detected in 1 ml of coculture of strain TB and a sheath-less mutant of \( S. \text{natans} \) (Fig. 3). This value is much less than that observed in coculture with a sheath-forming strain of \( S. \text{natans} \), indicating that the growth of strain TB in coculture depends on intact sheaths. In a 48-h pure culture of strain TB, however, the cell concentration did not exceed \( 5 \times 10^5 \) cells/ml. The sheath-less mutant of \( S. \text{natans} \) might produce a small amount of sheath or its intermediate and slightly supported the growth of strain TB. It should be also noted here that all of the sheath in the coculture was not degraded by strain TB. In contrast, purified sheath is readily degraded by strain TB (Fig. 1). As mentioned already, it was revealed that a growth-permitting factor is not released by \( S. \text{natans} \). Therefore, it can be assumed that structural alteration or dissociation of some protective material might be occurring in the procedure of sheath isolation.

We attempted to estimate the abundance ratio of \( S. \text{natans} \) and strain TB in coculture by competitive PCR targeted on a region of the 16S rRNA gene. DNA was extracted and purified by the method described elsewhere\(^{10–12} \) with modifications as follows: Cells were washed with and suspended in TE buffer (0.5 ml). The suspension was frozen at –70°C and then heated at 70°C for 2 min. Lysozyme (0.5 mg) was added to the suspension, incubated at 37°C for 1 h, 30 \( \mu \)l of 10% (w/v) SDS solution was added, and vortexed. To the mixture, NaCl and CTAB (hexadecyltrimethyl ammonium bromide)
Fig. 4. Partial 16S rDNA Sequences of Sphaerotilus natans and Strain TB.

Sequences amplified by 907F and 1522R were aligned using the Clustal W program. Consistent nucleotides are indicated by asterisks.

Binding positions of the forward and reverse primers for competitive PCR are boxed with broken and full lines, respectively. White characters on a black background shows the Sph site.

were added to give final concentrations of 5 M and 1.4% (w/v), respectively. After heating at 65°C for 10 min, 0.7 ml of chloroform 2-propanol mixture (24:1) was added, vortexed, and centrifuged. To the supernatant, 0.7 ml of phenol chloroform 2-propanol mixture (25:24:1) was added, vortexed, and centrifuged. The supernatant was recovered and 0.8 ml of 2-propanol was added. The precipitate was harvested by centrifugation, and washed with cold 70% ethanol. To evaluate the purity and amount of the DNA, it was electrophoresed, stained with SYBR Green I, and scanned with a fluorescence and radioisotope image analyzer (FLA-2000, Fuji Photo Film). The 16S rRNA gene of both bacteria was partially sequenced (Fig. 4) using a pair of primers of 5′-CCGTCAATTCATTTGAGTTT-3′ (binding position 907-926) and 5′-AAGGAGGTGATCCAGCCG-CA-3′ (binding position 1541-1522). The 16S rDNA sequences determined in this study have DDBJ/EMBL/Genbank accession numbers of AB072236 (Sphaerotilus natans IFO 13543’)) and AB072237 (Paenibacillus sp. JCM 11186=IAM 14962=KCTC 13912). Sequences were aligned using the Clustal W program. Based on the aligned sequences, a desirable region for competitive amplification was selected. We designed a forward primer (5′-GGGCTACACAGTG-3′) and a reverse primer (5′-TACGACTTCCTCC-3′) for competitive amplification of 281-bp fragments. Binding positions of these primers are shown in Fig. 4. After PCR amplification, the mixture of amplified products was digested with Sph1, which acts only on the product from strain TB, cutting it into fragments of 135 and 146 bp (Fig. 4). The fragments from strain TB was detected at 7–8 min as a single peak, while a peak of the product from S. natans (281 bp) was detected at 10–11 min on ion exchange HPLC. The HPLC conditions were as follows: column, TSKgel DEAE-NPR (4.6 mm ×
35 mm, Tooh); temperature, ambient; mobile phase, 20 mm Tris-HCl (pH 9.0) with linear gradient of NaCl from 0.5 to 0.65 M (20 min); detection, absorbance at 260 nm. To clarify the correlation between the ratios of the peak area of each PCR product and the composition of template DNA, PCR was done by various DNA compositions. DNAs of both bacteria were prepared individually and their amounts were measured using the image analyzer after electrophoresis. Standard DNA mixtures were prepared by mixing DNA solutions of known concentration. A satisfactory linear relation between the template DNA composition and PCR product composition was observed up to the S. natans/strain TB ratio of 200 with a coefficient of almost 1. When the DNA ratio (S. natans/strain TB) exceeded 200, the peak of the PCR product from strain TB was not detected by HPLC. The PCR product from strain TB was not detected in a 24 h-old coculture, indicating that the S. natans/strain TB ratio based on DNA was much more than 200 at this period. A ratio of 12.8 was obtained at 48 h and it decreased to 6.7 at 72 h. We examined five different 72 h-old cocultures and obtained S. natans/strain TB ratios of 6–15. It is confirmed that S. natans is dominant throughout cocultivation and the growth of strain TB occurs subsequently to the proliferation of S. natans.

Several ecological relations of bacteria have been discovered and studied so far. A producer of heat-stable tryptophanase and β-tyrosinase, Symbiobacterium thermophilum, grows only in mixed cultures with Bacillus strains, Escherichia coli, or Thermus thermophilus possibly being supplied with some ubiquitou metabolite(). Microbial degradation of polyvinyl alcohol (PVA) is achieved only in mixed cultures. A PVA-oxidizing bacterium, a Pseudomonas strain, requires pyrroloquinoline quinone (PQQ); the partner bacterium, also a Pseudomonas strain, supplies PQQ and uses oxidized PVA(). The former case is an example of a bacterial commensal relationship and the latter is that of bacterial symbiotic interaction. In both cases, interactions between two bacteria are achieved by particular growth factors of small molecular weight. Another type of ecological interaction is an example of a bacterial commensalism mediated by extracellular macromolecules, though it is not confirmed that the distributions of those bacteria in natural environment overlap each other. Gellan-degrading Bacillus sp. GL1 was reported to be isolated accompanied by gellan-producing Sphingomonas paucimobilis(). This may be another example of a commensal or symbiotic interaction between bacteria in which extracellular macromolecules are involved. The importance of production and degradation of extracellular polymers among bacterial ecosystem should be recognized.

References

Commensalism of a Sheath-degrading Bacterium


