**Note**

A Spatial Relationship between Sheath Elongation and Cell Proliferation in *Sphaerotilus natans*

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*Sphaerotilus natans* is a filamentous sheath-forming bacterium. A method of selective fluorescent-labeling of its sheath using conventional reagents was developed. Terminal expansion of the sheath was confirmed by this method. In addition, ubiquitous cell growth was revealed by sequential phase-contrast microscopy of a filament. Based on this and earlier reports, a model of the sheath formation is proposed.

**Key words:** cell proliferation; fluorescent labeling; sheath elongation; *Sphaerotilus natans*

*Sphaerotilus natans* is often found in activated sludge, and it causes poor settling due to filamentous bulking.1–3) Control of its propagation in wastewater treatment plants requires investigation.4–7) The filamentation of the bacterium is attributed to sheath formation.8,9) Because the sheath is a causative agent of bulking, study of the sheath-forming mechanism is important in order to improve the performance of activated sludge. From a morphological perspective, the sheath is an extracellular microtube that covers a line of cells and has a nonwoven fabric-like ultrastructure, which can be identified by electron microscopy.9,10) Elongation of the sheath is synchronized with cell growth within the sheath. This synchronicity raises a question regarding the spatial relationship between sheath generation and cell proliferation. Romano and Geason found that the sheath of *S. natans* extends at both terminals with application of a polyclonal antibody against the sheath,11) but the sheath used in their study was later found to be contaminated with cellular proteins and lipids.12,13) Moreover, no report has described the pattern of cell proliferation in *S. natans* filaments. The present study was designed in an attempt to confirm terminal elongation of the sheath and to determine its spatial relationship with cell proliferation. From a chemical perspective, the *Sphaerotilus* sheath is a supermolecule formed by self-assembly of thiol-rich polymers, thiopeptidoglycan molecules.14) A thiopeptidoglycan-based sheath is also produced by *Leptothrix cholodnii*, a close relative of *S. natans*.15,16) Terminal elongation of the *Leptothrix* sheath has recently been demonstrated by the thiol-selective labeling method.17) Hence, we applied a similar method to visualize the *Sphaerotilus* sheath.

*S. natans* JCM 20382 (IFO 13543) was precultured in a synthetic medium (Armbruster medium)8 at 30 °C for 18 h with shaking. Filaments from 1 mL of culture were collected by centrifugation, washed with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-NaOH buffer (pH 7.2), and then suspended in 1 mL of the same buffer. A portion of the suspension was dropped onto an amino-coated glass slide (MAS coat; Matsunami, Osaka, Japan) and subjected to a 2-step fluorescent labeling process.17) This method involves, the biotinylation of thiol groups with *N*-biotinyl-2-[(N-maleimidomethyl)piperazine (Biotin-PE-maleimide; Dojindo, Kumamoto, Japan) and subsequent immunostaining with a fluorescein isothiocyanate (FITC)-conjugated anti-biotin antibody (anti-Biotin-FITC; Miltenyi Biotec, Bergisch Gladbach, Germany). Microscopic observation was done using a BX51 microscope with a U-MNIBA3 mirror unit (Olympus, Tokyo). As expected, this technique made possible easy distinction of the sheath from the cells inside (Fig. 1a–c) and indicated that the sheath was open-ended (Fig. 1b, c). An empty sheath was also successfully stained (Fig. 1a–c). Next, S-biotinylated filaments fixed on the glass slide were caused to grow by soaking in Armbruster medium at 30 °C for 1 h. The slide was then immunostained, as previously reported.17) The middle regions of the grown filaments exhibited fluorescence, indicating that the sheath elongates in terminal regions (Fig. 1d–f), but the results did not exclude the possibility that sheath synthesis was interrupted by S-biotinylation. To confirm terminal elongation, a 3-step labeling method17) was applied, as follows: The thiol groups in the filaments on the slide were inactivated with 3-maleimidopropionic acid (MPA, Tokyo Chemical Industry, Tokyo). After cultivation in Armbruster medium at 30 °C for 1 h, the filaments were S-biotinylated and immunostained. The sheath was expected to exhibit fluorescence only in the newly synthesized regions, but the filaments exhibited no fluorescence, because cell growth was inhibited by MPA. As an alternative, the following double fluorescent staining technique was contrived. *S. natans* filaments immobilized on an amino-coated glass slide were S-biotinylated, cultured in Armbruster medium at 30 °C for 1.5 h, and then washed with HEPES-NaOH buffer. The grown filaments were labeled with a 1 mm aqueous solution of maleimide-Cy3 (HyLite Flow 555 C2 maleimide; AnaSpec, Fremont, CA) at 25 °C for

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15 min, followed by washing with HEPES-NaOH buffer (pH 7.4) containing 1 g/L of bovine serum albumin (BSA). The Cy3-labeled filaments were immunostained at 25°C for 10 min with anti-Biotin-FITC antibody (1:10 in the buffer containing BSA). After washing with the BSA-containing buffer, the double-stained filaments were observed under a microscope equipped with a mirror unit for dual fluorescence of FITC and Cy3 (U-MWIB3, Olympus, Tokyo). Pre-existing and newly synthesized regions of the filaments were expected to exhibit FITC (green) and Cy3 (orange) fluorescence respectively. The images (Fig. 1g–i) clearly indicated terminal elongation, confirming the results of Romano and Geason.11)

To investigate the spatial relationship between sheath elongation and cell proliferation, successive phase-contrast observations of a filament in the microculture were performed. A microculture was set up on an amino-coated glass slide as previously reported,17) with slight modifications. *S. natans* was precultured in a medium containing Proteose Peptone no. 3 (2 g/L), yeast extract (0.2 g/L), and MgSO₄•7H₂O (0.2 g/L) to promote cell growth. Filaments from 1 mL of the culture were washed with Armbruster medium and fixed on the slide within a grid formed with petroleum gel. Armbruster medium (0.3 mL) was added to the grid as previously described.17) Washing and cultivation with Armbruster medium were performed to facilitate immobilization of the *S. natans* filaments on the glass slide. The microculture was intermittently observed under an inverting microscope (DMI3000B; Leica Microsystems, Wetzlar, Germany) at 25°C. As indicated in the serial phase-contrast images (Fig. 2) of a filament, the cells elongated and divided regardless of position. The cellular elongation rate varied by filament and microculture from 0.02 to 0.09 µm/min (average rate, 0.055 µm/min). It was found that sheath elongation and cell proliferation in a filament progress at different locations. Similar observations have been reported for *L. chologndii* filaments, and a 2-stage, semi-ordered assembly model was proposed as the sheath elongation mechanism.17) In the initial assembly stage, reactive thiopeptidoglycan molecules assemble to form microfibrils by ordered aggre-
The microfibrils are metastable and do not further aggregate within the sheath, but have the potential to aggregate at the tip of the sheath. They then diffuse to the tip of the sheath. At the second assembly stage, the microfibrils at the tip of the sheath aggregate randomly to elongate the sheath. This model is based on five characteristic properties: (i) thiopeptidoglycan molecules have reactive thiol groups, (ii) the sheath has a nonwoven fabric-like substructure, (iii) sheath elongation is synchronized with cell proliferation, (iv) the sheath extends at its terminals, and (v) cell growth is uniform regardless of position in the filament. These properties have also been reported for S. natans, and hence the model is likely to be common and thus applicable to all thiopeptidoglycan-type sheath formers. Considering that the Sphaerotilus sheath does not have an outer diffusive layer, a slightly modified model is proposed, as illustrated in Fig. 3. However, it is still unclear whether assembly proceeds spontaneously or enzymatically. Another result of this study is the establishment of a convenient method for selective fluorescent labeling of the Sphaerotilus sheath while maintaining its viability. We believe that our method can be used in environmental sampling to detect the Sphaerotilus sheath, and especially to investigate the population and location of the Sphaerotilus filaments in activated sludge.

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