

Identification of a Gene Essential for Sheathed Structure Formation in *Sphaerotilus natans*, a Filamentous Sheathed Bacterium

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***Sphaerotilus natans*, a filamentous bacterium that causes bulking in activated sludge processes, can assume two distinct morphologies, depending on the substrate concentration for growth; in substrate-rich media it grows as single rod-shaped cells, whereas in substrate-limited media it grows as filaments. To identify genes responsible for sheath formation, we carried out transposon Tn5 mutagenesis. Of the approximately 20,000 mutants obtained, 7 did not form sheathed structures. Sequencing of the Tn5-flanking regions showed that five of the seven Tn5 insertions converged at the same open reading frame, designated *sthA*. The deduced amino acids encoded by *sthA* were found to be homologous to glycosyltransferase, which is known to be involved in linking sugars to lipid carriers during bacterial exopolysaccharide biosynthesis. Disruption of the gene of the wild-type strain by inserting a kanamycin resistance gene cassette also resulted in sheathless growth under either type of nutrient condition. These findings indicate that *sthA* is a crucial component responsible for sheath formation.**

Activated sludge systems are used worldwide for wastewater treatment. One of the major operational problems of these systems is excessive growth of filamentous bacteria (5, 9, 25). This results in poor settlement of activated sludge flocs, a problem that is commonly referred to as bulking. For years, *Sphaerotilus natans* has been considered a primary organism that causes bulking, and a number of studies have been carried out with this species (3–7, 16, 18, 21, 22). Although other types of filamentous bacteria are also known to be involved in bulking (4, 5, 8, 10), Jenkins et al. reported that *S. natans* was the dominant filamentous organism in 12% of 525 bulking and foaming sludge samples in the United States (9). Thus, *S. natans* is still a noteworthy causative agent of sludge bulking.

S. natans is characterized by a sheathed structure in which long chains of rod-shaped cells are enclosed (7, 12, 24). However, under some culture conditions, this organism grows as individual cells without forming a sheath. Since only the filamentous growth causes bulking of activated sludge, the growth conditions which determine the cell form have been studied. Gaudy and Wolfe reported that *S. natans* grew as single cells in the presence of 0.5% glucose and 0.5% peptone but grew as filaments in the presence of 0.1% glucose and 0.1% peptone (6). However, neither additional studies of sheath formation nor the pathway of sheath biosynthesis has been described; hence, nothing is known about the direct trigger that regulates expression of the genes for sheath biosynthesis.

To date, the only available information is the chemical structure of the sheath of *S. natans*. The sheath was initially found

to be a complex composed of polysaccharide, protein, and lipid, with the polysaccharide component consisting of glucose, hexosamine, and various other sugars (18). A more recent study showed that the sheath of *S. natans* is composed of polysaccharide and protein but not lipid, with the polysaccharide component consisting of glucose and *N*-acetylgalactosamine at a 1:4 molar ratio and the protein component consisting primarily of glycine and cysteine (22). These reports suggested that synthesis and the subsequent assembly of the sugar and amino acid components of the sheath occur in multiple steps. At present, however, little is known about the synthetic pathway and genes responsible for sheath formation.

In this study, in order to examine the pathway of sheath formation and to find a way to prevent bulking, we analyzed the gene that is crucial for sheath formation by obtaining sheath-deficient transposon mutants of *S. natans*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The strains and plasmids used are listed in Table 1. *S. natans* IAM 12068 was obtained from the Institute of Molecular and Cellular Biosciences, University of Tokyo. This strain was routinely maintained in 0.1% (wt/vol) nutrient broth (NB) (Difco) plates. A rifampin-resistant *S. natans* mutant was obtained by successively transferring the wild-type strain to 0.1% NB agar plates supplemented with rifampin (20 $\mu\text{g ml}^{-1}$). This mutant, designated *S. natans* S-1, was used as the recipient for transposon mutagenesis as described below. *Escherichia coli* strains were cultured at 37°C in L broth (LB) or on LB agar plates (19). When necessary, the antibiotics ampicillin (50 $\mu\text{g ml}^{-1}$), kanamycin (50 $\mu\text{g ml}^{-1}$), and rifampin (20 $\mu\text{g ml}^{-1}$) were used.

DNA techniques. Standard genetic techniques (i.e., restriction enzyme digestion, ligation, plasmid isolation, and transformation of DNA into *E. coli*) were carried out as described by Sambrook et al. (19). Restriction enzymes and T4 DNA ligase were purchased from Takara Shuzo, Kyoto, Japan. Nucleotide sequencing was performed by the dideoxy chain termination method with a BigDye terminator cycle sequencing Ready Reaction kit and a model 310 automated DNA sequencer (PE Applied Biosystems).

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Pertinent feature(s)	Source ^a
<i>Sphaerotilus natans</i> strains		
IAM 12068	Wild type	IAM
S-1	Rif ^r derivative of IAM 12068	This study
TM1 to TM5	Tn5-induced sheath-deficient mutant (<i>sthA</i> ::Tn5) of S-1, Rif ^r Km ^r	This study
TM6	Tn5-induced sheath-deficient mutant of S-1, Rif ^r Km ^r	This study
TM7	Tn5-induced sheath-deficient mutant of S-1, Rif ^r Km ^r	This study
SKM01	S-1 <i>sthA</i> ::Km ^r Rif ^r	This study
SGK01	S-1 <i>sthA-gfp</i> ::Km ^r Rif ^r	This study
<i>Escherichia coli</i> strains		
DH5 α	<i>hsdR17 endA1 recA1 gyrA1 thi relA1 supE44 ϕ80dlacZΔM15 Δ(lacZ-argF)U169</i>	BRL
S17-1	<i>recA pro thi hsdR⁻M⁺ chr::RP4-2</i>	DSMZ
Plasmids		
pUC118	Cloning vector, Ap ^r	Takara Shuzo
pT7-Blue	Cloning vector for PCR product, Ap ^r	Novagen
pSUP5011	pBR325 derivative::Tn5- <i>mob</i> , Ap ^r Km ^r	DSMZ
pUC4K	Source of Km ^r cassette	Amersham
pUTminiTn5gfp	<i>gfp</i> reporter on mini-Tn5, source of <i>gfp</i> , Ap ^r Tc ^r	ATCC
pSTH10	Used for disruption of <i>sthA</i> , <i>mob</i> ⁺ Ap ^r Km ^r	This study
pSTH16	Used for monitoring <i>sthA</i> expression in vivo, <i>mob</i> ⁺ <i>gfp</i> ⁺ Ap ^r Km ^r	This study

^a IAM, Institute of Applied Microbiology; BRL, Bethesda Research Laboratories; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen; ATCC, American Type Culture Collection.

Transposon mutagenesis. Transposon mutagenesis with Tn5 was accomplished through biparental mating as follows. Exponentially grown *E. coli* S17-1(pSUP5011) cells and *S. natans* S-1 cells grown at 30°C in 1.0% NB to the stationary phase were harvested and resuspended in minimal volumes of saline (0.9% NaCl). The two suspensions were mixed and dispensed onto a sterile nitrocellulose filter on a 1.0% NB agar plate, which was then incubated overnight at 30°C. The cells were then washed with a minimal volume of saline, plated onto 0.1% NB agar plates containing rifampin and kanamycin, and incubated for 3 to 4 days at 30°C. Sheath-deficient mutants were obtained by visually selecting colonies with a smooth morphology.

Southern blot hybridization. Chromosomal DNA was prepared by the procedure of Murray and Thompson (13). DNA digested with a restriction enzyme was separated by agarose gel electrophoresis and transferred onto a nylon membrane (Hybond N+; Amersham Pharmacia Biotech). Hybridization was carried out at 55°C with an AlkPhos direct labeling and detection kit (Amersham Pharmacia Biotech) by using a 1.8-kb *Bam*HI fragment of pSUP5011 as a Tn5-specific probe according to the manufacturer's instructions. The membrane was then washed at the same temperature.

Cloning of Tn5-inserted regions from the sheath-deficient mutants. Chromosomal DNA was digested with *Sac*I and ligated into the *Sac*I site of pUC118. Transformants were selected on LB agar plates containing kanamycin.

Disruption of the *sthA* gene. The fact that *sthA* is essential for sheath formation was confirmed by disrupting the gene by insertion of the kanamycin resistance (Km^r) gene. The plasmid used for double-crossover integration was constructed as follows. A 2.3-kb DNA fragment situated entirely within the *sthA* gene was amplified by PCR by using primers 5'-TGACGCGATTGGTACAAGTC-3' (upstream region of *sthA*) and 5'-AGATCCTTCAGGCGGATGCT-3' (downstream region of *sthA*) and PyroBEST DNA polymerase according to the instructions of the supplier (Takara Shuzo). The thermal cycling protocol consisted of an initial denaturation step at 95°C for 1 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 2 min. Adenine was added to the 3' terminus of the PCR product by using *Taq* polymerase (Takara Shuzo), and the product was cloned into pT7Blue by TA cloning. A unique *Ase*I site within *sthA* in the PCR product was used as the insertion site of the Km^r gene cassette as follows. A 1.2-kb *Hinc*II fragment carrying Km^r prepared from pUC4K was inserted into the *Ase*I site (blunted by T4 DNA polymerase) of *sthA* in the same orientation without producing a polar mutation. The 1.8-kb *Bam*HI fragment carrying the *mob* site from pSUP5011 was then inserted at the *Bam*HI site of the construct to generate pSTH10. *E. coli* S17-1 was then transformed by pSTH10. Plasmid pSTH10 was introduced into *S. natans* S-1 by conjugation with *E. coli* S17-1 harboring pSTH10 as described above (transposon mutagenesis). The transconjugant was selected on 0.1% NB agar plates supplemented with rifampin and kanamycin. Km^r insertion into *sthA*

was confirmed by Southern blot hybridization by using the Km^r gene cassette as the probe and PCR amplification performed with the primers mentioned above.

RNA extraction and quantification of *sthA* mRNA by real-time quantitative RT-PCR. Filamentous *S. natans* cells (strain S-1) were grown in 0.1% NB and harvested at the exponential phase. Single cells of the same strain were grown on a 1.0% NB agar plate for 10 h and harvested. Total RNAs were isolated by using ISOGEN-LS according to the instructions of the supplier (Nippon Gene, Tokyo, Japan). DNA contamination was then eliminated with RNase-free DNase I (Promega, Madison, Wis.), after which each isolated RNA was quantified with a RiboGreen RNA quantification kit (Molecular Probes, Eugene, Oreg.) and stored at -80°C until it was used.

Reverse transcription (RT)-PCR was carried by using a two-step protocol. A 500-ng portion of total RNA and 20 pmol of antisense primer *sthAREV* (5'-ATGCGGGTCTTGGCGATGAA-3'; positions 1093 to 1084 of the predicted coding region) were added to an RTG RT-PCR kit (Amersham Pharmacia Biotech) to obtain a final volume of 50 μ l. Although this kit is ordinarily used for one-step RT-PCR, in this case only the RT reaction was carried out. Samples were incubated for 30 min at 42°C and then heated at 95°C for 5 min to inactivate the reverse transcriptase. Aliquots (1 μ l) of the RT reaction mixture were then added to a LightCycler FastStart DNA Master SYBR Green I kit (Roche Diagnostics) containing 0.5 μ M primer *sthAFOR* (5'-ATCGGCACCGCAATC TCGGT-3'; positions 220 to 240 of the predicted coding region) and 0.5 μ M primer *sthAREV*. The thermal cycling protocol consisted of an initial denaturation step at 95°C for 10 min, followed by 55 cycles of denaturation at 94°C for 15 s, annealing at 60°C for 5 s, and extension at 72°C for 40 s. The PCR and monitoring of the PCR products were carried out with the LightCycler apparatus (Roche Diagnostics). Samples containing known amounts of an *sthA* fragment were used to generate a standard curve from which mRNA levels were determined.

In vivo monitoring of *sthA* expression with the GFP gene. To examine expression of *sthA* in living cells, the green fluorescent protein (GFP) gene was inserted downstream of *sthA* in the chromosome in order to be transcribed by readthrough from *sthA*. To do this, plasmid pSTH16 was constructed as shown schematically in Fig. 1A. This construct was introduced into *S. natans* S-1 by conjugation with *E. coli* S17-1(pSTH16), as described above, and the transconjugants were selected on 0.1% NB agar plates supplemented with rifampin and kanamycin (Fig. 1B). The double-crossover event at the desired position was verified by Southern blot hybridization by using the 1.3-kb Km^r cassette as the probe and PCR amplification performed with primers UP1 and DP4 (Fig. 1). GFP fluorescence was detected with an Olympus AX-80TR microscope equipped with a high-pressure mercury bulb and a fluorescence mirror unit (U-MWIBA/GFP; Olympus Optical, Tokyo, Japan).

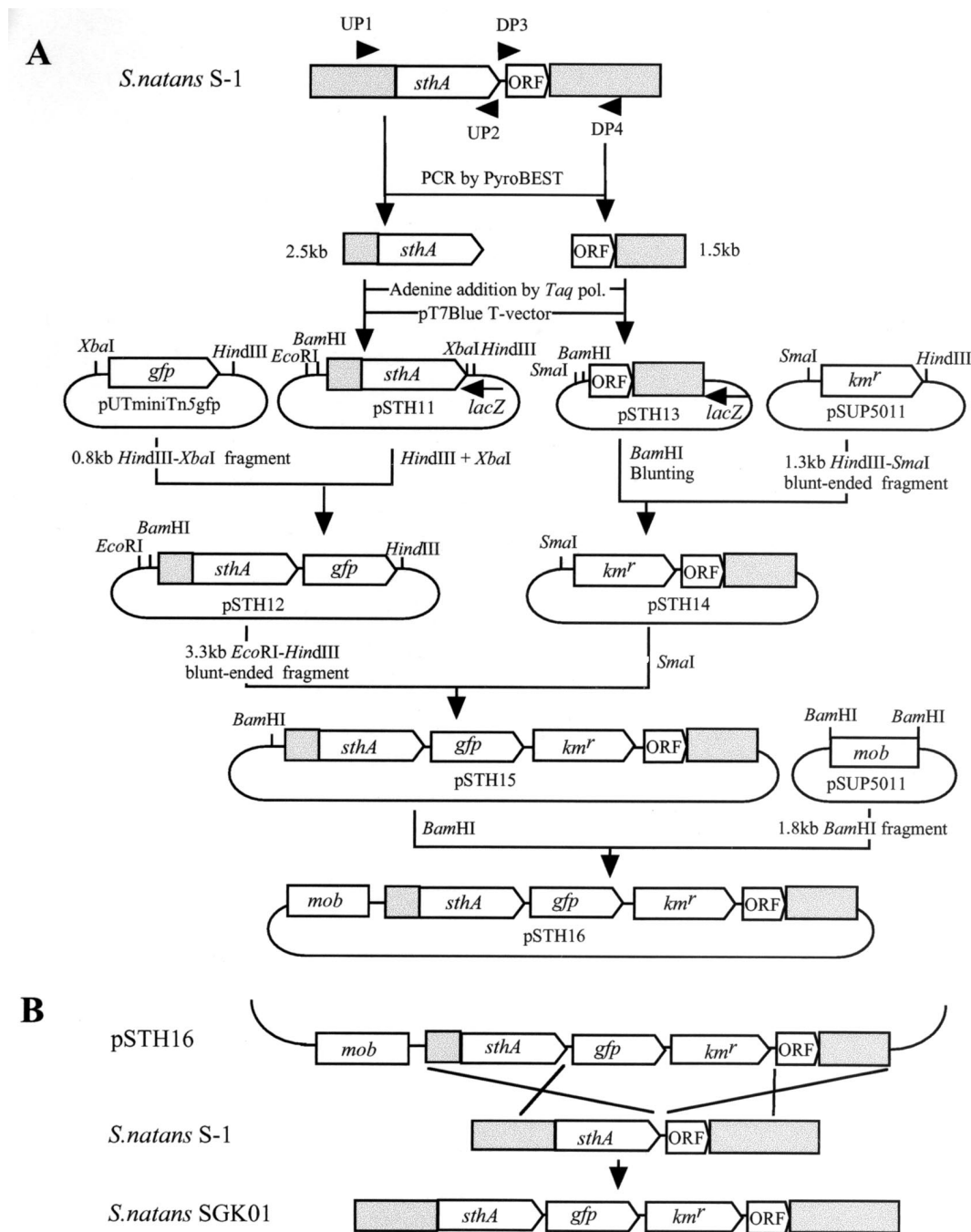


FIG. 1. Schematic representation of a method for monitoring *sthA* expression in vivo. (A) Construction of pSTH16, which was used to insert *gfp* and the Km^r cassette downstream of *sthA* by double-crossover integration. A 2.5-kb fragment containing *sthA* (open box) and its upstream region (shaded box) was amplified from genomic DNA by using PyroBEST polymerase and primers UP1 (5'-ATGAGCGATACCCACTTCGG CTTC-3') and UP2 (5'-TGTGGCGTGCACGACATGTTTCGAT-3'). Adenine was added to the 3' termini of the PCR products by using *Taq* polymerase, and the products were cloned into pT7Blue by the TA cloning procedure. A plasmid in which the orientation of the insert was opposite that of *lacZ* was then selected and designated pSTH11. A 0.8-kb *Xba*I-*Hind*III fragment, including promoterless *gfp*, was prepared from pUTminiTn5gfp and ligated into pSTH11 which had been digested with *Xba*I and *Hind*III to generate pSTH12. A 1.5-kb fragment that included the region downstream of the *sthA* gene (ORF and shaded boxes) was amplified from genomic DNA by using PyroBEST polymerase with primers DP3 (5'-TGAAGGCTGCGGGACGACAGAAGC-3') and DP4 (5'-TGCTCGTCGATGAAGCGGCTGGCCT-3'), and the PCR product was cloned into pT7Blue as described above. A plasmid in which the orientation of the insert was opposite that of *lacZ* was selected and designated pSTH13. A 1.3-kb *Hind*III-*Sma*I fragment carrying Km^r prepared from pSUP5011 was blunted and ligated into pSTH13 which had been digested with *Bam*HI and blunted in order to generate pSTH14. pSTH12 was digested with *Eco*RI and *Hind*III, and the 3.3-kb *Eco*RI-*Hind*III fragment containing *sthA-gfp* of pSTH12 was blunted and ligated into the *Sma*I site of pSTH14. A plasmid in which the insert was oriented properly was selected and designated pSTH15. Finally, a 1.8-kb *Bam*HI fragment carrying the *mob* site prepared from pSUP5011 was inserted into the *Bam*HI site of pSTH15, yielding pSTH16, in which *gfp* and the Km^r cassette were situated between *sthA* and its downstream ORF, without disrupting either gene. pol., polymerase. (B) Strategy for insertion of *gfp* and the Km^r cassette between *sthA* and its downstream ORF.

Scanning electron microscopy. Single cells of strain S-1 and an *sthA* disruptant (*sthA*::Tn5, strain TM1) grown on 1.0% NB agar plates were harvested and fixed with 2% glutaraldehyde for 1 h at room temperature, rinsed with phosphate buffer, and postfixed with 1% osmium tetroxide overnight at 4°C. The cells were then dehydrated by using a graded series of ethanol solutions, critical point dried, and coated with platinum. Samples were observed with a model S-4500 field emission scanning electron microscope (Hitachi, Tokyo, Japan).

Nucleotide sequence accession numbers. The nucleotide sequence data determined in this study have been deposited in the DDBJ/GenBank/EMBL nucleotide sequence database under accession numbers AB050638 to AB050640.

RESULTS

Morphological traits of *S. natans* IAM 12068. A previous study showed that *S. natans* can have two distinct morphologies, depending upon the substrate concentration (6). We found that the strain used in this study formed rough colonies comprised of filamentous cells when it was grown on 0.1% NB agar plates, whereas it formed smooth colonies comprised mostly of single cells when it was grown on 1.0% NB agar plates, although some of the cells formed filaments, particularly after prolonged incubation. These morphological traits were highly reproducible; therefore, all mutation experiments and subsequent verification were performed under these conditions.

Isolation of sheath-deficient mutants. Rifampin-resistant strain S-1, which was derived from *S. natans* IAM 12068, was used for all mutation experiments. The shape of strain S-1 cells was identical to the shape of the wild-type strain cells, and the characteristic NB concentration-dependent morphological features were also identical. When Tn5 transposon mutagenesis was used, approximately 20,000 kanamycin-resistant mutants were obtained. Seven of these mutants (TM1 to TM7) had smooth colony morphology on 0.1% NB agar plates. Microscopic observation revealed that all of the cells were rod shaped and did not form sheathed structures. The inability to form sheaths was confirmed by culturing the mutants in 0.1% NB (strain S-1 formed sheaths in the same medium). Without exception, these mutants grew continuously as single cells without forming sheaths (Fig. 2); no revertants (sheath-forming cells) were observed.

Cloning and sequencing of Tn5-containing DNA fragments from sheath-deficient mutants. The Tn5-containing genomic region was identified by Southern blot analysis with a Tn5-specific probe. The restriction profiles of *KpnI*, *EcoRI*, and *SacI* were identical for the genomes of five of the mutants (TM1 to TM5), suggesting that in these cases the Tn5 insert was located in the same specific region (data not shown). By contrast, the Tn5 insertions in the remaining two mutants (TM6 and TM7) were found to be located elsewhere.

To identify the gene disrupted by the Tn5 insertion, *SacI* fragments harboring Tn5 were cloned into pUC118, and their Tn5-flanking regions were sequenced. Consistent with the Southern blot analysis results, DNA sequencing revealed that five of the insertions occurred in the same open reading frame (ORF), designated *sthA*, which consisted of 1,407 bp encoding a 469-amino-acid protein (accession number AB050638). In addition, a putative Shine-Dalgarno sequence (AAGG) was found 9 bp upstream from the ATG codon.

One of the two remaining Tn5 insertions, TM6 (accession number AB050640), was situated in a region homologous to genes encoding the putative glycosyltransferases in the data-

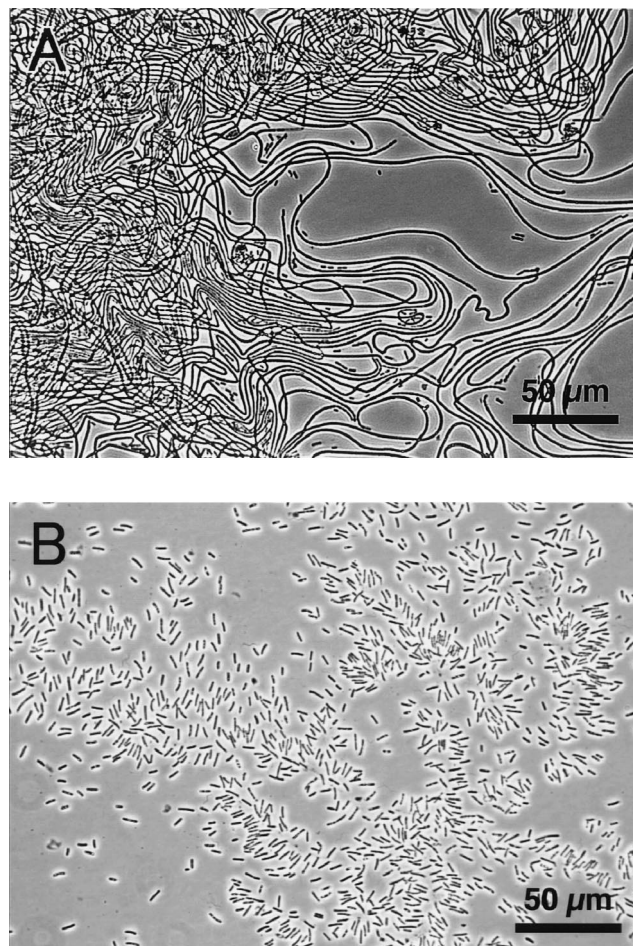


FIG. 2. Photomicrographs of *S. natans* cells. (A) Parental strain S-1 cells; (B) sheath-deficient mutant TM1 cells. Both strains were cultured in 0.1% NB.

base (e.g., WbpT in *Pseudomonas aeruginosa* [28% amino acid similarity], which is thought to be involved in exopolysaccharide [EPS]) synthesis [1]). The other Tn5 insertion, TM7 (accession number AB050639), was situated in a region homologous to genes encoding the putative RNA helicases or RNA polymerase-associated proteins (e.g., HepA in *E. coli* [43% amino acid similarity] [14]). Southern blot analyses suggested that these genes were located some distance from *sthA* and thus were not clustered with *sthA* on the genome (data not shown). In subsequent experiments we focused on the *sthA* gene.

Inactivation of *sthA*. Whether disrupting *sthA* blocked sheath formation was investigated by inactivating *sthA* by insertion of a Km^r cassette. The cassette was inserted at a unique *AseI* site in *sthA*, and successful insertion was confirmed by Southern blot hybridization and PCR amplification. The disruptant constructed (SKM01) grew as smooth colonies on 0.1% NB agar plates and as single cells in 0.1% NB and never formed a sheathed structure. This result, together with the results of the Tn5 mutagenesis experiments, strongly indicates that *sthA* is essential for sheath formation.

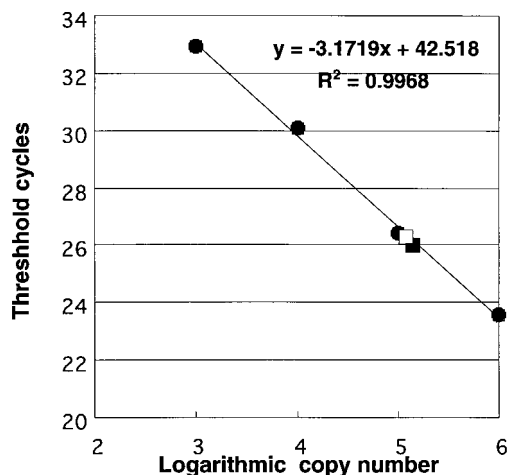


FIG. 3. Real-time quantitative RT-PCR assay for evaluating *sthA* transcription. A standard curve was generated by plotting the threshold cycle (the cycle at which the fluorescent signal became detectable above the background signal) as a function of the log of the template copy number. *sthA* mRNA levels in filamentous cells (□) and single cells (■) were calculated from the standard curve.

Analysis of *sthA* transcription. To determine whether *sthA* was expressed only by filamentous cells, we first attempted to detect the gene transcript by Northern blot hybridization. However, all of our attempts were unsuccessful, most likely because

the mRNA was either too unstable or too long to be transferred onto a nylon membrane. Therefore, we performed quantitative RT-PCR to determine the level of *sthA* transcription using strain S-1. As shown in Fig. 3, mRNAs encoding the *sthA* gene product were found in both single cells (grown on 1.0% NB agar plates) and filamentous cells (grown in 0.1% NB); in each case, approximately 1.0×10^5 copies per 10 ng of total RNA were detected, suggesting that the *sthA* gene is expressed at the same level in both morphotypes of cells.

To confirm this finding, we examined expression of *sthA* in living cells using the GFP gene as a reporter gene. The morphology of the mutant constructed (SGK01) was identical to that of the parental strain (S-1), and the ability to alter its morphology by using different NB concentrations was retained. The mutant was found to express GFP in both single and filamentous cells (Fig. 4), confirming that *sthA* was expressed regardless of the cell morphology.

Microscopic observation of single cells of the parental strain and the *sthA*-deficient mutant. Scanning electron microscopy revealed that the single-cell morphotype of parental strain S-1 (grown on a 1.0% NB agar plate) produced much larger amounts of extracellular matrix-like materials than an *sthA*-deficient mutant (TM1) produced (Fig. 5).

DISCUSSION

The results of Tn5 mutagenesis and a subsequent gene disruption analysis made it clear that the *sthA* gene is crucial for

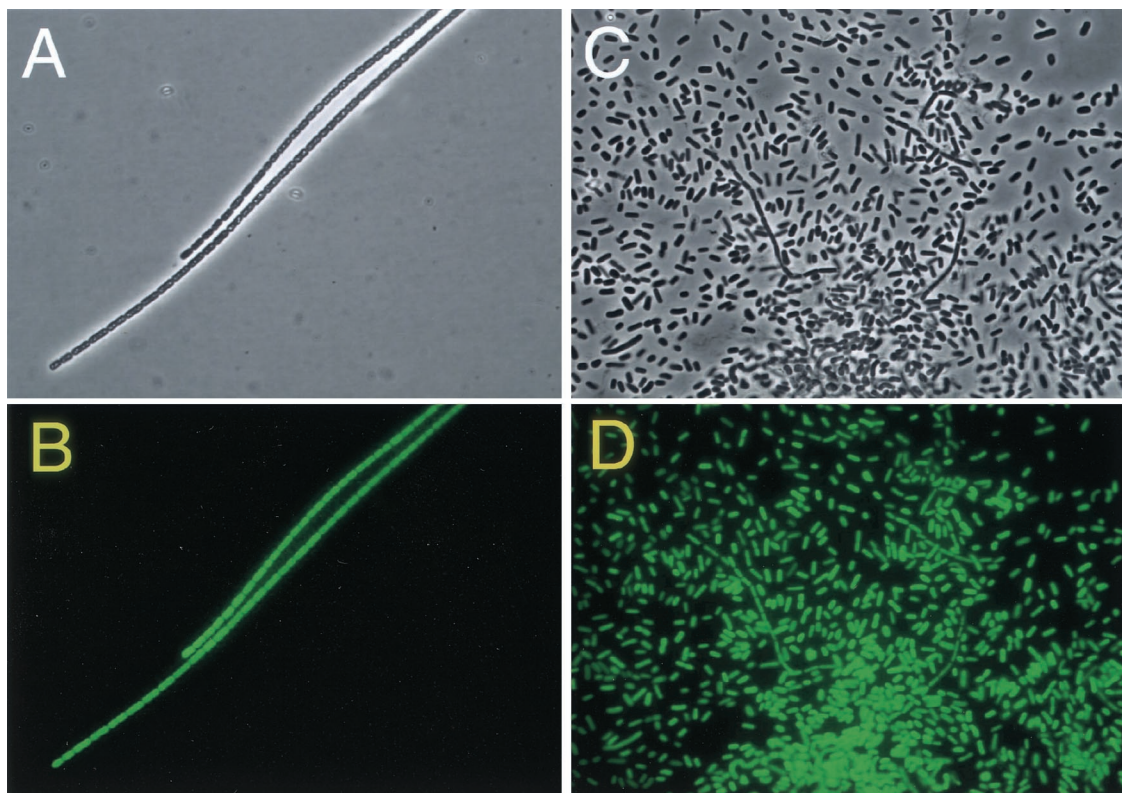


FIG. 4. Expression of GFP in filamentous and single cells. Filamentous cells (A and B) were obtained by growing the mutant with *gfp::Km^r* inserted (SGK01) in 0.1% NB, and single cells (C and D) were obtained by culturing the same mutant on 1.0% NB agar plates. (A and C) Phase-contrast micrographs; (B and D) GFP fluorescence emitted by the cells in panels A and C, respectively.

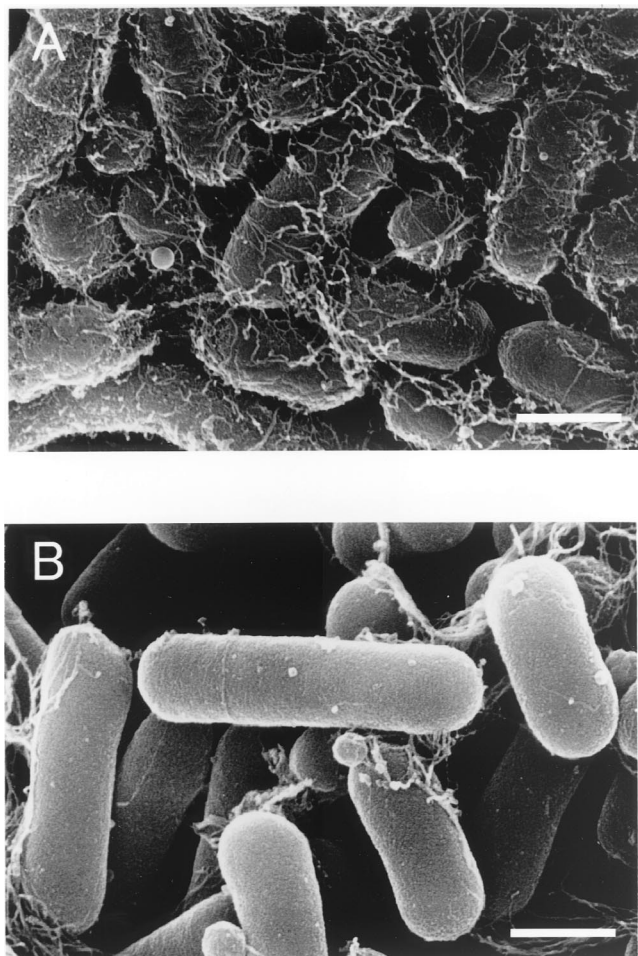


FIG. 5. Scanning electron micrographs of single *S. natans* cells. (A) Parental strain S-1 cells; (B) *sthA*-deficient mutant TM1 cells. Both strains were grown on 1.0% NB agar plates. Bars, 1 μ m.

sheath construction by *S. natans*. The putative amino acid sequence indicated that the *sthA* gene product is homologous to glycosyltransferases involved in bacterial EPS biosynthesis, including GumD from *Xanthomonas campestris* (31.6% amino acid identity) (2), EpsD from *Lactococcus lactis* (35.0% identity) (23), and ExoY from *Sinorhizobium meliloti* (37% identity) (17). These enzymes catalyze the transfer of sugar to undecaprenol phosphate, which acts as a sugar carrier in the membrane (15, 26). Considering that disrupting *sthA* by insertion of either Tn5 or a Km^r cassette blocked formation of the sheathed structure, the data strongly suggest that *sthA* encodes a similar enzyme catalyzing the glycosyl transfer essential for sheath formation.

Interestingly, transcriptional analyses based on quantitative RT-PCR or expression of the *gfp* reporter gene showed that *sthA* is transcribed in both filamentous and single cells. It has been reported that *S. natans* produces EPS at the outermost layer of both sheathed and single cells (6, 21). Our scanning electron microscopy results revealed that the single-cell morphology of the parental strain produced extracellular matrix-like material, which appeared to be EPS, whereas the *sthA*-deficient mutant produced far less extracellular material.

Disruption of *sthA* did not completely suppress extracellular polymer production, but it resulted in an obvious difference between the wild-type and *sthA*-deficient cells.

Our results strongly indicate that *sthA* is involved in both sheath synthesis and EPS synthesis. Moreover, genetic analysis of its downstream region revealed that *sthA* is followed by an ORF homologous to genes encoding EPS-exporting proteins (unpublished results). Thus, sheath formation and EPS formation very likely share common synthetic processes involved in the formation of extracellular materials.

As mentioned above, we also obtained two other transposon insertions that yielded sheathless mutants. Without doubt, sheath formation and EPS formation involve a number of synthetic steps and hence a number of genes, virtually none of which have been characterized. Still, the fact that five of the seven sheathless mutants were found to have disrupted *sthA* genes suggests that the *sthA* gene product is a crucial component of the mechanism responsible for sheath biosynthesis and subsequent assembly.

The filamentous form of *S. natans* is often observed in activated sludges, and a certain number of filaments are thought to be required for proper floc formation in order to retain the settleability of sludges (11, 20). However, excessive growth of the filamentous form of *S. natans* results in poor settling of sludge flocs (bulking). One effective way to prevent bulking would be to inhibit filamentous growth of this bacterium. If we could inhibit expression of the *sthA* gene or the activity of the *sthA* gene product with chemicals, we could temporarily inhibit the excess growth of filaments and thus prevent bulking when the size of the *S. natans* population approaches a bulking level. If similar genes are present in other filamentous bacteria, this method could be widely applicable for preventing bulking. In preliminary experiments, we found that *Leptothrix discophora*, which is phylogenetically affiliated with *S. natans*, also harbors *sthA* and its downstream genes (H. Yoshihara, T. Suzuki, and Y. Kamagata, unpublished data). Inhibition of filament formation is a new approach for preventing bulking. To better understand the pathway of filament formation, we should determine the in situ function of the products of *sthA* and its downstream genes, and such a study is under way.

REFERENCES

1. Belanger, M., L. L. Burrows, and J. S. Lam. 1999. Functional analysis of genes responsible for the synthesis of the B-band O antigen of *Pseudomonas aeruginosa* serotype O6 lipopolysaccharide. *Microbiology* **145**:3505–3521.
2. Chou, F. L., H. C. Chou, Y. S. Lin, B. Y. Yang, N. T. Lin, S. F. Weng, and Y. H. Tseng. 1997. The *Xanthomonas campestris* gumD gene required for synthesis of xanthan gum is involved in normal pigmentation and virulence in causing black rot. *Biochem. Biophys. Res. Commun.* **233**:265–269.
3. Dondero, N. C., R. A. Phillips, and H. Heukelekian. 1961. Isolation and preservation of culture of *Sphaerotilus*. *Appl. Microbiol.* **9**:219–227.
4. Eikelboom, D. H. 1975. Filamentous organisms observed in active sludge. *Water Res.* **9**:365–388.
5. Eikelboom, D. H., and H. J. J. van Buijsen. 1981. Microscopic sludge investigation manual. Report A94a. IMG-TNO, Delft, The Netherlands.
6. Gaudy, E., and R. S. Wolfe. 1961. Factors affecting filamentous growth of *Sphaerotilus natans*. *Appl. Microbiol.* **10**:1962–1968.
7. Hoeniger, J. F., H. D. Tauschel, and J. L. Stokes. 1973. The fine structure of *Sphaerotilus natans*. *Can. J. Microbiol.* **19**:309–313.
8. Howarth, R., R. F. Unz, E. M. Seviour, R. J. Seviour, L. L. Blackall, R. W. Pickup, J. G. Jones, J. Yaguchi, and I. M. Head. 1999. Phylogenetic relationships of filamentous sulfur bacteria (*Thiothrix* spp. and Eikelboom type 021N bacteria) isolated from wastewater-treatment plants and description of *Thiothrix eikelboomii* sp. nov., *Thiothrix unzii* sp. nov., *Thiothrix fructosivorans* sp. nov. and *Thiothrix defluvi* sp. nov. *Int. J. Syst. Bacteriol.* **49**:1817–1827.
9. Jenkins, D., M. G. Richard, and G. T. Daigger. 1993. Manual on the causes

- and control of activated sludge bulking and forming, 2nd ed. Lewis Publishers, Chelsea, Mich.
10. **Kanagawa, T., Y. Kamagata, S. Aruga, T. Kohno, M. Horn, and M. Wagner.** 2000. Phylogenetic analysis of and oligonucleotide probe development for Eikelboom type 021N filamentous bacteria isolated from bulking activated sludge. *Appl. Environ. Microbiol.* **66**:5043–5052.
 11. **Lao, A. O., P. F. Strom, and D. Jenkins.** 1984. Growth kinetics of *Sphaerotilus natans* and a floc former in pure and dual continuous culture. *J. Water Pollut. Control Fed.* **56**:41–51.
 12. **Mulder, E. G.** 1989. Sheathed bacteria, p. 1994–2008. In J. T. Staley, M. P. Bryant, N. Pfennig, and J. G. Holt (ed.), *Bergey's manual of systematic bacteriology*, vol. 3. Williams and Wilkins, Baltimore, Md.
 13. **Murray, M. G., and W. F. Thompson.** 1980. Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res.* **8**:4321–4325.
 14. **Muzzin, O., E. A. Campbell, L. Xia, E. Severinova, S. A. Darst, and K. Severinov.** 1998. Disruption of *Escherichia coli* HepA, an RNA polymerase-associated protein, causes UV sensitivity. *J. Biol. Chem.* **273**:15157–15161.
 15. **Reeves, P. R., M. Hobbs, M. A. Valvano, M. Skurnik, C. Whitfield, D. Coplin, N. Kido, J. Klena, D. Maskell, C. R. Raetz, and P. D. Rick.** 1996. Bacterial polysaccharide synthesis and gene nomenclature. *Trends Microbiol.* **4**:495–503.
 16. **Rensink, J. H.** 1974. New approach to preventing bulking sludge. *J. Water Pollut. Control Fed.* **46**:1888–1894.
 17. **Reuber, T. L., and G. C. Walker.** 1993. Biosynthesis of succinoglycan, a symbiotically important exopolysaccharide of *Rhizobium meliloti*. *Cell* **74**:269–280.
 18. **Romano, A. H., and J. P. Peloquin.** 1963. Composition of the sheath of *Sphaerotilus natans*. *J. Bacteriol.* **86**:252–258.
 19. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
 20. **Sezgin, M., D. Jenkins, and D. S. Parker.** 1978. A unified theory of filamentous activated sludge bulking. *J. Water Pollut. Control Fed.* **50**:362–381.
 21. **Takada, M., K. Iohara, S. Shinmaru, I. Suzuki, and J. Koizumi.** 2000. Purification and properties of an enzyme capable of degrading the sheath of *Sphaerotilus natans*. *Appl. Environ. Microbiol.* **66**:4998–5004.
 22. **Takeda, M., F. Nakano, T. Nagase, K. Iohara, and J. Koizumi.** 1998. Isolation and chemical composition of the sheath of *Sphaerotilus natans*. *Biosci. Biotechnol. Biochem.* **62**:1138–1143.
 23. **van Kranenburg, R., J. D. Marugg, S. van Swam II, N. J. Willem, and W. M. de Vos.** 1997. Molecular characterization of the plasmid-encoded *eps* gene cluster essential for exopolysaccharide biosynthesis in *Lactococcus lactis*. *Mol. Microbiol.* **24**:387–397.
 24. **van Veen, W. L., E. G. Mulder, and M. H. Deinema.** 1978. The *Sphaerotilus-Leptothrix* group of bacteria. *Microbiol. Rev.* **42**:329–356.
 25. **Wanner, J.** 1994. *Activated sludge bulking and foaming control*. Technomic Publishing Company, Lancaster, Pa.
 26. **Whitfield, C., and M. A. Valvano.** 1993. Biosynthesis and expression of cell-surface polysaccharides in gram-negative bacteria. *Adv. Microb. Physiol.* **35**:135–246.