Characterization of cytoplasmic fibril structures found in gliding cells of *Saprospira* sp.

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Abstract: The cytoplasmic fibril structures of *Saprospira* sp. strain SS98-5 grown on a low-nutrient agar medium were purified from cell lysates treated with Triton X-100 and were observed by electron microscopy to be about 7 nm in width and 200–300 nm in length. SDS–PAGE of the fibril structures exhibited a single protein band with a molecular mass of 61 kDa. A *Saprospira* cytoplasmic fibril protein (SCFP), which is a subunit of the fibril structures, was digested with trypsin to oligopeptides and analyzed for amino acid sequences. A partial nucleotide sequence of the SCFP gene was determined after PCR using primers designated from the amino acid sequences of the oligopeptides. SCFP gene including DNA fragments were detected by Southern hybridization using the PCR product for an SCFP gene as a probe and were cloned to determine whole nucleotide sequences. The SCFP gene indicated relatively higher similarity to conserved hypothetical phage tail sheath proteins. A Western immunoblotting analysis showed that SCFP was significantly expressed in gliding cells as compared with nongliding cells. The above findings with the previously reported results suggest that the cytoplasmic fibril structures are possibly related to the gliding motility of *Saprospira* sp. strain SS98-5.

Key words: Saprospira, gliding motility, *Saprospira* cytoplasmic fibril protein (SCFP).

Résumé : Les structures cytoplasmiques fibrillaires de la souche SS98-5 de *Saprospira* sp. cultivée dans un milieu gélosé à basse teneur en nutriments ont été purifiées de lysats cellulaires traités au Triton X-100 et leur taille telle que mesurée par microscopie électronique était d'environ 7 nm de largeur et de 200–300 nm de longueur. Une seule bande de protéine d'un masse moléculaire de 61 kDa fut révélée par un SDS–PAGE des structures fibrillaires. La protéine cytoplasmique fibrillaire de *Saprospira* (SCFP), qui est une sous-unité des structures fibrillaires, fut digérée avec de la trypsine pour produire des oligopeptides et analysée par séquençage des acides aminés. Une séquence de nucléotides partielle du gène de la SCFP fut déterminée par PCR en utilisant des amorces élaborées à partir des séquences en acides aminés des oligopeptides. Des fragments d'ADN renfermant le gène de la SCFP furent détectés par hybridation de type Southern en utilisant les produits de PCR de gènes de la SCFP comme sondes et furent clonés afin de déterminer la séquence nucléotidique complète. Le gène de la SCFP a démontré une similitude relativement élevée à des protéines hypothétiques conservées d'enveloppes de queues phages. Une analyse par immunobuvardage de type Western a démontré que la SCFP était significativement exprimée chez les cellules glissantes comparativement aux cellules non glissantes. Les découvertes présentées ici de concert avec des résultats mentionnés précédemment indiquent que les structures cytoplasmiques fibrillaires sont probablement liées à la mobilité par glissement de la souche SS98-5 de *Saprospira* sp.

Mots clés : Saprospira, mobilité par glissement, protéine cytoplasme fibrillaire de *Saprospira* (SCFP).

[Traduit par la Rédaction]

Introduction

Bacterial gliding motility is defined as a smooth movement of cells on a biotic or abiotic surface without flagella and is considered to be involved in surface colonization, biofilm formation, and host invasion (Harshey 2003). Bacteria that exhibit gliding motility are taxonomically diverse and belong to the myxobacteria, the mycoplasma, the cyanobacteria, and the *Cytophaga–Flavobacterium–Bacteroides* group. Many different models have been proposed to explain bacterial gliding motility. For example, three major cellular components, such as type IV pili, extracellular fibril materials consisting of polysaccharides and proteins, and lipopolysaccharide O antigen, are involved in S motility of *Myxococcus xanthus* (Wall and Kaiser 1999; Wu and Kaiser 1995; Bowden and Kaplan 1998; Sporman 1999). Type IV pili are also connected with the gliding motility of *Synechocystis* PCC6803, a unicellular cyanobacterium (Bhaya et al. 1999). In addition, *Phormidium uncinatum*, a filamentous cyanobacteria, and *M. xanthus* in A-motility are propeled by propulsion of polysaccharides outside the cell from an organelle called the junctional pore complex (Wolgemuth et al. 2002; Wolgemuth and Oster 2004). In the case of *Mycoplasma mobile*, the Gli349 protein, which is likely to form the spike structures, is known to be involved in glass binding and gliding (Uenoyama et al. 2004; Kusumoto et al.

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2004; Miyata and Petersen 2004). On the other hand, specific structures involved in gliding motility have never been reported in *Cytophaga–Flavobacterium–Bacteroides* bacteria, which include *Saprospira* spp. In *Flavobacterium johnsoniae*, nine *gld* genes (*gldA*, *gldB*, *gldC*, *gldD*, *gldE*, *gldF*, *gldG*, *gldH*, and *gldI*) and *ftsX* that are required for gliding motility have been identified (Agarwal et al. 1997; Hunnicutt and McBride 2000, 2001; Hunnicutt et al. 2002; McBride et al. 2003; McBride and Braun 2004; Kempf and McBride 2000). GldA, GldF, and GldG are found to compose ATP-binding cassette transport proteins. GldB, GldD, and GldH are lipoprotein components, which are localized in the cytoplasmic membrane, and their exact role in gliding motility is unknown. GldI is similar to peptidyl–prolyl *cis/trans*-isomerases of the FK506-binding protein family and may be involved in folding cell envelope protein components of the motility machinery.

Saprospira spp. isolated from marine environments showed algicidal activity against microalgal cells and remarkable gliding motility (Sakata 1990; Furusawa et al. 2003). While their gliding motility was thought to play an important role in the attacking microalgae, their relationship was not yet elucidated. In a previous study (Furusawa et al. 2003), it was demonstrated that the gliding motility of *Saprospira* sp. strain SS98-5 was stimulated by incubating the cells on agar plates with low concentrations of polypeptone (0.05%), while it was suppressed by increasing the polypeptone concentration to 0.5%. Furthermore, transmission electron microscopy showed numerous fibril structures in the gliding cells of SS98-5 but not in nongliding cells. When 100 μg podophyllotoxin/mL, an inhibitor of microtubule polymerization, was added to the culture medium, gliding motility was suppressed and the fibril structures were not observed in the cells. These findings indicate that the cytoplasmic fibril structures were related to cell gliding motility and that podophyllotoxin inhibited the polymerization and assemblage of the fibril structure components as well as those of eukaryotic microtubules (Furusawa et al. 2003).

In this study, we designated a subunit protein constituting the fibril structure *Saprospira* cytoplasmic fibril protein (SCFP), and to determine the involvement of the fibril structures in gliding motility, its expression was analyzed using an anti-SCFP antibody in gliding and nongliding cells of *Saprospira* sp. strain SS98-5 as well as the newly isolated *Saprospira* spp. strains SS03-3 and SS03-4. Furthermore, we determined a nucleotide sequence of the gene encoding SCFP.

Materials and methods

Bacterial strains and culture media

Saprospira sp. strain SS98-5, described in a previous paper, (Furusawa et al. 2003) was mainly used in this study. Algicidal and gliding strains SS03-3 and SS03-4 were newly isolated from coastal regions of Kagoshima Bay, Kagoshima, Japan, in 2003 and compared with a *Saprospira* sp. strain SS98-5. Gliding and nongliding cells of *Saprospira* spp. were obtained by incubating them on ESS agar media containing either 0.05% or 0.5% polypeptone, respectively, at 25 °C for 3 days, as described in a previous paper (Furusawa et al. 2003).

SCFP purification

Gliding cells of strain SS98-5 were suspended in 10 mmol Tris–HCl/L (pH 6.8). The cells were disrupted by ultrasonication on ice for 3 min. The resulting homogenate was centrifuged at 5000*g* for 15 min at 4 °C. To remove membrane fractions, the supernatant was collected, mixed with an equal volume of 2% Triton X-100 solution, and stirred overnight at 4 °C. The lysate treated with Triton X-100 was centrifuged at 100 000*g* for 1 h, and Tris–HCl buffer was added to the precipitate to wash the fibril structures. The suspension of the precipitate was kept on ice overnight and centrifuged at 5000*g* for 20 min. The supernatant obtained was centrifuged at 100 000*g* for 1 h. The precipitate was subjected to negative staining with uranyl acetate and observed under a transmission electron microscope (Hitachi 7100-F; Hitachi Ltd., Tokyo, Japan) and subjected to 12.5% SDS–PAGE (Laemmli 1970) to check its purity.

Preparation of anti-SCFP antibody

The precipitates containing the fibril structures were subjected to 7.5% SDS–PAGE, and then the protein band (61 kDa) including SCFP was extracted using a Biotrap electron-separation system for elution and purification of charged macromolecules (Schleicher & Schuell, Dassel, Germany). A polyclonal antibody against purified SCFP was prepared from rabbits by QIAGEN (Tokyo, Japan).

Western immunoblotting analysis

Three milligrams each of gliding or nongliding cells of SS98-5, SS03-3, and SS03-4 was suspended in 100 μL of SDS–PAGE sampling buffer (0.125 mol Tris–HCl/L, 10% 2-mercaptoethanol, 4% SDS, 10% sucrose, and 0.004% bromophenol blue) (Laemmli 1970) and the cells were heat treated at 100 °C for 10 min. Five microlitres of each sample was applied to 10% SDS–PAGE, and protein bands were transferred to a polyvinylidene fluoride membrane using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad, Hercules, California). Blocking was performed using 0.5% skim milk in PBS (137 mmol NaCl/L, 8.1 mmol Na_2HPO_4/L, 2.68 mmol KCl/L, and 1.47 mmol KH_2PO_4/L), and after shaking at room temperature for 1 h, the membrane was washed three times using a washing buffer (PBS and 0.5% Tween 20). After allowing the proteins to react with the polyclonal anti-SCFP antibody (1/5000 dilution) at room temperature for 1 h, the polyvinylidene fluoride membrane was again washed three times using the washing buffer and allowed to react with anti-rabbit IgG goat antibody labeled with horseradish peroxidase (1/10 000 dilution). Detection was performed using ECL Plus Western Blotting Reagents (Amersham Biosciences, Piscataway, New Jersey) and Lumino Image Analyzer LAS-1000 (Fuji Film, Tokyo, Japan).

Amino acid sequence analysis of SCFP

SCFP transferred to the polyvinylidene fluoride membrane was digested with trypsin to oligopeptides. These oligopeptides were separated by reversed phase chromatography, and two oligopeptides (fragments 28 and 43) were chosen

Table 1. Primers used for nested PCR amplification of the *Saprospira* sp. strain SS98-5 SCFP gene.

Amino acid sequence and primer	Nucleotide sequence
QYATPGVY	
28-S1	5′-CARTAYGCNACNCCNGG-3′
28-S2	5′-TCACTTCCTGCAGGCNACNCCNGGNGTNTA-3′
28-AS1	5′-TANACNCCNGGNGTNGC-3′
28-AS2	5′-TCACTTCCTGCAGGCCNGGNGTNGCRTAYTG-3′
VAISRPAEFIVI	
43-S1	5′-GTNGCNATHWSNMGNCC-3′
43-S2	5′-ACTTGCGGCCGCATHWSNMGNCCNGCNGA-3′
43-AS1	5′-ATNACDATRAAYTCNGC-3′
43-AS2	5′-ACTTGCGGCCGCATRTTYTCNGCNGGNCK-3′

Note: H = A + T + C, W = A + T, S = C + G, M = A + C, D = A + T + G, R = A + G, Y = C + T, K = T + G, and N = A + C + G + T.

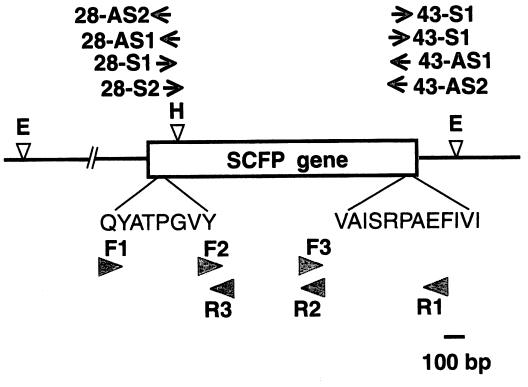
for amino acid sequence analysis (customer service of protein primary structures, Takara Bio, Otsu, Japan).

Nucleotide sequence analysis of the SCFP gene

To determine a partial nucleotide sequence of the SCFP gene, we designed forward primers 28-S1, 28-S2, 43-S1, and 43-S2 and reverse primers 28-AS1, 28-AS2, 43-AS1, and 43-AS2 from the partial amino acid sequences as shown in Table 1. In the first step of the PCR, the primers 28-S1, 43-AS1, 43-S1, and 28-AS1 were used, and after electrophoresis, PCR products were removed from the gel and purified to be used as a template for the second step of the PCR. In the second step of the PCR, the primers 28-S2, 43-AS2, 43-S2, and 28-AS2 were used (Fig. 1). PCR products obtained with the primers 28-S2 and 43-AS2 were electrophoresed and then recovered from the gel to be cloned into a pGEM®-T Easy Vector (Promega, Madison, Wisconsin). Determination of the sequence was performed using a BigDye Terminator v1.1 Cycle Sequencing Kit and ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, California). To obtain a whole sequence of the SCFP gene, partial libraries, in which SCFP gene including fragments were enriched, were constructed. Briefly, total DNA from SS98-5 was treated with *Eco*RI and *Hin*dIII and subjected to agarose gel electrophoresis. The sizes of the SCFP gene including DNA fragments were checked by a Southern hybridization analysis with the PCR-amplified partial SCFP gene fragments as a probe, and two fragments (1.8 and 3.0 kb) containing the SCFP gene were recovered from the gel. The DNA fragments were cloned into pUC19 to construct partial libraries and screened by Southern hybridization with the same probe as mentioned above. Nucleotide sequences of cloned DNA fragments were determined with an ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit and ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

Finally, to confirm the continuity of the obtained nucleotide sequences, PCR amplification was carried out with primers designed at both termini of the sequenced regions as follows: F1 (5′-TGGTTGACTTTTTTGGGCCGAG-3′), F2 (5′-TGCTCAATTGATCGGTGCCG-3′), F3 (5′-CCTGCAAAC GTAAGCATCTC-3′), R1 (5′-CGACTCGCTATAATCAAC AG-3′), R2 (5′-ATGCTTACGTTTGCAGGAGC-3′), and R3 (5′-TGATACCTCCACCATTAGCG-3′) (Fig. 1).

Fig. 1. SCFP gene structure of *Saprospira* sp. strain SS98-5. Primers 28-S2 and 43-AS2 were designed based on a partial sequence of the SCFP gene. F1, F2, F3, R1, R2, and R3 indicate sequencing primers. Restriction sites: E, *Eco*RI; H, *Hin*dIII.



Accession numbers

Accession numbers of the SCFP gene nucleotide sequence of SS98-5 and the small subunit ribosomal DNA (SSU rDNA) nucleotide sequences of SS03-3 and SS03-4 in the DNA Databank Japan (DDBJ) database were AB190931, AB191039, and AB191040, respectively.

Results

Characterization of newly isolated strains SS03-3 and SS03-4

The cells of bacterial strains SS03-3 and SS03-4 grew as helical multicellular filaments and possessed algicidal activity against *Chaetoceros ceratosporum* (Bacillariophyceae) on double-layered agar plates. They were Gram-negative, moved by gliding, yellow–orange pigmented, and not fermented in OF medium with glucose, suggesting that they belong to the genus *Saprospira* similar to SS98-5. A phylogenetic analysis based on SSU rDNA demonstrated that SS03-3 and SS03-4, whose sequences were completely identical, fell into the same cluster as *Saprospira grandis* and SS98-5, supporting their close taxonomical relationship (data not shown).

Isolation of cytoplasmic fibril structures and SCFP

The cytoplasmic fibril structures observed in the SS98-5 cells were obtained by Triton X-100 treatment and ultracentrifugation of the cell homogenate after ultrasonic disintegration. The straight fibril structures about 7 nm in diameter and 200–300 nm in length were observed in the precipitate preparations negatively stained by uranium acetate under a transmission electron microscope, infrequently associated with a hairpin configuration (Fig. 2). This result is consistent with transmission electron microscopic observation for ultrathin sections of SS98-5 cells demonstrated in a previous paper (Furusawa et al. 2003). The pellets including the fibril structures were analyzed using 12.5% SDS–PAGE, and a major band with a molecular mass of 61 kDa was detected (Fig. 3B). This band was also identified as a major band in SDS–PAGE of whole-cell proteins of gliding cells of SS98-5 (Fig. 3A). These results suggested that this subunit protein, designated as SCFP, was a component of the fibril structures.

Western immunoblotting analysis of SCFP

To compare the expression of the SCFP gene between gliding and nongliding cells of strains SS98-5, SS03-3, and SS03-4, Western immunoblotting analysis was performed using a polyclonal anti-SCFP antibody. The analysis demonstrated that SCFP was highly detected in gliding cells of these strains in comparison with nongliding cells (Fig. 4). As described in a previous paper (Furusawa et al. 2003), transmission electron microscopic observation showed that fibril structures were almost not observed in nongliding cells of strain SS98-5. SCFP was detected by Western immunoblotting analysis for nongliding cells of this strain, although the concentration was considerably lower than that of gliding cells.

Sequence analysis of the SCFP gene

The nucleotide sequences of the SCFP gene and its flanking regions were examined and an open reading frame (1500 bp) was found. The open reading frame included the same deduced amino acid sequences as QYATPGVY and VAISRPAEFIVI determined by partial amino acid sequencing of SCFP, indicating that the open reading frame corresponded to an SCFP gene. BLAST and MOTIF analyses were conducted based on the deduced amino acid sequence of the SCFP gene. There was no homology with already-known cytoskeleton-related proteins, and any motifs or domains of known cytoskeleton components were not found. However, SCFP gene indicated the deduced amino acid sequence similarity to *Photorhabdus luminescens* subsp. *laumondii* TT01 hypothetical protein plu2387 (32% identity over 497 amino acids), *Nostoc* sp. PCC 7120 hypothetical protein all3325 (37% identity over 518 amino acids), and *Serratia entomophila* Afp3 (30% identity over 519 amino acids).

Fig. 2. Transmission electron micrograph of negatively stained fibril structures collected from *Saprospira* sp. strain SS98-5. The fibril structures were obtained by ultracentrifugation of Triton X-100 treated cell homogenate. The sizes were 7 nm in diameter and 200–300 nm in length. The arrowheads indicate a hairpin configuration. Scale bar = 200 nm.

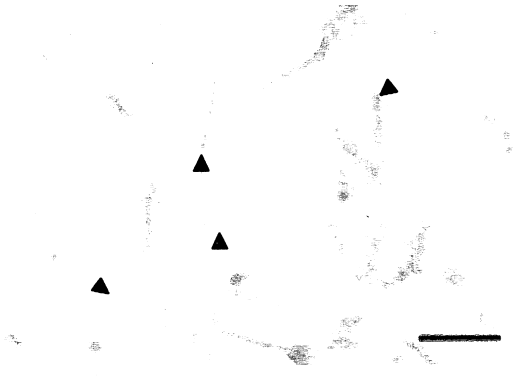


Fig. 3. SDS–PAGE of whole-cell proteins and fibril structures obtained from *Saprospira* sp. strain SS98-5. (A) Whole-cell proteins of nongliding cells (lane 1) and gliding cells (lane 2). The arrowhead indicates a major band that seems to be a fibril structure component. (B) Fibril structure component (arrow) purified from gliding cells. Molecular masses are shown on the left.

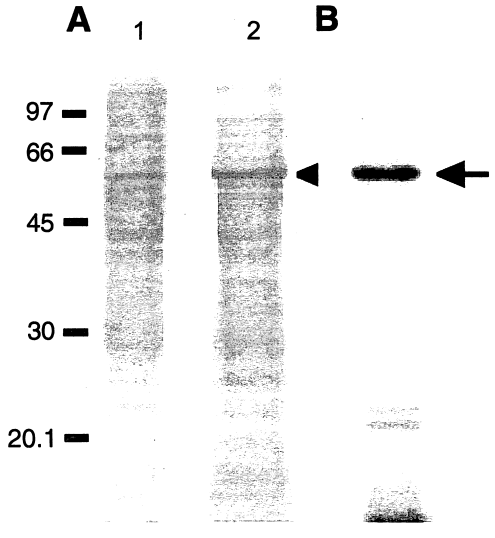
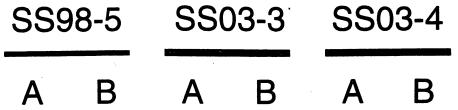


Fig. 4. Western immunoblotting analyses of SCFP in gliding and nongliding cells of *Saprospira* sp. strains SS98-5, SS03-3, and SS03-4. A and B indicate nongliding and gliding cell samples, respectively.



Discussion

Recently, various kinds of intracellular filament structures were observed in different prokaryotes. In known eukaryotic cells, microtubules are composed of different types of tubulin and have an outer diameter of approximately 24 nm, while microfilaments consist mainly of globular actin and are observed as a filamentous structure 7 nm in diameter. On the other hand, microtubule-like and microfilament-like struc-

tures of some prokaryotes have been reported to occur in the cells, although the amino acid sequences among the element proteins of eukaryotes and prokaryotes are not homologous. For example, *Mycoplasma gallisepticum* was found to have tubular structures that cross-reacted with anti-pig brain tubulin antibodies, suggesting that the tubular system is involved in mycoplasma motility (Korolev et al. 1994). The genus *Prosthecobacter* was shown to have two tubulin-like genes quite divergent from those of eukaryotes (Jenkins et al. 2002). Furthermore, some spiroplasmas contain fibril structures in their cytoplasm that are smaller than microtubules and more similar to actin. Fibril structures, approximately 5 nm in diameter, were released from a spiroplasma by treatment with detergents and associated in pairs, which were composed of a single protein of molecular mass 59 kDa (Trachtenberg 1998; Trachtenberg et al. 2003). A hypothesis that the association of helically wound 5-nm fibrils with the inner surface of the cytoplasmic membrane are involved in generating a helix and producing rotary movement of spiroplasma has been proposed

In addition, FtsZ, MreB, ParM, and CreS are known as components of cytoskeletal structures in prokaryotes. FtsZ, which is speculated to be an ancestor of eukaryotic tubulin, plays a key role in the division of bacterial cells and eukaryotic mitochondria and chloroplasts and assembles to form a Z ring at division septa (Carballido-López and Errington 2003; Dai and Lutkenhaus 1991; Bi and Lutkenhaus 1991). MreB is involved in the regulation of bacterial cell shape by forming dynamic helical filaments underneath the cytoplasmic membrane (Jones et al. 2001). ParM forms F-actin-like filaments and is necessary for segregation of a plasmid R1 to *Escherichia coli* daughter cells during cell division (Møller-Jensen et al. 2002). CreS is required for the vibrioid and helical shapes of *Caulobacter crescentus*, and the localization pattern of CreS is similar to that of MreB and ParM (Ausmees et al. 2003; Margolin 2004). However, the cytoplasmic fibril structures of *Saprospira* strain SS98-5 were found to form a bundle of more than 10 fibril structures longitudinally, and these structures are apparently different from those of FtsZ, MreB, ParM, and CreS.

Algicidal multicellular filaments of *Saprospira* spp. possess cytoplasmic fibril structures under gliding conditions, and a subunit protein designated as SCFP exhibits the deduced amino acid sequence similarity to *P. luminescens* subsp. *laumondii* TT01 hypothetical protein plu2387, *Nostoc* sp. PCC 7120 hypothetical protein all3325, and *S. entomophila* Afp3. Proteins plu2387 and Afp3 showed high similarity to the tail sheath protein domains of the bacteriophages T4 and P2 (Hurst et al. 2004), and Afp3 is encoded by a gene of a putative defective prophage that is essential to antifeeding activity against the grass grub *Costelytra zealandica* (Hurst et al. 2004).

In addition, InteroPro Scan showed that open reading frames found at downstream regions on the SS98-5 and SS03-4 SCFP genes had relatively higher similarity (about 30%) to conserved hypothetical phage tail region proteins (accession No. IPR0117). Genes encoding these hypothetical proteins were considered to be in prophages or laterally transferred regions of some bacterial genomes, and tentatively identified neighboring genes tend to encode phage tail region proteins. It is suggested that SCFP is expressed from a phage tail protein gene of a defective prophage, which is overproduced by SS98-5 under low-nutrient conditions. There is also a possibility that the cytoplasmic fibril structures composed from SCFP are related to gliding motility of *Saprospira* sp. strain SS98-5.

In a previous paper (Furusawa et al. 2003), it was suggested that the growth response of SS98-5 cells in high or low polypeptone concentrations offered a clue to elucidate a relationship between the cytoplasmic fibril structures and the gliding motility. However, this phenomenon does not provide any direct evidence for the involvement of these structures in the gliding motility. Although some models for gliding motility of prokaryotes have been proposed in myxobacteria, mycoplasma, and cyanobacteria, the mechanism of gliding motility connected with cytoplasmic fibril structures remains unexplained. Further studies are expected to elucidate the function and relationship of SCFP to the cell motility of *Saprospira* spp.

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