

# Algicidal activity and gliding motility of *Saprospira* sp. SS98-5

Gou Furusawa, Takeshi Yoshikawa, Akihiro Yasuda, and Taizo Sakata

**Abstract:** A marine bacterium, *Saprospira* sp. SS98-5, which was isolated from Kagoshima Bay, Japan, was able to kill and lyse the cells of the diatom *Chaetoceros ceratosporum*. The multicellular filamentous cells of this bacterium captured the diatom cells, formed cell aggregates, and lysed them in an enriched sea water (ESS) liquid medium. Strain SS98-5 also formed plaques on double layer agar plates incorporating diatom cells. The diatom cell walls were partially degraded at the contact sites with the bacteria, the bacteria invaded from there into the diatom cells, and then the diatom cells were completely lysed. The strain possessed gliding motility and grew as spreading colonies on ESS agar plates containing lower concentrations of polypeptone (below 0.1%) while forming nonspreading colonies on ESS agar plates containing 0.5% polypeptone. Electron micrographs of ultrathin sections demonstrated that microtubule-like structures were observable only in gliding motile cells. Both the gliding motility and the microtubule-like structures were diminished by the addition of podophyllotoxin, an inhibitor of microtubule assembly, suggesting that the microtubule-like structures observed in these bacterial cells are related to their gliding motility.

**Key words:** *Saprospira* sp., *Chaetoceros ceratosporum*, gliding motility, algicidal activity, microtubule-like structure.

**Résumé :** La souche SS98-5 de *Saprospira* sp. est une bactérie marine isolée de la Baie de Kagoshima au Japon. Ce microorganisme est capable de lyser et de tuer la diatomée *Chaetoceros ceratosporum*. Les cellules filamenteuses de cette bactérie sont capables de capter les diatomées, de former des agrégats cellulaires et de causer leur lyse dans un milieu liquide ESS. La souche SS98-5 entraîne la formation de plages sur des géloses à double couche contenant des diatomées incorporées. Les parois de cellules de diatomées étaient partiellement dégradées au point de contact avec les bactéries qui par la suite envahissaient les cellules de diatomées à partir de ce site et finalement lysaient totalement les cellules de diatomées. Cette souche présentait une mobilité par glissement et formait des colonies envahissantes sur des géloses ESS qui contenaient des concentrations faibles de polypeptone (moins de 0,1 %), mais les colonies n'étaient plus envahissantes sur des géloses contenant 0,5 % de polypeptone. Les micrographies électroniques de coupes minces ont permis d'observer des structures en forme de tubules uniquement chez les cellules mobiles par glissement. Cette mobilité par glissement et les structures en forme de microtubules étaient réduites lors de l'addition de podophyllotoxine, un inhibiteur de l'assemblage des microtubules. Ces observations suggèrent que les structures en forme de microtubules observées chez ces cellules bactériennes seraient apparentées à la mobilité par glissement.

**Mots clés :** *Saprospira* sp.; *Chaetoceros ceratosporum*; mobilité par glissement, activité algicide; structure en forme de microtubule.

[Traduit par la Rédaction]

## Introduction

Some unicellular microalgae such as diatoms are known to be suitable for feeding to juvenile fish in aquaculture and are cultivated massively in culture tanks to be supplied as primary live feed. However, cultured microalgal cells often aggregate and precipitate rapidly and are discarded as waste. Filamen-

tous multicellular bacteria often isolated from the culture tanks are suspected to cause this phenomenon. Some investigators reported that helical filamentous bacteria belonging to the genus *Saprospira*, which are commonly isolated from freshwater or marine environments (Lewin 1965a, 1965b; Lewin and Mandel 1967), prey on prokaryotic microorganisms such as bacteria and cyanobacteria (Ashton and Robarts 1987; Sangkhobol and Skerman 1981). We were able to isolate algicidal *Saprospira*-like bacteria from coastal seawater of Kagoshima Bay, Japan, by using double layer agar plates containing the diatom *Chaetoceros ceratosporum* (Sakata 1990; Sakata et al. 1991; Iwamoto et al. 2001). In general, algicidal bacteria can be categorized into direct and indirect attack types. The indirect attack type bacteria may kill algal cells through the extracellular production of algicidal substances. For example, the culture supernatant of marine bacteria, such as *Pseudomonas* sp. (Baker and Herson 1978) and *Pseudoaltermonas* sp. (Lovejoy et al. 1998; Lee et al. 2000), exhibits a killing activity against marine microalgae. The

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direct attack type bacteria include *Myxobacter* spp. (Shilo 1970; Yamamoto and Suzuki 1977), *Cytophaga* spp. (Imai et al. 1993; Mitsutani et al. 1992), and *Saprospira* spp. (Ashton and Roberts 1987; Lewin 1997; Sakata et al. 1991). The lysis of cyanobacteria by *Myxobacter* spp. has been observed under phase-contrast microscopy (Shilo 1970) and electron microscopy (Yamamoto and Suzuki 1977). Since direct attack type bacteria necessitate contact with microalgal cells for algicidal activity, their cell motility is critical. *Saprospira* spp. are unique in exhibiting gliding motility of multicellular filaments. The actual algicidal mechanisms of these bacteria, however, remain unclear.

In this report, we examine the diatom cell aggregation in liquid cultures, the plaque formation on double layer agar plates with diatom cells, and the colony formation on peptone agar plates. The ultrastructure of gliding and nongliding bacterial cells was also compared by using a transmission electron microscope to elucidate the algicidal process of strain SS98-5 against diatom cells.

## Materials and methods

### Cultivation of bacteria and diatoms

A bacterial strain SS98-5 showing algicidal activity was isolated from Kagoshima Bay, Japan, in 1998 by a double layer agar technique. The strain was cultivated in a yeast extract-enriched sea water (YES) medium (Sakata and Yasumoto 1991) containing 0.5% agar at 25°C for 3 days and stored at 16°C. The bacterial cells were pre-incubated at 25°C with shaking in ZoBell enriched sea water (ZE-CI) liquid medium (Sakata and Yasumoto 1991) for 3 days before being used in the following experiments. A *C. ceratosporum* strain (provided by Dr. Fukami of Kochi University, Japan) was maintained at 23°C under white fluorescent lamps (5000 lx, 12 h light : 12 h dark cycle) in 10 mL of enriched sea water (ESS) liquid medium as described previously (Sakata et al. 1991). The diatom cells were incubated for 7–10 days in flasks containing 300 mL of ESS liquid medium with aeration.

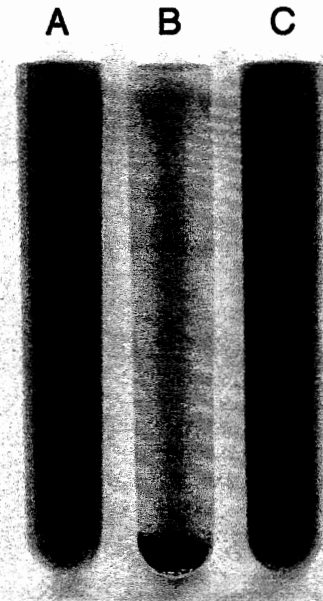
### Formation of cell aggregates

Bacterial cells grown in 30 mL of ZE-CI liquid medium for 3 days were harvested by centrifugation at 12 000 × g for 15 min at 4°C. The obtained cell pellets were washed twice and resuspended in 30 mL of ESS medium. This suspension was inoculated into 300 mL of diatom cell culture and incubated with aeration for 3 days. Aggregates formed by bacterial and diatom cells in the culture were collected for examination by electron microscopy.

### Effect of polypeptone and podophyllotoxin on algicidal activity and gliding motility

Plaque formation was observed using double layer agar plates prepared with a top layer of 1 mL of the diatom cell suspension and with 0.1 mL of bacterial culture added to 2 mL of ESS medium containing 0.75% agar and then laid on an ESS agar plate (1.5% agar). To examine the effect of polypeptone on plaque formation, 0.05, 0.1, 0.15, or 0.2% of polypeptone (Nihon Seiyaku Ltd., Tokyo, Japan) were added to double layer agar plates. Plaque diameter was measured

**Fig. 1.** Co-cultures of *Chaetoceros ceratosporum* and strain SS98-5 in ESS liquid media. (A) Axenic culture of *C. ceratosporum*. (B) Co-culture of *C. ceratosporum* and SS98-5 in enriched sea water (ESS) medium. (C) Co-culture in ESS medium containing 0.5% polypeptone. The cultures (300 mL) were incubated with aeration at 23°C for 3 days.

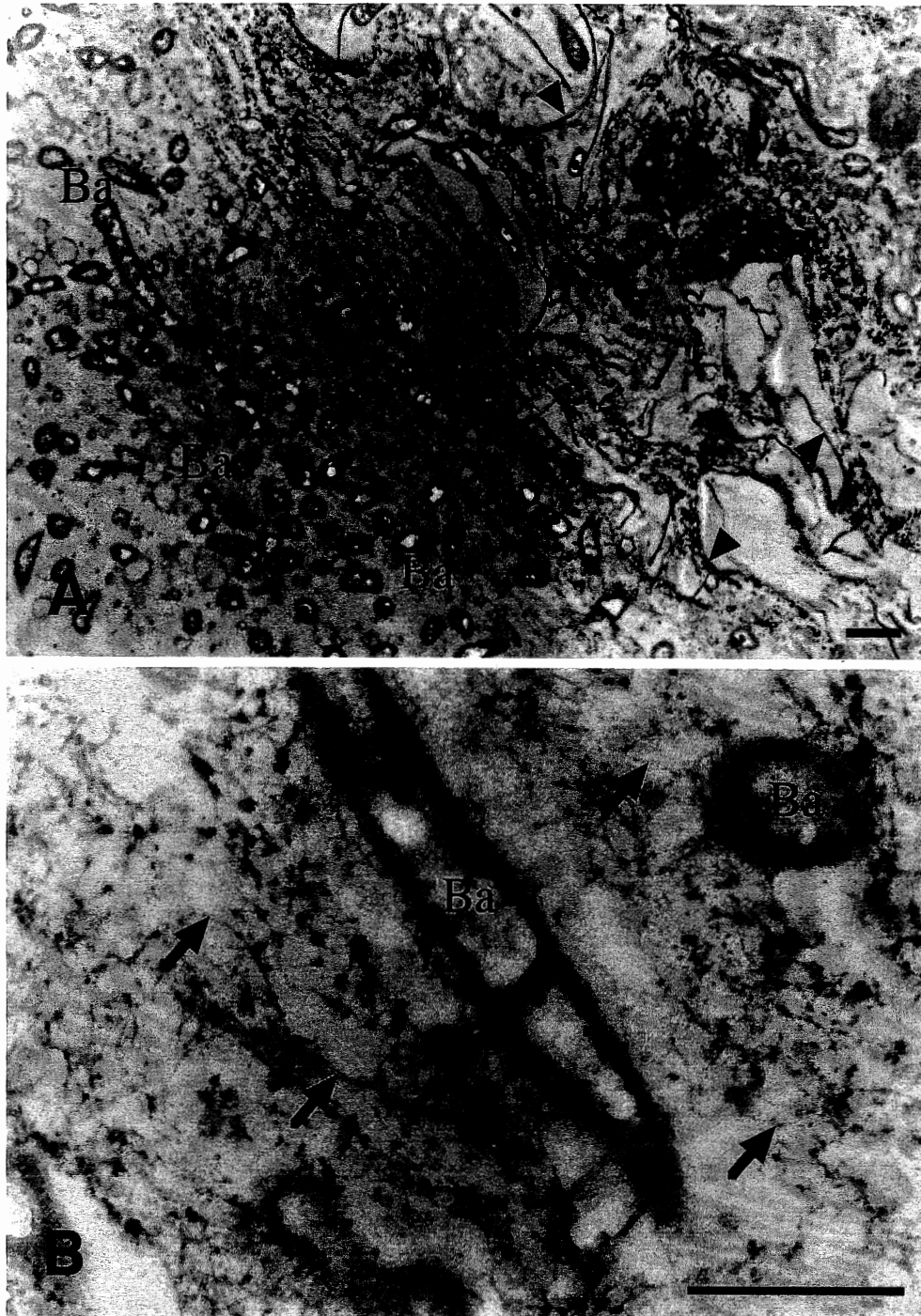


after incubation at 23°C under illumination for 8 days. To examine the effect of polypeptone on gliding motility, the bacterial cells were inoculated on the center of ESS agar plates (1.5% agar medium without diatom cells) containing 0.01, 0.05, 0.1, or 0.5% of polypeptone. Colony diameter was measured after incubating at 25°C for 4 days. To investigate the effect of podophyllotoxin on gliding motility, bacterial cells were inoculated with a needle on the center of ESS agar plates containing 0.05% polypeptone and podophyllotoxin (50 and 100 µg/mL) (Wako Pure Chemical Industries Ltd., Osaka, Japan), which was dissolved in dimethylsulfoxide (Wako Pure Chemical Industries Ltd.), and colony diameter was measured after 5 days incubation.

### Transmission electron microscopy

Agar blocks prepared from cell aggregates and the boundary area of plaques on plate cultures were treated with 2% glutaraldehyde (Nacalai Tesque Inc., Kyoto, Japan) in ESS liquid medium overnight on ice and washed three times with 10 mM phosphate buffer (pH 7.0). The samples were exposed to ESS liquid medium containing 1% OsO<sub>4</sub> (Wako Pure Chemical Industries Ltd.) for 3 h on ice and then washed three times with the same buffer. They were dehydrated in gradient ethanol solutions and transferred to propylene oxide and then embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead nitrate and observed using a transmission electron microscope (TEM), Hitachi 7100-F (Hitachi Ltd., Tokyo, Japan). Bacterial cells grown on agar plates were embedded in agar blocks and fixed for TEM observation.

**Fig. 2.** Transmission electron micrographs of the cell aggregates formed by *Chaetoceros ceratosporum* cells and strain SS98-5 in an enriched sea water (ESS) liquid culture. (A) The surface area surrounding a cell aggregate; arrow heads indicate cell wall structures of *C. ceratosporum*. (B) The surface area of a cell aggregate; arrows indicate electron-dense fibrils and particles. Ba indicates bacterial cells. Scale bar = 1  $\mu$ m.

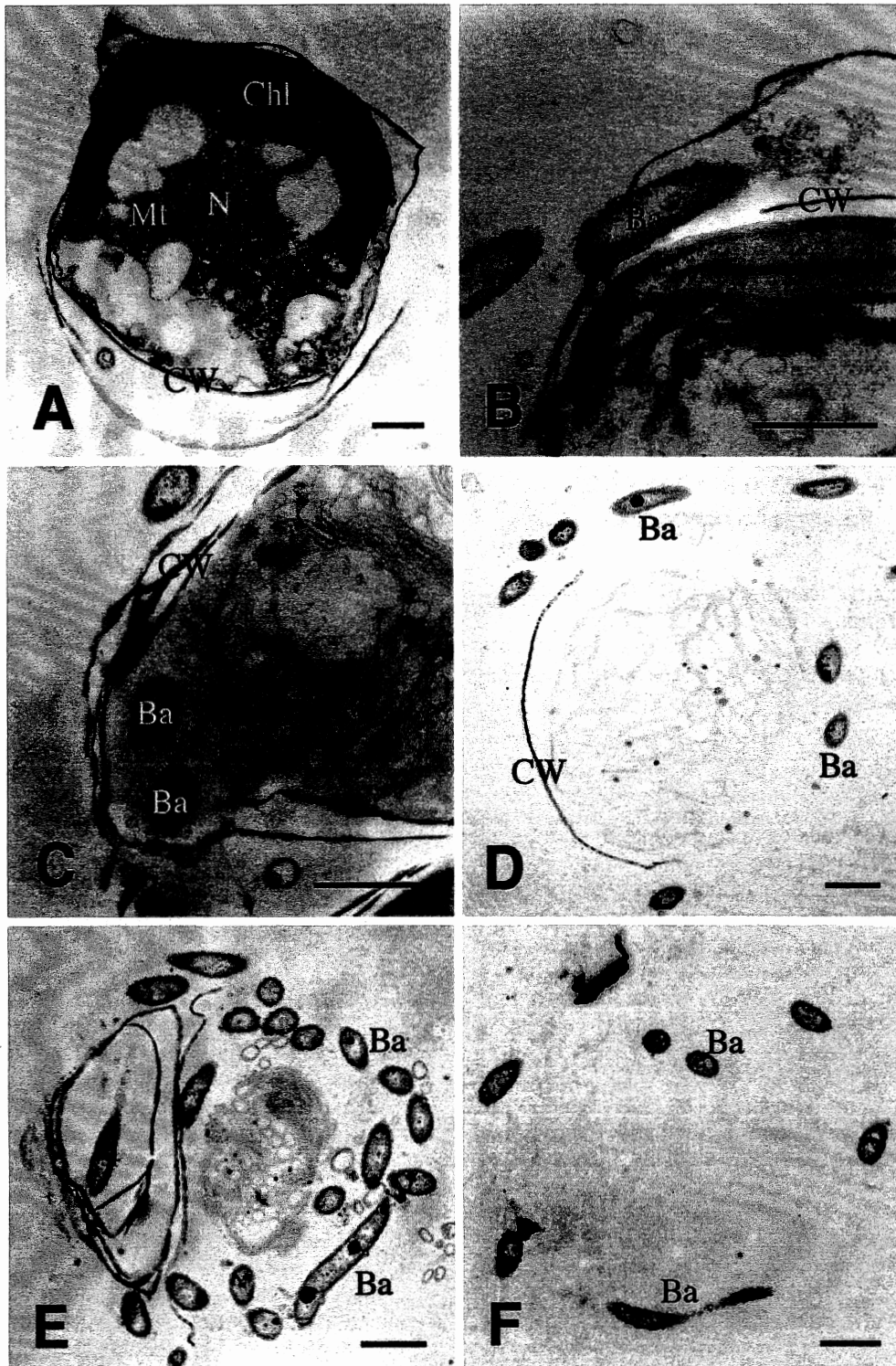


#### Sequence analysis and construction of the phylogenetic tree

Bacterial DNA of the strain SS98-5 was extracted and 16S rDNA was amplified by PCR as described by Iwamoto et al. (2001). Nucleotide sequences of PCR products were determined by using an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, Calif.). The 16S rDNA

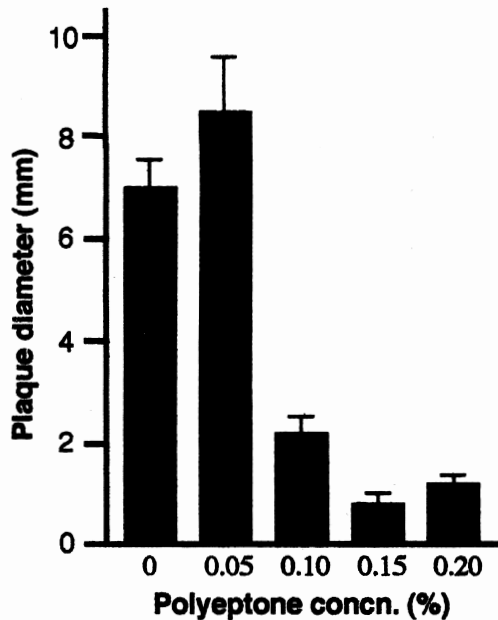
nucleotide sequence of strain SS98-5 was aligned by the Clustal X multiple sequence alignment program version 1.8 (Thompson et al. 1997) with those of the representative strains of the *Cytophaga-Flexibacter-Bacteroides* (CFB) group. The loci containing gaps were excluded from further calculation. The multiple alignments obtained were used for bootstrap resamplings of 1000 replications (Felsenstein

**Fig. 3.** Transmission electron micrographs of the lysing process of *Chaetoceros ceratosporum* by strain SS98-5 on a double layer agar plate. (A) An intact diatom cell on the outside of a plaque. (B) A diatom cell invaded by bacteria on the boundary of a plaque. (C) A diatom cell containing the bacteria within the cytoplasm. (D, E) Diatom cells degraded by the bacteria in the inner region of a plaque. (F) Bacterial cells left in a circle. N, nucleus; Chl, chloroplast; Mt, mitochondrion; CW, cell wall of a diatom; Ba, bacterial cells of strain SS98-5. Scale bar = 1  $\mu\text{m}$ .





**Fig. 4.** Effect of polypeptone concentration on plaque formation of strain SS98-5 on double layer agar plates. The diameter of plaques was measured after the plates were incubated at 23°C for 8 days.



1985), distance matrix calculations by Kimura's two-parameter model (Kimura 1980), and the construction of a phylogenetic tree by the neighbor-joining method (Saitou and Nei 1987) using the PHYLIP program package version 3.572c (Felsenstein 1989).

## Results

### Cell aggregation

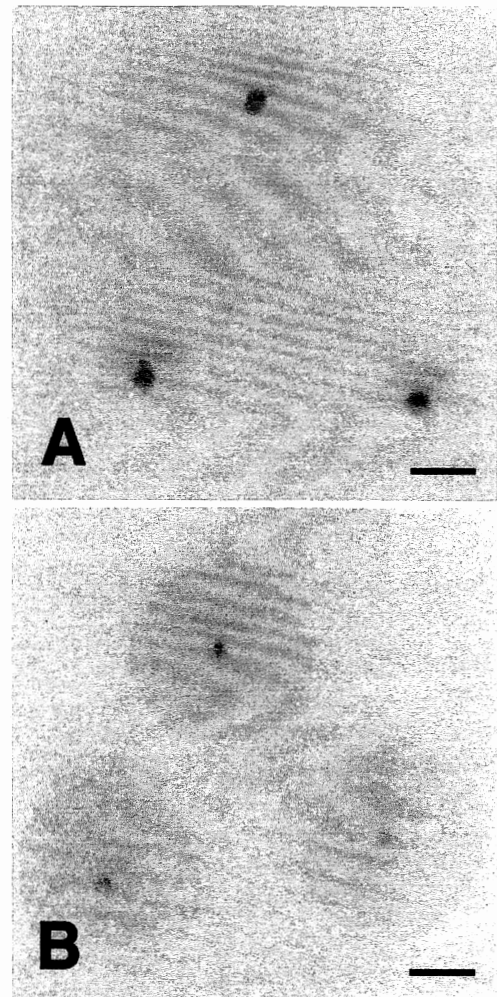
Diatom and bacterial cells were co-cultured in ESS liquid medium to clarify cell-aggregate formation. Small-size aggregates were visible after 6 h incubation. When aeration was stopped after 3 days incubation, these aggregates precipitated (Fig. 1B). These aggregates disappeared after 8 days incubation. This phenomenon was not observed in the axenic culture of the diatom (Fig. 1A) or in the co-culture containing 0.5% polypeptone, in which diatom cells grew normally during incubation (Fig. 1C).

### Ultrastructure of diatom and bacterial cells

The inside of the cell aggregates formed after 3 days incubation was mainly composed of cell walls of broken diatom cells and a few bacterial cells. Many bacterial cells were observed outside of the cell aggregates (Fig. 2A). A large number of electron-dense particles and fibril materials were also detected in the area between the bacterial cell layer and the diatom cell aggregates (Fig. 2B).

Diatom cells at the perimeter of the plaques formed on double layer agar plates were sometimes at a binary fission stage, suggesting that the condition was suitable for diatom cell growth. Their cell walls, nuclei, chloroplasts, and mitochondria could be clearly observed (Fig. 3A). On the boundary area of the plaques, diatom cells had just been contacted with bacteria, and their cell walls at the contact sites were

**Fig. 5.** Colony morphology of strain SS98-5 grown on enriched sea water (ESS) agar plates. (A) Bacterial colonies on an ESS agar plate containing 0.5% polypeptone. (B) Bacterial colonies on an ESS agar plate containing 0.05% polypeptone. The plates were incubated at 25°C for 4 days. Scale bar = 1 cm.

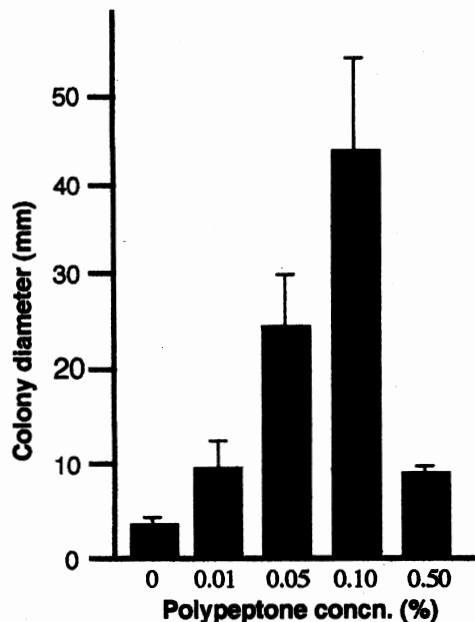


partially degraded (Figs. 3B and 3D). Bacterial invasion of the diatom cells was observed, and the diatom organelles were vesiculated (Fig. 3C). In the inner region of a plaque, many bacteria were observed around diatom cells, and the diatom cell walls were partially degraded (Figs. 3D and 3E). Finally, the diatom cells were lysed completely, leaving the bacteria in a circle in the agar (Fig. 3F).

### Effect of polypeptone on plaque and colony formation

When strain SS98-5 and diatoms were co-cultured on the double layer agar plates, small plaques appeared after 4 days incubation. After 8 days, plaques increased to about 7 mm in diameter. Plaque formation was inhibited by the addition of 0.1% polypeptone to the double layer agar plate (Fig. 4). When only bacterial cells were inoculated on the center of the ESS agar plates containing various concentrations of polypeptone, spread-type colonies were found on the agar plates with lower concentrations of polypeptone (Fig. 5B). Gliding motility and colony spread were highest on 0.1%

**Fig. 6.** Effect of polypeptide concentration on colony formation of strain SS98-5 on enriched sea water (ESS) agar plates. The bacterial cells were inoculated on the agar plates and incubated at 25°C for 4 days, and the diameter of colonies was measured.



polypeptide medium and completely inhibited on the 0.5% polypeptide medium (Fig. 6).

#### Microtubule-like structure in bacterial cells

To compare the morphology of gliding and nongliding bacteria, bacterial cells were sampled from the spread-type colonies formed on ESS agar plates with 0.1% polypeptide and from the nonspread-type colonies formed on ESS agar plates containing 0.5% polypeptide. Gliding speeds of spread-type and nonspread-type colonies on these plates were about 10 and 0 mm/day (colony diameter), respectively. TEM observations indicated a large amount of microtubule-like structures that could only be found in the cytoplasm of the gliding cells (Figs. 7C and 7D). Such intracellular structures were not detected in nongliding cells (Figs. 7A and 7B), while the surface structure of cells was similar between gliding and nongliding cells.

The microtubule-like structures observed in the cells of strain SS98-5 were diminished (Figs. 7E and 7F), and the gliding motility was strongly inhibited by the addition of 50 µg/mL podophyllotoxin (Table 1), and this concentration did not have an effect on bacterial growth (data not shown).

#### Phylogenetic analysis on 16S rDNA sequences

The PCR-amplified 16S rDNA nucleotide sequences of strain SS98-5 and *Saprospira grandis* ATCC 23116 were determined and aligned with representative small subunit rDNA sequences of the CFB group from the Ribosome Database Project II and the DDBJ, and their genetic distances were calculated. As shown in Fig. 8, the unrooted phylogenetic tree inferred from the neighbor-joining method indicated that strain SS98-5 (accession No. AB088635) is clustered with *S. grandis* ATCC 23116 (acc. No. AB088636) and ATCC 23119 strains and belongs to the genus *Saprospira*.

**Table 1.** Effect of podophyllotoxin on colony formation of SS98-5.

Concn. (µg/L)	Colony diameter (mm)
0	44.0±7.8
50	19.1±4.7
100	6.8±4.9

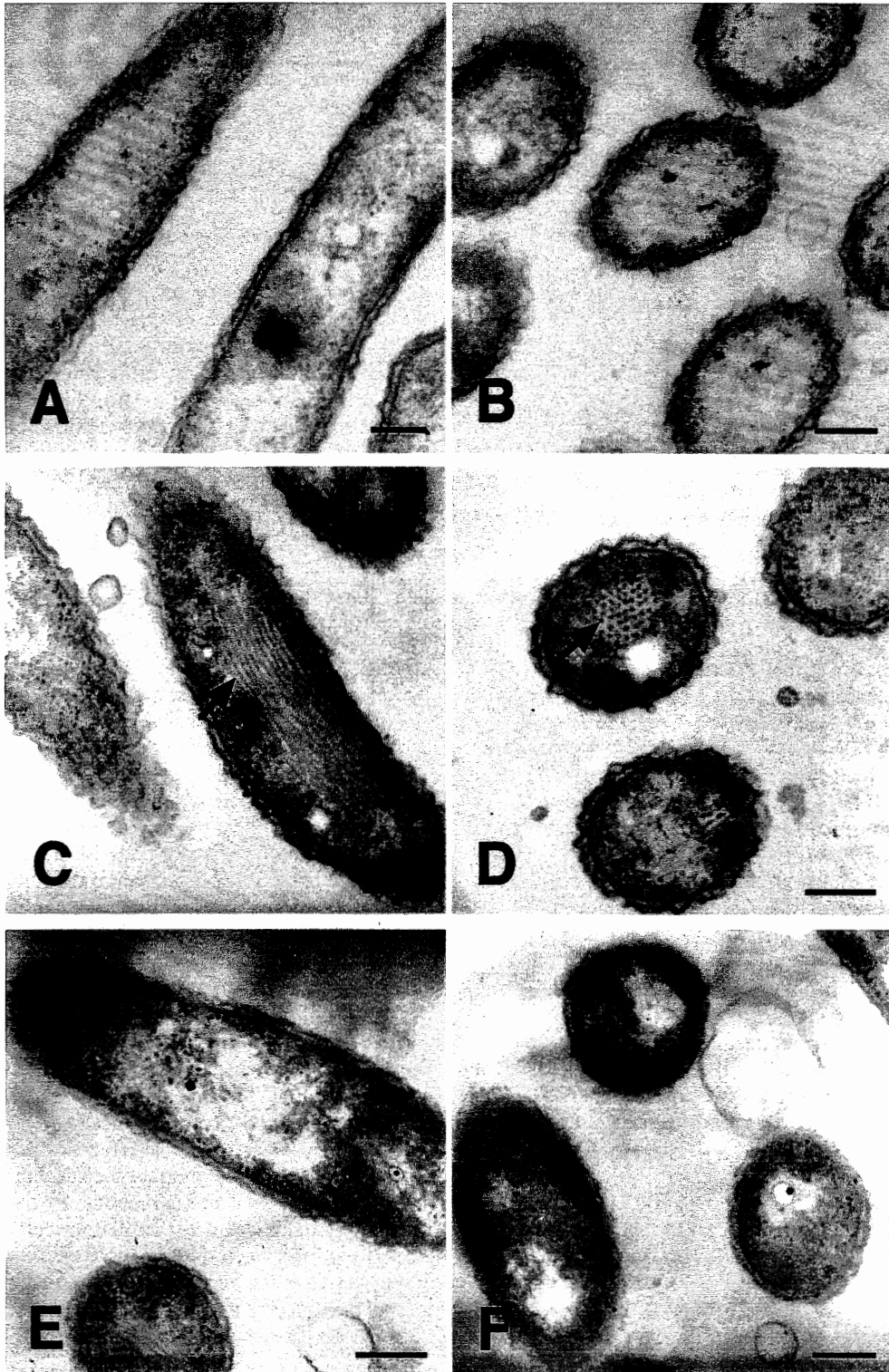
Note: Values are means ± SD (number of colonies measured is 10).

## Discussion

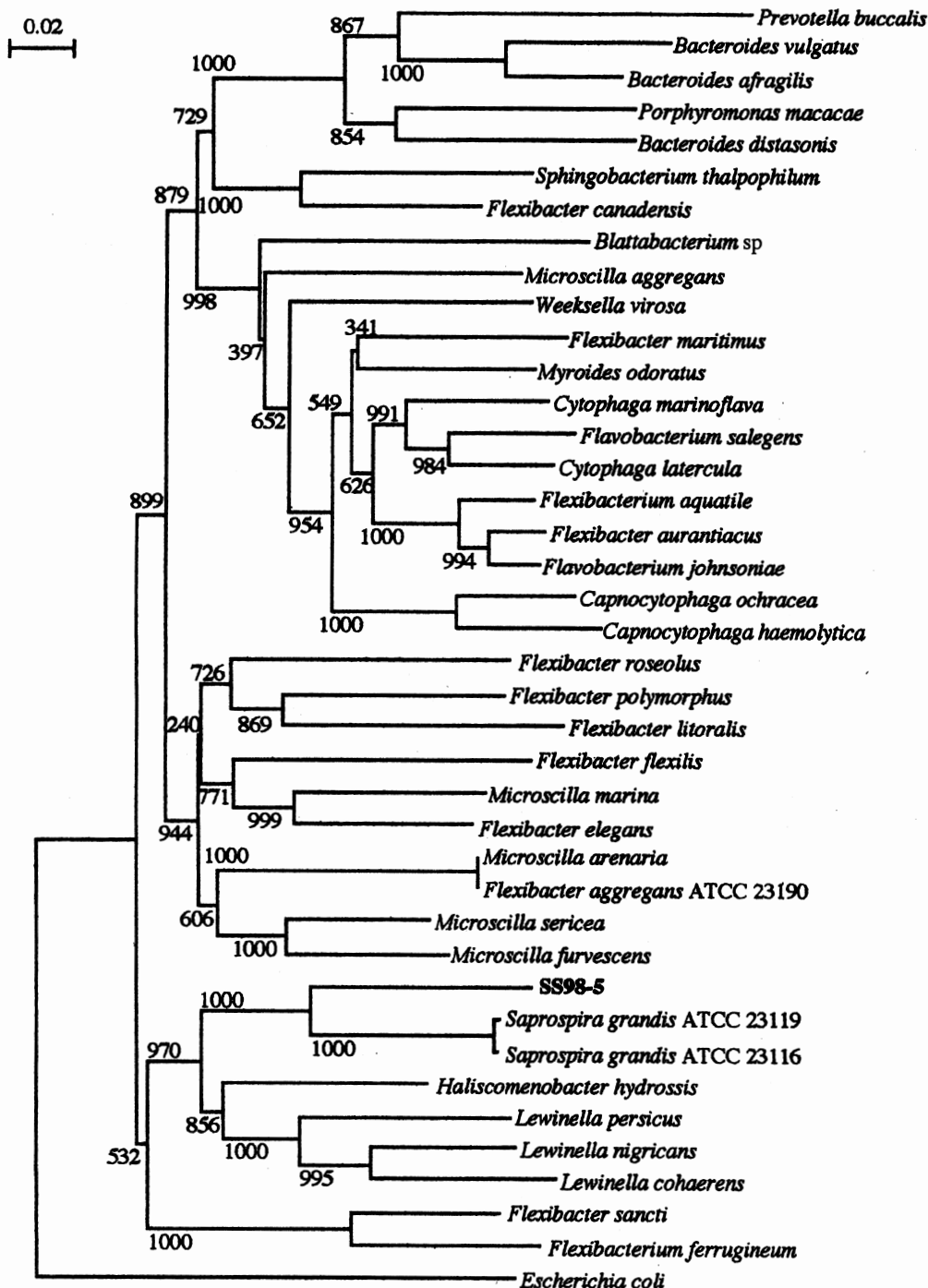
Algicidal filamentous bacteria isolated from Kagoshima Bay in Japan were able to kill the diatom *C. ceratosporum* and formed cell aggregates when co-cultivated with diatoms in a liquid culture under experimental conditions. The results of the phylogenetic analysis of 16S rDNA suggested that this bacterium belongs to the genus *Saprospira*. *Saprospira* spp. have been described as strictly aerobic bacteria with a helical multicellular filamentous form and gliding motility. These species have been isolated from freshwater and marine environments (Lewin 1965a, 1965b; Lewin and Mandel 1967). *Saprospira* spp. were shown to have algicidal activity, but any active substances had not yet been found in the culture filtrate.

Cell aggregates were formed in liquid cultures, and they contained a mass of cell walls (frustles) of diatoms, a few bacterial cells, and were surrounded by a large number of filamentous bacterial cells. Diatom cells cultured with the bacteria died, the cell contents were released, and consequently, many bacteria surrounding the cell aggregates could multiply. Finally, their frustles were digested and the cell aggregates had disappeared 8 days after incubation. However, the aggregate formation of diatom cells in liquid culture was completely inhibited by the addition of 0.5% polypeptide, and the number of diatom cells in the culture remained unchanged during 3 days incubation (data not shown), suggesting that algicidal substances were not released from bacterial cells and that this bacterium is a facultative saprophyte. Lewin (1997) reported that *S. grandis* can catch a prey by a process known as "ixotrophy". Slimy material was isolated from viscous liquid cultures of *Saprospira thermalis* (Lewin 1965a). On the basis of these results, it was suggested that electron-dense materials surrounding cell aggregates have a role in catching diatom cells because such materials were not detected when the bacterial cells were incubated in a liquid culture containing 0.5% polypeptide. Intact diatom cells at the outside of the plaques before being attacked by the bacteria had normal cell structures. In the boundary area of the plaques, the cell walls of diatom cells were partially decomposed, and bacterial invasion of the diatom cells could be observed at the contact sites. The cell aggregates were digested completely and had disappeared after 8 days incubation in the liquid culture. These findings suggest that the algal-lysing activity of the bacterium promotes the degradation of the frustles and cell contents. At present, the nature and function of the algal lysing factors of strain SS98-5 remain to be clarified. However, it was shown recently that a set of polycationic peptides (silaffins) and long-chain

**Fig. 7.** Transmission electron micrographs of gliding and nongliding cells of strain SS98-5. (A, B) Nongliding cells on an enriched sea water (ESS) agar plate, containing 0.5% polypeptone. (C, D) Gliding cells on an ESS agar plate containing 0.05% polypeptone. (E, F) Bacterial cells on an ESS agar plate containing 0.05% polypeptone and 100  $\mu\text{g}/\text{mL}$  podophyllotoxin. Bacteria were inoculated on each plate and incubated at 25°C for 5 days. Bacterial cells on agar plates were embedded in agar blocks and fixed for transmission electron micrograph (TEM) preparation. Arrows indicate microtubule-like structures. Scale bar = 0.2  $\mu\text{m}$ .



**Fig. 8.** Phylogenetic tree inferred from the 16S rDNA sequences of *Cytophaga*–*Flexibacter*–*Bacteroides* (CFB) representatives and the test strains. The tree was constructed by the neighbor-joining method. Scale bar indicates genetic distance.



polyamines isolated from diatom cell walls generate networks of silica nanospheres within a few seconds when added to a solution of silicic acid (Kroger et al. 1999, 2000). Silaffins and polyamines are considered to catalyze silicic acid polymerization and silica flocculation in diatom cell walls. It is inferred from these facts that strain SS98-5 may degrade the cell wall of diatoms by the decomposition of cell wall components, such as silaffins and polyamines.

Some prokaryotic cells such as *Myxobacter*, *Mycoplasma*, CFB group bacteria, and cyanobacteria are known

to possess gliding motility (Pate 1988). In the case of *Mycoplasma gallisepticum*, submembrane tubular structures in the cells were observed by electron microscopy, and the possible involvement of the tubular system in *Mycoplasma* motility was also suggested (Korolev et al. 1994). Strain SS98-5 was also found to show gliding motility, which was controlled by the addition of polypeptide and podophyllotoxin, an inhibitor of microtubule assembly (Dustin 1984). The microtubule-like structures were detectable only in gliding motile cells, and these structures were



diminished in the cells grown on the agar plate added with 100 µg/mL podophyllotoxin, an inhibitor of microtubule assembly, suggesting that the structures may be related to the gliding motility of this bacterium.

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