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Involvement of Periplasmic Fibrils in Motility of Spirochetes

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Nonmotile (Mot$^-$) strains of *Spirochaeta aurantia* and *Spirochaeta halophila* were isolated with a procedure involving mutagenesis of motile wild-type cells. Electron microscopy showed that a Mot$^-$ mutant strain of *S. halophila* possessed incomplete periplasmic fibrils, inasmuch as most or all of the filamentous portion of the periplasmic fibrils was absent. Some of the cells of this Mot$^-$, fibril-defective mutant strain lacked the filamentous portion of the periplasmic fibrils and formed proximal hooks, whereas other cells appeared to have a very small segment of the filamentous portion of the periplasmic fibrils attached to the proximal hooks. Motile revertants were isolated repeatedly from cultures of the Mot$^-$, fibril-defective mutant and from *S. halophila* Mot$^-$ mutants that completely lacked periplasmic fibrils. The motile revertants possessed periplasmic fibrils ultrastructurally indistinguishable from wild-type periplasmic fibrils. This study indicates that periplasmic fibrils play an essential role in the motility of spirochetes.

Spirochetes perform three main types of movement in liquid environments: translational motility, rotation about their longitudinal axis, and flexing motions (1, 3). Furthermore, spirochetes are able to creep or crawl on solid surfaces (3). Spirochetes possess characteristic fibrilliform organelles that have been called axial fibrils, axial filaments, flagella, endoflagella, and, more recently, periplasmic fibrils (3). Periplasmic fibrils are similar to bacterial flagella in fine structure and in certain chemical characteristics (3, 8). However, they differ from bacterial flagella because they are permanently wound around the cell body and because they are entirely endocellular, being enclosed (together with the coiled protoplasmic cylinder of spirochetes) by a multilayered membrane called outer sheath (3, 7). It has been suspected for many years that periplasmic fibrils play a role in the motility of spirochetes. However, direct experimental evidence was not available in support of this possible role of periplasmic fibrils.

Recently, Bromley and Charon (2) isolated nonmotile mutants of *Leptospira interrogans* serovar *illini*. One class of nonmotile mutants (linear mutants) lacked the hook- or spiral-shaped cell ends usually present in *L. interrogans* cells. Periplasmic fibrils isolated from cells of these linear mutants lacked the coiled configuration observed in periplasmic fibrils isolated from wild-type cells. Revertant analysis of two linear mutants indicated that the mutations were pleiotropic with respect to motility, periplasmic fibril coiling, and hook- and spiral-shaped cell ends. The authors concluded that the periplasmic fibrils, as well as the hook- and spiral-shaped ends of *L. interrogans*, are involved in the motility of this spirochete.

In this article we describe the isolation of nonmotile (Mot$^-$) spirochete mutants that lack periplasmic fibrils or have incomplete periplasmic fibrils, and of motile revertants with complete periplasmic fibrils. The work we report provides direct evidence indicating that periplasmic fibrils are implicated in the motility of spirochetes.

(A preliminary report of part of this work was previously presented [B. J. Paster and E. Canale-Parola, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, I61, p. 105].)

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Spirochaeta halophila* RS1 and *Spirochaeta aurantia* M1 (wild-type strains) were grown aerobically as described elsewhere (4, 5) and were harvested during the late exponential phase of growth by centrifugation at 5,000 × *g* for 30 min at 5°C. Mutant and revertant strains were grown under conditions identical to those used for the wild-type strains.

Isolation of nonmotile (Mot$^-$) mutants and of motile revertants. Mot$^-$ strains of *S. halophila* and *S. aurantia* were obtained by mutagenizing motile wild-type cells with UV or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Cultures of wild-type cells in broth were irradiated for 30 to 45 s (6 ml of bacterial culture in the bottom half of a plastic petri dish rotated at 150 rpm was exposed to light from two General Electric 15-W germicidal lamps at a distance of 40 cm) or treated for 15 to 30 min with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Aldrich Chemical Co., Milwaukee, Wisconsin) at final concentrations of 100 μg/ml to 1 mg/ml. Appropriate dilutions were then plated in soft agar overlays. Mot$^-$ strains were selected on the basis

of colony morphology. Colonies of Mot$^-$ cells developed on the agar medium surface and did not spread, in contrast to wild-type colonies which diffused through the agar medium (6). Exposure to the mutagens resulted in 90 to 99.99% cell killing. Colonies of Mot$^-$ cells appeared at a frequency of approximately 10^{-3} to 10^{-2} among survivors. Mot$^-$ strains were cloned by streaking cells on plates of appropriate media. Mot$^-$ mutants isolated by this procedure did not exhibit any form of motility (3). The ultrastructural features of Mot$^-$ strains were examined by electron microscopy.

Spontaneous motile revertants were isolated from cultures of Mot$^-$ mutants by a procedure including spot inoculation of mutant cells on migration medium plates (6). After 4 to 8 weeks of incubation, sectors of some of the colonies of Mot$^-$ strains on the spot-inoculated plates began to diffuse through the agar medium. Cells from these sectors were cloned on plates of appropriate media. Strains of motile revertants thus isolated were examined by electron microscopy.

Purification of periplasmic fibrils and of proximal hook-like structures. Periplasmic fibrils and proximal hook-like structures (see below) were purified by a modification of the method of Joseph and Canale-Parola (8). Twenty-five to fifty grams (wet weight) of cells was suspended in 100 ml of buffer solution (0.01 M potassium phosphate buffer, pH 7.2). An equal volume of 4% (wt/vol) sodium deoxycholate in buffer solution was added to the cell suspension, and the preparation was mixed with a magnetic stirring bar at 5°C for 12 to 14 h. RNase (1 μg/ml, final concentration) and DNase (10 μg/ml, final concentration) were added to the mixture at the beginning of the incubation. The suspension was centrifuged at 5,000 × *g* for 15 min at 5°C (all subsequent centrifugations were performed at this temperature). Then the supernatant liquid was centrifuged at 105,000 × *g* for 1 h to pellet the periplasmic fibrils or proximal hook-like structures and any remaining cellular debris. The pellets from this centrifugation step were suspended in 60 ml of buffer solution, and 30 g of KBr was added to the mixture and dissolved with stirring. The mixture was added to thin-walled polypropylene centrifuge tubes and centrifuged in an International Preparative Ultracentrifuge (International Equipment Co., Div. of Damon Corp., Needham Heights, Mass.), model B-60, utilizing an SB-283 rotor at 260,000 × *g* for 40 h. Fibril or proximal hook-like material banded at approximately one-half the distance between the meniscus of the liquid and the bottom of the tube. The bands were removed with a hypodermic syringe, and the pooled bands were diluted to 18 ml with buffer solution. Then the mixture was centrifuged at 200,000 × *g* for 1 h to pellet the fibril or proximal hook-like material. The pellets were suspended in 14.5 ml of buffer solution, and 7.25 g of KBr was added to the mixture and dissolved. The suspension was centrifuged in cellulose nitrate tubes in a model L2-65B ultracentrifuge (Beckman Instruments Inc., Fullerton, Calif.) utilizing an SW-39L rotor at 134,000 × *g* for 24 h. The bands of purified fibril or proximal hook-like material were removed with a syringe, and the material was washed three times with buffer solution by centrifugation at 200,000 × *g* for 1 h. The washed fibril or proximal hook-like material was suspended in buffer solution and stored at 5°C.

Additional periplasmic fibrils were salvaged from the first KBr density gradient step as follows. After this step, pellicles of pigmented cellular debris containing trapped periplasmic fibrils were at the top of the KBr gradient tubes. The pellicles were collected, suspended in 18 ml of buffer solution, and centrifuged at 200,000 × *g* for 1 h. The resulting pellets were suspended in 14.5 ml of buffer solution, and 7.25 g of KBr was added to the mixture and dissolved. The suspension was subjected to the ultracentrifugation step (260,000 × *g*, 40 h) described above. The bands of fibril material were removed, the material was washed once in buffer solution, and the KBr density gradient step at 134,000 × *g* for 24 h (see above) was performed. Finally, the bands of purified fibril material were removed with a syringe, and the material was washed three times with buffer solution by centrifugation (200,000 × *g*, 1 h). After the washing steps, the fibril material was suspended in buffer solution and stored (5°C).

Acid treatment of whole cells and of fibril or proximal hook-like material. A 5-ml culture of *S. halophila* was centrifuged at 3,000 × *g* for 10 min, and the pellet was suspended in 1 ml of ISM salt solution (4). The pH of the suspension was adjusted to 2.25 with 0.2 N HCl, and the suspension was incubated for 30 min at room temperature. The suspension was centrifuged at 3,000 × *g* for 10 min, and the pellet was suspended in 1 ml of 0.5 M potassium phosphate buffer, pH 7.0. Finally, the cells were examined by electron microscopy.

Fibril or proximal hook-like material was suspended in buffer solution (0.01 M potassium phosphate, pH 7.2), and the pH of the suspension was adjusted to 2.25 with 0.2 N HCl (8). The mixtures were incubated for 30 min at room temperature and then centrifuged at 200,000 × *g* for 1 h. The resulting pellets were suspended in 0.1 ml of buffer solution, and samples were examined by electron microscopy.

Electron microscopy. Negatively stained specimens of whole cells for electron microscopy were prepared as follows. Five-milliliter cultures were centrifuged at 3,000 × *g* for 10 min, and the resulting pellets were suspended in 1 ml of 0.2 N ammonium acetate or 0.5 M potassium phosphate buffer, pH 7.2. A drop of suspension was quickly placed on a collodion-coated, carbon-reinforced copper grid (300 mesh), and the excess fluid was drawn off with tissue paper after 1 min. Then the cells on the grid were stained with 1% (wt/vol) phosphotungstic acid (pH 6.5) for 15 s.

Specimens of purified fibrils or proximal hook-like structures were used directly for negative staining. The samples were placed on grids and stained as described above. However, these samples were stained for 40 s.

All samples were examined in a Philips (Einhoven, The Netherlands) EM 200 electron microscope equipped with a 30-μm objective aperture operating at 60 kV. A liquid nitrogen cold finger at the level of the specimen was used to reduce contamination.

RESULTS

Isolation of nonmotile mutants. Four types of nonmotile (Mot$^-$) spirochete mutants were isolated. The Mot$^-$ mutants lacked translational

motility and all other forms of motility.

Mot⁻ mutants of the type most frequently isolated (type 1) were obtained by means of UV and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis of wild-type *S. halophila* and *S. aurantia* cells. Mutants of this type possessed periplasmic fibrils and an outer sheath and were identical in gross morphology to their parental strains.

A second type of Mot⁻ mutant (type 2) was represented by three strains isolated by UV mutagenesis of *S. halophila* wild-type cells. Cells of these mutant strains were two to four times as long as wild-type cells and lacked the periplasmic fibrils (proximal hook and filamentous portion of the fibrils). Furthermore, the cells possessed abnormal coiling inasmuch as some regions of the cells had little or no coiling.

A strain (RS1-3) isolated by UV mutagenesis of *S. halophila* wild-type cells represented another type (type 3) of Mot⁻ mutants. The cells of this strain had defective periplasmic fibrils inasmuch as the filamentous portion of the periplasmic fibrils was absent. These fibril-defective (Df) mutants had only a short stublike structure, resembling a proximal hook, inserted near the ends of the cells (Fig. 1). It was not readily apparent by electron microscopy examination whether the stublike structure consisted only of a proximal hook, or of a proximal hook to which a very short segment of the filamentous portion of the periplasmic fibril was attached (Fig. 1). The stublike structures were referred to as proximal hook-like structures. Fragments of the filamentous portion of periplasmic fibrils were not observed among background debris of electron microscopy preparations of type 3 mutants, whereas such fragments were usually seen in electron microscopy preparations of wild-type cells. Type 3 mutant cells possessed an outer sheath (Fig. 1) that appeared to be identical to that of wild-type cells. Furthermore, type 3 mutant cells were indistinguishable in gross morphology and in size from wild-type cells. Mutants of type 3 were designated Mot⁻ Df mutants.

A fourth type of Mot⁻ mutant (type 4), isolated by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis of *S. aurantia* wild-type cells, was represented by a strain (NP-7) that had defective periplasmic fibrils, as described above for type 3 mutants. However, cells of type 4 mutants were not helical in shape, but were relatively straight, often with one or both ends bent or hooked. Mutants with a similar cell shape (Hel⁻ mutants) were described by Greenberg and Canale-Parola (6).

Isolation of motile revertants. Spontaneous motile revertants were isolated repeatedly from cultures of the type 3 mutant (Mot⁻ Df, strain RS1-3). Isolations were accomplished by transferring cells from spot-inoculated plate cultures of the Mot⁻ Df mutant, as described above. Two types of revertants differing in colony morphology were isolated. The type of revertant most frequently isolated formed colonies indistinguishable in appearance from those of the

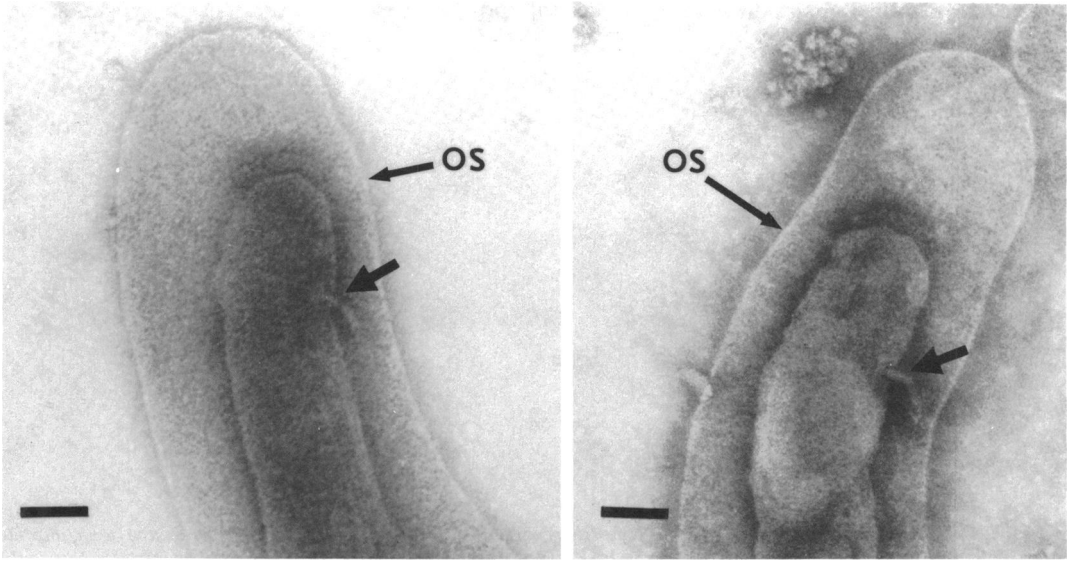

FIG. 1. *Electron micrographs of Mot⁻ Df mutant cells (strain RS1-3, type 3 mutant cells). The proximal hook-like structures are indicated by the short arrows. Notice that an outer sheath (OS) encloses each cell. The marker bars represent 0.1 μm.*

wild type, i.e., semitransparent colonies that diffused through the agar medium. The second type of revertant formed colonies that diffused at a markedly slower rate and, therefore, were smaller in size. Cells of both types of motile revertants isolated from type 3 (Mot⁻ Df) cultures possessed complete periplasmic fibrils and an outer sheath (Fig. 2) and appeared morphologically identical to wild-type cells. Furthermore, the motility behavior (3) of the revertants was the same as that of the wild-type strain.

Motile revertants were also isolated from cultures of type 2 Mot⁻ mutants. Whereas the cells of type 2 mutants were longer than those of the wild-type strain and had abnormal coiling, the motile revertant cells had the same length and the same cell-coiling pattern as the wild-type strain. The revertant cells possessed normal motility, had complete periplasmic fibrils, and formed colonies identical to those of wild-type cells.

Attempts to isolate motile revertants of the type 4 mutant (strain NP-7) were not successful.

Proximal hook-like structures. Proximal hooks were isolated from wild-type cells of *S. halophila* to compare their ultrastructure with that of proximal hook-like structures isolated from type 3 (Mot⁻ Df) mutants. To obtain proximal hooks, a homogeneous preparation of isolated periplasmic fibrils (Fig. 3A, B) was acid treated. The acid treatment resulted in disintegration of the filamentous portion of the periplasmic fibrils, whereas the morphological integrity of the proximal hooks did not seem to be affected (Fig. 4B). The isolated proximal hooks appeared identical in size and ultrastructure to proximal hook-like structures isolated from strain RS1-3 (Fig. 4A). Acid treatment did not seem to affect the morphology of isolated proximal hooklike structures. When wild-type cells of *S. halophila* were acid treated, the filamentous portion of the periplasmic fibrils was degraded, but the proximal hooks remained present at the points of fibril insertion (Fig. 5). These inserted proximal hooks were morphologically similar to proximal hook-like structures inserted in cells of the type 3 (Mot⁻ Df) mutant (Fig. 1), although the latter structures sometimes appeared slightly longer.

DISCUSSION

Our work shows that spirochete mutants (type 3) which lack the filamentous portion of the periplasmic fibrils are not motile, whereas revertants with complete periplasmic fibrils are motile. These observations indicate that periplasmic fibrils are components of the motility apparatus of spirochetes and that they perform a function (or functions) essential for the locomotion and for the other movements characteristic of these bacteria.

Ultrastructural comparisons and experiments involving treatment with acid indicated that the stublike structures inserted near the cell ends of type 3 (Mot⁻ Df) spirochete mutants were proximal hooks to which, in some of the cells, a very short segment of the filamentous portion of the periplasmic fibrils may have been attached.

Spontaneous motile revertants were also isolated from cultures of type 2 Mot⁻ mutants. It is possible that the mutations in type 2 strains

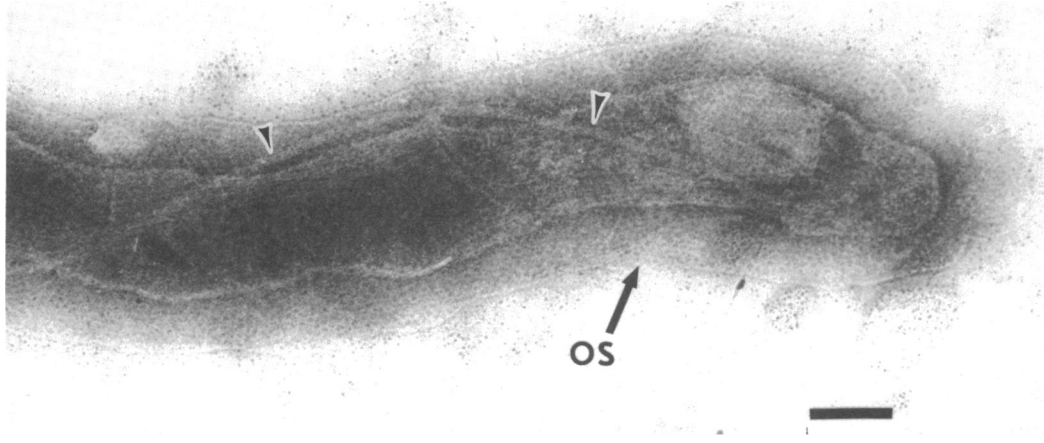

FIG. 2. *Electron micrograph of a cell of a motile revertant of strain RS1-3. Notice the presence of a complete periplasmic fibril (indicated by the arrow heads) and of an outer sheath (OS). The marker bar represents 0.1 μm.*

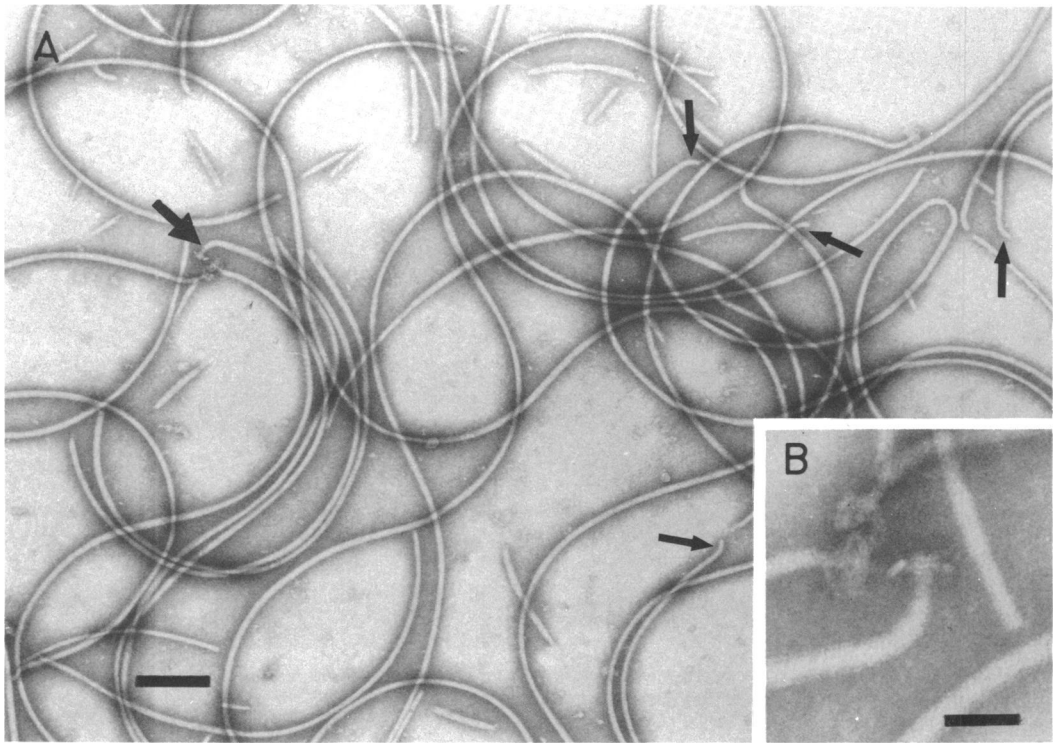

FIG. 3. *(A) Electron micrograph of purified periplasmic fibrils isolated from wild-type cells of S. halophila. Note that many of the fibrils have retained their proximal hooks (arrows) through the purification procedure. The marker bar represents 0.2 μm. (B) Electron micrograph of a proximal hook and disklike insertion structures. Enlarged from (A) (large arrow). The marker bar represents 0.05 μm.*

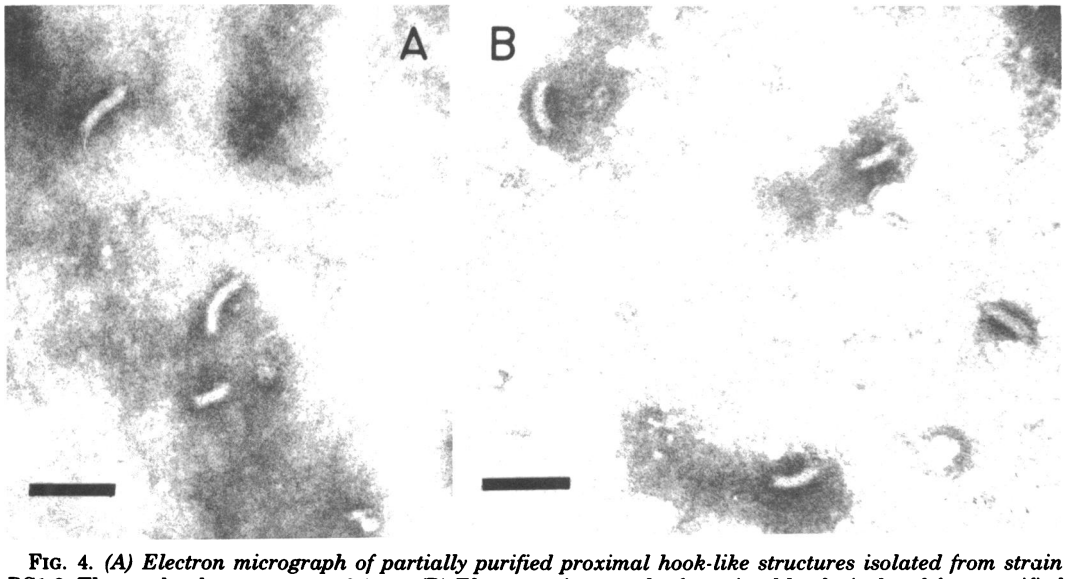

FIG. 4. *(A) Electron micrograph of partially purified proximal hook-like structures isolated from strain RS1-3. The marker bar represents 0.1 μm. (B) Electron micrograph of proximal hooks isolated from purified periplasmic fibrils of wild-type cells of S. halophila. The marker bar represents 0.1 μm.*

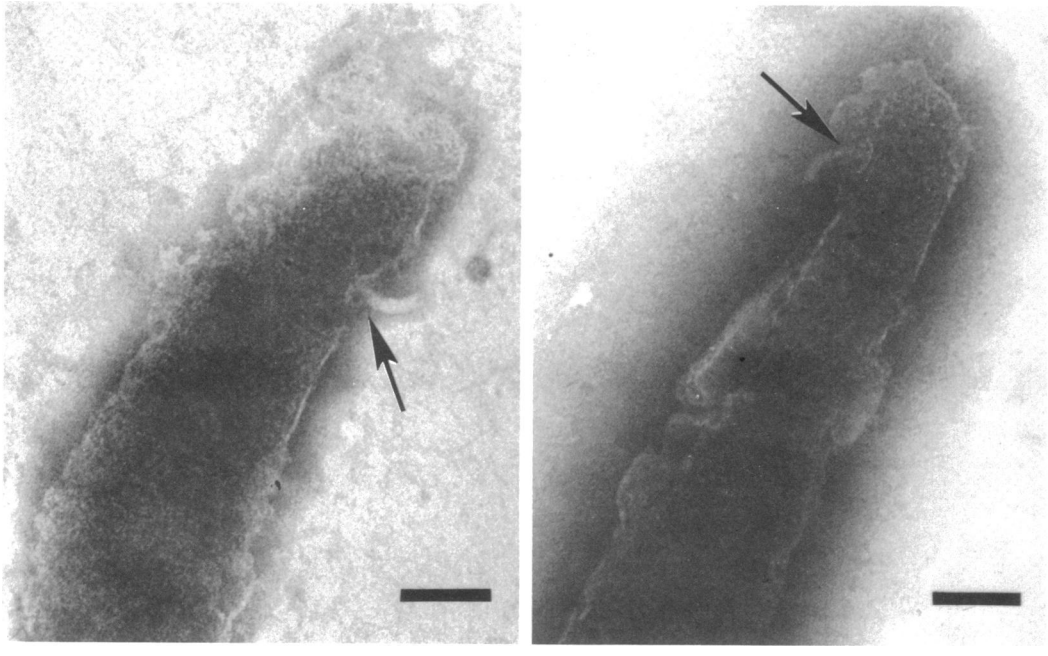

FIG. 5. *Electron micrographs of acid-treated cells of wild-type S. halophila. Notice the proximal hooks still present at the points of fibril insertion (arrows) in the protoplasmic cylinders of the cells. The marker bars represent 0.1 μm.*

were pleiotropic with respect to periplasmic fibrils, cell length, and cell coiling.

Type 1, type 2, and type 3 spirochete mutants may be equivalent, in a general sense, to Mot^-, Fla^-, and Hag^- mutant strains of *Escherichia coli*, respectively. *E. coli* Mot^- strains have flagella that appear to be ultrastructurally complete but are "paralyzed," some Fla^- strains have no apparent flagellar structures, and some Hag^- strains lack the flagellar filaments but have flagellar basal structures and hooks (9).

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LITERATURE CITED

1. **Berg, H. C.** 1976. How spirochetes may swim. J. Theor. Biol. **56**:269–273.
2. **Bromley, D. B., and N. W. Charon.** 1979. Axial filament involvement in the motility of *Leptospira interrogans*. J. Bacteriol. **137**:1406–1412.
3. **Canale-Parola, E.** 1978. Motility and chemotaxis of spirochetes. Annu. Rev. Microbiol. **32**:69–99.
4. **Greenberg, E. P., and E. Canale-Parola.** 1976. *Spirochaeta halophila* sp. n., a facultative anaerobic spirochete from a high-salinity pond. Arch. Microbiol. **110**: 185–194.
5. **Greenberg, E. P., and E. Canale-Parola.** 1977. Chemotaxis in *Spirochaeta aurantia*. J. Bacteriol. **130**:485–494.
6. **Greenberg, E. P., and E. Canale-Parola.** 1977. Relationship between cell coiling and motility of spirochetes in viscous environments. J. Bacteriol. **131**:960–969.
7. **Holt, S. C.** 1978. Anatomy and chemistry of spirochetes. Microbiol. Rev. **42**:114–160.
8. **Joseph, R., and E. Canale-Parola.** 1972. Axial fibrils of anaerobic spirochetes: ultrastructure and chemical characteristics. Arch. Mikrobiol. **81**:146–168.
9. **Silverman, M., and M. I. Simon.** 1977. Bacterial flagella. Annu. Rev. Microbiol. **31**:397–419.