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Treponema phagedenis Has at Least Two Proteins Residing Together on Its Periplasmic Flagella

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Treponema phagedenis **is an anaerobic, motile spirochete with several periplasmic flagella (PFs) at each cell end. This study provides the first genetic evidence that multiple protein species are associated with the PFs. In addition, these proteins were found to reside together on a given PF. Nonmotile mutants which lacked the PFs were isolated, and spontaneous revertants to motility regained the PFs. These results suggest that the PFs are involved in the motility of *T. phagedenis*. Isolated PFs had two major protein bands with molecular weights of 33,000 and 39,800, as revealed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Western blots with monoclonal and polyclonal antibodies indicated that both proteins were absent in the PF mutants but present in the revertants. Immunoelectron microscopy revealed that the 39,800-molecular-weight protein was distributed along the entire PF. Immunoprecipitation analysis suggested that the 39,800- and 33,000-molecular-weight proteins were closely associated in situ.**

Treponema phagedenis is an anaerobic spirochete that is cultivable on enriched media (36). The cells are approximately 0.2 μm wide and consist of a right-handed helical cell cylinder (23) surrounded by an outer membrane sheath (9, 17–21, 31, 36, 38). Inserted subterminally at each end of the cell, between the helical cell cylinder and the outer membrane sheath, are several periplasmic flagella (PFs), also referred to as axial filaments, endoflagella, and periplasmic fibrils (9, 17–20, 31, 36, 38).

The PFs of various spirochetes have been analyzed in some detail. Whereas most bacterial flagella consist of a single major protein, the major proteins isolated from PFs vary from one to six (5, 6, 16, 22, 28, 30, 32). In these studies, the major criterion used for the identification of PF proteins was enrichment during purification. In this paper, we present the first genetic evidence that multiple protein species are indeed associated with PFs.

The finding of multiple protein species associated with PFs is consistent with three possibilities. First, there could be two types of PFs, with each type consisting of a different protein. Both types of PFs could be found on each cell. This situation is analogous to the morphological flagellar types found in *Vibrio parahaemolyticus* (33). Second, phase variation could be occurring in the cell population. Each cell could be synthesizing only one or the other PF type. *Salmonella* species undergo such a phase variation in their flagella (34). Third, there could be one type of PF, with each PF consisting of multiple protein species. Both *Bacillus pumilis* (24, 37) and *Caulobacter crescentus* (26, 41) have multiple protein species associated on a given flagellum. We present evidence in this paper in support of the third explanation for *T. phagedenis*, i.e., there is more than one protein species residing on a given PF in *T. phagedenis*.

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MATERIALS AND METHODS

Organism and culture conditions. *T. phagedenis* Kazan 5 was obtained from R. Smibert, Virginia Polytechnic Institute and State University, Blacksburg. Cells were grown in a peptone-yeast extract-glucose medium supplemented with 10% heat-inactivated rabbit serum (PYG-RS [21]). Cells were plated on PYG-RS plus 0.5% Bacto-Agar (Difco Laboratories, Detroit, Mich.) by using overlays made with 0.5% SeaPlaque agarose (FMC Corp., Marine Colloids Div., Rockland, Maine) in peptone-yeast extract-glucose medium. Cultures were incubated at 35°C in an anaerobic chamber with an atmosphere of 85% nitrogen–10% carbon dioxide–5% hydrogen. Cultures were also manipulated by using the Virginia Polytechnic Institute anaerobic culture system (Bellco Glass, Inc., Vineland, N.J.). Cell concentrations were monitored by using a Coleman model 7 nephelometer (Coleman Instruments Inc., Oak Brook, Ill.). Nephelometer readings were correlated to total cell counts by using a Petroff-Hausser counting chamber and to viable cell counts (G. Caudill, M.S. thesis, West Virginia University, Morgantown, 1978; R. Limberger, Ph.D. thesis, West Virginia University, Morgantown, 1984).

Isolation of motility mutants and revertants. A clone from the *T. phagedenis* stock that consistently yielded large colonies approximately 10 mm in diameter at 6 days of incubation was selected. Progeny from this clone were used throughout this study. Motility mutants and revertants were obtained by using procedures similar to those described for *Leptospira illini* (7). A 40-ml quantity of logarithmic-phase cells (2.5×10^7 cells per ml; generation time, 5 h) was incubated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Aldrich Chemical Co., Inc., Milwaukee, Wis.) at a concentration of 50 μg/ml at 35°C. Samples (4 ml) were removed every 15 min for 75 min and diluted with 14 ml of peptone-yeast extract-glucose broth. Cells were centrifuged at 1,600 × *g* for 10 min at 25°C, resuspended in 4 ml of peptone-yeast extract-glucose broth, serially diluted, and plated. After 6 days of incubation, colonies with a diameter of 1 mm or less were regarded as potential nonmotile mutants. These colo-

nies were picked and grown in PYG-RS broth. Clones which consistently yielded small colonies after being replated were further analyzed. To obtain spontaneous revertants to motility, we spread mutant cells onto PYG-RS agar and incubated the plates for appoximately 1 month. The outer edges of the diffuse area of growth surrounding dense colonies were picked and grown in PYG-RS broth. Revertants were subsequently subcloned on PYG-RS plates.

Dark-field and electron microscopy. The motility of cells in PYG-RS broth was analyzed under dark-field illumination at 400× or 900×. For electron microscopy, specimens were incubated on carbon-coated Formvar-copper grids (300 mesh) for 1 min, washed with 0.1 M potassium phosphate buffer (pH 7.3), and stained for 30 s with 1% phosphotungstate (pH 5.0). PFs were detected by disrupting the outer membrane sheath with 1% deoxycholate and then staining. Isolated PF preparations were stained for 45 s. All specimens were examined with a Philips 201 electron microscope at an accelerating voltage of 60 kV.

Isolation of PFs. PFs were isolated by using a procedure based on methods described for other bacteria (11, 12). Cells were first harvested and then lysed as follows. A 500-ml quantity of late-logarithmic-phase cells (1.5×10^8 cells per ml) was centrifuged at 12,000 × *g* for 25 min at 4°C. The pellet was suspended in 0.1 M Tris (all Tris buffers were at pH 7.8) and centrifuged at 12,000 × *g*. Washed cells were suspended in 15 ml of 20% sucrose (wt/wt) in 0.1 M Tris, followed by the addition of 5 ml of 1.0 M Tris. The outer membrane sheath was removed by the addition of 4 ml of 20% Triton X-100 (vol/vol in 0.1 M Tris), followed by 5 min of incubation of 25°C. After centrifugation at 12,000 × *g* for 25 min at 4°C, cells were resuspended in 15 ml of the Tris-sucrose buffer–5 ml of 1.0 M Tris. Cells were digested by the addition of 2 ml of lysozyme (10 mg/ml) and 6 ml of 0.1 M Tris–0.1 M sodium EDTA. After incubation at 37°C for 30 min, another 2 ml of lysozyme was added, and the mixture was further incubated for 30 min at 37°C. DNase and RNase (3 mg each; Sigma Chemical Co., St. Louis, Mo.) were added, and the mixture was incubated for 20 min at 37°C. Deoxycholate was added to a final concentration of 1%, and the mixture was incubated for 18 to 24 h at 25°C.

PFs were isolated as follows, with electron microscopy and polyacrylamide gel electrophoresis (PAGE) being used to monitor purification. The lysate was cooled to 4°C, followed by the addition of cold saturated ammonium sulfate to a final concentration of 25%. This mixture was stirred at 4°C for 2 h and became quite viscous. Triton X-100 was added to a final concentration of 1%, and the mixture was centrifuged at 12,000 × *g* for 20 min at 4°C. The crude PFs were located in the slightly opaque material at the surface which was collected and dissolved in 1 ml of 0.1 M Tris. Occasionally, PF material would pellet to the bottom of the tube. The crude PFs were dialyzed against 0.1 M Tris–0.01 M EDTA overnight at 4°C. The PFs were then layered onto a 10 to 66% linear Hypaque 76 gradient (Winthrop Laboratories, Div. Sterling Drug Inc., New York, N.Y.) and centrifuged at 100,000 × *g* for 2 h at 20°C. The PFs migrated as a single sharp band that was collected and dialyzed overnight against the Tris-EDTA buffer. Discontinuous sodium dodecyl sulfate (SDS)-PAGE of cell extracts and purified PFs was done as described by Laemmli (25) by using a 5% stacking gel and a linear 5 to 15% gradient resolving gel. Molecular weights were estimated by using the method of Weber and Osborne (40).

Monoclonal antibody production. The procedure described by Caterson et al. (10) was used to produce monoclonal antibodies to the PFs. This procedure uses draining lymph nodes as the source for primed lymphocytes for fusion. Briefly, three BALB/c mice were injected with PF antigen at six sites (hind footpads and lateral thoracic and inguinal regions). Each mouse received approximately 80 μg of antigen on days 1, 3, 6, 9, and 12 for a total of five injections. The antigen was suspended in Freund complete adjuvant for the first injection and in Freund incomplete adjuvant for the second injection. For the remaining injections, the antigen was suspended in Dulbecco modified phosphate-buffered saline. At day 14, the draining lymph nodes were removed, and the lymphocytes were fused with the mouse myeloma cell line X63-Ag8.653 by using 20% polyethylene glycol. After 2 weeks of incubation, hybridoma culture supernatant fluids were screened for antibodies to the PFs by a modified enzyme immunoassay (10). Approximately 250 μg of purified PFs fixed in 0.05% glutaraldehyde was used as the antigen on each microtiter plate. Those cell cultures secreting PF antibodies were cloned by limiting dilution. Antibodies were obtained from cell culture supernatant fluid or ascites fluid induced with pristane (2,6,10,14-tetramethylpentadecane; Aldrich Chemical Co.).

Purification of PF subunit proteins. PF proteins were extracted from gels by using a procedure similar to that used by Caldwell and Schachter (8). After SDS-PAGE of purified PFs, the gel was stained with Coomassie blue for 30 min and destained for 30 min with 45% methanol–9% acetic acid. The gel was briefly washed in water, and the 33,000- and 39,800-molecular-weight protein bands were cut from the gel. The proteins were extracted from the gel matrix with an ISCO model 1750 electrophoretic concentrator (ISCO, Lincoln, Nebr.). The sample buffer consisted of 0.125 M Tris, 0.2 M glycine, and 0.1% SDS; inner-compartment buffer consisted of 0.025 M Tris, 0.2 M glycine, and 0.1% SDS. The concentration procedure was done at 3 W for 4 h at 25°C. Each purified PF protein migrated as one band in Coomassie blue-stained gels.

Rabbit immunization. New Zealand White rabbits were immunized with PF proteins in Freund incomplete adjuvant (15). Each rabbit was injected with approximately 200 to 260 μg of either the 33,000- or the 39,800-molecular-weight denatured PF protein. All rabbits were injected subcutaneously in the back of the neck on days 0, 14, 28, and 42 and bled on day 50. The serum was collected and stored at −70°C.

Western blotting. The general procedures were those described by Towbin et al. (39). A Transphor system (Hoeffer Scientific, San Francisco, Calif.) was used to transfer proteins from acrylamide gels to nitrocellulose paper (pore size, 0.2 μm; Schleicher & Schuell, Inc., Keene, N.H.). The buffer consisted of 0.025 M Tris, 0.192 M glycine, and 20% methanol. Transfer was performed at 0.1 to 0.2 A overnight at 4°C and then at 1 A for 0.5 h. Either anti-mouse or anti-rabbit immunoglobulin conjugated to horseradish peroxidase (Southern Biotechnology Associates, Birmingham, Ala.) was used as the second antibody. Bound antibodies were visualized by development in 4-chloro-1-naphthol with hydrogen peroxide.

Immunoelectron microscopy. Protein A-coated colloidal gold was used for immunolocalization as described by Barbour et al. with some modifications (2). Gold chloride (Sigma Chemical Co.) was reduced to gold sol with sodium citrate (14) and conjugated to protein A (35). A 10-ml quantity of late-logarithmic-phase cells (2.0×10^8 cells per ml) was washed once in 10 ml of 0.01 M phosphate-buffered saline (pH 7.5) with 0.03% sodium azide (PBS-azide) and

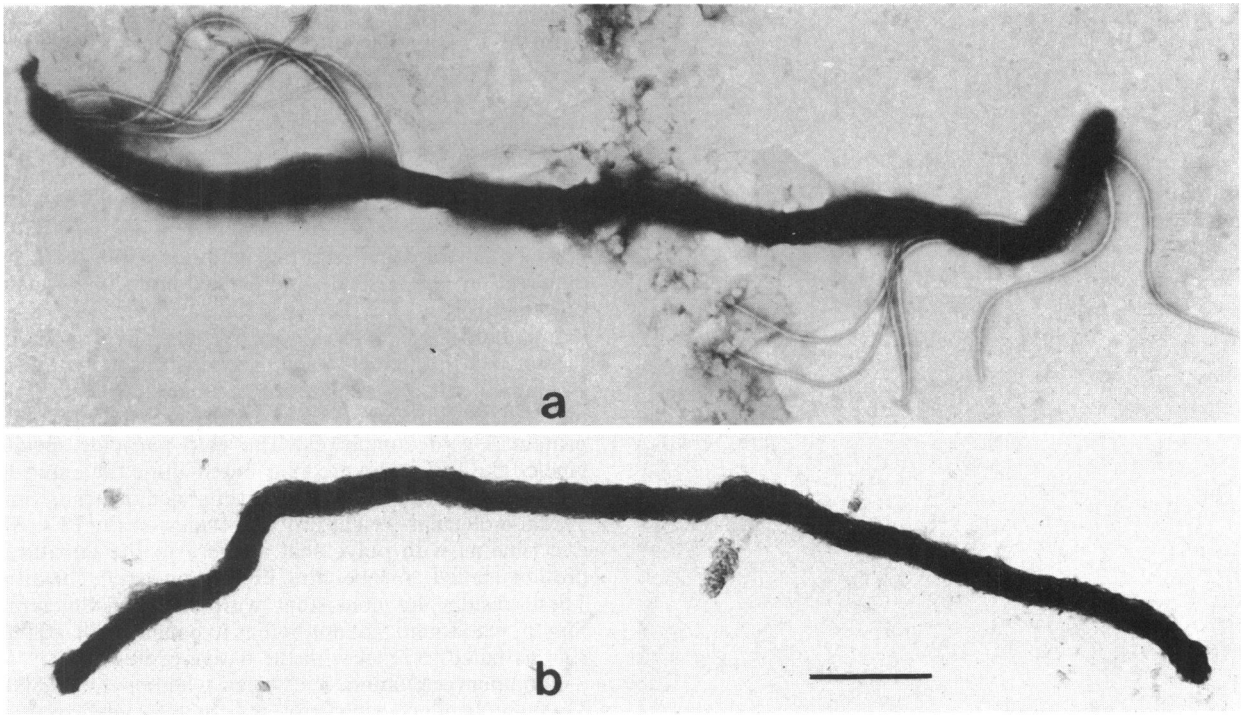

FIG. 1. Electron micrographs of the wild type (a) and PF mutant T-55. Cells were disrupted with 1% deoxycholate to free PFs. Mutant T-40 appeared to be identical to mutant T-55 (data not shown). Bar, 1 μm.

resuspended in 0.5 ml of this same buffer. All centrifugations were done at 1,600 × *g* for 10 min at 25°C. The PFs were exposed by disruption of the outer membrane sheath with 1% deoxycholate, and the cells were washed twice in 1 ml of PBS-azide. The final pellet was washed in 1 ml of 1% bovine serum albumin in PBS-azide and resuspended in 0.5 ml of this same buffer. Ascites fluid monoclonal antibody was added to the cells (final dilution, 1:10), and the mixture was incubated for 1 h at 25°C. Cells were washed twice in 1 ml of PBS-azide, and the final pellet was resuspended in 0.25 ml of PBS-azide. Protein A-gold (0.25 ml) was added to the cells, and the mixture was incubated for 30 min at 25°C. Cells were washed three times in 5 ml of PBS-azide, and the final pellet was resuspended in 0.5 ml of PBS-azide and viewed in the electron microscope after being stained with phosphotungstate.

Immunoprecipitation. The immunoprecipitation procedure was modified from the method described by Barbour et al. (1). A 10-ml quantity of late-logarithmic-phase cells of *T. phagedenis* was centrifuged at 1,600 × *g* for 10 min at 25°C and washed once in 10 ml of TBS buffer (0.02 M Tris, 0.05 M NaCl [pH 7.5]), and the pellet was resuspended in 1.5 ml of TBS. Cells were placed on ice and sonicated intermittently for 15 min. Large cell debris was removed by centrifugation at 1,600 × *g* for 15 min at 25°C. Ascites fluid monoclonal antibody 1C6 diluted 1:6 in 20% bovine serum albumin (0.6 ml) was added to the clear supernatant fluid, and the mixture was incubated for 1 h at 25°C. As a control, normal ascites fluid was also diluted 1:6 and incubated with the sonicate. In addition, the sonicate was incubated in bovine serum albumin diluent alone. Pansorbin (150 μl; *Staphylococcus aureus* Cowan 1; Calbiochem Behring, La Jolla, Calif.) was added to the samples, which were then incubated for 1 h at 25°C. The immunoprecipitate and controls were centrifuged at 1,600 × *g* for 5 min at 25°C and washed three times in 2 ml of TBS. The final pellet was suspended in 100 μl of electrophoresis buffer, boiled for 5 min, and briefly centrifuged. For Western blotting, approximately 30 μl of the supernatant fluids were applied to each lane of an SDS-polyacrylamide gel.

RESULTS

Isolation and characterization of motility mutants and their revertants. Electron-microscopic examination of growing *T. phagedenis* revealed 5.5 ± 1 PFs attached at each of the cell ends (mean ± standard deviation; range, 4 to 8 for 10 cells examined; Fig. 1a). Parental *T. phagedenis* had diffuse colonies with well-defined borders and a diameter of 10 mm after 6 days of incubation. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis yielded 60 colonies with a diameter of less than 1 mm after 6 days of incubation. Of these 60, 2 (mutants T-40 and T-55) were completely nonmotile, as assayed by dark-field microscopy. Electron microscopy revealed that they no longer formed PFs (Fig. 1b). The loss of motility and PFs in *T. phagedenis* occurred together. To determine if single or multiple mutations were involved in these altered phenotypes, we isolated spontaneous revertants to motility after prolonged incubation on agar plates. Ten motile revertants were isolated from mutant T-55, and four were isolated from mutant T-40. All 14 had regained their PFs. These results suggest that a single mutation which led to the loss of motility and the inhibition of PF synthesis occurred in each mutant. Three revertants from each mutant were studied in detail (see below).

Analysis of PF proteins. To further characterize the PF mutants, we first analyzed the PF proteins of the wild type. Electron microscopy revealed that isolated PFs were relatively free from contaminating cell material (Fig. 2). No PFs could be isolated from mutants T-40 and T-55 by using the same procedures as used for the wild type. Wild-type PFs

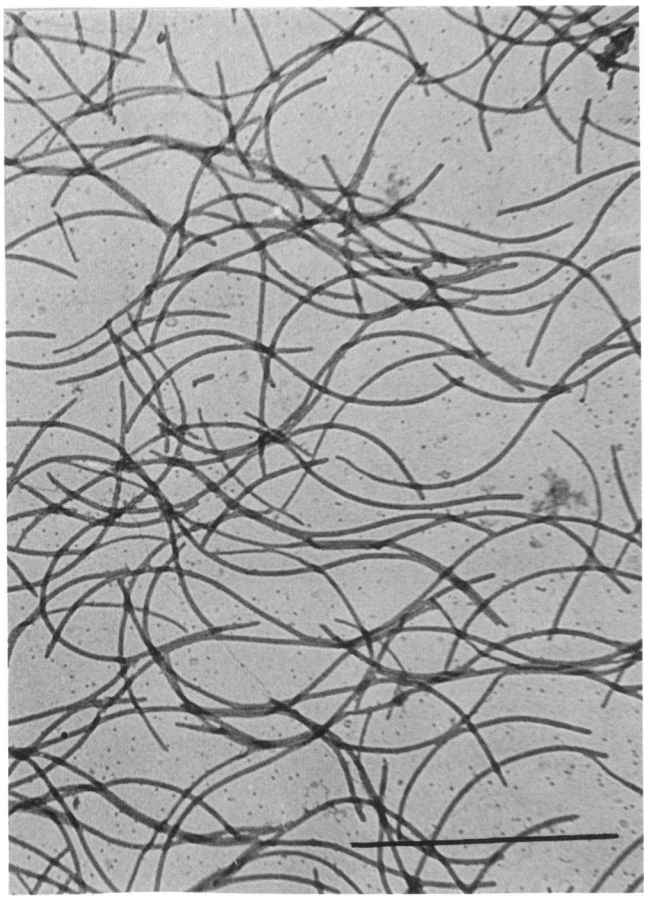

FIG. 2. Electron micrograph of isolated PFs. Bar, 1 μm.

had a wavelike morphology, as reported by others for *T. phagedenis* Reiter (16, 18, 19), and were readily dissociated in 8 M urea, 0.05 N NaOH, or 0.05 M HCl and by heat (boiling for 5 min). SDS-PAGE of the PFs from the wild type and the revertants from each mutant resulted in two major protein bands with molecular weights of 33,000 and 39,800 (Fig. 3). Samples taken for SDS-PAGE analysis during purification indicated that these proteins were enriched by the procedure. These results suggest that at least two major proteins with molecular weights of 33,000 and 39,800 constitute the PFs of *T. phagedenis*.

Western blotting. The PF mutants were tested by Western blotting for the presence of the 33,000- and 39,800-molecular-weight proteins. Two approaches were used to obtain PF antibodies. First, monoclonal antibodies were prepared against wild-type PFs. Of five clones secreting PF antibodies, only one (clone 1C6) produced antibodies (class IgG2b) which reacted in Western blotting. When tested against whole-cell lysates, monoclonal antibody 1C6 specifically reacted with the 39,800-molecular-weight PF protein of the wild type and the six motile revertants (Fig. 4). No reactions occurred with lysates from mutants T-40 and T-55. These results suggest that both PF mutants failed to synthesize detectable amounts of the 39,800-molecular-weight protein.

The second approach involved preparing polyclonal antibody probes to both the denatured 33,000- and the denatured 39,800-molecular-weight PF proteins extracted from SDS-polyacrylamide gels. The antiserum to the 39,800-molecular-weight protein reacted strongly with the 39,800- and weakly with the 33,000-molecular-weight proteins (Fig. 5a). However, the antiserum to the 33,000-molecular-weight protein reacted specifically with the 33,000-molecular-weight protein. Occasionally, a doublet band could be seen at this lower-molecular-weight area in the gel. As with monoclonal antibody 1C6, no reactions were detected with mutants T-40 and T-55, but all six revertants were positive (Fig. 5b). Taken together, the results with both monoclonal and polyclonal antibodies indicate that mutants T-40 and T-55 are deficient in the 33,000- and 39,800-molecular-weight PF proteins.

Immunoelectron microscopy. We directly tested for the presence of the 39,800-molecular-weight protein on the PFs. Partially disrupted cells were incubated with monoclonal antibodies from clone 1C6, washed, and then treated with protein A-gold complexes. The gold particles specifically labeled the PFs and were distributed along the entire length of the PFs (Fig. 6). These results demonstrate that the 39,800-molecular-weight protein resides on the PFs. Similar experiments with polyclonal antisera to the denatured PF proteins failed to label the PFs or any cell constituents. These results are consistent with those of Emerson and Simon, who found that antibodies to denatured flagellin of *B. subtilis* failed to react with the native protein (13).

Immunoprecipitation. We tested whether the 39,800- and 33,000-molecular-weight proteins are associated in situ. The supernatant fluid from a cell sonicate of *T. phagedenis* was

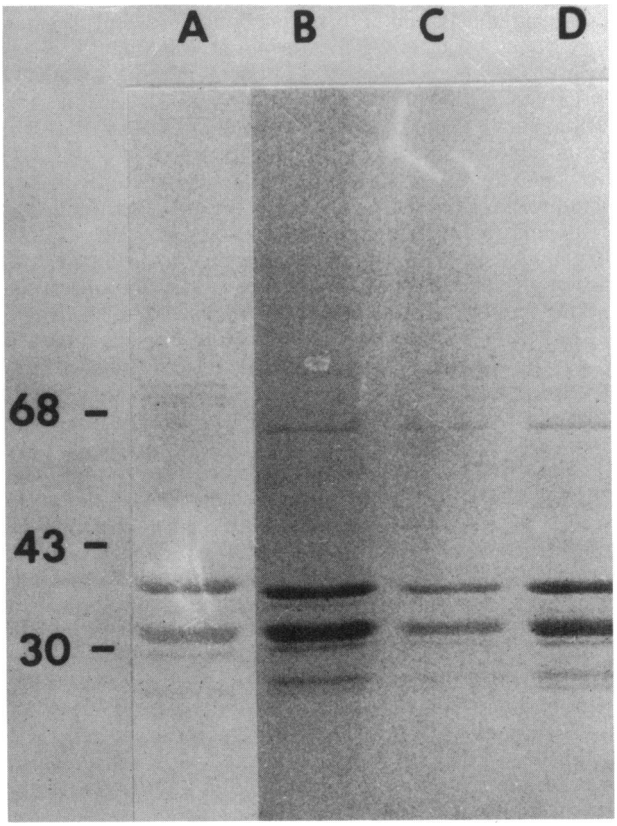

FIG. 3. SDS-PAGE of PFs isolated from the wild type (lane A) and motile revertants (lanes B to D). Other motile revertant PFs had identical electrophoretic patterns. Numbers at left indicate molecular weights in thousands.

reacted with monoclonal antibodies from clone 1C6. As control, the sonicate supernatant fluid was incubated with buffer alone (no antibody) and with normal ascites fluid. After precipitation with protein A, boiling, and SDS-PAGE, examination of the supernatant fluid obtained with monoclonal antibody 1C6 revealed that the 39,800- and 33,000-molecular-weight proteins were among the major proteins detected by Coomassie blue staining (Fig. 7, lane A). These proteins were not detected in the controls. The other major proteins detected in the monoclonal antibody reaction mixture had the same molecular weight as those found in ascites fluid precipitated with protein A alone and are thus unlikely to be *T. phagedenis* proteins (Limberger, Ph.D. thesis). Western blotting of the immunoprecipitate and controls probed with a mixture of polyclonal PF antisera revealed that the only positive reaction occurred with the incubation mixture containing monoclonal antibody 1C6; the controls were negative. In addition, both the 39,800- and 33,000-molecular-weight proteins reacted with the polyclonal antisera (Fig. 7, lane B). These results suggest that the PF proteins do not nonspecifically bind to protein A. Moreover, the results indicate a close association of the 33,000- and 39,800-molecular-weight proteins.

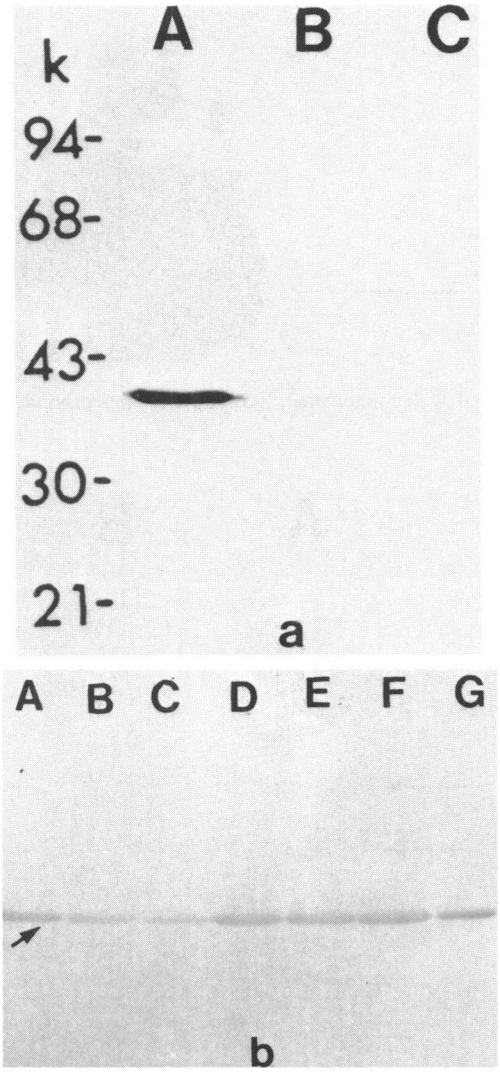

FIG. 4. (a) Western blotting of the wild type and PF mutants probed with monoclonal antibody 1C6. Lanes: A, wild type; B, mutant T-40; C, mutant T-55. Numbers at left indicate molecular weights in thousands (k). (b) Western blotting of six motile revertants probed with monoclonal antibody 1C6. Lanes: A to F, motile revertants; G, wild type. The arrow points to the 39,800-molecular-weight protein.

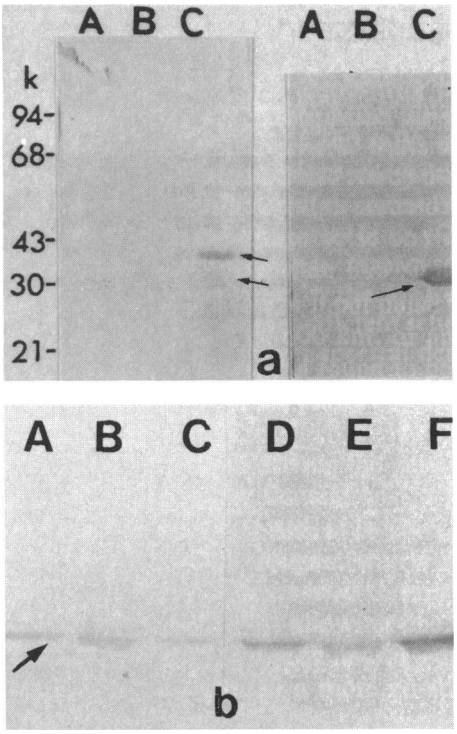

FIG. 5. (a) Western blotting of the wild type and PF mutants probed with polyclonal antisera to denatured PF proteins. Left panel, Antiserum directed against the 39,800-molecular-weight protein; right panel, antiserum directed against the 33,000-molecular-weight protein. Lanes (for both panels): A, mutant T-40; B, mutant T-55; C, wild type. Arrows point to the 39,800 (top)- and 33,000 (bottom)-molecular-weight proteins. Numbers at the left indicate molecular weights in thousands (k). (b) Western blotting of six motile revertants (lanes A to F) probed with polyclonal antiserum to the 33,000-molecular-weight protein. The arrow points to the 33,000-molecular-weight protein. The polyclonal antiserum to the 39,800-molecular-weight protein reacted similarly with the revertants, except that a weak reaction to the 33,000-molecular-weight protein was also present (data not shown).

DISCUSSION

The structural and biochemical analyses of mutants T-40 and T-55 indicate that the PFs are involved in the motility of *T. phagedenis*. PFs have also been shown to be involved in the motility of *L. illini* and *Spirochaeta halophila* (7, 29). Each of these spirochetes has only one PF at each cell end, whereas *T. phagedenis* has several.

The PFs of *T. phagedenis* were found to contain at least two different major proteins. Western blotting indicated that both proteins are present in freshly disrupted cells and thus are not the result of degradation upon isolation. The 39,800-molecular-weight protein was clearly shown by immunoelectron microscopy to be part of the PFs. The 33,000-molecular-weight protein is unlikely to be a contaminant, as it was purified along with the 39,800-molecular-weight protein, was absent in the PF mutants, and immunoprecipitated along with the 39,800-molecular-weight protein.

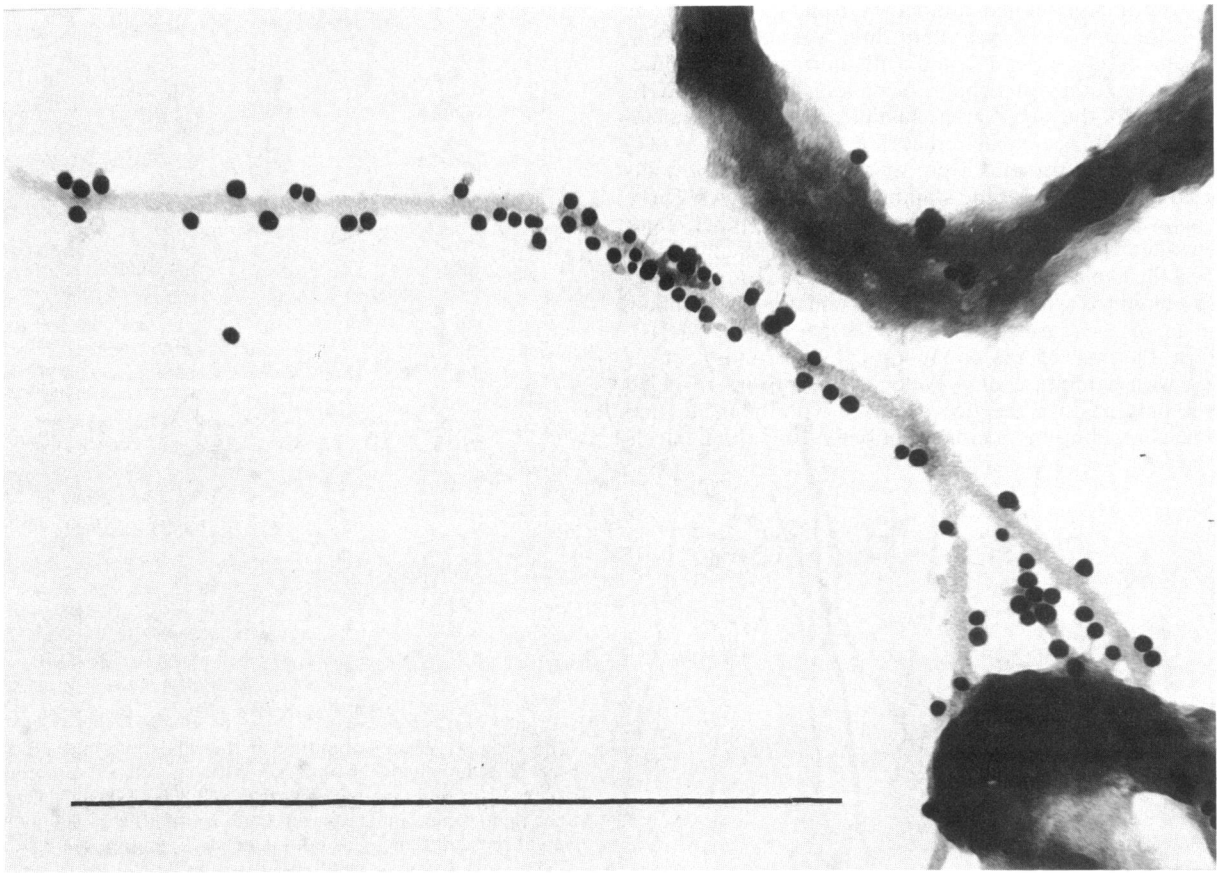

FIG. 6. Electron micrograph of partially disrupted *T. phagedenis* cells stained with monoclonal antibody 1C6 and protein A-coated colloidal gold. Bar, 1 μm.

Multiple major PF proteins have been reported in other spirochetes. Six were observed in *L. biflexa* B16 (28), two were observed in *Spirochaeta* spp. (22; B. Brahamsha and E. P. Greenberg, personal communication), and three were observed in *T. pallidum* (30). In *T. phagedenis* Reiter, which is genetically very similar to *T. phagedenis* Kazan 5 (27), three to four proteins have been observed to be associated with the PFs (5, 16, 30, 32). Of several spirochete species and strains we tested by using the monoclonal and polyclonal antisera described here, only *T. phagedenis* Reiter had Western blot reactions identical to those of *T. phagedenis* Kazan 5 (Limberger and Charon, Abstr. Annu. Meet Am. Soc. Microbiol. 1985; R. Limberger and N. Charon, manuscript in preparation). These results suggest that the PF proteins in both strains are very similar. Our results with *T. phagedenis* Kazan 5 are most similar to those reported by Bharier and Allis (5) and by Penn et al. (30). They found one protein with a molecular weight of 36,500 to 38,500 and a doublet of 33,000 to 34,000. Occasionally, we saw a doublet band at this lower molecular weight. Future experiments with two-dimensional gel electrophoresis and Western blotting should enable us to resolve the number of PF proteins in *T. phagedenis*.

T. phagedenis is one of only a few bacterial species shown to have more than one major protein residing on a given flagellum. It is unlikely that there are two types of PF in *T. phagedenis* which aggregate together during immunoprecipitation. The cells were extensively sonicated, and large cell debris was removed before reaction of the sonicate with

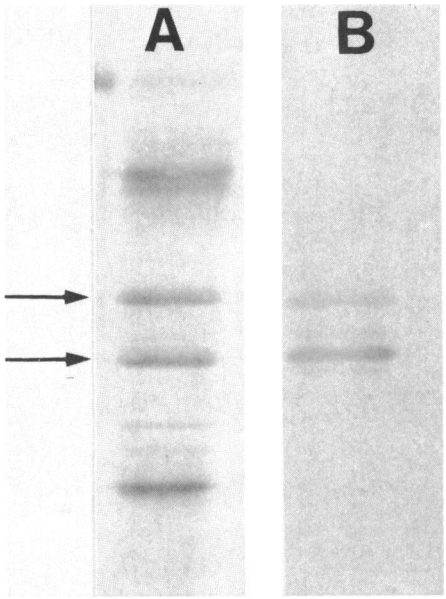

FIG. 7. Cell sonicates incubated with monoclonal antibody 1C6 and precipitated with protein A. Lanes: A, Coomassie blue-stained SDS-PAGE pattern of the immunoprecipitate; B, Western blotting of the immunoprecipitate probed with polyclonal PF antisera. The arrows point to the 39,800 (top)- and 33,000 (bottom)-molecular-weight proteins.

monoclonal antibody 1C6. Sonication breaks PFs into small fragments (16), which should minimize aggregation and also free the PFs from the cells. Along these lines, Coomassie blue staining of the immunoprecipitate indicated that the 39,800- and 33,000-molecular-weight proteins were the principal *T. phagedenis* proteins in the immunoprecipitate (Fig. 7, lane A). Our immunoprecipitation results suggest that the presence of multiple PF protein species is not due to phase variation or to different types of PFs residing on a given cell. The results presented here indicate that both proteins reside on a given PF in *T. phagedenis*, in analogy to *C. crescentus* (26, 41) and *B. pumilis* (24, 37).

It is too early to explain why multiple protein species are found on the PFs of *T. phagedenis* and other spirochetes. We propose that *T. phagedenis* swims by rotating the PFs (N. Charon, R. Limberger, and C. Maloney, manuscript in preparation) in a manner analogous to that of other bacterial flagella (34); this model is a variation of those proposed for other spirochetes (3, 4, 9). Perhaps one or more of the PF protein species augment rotation between the outer membrane sheath and the helical cell cylinder. Clearly, defining the function of these proteins is likely to be coupled to a better understanding of the mechanism of motility of these spirochetes.

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