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# Characterization of the Periplasmic Flagellum Proteins of *Leptospira interrogans*

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**The structure and composition of periplasmic flagella (PF) from *Leptospira interrogans* serovar pomona type kennewicki were characterized. Electron microscopic observations showed that leptospiral PF were complex structures composed of an 11.3-nm-diameter core surrounded by two sheath layers with 21.5- and 42-nm diameters. Two-dimensional gel electrophoresis of isolated PF showed the presence of seven different proteins ranging in mass from 31.5 to 36 kDa. Rabbit polyclonal and mouse monoclonal antibodies against PF proteins were prepared and were used to localize specific proteins to portions of the PF structure by immunoelectron microscopy. A 34-kDa protein was associated with the 11.3-nm-diameter core filament, while a 36-kDa protein was associated with a PF sheath (21.5-nm-diameter filament). The amino termini of the 34- and 35.5-kDa proteins were homologous to PF core proteins of other spirochetes. The experimental data suggested that *L. interrogans* PF contains 2 proteins (34 and 35.5 kDa) in the PF core.**

Spirochetes possess an unusual cellular structure. These bacteria are long, slender, and helical (13). Spirochetes, like gram-negative bacteria, possess a cytoplasmic membrane, peptidoglycan, and an outer membrane (13). Flagellar filaments are located in the periplasmic space (13). Because of their location, these structures are referred to as periplasmic flagella (PF). In *Leptospira* spp., one flagellum is inserted at each end of the protoplasmic cylinder and extends along the helical axis of the protoplasmic cylinder without overlapping (4, 11).

The mechanisms of spirochete motility are thought to differ significantly from those of other bacteria. Research directed at determining the mechanisms involved in spirochete motility has often used *Leptospira* spp. as models. Berg et al. (3) suggest that the PF rotate between the outer sheath and the protoplasmic cylinder. Rotation of the PF propagates a helical wave motion along the cell cylinder and causes rolling of the protoplasmic cylinder within the outer membrane (3, 7, 11). Rolling of the cell cylinder results in translational migration of the cell through gel-like medium in a manner similar to the movement of a corkscrew (3, 11). Both *Leptospira interrogans* and *Borrelia burgdorferi* undergo rapid translational movement through highly viscous solutions of methylcellulose (15, 17). Under these conditions, most bacteria with external flagella are typically nonmotile (17). The ability of pathogenic spirochetes, such as *L. interrogans*, *B. burgdorferi*, and *Treponema pallidum*, to penetrate many different types of tissue and basement membranes is thought to be directly related to the motility of these bacteria in viscous solutions (15, 17, 29, 32).

The PF from several different spirochetes have been characterized, and they are found to be complex structures containing several different proteins (5, 9, 16, 19, 23, 24, 27). Typically, a core filament is surrounded by one sheath layer (5, 9, 24). Sequence analyses of several spirochete PF genes and proteins show that there are two distinct classes (A and B) of PF proteins. Class A proteins are associated with the sheath surrounding core PF filaments, and they contain signal sequences at the amino terminus, and have short regions of sequence similarity with class A proteins from other spirochetes (6, 14, 24, 27). Class B proteins are flagellinlike proteins which form the PF core. Class B proteins lack signal sequences, are highly conserved among spirochetes, and are homologous to flagellum proteins from other eubacteria (8, 21, 24, 26, 27). Multiple class B proteins are present in the PF core (24, 27).

*Leptospira* spp. were shown previously to possess PF composed of four to six proteins (16, 23) and a core filament surrounded by at least two sheath layers (23). The purpose of this study was to characterize proteins associated with the leptospiral PF and localize some of these proteins to core and sheath structures.

## MATERIALS AND METHODS

**Bacteria.** *L. interrogans* serovar pomona type kennewicki RZ11 (37) was propagated in bovine serum albumin-Tween 80 medium (10) at 30°C.

**Electron microscopy.** Intact bacterial cells were examined by shadow casting as described elsewhere (2). Cells partially disrupted by Triton X-114 or by cold osmotic shock (36) and purified PF were placed on Parlodion-coated copper grids, negatively stained with 3% ammonium molybdate (pH 7.5) for 10 s (5), and examined in a Philips 410 electron microscope. The diameters of PF structures were determined by computer image analysis and by direct measurement of structures on electron micrographs. Standards for these measurements were 5-nm-diameter gold particles (Biocell Research Ltd., Cardiff, United Kingdom).

**PF enrichment.** Two liters of stationary-phase cultures of RZ11 was harvested by centrifugation and washed twice with 0.01 M phosphate-buffered saline (pH 7.2). The cell pellet was resuspended in 20 ml of TA buffer (0.1 M Tris-HCl, 4.6 mM $NaN_3$ [pH 7.8]). The cell suspension was brought to 1% Triton X-114 with a 10% Triton X-114 stock solution, and the outer membrane was extracted for 1 h on

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ice. Soluble outer membrane material was separated from the protoplasmic cylinders by centrifugation (38).

Protoplasmic cylinders were resuspended in 10 ml of 0.1 M Tris-HCl (pH 7.8) and digested with lysozyme (0.1 μg/ml) for 1 h on ice. The PF were stripped from protoplasmic cylinders by homogenization at high speed for 20 min at 4°C in a Sorvall OmniMixer. The PF were separated from the protoplasmic cylinders by centrifugation at 34,000 × *g* for 20 min, the supernatant (PF) was held on ice, and the pellet was extracted again. The supernatants were combined, and PF were pelleted by centrifugation at 450,000 × *g* for 4 h at 4°C. The PF were resuspended in 1 ml of TA buffer, and the mixture was partitioned into aqueous and detergent phases by increasing the concentration of Triton X-114 to 2%, as previously described (38). The aqueous phase was extracted twice with 2% Triton X-114 and then stored at −20°C. Purification steps were monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electron microscopy.

**Protein electrophoresis.** Protein samples were analyzed by electrophoresis in Tris-Tricine-buffered SDS-polyacrylamide gels (30). For two-dimensional (2D) PAGE, isoelectric focusing in the first dimension was performed by using 1.8% ampholytes, pH 5 to 7, and 0.2% ampholytes, pH 3 to 10 (25), and was followed by SDS-PAGE using Tris-Tricine-buffered gels (30). Molecular weight standards were purchased from GIBCO-BRL, Life Technologies, Inc. (Gaithersburg, Md.).

**Development of monoclonal and polyclonal antisera against PF proteins.** Monoclonal antibodies (MAbs) were prepared from BALB/c mice immunized with purified PF emulsified in Ribi adjuvant (Ribi Immunochem Research Inc., Hamilton, Mont.) as described elsewhere (35). Antibody production of hybridoma cultures was analyzed with an enzyme immunoassay (34) using the PF extract as antigen. Polyclonal antisera were produced in New Zealand White rabbits by using purified PF proteins extracted from Coomassie blue-stained preparative SDS-polyacrylamide gels as described elsewhere (20). Polyclonal antiserum to the 33-kDa *Treponema phagedenis* PF (20, 24) core protein was provided by Nyles Charon. Polyclonal antiserum to *T. pallidum* PF was provided by David Haake.

**Immunoblot analysis.** Proteins were electrophoretically transferred to Immobilon (Millipore Corp., Bedford, Mass.) membranes, and immunoblot analysis was performed as described previously (38). Rabbit antisera were diluted 1:100 before use. MAbs were used as undiluted hybridoma supernatants. Secondary antibodies were horseradish peroxidase-labeled goat anti-rabbit immunoglobulin G (IgG) or biotin-labeled goat anti-mouse IgA, IgG, and IgM (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) and were diluted 1:1,000. Immunoblots with goat anti-mouse antisera were incubated with horseradish peroxidase-labeled streptavidin (Kirkegaard & Perry). Immunoreactive proteins were identified by development with a solution containing hydrogen peroxide and 4-chloro-1-naphthol (38). Following development, additional proteins on the membranes were localized by staining with 0.1% amido black (31).

**Immunoelectron microscopy.** Protoplasmic cylinders from 10 ml of mid-logarithmic-phase cultures of RZ11 were prepared as described above. Protoplasmic cylinders were washed twice with 0.1 M Tris-HCl (pH 7.8) and reacted for 2 h at 24°C with either a 1:100 dilution of rabbit anti-PF antibody in TST buffer (10 mM Tris, 150 mM NaCl, and 0.05% Tween 20 [pH 7.4]) or undiluted hybridoma culture supernatants. Protoplasmic cylinders were washed twice in TST buffer, incubated for 1 h with a 1:10 dilution of either gold-labeled goat anti-rabbit IgG (5 nm-diameter particles) or gold-labeled goat anti-mouse IgM and IgG (5-nm-diameter particles) (Biocell Research Ltd.), and then washed twice in TST buffer.

Double labeling of PF was carried out by combining anti-PF mouse MAb 2A5 and rabbit polyclonal antibodies to the 34- and 34.5-kDa PF protein bands and reacting this mixture with the partially disrupted cells. In this experiment, a secondary-antibody mixture containing gold-labeled goat anti-mouse IgG (5-nm-diameter particles) and gold-labeled goat anti-rabbit IgG (10-nm-diameter particles) (1:10 dilution of each) was used. Grids were negatively stained with ammonium molybdate.

**Amino acid sequence analysis of PF proteins.** Protein samples from *L. interrogans* whole cells were separated by 2D PAGE, transferred to Immobilon membranes, and stained with amido black. Portions of the membrane containing the 34- and 35.5-kDa PF proteins were isolated, and amino-terminal sequence was determined by Edman degradation (1) with a 477A protein sequencer/120A analyzer (Applied Biosystems Inc., Foster City, Calif.) by the Iowa State University protein sequence service. Each protein was isolated and was sequenced twice.

**Nucleotide sequence accession number.** Protein sequences reported here have been deposited with the National Biomedical Research Foundation and are available from GenBank under accession no. A 41210 (34-kDa protein) and B 41210 (35.5-kDa protein).

## RESULTS

**Electron microscopy of *L. interrogans* PF.** The structure of *L. interrogans* PF was characterized by electron microscopy. Samples of intact and detergent-disrupted cells and of purified PF were examined. Analysis of shadow-cast intact cells showed that the PF extended along approximately one-third of the length of the protoplasmic cylinder. In intact cells, the PF were approximately 25 nm in diameter (Fig. 1A). PF filaments of various widths were seen in cells disrupted with Triton X-114. The largest of these PF filaments possessed a helical sheath, approximately 42 ± 5 nm in diameter, which surrounded a filament 21.5 ± 1.5 nm in diameter. (Fig. 1B). Electron microscopic analysis of a subcellular fraction enriched for PF showed the presence of both the 21.5-nm-diameter filament (Fig. 1C) and a filament 11.3 ± 0.3 nm in diameter (Fig. 1D). Although the PF-enriched fraction did not contain 42-nm-diameter filaments, coiled structures approximately 42 nm in diameter and free of PF were observed in cells disrupted by Triton X-114 or osmotic shock (data not shown).

**Preparation of *L. interrogans* PF.** To determine the protein composition of *L. interrogans* PF, PF structures were isolated and characterized by PAGE. Approximately 10 proteins with masses ranging from 15 to 62 kDa were seen in a PF-enriched fraction of *L. interrogans* (Fig. 2A). Four protein bands of relative molecular masses of 31.5, 34, 34.5, and 35.5 to 36 kDa were the most prominent. Since these proteins were similar in molecular mass to other spirochete PF proteins (5, 9, 16, 19, 24), they were characterized in detail. 2D PAGE demonstrated that at least four proteins form the 35.5- to 36-kDa protein band (Fig. 2B).

**Immunoblot analysis.** Polyclonal antisera and MAbs to PF proteins were prepared to characterize PF structures. Initially, the specificities of prepared antibodies were deter-

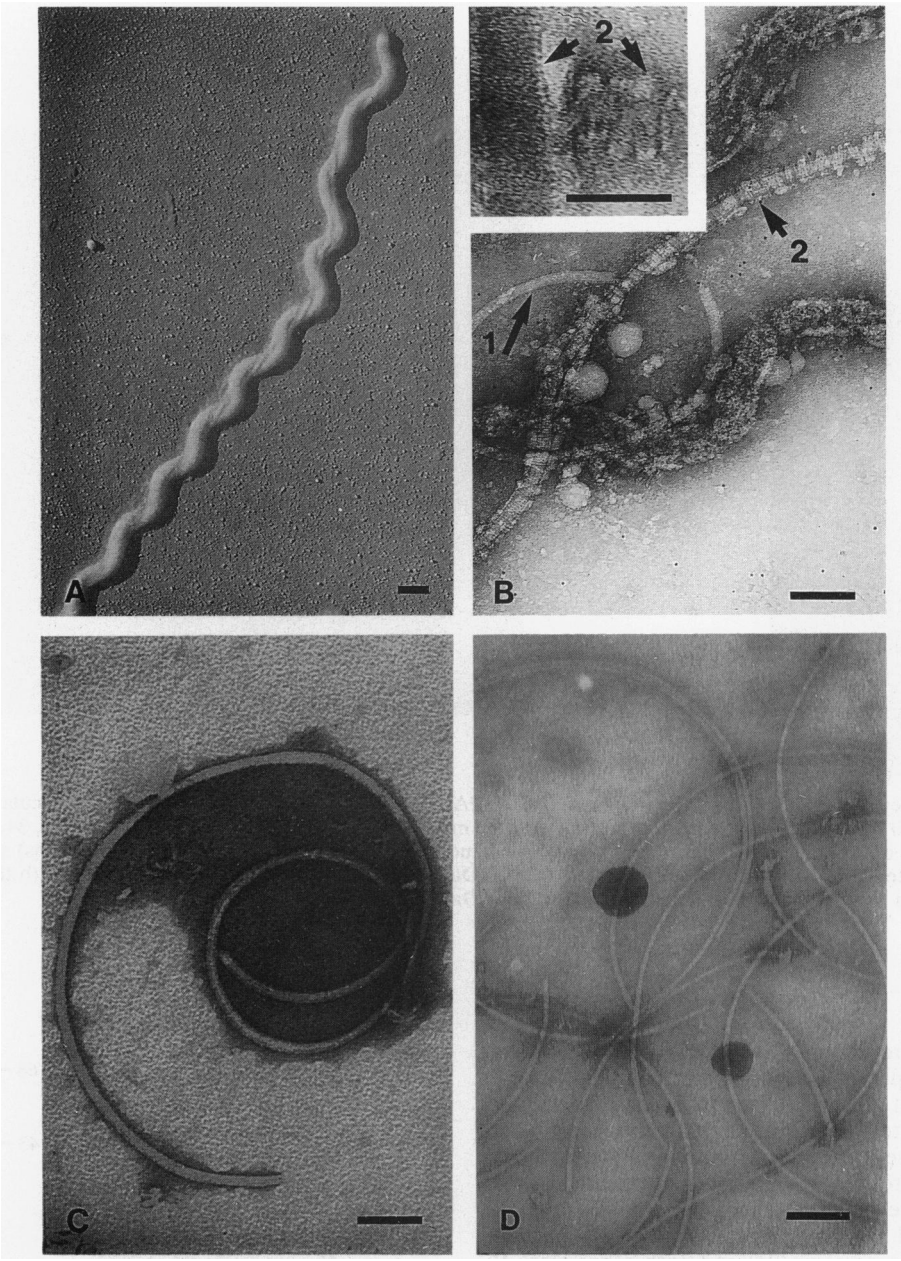


FIG. 1. Electron microscopic analysis of *L. interrogans* PF. (A) Shadow-cast preparation of *L. interrogans* showing PF. Bar, 200 nm. (B) Negative stain of Triton X-114-disrupted leptospiral cells showing the 21.5-nm-diameter filaments (arrow 1), and the 42-nm-diameter sheath (arrow 2). Bar, 100 nm; inset bar, 50 nm. (C) Negative stain of purified PF extract showing the 21.5-nm-diameter filament. Bar, 100 nm. (D) Negative stain of purified PF showing the 11.3-nm-diameter filament. Bar, 100 nm.

mined by immunoblot analysis with SDS-PAGE-fractionated PF preparations. The 31.5- and 34-kDa proteins were recognized by MAbs 4A9 and 3C12, respectively (Fig. 3 and 4). Two MAbs (2A5 and 1H8) reacted with a 36-kDa protein (Fig. 3 and 4). Polyclonal antiserum against the 35.5- to 36-kDa protein band, polyclonal antiserum against a mixture of the 34- and 34.5-kDa protein bands, and polyclonal antiserum against the *T. phagedenis* 33-kDa PF protein reacted with the 34- and 35.5-kDa proteins of *L. interrogans* (Fig. 3 and 4). Polyclonal antiserum to *T. pallidum* PF reacted with both the 34- and 35.5- to 36-kDa proteins and with a 60-kDa protein (Fig. 3 and 4).

**Immunoelectron microscopic analysis of PF.** Antibodies to PF proteins were used to localize specific proteins in the PF structure by immunoelectron microscopy. MAbs 1H8 and 2A5 reacted with the 21.5-nm-diameter filaments (Fig. 5). MAb 3C12 reacted with the 11.3-nm-diameter filament. Polyclonal antisera to the 35.5- to 36-kDa protein band and antiserum against the mixture of the 34- and 34.5-kDa protein bands reacted with the 11.3-nm-diameter filament (Fig. 5). Specific interaction between antibodies directed against treponemal PF proteins, or MAb 4A9, and cellular or PF structures was not observed by using immunoelectron microscopy. The results of immunoblot and immunoelectron

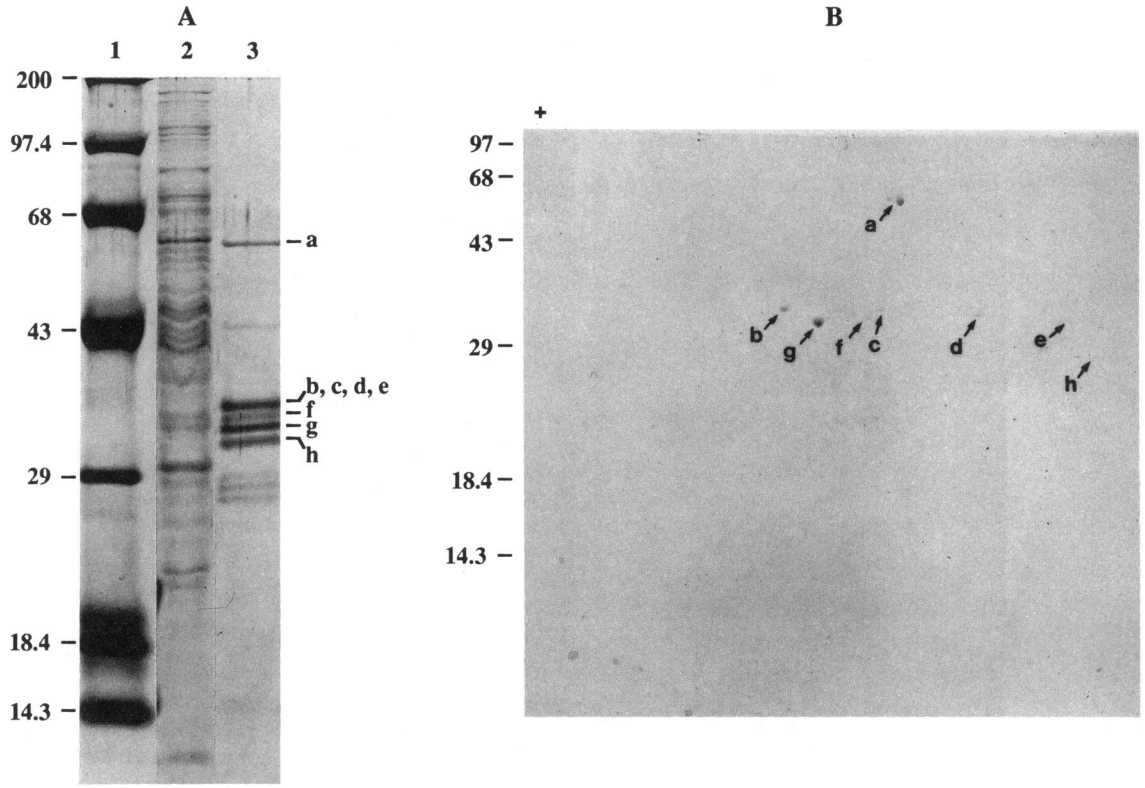


FIG. 2. Protein profiles of purified *L. interrogans* PF. (A) SDS-PAGE (10% polyacrylamide). Lanes: 1, molecular mass standards (in kilodaltons); 2, whole-cell lysate; 3, PF-enriched preparation showing major bands of 62 (a), 35.5 to 36 (b through e), 34.5 (f), 34 (g), and 31.5 (h) kDa and minor bands of 42.5, 28, and 27 kDa (Coomassie blue stained). Relative molecular masses (in kilodaltons) are shown on the left. (B) 2D PAGE of PF proteins showing eight proteins: one in the 62-kDa band (a), four in the 35.5- to 36-kDa band (b through e), one in the 34.5-kDa band (f), one in the 34-kDa band (g), and one in the 31.5-kDa band (h).

microscopic analyses of PF proteins are summarized in Table 1.

**Amino-terminal sequences of PF proteins.** Since the 34- and 35.5-kDa proteins appeared to be associated with the PF core, we expected that these proteins would have homology with class B spirochete PF proteins. The amino termini of both proteins were sequenced and compared with other spirochete class B proteins. The amino termini of the 34- and 35.5-kDa proteins were similar to each other and had homology with the deduced sequence for a 31.5-kDa PF protein from *Leptospira borgpetersenii* (21) and with other class B spirochete PF proteins (Fig. 6). Attempts to obtain the amino-terminal sequences of the other PF proteins were unsuccessful.

## DISCUSSION

In this study, *L. interrogans* PF structure and composition were analyzed. Leptospiral PF filaments from detergent-treated or osmotically disrupted cells fell into three distinct classes on the basis of filament diameter: 11.3, 21.5, and 42 nm. The 11.3-nm-diameter PF filament formed a core structure surrounded by a sheath, yielding the 21.5-nm-diameter filament. The 21.5-nm-diameter filament was surrounded by a second sheath layer, forming the 42-nm-diameter filament. Electron microscopic analysis showed that the 42-nm-diameter filaments were organized in a uniform helical array (Fig. 1). Since PF filaments in intact cells are more compact (25 nm) than those in disrupted cells, the 42-nm-diameter fila-

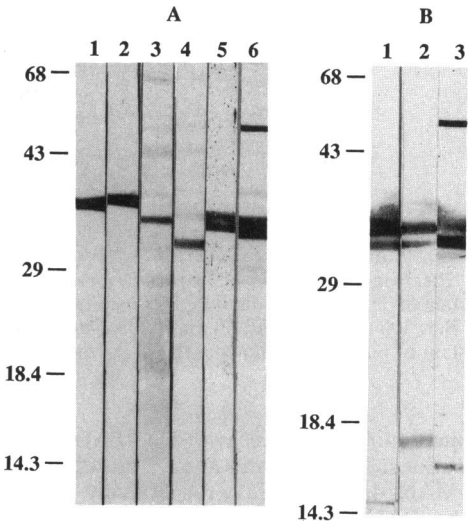


FIG. 3. Immunoblot analysis of whole-cell lysate of *L. interrogans* reacted with anti-PF antibodies. (A) Lanes 1 through 4, MAbs 1H8, 2A5, 3C12, and 4A9, respectively; lane 5, rabbit anti-*T. phagedenis* 33-kDa PF core protein; lane 6, rabbit anti-*T. pallidum* PF. (B) Lane 1, rabbit antibody to the 35.5- to 36-kDa *L. interrogans* PF protein band; lane 2, rabbit antibody to a mixture of the 34- and 34.5-kDa *L. interrogans* PF protein bands; lane 3, rabbit antibody to *T. pallidum* PF. Molecular masses (in kilodaltons) are shown on the left.

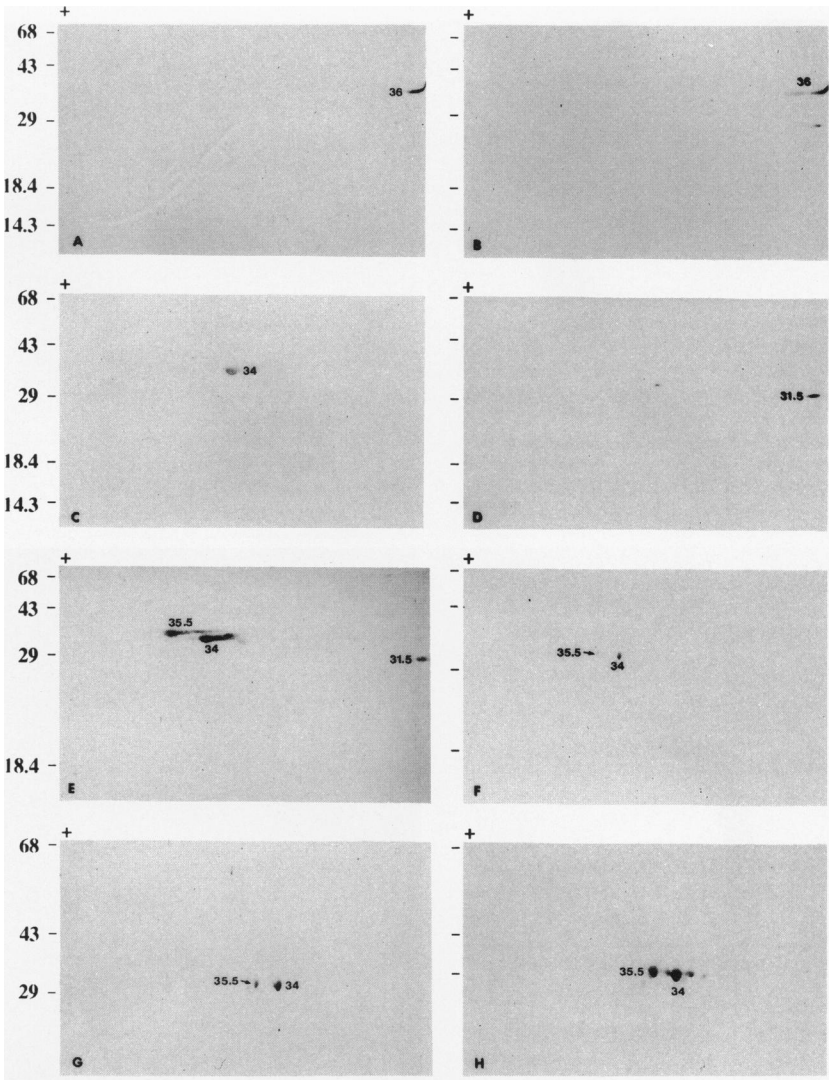


FIG. 4. Immunoblot of 2D PAGE of *L. interrogans* whole-cell lysate reacted with the following anti-PF antibodies: MAbs 1H8 (A), 2A5 (B), 3C12 (C), and 4A9 (D); rabbit antibodies to the 35.5- to 36-kDa leptospiral PF protein band (E) and to a mixture of the 34.5- and 34-kDa leptospiral PF protein bands (F); rabbit anti-*T. phagedenis* 33-kDa PF core protein (G); and rabbit anti-*T. pallidum* PF (H).

ments probably represent relaxed PF structures rather than PF in a truly native state. The results of this study confirm and expand on the findings of Nauman et al. (23).

The 11.3- and 21.5-nm-diameter PF filaments were isolated, and the protein composition of these filaments was determined. Attempts to isolate the 42-nm-diameter filaments and to separate the 11.3- from the 21.5-nm-diameter PF filaments were unsuccessful. Four PF protein bands (31.5, 34, 34.5, and 35.5 to 36 kDa) were detected by SDS-PAGE analysis of the subcellular fraction containing the 11.3- and the 21.5-nm-diameter PF filaments (Fig. 2A). Similar results were reported elsewhere (16). However, 2D PAGE separation of this fraction resolved seven proteins in the same molecular mass range (Fig. 2B).

At least two PF proteins (34 and 35.5 kDa) are thought to make up the 11.3-nm-diameter core for several reasons. First, both proteins were antigenically related to *Treponema* sp. PF core proteins (Fig. 3 and 4). Second, the 34-kDa protein was localized to the PF core by immunoelectron microscopy with a specific MAb (Fig. 5). Third, the amino termini of both the 34- and 35.5-kDa proteins were sequenced and found to be similar to class B proteins from other spirochetes (Fig. 6). Additional proteins may also be located in the PF core. Rabbit anti-*T. pallidum* PF serum reacted with several of the minor *L. interrogans* PF proteins (Fig. 4), indicating that these proteins are conserved. Although anti-treponemal-PF antibodies did not bind to PF structures, it is possible that their epitopes may have been masked. Conserved amino acid sequences often are located in the interiors of eubacterial flagellar filaments (22). Although a 31.5-kDa protein was associated with the PF, its location in the PF structure could not be determined by immunoelectron microscopic analysis. We believe that this 31.5-kDa protein may be related to a 31.5-kDa *L. borgpetersenii* class B protein and that MAbs against *L. interrogans* 31.5-kDa protein failed to bind to the PF because the reactive epitopes were buried in the core of the PF. Similar problems were encountered during the analysis of the 31.5-kDa PF core protein of *Spirochaeta aurantia* (5). The results of this study show that *L. interrogans* contains more than

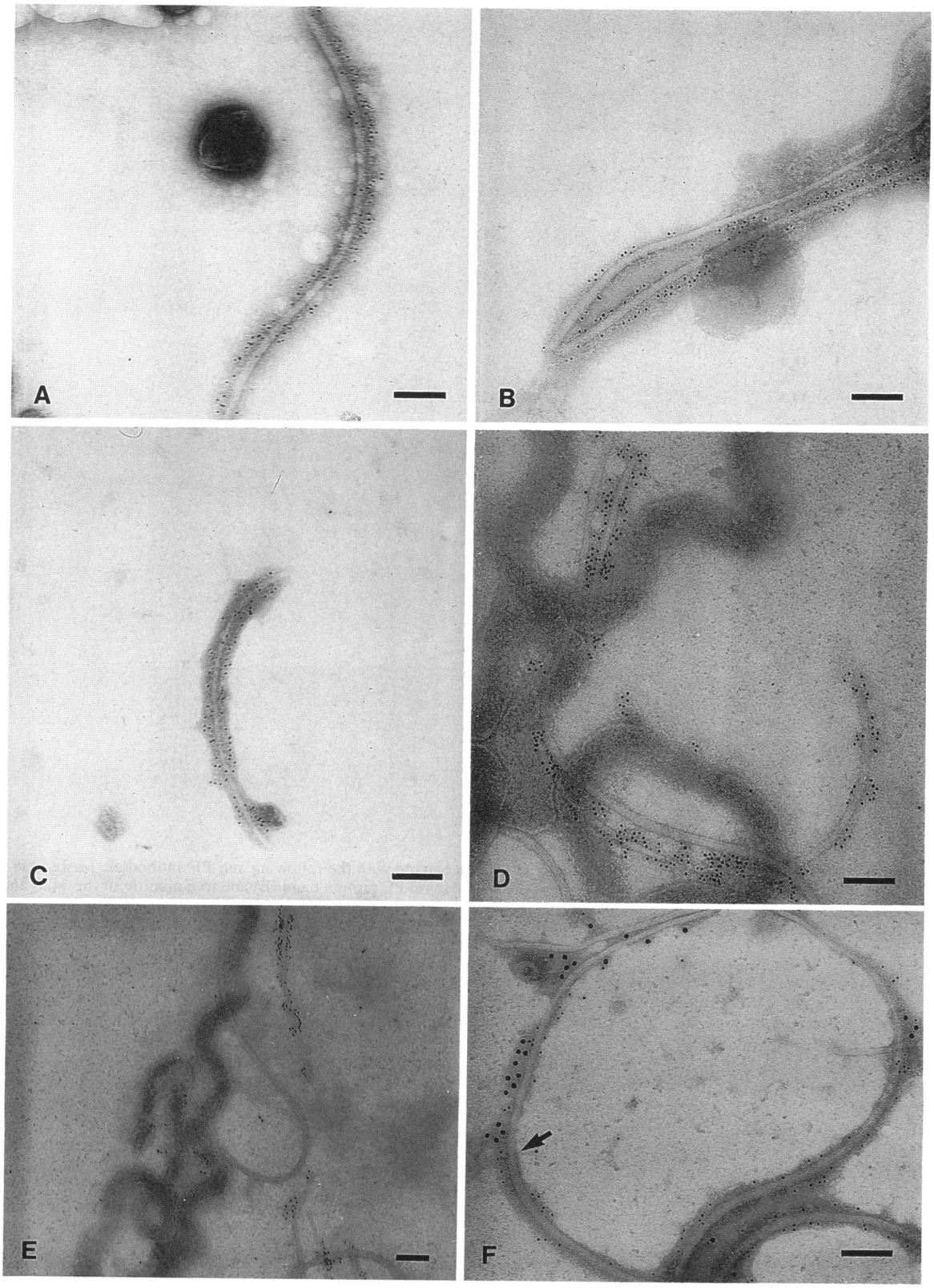


FIG. 5. Immunoelectron microscopy of Triton X-114-disrupted *L. interrogans* cells reacted with MAbs 1H8 (A), 2A5 (B), and 3C12 (C) and polyclonal antisera against *L. interrogans* 35.5- to 36-kDa PF protein band (D) and against a mixture of *L. interrogans* 34- and 34.5-kDa PF protein bands (E). (F) Double labeling of PF by polyclonal antibodies against the leptospiral 34- and 34.5-kDa PF protein bands, labeling the 11.3-nm-diameter filament (10-nm-diameter gold particles), and by MAb 1H8 against the 21.5-nm-diameter sheath (5-nm-diameter gold particles). Arrow, place where the 21.5-nm-diameter sheath is disrupted. Bar, 100 nm.

TABLE 1. Results of immunoblot and immunoelectron microscopic analyses

| Antibody | Reactivity | |
|---|---|---|
| | Protein size(s) (kDa)[a] | PF filament structure (diam [nm]) |
| Monoclonal (isotype) | | |
| 1H8 (IgG2b) | 36 (e) | Sheath (21.5) |
| 2A5 (IgG1) | 36 (e) | Sheath (21.5) |
| 3C12 (IgG) | 34 (g) | Core (11.3) |
| 4A9 (IgM) | 31.5 (h) | —[b] |
| Polyclonal, against PF from: | | |
| *T. phagedenis* (core protein) | 34 (g) and 35.5 (b) | — |
| *T. pallidum* | 34 (g) and 35.5 (b) | — |
| *L. interrogans* (35.5- to 36-kDa band) | 34 (g) and 35.5 (b) | Core (11.3) |
| *L. interrogans* (34- and 34.5-kDa bands) | 34 (g) and 35.5 (b) | Core (11.3) |

[a] Separated by 2D PAGE. Letters in parentheses correspond to those in Fig. 2B.
[b] —, No reaction.

one protein in the PF core. In this respect the PF of *L. interrogans* are similar to those of *T. phagedenis* (24), *T. pallidum* (24), and *S. aurantia* (27).

The *L. interrogans* PF sheath forming the 21.5-nm-diameter filament contains at least one protein with a molecular mass of 36 kDa. The size of this protein is similar to those of sheath proteins from *S. aurantia* (5, 27), *T. pallidum* (9, 14, 24), and *T. phagedenis* (24). Since the sheath layer forming the 21.5-nm-diameter filament remained with the PF core during purification, proteins making up this sheath layer are probably tightly associated with the core. In contrast, the material surrounding the 21.5-nm-diameter filament leading to the formation of the 42-nm-diameter filament readily disassociated from the 21.5-nm-diameter filament. The functions of both sheath layers are unknown, although the

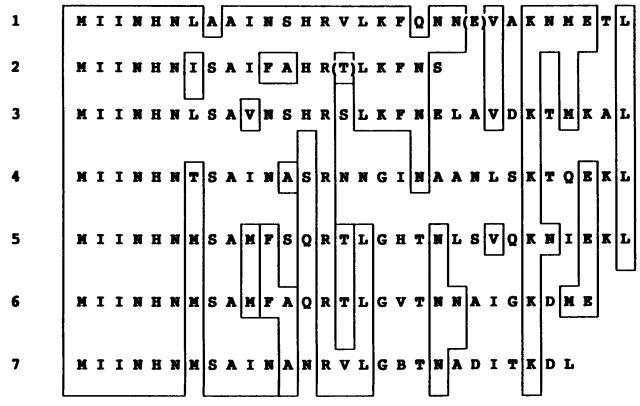


FIG. 6. Amino-terminal amino acid sequence of spirochetal PF core proteins. Line 1, *L. interrogans* 34-kDa protein (this study); line 2, *L. interrogans* 35.5-kDa protein (this study); line 3, *L. borgpetersenii* 31.5-kDa protein (21); line 4, *B. burgdorferi* 41-kDa protein (12); line 5, *T. pallidum* 31.3-kDa protein (26); line 6, *T. phagedenis* 34-kDa protein (24); line 7, *S. aurantia* 31.5-kDa protein (27). Regions in which the 34- and 35.5-kDa proteins show homology with other spirochetal PF class B proteins are boxed. Most likely amino acids are in parentheses.

presence of complex flagella in some bacteria is associated with the ability to swim in highly viscous environments (18, 28, 33). Of particular interest is the second sheath layer, as it appears to be a structure unique to *Leptospira* spp. (23; also this study). Further studies aimed at determining the composition of the second sheath layer and the nature of interactions between the different PF layers may provide insight into the mechanisms involved in *Leptospira* motility.

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