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Antigenic Relatedness and N-Terminal Sequence Homology Define Two Classes of Periplasmic Flagellar Proteins of *Treponema pallidum* subsp. *pallidum* and *Treponema phagedenis*

STEVEN J. NORRIS,[1]* NYLES W. CHARON,[2] RICHARD G. COOK,[3] MARIE D. FUENTES,[1] AND RONALD J. LIMBERGER[2]†

Department of Pathology and Laboratory Medicine, Medical School, University of Texas Health Science Center at Houston, Houston, Texas 77225[1]; Department of Microbiology and Immunology, Health Science North, West Virginia University, Morgantown, West Virginia 26506[2]; and Howard Hughes Medical Institute and Department of Microbiology and Immunology, Baylor College of Medicine, Houston, Texas 77030[3]

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The periplasmic flagella of many spirochetes contain multiple proteins. In this study, two-dimensional electrophoresis, Western blotting (immunoblotting), immunoperoxidase staining, and N-terminal amino acid sequence analysis were used to characterize the individual periplasmic flagellar proteins of *Treponema pallidum* subsp. *pallidum* (Nichols strain) and *T. phagedenis* Kazan 5. Purified *T. pallidum* periplasmic flagella contained six proteins (M_rs = 37,000, 34,500, 33,000, 30,000, 29,000, and 27,000), whereas *T. phagedenis* periplasmic flagella contained a major 39,000-M_r protein and a group of two major and two minor 33,000- to 34,000-M_r polypeptide species; 37,000- and 30,000-M_r proteins were also present in some *T. phagedenis* preparations. Immunoblotting with monospecific antisera and monoclonal antibodies and N-terminal sequence analysis indicated that the major periplasmic flagellar proteins were divided into two distinct classes, designated class A and class B. Class A proteins consisted of the 37-kilodalton (kDa) protein of *T. pallidum* and the 39-kDa polypeptide of *T. phagedenis*; class B included the *T. pallidum* 34.5-, 33-, and 30-kDa proteins and the four 33- and 34-kDa polypeptide species of *T. phagedenis*. The proteins within each class were immunologically cross-reactive and possessed similar N-terminal sequences (67 to 95% homology); no cross-reactivity or sequence homology was evident between the two classes. Anti-class A or anti-class B antibodies did not react with the 29- or 27-kDa polypeptides of *T. pallidum* or the 37- and 30-kDa *T. phagedenis* proteins, indicating that these proteins are antigenically unrelated to the class A and class B proteins. The lack of complete N-terminal sequence homology among the major periplasmic flagellar proteins of each organism indicates that they are most likely encoded by separate structural genes. Furthermore, the N-terminal sequences of *T. phagedenis* and *T. pallidum* periplasmic flagellar proteins are highly conserved, despite the genetic dissimilarity of these two species.

Bacteria of the order *Spirochaetales* possess periplasmic flagella (also termed axial filaments, periplasmic filaments, endoflagella, or flagella [39]) which originate at each end of the helical cell cylinder and extend toward the center of the cell (7, 19). These periplasmic flagella are situated between the outer membrane sheath and the cell cylinder (7, 19) and are involved in spirochete locomotion (6, 12, 27, 34). A distinctive feature of *Leptospira* (30; N. Charon and C. Maloney, unpublished data), *Treponema* (1–4, 8, 16, 27, 28, 31, 35, 37, 38), and *Spirochaeta* (13, 24) periplasmic flagellar filaments is that they are composed of between two and six major species of polypeptides, whereas most bacterial flagella have only one major filament protein (22, 41). In *Treponema phagedenis* (27) and *Treponema pallidum* (8), multiple protein species have been shown to reside together on the same flagellar filament, and in this respect these treponemal periplasmic flagella are similar to the flagella of *Caulobacter crescentus* (11, 26, 44).

Previous studies utilizing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (immunoblotting) techniques indicate that the periplasmic flagellar proteins of *T. phagedenis* and *T. pallidum* have structural and antigenic similarities (1–4, 8, 16, 27, 28, 35, 37, 38). Both species have a high-M_r periplasmic flagellar protein (35,000 to 39,800), a cluster of proteins at a lower M_r (30,000 to 35,300), and additional minor polypeptides (27,000 to 30,000 M_r). Within each species, the high- and low-M_r types of major periplasmic flagellar proteins appear to have different epitopes (1, 3, 4, 8, 27, 28, 35, 37, 38), structural locations (3, 4, 8, 35, 37), and N-terminal amino acid sequences (3). Antisera against the periplasmic flagellar proteins of one species react with those of the other, indicating antigenic similarities between the two organisms.

The aim of the present study was to characterize and compare the periplasmic flagellar proteins of *T. phagedenis* Kazan 5 and *T. pallidum* Nichols in detail by two-dimensional gel electrophoresis, Western blotting, and N-terminal amino acid sequencing. A major limitation of the previous studies was that SDS-PAGE failed to clearly resolve the multiple periplasmic flagellar protein species (especially the 33- to 35-kilodalton [kDa] proteins), and two-dimensional electrophoresis has been found to be useful in resolving these polypeptides (31). We report here that two-dimensional electrophoresis permitted the identification of six periplasmic flagellum-associated proteins in *T. pallidum* and at least five polypeptide moieties associated with the periplasmic flagella of *T. phagedenis*. Moreover, two classes of periplasmic flagellar proteins were identified based on immu-

* Corresponding author.
† Present address: Molecular Parasitology Group, New England BioLabs, Inc., Beverley, MA 01915.

nologic cross-reactivity and N-terminal amino acid sequence homology.

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MATERIALS AND METHODS

Bacteria. The Nichols strain of virulent *T. pallidum* subsp. *pallidum* was originally obtained from James N. Miller. *T. pallidum* was propagated by intratesticular infection of rabbits and was purified by Percoll density centrifugation as previously described (15). The Kazan 5 strain of *T. phagedenis* was propagated by in vitro culture (27) and washed three times by centrifugation and suspended in phosphate-buffered saline. Bacterial preparations were stored at −70°C prior to use in electrophoretic analysis.

Purification of periplasmic flagella. The periplasmic flagella of *T. phagedenis* were purified as described previously (27). *T. pallidum* periplasmic flagella were purified by a sucrose density gradient sedimentation technique based on the membrane purification procedure of Osborn et al. (33; S. J. Norris, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, J29, p. 153; S. J. Norris, manuscript in preparation). Roughly 10^{10} freshly prepared Percoll-purified *T. pallidum* organisms were centrifuged at 13,000 × *g* for 10 min at 4°C and suspended in 3.3 mM Tris–1.0 mM (EDTA)–0.2 mM dithiothreitol–0.25 M sucrose (pH 7.5). After recentrifugation, the organisms were suspended in 350 μl of 25% sucrose (wt/vol) with 5 mM EDTA and 0.2 mM dithiothreitol, sonically disrupted (three 10-s pulses at full power with a Micro-Ultrasonic Disrupter [Kontes, Vineland, N.J.]), and overlaid onto a 5-ml 30 to 55% (wt/vol) discontinuous sucrose gradient containing the same concentrations of EDTA and dithiothreitol. The gradient was centrifuged at 180,000 × *g* at 4°C for 14 to 18 h, and 0.25-ml fractions were collected. The periplasmic flagella sedimented at a density of approximately 1.24 g/ml, as determined by SDS-PAGE and electron microscopy of the fractions. Fractions containing the periplasmic flagella were diluted to 25% sucrose and resedimented over sucrose gradients to further ensure their purity. Electron microscopy and SDS-PAGE and silver staining (14) were used to monitor the purity of the periplasmic flagellar preparations.

Two-dimensional electrophoresis. Bacteria (3×10^8 to 5×10^8) equivalent to approximately 60 μg of protein as determined by the Bradford protein assay (5) were subjected to isoelectric focusing (IEF) followed by SDS-PAGE as described by O'Farrell (32). The bacteria were sonically disrupted and solubilized in 30 to 40 μl of lysis buffer consisting of 8 M urea, 2% Nonidet P-40, 5% 2-mercaptoethanol, 1.6% pH 5 to 7 Ampholines, and 0.4% pH 3.5 to 10 Ampholines (LKB, Uppsala, Sweden); RNase and DNase as used in the original procedure (32) were omitted. Samples were applied to tube gels (11.5 by 0.25 cm) containing the same Ampholine mixture and focused for 16 h at 400 V and for 1 h at 800 V. The second dimension consisted of SDS-PAGE utilizing 8 to 20% polyacrylamide linear gradient gels (31). Single-dimension lanes containing solubilized *T. pallidum* or *T. phagedenis* (30 μg of protein) and molecular weight standards (Bio-Rad Laboratories, Richmond, Calif.) were run in wells at either end of the IEF gel. The distribution of proteins was visualized by silver staining (14) or by staining with Coomassie brilliant blue R-250.

Consensus M_r values as reported by Norris et al. (31) were used to identify the *T. pallidum* proteins in this study. For comparison, M_r values were determined in our laboratory by the method of Weber and Osborn (42), using interpolation between neighboring molecular weight standards. M_rs obtained in this study corresponding to the consensus M_rs (in parentheses) were as follows: 80,000 (for consensus M_r = 83,000), 60,000 (61,000), 47,000 (47,000), 41,000 (41,000), 37,800 (37,000), 35,000 (34,500), 34,500 (33,000), 32,000 (30,000), 30,500 (29,000), 29,700 (27,000), and 30,000 to 38,000 (29,000 to 35,000, TpD [31]).

Antisera and monoclonal antibodies. Pooled anti-*T. pallidum* rabbit serum was obtained from rabbits at least 3 months after intratesticular infection with 2×10^7 to 5×10^7 *T. pallidum* Nichols. Monospecific rabbit antisera and monoclonal antibodies against *T. phagedenis* Kazan 5 periplasmic flagellar proteins were prepared as described by Limberger and Charon (27). These antibodies consisted of monoclonal antibody 1C6-E11 directed to the 39.8-kDa periplasmic flagellar protein (referred to as 39 kDa in this study) of *T. phagedenis* and polyclonal antisera directed to the 33- to 34-kDa periplasmic flagellar protein doublet, also of *T. phagedenis*. Rabbit antisera directed against the 37-, 34.5-, 33-, and 30-kDa polypeptides of *T. pallidum* Nichols were elicited with proteins purified by two-dimensional electrophoresis. *T. pallidum* proteins from 10 to 24 IEF gels (prepared as described above) were detected by staining with Coomassie blue G (23). The bands corresponding to the proteins of interest were identified by comparison of the IEF pattern with the corresponding two-dimensional gel pattern and were carefully cut out of the gel. To further purify the proteins, the slices corresponding to a given protein were incubated for 5 to 10 min with equilibration buffer (32) and then sealed in an agarose overlay and subjected to SDS-PAGE, as described for standard two-dimensional electrophoresis (31). The gels were stained with Coomassie brilliant blue R-250, and the purified protein was excised from the gel. The protein was electroeluted from the gel pieces in Tris-glycine buffer (0.125 M Tris base, 0.2 M glycine, 0.1% SDS, pH 8.5) with a model 1750 electroelution unit (Isco, Lincoln, Nebr.) at 3 W for 3 h, cooled on ice. Each preparation was examined for purity by SDS-PAGE and silver staining. Rabbits were immunized intradermally and intramuscularly with the purified proteins (equivalent to approximately 5×10^9 organisms) emulsified in complete Freund adjuvant (Difco Laboratories, Detroit, Mich.). The animals were boosted at 1-month intervals with an equivalent quantity of protein by intravenous injection or by intradermal and intramuscular administration of antigen emulsified in incomplete Freund adjuvant. Sera were obtained 7 days after immunization and tested for reactivity by immunoperoxidase staining of *T. pallidum* electroblots.

Immunoperoxidase staining. Proteins were transferred to nitrocellulose sheets (0.45-μm exclusion limit; Millipore Corp., Bedford, Mass.) by the method of Towbin et al. (40). Immunoperoxidase staining was performed as previously described (31), utilizing a 1:200 dilution (0.25 ml in 50 ml) of rabbit antisera or ascites fluid containing monoclonal antibodies. A 1:1,000 dilution of a horseradish peroxidase conjugate of either goat anti-rabbit immunoglobulin G (heavy and light chain specific; Boehringer Mannheim Biochemicals, Indianapolis, Ind.) or goat anti-mouse immunoglobulin G (heavy and light chain specific; Bio-Rad) was used as the second antibody, and 4-chloronaphthol was used as the color substrate (31).

Protein sequencing. For N-terminal sequence determinations, periplasmic flagellar proteins were purified by large-scale two-dimensional electrophoresis as described above,

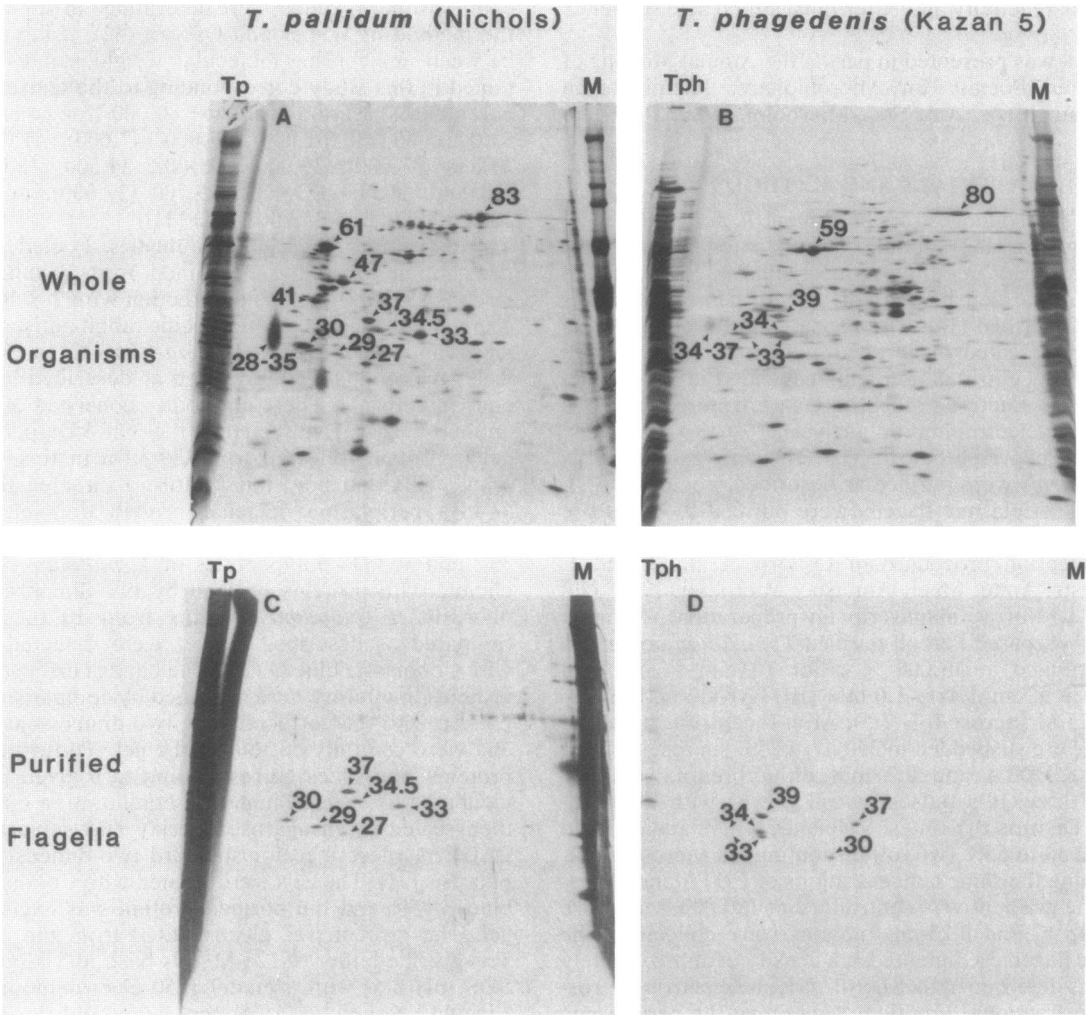


FIG. 1. Distribution of proteins after two-dimensional electrophoresis of whole *T. pallidum* Nichols (A), whole *T. phagedenis* Kazan 5 (B), purified periplasmic flagella from *T. pallidum* (C), and purified *T. phagedenis* periplasmic flagella (D) as visualized by silver staining (14). Samples were subjected to isoelectric focusing (pH 5 to 7 gradient) followed by SDS-PAGE (8 to 20% linear gradient gels) by the method of O'Farrell (32). The acid ends of the IEF gel are placed to the left. Single-dimension SDS-PAGE standard lanes on either side of the gels consist of whole *T. pallidum* (Tp), whole *T. phagedenis* (Tph), or Bio-Rad molecular weight standards (M). M_r values (10^3) (as described in the text) are indicated for the periplasmic flagellar proteins and other major polypeptides.

with some modifications as described by Hunkapiller et al. (21). *T. pallidum* proteins were purified from 5×10^9 to 1×10^{10} whole Percoll-purified organisms, whereas the *T. phagedenis* proteins were prepared from approximately 600 μg of purified periplasmic flagella. High-purity SDS (prepared as described previously [21] or purchased from Polysciences, Warrington, Pa.) was utilized at all stages, and 0.1 mM sodium thioglycolate was added to the electrode buffer during the SDS-PAGE step to reduce amino acid hydrolysis. Electroelution was done as described previously (21), using an Isco electroelution unit modified to allow continuous buffer replacement at a rate of 100 to 150 ml per h. Following electroelution, the protein samples were dried on a Speedvac rotary evaporator (Savant Instruments, Inc., Hicksville, N.Y.), dissolved in 100 μl of distilled water, and precipitated overnight at −20°C by the addition of 1 ml of ethanol. The proteins were pelleted by centrifugation at 12,000 × *g* in a microcentrifuge for 20 min, washed once in 80% ethanol, and dissolved in 100 μl of 0.05 M $NaHCO_3$ with 0.1% (wt/vol) SDS. A 5-μl sample of each protein preparation was analyzed for purity by SDS-PAGE and silver staining. The remainder was applied to a Polybrene-treated glass fiber filter, air dried, and subjected to Edman degradation analysis on a model 470A gas-phase sequenator with an inline model 120A phenylthiohydantoin (PTH) derivative analyzer (Applied Biosystems, Foster City, Calif.). Sequence analysis of separate preparations of the same proteins yielded identical results.

RESULTS

Two-dimensional electrophoretic analysis of periplasmic flagellar proteins. The two-dimensional electrophoretic profiles of whole *T. pallidum* Nichols and *T. phagedenis* Kazan 5 are shown in Fig. 1A and B. For the *T. pallidum* polypeptides, the consensus M_r values as given in reference 31 are used throughout this report. There were some similarities in the two-dimensional electrophoretic patterns of these two organisms, most notably the presence of apparently related proteins with M_rs of 80,000 to 83,000 and 59,000 to 61,000

and a diffusely distributed spot at M_r 29,000 to 35,000. However, the overall patterns were quite different, reflecting significant genetic divergence in terms of the relative molecular weights and isoelectric points of many of the proteins. For example, *T. pallidum* possesses a major 47-kDa protein which is highly antigenic (see Fig. 2A), whereas *T. phagedenis* appears to lack a protein with migration characteristics similar to the 47-kDa *T. pallidum* polypeptide. The two-dimensional electrophoresis profile of the Reiter strain of *T. phagedenis* closely resembles that of the Kazan 5 strain (data not shown).

Periplasmic flagella were purified by sucrose density gradient sedimentation for *T. pallidum* or by a procedure involving differential solubilization and sedimentation for *T. phagedenis*. In each case, purity was verified by electron microscopy and by monitoring by SDS-PAGE the removal of extraneous proteins. The polypeptides present in the periplasmic flagellar preparations were examined by two-dimensional electrophoresis (Fig. 1C and D). The periplasmic flagellum-associated proteins of *T. pallidum* consisted of a major 37-kDa protein, three moderately abundant proteins with sizes of 34.5, 33, and 30 kDa, and two relatively minor polypeptides at 29 and 27 kDa (Fig. 1C). The *T. phagedenis* periplasmic flagellar preparations consistently contained a 39-kDa protein and a cluster of four 33- to 34-kDa proteins (Fig. 1D); the minor 33- and 34-kDa polypeptide species stained faintly and are more readily visible in Fig. 1B (see also Fig. 2H and J). In some preparations, two additional proteins at 37 and 30 kDa were also present (Fig. 1D); it is unclear at this time whether these proteins represent contaminants or true periplasmic flagellum-associated proteins. The cluster of four 33- to 34-kDa proteins was distinctive because it consisted of two doublets, a major one with an isoelectric focusing point (pIEF) similar to that of the 39-kDa protein and a relatively less abundant doublet with the same M_rs but a more acidic pIEF. Determination of pIEFs for these proteins was attempted, but the inconsistency of pH measurements obtained with different IEF gels precluded the assignment of accurate pIEF values. In general, the periplasmic flagellar proteins of both organisms had pIEF values in the range of 5.6 to 6.3, and the pIEFs of the *T. phagedenis* proteins appeared to differ somewhat from those of the *T. pallidum* proteins. The positions of the periplasmic flagellum-associated proteins in the two-dimensional patterns of whole organisms are indicated in Fig. 1A and B.

Antigenic reactivity of periplasmic flagellar polypeptides. The antigenic relatedness of the periplasmic flagellar proteins was examined by exposing electroblots of two-dimensional electrophoresis patterns of the whole organisms to antisera and monoclonal antibodies and visualizing the binding of antibodies by immunoperoxidase staining. Pooled anti-*T. pallidum* antisera obtained from *T. pallidum*-infected rabbits reacted with nearly all the proteins of *T. pallidum* detectable by silver staining, including prominent reactions with the periplasmic flagellar proteins (Fig. 2A); similar results have been obtained with sera from syphilis patients (30a). The anti-*T. pallidum* antisera also reacted with a relatively small number of *T. phagedenis* proteins, most notably the 39- and 33- to 34-kDa periplasmic flagellar proteins and also 59- and 80-kDa proteins (Fig. 2B). Thus, as previously noted (1, 4, 16, 30a, 35, 37, 38), *T. pallidum* infection induces the expression of anti-periplasmic flagellum antibodies, including those which react strongly with *T. phagedenis* periplasmic flagellar protein epitopes.

The antigenic relationship of the periplasmic flagellar proteins was assessed in greater detail with monoclonal and monospecific antibodies. The monoclonal antibody 1C6-E11 was originally induced by immunization of mice with *T. phagedenis* Kazan 5 periplasmic flagella (27). It reacted specifically with the 39-kDa protein of the Kazan strain (Fig. 2D); no reaction with the lower-molecular-weight periplasmic flagellar proteins was noted. When reacted with two-dimensional gels of *T. pallidum*, this monoclonal antiserum also reacted with the 37-kDa periplasmic flagellar protein and an additional 41-kDa protein (Fig. 2C). The reaction against the 37-kDa periplasmic flagellar protein of *T. pallidum* was weaker than with *T. phagedenis*, requiring prolonged color reagent development for visualization. We also tested a monospecific antiserum prepared against the 37-kDa periplasmic flagellar protein of *T. pallidum*. This antiserum reacted with the 37-kDa protein of *T. pallidum*, and it also reacted with the same *T. pallidum* 41-kDa protein detected with monoclonal antibody 1C6-E11 (Fig. 2E). When tested with two-dimensional gels of *T. phagedenis*, this monospecific antiserum reacted significantly with the 39-kDa periplasmic flagellar protein (Fig. 2F). As with the monoclonal antibody 1C6-E11, no reactivity was noted with the lower-molecular-weight periplasmic flagellar proteins.

An interesting antigenic relationship among a group of lower-molecular-weight periplasmic flagellar proteins of *T. pallidum* was also revealed by the reactivities of polyclonal monospecific antisera. Rabbit antisera were prepared against each of the 34.5-, 33-, and 30-kDa polypeptides of *T. pallidum* purified by large-scale two-dimensional electrophoresis. Each of these monospecific antisera reacted with all three of these periplasmic flagellar proteins rather than just the one used to elicit the antiserum, as exemplified by the anti-34.5-kDa protein antiserum (Fig. 2G). No reactivity was noted with the 37-kDa *T. pallidum* periplasmic flagellar protein. Thus, the 34.5-, 33-, and 30-kDa proteins of *T. pallidum* appear to have shared epitopes, but lack antigenic cross-reactivity with the 37-kDa protein.

The lower-M_r periplasmic flagellar proteins of *T. pallidum* had their analogous antigenic counterparts in *T. phagedenis* periplasmic flagella. The monospecific antisera against the 34.5-, 33-, and 30-kDa periplasmic flagellar proteins of *T. pallidum* reacted with each of the *T. phagedenis* periplasmic flagellar proteins in the 33- to 34-kDa cluster (Fig. 2H). Similarly, rabbit antisera against the 33- to 34-kDa *T. phagedenis* periplasmic flagellar proteins (Fig. 2J) also cross-reacted with each of the three antigenically related *T. pallidum* periplasmic flagellar proteins (Fig. 2I). Therefore, these groups of antigenically related proteins not only share epitopes within each species, but also cross-react with the polypeptides of the corresponding cluster in the other species. None of the antisera induced by these lower-M_r proteins reacted with the 39-kDa protein of *T. phagedenis* or the 37-kDa protein of *T. pallidum*.

It could be argued that the *T. pallidum* protein preparations used to induce the monospecific antisera were contaminated with the other class B periplasmic flagellar proteins, resulting in the presence of antibodies against the other proteins. This explanation is unlikely, since SDS-PAGE and silver staining (Fig. 3), sequence analysis (Fig. 4), and the lack of antiserum reactivity with other potential contaminants with similar M_rs and IEF mobilities (Fig. 2E) all indicate that the proteins were apparently free of significant contaminants.

On the basis of the observed pattern of immunologic cross-reactivity, we designated the 37-kDa protein of *T. pallidum* and the 39-kDa protein of *T. phagedenis* as class A periplasmic flagellar proteins, whereas the 34.5-, 33-, and

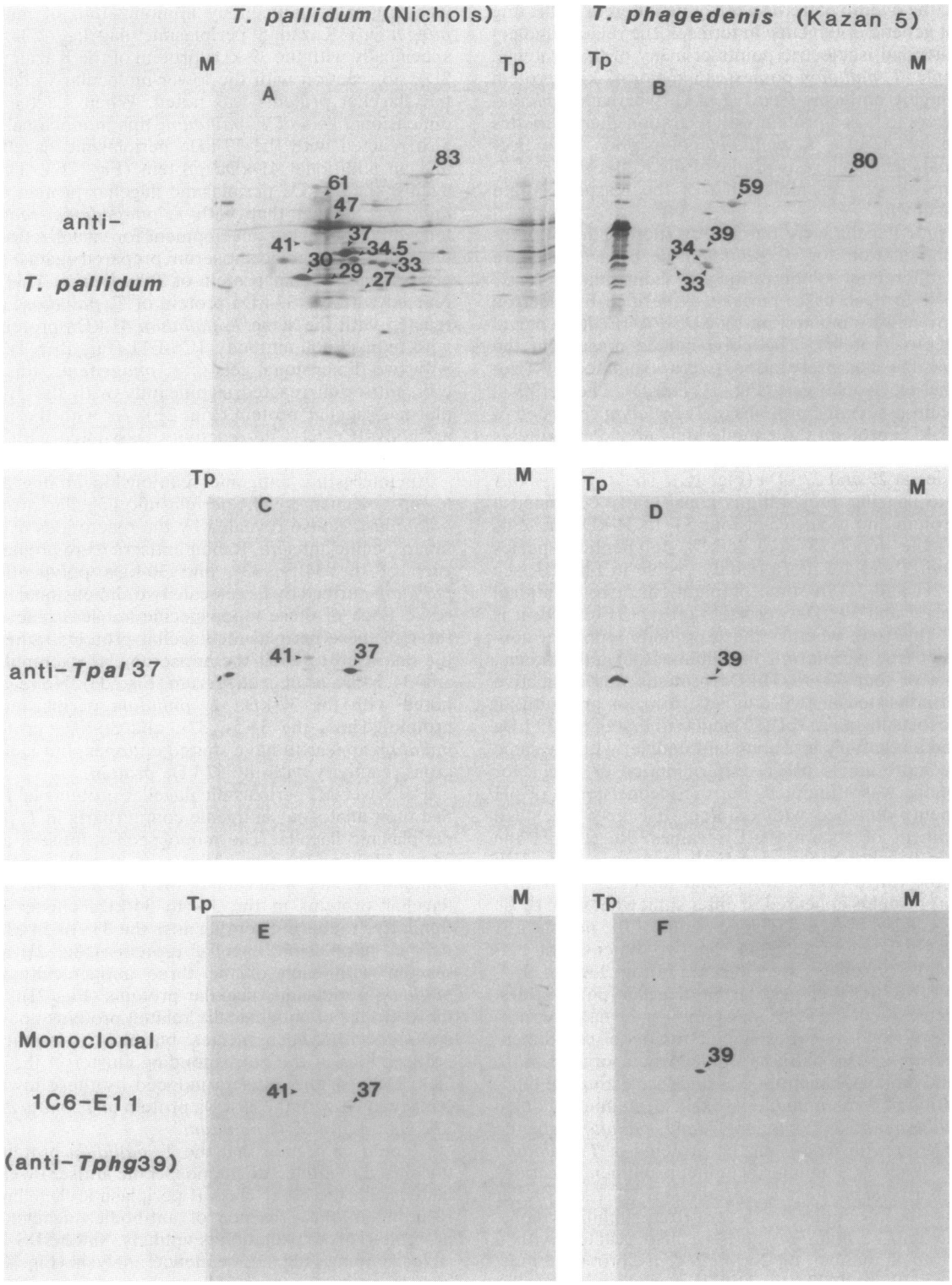
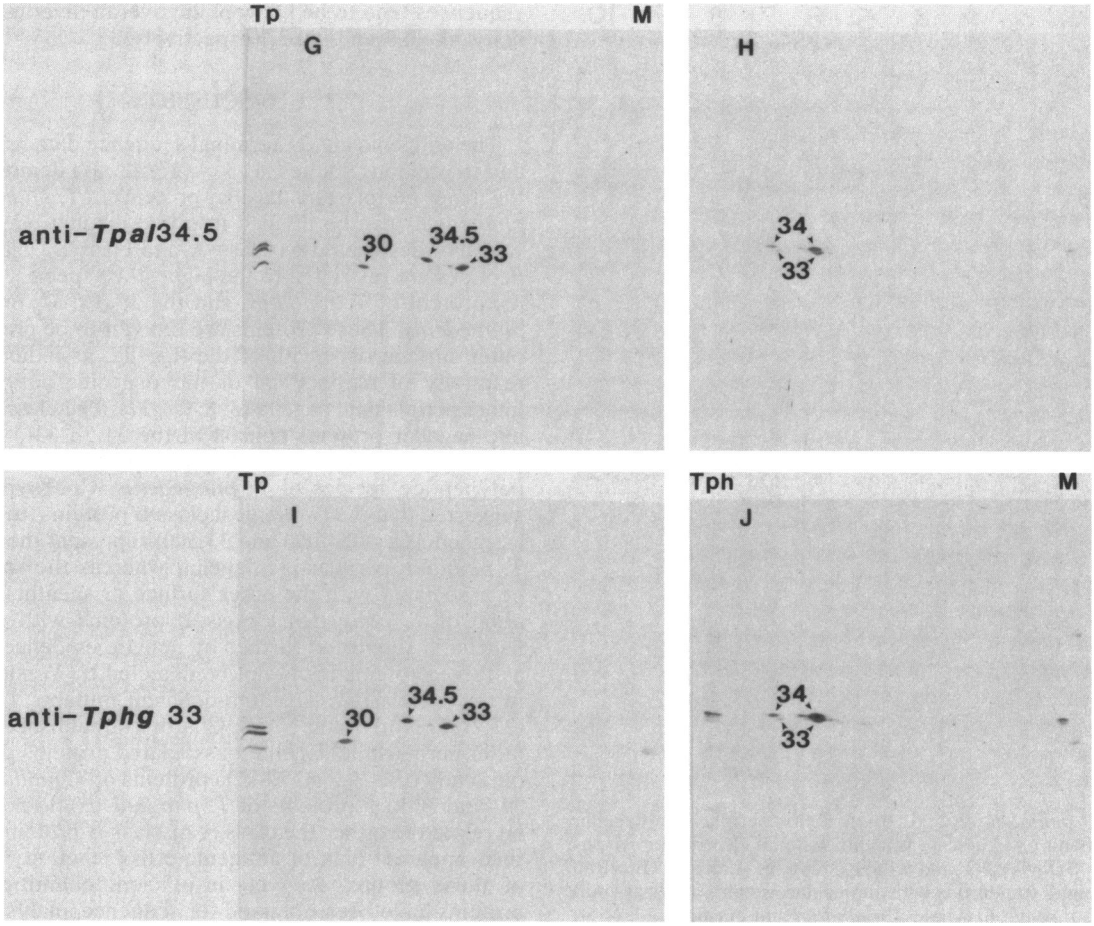


FIG. 2. Reactivity of antisera and a monoclonal antibody with the two-dimensional electrophoresis patterns of whole *T. pallidum* Nichols (A, C, E, G, and I) and whole *T. phagedenis* Kazan 5 (B, D, F, H, and J), as revealed by immunoperoxidase staining of electroblots. Reactions resulting from incubation with the following antibodies are shown: rabbit anti-*T. pallidum* antiserum (A, B); monoclonal antibody 1C6-E11 (E, F); and monospecific rabbit antisera against *T. pallidum* (Tpal) 37-kDa protein (C, D) and 34.5-kDa protein (G, H) and *T. phagedenis* (Tphg) 33- to 34-kDa periplasmic flagella proteins (I, J). Single-dimension SDS-PAGE lanes and M_r values correspond with those shown in Fig. 1.



30-kDa polypeptides of *T. pallidum* and the cluster of 33- and 34-kDa proteins of *T. phagedenis* were called class B periplasmic flagellar proteins. The *T. pallidum* 29- and 27-kDa periplasmic flagellar proteins and the 37- and 30-kDa proteins of *T. phagedenis* did not react with antibodies against the class A and class B proteins and therefore apparently do not share epitopes with either of these antigenic groups.

N-terminal sequence homology. To define more clearly the relationships among the periplasmic flagellar proteins, we analyzed the N-terminal sequences of the 37-, 34.5-, 33-, and 30-kDa periplasmic flagellar proteins of *T. pallidum* Nichols and the 39-kDa and the major 34- and 33-kDa periplasmic flagellar proteins of *T. phagedenis*. The proteins were purified by large-scale two-dimensional electrophoresis, and the resulting preparations were free of significant contamination with other proteins as determined by SDS-PAGE and silver staining (Fig. 3). The estimated quantity of sequencable material ranged from 60 to 300 pmol (2 to 10 μg) per protein. The *T. phagedenis* 34- and 33-kDa proteins comigrated in the IEF dimension and migrated at very similar R_fs in the SDS-PAGE dimension. For these proteins, it was necessary to first purify the 34- to 33-kDa doublet by two-dimensional electrophoresis, electroelute the two proteins together, and apply the mixture over the entire length of two combless SDS-PAGE gels. By spreading out the protein over two gels, it was possible to decrease the band width so that the two protein bands could be resolved.

The results of the N-terminal sequence analyses are given in Fig. 4 and Table 1. A high degree of homology was found to exist between the N-terminal amino acid sequences of the class B *T. pallidum* and *T. phagedenis* periplasmic flagellar proteins. The *T. pallidum* 34.5-, 33-, and 30-kDa sequences were from 71 to 95% homologous to one another (Table 1). The *T. phagedenis* 34- and 33-kDa sequences shared 21 of 27 amino acids, a 78% homology. In addition, the N-terminal sequences of the *T. pallidum* and *T. phagedenis* class B proteins were also highly homologous between the two species; the interspecies homology ranged from 67 to 95%, and the first 11 amino acids were identical within this group.

The class A periplasmic flagellar protein N-terminal sequences were also highly homologous to one another and were distinct from those of the class B periplasmic flagellar proteins (Fig. 4). The *T. pallidum* 37-kDa and the *T. phagedenis* 39-kDa proteins shared 16 of 25 amino acids at their N-terminal amino acid sequences, indicating a 64% homology in this region (Table 1). The *T. pallidum* 37-kDa and the *T. phagedenis* 39-kDa N-terminal sequences lacked homology (0 to 7% identity) with any region of the class B N-terminal sequences (Fig. 4; Table 1). The N termini of the class A proteins are apparently cleaved, as indicated by the absence of a terminal methionine residue.

Computer-assisted comparison of the treponemal protein sequences with a data bank of 5,415 proteins (Molecular Biology Information Resource, Baylor College of Medicine, Houston, Tex.) did not identify any proteins with extensive homology with the treponemal periplasmic flagellar se-

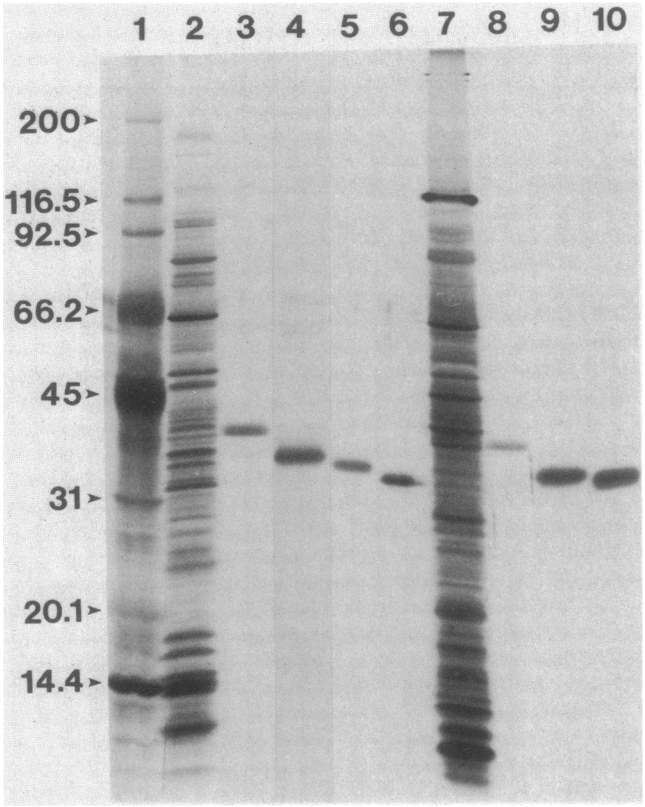


FIG. 3. *T. pallidum* and *T. phagedenis* periplasmic flagellar proteins purified by large-scale two-dimensional electrophoresis, as analyzed by SDS-PAGE and silver staining. Lane 1, Bio-Rad molecular weight standards, with molecular weights (10^3) as indicated; lanes 2 through 6, whole *T. pallidum* and purified 37-, 34.5-, 33-, and 30-kDa periplasmic flagellar proteins, respectively; lanes 7 through 10, whole *T. phagedenis* and purified 39-, 34-, and 33-kDa periplasmic flagellar proteins, respectively.

quences. However, the N-terminal sequence of the *Bacillus subtilis* flagellin protein (9) shared 6 of the first 10 residues with the class B periplasmic flagellar polypeptides (Fig. 4c); two of the four amino acid differences in this region were conservative (residues 7 and 10). The overall identity over the 30 N-terminal amino acids of the 30-kDa protein of *T. pallidum* and the same region of the *B. subtilis* flagellin molecule was 43% (Fig. 4c). The treponemal N-terminal sequences lacked significant homology with the protein sequences (inferred from DNA) of the *Escherichia coli* K-12 flagellin (25), the *Salmonella typhimurium* H-1^a flagellin (43), or the 28.5-kDa protein of *C. crescentus* flagella (11).

Analysis of secondary structure by the method of Garnier et al. (10) indicated a predilection for α-helical structure for the first 13 to 15 amino acids of the class B proteins (data not shown); this region is also hydrophobic, as determined by hydrophilicity analysis (20). This sequence is followed by a region (amino acids 14 to 21) of low predictability in terms of structure which contains possible random coil, reverse turn, and beta-pleated sheet configurations. Amino acids 12 to 21 also correspond to an area of probable hydrophilicity (20). Overall, however, the N-terminal sequences of this group of proteins tend to be hydrophobic (average hydrophilicity per sequence = −0.5 to −0.2). The class A proteins also begin with regions of presumed α-helical structure and contain an area of high hydrophilicity (residues 17 through 22); in contrast to the class B N termini, however, these two sequences tend to be hydrophilic overall (average hydrophilicity = +0.3 and +0.2, respectively).

DISCUSSION

The antigenic and N-terminal sequence data reported here and in previous studies (1, 3, 4, 27, 28, 37) demonstrate that the major periplasmic flagellar proteins of *T. pallidum* and *T. phagedenis* can be divided into two distinct classes, which we have designated as class A and class B. Class A polypeptides are restricted to the highest-M_r species, the 37,000-M_r protein of *T. pallidum* and the 39,000-M_r protein of *T. phagedenis*. These proteins are thought to be present on the outer surface or sheath of the flagella, as indicated by the reactivity of monoclonal or monospecific antibodies with intact periplasmic flagella (4, 8, 27, 37). The class B periplasmic flagellar proteins consist of the 34.5-, 33-, and 30-kDa proteins of *T. pallidum* and the cluster of four 33- and 34-kDa polypeptide species of *T. phagedenis*. Cockayne et al. (8) suggested that the two largest class B proteins (to which they assigned M_rs of 34,000 and 33,500) represent the core of the *T. pallidum* periplasmic flagella, whereas the smallest may be associated with the outer surface or sheath. The association of *T. phagedenis* class B proteins with periplasmic flagella is supported further by genetic evidence, inasmuch as these proteins could not be detected by Western blotting in the periplasmic flagellum-deficient mutants T40 and T55 (27; S. J. Norris and N. W. Charon, unpublished data). The other periplasmic flagellum-associated proteins described in our study (the 29- and 27-kDa proteins of *T. pallidum* and the 37- and 30-kDa proteins of *T. phagedenis*) do not appear to be related to either the class A or class B proteins, based on their apparent lack of antigenic cross-reactivity with either of these groups. As yet, insufficient quantities of these proteins have been obtained for sequence analysis or immunization. These proteins could be part of the periplasmic flagellum hook or basal assembly, or they may simply represent contaminants. Overall, the structural complexity of the periplasmic flagella of *Treponema* species and other spirochetes is gradually being elucidated, but the functional importance of this complex arrangement in motility is still unknown.

Comparison of the two-dimensional electrophoresis patterns of these two organisms reveals both similarities and differences. In terms of their periplasmic flagellar proteins, more differences are apparent on two-dimensional gels than by SDS-PAGE analysis, particularly with regard to the class B proteins. Each of the *T. pallidum* class B proteins exhibits a different isoelectric point, whereas those of *T. phagedenis* are present as two major polypeptides with the same pIEF and two minor polypeptides at a more acidic pIEF. We know that the *T. pallidum* and the two major *T. phagedenis* class B proteins each have distinctive N-terminal sequences (Fig. 4), but the minor *T. phagedenis* species have not been sequenced. The two minor polypeptide species could be post-translationally modified versions of the major 34- and 33-kDa species, or they could represent products of separate genes; this question could be answered by either protein sequencing or DNA studies. In terms of the nonperiplasmic flagellar proteins, *T. pallidum* and *T. phagedenis* seem to be quite dissimilar, with the following exceptions: the major 61- and 59-kDa antigens (which represent the common antigens described by Hindersson et al. [17, 18] and have been characterized as *E. coli* groEL homologs [16a] by L. S. Houston, R. G. Cook, and S. J. Norris [manuscript in preparation]); large, diffuse 29- to 35-kDa and 34- to 37-kDa spots

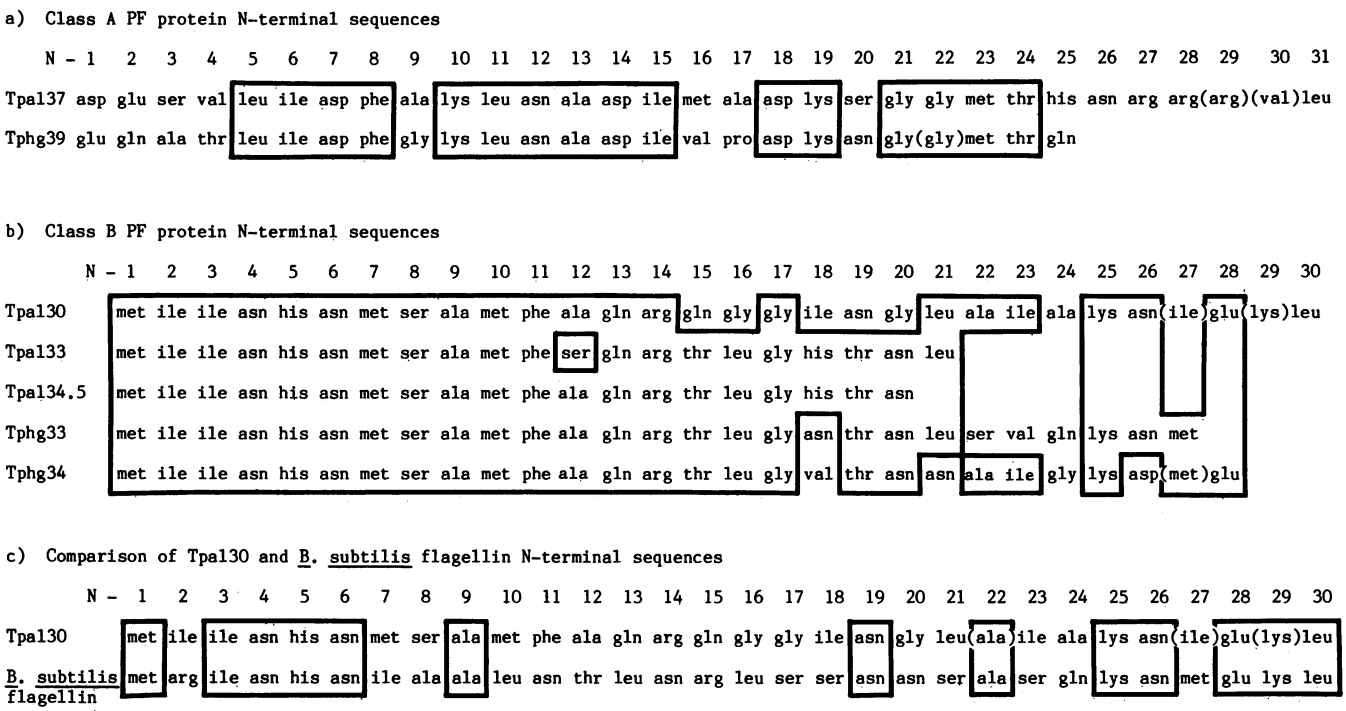


FIG. 4. N-terminal amino acid sequences of the major class A and class B periplasmic flagellar (PF) proteins of *T. pallidum* subsp. *pallidum* Nichols (as indicated by Tpal prefix and M_r) and *T. phagedenis* Kazan 5 (Tphg prefix and M_r) and comparison with the *B. subtilis* flagellin N-terminal sequence (9). Boxes indicate regions of amino acid identity. Tentative identifications are indicated by parentheses.

on the acid end of the gel (TpD; see reference 31); and other major antigens at 83 and 80 kDa (Fig. 1). Differences between the two-dimensional patterns are particularly apparent in the M_r range of 35,000 to 50,000 and include the lack of a *T. phagedenis* molecule with migration characteristics similar to the major 47-kDa antigen of *T. pallidum* (see reference 31). The electrophoretic dissimilarities observed are consistent with the low DNA homology and disparate G+C ratios reported for these organisms (29).

The periplasmic flagellar protein composition of *T. phagedenis* and *T. pallidum* reported here is similar to that previously reported in the literature, as indicated in the tentative correlation shown in Table 2. In an attempt to avoid confusion, the consensus M_r values of *T. pallidum* proteins (as determined by comparison of SDS-PAGE results from 16 laboratories [31]) were used to identify the proteins in this study. The correlation of periplasmic flagellar proteins in the different reports (Table 2) was simplified by the consistent presence of a polypeptide doublet corresponding to the two highest-M_r class B proteins. These results share a fair amount of consistency from laboratory to laboratory, and the M_rs when averaged are generally close to the consensus values (31) used in this study. The variation noted is most likely related to the different methods of isolation of the periplasmic flagella and subsequent electrophoretic analysis. The molecular weight standard values and method of M_r determination used can also affect these values (31). These differences are probably not due to strain differences, inasmuch as identical two-dimensional electrophoresis and SDS-PAGE Western blotting results were obtained with periplasmic flagellum-specific antisera tested against both the Kazan 5 and Reiter strains (28; R. Limberger, Ph.D. dissertation, West Virginia University, Morgantown 1984).

The immunologic studies shown in Fig. 2 support the division of periplasmic flagellar proteins into two antigenically distinct groups. These results indicate that the proteins within each group (class A and class B) share epitopes with one another, but lack antigenic cross-reactivity with the other group. The monoclonal antibody reactivity patterns described by Bailey et al. (1) provide additional evidence for

TABLE 1. Homology among the N-terminal amino acid sequences of the major periplasmic flagellar proteins of *T. pallidum* subsp. *pallidum* Nichols and *T. phagedenis* Kazan 5

Protein[a]	Protein sequence identity (no. of amino acids identical/total)					
	Tphg39	Tpal37	Tphg34	Tphg33	Tpal34.5	Tpal33
Tpal30	0 (0/25)	7 (2/30)	67 (18/27)	69 (18/26)	75 (15/20)	71 (15/21)
Tpal33	5 (1/21)	5 (1/21)	86 (18/21)	90 (19/21)	95 (19/20)	
Tpal34.5	5 (1/20)	5 (1/20)	95 (19/20)	95 (19/20)		
Tphg33	4 (1/25)	4 (1/27)	78 (21/27)			
Tphg34	4 (1/25)	4 (1/26)				
Tpal37	64 (16/25)					

[a] Tpal, *T. pallidum*; Tphg, *T. phagedenis*; number shows $M_r \times 10^3$.

TABLE 2. Probable correlation of M_rs of periplasmic flagellar proteins from *T. pallidum* subsp. *pallidum* Nichols and *T. phagedenis* biotypes Kazan 5 and Reiter

Proteins	M_r										
	Consensus (31)	This study	Blanco et al. (3)	Cockayne et al. (8)	This study (Kazan 5)	Limberger and Charon (27) (Kazan 5)	Bharier and Allis (2) (Reiter)	Hardy et al. (16) (Reiter)	Sand Peterson et al. (34) (Reiter)	Radolf et al. (38) (Reiter)	Cockayne et al. (8) (Reiter)
T. pallidum	37	37.8	35	38.5							
	34.5	35.3	33	33.75							
	33	34.5	33	33.25							
	30	32	30	30							
	29	30.5		27							
	27	29.7									
T. phagedenis					39	39.8	36.5	25	34	37	38.5
					34	33	33	15	30	33	33.75
					33	33	33		30	33	33.25
					30				28	30	31.75
										27	

this antigenic relationship. Hybridomas induced by immunization of mice with purified *T. pallidum* periplasmic flagella expressed antibodies which reacted with either class A or class B proteins, but not with both. Several monoclonal antibodies were identified which reacted with the 37-kDa periplasmic flagellar protein of *T. pallidum*, none of which reacted with any of the lower-molecular-weight periplasmic flagellar proteins. On the other hand, one monoclonal antibody recognized epitopes on all three of the class B proteins; another antibody was found to react with only the 31.5-kDa periplasmic flagellar protein of this group, whereas a third bound to both the 31.5- and 33.5-kDa periplasmic flagellar antigens. None of these monoclonal antibodies reacted with the periplasmic flagella of *T. phagedenis*. The results of Bailey et al. (1) are consistent with the existence of little or no immunologic cross-reactivity between class A and class B proteins and the presence of both shared and unique epitopes among the class B proteins of *T. pallidum* and *T. phagedenis*.

Our results differed somewhat from those of Radolf et al. (37), who reported that all the *T. phagedenis* biotype Reiter periplasmic flagellar proteins are antigenically related to various degrees. They found that affinity-purified antibodies eluted from their *T. phagedenis* 37-kDa protein reacted with all the other *T. phagedenis* periplasmic flagellar proteins, with the strongest cross-reaction occurring with the 27-kDa protein. On the other hand, affinity-purified antibodies against the 37-kDa (class A) and 33-kDa (class B) *T. phagedenis* antigens reacted specifically with the corresponding class A and class B molecules of *T. pallidum*, in agreement with our results. Chymotrypsin peptide maps were interpreted by Radolf et al. (37) as indicating a moderate degree of homology between the 37- and 33-kDa *T. phagedenis* proteins.

N-terminal sequence analysis provided the strongest evidence for relatedness among the periplasmic flagellar proteins (Fig. 4; Table 1). Because of the structural and antigenic relationship of these spirochetal proteins, it is likely that the observed sequence identity represents true homology (i.e., reflecting common ancestral genes) rather than convergent evolution or coincidental sequence identity. The class B sequences exhibited a particularly high degree of homology, ranging from 67 to 95%. This relatedness extended between the two treponemal species, as exemplified by the 34.5-kDa protein of *T. pallidum* and the major 34-kDa *T. phagedenis* periplasmic flagellar polypeptide, which shared 19 of 20 N-terminal amino acids. The homology among the first 14 amino acids was particularly striking, with four of five class B proteins having an identical sequence and the other differing by only one amino acid. The more acidic, minor 33- and 34-kDa class B polypeptides of *T. phagedenis* were not available in sufficient quantities for sequencing, so it is not known whether these represent separate gene products or posttranslational modifications of the major class B proteins.

The two class A proteins also exhibited homology at 16 of 25 positions, consistent with their antigenic cross-reactivity. The class A N-terminal sequences were unrelated to those of the class B proteins in all possible alignments, again emphasizing the differences between these groups of proteins.

Recently, Blanco et al. (3) reported N-terminal amino acid sequences obtained from Edman degradation of whole purified *T. pallidum* periplasmic flagella. In the analysis of PTH derivatives from the periplasmic flagellar sequences, they identified a major and a minor sequence component. They equated these sequences with the 33-kDa doublet (which they considered one protein) and the 35-kDa protein, respectively. Because they detected only two sequences, Blanco et al. (3) concluded that *T. pallidum* periplasmic flagella most likely contain only two proteins and that the additional proteins (the second band of the 33-kDa doublet and the 31-, 29-, and 27-kDa bands) were altered forms or degradation products of a single 33-kDa protein. Our results indicate that this conclusion is incorrect. By using whole flagella for sequence analysis, Blanco et al. (3) were actually determining the sequences of the class A protein (the minor component) and the sum of the sequences of the three class B proteins (the major component). Because the N-terminal sequences of the class B proteins are closely related overall and identical over the first 11 amino acids (Fig. 4b), a mixture of these proteins would appear to yield a single sequence. However, the results we obtained with two-dimensional electrophoresis-purified proteins indicated that each of the three *T. pallidum* class B proteins has a unique N-terminal sequence; the serine at position 12 which distinguishes the 33-kDa protein sequence from that of the 34.5-kDa protein was confirmed by sequence analysis of two separate preparations of the 33-kDa protein. Despite the differences in interpretation, the sequences reported by Blanco et al. (3) are in close agreement overall with our *T.*

pallidum 37-kDa and 33-kDa sequences, except that we identified an alanine at position 17 of the 37-kDa protein (instead of a glycine in their study) and a threonine at position 19 of the *T. pallidum* 33-kDa protein (instead of a lysine residue) (3).

More sequence information will be required to determine whether the homology patterns observed among the class A and class B periplasmic flagellar polypeptides extend over the entire length of these proteins. However, antigenic and sequence information suggest that at least certain regions of the periplasmic flagellar proteins are highly conserved in spirochetes. As an indication of the extent of this conservation, antisera against the 33- to 34-kDa *T. phagedenis* periplasmic flagellar antigens cross-react with periplasmic flagellar proteins of eight *Treponema* species and *Spirochaeta aurantia* (28). Similarly, monoclonal antibodies against *S. aurantia* periplasmic flagellar proteins react with *T. phagedenis* Kazan 5 periplasmic flagella (5a). As suggested previously (28), conserved epitopes may be involved in functions related to motility. The genetic range of this conservation may extend far beyond the spirochetes, if additional sequence data indicates that the homology between class B proteins and *B. subtilis* flagellin (Fig. 4c [9]) is indeed significant (see the Addendum in Proof).

The lack of an N-terminal methionine in the class A sequences indicates that the translation initiation site has been removed posttranslationally, perhaps as a signal peptide. The gene of the 37.5-kDa periplasmic flagellar protein of *S. aurantia*, which has recently been cloned and sequenced by B. Brahamsha and E. P. Greenberg (Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, I72, p. 122; B. Brahamsha and E. P. Greenberg, manuscript in preparation), provides information supporting this conclusion. The first 21 amino acids of the deduced amino acid sequence of this gene constitute a predicted signal peptide (36); 10 of the next 11 amino acids are identical to residues 1 through 8, 10, and 11 of the mature *T. phagedenis* 39-kDa protein (Fig. 4), indicating that the *S. aurantia* 37.5-kDa protein is related to the treponemal class A proteins. Because the flagellin genes of *E. coli* (25), *S. typhimurium* (43), and *B. subtilis* (9) lack signal peptide sequences, spirochetal class A periplasmic flagellar proteins may be processed, transported, and assembled in a manner different from that of other bacterial flagellins (22).

For the class B proteins, the N-terminal methionine appears to represent an unmodified translation initiation site. Similar to signal peptides (36), these N-terminal regions contain a predicted hydrophobic α-helical segment. However, unlike signal peptides, these regions lack highly charged residues at the N terminus and are apparently not cleaved in the mature proteins. The highly conserved nature of the first 14 amino acids may mean that this region is important in the processing or function of these proteins.

Although the amount of sequence information is limited, secondary structure and hydrophilicity analyses suggest that the periplasmic flagellar protein N termini possess domains consisting of α-helical regions followed by segments of relatively random and hydrophilic structures. Hydrophilic regions are thought to be more likely to represent immunogenic epitopes (20). The class A N termini tended to be more hydrophilic overall than those of the class B proteins, but the amount of data is too small to speculate on whether this tendency is related to their putative sheath or core structural locations.

The differences between the N-terminal sequences among the class B proteins of *T. pallidum* and *T. phagedenis* indicate that these polypeptides are most likely encoded by separate structural genes, although other possibilities exist. When DNA segments corresponding to the periplasmic flagellar class B protein genes are isolated, these probes could be used to determine the number, distribution, arrangement, and regulation of the members of this putative gene family. The existence of periplasmic flagellar regulons consisting of coordinately regulated class A and class B genes is an intriguing possibility.

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ADDENDUM IN PROOF

J. H. Martin and D. C. Savage (J. Bacteriol. **170**:2612–2617, 1988) recently published the structural gene sequence and the N-terminal amino acid sequence of the flagellin of *Roseburia cecicola*. The N-terminal sequence of *R. cecicola* flagellin has a high sequence identity with the N-terminal sequences of all of the *T. pallidum* and *T. phagedenis* class B periplasmic flagellar proteins (37 to 53%), as well as with the N-terminal sequences of *B. subtilis* and *S. typhimurium* flagellins. The treponemal class B N-terminal sequences also exhibited a moderate sequence identity (38 to 43%) with *B. subtilis* flagellin (Fig. 4) and, upon reexamination, a low to moderate identity with the *S. typhimurium* H-1^a (18 to 29% [44]) and *E. coli hag* (15 to 27% [25]) N-terminal sequences. These results indicate that the treponemal class B proteins possess some degree of sequence identity with a broad class of flagellins present in a wide variety of bacteria.

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