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Polypeptides of *Treponema pallidum*: Progress toward Understanding Their Structural, Functional, and Immunologic Roles†

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INTRODUCTION

Treponema pallidum is an unusual bacterium in terms of its structure, physiology, and host-parasite interactions. All of these features are related, either directly or indirectly, to its proteins, which are responsible for most of its functional activities. Over the past 15 years, there has been increasing recognition of the need to characterize the components of *T. pallidum* according to their antigenic, structural, and functional roles. The identification of individual *T. pallidum* polypeptides began in 1975, with the eventual application of many electrophoretic techniques, including sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), crossed immunoelectrophoresis, and two-dimensional gel electrophoresis (2DGE). In addition, the use of recombinant DNA and hybridoma technologies has greatly enhanced our knowledge of the protein components of *T. pallidum*. The rapid accumulation of new information has produced a need to correlate the results from different laboratories and fuse the new information into a more easily interpretable body of knowledge. In this monograph we attempt to correlate the rapidly expanding information regarding the proteins of *T. pallidum* with the structural, physiologic, pathogenic, and immunologic properties of this spirochete. Excellent reviews by Strugnell et al. (207) on the molecular and antigenic aspects of treponemes and by Schouls (188) on the use of recombinant DNA techniques to characterize *T. pallidum* proteins are also available.

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NEED FOR *T. PALLIDUM* RESEARCH

Although effective therapies have been available since the World War II era, syphilis and related treponematoses still constitute important global health problems (31, 32). In the United States, the total reported incidence of syphilis peaked at 963,647 cases in 1946. With the introduction of penicillin, the total reported incidence decreased rapidly and eventually reached an all-time low of 64,621 cases in 1977. An urban epidemic of syphilis occurred in 1986 to 1990, culminating in 134,255 reported cases in 1990 and an 80% increase in the reported rate per 100,000 population. In 1991, 4,332 cases of early congenital syphilis were reported, reflecting an increased proportion of early syphilis cases in female patients (31). A puzzling aspect of this recent epidemic is that it coincided with a dramatic 45% reduction in the number of reported cases of gonorrhea (decreasing from 911,419 to 491,447 from 1985 to 1992), a trend more consistent with the anticipated (but probably unrealistic) institution of safe-sex practices.

Despite its importance as an infectious agent and the recent application of biochemical and molecular biology techniques to its characterization, relatively little information about *T. pallidum* is available in comparison with other bacterial pathogens. The organism cannot be cultured continuously in vitro, available diagnostic tests are suboptimal, no vaccine is available, and the mechanisms of pathogenesis are not well understood (as discussed below). In addition, it is thought that syphilitic lesions may facilitate the transmission of human immunodeficiency virus (83), and syphilis has more severe manifestations in AIDS patients (83, 124, 136). It is clear that a better understanding of the biological properties, host-parasite relationships, and epidemiology of this organism is needed.

UNUSUAL FEATURES OF *T. PALLIDUM*

Genetics and Relationship to Other Treponemal Organisms

Treponemes pathogenic to humans were reclassified in 1984 as *T. pallidum* subsp. *pallidum* (venereal syphilis), *T. pallidum* subsp. *endemicum* (endemic syphilis), and *T. pallidum* subsp. *pertenue* (yaws); because of a lack of genetic information, *Treponema carateum* (pinta) and related organism, *Treponema paraluiscuniculi* (venereal spirochetosis of rabbits), were retained as separate species (194). These organisms are virtually identical in terms of morphology, DNA-DNA homology (where tested) (55, 119–121), and protein composition, although they exhibit different patterns of pathogenesis in humans and laboratory animals (222). *T. pallidum* subsp. *pallidum*, *pertenue*, and *endemicum* were found to have >95% homology by DNA-DNA hybridization (55, 119, 120). Significant differences exist between the *T. pallidum* subspecies and other spirochetal species. *T. pallidum* subsp. *pallidum* shares less than 5% DNA homology with *Treponema phagedenis*, *Treponema refringens*, and *Serpulina hyodysenteriae* (formerly *Treponema hyodysenteriae*) (119, 121). Neither the *Treponema* species pathogenic to humans nor *Treponema paraluiscuniculi* have been cultivated continuously in vitro, although progress has been made in this area during the past decade (see the section on physiology, below). The other treponemal species can be cultured quite easily in liquid media under either anaerobic

or microaerobic conditions. rRNA sequence data (159b) confirm the close phylogenetic relationship between *T. pallidum*, *T. phagedenis*, and other cultivable spirochetes.

Noordhoek et al. (140–142) have made a concerted effort to develop methods for distinguishing between *T. pallidum* subsp. *pallidum* and *T. pallidum* subsp. *pertenue* for diagnostic and epidemiologic purposes. They showed that a homologous gene in the two subspecies (described as *tpf1* and *tyf1*, corresponding to *tpn19* in this article) varied at only 1 of 531 bp (141) resulting in a single-amino-acid difference in the deduced sequence. Three of four syphilis strains had an A at residue 122 (with the other strain having a G), whereas five of six yaws isolates had a G at that position (the other having an A) (142). Thus the correlation between subspecies and sequence is not absolute even in this 1-bp difference. In another approach, Noordhoek et al. (140) screened 67 monoclonal antibodies against 17 different polypeptides for their preferential reactivity with syphilis or yaws isolates. All of these monoclonal antibodies reacted with polypeptides of the same apparent molecular weight in both sets of organisms. These results, together with previous electrophoretic and antigenic comparisons (13, 195, 218) (see Fig. 3), underscore the similarities of these *T. pallidum* variants and leave unresolved the reasons for their distinct patterns of pathogenesis.

A previous estimate of the *T. pallidum* genome size based on renaturation kinetics indicated that it was 9.05×10^9 Da or ~13,700 kbp, approximately three times larger than the *E. coli* genome (55, 119). Recent pulsed-field gel electrophoresis studies have shown that the genome is in the form of a circular chromosome of approximately 1,000 kbp, making it one of the smallest procaryotic genomes (185, 232). This latter finding is more congruous with the fastidious nature of *T. pallidum*, which may be due in part to a dependence on the host for nutritional requirements or to a lack of adaptability.

Structure

T. pallidum is structurally unique (Fig. 1). Like other spirochetes, it is spiral shaped and has outer and cytoplasmic membranes, a thin peptidoglycan layer, and periplasmic flagella (also called endoflagella) which lie in the periplasmic space and extend from both ends toward the middle of the organism (85–87). The flagellar filament has a sheath and core structure and is composed of at least four major polypeptides. Members of the genus *Treponema* also contain cytoplasmic filaments, also known as cytoplasmic fibrils (87, 126). These ribbon-shaped filaments are 7.0 to 7.5 nm in width. They run the length of the organism just underneath the cytoplasmic membrane and parallel to the periplasmic flagella; their function is unknown. *T. pallidum* possesses at least eight major lipoproteins; they appear to be membrane associated, but their functions and precise structural locations (i.e., internal versus external surfaces of the outer or cytoplasmic membranes) have not been established.

The most unusual structural feature of *T. pallidum* is the paucity of intramembranous protein particles in the outer membrane. Recent freeze fracture studies (180, 233, 234) have revealed that the concave (outer leaflet) fracture face of the outer membrane contains ~70 intramembranous particles per μm^2, compared with severalfold more outer leaflet-associated particles in other spirochetes (233) and in gram-negative bacteria (234) (Fig. 2). The *T. pallidum* outer membrane particles appear to be uniform in size, leading to the hypothesis that there are only a few different protein species in the outer membrane (180, 234). It is thought that a low concentration of surface-exposed protein antigens may permit *T. pallidum* to evade the immune response, thereby contributing to its pathogenesis (25, 43, 160, 164, 180, 198, 233, 234).

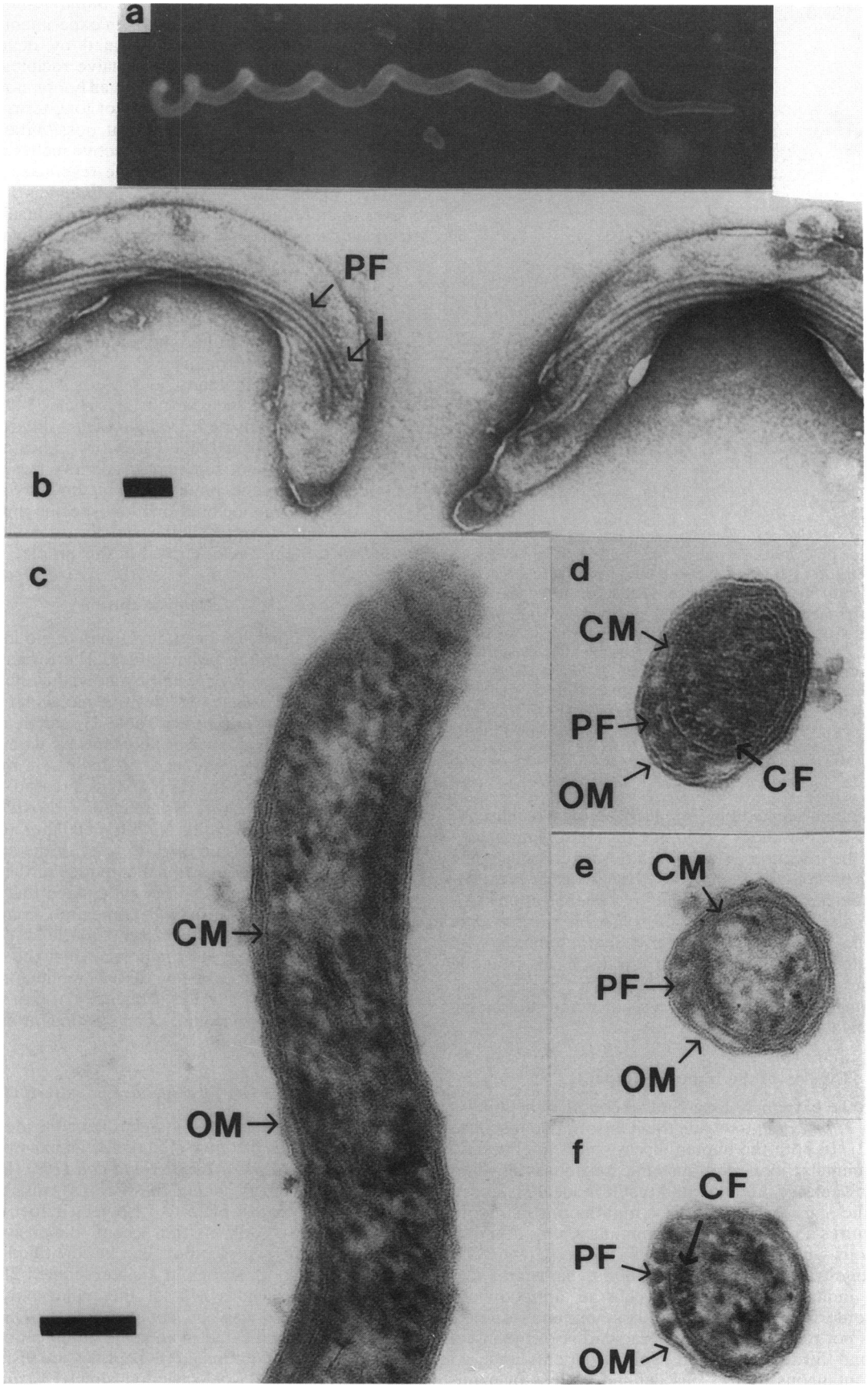
Physiology

T. pallidum is an obligate parasite of humans and is one of the few bacteria that are pathogenic to humans and have not been cultured continuously in vitro. *T. pallidum* is propagated by intratesticular infection of rabbits for research and diagnostic purposes. Long thought to be an obligate anaerobe, *T. pallidum* is now known to be a microaerophilic organism. By incubating *T. pallidum* with Sf1Ep cottontail rabbit epithelial cell cultures in a special tissue culture medium under microaerobic conditions, Fieldsteel et al. (56, 57) in 1982 were able to achieve up to 100-fold multiplication of the bacterium, as confirmed by other laboratories (151, 152). Unfortunately, attempts at subculture and long-term in vitro growth have not been fruitful (44, 152), although we are gradually gaining a better understanding of the physiology and growth requirements of this organism (42, 44, 57, 151, 152). The fastidious nature and slow growth of *T. pallidum* in vivo (45, 118) and in vitro (56) (doubling time, ≥30 h) indicate that it may have metabolic limitations and requirements for as yet unidentified host products. Previous studies have shown, however, that *T. pallidum* is fully capable of glucose metabolism and DNA, RNA, and protein synthesis (42), and a recent report by Gherardini et al. (65) provides genetic evidence for its ability to synthesize at least some amino acids. It is possible that *T. pallidum* is dependent on host cells for protection against oxygen radicals, because the bacterium requires oxygen for its metabolism but is highly sensitive to its toxic effects (42, 44, 203).

Pathogenesis

The human treponemal diseases are characterized by multiple clinical stages and long periods of latent (asymptomatic) infection (146, 192, 223). In syphilis, the chancre represents a localized, primary infection from which the organism disseminates. *T. pallidum* is extremely invasive, rapidly attaching to mammalian cell surfaces and penetrating endothelial junctions and tissue layers (58, 183, 216, 217). Secondary syphilis, which occurs in roughly one-fourth of all untreated syphilis cases, results from the multiplication of disseminated *T. pallidum* and formation of lesions at multiple sites in the skin and internal organs, despite the presence of a vigorous antibody response. Tertiary signs, including neurosyphilis, cardiovascular syphilis, and gummatous syphilis, develop in about one-third of untreated syphilis cases, often several years or even decades after the initial

FIG. 1. Morphology of *T. pallidum* subsp. *pallidum* Nichols. (A) Scanning electron micrograph showing the spiral shape. (B) Negatively stained view of the tips of two organisms. Note the insertion points (I) of periplasmic flagella (PF) near the ends. (C to F) Electron micrographs of ultrathin sections, showing the outer membrane (OM), the cytoplasmic membrane (CM), the periplasmic flagella (PF), and the location of the cytoplasmic filaments (CF). Bars, 0.1 μm. Adapted from reference 146 with permission of the publisher.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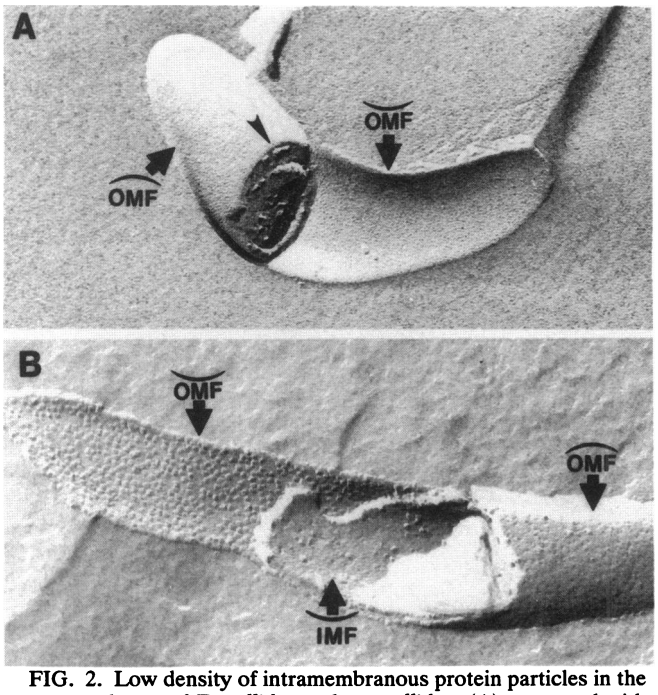

FIG. 2. Low density of intramembranous protein particles in the outer membrane of *T. pallidum* subsp. *pallidum* (A) compared with *Spirochaeta aurantia* (B), demonstrated by freeze fracture electron microscopy. Very few particles are visible in the inner face (*OMF*) and outer face (*OMF*) of the outer membrane of *T. pallidum*, whereas a nearly confluent array of particles are visible in the outer membrane of *Spirochaeta aurantia*. Periplasmic flagella (arrowhead) are apparent in a fracture face across the *T. pallidum* cell body. Intramembranous particles are also evident in the inner or cytoplasmic membrane of *Spirochaeta aurantia* (*IMF*) and *T. pallidum* (not shown). Freeze fracture electron micrographs kindly provided by E. M. Walker.

infection. Syphilis seems to be primarily an invasive disease with most of the pathology being due to host inflammatory reactions to the infection (146, 192), although there is some evidence for pathologic reactions and necrosis of mammalian cells at extremely high *T. pallidum* concentrations (63, 154). Lyme disease (caused by *Borrelia burgdorferi* and other *Borrelia* species) shares many characteristics with syphilis, including the evolution from local to systemic infection, the capacity for long-term latency, and multiple pathologic stages, including cardiovascular and neurologic manifestations (201).

Evasion of the Immune Response

Related to the extremely long clinical course of syphilis is the ability of *T. pallidum* to evade the host immune response (15, 146, 192). In both the human disease and experimental syphilis in animals, local and systemic responses develop within a few weeks of infection and result in local clearance of most of the spirochetes. Within 6 months postinfection, the host acquires so-called chancre immunity, i.e., does not develop lesions when reinfected with *T. pallidum* (146, 192, 222). Adoptive-transfer studies in rabbits, hamsters, and guinea pigs indicate that either T cells or antibody are capable of conferring resistance. The host immune response usually does not result in complete eradication of the infection. Only one-third of untreated syphilis patients undergo apparent spontaneous "cure," as defined by loss of manifestations and serologic reactivity. Small numbers of viable *T. pallidum* cells persist for years in experimentally infected laboratory animals, as demonstrated by development of lesions or serologic reactivity in naive recipients following injection of extracts of tissue (such as lymph nodes) from an infected animal. The mechanisms of long-term latent infection have not been elucidated, but possibilities include (i) localization of organisms in "protective niches," i.e., tissue regions inaccessible to the immune response; (ii) low antigenicity of the outer surface of *T. pallidum* as a result of low outer membrane protein content; (iii) masking of the treponemal surface by host proteins or glycosaminoglycans; and (iv) intracellular localization.

POLYPEPTIDES OF *T. PALLIDUM*

The need to standardize the identification of *T. pallidum* polypeptides was recognized at a meeting on Treponemal Pathogenesis and Immunity held in Los Angeles, Calif., 3 to 5 December 1985. As a result, an article (149) correlating SDS-PAGE patterns of *T. pallidum* from different laboratories was published in 1987. This work indicated that SDS-PAGE alone was not sufficient to identify treponemal polypeptides definitively; however, a method involving 2DGE (155, 156) and monoclonal or monospecific antibodies was outlined which permitted the specific identification of *T. pallidum* proteins, as described in this article.

Nomenclature

A nomenclature has been developed to aid in the identification of *T. pallidum* polypeptides. The format consists of the prefix TpN (for *T. pallidum* Nichols, the reference strain) followed by a consensus M_r (relative molecular mass, based on SDS-PAGE results) (see Table 1) and, if necessary, a letter to distinguish between polypeptides with similar M_rs. The corresponding gene can be indicated by lowercase italics; for example, the TpN47 is expressed by the gene *tpn47*. The nomenclature is intended to provide a standard for correlating the results of different laboratories, which may use different terminology to refer to the same protein. As knowledge of the functional activities of the *T. pallidum* polypeptides accumulates, it is anticipated that other, more descriptive nomenclature will come into common usage, e.g., the FlaA, FlaB1, FlaB2, and FlaB3 designations for flagellar polypeptides. It is important that the identity of a polypeptide be established by 2DGE, monoclonal or monospecific antibody reactivity, sequence data, or other definitive means (149) prior to use of an existing (or assignment of a new) TpN designation.

Two-Dimensional Gel Electrophoresis Pattern of *T. pallidum*

2DGE has been useful in standardizing the identification of the polypeptides of other organisms, as exemplified by the gene-protein index for *Escherichia coli* (169). In this study, *T. pallidum* subsp. *pallidum* Nichols was subjected to 2DGE, consisting of pH 5 to 7 isoelectric focusing gradient gels and SDS-PAGE in the second dimension. Approximately 100 polypeptide spots can be identified consistently by their relative positions in a silver-stained 2DGE pattern (Fig. 3A). Consensus M_rs of the major polypeptides as determined previously by comparison of SDS-PAGE patterns (149) are indicated, with some modifications and additions (Table 1). Additional polypeptides are visible in gels in which pH 3.5 to 10 isoelectric focusing (IEF) gradients (Fig.

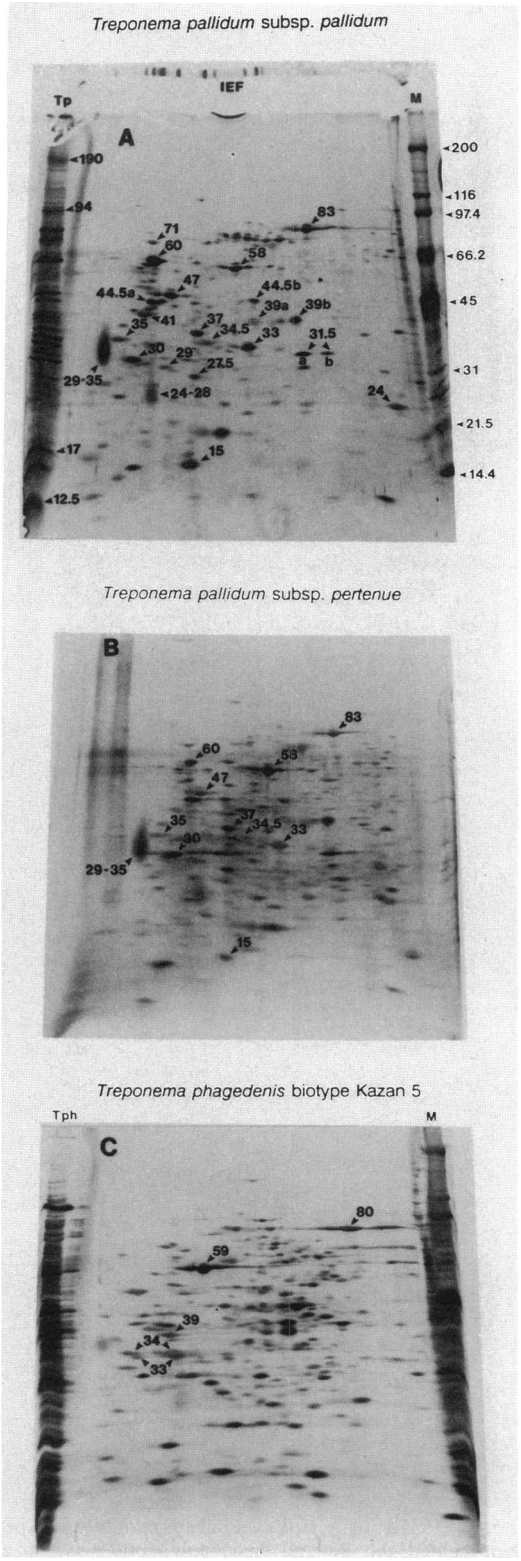

FIG. 3. 2DGE patterns of *T. pallidum* subsp. *pallidum* Nichols (A), *T. pallidum* subsp. *pertenue* Gauthier (B), and *T. phagedenis* biotype Kazan 5 (C), as revealed by silver staining (66). *T. pallidum* was purified by Percoll density gradient sedimentation (70). Whole organisms were dissociated and subjected to IEF (pH 5 to 7) followed by SDS-PAGE (8 to 20% gradient gel) (149). In this and subsequent figures, the acidic side of the gel in the IEF dimension is on the left side. Numbers indicate consensus M_rs (in thousands) as determined by comparison of the SDS-PAGE results of 16 laboratories (149) and correspond to the polypeptides described in Table 1. Single-dimension SDS-PAGE lanes on either side of the 2DGE contain *T. pallidum* (Tp) and Bio-Rad high- and low-molecular-mass standards (M). A Coomassie blue G-stained IEF gel is shown at the top of panel A. Polypeptides marked on the SDS-PAGE profile have not been identified in the 2DGE pattern. *T. pallidum* subsp. *pallidum* Nichols (panel A) and *T. pallidum* subsp. *pertenue* Gauthier (panel B) have nearly identical electrophoretic profiles, as indicated by representative proteins in the *T. pallidum* subsp. *pertenue* pattern. In contrast, the *T. phagedenis* profile (panel C) is quite different except for conserved polypeptides such as the flagellar proteins (39, 34, and 33 kDa), the GroEL-equivalent protein (59 kDa), and the cytoplasmic filament protein (80 kDa).

4) or nonequilibrium pH gradient electrophoresis (Fig. 5) are used in the first dimension. The distribution of spots in the 2DGE pattern is highly reproducible. Similar *T. pallidum* 2DGE patterns have been reported by Thornburg and Baseman (218) and by Altaie and Cox (6) and have also been obtained in the laboratories of Baughn, Lukehart, Penn, and Radolf.

Comparison of *T. pallidum* subsp. *pallidum* (Nichols strain) (Fig. 3A) with *T. pallidum* subsp. *pertenue* (Gauthier strain) (Fig. 3B) indicated only minor differences in their protein profiles, consistent with previous reports (13, 198, 218). All of the major polypeptides observed in the 2DGE patterns of the two subspecies have similar electrophoretic mobilities; TpN58 (corresponding to TpA [229] [Table 1]) seemed to be expressed in greater quantities in the yaws isolate. In contrast, the 2DGE pattern of the easily cultivable, saprophytic spirochete *Treponema phagedenis* Kazan 5 was quite different from those of the *T. pallidum* strains (Fig. 3C). Only a few polypeptides have M_rs and isoelectric properties similar to those in *T. pallidum*. These include proteins with M_rs of 80,000, 59,000, and 34,000 to 39,000, which appear to correspond to the cytoplasmic filament protein (126), the GroEL protein (76, 78, 80, 84), and flagellins (24, 106, 107, 150, 172), respectively.

Correlation of Two-Dimensional Gel Electrophoresis and SDS-PAGE Patterns

Internal-standard 2DGE gels were used to correlate spots in the 2DGE pattern with the corresponding bands in the SDS-PAGE profile. In this method, *T. pallidum* (8×10^8 cells per gel) solubilized in equilibration buffer (155) was added to the agarose overlay used to seal the IEF gel in place. After electrophoresis and staining, lines corresponding to each polypeptide were formed which connected the spots in the 2DGE pattern with the band in a single-dimension SDS-PAGE lane (Fig. 4A). In the experiment shown, an electroblot was stained by the immunoperoxidase method with rabbit anti-*T. pallidum* antiserum to reveal the distribution of antigenic proteins; very similar results were obtained by silver staining. By using this technique, a definitive correlation between the SDS-PAGE and 2DGE distributions could be made for most of the major polypeptides.

The internal-standard technique was also used to corroborate the relative molecular weights of the 2DGE spots by placing molecular weight standards in the agarose overlay (Fig. 4B). In this case, the spot position relative to the molecular weight standard "bands" was measured directly and M_rs were calculated by the method of Weber and

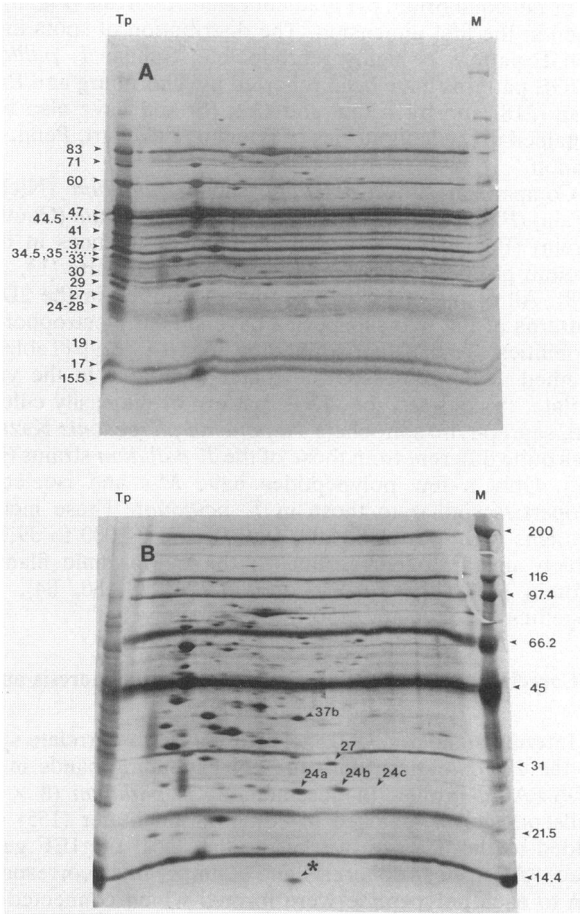

FIG. 4. Internal-standard 2DGE of *T. pallidum* subsp. *pallidum* Nichols. (A) Correlation of SDS-PAGE and 2DGE patterns by inclusion of solubilized *T. pallidum* in the agarose overlay used to hold the IEF gel in place during the second-dimension run. The gel pattern was transferred electrophoretically to nitrocellulose and stained immunologically with rabbit anti-*T. pallidum* antiserum (149, 220). This method has a sensitivity similar to that of silver staining and permits the correlation of SDS-PAGE and 2DGE migration patterns of antigenic proteins. (B) Use of molecular mass standards in the agarose overlay, as revealed by silver staining. Tp and M represent the single-dimension SDS-PAGE patterns of *T. pallidum* and Bio-Rad high- and low-molecular-mass standards, respectively. The IEF dimension consisted of pH 3.5 to 10 gels; polypeptides not visible in the pH 5 to 7 pattern are indicated. The asterisk corresponds to a spot with an M_r of ~12,000 also visible in Fig. 3.

Osborn (236). The gel shown was silver stained; Coomassie blue R staining provided narrower molecular weight standard bands but did not reveal as many spots in the 2DGE pattern. This procedure permits more accurate determination of M_rs for polypeptides which comigrate by SDS-PAGE and also minimizes error due to curvature of the gel pattern.

Nonequilibrium pH Gradient Electrophoresis

Basic polypeptides often cannot be identified by standard isoelectric focusing (IEF), because they either do not enter the gel or are not well focused because of cathodic drift. Nonequilibrium pH gradient electrophoresis (NEPHGE) as described by O'Farrell et al. (156) is a simple modification of the IEF technique which permits resolution of basic proteins. In this case, the protein sample is electrophoresed

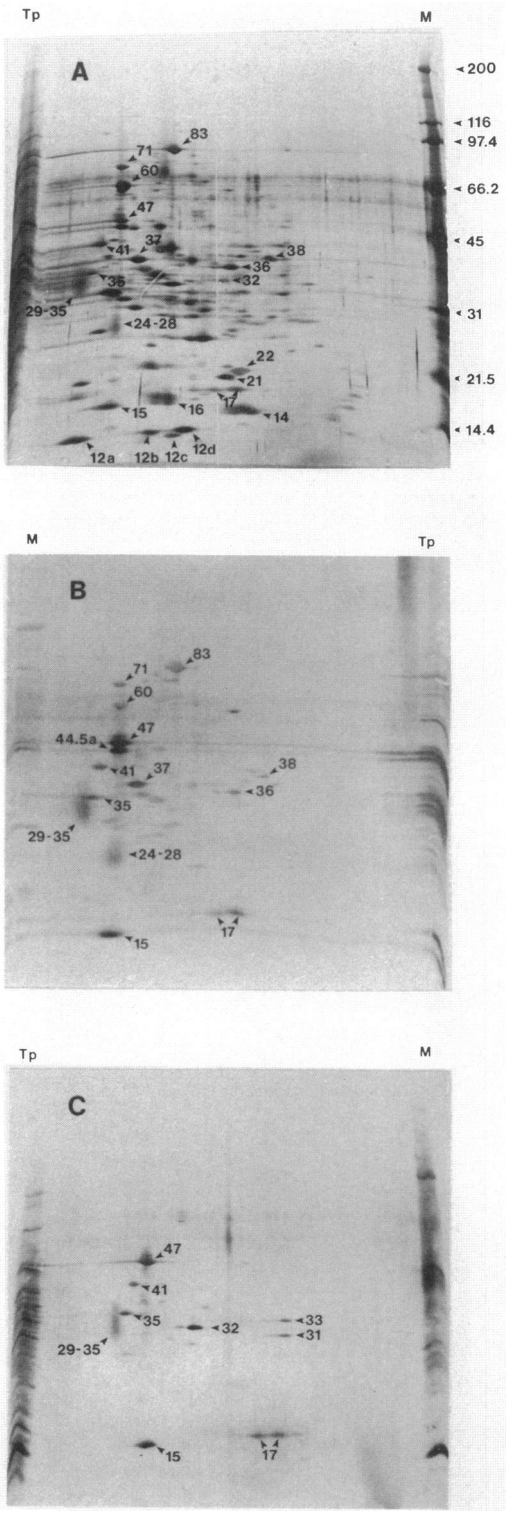

FIG. 5. NEPHGE of *T. pallidum* subsp. *pallidum* polypeptides. (A) Silver-stained gel. Several basic polypeptides which are not resolved in IEF gels are indicated (molecular weight in thousands). Also visible are several additional low-M_r spots (marked 12a to d and 16). (B) Reactivity of *T. pallidum* polypeptides with serum antibodies from patients with secondary syphilis, as determined by immunoperoxidase staining of electroblots (127, 220). (C) Polypeptides partitioning with Triton X-114, a biphasic detergent. Most of the spots thus identified correspond to known lipoproteins (see Tables 1 and 3).

from the acidic toward the basic end of the gel for a limited time (4 h at 400 V), so that the polypeptides are still migrating in the gel and do not reach their equilibrium position.

NEPHGE of *T. pallidum* reveals several polypeptides which are not detected on either pH 5 to 7 or pH 3.5 to 10 IEF gels (Fig. 5A). For the most part, the pattern to the left (acidic) side of the gel resembles a compressed version of a typical IEF-2DGE pattern. Basic polypeptides revealed by NEPHGE include TpN38 (21), TpN34 (TmpB [71, 189, 190, 229, 247]), and TpN17 (1, 33). Also present are heretofore unidentified spots with M_rs of 16,000 and 14,000 and a series of prominent spots with an estimated M_r of 12,000. Interestingly, the 12-kDa spots (TpN12a to TpN12d) are located in the region of the gel corresponding to the normal IEF range yet are not clearly visible (except for one spot marked by the asterisk in Fig. 4B) in a standard IEF two-dimensional gel. These results suggest that these moieties may possess anomalous migration properties under either IEF or NEPHGE conditions. Treatment with proteinase K destroyed all of the spots visible in silver-stained NEPHGE-2DGE patterns, indicating that they are proteinaceous and do not include any apparent lipopolysaccharides (LPS) or lipooligosaccharides (148).

Reactivity with Serum Antibodies from Syphilis Patients

Infection with *T. pallidum* results in the induction of antibody and cellular responses against its protein components, as discussed in "Immune Response to *T. pallidum* Proteins." Figure 5B shows a representative immunoperoxidase pattern obtained with pooled sera from patients with secondary syphilis, the stage in which the antibody response approaches its peak intensity. Antibodies against most of the major polypeptides of *T. pallidum* are generated during infection and are detectable by these means (Fig. 5B). Western immunoblot reactivity against the spots with M_r 12,000, 14,000, and 16,000 was not detected; the reason for this apparent lack of reactivity has not been determined.

Hydrophobicity and Membrane Localization

Recently, there has been a great deal of interest in identifying the membrane proteins of *T. pallidum* because of their potential importance in protective immunity. One approach to identifying membrane-associated proteins is by detergent solubilization, which can selectively solubilize hydrophobic proteins under certain conditions. The nonionic detergent Triton X-114 has been found to be particularly useful in this regard (33, 47, 174); because of its low cloud point, the detergent phase can be separated easily from the aqueous phase, permitting the purification of detergent-soluble (hydrophobic) proteins from those which remain in the aqueous phase (26).

Triton X-114 soluble polypeptides detectable by NEPHGE-2DGE and silver staining are shown in Fig. 5C. Many of these have been identified previously as putative membrane proteins by detergent solubilization and other means; they include TpN47 (34), TpN44.5a (TmpA [71, 189, 190, 229, 247]), TpN35 (TmpC [90, 191, 229]), TpN29-35 (TpD [77, 190], 34 K [213, 214]), TpN24-28 (TpE [190, 229]; not visible in this gel pattern), TpN17 (1, 33), and TpN15 (33, 170, 171). These results will be discussed below in the context of outer membrane and cytoplasmic membrane composition and protein content.

Structural Fractionation of *T. pallidum*

Purification of the structural components of *T. pallidum* is an important step in the identification of proteins associated with the outer membrane, cytoplasmic membrane, periplasmic flagella, and other structures. One approach is to separate components by density sedimentation, as exemplified by the method of Osborn et al. (157) for purifying the outer and cytoplasmic membranes of *Salmonella typhimurium* by sucrose gradient sedimentation. In attempts to apply this approach to *T. pallidum* (148), the spirochetes did not form spheroplasts on treatment with lysozyme and EDTA; instead, the cells were disrupted by sonication or by use of a French pressure cell. The results of centrifugation of disrupted *T. pallidum* at 180,000 × *g* for 16 h in a 25 to 55% sucrose gradient followed by SDS-PAGE are shown in Fig. 6. Fragments of periplasmic flagella sediment near the bottom of the gradient, as demonstrated by the distribution of the 37-kDa FlaA protein (Fig. 6D) and confirmed by electron microscopy (150). The *T. pallidum* homolog of the GroEL protein (an oligomer of TpN60 subunits) also appears as a discrete band in the sucrose gradient; this property was used in its purification and characterization (84). TpN83 and a 75-kDa band (which appears to be TpN83 with altered migration properties [161]) comigrate in the gradient and appear to represent the cytoplasmic filaments (126, 160), although this correlation has not been proven as yet for *T. pallidum* (148). The 190-kDa oligomer of the 4D protein (TpN19) is visible in silver-stained gels (Fig. 6B) in fractions 17 and 18 of this gradient. Most of the proteins visible in the upper part of the gradient are thought to be associated with the cytoplasmic and outer membranes, which, on the basis of electron microscopy of gradient fractions, appear to sediment as overlapping peaks. NADH oxidase activity, typically associated with the cytoplasmic membrane (157), sediments as a distinct peak in this region of the gradient (Fig. 6D). The outer membranes of gram-negative bacteria migrate near the bottom of sucrose gradients (157), well separated from the cytoplasmic membranes, because of their high LPS content and hence higher density. Although we have no definitive markers for identifying the outer membrane of *T. pallidum*, it appears to sediment at a density similar to that of the cytoplasmic membrane because of its lack of LPS. When fractions were diluted and recentrifuged for 1 h to pellet large structural components (such as membranes, flagella, and cytoplasmic filaments), the 4D 190-kDa complex, the GroEL homolog, and a 12.5-kDa polypeptide remained in the supernatant, indicating that they are not associated with the membranes or other large structures (Fig. 6C). Thus the 4D protein does not appear to be associated with the outer membrane as proposed previously (175).

Through modifications of this or other density gradient procedures, it may be possible to purify and characterize the outer membrane, cytoplasmic membrane, and cytoplasmic filaments. Selective detergent solubilization (see the section on outer and cytoplasmic membrane composition and ultrastructure, below) and other techniques may also yield valuable information on the protein content and other structural properties of these interesting treponemal components.

Specific Identification with Monoclonal Antibodies and Monospecific Antiserum

A total of 41 specific antibodies provided by 12 laboratories were analyzed for reactivity with 2DGE electroblots of

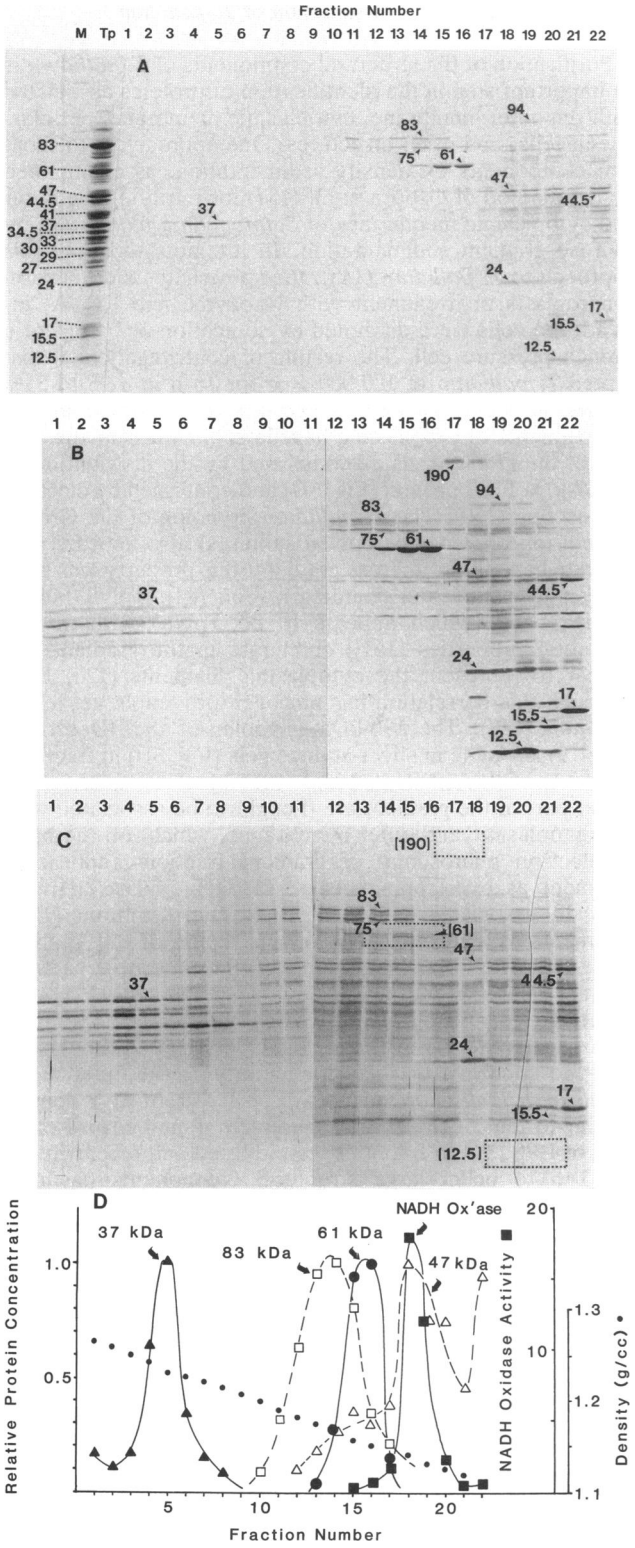

FIG. 6. Association of *T. pallidum* subsp. *pallidum* polypeptides with structural components, as determined by sucrose density gradient sedimentation followed by SDS-PAGE (84, 148, 150, 157). Panels A to C show fractions 1 to 22 of the same sucrose gradient, with fraction 1 being from the bottom. M, molecular weight standards (in thousands); Tp = whole *T. pallidum*. (A) Coomassie blue-stained pattern, showing the distribution of major polypeptides. (B) Silver-stained pattern of the same gel shown in panel A. The 190-kDa oligomer of TpN19 (the 4D antigen) is visible. (C) Particulate material sedimented from the gradient fractions by dilution followed by centrifugation in an Airfuge at 180,000 × *g* for 1 h. Note the absence of bands corresponding to the 190-kDa complex of TpN19, the GroEL equivalent (84) TpN60 (marked here as 61), and a 12.5-kDa polypeptide. (D) Distribution of TpN37 (FlaA, a representative flagellar protein), TpN83 (the putative cytoplasmic filament monomer), TpN60 (the GroEL equivalent), TpN47 (a major membrane lipoprotein), and NADH oxidase activity (157) as a measure of cytoplasmic membrane sedimentation. Values are based on densitometry measurements from the Coomassie blue-stained gel shown in panel A and are normalized to a peak value of 1.0 for each polypeptide.

T. pallidum subsp. *pallidum* Nichols by the immunoperoxidase method. After photography and determination of the coordinates of the reactive spot, the same electroblot was counterstained with rabbit anti-*T. pallidum* antiserum. Because this antiserum reacts with most of the major polypeptides, it was possible to determine the location of the spot reacting with the specific reagent in the overall 2DGE pattern. Examples of monoclonal antibody reactivity patterns are shown in Fig. 7. In nearly all cases, monoclonal antibodies reacted specifically with a single spot in the 2DGE pattern, although some exceptions were found (Fig. 7C).

A summary of antibody reactivities and other available information on the major polypeptides of *T. pallidum* is presented in Table 1. The 41 antibody reagents tested to date reacted with a total of 15 different polypeptides. The availability of multiple monoclonal antibodies (as with TpN47) provides an excellent resource for cross-referencing results obtained by different laboratories. Also listed are recombinant DNA vectors expressing *T. pallidum* proteins (including several which have been sequenced), as well as a summary of known structural, functional, and biochemical properties.

STRUCTURE AND FUNCTION OF *T. PALLIDUM* POLYPEPTIDES

Flagellar Proteins

Multiple polypeptides of *T. pallidum* periplasmic flagella. The periplasmic flagella or endoflagella are presently the best-characterized structural components of *T. pallidum* (38). The filaments of the flagella of most bacteria consist of polymers of a single polypeptide subunit (117, 137). In contrast, the endoflagellar filament of *T. pallidum* subsp. *pallidum* is composed of multiple polypeptides arranged into an outer sheath and a central core (22, 24, 39, 150, 162, 172). A similar protein composition and ultrastructure have been described for the endoflagella of *T. phagedenis* (24, 87, 106, 150, 172), *Treponema denticola* (40), *S. hyodysenteriae* (100, 101), *Leptospira* species (99, 138, 221), *Spirochaeta aurantia* (29, 30, 159, 159a), and other *Spirochaeta* species (98). The flagellar filaments of *B. burgdorferi* are composed of a single flagellin (235), unlike the other spirochetes thus far examined.

Up to six flagellum-associated polypeptides have been described in *T. pallidum*, corresponding to TpN37a, TpN34.5, TpN33, TpN30, TpN29, and TpN27.5. Most of these have been well characterized at the protein and genetic levels and have been subdivided into class A and class B

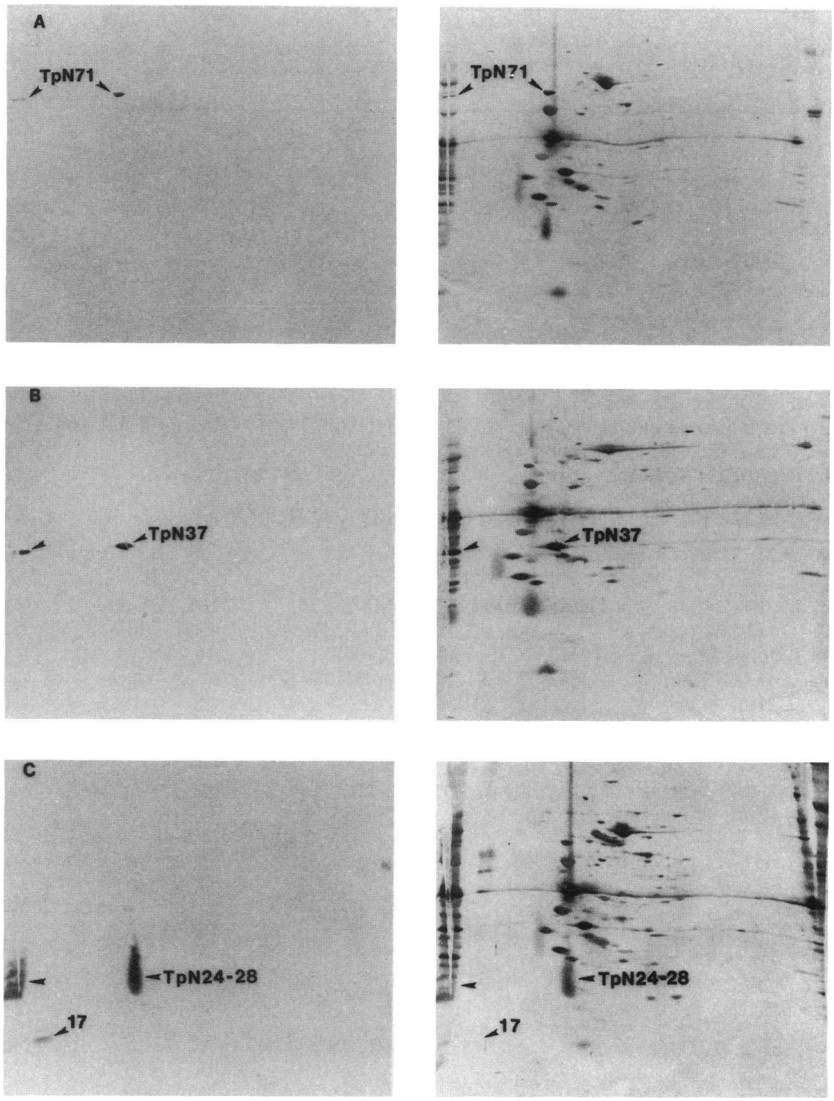

FIG. 7. Examples of protein identification by reactivity with monoclonal antibodies and monospecific antisera, as demonstrated by immunoperoxidase staining. In each case, the two-dimensional immunoblots were first stained with the polypeptide-specific antibody preparation (left side) and then counterstained with anti-*T. pallidum* antiserum (right side). A single-dimension SDS-PAGE pattern of *T. pallidum* polypeptides is present on the left side of each immunoblot. (A) Identification of TpN71, the putative DnaK equivalent, with the monoclonal antibody HATR 1-27 (75). (B) Reaction of the 37-kDa flagellar sheath protein (FlaA) with monoclonal antibody H9 (114). (C) Binding of monoclonal antibody 3F3-2E6 (28) to TpN24-28 (TpE [10, 229]) and a minor 17-kDa polypeptide.

flagellins on the basis of their N-terminal amino acid sequences (22, 93, 150), gene sequences (37, 93, 95, 158), and antigenic relatedness (22, 107, 150, 162, 172). TpN37a has been classified as a class A flagellin and designated FlaA (93, 95), whereas the structurally and antigenically related class B polypeptides TpN34.5, TpN33, and TpN30 are named FlaB1, FlaB2, and FlaB3, respectively (37, 158).

Ultrastructure of periplasmic flagella. *T. pallidum* endoflagella are ~17 nm in diameter and can extend more than half the length of the organism (i.e., up to 12 μm) within the periplasmic space (85–87). Two to four endoflagella originate at each end and entwine the cell body as they extend toward the middle of the cell. The endoflagella retain a helical conformation when released from the periplasmic space by treatment with detergents, indicating that the spiral shape is a stable structural property of endoflagella and is not dependent on association with the cell body. Treatment of endoflagella with trypsin and 6 M urea (39) or repeated centrifugation and resuspension in distilled water (86, 87) results in release of the outer layer or sheath, leaving a core ~12 nm in diameter. Cockayne et al. (39) showed by immunoelectron microscopy that monoclonal antibodies directed against FlaA reacted with intact flagella but had reduced reactivity when the outer sheath was removed. Similar studies with *T. phagedenis* (106) and *Spirochaeta aurantia* (29) also indicate that the homologous FlaA proteins of these organisms are associated with the sheath whereas some or all of the FlaB polypeptides are associated with the core.

TpN29 and TpN27.5 are two additional polypeptides which have been detected in some *T. pallidum* endoflagellum preparations (150). Little information on these proteins is

TABLE 1. Properties of major *T. pallidum* subsp. *pallidum* polypeptides

Designation[a]	Internal-standard M_r[b]	Designation in ref. 149	Other designations[c]	Monoclonal/monospecific antibodies[c]	Recombinant DNA vectors	Sequence known[c,d]	Properties[c]
TpN94	94,000	b					Penicillin-binding protein?[e] (46, 177); not identified in 2DGE pattern
TpN83	82,000	c	78,000 (160), P1?[e] (17, 168)		P1?[e] (168)		Fibronectin-binding protein (16, 18, 165); cytoplasmic filament protein?[e] (126, 148, 160)
TpN71	70,000	d		HATR 1-27 (75), rabbit anti-DnaK (148)			Reacts with antiserum against *E. coli* DnaK protein (148)
TpN60	59,000	e	Tp4, common antigen (78, 80), GroEL (76, 84)	HATR 1-24 (75), rabbit antiserum (84)	pHI1001 (75, 76)	DNA (76), AA (84)	Homolog of GroEL heat shock protein (chaperonin) (76, 84)
TpN57	57,000		TpA (229)		pRIT9320 (229)		Apparent homolog prominent in *T. pallidum* subsp. *pertenue* (Fig. 3B)
TpN47	45,000	f	47,000 (35, 88, 143), 48,000 (114), P6, 45,000 (166, 219), 46,000 (135, 224), TpS (75)	11E3, 8G2 (97, 122, 123), A8, C2, D10, 2G10, H9-1 (114), 13F3 (219), 4a (135), C3E5 (224), AD5 (8), HATR 1-4 (75)	pMN23 (35, 144), P6 (166), pHI1030 (75)	DNA, AA (88, 238); DMW = 46,740	Major membrane protein; highly immunogenic; lipoprotein (33, 34); activates endothelial cells (182)
TpN44.5(a)	42,000	(g)	TmpA, 42,000 (229); 44,000 (8)	1-14M1 (224), 2D7 (8, 10)	pRIT4694 (71, 189, 229), pMJB10 (10)	DNA (71), DMW = 42,000	Membrane lipoprotein with signal peptide (71, 189); Homolog in *T. phagedenis* (247)
TpN44.5(b)	42,000	(g)					More basic than TpN44.5(a)
TpN41	39,500	h					Reacts with anti-TpN37 rabbit antiserum (150)
			TpF2, 41,000 (229)		pRIT7100 (229)		Not as yet identified by 2DGE; gene adjacent to *tpn24-28* (229)
TpN39(a)	38,000 40,000	(i)	38-kDa lipoprotein (21), 41,000 (10)	MAbs[f] 4G7, 1C5, 7C6, 1A1, 5D6, 8B8 (21), 33a (10)	pMN25, pPB7 (21) pMJB20 (10)	DNA, AA (21), DMW = 38,770 Restriction map (10)	Doublet (Fig. 3A); membrane lipoprotein with signal peptide (21)
TpN39(b)	38,000	(i)	Basic membrane protein (39 kDa) (48)	Rabbit antiserum (48)	pLVS3 (48, 196, 199)	DNA (48), DMW = 37,880	Basic membrane protein with signal peptide (48)
TpN38			38-kDa antigen (53), 37-kDa antigen (184)		pAW305 (53, 184, 230)	Restriction map (184)	Not as yet identified by 2DGE; reactive with human syphilitic sera (184)
	37,000		P2 (165, 168)		P2 (168)		Putative fibronectin-binding adhesin protein (16, 17, 165, 168)
TpN37	37,000	j	FlaA (93, 95), 37,000 (22, 150)	H9-2 (114), IB8 (9)	pRI4 (93, 95)	DNA (93, 95), AA (22, 93, 150), DMW = 36,948	Flagellin (9, 22, 150, 162) with signal peptide (95); sheath associated (39); sequence similarity with *S. aurantia*, *T. phagedenis*, *S. hyodysenteriae* flagellar sheath proteins (30, 101, 150, 159a)

Continued on following page

TABLE 1—*Continued*

Designation[a]	Internal-standard M_r[b]	Designation in ref. 149	Other designations[c]	Monoclonal/monospecific antibodies[c]	Recombinant DNA vectors	Sequence known[c,d]	Properties[c]
TpN36	36,000		TmpB, 35,000 (229, 247); 33,000 (135); 34,000 (75)	K1782 (rabbit) (228); 2a (135), HATR 1-29 (75)	pRIT4694 (75, 189, 229), pMJB10 (10)	DNA (71, 247), DMW = 37,000	Basic polypeptide visible in NEPHGE-2DGE patterns; Nonlipidated membrane protein (190, 247)
TpN35	35,500		TmpC, 35,000 (229); 35.5-kDa lipoprotein (90)	Rabbit antiserum (228), MAb 15 (10)	pRIT9000 (191, 229), pMJB40 (10), pCH2 (90)	DNA (191), DMW = 35,600	Membrane lipoprotein (90, 190, 229) with signal peptidase II recognition sequence (190)
TpN34.5	35,000	k	FlaB1 (37)	Rabbit antiserum (150)	pCC1 (37)	DNA (37), AA (22, 150), DMW = 31,179	Flagellin (22, 37, 150, 162, 172); localized in flagellar core (39); class B flagellin related to TpN33, TpN30, and flagellins of other bacteria (Table 2, Fig. 8)
TpN33	33,000	l	FlaB2 (158), 33,000 (150)	Rabbit antiserum (150)	pLPA105 (158)	DNA (158), AA (22, 150), DMW = 31,352	Flagellin (22, 37, 150, 162, 172); localized in flagellar core (39); Class B flagellin related to TpN34.5, TpN33, and flagellins of other bacteria (Table 2, Fig. 8).
TpN29-35	30-38,000		TpD (77, 229), 26,000–32,000; 34,000 (144, 213–215)	5522 (rabbit) (77); 10G2, 3B5, 9B12, (123, 215); 10c (10); HATR 1-3 (77)	pRIT3217 (77, 190, 229), pMN20 (144, 213–215), pMJB30 (10), pHI1002 (77)	DNA (190, 214), DMW = 20,123	Diffuse, acidic spot by 2DGE, smear by SDS-PAGE; lipoprotein with signal sequence (191, 213, 214)
TpN31.5(a), TpN31.5(b)	33,000						Two spots differing by isoelectric point
TpN30	32,000	m	FlaB3 (37), 30,000 (150), 31,500 (39)	JD11 (9), HATR 1-21, HATR 1-19, HATR 1-14 (75)	pCC1 (37)	DNA (37), AA (150), DMW = 31,069	Flagellin (22, 37, 150, 162, 172); class B flagellin related to TpN34.5, TpN33, and flagellins of other bacteria (Table 2, Fig. 8)
TpN29	30,000		29,000 (150)				Flagellum-associated protein (150)
TpN27.5	29,000		27,500 (150)				Flagellum-associated protein (150)
TpN24-28	24,000–30,000		TpE, 19–24 kDa (229)	HATR 125 (75), 3F3-2E6 (28)	pRIT9100 (229), pMJB50 (10)	Restriction map (10)	Diffuse spot by 2DGE; lipoprotein with signal sequence (191)
TpN24(a,b)	24,000	(n)	TpT, 21,000 (75)	HATR 1-17 (75)	pHI1004 (75)		MAb reacts with two basic spots on 2DGE pattern
TpN24(c)	24,000	(n)	p24 (89)	Rabbit antiserum (89)	pPH21 (89)		Same M_r as TpN24(a,b) but more basic; barely detectable in 2DGE pattern; secreted in *E. coli* (89)
TpN20	20,000						Large, diffuse spot by 2DGE
TpN19		a	4D (230), TpF1 (141, 229)	Rabbit antiserum (27, 54)	pAW329 (230), pRIT7100 (141, 229), pSY888 (231)	DNA (141, 231), DMW = 19,300	190-kDa homooligomer of 19-kDa subunits; ring-like structure with interchain disulfide bonds (52, 54, 173); immunogenic (27); identity in 2DGE pattern not established

Continued on following page

TABLE 1—*Continued*

Designation[a]	Internal-standard M_r[b]	Designation in ref. 149	Other designations[c]	Monoclonal/monospecific antibodies[c]	Recombinant DNA vectors	Sequence known[c,d]	Properties[c]
TpN17	17,000		Tpp17 (1), 14 kDa (12)	19G3 (181)	pMN7, pAE47 (1)	DNA, AA (1), DMW = 16,441	Large, basic spot detectable by NEPHGE-2DGE; major membrane lipoprotein (33) with signal sequence (1)
	17,000				pMJB60 (10)	Restriction map (10)	Restriction map differs from that of *tpp17*; genetically linked to *tpn15* (10)
TpN15	15,000		15.5 kDa (10), 12 kDa (114), 12 kDa (135)	3 (10), F5 (114), 1b (135)	pMJB60 (10), pMN40 (170, 171)	DNA (171), DMW = 13,967, restriction map (10)	Lipoprotein (33) with signal peptide (171); Induces antibody response during infection
TpN12a, TpN12b, TpN12c, TpN12d	<14,400						Cluster of spots detectable in NEPHGE gels by silver staining (Fig. 5A); Do not bind anti-*T. pallidum* antibody; migrate below lysozyme (14.4 kDa)

[a] Nomenclature format: TpN prefix, consensus M_r value, and suffix (a,b) as necessary to distinguish polypeptides with similar M_rs.
[b] M_r estimated from internal-standard 2DGE gels (Fig. 2B).
[c] References are given in parentheses.
[d] DNA, complete DNA sequence (unless otherwise indicated); AA, partial amino acid sequences; DMW, deduced molecular weight of mature polypeptide based on DNA sequence (does not include cleaved signal sequences, attached lipids, carbohydrates, etc.).
[e] Subject to confirmation.
[f] MAb, monoclonal antibody.

available, and their structural and functional roles in the endoflagella are unknown. They do not appear to be degradation products of the other flagellins, however, because monospecific antisera and monoclonal antibodies directed against FlaA and the FlaB proteins do not show any reactivity with TpN29 and TpN27.5 (150).

Flagellin gene structure and expression. The *T. pallidum* flagellin genes *flaA* (93, 95), *flaB2* (158), *flaB1* and *flaB3* (37) have been cloned and sequenced, thereby confirming that each of the polypeptides is expressed by a separate structural gene. The *flaA* gene sequence encodes a hydrophobic signal peptide with a consensus signal peptidase I sequence (95), which is cleaved off in the mature protein (22, 93, 150), indicating that FlaA is transported across the cytoplasmic membrane by standard polypeptide export mechanisms. The *flaA* promoter region contains −35 and −10 sequences typical of those recognized by σ^{70} transcriptional factors (95), rather than the σ^{28} recognition sequences associated with most motility-related genes. Interestingly, expression of the cloned, intact *flaA* gene in *E. coli* is lethal to the host organism, apparently because large quantities of unprocessed FlaA accumulate in the cytoplasm (93, 95). The flagellar sheath protein appears to be unique to spirochetes.

In contrast, the *T. pallidum flaB* structural genes and amino acid sequences (37, 158) closely resemble those of other eubacterial flagellins (Fig. 8). The *flaB1*, *flaB2*, and *flaB3* genes encode polypeptides of 286, 286, and 285 amino acids, respectively, and possess a high degree of homology. The deduced amino acid sequences exhibit sequence identity of 76% (FlaB1 and FlaB2), 72% (FlaB1 and FlaB3), and 67% (FlaB2 to FlaB3) when short gaps are introduced; consideration of conservative amino acid substitutions boosts the similarity to 89, 83, and 80%, respectively (37, 158). The *flaB1* and *flaB3* open reading frames are closely linked (separated by 281 bp) and were cloned as a single DNA fragment by Champion et al. (37). It is possible that the *T. pallidum* motility genes are clustered in certain regions of the genome, although this remains to be determined.

Both *flaB1* and *flaB2* (37, 158) have been shown to have an upstream nucleotide sequence resembling the σ^{28} promoter sequence characteristic of bacterial motility genes (72, 104, 130); *flaB3* is located downstream from *flaB1* and lacks a promoter sequence, raising the possibility that the two genes are expressed as a polycistronic mRNA. Like the flagellins of other bacteria (117, 137), the FlaB proteins of *T. pallidum* lack a signal peptide sequence and are secreted with an intact N terminus (150). Therefore the FlaB subunits are apparently transported through the core of the periplasmic flagella and added to the end, as has been described previously for other bacteria (137). FlaA secreted into the periplasmic space may then be overlaid onto the growing flagellar core. The three FlaB proteins and FlaA seem to be expressed in nearly equimolar amounts (148), suggesting a stoichiometric relationship and regular structural array of the four proteins. However, we do not yet understand the spatial orientation of *T. pallidum* flagellins (other than the sheath and core structure). It is even uncertain whether FlaB1, FlaB2, and FlaB3 are present on the same flagella or are assembled into separate flagella. An interesting observation in this regard was made by Cockayne et al. (41), who found that the venereal syphilis isolate SS2, although fully motile, lacked FlaB1. Thus the FlaB proteins may be able to substitute for one another, making it nonessential for all three to be expressed for proper flagellar assembly and function.

Relationship with flagellins of other bacteria. A comparison

of *T. pallidum* FlaB and other published bacterial flagellin sequences is shown in Fig. 8. The FlaB proteins are highly homologous to the flagellins found in a broad spectrum of bacteria, including both gram-positive and gram-negative organisms (Table 2). In particular, the N termini (corresponding to amino acids 1 to 143 in *T. pallidum* FlaB2) and C-terminal regions (amino acids 206 to 236) are highly conserved across all of the bacterial species shown, whereas the central portion is variable in length as well as amino acid sequence. For example, the *E. coli hag* gene encodes a 497-amino-acid polypeptide (deduced molecular weight, 51,172), in contrast to the 286-amino-acid FlaB1 (deduced molecular weight, 31,178), with most of the difference being due to a 208-amino-acid "spacer" in the *E. coli* sequence. It is unclear whether the ancestral gene was short like the *T. pallidum* gene and underwent expansion through insertions, or if a longer gene, like that in the members of the family *Enterobacteriaceae*, became truncated in treponemes and other bacteria. The former seems more likely, however, since the flagellar gene products of *B. burgdorferi* and *Bacillus subtilis* (which are intermediate in length) have little detectable homology with *E. coli* in the spacer region. Kuwajima (102) found that functional flagella were still produced by *E. coli* deletion mutants which expressed flagellin lacking the central 187 amino acids. Recent X-ray diffraction studies (137) indicate that the N- and C-terminal regions are responsible for the subunit interactions in bacterial flagella. The C-terminal region has been shown to be essential in both the excretion and assembly stages (82, 104). Therefore it is reasonable that these regions are conserved phylogenetically whereas the middle region is divergent.

The sheath protein FlaA appears to be present only in spirochetes and is highly conserved in the sequences studied to date (Table 2). The gene encoding the *Spirochaeta aurantia* class A protein (*flaA*) has been cloned and sequenced (30, 159a). Recent studies have indicated that the initially published sequence (30) was incorrect beyond the first 508 bp, and the correct sequence has been determined (159a). The *Spirochaeta aurantia flaA* gene encodes a predicted gene product which has 42% amino acid sequence identity with the deduced *T. pallidum* FlaA sequence (93, 95, 159a). The N-terminal amino acid sequence of the *T. phagedenis* FlaA protein is identical with the *T. pallidum* sequence at 16 of 25 positions (150) and with the mature (postcleavage) *S. aurantia* N terminus 10 of the first 11 residues (30, 150, 159). N-terminal amino acid and DNA sequence analyses by Koopman et al. (101) indicate that *S. hyodysenteriae* possesses a 43-kDa FlaA homolog, as well as four distinct FlaB proteins (37, 35, 34, and 32 kDa). It is likely that other spirochetes with multiple flagellins, including members of the family *Leptospiraceae*, possess a similar flagellar sheath protein.

Are *T. pallidum* flagellins glycosylated? FlaB1 and FlaB2 exhibit M_rs (on the basis of internal-standard gels [Table 1]) of 35,000 and 34,000, respectively, although their deduced molecular weights from DNA sequence data are 31,179 and 31,352, respectively (37, 158). The M_r of FlaB3 (32,000) is much closer to its predicted molecular weight of 31,069. Although there are many possible causes of anomalous electrophoretic mobility, it is possible that FlaB1 and FlaB2 are glycosylated. In 1984, Moskophidis and Müller (134, 135a) reported the *N*-[^{14}C]acetylglucosamine was incorporated preferentially into two *T. pallidum* polypeptides, with migration characteristics similar to those of FlaB1 and FlaB2; the radiolabeling pattern of *T. phagedenis* was also consistent with preferential *N*-acetylglucosamine incorporation into the FlaB polypeptides. In studies of the *Spirochaeta aurantia* flagellins, Brahamsha and Greenberg (30) found that the lectin concanavalin A (which has a high affinity for D-mannose residues) bound to some of the low-M_r flagellins that are now known to have sequence similarities to the FlaB proteins (159). The evidence is only suggestive at this point (particularly since protein glycosylation is rare in procaryotes), but the glycosylation of FlaB polypeptides represents an intriguing possibility. Interestingly, the flagellins of *Halobacterium* species (244) and other archebacteria are sulfated glycoproteins, although they lack significant sequence similarity with the FlaB proteins and other members of the eubacterial flagellin family.

Outer and Cytoplasmic Membrane Ultrastructure and Composition

The composition of the outer membrane of *T. pallidum* has been investigated intensively, because of its perceived importance in the immune clearance of the organism. The cytoplasmic membrane, as the site of transport and oxidative phosphorylation, is also of interest in terms of the complex physiology of *T. pallidum*. At the time of this writing, we do not have a clear picture of the composition and protein content of these membranes.

Paucity of intramembranous particles in the outer membrane. Recent freeze-fracture studies by Radolf et al. (180) and Walker et al. (233, 234) have shown that the outer membranes of *T. pallidum* subsp. *pallidum* and *T. pallidum* subsp. *pertenue* contain extremely small numbers of intramembranous particles corresponding to integral membrane proteins. As shown in Fig. 2, concave (outer-leaflet) fracture faces of the outer membranes of *T. pallidum* subspecies contain fewer than 100 particles per μm^2 on average, compared with estimates of 800 particles per μm^2 for *T. phagedenis* biotype Reiter, 2,601 ± 242 for *S. hyodysenteriae*, 1,860 ± 132 for *B. burgdorferi* B31, 382 ± 84 for *B. hermsii*, and ~5,000 for *Spirochaeta aurantia*, a free-living spirochete (233, 234). The outer membrane of *E. coli* exhibits a nearly confluent array (6,000 to 10,000 particles per μm^2) of intramembranous particles which vary greatly in size (110), whereas the outer membrane particles of *T. pallidum* are sparsely distributed and appear to be uniform in size. Fracture faces of the *T. pallidum* cytoplasmic membrane have a particle density and size variability similar to that found in the inner membranes of other bacteria (180, 233, 234). Freeze-etching of the *T. pallidum* outer surface also indicates a low particle density (180, 233), but this procedure is not a reliable means of detecting surface-localized membrane proteins.

The putative proteins corresponding to the intramembranous particles have been termed treponemal rare outer membrane proteins (TROMPs) (25). It has been postulated that the apparently low content of surface-localized proteins could decrease the reactivity of antibodies and immune cells with intact *T. pallidum*, permitting the organism to evade the immune response (see the section on immune response to *T. pallidum* polypeptides, below).

In search of the *T. pallidum* outer membrane proteins. Several biophysical techniques have been applied to the characterization of the outer membrane. Surface radioiodination has yielded highly variable results and seems to be unreliable in identifying surface-localized proteins (3, 135, 153, 161). Treatment of *T. pallidum* with Triton X-100 or Triton X-114 appears to selectively solubilize the outer membrane (34, 47, 160, 161, 165, 174, 178). The solubilized

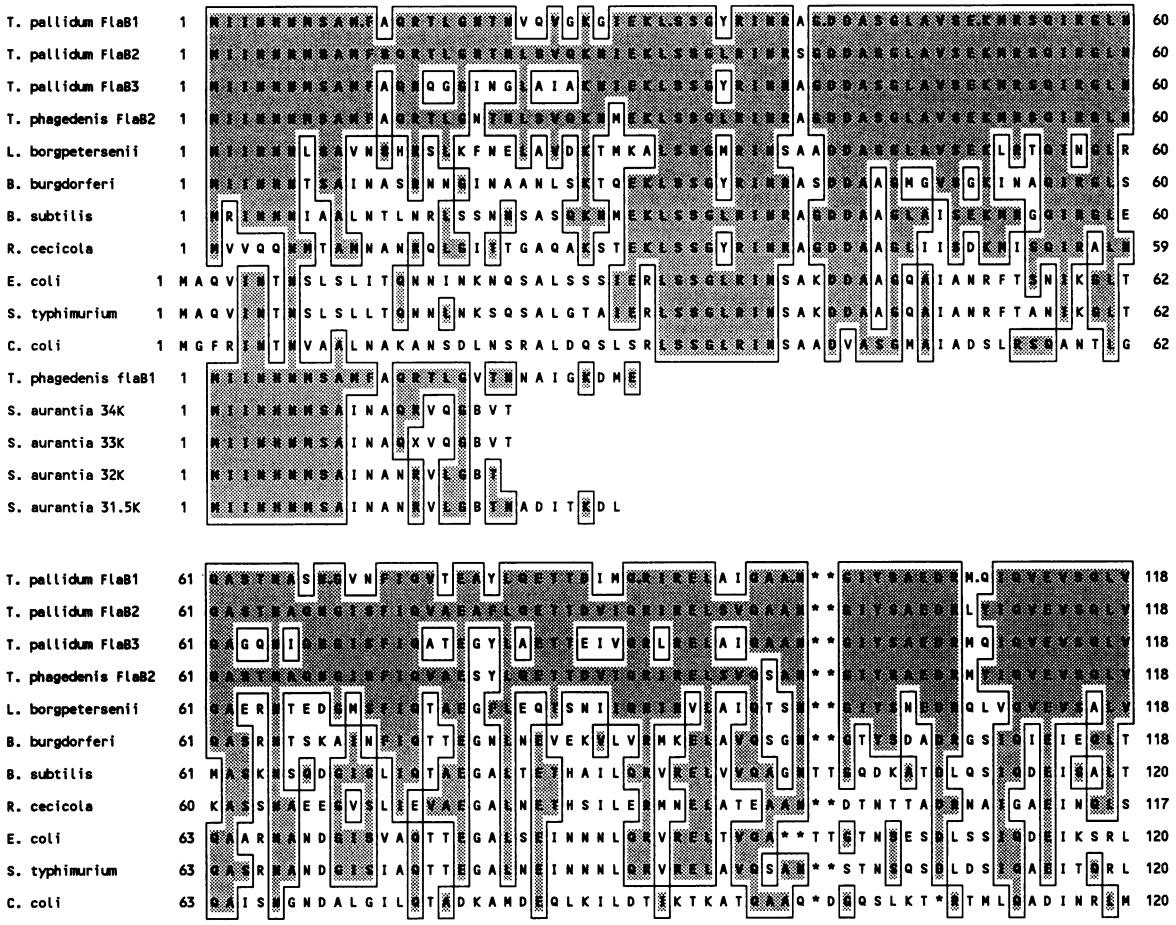

FIG. 8. Comparison of the class B flagellin polypeptides of *T. pallidum* subsp. *pallidum* Nichols with flagellins of other bacteria. Deduced sequences of FlaB1, FlaB2, and FlaB3 (37, 158) were aligned with the amino acid sequence of the *Bacillus subtilis* flagellin (49) and the deduced sequences of FlaB2 from *T. phagedenis* Kazan 5 (108) and flagellins from *L. borgpetersonii* (131), *B. burgdorferi* (235), *Roseburia cecicola* (125), *E. coli* (72, 103), *Salmonella typhimurium* (72, 237), and *Campylobacter coli* (109). Also shown are the N-terminal sequences of class B flagellins from *T. phagedenis* biotype Kazan 5 (150) and *Spirochaeta aurantia* (159). Amino acids identical to *T. pallidum* FlaB2 are boxed and shaded. Numbers indicate amino acid position, and asterisks represent gaps introduced to maximize alignment. The *B. burgdorferi*, *Bacillus subtilis*, *E. coli*, *Salmonella typhimurium*, and *C. coli* flagellins possess spacer regions in the middle of their sequences, which are not conserved (except for homology between the *E. coli* and *Salmonella typhimurium* sequences).

material contains TpN47, TpN35 (TmpC), and several other polypeptides (Fig. 6C). It is uncertain whether these proteins are specifically associated with the outer membrane or are released from the cytoplasmic membrane, the periplasm, or other structures as the result of detergent treatment. Low concentrations of SDS (0.04%) result in dissociation of the outer membrane from the protoplasmic cylinder (198). The pellet obtained from the supernatant following SDS treatment contains membranous material, but the possibility that these membranes represent rabbit tissue contaminants has not been excluded. This membranous pellet contains little or no detectable protein other than flagellar polypeptides, consistent with the freeze fracture results. However, many proteins are present in the supernatant phase following treatment with 0.04% SDS (148); it is possible that some of these represent solubilized membrane proteins.

Definitive information about the composition of the outer membrane cannot be obtained from detergent treatment studies, because of the possibility that proteins from other structures are nonspecifically released by these procedures or that membranes released by detergents may be altered in composition. Future emphasis should be placed on the development of procedures for the isolation of intact outer membranes and cytoplasmic membranes without detergent treatment.

A new approach to identifying translocated proteins (and hence possible outer membrane proteins) is the use of *T. pallidum* recombinant DNA clones expressing *phoA* (alkaline phosphatase) fusion products, as exemplified by the work of Blanco et al. (23). In their study, *T. pallidum* DNA was inserted into vectors containing *phoA* lacking its signal sequence. *T. pallidum* DNA possessing an active signal sequence inserted next to (and in frame with) the *phoA* gene results in expression, translocation, and detection of an active PhoA product. By this means, three types of *T. pallidum* signal sequences were identified: those encoding signal peptidase I sites, those encoding signal peptidase II sites, and those encoding highly hydrophobic leader sequences (23). Some of the *T. pallidum* gene segments detected by this method may represent the elusive TROMPs. At present, however, there are no definitive means of determining whether the clones are associated with cytoplas-

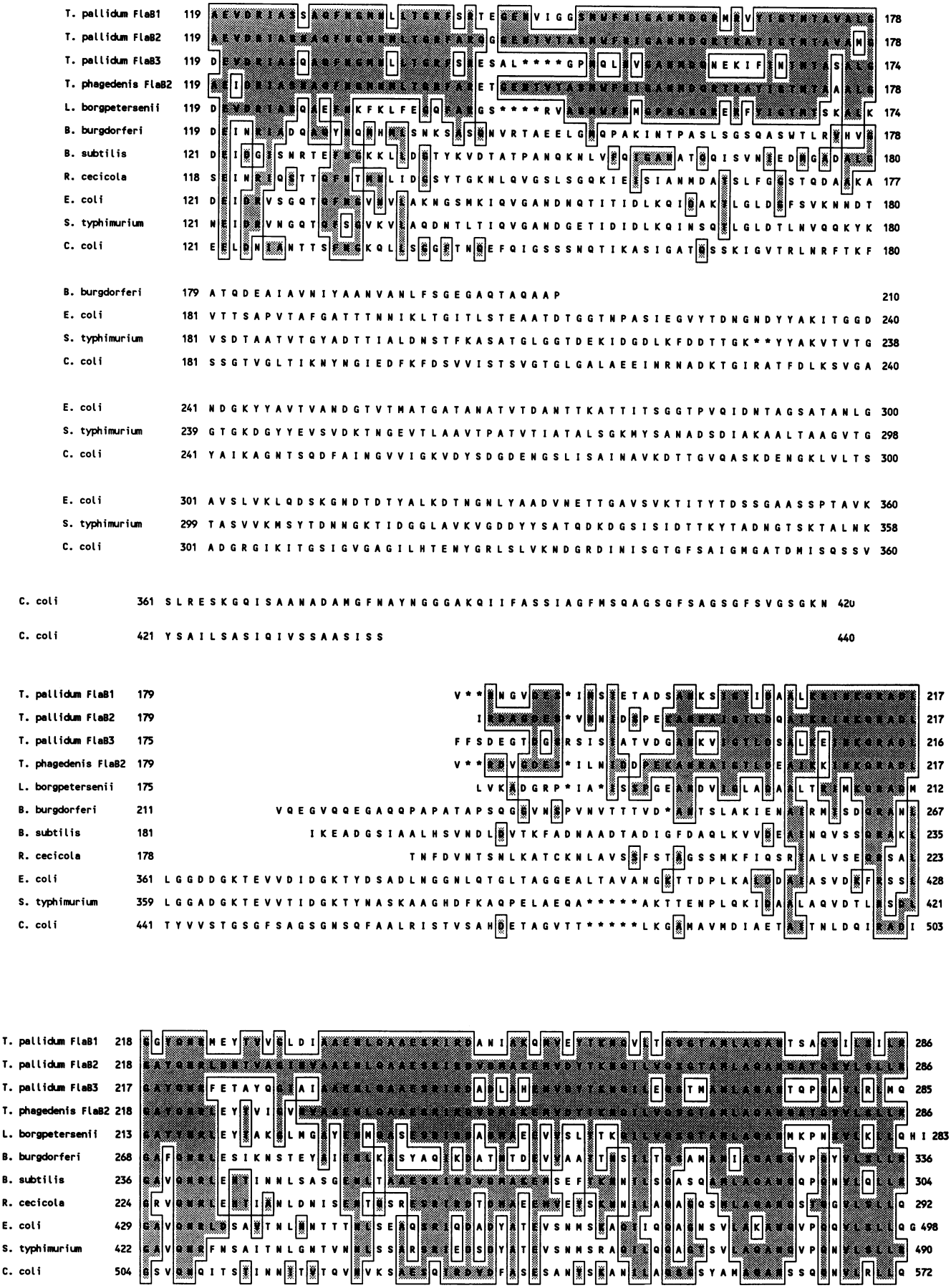

Figure 8—*Continued*

TABLE 2. Endoflagellum-associated polypeptides and related proteins

Flagellin type	Organism	Polypeptide	M_r[a]	Deduced MW[b]	% Amino acid sequence identity with *T. pallidum* protein[c]	Reference(s)
Sheath associated (class A)	*T. pallidum* subsp. *pallidum*	FlaA (TpN37)	37,000	38,860, 36,948	(100)	22, 39, 93, 95, 150
	S. aurantia	FlaA	37,000	36,816, 34,627	42	29, 30, 159a
	T. phagedenis	39K	39,000		64[d]	150
	T. denticola	–[e]				40
	S. hyodysenteriae	FlaA	44,000	36,000, 33,800	22	100, 101
	Members of the *Leptospiraceae*	36K				99, 138, 221
Core associated (class B)	*T. pallidum* subsp. *pallidum*	FlaB1 (TpN34.5)	35,000	31,179	78, 74	22, 37, 39, 150
		FlaB2 (TpN33)	34,000	31,352	(100), (100)	22, 37, 150, 158
		FlaB3 (TpN31)	31,000	31,069	71, 73	22, 37, 39, 150
	T. phagedenis	FlaB1	34,000		71[d]	150
		FlaB2	33,000	31,500	93, 93	108
	S. aurantia	36K (minor)	36,000			159
		34K (major)	34,000		60[d]	159
		33K (minor)	33,000		55[d]	159
		32K (minor)	32,000		68[d]	159
		31.5K (major)	31,500		55[d]	159
	L. borgpetersenii	FlaB	32,000	31,300	61, 67	131
	L. interrogans	35.5K	35,500		60[d]	221
		34K	34,000		53[d]	221
Related flagellins	*B. burgdorferi*	41K	41,000	35,757	51, 53	235
	B. subtilis	*hag* product		32,631	55, 64	49, 104, 130
	R. cecicola	*flaH* product	42,000	31,370	49, 59	125
	E. coli	hag_E product	55,000	51,172	40, 49	102, 103
	S. typhimurium	*H1*[a] product	55,000	51,334	40, 47	72, 82, 237
	C. coli	P1	61,500	58,814	39, 47	109

[a] Apparent molecular weight by SDS-PAGE.
[b] Deduced molecular weight (MW) based on DNA sequence (molecular weights for precursor and mature forms indicated for FlaA sequences).
[c] For class A polypeptides, the percent sequence identity with FlaA is given. For class B polypeptides and related proteins, the percent sequence identity with FlaB2 is given. When two numbers are given, the first is for the *T. pallidum* N-terminal area of FlaB2 (amino acids 1 to 143) and the second is for the C-terminal area (amino acids 206 to 286) (Fig. 8).
[d] Based on N-terminal amino acid sequences (19 to 30 amino acids).
[e] Homologous proteins not yet identified.

mic membrane or outer membrane protein genes, indicating once again the importance of developing such procedures.

Multiple lipoproteins of *T. pallidum*. In the process of characterizing hydrophobic, membrane-associated proteins of *T. pallidum*, it has been shown that a large number of these proteins are lipidated (Table 3). Thus far, eight major *T. pallidum* subsp. *pallidum* proteins have been identified as lipoproteins, on the basis of the globomycin-sensitive incorporation of radiolabeled fatty acids either in *T. pallidum* or in *E. coli* recombinants containing the *T. pallidum* genes (1, 33, 34, 90, 171, 190, 213). The gene sequences of seven of these proteins have been reported (1, 71, 88, 171, 190, 214, 238, 247). The 5′ region of each gene encodes a hydrophobic, 17- to 21-amino-acid signal peptide ending with a 4- or 5-amino-acid motif (L-X-Y-C, S-X-Y-C, or L-X-Y-Z-C) resembling the signal peptidase II recognition sequence (51, 110, 246) exemplified by the murein lipoprotein of *E. coli* (Table 3). Acid or alkaline hydrolysis of *T. pallidum* lipoproteins labeled with [^{3}H]palmitate resulted in the release of radiolabeled palmitate (33, 213). These results are consistent with the signal peptidase II-mediated lipidation pathway, involving the addition of fatty acids to the cysteine at the cleavage site through an alkali-labile amide linkage, as well as the formation of an acid-labile diacylglyceryl thioester bond (51). Schouls et al. (191) showed that conversion of the cysteine codon in the signal sequence to a serine in the *tmpA*, *tmpC*, and *tpD* genes resulted in the loss of lipidation; "grafting" the *tmpA* signal sequence onto the *tmpB* gene resulted in acylation of the ordinarily nonlipidated product. These findings confirm a signal peptidase II-like mechanism.

The *tpn47* gene was originally thought to lack a signal peptide sequence (88). Reanalysis of the 5′ sequence (238) revealed the presence of an additional nucleotide (an added G between nucleotides 132 and 133 in the previously published sequence [88]), changing the deduced N-terminal sequence to that shown in Table 3. The remaining sequence beyond nucleotide 133 is correct and in the proper reading frame, as demonstrated by sequence analysis of several tryptic and CNBr cleavage fragments (88). The putative start codon is the alternative *N*-formylmethionine codon GTG, which lies 7 nucleotides downstream from a consensus ribosome-binding site sequence (238).

Lipoprotein activities and structural locations. The gene for a 38,000-molecular-weight lipoprotein corresponding to TpN39(a) has been cloned and sequenced by Becker and Norgard (21). Homolog proteins have been detected in *T. phagedenis* biotype Reiter, *T. denticola*, and *T. refringens* by Western blot analysis (21). The deduced amino acid sequence of this protein has regions of sequence identity with the galactose/glucose-binding protein (MglB) of *E. coli*,

TABLE 3. Membrane-associated polypeptides of *T. pallidum*

Protein type and designation	M_r[a]	Deduced MW[b]	N-terminal sequence[b]	Reference(s)
Lipoproteins				
TpN47 (47K)	47,000	46,740	MKVKYALLSAGALQLLVVG↓CG . . .	34, 88, 238
TpN44.5a (TmpA)	42,000	42,000	MNAHTLVYSGVALACAAMLGS↓CA . . .	71, 190, 247
TpN39 (a)	38,000	36,194	MKENSCTACSRRLALFVGAAVLVVG↓CS . . .	21, 81
TpN35 (TmpC)	35,500	35,600	VREKWVRAFAAVFCAMLLIG↓CS . . .	90, 191
TpN29-35 (34K, TpD)	30,000–38,000	22,087	MKRVSLLGSAAIFALVFSA↓CG . . .	190, 213–215
TpN24-28 (TpE)	24,000–30,000	ND[c]		10, 229
TpN17 (Tpp17)	17,000	14,438	MKGSVRALCAFLGVGALGSAL↓CV	1, 33
TpN15 (Tpp15)	15,000	13,967	MVKRGRFALCLAVLLGA↓CS . . .	170, 171
E. coli lipoprotein	7,200	6,372	MKATKLVLGAVILGSTLLAG↓CS . . .	246
Nonlipidated memrane proteins				
TpN39b (basic membrane protein)	38,000	37,880	MGRYIVPALLCVAGMGFAHA↓QSALQPIAD	48[d]
TpN36 (TmpB)	34,000	37,000	MKTRNFSLVSALYVLLGVPLFVSAAS . . .	71, 189, 190, 247

[a] Relative molecular weight based on SDS-PAGE.
[b] Deduced molecular weights of mature polypeptides (without lipid moieties) and N-terminal sequences, based on DNA sequence data, for polypeptides whose gene sequence has been determined; the *E. coli* lipoprotein sequence is included for comparison. Putative recognition sequences (boldface) and cleavage sites (arrows) for signal peptidase I (TpN36) and signal peptidase II (lipoproteins) are indicated for the N-terminal sequences.
[c] ND, not determined.
[d] Cleavage site confirmed by N-terminal amino acid sequence (48).

a periplasmic protein involved in transport and chemotaxis (21). Hindersson et al. (81) have independently cloned and sequenced an *mglB* homolog (81), and it is likely that this is the same gene described by Becker and Norgard (21). Further study of this *T. pallidum* gene may elucidate hitherto unknown mechanisms of transport and chemotaxis in this organism.

The functions and structural locations of the other *T. pallidum* lipoproteins are unknown. None of these proteins have significant homology with other bacterial or eucaryotic genes in GenBank or the other sequence data bases. Except for the N-terminal signal peptides, the protein sequences are hydrophilic, indicating that their hydrophobic (e.g., detergent phase-partitioning) characteristics are due entirely to lipidation. Thus it is likely that the lipoproteins are anchored into the *T. pallidum* membranes via the lipid tail, similar to other bacterial lipoproteins. Four possible locations exist, on the inner or outer surfaces of the cytoplasmic and outer membranes. Because of the current inability to obtain intact, purified inner and outer membranes, it has not been possible to determine conclusively whether any of the lipoproteins are associated with the outer membrane. However, the results of the detergent solubilization and freeze-etching studies suggest that they are predominantly cytoplasmic membrane constituents.

The lipoproteins of *T. pallidum* may have significant immunomodulatory effects. Radolf et al. (179) found that Triton X-114 extracts enriched for lipoproteins stimulated the expression of tumor necrosis factor by murine macrophages. Riley et al. (182) showed that similar extracts and purified TpN47 activated endothelial cells. The lipidated form of TpN17 has been shown to activate macrophages, whereas the nonlipidated protein did not (1). Thus the lipoproteins may be important in host cell activation and may stimulate local, nonspecific inflammatory responses.

Other membrane-associated proteins. Two nonlipidated, hydrophobic membrane proteins have been thoroughly characterized: TpN39b (basic membrane protein) and TpN34 (TmpB) (Table 3). The basic membrane protein was so named because of its high positive charge and hydrophobic nature. The complete gene sequence of basic membrane protein was determined by Dallas et al. (48). The deduced N-terminal sequence contains a signal peptide sequence and a consensus cleavage site, consistent with processing by signal peptidase I (Table 3). The *tmpB* open reading frame (247) overlaps with the 3′ encoding region of *tmpA* by one nucleotide, and the gene pair is expressed as a polycistronic mRNA from a single RNA transcription initiation site (71, 190, 247). A cluster of overlapping genes present in the nonpathogen *T. phagedenis* which closely resemble *tmpA* and *tmpB* were recently identified, cloned, and sequenced (247). A high degree of sequence identity exists between the *T. pallidum* and *T. phagedenis* versions of these genes (247), indicating a closer relationship between these organisms than shown by DNA-DNA hybridization and percent G+C content (119, 159b).

Several fibronectin-binding proteins have been described (16, 18, 19, 165, 168), some of which are proposed to be surface localized and implicated in the attachment of *T. pallidum* to mammalian cells (2, 16, 18, 61, 168, 202). The genes encoding two of these proteins, P1 and P2, have been cloned in *E. coli* (168). One of the fibronectin-binding proteins (P1) appears to be the same as TpN83, which is also the prime candidate for the intracellular cytoplasmic filament subunit (126, 148, 160). If this identification is correct, it is unlikely that TpN83 could serve as a surface-localized

fibronectin receptor. A recombinant DNA vector expressing another *T. pallidum* polypeptide (P2*) that comigrates with P2 has also been described (167). Further studies are required to determine the structural locations of these proteins and their functional roles.

Other *T. pallidum* Polypeptides

Tpn19 (4D antigen). The 4D antigen (also known as TpF1 [229]) was one of the first *T. pallidum* proteins cloned in *E. coli* (229, 230) and is one of the best characterized. The native 4D molecule has an M_r of ~190,000 and consists of a ring-like structure 6 nm in diameter formed of ~10 identical 19-kDa subunits (52, 54). Sequence data for the 4D gene have been obtained for *T. pallidum* Nichols (141, 231) and for several other isolates that cause syphilis and yaws (141, 142). The sequences examined differed by at most one nucleotide, resulting in one amino acid difference, underscoring the extreme relatedness among this group of organisms. The deduced amino acid sequence contains two cysteine residues (141, 231), which are presumably involved in interchain disulfide bonds whose reduction is required for dissociation of the oligomeric structure (173). The ring structure of the 4D molecule at least superficially resembles that of the chaperone GroES (74, 139), but there is no sequence similarity between the two proteins. Although postulated to be surface localized (175), the 4D molecule sediments as a distinct moiety (separate from putative membrane proteins) on sucrose density gradients (Fig. 6); therefore it does not seem to be an integral membrane protein. The function of the 4D antigen is not known, and to date homologous sequences have not been identified in other organisms.

TpN60 (GroEL, common antigen). A major 60-kDa polypeptide (TpN60) is one of the most abundant proteins in *T. pallidum*, representing ~6% of the total protein content (148). TpN60 cross-reacts immunologically with similar proteins in a wide variety of bacteria, leading to its description as the common antigen (78, 80). The native protein is a ring-like structure with a molecular weight of approximately 800,000 (84). Structural, antigenic, amino acid sequence (84), and DNA sequence (76) data indicate that TpN60 is homologous to the GroEL protein of *E. coli*, a ubiquitous heat shock protein involved in protein processing and assembly (73, 139). Interestingly, *T. pallidum* seems to lack a detectable heat shock response, as exemplified by the absence of a significant amplification of TpN60 or other proteins at elevated temperatures (147, 197). This finding correlates with the lack of consensus binding sites for σ^{32} (76), a transcription initiation factor involved in enhanced expression of heat shock proteins (139). TpN71, which reacts specifically with antiserum against the *E. coli* heat shock protein DnaK, is also consistently expressed at high levels (148). The need for a heat shock response may be overriden by a constitutively high expression of TpN60 and other members of this family; the proportion of TpN60 in *T. pallidum* is over three times that of GroEL in *E. coli* grown at 37°C (139, 148). Alternatively, the fastidious nature and temperature sensitivity (57, 222) of *T. pallidum* may be due in part to a deficiency in the heat shock response.

Cytoplasmic filaments. As noted above, preliminary evidence indicates that the cytoplasmic filaments of *T. pallidum* are composed of the polypeptide TpN83. Masuda and Kawata (126) described the purification and characterization of the cytoplasmic filaments of *T. phagedenis* and other cultivable treponemes. The cytoplasmic filaments of the organisms are composed of a single polypeptide with an M_r of 82,000 whose gel migration properties and abundance are very similar to those of TpN83 (Fig. 3A and C). Thus it seems likely that TpN83 is the cytoplasmic filament subunit of *T. pallidum*. The function of these intriguing structures is not known, but their close juxtaposition to the periplasmic flagella suggests that they may serve some function in motility. Further analysis of composition and function of these unique intracellular filaments is warranted.

Penicillin-binding proteins. Isolation of the peptidoglycan sacculus of *T. pallidum* was recently reported (177), and several penicillin-binding proteins (of 94, 80, 63, and 58 kDa) have been identified (46, 177). The penicillin-binding proteins appear to be associated with the cytoplasmic membrane and are most probably involved in peptidoglycan synthesis.

Extracellular proteins. Stamm et al. (195, 198) found that *T. pallidum* subsp. *pallidum* Nichols and *T. pallidum* subsp. *pertenue* Gauthier released at least four low-molecular-weight polypeptides into the medium during in vitro incubation. These polypeptides, with M_rs of 10,000, 14,000, 15,500, and 17,000, were radiolabeled in the presence of [^{35}S]methionine; incorporation was blocked by addition of chloramphenicol, showing that the proteins were synthesized by treponemes rather than by contaminating host cells. The extracellular proteins were present in very small quantities but were clearly demonstrable by radioimmunoprecipitation with rabbit anti-*T. pallidum* serum, SDS-PAGE, and fluorography. Most sera from patients beyond the primary stage of syphilitic infection contained antibodies that precipitated these proteins, consistent with the production of these proteins during human infection and the elicitation of a specific host response. Hsu et al. (89) also reported that small quantities of a 24-kDa *T. pallidum* polypeptide were secreted by *E. coli* recombinants containing the *tpn24(c)* gene. At present, no functional properties for these extracellular proteins have been identified, nor has it been shown conclusively that they are released by a specific secretory mechanism. However, it is possible that such proteins possess enzymatic activities or are factors involved in invasion or pathogenesis.

Enzymatic Activities

There is clearly a deficiency in our knowledge of the functional activities of *T. pallidum* polypeptides. However, a number of enzymatic activities, which should eventually be attributable to certain proteins, have been described (42). In terms of catabolic activities, Cox and coworkers (42, 115, 116, 187) identified many *T. pallidum* enzyme activities associated with the Embden-Meyerhoff-Parnas pathway, the hexose monophosphate shunt, and a terminal electron transport chain; interestingly, most Krebs cycle activities were not detectable, indicating that other means of generating NADH for oxidation may be active. Flavoproteins and cytochromes *b*, *c*, and *o* were also present (115, 116). Despite its reluctance to grow in vitro, *T. pallidum* is fully capable of DNA, RNA, and protein synthesis and therefore must possess the requisite enzyme activities. Although the presence of catalase in *T. pallidum* prepared from rabbits has been reported (7), Steiner et al. (203) showed that the catalase detected was mostly probably of rabbit origin.

Gherardini et al. (65) used gene complementation to isolate a *T. pallidum* recombinant DNA segment which complemented *E. coli* proline auxotrophs. The cloned segment was found to be a homolog of the *proC* gene (65). A *T.*

pallidum homolog of the *serB* gene was also isolated by the same approach (163). Further analysis of the genetic content of *T. pallidum* by gene complementation and other techniques will help delineate its metabolic and functional activities and deficiencies.

Stamm et al. (200) were unable to detect the recombination-mediating protein RecA in *T. pallidum* and *B. burgdorferi* by immunoprecipitation with antibodies against *E. coli* RecA, although the same antiserum detected RecA in *Leptospira interrogans*. Further studies are needed to substantiate this unusual finding. However, it is possible that these organisms are deficient in homologous recombination and DNA repair mechanisms, which may account in part for their obligate parasitism, fastidious nature, and apparent genetic stability.

It has long been noted that the extracellular matrix increases in quantity during syphilitic infection, particularly in experimentally infected rabbits (222, 225, 226). It has been hypothesized that this material represents a capsular substance that is produced by *T. pallidum* and that protects it from the host response. Wos and Wicher (245) found that the mucoid fluid present in infected rabbit testes is virtually indistinguishable from extracts from normal testes in terms of protein and carbohydrate content, although uronic acid levels were elevated slightly. Antigenic analysis indicated the presence of host proteins, but treponemal antigens were not detected (245). Strugnell et al. (208, 209) showed that synthesis of glycosaminoglycans in slices of infected testes was blocked by inhibitors of eucaryotic metabolism but was unaffected by procaryotic inhibitors. Therefore the accumulation of this substance in lesions apparently represents a host reaction rather than a treponemal activity. Fitzgerald et al. (58, 60, 62) showed that *T. pallidum* preparations contain hyaluronidase, but it is unclear whether the activity is produced by the bacterium or the host (60).

IMMUNE RESPONSE TO *T. PALLIDUM* PROTEINS

Antibody Responses

Serum immunoglobulin G (IgG) and IgM responses to *T. pallidum* proteins in syphilis patients and in experimentally infected animals have been studied extensively by both Western blot (12, 41, 50, 68, 69, 105, 112, 113, 153, 227, 242, 243) and radioimmunoprecipitation (4, 17, 18, 133, 195, 198, 218) techniques. Although it is difficult to correlate the results of these studies precisely, the following general observations appear to be consistent. Normal human sera often contain small amounts of antibody reactive with *T. pallidum* polypeptides, particularly TpN47 and some of the flagellar proteins (most notably TpN33 and TpN30) (12, 68). Both IgM and IgG anti-*T. pallidum* antibodies are present during active primary and secondary syphilis, but IgM reactivity diminishes during later stages of the disease and also following treatment. These early IgM and IgG responses appear to recognize roughly the same group of treponemal proteins; no consistent differences in the reactivity pattern of the two Ig isotypes have been observed. During symptomatic primary syphilis (chancre development), the earliest responses detected by Western blotting are against TpN47 and the flagellins, including TpN37, TpN33, and TpN30. Interestingly, Baker-Zander et al. (12) found that anti-TpN47 IgG and anti-TpN83 IgM antibodies were clearly detectable in contacts of syphilis patients who had not themselves developed clinical symptoms; therefore humoral immune responses are apparently activated prior to clinical disease, contrary to the concept of a "slow" immune response to syphilitic infection. Serum samples from patients with secondary and early latent syphilis contain antibodies reactive with most, if not all, of the major polypeptides of *T. pallidum*, with the greatest Western blot reactivity being observed against TpN47, TpN44.5a, TpN37, TpN34.5, TpN33, TpN30, TpN17, and TpN15. Overall reactivity is decreased in sera from untreated late latent (>2 years duration) and late syphilis patients, as reflected in highly variable immunofluorescence titers (1:8 to 1:256) (12) and differences in the reactivity patterns observed with individual sera. However, some IgG reactivity remains and is responsible for the serofast nature of treponemal tests compared with nontreponemal (cardiolipin) tests for syphilis (223). Similar results have been obtained in time course experiments with rabbits, guinea pigs, and hamsters infected with *T. pallidum* subsp. *pallidum* or other *T. pallidum* subspecies (4, 64, 67, 112, 113, 239, 242, 243). Successful treatment of syphilis causes a gradual diminution of anti-*T. pallidum* antibodies, particularly the IgM response (113). Strugnell et al. (212) found that the presence of antibodies against a 45-kDa antigen (most probably TpN44.5a or TmpA) correlates with the development of resistance to reinfection in *T. pallidum*-infected rabbits.

Similar antibody responses have been shown to occur in response to infection by *T. pallidum* subsp. *pertenue* (yaws) (13, 141, 218) and *T. carateum* (pinta) (64). Sera from patients with these infections or rabbits infected with *T. paraluiscuniculi* react with a large number of *T. pallidum* subsp. *pallidum* polypeptides, indicating a high degree of antigenic conservation among these organisms (13, 64, 141, 218). In addition, Baker-Zander and Lukehart (14) have shown that rabbit antisera against *S. hyodysenteriae*, *L. interrogans*, and *B. hermsii* contained antibodies which reacted prominently with a smaller number of *T. pallidum* polypeptides, most notably those corresponding to the flagellins and the GroEL homolog TpN60. Interestingly, antisera against a virulent strain of *S. hyodysenteriae* also reacted with a prominent 48-kDa *T. pallidum* protein (corresponding to TpN47), whereas antiserum against a nonvirulent strain did not (14). The possibility that virulent *S. hyodysenteriae* contains a protein which is antigenically cross-reactive with TpN47 is of interest, because TpN47 is thought to be a pathogen-specific polypeptide of *T. pallidum* and closely related species and subspecies (122, 143).

The Western blot and radioimmunoprecipitation results are, of course, affected by the amount of each antigen present in the immunoblots and solubilized *T. pallidum* used for immunoprecipitations, and they tend to favor the detection of antibodies against abundant proteins. Therefore, antibody responses against relatively minor polypeptides may be of equal importance in immunodiagnosis and the development of immunologic resistance but may not detected by these procedures.

Use of Polypeptides for Immunodiagnosis

Commercially available treponemal tests (i.e., assays for detection of anti-*T. pallidum* antibodies) use either intact or sonically disrupted *T. pallidum* as the source of antigen. In recent years, there has been considerable interest in the development of immunoassays that use either treponemal structural components or individual *T. pallidum* polypeptides produced by recombinant DNA techniques (reviewed in references 94 and 188). A list of such assays is provided in Table 4.

TABLE 4. Immunoassays for anti-*T. pallidum* antibodies by using treponemal structural components or polypeptides as antigens

Antigen	Assay type[a]	Reference(s)
Assays using structural components		
T. phagedenis flagella	EIA	205, 206
T. phagedenis rRNA	EIA	204
T. phagedenis 37K flagellin (FlaA)	RIA	20
Fibronectin-binding proteins P2 and P3	EIA	132
Fibronectin-binding proteins	Fibronectin capture EIA	5
Assays using recombinant DNA products or monoclonal antibodies		
TpN38	RIA	184
TpN19 (4D)	EIA	176
TpN44.5a (TmpA)	EIA	91
TpN47 or TpN44.5a (TmpA)	Monoclonal antibody binding inhibition	92
TpN35 (TmpC)	EIA	191

[a] EIA, enzyme immunoassay; RIA, radioimmunoassay.

Several structural components and polypeptides have been tested as antigens in enzyme-linked immunosorbent assays (ELISAs) or radioimmunoassays, including purified *T. phagedenis* biotype Reiter flagella (205, 206); fibronectin-binding proteins (5, 132); the proteins TpN44.5a (TmpA) (91), TpN38 (184), TpN35 (TmpC) (189), and TpN19 (4D) (176); and even *T. phagedenis* rRNA (204). With the exception of the TmpC and rRNA assays, they have been reported to have a high overall sensitivity (≥91%) and specificity (≥98%). Although sensitivities of these assays were generally lower than that of the fluorescent treponemal antibody-absorbed (FTA-ABS) test in cases of untreated primary syphilis, tests for detecting IgM antibodies (such as the Reiter flagellum IgM ELISA [205]) can be equally sensitive in verifying early cases of infection.

Ijsselmuiden et al. (92) described a unique monoclonal antibody inhibition ELISA for detection of anti-*T. pallidum* antibodies. In this system, antibodies present in the serum of syphilis patients block the binding of horseradish peroxidase-conjugated anti-TpN47 or anti-TpN44.5a monoclonal antibodies to *T. pallidum*-coated wells, thus decreasing the amount of peroxidase substrate converted to a colored reaction product. When tested in parallel with FTA-ABS and microhemagglutination-*T. pallidum* (MHA-TP) assays with fresh serum samples, the sensitivity of the monoclonal antibody inhibition assay was unfortunately much lower (64%) than those of the conventional assays (86 and 92% respectively).

Because of the diagnostic dilemmas presented by congenital syphilis and early primary syphilis, some researchers have advocated the use of Western blotting techniques for the diagnosis of problem cases (50, 74, 105, 186). In particular, reactivity with bands corresponding to TpN47, TpN17, and TpN15 appears to correlate well with syphilitic infection and provides a low level of false-positive reactions. Lewis et al. (105) found that an IgM-specific Western blot technique yielded positive reactions (as judged by the presence of at least five distinct bands) in 19 of 21 patients with symptomatic congenital syphilis and 11 of 15 asymptomatic at-risk infants compared with 16 of 21 and 9 of 15, respectively, for an IgM-specific FTA-ABS test. Sanchez et al. (186) obtained similar Western blot results, with reactivity against TpN47 being consistently present in the IgM fraction of sera from 12 of 12 symptomatic patients with congenital syphilis and 2 of 9 asymptomatic infants of mothers with active syphilis. The IgM Western blot reactivity of cerebrospinal fluid (CSF) samples also correlated well with the CSF-VDRL test (105), which at present is the only standardized serologic test available for diagnosis of neurosyphilis (223).

As another adjunct to diagnosis, monoclonal antibodies against *T. pallidum* proteins have been shown to be useful in the specific detection of treponemes in tissue samples and exudates (96, 114, 145).

Cellular Responses to *T. pallidum* Proteins

T-cell responses are undoubtedly important in the development of protective immunity to *T. pallidum* (as reviewed in references 146 and 192). In 1988, Baker-Zander et al. (11) demonstrated that *T. pallidum*-infected rabbits exhibited specific lymphocyte proliferation responses to *T. pallidum* polypeptides. SDS-PAGE bands corresponding to TpN47, TpN37, TpN34.5, TpN33, TpN30, TpN17, and TpN15 (the last two called 14 kDa and 12 kDa [11]) were electroeluted and used as stimulating antigens in an in vitro assay with [^{125}I]iododeoxyuridine incorporation to detect proliferation. Rabbit splenic lymphocytes were tested for reactivity 6, 10, 17, 30, and 210 days post-intratesticular infection with *T. pallidum*. Responses above background were observable as soon as 6 days postinfection, at the same time that anti-*T. pallidum* immunoglobulins were first detectable. Lymphocyte proliferation in response to TpN37, TpN30, and TpN17 was consistently high 10 to 210 days postinfection, whereas responses against TpN47 and the TpN34.5 and TpN33 doublet were variable, yielding a low level of incorporation 30 days postinfection. TpN15 provided a high degree of stimulation only after 210 days. In a separate study, Borenstein et al. (27) showed that splenic lymphocytes from rabbits immunized 6 to 10 months previously with the recombinant antigen 4D (TpN19) had strong proliferative responses to the antigen in vitro; this cellular reactivity may correlate with the altered course of *T. pallidum* lesion development seen in 4D-immunized animals (see the section on vaccination, below). Further analysis of the cellular responses to *T. pallidum* antigens should shed light on both the importance of T-cell reactions and the relative roles of individual polypeptides in the stimulation of protective immunity.

T. pallidum Polypeptides as Vaccinogens

One of the goals of *T. pallidum* research is to determine the potential efficacy of vaccination in the prevention of syphilis (111, 193). Immunization of rabbits with nonviable *T. pallidum* cells has led to demonstrable protection against experimental infection in only two studies, one using *T. pallidum* inactivated by gamma irradiation (129) and the other using treponemes rendered noninfectious by prolonged storage at 4°C (128). Both procedures required large numbers of organisms and repeated injections, rendering these approaches impractical for human use. Fitzgerald (59) reported in 1991 that treatment of rabbits with immunomodulatory agents (cyclophosphamide, monophosphoryl lipid A, trehalose dimycolate, and indomethacin) enhanced the efficacy of a single vaccination with heat-inactivated *T. palli-*

TABLE 5. Effects of immunization with treponemal structural components and recombinant-derived proteins on the course of experimental syphilis

Immunizing antigen	Animal model	Effects on lesion development following infection with *T. pallidum*[a]	Reference(s)
TpN19 (4D)	Rabbit	Accelerated lesion development; atypical lesions; DF-negative lesions	27
T. pallidum subsp. *pallidum* endoflagella	Rabbit	Accelerated lesion development; atypical lesions; DF-negative lesions	36
TpN36 (TmpB)	Guinea pig (C4D Strain)	Reduced lesion severity and duration; decreased numbers of *T. pallidum*; DTH reaction	240
TpN36 (TmpB)	Guinea pig (Strain 2)	No effect	241
TpN44.5a (TmpA)	Guinea pig (strain 2 and C4D strain)	No effect	240, 241
TpN35 (TmpC)	Guinea pig (strain 2 and C4D strain)	No effect	240, 241
T. phagedenis biotype Reiter endoflagella	Rabbit	No effect	79
TpN60	Rabbit	No effect	84
TpN24(c)	Rabbit	No effect	89

[a] Accelerated lesion development: mean incubation period before occurrence of lesions was reduced (TpN19 immunized = 9.6 days, versus 15.1 days for nonimmunized controls [27]; endoflagella immunized = 8.0 days versus 13.6 days for nonimmunized controls [36]). Atypical lesions: flat, nonindurated. DF-negative lesions: spirochetes not detected by dark-field microscopy in needle aspirates of lesions. DTH: delayed-type hypersensitivity (erythema and induration within 24 to 48 h).

dum cells, resulting in reductions in the severity of dermal lesions and in the dissemination to other tissues; he suggested that this effect was due to the inhibition of suppressive activity (59).

The advent of molecular biological techniques and advances in subcellular fractionation of *T. pallidum* and related organisms have introduced the possibility of vaccination with relatively large quantities of polypeptides and purified structural components. A summary of publications to date involving immunization with recombinant DNA-derived polypeptides, endoflagella, and other proteins is provided in Table 5.

Immunization with TpN19. Borenstein et al. (27) provided a thorough examination of immunogenicity of the *T. pallidum* antigen 4D (TpN19) in the rabbit model. The oligomeric protein was produced in an *E. coli* recombinant and purified to homogeneity by combination of ammonium sulfate precipitation with ion-exchange and gel filtration chromatography. Three immunization protocols were tried: intramuscular injections with Freund's adjuvant (500, 500, and 250 μg of antigen in three sequential injections); combined intramuscular and intravenous immunization (1 mg of 4D antigen in incomplete Freund's adjuvant followed by five intravenous injections with increasing dosages of antigen); and intravenous immunization with 4D antigen with a Ribi adjuvant, consisting of cell wall skeleton and trehalose dimycolate constituents from the BCG bacterium (three injections, each containing 200 μg of 4D). All immunized rabbits had high titers of anti-4D antibodies. Rabbits immunized with 4D-Ribi adjuvant combination were tested for cell-mediated reactivity and exhibited a strong in vitro lymphocyte proliferative response to 4D. To determine their resistance to infection, rabbits were challenged with 10^3 *T. pallidum* at four intradermal sites.

The most promising results were obtained with the combined intramuscular and intravenous injection protocol and the Ribi adjuvant protocol. Although *T. pallidum* infection was not prevented, the course of lesion development was altered. In comparison with nonimmunized or adjuvant-immunized controls, lesions were atypical, i.e., smaller, flattened, and relatively nonindurated; also, fewer sites underwent ulceration. In the rabbits immunized intravenously with 4D antigen and Ribi adjuvant, lesion development was accelerated. The incubation period was 9.6 days compared with 13.2 days for controls. No *T. pallidum* cells were detected in needle aspirates of lesions 14 days postinfection in the immunized animals, but organisms were observed at 20 days. Surprisingly, anti-4D IgG titers actually decreased following challenge with *T. pallidum*.

Immunization with the 4D antigen, although not completely protective under the conditions tested, has a significant impact on the course of lesion development. The accelerated appearance of lesions has been observed in previous immunization experiments (192) and may be due to an enhanced cellular immune response (27).

Vaccination with flagellar antigens. Champion et al. (36) determined the effects of immunization with purified *T. pallidum* endoflagella. Rabbits were given intramuscular and subcutaneous injections of 100 μg of endoflagella emulsified in complete Freund's adjuvant, and booster doses were administered seven times at 4-week intervals with 50 μg of antigen in incomplete Freund's adjuvant. Sera from the animals contained a high titer of anti-endoflagellar IgG as determined by ELISA and also exhibited some complement-dependent treponemicidal activity in the *T. pallidum* immobilization assay. On intradermal challenge with *T. pallidum*, lesions developed at all inoculation sites. However, lesion development occurred earlier in some immunized animals and was atypical compared with controls; this is similar to what had occurred with 4D-immunized rabbits (27). In addition, motile treponemes were not detected in needle aspirates of seven of eight lesions examined, indicating that smaller numbers were present than in similar sites in nonimmunized animals. These results are consistent with incomplete protection coupled with a accelerated cellular reaction.

Hindersson et al. (79) attempted to vaccinate rabbits with purified endoflagella from *T. phagedenis* biotype Reiter, using three weekly subcutaneous injections of 50 μg of purified flagella in incomplete Freund's adjuvant. The time and severity of lesion development following intradermal infection were unaltered compared with those in control rabbits. It is unclear whether the different results obtained with Reiter preparation compared with the *T. pallidum* endoflagella was due to antigenic differences or to variations in the immunization protocols. The latter possibility seems more likely, since the flagellins of the two organisms are

antigenically cross-reactive and have a high degree of sequence identity (37, 93, 95, 107, 108, 150, 158, 172).

Immunization with TmpA, TmpB, and TmpC. Schouls and colleagues (190, 240, 241) have investigated the immunogenicity of three *T. pallidum* membrane-associated polypeptides in the guinea pig model. When crude fractions of TmpA and TmpB or purified TmpC were used for immunization of inbred strain 2 guinea pigs, no protection was observed (241). More promising results were obtained with immunization of strain C4D guinea pigs (240), which are deficient in the complement component C4 and are more susceptible to *T. pallidum* infection. C4D guinea pigs were immunized at monthly intervals with six 100-μg doses of the partially purified recombinant proteins; in addition, the antigens used were more highly purified, and the Ribi adjuvant used contained mycobacterial cell wall skeletons as well as the monophosporyl lipid A and trehalose dimycolate components used in the earlier study (241). The challenge dosage was 3×10^6 *T. pallidum* cells. All animals immunized with TmpA, TmpB, or TmpC developed a delayed-type hypersensitivity reaction within 24 h at the site of *T. pallidum* injection, indicative of an active T-cell response. Vaccination with recombinant TmpB resulted in reduction of the size and severity of the *T. pallidum* lesions. Of 10 TmpB-immunized animals, 2 did not develop lesions (beyond the initial delayed-type hypersensitivity reaction), 5 had induration only, and 3 developed induration and ulceration. In contrast, all 10 unimmunized control animals developed long-lasting ulcerative lesions. The numbers of treponemes observed in histologic sections by silver staining were also reduced in the four TmpB-immunized animals examined. As in the previous study (241), immunization with TmpA or TmpC had little effect on the *T. pallidum* infection (240). Thus TmpB seems to induce partial protection in this model, although further studies are needed to show whether symptomatic, latent, and systemic infection can be prevented consistently.

Immunization with other proteins. In a limited study involving two rabbits, Houston et al. (84) found that immunization with TpN60 had no effect on the course of *T. pallidum* infection. TpN24(c) was also nonprotective in a similar experiment (89).

Prospects for vaccine development. Immunization with recombinant-derived polypeptides or treponemal structural components warrants further investigation, inasmuch as altered lesion development occurred in studies in which the 4D antigen, *T. pallidum* endoflagella, or TmpC was used as the immunogen. However, these observations must be interpreted with caution, because the changes conceivably could be due solely to differences in the immunopathologic reaction without significant inhibition of *T. pallidum* growth and dissemination. For instance, more accurate indicators of the numbers of organisms per site should be monitored in future experiments in which apparent partial or complete protection is observed. In addition, distant sites (e.g., lymph nodes) should be examined for the presence of infectious treponemes.

Why is artificial immunization against *T. pallidum* infection so difficult, and how can the outcome be improved? Several possibilities exist. On the basis of recent in vitro immobilization studies, Blanco et al. (25) hypothesize that antibodies against the TROMPs are required for antibody-dependent killing. In their studies, aggregation of TROMPs on exposure to antibodies present in immune serum and extracts from infected rabbit tissue appeared to be necessary for antibody- and complement-mediated immobilization and susceptibility to antibodies against internal antigens. This aggregation, which requires several hours in vitro, may provide the required concentration of IgG-antigen complexes for C1q binding and complement activation. Therefore, expression of anti-TROMP antibodies may be critical in the development of resistance to *T. pallidum* infection.

Other factors which may also affect the efficacy of vaccination include optimization of immunization protocols, generation of cellular as well as antibody responses against the antigen(s), and use of multiple immunogens. One promising approach described by Strugnell et al. (210) is vaccination with attenuated *Salmonella* cells expressing *T. pallidum* antigens. Incorporation of the gene for TpD (TpN28-35) into the chromosomal *aroC* locus of *Salmonella typhimurium* BRD207 *polA* resulted in production of low levels of TpD. Stable expression of *T. pallidum* antigens by an agent capable of persistent colonization may lead to enhanced immunity. In a preliminary study, the immunoprotective effects of intravenous administration of *Salmonella typhimurium* expressing *T. pallidum* polypeptides were examined (211). Immunization with salmonellae expressing TmpA (TpN44.5a), TmpB (TpN34), TmpC (TpN35), or TpD (TpN28-35) caused a 2- to 3-day acceleration of *T. pallidum* lesion development but no protective effect. Immunization with strains expressing TpE (TpN24-28), TpF1 (TpN19), or TpF2 (no designation) had no effect on lesion development (211).

FUTURE DIRECTIONS IN *T. PALLIDUM* POLYPEPTIDE RESEARCH

Much progress has been made during the past decade in terms of the identification of *T. pallidum* polypeptides, production of recombinant DNA reagents and monoclonal antibodies for their study, and characterization of certain subsets, such as the flagellins and membrane-associated lipoproteins. Major areas for future study include (i) further determination of the structural locations and functional activities of *T. pallidum* polypeptides; (ii) isolation and characterization of the outer membrane and cytoplasmic membrane of *T. pallidum*, identification of their associated proteins, and determination of the role of the outer membrane proteins in immunity; (iii) elucidation of important metabolic and biosynthetic pathways and associated enzymes in *T. pallidum*, and their relationship with the unusual physiology of the organism; (iv) continued study of the induction of antibody and cellular responses to *T. pallidum* proteins and the potential for immunoprotection and improved diagnostic techniques; (v) clarification of the roles of toxins, secreted enzymes, adhesins, or other factors in the pathogenesis of *T. pallidum*; (vi) definition of the subtle differences between treponemal subspecies and species that are responsible for the distinctions between venereal syphilis, endemic syphilis, yaws, and pinta; and (vii) further characterization of the nonproteinaceous components of *T. pallidum*, including membrane-associated lipids and cell wall components.

We hope that the next decade of *T. pallidum* research will provide valuable information in each of these areas.

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