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Isolation of the Outer Membranes from *Treponema pallidum* and *Treponema vincentii*

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The outer membranes from *Treponema pallidum* subsp. *pallidum* and *Treponema vincentii* were isolated by a novel method. Purified outer membranes from *T. pallidum* and *T. vincentii* following sucrose gradient centrifugation banded at 7 and 31% (wt/wt) sucrose, respectively. Freeze fracture electron microscopy of purified membrane vesicles from *T. pallidum* and *T. vincentii* revealed an extremely low density of protein particles; the particle density of *T. pallidum* was approximately six times less than that of *T. vincentii*. The great majority of *T. vincentii* lipopolysaccharide was found in the outer membrane preparation. The *T. vincentii* outer membrane also contained proteins of 55 and 65 kDa. ^{125}I-penicillin V labeling demonstrated that *T. pallidum* penicillin-binding proteins were found exclusively with the protoplasmic cylinders and were not detectable with purified outer membrane material, indicating the absence of inner membrane contamination. Isolated *T. pallidum* outer membrane was devoid of the 19-kDa 4D protein and the normally abundant 47-kDa lipoprotein known to be associated with the cytoplasmic membrane; only trace amounts of the periplasmic endoflagella were detected. Proteins associated with the *T. pallidum* outer membrane were identified by one- and two-dimensional electrophoretic analysis using gold staining and immunoblotting. Small amounts of strongly antigenic 17- and 45-kDa proteins were detected and shown to correspond to previously identified lipoproteins which are found principally with the cytoplasmic membrane. Less antigenic proteins of 65, 31 (acidic pI), 31 (basic pI), and 28 kDa were identified. Compared with whole-organism preparations, the 65- and the more basic 31-kDa proteins were found to be highly enriched in the outer membrane preparation, indicating that they may represent the *T. pallidum* rare outer membrane proteins. Reconstitution of solubilized *T. pallidum* outer membrane into lipid bilayer membranes revealed porin activity with two estimated channel diameters of 0.35 and 0.68 nm based on the measured single-channel conductances in 1 M KCl of 0.40 and 0.76 nS, respectively.

The outer membranes of spirochetes, including that of *Treponema pallidum* subsp. *pallidum*, the agent of syphilis, are fragile compared with those of typical gram-negative bacteria (17, 22, 24, 39, 42, 49). Spirochetal outer membranes bleb from the underlying protoplasmic cylinder under relatively mild conditions, including dilute detergents and hypotonic environments (24). Freeze fracture electron microscopy has revealed that the outer membranes of pathogenic spirochetes contain amounts of integral membrane protein that are 1 to 2 orders of magnitude less than those of gram-negative bacteria (45, 54, 55). This striking feature has been suggested to be a key factor in the pathogenicity of these organisms by contributing to their ability to evade the host immune response and to establish chronic infection (11, 15, 38, 45, 54, 55).

The outer membrane of *T. pallidum* has an extremely low content of membrane-spanning protein (45, 55), a finding which has explained the surface antigenic inertness (18, 20, 25, 40, 43) of this spirochete and its relative resistance to bactericidal antibody (9, 20, 32, 33, 43). Freeze fracture electron microscopy has shown that the *T. pallidum* rare outer membrane protein (TROMP) molecules have surface-exposed antigenic sites, evidenced by immune-serum-mediated aggregation of TROMP molecules (11). Immune-serum antibody was also shown to effect the aggregation of TROMP at a time concurrent with complement activation (11), suggesting that TROMP represents key surface-exposed targets for treponemicidal antibody.

There has been a clear need for a means of purification of the *T. pallidum* outer membrane and identification of TROMP as a key step in understanding the molecular basis of syphilis pathogenesis. However, the limited numbers of organisms that can be obtained from infected animals, the potential for contamination of treponemes by rabbit tissue components (2, 4, 6, 14), the fragility of the *T. pallidum* outer membrane (17, 22, 24, 39, 42, 49), and the paucity of TROMP (11, 45, 55) have posed significant difficulties for isolation and purification of the *T. pallidum* outer membrane and identification of its constituent protein.

In this report, we describe a novel method which has resulted in isolation of the outer membrane of *T. pallidum*. The method was also applied to *Treponema vincentii*, a cultivatable treponeme that, unlike *T. pallidum*, has an outer membrane containing lipopolysaccharide (LPS) (27). By this procedure, LPS was released and copurified with the *T. vincentii* outer membrane. The outer membrane isolated from *T. pallidum* did not contain penicillin-binding proteins (PBPs) or the normally abundant 47-kDa lipoprotein (12, 13), indicating the absence of inner membrane contamination. Freeze fracture electron microscopy showed that purified outer membrane vesicles of both *T. pallidum* and *T. vincentii* had extremely low protein particle densities, consistent with outer membrane freeze fracture analysis of each whole organism. Sodium dodecyl

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sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis of outer membrane vesicles from *T. pallidum* and *T. vincentii* showed the presence of relatively few protein species, of which candidate TROMP molecules are described. In addition, reconstitution of detergent-solubilized *T. pallidum* outer membrane into lipid bilayer membranes resulted in porin activity, indicating that at least one TROMP species functions as an outer membrane porin.

MATERIALS AND METHODS

Treponemes. *T. pallidum* subsp. *pallidum*, Nichols strain, was maintained by testicular passage in New Zealand White rabbits as described previously (31). Animals used for *T. pallidum* inoculation were injected intramuscularly with 10 mg of cortisone acetate (Merck, Sharp & Dohme, Rahway, N.J.) per kg of body weight from days 3 through 12 after infection.

T. vincentii was grown at 34°C in spirolate broth (GIBCO, Grand Island, N.Y.) supplemented with 10% heat-inactivated rabbit serum. Approximately 300 ml of culture containing 2×10^8 organisms per ml, enumerated by dark-field microscopy as described previously (31), was centrifuged at 10,000 × *g* for 15 min. The resulting treponemal pellet was resuspended in 140 ml of phosphate-buffered saline (PBS) (pH 7.2) and used for outer membrane isolation.

Purification of *T. pallidum*. A total of 300 ml of PBS (pH 7.2) in 50-ml volumes was used to extract treponemes from the testicles of 10 infected rabbits. The treponemal suspension, containing approximately 6×10^{10} organisms, was subjected to two low-speed centrifugations at 400 × *g* in order to remove gross tissue debris. Treponemes were then pelleted by centrifugation at 20,000 × *g* for 15 min followed by resuspension in 40 ml of PBS containing 0.5% bovine serum albumin (Intergen Co., Purchase, N.Y.) and 7% Ficoll (Pharmacia, Piscataway, N.J.). Ten milliliters (10 ml) of suspension was layered onto 25 ml of a discontinuous Ficoll-PBS gradient with increasing buoyant densities of 1.045, 1.055, 1.065, 1.075, and 1.085 g/ml. After centrifugation at 7,000 × *g* for 15 min, several bands were observed in the gradient. Previous studies using dark-field and electron microscopy of the four interdensity zones have shown "clean" single, motile treponemes within the two upper zones (1.065 to 1.055 and 1.055 to 1.045 g/ml) and some aggregated and single treponemes plus host cell debris within the two lower zones (1.085 to 1.075 and 1.075 to 1.065 g/ml) (30). Only treponemes from the uppermost zone, which were recovered from the gradient by needle aspiration followed by a fourfold dilution in PBS, were used for subsequent experiments. The resulting treponemal suspension was used immediately for extraction of the outer membrane.

***T. pallidum* and *T. vincentii* outer membrane isolation.** A 200-μl volume of 20 μM octadecyl rhodamine B chloride salt (R18) (Molecular Probes, Eugene, Oreg.) was added to 140 ml of treponemal suspension containing approximately 5×10^{10} treponemes. The suspension was incubated at room temperature for 10 min and then centrifuged at 8,000 × *g* for 20 min. For removal of the outer membrane, the treponemal pellet was resuspended in 60 ml of ice-cold 0.05 M sodium citrate buffer (pH 3.2) and incubated on a rocker with occasional vortexing for 1 h at room temperature. The suspension was then centrifuged successively at 2,000, 4,000, and 8,000 × *g* for 15 min each in order to remove and recover treponemal protoplasmic cylinders. The supernatant containing released outer membrane was then adjusted to pH 7.0 with 1 M Tris-HCl (pH 9.0) and centrifuged at 150,000 × *g* for 16 h at 15°C. The resulting membrane pellet was resuspended in 2 ml of PBS, layered onto 36 ml of a continuous 5 to 40% (wt/wt) sucrose-PBS gradient for *T. pallidum* or a 10 to 40% (wt/wt) gradient for *T. vincentii*, and centrifuged at 100,000 × *g* for 16 h at 15°C. Following centrifugation, the membrane band, identified visually by its rhodamine labeling, was needle aspirated, diluted sevenfold with PBS, and recentrifuged at 150,000 × *g* for 5 h. The final purified membrane pellet was resuspended in 100 μl of PBS containing 1 mM EDTA–1 mM phenylmethylsulfonyl fluoride and stored at −70°C.

Electron microscopy and freeze fracture electron microscopy. For electron microscopy, Parlodion (Mallinckrodt, Inc., St. Louis, Mo.) and carbon-coated 300-mesh copper grids (Ted Pella Inc., Tustin, Calif.) were floated for 5 min on 40-μl specimen drops. After three washes in PBS and two washes in doubled-distilled water, the grids were negatively stained with 1% uranyl acetate and examined in an electron microscope (JEOL 100 CX) at 80 kV accelerating voltage. Freeze fracture electron microscopy of outer membrane vesicles was performed as follows. Fifty microliters of membrane suspension was pelleted by centrifugation at 200,000 × *g* for 3 h and resuspended in 1 μl of 20% glycerol in double-distilled water. A 0.5-μl sample was placed on a standard specimen holder (Balzars Co., Redding, Calif.) and frozen by immersion in liquid propane (−190°C) with a gravity freeze plunger. Frozen samples were transferred under liquid nitrogen to the specimen stage of a Balzars 400K freeze fracture apparatus precooled to −150°C. Frozen samples were fractured at −120°C by using a knife cooled at the temperature of liquid nitrogen. The fracture surface was immediately replicated with platinum-carbon at 45° and carbon at 90°. The replicas were floated in 3 to 4% sodium hypochlorite to bleach the organic material and washed three times in double-distilled water. The replicas were then placed on Formvar-coated freeze fracture grids (Ted Pella Inc.) and observed by electron microscopy as described above.

One- and two-dimensional SDS-PAGE and immunoblotting. SDS-polyacrylamide slab gels were run by using the discontinuous buffer system of Laemmli (28). Samples containing 3×10^8 to 5×10^8 whole organisms or 1×10^9 to 5×10^9 treponemal equivalents of membrane material were boiled for 10 min in final sample buffer consisting of 4% SDS, 10% 2-mercaptoethanol, and 0.01% bromphenol blue in 62.5 mM Tris buffer, pH 6.8 (FSB); for some samples, urea at a final concentration of 8 M (FSB-U) was included. In some experiments, samples were solubilized in FSB containing proteinase K (Sigma Chemical Co., St. Louis, Mo.) at a concentration of 100 μg/ml and incubated for 1 h at 37°C before being boiled. Two-dimensional gel electrophoresis was performed as described by O'Farrell (37), with minor modifications. Outer membrane material derived from 5×10^9 to 3×10^{10} treponemes was first solubilized for 1 h at room temperature in lysis buffer containing 9 M urea, 2% Nonidet P-40, and 2% carrier ampholytes at pH 9.5. Isoelectric focusing was carried out for 18 h at a constant voltage of 600 V in polyacrylamide tube gels (0.2 by 12 cm) containing 2% pH 5 to 7 and 0.8% pH 3 to 10 Ampholines (Bio-Rad, Richmond, Calif.), 2% Nonidet P-40, and 9 M urea. The second dimension consisted of standard SDS-PAGE as described above. After electrophoresis, gels were stained with Coomassie brilliant blue or transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, Mass.) as previously described (51). Following transfer, PVDF membranes were stained with 1% amido black or Aurodye Forte (Amersham UK). For immunoblotting, PVDF membranes were incubated for 1 h with serum diluted 1:1,000 in PBS containing 5% nonfat dry milk (Carnation Co., Los Angeles, Calif.) and 0.1% Tween-20 (Sigma Chemical Co.) (MT-PBS). Antibody-antigen binding was detected by using

the enhanced chemiluminescence (ECL) system of Amersham UK. Blots were incubated for 1 h in anti-rabbit immunoglobulin or anti-mouse immunoglobulin conjugated to horseradish peroxidase (Amersham UK) diluted 1:2,500 in MT-PBS. Blots were next washed in PBS containing 0.1% Tween-20, incubated for 1 min in the ECL developing reagents (Amersham UK), and then chemilumigraphed with Kodak X-AR5 film.

Detection of *T. pallidum* PBPs. PBPs of *T. pallidum* were identified by using ^{125}iodine-labeled penicillin V as follows. Sodium *p*-(trimethylstannyl)phenoxyacetamidopenicillin, kindly provided by Larry C. Blaszczak, Lilly Laboratories, Eli Lilly and Company, Indianapolis, Ind., was labeled with Na^{125}I by using chloramine-T as previously described (41). Equal volumes of ^{125}I-penicillin V were combined with 1×10^8 Ficoll-purified treponemes, 1×10^8 protoplasmic cylinders, and outer membrane derived from 5×10^9 treponemes. Suspensions were incubated at room temperature for 30 min prior to centrifugation at 10,000 × *g* for 15 min for whole organisms and protoplasmic cylinders and at 100,000 × *g* for 1 h for outer membrane material. Pellets were resuspended in FSB and electrophoresed under the SDS-PAGE conditions described above. Following electrophoresis, the gel was vacuum dried and then autoradiographed with Kodak X-AR5 film at room temperature for 24 h.

Antisera. Serum from syphilitic rabbits immune to challenge (immune rabbit serum) (IRS) was acquired after 6 months from animals infected intratesticularly with 4×10^7 *T. pallidum* organisms. Preimmune serum was obtained from rabbits prior to infection with *T. pallidum*. Antiserum against the *T. pallidum* recombinant 4D protein has been described previously (43). Monoclonal antibodies against the *T. pallidum* 47-kDa lipoprotein (antibody 11E3) and against the 42-kDa TmpA lipoprotein were kindly provided by Michael V. Norgard, University of Texas (13), and by Jan van Emben and Leo Schouls, National Institute of Public Health and Environmental Protection, Bilthoven, Netherlands (46), respectively. Anti-rabbit serum proteins was purchased from Sigma Chemical Co. Antiserum against *T. vincentii* was generated in rabbits as follows. Approximately 10^9 PBS-washed organisms were disrupted by sonication, combined with Freund's complete adjuvant, and injected both intramuscularly and subcutaneously. After 3 weeks, animals were given intramuscular and subcutaneous booster injections of a similarly prepared suspension in Freund's incomplete adjuvant. Animals were bled 1 week following the booster immunization.

Black lipid bilayer experiments. The pore-forming ability of protein from the *T. pallidum* outer membrane preparation was examined by using planar lipid bilayers (7). Lipid bilayers made from 1.5% (wt/vol) oxidized cholesterol in *n*-decane were formed across a 0.2-mm^2 hole separating two compartments of a Teflon chamber containing a 1.0 M KCl solution. Bilayer formation was recognized by the fact that the membrane appeared black when viewed by incident light. Calomel electrodes were implanted in each compartment, one of which was connected to a voltage source and the other of which was connected to a current amplifier with the output monitored on an oscilloscope. Conductance data were recorded on a strip chart recorder for further analysis. *T. pallidum* outer membrane derived from 5×10^9 treponemes (approximately 2.5 to 10 ng of each outer membrane protein) was solubilized in 0.1% Triton X-100 (Sigma Chemical Co.) before addition to one of the compartments. The pore-forming ability of the outer membrane proteins was then assessed by applying a voltage of 50 mV across the lipid bilayer and measuring increases in conductance. Pore channel diameters were calculated from the measured conductance increases as described previously (7, 19, 26).

RESULTS

Isolation of the *T. pallidum* and *T. vincentii* outer membranes. The key steps in outer membrane isolation were Ficoll density gradient centrifugation to purify *T. pallidum* from rabbit tissue, the use of octadecyl rhodamine B chloride to label membranes, and the selective removal of the outer membrane with 0.05 M citrate, pH 3.2.

Ficoll purification of *T. pallidum* resulted in significant removal of host contaminating proteins, as determined by SDS-PAGE (Fig. 1A) and immunoblotting with anti-rabbit serum proteins (Fig. 1B). Ficoll-purified *T. pallidum* and PBS-washed *T. vincentii* organisms were treated with 0.05 M citrate buffer, which resulted in the release of membrane as monitored by fluorescence microscopy of rhodamine-labeled material (data not shown) and by electron microscopy (Fig. 2B). After 1 h, the majority of treponemes had significantly narrower diameters, consistent with the removal of their outer membranes. The diameters of whole *T. pallidum* and *T. vincentii* organisms (means ± standard deviations) were 0.126 ± 0.01 and 0.166 ± 0.008 μm, respectively. By comparison, the diameters of *T. pallidum* and *T. vincentii* protoplasmic cylinders following outer membrane removal were 0.067 ± 0.006 and 0.071 ± 0.003 μm, respectively. Depolymerization of endoflagellar filaments at pH 3.2 (10) may have also contributed to the release of outer membrane material. Comparison by SDS-PAGE of the protoplasmic cylinders from citrate-treated treponemes with those of whole treponemes showed similar profiles and intensities of stained proteins (data not shown), indicating that treponemes were not disrupted by this procedure. Sucrose gradient purification of membrane material yielded a single rhodamine-labeled band at the densities of 7% sucrose for *T. pallidum* and 31% sucrose for *T. vincentii*, as determined by refractive index analysis (data not shown). The membranous nature of this material was demonstrated by electron microscopy, which showed membrane vesicles that ranged in diameter from approximately 300 to 700 nm (Fig. 2C).

Freeze fracture electron microscopy of membrane vesicles. Purified membrane vesicles were analyzed by freeze fracture electron microscopy in order to determine intramembranous particle composition (Fig. 3). Membrane vesicles from both *T. pallidum* and *T. vincentii* contained extremely few protein particles. Of 200 *T. pallidum* vesicles observed, only 8 were found to have fracture faces containing particles; the number of particles in these fracture faces ranged from one to three. By comparison, of 50 *T. vincentii* vesicles observed, 22 had fracture faces containing at least one particle. Total particle enumeration showed that the membrane particle density of *T. pallidum* was approximately six times less than that of *T. vincentii*. In contrast, the fracture faces of *T. pallidum* and *T. vincentii* protoplasmic cylinder inner membranes (data not shown) and of host tissue membranous material acquired from noninfected rabbits (Fig. 3C) contained a high density of particles (>1,000 particles per μm^2 of membrane fracture face).

Composition of *T. vincentii* outer membrane vesicles. The detection of the *T. vincentii* LPS stepladder by immunoblot analysis of proteinase K-treated membrane, protoplasmic cylinder, and whole-organism fractions was used to assess the efficiency of outer membrane recovery (Fig. 4). The number and intensity of LPS bands detected in outer membrane material derived from 1×10^9 organisms were similar to those detected in 2×10^8 whole organisms. By comparison, proto-

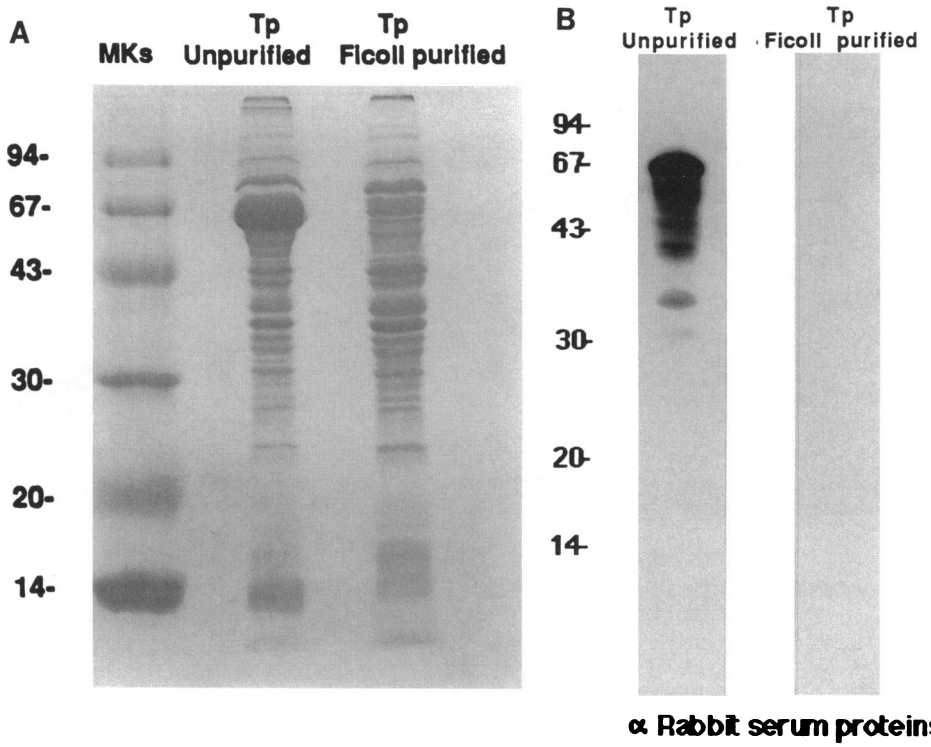

FIG. 1. SDS-PAGE analysis of 5×10^8 unpurified and Ficoll-purified *T. pallidum* organisms. (A) Coomassie-stained gel. (B) Immunoblots probed with anti-rabbit serum proteins. Molecular weights (in thousands) of marker standards (MKs) are indicated on the left.

plasmic cylinders derived from 2×10^8 organisms showed a marked decrease in the number and intensity of the LPS stepladder bands. These results indicate that approximately 20% of the *T. vincentii* outer membrane was recovered and that the vast majority of the LPS-containing outer membrane was released by treatment with 0.05 M citrate, pH 3.2.

Immunoblot analysis (Fig. 4) and Coomassie-stained SDS-PAGE (data not shown) of the proteinase K-untreated *T. vincentii* outer membrane also revealed two antigenic proteins with molecular masses of approximately 65 and 55 kDa.

Composition of *T. pallidum* outer membrane vesicles. The detection of the *T. pallidum* inner membrane-associated PBPs (16, 44) was used to assess the purity of isolated outer membrane (Fig. 5). Major PBPs of 94, 80, 58, 43, and 38 kDa were detected in 1×10^8 whole-organism and protoplasmic cylinder preparations but not in outer membrane material derived from 5×10^9 treponemes, indicating the absence of detectable inner membrane contamination.

In order to further evaluate the possibility of cytoplasmic membrane protein and periplasmic protein contamination, immunoblots of outer membrane vesicles derived from 5×10^9 *T. pallidum* organisms and 1×10^8 whole organisms were probed with antiserum against the 19-kDa protoplasmic cylinder-associated protein 4D (43), with antiserum against the endoflagella (10), and with a monoclonal antibody against the abundant 47-kDa lipoprotein (13) (Fig. 6). The outer membrane preparation was found to have no detectable 47-kDa lipoprotein or 4D protein. In addition, only trace amounts of endoflagella were detected, corresponding to approximately 0.2% endoflagellar contamination based on a 10-fold decrease in the relative intensities of the bands detected compared with those of 10^8 whole organisms.

Consistent with the known paucity of outer membrane protein in *T. pallidum* (45, 55) and the freeze fracture electron microscopy findings described above, Coomassie-stained SDS-PAGE of outer membrane vesicles derived from approximately 5×10^9 treponemes did not detect any protein bands (data not shown). The initial identification of membrane-associated protein was determined on the basis of antigenicity, using IRS and ECL. As shown in Fig. 7, three prominently reacting bands at 17, 32, and 45 kDa were detected by immunoblotting. Solubilization of the outer membrane material in the presence of 8 M urea resulted in the loss of the 32-kDa band but not the 17- and 45-kDa proteins, suggesting that the 32-kDa protein is an oligomer composed of lower-molecular-mass monomeric forms. Two-dimensional immunoblot analysis (Fig. 8) revealed that the 17-kDa protein and proteins of 32 and 45 kDa had the same pI of >7. In addition, a second 45-kDa protein was also identified at a pI of approximately 5.5. This 45-kDa protein was subsequently identified as the TmpA lipoprotein (23, 46) by using specific monoclonal antibodies (data not shown). Exposure of chemilumigrams of the two-dimensional immunoblot for longer periods also identified spots corresponding to the molecular weights and pIs of the endoflagellar proteins (34) (data not shown).

In addition to the 17-kDa protein and its oligomeric forms, the 45-kDa TmpA protein, and the endoflagellar proteins, two-dimensional blots containing outer membrane derived from 3×10^{10} treponemes showed five additional proteins when stained with Aurodye Forte, including two separate spots with different pIs at 31 kDa and single spots at 28, 65, and 68 kDa (Fig. 9A). While the 68-kDa protein was shown by immunoblot analysis to be rabbit albumin (data not shown), all of the other proteins specifically bound antibodies present in low dilutions of IRS, but not preimmune serum, indicating

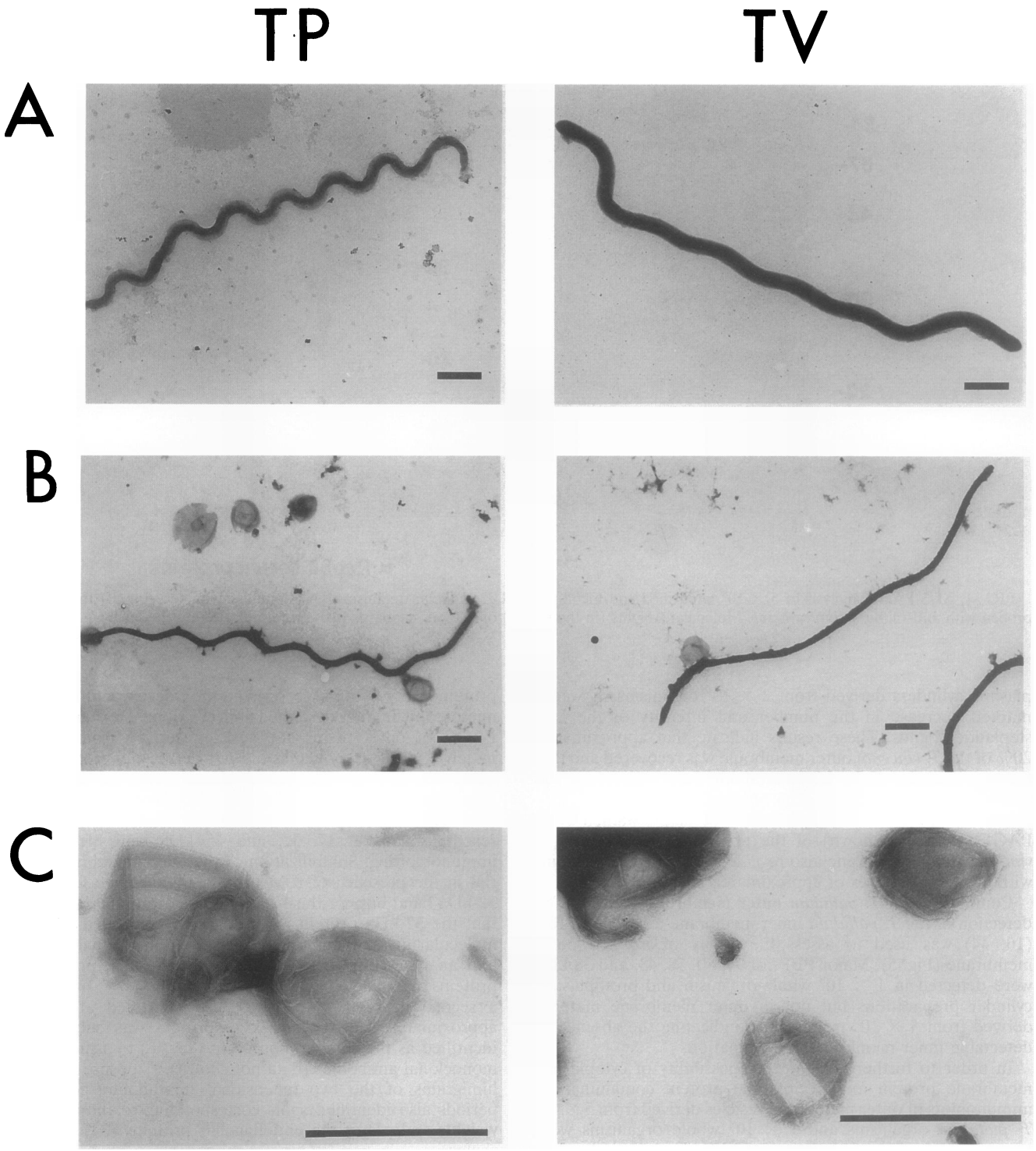

FIG. 2. Whole-mount electron microscopy of outer membrane material isolated from *T. pallidum* (TP) and *T. vincentii* (TV). (A) Ficoll-purified and rhodamine-labeled *T. pallidum* and PBS-washed and rhodamine-labeled *T. vincentii*. (B) Citrate buffer-treated organisms, showing release of outer membrane. (C) Sucrose gradient-purified outer membrane vesicles. Bars, 0.5 μm.

their *T. pallidum* origin (data not shown). Identification and comparison of these proteins with those on amido black-stained two-dimensional blots containing 3×10^8 whole organisms showed that the 31-kDa (acidic-pI) and the 28-kDa proteins corresponded to readily identifiable protein spots (Fig. 9B). In contrast, the 31-kDa (basic-pI) protein corresponded to a minor and faintly detectable protein on the two-dimensional blot of whole organisms while the 65-kDa

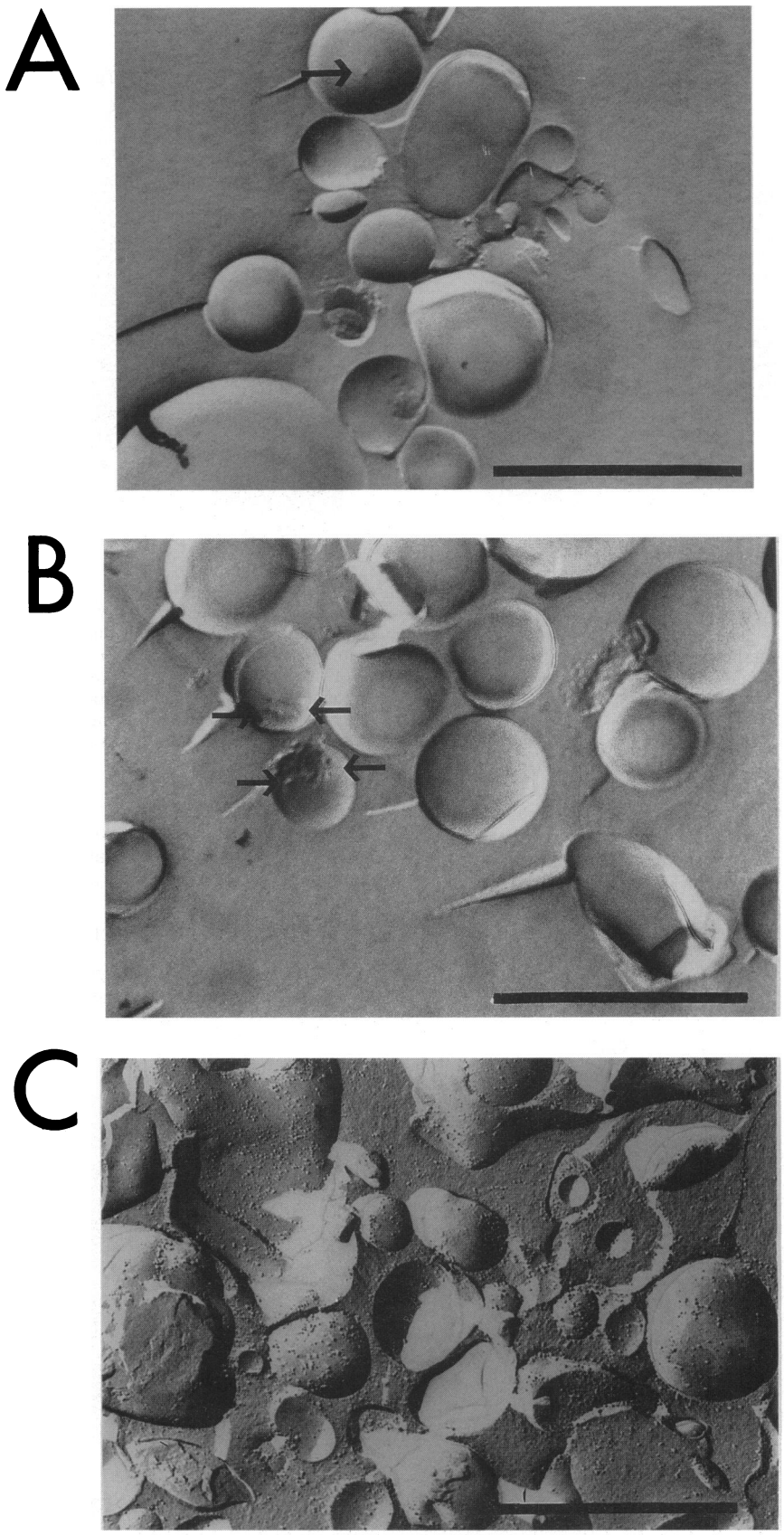

FIG. 3. Freeze-fracture electron microscopy of purified outer membrane vesicles from *T. pallidum* (A) and *T. vincentii* (B) in comparison with host tissue membranous material (C). Note the high density of intramembranous protein particles in fracture faces of host tissue membranous material compared with rare intramembranous protein particles (arrows) in fracture faces of outer membrane vesicles from *T. pallidum* and *T. vincentii*. Bars, 0.5 μm.

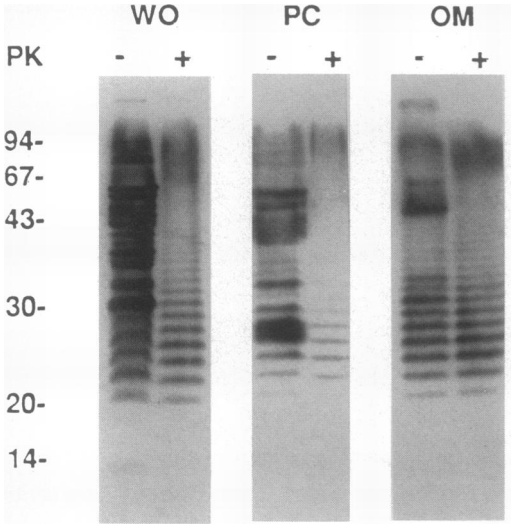

FIG. 4. Composition of the *T. vincentii* outer membrane. Immunoblot analysis of untreated (−) and proteinase K (PK)-treated (+) whole organisms (WO) (2×10^8) and protoplasmic cylinders (PC) and outer membrane (OM) derived from 1×10^9 organisms. Immunoblots were probed with antisera generated against whole organisms as described in Materials and Methods. Molecular weights (in thousands) of marker standards are indicated on the left.

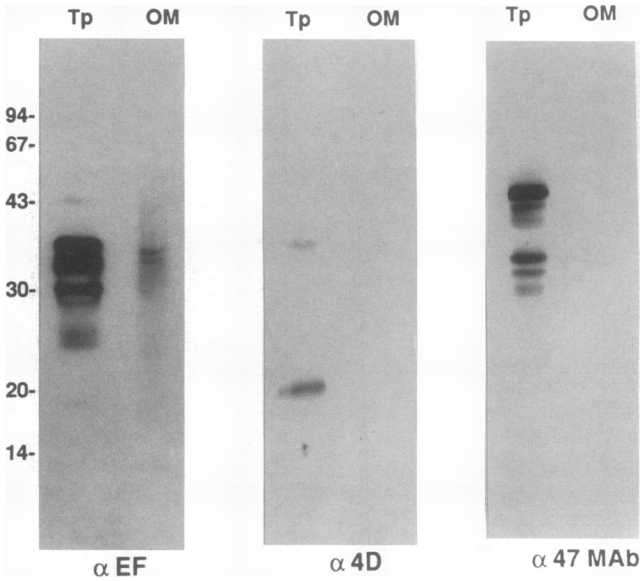

FIG. 6. Identification of *T. pallidum* periplasmic and cytoplasmic membrane-associated proteins during outer membrane isolation. Immunoblots of 1×10^8 whole organisms (Tp) and outer membrane (OM) derived from 5×10^9 organisms were reacted with anti-endoflagellar serum (αEF), anti-19-kDa 4D serum (α4D), and a monoclonal antibody against the 47-kDa lipoprotein (α47 MAb). Molecular weights (in thousands) of marker standards are indicated on the left.

protein did not show any corresponding *T. pallidum* protein at that molecular mass and pI.

Porin activity of the *T. pallidum* outer membrane. The addition of solubilized *T. pallidum* outer membrane derived from 5×10^9 treponemes to the model membrane system resulted in channel formation which was demonstrated by stepwise conductance increases across a lipid bilayer (Fig. 10A). A total of 197 membrane insertion events were observed, and the measurement of conductance increases showed a distinct distribution about two means of 0.40 and 0.76 nS (Fig. 10B). This observation indicates that there are two different channel sizes, corresponding to 0.35 and 0.68 nm.

DISCUSSION

The isolation of the *T. pallidum* outer membrane and identification of its protein constituents have been complicated

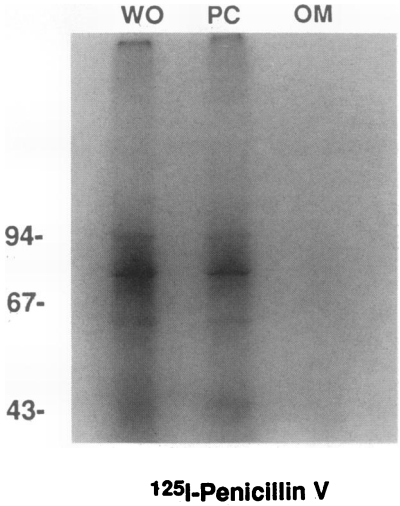

FIG. 5. Identification of *T. pallidum* PBPs during outer membrane (OM) isolation. Samples containing 1×10^8 whole organisms (WO), protoplasmic cylinders (PC), and OM derived from 5×10^9 organisms were incubated with ^{125}I-penicillin V and analyzed by SDS-PAGE and autoradiography. Molecular weights (in thousands) of marker standards are indicated on the left.

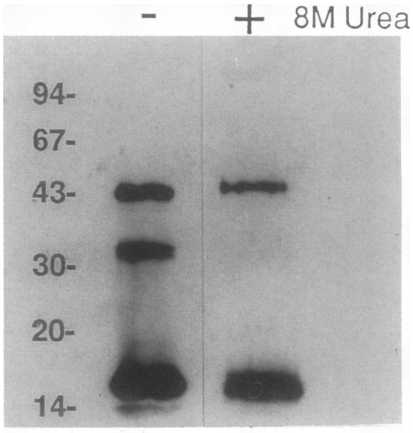

FIG. 7. Antigenicity of *T. pallidum* outer membrane-associated proteins. Immunoblots of *T. pallidum* outer membrane material derived from 5×10^9 organisms, solubilized in sample buffer with (+) and without (−) 8 M urea, were probed with IRS. Molecular weights (in thousands) of marker standards are indicated on the left.

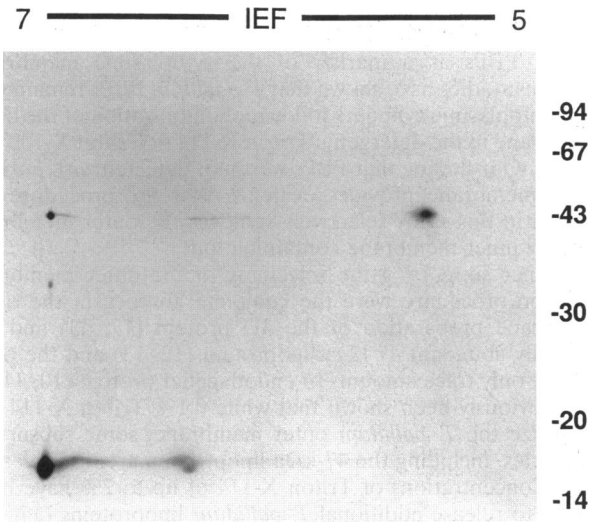

FIG. 8. Antigenicity of *T. pallidum* outer membrane-associated proteins separated by two-dimensional gel electrophoresis. *T. pallidum* outer membrane material derived from 5×10^9 organisms was subjected to isoelectric focusing (IEF) (pH 5 to 7) followed by SDS-PAGE and immunoblotting with IRS. Molecular weights (in thousands) of marker standards are indicated on the right.

by the fragility of this structure, the limited number of treponemes that can be acquired by rabbit infection, and the potential for contaminating protein of host origin following extraction of organisms from infected rabbits. Moreover, freeze fracture electron microscopy has revealed that the outer membrane of *T. pallidum* contains 2 orders of magnitude less membrane-spanning protein than typical gram-negative bacteria (45, 55). Because of the paucity of TROMP, it is likely that previous studies using detergent extraction of *T. pallidum* to identify transmembrane outer membrane proteins have instead identified abundant subsurface molecules, including inner membrane-anchored lipoproteins which are released by such treatments (12, 17, 39, 40, 42).

In this study, we have developed a procedure to isolate the outer membranes from treponemes in the absence of detergents. In addition to *T. pallidum*, we applied this procedure to *T. vincentii* because the LPS contained in its outer membrane could be used as a marker for the fate of the outer membrane upon cellular fractionation. Preliminary studies showed that while a hypotonic osmotic environment caused significant blebbing of the treponemal outer membrane, only a small amount of outer membrane was released. Because it has been suggested that endoflagellar filaments physically interact with the outer membrane in the process of motility (8), we speculated that the endoflagella may limit the release of outer membrane under hypotonic conditions. The use of 0.05 M citrate (pH 3.2), known to dissociate endoflagellar filaments (10), resulted in the complete release of outer membrane as viewed by electron microscopy. *T. pallidum* was purified with a discontinuous Ficoll step gradient (30) which not only was found to remove contaminating host components but also was compatible with the subsequent low-pH incubation. The physical characteristics of Percoll, which is widely used to purify *T. pallidum* from rabbit testicular material (22), change at pH 3.2, making Percoll incompatible with this procedure.

The treatment of treponemes with octadecyl rhodamine B chloride salt, a lipid-conjugated chromophore which intercalates into biological and liposomal membranes (50), provided a key marker with which to visually monitor released outer membrane. Because octadecyl rhodamine has the property of intercalating into membranes, we determined whether this reagent alters membrane particle density. Using *Borrelia burgdorferi*, a pathogenic spirochete which has an amount of outer membrane protein 1 order of magnitude greater than that of *T. pallidum* (54), we verified that octadecyl rhodamine did not change the outer membrane particle density, as judged by freeze fracture electron microscopy (data not shown). Implicit

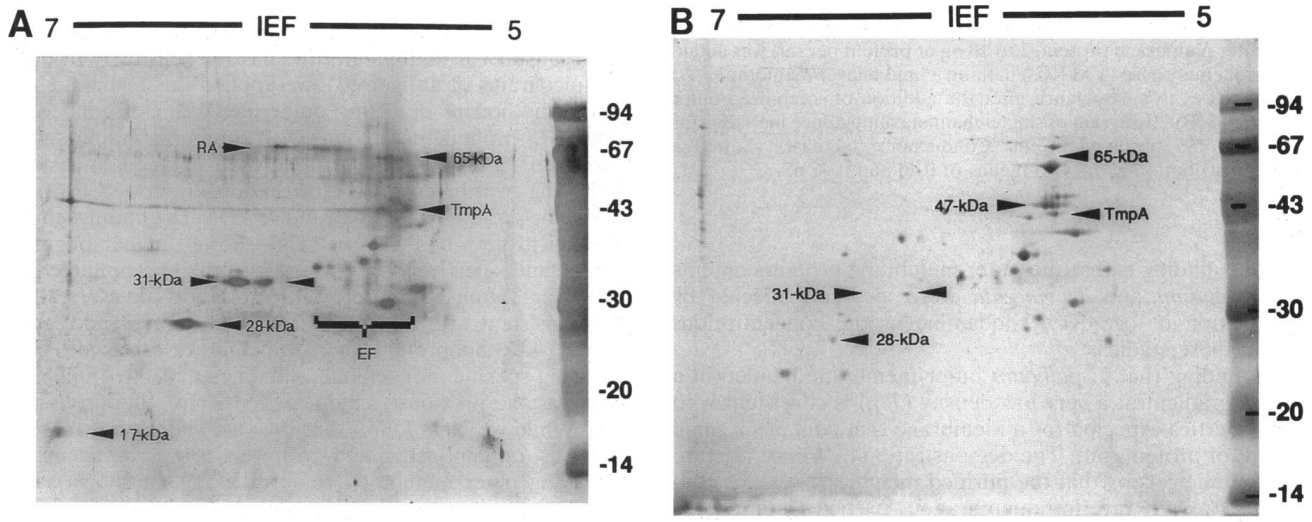

FIG. 9. Comparison of the protein composition of the *T. pallidum* outer membrane with that of whole organisms. (A) Outer membrane material from 3×10^{10} organisms and (B) 3×10^8 whole organisms were subjected to isoelectric focusing (IEF) (pH 5 to 7) followed by SDS-PAGE and transfer to a PVDF membrane. Separated proteins from the outer membrane and from whole organisms were visualized by being stained with Aurodye Forte and amido black, respectively. The *T. pallidum* proteins identified are indicated, including endoflagellar proteins (EF) indicated by the bracket. Rabbit albumin (RA) was identified by specific antibody (data not shown). Molecular weights (in thousands) of marker standards are indicated on the right.

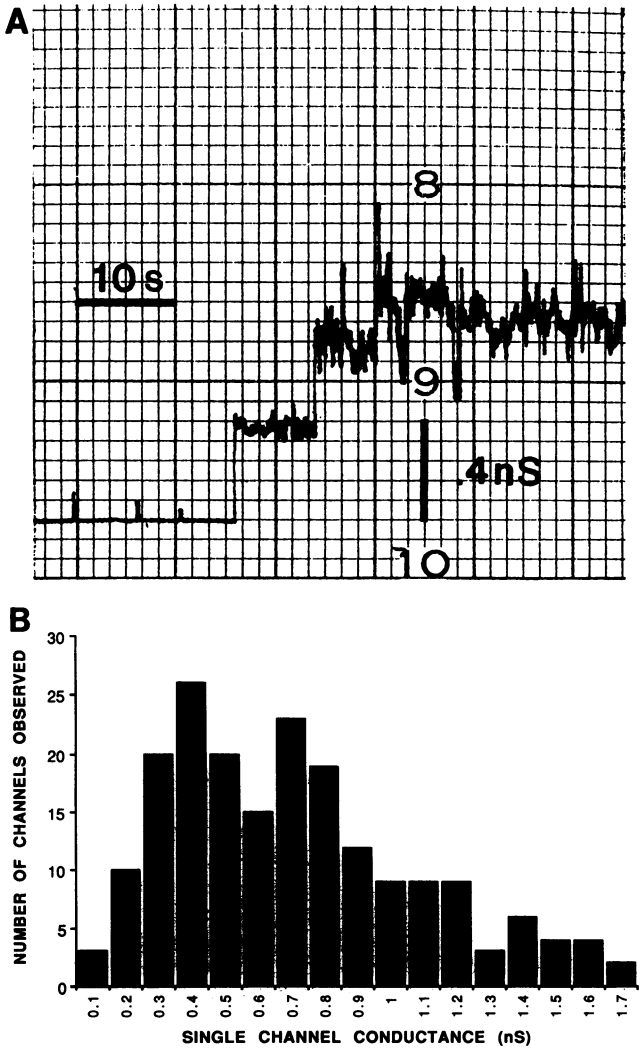

FIG. 10. Porin activity of purified *T. pallidum* outer membrane. Triton X-100-solubilized outer membrane (final concentrations of each outer membrane protein, 2 to 10 ng of protein per ml) was added to the aqueous phase (1 M KCl) bathing a lipid bilayer membrane. (A) Step increases in conductance after the addition of solubilized outer membrane. (B) Histogram of single-channel conductance increases for a total of 197 observed events. Conductance increases showed a distinct distribution about two means of 0.40 and 0.76 nS.

from this finding is that the outer membrane protein contents of *T. pallidum* and *T. vincentii* were also not affected by intercalation of octadecyl rhodamine at the concentrations used in these studies.

The finding that *T. pallidum* outer membrane banded in a sucrose gradient at a very low density (7%) is consistent with the properties expected for a membrane that contains a small amount of protein (50). The demonstration by freeze fracture electron microscopy that the purified membrane vesicles contained extremely rare intramembranous particles is in accord with the low particle density observed for the native outer membrane of *T. pallidum* (45, 55). By comparison, the *T. vincentii* outer membrane banded in a sucrose gradient at a higher density (31%), which may be explained by the greater amount of intramembranous particles observed in its membrane and by the presence of LPS.

Evidence for selective isolation of the *T. pallidum* outer membrane from the protoplasmic cylinder was obtained by the use of PBPs as a marker of the cytoplasmic membrane. Previous studies have shown that *T. pallidum* PBPs remain with the protoplasmic cylinders following solubilization of the outer membrane in the detergents Triton X-114 or Triton X-100 (16, 17, 44). Our finding that PBPs were not detected with purified outer membrane provides evidence that the procedure described in this study selectively removes the outer membrane without inner membrane contamination.

Further supporting the selectivity of the outer membrane isolation procedure were the complete absence in the outer membrane preparation of the 4D protein (17, 43) and the normally abundant 47-kDa lipoprotein (12, 13) and the presence of only trace amounts of endoflagellar protein (10, 34). It has previously been shown that while 0.1% Triton X-114 can solubilize the *T. pallidum* outer membrane, some subsurface molecules, including the 47-kDa lipoprotein, are also released (42). Concentrations of Triton X-114 of up to 2% have been shown to release additional *T. pallidum* lipoproteins (17, 42). Our finding that the 47-kDa lipoprotein, one of the most abundant *T. pallidum* molecules, was not detected in the outer membrane preparation suggests that inner membrane-anchored lipoproteins were not released by this procedure.

Coomassie-stained SDS-PAGE and immunoblot analysis of 10^9 *T. vincentii* equivalents of outer membrane revealed two major antigenic protein species of 65 and 55 kDa. In contrast, Coomassie-stained SDS-PAGE of a fivefold-greater amount of *T. pallidum* outer membrane showed no detectable protein. These findings are consistent with the observations of freeze fracture electron microscopy, indicating that the outer membrane particle density of *T. pallidum* is six times less than that of *T. vincentii*. The outer membrane particle density of *T. pallidum*, which has been determined to be 170 particles per μm^2 (54, 55), together with the surface area of *T. pallidum*, which is approximately 4 μm^2, has allowed for estimation of the total amount of outer membrane protein. We have calculated that 5×10^9 *T. pallidum* organisms should contain only 250 ng of outer membrane protein on the basis of a single species of 50 kDa. Therefore, the amount of a single species of TROMP is estimated to be several hundred times less than those of previously identified *T. pallidum* proteins (35, 36).

Because ECL immunoblotting has the sensitivity of detecting picograms of antigen (3), we applied this technique as an initial method of detecting outer membrane-associated protein. IRS-probed immunoblots of outer membrane samples prepared in urea and electrophoresed in one dimension or following two-dimensional electrophoresis showed two major antigenic protein bands at 17 and 45 kDa. The findings that the 17-kDa protein had a pI of >7.0, showed higher oligomeric forms, and selectively partitioned into the hydrophobic phase following Triton X-114 detergent extraction (data not shown) are consistent with the properties of the native and recombinant 17-kDa lipoproteins of *T. pallidum* (1). It was also shown by using specific monoclonal antibodies that the 45-kDa protein was the previously characterized TmpA lipoprotein (23, 46). While we have found that the vast majority of these two proteins remain associated with the protoplasmic cylinder following outer membrane removal (data not shown), it appears that some of the 17- and 45-kDa lipoproteins are specifically outer membrane associated, given the absence of the normally abundant 47-kDa cytoplasmic membrane lipoprotein. The function of these outer membrane lipoproteins remains to be determined.

In addition to the strongly antigenic 17- and 45-kDa lipoproteins, gold-stained two-dimensional blots of 3×10^{10} trepone-

mal equivalents of outer membrane revealed four additional *T. pallidum* proteins, including one each at 28 and 65 kDa and two at 31 kDa with distinct pI migration patterns. All of these proteins were found to be antigenic when low dilutions of IRS were used. Comparison of the pIs of these four proteins with those on two-dimensional blots of 3×10^8 whole organisms showed that the 31-kDa (acidic-pI) and 28-kDa proteins corresponded to readily identifiable *T. pallidum* protein spots. These proteins may be additional outer membrane-associated lipoproteins which have not been previously described. In contrast, the 31-kDa (basic-pI) protein corresponded to a minor and faintly detectable protein spot on two-dimensional blots of whole organisms, while the 65-kDa protein did not correspond to any previously identified *T. pallidum* protein. It is therefore conceivable that the 31-kDa (basic-pI) and 65-kDa proteins, in view of their significant enrichment following outer membrane isolation, represent the TROMP molecules viewed by freeze fracture electron microscopy.

The demonstration of porin activity by using *T. pallidum* outer membrane material containing approximately 2 to 10 ng each of the 28- and 65- and both 31-kDa proteins, which is the minimum amount of protein necessary to achieve porin function in the black lipid bilayer assay (7, 19, 26), suggests that at least one of these proteins functions as an outer membrane porin. Conductance measurements showed two distinct mean distributions of 0.40 and 0.76 nS, indicating two calculated pore channel sizes of 0.35 and 0.68 nm, respectively. This suggests that the *T. pallidum* outer membrane may possess two different porin proteins. However, because the larger channels have approximately twice the pore size of the smaller channels, it is also possible that the larger channels are simply dimeric aggregates of the smaller ones. The finding of porin activity in the *T. pallidum* outer membrane is in accordance with that for other spirochetes, including *Spirochaeta aurantia* (26) and *Treponema denticola* (19). While the pore sizes for *S. aurantia* and *T. denticola* are noted to be large and are approximately 6.5- and 10-fold greater than the *T. pallidum* 0.35-nm pore size, recent studies using solubilized outer membrane from *Leptospira alstoni* indicate a porin protein which has a pore size nearly identical (0.30 nm) to that of *T. pallidum* (47). It therefore appears that both nonpathogenic and pathogenic spirochetes possess a wide range in size of their porin protein channels, the relationship of which in the adaptation of spirochetes to various environmental and host milieus remains to be determined.

It is presumed that the TROMP molecules have properties similar to those of outer membrane proteins of typical gram-negative bacteria. These include an export signal cleaved by leader peptidase I (53) and amphipathic beta-pleated sheet structure characterizing membrane-spanning regions (52, 56). Recently, the gene encoding a surface-exposed 31-kDa protein of *Leptospira alstoni*, designated OmpL1 (outer membrane protein of *Leptospira*), has been cloned, sequenced, and expressed (21). The deduced amino acid sequence of this protein shows an export signal with a leader peptidase I cleavage site and amphipathic beta-pleated sheet topology resulting in 10 membrane-spanning domains. It has also been determined that this protein has a terminal membrane-spanning region which possesses significant homology and features similar to those of other gram-negative outer membrane proteins (21). The architecture of OmpL1 suggests that outer membrane proteins of pathogenic spirochetes follow the same structural motif as outer membrane proteins of typical gram-negative bacteria. We have recently applied the procedure described in this study to the isolation of the *Leptospira* outer membrane. The membrane material purified was found to selectively contain lipopolysaccharide-like substance, a constituent of the *Leptospira* outer membrane (48, 57), and several proteins, including OmpL1. These findings provide additional evidence that this outer membrane isolation procedure can result in selective recovery of the outer membrane and associated protein from several spirochetes.

We have demonstrated previously that immune-serum antibody binding to virulent *T. pallidum* results in TROMP particle aggregation, as viewed by freeze fracture electron microscopy (11). These findings have recently been confirmed and extended by using serum obtained from infected animals with various degrees of challenge immunity. We have shown that particle aggregation directly correlates with the development of challenge immunity, suggesting that TROMPs represent key targets for a protective host immune response (29). The ability to isolate and purify the *T. pallidum* outer membrane now provides a basis for the eventual identification and cloning of TROMP molecules. We hope to use recombinant TROMP to address directly the molecular basis of syphilis pathogenesis and the relationship of outer membrane proteins to the protective immunity which develops during the course of syphilitic infection.

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