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Recombinant *Treponema pallidum* Rare Outer Membrane Protein 1 (Tromp1) Expressed in *Escherichia coli* Has Porin Activity and Surface Antigenic Exposure

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We recently reported the cloning and sequencing of the gene encoding a 31-kDa *Treponema pallidum* subsp. *pallidum* rare outer membrane porin protein, designated Tromp1 (D. R. Blanco, C. I. Champion, M. M. Exner, H. Erdjument-Bromage, R. E. W. Hancock, P. Tempst, J. N. Miller, and M. A. Lovett, J. Bacteriol. 177:3556–3562, 1995). Here, we report the stable expression of recombinant Tromp1 (rTromp1) in *Escherichia coli*. rTromp1 expressed without its signal peptide and containing a 22-residue N-terminal fusion resulted in high-level accumulation of a nonexported soluble protein that was purified to homogeneity by fast protein liquid chromatography (FPLC). Specific antiserum generated to the FPLC-purified rTromp1 fusion identified on immunoblots of *T. pallidum* the native 31-kDa Tromp1 protein and two higher-molecular-mass oligomeric forms of Tromp1 at 55 and 80 kDa. rTromp1 was also expressed with its native signal peptide by using an inducible T7 promoter. Under these conditions, rTromp1 fractionated predominantly with the *E. coli* soluble and outer membrane fractions, but not with the inner membrane fraction. rTromp1 isolated from the *E. coli* outer membrane and reconstituted into planar lipid bilayers showed porin activity based on average single-channel conductances of 0.4 and 0.8 nS in 1 M KCl. Whole-mount immunoelectron microscopy using infection-derived immune serum against *T. pallidum* indicated that rTromp1 was surface exposed when expressed in *E. coli*. These findings demonstrate that rTromp1 can be targeted to the *E. coli* outer membrane, where it has both porin activity and surface antigenic exposure.

The ability of *Treponema pallidum* subsp. *pallidum*, the agent of venereal syphilis, to evade host immune defenses and cause a prolonged chronic infection has been related to its unique outer membrane, which contains 100-fold-less membrane-spanning protein compared with typical gram-negative bacteria (22, 30). These *T. pallidum* rare outer membrane proteins (TROMPs) have been shown to have surface antigenic exposure (9), which suggests that they are the potential mediators of *T. pallidum* virulence and the surface targets of the host immune response. While the identification and study of TROMPs have been deemed essential to a molecular understanding of *T. pallidum* virulence, the extreme rarity of these proteins has hampered their past identification and has made such research efforts unattainable with native TROMPs.

We recently reported the cloning of the gene encoding the 31-kDa TROMP which we have designated Tromp1 (6). The demonstration of porin activity using purified native Tromp1 has confirmed that it is a membrane-spanning outer membrane protein, the first such protein to be identified from *T. pallidum*. Tromp1 was also found to possess significant amino acid sequence homology (27% identity) to a family of streptococcal surface adhesins (18), suggesting that Tromp1 has a similar function in *T. pallidum.*

The use of recombinant porin proteins for the study of porin function, bacterial virulence, and host immunity has long been limited largely because of the lethality of their expression in *Escherichia coli* and the correspondingly small amount of recoverable protein (10, 12, 17). Moreover, while recombinant porins with porin activity initially expressed as cytoplasmic inclusion bodies have previously been reported (27), the demonstration of functionally active recombinant porins from the *E. coli* outer membrane has been shown only for the Opr porins of *Pseudomonas aeruginosa* (14, 16, 26, 31) and for OmpL1 of *Leptospira kirschneri* (25). Because porin function and immune response recognition are likely to require native membrane conformation of the protein, the study of recombinant porins that have biological activity would appear to be essential in identifying surface-exposed domains which have relevance to bacterial virulence and host immunity.

Here, we report the stable expression of recombinant Tromp1 (rTromp1) in *E. coli*. rTromp1 expressed in *E. coli* was targeted to the outer membrane as identified by using specific antiserum generated to a fast protein liquid chromatography (FPLC)-purified rTromp1 fusion protein. rTromp1 isolated from the *E. coli* outer membrane was also shown to have porin activity similar to that reported previously for native Tromp1 (6), suggesting a protein conformation of the recombinant that

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is similar to that of the native protein. We also showed by immunoelectron microscopy that antibody from infection-derived immune rabbit serum specifically bound to the surfaces of intact *E. coli* cells expressing rTromp1, indicating a membrane-spanning topology and surface antigenic exposure of outer-membrane-associated rTromp1.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *E. coli* BL21 DE3(pLysE) (Novagen, Inc., Madison, Wis.) was used as the host strain for transformations with recombinant plasmid DNA. The pET17b plasmid (Novagen, Inc.) was used for subcloning the *tromp1* structural gene both containing and lacking the region encoding its signal peptide. *E. coli* transformants were grown at 34°C in Luria-Bertani medium containing 100 μg of ampicillin per ml and 34 μg of chloramphenicol (all from Sigma Chemical Co., St. Louis, Mo.) per ml.

PCR. PCR was used to amplify the *tromp1* porin gene. For generating a PCR product lacking the region encoding the Tromp1 signal peptide, the primers used were 5′-CGCGGATCCATTCGGTAGCAAGGATGCCGCA-3′ and 5′-CCGGAATTCCTAGCGAGCCAACGCAGCAAC-3′, containing *Bam*HI and *Eco*RI restriction endonuclease sites, respectively (underlined regions indicate *tromp1* gene sequences). For generating a PCR product encoding Tromp1 including its signal peptide, the primers used were 5′-GGAATTCCATATGCATCAAAATTCACCCAA-3′ and 5′-CGCGGATCCCTAGCGAGCCAACGCAGCAA-3′, containing *Nde*I and *Bam*HI restriction endonuclease sites, respectively. PCR was performed according to the manufacturer's instructions by using AmpliTaq (GeneAmp; Perkin-Elmer Cetus, Norwalk, Conn.) and a programmable thermal controller (TTC-100; M. J. Research, Inc., Watertown, Mass.). Reactions with 50-μl volumes were performed in 10 mM Tris-HCl (pH 8.3)–50 mM KCl–2.0 mM $MgCl_2$–0.001% (wt/vol) gelatin–0.5 μM (each) primer–200 μM (each) deoxynucleotide triphosphate–10 ng of *T. pallidum* genomic DNA template–1.25 U of AmpliTaq. After a mineral oil overlay, the reactions were performed for 30 cycles, beginning with an initial denaturation step of 2 min at 94°C followed by 30 s at 94°C, 30 s at 45°C, 30 s at 72°C, and a final extension step of 72°C for 10 min. The amplification products were analyzed by agarose gel electrophoresis and purified with Geneclean II (Bio 101, La Jolla, Calif.).

Cloning of *tromp1* for expression in *E. coli*. The *tromp1* PCR products described above were ligated into pET17b (Novagen, Inc.), previously digested with *Bam*HI and *Eco*RI or *Nde*I and *Bam*HI. The resulting constructs were transformed into *E. coli* BL21 DE3(pLysE) (Novagen, Inc.) by using cells made competent by $CaCl_2$ as previously described (20).

FPLC purification of the rTromp1 fusion protein expressed from pET17b. One hundred milliliters of Luria-Bertani–ampicillin–chloramphenicol was inoculated with 4 ml of an overnight culture of the BL21 DE3(pLysE/pET17b) rTromp1 transformant. The inoculated culture was shaken at 34°C until an optical density at 600 nm (OD_{600}) of 0.6 at which time IPTG (isopropyl-β-D-thiogalactopyranoside) (Gibco BRL, Gaithersburg, Md.) was added to a final concentration of 1 mM. After an additional incubation for 30 min, rifampin (Sigma Chemical Co.) was added at a final concentration of 0.2 μg/ml with a subsequent additional 2 h of incubation. The culture was then centrifuged at 3,000 × *g* for 15 min, and the bacterial pellet was resuspended into 10 ml of TEN buffer (50 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, [pH 8.0]). To this suspension was added 100 μl of lysozyme (10-mg/ml stock solution), 10 μl of phenylmethylsulfonyl fluoride (100 mM stock solution), 100 μl of sodium deoxychlolate, (10% stock solution), 30 μl of DNase I (1-mg/ml stock solution), and 100 μl of 1 M $MgCl_2$. This mixture was stirred until the solution was no longer viscous and then centrifuged at 12,000 × *g* for 20 min. Both the supernatant (soluble fraction) and pellet (whole-cell debris) were found to have rTromp1 as detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (data not shown). To 5 ml of the soluble fraction containing approximately 4.2 mg of total protein (determined by OD_{280}; 1 OD unit is equivalent to 1 mg of protein) per ml was added 50 μl of a 10% stock solution of Triton X-100 (Calbiochem, La Jolla, Calif.). The mixture was then dialyzed for 3 h against 1 liter of 50 mM Tris, pH 8.0, with subsequent centrifugation at 13,000 × *g* for 5 min prior to being loaded onto a 1-ml Mono Q HR 5/5 FPLC column (Pharmacia LKB Biotechnology, Uppsala, Sweden). The column was washed with 50 mM Tris-HCl–0.1% Triton X-100, pH 8.0, prior to elution in the same buffer with increasing step concentrations of NaCl (30 mM steps from 0 to 600 mM). Approximately 2 mg of purified rTromp1 (determined by OD_{280} measurement) eluted completely in the salt gradient steps from 150 to 210 mM NaCl (data not shown).

Fractionation of *E. coli* cells expressing rTromp1. The outer membrane, inner membrane, and soluble fractions were isolated from *E. coli* cells expressing rTromp1 containing its native signal peptide. A 5-ml overnight culture of a BL21 DE3(pLysE/pET176) *rtromp1* transformant (OD_{600} = 3.2) was centrifuged at 3,000 × *g* for 15 min. The pellet was resuspended into 5 ml of phosphate-buffered saline (PBS; pH 7.4), frozen at −20°C, and then thawed at room temperature. Bacteria were disrupted by three passages through a French pressure cell set at 600 lb/in^2. The resulting disrupted bacterial suspension was centrifuged at 13,000 × *g* for 1 min in order to remove unbroken cells and then at 13,000 × *g* for 30 min in order to pellet the total membrane fraction (the supernatant was saved and represents the total soluble fraction). Then the membrane pellet was resuspended in 1 ml of 2% Triton X-100 in PBS and incubated with rocking for 1 h at 4°C followed by 1 h at room temperature. After incubation, the suspension was centrifuged at 13,000 × *g* for 30 min. The supernatant represents the inner membrane Triton X-100-soluble fraction. The outer membrane pellet was washed once in 1 ml of PBS and recentrifuged as described above. The final outer membrane pellet was resuspended in 20 μl of PBS. Samples were prepared for SDS-PAGE by being boiled for 10 min in final sample buffer consisting of 4% SDS, 10% 2-mercaptoethanol, 10% glycerol, and 0.01% bromphenol blue in 62.5 mM Tris buffer (pH 6.8) or by being incubated at room temperature for 30 min in 0.2% SDS–10% glycerol–0.01% bromphenol blue in 62.5 mM Tris buffer (pH 6.8). Then samples were separated by SDS-PAGE (12.5 or 15% acrylamide gels) and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, Mass.) as previously described (29).

Antisera. Antiserum against FPLC-purified rTromp1 was generated as follows. A total of 100 μg of rTromp1 (100 μg/100 μl) was combined with 100 μl of final sample buffer and boiled for 10 min before separation by SDS-PAGE using a slab gel (10 by 7 cm) apparatus. After electrophoresis, the gel was stained for 10 min with 0.05% Coomassie brilliant blue in H_2O in order to visualize protein. The rTromp1 protein band containing approximately 100 μg was excised from the gel with a scalpel, cut into small pieces, combined with Freund's complete adjuvant, and inoculated subcutaneously and intramuscularly into a New Zealand White male rabbit. Three weeks after the initial immunization, the rabbit was given booster injections by the same routes with similarly prepared materials combined in Freund's incomplete adjuvant. The serum obtained was absorbed three times with sonicated and whole-organism preparations of BL21 DE3 (pLysE/pET17b). Anti-rTromp1 serum was used on immunoblots at a 1:2,500 dilution in M-PBS (5% nonfat dry milk [Carnation Co., Los Angeles, Calif.] and 0.1% Tween 20 [Sigma] in PBS).

Serum samples from syphilitic rabbits immune to challenge (immune rabbit sera [IRS]) were acquired after 6 months from animals infected intratesticularly with 4×10^7 *T. pallidum* organisms. IRS was absorbed three times with sonicated and whole-organism preparations of BL21 DE3(pLysE/pET17b).

Antiserum against the *E. coli* outer membrane protein OmpA was kindly provided by W. Wickner, Dartmouth College, and used on immunoblots at a 1:10,000 dilution in M-PBS. Antiserum against the *E. coli* inner membrane protein F_1F_0 ATPase c subunit was kindly provided by J. Hermolin and R. Fillingame, University of Wisconsin, Madison, and used on immunoblots at 1:5,000 dilution in M-PBS.

Black lipid bilayer experiments using rTromp1 gel purified from *E. coli* outer membranes. Outer membrane fractions prepared as described above from *E. coli* cells expressing rTromp1 and *E. coli* cells harboring a nonrecombinant pET17b plasmid control were solubilized at room temperature for 30 min in SDS-PAGE sample buffer consisting of 0.2% SDS, 10% glycerol, and 0.01% bromphenol blue in 62.5 mM Tris buffer (pH 6.8). After SDS-PAGE, the 31- to 35-kDa region of each gel lane, determined from prestained molecular weight protein standards (Gibco BRL), was excised with a scalpel, cut into small pieces, and eluted overnight at 4°C in 100 μl of 0.1% Triton X-100–100 mM NaCl. Ten microliters from each eluted sample was separated by SDS-PAGE, transferred to polyvinylidene difluoride nylon membranes as previously described (29), and then probed with antiserum generated against FPLC-purified rTromp1. Antibody-antigen binding on immunoblots was detected with anti-rabbit immunoglobulin conjugated to horseradish peroxidase (Amersham) and the enhanced chemiluminescence system (Amersham) as described previously (8). Dilutions in 1 M KCl of the remaining 90-μl samples were tested for porin activity by the black lipid bilayer assay as described previously (4, 6, 8, 13). Pore-forming ability was assessed by applying a voltage of 50 mV across the lipid bilayer to which samples were added and measuring the increases in conductance.

Immunoelectron microscopy. Whole-mount immunoelectron microscopy of *E. coli* cells expressing and exporting rTromp1 was performed as follows. Five milliliters of Luria-Bertani–ampicillin–chloramphenicol was inoculated with 100 μl of an overnight culture of the BL21 DE3(pLysE/pET176) rTromp1 transformant or a BL21 DE3(pLysE) nonrecombinant pET17b control. The cultures were grown in a shaker at 34°C to an OD_{600} of 0.4, at which time they were centrifuged at 13,000 × *g* for 5 min with subsequent resuspension of the pellets in 5 ml of PBS. Cells (equivalent to an OD_{600} of 0.2) were pelleted by centrifugation at 13,000 × *g* for 5 min, resuspended in 50 μl of PBS, and combined with 50 μl of absorbed IRS. Then mixtures were incubated at 37°C in a shaker for 3 h, centrifuged at 7,000 × *g*, washed three times with centrifugation and resuspension in 1 ml of PBS, and resuspended in 50 μl of PBS. For immunoelectron microscopy, Parlodion (Mallinckrodt, Inc., St. Louis, Mo.) and carbon-coated 300-mesh copper grids (Ted Pella, Inc., Tustin, Calif.) were floated for 15 min on 50-μl specimen drops. The grids were washed once in PBS and then blocked for 30 min by incubation at room temperature in 0.5% bovine serum albumin (BSA; Sigma Chemical Co.) in PBS. After the blocking step, the grids were washed three times in PBS with subsequent incubation for 1 h at room temperature in goat anti-rabbit immunoglobulin conjugated to 10-nm colloidal gold particles (Amersham) diluted 1:10 in 0.01% BSA–PBS. Grids were washed five times in SCM buffer (0.15 M NaCl, 10 mM $CaCl_2$, 10 mM $MgCl_2$) and two times in double-distilled water, negatively stained for 2 s in 1% uranyl acetate, washed once in double-distilled water, and examined in an electron microscope (JEOL 100 CX) at 80 kV accelerating voltage. The average number and standard

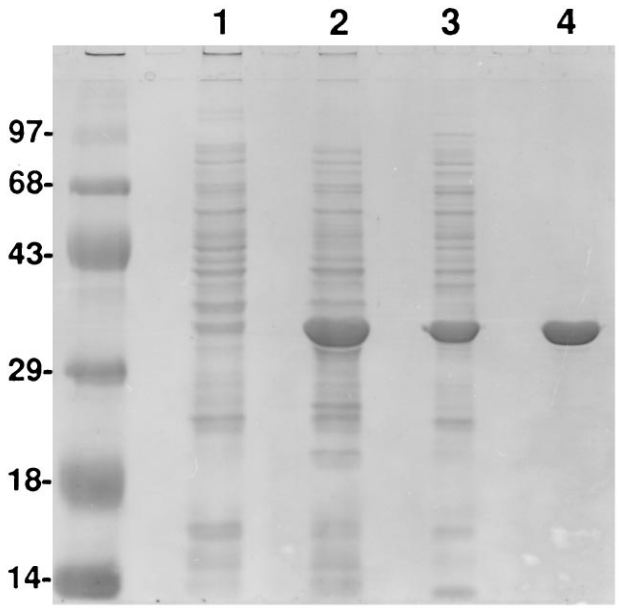

FIG. 1. Expression and purification of rTromp1 fusion protein. The gene encoding the mature Tromp1 protein lacking its signal peptide was cloned into the pET17b plasmid and expressed from an inducible T7 promoter in *E. coli*. Coomassie blue-stained SDS-PAGE gel of 2×10^8 uninduced cells harboring the rTromp1/pET17b plasmid (lane 1), 2×10^8 cells after 2 h of IPTG induction (lane 2), 2×10^8 cell equivalents of the soluble fraction from induced cells (lane 3), and 10 μg of FPLC-purified rTromp1 fusion (lane 4). The positions of molecular mass (in kilodaltons) markers are on the left.

deviation of bound gold particles on test and control *E. coli* strains were determined by counts made from 10 organisms.

RESULTS

Isolation and purification of rTromp1 fusion protein. Our initial studies showed that recombinant expression of the *tromp1* gene from a high-copy-number plasmid was lethal to *E. coli* (6), an observation consistent with many recombinant porin proteins expressed in *E. coli* (10, 17). In order to stably express rTromp1 in amounts sufficient for isolation and purification, the *tromp1* gene lacking the region encoding its signal peptide was cloned into pET17b, an inducible and relatively low-copy-number expression plasmid. The cloned construct in pET17b encoded the entire mature Tromp1 protein fused at the N terminus to 22 residues of the T7 capsid protein. Expression under the control of an inducible T7 promoter resulted in high levels of nontoxic expression of rTromp1 fusion protein, the majority of which was soluble (Fig. 1, lane 3). The amount of rTromp1 produced after induction was estimated to be 10% of the total cellular protein. The isolation of rTromp1 from the soluble fraction by FPLC resulted in a highly purified preparation (Fig. 1, lane 4), which was used for rabbit immunizations to produce antiserum for subsequent studies.

Identification of native Tromp1 oligomers using antiserum against FPLC-purified rTromp1. Because most gram-negative bacterial porin proteins exist in the outer membrane in either an SDS-unstable or -stable trimer configuration (13), we tested whether native Tromp1 would also be organized in an oligomeric form in the outer membrane of *T. pallidum*. As shown in Fig. 2A, lane 1, antiserum generated against the FPLC-purified rTromp1 fusion protein identified both 55- and 80-kDa forms of Tromp1 in addition to the 31-kDa form from a *T. pallidum* preparation that was solubilized in 4% SDS–10% 2-mercaptoethanol. The absence of a reducing agent in these experiments did not alter the electrophoretic profile of these proteins (data not shown). When sample buffer containing 8 M urea was used to solubilize *T. pallidum*, only a single band migrating slightly more slowly than 31 kDa was observed (Fig. 2A, lane 2). When low concentrations of SDS (0.02%) at room temperature were used to solubilize and separate *T. pallidum* proteins, only a 98-kDa band was detected with the rTromp1 antiserum (Fig.

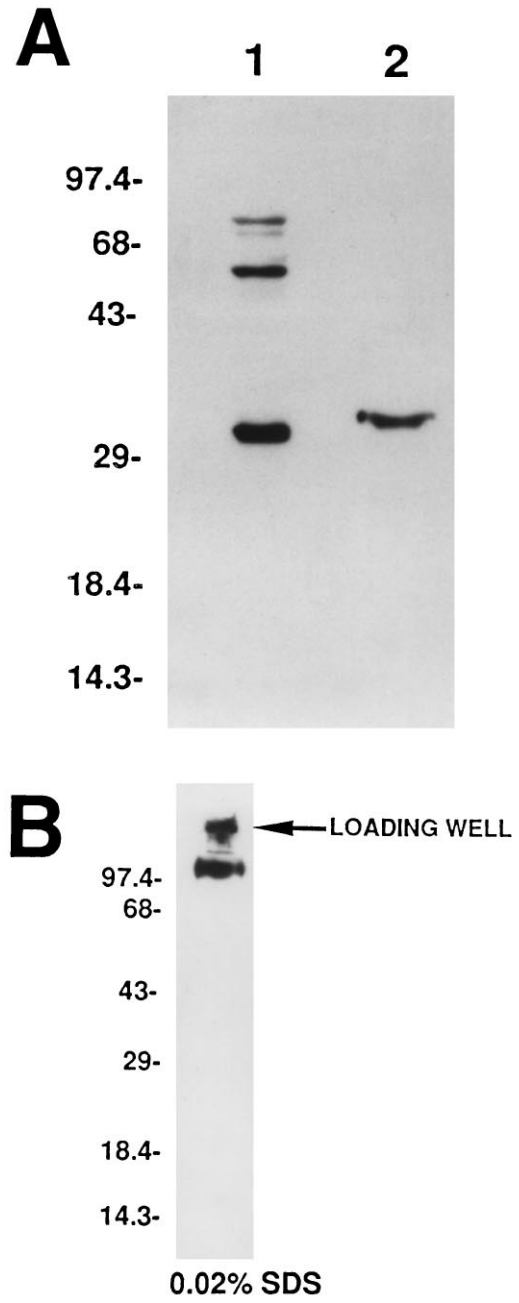

FIG. 2. Identification of native Tromp1 oligomers. Immunoblots containing 10^9 *T. pallidum* organisms per lane were probed with antiserum generated to the FPLC-purified rTromp1 fusion. (A) *T. pallidum* organisms were solubilized in sample buffer without (lane 1) and with (lane 2) 8 M urea. Note higher oligomeric forms at approximately 55 and 80 kDa in the absence of 8 M urea. (B) *T. pallidum* organisms were solubilized in sample buffer containing 0.02% SDS and electrophoresed in an SDS-PAGE system containing 0.02% SDS. The positions of molecular mass (in kilodaltons) markers are on the left.

2B). These same SDS-PAGE conditions did not alter the electrophoretic migration of *T. pallidum* endoflagellar proteins in previous studies (7). Thus, these findings suggest that native Tromp1 exists in *T. pallidum* in an SDS-unstable oligomeric conformation, which is similar to the porin proteins from several other gram-negative bacteria (13).

Fractionation of *E. coli* cells expressing and exporting rTromp1. In order to determine if rTromp1 expressed in *E. coli* can be targeted to the outer membrane, the gene encoding the mature Tromp1 protein sequence including its signal peptide was cloned into the expression plasmid pET17b. When transformants were grown overnight under noninducing conditions, low levels of rTromp1 were produced because of basal-level expression of T7 RNA polymerase. Under these conditions, basal-level expression of rTromp1 was not lethal to *E. coli*. As shown in Fig. 3A, rTromp1 ranging in molecular mass from 31 to 35-kDa was detected with the anti-rTromp1 serum on an immunoblot containing whole-cell and soluble extracts of *E. coli* (lanes 1 and 2). rTromp1 was also prominently detected in the outer membrane fraction, although only the higher-molecular-mass form was observed (Fig. 3A, lane 4). When outer membrane samples were solubilized in 0.2% SDS and not boiled, a small amount of rTromp1 was further detected at approximately 75 to 80 kDa (Fig. 3A, lane 5), which is similar to the oligomeric size described above for native Tromp1 (Fig. 2B). By comparison, only a small amount of rTromp1 was detected in the inner membrane fraction (Fig. 3A, lane 3), whose identity and purity were confirmed by the reactivity of antiserum to the *E. coli* inner membrane protein F_1F_0 ATPase (Fig. 3C, lane 3) and absence of reactivity with antiserum to OmpA (Fig. 3B, lane 3).

Porin activity of rTromp1 isolated from the *E. coli* outer membrane. In order to determine if outer-membrane-associated rTromp1 has porin activity, rTromp1 was isolated from *E. coli* outer membranes by low-concentration SDS solubilization followed by separation by SDS-PAGE. Under these conditions, *E. coli* porins migrated to approximately 97 to 110 kDa because of their SDS-stable trimer conformations and OmpA migrated to 28 kDa because of its heat-modifiable nature (Fig. 3B, lane 5). The 31- to 35-kDa region of SDS-PAGE-separated *E. coli* outer membranes, from both a nonrecombinant control and rTromp1-expressing organisms, was excised and eluted in 0.1% Triton X-100–100 mM NaCl. The recovery of rTromp1 was confirmed by immunoblot analysis (Fig. 4, lane 2). The rTromp1-containing sample and the control sample were tested for porin activity by the black lipid bilayer assay. The addition of the sample containing rTromp1 to the model membrane system resulted in channel formation, which was demonstrated by stepwise conductance increases (Fig. 5A). Histogram analysis of the data for over 56 membrane insertion events showed a relatively equal distribution of conductance increases of about 0.4 and 0.8 nS (Fig. 5B). In contrast, an equivalent amount and a 10-fold-greater amount of control sample failed to show any increase in conductance. These findings demonstrate that rTromp1 isolated from the *E. coli* outer membrane has porin activity.

Surface antigenic exposure of rTromp1 expressed in *E. coli*. Whole-mount immunoelectron microscopy was used to determine if epitopes of rTromp1 could be detected on the surfaces of intact *E. coli* cells. As shown in Fig. 6B, IRS antibody specifically bound to the surfaces of *E. coli* cells expressing rTromp1 (average and standard deviation of 72 ± 12 gold particles per organism). The specificity of this reaction was demonstrated (Fig. 6A) by the limited amount of antibody binding to an *E. coli* cell harboring a nonrecombinant plasmid control (average and standard deviation of 10 ± 3 gold parti-

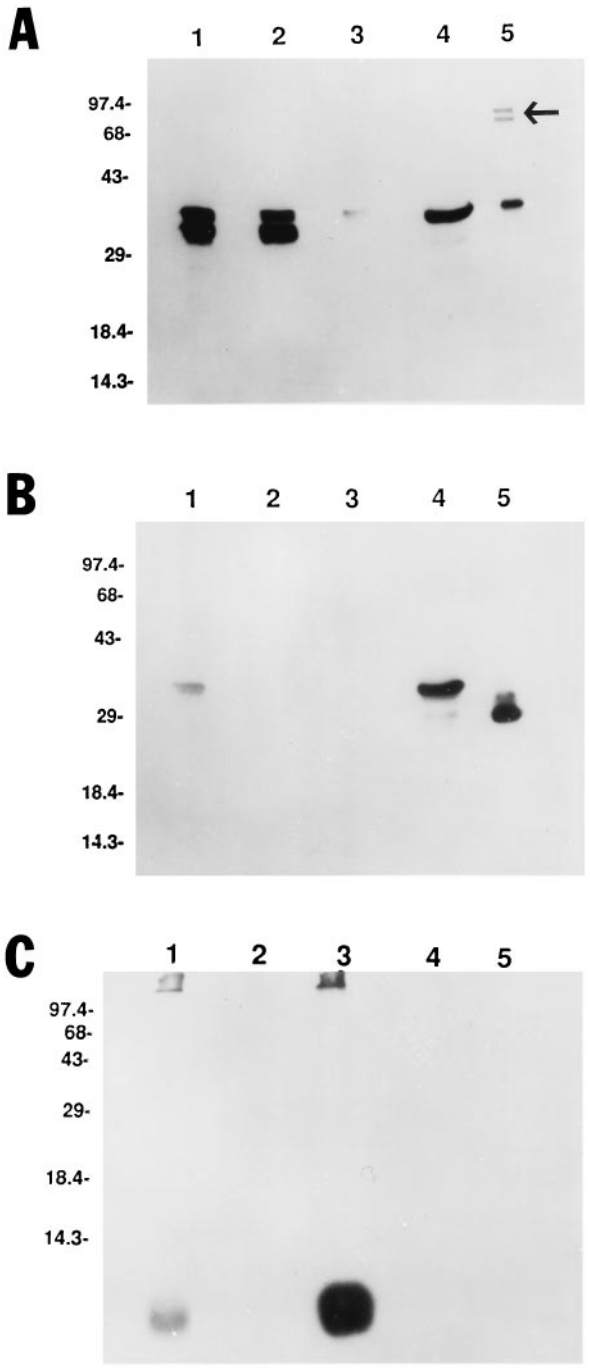

FIG. 3. Fractionation of *E. coli* cells expressing and exporting rTromp1. The gene encoding the Tromp1 protein including its signal peptide was cloned into the pET17b plasmid and expressed in *E. coli*. Immunoblots containing 10^8 *E. coli* cells harboring the rTromp1/pET17b plasmid (lanes 1), 10^8 cell equivalents of the soluble fraction (lanes 2), 10^9 cell equivalents of the inner membrane fraction (lanes 3), 10^9 cell equivalents of the outer membrane fraction (lanes 4), and 10^9 cell equivalents of the outer membrane fraction solubilized at room temperature in 0.2% SDS (lanes 5). Immunoblots were probed with antiserum to the FPLC-purified rTromp1 fusion (A), antiserum to the *E. coli* outer membrane protein OmpA (B), and antiserum to the *E. coli* inner membrane protein F_1F_0 ATPase (C). The arrow identifies oligomeric forms of rTromp1 when the sample was solubilized in 0.2% SDS and not boiled. The positions of molecular mass (in kilodaltons) markers are on the left.

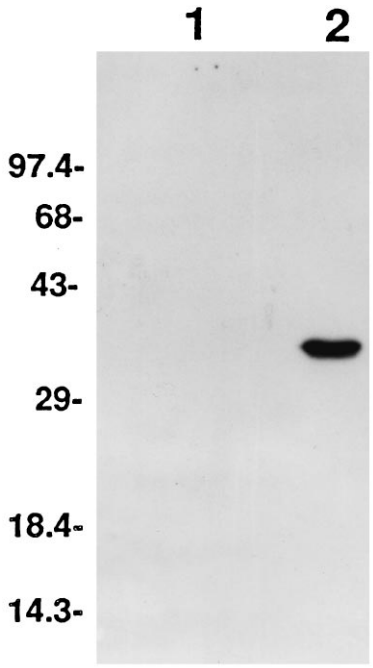

FIG. 4. Isolation of rTromp1 from the *E. coli* outer membrane. rTromp1 was isolated from the *E. coli* outer membrane by SDS-PAGE (outer membrane was solubilized at room temperature in 0.2% SDS) followed by gel elution of the 31- to 35-kDa region in 100 μl of 100 mM NaCl–0.1% Triton X-100. An immunoblot of SDS-PAGE-separated material containing 10 μl of a gel-eluted outer membrane control sample (lane 1) and 10 μl of gel-eluted outer membrane containing rTromp1 was probed with antiserum generated to the FPLC-purified rTromp1 fusion. The positions of molecular mass (in kilodaltons) markers are on the left.

cles per organism). Interestingly, the anti-rTromp1 serum generated against the FPLC-purified rTromp1 fusion protein failed to show significant binding of the antibody to the surfaces of *E. coli* cells expressing rTromp1 (data not shown).

DISCUSSION

Porin proteins of gram-negative pathogens not only function as portals for nutrient acquisition across the outer membrane but have also been shown to play a role in pathogenesis by acting as adhesins (3, 28) and targets of bactericidal antibodies (11, 12, 21, 24). The conformation of these membrane-spanning proteins is believed to be particularly important to their biological function. Many surface-exposed epitopes on porins shown to be targets for bactericidal antibodies are, in fact, conformational (11, 21). Thus, correct outer membrane protein conformation should be a key consideration in the study of porins as they relate to bacterial virulence and host immunity.

Our recent cloning of the gene encoding the 31-kDa Tromp1 porin (6), the first *T. pallidum* membrane-spanning outer membrane protein to be identified, has now provided the basis for studies to address directly the relationship of a *T. pallidum* outer membrane protein to syphilis pathogenesis and host immunity. The finding that Tromp1 has significant amino acid sequence homology to a family of streptococcal surface adhesins (18) has suggested the potential relevance of Tromp1 to syphilis pathogenesis and is an area of research which we are currently investigating. Moreover, the previous demonstration of IRS antibody-mediated aggregation of TROMPs, as viewed by freeze fracture electron microscopy of the *T. pallidum* outer membrane (9), further suggests that Tromp1 could be a key surface-exposed target for antibody which mediates killing (5, 9) or opsonization (1, 19). Because of these possibilities, we initiated studies with the goal of expressing a functional form of rTromp1 in amounts sufficient for its use in experimental biology.

Since the entire *tromp1* gene expressed from a high-copy-number plasmid was lethal to *E. coli* (6), similar to other recombinant porins (10, 17), we initially sought to express a nonlethal form of rTromp1 in an effort to recover amounts for purification and antibody production. Expression of rTromp1 as a fusion protein that was not exported across the cytoplasmic membrane resulted in the overproduction of rTromp1, which was subsequently purified by FPLC. Not surprisingly,

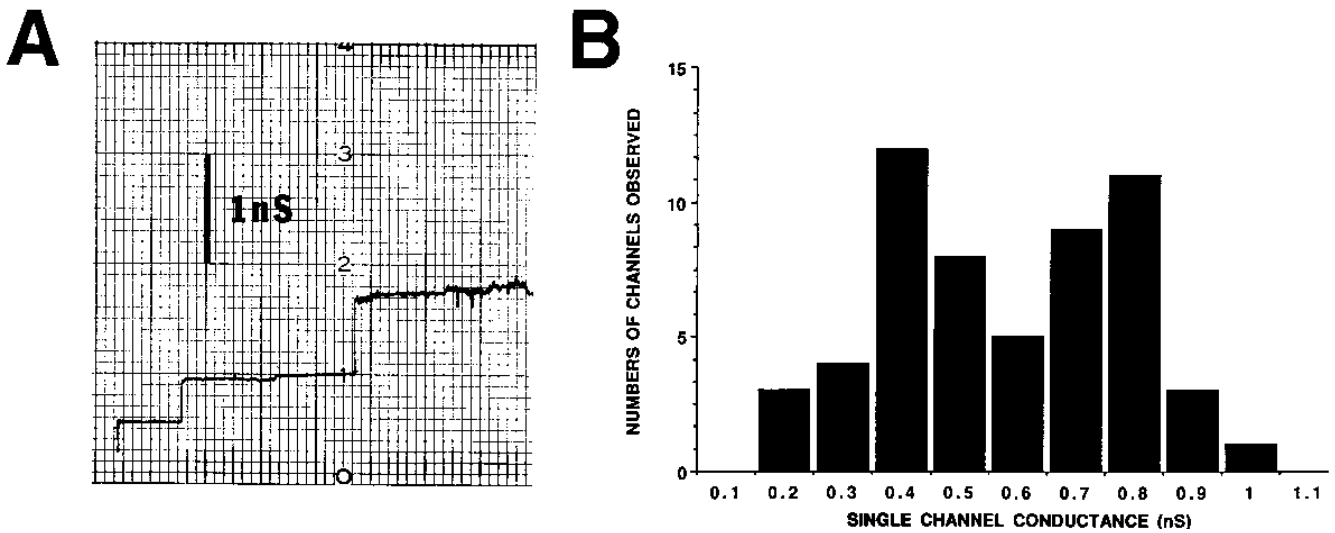

FIG. 5. Porin activity of rTromp1. rTromp1 isolated from the *E. coli* outer membrane was added to the aqueous phase (1 M KCl) bathing a lipid bilayer membrane. (A) Step increases in conductance at 50 mV after the addition of rTromp1. (B) Histogram of single-channel conductance increases for over 56 observed events. Conductance increases showed a mean distribution of about 0.4 and 0.8 nS. Gel-eluted material from the outer membrane control sample had no porin activity (data not shown).

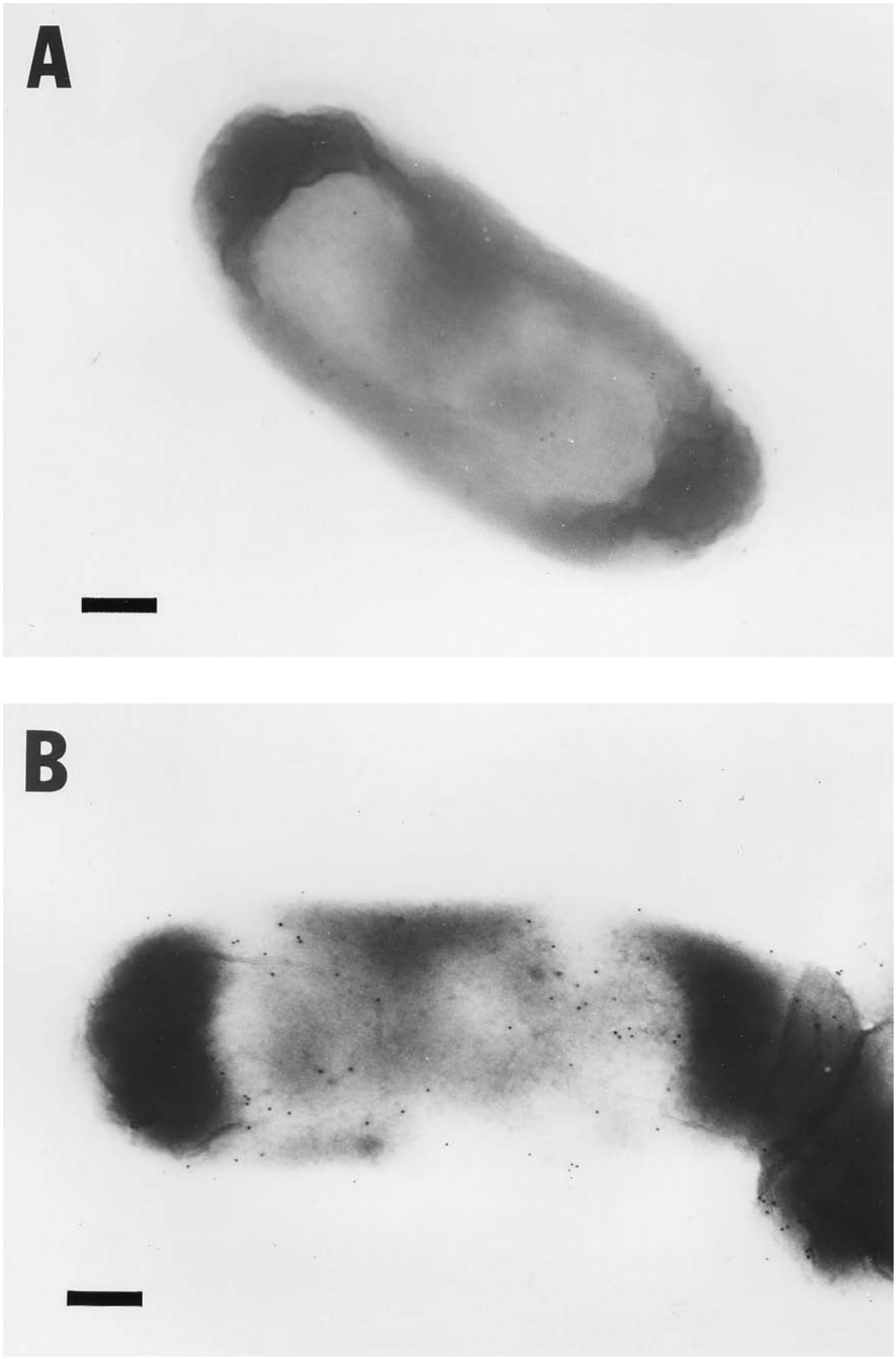

FIG. 6. Surface antigenic exposure of rTromp1 expressed in *E. coli*. Whole-mount immunoelectron microscopy was used to detect surface-exposed epitopes of rTromp1. *E. coli* cells were incubated in serum from a syphilitic rabbit immune to challenge reinfection. Antibody bound to the surfaces of *E. coli* cells was detected by anti-rabbit immunoglobulin G conjugated to 10-nm colloidal gold particles. (A) *E. coli* cell harboring a nonrecombinant plasmid control; (B) *E. coli* cell expressing rTromp1. Bar, 0.2 μm.

this soluble form of rTromp1 did not have any demonstrable porin activity when tested by the black lipid bilayer assay (data not shown). However, antiserum against this purified recombinant did identify two higher-molecular-mass (approximately 55- and 80-kDa) forms of native Tromp1 on immunoblots of *T. pallidum*. A 98-kDa form was further found to be the only detectable form of Tromp1 when *T. pallidum* was solubilized at room temperature in a low concentration of SDS. We believe that these findings are consistent with the idea that native Tromp1 exists in the *T. pallidum* outer membrane in an SDS-unstable trimer conformation, a property which would be similar to several other gram-negative porins (13).

In an effort to ultimately produce a form of rTromp1 with porin activity, we expressed Tromp1 containing its native signal peptide in *E. coli* to determine whether rTromp1 would localize to the outer membrane. The controlled, nonlethal expression of exported rTromp1 was accomplished with an inducible T7 promoter. Uninduced basal levels of T7 RNA polymerase resulted in the stable expression, export, and outer membrane localization of rTromp1. In contrast, inducing conditions for rTromp1 expression resulted in immediate lethality for *E. coli* (data not shown). Expression was also found to result in several forms of rTromp1 which ranged in molecular mass from 31 to 35 kDa. These different sizes of rTromp1 may represent different conformations of the protein. Interestingly, only the highest-molecular-mass form, at approximately 35 kDa, was present in the outer membrane fraction. One explanation for this observation is the possible association of rTromp1 with lipopolysaccharide (LPS), which is known to form a complex with gram-negative outer membrane porin proteins (23) and would therefore result in an increase in the apparent molecular mass of outer-membrane-associated rTromp1. This putative interaction would certainly be novel for Tromp1 since the *T. pallidum* outer membrane does not contain LPS (2, 15). It was also observed that both native Tromp1 and rTromp1 showed oligomeric forms of relatively equal size, ranging from 75 to 80 kDa. The ability of rTromp1 to form an oligomer similar in size to that of native Tromp1 suggests that their oligomeric conformations are similar.

The demonstration of porin activity using rTromp1 isolated from *E. coli* outer membranes has shown that its biological activity can be successfully generated from recombinant expression. These findings also imply that Tromp1 possesses signals similar to those of *E. coli* outer membrane proteins for export and targeting to the outer membrane. Single-channel conductance measurements of rTromp1 in planar lipid bilayers showed two distinct distributions of activity at about 0.4 and 0.8 nS. The 0.8-nS conductance measurement is similar to the 0.7-nS conductance previously determined for native Tromp1 (6), suggesting that the pore-forming conformation of a portion of rTromp1 is similar to that of the native protein. Although it is not known at this time why rTromp1 also showed a 0.4-nS conductance measurement, it is interesting that our previous porin studies using purified *T. pallidum* outer membranes also demonstrated a conductance of 0.4 nS (8). One interpretation for this observation is that the 0.4-nS conductance measurement represents a breakdown of porin channel activity, possibly due to partial damage during isolation and purification. This would therefore suggest that our original porin conductance determinations for purified *T. pallidum* outer membranes (8) were the conductance activities associated with only Tromp1 and conformationally modified Tromp1.

The importance of proper porin protein conformation to biological function was further revealed in our studies using rTromp1. It has been suggested from the primary amino acid sequence of Tromp1 that there are two possible signal peptide processing sites, one at threonine-histidine-alanine (residues 30 through 31) and the other at alanine-alanine-alanine (residues 38 through 40) (6). We recently made signal peptide fusion constructs at these two possible cleavage sites by using the OmpT signal peptide of *E. coli* and found that both constructs resulted in an exported form of rTromp1 targeted to the *E. coli* outer membrane (unpublished observations). However, only the OmpT signal fused at the alanine-alanine-alanine position resulted in an exported product which migrated during SDS-PAGE most closely with that of native Tromp1. Moreover, while the outer membrane form of rTromp1 processed at threonine-histidine-alanine showed porin activity, the average channel conductance was 3.2 nS, which is considerably larger than the 0.7- to 0.8-nS channel observed for native Tromp1 and rTromp1 exported with its native signal. Unfortunately, the greater lethality and limited recovery of the OmpT-Tromp1 construct processed at alanine-alanine-alanine precluded the ability to test this outer membrane form for porin activity. We believe that these findings suggest that alanine-alanine-alanine is the likely processing site in the Tromp1 sequence for leader peptidase I. More importantly, these findings indicate that subtle changes in the length of the primary amino acid sequence of processed Tromp1 can have significant effects upon porin activity, which are likely to be the result of an altered conformation of the protein. Thus, we believe that proper Tromp1 conformation represents a significant issue in regard to future studies addressing its role in syphilis pathogenesis.

A further issue regarding the outer membrane localization of porin-active rTromp1 was whether this conformation resulted in surface-exposed antigenic domains. The results of whole-mount immunoelectron microscopy demonstrated the surface binding of IRS antibody on *E. coli* cells expressing rTromp1, suggesting that rTromp1 surface-exposed epitopes result from a membrane-spanning topology. Interestingly, antiserum to FPLC-purified, denatured or nondenatured rTromp1, which was found to be 100-fold more sensitive in detecting rTromp1 by immunoblot analysis than was IRS (data not shown), did not show any appreciable surface binding in these experiments. It is again pertinent to note that the FPLC-purified soluble form of rTromp1 did not have porin activity when tested in the black lipid bilayer assay. Therefore, a reasonable explanation for these immunoelectron microscopy observations is that the bound IRS antibodies recognize conformational epitopes of rTromp1. This further suggests that the surface-exposed epitopes on native Tromp1 are also conformational. Thus, these results stress again the importance of protein conformation as it relates to biological function.

As previously mentioned, freeze fracture electron microscopy has demonstrated the ability of IRS antibody to aggregate TROMPs, which has been suggested to be a prerequisite for complement activation and killing of *T. pallidum* (9). These studies have been extended by using sera obtained from infected animals with various degrees of challenge immunity and have shown that TROMP aggregation correlates directly with the development of challenge immunity (unpublished observations). Implicit from these findings is that TROMPs represent the key surface-exposed targets for a protective host immune response. The findings presented here demonstrate that rTromp1 can be expressed in its *E. coli* host in a functional porin-active form and with surface antigenic exposure. We are currently engaged in studies using the porin-active form of rTromp1 to address the ability of this *T. pallidum* outer membrane protein to elicit protective immunity in experimental syphilis.

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