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Sequence Analysis and Recombinant Expression of a 28-Kilodalton *Treponema pallidum* subsp. *pallidum* Rare Outer Membrane Protein (Tromp2)†

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In this study, we report the cloning, sequencing, and expression of the gene encoding a 28-kDa *Treponema pallidum* subsp. *pallidum* rare outer membrane protein (TROMP), designated Tromp2. The *tromp2* gene encodes a precursor protein of 242 amino acids including a putative signal peptide of 24 amino acids ending in a type I signal peptidase cleavage site of Leu-Ala-Ala. The mature protein of 218 amino acids has a calculated molecular weight of 24,759 and a calculated pI of 7.3. The predicted secondary structure of Tromp2 shows nine transmembrane segments of amphipathic beta-sheets typical of outer membrane proteins. Recombinant Tromp2 (rTromp2) was expressed with its native signal peptide, using a tightly regulated T7 RNA polymerase expression vector. Under high-level expression conditions, rTromp2 fractionated exclusively with the *Escherichia coli* outer membrane. Antiserum raised against rTromp2 was generated and used to identify native Tromp2 in cellular fractionations. Following Triton X-114 extraction and phase separation of *T. pallidum*, the 28-kDa Tromp2 protein was detected prominently in the detergent phase. Alkali and high-salt treatment of purified outer membrane from *T. pallidum*, conditions which remove peripherally associated membrane proteins, demonstrated that Tromp2 is an integral membrane protein. Whole-mount immunoelectron microscopy of *E. coli* cells expressing rTromp2 showed specific surface antibody binding. These findings demonstrate that Tromp2 is a membrane-spanning outer membrane protein, the second such protein to be identified for *T. pallidum*.

Based on freeze fracture analysis, the outer membrane of the pathogenic spirochete *Treponema pallidum* subsp. *pallidum* (herein referred to as *T. pallidum*) contains an extremely low content of membrane-spanning proteins (28, 39). It has been speculated that this property contributes to the chronicity of syphilitic infection by permitting *T. pallidum* to evade host immune defenses. Due to the low molar concentration of *T. pallidum* rare outer membrane proteins (TROMPs), their identification and isolation has been challenging. Recently, we described a novel procedure for the isolation and purification of the outer membrane of *T. pallidum* along with the identification of its constituent proteins, including 31- and 28-kDa species (5). The 31-kDa protein, termed Tromp1, has been cloned, sequenced, and expressed. Both native and recombinant Tromp1 (rTromp1) have porin activity, confirming that it is a membrane-spanning outer membrane protein (3, 4).

In this study, we describe the cloning, sequencing, and expression of the gene encoding the 28-kDa outer membrane protein, designated Tromp2. Antiserum generated to rTromp2 was used to identify native Tromp2 in cellular fractionations. Results of immunoblot analysis after Triton X-114 extraction and phase separation of *T. pallidum* indicated that Tromp2 was detected prominently in the detergent phase, consistent with a hydrophobic outer membrane protein. Tromp2 was also detected in the protoplasmic cylinders following Triton X-114 extraction, suggesting that Tromp2 may also be anchored to a subsurface location. Expression of Tromp2 in *Escherichia coli* resulted in its exclusive targeting to the outer membrane and its surface exposure. These findings indicate that Tromp2 is a membrane-spanning outer membrane protein, the second such protein to be identified for *T. pallidum.*

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *T. pallidum* subsp. *pallidum*, Nichols strain, was maintained by testicular passage in New Zealand White rabbits as described previously (22). *T. pallidum* used for all experiments was extracted from infected animals as previously described (5) and washed once in phosphate-buffered saline, pH 7.4 (PBS). The plasmids Bluescript KS (Stratagene, La Jolla, Calif.) and pET17b (Novagen, Inc., Madison, Wis.) were used as vectors for cloning, sequencing, and expression of *tromp2. E. coli* DH5α (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) was used as the host strain for transformation with Bluescript KS recombinant plasmid DNA. *E. coli* BL21 DE3 pLysE (Novagen, Inc.) was used as the host strain for transformations with pET17b recombinant plasmid DNA. *E. coli* PLK-F′ (Stratagene) was used as the host strain for infection with the lambda Zap II phage vector. *E. coli* SOLR (Stratagene) was used as the host strain for infection with in vivo-excised filamentous lambda Zap II. *E. coli* transformants were grown at 37°C in Luria-Bertani (LB) liquid medium or on LB agar containing 100 μg of ampicillin per ml and 34 μg of chloramphenicol per ml (LB/amp/cam; Sigma Chemical Co., St. Louis, Mo.).

Isolation, cloning, and sequencing of the *tromp2* gene. The 28-kDa Tromp2 protein was prepared and sequenced as previously described for Tromp1 (4). Analysis of two peptides, designated 28-A and 28-B, yielded the sequences SDYEIP and DFYVFFDO, respectively. The underlined region of peptide 28-B was used to generate an oligonucleotide having 128-fold degeneracy to be used in identifying the gene encoding the 28-kDa protein.

Approximately 10^{11} *T. pallidum* cells were used to prepare genomic DNA as previously described (7) for Southern blot analysis (20) and isolation of the *tromp2* gene as follows. The oligonucleotide 26-B was labeled at its 5′ end with [γ-^{32}P]ATP (5,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) and T4 polynucleotide kinase and then purified over a Biospin 6 column (Bio-Rad

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† This paper is dedicated to the memory of Philip A. Hanff, whose many contributions to the syphilis field will long be remembered.

Laboratories, Hercules, Calif.). The membrane was hybridized overnight at 37°C in hybridization buffer containing 10^6 cpm per ml of labeled probe. The filter was washed at 45°C in 3.0 M tetramethylammonium chloride (Aldrich)–50 mM Tris (pH 8.0)–2.0 mM EDTA–1% sodium dodecyl sulfate (SDS) as previously described (40). *Eco*RI fragments of *T. pallidum* genomic DNA in the molecular weight range identified by Southern blot analysis with the oligonucleotide 28-B were ligated into the arms of the lambda ZAP II vector (Stratagene). Following ligation of the DNA, the library was packaged, plated, and amplified according to the manufacturer's recommendations. Approximately 10,000 plaques were plated, transferred to filters in duplicate, and processed as previously described (20). The oligonucleotide 28-B was radiolabeled as described above and used for plaque hybridizations. Positive recombinant pBluescript SK(−) clones were recovered by in vivo excision according to the manufacturer's instructions. Following restriction mapping, appropriate DNA fragments were subcloned into pBluescript KS and sequenced by primer walking on both strands, using the dideoxynucleotide chain termination method described by Sanger et al. (31).

DNA and protein sequence analysis. The DNA sequence was analyzed by the DNA Strider version 1.0 program (21). Homology searches were performed with the FASTA and Profilesearch programs, which are found in the University of Wisconsin Genetics Computer Group, Inc., package, version 7.0 (10). Hydrophobicity and beta-moment analysis were determined by using the Moment program at the laboratory of David Eisenberg, Molecular Biology Institute, University of California at Los Angeles (11).

Synthetic oligonucleotides and PCR. Oligonucleotides were synthesized with an Applied Biosystems model 470B automated DNA synthesizer (Foster City, Calif.). PCR was used to amplify the *tromp2* gene. For generating a PCR product encoding Tromp2 including its signal peptide, the primers used were 5′-GGAA TTCCATATGAAACAGGGCTGTTTTATG-3′ and 5′-CCGGAATTCTCATT TGCCGCTCTCTCCTTCC-3′ containing *Nde*I and *Eco*RI restriction endonuclease sites, respectively. PCR was performed according to the manufacturer's instructions by using AmpliTaq (GeneAmp; Perkin-Elmer Cetus, Foster City, Calif.) and a programmable thermal controller (TTC-100; M. J. Research Inc., Watertown, Mass.). Briefly, 50-μl PCR mixtures were incubated with 5 μl of recombinant phage stock containing the *tromp2* gene as the template, 0.5 μM primers, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.001% (wt/vol) gelatin, 200 μM each deoxynucleotide triphosphate, and 1.25 U of AmpliTaq. After being overlaid with 50 μl of mineral oil, the PCR mixtures were incubated 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 58°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 *tromp2* for expression in *E. coli*. The *tromp2* PCR products described above were ligated into pET17b (Novagen, Inc.) previously digested with *Nde*I and *Eco*RI. The resulting constructs were transformed into *E. coli* BL21 DE3 pLysE (Novagen, Inc.) with cells made competent by $CaCl_2$ as previously described (20).

***E. coli* fractionation of cells expressing rTromp2.** The outer membrane, inner membrane, and soluble fractions were isolated from *E. coli* expressing rTromp2 containing its export signal peptide. One hundred microliters of a 5-ml overnight culture of a BL21 DE3 pLysE *tromp2* transformant (optical density at 600 nm [OD_{600}] = 2.1) was used to inoculate 5 ml of LB/amp/cam. The culture was grown to an OD_{600} of 0.5 and then induced with IPTG (isopropyl-β-D-thiogalactopyranoside) at a final concentration of 1 mM for 2 h. Organisms were centrifuged at 3,000 × *g* for 15 min, and the pellet was resuspended into 5 ml of PBS, frozen at −20°C, and then thawed at room temperature. The bacteria were disrupted by three passages through a French pressure cell at a gauge setting of 600. The resulting disrupted bacterial suspension was centrifuged at 13,000 × *g* for 1 min in order to remove unbroken cells and then for 30 min to pellet the total membrane fraction (the supernatant was saved and represents the total soluble fraction). The membrane pellet was then resuspended in 1 ml of 2% Triton X-100 in PBS and incubated 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 resulting 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 into 20 μl of PBS. Purity of the inner and outer membrane fractions with this protocol has been shown recently with antisera to the *E. coli* outer membrane protein OmpA and the *E. coli* inner membrane protein F_1F_0 ATPase C subunit, respectively (4). Samples were prepared for SDS-polyacrylamide gel electrophoresis (PAGE) by boiling for 10 min in a final sample buffer (FSB) consisting of 4% SDS, 10% 2-mercaptoethanol, 10% glycerol, and 0.01% bromophenol blue in 62.5 mM Tris buffer, pH 6.8, or by incubating at room temperature for 30 min in 0.2% SDS, 10% glycerol, and 0.01% bromophenol blue in 62.5 mM Tris buffer, pH 6.8. Samples were then separated by SDS-PAGE (12.5% acrylamide gels) and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, Mass.) as previously described (37).

Antisera. Antisera against *E. coli* outer membranes with and without rTromp2 were generated as follows. Outer membrane fractions were prepared from nonrecombinant and *tromp2* recombinant in *E. coli* as described above. Approximately 100 μg of rTromp2 in *E. coli* outer membrane, as determined by SDS-PAGE Coomassie blue-stained visualization, and a similar amount of control outer membrane material were inoculated subcutaneously and intramuscularly into New Zealand White male rabbits without the addition of adjuvant. Four weeks after the initial immunization, the rabbits were boosted by the same routes with similar prepared material. Serum obtained was adsorbed 10 times with whole-organism preparations of BL21 DE3 pLysE/pET17b.

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* cells. IRS was adsorbed 10 times with sonicated and whole-organism preparations of BL21 DE3 pLysE/pET17b.

Alkali and salt treatment of purified outer membrane from *T. pallidum*. The isolation and purification of the *T. pallidum* outer membrane were performed as previously described (5). Approximately 10^9 *T. pallidum* outer membrane equivalents were used for each reaction. The outer membrane was concentrated by centrifugation at 47,800 × *g* for 45 min followed by resuspension in 200 μl of either 1% SDS, 0.1 M Na_2CO_3 (pH 11.5), 0.1 N NaOH (pH 11.0), or 1 M NaCl. Following incubation for 15 min at room temperature, the samples were centrifuged at 47,800 × *g* for 45 min. The supernatants were carefully removed, and the proteins were acetone precipitated and resuspended in 15 μl of FSB. The pellets were resuspended in 15 μl of 2× FSB. All samples were analyzed by SDS-PAGE and immunoblotting as described below.

Black lipid bilayer experiments using rTromp2 gel purified from *E. coli* outer membranes. Outer membrane fractions prepared as described above from *E. coli* cells expressing rTromp2 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% bromophenol blue in 62.5 mM Tris buffer, pH 6.8. Following SDS-PAGE, the 25- to 28-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 250 μl of 0.1% Triton X-100 and 100 mM NaCl. Ten microliters from each eluted sample was separated by SDS-PAGE, transferred to polyvinylidene difluoride nylon membranes as previously described (37), and then probed with the adsorbed rTromp2 antiserum. Antibody-antigen binding on immunoblots was detected with anti-rabbit immunoglobulin conjugated to horseradish peroxidase (Amersham, Buckinghamshire, United Kingdom) and the enhanced chemiluminescence system of Amersham. Dilutions in 1 M KCl of the remaining 240-μl samples were tested for porin activity with the black lipid bilayer assay as described previously (1, 4, 5, 16). Pore-forming ability was assessed by applying a voltage of 50 mV across the lipid bilayer to which samples were added and measuring increases in conductance.

Triton X-114 extraction of *T. pallidum* cells. Triton X-114 extraction and phase separation of 10^9 *T. pallidum* cells were carried out as previously described (8). Briefly, organisms were incubated for 2 h at 4°C in 1 ml of 1% Triton X-114 in PBS, pH 7.2. Following incubation, the suspension was centrifuged for 20 min at 13,000 × *g* in order to pellet the insoluble protoplasmic cylinders. Added to the supernatant was 100 μl of 10% Triton X-114, to give a final detergent concentration of 2%. The supernatant was then incubated at 37°C for 5 min, and the aqueous and detergent phases were separated by centrifugation at 13,000 × *g* for 5 min. Aqueous and detergent phases were extracted three times with 1% Triton X-114 and PBS, respectively. Protein contained in the final aqueous and detergent phases was precipitated with 10 volumes of ice-cold acetone and resuspended in FSB. Equal amounts of each fraction were analyzed by SDS-PAGE and immunoblotting as described above.

Immunoelectron microscopy. Whole-mount immunoelectron microscopy was performed with *E. coli* cells expressing rTromp2 as follows. Five milliliters of LB/amp/cam was inoculated with 100 μl of an overnight culture of the BL21 DE3 pLysE *tromp2* transformant or a BL21 DE3 pLysE nonrecombinant pET17b control. The cultures were grown in a shaker at 37°C to an OD_{600} of 0.4, at which time the cultures were induced with IPTG at a final concentration of 1 mM and grown for an additional 2 h. After incubation, the cultures were centrifuged at 13,000 × *g* for 5 min followed by resuspension of the pellets in 5 ml of PBS. The cells from a total of 0.2 OD_{600} units from each suspension were pelleted by centrifugation at 13,000 × *g* for 5 min, resuspended into 50 μl of PBS, and combined with 50 μl of adsorbed IRS. The mixtures were then incubated at 37°C in a shaker for 3 h followed by three washes involving centrifugation and resuspension in 1 ml of PBS and a final resuspension 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 of specimen drops. The grids were washed once in PBS and then blocked by incubation at room temperature in 0.5% bovine serum albumin (Sigma Chemical Co.)–PBS. After the blocking step, the grids were washed three times in PBS followed by incubation for 1 h at room temperature in goat anti-rabbit immunoglobulin conjugated to 10-nm-diameter colloidal gold particles (Amersham) diluted 1:10 in 0.01% bovine serum albumin–PBS. The grids were then 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 an additional time in double-distilled water, and then examined in an electron microscope (JEOL 100 CX) at an 80-kV accelerating voltage. The average number and standard deviation of bound gold particles on test and control *E. coli* cells were determined by counts made from 10 organisms.

Nucleotide sequence accession number. The DNA sequence reported here has been deposited in GenBank under the accession number U65743.

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                                                    -36
                                                    CCTCGAGCGTTAGGGGTGGTGAAAAGGAGTTTGCGA
1                                                                                       60
ATG AAA CAG GGC TGT TTT ATG GTG GCG GGC TTT GCG CTG ACG TGC GCG TTT TTG GTG TCC
 M   K   Q   G   C   F   M   V   A   G   F   A   L   T   C   A   F   L   V   S
61                                                                                     120
CCC CTT GCG GCG CAA AGG TCG AAG GTC AAT TAC CAG GCA TAC TTC ATA GAT GAC TTT GAC
 P   L   A   A ↑ Q   R   S   K   V   N   Y   Q   A   Y   F   I   D   D   F   D
121                                                                                    180
GGT GCC TCC GAG GAT CAG GGT CTC GCC TGG CGT GCT GCG GGG AGC AAG TTC ATC ACA AAG
 G   A   S   E   D   Q   G   L   A   W   R   A   A   G   S   K   F   I   T   K
181                                                                                    240
GGC TTC CCT ATC CTC AAG TAT TTC GAG GGC ATG CCA CAG GCG GTG CGC ATG GCG GGC TCG
 G   F   P   I   L   K   Y   F   E   G   M   P   Q   A   V   R   M   A   G   S
241                                                                                    300
TGG CAA GGG AAG GAC AAG GAA GCC CGG TTC ATC GGA GTA GAG TGC AAG TTC AAT CGA CAG
 W   Q   G   K   D   K   E   A   R   F   I   G   V   E   C   K   F   N   R   Q
301                                                                                    360
GGG AAT AAC TGG CTG GAC CTA ATT CCG ACT AAG GGT GGT AGC GAT TAC GAG ATC CCC CTG
 G   N   N   W   L   D   L   I   P   T   K   G   G   S   D   Y   E   I   P   L
361                                                                                    420
CGT GGG GTG GTC AGT GGG TTC GAC GTG TGG GTG TGG GGT GCA GGT TAT CAG TAC TCG CTC
 R   G   V   V   S   G   F   D   V   W   V   W   G   A   G   Y   Q   Y   S   L
421                                                                                    480
GAG GCT TTG GTT AGG GAC TGC ACG GGA CGA GTC CAC ACC CTC CTA ATA GGC AAC CTC GAC
 E   A   L   V   R   D   C   T   G   R   V   H   T   L   L   I   G   N   L   D
481                                                                                    540
TTC CAA GGG TGG AAG AAC CTT AGT GTT TCG GTT CCC ACA CAC ATC CCA CAG ACG TCG CGC
 F   Q   G   W   K   N   L   S   V   S   V   P   T   H   I   P   Q   T   S   R
541                                                                                    600
TAT TTG GGG AGC GCG CAA CAC CTG AGT TTT GTC GGT TTC AGG ATC CGT ACT AGC CCA TCA
 Y   L   G   S   A   Q   H   L   S   F   V   G   F   R   I   R   T   S   P   S
601                                                                                    660
GAG CGG GTG GAT GAT TTC TAC GTG TTC TTT GAC CAG TTC AAG GCG CTT GCT AAC ATG CAT
 E   R   V   D   D   F   Y   V   F   F   D   Q   F   K   A   L   A   N   M   H
661                                                                                    720
ATC GAC TTT TAC GAT GGA CAC GAG TTG AGA AAT GCT CAG TTC AAG GAG GAA GGA GAG AGC
 I   D   F   Y   D   G   H   E   L   R   N   A   Q   F   K   E   E   G   E   S
721                                                                                    780
GGC AAA TGA
 G   K
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FIG. 1. Nucleotide sequence of the *T. pallidum tromp2* gene. The deduced amino acid sequence is shown below the nucleotide sequence. The putative ribosome-binding site is indicated by the double-underlined sequence. Sequences obtained from the tryptic peptides are indicated by the single-underlined regions. The vertical arrow indicates the putative position of signal peptide processing.

RESULTS

Cloning of the *tromp2* structural gene. To isolate the *tromp2* gene, a mixed oligonucleotide was generated based on amino acid sequences obtained from tryptic digestion of the native 28-kDa outer membrane protein. The mixed oligonucleotide hybridized to a 4.2-kb *Eco*RI restriction fragment identified by Southern blot analysis of *T. pallidum* genomic DNA (data not shown). DNA fragments in the size range of 3 to 5 kb were excised from the agarose gel and ligated into the lambda ZAP II vector (Stratagene). After plating, hybridization using again the mixed oligonucleotide identified four positive plaques which were converted to the pBluescript SK(−) recombinant plasmid by in vivo excision. All four clones contained the same 4.2-kb *Eco*RI insert. One clone, designated pTpT2-d, was chosen for further analysis. A partial restriction map of pTpT2-d was constructed, and Southern analysis with the mixed oligonucleotide identified a region of hybridization to a 1.1-kb *Bam*HI-*Eco*RI fragment (data not shown).

DNA sequence analysis. DNA sequence analysis of the 4.2-kb insert revealed an open reading frame of 726 bp encoding a 242-residue protein of 27,216 Da (Fig. 1). A Shine-Dalgarno ribosome-binding sequence was identified 12 bp upstream from the ATG start codon (Fig. 1). As shown in Fig. 2A, Kyte-Doolittle hydropathy analysis of the deduced amino acid sequence showed that the primary sequence was predominantly hydrophilic except for a 24-residue hydrophobic N-terminal region consistent with an export signal peptide. This putative signal peptide terminates in a typical leader peptidase I cleavage site of leucine-alanine-alanine (38), indicating that Tromp2 is not a lipoprotein. The processed Tromp2 protein of 218 residues would have a calculated mass of 24,759 Da and a calculated pI of 7.3. A search of the GenBank database did not reveal any significant amino acid sequence homologies.

Further analysis of the mature protein sequence revealed characteristics in common with other gram-negative outer membrane proteins. Secondary structure analysis identified nine regions of amphipathic beta-sheets, most (six of nine) of which were reflected as peaks by hydrophobic-moment analysis (Fig. 2B). As shown in Fig. 3, a topological outer membrane model of Tromp2 with nine membrane-spanning regions which conform to an alternating patter of hydrophobic residues is proposed.

Localization of Tromp2 in *T. pallidum* cells following Triton X-114 extraction and phase separation. Triton X-114 extraction of *T. pallidum* cells was carried out as described previously under conditions which have demonstrated the complete sol-

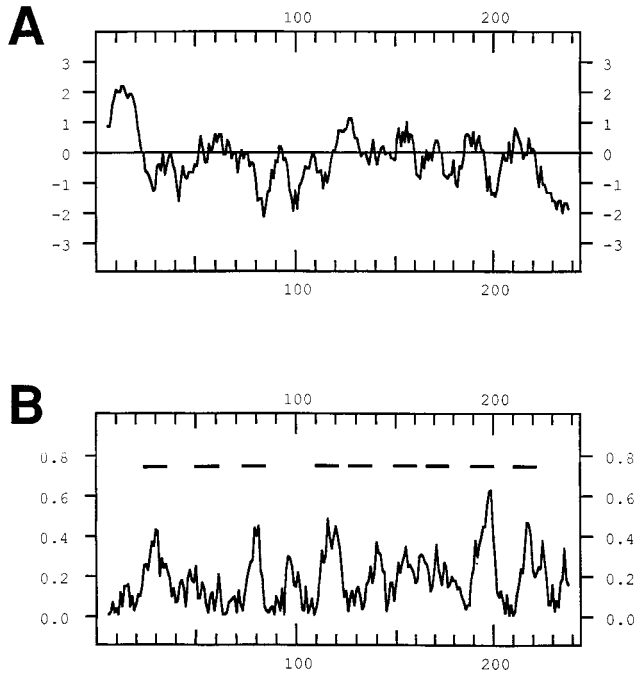

FIG. 2. Kyte-Doolittle and beta-moment plots of the derived amino acid sequence of Tromp2. (A) Kyte-Doolittle analysis. Positive values indicate regions of hydrophobicity. (B) Beta-moment plot. Horizontal bars are predicted regions where transmembrane segments may occur in Tromp2.

ubilization of the *T. pallidum* outer membrane and the release of some inner membrane-anchored lipoproteins (8, 29). A characteristic of integral membrane proteins, including outer membrane proteins from gram-negative bacteria, is their selective partitioning into the Triton X-114 detergent phase (36). As shown in Fig. 4B, anti-rTromp2 serum detected Tromp2 in whole-cell extracts (lane W) and in the detergent-phase fraction (lane D). Unlike Tromp1 (Fig. 4C, lane A), a small amount of Tromp2 was also detected in the aqueous phase (Fig. 4B, lane A), suggesting that the hydrophobic nature of Tromp2 is sensitive to these extraction conditions, and was presumably no longer folded in a hydrophobic conformation. Again unlike Tromp1 (Fig. 4C, lane P), a small amount of Tromp2 also remained with the protoplasmic cylinders (Fig. 4B, lane P). These findings indicate that Tromp2 is a protein which has hydrophobic properties consistent with those of other outer membrane proteins.

Evidence that Tromp2 is an integral membrane protein. To demonstrate that Tromp2 was integrated rather than peripherally associated with the outer membrane of *T. pallidum*, purified outer membranes were treated with alkali and high salt concentrations, conditions which release nonintegral membrane proteins while leaving the membrane lipid bilayer structure intact (14, 18, 35). Immunoblot analysis of soluble fractions and insoluble membrane fractions revealed that Tromp2 remained anchored to the insoluble membrane fraction (Fig. 5, lanes P) when treated with 0.1 M Na_2CO_3, 0.1 N NaOH, and 1.0 M NaCl. By comparison, when membranes were solubilized with 1% SDS, Tromp2 was found in the soluble fraction (lane S). As a further test, the same blot was incubated with anti-Tromp1 serum which showed results identical to those of Tromp2 (data not shown). These findings further demonstrate that Tromp2 and Tromp1 are integral membrane proteins.

Expression and fractionation of Tromp2 in *E. coli* cells. In order to determine if rTromp2 expressed in *E. coli* cells can target the outer membrane, the *tromp2* gene, including its signal peptide, was cloned into the expression plasmid pET17b and transformed into *E. coli* BL21 DE3 pLysE. Based on immunoblot analysis with anti-rTromp2 serum and IRS, overexpression under the control of an inducible T7 promoter resulted in high levels of nontoxic expression of

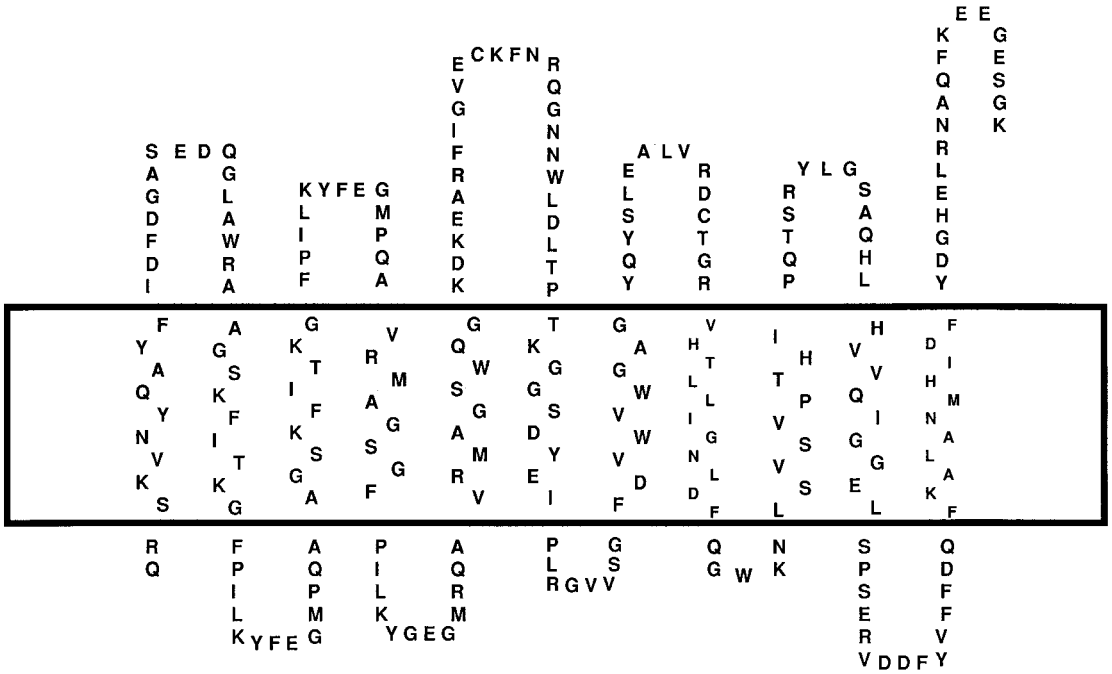

FIG. 3. Proposed topology of Tromp2. Sequences within the rectangle indicate nine membrane-spanning regions having amphipathic beta-sheet structures. Sequences above the rectangle indicate six surface-exposed loops. Sequences below the rectangle indicate regions exposed to the periplasmic space.

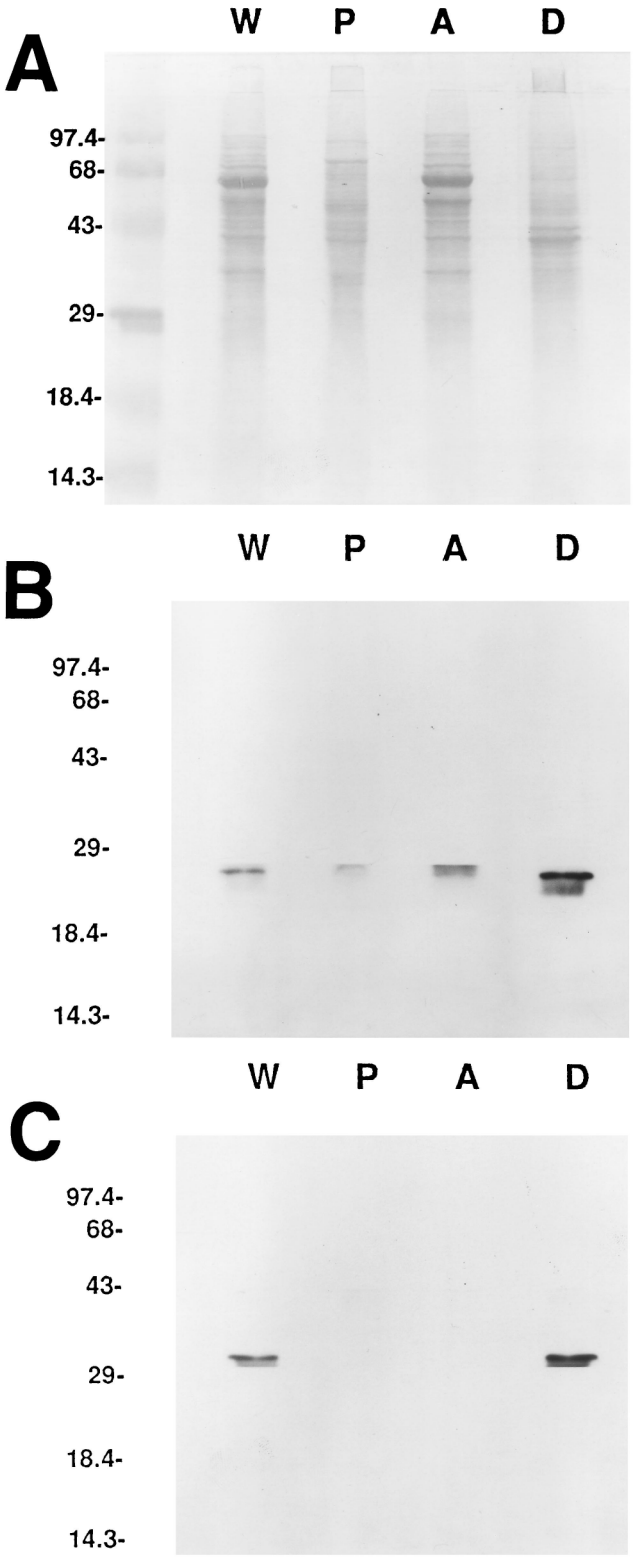

FIG. 4. Immunoblot analysis of Triton X-114-extracted and phase-partitioned material from 10^9 *T. pallidum* cells. W, whole organisms; P, protoplasmic cylinders; A, Triton X-114 aqueous-phase proteins; D, Triton X-114 detergent-phase proteins. Shown are an amido black-stained immunoblot (A), the immunoblot probed with a 1:1,000 dilution of anti-rTromp2 serum (B), and the immunoblot shown in panel B stripped of antibody and reprobed with a 1:1,000 dilution of anti-rTromp1 serum (C). The numbers to the left of each blot are the molecular masses of protein standards (in kilodaltons).

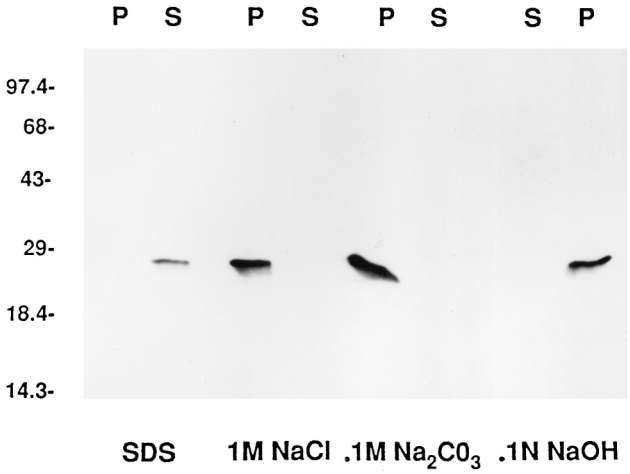

FIG. 5. Immunoblot of alkali and high-salt concentration treatments of purified *T. pallidum* outer membrane. The outer membrane was treated with 1.0 M NaCl, 0.1 M Na_2CO_3 (pH 11.5), and 0.1 N NaOH (pH 11.0) followed by centrifugation to separate soluble material (S) from the membrane-pelleted (P) material. As a solubilization control, the outer membrane was treated with 1% SDS. The samples were separated on an SDS–12.5% polyacrylamide gel, transferred to Immobilon-P, and incubated with a 1:1,000 dilution of anti-rTromp2 serum. Molecular masses (in kilodaltons) of prestained protein markers are indicated on the left.

rTromp2 (Fig. 6A, lane 2, and Fig. 6B, lane WO). Both native and recombinant Tromp2 migrated at the same molecular mass, although a small amount of rTromp2 was observed at a slightly higher form, which may represent the unprocessed form in *E. coli*. Following *E. coli* fractionation, rTromp2 was exclusively detected in the outer membrane fraction (Fig. 6B, lane OM) and not in the soluble (lane SOL) or inner membrane (lane IM) fractions. When low concentrations of SDS at room temperature were used to solubilize the outer membrane, two higher forms of rTromp2 at approximately 50 and 75 kDa (Fig. 6B, lane OM, 0.2% SDS) resulted.

Black lipid bilayer analysis of rTromp2 isolated from the *E. coli* outer membrane. Because sufficient amounts of native Tromp2 for the porin assay cannot be obtained at this time, we tested the recombinant form for porin activity. rTromp2 was isolated from *E. coli* outer membranes by low-concentration SDS solubilization followed by separation by SDS-PAGE. Under these conditions, *E. coli* porins migrate at approximately 97 to 110 kDa due to their SDS-stable trimer conformation. The 25- to 28-kDa region of SDS-PAGE-separated *E. coli* outer membranes, from both the nonrecombinant control and rTromp2-expressing clone, were excised and eluted into 0.1% Triton X-100 and 100 mM NaCl. The recovery of rTromp2 was confirmed by immunoblot analysis (data not shown). The rTromp2-containing sample and the control sample were tested for porin activity by the black lipid bilayer assay (1, 4, 5, 16). Unlike native Tromp1 or rTromp1 (3, 4), the addition of the sample containing rTromp2 to the model membrane system resulted in only a small number of insertional events (data not shown). A similar and greater amount of control sample showed no porin activity. While some activity was demonstrated for rTromp2, the total number of insertional events for rTromp2 was considerably less than expected for the amount of protein present in the sample. Therefore, a definitive statement as to whether Tromp2 is a porin protein cannot be made at this time.

Surface antigenic exposure of rTromp2 expressed in *E. coli*. The detection of surface-exposed rTromp2 epitopes on *E. coli*

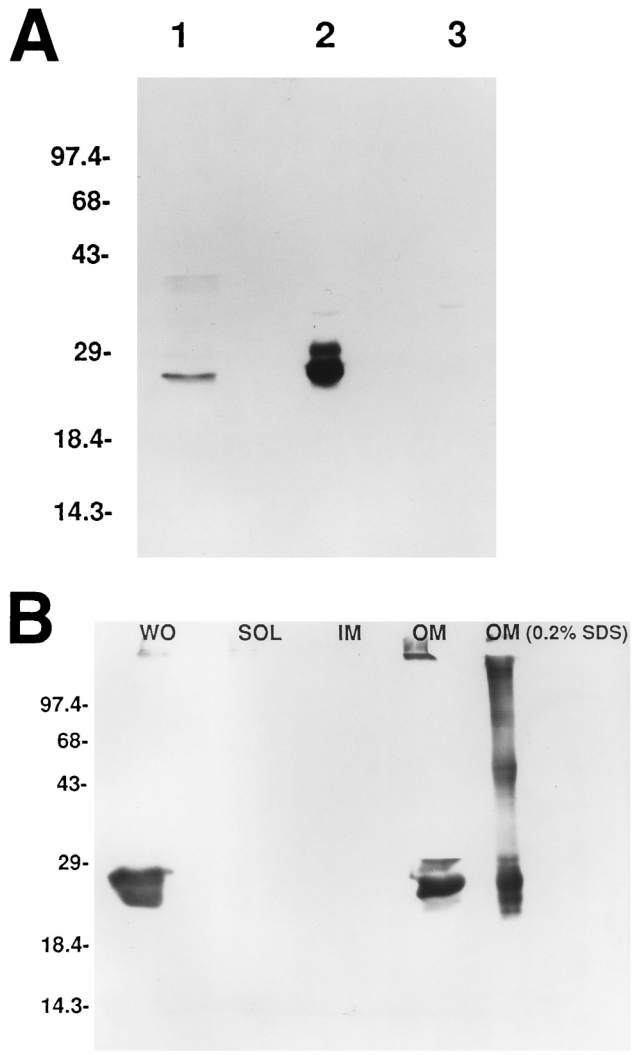

FIG. 6. Expression of rTromp2. The gene encoding the Tromp2 protein, including its signal peptide, was cloned into the pET17b plasmid and expressed in *E. coli* cells. (A) Comparison of native Tromp2 and rTromp2. The immunoblot contains 10^9 *T. pallidum* cells (lane 1), 5×10^8 *E. coli* cells expressing rTromp2 (lane 2), and 5×10^8 *E. coli* cells harboring a nonrecombinant plasmid control (lane 3). (B) Fractionation of *E. coli* cells expressing rTromp2. The immunoblot contains 5×10^8 *E. coli* whole organisms expressing rTromp2 (WO), 5×10^8 cell equivalents of the soluble fraction (SOL), 1×10^9 cell equivalents of the inner membrane fraction (IM), 1×10^9 cell equivalents of the outer membrane fraction (OM), and 1×10^9 cell equivalents of the outer membrane fraction solubilized at room temperature in 0.2% SDS (OM 0.2% SDS). The immunoblots in panels A and B were probed with a 1:1,000 dilution of anti-rTromp2 serum and IRS, respectively. The numbers to the left of each blot are the molecular masses of protein standards (in kilodaltons).

cells was investigated by whole-mount immunoelectron microscopy. As shown in Fig. 7B, adsorbed IRS bound to the surface of *E. coli* cells expressing rTromp2 (average and standard deviation of 31.8 ± 4.07 gold particles/organism). The specificity of this reaction was demonstrated by the limited amount of antibody binding to *E. coli* cells harboring a nonrecombinant plasmid control (average and standard deviation of 1.1 ± 0.73 particles/organism) (Fig. 7A).

DISCUSSION

The outer membranes of spirochetes and gram-negative bacteria function as selective permeable barriers, allowing the entrance of nutrients into the cell and at the same time protecting the cell from the harsh environment. The outer membrane of *T. pallidum* is unusual compared to those of gram-negative bacteria in that it contains 100-fold less membrane-spanning protein. Our recent ability to isolate and purify the outer membrane of *T. pallidum* (5) has been a crucial step forward in the characterization of its protein constituents as well as in providing a foundation for future understanding of the molecular basis of syphilis pathogenesis. In that study, porin activity was demonstrated in the purified outer membrane of *T. pallidum*. Based on the fact that most porin proteins have molecular masses between 28 and 48 kDa (25), we focused on the isolation of two outer membrane protein constituents of 28 and 31 kDa in size. We recently reported on the cloning of the gene encoding the 31-kDa rare outer membrane protein, designated Tromp1, and demonstrated that purified native Tromp1 and rTromp1 showed porin activity (3, 4).

In this study, we report the cloning, sequencing, and expression of the 28-kDa rare outer membrane protein of *T. pallidum*, designated Tromp2. As was the case with Tromp1, conventional N-terminal amino acid sequence analysis of Tromp2 was not possible, due to the extremely small amount of Tromp2 in the outer membrane. In fact, we estimate that the amount of Tromp2 in the outer membrane of *T. pallidum* is three- to fourfold less than that of Tromp1. In order to obtain an amino acid sequence, internal peptide sequences were generated with as little as 10 pmol of trypsin-digested Tromp2. The amino acid sequences obtained from Tromp2 were used to make degenerate mixed oligonucleotides for use as probes to identify and ultimately clone the *tromp2* gene from a *T. pallidum* genomic library.

The *tromp2* gene encodes a protein whose deduced amino acid sequence and secondary structure is consistent with that of an outer membrane protein. First, the predicted N-terminal amino acid sequence is a typical procaryotic signal peptide ending in a leader peptidase I cleavage site (38), allowing export through the inner membrane. Next, application of the Kyte-Doolittle algorithm indicates no strongly hydrophobic regions of the mature Tromp2 sequence, as is the case for gram-negative outer membrane proteins as well as for the two previously described pathogenic spirochetal outer membrane porin proteins, OmpL1 from *Leptospira kirschneri* and Tromp1 (4, 15). Last, a beta-moment plot of the mature sequence of Tromp2 predicted nine membrane-spanning amphipathic beta-sheet segments, similar to those found for other gram-negative outer membrane proteins, including porins (19).

Unlike rTromp1 expression (3), overexpression of rTromp2 containing its native signal peptide in *E. coli* was not lethal. Results of immunoblot analysis showed that T7 RNA polymerase-induced conditions resulted in the stable expression, export, and exclusive outer membrane localization of rTromp2. By comparison, we have previously shown that rTromp1, which also localizes to the outer membrane, was detected in the soluble fraction of *E. coli* preparations (3). Thus, in this regard, rTromp2 was much more efficient in outer membrane targeting than rTromp1. The use of IRS to detect rTromp2 in these experiments indicates that native Tromp2 is recognized by the host immune response during the course of rabbit syphilitic infection. We have also found by immunoblot analysis that serum from syphilitic humans recognizes rTromp2 (data not shown). However, like Tromp1, Tromp2 is also relatively weakly antigenic compared to the more abundant and strongly antigenic lipoproteins and endoflagellar proteins of *T. pallidum.*

Evidence to support the outer membrane location of Tromp2 was demonstrated by Triton X-114 detergent extraction and phase separation of whole *T. pallidum* cells. It was found that Tromp2 fractionated preferentially into the deter-

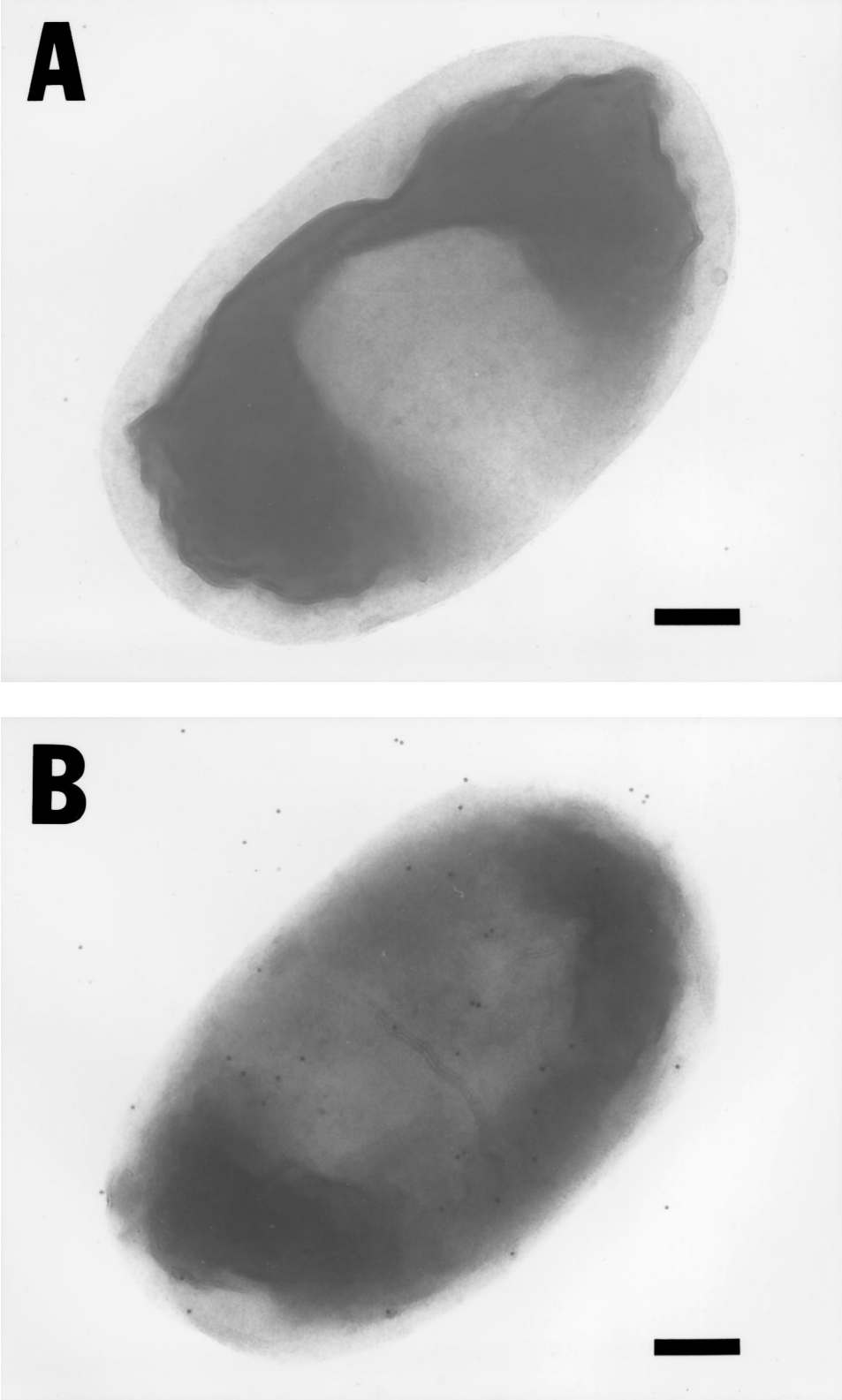

FIG. 7. Surface antigenic exposure of rTromp2 expressed in *E. coli* cells. Whole-mount immunoelectron microscopy was used to detect surface-exposed epitopes of rTromp2. *E. coli* cells were incubated in serum from a syphilitic rabbit immune to challenge reinfection. Antibody bound to the surface of *E. coli* cells was detected by anti-rabbit immunoglobulin G conjugated to 10-nm-diameter colloidal gold particles. (A) *E. coli* cells harboring a nonrecombinant plasmid control; (B) *E. coli* cells expressing rTromp2. Bars, 0.2 μm.

gent phase, although some was also detected in the aqueous phase. These results demonstrate both a hydrophobic and a hydrophilic nature for Tromp2 when fractionated in Triton X-114. The fact that Tromp2 does partition to the detergent phase indicates its hydrophobic character, which is consistent with an outer membrane protein. The lesser amount of Tromp2 associated with the aqueous phase may suggest that its hydrophobic property is detergent sensitive and perhaps modified conformationally during Triton X-114 extraction. This phenomenon has been reported for other spirochetal membrane-spanning proteins. Recently, both Probert et al. and Skare et al. reported on surface-exposed outer membrane proteins from *Borrelia burgdorferi*, p66 and Oms28, respectively, which partition into the Triton X-114 aqueous phase (27, 32). Of further interest was the finding that Triton X-114 extraction did not result in the complete release of Tromp2, as indicated by the presence of Tromp2 associated with the protoplasmic cylinders. In contrast, Tromp1 was completely released by Triton X-114 and partitioned exclusively into the detergent phase as previously observed (4). This result may suggest a possible interaction of Tromp2 with some subsurface molecule or structure. Alternatively, Tromp2 may not be as efficiently exported as Tromp1, resulting in detectable amounts of Tromp2 associated with the inner membrane.

Further evidence to support the integral membrane association of both Tromp2 and Tromp1 was provided by treatment of purified *T. pallidum* outer membrane with alkali and high salt concentrations, conditions which release peripherally associated membrane proteins (14, 18, 35). Neither 1 M NaCl, 0.1 M Na_2CO_3 (pH 11.5), nor 0.1 N NaOH (pH 11.0) was found to release Tromp2 or Tromp1 (data not shown) from *T. pallidum* outer membrane, supporting the membrane-spanning topology of these two proteins.

Using the black lipid bilayer porin assay, we attempted to demonstrate porin activity with native Tromp2. However, because Tromp2 is present in such small amounts, efforts to obtain enough purified native material for the porin assay were unsuccessful. As an alternative approach, we tested rTromp2 in the black lipid bilayer assay, using samples from both *E. coli* control outer membranes (no rTromp2) and *E. coli* outer membranes with rTromp2. Single-channel conductance measurements of rTromp2 showed some activity, whereas the control had no activity. However, it was observed that the total number of insertional events for rTromp2 was extremely small compared to the amount of rTromp2 tested. This is in contrast to our previous studies using both native Tromp1 and rTromp1, where numerous insertional events were observed. One explanation may be that Tromp2 porin confirmation is detergent sensitive. In support of this possibility, some Tromp2, but not Tromp1, was found in the aqueous phase following phase separation with Triton X-114. As previously mentioned, unlike rTromp1, rTromp2 expression was not found to be lethal to *E. coli*, even under maximum inducing conditions. One interpretation of this result is that rTromp2 expression is not lethal because it does not form a pore upon insertion into the *E. coli* outer membranes. Taken together, a definitive statement as to whether rTromp2 functions as a porin is not possible at this time.

A further issue regarding the outer membrane localization of rTromp2 was whether antigenic epitopes were surface exposed. The results of whole-mount immunoelectron microscopy in fact demonstrated specific binding to the surface of *E. coli* cells expressing rTromp2, indicating that rTromp2 has surface-exposed epitopes.

As previously mentioned, the outer membrane of *T. pallidum* has an extremely low content of membrane-spanning protein (28, 39), a finding which has explained the surface antigenic inertness (9, 13, 17, 26, 28) of this spirochete and its relative resistance to bactericidal antibody (2, 13, 23, 24, 30). 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* cells (6). These studies have been extended, using serum obtained from infected animals with varying degrees of challenge immunity, and have shown that TROMP aggregation correlates directly with the development of challenge immunity (unpublished data). Implicit in these findings is that TROMPs represent the key surface-exposed targets for a protective host immune response. The ability now to express both rTromp1 and rTromp2 with surface antigenic exposure and in sufficient amounts provides an opportunity to assess directly the ability of these outer membrane proteins to elicit protective immunity in experimental syphilis. However, it should be noted that the correct outer membrane conformation of the TROMPs may prove to be a key factor in whether protective immunity results, as is the case for several bacterial porins (12, 33, 34).

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