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# Porin Activity and Sequence Analysis of a 31-Kilodalton *Treponema pallidum* subsp. *pallidum* Rare Outer Membrane Protein (Tromp1)†

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**We have recently reported the isolation and purification of the *Treponema pallidum* outer membrane and the identification of its rare protein constituents, including a 31-kDa protein markedly enriched in the outer membrane preparation (D. R. Blanco, K. Reimann, J. Skare, C. I. Champion, D. Foley, M. M. Exner, R. E. W. Hancock, J. N. Miller, and M. A. Lovett, J. Bacteriol. 176:6088–6099, 1994). In this study, we report the cloning, sequencing, and expression of the structural gene which encodes the 31-kDa outer membrane protein, designated Tromp1. The deduced amino acid sequence from the *tromp1* gene sequence encodes a 318-amino-acid polypeptide with a putative 40-amino-acid signal peptide. Processing of Tromp1 results in a mature protein with a predicted molecular mass of 30,415 Da and a calculated pI of 6.6. Secondary-structure predictions identified repeated stretches of amphipathic beta-sheets typical of outer membrane protein membrane-spanning sequences. A topological model of Tromp1 containing 14 transmembrane segments is proposed. Specific antiserum against a recombinant Tromp1 fusion protein was generated and was used to identify native Tromp1 in cellular fractionation. Upon Triton X-114 extraction and phase separation of *T. pallidum*, the 31-kDa Tromp1 protein was detected in the detergent-phase fraction but not in the protoplasmic cylinder or aqueous-phase fractions, consistent with a hydrophobic outer membrane protein. Anti-Tromp1 antiserum was also used to identify native Tromp1 purified from whole *T. pallidum* by Triton X-100 solubilization followed by nondenaturing isoelectric focusing. Reconstitution of purified Tromp1 into planar lipid bilayers showed porin activity based on the measured single channel conductances of 0.15 and 0.7 nS in 1 M KCl. These findings demonstrate that Tromp1 is a transmembrane outer membrane porin protein of *T. pallidum*.**

Syphilis is marked by months of clinically evident disease followed by years of latency, with the possibility of relapse if untreated. This chronic infection by *Treponema pallidum* subsp. *pallidum* (hereafter referred to as *T. pallidum*), the agent of syphilis, has been related to a striking property of its outer membrane. The *T. pallidum* outer membrane contains 2 orders of magnitude less membrane-spanning protein than do outer membranes of typical gram-negative bacteria (32, 40). The observation that these surface-exposed proteins are so rare as to be undetectable by conventional means (19, 29, 31) is in accord with the relative resistance of *T. pallidum* to complement-dependent antibody-mediated killing (5, 17, 27, 31). Freeze fracture electron microscopy has been the only method by which *T. pallidum* rare outer membrane proteins (TROMPs) have been directly visualized (32, 40). Immune serum-mediated aggregation of TROMP molecules has provided evidence that these proteins have surface-exposed antigenic sites (7). TROMP aggregation has also been suggested to be the prerequisite for complement activation and killing of *T. pallidum* cells (7). These observations imply that TROMPs represent targets essential for a protective humoral immune response and are the potential mediators of *T. pallidum* virulence.

Identification and study of TROMPs has been considered essential to the understanding of syphilis pathogenesis and immunity in molecular terms. However, the limited numbers of *T. pallidum* cells available from its cultivation in rabbits, the rarity of TROMP, and the lack of a procedure to isolate and purify the *T. pallidum* outer membrane have vastly complicated attempts to identify these proteins. We have recently reported a novel procedure to purify the *T. pallidum* outer membrane and define its protein constituents (6). The purified outer membrane exhibited porin activity and the enrichment of two proteins of 31 and 65 kDa, suggesting that these proteins were potential TROMP porin candidates.

In this report, we describe the cloning, sequencing, and expression of the gene which encodes the 31-kDa *T. pallidum* outer membrane protein, which we have designated Tromp1. Antiserum raised against recombinant Tromp1 was used to identify and isolate native Tromp1, which was shown to have porin activity similar to that of the purified outer membrane (6). This finding has confirmed that Tromp1 is a transmembrane outer membrane protein, the first to be identified for *T. pallidum.*

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† This paper is dedicated to the memory of Philip J. Bassford, Jr., a pioneer in studying the *T. pallidum* outer membrane.



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## MATERIALS AND METHODS

***T. pallidum.*** *T. pallidum*, subsp. *pallidum*, Nichols strain, was maintained by testicular passage in New Zealand White rabbits as described previously (25). *T. pallidum* used for all experiments was purified from contaminating host tissue material by a discontinuous Ficoll gradient as previously described (6).

***Escherichia coli.*** *E. coli* DH5α (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) was used as the host strain for transformations with recombinant DNA. *E. coli* PLK-F′ (Stratagene, La Jolla, Calif.) 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.

**Tromp1 isolation and amino acid sequencing.** The 31-kDa Tromp1 protein was prepared from purified *T. pallidum* outer membrane by two-dimensional electrophoresis as described previously (6). Electrophoresed outer membrane proteins from $3 \times 10^{10}$ treponemes were transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, N.H.) as previously described (6) and stained with 1% amido black. The stained 31-kDa protein spots from several transfers were excised, pooled, and processed for internal amino acid sequence analysis as described elsewhere (37), with modifications as follows. In situ proteolytic cleavage was performed with 0.5 μg of trypsin (Promega, Madison, Wis.) in 25 μl of 100 mM $NH_4HCO_3$ (supplemented with 10% acetonitrile and 0.3% Tween 80) at 37°C for 3 h. The resulting peptide mixture was reduced and S alkylated with 0.1% 2-mercaptoethanol (Bio-Rad) and 0.3% 4-vinyl pyridine (Aldrich, Milwaukee, Wis.) and fractionated by reversed-phase high-performance liquid chromatography (HPLC) (15). Identification of tryptophan- or cysteine-containing peptides was done by manual ratio analysis of $A_{297}$ and $A_{277}$, monitored with an Applied Biosystems (Foster City, Calif.) model 1000S diode-array detector. Chemical sequencing of two selected peptides was done with a model 477A amino acid sequencer (Applied Biosystems) with on-line analysis (120A HPLC system with PTH $C_{18}$ column [2.1 by 220 mm]; Applied Biosystems). Instruments and procedures were optimized for femtomole-level phenylthiohydantoin amino acid analysis as described previously (36). Analysis of the two selected peptides, designated C and A, respectively, yielded the following sequences: LSLEEAEFDPHVXF and GLQGVSTASEASAHDMQELAAFI. The underlined regions of each sequence were used to generate two sets of complementary oligonucleotides (designated C and C′ and A and A′), all having 64-fold degeneracy, to be used in identifying the gene encoding the 31-kDa protein, as described below.

**DNA purification and manipulations.** Approximately $10^{11}$ *T. pallidum* cells were used to prepare genomic DNA as previously described (9).

**Synthetic oligonucleotides.** Oligonucleotides used for PCR primers were synthesized with an Applied Biosystems model 470B automated DNA synthesizer and purified by oligonucleotide purification cartridge chromatography (Applied Biosystems).

**PCR.** PCR was performed according to the manufacturer's instructions by using AmpliTaq (GeneAmp; Perkin-Elmer Cetus, Norwalk, Conn.) and a Programmable Thermal Controller (PTC-100; M. J. Research Inc., Watertown, Mass.). Reactions with 50-μl-volume reaction mixtures 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, and 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 and sequencing of the *tromp1* gene.** A *T. pallidum* genomic library was constructed with the lambda ZAP II vector (Stratagene) as follows. Partial digests of *T. pallidum* genomic DNA using *Rsa*I, *Alu*I, *Hae*III, and *Sau*3A, each with a size range of 0.8 to 4 kb, were ligated to *Eco*RI adaptors (Bethesda Research Laboratories) and cloned into the arms of the lambda ZAP II vector. The ligated DNA was packaged with Gigapack II Gold packaging extract (Stratagene) and stored in 0.3% chloroform at 4°C. Approximately 25,000 plaques were plated, transferred to nitrocellulose filters (Schleicher and Schuell) in duplicate, and processed as previously described (23). Oligonucleotide probes C and A′ described above were used as PCR primers with *T. pallidum* genomic DNA to generate a 320-bp PCR product designated 31CA′, which was blunt ended with the Klenow fragment (Bethesda Research Laboratories) and ligated into *Eco*RV-digested pBluescript KS (Stratagene). The 31CA′ PCR product was labeled with [α-$^{32}$P]dATP by the random priming method (United States Biochemicals), and $10^6$ cpm of hybridization mixture was incubated overnight at 42°C with the nitrocellulose plaque lifts representing the lambda ZAP II *T. pallidum* genomic library. Following hybridization, the filters were washed at room temperature in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and were then washed successively at 65°C in 1× SSC, 0.5× SSC, and 0.1× SSC. The filters were then autoradiographed for 4 h with intensifying screens at −70°C with Kodak X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.). Phage plaques which hybridized with the 31CA′ PCR product were converted to pBluescript SK(−) plasmid DNA by in vivo excision according to the manufacturer's instructions (Stratagene). After restriction mapping, appropriate DNA fragments were subcloned into pBluescript KS (Stratagene) and sequenced by primer walking on both strands, using the dideoxynucleotide chain termination method described by Sanger et al. (33).

**DNA and protein sequence analysis.** The DNA sequence was analyzed by the DNA Strider version 1.0 program (24). 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 (12). 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 (14).

**Antisera.** Serum from syphilitic rabbits immune to challenge (immune rabbit serum; IRS) was acquired after 6 months from animals infected intratesticularly with $4 \times 10^7$ *T. pallidum* cells. Specific rabbit antiserum against Tromp1 was generated against a recombinant glutathione *S*-transferase (GST)-Tromp1 fusion protein as follows. The 31CA′ pBluescript recombinant was digested with *Bam*HI and *Hin*cII and ligated into pGEX-2T (Pharmacia Biotech Inc., Piscataway, N.J.) previously digested with *Bam*HI and *Sma*I. The resulting construct, 31CA′/pGEX-2T, was then transformed into *E. coli* DH5α. Expression of a 38-kDa GST-Tromp1 fusion protein (26 kDa of GST plus 12 kDa of 31CA′) was performed according to the manufacturer's instructions (Pharmacia Biotech Inc.). The GST-Tromp1 fusion protein was separated by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by staining for 10 min with 0.05% Coomassie brilliant blue in $H_2O$. The GST-Tromp1 band, containing approximately 100 μg of protein, was combined with Freund's complete adjuvant and inoculated subcutaneously and intramuscularly into a male New Zealand White rabbit. Four and eight weeks after the initial immunization, the rabbit was boosted by the same routes, using similarly prepared material combined in Freund's incomplete adjuvant. The serum obtained was absorbed three times with DH5α harboring the pGEX vector and purified GST protein.

IRS and absorbed anti-GST-Tromp1 serum were diluted 1:1,000 in phosphate-buffered saline (PBS) containing 5% nonfat dry milk (Carnation Co., Los Angeles, Calif.) and 0.1% Tween 20 (Sigma) prior to use on immunoblots. Antibody-antigen binding was detected with the enhanced chemiluminescence system of Amersham (Little Chalfont, Buckinghamshire, England) as described previously (1).

**Triton X-114 extraction of *T. pallidum* cells.** Triton X-114 extraction and phase separation of $10^9$ Ficoll-purified *T. pallidum* cells was carried out as previously described (11).

**Black lipid bilayer experiments using purified native Tromp1.** Approximately $2 \times 10^9$ *T. pallidum* cells in 100 μl of PBS were solubilized in 1% Triton X-100 (Sigma Chemical Co.) for 2 h at 4°C. The suspension was centrifuged at 13,000 × *g* in order to remove protoplasmic cylinders, and the supernatant was recovered and concentrated to 45 μl. To the sample was added CHAPS {3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate} detergent, Ampholines (Bio-Rad), pH 5 to 7, and glycerol, all at final concentrations of 2%. The sample was then subjected to nondenaturing isoelectric focusing (IEF) for 16 h at a constant voltage of 600 V in a polyacrylamide tube gel (0.2 by 12 cm) containing 2% Ampholines (Bio-Rad), pH 5 to 7, 9% sorbitol, and 2% CHAPS. Following electrophoresis, the IEF tube gel was cut into fractions and material from each fraction was eluted overnight at 4°C into 150 μl of 0.1% Triton X-100–100 mM NaCl. Twenty microliters from each fraction was dot blotted onto nitrocellulose (Schleicher and Schuell), using a Bio-Dot apparatus (Bio-Rad), according to the manufacturer's instructions. The membrane was then probed with antiserum against GST-Tromp1 and developed by enhanced chemiluminescence as described above. In order to assess the purity of isolated Tromp1, 100 μl of the fraction containing Tromp1 was subjected to denaturing two-dimensional gel electrophoresis (6) and to enhanced chemiluminescence immunoblot analysis, which has a sensitivity of detection to picogram levels (1). The immunoblot was sequentially probed with anti-GST-Tromp1 serum and IRS. As a control, IRS was also used to probe a two-dimensional immunoblot of Triton X-100-solubilized material released from $2 \times 10^9$ *T. pallidum* cells. To test for porin activity, 10 μl from the remaining 20 μl of the fraction containing Tromp1 (approximately 2 to 10 ng of protein) was diluted 1:50 in 1 M KCl and 10 μl of the diluted sample was added to 6 ml of 1 M KCl bathing solution used in the black lipid bilayer assay described previously (3, 6). Pore-forming ability was assessed by applying a voltage of 50 mV across the lipid bilayer and measuring increases in conductance.

**Nucleotide sequence accession number.** The DNA sequence reported here has been deposited in GenBank under the accession number U16363.

## RESULTS

**Cloning of the *tromp1* structural gene.** Amino acid sequence analysis following a tryptic digestion of the 31-kDa outer membrane protein yielded two sequences from which degenerate oligonucleotides were created. In order to use a specific DNA probe to identify the gene encoding the 31-kDa protein, a PCR product was generated from genomic *T. pallidum* DNA with the degenerate oligonucleotides as primers. The resulting 320-bp PCR product was cloned into pBluescript and se-

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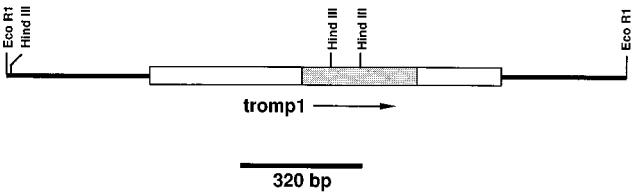


FIG. 1. Partial restriction map of the 1.67-kb *Eco*RI fragment containing the *tromp1* gene. The boxed area shows the coding region for Tromp1, and the shaded box indicates the position of the 320-bp PCR product.

quenced. The deduced amino acid sequence was identical, at the N- and carboxy-terminal regions, to the amino acid sequences determined for the C and A peptides from the 31-kDa protein. Southern blot analysis with endonuclease-digested *T. pallidum* genomic DNA and the PCR product as a probe indicated that the gene encoding the 31-kDa protein was present as a single copy in the *T. pallidum* genome (data not shown). The PCR product was then used as a probe to screen a lambda ZAP II phage library of *T. pallidum* genomic DNA.

Five positive plaques of the 25,000 plaques screened were picked, replated, and reprobed in order to purify phage bearing the hybridizing DNA fragment. One plaque, designated clone 2A, was selected for further analysis. Clone 2A recombinant phage was converted to pBluescript SK(−) plasmid by in vivo excision and recircularization. It was observed, however, that growth of *E. coli* harboring the rescued recombinant plasmid was poor, and the cells could not be cultured continuously either on solid media or in broth. As a result, recombinant plasmid DNA from clone 2A could be acquired only from cells scraped from solid media immediately following growth after in vivo excision and rescue. *Hin*dIII and *Eco*RI restriction analysis of recombinant plasmid DNA revealed an insert size of 1.67 kb and provided information relevant to subcloning this insert into three stable constructs: 850- and 75-bp *Hin*dIII-*Hin*dIII fragments and a 750-bp *Hin*dIII-*Eco*RI fragment (Fig. 1).

**Sequence analysis.** Sequence analysis of the 1.67-kb insert revealed a single open reading frame of 954 bp encoding a 318-residue protein of 34,722 Da (Fig. 2). *E. coli*-like −35 (TTATGC) and −10 (TATCAT) promoter regions and a putative ribosome binding site (GGGT) were identified upstream from the ATG start codon. The deduced amino acid sequence begins with a 40-residue hydrophobic region presumed to be a signal peptide, which is consistent with a protein exported across the cytoplasmic membrane. This region conforms to the rules for procaryotic signals (39), including a basic amino-terminal region (amino acids 1 through 15), a hydrophobic core (amino acids 16 through 30), and a carboxy-terminal region with a putative leader peptidase I cleavage site of Ala-X-Ala. *E. coli* expression of a fusion construct of the *E. coli* OmpT signal peptide fused to the Tromp1 sequence following Ala-X-Ala results in a processed protein which migrates in SDS-PAGE in a manner identical to that of native Tromp1, supporting this site as the location of leader peptidase I processing (data not shown).

The processed Tromp1 protein of 278 residues would have a calculated mass of 30,415 Da and a calculated pI of 6.6, both of which are in accord with the observed properties of native Tromp1 upon two-dimensional gel electrophoresis (6). Downstream of the termination codon is a 5-bp inverted repeat not followed by deoxyribosylthymine nucleotides, which may represent a rho-dependent transcription terminator (21). A search of the GenBank database showed that Tromp1 has 27.9% identity to the type 1 fimbrial adhesin of *Streptococcus parasanguis* and 27.1% identity to the adhesin B of *Streptococcus sanguis*. These adhesins are part of a larger family of related streptococcal adhesins, including those from *Streptococcus pneumoniae* and *Enterococcus faecalis* (22).

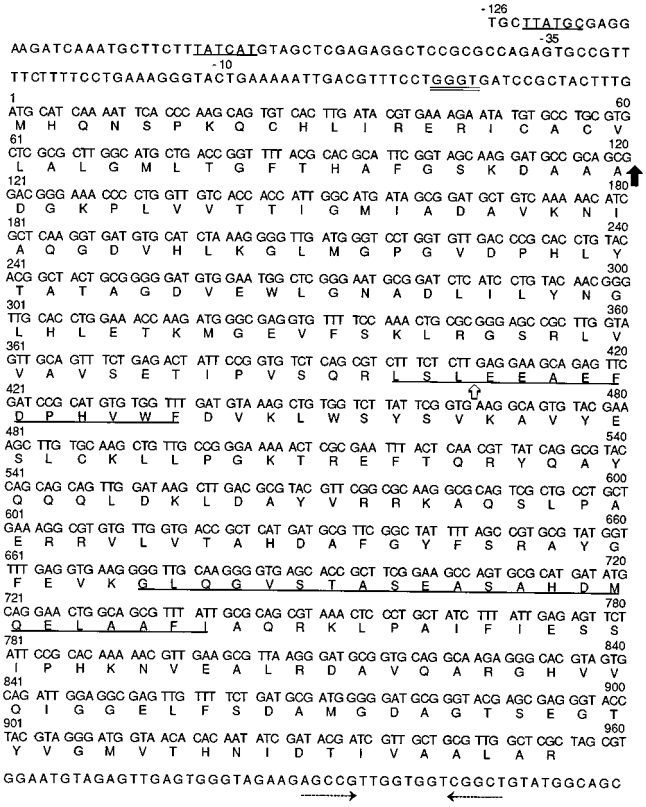


FIG. 2. Nucleotide sequence of the *T. pallidum tromp1* gene. The deduced amino acid sequence is shown below the nucleotide sequence. Putative −35 and −10 promoter regions are shown. The putative ribosome binding site is indicated by the double-underlined sequence. Sequences obtained from the tryptic peptides are indicated by the single-underlined amino acid residues. The solid vertical arrow indicates the putative position of signal peptide processing. The open vertical arrow indicates the position of the GST fusion. The inverted horizontal arrows indicate a region of potential stem-loop formation.

The mature protein sequence had characteristics in common with gram-negative outer membrane proteins. Secondary-structure analysis identified 14 regions of amphipathic beta-sheets, most of which were reflected as peaks by beta-moment analysis (data not shown). As shown in Fig. 3, a topological model with 14 membrane-spanning regions which conform to an alternating pattern of hydrophobic residues is proposed for Tromp1. Also proposed are seven surface-exposed loops of various lengths, which contain all of the regions with high surface probability, hydrophilicity, and antigenic indices determined by using the PLOTSTRUCTURE program (data not shown). Lastly, six periplasmic loops, which are typical of those found in outer membrane proteins in that they are short and usually contain the turn-promoting residues of proline, glycine, serine, or arginine, are proposed.

**Identification of native and recombinant Tromp1.** Specific antiserum generated against a 38-kDa GST-Tromp1 fusion protein was used to identify native and recombinant Tromp1 (Fig. 4B). The 31-kDa native Tromp1 protein was detected in a sample of $10^9$ *T. pallidum* cells (Fig. 4B, lane 1). The 31-kDa recombinant Tromp1 protein was also detected in whole-cell

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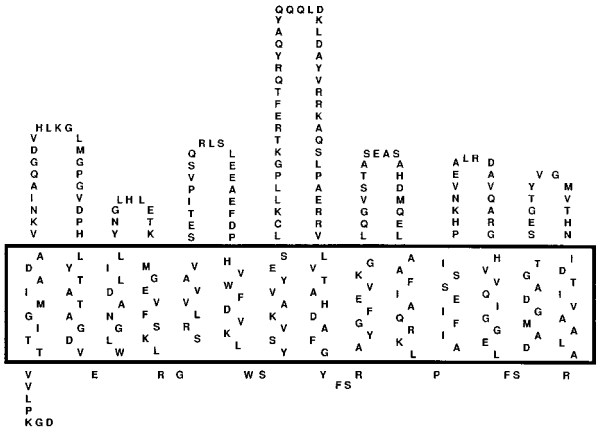

FIG. 3. Proposed topology of Tromp1. Sequences within the rectangle indicate 14 membrane-spanning regions having amphipathic beta-sheet structures. Sequences above the rectangle indicate seven surface-exposed loops. Sequences below the rectangle indicate regions exposed to the periplasmic space.

extracts of clone 2A (Fig. 4B, lane 2) but not in *E. coli* harboring a nonrecombinant pBluescript plasmid control (Fig. 4B, lane 3). Also shown are results of the reaction against the 38-kDa GST-Tromp1 fusion protein and several lower-molecular-weight breakdown products (Fig. 4B, lane 4).

**Localization of Tromp1 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 solubilization of the *T. pallidum* outer membrane and the release of some inner membrane-anchored lipoproteins (11). 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 (38). As shown in Fig. 4C, anti-GST-Tromp1 serum specifically detected the 31-kDa Tromp1 protein in whole-cell extracts (lane W) and in the detergent-phase fraction (lane D) but not in the protoplasmic cylinder fraction (lane P) or in the aqueous-phase fraction (lane A). These findings indicate that Tromp1 is completely released by Triton X-114 solubilization of the *T. pallidum* outer membrane and that it is a protein which has hydrophobic properties consistent with those of an outer membrane protein.

**Tromp1 is a porin protein.** In order to be tested for porin activity, native Tromp1 was isolated by IEF from *T. pallidum* cells and identified by using the antiserum generated against the recombinant GST-Tromp1 fusion protein. As shown in Fig. 5A, IEF fraction 5 specifically reacted with anti-GST-Tromp1 serum. A pH measurement of adjacent fractions showed that fraction 5 corresponded to a pH between 6.1 and 6.8, which is the expected position of IEF migration for Tromp1, given its calculated pI of 6.6. The determination that Tromp1 was the only major constituent of this fraction was demonstrated by immunoblot analysis of a denaturing two-dimensional gel, using anti-GST-Tromp1 serum and IRS, both of which showed the detection of only Tromp1 (Fig. 5B). As a control to demonstrate the sensitivity of IRS to detect contaminating *T. pallidum* proteins, proteins released from $2 \times 10^9$ *T. pallidum* cells by Triton X-100 solubilization were separated in a similar gel system and reacted with IRS (Fig. 5C and D). All of the two-dimensional gel-separated *T. pallidum* proteins visualized by amido black staining were reactive with IRS at a 1:1,000 dilution. Tromp1 was also detected, but only upon using IRS at a 1:50 dilution (data not shown). Therefore, under conditions

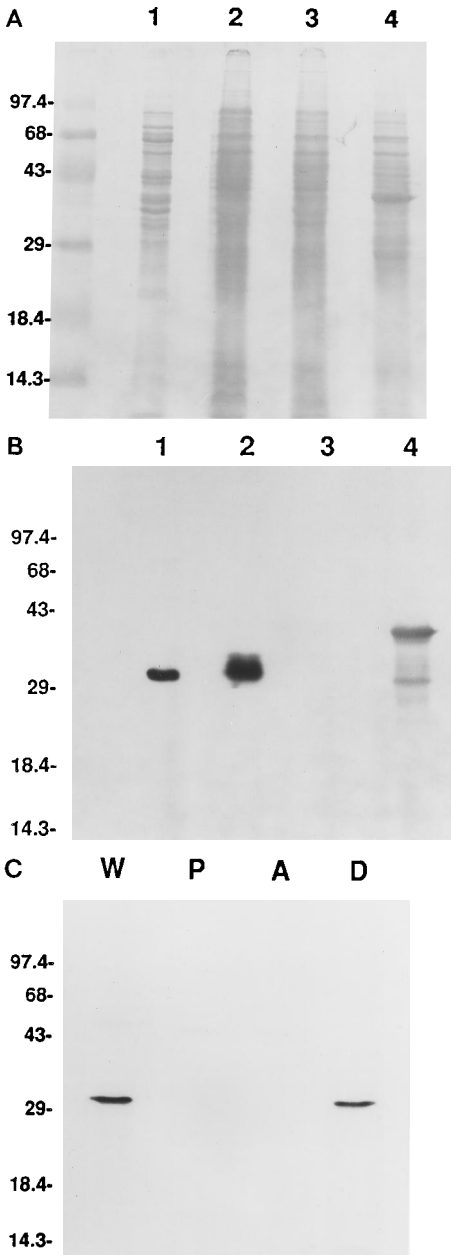


FIG. 4. Identification of native and recombinant Tromp1. (A) Amido black-stained immunoblot containing $10^9$ *T. pallidum* cells (lane 1), $5 \times 10^8$ *E. coli* cells harboring the recombinant 2A plasmid encoding Tromp1 (lane 2), $5 \times 10^8$ *E. coli* cells harboring a nonrecombinant plasmid control (lane 3), and $5 \times 10^8$ *E. coli* cells expressing the GST-Tromp1 fusion (lane 4). (B) The immunoblot shown in panel A was probed with antiserum generated to the GST-Tromp1 fusion protein. (C) Antiserum generated to the GST-Tromp1 fusion was used to probe an immunoblot of Triton X-114-extracted and phase-partitioned material from $10^9$ *T. pallidum* cells. W, whole organisms; P, protoplasmic cylinders; A, aqueous phase; D, detergent phase. The numbers on the left indicate the positions of molecular weight standards ($10^3$).

under which IRS binding to Tromp1 is demonstrated, IRS has a far greater capacity to bind to other *T. pallidum* proteins released by Triton X-100 solubilization. The stained blot also showed a 68-kDa protein, which was demonstrated by specific antiserum to be rabbit albumin (data not shown).

Purified Tromp1 was then tested for porin activity by using the black lipid bilayer assay (3, 6). The addition of purified

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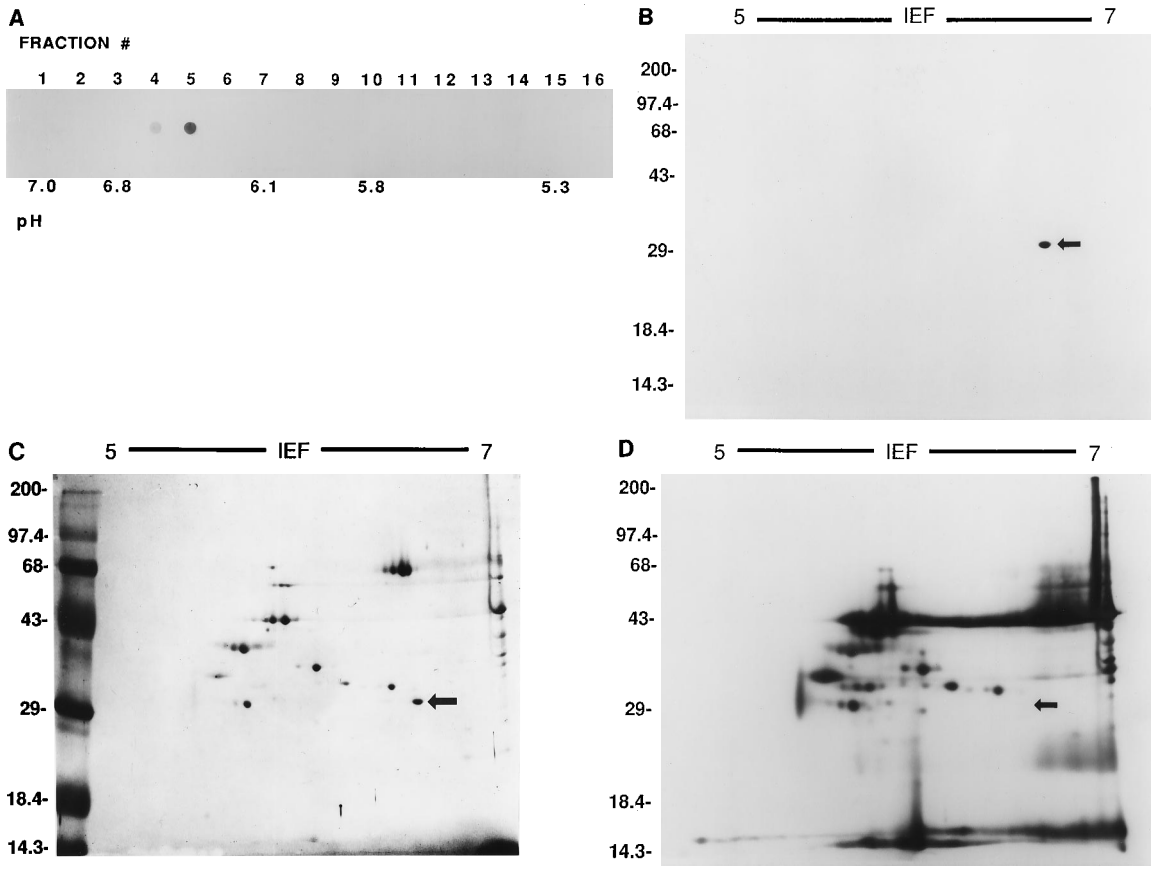


FIG. 5. Purification of native Tromp1 by nondenaturing IEF. (A) Dot blot analysis of IEF fractions reacted with anti-GST-Tromp1 serum. (B) Two-dimensional immunoblot analysis of IEF-purified Tromp1 probed sequentially with anti-GST-Tromp1 serum and IRS. (C) Amido black-stained two-dimensional immunoblot of Triton X-100-solubilized material from $2 \times 10^9$ *T. pallidum* cells. (D) The stained immunoblot from panel C reacted with IRS. The arrows indicate the position of Tromp1. The numbers on the left indicate the positions of molecular weight standards ($10^3$).

Tromp1 to the model membrane system resulted in channel formation, which was demonstrated by stepwise conductance increases across a lipid bilayer (Fig. 6A). More than 127 membrane insertion events were observed (Fig. 6B), and the measurement of conductance increases showed a distinct distribution about two means of 0.15 and 0.7 nS. These observations indicate that Tromp1 is a porin.

## DISCUSSION

Recently, we reported the purification of the *T. pallidum* outer membrane and the identification of 31- and 65-kDa proteins enriched in this preparation (6). It was also demonstrated that the purified outer membrane had porin activity. Because most porin proteins have molecular masses between 28 and 48 kDa (28), we focused on the isolation of the 31-kDa outer membrane-associated protein as a first TROMP candidate.

The extreme difficulty in acquiring sufficient amounts of individual TROMPs from purified outer membrane for conventional N-terminal amino acid sequence analysis prompted our approach to acquire internal amino acid sequences which were obtained from as little as 10 pmol of trypsin-digested TROMP candidates. The amino acid sequences obtained for the 31-kDa protein were used to clone the encoding structural gene, which we have designated *tromp1*.

Antiserum generated against a recombinant Tromp1 fusion protein was used to identify native Tromp1, which was purified by nondenaturing IEF. The demonstration of porin activity with purified Tromp1 has confirmed that Tromp1 is a transmembrane outer membrane protein of *T. pallidum*, the first such protein to be identified for this organism. The lethality for *E. coli* transformants harboring the intact *tromp1* structural gene is similar to that observed for many recombinant gram-negative porin proteins expressed in *E. coli* (8). In this context, cloning of *tromp1* was greatly facilitated by having sequences from two regions of the protein derived from its tryptic digestion.

Tromp1 single channel conductance measurements revealed two distinct distributions around means of 0.15 and 0.7 nS. The larger of these channels was similar in mean conductance to one observed in our previous studies with purified outer membranes (6). These previous studies were performed under conditions in which the 0.15 nS channel would not be readily observed (i.e., where a 0.7 nS channel caused a 4% deflection on the chart recorder). There are several potential explanations for this heterogeneity. For example, such heterogeneity could result from contamination by another porin. However, the relatively common observation of both channel sizes (Fig. 6B), the high purity of the Tromp1 utilized (Fig. 5B), and the very small amounts of porin (nanogram amounts) required to form channels argue against this possibility. The porin activity observed only from Tromp1 is also substantiated by the fact that the diluted amount of Tromp1 used in the black lipid

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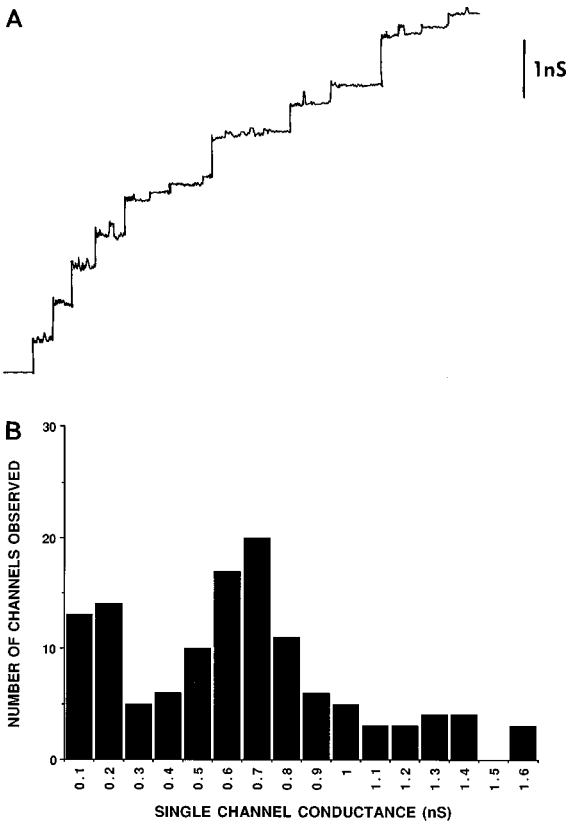


FIG. 6. Porin activity of purified native Tromp1. Purified Tromp1 was added to the aqueous phase (1 M KCl) bathing a lipid bilayer membrane. (A) Step increases in conductance after the addition of purified Tromp1. (B) Histogram of single channel conductance increases for more than 127 observed events. Conductance increases showed a distinct mean distribution about 0.15 and 0.70 nS.

bilayer assay was at the threshold level of detectable porin activity. Therefore, a contaminating porin could account for porin activity only if present in amounts relatively equal to those of Tromp1, which was not detected by two-dimensional SDS-PAGE analysis of the purified Tromp1 preparation (Fig. 5). A second possibility is that Tromp1 intrinsically forms two channels of different sizes, as has been seen with OprF of *Pseudomonas aeruginosa* (41) or with cecropin peptides (10). Alternatively, the large channel could be an aggregate or oligomer of the small channel, or the small channel could be a substrate of the larger channel, possibly caused by the application of a voltage (4) or due to partial damage during isolation and purification.

Our previous studies using purified *T. pallidum* outer membrane also showed a prominent mean single channel conductance measurement of 0.4 nS (6), which was not appreciably detected in the present study using purified Tromp1. Taken together, these observations suggest the possibility of a second *T. pallidum* porin protein. In view of this possibility, it is pertinent to note that Hardham and Stamm (18) have recently cloned a gene encoding a 50-kDa *T. pallidum* protein shown to have significant amino acid sequence homology with OmpA from *E. coli*. In our laboratory, we are currently investigating whether a 28-kDa protein identified in our purified outer membrane preparation (6) has porin activity.

The deduced Tromp1 amino acid sequence supports the idea that Tromp1 topology is in accord with the structural paradigms of other porins from gram-negative bacteria. A beta-moment plot of the mature protein sequence predicts that Tromp1 has 14 membrane-spanning amphipathic beta-sheet segments typical of gram-negative outer membrane proteins, including other porins (20). It is interesting to note that the predicted terminal transmembrane segment of Tromp1 ends in a positively charged residue (arginine), as is the case for the porin OprF of *P. aeruginosa*, which terminates in lysine (13).

Antiserum against the Tromp1 fusion protein was also used to follow the fate of native Tromp1 after Triton X-114 detergent extraction and phase separation of whole *T. pallidum* cells. Previous studies showed the selective solubilization of the *T. pallidum* outer membrane, although some inner membrane-anchored lipoproteins are also released, when using the phase-separating, nonionic detergent Triton X-114 (11, 30). Under these conditions, Tromp1 was completely released from the underlying protoplasmic cylinder and exclusively partitioned into the Triton X-114 detergent phase, indicating the hydrophobic nature of Tromp1.

Immunoblot analysis demonstrated that Tromp1 is recognized by antibody present in low dilutions (1:50) of immune serum from rabbits with latent syphilitic infection. Because many gram-negative porin proteins are targets for bactericidal antibody (16, 26, 34), it is tempting to speculate that Tromp1 represents a surface-exposed target on *T. pallidum* cells for treponemicidal antibody. The proposed topological model of Tromp1 includes seven surface-exposed regions, many of which show high antigenic indices. Preliminary studies using the antibodies generated against the GST-Tromp1 fusion, which includes 42 residues of Tromp1 corresponding to surface loop 4, have shown significant complement-dependent treponemicidal activity for *T. pallidum* (data not shown). We are currently engaged in studies to further identify and characterize Tromp1 epitopes relevant to protective immunity.

The finding that Tromp1 shares significant homology (27% identity) with a family of streptococcal adhesins is intriguing and suggests a possible similar function in *T. pallidum*. It is interesting to note that porin proteins from other gram-negative pathogens, including *Chlamydia trachomatis* and *Legionella pneumophila*, are involved in bacterial adherence to host cells (2, 35). As recombinant Tromp1 becomes available for experimental biology studies, we plan to investigate the involvement of this outer membrane protein in the host cell adherence and invasion properties associated with *T. pallidum*.

We have demonstrated previously that immune serum antibody binding to virulent *T. pallidum* cells results in TROMP particle aggregation as viewed by freeze fracture electron microscopy (7). These findings have recently been confirmed and extended, using serum obtained from infected animals with various degrees of challenge immunity. We have shown that particle aggregation correlates directly with the development of challenge immunity, suggesting that TROMPs represent key targets for a protective host immune response (unpublished data). The cloning of the first TROMP protein now provides the means to address directly the relationship of a *T. pallidum* outer membrane protein to the pathogenesis of syphilis and to the protective immunity which develops during the course of syphilitic infection.

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An equal contribution to this study was made by D. R. Blanco and C. I. Champion, who should be considered co-first authors of this work.

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