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Identification of Homologs for Thioredoxin, Peptidyl Prolyl *cis-trans* Isomerase, and Glycerophosphodiester Phosphodiesterase in Outer Membrane Fractions from *Treponema pallidum*, the Syphilis Spirochete

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In this study, we characterized candidate rare outer membrane (OM) proteins with apparent molecular masses of 19, 27, 38, and 38.5 kDa, which had been identified previously in OM fractions from *Treponema pallidum* (J. D. Radolf et al., Infect. Immun. 63:4244–4252, 1995). Using N-terminal and internal amino acid sequences, a probe for the 19-kDa candidate was PCR amplified and used to screen a *T. pallidum* genomic library in Lambda Zap II. The corresponding gene (*tlp*) encoded a homolog for periplasmic thioredoxin-like proteins (Tlp), which reduce *c*-type cytochromes. A degenerate oligonucleotide derived from the N terminus of the 27-kDa protein was used to PCR amplify a duplex probe from a *T. pallidum* genomic library in pBluescript II SK+. With this probe, the corresponding gene (*ppiB*) was identified and found to code for a presumptive periplasmic cyclophilin B-type peptidyl prolyl *cis-trans* isomerase (PpiB). We postulate that PpiB assists the folding of proteins within the *T. pallidum* periplasmic space. The N terminus of the 38-kDa candidate was blocked to Edman degradation. However, internal sequence data revealed that it was basic membrane protein (Bmp), a previously characterized, signal peptidase I-processed protein. Triton X-114 phase partitioning revealed that despite its name, Bmp is hydrophilic and therefore likely to be periplasmic. The final candidate was also blocked to Edman degradation; as before, a duplex probe was PCR amplified with degenerate primers derived from internal sequences. The corresponding gene (*glpQ*) coded for a presumptively lipid-modified homolog of glycerophosphodiester phosphodiesterase (GlpQ). Based upon findings with other treponemal lipoproteins, the hydrophilic GlpQ polypeptide is thought to be anchored by N-terminal lipids to the periplasmic leaflet(s) of the cytoplasmic membrane and/or OM. The discovery of *T. pallidum* periplasmic proteins with potentially defined functions provides fresh insights into a poorly understood aspect of treponemal physiology. At the same time, however, these findings also raise important issues regarding the use of OM preparations for identifying rare OM proteins of *T. pallidum*.

Syphilis is a sexually transmitted infection caused by the noncultivatable spirochetal pathogen *Treponema pallidum*. Beginning as an ulcer (chancre) at the site of inoculation (usually in the genital area), untreated disease typically progresses through secondary (disseminated), latent, and tertiary (recrudescent) stages (37). Despite the availability of effective antimicrobial therapy for nearly 5 decades, syphilis remains a significant public health problem in both industrialized and underdeveloped countries (40, 44). Most recently, genital ulcers caused by syphilis have been recognized as cofactors for the sexual transmission of human immunodeficiency virus (20). These trends emphasize the need to identify protective immunogens of *T. pallidum* in order to develop a safe and effective syphilis vaccine.

Like all spirochetes, *T. pallidum* is a highly motile bacterium composed of an outer membrane (OM) which surrounds a periplasmic space, a peptidoglycan-cytoplasmic membrane (CM) complex, and a protoplasmic cylinder; within the periplasmic space are the endoflagella, the structures responsible for the bacterium's distinctive motility (30). It is well established that the OMs of *T. pallidum* and gram-negative bacteria differ markedly with respect to physical properties, composition, and molecular architecture (12, 42, 47, 48, 62). One of the most important differences is the strikingly lower density of integral membrane proteins in the *T. pallidum* OM (10, 42, 47). The paucity of surface-exposed proteins is believed to explain at least in part how *T. pallidum*, an extracellular pathogen, so successfully evades the robust humoral immune responses it evokes during persistent infection (4, 25). In contrast to the protein-deficient OM, the majority of *T. pallidum* cell envelope constituents, including integral membrane proteins, appear to be associated with the peptidoglycan-CM complex (17, 42, 47, 48). Interestingly, comparatively little is known about the soluble periplasmic constituents of *T. pallidum* (42, 55).

Since the discovery of rare *T. pallidum* OM proteins by freeze-fracture electron microscopy (48, 62), molecular characterization of these morphological entities has been an important goal of syphilis research (10, 47). The recent development of improved methodologies for the fractionation of *T. pallidum* and isolation of OMs has been considered a major advance toward this objective (11, 47, 49). When protocols for isolating *T. pallidum* OMs were developed, however, it was not known which, if any, of the OM-associated polypeptides visualized by sodium dodecyl sulfate-polyacrylamide gel electro-

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TABLE 1. Key oligonucleotides used in this study

Designation	Sequence	Description
tlp-5′NT	5′-GCNGAYGTIGCICAYAAYGCIGAYGTNCC-3′	Degenerate primer corresponding to Tlp N terminus
tlp-3′IN	5′-TTICCNCKNGCDATRAA-3′	Degenerate primer corresponding to Tlp internal sequence
tlp-prime	5′-ACCAGGTCGGAACGGAACACAAGG-3′	Oligonucleotide used for *tlp* primer extension
ppi-5′NT	5′-GARGARAARATGGTIMGIGARGARGG-3′	Degenerate primer corresponding to PpiB N terminus
glp-5′IN	5′-GGNTAYGTNCCNGA-3′	Degenerate primer corresponding to GlpQ internal sequence
glp-3′IN	5′-TANGTYTGNACRTANAC-3′	Degenerate primer corresponding to GlpQ internal sequence
bmp-5′B	5′-GCGGGATCCGTGAATTTATTCCGGCGTGAGCC-3′	Forward primer used to generate Bmp fusion; also contains *Bam*HI site
bmp-3′E	5′-GCGAATTCTCACCAGTCGAGCACCTTGCCG-3′	Reverse primer used to generate Bmp fusion; also contains *Eco*RI site
T7	5′-GTAATACGACTCACTATAGGGCGAA-3′	Reverse primer used to generate *ppiB* duplex probe
T3	5′-GCAATTAACCCTCACTAAAGGGAAC-3′	Reverse primer used to generate *ppiB* probe

phoresis (SDS-PAGE) were authentic rare OM proteins, nor did there exist immunologic reagents specific for suspected rare OM proteins. As an alternative approach, OM-associated proteins which appeared to be highly enriched in OM fractions (and thus thought more likely to be rare OM proteins) were identified and slated for subsequent genetic characterization (10, 11, 49). In this study, we used microsequence analysis as the basis for molecular analysis of four of these candidate rare OM proteins, three of which were found to be newly recognized treponemal polypeptides. Somewhat surprisingly, all four candidates appear to be periplasmic constituents rather than integral OM proteins. The discovery of *T. pallidum* periplasmic proteins with potentially defined functions provides fresh insights into a poorly understood aspect of treponemal physiology. At the same time, however, these findings also raise important issues regarding the use of OM preparations for identifying rare OM proteins of *T. pallidum.*

MATERIALS AND METHODS

Bacterial strains, vectors, and construction of *T. pallidum* genomic libraries. *T. pallidum* was propagated by intratesticular inoculation of New Zealand White rabbits, harvested from testes at room temperature in an enriched medium (56), and purified by Percoll density gradient centrifugation (26). *Escherichia coli* XL-2 (Stratagene, La Jolla, Calif.), SOLR (Stratagene), and INVαF′ (Invitrogen, San Diego, Calif.) were used for all cloning experiments and were cultivated in Luria broth with or without 100 μg of ampicillin per ml. PCR products were cloned into plasmid vector pCR 2.1 (Invitrogen) or pProEx-HTb (Gibco/BRL, Bethesda, Md.). Two *T. pallidum* genomic libraries were used in this study. One was constructed in Lambda Zap II (Stratagene) as previously described (61). The second was produced from a partial *Sau*3AI digest of *T. pallidum* DNA; fragments of between 0.4 and 3 kb were gel purified and ligated into the *Bam*HI site of pBluescript II SK+.

Isolation of *T. pallidum* OMs and protein microsequence analysis. *T. pallidum* OMs were obtained by a previously described plasmolysis-based fractionation procedure (49). Amino acid sequence analysis was performed at the University of Texas Southwestern Medical Center Protein Chemistry Core Facility. Polypeptides in OMs from 4×10^{11} treponemes were separated by SDS-PAGE (as described below), transferred to a polyvinylidene difluoride membrane, and stained with Ponceau S. Bands were excised for amino acid microsequencing by automated Edman degradation (38). The internal amino acid sequence was obtained after either trypsin or LysC digestion of the protein and separation of the resultant peptides by high-performance liquid chromatography as previously described (1).

Nucleotide sequence analysis. The nucleotide sequences of double-stranded DNA templates were determined by the dideoxy chain termination method of Sanger et al. (52) with an automated DNA sequencer (model 373A; Applied Biosystems Inc., Foster City, Calif.) and a PRISM ready reaction DyeDeoxy terminator cycle sequencing kit (Perkin-Elmer, Foster City, Calif.). Both DNA strands were sequenced to ensure accuracy. To rule out possible chimeric rearrangements during the construction of *T. pallidum* libraries, PCR was used to confirm the arrangement and contiguity of open reading frames (ORFs), as predicted by the sequence data. Nucleotide and deduced amino acid sequences were analyzed by using the MACVECTOR sequence analysis package (version 4.1.1; International Biotechnologies, Inc., New Haven, Conn.), Sequence Editor (version 1.0.3; Applied Biosystems), and the University of Wisconsin Genetics Computer Group package (version 8.1) (19). Sequence homologies were determined by using the BLAST server of the National Center for Biotechnology Information. Multiple-sequence alignments of the various *T. pallidum* OM-associated proteins and their homologs were performed by using the Genetics Computer Group PILEUP and BESTFIT algorithms with gap weights of 3.0 and gap length weights of 0.1. Potential transcriptional terminators were identified by using the algorithm of Brendel and Trifonov (13).

Primer extension. RNA for primer extension analysis was extracted from approximately 10^{10} freshly harvested *T. pallidum* spirochetes by using the Ultraspec RNA isolation system (Cinna/Biotecx Laboratories Inc., Houston, Tex.). Primer extension was performed as previously described (45, 63).

Generation of recombinant Bmp. The portion of basic membrane protein (Bmp) encoding amino acids 30 to 361 (18) was amplified from *T. pallidum* DNA with primer pair bmp-5′B and bmp-3′E (Table 1) and directionally cloned into the *Bam*HI and *Eco*RI sites of pProEx-HTB. The resulting His_6-Bmp fusion was purified with a nickel resin and then cleaved with rTEV protease according to the instructions of the manufacturer (Gibco/BRL).

Generation of anti-Bmp antiserum. Two 6-week-old Sprague-Dawley rats were primed by intraperitoneal injection with 15 μg of Bmp in a 1:1 mixture of phosphate-buffered saline (PBS; pH 7.4) and Freund's complete adjuvant. This was followed 4 and 6 weeks later by intraperitoneal booster injections consisting of 10 μg of antigen in a 1:1 mixture of PBS and Freund's incomplete adjuvant.

Triton X-114 phase partitioning. *T. pallidum* (10^9) was solubilized for 2 h with agitation at 4°C in 1 ml of 2% Triton X-114 in PBS. The insoluble material was removed by centrifugation for 15 min in a microcentrifuge, and the supernatant was phase separated as previously described (14). The proteins in the aqueous and detergent-enriched phases were concentrated by acetone precipitation prior to SDS-PAGE.

SDS-PAGE and immunoblot analysis. Samples were boiled for 5 min in final sample buffer consisting of 62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 5% (vol/vol) 2-mercaptoethanol, and 0.001% bromophenol blue prior to electrophoresis through 2.4% stacking and 12.5% separating SDS-polyacrylamide gels. Gels were either stained with Coomassie brilliant blue or transferred electrophoretically to 0.2-μm-pore-size nitrocellulose (Schleicher and Schuell, Keene, N.H.) for immunoblotting. Immunoblots were incubated with a 1:500 dilution of rat anti-Bmp antiserum, followed by sequential incubations with 1:1,000 dilutions of horseradish peroxidase conjugates of goat anti-rat and rabbit anti-goat immunoglobulins (Zymed Laboratories, Inc., South San Francisco, Calif.). Blots were developed with 4-chloro-1-naphthol as the substrate.

Nucleotide sequence accession numbers. The GenBank accession numbers for the *T. pallidum* genomic fragments containing the *tlp*, *ppiB*, and *glp* genes are U95250, U97573, and U95744, respectively.

RESULTS AND DISCUSSION

Candidate rare OM proteins in *T. pallidum* OM fractions. Because of the paucity of information and reagents for potential OM proteins in *T. pallidum*, we began our molecular characterization studies by identifying a subset of OM-associated proteins which appeared to be enriched in OM fractions compared to cell cylinders and whole cells (49). These molecules, the candidate rare OM proteins (Fig. 1), were selected for further analysis on the assumption that one or more may be the low-density intramembranous particles visualized during freeze-fracture electron microscopy of intact treponemes and *T. pallidum* OM preparations (48, 49). TpLRR, a candidate with an apparent SDS-PAGE mobility of 26 kDa, has already been characterized and localized to the periplasmic space (55).

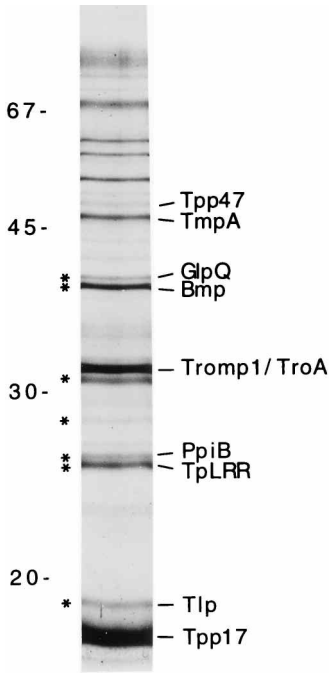


FIG. 1. Polypeptide profile of *T. pallidum* OM fractions. *T. pallidum* OMs, obtained from 7×10^9 spirochetes, were separated on an SDS–12.5% polyacrylamide gel and stained with silver. Asterisks on the left indicate previously identified candidate OM proteins (49). The designations on the right are those for previously characterized *T. pallidum* polypeptides (8, 28, 42, 55) and the three novel candidates (Tlp, PpiB, and GlpQ) characterized in this study. Molecular mass markers (in kilodaltons) are shown on the left.

Although it was a major constituent in our OM fractions (Fig. 1), Tromp1 (8) was not designated a candidate in our prior study because it did not appear to be enriched in OM fractions (49). This assignment is supported by recent evidence that Tromp1 is anchored to the *T. pallidum* CM by an uncleaved signal peptide, where it most likely functions as a substrate-binding protein component for an ABC transporter (hence, the alternative designation of transport-related operon protein A or TroA [Fig. 1]) (2, 28). To pursue the analysis of additional candidates, automated Edman degradation was used to obtain amino acid sequence information from the OM-associated proteins with apparent molecular masses of 19, 27, 38, and 38.5 kDa (designated Tlp, PpiB, Bmp, and GlpQ, respectively [Fig. 1]).

Thioredoxin-like protein (Tlp). Both N-terminal and internal (a LysC digestion product) sequences were obtained for the 19-kDa OM-associated protein (Fig. 2A). No matches were found for either in protein databases, suggesting that they were derived from a novel treponemal polypeptide. Degenerate forward and reverse primers tlp-5′NT and tlp-3′IN (Table 1), respectively, were used to amplify a 323-bp fragment from *T. pallidum* chromosomal DNA. Nucleotide sequence analysis of this fragment revealed that the coding sequences immediately downstream from the forward and reverse primers were exact matches for amino acids which had been identified by microsequencing but not included in the primers (Fig. 2A). This confirmed that the amplicon was in fact derived from the gene of interest. The PCR product was used to screen a *T. pallidum* genomic library in Lambda Zap II.

A single 200-amino-acid ORF was identified within the 858-bp sequence obtained from the insert of one hybridizing clone. However, this ORF's translational start (the ATG at bp 77 to 79) was deemed to be problematic because there was no ribosomal binding site (RBS) immediately upstream and the predicted 33-amino-acid leader sequence was atypical in several respects for a signal peptide (60). Therefore, primer extension (with oligonucleotide tlp-primer [Table 1]) was performed to help determine whether this ATG was the true translational start. As shown in Fig. 3, the start of transcription was found to be the same A at bp 77 which originally had been thought to be part of the translational start codon. Based upon this finding, the GTG at bp 122 to 124 was selected as the probable translational start (Fig. 2A). It is worth noting that two other *T. pallidum* proteins (Tpp47 and TpN50) are believed to be synthesized from GTG translational start codons (27, 63). The revised ORF (Fig. 2A) codes for a polypeptide of 185 amino acids, including an 18-amino-acid signal peptide. The mature polypeptide has a calculated molecular weight of 18,548 and a theoretical pI of 8.3. Searches of the region upstream from the predicted RBS failed to identify any sequences which were good matches for consensus σ^{70}, σ^{32}, σ^{54}, and σ^{28} promoter elements. It was noted, however, that the 6-bp sequence at positions 65 to 70 matched the σ^{38} (σ^{s} or RpoS) consensus at 4 of 6 positions (Fig. 2A) (57). No good rho-independent transcriptional termination signals were found downstream of the coding sequence.

A BLASTP search revealed that the 19-kDa protein showed a high degree of sequence relatedness to known or presumptive prokaryotic disulfide oxidoreductases. The best matches were with ORFX14 of *Bacillus subtilis* (51% similar and 28% identical), TlpA of *Bradyrhizobium japonicum* (52% similar and 29% identical), and HelX of *Rhodobacter capsulatus* (54% similar and 31% identical). The latter two are periplasmic proteins which have previously been shown to be essential for the biogenesis of *c*-type cytochromes, which also are periplasmic (6, 36). The prediction of functional similarities between these polypeptides and the 19-kDa *T. pallidum* OM-associated protein was strengthened by the identification in the treponemal homolog of a sequence (W-C-P-P-C-R) at amino acids 81 to 86 (Fig. 2A) which matches the consensus (W-C-X-P-C-R) for the active site of a prokaryotic thioredoxin (6). Furthermore, when a multiple-sequence alignment was performed, it was apparent that the putative active sites of Tlp and the *B. japonicum* and *R. capsulatus* homologs were similarly located within the central, most highly conserved regions of the proteins (data not shown). Based upon these findings and pending further biochemical and/or genetic complementation studies, the OM-associated protein was designated Tlp (for thioredoxin-like protein). By using the algorithm of Kyte and Doolittle (34), hydrophilicity analysis revealed that the mature protein is predominantly hydrophilic (Fig. 2B). Therefore, like its gram-negative homologs, Tlp is presumed to be a periplasmic protein.

If the *tlp* gene product functions as predicted by its sequence homologies, then *T. pallidum* should also express periplasmic *c*-type cytochromes. In this regard, it is interesting that *c*-type cytochromes are induced in *E. coli* during cultivation under anaerobic, nonfermentative conditions (58). *T. pallidum* is microaerophilic and therefore may constitutively express such *c*-type cytochromes (41). In a prior study, we demonstrated that the polypeptide portion of the 17-kDa lipoprotein immunogen of *T. pallidum* (Tpp17), which is located within the periplasmic space (17), consists of disulfide-bonded multimers (1). Disulfide bond formation within the periplasmic space of *E. coli* and other gram-negative bacteria is catalyzed by another class of oxidoreductase, represented by the protein DsbA (5), which lacks overall sequence homology with thioredoxins

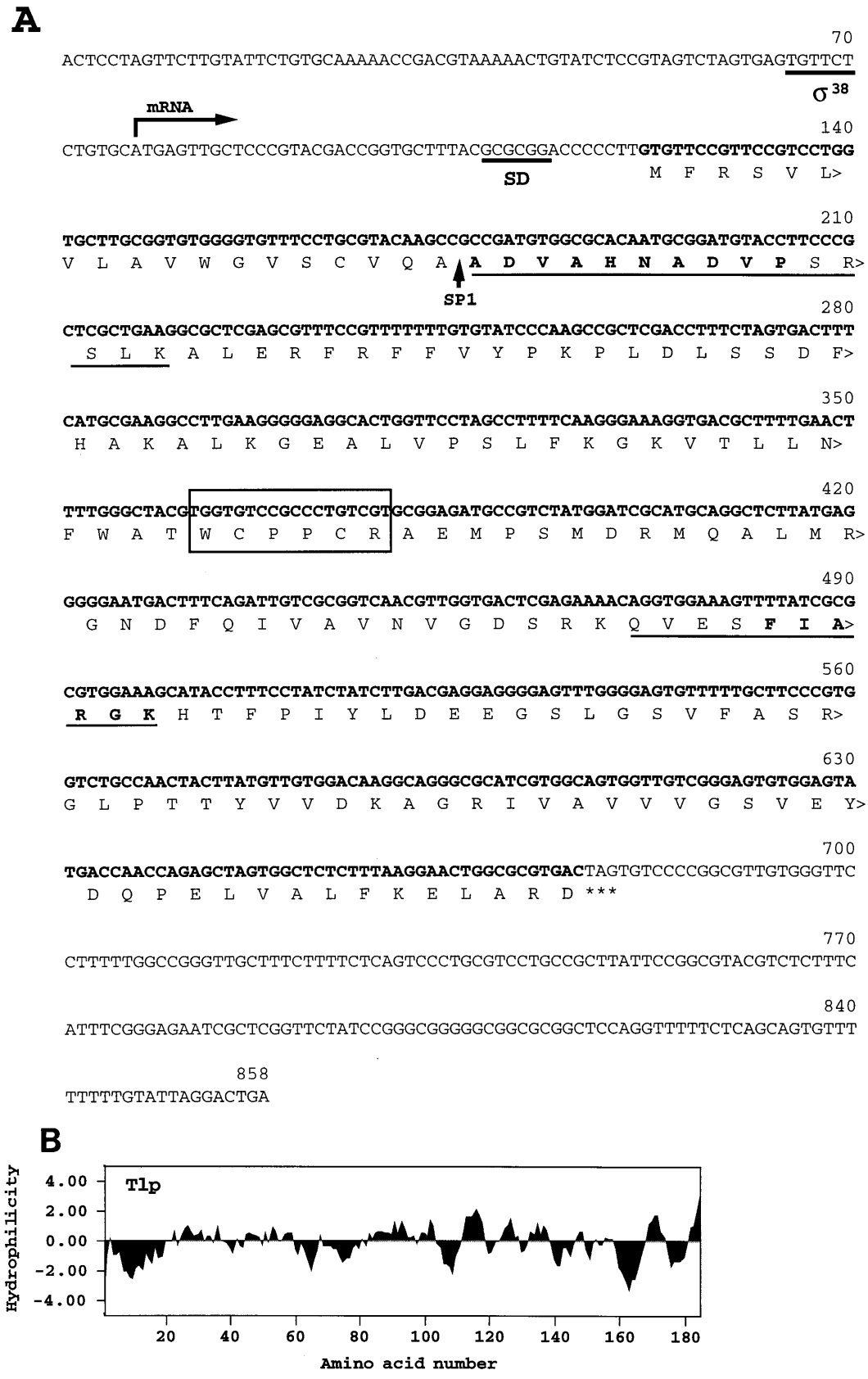


FIG. 2. Molecular analysis of Tlp, a processed 19-kDa thioredoxin-like protein of *T. pallidum*. (A) Nucleotide and deduced amino acid sequences. The proposed σ^{38} and Shine-Dalgarno (SD) sequences are underlined. The site of transcriptional initiation, as determined by primer extension reaction (Fig. 3), is indicated by an arrow labeled mRNA. The SPaseI cleavage site is indicated by a vertical arrow labeled SP1. The peptide sequences obtained by automated Edman degradation are underlined; the amino acids in boldface were used to design the degenerate primers tlp-5′NT and tlp-3′IN (Table 1). The amino acids corresponding to the putative enzymatic active site are boxed. Asterisks indicate the translational stop codon. (B) Hydrophilicity analysis of Tlp by the algorithm of Kyte and Doolittle (34) with a window size of 7. Hydrophobic regions are below the baseline, and hydrophilic regions are above the baseline.

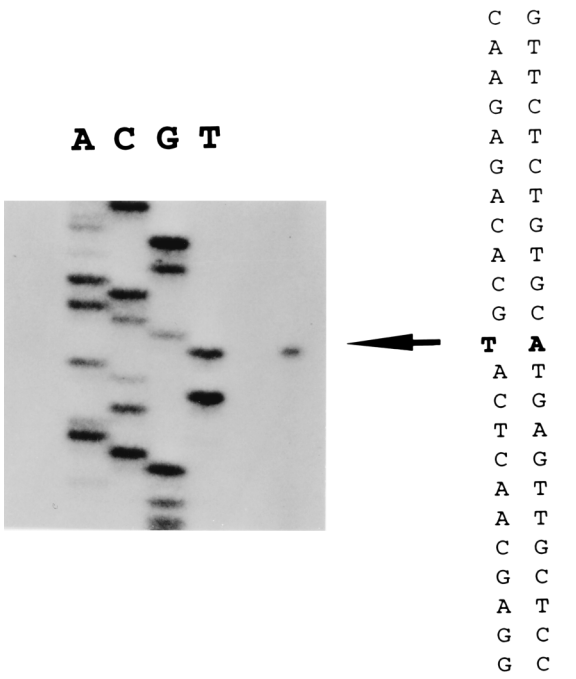


FIG. 3. Determination of the transcriptional start of the *tlp* gene. Shown are the primer extension product of the *tlp* mRNA and the sequencing ladder of the homologous region of the *tlp* gene coding strand. Lanes loaded with individual dideoxy sequencing reaction mixtures are labeled A, C, G, and T. The arrow indicates the position of the primer extension product in relation to the sequencing ladder.

(6). Thus, the *T. pallidum* periplasmic space is predicted to contain two types of oxidoreductases.

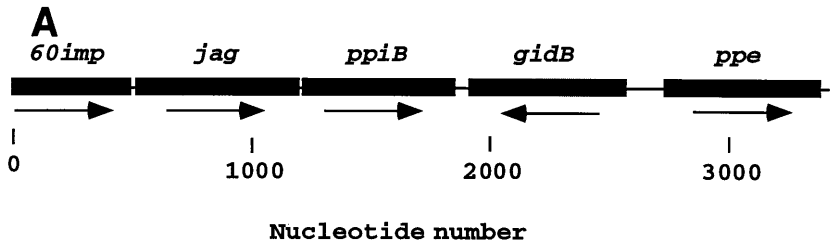
Peptidyl prolyl *cis-trans* isomerase (PpiB). Only the N-terminal sequence (without database matches) was obtained for the 27-kDa protein (Fig. 4B). Using a degenerate oligonucleotide probe derived from this sequence (ppi-5′NT [Table 1]), we were unable to identify Lambda Zap II clones containing the corresponding gene. To circumvent this problem, we utilized an alternative PCR-based strategy to obtain a larger, duplex probe (32, 33). First, a genomic library was constructed by ligating 0.4- to 3-kb fragments from a partial *Sau*3AI digest of *T. pallidum* DNA into pBluescript II SK+. Next, two separate PCRs were performed with ppi-5′NT as the forward primer and the reverse primers derived from the T3 and T7 promoters of the vector (Table 1). A 222-bp amplicon containing a 74-amino-acid ORF was obtained with the ppi-5′NT–T7 primer pair. Immediately downstream from the sequence corresponding to primer ppi-5′NT was the portion of the N terminus not included in the primer, thereby confirming that the amplicon was derived from the gene of interest. This fragment was used to screen the *T. pallidum* Lambda Zap II library. Four complete ORFs and a truncated fifth ORF (Fig. 4A) were identified within the 3,400-bp sequence obtained from a clone which hybridized with the probe.

The ORF corresponding to the 27-kDa protein had a high degree of sequence similarity to numerous peptidyl proline *cis-trans* isomerases (PPIases), a ubiquitous group of prokaryotic and eukaryotic enzymes which contribute to protein folding by catalyzing *cis-trans* isomerization about proline residues (22). Among prokaryotic enzymes, the strongest matches were for the cyclophilin B PPIases of *Mycobacterium tuberculosis* (62% similar and 51% identical), *Mycobacterium leprae* (61% similar and 46% identical), and *B. subtilis* (67% similar and 47% identical); hence, the designation PpiB was given to the treponemal homolog. The translational start for the treponemal *ppiB* gene product was predicted to be either the ATG at positions 92 to 95 or the GTG at positions 104 to 106. The former start seemed to be preferable because it yielded a better RBS (Fig. 4B). With this start, the ORF codes for a polypeptide of 215 amino acids, the first 24 of which comprise a typical cleaved signal peptide. The molecular mass and pI of the mature protein were predicted to be 20,598 Da and 8.3, respectively, indicating that PpiB migrates somewhat aberrantly during SDS-PAGE (Fig. 1). No promoter elements were identified upstream of the RBS, and no obvious transcriptional terminator signals were identified downstream from the *ppiB* ORF. By hydrophilicity analysis, PpiB is mainly hydrophilic (Fig. 4C); therefore, the protein is predicted to be periplasmic. Although the PPIase activity of the *T. pallidum* protein remains to be demonstrated, it is tempting to speculate that like its homologs, this enzyme functions within the periplasmic space to facilitate proper folding of *T. pallidum* cell envelope constituents.

BLASTP searches identified homologs for the other three complete ORFs and one incomplete ORF which flanked *ppiB* (Fig. 4A). The ORF designated *jag* codes for a 191-amino-acid polypeptide which is 56% similar and 33% identical to the protein encoded by the *B. subtilis jag* gene, which is found in a sporulation locus. The ORF designated *gidB* encodes a 222-amino-acid protein with a high degree of sequence similarity to glucose-inhibited division B protein (GidB) (53% similar and 32% identical to GidB of *E. coli*, the best match). The 218-amino-acid ORF designated *ppe* is 62% similar and 45% identical to the pentose-5-phosphate-3-epimerase (Ppe) of a *Synechocystis* sp. A 60-kDa hypothetical inner membrane protein of *Haemophilus influenzae* was the best match (67% similar and 41% identical) for the 167-amino-acid truncated ORF designated *60imp*. However, a good match (56% similar and 28% identical) was also made with the product of *SpoIIIJ*, a sporulation gene which is immediately upstream of and transcriptionally linked to *jag* in *B. subtilis* (21). Interestingly, unlike the *gidB* genes of *E. coli* (15), *B. subtilis* (43), *Pseudomonas putida* (43), and *Borrelia burgdorferi* (GenBank accession no. Z12160 and X95668), the *gidB* gene in *T. pallidum* is not linked to a *gidA* homolog (which has yet to be identified). The locations of these two genes within the *T. pallidum* chromosome are of considerable importance because of their proximity in other bacteria to *oriC*, the chromosomal origin of replication (43). It must be emphasized that the arrangement of the genes depicted in Fig. 4A was confirmed by PCR analysis with *T. pallidum* DNA as the template.

Bmp. The N terminus of the 38-kDa protein was blocked to Edman degradation. Surprisingly, internal sequences for three LysC digestion products (data not shown) were exact matches for Bmp, a *T. pallidum* polypeptide previously shown by Dallas and coworkers to be processed by signal peptidase I (SPaseI) (18). From an examination of the published sequence, glutamine was predicted to be the first amino acid of the mature protein (18). N-terminal glutamines can cyclize to pyroglutamate, which is not susceptible to Edman degradation (23). Because of *T. pallidum*'s abundance of lipoproteins, it is often assumed that a protein with a blocked N terminus is lipid modified. The finding for Bmp sounds a cautionary note against such assumptions.

Bmp was reported to be a membrane protein because it was recovered in a putative *T. pallidum* membrane fraction (18). However, those experiments were performed well before the development of contemporary methods for fractionating *T. pallidum*. Furthermore, it has never been determined whether



B

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                                                                      70
CAACGCTGCATGGGATCAGTGGGGTAGAGACCAGAAGTGAGGGGGAAGGGCTTTACAAGCAGGTGCGGGT

                                                                     140
GTGTCACACCGGGGGTGCGATATGAATACGCAGGTGTGGCGCGTCTGTGTGGGGGTGATGTTGTTCTGCT
                 SD          M  N  T  Q  V  W  R  V  C  V  G  V  M  L  F  C>

                                                                     210
TCGTTGGCAGGATCGGGTGTGCGGAGGAAAAAATGGTACGCGAGGAAGGGTTGGCGGTCGCTGACGGTAT
F  V  G  R  I  G  C  A  E  E  K  M  V  R  E  E  G  L  A  V  A  D  G  I>
                       SP1
                                                                     280
ATATGCGGTAATGGAAACAAATCGGGGAACGATTGTTCTCTCGCTTTTTTTTGAAAAGGCGCCGCTTACG
  Y  A  V  M  E  T  N  R  G  T  I  V  L  S  L  F  F  E  K  A  P  L  T>

                                                                     350
GTGTGTAATTTTGTGGGGTTAGCAGAGGGAACGTTGGCGGTGTGCAAGGGTCGTCCTTTTTATCAGGGGT
 V  C  N  F  V  G  L  A  E  G  T  L  A  V  C  K  G  R  P  F  Y  Q  G>

                                                                     420
TAACGTTTCACCGTGTTATCAAAGATTTTATGATCCAGGGGGGAGACCCGCAGGGAAATGGGACGGGAGG
L  T  F  H  R  V  I  K  D  F  M  I  Q  G  G  D  P  Q  G  N  G  T  G  G>

                                                                     490
TCCTGGCTATCAATTCCCCGATGAATGTGATCCTGCGTTGCGACACGACAGCCCAGGAGTGTTGTCAATG
  P  G  Y  Q  F  P  D  E  C  D  P  A  L  R  H  D  S  P  G  V  L  S  M>

                                                                     560
GCGAATGCAGGACCAGGAACGAACGGTTCGCAGTTTTTTATTACCCACGTGGCTACGCCGTGGTTGGATG
 A  N  A  G  P  G  T  N  G  S  Q  F  F  I  T  H  V  A  T  P  W  L  D>

                                                                     630
GAAAGCATACCGTGTTTGGCAAAGTCGTTGAGGGTATGGAAGTGGTGCATGCAATTATAGCGGGAGATAC
G  K  H  T  V  F  G  K  V  V  E  G  M  E  V  V  H  A  I  I  A  G  D  T>

                                                                     700
GATTCGCTCACTCAAAATAGTGCGCCGTGGTGCGGCAGCGAAGCGTTTTGTCTGCGATCAGGCGCAGTTC
  I  R  S  L  K  I  V  R  R  G  A  A  A  K  R  F  V  C  D  Q  A  Q  F>

                                                                     770
GACCAGCTGCGAAAACGGGTATCCGCAGCGTCCAAATAATGGGCATGCCGTTTGTATGTTCTCCTCTCTG
 D  Q  L  R  K  R  V  S  A  A  S  K  ***
```

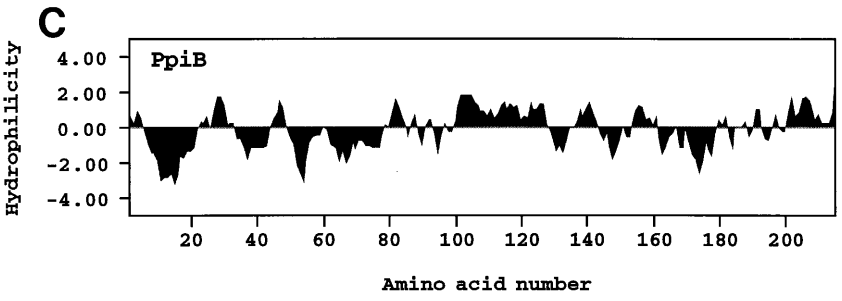


FIG. 4. Molecular analysis of PpiB, a *T. pallidum* cyclophilin B peptidyl prolyl *cis-trans* isomerase homolog. (A) Schematic diagram of *ppiB* and flanking genes. *60imp*, *jag*, *gidB*, and *ppe* denote *T. pallidum* homologs for (i) a gene encoding an *H. influenzae* hypothetical 60-kDa inner membrane protein, (ii) the *spoIIIJ*-associated gene of *B. subtilis*, (iii) the glucose-inhibited division protein B gene of *E. coli*, and (iv) the pentose-5-phosphate-3-epimerase gene of *E. coli*, respectively. Arrows indicate the directions of transcription. (B) Nucleotide and deduced amino acid sequences of *ppiB* and the corresponding protein. The proposed Shine-Dalgarno (SD) sequence is underlined. The SPaseI cleavage site is indicated by a vertical arrow labeled SP1. The peptide sequences obtained by automated Edman degradation are underlined; the amino acids in boldface were used to design the degenerate primer ppi-5′NT (Table 1). Asterisks indicate the translational stop codon. (C) Hydrophilicity analysis of PpiB with a window size of 7; hydrophobic regions are below the baseline, and hydrophilic regions are above the baseline.

Bmp possesses the amphiphilicity characteristic of an integral membrane protein. To address this issue, we used a monospecific polyclonal antiserum to probe *T. pallidum* whole cells after solubilization in Triton X-114 and phase partitioning (14). As shown in Fig. 5, Bmp partitioned exclusively into the aqueous phase. Consistent with this finding, Kyte-Doolittle analysis predicted that Bmp is predominantly hydrophilic (data not shown). These two pieces of information, coupled with the knowledge that Bmp is processed by SPaseI, suggest that it too is periplasmic.

Bmp was discovered in the mid-1980s by screening a *T. pallidum* genomic library with human syphilitic serum (18). During that period of treponemal research, it was uncommon to find matches in databases for newly sequenced *T. pallidum* genes and proteins. Consequently, it was of interest to see whether a putative function for Bmp could be deduced by searching contemporary databases; no significant matches were found. This situation, that is, a lack of homology with proteins in the database, pertains to a number of other *T. pallidum* proteins which were also originally identified by their reactivities with syphilitic serum (42).

Glycerophosphodiester phosphodiesterase (GlpQ). The N terminus of the 38.5-kDa protein was blocked to Edman degradation. However, sequences for two internal tryptic fragments were obtained (Fig. 6B). With degenerate primers derived from these sequences (glp-5′IN and glp-3′IN [Table 1]), a 509-bp fragment was amplified. After sequence analysis confirmed that the amplicon was derived from the gene of interest, it was used to screen the Lambda Zap II genomic library. Three complete ORFs were identified within the 2,204-bp sequence obtained from the insert of the hybridizing clone selected for further analysis (Fig. 6A).

The 356-amino-acid ORF corresponding to the 38.5-kDa OM-associated protein (Fig. 6B) was an extremely strong match for the glycerophosphodiester phosphodiesterase (GlpQ) of *H. influenzae* (75% similar and 55% identical) and that of *E. coli* (72% similar and 51% identical). This enzyme, a member of the *glp* regulon involved in the catabolism of glycerol and glycerol-3-phosphate (G3P), hydrolyzes deacylated phospholipids to alcohol plus G3P (35, 39). A weaker, though still highly significant, match was obtained for the recently described *Borrelia hermsii* GlpQ (63% similar and 41% identical), which interestingly was also identified in OMs obtained from that spirochete (53, 54). The *T. pallidum* GlpQ polypeptide has a typical 20-amino-acid signal sequence terminated by a putative SPaseII cleavage site (L-V-A-G-C) (Fig. 6B); lipid modification is also consistent with the protein's N terminus being blocked to Edman degradation. Cleavage of this signal peptide results in a mature protein of 38,957 Da (exclusive of lipids), which, like Tlp, PpiB, and Bmp, is predicted to be relatively basic (theoretical pI of 9.12). In *E. coli*, GlpQ is synthesized as a precursor containing an SPaseI cleavage site (59). The *H. influenzae* enzyme has previously been shown to be lipid modified, and that of *B. hermsii* is presumed to be a lipoprotein (31, 54). As shown in Fig. 6C, Kyte-Doolittle analysis revealed that the GlpQ of *T. pallidum* is highly hydrophilic (Fig. 6C).

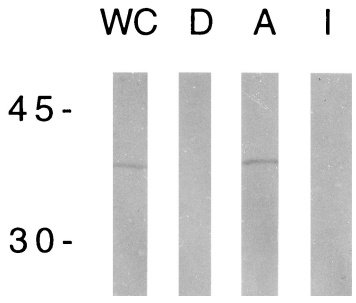
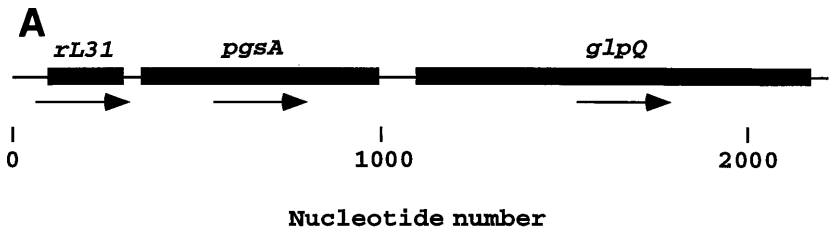


FIG. 5. Amphiphilic analysis of native Bmp. *T. pallidum* (10^9) was solubilized in 2% Triton X-114 and phase partitioned. Polypeptides in whole cells (WC), detergent-enriched phase (D), aqueous phase (A), and Triton X-114-insoluble material (I) were separated on an SDS–12.5% polyacrylamide gel, transferred to nitrocellulose, and immunoblotted with a 1:500 dilution of rat anti-Bmp antiserum generated with recombinant Bmp. Molecular mass markers (in kilodaltons) are shown on the left.

Ninety-nine base pairs upstream from the translational start codon of GlpQ was a 216-amino-acid ORF with homology to a number of bacterial phosphatidylglycerophosphate synthases (PgsA), enzymes which catalyze the committed step in the biosynthesis of acidic phospholipids (50) (Fig. 6A). The best match was for the PgsA protein of *B. subtilis* (58% similar and 32% identical). Hydrophilicity analysis predicted that similar to the PgsA of *E. coli* (50), the treponemal homolog possesses multiple hydrophobic domains consistent with a polytopic integral CM protein (data not shown). No promoter elements could be identified in front of *glpQ*. However, appropriately located putative σ^{70} promoter elements were found upstream of the *pgsA* translational start (data not shown). This finding, along with the lack of a transcriptional terminator in the intergenic region, suggests that *glpQ* and *pgsA* are transcriptionally linked. In *E. coli*, *B. subtilis*, *H. influenzae*, and *B. hermsii*, the *glpQ* gene is in close proximity to the gene (*glpT*) which codes for the G3P transporter, whereas *pgsA* (which is not a member of the *glp* regulon) is located elsewhere on the chromosomes of the respective organisms (7, 24, 29, 54, 59). Given that G3P cannot passively traverse the CM, the existence of a *T. pallidum* homolog for GlpQ leads us to predict that the spirochete that causes syphilis also possesses a GlpT homolog. Because only 39 bp downstream of *glpQ* were sequenced, no statements about linkage with other members of the *glp* regulon or the presence of transcriptional terminators can be made.

The ORF upstream of *pgsA* codes for a 67-amino-acid protein with a high degree of sequence relatedness to ribosomal protein L31 (Fig. 6A); the strongest match (77% similar and 55% identical) was for the *H. influenzae* homolog. The presence of possible σ^{70} promoter elements in front of this ORF suggests that it is separately transcribed from the putative *pgsA*/*glpQ* operon (data not shown).

***T. pallidum* OM fractions and the quest for rare OM proteins—a reappraisal.** The primary objective of this study was to determine whether one or more candidates in our OM preparations are bona fide rare OM proteins of *T. pallidum*. Three of the candidates, Tlp, PpiB, and Bmp, do in fact possess



B

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                                                                      70
ACTGAAGGTAGGATCAGCGCTCAGTGGAGATATGCGGCGTGCTACTATGCACGGTGACGATCTGTGCATT

                                                                     140
CTATAACAGGGGAGGAGAGAAGTTTATGCGGGGAACATATTGTGTGACGCTTTGGGGGGGGGTGTTTGCG
                  SD                 M  R  G  T  Y  C  V  T  L  W  G  G  V  F  A>

                                                                     210
GCATTGGTTGCAGGCTGTGCGTCCGAACGTATGATAGTTGCGTATCGGGGCGCTGCAGGATATGTGCCCG
 A  L  V  A  G  C  A  S  E  R  M  I  V  A  Y  R  G  A  A  G  Y  V  P>

                                                                     280
AGCACACCTTTGCCTCGAAAGTTCTTGCTTTTGCACAAGGAGCAGATTACCTGCAGCAGGATGTCGTGCT
E  H  T  F  A  S  K  V  L  A  F  A  Q  G  A  D  Y  L  Q  Q  D  V  V  L>

                                                                     350
TTCAAAGGATAATCAGCTTATCGTAGCGCAAAGCCATATTCTGGATAATATGACTGACGTGGCAGAAAAA
  S  K  D  N  Q  L  I  V  A  Q  S  H  I  L  D  N  M  T  D  V  A  E  K>

                                                                     420
TTTCCACGCCGGCAGCGTGCGGATGGGCATTTCTATGTCATAGATTTTACGGTAGAAGAACTTTCCCTCC
 F  P  R  R  Q  R  A  D  G  H  F  Y  V  I  D  F  T  V  E  E  L  S  L>

                                                                     490
TCCGTGCAACCAATAGTTTCTATACGCGCGGTAAGCGACATACGCCGGTGTATGGCCAGCGCTTTCCTCT
L  R  A  T  N  S  F  Y  T  R  G  K  R  H  T  P  V  Y  G  Q  R  F  P  L>

                                                                     560
TTGGAAGCCTGGTTTTAGGCTGCACACTTTTGAAGAGGAGTTGCAGTTTATCCGTGGGTTGGAACAGACA
  W  K  P  G  F  R  L  H  T  F  E  E  E  L  Q  F  I  R  G  L  E  Q  T>

                                                                     630
ACCGGGAAAAAGATTGGAATTTACTCTGAAATAAAGGTGCCGTGGTTTCATCATCAGGAAGGAAAAGACA
 T  G  K  K  I  G  I  Y  S  E  I  K  V  P  W  F  H  H  Q  E  G  K  D>

                                                                     700
TCGCAGCGCTTACCCTCGCTCTGTTGAAAAAATACGGTTACCAAAGTCGATCGGATCTAGTGTATGTGCA
I  A  A  L  T  L  A  L  L  K  K  Y  G  Y  Q  S  R  S  D  L  V  Y  V  Q>

                                                                     770
AACGTATGATTTTAACGAGCTGAAGCGTATCAAACGAGAACTTTTACCAAAGTACGAAATGAACGTGAAG
  T  Y  D  F  N  E  L  K  R  I  K  R  E  L  L  P  K  Y  E  M  N  V  K>

                                                                     840
CTGATTCAGCGTGTTGCTTACACAGATCAACGTGAAACACAGGAGAAGGACTCGCGTGGGAAATGGATAA
 L  I  Q  R  V  A  Y  T  D  Q  R  E  T  Q  E  K  D  S  R  G  K  W  I>

                                                                     910
ACTACAATTACAATTGGATGTTTGAGCCCGGTGGTATGCAGAAAATAGCAAAATATGCAGACGGCGTGGG
N  Y  N  Y  N  W  M  F  E  P  G  G  M  Q  K  I  A  K  Y  A  D  G  V  G>

                                                                     980
TCCTGACTGGAGGATGCTCATAGAGAATGAATGGTCGAAGGTGGGCGCTGTTCGCCTGAGTCCGATGGTT
  P  D  W  R  M  L  I  E  N  E  W  S  K  V  G  A  V  R  L  S  P  M  V>

                                                                    1050
TCTGCAATCCAAGATGCGAAATTGGAATGTCATGTGCACACGGTACGGAAAGAAACACTGCCTAGCTACG
 S  A  I  Q  D  A  K  L  E  C  H  V  H  T  V  R  K  E  T  L  P  S  Y>

                                                                    1120
CGCGCACCATGGACGAGATGTTTTCCATTTTGTTCAAACAGACGGGCGCAAACGTGGTGCTCACGGATTT
A  R  T  M  D  E  M  F  S  I  L  F  K  Q  T  G  A  N  V  V  L  T  D  F>

                                                                    1190
TCCTGATCTTGGGGTAAAGTTTCTGGGCAAACCCGCCCGCTATTGACCGGCTTCTGTGTAGTCCAGGCGA
  P  D  L  G  V  K  F  L  G  K  P  A  R  Y  ***
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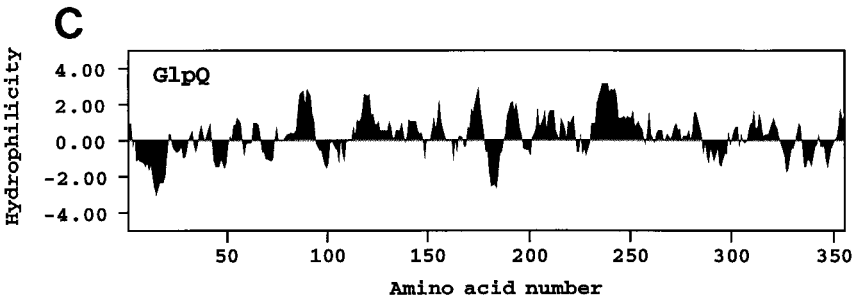

FIG. 6. Molecular analysis of *glpQ*, the glycerophosphodiester phosphodiesterase gene. (A) Schematic diagram of *glpQ* and upstream genes encoding phosphatidylglycerophosphate synthase (*pgsA*) and 50S ribosomal protein L31 (*rL31*). Arrows indicate the directions of transcription. (B) Nucleotide and deduced amino acid sequences of *glpQ*. The proposed Shine-Dalgarno (SD) sequence is underlined. The putative GlpQ SPaseII processing site is boxed. The peptide sequences obtained by automated Edman degradation of internal tryptic fragments are underlined. The amino acids in boldface were used to design the degenerate primers glp-5′IN and glp-3′IN (Table 1). Asterisks indicate the translational stop codon. (C) Hydrophilicity analysis of GlpQ with a window size of 7; hydrophobic regions are below the baseline, and hydrophilic regions are above the baseline.

some properties typical of OM proteins of gram-negative bacteria. All three are synthesized with leader peptides cleaved by SPaseI, and all three lack hydrophobic stretches long enough to act as typical CM-spanning domains (46). Nevertheless, the sequence homology data for Tlp and PpiB and the Triton X-114 phase partitioning results for Bmp indicated that all three are almost certainly periplasmic. As a putative lipoprotein, GlpQ would be amphiphilic due to the lipid modification of its N terminus; as with other spirochetal lipoproteins (47), its hydrophilic polypeptide presumably lacks transmembrane domains and is extrinsic to the lipid bilayer.

Given the considerable time and effort required to genetically analyze these proteins, it was indeed disappointing that none appears likely to be a rare OM protein. On the other hand, prior to this work, little was known about nonlipidated periplasmic proteins in *T. pallidum* (42). Findings here and elsewhere suggest that this is probably due to the fact that these proteins are relatively low in abundance as well as poorly immunogenic during syphilitic infection (42, 47). With the exception of the flagellar proteins, Tlp and PpiB are the first nonlipidated periplasmic proteins with putative physiological roles to be described for the syphilis spirochete (42). As noted earlier, the expression of treponemal Tlp and GlpQ homologs also implies the existence of physiologically linked genes. The availability of the *T. pallidum* genomic sequence in the near future will determine whether these predictions are accurate.

If these four candidate polypeptides are not rare OM proteins, why were they found in OM fractions? One potential explanation is that each possesses one or more domains which foster a stable association with the OM. With respect to GlpQ, this would occur if the polypeptide was lipid anchored to either the periplasmic or external leaflet of the OM. Although GlpQ is periplasmic in *E. coli* (35), it is worth noting that in *H. influenzae*, GlpQ (protein D) is believed to be surface exposed in addition to being lipid modified (31, 39, 51). For two of the nonlipoproteins, Tlp and PpiB, hydrophilicity analyses revealed small hydrophobic domains which conceivably could facilitate stable OM associations. However, the alternative explanation, namely, that these candidates are contaminants from the periplasm (i.e., Tlp, PpiB, and Bmp) or CM (i.e., GlpQ), also merits consideration. It is already known that *T. pallidum* OM fractions obtained by either of the two published procedures (11, 49) contain small amounts of periplasmic and CM contaminants. As shown in Fig. 1, these contaminants may appear to be major OM constituents when relatively large amounts of OMs are subjected to SDS-PAGE analysis. Moreover, distinguishing between authentic OM constituents and contaminants becomes truly problematic when previously uncharacterized, low-abundance proteins are being examined. This uncertainty is compounded by the fact that the only reliable method so far for detecting rare OM proteins, freeze-fracture electron microscopy, does not permit accurate estimates of the number of distinct OM protein species or their relative abundances in whole treponemes or OM fractions. In other words, it may be assumed incorrectly that rare OM proteins are enriched sufficiently in OM fractions to be easily detectable by SDS-PAGE and therefore easily distinguishable from periplasmic and CM contaminants.

The recognition of these caveats and of their attendant risks for misidentification of rare OM proteins has given rise to divergent interpretations of the molecular data generated by fractionation-based studies of candidate OM proteins. One viewpoint is that *T. pallidum* has multiple OM proteins, many of which are among the candidates identified in OM preparations (10). Two of them, Tromp1 and Tromp2, have already been described, and a third, Tromp3, is currently under investigation (8–10, 16). The alternative viewpoint, supported by studies here and elsewhere (2, 28, 47, 55), is that most (perhaps all) of the candidate OM proteins identified thus far are derived from cellular compartments other than the OM. The inability so far to localize native Tromps in *T. pallidum* and our recent finding that Tromp2 possesses sequence relatedness (49% similarity and 23% identity) to FlaA (3) are also consistent with this interpretation. The resolution of these discordant notions is clearly of considerable importance for our understanding of *T. pallidum* membrane biology and its relationship to syphilis pathogenesis.

If most candidate OM proteins are contaminants, then a critical question is whether the use of OM preparations remains a viable strategy for characterizing rare OM proteins. This question has two possible answers. The first is affirmative, provided that approaches can be developed to improve our ability to select candidates for subsequent molecular characterization and cellular localization. The alternative possibility is that the low abundances of rare OM proteins in *T. pallidum* and/or OM fractions preclude the accumulation of bona fide OM proteins in amounts sufficient for microsequencing. The presence of unidentified extremely-low-abundance polypeptides in OM preparations (Fig. 1) lends credence to this possibility. If this turns out to be the case, then an entirely different approach, most likely one employing computer-based analysis of the *T. pallidum* genomic sequence to identify candidate molecules, will be needed in the ongoing search for rare OM protein(s) of the syphilis spirochete.

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