Identification and Purification of a Recombinant *Treponema pallidum* Basic Membrane Protein Antigen Expressed in *Escherichia coli*

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A recombinant plasmid designated pLVS3 previously was described that harbored a 14-kilobase insert of *Treponema pallidum* genomic DNA. *Escherichia coli* maxicells programmed with this plasmid synthesized three treponemal protein antigens of molecular weights 39,000, 35,000, and 25,000 (39K, 35K, and 25K proteins, respectively). In this study, a detailed deletion analysis of pLVS3 demonstrated that the genetic information for all three protein antigens is contained within a 1.5-kilobase *Eco*RI-*Hpa*I restriction fragment. The DNA sequence of this fragment revealed a single open reading frame of 361 codons that most likely encodes a signal peptide-bearing precursor to the 39K protein that can be transiently detected in *E. coli* maxicells. Evidence indicated that the 35K and 25K protein antigens are derivatives of the larger protein and are only produced in maxicells. A significant elevation in expression of the 39K treponemal protein antigen in *E. coli* was obtained by using the *E. coli lpp* and *lac* promoters and a genetic construction in which the signal peptide and first four residues of the "mature" 39K protein were replaced by six amino acids encoded by the vector. This hybrid protein exhibited an unusually high pI, which greatly facilitated its purification to homogeneity. By using antibody prepared against the hybrid protein, the native treponemal protein counterpart, also of molecular weight 39,000, was identified as a membrane component of *T. pallidum*. Since the native protein also exhibited a net positive charge, it has been designated the *T. pallidum* basic membrane protein.

The bacterium *Treponema pallidum*, the etiological agent of syphilis, cannot be continuously cultivated in vitro. To obtain treponemes for experimental analyses, the organism usually is cultivated in rabbit testes, from which it can be extracted and purified in only limited quantities. For this reason, little has been learned concerning the virulence determinants of *T. pallidum* or about the immunogenic determinants responsible for eliciting a protective host immune response. Despite the experimental difficulties, researchers have extensively compared the protein profiles of various noncultivable and cultivable treponemal strains by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and defined the various protein antigens of *T. pallidum* that are reactive with sera from both infected humans and rabbits at different times after infection (3, 5, 15, 16, 26, 30, 44, 47, 50). Several studies have attempted to identify those protein antigens that reside on the treponemal cell surface (1, 2, 21, 33, 35, 39, 44, 48), and it has been suggested that several proteins serve as ligands involved in the adherence of treponemes to host tissues (6, 37, 49). In addition, a unique class of extracellular protein antigens of *T. pallidum* has been identified (44).

In recent years, several laboratories have begun applying recombinant DNA technology to the study of *T. pallidum*, in hopes of at least partially circumventing problems associated with the cultivation of this organism (11, 17, 31, 39, 43, 45, 46, 54). It was anticipated that recombinant treponemal

proteins would be conveniently produced in sufficient quantities such that their role in syphilis and possibly other human treponematoses could be defined and their applicability as vaccinogens or improved serodiagnostic reagents could be investigated.

A recombinant plasmid, pLVS3, was initially identified by screening *Escherichia coli* clones for the expression of antigens recognized in an in situ assay by high-titer rabbit and human syphilitic sera (45). Subsequently, this plasmid was found to encode three *T. pallidum* protein antigens of molecular weights 39,000, 35,000, and 25,000 (39K, 35K, and 25K proteins, respectively) (46). Although the native *T. pallidum* counterparts to these proteins were not known, the 39K protein was of particular interest for several reasons. First, there was an indication that this protein is initially synthesized with an amino-terminal signal peptide that would direct this protein to some extracytoplasmic compartment in *E. coli* and *T. pallidum* (46). Second, the evidence indicated that *E. coli* cells harboring plasmid pLVS3 expressed treponemal antigens on their cell surface (43, 45). Third, this protein was recognized by virtually every human syphilitic serum tested, including sera from patients with known primary, secondary, latent, and late cardiovascular syphilis (46). Thus, it was thought that this protein might have value as a serodiagnostic reagent for syphilis.

In this study, the nucleotide sequence of the gene encoding the 39K protein has been determined. From this sequence and other data, the presence of a signal peptide is strongly indicated. The coding sequence has been cloned into an *E. coli* expression vector, facilitating purification of a hybrid protein that includes nearly the entire "mature" 39K protein. Rabbit immunoglobulin G (IgG) antibodies prepared

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against the purified protein precipitated a single, 39K membrane protein from a solubilized, radiolabeled *T. pallidum* cell extract. The purified, recombinant protein was found to have an unusually high pI. Additional evidence indicated that the native 39K protein also was quite basic. For these reasons, the 39K protein encoded by plasmid pLVS3 has been designated the *T. pallidum* basic membrane protein (BMP).

MATERIALS AND METHODS

Bacterial strains, phage, and plasmids. M13 phage derivatives and pUC8 derivatives were propagated in *E. coli* JM103 (32). All other plasmids were propagated in strain MM294 (ATCC 33625). Strain SE5000 (46) was used for maxicell analyses in pulse-chase experiments. Strain MC4100 (9) was used for protein purification. Phage M13mp18 was used for determining the *bmp* DNA sequence (32). The plasmid pLVS3 is a pBR322 derivative harboring a 14-kilobase insert of *T. pallidum* genomic DNA (27), and pUC8 is a plasmid with a multiple cloning site region (52). The expression vectors pIN-III-A2 and pIN-III-A3 have been described previously (27). The source and cultivation of *T. pallidum* (Nichols strain) were previously described (46).

DNA isolation, enzyme reactions, gel electrophoresis, and transformation. Plasmid DNA was isolated using an alkaline lysis method for both minipreparations and equilibrium density centrifugation (38). All enzymes were purchased from Bethesda Research Laboratories, Gaithersburg, Md., and restriction enzyme and ligase reaction conditions were as described by O'Farrell et al. (34). Restricted DNA samples were analyzed on vertical agarose slab gels in Tris-acetate buffer. Plasmid DNA was transformed into bacterial strains by the method of Hanahan (14). DNA fragments were isolated from agarose gels by the procedure of Lizardi et al. (24).

Identification of recombinant *T. pallidum* protein antigens. Plasmids were transformed into *E. coli* SE5000, and [^{35}S]methionine-radiolabeled maxicell extracts were prepared as previously described (46). Recombinant treponemal protein antigens were immune precipitated from solubilized maxicell extracts by using high-titer experimental rabbit syphilitic serum and analyzed by SDS-PAGE and fluorography as previously described (46). [^{35}S]methionine (translation grade; 1,154 Ci/mol) was obtained from New England Nuclear Corp., Boston, Mass.

Plasmid derivatives of pLVS3. Deletion derivatives of pLVS3 were isolated in two steps. Initially, pLVS3 was digested with either *Hpa*I, *Sst*I, or *Eco*RI. The enzyme was heat inactivated, the reaction mixture was diluted to a DNA concentration of 20 μg/ml, and the ends of the plasmid were rejoined with ligase. After transformation of the ligation mix into strain MM294 cells, colonies were screened (38) for the presence of plasmids that represented deletions of pLVS3. The plasmid isolated after *Eco*RI deletion, designated pTP020 (possessing only one *Eco*RI site), was used in making additional deletions. The plasmid was linearized by restriction with *Sal*I, for which there is a single site within the vector. The plasmid was then subjected to a second digestion with either *Sst*I, *Hpa*I, or *Hin*dIII. All of the ends were made blunt by treating the DNA with Klenow fragment of DNA polymerase I in the presence of all four deoxyribonucleotide triphosphates (8). As described above, the ends of the plasmid were joined, and subsequent transformants were analyzed for plasmid. Plasmid pTP022 (see Results) was the plasmid derived from the *Sal*I-*Sst*I digest and was

shown to have a regenerated *Sal*I site but no *Sst*I site. As predicted from the DNA sequence (see below), plasmids from the other two double digests did not regenerate the restriction sites used to make the deletions.

The unique *Hpa*I site in pTP020 was converted to an *Eco*RI site with an oligonucleotide (GGAATTCC) from New England Biolabs, Beverly, Mass., that was incorporated as described by Bahl et al. (4). A transformant having a plasmid with two *Eco*RI sites and no *Hpa*I site was identified and was designated pTP027.

DNA sequence determination. An *Eco*RI fragment from plasmid pTP027 was isolated and inserted into M13mp18. The insert could only be isolated in the orientation that would not result in transcription of *bmp*. To sequence the opposite strand with M13mp18, the *Eco*RI fragment was cloned as two *Eco*RI-*Mst*I blunt-ended fragments into the *Sma*I site. The DNA sequence was determined by the strategy of Henikoff (19) and the chain-terminating method of Sanger et al. (41). DNA was radiolabeled with ^{35}S, and reaction mixtures were electrophoresed on buffer gradient gels (7). The sequence at the junction of expression vectors and *bmp* was determined by the method of Maxam and Gilbert (28).

Construction of plasmids pTP106 and pTP107. The generation of plasmid pTP106 is presented diagrammatically in Fig. 1. A *Dde*I fragment (which included a *Pst*I site) was isolated from pTP025 DNA and cloned into plasmid pUC8 by filling in the *Dde*I ends and inserting the fragment into the *Sma*I site. The insert orientation was determined by using a *Hinf*I site (which overlapped the *Dde*I site by 3 base pairs [bp]). The small *Pst*I-*Hpa*I fragment was cloned from pTP020 into pUC8 between the *Pst*I and *Hinc*II sites, forming pTP007. A three-fragment ligation of the *Eco*RI-*Pst*I fragment from the first construction, the *Pst*I-*Bam*HI fragment from pTP007, and *Eco*RI-*Bam*HI-cleaved pIN-III-A2 DNA generated plasmid pTP106. Determination of the DNA sequence from the *Xba*I site through the *Pst*I site confirmed the plasmid structure. Plasmid pTP107 was a 25-bp deletion derivative of pTP106. The latter was restricted with both *Xba*I and *Eco*RI, the ends were repaired, and the plasmid was recircularized as described above. Both enzyme sites were regenerated (as predicted, TCTAGAATTCC) in pTP107, and the *Xba*I-*Pst*I small fragment was shown to be the same size as the *Eco*RI-*Pst*I small fragment by agarose gel electrophoresis.

Construction of plasmid pTP102. Plasmid pTP007 DNA was restricted with *Pst*I, the ends were made blunt, and the *Eco*RI linker was inserted. The treponemal DNA was then excised from the plasmid with *Eco*RI and *Bam*HI and inserted into the expression vector pIN-III-A3, forming pTP102. The structure of the plasmid was confirmed by DNA sequencing.

Pulse-chase experiments. Vigorously aerated 6-ml cultures were grown in M63 minimal medium (29) with 0.2% glycerol as the carbon source at 37°C to an optical density at 600 nm of 0.2 and then induced with 2 mM isopropyl-β-D-thiogalactopyranoside. At 1 h postinduction, cells were pulse-labeled with [^{35}S]methionine (32.5 μCi/ml). The chase was initiated by the addition of 1/10 volume of 4% unlabeled methionine. Samples (0.7 ml) were removed at the indicated times and added to an equal volume of ice-cold 10% trichloroacetic acid. The acid precipitates were pelleted, washed once with 0.5 ml of acetone, and air dried. The pellets were suspended, with a Bransonic 12 water bath sonifier, in 140 μl of 10 mM Tris (pH 7.5)–1 mM EDTA–1% SDS and solubilized by heating for 4 min in a boiling water bath. A portion

(50 μl) of the clarified supernatant of these samples was used as the antigen extract in an immune precipitation procedure identical to one previously described (46). Samples of 10 μl of rabbit anti-hybrid BMP (BMP*; see Results) IgG were used for each precipitation reaction. Precipitates were analyzed by SDS-PAGE and fluorography as previously described (46).

Purification of the BMP*. *E. coli* MM294 harboring plasmid pTP102 was grown in L broth (29) containing 50 mg of ampicillin per liter at 35°C under forced aeration. The cells were grown in 15-liter carboys to an optical density at 600 nm of 0.8 to 1.0, at which time isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1.3 mM. After an additional 2 h of incubation with inducer, cells (150 liters)

FIG. 1. Schematic diagram of the steps involved in constructing expression plasmids. The *Eco*RI-*Hpa*I fragment from pLVS3 is shown at the top. A *Dde*I fragment was inserted into pUC8 at the *Sma*I site, forming pTP089. A *Pst*I-*Hpa*I fragment was inserted into pUC8 at the *Pst*I and *Hinc*II sites, forming pTP007. For clarity, the orientation of pTP007 is shown as the reverse of pTP089 (note the arrows indicating the direction of transcription from the *lac* promoter). An *Eco*RI-*Pst*I fragment from pTP089, a *Pst*I-*Bam*HI fragment from pTP007, and *Eco*RI-*Bam*HI-cleaved pIN-III-A2 DNA were joined to form pTP106. An expanded region of pIN-III-A2 is shown with the lipoprotein gene and lactose operon (*lppP* and *lacP*), the lactose operon operator (*lacO*), and the lipoprotein gene ribosome-binding site and initiation codon (rb and ATG). Arrows in pTP106 indicate the direction of transcription for the ampicillin resistance gene (*bla*), the lactose repressor gene (*lacI*), and the *lppP-lacP* double promoter. An *Xba*I-*Eco*RI deletion was made in pTP106 to form pTP107. Abbreviations for restriction enzymes: E, *Eco*RI; D, *Dde*I; P, *Pst*I; Hp, *Hpa*I; B. *Bam*HI; Sm, *Sma*I; Hc, *Hinc*II; X, *Xba*I.

were harvested with the Pellicon (Millipore Corp.). After concentration to 3 liters, cells were centrifuged at 13,000 × *g* for 15 min. The cell pellet (574 g) was suspended in a final volume of 1,000 ml of 50 mM KPO$_4$ buffer (50 mM dipotassium phosphate adjusted to pH 7.3 with 50 mM monopotassium phosphate) and disrupted by sonication (10 30-s bursts). The total cell extract was centrifuged at 23,000 × *g* for 1 h. The pellet was suspended in 400 ml of buffer with an Omnimixer (Sorvall) and centrifuged again. The supernatants were combined (900 ml) and 342 g of solid (NH$_4$)$_2$SO$_4$ was added to the crude extract as the extract was stirred at 4°C. After 30 min, the suspension was centrifuged at 13,000 × *g* for 20 min, and the supernatant was discarded. The pellet was suspended in 200 ml of 50 mM KPO$_4$ buffer and dialyzed four times against 4 liters of the same buffer.

The dialyzed extract (415 ml) was pumped onto a 2.5- by 20-cm column of phosphocellulose that had been equilibrated with the same buffer at a flow rate of 2.0 ml/min. The column was washed with 300 ml of the same buffer. The protein of interest was eluted from the column with 500 ml of 50 mM KPO$_4$ buffer containing 0.2 M LiCl, and 20-ml fractions were collected. The fractions were analyzed by SDS-PAGE (23); the fractions containing the protein were pooled (450 ml), precipitated with (NH$_4$)$_2$SO$_4$, and dialyzed three times against 2 liters of 25 mM KPO$_4$ buffer (pH 7.4). The dialyzed solution was passed through a 2.5- by 10-cm column of DE-52, which was subsequently washed with the same buffer. Fractions of 10 ml were collected, and the absorbance at 280 nm was monitored. Those fractions with an absorbance of greater than 0.05 were pooled and lyophilized. The residue was resuspended in H$_2$O to a total volume of 40 ml; 10-ml samples were chromatographed on a 2.5- by 100-cm G-200 Sephadex column equilibrated in 25 mM KPO$_4$ (pH 7.4) at a flow rate of 12.5 ml/h, and 5-ml fractions were collected. The absorbance at 280 nm was determined, and once again those fractions with an absorbance greater than 0.05 were assayed by SDS-PAGE. Those fractions containing the BMP* were pooled, dialyzed against 4 liters of 10 mM EDTA for 12 h, then dialyzed against H$_2$O for 24 h, and lyophilized.

Protein concentrations were determined by the method of Lowry et al. (25) with bovine serum albumin as the standard. The presence of the protein and its purity were determined by SDS-PAGE (23); protein bands were stained and destained by the method of Ferguson (12). Molecular weight markers were obtained from Pharmacia Fine Chemicals, Piscataway, N.J. The isoelectric point (pI) of the BMP* was determined by isoelectric focusing as previously described (40) with carrier ampholytes (Pharmylate pH 8 to 10.5 with 10% Pharmylate pH 6 to 8) obtained from Pharmacia.

Preparation of rabbit anti-BMP* serum. Antiserum to the BMP* was obtained from male New Zealand White rabbits. Purified BMP* (1 mg) was suspended in phosphate-buffered saline and mixed 1:1 with incomplete Freund adjuvant to a total volume of 1.0 ml. A 0.2-ml sample was injected into the muscle of each hind leg; booster injections of purified BMP* in phosphate-buffered saline alone were administered at 2-week intervals. After rabbits were bled, the IgG antibody fraction was purified as previously described (10).

Identification of native BMP. Cells of *T. pallidum* that had been freshly extracted from infected rabbit testes were intrinsically radiolabeled with [^{35}S]methionine as previously described (44). Treponemal fractions were prepared and solubilized, and the native BMP was precipitated with rabbit anti-BMP* IgG and analyzed by SDS-PAGE and fluorography as previously described (44).
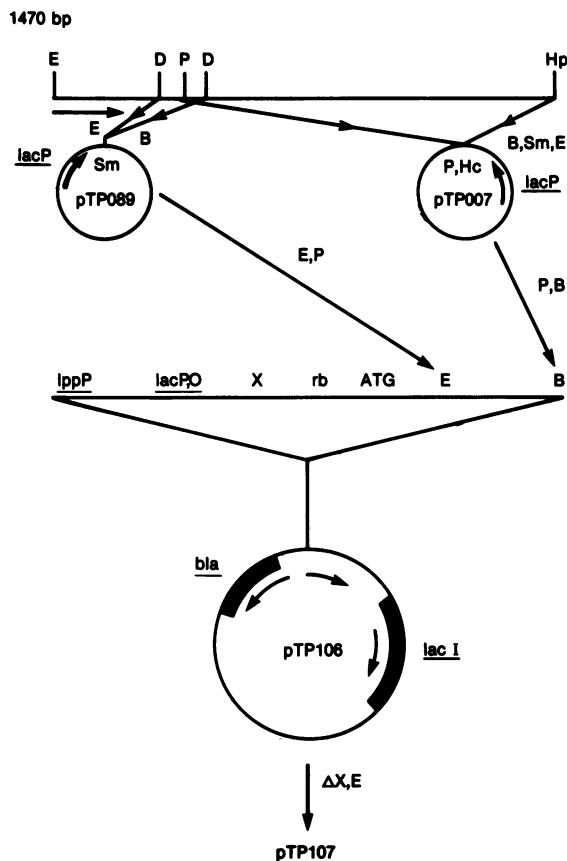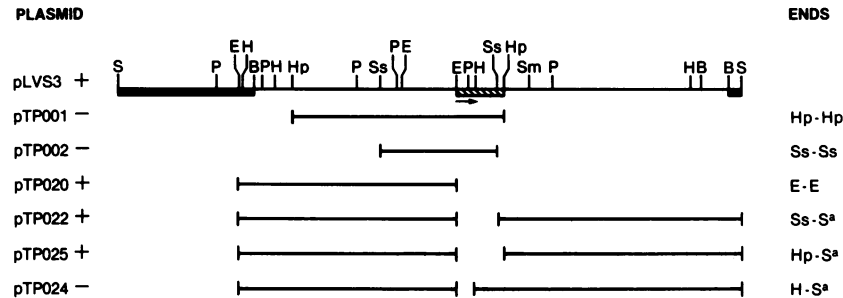
FIG. 2. Schematic diagram of restriction enzyme recognition sites and deletion derivatives of plasmid pLVS3. The plasmid has been linearized by cleavage with *Sal*I, which cuts the plasmid once in the vector portion. The thickened line represents pBR322. The cross-hatched line demarcates that region coding for the *T. pallidum* protein antigens, and the underlying arrow indicates the direction of transcription. Listed at the left are deletion derivatives of pLVS3, and the + or − indicates whether or not cells carrying the plasmid express the 39K protein. Solid bars show the extent of each deletion, and the column at right denotes the ends of each deletion. The superscript a indicates plasmids that are deletion derivatives of pTP020 (see Materials and Methods). Abbreviations for restriction enzymes: S, *Sal*I; P, *Pst*I; E, *Eco*RI; H, *Hin*dIII; B, *Bam*HI; Hp, *Hpa*I; Ss, *Sst*I; Sm, *Sma*I.

RESULTS

Restriction map and deletion analysis of pLVS3. The recombinant plasmid pLVS3 was previously shown to consist of two *Bam*HI fragments of *T. pallidum* DNA (13,700 and 900 bp) inserted into the single *Bam*HI site of pBR322 (45, 46). To initiate this study, a more detailed restriction map of pLVS3 was generated (Fig. 2). By selectively deleting specific DNA fragments with restriction enzymes, a series of smaller plasmids was obtained, some of which are diagramatically presented in Fig. 2. The effect of each deletion on the expression of the three treponemal protein antigens was then evaluated by using *E. coli* maxicells to detect plasmid-encoded proteins. The identity of the treponemal proteins was authenticated by immunoprecipitation of radiolabeled maxicell extracts with experimental rabbit syphilitic serum (Fig. 3). Deletion of either a 6,500-bp *Hpa*I fragment (plasmid pTP001) or a 3,600-bp *Sst*I fragment (plasmid pTP002) resulted in the concomitant loss of synthesis of all three proteins. In contrast, all three proteins were still expressed, albeit in lesser amounts, after deletion of two *Eco*RI fragments totaling 6,700 bp (plasmid pTP020). This latter plasmid was then used to generate additional deletions, each extending from the unique *Sal*I site located in the vector portion to an *Sst*I site (plasmid pTP022), an *Hpa*I site (pTP025), or an *Hin*dIII site (pTP024). Maxicell analysis of each of these plasmids revealed that only the 8,500-bp *Sal*I-*Hin*dIII deletion abolished expression of all three protein antigens. Neither of the other deletions eliminated expression of any of the proteins, although the 8,000-bp *Sal*I-*Sst*I deletion in plasmid pTP022 did result in the appearance of an additional higher-molecular-weight immunoreactive protein antigen (Fig. 3). Note that pTP025 was the smallest plasmid that, when analyzed in maxicells, encoded each of the three treponemal protein antigens of the parental plasmid pLVS3. The size of the DNA insert in pTP025 was estimated to be approximately 1,470 bp, which is insufficient to encode successive genes for the three treponemal protein antigens. However, it would be adequate to encode the largest of the three, a protein of molecular weight 39,000. Note that there have been at least two distinct treponemal proteins identified in different laboratories as the "39K" protein (33, 46). Since the 39K protein encoded by plasmid pLVS3 has been shown to exhibit an unusually high pI (see below), this protein will henceforth in the text be referred to

as the BMP of *T. pallidum*. The DNA sequence encoding this protein has been designated the *bmp* gene.

Elevated expression of the BMP. The 1,470-bp fragment was bounded by an *Eco*RI site and an *Hpa*I site. In an effort to obtain elevated synthesis of the recombinant treponemal proteins in *E. coli*, this fragment was inserted into the expression vector pIN-III-A2, which uses the *E. coli* lipoprotein (*lppP*) and *lacUV5* promoters to enhance transcription of cloned DNA (27). These promoters are followed by the lactose operator (*lacO*), which permits the regulation of transcription of distal genes by the lactose repressor, whose structural gene is also included on the vector. The *Hpa*I site at one end of the 1,470-bp fragment was converted to an *Eco*RI site by adding an *Eco*RI linker to pTP020, thus permitting insertion of the fragment into the unique *Eco*RI site of plasmid pIN-III-A2 in both possible orientations. Plasmid isolates corresponding to the two insert orientations were identified by restricting with *Hin*dIII. Gene expression from two representative plasmids was then analyzed in maxicells (Fig. 4). For just one of these plasmids (pTP109), the presence of isopropyl-β-D-thiogalactopyranoside resulted in enhancement of expression of the BMP, indicating
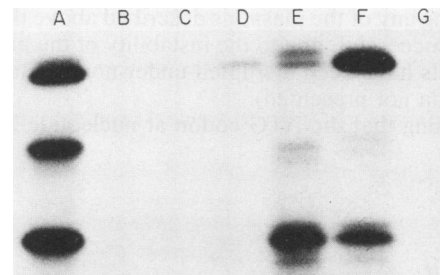
FIG. 3. *T. pallidum* protein antigens epressed by plasmid pLVS3 and its deletion derivatives. Plasmid-encoded, [^{35}S]methionine-radiolabeled recombinant treponemal protein antigens were immune precipitated from solubilized *E. coli* maxicell extracts and analyzed by SDS-PAGE and fluorography. Only the relevant portion of the gel is shown. Lanes: A, pLVS3; B, pTP001; C, pTP002; D, pTP020; E, pTP022; F, pTP025; G, pTP024. Protein antigens of 39K, 35K and 25K can easily be discerned in lane A. Note that longer exposure of this gel clearly revealed synthesis of all three protein antigens by plasmid pTP020 (lane D).

that the direction of transcription of the *bmp* gene is from the *Eco*RI site toward the *Hpa*I site (Fig. 2).

The nucleotide sequence of the entire *Eco*RI-*Hpa*I fragment of pLVS3 was determined (Fig. 6). An ATG codon was identified at nucleotide 285 (numbering from the *Eco*RI site), which was preceded by a putative ribosome binding site (GGAG; nucleotides 276 through 279 [42]) and followed by a long open reading frame capable of encoding a protein of 361 residues. This was the only large open reading frame encountered in this DNA fragment and thus must represent the *bmp* gene. Interestingly, note that the *Sst*I site used to generate plasmid pTP022 resides just within the 3' end of the coding sequence. Thus, the somewhat anomalous gel behavior of the recombinant BMP in maxicells programmed with this plasmid must result from generation of a recombinant protein with a new carboxy terminus (see above).

As described in Materials and Methods, plasmid pTP107 was constructed, in which the BMP coding sequence from plasmid pLVS3 was placed just downstream from the *lacO* sequence of plasmid pIN-III-A2. Maxicells programmed with plasmid pTP107 produced a somewhat greater quantity of the BMP than did maxicells programmed with plasmid pTP109 (Fig. 4). This result suggested that the ATG codon at nucleotide 285 was indeed the initiating codon for the BMP.

BMP is initially expressed in *E. coli* in a precursor form. When the production of the BMP was assayed in whole *E. coli* cells containing plasmid pTP107, only a small amount of this protein was made. This may be due to an inefficient ribosome-binding site provided by the native treponemal gene. Also, the possibility was considered that low expression was due in part to the localization of this protein when expressed in *E. coli*. It previously had been suggested that the BMP was synthesized in *E. coli* with an amino-terminal signal peptide that was subsequently cleaved (46). Pulse-chase studies revealed that the BMP was initially synthesized in maxicells as a larger-molecular-weight precursor form that was slowly processed to its 39K "mature" size (Fig. 5A). Evidence that this higher-molecular-weight form represents an unprocessed signal peptide-bearing precursor is supported by the observation that only this form of the protein was observed when maxicells were labeled in the presence of membrane perturbants, either 0.3% phenethyl alcohol or 10% ethanol, treatments which are known to inhibit signal peptide processing in *E. coli* (13; data not presented). Attempts to unambiguously determine the intracellular location of the BMP synthesized in *E. coli* maxicells containing any of the plasmids described above thus far have been unsuccessful, due to the instability of the protein when these cells have been disrupted under nondenaturing conditions (data not presented).
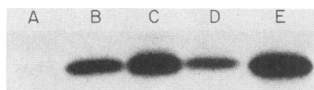
Assuming that the ATG codon at nucleotide 285 initiates
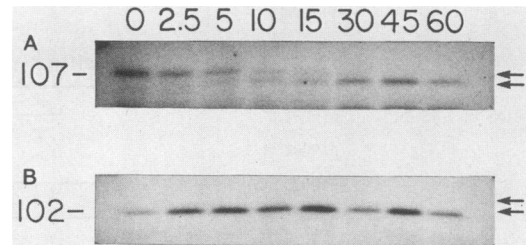

FIG. 5. Pulse-chase analysis of recombinant treponemal protein antigens expressed in *E. coli*. Cells of *E. coli* SE5000 harboring either plasmid pTP107 (A) or pTP102 (B) were pulse-labeled for 30 s with [35S]methionine. At the indicated chase times, samples were taken, total protein was acid precipitated, the recombinant treponemal protein antigen was immune precipitated, and the precipitates were analyzed by SDS-PAGE and fluorography (see Materials and Methods).

translation of the BMP, translation of the downstream DNA sequence revealed a primary amino acid sequence of 20 residues that is fairly typical of procaryotic signal peptides (Fig. 6) (53). This putative signal peptide includes a charged residue very near its amino terminus followed by a fairly long hydrophobic core and, last, the sequence Ala-X-Ala, which precedes the cleavage site in many nonlipoprotein signal peptides in *E. coli* (36, 53). Just beyond this coding region, the DNA sequence identified a *Pst*I site (Fig. 6). Using this restriction site we generated in vitro a deletion that removed the putative signal peptide-coding region, and subsequent manipulations directed translational initiation of the remaining sequence at a site early in the coding region for the mature BMP (see Materials and Methods). In this plasmid, designated pTP102, translation of the 39K protein was initiated at the lipoprotein ribosome-binding site, and the first four residues of the mature BMP were replaced by six amino acids encoded by the vector (Fig. 7). This hybrid protein was designated BMP*. When plasmid pTP102 was analyzed in maxicells, enhanced expression of the BMP* was observed (Fig. 4). As expected, a precursor form for this protein was not discerned in a pulse-chase experiment (Fig. 5B).

Purification of the recombinant BMP* encoded by plasmid pTP102. When whole cells of strain MM294 harboring plasmid pTP102 were analyzed by SDS-PAGE and Coomassie blue staining, the BMP* could easily be discerned (Fig. 8). Interestingly, synthesis of the 25K and 35K treponemal protein antigens was not detected. We estimated that production of this protein was elevated approximately 10-fold above the level observed in cells harboring plasmid pTP107. Cells of strain MM294 (pTP102) were disrupted by sonication, and crude membrane and soluble fractions were prepared and analyzed by SDS-PAGE. Under these conditions, the bulk of the BMP* was clearly in the soluble fraction.

The BMP* was purified to homogeneity by conventional techniques, as described in detail in Materials and Methods. Initially after the soluble and membrane wash fractions were pooled and precipitated with $(NH_4)_2SO_4$, the dialyzed extract was applied to a chromatofocusing column. The protein did not bind to this resin, suggesting that the isoelectric point of this protein was above 7.5. A phosphocellulose resin was then utilized, since it has a high capacity for basic proteins and most native *E. coli* proteins are acidic. Binding of the protein to phosphocellulose and batch elution of the protein with 0.2 M LiCl resulted in approximately a 100-fold purification. Further purification of the protein to apparent homo-

FIG. 4. Synthesis of BMP and BMP* by recombinant plasmids. Plasmid-encoded [35S]methionine-radiolabeled recombinant treponemal protein antigens were immune precipitated from solubilized *E. coli* maxicell extracts and analyzed by SDS-PAGE and fluorography. Only the relevant portion of the gel is shown. Lanes: A, pTP111; B, pTP109; C, pTP107; D, pTP106; E, pTP102. Note that plasmids pTP111 and pTP109 harbor the 1,470-bp *Eco*RI-*Hpa*I fragment in opposite orientations. Plasmid pTP106 was an intermediate in the construction of plasmid pTP102 (see Materials and Methods).

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EcoRI      •         •         •         •         •         •         •        80
GAATTCTGGTGCCGTGGCGTTGTTTGGTGAGAAATACGAATCAACTGTAAAGGTGTACTCCATAGGAACTTTTTCTCGGG

     •         •         •         •         •         •         •         •    160
AAGTGTGTGGCGGTCCACATGTAGCACGTACCGGGCAGCTCGGGCGGTTTTCTATTCAAAAGGAACAGTCGAGTGCGGCG

     •         •         •         •         •         •         •         •    240
GGGGTGCGGCGTATTCGCGCGATTCTTGAGAAAAGCGGGGAAAAGTCGTAACTTGCCCTGCTGCGTATAGTTTCTCCGGC

     •     DdeI •         •         •         •         •         •         •   320
GTGTGTGCTGGTGACTGAGTCGCGGTTGTTTACGGGGAGTTCTCATGGGCAGATACATAGTTCCCGCTCTGTTGTGCGTG
                                              MetGlyArgTyrIleValProAlaLeuLeuCysVal

     •         •         •    PstI •         •         •         •         •    400
GCGGGTATGGGGTTTGCGCACGCGCAGTCGGCGCTGCAGCCTATCGCCGAAGTGAATTTATTCCGGCGTGAGCCGGTGAC
AlaGlyMetGlyPheAlaHisAlaGlnSerAlaLeuGlnProIleAlaGluValAsnLeuPheArgArgGluProValTh

     •         •         •         •         •         •    DdeI•         •    480
GCTCGGACAGATTAAGGCAAGAATTTCTGCGATTGAGAAAGAGATGGGAAAGAAGCTCAGCACTGCTGAGCGTAGACAAT
rLeuGlyGlnIleLysAlaArgIleSerAlaIleGluLysGluMetGlyLysLysLeuSerThrAlaGluArgArgGlnP

     •         •    HindIII•         •         •         •         •         •  560
TCATGGACAGTCTTATTGACGAAAAGCTTTTTGCTCAAGCGGCAGAAAAGGCGGGTATCCAGGTAACGGATGCTGAAGTG
heMetAspSerLeuIleAspGluLysLeuPheAlaGlnAlaAlaGluLysAlaGlyIleGlnValThrAspAlaGluVal

     •         •         •         •         •         •         •         •    640
AATCAGTATTTTAATGGAATGCTTTCTCAACAGATTGGACGTGCGGTAACGGAGGCTGAGTTCGCCAATTACGTCAAGGA
AsnGlnTyrPheAsnGlyMetLeuSerGlnGlnIleGlyArgAlaValThrGluAlaGluPheAlaAsnTyrValLysGl

     •         •         •         •         •         •         •         •    720
GAAGCAGAATATCTCGCTCGATCAGTTCATGAAGCAGCAAAACGGCATGACTATGCAGAATACAAAAAGTTTCTAAAGA
uLysGlnAsnIleSerLeuAspGlnPheMetLysGlnGlnAsnGlyMetThrMetAlaGluTyrLysLysPheLeuLysT

     •         •         •         •         •         •         •         •    800
CGGCAGGTGAGCACGCAGCGCTACGTGGCACAAAAGAAGGCAGACGAGTTTCGGAATTTGAAGGGACCTGAGGACTCTCAA
hrGlnValSerThrGlnArgTyrValAlaGlnLysLysAlaAspGluPheArgAsnLeuLysGlyProGluAspSerGln

     •         •         •         •         •         •         •         •    880
ATCCGTTCCTACTACGAGCTTAATAAACAGGCCTTTTTCCGCCCTGACACGGTTAAGTTGTTTCTTATTTCTGTCCCGAA
IleArgSerTyrTyrGluLeuAsnLysGlnAlaPhePheArgProAspThrValLysLeuPheLeuIleSerValProLy

     •         •         •         •         •         •         •         •    960
GGGTTCAGGTCCTGCTGCTGCAAAGGCGAAAGCGCAAGAGTTTGTTAAGAAACTTAAAAGTTCCGGTGTCAAAGCCACGG
sGlySerGlyProAlaAlaAlaLysAlaLysAlaGlnGluPheValLysLysLeuLysSerSerGlyValLysAlaThrA

     •         •         •         •         •         •         •         •    1040
CTGACATTAAGAGTAAGGCAAATGGTGCACAGGCGGGTTACTCTGCCGGGGAAATATACCTGGGGAAGACTGCGGTTACT
laAspIleLysSerLysAlaAsnGlyAlaGlnAlaGlyTyrSerAlaGlyGluIleTyrLeuGlyLysThrAlaValThr

     •         •         •         •         •         •         •         •    1120
GCCACGCAGCTTGGTCTGACGATGGAGGCGTTGCTTGAAATTTTTGGCATGGGAGTTGGTGCTGTTTCGGACGTGAATGA
AlaThrGlnLeuGlyLeuThrMetGluAlaLeuLeuGluIlePheGlyMetGlyValGlyAlaValSerAspValAsnGl

     •         •         •         •         •         •         •         •    1200
AACGGCTAATGACTATCAGTGCTTCATAGTTTTGAAGAAAGAGGAAGCGAAGATCCTAACGCTCAGCGATTTGGTGGAGC
uThrAlaAsnAspTyrGlnCysPheIleValLeuLysLysGluGluAlaLysIleLeuThrLeuSerAspLeuValGluP

     •         •         •         •         •         •         •         •    1280
CTGACAAGACCGTCTCGCTTTACGAGTTTATCAAGAATCTTCTTACTTCCCAAATCCAACAAAAAGCGCTCGAGGACGCT
roAspLysThrValSerLeuTyrGluPheIleLysAsnLeuLeuThrSerGlnIleGlnGlnLysAlaLeuGluAspAla

     •         •         •         •         •         •    SstI •    1360
ATCCGGGAAGTGTCGGCGGAGCTGAGGAAGTCTGGTACCTATAAGGTATTCTTGGCTGACGCAGAGCTCGGCAAGGTGCT
IleArgGluValSerAlaGluLeuArgLysSerGlyThrTyrLysValPheLeuAlaAspAlaGluLeuGlyLysValLe

     •         •         •         •         •         •         •         •    1440
CGACTGGTGAGTGAAGTATTCCCAAAGATAGGGAGGAGGCGCGCTCGGATTCTGGCTTTTCAGGCTCTCTTTGCCTGGGA
uAspTrp

     •         •         •
CGCGGCGGGCATCACGCCGGAGACGTTAAC
                          HpaI
```

FIG. 6. Nucleotide sequence of the *Eco*RI-*Hpa*I 1,470-bp fragment from pLVS3. Only the sense strand is given. The locations of restriction enzyme recognition sites used to make deletions or to construct expression plasmids are indicated. The predicted amino acid sequence of the *bmp* translation product is shown below the DNA sequence.

geneity was obtained by DE-52 column chromatography (the BMP* does not bind) and by molecular sieving (the BMP* migrates either as a monomer or dimer but not as an aggregate).

A summary of the purification procedure is shown in Table 1, and an SDS-PAGE analysis of the purified BMP* is presented in Fig. 8. The purified protein has an isoelectric point of 9.5 and an apparent monomer molecular weight of 39,000 as determined by SDS-PAGE. After purification, the protein tended to aggregate and precipitate from solution

upon standing, even in the presence of 50 mM KPO₄ (pH 7 to 9) and 1% Triton X-100. The sequence of the amino-terminal 25 residues of the purified protein has been determined (data not presented). This sequence corresponded with what was predicted from the DNA sequence and the genetic construction that yielded plasmid pTP102.

Identification of the native BMP of *T. pallidum*. Rabbit IgG antibody directed against the purified recombinant BMP* was prepared as described in Materials and Methods. The antibody precipitated each of the three treponemal protein

antigens detected in maxicell extracts of plasmid pLVS3 (data not presented), further indicating that the two smaller proteins are somehow derived from the larger 39K BMP. In addition, this same IgG preparation was used in an immune precipitation reaction with an antigen extract prepared from whole cells of *T. pallidum* that had been radiolabeled in vitro with [^{35}S]methionine (see Materials and Methods). A single treponemal protein of approximately 39K was precipitated (Fig. 9). This protein migrated on SDS-PAGE to the same position as the processed BMP encoded by plasmid pTP107 (data not presented). The native BMP was found almost exclusively in the particulate treponemal fraction (Fig. 9). A treponemal membrane extract and purified BMP* were solubilized in a buffer containing Triton X-100 and electro-phoresed on an analytical agarose gel at pH 7.8. Subse-quently, proteins were transferred from the agarose gel to nitrocellulose paper and probed with anti-BMP* IgG. Both the native BMP and recombinant BMP* were found to migrate in an identical manner toward the cathode, indicat-ing that both proteins exhibited what appeared to be an identical net positive charge (data not presented). Finally, attempts to precipitate a protein from solubilized extracts prepared from radiolabeled cells of the cultivable treponemal strain *T. phagedenis* biotype Reiter were unsuccessful (data not presented).

DISCUSSION

The nucleotide sequence indicates that the 1,470-bp *Eco*RI-*Hpa*I fragment of recombinant plasmid pLVS3 en-codes a single treponemal protein of 361 residues with a compositional molecular weight of 39,940. It appears that this protein includes an amino-terminal signal peptide of 20 residues, assuming that processing occurs immediately after the Ala-His-Ala sequence that resembles the processing site for *E. coli* leader peptidase (36, 53). Assuming that this is correct, then the processed form of the protein would have 341 residues with a compositional molecular weight of 37,880, which is very close to the 39,000 molecular weight estimated for this protein from analysis on SDS-PAGE. The recombinant BMP* that was purified from *E. coli* and that differs only slightly from the native treponemal protein exhibited an unusually high isoelectric point of 9.5, which greatly facilitated its purification. The high pI is not reflected in the protein's primary amino acid sequence, where basic residues outnumber acidic residues only slightly (48 versus 43).

In both this study and a previous study (46), *E. coli* maxicells programmed with plasmid pLVS3 produced three treponemal protein antigens, 39K, 35K, and 25K. However, a deletion analysis of this plasmid clearly demonstrated that all three proteins were encoded on a DNA fragment of insufficient size to include separate genes for these three proteins. Since the DNA sequence of this fragment revealed only a single large open reading frame, and since all three
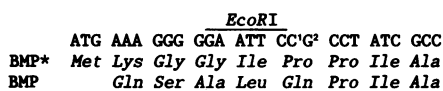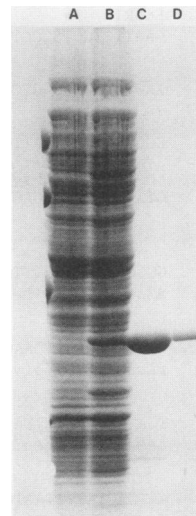

FIG. 8. SDS-PAGE of purified BMP*. Cells of *E. coli* MM294 harboring either plasmid pIN-III-A3 (vector control) or pTP102 were grown, induced, and disrupted as described in Materials and Methods. The crude extract was centrifuged at 13,000 × *g* for 15 min, and a 5-µl sample of the clarified extract obtained from the control strain (lane A) or the extract containing the BMP* (lane B) were mixed with sample buffer, boiled, and analyzed by SDS-PAGE. BMP* was purified as described in Materials and Methods; samples of 15 µg (lane C) and 2.5 µg (lane D) of the purified material (Table 1) were analyzed on the same gel. Note that there is a second protein species that migrates just below the BMP* (lane B) that also appears to be encoded by plasmid pTP102. This is believed to represent a breakdown product of the BMP*.

proteins can be precipitated by antibody prepared against the purified BMP*, one must conclude that the 25K and 35K proteins are derivatives of the larger 39K BMP. In pulse-chase studies with maxicells, all three proteins radiolabeled simultaneously and were quite stable; there was no evidence that the smaller proteins arose from the proteolytic break-down of the larger protein (data not presented). Only the 39K BMP was detected in untreated *E. coli* cells (i.e., not maxicells) and radiolabeled cells of *T. pallidum*. Thus, it appears that the synthesis of the 35K and 25K protein antigens is an artifact of the maxicell procedure. It could be that the smaller proteins result from translational restarts within the BMP message when it is expressed in *E. coli* maxicells. There are ATG codons at nucleotides 444 and 669 of the *Eco*RI-*Hpa*I fragment that might serve as translation initiation sites for synthesis of the 35K and 25K proteins, respectively.

The very slow processing observed for the BMP when it is synthesized in *E. coli* cells is somewhat intriguing. Similar kinetics of processing have been noted in this laboratory for a number of cloned treponemal proteins expressed in *E. coli*

```
                    EcoRI
      ATG AAA GGG GGA ATT CC'G² CCT ATC GCC
BMP*  Met Lys Gly Gly Ile Pro    Pro Ile Ala
BMP       Gln Ser Ala Leu Gln    Pro Ile Ala
```

FIG. 7. Nucleotide sequence at the junction of the expression vector and *bmp* in pTP102. C¹ is the last nucleotide of the synthetic *Eco*RI octomer that was added at the *Pst*I site of *bmp*. G² is the last nucleotide of the *Pst*I site (CTGCAG²). The amino-terminal se-quence for BMP* is shown below the nucleotide sequence. The predicted amino terminal sequence for mature BMP is also provided.

TABLE 1. Purification of the recombinant BMP* produced in cells of *E. coli* MM294[a]

Fraction	Vol (ml)	Protein (g)
Crude lysate	900	68.8
Phosphocellulose load	415	39.3
Phosphocellulose pool	450	0.357
DE-52 effluent	600	0.240
G-200 pool	164	0.174

[a] The purification of the BMP* was followed by SDS-PAGE (Fig. 8). The overall yield based on scanning the crude lysate was approximately 50%.
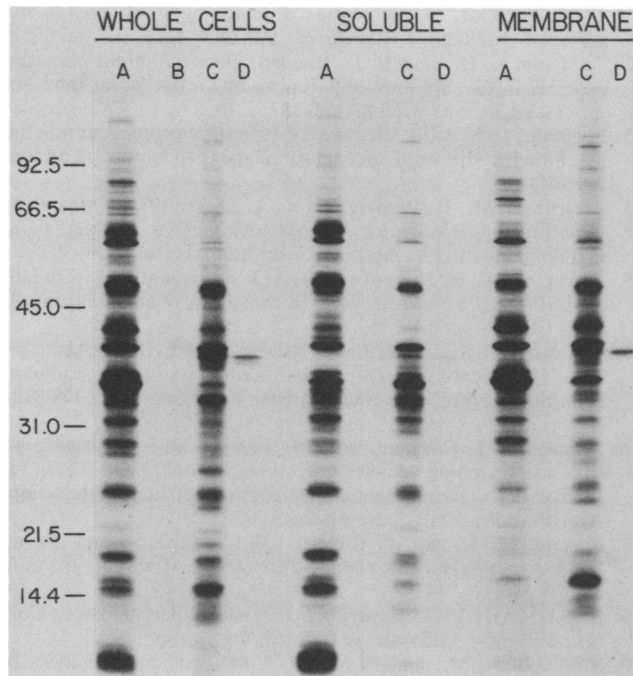
FIG. 9. Identification and cellular location of native BMP. Whole cell, soluble, and membrane fractions of [³⁵S]methionine-labeled treponemes were prepared, and the presence of the native BMP was identified in each fraction by radioimmunoprecipitation, SDS-PAGE, and fluorography. For each fraction, the order of presentation is as follows: A, treponeme fraction solubilized in buffer containing 1% SDS; B, precipitate obtained with normal rabbit serum; C, precipitate obtained with high-titer experimental rabbit syphilitic serum; and D, precipitate obtained with rabbit anti-BMP* IgG. Note that normal rabbit serum control is presented only for the whole cell fraction.

(Stamm, unpublished data). Since *E. coli* signal peptide-bearing proteins are normally exported and processed either cotranslationally or very shortly after the completion of translation (22), such a result strongly suggests that these treponemal proteins are not efficiently recognized by the *E. coli* export machinery. It could well be that the various signals used for determining the ultimate cellular location for envelope proteins in *E. coli* and *T. pallidum*, two procaryotes which are not closely related, are considerably different.

Rabbit polyclonal antibody prepared against the purified recombinant protein precipitated a single protein from solubilized, radiolabeled *T. pallidum* cell extracts that migrated identically on SDS-PAGE, as did its cloned counterpart produced in *E. coli* maxicells. The finding that this protein is synthesized in *E. coli* with a signal peptide that is subsequently processed and that the great majority of this protein appears to reside in the particulate treponemal fraction strongly indicates that the BMP is a component of the *T. pallidum* cell envelope. A hydrophilicity profile of the primary amino acid sequence (not shown) reveals a protein that is predominantly hydrophilic in character but does include several small hydrophobic regions and one large hydrophobic region that extends from residues 214 through 255 of the mature sequence. To date, techniques have not been developed to prepare separate inner and outer membrane fractions from *T. pallidum* cells, but preliminary data have indicated that the BMP* is prominantly exposed on the

surface of treponemes that have been radiolabeled in vitro (Stamm, unpublished data). Because of its high pI and possible cell surface location, it is tempting to speculate that this protein could be involved in the attachment of treponemes to host tissues. Although the BMP does not appear to be a major treponemal protein, previous studies indicated that it is quite immunogenic in infected humans and rabbits (46). Potential functional roles for this BMP of *T. pallidum* in the pathogenesis of syphilis are currently being investigated.

There also is a potential application for the purified BMP* treponemal antigen produced by *E. coli* as a highly specific reagent for the serodiagnosis of syphilis and other human treponematoses. Previously, it was demonstrated that the great majority of human syphilitic and yaws sera tested, regardless of disease stage, were capable of precipitating the BMP from maxicell extracts programmed with plasmid pLVS3 (46). All of the normal human sera tested in like manner were nonreactive. In addition, sera from rabbits immunized with cells of the cultivable treponeme *T. phagedenis* biotype Reiter did not recognize this protein, suggesting that the BMP may represent a pathogen-specific antigen (46). This was further established by the failure of antisera prepared against the purified recombinant protein to precipitate any proteins from solubilized Reiter cell extracts. Many normal human sera contain antibodies that cross-react with *T. pallidum* antigens and presumably arise in response to antigens of the normal bacterial flora (18). The use of a pathogen-specific antigen would eliminate the requirement for absorbing human sera with an extract prepared from Reiter treponemes, as is now done for most commonly used confirmatory tests for syphilis (20, 51).

This study does not include data concerning the analysis of the cloned *T. pallidum* DNA sequence for the identification of potential transcription and translation signals that might be employed by this organism or for preferential codon usage. The sequences of cloned DNA fragments encoding two additional *T. pallidum* protein antigens of 56K and 34K recently have been determined (Dallas, unpublished data). A detailed analysis of all three treponemal genes in this regard will be presented elsewhere.

Finally, this study represents the third instance in which a protein antigen of *T. pallidum* has been expressed in *E. coli* and the recombinant protein has been purified. Lovett and co-workers (11, 39) have purified and characterized a protein antigen designated 4D, a polymer of identical 19K monomers that forms an ordered ring structure of 10 nm diameter and that appears to coat the treponemal outer surface. van Embden and co-workers have cloned and sequenced a treponemal gene designated *tmpA* that encodes a 44K treponemal membrane protein, which appears also to exhibit slow signal peptide processing when expressed in *E. coli* (17). A *cro*-TmpA-β-galactosidase sandwich protein was engineered and purified and currently is being tested for possible uses as a serodiagnostic reagent (17). Thus, it is apparent that the difficulties in obtaining suitable quantities of *T. pallidum* cells for experimental studies can be at least partially circumvented by employing recombinant DNA technology. We anticipate that the analysis of recombinant treponemal antigens will provide new insights into the pathogenesis and immunobiology of *T. pallidum* infection.

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