Characterization of the 35-Kilodalton *Treponema pallidum* subsp. *pallidum* Recombinant Lipoprotein TmpC and Antibody Response to Lipidated and Nonlipidated *T. pallidum* Antigens

LEO M. SCHOULS,* HAN G. J. VAN DER HEIDE, AND JAN D. A. VAN EMBDEN

Unit Molecular Microbiology, National Institute of Public Health and Environmental Protection, P.O. Box 1, 3720 BA Bilthoven, The Netherlands

Received 19 February 1991/Accepted 16 July 1991

The gene encoding the 35-kDa immunogenic *Treponema pallidum* subsp. *pallidum* (*T. pallidum*) membrane protein C, TmpC, was cloned, sequenced, and expressed in *Escherichia coli*. The deduced amino acid sequence carries an N-terminal signal sequence with a four-amino-acid motif, which is characteristic for bacterial lipoproteins. Metabolic labeling with radioactive palmitic acid of *E. coli* expressing TmpC revealed incorporation of the fatty acid into the antigen. The antigen was overproduced, purified to near homogeneity and used in an enzyme-linked immunosorbent assay (ELISA) to evaluate its potential for the serodiagnosis of syphilis. Although all sera from untreated secondary syphilis patients were reactive in this TmpC ELISA, only a minority of the serum samples from untreated patients in the primary or early latent stage of the disease contained significant anti-TmpC antibodies. To study the influence of the lipid moiety on the antigenic properties of the TmpC, TmpA, and TpD lipoproteins, plasmids encoding nonlipidated forms of these antigens were constructed. In addition, a plasmid expressing a lipidated form of the otherwise non-lipid-modified antigen TmpB was constructed. Immunization and absorption experiments with these lipidated and nonlipidated antigens showed that antibodies against the lipid moiety of lipoproteins could not be detected on immunoblots, neither in sera from infected rabbits nor in sera from animals immunized with the lipoproteins. In addition, we were unable to demonstrate cross-reactivity between antibodies against the *T. pallidum* lipoproteins and those reactive to the Venereal Diseases Research Laboratories test, suggesting that antibodies reactive to the Venereal Diseases Research Laboratories test are unrelated to antilipoprotein antibodies.

Infection with *Treponema pallidum* subsp. *pallidum* (*T. pallidum*), the causative agent of syphilis, leads to a powerful cellular and humoral immune response. Despite this vigorous immune response, *T. pallidum* is not fully eliminated, as manifested by relapses of secondary syphilis and/or late syphilitic processes. A possible explanation for this incomplete clearance of *T. pallidum* may be the near lack of antibody binding to the surface of the treponemes. The inaccessibility to antibodies has been appreciated since the studies of Hardy and Nell in 1957 on the nonagglutinability with anti-*T. pallidum* antibodies of freshly isolated treponemes. The ability of *T. pallidum* cells to bind a large variety of host proteins (1) is presumed to contribute to this inaccessibility to antibodies.

Studies on the structure of the surface of *T. pallidum* have been greatly hindered by the inability to cultivate the organism and to separate the outer and inner membranes. However, the extraction of *T. pallidum* with low concentrations of detergents like sodium dodecyl sulfate (SDS) and Triton X-114 suggests that the outer membrane contains a paucity of proteins (22). This is consistent with recent observations on freeze-fractured *T. pallidum* cells which suggest an unusually low density of intramembranous particles in the outer membrane (24, 33). Although no direct data are available on the structure of the outer surface of *T. pallidum*, the apparent paucity of proteins likely contributes to the relative inaccessibility of *T. pallidum* to antibodies.

Another unusual property of *T. pallidum* is the posttranslational lipidation of many of the major immunogenic membrane-associated proteins of *T. pallidum* (4, 29). The lipid moiety of lipoproteins likely acts as a membrane anchor, with the hydrophilic peptide part protruding into the periplasmic space, leaving the protein unexposed to the cell surface. At present, it is unclear whether *T. pallidum* lipoproteins are located in the inner or outer membrane.

At least eight lipid-modified proteins in *T. pallidum* have been identified, either by direct chemical analysis of whole organisms or detergent phase-partitioned proteins, by the in vivo incorporation of ^3H-palmitic acid in *T. pallidum* proteins, or indirectly by the in vivo lipidation of cloned *T. pallidum* proteins expressed in *Escherichia coli*. Although the sequences of four lipoproteins (the 47-kDa protein, TmpA, TpD, and the 15-kDa protein) have been described, only one of these proteins, TmpA, has been overexpressed and purified to homogeneity from *E. coli* K-12. Therefore, the humoral immune response of a large number of syphilis patients to only TmpA has been investigated (7, 12). In the present study, we describe the purification of the 35-kDa lipoprotein TmpC and the reactivity of serum samples from syphilis patients to this protein.

The role of the unusually large number of lipoproteins in *T. pallidum* is unclear. Perhaps these proteins provide the outer membrane with rigidity or other functions without exposing parts of the protein to the surface of the bacterial cell. Thus, lipoproteins would not be accessible to antibodies. It is well known that the lipid moiety on bacterial lipoproteins strongly potentiates the humoral as well as the cellular immune response to the peptide moiety of these proteins (6, 13). To investigate the role of the lipid part of *T. pallidum* lipoproteins in the immune response, we constructed and analyzed mutants of *T. pallidum* lipoproteins,
which as a result of site-directed mutagenesis are no longer lipidated in vivo, and a mutant expressing a lipidated \textit{T. pallidum} protein, which is normally not lipid modified.

\textbf{MATERIALS AND METHODS}

\textbf{Bacterial strains and plasmids.} \textit{T. pallidum} Nichols was cultivated and isolated as described previously (31). The \textit{E. coli} K-12 strains and plasmids used throughout this study are listed in Table 1.

\textbf{Media and reagents.} NZYM medium and NZYM agar (17), supplemented with ampicillin (100 \mu g/ml in solid medium and 200 \mu g/ml in liquid medium) and kanamycin (30 \mu g/ml), were used for cultivating \textit{E. coli} K-12.

The enzymes used in this study were obtained from Boehringer GmbH (Mannheim, Germany), New England Biolabs, Inc. (Beverly, Mass.), or the Pharmacia Molecular Biology Division (Uppsala, Sweden) and were used as recommended by the manufacturers.

\textbf{1-\textsuperscript{14}C}palmitic acid (3.7 MBq/ml) was from Dupont, NEN Research Products. The solvent in the palmitic acid solution was removed by evaporation, and the fatty acid was redissolved in 0.4% Triton X-100 to 18.5 MBq/ml.

\textbf{Immunological techniques.} The Western blotting (immunoblotting) technique has been described previously (31). SDS-polyacrylamide gel electrophoresis (PAGE) was done with 13% polyacrylamide gels as described by Laemmli (16).

An enzyme-linked immunosorbent assay (ELISA) using TmpC as an antigen was performed similarly to the ELISA with TmpA as an antigen (12) except that TmpC was used at a concentration of about 1 \mu g/ml.

A panel of serum samples, used previously to measure their reactivities to the recombinant proteins TmpA and TmpB (28), was used to detect anti-TmpC antibodies. The negative control group consisted of serum samples negative in the classical syphilis tests. The second group of samples originated from syphilis patients treated with antibiotics.

The latter group was subdivided into samples reactive in the Venereal Diseases Research Laboratories (VDRL) test and into samples nonreactive in the VDRL test. The last group consisted of serum samples from untreated syphilis patients, subdivided into primary, secondary, and early latent syphilis.

\textbf{DNA technology.} Plasmid isolation and DNA manipulations were performed essentially as described by Maniatis et al. (17).

The dideoxy chain termination technique for nucleotide sequencing of double-stranded DNA (9, 26) was used to determine the nucleotide sequence of both strands of the DNA. For this purpose, a 1.2-kb fragment from pRIT9070 carrying the \textit{tmpC} gene was inserted into pEMBL9 to form pRIT9091. The sequence was then determined by using the M13 forward and reverse primers and custom-made oligonucleotides. The DNA sequence of the regions upstream and downstream of the \textit{tmpC} gene was derived from plasmids pRIT9092 and pRIT9093 by using custom-made oligonucleotides.

The polymerase chain reaction was basically performed by using the protocol and buffers described by Noorderkoek et al. (18). Amplification of the plasmid fragments was done in 20 cycles with 50 ng of plasmid as the template DNA.

\textbf{Lipoprotein labeling with radioactive palmitic acid.} \textit{E. coli} cells carrying either recombinant plasmids or vector DNA were grown in NZYM at 28°C. When the cells reached the log phase of growth, 20 \mu l of [1-\textsuperscript{14}C]palmitic acid (18.5 MBq/ml) in 0.4% Triton X-100 was added to 1 ml of the \textit{E. coli} cells. The temperature was raised to 42°C, and the culture was incubated at this temperature for 3 h. Cells were centrifuged and lysed in SDS-PAGE sample buffer (50 mM Tris-HCl [pH 8.3], 2% SDS, 2% dithiothreitol, 10% glycerol, 0.01% bromophenol blue).

\textbf{Metabolic labeling of TmpC protein.} \textit{E. coli} cells carrying pRIT9070 were grown at 28°C in NZYM medium until the

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
Strain or plasmid & Relevant properties & Reference \\
\hline
M1034 & SC 181 carrying pCI857 & 28 \\
JM101 & (lac pro) supE thi F' traD36 proAB lacF' Z M15 & 29 \\
M1070 & Strain 1046 carrying plasmid pCI857 & 28 \\
\hline
Plasmid pEMBL9 & Cloning vector, amp, lacZ & 29 \\
pPLc236 & Expression vector, amp, carries lambda promoter P1 & 25 \\
pCI857 & Repressor plasmid, kan, carries thermosensitive cI857 repressor gene & 28 \\
pRIT9000 & Cosmid carrying the \textit{tmpC} gene & This study \\
pRIT9001 & Sau3A deletion derivative of pRIT9000 & This study \\
pRIT9003 & Clal deletion derivative of pRIT9001 & This study \\
pRIT9035 & BstEII deletion derivative of pRIT9001, obtained by partial digestion & This study \\
pRIT9036 & BstEII deletion derivative of pRIT9001, obtained by complete digestion & This study \\
pRIT9050 & pPLc236 carrying the \textit{HindIII} fragment of pRIT9001 & This study \\
pRIT9060 & pPLc236 carrying the \textit{Avai-HindIII} fragment of pRIT9050 & This study \\
pRIT9070 & Bal31 deletion of pRIT9060 & This study \\
pRIT9091 & pEMBL9 carrying the 1.2-kb EcoRI-SauI fragment of pRIT9070 & This study \\
pRIT9092 & pEMBL9 carrying the Clal-SauI fragment of pRIT9001 & This study \\
pRIT9093 & pEMBL9 carrying the Smal-EcoRI fragment of pRIT9001 & This study \\
pRIT9091 & Cys--Ser mutant of pRIT9070 (TmpC*) & This study \\
pRIT4661 & Expression plasmid carrying the \textit{tmpA}--\textit{tmpB} operon & 28 \\
pRIT4683 & Cys--Ser mutant of pRIT4661 (TmpA*) & This study \\
pRIT9932 & Nael-SnaI deletion derivative of pRIT4661 (TmpB*) & This study \\
pRIT2885 & Expression plasmid carrying the tpd gene & 29 \\
pRIT2801 & Cys--Ser mutant of pRIT2885 (Tpd*) & This study \\
\hline
\end{tabular}
\caption{\textit{E. coli} K-12 strains and plasmids used in this study}
\end{table}
log phase was reached. Cells were spun down and resuspended in minimal medium supplemented with 0.05% Casamino Acids, 0.05% yeast extract, and 0.2% glucose. After 20 min of incubation at 28°C, the culture was split into two fractions. One part of the culture was further incubated at 28°C, and the temperature of the other part was raised to 42°C. After 1, 2, and 3 h, respectively, 14C-amino acids (95 GBq/ml, final concentration) were added to the cells and the culture was labeled for 10 min, after which the cells were harvested by centrifugation. Cells were lysed with SDS-PAGE sample buffer and subjected to SDS-PAGE and fluorography.

Construction of plasmids hyperexpressing TmpC. The 45-kb cosmid recombinant pRIT9001 (31) was cut with Sall and religated to give the 11-kb deletion derivative pRIT9001. The pRIT9001 derivatives pRIT9003, pRIT9035, and pRIT9036 were made by complete ClaI, partial BstEII, and complete BstEII digestions, respectively. The expression plasmid pRIT9050 was constructed by inserting the 4-kb HindIII fragment of pRIT9001 into the HindIII site of the expression vector pLCL236 (25). To construct a plasmid with tmpC inversely oriented to the P2 promoter, pRIT9050 was partially digested with BstEII and cut with HindIII, and the fragment carrying the tmpc gene was inserted into Aval- and HindIII-cleaved pLCL236. The resulting plasmid was designated pRIT9060. To construct a high-level expression plasmid, pRIT9060 was cut with BamHI and treated with DNA polymerase in a mixture of dTTP, dCTP, dGTP, and sulfonated dATP. The linear plasmid was digested with HindIII, exonuclease Bal 31, and S1 nuclease. Because the sulfonated dATP was incorporated at the BamHI end, the nuclease Bal 31 affected only the HindIII end. After religation of the mixture, a number of plasmids with unidirectional deletions were obtained. One of these, pRIT9070, was selected for further study.

Site-directed mutagenesis of leader sequences. To mutagenize theTmpA leader sequence, a 190-bp fragment of plasmid pRIT4661, which carries the tmpa gene (28), was amplified by the polymerase chain reaction, driven by two oligonucleotides, one with a perfect match upstream from the EcoRI site and one overlapping the cysteine codon in the tmpa gene and the NarI site, but with a mismatch in the codon for cysteine. The mismatch causes a 1-bp mutation resulting in a single amino acid substitution from cysteine to serine (TGT→TCT). The amplified fragment carrying the mutation was then cut with the restriction enzymes EcoRI and NarI and introduced into EcoRI- and NarI-digested pRIT4661. The resulting plasmid was designated pRIT4663.

A similar procedure could not be employed for the mutagenesis of plasmids carrying the tmpc and tmpd genes because of the lack of suitable restriction sites close to the mutation. To circumvent this problem, a two-step, PCR-mediated, site-directed mutagenesis procedure proposed by Kadowaki et al. (14), in which a 5′ FokI site is located on the oligonucleotide used for mutagenesis, was applied. This procedure was used to mutate the leader sequence in pRIT9070, carrying the tmpc gene, and in pRIT2885, which carries the tmpd gene. The mutant plasmids expressing the nonlipidated productsTmpC* and TmpD* were designated pRIT9012 and pRIT2801, respectively. In both cases, a TGC→AAG mutation resulted in a single amino acid substitution from cysteine to serine.

To obtain a lipidated form of TmpB, plasmid pRIT4661 carrying the tmpa-tmpb operon was cut with NarI and treated with DNA polymerase (Klenow) in the presence of dCTP. The fragment was then cut with SnaBI and religated.

The plasmid expressing the lipidated TmpB* fusion product was designated pRIT9392.

All plasmid derivatives were analyzed by DNA sequencing, and it was found that the desired 1-bp substitutions in the tmpa, tmpc, and tmpd genes had been achieved. However, in plasmid pRIT9392, a 132-bp deletion of the tmpb gene was found.

Purification of TmpC. E. coli K-12 carrying plasmid pRIT9070 was cultured at 28°C in 7-liter fermentors in NZYM medium containing ampicillin (200 μg/ml). The partial O2 pressure was monitored, and the oxygen concentration was kept at 10% saturation. The pH was kept at 7.5 with 10 M NaOH. Glucose was added slowly during cultivation at a rate sufficient to maintain a glucose concentration of 1 g/liter. At 24 h postinoculation, the temperature was raised to 42°C and the culture was incubated for another 4 h at 42°C. Cells were harvested by centrifugation, resulting in a yield of approximately 135 g (wet weight) of cells. The cells were suspended in 500 ml of 150 mM NaCl and stored at −70°C in 40-ml fractions.

Frozen cells (40 ml) were thawed, lysozyme was added (1 mg/ml), and the mixture was incubated for 20 min at room temperature. EDTA was added to a concentration of 20 mM and incubated for 10 min at room temperature. The cells were disrupted by ultrasonic treatment using a sonifier (B-12; Branson Sonic Power Co., Danbury, Conn.) operating at 100 W. Cellular debris was removed by centrifugation for 10 min at 8,000 × g. Membranes and other particulate material were collected by centrifugation at 20,000 × g for 20 min. The pellet was resuspended in 40 ml of 150 mM NaCl and centrifuged again for 20 min at 20,000 × g, and the pellet was suspended in 40 ml of 150 mM NaCl. This crude cell envelope preparation was then incubated for 2 h at room temperature with 0.25% N-tetradecyl-N,N-dimethylammonio-1-propanesulfonate (SB-14; Serva) in the presence of 10 mM MgCl2 and 10 mM Tris, pH 8.0, under constant stirring. This treatment solubilized about 90% of the TmpC.

After centrifugation at 20,000 × g for 30 min, the protein mixture was subjected to anion exchange (DEAE-Trisacryl; IBF) and fractions containing TmpC were pooled. TmpC was purified to near homogeneity by preparative isoelectric focusing as described previously (28). TmpC was extracted from a gel fraction in the pH 4.5 region of the focusing gel.

Fractionation of cell envelopes of T. pallidum and E. coli. The preparation of T. pallidum and E. coli cell envelopes was done as described previously (28). Briefly, cells were treated with lysozyme and disrupted by ultrasonic treatment, unbroken cells were removed by low-speed centrifugation, and the membranes were collected by ultracentrifugation. This membrane fraction was washed twice and used for further analysis.

The method for determining the peptidoglycan association of proteins was basically the same as that of Hazumi et al. (10). Crude cell envelopes of T. pallidum and E. coli recombinants were prepared as described above, except that the lysozyme treatment was omitted. The cell envelope fraction was then treated with extraction buffer (10 mM Tris-HCl, 2% dithioerythritol, 2% SDS, 10% glycerol, pH 7.5) for 30 min at 60°C. The solubilized proteins were separated from the peptidoglycan fraction by centrifugation for 15 min at 13,000 × g. For SDS-PAGE analysis, samples were pretreated with lysozyme (250 μg/ml) or trypsin (250 μg/ml) or mixed directly with the SDS-PAGE sample buffer.

Processing of the lipidated and nonlipidated T. pallidum antigens. To study the posttranslational cleavage of the T. pallidum precursor molecules in E. coli, cells carrying the
various recombinant plasmids were grown to log phase at 28°C. Cultures were split in two fractions, with 6% ethanol added to one part of the culture and no ethanol added to the other part. Cells were incubated for 20 min at 28°C, after which the temperature was raised to 42°C and cells were further incubated at 42°C for 4 h. Samples of the cultures were separated by SDS-PAGE, electroblotted, and immunostained with monoclonal antibodies.

**Nucleotide sequence accession number.** The nucleotide sequence of the *T. pallidum* tmpC gene will appear in the EMBL, GenBank, and DDBJ nucleotide sequence data bases under accession no. X57836.

### RESULTS

**Inducible expression of TmpC.** As previously described, pRIT9000 expresses TmpC in *E. coli* from transcriptional signals located on the *T. pallidum* DNA insert in this plasmid (31). To locate the *tmpC* gene on the physical map of the cloned *T. pallidum* DNA, various deletion derivatives of the 45-kb cosmid pRIT9000 were made and the TmpC expression of these derivatives was monitored on Western blots. The results, as summarized in Fig. 1, indicate that the *tmpC* gene is located between 0 and 3.2 kb on the physical map of the *T. pallidum* DNA insert.

To determine the direction of transcription and to obtain hyperexpression of TmpC, the heat-inducible lambda promoter P₂ was inserted at various sites of the TmpC plasmid DNA. The position of P₂ in three of the plasmids obtained and the expression of TmpC at 28°C and 42°C are shown in Fig. 1. One of these, plasmid pRIT9070, expressed TmpC at high levels at 42°C, whereas no detectable expression was found at 28°C, apparently due to binding of the λ cl857 repressor, which shuts off transcription by binding to the λ operator at 28°C (Fig. 2). This indicates that the transcription of *tmpC* is from right to left, as depicted in Fig. 1, and that the treponemal promoter apparently is not present or not functional in plasmid pRIT9070. This is consistent with the observation that deletion of *T. pallidum* DNA rightwards from the left BstEII site at 1.8 kb on the physical map results in loss of TmpC expression (Fig. 1) and with the identification of putative promoter sequences (see below).

Metabolic labeling with radioactive amino acids during heat induction of *E. coli* producing TmpC showed that protein synthesis was almost exclusively limited to TmpC and that the protein synthesis stopped after about 1 h of heat induction (Fig. 2B). This is probably due to the toxicity of high levels of TmpC to *E. coli*, as viability of the cells decreased about 10⁴-fold after 1 h of temperature induction. The TmpC protein band expressed by *E. coli* displays the same mobility on SDS-PAGE gels as TmpC expressed in *T. pallidum* (31). As TmpC is posttranslationally modified (see below), this suggests that this processing in *E. coli* is similar to that in *T. pallidum*.

**Antibodies to TmpC in syphilis patients.** TmpC was purified from heat-induced, fermentor-grown *E. coli* cells by selective detergent extraction, anion-exchange chromatography, and preparative isoelectric focusing as described in Materials and Methods. The purified TmpC protein was used as antigen in an ELISA to measure the presence of anti-TmpC antibodies in serum samples from various groups of syphilitic patients and from a control group. The results are shown in Fig. 3. Although all sera from untreated patients with secondary syphilis contained anti-TmpC antibodies, only 46 and 75% of the untreated patients with primary and early latent syphilis, respectively, showed anti-TmpC reactivity. A minor fraction of the serum samples from syphilis patients who had been treated with penicillin contained detectable anti-TmpC antibodies.

**Nucleotide sequence of tmpC.** The nucleotide sequence of a 1,179-bp DNA fragment carrying the *tmpC* gene was determined (Fig. 4). Only one open reading frame, long enough to accommodate a gene the size of *tmpC*, is present in this 1,179-bp region. The most-proximal potential initiation codon is located at position 64. The translated sequence starting at this GTG codon would encode a protein of 353

---

**FIG. 1.** Physical map of plasmids carrying the *tmpC* gene and expression of TmpC. Treponemal DNA is indicated by thick lines, open boxes represent vector DNA, heavy arrows denote the position and direction of transcription of the lambda promoter in the expression vector pPLc236, and the open arrow indicates the position and direction of transcription of the *tmpC* gene. –, no reactivity on Western blot; ±, weakly positive on Western blot; +, strongly positive on Western blot; ++++, intense band on Coomassie-stained gel.
Amino acids with a calculated mass of 37.8 kDa and an isoelectric point of 4.57. This predicted isoelectric point is consistent with that reported for TmpC by Norris et al. (19) and with the observation that TmpC is purified from a pH 4.5 fraction by isoelectric focusing. The sequence upstream from the designated start codon contains putative Shine-Dalgarno and -35 and -10 promoter sequences upstream from the start codon. Conclusive evidence for the in vivo translation of this open reading frame stems from the loss of lipidation after site-directed mutagenesis as described below.

The region downstream from the stop codon starting at position 1137 carries a 26-bp DNA sequence which may form a stem-loop structure centered on base 1150. This relatively stable structure (−19.8 kcal [82,843.2 J]) may function as a transcriptional terminator, indicating that the TmpC mRNA is a monocistronic messenger.

In an effort to elucidate the function of this lipoprotein, the translated TmpC sequence and the tmpC DNA sequence were compared with sequences from the protein data banks (Swiss-Prot v14 and GenPept v63), DNA data bases (EMBL v24 and GenBank v65), and all T. pallidum protein and DNA sequences that have been published (20). This homology search, however, did not reveal any significant homology with other known sequences.

In vivo lipidation of TmpC. The deduced amino acid sequence of TmpC revealed a potential N-terminal signal sequence, characteristic for prokaryotic proteins that are transported across the inner membrane (15). In addition, the four-amino-acid sequence Leu-Ile-Gly-Cys, in the C-terminal part of this signal sequence, is a motif characteristic of bacterial lipoproteins, suggesting that TmpC is lipid modified (32, 34). In order to determine whether TmpC is indeed a lipoprotein, metabolic labeling of TmpC-producing E. coli with radioactive palmitic acid was performed. An analysis of lysates from the labeled cultures on SDS-PAGE and subsequent fluorography revealed that the cells indeed incorporated the radioactive fatty acid into the TmpC antigen (Fig. 5A, lane C). Consistent with the presence of the hydrophobic lipid groups on TmpC, we found that TmpC partitioned in the detergent fraction after Triton X-114 extraction of T. pallidum (data not shown).

Construction of recombinants expressing homologous lipoplated and nonlipoplated T. pallidum proteins. To obtain pairs of homologous lipoplated and nonlipoplated T. pallidum proteins, we engineered the 3′-proximal sequences of the genes encoding TmpA, TmpB, TmpC, and TpD. As previously described, the leader sequences of TmpA, TmpC, and TpD carry a tetrapeptide lipidation motif containing a cysteine residue (Fig. 6). This cysteine is essential for recognition by signal peptidase II and for attachment of the lipid moiety. In order to obtain mutants which have lost the capacity to be lipoplated, we substituted serine for this cysteine in the lipidation motif in TmpA, TmpC, and TpD. As expected, radioactive palmitic acid was not incorporated during in vivo labeling into the mutant proteins, which were designated TmpA*, TmpC*, and TpD*, respectively (Fig. 5A).

In contrast to the above-mentioned lipoproteins, the TmpB antigen is not lipid modified (28), as can be expected from the deduced signal sequence of the tmpB gene, which does not carry the motif characteristic of lipoproteins. In order to obtain a lipid-modified TmpB protein, the signal sequence of the tmpA gene, including three codons downstream from the signal peptidase II cleavage site, was fused
to the tmpB gene. As shown in Fig. 5A, radioactive palmitic acid was incorporated during in vivo labeling into the fusion product, designated TmpB*. The TmpB* protein has an apparent molecular mass of 32 kDa on SDS-PAGE gels, which is significantly less than the expected molecular mass. Sequence analysis of the TmpB* plasmid revealed that probably because of incomplete ligation during the construction, a 132-bp deletion had occurred, resulting in a loss of the first 48 amino acids of the TmpB antigen. Analysis of lysates from E. coli producing TmpA, TmpB, TmpC, or TpD or their mutant forms on Western blots revealed that both lipidated and nonlipidated proteins retained their reactivity with polyclonal and monoclonal antibodies (Fig. 5B and 7).

Posttranslational processing of the mutant proteins. To obtain information on the effect of the leader sequence mutation on the processing of the T. pallidum antigens, E. coli cells carrying the various recombinant proteins were induced for antigen production in the presence or absence of ethanol, a compound known to inhibit the posttranslational cleavage of the signal peptide. Western blot analysis (Fig. 7) revealed that all antigens except TpD* are posttranslationally cleaved in E. coli. The processing of TmpA* and TmpC* is remarkable, as the cleavage site for signal peptidase II has been destroyed by the amino acid substitution. However, inspection of the N-terminal part of these proteins revealed an alternate processing site for signal peptidase I in both proteins, which might explain the processing of the nonlipidated antigens (Fig. 6). There is precedence for alternate cleavage by signal peptidase I of lipoproteins if the signal peptidase II cleavage site is inactivated by mutation (34). Although a weak alternate cleavage site is also present in the TpD antigen, no processing of TpD* was observed. Interestingly, the non-lipid-modified TpD* antigen did not display the heterogeneous mobility on SDS-PAGE characteristic of TpD, suggesting that posttranslational modification is responsible for the heterogeneity (Fig. 5B and 7).

Although the presence of peptidoglycan has been demonstrated in T. pallidum (23), no data have been published on the association of proteins or lipoproteins with this matrix structure. Cell envelopes of E. coli producing the lipoprotein TmpA, TmpC, or TpD and cell envelopes of T. pallidum were treated with SDS at 60°C, and the nonsolubilized peptidoglycan skeleton with the associated protein was spun down. The soluble and particulate fractions were then analyzed on SDS-PAGE gels and by Western blotting. To release any proteins covalently bound to the peptidoglycan, the particulate fraction was treated with either lysozyme or trypsin. No T. pallidum proteins were found to be associated with the SDS-insoluble, lysozyme-treated fraction, either by gold staining of proteins electroblotted after SDS-PAGE (data not shown) or by immunoblotting with polyclonal anti-T. pallidum serum (Fig. 8) or anti-Braun’s lipoprotein antibodies (data not shown). Furthermore, none of the recombinant lipoproteins TmpA, TmpC, and TpD was detected in the Coomassie blue-stained peptidoglycan E. coli fraction (Fig. 8A). Immunoblots incubated with the anti-Braun’s lipoprotein antibodies showed that this protein was strongly represented in the peptidoglycan E. coli fraction.

FIG. 3. (A) Anti-TmpC antibodies in patient sera measured by ELISA. Wells of the microdilution plates were coated with 100 ng of purified TmpC in 100 μl of ELISA buffer. Sera were tested in 1:100 dilutions. Serum samples: •, nonsyphilitic control sera; VDRL− and VDRL+, positive serum samples from treated patients with negative or positive reactivity in the VDRL test, respectively; L1, LII, and LL, serum samples from untreated patients with primary, secondary, and early latent syphilis, respectively. Each dot represents an individual serum sample. (B) Purity of the TmpC antigen after isolation and purification. Samples were separated by SDS-PAGE and stained with Coomassie brilliant blue. Lanes: A, molecular mass markers (kilodaltons); B, total lysate of E. coli producing TmpC after induction; C, purified TmpC.
(data not shown). None of the analyzed recombinant lipoproteins, but also none of the other T. pallidum antigenic proteins, seems to be associated with the peptidoglycan fraction (Fig. 8).

Absorption with lipided and nonlipided antigens. In order to obtain information on whether the development of VDRL antibodies during syphilis was caused by T. pallidum lipoproteins, several absorption experiments were performed. For this purpose, anti-T. pallidum antibodies, from experimentally infected rabbits, or anti-TmpA and anti-TmpC antisera, obtained by immunizing rabbits with purified TmpA or TmpC, were absorbed with either lipided or nonlipided TmpA and TmpC and the remaining nonabsorbed antibodies were analyzed by immunoblotting. The absorption procedures, with lipided or nonlipided antigen, removed all reactivity against the lipided forms of TmpA and TmpC (Table 2). An analysis of sera from rabbits immunized with lipided, purified recombinant TmpA or TmpC showed that these lipoproteins elicited no VDRL-reactive antibodies. In addition, the lipided fusion product TmpB*, carrying the TmpA signal sequence, does not react with antibodies directed against lipided TmpA nor does it elicit VDRL antibodies after the immunization of rabbits.

**DISCUSSION**

In this study, we report the DNA sequence of a cloned T. pallidum gene encoding the 35-kDa lipoprotein TmpC.

By replacing the treponemal tmpC promoter by the thermoinducible lambda promoter, a high level of temperature-dependent TmpC production was obtained. This enabled us to isolate and purify the TmpC antigen for use in an ELISA to determine its usefulness for the serodiagnosis of syphilis.

Serum samples from untreated syphilis patients in different stages of the disease and from antibiotic-treated patients were investigated. Although the serum samples from untreated secondary syphilis patients were reactive without exception, a minority (46%) of the serum samples from untreated patients in the primary stage displayed reactivity with the TmpC antigen. In addition, 25% of the serum
samples from early-latent-stage syphilis patients were non-reactive in the TmpC ELISA. The relatively poor reactivity of samples from primary and latent syphilis patients suggests that the antibody response against TmpC occurs late in infection and that the antibody level declines during latency. Apparently, TmpC is poorly presented to the immune system during natural infection with T. pallidum. This is probably not due to the intrinsically poor immunogenicity of TmpC, because recombinant TmpC is extremely immunogenic in rabbits, evoking a strong antibody response even after a single vaccination with this antigen without adjuvant (27).

Only a few serum samples from antibiotic-treated patients were reactive with TmpC. This suggests that the anti-TmpC antibody level, similar to that of antibodies reactive in the VDRL test and the level of anti-TmpA antibodies, drops after antibiotic treatment. In contrast, antibodies measured in the fluorescent treponemal antibody (FTA)-ABS and the TPHA generally remain detectable for many years after treatment.

Unlike antibodies from rabbits immunized with purified

\[ \text{TmpA} \quad \text{MNAHTLVYSVALCAAM} \quad \text{LGS} \quad \text{ASGKEE} \]

\[ \text{TmpA}^* \quad \text{---} \quad \text{---} \quad \text{---} \quad \text{---} \quad \text{---} \]

\[ \text{TmpC} \quad \text{MREKWVRAAFVACML} \quad \text{LGS} \quad \text{SKSDEPQM} \]

\[ \text{TmpC}^* \quad \text{---} \quad \text{---} \quad \text{---} \quad \text{---} \quad \text{---} \]

\[ \text{TpD} \quad \text{MKRSLGLSADFALV} \quad \text{FSA} \quad \text{GGGEHQ} \]

\[ \text{TpD}^* \quad \text{---} \quad \text{---} \quad \text{---} \quad \text{---} \quad \text{---} \]

\[ \text{TmpB}^* \quad \text{MNAHTLVYSVALCAAM} \quad \text{LGS} \quad \text{ASGDEY} \]

\[ \text{TmpB} \quad \text{MKTRNFSLVWADLYLGGVLFVSAASDY} \quad \text{---} \quad \text{---} \]

FIG. 6. N-terminal amino acid sequences of the various recombinant proteins and their mutant forms. The open triangle indicates the signal peptidase II cleavage site; closed triangles indicate alternative cleavage sites for signal peptidase I; boxed sequences indicate leader sequences carrying tetrapeptide lipidation motifs and substitutions of serines for cysteines to obtain mutants.

TmPA, TmPB, or TpD, antibodies from rabbits or guinea pigs immunized with recombinant TmpC did not react in the FTA-ABS with either acetone-fixed or unfixed T. pallidum cells. These antibodies, however, display strong reactivity on Western blots and in the TmpC and T. pallidum ELISAs. Possibly the TmpC epitopes are buried in the T. pallidum membrane and therefore are unable to react with antibodies if the membrane is not solubilized.

Chamberlain et al. (4) found that in vivo labeling of T. pallidum with radioactive fatty acids resulted in the incorporation of the radioactive fatty acid in 5 of the major immunogens. These lipid-modified T. pallidum proteins had molecular masses of 47, 38, 36, 17, and 15 kDa. As the apparent molecular mass of TmpC is about 35 kDa, the 36- or 38-kDa lipoprotein of T. pallidum identified by Chamberlain et al. might represent the TmpC antigen. This study shows that TmpC is characteristic of prokaryotic lipoproteins, produced as a precursor with a signal peptide carrying a four-amino-acid sequence, Leu-Ile-Gly-Cys. In prokaryotic lipoproteins, the cysteine residue in this tetrapeptide is the acceptor for a thioether bond with glycerol, which is subsequently modified by the addition of fatty acids. After this

FIG. 7. Processing of lipidated and nonlipidated antigens in the absence (a) or presence (b) of ethanol. Exponentially growing E. coli cells carrying the recombinant plasmids were heat induced for antigen synthesis, separated on SDS-PAGE gels, and immunostained with a mixture of monoclonal antibodies directed against TmPA, TmPB, TmpC, and TpD. See the legend to Fig. 5A for lane identifications. Arrowheads indicate the precursor and processed forms of the antigens.
lipidation, the signal peptidase II cleaves off the lipoprotein signal sequence, leaving an N-terminal cysteine, which is then linked to a third fatty acid via an amide bond. The TmpA (29), TpD (29, 30), and 15-kDa (21) T. pallidum antigens also seem to follow this lipid modification pathway, which was first described for the major outer membrane lipoprotein in E. coli, Braun’s lipoprotein (3, 34). Other pathways for protein lipidation might exist in T. pallidum, because the 47-kDa antigen of T. pallidum, despite the lack of a characteristic signal sequence with the lipidation motif, has also been shown to be lipid modified in both T. pallidum and E. coli (5, 11).

The signal sequences of four T. pallidum protein genes were genetically manipulated, resulting in strains that enabled the isolation of TmpA, TmpB, TmpC, and TpD as lipidated and nonlipidated antigens. These antigens will be used for further studies on the role of lipid modification in the regulation of the immune response to infections with T. pallidum. In this study, we took advantage of the availability of both types of antigens to answer the question of whether the lipid moiety on lipoproteins plays a role in the induction of antilipoidal antibodies, which are invariably found during active syphilis. Such antibodies react with the VDRL antigen, a complex of the phospholipids cardiolipin, cholesterol, and lecithin. The results of this study suggest that the lipoprotein plays no direct role in the induction of VDRL-reactive antibodies because (i) immunization with purified, lipidated TmpA and TmpC did not elicit a VDRL response, (ii) reactivity to lipidated TmpA and TmpC was lost after absorption of sera with nonlipidated TmpA and TmpC, and (iii) affinity-purified VDRL antibodies do not react with T. pallidum antigens on Western blots (2). The origin of the antigen inducing the production of VDRL-reactive antibodies therefore remains obscure.

Integral membrane proteins are inserted into the membrane by hydrophobic transmembrane regions. Most lipoproteins lack such hydrophobic regions, and it is presumed that the lipid moiety serves as an anchor for insertion into the lipid bilayer of the membrane. The observation that the nonlipidated TmpB antigen is a soluble protein and that its mature lipidated homolog, TmpB*, is strongly membrane associated certainly corroborates this hypothesis. Such lipidation-dependent localization was not observed for TmpA, TmpC, and TpD, as the lipidated as well as the nonlipidated homologs of these antigens were found to be associated with the E. coli membrane during isolation. Possibly these nonlipidated antigens, which are poorly processed, associate with the E. coli membrane by means of their hydrophobic signal sequence.

E. coli and a number of other members of the family Enterobacteriaceae contain a low-molecular-weight lipoprotein which is the most abundant protein in the cell. In E. coli, this so-called Braun’s lipoprotein performs an important

---

**TABLE 2. Reactivity of rabbit sera after absorption with lipidated and nonlipidated antigens**

<table>
<thead>
<tr>
<th>Sorbent</th>
<th>Reactivity of serum with indicated antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anti-T. pallidum</td>
</tr>
<tr>
<td></td>
<td>TmpA*</td>
</tr>
<tr>
<td>None</td>
<td>+</td>
</tr>
<tr>
<td>TmpA</td>
<td>-</td>
</tr>
<tr>
<td>TmpA*</td>
<td>-</td>
</tr>
<tr>
<td>TmpC</td>
<td>+</td>
</tr>
<tr>
<td>TmpC*</td>
<td>+</td>
</tr>
</tbody>
</table>

* Reactivity measured by Western blotting.
* VDRL titer (reciprocal value of the dilution of serum giving detectable agglutination).
* ND, not done.
function in maintaining the shape and rigidity of the cell. Braun’s lipoprotein is anchored into the outer membrane by means of the N-terminal lipid moiety and is coupled to the peptidoglycan layer by means of a covalent bond between a C-terminal lysine of the protein and the meso-diaminopimelic acid of the peptidoglycan. As expected from the lack of a C-terminal lysine in TmpA, TmpC, and TpD, none of these lipoproteins was found to be covalently coupled to the peptidoglycan layer in T. pallidum or in E. coli. In most gram-negative bacteria, a number of other proteins, mainly porins, are also strongly, but not covalently, associated with the peptidoglycan layer. In addition to their porin properties, these proteins also contribute to the stability of the bacterial cell. Such proteins, however, could not be detected in the peptidoglycan fraction of T. pallidum, suggesting an unusually low concentration or absence of peptidoglycan-associated proteins. This finding would be consistent with the observation that the T. pallidum outer membrane contains a paucity of proteins (24). Together with the lack of lipopolysaccharide, the lack or paucity of outer membrane proteins such as Braun’s lipoprotein and porins might explain the extreme fragility of T. pallidum.

The lipid moiety on bacterial lipoproteins possesses strong immune-modulating properties. A synthetically prepared dipetide-lipid conjugate (Pam$_3$Cys-Ser), in which three palmitic acid molecules are bound to cysteine in a fashion similar to the bacterial lipoprotein, can be covalently coupled to peptides. It has been shown that linkage of Pam$_3$Cys-Ser to peptides greatly enhances the antibody response to these peptides, making coupling to large carrier molecules and the addition of adjuvants unnecessary (13). A recent study shows that Pam$_3$Cys-Ser-conjugated peptides can also elicit strong, protective cytotoxic T-cell responses (6). These findings show that the lipidation of proteins potentiates both humoral and cellular immune responses against these proteins. The mechanism for this potentiation of an immune response by covalently attached lipids is unclear. Possibly, the lipid moiety stabilizes the peptide, extending the half-life of the peptide. Alternatively, lipid modification might facilitate the association of the peptide with the major histocompatibility complex molecules and the insertion into the membrane of the antigen-presenting cell. Whatever the mechanism, the possible stimulation of an immune response by treponemal lipoproteins makes these antigens particularly interesting for the study of the immune response in patients with syphilis. The availability of pairs of lipidated and nonlipidated T. pallidum antigens described in this study will make possible future studies on the role of lipid modification in the immune response to T. pallidum infection.

ACKNOWLEDGMENTS

We thank G. T. Noordoek and J. Top for assistance in the construction and analysis of the lipoprotein mutants and B. Wielies for assistance in DNA sequencing of the tmpC gene.

REFERENCES


27. Schouls, L. M. Unpublished observations.


