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Native Surface Association of a Recombinant 38-Kilodalton *Treponema pallidum* Antigen Isolated from the *Escherichia coli* Outer Membrane

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A recombinant plasmid designated pAW305, containing a 6-kilobase insert of *Treponema pallidum* DNA, directed the expression of a 38-kilodalton (kDa) treponemal antigen in *Escherichia coli*. The 38-kDa antigen copurified with the outer membrane fraction of the *E. coli* cell envelope after treatment with nonionic detergents or sucrose density gradient centrifugation. Rabbits immunized with the recombinant 38-kDa antigen developed antibodies which reacted specifically with a 38-kDa *T. pallidum* antigen on immunoblots, and 38-kDa antisera specifically immobilized *T. pallidum* in a complement-dependent manner in the *T. pallidum* immobilization test. Antisera to the 38-kDa recombinant antigen were also used to demonstrate its native surface association on *T. pallidum* by immunoelectron microscopy.

It is likely that important steps in the pathogenesis of syphilis and in the acquisition of resistance to *Treponema pallidum* infection are mediated by specific treponemal surface molecules. Identification of these surface molecules and determination of their biological functions have been major, but elusive, goals which have been complicated by the problems of yield and purity inherent in the cultivation of *T. pallidum* in rabbit testicles. Although electron microscopy has demonstrated that an outer membrane encloses the periplasmic flagella and the cytoplasmic cylinder of the spirochete (14), this membrane has not been physically isolated, nor has its composition been directly determined. There is evidence which suggests that, compared with the stability of the outer membrane of gram-negative bacteria, the *T. pallidum* outer membrane has a more labile association with the cytoplasm (12). We believe that studies directed at the definition of the *T. pallidum* surface must use methods which preserve motility and virulence to prevent the potential loss of functionally significant surface-located molecules.

In the accompanying study (25), we reported the use of the *T. pallidum* immobilization (TPI) test conditions for study by immunoelectron microscopy. It is well established that treponemal motility and virulence are preserved during the TPI test unless both specific antibody and complement are present (3, 21, 27, 28). We found that the surface of virulent *T. pallidum* is remarkably resistant to demonstrable antibody binding; active complement as well as specific syphilitic antibody must be present before antibody coating of the treponemal surface can be detected (25). Using identical conditions, we localized another recombinant treponemal antigen, 4D (8), on the surface of *T. pallidum*.

In this study, we describe a recombinant 38-kilodalton (kDa) *T. pallidum* antigen which copurified with outer membrane fractions of *Escherichia coli*. Like antibody to the 4D antigen (8), antiserum to the 38-kDa antigen immobilized virulent *T. pallidum* in the TPI test and was used to demonstrate the association of this antigen with the surface of *T. pallidum* by immunoelectron microscopy.

MATERIALS AND METHODS

Expression of *T. pallidum* antigens in *E. coli*. Our Charon 30 gene bank of *T. pallidum* DNA, constructed with *Sau*3A partial digestion products, was screened for plaques specifically reactive with syphilitic serum as previously described (8, 29). Plasmid pAW305, which expresses the 38-kDa *T. pallidum* antigen in *E. coli* RR1, is described in Results.

SDS-PAGE and immunoblotting. Sodium dodecyl sulfate-polyacrylamide running gels of 12.5% or linear gradient gels of 8 to 20%, each with 2.6% cross-linking, were prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (8). Samples were mixed 1:2 with final sample buffer and placed in a boiling-water bath (100°C) for 10 min before SDS-PAGE. After SDS-PAGE, gels were stained with Coomassie blue or immunoblotted (8). Nitrocellulose sheets or strips were then probed with serum and ^{125}I-labeled protein A then and autoradiographed (8).

Partial purification of the recombinant 38-kDa antigen. Cultures of *E. coli* RR1(pAW305) cells were grown overnight to stationary phase in NZY broth containing 50 μg of ampicillin per ml to an approximate density of 5×10^9/ml, pelleted, washed, and suspended in 50 mM Tris, pH 8.4. The cells were lysed by sonication on ice for three 2-min periods with 5-min rests between bursts. The disrupted cells were centrifuged for 10 min at 1,912 × *g* to remove unlysed cells, and the supernatant from this low-speed centrifugation was centrifuged again at 47,800 × *g* for 20 min to separate soluble proteins from the cell envelopes (20K pellets). Unless otherwise noted, all centrifugations were conducted at 4°C with a Sorvall SS-34 rotor. The 20K pellet, enriched in the cell envelope fractions, was further separated by sucrose density centrifugation (10) or extracted with detergents as described below. Cultures of *E. coli* RR1 containing the plasmid pBR322 without the *T. pallidum* DNA insert were also grown and processed as described above for parallel com-

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parisons of the protein profiles of respective cell fractions from both cell types.

The 20K pellets enriched in cell envelope fractions were detergent extracted as follows. The 20K pellet was resuspended with a Dounce homogenizer in 50 mM Tris buffer (pH 8.4) at an approximate volume of 3 ml of Tris buffer per liter of original culture. To this was added 10% (vol/vol) Nonidet P-40 (NP-40; Shell Oil, Inc., Chemical Division, Lafayette, Calif.) detergent in the same buffer to a final concentration of 2%; after gentle mixing, the sample was incubated for 2 h at 37°C. The sample was then centrifuged at 47,800 × *g* for 20 min at 4°C to separate soluble from insoluble fractions. The resulting pellet was resuspended in a volume of Tris buffer (pH 8.4) equivalent to the starting volume before extraction with a Dounce homogenizer. NP-40-extracted 20K pellets were further treated with the following detergents at a 2% final concentration in Tris buffer, pH 7.5, either individually or in combination as noted in Results: *N*-lauroylsarcosine (sarcosyl), sodium deoxycholate, and *n*-octylglucoside (Sigma Chemical Co., St. Louis, Mo.); lithium diiodosalicylate and 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS; Pierce Chemical Co., Rockford, Ill.); and Zwittergent 3-14 (Calbiochem-Behring, La Jolla, Calif.). After incubation at 37°C, the detergent-treated samples were centrifuged at 47,800 × *g*, and the resulting supernatants and pellets were analyzed by SDS-PAGE.

Separation of *E. coli* membranes on sucrose gradients. The cell envelope-enriched fraction (20K pellet) of *E. coli* RR1 (pAW305) cells was separated into inner (cytoplasmic) and outer membrane fractions by the procedure of Fox et al. (10), with minor modifications. Linear 6-ml gradients of 30 to 60% sucrose in 10 mM Tris buffer (pH 7.5) containing 3 mM EDTA were prepared at 4°C. Cell envelope-enriched fractions, each containing approximately 1.5 mg of protein, were layered on top of the gradients. The gradients were then centrifuged for 16 h at 288,000 × *g* at 4°C in a Spinco SW 41 rotor (Beckman Instruments, Inc., Palo Alto, Calif.). Fractions (25 drops each) were collected with a fraction collector (Gilson Medical Electronics, Inc., Middleton, Wis.) by puncturing the bottoms of the tubes, pumping 75% (wt/vol) sucrose into the gradient tubes, and collecting drops from the tops of the capped tubes. Approximately 24 fractions were collected from each tube, and each fraction was analyzed by SDS-PAGE.

Production and characterization of rabbit antisera to the recombinant 38-kDa antigen. Adult male New Zealand White rabbits with nonreactive Veneral Disease Research Laboratory (VDRL) and TPI tests were immunized with the partially purified, cloned, 38-kDa protein as follows. For each rabbit, a 0.5-ml amount of the NP-40-extracted cell envelope pellet of *E. coli* RR1(pAW305) cells containing approximately 1.0 mg of protein was mixed with an equal volume of Freund incomplete adjuvant in a Sorvall Omnimixer. Each rabbit was injected intramuscularly at two sites on the hindquarters with 0.5 ml of the antigen suspension in each site. The rabbits were given a booster immunization with the same protocol 7 weeks after the initial injection. The rabbits were bled at 2, 5, 12, and 15 weeks after immunization, and the serum was stored at −70°C before being tested. TPI and VDRL tests were performed as previously described (8).

Demonstration of a 38-kDa antigen in *T. pallidum*. Samples of Percoll-purified *T. pallidum* (12) were mixed with an equal volume of final sample buffer and separated on preparative 8 to 20% linear gradient SDS-PAGE gels as previously described (8). An immunoblot was reacted with rabbit antiserum raised to the partially purified, cloned, 38-kDa protein and probed with ^{125}I-labeled protein A followed by autoradiography.

Localization by immunoelectron microscopy of the 38-kDa antigen in *T. pallidum*. Freshly extracted, virulent *T. pallidum* cells were incubated under TPI test conditions at a concentration of approximately 1.5 × 10^7/ml as previously described (8). After a 16-h incubation, motility was determined by dark-field microscopy. Incubation mixtures were then transferred to Microfuge tubes with siliconized Pasteur pipettes and centrifuged for 4 min at 12,800 × *g* in a Beckman Microfuge. The pellets were washed in 0.5 ml of phosphate-buffered saline, pH 7.4, and centrifuged again at 12,800 × *g* for 4 min, and the final pellets were resuspended in 25 μl of phosphate-buffered saline. Parlodion (Mallinckrodt, Inc., St. Louis, Mo.)-covered 400-mesh copper grids (Ted Pella Inc., Tustin, Calif.) were floated on 5-μl drops of the treponemal suspension, washed, and floated on 5-μl drops of 1:5,000 ferritin-conjugated goat anti-rabbit immunoglobulin G (Cooper Biomedical, Inc., West Chester, Pa.). Electron microscopy was performed with a Phillips EM 200 microscope at 60-kV accelerating voltage.

RESULTS

Clones expressing the 38-kDa antigen. Charon 30 clones expressing *T. pallidum* antigens in *E. coli* K802 have been described previously (8, 29). A clone designated Tp3A (29) expresses the 38-kDa antigen. Clones designated Tp1C and Tp2D (8) also express the 38-kDa antigen. Partial restriction maps of these bacteriophages are presented in Fig. 1. A 6-kilobase *Hin*dIII fragment of Tp2D was ligated to *Hin*dIII-cleaved pBR322 to create pAW305, which expresses the 38-kDa antigen in *E. coli* RR1. The 6-kilobase *Hin*dIII-*Sau*3A fragment hybridizes specifically to a 5.3-kilobase *Hin*dIII fragment of Tp3A as well as to the 6-kilobase *Hin*dIII fragment of Tp1C (data not shown).

Identification of the recombinant 38-kDa antigen in *E. coli*. *E. coli* RR1(pAW305) and *E. coli* RR1(pBR322) cells were sonically disrupted and separated into soluble and cell envelope-enriched fractions (20K pellet) by centrifugation at

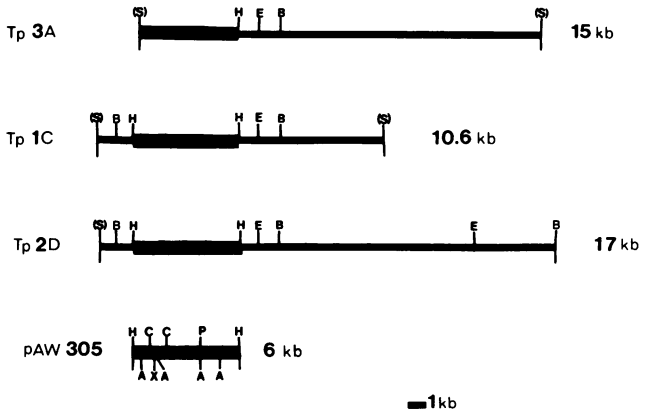


FIG. 1. Clones expressing the 38-kDa antigen. Restriction maps of the *T. pallidum* DNA inserts of Charon 30 bacteriophage clones Tp3A, Tp1C, and Tp2D and the pBR322 plasmid subclone of Tp2D, pAW305, are shown. Heavy lines represent fragments found to be homologous by Southern blotting. Abbreviations: A, *Ava*I; B, *Bam*HI; C, *Cla*I; H, *Hin*dIII; X, *Xba*I; P, *Pst*I; E, *Eco*RI; (S), *Sau*3A (insert not entirely mapped for *Sau*3A). There are no restriction sites for the following endonucleases on the insert DNA: *Hin*dIII, *Bam*HI, *Eco*RI, *Bgl*II, *Kpn*I, and *Sac*I.

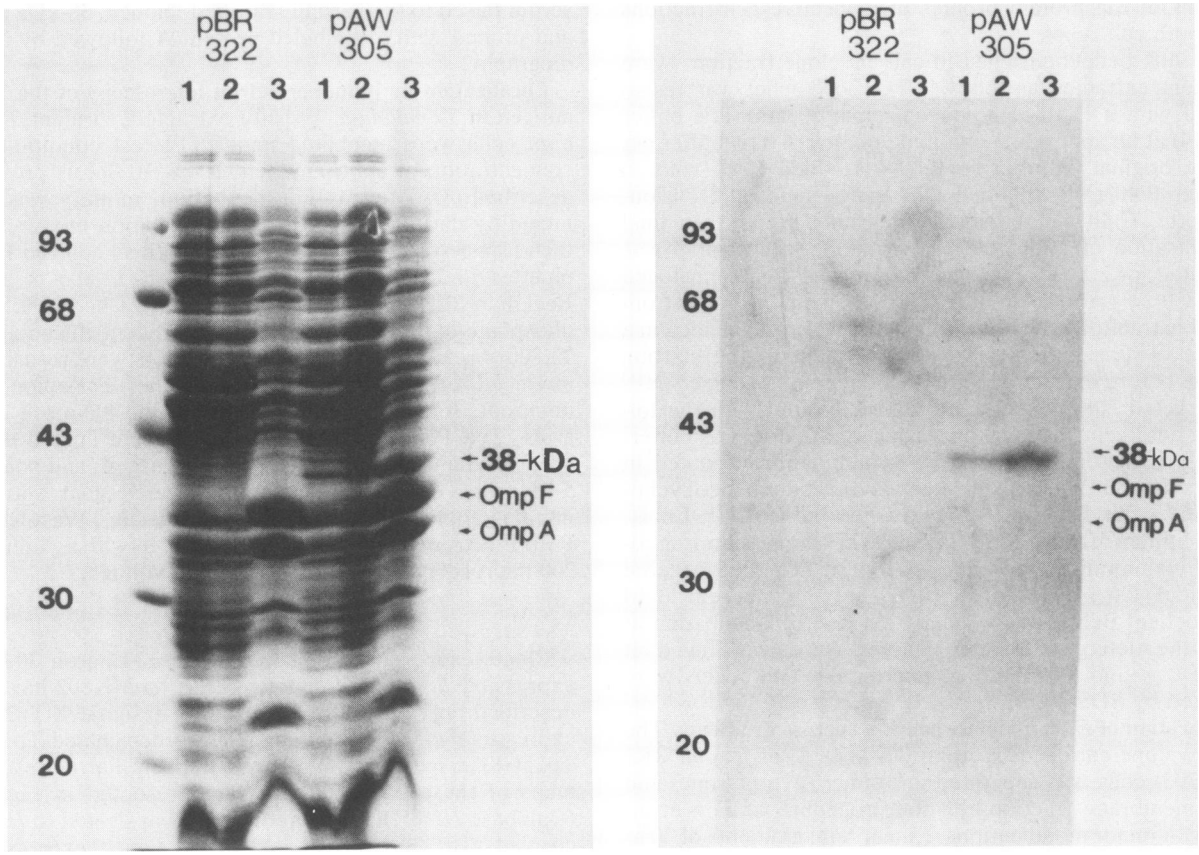
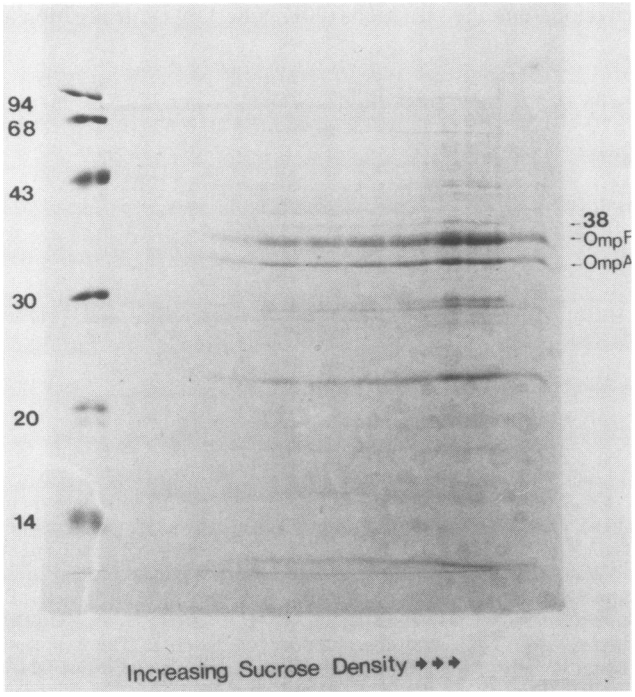


FIG. 2. Identification of the recombinant 38-kDa antigen in *E. coli* cell and subcellular fractions. Molecular sizes (in kilodaltons) are listed to the left of each panel. (A) Fractions of sonicated *E. coli* RR1(pBR322) and *E. coli* RR1(pAW305) cells analyzed by SDS-PAGE. Lanes 1, crude whole cell sonicate; lanes 2, supernatant of cell envelope-enriched pellet; lanes 3, cell envelope-enriched pellet (20K pellet). (B) Immunoblot of the same samples against human syphilitic serum.



47,800 × *g* as described in Materials and Methods. These fractions were then analyzed by SDS-PAGE. A Coomassie blue-stained SDS-PAGE gel of fractions of *E. coli* RR1(pBR322) and *E. coli* RR1(pAW305) cells is shown in Fig. 2A. The 38-kDa *T. pallidum* polypeptide was found exclusively in the *E. coli* RR1(pAW305) cells. Samples of the 20K pellets of the *E. coli* RR1(pAW305) cells (Fig. 2A, lane 3) showed enrichment for the 38-kDa protein as well as for *E. coli* protein bands considered to be the 37-kDa OmpF/C (4) and the 33-kDa OmpA (5). The OmpF/C and OmpA proteins of *E. coli* were identified by virtue of their molecular weights, resistance to detergent solubilization, and the heat-modifiable molecular weight of OmpA in this system (18) (data not shown). An SDS-PAGE gel containing identically prepared samples of pBR322 and pAW305 was immunoblotted with syphilitic serum. Antibodies present in the syphilitic serum clearly bound specifically to the cloned 38-kDa antigen present in pAW305-containing cell fractions,

FIG. 3. Copurification of the 38-kDa antigen with *E. coli* outer membranes. The cell envelope-enriched fraction of sonically disrupted pAW305 was separated by sucrose density gradient ultracentrifugation. Individual fractions were analyzed by SDS-PAGE. The 38-kDa antigen in *E. coli* copurified with outer membrane proteins OmpF/C and OmpA. Less-dense fractions containing inner membrane material are not shown. Molecular sizes (in kilodaltons) are listed to the left of the figure.

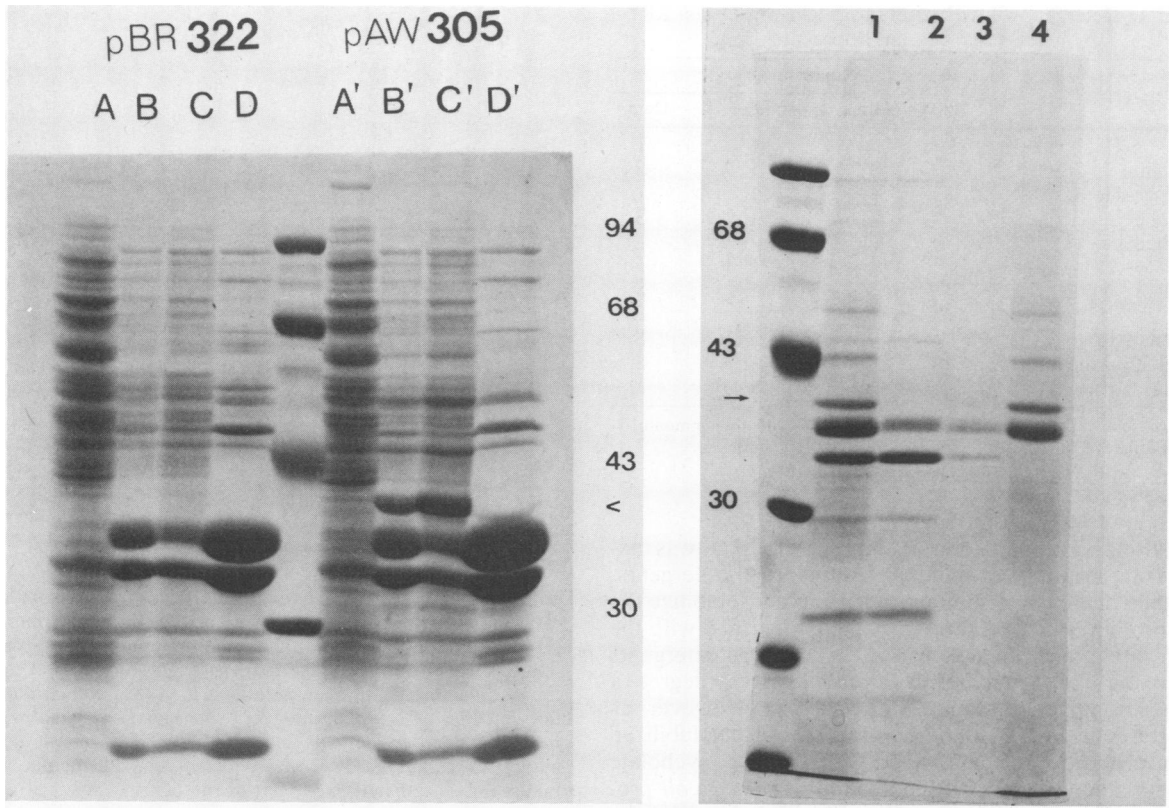


FIG. 4. Partial purification of the 38-kDa antigen from *E. coli* membranes. Molecular sizes (in kilodaltons) are listed on the sides of the panels. (A) After extraction of the cell envelope fractions of pAW305 with 2% NP-40, the 38-kDa antigen was found in the NP-40-insoluble material (lane B') but not in the NP-40-soluble supernatant (lane A'). Most of the 38-kDa antigen was solubilized in 2% sarcosyl at 37°C (lane C'), whereas the majority of OmpF/C and OmpA remained in the sarcosyl-insoluble material (lane D'). An equivalent amount of OmpF/C and OmpA was sarcosyl extracted (lane C') along with the 38-kDa antigen. Identically manipulated cell envelope fractions of pBR322 (lanes A to D) showed identical protein profiles except for the absence of the 38-kDa antigen. (B) The NP-40-insoluble fraction of pAW305 outer envelopes containing the 38-kDa antigen (lane 1) was extracted (lane 2) and then reextracted (lane 3) with a mixture of 2% deoxycholate and octylglucoside. All of the OmpA and much of the OmpF/C proteins were solubilized by these procedures. The deoxycholate-octylglucoside-insoluble material (lane 4) included all of the 38-kDa antigen and a comparable amount of OmpF/C. →, 38-kDa polypeptide.

particularly the cell envelope-enriched pellet (lane 3), but not *E. coli* outer membrane proteins (Fig. 2B).

Identification of the recombinant 38-kDa protein in *E. coli* outer membrane fractions. To study the association of the 38-kDa antigen with *E. coli* membranes, the cell envelope of pAW305 cells were analyzed by sucrose density gradient centrifugation as described in Materials and Methods. Sequential fractions of the sucrose gradients were analyzed by SDS-PAGE. The 38-kDa protein copurified with denser outer membrane fractions which contained the OmpF/C and OmpA proteins (Fig. 3). Neither the 38-kDa protein nor the OmpF/C and OmpA proteins were seen in less-dense fractions which contained *E. coli* inner membranes (data not shown).

Partial purification of the 38-kDa antigen from *E. coli* membranes. The strategy adopted to purify the 38-kDa protein from the membrane fractions of the *E. coli* RR1 (pAW305) cells used relatively mild procedures to preserve native structural features on the 38-kDa protein. After sonication, the cell envelope-enriched 20K pellet was extracted with 2% NP-40 at 37°C to create an outer membrane fraction (18). After NP-40 detergent extraction, the cloned 38-kDa antigen was present in the insoluble fraction (lane B') along with the OmpF/C and OmpA proteins, whereas the soluble fraction (lane A') contained many of the cell envelope proteins which previously were insoluble before NP-40 detergent extraction (Fig. 4A).

Treatment of the NP-40-insoluble outer membrane fraction (lane B') with 2% sarcosyl at 37°C solubilized most of the recombinant 38-kDa protein and a variety of other proteins, including a fraction of the OmpF/C and OmpA proteins (lane C'); it can be seen that the apparent amount of OmpF/C and OmpA proteins released was similar to the amount of the 38-kDa protein released (Fig. 4A). Most OmpA and OmpF/C proteins remained insoluble with the sarcosyl treatment (Fig. 4A, lane D'). Identically prepared cell envelopes of control *E. coli* RR1(pBR322) cells (lanes A to D) showed protein profiles which were virtually indistinguishable from the fractions of the pAW305 cells (lanes A' to D'), except for the absence of the 38-kDa polypeptide (Fig. 4A).

Both the original 20K cell envelope-enriched pellet and the NP-40-insoluble pellet were reextracted with NP-40 in the presence of 10 mM EDTA. Neither the cloned 38-kDa protein nor the OmpF/C protein was further solubilized by the detergent treatment in the presence of EDTA. However, the OmpA protein was solubilized under these conditions (data not shown).

TABLE 1. Detergent solubilization of proteins, in *E. coli* RR1 (pAW305) cell envelopes

Detergent	Solubility of[a]:		
	38-kDa protein	OmpF	OmpA
NP-40	I	I	I
Deoxycholate	I	I	S
n-Octylglucoside	I	I	I
Deoxycholate + octylglucoside	I	I	S
Tween 20	I	P	I
SDS (100°C)	S	S	S
Sarcosyl	S	P	P
Zwittergent 3-14	I	P	S
Lithium diiodosalicylate	I	P	S
CHAPS	I	I	I

[a] Abbreviations: CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate; I, insoluble; S, soluble; P, partially soluble.

The NP-40-insoluble outer membrane fraction was extracted with a variety of nonionic and charged detergents, individually and in combination, to selectively solubilize the 38-kDa protein or *E. coli* proteins which copurified with the 38-kDa protein to this stage. The effects of these detergents are listed in Table 1. Only sarcosyl provided some degree of selective solubilization of the 38-kDa antigen. However, as demonstrated in Fig. 4A (lane B'), sarcosyl also solubilized a certain proportion of OmpA and OmpF/C. Deoxycholate and *n*-octylglucoside solubilized several of the *E. coli* proteins which copurified with the cloned 38-kDa protein in the NP-40-insoluble pellet.

When the NP-40-insoluble fraction (Fig. 4B, lane 1) was extracted twice with a mixture of deoxycholate and *n*-octylglucoside, the proteins released after the first (lane 2) and second wash (lane 3) included all of OmpA and a lesser proportion of OmpF/C. The remaining detergent-insoluble material (lane 4) was enriched for the 38-kDa protein.

Induction of immobilizing antibody by immunization with the cloned antigen. Two rabbits were immunized intramuscularly with the partially purified, cloned, 38-kDa protein (Fig. 4B, lane 4) and were boosted 7 weeks later. Serum was obtained from both rabbits 2, 5, 12, and 15 weeks after the initial immunization, and the sera were tested in the TPI test. One rabbit exhibited weakly reactive TPI activity 5 weeks after the first injection of the recombinant 38-kDa antigen (Table 2). After booster immunization, both animals developed TPI reactivity.

TABLE 2. Induction of complement-dependent immobilizing (TPI) antibody by the recombinant 38-kDa protein

Time after immunization (weeks)	Antibody activity of rabbit no.[a]:	
	101	110
Initial injection		
0	NR	NR
2	NR	NR
5	WR	NR
Booster injection[b]		
12	R, WR	WR
15	R	R

[a] Abbreviations: NR, nonreactive TPI; WR, weakly reactive TPI; R, reactive TPI.

[b] The booster injection was given 7 weeks after the initial injection.

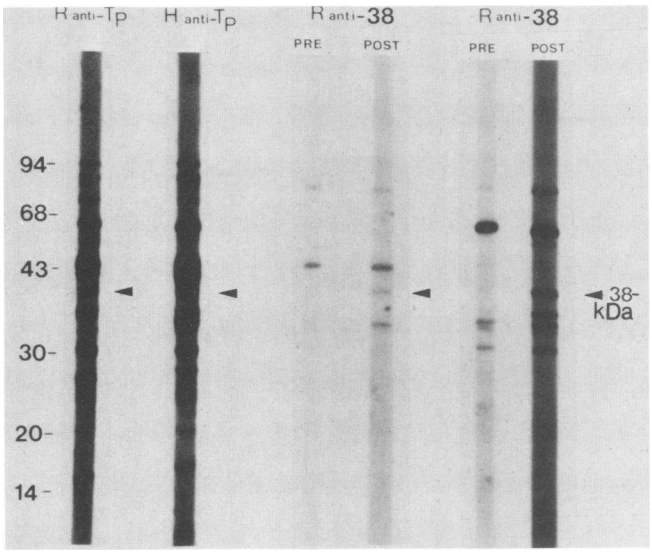


FIG. 5. Identification of the native 38-kDa antigen in *T. pallidum*. Immunoblots of *T. pallidum* polypeptides were reacted with rabbit immune syphilitic serum (R anti-Tp) human syphilitic serum (H anti-Tp), and the pre- and postimmune sera of two rabbits immunized with the 38-kDa antigen (R anti-38). Rabbit antisera to the purified 38-kDa antigen identified a 38-kDa *T. pallidum* antigen not detected with preimmune sera. An indistinguishable 38-kDa *T. pallidum* polypeptide was identified by both rabbit and human syphilitic sera. ◀, 38-kDa polypeptides. Molecular sizes (in kilodaltons) are listed to the left of the figure.

Identification of a 38-kDa protein antigen in *T. pallidum*. Individual strips of nitrocellulose containing transferred *T. pallidum* proteins were reacted with rabbit antiserum raised to the partially purified, cloned, 38-kDa protein described above. Antibody to a 38-kDa *T. pallidum* protein was demonstrable in both immunized rabbits and was not present in the preimmune serum of either rabbit (Fig. 5). Antibody which reacted with other *T. pallidum* proteins was detectable in both pre- and postimmune serum samples from both rabbits. These bands represent natural antibody to host-indigenous nonpathogenic treponemes which share epitopes with *T. pallidum* proteins (11, 19). Human and rabbit immune syphilitic sera detected a 38-kDa *T. pallidum* antigen indistinguishable by molecular size in this one-dimensional SDS-PAGE analysis from the recombinant 38-kDa antigen.

Native surface association of the 38-kDa antigen. *T. pallidum* cells incubated under TPI test conditions were processed for immunoelectron microscopy as described in Materials and Methods. Organisms incubated with rabbit immune syphilitic serum and heat-inactivated complement retained their motility and did not demonstrate significant immunoferritin labeling (Fig. 6B). Organisms immobilized during incubation with rabbit immune syphilitic serum and active complement were heavily and uniformly ferritin labeled (Fig. 6A), indicating large amounts of surface-bound immunoglobulin G. Organisms immobilized with 38-kDa antiserum and active complement were heavily ferritin labeled and were indistinguishable from ferritin-labeled rabbit immune syphilitic serum-immobilized *T. pallidum* cells (Fig. 6C). In contrast, organisms which retained their motility during incubation with 38-kDa antiserum and heat-inactivated complement were unlabeled (Fig. 6D). These experiments demonstrate the surface association of the 38-kDa antigen on *T. pallidum*.

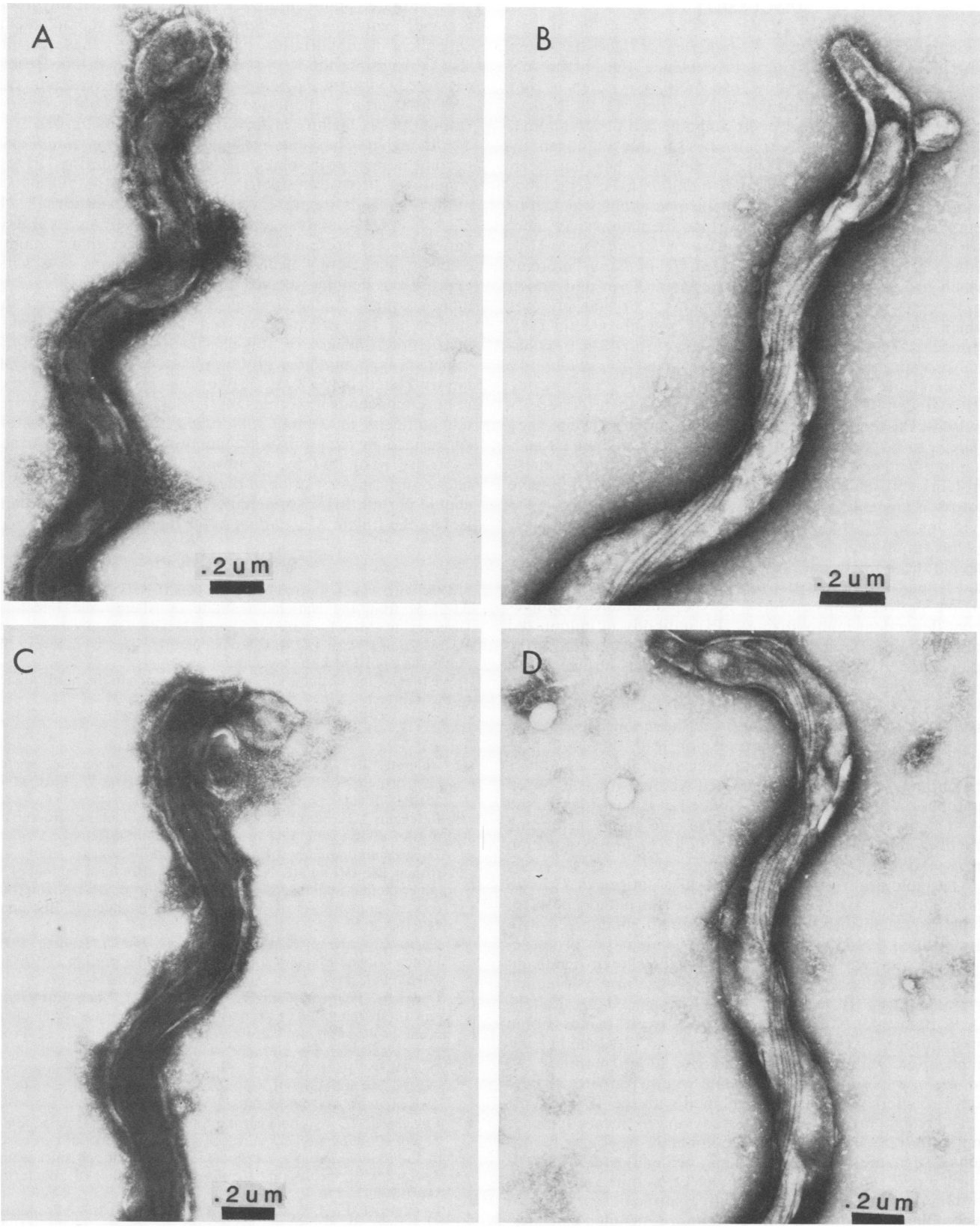


FIG. 6. Native surface association of the 38-kDa antigen. Electron micrographs of *T. pallidum* incubated under TPI test conditions with rabbit immune syphilitic serum and active complement (A) or heat-inactivated complement (B) and *T. pallidum* incubated with 38-kDa antiserum with (C) or without (D) active complement. Ferritin-conjugated goat anti-rabbit immunoglobulin G was used to visualize bound rabbit antibody.

DISCUSSION

Characterization of the *T. pallidum* surface has been particularly complex. There is evidence that organisms manipulated in vitro readily lose motility, virulence, and outer membrane structure (12; J. D. Radolf, D. R. Blanco, J. N. Miller, and M. A. Lovett, unpublished observations). Definition of the surface molecules of this pathogen by physical isolation of an outer membrane has not been reported. Surface iodination of *T. pallidum* by several laboratories has led to disparate conclusions about which proteins are labeled (2, 16, 23). Surface iodination may label cytoplasmic proteins, as in the case of *H. influenzae* (17). Other investigators have identified *T. pallidum* proteins which are present in sonication-resistant form and which may represent membrane components (26) and proteins which attach to tissue culture cells and fibronectin (24). Monoclonal antibodies which recognize a 47-kDa treponemal protein react with the surface of fixed *T. pallidum* by immunofluorescence (20) and immunoelectron microscopy (21a). Recently, Hansen and co-workers have described 35- and 44-kDa recombinant *T. pallidum* antigens which they believe to be membrane proteins (13). However, data substantiating the *E. coli* outer membrane location of these proteins and their native treponemal surface location have not been presented.

In this report, we have described a recombinant 38-kDa *T. pallidum* polypeptide which copurified with the *E. coli* outer membrane both by selective detergent treatment (Fig. 4A and B) and by sucrose density gradient centrifugation (Fig. 3). We showed that the 38-kDa polypeptide induces complement-dependent immobilizing (TPI) antibody in immunized rabbits (Table 2), and we used immunoelectron microscopy to localize the 38-kDa polypeptide on the surface of *T. pallidum* (Fig. 6). Engleberg et al. reported recently (7) that cloned *Legionella* outer membrane proteins are translocated to the *E. coli* outer membranes and have surface-exposed epitopes. Although our results demonstrate the copurification of the 38-kDa treponemal protein with the *E. coli* outer membrane, we have not yet studied whether it has exposed epitopes on the *E. coli* surface. Indeed, it is possible that the 38-kDa protein copurified with *E. coli* outer membrane fractions for reasons unrelated to its likely surface location in *T. pallidum*.

The biological function of the 38-kDa antigen in *T. pallidum* and its possible contribution to the pathogenesis of syphilis remain to be determined. Rabbits immunized with the purified 190-kDa 4D antigen (8), which is a protease-resistant ordered-ring structure (7a), have shown significant partial protection against dermal challenge in experimental syphilis (L. A. Borenstein, T. E. Fehniger, J. D. Radolf, D. R. Blanco, J. N. Miller, and M. A. Lovett, manuscript in preparation). Experiments are planned to test whether immunization with the 38-kDa antigen alone modifies the course of experimental syphilis and whether greater protection is conferred when it is combined with the 4D antigen.

The results presented in this study and in the accompanying study (25) show that the recombinant treponemal antigens designated 4D and 38 kDa can be demonstrated on the surface of *T. pallidum* by immunoelectron microscopy. The system we used to determine native surface association of recombinant treponemal antigens requires detailed consideration. We showed that motile *T. pallidum* cells are remarkably resistant to antibody interaction in the absence of complement under the conditions of the TPI test, confirming and extending the work of Hovind-Hougen et al. (15). They showed that syphilitic antibodies do not demonstrably interact with virulent *T. pallidum* cells unless complement-dependent immobilization has taken place. These results also corroborate the long-standing observation that freshly extracted, virulent *T. pallidum* cells are not reactive with syphilitic serum in the fluorescent treponemal antibody test without prior "aging" or fixation of organisms (6, 22).

Although it is possible that the surface of *T. pallidum* may become nonnative in some unrecognized way during TPI test conditions, it is well established that motility and virulence of the organisms are preserved under these conditions (3, 21, 27, 28), unless active complement as well as syphilitic antibodies are present. We therefore believe that immunoelectron microscopy using TPI test conditions is a valid approach to defining surface components of *T. pallidum*. A more-complete understanding of the means by which the surface of this organism so remarkably resists antibody interaction is necessary. It is possible that the surface of the organism is immunologically nonreactive by virtue of coating with host components (1, 9) or the relative abundance of nonproteinaceous molecules on the surface. "Surface" protein antigens of *T. pallidum* might then be immediately subsurface in location, with temporally restricted surface exposure permitted by membrane fluidity. Demonstrated native surface association of the 38-kDa and 4D antigens (25) should permit their use as markers in attempts to physically isolate the fragile *T. pallidum* outer membrane.

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LITERATURE CITED

1. **Alderete, J. F., and J. B. Baseman.** 1979. Surface-associated host proteins on virulent *Treponema pallidum*. J. Exp. Med. **26**:1048–1056.
2. **Baseman, J. B., and E. C. Hayes.** 1980. Molecular characterization of receptor-binding proteins and immunogens of virulent *Treponema pallidum*. J Exp. Med. **151**:573–586.
3. **Bishop, N. H., and J. N. Miller.** 1976. Humoral immunity in experimental syphilis. II. The relationship of neutralizing factors in immune serum to acquired resistance. J. Immunol. **117**:197–207.
4. **Chen, R., C. Kramer, W. Schmidmayer, and U. Henning.** 1979. Primary structure of major outer membrane protein I of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **76**:5014–5017.
5. **Chen, R., W. Schmidmayer, C. Kramer, U. Chen-Schmeisser, and U. Henning.** 1980. Primary structure of major outer membrane protein II (OmpA protein) of *Escherichia coli* K-12. Proc. Natl. Acad. Sci. USA **77**:4592–4598.
6. **Deacon, W. F., J. B. Lucas, and F. V. Price.** 1966. Fluorescent treponemal antibody absorption (FTA-ABS) test for syphilis. J. Am. Med. Assoc. **198**:624–628.
7. **Engelberg, N. C., E. Pearlman, and B. I. Eisenstein.** 1984. *Legionella pneumophila* surface antigens cloned and expressed in *Escherichia coli* are translocated to the host cell surface and interact with specific anti-*Legionella* antibodies. J. Bacteriol. **160**:199–203.

7a. **Fehniger, T. E., J. D. Radolf, and M. A. Lovett.** 1986. Properties of an ordered ring structure formed by recombinant *Treponema pallidum* surface antigen 4D. J. Bacteriol. **165**:732–739.

8. **Fehniger, T. E., A. M. Walfield, T. M. Cunningham, J. D. Radolf, J. N. Miller, and M. A. Lovett.** 1984. Purification and characterization of a cloned protease-resistant *Treponema pal-*

lidum-specific antigen. Infect. Immun. **46**:598–607.
9. **Fitzgerald, T. J., and R. C. Johnson.** 1979. Surface mucopolysaccharides of *Treponema pallidum*. Infect. Immun. **24**: 244–251.
10. **Fox, C. F., J. Law, N. Tsukagoshi, and G. Wilson.** 1970. A density label for membranes. Proc. Natl. Acad. Sci. USA **67**:598–604.
11. **Hanff, P. A., J. N. Miller, and M. A. Lovett.** 1983. Molecular characterization of common treponemal antigens. Infect. Immun. **40**:825–828.
12. **Hanff, P. A., S. J. Norris, M. A. Lovett, and J. N. Miller.** 1984. Purification of *Treponema pallidum*, Nichols strain, by Percoll density gradient centrifugation. Sex. Transm. Dis. **11**:275–286.
13. **Hansen, E. B., P. E. Pedersen, L. M. Schouls, E. Severin, and J. D. R. van Embden.** 1985. Genetic characterization and partial sequence determination of a *Treponema pallidum* operon expressing two immunogenic membrane proteins in *Escherichia coli*. J. Bacteriol. **162**:1227–1237.
14. **Hovind-Hougen, K.** 1983. Morphology, p. 3–28. *In* R. F. Schell and D. M. Musher (ed.), Pathogenesis and immunology of treponemal infection. Marcel Dekker, Inc., New York.
15. **Hovind-Hougen, K., A. Birch-Andersen, and H. A. Nielsen.** 1979. Electron microscopy of treponemes subjected to the *Treponema pallidum* Immobilization (TPI) test. Acta. Pathol. Microbiol. Scand. Sect. C **87**:263–268.
16. **Jones, S. A., K. S. Marchitto, J. N. Miller, and M. V. Norgard.** 1984. Monoclonal antibody with hemagglutination, immobilization and neutralization activities defines an immunodominant 47,000 mol. wt. surface-exposed immunogen of *Treponema pallidum* (Nichols). J. Exp. Med. **160**:1404–1420.
17. **Loeb, M. R., and D. H. Smith.** 1983. Lactoperoxidase and Iodo-Gen-catalyzed iodination labels inner and outer membrane proteins of *Haemophilus influenzae*. J. Bacteriol. **155**:443–446.
18. **Lugtenberg, B., and L. Van Alphen.** 1983. Molecular architecture and functioning of the outer membrane of *Escherichia coli* and other gram negative bacteria. Biochim. Biophys. Acta. **465**:571–578.
19. **Lukehart, S. A., S. A. Baker-Zander, and E. R. Gubish.** 1982. Identification of *Treponema pallidum* antigens: comparison with a nonpathogenic treponeme. J. Immunol. **129**:833–838.
20. **Lukehart, S. A., M. R. Tam, J. Hom, S. A. Baker-Zander, K. K. Holmes, and R. C. Nowinski.** 1985. Characterization of monoclonal antibodies to *Treponema pallidum*. J. Immunol. **134**:585–592.
21. **Magnuson, H. J., F. A. Thompson, and C. P. McLeod.** 1951. Relationship between treponemal immobilizing antibodies and acquired immunity in experimental syphilis. J. Immunol. **67**:41–48.
21a. **Marchitto, K. S., S. A. Jones, R. F. Schell, P. L. Holmans, and M. V. Norgard.** 1984. Monoclonal antibody analysis of specific antigenic similarities among pathogenic *Treponema pallidum* subspecies. Infect. Immun. **45**:660–666.
22. **Metzger, M., and J. Ruczkowska.** 1964. Influence of lysozyme upon the reactivity of *Treponema pallidum* in the fluorescent antibody reaction. Arch. Immunol. Ther. Exp. **12**:702–708.
23. **Norris, S. J., and S. Sell.** 1984. Antigenic complexity of *Treponema pallidum*: antigenicity and surface localization of major polypeptides. J. Immunol. **133**:2686–2692.
24. **Peterson, K. M., J. B. Baseman, and J. F. Alderete.** 1983. *Treponema pallidum* receptor binding proteins interact with fibronectin. J. Exp. Med. **157**:1958–1970.
25. **Radolf, J. D., T. E. Fehniger, F. J. Silverblatt, J. N. Miller, and M. A. Lovett.** 1986. The surface of virulent *Treponema pallidum*: resistance to antibody interaction in the absence of complement and surface association of recombinant antigen 4D. Infect. Immun. **52**:579–585.
26. **Stamm, L. V., and P. J. Bassford, Jr.** 1985. Cellular and extracellular protein antigens of *Treponema pallidum* synthesized during in vitro incubation of freshly extracted organisms. Infect. Immun. **47**:799–807.
27. **Turner, T. B., and D. H. Hollander.** 1957. Biology of the treponematoses. WHO Monogr. Ser. **35**:138–141.
28. **Turner, T. B., and R. A. Nelson.** 1950. The relationship of treponemal immobilizing antibody to immunity in syphilis. Trans. Assoc. Am. Phys. **63**:112–117.
29. **Walfield, A. M., P. A. Hanff, and M. A. Lovett.** 1982. Expression of *Treponema pallidum* antigens in *Escherichia coli*. Science **216**:522–523.