

## Identification of a *Treponema pallidum* Laminin-Binding Protein

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**Host extracellular matrix (ECM) components represent ideal microbial adhesion targets that many pathogens use for colonization of tissues and initiation of infection. This study investigated the interaction of the spirochete *Treponema pallidum* with the ECM component laminin. To identify candidate laminin-binding adhesins, the *T. pallidum* genome was analyzed to predict open reading frames that encode putative outer membrane proteins, as these proteins interact directly with host ECM components. Subsequent recombinant expression of these proteins and analysis of their laminin-binding potential identified one protein, Tp0751, that demonstrated specific attachment to laminin. Tp0751 attached to laminin in a dose-dependent, saturable manner but did not attach to the ECM component collagen type I or IV or to the negative control proteins fetuin or bovine serum albumin. Sodium metaperiodate treatment of laminin reduced the Tp0751-laminin interaction in a concentration-dependent manner, suggesting that oligosaccharides play a role in this interaction. In addition, Tp0751-specific antibodies were detected in serum samples collected from both experimental and natural syphilis infections, indicating that Tp0751 is expressed in vivo during the course of infection. Collectively, these experiments identified Tp0751 as a laminin-binding protein that is expressed during infection and may be involved in attachment of *T. pallidum* to host tissues.**

Syphilis, which is caused by the spirochete bacterium *Treponema pallidum* subsp. *pallidum*, is a chronic bacterial infection that remains a public health concern worldwide. In 1995, the World Health Organization's global estimate of annual new syphilis infections was 12 million, with a global syphilis burden of at least 25 million cases (26). Syphilis is a chronic infection that fluctuates between symptomatic stages and prolonged asymptomatic stages. If left untreated, syphilis can cause serious late sequelae that may involve any organ system and lead to loss of function and destruction of tissue.

As observed for other pathogenic bacteria, the critical first steps in the establishment of a *T. pallidum* infection are attachment to and colonization of epithelial cell surfaces. Numerous studies have confirmed that *T. pallidum* attaches to host cells (3, 4, 16–20, 34, 64, 67, 77), although neither the host cell nor the *T. pallidum* components mediating such attachment have been clearly defined. Nonpathogenic *T. phagedenis* biotype Reiter does not adhere to cultured mammalian cells (8, 67), suggesting that attachment to host cells is a function unique to pathogenic treponemes. Previous studies have demonstrated that adherence appears to contribute to the prolonged survival, motility, and virulence of *T. pallidum* (18, 19). Results from experimentally induced *T. pallidum* infections (12, 59) and in vitro studies (67) have shown that *T. pallidum* adheres to and invades epithelial surfaces, traverses the tissue barrier, and enters the circulation by invading the tight junctions between endothelial cells. Treponemal invasion results in widespread bacterial dissemination, which in turn sets the stage for establishment of chronic infection. Similarly, in a natural syphilis infection, *T. pallidum* is believed to attach to and enter mucosal surfaces or epidermal abrasions, become widely dissemi-

nated throughout the tissues of the body, and establish a chronic infection (44, 45, 72). The invasive nature of *T. pallidum* is well illustrated by the fact that invasion of the central nervous system occurs in greater than 40% of syphilis patients (43) and that syphilis can be transmitted by an infected woman to her fetus in utero. Host cell attachment is critical to this ability of *T. pallidum* to invade and establish infection in many diverse tissues. These observations demonstrate the importance of treponemal attachment to host cells, not only in the initial stages of infection but also throughout the course of *T. pallidum* infection.

Adherence to and colonization of epithelial surfaces are the primary events in the pathogenesis of many bacterial infections (5, 52). This process involves the interaction of specific bacterial surface structures, known as adhesins, with host components. Extracellular matrix (ECM) molecules represent ideal microbial adhesion targets that many pathogens use for colonization of tissues and initiation of infection. The term MSCRAMM (microbial surface components recognizing adhesive matrix molecules) has been introduced to describe microbial molecules that recognize ECM components, including fibronectin, fibrinogen, collagens, laminins, vitronectin, and heparan sulfate (52). Bacterial adhesins that specifically recognize ECM components have been characterized in many pathogens, including the spirochetes *Borrelia burgdorferi* (10, 30, 31, 56), *B. garinii* (38), *Leptospira interrogans* (47), and *T. denticola* (14, 15, 74).

Similarly, numerous studies have documented the specific interaction of *T. pallidum* with ECM components. Previous literature suggests that fibronectin and laminin are ECM molecules that are central to treponemal cytoadherence. *T. pallidum* has been shown to attach to fibronectin- and laminin-coated surfaces (20, 34). Attachment is not a nonspecific association, as shown by the inability of heat-killed *T. pallidum* (20) and nonpathogenic *T. phagedenis* biotype Reiter (54, 75) to bind to these ECM components. *T. pallidum* has been re-

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TABLE 1. ORF-specific primers used to amplify fragments for recombinant expression

ORF	Sense primer(s) <sup>a</sup>	Antisense primer(s) <sup>a</sup>	Amplicon details	
			Size (bp)	Portion of ORF
Tp0155	5'- <u>ggatccaaccattgacacctgcc</u>	5'- <u>gaattctgcagctgaattatagaac</u>	428	5' end
	5'- <u>ggatccttaacacgcccgtctcttc</u>	5'- <u>gaattccggaagggtacgcatac</u>	630	3' end
Tp0316 (TprF)	5'- <u>ggatcctatgcagcgctactcaactccg</u>	5'- <u>gaattctcagcaagcaccacctgttcc</u>	1,062	Internal
Tp0326 (Tp92)	5'- <u>caggcaaacgacaattgg</u>	5'- <u>caattatttaccgtgaacg</u>	2,448	Full length
Tp0453	5'- <u>ggatcccgtggaaggcatcagtag</u>	5'- <u>gaattccgaactccctttttggag</u>	758	Full length
Tp0483	5'- <u>ggatcccacgcccgcctcaaaaccg</u>	5'- <u>gaattcgttatgaaagcgatagccg</u>	590	3' end
Tp0557	5'- <u>ggatccatccgcacgtttttatccgcac</u>	5'- <u>gaattcggggcagtgtagccgag</u>	636	Full length
Tp0620 (Tpr1)	5'- <u>ggatcccgcactcaccctgaaacca</u>	5'- <u>gaattcgggtgagcaggtgggtgtag</u>	633	Internal
Tp0751	5'- <u>ggatcccggcaccgccgcacac</u>	5'- <u>gaattccttggcgtgtgtgtgcgc</u>	360	5' end
Tp0856	5'- <u>gcgcgcaagactcgctc</u>	5'- <u>cttgcgtccgacgag</u>	1,086	Full length
Tp0952	5'- <u>ggatcctttccaagaagtcttcgaagt</u>	5'- <u>gaattcagcgcacctgttaaagg</u>	502	5' end
	5'- <u>ggatcctgttgggactcagatggg</u>	5'- <u>gaattcattgaaatgtttgctcacag</u>	542	3' end
<i>S. aureus</i> LBP <sup>b</sup>	5'- <u>ggatcctgcgaattattacagatg</u>	5'- <u>aagcctttattatctaagttataga</u>	1,304	Full length

<sup>a</sup> Restriction sites incorporated into the primers are underlined.

<sup>b</sup> LBP, laminin-binding protein.

ported to bind to cultured mammalian cells via the C-terminal cell-binding domain of fibronectin (64). Pretreatment of host cells with antiserum to fibronectin, but not pretreatment with control irrelevant antiserum, inhibits attachment of *T. pallidum* (34, 54, 64). Likewise, the immunoglobulin G (IgG) fraction from immune serum blocks treponemal attachment to both fibronectin and laminin (20).

Laminins are large multidomain glycoproteins that are important constituents of condensed, polymer-like aggregates of the ECM termed basement membranes (68). Since the discovery of laminin in 1979 (70), a growing number of pathogens have been shown to bind specifically to this ECM component, including *Helicobacter pylori* (76), *Mycobacterium leprae* (51, 60), *Enterococcus faecalis* (50, 79), *Trichomonas vaginalis* (11), *Streptococcus pyogenes* (35), *Escherichia coli* (62), and *T. denticola* (14). The results reported herein confirm and extend the studies performed by Fitzgerald et al. (20) documenting *T. pallidum* attachment to laminin by describing the identification of a *T. pallidum* laminin-binding protein.

#### MATERIALS AND METHODS

**ECM proteins.** Collagen types I and IV, fibronectin, fibrinogen, and laminin isolated from the Engelbreth-Holm-Swarm murine sarcoma, as well as the control proteins bovine serum albumin (BSA) and fetuin, were purchased from Sigma Chemical Co. (St. Louis, Mo.).

**Purification of *T. pallidum*.** *T. pallidum* subsp. *pallidum* (Nichols strain) was propagated in New Zealand White rabbits as described elsewhere (42). All animal studies were approved by the local institutional review boards and conducted in accordance with standard accepted principles. Treponemes were purified to remove contaminating rabbit proteins by the protocol described by Hanff et al. (33). Briefly, treponemes were extracted in saline and subjected to two 400 × g spins to remove gross cellular debris, and 4 × 10<sup>8</sup> *T. pallidum* cells in a total volume of 10 ml were gently overlaid onto a 20-ml cushion of 43% Percoll (Sigma). Treponemes were purified by density centrifugation at 34,800 × g for 30 min, followed by removal of the visible treponeme band and resuspension in 25 ml of saline. The treponemes were subsequently separated from the Percoll by centrifugation at 100,000 × g for 1 h. Percoll-purified treponemes were shown by dark-field microscopy to be viable and fully motile.

**Treponemal adherence assay.** Adherence assays were performed on the basis of previously published protocols (20, 53, 54, 64, 66). Briefly, 4 μg of attachment matrices, including fibronectin, collagens I and IV, fibrinogen, laminin, and the attachment-negative control proteins fetuin and BSA, were coated in phosphate-buffered saline (PBS) on Lab-Tek II chamber slides (Nunc, Rochester, N.Y.) by incubation for 16 h at room temperature. After being washed with PBS, slides were blocked for 2 h with 3% BSA, washed a second time, and then incubated

for 2 h at 34°C under anaerobic conditions with 10<sup>8</sup> Percoll-purified *T. pallidum* cells in saline. After gentle washing with saline (10 times for 5 min each time), the attached spirochetes were visualized by dark-field microscopy and quantitative attachment was determined by calculating the number of attached treponemes per field. The assay was blinded, and a total of six fields were read for each attachment condition. Statistical analyses were performed by using the Student two-tailed *t* test.

**Computer analyses.** Computer analyses were performed on the published *T. pallidum* genome (22) to identify putative outer membrane proteins. Such proteins have the potential to reside on the bacterial surface and thus are the most likely to possess cellular functions such as host cell attachment. *T. pallidum* open reading frames (ORFs) were retrieved from the sexually transmitted disease sequence database (<http://stdgen.lanl.gov/stdgen/>) and used for sequence analyses. The PSORT program (49) (<http://psort.nibb.ac.jp>) was used to predict the cellular localization of proteins encoded by the ORFs. Briefly stated, PSORT assigns a cellular localization based on the presence or absence of a signal sequence; the nature of the signal sequence, if one is present (i.e., cleavable or uncleavable); the presence or absence of hydrophobic transmembrane segments; and the presence of characteristic amino acid compositions (49) (<http://psort.nibb.ac.jp/helpwww.html>). By using these criteria, PSORT correctly classifies 83% of gram-negative bacterial proteins (*n* = 106) into one of the four possible localization sites (outer membrane, inner membrane, periplasmic space, cytoplasm) (<http://psort.nibb.ac.jp/helpwww.html>).

*T. pallidum* translated ORFs with a greater than 50% calculated likelihood of outer membrane localization, as identified through PSORT analysis, were subjected to additional sequence analyses. These included screening of the ORFs against the 3D-PSSM database (36) (<http://www.sbg.bio.ic.ac.uk/~3dpssm/>) to identify homologous proteins with known tertiary structures, as well as the PROSITE database (<http://www2.ebi.ac.uk/ppsearch>) for identification of characteristic outer membrane protein motifs.

**Recombinant expression.** ORFs encoding computer-predicted putative outer membrane proteins were PCR amplified from *T. pallidum* subsp. *pallidum* (Nichols strain) genomic DNA by using primers designed from the coding sequence of each gene in Table 1. To ensure optimal expression of each recombinant molecule within *E. coli*, DNA sequences encoding predicted N-terminal signal sequences were excluded from the primer design and, as a result, from the expressed recombinant proteins. The positive control laminin-binding protein (SwissProt accession number 069174) was amplified from *Staphylococcus aureus* genomic DNA by using the primers listed in Table 1. Following amplification, PCR products (except Tp0326 and Tp0856 [see below]) were digested with *Bam*HI and either *Hind*III (positive control laminin-binding protein) or *Eco*RI (all other products), ligated to a similarly digested pRSETc T7 expression vector (Invitrogen, Carlsbad, Calif.), and transformed first into *E. coli* XL-1 Blue and then into *E. coli* expression strain BL21(DE3)/pLysS. ORFs Tp0326 and Tp0856 were directly cloned into the pBAD TOPO TA expression vector and expressed in *E. coli* strain TOP10 (both from Invitrogen). The sequence and reading frame of the expression constructs were verified by DNA sequencing with vector-specific primers, as well as internal primers based on each of the ORFs. Expression of the constructs and purification of the resulting six-histidine-tagged recombinant proteins were performed as previously described (6). The expressed

proteins were renatured by dialysis on the basis of the renaturation protocol described by Qi et al. (57) by using the dialysis modification described by Zhang et al. (80). This procedure has been shown to produce recombinant proteins that closely resemble native *T. pallidum* proteins (80). Briefly, the detergent Zwittergent 3-12 (Calbiochem, San Diego, Calif.) was added to the expressed proteins to a final concentration of 0.5% prior to dialysis against 100 mM Tris (pH 8.0)–200 mM NaCl–10 mM EDTA. Quantitation of each of the recombinant proteins was performed with the BCA Protein Assay kit (Pierce, Rockford, Ill.).

**Laminin-binding adherence assays.** To test for adherence of the recombinant proteins to laminin, enzyme-linked immunosorbent assay (ELISA)-based assays were performed. ELISA plates (Nunc-Immuno Plate MaxiSorp Surface; Nalge Nunc International, Rochester, N.Y.) were coated with 0.5  $\mu$ g of either laminin or the negative control protein BSA in PBS and incubated for 1.5 h at 37°C. The specificity profile of the laminin-binding recombinant protein was determined by coating with the ECM components collagens I and IV and the negative control proteins fetuin and BSA under identical conditions. To test for the dose-dependent attachment of laminin-binding recombinant proteins to laminin, wells were coated with 100  $\mu$ l of various laminin concentrations ranging from 0 to 5  $\mu$ g/ml in PBS. Wells were washed three times with PBS–0.05% Tween 20 (PBST) and blocked for 30 min at 37°C with 1% BSA, followed by addition of either the recombinant *T. pallidum* proteins or the positive control laminin-binding protein from *S. aureus*. For the adherence assays, 100  $\mu$ l of the recombinant proteins was added per well at a constant concentration of 20  $\mu$ g/ml in PBS, while for determination of the dose-dependent binding of Tp0751 to immobilized laminin, a range of recombinant Tp0751 concentrations of 0 to 5,000 nM in PBS was used. After incubation for 1.5 h at 37°C, wells were washed six times with PBST. Adherent recombinant proteins were detected with nickel-labeled horseradish peroxidase (Ni-HRP; Kirkegaard & Perry Laboratories, Gaithersburg, Md.) in accordance with the manufacturer's instructions. Briefly, wells were treated for 5 min at room temperature with 2% sucrose and dried for 1 h at 37°C. A 1:2,000 dilution of Ni-HRP in 1% BSA was added to the wells, and the plate was incubated for 30 min at room temperature. Wells were washed six times with PBST and developed with the TMB peroxidase substrate system (Kirkegaard & Perry Laboratories). The plates were read at 600 nm with an ELISA plate reader (Bio-Tek Instruments, Winooski, Vt.). Statistical analyses were performed with the Student two-tailed *t* test.

**Laminin chemical oxidation.** Chemical oxidation of laminin was performed with sodium metaperiodate as previously described (78). Briefly, ELISA plates were coated with 0.5  $\mu$ g of laminin in 50 mM sodium acetate buffer, pH 5.0, and incubated for 1.5 h at 37°C. Wells were washed three times with 50 mM sodium acetate buffer, pH 5.0, prior to exposure to various concentrations of periodate (0 to 100 mM) for 1 h at room temperature in the dark. Wells were washed three times with 50 mM sodium acetate buffer, pH 5.0, and blocked for 30 min at 37°C with 1% BSA. To assess the attachment of the recombinant laminin-binding protein to periodate-treated laminin, 100  $\mu$ l of the recombinant protein was added to the wells at a constant concentration of 20  $\mu$ g/ml in PBS. After incubation for 1.5 h at 37°C, wells were washed six times with PBST and developed with a 1:2,000 dilution of Ni-HRP as outlined above.

**Syphilis serum reactivity.** The reactivity of human and rabbit syphilitic serum samples against the *T. pallidum* recombinant protein was tested with an ELISA-based assay. Human serum samples (kindly provided by Bruno Schmidt, Ludwig Boltzmann Institut für Dermato-Venerologische Serodiagnostik, Vienna, Austria) were collected from individuals with confirmed syphilis infections and corresponded to various stages of infection, including primary syphilis, secondary syphilis, early latent infection, and neurosyphilis ( $n = 43$ ). Uninfected human serum samples ( $n = 15$ ) were obtained from laboratory personnel in Seattle, Wash. Sequential rabbit serum samples were collected at days 3 through 90 postinfection from New Zealand White rabbits that had been infected with *T. pallidum* subsp. *pallidum* (Nichols strain) as described elsewhere (42). Briefly, ELISA plates were coated at 4°C with the *T. pallidum* recombinant protein at a concentration of 4  $\mu$ g/ml in PBS and incubated overnight. Wells were blocked for 2.5 h at room temperature with 4% milk powder–0.2% Triton X-100 and washed four times with Tris-buffered saline with 0.05% Tween 20 (TTBS). Wells were incubated with a 1:50 dilution of either human or rabbit syphilitic serum (1:50 dilution in blocking reagent), washed four times with TTBS, and incubated for 1 h at room temperature with a 1:2,000 dilution of either goat anti-human IgG ( $\gamma$  chain specific) F(ab')<sub>2</sub>-peroxidase or goat anti-rabbit IgG (whole molecule) F(ab')<sub>2</sub> fragment-peroxidase (both from Sigma). Wells were washed four times with TTBS and developed with the TMB peroxidase substrate system. Optical densities were read at 600 nm with an ELISA plate reader. For statistical analyses, the cutoff for assignment of a negative or positive result was standardized from the absorbance values obtained from the 15 uninfected control serum samples. Negative serum samples were defined as those that yielded absorbance

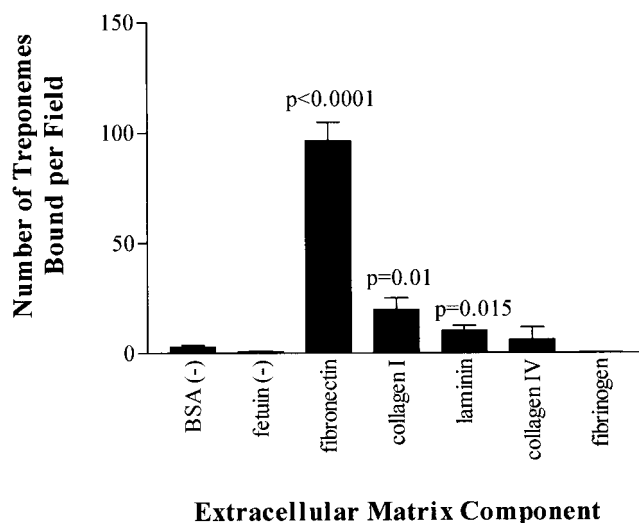


FIG. 1. *T. pallidum* attachment to various ECM components. Each bar represents the average number of treponemes bound  $\pm$  the standard deviation for six microscopic fields, and the results are representative of three independent experiments. For statistical analyses, attachment to each ECM component was compared to attachment to the negative control BSA by the Student two-tailed *t* test.

values less than the mean plus two times the standard deviation of the absorbance values of the uninfected serum samples, while positive serum samples were defined as those that gave absorbance values greater than this cutoff. All human studies were approved by the local institutional review boards and conducted in accordance with standard accepted principles.

## RESULTS

**Analysis of *T. pallidum* attachment to ECM components.** To confirm and extend previous studies investigating the attachment of *T. pallidum* to ECM components (1, 20, 34, 53, 54, 64–66, 75), attachment assays were performed with purified *T. pallidum* preparations and various ECM components. For these studies, *T. pallidum* was purified by Percoll density centrifugation (33) to minimize contaminating rabbit proteins. Treponemes purified in this manner are viable and motile and have been demonstrated by Hanff et al. (33) to remain fully virulent. This purification step was performed because treponemes freshly harvested from rabbit testicles are extensively coated with host proteins that may themselves contribute to ECM attachment. By use of a purified source of *T. pallidum*, the contribution of treponemal proteins to ECM attachment can be more accurately determined.

As demonstrated in Fig. 1, high numbers of purified *T. pallidum* bacteria were attached to fibronectin-coated surfaces ( $P < 0.0001$ ). In addition, statistically significant numbers of organisms were attached to collagen I ( $P = 0.01$ ) and laminin ( $P = 0.015$ ). No significant level of attachment to the other two ECM components tested, collagen IV ( $P = 0.5663$ ) and fibrinogen ( $P = 0.1574$ ), or to the attachment-negative control proteins fetuin and BSA was observed. These results suggest that *T. pallidum* possesses adhesins that bind specifically to fibronectin, collagen I, and laminin. The identities of the treponemal adhesins that mediate binding to the ECM components fibronectin and collagen I are the focus of separate studies.



TABLE 2. Recombinant expression of the *T. pallidum* ORFs

<i>T. pallidum</i> ORF	PSORT outer membrane prediction <sup>a</sup>	Portion(s) of ORF expressed	
		Amino acid residues	Fragment of protein
0155	86	46–187 162–371	N terminal C terminal
0316 (TprF)	93	16–369	Internal
0326 (Tp92)	85	22–837	Full length
0453	78	30–287	Full length
0483	69	179–374	C terminal
0557	72	26–237	Full length
0620 (TprI)	92	259–469	Internal
0751	92	54–173	N terminal
0856	94	33–394	Full length
0952	87	34–199 166–345	N terminal C terminal

<sup>a</sup> Percent probability that the protein resides in the outer membrane.

The present investigation focused on the identification of the *T. pallidum* adhesin(s) that mediates attachment to the ECM component laminin.

**Computer prediction of potential *T. pallidum* adhesins.** The release of the *T. pallidum* genome sequence (22) has provided readily accessible information on the complete repertoire of ORFs contained within the genome. To identify potential *T. pallidum* adhesins, computer analyses of the published *T. pallidum* genome (<http://www.stdgen.lanl.gov/stdgen/>) were performed to identify ORFs predicted to encode outer membrane proteins. Such proteins may reside on the bacterial surface and thus are most likely to possess cellular functions such as host cell attachment. These analyses identified 27 *T. pallidum* ORFs that have the potential to encode outer membrane proteins and thus may encode proteins involved in host attachment. Ten ORFs were given priority for expression on the basis of either their strong PSORT predictions for potential outer membrane location ( $\geq 69\%$ ; Table 2), the predicted structural similarity of these proteins to known  $\beta$ -barrel-containing proteins (3D-PSSM analysis), or the presence of motifs characteristic of outer membrane proteins (PROSITE analysis).

**Recombinant expression.** As shown in Table 2, the selected 10 of the 27 *T. pallidum* ORFs encoding potential outer membrane proteins have been expressed as recombinant proteins in *E. coli*. Where possible, the entire ORF was expressed; however, in many cases, toxicity prevented expression of the full-length ORF and, instead, fragments were expressed as outlined in Table 2. In addition, a laminin-binding protein from *S. aureus* was expressed in parallel as a positive control. The expressed proteins (except TprF and TprI) were renatured by dialysis.

**Laminin-binding adherence assay.** The recombinant *S. aureus* laminin-binding protein and the 10 *T. pallidum* ORFs expressed as recombinant proteins were examined for the abil-

ity to bind to laminin with an ELISA-based assay. As observed in Fig. 2A, the *S. aureus* laminin-binding protein demonstrated a significant level of attachment to laminin ( $P < 0.0001$ ). Of the panel of expressed *T. pallidum* ORFs tested for attachment to laminin, one protein, Tp0751, exhibited a significant degree of laminin binding ( $P < 0.0001$ ). In each case, the statistical significance of attachment was determined by comparison with the ability of the recombinant protein to attach to BSA. As shown in Fig. 2B, in all cases, the panel of expressed proteins exhibited minimal binding to BSA.

**Specificity of recombinant Tp0751 attachment to laminin.** The attachment of recombinant *T. pallidum* protein Tp0751 to laminin was investigated in several experiments. The specificity profile of Tp0751 was determined by testing attachment to the ECM components laminin and collagens I and IV and the negative control proteins fetuin and BSA. As shown in Fig. 3, Tp0751 showed a statistically significant level of attachment to laminin ( $P < 0.0001$ ) but did not demonstrate significant attachment to either collagen I, collagen IV, fetuin, or BSA. These results demonstrate the attachment specificity of Tp0751 for laminin. To further investigate the Tp0751-laminin interaction, ELISA analyses were performed to assess the binding of recombinant Tp0751 to immobilized laminin as a function of both varying laminin concentrations and varying recombinant Tp0751 concentrations. As shown in Fig. 4A, Tp0751 bound to increasing concentrations of laminin in a dose-dependent, saturable manner, compared to the minimal level of laminin attachment observed for the negative control expressed *T. pallidum* ORF, Tp0557. The positive control *S. aureus* laminin-binding protein also bound to laminin at a consistently higher level than the negative control recombinant protein. Further, the saturated nature of the positive control binding curve suggests that the dose-dependent attachment of this recombinant protein to laminin is outside the laminin concentration range used for optimal attachment of Tp0751. In addition, as shown in Fig. 4B, increasing concentrations of recombinant Tp0751 bound to immobilized laminin in a dose-dependent, saturable manner.

**Contribution of carbohydrate moieties to Tp0751-laminin attachment.** The oxidative effect of sodium metaperiodate was exploited to determine the contribution of carbohydrate moieties to the interaction of laminin with recombinant Tp0751. Sodium metaperiodate treatment of laminin was conducted under mild conditions previously shown to cleave carbohydrate vicinal hydroxyl groups without altering the polypeptide chain structure (78). As shown in Fig. 5, recombinant Tp0751 exhibited decreased attachment to metaperiodate-treated laminin. Attachment decreased in a statistically significant manner as a function of increasing sodium metaperiodate concentration (Fig. 5), with a reduction of 73% of Tp0751 attachment observed at the highest metaperiodate concentration (100 mM). These results suggest that laminin carbohydrate groups play a role in the interaction of Tp0751 with laminin.

**Syphilis serum reactivity to recombinant Tp0751.** The antibody reactivity of serum samples collected from syphilis patients and experimentally infected rabbits against recombinant Tp0751 was determined by ELISA analysis. Sequential rabbit serum samples collected from experimentally infected rabbits demonstrated reactivity against recombinant Tp0751 (Fig. 6A). Reactivity first appeared at day 10 postinfection, and consis-

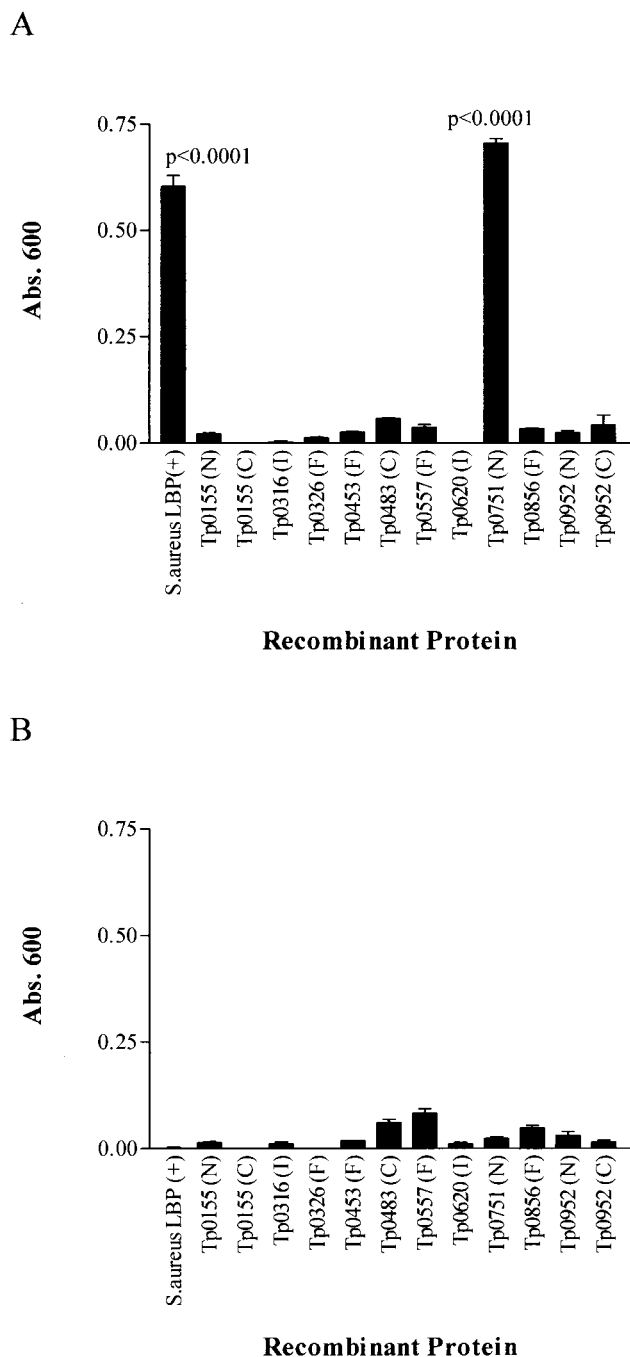


FIG. 2. Binding of recombinant proteins to human laminin (A) and BSA (B). Each bar represents the mean absorbance (Abs.) value at 600 nm ± the standard deviation for three wells, and the results are representative of three independent experiments. For statistical analyses, the attachment of each of the recombinant proteins to laminin was compared to the attachment of the protein to BSA by the two-tailed *t* test. LBP, laminin-binding protein; F, full-length protein; N, N-terminal fragment; C, C-terminal fragment; I, internal fragment.

tent levels of antibody reactivity were observed through day 90 postinfection. Similarly, serum samples from individuals with syphilis infections (*n* = 43) and a control group of uninfected individuals (*n* = 15) were analyzed for reactivity against re-

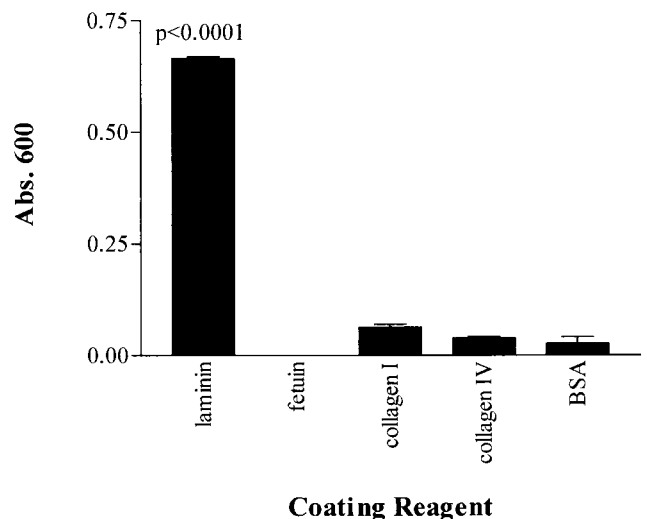


FIG. 3. Specificity profile of attachment of recombinant Tp0751 to various coating reagents. Each bar represents the mean absorbance (Abs.) value at 600 nm ± the standard deviation for three wells, and the results are representative of three independent experiments. For statistical analyses, attachment to each component was compared to attachment to the negative control BSA by the Student two-tailed *t* test.

combinant Tp0751. While none of the serum samples from the control group of uninfected individuals demonstrated reactivity to Tp0751 (data not shown), serum samples from 42% of syphilis patients demonstrated Tp0751 reactivity (Fig. 6B). The reactivity did not appear to correlate with the stage of infection at which serum was collected (Fig. 6B). The presence of antibodies against Tp0751 in serum samples collected from both experimental and natural syphilis infections is indicative of expression of the protein in vivo during the course of infection.

DISCUSSION

The critical first step in the establishment of infection by pathogenic bacteria is attachment to host components. Laminins are major constituents of basement membranes and, as such, are widely distributed throughout the host. Specifically, laminins are associated with basement membranes that underlie epithelial and endothelial cell layers and surround muscle cells and peripheral nerves (69). Attachment to the ECM component laminin could be an important portal for the initiation of *T. pallidum* infection, since tissue injury that leads to the degradation of the epithelial layer would expose the laminin-rich basement membrane. In addition, *T. pallidum* attachment to laminin may also play a role in spirochetal dissemination and tissue invasion. In this respect, it is relevant that in infected tissues *T. pallidum* localizes to perivascular areas and that spirochetes have been shown by phase-contrast and scanning electron microscopy to attach to isolated basement membranes of retinal and kidney tissues (20).

Attachment to laminin has been suggested to play a role in the pathogenesis of a number of microorganisms, including various species of bacteria (14, 35, 50, 55, 62, 76), parasites (11, 25, 27, 39), and fungi (40, 46, 71). Previous investigations have studied the attachment of *T. pallidum* to several ECM compo-

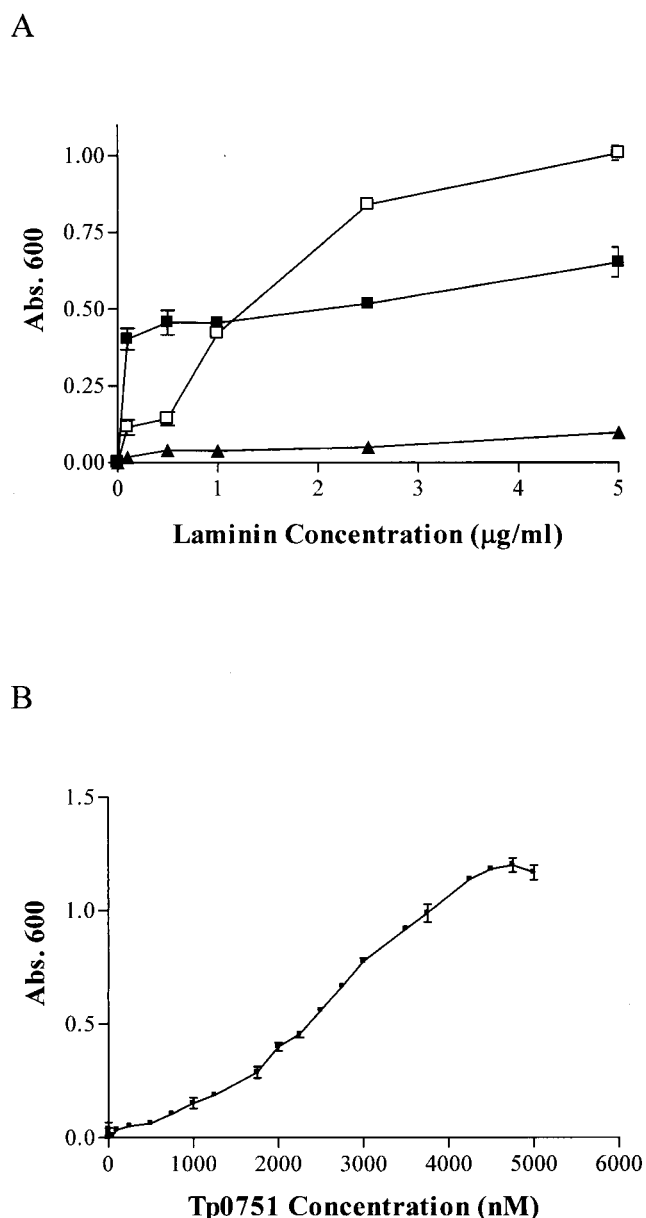


FIG. 4. Binding of recombinant Tp0751 (□) to immobilized laminin as a function of varying laminin concentrations (A) and varying Tp0751 concentrations (B). Each datum point represents the mean absorbance (Abs.) value at 600 nm  $\pm$  the standard deviation for three wells, and the results are representative of three independent experiments. Positive and negative control recombinant proteins tested in panel A were the *S. aureus* laminin-binding protein (■) and *T. pallidum* ORF Tp0557 (▲), respectively.

nents, including laminin (20), but the identity of the spirochete molecule(s) mediating this attachment was not determined. In this report, we have identified *T. pallidum* ORF Tp0751 as encoding a protein that attaches to immobilized laminin. The binding of Tp0751 to laminin is characteristic of a specific receptor-ligand interaction: (i) it is dependent on the concentration of both laminin and Tp0751, (ii) it is saturable, and (iii) it is specific for laminin and is not observed with other highly glycosylated proteins.

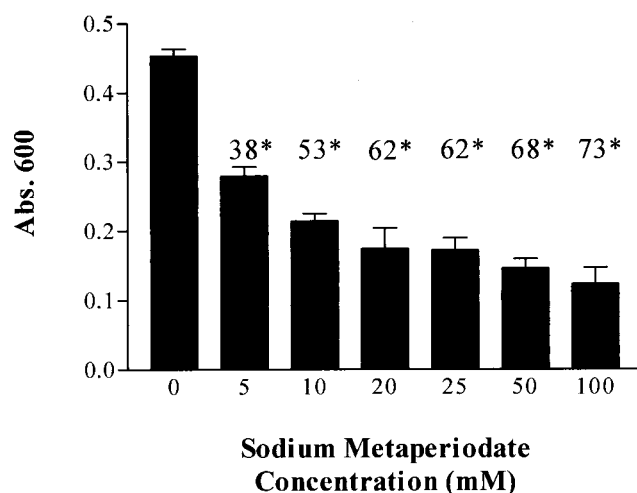


FIG. 5. Contribution of laminin carbohydrate moieties to recombinant Tp0751-laminin interaction. Shown is a bar graph representing Tp0751 attachment to untreated laminin (0 mM bar) and laminin treated with various concentrations of sodium metaperiodate (5 to 100 mM). Each bar represents the mean absorbance (Abs.) value at 600 nm  $\pm$  the standard deviation for three wells, and the results are representative of three independent experiments. Shown above each bar is the percent reduction in recombinant Tp0751 attachment to sodium metaperiodate-treated laminin as a function of the sodium metaperiodate concentration. The data are expressed as percent reduction in attachment compared to the level of attachment to untreated laminin. In each case (\*), the *P* value, as measured by the Student two-tailed *t* test, was  $\leq 0.0009$ .

Several reports have suggested, on the basis of immunological cross-reactivity, the evolutionary conservation of at least some sequences in both prokaryotic and eukaryotic laminin-binding proteins (41, 48). In general, however, laminin-binding proteins vary widely in molecular size and amino acid sequence, and to date, no consensus laminin-binding motif has been identified within the documented laminin adhesins. Along these lines, the Tp0751 sequence demonstrates no shared homology with sequences contained within the protein databases, including the predicted ORFs from the related spirochete *B. burgdorferi*. In addition, a search of the unfinished *T. denticola* genome (<http://tigrblast.tigr.org/ufmg/>) identified a low-specificity match to a translated ORF contained within the genome (16% identity, 29% similarity). These results suggest that Tp0751 may be unique to *Treponema*.

Laminin is a highly glycosylated molecule that possesses about 40 N-linked oligosaccharides with repeating units of poly-*N*-acetylglucosaminyl side chains attached to the trimannosyl core portion of bi-, tri-, and tetra-antennary complex-type oligosaccharides (2, 23, 37). These complicated N-linked oligosaccharides result in many different structures, some of which are unique to laminin (63). For the majority of pathogenic organisms that demonstrate attachment to laminin, glycosylation plays a key role in the laminin-pathogen interaction. Specifically, carbohydrate groups have been shown to be important for the interaction of laminin with *H. pylori* (76), *M. leprae* (61), *L. donovani* (27), *S. aureus* (7), *T. vaginalis* (11), and *E. coli* (62). In addition, the interaction of an  $\alpha 6/\beta 1$  integrin, designated gp120/140, with laminin is also dependent on N-linked oligosaccharides (9). Consistent with these findings,

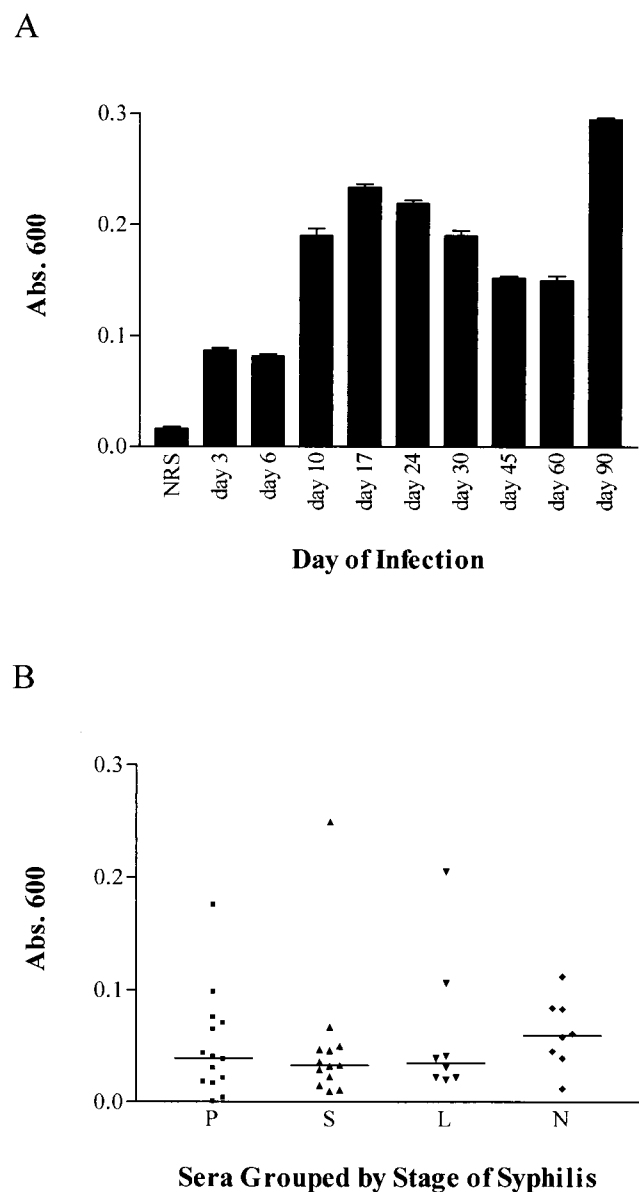


FIG. 6. Reactivity of syphilitic serum samples to recombinant Tp0751. (A) Reactivity of sequential rabbit serum samples collected from experimentally infected rabbits. The serum samples on the x axis represent pools of serum samples collected from rabbits infected with *T. pallidum* for the indicated time periods. NRS, normal rabbit serum. (B) Reactivity of human serum samples from different stages of syphilis: P, primary ( $n = 14$ ); S, secondary ( $n = 13$ ); L, latent ( $n = 8$ ); N, neurosyphilis ( $n = 8$ ). The overall mean absorbance (Abs.) of each group is represented by a horizontal line.

reduced attachment of Tp0751 to metaperiodate-treated laminin was observed, indicating that carbohydrate groups present on laminin are important for the Tp0751-laminin interaction. This finding is also consistent with previous investigations showing reduced attachment of *T. pallidum* to cultured mammalian cells pretreated with periodate (1).

Within the context of mammalian cells, the interaction of both integrin (28) and non-integrin (24) receptors with laminin has been correlated with the invasive ability of tumor cells. In

each case, the receptor-laminin interactions have been shown to result in signal transduction and subsequent synthesis and release of proteases by tumor cells. These proteases promote the invasion process of tumor cells by degrading ECM components. A similar scenario may exist for *T. pallidum*, in which attachment to laminin may induce the production of proteases and, in this way, increase the invasive capabilities of this pathogen. Consistent with this hypothesis are the observations that tissue degradation is a well-documented phenomenon associated with syphilis infection and that previous investigations have documented the morphological destruction of cultured cells upon attachment of *T. pallidum* (21). In addition, the related oral spirochete *T. denticola* has been shown to both attach to laminin (14, 32) and produce proteases that result in basement membrane degradation and treponemal invasion (29, 58, 73). Further, in *T. denticola* the protein identified as mediating attachment to laminin, the major surface protein (14, 32), has been shown to be closely associated in vivo with the chymotrypsin-like protease implicated in basement membrane degradation (13). The questions of whether a similar situation exists for *T. pallidum* and whether this correlation parallels the method of tumor invasiveness await further experimentation.

In conclusion, this report details the identification of a *T. pallidum* adhesin that exhibits specific attachment to laminin. Although the complete picture of *T. pallidum* attachment to host cells remains to be determined, it is likely that spirochete attachment and infection depend on complex interactions of several bacterial proteins with various ECM, and perhaps other host cell, components.

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