Heterologous Expression of the *Treponema pallidum* Laminin-Binding Adhesin Tp0751 in the Culturable Spirochete *Treponema phagedenis*

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*Treponema pallidum* subsp. *pallidum*, the causative agent of syphilis, is an unculturable, genetically intracellular bacterium. Here we report the use of the shuttle vector pKMR4PEMCS for the expression of a previously identified *T. pallidum* laminin-binding adhesin, Tp0751, in the nonadherent, culturable spirochete *Treponema phagedenis*. Heterologous expression of Tp0751 in *T. phagedenis* was confirmed via reverse transcriptase PCR analysis with tp0751 gene-specific primers and immunofluorescence analysis with Tp0751-specific antibodies; the latter assay verified the expression of the laminin-binding adhesin on the treponemal surface. Expression of Tp0751 within *T. phagedenis* was functionally confirmed via laminin attachment assays, in which heterologous Tp0751 expression conferred upon *T. phagedenis* the capacity to attach to laminin. Further, specific inhibition of the attachment of *T. phagedenis* heterologously expressing Tp0751 to laminin was achieved by using purified antibodies raised against recombinant *T. pallidum* Tp0751. This is the first report of heterologous expression of a gene from an unculturable treponeme in *T. phagedenis*. This novel methodology will significantly advance the field of syphilis research by allowing targeted investigations of *T. pallidum* proteins purported to play a role in pathogenesis, and specifically host cell attachment, in the nonadherent spirochete *T. phagedenis*.

Syphilis is a chronic sexually transmitted disease caused by infection with the spirochete bacterium *Treponema pallidum* subsp. *pallidum*. An estimated 12 million new syphilis cases occur globally each year, the majority of which are in developing nations (20). In recent years, there has been a resurgence of syphilis in many developed countries, particularly among populations of men who have sex with men, with notable outbreaks spreading rapidly across North America, Europe, and Australia (1, 8, 11, 25, 44). Existing public health measures are not successfully controlling the spread of this disease, and alternative means of syphilis prevention are needed. In particular, in order to design an effective syphilis vaccine it is necessary to gain a deeper understanding of the pathogenic mechanisms used by *T. pallidum* to establish and sustain infection.

At the molecular level, little is currently known about *T. pallidum* pathogenesis. The bacterium gains entry to the host through intact mucosal barriers or microscopic epidermal abrasions (42). The organism is highly invasive, with in vitro studies showing that *T. pallidum* is able to penetrate endothelial cell monolayers to enter the bloodstream (45, 50) within hours of infection (13, 43). Treponemal invasion results in widespread bacterial dissemination, which, in turn, sets the stage for establishment of chronic infection. One key factor that has severely hampered studies of the pathogenic mechanisms used by this important human pathogen is that it cannot be continuously cultured in vitro. Although limited multiplication has been achieved in an in vitro tissue culture system (12, 17, 32, 34–37), *T. pallidum* is a fastidious, obligate human pathogen for which intratesticular inoculation of rabbits is the only reliable method of bacterial propagation. This organism remains refractory to genetic manipulations, thus preventing direct investigation of the functions of individual gene products. To circumvent this issue, investigators in the field must use heterologous systems to express candidate genes that are hypothesized to be involved in *T. pallidum* pathogenesis or virulence. Successful expression of *T. pallidum* genes has been accomplished in *Escherichia coli* (9, 16, 22, 47, 49); however, the dissimilarity in outer membrane ultrastructure and physiology between the two bacteria has yielded limited functional data from these studies (23). Expression of *T. pallidum* genes has also been performed in the bacterium *Treponema denticola*, a cultivable spirochete found in subgingival plaques associated with periodontitis (10, 48). Although expression studies using this related treponeme are physiologically more relevant than those performed with *E. coli*, interpretation of functional studies is complicated by the pathogenic nature of *T. denticola*. Specifically, *T. denticola* attaches to host cells and cellular components (7, 14, 15, 21, 26, 40, 41), penetrates endothelial cell monolayers (41), and invades the gingival connective tissue in some forms of periodontal disease (18, 28, 31, 46). Therefore, expression within *T. denticola* of *T. pallidum* molecules that are predicted to be involved in adhesion or tissue invasion, and subsequent determination of the effects of these heterologously expressed virulence factors on the overall pathogenicity of *T. denticola*, is not straightforward.

*Treponema phagedenis*, another member of the *Treponema*
genus, is a strict anaerobe that does not attach to host cells or invade cell monolayers (7, 41). We hypothesized that the non-adherent, non-invasive phenotype of T. phagedenis, combined with its ease of cultivation, would make this treponeme an ideal candidate for heterologous expression of T. pallidum virulence factors and establishment as a model treponeme.

Further, T. phagedenis has a G+C ratio similar to that of T. denticola (39), and therefore it was predicted that a shuttle vector developed for use in T. denticola would also function in T. phagedenis.

Previously, we identified a T. pallidum adhesin, designated Tp0751, that attaches to the extracellular matrix protein laminin (3, 5), a major component of basement membranes that underlie endothelial cell layers (51). This adhesin is purported to be involved in treponeme dissemination and pathogenesis (3, 5), although direct evidence of the involvement of this adhesin in the treponemal invasion process is currently lacking due to the experimental limitations associated with research on T. pallidum. Here we report the expression of this T. pallidum laminin-binding protein on the surface of T. phagedenis. This methodology will advance the field of syphilis research by opening new possibilities for functional investigations of T. pallidum proteins involved in pathogenesis and will directly facilitate the investigation of T. pallidum adhesins within a nonadherent, related treponeme.

MATERIALS AND METHODS

Extracellular matrix proteins. Laminin isolated from the Engelbreth-Holm-Swarm murine sarcoma and the negative control protein fetuin were purchased from Sigma Chemical Co. (Oakville, Ontario, Canada).

Bacteria. T. pallidum subsp. pallidum (Nichols strain) was propagated in New Zealand White rabbits as described elsewhere (29). All animal studies were approved by the local Institutional Review Boards and conducted according to standard accepted principles. T. phagedenis biotype Kazan was grown in either TYGVs medium (38) or thiochololate broth (Sigma) supplemented with 20% heat-inactivated rabbit serum (R-7136; Sigma) in the presence of 10 μg/ml rifampin and 40 μg/ml erythromycin (the latter antibiotic was added to all cultures except wild-type T. phagedenis) (Sigma) at 37°C in an anaerobic chamber (Oxoid, Hampshire, United Kingdom). The identity of T. phagedenis was confirmed by 16S rRNA gene sequencing with the primers 5′-CACACCCCGGGCTCACCC-3′ and 5′-GCTGACTACTGGCGTGCAAGGAAGCGTC (SalI site underlined). Transformants of T. phagedenis subsp. pallidum (Nichols strain) genomic DNA with the primers 5′-GTCGACTACTGGCGTGCAAGGAAGCGTC (SalI site underlined) and 5′-AGATCTTCAGGGCGAAGGAGCACTAG (BglII site underlined). Following amplification, the PCR product was digested with SalI and BglII and ligated to a similarly digested pKMR4PEMCs shuttle vector. This vector is related to the previously reported pKMR4PE shuttle vector (10) but is more versatile with the introduction of a multiple cloning site (MCS). The map of the shuttle vector and resulting Tp0751/pKMR4PEMCs construct is illustrated in Fig. 1. The construction was transformed into E. coli Top10 (Invitrogen), and the sequence of the Tp0751 ORF was verified by DNA sequencing with pKMR4PEMCs vector-specific primers, as well as internal primers designed from the Tp0751 ORF. A large-scale DNA isolation was performed with the Qiagen Miniprep kit (Qiagen, Valencia, CA), and the purified DNA was used to transform T. phagedenis by the electroporation protocol previously reported for the transformation of T. denticola (10, 27). Briefly, ≥ 108 freshly prepared competent T. phagedenis cells were mixed with 2 μg of the plasmid preparations on ice in a 0.1-mm electroporation cuvette. Electroporation was accomplished with a Gene Pulser (Bio-Rad Laboratories, Melville, NY) set at 1.8 kV, 25 μF, and 200 Ω, producing a time constant of 4 to 4.5 μs, after which 1 ml of TYGVs medium was added and the cells were transferred to a culture tube and incubated overnight. All culturing was performed at 37°C under anaerobic conditions. Transformants were selected on TYGVs plates supplemented with 0.3% SeaPlaque agarose (FMC Bioproducts, Rockland, ME) and 40 μg/ml erythromycin. Transformants appeared within 7 to 10 days at an efficiency of approximately 1 colony/μg of plasmid DNA. Individual colonies were inoculated into TYGVs broth and grown to mid-logarithmic phase, and plasmid DNA was isolated with a Qiagen miniprep kit to verify plasmid presence; in all cases, the plasmid was observed. Transformants of T. phagedenis containing the pKMR4PEMCs plasmid alone or the Tp0751/pKMR4PEMCs construct displayed growth profiles and generation times similar to those of wild-type T. phagedenis.

RNA isolation and RT-PCR analysis. T. phagedenis transformants were freshly harvested by centrifugation. RNA was extracted with the TRIZOL reagent (Invitrogen) and the Fastprep system (Krackeler Scientific Inc., NY) and treated with DNase I (Sigma). T. phagedenis first-strand cDNA was synthesized from 1 μg total RNA with SuperScript III reverse transcriptase (RT; Invitrogen) using a specific primer complementary to the 3′ end of the Tp0751 gene (5′-TCAAGGCCAAAGGAGCACTAG-3′). Specific oligonucleotide primer sets were designed to amplify a 700-bp internal region of the Tp0751 gene (5′-TACTCGGTCGAAGAAGGCT-3′ and 5′-GTGATATCCTGTTATGCATACCGG-3′) and RT-PCR was performed with 2 μl of the cDNA and a 0.5 μM concentration of the oligonucleotide primer sets established by techniques (30). The RT-PCR was accompanied by positive and negative control reactions that used the same set of primers and the Tp0751/pKMR4PEMCs plasmid and CDNA synthesized from a control pKMR4PEMCs/T. phagedenis transformant, respectively. To confirm the absence of residual DNA in each RNA sample, additional negative control reactions were performed in which T. phagedenis transformant RNA samples were used as templates without the RT step. For analysis, 1 μg of each of the RT-PCR products, or total RNA for the control reactions, was separated on a 1.5% agarose gel and visualized by ethidium bromide staining.

Immunofluorescence studies. Twenty-five microliters of mid-logarithmic growth phase culture of either wild-type T. phagedenis or T. phagedenis transformed with pKMR4PEMCs or Tp0751/pKMR4PEMCs (approximately 2 × 1010 bacteria) was used to coat, in duplicate, polyl-L-lysine-coated 10-well, 5-mm microscope slides (Takeda Incorporated, Myakka City, FL) for 1 h at room temperature. Wells were washed with saline three times for 5 min each by immersion of the slide in a Coplin jar (Tekton Incorporated, Myakka City, FL) and gentle agitation at 50 rpm. To reduce background fluorescence levels, polyclonal Tp0751-specific IgY antibodies were preadsorbed with a T. phagedenis lysate as follows. Ten milliliters of mid-logarithmic growth phase wild-type T. phagedenis culture was harvested by centrifugation at 9,300 × g for 10 min; the pellet was resuspended in PBS containing 1

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mg/ml lysozyme, 2 μg/ml aprotinin, 0.2 μg/ml pepstatin A, and 100 μg/ml phenylmethylsulfonyl fluoride; and the mixture was incubated for 30 min on ice, followed by sonication on ice (three 30-s bursts). A 1:12.5 dilution of the polyclonal Tp0751-specific IgY antibodies was adsorbed overnight at 4°C with 10% (vol/vol) of the T. phagedenis lysate, samples were centrifuged at 12,000 × g for 10 min at 4°C, and 25 μl of the preadsorbed antibodies was diluted to 1,800 μl and added to the wells. To rule out the possibility that detected fluorescence was due to a breach in the integrity of the T. phagedenis outer membrane, control slides were included that consisted of a 1:100 dilution of polyclonal Tp0249-specific antibodies (directed against the T. pullulum periplasmic flagellar protein FhaA) incubated with wells coated with either wild-type T. phagedenis (negative control) or wild-type T. phagedenis that had first been subjected to a membrane permeabilization step by incubation for 1 h with 25 μl of 2% Triton X-100 (positive control). All antibody incubation steps were carried out for 1 h at room temperature. Negative control wells consisting of each construct with no primary antibody were also included. After washing four times with saline, 25 μl of a 1:500 dilution of fluorescein-labeled goat-anti-chicken IgY (Aves Labs, Inc.) was added to the wells and incubated in the dark for 1 h. All antibody incubation steps were carried out for 1 h at room temperature. Negative control wells consisting of each construct with no primary antibody were also included. After washing four times with saline, 25 μl of a 1:500 dilution of fluorescein-labeled goat-anti-chicken IgY (Aves Labs, Inc.) was added to the wells and incubated in the dark for 1 h. After one wash with saline, 30 μl of 250 μg/ml 4',6'-diamidino-2-phenylindole (DAPI) dilactate (Invitrogen) was added to each well, and the attached spirochetes were visualized by dark-field microscopy. Quadruplicate assays were performed, the assays were blinded, and in each case the average number of attached treponemes per field was calculated by reading a total of eight fields for each attachment condition. Statistical analyses were performed with the two-tailed Student t test.

RESULTS

Preparation of the tp0751/pKMR4PEMCS expression construct. Previous investigations used the E. coli-T. denticola
shuttle vector pKMR4PE to successfully express the *T. pallidum* flagellar gene *flaA* in *T. denticola* (10). To extend the usefulness of this vector, an MCS was introduced to create the shuttle vector pKMR4PEMCS, as shown in Fig. 1. The MCS was placed downstream of the erythromycin resistance (Em') gene cassette, which was, in turn, located downstream of the constitutively expressed *T. denticola* prtB promoter (2). In previous studies, this genetic organization resulted in the favorable expression of *flaA* in *T. denticola* (10) with no major polar effects observed downstream of the Em' gene cassette (24). The entire tp0751 ORF, including the putative signal sequence, and upstream ribosome-binding site were PCR amplified and cloned into the MCS of the pKMR4PEMCS shuttle vector by using the SalI and BglII sites, as illustrated in Fig. 1, to create the construct tp0751/pKMR4PEMCS.

**Expression of T. pallidum tp0751 in T. phagedenis.** *T. phagedenis* was transformed with either the tp0751/pKMR4PEMCS construct or the shuttle vector alone by the electroporation protocol developed for *T. denticola* (10). Transformants harboring the correct chimeric plasmid were verified by antibiotic selection and DNA sequencing. RT-PCR analysis of total RNA extracted from *T. phagedenis* transformed with the tp0751/pKMR4PEMCS construct revealed the presence of an mRNA transcript corresponding to the tp0751 gene (Fig. 2), thus demonstrating that the heterologous *T. pallidum* tp0751 gene was successfully transcribed in *T. phagedenis*. To confirm the expression of Tp0751 on the *T. phagedenis* surface, immunofluorescence assays were performed. In these assays, polyclonal antibodies raised against recombinant *T. pallidum* Tp0751 were reacted with intact wild-type *T. phagedenis* or *T. phagedenis* transformed with either the tp0751/pKMR4PEMCS construct or the pKMR4PEMCS shuttle vector alone. Control antibodies included preimmune IgY antibodies, as well as antibodies raised against the periplasmic *T. pallidum* FlaA sheath protein (designated Tp0249) (19) which exhibit cross-reactivity.
with *T. phagedenis* biotype Kazan (33). As shown in Fig. 3, immunofluorescence was observed with antibodies against the periplasmic flagellar protein Tp0249 incubated with wild-type *T. phagedenis* whose outer membrane had been permeabilized by treatment with the detergent Triton X-100 (Fig. 3B), while no fluorescence was observed with Tp0249-specific antibodies on nonpermeabilized wild-type *T. phagedenis* (Fig. 3D), thus confirming the maintenance of treponemal structural integrity during experimentation. Importantly, Tp0751-specific antibodies demonstrated fluorescence with nonpermeabilized, intact *T. phagedenis* transformed with tp0751/pKMR4PEMCS (Fig. 3F), while no fluorescence was observed with Tp0751-specific antibodies incubated with either *T. phagedenis* transformed with the shuttle vector alone (Fig. 3H) or with wild-type *T. phagedenis* (Fig. 3J). Further, analysis of fluorescent *T. phagedenis* expressing Tp0751 by DAPI staining confirmed that all of the treponemes present displayed fluorescence (compare Fig. 3E and F). These results definitively demonstrate successful heterologous expression of the *T. pallidum* Tp0751 protein on the surface of *T. phagedenis*.

**Laminin attachment assays.** To investigate the laminin attachment capacity of *T. phagedenis* transformed with the tp0751/pKMR4PEMCS construct, laminin attachment assays were performed with laminin-coated or fetuin-coated (negative control) chamber slides. As shown in Fig. 4A, *T. phagedenis* transformed with tp0751/pKMR4PEMCS demonstrated attachment to laminin (frame vi), while wild-type *T. phagedenis* (frame ii) and *T. phagedenis* transformed with the shuttle plasmid alone (frame iv) exhibited only minimal levels of background attachment to laminin. Further, neither wild-type *T. phagedenis* (frame i), *T. phagedenis* transformed with the shuttle vector alone (frame iii), nor *T. phagedenis* transformed with the tp0751/pKMR4PEMCS construct (frame v) attached to the highly glycosylated negative control protein fetuin. Figure 4B reports the quantitation of the number of treponemes attached per field to each of the coated surfaces. *T. phagedenis* transformed with tp0751/pKMR4PEMCS demonstrated an 8-fold increase in attachment to laminin over the attachment of wild-type *T. phagedenis*, an 8-fold increase in attachment to laminin over that of *T. phagedenis* transformed with the shuttle vector alone, and a 52-fold increase in attachment to laminin compared to the attachment to fetuin. To further define the laminin attachment potential of *T. phagedenis* transformed with the tp0751/pKMR4PEMCS construct, and to verify that this observed attachment capacity was conferred by heterologous expression of Tp0751, attachment inhibition experiments were performed. As shown in Fig. 4C, a 7-fold inhibition of the attachment of *T. phagedenis* expressing Tp0751 to laminin was observed upon bacterial pretreatment with Tp0751-specific polyclonal antibodies, compared to the 1.8-fold and 2.3-fold inhibitions observed for bacteria pretreated with the control preimmune and Tp0249-specific IgY antibodies, respectively. The minimal level of inhibition observed with the control antibodies likely represents cross-reactive chicken antibodies that bind to *T. phagedenis*, thereby providing a small degree of steric hindrance and thus a low level of inhibition of attachment.

**FIG. 4.** Attachment of wild-type and transformed *T. phagedenis* to laminin and fetuin (negative control). (A) Wild-type (WT) *T. phagedenis* (i and ii), *T. phagedenis* transformed with the pKMR4PEMCS shuttle vector (iii and iv), and *T. phagedenis* transformed with the tp0751/pKMR4PEMCS shuttle vector (v and vi) were incubated with either fetuin-coated negative control slides (i, iii, and v) or laminin-coated slides (ii, iv, and vi), and spirochetes were visualized by dark-field microscopy with a Nikon Eclipse E600 microscope. (B) Quantitation of *T. phagedenis* attachment. Statistical analyses compared the level of attachment of each *T. phagedenis* construct to laminin with that of attachment to fetuin by the two-tailed Student *t* test (*, *P* < 0.0001). (C) Quantitation of attachment of *T. phagedenis* transformed with the tp0751/pKMR4PEMCS shuttle vector to laminin-coated slides following no pretreatment or pretreatment of bacteria with either preimmune IgY antibodies or polyclonal Tp0249-specific IgY antibodies (negative controls) or with polyclonal Tp0751-specific IgY antibodies. Statistical analyses compared the level of attachment of nonpretreated bacteria to that of bacteria pretreated with Tp0751-specific IgY antibodies by the two-tailed Student *t* test (*, *P* < 0.0001).
DISCUSSION

The results presented herein extend the usefulness of the pKMR4PEMCS shuttle vector, previously used for the expression of heterologous genes in *T. denticola* (10), to the expression of such genes in the nonadherent treponeme *T. phagedenis*, a significant advancement in the field of heterologous spherogone gene expression. Specifically, we describe the novel expression of the *T. pallidum* tp0751 gene encoding the laminin-binding adhesin within *T. phagedenis*. Expression of the tp0751 gene within *T. phagedenis* was verified at the mRNA level via RT-PCR analysis and at the protein level by immunofluorescence assays performed on the heterologous *T. phagedenis* construct with Tp0751-specific polyclonal antibodies. The latter assay verified the expression of the laminin-binding adhesin on the *T. phagedenis* surface. Expression of Tp0751 within *T. phagedenis* was functionally confirmed by performing treponemal laminin attachment assays. In these studies, Tp0751 expression within *T. phagedenis* conferred an adherent phenotype upon this nonadherent treponeme, with an eightfold enhancement of attachment to laminin being observed compared to controls. This laminin attachment capability could be specifically inhibited with polyclonal antibodies directed against Tp0751, with a threefold inhibition of attachment of the heterologously expressed protein within *T. phagedenis*. This result suggests proper recognition of the ribosome-binding site upstream of the tp0751 gene sequence and proper processing of the heterologously expressed protein within *T. phagedenis*, as evidenced by the laminin-binding capability conferred upon the heterologous *T. phagedenis* construct. Second, the observed attachment of viable *T. phagedenis* expressing Tp0751 to laminin-coated slides, combined with the immunofluorescence analyses showing surface immunofluorescence in non-detergent-treated, viable treponemes, verifies the expression of Tp0751 on the treponemal surface. The definitive identification of proteins that reside on the *T. pallidum* surface has been elusive due to the experimental limitations associated with research on *T. pallidum* (4), and evaluation of the surface exposure of a particular protein via direct extrapolation from experiments performed with *T. phagedenis* is useful in this regard.

Similarly, the nonculturable nature and genetic intractability of *T. pallidum* preclude the direct study of the function of Tp0751 within its native organism. The generation of a *T. phagedenis* construct heterologously expressing Tp0751 provides an important tool for investigating the hypothesized role of the Tp0751-laminin interaction in treponemal attachment and dissemination. Specifically, the creation of this nonadherent “model” treponeme will allow determination of the role of Tp0751, both alone and in combination with other *T. pallidum* and host proteins, in treponemal dissemination.

To our knowledge, these studies represent the first report of the successful transformation of *T. phagedenis*. This will advance our understanding of the pathogenesis of unculturable spirochetes, including *T. pallidum*, by allowing functional and ultrastructural investigations to be performed on putative outer membrane proteins, adhesins, and virulence factors within the context of a nonadherent treponeme. Although both the knowledge of treponemal molecules involved in pathogenesis and the process of treponemal genetic manipulation are still in their infancy, results presented here lay the foundation for future studies in these important, interrelated areas of research.

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