INFECTION AND IMMUNITY, July 1999, p. 3653–3656 Vol. 67, No. 7
0019-9567/99/$04.00+0

Development of a System for Expressing Heterologous Genes in the Oral Spirochete *Treponema denticola* and Its Use in Expression of the *Treponema pallidum flaA* Gene

BO CHI, SARITA CHAUHAN,† AND HOWARD KURAMITSU*

Department of Oral Biology, State University of New York at Buffalo, Buffalo, New York 14214

Received 16 February 1999/Returned for modification 8 April 1999/Accepted 19 April 1999

The present communication describes the construction of a new *Escherichia coli-Treponema denticola* shuttle vector based on the naturally occurring spirochete plasmid pTS1 and the expression of the heterologous *T. pallidum flaA* gene from the plasmid in *T. denticola*. This new shuttle vector system should prove useful in characterizing virulence factors from unculturable pathogenic spirochetes.

Spirochetes have unique morphology and motility. Their periplasmic flagella, located between the outer membrane and the cytoplasmic membrane, play an important role in cellular morphology and motility (5, 15). The *Treponema* genus contains several important pathogens, and many of these pathogenic spirochetes cannot be cultured in vitro. One of the most important spirochete pathogens is *Treponema pallidum*, the causative agent of syphilis, which can be grown experimentally only in rabbit testes, but no gene transfer system for the organism is available. For identifying the virulence factors of these pathogens, potential virulence genes must be expressed in heterologous systems. Although some *T. pallidum* genes can be expressed in *Escherichia coli* (6, 15), the distinct physiological differences between spirochetes and *E. coli* limits the use of the *E. coli* system for functional investigations.

One of the oral spirochetes, *Treponema denticola*, which has been shown to be associated with periodontitis (11, 12, 16), can be cultured in the laboratory. In addition, a gene transfer system for *T. denticola* was recently developed in our laboratory (8, 9). These advantages together with the similarity of *T. denticola* with other spirochetes suggest that *T. denticola* may serve as a suitable host for expressing heterologous spirochete genes.

Previously, the broad-host-range plasmid pKT210 was shown to serve as a shuttle vector in a variety of bacterial hosts, including *T. denticola* (8). However, this plasmid proved to be unstable in several host systems. Therefore, in the present study we constructed a novel *E. coli-T. denticola* shuttle vector based on the naturally occurring spirochete plasmid pTS1 (3) and demonstrated the expression of the heterologous *T. pallidum flaA* gene from the plasmid.

Construction of a novel shuttle vector and transformation of *T. denticola*. The cryptic plasmid pTS1 of *T. denticola* ATCC u9b (3) was used for shuttle vector construction. The sequence of pTS1 (3a) revealed an open reading frame homologous to a gene on plasmid pJDB23, a cryptic plasmid of *Selenomonas ruminantium* subsp. *lactilytica* (2). The fact that the gene on pJDB23 is responsible for the plasmid replication in *E. coli* (2) suggested that the open reading frame on pTS1 encodes a Rep protein. *Bam*HI digestion of the pTS1 plasmid generated two fragments, and the larger, 2.8-kb fragment, which contains the potential Rep-encoding gene, was ligated into the *Bam*HI site of an *E. coli* plasmid, pKMOZ19 (14), yielding the chimeric plasmid pKMRep4, which should replicate in both *T. denticola* and *E. coli* (Fig. 1A). The erythromycin resistance gene cassette (4), which has been shown to be expressed in *T. denticola* (9), was chosen as the selective marker for the shuttle vector. To ensure the transcription of the Em^r cassette in *T. denticola*, the promoter of a *T. denticola* proteinase gene, *prtB* (1), was placed upstream of the Em^r cassette. Both the Em^r cassette and the *prtB* promoter were PCR amplified and cloned into the *E. coli* plasmid pBK-CMV (Stratagene, La Jolla, Calif.). The fragment which contained the promoter and the Em^r cassette was removed from pBK-CMV, blunt ended, and ligated into the *Hin*cII site of plasmid pKMRep4 to generate the 7.7-kb pKMR4PE (Fig. 1A).

pKMR4PE was then transformed into *T. denticola* ATCC 33520 by electroporation as described previously (8). Ten micrograms of pKMR4PE plasmid (2 μg/μl) was used to transform 80 μl of fresh competent cells (about 4×10^9 cells). Transformants were selected on TYGVS plates supplemented with 0.8% SeaPlaque agarose (FMC BioProducts, Rockland, Maine) and erythromycin (40 μg/ml). All culturing was carried out at 37°C under anaerobic conditions. The erythromycin-resistant colonies began to appear after 7 to 10 days. The transformation efficiency was approximately 0.5 to 1 colony per μg of pKMR4PE. The individual colonies were then inoculated into 2 ml of TYGVS-erythromycin broth 2 to 3 days after their appearance and diluted to 10 ml at the mid-logarithmic growth phase. Plasmid DNA was isolated from *T. denticola* by using the Wizard Minipreps kit (Promega, Madison, Wis.) according to manufacturer's protocol.

As demonstrated in Fig. 2, the wild-type strain ATCC 33520 carried the cryptic plasmid pTD1 of approximately 2.6 kb (7) (Fig. 2, lane 2). The pKMR4PE transformant also contained an additional plasmid (Fig. 2, lane 3). The linearized pKMR4PE in the transformant had the same size as the original pKMR4PE following cleavage with *Sma*I (Fig. 2, lanes 6 and 7). The *T. denticola* plasmids were next reintroduced into *E. coli* XL1-Blue cells (Stratagene). The rescued plasmids isolated from the erythromycin-resistant XL1-Blue colonies were characterized by restriction enzyme digestion. The analysis revealed that the rescued plasmids were indistinguishable from the original plasmids (data not shown). These results confirmed that the new shuttle vector pKMR4PE is capable of

* Corresponding author. Mailing address: Department of Oral Biology, State University of New York at Buffalo, 3435 Main St., Buffalo, NY 14214-3092. Phone: (716) 829-2068. Fax: (716) 829-3942. E-mail: kuramits@acsu.buffalo.edu.

† Present address: Dupont Central Research and Development, Wilmington, DE 19880.

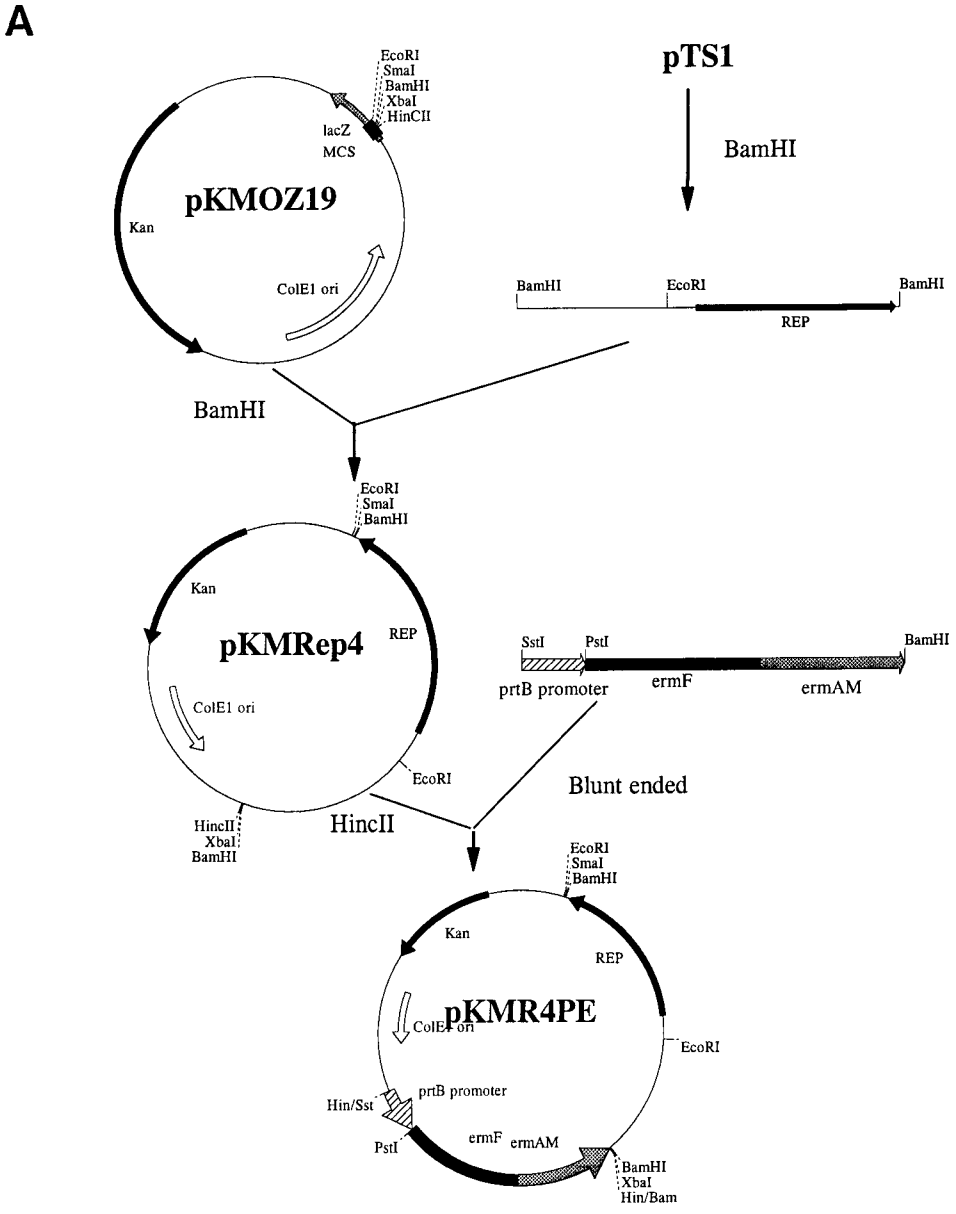

FIG. 1. Construction of shuttle vectors pKMR4PE (A) and pKMflaA (B). The position and orientation of the putative Rep-encoding gene of pTS1 (Rep), the *T. denticola prtB* promoter (prtBp), and the Em^r cassette (ermF and ermAM) are shown. Relevant restriction sites are indicated.

replicating independently and stably in *T. denticola* and that the open reading frame on the *Bam*HI fragment of pTS1 encodes the Rep protein.

The transformation efficiency of *T. denticola* with the shuttle vector following electroporation is more than 100-fold higher when the plasmid isolated from *T. denticola* is used compared to the same plasmid isolated from *E. coli*. In addition, our experience (data not shown) and a previous report (7) have demonstrated that the *Eco*RI site of the plasmid is modified, probably methylated, in *T. denticola* but not in *E. coli*. Taken together, these results suggested that the restriction and modification systems are different in *T. denticola* and *E. coli* and that the DNA isolated from *E. coli* can be degraded by *T. denticola* restriction systems.

Expression of the *T. pallidum flaA* gene in *T. denticola*. Our next step was to use the new shuttle vector to express heterologous spirochete genes. The gene of *T. pallidum* endoflagellum protein FlaA was chosen as a suitable gene because its sequence is known (5) and a monoclonal antibody, H9-2 (13), is available (gift from Sheila Lukehart, Harborview Medical Center, Seattle, Wash.). PCR primers were designed according to the *T. pallidum flaA* gene sequence (5), and the *flaA* gene was amplified from *T. pallidum* genomic DNA (gift from Kayla Hagman, University of Texas, Dallas). Our first attempt to clone the *flaA* gene together with its native promoter onto pKMR4PE in *E. coli* was not successful. This is consistent with previous reports that the strong expression of this *flaA* gene cannot be tolerated by *E. coli* (5). It was also known that the Em^r cassette does not have transcriptional termination signals (10). The *flaA* gene was then placed downstream of the Em^r cassette to be expressed from the *prtB* promoter. By using the *Xba*I restriction sites (underlined) which were incorporated

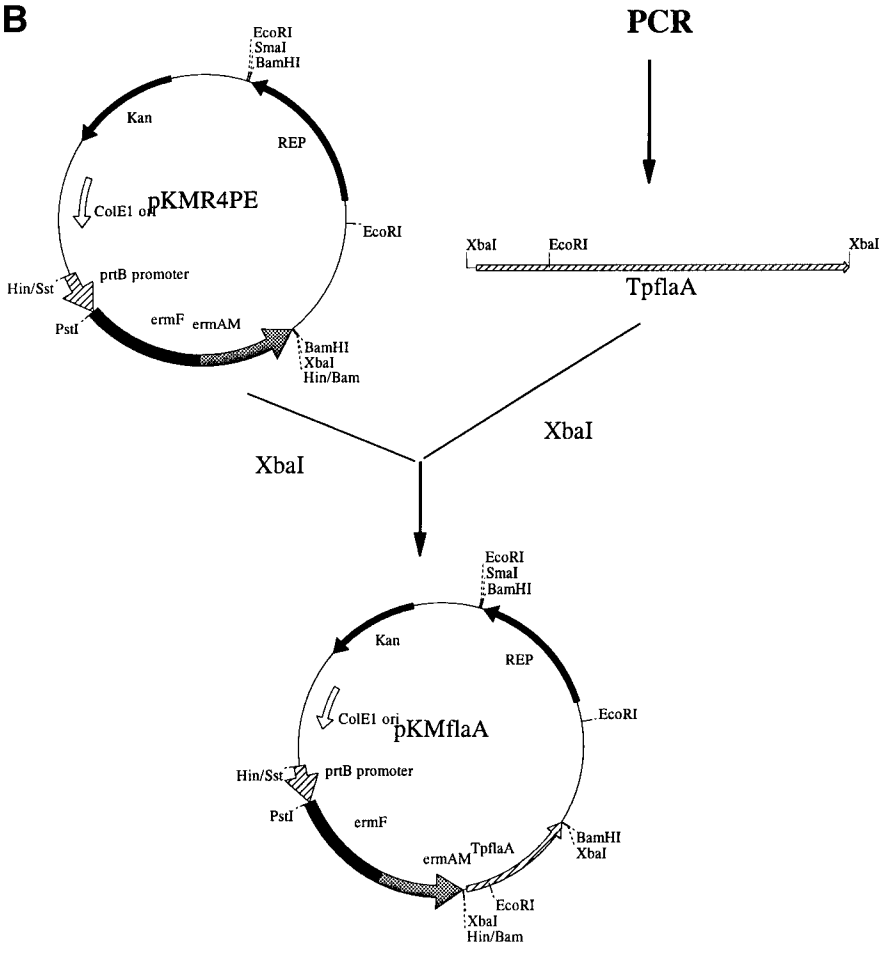

FIG. 1—*Continued.*

into the forward and reverse PCR primers (5′-TTTTTTCTAGAGAGTGGTTATCTTATTGTGCG-3′ and 5′-TTTTTTCTAGATAGCCATCCTACCACGCATCC-3′, respectively) the amplified 1.25-kb *flaA* gene, which begins 24 bp upstream of its ribosome-binding site, was inserted into the unique *Xba*I site of pKMR4PE (Fig. 1B). The *E. coli* XL1-Blue colonies were screened by restriction endonuclease mapping for the *flaA* gene inserted in the same orientation as the Em^r cassette. The resulting plasmid, pKMflaA, was transformed into *T. denticola*, and erythromycin-resistant *T. denticola* colonies were analyzed for plasmids and *flaA* gene expression. As shown in Fig. 2, pKMflaA-transformed *T. denticola* contained an additional band larger than that in pKMR4PE (Fig. 2, lane 4). Linearization of the plasmid with *Sma*I indicated that the plasmid had the same size as the original pKMflaA plasmid (Fig. 2, lanes 8 and 9). The pKMflaA plasmid from *T. denticola* was next retransformed into *E. coli* XL1-Blue cells. The rescued plasmids were further analyzed by restriction endonuclease mapping and proved to be identical to the original pKMflaA plasmid (data not shown).

The *T. denticola* pKMflaA transformants were then examined for expression of the *T. pallidum* FlaA protein by Western blot analysis. Monoclonal antibody H9-2, which is specific for *T. pallidum* FlaA protein (5), was used as the primary antibody. As shown in Fig. 3, H9-2 reacts with the 37-kDa FlaA band in the *T. pallidum* cell extract (gift from Kayla Hagman) (Fig. 3,

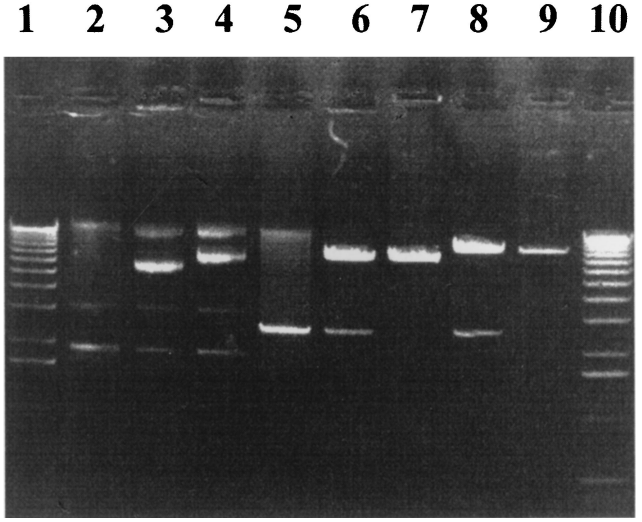

FIG. 2. Electrophoretic analysis of plasmids isolated from *T. denticola* pKMR4PE and pKMflaA transformants. Lane 2, plasmid from wild-type *T. denticola* 33520; lane 3, plasmid from pKMR4PE transformants; lane 4, plasmid from pKMflaA transformants; lanes 5 to 9, *Sma*I digestions of plasmids from lane 2, lane 3, original pKMR4PE, lane 4, and original pKMflaA, respectively; lanes 1 and 10, 1-kb DNA ladder.

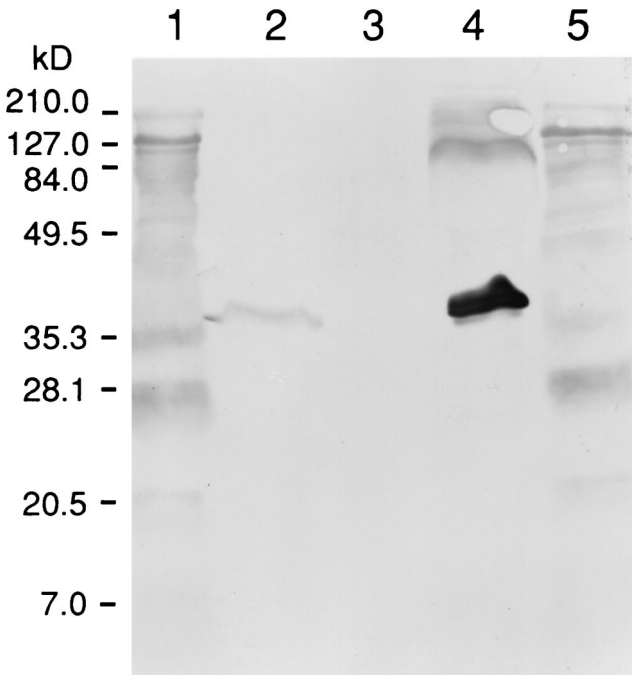

FIG. 3. Western blot analysis of *T. denticola* pKMflaA transformants. Cell extracts were separated on a sodium dodecyl sulfate–12.5% polyacrylamide gel and transferred to a nitrocellulose membrane. Monoclonal antibody H9-2 of *T. pallidum* FlaA protein (1:10 dilution) was used as the primary antibody. Lanes 1 and 5, prestained sodium dodecyl sulfate-polyacrylamide gel electrophoresis standards (Bio-Rad, Hercules, Calif.); lane 2, cell extract of *T. denticola* pKMflaA transformants; lane 3, cell extract of *T. denticola* pKMR4PE transformants; lane 4, cell extract of *T. pallidum*.

lane 4) (5). A band with the same size was also detected by the H9-2 antibody in the *T. denticola* pKMflaA transformant cell extract (Fig. 3, lane 2). As a control, the *T. denticola* pKMR4PE cell extract doesn't react with H9-2 (Fig. 3, lane 3). Compared to the wild type, ATCC 33520, the *T. denticola* pKMflaA transformants did not show any difference in growth rate or morphology under phase-contrast microscopy. The *T. denticola* pKMflaA transformants after three passages still expressed the *T. pallidum* FlaA protein (data not shown). While the size of the *T. pallidum* FlaA protein expressed in *T. denticola* corresponds to that of the protein expressed in the former organism, we cannot formally rule out the possibility of minor alternations in the protein expressed in the heterologous spirochete.

To our knowledge, this is the first report of heterologous gene expression from a shuttle vector in a spirochete. Therefore, *T. denticola* can serve as a potential system for characterizing virulence genes from unculturable spirochetes. PCR fragments of potential virulence genes from other spirochetes could be inserted into the shuttle vector, and the function of the expressed proteins could be examined. At present, the virulence factors of pathogenic spirochetes remain largely undefined. This new shuttle vector system should prove useful in identifying virulence factors from these organisms.

This investigation was supported by National Institutes of Health grant DE09821.

Editor: J. R. McGhee

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