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Gene Inactivation in the Oral Spirochete *Treponema denticola*: Construction of an *flgE* Mutant

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Treponema denticola **is implicated in the etiology of periodontal diseases. We now report the construction of a specific *flgE* mutant of *T. denticola* ATCC 35405 following electroporation utilizing an erythromycin resistance cassette inserted into an *flgE* DNA fragment. The resulting mutant displays no visible motility and lacks periplasmic flagella as would be predicted from inactivation of the gene for the flagellar hook protein.**

Oral spirochetes have been widely recognized as important agents in the pathogenesis of periodontitis as well as other oral infections (5, 11, 12). Recent investigations have demonstrated that a variety of potential virulence factors have been detected in this group of bacteria (1, 2, 8, 20, 21). Among these, their unique locomotive characteristics may enable them to penetrate and invade periodontal tissues (15). For spirochetes, the motile organelles are periplasmic flagella which originate near each end of the cell and extend toward the center of the cell (4, 13). Unlike the case with the other extracellularly flagellated eubacteria, the periplasmic flagella of spirochetes are situated between the outer membrane sheath and the cytoplasmic membrane. A flagellum consists of a corkscrew-like propeller (filament) connected via a universal joint (hook) to a transmission shaft, motor, and bushings (basal body) embedded in the cell envelope (22).

Despite increasing interest in oral spirochetes, limited information regarding their virulence properties is available, since spirochetes are difficult to cultivate and gene transfer systems for genetic evaluation are lacking. *Treponema denticola*, a small obligately anaerobic oral spirochete, has been strongly associated with the pathogenesis of periodontal diseases (14, 19). Recently, a gene transfer system for *T. denticola* utilizing electroporation has been developed by us (8). This system has now made possible a direct approach toward the specific mutagenesis of potential virulence factors in *T. denticola*. The *T. denticola flgE* gene coding for the flagellar hook protein was initially chosen for mutagenesis since *flgE* mutants of other bacteria lack intact flagella and are nonmotile (13) and therefore the phenotype produced by the gene can be readily detected.

Construction of *T. denticola* mutant. On the basis of the nucleotide sequence of the *Treponema phagedenis flgE* gene (10), two DNA primers were synthesized in order to isolate the homologous sequence of a 550-bp internal fragment of the *T. denticola flgE* gene following PCR amplification. The resulting DNA fragment was cloned in the plasmid vector pCRII (Invitrogen Corporation, San Diego, Calif.) and then sequenced to confirm its identity. As depicted in Fig. 1, the plasmid pHLfE harboring an *flgE* gene fragment insertionally inactivated by an erythromycin resistance (Em^r) cassette containing the *ermF* and *ermAM* genes (6) was constructed. The linearized interrupted *flgE* fragment (*Xho*I-digested pHLfE) was used to transform *T. denticola*. Electroporation was carried out as described previously (8). Briefly, 80 μl of competent cells (about 5×10^{10} cells) was mixed with 10 μg of plasmid preparation and electroporated in a 1.0-mm-diameter cuvette by utilizing a Gene Pulser (Bio-Rad Laboratories, Melville, N.Y.) with the pulse controller set at 1.8 kV, 25 μF, and 200 Ω, producing a time constant of approximately 4 to 4.5 ms. Two milliliters of TYGVS medium (1) was then added immediately to the treated cells. After overnight anaerobic incubation without antibiotics, 1.0 ml of the culture was mixed with 35 ml of precooled medium supplemented with 0.8% SeaPlaque agarose (FMC BioProducts, Rockland, Maine) and 40 μg of erythromycin per ml. The mixture was poured into a petri dish and transferred into an anaerobic chamber (Coy Laboratory Products, Inc., Ann Arbor, Mich.). The Em^r transformant colonies were visible on the plates after 8 to 10 days of incubation. For *T. denticola* ATCC 35405, the transformation efficiency for the linearized pHLfE was approximately 0.9 colonies per μg of pHLfE, and similar results were obtained for strain ATCC 33520. The individual colonies were then isolated, inoculated into 10 ml of TYGVS-erythromycin broth, and grown to the mid-logarithmic growth phase. The purity of the cultures was confirmed by phase-contrast microscopy before harvesting.

Following electroporation of strain 35405, the Em^r transformants of *flgE* mutants appeared as small, dense, and pinpoint-shaped colonies. The edges of the colonies were defined instead of diffusing outwardly like those of the wild-type strain. When spread on the surface of the plates, the wild-type bacteria migrated into the interior of the agarose layers. On the other hand, the *flgE* mutant colonies grew on the surface of the agarose plates. Moreover, as viewed by both dark-field and phase-contrast microscopy, the *flgE* mutant completely lacked motility, whereas the wild-type 35405 showed noticeable cell movement. In addition, most of the cells of the wild type had an irregular morphology. In contrast, the *flgE* mutant demonstrated helical morphology. Similar results have been found for a spontaneously occurring nonmotile mutant of strain 33520 which lacked periplasmic flagella (17, 18). All of these morphologic differences between the parental and the mutant cells are consistent with the absence of flagella in the latter due to the loss of the flagellar hook protein.

Southern blot analyses. In order to confirm the allelic exchange and insertional inactivation of the *flgE* gene, Southern blot hybridization was conducted with the ECL system (Amersham Life Science, Inc., Arlington Heights, Ill.) as described recently (8). A 0.55-kb *Eco*RI *flgE* fragment from pCRflgE and

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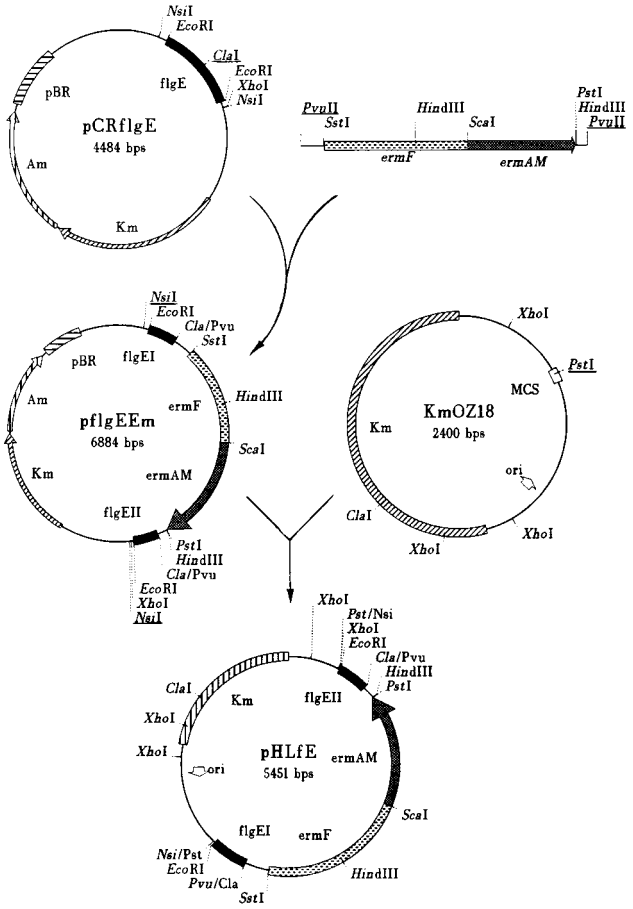

FIG. 1. Construction of plasmid pHLfE containing an *flgE* gene fragment insertionally inactivated with an Em^r cassette (diagramatic representation). Plasmid pCRflgE containing a portion of the *T. denticola* 35405 *flgE* gene was cut with *Cla*I within the *flgE* fragment and blunt ended with Klenow DNA polymerase. A *Pvu*II-*Pvu*II *ermF-ermAM* cassette from pVA2198 (6) was then inserted into the blunt-ended *Cla*I site of pCRflgE to construct plasmid pflgEEm. The latter was digested with *Nsi*I, and the 3-kb fragment containing the *flgE*-Em^r region was isolated and subcloned into pKmOZ18 at the *Pst*I site to produce plasmid pHLfE.

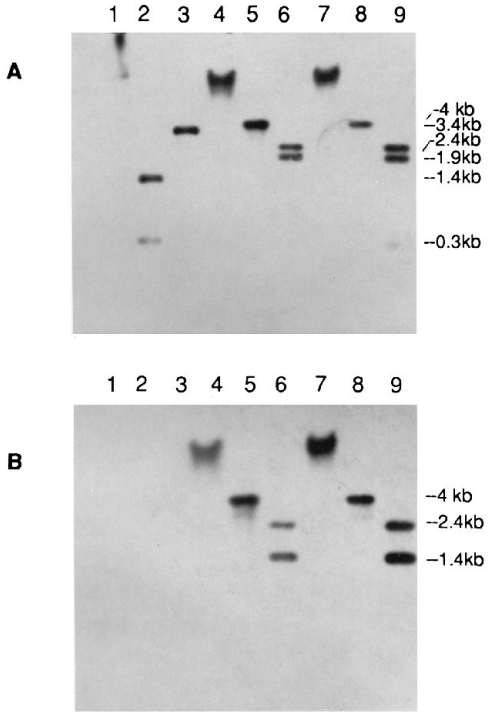

FIG. 2. Southern blot analyses of chromosomal DNA extracted from parental strain 35405 and the *flgE* mutants HL51 and HL52. Lanes: 1, strain 35405, uncut; 2, strain 35405, *Cla*I cut; 3, strain 35405, *Hin*dIII cut; 4, *flgE* mutant HL51, uncut; 5, HL51, *Cla*I cut; 6, HL51, *Hin*dIII cut; 7, *flgE* mutant HL52, uncut; 8, HL52, *Cla*I cut; 9, HL52, *Hin*dIII cut. The probe for blot A was the 0.55-kb *Eco*RI-*Eco*RI *flgE* region from pCRflgE. The probe for blot B was the 2.1-kb *Sst*I-*Pst*I Em^r cassette. Numbers on the right indicate the molecular sizes of the hybridizing bands.

the 2.1-kb *Sst*I-*Pst*I Em^r cassette (Fig. 1) were labeled as probes. As demonstrated in Fig. 2A, the uncut chromosomal DNA from strain 35405 (lane 1) and the DNAs from two randomly isolated *flgE* mutants, HL51 and HL52 (lanes 4 and 7, respectively), all reacted with the *flgE* probe. When the DNA samples were digested with *Cla*I and *Hin*dIII, the hybridization patterns were, however, dramatically different between the wild-type strain and the *flgE* mutants. For the wild type, *Cla*I yielded two fragments (0.3 and 1.4 kb) (lane 2) which reacted with the *flgE* probe. In contrast, for the two *flgE* mutants, *Cla*I cleavage yielded a single 4-kb hybridizing fragment (lanes 5 and 8). Similarly, following *Hin*dIII digestion, strain 35405 yielded a 3.4-kb *Hin*dIII positive fragment (lane 3). However, the two *flgE* mutants demonstrated two bands (1.9 and 2.4 kb) which reacted with the *flgE* probe (lanes 6 and 9). Furthermore, when the same blot was reprobed with the Em^r cassette (Fig. 2B), the samples derived from the parental strain showed no positive hybridization signals (lanes 1 to 3). However, for the two *flgE* mutants, the Em^r probe reacted with the uncut DNA (lanes 4 and 7) and also with the 4-kb *Cla*I fragment which reacted with the *flgE* probe (lanes 5 and 8), as well as with the two 1.4- and 2.4-kb *Hin*dIII bands (lanes 6 and 9).

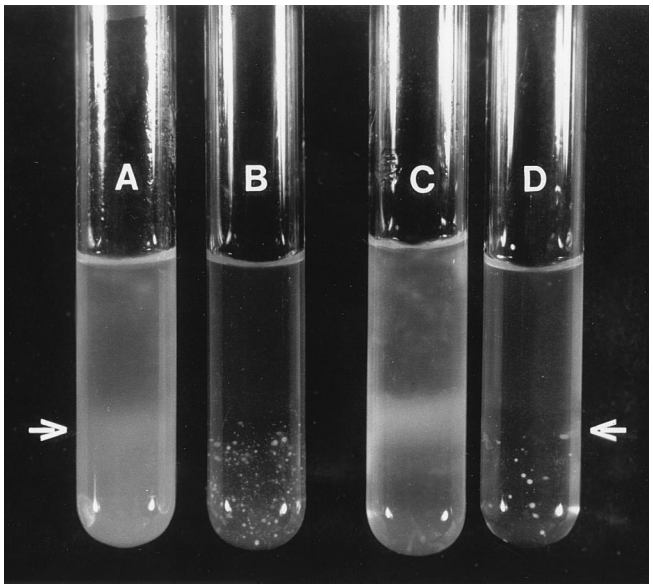

FIG. 3. Motility of the *flgE* mutants. In tubes A and B, the bottom layers consisted of complete TYGVS-Noble agar medium inoculated either with wild-type cells (tube A) or with *flgE* mutant HL51 cells (tube B). In tubes C and D, the bottom layers consisted of TYGVS-Nobel agar medium without serum and supplements inoculated either with wild-type cells (tube C) or with *flgE* mutant HL51 cells (tube D). The top layers of all four tubes consisted of complete TYGVS-SeaPlaque agarose media. The arrows indicate the original boundaries between the two layers.

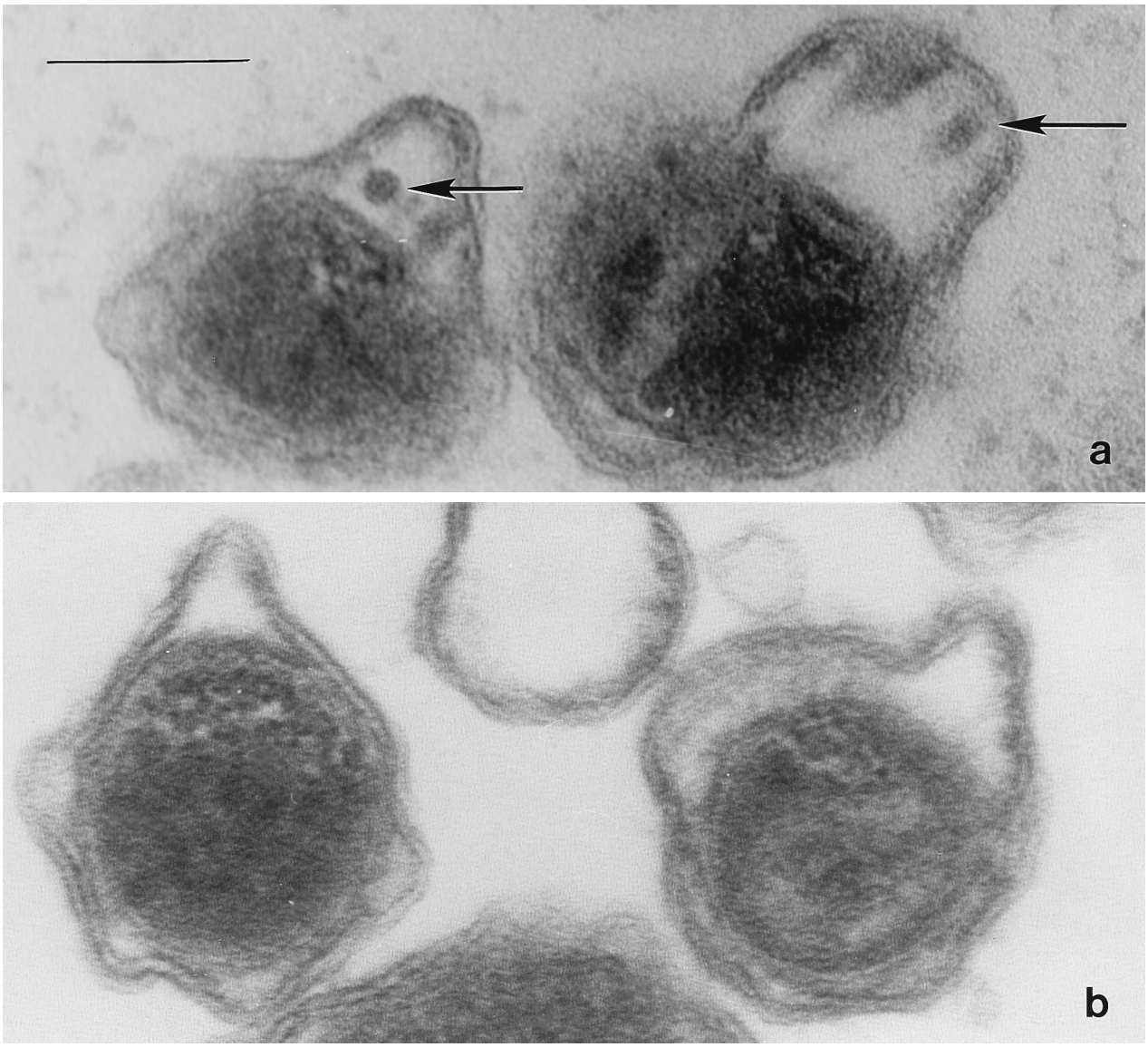

FIG. 4. Electron microscopy. Thin-section electron micrographs of wild-type (a) and *flgE* mutant HL51 (b) strains of *T. denticola* are shown. Arrows point to periplasmic flagella. Periplasmic flagella are not evident in the *flgE* mutant HL51 strain. Bar, 0.1 μm.

These results confirmed the allelic exchange of the insertionally inactivated *flgE* fragment with the strain 35405 chromosomal copy of the gene following a double-crossover event. Further confirmation of the insertional inactivation of the *flgE* gene was provided by PCR analyses (data not shown). DNA primers based on the sequence of the *flgE* gene fragment and the Em^r cassette amplified DNA fragments of the predicted sizes from the *flgE* mutants but not from the parental strain.

Motility assays. For direct confirmation of the loss of motility in the transformants, the mutant strain was compared with the parental strain in a repellent assay. Recently, it has been demonstrated that *T. denticola* migrates from agar to agarose layers because of the presence of unknown inhibitory substances within the former media (3). On the basis of this property, a two-phase agar-agarose system modified from the method of Chan et al. (3) was developed to compare the motilities of the mutant and wild-type organisms (Fig. 3). The cells were initially inoculated into the lower layer consisting of 0.5% Noble agar supplemented with (tubes A and B) or without (tubes C and D) 10% rabbit serum and 10% nutrient supplements. Following solidification of the agar, the top layer containing 0.8% SeaPlaque agarose and complete medium was added. After incubation for 7 days, strain 35405 migrated into the upper agarose layers (tubes A and C). However, the *flgE* mutant HL51 cells remained in the lower layers (tubes B and D) and formed dense individual colonies which were incapable of migrating into the upper layer containing agarose and nutrient supplements. Therefore, this assay system demonstrated that the *flgE* mutant cells had lost their locomotive capability as would be predicted for mutagenesis of the *flgE* gene.

Electron microscopy of *flgE* mutants. Direct confirmation of the absence of flagella in the *flgE* mutants was provided by electron microscopy. Both cells negatively stained with 2% uranyl acetate or 1% phosphotungstate (9) and thin sections of imbedded cells (7) were used to analyze for the presence of periplasmic flagella. Individual cells of the parental strain

35405 contained periplasmic flagella characteristic of these organisms (Fig. 4a). However, no flagella could be observed for the *flgE* mutant HL51 cells (Fig. 4b).

In conclusion, we have constructed a plasmid, pHLfE, containing a portion of the *flgE* gene which has been interrupted by an Em^r cassette. Linearized pHLfE was introduced into *T. denticola* via electroporation, and nonmotile mutants defective in *flgE* expression were isolated following homologous recombination. Recently, Rosey et al. (16) have described the inactivation of flagellar genes from another spirochete, *Serpulina hyodysenteriae*. To our knowledge, the present communication represents the first description of a gene inactivation system for an oral spirochete. In addition, this is the first demonstration that inactivation of a putative motility gene results in both inhibition of periplasmic flagellum synthesis and complete loss of motility in a spirochete. This system should prove useful in identifying potential virulence factors in *T. denticola*. Likewise, the nonmotile mutants constructed in this investigation can now be utilized to examine the role of motility in putative virulence properties of the organisms.

Nucleotide sequence accession number. The sequence of the 550-bp *T. denticola flgE* fragment isolated in this study has been submitted to GenBank under accession no. L75953.

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