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FlaA Proteins in *Leptospira interrogans* Are Essential for Motility and Virulence but Are Not Required for Formation of the Flagellum Sheath

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Spirochetes have periplasmic flagella composed of a core surrounded by a sheath. The pathogen *Leptospira interrogans* has four *flaB* (proposed core subunit) and two *flaA* (proposed sheath subunit) genes. The *flaA* genes are organized in a locus with *flaA2* immediately upstream of *flaA1*. In this study, *flaA1* and *flaA2* mutants were constructed by transposon mutagenesis. Both mutants still produced periplasmic flagella. The *flaA1* mutant did not produce FlaA1 but continued to produce FlaA2 and retained normal morphology and virulence in a hamster model of infection but had reduced motility. The *flaA2* mutant did not produce either the FlaA1 or the FlaA2 protein. Cells of the *flaA2* mutant lacked the distinctive hook-shaped ends associated with *L. interrogans* and lacked translational motility in liquid and semisolid media. These observations were confirmed with a second, independent *flaA2* mutant. The *flaA2* mutant failed to cause disease in animal models of acute infection. Despite lacking FlaA proteins, the flagella of the *flaA2* mutant were of the same thickness as wild-type flagella, as measured by electron microscopy, and exhibited a normal flagellum sheath, indicating that FlaA proteins are not essential for the synthesis of the flagellum sheath, as observed for other spirochetes. This study shows that FlaA subunits contribute to leptospiral translational motility, cellular shape, and virulence.

Leptospira interrogans is a pathogenic spirochete and the causative agent of leptospirosis, a zoonosis contracted through contact with animal urine or contaminated water. The disease ranges in severity from a mild, influenza-like illness to a severe syndrome with multiple-organ failure (1).

Spirochete motility enables highly effective translocation through viscous substrates and tissues, allowing access to otherwise inaccessible host niches (7). Leptospires disseminate rapidly during infection and are isolated from blood and target organs within minutes after inoculation in animal models. Mutations affecting motility severely reduce virulence in several spirochete genera, illustrating the importance of motility to infection (16, 17, 19, 31).

Spirochete motility is mediated by multiple periplasmic flagella (endoflagella). Leptospires differ from other spirochetes in that they have a single flagellum inserted subterminally at each pole, wrapping around the protoplasmic cylinder and extending toward the middle of the cell without overlapping (12). In nonviscous media, the ends of *L. interrogans* cells are deformed into distinctive hook- or spiral-shaped ends depending on the direction of flagellar rotation. The shape of the cell end in turn determines the direction of motility. In viscous media, the bacteria move through the substrate with a screw-like motion (reviewed in reference 12).

The basal body and hook structures of spirochete flagella are similar to those of the model systems of *Escherichia coli* and *Salmonella enterica* (see Table S1 in the supplemental material). However, in contrast to the flagellin polymer of enterobacteria, the flagellar filaments of spirochetes investigated to date have a complex structure comprising a FlaB core surrounded by a FlaA protein sheath (5, 11, 14, 15). *Brachyspira hyodysenteriae* flagella without FlaA lack a typical flagellar sheath and have an altered shape (14, 15). *L. interrogans* has four *flaB* genes and two *flaA* genes, all of which are expressed with FlaA1 and FlaA2 present at similar levels (20). The leptospiral FlaB proteins share a high degree of identity (>70%) and are essentially the same length (281 to 285 amino acids), suggesting a similar or interchangeable function. In contrast, the FlaA proteins are only 28% identical and differ in length (315 and 239 amino acids), suggesting distinct roles in the flagellum structure and/or function.

The configuration of the subunits within the leptospiral flagellum and their role in motility have not been determined. This is largely due to a lack of genetic techniques that may be applied to *L. interrogans*. The recent development of mutagenesis techniques (4, 8, 24) has revealed interesting features of leptospiral biology, including important aspects of the molecular basis of pathogene-

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sis (2). In this study, transposon mutagenesis was applied to demonstrate that the FlaA proteins contribute to the cell shape, translational motility, and virulence of *L. interrogans*. Findings also suggest that FlaA subunits are not the only components of the flagellum sheath.

(This work is part of the doctoral thesis of A. Lambert.)

MATERIALS AND METHODS

Leptospiral strains and culture conditions. The pathogens *L. interrogans* serovar Manilae strain L495 and *L. interrogans* serovar Lai strain Lai 56601 were cultivated in liquid Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (Difco) or on 1% agar EMJH plates at 30°C. When appropriate, kanamycin was added to culture media at 50 μg ml^{-1}. Growth curves were determined by the optical density at 420 nm (OD_{420}) in liquid EMJH medium at 30°C with shaking.

Transposon mutagenesis of *L. interrogans*. The mutagenesis of *L. interrogans* serovars Lai and Manilae with the transposon *Himar1* was conducted as described previously (4). The location of the transposon insertion was determined by either direct sequencing from genomic DNA or semirandom PCR (23). The confirmation of genotypes was performed by PCR with primers located in the flanking sequences of the insertion site of the transposon and Southern blots of EcoRI-digested DNA probed for hybridization with the kanamycin resistance cassette (data not shown).

Preparation of periplasmic flagella. Initial experiments using sodium deoxycholate for cell disruption were found to deplete the flagellar sheath (data not shown). Accordingly, flagella were isolated by a milder preparation method employing Triton X-100, similar to that used to purify endoflagella from other spirochetes (11, 14). Forty milliliters of a logarithmic-phase culture (approximately 10^8 cells/ml) was centrifuged at 8,000 × *g* for 20 min at 4°C. The cell pellet was washed with phosphate-buffered saline (PBS) and then washed in a cold sucrose solution (0.5 M sucrose, 0.15 M Tris [pH 8]). Cells were resuspended in 3 ml cold sucrose solution, and 0.3 ml of 10% Triton X-100 was added while stirring with a magnetic bar. The mixture was stirred for 30 min at room temperature; next, 30 μl of lysozyme was added, and the mixture was incubated on ice for 5 min. Three hundred microliters of 20 mM EDTA (pH 8) was added dropwise with swirling. After 2 h of incubation at room temperature, 60 μl of 0.1 M $MgSO_4$ followed by 60 μl of 0.1 M EDTA was added to the mixture. The lysate was then centrifuged at 17,000 × *g* for 15 min at 4°C, and the supernatant was resuspended in 0.4 ml of a polyethylene glycol 8000 (PEG 8000) solution (20% PEG, 1 M NaCl). The mixture was incubated on ice for 30 min and then centrifuged by using a Beckman Optima Max centrifuge equipped with a TLA110 rotor at 27,000 × *g* for 30 min at 4°C. The flagellar extract was resuspended in 1 ml of water, incubated overnight at 4°C, and then resuspended in 200 μl of water after centrifugation at 80,000 × *g* in a Beckman TLA55 rotor for 45 min at 4°C. The flagellar extracts were stored at 4°C. Under these storage conditions, flagella remained intact.

Western blotting. Rabbit antisera against leptospiral FlaA1 (9), FlaA2, and ImpL63 (13) were prepared as described previously. The protein concentration of whole-cell lysates or flagellum extracts was determined by a Bradford protein assay (Coomassie Plus protein assay; Pierce). Six micrograms of whole-cell lysate or 2 μg of flagellum extracts was separated by using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred onto Immobilon polyvinylidene difluoride (PVDF) membranes and probed with *L. interrogans* FlaA1 and FlaA2 antisera at a dilution of 1:2,000. ImpL63 antiserum diluted 1:3,000 was also used as a loading control. The secondary antiserum used was peroxidase-conjugated goat anti-rabbit IgG at a dilution of 1:100,000. Blots were developed with a chemiluminescence kit (SuperSignal West Pico chemiluminescent substrate; Thermo Scientific, IL) according to the manufacturer's instructions.

Electron microscopy. For scanning electron microscopy, leptospires in the exponential growth phase (OD_{420} of ~0.2) were centrifuged, washed once in PBS, and resuspended in 2.5% glutaraldehyde in cacodylate buffer (0.1 M, pH 7.2). Samples were then processed for scanning electron microscopy as described previously (29). To determine the flagellum diameter, flagellar extracts were applied onto glow-discharged, carbon-coated copper grids and negatively stained with 2% uranyl acetate. Samples were viewed with a Jeol JEM-1200 EXII transmission electron microscope operating at 80 kV. For measurements, 20 individual flagella in the transmission electron microscopy images were chosen randomly, and diameters were calculated manually by using the ImageJ program (http://rsb.info.nih.gov/ij/). Measurements of flagellar diameters were made at the widest point, as the thicknesses of individual flagella were found to vary along their lengths.

Immunofluorescence. Cells were air dried on glass slides and permeabilized with methanol. Primary antibody was added at a dilution of 1:10 (anti-FlaA2 antibody) or 1:20 (anti-FlaA1 antibody), incubated for 1 h at 37°C in a humidified chamber, and then washed three times in PBS, followed by incubation for 1 h at 37°C with a 1:20 dilution of Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen). Cells were mounted with ProLong Gold mounting medium (Invitrogen) containing a 4′,6′-diamidino-2-phenylindole (DAPI) counterstain, covered with a coverslip, and sealed with nail polish. Slides were viewed with a model BX51TF fluorescence microscope using DP Controller software (Olympus Optical Co.).

Assessment of leptospiral motility. The cell motility of exponential-phase cultures in liquid EMJH medium was analyzed by dark-field microscopy. Motility was also evaluated by inoculating leptospiral cells (approximately 10^6 bacteria) onto 0.3% and 0.5% agar semisolid EMJH medium. The plates were incubated for 15 days at 30°C, and the diameters of the zones of spread were measured. Plate assays were repeated at least three times; similar results were obtained each time.

Virulence test. Leptospires (10^6 or 10^3) were injected intraperitoneally into groups of 8 or 10 28-day-old male hamsters (Janvier) (*L. interrogans* serovar Manilae 50% infective dose [ID_{50}] of <10 [25]), which were monitored for up to 21 days. Protocols for animal experiments conformed with the guidelines of the Animal Care and Use Committees of the Institut Pasteur (France) and Khon Kaen University (Thailand). Data were graphed and analyzed by using GraphPad Prism 5.0c (GraphPad Software, San Diego, CA). Survival curves were compared by using a log rank (Mantel Cox test) analysis.

Quantitative PCR (qPCR). Groups of four hamsters were infected intraperitoneally with 10^6 leptospires (wild-type *L. interrogans* serovar Manilae or the *L. interrogans* serovar Manilae *flaA1* or *flaA2* mutant strain) and euthanized at 5 days postinfection. Surviving hamsters infected with the *L. interrogans* serovar Manilae *flaA2* mutant (see above) were also euthanized at 25 days postinfection. The liver and kidneys were collected, and approximately 50 mg of tissues was homogenized in PBS. Total genomic DNA was then extracted by using a tissue DNA purification kit (Maxwell; Promega). The concentration of leptospires in tissues was quantified by a TaqMan assay targeting the *lipL32* gene (32), using the CFX96 real-time PCR detection system (Bio-Rad). The standard curve for genome equivalents per gram of tissue was determined by the 10-fold serial dilution of purified DNA of a known concentration of *L. interrogans* serovar Manilae strain L495 cells. All PCRs were performed in duplicate, and control reactions without a template were included in each assay.

RESULTS

Generation of *flaA* mutants by transposon mutagenesis. The *L. interrogans flaA2* (LA3380/LIC10787) and *flaA1* (LA3379/LIC10788) genes form a putative operon separated by an intergenic region of 23 bp (Fig. 1). The mutagenesis of *L. interrogans* serovars Lai and Manilae was conducted with the transposon *Himar1* (4, 23). An *L. interrogans* serovar Manilae mutant (laboratory reference Man22) and an *L. interrogans* serovar Lai mutant (laboratory reference L537) had transposon insertions in *flaA2* (634 bp and 712 bp into the 720-bp gene, respectively); these were designated the *L. interrogans* serovar Manilae *flaA2* mutant and the *L. interrogans* serovar Lai *flaA2* mutant. An *L. interrogans* ser-

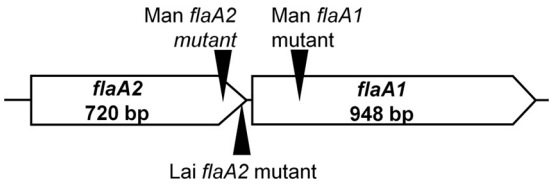

FIG 1 Location of transposon insertions in the *flaA* locus of *Leptospira interrogans*. Black triangles indicate transposon insertion points. Man, *L. interrogans* serovar Manilae.

ovar Manilae mutant (laboratory reference M718) had a transposon insertion in *flaA1* (163 bp into the 948-bp gene) (Fig. 1); this strain was designated the *L. interrogans* serovar Manilae *flaA1* mutant.

The *flaA* mutants were examined for flagellar subunit production by immunoblotting and immunofluorescence microscopy (Fig. 2). Neither of the *flaA2* mutants produced the FlaA1 or FlaA2 protein, whereas the *L. interrogans* serovar Manilae *flaA1* mutant lacked FlaA1 but still produced FlaA2. Both subunits were detected in their respective parental strains. The *flaA2* mutants were found to produce FlaB (data not shown). All three *flaA* mutants produced morphologically intact flagella, as observed by electron microscopy (see below). This finding indicates that in the absence of FlaA, flagella are still assembled but are not fully functional (see below).

Flagellum rotation transiently distorts the end of the leptospiral cell, conferring a hook or helical superstructure to the cell end (34). The mutants were examined to determine if the loss of FlaA subunits impacts cell morphology. Wild-type bacteria of both serovars were observed as individual cells and had distinct hook- or helix-shaped ends. The *L. interrogans* serovar Manilae *flaA1* mutant retained a normal appearance, with hook structures observed at the ends of cells. In contrast, *flaA2* mutants were generally observed in chains of 2 to 5 bacteria; approximately 70% of cells of the *flaA2* mutants were found as chains of 2 cells (~48%), 3 cells (~14%), 4 cells (~6%), or 5 cells (~2%), in comparison to ~13% of cells of the wild-type strains (only as chains of 2 cells). In addition, hooked or helical ends were absent in the *flaA2* mutants (Fig. 2C). This observation indicates that FlaA2, but not FlaA1, is required for the maintenance of leptospiral cell hooked ends. When examined by electron microscopy, all mutants retained the typical helical morphology of wild-type strains (Fig. 2C).

The *flaA2* mutants lack translational motility. The wild-type, *flaA1* mutant, and *flaA2* mutant strains had similar cell growth kinetics under normal *in vitro* growth conditions (aerobic conditions in liquid EMJH medium at 30°C) (data not shown). The motility of mutant and wild-type bacteria in semisolid and liquid media was examined (Fig. 3). In semisolid plates of either 0.3% or 0.5% agar, the *flaA2* mutants showed greatly reduced motility compared to their respective parent strains. The *L. interrogans* serovar Manilae *flaA1* mutant demonstrated an intermediate level of motility on 0.3% agar plates compared to the wild type and the *flaA2* mutant.

When observed by dark-field microscopy in liquid EMJH medium, wild-type bacteria and *L. interrogans* serovar Manilae *flaA1* mutant bacteria displayed vigorous rotational or gyrational motility with occasional translational motility. In contrast, the *flaA2* mutants exhibited motility around the longitudinal axis but

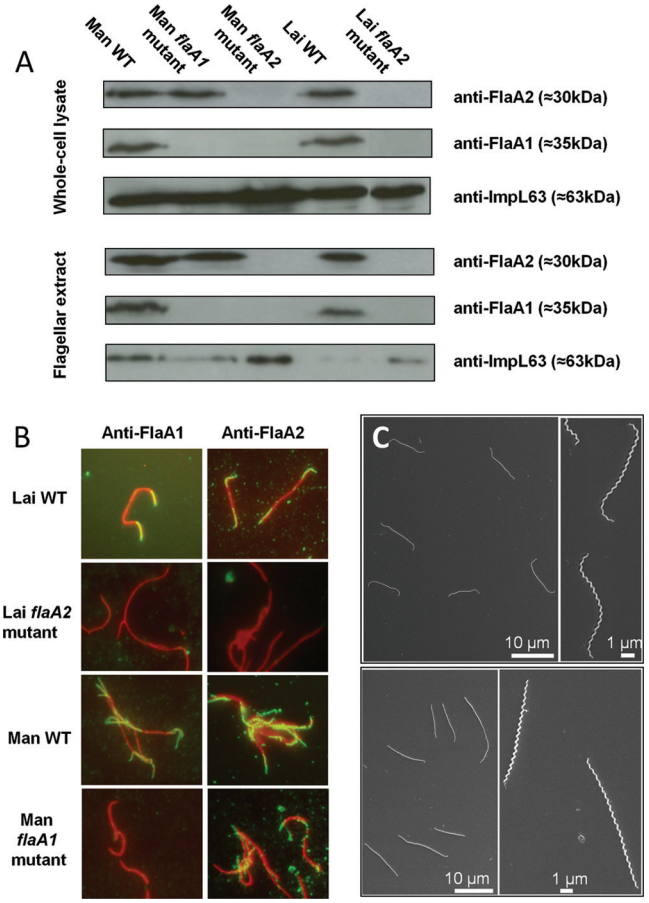

FIG 2 Characterization of *flaA* mutants. (A) Analysis of FlaA1 and FlaA2 expression by immunoblotting. Approximately 6 μg of whole-cell lysate or 2 μg of flagellum extracts was loaded into each lane. Samples were separated by SDS-PAGE and transferred onto a PVDF membrane. Detection was performed with rabbit anti-FlaA1 or anti-FlaA2 antisera. ImpL63 (*L. interrogans* transmembrane protein) antiserum was used as a loading control. (B) Immunofluorescence analysis of FlaA expression. Methanol-fixed cells were probed with anti-FlaA1 or anti-FlaA2 antisera, detected with Alexa Fluor 488-labeled secondary antibodies, and then mounted with a DAPI counterstain (recolored in red). Merged images are presented. (C) Analysis of cell morphology by electron microscopy. Shown are scanning electron microscope images of the *L. interrogans* serovar Manilae wild-type (WT) strain (top) and the *L. interrogans* serovar Manilae *flaA2* mutant (bottom).

lacked translational movement. These results demonstrate that both FlaA proteins contribute to motility.

FlaA-deficient flagella retain a sheath but do not form supercoils. Previous analyses of some spirochetes found that FlaA forms a sheath around the FlaB core and contributes to flagellum morphology (5, 14, 15). The loss of leptospiral FlaA may therefore result in thinner flagella with an altered flagellum shape.

Electron microscopy was used to examine the flagella of the *L. interrogans* serovar Manilae *flaA1* mutant and *flaA2* mutants (Fig. 4). Wild-type flagellum preparations were confirmed to contain both FlaA proteins by immunoblotting prior to analysis (Fig. 2A). The *flaA1* mutant and *flaA2* mutants had periplasmic flagella that were indistinguishable from those of the wild-type parent strain; there were no obvious differences in flagellum diameters (Table 1). Isolated leptospiral flagella typically form a supercoiled structure (26). However, while supercoiled flagellum forms were ob-

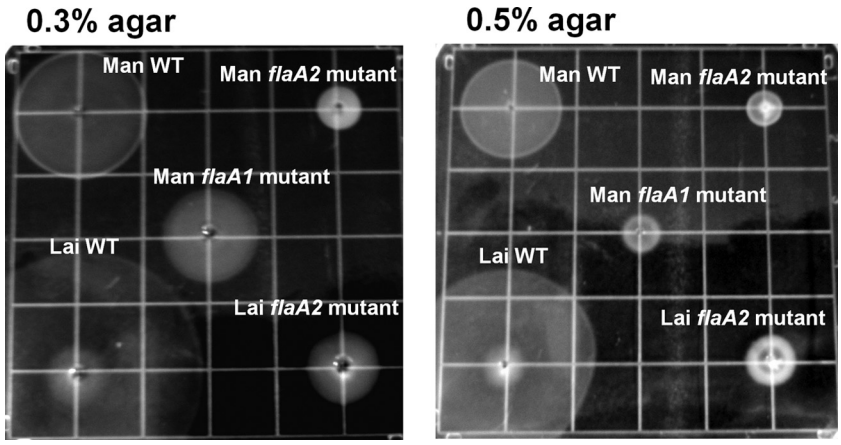

FIG 3 Analysis of bacterial motility. EMJH plates solidified with 0.3% or 0.5% agar were inoculated with the wild-type strain or the *flaA* mutant, and the colony diameter was measured after 19 days. A representative plate from one of three experiments is shown.

served for the wild-type and *flaA1* mutant strains (Fig. 4A), they were not found in the *flaA2* mutants, suggesting a loss of the rigidity of flagella of strains not expressing both the FlaA1 and FlaA2 proteins. Occasionally, two distinct structures (a normal diameter and a thinner one of approximately 16 nm) were seen for flagella from the mutant and wild-type strains (Fig. 4B). The normal-diameter structure is most likely a sheathed flagellum, while the thinner structure is the flagellar core with the sheath removed. This observation was consistent with the notion that leptospiral endoflagella consist of a core surrounded by a protein sheath.

The FlaA2 protein is required for virulence. The *L. interrogans* serovar Manilae *flaA1* and *flaA2* mutants were tested for virulence by intraperitoneal infection with 10^6 organisms in hamsters (Fig. 5A). The *L. interrogans* serovar Manilae *flaA2* mutant had a complete loss of virulence in the hamster model, while the wild type and the *L. interrogans* serovar Manilae *flaA1* mutant caused death 5 to 7 days after infection. No *flaA2* mutants were detected by qPCR in target organs (kidney and liver) at 25 days postinoculation (data not shown). The *L. interrogans* serovar Manilae *flaA1* mutant retained its virulence at a lower dose of infection (10^3 leptospires), with no significant increase in the hamster survival rate ($P < 0.0001$) or delay in the time of death compared to the wild type. The virulence of the *L. interrogans* serovar Lai *flaA2* mutant could not be tested because the *L. interrogans* serovar Lai parent strain was not virulent (data not shown). This result indicates that the production of FlaA2 alone is sufficient for virulence. However, we cannot exclude the possibility that a strain producing FlaA1, but not FlaA2, may retain virulence; such a mutant is not available.

To investigate the impact of the *flaA* deletion on dissemination *in vivo*, we assessed the bacterial burden by qPCR in the kidneys and liver. Groups of 4 hamsters infected intraperitoneally with 10^6 organisms (*L. interrogans* serovar Manilae wild-type, *flaA1*, and *flaA2* mutant strains) were euthanized at 5 days postinoculation (before the appearance of the first death among animals infected with the wild-type and *flaA1* strains). The bacterial burden in tissues of animals infected with the *L. interrogans* serovar Manilae *flaA1* mutant was similar to that in tissues of animals infected with the *L. interrogans* serovar Manilae wild-type strain; a higher number of leptospires was found in the liver than in the kidneys. In contrast, leptospires were at or below the detection limit in tissues of hamsters infected with the *L. interrogans* serovar Manilae *flaA2* mutant (Fig. 5B). These results suggest that the *L. interrogans* serovar Manilae *flaA2* mutant did not reach the tissues and/or was cleared from the body.

DISCUSSION

Leptospiral genomes contain about 50 motility-related genes, not including those involved in chemotaxis. The genes of the periplasmic flagellum are conserved in saprophytic and pathogenic *Leptospira* species and are considered core genes of the genus *Leptospira* (28). Most of these flagellar genes are homologous to structural proteins described previously for *Salmonella* and *E. coli* flagella (see Table S1 in the supplemental material). The leptospiral flagellum, like those of other spirochetes, was suggested to comprise a core of FlaB subunits (4 *flaB* genes in *L. interrogans*, 3 in *Treponema pallidum* and *B. hyodysenteriae*, and 1 in *Borrelia burgdorferi*) surrounded by a sheath of FlaA subunits (2 *flaA* genes in *L. interrogans* and *T. pallidum* and 1 *flaA* gene in *B. burgdorferi* and *B. hyodysenteriae*). However, there is limited experimental information on the structure and function of leptospiral flagella, in part due to the difficulty in genetically manipulating these bacteria. Some work has been conducted with the saprophyte *Leptospira biflexa*, where the mutagenesis of *flaB* resulted in aflagellate, nonmotile bacteria (27). The current study has examined the role of FlaA in the flagellum function, motility, and virulence of the pathogen *L. interrogans*.

The *flaA1* and *flaA2* transposon mutants generated in this study produced flagella, indicating that FlaA proteins are not required for the stable production of flagella, although flagella lacking FlaA were not fully functional. Similar results were obtained for *B. hyodysenteriae* and *B. burgdorferi* (22, 30). The absence of FlaA1 in the *flaA2* mutant is likely due to polar effects, although it could also be a result of FlaA1 protein instability resulting from the absence of an interacting protein partner in the flagellum (22). Interestingly, the *flaA2* mutants grew in chains of bacteria. This phenotype may be a result of reduced flagellum function or alternatively may be a stress response; previously, a *B. burgdorferi flaB* mutant was also observed to grow in long chains (21). It was also shown that polar flagellum biosynthesis influences cell division in *Campylobacter jejuni* (3).

Models for leptospiral motility differ depending on the sub-

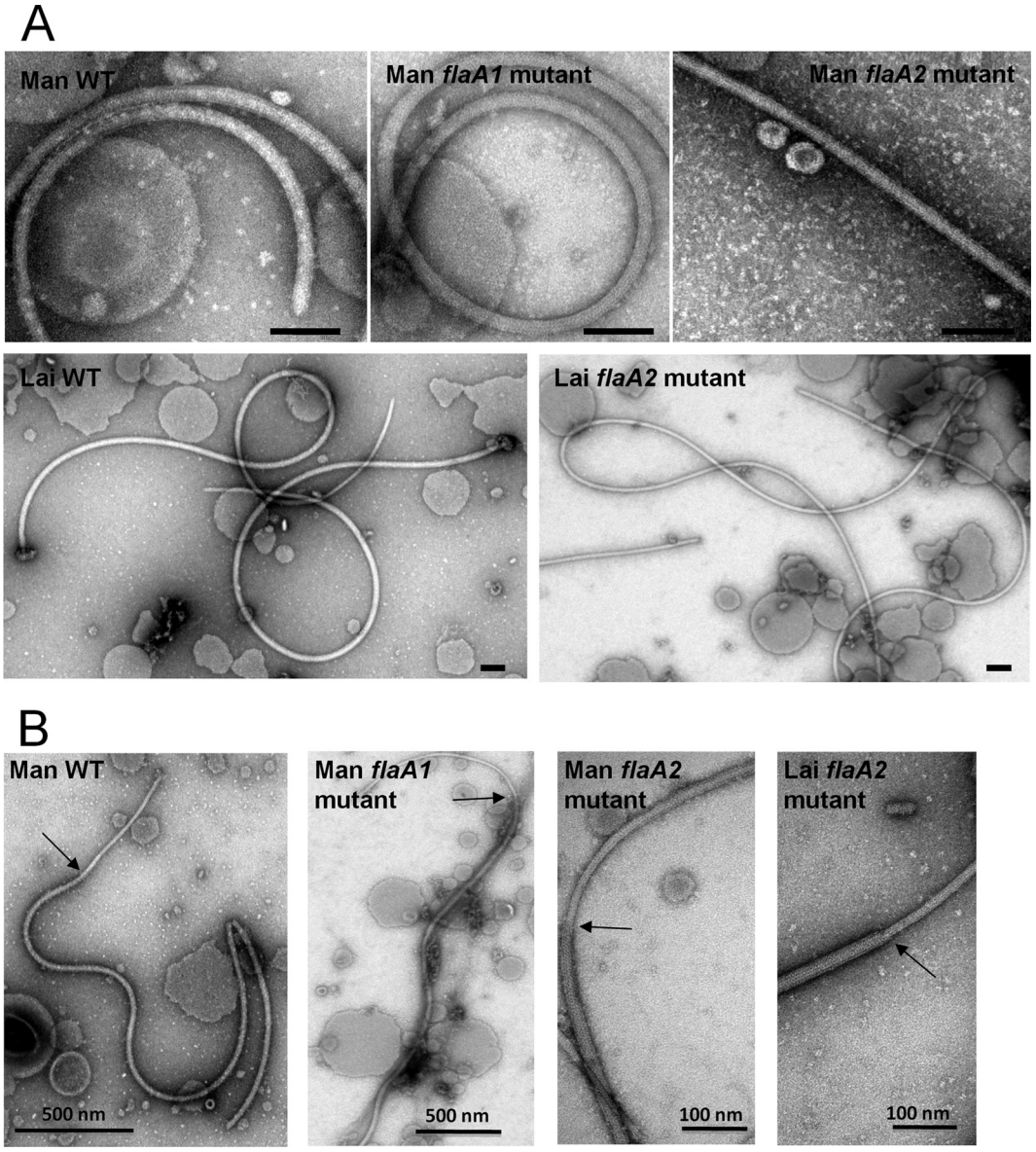

FIG 4 Analysis of flagella by transmission electron microscopy. (A) Periplasmic flagella of wild-type and mutant strains have the same diameter of approximately 22 nm. Bars represent 100 nm. (B) The outer sheath was partially removed (arrows) in some flagella from wild-type and mutant strains.

strate (reviewed in reference 12). In nonviscous liquids, *Leptospira* cells are temporarily distorted by flagellum revolution, deforming the end of the cell into either a hook or spiral (34). Bacteria undergoing translational movement have a spiral-shaped anterior end and a hook-shaped posterior end. Movement is thought to occur via the gyration of the anterior helical end of the cell, propagating a wave down the cell that drives it forward (6). FlaA2, but not FlaA1, was required for the formation of a hook/helix-shaped end to the cell. The absence of the helix at the end of the *flaA2* mutant cells prevented the propagation of the motive wave, thereby ablating motility in liquid media. In semisolid or viscous media, bacteria are thought to move by the counterrotation of the cell body around the flagellum, resulting in forward movement through a screw-like motion without slippage on the substrate (12). The *flaA2* mutants were nonmotile in semisolid medium yet retained a helical shape (i.e., they are still a "screw"), suggesting that the bacteria no longer rotate around the flagella but gyrate instead. The *flaA1* mutant had reduced motility in plates, suggesting that it had lost some rotational motility in viscous media.

TABLE 1 Flagellum diameters for wild-type leptospires and *flaA* mutants

L. interrogans serovar	Description	FlaA phenotype	Flagellum diam (nm) (SD)
Lai	Wild type	FlaA1$^+$ FlaA2$^+$	25.8 (1.4)
Lai	*flaA2* mutant	FlaA1$^-$ FlaA2$^-$	23.9 (3.3)
Manilae	Wild type	FlaA1$^+$ FlaA2$^+$	24.5 (2.4)
Manilae	*flaA1* mutant	FlaA1$^-$ FlaA2$^+$	24.1 (2.6)
Manilae	*flaA2* mutant	FlaA1$^-$ FlaA2$^-$	25.2 (2.0)

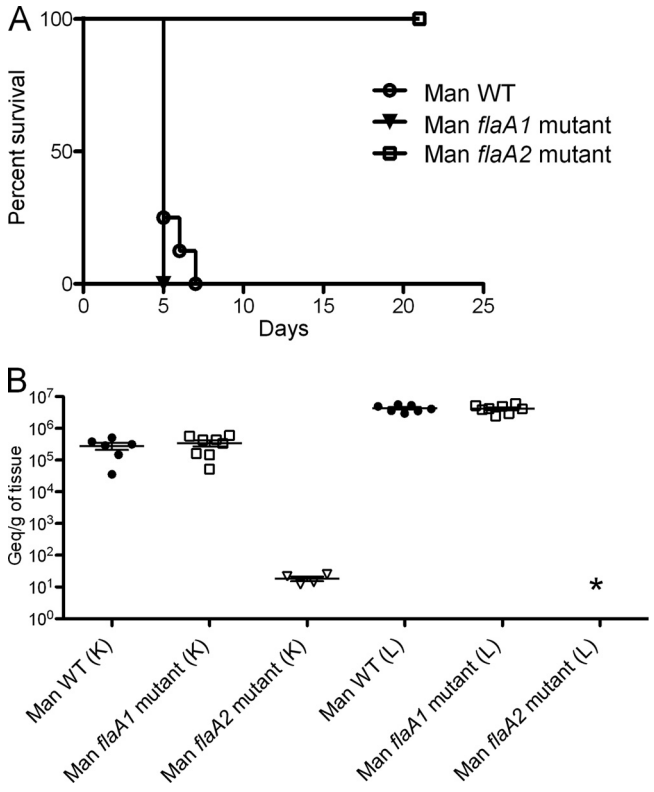

FIG 5 Virulence test for *L. interrogans flaA* mutants. (A) Survival curve of hamsters infected with wild-type *L. interrogans* serovar Manilae, the *L. interrogans* serovar Manilae *flaA1* mutant, or the *L. interrogans* serovar Manilae *flaA2* mutant. Groups of eight animals were injected intraperitoneally with 10^6 leptospires of either wild-type *L. interrogans* serovar Manilae or *L. interrogans* serovar Manilae *flaA* mutants. (B) Bacterial burden of organs after infection of hamsters with 10^6 leptospires. At 5 days postinoculation, the organs (K, kidney; L, liver) of animals were recovered, genomic DNA was extracted, and real-time PCR was carried out. Geq, genome equivalent; *, not detected (threshold cycle [C_T] values of >40).

The above-described motility defects may result from reduced flagellum stiffness in the *flaA* mutants. *B. hyodysenteriae flaA* mutants show reduced flagellum helicity, resulting in straighter flagella; this is thought to result from a diminished flagellum rigidity (15). The *L. interrogans* flagella lacking FlaA were also straighter. Reduced flagellum rigidity could diminish the ability of flagella to deform the cytoplasmic cylinder, resulting in less thrust (15). It is also possible that the FlaA proteins could stabilize one or more helical conformations of flagella, as found previously for *Brachyspira* (7). As the FlaA1 mutant (which still produces FlaA2) had a phenotype showing intermediate motility, hooked ends, and translational motility in liquid, each FlaA subunit may contribute to rigidity in a cooperative manner.

Our data are consistent with previous estimations of leptospiral flagellum diameters, ranging from 11.3 nm (core) and 21.5 nm (sheath) (33) to 13 to 16 nm (core) and 20 to 25 nm (sheath) (26). FlaA subunits were proposed to form the sheath of the leptospiral flagellum; however, we found that FlaA-deficient flagella retained the distinct sheath seen in wild-type bacteria and did not differ in thickness from wild-type flagella. This finding suggests that FlaA subunits are not the only components of the flagellum sheath. FlaA proteins were indeed part of the flagellum structure, as demonstrated by the presence of FlaA in flagellum preparations and in flagellum structures observed by immunofluorescence. However, the FlaA proteins could be readily removed from wild-type flagella by using a strong detergent (deoxycholate), as found previously for *B. burgdorferi* (11), suggesting that they are not integrated into the flagellum structure. These data contrast with results for *B. hyodysenteriae*, where a *flaA* mutant lacked a flagellar sheath and had significantly thinner flagella than the wild type (14, 15) but are similar to *B. burgdorferi*, where there was no change in flagellar thickness upon the loss of FlaA (22).

We found that the motility defect of the *flaA2* mutants was associated with reduced virulence in a hamster model of acute infection. This result was not surprising because leptospires are highly motile during infection and disseminate rapidly to all tissues of the host. In contrast, the nonmotile *flaA2* mutant appeared to be incapable of dissemination, as suggested by the absence of the mutant in target organs at 5 and 25 days postinfection. A recent study found that the mutagenesis of a flagellar motor switch protein resulted in a loss of motility and virulence in *L. interrogans* serovar Lai cells (17). Other spirochetes are also highly attenuated when motility is ablated (16, 19, 31).

Motility mutants of the *Leptospiraceae* were described previously. Faine and Vanderhoeden (10) found that an *L. interrogans* serovar Icterohaemorrhagiae isolate readily converted between a normal diffuse colony type and a compact colony. As described in the present study, motility-deficient bacteria formed compact colonies, had reduced hooks, grew in chains, had straighter flagella, and exhibited vigorous rotational or gyrational motility without translational motility. Similarly, Bromley and Charon (6) found that a strain of *Leptonema illini* (formerly *Leptospira interrogans* serovar Illini) could be forced to convert to a similar nonmotile form after treatment with mutagens, with similar changes in bacterial, flagellar, and colony morphologies. It is plausible that the motility defects described in the above-mentioned papers resulted from a mutation of the *flaA* genes or indeed other flagellum- or chemotaxis-related genes.

In summary, *flaA* transposon mutants were able to assemble periplasmic flagella, but their motility, virulence, and flagellar and cellular morphologies were affected, depending on whether FlaA1 or both FlaA proteins were expressed. Surprisingly, our data do not support the standard model of the spirochetal flagellar structure with a core composed of one or more FlaB proteins surrounded by a sheath of one or two FlaA proteins. Our results indicate that the structure of leptospiral flagella is more complex than those of other organisms. Further studies will be required to identify the components of the flagellum and their arrangement, role, and function.

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