The Spirochete FlaA Periplasmic Flagellar Sheath Protein Impacts Flagellar Helicity

CHUNHAO LI,1 LINDA CORUM,1 DAVID MORGAN,2 EVERETT L. ROSEY,3 THADDEUS B. STANTON,4 AND NYLES W. CHARON1*

Department of Microbiology and Immunology, Health Sciences Center, West Virginia University, Morgantown, West Virginia 26506-9177,1 Department of Biophysics, Boston University School of Medicine, Boston, Massachusetts 02118,2 Pfizer Central Research, Groton, Connecticut 06340,3 and National Animal Disease Center, United States Department of Agriculture, Agricultural Research Service, Ames, Iowa 5001014

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Spirochete periplasmic flagella (PFs), including those from Brachyspira (Serpulina), Spirochaeta, Treponema, and Leptospira spp., have a unique structure. In most spirochete species, the periplasmic flagellar filaments consist of a core of at least three proteins (FlaB1, FlaB2, and FlaB3) and a sheath protein (FlaA). Each of these proteins is encoded by a separate gene. Using Brachyspira hyodysenteriae as a model system for analyzing PF function by allelic exchange mutagenesis, we analyzed purified PFs from previously constructed flaA::cat, flaA::kan, and flaB1::kan mutants and newly constructed flaB2::cat and flaB3::cat mutants. We investigated whether any of these mutants had a loss of motility and altered PF structure. As formerly found with flaA::cat, flaA::kan, and flaB1::kan mutants, flaB2::cat and flaB3::cat mutants were still motile, but all were less motile than the wild-type strain, using a swarm-plate assay. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis indicated that each mutation resulted in the specific loss of the cognate gene product in the assembled purified PFs. Consistent with these results, Northern blot analysis indicated that each flagellar filament gene was monocistronic. In contrast to previous results that analyzed PFs attached to disrupted cells, purified PFs from a flaA::cat mutant were significantly thinner (19.6 nm) than those of the wild-type strain and flaB1::kan, flaB2::cat, and flaB3::cat mutants (24 to 25 nm). These results provide supportive genetic evidence that FlaA forms a sheath around the FlaB core. Using high-magnification dark-field microscopy, we also found that flaA::cat and flaA::kan mutants produced PFs with a smaller helix pitch and a stronger fluorescence signal compared to those of the wild-type strain and flaB mutants. These results indicate that the interaction of FlaA with the FlaB core impacts periplasmic flagellar helical morphology.

The spirochetes are a phylogenetically and morphologically unique group of bacteria (42). This phylum contains not only many medically important species such as Treponema pallidum and Borrelia burgdorferi but others that are commensal with arthropods such as termites and some that are free-living and reside in soil and water (5, 17, 28, 42). The distinctive spirochete structure has been characterized in detail. Outermost is a membrane sheath, and within this sheath is the cell cylinder and periplasmic flagella (PFs). The PFs reside between the outer membrane sheath and cell cylinder in the periplasmic space. Each PF is subterminally attached to one end of the cell cylinder. Several lines of evidence indicate that the PFs are directly involved in spirochete motility and that these organelles rotate in a manner similar to that of flagella of other bacteria (6, 8, 30). The size of the spirochete, the number of PFs attached at each end of the cell cylinder, and whether the PFs overlap in the center of the cell vary from species to species (5).

The protein composition of PFs is complex (see 29 for recent review). In contrast to the flagella of most bacterial species, PFs are comprised of two classes of proteins termed FlaA and FlaB (3, 8, 39). PFs consist of one to two FlaA proteins and three to four FlaB proteins. B. burgdorferi is an exception, as this species has one FlaA protein and only one FlaB protein (11, 14). These proteins share immunological and sequence similarity within a given class but not between classes. In addition, these classes show extensive conservation within the spirochete phylum (3, 8, 9, 14, 26, 33, 39, 40, 47, 52). Nucleotide sequence data and N-terminal amino acid sequence information indicate that FlaA is likely to be secreted into the periplasmic space via the general secretory pathway (4, 39, 40, 43). In contrast, FlaB proteins have significant homology to flagellin of other bacteria in the N-terminal and C-terminal regions. These proteins are not cleaved at the N terminus and are most likely secreted into the periplasmic space by a type-III secretion system (3, 8, 39, 40).

The structure of the spirochete PF is atypical compared to the flagella of other bacteria. In fact, it is among the most complex of bacterial flagellar filaments so far studied (8). PFs consist of a core surrounded by a protein sheath (18, 38, 52). The intact PF has a diameter of 18 to 25 nm and a core of approximately 11 to 16 nm in diameter (18, 38, 52). Several lines of evidence indicate that FlaA comprises the sheath and that the several FlaB proteins form the core. The evidence includes immunoelectron microscopy of PFs from Spirochaeta aurantia, Leptospira interrogans, and Brachyspira (formerly Serpulina, Treponema) hyodysenteriae (3, 26, 31, 52), partially disrupted cells of T. pallidum analyzed by electron microscopy and Western blot analysis (9), and analysis of T. pallidum purified PFs by a Western blotting method termed epitope bridging (2).

The function of the individual PF proteins is poorly understood, as gene transfer systems have only recently been estab-
DNA Primers

TABLE 1. Bacteria, plasmids, and oligonucleotides

| Strains, plasmids, or primers | Description | Source or reference |
|-------------------------------|-------------|---------------------|
| **E. coli**                    |             |                     |
| DH 5α                         |            | Gibco-BRL          |
| JM109                         | F⁻ hsdR17 supE44 thi-1 recA1 Δ(argF-lac)U169 φ80dlacZ ΔM15 λ⁻ endA1 recA1 gyrA96 thi hsdR17(rK mK) relA1 supE44 λ⁻ ΔlacF-prl ΔproAB lacFZΔM15 | Promega |
| **B. hyodysenteriae**          |             |                     |
| A20                          | Wild-type B204 serotype 2 (ATCC 31212) | T. Stanton |
| A203                         | Homologous recombination of pER199 at A201 chromosomal flaA locus | 44 |
| A204                         | Homologous recombination of pER 158 at A201 chromosomal flaA locus | 44 |
| A205†                        | Homologous recombination of pER 141 at A120 chromosomal flaB1 locus | This study |
| A208                         | Homologous recombination of pLB22 at A120 chromosomal flaB2 locus | This study |
| A211                         | Homologous recombination of pLN3 at A120 chromosomal flaB3 locus | This study |
| **Plasmids**                  |             |                     |
| pER187                       | The NiaIV 852-bp cat gene cloned into pUC18 Smal site | 45 |
| pTrep7                       | pUC19 with a 2,350-bp HindIII chromosomal fragment containing flaB1 | 44 |
| pER141                       | pTrep7 with kan gene replacing 589-bp BlgI fragment | 44 |
| pGEM-T                       | Ampβ, PCR cloning vector | Promega |
| pGEM2                        | pGEM-T with a 910-bp PCR fragment containing flaB2 gene | This study |
| pLNB2                        | pGEM-T with cat gene replacing 345-bp EcoRI and HindIII fragment | This study |
| pGFB2                        | pGEM-T with 1,185-bp PCR fragment containing flaB3 gene | This study |
| pLNB3                        | pGFB3 with cat replacing 278-bp EcoRI and HindIII fragment | This study |
| **DNA Primers**              |             |                     |
| CHB1                         | 5'-TTTGCACATGCGGTACG-3' (flaB2, 5'→) | This study |
| CHB2                         | 5'-TTAGTTTCTGATAAGCAAC-3' (flaB3, 3'→) | This study |
| CHB3                         | 5'-TTGCACATGCGGTACG-3' (flaB2, 5'→) | This study |
| CHB4                         | 5'-TTAGTTTCTGATAAGCAAC-3' (flaB3, 3'→) | This study |
| CHB5                         | 5'-TTGCACATGCGGTACG-3' (flaB2, 5'→) | This study |
| CHB6                         | 5'-TTGCACATGCGGTACG-3' (flaB3, 3'→) | This study |
| CHB7                         | 5'-TTGCACATGCGGTACG-3' (flaB2, 5'→) | This study |
| CHB8                         | 5'-TTGCACATGCGGTACG-3' (flaB3, 3'→) | This study |
| CAT1                         | 5'-TTGCACATGCGGTACG-3' (flaB2, 5'→) | This study |
| CAT2                         | 5'-TTGCACATGCGGTACG-3' (flaB3, 3'→) | This study |
| DFB3                         | 5'-TTGCACATGCGGTACG-3' (flaB2, 5'→) | This study |
| DFB3                         | 5'-TTGCACATGCGGTACG-3' (flaB3, 3'→) | This study |
| 16S-rRNA-1                   | 5'-TTGCACATGCGGTACG-3' (16S-rRNA, 5'→) | This study |
| 16S-rRNA-2                   | 5'-TTGCACATGCGGTACG-3' (16S-rRNA, 3'→) | This study |

* The A205 strain originally constructed in (44) was lost, and it was reconstructed using B204 as described in reference 44. flaAI previously described in (44) is referred to here as flaA.*

lished for spirochetes so that specific genes can be inactivated (19, 30, 49, 51). Recently, Rosey et al., using allelic exchange mutagenesis, inactivated two PF filament genes of the spirochete *B. hyodysenteriae*, the etiological agent of swine dysentery (44, 45). This spirochete has approximately eight to nine PFs, and those from the previous mutants in detail. We show that high-magnification dark-field microscopy. We show that FlaA influenced the helical shape of the assembled PFs.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** *Esherichia coli*, *Salmonella enterica* serovar Typhimurium LT2, and *B. hyodysenteriae* B204 and derived mutant strains are listed in Table 1. For convenience, the flaA1 gene (and its encoded protein) described by Rosey et al. (44) is referred to as flaA, as no other FlaA proteins have been detected in both analyses. *E. coli* and *S. enterica* serovar Typhimurium strains were grown in Luria Bertani medium or 2YT medium (35). *B. hyodysenteriae* cells were cultured in a Coy chamber using a premixed gas mixture of 90% N₂ with 10% CO₂ at 38°C. The equilibrated chamber contained 1 to 2% O₂ (measured with a model 3100 oxygen sensor [Bio systems, Inc., Middlefield, Conn.]) which is optimal for *B. hyodysenteriae* (50). *B. hyodysenteriae* cells were grown in brain heart infusion broth supplemented with 10% fetal bovine serum (BHI-FBS) (44). Kanamycin (200 µg/ml) and/or chloramphenicol (20 µg/ml) were added to the media of appropriate mutant strains. Mutant strains were cloned by harvesting individual colonies from Trypticase soy agar plates supplemented with 5% whole bovine blood (TSAB) instead of the previously used sheep blood (44).

**DNA manipulation and PCR conditions.** Enzyme modification, subcloning, and transformation were carried out by standard procedures (35). *B. hyodysenteriae* chromosomal DNA was isolated as previously described (44). For amplification of target genes, the primer sequences, target loci, and binding sites for target genes are listed in Table 1. DNA amplifications were performed with Taq (Promega) or Vent polymerases. PCR was carried out at 95°C for 3 min, followed by 35 cycles of 94°C for 1 min, 52°C for 1 min, 72°C for 2 to 3 min, and a final extension at 72°C for 8 min. The amplified DNA products were purified by using Qiagen PCR purification kits.
Electrotransformation of *B. hyodysenteriae*. The preparation of competent cells of *B. hyodysenteriae* and electroporation were carried out essentially as previously described (44). All manipulations except centrifugations and electroporations were performed in a cold chamber. M. Jacques (Université de Montréal, Montreal, Quebec, Canada) provided the Bio-Rad Gene Pulser II system at 1.5 kV, 25 μF, and 200 Ω resistance. Samples were prepared for electroporation by mixing 90 μl of electroporation buffer 15% glycerol to 2 mM sucrose. Following electroporation, 1 ml of prewarmed BHI-FBS was immediately added to caudetubes and transferred to a screw-cap tube (Falcon; 16 by 125 mm) containing a small stir bar. The recovered cells were incubated in the Coy chamber overnight with constant stirring. Chloramphenicol was added, and the cells were cultured for another 18 h. Approximately 0.5-ml samples were spread onto TSA plates containing chloramphenicol. Colonies recovered after 5 to 7 days incubation were inoculated into 3 ml of BHI-FBS broth containing chloramphenicol.

DNA cloning techniques and screening of the genomic library. Two degenerative primers were used to clone flaB3. The first primer was deduced from the variable region near the N-terminal amino acid sequence obtained by Edman degradation of the gel-extracted purified protein (C. Slaughter, Southwestern Medical School, Dallas, Tex.) (amino acid sequence, TGNSMT [Table 1, primer DFB2]). The second primer, which was directed to a conserved sequence in the C-terminal region common (amino acid sequence, MIATEN [Table 1 primer DRBI]) to both FlaB1 and FlaB2 of *B. hyodysenteriae*, was obtained using the PepTools alignment program. The 700-bp PCR product obtained after amplification was cloned into pGEM-T. Double-stranded DNA sequence analysis, BLAST comparisons with other FlaB proteins, and comparisons with peptide sequences obtained after trypsin digestion confirmed that the amplified DNA corresponded to flaB3. To obtain the entire flaB3 gene, a digoxigenin-labeled 1.5-mer PCR probe (random labeling kit [Boehringer Mannheim]) was used to screen a lambda ZAP II library (Stratagene) of *B. hyodysenteriae* DNA by standard methods (35). DNA from plasmids derived from positive plaques were further analyzed using Southern blotting and DNA sequencing. The GenBank Accession Number for *B. hyodysenteriae flaB3* is flh241832.

Northern blot analysis. Northern blot analysis was carried out using standard procedures (35). Approximately 500 ml of a culture of *B. hyodysenteriae* was harvested and washed with cold 0.067 M phosphate-buffered saline (pH 7.5 [PBS]). Total RNA was isolated and purified using Qiagen RNeasy midikit. The resulting cells (4.5 × 1010 cells/ml) were resuspended and washed twice with cold PBS. Following the second wash, the volume. The resulting cells (4.5 × 1010 cells/ml) were maintained on ice until use or stored at −80°C. Samples were prepared for electroporation by mixing 90 μl of electroporation buffer 15% glycerol to 2 mM sucrose. Following electroporation, 1 ml of prewarmed BHI-FBS was immediately added to cuvettes and transferred to a screw-cap tube (Falcon; 16 by 125 mm) containing a small stir bar. The recovered cells were incubated in the Coy chamber overnight with constant stirring. Chloramphenicol was added, and the cells were cultured for another 18 h. Approximately 0.5-ml samples were spread onto TSA plates containing chloramphenicol. Colonies recovered after 5 to 7 days incubation were inoculated into 3 ml of BHI-FBS broth containing chloramphenicol.

Proteins were isolated from *T. pallidum* by the Bio-Rad Gene Pulser II system at 1.5 kV, 25 μF, and 200 Ω resistance. Samples were prepared for electroporation by mixing 90 μl of electroporation buffer 15% glycerol to 2 mM sucrose. Following electroporation, 1 ml of prewarmed BHI-FBS was immediately added to cuvettes and transferred to a screw-cap tube (Falcon; 16 by 125 mm) containing a small stir bar. The recovered cells were incubated in the Coy chamber overnight with constant stirring. Chloramphenicol was added, and the cells were cultured for another 18 h. Approximately 0.5-ml samples were spread onto TSA plates containing chloramphenicol. Colonies recovered after 5 to 7 days incubation were inoculated into 3 ml of BHI-FBS broth containing chloramphenicol.

Electron microscopy. PFs and thin sections were examined by standard methods (36). In our analysis, we found a single wide band reacting with the FlaB antiserum (FlaB1 [37 kDa], FlaB2 [34 kDa], and FlaB3 [32 kDa]) (Fig. 1, lane a). Occasionally, the FlaB band appeared as a doublet by SDS-PAGE and Western blot. This FlaB doublet has also been observed by Rosey et al. (40). Proteins with molecular masses of 27, 35, and 29 kDa were consistently found in the PF preparations (Fig. 1, lane b), but these proteins failed to react with the FlaA or FlaB antiserum. Similar findings of a 29- to 30-kDa protein in PF preparations have been reported for PFs of *T. pallidum*, *T. phage- denis*, and *T. denticola* (39, 47). Because the 39- and the 29-kDa proteins were not detected on CsCl purification of the PFs (Fig. 1, lane c), these two proteins are likely to be loosely associated with the PFs. Two-dimensional gel electrophoresis and Western blot analysis clearly resolved the wide band at 44...
kDa into a smeared doublet (Figure 1, lane d). In contrast to T. phagedenis and T. denticola (39, 47), no additional major FlaB proteins were resolved with two-dimensional gel electrophoresis and Western blotting (Figure 1, lane d). The minor spot to the left of FlaB3 was analyzed in some detail. No differences were detected with this protein compared to FlaB3 by mass spectroscopy of digested peptides and N-terminal amino acid sequence analysis. In addition, mutations in the flaB3 gene led to its disappearance, indicating that the spot was encoded by flaB3. We consider that this spot is likely an artifact of two-dimensional gel electrophoresis.

Construction of flaB2 and flaB3 allele replacement vehicles and mutagenesis. Besides the flaA::cat, flaA::kan, and flaB1::kan mutants already reported (44), we constructed and also analyzed flaB2::cat and flaB3::cat deletion mutants. The sequence of the flaB2 gene has recently been determined (25). To target flaB2 for allelic exchange mutagenesis, it was first amplified by PCR using primers CHB1/CHB2 (Table 1). The product obtained was cloned into pGEM-T to form plasmid pGFB2 (Fig. 2a). flaB2 was disrupted by replacement of an internal 345-bp EcoRI/HindIII fragment with a 922-bp cat cassette. The insert was flanked with 318-bp upstream and 246 bp downstream of B. hyodysenteriae DNA. The orientation of the insert was confirmed by PCR and DNA sequence analysis, and the resultant linearized plasmid pLN2B was used for allelic-exchange mutagenesis.

The entire flaB3 gene was cloned and sequenced as described in Materials and Methods. Using the lambda ZAP II library and a labeled 615-bp flaB3 PCR-amplified product as probe, only one of several thousand plaques was positive. Sequencing the plasmid insert revealed an 840-bp open reading frame corresponding to a protein of 280 amino acids and a predicted mass of 30.5 kDa. This predicted size of FlaB3 ap-
impact on the assembly of the other FlaA and FlaB proteins in forming the PF. To further analyze the composition and structure of the PFs of these mutants, along with the newly constructed flaB2::cat and flaB3::cat mutants, the PFs of the mutants were purified and compared to the wild type. As with the previously described flaA::cat, flaA::kan, and flaB1::kan mutants, the flaB2::cat and flaB3::cat mutants were still motile as determined by dark-field microscopy. Western blot analysis of the PFs indicated that both flaA::cat and flaA::kan were deficient in the FlaA protein found in the wild type (Fig. 4). In addition, PFs from the flaB1::kan, flaB2::cat, and flaB3::cat mutants were specifically deficient in their respective cognate proteins. A similar pattern was obtained when whole-cell lysates were probed with the FlaA and FlaB antiserum (data not shown). These results indicated that not only do all mutants fail to specifically express the mutant gene product but intact PFs are assembled from the remaining PF proteins. We also determined the ratios of the Flab proteins relative to FlaA in the wild type and flaB1::kan and flaB2::cat mutant in purified PFs. We found that the wild type had a ratio of Flab1 to FlaA of 0.42±0.01, of Flab2 to FlaA of 0.22±0.04, and of Flab3 to FlaA of 0.45±0.07. In both the flaB1::kan and flaB2::cat mutants, these ratios did not markedly differ from those of the wild type. For example, in the flaB1::kan mutant, the ratio of Flab2 to FlaA was 0.34±0.04 and of Flab3 to FlaA was 0.56±0.04. Similar results were found with the flaB2::cat mutant (data not shown). These results suggest that the mutants did not compensate for a given Flab mutation by producing more of another Flab protein.

Northern blot analysis. The analysis of the PF proteins indicated that a mutation in one gene did not have a polar effect on the expression of the other flaA and flaB genes. These results suggested that these genes are not part of an operon. To further understand the expression of these genes, we analyzed the transcripts of flaA, flaB1, flaB2, and flaB3 using Northern blot analysis (Fig. 5). Each of these genes was found to synthesize a relatively small-sized mRNA of approximately 1 kb. These results indicated that each of the flagellin genes is transcribed as a single gene transcript and are consistent with flaB

![FIG. 3. PCR analysis of newly constructed flaB mutants compared with the wild-type strain B204. Ethidium bromide electrophoresis of DNA-amplified products using primers for analysis of (a) wild type and flaB2::cat mutant A208 and (b) flaB3::cat mutant A211 and wild type. PCR primers are listed in Table 1.](image)

![FIG. 4. Western blot analysis of purified PFs of mutants and wild type using B. hyodysenteriae FlaA and FlaB polyclonal antisera.](image)

![FIG. 5. Northern blot analysis of wild-type B204. Lanes indicate DNA probes used for hybridization.](image)
Ultransructure of the wild-type and mutant PFs. The PFs of the wild-type and \textit{flaA::cat} and \textit{flaA::kan} mutants have been reported to have the same diameter of approximately 15 nm (44, 45). These results, which were obtained by examining PFs from partially disrupted cells, are inconsistent with other evidence that FlaA forms a sheath around the FlaB core. Accordingly, we examined purified PFs of the wild-type and mutants by negative staining and electron microscopy. The PFs of the wild type consisted of filaments of approximately 25.5 nm in diameter (Fig. 6, left panel). Occasionally, some PFs were seen that were obviously thinner. In addition, some appeared thicker at one region and thinner at another region. These thin PFs and the thin regions on the thicker PFs had diameters of approximately 18.4 nm. The diameters of the PFs from the \textit{flaB1::kan} and \textit{flaB2::cat} mutants were similar to that of the wild type (Fig. 6). In contrast, the diameters of the PFs of the \textit{flaA::cat} mutant (19.6 ± 1.8 nm [Fig. 6, right panel]) were significantly thinner than those of the wild type and \textit{flaB1::kan} and \textit{flaB2::cat} mutants (Fig. 6). In addition, the \textit{flaA::cat} mutant PFs were similar in diameter to the occasional thin PFs seen in the wild type. Taken together, these genetic results support the immunoelectron microscopy findings that FlaA forms a sheath around the FlaB core in \textit{B. hyodysenteriae} (26, 31). They also support a similar conclusion reached with other spirochete species PFs (2, 3, 9, 52).

Periplasmic flagellar shape. Bacterial flagella, as well as PFs from several spirochete species, have been shown to be helical as determined by high-magnification dark-field microscopy (6, 7, 34). Each spirochete species has been shown to have left-handed PFs with a distinct helix pitch, helix diameter, and handedness. Because the PFs consist of multiple proteins, it is not known what role each protein species plays with respect to overall filament morphology. Accordingly, we determined the handedness, helix pitch, and helix diameter of the wild-type and mutant PFs by dark-field microscopy (Fig. 7a through c). We found that the PFs of the wild-type and mutants were all left handed. The helix pitch of the wild type was 2.84 ± 0.09 μm with a helix diameter of 0.83 ± 0.04 μm. The PFs from the \textit{flaB1::kan}, \textit{flaB2::cat}, and \textit{flaB3::cat} mutants were slightly smaller in helix pitch and diameter as compared to those of the wild type. In contrast, the PFs of both \textit{flaA::cat} and \textit{flaA::kan} mutants had a helix pitch and helix diameter significantly less than those of the wild type (Tukey’s honestly significant difference (HSD), \( P < 0.0001 \)). For example, the \textit{flaA::cat} mutant A203 had a helix pitch of 2.43 ± 0.15 μm and a helix diameter of 0.61 ± 0.07 μm. These results indicated that mutants deficient in FlaA have PFs with a markedly altered helical shape.

Number of PFs from wild type and mutants. The location of the individual FlaB proteins in a given PF is unknown. A number of possible structural models have been postulated (44). One model states that the PFs are heterogenous within a given cell, but homogeneous with respect to a given filament (44). Thus, for example, some of the PFs in a given cell could be composed of FlaAFlaB1, while others could be composed of FlaAFlaB2 and FlaAFlaB3. If PFs from the mutants were heterogeneous within a given cell, the \textit{flaB1::kan}, \textit{flaB2::cat}, and \textit{flaB3::cat} mutants should have fewer PFs than the wild type. To test for this possibility, we examined \textit{flaB1::kan} and \textit{flaB2::cat} mutant cells by thin-section electron microscopy. We found that the number of PFs per cell did not significantly differ from that of the wild type (Fig. 8). The mean number of PFs varied between by eight or nine PFs per cell. Because each mutant did not significantly produce more of the other FlaB proteins to compensate for its respective mutation (see above), it is unlikely that this number was achieved by overproducing one of the other FlaB proteins. These results are not consistent with the proposal that a given PF is composed of a single FlaB protein in association with FlaA; rather, the data suggest that a given PF contains FlaA and several different FlaB proteins (44).

Motility of wild type and mutants. Rosey et al. (44) previously reported that \textit{flaA::cat}, \textit{flaA::kan}, and \textit{flaB1::kan} mutants were motile as determined by light microscopy, but the behavior of these mutants as observed by dark-field microscopy appeared different than that of the wild type. We found that these mutants, along with the newly obtained \textit{flaB2::cat} and \textit{flaB3::cat} mutant strains, could translate both in PBS and in PBS containing 1% methylcellulose as observed by dark-field

![FIG. 6. Electron microscopic analysis of purified PFs from wild type and \textit{flaA::cat} mutant A203. The PF diameters in nanometers (mean plus or minus SEM) of the wild type and mutants were as follows: wild type, 25.57 ± 0.23; \textit{flaA::cat}, 19.62 ± 0.35; \textit{flaB1::kan}, 24.37 ± 0.28; \textit{flaB2::cat}, 24.02 ± 0.26; \textit{flaB3::cat}, not determined. \textit{flaA::cat} was significantly smaller than the wild type (\( P < 0.0001 \)).](http://jb.asm.org/Downloaded from)
microscopy. Thus, we found that inactivation of each of the genes which encode the major filament proteins still results in motile cells. Swarm plate assays were used to quantitatively assay motility. We found that the swarm diameters of the \textit{flaA::cat}, \textit{flaA::kan}, and \textit{flaB1::kan} mutants were slightly less than that of the wild type (Fig. 9). These results support those of Rosey et al. that the motility of the mutants was altered (44). The newly obtained \textit{flaB2::cat} and \textit{flaB3::cat} mutants also showed smaller swarms than that of the wild type. Of all the mutants analyzed, \textit{flaB2::cat} consistently showed a smaller swarm diameter than the diameters of the other mutants. These results indicated that although all the mutants can still assemble PFs, each appears somewhat deficient in motility, with the \textit{flaB2::cat} mutant having the most deficiency.

\textbf{DISCUSSION}

Spirochetes have historically been a difficult phylum of bacteria to study. These organisms generally require a rich medium for growth and have long generation times. Several important spirochete pathogens, including \textit{T. pallidum} and many of the oral \textit{Treponema} species, have yet to be continuously cultured (5, 42). Only in \textit{B. hyodysenteriae} and \textit{B. burgdorferi} has a gene exchange system been shown to occur by a mechanism other than via electroporation (19; D. Samuels, personal communication). Compared to the genetic techniques used to analyze function in other bacteria, the tools now available for spirochete gene analysis are relatively primitive. The experiments reported here exploit the use of electroporation and allelic-exchange mutagenesis as a means to better understand the function of specific PF genes in \textit{B. hyodysenteriae}. Because the structure and composition of the PFs of \textit{B. hyodysenteriae} (13, 25–27) are so similar to those of \textit{T. denticola} (47), \textit{L. interrogans} (37, 52), \textit{S. aurantia} (3, 4, 8, 41), and the more intractable \textit{T. pallidum} (39, 40), the results obtained are likely to be relevant to these other species.

Motility is likely to be an important virulence factor for spirochetes (22, 45, 48). These organisms can swim through gel-like viscous media, such as connective tissue, which inhibit the motility of most bacteria (1, 16, 24, 46). \textit{B. burgdorferi} penetrates the skin after a tick bite, and \textit{T. pallidum}, \textit{L. interrogans}, and \textit{B. burgdorferi} infect many tissues of the host, even the eye, which other organisms fail to invade (20, 24). In addition, genomic analyses of \textit{B. burgdorferi} and \textit{T. pallidum} indicate that these spirochetes have at least 4 to 6% of their
genes dedicated to motility and chemotaxis (11, 12). This relatively large percent of the genetic material aimed toward these functions reinforces the role of motility and chemotaxis in the survival of these bacteria within the hosts that they parasitize and in which, in some cases, they cause disease. For *B. hyodysenteriae*, mutants which have altered PFs and motility also are less virulent in mice, which again reinforces the role of motility for certain species of spirochetes (22, 45).

In our analysis of the PF proteins of *B. hyodysenteriae*, we found that two-dimensional gel electrophoresis enhanced resolution of the FlaA PF proteins, but no new FlaB proteins were detected. FlaA appeared as a smeared doublet of approximately 43 to 44 kDa. Our results are similar to those of Rosey et al., who found a FlaA doublet of approximately the same molecular masses (44). One of the proteins in the doublet is likely to be a precursor to the other rather than each being encoded by a separate gene. Specifically, inactivation of flaA inhibits synthesis of both proteins in the doublet. Because Northern blot analysis indicates that flaA is synthesized as a monocistronic mRNA, it is unlikely that the results obtained are related to a polar effect on gene expression. The broad band observed for FlaA may be related to post-translational modification. Along these lines, Li et al found that FlaA is likely to be glycosylated (31). Glycosylated proteins often appear smeared in two-dimensional gel electrophoresis. Our results differ from those of Koopman et al., who found two FlaA proteins with masses of 44 and 35 kDa (26). One possible explanation for this difference may be related to the strains tested; we used strain B204 as did Rosey et al. (44), whereas Koopman used strain C5 (26).

Our results, in conjunction with the previous results of Rosey et al. (44), indicate that mutants with single mutations in flaB1, flaB2, or flaB3 were still motile and could synthesize PFs. Moreover, based on the parameters measured, the morphology of the PFs was not markedly altered in the flaB mutants. Alignment of the deduced amino acid sequences of the three FlaB proteins shows between 37 and 51% identity, with the N-terminal region most conserved (data not shown). These findings, coupled with the mutational analysis, indicate that the FlaB proteins are at least somewhat redundant with respect to function. These results are analogous to flagella formation in *Caulobacter*, *Helicobacter*, and *Campylobacter* spp. Each of these species synthesizes multiple flagellin filament species, and inactivation of one of the encoding genes still results in the retention of motility and filament synthesis, although in each species the motility of the mutants is altered (21, 36, 54).

FlaA impacts the shape of the PFs as determined by measuring the helix pitch and helix diameter of the purified PFs. Because the helix pitch and diameter of the PFs of the flaA::cat and flaA::kan mutants were markedly less than those of the wild type, evidently the presence of FlaA results in an increase in helicity. It may be that the sheath structure per se influences the overall shape of the PFs. Along these lines, preliminary evidence with a double flaB mutant and with a fliG mutant indicate that the sheath can form a hollow tube independently of the core in *B. hyodysenteriae* (C. Li and N. W. Charon, unpublished data). Because bacterial flagella are often quasi-rigid and undergo helical transformations (34, 53), perhaps the sheath helps stabilize the FlaB helical core into one of these configurations for optimal thrust as it rotates between the outer membrane sheath and cell cylinder. We anticipate that future genetic experiments will allow us to determine the nature of the interaction of FlaA with the multiple FlaB proteins and how these proteins participate in achieving optimal cell motility.

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