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# Differential Regulation of the Multiple Flagellins in Spirochetes▽

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**The expression of flagellin genes in most bacteria is typically regulated by the flagellum-specific sigma[28] factor FliA, and an anti-sigma[28] factor, FlgM. However, the regulatory hierarchy in several bacteria that have multiple flagellins is more complex. In these bacteria, the flagellin genes are often transcribed by at least two different sigma factors. The flagellar filament in spirochetes consists of one to three FlaB core proteins and at least one FlaA sheath protein. Here, the genetically amenable bacterium *Brachyspira hyodysenteriae* was used as a model spirochete to investigate the regulation of its four flagellin genes, *flaA*, *flaB1*, *flaB2*, and *flaB3*. We found that the *flaB1* and *flaB2* genes are regulated by sigma[28], whereas the *flaA* and *flaB3* genes are controlled by sigma[70]. The analysis of a flagellar motor switch *fliG* mutant further supported this proposition; in the mutant, the transcription of *flaB1* and *flaB2* was inhibited, but that of *flaA* and *flaB3* was not. In addition, the continued expression of *flaA* and *flaB3* in the mutant resulted in the formation of incomplete flagellar filaments that were hollow tubes and consisted primarily of FlaA. Finally, our recent studies have shown that each flagellin unit contributes to the stiffness of the periplasmic flagella, and this stiffness directly correlates with motility. The regulatory mechanism identified here should allow spirochetes to change the relative ratio of these flagellin proteins and, concomitantly, vary the stiffness of their flagellar filament.**

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Spirochetes are motile bacteria that are able to swim in highly viscous gel-like environments. The medically important spirochetes include *Borrelia* spp. (relapsing fever and Lyme disease), *Brachyspira* spp. (formerly known as *Treponema* and *Surpulina* spp., which cause human and animal gastrointestinal diseases), *Leptospira* spp. (leptospirosis), *Treponema pallidum* and related subspecies (syphilis, pinta, and yaws), and oral *Treponema* spp. (periodontal disease) (5, 12, 33, 45, 59). The spirochetes swim by means of rotating periplasmic flagella (see references 6, 36, and 38 for recent reviews). These organelles reside in the periplasmic space and are attached subterminally to the ends of the cell cylinder. Periplasmic flagella (PFs) are structurally similar to the flagella of other bacteria, as each consists of a basal body-motor complex, hook, and filament (8, 27, 28, 39, 48, 55). However, the periplasmic flagellar filament is unique and is among the most complex of bacterial flagella. Specifically, in most spirochete species, the PFs contain at least one flagellar sheath protein, referred to as FlaA, and one to three core proteins, designated FlaB1, FlaB2, and FlaB3 (6, 35–37). In any given spirochete species, each FlaA and FlaB protein is encoded by an individual gene. There is no sequence similarity or antigenic cross-reactivity between FlaA and FlaB proteins (2, 15, 16, 35, 36, 49, 50, 56).

The individual periplasmic flagellar proteins have been studied in detail. FlaA proteins are 37 to 44 kDa and are similar between species based on amino acid sequences and antigenic cross-reactivity (6, 36, 38, 49). These proteins are likely exported to the periplasmic space by the type II secretion pathway, as their N-terminal amino sequences are cleaved and a typical peptidase I cleavage site is present near the N terminus (4, 18, 49). In contrast, FlaB proteins are exported to the periplasmic space most likely via the flagellum-mediated type III secretion pathway (6, 49). FlaB proteins comprise a family of well-conserved proteins. For example, the FlaB proteins of *T. pallidum*, *Treponema denticola*, and *Brachyspira hyodysenteriae* share 57 to 84% amino acid sequence identities (2, 16, 56). FlaB proteins are generally 33 to 41 kDa, and these proteins immunologically cross-react between one another in a given species and also between species (2, 6, 36, 49). Because FlaB proteins have sequence similarities to the flagellins of other bacteria, especially at the N- and C-terminal regions, they are considered to have an identical function in forming the helical flagellar filaments that rotate (36, 37, 64). Several studies have shown that the PFs and the PFs devoid of the FlaA sheath are left-handed helices and do indeed rotate (7, 21, 35, 37).

The regulation of flagellar synthesis is complex (1, 10). Studies with the paradigm models *Escherichia coli* and *Salmonella enterica* serovar Typhimurium indicate that a cascade control mechanism is involved in the regulation of flagellar genes. Within this hierarchy, the class I genes (*flhCD*) initiate the expression of the class II genes that encode structural proteins involved in the synthesis of the motor-basal body-hook complexes and the regulatory proteins. These regulatory proteins, FliA (also called sigma[28]) and FlgM (anti-sigma[28]), control the expression of the class III genes, which include the genes encoding flagellin and chemotaxis proteins. FlgM and FliA remain bound as a complex in the cytoplasm during basal body and hook synthesis. As FlgM exits cells via the flagellum export apparatus after completion of the hook, FliA is free to initiate

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the transcription of late genes. In addition, the disruption of one of the class II genes involved in the synthesis of the hook-basal body complex typically results in nonflagellated cells, as FlgM cannot be excreted.

The above control scheme is common to many motile bacteria, but there are some variations in several bacterial species that contain multiple flagellin units (13, 14, 24, 43, 51, 60, 63). For instance, *Helicobacter pylori* and *Campylobacter jejuni* have two flagellin genes (*flaA* and *flaB*), and *Agrobacterium tumefaciens* contains four flagellin genes (*flaA*, *flaB*, *flaC*, and *flaD*). In these organisms, at least two different sigma factors are involved in the regulation of their multiple flagellin genes (9, 22, 60). Similar to the above organisms, spirochetes also have multiple flagellin genes, and previous studies suggest that the regulatory hierarchy in spirochetes may be different from the well-studied paradigm model of the enteric bacteria (6, 36). For example, although the homolog of *fliA* has been found in *T. denticola*, *T. pallidum*, and *L. interrogans* (16, 52, 56) and $sigma^{28}$ consensus sequences have been identified upstream of several *flaB* genes, the FlgM homolog has not been found in any spirochete species. In contrast to the case for *flaB* genes, the promoters for *flaA* genes have $sigma^{70}$ consensus sequences (4, 18, 26, 32). The regulation of these flagellin genes has not been systematically investigated for any spirochete species that contains multiple FlaB proteins, such as *Treponema* and *Brachyspira* species.

The spirochete *B. hyodysenteriae*, the causative agent of swine dysentery, can be genetically manipulated by both allelic exchange mutagenesis and generalized transduction via a defective transducing phage (23, 58). These genetic tools and the recent availability of its genomic sequence have led this species to emerge as a model spirochete for genetic analysis (2). In addition, its PFs are similar in composition and structure to those of *T. denticola* and the uncultivable *T. pallidum* (3, 30–32, 49). As such, *B. hyodysenteriae* has been used to analyze the complex structure of the PFs and the contribution of the individual filament proteins to filament organization, filament stiffness, and motility (35, 37, 53). Previous experiments have shown that single and most double mutants have decreased motility. Only a *flaB1 flaB2* double mutant is completely nonmotile (35, 37). In this report, we continued to use *B. hyodysenteriae* to investigate the regulation of its multiple flagellin genes and to better understand its flagellar filament assembly. We found that the flagellin genes are controlled by two different sigma factors: *flaB1* and *flaB2* are regulated by $sigma^{28}$, and *flaA* and *flaB3* are controlled by $sigma^{70}$. These results were further supported by the analysis of a flagellar motor switch *fliG* mutant. In addition, in a manner somewhat similar to that seen with the recently characterized *flaB1 flaB2* double mutant (37), incomplete hollow filament structures composed primarily of FlaA continued to be synthesized in the *fliG* mutant.

## MATERIALS AND METHODS

**Bacteria strains and culture conditions.** *E. coli* and *S. enterica* serovar Typhimurium strains were grown in Luria-Bertani medium. *S. enterica* wild-type strain *SJ1103* and the nonflagellated *fliG* mutant *SJ1368* were provided by D. Blair, University of Utah (25, 29). The origin and culture conditions for *B. hyodysenteriae* strain B204 (wild type) have been previously described (35, 37, 53). *B. hyodysenteriae* strains were grown in brain heart infusion broth supplemented with 10% fetal bovine serum (BHI-FBS) or Trypticase soy agar plates supplemented with 5% whole blood (TSAB) in an atmosphere of 90% $N_2$, 10% $CO_2$, and 1 to 2% $O_2$. Chloramphenicol (20 μg/ml) was added to the medium for selecting *B. hyodysenteriae* mutants.

**Primer extension analysis.** Primer extension analysis was performed according to our previous studies (20, 34, 65). For the isolation of RNA, 500 ml of mid-exponential-phase *B. hyodysenteriae* cells were harvested and washed twice with phosphate-buffered saline (PBS; pH 7.4). Total RNA was isolated with Tris reagent (Sigma-Aldrich) according to the manufacturer's instructions, followed by treatment with RNase-free DNase I (Promega, Madison, WI). Further purification of RNA was performed using a Qiagen RNA isolation kit (Qiagen, Valencia, CA). Primer extension was carried out by using an AMV reverse transcriptase primer extension system (Promega) with a slight modification. Briefly, 20 pmol of the following reverse primers were used (the primers are approximately 80 to 150 bp from the putative promoter consensus): *flaA*, 5′-TAAACCGTAAGCAGTGC-3′; *flaB1*, 5′-TGCGTTGTGCGTTTATAGC-3′; *flaB2*, 5′-TGCATTTATAGCACTAATG-3′; *flaB3*, 5′-TATTGTTGATAATCATAC-3′. These primers were labeled with [$^{32}$P]ATP at 37°C and purified with a Qiagen nucleotide removal kit. For reverse transcription, 1 μl of labeled primer was mixed with 20 μg RNA and incubated at 58°C for 20 min. AMV reverse transcriptase and other reagents were then added, and the extension reaction was carried out at 42°C for 45 min. The products were precipitated with ethanol and dissolved in 5 μl standard DNA loading buffer. The primer extension products were run along with the sequence ladder generated from a *flaA* primer and a plasmid containing the *flaA* gene on a sequencing gel with a DNA sequencing kit (GE Healthcare, Piscataway, NJ).

**Cloning and sequencing of *B. hyodysenteriae fliG*.** To clone *B. hyodysenteriae fliG*, a pair of degenerative primers was designed based on two highly conserved regions of FliG (N terminus, QQEHP; C terminus, MFVFED) found in other bacteria. These two primers were designated DFG1 (5′-CADCADGADCAYCCNCADAC-3′) and DRG1 (5′-TYYTCDAANACDAACAT-3′). By using these two primers and *B. hyodysenteriae* chromosomal DNA as a template, a 740-bp PCR product was obtained and further cloned into pGME-T vector (Promega). A DNA sequence analysis and a BLAST comparison indicated that this PCR fragment shared high homology to *fliG* genes from other bacterial species. To obtain the entire *fliG* gene, a 433-bp *fliG* gene fragment was amplified by PCR (forward primer, 5′-CTAACCAACTGTACCTGC-3′; reverse primer, 5′-GGATACGATGCCAAAGAT-3′) and labeled with digoxigenin (Boehringer Mannheim, Germany). The labeled PCR fragment was used as a probe to screen the *fliG* gene from a lambda ZAP II DNA library of *B. hyodysenteriae*, which was kindly provided by T. Stanton (35).

A positive clone containing approximately 7 kb of *B. hyodysenteriae* chromosomal DNA was obtained and sequenced. One open reading frame (1,026 bp) encodes a 342-amino-acid protein with a calculated molecular mass of 38.9 kDa. BLAST analysis revealed that this protein shares a sequence identity of 29% to *E. coli* FliG and a 100% identity to the FliG homolog (BHWA1_00668) in the recently sequenced genome of the *B. hyodysenteriae* WA1 strain (2). In addition, the C-terminal region of *B. hyodysenteriae* FliG, which is essential for binding to other motor proteins, such as MotB and FliM, contains all the key residues (data not shown) that were identified in the FliG proteins of the enteric bacteria (41). The data, taken together, indicate that the sequenced gene is a *fliG* homolog.

**Construction of a plasmid for the targeted mutagenesis of *fliG*.** We used a previously described strategy to inactivate *fliG* (35, 37). Briefly, a 1,255-bp fragment containing the entire *fliG* gene was amplified by PCR with primers $MG_1/MG_2$ ($MG_1$, 5′-ACAAGCTATGATGAATGC-3′; $MG_2$, 5′-CACAACCATTTCATCTTC-3′) and cloned into pGEM-T vector (Promega). A 415-bp EcoRI/ClaI fragment was deleted and replaced by 920 bp of a chloramphenicol-resistant marker (*cat*), which was amplified by PCR with primers $CAT_1/CAT_2$ ($CAT_1$, 5′-GAATTCGAGCTCGGTAC-3′; $CAT_2$, 5′-ATCGATGCCTGCAGGTCGAC-3′). The insert was flanked by 433 bp upstream and 407 bp downstream. The orientation of the insert was confirmed by PCR.

**Purification of PFs, gel electrophoresis, and Western blotting.** The PFs were purified as previously described (35, 37). The *B. hyodysenteriae* FlaA and FlaB antisera and the monoclonal antibody against *B. hyodysenteriae* FlaBs were provided by M. Jacques (University of Montreal) and G. Duhamel (University of Nebraska), respectively. The *S. enterica* FliG antibody was obtained from the late R. Macnab (Yale University). The monoclonal antibody against the *B. hyodysenteriae* 35-kDa protein was prepared using standard methodology by injecting purified PFs derived from the mutant A204 (53) lacking the FlaA protein into mice. Lymphocyte fusions were carried out with myeloma cell line P3.X63.Ag8.653 (62). Immunoblots were developed by using horseradish peroxidase secondary antibody with an ECL luminol assay (GE Healthcare).

**Standard TEM and cryo-TEM.** Outer membrane-disrupted cells, thin sections, and purified PFs from the wild type and the *fliG* mutant LC-1 were examined by transmission electron microscopy (TEM) and cryo-TEM as previously described

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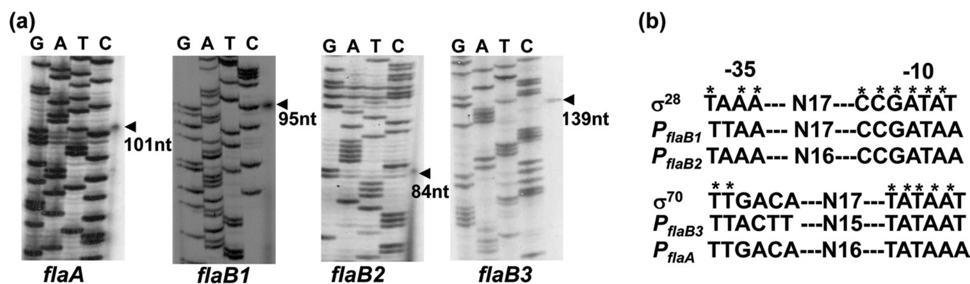


FIG. 1. The expression of four flagellin genes is controlled by two different promoters. (a) Primer extension assay to determine the transcriptional start sites of *flaA*, *flaB1*, *flaB2*, and *flaB3*. The DNA ladders were generated with DNA sequencing reactions using the *B. hyodysenteriae flaA* gene as a template. The primer extension products were run along with the DNA ladder on 6% DNA sequencing gels. The numbers are the sizes of cDNA products generated from the primer extension assay. (b) Comparison of the identified sequences to those of the consensus $\sigma^{28}$ and $\sigma^{70}$ promoters of *E. coli*. nt, nucleotide.

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(35, 37). Tobacco mosaic virus (TMV) was used as an internal control to measure flagellar diameter. The diameters of the PFs were directly measured from the digital images by using Openlab software (Improvision, Inc., Coventry, United Kingdom). No more than two measurements were made per filament, and at least 30 filaments were measured per sample. The measurements were calibrated by the diameters of TMV and were expressed as means plus or minus standard errors of the means. Significant differences were evaluated using analysis of variance (ANOVA) and the *post hoc* Tukey-Kramer test.

**qRT-PCR.** RNA sample preparations and quantitative reverse transcriptase PCR (qRT-PCR) were performed as previously described (55). Briefly, RNA samples were purified from the wild type and LC-1 as described above. Equal amounts of total RNA (approximately 0.5 μg) were used to generate cDNA by using an AMV reverse transcriptase primer extension system according to the manufacturer's protocol (Promega). To ensure that the RNA preparations were free of genomic DNA, reverse transcription was performed with or without reverse transcriptase. Quantitative PCR was carried out in triplicate on cDNA with SYBR green master mix (Qiagen) and a light cycler (Roche, Indianapolis, IN). The 16S rRNA gene was used as an internal control, and its reverse transcription product was diluted 5-fold for quantitative PCR analysis. The results are expressed as the threshold cycle at which the measured fluorescence increased above background. In addition, the products derived from qRT-PCR analysis were directly detected by electrophoresis using a 1% agarose gel. The following primers were used for qRT-PCR analysis: *flaB1* (forward, 5′-GATTC TGCTATGATTTCTAG-3′; reverse, 5′-TAGATACCGTTAGCAGCTTG-3′), *flaB2* (forward, 5′-TTCGCGGTTTACGTCAGGCT-3′; reverse, 5′-CAGCATC AGTATAGATACCG-3′), *flaB3* (forward, 5′-AACTGGAAATTCAATGAC-3′; reverse, 5′-GTGCGTTTCTAGTAGCTTG-3′), and 16S rRNA gene (GenBank accession number M57741) (forward, 5′-AGCAATCCGCTTTACGATG-3′; reverse, 5′-CGATGTCGCTCCATCAGACT-3′).

**Nucleotide sequence accession number.** The GenBank accession number for the *fliG* gene of *B. hyodysenteriae* is AAS89035.

## RESULTS

**Mapping the promoters of the four flagellin genes.** Our previous studies have shown that the four flagellin genes of *B. hyodysenteriae* are monocistronic (35). Sequence analysis from other laboratories postulated that upstream of *flaA* there is a sigma[70] promoter consensus sequence and that *flaB1* has a consensus sigma[28] promoter (17, 32). The promoter consensus sequences for *flaB2* and *flaB3* remain unidentified. We used primer extension analysis to accurately map the transcription start sites for all four of these filament genes. Sigma[70]-like promoter sequences were identified as transcriptional start sites in the upstream regions of *flaA* and *flaB3* (Fig. 1a and b). The −10 and −35 regions of *flaA* and −10 region of *flaB3* were well conserved compared to the typical sigma[70] consensus sequences; there was some degree of variation at the −35 region of *flaB3* (Fig. 1b). In contrast, typical sigma[28] promoter consensus sequences were found upstream of both *flaB1* and *flaB2*. These two promoters showed strong conservation at the −10 and −35 regions (Fig. 1b). These results indicate that the flagellar filament genes in *B. hyodysenteriae* are differently regulated: *flaA* and *flaB3* by sigma[70] and *flaB1* and *flaB2* by sigma[28].

**Analysis of a *fliG* targeted mutant.** The flagellar motor switch complex protein FliG plays a critical role in flagellar biosynthesis and motility. In the enteric bacteria, the inactivation of *fliG* disrupts the flagellum export apparatus and consequently turns off the expression of class III genes, such as *fliC*, due to the increase of intracellular FlgM (1, 10, 29). We tested whether the expression of the four filament genes in *B. hyodysenteriae* would also be inhibited in a *fliG* mutant. The *fliG* gene of *B. hyodysenteriae* was targeted using allelic exchange mutagenesis by replacing a 415-bp EcoRI/ClaI fragment with a chloramphenicol resistance cassette (*cat*) (35, 53). Ten independent clones were analyzed, and all of them contained *cat* inserted into *fliG*, as determined by PCR (data not shown). One clone (LC-1) was analyzed in detail. Western blot analysis indicated that whereas wild-type *B. hyodysenteriae* (strain B204) and *S. enterica* (strain *SJ1103*) cell lysates strongly reacted with the FliG antiserum, no reactivity was detected from the lysates of LC-1 and the *S. enterica fliG* mutant *SJ1368* (Fig. 2a). These results suggest that the synthesis of FliG was completely inhibited in LC-1.

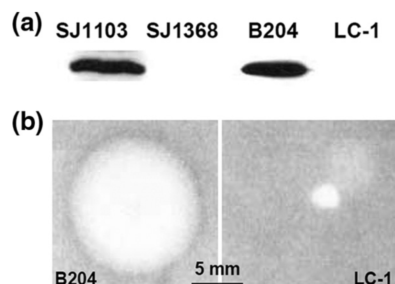


FIG. 2. Western blot and swarm plate assay. (a) Western blot analysis of the *B. hyodysenteriae* wild type (B204) and the LC-1 mutant using an antibody against *S. enterica* FliG. As controls, *SJ1103*, a wild-type strain, and *SJ1368*, a null *fliG* mutant of *S. enterica*, were used (25, 29). (b) Swarm plate assay. Four microliters of late log-phase *B. hyodysenteriae* cultures were deposited on Trypticase soy agar (0.3%) plates supplemented with 5% whole sheep blood (35). Diameters (in millimeters) of swarms were measured after 72 h of incubation.

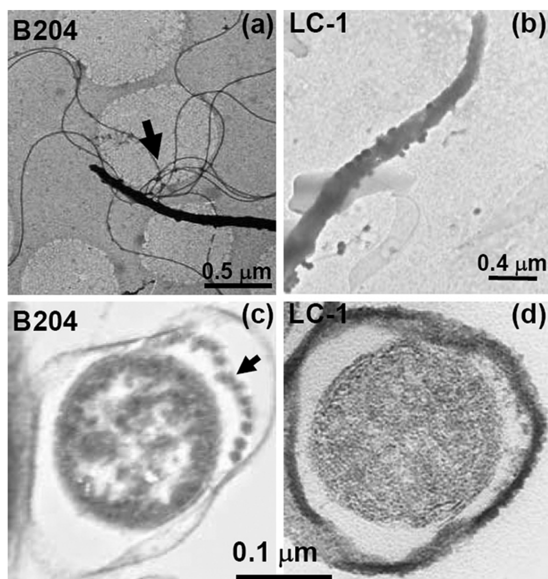


FIG. 3. Electron microscopy analysis of the LC-1 mutant. TEM of outer membrane-disrupted (a) wild-type (B204) and (b) LC-1 mutant cells. The outer membrane was removed by prior treatment with 1% Triton X-100. The samples were stained with 1% phosphotungstate as previously described (35, 53). TEM of thin sections of (c) wild-type and (d) LC-1 mutant cells. Arrows point to PFs.

The phenotype of LC-1 was analyzed in detail. Null *fliG* mutants in other bacterial species are typically nonmotile and lack flagella (29). We found that LC-1 was similar to such mutants. Dark-field microscopy revealed that LC-1 was completely nonmotile in all the standard tests for assaying *B. hyodysenteriae* motility (35, 53): liquid media, liquid media containing 1% methylcellulose, and 1% saline with or without methylcellulose. Swarm plate analysis revealed that the wild-type swarm diameter was 14.2 mm after a 3-day incubation. In contrast, the region of LC-1 remained at the inoculum site and was 2.8 mm in diameter (Fig. 2b). These results indicate that LC-1 was completely deficient in swarming. Transmission electron microscopy (TEM) revealed that no filaments attached to the cell cylinders (over 20 cells examined) were evident in the LC-1 mutant (Fig. 3a and b). In addition, whereas the PFs were seen in the cross sections of the wild type, these organelles were absent in LC-1 (Fig. 3c and d). These results indicate that FliG is essential for the motility and the assembly of PFs in *B. hyodysenteriae*.

We tested whether all four filament proteins accumulated in LC-1 by using Western blot analysis. As expected, all four filament proteins (FlaA, FlaB1, FlaB2, and FlaB3) were detected in the whole-cell lysates of the wild type. However, only FlaA and FlaB3 were found in the lysates of LC-1 (Fig. 4). The amount of FlaA in LC-1 was similar to that of the wild type. Although FlaB3 was detected in the mutant, its level was decreased by approximately 75%. In contrast to FlaA and FlaB3, the accumulation of FlaB1 and FlaB2 was completely inhibited in LC-1. These results are consistent with the above primer extension analysis and what is found for other bacteria: in a *fliG* null mutant background, the accumulation of proteins that are regulated by sigma$^{28}$ is specifically inhibited (1, 10, 29).

We directly tested if the complete inhibition of FlaB1 and

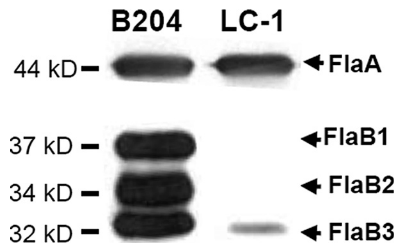


FIG. 4. Western blot of filament proteins of the LC-1 mutant. Approximately 10 μg of whole-cell lysates of B204 and the LC-1 mutant were loaded into each lane of an SDS-PAGE gel. The presence of flagellar filament proteins was detected using the polyclonal antiserum against the *B. hyodysenteriae* FlaA protein and a monoclonal antibody against FlaB.

FlaB2 accumulation in LC-1 occurs at the transcriptional level. We also tested whether the 75% decrease in the accumulation of FlaB3 is a reflection of a decrease in the transcription of *flaB3*. RT-PCR and quantitative RT-PCR analysis were used to measure *flaB1*, *flaB2*, and *flaB3* mRNA. We found that the expression of *flaB1* and *flaB2* genes was completely blocked in the LC-1 mutant, whereas the *flaB3* messenger was detected in this mutant (Fig. 5). qRT-PCR analysis confirmed these results, and it revealed that the *flaB3* mRNA in the mutant remained at the same level as that of wild type ($\Delta C_T$, the difference between threshold cycles of the wild type and LC-1, was 0.1). These results indicate that the inhibition of FlaB1 and FlaB2 accumulation in LC-1 occurs at the transcriptional level. In contrast, because the levels of *flaB3* mRNA in the wild type and the mutant were equivalent, the decrease in accumulation of FlaB3 in LC-1 takes place at the posttranscriptional level. Similar posttranscriptional modulation of FlaA and FlaB accumulation in certain mutants of *Borrelia burgdorferi* has been noted (47, 55). The data, taken together, strongly indicate that the flagellin genes of *B. hyodysenteriae* are differentially regulated.

**FlaA forms hollow tubular filaments.** We recently found that the *flaB1 flaB2* nonmotile mutant of *B. hyodysenteriae* synthesized incomplete hollow filaments composed primarily

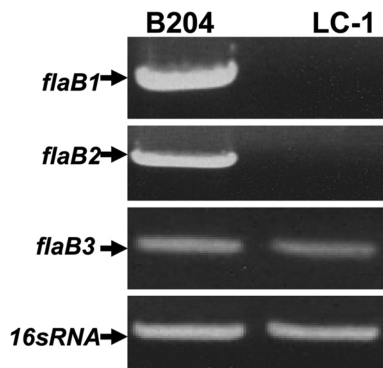


FIG. 5. Detection of *flaB1*, *flaB2*, and *flaB3* mRNA in the LC-1 mutant. cDNA was generated by reverse transcription reactions with random hexamer primers using total RNA prepared from wild-type and mutant LC-1 cells. For electrophoresis, PCR samples amplified for 30 cycles were analyzed with a 1% agarose gel and visualized with ethidium bromide.

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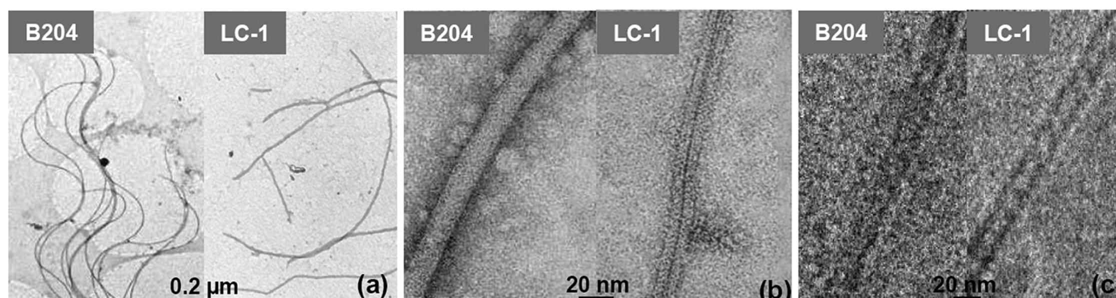


FIG. 6. TEM of the filaments isolated from the LC-1 mutant. Filament fractions isolated from wild-type and mutant LC-1 cells visualized (a) using low magnification and negatively stained with phosphotungstate and (b) using high magnification and negatively stained with uranyl acetate. (c) High magnification using cryo-TEM of the same fractions. Filament diameters were approximately 19 nm.

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of FlaA, a 35-kDa protein, and reduced the amount of FlaB3 (37). Since FlaA and a decreased amount of FlaB3 still accumulated in LC-1, we tested whether the remaining proteins form structures similar to those observed with the *flaB1 flaB2* mutant. TEM and negative-staining analyses revealed that the filaments purified from LC-1 were less helical than those of the wild type (Fig. 6a), which is similar to the filaments obtained from the *flaB1 flaB2* mutant. The internal region of the filaments from the LC-1 mutant stained dark, whereas the wild-type filaments stained primarily on the periphery (Fig. 6b). These results suggest that the filaments from LC-1 are hollow. We further analyze the filaments by cryo-TEM. The wild-type PFs appeared dense throughout the filament image, whereas only along the periphery of the filaments of LC-1 was there obvious protein deposition (Fig. 6c). These results are similar to those with the *flaB1 flaB2* mutant filaments and further substantiate the idea that the filaments from LC-1 are hollow. We measured the diameter of the filaments observed for LC-1. The diameter of the filaments from the mutant (19 $\pm$ 0.9 nm) was similar to that of the wild type (21 $\pm$ 1.2 nm). These results suggest that the filaments from LC-1 form hollow tube-like structures with the same diameter as the intact PFs of *B. hyodysenteriae* wild-type cells (37).

We analyzed the protein composition of the purified hollow filaments from LC-1 by SDS-PAGE and Western blotting. As shown in Fig. 7, only FlaA and a 35-kDa protein were detected in the filament preparation. The 35-kDa protein purifies along with the PFs, but its function is unknown (35, 37). The ratio between FlaA and the 35-kDa protein was approximately 1:0.5, which was similar to that of the hollow tubes isolated from the *flaB1 flaB2* mutant (37). In contrast to the *flaB1 flaB2* mutant, no FlaB3 was associated with the filaments in LC-1 even though FlaB3 was detected in its whole-cell lysates (Fig. 4). These results suggest that although FlaB3 is synthesized in the mutant, it was not assembled in the hollow tubes in LC-1. Taken together, these results indicate that FlaA in association with the 35-kDa protein polymerizes into the hollow tubular filaments.

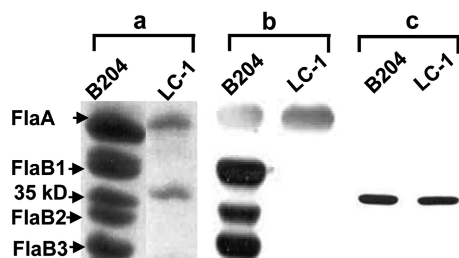


FIG. 7. Protein composition of the LC-1 mutant PF fractions. (a) Coomassie blue-stained gel of the purified PF fractions from the wild type and the LC-1 mutant. (b and c) Western blot of these fractions from the wild-type and mutant LC-1 cells probed with antibodies against FlaA and FlaB as described for Fig. 2 (b) or probed with a monoclonal antibody against the 35-kDa protein (c).

## DISCUSSION

In this study, we found that both sigma$^{28}$ and sigma$^{70}$ factors are involved in the regulation of the filament genes in *B. hyodysenteriae*. Primer extension analysis revealed that sigma$^{70}$ promoter sites mapped upstream of both *flaA* and *flaB3*, whereas sigma$^{28}$ promoters were identified upstream of both *flaB1* and *flaB2*. These results suggest that the expression of flagellin genes is regulated by different mechanisms. In support of this proposition, we found that both the *flaB1*- and *flaB2*-encoded proteins and mRNA were completely inhibited in the LC-1 mutant. Concomitantly, there was no inhibition of *flaA* and *flaB3* message. These results are consistent with the *flaB1* and *flaB2* genes being transcribed by sigma$^{28}$ and *flaA* and *flaB3* being transcribed by sigma$^{70}$. In other bacteria, the anti-sigma$^{28}$ factor FlgM modulates expression of sigma$^{28}$ consensus promoters (1, 10, 11, 54). Based on immune cross-reactivity with *S. enterica* FlgM, we recently identified a putative FlgM homolog in cell lysates of *B. hyodysenteriae* of the same mass (12 kDa) as that of *S. enterica* FlgM (C. Li and N. W. Charon, unpublished data). These results suggest that such a factor potentially modulates expression of *flaB1* and *flaB2* in *B. hyodysenteriae*.

The control of flagellin gene regulation in *B. hyodysenteriae* is different in many respects from that of the spirochete *B. burgdorferi* but shows some similarity. *B. burgdorferi* differs from other spirochetes as its PFs consist of only one FlaA and one FlaB species (19, 46). In addition, *B. burgdorferi* is unique among bacteria and other spirochete species, as no sigma$^{28}$ factor is present in its genome and all of its motility and chemotaxis genes are controlled by consensus sigma$^{70}$-like promoters (6). Moreover, posttranscriptional control is involved in flagellar gene expression (19, 47, 55). For example, the mutants

that fail to synthesize its hook-basal body complex have a marked inhibition in the accumulation of both FlaB and FlaA (55). However, its *flaA* and *flaB* mRNA levels are equivalent to that of the wild type, which strongly points toward posttranslational control. In contrast, the decrease in the accumulation of FlaB1 and FlaB2 in the LC-1 mutant directly correlates with a decrease in the corresponding mRNA. As noted, the decrease in FlaB3 shows posttranslational processing, which is similar to that found with *B. burgdorferi*.

Does the control pattern observed with *B. hyodysenteriae* flagellin proteins have any obvious significance? In the wild-type cells, the ratio of FlaA to FlaB1, FlaB2, and FlaB3 is 1:0.42:0.22:0.45 (35). Genetic and structural evidence suggests that FlaB1, FlaB2, and FlaB3 are distributed throughout the filament core and that each flagellin does not reside in one specific domain within the core. Each flagellin also contributes to the overall structure of the filament and optimal motility (35, 37). *B. hyodysenteriae* is one of the few species of bacteria with multiple flagellins for which the relative stiffness of its flagella has been determined for specific flagellin mutants (35). In addition, this flagellar stiffness has been shown to positively correlate with motility. The precise function of the FlaA sheath remains unknown, but it adds stiffness to the PFs (37). The three FlaB proteins also vary with respect to both their contribution to the overall stiffness of the flagellar filaments and their contribution to motility (37). For example, the PFs of *flaB1* mutant are 19% less stiff than those of the wild type, and this mutant shows a concomitant decrease in speed of 28%. In contrast, the PFs of the *flaB3* mutant are 7% less stiff than those of the wild type, and this mutant has a decrease of 13% in cell speed (37). Thus, FlaB1 contributes to flagellar stiffness and motility more than FlaB3. With FlaB1 under the control of sigma$^{28}$, and FlaB3 under the control of sigma$^{70}$, this ratio is likely to be modulated via the different control systems. Thus, *B. hyodysenteriae* has the potential to modulate the stiffness of the PFs in response to environmental signals by changing the ratios of FlaB1, FlaB2, and FlaB3 to one another. For *H. pylori*, a similar suggestion has been made with respect to modulating its multiple flagellin proteins in response to environmental stimuli (42, 44, 61). Conceivably, *B. hyodysenteriae* cells could synthesize PFs that are stiffer early in infection (higher ratio of FlaB1 and FlaB2 to FlaB3) before colonization. During the later stages of infection, once colonization and attachment of the cells to the intestine has occurred, the cells could switch to synthesizing PFs with a lower ratio of FlaB1 and FlaB2 to FlaB3. Such cells would have PFs that are less stiff, and the cells would be less motile than those in the early stage of infection.

In other spirochete species, both sigma$^{70}$ and sigma$^{28}$ consensus sequences have been identified upstream of several flagellin genes by both sequence (Table 1) and primer extension analysis. *T. denticola* has sigma$^{70}$ promoters at upstream of its *flaA* (TDE1408) and *flaB3* (TDE1457) genes and sigma$^{28}$ consensus sequences upstream of its *flaB1* (TDE1004) and *flaB2* (TDE1477) genes. Thus, the pattern in *T. denticola* is similar to one found with *B. hyodysenteriae*, but confirmation that these are indeed the start sites will depend on promoter mapping. With respect to *T. pallidum*, sigma$^{70}$ consensus sequences are upstream of *T. pallidum flaA* (TP0663), whereas a sigma$^{28}$ sequence is upstream of both *flaB1*, and a putative

TABLE 1. Putative promoters upstream of flagellin genes of *T. denticola* and *T. pallidum*

| Flagellin gene[a] | Consensus sequence |
|---|---|
| $\sigma^{70}$ | TTGACA--N17-----TATAAT |
| *T. pallidum flaA* | TTGACA--N18-----TAGAAT |
| *T. denticola flaA* | TTGACA--N17-----TATAAT |
| *T. denticola flaB3* | TTGTTA--N16-----TATAAT |
| $\sigma^{28}$ | TAAA----N17-----CCGATAT |
| *T. pallidum flaB1* | TCAA----N16-----CCGAAAA |
| *T. pallidum flaB2* | TCAA----N16-----CCGATAC |
| *T. denticola flaB1* | TTAA----N16-----CCGATAA |
| *T. denticola flaB2* | TAAA----N17-----CCGAAGA |

[a] The consensus sequences for $\sigma^{70}$ and $\sigma^{28}$ are those identified for *E. coli*.

*flaB2 flaB3* operon. Taken together, these studies (Table 1) and the promoter analysis of other spirochete flagellin proteins (4, 18, 26) suggest that *flaA* genes are transcribed by sigma$^{70}$, whereas there is variation in whether a given *flaB* gene is transcribed by sigma$^{28}$ or sigma$^{70}$.

The motor switch complex protein FliG plays an essential role in flagellar assembly and motility (1, 10, 29, 40). Null mutants of *fliG* are nonmotile and lack flagella, and we found results similar to those reported here with *B. hyodysenteriae*. A deletion mutant of *fliG* (*TDE1216*) in *T. denticola* was reported to be nonmotile. This mutant still synthesized PFs, but their number was markedly decreased (57). In most motile bacteria, only one homolog *fliG* is present. However, in several spirochete species, including *T. denticola*, two *fliG* homologs have been detected (15, 16, 52, 56). *B. hyodysenteriae* has only one *fliG* homolog (2) (R. Zuerner, personal communication). One possible explanation for the *T. denticola* results is that there is a redundancy between its two *fliG* genes such that mutation in one weakly complements the other.

The assembly of the PFs in *B. hyodysenteriae* and other spirochetes is complex. Hollow tubes composed primarily of FlaA were found in the flagellar fractions prepared from LC-1 and also from the *flaB1 flaB2* mutant (37). Moreover, the diameters of the hollow tubes from both mutants were identical to those of the intact PFs. The simplest model is that FlaA, which is secreted into the periplasmic space by a type II secretion pathway, polymerizes around the FlaB proteins, as these core proteins emerge through the hook-basal body complex via the type III secretion pathway. If there are insufficient FlaB proteins being secreted, or if FlaA forms the sheath at a higher rate than the secretion and polymerization of the multiple FlaB proteins, FlaA continues to polymerize and forms hollow tubes. As a corollary, if FlaB polymerizes faster than FlaA is able to decorate the core surface or if there is less FlaA than FlaB that is synthesized by the cells (as occurs in *B. burgdorferi* [17]), then the filaments will be partially unsheathed. Our results with LC-1 suggest that FlaA is able to form hollow tubes without detectable FlaBs. It may be that a small amount of FlaB3 is present to initiate polymerization of FlaA, but this limited amount may be beyond the level of detection. In addition, both this report and previous studies have shown that the 35-kDa protein is tightly associated with the PF preparations in *B. hyodysenteriae* (35, 37). Preliminary peptide mapping results indicate that the 35-kDa protein is a variable membrane protein, suggesting that there may be an interaction

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between the FlaA sheath and the outer membrane. We anticipate that further genetic studies will help define the function of the 35-kDa protein and determine the precise steps in the assembly of the PFs.


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