The Decrease in FlaA Observed in a *flaB* Mutant of *Borrelia burgdorferi* Occurs Posttranscriptionally

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The Lyme disease bacterium *Borrelia burgdorferi* is a motile spirochete with a flat-wave morphology. The periplasmic flagella, which are situated between the outer membrane sheath and cell cylinder, are essential for both the cell's wavy shape and motility. Here we focus on the structure and regulation of its periplasmic flagella. Previous studies have suggested that the periplasmic flagella consist of a polymer of the major filament protein FlaB and a minor protein, FlaA. We used immunoprecipitation methodology to present further evidence that FlaA is indeed a flagellar protein. In addition, in contrast to FlaA of the spirochete *Brachyspira hyodysenteriae*, *B. burgdorferi* FlaA did not impact the overall helical shape of the periplasmic flagella. We have previously shown that *B. burgdorferi* lacks the sigma factor-dependent cascade control of motility gene transcription found in other bacteria. To begin to understand motility gene regulation in *B. burgdorferi*, we examined the effects of an insertion mutation in *flaB* on the amounts of proteins encoded by motility genes. Of several motility gene-encoded proteins examined, only the amount of FlaA was decreased in the *flaB* mutant; it was 13% compared to the wild-type amount. Real-time reverse transcriptase PCR analysis indicated that this inhibition was not the result of a decrease in *flaA* mRNA. In addition, protein stability analysis suggested that FlaA was turned over in the *flaB* mutant. Our results indicate that the lack of FlaB negatively influences the amount of FlaA found in the cell and that this effect is at the level of either translational control or protein turnover.

Borrelia burgdorferi is a motile spirochete that is the causative agent of Lyme disease. The organelles for motility, the periplasmic flagella (PFs), reside between the outer membrane sheath and protoplasmic cell cylinder. Approximately 7 to 11 PFs are subterminally attached at each end of the cell, and these filaments overlap in the center of the cell (4, 27, 31). Motility is likely to be an important virulence factor for these spirochetes, as *B. burgdorferi* penetrates into tissues where other organisms fail to invade (11, 34). Moreover, motility has been postulated to be essential for the life cycle of these spirochetes in both the tick and the mammalian hosts (45, 55).

We are beginning to understand the dynamics of *B. burgdorferi* motility (see references 11, 42, and 45 for recent reviews). Recent results indicate that the PFs have both skeletal and motility functions (50, 61). Cells of mutants that are targeted in *flaB*, which encodes the major PF protein, are nonmotile and are rod shaped instead of being a flat wave as are wild-type cells (50, 61). During translational motility, backward moving waves are generated that are responsible for cell displacement (28). These waves result from the rotation of the relatively rigid left-handed helically shaped PFs closely juxtaposed to and interacting with the flexible protoplasmic cell cylinder: the PFs act as a gear generating waves along the cell cylinder from the anterior to the posterior end of the cell (11, 27, 28, 42). The bundles of PFs rotate asymmetrically, with the anterior bundle

rotating counter-clockwise and the posterior one clockwise as viewed along the length of the PFs from their distal ends towards their insertion points on the cell (11, 28, 40).

The PFs of *B. burgdorferi* have been analyzed in some detail. These organelles have been shown to be left-handed, with a defined helix pitch and diameter (12). Their major constituent is a polymer of FlaB, which is similar in sequence at the N- and C-terminal domains to PFs of other spirochete species as well as to flagellin of other bacteria (54, 63). Based on purification of PFs, FlaB was considered for several years to be the only filament protein. However, DNA sequence analysis indicated the presence of a *flaA* homolog (17, 20). In other spirochete species, FlaA forms a sheath around the FlaB core. In addition, FlaA markedly affects the shape of the PFs in *Brachyspira hyodysenteriae* with respect to helix pitch and helix diameter (9, 38, 41, 42, 54). We found that *flaA* was expressed in *B. burgdorferi*, and when a gentler method of isolation was employed FlaA copurified with the PFs (23). However, in contrast to results seen with other spirochete species (9, 38, 41, 42, 54, 59) considerably less FlaA relative to FlaB was present in these purified PFs. Using immunoprecipitation, we provide further evidence that FlaA is associated with FlaB. In addition, we find that in contrast to other spirochetes there is considerably less FlaA relative to FlaB not only in purified PFs but also within the whole cell. Finally, we show that FlaA does not affect the PF shape for *B. burgdorferi* as it does for *B. hyodysenteriae*.

Bacterial flagella are complex organelles that often comprise a disproportionately large amount of protein in a motile cell. As a result, bacteria have evolved elegant mechanisms of flagellar assembly and control. Specifically, motile bacteria possess a cascade system of transcriptional control (1–3, 14, 48,

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49). For example, in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium the cell senses specific environmental signals to commence expression of class 1 genes *flhC* and *flhD*. FlhC and FlhD then promote the initiation of transcription of class 2 motility genes (2, 14). These genes include, among others, those that encode the proteins that comprise the basal body apparatus. Two of the class 2 genes, *fliA* and *flgM*, play critical roles in regulating class 3 gene transcription. Specifically, *fliA* encodes sigma[28] that promotes transcription of class 3 genes, including the flagellar filament gene *fliC*. *flgM* encodes an anti-sigma[28] factor, FlgM (2, 14), which is excreted into the medium when the flagellar hook-basal body is complete. FlgM insures that the class 3 genes are not transcribed until the initial flagellar structure is intact, thus providing the cell with a mechanism to sense the state of flagellar synthesis and adjust its transcription accordingly (2, 14).

The regulation of motility gene expression in *B. burgdorferi* is unique, as it lacks the cascade control of flagellar gene transcription found in other bacteria. The results of several studies indicate that gene transcription of the numerous motility operons is initiated by the housekeeping transcription factor sigma[70] (11, 17, 20, 22, 24, 25, 42). Furthermore, no *fliA* homolog has been identified in its genome (17, 42). To begin to understand how the motility genes are regulated in *B. burgdorferi*, we are examining the effects of specific PF mutations on the synthesis of several motility and chemotaxis gene products. In this communication, we report our results determined with a previously described insertion mutant in *flaB* (50, 61).

MATERIALS AND METHODS

Bacterial strains and growth conditions. High-passage *B. burgdorferi* sensu stricto strain B31A, the *flaB* mutant MC-1, and the complemented *flaB* mutant MS17 have been previously described (8, 50, 61). Briefly, strain MC-1 has a *flgB* promoter-kanamycin cassette inserted into *flaB* of *B. burgdorferi*. This mutant is nonmotile and fails to synthesize PFs (50). Strain MS17 is a derivative of MC-1 containing a wild-type copy of *flaB* in the plasmid pED3. pED3 complements the *flaB* mutation in *cis*, i.e., only when the plasmid is integrated into the chromosome by a Campbell-type recombination event (61). Cells were grown in Barbour-Stoenner-Kelly II (BSK-II) broth or on plates at 32 to 34°C as previously described (50). *S. enterica* serovar Typhimurium strain SJW1103 is wild type for chemotaxis and flagellation, and strain SJW1368 Δ(*cheW-flhD*) is a master operon mutant which is aflagellate (18). These strains were the kind gift of the late R. Macnab, Yale University.

Gel electrophoresis and Western blot analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting with an enhanced chemiluminescent detection method (ECL; Amersham Pharmacia) were carried out as previously reported (23). Equal amounts (except as noted) of cellular protein in lysates, as determined by a Bio-Rad protein assay kit, were applied to each lane. Several monoclonal and polyclonal antibodies kindly provided by other investigators included the following: monoclonal anti-FlaA (B. Johnson, Centers for Disease Control and Prevention, Atlanta, Ga.), monoclonal anti-OspA and DnaK (J. Benach, State University of New York, Stony Brook), monoclonal anti-FlaB (H9724; A. Barbour, University of California, Irvine), rabbit anti-MotB (J. Carroll, Rocky Mountain Laboratories, Hamilton, Mt.), rabbit polyclonal anti-FlaA of *Treponema pallidum* (S. Norris, University of Texas, Houston), rabbit anti-CheA of *E. coli* (P. Matsumura, University of Illinois, Chicago), and rabbit anti-FliM of *E. coli* (D. Blair, University of Utah, Salt Lake City). Rabbit anti-CheY2 and anti-FlgE will be described elsewhere (M. Motaleb, M. Sal, and N. Charon, manuscript in preparation). Specific monoclonal or polyclonal reactivity to *B. burgdorferi* DnaK, FlaA, FlaB, FliI, MotB, CheA1, CheA2, and CheY3 has been previously reported (5, 10, 15, 25, 26, 40, 50, 53). Insertion mutations in *flaA*, *flaB*, *flgE*, *motB*, *cheA1*, *cheA2*, and *cheY3* verified antibody specificity by Western blotting (40, 50; M. Sal, M. A. Motaleb, and N. W. Charon, Abstr. Bacterial Locomotion and Signal Transduction Meeting VII, Cuernavaca, Mexico, p. 87, 2003; M. Motaleb, M. Sal, and N.

Charon, unpublished data). Anti-FliM reactivity was verified using *S. enterica* serovar Typhimurium strains SJW1103 and SJW1368.

Quantitative immunoblot assays. To quantify FlaA and FlaB in cell lysates, PFs were purified using the Triton X-100 method as previously described (23). PF proteins were separated on SDS-PAGE gels, and the bands corresponding to FlaA and FlaB were cut from the gels which were then electroeluted using ExElutor (Labnet International Inc., Woodbridge, N.J.). FlaA and FlaB were concentrated by centrifugation in Amicon Ultra concentrators (Millipore, Bedford, Mass.). Protein concentrations were determined using a Bio-Rad protein assay kit as well as SDS-PAGE gels stained with Coomassie brilliant blue R-250 with bovine serum albumin as a standard. Different amounts of purified FlaA, FlaB, and cell lysates were probed with monoclonal anti-FlaA and anti-FlaB by the use of Western blotting. Signals were quantified by FluorChem spot densitometry (Alpha Innotech, San Leandro, Calif.).

Immunoprecipitation and PF morphology. Immunoprecipitation was carried out as previously described (43). Approximately 5×10^9 *B. burgdorferi* cells were washed twice with 150 mM phosphate-buffered saline (pH 7.5)–5 mM MgCl$_2$ and were lysed by incubation at 37°C for 1 h in TSEA buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 0.05% sodium azide, pH 7.5) containing 1% Nonidet P-40 and 50 μg of phenylmethylsulfonyl fluoride/ml. The lysate was centrifuged (1,600 × *g* for 30 min, 25°C), and the pellet was resuspended in phosphate-buffered saline and sonicated for 1 min intermittently for 20 min in ice followed by centrifugation (14,800 × *g* for 30 min, 25°C). Approximately 200 μl of the supernatant fluid was incubated with 15 μl of the monoclonal anti-FlaB for 1 h at 25°C in the presence of 1% bovine serum albumin. Controls consisted of anti-FlaB incubated with no cell sonicate and the cell sonicate incubated with no antibody. After incubation, approximately 50 μl of protein A (Pansorbin [*Staphylococcus aureus* Cowan 1]; Calbiochem Behring, La Jolla, Calif.) was added to each sample and further incubated for 1 h at 25°C. The immunoprecipitates and controls were centrifuged at 1,600 × *g* at 25°C and washed three times with 1 ml of TSEA buffer containing 0.05% Tween-20. The final pellets were suspended in 60 μl of electrophoresis sample buffer, boiled for 5 min, and briefly centrifuged as before. For Western blotting, 10 μl of the supernatant fluids was applied to each lane of a SDS-PAGE gel. The left- or right-hand orientation, helical pitch, and helical diameter of the purified PFs were determined using dark-field microscopy as previously described (12, 23, 41). PFs containing both FlaA and FlaB were purified using the Triton X-100 method as previously described (23). PFs containing FlaB and no FlaA were purified with Sarkosyl as previously reported (12, 23).

Real-time quantitative RT-PCR and RNase protection assays. Total cellular RNA was extracted using an RNeasy kit from QIAGEN and then digested with Turbo DNase I (Ambion) at 37°C for 2 h. Equal amounts of total RNA (approximately 0.3 μg) were used to generate cDNA according to the protocol of the manufacturer (Promega). To ensure that the RNA preparations were free from genomic DNA, reverse transcription was performed with or without reverse transcriptase. Quantitative PCR was carried out in duplicate on cDNA with SYBR Green master mix (QIAGEN) and a LightCycler (Roche Biochemicals). A standard curve was generated using 10-fold serial dilutions (1.0 pg to 0.1 fg) from the *flaA* reverse transcriptase PCR (RT-PCR) product. 16S rRNA was used as an internal control. PCR primers for *flaA* (BB0668; GenBank accession no. U62900) were the following: forward primer, 5′-CACTAGTTCTTTTTGCTCAA-3′; reverse primer, 5′-ACTGCAGTACCTTTTTGACTC-3′. For 16S rRNA (GenBank accession no. L40596), the primers were the following: forward primer, 5′-GTGGCGAACGGGTGAGTAAC-3′; reverse primer, 5′-CCGTCAGCTTTCGCCATTGC-3′. Results are expressed with respect to the threshold cycle at which the measured fluorescence increased above background (57).

RNase protection assays were carried out using a Hybspeed RPA kit from Ambion according to standard procedures (23). Briefly, primers for the *B. burgdorferi flaA* and 16S rRNA were used to amplify specific regions by PCR. The amplified regions (305-bp *flaA* and 296-bp 16S rRNA) were cloned into pGEM-T Easy vector (Promega), and the resultant plasmids were linearized with SpeI (*flaA*) or NcoI (16S rRNA). Antisense riboprobe was synthesized with [α-^{32}P]UTP (Amersham Pharmacia) and a transcription kit (Ambion) and purified using a 5% polyacrylamide–8 M urea gel. Hybridization and digestion were done as described previously (23). Transcript levels were quantified using a Molecular Dynamics PhosphorImager.

Protein turnover. Techniques previously employed for other bacteria (33) were used to determine the stability of FlaA in growing cells. Wild-type and MC-1 strains were grown in BSK-II medium at 33°C until cells reached a density of 5×10^8 cells per ml. Cells (2 ml) were added to 40 ml of BSK-II medium containing spectinomycin (final concentration, 100 μg/ml) and incubated at 33°C. Samples were removed and processed for Western blotting at indicated time points.
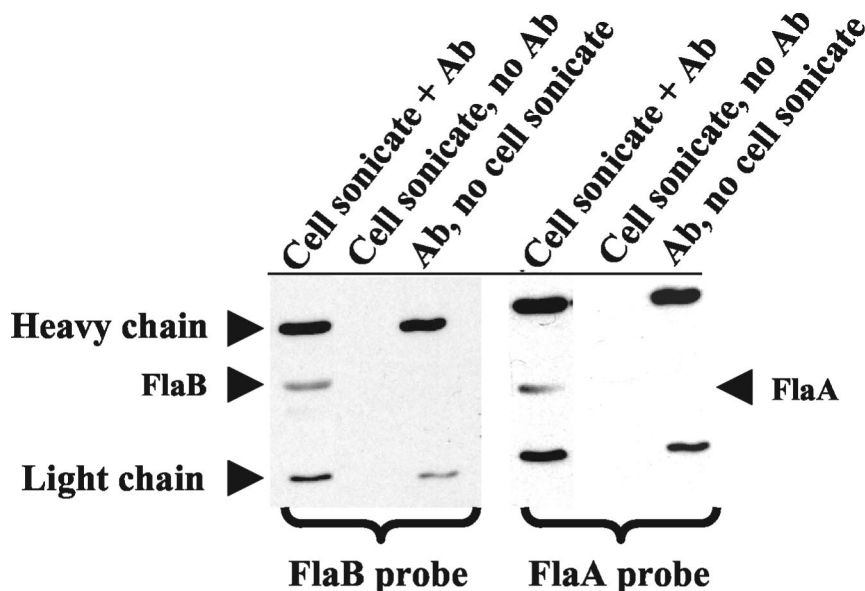
FIG. 1. Immunoprecipitation of FlaA with monoclonal H9724 anti-FlaB. Wild-type-cell sonicates were immunoprecipitated with FlaB antibody (Ab) and then probed with monoclonal antibody to FlaB (left panel) and FlaA (right panel) by Western blotting. The upper and lower bands of heavy and light chains of the primary antibody are identified. As controls without FlaB antibody, neither FlaB nor FlaA was precipitated (middle lanes).

RESULTS

Association of FlaA with FlaB. Ge et al. previously found that FlaA and FlaB copurified when the Triton X-100 procedure was used for PF isolation (23). To confirm that these two proteins were actually associated with one another, FlaB was immunoprecipitated from cell lysates of *B. burgdorferi* and the resulting immunoprecipitate was probed with anti-FlaB (Fig. 1, left panel) and anti-FlaA (Fig. 1, right panel) in Western blotting. We found that FlaA coprecipitated with FlaB (Fig. 1, right panel), while no coprecipitation occurred with another abundant *B. burgdorferi* protein, OspA (data not shown). These results suggest that there is a specific association of FlaA with FlaB in situ.

FlaA is a minor PF protein. SDS-PAGE of purified PFs was previously used to estimate the relative amounts of FlaA and FlaB in *B. burgdorferi*. In these studies, FlaA was found to be a minor component (23). These results are in contrast to those obtained with other spirochete species, for which the amount of FlaA is approximately equal to that of FlaB (9, 41, 53, 54, 59). One concern is that perhaps much of the *B. burgdorferi* FlaA was lost during the purification of PFs. Accordingly, we determined the amounts of both FlaA and FlaB in total cell lysates by the use of quantitative immunoblotting. Western blot results indicated that FlaA contributed less than 0.5% to the total protein of the cell (Fig. 2a). In contrast, FlaB was found to contribute 10 to 14% of the total cell protein (Fig. 2b). This relatively large amount of FlaB is in agreement with the results seen with other multiflagellated bacteria. In *S. enterica* serovar Typhimurium, which has approximately eight flagella per cell, the filament protein FliC contributes 8% of the total cell protein (51). *B. hyodysenteriae* has approximately 10 to 13 PFs per bundle, and these PFs have been estimated to contribute 5 to 10% of the total protein (39). Our results are

consistent with the PFs being a major constituent of *B. burgdorferi* and with these organisms accumulating considerably less FlaA than FlaB.

Influence of FlaA on PF structure. FlaA has been shown to affect the PF structure of *B. hyodysenteriae*. Specifically, PFs from mutants that lack FlaA have a helix pitch and helix diameter markedly different from those of the wild type (41). *B. burgdorferi* PFs were previously shown to be left-handed and have a helix pitch of 1.48 μm ± 0.03 μm and helix diameter of 0.28 μm ± 0.03 μm. These measurements were made with PFs isolated using Sarkosyl; such PFs lack FlaA (12). To examine whether FlaA influenced the PF shape of *B. burgdorferi*, we isolated PFs containing both FlaA and FlaB by the use of the gentler technique that replaces Sarkosyl with Triton X-100 (23). We found that PFs with both FlaA and FlaB were identical in shape to those isolated without FlaA (left-handed; helix pitch, 1.5 μm ± 0.028 μm; helix diameter, 0.33 μm ± 0.027 μm) (Fig. 2c). The results indicate that in contrast to the results seen with *B. hyodysenteriae*, FlaA does not influence the helical shape of the PFs.

Influence of *flaB::kan* on the synthesis of select gene products in motility operons. Previous results indicated that *B. burgdorferi* lacks the typical cascade control of flagellar gene regulation seen in other bacteria (11, 17, 20, 22, 24, 25, 42). To begin to understand how motility genes are regulated in *B. burgdorferi*, we examined the effects of a *flaB::kan* mutation (mutant MC-1) on the synthesis of other flagella and chemotaxis proteins. The flagellar filament gene *flaB* maps far to the left on the linear *B. burgdorferi* chromosome (17) and is transcribed as a monocistronic mRNA (19, 60). We first examined protein synthesis with respect to select genes in the large 21-kb *flgB* operon that encodes most of the hook, basal body, and motility genes. This operon maps approximately 180 kb away
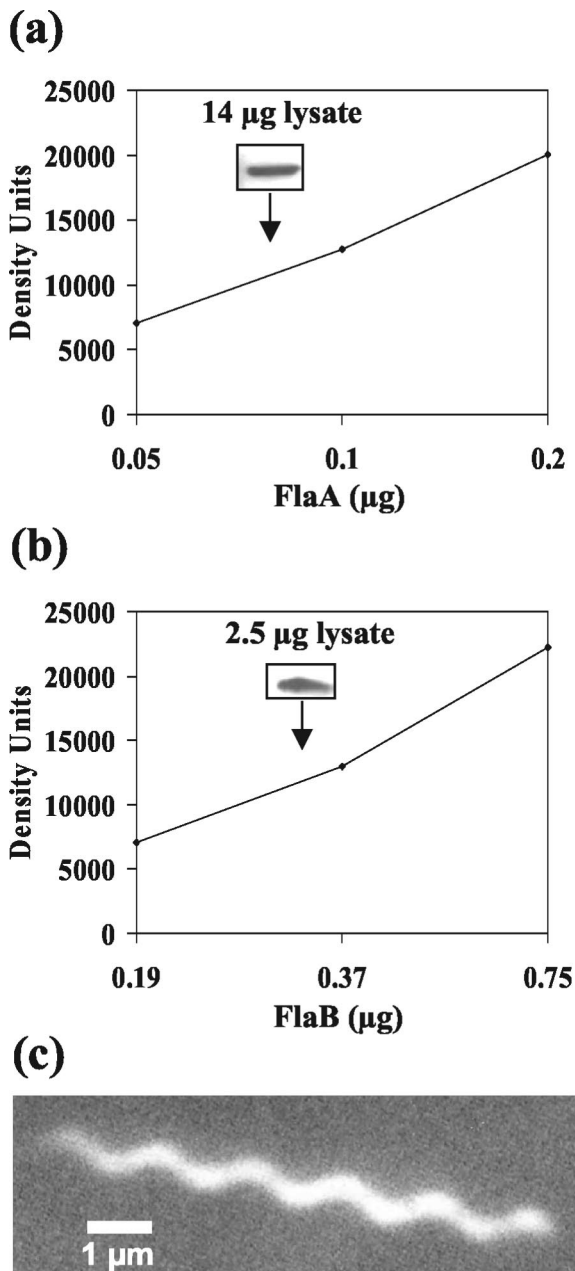
(a)

(b)

(c)

FIG. 2. Quantitative Western blot analysis of periplasmic flagellar protein levels in wild-type *B. burgdorferi*. (a and b) Different amounts of gel-purified FlaA, FlaB, and cell lysates were separated and probed with monoclonal anti-FlaA (a) and anti-FlaB (b) antibodies. The amounts of FlaA and FlaB in the cell lysates were calculated from the standard curves obtained from purified FlaA (a) and FlaB (b), respectively. Inserts illustrate Western blot reactivity at a specific amount of protein lysate. (c) Dark-field micrograph of a PF bundle containing FlaA and FlaB, which were isolated using the Triton X-100 method (23).

from *flaB* (17). Those genes chosen for analysis were *fliI*, *flgE*, *fliM*, and *motB*; each maps several open reading frames (ORFs) away from the others. FliI in other bacteria is associated with both basal body protein and flagellin export. FlgE is the flagellar hook structural protein which serves as a universal joint between the flagellar basal body and the filament (6).
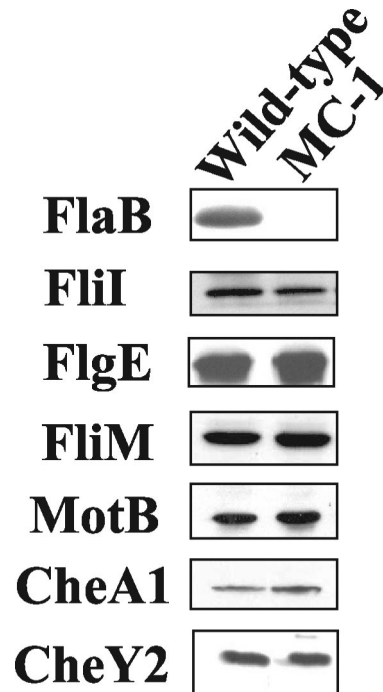

FIG. 3. Effect of *flaB::kan* on other motility and chemotaxis proteins. Equal amounts (10 μg) of cell lysates from wild-type and the *flaB::kan* mutant MC-1 strains were probed by Western blotting with antibodies specific for the indicated proteins. DnaK was used as an internal control as shown in Fig. 4b. The apparent sizes (in kilodaltons) of the proteins are as follows: FlaB, 41; FliI, 48; FlgE, 40; FliM, 37; MotB, 24; CheA1, 80; CheY2, 12.

FliM is part of the rotary apparatus of the motor, and MotB is the stator involved in energy transduction to the motor (6). Insertion mutations in *flgE* and *motB* led to the expected nonmotile phenotypes (Sal et al., Abstr. Bacterial Locomotion and Signal Transduction Meeting VII; Motaleb, Sal, and Charon, unpublished), indicating that these genes function in motility as predicted from their sequence. Western blot analysis indicated that although the synthesis of FlaB was inhibited in MC-1 as reported previously (50), no decrease in the amount of protein is evident with respect to FlgE, FliI, FliM, and MotB (Fig. 3). These results suggest that *flaB::kan* did not alter the synthesis of the encoded proteins for the select motility genes assayed in this operon.

We examined the effect of the *flaB* mutation on a putative chemotaxis operon, which is approximately 400 kb away from *flaB*. This operon consists of *cheW2*, *orf566*, *cheA1*, *cheB2*, *orf569*, and *cheY2*. Preliminary results indicate that mutations in *cheW2*, *orf569*, and *cheY2* negatively impact chemotaxis (C. Li, M. Motaleb, and N. Charon, unpublished data). However, although Western blot analysis indicated that CheA1 was produced it was found not to be essential for chemotaxis (40). We tested whether *flaB::kan* affected the expression of CheA1 and CheY2. Western blot analysis indicated that the levels of CheA1 and CheY2 in the MC-1 mutant were equal to those in the wild type (Fig. 3). These results indicate that *flaB::kan* did not influence the amounts of CheA1 and CheY2.
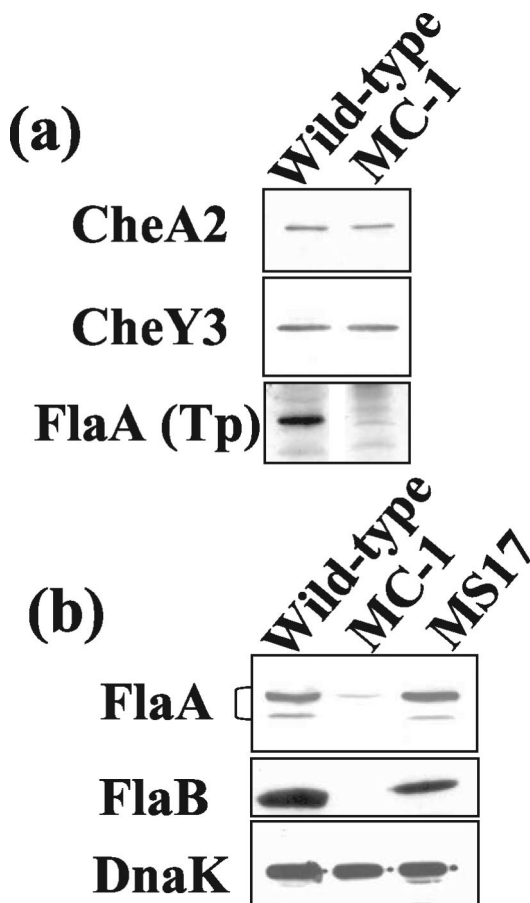
FIG. 4. Effect of *flaB*::*kan* on proteins encoded by genes in the *flaA* operon. (a) Western blot analysis of wild-type and MC-1 strains. FlaA was probed with polyclonal *T. pallidum* FlaA antiserum (Tp). (b) Western blot probed with monoclonal antibodies anti-FlaA, FlaB, and DnaK to the wild-type strain, MC-1, and the complemented strain MS-17. DnaK was used as an internal control. For FlaA, FlaB, and DnaK detection in the wild-type and MS-17 strains, approximately 10 μg of cell lysate was loaded into each lane. For strain MC-1, 20 μg of cell lysate was loaded into the lane for FlaA and 10 μg was loaded for DnaK. The apparent sizes (in kilodaltons) of the proteins are as follows: CheA2, 98; CheY3, 10; FlaA, 38; DnaK, 72.

Protein synthesis of select genes in the *flaA* operon. Another relatively large operon consists of *flaA-cheA2-cheW3-cheX-cheY3* (20, 22, 23). It maps approximately 550 kb away from *flaB*. Targeted mutations in *cheA2*, *cheX*, and *cheY3* markedly affect chemotaxis in *B. burgdorferi* (40; M. A. Motaleb and N. W. Charon, unpublished data). We tested the effect of the *flaB*::*kan* mutation on the synthesis of FlaA, CheA2, and CheY3. Western blot analysis indicated that both CheA2 and CheY3 in strain MC-1 were synthesized at levels equivalent to that for the wild type (Fig. 4a). However, the results obtained with Western blots probed with either a heterologous polyclonal FlaA antiserum (Fig. 4a) or a monoclonal antibody directed to *B. burgdorferi* FlaA (Fig. 4b) indicated that the level of FlaA in MC-1 was markedly decreased. When the monoclonal antibody was used, FlaA often formed a doublet, with the major band migrating to approximately 38 kDa and the minor band at 37 kDa. With the monoclonal antibody, approx-
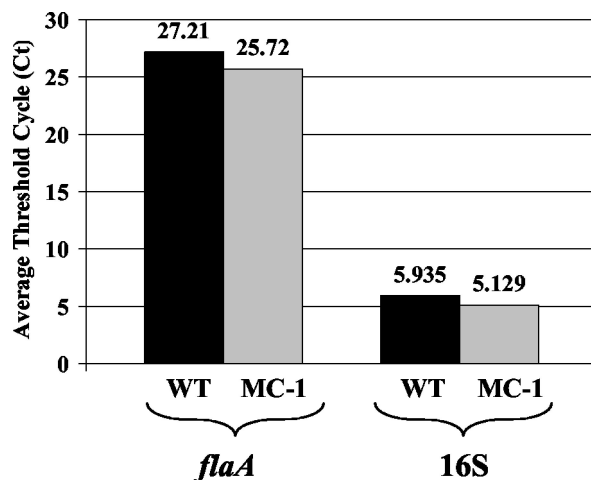

FIG. 5. Detection and quantification of relative amounts of *flaA* transcript in the wild-type (WT) and MC-1 strains using real-time RT-PCR. Average threshold cycle (Ct) values were calculated from duplicate samples in each experiment.

imately 15 μg of protein lysate loaded in the gel lane was necessary for detection of FlaA in strain MC-1. No FlaA in strain MC-1 was seen with the polyclonal antiserum under the same conditions. Quantitative immunoblotting analysis indicated that the amount of FlaA in MC-1 was approximately 13% of the amount seen with the wild-type strain (Fig. 4b). To further test whether this inhibition of FlaA accumulation was specifically related to *flaB*::*kan*, we tested the *flaB*::*kan*-complemented strain MS17. This strain is wavy and motile and has PFs (61). We found that the level of FlaA in strain MS17 was equivalent to that seen with the wild-type strain (Fig. 4b). These results suggest that the *flaB*::*kan* mutation specifically decreases FlaA accumulation in *B. burgdorferi*.

Transcription of *flaA* in strain MC-1. One mechanism that could explain the reduced FlaA protein levels in the *flaB*::*kan* mutant is inhibition of *flaA* transcription. To test for this possibility, mRNA levels were measured for the wild-type strain and *flaB*::*kan* mutant by both quantitative RT-PCR and RNase protection assays. 16S rRNA was used as a control in both assays. Quantitative RT-PCR results indicated that the level of *flaA* transcript in MC-1 was similar to or slightly greater than that seen with the wild-type strain (Fig. 5). Furthermore, RNase protection assay results yielded PhosphorImager units for the wild-type and MC-1 strains of 2,150 and 2,020 for *flaA* and 1,175 and 1,015 for 16S rRNA, respectively. These results taken together reveal that the *flaB*::*kan* mutation did not negatively influence *flaA* mRNA synthesis. The results are also consistent with the lack of inhibition of downstream gene expression in this operon in strain MC-1, as exemplified by the results seen with CheA2 and CheY3 (Fig. 4a).

Posttranscriptional control of FlaA. Two explanations are evident with respect to the lack of FlaA protein detected in MC-1. One hypothesis states that FlaA synthesis is controlled at the translational level. For example, perhaps FlaB is an activator for translation of FlaA. Alternatively, perhaps FlaA is rapidly degraded without FlaB. To test for possible turnover, spectinomycin was added to growing cells and the stability of
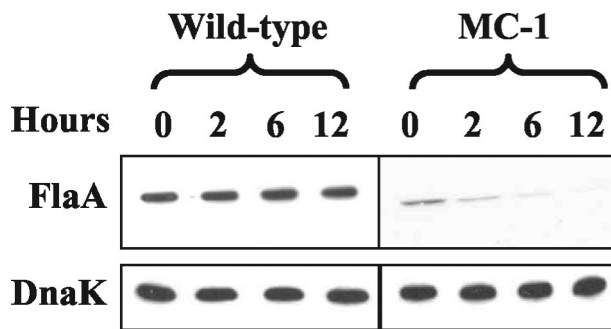
FIG. 6. Degradation of FlaA. Stability of FlaA was determined by adding spectinomycin (100 μg/ml of culture) to growing *B. burgdorferi* cells. At different time points, samples were subjected to Western blotting with monoclonal anti-FlaA. DnaK was used as an internal control. Approximately 5 μg of lysate was loaded into each lane for the wild-type strain. For strain MC-1, approximately 20 μg was loaded for detection of FlaA and 5 μg was loaded for detection of DnaK.

FlaA was monitored by Western blot analysis (Fig. 6). Spectinomycin at 100 μg/ml was found to rapidly inhibit protein synthesis and growth, and the cells remained intact for several hours. DnaK was used as a control, as transcription of this protein has been previously shown to be relatively constant under different growth conditions (56). We found that the level of DnaK in spectinomycin-treated cells was stable in both the wild-type and MC-1 strains for as long as 12 h (Fig. 6). Similar results were found with respect to FlaA in the wild-type strain. However, the level of FlaA in strain MC-1 showed an approximately 50% decrease by 2 h. By 12 h, there still was a trace of FlaA present. These results indicate that FlaA is degraded in the MC-1 strain, with a half-life of approximately 2 h.

DISCUSSION

Motility is energetically costly to *B. burgdorferi*. We found that the contribution of the major PF filament protein FlaB to the organism is substantial; it constitutes between 10 and 14% of the cellular protein total. Furthermore, at least 5% of the genome is dedicated to motility and chemotaxis (17). Specifically, approximately 42 ORFs homologous to motility genes of other bacteria have been identified; this estimate includes the multiple homologs of certain chemotaxis (*cheY*, *cheA*, *cheW*, *cheR*, and *cheB*) and flagellar genes (*fliG*). Targeted mutagenesis of many of these genes indicates that they function in motility and chemotaxis (40; M. Motaleb, C. Li, M. Sal, R. Bakker, and N. Charon, unpublished data). In addition, eight more ORFs of undetermined function map within the major motility operons. Because of their location, these ORFs are also likely to be involved in motility and chemotaxis. Thus, we estimate that approximately 50 of the total of 1,000 ORFs encoded by the *B. burgdorferi* chromosome are involved in these functions.

We found that there was considerably less FlaA than FlaB in *B. burgdorferi*, with FlaA contributing less than 0.5% of the total cellular protein. The function of FlaA in spirochete motility is presently unknown. In several spirochete species, FlaA forms a protein sheath around the FlaB core (9, 11, 38, 41). Null mutants in *flaA* in *B. hyodysenteriae* have been charac-

ized (41, 58). These mutants lack the flagellar protein sheath and have an altered PF helical shape, but they still retain motility (41). Microscopic observations and swarm plate assays indicate that these *flaA* mutants of *B. hyodysenteriae* are less motile than the wild type (41, 58). In *B. burgdorferi*, no sheath is evident in electron microscopic observation (Motaleb and Charon, unpublished). As shown by immunoprecipitation, FlaA associates with FlaB, confirming that it is a flagellar protein. In contrast to the results seen with *B. hyodysenteriae* (41), FlaA did not influence the helical shape of the PFs from *B. burgdorferi*. Preliminary results with a *flaA*::*kan* mutant yielded a PF shape identical to that seen with the wild type (Motaleb and Charon, unpublished). Future experiments will focus on localizing FlaA on the PF filament by immunogold technology.

We found by Western blot analysis that FlaA in *B. burgdorferi* often migrated as a doublet (Fig. 4b). These doublets were most commonly observed when large amounts of protein were loaded in the gels. The major protein was approximately 38 kDa in size, which is consistent with our published results obtained with strain 212 and the results of others (23, 26). The minor protein of approximately 37 kDa was often not seen (Fig. 1, 2, and 6), and we could only detect it by Western blotting. The minor 37-kDa band has not been previously reported (23, 26). Several possible explanations could account for the lack of previously detection of the minor FlaA protein. One possibility is differences with anti-FlaA reagents. In our previous studies, we used a heterologous polyclonal antibody to *T. pallidum* FlaA (23). As shown in Fig. 4, the minor protein was not detected using this antiserum; it was only detected using the monoclonal anti-FlaA of *B. burgdorferi*. Furthermore, when we used the monoclonal antibody to test strain 212, the Western blot pattern was identical to that seen with strain B31 (Motaleb and Charon, unpublished). Thus, the 37-kDa band is not strain specific. In addition, this minor protein was not detected by Gilmore et al., who first obtained and analyzed the FlaA monoclonal antibody (26). One possible explanation is that these investigators used the less-sensitive colorimetric assay for immunostaining. Because the 37-kDa protein was present in relatively small amounts, it may have gone undetected (26). Motaleb and Charon found that prolonged incubation of cell lysates at 23 or 37°C did not change the ratio of the two FlaA bands (Motaleb and Charon, unpublished); thus, it is unlikely that the 37-kDa protein is the result of protein degradation. Both proteins are likely to be encoded by *flaA*, as a *flaA*::*kan* mutant lacks both proteins but still synthesizes FlaB at a level similar to that seen with the wild-type strain (Motaleb and Charon, unpublished). Our preliminary experiments performed to characterize the minor band by mass spectroscopy have been unsuccessful. It will be interesting in future experiments to sort out the relationship of the two proteins. Several lines of evidence suggest that the N terminus of the FlaA is cleaved by signal peptidase I (20, 21, 23, 26) and that this protein is also glycosylated (23). Perhaps the lower-molecular-weight form is unglycosylated FlaA. Alternatively, others have shown that the carboxy-terminal end of certain outer membrane proteins of *B. burgdorferi* is cleaved (52); thus, the low-molecular-weight protein could be the result of specific protease digestion.

We examined the effects of an insertion mutation in *flaB* on

the synthesis of other motility proteins. Because *B. burgdorferi* lacks sigma-dependent cascade control of flagellar gene regulation (11, 17, 20, 22, 24, 25, 42), we could not predict how a *flaB* mutation would influence the expression of other motility genes. Western blot analysis indicates that eight of nine putative chemotaxis and motility proteins tested had unaltered levels of accumulation compared to the wild-type results. Of those tested, the only gene product found to be significantly reduced was FlaA at a level of approximately 13% of the wild-type level (Fig. 4b). In *E. coli* and *S. enterica* serovar Typhimurium, mutations in *fliC*, which encodes the only protein found in the filament, do not effect the expression of other flagellar genes (14). Thus, the results obtained are similar in some respects to those obtained with *E. coli*. However, several species of bacteria have multiple filament proteins. For example, *Caulobacter crescentus* and *Vibrio parahaemolyticus* have six (16, 47–49), *B. hyodysenteriae* has four (41, 58), and *Helicobacter pylori*, *H. mustelae*, and *Campylobacter jejuni* have two (35, 62, 65). In these species, mutants with mutations in the genes that encode specific filament proteins still synthesize the other filament proteins at levels close to those seen with the wild-type strain (35, 41, 49, 62). Thus, *B. burgdorferi* is different from these other species of bacteria, as *flaB*::*kan* did decrease the amount of FlaA.

We investigated the basis of FlaA inhibition in the *flaB*::*kan* mutant strain. The results indicated that there was no change in the amount of *flaA* message. These results suggest that the inhibition of FlaA in the MC-1 is posttranscriptional. Posttranscriptional control of flagellar gene expression in other species has been noted. For example, in *S. enterica* serovar Typhimurium this control is coupled to the secretion and assembly of basal body proteins and flagellin (2, 7, 36, 37). In addition, the membrane protein FlK regulates translation of the flagellum-specific anti-sigma factor FlgM in response to flagellar ring assembly (37). In *C. crescentus*, synthesis of both FljK and FljL flagellar filament proteins have been shown to be under translational control at specific checkpoints in flagellar assembly (3, 49). Recently, Limberger et al. isolated a *T. denticola* mutant with a mutation in *tap1* (44). *tap1* is a homolog to *fliK*, the flagellar hook assembly gene found in other bacteria. This mutant failed to make flagellar filament FlaB proteins, but RT-PCR results indicated that *flaB* continued to be transcribed. Because the relative amount of *flaB* mRNA in *tap1* was not determined, it is unclear whether the *flaB* mRNA in the mutant achieved wild-type levels.

There are two likely explanations for the inhibition of FlaA accumulation in the FlaB mutant. First, FlaA could be degraded by cell proteases. Spectinomycin inhibition results indicate that whereas this protein is stable in the wild-type strain, the small amount detected in the *flaB*::*kan* mutant was degraded, with a half-life of approximately 2 h. This turnover rate relative to the 8- to 10-h generation time would readily explain the small amount of FlaA detected in the *flaB* mutant. To our knowledge, these results are the first evidence for protein turnover in *B. burgdorferi*. Degradation of FlaA in this mutant could be related to the complicated assembly of spirochete flagellar filaments. Specifically, FlaB has no signal sequence and is excreted into the periplasmic space by a type III secretory pathway in a manner similar to that of other bacterial flagellin (11, 13, 20, 46). On the other hand, FlaA of *B. burg-*

dorferi and other spirochetes contains a signal peptidase I cleavage site near its N terminus (20). Several lines of evidence indicate that it reaches the periplasmic space by the type II pathway (13, 20, 21, 23, 26, 42). Both proteins evidently form a complex in the periplasmic space for filament formation. Perhaps in the absence of FlaB, FlaA is more vulnerable to protease digestion. In other systems, proteins that comprise a complex but fail to pair with their partners, e.g., RcsA and RcsB in *E. coli*, are often unstable and readily degradable (29). This hypothesis is further supported by our finding that among the downstream genes in the *flaA* operon, the amounts of CheA2 and CheY3 were unaltered in strain MC-1 compared to the results seen with the wild-type strain (Fig. 4a).

Another possible explanation is translational control of FlaA. For example, perhaps FlaB or another factor acts as a positive effector for translation of the *flaA* message. This type of control has been noted in other systems, such as the Com protein stimulating translation of *mom* mRNA in bacteriophage Mu (64). Genomic analysis of *B. burgdorferi* indicates that it has only a few two-component signal transduction systems, and obvious operator-repressor systems are not evident (17). Recent evidence indicates that RpoN and RpoS are both involved in the regulation of gene expression in tick and mammalian hosts (32). Perhaps *B. burgdorferi* evolved specific adaptive strategies for these hosts and consequently shed many of the control mechanisms found in other bacteria. Along these lines, several other genes (e.g., genes *Bbk2.10* and *elpA.1* and genes *flaA* and *flaB* in a *flgE* mutant) have been identified in *B. burgdorferi* for which there was abundant mRNA present but the level of the encoded protein was undetectable or markedly decreased (30; Sal et al., Abstr. Bacterial Locomotion and Signal Transduction Meeting VII). The results presented here and elsewhere do not allow us to differentiate which type of mechanism (translational control versus degradation) is occurring with respect to FlaA regulation and these other proteins; perhaps both are occurring simultaneously. To precisely sort this out, rapid pulse-labeling experiments would be helpful. However, because of the rich nutrient requirements of *B. burgdorferi* our attempts to use this approach have so far been unsuccessful.

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