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An Unexpected *flaA* Homolog Is Present and Expressed in *Borrelia burgdorferi*

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Most investigators have assumed that the periplasmic flagella (PFs) of *Borrelia burgdorferi* are composed of only one flagellin protein. The PFs of most other spirochete species are complex: these PFs contain an outer sheath of FlaA proteins and a core filament of FlaB proteins. During an analysis of a chemotaxis gene cluster of *B. burgdorferi* 212, we were surprised to find a *flaA* gene homolog with a deduced polypeptide having 54 to 58% similarity to FlaA from other spirochetes. Like other FlaA proteins, *B. burgdorferi* FlaA has a conserved signal sequence at its N terminus. Based on reverse transcription-PCR and primer extension analysis, this *flaA* homolog and five chemotaxis genes constitute a motility-chemotaxis operon. Immunoblots using anti-FlaA serum from *Treponema pallidum* and a lysate of *B. burgdorferi* showed strong reactivity to a protein of 38.0 kDa, which is consistent with the expression of *flaA* in growing cells.

Lyme disease, which is caused by *Borrelia burgdorferi*, is the most common arthropod-borne human infection in the United States (1). *B. burgdorferi* has a morphology similar to that of other spirochetes; it has a protoplasmic cell cylinder and periplasmic flagella (PFs) surrounded by an outer membrane sheath (15, 17). Approximately seven PFs are attached at each end of the protoplasmic cell cylinder, and these PFs have been shown to be involved in motility (16, 17, 34). *B. burgdorferi* motility is likely to play a role in the development of Lyme disease. These organisms are highly motile in viscous gel-like environments such as connective tissue and thus can penetrate tissues when other bacteria fail to invade (16, 19, 20, 37). In addition, an analysis of a spontaneously occurring PF mutant has indicated that motility augments cell penetration (34). Finally, over 35 motility genes spanning approximately 35 kb have been identified in *B. burgdorferi* (GenBank accession no. L76303, L75945, U28962, U43739, U61498, U62900, U62901, and U66699) (12, 14); we have estimated that at least 3% of its genome, which is approximately 945 kb (7, 35), is involved in motility and chemotaxis.

The structure of PFs is distinct from that of the flagella of other motile bacteria. Specifically, the PFs of most spirochetes are composed of multiple protein species referred to as FlaA and FlaB proteins (4, 8, 21, 29, 30, 38). FlaA proteins form the PF sheath, and FlaB proteins comprise the core (4, 8, 21, 30). Within a given species, there are one or two different FlaA proteins and three or four different FlaB proteins (8, 21, 29, 30, 33, 38). FlaB proteins show amino acid sequence similarities to the flagellin proteins of rod-shaped bacteria (29, 30, 41). Because FlaB proteins contain no typical signal sequences, these proteins are evidently excreted by the flagellum-specific pathway (8, 27). On the other hand, FlaA proteins possess an N-terminal signal sequence recognized by the typical SecA-dependent excretion pathway (5, 8, 18, 22).

In contrast to other spirochetes, only one flagellin protein of 41 kDa has been reported in *B. burgdorferi* (3, 8, 9, 40). This protein, encoded by the *fla* gene (hereafter referred to as the *flaB* gene), serves as a major antigen in the diagnosis of Lyme disease; high titers of anti-FlaB antibodies are detected in all disease stages (2, 9, 40). Based on electron microscopic observations (17) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis profiles of purified PFs, no apparent PF sheath or proteins other than the 41-kDa protein have been identified. During an analysis of a chemotaxis gene cluster in *B. burgdorferi* 212, we were surprised to find a *flaA* homolog directly upstream of the putative *cheA* gene. Here we present our characterization of this *flaA* gene. We show that this gene is part of a putative motility-chemotaxis operon and that it is expressed in growing cells.

Trueba et al. recently sequenced and mapped a *cheA* homolog in *B. burgdorferi* B31 which mapped at 722 to 737 kb from the 0 telomere (39). We cloned and analyzed the region downstream of *cheA* in *B. burgdorferi* 212 and found four other putative chemotaxis genes, including *cheW*, *cheR*, *cheX*, and *cheY* (GenBank accession no. U61498) (11). Since motility and chemotaxis genes are generally clustered to form a functional operon, we hypothesized that there would be additional chemotaxis genes upstream of *cheA*. To clone and sequence the DNA segment upstream of the *cheA* gene, we used a method referred to as "semi-random PCR chromosome walking," which has been successfully used in the analysis of other motility operons of *B. burgdorferi* (10, 13). To sequence the upstream region, we first amplified cellular DNA under low-stringency conditions by using a primer (pcheA3) derived from the B31 *cheA* sequence (Fig. 1). We obtained a 0.7-kb fragment, and sequence analysis indicated that it was upstream of *cheA*. We then did a second PCR amplification under low-stringency conditions by using a primer, pflaA1, based on the sequence data obtained from the first 0.7-kb clone; we obtained a 0.9-kb fragment which was also upstream of *cheA*. Thus, the region extending approximately 1.6 kb upstream of *cheA* was cloned and sequenced. Immediately upstream of *cheA*, we identified a 1,023-bp open reading frame encoding a 341-amino-acid protein which has an estimated size of 38.0 kDa (Fig. 1). The GC content of this gene was 34%, which is similar to that found for the *B. burgdorferi* genomic DNA. The results of multiple alignments indicated that the protein encoded by this open reading frame was a well-conserved *flaA*

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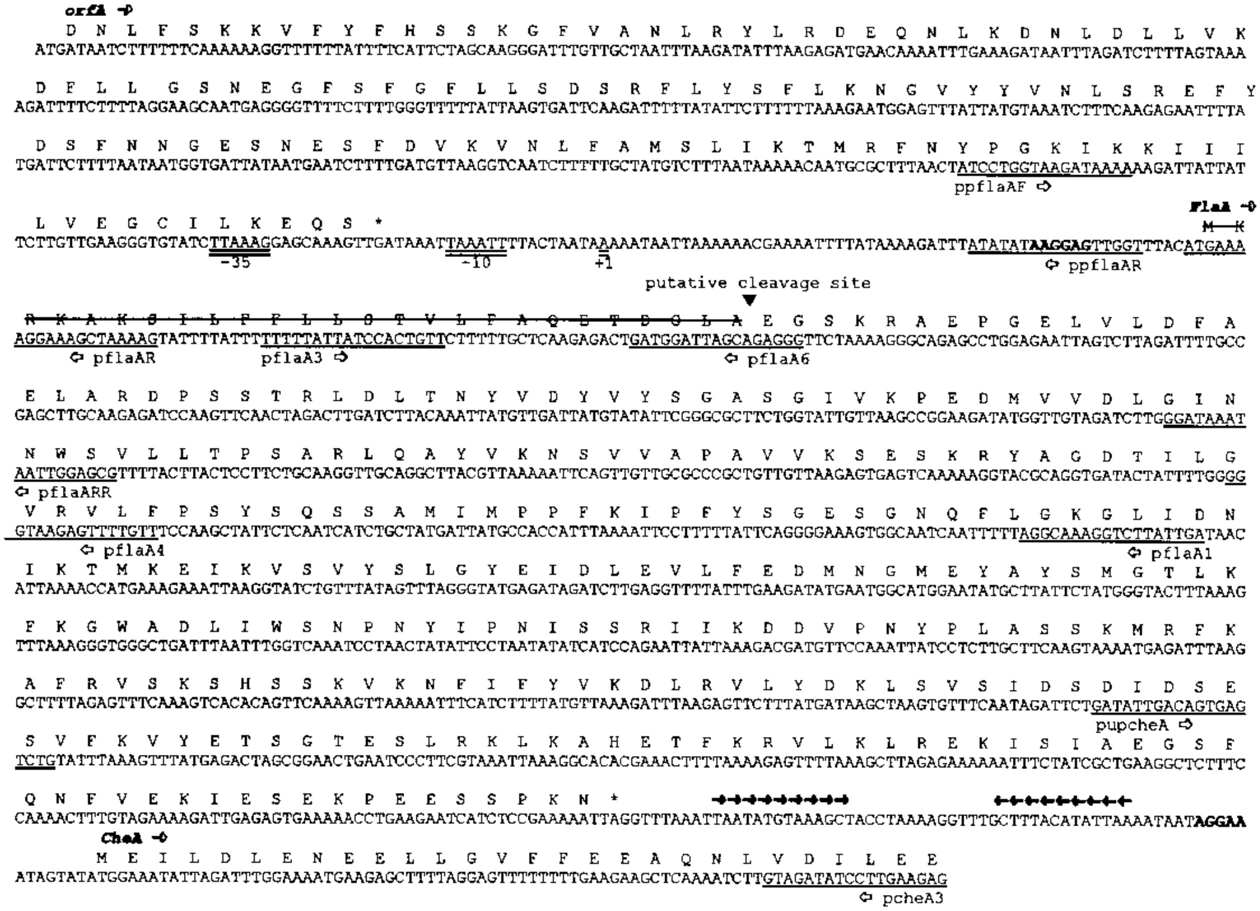

FIG. 1. DNA and protein sequences upstream of the *cheA* gene. The putative ribosom-binding site of each gene is in boldface. The double underlined regions are the promoter sequences corresponding to the mapped transcriptional start site and the −10 and −35 regions. The polypeptide with letters struck out represents the putative signal sequence cleaved before export of the mature protein. The nucleotides involved in the stem-loop structure are indicated by opposing arrows. The nucleotides used to generate the primers for cloning, RT-PCR, and primer extension are underlined, and the orientations of the primers are marked.

gene homolog (Fig. 2); *B. burgdorferi* FlaA showed identity and similarity of 31 and 58% to *Serpulina hyodysenteriae* FlaA, 29 and 54% to *Treponema pallidum* FlaA, and 31 and 59% to *Spirochaeta aurantia* FlaA. No significant homology with other bacterial proteins was found by BLAST analysis. The alignment of the four FlaA proteins from these species reveals that most of the proteins were fairly well conserved, except at the N and C termini. An important feature of the *B. burgdorferi* FlaA homolog is that it contains a typical signal sequence at its N terminus (Fig. 1), including a positively charged N-terminal domain, a central hydrophobic segment, and a signal peptidase I cleavage site (32). After cleavage, the mature protein has a predicted size of approximately 36.0 kDa. The *B. burgdorferi* FlaA homolog has an unusually high content of serine residues, comprising 12% of the total amino acids, which is higher than those of other FlaA proteins. Upstream of *flaA*, only a partial sequence of one open reading frame (*orfA*) was cloned and sequenced, as shown in Fig. 1. This open reading frame does not share homology with other known proteins.

One possible reason that the FlaA homolog has been undetected is that, like several other *B. burgdorferi* genes (36), it could conceivably be synthesized only in vivo. To test this, reverse transcription (RT)-PCR was used to determine whether *flaA* was transcribed in cells growing in culture and whether it was transcribed together with *orfA* and *cheA*. RT-PCR analysis has been successfully used in several laboratories to analyze spirochete mRNA (25, 28, 36). We and others have found that Northern blots of large transcripts from spirochetes are difficult to interpret, possibly due to degradation (24, 28). Between *orfA* and *flaA*, there is a 78-bp intergenic gap with no stem-loop and terminator-like sequence, while the region (68 bp) between *flaA* and *cheA* has a stem-loop structure (Fig. 1). Neither region has readily identifiable terminator sequences. To obtain purified RNA free of DNA for RT-PCR and primer extension analysis, total bacterial RNA was isolated by using TRI REAGENT (Sigma), followed by twice-repeated DNA digestion and use of a Qiagen RNA purification kit. Three controls were used for RT-PCR. Two controls consisted of primers (pftsZ1 [5′-GGAAGACATAGATGTTATAC-3′] and pftsZ4 [5′-AGATGCAAAACCTGTAGC-3′]) obtained from the central region of the housekeeping gene *ftsZ* (GenBank accession no. L76303) with and without reverse transcriptase. *ftsZ* has been shown to be necessary for cell division in other bacteria (26). A positive reaction without reverse transcriptase would indicate DNA contamination. Another control was a pair of primers (pflgK1 [5′-CGAATCACACATAAAGCAC-3′] and pflgK2 [5′-CTGAAAATGTTGAATCCACTC-3′]) derived from a region which is divergently transcribed (*thdF* to *flgK*) (GenBank accession no. U62901) and should yield a negative result. For RT-PCR analysis, appropriate primer

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Sh MK_K____LFVVLTSIFIAASAYGLTN_ST_______LIDFA______LTGNADNLQAGEGDTNEVVPVAENLYND__
Sa MK_K_____AVVLSAVALLSGVCAVADESV_______LIDFAKLNADIMADKSGGMTHNRRTVLDYASLADTSYTDEQ
Tp MKRF____FFAILGAALFVGNSGAFAEQAT_______LIDFSKLVGE______GNTGLHAPTTIDYSRQAGSAYSAED
Bb MKRKAKSILFFLLSTVLFAQETDGLAEGSKRAEPGELVLDFAELARD___________PSSTRLDLTNYVDYVYSGAS
    *..    .. .*......    .... ...  .. ...**...  . .   ..       ....    ..  *...

Sh _______________NWVVWLNESARLTENRRNSYVTNVDSKGNNGAWEAGKVLGVRVHFPLAAWNSYALVKPVYELE
Sa RAL____MRSSLAVAQWEVVLNSSARNPVAHAASRVIEAPVSEGAKSFAGERVLGVRVLFPTWDSNANAMIKPAFVIP
Tp RAA____MKISLAIPSWEIELASSSQTVENQTLSLVTAAPVKQDAARYGGETVMGVRIHFPSFGINSFAVIKPPFVIP
Bb GIVKPEDMVVDLGINNWSVLLTPSARLQAYVKNSVVAPAVVKSESKRYAGDTILGVRVLFPSYSQSS_AMIMPPFKIP
   ..  . ..  .... .*.. *..*... ..  .* *. .... . .........***..**.   ...*...*.. ..

Sh MYG___________________GADGTKYTEGKGVIHNVGEIKSISSWVYGRNYLISYFVNLQNEFGELKSYPMGTVY
Sa AYEVMAQVDDQGNVQAPTEEEKASGKGRFEDGYGVVKNVGVLKSIAVNTYGMNYPHGLYVMMRDQDGEVHRYFMGYLL
Tp AYATLGDATAQNAVAG__________GQF_DGFGVLKNVGVIKSIQINILGRNYLNRLSLLLEDQNGDEREIVMGYLN
Bb FYS___________________GESGNQFL_GKGLIDNIKTMKEIKVSVYSLGYEIDLEVLFEDMNGMEYAYSMGTLK
    *  ..    .  . ..     .  . .. .*.*...*....*.* .......*.. .........*..  . **..

Sh FNGWRQVRWENREYLPNVRDRVLV_REPLYPRMIPSVKLDSLGFYRTKDTKGGDFITYVKDVTLEYDVVVVDFEEDID
Sa FDSWKELVWNNPSYISDVRSREVR_LYPVYPASTPHVVFEGFMVTRDAAHAGGDYVGYFKDVKIIYDKAVLSTVRDFA
Tp FDGWKSLQWNNPNYQTEVRNRDLQ_IVPLYPRSAPLIKLKGIKIHRDGSQEGGDIVSYIKDIKVIYDQAVVDRNSDVD
Bb FKGWADLIWSNPNYIPNISSRIIKDDVPNYPLASSKMRFKAFRVSKSHSSKVKNFIFYVKDLRVLYDKLSVSIDSDID
   *..*. . *.*..*......* . . .*.**.. .  ..... . .  . ...... *.**....**. ...  .*..

Sh DEATWQLLKTENDRKQAIESARIREQAELRDLEQRRIGDGTAADQGAAANTGAADTGAAQEQAQ
Sa DEDLWGIQARREAERKRVEVARFGQQQVLRYIEQEKLATEVGFTPSGGAQRQEEQQ
Tp DEAIWGILRQREEQYRNFELAKLGNLQVLRSLEKKKMAKEADFDQAAPAAAAARAPATN
Bb SESVFKVYETSGTESLRKLKAHETFKRVLKLREKISIAEGSFQNFVEKIESEKPEESSPKN
   .*. . .             *.      *.  *  ...  .  .  .  .
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FIG. 2. Multiple alignment of FlaA sequences of different spirochetes. Multiple alignment was done with the PepPepSearch Program. Alignment gaps are represented by lines. The asterisks indicate identical residues. The dots indicate conserved amino acid substitutions. The blanked residue is not conserved among the four FlaA proteins. *Sh*, *S. hyodysenteriae*; *Tp*, *T. pallidum*; *Sa*, *S. aurantia*; *Bb*, *B. burgdorferi*.

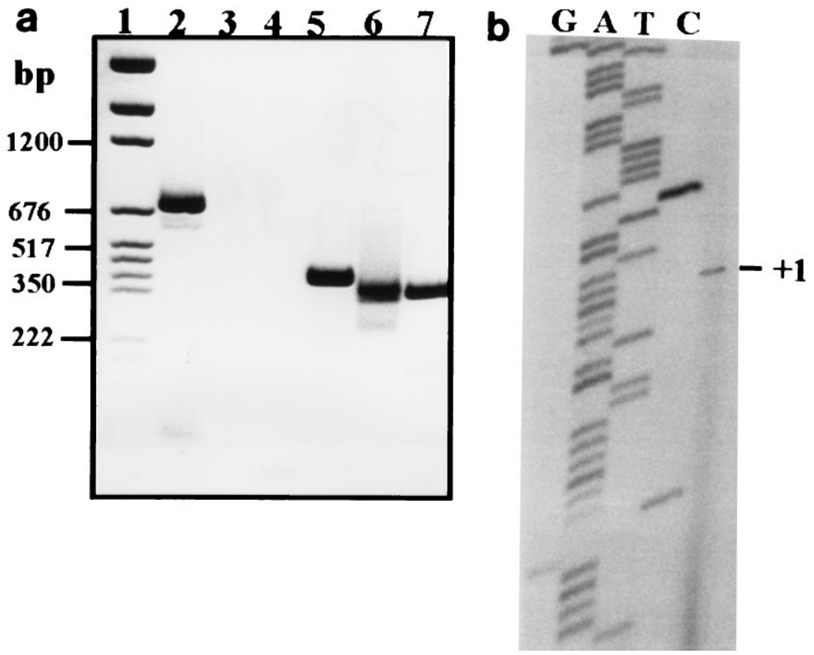

FIG. 3. RT-PCR and primer extension. (a) Products of RT-PCR examined in a 2% agarose gel with the Eagle Eye system (Stratagene). RT-PCR was done by using the Access RT-PCR System from Promega in accordance with manufacturer's instructions. The amplified products from appropriate primers were loaded in the following lanes: 1, pGEM marker; 2, a pair of primers for the middle of the *ftsZ* gene; 3, same as lane 2 except no reverse transcriptase; 4, a pair of primers from the divergently transcribed *thdF* and *flgK* regions; 5, primers ppflaAF and pflaARR; 6, primers pflaA3 and pflaA4; 7, primers pupcheA and pcheA3. (b) Results of primer extension measured in a 5% polyacrylamide gel. The primer pflaA6 was used for extension. +1 indicates the transcriptional start site.

	-35		-10		+1
σ^{28}	CTAAA	---N_{16}---	CCGATAT	--N_7--	A
σ^{70}	TTGACA	---N_{17}---	TATAAT	-----N_7--	A
flaA	TTAAAG	---N_{17}---	TAAATT	-----N_9--	A

FIG. 4. Comparison of the *flaA* promoter with consensus σ^{70} and σ^{28} promoters.

pairs were designed to connect the adjacent genes *orfA* and *flaA* (ppflaAF and pflaARR), the middle of *flaA* (pflaA3 and pflaA4), and *flaA* and *cheA* (pupcheA and pcheA3; Fig. 1). RT-PCR was carried out with an Access RT-PCR System from Promega. Positive results were obtained with all three pairs of primers (Fig. 3a, lanes 5, 6, and 7) with the controls working as predicted (lanes 2, 3, and 4). These results indicate that *flaA* is transcribed in cultured cells and that it is transcribed together with *orfA* and *cheA* as one unit. Identical results (data not shown) were obtained with strain HB19.

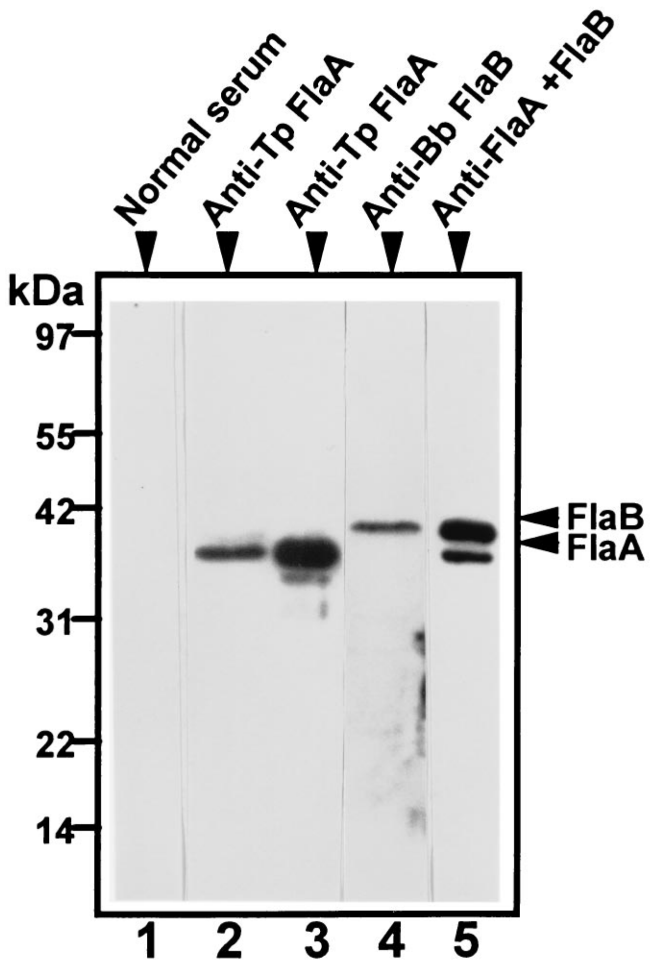

FIG. 5. Western blotting analysis of *B. burgdorferi* (Bb) FlaA protein. The assay was done with an ECL Western blotting system from Amersham. The anti-*T. pallidum* (Tp) 37.0-kDa FlaA serum was diluted 1:3,000, and the anti-*B. burgdorferi* 41.0-kDa FlaB monoclonal antibody was diluted 1:1,000. Lanes: 1, 2, 4, and 5, whole-cell lysate of *B. burgdorferi* 212; 3, purified *T. denticola* PFs (33). Lane 1 was blotted with normal rabbit serum, lanes 2 and 3 were blotted with anti-*T. pallidum* FlaA serum, lane 4 was blotted with anti-*B. burgdorferi* FlaB monoclonal antibody, and lane 5 was blotted with anti-*T. pallidum* FlaA serum and anti-*B. burgdorferi* FlaB monoclonal antibody.

By using primer extension (Promega AMV Primer Extension System), we determined whether there are any promoters upstream of *flaA* or *cheA*. No detectable signal was identified from the region immediately upstream of the *cheA* gene (data not shown). In contrast, a transcriptional start signal was observed from the region immediately upstream of *flaA* (Fig. 3b). This conclusion was verified by using two different primers (pflaA6 and pflaAR). As illustrated in Fig. 1, the *flaA* promoter has the sequences TAAATT at −10 and TTAAAG at −35, which have weak homology with σ^{70}-like promoters (Fig. 4). To test the strength of this promoter element in *Escherichia coli*, we amplified and cloned the promoter region (the segment between ppflaAF and ppflaAR) into a promoter probe vector (pKK232-8) with a promoterless *cat* gene (6). However, we could not detect chloramphenicol resistance in the transformed *E. coli* cells, indicating that this promoter was not functional in *E. coli*. Although we identified this promoter immediately upstream of *flaA*, we also detected a transcript spanning the region between *orfA* and *flaA* (Fig. 3a, lane 5). These results suggest that the *flaA-cheA* operon is controlled by at least two promoters. A similar transcriptional regulation has been described in *Salmonella typhimurium*, in which a class III gene (*flgK*) operon is controlled by both its own promoter and an upstream class II promoter (23). Together with our previous finding that all five chemotaxis genes and *flaA* are transcribed as one transcript (11), our results suggest that *flaA*, *cheA*, *cheW*, *cheR*, *cheX*, and *cheY* constitute a motility-chemotaxis operon.

We directly tested whether a protein compatible with *flaA* expression is synthesized in cultured cells. Our approach was to examine whether cell lysates of *B. burgdorferi* reacted with an antiserum specific to the *T. pallidum* FlaA protein. This antiserum has been shown to react specifically with the FlaA proteins of *T. pallidum*, *T. phagedenis*, and *T. denticola* (29, 33). Western blot analysis of cell lysates of *B. burgdorferi* indicated that a single band of approximately 38.0 kDa reacted with this antiserum (Fig. 5, lane 2). Three controls were used. No reaction was detected when normal rabbit serum was used (Fig. 5, lane 1). In addition, the antiserum reacted with FlaA in purified *T. denticola* PFs as expected (Fig. 5, lane 3). Finally, monoclonal antibody H9724, specific to *B. burgdorferi* FlaB (3), reacted with a protein with a size (41 kDa) distinct from that of FlaA (Fig. 5, lanes 4 and 5). These results suggest that a FlaA homolog is synthesized in *B. burgdorferi*, that it is distinct from FlaB, and that its size approximates that of the deduced *flaA* gene.

The precise function of *B. burgdorferi* FlaA is unclear. Because of its marked similarity to FlaA proteins of other spirochetes and because it is part of an operon involved in chemotaxis, it is likely to be involved in motility. However, in both *T. pallidum* and *S. aurantia*, *flaA* genes are transcribed from σ^{70} promoters and are monocistronic (18, 31), in contrast to our findings on *B. burgdorferi*. Based on sequence analysis, the *B. burgdorferi flaA* homolog likely encodes a flagellar filament sheath protein. It is not clear why *B. burgdorferi* FlaA has not been found to be associated with purified PFs. Perhaps upon purification of the PFs, FlaA readily dissociates from the filaments. Alternatively, *B. burgdorferi* FlaA could conceivably have a function other than being a PF sheath protein. Future experiments using cell fractionation and immunolocalization studies should allow us to determine the location of FlaA in *B. burgdorferi* cells.

Nucleotide sequence accession number. The sequence reported in Fig. 1 has been assigned GenBank accession no. U62900.

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