

The Hook Protein of *Borrelia burgdorferi*, Encoded by the *flgE* Gene, Is Serologically Recognized in Lyme Disease

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The periplasmic flagellum of *Borrelia burgdorferi* consists of a unipeptide flagellar filament, a hook, and a basal body. Here, we report the cloning and expression of the hook gene, *flgE*, of *B. burgdorferi* N40. The *flgE* gene is 1,119 nucleotides long and is located on the 950-kb linear chromosome of *B. burgdorferi*. The primary protein sequence of FlgE shows 73% similarity to the FlgE protein of *Treponema phagedenis* and ~50% similarity to the FlgG proteins of both gram-positive and gram-negative bacteria. The *flgE* gene was cloned into an *Escherichia coli* expression plasmid, pMX, to produce FlgE protein. Subsequently, FlgE murine antiserum was prepared by immunizing mice with the partially purified *B. burgdorferi* FlgE protein. By Western blot (immunoblot) analysis, the antiserum was found to react with a 40-kDa peptide in the whole-cell lysates, confirming the expression of the *flgE* gene in *B. burgdorferi*. Additionally, antibodies to FlgE were found in serum specimens from 19 of 42 patients with Lyme disease. Moreover, when other antigens, including 41G (the immunodominant domain of flagellin), OspE, OspF, and p22, were used to test for the development of corresponding antibodies in these patients, 67% of these patients (28 of 42) reacted to at least one of these five antigens, suggesting that a combination of FlgE with other available *B. burgdorferi* recombinant proteins is a good candidate for substrates in assays to aid in the diagnosis of Lyme disease.

Borrelia burgdorferi is the pathogenic spirochete that causes Lyme disease (4). During Lyme disease infection (25), patients may develop cardiac, neurologic, and rheumatologic abnormalities following the characteristic skin lesions, erythema migrans. The motility of *B. burgdorferi* and invasion of the tissue of an infected host (24, 26) are important in the initiation of infection. So far, the flagella, the key component in the motility of spirochetes, have been studied mostly by electron microscopy. With the exception of the *fla* gene, which encodes flagellin, the unipeptide of the flagellar filament, the genes involved in the flagellum assembly of *B. burgdorferi* have not yet been described.

B. burgdorferi, like other pathogenic spirochetes, such as *Treponema pallidum* and *Borrelia hermsii*, has a membrane structure which resembles that of gram-negative bacteria, namely, it has an outer membrane, a peptidoglycan layer, and a cytoplasmic membrane. On the outer membrane, there reside many outer surface proteins, including OspA, OspB, OspC, OspD, OspE, OspF, and lipoprotein p27, that induce immune responses in the host (2, 8, 13, 17, 19). In the periplasmic space, which is situated between the outer membrane and the peptidoglycan layer, several flagellar filaments are present. In contrast to the flagella of *Escherichia coli*, the flagella of *B. burgdorferi* are not located externally; nevertheless, the flagella remain functional and responsible for the motility of the spirochete (for reviews, see references 5, 6, and 9). The flagella of spirochetes and other bacteria consist of a helical filament, a hook, and a basal body (for a review, see reference 12). The genes which encode the structural components and the regulatory factors for flagella are usually found to be clustered in the bacterial chromosome (15). Unlike the filament of other

spirochetes, such as the treponemes, the flagellar filament of *B. burgdorferi* consists of only one protein, flagellin. The filament is connected to the basal body, which is embedded in the cytoplasmic membrane, via the hook. Recently, the *flgE* gene that encodes the hook protein of *Treponema phagedenis* has been identified (14), and the hook protein sequence has been shown to be homologous to that of the *Salmonella typhimurium* hook protein. Possibly, most of the proteins involved in flagellar formation are conserved among bacteria.

The knowledge of the hook and basal body complex comes mainly from studies of *E. coli* and *S. typhimurium* (for a review, see reference 15). Several additional hook-associated proteins were found to be located at the junction of the hook and the filament. The basal body is an assembly of many proteins, specifically, MS ring (FliG) at the cytoplasmic membrane, P ring (FlgI) and L ring (FlgH) at the periplasmic space and outer membrane, and a central rod connecting these rings to the hook. The rod can be further disassembled into proximal rod proteins (FlgB, FlgC, and FlgF) and a distal rod protein (FlgG) with respect to the position of the cell membrane. Since the flagella of spirochetes are endoplasmic, the basal body may contain only one ring (the basal disk), as opposed to the two-ring construction in gram-negative bacteria. In *Bacillus subtilis* (1, 18, 27), several proteins were also found to be homologous to the structural components of the basal body in *S. typhimurium*, although characterization of these homologs remains to be done. As will be shown in this paper, it is interesting that the sequence conservation of these structural proteins extends to the spirochete, a bacterium evolutionarily distant from those mentioned above. Here, we report an FlgE homolog in *B. burgdorferi* which is the putative hook protein connecting the flagellar filament and the basal body embedded in the inner membrane. We further show that this molecule is serologically recognized by some patients with either early- or late-stage Lyme disease.

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MATERIALS AND METHODS

Screening of a *B. burgdorferi* genomic library. A *B. burgdorferi* N40 genomic library was constructed in λ Zap II vector as described previously (13). The expression library was screened by Western blotting (immunoblotting) (20) with antiserum from a patient with late-stage Lyme disease marked by arthritis. In brief, the patient's antiserum was first absorbed with *E. coli* and λ phage lysate to eliminate most background signals. Subsequently, a 1:50 dilution of the patient serum was incubated with nitrocellulose filters containing the proteins synthesized from the N40 *B. burgdorferi* DNA library. A second antibody, a 1:2,000 dilution of alkaline phosphatase-conjugated goat anti-human immunoglobulin G (IgG), IgA, and IgM, was then used to detect bound patient antibody (Organon Teknica Corp., West Chester, Pa.). Approximately 100,000 phage clones were screened, and 0.05% of the total clones were responsive to this patient's antiserum. The clone that gave the strongest signal after Western blotting was chosen for further characterization.

Subcloning and DNA sequencing. The pBluescript phagemid containing the cloned *B. burgdorferi* DNA insert was excised from the λ Zap II phage vector with helper phage VCSM13 (Stratagene). The phagemid DNA was further mapped with restriction endonucleases for subcloning. Several subclones were generated by cleaving with restriction endonuclease *Bam*HI, *Pst*I, *Sac*I, or *Eco*RI and ligating the cleaved vector to remove part of the inserted *B. burgdorferi* fragment. DNA sequencing was performed by the dideoxy method (21) with a Sequenase kit (U.S. Biochemical Corp.). Sequencing of the clone and the subclones gave rise to two incomplete open reading frames (ORFs) missing either the 5'-end region (ORF-A) or the 3'-end region (ORF-2). Hence, DNA fragments from each ORF were ³²P labeled by using a Prime-It kit (Stratagene) to search for the corresponding missing portion of the two ORFs from the N40 genomic library. Southern hybridization was then done as described previously (20). Sequences were analyzed with the software program MacVector (International Biotechnology, Inc.), and a homology search through Entrez databases loaded with DDBJ/EMBL/GenBank/PIR/SWISS-PROT Nucleotide/Peptide Sequence Databases was done. The alignment shown below (see Fig. 2) was performed by using Prettybox (University of Wisconsin Genetics Computer Group package).

Cloning of the *flgE* gene and expression of the recombinant FlgE protein. The full-length *flgE* gene was PCR amplified from *B. burgdorferi* N40 by using oligonucleotide primers, 31 nucleotides in length, containing the 5' end or the 3' end of the *flgE* gene in addition to a restriction site (*Bam*HI for the 5'-end primer and *Hind*III for the 3'-end primer) designed for cloning. Vent polymerase (New England Biolabs) was used to reduce the potential of incorporation errors. The *B. burgdorferi* DNA was denatured at 94°C for 1 min, annealed at 50°C for 1 min, and primer extended at 72°C for 3 min, and the cycle was repeated 30 times. The PCR-amplified *flgE* gene was cloned in frame with the glutathione *S*-transferase (GT) gene into pMX vector (23), a derivative of pGEX-2T (Pharmacia), and was then transformed into *E. coli* DH5 α cells by electroporation. The GT-FlgE fusion protein was then synthesized by the induction of 1 mM isopropyl-1-thio- β -D-galactoside (IPTG).

Production of murine anti-FlgE sera. Because of the insolubility of the GT-FlgE fusion protein, large quantities of purified GT-FlgE protein could not be obtained with a glutathione-Sepharose 4B column. The *E. coli* cells containing GT-FlgE fusion protein were therefore extracted with phosphate-buffered saline (PBS) and 1% Triton X-100 to remove most of the soluble *E. coli* proteins. The pellet containing the insoluble proteins was then extracted with PBS and 1% sodium dodecyl sulfate (SDS), and the GT-FlgE fusion protein was the predominant protein in the extracted mixture (see Fig. 5A). Subsequently, C3H/HeJ mice were immunized subcutaneously with 10 μ g of the *E. coli* lysate containing the enriched GT-FlgE protein and with *E. coli* lysate containing GT protein (as a negative control) separately in complete Freund's adjuvant and were given booster injections of the same amount of lysate in incomplete Freund's adjuvant at 14 and 28 days. The final concentration of SDS in the injected immunization mixture was 0.25%. Immunoblotting (see Fig. 5B and C) was performed according to the manufacturer's protocol (Amersham Corp.) with the amounts of serum described below. The primary antibody used was a 1:1,000 dilution of the murine antiserum, and the secondary antibody used to detect the bound murine antibody was a 1:5,000 dilution of goat anti-mouse IgG conjugated with horseradish peroxidase (Southern Biotechnology Associates, Inc., Birmingham, Ala.).

Collection of serum samples from 42 patients. All patients were seen at the Yale Lyme Disease Study Clinic. Patients with early-stage Lyme disease had erythema migrans, and patients with late-stage disease had previous erythema migrans and a persistent history of arthritis for more than 6 months. Samples of sera from patients who tested positive by an enzyme-linked immunosorbent assay (ELISA) using whole-spirochete lysate were chosen randomly. The ELISA was performed as described previously (7). Of the 42 serum specimens collected, 21 were from patients with early Lyme disease and the other 21 were from patients with late Lyme disease. A pool of serum samples collected from eight healthy human volunteers was used as a background control.

Nucleotide sequence accession number. ORF-2 was designated *flgE* because of its considerable homology with the *flgE* sequence of *T. phagedenis*. The sequence of *flgE* has been deposited in GenBank under accession no. U12870.

TABLE 1. Amino acid sequence comparison of *B. burgdorferi* FlgE protein with its homologs and other FlgG proteins^a

Organism	Protein	Size (amino acids)	% Similarity	% Identity
<i>B. burgdorferi</i>	FlgE	372	100	100
<i>T. phagedenis</i>	FlgE	463	73	56
<i>S. typhimurium</i>	FlgE	402	52	33
<i>B. subtilis</i>	FlgG	264	62	39
<i>S. typhimurium</i>	FlgG	260	55	29
<i>C. crescentus</i>	FlgG	262	48	25

^a The sequence comparison was performed with Pileup (University of Wisconsin Genetics Computer Group package).

RESULTS AND DISCUSSION

The nucleotide sequence and deduced amino acid sequence of *flgE*. A *B. burgdorferi* N40 genomic library was screened with antiserum from a patient with late-stage Lyme disease by Western blotting, and the clone that gave the strongest signal was selected. This clone contained a ~2.5-kb insert of *B. burgdorferi* DNA in which two incomplete ORFs were identified. We focused on the ORF (ORF-2) that was responsible for the immunogenicity of the clone (data not shown), and the missing portion of ORF-2 was retrieved from the N40 genomic library by plaque lift using the sequenced portion of ORF-2 as a probe to give a complete ORF. ORF-2 was designated *flgE* because of its considerable homology with the *flgE* sequence of *T. phagedenis* (Table 1).

The complete sequence of the *flgE* gene is shown in Fig. 1. The gene is preceded by a ribosomal binding site, GGAGG. One partial ORF (ORF-1) was identified ahead of the *flgE* gene sequence; however, whether ORF-1 encodes an authentic protein needs more investigation. This partial ORF-1 has some homology with the ORF-1 upstream from the *flgE* gene of *T. phagedenis* (data not shown). It is very likely that the *flgE* gene is in the same operon as ORF-1. Downstream from the *flgE* gene, the flanking 180-nucleotide sequence does not contain any authentic ORF. The *flgE* gene is 1,119 nucleotides long and has a base composition of 36% G+C, which is consistent with the observation that *B. burgdorferi* DNA is highly AT rich (11, 22). The AT-rich feature of *B. burgdorferi* DNA is reflected in the codon usage pattern of FlgE as well as other *B. burgdorferi* proteins; in particular, the wobble position of the FlgE codon is often an A or U (Fig. 1). We have assigned the gene the name *flgE* on the basis of the observation that it is a close homolog of other *flgE* sequences. A sequence alignment of three FlgE proteins is shown in Fig. 2A. As summarized in Table 1, the deduced amino acid sequence of ORF-2 displays a high degree of homology (73%) with that of the FlgE protein of *T. phagedenis* (14). There is somewhat less homology with the FlgE protein of *S. typhimurium*. Interestingly, *B. burgdorferi* FlgE protein also has homology with FlgG proteins of both gram-positive and gram-negative bacteria (Fig. 2B). FlgG protein is a structural component located in the flagellar basal body complex (15). The homologous domains are clustered at the N-terminal regions of the FlgG proteins, especially the first ~150 amino acid residues. Previously, it was found that the components in the flagellar basal body rods of *S. typhimurium* all share homology to various extents. A consensus signal peptide, ANNLAN, was thus deduced from the alignment of these rod proteins (10). The *B. burgdorferi* FlgE protein also contains the signal peptide GNNIAN. However, the FlgE protein is about 30% bigger than other FlgG proteins.

Localization of *flgE*. In addition to the linear chromosome with a size of approximately 1 megabase, *B. burgdorferi* has a

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1      AAATGGCGCAATTTTCGGCTCTTGAGCAAATGGCTAATATGAGTAGATCGTTTGAAAAC
ORF1   M A Q F S A L E Q M A N M S R S F E K L
61     TTTCATCTTCTTTAGAAATAAGAAAAGATTGGATTATTAGGTAAAGTAATTAATTTTC
      S S S L E I R K D L D L L G K V I K F Q
121    AGCATGGTGATGGGGAGATTGTTGAGGGTCGCGTCACAAACATTAAAGACAGCGCAATAC
      H G D G E I V E G R V T N I K T G A I P
181    CTCAAGTTATGGTTAATGGTAATTATATGTATATAAAAAATATATTATCGGTAGGTTTGG
      Q V M V N G N Y Y V Y K N I L S V G L E
241    AGGAATAATTATATGATGAGGTCCTTATATTCTGGTGTTTCTGGGCTTCAGAATCATCAA
ORF2   E * M M R S L Y S G V S G L Q N H Q
301    ACAAGAATGGATGTTGTTGGTAACAATATCGCCAATGAAATACAATTGGCTTTAAAAAG
      T R M D V V G N N I A N V N T I G F K K
361    GGAAGAGTAAATTTCAAGATATGATATCGCAATCTATTTCTGGAGCTTCTCGCCCTACT
      G R V N F Q D M I S Q S I S G A S R P T
421    GATGCTCGTGGTGGGACTAATCCCAAGCAAGTTGGATTAGGCATGAATGTGCCTCAATT
      D A R G G T N P K Q V G L G M N V A S I
481    GACACTATTACACTCAAGGAGCTTTTCAAAGCACTCAAAAAGCATCTGATCTTGGGGTT
      D T I H T Q G A F Q S T Q K A S D L G V
541    AGTGGCAACGGATTTTTTATTTTAAAAGAAGGTAAAAAATTTGTTTTATACAAGACCGGT
      S G N G F F I L K E G K N L F Y T R A G
601    GCTTTGACGTGGACTCTGATCGACATCTTGTAATCCTGCAAAATGGAATGCGAATTCAA
      A F D V D S D R H L V N P A N G M R I Q
661    GGTTGGATGGCAAGAGATTTAGAAGGTAAAAGGTTATAAATACAGCTTCTGATATTGAG
      G W M A R D L E G E K V I N T A S D I E
721    GATCTGATTATCCGATTTGGAGATAAAGAGGGAGCAAAGTCTACTAAAAATATTACTTTTT
      D L I I P I G D K E G A K S T K N I T F
781    GCTTGAATCTTGATAAGAGATTGCCCTTAATTCAGAAGGTGCGAGTCTGCAGATATT
      A C N L D K R L P L I Q E G A S P A D I
841    GCACGCGGAACCTGGGTTGTCAATAAATCATTGTATGACAGTTTTTGAATGTTAGTGTT
      A R G T W V V N K S L Y D S F G N V S V
901    CTTGAGCTTAGAGTTTAAAGATTAAATACGCCTAATTATGGAATGCAACAGTATTA
      L E L R V V K D L N T P N L W N A T V L
961    ATAAATGGTGAGCAAAATTCAAATTTTACACTTGGGTTTGACAAATGAAGGAGCATTGGCG
      I N G E Q N S N F T L G F D N E G A L A
1021   TCTTTAAATGGTCAACCAGGGCAAAAAGGAGATATCTTCAAAATCCTATAACATTTAAT
      S L N G Q P G Q K G D I L Q I P I T F N
1081   GTTTTGGGTGCAAAATGTAGGTGAAGTTGGTGAGCAGCAAACTGTAATTTGAAATTTGGGA
      V L G A N V G E V G E Q Q T V N L K L G
1141   ACAGTTGGAAGTTACACTGATTCAATTAATCTAGTTTCTGATCTTAGTAGCACAAGGCT
      T V G S Y T D S I T Q F A D S S S T K A
1201   ATTATTCAAGATGGATATGGCATGGGATATATGGAAAATTATGAAATTTGATCAAAATGGT
      I I Q D G Y G M G Y M E N Y E I D Q N G
1261   GTTATAGTTGGCATTATTCAAATGGCATAAGACGAGATCTGGCAAGATTGCTCTTGCT
      V I V G I Y S N G I R R D L G K I A L A
1321   TCTTTTATGAATCCCGGAGACTTGCAAAATCAGGCGATACATATTTGTAGAAAACAGCA
      S F M N P G D L Q N Q A I L I L *
1381   ATTCAGTCAAGTTAGAATAGGCGAAACTGGACTTGTGGACTTGGTGATATTAGATCTG
1441   GTGTTTTAGAAAATGGCCAATGTTGATCTTGCAGAGCATTTACAGATATGATAGTGACCCA
1501   AAGAGGATTTCAAGCAAAATGCAAAACCATTACCCTCTGATCAATTATTAC

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FIG. 1. Nucleotide and deduced amino acid sequences of the *flgE* gene and the flanking regions.

large number of linear and circular plasmids. We therefore determined whether the *flgE* gene was located on the chromosome or on a plasmid. Total DNA of *B. burgdorferi* was separated into chromosomal DNA and plasmid DNA by pulsed-field gel electrophoresis performed as described previously (13). One control lane was probed with an *ospA* gene fragment, which confirmed that *ospA* is located on the 49-kb plasmid. As shown in Fig. 3, the *flgE* gene is detected comigrating with the chromosome and the chromosomal *fla* gene encoding p41 flagellin. In addition, a smear was observed in the strip probed with flagellin DNA, and two faint bands in the lower plasmid region of the strip hybridized with *flgE* DNA. These are probably the result of cross-hybridizing plasmid sequences or non-specific binding of the radioactive probes to the plasmid DNA of *B. burgdorferi* N40.

Cloning of *flgE* and expression of GT-FlgE fusion protein. The full-length DNA fragment of *flgE* was amplified from *B. burgdorferi* N40 and cloned into pMX, a modified GT fusion vector; the *flgE* gene was ligated in frame with GT to facilitate protein purification. One clone was chosen, and recombinant GT-FlgE fusion protein of the clone was expressed upon IPTG induction. As shown in Fig. 4A, a 66-kDa band appeared

clearly with IPTG induction in clone 2. Clone 1, which contained only the GT fusion without the *flgE* insert, gave a band with a size of 26 kDa upon IPTG induction. The corresponding immunoblot (Fig. 4B) showed that the 66-kDa band was reactive to the patient antiserum which was originally used to screen for immunogenic proteins of *B. burgdorferi*, whereas the GT-FlgE fusion protein was nonresponsive to normal human serum (Fig. 4C).

Expression of *flgE* gene in *B. burgdorferi*. The PCR-amplified *flgE* DNA fragment was fused in frame with GT in order to purify the fusion protein with an affinity column. However, the GT-FlgE fusion protein was found to be insoluble in the loading buffer, similar to our experience with the GT-flagellin fusion protein, so it was difficult to obtain large amounts of GT-FlgE fusion protein purified to homogeneity for immunization. The murine polyclonal antibody was therefore generated by immunizing mice with partially purified GT-FlgE (Fig. 5A) (see figure legend for details). The two murine serum samples were diluted 1:100 for the immunoblotting whose results are shown in Fig. 5B and C. The polyclonal GT-FlgE antiserum reacted with an ~40-kDa polypeptide, consistent with the predicted size of FlgE, in the lysate of *B. burgdorferi*

A

Bb_FlgE	M M R S L Y S G V S	G L Q N H O T R M D	V V G N N I A N V N	T I G F K K G R V N	F Q D M I S Q S I S	G A S R P T D A R G	60
Tp_FlgE	M M R S L F S G V S	G M Q N H O T R M D	V V G N N V I A N V N	T T G F K R G R V N	F O D L I S Q Q L S	G A S R P N E E V G	60
St_FlgE	. . M S F S Q A V S	G L N A A A T N L E D	V I G N N I A N S A	T Y G F K S G T A S	F A D M F A G S	46
Bb_FlgE	G T N P K Q V G L G	M N V A S I D T I H	T Q G A F Q S T Q K	A S D L G V S G N G	F F I L K E G K N L	. F Y T R A G A F D	119
Tp_FlgE	G V N P K E V G L G	V M V A S I D T V H	T Q G A L Q T T Q I	N T D I A I Q G N G	F F I L K D G E K S	. F Y T T A G A F G	119
St_FlgE K V G L G	V K V A G I T Q D F	T D G T T T N T G R	G L D V A I S Q N G	F F R L L V D S N G S	V F Y S R N G Q F K	101
Bb_FlgE	V D S D R H L V N P	A N G M R I Q G W M	A R D L E G E K V I	N T A S D I E D L I	I P I G D K E G A K	S T K N I T F A C N	179
Tp_FlgE	V D R D G T L V N P	A N G M R V Q G W M	A E D I E G Q Q I I	N T S D Q T E D L I	I P I G Q K I D A K	A T T D V A Y A C N	179
St_FlgE	L D E N R N L V N .	M Q G M Q L T G Y P	A T G . . T P P T I	Q Q G A N P A P I T	I P . N T L M A A K	S T T T A S M Q I N	157
Bb_FlgE	L D K R L P L I Q E	G A . S P A D I A R	G T W V V N K S L Y	D S F G N V S V L E	L R V V K D L N T P	N L W N A T V L I N	238
Tp_FlgE	L D K R L P E L P E	G A . N Q A D I L R	S T W A T D F N V Y	D T F G E Q H K L Q	M V F S R V P G T N	N Q W L A T V N V D	238
St_FlgE	L N S T D P V P S K	T P F S V S D A D S	Y N K K G T V T V Y	D S Q G N A H D M N	V Y F V K . . T K D	N E W A V Y T H D S	215
Bb_FlgE	G E Q N S N V G I G T T D G T E	. . F T L G F D N E	G A L A S L N G Q P	G Q . . . K G D I L	Q I P I T F N V L G	279
Tp_FlgE	P E N Q A G T E T R	. . V G I G T T D G T E	N T F I V S F D N Y	G H L A S V T D T A	G N V T A P A G Q V	L V Q A S Y N V V G	298
St_FlgE	S D P A A T A P T T	A S T L T L K E N E N	G I L E S G G T V N	245
Bb_FlgE	A N V G E V G E . .	Q Q T V N L K L G T	V G S Y T D S I T Q	F A D S S S T K A I	I Q D G Y G M G Y L	E N Y E I D Q N G V	337
Tp_FlgE	A N P D E G G A P T	R H T F N I N L G E	I G T S R R N T I T Q	F A E R S S T T K A Y	Q Q D G Y A M G Y L	E N F K I D Q S G I	358
St_FlgE	I T T G T I N G A T	A A T F S L S F L N S M Q Q	N T G A N N I V A T	N Q N G Y K P G D L	V S Y Q I N N D G T	299
Bb_FlgE	I V G I Y S N G I R	R D L G K I A L A S	F M N P G D L Q N Q	A I L I L	372
Tp_FlgE	I T G V Y S N G A S	R E I G Q L A L A G	F A N Q G G L E K A	G E N T Y I Q S N N	S G I A N I T V S G	V M G K G K L I A G	418
St_FlgE	V V G N Y S N E Q E	Q V L G Q I V L A N	F A N N E G L A S Q	G D N V W A A T Q A	S G V A L L G T A G	S G N F G K L T N G	359
Bb_FlgE	372
Tp_FlgE	T L E M S N V D L T	D Q F T D M I I T Q	R G F Q A G A K T I	Q T S D T M L E T V	L N L K R 463		
St_FlgE	A L B A S N V D L S	K E L V N M I V A Q	R N Y Q S N A Q T I	K T Q D Q I L N T L	V N L R * 403		

B

Bb_FlgE	M M R S L Y S G V S	G L Q N H O T R M D	V V G N N I A N V N	T I G F K K G R V N	F Q D M I S Q S I S	G A S R P T D A R G	60
Bs_FlgG	M L R S L Y S G I S	G M K N F O T K L D	V I G N N I A N V N	T V G F K K S R V T	F K D M V S Q T I A	G G S A A G A T I G	60
Cc_FlgG	. M Q A L R T A A S	G M A A Q Q L N V E	V I S N N I A N M N	T V G F K R Q R A E	F O D L L Y Q T I E	R A G S Q S S S D G	59
St_FlgG	M I S S L W I A K T	G L D A Q Q T N M D	V I A N N L A N V S	T N G F K R Q R A V	F E D L L Y Q T I R	Q P G A Q . S S E Q	59
Bb_FlgE	G T . . N P K Q V G	L G M N V A S I D T	I H T Q G A F Q S T	Q K A S D L G V S G	N G F F . I L K E G	K N L F Y T R A G A	117
Bs_FlgG	G T . . N S K Q V G	L G S S S G T I D T	I H S T S A T Q S T	G R T L D L A I D G	D G Y F R I D T G D	G . T A Y T R A G N	117
Cc_FlgG	N I V P T G V Q I G	G G V K A G S V Y R	I T E Q G T P T L T	D S P L D L A I D G	K G Y M P I L L P S	G E T A Y T R A G N	119
St_FlgG	T T L P S G L Q I G	T G V R P V A T E R	L H S Q G N L S Q T	N N S K D V A I K G	Q G F F Q V M L P D	G T S A Y T R D G S	119
Bb_FlgE	F D V D S D R H L V	N P A N G M R I Q G	W M A R D L E G E K	V I N T A S D I E D	L I I P I G D K E G	A K S T K N I T F A	177
Bs_FlgG	F Y L D N T G T L V	T G D G	Y H V L N M N G G .	T I K I P T D A Q S	F S I G S D C K V S	I	161
Cc_FlgG	F S T N D Q G Q I V	T E D G	Y L V Q P .	G I T I P Q N A T D	I P I S K S G L V Q	V K	160
St_FlgG	F Q V D Q N G Q L V	T A G G	F Q V Q P .	A I T I P A N A L S	I T I G R D G V V S	V T	160
Bb_FlgE	C N L D K R L P L I	Q E G A S P A D I A	R G T W V V N K S L	Y D S F G N V S V L	E L R V V K D I N T	P N L W N A T V L I	237
Bs_FlgG V	D A E G K T Q D G G	Q I G I V T F A N S D G L	D . K I G S N L Y R	E S L N S G T A S A	204
Cc_FlgG L	D G Q P Q P Q T V G	Q I Q L A N F L N E G G L	E . A I G D N L F L	E T A A S G . A A T	202
St_FlgG Q	Q G Q A A P V Q V G	Q L N L T T E M N D T G L	E . S I G E N L Y I	E T Q S S G . A P N	202
Bb_FlgE	N G E Q N S N F T L	G F D N E G A L A S	L N G Q P G Q K G D	I L Q I P I T F N V	L G A N V G E V G E	. . Q Q T V N L K L	295
Bs_FlgG	A N Q P G D G G T G	A L K S G F L E M S	N V D L T D E F T E	M I T A Q R G F Y S	N S K I I T S D D E	I L Q E L V N L K R	264
Cc_FlgG	L V R R A S R A L A	C C C S T D T E A S	N V D A V S E I T A	L I T A Q R A Y E M	N S K V I S T A D Q	M L Q A S Q L R S	262
St_FlgG	E S T P G L N G A G	L L Y Q G Y V E T S	N V N V A E E L V N	M I Q V Q R A Y E I	N S K A V S T T D Q	M L Q K L T Q L *	260
Bb_FlgE	G T V G S Y T D S I	T Q F A D S S S T K	A I I Q D G Y G M G	Y M E N Y E I D Q N	G V I V G I Y S N R	I R R D L G K I A L	355
Bs_FlgG	*	264
Cc_FlgG	*	262
St_FlgG	260
Bb_FlgE	A S F M N P G D L Q	N Q A I L I L 372					
Bs_FlgG 264					
Cc_FlgG 262					
St_FlgG 260					

FIG. 2. (A) Alignment of the *B. burgdorferi* (Bb) FlgE primary protein sequence with its homologs in *T. phagedenis* (Tp) and *S. typhimurium* (St). (B) Alignment of *B. burgdorferi* FlgE protein with the FlgG proteins of *Bacillus subtilis* (Bs), *S. typhimurium* (St), and *Caulobacter crescentus* (Cc). The black areas highlight the identical sequences of the flagellar proteins among different organisms, whereas the gray areas show those conserved residues in alignment with the consensus residues.

(Fig. 5C, lane 2). The murine antiserum also displayed specificity to the purified GT-FlgE (Fig. 5C, lane 3). In contrast, control mouse antiserum acquired by immunization with *E. coli* lysate containing overexpressed GT protein did not show

significant reactivity to the FlgE homolog in the *B. burgdorferi* lysate (Fig. 5B).
FlgE antibodies in patients with Lyme disease. Antiserum samples from 42 patients with Lyme disease were examined for

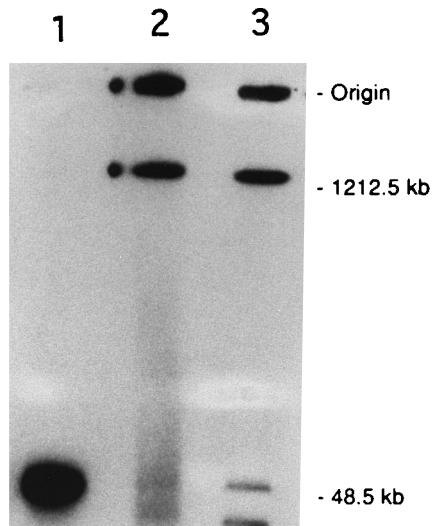


FIG. 3. Localization of the *flgE* sequence. The chromosomal and plasmid DNAs of *B. burgdorferi* N40 were separated by pulsed-field gel electrophoresis (13) (as indicated by size markers on the right) and were then transferred from the agarose gel to a nitrocellulose membrane. The corresponding nitrocellulose strips were subsequently probed with *ospA* (lane 1), flagellin (lane 2), and *flgE* (lane 3) DNA fragments by Southern hybridization.

the production of antibodies against FlgE protein by immunoblotting (Table 2). Half of the patients were in the early stages of Lyme infection and the other half of the patients were in late stages of the disease, on the basis of clinical manifestations. All the patients were found to be seropositive for exposure to *B. burgdorferi* by ELISA. The average IgM titers are 1,300 for early-stage Lyme disease patients and 400 for late-stage patients, whereas the average IgG titers are 450 for early-stage Lyme disease patients and 3,200 for late-stage patients. Interestingly, 19 of 42 patients had serum antibodies against the FlgE protein. However, FlgE appeared to be an antigen that is generated at the later stages of infection, because the level of production of FlgE antibodies was slightly higher in patients with late-stage infection (57%) than in patients with early-stage infection (33%). Like flagellin, FlgE protein shares sequence similarity with its homologs in other bacteria. Therefore, the use of whole FlgE protein as a diagnostic marker for Lyme disease may give false-positive results. Cross-reactivity of FlgE with antibodies elicited during infection with other bacteria is an important issue that must be addressed in the future. For example, determination of the immunodominant epitopes of FlgE protein that do not share significant homology with other pathogenic bacteria, similar to previous work with flagellin (3), may be considered. It should be noted, however, that in this study we have tested the possibility that the antibodies generated in response to the bacteria normally present in the human body (for example, mouth, gut, conjunctival, or urogenital flora) may react with the *B. burgdorferi* FlgE protein. More specifically, we found that a pool of eight normal human serum samples failed to show reactivity with the GT-FlgE fusion protein (data not shown), demonstrating that the antibodies raised against the nonpathogenic bacteria living in humans do not cross-react with the FlgE protein.

The reactivity of the FlgE antibodies in these patient sera is similar to that of the 41G fragment, which contains the immunodominant region of the flagellin comprising amino acids 197 to 241 (3). The 41G fragment can detect both early and late stages of Lyme disease, as previously shown (7, 16). The lower

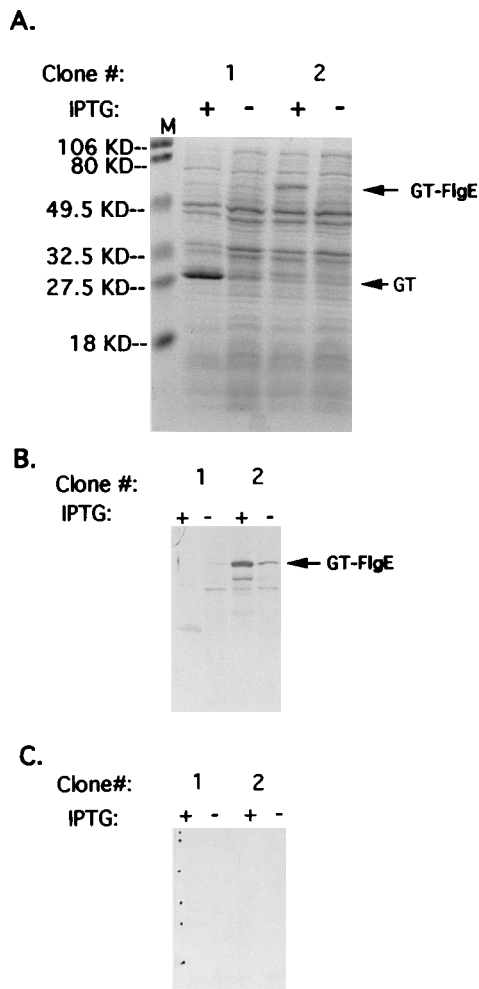


FIG. 4. Expression of the *flgE* gene in *E. coli* DH5 α cells. (A) Coomassie blue-stained protein gel. Lane M, molecular mass markers. (B) Corresponding Western blot probed with antiserum from a patient with late-stage Lyme disease. The clone containing the *flgE* gene was originally selected by this antiserum (see Materials and Methods). (C) Western blot corresponding to the gel in panel A probed with normal human serum as a negative control.

level of detection of 41G in the patients with early-stage disease may be because the infection of these patients is at a very early stage of the disease. Indeed, while 41G failed to be recognized by the sera obtained from most of the patients who were clinically ill for less than 10 days, antibodies to 41G did eventually appear in those patients reexamined 10 days following the diagnosis of erythema migrans (7). It is possible that the FlgE protein may have a similar scenario in the course of antibody development. To confirm this possibility, we tested for the development of antibodies against the FlgE protein in the infected host. Interestingly enough, we found that FlgE antibodies were generated as early as 14 days in mice challenged with 10^4 spirochetes of *B. burgdorferi* and that the antibody titer remained high at 28 days, 90 days, and 6 months (data not shown), suggesting that FlgE, as well as flagellin, can be used as an early marker for Lyme disease. We further examined the serologic reactivities of the 42 patients to three other *B. burgdorferi* antigens originally cloned in this laboratory, including OspE, OspF, and p22. Acting together, these five antigens were able to detect 67% of the total Lyme patients. In late-stage disease, combination of the five antigens

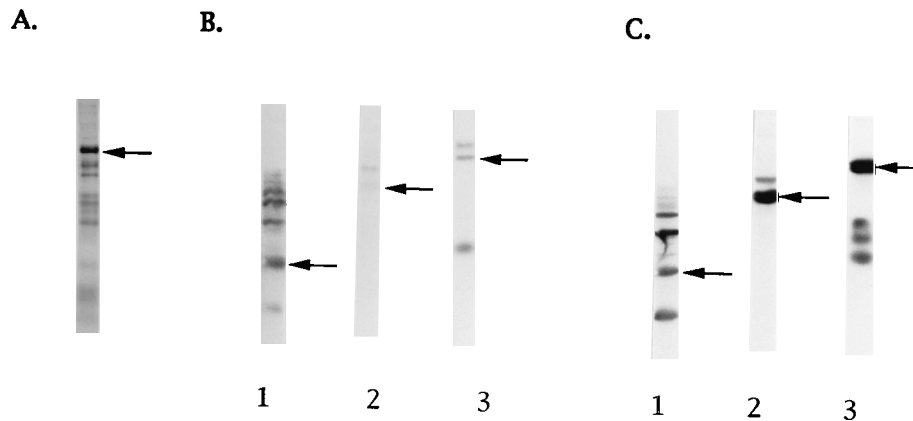


FIG. 5. (A) The *E. coli* lysate containing the GT-FlgE protein was used for immunizing mice to obtain murine antiserum. Most of the soluble proteins in this *E. coli* lysate have been removed. The position of the 66-kDa GT-FlgE protein (arrow) is indicated. (B) Immunoblots probed with the polyclonal anti-GT mouse serum as a negative control. (C) Immunoblots probed with the polyclonal anti-GT-FlgE mouse serum. Lanes 1, lysate of *E. coli* cells containing GT protein; lanes 2, *B. burgdorferi* N40 lysate; lanes 3, ~100 ng of purified GT-FlgE protein. The positions of the 26-kDa GT protein (lanes 1), the 40-kDa FlgE homolog (lanes 2), and the 66-kDa GT-FlgE fusion protein (lanes 3) are indicated (arrows).

gave a detection rate as high as 86% (18 of 21), suggesting that a cocktail of several *B. burgdorferi* antigens can be used for accurate diagnosis of Lyme disease. However, the 42 patients in this study were selected by positive ELISA using sonicated whole-cell *B. burgdorferi* lysate. The sensitivity of diagnosis using a combination of these five antigens might be lower if the patients are not prescreened by ELISA. To validate both the sensitivity and the specificity, the idea of using a cocktail of *B. burgdorferi* antigens as a diagnostic tool for Lyme disease still needs further testing.

In conclusion, the cloning, DNA sequencing, and protein expression of the gene encoding FlgE, a putative hook protein that is part of flagella, were performed. *B. burgdorferi* FlgE is 40 kDa in size and displays a high degree of homology with *T. phagedenis* FlgE (Fig. 2A). Direct evidence showing that this protein is the hook protein involved in the motility and chemotaxis of *B. burgdorferi* awaits future studies. The *flgE* gene in the original clone selected by a patient's antiserum was constitutively expressed in *E. coli* with or without IPTG induction, implying that the 2.5-kb *B. burgdorferi* DNA insert carried the promoter elements of the *flgE* gene that were recognized by the *E. coli* transcription machinery. However, no consensus

sequence for potential promoter elements could be located ahead of the ribosomal binding site of the *flgE* sequence. Quite likely, the *flgE* gene is in the same operon as ORF-1. It is interesting that the partial ORF-1 is homologous to the partial ORF-1 ahead of the *flgE* gene of *T. phagedenis* (14). Two-thirds of the 81-amino-acid sequence is homologous to the partial ORF-1 of *T. phagedenis*, strongly confirming that the *B. burgdorferi* *flgE* gene is the homolog of the *T. phagedenis* *flgE* gene and that the two genes are organized similarly. Like the *flgE* gene, the *fla* gene is localized on the linear chromosome (Fig. 3), but the physical distance between these two functionally related genes has not yet been determined. Although the flagellar genes are often clustered in the genome, our attempts to amplify the DNA fragment between the *fla* locus and the *flgE* locus by improved PCR (extender activity purchased from Stratagene) failed to show that the two loci were located within 10 kb (data not shown). The murine antibody against FlgE binds to a 40-kDa polypeptide in *B. burgdorferi* lysate (Fig. 5), confirming that the *flgE* gene is indeed expressed in *B. burgdorferi*. However, in the Western blot from SDS-polyacrylamide gel electrophoresis of *B. burgdorferi*, we did not observe a ladder of bands as found in the study of the *T. phagedenis* hook protein (14), suggesting that the *B. burgdorferi* FlgE peptide is not cross-linked. FlgE appears to elicit humoral immune responses in patients with Lyme disease, suggesting that a cocktail of several *B. burgdorferi* antigens containing recombinant FlgE protein (Table 2) can be used as a diagnostic tool for Lyme disease. FlgE is the first protein of the hook-basal body complex that is potentially involved in the motility and chemotaxis machinery of *B. burgdorferi* to be found. Further identification and characterization of other components involved in the flagella of *B. burgdorferi* will facilitate the understanding of the motility, chemotaxis, and pathogenesis of spirochetes.

TABLE 2. Antibodies to recombinant FlgE protein and 41G protein in patients with Lyme disease as detected by immunoblotting^a

Stage of disease ^b	No. of patients	No. (%) of patients with antibodies to:					Sum
		FlgE	41G	OspE	OspF	p22	
Early	21	7 (33)	4 (19)	5 (24)	5 (24)	0 (0)	10 (48)
Late	21	12 (57)	11 (52)	6 (29)	3 (14)	6 (29)	18 (86)
Total	42	19 (45)	15 (36)	11 (26)	8 (19)	6 (14)	28 (67)

^a Lysates of *B. burgdorferi* N40 and *E. coli* overexpressing GT or GT-FlgE were electrophoresed on protein gels and transferred to nitrocellulose membranes as described previously (20). Each strip contained ~100 ng of GT or GT-FlgE protein. Immunoblotting was performed with a 1:2,000 dilution of each patient's antiserum, and normal human serum at the same dilution was included as a background control. In the case of 41G, ~100 ng of purified protein was spotted on a nitrocellulose membrane directly for immunoblotting.

^b All the patients were seropositive for exposure to *B. burgdorferi* as determined by ELISA (7). Patients with early-stage Lyme disease were classified by the manifestation of erythema migrans, whereas patients with late-stage infection were diagnosed by symptoms of arthritis.

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