The *flgK* motility operon of *Borrelia burgdorferi* is initiated by a σ^{70} -like promoter

Yigong Ge,¹ Iain G. Old,² Isabelle Saint Girons² and Nyles W. Charon¹

Author for correspondence: Nyles W. Charon. Tel: +1 304 293 4170. Fax: +1 304 293 7823.
e-mail: charon@wvnmms.wvnet.edu

¹ Department of Microbiology, West Virginia University, Health Sciences Center, Box 9177, Morgantown, West Virginia 26506-9177, USA

² Unité de Bactériologie Moléculaire et Médicale, Institut Pasteur, 75724 Paris, Cédex 15, France

A cluster of flagellar genes of *Borrelia burgdorferi* was identified and sequenced. This cluster comprises an operon, designated the *flgK* operon, which is initiated by a σ^{70} -like promoter. The *flgK* operon consists of *flbF* (function unknown), *flgK* (encoding HAP1), *flgL* (encoding HAP3) and *orfX* (function unknown), and maps at 185 kb on the chromosome. In other bacteria, the hook-associated proteins HAP1 and HAP3 connect the flagellar filament to the hook and are required for the last stage of flagellar assembly. Reverse transcriptase-PCR analysis indicated that *flbF* through to *orfX* are transcribed as a single mRNA, and primer extension analysis revealed that transcription of the *flgK* operon is initiated by a σ^{70} -like promoter upstream of *flbF*. Subcloning the *flgK* promoter element into a promoter probe *cat* vector revealed that the *flgK* promoter element had strong activity in both *Escherichia coli* and *Salmonella typhimurium*. In addition, when this construct was transformed into a *fliA* mutant of *S. typhimurium* which lacked a functional flagellar-specific σ^{28} factor, the *flgK* promoter was still functional. Based on these results, the promoter element of the flagellin gene (*fla*, hereafter referred to as *flaB*) was re-examined. *flaB* encodes the flagellar filament protein, and a σ^{33-34} -like promoter has been reported to be involved in the transcription of this gene. A transcriptional start point was found 1 bp downstream of the reported start site. The sequence around –10 and –35 are consistent with the presence of a σ^{70} -like promoter in addition to the putative σ^{33-34} -like promoter for *flaB*. In contrast to the *flgK* promoter element, no activity was detected after subcloning a *flaB* promoter element into the promoter probe *cat* vector. Because a σ^{70} -like promoter rather than a unique flagellar sigma factor is involved in the later stage of flagellar assembly, the regulation of *B. burgdorferi* flagellar genes is evidently different from that of other bacteria.

Keywords: *Borrelia burgdorferi*, motility, flagella, hook-associated protein, sigma factor

INTRODUCTION

The flagellum is a complicated organelle essential for the motility of many species of bacteria. A typical flagellum consists of a terminal cap protein, filament, hook-associated proteins and a hook-basal body complex. Located at the cell membrane and within the cytoplasm

are the components required for flagellar synthesis, chemotaxis and motility. Over forty motility and chemotaxis genes have been identified in *Escherichia coli* and *Salmonella typhimurium*; these genes are arranged in large clusters on the chromosome. In *E. coli* these clusters include four motility regions (region I, II, IIIa and IIIb), which consist of 15 operons (Aizawa, 1996; Macnab, 1996).

In several species of bacteria, expression of flagellar genes is tightly controlled in a transcriptional hierarchy (Fredrick *et al.*, 1995; Helmann, 1991; Kutsukake & Ide, 1995; Macnab, 1996; Marquez-Magana & Chamberlin, 1994; Mohr *et al.*, 1996; Wu *et al.*, 1995).

Abbreviations: CAT, chloramphenicol acetyltransferase; HAP, hook-associated protein; PF, periplasmic flagellum; RT-PCR, reverse transcriptase-PCR; SRPCW, semi-random PCR chromosome walking.

The GenBank accession numbers for the sequences of the *flgK* operon and of the upstream region of *flaB* reported in this paper are U62901 and U66699, respectively.

Specifically, the expression of early-stage genes is a prerequisite for activation of the later-stage genes during flagellar synthesis. For example, in *E. coli* and *S. typhimurium*, expression of the class 1 master genes is essential for activation of the class 2 operons which encode proteins required for the early assembly of the flagellum (Helmann, 1991). The flagellar-specific σ^{28} factor, which is encoded by a class 2 gene, augments transcription of the class 3 operons which encode flagellin, hook-associated proteins (HAPs), proteins involved in chemotaxis and negative regulatory proteins (Aizawa, 1996; Helmann, 1991; Macnab, 1996). A similar regulatory cascade is observed in *Caulobacter crescentus* and *Bacillus subtilis*, but in *C. crescentus* flagellar synthesis is tightly coupled to cell division (Mohr *et al.*, 1996; Quon *et al.*, 1996; Zhuang & Shapiro, 1995). Like other bacterial sigma factors, flagellar-specific sigma factors recognize a specific consensus sequence (Helmann, 1991; Macnab, 1996). The well conserved σ^{28} -like promoter elements are involved in the transcription of most class 2 and class 3 operons in enteric bacteria and *B. subtilis* (Fredrick *et al.*, 1995; Fredrick & Helmann, 1994; Macnab, 1996; Marquez-Magana & Chamberlin, 1994; Mirel *et al.*, 1992). In *C. crescentus*, a similar σ^{54} promoter is required for class 3 and 4 operons (Anderson *et al.*, 1995; Ramakrishnan *et al.*, 1994; Wu *et al.*, 1995).

Borrelia burgdorferi is a motile, pathogenic spirochaete which causes Lyme disease. The outermost layer of the cell is a membrane sheath, and within this sheath is the cell cylinder and periplasmic flagella (PFs). The PFs are attached near each end of the cell cylinder and are morphologically similar to the flagella of other bacteria (Hovind Hougen, 1984). Genetic evidence indicates that the PFs are involved in motility and rotate in a manner similar to the flagella of rod-shaped bacteria (Goldstein *et al.*, 1994; Sadziene *et al.*, 1991). Several lines of evidence suggest that motility is important for this organism to infect the mammalian host. First, after being deposited in the skin as a consequence of a tick bite, the spirochaetes must traverse the intercellular matrix and eventually enter the bloodstream (Kimsey & Spielman, 1990). In fact, *B. burgdorferi* and other spirochaetes have the unique ability to efficiently swim in gel-like media such as collagen fibres and hyaluronic acid (Canale-Parola, 1978; Goldstein *et al.*, 1994; Kimsey & Spielman, 1990). Second, genetic experiments implicate motility as an important virulence factor. Specifically, Sadziene *et al.* (1991) found that a spontaneous nonmotile mutant (HB19Fla⁻) is less invasive than wild-type *B. burgdorferi* in cell penetration assays. Finally, because more than 38 genes, or approximately 3.5% of its genome, are dedicated to motility and chemotaxis (our unpublished results; Gassmann *et al.*, 1991; Ge *et al.*, 1996), motility may be presumed vital for *B. burgdorferi* to survive in nature (Armitage, 1992).

The mechanism underlying motility of *B. burgdorferi* is poorly understood. Most of the information obtained has been based primarily on morphological observations of swimming cells and an analysis of PF structure

(Goldstein *et al.*, 1994, 1996; Hovind Hougen, 1984). The absence of a gene exchange system in *B. burgdorferi* has severely impaired the study of motility at a molecular level. To elucidate the role of motility in the development of Lyme disease, we initiated a genetic analysis of the flagellar system of *B. burgdorferi* (Ge *et al.*, 1996).

In this report we describe a flagellar operon containing at least four genes. Within this operon, three putative flagellum-related genes were sequenced and its promoter region analysed in detail. In contrast to the flagellar genes of other bacteria, a σ^{70} -like promoter was found to be involved in the transcription of this operon. In addition, because the promoter of the flagellin operon (*fla*, hereafter referred to as *flaB*) of *B. burgdorferi* has been reported to utilize a σ^{p33-34} promoter sequence (Noppa *et al.*, 1995), we re-examined this promoter and also found it to be σ^{70} -like. This is the first report to our knowledge of σ^{70} -like promoters being involved in the transcription of a flagellar hook-basal body operon in bacteria.

METHODS

Bacterial strains, plasmids and growth conditions. French isolate of *B. burgdorferi sensu stricto* strain 212, Northern American isolate of *B. burgdorferi* strain HB19 and a spontaneous PF mutant obtained from strain HB19 (HB19Fla⁻) were used in this study (Sadziene *et al.*, 1991). *B. burgdorferi* were grown in BSK-II medium (Barbour, 1984). *E. coli* DH5 α (Bethesda Research Laboratory) and HB101 (Promega) were used for propagation of plasmids or for the chloramphenicol acetyltransferase (CAT) assay. *E. coli* and *S. typhimurium* strains were grown in LB medium or 2YT medium (Sambrook *et al.*, 1989). Bacteria carrying recombinant plasmids were maintained in the presence of antibiotics at the relevant concentrations (ampicillin, 50 $\mu\text{g ml}^{-1}$ and/or chloramphenicol, 25 $\mu\text{g ml}^{-1}$). The plasmids used in this study are listed in Table 1.

DNA analysis. Restriction enzymes and other enzymes were used as directed by the suppliers (New England Biolabs, Life Technology and Promega). Plasmids were isolated using Qiagen Mini or Midi plasmid isolation kits. *B. burgdorferi* genomic DNA was isolated as described by Ausubel *et al.* (1995). Inserts were sequenced on both strands by the Sanger method (Sanger *et al.*, 1977). The sequencing strategy was subcloning and internal primer walking. DNA sequence data were analysed using DNASTAR and DNASIS, along with on-line BLAST.

Cloning and mapping the genes upstream of *flgK*. By random sequencing of a previously constructed partial *EcoRI* bank from *B. burgdorferi* strain 212, we isolated the *gidA*, *gidB* and *thdF* genes of strain 212 which mapped at 185 kb on the chromosome and are transcribed toward the 0 telomer (Saint Girons *et al.*, 1994). Extended sequencing of the region adjacent to *thdF* revealed an ORF with identity to *flgK* of *B. subtilis*, and that this ORF is transcribed in the opposite orientation to that of *thdF* (I. Saint Girons & I. G. Old, unpublished).

Semi-random PCR chromosome walking (SRPCW). To sequence part of *flgK* and the downstream genes, we used the new method of SRPCW (unpublished). This method is a modification of that described by Parker *et al.* (1991). A specific primer (5'-CCCAATGAATTTTAAATCC-3') gen-

Table 1. Plasmids used in this study

Plasmid	Description	Reference or source
pNoTA/T7	Amp ^r PCR cloning vehicle	5' → 3', Inc.
pGEM-T	Amp ^r PCR cloning vehicle	Promega
pCR II	Amp ^r and Kan ^r PCR cloning vehicle	Invitrogen
pKK232-8	Amp ^r promoter probe vehicle with promoterless <i>cat</i> gene	Brosius (1984)
pER187	Amp ^r derived from pUC19 with a mobile <i>cat</i> gene from <i>S. aureus</i>	E. Rosey, Pfizer, Groton, CT, USA
pflgKL	3.0 kb PCR fragment containing <i>flgK</i> and <i>flgL</i> genes cloned into pNoTA/T7 vector	This study
pf1aBp	1.0 kb PCR fragment containing the sequence upstream of <i>flaB</i> gene cloned into pGEM-T	This study
ppflgK	pKK232-8 with 230 bp insert from <i>flgK</i> promoter element	This study
ppflaB	pKK232-8 with 130 bp insert from <i>flaB</i> promoter element	This study

erated from the 5'-end of *flgK* was paired with a random primer (5'-GCGTGAAAGA-3') for the amplification of the 3'-end of *flgK* under low-stringency PCR conditions. The nucleotide composition of the random primers is slightly higher in G+C content than that of the *B. burgdorferi* genome, which is 27 mol% G+C (Johnson *et al.*, 1984). For amplification, the PCR reaction was carried out with 200 ng of *B. burgdorferi* DNA and 100 pmol each of the specific primer and the random primer using PrimeZyme (Biometra). Amplification was carried out at 94 °C for 1 min, 43 °C for 1 min and 72 °C for 5 min for 35 cycles in 5 mM MgCl₂. The PCR products were analysed in 1% agarose gels, purified using a Qiagen PCR purification kit and cloned into pGEM-T. After selecting five clones for DNA sequencing, one positive clone (pflgKL) with a 3.0 kb insert was found to contain the remaining portion of the *flgK* gene and an intact *flgL* gene (Table 1). The sequence of the fragment was further verified by repeating the PCR amplification under high-stringency conditions using *Pfu* DNA polymerase (Stratagene) which has higher fidelity, together with specific primers generated from the new sequence data from the 3'-end of *flgL*. The amplified products were cloned into the pNoTA/T7 vector. For the high-stringency reaction, the annealing temperature was raised to 52 °C and the concentration of MgCl₂ was decreased to 1.5 mM. The same strategy was utilized to amplify a segment upstream of the *flaB* operon. By using a specific primer, 5'-TGAAGCATTAATAGCTGATG-3', and a random primer, (5'-CCTTTAAGGAA-3'), a 1.0 kb PCR fragment was amplified and cloned into pGEM-T, resulting in the plasmid pflaBp.

Isolation of total RNA, primer extension and reverse transcriptase-PCR (RT-PCR). For the isolation of RNA, 500 ml of mid-exponential phase *B. burgdorferi* cells (10^7 cells ml⁻¹) were harvested and washed with cold 0.13 mM phosphate-buffered saline (0.145 M sodium chloride, 0.15 M sodium phosphate, pH 7.4) twice. Total RNA was isolated with Tri Reagent (Sigma) according to the manufacturer's instructions and treated with DNase (Promega). Further purification of RNA was completed using a Qiagen RNA isolation kit. Primer extension was carried out by using AMV Reverse Transcriptase Primer Extension System (Promega) with modification. Briefly, 15 pmol of reverse primer (5'-GCG-ACCTCTTTAAGTCCT-3' for the *flgK* promoter and 5'-TGTAATCAATCTACGAGC-3' for the *flaB* promoter) were labelled with [γ -³²P]ATP at 37 °C for 30 min, and purified with a Qiagen Nucleotide Cleaning kit. For primer extension, 1 μ l (50 fmol) ³²P-labelled primer was mixed with 50 μ g RNA and incubated at 58 °C for 20 min. AMV reverse transcriptase and other reagents were then added and the extension reaction was

flgK

Fig 6

L F P T A L A V I D D D R E F F K S M *thdF*

TTTCCACACGTTCTTCGATGTATATAGTAGTAGAGAGAGTTTTTGAATGAGTA

AAAGGTGTTGCAAGAGCTACAAATATCATCATCTCTCTCAAAAACCTTACTATATTAATGTATAT

ppflqR $\rightarrow$ -35 -10 +1

TATAACACAAATAAAAAAGACAAATTTGATTTGTAATATGTTTATAATAGCTTATAATTAATTTTCAT

FibF $\rightarrow$

M V E

AAAAATGAACAACAACTTTGAACCTGAACTAAAGGAAGTCTTAAAAAAGAAGCTCTCTTGTAGAAG

$\leftarrow$ ppflqR

flaB

ppflaBF →
 ACATTAAAGGCTTTTGATTTTAATCAAAGAAATAAATAATTAATTTTATATGCTATTGCTAT
 -35 -10 +1
 TTGCGTTATTTTAAATTTTGTGCTATCTTTTAAACAGGAAAGGATTGCGAAAGTCAA
 flaB →
 AATTTTATTTATCATGGAGGAATGATATATGATTATCAATCAATACATCAGCTATTATAGCTTC
 0 ppflaBR M I I N H N T S A I N A S

Fig. 1. Promoter elements of *flgK* and *flaB* operons which were amplified and cloned into the promoter probe vector. The double-underlined sequences are the amplified regions.

carried out at 41 °C for 30 min. The products were precipitated with ethanol and resuspended in 4 µl loading buffer. The primer products were run along with a sequence ladder generated from the same primers.

RT-PCR was carried out by the method described by Limberger *et al.* (1996). Briefly, 500 ng total RNA was mixed with appropriate reverse primers, 1 unit RNase inhibitor (Promega), 1 unit Superscripts-H reverse transcriptase (Life Technology) and the required reagents according to the manufacturer's protocol. The mixture was incubated for 25 min at 42 °C followed by 5 min at 99 °C to inactivate reverse transcriptase. The synthesized cDNA was amplified after adding the appropriate forward primer by a PCR reaction of 35 cycles consisting of 1 min at 94 °C, 1 min at 52 °C and 1 min at 72 °C. The amplified products were analysed in 2% agarose gels.

Analysis of *flgK* and *flaB* promoter elements in *E. coli*. A plasmid, pKK232-8, containing a promoterless *cat* gene was used to test for function and strength of the promoter elements from two flagellar operons (Brosius, 1984). The regions containing putative *flgK* and *flaB* promoter elements were amplified by using primer pairs, ppflgKF/ppflgKR and ppflaBF/ppflaBR, respectively (Fig. 1). Both pairs of primers have a *Bam*HI site at the 5'-end and a *Hind*III site at the 3'-end.

The amplified PCR products were cloned into the *Bam*HI/*Hind*III sites of pKK232-8, resulting in the plasmids p*flgK* (*flgK* promoter) and p*flaB* (*flaB* promoter, Table 1). After transformation, the recombinants were selected on ampicillin plates with or without chloramphenicol. An MIC assay was used to determine relative promoter strength. In brief, the *E. coli* HB101 transformants harbouring promoter probe constructs were grown at 37 °C in 5 ml 2YT broth containing 100 µg ampicillin ml⁻¹ to an OD₆₀₀ of 1.0. Diluted cultures were plated onto 2YT medium containing 2–1000 µg chloramphenicol ml⁻¹ and 100 µg ampicillin ml⁻¹, and examined for growth after incubation at 37 °C. Each test was performed in duplicate. The MIC was calculated as the lowest concentration of chloramphenicol at which no growth was discernible. The quantity of CAT protein in the transformed cells was determined in two separate experiments using an ELISA kit from Boehringer Mannheim. Five millilitres of exponential-phase cells were collected by centrifugation, and after lysing the cells, the concentration of CAT protein was determined according to the manufacturer's protocol. Results are expressed as pg CAT protein (mg total cell protein)⁻¹.

RESULTS AND DISCUSSION

Mapping and orientation of *flgK* operon

In a clone containing the region around the 185 kb point of the chromosome, a partial ORF (*flgK*) encoding a protein with similarity to HAP1 was identified. We sequenced the upstream region of the clone and identified an ORF (*flbF*, see below). We used SRPCW to amplify the remaining portion of the *flgK* gene and downstream region. After testing several specific and random primers, successful amplification of the desired region was achieved. A 3.0 kb fragment containing part of *flgK* and the downstream region was obtained and cloned into an appropriate vector for sequencing. The fragment was further verified as containing *flgK* by PCR using specific primer pairs. Two other genes, *flgL* and *orfX*, were identified downstream of *flgK*.

The *flgK* gene cluster is transcribed in an opposite direction to the adjacent non-flagellar *thdF* gene (I. Saint Girons & I. Old, unpublished; Fig. 2). The *flgK* gene cluster is the fourth flagellar gene cluster identified in *B. burgdorferi*. The others include a single *flaB* gene operon localized at 124 kb (Saint Girons *et al.*, 1994), a large *flgB* operon at 300–310 kb (Ge *et al.*, 1996; Saint Girons *et al.*, 1994; unpublished) and a *cheA*–*cheW* operon at 725 kb (G. Treuba, I. Old, I. Saint Girons & R. C. Johnson, unpublished). These results suggest that, like

Fig. 2. Gene order and orientation of the *flgK* operon and adjacent *thdF* gene on *Borrelia burgdorferi* 212 chromosome.

flagellar genes in other bacteria (Macnab, 1996; Zuberi *et al.*, 1991), *B. burgdorferi* flagellar genes constitute large gene clusters and are distributed on the chromosome at specific loci. These loci are likely to be part of a large flagellar motility-chemotaxis regulon.

Sequence analysis of the *flgK* cluster

The first gene in this region is an ORF of 435 bp encoding a deduced protein of 145 amino acids. In order to simplify the nomenclature of this gene of unknown function, and because it is adjacent to *flgK*, we designated this gene *flbF* (flagellar gene of *Borrelia*). *flbF* begins with the start codon TTG (Fig. 1). There are several reasons for assigning TTG rather than ATG as the translational start site. First, initiation at TTG is consistent with primer extension analysis (see below). The ATG at 51 bp upstream of TTG is incorrectly positioned relative to the transcriptional start site to serve as the initiation codon. Second, there is a putative RBS, AAAGA, immediately before the TTG codon; an RBS is not present in front of the ATG sequence. Finally, the start codons of other flagellar genes from *B. burgdorferi* and other bacteria, in particular *B. subtilis*, are TTG or GTG (Ge *et al.*, 1996; Hardham *et al.*, 1995; Zuberi *et al.*, 1991). The putative gene product of *flbF* shows weak homology (40% similarity) with a putative flagellar protein, Orf139, from a homologous flagellar operon of *B. subtilis* (Mirel *et al.*, 1994). Since flagellar genes are clustered by function, *flbF* is most likely involved in the final stage of flagellar assembly.

Immediately after *flbF* is a large 1881 bp ORF encoding a 627 amino acid protein with a molecular mass of 71 kDa. The deduced protein product of this gene, designated *flgK*, shows highest homology with the N-terminal region of *B. subtilis* HAP1 (35% identity, 76% similarity; only the N-terminal sequence of *B. subtilis* HAP1 is accessible). It also shows weak homology with HAP1 of *Vibrio parahaemolyticus* (22% identity, 53% similarity) and *S. typhimurium* (26% identity, 52% similarity). *B. burgdorferi* HAP1 is similar in size to its counterparts found in *V. parahaemolyticus* (646 amino acids) but larger relative to that of *S. typhimurium* (553 amino acids). The *B. burgdorferi* *flgL* homologue (1278 bp) is immediately downstream of *flgK* (Fig. 2). Its putative gene product, HAP3, a 425 amino acid polypeptide, also demonstrated homology with its counterparts from *V. parahaemolyticus* (17% identity, 54% similarity) and *S. typhimurium* (18% identity, 46% similarity). In addition, *B. burgdorferi* HAP3 shows weak sequence homology to flagellin proteins (FlaB) from many species of bacteria, including *B. burgdorferi* and *Treponema pallidum* (data not shown). Immediately downstream of *flgL* a potential ORF was identified and designated as *orfX*. Only one bp separates the *flgL* stop codon and the *orfX* start codon. Because only limited sequence data is available (67 bp), we tentatively designate *orfX* as a potential ORF.

B. burgdorferi *flgK* and *flgL* are likely to have functions similar to their counterparts in other bacteria. We and

Fig. 3. RT-PCR products after electrophoresis in a 2% agarose gel and staining with ethidium bromide. The image was obtained with the Eagle Eye system (Stratagene). Lane 0 contains digested pGEM markers; lanes 1, 3, 5 and 7 contain total RNA from the HB19Fla⁻ mutant; and lanes 2, 4, 6 and 8 contain total RNA from wild-type *B. burgdorferi* cells. The amplified products were obtained from pairs of primers which span the intergenic regions: *flbF*–*flgK* (lanes 3 and 4), *flgK*–*flgL* (lanes 5 and 6) and *flgL*–*orfX* (lanes 7 and 8). Lanes 1 and 2 are negative controls using primers generated from *thdF* and *flbF*. Arrows point to amplified products of predicted size.

others have found that hook-basal body complexes of *B. burgdorferi* are very similar in structure to those of *E. coli* or *S. typhimurium* but lack the L and P rings associated with the Gram-negative outer membrane (Hovind Hougen, 1984; Kreiling, 1995). HAP1 and HAP3 form the junction between the flagellar hook and filament in *S. typhimurium* (Macnab, 1996). Both proteins are necessary for filament assembly onto the hook. HAP1 and HAP3 serve as an adaptor to connect the inner domains of the flagellar hook to the filament (Aizawa, 1996; Homma & Iino, 1985; Homma *et al.*, 1984; Macnab, 1996).

The *flgK* cluster is organized as an operon

Examination of the DNA sequence of *flbF*, *flgK*, *flgL* and *orfX* indicates that these genes are likely to be transcribed as a polycistronic message. To test this, we determined if a single message encoded the proteins using RT-PCR. We chose DNA primers specific to two adjacent genes, and determined whether the message extended through both genes. To ensure the validity of this assay, we used primers from *flbF* and *thdF* which are obviously transcribed in opposite directions as a negative control (Fig. 1). The results obtained indicated that the assay was valid; the control did not yield products. On the other hand, using the RT-PCR products, the predicted values were obtained between *flbF* and *flgK*, *flgK* and *flgL*, and *flgK* and *orfX* (Fig. 3). These results

Fig. 4. Comparison of the *flgK* operon of *B. burgdorferi* with that of other bacteria (Macnab, 1996; McCarter, 1995; Mirel *et al.*, 1994; Ramakrishnan *et al.*, 1994). Transcriptional directions are marked with arrows. The appropriate promoter elements are presented upstream of the first gene for each operon.

Fig. 5. Primer extension of the *flgK* (a) and *flgB* (b) operons in a 6% polyacrylamide gel. The start site is marked by +1. The exposure times for p1 and p2 were 10 h and 48 h, respectively.

suggest that these genes are transcribed as a single polycistronic mRNA.

In comparing the organization of the *flgK* operon to those of other bacteria, we found several interesting features (Fig. 4). *flgK* and *flgL* were always adjacent to one another, and *flgK* always preceded *flgL*. In contrast, the flanking regions demonstrated many variations from one species to another. In *E. coli* and *S. typhimurium*, *flgK* and *flgL* constitute the last operon within a flagellar gene cluster (region I), and there are two operons (*flgA* and *flgB*) upstream of *flgK* (Macnab, 1996); in *V. parahaemolyticus*, *flaE* (the *flgK* homologue) with *flaF* (*flgL* homologue) comprises the first operon followed by two single flagellin gene operons (*flaC* and *flaD*) (McCarter, 1995). In *C. crescentus*, *flgK* and *flaQ* (function unknown) form an operon with an orientation opposite to a hook-related operon containing *flbG*, *flgD*

	-35	-10	+1	Reference
σ^{28} (<i>fliA</i>)	----CTAAA-----	CCGATAT-----	A--	Macnab (1992)
σ^{70}	---TTGACA----- *****	TATAAT----- *****	A-- *	Harley & Reynolds (1987)
<i>flgK</i> (<i>Bb</i>)	<u>GATTTGTAAATATGTTTATAATAGCTTATAATTAATTCATA</u> *** *	*****	*	This study
<i>flaB</i> (<i>Bb</i>)	<u>CGTTTCCTTTTAAATTTTGTGCTATTCCTTTTAACAGG</u> **	*** *	*	This study
<i>flaB</i> (<i>Bb</i>)	<u>GCGTTTCCTTTTAAATTTTGTGCTATTCCTTTTAACAG</u>			Noppa et al. (1995)
<i>flaA</i> (<i>Bb</i>)	<u>CTTAAAGGAGCAAAGTTGATAAAATTAATTTTACTAATAAAA</u> ** *	*** *	●	Ge & Charon (1997)
<i>flaA</i> (<i>Sa</i>)	<u>GTCGTTGACAAAGGATCAAGCATCCATATAATGAATTCAG</u> *****	*****	●	Parales & Greenberg (1993)
<i>flaA</i> (<i>Tp</i>)	<u>TCAGTTGACAAATAGGCGGTGAGCGTATACTCGAAGAGTGG</u> *****	*****	*	Issacs & Radolf (1990)
<i>flaA</i> (<i>Sh</i>)	<u>TATTTTGGCAACTTCATATAGTCAGTTAGATTGTCGGAATAC</u> *** **	*** *	*	Koopman et al. (1993)

Fig. 6. Comparison of the sequences of *B. burgdorferi* (*Bb*) *flgK*, *flaB* and *flaA* promoters with those of σ^{28} and σ^{70} and with other reported spirochaete motility promoter sequences: *flaA* from *Spirochaeta aurantia* (*Sa*), *flaA* from *Treponema pallidum* (*Tp*) and *flaA* from *Serpulina hyodysenteriae* (*Sh*). The nucleotides marked with an asterisk are those identical to the σ^{70} consensus sequence.

and *flgE* (Anderson et al., 1995; Ramakrishnan et al., 1994). In contrast, the structure of the *B. subtilis* *flgK* operon resembles that of *B. burgdorferi*. Four *B. subtilis* genes have been identified with the order *orf139*, *flgM*, *orf160* and *flgK* (Mirel et al., 1994). *orf139* and *orf160* have unknown function, but *orf139* has homology with *B. burgdorferi* *flbF*. Apparently, *orf160* and *flgM*, the latter of which encodes an anti- σ^{28} factor (Hughes et al., 1993; Iyoda & Kutsukake, 1995; Macnab, 1996), are missing from this operon in *B. burgdorferi*. These results are consistent with the evidence presented below that *B. burgdorferi* lacks a flagellar-specific σ factor.

Determination of *flgK* and *flaB* operon promoters

To investigate the transcriptional regulation of the *flgK* operon in *B. burgdorferi*, transcriptional initiation was analysed by primer extension. A clear start signal was detected upstream of *flbF* 59 bp relative to its deduced initiation codon, TTG. Analysis of the upstream sequence indicated a typical σ^{70} -like promoter at the -10 and -35 region (Fig. 5a). Significantly, the -10 region is 100% identical to the *E. coli* consensus Pribnow box (Harley & Reynolds, 1987) (Fig. 6), but there is some degree of variation in the -35 region. Because transcription of housekeeping genes is often initiated by σ^{70} -like promoters (Gralla & Collado-Vides, 1996), the genes in this *flgK* operon could be continuously ex-

pressed in *B. burgdorferi*. Consistent with these results is the finding that the PF proteins of *B. burgdorferi* are present throughout its life cycle as surveyed in bacteria from ticks (T. Schwan, personal communication), animals and those cultured *in vitro* (Magnarelli et al., 1996). The distinct -35 region could be related to modulation of transcription, as this region binds factors which influence transcriptional expression (Ishihama, 1992). Along these lines, a *B. burgdorferi* gene encoding a σ^{70} -like factor has been identified and sequenced (Tsai & Pan, 1996). Analysis of this gene indicated that the -10 binding region was well conserved when compared to that of other bacteria, whereas the -35 binding region was less conserved (Tsai & Pan, 1996).

This is the first report to our knowledge of a σ^{70} -like promoter committed to transcription of a typical 'class 3' flagellar operon in bacteria. As illustrated in Fig. 4, four other bacterial *flgK* operons from *E. coli*/*S. typhimurium*, *B. subtilis*, *C. crescentus* and *V. parahaemolyticus* have been characterized (Macnab, 1996; McCarter, 1995; Mirel et al., 1992; Ramakrishnan et al., 1994). Transcriptional analyses indicate that expression of the class 3 *flgK* and *fliD* operons in *S. typhimurium* are dependent on the σ^{28} factor along with class 2 transcription initiation (Kutsukake & Ide, 1995). In both *B. subtilis* and *V. parahaemolyticus*, the *flgK* operon is initiated from a typical σ^{28} consensus promoter

Table 2. Analysis of promoter strength by MIC and CAT assays

Host cell	MIC ($\mu\text{g ml}^{-1}$)/CAT concn [$\text{pg } (\mu\text{g total cell protein})^{-1}$]			
	Plasmid ...	pKK232-8	pER187*	ppflgK ppflaB
<i>E. coli</i> HB101 (wild-type)		2/ND	64/ND	1000/ND 4/ND
<i>S. typhimurium</i> KK1203 (wild-type)†		8/0	86/10	1000/3030 4/0
<i>S. typhimurium</i> KK1401 (<i>flaA</i> mutant)†		4/0	86/10	1000/3060 2/0

ND, Not determined.

* Provided by K. Kutsukake, Hiroshima University, Japan.

† Provided by E. Rosey, Pfizer, Groton, CT, USA.

(McCarter, 1995; Mirel *et al.*, 1994). A similar flagellar-specific sigma factor (σ^{54}) is involved in the transcription of the *flgK* operon in *C. crescentus* (Ramakrishnan *et al.*, 1994). Clearly, transcription of all previously characterized *flgK* operons is initiated by class 2 and class 3 promoters and by a family of flagellar-specific sigma factors (σ^{28} or σ^{54}), which is very distinct from what is found with *B. burgdorferi*.

Recently, primer extension analysis yielded different conclusions concerning the promoter sequences involved in transcription of the *flaB* gene in *B. burgdorferi*. Gassmann *et al.* (1991) suggest that a σ^{70} -like promoter is involved in *flaB* transcription. On the other hand, Noppa *et al.* (1995) suggested that a *B. subtilis* bacteriophage $\sigma^{\text{gp33-34}}$ -like promoter is involved in transcription of this gene. The results which show the *B. burgdorferi flab* promoter being transcribed from a $\sigma^{\text{gp33-34}}$ -like promoter were considered to be inconsistent with our results with the *flgK* operon, as we would expect this class 3 operon also to be regulated by a σ^{70} -like promoter. Along these lines, we recently identified σ^{70} -like promoters involved in the transcription of several other *fla/che* operons of *B. burgdorferi* (unpublished). To examine whether *flaB* is transcribed from a $\sigma^{\text{gp33-34}}$ -like promoter, we re-examined the promoter element of *B. burgdorferi flab*. To obtain the upstream region of *flaB* of strain 212, we used SRPCW and a primer based on the published sequence of *flaB* (Wallich *et al.*, 1990). The promoter region we obtained for strain 212 is essentially identical to that of strain B31 except for a single base pair difference (Noppa *et al.*, 1995). Our primer extension results indicate that the transcriptional start site is 1 bp downstream of the reported start site as shown in Fig. 5(b). The sequence around the -10 region revealed a putative σ^{70} -like promoter (TATTCT) 7 bp upstream of the start site, although this region is not as well conserved as that of the *flgK* operon. Gassmann *et al.* (1991) also propose these same nucleotides as the -10 region for *flaB*. As with the *flgK* operon, the -35 region of the *flaB* operon is different from that of a typical σ^{70} -like promoter. These results suggest that a σ^{70} -like rather than a $\sigma^{\text{gp33-34}}$ -

like promoter could be involved in the transcription of *flaB* operon.

Little is known about transcriptional regulation of the flagellar genes of spirochaetes. A σ^{28} -like promoter sequence was localized by primer extension at a region upstream of a large flagellar cluster in *T. pallidum* and *Treponema phagedenis* (Limberger *et al.*, 1996). In *T. pallidum*, *T. phagedenis*, *Serpulina hyodysenteriae*, and *Spirochaeta aurantia*, the flagellar *flaB* filament genes are transcribed from a σ^{28} -like promoter (Champion *et al.*, 1990; Charon *et al.*, 1992; Koopman *et al.*, 1993; Limberger *et al.*, 1992; Pallesen & Hindersson, 1989). In addition, the *flaA* gene, which encodes the flagellar sheath in *Se. hyodysenteriae*, *T. pallidum* and *Sp. aurantia* has a very well conserved σ^{70} -like promoter (Fig. 6) (Isaacs & Radolf, 1990; Koopman *et al.*, 1992; Parales & Greenberg, 1993).

Functional analyses of *flaB* and *flgK* promoter

We examined whether promoter elements from *flgK* and *flaB* function to initiate transcription in *E. coli* and *S. typhimurium*. We also examined whether expression occurred in a *flaA* motility mutant of *S. typhimurium* defective in a σ^{28} factor. We used the promoter selection vector pKK232-8 in this analysis (Brosius, 1984). This plasmid vector contains a promoterless *cat* gene with stop codons in three different frames between the polylinker site and *cat* gene to prevent translational read through from vector genes. It also has a strong transcriptional terminator upstream of the polylinker site. Several different constructs were cloned into pKK232-8 and transformed into *E. coli* or *S. typhimurium*. The *flgK* promoter elements enabled both the *E. coli* and *S. typhimurium* parents and the *flaA* mutant to be resistant to an extremely high concentration of chloramphenicol with MICs of 1 mg ml^{-1} , while the cells harbouring the *flaB* promoter showed almost no resistance to chloramphenicol (Table 2). The activity of the *flgK* promoter was even higher than the positive control (pER187). The plasmid pER187 contains a *cat* gene of *Staphylococcus aureus* with its own promoter. ELISA assays agreed with the MIC results, as high concentrations of CAT protein

were detected in the *ppflgK*-transformed cells, a lower level in the control, but none in cells transformed by *ppflaB* (Table 2). These results suggest that the *flgK* promoter of *B. burgdorferi* is functional in *E. coli* and *S. typhimurium*. In addition, because CAT expression was high in the *fliA* mutant, a σ^{70} -like rather than a σ^{28} factor is most likely involved in the transcription of these genes. It is not clear why the *flaB* promoter is not functional in *E. coli* or *S. typhimurium*; perhaps there are other transcriptional factors involved in the expression of *B. burgdorferi flab*. In addition, we cannot exclude the role of the putative $\sigma^{gp33-34}$ -like promoter in its transcription.

Analysis of a flagella-less mutant

Several years ago Sadziene *et al.* (1991) isolated a spontaneously occurring nonmotile mutant from *B. burgdorferi* strain HB19 (HB19Fla⁻). Although this mutant lacked detectable FlaB protein, the *flaB* gene was transcribed at a level identical to the wild-type. Although the location of the gene which is altered in this mutant is unknown, it is likely that it suffered a mutation in a gene involved in the late stage of flagellar synthesis. Specifically, electron microscopy revealed that this mutant had intact hook-basal body structures (Sadziene *et al.*, 1991). Because mutations in either *flgK* or *flgL* result in the flagella-less mutants with intact hook-basal bodies in other bacteria (Jones & Macnab, 1990), we hypothesized that the *flgK* operon could be altered in HB19Fla⁻. To test this, we amplified the region between *flbF* and *orfX* in both wild-type HB19 and HB19Fla⁻. Neither an insertion nor a deletion in this region was detected in HB19Fla⁻ (data not shown). In addition RT-PCR analysis indicated that HB19Fla⁻ produced detectable levels of mRNA from this region (Fig. 3). Although we are unable to exclude the possibilities that point mutations, or small insertions or deletions exist in this region, our results indicate that there is no obvious transcriptional defect, insertion or deletion present in the *flgK* operon in HB19Fla⁻.

The study of the transcriptional regulation in spirochaetes has just begun, and many questions need to be answered. For example, we do not understand the mechanism underlying the transcriptional cascade of flagellar synthesis of *B. burgdorferi*. Our results suggest that a σ^{70} -like rather than a σ^{28} -like factor is involved in flagellar gene transcription. The conclusion drawn from this and our previous analyses must be that the transcriptional pathway of the *B. burgdorferi* flagellar operons is distinct from those of other bacteria, including other spirochaete species.

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