Expression of the flagellin gene in *Borrelia* is controlled by an alternative σ factor

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The flagellin genes from six *Borrelia* species were cloned, sequenced and characterized at the molecular level. The flagellin genes of two relapsing fever *Borrelia* species, *B. hermsii* and *B. crocidurae*, three Lyme disease genomic species, *B. burgdorferi*, *B. afzelii* and *B. garinii*, and the avian borreliosis agent, *B. anserina*, were compared and showed an 85–93% sequence identity to each other. Comparison of the *fla* genes from the different Lyme borreliosis spirochaetes revealed that they were 94–99% identical. Nucleotide sequencing of the *fla* gene and primer extension on isolated mRNA from both *B. hermsii* (as transcribed in *Escherichia coli*) and *B. burgdorferi* (as transcribed in the natural host) identified the putative transcriptional start points, the ribosome-binding sites and the promoter regions of these genes. The deduced promoter of the *Borrelia* flagellin gene resembled neither the σ^{70} promoter of prokaryotes, as seen for the genes for the outer-surface proteins A and B in Lyme disease *Borrelia* and the genes for the variable major proteins 7 and 21 of *B. hermsii*, nor the σ^{28} consensus promoter region of motility genes from other bacteria. Instead, the promoter of the *fla* gene in *Borrelia* has most similarity to the bacteriophage SP01 $\sigma^{9p33-34}$ promoter sequence of *Bacillus subtilis*.

Keywords: *Borrelia*, spirochaetal flagella, flagellin

INTRODUCTION

Spirochaetes, distinct from other bacteria in their morphology and motility, constitute a separate phylum of bacteria that are only remotely related to Gram-negative and Gram-positive bacteria (Woese, 1987). The genus *Borrelia* contains several major pathogenic groups of organisms. Among these are the relapsing fever borreliae, Lyme borreliosis borreliae, and the aetiological agent of avian spirochaetosis, *B. anserina*. The Lyme disease agent *B. burgdorferi* has recently been divided into different genomic species, including *B. burgdorferi*, *B. garinii* and *B. afzelii* (Baranton *et al.*, 1992; Marconi & Garon, 1992a; Canica *et al.*, 1993).

The motility-promoting flagellar apparatus is a cell structure that spirochaetes have in common with other motile bacteria (Canale-Parola, 1978; Goldstein & Charon, 1988; Charon *et al.*, 1992). Unlike other bacterial

flagella, those of the spirochaetes are situated within the periplasm and are referred to as periplasmic flagella. Flagella are inserted at each end of the protoplasmic cylinder and extend along its axis, overlapping each other in the middle. The flagella of borreliae are different from those of other spirochaetes in that they are not surrounded by an outer sheath layer (Holt, 1978), and in *B. burgdorferi* the flagella are comprised of a single flagellin protein (Barbour *et al.*, 1986).

Immunochemical analysis of borreliae has revealed that the flagellin is one of the immunodominant antigens during infection in both humans and animals. Antibodies against the flagellin appear early in the sera of most patients and are prominent during all stages of the disease (Barbour, 1984b; Craft *et al.*, 1984, 1986). Therefore, the flagellins in *Borrelia* have been studied extensively with the purpose of developing diagnostic tools for Lyme borreliosis (Malloy *et al.*, 1990; Marconi & Garon, 1992b; Picken, 1992; Rosa & Schwan, 1989; Rosa *et al.*, 1991). Previously, it has been shown that an aflagellate mutant of *B. burgdorferi* had reduced ability to penetrate human endothelial cell layers *in vitro* (Sadziene *et al.*, 1991),

The EMBL accession numbers for the nucleotide sequences reported in this paper are X75200 for *B. burgdorferi* HB19, X75202 for *B. afzelii* ACAI, X75203 for *B. garinii* Ip90, X75204 for *B. crocidurae* and X75201 for *B. anserina*.

Table 1. Oligonucleotides used for PCR, screening of plasmid gene libraries, nucleotide sequencing and primer extension analysis

Oligonucleotide	Strain of origin	Position*	Sequence 5' to 3'
FB1	B31	-73-53	GTGCTATTCTTTTAAACAGGC
FB2	B31	1-25	ATGATTATCAATCATAATACATCAG
FB3	HS1	88-105	TCTATGCCCACTAGAAAG
FB4	B31	116-135	GCCAGCAGCATCATCAGAAG
FB5	B31	438-459	AACAGCTGAAGAGCTTGAATG
FB6	B31	572-593	AAAAGATTTGCAACATTAGCTG
FB7	B31	989-1013	TTATCTAAGCAATGACAAAACATAT
FB8†	B31	1157-1176	CCCTACTCAAAGCAAACCTCC

* All positions are derived from the *B. burgdorferi* B31 *fla* gene sequence in Fig. 2.

† Oligonucleotide FB8 is located downstream of the *fla* gene and therefore has no match in Fig. 2.

indicating that the flagella have a role in the invasion of human tissue.

The genes encoding the flagellin polypeptides, *flaA* and *flaB*, of several spirochaetes, including *Spirochaeta aurantia*, *Treponema pallidum* and *Serpulina hyodysenteriae*, have been cloned and sequenced (Brahamsha & Greenberg, 1989; Parales & Greenberg, 1993; Isaacs & Radolf, 1990; Isaacs *et al.*, 1989; Koopman *et al.*, 1992, 1993). The *flaA* gene products constitute the flagellar sheath layer and have the unique characteristic, unlike flagellins of other eubacteria, of an N-terminal signal peptide. These flagellins show no sequence homologies with those of eubacteria. However, the core polypeptides, encoded by the *flaB* genes lack a signal peptide and share homologies with the flagellins of *Salmonella typhimurium* and *Escherichia coli*. The expression of flagellin and chemotactic genes of many eubacteria is controlled by alternative σ -factors (Arnosti & Chamberlin, 1989; Helmann, 1991). In the enteric bacteria, the promoter sequences preceding the flagellin genes are very similar to the *Bacillus subtilis* σ^{28} recognition site (Helmann & Chamberlin, 1987; Helmann, 1991).

In this study, we have analysed and compared the flagellin genes and gene products from different representatives of six genomic *Borrelia* species. The transcriptional start points in two species, *B. burgdorferi* and *B. hermsii*, have been determined. We have also analysed the upstream control regions of the respective *fla* genes and compared them to those of other eubacterial flagellin genes.

METHODS

Bacterial strains and plasmids. The Lyme borreliosis strains investigated, B31, HB19, ACAI and Ip90 were chosen to represent a wide geographical distribution and to include one of each of the three newly defined genomic species, *B. burgdorferi*, *B. garinii* and *B. afzelii* (Marconi & Garon, 1992a; Baranton *et al.*, 1992; Canica *et al.*, 1993). *B. burgdorferi* is represented by the North American reference strain B31 (ATCC 35210) isolated from an *Ixodes scapularis* tick, and strain HB19, a blood isolate from a patient with Lyme disease in Connecticut, USA. *B. afzelii* strain ACAI, isolated from a patient with acrodermatitis chronica atrophicans, was obtained from Dr Eva Åsbrink

(Södersjukhuset, Stockholm, Sweden; Åsbrink *et al.*, 1984), and *B. garinii* strain Ip90, isolated from an *Ixodes persulcatus* tick, was kindly provided by Drs E. I. Korenberg and V. N. Kryuchevnikov of the Gamaleya Institute, Moscow, Russia. *B. hermsii* strain HS1, serotype C (ATCC 35209), *B. crociduræ* and *B. anserina* were obtained from the strain collection of Alan G. Barbour. Plasmids pBR322 and pUC18 were purchased from Bio Labs, Finnzymes.

Media and growth conditions. All *Borrelia* species were cultivated in BSKII medium at 34 °C (Barbour, 1984a). The cells were harvested at the mid-exponential phase and prepared for DNA or protein analysis as described previously (Barbour, 1988; Barbour & Garon, 1987; Howe *et al.*, 1985; Jonsson *et al.*, 1992). The *E. coli* strains DH5 and DH5 α (Life Technologies) were used for propagation of recombinant plasmids.

Cloning of the flagellin gene from four different *Borrelia* species. A flagellin-bearing clone originating from *B. burgdorferi* HB19 was constructed as follows. An oligonucleotide representing a conserved sequence (FB4 in Table 1), of the previously sequenced *fla* genes of *B. burgdorferi* GeHo and B31 (Gassmann *et al.*, 1989a, 1991; Wallich *et al.*, 1990), was used to identify *fla*-containing clones in a λ -fixII library of genomic DNA from *B. burgdorferi* HB19. A 5.0 kb *Bgl*II fragment containing the complete *fla* gene was subcloned into pBR322 to yield pASF01.

To clone the flagellin genes from *B. anserina* and *B. crociduræ*, plasmid gene libraries were constructed by partial *Hind*III digestion of total borrelial DNA (Jonsson *et al.*, 1992). The oligonucleotides FB2, FB5 and FB6 were designed and synthesized from the published *fla* sequences (Table 1). The oligonucleotides were end-labelled with [γ -³²P]dATP and used for screening libraries as described by Maniatis *et al.* (1982). The hybridization was performed at moderate stringency at 55 °C, as some differences in the nucleotide sequences of the different species were expected. Plasmids obtained were named pLNA01 (*B. anserina*), pLNC01 and pLNC02 (*B. crociduræ*). Oligonucleotide FB5 was used to detect pLNA01, which had a 5.8 kb insert containing the complete *fla* gene. Oligonucleotide FB2 was used to detect pLNC01, which covers the 5' part of the *fla* gene, while pLNC02 covers the middle part and was detected with FB5.

The last 3' part of the flagellin gene from *B. crociduræ* was constructed by cloning a PCR fragment, which was amplified using the FB5 and FB7 oligonucleotides as primers. Amplification of DNA was according to the manufacturer's instructions

Fig. 1. SDS-PAGE (12.5% acrylamide) and Western blots of whole-cell lysates of *Borrelia* spirochaetes. (a) Coomassie-brilliant-blue-stained protein gel of *Borrelia* species: lanes: 2, *B. burgdorferi* B31; 3, *B. afzelii*; 4, *B. garinii*; 5, *B. crocidurae*; 6, *B. hermsii*; 7, *B. anserina*. Molecular mass standards are in lane 1. (b) Western blot analysis of the same *Borrelia* species using anti-flagellin monoclonal antibody H9724. Lanes 2–7, as for (a). The flagellin proteins are arrowed.

(Perkin-Elmer Cetus). The first five cycles of denaturation were done at 94 °C for 2 min, annealing at 45 °C for 1 min, and elongation at 72 °C for 1 min. In the next 30 cycles, the denaturation time was shortened to 30 s and the annealing temperature was raised to 55 °C, otherwise the conditions were the same. Taking advantage of the 3' adenine overhang left by many *Taq* DNA polymerases, we were able to ligate fragments containing the last part of the *fla* gene into the pT7blue T-vector (Novagene), containing compatible single thymine overhangs. Three positive clones were isolated, each originating from different PCR reactions, and were designated pLNC03, pLNC04 and pLNC05. The same approach was used to clone the whole *fla* gene from *B. burgdorferi* strain Ip90. FB1 and FB8 oligonucleotides, located upstream and downstream, respectively, of the *fla* gene, resulted in plasmid constructs of pLNI01, pLNI02 and pLNI03. The flagellin gene of *B. hermsii* was also cloned by PCR. An oligonucleotide primer, located upstream of the gene, was designed: 5'-TTTGAATTCACAGCGACAAAACA-3' containing a mismatch creating an *EcoRI* site (underlined). The second primer, 5'-ATAAGGATCCATTAAGACCC-3', located downstream of the gene, contains a mismatch creating a *BamHI* site (underlined). This enabled us to digest the PCR product with the same enzymes and directly ligate it into the corresponding sites of pUC18. A positive clone was obtained and named pLNI01. The cloned gene was further characterized by restriction enzyme analysis and mini plasmid preparations (Saunders & Burke, 1990) followed by nucleotide sequencing (Sanger *et al.*, 1977).

Protein preparation, SDS-PAGE and Western blotting. Extraction of proteins from four different *Borrelia* species was performed as described before (Jonsson *et al.*, 1992). Protein

samples (10 µg per well) were analysed by SDS-PAGE (12.5% acrylamide) (Maniatis *et al.*, 1982). Molecular mass standards ranging in size from 14.4 to 94 kDa were obtained from Pharmacia. The gels were either fixed and stained by Coomassie brilliant blue (Sigma), or processed for immunoblotting. The proteins were electrophoretically transferred to a Fluorotrans transfer membrane (Pall Europe) for 45 min at 0.8 mA cm⁻². The blocking procedure, incubation and visualization of the bound antibodies were performed as described previously (Jonsson *et al.*, 1992). Monoclonal antibody H9724, which has a murine immunoglobulin G2a isotype and is specific for *Borrelia* flagellins (Barbour *et al.*, 1986), was used.

DNA techniques. Restriction enzymes, T4 DNA ligase (Bio Labs, Finzymes), reverse transcriptase (Pharmacia, and Life Science), Sequenase (USB), and the T7 sequencing kit (Pharmacia) were used as recommended by the manufacturers. *Borrelia* genomic DNA was isolated as described previously (Barbour, 1988; Barbour & Garon, 1987; Howe *et al.*, 1985).

Isolation of mRNA and primer extension. A plasmid, pLNI01, containing the *fla* gene from *B. hermsii* was introduced into *E. coli* DH5α cells, and total mRNA was prepared as described previously (von Gabain *et al.*, 1983). The isolated mRNAs were subjected to primer extension in an *in vitro* reaction with a specific ³²P-labelled oligonucleotide primer FB3 (Table 1). The oligonucleotide was end-labelled using T4 polynucleotide kinase (Pharmacia) and [^γ-³²P]ATP (> 5 kCi mmol⁻¹; 185 TBq mmol⁻¹, Amersham) as described by Maniatis *et al.* (1982). AMV reverse transcriptase (Pharmacia) was used in DNA synthesis as described by Göransson *et al.* (1989). Total RNA from *B. burgdorferi* HB19 was isolated (Burman *et al.*, 1990) and subjected to primer extension with the use of primer FB4 (Table 1) according to Hamlyn *et al.* (1981). In both cases, only full-length extension products were synthesized, and the sizes of the transcripts were compared with a DNA sequence ladder (Sanger *et al.*, 1977), directed by the same oligonucleotide primers, using plasmids pLNI01 and pASF1 as templates for *B. hermsii* HS1 and *B. burgdorferi* HB19, respectively.

Nucleotide sequence analysis. Single-stranded DNA from the flagellin genes of *B. hermsii* HS1, *B. burgdorferi* B31 and *B. afzelii* ACAI was obtained by asymmetric PCR (Gyllenstein & Erlich, 1988) using 10 ng of the double-stranded PCR products that were purified from agarose gels (Zhu *et al.*, 1985). The final concentration of the limiting primer was 0.015 mM, and the concentration of the primer in excess was 1.0 mM. The running conditions for the PCR were as described above. Single-stranded DNA from the asymmetric PCR reactions was precipitated and sequenced directly using the dideoxy chain-terminating method (Sanger *et al.*, 1977). The same method was used to sequence the double-stranded recombinant plasmids containing the complete or partial fragments of the *fla* genes (described in detail above) from *B. anserina*, *B. crocidurae*, *B. hermsii* (HS1), *B. burgdorferi* (HB19) and *B. garinii* (Ip90). The nucleotide sequences of both strands were determined partly with the primer oligonucleotides listed in Table 1 and partly with other oligonucleotides derived from the sequence data of Gassmann *et al.* (1989a). Sequences were assembled using GENEUS (Harr *et al.*, 1986) software for VAX computers (DEC). Further nucleotide sequence and protein sequence analyses were performed using the University of Wisconsin GCG sequence analysis software for VAX computers.

Phylogenetic analysis. DNA phylogeny predictions of the *fla* gene between the different species were performed by the parsimony method in the PAUP program for the Apple Macintosh computer (Swofford, 1990). Support for these groups was assessed using bootstrap analysis (1000 replicates), which is included in the PAUP package. The resulting

-35° -10° TT

B.b B31 -106 ATGCTATTGGCTTTCCTTTTATTTTGTCATTCCTTTTAACA GGC AAAAGGATTGCCAAGTCAG AAATTTAAAT TTTATC
B.b HB19 -106 C-----A-----A-----G-----AC--A--T-A-----
B.af ACAI -106 -----C-A-----A-T-----AAT-A--T-A-----C-----
B.g Ip90 -76 -----A-----A-----A-----A-GAC-A--T-----T-G---CCAC-T-A-----C-----
B.c -112 --GAA-- --TA--A-----C-TG-----A-C-----A-A-----T-----T-G---AA--TC--A--T--TAA-----
B.h HS1 -108 --AA-- --TA--A-----C-TG-----A-C-----A-A-----A--T-----T-T--A-ATT--A--T--TAA-----
B.a -108 -G-- A-TA--A-----C-TG-----A-C-----A-G--A--T-----T--AA--TCA--A--T--TAAC-----

RBS

B.b B31 -15 ATGCGG AATGATATAAGATTATCAATCATATACATCAGCTATTATGCTTCAAGAAATAATGCCATTAAAGCTGCTAATCTTAGTAAAACCTCAAGA
B.b HB19 -15 -----A-----C-----T-----C-----G-----
B.af ACAI -15 -----A-----T-----T-----C-----G-----
B.g Ip90 -15 -----A-----A-----G-----A-----CT--T-T-----C-----G-----
B.c -16 -C--A-----C-A-----G-----A-----A-----T-----T-A-----C-----
B.h HS1 -16 -C--A-----C-A-----G-----A-----A-----T-----T-A-----C-----
B.a -16 -C--A-----C-A-----G-----A-----T-----T-A-----C-----

B.b B31 85 AAGCTTTCTAGTGGGTACAGAAATTAATCGAGCTTCTGATGATGCTGCGCATGGGAGTTCTGCTGAAGATTAACTCTCAATTAAGAGGTOTTCACAA
B.b HB19 85 -----T-----T-----G-----C-----C-----A-----
B.af ACAI 85 -----T-----A-----T-----G-----G-----A-----
B.g Ip90 85 -----A-----C-T-----T-A-----T-----G-----G-A-----C-----T-----G-A-C-----
B.c 85 -----A-----C-T-----T-A-----T-----C-----G-A-A-----T-----G-----T-G-----
B.h HS1 85 -----A-----A-T-----T-A-----T-----T-----G-A-A-----T-----G-----A-A-T-----
B.a 85 -----A-----A-T-----T-A-----T-----T-----G-A-----T-----A-----A-T-----

B.b B31 185 CTCTAGAAATACTTCAAAGCTATTAAATTTATTCAGACAACAGAGGGAATTTAAATGAAGTAGAAAAAGTCTTAGTAAAGATGAAGGAATGGCAGT
B.b HB19 185 -----C-----A-----C-----A-----T-----A-----A-----
B.af ACAI 185 -----C-----A-----A-----G-----T-----A-----A-----
B.g Ip90 185 -----A-----G-A-----A-----A-----GG-----G-----G-G-G-----A-OC-T-T-----
B.c 185 -----A-----A-----A-----A-----A-----G-G-----A-C-----C-T-T-----
B.h HS1 185 -----A-----A-----C-----A-----A-----G-----G-----A-----A-----C-T-T-----
B.a 185 -----A-----A-----C-----A-----A-----G-----G-----A-----A-----C-T-T-----

B.b B31 285 TCAATCAGGTAAAGCACATATTCAGATGCAGACAGAGGTTCTATACAAATGAAATAGAGCAACTTACAGACGAAATTAAGAAATGCTGATCAAGCT
B.b HB19 285 -----A-G-----CT-----G-----G-----A-----
B.af ACAI 285 -----T-----C-----A-----G-----T-----G-----C-----A-----
B.g Ip90 285 -----T-----T-----C-----T-----G-----G-T-A-----T-----G-C-----A-----
B.c 285 -----T-----T-----T-----T-----G-----G-T-A-----T-----G-C-----A-----
B.h HS1 285 -----T-----T-----T-----T-----T-----T-----T-----C-C-----G-----
B.a 285 -----T-----T-----G-----T-----C-----T-----T-----T-----C-----

B.b B31 385 CAATATAACCAAAATGCACATGTTATCAAAACAAATCGCTTCTCAAAATGAAAGAACAGCTGAAGAGCTTGAATGCAGCTGCAAAAATTAACACACCAG
B.b HB19 385 -----G-----C-----A-----A-----A-----C-----
B.af ACAI 385 -----G-----C-----A-----A-----A-----C-----
B.g Ip90 385 -----T-----T-----T-G--AT--G--G-OC-----A-----T-----G-----T-----
B.c 385 -----C-----T-----G-C-----G-A--G-----A-----A-C-----
B.h HS1 385 -----T-----T-----G-T-----G-A--G-----A-----T-----
B.a 385 -----T-----T-----G-T-----G-A--G-----A-----T-----

B.b B31 485 CATCACTTTCAGGCTTCAAGGCTCTGGACTTTAAGAGTTCAATGTTGGAGCAACCAAGATGAAGCTATTGCTGTAATATTTATGACAGCTAATGTTGC
B.b HB19 485 -----A-----T-----G-----CC-----A-----
B.af ACAI 485 -----G-----A-----T-----C-----G-----T-----G-----A-----T-----
B.g Ip90 485 -----T-A--T-A-A-----T-A-----A-----A-----G-T-----T-----G-----A-----T-----T-----
B.c 485 -----AG-T-A-A-----T-A-----A--G-----A--G-C-----T-----G-----A-----T-----T-----
B.h HS1 485 -----A--T-A-A-----T-A-----A-----A-----G-T-----T-----G-----A-----T-----
B.a 485 -----A--T-A-A-----T-A-----A-----A-----G-T-----T-----G-----A-----T-----

B.b B31 585 AAATCTTTCTCTGGTGAGGGAGCTCAAACTGCTCAGGCTGCACCGGTTCAAGAGGGGTGTTCAACAGGAAGAGGCTCAA CAG CCAGCACCTGCTACA
B.b HB19 585 -----TG-----G-----A-----T-----C-----G-A-----G-----A-----A-----
B.af ACAI 585 -----A-----A-----A-----GG-----A-----T-----A-A-C-----A-----A-----T-----
B.g Ip90 585 -----T-A-----A-----T-----A-TA--T-A-C-----CA--G-A-----A-----G-----CT-----T-A--AT-----
B.c 585 -----TG-A-----A-----C-----T-A-G-----ATA-GA--G-A-G-A-G-----G-----CT-----T-A--AG-----
B.h HS1 585 -----TG-A-----A-----T-----T-A-C-----A-CA-----A-G-----TG-----G-A--G-----T-----A--TAG-T-----
B.a 585 -----TG-A-----A-----T-----T-A-C-----A-CA-----A-G-----TG-----G-A--G-----T-----A--TAG-T-----

B.b B31 682 GCACCTTCTCAAGGCGGAGTTAATTTCTCTGTTAATGTTTACAACTACAGTTGATGCTTAATACATCACTTGCTAAAATGAAAATGCTATTAGAATGATAA
B.b HB19 682 -----A-----T-----C-----A-----A-----
B.af ACAI 682 -----G-----G-----T-----C-----A-----G-----T-----A-----A-----A-----G-----
B.g Ip90 682 -----T--AG-----T-----C-----A-----G-----T-----TG-----A-A-----A-----G-----
B.c 682 -----T--AG-----T-----C-----A-----G-----T-----TG-----A-G-A-----GGG-----G-----G-----
B.h HS1 679 -----T--AG-----C-----T-----G-----AA-----T-----G-----T-----TG-----A-G-A-----G-----G-----
B.a 679 -----T--AG-----C-----T-----G-----AA-----T-----G-----T-----TG-----A-G-A-----G-----G-----

B.b B31 782 GTGATCAAAAGGCAAAATTTAGGTGCTTTTCAAAATAGACTTGAATCTATAAAGAAATAGTACTGAGTATGCAATGAAAATCTAAAAGCATCTTTATGCTCA
B.b HB19 782 -----AC-----G-----C-----T-----C-----
B.af ACAI 782 -----A-----A-----G-----G-----A-----T-----C-----G-----
B.g Ip90 782 -----A-----A-----G-----G-----A-----T-----C-----G-----
B.c 782 -----A-----C-T-----C-----G-----T-----AGC-----C-----A-----T-----T-----G-----
B.h HS1 779 -----A-----C-T-----C-----G-----T-----G-----A-----A-----T-----CT-G-----A-----
B.a 779 -----A-----CC-T-----C-----G-----T-----G-----C-----A-----A-----T-----CT-----

B.b B31 882 AATAAAGATGCTACAATGACAGATGAGGTTGTAGCAGCAACAACCTAATAGTATTTTAAACAACATCTGCAATGGCAATGATTOGCGAGGCTAATCAAGCA
B.b HB19 882 -----T-----T-----A-----C-----
B.af ACAI 882 -----T-----T-----A-----A-----
B.g Ip90 882 -----T-----A-----AA-----T-----A--C-----G-----G-----T-----A--A--A-----
B.c 882 -----T-----A-----A-----T-----C-----G-----T-----T-----A--A--A-----
B.h HS1 879 -----T-----A-----A-----T-----C-----G-----T-----T-----A--A--A-----
B.a 879 -----T-----A-----AA-----T-----T-----C-----C-----T-----A--A--A-----

B.b B31 982 TTCGCCAATATGTTTGTTCATGCTTAGATAA
B.b HB19 982 -----T-----
B.af ACAI 982 -----T-----
B.g Ip90 982 -----G-T-----A-----C-----
B.c 982 -----A-----T-----A-----A-----
B.h HS1 979 -----G-T-----A-----A-----
B.a 979 -----G-T-----A-----A-----

Fig. 2. For legend see facing page.

dendrogram had the same clustering order as that obtained from pairwise alignments performed by the program PILEUP in GCG.

RESULTS AND DISCUSSION

The *Borrelia* flagellin protein

Whole-cell protein extracts prepared from *B. burgdorferi* B31, *B. afzelii*, *B. garinii*, *B. crocidurae*, *B. hermsii* and *B. anserina* were separated by 12.5% SDS-PAGE (Fig. 1a). The separated proteins were subjected to Western blot analysis using the *Borrelia*-specific anti-flagellin monoclonal antibody H9724. For all strains, a protein of about 40 kDa bound the antibody (Fig. 1b). The small differences in sizes between the flagellin proteins from the different strains are in accordance with the respective *fla* gene products deduced from the nucleotide sequence (see below).

Nucleotide sequence analysis of flagellin genes

The *fla* genes of the two relapsing fever species, *B. hermsii* and *B. crocidurae*, of the Lyme borreliosis genomic species, *B. afzelii*, *B. burgdorferi* and *B. garinii*, and of the avian borreliosis isolate *B. anserina* were sequenced. The sequences and their alignments are shown in Fig. 2. An overall sequence identity of 85–86% was seen when comparing the *fla* gene of the Lyme borreliosis genomic species with the two relapsing fever species and *B. anserina*. The Lyme borreliosis *Borrelia* species showed 94–95% identity to each other. The *fla* sequences of *B. burgdorferi* strains HB19 and B31 were > 99% identical. The *fla* sequence of *B. anserina* was more similar to the sequences from the two different relapsing fever species than to the Lyme borreliosis genomic species: 91% to *B. crocidurae*, 93% to *B. hermsii*, while only 86–88% identical to the different Lyme borreliosis genomic species. Within the relapsing fever species, *B. hermsii* and *B. crocidurae*, the identity was 90.5%. The *fla* gene of *B. crocidurae* is the first sequenced gene of this species.

We also compared the flagellin gene sequences of *B. burgdorferi* B31 and *B. hermsii* HS1 to the previously published sequences of these strains (Gassmann *et al.*, 1989a; Wallich *et al.*, 1990; Picken, 1992). One base pair difference in the *B. burgdorferi* B31 *fla* gene was seen at position 539 (Fig. 2): Gassmann *et al.* (1989a) have reported a C in this position, where our sequence has an A. The *B. hermsii* *fla* sequence was identical to the previously published sequence.

Further analysis of the nucleotide sequences revealed that the upstream control regions were similar in all six genomic species investigated, which indicates that all six *Borrelia* species use similar regulatory signals. A putative

ribosome-binding site, 5'-GGAGG-3', was observed 13 bp upstream of the initiation codon, ATG, in all of the strains sequenced by us (Fig. 2).

From the sequence data obtained, we conclude that the *fla* genes from different *Borrelia* species show a high degree of similarity. However, some significant differences were seen, especially in positions 606–614 in *B. hermsii* and *B. anserina*, and positions 613–618 in *B. crocidurae*, where a duplication of 9 bp or a deletion of 6 bp, respectively, has occurred. These regions of sequence heterogeneity were particularly useful for species determination in *Borrelia* spirochaetes found in seabirds and *Ixodes uriae* ticks (Olsen *et al.*, 1993). They may also be of practical benefit for the construction of oligonucleotide primers for PCR diagnosis of Lyme, avian, and relapsing fever borreliosis (Picken, 1992).

Determination of the transcriptional start point of *fla*

To locate the *fla* promoter, the transcriptional start site of the *fla* gene of *B. hermsii* HS1 was determined from a *fla* gene expressed in *E. coli*. The transcriptional start point of the *fla* gene of *B. burgdorferi* HB19 was determined on mRNA prepared directly from whole cells of *Borrelia* bacteria. The transcriptional initiation sites of *B. hermsii* HS1 and *B. burgdorferi* HB19 *fla* genes were shown to be an A at base 60 and a C at base 57 upstream of the AUG, respectively (Fig. 2). This is consistent with the findings of Gassmann *et al.* (1991) using strain GeHo. Since we analysed the transcriptional start points from *Borrelia* as well as from *E. coli* mRNA, the results suggest that the *fla* gene expression uses the same promoter sequence regardless of whether the *fla* gene is expressed in *Borrelia* or in *E. coli*.

All *fla* genes that were sequenced had similar regulatory sequences upstream of the structural genes. Of particular interest was the apparent lack of the typical consensus σ^{70} promoter recognition site found in other sequenced *Borrelia* genes, i.e. the *ospAB* operons of *B. burgdorferi*, *B. afzelii* and *B. garinii*, or the *vmp* genes of *B. hermsii* (Bergström *et al.*, 1989; Jonsson *et al.*, 1992; Burman *et al.*, 1990). Conserved elements upstream of the *flaB* genes of *Treponema pallidum* and *T. phagedenis*, and of the flagellar and chemotaxis operons in *E. coli* and *Salmonella typhimurium*, have been found to be similar to the consensus sequences that determine the σ^{28} function in *Bacillus subtilis* (Pallesen & Hindersson, 1989; Champion *et al.*, 1990; Limberger *et al.*, 1992; Helmann, 1991). Earlier reports concerning the promoter sequences of the *fla* gene in *Borrelia* have suggested that a Pribnow box is located at positions -19 to -13 (Wallich *et al.*, 1990), while Gassmann *et al.* (1991) suggested that a presumptive

Fig. 2. Comparison of nucleotide sequences of the flagellin genes from *B. burgdorferi* (B. b) B31 and HB19, *B. afzelii* (B. af), *B. garinii* (B. g), *B. crocidurae* (B. c), *B. hermsii* (B. h) HS1 and *B. anserina* (B. a). The flagellin sequence of *B. burgdorferi* B31 is shown in the top line. Hyphens indicate homology between the *fla* genes of the different species and strains, capital letters indicate differences, and gaps indicate missing (or inserted) bases. The '-10' and '-35' regions of the promoter and the ribosome-binding sites (RBS-Shine-Dalgarno sequences) are overlined. The transcriptional startpoints, as determined by primer extension (data not shown), are indicated by arrowheads, while the ATG start codon is marked with an arrow.

Table 2. Comparison of the promoter region preceding the *fla* genes in *Borrelia*, *Leptospira* and *Treponema* with *Bacillus subtilis* $\sigma^{gp33-34}$ and σ^{28} recognition sequences

Organism	Gene	Promoter sequence	Reference/source
<i>Bacillus subtilis</i>, phage SP01*			
		CGTTAG GATATT	Johnson <i>et al.</i> (1983)
<i>B. burgdorferi</i> B31	<i>fla</i>	CGTTTC tttttttttaatttttgt GCTATT	Gassmann <i>et al.</i> (1989a)
<i>B. burgdorferi</i> HB19	<i>fla</i>	CGTTTC tttttttttaatttttgt GCTATT	This study
<i>B. afzelii</i> ACAI	<i>fla</i>	CGTTTC tttttttttaatttttgt GCTATT	This study
<i>B. garinii</i> Ip90	<i>fla</i>	TTC tttttttttaatttttgt GCTATT	This study
<i>B. crocidurae</i>	<i>fla</i>	ATTTAT tttt ttcttggtttttgt GATATC	This study
<i>B. hermsii</i> HS1	<i>fla</i>	ATTTAT tttt ttcttggtttttgt GATATC	This study, Picken (1992)
<i>B. anserina</i>	<i>fla</i>	ATTTAT tttt ttcttggtttttgt GATATC	This study
<i>L. borgpetersenii</i>	<i>flaB</i>	CGCCTG gtctgtcagggtgtctg AATATT	This study, Mitchison <i>et al.</i> (1991)
<i>S. hyodysenteriae</i>	<i>flaB2</i>	AGTTAG attgtcgaataacct TAAATT	This study, Koopman <i>et al.</i> (1993)
<i>Bacillus subtilis</i>†			
		CTAAA N₁₆ CCGATAT	Gilman <i>et al.</i> (1981)
<i>T. pallidum</i>	<i>flaB2</i>	CTCAA gtgcacccggcgatt CCGATAC	Pallesen & Hindersson (1989)
<i>T. pallidum</i>	<i>flaB1</i>	CTCAA gtcccttttcggtttt CCGATT	Champion <i>et al.</i> (1990)
<i>T. phagedenis</i>	<i>flaB2</i>	CTTAA gtgttttttcgccat CCGATAC	Limberger <i>et al.</i> (1992)

* $E\sigma^{gp33-34}$ promoter sequence of bacteriophage SP01 of *B. subtilis*.† *B. subtilis* $E\sigma^{28}$ consensus promoter sequence.

<i>B. burgdorferi</i> B31	1	MIINHNTSAINASRNNGINAANLSKTQELSSGYRINRASDDAAGMGVSGKINAQIRGLSQASRNTSKAINFIQTTEGNLNEVEKVLVRMKELAVQSGNG	51
<i>B. burgdorferi</i> HB19		-----A-----	
<i>B. afzelii</i> ACAI		-----A-----	
<i>B. garinii</i> Ip90		-----A-----	
<i>B. crocidurae</i>		-----A-V-----H-----A-----D-----	
<i>B. hermsii</i> HS1		-----S-T-----H-----A-----R-----	
<i>B. anserina</i>		-----T-----A-----	
<i>B. burgdorferi</i> B31	101	TYSDADRGSIQIEIQLTDEINRIADQAQYNQMHLNKSASQNVRTAEELGMQPAKINTPASLSGSQASWTLRVHVGANQDEATAVNIYAANVANLPSG	151
<i>B. burgdorferi</i> HB19		-----S-----K-----P-----	
<i>B. afzelii</i> ACAI		-----S-----K-----A-----	
<i>B. garinii</i> Ip90		-----S-----K-----A-----	
<i>B. crocidurae</i>		-----V-----H-----R-SAEH-----V-----A-----	
<i>B. hermsii</i> HS1		-----A-----K-----A-----S-----A-----	
<i>B. anserina</i>		-----A-----A-----	
<i>B. burgdorferi</i> B31	201	EGAQTAQAAPVQEGVQGEQAQQ PAPATAPSQGVNSPVNVTITVDANTSIAKIAENIRMSIDQRANLGAFOQRLESIKNSTEYAIENLKASYAIKDAT	251
<i>B. burgdorferi</i> HB19		-----A-----A-E-----T-----T-----P-----D-----	
<i>B. afzelii</i> ACAI		-----A-----A-E-----T-----T-----P-----D-----	
<i>B. garinii</i> Ip90		-----A-T-----A-----V-----D-----D-----	
<i>B. crocidurae</i>		-----V-A-----A-----S-A-----A-----M-T-----D-----T-----V-A-----	
<i>B. hermsii</i> HS1		-----IG-----EG-AA-----A-A-----I-----A-----M-----G-----V-----D-----	
<i>B. anserina</i>		-----A-A-----V-AT-----VA-AP-----I-I-A-----M-----D-----V-----D-----	
<i>B. burgdorferi</i> B31	300	MTDEVVAATTNSILTQSAMAMIAQANQVPQYVLSLLR	
<i>B. burgdorferi</i> HB19		-----L-----	
<i>B. afzelii</i> ACAI		-----L-----	
<i>B. garinii</i> Ip90		-----L-----	
<i>B. crocidurae</i>		-----I-S-----	
<i>B. hermsii</i> HS1		-----S-H-----	
<i>B. anserina</i>		-----I-S-----	

Fig. 3. Comparison of deduced amino acid sequences of the flagellin proteins from *B. burgdorferi*, *B. afzelii*, *B. garinii*, *B. crocidurae*, *B. hermsii* HS1 and *B. anserina*. All sequences are compared and aligned with the *B. burgdorferi* B31 sequence. Hyphens indicate homology between the strains, capital letters indicate differences, and gaps indicate missing amino acids.

control region that exhibited a closer homology to σ^{70} promoters of *E. coli* could be found. However, our sequence data for the *fla* gene promoter region did not show any sequence similarity in the -10, -35 or other regions upstream of the consensus sequences for σ^{70} , σ^{54} , σ^{32} or σ^{28} -like promoters. Instead, the putative promoters for the *fla* genes of *B. burgdorferi* and *B. hermsii* most closely resembled the '-10' and '-35' regions of the $\sigma^{gp33-34}$ promoter sequence of the late genes of

bacteriophage SP01 of *Bacillus subtilis* (Table 2) (Johnson *et al.*, 1983; Costanzo *et al.*, 1984). The matches with the respective putative promoters are indicated in Fig. 2 and summarized in Table 2. The '-10' element is almost identical (5 out of 6 nucleotides) to the consensus -10 region of the late SP01 genes, whereas the '-35' element has only 4 out of 6 nucleotides or 3 out of 6 nucleotides identical to the consensus sequence for *B. burgdorferi* and *B. hermsii*, respectively. Also, the putative promoter

regions of *B. afzelii*, *B. garinii*, *B. crocidurae* and *B. anserina* most closely resemble the SP01 promoter (Table 2).

In *Treponema* spp., the flagellum is composed of a core protein encoded by the *flaB* gene, surrounded by a sheath protein encoded by the *flaA* gene (Norris *et al.*, 1993). The promoter sequences of the *Treponema flaA* genes are similar to the consensus σ^{70} promoter, while the *flaB* genes appear to use a σ^{28} -like promoter as for other eubacterial motility genes (Table 2). Recently, the *flaB* gene of *Leptospira borgpetersenii* and the *flaB2* gene of *Serpulina* (*Treponema*) *hyodysenteriae* were sequenced and no promoter sequences were found (Mitchison *et al.*, 1991; Koopman *et al.*, 1993). No transcriptional start point has been determined for these genes, but when we examined the *flaB* 5'-noncoding region of *L. borgpetersenii* and *S. hyodysenteriae*, we discovered a putative promoter sequence, 82 and 65 bp, respectively, upstream of the ATG start codon. These suggested promoter sequences are similar to both the $\sigma^{gp33-34}$ promoter and the promoter sequences proposed here for the *Borrelia fla* genes (Table 2). This finding implies that the expression of the *fla* gene of *Borrelia* and the homologous *flaB* of *Leptospira* and *Serpulina* might be controlled by a σ factor similar to $\sigma^{gp33-34}$.

Sequence analysis of the deduced flagellar proteins

The deduced translational products of the *fla* gene from the different species were compared to the deduced translational product of *B. burgdorferi* B31. The comparison of the different flagellar proteins in an optimal alignment is shown in Fig. 3. The deduced flagellins of the Lyme disease genomic species, strains B31, HB19, ACAI and Ip90, were 336 amino acids long with a calculated molecular mass of 36960 Da, and the protein for *B. crocidurae* was 335 amino acids long with a molecular mass of 36850 Da. *B. hermsii* HS1 and *B. anserina* were 334 amino acids long with a molecular mass of 36740 Da. The Lyme borreliosis species were 94–95% identical to the relapsing fever species at the deduced amino acid level, compared to 85–86% at the DNA level. From the amino acid sequence comparison of the flagellar protein, it was evident that the N- and C-terminal parts were conserved between different species, while central parts of the protein showed more sequence variation. This feature of having conserved N- and C-terminal parts of the polypeptide chain and heterology in the central region has been observed in all sequenced flagellin proteins when comparing them either within the species or to related genera. Furthermore, N-terminal amino acid sequence comparisons of the *B. burgdorferi* flagellar protein revealed a remarkable similarity to the N-terminal regions of class B (core) flagellin polypeptides from other spirochaetes (Gassmann *et al.*, 1989b; Norris *et al.*, 1993).

Fedorov & Efimov (1990) proposed a model for the *E. coli* flagellin where the conserved regions, i.e. the terminal parts, are involved in flagellin export and flagellum assembly, whereas the variable middle region seems to be less important for the structure, and may therefore represent variable antigenic determinants. Results sup-

Fig. 4. Evolutionary relationship of the flagellar proteins between different relapsing fever borreliae, *B. hermsii* and *B. crocidurae*; Lyme borreliae, *B. burgdorferi*, *B. afzelii* and *B. garinii*; and the avian borreliosis agent *B. anserina*. The phylogenetic tree was derived from the parsimony analysis performed with the PAUP package. The numbers on each branch represent the unique characters (recognized by PAUP) that differ between the bars.

porting this model in *Borrelia* have been reported by different authors. Berland *et al.* (1991) reported that sera from 17 out of 18 patients with late-stage Lyme disease reacted with the variable middle part of the *fla* gene product from *B. burgdorferi* strain N40. Furthermore, antisera against native or denatured flagellar proteins derived from different animal species immunized with *B. burgdorferi* GeHo, reacted strongly with epitopes located in the variable domain of the flagellin, while cross-reactive antisera raised against *Bacillus subtilis* and *T. phagedenis* bound exclusively to the N- and C-terminal regions (Gassmann *et al.*, 1991; Rasiah *et al.*, 1992). A 14 kDa fragment covering part of the variable domain (amino acids 131–266) does not cross-react with sera from *Bacillus subtilis*, *E. coli*, *Proteus mirabilis*, *S. typhimurium* and *T. phagedenis* (Rasiah *et al.*, 1992).

Evolutionary relationships

The evolutionary relationships between the deduced flagellar proteins from the four *Borrelia* species were further investigated using the programs PAUP and PILEUP. These computer analyses revealed that the Lyme borreliosis genomic species were evolutionarily more closely related to each other than to the relapsing fever species or avian borreliosis species (Fig. 4). *B. hermsii* and *B. anserina* can be distinguished as a group distant from the Lyme disease species *B. burgdorferi*, *B. afzelii* and *B. garinii*. These results are in agreement with those obtained by Marconi & Garon (1992a), who investigated the re-

relationship between 16S rRNA sequences on the basis of signature or diagnostic nucleotide positions.

In the Lyme borreliæ branch of the tree, the species delineation into three different groups can be clearly seen, which is in agreement with earlier reports (Baranton *et al.*, 1992; Canica *et al.*, 1993). Interestingly, the North American relapsing fever species *B. hermsii* is more closely related to the avian borreliosis species *B. anserina* than to *B. crociduræ*, which is a relapsing fever agent from West Africa; a likely explanation is that our study used *B. hermsii* and *B. anserina* isolated from North America. Therefore, these two species probably share some properties. The partial *fla* gene sequences (Picken, 1992) from the North American relapsing fever species *B. turicatae* and *B. parkeri* were grouped into the same branch as *B. hermsii* and *B. anserina* (data not shown).

The fact that the *fla* gene is highly conserved among the different *Borrelia* species makes the molecular studies, particularly nucleotide sequence analysis, an important tool for studies of evolution of *Borrelia* and also for practical use in the diagnosis of borreliosis in mammals and birds.

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