The *Borrelia burgdorferi* Flagellum-Associated 41-Kilodalton Antigen (Flagellin): Molecular Cloning, Expression, and Amplification of the Gene

REINHARD WALLICH,1* SABINE E. MOTEK,2 MARKUS M. SIMON,3 KLAUS EBNET,3 ANTJE HEIBERGER,1 AND MICHAEL D. KRAMER2

*Department of Applied Immunology, Institute for Radiology and Pathophysiology, German Cancer Research Center, Im Neuenheimer Feld 280, D-6900 Heidelberg, Federal Republic of Germany; Dermatological Clinic, University of Heidelberg, D-6900 Heidelberg, Federal Republic of Germany; and Max-Planck-Institute for Immunobiology, D-7800 Freiburg, Federal Republic of Germany

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Monoclonal antibodies directed against the major *Borrelia burgdorferi* flagellar protein, the 41-kilodalton (kDa) protein flagellin, were used to monitor cloning and expression of the flagellin gene from a *Borrelia burgdorferi* genomic library. The structure of the gene was analyzed, and recombinant nonfusion flagellin was produced in *Escherichia coli*. A DNA sequence analysis of the 41-kDa flagellin gene revealed the presence of an open reading frame that encoded a protein having 336 amino acid residues and a calculated molecular mass of 35.8 kDa, indicating that there was posttranslational modification of the natural 41-kDa flagellin protein. Upstream from the AUG start codon sequence we identified motifs corresponding to consensus procaryotic promoter elements which could be utilized by the cloned flagellin gene when it was expressed in *E. coli* MC1061. The deduced flagellin protein sequence exhibited high levels of homology to sequences of flagellin proteins from *Bacillus subtilis* and *Salmonella typhimurium*. The levels of sequence similarity for the amino- and carboxy-terminal portions were about 65 and 56%, respectively. DNA sequence information on the flagellin gene was used to design oligonucleotides for gene amplification by the polymerase chain reaction method, and by using this method 0.01 pg of *Borrelia burgdorferi* DNA could be detected. Our results provide a basis for further biochemical analysis of the 41-kDa flagellin protein, investigation of the role of this protein in host-pathogen interactions, and development of a standardized reagent for diagnostic systems for *Borrelia burgdorferi* infections.

*Borrelia burgdorferi* is the etiological agent of Lyme disease, which is a common tick-borne infection in Europe and the United States. In humans Lyme disease appears as a chronic progressive disease that involves multiple organs, including the heart, the liver, the kidneys, the musculoskeletal system, the skin, and the central and peripheral nervous systems (5, 12, 35). Diagnosis of the disease is largely clinical, and since isolation and cultivation of *borreliae* from patients is not practical for routine purposes, positive diagnoses are supported mainly by detection of anti-*Borrelia burgdorferi* antibodies in patient sera (6–8, 13, 34).

Immunochromatographic analyses of *Borrelia burgdorferi* have revealed the presence of several immunodominant antigens that are recognized during infection in humans (13). In immunoblotting studies an early, consistent immune response against the genus-specific 41-kilodalton (kDa) flagellin antigen, the major constituent of the endoplasmic flagella, has been observed (1, 7). The flagella are contained within the outer envelope of the spirochete, and therefore flagellin is not readily exposed on the surface of the organism. However, since the first antibodies detected soon after the onset of Lyme disease are specific for the 41-kDa flagellin, spirochetes are probably processed via host defense mechanisms to expose flagellin, resulting in the development of specific antibodies (7, 8).

The 41-kDa flagellin of *Borrelia burgdorferi*, which is encoded by a single-copy gene of chromosomal origin, shows only marginal (if any) strain variation and therefore represents the prime candidate for routine serological diagnosis of Lyme disease. At present, whole cells and whole-spirochete sonic extracts are used as antigen sources in serological tests, such as the immunofluorescence assay and the ELISA, respectively (24). However, both of these test systems yield ambiguous results because of interference with cross-reacting antibodies and because of the fact that in general antibody responses against surface structures in patients appear only later during infections (6–8). In middle Europe the cross-reactive antibodies in patient sera are primarily those induced by the related pathogen *Treponema pallidum*, the etiological agent of syphilis (15).

Thus, to allow further analysis of the *Borrelia burgdorferi* flagellin, we performed molecular cloning and DNA sequence analysis of the 41-kDa flagellin gene of *Borrelia burgdorferi* B31 (= ATCC 35210) and determined expression of the recombinant gene in *Escherichia coli* MC1061. Furthermore, the polymerase chain reaction was used to establish a method for amplification of a precisely defined segment of the *Borrelia burgdorferi* flagellin gene.

MATERIALS AND METHODS

Abbreviations. ELISA, Enzyme-linked immunosorbent assay; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate. *Borrelia burgdorferi*. *Borrelia burgdorferi* B31 (= ATCC 35210) and ZS7 (isolated from a female tick in the Freiburg, Federal Republic of Germany, area) (33) were used in this study. Spirochetes were grown in modified Kelly medium containing CMRL-1066 (GIBCO), 3.5% bovine serum albu-
min (Sigma), and 6.4% heat-inactivated rabbit serum (5). After incubation in a humidified atmosphere containing 5% CO₂ in air at 33°C for 4 days, cultures were centrifuged at 10,000 x g (Sorvall) for 15 min. The cultures were usually grown to a density of 0.5 x 10⁸ to 1 x 10⁹ cells per ml. Sedimented bacteria were washed three times in PBS and were counted by using a dark-field microscope. To prepare soluble antigen, *Borrelia burgdorferi* cells were sonicated eight times (15 each) in an ice bath by using a cell disruptor set at level 4. After centrifugation at 10,000 x g for 15 min, the supernatant was filtered through a membrane filter, and the protein content was determined by using the BCA protein assay (Pierce).

**Monoclonal antibody production.** Immune spleen cells were obtained from BALB/c mice that were immunized with sonic extracts of *Borrelia burgdorferi* (100 μg in complete Freund adjuvant administered subcutaneously at time zero; 100 μg in PBS administered intraperitoneally on days 14, 28, and 42). Cell fusion on day 45 and selection and stabilization of hybridoma cell lines in culture were performed by using previously described methods (29; Kramer et al., unpublished data). Monoclonal antibodies that were reactive with *Borrelia burgdorferi* antigens were identified by using ELISA and sonic extracts of strain B31 and were further characterized by immunoblotting on whole *Borrelia burgdorferi* cells that were disintegrated by exposure to SDS sample buffer at 95°C and separated by SDS-PAGE. Several independent monoclonal antibodies (designated LA-1, LA-10, LA-21, LA-22, and LA-24) that reacted with the 41-kDa *Borrelia burgdorferi* flagellin were identified by Western blot (immunoblot) analysis. A mixture of these monoclonal antibodies was used for expression screening.

**DNA preparation and library construction.** A total of 10¹⁰ spirochete cells were washed in PBS and suspended in 10 ml of Tris-EDTA buffer. After treatment with lysozyme (5 mg/ml) for 15 min at 30°C, DNA was released by adding 1 ml of 20% SDS. After precipitation the pellet was dissolved in 0.5 ml of Tris-EDTA buffer, and the DNA was randomly sheared by sonication for 3 s. T4 DNA polymerase (30 min, 37°C) and Klenow enzyme (5 min, 37°C) were used to fill in the single-stranded regions of the DNA fragments generated. Blunt-ended DNA was ligated into the BamHI site of the bacterial expression vector pUEX1 by using an adaptor cloning strategy (4). After transformation of competent *E. coli* MC1061 host cells, the percentage of recombinant clones and the average size of the inserted *Borrelia burgdorferi* DNA were determined. More than 60% of the plasmids analyzed were found to contain insertions with an average size of ≥1.5 kilobases.

**Expression screening of Borrelia burgdorferi library.** Transformed cells were plated onto dishes (24 by 24 cm) containing Luria-Bertani agar supplemented with ampicillin at a density of approximately 7,000 colonies per plate and were incubated overnight at 30°C. Bacterial colonies were transferred to nitrocellulose filters, and expression of β-galactosidase fusion proteins was induced by temperature shifting to 42°C for 2 h. The filters were placed onto Whatman 3MM filter paper impregnated with 5% SDS and kept for 25 min in an oven at 95°C. After electroblotting with a Millipore-SDE transfer system (Millipore), the nitrocellulose filters were treated with DNase (10 mg/ml) and washed with PBS containing 0.1% Nonidet P-40, 0.5% gelatin, and 1 mM benzamidine. The nonspecific binding sites of the nitrocellulose filters were then blocked by incubating the filters in PBS containing 0.1% Tween 20, 0.2% gelatin, and 0.01% NaN₃ for 1 h at room temperature. The filters were incubated overnight in a monoclonal antibody mixture diluted 1:100 in PBS containing 0.1% Tween 20 and processed as described below for immunoblotting.

**DNA sequencing.** *Borrelia burgdorferi* DNA insertions were excised from pUEX1 plasmids by digestion with *Bam*HI and then subcloned into pGEM-3Z. To obtain unidirectional nested deletions in the DNA insertion, we used an ExoIII/mung bean deletion kit (Stratagene). After relegation of ExoIII/mung bean-treated plasmids, competent *E. coli* HB101 cells were transformed. Colonies from each of the transformation preparations were subjected to the alkaline lysis procedure and were analyzed on an 0.8% agarose gel to verify successful deletion (25). Plasmids containing progressive deletions (200-base-pair intervals) were sequenced by using the step-by-step protocol and a Sequenase kit (USB Corp.).

**Extraction of recombinant *E. coli* expressing the 41-kDa flagellin.** Bacterial clones that expressed flagellin were grown overnight at 30°C in 10 ml of Luria-Bertani broth supplemented with ampicillin. Samples (1 ml) of the overnight cultures were placed into 100-ml portions of medium and grown at 30°C with good aeration to a density of 8 x 10⁸ cells per ml (A₅₀₀nm 0.2). Expression of recombinant proteins was achieved by transferring the cells to 42°C for 2 h. After the cells were washed in STE buffer (10 mM Tris, 100 mM NaCl, 1 mM EDTA, pH 8.0), the pellet was suspended in 0.6 ml of lysis buffer (25% sucrose, 50 mM Tris, pH 8.0). A 150-μl portion of lysozyme (10 mg/ml) was added, and the mixture was incubated for 15 min on ice; this was followed by 15 min of incubation on ice with 18 μl of DNase I (10 mg/ml) and 5 μl of 1 M MgCl₂. Finally, 250 μl of a 4× detergent mixture (1% Triton X-100, 0.5% deoxycholate, 0.1 M NaCl, 10 mM Tris, pH 7.4) was added, and the preparation was incubated for 5 min on ice. After centrifugation, the resulting pellets were washed twice in buffer A (50 mM Tris, 50 mM NaCl, 1 mM EDTA, pH 8), suspended in 9 volumes of buffer A containing 8 M urea, and incubated for 1 h at room temperature. The samples were diluted with 9 parts of buffer B (50 mM KH₂PO₄-K₂HPO₄ [pH 10.7], 50 mM NaCl, 1 mM EDTA and stirred for 30 min at room temperature while the pH was kept at 10.7 by adding KOH. After the pH of the solution was adjusted to 7.0 by adding HCl, the samples were dialyzed overnight in the cold against buffer A. Solutions were centrifuged for 10 min at 10,000 x g and 4°C, and the resulting supernatants were stored at −20°C.

**SDS-PAGE and Western blotting.** SDS-PAGE was carried out as described by Laemmli (19) by using 12% acrylamide running gels and 4% acrylamide stacking gels. The gels were either fixed and stained with Coomassie blue or processed for immunoblotting. Natural or recombinant *Borrelia burgdorferi* proteins were transferred to nitrocellulose paper by electrophoresis at 400 mA and 140 V for 2 h. The nonspecific binding sites of the nitrocellulose sheets were then blocked with PBS-0.1% Tween 20 containing 0.2% gelatin. After this, the sheets were incubated overnight with monoclonal antibodies (1:200 dilution of the culture supernatant in PBS-0.1% Tween 20) or patient sera (1:400 dilution in PBS-0.1% Tween 20). After extensive washing, an appropriate peroxidase-labeled species-specific antibody (1:10,000 dilution in PBS-0.1% Tween 20) was added for 1 h with continuous shaking. Bound antibodies were then visualized by using ethylenediaminetetraacetic acid (pH 8.0) as the peroxidase substrate.

**Polymerase chain reaction.** DNA was extracted from *borreliae* as described above. Oligonucleotide primers for the polymerase chain reaction were chemically synthesized by using an Applied Biosystems (ABI) DNA synthesizer and
β-cyanoethyl chemistry (2, 26). The following sequences of oligonucleotides were used throughout the experiments: prB31/41-1, 5'-CATATTCAAGTCAGACAGAGG-3'; prB 31/41-2, 5'-GCTGCTAGCTACTGAACATGTA-3'; prB31/ 41-3, 5'-TCTGATAGTCTGCCTCTGACT-3'; prB31/41-4, 5'- CTGCTGCACTGGATTTCT-3'; and prB31/41-5, 5'- TCAATTGCATACTCAFTACT-3'. Each oligonucleotide was purified by using oligonucleotide purification cartidges (ABI) according to the instructions of the supplier. GeneAmp reagents (Perkin Elmer Cetus, Norwalk, Conn.) were used with an automated DNA thermal cycler (IHB). Each polymerase chain reaction mixture consisted of 800 μM deoxynucleoside triphosphate, 4.2 U of Taq DNA polymerase (Amersham), 1.5 mM MgCl₂, and 0.5 μM primer. Serial dilutions of DNA were made in Tris-EDTA buffer. For polymerase chain reaction assays in the presence of eucaryotic DNA, 100 ng of human genomic DNA was added to the samples. The reaction volume was 50 μl. Samples were denatured at 94°C for 1.2 min, annealed at 42°C for 2 min, and extended at 72°C for 2 min. The total number of cycles was 35. Portions (5 μl) of the reaction products were separated by electrophoresis on 1.5% agarose gels containing ethidium bromide (0.5 μg/ml).

Southern blots onto nitrocellulose filters were performed essentially as described previously (25). Prehybridization and hybridization buffer contained 5× SSPE (1× SSPE is 0.15 M NaCl, 10 mM sodium phosphate, and 1 mM EDTA, pH 7.4), 5× Denhardt solution, 0.1% SDS, 0.2 mg of denatured salmon sperm DNA per ml, and 50% formamide (25). By using BamHI digestion a 0.7-kilobase flagellin probe was derived from plasmid pB31/41-3, and this probe was labeled with [α-32P]dCTP (Amersham) by using a random primed DNA labeling kit (Boehringer GmbH, Mannheim, Federal Republic of Germany). Hybridization was done overnight at 42°C. Filters were washed for 20 min at 55°C in 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.1% SDS and then for 2 h at 65°C in 0.02× SSC-0.1% SDS. The autoradiogram was exposed for 30 min with a single intensifying screen.

RESULTS

Monoclonal antibodies that recognize Borrelia burgdorferi flagellin. Monoclonal antibodies specific for the 41-kDa antigen (flagellin) of Borrelia burgdorferi were generated by cell fusion and were identified by Western blot analysis on electrophoretically separated strain B31 antigens. As shown in Fig. 1, monoclonal antibodies LA-1, LA-10, LA-21, LA-22, and LA-24 recognized conserved antigenic epitopes expressed by Borrelia burgdorferi B31, as well as by a variety of European isolates (data not shown). For comparison, Fig. 1 also shows the results obtained with serum from a patient suffering from acrodermatitis chronica atrophicans, a manifestation of chronic Lyme disease that also expresses antibodies that react with the 41-kDa flagellin.

Recombinant plasmids carrying the 41-kDa flagellin gene. A total of 4×10⁴ independent clones of the expression library were plated and protein expression was analyzed by using a mixture containing monoclonal antibodies LA-1, LA-10, LA-21, LA-22, and LA-24. The physical maps of different plasmids are shown in Fig. 2. Plasmid pB31/41-1 comprised a Borrelia burgdorferi DNA insertion in which the flagellin gene was fused to the 3′ end of the lacZ (β-galactosidase) gene at a position corresponding to amino acid 38 of the 41-kDa flagellin, leading to expression of a β-galactosidase-flagellin fusion protein. Similarly, plasmids pB31/41-2 and pB31/41-3 were fused to the lacZ gene at positions corresponding to amino acids 70 and 83, respectively (Fig. 3, open arrowheads). In contrast, plasmids pB31/41-9 and pB31/ 41-11 expressed the following nonfusion proteins: 116-kDa β-galactosidase and the complete 41-kDa flagellin protein (as shown for recombinant protein rB31/41-9 in Fig. 3 [solid arrowheads]). In these constructions the lacZ gene was fused to a position in the 5′ region upstream from the initial ATG start codon of the 41-kDa flagellin gene.

DNA sequence analysis of the flagellin gene. An extended version of our preliminary sequence of the flagellin gene is shown in Fig. 4 (11). The previously published nucleotide sequences demonstrated the existence of two independently isolated 41-kDa flagellin genes derived from an American isolate (strain B31) and a European isolate (strain GeHO) of Borrelia burgdorferi that exhibited four nucleotide differences in the coding portion of the gene, leading to two amino acid differences (11). The mRNA starting point is preceded by a TTGCCA region (base pairs −44 to −39) that may function in the initial recognition by the RNA polymerase. An RNA polymerase start site TATCATG, also known as a Prbnow box, is found at positions −19 to −13. A ribosome- binding (Shine-Dalgarno) sequence, GAGGAAATGAT (base pairs −12 to −1), is located adjacent to the start codon, ATG (Fig. 4). The open reading frame begins at nucleotide +1 with a methionine start and ends after nucleotide 1008 with a TAA stop codon. At the 3′ end of the flagellin gene, a perfect inverted repeat sequence of 28 nucleotides (base pairs 1043 to 1070) occurs. The guanine-plus-cytosine content of the gene that encodes the 41-kDa flagellin is 36.9

FIG. 1. Immuno blot analysis in which whole Borrelia burgdorferi cells were used. Whole Borrelia burgdorferi cells (10μg per lane) were disintegrated by heating in SDS-PAGE sample buffer and were then separated by SDS-PAGE. Separated proteins were transferred to nitrocellulose filters and reacted either with a 1:400 dilution of ascites fluid obtained from a patient suffering from acrodermatitis chronica atrophicans (ACA) or with 1:500 dilutions of culture supernatants from monoclonal antibodies LA-1, LA-10, LA-21, LA-22, and LA-24. The reaction was visualized as described in the text.
Flagellin protein sequence. The amino-terminal end of the 41-kDa flagellin protein was independently verified by determining the amino acid sequence (10, 23). The hydrophobicity profile revealed that the flagellin protein was usually a hydrophilic protein, as calculated by the method of Kyte-Doolittle (Fig. 5B). Two larger hydrophobic domains were located at the carboxy-terminal portion (~30 amino acid residues) and at a region in the middle part of the protein around amino acid 190. On the basis of its amino acid composition, the flagellin protein seems to be a rather neutral protein with 8% acidic and 8% basic residues and an isoelectric point of 6.7; the observed isoelectric point was 6.8 (data not shown) (23). The calculated molecular mass (35.8 kDa) was somewhat lower than the molecular masses of immunoprecipitated 41-kDa flagellin proteins from *Borrelia burgdorferi*, indicating that the protein was posttranslationally modified. No cysteine residue and only one tryptophan residue were found in the deduced amino acid sequence. In a comparison with previously published flagellin protein sequences (22; NBFR protein database, release 23.0) for other bacteria, such as *Salmonella typhimurium* (18) and *Bacillus subtilis* (9), a high degree of homology (between 52 and 55%) was observed (Fig. 5A). The amino-terminal domain that encompassed amino acids 1 to 130 and the carboxy-terminal region (~85 amino acids) exhibited even higher levels of conservation (65 and 58%, respectively). In the middle part of the flagellin protein, a level of sequence homology of only 31 to 37% and a remarkable difference in size were observed. The length of this amino acid stretch in the three flagellum proteins compared in Fig. 5A varies between 90 and 275 residues.

Expression of recombinant flagellin proteins. As described above, the recombinant proteins were expressed in *E. coli* MC1061. The β-galactosidase fusion proteins reacted with monoclonal antibody LA-21 in immunoblotting experiments (Fig. 3, immunoblots rB31/41-1, rB31/41-2, and rB31/41-3, open arrowheads). Molecular constructions containing the *Borrelia burgdorferi* promoter elements expressed the unmodified β-galactosidase molecule and an additional protein having a molecular mass of about 41 kDa. As shown in Fig. 3 (rB31/41-9 protein stain), the urea extract derived from bacteria transformed with plasmid pB31/41-9 contained three major bands, a 116-kDa protein band representing β-galactosidase and two protein bands with molecular masses of approximately 41 and 35 kDa. As determined by immuno-

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**FIG. 2.** Plasmid map of *Borrelia burgdorferi* DNA segments cloned into the pUEX1 expression vector. Plasmid pUEX1 contains the gene for β-lactamase (which provides ampicillin drug resistance) pBR322 replication origin, c1857 (the mutant repressor gene of bacteriophage λ) and the lacZ gene (which is fused at its 5' end to the P_R promoter of bacteriophage λ together with the coding sequence for the first 9 amino acids of its natural gene, cro, thus ensuring a high level of expression). Into the BamHI cloning site at the 3' end of the lacZ gene, segments of the *Borrelia burgdorferi* 41-kDa flagellin gene were ligated using BamHI blunt adaptors. The lengths of the *Borrelia burgdorferi* DNA insertions varied between 0.7 and 3.5 kilobases (kb). Plasmids pB31/41-1, pB31/41-2, and pB31/41-3 overproduced β-galactosidase fusion proteins at 42°C; in contrast, plasmids pB31/41-9 and pB31/41-11, which contained the entire 41-kDa flagellin gene, including parts of the 5' promoter region, overproduced both β-galactosidase and 41-kDa flagellin at 42°C.
blotting, the latter two proteins were reactive with anti-flagellin monoclonal antibody LA-21 (Fig. 3, rB31/41-9 immunoblot). The upper immunoreactive compound comigrated with the immunoreactive native 41-kDa flagellin associated with *Borrelia burgdorferi* cells (Fig. 3, *Borrelia burgdorferi* immunoblot).

**Amplification of flagellin gene segments by the polymerase chain reaction.** As shown in Fig. 6, a 0.7-kilobase region of the *Borrelia burgdorferi* B31 flagellin gene with a low level of homology to the related bacterial flagellin genes (*Bacillus subtilis* and *S. typhimurium*) was used to design oligonucleotides for gene amplification by the polymerase chain reaction. The reaction was performed as described above. Several combinations of oligonucleotides were used throughout the experiments, from which one oligonucleotide pair (namely, prB31/41-4 and prB31/41-5) worked best in our hands. After 35 cycles of amplification the 0.7-kilobase flagellin gene segment encompassed by these oligonucleotide primers could be visualized by ethidium bromide staining in agarose gels (10% of each reaction mixture was analyzed). The detection limit of this method was about 0.2 pg of *Borrelia burgdorferi* ZS7 DNA per reaction mixture (Fig. 6A, lanes 6 through 14). Identical results were obtained in the presence of excess eucaryotic DNA (100 ng of human genomic DNA per reaction mixture); no staining was observed when human genomic DNA was omitted (data not shown). To verify the identity of the amplified products, the gel was blotted onto nitrocellulose filters and hybridized to a radiolabeled flagellin gene DNA probe. When this more sensitive method was used, a specific amplified product was detected with 0.01 pg of *Borrelia burgdorferi* DNA (Fig. 6B, lanes 2 through 14).

**DISCUSSION**

In this paper we describe the cloning and expression of *Borrelia burgdorferi* flagellin and its usefulness as a target for DNA amplification when the polymerase chain reaction is used.

We found that the nucleotide sequence contains all of the classical regulatory features of bacterial genes. A ribosome-binding (Shine-Dalgalorno) site is located immediately upstream from the initial coding nucleotide. Adjacent to this sequence we found an RNA polymerase start site (Pribnow box), which was preceded by a recognition region (30, 32, 36). This finding indicates that at least for the 41-kDa flagellin gene very similar regulatory elements are used in *Borrelia burgdorferi* and *E. coli*. This may explain the relatively high production of *Borrelia burgdorferi* flagellin in *E. coli* MC1061, although compared with the codon usage pattern of *E. coli* (Table 1), the 41-kDa flagellin gene of *Borrelia burgdorferi* is significantly different, with the exception of codons having a guanine-residue in the first position (14). At the 3' end of the gene a putative hairpin loop represents a potent secondary structure for termination of transcription (30, 32, 36).
When

\[
\begin{align*}
-183 & \text{ctgagagaat ctaagaaata cattaaaggg tgtattgtt aataaaagaaggtcagttg} \\
-133 & \text{tatgatttt gctgatttga tgggaaagttc gtttataataataaat} \\
- 83 & \text{tttattt attcgttcat tcattcaagattttggtcagttttaggt} \\
- 33 & \text{agaattaa atttiiacat gcppggata tattgtagt attacattact}
\end{align*}
\]

\( \text{bp} \)
\( \text{amino acid} \)

\(-183\) cat aca tca gca gct gct gct gta 
\(-133\) tca caa gaa gct gct gct gta 
\(- 83\) tca aac cat gca 
\(- 33\) aac caa gac 

\( S / D \) 
\( M \)

13 cat aca tca gca gct gct gct gta 
18 aag ctt tct ggt tgg ttc att aag aat 
21 gat gtt gct gct gct gaa gct gct gga 
28 gna gca gta gaa gct gct gct gtt gtt 
35 att ggt gca att att atc gca 
43 ttc tct tct ggt ggt ggt gaa gct gat gct 
50 lta tca 
57 gna gca ggt gca ggt ggt gga gca 
64 gca 
71 tta gca gaa gca 
78 aac caa gac 
85 aac caa gac 
92 gca aac 
99 aac caa gac 
102 gca aac 

DNA sequence information for the Borrelia burgdorferi B31 41-kDa flagellin gene was used to design oligonucleotide primers. When oligonucleotide primers prB31/41-4 and prB31/41-5 were used in combination with radioactive hybridization techniques, about 0.01 pg of Borrelia burgdorferi ZS7 DNA, equivalent to the DNA in about 2 cells, could be amplified (based on an estimated DNA content of 5 × 10^9 mg per Borrelia burgdorferi cell). Two groups of primer pairs. The usefulness of the polymerase chain reaction as a specific and sensitive method to detect Borrelia burgdorferi DNA by amplifying a DNA segment of unknown function (31). In this study we chose a single-copy gene of chromosomal origin (G. Gassmann, personal communication) that encodes a well-defined fundamental structural element, the major subunit of flagellum filaments. The flagellin-derived oligonucleotide primers are primary candidates to react with all Borrelia burgdorferi strains since the flagellin gene sequence is extremely conserved in this species (e.g., there are four nucleotide differences in the coding regions of an American isolate and a European isolate) (11). Although we have found that oligonucleotide primers deduced from the flagellin gene sequence of American Borrelia burgdorferi strain B31 can be used to amplify DNA derived from European Borrelia burgdorferi strain ZS7, the species specificity has not been established by using a test with flagellin oligonucleotide primers on a series of different Borrelia burgdorferi strains and other closely related members of the genus. In order to develop an accurate and sensitive assay for the detection of small numbers of Borrelia burgdorferi cells in tissue specimens, sensitivity of the polymerase chain reaction will have to be demonstrated by using crude DNA extracts derived from biopsies of infected tissues (20, 21). Gene amplification by the polymerase chain reaction should be compared with the results of other methods, such as immunohistology (28) or in situ hybridization, which can also be used for detection of Borrelia burgdorferi in tissue samples.

Analysis of the deduced amino acid sequence has revealed that the Borrelia burgdorferi 41-kDa flagellin is an overall hydrophilic protein. One hydrophobic domain that might account for interaction of the molecule with other flagellum structures is localized to the carboxy-terminal portion of the protein (Fig. 3B). An additional hydrophobic domain is present in the variable middle part of the molecule (around amino acid 190). The Borrelia burgdorferi flagellin contains no cysteine residue and only one tryptophan residue, which is reminiscent of previously described flagellin amino acid sequences (16, 18).

To date, recombinant intact flagellin has been produced in large quantities either as β-galactosidase fusion protein (rB31/41-1, 4B31/41-2, and rB31/41-3) or as a nonfusion protein (i.e., as a nonfusion protein, rB31/41-9). The rB31/41-9 antigen preparation consisted of two major proteins that reacted with anti-flagellin monoclonal antibody LA-21 (Fig. 3, immunoblot). One protein had an apparent molecular mass of 41 kDa and comigrated with natural flagellin recognized by monoclonal antibody LA-21 in a total sonic extract of Borrelia burgdorferi cells. The second protein had a molecular mass of approximately 35 kDa. Appearance of this second immunoreactive protein with a lower molecular mass could have been due to the following reasons: (i) the 41-kDa product was proteolytically modified; (ii) the 35-kDa protein was somehow posttranslationally processed to yield a 41-kDa product; or (iii) a different ATG site served as a start codon for detection of Borrelia burgdorferi in tissue samples.

Our amino acid sequence comparisons (Fig. 5A) revealed a high degree of homology with previously described flagellin sequences of Bacillus subtilis (9) and S. typhimurium (18), especially in the amino- and carboxy-terminal portions of the molecule. Since differences in size and higher variations in sequence were observed mainly in the middle part of the molecule, we suspect that species-specific antigenic epitopes should be expected more frequently in this part of the protein. Recently, Namba et al. (27) have described the X-ray fiber diffraction analysis of bacterial flagellum filaments. The predicted secondary structure of flagellin fila-

FIG. 4. Nucleotide sequence of the 41-kDa flagellin gene of Borrelia burgdorferi B31. The amino acid sequence of the flagellin protein is indicated along the nucleotide sequence. The proposed RNA polymerase binding site (Pribnow box) is underlined. The nucleotide sequence in italics represents a potential Shine-Dalgarno motif. Inverted repeats are indicated by arrows along the sequence. These sequence data have been submitted to the European Molecular Biology Laboratory-GenBank-DDBJ Data Libraries under the accession number X16833. bp, Base pair.
TABLE 1. Codon frequency observed in the *Borrelia burgdorferi* B31 41-kDa flagellin gene

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Codon</th>
<th>No.</th>
<th>Amino acid</th>
<th>Codon</th>
<th>No.</th>
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<td>Gly</td>
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<td>AGG</td>
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<td>Trp</td>
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<td>1</td>
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<tr>
<td>Gly</td>
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<td>6</td>
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* The numbers indicate the numbers of times that the codons are used in the 41-kDa flagellin gene. The underlined codons are the codons that are used frequently in *E. coli* (14).

ments revealed that the filament backbone is formed by the terminal regions of flagellin monomers. These domains are located toward the central hole of native filaments. On the other hand, the middle portions of flagellin seem to have no influence on the filament conformation but rather represent the domains of high variability localized on the outside of the filament structure. Presumably, the exposed middle domain may function in an infected host as an inducer of the immune response.
response. These data for *S. typhimurium* flagellin support our hypothesis that species-specific antigenic epitopes may be located preferentially in the middle part of *Borrelia burgdorferi* flagellin. Future attempts will be made to achieve expression and production of the middle part of flagellin (amino acids 130 to 250) in *E. coli*. This truncated *Borrelia burgdorferi* flagellin will be used to determine its usefulness as an antigen in an ELISA system.

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LITERATURE CITED