

Molecular Characterization of a 35-Kilodalton Protein of *Borrelia burgdorferi*, an Antigen of Diagnostic Importance in Early Lyme Disease

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Antibodies against a 35-kDa antigen of *Borrelia burgdorferi* are detectable in the serum of about half of patients with early Lyme disease. The gene encoding this antigen was isolated from a genomic library of *B. burgdorferi* B31 (low passage), and full-length expression of the recombinant gene product was achieved in *Escherichia coli*. Antiserum raised against the recombinant protein was reactive with a *B. burgdorferi* protein of the same molecular size as the diagnostic 35-kDa antigen cited in an earlier study of criteria for the serodiagnosis of early Lyme disease. Also, the recombinant protein was reactive with serum from patients with early Lyme disease who were seropositive for the 35-kDa antigen. DNA sequence analysis of the gene indicated an open reading frame of 909 bp encoding a protein with a calculated molecular mass of 34.3 kDa. This gene did not possess the usual initiation codon ATG but rather probably used a TTG codon. The deduced amino acid sequence of the N terminus exhibited a motif similar to that for signal peptides of lipoproteins. Southern blotting revealed a chromosomal location for this gene; and it was specific for *B. burgdorferi*, *B. afzelii*, and *B. garinii* but not for *B. hermsii*, *B. coriaceae*, or *B. turicatae*.

Lyme disease is the most prevalent vector-borne disease of humans in the United States and is transmitted by the bite of *Ixodes* ticks. Infection is caused by the bacterium *Borrelia burgdorferi*, resulting in an illness affecting various organ systems of the body. The clinical manifestations of Lyme disease are diverse and sometimes nonspecific (19). Serologic tests can therefore provide critical support to clinical diagnosis.

Current recommendations for the serodiagnosis of Lyme disease in the United States involve a two-tiered approach. A relatively inexpensive test, such as an enzyme-linked immunosorbent assay, is used first. Samples scored positive or equivocal in the first test are then evaluated by a more specific Western immunoblot (2). The work of Dressler et al. (5), confirmed by Kowal and Weinstein (11) and Johnson et al. (8), led to the interim adoption of criteria for the interpretation of immunoglobulin G (IgG) immunoblots by public health laboratories (2). Subsequent work has suggested that the criteria for IgG immunoblot interpretation may be simplified without sacrificing test sensitivity (6, 11). In one of the proposed simplifications, an antigen of 35 kDa is important in the scoring (6).

This report describes the isolation and characterization of the gene encoding a 35-kDa antigen (P35) of *B. burgdorferi*, which has been shown to be a significant marker of early Lyme disease. The P35 protein, together with other diagnostically important recombinant antigens, may be useful in efforts to eventually supplant immunoblotting with simpler tests of high specificity.

MATERIALS AND METHODS

Borrelia strains. *B. burgdorferi* sensu stricto strains B31 (passages <10 and >50) and cloned HB19 (passage 2) and *B. hermsii* HS1 serotype C were donated

by A. Barbour (University of Texas Health Sciences Center, San Antonio). *B. garinii* 20047 was the gift of J. Anderson (Connecticut Agricultural Experiment Station, New Haven). *B. afzelii* PGau was from B. Luft (State University of New York at Stony Brook, Stony Brook), and IP3 was from V. Kryuchevnikov (Gamaleya Institute, Moscow, Russia). *B. turicatae* was provided by T. Schwan (Rocky Mountain Laboratories, National Institutes of Health, Hamilton, Mont.), and *B. coriaceae* was donated by R. LeFebvre (University of California, Davis). Except where specified, strains were of unknown passage.

Serum samples. Lyme disease case serum samples were from six persons with erythema migrans who resided in New York, Massachusetts, or Wisconsin. *B. burgdorferi* was isolated from a skin biopsy sample from each of four of these patients; in two cases, culture was not attempted. Paired acute- and convalescent-phase serum samples were tested for five of the six patients. The acute-phase sample was obtained at the time of first visit to a physician's office, when erythema migrans was diagnosed. The convalescent-phase sample was obtained 2 to 4 weeks later. Control serum samples were from healthy blood donors residing in an area (Ohio) not endemic for this disease. These serum samples possessed various ranges of immunoreactivity against the 35-kDa antigen, as determined by Western blot.

Cloning of P35 antigen gene. Genomic DNA was purified from *B. burgdorferi* B31 low-passage strain cultured in vitro and used in the construction of a LambdaZap II (Stratagene, La Jolla, Calif.) genomic library as described previously (7). Lambda phage from the unamplified genomic library were plated according to manufacturer's directions. Following an incubation period, when the plaques were just visible, the plates were overlaid with nitrocellulose filters previously dampened with 1 mM isopropyl-1-thio- β -D-galactopyranoside (IPTG) and allowed to incubate at 37°C overnight. The next day, the filters were removed from the plates and probed with a mouse polyclonal antibody against P35. The generation of mouse polyclonal anti-P35 serum created by immunization with a preparative gel protein fraction of *B. burgdorferi* has been described (7). Immunoreactive plaques were detected either colorimetrically (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) or by chemiluminescence (ECL; Amersham, Arlington Heights, Ill.), and were plaque purified. Cloned inserts were subcloned from the LambdaZap II vector into the plasmid pBluescript (Stratagene) by the phagemid rescue method of the manufacturer into *Escherichia coli* XLI Blue MRF' or SURE (Stratagene).

Expression of recombinant P35 gene product and Western blotting. *E. coli* clones containing the P35 antigen gene were grown in Luria-Bertani broth culture to late log or stationary phase with or without the addition of 0.5 to 1 mM IPTG during mid-log phase. Cells were pelleted, and an aliquot was resuspended in 2 \times sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample loading buffer (containing 5% 2-mercaptoethanol and 4% SDS), boiled for 5 min, and loaded onto a 10% polyacrylamide gel for SDS-PAGE protein

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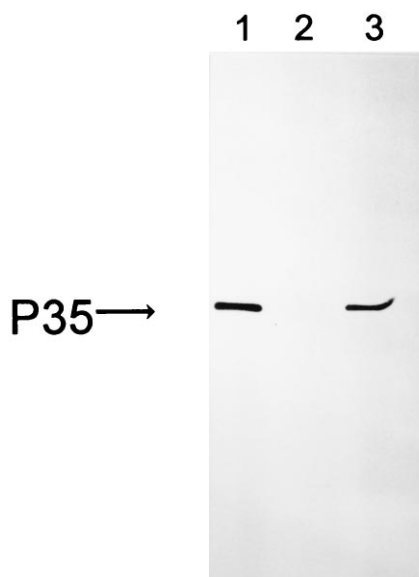


FIG. 1. Western blot showing expression of recombinant P35 in *E. coli*. Lanes: 1, *B. burgdorferi* B31 lysate; 2, *E. coli* containing pBluescript with no insert; 3, *E. coli* containing the P35 gene. The blot was reacted with P35 monoclonal antibody diluted 1:2,000.

analysis. Gels were blotted electrophoretically onto Immobilon (Millipore Corp., Bedford, Mass.) polyvinylidene difluoride membranes, and Western blot analyses were performed according to standard procedures (20). A monoclonal antibody against P35 provided by the laboratory of Mario Philipp, Tulane Regional Primate Research Center, Covington, La., was used to detect recombinant protein expression in the *E. coli* lysates.

DNA sequencing. Plasmid DNA containing the P35 gene insert to be sequenced was purified from *E. coli* cells by QIAprep-spin Plasmid Kit (Qiagen Inc., Chatsworth, Calif.). The 2-kDa insert DNA was sequenced by using a strategy of creating unidirectional deletion clones from the multiple cloning site of the vector using the Erase-a-Base kit (Promega, Madison, Wis.). Resultant DNA was sequenced by using M13/pUC universal primers and custom primers (made by Macromolecular Resources, Fort Collins, Colo.) utilizing the Taq DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems Inc., Foster City, Calif.). Sequencing reactions were run and analyzed by the automated sequencing apparatus Model 373A (Applied Biosystems).

Southern blotting. *Borrelia* genomic DNAs were electrophoresed in a 0.35% Tris-acetate buffered agarose gel overnight at 15 V constant voltage in a BioRad Mini-Sub gel apparatus (Hercules, Calif.). Following electrophoresis, the gel was photographed and subsequently treated in depurination, denaturation, and neutralization solutions, according to standard Southern blotting procedures. Following neutralization of the gel, the DNA was transferred to a Nytran membrane (Schleicher & Schuell, Inc., Keene, N.H.) by capillary blotting overnight. The DNA was fixed to the membrane by UV light in a UV Stratalinker 2400 (Stratagene, La Jolla, Calif.). Hybridization conditions, probe generation, and signal detection were done according to the manufacturer's directions using an ECL direct nucleic acid labelling and detection system (Amersham Life Science, Buckinghamshire, England), with 0.5 M NaCl in the hybridization buffer, and washed with a stringency of 0.5× sodium chloride-sodium citrate. The probe was generated by PCR amplification of the P35 gene from *B. burgdorferi* genomic DNA as described below.

PCR. PCR primers to amplify the P35 gene open reading frame were made by Macromolecular Resources, Fort Collins, Colo. The upstream primer was 5' TTGAAGGATAACATTTTG 3' (position 91 to 108). The downstream primer was 5' TCTCCCCCTTTTGAATG 3' (position 1043 to 1026). *Borrelia* genomic DNA was amplified under the following conditions: 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 1.5 mM MgCl₂; 0.001% gelatin; 200 μM each dATP, dCTP, dGTP, and dTTP; 1 μM each primer; and 2.5 U of *Taq* DNA polymerase (AmpliTaq, Perkin-Elmer Cetus, Norwalk, Conn.). Amplification was performed in a GeneAmp PCR System 9600 thermocycler (Perkin-Elmer Cetus) with the parameters of 94°C, 1 min; 37°C, 30 s; 72°C, 2 min for 35 cycles. The amplified P35 gene open reading frame PCR fragment was ligated into the expression vector, pSCREEN-1b(+) (Novagen, Madison, Wis.) according to manufacturer's directions. This construct produced a recombinant fusion protein when expressed in *E. coli* NovaBlue (DE3) (Novagen).

GenBank accession number. The DNA sequence was deposited in the GenBank database with the accession number U59487.

RESULTS

Cloning and expression of the P35 gene. Recombinant lambda plaques immunoreactive with antiserum against the 35-kDa antigen were identified, isolated, and plaque purified. The resultant *E. coli* clone was assayed by Western blotting for the production of the recombinant gene product. Figure 1 shows that the recombinant P35 was expressed as a full-length peptide. The gene was located internally on a 2-kb insert in the *E. coli* clone; therefore expression was probably driven by the borrelia promoter since the protein was not expressed as a β-galactosidase fusion protein from the pBluescript expression vector, nor was expression induced by IPTG (data not shown). The soluble fraction of the recombinant P35 *E. coli* sonicated lysate was used to immunize mice. After one booster injection, the mice seroconverted against a *B. burgdorferi* 35-kDa peptide as seen on Western blot (Fig. 2). This recognized antigen is one that comigrates on SDS-PAGE with the 35-kDa diagnostic antigen scored in a serological study of early Lyme disease patients by Engstrom et al. (6, 9).

DNA sequence of P35 gene. The DNA sequence of this gene, including upstream and downstream noncoding regions, is shown in Fig. 3. The open reading frame from the presumed start site consisted of 909 nucleotides encoding a protein with a calculated molecular mass of 34,347 Da. An unusual feature of this gene was that the translation initiation codon was not ATG. TTG has been described as an alternate initiation codon in other genes (13), and a TTG may act as the initiator here. Another *B. burgdorferi* gene, *bmpC*, has been described as having a TTG start codon (1). The upstream noncoding portion was a very rich A-T region, which is typical of prokaryotic promoters. There are several areas of consensus -10 and -35 sequences that may serve as the promoter for this gene, as well

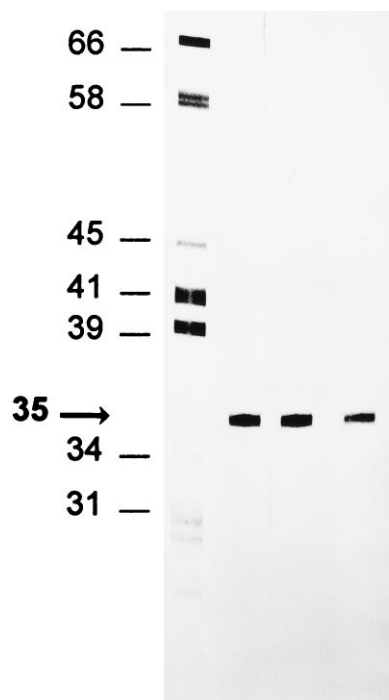


FIG. 2. Western blot showing reactivity of three mouse serum samples following immunization with recombinant P35 *E. coli* lysate. Serum samples were diluted 1:100 and blotted against *B. burgdorferi* B31 antigens. The left lane is a marker lane of human Lyme disease serum reactivity. Apparent molecular masses are indicated in kilodaltons.

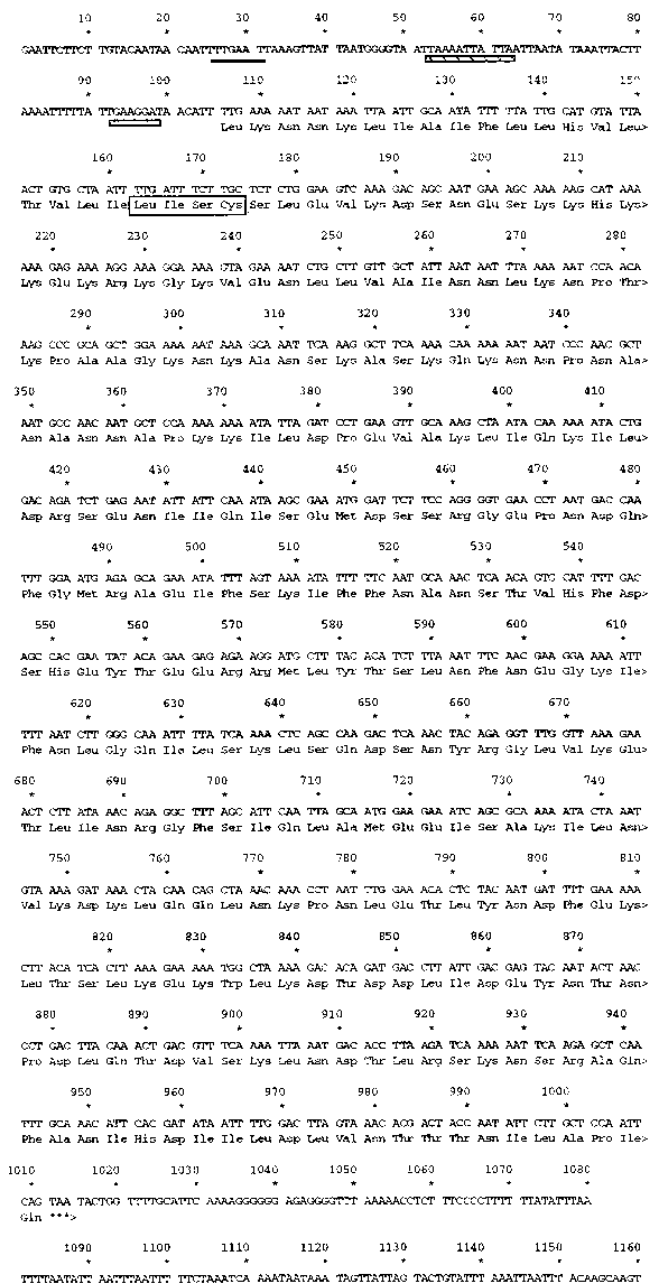


FIG. 3. The DNA sequence and deduced amino acid sequence of the P35 gene. The open reading frame is shown beginning with the putative initiation site of TTG at position 106. The proposed ribosome binding site is underlined with a stippled bar at position 92 to 99. Potential -10 and -35 promoter sites are denoted by the cross-hatched bar and solid line, respectively. The putative Lipoprotein signal peptidase site (LISC) is boxed at position 163 to 174.

as one A-G-rich region that is a putative ribosomal binding site. The putative promoter sites are marked in Fig. 3. A search of the GenBank database revealed significant homology (>75%) to upstream flanking DNA of the D6 protein gene of *B. garinii* (strain VS102; GenBank accession number U50840). Apparently, in the recording of the D6 gene, a truncated version of a P35 analog was deposited as well, in the form of upstream sequence. DNA sequence data downstream of our P35 gene revealed the presence of a D6 gene analog in *B. burgdorferi* (data not shown).

The amino terminus of the P35 protein contained sequences that are similar for signal peptides and contained a motif consistent with a signal peptidase site (LISC) commonly seen in other prokaryotic lipoproteins (Fig. 3). A number of lipoproteins have been described for *B. burgdorferi* (3, 4, 10, 12, 14, 17, 21, 23), and P35 may also share this characteristic. The hydrophilicity plot shows a hydrophobic domain at the amino end of the protein, representing the leader peptide, which is consistent with the membrane anchoring portion of lipoproteins, followed by the mostly hydrophilic and alpha-helical regions (Fig. 4). The native protein was also subjected to N-terminal amino acid sequencing but was blocked, probably because of lipidation.

Southern blotting. Genomic DNA preparations were probed with PCR-amplified P35 open reading frame DNA fragment as seen in Fig. 5. The gene hybridized to the chromosomal DNA of the *B. burgdorferi* sensu stricto strains B31 and HB19, as well as to the European species *B. garinii* and *B. afzelii*. There was no hybridization under moderate stringency washes to borrelia species *B. hermsii*, *B. coriaciae*, or *B. turicatae*, suggesting a specificity for the P35 gene to sensu lato strains.

Reactivity of P35 recombinant protein with human Lyme disease serum samples. Figure 6A shows serum samples from human Lyme disease patients that reacted with the *B. burgdorferi* 35-kDa antigen on immunoblot. Samples 1, 3, 5, 6, and 7 gave the strongest signals. Samples 2, 8, 11, 12, and 13 had weaker, but detectable, anti-35 signals. Samples 4, 9, and 10 were negative. These serum samples were immunoblotted against a crude *E. coli* lysate, which contained recombinant P35 (Fig. 6B). The human samples that were reactive with native 35-kDa antigen also reacted with the recombinant P35, while those samples that were negative in their immunoreactivity to the 35-kDa antigen failed to react with the recombinant. Samples that gave the weaker anti-P35 signals when blotted against native antigen (samples 2, 8, 11, 12, and 13; Fig. 6A) produced a detectable, distinct signal against the recombinant antigen (Fig. 6B). These results illustrate the potential that recombinant P35, once purified from host *E. coli* proteins, could serve in serodiagnostic assays for early Lyme disease.

DISCUSSION

A number of *B. burgdorferi* antigens that are useful in the serodiagnosis of Lyme disease have been cloned, sequenced, and expressed in *E. coli* (3, 15, 16, 18, 22). This characterization has been valuable in the process of generating monoclonal antibodies that identifies proteins of diagnostic importance and permits standardization of immunoblots from different clinical laboratories. The 35-kDa antigen has been shown to be a statistically significant marker in IgG immunoblots in a study of 55 patients with early Lyme disease who presented with erythema migrans (6). The study, by Engstrom et al., demonstrated that the most sensitive and specific criterion for interpretation of IgG immunoblots in the samples surveyed for early Lyme disease involves recognition of two of five antigens, one of which is P35. Preliminary studies with human Lyme patient serum samples, which recognize the 35-kDa antigen, demonstrated reactivity against the recombinant P35 from crude *E. coli* sonicates. Recombinant P35 protein may be useful as a diagnostic reagent, especially in combination with other antigens that have been deemed relevant in serodiagnosis of early Lyme disease.

We have determined the nucleotide sequence of the P35 gene, which revealed some interesting characteristics of the gene and protein. First, the initiation codon for this gene was not the standard ATG but probably a TTG. This presumption

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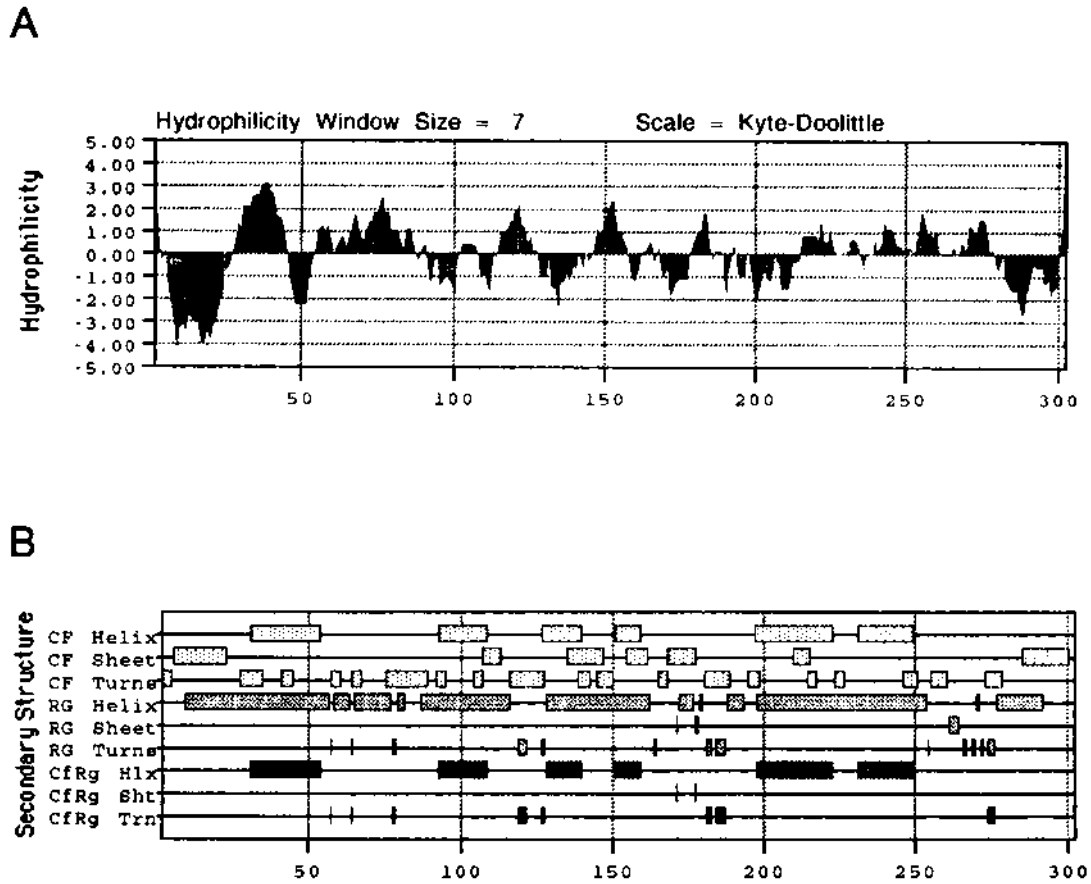


FIG. 4. (A) The hydrophilicity plot of Kyte-Doolittle for the P35 protein. Note the hydrophobic nature of the amino terminus, indicating the signal peptide and putative membrane anchoring domain (amino acids 1 to 25). (B) Secondary structure predictions using Chou-Fasman (CF) and Robson-Garnier (RG) algorithms, and a consensus of the two (CfRg) showing the alpha helix characteristics of this protein. The figures were generated by MacVector sequence analysis software (Eastman Kodak Company, Rochester, N.Y.).

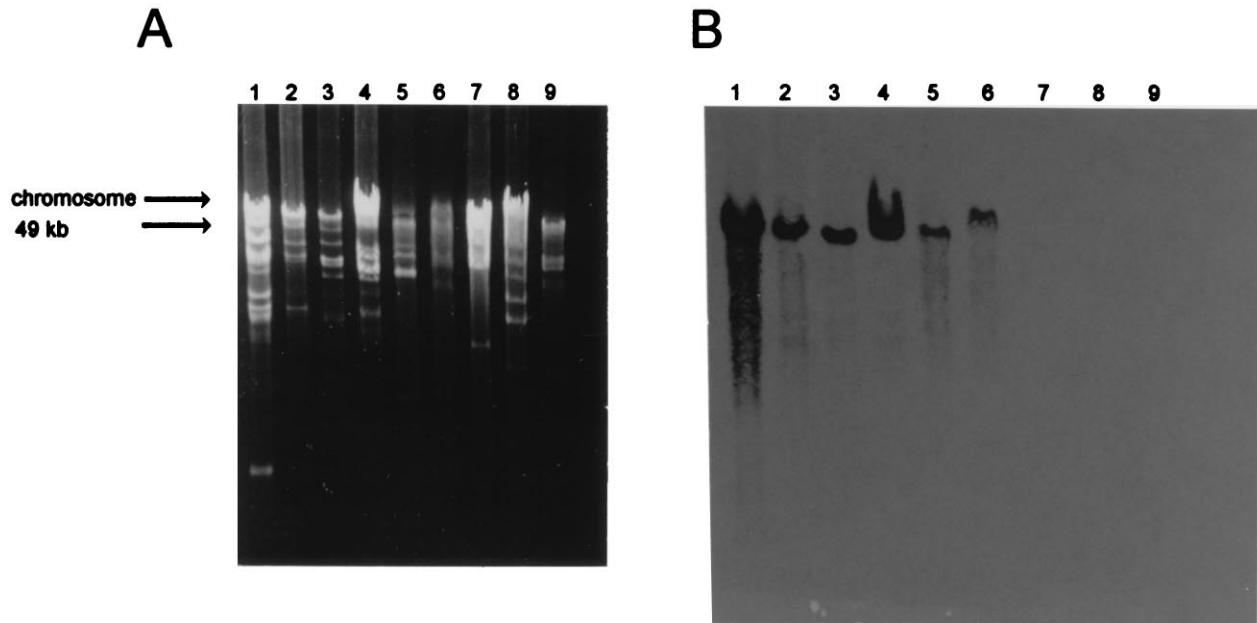


FIG. 5. Southern blot of borreliac genomic DNAs showing the chromosomal location of the P35 gene. (A) Ethidium bromide stained 0.35% agarose gel of the genomic DNA samples; (B) Southern blot of the samples in A with a P35 DNA probe. Lanes: 1, *B. burgdorferi* B31 low passage; 2, *B. burgdorferi* B31 high passage; 3, *B. burgdorferi* HB19; 4, *B. afzelii* IP3; 5, *B. afzelii* Pgau; 6, *B. garinii* FR20047; 7, *B. hermsii*; 8, *B. turicatae*; 9, *B. coriaciae*.

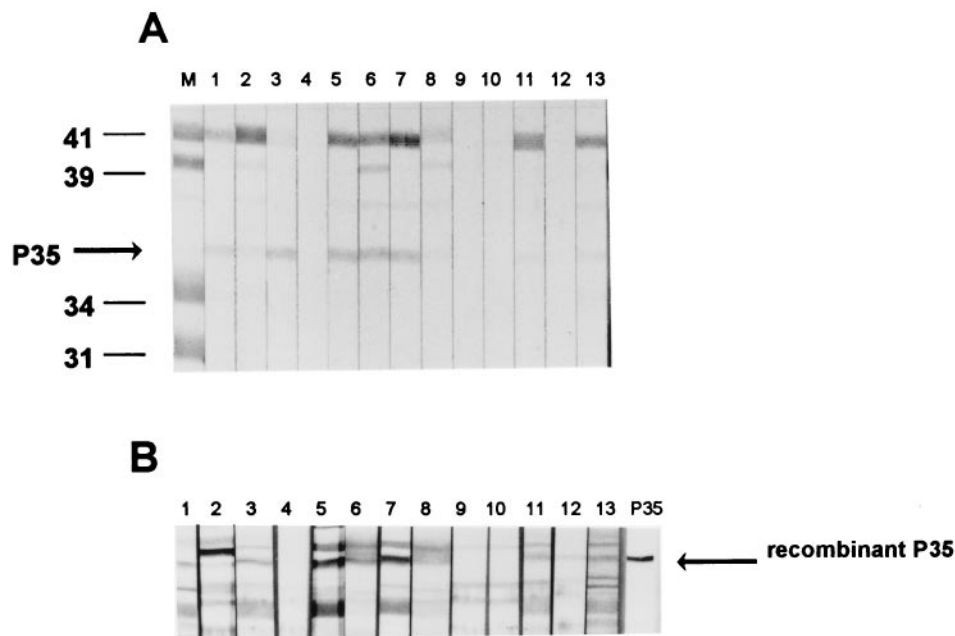


FIG. 6. Immunoblot of human Lyme patient sera and their reactivities against P35 in *B. burgdorferi* B31 low passage (panel A) and recombinant P35 *E. coli* lysate (panel B). The patient sera are numbered 1 to 13. Lanes 1 to 6, acute samples from patients with early Lyme disease; lanes 9 and 10, negative control samples from healthy people residing in an area in which Lyme disease is nonendemic; remaining lanes, convalescent samples. Paired acute- and convalescent-phase samples are lanes 1 and 13, 3 and 11, 4 and 12, 5 and 7, and 6 and 8. Lane M in panel A is a serum standard serving as a marker lane with the antigen molecular mass designations in kilodaltons denoted on the left. Lane P35 in panel B is an anti-P35 monoclonal antibody. The arrow in panel B denotes the immunoreactive recombinant P35 as calibrated by the anti-P35 marker.

is based partly upon the presence of the only A-G-rich region upstream of a potential start codon that could serve as a Shine-Dalgarno ribosome binding site. Also, within the amino-terminal region of the open reading frame were sequences consistent with those of leader peptides of lipoproteins, with a putative signal peptidase site at the LISC motif (24). A cleavage site such as this is generally around 20 amino acids from the initiation site, which is more evidence for the TTG start. Second, the deduced amino acid sequence of the N terminus shares features of other borrelia proteins that are lipoproteins.

Southern blot hybridization, using a probe specific to the entire reading frame of the P35 gene under moderate stringency washing conditions, indicated the presence of the P35 gene (or related sequence) in the representative *B. burgdorferi*, *B. garinii*, and *B. afzelii* strains analyzed. The lack of P35 gene sequence hybridization to *B. hermsii*, *B. coriaciae*, and *B. turicatae* would suggest a specificity of the P35 gene to *B. burgdorferi* sensu lato.

In conclusion, this report describes the molecular characterization of the P35 antigen of *B. burgdorferi* and proposes that it may be valuable in concert with other recombinant antigens in second-generation serodiagnostic assays for Lyme disease.

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