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Characterization of a Chromosomal Gene and the Antigen It Expresses from the Lyme Disease Agent, *Borrelia burgdorferi*

RANCE B. LEFEBVRE,[1]* WILLIAM S. PROBERT,[1] AND GUEY-CHUEN PERNG[2]

Department of Veterinary Microbiology and Immunology, School of Veterinary Medicine, University of California, Davis, Davis, California 95616,[1] and Ophthalmology Research Lab, Cedars Sinai Medical Center, Los Angeles, California 90048[2]

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The sequence and characterization of a chromosomal gene from the Lyme disease agent *Borrelia burgdorferi* and the antigen it encodes are described. The gene was cloned and expressed in transformed *Escherichia coli* cells. The gene is composed of 597 bases and expresses a predicted protein of 199 amino acids. Antibodies specific for the recombinant antigen reacted with a single *B. burgdorferi* protein with a molecular mass of approximately 22 kDa. The protein was not susceptible to proteinase digestion but was extracted by *n*-butanol phase partitioning, suggesting a periplasmic location of the antigen. Sera from humans and canines seropositive for *B. burgdorferi* reacted with the recombinant antigen. The antigen characterized in this report appears to be immunologically significant in naturally infected hosts.

Borrelia burgdorferi, the causative agent of Lyme borreliosis, inhabits a global niche, having been identified as a pathogenic agent in many parts of the world. The protean clinical manifestations of the disease and the similarities, in terms of symptoms, to a host of other diseases and maladies often make proper diagnosis difficult. *B. burgdorferi* isolates are routinely characterized by specific monoclonal antibodies (4, 6) or genetic analysis (2, 20, 21, 26, 31, 34). On the basis of several reports describing a number of isolates, it is now appreciated that a wide range of diversity exists among *B. burgdorferi* isolates at both the genetic (20, 21, 25, 26, 33) and antigenic (3, 4, 7, 9, 10, 19, 20, 37, 45) levels. Genetically, this diversity exists at both the chromosomal and plasmid levels (2, 20, 38). Multiple linear plasmids, unique to the *Borrelia* species, are known to express major surface antigens (5, 15, 16, 28). Linear plasmids in the borreliae that cause relapsing fever encode the genes responsible for antigenic variation (5, 30). In addressing Lyme borreliosis with a view toward reliable diagnostic, therapeutic, and prophylactic reagents, conserved genes expressing key proteins of *B. burgdorferi* were targeted. In this report we describe the cloning, characterization, and sequence of a *B. burgdorferi* chromosomal gene and the 22-kDa protein which it encodes.

MATERIALS AND METHODS

Bacterial strains. Reference strain *B. burgdorferi* B31 was used to isolate chromosomal DNA and was maintained as described previously (1).

Chromosomal DNA isolation and cloning. A chromosomal DNA-enriched preparation was made as described previously (22). Briefly, 20 ml of logarithmic-growth-phase cultures was washed three times in phosphate-buffered saline (PBS; pH 7.2) with 5 mM $MgCl_2$. The pellets were suspended in 100 μl of 15% sucrose in 50 mM Tris hydrochloride (pH 8.0)–50 mM EDTA, to which an equal volume of 5 M NaCl was added. To this solution was added 1/10 volume 1% sodium deoxycholate. The solution was placed on ice for 20 min; this was followed by centrifugation in a microcentrifuge for 5 min. The pellet containing the chromosomal DNA was suspended in 100 μl of 15% sucrose in 50 mM Tris hydrochloride (pH 8.0)–50 mM EDTA. To this solution was added 50 μl of 10% sodium dodecyl sulfate (SDS) and 25 μl of 20 mg of proteinase K per ml. The solution was incubated for 30 min at 37°C; this was followed by phenol extraction and ethanol precipitation. The chromosomal DNA was digested with *Cla*I and ligated into a *Cla*I-digested Pev-Vrf expression vector (11). *Escherichia coli* RRI was transformed with the recombinant plasmids. Expression of cloned genes into this vector was controlled as described previously (11) (heat shock 42°C for 2 h). Recombinant colonies were screened for expression of *B. burgdorferi* antigens by colony blot immunoassay by standard procedures (24).

DNA sequencing and analysis. The gene expressing the 22-kDa antigen was determined to be contained within a 1.65-kbp fragment of a *Cla*I insert. The recombinant plasmid expressing this antigen was designated pLP2. The gene was sequenced by standard procedures (36). The amino acid sequence of the antigen was derived from the Genetics Computer Group software (12).

SDS-PAGE and immunoblotting. SDS-polyacrylamide gel electrophoresis (PAGE) was performed as described previously (18, 43). Approximately 15 μg of a whole-cell lysate or a butanol extract (see below) was added to a 12% gel. The gel was run at 200 V of constant voltage for 45 min. Transfer of the electrophoresed proteins to nitrocellulose membranes was performed with a Pharmacia/LKB semidry transfer system according to the manufacturer's specifications (Pharmacia, Uppsala, Sweden). The membrane was blocked and probed by standard procedures (43). Rabbit antiserum raised against *B. burgdorferi* cells by intradermal injection (titer, 1:10,000) by enzyme-linked immunosorbent assay (ELISA) was used at a dilution of 1:1,000 as a positive control.

Antibody preparation specific for the 22-kDa antigen. Rabbit antiserum to whole-cell *B. burgdorferi* was prepared as described previously (22). Rabbit antibodies specific for the antigen were affinity purified. Briefly, 15 μg of a lysate of transformed *E. coli* cells containing the recombinant pLP2 plasmid and expressing the antigen was fractionated in a 12% acrylamide gel (18), and the proteins were transferred to nitrocellulose by standard semidry blot procedures (43). The

* Corresponding author.

membrane was blocked and probed with a 1:100 dilution of the polyclonal rabbit anti-*B. burgdorferi* serum by standard procedures. The antigen was identified and excised, and the bound antibodies were eluted by immersion in 100 mM glycine (pH 2.5) (27, 42). The eluent was then neutralized by the addition of 1.5 M Tris (pH 8.8) and was used as monospecific, polyclonal antibodies in subsequent experiments.

Canine serum assays. Canine sera submitted from veterinary clinics in Northern California for serodiagnosis of *B. burgdorferi* were used for the canine serum assay. Samples positive for *B. burgdorferi* by ELISA were used to screen immunoblots (see below). ELISAs were performed with supernatant fractions from sonicated B31 cells. Canine serum was diluted 1:200, and the optical density reading cutoff for a positive sample was >0.5. Positive sera were tested for their reactivities with the 22-kDa antigen expressed in the pLP2 plasmid in transformed *E. coli* cells. This was performed on immunoblots, and each serum sample was used at a dilution of 1:50 after preadsorption with a negative control *E. coli* lysate. Protein A conjugated to alkaline phosphatase (1:10,000; Sigma, St. Louis, Mo.) was used as the secondary reactant in these assays. The enzyme substrate for the alkaline phosphatase was 5-bromo-4-chloro-3-indolylphosphate.

Butanol extraction of *B. burgdorferi*. Enrichment for outer surface proteins from *B. burgdorferi* was performed as described previously (14). Briefly, 500 ml of a logarithmic-phase *B. burgdorferi* B31 culture was harvested, washed in PBS, and sonicated for a total of 5 min. The sonicate was centrifuged at 27,000 × *g* for 60 min at 4°C, and the pellet was resuspended in 2.5 ml of PBS. Five milliliters of *n*-butanol (Fisher Scientific, Pittsburgh, Pa.) was added, and the entire suspension was mixed for 1 h at 4°C. The solution was again centrifuged at 27,000 × *g* for 60 min at 4°C. The supernatant fraction was harvested and used in subsequent experiments as an outer surface protein-enriched fraction.

Nucleotide sequence accession number. The GenBank/EMBL accession number for the DNA sequence of the gene expressing the 22-kDa antigen is M90084.

RESULTS AND DISCUSSION

A gene from *B. burgdorferi* encoding an antigen of approximately 22 kDa was cloned and the sequence of the gene was determined. The amino acid sequence of the derived protein translation sequence was also determined. The gene was shown to be chromosomally encoded by hybridization studies with a specific probe and whole-cell *B. burgdorferi* DNA in pulsed-field gels (data not shown). The methods used to locate this gene on the chromosome were described previously in a report characterizing the gene expressing a 79.8-kDa antigen from *B. burgdorferi* (22, 29). Figure 1 lists the DNA sequence of the gene expressing the 22-kDa antigen and the putative amino acid sequence of the encoded protein. The ATG start codon is followed by 596 bases encoding a protein of 199 amino acids with a molecular size of approximately 26 kDa. A putative −35 sequence (ttgctct) (35) and a −10 sequence (tattat) (32) were identified. No consensus ribosome-binding site was identified. A putative signal sequence was identified in the protein (44). This consisted of a short, positively charged amino-terminus sequence (M-G-K) followed by a hydrophobic region (amino acids 4 to 16), a polar C terminus, a valine at −3 (amino acid 23), and a threonine at −1 (amino acid 25). The protein had a predicted isoelectric point of 8.37 on the basis of the derived amino acid sequence.

Figure 2A is a Coomassie-stained acrylamide gel, and Fig. 2B is an immunoblot of a gel similar to the one in Fig. 2A illustrating proteins representative of *B. burgdorferi* whole-cell and outer surface protein enrichment protocols (lanes 4 and 5, respectively). Also included in Fig. 2B are whole-cell lysates of *E. coli* JM83 and *E. coli* cells transformed by the pLP2 recombinant plasmid and expressing the 22-kDa antigen (lanes 2 and 3, respectively). (The 22-kDa antigen is not expressed in the *E. coli* cells to a concentration at which it is readily visible in stained gels.) The blot (Fig. 2B) was reacted with rabbit anti-*B. burgdorferi* B31 polyclonal antiserum. The recombinant antigen expressed in the transformed *E. coli* cells was identified at approximately 22 kDa. An antigen of this molecular size was also visible in the lane containing whole-cell *B. burgdorferi* and in the lane containing *n*-butanol-extracted materials, thus indicating that it may be situated on the outer surface or in the periplasmic space (14). Evidence for residence in the periplasmic space is that the 22-kDa antigen in *B. burgdorferi* was not degraded by proteinase K treatment of intact cells (data not shown), as were the outer surface proteins A and B (OspA and OspB) (6). Additionally, the 22-kDa protein expressed by the recombinant plasmid in *E. coli* cells was extracted by butanol and reacted with a monoclonal antibody raised against the 22-kDa periplasmic protein described by Simpson et al. (41) (data not shown). Thus, in light of the fact that the two antigens share an epitope that is reactive with a monoclonal-specific antibody, the evidence supporting the translocation of the two antigens to the periplasmic space suggests strongly that the 22-kDa antigen and the sequenced gene that expresses it, as described here, are the same as that as described by Simpson et al. (41). However, without sequence analysis of their gene or hybridization assays between the two genes, an unequivocal relationship between the two genes and their expressed proteins cannot be confirmed.

Figure 2C represents an immunoblot probed with affinity-purified, monospecific polyclonal antibodies directed against the recombinant antigen as expressed in transformed *E. coli* cells. Figure 2C demonstrates that the protein expressed by pLP2 in *E. coli* is also expressed by *B. burgdorferi* and is of the same molecular size in both organisms. The blot also demonstrates the specificity of the affinity-purified antibodies to the antigen and the fact that there is no cross-reactivity with other *B. burgdorferi* antigens.

Figure 3 shows immunoblot strips containing lysed *E. coli* cells expressing the recombinant *B. burgdorferi* antigen. The blot was probed for *B. burgdorferi* with ELISA-positive canine serum. The recombinant antigen reacted strongly with the eight canine serum specimens represented on the blot. To date, 9 of 10 canine serum specimens previously determined by ELISA to be seropositive for *B. burgdorferi* reacted strongly with the recombinant antigen. (The nonreactive sample elicited a very weakly positive sample to ELISA and immunoblot assays [data not shown].) Currently, a larger number of samples from a wider geographical distribution are being screened in order to determine the potential of this antigen as a diagnostic or prophylactic reagent. Notwithstanding, in the present preliminary study, it is apparent in cases of natural exposure that the *B. burgdorferi* 22-kDa antigen is expressed and elicits a definitive host immunoglobulin response in dogs. The antigen also reacted strongly with serum from a human patient serologically positive by ELISA for *B. burgdorferi* (data not shown).

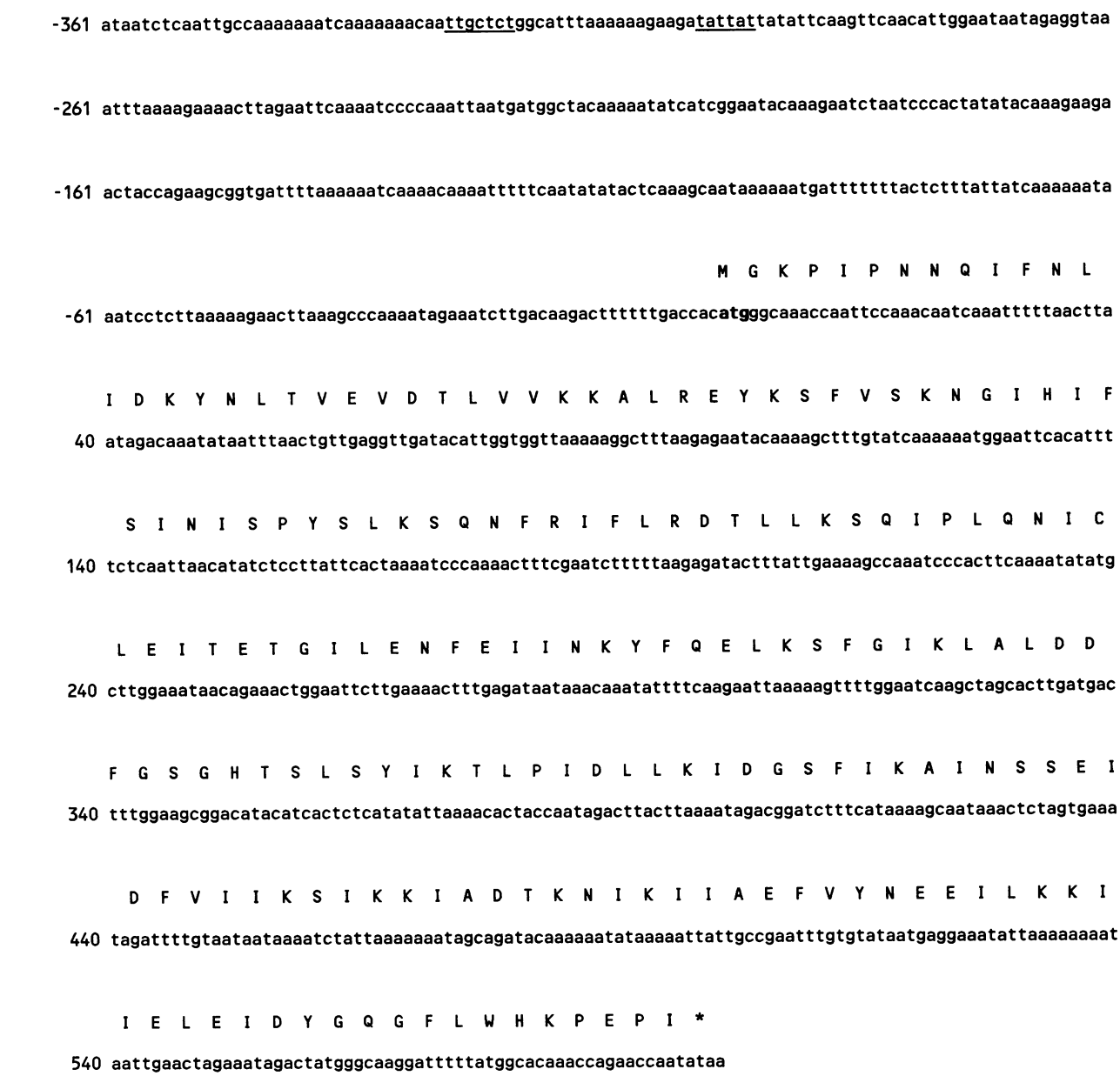

FIG. 1. DNA and predicted amino acid sequences of the gene encoding the 22-kDa antigen. The open reading frame consists of 597 bases encoding a deduced protein of 199 amino acids. Both −35 (ttgctct) and −10 (tattat) sequences are underlined. The ATG start codon is highlighted in bold. The asterisk denotes a stop codon at the end of the gene.

The protein, and the gene encoding it, described in this report represents a significant antigen expressed by *B. burgdorferi*. Fuchs et al. (13) recently analyzed the sequence of a gene expressing a 22-kDa antigen (pC) in *B. burgdorferi* (13). However, on the basis of comparison of the sequences of the genes and their respective proteins, the two are distinct and unrelated to each other. The protein described here is also different from the 22-kDa protein described by Luft et al. (23) on the basis of a comparison of our predicted N-terminal sequence with their sequenced protein. The protein which they described also shared cross-reactive epitopes with other surface proteins of *B. burgdorferi* (17). As demonstrated in Fig. 2C, the antigen did not share cross-reactive epitopes with other antigens.

A 22-kDa antigen was described by Coleman and Benach (8). That antigen demonstrated extensive cross-reactivity with other bacterial antigens. It is unlikely that the antigen described here is the same protein described in their report. No cross-reactivity with *E. coli* proteins was observed (Fig. 2A, B, and C). Additionally, their 22-kDa protein was acidic in overall amino acid composition, whereas the 22-kDa antigen reported here is basic, with a predicted isoelectric point of 8.37. It is possible that the antigen described herein is the same protein as that described by Simpson et al. (41). They reported a recombinant plasmid expressing a 22-kDa antigen. The chromosomal gene and the expressed antigen were reported to be conserved within the *B. burgdorferi* species. They also demonstrated that *B. burgdorferi* ex-

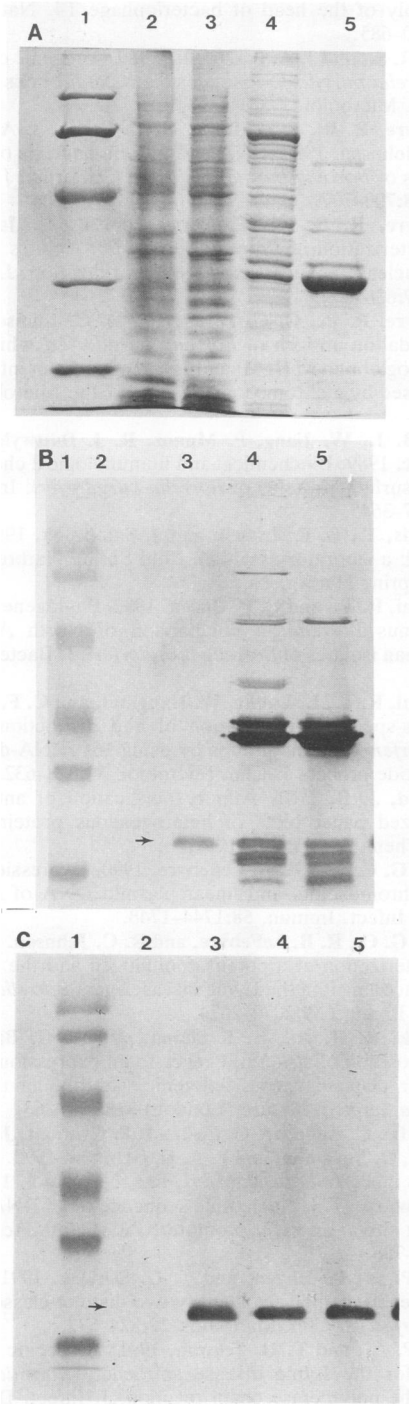

FIG. 2. (A) SDS–12% PAGE stained with Coomassie blue (see panel B description for lane designations). (B) Immunoblot of a gel similar to the one described in panel A. Approximately 15 μg of protein was added to each lane. Lane 1, prestained molecular size standards (sizes from top to bottom are approximately 106, 80, 50, 33, 28, and 19 kDa); lane 2, lysed whole-cell *E. coli*; lane 3, lysed *E. coli* containing the pLP2 recombinant plasmid; lane 4, lysed whole-cell *B. burgdorferi*; lane 5, butanol-extracted, outer membrane protein-enriched *B. burgdorferi*. The blot was reacted with polyclonal rabbit anti-whole-cell *B. burgdorferi* antiserum. The arrow indicates the 22-kDa antigen. (C) Immunoblot demonstrating the similar sizes of the recombinant expressed in *E. coli* and the antigen expressed in *B. burgdorferi* and the lack of epitope cross-reactivity of anti-22-kDa antigen antibodies with other proteins of *B. burgdorferi*. For lanes, see the legend for panel B.

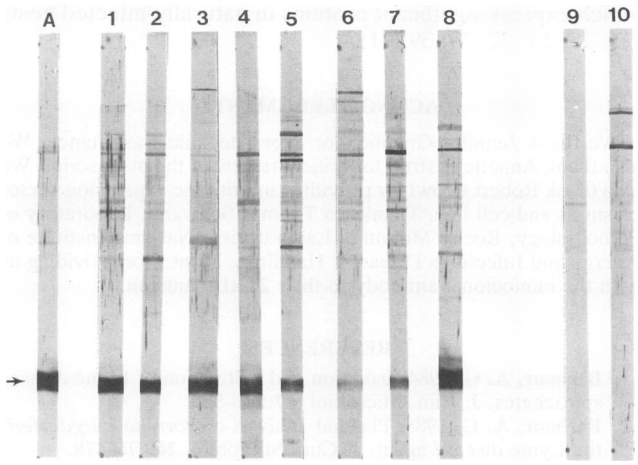

FIG. 3. Immunoblot of lysed *E. coli* cells containing the pLP2 recombinant plasmid reacted with rabbit serum raised against whole-cell *B. burgdorferi* (lane A) and eight canine serum samples previously determined to be ELISA positive for *B. burgdorferi* B31 (lanes 1 to 8). Lanes 9 and 10, canine sera that were ELISA negative for *B. burgdorferi*. The arrow indicates the 22-kDa antigen.

[p]ressed the antigen in infected mammalian hosts. The fact that a specific monoclonal antibody raised against their protein reacted with the protein expressed by our recombinant reinforces the suspicion that the two antigens are one and the same. However, without sequence analysis of the genes and their expressed proteins and because of the number of reactive antigens of *B. burgdorferi* that resolve within the molecular size range of 20 to 34 kDa, it is difficult to determine whether the same proteins are being described.

The molecular size of the protein expressed by the pLP2 recombinant was calculated to be 26 kDa on the basis of the predicted amino acid sequence. However, when SDS-PAGE is performed on *B. burgdorferi* proteins or on the expressed antigen in transformed *E. coli* cells, the molecular size approximation is 22 kDa. This discrepancy may be due to the lack of resolution in the electrophoretic system at this size range. Additionally, it is possible that the protein visualized by SDS-PAGE represents a processed version of the antigen because of the removal of the signal sequence (amino acids 1 to 26) which is cleaved off during processing. Removal of the signal sequence leaves a protein with a calculated molecular size of 23 kDa, which is more consistent with the resolution of the protein in SDS-polyacrylamide gels.

Medical approaches to spirochetal diseases share the difficulties of achieving reliable vaccine prophylaxis, accurate diagnosis, and successful therapy. A large component of these obstacles is the time required and the difficulty involved in culturing the pathogenic organisms. Furthermore, cultured spirochetes may lose or no longer express key virulence factors and immunogens in vitro (37, 38, 40). Also of concern is the wide range of genetic and antigenic diversity in *B. burgdorferi* isolates (4, 10, 19, 20, 21, 45). Thus, new and improved strategies are being developed for treating Lyme borreliosis. What may be the most promising of these approaches is the identification and cloning of genes, such as the one reported here, from *B. burgdorferi*

which express significant proteins in naturally infected hosts (13, 15, 16, 22, 29, 39, 41).

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