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A Chromosomal *Borrelia burgdorferi* Gene Encodes a 22-Kilodalton Lipoprotein, P22, That Is Serologically Recognized in Lyme Disease

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We describe the isolation of the gene encoding a 22-kDa antigen from *Borrelia burgdorferi*, the etiologic agent of Lyme disease. The *p22* gene is 582 nucleotides in length and encodes a protein of 194 amino acids with a predicted molecular mass of 21.8 kDa. The leader signal sequence of P22 consists of a positively charged short amino terminus, a central hydrophobic domain, and at the carboxyl terminus, a cleavage site that is presumably recognized and cleaved by a *B. burgdorferi* signal peptidase. P22 has 98.5% homology with the recently described *B. burgdorferi* protein IpLA7. P22 is processed as a lipoprotein, as demonstrated by [^{3}H]palmitate labeling. Pulsed-field gel electrophoresis showed that *p22*, like *LA7*, is localized to the linear chromosome of *B. burgdorferi*. Examination of sera from patients with Lyme disease revealed that antibodies to P22 are rarely detected in patients with early-stage disease characterized by erythema migrans (2 of 20), and 35% of the patients with late-stage disease characterized by arthritis (9 of 26) developed antibodies to P22. Sera from patients with syphilis did not react with P22. When patients with late-stage disease were tested for their antibody reactivities to four other outer surface proteins (OspA, OspB, OspE, and OspF), 75% of these patients responded to P22 or to one or more outer surface proteins.

Lyme disease is a multisystem infection caused by the tick-borne spirochete *Borrelia burgdorferi* (7), which is transmitted to humans by *Ixodes scapularis* ticks (30, 46). Following the bite of a spirochete-infected tick, *B. burgdorferi* can cause local and systemic manifestations (44). *B. burgdorferi* infection may begin with a skin rash called erythema migrans (9, 45). In the weeks to months that follow the initial infection, *B. burgdorferi* can disseminate to systemic organs and cause cardiac, neurologic, and rheumatologic abnormalities (44). Since its recognition in 1975, Lyme disease has been shown to be the most common tick-borne infectious disease in the United States (8). Identified in at least 46 states, the disease has also been described in most of Europe, Russia and the republics of the former Soviet Union, Australia, the People's Republic of China, and Japan (36, 41). Lyme disease represents a significant worldwide health issue, and strategies to curtail its spread are needed.

The genome of *B. burgdorferi* consists of a linear chromosome (11) and a series of linear and circular plasmids (20). The linear plasmids have hairpin telomeres (3, 19). In *B. burgdorferi*, the genes encoding for known surface-exposed lipoproteins have been localized to either linear or circular plasmids. The *ospA-ospB* operon, encoding two immunodominant outer surface proteins, OspA and OspB, is located on a 49-kb linear plasmid (4, 5). The gene for outer surface protein C, OspC, a 22-kDa protein previously identified as pC, was found on a 27-kb circular plasmid and was demonstrated to be actively transcribed in only certain *B. burgdorferi* isolates (16, 27, 33). The gene for another outer surface lipoprotein, OspD, is located on a 38-kb linear plasmid and is preferentially expressed by low-passage, virulent strains of *B. burgdorferi* B31 (29). The gene for a fifth lipoprotein, P27, has been localized to a 55-kb linear plasmid and has been shown to be expressed in the European strain, *B. burgdorferi* B29, but not in the U.S. strain, B31 (31). Moreover, the cloning of a sixth *B. burgdorferi* lipoprotein, IpLA7, was recently reported (48). IpLA7 from strain ZS7 and P22 from strain N40 share a 98.5% amino acid identity.

B. burgdorferi antigens may be useful as substrates in diagnostic assays or as vaccine candidates. However, because genetic (1, 26, 47) and antigenic (21, 26, 49) variability between different *B. burgdorferi* isolates exists, the diagnosis of Lyme disease and protective immunity offered by vaccination against all isolates may prove to be difficult. Because all known *B. burgdorferi* outer surface lipoproteins are encoded by genes located on plasmids, which may be lost during repeated passage, we were interested in identifying chromosomal gene products which may serve as reliable diagnostic markers and/or vaccine candidates. We characterized the gene encoding a newly discovered *B. burgdorferi* antigen, P22, unrelated to OspC, and defined the amino acid sequence of the protein. We present data showing that P22 is a lipoprotein and that it is encoded by a gene located on the linear chromosome of *B. burgdorferi*. We show that P22 is recognized by sera from some patients with Lyme disease.

MATERIALS AND METHODS

Construction of *B. burgdorferi* library. A bacteriophage lambda ZAP II custom genomic DNA expression library was constructed by Stratagene (La Jolla, Calif.). A low-passage (second-passage), infectious strain of *B. burgdorferi*, designated N40, was grown in modified Barbour-Stoener-Kelly medium at 32°C for 7 days, harvested by centrifugation at 5,000 × *g* for 30 min, and lysed with sodium dodecyl sulfate (SDS) (2). Genomic DNA was then isolated and purified by phenol-

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chloroform extraction. To construct the library, 200 μg of DNA was randomly sheared, the ends were blunted with S1 nuclease, and the *Eco*RI sites on the fragments were then methylated with *Eco*RI methylase. *Eco*RI linkers were ligated to the ends of the DNA, and the fragments were then digested with *Eco*RI. Following purification through a sucrose gradient, fragments varying from 1 to 9 kb were isolated and ligated to *Eco*RI-digested lambda ZAP II arms.

Production of rabbit anti-*B. burgdorferi* N40 serum. Treponeme-free, Venereal Disease Research Laboratory test-negative New Zealand rabbits (Millbrook, Amherst, Mass.) were used. Polyclonal rabbit anti-N40 serum was raised by intravenous injection of an inoculum of 10^8 live *B. burgdorferi* N40, a strain known to be pathogenic in C3H/HeJ mice (12), in phosphate-buffered saline (PBS) via the marginal ear vein, and the rabbits were given booster injections at 14, 21, and 49 days with another inoculum of 10^8 live *B. burgdorferi* N40 in PBS. Two weeks following the last booster injections, the rabbits were sacrificed and bled. The rabbit anti-N40 serum was separated by centrifugation of the blood at 1,000 × *g* for 15 min.

Absorption of rabbit anti-N40 serum with *Escherichia coli*-phage lysate. To remove from the rabbit antiserum antibodies that recognize *E. coli* and phage proteins, the antiserum was absorbed with an *E. coli*-phage lysate (Stratagene). The lysate was first diluted 1:10 in Tris-buffered saline (TBS) with 0.05% Tween 20. Nitrocellulose (NC) filters with a pore size of 0.45 μm (Millipore, Bedford, Mass.) were incubated in the lysate for 30 min at room temperature. The NC filters were then removed, air dried on Whatman filter paper (Whatman International Ltd., Maidstone, England), and washed three times with TBS for 5 min each time. The NC filters were then immersed in blocking solution (1% bovine serum albumin in TBS) for 1 h at room temperature and were then rinsed three times with TBS-Tween 20. The rabbit anti-*B. burgdorferi* N40 serum was diluted 1:5 in TBS-Tween 20, and the mixture was incubated with the NC filters with shaking for 10 min at 37°C. The NC filters were then removed and discarded.

Bacterial strains. The host cell used for screening the *B. burgdorferi* N40 expression library was *E. coli* SURE (Stratagene). A culture inoculated from a single colony of *E. coli* SURE picked from a Luria broth-tetracycline agar plate was grown overnight with vigorous shaking at 30°C in LB medium supplemented with 0.2% maltose and 10 mM $MgSO_4$. The bacterial cells were spun down at 1,000 × *g* for 10 min. The supernatant was carefully discarded, and the pellet was resuspended in 10 mM $MgSO_4$. Before use, the bacterial cells were diluted with 10 mM $MgSO_4$ to an optical density at 600 nm of 0.5.

Cloning procedure. The *B. burgdorferi* N40 expression library was screened with the polyclonal rabbit anti-N40 serum as described in the insert to the picoBlue immunoscreening kit (Stratagene). Briefly, lambda ZAP II phages were plated onto a lawn of *E. coli* SURE. Protein expression was induced with 10 mM isopropyl-1-thio-β-D-galactoside (IPTG). NC filters containing the expressed proteins were incubated with a 1:200 dilution of the absorbed rabbit anti-N40 serum. Following washing, the NC filters were incubated with a 1:5,000 dilution of alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G antibody (Organon Teknika Corp., West Chester, Pa.). Substrates used in color development were Nitro Blue Tetrazolium (NBT; Stratagene) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Stratagene). Any clone that reacted with the rabbit antiserum was screened with *ospA*, *ospB*, and *fla* DNA probes by Southern blotting to exclude these previously identified genes (43). The DNA probes were radioactively labeled by using the Prime-It random primer kit (Stratagene).

In vivo excision of the pBluescript plasmid from reactive clones was carried out by the simultaneous infection of XL1-Blue *E. coli* cells with the reactive clones and with R408 helper phage (Stratagene). Inside *E. coli*, the helper phage synthesizes proteins which recognize the initiation DNA sequence in the lambda phage genome, replicates all of the DNA downstream until the termination DNA sequence positioned 3′ of the initiator signal is encountered, and recircularizes the replicated single-stranded DNA molecule, including all of the sequences of the pBluescript plasmid and the cloned insert. Reinfection of fresh XL1-Blue *E. coli* cells produces a double-stranded plasmid.

DNA sequence analysis. To sequence *B. burgdorferi* genes encoding antigens that reacted with the rabbit antiserum, a nested set of deletions in the target DNA of the clone was generated with the Erase-A-Base system (Promega, Madison, Wis.). For the clone isolated and reported here (clone 10), the pBluescript plasmid was digested with *Xho*I, generating a 5′ blunt end (susceptible to *Exo*III digestion) proximal to the target DNA, and with *Kpn*I, creating a 3′ overhanging end (resistant to *Exo*III attack) next to the primer-binding site. The subclones generated by the nested deletions were sequenced by the dideoxynucleotide chain-termination method (34) by using the Sequenase kit (United States Biochemical Corp., Cleveland, Ohio). Analysis of DNA and protein sequences and homology searches of GenBank were performed by using the software program MacVector (International Biotechnology, Inc., New Haven, Conn.).

Pulsed-field gel electrophoresis. Pulsed-field gel electrophoresis was performed with total *B. burgdorferi* N40 DNA as described previously (11), with minor modifications. In brief, DNA plugs containing approximately 10^8 *B. burgdorferi* N40 were loaded onto an 0.8% agarose gel which was prepared and run in Tris-borate-EDTA buffer (0.025 M Tris, 0.5 mM EDTA, 0.025 M boric acid) by using the CHEF-DRII system (Bio-Rad Laboratories, Richmond, Calif.). The gel was run at 14°C for 18 h at 198 V, with ramped pulse times from 1 to 30 s. Southern blotting was carried out as described previously (43). The full-length *ospD* and *fla* genes were obtained from *B. burgdorferi* N40 by PCR by using oligonucleotide primers derived from the published DNA sequences of these genes (29). The *ospD* primers correspond to nucleotides 63 to 83 and 751 to 771, and the *fla* primers correspond to nucleotides 1 to 20 and 988 to 1008. The *p22* primers are as described below. The *ospD*, *fla*, and *p22* DNA probes used in Southern blots were labeled with [α-^{32}P]dCTP by using the Prime-It random primer kit according to manufacturer's protocol (Stratagene).

Amplification and cloning of the *p22* gene. The *p22* gene lacking the sequences encoding for the hydrophobic leader peptide was amplified by PCR by using oligonucleotide primers based on the *p22* DNA sequence, such that P22 is soluble when expressed, as described previously for the purification of OspA (10). The 5′-end and 3′-end primers correspond to nucleotides 67 to 87 and 562 to 582 of the *p22* gene, respectively. In brief, the template was denatured at 94°C for 1 min, annealed at 40°C for 2 min, and extended at 72°C for 3 min, and the process was repeated for 30 cycles. The amplified *p22* gene was cloned in frame with the glutathione *S*-transferase (GT) gene into the PMX vector, a pGEX-2T vector (Pharmacia, Pistacaway, N.J.) with a modified polylinker (40). The DNA sequence of the amplified *p22* gene was confirmed.

Expression and purification of recombinant P22. The GT-P22 fusion protein was expressed in *E. coli* DH5α under IPTG induction and was purified by using a glutathione-Sepharose

4B column (Pharmacia), with minor modifications from the published method (12). In brief, P22 was cleaved from the fusion protein by using thrombin, according to the manufacturer's protocol (Pharmacia). Thrombin (25 U) was added to the glutathione column containing the fusion proteins and was allowed to incubate overnight at room temperature. The cleaved P22 was eluted with 50 mM Tris-2.5 mM $CaCl_2$-150 mM NaCl and was treated with antithrombin beads.

Production of anti-P22 serum. C3H/HeJ mice (Jackson Laboratory, Bar Harbor, Maine) were immunized subcutaneously with 10 μg of purified, cleaved P22 in complete Freund's adjuvant and were boosted at 14 and 28 days with the same amount in incomplete Freund's adjuvant. Control mice were similarly immunized with bovine serum albumin (BSA).

SDS-PAGE. One dimensional SDS-polyacrylamide gel electrophoresis (PAGE) was performed as described previously (22). Approximately 1 μg of the cleaved P22 protein was electrophoresed through a 12% gel and, after separation, was examined by staining with Coomassie brilliant blue.

[^{3}H]palmitate labeling and immunoprecipitation. *B. burgdorferi* N40 was grown in the presence of [9,10(*n*)-^{3}H]palmitic acid (specific activity, 54 Ci/mmol; Amersham, Arlington Heights, Ill.), and radiolabeled lipoproteins were extracted by Triton X-114 phase partitioning as described previously (6). For immunoprecipitation of P22, an aliquot of the detergent phase was diluted 1:5 and was then precleared by incubation with normal rabbit serum (GIBCO, Grand Island, N.Y.) and protein G-Sapphires (Pharmacia). [^{3}H]palmitate-labeled P22 was immunoprecipitated from the precleared detergent phase by the sequential addition of murine anti-P22 serum and protein G-Sapphires. These samples were incubated by rotating them end over end for 1 h at 4°C. Following incubation, the antigen-antibody-protein G-Sapphires complexes were harvested by centrifugation at 1,500 × *g* for 15 s, washed three times with ice-cold 0.2% Triton X-114 in PBS, and boiled in 2× SDS sample buffer for SDS-PAGE on 12% gels (Bio-Rad Laboratories, Hercules, Calif.). After electrophoresis, the gels were fixed in a 7.5% acetic acid–5% methanol solution for 30 min, soaked in Enlightening Fluid (DuPont, Boston, Mass.) for 30 min, dried under vacuum, and exposed to film at −70°C.

Human serum. All sera from patients with early-stage and late-stage Lyme disease were collected at the Yale University Medical Center Lyme Disease Clinic (15). Patients classified as having early Lyme disease had erythema migrans, which was present on the day of diagnosis. Serum was collected from 14 to 30 days after diagnosis. In most cases, the skin rash had been present for less than 1 week. Patients classified as having late Lyme disease had previous erythema migrans, positive enzyme-linked immunosorbent assay result for *B. burgdorferi* antibody, and a 6-month history of arthritis. Five patients had recurrent bouts of intermittent arthritis. Serum samples from patients with late-stage Lyme disease were collected 1 to 4 years after the initial diagnosis. Serum samples from patients with syphilis were obtained from the Connecticut State Laboratory. All sera from patients with syphilis had Venereal Disease Research Laboratory test titers of 1:16 to 1:128 and were positive by fluorescent treponemal antibody adsorption.

Immunoblots. *B. burgdorferi* N40 proteins or cleaved P22 proteins were resolved in 12% gels by SDS-PAGE and were transferred to NC membranes. NC strips containing *B. burgdorferi* N40 proteins were probed with mouse anti-P22 and anti-BSA sera diluted 1:100. The secondary antibody was alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (Stratagene). The substrates used in color development were NBT (Stratagene) and BCIP (Stratagene). Immunoblots were also performed on P22-containing strips with sera (diluted 1:200) from patients with early- and late-stage Lyme disease or syphilis by using the enhanced chemiluminescence kit (Amersham) according to the manufacturer's protocol. The secondary antibody used in the enhanced chemiluminescence kit was horseradish peroxidase-labeled goat anti-human immunoglobulins (all immunoglobulins) (Southern Biotechnology Associates, Inc., Birmingham, Ala.).

Nucleotide sequence accession number. The nucleotide sequence of the *p22* gene from *B. burgdorferi* N40 reported here has been deposited in the GenBank database under the accession number L22530.

RESULTS AND DISCUSSION

Cloning of the *p22* gene. The *B. burgdorferi* N40 genomic DNA expression library was screened by immunoblotting by using the rabbit anti-*B. burgdorferi* N40 serum. From 10,000 PFU that was plated, induced with IPTG, and probed with the rabbit anti-N40 serum, a clone designated clone 10 (from over 100 clones) expressed a *B. burgdorferi* N40 antigen that was reactive to the antiserum.

DNA sequence analysis of the *p22* gene. The nucleotide sequence of the *p22* gene, the sequences of its 5′- and 3′-flanking regions, and the deduced amino acid sequence of P22 are presented in Fig. 1. The 582-bp open reading frame starts with the ATG initiation codon at position 1 and terminates with the stop codon TAA at nucleotides 583 to 585. Located 8 bp upstream of the ATG start codon of the *p22* gene is a ribosomal binding site with the sequence -AA*GGAG- that almost perfectly matches the consensus Shine-Dalgarno sequence (17). Further upstream of this translational initiation sequence are the −10 region (-TTTAAT-) and the −35 region (-TTGTAA) that closely resemble the promoter elements found in *E. coli* (28) and other *B. burgdorferi* genes (Fig. 2). The TAA stop codon of the *p22* gene is followed by a typical prokaryotic transcriptional termination signal sequence with dyad symmetry. This suggests that the *p22* gene is transcribed as a monocistronic mRNA. These data are virtually identical to and confirmatory of those for the recently described LA7 gene (48).

Amino acid sequence analysis of the P22 protein. The *p22* gene encodes a protein of 194 amino acids with a calculated molecular mass of 21,821 Da. The deduced amino acid sequence of P22 shows that there is a comparatively high content of lysine (10.3%), serine (10.3%), and isoleucine (9.8%) (Table 1). Other preferential codons found in P22 are glutamic acid, asparagine, aspartic acid, leucine, and phenylalanine. On the basis of amino acid composition, the isoelectric point of P22 is predicted to be 5.1.

Analysis of the amino acid composition of P22 shows that there is a preference toward the use of certain codons to represent given amino acids in *B. burgdorferi*, as shown in Table 2. The codon usage pattern of the P22 protein is similar to the codon usage patterns of the OspA, OspB, OspC, OspD, and P27 proteins (5, 16, 29, 31). For example, lysine is frequently represented by AAA, asparagine is frequently represented by AAU, and glutamic acid is frequently represented by GAA. As suggested by earlier analysis of OspA and OspB, this observation supports the hypothesis that the preferred codon usage of the highly expressed outer surface proteins may be representative of the overall codon usage in *B. burgdorferi* (5).

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TCATATTAATAAGACCTCCTGTTTCATTTTAACATTTTAATTGTTTTTAAAGTGTGTACAAAATA

AATTATTTATTGTAAACTTACTTTTAATTTTAATATGATTAATAAATTATAAGGGAGAATTTTT

                15                  30                  45
ATG TAT AAA AAT GGT TTT TTT AAA AAC TAT TTG TCA TTG CTT TTA ATT TTT
Met Tyr Lys Asn Gly Phe Phe Lys Asn Tyr Leu Ser Leu Leu Leu Ile Phe

        60                  75                  90
TTA GTA ATT GCT TGT ACT TCA AAA GAC AGC TCA AAT GAA TAT GTT GAG GAG
Leu Val Ile Ala Cys Thr Ser Lys Asp Ser Ser Asn Glu Tyr Val Glu Glu

105                 120                 135                 150
CAA GAA GCG GAG AAC TCT TCT AAG CCT GAT GAT TCT AAA ATA GAT GAA CAT
Gln Glu Ala Glu Asn Ser Ser Lys Pro Asp Asp Ser Lys Ile Asp Glu His

            165                 180                 195
ACT ATT GGG CAT GTT TTT CAC GCT ATG GGA GTA GTT CAT TCA AAA AAG GAT
Thr Ile Gly His Val Phe His Ala Met Gly Val Val His Ser Lys Lys Asp

    210                 225                 240                 255
CGA AAA AGT TTA GGA GAA AAT ATA AAG GTT TTT TAT TTT TCT GAA GAA GAT
Arg Lys Ser Leu Gly Glu Asn Ile Lys Val Phe Tyr Phe Ser Glu Glu Asp

                270                 285                 300
GGA CAT TTT CAA ACA ATA CCC TCA AAA GAG AAT GCA AAG TTA ATA GTT TAT
Gly His Phe Gln Thr Ile Pro Ser Lys Glu Asn Ala Lys Leu Ile Val Tyr

        315                 330                 345
TTT TAT GAC AAT GTT TAT GCA GGA GAG GCT CCA ATT AGT ATC TCT GGA AAA
Phe Tyr Asp Asn Val Tyr Ala Gly Glu Ala Pro Ile Ser Ile Ser Gly Lys

360                 375                 390                 405
GAA GCC TTT ATT TTT GTT GGG ATT ACC TCT GAC TTT AAA AAG ATT ATA AAC
Glu Ala Phe Ile Phe Val Gly Ile Thr Ser Asp Phe Lys Lys Ile Ile Asn

            420                 435                 450
AGC AAT TTA CAT GGC GCT AAA AGT GAT CTT ATT GGT ACT TTT AAA GAT CTT
Ser Asn Leu His Gly Ala Lys Ser Asp Leu Ile Gly Thr Phe Lys Asp Leu

    465                 480                 495                 510
AAT ATT AAA AAT TCA AAA TTG GAA ATT ACA GTT GAT GAG AAT AAT TCA GAT
Asn Ile Lys Asn Ser Lys Leu Glu Ile Thr Val Asp Glu Asn Asn Ser Asp

                525                 540                 555
GCC AAG ACT TTC CTT GAA TCT GTT AAT TAC ATT ATC GAC GGC GTT GAA AAA
Ala Lys Thr Phe Leu Glu Ser Val Asn Tyr Ile Ile Asp Gly Val Glu Lys

        570                 585
ATT TCA CCT ATG TTA ACG AAT TAA TTTATATTTTTGATTTTATAGGCTTTAATCTAAAT
Ile Ser Pro Met Leu Thr Asn ***

TAAAGCCTATTTTAAAAAATCAAGCTCTCAAGTCCTTTTATTAAAATTTCTGCTGT
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FIG. 1. Nucleotide sequence of the *p22* gene and deduced amino acid sequence of the P22 protein. The numbers above the sequence indicate nucleotide positions. The ribosomal binding site, and the possible −10 and −35 promoter elements further upstream of the 582-bp open reading frame are underlined. The stop codon is indicated by asterisks. A putative transcriptional termination signal downstream of the stop codon is underlined and shown in italicized letters.

The hydrophilicity profile and amino acid sequence of P22 reveal a leader signal peptide that is found in typical prokaryotic lipoprotein precursors (Fig. 3) (18). The leader signal sequence of P22 begins with the short positively charged peptide M-Y-K-N-G-F-F at the amino terminus; this is followed by a hydrophobic domain of 16 amino acids. At the carboxyl terminus of the hydrophobic core is a signal peptidase II cleavage site that is similar to those of typical bacterial prolipoproteins. The lipoprotein processing site of P22 is immediately followed by a stretch of about 30 hydrophilic amino acids. Beyond this large hydrophilic domain, the hydrophilicity plot of P22 shows mixed intervals of hydrophobic and hydrophilic regions.

The consensus sequence around the cleavage site of typical bacterial lipoprotein precursors is L-X-Y-C, where X and Y are predominantly neutral small amino acids such as alanine, glycine, serine, and valine (18). Recently, it has been recognized that variabilities in the consensus tetrapeptide sequence given above, such as L-X-Y-Z-C, are observed (18). Indeed, the sequence around the cleavage site of P22 is L-V-I-A-C. A *B. burgdorferi* signal peptidase presumably recognizes this site and cleaves between the alanine at position 21 and the cysteine at position 22. In known *B. burgdorferi* outer surface lipoproteins and other prokaryotic lipoproteins, the cleavage site is also in front of a conserved cysteine residue (18). The biosynthetic pathway of lipoproteins has been elucidated and reveals that this cysteine is covalently modified through a thioether linkage with a diacylglyceride; this is followed by the cleavage reaction in front of the cysteine by signal peptidase II and then by the addition of a fatty acid moiety, usually palmitate, to the α-NH_2 group of diacylglyceride-cysteine in an amide linkage (18).

	"-35 region"	"-10 region"	Ribosomal binding site
Consensus	TTGACA	TATAAT	AAAGGAGGTGATC
OspA	TTGTTA	TATAAT	AAAGGAG
OspB			AAGGAG
OspC	TTGAAA	TATAAA	AAAGGAGG
OspD	TTGATA	TATAAT	AAGGAG
P27	TTTAAA	TATAAT	AAAGGAG
P22	TTGTAA	TTTAAT	AA*GGAG

FIG. 2. Comparison of the control regions of transcription and translation in five known *B. burgdorferi* outer surface proteins with that of P22. The consensus −35 and −10 σ70-like promoter elements and the consensus ribosomal binding site (Shine-Dalgarno sequence) are from *E. coli*. The sequence of the control region of the *ospA-ospB* operon is derived from strain B31, that of *ospC* is derived from strain PKo, that of *ospD* is derived from strain B31, and that of *p27* is derived from strain B29.

TABLE 1. Amino acid composition of P22[a]

Amino acid	No. (%) of P22[a]
Alanine (A)	9 (4.6)
Arginine (R)	1 (0.5)
Asparagine (N)	15 (7.7)
Aspartic acid (D)	13 (6.7)
Cysteine (C)	1 (0.5)
Glutamic acid (E)	16 (8.2)
Glutamine (Q)	2 (1.0)
Glycine (G)	11 (5.6)
Histidine (H)	6 (3.1)
Isoleucine (I)	19 (9.8)
Leucine (L)	13 (6.7)
Lysine (K)	20 (10.3)
Methionine (M)	3 (1.5)
Phenylalanine (F)	13 (6.7)
Proline (P)	4 (2.0)
Serine (S)	20 (10.3)
Threonine (T)	8 (4.1)
Tryptophan (W)	0 (0.0)
Tyrosine (Y)	8 (4.1)
Valine (V)	12 (6.1)
Total	194

[a] The first number of each line indicates the frequency of occurrence of each amino acid, whereas the numbers in parentheses refer to the percentage of occurrence.

TABLE 2. Codon usage of the *p22* gene in *B. burgdorferi* N40[a]

UUU	Phe	12	**UCU**	Ser	7	**UAU**	Tyr	7	UGU	Cys	1
UUC	Phe	1	UCC	Ser	0	UAC	Tyr	1	UGC	Cys	0
UUA	Leu	6	**UCA**	Ser	8	UAA	***	1	UGA	***	0
UUG	Leu	3	UCG	Ser	0	UAG	***	0	UGG	Trp	0
CUU	Leu	4	**CCU**	Pro	2	**CAU**	His	5	CGU	Arg	0
CUC	Leu	0	CCC	Pro	1	CAC	His	1	CGC	Arg	0
CUA	Leu	0	CCA	Pro	1	**CAA**	Gln	2	CGA	Arg	1
CUG	Leu	0	CCG	Pro	0	CAG	Gln	0	CGG	Arg	0
AUU	Ile	12	**ACU**	Thr	4	**AAU**	Asn	12	**AGU**	Ser	3
AUC	Ile	2	ACC	Thr	1	AAC	Asn	3	AGC	Ser	2
AUA	Ile	5	**ACA**	Thr	2	**AAA**	Lys	14	**AGA**	Arg	0
AUG	Met	3	ACG	Thr	1	AAG	Lys	6	AGG	Arg	0
GUU	Val	10	**GCU**	Ala	4	**GAU**	Asp	9	**GGU**	Gly	2
GUC	Val	0	GCC	Ala	2	GAC	Asp	4	GGC	Gly	2
GUA	Val	2	**GCA**	Ala	2	**GAA**	Glu	10	**GGA**	Gly	5
GUG	Val	0	GCG	Ala	1	GAG	Glu	6	GGG	Gly	2

[a] Underlined and emboldened letters are the preferred codons in *B. burgdorferi* genes *ospA* from strain B31, *ospB* from strain B31, *ospC* from strain PKo, *ospD* from strain B31, and *p27* from strain B29 (5, 17, 30, 32). Each number indicates the frequency of occurrence of each codon in the *p22* gene. Stop codons are marked by asterisks. Notice that the wobble position of the preferentially used codons in these *B. burgdorferi* outer surface proteins is either an A or an U.

The leucine and cysteine residues in the consensus signal peptidase II cleavage sequence L-X-Y-C are usually separated by two neutral small amino acids (18). Indeed, there are two amino acids between the leucine and cysteine in the cleavage sequence of OspA from *B. burgdorferi* B31 (L-I-A-C) and OspB from strain B31 (L-I-G-C) (5). The leucine and cysteine residues in P22 from strain N40 (L-V-I-A-C) are separated by three amino acids. In addition, there are three amino acids in the cleavage site of OspC from strain PKo (L-F-I-S-C) (16), OspD from strain B31 (L-S-I-S-C) (29), and P27 from strain B29 (L-I-L-S-C) (31). Despite this variation in the cleavage sequence, OspA and OspB (6), as well as OspD (29) and P27 from strain B29 (31), have been shown by the established [^{3}H]palmitate-labeling procedure to be lipoproteins. Furthermore, we reported here that P22 is also a lipoprotein. The lipoprotein identity of OspC remains to be elucidated, but the features of its leader signal sequence suggest that it may be processed as a lipoprotein as well.

The deduced amino acid sequence of P22 was compared with those of known *B. burgdorferi* outer surface proteins (OspA to OspD; P27). P22 was found to share no sequence similarity to these outer surface proteins. A homology search of GenBank revealed that P22 shares a 98.5% amino acid sequence identity with a recently identified *B. burgdorferi* lipoprotein called IpLA7 (GenBank accession number X70826) (48). The *LA7* gene was isolated from the European strain ZS7 of *B. burgdorferi* and is, in all likelihood, the same as the *p22* gene from *B. burgdorferi* N40, isolated from a patient in New York State. As expected, the findings reported for IpLA7 were confirmed in the present study.

Mapping of the *p22* gene. The plasmids and the linear chromosome of *B. burgdorferi* N40 were separated by pulsed-field gel electrophoresis and were transferred to an NC filter (Fig. 4). Control Southern blot analysis showed that the *fla* gene is located on the linear chromosome and that the *ospD* gene localizes to the 38-kb linear plasmid, as shown earlier (29). The *p22* DNA probe hybridized to the band corresponding to the linear chromosome of *B. burgdorferi*. Because all of the genes encoding for known *B. burgdorferi* outer surface lipoproteins have been localized to either linear or circular

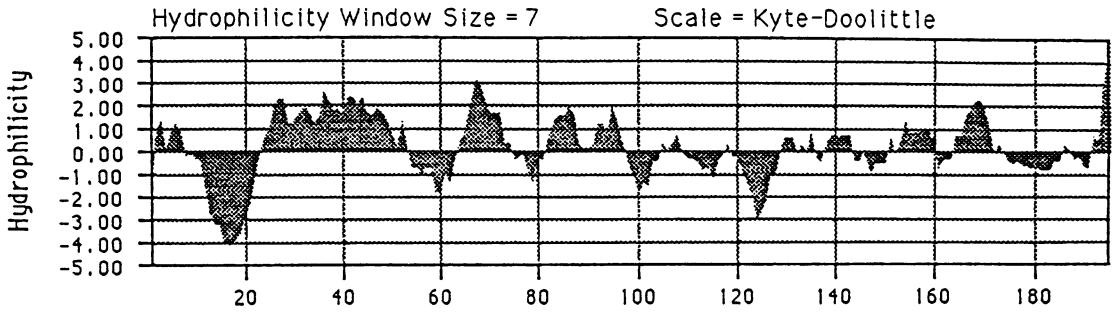

FIG. 3. Hydrophilicity profile of the P22 protein. The hydrophilicity window size is 7, and the hydrophilicity scale is that of Kyte-Doolittle. The leader peptide corresponds to the first 22 amino acids of P22. The leader signal sequence begins with a positively charged hexapeptide; this is followed by a 16-amino-acid hydrophobic core. A *B. burgdorferi* signal peptidase presumably recognizes the sequence L-V-I-A-C and cleaves in front of the conserved cysteine residue at position 22.

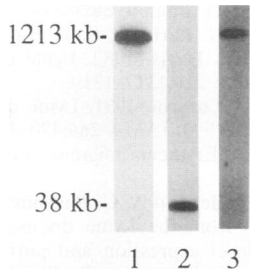

FIG. 4. Southern blot of the pulsed-field gel separating the chromosomal and plasmid DNAs of *B. burgdorferi* N40. The Southern blot was hybridized separately with radiolabeled *fla* (lane 1), *ospD* (lane 2), and *p22* (lane 3) DNA probes. Lane 3 shows that the *p22* gene is located on the linear chromosome of *B. burgdorferi* N40.

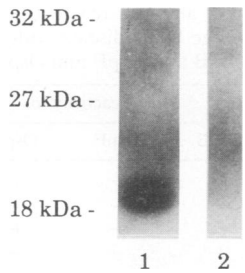

FIG. 6. Fluorograph of the [^{3}H]palmitate-labeled P22 protein (lane 1), which was immunoprecipitated with murine anti-P22 serum and separated by SDS-PAGE. The native *B. burgdorferi* P22 lipoprotein migrated at an apparent molecular mass of 20 kDa (lane 2). A control study was performed without murine anti-P22 serum.

plasmids, P22 appears to be the first lipoprotein that has been shown to be encoded by a *B. burgdorferi* chromosomal gene. The *ospA-ospB* operon, the *ospD* gene, and the *p27* gene are located on the 49-, 38-, and 55-kb linear plasmids, respectively (3, 4, 29, 31). The gene for outer surface protein C is located on the 27-kb circular plasmid (27, 33). In vitro cultivation of *B. burgdorferi* in the presence of monoclonal antibodies to either OspA or OspB has been shown to produce a class of mutants lacking OspA and OspB and the 49-kb linear plasmid that encodes the two proteins (32). Furthermore, repeated in vitro cultivation of *B. burgdorferi* has demonstrated a reduction in the number of detectable plasmids and the loss of infectivity (37, 38). Because active immunization with OspA (12, 13, 42) or OspB (14) and passive immunization with polyclonal (12) and monoclonal (12, 35) antibodies to OspA have been shown to protect mice from *B. burgdorferi* infection, it is of paramount importance that a Lyme disease vaccine be able to confer total protective immunity from any *ospA-ospB* operon-lacking mutants of *B. burgdorferi* that may exist in nature. The fact that the *p22* gene is located on the chromosome and encodes a lipoprotein warrants further research into the role of P22 in the protection from and diagnosis of Lyme disease.

Expression and purification of P22. The *p22* gene lacking the sequences encoding for the hydrophobic leader peptide was overexpressed in *E. coli* as a fusion protein with GT. Expression of the full-length *p22* gene in *E. coli* would have been expected to yield a low level of recombinant P22 because of the retention of the protein in the membrane fraction of *E. coli* (39). As Fig. 5 shows, the cleaved P22 protein lacking the leader peptide migrated at an apparent molecular mass of 22 kDa, which is slightly greater than the predicted molecular mass of 21.8 kDa for the full-length P22 protein.

[^{3}H]palmitate labeling. To determine whether P22 is a lipoprotein and expressed by *B. burgdorferi* N40, lipoproteins were radiolabeled with [^{3}H]palmitate. Radiolabeled P22 was then separated by SDS-PAGE and visualized by fluorography. The fluorograph in Fig. 6 shows that murine anti-P22 serum recognizes a lipoprotein with an apparent molecular mass of 20 kDa, which presumably represents P22. As expected, a control experiment performed without the addition of anti-P22 serum showed no reactivity.

Serologic recognition of P22 in patients with Lyme disease. The production of antibodies to P22 in patients with Lyme disease was examined. Two of the 20 serum samples from patients with early-stage Lyme disease were found to have antibodies to the P22 protein by enhanced chemiluminescence. Ten samples of normal human serum were tested to provide background for comparison. Among the 26 serum samples from patients with late-stage Lyme disease analyzed, 9 had antibodies which reacted with P22 (Fig. 7). Sera from 16 selected patients with late-stage Lyme disease were tested for antibody reactivities to OspA and OspB (15) and to two recently described outer surface proteins, OspE and OspF (23) (Table 3). Cumulatively, serologic reactivity to these five outer surface proteins permited the detection of 75% (12 of 16) of all patients with late-stage Lyme disease. No reactivity of P22 with sera from five patients with syphilis was noted. The sensitivity of these five outer surface proteins for the accurate detection of late-stage Lyme disease is not yet sufficient for use as a reliable diagnostic test. We hypothesize that a cocktail mixture of several *B. burgdorferi* antigens may be needed to increase the sensitivity of a diagnostic assay based on recombinant antigens. The specificity of such a combination of antigens in

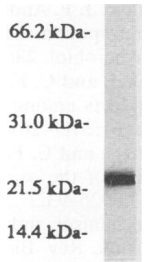

FIG. 5. Coomassie brilliant blue-stained SDS-polyacrylamide gel of the recombinant P22 protein. P22 migrated at an apparent molecular mass of 22 kDa. The predicted molecular mass of the full-length P22 gene is 21.8 kDa.

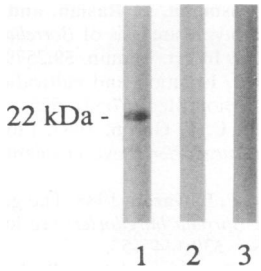

FIG. 7. Representative examples of antibody reactivity to P22 as detected by enhanced chemiluminescence. Lane 1, serum from a patient with late-stage Lyme disease; lane 2, serum from a patient with early-stage Lyme disease; lane 3, normal human serum. All serum samples were diluted 1:200.

TABLE 3. Comparison of antibody reactivities to P22 of sera from patients with late-stage Lyme disease with those to OspA and OspB and OspE and OspF[a]

Patient no.	Reactivity to:					
	OspA	OspB	OspE	OspF	P22	Sum
1	−	−	+	+	−	+
2	−	−	−	+	+	+
3	−	−	−	+	−	+
4	+	+	−	−	+	+
5	−	−	+	−	−	+
6	−	+	−	−	−	+
7	−	−	−	−	−	−
8	+	+	−	+	+	+
9	−	−	−	−	+	+
10	−	−	−	−	−	−
11	+	+	−	−	+	+
12	+	−	−	+	−	+
13	−	−	−	−	−	−
14	+	+	−	+	−	+
15	+	−	−	−	−	+
16	−	−	−	−	−	−

[a] Sera were used to probe recombinant OspA, OspB, OspE, OspF, and P22. Sum refers to antibody reactivities to one or more of the five recombinant antigens. Reactivities to OspA and OspB (14) were described previously.

the diagnosis of late-stage Lyme disease must also be explored further, since cross-reactivity in serologic tests for other spirochetal infections, such as syphilis and periodontitis, has been a major concern (24, 25). The roles of this lipoprotein in the immune response to *B. burgdorferi* infection and in the pathogenesis of Lyme disease can now be examined.

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