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Lyme Disease-Causing *Borrelia* Species Encode Multiple Lipoproteins Homologous to Peptide-Binding Proteins of ABC-Type Transporters

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To identify cell envelope proteins of Borrelia burgdorferi, the causative agent of Lyme disease, we constructed a library of *B. burgdorferi* genes fused to the Escherichia coli phoA gene, which expresses enzymatically active alkaline phosphatase. One such gene, oppA-1, encodes a predicted polypeptide with significant similarities to various peptide-binding proteins of ABC-type transporters. Immediately downstream of oppA-1 are two genes, oppA-2 and oppA-3, whose predicted polypeptide products show strong similarities in their amino acid sequences to OppA-1, including a sequence that resembles the most highly conserved region in peptide-binding proteins. By labeling with [^{3}H]palmitate, OppA-1, OppA-2, and OppA-3 were shown to be lipoproteins. DNA hybridization analysis showed that the oppA-1 oppA-2 oppA-3 region is located on the linear chromosome of *B. burgdorferi*, and the genes are conserved among different Borrelia species that cause Lyme disease (*B. burgdorferi*, B. garinii, and B. afzelli), suggesting that all three homologous genes are important to the maintenance of Lyme disease spirochetes in one or more of their hosts.

Lyme disease is caused by members of a group of closely related spirochetes of the genus Borrelia: *B. burgdorferi*, B. garinii, B. afzelli, B. japonica, and several other possible species that lack formal names (16). *B. burgdorferi* was the first of these species to be isolated and is the prototypical Lyme disease spirochete (14, 16). Following their transmission to human hosts by Ixodes ticks, the spirochetes cause an infection that may result in arthritis, cardiac abnormalities, neurological complications, or chronic skin disease (acrodermatitis chronica atrophicans) (74).

Cell envelope proteins of bacterial pathogens play important roles in the host-parasite interactions that occur during infection, including cell adherence, cell invasion, and immune cell activation or evasion (26). Characterization of *B. burgdorferi* envelope proteins is therefore necessary to understand the mechanism of pathogenesis as well as to develop effective vaccines and immunodiagnostic tests for Lyme disease. Among the various cell envelope proteins of *B. burgdorferi* that have been described are the outer surface proteins OspA (29 kDa) (11), OspB (32 kDa) (11), OspC (23 kDa) (30), OspD (28 kDa) (57), OspE (19 kDa) (48), and OspF (26 kDa) (48), the 41-kDa flagellin protein (83), and other proteins with sizes of 18 kDa (17), 22 kDa (84), 27 kDa (65), 28 kDa (73), 35 kDa (33), 36 kDa (86), 39 kDa (72), 55 kDa (25), 66 kDa (13), 80 kDa (63), and 93 kDa (51). OspA has been shown to bind to human plasminogen (29). The flagellin protein is the major component of the periplasmic flagella (83). Although functional roles for the other cell envelope proteins are currently unknown, the 36-kDa surface-exposed lipoprotein VlsE undergoes extensive antigenic variation that may contribute to the ability of *B. burgdorferi* to evade the host immune response (86). In addition, several putative envelope proteins of *B. burgdorferi* appear to be expressed only in the infected mammalian host (17, 77, 82).

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To identify novel cell envelope proteins of *B. burgdorferi*, we constructed fusions of *B. burgdorferi* genes to the Escherichia coli phoA gene, which encodes alkaline phosphatase. Because alkaline phosphatase is enzymatically active only after it is exported across the cytoplasmic membrane, it acts as a sensor for proteins that carry export signals (52). Using this genetic approach, we have identified a number of novel *B. burgdorferi* genes that encode putative cell envelope proteins. Here we present our studies of three genes, oppA-1, oppA-2, and oppA-3, which encode polypeptides that have remarkable similarity to peptide-binding proteins of peptide transport systems. The products of these genes were identified as lipoproteins, and the oppA-1 oppA-2 oppA-3 operon was shown to be conserved in Borrelia species that cause Lyme disease. We discuss the potential significance of multiple peptide-binding proteins in Borrelia cell physiology and pathogenesis.

MATERIALS AND METHODS

Bacterial strains. *B. burgdorferi* B31 (ATCC 35210) (7) and N40 (48), B. garinii Ip90 (71), and B. afzelli ACAI (71) were obtained from A. Barbour (University of California at Irvine). B. hermsii (type C) and B. turicatae (type A), which cause relapsing fever in humans (8), and B. anserina, which causes avian spirochetosis (9), were also supplied by A. Barbour. E. coli SCS1, XL1-Blue MRF′, and SOLR were obtained from Stratagene (La Jolla, Calif.). E. coli BL21(DE3) contains the gene for bacteriophage T7 RNA polymerase in the chromosome under the control of the lacUV5 promoter (76), and CC118.1 has a deletion of the chromosomal phoA gene and contains the F′ (*lacI*^{q} *lacZ*::Tn*5*) plasmid (35).

Media and reagents. Borrelia strains were grown in BSKII medium (7) supplemented with 50 μg of rifampin per ml and 6 to 12% rabbit serum (Sigma). Media for growth of E. coli were LB (68) and M9 (68) supplemented, when necessary, with ampicillin at 100 μg/ml and chloramphenicol at 25 μg/ml. When required, media contained 5-bromo-4-chloro-3-indolylphosphate (XP) at 20 μg/ml to identify bacterial colonies that express alkaline phosphatase activity.

DNA methodologies. Total genomic DNA was isolated from Borrelia species by a published procedure (42). Plasmid DNA was isolated from E. coli and *B. burgdorferi* by using a Qiagen plasmid purification kit (Qiagen Inc., Chatsworth, Calif.). Specific DNA fragments were amplified in vitro by PCR using AmpliTaq DNA polymerase (Perkin-Elmer, Norwalk, Conn.) and a Perkin-Elmer DNA Thermal Cycler with the following cycling conditions: 30 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min, followed by 72°C for 30 min. Oligonucleotides were custom synthesized by commercial suppliers. The following oligonucleotides were used in this study: D53, GAGTATCAAACTTAAGCGAGCCA TCATCAC (nucleotides 89 to 118 of oppA-1); ospA-214, GGATCTGGAGTA

CTTGAAGG (nucleotides 214 to 233 of ospA [11]); ospB-43, GGATGTGCA CAAAAAGGTGC (nucleotides 43 to 62 of ospB [11]); phoA-181, CGCTAAG AGAATCACGC (nucleotides 181 to 165 of phoA [18]); oppA-1-Nde, cgcgtgac catATGAAATATATAAAAATAGCC (nucleotides 1 to 21 of oppA-1); oppA-1-Bam, gcaggatccTTTCTTTCCGTAGATATTAAT (sequence located 63 to 43 bp downstream of oppA-1); oppA-1-603, TGTTAGTGGCGCATACAAACT TAA (nucleotides 603 to 626 of oppA-1); oppA-2-Nde, cgcgtaggcatATGAAAT TACAAAGGTCATTA (nucleotides 1 to 21 of oppA-2); oppA-2-Bam, gcaggat ccAAACCGTCCATAAGGAATAAA (sequence located 71 to 51 bp downstream of oppA-2); oppA-2-838, TCATCAGCTGTTAATGCCATATAC (nucleotides 838 to 861 of oppA-2); oppA-3-Nde, cgcgtgaccatATGAGCTTTA ATAAAACTAAA (nucleotides 1 to 21 of oppA-3); oppA-3-Bam, gcaggatccCA TAGAATCTTACACATTATT (sequence located 120 to 100 bp downstream of oppA-3); and oppA-3-865, CAACACAAAAGTAATGCAATTTAT (nucleotides 865 to 888 of oppA-3) (lowercase letters denote 5′ nucleotides used to create NdeI or BamHI sites during PCR DNA amplification). Restriction endonucleases and T4 DNA ligase were obtained from commercial suppliers and used as recommended. Agarose gel electrophoresis (68) and pulsed-field gel electrophoresis (35) were done according to published procedures. Transformation of E. coli was by the method of Cohen et al. (20).

Colony blots, phage blots, and Southern blots were prepared according to published procedures (68). Blot hybridizations were done with oligonucleotide or full-length gene probes. Oligonucleotide probes were end labeled with ^{32}P, using [γ-^{32}P]ATP and T4 polynucleotide kinase (68). A full-length gene probe for oppA-1 was synthesized by PCR using ^{32}P-labeled oligonucleotides oppA-1-Nde and oppA-1-Bam. Hybridization solutions contained 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.05× BLOTTO, and hybridization washes were done in 2× SSC–0.1% sodium dodecyl sulfate as described previously (68). The hybridization and washing temperature for an oligonucleotide probe was 15°C less than the calculated melting temperature for the oligonucleotide (68); the hybridization and washing temperature for the full-length oppA-1 probe was 68°C.

Plasmids. Plasmid pET-3a (pBR322 replicon, ampicillin resistance) contains the bacteriophage T7 ϕ10 promoter and translation initiation signals (76); pLysS (P15A replicon, chloramphenicol resistance) carries the gene for T7 lysozyme, an inhibitor of T7 RNA polymerase (76). Plasmids pD53-λ11, pD53-λ17, and pD53-λ20 were excised from Lambda ZAP II clones (see below) and carry *B. burgdorferi* DNA fragments that encode full-length oppA-1, oppA-2, and oppA-3, respectively.

Plasmids were constructed as follows: pD5303, PCR amplification of an oppA-1-containing fragment from pD53-λ11 by using oligonucleotides oppA-1-Nde and oppA-1-Bam, cleavage with NdeI and BamHI, and insertion into pET-3a; pD5306, PCR amplification of an oppA-2-containing fragment from pD53-λ17 by using oligonucleotides oppA-2-Nde and oppA-2-Bam, cleavage with NdeI and BamHI, and insertion into pET-3a; pD5318, PCR amplification of an oppA-3-containing fragment from pD53-λ20 by using oligonucleotides oppA-3-Nde and oppA-3-Bam, cleavage with NdeI and BamHI, and insertion into pET-3a; pUC-phoA1, pUC-phoA2, and pUC-phoA3, insertion of phoA-containing PstI fragments of pCH39, pCH2, and pCH40 (43), respectively, into pUC18 (85).

***B. burgdorferi* gene libraries.** An expression library of *B. burgdorferi* genes fused to the E. coli phoA gene was constructed by insertion of *B. burgdorferi* DNA fragments into the vectors pUC-phoA1, pUC-phoA2, and pUC-phoA3. Total genomic DNA from *B. burgdorferi* B31 was partially digested with a combination of Sau3AI and TaqI to produce DNA fragments with sizes of up to about 5 kb. The *B. burgdorferi* DNA fragments were ligated with an equimolar mixture of the three pUC-phoA vectors that had been treated with BamHI and AccI. E. coli SCS1 was transformed with the ligation mixture, and the transformants were pooled and grown for several generations. Plasmid DNA was then prepared from the total library and used to transform the E. coli ΔphoA strain CC118.1. Transformants expressing alkaline phosphatase activity were identified as blue colonies on selective media containing the chromogenic indicator XP (52).

A Lambda ZAP II DNA expression library of the low-passage-number, infectious *B. burgdorferi* strain N40 has been described previously (48) and was obtained from the laboratory of Erol Fikrig (Yale University). After identification and isolation of specific Lambda ZAP II clones, the pBluescript plasmid containing the cloned DNA fragment was excised in vivo from the lambda vector. In vivo excision was performed according to the Lambda ZAP II instruction manual (Stratagene). In brief, E. coli XL1-Blue MRF′ cells were simultaneously infected with Lambda ZAP II phage and ExAssist helper phage to produce single-stranded plasmid packaged as a filamentous phage. E. coli SOLR cells were then infected with the filamentous phage and plated on LB-ampicillin plates to produce bacterial colonies that contain the double-stranded plasmid.

DNA sequence analysis. Nucleotide sequences were determined by the dideoxynucleotide chain termination method (69), using a Sequenase DNA sequencing kit (Amersham Corp., Arlington Heights, Ill.). Sequencing products were labeled with [α-^{35}S]thio-dATP (Amersham), separated by gel electrophoresis, and visualized by autoradiography as described in the Sequenase protocol. The gene fusion joints in *B. burgdorferi* PhoA$^+$ clones were sequenced by using double-stranded plasmid templates and the oligonucleotide phoA-181. The nucleotide sequence of the 6.3-kb region encoding oppA-1, oppA-2, and oppA-3 was generated by using several overlapping clones that were isolated from the Lambda ZAP II library of *B. burgdorferi* N40. The pBluescript plasmid containing the cloned DNA fragment was excised from each Lambda ZAP II clone and used as a double-stranded template for the sequencing reactions. The complete sequence for both DNA strands of the oppA-1 oppA-2 oppA-3 region was determined by using the method of progressive oligonucleotide primers (68). The computer program DNA Strider (23) was used for basic DNA sequence analysis. Protein database searches were performed by using the BLAST alignment program (6). Multiple sequence alignments were done with the MegAlign program (19).

Polypeptide analysis. The bacteriophage T7 RNA polymerase-dependent expression system (76) was used to express the polypeptide products of oppA-1, oppA-2, and oppA-3. Each gene was cloned individually downstream of the T7 ϕ10 promoter in pET-3a (creating pD5303, pD5306, and pD5318). The host strain, E. coli BL21(DE3), also contained plasmid pLysS to prevent deleterious expression of the cloned genes (76). To label preferentially the product of the cloned gene, a 20-ml culture of cells was grown in M9 (supplemented with ampicillin and chloramphenicol) to mid-logarithmic phase (optical density at 600 nm of 0.6) and then split into two 10-ml cultures. One of the 10-ml cultures was supplemented with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to induce expression of the lacUV5 promoter. After 60 min of incubation, both cultures were supplemented with rifampin (200 μg/ml), and incubation was continued for 90 min. Cells from 1 ml of each culture were labeled at 37°C with either 5 μCi of L-[U-^{14}C]amino acid mixture (>50 mCi/mg · atom of carbon; Amersham) for 5 min or 100 μCi of [9,10(*n*)-^{3}H]palmitic acid (43 Ci/mmol; Dupont NEN, Boston, Mass.) for 20 min, collected by centrifugation, and suspended in 0.2 ml of protein sample buffer (0.0625 M Tris, 2% sodium dodecyl sulfate, 10% glycerol, 5% β-mercaptoethanol [pH 6.8]). Samples of 15 μl were heated to 100°C for 5 min and analyzed by electrophoresis through a sodium dodecyl sulfate–10% polyacrylamide gel (with a 5% stacking gel) with the discontinuous buffer system of Laemmli (47). After electrophoresis, the gel was fixed and stained in 50% methanol–10% acetic acid–0.25% Coomassie blue, destained in 30% methanol–10% acetic acid, and prepared for fluorography with ENTENSIFY autoradiography enhancer (Dupont NEN). The gel was then dried, placed against Kodak X-Omat LS film, and exposed at −80°C. ^{14}C-labeled protein molecular size markers (Amersham) were phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), ovalbumin (46 kDa), and carbonic anhydrase (30 kDa).

Nucleotide sequence accession. The GenBank accession number for the sequence of the oppA-1 oppA-2 oppA-3 operon is AF043071.

RESULTS

Construction of a *B. burgdorferi* PhoA$^+$ library. We constructed a set of plasmid vectors (pUC-phoA1, pUC-phoA2, and pUC-phoA3) that permit gene fusions to phoA (lacking its signal sequence) in each of the three possible translational reading frames. *B. burgdorferi* B31 DNA fragments were cloned into these vectors to generate a library consisting of about 50,000 clones, which we estimate contains >50 genome equivalents of *B. burgdorferi* DNA. To identify those clones that encode *B. burgdorferi* protein export signals fused to alkaline phosphatase, plasmid DNA from the total library was used to transform the E. coli ΔphoA strain CC118.1. Approximately 1,200 of 50,000 transformants were blue on media containing XP, indicating the presence of enzymatically active alkaline phosphatase. This collection of PhoA$^+$ clones was designated the *B. burgdorferi* PhoA$^+$ library.

To assess the complexity of the *B. burgdorferi* PhoA$^+$ library, we screened the clones by colony blot hybridizations with oligonucleotide probes specific for genes that encode the known outer surface proteins OspA and OspB. From the results (not shown), we estimate that (i) the library contains segments of approximately 100 different *B. burgdorferi* genes that encode cell envelope proteins and (ii) there is about a 10-fold redundancy of each gene in the library.

DNA sequence analysis of *B. burgdorferi*-PhoA$^+$ clones. We determined the nucleotide sequences around the gene fusion joints of various *B. burgdorferi* PhoA$^+$ clones that did not hybridize to probes for ospA and ospB. The sequences revealed 22 clones that were predicted to encode unique *B. burgdorferi* polypeptide segments involved in promoting protein export. These clones were grouped into two categories: (i) 13 clones were predicted to encode relatively short *B. burgdorferi* polypeptide segments (22 to 68 amino acids) fused to

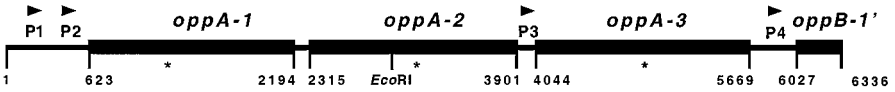

FIG. 1. Structure of the oppA-1 oppA-2 oppA-3 region of *B. burgdorferi*. The nucleotide sequence was determined for a 6,336-bp region (GenBank accession no. AF043071). Numbers refer to nucleotide positions, and the unique EcoRI site at nucleotide position 2949 is indicated. The locations of oppA-1, oppA-2, and oppA-3 are shown with nucleotide positions for the translational initiation and termination codons; oppB-1′ represents the 5′ end of an adjacent gene. P1 through P4 indicate sequences that resemble σ^{70}-type promoters of E. coli (37); all contain reasonable −35 and −10 regions that are spaced appropriately for efficient initiation of transcription. The −10 regions of the potential promoters are located at nucleotide positions 272, 370, 4004, and 5981. Arrowheads show the predicted directions of transcription. Asterisks indicate locations of sequences that comprise the gene-specific oligonucleotide probes oppA-1-603, oppA-2-838, and oppA-3-865.

PhoA, and the sequences allowed the fusion proteins to be defined; and (ii) 9 clones were predicted to have longer polypeptide segments fused to PhoA and required further sequence analysis to completely define the fusion proteins. All 13 of the former clones had N-terminal sequences that were predicted to be signal peptides for protein export (64), five ending with a consensus signal peptidase I cleavage site (81) and eight ending with a consensus signal peptidase II cleavage site (38). We compared the amino acid sequences of the 22 unique *B. burgdorferi* polypeptide segments to sequences of known *B. burgdorferi* proteins. Only one polypeptide, which was identical to the 22-kDa lipoprotein IpLA7 (84), had been described prior to the release of the *B. burgdorferi* genome sequence (28).

Analysis of oppA-1. One novel *B. burgdorferi* polypeptide segment, which contains a putative signal peptide with a signal peptidase II cleavage site, is encoded by *B. burgdorferi* PhoA$^+$ clone D53. The nucleotide sequence of the gene fusion joint in this clone showed a partial *B. burgdorferi* gene of 41 codons fused in frame to phoA. To clone the full-length gene, an oligonucleotide probe (D53) to the 5′ end of the gene was used in plaque hybridizations to isolate clones from a Lambda ZAP II genomic library of *B. burgdorferi* N40. We determined the nucleotide sequence of the cloned region, and sequence analysis showed that the complete gene consists of 524 codons (designated oppA-1 in Fig. 1). The potential ATG translational start codon is preceded by a good Shine-Dalgarno sequence for ribosome binding, 5′-AAAGGA-3′, that is complementary to the 3′ end of the 16S rRNA of both *B. burgdorferi* (31) and E. coli (70). The spacing between the Shine-Dalgarno sequence and the ATG codon is seven nucleotides, which is suitable for efficient initiation of translation in E. coli (75).

Translation of the gene sequence results in a predicted polypeptide of 523 amino acids with a molecular mass of 59,875 Da (designated OppA-1 in Fig. 2). Homology searches of protein databases revealed significant similarities of this polypeptide to various substrate-binding proteins of ABC-type transporters (40, 78) (Table 1). This family of proteins consists mostly of peptide-binding proteins of peptide transporters, which promote the transfer of short peptides (two to eight amino acids) into the cell (40, 60, 78). Such systems have been shown recently to be important in bacterial cell signaling and virulence (24, 44, 59, 78). Thus, the *B. burgdorferi* polypeptide was designated OppA-1 (oligopeptide permease), in keeping with this homology.

oppA-1 is a member of a gene family. Peptide transport systems are generally composed of a single peptide-binding protein, two integral membrane proteins, and two ATP-binding proteins (40, 60, 78). The genes for these proteins are usually expressed as part of a multicistronic operon, with the gene for the peptide-binding protein typically being the first gene in the operon, followed by the genes for the membrane transporter and ATP-binding proteins (1, 60). We therefore continued sequencing downstream of oppA-1 to determine if it is located in an operon with genes for the other transporter components. The nucleotide sequence of the downstream region revealed two large open reading frames, designated oppA-2 and oppA-3 (Fig. 1). Both have potential ATG translational start codons preceded by appropriately spaced Shine-Dalgarno sequences for ribosome binding (5′-GGAGGT-3′ and 5′-AGGT-3′, respectively).

The predicted polypeptide products of oppA-2 and oppA-3 consist of 528 amino acids (60,624 Da) and 541 amino acids (62,315 Da), respectively (Fig. 2). Surprisingly, we found that the amino acid sequences of both OppA-2 and OppA-3 are highly similar to that of OppA-1 (Fig. 2). Pairwise comparisons of the three polypeptides revealed amino acid identities of 56% for OppA-1 and OppA-2, 52% for OppA-1 and OppA-3, and 59% for OppA-2 and OppA-3. In addition, all three polypeptides contain amino acid sequences that are analogous to the most highly conserved region found in peptide-binding proteins (Fig. 2). Thus, OppA-1, OppA-2, and OppA-3 constitute a family of *B. burgdorferi* polypeptides that have sequence similarity to peptide-binding proteins of peptide transport systems.

The nucleotide sequence downstream of oppA-3 contains a partial open reading frame, designated oppB-1′ (Fig. 1). Because oppB-1′ is preceded by a good Shine-Dalgarno sequence for ribosome binding (5′-AAAGGA-3′), it may represent the 5′ end of another *B. burgdorferi* gene. We compared the predicted OppB-1′ polypeptide to sequences in protein databases and found strong similarities to N-terminal segments of various integral membrane protein components of peptide transport systems. Proteins that have the greatest similarity to OppB-1′ include OppB of *Haemophilus influenzae* (49% identity) (27), OppB of *Salmonella typhimurium* (48% identity) (41), OppB of *Bacillus subtilis* (43% identity) (62, 66), and DppB of B. subtilis (35% identity) (54). Thus, this particular region appears to contain genes that encode a peptide transporter with multiple peptide-binding proteins.

OppA-1, OppA-2, and OppA-3 are lipoproteins. The N-terminal regions of OppA-1, OppA-2, and OppA-3 have features that are typical for signal peptides of lipoprotein precursors: a positively charged amino terminus, a hydrophobic core, and a potential site for lipid attachment and cleavage by signal peptidase II (Leu-X-Y-Cys or Leu-X-Y-Z-Cys, where X, Y, and Z are preferably small neutral amino acids) (38, 64) (Fig. 2). To determine whether the mature polypeptides are lipoproteins when produced in E. coli, oppA-1, oppA-2, and oppA-3 were each cloned downstream of a phage T7 promoter (generating pD5303, pD5306, and pD5318, respectively) and expressed preferentially (Fig. 3). By labeling with ^{14}C-amino acids and suppressing host gene expression with rifampin, each gene was found to express a polypeptide with an observed mass that corresponds closely to the predicted product. In addition, the OppA proteins appeared to be somewhat unstable since several lower-molecular-weight species were observed after IPTG induction, and the proteins did not accumulate to high levels after induction with IPTG in the absence of rifampin (results not shown). To specifically label the lipid moieties of the putative lipoproteins, the genes were expressed in the presence of

FIG. 2. Predicted amino acid sequences of OppA-1, OppA-2, and OppA-3. The sequences were aligned by using the Clustal method of the MegAlign program. Gaps, indicated by dashes, were introduced into the sequences to permit optimal alignment. Boxes indicate amino acids that are present in at least two of the three polypeptides. A consensus signal peptidase II processing site (SPase II) and a region that has high sequence identity to the most highly conserved region in peptide-binding proteins of peptide transporters (conserved region) are noted. The consensus sequence for the conserved region is (LIVM)**A**X_2 (WI)$X_{1\ or\ 2}$ (SN)(KE)**D** X_4**T**(FY) X(LIV)**R**X_3**K** (78), where X indicates any amino acid residue, alternate amino acid residues are given in parentheses, and residues in boldface are invariant.

[^{3}H]palmitate, which is the predominant fatty acid in *B. burgdorferi* lipoproteins (10). Because the three polypeptides were radiolabeled under these conditions (Fig. 3), we conclude that OppA-1, OppA-2, and OppA-3 are lipoproteins when expressed in E. coli. Comparison of the published pattern of lipoproteins for *B. burgdorferi* B31 indicates that there are lipoprotein species in the appropriate molecular mass range (~58 kDa) to comprise members of the OppA protein family (12). However, verification of the lipoprotein status of these proteins in *B. burgdorferi* awaits development of specific antisera.

Conservation of oppA-1, oppA-2, and oppA-3 among Lyme disease spirochetes. To determine whether oppA-1, oppA-2, and oppA-3 are conserved among different species of Lyme disease spirochetes, DNA from various Borrelia species was digested with EcoRI and hybridized with specific probes for each of the genes (Fig. 4). A full-length oppA-1 probe detected two DNA fragments (6.5 and 5.5 kb) from *B. burgdorferi* N40. This result was expected because the probe cross-hybridizes with oppA-2 (results not shown), and the oppA-1 oppA-2 oppA-3 region of *B. burgdorferi* contains a single EcoRI site (Fig. 1). The probe also detected DNA fragments from two other species of Lyme disease spirochetes (B. afzelli and B. garinii) but did not detect any fragments from three Borrelia species that do not cause Lyme disease (B. hermsii, B. turicatae, and B. anserina). B. afzelli showed two hybridizing fragments of the same sizes as those detected in *B. burgdorferi*. For B. garinii, three fragments (6.5, 4.3, and 1.3 kb) were observed, but only one (6.5 kb) has the same size as a fragment detected in *B. burgdorferi* and B. afzelli. However, the combined size of the other two fragments (4.3 and 1.3 kb) approximates the size of the second fragment (5.5 kb) found in *B. burgdorferi* and B. afzelli, suggesting that B. garinii has an additional EcoRI site in its oppA region. These results indicate that the three species of Lyme disease spirochetes (*B. burgdorferi*, B. garinii, and B. afzelli) have similar oppA regions.

We also examined EcoRI-digested DNA from the three species of Lyme disease spirochetes by hybridization with gene-specific oligonucleotide probes (Fig. 4). Each probe was designed to have a unique sequence from either oppA-1, oppA-2, or oppA-3 (Fig. 1) that does not cross-hybridize with the other two genes (results not shown). The oppA-1-specific probe (oppA-1-603) detected a 5.5-kb fragment from *B. burgdorferi* and B. afzelli and a 4.3-kb fragment from B. garinii. These are the same fragments that exhibited the strongest hybridization signals with the full-length oppA-1 probe. The oppA-2- and oppA-3-specific probes (oppA-2-838 and oppA-3-865, respectively) both detected a 6.5-kb fragment in all three species. Because the two probes are unique, do not cross-hybridize, and correspond to an analogous region in oppA-2 and oppA-3, the results indicate specific hybridization to each of the two genes located on the same DNA fragment. We

TABLE 1. Substrate-binding proteins of ABC-type transporters that have similarity to OppA-1 of *B. burgdorferi*

Protein[a]	Organism or plasmid	Substrate	No. of residues	% Identity to OppA-1	% Similarity to OppA-1[b]	Reference(s)
OppA	*Salmonella typhimurium*	Oligopeptide	542	33	41	41
OppA	*Escherichia coli*	Oligopeptide	543	32	40	45
DppE (DciAE)	*Bacillus subtilis*	Dipeptide	543	30	39	54
PrgZ	pCF10 (*Enterococcus faecalis*)	Peptide pheromone	545	30	37	67
TraC	pPD1 (*E. faecalis*)	Peptide pheromone	545	29	38	55
TraC	pAD1 (*E. faecalis*)	Peptide pheromone	543	28	35	79
OppA (Spo0KA)	*B. subtilis*	Oligopeptide	545	28	38	62, 66
DppA	*E. coli*	Dipeptide	535	26	36	2, 58
Has	*Streptococcus equisimilis*	Hyaluronate	522	26	33	49
HbpA	*Haemophilus influenzae*	Heme	547	25	33	36
NikA	*E. coli*	Nickel	524	25	33	56
AliA (PlpA)	*Streptococcus pneumoniae*	Oligopeptide	660	23	33	4, 61
SapA	*S. typhimurium*	Oligopeptide	549	23	33	59
AliB	*S. pneumoniae*	Oligopeptide	652	23	32	4
AppA	*B. subtilis*	Oligopeptide	543	23	31	46
AccA	pTiC58 (*Agrobacterium tumefaciens*)	Agrocinopine	521	21	32	39
SapA	*H. influenzae*	Unknown	565	21	30	27
AmiA	*S. pneumoniae*	Oligopeptide	659	20	31	5
OppA	*Lactococcus lactis*	Oligopeptide	600	18	27	80
XP55	*Streptomyces lividans*	Unknown	542	18	27	15

[a] Listed in order of percent identity to OppA-1.
[b] Determined by allowing conservative amino acid substitutions.

conclude that all three species of Lyme disease spirochetes contain oppA-1, oppA-2, and oppA-3.

The genome of *B. burgdorferi* consists of a 911-kb linear chromosome and a number of circular and linear plasmids that range in size from about 9 to 56 kb (16, 28). To determine the genomic location of the oppA-1 oppA-2 oppA-3 region, total DNAs of *B. burgdorferi* B31 (high passage number) and N40 (passage 2) were separated by pulsed-field gel electrophoresis or conventional agarose gel electrophoresis and hybridized with an oligonucleotide probe specific for oppA-1, oppA-2, or oppA-3. The results showed specific hybridization of the probes to only the chromosomal DNA band (results not shown), consistent with the chromosomal location of these genes on the recently published genome sequence of *B. burgdorferi* (28). However, since the latter report did not provide the sequences for all of the *B. burgdorferi* B31 plasmids, and certain plasmids are notoriously unstable, we cannot exclude the presence of an oppA-1, oppA-2, or oppA-3 homolog on particular plasmids in either of these two strains.

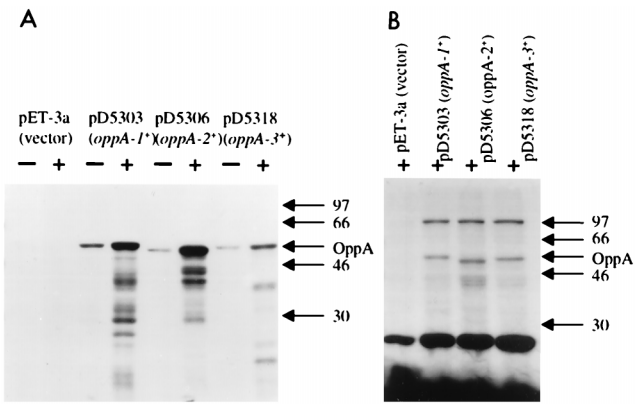

FIG. 3. Polypeptide products of oppA-1, oppA-2, and oppA-3. Each gene was cloned individually and expressed in vivo from the bacteriophage T7 ϕ10 promoter. Polypeptides specified by the cloned genes were selectively labeled with either ^{14}C-amino acids (A) or [^{3}H]palmitic acid (B), separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and visualized by autoradiography. + or − indicates expression in the presence or absence, respectively, of IPTG. The abundant small lipoprotein is presumed to be Braun's lipoprotein of E. coli (38), while the 97-kDa species is of unknown origin. Sizes are indicated in kilodaltons.

DISCUSSION

phoA fusion technology has been widely used to create recombinant DNA libraries specifically encoding portions of cell envelope proteins from a variety of microorganisms (52). This approach is particularly valuable for a molecular analysis of pathogenesis since envelope proteins that contribute to virulence, such as cell adhesins and invasins, factors contributing to immune cell evasion or suppression, and various exoenzymes and toxins, can be identified and characterized. Several novel *B. burgdorferi* cell envelope proteins have been previously identified by constructing gene fusions to phoA (3, 17, 32). Using this strategy, we identified 22 *B. burgdorferi* genes that encode putative envelope proteins. Comparisons of the 22 predicted amino acid sequences to the recently released *B. burgdorferi* genome sequence (28) indicated that 14 sequences match predicted proteins with a variety of suggested functions (73 to 100% identity), 3 sequences match predicted proteins with no suggested function (93 to 100% identity), and 5 sequences show no significant matches at the amino acid level. This latter category may represent regions not yet sequenced by Fraser et al. (28) (e.g., plasmids cp32 and lp56), or alternatively, they may represent genes whose sequence has diverged significantly between the different isolates of *B. burgdorferi* B31 used (high versus low passage number). Because only one of the 22 genes was described prior to the release of the *B. burgdorferi* genome sequence, a phoA fusion strategy proves to be an economical and effective way of identifying genes encoding novel envelope proteins unless a substantial effort is going to be made to acquire the complete genome sequence of a particular microorganism. In addition, phoA fusion technology remains a useful method to confirm the presence of predicted export signals, given the existence of a genome sequence.

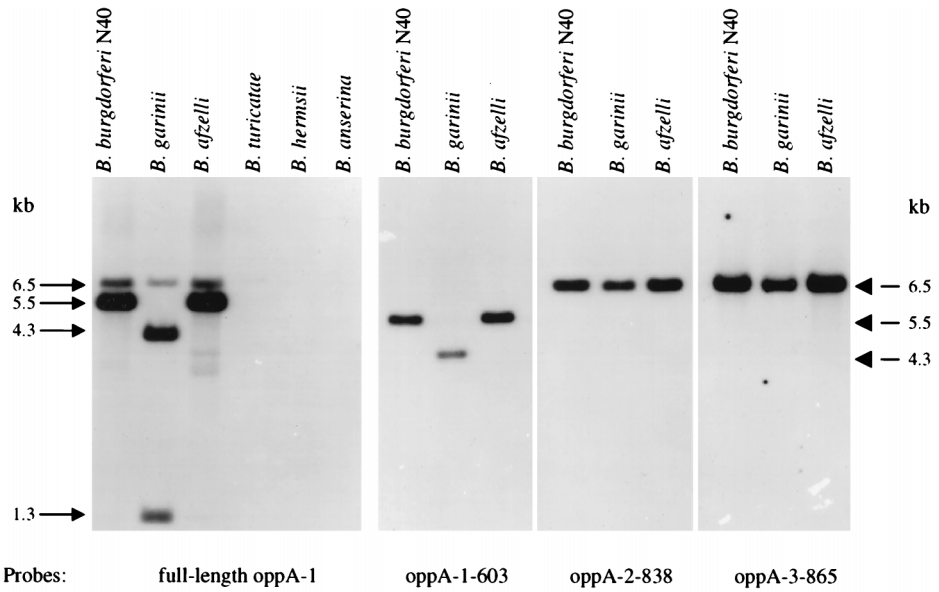

FIG. 4. Presence of oppA-1 oppA-2 oppA-3 in Borrelia species. Total genomic DNA from the indicated Borrelia species was digested with EcoRI, separated by agarose gel electrophoresis, and transferred to nitrocellulose filters. The filters were hybridized with the indicated ^{32}P-labeled full-length oppA-1 probe or gene-specific oligonucleotide probes and visualized by autoradiography.

In this study, we have described a family of three genes (oppA-1, oppA-2, and oppA-3) that encode predicted peptide-binding proteins. The recently published genome sequence of *B. burgdorferi* (28) reveals that the oppA genes reside in a seven-gene operon that encodes components of a predicted peptide transport system. The first three genes of the operon are 99.8, 100, and 99.6% identical to oppA-1, oppA-2, and oppA-3, respectively. They are followed by two genes (oppB-1 and oppC-1) predicted to encode integral membrane proteins of the peptide transporter and two genes (oppD and oppF) predicted to encode ATP-binding proteins for driving transport. The gene arrangement of this operon, with multiple tandem genes for peptide-binding proteins, is unique among operons that encode peptide transporters (60, 78).

Peptide-binding protein-dependent transport systems are now recognized to play important roles in microbial cell signaling and virulence (24, 44, 59, 78). These systems have been shown to be important for recycling of cell wall peptides and chemotaxis in E. coli and S. typhimurium (2, 34, 53), resistance to antimicrobial cationic peptides in S. typhimurium (59), pheromone-mediated conjugation in Enterococcus faecalis (24, 50), initiation of sporulation and natural competence in B. subtilis (44, 46, 62, 66), and cell adherence, natural transformation, and altered drug susceptibility in Streptococcus pneumoniae (4, 5, 21, 61). What functions might we ascribe to the OppA family of peptide-binding proteins? Given the fact that they are most similar to oligopeptide-binding proteins, it seems nearly certain that oligopeptides are their natural substrate. It also makes sense that *B. burgdorferi* would possess a peptide transport system, given its limited biosynthetic capability (28). Clearly, the presence of more than one peptide-binding protein would expand the repertoire of peptides that could be effectively taken up by the spirochete from tick or mammalian hosts, which may have very different extra- or intracellular peptide profiles. Because peptide transport systems are associated with virulence of S. typhimurium (59), S. pneumoniae (21), and E. faecalis (24), it is reasonable to hypothesize that the OppA proteins may have a role in the virulence of *B. burgdorferi*. For example, this family of peptide-binding proteins may be important in regulating spirochetal chemotaxis or gene expression for targeting and penetration of specific tissues in invertebrate and vertebrate hosts.

We note that the *B. burgdorferi* genome sequence shows that, in addition to three chromosomally encoded peptide-binding proteins that we have described here, there are two other predicted peptide-binding proteins, OppA-4 and OppA-5, which are encoded on the 54-kb linear plasmid and 26-kb circular plasmid, respectively (28). Molecular phylogenetic analysis of these latter two proteins indicated that OppA-4 is most closely related to OppA-2 (62.7% similarity), whereas OppA-5 appears to have diverged somewhat more from OppA-1 OppA-2 OppA-3 (43 to 50% similarity). It seems reasonable to speculate that OppA-4 and OppA-5 may utilize the other components of the chromosomally encoded oligopeptide transporter for function, by analogy to a similar system in E. faecalis (50). We think that it is significant that *B. burgdorferi* has no fewer than five predicted oligopeptide-binding proteins, with two of these encoded on plasmids, where gene loss would be expected to be frequent unless such genes encode important functions. Similarly, our finding that oppA-1, oppA-2, and oppA-3 are present in three genospecies of Lyme disease-causing Borrelia further underscores the importance of this family of genes in Borrelia cell biology and physiology. These observations suggest that in addition to having a general housekeeping function, at least certain members of this family of peptide-binding proteins play roles in the pathobiology of the Lyme agent.

A recent report described an apparent 30-kDa protein of *B. burgdorferi*, P30, that is homologous to peptide-binding proteins and is recognized by antibodies in sera from a subset of patients with Lyme disease and from *B. burgdorferi*-infected mice (22). Immunofluorescence studies suggested that this antigen is located on the cell surface of spirochetes. Comparison of our sequence with that for p30 (both of which were obtained from the same *B. burgdorferi* N40 library) indicated that p30 is 93% identical to the 5′ portion of oppA-2 and is missing a T at

nucleotide 783 (relative to the start codon), leading to the presence of a premature termination codon in p30. This observation suggests that the p30 sequence may have resulted from a cloning or sequencing artifact. This notion is consistent with the lack of any other sequence that is highly homologous to p30 (besides oppA-2) in the genome sequence of *B. burgdorferi* B31 (28). Furthermore, the surface localization of this antigen, which we assume corresponds to OppA-2 or members of the OppA family of lipoproteins, appears problematic, since peptide-binding proteins have always been shown to be periplasmically localized in eubacteria comprised of double-membrane systems, where they are able to interact with transport components located in the plasma membrane (40).

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