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Outer Surface Proteins E and F of *Borrelia burgdorferi*, the Agent of Lyme Disease

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We report the cloning and characterization of two outer surface proteins (Osps), designated OspE and OspF, from strain N40 of *Borrelia burgdorferi*, the spirochetal agent of Lyme disease. The *ospE* and *ospF* genes are structurally arranged in tandem as one transcriptional unit under the control of a common promoter. The *ospE* gene, located at the 5′ end of the operon, is 513 nucleotides in length and encodes a 171-amino-acid protein with a calculated molecular mass of 19.2 kDa. The *ospF* gene, located 27 bp downstream of the stop codon of the *ospE* gene, consists of 690 nucleotides and encodes a protein of 230 amino acids with a calculated molecular mass of 26.1 kDa. Pulsed-field gel electrophoresis showed that the *ospE* and *ospF* genes are located on a 45-kb plasmid. Comparison of the leader sequences of OspE and OspF with those of the four known *B. burgdorferi* Osps (OspA, OspB, OspC, and OspD) reveals a hydrophobic domain and a consensus cleavage sequence (L-X-Y-C) recognized by signal peptidase II, and [^{3}H]palmitate labeling shows that OspE and OspF are lipoproteins. Immunofluorescence studies demonstrated that both the OspE and OspF proteins are surface exposed. These features are consistent with the finding that OspE and OspF are *B. burgdorferi* surface lipoproteins.

Lyme disease is a multisystem infectious disease caused by the tick-borne spirochete *Borrelia burgdorferi*, which is transmitted to humans by *Ixodes* ticks (6, 35). The onset of Lyme disease is heralded by the characteristic bull's-eye-shaped skin lesion termed erythema migrans and accompanied by nonspecific symptoms such as fatigue, headache, and fever (8, 34). In the weeks to months that follow *B. burgdorferi* infection, cardiac, neurologic, and rheumatologic abnormalities may ensue (19). Since its recognition in 1975, Lyme disease has become the most common tick-borne infectious disease in the United States (7). Identified in at least 43 states, the disease has also been found in most of Europe, the Soviet Union, Australia, China, and Japan (28, 31). Lyme disease represents a significant health problem, and strategies to prevent its spread are needed.

B. burgdorferi antigens may be useful as substrates in diagnostic assays or as vaccine candidates. Several *B. burgdorferi* outer surface antigens have been characterized and shown to be lipoproteins. The genes for outer surface proteins A and B (OspA and OspB), two major membrane antigens, are encoded in a single operon on a 49-kb linear plasmid (4). The gene for OspC, a 22-kDa protein previously identified as pC, is located on a 27-kb circular plasmid (15, 25). The gene for OspD is located on a 38-kb linear plasmid and is preferentially expressed by low-passage, virulent strains of *B. burgdorferi* B31 (22). These outer surface antigens have been shown to be lipoproteins by labeling with [^{3}H]palmitate (5). The 41-kDa protein (flagellin) is associated with the flagellum and has regions of homology with other bacterial flagellins (3, 16). The 93-kDa immunogenic protein is localized to the periplasmic space (36). Neither the 41- nor the 93-kDa antigen is a lipoprotein.

Research on immunity to Lyme disease has shown that outer surface lipoproteins may be vaccine candidates. Active immunization of C3H/HeJ mice with recombinant OspA from *B. burgdorferi* N40 elicited antibodies to OspA and conferred long-lasting protection against challenge with several *B. burgdorferi* strains (11, 12). Passive immunizations of the immunity-deficient *scid* mice with monoclonal and polyclonal antibodies to OspA also prevented spirochetal infection (27, 32). Immunization with OspB elicited a protective immune response in C3H/HeJ mice, although the degree of protection was not as great as with OspA (13). Furthermore, in a gerbil model of Lyme disease, active immunization with OspC protected rodents from infection, particularly against challenge with European *B. burgdorferi* strains that do not express or express low levels of OspA (23). We now characterize a putative operon containing the genes for two *B. burgdorferi* antigens, OspE and OspF, and define the amino acid sequences of the two proteins. We present data showing that the *ospE* and *ospF* genes are located on a 45-kb plasmid and that they encode two lipoproteins which are surface exposed.

MATERIALS AND METHODS

Construction of *B. burgdorferi* library. A 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 8,000 × *g* for 30 min, and lysed with sodium dodecyl sulfate (SDS) (1). 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 *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. Polyclonal rabbit anti-N40 serum was raised by intravenous injection of an inoculum of 10^8 live *B. burgdorferi* N40 organisms, a strain known to be pathogenic in C3H/HeJ mice (11), in phosphate-buffered saline (PBS) via the marginal ear vein and boosted at 14, 21, and 49 days with another inoculum of 10^8 live *B. burgdorferi* N40 organisms in PBS. Two weeks following the last boost, 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 antibodies in the rabbit antiserum 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 (TBST). Nitrocellulose (NC) filters of 0.45-μm pore size (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 then rinsed three times with TBST. The rabbit anti-*B. burgdorferi* N40 serum was diluted 1:5 in TBST and incubated with the NC filters with shaking for 10 min at 37°C. The NC filters were then removed and discarded.

Bacterial strains. The recommended host cell for screening the *B. burgdorferi* N40 expression library is *E. coli* SURE (Stratagene). A culture inoculated from a single colony of *E. coli* SURE picked from Luria broth-tetracycline agar plate was grown overnight with vigorous shaking at 30°C in Luria broth medium supplemented with 0.2% maltose and 10 mM magnesium sulfate. 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 magnesium sulfate. Before use, the bacterial cells were diluted with 10 mM magnesium sulfate 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 instructions provided with the picoBlue Immunoscreening Kit (Stratagene). Briefly, Lambda ZAP II phages were plated on 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 nitroblue tetrazolium (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 *flagellin* DNA probes by Southern blotting to exclude these previously identified genes (33). The DNA probes were radioactively labeled with 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, replicate all the DNA downstream until the termination DNA sequence positioned 3′ of the initiator signal is encountered, and recircularize the replicated single-stranded DNA molecule, including all the sequences of the pBluescript plasmid and the cloned insert. The single-stranded recircularized plasmid is packaged, secreted from *E. coli*, and made double stranded by reinfection with fresh XL1-Blue *E. coli* cells.

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.). The pBluescript plasmid was digested with *Sma*I, generating a 5′ blunt end (susceptible to exonuclease III digestion) proximal to the target DNA, and with *Bst*XI, creating a 4-base 3′ overhanging end (resistant to exonuclease III attack) next to the primer binding site. The subclones generated by the nested deletions were sequenced by the dideoxynucleotide chain termination method (26) with the Sequenase kit (United States Biochemical Corp., Cleveland, Ohio). Analysis of DNA and protein sequences was performed by using the software program MacVector (International Biotechnology, Inc., New Haven, Conn.). Within the sequence of the insert, putative genes which encode proteins of a specified minimum size, as identified by searching for open reading frames through MacVector, were amplified by PCR. The PCR-amplified DNA sequences of these putative genes were confirmed.

Amplification and cloning of the *ospE* and *ospF* genes. The *ospE* and *ospF* genes lacking the sequences encoding the hydrophobic, N-terminal leader peptide were PCR amplified with oligonucleotide primers based on the *ospE* and *ospF* DNA sequences, such that OspE and OspF are soluble when expressed, as previously described for the purification of OspA (9). The *ospE* primers correspond to nucleotides 61 to 81 and 493 to 513. The *ospF* primers correspond to nucleotides 55 to 75 and 670 to 690. 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 *ospE* and *ospF* genes were cloned in frame with the glutathione *S*-transferase (GT) gene into the PMX vector, a pGEX-2T vector (Pharmacia, Piscataway, N.J.) with a modified polylinker (30).

Expression and purification of recombinant OspE and OspF. The GT-OspE and GT-OspF fusion proteins were expressed in *E. coli* DH5α and purified by using a glutathione-Sepharose 4B column (Pharmacia), with minor modifications of the published method (11). In brief, OspE and OspF were cleaved from the fusion proteins with thrombin, according to the manufacturer's protocol (Pharmacia). A 25-U amount of thrombin was added to the glutathione column containing the fusion proteins, and the column was allowed to incubate overnight at room temperature. The cleaved OspE and OspF were eluted with 50 mM Tris-$CaCl_2$-NaCl and treated with antithrombin beads.

Production of anti-OspE and anti-OspF sera. New Zealand rabbits (Millbrook, Amherst, Mass.) were immunized subcutaneously with 50 μg of purified OspE or OspF in complete Freund's adjuvant and boosted at 14 and 28 days with the same amount in incomplete Freund's adjuvant. C3H/HeJ

mice (Jackson Laboratory, Bar Harbor, Maine) were similarly immunized but with 10 μg of the purified antigen.

Protein expression and Western blotting (immunoblotting). XL1-Blue cells containing the pBluescript plasmid were grown to an optical density at 600 nm of 0.5 (about 3 h), and recombinant fusion proteins were induced by the addition of IPTG to a final concentration of 1 mM (about 2 h). The bacterial cells were centrifuged at 13,000 rpm for 1 min, and the pellet was resuspended in a 1/10 volume of PBS with 1% Triton X-100 and a 1/10 volume of 2× SDS sample buffer. The samples were boiled for 5 min and then electrophoresed through SDS–12% polyacrylamide gels, which were transferred overnight to NC filters. The filters were blocked for 1 h with blocking solution, incubated with the combined, absorbed rabbit anti-OspE and anti-OspF sera (diluted 1:200) for 1 h, washed three times with TBST for 5 min each, incubated with a 1:5,000 dilution of alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G antibody (Stratagene), washed three times with TBST for 5 min each, and developed with nitroblue tetrazolium and BCIP (Stratagene). DH5α cells containing the PMX-OspE and PMX-OspF plasmids were similarly grown, expressed, and probed with the combined rabbit anti-OspE and anti-OspF sera.

B. burgdorferi N40 proteins (1 μg) were resolved in SDS–12% polyacrylamide gels by electrophoresis and transferred to NC membranes, which were cut into strips for probing. Immunoblotting was performed by incubating the *B. burgdorferi* strips with murine anti-OspE or anti-OspF sera (diluted 1:5,000). The secondary antibody was alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (Stratagene).

Pulsed-field gel electrophoresis. Pulsed-field gel electrophoresis was performed with total *B. burgdorferi* N40 DNA as described previously, with minor modifications (10). In brief, DNA plugs containing approximately 10^8 *B. burgdorferi* N40 organisms 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 elsewhere (33). The full-length *ospA*, *ospD*, *ospF*, and flagellin genes were obtained from *B. burgdorferi* N40 by PCR with oligonucleotide primers derived from published DNA sequences of these genes. The *ospA* primers correspond to nucleotides 1 to 20 and 800 to 819, the *ospD* primers correspond to nucleotides 63 to 83 and 751 to 771, the *ospF* primers correspond to nucleotides 55 to 75 and 670 to 690, and the flagellin primers correspond to nucleotides 1 to 20 and 988 to 1008. The *ospA*, *ospD*, *ospF*, and flagellin DNA probes used in Southern blots were labeled with [α-^{32}P]dCTP by using the Prime-It random primer kit according to the manufacturer's protocol (Stratagene).

[^{3}H]palmitate labeling and immunoprecipitation. *B. burgdorferi* N40 organisms were 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 (5). For immunoprecipitation of OspE and OspF, an aliquot of the detergent phase was diluted 1:5 and then precleared by incubation with normal rabbit serum (GIBCO, Grand Island, N.Y.) and protein G-Sepharose (Pharmacia). [^{3}H]palmitate-labeled OspE and OspF were immunoprecipitated from the precleared detergent phase by the sequential addition of rabbit anti-OspE or anti-OspF sera and protein G-Sepharose. These samples were incubated by end-over-end rotation for 1 h at 4°C. Following incubation, the antigen–antibody–protein G-Sepharose complexes were harvested by centrifugation at 4,000 rpm 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-polyacrylamide gel electrophoresis (PAGE) on 12% acrylamide gels. 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 a vacuum, and exposed to film at −70°C.

Fluorescence microscopy. *B. burgdorferi* N40 organisms were maintained and fixed as described previously (21). To wash away the Barbour-Stoener-Kelly culture medium, spirochetes were pelleted in an Eppendorf microcentrifuge for 2 min at room temperature and resuspended in PBS (Ca^{2+} and Mg^{2+} free). Spirochetes were fixed in suspension in 4% paraformaldehyde in PBS in the presence or absence of 0.01% saponin for 20 min at room temperature. The detergent allows permeabilization of cells and was present in all incubations and washes for those samples (37). Samples were washed twice by pelleting them in a microcentrifuge for 2 min each time and resuspending them in PBS, and then they were incubated for 20 min in PBS supplemented with 10% goat serum. Primary antibody (rabbit anti-OspE or anti-OspF sera) at a dilution of 1:100 was added in the PBS–10% goat serum for 1 h at room temperature. Samples were washed twice in PBS and then incubated again for 20 min in PBS–10% goat serum at room temperature. Antibodies were visualized with a fluorescein-conjugated goat anti-rabbit secondary antibody (Tago, Inc., Burlingame, Calif.) diluted 1:200 in PBS–10% goat serum and incubated with the spirochetes for 1 h at room temperature. Stained spirochetes were mixed 1:1 with moviol (Calbiochem, San Diego, Calif.), mounted on glass slides under coverslips, and examined on a Zeiss Axioskop microscope.

Nucleotide sequence accession numbers. The nucleotide sequences of the *ospE* and *ospF* genes from *B. burgdorferi* N40 reported in this paper have been deposited in the GenBank data base under the accession numbers L13924 and L13925, respectively.

RESULTS

Cloning of *ospE* and *ospF*. The *B. burgdorferi* N40 genomic DNA expression library was screened by immunoblotting with 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 no. 11 expressed *B. burgdorferi* N40 antigens that were reactive to the antiserum. To determine whether it contains the *ospA*, *ospB*, or flagellin gene, clone 11 was screened with radiolabeled DNA probes made from these three genes. Clone 11 did not hybridize to the three probes and was therefore excised in vivo with R408 helper phage to rescue the pBluescript plasmid that contained the cloned insert.

To identify the proteins encoded by clone 11, the bacteria containing the clone 11 plasmid were evaluated for the production of recombinant proteins. Analysis of the products by SDS-PAGE and immunoblotting with the combined rabbit anti-OspE and anti-OspF sera revealed two bands in a lysate of bacteria containing the plasmid (Fig. 1B). Western blotting with rabbit anti-N40 serum yielded the same result (data not shown). The two bands were not present in the control lysate of bacteria lacking the plasmid and corresponded to two proteins with the apparent molecular masses

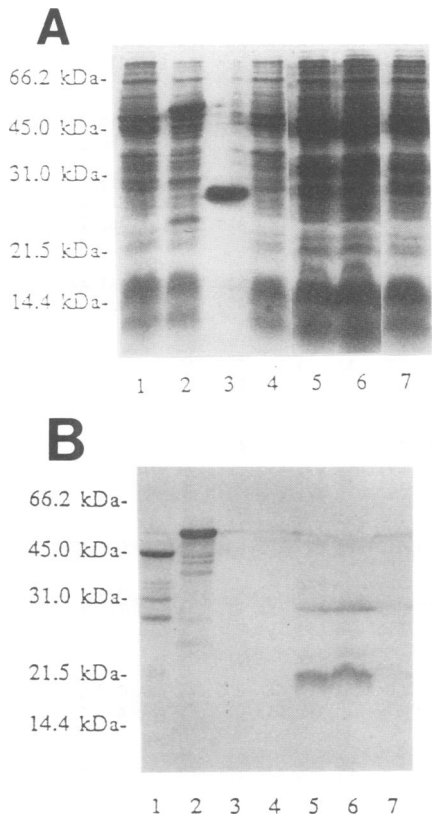

FIG. 1. Coomassie blue-stained gel (A) and the corresponding immunoblot (B) of various lysates of bacteria probed with the combined rabbit anti-OspE and anti-OspF sera. Lanes: 1, PMX-OspE plasmid in DH5α induced in 1 mM IPTG; 2, PMX-OspF plasmid in DH5α induced in 1 mM IPTG; 3, GT protein; 4, DH5α cells induced in 1 mM IPTG; 5, clone 11 plasmid in XL1-Blue induced in 1 mM IPTG; 6, clone 11 plasmid in XL1-Blue grown without IPTG; 7, uninduced XL1-Blue cells.

of 19 and 29 kDa. The two proteins were present in bacteria that were not induced with IPTG, as well as in those that were induced in 1 mM IPTG. In the Coomassie blue-stained gel of the corresponding immunoblot, a band representing the 19-kDa protein was prominent in both the induced and uninduced fractions of bacteria harboring the clone 11 plasmid, but this band was absent in the control lysate (Fig. 1A). Because of the clustering of proteins in the 30-kDa range, the band for the 29-kDa protein was not easily discernible in all lysates of bacteria.

DH5α cells containing the PMX-OspE and PMX-OspF plasmids were expressed under IPTG induction, and the recombinant GT-OspE and GT-OspF fusion products were analyzed by SDS-PAGE and Western blotting. The Coomassie blue-stained gel showed the GT protein at 26 kDa, as well as the GT-OspE and GT-OspF fusion proteins with the apparent molecular masses of 45 and 55 kDa, respectively (Fig. 1A). The combined rabbit anti-OspE and anti-OspF sera recognized the same two bands at 45 and 55 kDa, which represent the two fusion proteins (Fig. 1B). The sizes of OspE and OspF without their GT fusion partners are 19 and 29 kDa, respectively, which are consistent with the sizes of the proteins observed in the immunoblot of the clone 11 plasmid.

DNA sequence analysis of *ospE* and *ospF*. The DNA sequence of clone 11 revealed a putative bicistronic operon. We designated the antigens encoded by the two genes OspE and OspF. The nucleotide sequences of *ospE* and *ospF* and their flanking regions, together with the deduced amino acid sequences of OspE and OspF, are shown in Fig. 2. A restriction map of *ospE* and *ospF* and their flanking sequences is provided in Fig. 3. The ATG start codon of the *ospE* gene at position 1 is followed by an open reading frame of 513 nucleotides, which corresponds to a 171-amino-acid protein with a calculated molecular mass of 19.2 kDa. The *ospF* gene, located 27 bp downstream of the TAG stop codon of the *ospE* gene, consists of 690 nucleotides and encodes a protein of 230 amino acids with a calculated molecular mass of 26.1 kDa. The TAA stop codon of the *ospF* gene is followed by a putative stem-and-loop structure with dyad symmetry.

Located 10 bp upstream of the ATG start codon of the *ospE* gene is a consensus ribosome binding site with the sequence GGAG (Shine-Dalgarno sequence) (17). Further upstream of this translational initiation sequence are the −10 region (TATATT) and the −35 region (TTGTTA) that closely resemble those found in *E. coli* and other *B. burgdorferi* genes (Table 1). In the region between the *ospE* and *ospF* genes and 14 bp upstream of the ATG start codon of the *ospF* gene is another ribosome binding site with the sequence AGGAG.

The G+C contents of the *ospE* and *ospF* genes are 28.1 and 29.3%, respectively. As expected for an organism with a low G+C content, the wobble position of the preferentially used codons of *ospE* and *ospF* is often an A or U (Table 2) (18, 29). This unique feature is also found in the *ospA*, *ospB*, *ospC*, and *ospD* genes, which have a low G+C content as well (4, 15, 22).

Amino acid sequence analysis of OspE and OspF. The deduced amino acid sequences of OspE and OspF show that there is a comparatively high content of lysine (14.53% in OspE and 17.75% in OspF) and glutamic acid (9.88% in OspE and 14.72% in OspF). Other preferential codons found in OspE and OspF are leucine, isoleucine, glycine, and serine. On the basis of amino acid composition, the isoelectric points of OspE and OspF are predicted to be 8.05 and 5.33, respectively.

The hydrophilicity profile of OspE and OspF suggests that both proteins are hydrophilic proteins (Fig. 4). However, one large hydrophobic domain of about 20 amino acids is identified at the amino-terminal portion of both OspE and OspF. This N-terminal peptide corresponds to the leader signal peptide found in typical prokaryotic lipoprotein precursors (5, 38). At the carboxyl terminus of the hydrophobic core is a cleavage site presumably recognized by a *B. burgdorferi* signal peptidase. The potential cleavage site in OspE is found between alanine at position 19 and cysteine at position 20, and that in OspF is located between serine at position 17 and cysteine at position 18. Both OspE and OspF have a sequence around the cleavage site that is similar to the consensus signal sequence of bacterial lipoprotein precursors. This consensus sequence is L-X-Y-C, in which X and Y represent any small, neutral amino acid such as alanine, valine, glycine, or serine (38). The sequence around the cleavage site of OspE is L-I-G-A-C, and that around the cleavage site of OspF is L-I-V-S-C.

The amino acid sequences of OspE and OspF begin with the basic N-terminal peptide M-N-K-K-M, followed by a hydrophobic domain and the cleavage sequence recognized by a signal peptidase. The hydrophilicity plot shows that besides the large hydrophobic region in the amino terminus,

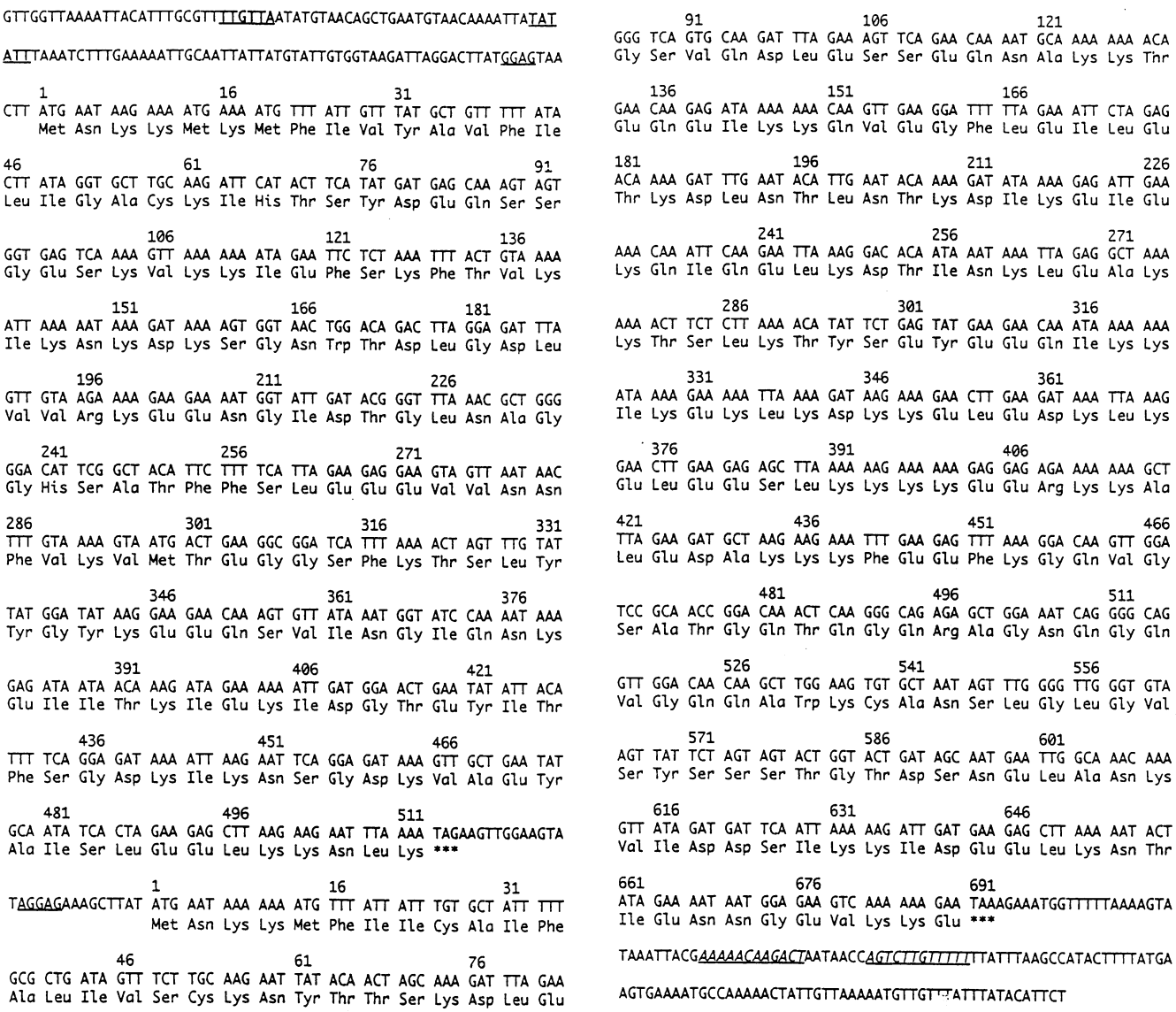

FIG. 2. Nucleotide sequence of the putative *ospE-ospF* operon and the deduced amino acid sequences of OspE and OspF. The numbers above the sequence indicate the nucleotide positions. The possible −10 and −35 promoter elements upstream of the *ospE* sequence are underlined. The ribosomal binding sites are also underlined. A putative hairpin loop structure downstream of the *ospF* sequence is underlined and shown in italics. Stop codons are indicated by asterisks.

both OspE and OspF contain long hydrophilic domains separated by short stretches of hydrophobic segments. The first 30 amino acids of OspE and OspF are 60% homologous. However, beyond the N-terminal portion, the amino acid sequences of OspE and OspF have no significant homology with each other.

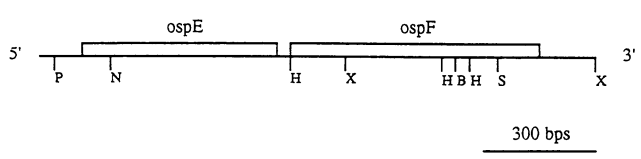

FIG. 3. Restriction map of the putative *ospE-ospF* operon and its flanking sequences. The two coding regions are boxed. The restriction sites for *Pvu*II (P), *Nde*I (N), *Hin*dIII (H), *Xba*I (X), *Bam*HI (B), and *Sca*I (S) are shown.

Mapping of *ospE* and *ospF*. By pulsed-field gel electrophoresis, the plasmid and chromosomal DNAs of *B. burgdorferi* N40 were separated (Fig. 5A). Control Southern blot analysis showed that the *flagellin* gene is located on the chromosome and that the *ospA* and *ospD* genes localize to the 49- and 38-kb plasmids, respectively, in agreement with earlier findings (2, 22). The *ospF* DNA probe bound to a plasmid that migrated faster than the 49-kb plasmid but more slowly than the 38-kb plasmid. The *ospE* probe also bound to the same plasmid (data not shown). On the basis of the locations of these two plasmids, we estimated the size of the plasmid containing the putative *ospE-ospF* operon to be approximately 45 kb. Weak cross-reactivity of the *ospF*

TABLE 1. Comparison of the control regions of transcription and translation in six known *B. burgdorferi* Osps[a]

Protein	Sequence of:		
	−35 Region	−10 Region	Ribosomal binding site
Consensus Osp	TTGACA	TATAAT	AAAGGAGGTGATC
OspA	TTGTTA	TATAAT	AAAGGAG
OspB			AAGGAG
OspC	TTGAAA	TATAAA	AAAGGAGG
OspD	TTGATA	TATAAT	AAGGAG
OspE	TTGTTA	TATATT	GGAG
OspF			AGGAG

[a] The consensus −35 and −10 sigma 70-like promoter sequences and consensus ribosomal binding sequence are from *E. coli*. The sequence of the control region of the *ospA-ospB* operon is derived from strain B31, the sequence of *ospC* is derived from strain PKo, that of *ospD* is derived from strain B31, and that of the putative *ospE-ospF* operon is derived from strain N40.

probe with the 49-kb plasmid or a comigrating plasmid was noted (Fig. 5B).

Expression of OspE and OspF in *B. burgdorferi* N40. To characterize the expression of OspE and OspF in *B. burgdorferi* N40, murine anti-OspE and anti-OspF sera were used to probe NC strips containing *B. burgdorferi* proteins. As shown in Fig. 6, the immunoblot of murine anti-OspE sera staining *B. burgdorferi* strips showed a 19-kDa band, corresponding to OspE. Murine anti-OspF sera, however, recognized a 29-kDa band, corresponding to OspF, and another band at approximately 36 kDa.

[^{3}H]palmitate labeling. To determine whether OspE and OspF are lipoproteins, *B. burgdorferi* N40 lipoproteins were radiolabeled with [^{3}H]palmitate. Radiolabeled OspE and OspF were immunoprecipitated, separated by SDS-PAGE, and visualized by fluorography. The fluorograph in Fig. 7 showed that rabbit anti-OspE sera immunoprecipitated a 19-kDa protein, corresponding to OspE. Rabbit anti-OspF sera, however, immunoprecipitated a 29-kDa protein, corresponding to OspF, as well as another 19-kDa protein.

Localization of OspE and OspF. We performed immunofluorescence studies to determine the localization of OspE and OspF on the spirochetes. Staining of individual spirochetes was bright and beaded in appearance with both OspE and OspF (Fig. 8); no staining was seen with normal rabbit serum. In addition, we added the detergent saponin to some samples to allow permeabilization of the organisms and thus the detection of the contribution of any internal OspE or OspF (37). As a control, we stained spirochetes with an antibody directed against the 41-kDa internal antigen, flagellin; staining was seen only when saponin was included (data not shown). No apparent differences in the fluorescence staining patterns of spirochetes stained for OspE or OspF with and without detergent (data shown with detergent only) were observed, suggesting that these newly identified proteins are predominantly expressed on the surface of spirochetes.

DISCUSSION

We have used a *B. burgdorferi* expression library to identify a putative bicistronic operon encoding new *B. burgdorferi* antigens. A Lambda ZAP II *B. burgdorferi* expression library, probed by immunoblotting with rabbit anti-*B. burgdorferi* N40 serum, revealed a bacterial clone (no. 11) consisting of two genes that encode two new putative *B. burgdorferi* antigens. These antigens, designated OspE and OspF, have the characteristics of surface-exposed lipoproteins.

ospE and *ospF* were constitutively expressed in *E. coli* with or without IPTG induction, implying that the expression of these genes in *E. coli* was driven not by the *lacZ* promoter but rather by their own constitutive *B. burgdorferi* promoter. This observation suggests that these *B. burgdorferi* regulatory sequences are functionally compatible with the *E. coli* transcriptional and translational apparatus. Given the above finding, it is not surprising that the upstream regulatory region of the *ospE* and *ospF* genes shows sequence homology with typical control regions of *E. coli* and other prokaryotic organisms (20). It is likely that these similarities allow the *E. coli* protein synthesis machinery to

TABLE 2. Codon usage of the *ospE* and *ospF* genes in *B. burgdorferi* N40[a]

Codon	Amino acid	Frequency	Codon	Amino acid	Frequency	Codon	Amino acid	Frequency	Codon	Amino acid	Frequency
UUU	Phe	7, 5	**UCU**	Ser	1, 4	**UAU**	Tyr	7, 4	UGU	Cys	0, 2
UUC	Phe	2, 0	UCC	Ser	0, 1	UAC	Tyr	0, 0	UGC	Cys	1, 1
UUA	Leu	5, 9	**UCA**	Ser	7, 3	UAA	***	0, 1	UGA	***	0, 0
UUG	Leu	1, 5	UCG	Ser	1, 0	UAG	***	1, 0	UGG	Trp	1, 1
CUU	Leu	2, 4	**CCU**	Pro	0, 0	**CAU**	His	2, 0	CGU	Arg	0, 0
CUC	Leu	0, 0	CCC	Pro	0, 0	CAC	His	0, 0	CGC	Arg	0, 0
CUA	Leu	1, 1	CCA	Pro	0, 0	**CAA**	Gln	3, 12	CGA	Arg	0, 0
CUG	Leu	0, 1	CCG	Pro	0, 0	CAG	Gln	0, 3	CGG	Arg	0, 0
AUU	Ile	7, 8	**ACU**	Thr	5, 6	**AAU**	Asn	8, 12	**AGU**	Ser	5, 5
AUC	Ile	1, 0	ACC	Thr	0, 1	AAC	Asn	3, 1	AGC	Ser	0, 3
AUA	Ile	8, 8	**ACA**	Thr	4, 7	**AAA**	Lys	18, 34	**AGA**	Arg	1, 2
AUG	Met	4, 2	ACG	Thr	1, 0	AAG	Lys	7, 9	AGG	Arg	0, 0
GUU	Val	7, 5	**GCU**	Ala	5, 7	**GAU**	Asp	7, 11	**GGU**	Gly	6, 2
GUC	Val	0, 1	GCC	Ala	0, 0	GAC	Asp	1, 1	GGC	Gly	1, 0
GUA	Val	5, 1	**GCA**	Ala	1, 3	**GAA**	Glu	12, 22	**GGA**	Gly	7, 7
GUG	Val	0, 1	GCG	Ala	0, 1	GAG	Glu	5, 10	GGG	Gly	1, 4

[a] Boldface letters are the preferred codons in *B. burgdorferi ospA*-B31, *ospB*-B31, *ospC*-PKo, and *ospD*-B31 genes (4, 15, 22). The two sets of numbers in the columns headed Frequency show the frequency of occurrence of each codon in the *ospE* and *ospF* genes, respectively. Stop codons are marked by asterisks. Notice that the wobble position of the preferentially used codons in these *B. burgdorferi* Osps is either an A or a U.

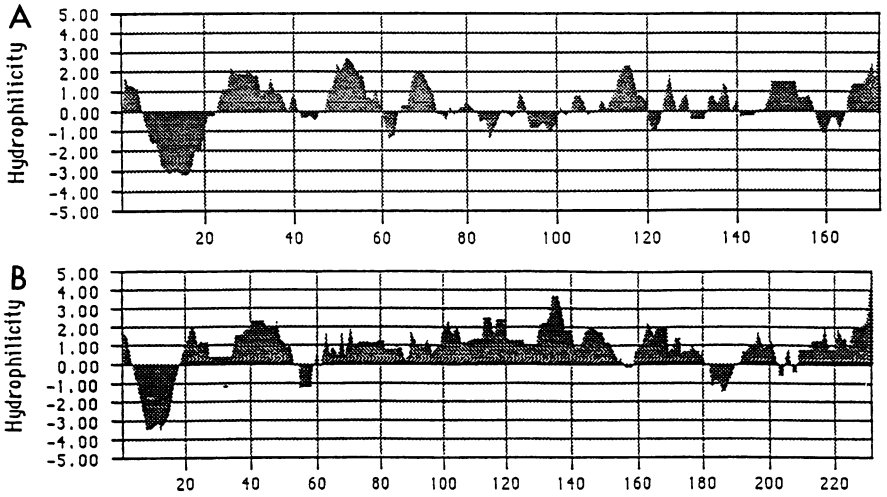

FIG. 4. Hydrophilicity profiles of OspE (A) and OspF (B). In both profiles, the hydrophilicity window size is 7 and hydrophilicity is measured by the Kyte-Doolittle scale.

recognize *B. burgdorferi* regulatory sequences. The control regions of the putative *ospA-ospB* operon (4), *ospC* gene (15), and *ospD* gene (22) are also similar to typical prokaryotic control signals, as indicated in Table 1.

Analysis of the amino acid composition of OspE and OspF shows that there is a preference towards using certain codons to represent given amino acids in *B. burgdorferi*, as shown in Table 2. There is a consistency in the codon usage pattern of OspE and OspF when compared with the codon usage pattern of OspA, OspB, OspC, and OspD. For example, glutamic acid is frequently represented by GAA, lysine is frequently represented by AAA, and tyrosine is frequently represented by UAU. The genes encoding the Osps are highly expressed, as determined by immunofluorescence. As suggested by earlier studies of OspA and OspB, this observation supports the hypothesis that the preferred codon usage of the highly expressed Osps may be representative of the overall codon usage in *B. burgdorferi* (4, 15, 22).

The consensus sequence of typical bacterial lipoprotein precursors recognized and cleaved by signal peptidase II is L-X-Y-C (38). The leucine and cysteine are usually separated by two small, neutral amino acids. Recently, it has been recognized that variations in the consensus tetrapeptide described above, such as L-X-Y-Z-C, are observed (38). There are two amino acids between the leucine and cysteine in the cleavage sequence of OspA of strain B31 (OspA-B31) (L-I-A-C) and OspB-B31 (L-I-G-C) (4). There are three amino acids in the cleavage sites of OspE-N40 (L-I-G-A-C) and OspF-N40 (L-I-V-S-C). The leucine and cysteine residues in OspC-PKo (L-F-I-S-C) (15) and OspD-B31 (L-S-I-S-C) (22) are separated by three amino acids as well. Despite

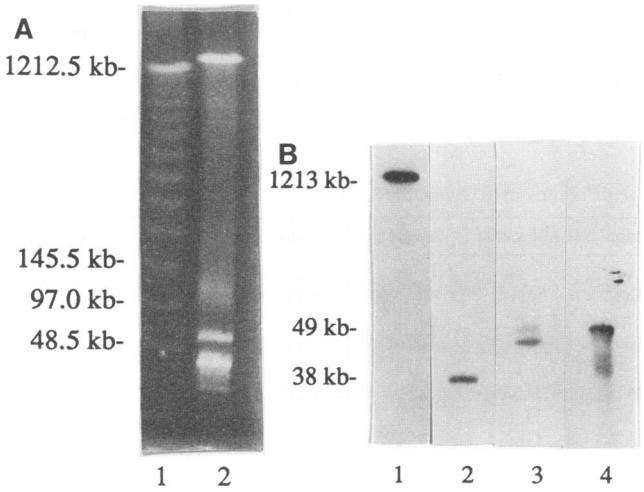

FIG. 5. (A) Pulsed-field gel electrophoresis separating the chromosomal and plasmid DNAs of *B. burgdorferi* N40. The gel was visualized by staining with ethidium bromide. Lane 1 contains the molecular size standard consisting of phage λ concatemers, and lane 2 consists of *B. burgdorferi* N40 DNA, with the chromosomal band slightly larger than the 1,212.5-kb marker, which is larger than that previously described (approximately 1,000 kb). Plasmids can be seen clearly at molecular sizes of 49 kb and smaller. (B) The putative *ospE-ospF* operon is located on a 45-kb plasmid of *B. burgdorferi* N40. The Southern blot of the pulsed-field gel described in the legend to panel A was hybridized separately with flagellin (lane 1), *ospD* (lane 2), *ospF* (lane 3), and *ospA* (lane 4) DNA probes.

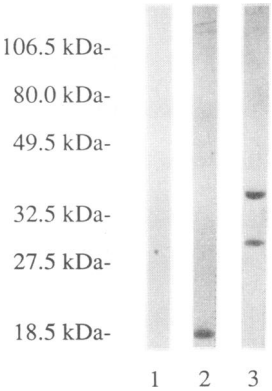

FIG. 6. Immunoblot of *B. burgdorferi* proteins probed with murine anti-GT (control), anti-OspE, and anti-OspF sera. Lanes: 1, control serum (diluted 1:500); 2, anti-OspE serum (diluted 1:5,000); 3, anti-OspF serum (diluted 1:5,000).

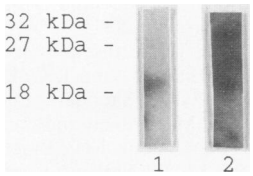

FIG. 7. Fluorograph of the [^{3}H]palmitate-labeled OspE (lane 1) and OspF (lane 2), which were immunoprecipitated with rabbit anti-OspE and anti-OspF sera, respectively, and separated by SDS-PAGE. The presence of two bands in lane 2 is explained in the Discussion.

this variation in the cleavage sequence, OspA and OspB (5), as well as OspD (22), have been shown by the established [^{3}H]palmitate labeling procedure to be lipoproteins. Furthermore, we report in this paper that OspE and OspF are also lipoproteins. The lipoprotein identity of OspC remains to be elucidated, but the features of its leader signal peptide suggest that it may be processed as a lipoprotein as well, by the addition of a lipid moiety at the cysteine residue (38).

The Southern blot of the pulsed-field gel showed that the *ospF* DNA probe bound to a 45-kb plasmid. In addition, faint binding to the *B. burgdorferi* 49-kb plasmid could be detected, suggesting some cross-hybridization with the *ospF* probe. Alternatively, this result suggests that the *ospF* gene may be present in duplicate copies in the *B. burgdorferi* N40 genome, but this is not very likely in light of the fact that the intensities of the two bands are significantly different.

The immunoblot of *B. burgdorferi* strips probed with murine anti-OspE and anti-OspF sera showed that both OspE and OspF are expressed in *B. burgdorferi* N40. Murine antibodies to OspF, however, also bound to a 36-kDa protein. Because of the unexpected cross-reactivity with the 36-kDa protein, we hypothesize that OspF may share epitopes with another *B. burgdorferi* protein of 36 kDa or that the 36-kDa protein may be a variant of OspF. It should be noted that the 36-kDa protein is not OspB, for monoclonal antibodies to OspB recognized a band at 34 kDa which clearly migrated below the 36-kDa band (data not shown).

The [^{3}H]palmitate labeling of lipoproteins and immunoprecipitation of radiolabeled OspE and OspF showed that both proteins are indeed lipoproteins. The immunoprecipitation of radiolabeled lipoproteins with rabbit anti-OspE sera yielded a protein of 19 kDa, which is the expected size of OspE. Rabbit anti-OspF sera immunoprecipitated the 29-kDa OspF as well as a 19-kDa protein. We hypothesize that the 19-kDa protein may be a truncated form of OspF which lacks the carboxyl terminus, such that the [^{3}H]palmitate moiety still remains in the amino terminus and is responsible for the 19-kDa band seen in the fluorograph. Alternatively, the 19-kDa protein may be another *B. burgdorferi* lipoprotein which shares epitopes with OspF. Why the 19-kDa protein was not recognized in the immunoblot of *B. burgdorferi* proteins probed with murine anti-OspF sera may be explained by the fact that the denaturing conditions of SDS-PAGE obliterated conformational epitopes normally recognized by murine antibodies to OspF. The failure to appear in the fluorograph of a 36-kDa band, which was observed in the immunoblot, may be because the 36-kDa protein is not a lipoprotein and hence could not be labeled with [^{3}H]palmitate and visualized by fluorography, even though the protein may have been immunoprecipitated and may be present in the SDS-PAGE gel.

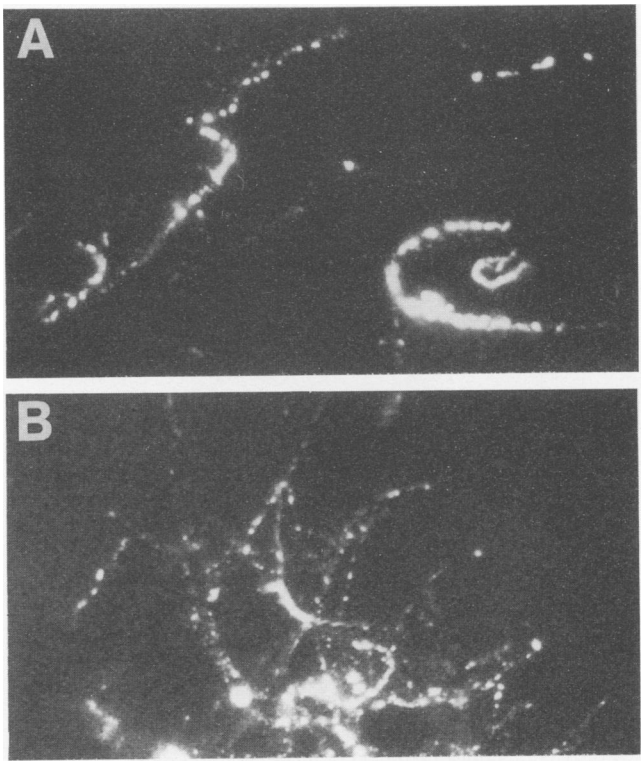

FIG. 8. Immunofluorescence of *B. burgdorferi*. Spirochetes were fixed with paraformaldehyde and stained in suspension with rabbit antisera, diluted 1:100, directed against OspE (A) and OspF (B), as described in Materials and Methods. Samples were photographed under oil immersion. Magnification, ×91.

Antibodies to OspE and OspF in sera obtained from rabbits immunized with OspE or OspF stain the surface of the spirochetes in a beaded pattern. No apparent differences between patterns were detected when staining was done in the presence and in the absence of detergent, suggesting that the expression of these proteins is predominantly on the surface. OspE and OspF can now be appropriately called outer surface lipoproteins.

The function of OspE and OspF and the development and importance of the host immune response to these antigens remain to be elucidated. It is likely that these putative *B. burgdorferi* lipoproteins will have important roles in our understanding of the immune response to *B. burgdorferi* and the pathogenesis of Lyme disease. Among the known *B. burgdorferi* lipoproteins, OspA, OspB, and OspC have all been shown to be important in protective immunity to *B. burgdorferi* infection (11, 13, 23). Indeed, *B. burgdorferi* mutants, with altered expression of these Osps, may evade immune-response destruction in vivo as well as in vitro (14, 24). Studies with OspD have suggested that it may be a virulence factor for spirochetal infection (22). The nature of the immune response to OspE and OspF in humans infected with Lyme disease and in animal models of Lyme disease can now be studied. The presence of these genes and their expression and variability in other *B. burgdorferi* isolates can also be assessed. The roles of OspE and OspF in Lyme disease diagnostics, protective immunity, and pathogenesis are areas for further research.

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