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Molecular Characterization of a 6.6-Kilodalton *Borrelia burgdorferi* Outer Membrane-Associated Lipoprotein (lp6.6) Which Appears To Be Downregulated during Mammalian Infection

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Isolated outer membranes of *Borrelia burgdorferi* 297 were utilized to obtain partial amino acid sequence information for a low-molecular-weight, outer membrane-associated polypeptide. Degenerate oligonucleotide primers based upon this information were used to amplify a 100-bp probe for detection of the corresponding full-length gene within a *B. burgdorferi* total genomic library. The relevant open reading frame (ORF) encoded a polypeptide comprised of a 17-amino-acid putative signal peptide terminated by LFVAC, a probable consensus sequence for lipoprotein modification, and a mature protein of 51 amino acids (predicted molecular mass of 5.8 kDa). The DNA sequences of the corresponding ORFs in *B. burgdorferi* 297 and B31 were identical; the corresponding ORF in strain N40 differed by only one nucleotide. Assuming conventional processing and acylation, the molecular weight of the lipoprotein, designated lp6.6, is about 6,600. The *lp6.6* gene, which was localized to the 49-kb linear plasmid of *B. burgdorferi*, subsequently was cloned and expressed in *Escherichia coli* as a fusion protein with glutathione *S*-transferase. Immunoblot analysis with monoclonal antibody 240.7 revealed that lp6.6 was identical to a low-molecular-weight, highly conserved *B. burgdorferi* lipoprotein reported previously (L. I. Katona, G. Beck, and G. S. Habicht, Infect. Immun. 60:4995–5003, 1992). Results of indirect immunofluorescence assays, growth inhibition assays, passive immunizations, and active immunizations indicated that this outer membrane-associated antigen is not surface exposed in *B. burgdorferi*. Particularly interesting was the finding that mice and rhesus monkeys chronically infected with *B. burgdorferi* failed to develop antibodies against this antigen. We propose that high-level expression of *lp6.6* is associated with the arthropod phase of the spirochetal life cycle and that expression of the gene is downregulated during mammalian infection.

Lyme disease is the most common arthropod-borne infection in the United States (20). The spirochetal etiologic agent of Lyme disease, *Borrelia burgdorferi*, is maintained in zoonotic cycles involving a diversity of wild mammals and ticks, primarily of the genus *Ixodes* (19, 38). The existence of this complex life cycle implies that the Lyme disease spirochete must adapt to diverse environmental conditions. Inasmuch as the bacterial cell envelope functions as the primary interface between the spirochete and its invertebrate and vertebrate hosts, cell envelope constituents should be highly relevant to these adaptive processes. In this regard, there is now a substantial body of evidence correlating differential expression of genes encoding cell envelope proteins with the arthropod and mammalian phases of the *B. burgdorferi* life cycle (3, 21, 41, 54, 57, 61). Thus far, most differentially expressed genes appear to be selectively expressed during mammalian infection; OspA and OspB are the only known *B. burgdorferi* gene products associated with the arthropod phase of the spirochetal life cycle.

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Technological advancements for the isolation of *B. burgdorferi* outer membranes have provided new strategies for identifying constituents potentially involved in borrelial adaptive responses (16, 47). Specifically, we have employed the procedure of Radolf et al. (47) to begin to survey the contents of outer membranes isolated from a number of *B. burgdorferi* strains. These efforts have led to the identification and characterization of a highly conserved low-molecular-mass (6.6-kDa) lipoprotein in outer membranes of *B. burgdorferi* 297, N40, and B31. Interestingly, this abundant and highly immunogenic antigen, whose gene localizes to the 49-kb linear plasmid (harboring the *ospAB* operon), does not generate an antibody response in *B. burgdorferi*-infected mice or rhesus monkeys. These findings have prompted us to hypothesize that high-level expression of *lp6.6* is associated with the arthropod phase of the spirochetal life cycle and that expression of the gene is downregulated during mammalian infection.

MATERIALS AND METHODS

Bacterial strains and plasmids. Low-passage uncloned *B. burgdorferi* 297 and N40 were obtained from Russell Johnson (Minneapolis, Minn.) and Stephen Barthold (New Haven, Conn.), respectively. Low-passage uncloned *B. burgdorferi* B31 and high-passage B313 (51) were provided by Alan Barbour (San Antonio, Tex.). All low-passage isolates were passaged in BSK II medium (7) not more than four times before experimental manipulations. The virulence of all isolates

TABLE 1. Primers used for PCR amplification

Gene	Primer derived from strain 297 DNA sequence information[a]	Purpose
lp6.6	GAYGARATGGARAAYAC[b]	5′ degenerate primer (DEMENT)
	WGTCATNGGTTGYTTCATHS[b]	3′ degenerate primer (complementary to SMKQPMT)
	CG**GGATCC**TGCGAAACTACAAGAATTTCAG[c]	5′ primer for GST fusion and PCR probe (nucleotides 52–73)
	CG**GAATTC**TTTTTCTAAATCAAAATAATAAATAGTTATTAG[d]	3′ primer for GST fusion and PCR probe (nucleotides 274–242)
	GC**TCTAGA**AAGAAGGAGAATAAACAATGACAAAATTAATGTACGC[e]	5′ primer for PCR from strains N40 and B31 (nucleotides −18–20)
	CGACTACCAATATTCTTGCTCCAATTCAG	3′ primer for PCR from strains N40 and B31 (nucleotides 389–361)
ospA	TT**GGAT**CCTGTAAGCAAAATGTTAGCAGC[c]	5′ primer for GST fusion and PCR probe
	TCTCCTTATTTTAAAGCGTT	3′ primer for GST fusion and PCR probe
ospC	CGC**GGATCC**AATTCAGGGAAAGGTGGG[c]	5′ primer for PCR probe
	GC**CTGCAG**GATCTTATTAAGGTTTTTTTGGACT[f]	3′ primer for PCR probe

[a] The direction of each sequence is from 5′ to 3′.
[b] Mixed sites: Y, C/T; R, A/G; W, A/T; N, A/G/C/T; H, A/T/C; S, G/C.
[c] The *Bam*HI site is in boldface.
[d] The *Eco*RI site is in boldface.
[e] The *Xba*I site is in boldface.
[f] The *Pst*I site is in boldface.

was confirmed by induction of carditis and arthritis following intradermal needle inoculation of 3-week-old C3H/HeJ mice (Jackson Laboratory, Bar Harbor, Maine) with 10^4 bacteria and/or by recovery from ear punch biopsies (56). *Escherichia coli* XL1-Blue (Stratagene, La Jolla, Calif.) and INV-αF′ (Invitrogen, San Diego, Calif.) were used as cloning hosts and were cultivated either in yeast-tryptone broth or on yeast-tryptone agar supplemented with 100 μg of ampicillin per ml. The cloning vector was either pGEX-4T-2 (Pharmacia LKB Biotechnology, Piscataway, N.J.) or pCRII (Invitrogen).

Fractionation of *B. burgdorferi* outer membranes. Outer membranes of *B. burgdorferi* 297 were isolated as previously described (47).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. Samples for protein analysis were boiled for 10 min in final sample buffer (62.5 mM Tris-HCl [pH 6.8], 10% [vol/vol] glycerol, 5% [vol/vol] 2-mercaptoethanol, and 0.001% [vol/vol] bromophenol blue) prior to electrophoresis through 2.4% stacking and 12.5 or 15% polyacrylamide resolving gels. Gels were then stained with either Coomassie brilliant blue or silver nitrate. Alternatively, proteins were transferred electrophoretically to a 0.45-μm-pore-size nitrocellulose filter (Schleicher & Schuell, Keene, N.H.) for immunoblotting. Immunoblots were incubated with either 1:100 dilutions of sera from *B. burgdorferi*-infected mice or rhesus monkeys, 1:10,000 dilutions of rat polyclonal antisera, or a 1:500 dilution of purified murine monoclonal antibody 240.7 (1.0 mg/ml) directed against a low-molecular-weight lipoprotein of *B. burgdorferi* (34). This was followed by sequential incubations with 1:1,000 dilutions of either goat anti-mouse, goat anti-human, or goat anti-rat immunoglobulin G (heavy- and light-chain-specific)–horseradish peroxidase conjugates and rabbit anti-goat immunoglobulin G-horseradish peroxidase conjugates (Jackson ImmunoResearch, West Grove, Pa.). Immunoblots were developed with 4-chloro-1-naphthol as the substrate.

Amino acid sequencing of individual borrelial outer membrane proteins. Amino acid sequencing of borrelial polypeptides was performed in the Protein Chemistry Core Facility (University of Texas Southwestern Medical Center). Briefly, *B. burgdorferi* outer membrane-associated polypeptides were separated by SDS-PAGE, transferred to nitrocellulose or polyvinylidene difluoride membranes, and subjected to amino acid microsequencing by standard methods (1, 2, 5, 40, 63); automated Edman degradation was employed in attempts to derive N-terminal amino acid sequences. Internal amino acid sequences were obtained by trypsin digestion and separation of the peptides by high-performance liquid chromatography.

Construction of a lambda ZAP II *B. burgdorferi* 297 genomic DNA library. A genomic DNA library was constructed by Stratagene using randomly sheared total genomic DNA from uncloned, low-passage (virulent) *B. burgdorferi* 297 and lambda ZAP II as the cloning vector. Briefly, genomic DNA was isolated from the spirochetes by using a DNA extraction kit (Stratagene) according to procedures recommended by the manufacturer. Borrelial DNA was then randomly sheared by passage multiple times through a 5/8-in.-long, 25-gauge needle. The DNA fragments were made blunt ended with *Pyrococcus furiosus* (*Pfu*) DNA polymerase, and then dephosphorylated *Eco*RI adapters were ligated onto the blunt-ended fragments. Fragments ranging in size from 5 to 10 kb were subsequently isolated by sucrose density gradient centrifugation and ligated onto *Eco*RI arms of the lambda ZAP II cloning vector.

Southern hybridization analysis. Hybridization probes for genes encoding the 6.6-kDa protein, OspA, and OspC were generated by PCR amplification with the primer pairs listed in Table 1. The resulting products were gel purified by using the QIAEX extraction kit (Qiagen, Chatsworth, Calif.) and labeled with [α-^{32}P]dCTP by using the Boehringer-Mannheim (Indianapolis, Ind.) random-primed DNA labeling kit.

B. burgdorferi total genomic DNA was isolated with a DNA extraction kit (Stratagene). Borrelial DNAs were digested to completion with *Sau*3A; 1.5-μg quantities of each sample were then loaded into wells of 1% agarose gels. After electrophoresis, gels were partially depurinated by being soaked in 0.25 M HCl (with rocking) for 30 min at room temperature. Gels were subsequently denatured for 30 min in 1.5 M NaCl–0.5 M NaOH (with constant agitation), rinsed in water, and neutralized for 1 h with 1 M Tris (pH 7.4)–1.5 M NaCl. DNA in the gels was then transferred to nylon membranes (Micron Separations, Westboro, Mass.). The membranes were hybridized overnight in 5× Denhardt's solution–5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1.0% SDS–1 mM EDTA–100 μg of sonicated salmon sperm DNA per ml at 65°C by using a rotating hybridization oven (Robbins Scientific Corp., Sunnyvale, Calif.). After hybridization, membranes were washed twice for 15 min each at 65°C in 2× SSC containing 0.1% SDS, followed by four washes at room temperature in 0.1× SSC containing 0.1% SDS. The membranes then were subjected to autoradiography at −70°C for 4 to 48 h.

Northern blot analysis. Northern blotting with *B. burgdorferi* 297 RNA was carried out as described by Porcella et al. (44). The hybridization probe, corresponding to the gene for the 6.6-kDa protein (*lp6.6*), was generated by PCR (see "Fusion proteins" below) with the glutathione *S*-transferase (GST) fusion primer pair given in Table 1.

Pulsed-field gel electrophoresis analysis. Agarose plugs containing spirochetes grown to a density of 10^8 organisms per ml were prepared essentially as described by Ferdows and Barbour (29). Solidified plugs were recovered from the molds, transferred to petri dishes, and then incubated for 18 to 24 h at 37°C in lysis buffer (50 mM Tris-HCl, 50 mM EDTA [pH 8.0]) containing 1 mg of proteinase K per ml and 1% (wt/vol) sodium lauroyl sarcosinate. Lysis buffer was decanted from the plugs and replaced with a solution of 10 mM Tris (pH 8.0)–1 mM EDTA (TE buffer). Plugs were washed three times in TE buffer at 37°C and then were stored at 4°C in TE buffer.

For pulsed-field gel electrophoresis, plugs containing approximately 5×10^6 spirochetes were loaded into wells of 1.2% agarose gels immersed in 0.5× TBE buffer (0.045 M Tris-borate, 1 mM EDTA, pH 8.0). Molten 1.2% agarose was overlaid to seal the plugs into the wells. Constant homogeneous electric field electrophoresis was carried out in a DRIII apparatus (Bio-Rad, Palo Alto, Calif.) at 9°C in 0.5× TBE buffer with continuous buffer recirculation. Gels were subjected to electrophoresis (6 V/cm) with 5-s pulses for 2 h followed by 0.5 to 1.5 s of ramping for an additional 18 h. For two-dimensional gel analyses (51), gels were rotated 90° and subjected to constant-field electrophoresis (6 V/cm) for an additional 2 h. Gels were then stained for 30 min in 1 μg of ethidium bromide per ml and irradiated with 60 mJ of UV light in a Stratalinker 1800 apparatus (Stratagene) to enhance capillary transfer of large DNA fragments. Separated DNAs were then transferred to 0.45-μm-pore-size nylon membranes and were UV cross-linked for subsequent Southern hybridizations. Labeled probes were

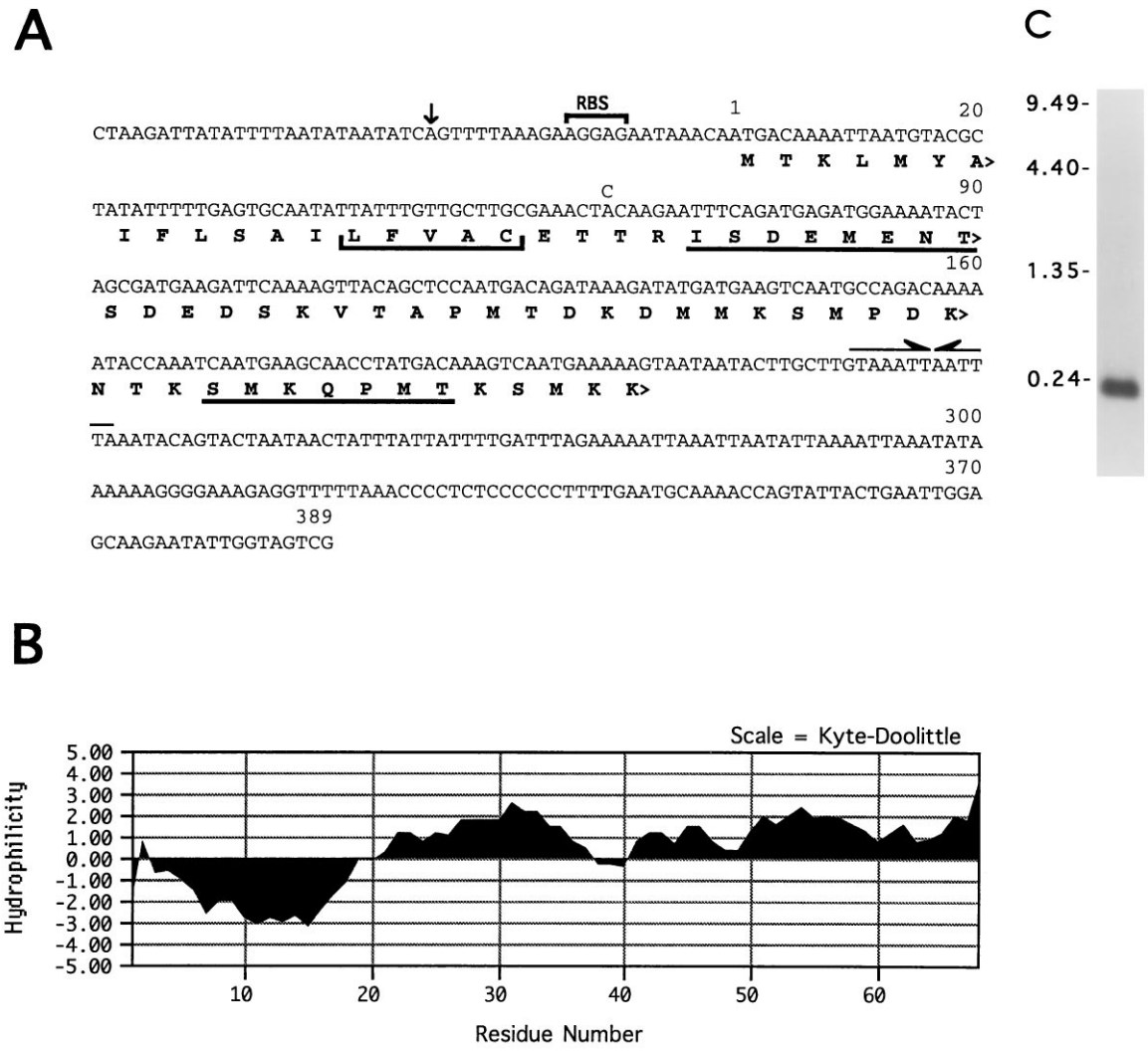

FIG. 1. Molecular characterization of lp6.6 from *B. burgdorferi*. (A) DNA and deduced amino acid sequences for lp6.6 of strains 297 and B31. For *B. burgdorferi* N40, the DNA sequence of the *lp6.6* gene differed by only one nucleotide (A→C at position 61). A putative ribosomal binding site (RBS) is denoted by the upper bracket. A consensus sequence for lipid modification and processing (LFVAC) is designated by the bracket below the sequence. The two underlined polypeptide sequences, obtained by amino acid sequencing of trypsin fragments from native lp6.6, were the bases for designing degenerate oligonucleotide primers for PCR amplification of a probe. A putative stem-loop structure is shown as inverted half-arrows. (B) Hydrophilicity profile generated according to the method of Kyte and Doolittle (36). (C) Northern blot analysis with strain 297 RNA. The hybridization probe used to detect an *lp6.6* transcript was generated by PCR with the GST fusion primer pair given in Table 1. Molecular size markers (in kilobases) are shown at the left.

hybridized to membranes as described above for 16 h in a rotating oven (Robbins Scientific Corp.). Washed membranes were covered in plastic wrap and exposed to X-ray film at −70°C.

DNA sequencing and computer analyses. Nucleotide sequencing was performed with an Applied Biosystems Inc. model 373A automated DNA sequencer and PRISM ready reaction DyeDeoxy terminator cycle sequencing kits according to the manufacturer's instructions (Applied Biosystems Inc., Foster City, Calif.). Nucleotide and deduced amino acid sequences were analyzed and manipulated by using the University of Wisconsin Genetics Computer Group version 7.3 (GenBank database release 82.0) (26), Lasergene (DNASTAR, Madison, Wis.), and MacVector version 4.1.1 (International Biotechnologies Inc.-Kodak, New Haven, Conn.) software packages.

Fusion proteins. GST fusion proteins with the 6.6-kDa protein (Fig. 1A) and OspA (GenBank accession number X85442) were generated by PCR amplification of the DNAs encoding the predicted mature portions of the proteins; the respective forward and reverse oligonucleotide primers are shown in Table 1. Conditions for PCR were 35 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. Amplification products were purified with a QIAEX gel extraction kit (Qiagen). The fragments were then ligated into the appropriate polylinker sites of pGEX-4T-2 and used for transformation of XL1-Blue host cells. The DNAs of all fusion constructs were sequenced to confirm that cloning junctions were as intended. Generation of fusion proteins corresponding to OspB, OspF, and BbK2.10 has been described previously (3, 46). Expression of recombinant fusion proteins was induced by the addition of IPTG (isopropyl-β-D-thiogalactopyranoside). The resultant fusion proteins were purified by affinity chromatography on an agarose-glutathione matrix according to the manufacturer's instructions (Pharmacia). In some experiments, the fusion protein bound to the glutathione matrix was cleaved with thrombin.

Antisera. To generate polyclonal antisera against fusion proteins, 4- to 6-week-old Sprague-Dawley rats were primed by intraperitoneal injection with 50 μg of purified fusion protein in a 1:1 mixture of phosphate-buffered saline (PBS) (pH 7.4) and Freund's complete adjuvant. This was followed at 2-week intervals by intraperitoneal booster injections with 25 μg of purified protein in a 1:1 mixture of PBS and incomplete Freund's adjuvant. To produce hyperimmune polyclonal antiserum directed against *B. burgdorferi* 297 total antigens, rats were primed by intraperitoneal injection with 10^9 heat-killed, sonicated spirochetes in a 1:1 mixture of PBS and Freund's complete adjuvant followed by intraperitoneal boosters at 2-week intervals with 10^8 spirochetes in a 1:1 mixture of PBS and incomplete Freund's adjuvant.

Sera from rhesus monkeys which were tick inoculated with the JD1 strain of *B. burgdorferi* (48) were generously provided by Mario T. Philipp. Serum samples used in this study were obtained from one chronically infected animal (designated K-383) (48) at 17 and 20 weeks postinfection. These sera lacked detectable

antibodies to OspA and OspB on immunoblots (see Fig. 6B) and thus paralleled the human antibody response to *B. burgdorferi* (6, 42, 48).

Triton X-114 phase partitioning. Triton X-114 extraction of the recombinant 6.6-kDa fusion protein as well as proteins in isolated outer membranes was carried out as described previously (18). Protein concentrations were determined by the bicinchoninic acid method (Pierce Chemical Co., Rockford, Ill.).

Murine model of Lyme borreliosis. The well-characterized murine model of Lyme borreliosis (11–14, 30) was used to assess the ontogeny of the antibody response during chronic infection as well as for passive and active immunization experiments. Briefly, six groups of five 3-week-old C3H/HeJ mice were needle inoculated intradermally with 10^4 *B. burgdorferi* 297 organisms in 50 μl of BSK II medium. At intervals of 1, 2, 4, 8, and 16 weeks and 6 months postinoculation, mice were sacrificed by CO_2 narcosis. Specimens of blood, ear pinna, heart, and urinary bladder from each sacrificed animal were cultured in BSK II medium supplemented with rifampin (50 μg/ml) and amphotericin B (25 μg/ml). Disseminated *B. burgdorferi* infection of mice was confirmed by recovery of spirochetes from any of the cultured sites. Sera from each culture-positive mouse at each time point were pooled and stored at −20°C until use.

In passive-immunization experiments (11), groups of four 3-week-old mice first were injected subcutaneously with 50 μl of rat antiserum against either recombinant 6.6-kDa–GST fusion protein, OspA-GST (positive control), or GST alone (negative control). Eighteen hours later, mice were intradermally needle inoculated with 10^3 low-passage *B. burgdorferi* 297 organisms. Two weeks postchallenge, mice were sacrificed and specimens of blood, ear pinna, heart, and urinary bladder were cultured in BSK II medium. Passive protection was indicated by the inability to recover spirochetes from any of these culture sites.

For active-immunization experiments (13, 30), similar groups of mice were immunized subcutaneously with 20 μg of either recombinant 6.6-kDa fusion protein, OspA, or GST suspended in PBS-complete Freund's adjuvant. Priming was followed at 2-week intervals by two booster injections of 10 μg of the respective protein in PBS-incomplete Freund's adjuvant. Two weeks after the final booster, mice were bled to assess antibody titers and then were challenged with *B. burgdorferi* 297 (as described above). Two weeks postchallenge, the level of immunoprotection was assessed by culturing tissue specimens (as described above).

***B. burgdorferi* growth inhibition assays.** Borreliacidal activities of antisera were determined by modifications of previously described procedures (39, 50). Rat antisera directed against recombinant 6.6-kDa fusion protein, OspA, or GST were heat-inactivated (56°C; 45 min), filter sterilized through a 0.22-μm-pore-size filter (Costar, Cambridge, Mass.), and serially diluted twofold in 100 μl of BSK II medium; the first dilution in the series was 1:8. Dilutions and assays were carried out in flat-bottomed, 96-well microtiter plates (Falcon/Becton-Dickinson, Lincoln Park, N.J.). Low-passage strain 297 organisms were grown until the culture reached late exponential phase. Cultures were diluted to 4×10^7 cells/ml by adding fresh BSK II medium. Aliquots of 100 μl, containing 4×10^6 borreliae, were mixed with diluted serum samples in individual wells. Guinea pig complement (Pel-Freez, Rogers, Ark.) was added to each well at a final concentration of 10 hemolytic units per ml of medium. The plates were sealed with laboratory film and incubated for 3 days at 34°C in a 1% CO_2 atmosphere (with additional humidity). Borrelial growth was indicated qualitatively by the color change of the medium from red to yellow. A lack of borrelial growth was noted by observing nonmotile, clumped, fragmented organisms in the wells by dark-field microscopy. Quantitative assessment of borrelial growth was performed with the aid of a Thermomax enzyme-linked immunosorbent assay reader (Molecular Devices, Sunnyvale, Calif.) by using dual-wavelength readings at 562 and 620 nm (39); an adjusted absorbance value ($A_{562} - A_{620}$) of ≥0.30 generally was reflective of borrelial growth in the assay system.

Indirect immunofluorescence assay of either prefixed (disrupted) or intact spirochetes. Antisera were either reacted with methanol-fixed borreliae on glass slides or added directly to 1-ml portions of mid-logarithmic-phase cultures of *B. burgdorferi*; spirochetes were processed for indirect immunofluorescence assay as previously described (22). Antiserum against *B. burgdorferi* endoflagella was described previously (22).

Indirect immunofluorescence of *B. burgdorferi* encapsulated in agarose microdroplets. Spirochetes in mid-logarithmic phase were encapsulated in gel microdroplets consisting of 2% low-melting-temperature agarose as described previously (23). Microdroplets containing spirochetes were then mixed with antiserum, washed, and processed for indirect immunofluorescence assay as described by Cox et al. (22, 23).

Nucleotide sequence accession numbers. The nucleotide sequences reported in this paper were submitted to GenBank and assigned accession numbers U59857 (strain 297), U59858 (strain N40), and U59859 (strain B31).

RESULTS

Identification of a low-molecular-weight protein in *B. burgdorferi* outer membranes and isolation of the respective gene. A *B. burgdorferi* 297 outer membrane-associated polypeptide of approximately 10 kDa (47) (see Fig. 4A) was recovered for amino acid sequence analysis. This molecule was blocked to Edman degradation; however, upon digestion with trypsin and separation of the resultant peptides, two peptide fragments yielded nonoverlapping sequences of ISDEMENT and SMK- QPMT (Fig. 1).

Four degenerate oligonucleotide primers were synthesized based upon the two peptide sequences, the two possible orientations of the respective DNA encoding regions, and the preferred codon usage for *B. burgdorferi* (37). Only one degenerate primer pair (Table 1) yielded a DNA amplification product (ca. 100 bp), indicating that the ISDEMENT peptide was N terminal to the SMKQPMT peptide. Screening of a *B. burgdorferi* 297 lambda ZAP II genomic library by Southern hybridization with the same radiolabeled 100-bp probe yielded a number of hybridizing clones. Hybridizing clones were isolated and processed for in vivo excision of the pBluescript phagemid from the lambda ZAP II vector; rescued plasmids were purified and used as templates for automated DNA sequence analysis (Fig. 1).

DNA and deduced amino acid sequences. The complete strain 297 nucleotide sequence of an open reading frame (ORF) encompassing the 100-bp probe is shown in Fig. 1A. Of note, six independently isolated recombinant clones originating from strain 297 DNA all had identical DNA sequences for a 204-bp ORF encoding a polypeptide of 68 amino acids. A number of putative promoter elements (−10 and −35 regions) for the ORF could be assigned, but the absence of supporting data precluded precise determinations. However, 13 nucleotides upstream of the ORF was a putative ribosomal binding site (AGGAG) (55). Furthermore, Northern blotting revealed that a PCR-generated probe corresponding to the ORF hybridized with a 230-nucleotide RNA transcript of *B. burgdorferi* 297 (Fig. 1C), indicating that the ORF was expressed by *B. burgdorferi* (replicating in vitro) but not as a component of an operon.

The N terminus of the molecule contained a 17-amino-acid hydrophobic domain (Fig. 1B) with a positive charge near the initial methionine residue, properties consistent with those of a signal peptide (60). At the distal portion of the presumptive signal peptide was the sequence LFVAC, suggestive of a consensus sequence for lipid modification (31). With the presumption that the gene translation product is processed, the calculated molecular weight of the mature, nonacylated 51-amino-acid molecule would be 5,848, with a theoretical pI of 6.5. The mature portion of the molecule was notably hydrophilic (36) (Fig. 1B). Assuming that the molecule is modified via the configuration typical of other bacterial lipoproteins (31) and that the three acyl chains most likely are palmitates (15), the actual molecular mass of the mature, lipid-modified molecule would be approximately 6.6 kDa. This molecular mass, therefore, was incorporated into the descriptive term (lp6.6) for the molecule. Fourteen nucleotides downstream of the UAA termination codon was a potential stem-loop structure (Fig. 1) that also may be involved in transcriptional termination (43).

PCR primers, based upon the strain 297 sequence (Fig. 1 and Table 1) and encompassing the predicted mature polypeptide, were used to amplify the corresponding genes from strains N40 and B31. In each case, the predicted approximately 400-bp PCR amplification product was obtained, isolated, and cloned into pCRII for subsequent DNA sequence analysis. The nucleotide sequence from strain B31 was identical to that from strain 297, while that from strain N40 differed by only one nucleotide at position 61 (relative to the ATG initiation codon), where A was replaced by C; this single base substitution would alter the translated amino acid from threonine to proline. BLASTp searches (4) via the National Center for Biotechnology Information did not reveal any homologies be-

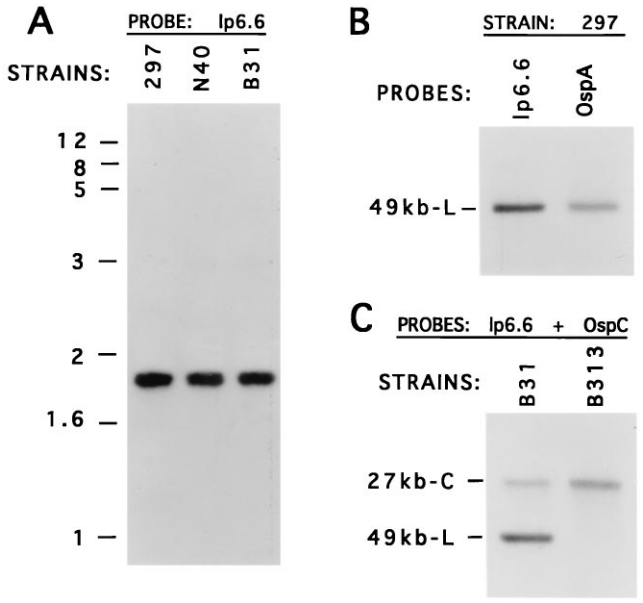

FIG. 2. Southern blot analysis of the *lp6.6* gene and localization to the 49-kb linear plasmid in *B. burgdorferi*. (A) Southern blot of *Sau*3A-restricted, total genomic DNAs of strains 297, N40, and B31. The probe was obtained by PCR amplification of *lp6.6* and then radiolabeled. Molecular size markers on the left are in kilobases. (B) Pulsed-field gel electrophoresis and DNA-DNA hybridization of genetic contents from *B. burgdorferi* 297. Probes (for *lp6.6* and *ospA* genes) were generated by PCR and then radiolabeled. 49kb-L, 49-kb linear plasmid. (C) Pulsed-field gel electrophoresis and DNA-DNA hybridization of genetic contents from *B. burgdorferi* B31 and B313. Genetic contents from each strain were hybridized with a mixture of PCR-generated, radiolabeled probes for *lp6.6* and *ospC*. 27kb-C, 27-kb circular plasmid. Note that whereas strains B31 and B313 each contained the 27-kb circular plasmid encoding *ospC* (51), *lp6.6* is present only in strain B31, harboring the 49-kb linear plasmid.

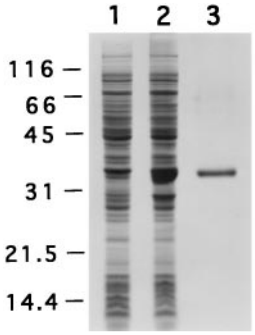

FIG. 3. Expression in *E. coli* and purification of the GST-lp6.6 fusion protein. An *E. coli* clone harboring *lp6.6* fused in frame with the GST gene was either uninduced (lane 1) or induced with IPTG (lane 2); total proteins were resolved by SDS-PAGE and stained with Coomassie brilliant blue. The 34-kDa fusion protein subsequently was purified (lane 3) by column chromatography on a glutathione matrix. Molecular mass markers on the left are in kilodaltons.

tween lp6.6 and other protein sequences in the databases. However, a BLASTn search indicated that the negative DNA strand of *lp6.6* was 70.6% homologous to DNA encoding the D6 gene (GenBank accession number U50840) located on a 70-kb plasmid of *Borrelia garinii* (BLAST score of 626; $P = 1.6 \times 10^{-84}$); the significance of this homology remains unknown. Computer searching also revealed another ORF (GenBank accession number U59487), encoding a 35-kDa polypeptide, 150 bp downstream and in the opposite orientation of *lp6.6*.

The *lp6.6* gene is located on the 49-kb linear plasmid of *B. burgdorferi*. Given reports of lipoprotein gene redundancy in *B. burgdorferi* (3, 44, 58), we next determined whether the *lp6.6* gene exists in single or multiple copies. First, total genomic DNAs of *B. burgdorferi* 297, N40, and B31 digested with *Sau*3A all yielded a single, 1.8-kb fragment which hybridized in Southern blots with a radiolabeled, PCR-generated *lp6.6* probe (Fig. 2A). Subsequent hybridization studies employing two-dimensional pulsed-field gel electrophoresis (51) indicated that the *lp6.6* gene was located exclusively on a linear plasmid(s) (not shown). Additional experiments using one-dimensional pulsed-field gel electrophoresis showed that the *lp6.6* gene hybridized to a linear plasmid which comigrated with the 49-kb linear plasmid harboring the *ospAB* operon (9) (Fig. 2B). Consistent with this finding, the *lp6.6* gene was absent in *B. burgdorferi* B313 (Fig. 2C), which lacks the 49-kb linear plasmid (Fig. 2C) (51), indicating that *lp6.6* and the *ospAB* operon are located on the same genetic element.

Characterization of lp6.6. The *lp6.6* gene (without the portion encoding the leader peptide) was cloned in frame with the gene encoding GST, and the resultant gene fusion was inducibly expressed in *E. coli* (Fig. 3, lane 2). This genetic construct was sequenced in its entirety to verify the DNA sequence. The resultant fusion protein was purified from *E. coli* (Fig. 3, lane 3) and used to generate a rat polyclonal antiserum. When the nonlipidated recombinant lp6.6 was cleaved from its GST fusion partner and subjected to SDS-PAGE and immunoblot analysis with this antiserum (Fig. 4, lanes 3), it migrated slightly slower than its native counterpart in *B. burgdorferi* (lanes 1), most likely due to the lesser binding of SDS. Of note, native lp6.6 also was readily detectable in isolated outer membranes by either silver staining (Fig. 4A, lane 2) or immunoblotting (Fig. 4B, lane 2). The highly similar electrophoretic mobilities of lp6.6 and a previously described low-molecular-weight lipoprotein (34) prompted us to determine whether these two polypeptides were identical. As shown in Fig. 4C, monoclonal antibody 240.7 directed against the low-molecular-weight lipoprotein described by Katona et al. (34) bound strongly to native and recombinant lp6.6.

Covalently bound lipids typically confer amphiphilicity to the polypeptide portions of spirochetal lipoproteins (32). Consistent with this, native lp6.6 in isolated borrelial outer membranes partitioned exclusively into the detergent-enriched phase upon extraction with Triton X-114 (Fig. 5A). In contrast, the nonacylated recombinant GST fusion protein partitioned exclusively into the aqueous phase upon extraction with Triton X-114 (Fig. 5B). The latter finding was not due to the presence of the GST fusion partner, inasmuch as the 6.6-kDa polypeptide cleaved from GST also partitioned exclusively into the aqueous phase (not shown). The combined findings are consistent with the prediction that the polypeptide portion of lp6.6 is hydrophilic (Fig. 1B) and that amphiphilic character is imparted by the acyl moieties.

Mammals infected with *B. burgdorferi* fail to produce antibodies directed against lp6.6. Recent studies have shown that the lack of an antibody response against OspA and OspB during low-dose needle or tick inoculation (6, 12, 49, 53) is due to downregulation during early borrelial infection of mammalian hosts (25, 41, 54). These observations prompted us to investigate whether *lp6.6* was expressed in vivo by examining the lp6.6-specific antibody responses of *B. burgdorferi*-infected animals. Groups of mice were needle inoculated with low doses (10^4 organisms) of virulent *B. burgdorferi* 297 and housed for various time intervals prior to sacrifice. Consistent with previous observations (3, 12, 53), antibodies directed against OspA

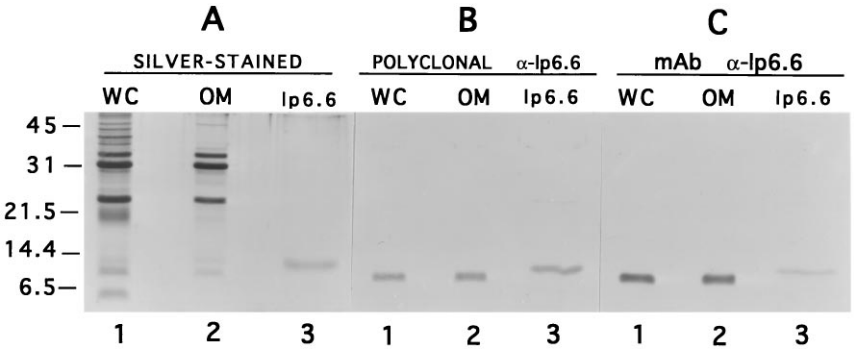

FIG. 4. SDS-PAGE and immunoblot analysis of *B. burgdorferi* whole cells (WC), isolated outer membranes (OM), and recombinant lp6.6 (cleaved from GST). (A) Silver-stained gel; (B) immunoblot probed with polyclonal rat anti-lp6.6 (α-lp6.6); (C) immunoblot probed with monoclonal antibody (mAb) 240.7 (34). Molecular mass markers at the left are in kilodaltons.

or OspB were not detected in any of the mouse sera (not shown). Antibodies directed against lp6.6 similarly were not detectable in any of the infection sera at dilutions as low as 1:100 (Fig. 6A, lane 3) (data not shown for 1-, 2-, 4-, 16-, and 26-week-postinfection sera). In contrast, lp6.6 was expressed by *B. burgdorferi* cultivated in vitro (Fig. 6A, lane 2), and the intrinsic immunogenicity of native lp6.6 was demonstrated by the observation that rats hyperimmunized with *B. burgdorferi* 297 produced specific anti-lp6.6 antibodies in high titers (>1:20,000) (Fig. 6A, lane 5).

Sera obtained from a tick-inoculated rhesus monkey (48) also were examined for antibodies directed against lp6.6. Analogous to the situation for low-dose-, needle-inoculated mice (3), sera from this monkey obtained after 17 or 20 weeks postinfection (with the JD1 strain of *B. burgdorferi*) lacked detectable antibodies to lp6.6 (Fig. 6B, lane 6) (as well as to OspA and OspB [Fig. 6B, lanes 2 and 3, respectively]) despite

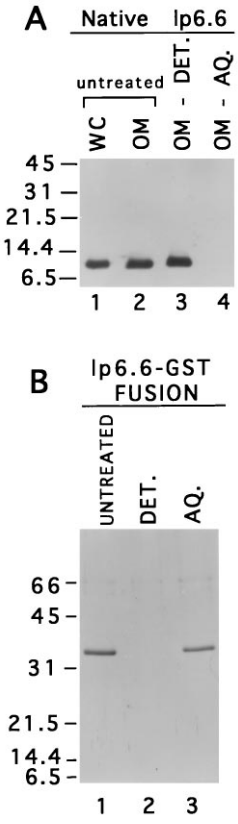

FIG. 5. Triton X-114 phase partitioning and SDS-PAGE of native (A) and recombinant (B) lp6.6. (A) Whole cells (WC) and isolated outer membranes (OM) of *B. burgdorferi* were untreated (lanes 1 and 2), or outer membranes were extracted with Triton X-114 (lanes 3 and 4). After SDS-PAGE, unextracted samples or proteins partitioning into either the detergent (DET.) or aqueous (AQ.) phase were immunoblotted with polyclonal anti-lp6.6 antiserum. (B) Purified recombinant GST-lp6.6 fusion protein was either untreated (lane 1) or extracted with Triton X-114 for partitioning into either the detergent (lane 2) or aqueous (lane 3) phase. Gels were then stained with Coomassie brilliant blue. Molecular mass markers at the left are in kilodaltons.

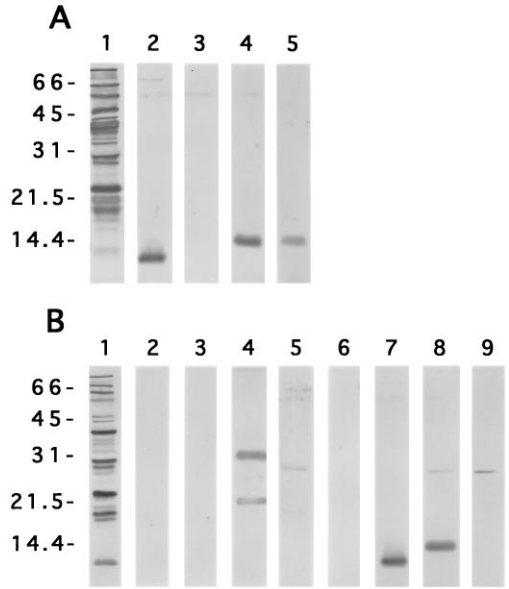

FIG. 6. Lack of anti-lp6.6 antibodies in immunoblots of sera from low-dose-, needle-inoculated mice (A) or a tick-inoculated rhesus monkey (B). (A) Lanes 1 and 2, whole-cell lysates of *B. burgdorferi* 297; lanes 3 to 5, recombinant lp6.6 cleaved from the GST fusion partner. Lanes 1 and 3 were probed with serum (1:100 dilution) from mice infected with *B. burgdorferi* 297 for 8 weeks. Lanes 2 and 4 were probed with rat anti-lp6.6 antiserum. Lane 5 was probed with serum from rats hyperimmunized with nonviable *B. burgdorferi* 297. Molecular mass markers at the left are in kilodaltons. (B) Lanes 1 and 7, whole-cell lysates of *B. burgdorferi* JD1; lanes 2, 3, 4, and 5, recombinant OspA, OspB, OspF, and BbK2.10, respectively; lanes 6, 8, and 9, recombinant lp6.6. Lanes 1 to 6 were probed with monkey serum (1:100 dilution) harvested 17 weeks after tick inoculation with *B. burgdorferi* JD1. Lanes 7 and 8 were probed with rat anti-lp6.6 antiserum. Lane 9 was probed with rat anti-GST antiserum. Molecular mass markers at the left are in kilodaltons.

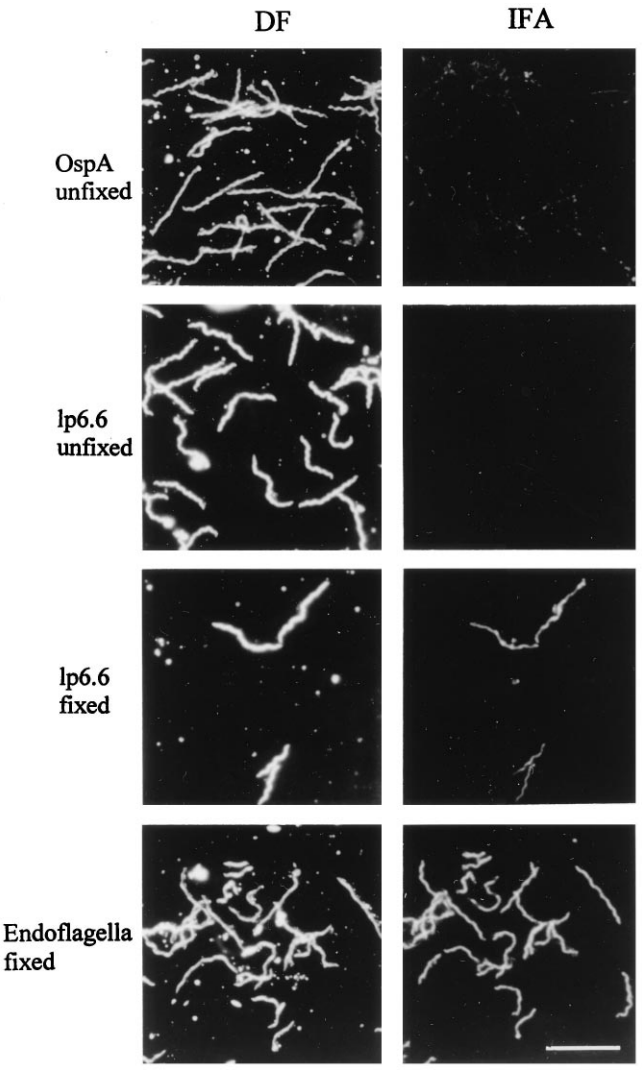

FIG. 7. Indirect immunofluorescence assays of unfixed (intact) and fixed (disrupted) *B. burgdorferi*. Photographs are from dark-field microscopy (DF) or immunofluorescence assays (IFA). Rows (top to bottom) correspond to unfixed (intact) borreliae incubated with antiserum to OspA, unfixed (intact) borreliae incubated with anti-lp6.6 antiserum, fixed borreliae incubated with anti-lp6.6 antiserum, and fixed borreliae incubated with antiserum directed against the periplasmic endoflagella. Bar, 20 μm.

the fact that *B. burgdorferi* JD1 abundantly expressed the lipoprotein when cultivated in vitro (Fig. 6B, lane 7) (data not shown for 20-week serum sample). By contrast, antibodies were produced against OspF (Fig. 6B, lane 4), a lipoprotein which appears to be expressed both in vitro and in vivo, and against BbK2.10 (Fig. 6B, lane 5), an OspF homolog which appears to be expressed exclusively during mammalian infection (3).

Localization of lp6.6 in *B. burgdorferi*. Further studies were warranted to investigate possible surface exposure of lp6.6. First, indirect immunofluorescence assays were performed with a format which enabled us to distinguish between surface-exposed and subsurface antigens (22). As reported previously (10, 25), intact spirochetes incubated with anti-OspA antibodies fluoresced lightly with a beaded pattern (Fig. 7); in contrast, no immunofluorescence was observed when intact *B. burgdorferi* was exposed to antibodies directed against either lp6.6 (Fig. 7) or the periplasmic endoflagella (not shown). However, spirochetes mounted on glass slides and then fixed with methanol (i.e., organisms with compromised outer membranes) fluoresced intensely when reacted with the same three antibodies (Fig. 7) (data not shown for OspA). Markedly enhanced immunolabeling of methanol-fixed spirochetes with anti-OspA antibodies was previously described (22).

Encapsulation of *B. burgdorferi* in agarose gel microdroplets was utilized as a secondary method for evaluating surface exposure of lp6.6 (22, 23). As in the immunofluorescence assays described above, virtually all organisms in microdroplets were lightly labeled by antibodies directed against OspA (Fig. 8). In contrast, a very small percentage of borreliae, presumably damaged organisms, fluoresced when exposed to anti-lp6.6 or antiendoflagellum antibodies (Fig. 8). However, virtually all organisms bound anti-lp6.6 antibody after pretreatment of encapsulated spirochetes with even a very low concentration (0.03%) of Triton X-100 (Fig. 8), a condition known to disrupt the borrelial outer membrane (22). Moreover, the pattern of increased binding of anti-lp6.6 antibody by encapsulated spirochetes exposed to graded concentrations of Triton X-100 closely paralleled that obtained by probing *B. burgdorferi* with antibody directed against endoflagella (Fig. 8).

The results of the immunofluorescence assays described above implied a periplasmic location for lp6.6. However, such methods are unable to establish whether the antigen resides on the inner leaflet of the outer membrane or on the outer leaflet of the cytoplasmic membrane. In attempts to clarify this, preparations of isolated *B. burgdorferi* outer membranes and protoplasmic cylinders (i.e., lacking outer membranes) from equivalent numbers of spirochetes were immunoblotted in parallel with anti-lp6.6 antiserum. As shown in Fig. 9, outer membranes contained only a small proportion of lp6.6 in comparison with the quantity in protoplasmic cylinders.

Growth inhibition and immunoprotection studies. Although the immunofluorescence studies described above indicated that lp6.6 was not surface exposed, it was possible that these methods were not sufficiently sensitive to detect minute amounts of surface-exposed antigen. For this reason, the potential borreliacidal activity of anti-lp6.6 antiserum also was examined (39, 50). High-titer (>1:20,000) rat antiserum directed against the lp6.6 antigen failed to demonstrate borreliacidal activity even at dilutions as low as 1:16. In fact, results derived from examining microtiter plate wells macroscopically, microscopically, and spectrophotometrically all were essentially identical to those obtained for the negative control wells (wells lacking antisera or wells containing anti-GST antiserum). In contrast, polyclonal rat antiserum directed against OspA produced marked agglutination of borreliae and inhibition of growth at dilutions as high as 1:2,048.

Passive- and active-immunization experiments also were performed. Groups of four mice were either passively or actively immunized with rat anti-lp6.6 antiserum or purified recombinant lp6.6-GST fusion antigen, respectively. Separate groups of positive-control mice were immunized with either rat anti-OspA antiserum or purified recombinant OspA-GST. Negative-control mice were immunized with either anti-GST antiserum or purified GST. Sera used in passive-immunization experiments contained comparable levels of antibodies in that all reacted with their respective antigens at dilutions of ≥20,000. Antibody titers elicited by active immunization with the respective purified antigens were as follows: OspA, 1:20,000; lp6.6, 1:10,000 to 20,000; and GST, 1:10,000. All mice (four of four) immunized either passively with 50 μl of anti-OspA antiserum or actively with OspA were protected against challenge with 10^3 virulent *B. burgdorferi* organisms. In con-

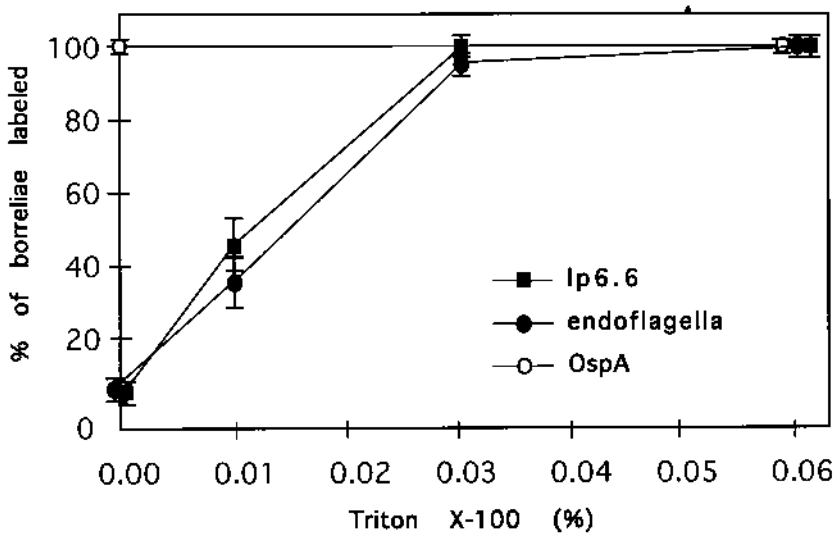

FIG. 8. Encapsulation of *B. burgdorferi* in agarose gel microdroplets and indirect immunofluorescence assays. The percent labeling of borreliae exposed to either rat anti-OspA, rat anti-lp6.6, or rat antiserum directed against the periplasmic endoflagella is shown as a function of Triton X-100 concentration. Data are expressed as the means for three experiments ± standard deviations.

trast, as in the cases of all negative-control mice, none of the mice immunized either passively with 50 μl of anti-lp6.6 antiserum or actively with purified lp6.6 were protected from challenge with *B. burgdorferi.*

DISCUSSION

In the present study, reactivity with monoclonal antibody 240.7 confirmed that lp6.6 has identity with the highly abundant, low-molecular-weight, phenol-chloroform-petroleum ether-extractable immunogen originally described by Katona et al. (34); its lipoprotein nature was established by intrinsic radiolabeling of *B. burgdorferi* with [^{3}H]palmitate (34). Katona et al. (34) also showed that the molecule was highly conserved among various (10 of 10) strains of *B. burgdorferi* (but was not detectable in *Borrelia anserina*, *Borrelia hermsii*, *Treponema pallidum*, or *Treponema phagedenis*). This degree of conservation was consistent with the findings of this study that the DNA sequence encoding lp6.6 differed by only one nucleotide (in strain N40) among three virulent strains of *B. burgdorferi* examined (297, N40, and B31).

During SDS-PAGE, lp6.6 typically migrates with an apparent molecular mass of about 10 kDa. By linear regression analysis, it was estimated previously that the molecular mass of the monomer actually was about 7.5 kDa (34). In the present study, DNA sequence analysis allowed us to determine that the molecular mass of the 51-amino-acid mature, acylated polypeptide is about 6.6 kDa. Other studies have noted the existence in *B. burgdorferi* of low-molecular-mass polypeptides and lipoproteins (28, 52, 62), but their relationship to lp6.6 remains uncertain. As a cautionary note, investigators should be aware of the need to shorten resolution times during SDS-PAGE in order to visualize lp6.6 or other low-molecular-mass borrelial polypeptide constituents.

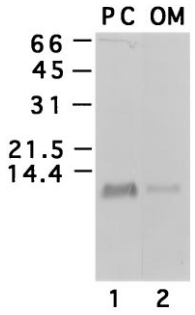

FIG. 9. Immunoblot analysis of *B. burgdorferi* protoplasmic cylinders (PC) and outer membranes (OM) isolated by sucrose density gradient centrifugation. Protoplasmic cylinders and outer membranes from equivalent numbers (ca. 5×10^7) of spirochetes were collected from sucrose density gradients (47), separated by SDS-PAGE, and immunoblotted with polyclonal rat anti-lp6.6 antiserum. Molecular mass markers on the left are in kilodaltons.

Although lp6.6 is readily detectable in isolated outer membranes, using several methodologies we were unable to obtain evidence for surface exposure. These findings were consistent with the observation by Katona et al. (34) that motile borreliae did not bind monoclonal antibody 240.7. Moreover, the preponderance of this lipoprotein was associated with protoplasmic cylinders. Such data are consistent with two possible interpretations. First, lp6.6 may be associated with both the outer and cytoplasmic membranes, with the majority residing in the cytoplasmic membrane. Along these lines, there is now considerable evidence for a dual-membrane distribution of other abundant *B. burgdorferi* lipoproteins (e.g., OspA and OspB) (16, 17, 22, 47). A second explanation of our data is that the portion of lp6.6 within isolated outer membranes represents contamination introduced during the outer membrane isolation procedure. Additional antigen localization methodologies (e.g., immunocryoultramicrotomy) (17) may help to distinguish these two possibilities.

lp6.6 is typical of spirochetal lipoproteins in that it consists of a hydrophilic polypeptide with covalently attached lipids that serve as membrane anchors. Recently, we showed by reconstitution of membrane vesicles with purified spirochetal lipoproteins that the polypeptide portions of these immunogens are extrinsic to the lipid bilayer (32). With the above-described topology in mind, the polypeptide portion of a subsurface lipoprotein, such as lp6.6, would have to be located in the periplasmic space regardless of whether it was tethered by its lipid moieties to the outer membrane, cytoplasmic mem-

brane, or both. Tethering of lp6.6 to the inner leaflet of the outer membrane would be analogous to the case for murein lipoprotein of *E. coli* (45). Currently, however, there is no information as to the potential role of lp6.6 in the membrane biology of *B. burgdorferi*; its predominance in the cytoplasmic membrane (to which the peptidoglycan is linked) could lead to the hypothesis that it has a Braun's lipoprotein-like function, although downregulation of lp6.6 during the growth of *B. burgdorferi* in vivo tends to argue against this notion. In any event, the periplasmic location of the lp6.6 polypeptide was corroborated by immunofluorescence studies with encapsulated spirochetes (22); the percentages of organisms labeled in the presence of graded concentrations of Triton X-100 closely paralleled those obtained with antibodies directed against flagellin, a periplasmic marker. We recently proposed that *B. burgdorferi* possesses a secretory apparatus which shuttles lipoproteins to the spirochetal surface and that this apparatus most likely can distinguish lipoproteins which can become surface exposed from those destined to remain entirely subsurface (22). The fact that lp6.6 is exclusively subsurface provides further indirect evidence for this conjecture.

DNA hybridization studies revealed that *lp6.6* is a single-copy gene which localizes to the *ospAB* operon-encoding 49-kb linear plasmid (9). Interestingly, like OspA and OspB, lp6.6 did not generate an antibody response in mice or rhesus monkeys following low-dose needle or tick inoculation, respectively, despite the fact that the native molecule is extremely immunogenic when administered in the context of killed borreliae. These findings appear to be relevant to human Lyme borreliosis in that antibodies directed against low-molecular-mass *B. burgdorferi* antigens typically are absent, particularly during early human infection (8, 24, 27, 28, 59). All of these findings lead us to postulate that, analogous to that of the OspA and OspB genes (25, 54), the expression of *lp6.6* occurs in the tick vector but is downregulated during mammalian infection. In support of this contention, preliminary results from immunoblotting have revealed the presence of lp6.6 in extracts of individual, field-collected (New York) ticks probed with monoclonal antibody 240.7 (35). Moreover, the lack of surface exposure implies that downregulation reflects a physiological adaptation(s) rather than immunological pressure from the mammalian host. In recent years, there has been increasing interest in understanding the antigenic changes, environmental stimuli, and regulatory mechanisms associated with the differential expression of *B. burgdorferi* genes and their roles in disease pathogenesis (3, 21, 33, 41, 54, 57, 61). Further studies with *lp6.6* as a model system could provide valuable insights into this new and important avenue of Lyme disease research.

(Consistent with the conclusions of this study, primer extension analysis [44] with *B. burgdorferi* 297 RNA and the primer 5′-CATCTGAAATTCTTGTAGTTTCGC-3′ revealed a transcriptional initiation site for *lp6.6* at nucleotide position −24 [Fig. 1A, arrow].)

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