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Molecular and Immunological Characterization of a Novel Polymorphic Lipoprotein of *Borrelia burgdorferi*

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We describe the cloning, expression, and molecular characterization of a novel polymorphic *Borrelia burgdorferi* lipoprotein recognized by monoclonal antibody LA7. Sequence analysis revealed an open reading frame encoding a 21,866-Da polypeptide (IpLA7). Comparison with other known proteins indicated sequence similarity between IpLA7 signal peptides and those of other prokaryotic lipoproteins, including the immunodominant *B. burgdorferi* outer surface proteins OspA, OspB, pC, and OspD. Both natural IpLA-7 and recombinant IpLA-7 could be biosynthetically labeled with [^{3}H]palmitate. Upon solubilization of intact *B. burgdorferi* with the nonionic detergent Triton X-114, IpLA7 was extracted together with other lipoproteins into the detergent phase. Indirect immunolabeling studies indicated that the epitope recognized by monoclonal antibody LA7 is mainly located in the periplasmic space. Two-dimensional gel electrophoresis and immunoblotting confirmed the calculated acidic pI of 5.7 for IpLA-7. The *LA7* gene was shown to be species specific and to be located on the linear chromosome of *B. burgdorferi*. The analysis of 40 individual spirochetal isolates on the basis of restriction fragment length polymorphisms revealed considerable genotypic heterogeneity of *LA7* corresponding to that previously found for *ospA*. Native IpLA-7 and recombinant IpLA-7 were recognized by immune sera from infected mice as well as some human sera derived from infected but healthy donors and may thus prove useful as an additional marker for the serodiagnosis of Lyme disease.

Lyme disease is the most common vector-borne infectious disease of regions with a temperate climate. The etiological agent, the spirochete *Borrelia burgdorferi*, causes a multisystemic illness in humans that may affect the skin, nervous system, joints, and heart (29). *B. burgdorferi* strains isolated from different biological sources and geographic areas are heterogeneous (3, 31, 35), and it is assumed that the patterns of disease manifestations are influenced by antigenic differences among the spirochetal strains. Although several attempts to classify *B. burgdorferi* on the basis of immunological or molecular criteria were recently reported, the taxonomy of *B. burgdorferi* is still a matter of controversy and active research (34).

To date, a variety of *B. burgdorferi* antigens, such as outer surface proteins OspA, OspB, pC, and p100, have been used for serological diagnosis and as putative candidates for vaccine development (25). However, because of their obvious heterogeneity, unambiguous criteria for the generation of a species-specific diagnostic standard and for the development of a polypeptide vaccine that would guarantee protection against any subspecies are still lacking (11, 13, 14). Therefore, more detailed information on the polymorphisms of the immunologically relevant *B. burgdorferi* component(s) is urgently required. We described previously a series of monoclonal antibodies (MAb) that recognize distinct *B. burgdorferi*-associated antigens (15). In the present paper, we describe the genetics and further biochemical characterization of a novel polymorphic *B. burgdorferi* lipoprotein recognized by MAb LA7.

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MATERIALS AND METHODS

***Borrelia* strains.** The *B. burgdorferi* strains used in this study (Table 1) were described elsewhere (26, 27). Borrelia strains were grown in modified Barbour-Stoenner-Kelly II (BSKII) medium (1) at 33°C. Spirochetes were harvested by centrifugation at 10,000 × *g* for 20 min at 4°C, washed two times in phosphate-buffered saline (PBS), and enumerated by dark-field microscopy.

Preparation and screening of a *B. burgdorferi* expression library. Genomic DNA was prepared from *B. burgdorferi* ZS7 by the lysozyme-sodium dodecyl sulfate (SDS) method, and DNA fragments were generated by sonication. Blunt-ended DNA was inserted into the pUEX1 vector by use of an adaptor cloning strategy (7, 33). The ligated DNA was transformed into *Escherichia coli* MC1061, and then expression screening was done with MAb LA7 (32).

Southern blot hybridization. Total genomic DNA was extracted from *Borrelia* organisms as described previously (32). Approximately 5 μg of DNA was digested with 100 U of restriction nuclease *Hin*dIII in accordance with the manufacturer's recommendations (Boehringer GmbH, Mannheim, Germany). Samples were subjected to electrophoresis with a 0.7% agarose gel. DNA fragments were transferred to a Hybond-N nylon membrane (Amersham, Braunschweig, Germany) and then subjected to UV crosslinking and hybridization as described previously (31). In brief, hybridization was done by use of ^{32}P-labeled probes overnight at 65°C with 0.5 M $NaHPO_4$–7% SDS (pH 7.2). After being washed in 40 mM $NaHPO_4$–1% SDS (pH 7.2) at room temperature for 30 min, the dry membrane was autoradiographed on Kodak XAR-5 film with intensifying screens at −80°C for 1 to 12 h. As a hybridization probe, a 500-bp DNA fragment encompassing the *LA7* coding region was used. The gene fragment of interest was recovered from low-melting-temperature

TABLE 1. *B. burgdorferi* isolates used in this study

B. burgdorferi isolate	Biological origin[a]	Geographic origin[b]	Fla, Hsp60, and Hsp70 genotype	OspA genotype	Reactivity of MAb LA7	IpLA7 genotype[c]
B31	Tick (*Ixodes dammini*)	USA	AAA	I	+	2.0
ZS7	Tick (*I. ricinus*)	Germany	AAA	I	+	2.0
Z37	Tick (*I. ricinus*)	Germany	AAA	I	+	ND
Getto	Skin (ECM)	Germany	AAA	I	+	ND
B1	Skin (ECM)	Germany	AAA	I	+	ND
B2	Skin (ECM)	Germany	AAA	I	+	ND
B3	Skin (AD)	Germany	AAA	I	+	ND
20004	Tick (*I. ricinus*)	France	AAA	I	+	ND
19535	Mouse (*Peromyscus* sp.)	USA	AAA	I	+	2.0
26816	Vole (*Microtus* sp.)	USA	AAA	I	+	ND
28691	Tick (*I. dammini*)	USA	AAA	I	+	2.0
21305	Mouse (*Peromyscus* sp.)	USA	AAA	I	+	2.0
21343	Mouse (*Peromyscus* sp.)	USA	AAA	I	+	ND
26815	Chipmunk	USA	AAA	I	+	2.0
297	Cerebrospinal fluid	USA	AAA	I	+	ND
Mac3	Skin	USA	AAA	I	+	ND
20001	Tick (*I. ricinus*)	France	AAA	I	+	ND
CTiP7	Dog tick	USA	AAA	I	+	ND
SH-2-82	Tick (*I. dammini*)	USA	AAA	I	+	ND
CA-2-87	Tick (*I. pacificus*)	USA	AAA	I	+	ND
S12/14	Tick (*I. ricinus*)	Germany	AAA	I	+	ND
IP1	Cerebrospinal fluid	France	AAA	I	+	2.0
NE2	Tick (*I. ricinus*)	Switzerland	AAA	I	+	ND
R7NE4	Tick (*I. ricinus*)	Switzerland	AAA	I	+	ND
LW2	Skin	Germany	AAA	I	+	ND
LW2.4	Skin	Germany	AAA	I	+	ND
ZQ1	Tick (*I. ricinus*)	Germany	BBB	II	−	0.8
NE4	Tick (*I. ricinus*)	Switzerland	BBB	II	−	0.8
NE58	Tick (*I. ricinus*)	Switzerland	BBB	II	−	0.8
NE11H	Tick (*I. ricinus*)	Switzerland	BBB	II	−	0.8
IP3	Cerebrospinal fluid	France	BBB	II	ND	0.8
R3NE2	Tick (*I. ricinus*)	Switzerland	BBB	II	−	ND
N34	Tick (*I. ricinus*)	Germany	BBB	II	−	ND
20047	Tick (*I. ricinus*)	France	BBB	V	+	0.6
S90	Tick (*I. ricinus*)	Germany	BBB	VI	−	>0.8
19857	Rabbit kidney	USA	B(A/B)A	III	+	0.75
21038	Larva (*I. dentatus*)	USA	B(A/B)A	III	+	0.75
ACA-1	Skin (ACA)	Sweden	BBA	IV	−	1.9
Bo23	Skin (ECM)	Germany	BBA	IV	−	1.9
So2	Tick (*I. ricinus*)	England	BBA	IV	ND	1.9

[a] AD, atrophoderma.
[b] USA, United States.
[c] Numbers indicate fragment sizes in kilobases. ND, not done.

agarose gels, precipitated by ethanol treatment, and radiolabeled by a random-primer reaction as described previously (8).

Triton X-114 phase partitioning. *B. burgdorferi* was grown in BSKII medium, and borrelial lipoprotein-enriched preparations were obtained by extraction and phase separation with the nonionic detergent Triton X-114 (6, 22, 23). In brief, washed bacteria were suspended in ice-cold 2% (vol/vol) Triton X-114 (Fluka Chemie, Buchs, Switzerland) in PBS (pH 7.4) at 5×10^9 organisms per ml of detergent. After incubation overnight at 4°C on an end-over-end rotating wheel, insoluble material was removed by centrifugation at 20,000 × *g* for 30 min at 4°C. The supernatant was phase separated by being warmed to 37°C for 15 min in a water bath and then was centrifuged for 15 min at room temperature in a microcentrifuge. The separated detergent and aqueous phases were then washed four times in the following manner. The detergent phase was suspended to its original volume in ice-cold PBS, while the aqueous phase was brought to a final concentration of 2% detergent by the addition of cold 10% Triton X-114 and then was phase separated as described above. Protein concentrations were determined by the bicinchoninic acid method (Pierce Chemical Co., Rockford, Ill.).

Intrinsic radiolabeling of *B. burgdorferi* proteins. *B. burgdorferi* proteins were labeled with [9,10-(*n*)-^{3}H]palmitate (Amersham; specific activity, ≈50 Ci/mmol). *B. burgdorferi* was grown in BSKII medium at 33°C to a density of 10^8 organisms per ml. Radiolabeled palmitate was then added to a final concentration of 0.25 mCi/ml, and incubation was continued for 2 days. Bacteria were collected by centrifugation, and Triton X-114 fractionation was then performed as described above.

Gel electrophoresis. For electrophoresis on one-dimensional SDS-polyacrylamide slab gels as described by Laemmli (17), 40 μl of each lysate (equivalent to ≈10^8 organisms) was mixed with 10 μl of 5× reducing sample buffer. Two-dimensional polyacrylamide gel electrophoresis (PAGE) was carried out as described by O'Farrel (21) with IEF (Pharmacia/LKB ampholytes: 1.45%, pH 3.5 to 10; 0.1%, pH 2.5 to 4.0; 0.2%, pH 4 to 6; 0.2%, pH 9 to 11) in the first dimension.

The same amount of lysate was applied as for one-dimensional gel electrophoresis. Gels were either silver stained or processed for Western blotting (immunoblotting) (15).

Surface proteolysis with proteinase K (Boehringer) was carried out by the method of Norris et al. (20). The proteins were then separated by SDS-PAGE, and individual antigens were identified by immunoblotting.

Western blotting. Following two-dimensional SDS-PAGE, proteins were electroblotted for 1 h at a constant current (60 mA) onto Hybond-C nitrocellulose sheets (Amersham) by use of a semidry electroblotting chamber (BIO-RAD, Munich, Germany) in accordance with the manufacturers' recommendations. Following overnight incubation in blocking buffer (50 mM Tris-HCl, 150 mM NaCl, 5% nonfat dried milk), immunoblots were incubated for 2 h at room temperature with a 1:100 (vol/vol) dilution of mouse and human antisera in dilution buffer (50 mM Tris-HCl, 150 mM NaCl, 1% dried milk, 0.2% Tween 20) or with the culture supernatant of a mouse MAb (LA7). The nitrocellulose filters were washed five times in dilution buffer and incubated for an additional 1 h with an alkaline phosphatase-conjugated goat anti-rabbit antiserum (1:400 [vol/vol]; Dianova, Hamburg, Germany). The blots were washed four times in the above-mentioned dilution buffer and twice in Tris-borate-saline (TBS), and immunoreactive bands were then visualized by the addition of 20 ml of DEA buffer (0.1 M diethanolamine [Sigma], 0.02% NaN_3, 5 mM $MgCl_2$ [pH 9.0]) supplemented with 5-bromo-4-chloro-3-indolylphosphate (Sigma; 165 μg/ml) and nitroblue tetrazolium (Sigma; 330 μg/ml) as a substrate. The reaction was stopped by washing of the membranes in TNE buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA).

Patient sera. Serum specimens used in the enzyme-linked immunosorbent assay (ELISA) and in Western blotting were from a clinically characterized serum bank. All the positive sera had an optical density of ≥0.3 in the ELISA with either immunoglobulin M or immunoglobulin G conjugates and were reactive with certain immunodominant *B. burgdorferi* proteins (p41, p60, and p20, etc.) in Western blotting. Five serum specimens were from patients with clinically verified acrodermatitis chronicum atrophicans (ACA). Seven serum specimens were from patients with erythema chronicum migrans (ECM). Three serum specimens were obtained from forest workers who had been exposed to *B. burgdorferi*, as evidenced by seroconversion. Five negative control serum specimens were selected from healthy blood donors (courtesy of the Heidelberg Blood Bank).

Immunofluorescence. *B. burgdorferi* organisms were washed twice in PBS, transferred to adhesion slides (Superior, Bad Mergentheim, Germany) (10^5 spirochetes per reaction field), fixed in absolute ethanol (2 min, −20°C), and air dried. The fixed spirochetes were incubated with the individual MAb diluted in PBS in a moist chamber for 30 min. After three washings in PBS, the spirochetes were incubated with a fluorescein isothiocyanate-labeled goat anti-mouse immunoglobulin antiserum (Medac, Hamburg, Germany) in a dark moist chamber for 30 min. After three washings in PBS, the preparations were examined with a fluorescence microscope and documented with 400-ASA black-and-white film.

ELISA. *B. burgdorferi*-specific antibodies were measured in a solid-phase ELISA system with *B. burgdorferi* B31 antigens as described previously (15).

DNA sequencing. *B. burgdorferi* genomic DNA fragments cloned in pUEX1 plasmid derivatives were sequenced by use of a T7 sequencing kit (Pharmacia) in accordance with the manufacturer's recommendations (32).

Amino acid sequence analyses. Simultaneous alignments for protein sequences were determined by use of HUSAR software (30).

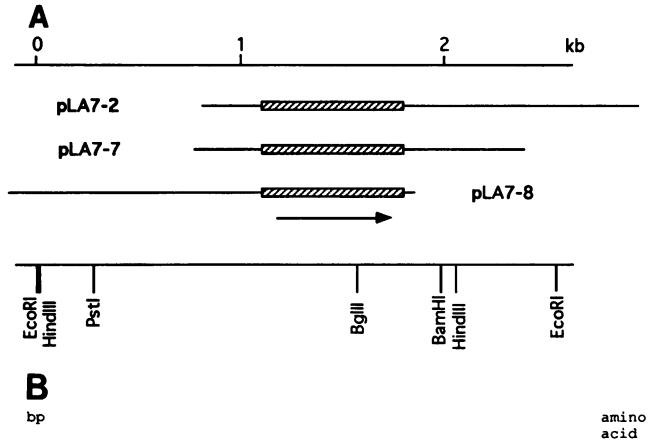
Nucleotide sequence accession number. The *LA7* gene sequence has been entered into the EMBL and GenBank data bases with the accession number X70826.

RESULTS

Cloning and sequence analysis of the *LA7* gene. A pUEX1 expression library of *B. burgdorferi* ZS7 genomic DNA was screened with MAb LA7. Three independent recombinant clones were identified. All three clones overlapped and constituted a colinear sequence encompassing the entire coding region of the putative *LA7* gene. The physical maps of these three plasmid inserts (pLA7-2, pLA7-7, and pLA7-8) and some restriction sites are shown in Fig. 1A. The *LA7* gene sequence is depicted in Fig. 1B. Located 6 bases upstream of the ATG start codon was a consensus ribosome binding site (AAGGGAGA). Located further upstream of this translational start site, at positions −34 to −28, was a putative RNA polymerase start site, also known as the −10 box (TAATATG); this box was preceded by a −35 region (TGTGTACAAAA), at positions −77 to −67 (24). Located downstream of the protein-encoding region was a perfect 12-mer palindromic sequence (ATAGGCTTTAAT), starting at nucleotide 603. The relatively high expression of IpLA7 in *E. coli* suggests that similar regulatory sequences function in both species.

The molecular mass of the protein predicted from the amino acid sequence of 194 residues is 21,865 Da. Molecular analysis and sequence comparison of IpLA7 with other proteins revealed sequence similarity to the signal peptides of lipoproteins (36, 37). As shown in Fig. 2, IpLA7 resembles other outer membrane proteins comprising a large portion of charged amino acid residues (>30%) and a single segment of hydrophobicity in the N-terminal region (19). Beyond the leader sequence, the deduced IpLA7 sequence is largely hydrophilic and predominantly alpha-helical, as predicted by secondary structure analyses (data not shown). While this manuscript was in preparation, the isolation of two additional low-molecular-weight proteins from *B. burgdorferi* (pC and OspD) was reported (10, 20). As shown in Fig. 3, a comparison of all three sequences indicated the presence of Leu-X-Y-Z-Cys at the COOH-terminal end of the signal sequence; this motif represents a common feature of lipoprotein precursors in bacteria (37). Cleavage at the cysteine residue by signal peptidase II would yield a polypeptide with a predicted molecular mass of 19,341 Da. The first 50 amino acids of IpLA7 show 30% homology to the N-terminal sequence of pC and 20% homology to that of OspD (Fig. 3). No significant homology to the N-terminal amino acid sequence of a low-molecular-mass (22-kDa) protein recently described by Luft et al. (18) was found.

RFLPs of the *LA7* gene. Restriction fragment length polymorphism (RFLP) analysis of *LA7* with endonuclease *Bam*HI (Fig. 4A) or *Hin*dIII (Fig. 4B) revealed at least six distinct hybridization patterns among the 40 *B. burgdorferi* isolates tested (Table 1). After digestion of *B. burgdorferi* whole-cell DNAs with *Hin*dIII (Fig. 4B), all isolates previously shown to express OspA genotype I antigens (e.g., B31 and ZS7; 31) were characterized by one hybridization fragment of 2 kb; those expressing OspA genotype II (e.g., ZQ1) were characterized by one fragment of 0.8 kb. Because of

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-133    tttttcatat taataagacc tcctgtttca ttttaacatt ttaattgttt
                "-35"
- 83    ttaaagtgtg tacaaaataa attatttatt gtaaacttac ttttaatttt
        "-10"                          -1 +1
- 33    aatatgatta ataaattata agggagaatt ttt atg tat aaa aat
                                 (RBS)      M   Y   K   N      4
  13    ggt ttt ttt aaa aac tat ttg tca ttg ttt tta att
        G   F   F   K   N   Y   L   S   L   F   L   I     16
  49    ttt tta gta att gct tgt act tca aaa gat agc tca
        F   L   V   I   A   C   T   S   K   D   S   S     28
  85    aat gaa tat gtt gag gag caa gaa gcg gag aac tct
        N   E   Y   V   E   E   Q   E   A   E   N   S     40
 121    tct aag cct gat gat tct aaa ata gat gaa cat act
        S   K   P   D   D   S   K   I   D   E   H   T     52
 157    att ggg cac gtt ttt cac gct atg gga gta gtt cat
        I   G   H   V   F   H   A   M   G   V   V   H     64
 193    tca aaa aag gat cga aaa agt ttg ggg aaa aat ata
        S   K   K   D   R   K   S   L   G   K   N   I     76
 229    aag gtt ttt tat ttt tct gaa gaa gat gga cat ttt
        K   V   F   Y   F   S   E   E   D   G   H   F     88
 265    caa aca ata ccc tca aaa gag aat gca aag tta ata
        Q   T   I   P   S   K   E   N   A   K   L   I    100
 301    gtt tat ttt tat gac aat gtt tat gca gga gag gct
        V   Y   F   Y   D   N   V   Y   A   G   E   A    112
 337    cca att agt atc tct gga aaa gaa gcc ttt att ttt
        P   I   S   I   S   G   K   E   A   F   I   F    124
 373    gtt ggg att acc cct gac ttt aaa aag att ata aat
        V   G   I   T   P   D   F   K   K   I   I   N    136
 409    agc aat tta cat ggc gct aaa agt gat ctt att ggt
        S   N   L   H   G   A   K   S   D   L   I   G    148
 445    act ttt aaa gat ctt aat att aaa aat tca aaa ttg
        T   F   K   D   L   N   I   K   N   S   K   L    160
 481    gaa att aca gtt gat gag aat aat tca gat gcc aag
        E   I   T   V   D   E   N   N   S   D   A   K    172
 517    acc ttc ctt gaa tct gtt aat tac att atc gac ggc
        T   F   L   E   S   V   N   Y   I   I   D   G    184
 553    gtt gaa aaa att tca cct atg tta acg aat taa ttt
        V   E   K   I   S   P   M   L   T   N   *        194
 589    atatttttga ttttataggc tttaatctaa attaaagcct attttaaaaa
                   ---------->     <----------
 639    atcaagctct caagtccttt tattaaaatt tctgctgttt ttacgttggt
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FIG. 1. (A) Restriction maps of three overlapping *B. burgdorferi* DNA fragments cloned in pUEX1. The region corresponding to the protein-encoding region of the *LA7* gene is shown as a hatched box. The arrow indicates the direction of transcription. (B) Complete nucleotide sequence and deduced amino acid sequence of the *B. burgdorferi* (strain ZS7) *LA7* gene. Nucleotides are numbered relative to the putative ATG start codon (+1). The following features of the DNA sequence are indicated: a consensus ribosome binding site (RBS); the putative active −10 and −35 promoter sequences (underlined); the termination codon at the 3′ end of the open reading frame (asterisk); and a 12-mer complementary region indicating the formation of a hairpin loop (-----> and <-----). The open reading frame encodes a 194-amino-acid polypeptide with a hydrophobic leader sequence and a typical signal peptidase II cleavage site.

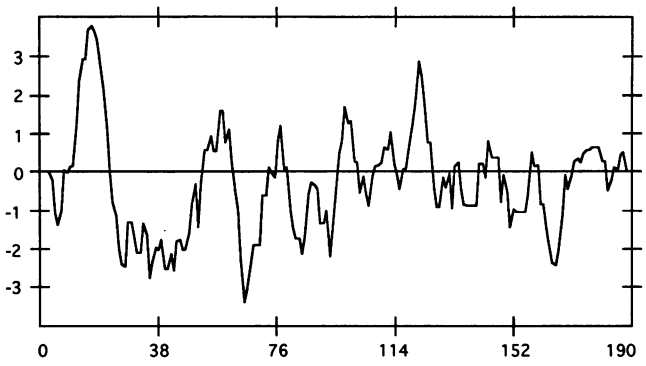

FIG. 2. Hydropathicity profile of the deduced *B. burgdorferi* IpLA7 amino acid sequence.

underloading, the 0.8-kb band was virtually invisible, but it became obvious after prolonged exposure (data not shown). *B. burgdorferi* isolates expressing OspA genotype III (19857 and 21038) exhibited one hybridizing fragment of 0.75 kb, and all isolates expressing OspA genotype IV (e.g., ACA-1) exhibited one fragment of 1.9 kb. *B. burgdorferi* isolates with OspA genotypes V (20047) and VI (S90) exhibited fragments of 0.6 and >0.8 kb, respectively (Table 1).

The distinct patterns of the *LA7*-specific RFLPs correlated with the differential expression of the epitope defined by MAb LA7. Only *B. burgdorferi* isolates of OspA genotypes I, III, and V (e.g., ZS7, 19857, and 20047) and not those of OspA genotypes II, IV, and VI (e.g., ZQ1, ACA-1, and S90) reacted with MAb LA7 (Table 1).

In contrast to the *ospA* gene, which is located on the 49-kb extrachromosomal linear plasmid, IpLA7 is encoded by a chromosomal gene, as elucidated by pulse field gel electrophoresis (data not shown). DNAs isolated from other *Borrelia* species, such as *B. coriaceae* Co53 (Fig. 4), *B. hermsii*, and *B. turicatae*, and from *Treponema pallidum* (data not shown) did not hybridize to the *LA7*-specific probe, indicating specificity for *B. burgdorferi*.

Two-dimensional immunoblot analysis of IpLA7. For analysis of the expression of IpLA7 in *B. burgdorferi* organisms, whole-cell lysates were separated on two-dimensional gels and subsequently either silver stained (Fig. 5A) or immunoblotted with MAb LA7 and, as controls, MAb directed against OspA, OspB, and flagellin (Fig. 5B). IpLA7 was clearly separated from other low-molecular-weight proteins. Another 20-kDa protein migrated at a charge position similar to that of OspB and reacted with OspB-specific MAb LA32 (15). Whether MAb LA32 recognizes a truncated version of OspB or whether it recognizes an antigenic epitope shared by two independent proteins needs further investigation. However, this finding is reminiscent of data recently published by Norris et al. showing that MAb H6831 recognizes a cross-reactive epitope expressed on both OspB and a 20-kDa polypeptide (20).

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OspD    MKK-LIKILLLSLFL-LLSISCVH-DKQELSSKSNLNNQKGYLDNEGANSNYE
pC      MKKNTLSAILMTLF---LFISCNNSGKGGDSASTNPADESAKGPNLTEISKKI
pLA7    MYKNGFFKNYLSLFLIFLVIACTSKDSSNEYVEEQEAENSSKPDDSKID---E
        * ** +    ++*** +* *+*   ++ +   +++ +++++   +    *
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FIG. 3. Comparison of the IpLA7, pC, and OspD amino acid sequences derived from *B. burgdorferi* ZS7, PKo, and B31, respectively. Positions at which all residues are identical are marked with an asterisk, and positions at which all residues are similar are marked with a plus sign. (Similarity of residues is defined as a score of ≥8 in a Dayhoff matrix of amino acid similarity [30].) A sequence similar to the consensus signal peptidase II cleavage sequence is underlined. The arrow indicates the N-terminal cysteine used for the covalent addition of fatty acids.

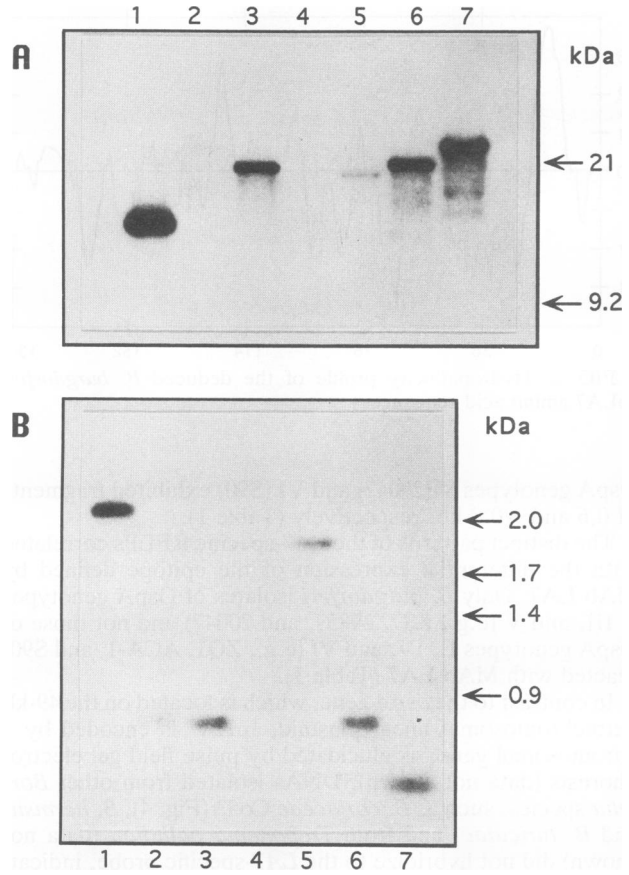

FIG. 4. Southern blot hybridization analysis. Whole-cell DNAs (5 μg) derived from the *B. burgdorferi* isolates were digested with *Bam*HI (A) or *Hin*dIII (B), electrophoretically separated on a 0.7% agarose gel, and transferred to a nylon membrane (Hybond-N). The DNAs were isolated from ZS7 (lane 1), ZQ1 (lane 2), 19857 (lane 3), *B. coriaceae* Co53 (lane 4), ACA-1 (lane 5), 21038 (lane 6), and 20047 (lane 7) (all *B. burgdorferi* isolates, unless otherwise indicated). Southern blots were probed with the *LA7*-specific probe.

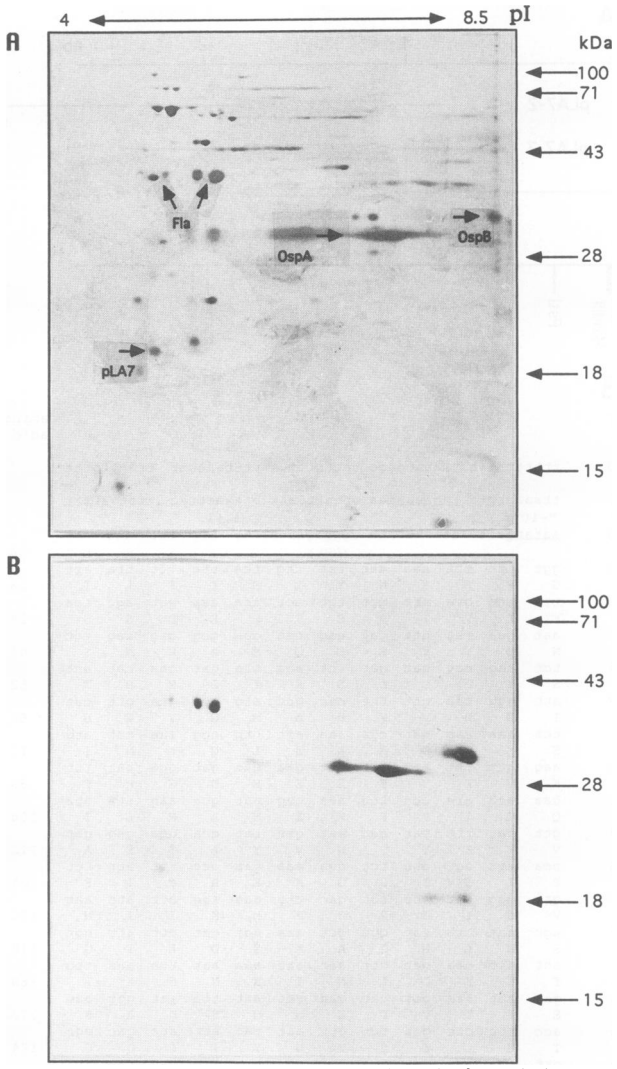

FIG. 5. Two-dimensional gels of *B. burgdorferi* whole-cell lysates (first dimension, pH 4 to 8.5; second dimension, SDS-PAGE [13% polyacrylamide]). Lysates were either silver stained (A) or electrophoretically transferred to a nylon membrane and reacted with a mixture of MAb LA7, LA21 (flagellin), LA2 (OspA), and LA32 (OspB) (B). Molecular masses are indicated on the right, and pI values are indicated across the top. The arrows in the blots indicate proteins subsequently identified by immunoblotting.

Analysis of IpLA7 by phase partitioning with Triton X-114. *B. burgdorferi* ZS7 was labeled with [^{3}H]palmitate and subsequently extracted with Triton X-114. Radiolabeled proteins were separated by one-dimensional SDS-PAGE and visualized by fluorography (Fig. 6A). From the unpartitioned preparations of strain ZS7, at least seven lipoproteins with apparent molecular masses of about 55, 34, 31, 24, 21, 19, and 14 kDa could be identified (Fig. 6A, lane 4). The most prominent polypeptides, with molecular masses of 31 and 34 kDa, are likely to represent the OspA and OspB antigens, respectively. All of the [^{3}H]palmitate-labeled polypeptides partitioned exclusively into the detergent phase. One of the low-molecular-mass proteins (about 20 kDa) presumably represents the IpLA7 antigen. For confirmation of this assumption, two-dimensional gel electrophoresis was performed with [^{3}H]palmitate-labeled *B. burgdorferi* ZS7. The proteins identified by two-dimensional SDS-PAGE of whole-cell lysates included IpLA7, OspA, and other as-yet-unidentified molecules (Fig. 6B). Because of the limited pI range (4 to 8), the OspB molecule was not seen; it has a calculated pI of 9.7.

Expression of IpLA7 in *E. coli*. We examined the expression of IpLA7 in *E. coli* transformed with plasmids carrying full-length (pLA7-2) or truncated (pLA7-A2) versions of the *LA7* gene. As controls, the full-length (pOspA) and truncated (pOspA-17) versions of the *ospA* gene were used in similar experiments (Fig. 7). The respective *E. coli* cells were extracted by detergent-phase partitioning and analyzed by gel electrophoresis. *E. coli* cells carrying plasmid pLA7-2 expressed one band with an apparent molecular mass of 21 kDa (Fig. 8, pLA7-2). *E. coli* cells harboring pOspA expressed a band of 31 kDa (Fig. 8, pOspA). In contrast, no lipoproteins with the expected molecular masses were formed from the truncated versions of *LA7* (pLA7-A2) and *ospA* (pOspA-17). However, expression of nonlipidic forms of OspA and IpLA7 by the two truncated clones, pOspA-17 and pLA7-A2, was revealed by immunoblotting (data not shown).

Subcellular localization of IpLA7. For exploration of the

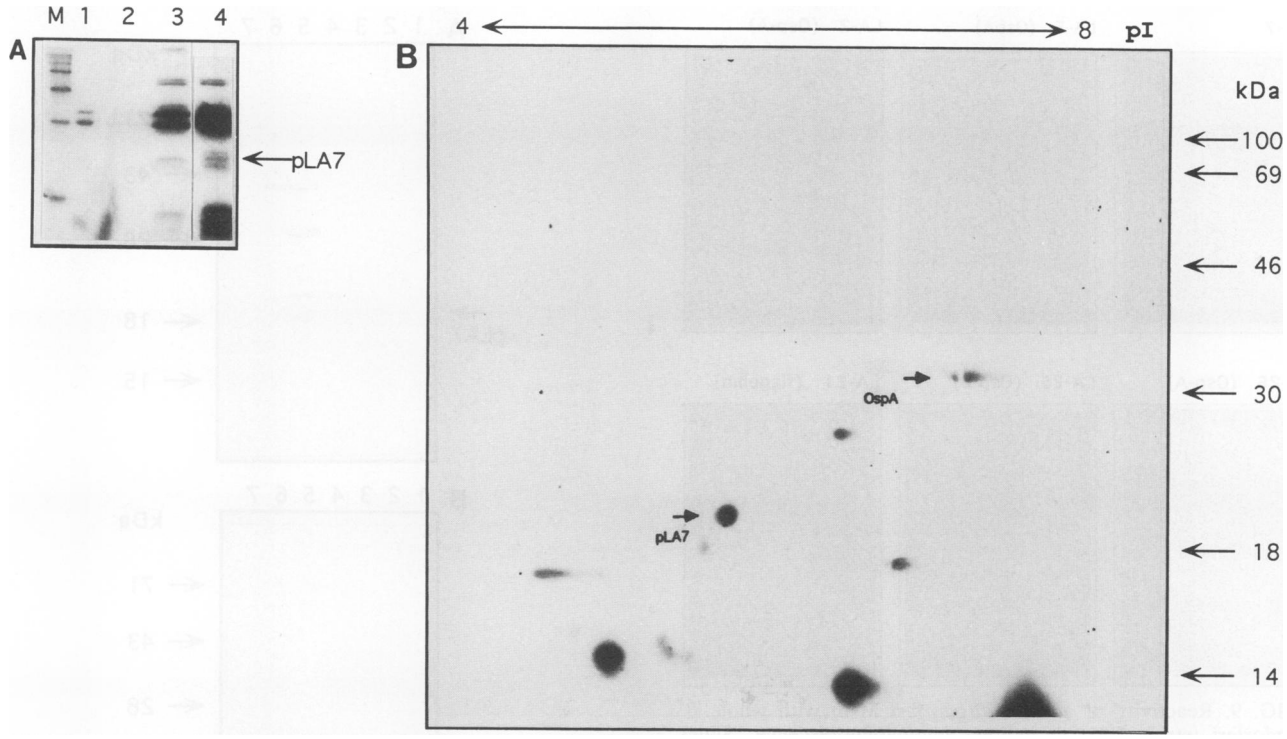

FIG. 6. (A) Triton X-114 fractionation of *B. burgdorferi* ZS7 proteins after biosynthetic labeling with [^{3}H]palmitate. Lanes: 1, insoluble proteins; 2 and 3, proteins in the aqueous and detergent phases, respectively; 4, whole *B. burgdorferi* cells. Molecular masses of marker proteins (M) were, from bottom to top, 14, 30, 46, 69, 100, and 200 kDa. (B) Two-dimensional gel of *B. burgdorferi* ZS7 lipoproteins, as identified by biosynthetic labeling with [^{3}H]palmitate. pLA7 = IpLA7.

localization of the LA7 epitope in intact spirochetes, MAb LA7 as well as a selected range of MAb directed against different *B. burgdorferi* structures were tested for staining of the bacteria by immunofluorescence (Fig. 9). As shown previously, two MAb specific for OspA (LA2 and LA26) and one MAb specific for OspB (LA25) yielded bright fluorescence staining, whereas another MAb specific for OspA (LA5) as well as flagellin MAb LA21 stained much more weakly. A patchy and weak staining pattern was also observed with MAb LA7, suggesting that the relevant epitope is not readily accessible to this MAb.

For further elucidation of the localization of the IpLA7 protein, intact *B. burgdorferi* ZS7 was treated with proteinase K and subsequently analyzed by SDS-PAGE (Fig. 10). The bands corresponding to OspA, OspB, and a 24-kDa protein showed decreased intensity compared with that of the untreated control (Fig. 10A). In contrast, IpLA7 was resistent to degradation under these conditions, as revealed by Western blot analysis with MAb LA7 (Fig. 10C). The fact

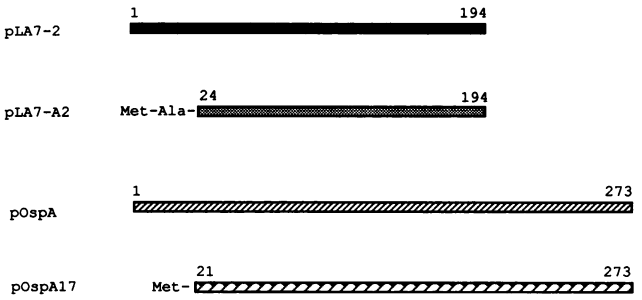

FIG. 7. Diagrammatic representation of the expressed recombinant *B. burgdorferi* proteins. The positions of the amino- and carboxy-terminal amino acids of the expressed recombinant proteins are indicated to the left and right of the bars. The truncated *LA7* gene (pLA7-A2) was amplified by the polymerase chain reaction from plasmid pLA7-2, which contains the full-length gene, by use of the following primer: 5′-TGCC ATG GCT TCA AAA GAT AGC TCA AAT GAA TAT GTT GAG GAG-3′. An *Nco*I restriction site (underlined) was placed at the 5′ end of the oligonucleotide to facilitate ligation of the amplified DNA into plasmid vector pTrcHisA (Invitrogen, San Diego, Calif.). The truncated *ospA* gene (pOspA17) was generated similarly from plasmid pOspA by use of the following primer: 5′-ACC ATG GTT AGC AGC CTT GAC GAG AAA AAC AGC-3′.

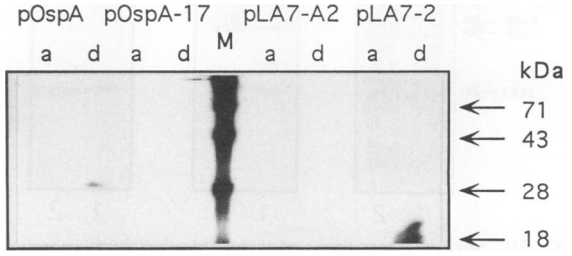

FIG. 8. Triton X-114 fractionation of recombinant proteins expressed in *E. coli* and biosynthetically labeled with [^{3}H]palmitate. Aqueous-phase (a) and detergent-phase (d) proteins of *E. coli* cells expressing the complete (pOspA) or truncated (pOspA-17) *ospA* gene and the complete (pLA7-2) or truncated (pLA7-A2) *LA7* gene were separated by SDS-PAGE and visualized by fluorography. Lane M shows marker proteins.

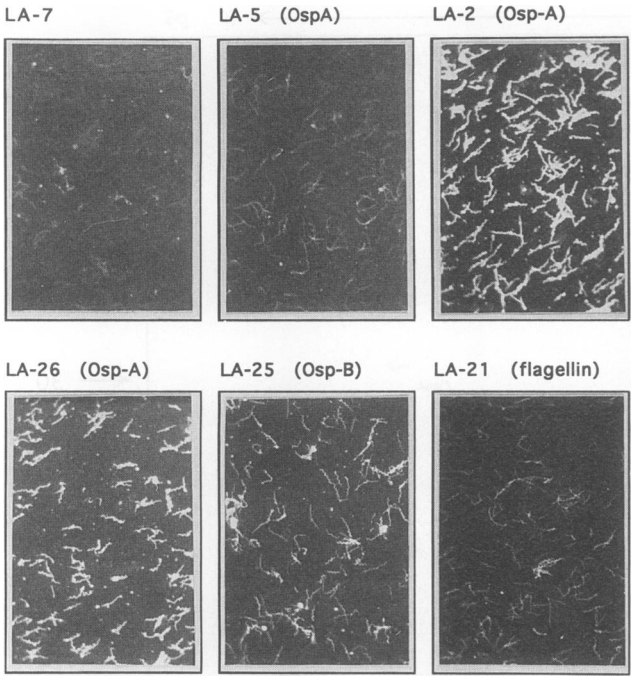

FIG. 9. Reactivity of anti-*B. burgdorferi* MAb with whole *B. burgdorferi* (strain B31) in indirect immunofluorescence. Slides were incubated with a 1:100 dilution in PBS (≈0.1 μg/ml) of hybridoma supernatants of the following MAb (this concentration was shown to be optimal under these conditions): LA7 and LA2 (OspA), LA5 (OspA), LA26 (OspA), LA25 (OspB), and LA21 (flagellin). Bound antibodies were visualized by use of fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin antibodies.

that other internally localized proteins, such as flagellin, also were not affected under similar conditions suggests that IpLA7 is also not accessible to exogenous proteolytic enzymes (Fig. 10B).

Immunoreactivity of IpLA7. Sera derived from mice previously inoculated with 10^8 *B. burgdorferi* (ZS7) cells were

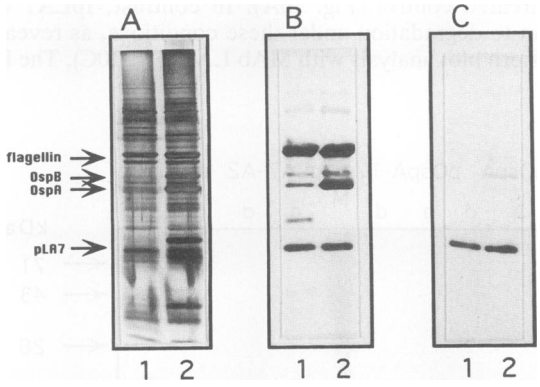

FIG. 10. Surface proteolysis of *B. burgdorferi* ZS7 indicating the degradation of OspA, OspB, and other proteins but not IpLA7. Borrelias were washed and exposed to proteinase K (0.5 mg/ml) for 40 min at 25°C (lanes 1) or left untreated (lanes 2). Proteins were separated by SDS-PAGE and visualized by Coomassie blue staining (A) or electrophoretically transferred to a nylon membrane and reacted with a mixture of MAb LA7, LA21 (flagellin), and LA2 (OspA) (B) or with MAb LA7 alone (C).

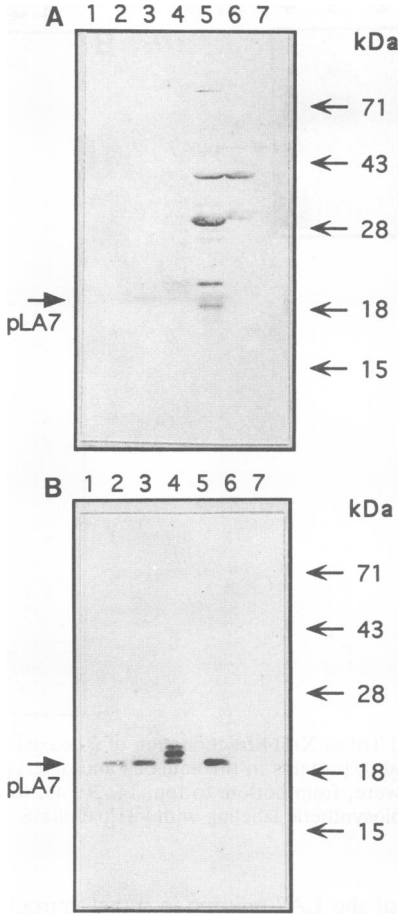

FIG. 11. Immunoblot analyses of IpLA7. Whole-cell lysates of *E. coli* carrying plasmids pLA7-A2 (lane 1), pLA7-8 (lane 2), and pLA7-2 (lane 3), affinity-purified recombinant IpLA7 derived from clone pLA7-2 (lane 4), whole-cell lysates of *B. burgdorferi* ZS7 (lane 5) and ZQ1 (lane 6), and whole-cell lysates of *E. coli* carrying the cloning vector (lane 7) were separated by SDS-PAGE and blotted onto nylon membranes. Blots were reacted with mouse anti-*B. burgdorferi* serum (A) or MAb LA7 (B).

shown to contain antibodies with specificity for IpLA7 (Fig. 11A) as well as the recombinant proteins encoded by pLA7-2 and pLA7-8; no reactivity was found with lysates from *E. coli* carrying only the cloning vector. In the affinity-purified preparation of recombinant IpLA7, two additional larger bands were stained with MAb LA7; these bands may represent artifacts generated during purification procedures (Fig. 11B).

In further experiments, human sera from (i) healthy donors, (ii) patients with ECM and ACA, and (iii) members of a high-risk group (forest workers) were similarly analyzed for the presence of IpLA7-reactive antibodies. As shown in Table 2, all serum specimens from healthy individuals (n = 5) were negative when tested in an ELISA (on B31 sonicate) and by Western blot analysis with a total *B. burgdorferi* lysate as well as recombinant IpLA7. All serum specimens from patients with ECM and ACA (n = 12) were positive when tested in an ELISA (on B31 sonicate) and by Western blot analysis with a total lysate but negative when tested by Western blot analysis with recombinant IpLA7. In contrast, three serum specimens from forest workers who were

TABLE 2. ELISA and immunoblot analyses of sera from healthy donors and patients with Lyme disease characterized by ECM or ACA

Serum specimens (*n*)	Result of:			
	ELISA[a]	Immunoblotting (total lysate)[b]	Immunoblotting (pLA7-2)[c]	Manifestation of Lyme disease
Normal seronegative (5)	−	−	−	None
Asymptomatic seropositive (3)	+	+	+	None
Symptomatic seropositive (5)	+	+	−	ACA
Symptomatic seropositive (7)	+	+	−	ECM

[a] Immunoglobulin G antibody responses to a whole, sonicated *B. burgdorferi* B31 lysate. The different sera were diluted 1:100. The cutoff point for a positive result was determined to be the mean of results from a normal population of 12 individuals with no known history of spirochetal infection plus 0.2 A_{492} unit.

[b] Criteria for positive blots were those recommended previously (11). Sera were reactive with the 41-kDa flagellin polypeptide and some antigens in the low-molecular-mass range (≤20 kDa).

[c] Sera were reactive with the recombinant IpLA7 polypeptide expressed in *E. coli* carrying plasmid pLA7-2.

healthy but were found seropositive in an ELISA (on B31 sonicate) and by Western blot analysis with a total lysate were also reactive with recombinant IpLA7 in Western blotting (Table 2).

DISCUSSION

In the present study, we cloned and characterized a novel 22-kDa chromosomally encoded lipoprotein of *B. burgdorferi*, termed IpLA7. Using an IpLA7-specific MAb, we observed differential expression of the respective epitope by various isolates. Furthermore, Southern blot analysis of 40 *B. burgdorferi* isolates indicated the existence of remarkable RFLPs among restriction fragments containing the gene and flanking sequences.

Like other bacterial lipoproteins, IpLA7 contains a signal sequence similar to the consensus sequence (Leu-X-Y-Cys) required for processing by signal peptidase II and for the subsequent covalent addition of fatty acids to the resulting N-terminal cysteine (37). The hydrophobic leader sequence further indicates that IpLA7 may be attached to membrane structures by its lipidic N terminus. IpLA7 was identified as a lipoprotein by its amphiphilic character and by its capacity to covalently attach to [^{3}H]palmitate. The deduced amino acid sequence of IpLA7 exhibited no significant homologies to those of other known borrelial proteins, such as OspA, OspB, pC, and OspD (4, 10, 16, 20).

The *LA7* gene was shown to be located on the chromosome of *B. burgdorferi*. Unlike other chromosomally derived genes (those for Fla, Hsp60, and Hsp70), which exhibit only limited genotypic variability, the *LA7* gene is highly variable and therefore represents the first example of a *B. burgdorferi* chromosomal gene with extensive polymorphisms. On the basis of RFLPs for the *LA7* gene, at least six distinct banding patterns defining six genomic groups of *B. burgdorferi* and corresponding to the previously described OspA genotypes could be observed (2, 31, 34). The fact that *LA7* is species specific, as judged by hybridization to DNAs from other species of the genus *Borrelia*, makes it a useful additional marker for the identification and typing of new isolates of *B. burgdorferi*.

Immunoblot analysis of individual *B. burgdorferi* isolates with MAb LA7 revealed differential expression of the epitope: all isolates of OspA genotypes I, III, and V stained positive, whereas all isolates of genotypes II, IV, and VI stained negative. The fact that DNAs from all *B. burgdorferi* isolates exhibited characteristic RFLP signals with the radiolabeled insert from plasmid pLA7-2 indicates that all tested isolates contained the respective gene but not necessarily the epitope recognized by MAb LA7.

It is known that lipid moieties act as hydrophobic membrane anchors that enable hydrophilic polypeptides to be positioned on appropriate membranes of the bacterium. Surface proteolysis of intact *B. burgdorferi*, which led to nearly complete degradation of OspA and OspB, did not significantly affect the reactivity of MAb LA7. This finding indicates that the majority of the IpLA7 lipoprotein is not surface exposed but rather is located in the periplasmic space. Whether this antigen is part of inner membranes or other structures of *B. burgdorferi* must be investigated by further analyses, e.g., by immunoelectron microscopy. Previous work demonstrated that additional 19- and 22-kDa lipoproteins of *B. burgdorferi* that are not exposed on the surface of the spirochete outer envelope exist (8a). In contrast to these antigens defined by MAb CB49 and CB625, which showed significant cross-reactivity when tested in an ELISA against bacterial antigens derived from other bacteria, e.g., *B. hermsii*, *T. phagedenis*, *Leptospira interrogans*, and *E. coli*, the antigen recognized by MAb LA7 showed species specificity (8a). Whether the antigens described by Coleman and Benach are similar to the IpLA7 lipoprotein or correspond to other, formerly described lipoproteins must be elucidated in further studies (6, 8a, 28).

The analysis of sera from infected mice clearly showed that native protein IpLA7 is immunogenic in this species. On the other hand, when human sera from uninfected individuals, individuals from a high-risk group (forest workers), or patients with ECM and ACA were tested, it was found that only sera from seropositive but healthy forest workers contained antibodies reactive with IpLA7. At present, there is no explanation for this discrepancy. Whether the coincidence of seroconversion and reactivity to IpLA7 with the asymptomatic status of the three infected forest workers is of significance and is a marker for protection against the development of Lyme disease must await further experimentation. However, it should be noted that adoptive transfer experiments with mice clearly demonstrated that immune sera to IpLA7, unlike those to OspA and OspB, were unable to protect *scid* mice against the development of arthritis (24a). In any case, the data clearly indicate that IpLA7 is also immunogenic, at least in part, in humans and suggest that this lipoprotein is a useful marker for the diagnosis of Lyme disease.

Finally, IpLA7 may be involved in the regulation of various host responses in mice and humans. The fact that lipoproteins of *E. coli* and *B. burgdorferi* have been shown to be mitogenic for B cells and to readily activate macrophages to produce inflammatory cytokines suggests a similar role for IpLA7 (5, 6, 9, 12, 23).

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