Regulated synthesis of the *Borrelia burgdorferi* inner-membrane lipoprotein IpLA7 (P22, P22-A) during the Lyme disease spirochaete’s mammal–tick infectious cycle

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Results of previous immunological studies suggested that *Borrelia burgdorferi* regulates synthesis of the IpLA7 lipoprotein during mammalian infection. Through combined use of quantitative reverse transcription PCR, immunofluorescence analyses, ELISA and immunoblotting, it is now demonstrated that IpLA7 is actually expressed throughout mammalian infection, as well as during transmission both from feeding ticks to naïve mice and from infected mice to naïve, feeding ticks. However, proportions of IpLA7-expressing *B. burgdorferi* within tick midguts declined significantly with time following completion of blood feeding. Cultured bacteria differentially expressed IpLA7 in response to changes in temperature, pH and concentration of 4,5-dihydroxy-2,3-pentanedione, the precursor of autoinducer 2, indicative of mechanisms governing IpLA7 expression. Previous studies also reported mixed results as to the cellular localization of IpLA7. It is now demonstrated that IpLA7 localizes primarily to the borrelial inner membrane and is not surface-exposed, consistent with the ability of these bacteria to produce IpLA7 throughout mammalian infection despite being the target of a robust immune response.

INTRODUCTION
The Lyme disease spirochaete, *Borrelia burgdorferi*, controls production of a large number of bacterial proteins to facilitate the many steps of its natural cycle between vertebrate hosts and tick vectors. The bacterium senses a variety of largely unknown environmental cues to determine its location throughout the infectious cycle and accordingly synthesizes proteins appropriate for each stage. Identification of regulated *B. burgdorferi* proteins and the mechanisms by which the spirochaete controls their expression are important for enhancing understanding of infectious mechanisms employed by the Lyme disease spirochaete.

Humans with late-stage Lyme disease frequently produce high levels of antibodies directed against a particular 22 kDa borrelial protein, while humans with early stages of the disease generally produce few or no immunoblot-detectable antibodies to this protein (Lam et al., 1994; Nowalk et al., 2006a). This antigen has been referred to as ‘IpLA7’, in that it is the antigen recognized by a monoclonal antibody named LA7 (Wallich et al., 1993), simply ‘LA7’ (Casjens et al., 1995; Crother et al., 2003; Nowalk et al., 2006a, b; Rauer et al., 2001), or ‘P22’, in reference to its approximate size (Lam et al., 1994). The gene encoding IpLA7 is annotated as open reading frame (ORF) BB0365, ORF BG0364 and ORF BAPKO0373 in the published genome sequences of strains B31, PBi and PKo, respectively (Fraser et al., 1997; Glöckner et al., 2004, 2006).
As do many other bacteria, Lyme disease spirochaetes produce 4,5-dihydroxy-2,3-pentanedione (DPD) and homocysteine from a byproduct of cellular methylation reactions, using the enzymes Pfs and LuxS (Babb et al., 2005; von Lackum et al., 2006). DPD and its spontaneously cyclized derivatives are collectively termed 'autoinducer 2' (AI-2), in that, for some bacteria, environmental levels of DPD affect gene expression (Bassler et al., 1994; Taga et al., 2001; Xavier & Bassler, 2003). The vast majority of eubacteria use homocysteine to regenerate methionine for cellular uses, but B. burgdorferi lacks that ability, suggesting that the LuxS enzyme is produced by B. burgdorferi solely for production of DPD (Babb et al., 2005; von Lackum et al., 2006). Further supporting that hypothesis, addition of DPD to B. burgdorferi culture medium leads to distinct changes in protein expression profiles, and comparisons between wild-type and luxS mutant B. burgdorferi likewise reveal differences in protein expression levels (Babb et al., 2005; Stevenson & Babb, 2002; Stevenson et al., 2003; von Lackum et al., 2006). In an extension of an earlier study (Babb et al., 2005), we tentatively identified IpLA7 as one of the B. burgdorferi proteins whose expression levels varied with addition of DPD to culture media. Those data, in addition to the apparent in vivo-regulated expression of IpLA7 suggested by previous immunological studies, led us to analyse IpLA7 protein and gene expression patterns throughout mammalian and arthropod infections. Cultured bacteria were also studied to provide insight into the mechanisms by which B. burgdorferi controls IpLA7 synthesis. In addition, previous labelling studies with radioactive lipids demonstrated that IpLA7 is a lipoprotein (Lam et al., 1994; Wallich et al., 1993), but conflicting results have been reported for cellular localization studies of this protein (Grewe & Nuske, 1996; Simpson et al., 1991; Wallich et al., 1993). Detailed analyses were performed, which indicated that IpLA7 is primarily anchored to the borrelial inner membrane.

**METHODS**

*B. burgdorferi*. Strain B31-MI is a non-clonal subculture of type strain B31, whose complete genome has been sequenced (Casjens et al., 2000; Fraser et al., 1997). Strain B31-MI-16 is a clonal derivative of B31-MI that is fully virulent, and contains all the plasmids carried by B31-MI (Miller et al., 2003). Strains A3tnra and A3tpoS are ΔpoN (tnra) and ΔpoS mutants, respectively, of strain B31 clone A3 (Elías et al., 2002; Fisher et al., 2005), and were gifts of Patricia Rosa and Mark Fisher (both of Rocky Mountain Laboratories, NIH, Hamilton, MT, USA). Strain B31-e2 is a non-infectious clonal subculture of B31 that lacks several of the parent's linear and circular plasmids (Casjens et al., 1997). Strain B313 is a non-infectious, clonal derivative of B31 that lacks all linear plasmids and most of the circular plasmids of the parental strain (Sädzień et al., 1993; Zückert et al., 1999). B. burgdorferi strain 297 is an infectious, wild-type bacterium (Stevere et al., 1983). Strain AH309 is a ΔluxS derivative of strain 297 (Hübner et al., 2003), and was a gift of Michael Norgard (University of Texas Southwestern, Dallas, USA). Unless otherwise noted, all B. burgdorferi were cultivated in Barbour–Stoenner–Kelly II (BSK-II) medium at 34°C (Barbour, 1984).

**Recombinant IpLA7.** Total bacterial DNA was purified from B. burgdorferi B31-MI-16 using DNAeasy (Qiagen). A 516 bp fragment of the B. burgdorferi B31 chromosome composed of the IpLA7-encoding gene (ORF BB0365), minus the leader sequence, was amplified by PCR using oligonucleotide primers KVL-LA7-7 (‘5'-GAGCAgGACAAgATGACCTTAAAgATagC-3’) and KVL-LA7-8 (‘5'-GAGGAAGACGCCCCGTTACGTtAAcATAGG-3’). The resulting amplicon was cloned into pET-30 Ek/Lic (Invitrogen) to produce plasmid pTagLPET, encoding an N- and C-terminal polyhistidine-tagged recombinant IpLA7 protein. The insert of this plasmid was completely sequenced to ensure that no mutations were introduced during cloning processes. Escherichia coli strain BL21(DE3)pLysS (Invitrogen) was transformed with pTagLPET, and recombinant IpLA7 purified using a MagneHis Protein Purification System (Promega). Protein purity was assessed by SDS-PAGE and staining with Coomassie brilliant blue. Protein concentration was assessed by bicinchoninic acid (BCA) protein assay (Pierce).

**Antibodies.** To generate antisera against IpLA7, a New Zealand White rabbit was injected intramuscularly and subcutaneously with 1.5 mg recombinant IpLA7 protein emulsified in Freund’s complete adjuvant. The rabbit was boosted 2 and 4 weeks after injection with a similar concentration of IpLA7 emulsified in Freund’s incomplete adjuvant, then exanguinated approximately 2 weeks after the final boost. Prior to use, antisera was preadsorbed with E. coli cellular lysates and passed through Hi-Trap protein A columns (Amersham). Antiserum specificity for IpLA7 was determined by immunoblot analyses of recombinant IpLA7 and total B. burgdorferi B31-MI-16 lysates.

Rabbit antisera raised against P22-A, the protein product of pSPR37 (Simpson et al., 1991), was provided by Tom Schwann (Rocky Mountain Laboratories, NIH, Hamilton, MT). Monoclonal antibodies recognizing B. burgdorferi FlaB, OspA and OspC were produced from hybridomas H9724, H5332 and B5, respectively (Barbour et al., 1983, 1986; Mbow et al., 2002). Polyclonal rabbit antisera specific for B. burgdorferi OppA-IV (Bono et al., 1998) was provided by Patricia Rosa.

**Immunoblot analyses.** Proteins were separated by SDS-PAGE using either a multi-well comb (for analyses of multiple samples) or a preparative, single-toothed comb (for multiple analyses of a single protein sample). Proteins were transferred to nitrocellulose membranes, blocked, then incubated with an appropriate antibody/serum. For analyses of IpLA7-directed antibodies in multiple serum samples, all sera were examined simultaneously using a Bio-Rad Mini-Protein II Multiscreen apparatus. Bound antibodies were detected using either enhanced chemiluminescence (Pierce), 1-Step NBT/BCIP (Nitro-blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate p-toluidine) colorimetric substrate (Pierce), or an Odyssey infrared imaging system (Li-Cor Biosciences). Immunoblot signal intensities were analysed using Image J 1.36b (USA National Institutes of Health), and reported as density of each IpLA7 signal relative to density of the control FlaB signal for that same sample. Each analysis was performed at least twice on samples from independent experiments.

**Mouse infections.** Thirty 4–6-week-old female BALB/c mice were infected by subcutaneous inoculation with 10⁸ B. burgdorferi B31-MI-16, a route and dose that results in antibody responses comparable to those following infection by natural tick-bite (de Souza et al., 1993; Schaible et al., 1993). This mode of infection was chosen to enable statistical comparisons between animals, since all were infected with identical numbers of B. burgdorferi. Although infection by tick bite is more natural, the numbers of bacteria transmitted by that route can vary wildly between animals, with variations in bacterial loads and immune system responses prohibiting statistical comparisons between groups of animals (our unpublished results,
and Miller, 2003). Nine animals each were euthanized 2 weeks, 4 weeks, or 2 months post-infection, and the remaining three mice after 5 months of infection, and all sera collected.

RNA isolation and analysis. One entire ear pinna and tibiotarsal joint, and the whole heart, were collected from each animal killed after 2 weeks or 5 months infection. Tissues were individually frozen in liquid nitrogen. DNA-free, total RNA was purified from all tissues (Miller, 2005). Control PCR analysis was used to confirm that each RNA sample was free of contaminating genomic DNA, as previously described (Miller, 2005). Next, 1 μg of each DNA-free RNA sample was reverse-transcribed using random primers and the First Strand cDNA Synthesis system (Roche Applied Science). Quantitative reverse transcription PCR (qRT-PCR) of B. burgdorferi ORF BB0365 and flaB (a constitutively expressed, internal control) mRNAs were performed on the first-strand cDNAs (Miller, 2005). The primer set KV-ORF BB0365-5 (5'-CAAGCTATGGAGTGATTTCAATCTC-3') and KV-ORF BB0365-6 (5'-GGAGCGCTTCCTCGCATACAAACATTG-3') was used to amplify a unique 150 bp segment of ORF BB0365, while primers FLA3 (5'-GGGTCTCAAGCGTCTTGG-3') and FLA4 (5'-GAACCGGTTGAGCAG-3') were used to detect flaB. All cDNA samples were analysed in triplicate. A negative control lacking primer template was included with each analysis to test for potential DNA contamination of reagents. Programmes used for melting curve analysis and cooling were as previously reported (Miller, 2005). To create a set of genomic standards, 1 ng B. burgdorferi B31-MI-16 genomic DNA was serially diluted tenfold (1 ng-100 fg) and included in every assay for both the ORF BB0365 and flaB primer sets. Results of the genomic standards were used to generate standard curves from which the amount of ORF BB0365 or flaB expression (ng) of each cDNA triplicate divided by the mean expression of control cDNA samples could be calculated using LightCycler 3.5.3 software (Roche). Primer specificity was tested by conducting melting curve analyses, examination of all reaction products for proper size and purity by agarose gel electrophoresis, and by sequencing of reaction products (Miller, 2005). Values for ORF BB0365 in each tissue sample are reported as the mean ORF BB0365 expression (ng) of each cDNA triplicate divided by the mean flaB expression (ng) of the same cDNA triplicate.

ELISA. IgM and IgG ELISAs were conducted on all collected mouse serum samples. Each well of 96-well plates was coated with 1 μg recombinant IpLA7, washed with PBS/0.5 % (v/v) Tween 20 (PBS-T), and blocked with PBS-T containing 10 % fetal bovine serum. Duplicate wells were then incubated with 200 μl of either 1:100-diluted B. burgdorferi-infected mouse sera or 1:100-diluted sera from two control, uninfected mice. Plates were washed and duplicate wells incubated with a 1:30000 dilution of either alkaline phosphatase-conjugated goat-anti-mouse IgM or goat anti-mouse IgG (Sigma). After washing again, 200 μl p-nitrophenyl phosphate was added to each well, and colour allowed to develop for 30 min. The absorbance at 405 nm was determined with a VersaMax microplate reader and Softmax Pro software (Molecular Devices). Mean values plus three standard deviations for uninfected sera were subtracted from mean values obtained for sera from the infected mice.

Ticks and mice. Egg masses laid by pathogen-free Ixodes scapularis ticks were obtained from the Department of Entomology, Oklahoma State University (Stillwater, OK, USA), and held in a humidified chamber until hatching (von Lackum et al., 2005). Larvae were then fed on cohorts of 6-8-week-old female BALB/c mice that had previously been infected by subcutaneous injection with 10^8 B. burgdorferi B31 MI-16. For studies of B. burgdorferi acquisition by ticks, feeding larvae were removed either 24, 48 or 72 h after placement on the infected mice. After 96 h, remaining ticks had engorged fully and naturally dropped off the mice. At that time point, some ticks were dissected immediately, while the remaining ticks were returned to the humidified chamber. Cohorts of larvae were dissected either 120, 144, 168, 192 or 264 h after the initiation of tick feeding, or were allowed to moult to the nymphal stage. Cohorts of unfed (flat) nymphs were also examined. For studies of B. burgdorferi transmigration from ticks to mice, 20 infected, flat nymphs each were fed on 4-6-week-old naïve female BALB/c mice. As above, feeding nymphal ticks were removed with forceps after 24, 48 or 72 h after placement on mice. Nymphs completed feeding and dropped off after 96 h and were either examined immediately or returned to the humidified chamber and examined at 120, 144, 168, 192 or 264 h after their placement on mice.

Indirect immunofluorescence analysis (IFA) of B. burgdorferi during tick and mammalian infections. Ticks were dissected and midguts removed as described previously (von Lackum et al., 2005). During forcible removal of a feeding tick, a piece of skin often remained attached to the tick hypostome. Those attached skin samples were removed from the tick and analysed separately. Tick midguts or mouse skin samples were immediately minced in 10 μl PBS on glass slides, and allowed to air dry overnight. Slides were processed essentially as previously described (von Lackum et al., 2005). Briefly, fixed and permeabilized tissues were sequentially incubated in a 1:350 dilution of IpLA7-specific rabbit polyclonal antisera, a 1:4 dilution of H9724 hybridoma supernatant (recognizing B. burgdorferi Flab), then 1:1000 dilutions of both Alexa Fluor 488-labelled goat anti-mouse IgG and Alexa Fluor 594-labelled goat anti-rabbit IgG (Molecular Probes). Slides were analysed at 400 × magnification using an Olympus BX51 epifluorescence microscope and a Q-Imaging Retiga 2000R Fast 1394 imaging system. Anti-FlaB-labelled bacteria within 25 random fields per slide were counted, and proportions of those bacteria also labelled with the IpLA7 antibody were calculated. As controls, slides of dissected tick midguts and skin biopsies were also incubated with either the IpLA7- or Flab-directed primary antibodies alone, or with only the secondary antibodies. A minimum of three ticks and three skin samples were examined at each time point.

Effects of culture conditions on B. burgdorferi protein expression. For temperature regulation studies, bacteria were first cultured at 25 °C in BSK-II to mid-exponential phase (approx. 10^7 bacteria ml^-1). Aliquots were then diluted 1:100 into fresh BSK-II, then incubated to mid-exponential phase at 34 °C (Schwan et al., 1993; Stevenson et al., 1995). For some studies, bacteria were repeatedly passaged at 34 °C by sequential 1:100 dilutions into fresh media.

To assess the effects of environmental pH, bacteria were cultivated at 34 °C in BSK-II medium plus 25 mM HEPES, adjusted to pH 6.5, 7.0 or 8.0 (Carroll et al., 1999). Cultures were harvested upon reaching mid-exponential phase and the pH of the used medium was retested.

DPP was synthesized using purified recombinant B. burgdorferi LuxS and PIs enzymes and 5-adenosylhomocysteine (Sigma) (Babb et al., 2005). AI-2 activities of DPP preparations were assessed by Vibrio harveyi luminescence bioassay (Taga, 2005). V. harveyi strains BB120 (luxS^-) and BB170 (ΔluxN) were obtained from Bonnie Bassler (Princeton University, Princeton, NJ, USA) (Bassler et al., 1997; Taga, 2005). Synthetic DPP/AI-2 was added to early exponential phase (approx. 10^6 bacteria ml^-1) cultures of B. burgdorferi B31-MI-16 to final concentrations of 1.0 μM (Schauder et al., 2001). Cultures were incubated at 34 °C for an additional 2 days (approx. four doublings), then bacteria were harvested by centrifugation (Babb et al., 2005).

Mapping the start of ORF BB0365 transcription. DNA-free, bacterial RNA was isolated from cultured B. burgdorferi B31-MI-16 (Miller, 2005). Control PCR analysis was used to confirm that samples were free of contaminating genomic DNA, as described above. The 5'-RACE (5'-rapid amplification of cDNA ends) System 2.0 (Invitrogen) was used to map the ORF BB0365 transcriptional initiation site. Briefly, single-stranded cDNA derived from ORF...
BB0365 mRNA was synthesized from total B. burgdorferi RNA using the ORF BB0365-specific primer 5′-ATCCTTTTGTAGAGAAG-3′. RNA was then degraded, the single-stranded cDNA product purified, and a poly-dC tail added to its 3′ end via terminal deoxynucleotidyl transferase. That product was then PCR amplified using a primer complementary to the poly-dC end (Invitrogen) and the nested ORF BB0365-specific primer 5′-CTCCTC-GCTTCTT-GCTCCT-3′. The double-stranded PCR product was purified by agarose gel electrophoresis, then both strands were sequenced. DNA sequences 5′ of the determined transcriptional start site were then compared with genomes of B. burgdorferi strain B31-M1 and other organisms using BLAST-N of the B31-M1 genome sequence (http://tigrblast.tigr.org/cmr-blast) and GenBank (http://www.ncbi.nlm.nih.gov/blast), respectively.

Cellular localization of IpLA7. Localization was first addressed by IFA determination of accessibility of antibodies to IpLA7 in intact B. burgdorferi (Zückert et al., 1999). Briefly, methanol-fixed or intact spirochaetes were incubated with antibodies specific for IpLA7, OspA, OspC or FlaB, followed by incubation with either FITC-labelled goat-anti-rabbit IgG (whole molecule) (Sigma-Aldrich) or goat-anti-mouse IgG (H+L) (Kierkegaard & Perry Laboratories). Cells were analysed by epifluorescence microscopy using a Nikon Eclipse E600 microscope fitted with a FITC-HYQ filter block and a Q-Imaging Micropublisher Digital CCD colour camera.

In a second series of experiments, intact, live B. burgdorferi were treated by surface proteolysis (Bunikis & Barbour, 1999; El-Hage et al., 2001). Briefly, mid-exponential phase cultures of B. burgdorferi were harvested, washed, then incubated in 200 μg proteinase K ml−1 for 1 h at room temperature. Proteolysis was stopped by addition of PMSF (Sigma) to a final concentration of 1.6 μg ml−1. Bacteria were lysed by immersion in boiling water, and proteins separated by SDS-PAGE followed by transfer to nitrocellulose membranes. Protease degradation of specific proteins was assessed by immunoblot analyses using antibodies specific for IpLA7 and control proteins OspA, OspC and FlaB.

In a third method, inner- and outer-membrane fractions of B. burgdorferi were isolated by isopycnic centrifugation as previously described (Carroll & Gherardini, 1996; Carroll et al., 2001; Skare et al., 1995). Proteins of each fraction were then separated by SDS-PAGE and analysed by immunoblotting, as described above.

RESULTS

Regulated expression of IpLA7 during the B. burgdorferi infectious cycle

Results of earlier studies suggested that synthesis of IpLA7 might be regulated during mammalian infection. To pursue that possibility in detail, cohorts of mice were infected with known quantities of B. burgdorferi, then tissues and sera were examined after either 2 weeks (early stage infection) or 5 months (disseminated, later-stage infection), and sera alone at 4 or 8 weeks post-infection. Immunoblot analyses of sera from these mice indicated patterns of IpLA7-directed antibody development similar to those seen from tick-borne infection in humans and mice (Fig. 1a) (Lam et al., 1994; Nowalk et al., 2006a). Weak immunoblot signals were obtained when examining serum from mice infected for 2 weeks, and very strong signals from animals infected for longer durations. In contrast, ELISA indicated early IgM and IgG antibody responses against IpLA7, which increased two- to threefold over 5 months of infection (Fig. 1b).

Tissues from those same animals were then analysed using qRT-PCR to assess mRNA levels during early (2 weeks) and a later stage (5 months) of infection. IpLA7-encoding transcript levels were normalized to those of the constitutively expressed B. burgdorferi flaB gene. These analyses indicated that ORF BB0365 is expressed during both early and later stages of mammalian infection, albeit with a trend toward increased ORF BB0365 expression as infection progressed (Fig. 2, P = 0.0251 by t-test).

As noted in the Introduction, several B. burgdorferi proteins have been observed to be expressed during mammalian infection but at reduced levels during colonization of vector ticks. We therefore employed IFA to examine IpLA7 expression during tick acquisition, colonization and transmission, points in the infectious cycle during which B. burgdorferi are readily visualized. As an initial step, we confirmed that the polyclonal antiserum raised against recombinant IpLA7 is specific for that protein, and does not recognize any other components of B. burgdorferi (data not

Fig. 1. Temporal analyses of IpLA7-directed immune responses of infected mice. (a) Immunoblot analyses of serum samples from different mice experimentally infected with B. burgdorferi B31 for 2 weeks (left four lanes) or 5 months (20 weeks, right four lanes). Purified recombinant IpLA7 protein was applied to the broad, single well of a preparative SDS-polyacrylamide gel. Following transfer to nitrocellulose, immunoblots were performed simultaneously using a multiwell apparatus. (b) Results of IgM (grey bars) and IgG (black bars) ELISA of serum samples from those same mice, plus others infected with B. burgdorferi for 4 or 8 weeks. Wells contained 1 μg recombinant IpLA7.
shown). Ticks and mouse skin tissues were then dissected and examined by IFA using the IpLA7 antiserum. All tissues were examined simultaneously by IFA using a monoclonal antibody recognizing the constitutively expressed \textit{B. burgdorferi} FlaB protein, to identify all \textit{B. burgdorferi} within those tissues. Essentially all spirochaetes expressed IpLA7 as they were acquired by tick larvae feeding on infected mice (Fig. 3a). Percentages of bacteria expressing IpLA7 remained elevated for 192 h (8 days) after initiation of feeding, then declined significantly over the next 3 days. Following the moult from larval to nymphal stage, fewer than half the \textit{B. burgdorferi} within infected tick midguts expressed detectable levels of IpLA7 (Fig. 3b). Feeding by tick nymphs was accompanied by an increase in overall IpLA7 expression, reaching a maximum at the time of engorgement and natural drop-off (Fig. 3b). Proportions of IpLA7-expressing bacteria then declined significantly 3–4 days following drop-off. Noting that \textit{B. burgdorferi} change their protein expression profiles during the transmission process, we also examined bacteria within mouse skin at the tick-bite site, which revealed that essentially all spirochaetes in those host tissues produced IpLA7, regardless of duration of tick feeding (Fig. 3c).

**Insight into mechanisms by which \textit{B. burgdorferi} controls IpLA7 expression**

Changes in many different culture conditions can lead the Lyme disease spirochaete to alter gene expression patterns. Comparisons of different culture conditions can point toward signals encountered by \textit{B. burgdorferi} in the natural...
environment that cause modulation of gene expression. Moreover, identification of conditions that affect gene expression in vitro enable molecular dissection of the mechanisms controlling gene expression levels. All analyses were repeated at least twice using independent cultures.

As do many other pathogens, *B. burgdorferi* regulates expression of many genes in response to culture temperature (Bykowski et al., 2006; Ojaimi et al., 2003; Revel et al., 2002; Schwan et al., 1995; Stevenson et al., 1995, 2006). *B. burgdorferi* was therefore cultured either at a constant 23°C or shifted from 23 to 34°C (Schwan et al., 1995; Stevenson et al., 1995). Bacteria grown at the warmer temperature expressed a mean of 2.3-fold greater levels of IpLA7 than did the spirochaetes maintained at 23°C (Fig. 4a). IpLA7 expression levels remained elevated during continual cultivation at 34°C (data not shown).

*B. burgdorferi* also regulates expression of many proteins in response to environmental pH, presumably reflecting the many pH changes encountered by Lyme disease spirochaetes during infection processes (Bykowski et al., 2006; Carroll et al., 1999; Revel et al., 2002; Stevenson et al., 2006; von Lackum et al., 2005; Yang et al., 2000). Cultivation at pH 8.0 led to 1.9- or 1.6-fold greater mean levels of IpLA7 than did cultivation at pH 6.5 or 7.0, respectively (Fig. 4a).

As noted above, *B. burgdorferi* cultured in the presence of additional DPD produce different levels of certain proteins than do bacteria cultivated in medium lacking DPD. Proteomic analyses of one such protein tentatively identified it as IpLA7 (data not shown). We therefore assessed the effect of DPD on IpLA7 expression. First, wild-type *B. burgdorferi* B31-MI-16 were grown either in the absence or presence of 1 μM DPD. Immunoblot analyses of whole-cell lysates indicated that addition of DPD led to a mean 1.7-fold increase in IpLA7 (Fig. 4b). Second, since *B. burgdorferi* produces DPD/AI-2 during cultivation in laboratory media (Babb et al., 2005), levels of IpLA7 produced by cultured wild-type and an isogenic luxS mutant were compared. Those analyses revealed that the mutant produced approximately 1.7-fold less IpLA7 than did the wild-type parental strain (Fig. 4b).

*B. burgdorferi* produces three RNA polymerase sigma subunits: the housekeeping σ70, and homologues of σ5 and σN (Fraser et al., 1997). Several *B. burgdorferi* genes induced during transmission from feeding ticks to mammals are regulated by one or both of those alternative sigma subunits (Caimano et al., 2004; Fisher et al., 2005; Hübner et al., 2001; Yang et al., 2005). We therefore examined whether either σ5 or σN is involved in IpLA7 synthesis, by examining production of that protein by wild-type strain B31-A3 and isogenic rpoS and rpoN mutants. Both mutant strains exhibited IpLA7 levels identical to the wild-type parent, at both 23 and 34°C culture temperatures (data not shown). Control immunoblots indicated that FlaB was also unaffected by the mutations, while OsPc was not expressed by either mutant, as previously reported (Hübner et al., 2001; Yang et al., 2005) (data not shown). Since transcription requires use of a sigma factor, these results indicate that ORF BB0365 mRNA synthesis must be directed by RNA polymerase holoenzyme containing the σ70 subunit.

### The ORF BB0365 promoter region

Next, the transcriptional start site of the IpLA7-encoding gene was mapped to enable comparisons with other characterized *B. burgdorferi* genes. A single start site was identified, with appropriately located potential −10 and −35 sequences (Fig. 5). Also 5’ of this region is a DNA sequence (TGTAACT) that differs by only 1 bp from the sequence recognized by the *B. burgdorferi* EbfC DNA-binding protein (TGT^N/TACA) (Babb et al., 2006). *B. burgdorferi* erp loci contain EbfC binding sites 5’ of transcriptional promoters, within a DNA region that is involved with regulation of transcription (Babb et al., 2006). BLAST-N analyses of the IpLA7-encoding gene promoter did not reveal appreciable similarities to any other sequences of *B. burgdorferi* or other bacteria.

### IpLA7 is identical to the ‘P22-A’ antigen

A previously produced polyclonal antiserum specifically recognizes a 22 kDa *B. burgdorferi* antigen, identified as ‘P22-A’ (Simpson et al., 1991). Restriction mapping of the *B. burgdorferi* chromosome indicated that the genes encoding P22-A and IpLA7 are closely linked, although a separate gene encoding a different ‘P22’ is also located nearby.
Surface proteins (Bunikis & Barbour, 1999; Noppa et al., 1995) block interactions between antibodies and other borrelial outer-surface proteins. This suggests that surface-exposed proteins are protected from proteases due to interactions with other outer-surface proteins. However, some B. burgdorferi surface-exposed proteins are protected from proteases due to interactions between those proteins and other borrelial outer-surface proteins (Bunikis & Barbour, 1999; Noppa et al., 2001; Sadziene et al., 1995). To examine that possibility, we also examined IpLA7 for susceptibility to in situ proteolysis. Surface-exposed proteins of intact bacteria can be degraded by proteases, whereas subsurface proteins are protected from proteolysis. Much as reported previously (Wallich et al., 1993), proteinase K was unable to degrade IpLA7 from intact B31 bacteria (Fig. 6b and data not shown). However, some B. burgdorferi surface-exposed proteins are protected from proteases due to interactions between those proteins and other borrelial outer-surface proteins (Bunikis & Barbour, 1999; Noppa et al., 2001; Sadziene et al., 1995). To examine that possibility, we also examined IpLA7 for susceptibility to in situ proteolysis.

### Localization of IpLA7 in B. burgdorferi

Previous reports on IpLA7 listed conflicting results of localization studies of the lipoprotein within the bacterium (Grewe & Nuske, 1996; Simpson et al., 1991; Wallich et al., 1993). Other studies have demonstrated limitations to some of the techniques used earlier (Bunikis & Barbour, 1999; Noppa et al., 2001; Sadziene et al., 1995), also clouding the picture of IpLA7 localization. Since accurate knowledge of the cellular localization of IpLA7 will be extremely important for studies of its function, and given the contradictory results of previous studies, we sought to answer that question using several different, independent techniques in two laboratories (those of W. R. Zücket and J. A. Carroll). Comparable results were obtained from both laboratories, strengthening our conclusions.

As a first step, intact B. burgdorferi were examined for ability to bind IpLA7-directed antibodies, which would be indicative of IpLA7 surface-exposure. There are precedents in which abundant B. burgdorferi surface proteins physically block interactions between antibodies and other borrelial surface proteins (Bunikis & Barbour, 1999; Noppa et al., 2001; Sadziene et al., 1995). For that reason, we examined both B31-e2, a subculture of strain B31 that expresses abundant surface proteins such as OspA, and B313, which produces very few surface proteins. Intact and permeabilized B. burgdorferi were examined by IFA using antibodies directed against IpLA7, the periplasmic FlaB protein, and either of two surface-exposed proteins, OspA and OspC. Regardless of the variant examined, unfixed, non-permeabilized bacteria were unable to bind IpLA7-directed antibodies (Fig. 6a). FlaB was also inaccessible, while surface OspA or OspC were readily detected. As a control, bacteria permeabilized by methanol were also examined by IFA, which confirmed that the examined spirochaetes did indeed produce IpLA7 and the other proteins.

Next, IpLA7 was examined for susceptibility to in situ proteolysis. Surface-exposed proteins of intact bacteria can be degraded by proteases, whereas subsurface proteins are protected from proteolysis. Much as reported previously (Wallich et al., 1993), proteinase K was unable to degrade IpLA7 from intact B31 bacteria (Fig. 6b and data not shown). However, some B. burgdorferi surface-exposed proteins are protected from proteases due to interactions between those proteins and other borrelial outer-surface proteins (Bunikis & Barbour, 1999; Noppa et al., 2001; Sadziene et al., 1995). To examine that possibility, we also...
tested *B. burgdorferi* strain B313 for susceptibility of its IpLA7 to proteinase degradation. Proteases were unable to digest IpLA7 from intact, live B313 bacteria (Fig. 6b), further strengthening the conclusion that IpLA7 is not exposed to the borrelial outer surface.

Finally, inner and outer membranes of cultured *B. burgdorferi* were purified and analysed for presence of IpLA7. The protein was primarily associated with the inner-membrane-enriched/protoplasmic cylinder fractions, although some was evident in outer-membrane fractions (Fig. 6c and data not shown). A control marker for purity of membrane preparations, OppA-IV (Bono *et al*., 1998), was found only in inner-membrane fractions. The outer-surface proteins OspA or OspC were found in both the outer-membrane- and inner-membrane-enriched/protoplasmic cylinder fractions, probably due to inclusion of intact bacteria in the latter fraction. These data lead to the conclusion that the IpLA7 lipoprotein is primarily tethered to the inner membrane.

**DISCUSSION**

*B. burgdorferi* expressed the IpLA7-encoding gene during both early and later stages of murine infection. Infected animals developed antibody responses consistent with a protein synthesized by *B. burgdorferi* throughout infection. Intriguingly, serum samples collected 2 weeks after infection contained levels of IgG antibodies that were approximately half the levels of IgG in sera collected after 5 months of infection, yet immunoblot analyses scarcely detected any of the antibodies in 2 week sera while showing robust signals from 5 month sera. Detection of antibodies by ELISA but not immunoblots has been described for at least one other *B. burgdorferi* protein synthesized during mammalian infection (Rossmann *et al*., 2006). A possible explanation for these observations is that antibodies produced early in infection may tend to be directed against conformational epitopes, and proportionally more linear epitopes are targeted as infection progresses (Rossmann *et al*., 2006). Earlier studies using immunoblots analyses reported that IpLA7-directed antibodies were primarily found in sera from humans and animals infected for long periods of time (Lam *et al*., 1994; Nowalk *et al*., 2006a; Wallich *et al*., 1993). Our data suggest that those results may have been due to the low sensitivity of immunoblotting for detecting conformational epitopes, rather than expression of IpLA7 only during late stages of mammalian infection.

During its natural vertebrate–tick infectious cycle, *B. burgdorferi* controls synthesis levels of many bacterial proteins. All borreliae transmitted from infected, feeding ticks to mammals expressed detectable levels of IpLA7, as also did bacteria acquired by naïve ticks feeding on infected mice, and qRT-PCR demonstrated that ORF BB0365 is transcribed both early and late in mammalian infection. Two previous, limited studies of transiently infected rabbits and SCID mice also detected early production of IpLA7 (Crother *et al*., 2003, 2004). Significant differences in IpLA7 expression levels were observed during colonization of vector ticks. Following acquisition by feeding tick larvae, the proportion of *B. burgdorferi* that produced IpLA7 diminished significantly. Reduced levels were maintained through the moult to the nymph stage. The percentage of *B. burgdorferi* synthesizing detectable levels of IpLA7 increased as those nymphs fed, but then declined significantly within a few days of engorgement and drop-off. Thus, *B. burgdorferi* regulates synthesis of IpLA7 during its mammal–tick infectious cycle, with an expression pattern suggesting a function of greater importance for transmission and/or infection of mammals than for colonizing ticks.

Increased temperature, pH and concentrations of DPD enhanced IpLA7 production by *B. burgdorferi*, phenomena that are proving important in ongoing studies to characterize the molecular basis of IpLA7 regulation. The effects of culture temperature are consistent with the increased proportion of IpLA7-expressing spirochaetes in feeding tick nymphs, in which bacteria are exposed to warm host blood, and with previous global array analyses of temperature-induced *B. burgdorferi* genes (Ojaimi *et al*., 2003; Revel *et al*., 2002). During transmission from tick to mammalian host, *B. burgdorferi* experiences an alkaline pH in the tick’s salivary glands (Sauer *et al*., 1995; Yang *et al*., 2000). Expression of *luxS* by *B. burgdorferi* within tick nymph midguts increases as the tick ingests blood (Narasimhan *et al*., 2002), suggesting that concentrations of DPD/AI-2 also increase during that time period. Also of note, the present study found that, 7 days after completion of tick feeding, less than 20% of bacteria in nymph midguts expressed detectable levels of IpLA7, while approximately 60% of spirochaetes in 7 day post-fed larval midguts expressed the protein. This may have been due to the differences between larval and nymphal midgut physiologies (Balashov, 1972). It is also possible that context plays a role in IpLA7 expression: bacteria in the larval midguts had recently been acquired from infected mammalian hosts, while bacteria in the post-fed nymphal midguts had colonized that organ for several weeks, then been exposed to mouse blood, but had remained attached to the midgut. Altogether, results of these in vivo and in vitro studies indicate that *B. burgdorferi* detects and responds to a complex array of stimuli to regulate expression of IpLA7.

As with so many other *B. burgdorferi* proteins produced during mammalian infection, the biological function of IpLA7 is not known. Homologues of ORF BB0365 have been identified in every Lyme disease spirochaete yet examined, but are lacking from other species of *Borrelia* (Simpson *et al*., 1991), indicating a function for IpLA7 that is specific for Lyme borreliae. **BLAST-P** (http://www.ncbi.nlm.nih.gov/blast/) analyses have not revealed significant sequence similarities between IpLA7 and any other known protein. Structural modelling using Phyre (http://www.sbg.bio.ic.ac.uk/phyre) suggests moderate similarities (35–40% estimated precision) with a staphylococcal cysteine protease
and a β-lactamase/penicillin-binding protein of the syphilis spirochaete Treponema pallidum (Cha et al., 2004; Deka et al., 2002; Filipek et al., 2004). Ongoing studies, including production and analyses of IpLA7-deficient bacteria, will help further our understanding of this protein’s function.

Studies from one of our laboratories (W. R. Zückert) demonstrated that B. burgdorferi exports lipoproteins to the outer leaflet of the outer membrane by default, but retains certain lipoproteins within the inner membrane when directed by as-yet-unidentified amino acid sequence cues (Schulze & Zückert, 2006). The sorting mechanism for these spirochaetes is distinctly different from that employed by Gram-negative, enteric bacteria such as E. coli. Very few inner-membrane lipoproteins have been identified in B. burgdorferi, so our finding that IpLA7 is such a protein will be very important for elucidation of the mechanisms responsible for lipoprotein trafficking in Lyme disease spirochaetes.

In conclusion, B. burgdorferi synthesizes IpLA7 during all stages of mammalian infection, and infected animals produce antibodies against IpLA7 throughout infection. However, bacterial levels of IpLA7 decreased significantly following acquisition by tick larvae. B. burgdorferi in midguts of feeding, infected tick nymphs increased production of IpLA7, and those bacteria remaining in the nymph midgut again decreased IpLA7 production following completion of blood feeding. In the laboratory, IpLA7 production was affected by changes in temperature, pH and levels of DPD/AI-2, data that will be important in dissecting the mechanisms by which the spirochaete controls expression of IpLA7. This lipoprotein localized to the B. burgdorferi inner membrane, a possible explanation for the ability of these bacteria to persistently infect immunocompetent mammals while continuing to express the antigenic IpLA7 protein. Since previous studies determined that IpLA7 is specific to Lyme disease borreliae (Simpson et al., 1991), continued characterization of this lipoprotein will provide further insights into the unique biology of B. burgdorferi and the pathogenesis of Lyme disease.

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