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The lipoprotein La7 contributes to *Borrelia burgdorferi* persistence in ticks and their transmission to naïve hosts

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Abstract

La7, an immunogenic outer membrane lipoprotein of *Borrelia burgdorferi*, produced during infection, has been shown to play a redundant role in mammalian infectivity. Here we show that La7 facilitates pathogen survival in all tested phases of the vector-specific spirochete life cycle, including tick-to-host transmission. Unlike wild type or *la7*-complemented isolates, isogenic La7-deficient spirochetes are severely impaired in their ability to persist within feeding ticks during acquisition from mice, in quiescent ticks during larval-nymphal inter-molt, and in subsequent pathogen transmission from ticks to naïve hosts. Analysis of gene expression during the major stages of the tick-rodent infection cycle showed increased expression of *la7* in the vector and a swift downregulation in the mammalian hosts. Co-immunoprecipitation studies coupled with liquid chromatography-mass spectrometry analysis further suggested that La7, a highly conserved and abundant inner membrane protein, is involved in protein-protein interaction with a discrete set of borrelial ligands although biological significance of such interactions remains unclear. Further characterization of vector-induced membrane antigens like La7 and its interacting partners will likely aid in our understanding of the molecular details of *B. burgdorferi* persistence and transmission through a complex enzootic cycle.

Keywords

Borrelia burgdorferi; Lyme disease; La7 protein; transmission; tick-borne

1. Introduction

Borrelia burgdorferi, the spirochete pathogen of Lyme borreliosis, survives in a complex enzootic life cycle involving *Ixodes scapularis* ticks and specific vertebrate hosts, primarily

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wild rodents. Humans, however, as well as a number of domesticated animals, are incidental hosts and risk becoming infected with *B. burgdorferi* during a blood meal engorgement by *I. scapularis* [1]. *Borrelia burgdorferi* replicates and persists locally at the site of the tick bite over the course of several days to several weeks [2]. It then spreads to several other internal organs such as, the heart, joints, and the central nervous system, where the pathogen often triggers clinical complications including, arthritis, carditis, and various neurological disorders [2, 3]. If detected and treated early on, the infection can be cured with antibiotics. Some patients, however, will still develop arthritis with antibiotic resistance, which is unrelated to the active infection [4]. Since a vaccine is currently unavailable for human use, the identification of borrelial gene-products that support the pathogen persistence *in vivo* and their characterization as potential vaccine candidates or drug targets remains a primary focus of Lyme disease research.

Despite its relatively small size of 1.5 Mb, the *B. burgdorferi* genome exhibits remarkable structural and functional redundancy [5-7]. This is apparent due to the presence of a significant number of paralogous gene clusters, and by experimental evidence that many borrelial genes are expressed *in vivo* yet play nonessential roles in infectivity [8-13]. On the other hand, a few unique gene-products have been identified, which are essential for infectivity [14, 15]. One of these, La7 (also known as BB0365 or P22) [16-18] has been suggested to play an important role in spirochete survival within feeding ticks [17]. This chromosomally-encoded and conserved gene encoding a 22kDa lipoprotein is unique among infectious *B. burgdorferi* isolates that cause Lyme disease [7, 19]. *la7* is expressed in the mammalian host and in ticks [17, 20, 21], and is serologically recognized in infected mammals [22], sometimes during the late stages of human Lyme disease [16]. Although, the deletion of *la7* did not affect the spirochete infection in murine hosts, the mutants exhibited an impaired ability to persist within feeding ticks [17]. These initial studies suggest that La7 plays an important role in infectivity in ticks, however, because of a lack of a genetic complementation [17], a conclusive role of this antigen supporting borrelial life cycle remains uncertain. Here, we show that a stable genetic complementation of La7 not only rescues the inability of *la7* mutants to survive within the feeding vector, but it also supports the ability of the spirochetes to persist throughout the intermolt stages of the ticks as well as their subsequent transmission back to naïve hosts. Although La7 is shown to localize primarily to the borrelial inner membrane [21], the protein is also detectable in the outer membrane [23], probably due to its ability to form protein complexes in the outer membrane [24]. Here we also provided additional evidences that La7 is involved in protein-protein interaction involving specific spirochetes proteins. Understanding the molecular function of membrane antigens that support microbial persistence throughout an enzootic infection cycle is not only important in our understanding of intriguing biology of spirochetes and pathogenesis of Lyme disease, but in developing effective preventative strategies to combat infection.

2. Materials and methods

2.1. *Borrelia burgdorferi*, mice and ticks

A *B. burgdorferi* infectious isolate B31-5A11 was used in this study [25]. Four to six-week-old C3H/HeN mice were purchased from the National Institutes of Health. *Ixodes scapularis* ticks used in this study originated from a colony that is maintained in the laboratory.

2.2. PCR

The primers used in PCR reactions are indicated in supplementary Table 1. For analysis of *la7* (also annotated as *bb0365*) expression, *B. burgdorferi*-infected *I. scapularis* were collected as feeding larvae at 66 hours of feeding on infected mice, as repleted larvae 21

days after feeding, and as freshly-molted infected feeding nymphs after 48 and 66 hours of attachment, as detailed earlier [26, 27]. To assess *la7* expression in the host, mice (5 animals/group) were inoculated with *B. burgdorferi*. The skin samples were collected at weekly intervals until four weeks of infection and were then pooled together [28]. Pathogen burden in ticks or in mice were detected using quantitative RT-PCR by measuring *B. burgdorferi flaB* levels, normalized against tick or murine *-actin* as previously described [26, 28].

2.3. Immunoblotting

Immunoblotting was performed as detailed [26, 28]. *B. burgdorferi* protein was separated in 12% SDS-PAGE gel, transferred to nitrocellulose membranes, and probed with various primary antibodies including La7 [17], OspA [29] or anti-*B. burgdorferi* antiserum, which was collected from mice infected with spirochetes for 21 days [17]. Signals were developed following incubation with HRP-conjugated IgG and chemo-luminescence substrate.

2.4. Generation of genetically-manipulated isolates of *B. burgdorferi*

A previously generated *la7*-deficient *B. burgdorferi* [17] was used for genetic complementation using re-insertion of a wild-type copy of *la7* gene. A DNA fragment including locus *la7* (*bb0365*), as well as 200 base pairs sequence upstream was amplified and sub-cloned into the *Bam*HI and *Sa*I sites of pKFSS1, which contained a streptomycin-resistance cassette (*aadA*) [30]. An insert containing both *la7* and *aadA* fusion was digested out from pKFSS1 and further cloned into the *Bam*HI and *Sma*I sites of the plasmid pXLF14301 [31]. The plasmid carries 5' and 3' arms required for homologous recombination into *B. burgdorferi* chromosomal locus *bb0444-bb0446*. Next, the plasmid construct was sequenced to confirm its identity, and 25 µg of the plasmid DNA was then electroporated into the *la7* mutant. The clones growing in BSK medium in the presence of both kanamycin (350 µg/ml) and streptomycin (100 µg/ml) were further analyzed using PCR to confirm the intended recombination events. One of the *la7*-complemented clones contained the same plasmid profiles as that of the wild type. For *in vitro* growth analysis, spirochetes were diluted to a density of 10⁵ cells/ml, grown until stationary phase (~10⁸ cells/ml), and counted by dark-field microscopy using a Petroff-Hausser cell counter [32].

2.5. Phenotypic analysis of wild type and genetically-manipulated isolates

For assessment of *B. burgdorferi* acquisition and persistence in *I. scapularis* ticks, C3H mice were infected with wild-type spirochetes, *la7*-mutants or *la7*-complemented *B. burgdorferi* (10⁵ spirochetes/mouse, 3 animals/group), as detailed [33]. Following two weeks of infection, larvae (25 ticks/mouse) were allowed to engorge on the mice. *B. burgdorferi* levels in one group of fed ticks were assessed at 66 hours of feeding using qRT-PCR by assessing *flaB* transcripts normalized to tick *-actin*. A second group of fed larvae were allowed to rest for 21 days in the incubator and were then analyzed using qRT-PCR. A third group of infected larvae were allowed to molt into nymphs; freshly-molted nymphs (5 ticks/mouse, 3 mice/group) were placed on naïve mice to replete. The nymphal ticks were collected at 24, 48, and 72 hours during feeding, then *B. burgdorferi* burden was detected using qRT-PCR, as detailed [33].

For transmission studies, naturally-infected nymphs as generated in the laboratory [33], or nymphs infected via microinjection [34] with wild type or genetically-manipulated *B. burgdorferi*, were allowed to feed on naïve mice (5 ticks/mice, 3 mice/group). *B. burgdorferi* burden was assessed in whole ticks before feeding and at 72hrs of feeding using qRT-PCR. After 60h of feeding, some of the ticks were forcibly detached from the mice. The salivary glands of the ticks (at least 3 ticks/mouse) were dissected and the spirochete burdens were assessed by qRT-PCR. Fourteen days following completion of tick feeding, mice were

euthanized, and skin, joint, and bladder tissues were collected and analyzed for spirochete burden using qRT-PCR. In addition, spleen samples were separately inoculated into BSK media in order to examine the presence of viable spirochetes.

2.6. Confocal microscopy and co-localization studies

Confocal immunofluorescence analysis of tick salivary glands were performed as described [33]. The salivary glands from a minimum of five ticks were collected at 60 hours of feeding and scanned at 0.6 μm intervals throughout the entire depth of the tissue. Spirochetes were detected and enumerated using FITC-labeled goat anti-*B. burgdorferi* IgG (KPL), whereas the tick tissues were labeled with propidium iodide (Sigma).

Co-localization assays in tick gut tissues were performed using anti-rabbit La7 polyclonal and anti-mouse OspA monoclonal antibodies as detailed [35]. Briefly, gut tissues were isolated from 24-hr fed nymphal ticks, fixed, and incubated with respected primary antibodies. After washing the samples, La7, OspA, and tick tissues were detected using Alexa 488-labeled anti-rabbit IgG, Alexa 568-labeled anti-mouse IgG, and DAPI (Invitrogen), respectively. Images were examined and acquired using the confocal 40 $\times$ objective lens of a confocal microscope.

2.7. Co-Immunoprecipitation and protein-protein interaction assays

Co-Immunoprecipitation (Co-IP) assay was performed as described earlier [24]. Briefly, *B. burgdorferi* cells (2×10^{10}) were collected by centrifugation at $5,000 \times g$ for 20 min and were washed four times in PBS (pH 7.4). Cell pellets were solubilized and lysed with BugBuster Reagent (EMD Biosciences, Inc., Darmstadt, Germany) and were supplemented with 2 μl Lysonase Bioprocessing Reagent (EMD Biosciences, Inc.) and 20 μl of protease inhibitor cocktail (Sigma, St. Louis, MO). The mixture was rocked for 20 min at room temperature, then centrifuged at $15,000 \times g$ for 15 min at 4 $^{\circ}\text{C}$. The supernatant was collected and used for Co-IP experiments using Protein G Immunoprecipitation Kit (Sigma) according to the manufacturer's instructions. The pre-cleared lysates were briefly incubated with either polyclonal rabbit anti-La7 or normal rabbit serum (NRS) for 4 hours. Next Protein G beads were added and incubated overnight at 4 $^{\circ}\text{C}$. After washing the beads several times, the bound proteins were eluted with 50 μl sample buffer [50 mM Tris-HCl (pH 6.8), 10% v/v glycerol, 100 mM DTT, 2% SDS, 0.001% bromophenol blue], subjected to SDS-PAGE, and analyzed by Coomassie staining or immunoblot analysis.

Interaction of recombinant La7 and OspA was assessed using a published procedure [35]. Briefly, BSA, or recombinant glutathione-S-transferase (GST) or OspA (0.5 $\mu\text{g}/\text{well}$) were coated on the microtiter plate, blocked with 5% goat serum, and incubated with recombinant La7 (0.5 $\mu\text{g}/\text{well}$). After washing with buffer (PBS supplemented with 0.5% Tween-20), bound proteins were detected with anti-La7 antibodies [17] followed by secondary antibodies.

2.8. Liquid chromatography-mass spectrometry (LC-MS/MS)

For protein identification, excised SDS-PAGE gel bands were subjected to tryptic in-gel digestion, which was further processed for liquid chromatography-mass spectrometry (LC-MS/MS) analysis, as detailed in our earlier publications [24, 26]. The LC-MS/MS data files were analyzed using two search engines: Sequest search engine via Bioworks (Thermo Electron) and Mascot search engine via an in-house Mascot Server (Matrix Science). Results were combined using Scaffold Distiller (Proteome Software) for the identification of proteins.

2.9. Statistical analysis

Results are expressed as the mean $\pm$ standard error (SEM). The significance of the difference between the mean values of the groups was evaluated by two-tailed Student *t* test.

3. Results

3.1. La7 is required for all tested phases of *B. burgdorferi* infection in the vector including tick-to-mouse transmission

Previous studies suggest that *la7* mutants retain full infectivity in mice, but are attenuated for their optimal survival in feeding nymphs [17]. However, given a lack of complementation evidence, it was unclear whether the observed phenotypic defect was due to the loss of La7, or because of an aberrant effect of the gene manipulation process. We therefore sought to genetically complement the mutant with a wild-type copy of the *la7* (*bb0365*) gene and perform infectivity studies involving ticks. For stable integration of the complemented construct (Fig. 1A), a DNA fragment encompassing the putative native promoter-*la7* fusion, and an antibiotic resistance cassette *aadA* was inserted into the *B. burgdorferi* chromosome via allelic exchange. The RT-PCR and immunoblotting analysis showed that one of the clones that grew in the presence of the antibiotics expressed *la7* mRNA (Fig. 1B) and protein (Fig. 1C). The PCR analysis later confirmed that the *la7*-complemented isolates retained all endogenous plasmids present in the parental isolate (data not shown). The isolate also displayed the same growth patterns *in vitro* as the wild-type spirochetes and *la7* mutants (data not shown).

To examine the requirement of La7 in *B. burgdorferi* acquisition from murine hosts to ticks, C3H mice were intradermally inoculated with the wild-type spirochete, *la7* mutant, or *la7*-complemented *B. burgdorferi*. In agreement with a previous study showing a redundant role of La7 in murine infectivity, all mice developed similar levels of infection within 14-days of infection (data not shown). Larval ticks were then allowed to feed on infected mice and pathogen levels were measured in fed ticks. Results showed that the level of *la7* mutants were significantly lower in 66h fed larvae compared to that of wild type or *la7*-complemented isolates (Fig. 1D, left panel). A similar result was also recorded when additional groups of fed larvae were kept in the incubator and assessed following 21 days of engorgement (Fig. 1D, middle panel), suggesting that La7 is necessary for the acquisition in feeding ticks as well as in persistence in quiescent intermolt ticks. To examine the role of La7 in *B. burgdorferi* transmission from infected ticks to naïve mice, parallel groups of fed infected larvae, molted into unfed nymphs, were allowed to engorge on the naïve mice. The ticks were then collected at 24, 48, and 72 hours of feeding. Compared to wild type and *la7*-complemented isolates, lower levels of *la7* mutants were detected in infected nymphs at all time points during borrelial transmission from ticks to mice (Fig. 1D, right panel). Consistent with a reduced level of mutants in ticks (Fig. 1D), the numbers of La7-deficient spirochetes that are localized in the salivary glands of feeding nymphs were also lower, as detected by confocal microscopy (data not shown) or using qRT-PCR analysis (Fig. 2A). Fourteen days after the tick engorgement, skin, joint, and bladder samples were collected from mice, and *B. burgdorferi* were detected using qRT-PCR and culture analysis. Results showed that both wild type and *la7*-complemented spirochetes persisted in all murine tissues, while levels of *la7* mutants were significantly lower ($P < 0.01$, Fig. 2B). While spirochetes were recovered by culture analysis of all mice infected with either wild type, or *la7*-complemented isolates (3 out of 3), mutant spirochetes were recovered from only one of three mice. Additionally, all mice fed on by wild type and *la7*-complemented infected ticks developed antibody responses against *B. burgdorferi*, although mice engorged by *la7* mutant infected ticks remained weakly seropositive (data not shown).

Because *la7* mutants exhibit a major defect in their ability to survive in unfed intermolt ticks (Fig. 1D), we performed additional studies to exclude the possibility that the phenotypic defect in *la7* mutants to transmit to mice (Fig. 2) was not due to initial low levels of the pathogen in the tick gut. To do this, separate groups of unfed nymphs were artificially infected with equal numbers of wild-type spirochetes, *la7* mutants and *la7*-complemented isolates via an established microinjection procedure [34]. Five days after injection, the spirochete burdens in the unfed ticks were tested using qRT-PCR, which confirmed that the levels of *la7* mutants were similar to that of the other isolates (Fig. 3A, $P > 0.05$). A parallel group of similar artificially-infected ticks were then allowed to engorge on naïve C3H mice. After 72 hours of feeding, analysis of the pathogen levels using qRT-PCR indicated that the level of *la7* mutants was apparently decreased although without a statistically significance difference with that of either wild type or *la7*-complemented isolates ($P > 0.05$, Fig. 3B). However, examination of murine tissues 14 days following tick feeding also indicated that a significantly lower level of *la7* mutants were detectable in all tested tissues including skin, joint, and bladder, compared to the wild-type spirochetes ($P < 0.05$, Fig. 3C) and in joint tissues compared to the complemented isolates ($P < 0.05$, Fig. 3C). Together, these data establish that La7 plays an important role for spirochete transmission via feeding ticks.

3.2. *la7* is predominantly expressed during *B. burgdorferi* infection in the vector

Although *la7* is expressed in both mammalian hosts and ticks [17, 20, 21], information regarding its temporal and spatial expression, particularly in the major phases of *B. burgdorferi* infection in ticks, is lacking. We, therefore, used quantitative RT-PCR assays to analyze *la7* expression during a *B. burgdorferi* experimental infection life cycle, including pathogen acquisition in ticks, and persistence in and transmission to naïve hosts. Results showed that *la7* is highly expressed during spirochete entry into the larval ticks and during persistence through the intermolt phase. Even though *la7* expression is dramatically induced in feeding nymphal gut during *B. burgdorferi* transmission, its expression is considerably reduced in the salivary glands and in the naïve hosts (Fig. 4). This suggests a predominant tick-specific expression of *la7*, which is consistent with the requirement of La7 in the vector-specific *B. burgdorferi* life cycle.

3.3. La7 interacts with other spirochete proteins including OspA

Previously, we have shown that La7 participates in the formation of *B. burgdorferi* outer membrane (OM) complexes [24]. However, its potential co-occurrence in multiple OM complexes [24], complicates the precise identity of borrelial protein(s) that are specifically involved in interactions with La7. To further identify possible La7-binding borrelial ligand(s), we used a co-immunoprecipitation assay followed by LC-MS-MS analysis. To achieve this, *B. burgdorferi* lysates were immunoprecipitated with La7 antibody, resolved on SDS-PAGE gels, and assessed by staining with Coomassie brilliant blue (Fig. 5A) or immunoblot analysis using anti-*B. burgdorferi* antiserum (Fig. 5B) or specific antibodies against OspA and La7. The excised proteins (arrows, Fig. 5A) were subsequently identified by LC-MS/MS analysis. The data shows that several proteins including outer surface protein A, putative enzymes and chaperones, were detected as La7-interacting proteins (Fig. 5C, Table 1). In a subsequent ELISA-based assay [35], recombinant La7 also directly interacted with OspA (Fig. 5D). Finally, immunofluorescence studies using specific antibodies demonstrated co-localization of OspA and La7 in the tick gut (Fig. 5E).

4. Discussion

The regulated synthesis of La7 [21] (also known as BB0365 or P22) has previously been demonstrated in spirochetes grown in culture or their persistence *in vivo* [16-18]. Although there is no definite evidence, it has been suggested that the protein facilitates spirochete

survival within feeding ticks, during pathogen acquisition from infected hosts [17]. Our studies have now confirmed and expanded on these initial observations, showing that La7 is, indeed, a feeding-induced gene that is predominantly expressed in ticks and that is rapidly downregulated during mammalian infection. Using naturally or artificially-infected ticks (to ensure equal delivery of mutants and parental isolates), we confirmed that the protein is required for pathogen persistence in all tested phases of tick infection (including in their transmission from vector to host). Understanding the biological significance of La7 could shed new light on the intricate mechanism of spirochete persistence in the enzootic infection cycle, which could further contribute to the development of new strategies to interfere with Lyme borreliosis.

The fact that *La7* displays a differential expression *in vivo*, and that *La7* expression in cultured spirochetes is effected by changes in temperature, pH, and levels of signal molecules linked to quorum sensing (DPD/AI-2) [14, 21], suggests that function of the gene-product is likely linked to the spirochete ability to adapt to a changing tissue environment. While La7 displays insignificant homology to known proteins in the database and its molecular function is currently unknown, previous studies have shown that this is primarily an inner membrane protein [21], yet forms multiple discrete protein complexes within borrelial OM [24], possibly via its ability to interact with discrete borrelial protein via protein-protein interaction. Our study further supports the latter speculation and further identified specific La7-interacting proteins within *B. burgdorferi*, including specific OM proteins, enzymes, and chaperones. It will be quite interesting to know in future what is the biological significance of such interactions. Presently, *in vivo* expression (and function) of most of the La7-interacting proteins are unknown, however, one of the La7-binding proteins, OspA, also displays a vector-specific expression. We also show that La7 and OspA directly interact with each other, and also co-localized in the tick gut. Interestingly, both of these proteins, among others, respond similarly to environmental cues [21, 36-38] and are subjected to a common genetic regulatory network [39] involving the sigma factor RpoS [15, 20]. Therefore, it is possible that La7 interaction with OspA, or other ligands, supports the specific aspects of the spirochete's ability to adapt to the vector environment, for example, colonization of the gut epithelium, signal transduction, nutrient uptake, and counteracting extreme digestive or immunity-related activities [17]. In addition, our current study suggests that La7 could interact with additional proteins including potential chaperones. Therefore, it is possible that *B. burgdorferi* may need to maintain La7 levels in the membrane and/or in the periplasm in a timely and regulated manner; alternatively, it is also possible that La7, itself, functions as a co-chaperone, contributing to the quality of target borrelial proteins.

Previous studies suggest that the maintenance of *B. burgdorferi* in the enzootic cycle requires its successful persistence in multiple developmental stages of the arthropod, as well as a well-orchestrated mechanism of transmission to hosts via coordinated dissemination through specific tick tissues [14]. The precise mechanisms by which *B. burgdorferi* exits the feeding gut and disseminates to the salivary glands during transmission to hosts, however, is largely unknown. In recent years, a new model concerning migration of non-motile spirochetes within the feeding tick gut, from apical (luminal) to basal (hemocoelic) surface of gut via adherence to epithelial cells, has been proposed [40]. Additionally, a few borrelial proteins, including La7 in our current study, have also been shown to assist in spirochete transmission [26, 33, 41-45] including their dissemination from the gut to the salivary glands, and eventually to the host via a yet-to-be identified mechanism. Continued study of the function of *B. burgdorferi* gene-products like La7 will shed new light on how selected proteins facilitate pathogen persistence and/or transmission and may contribute towards development of novel preventative strategies to combat Lyme borreliosis.

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Fig 1.

La7 is necessary for *B. burgdorferi* acquisition in ticks, their persistence and subsequent transmission to naïve hosts. (A). Construction of a genetically complemented isolate. A schematic diagram representing the DNA construct, pXLF14301-*la7*, for *cis*-integration of a wild type of *la7* in the mutant, is shown. The *la7* open reading frame including its potential promoter region and a streptomycin resistance gene (*aadA*) were cloned into pXLF14301 for homologous recombination and integration of *la7* in *B. burgdorferi* chromosome. (B). RT-PCR analysis of *la7* transcription. Total RNA was isolated from wild type (WT), *la7* mutant (*la7*⁻) or *la7*-complemented (*la7* Com) *B. burgdorferi*, reverse transcribed to cDNA and then subjected to RT-PCR analysis with *flaB* and *la7* primers. (C). *La7* production in *B. burgdorferi*. Lysates from spirochetes were separated on SDS-PAGE gels, which were either stained with Coomassie blue (left panel) or immunoblotted with antiserum against *La7* or *FlaB* (right panel). (D). *La7* is necessary for optimal *B. burgdorferi* acquisition in ticks, their persistence through intermolt stages, and subsequent transmission to naïve hosts. Mice (3 mice/group) were infected with wild type (WT), *la7* mutants (*la7*⁻) or *la7* complemented (*la7* Com) *B. burgdorferi* and, following 14 days of infection, naïve ticks (25 ticks/mice) were allowed to feed on mice. Levels of *B. burgdorferi* in fed ticks were determined by detecting pathogen levels at 66 hours of larval feeding, or in quiescent intermolt ticks following 21 days of engorgement by qRT-PCR analysis targeting *flaB* transcripts normalized to tick *-actin* levels. A parallel group of infected larvae were allowed to molt into nymphs and were then placed on naïve mice to engorge. The nymphal ticks were collected at 24, 48, and 72 hours during feeding, and *B. burgdorferi* levels were measured using qRT-PCR. Bars represent the mean $\pm$ SEM of three qRT-PCR analyses derived from three independent

infection experiments. The levels of *la7* mutants in infected ticks were significantly lower than that of wild type and *la7*-complemented *B. burgdorferi* (* $P < 0.01$).

Fig. 2.

La7 is required for *B. burgdorferi* transmission to murine hosts via dissemination from tick salivary glands. (A) Assessment of spirochete burden in the salivary glands. Infected ticks were collected from mice at 60 hours feeding, and salivary glands were isolated for measurement of *B. burgdorferi* via qRT-PCR. Bars represent the mean $\pm$ SEM from three independent experiments. The levels of *la7* mutants in infected ticks were significantly lower than that of wild-type spirochetes and *la7* complemented *B. burgdorferi* (* P < 0.05). (B). *La7* is required for efficient spirochete transmission from infected ticks to naïve mice. The *B. burgdorferi*-infected nymphs were fed on naïve mice and, 14 days post engorgement, the murine tissues including skin, joint, and bladder were collected. The burden of wild type (WT, white bar), *la7* mutants (*la7*⁻, black bar) and *la7* complement (*la7* Com, gray bar) *B.*

burgdorferi in mice was analyzed by quantitative RT-PCR by measuring copies of the *flaB* genes and normalized against mouse *-actin* levels. Bars represent the mean $\pm$ SEM of relative tissue levels of pathogen from three independent animal infection experiments. Differences between *la7* mutant burdens and wild type or *la7*-complemented isolates were significant at all time points and tissues ($P < 0.001$).

Fig. 3.

Unlike wild type or *la7*-complemented isolates, *la7* mutants were impaired to transmit to naïve hosts when equal levels of spirochetes were present in unfed ticks. (A). Similar levels of *B. burgdorferi* were introduced in unfed nymphs via microinjection. Ticks were microinjected with equal levels of wild type *B. burgdorferi* (WT), *la7* mutant (*la7*⁻) or *la7*-complemented *B. burgdorferi* (*la7* Com). Spirochete numbers in unfed ticks were analyzed 5 days after microinjection by qRT-PCR. (B). Levels of *la7* mutants after feeding. The ticks, infected via microinjection, were allowed to attach on naïve mice. Spirochete levels were analyzed by qRT-PCR at 72 hours of feeding. Bars represent the mean $\pm$ SEM of three independent experiments. The levels of *la7* mutants in infected ticks apparently declined but with a non-significant difference compared to wild type spirochetes and *la7*-complemented *B. burgdorferi* (* $P > 0.05$). (C). Impaired ability of *la7* mutants to transmit to naïve hosts. Nymphal ticks were infected with *B. burgdorferi* via microinjection, as described in panel A, and were allowed to engorge on naïve mice. Fourteen days after repletion, the murine tissues including skin, joint, and bladder were collected, and the levels of *B. burgdorferi* in mice were analyzed by quantitative RT-PCR. Bars represent the mean $\pm$ SEM of relative tissue levels of pathogen from three independent animal infection experiments. The levels of *la7* mutants (black bar) in infected murine tissues were significantly lower than that of wild-type spirochetes at all tissues (white bar, * $P < 0.05$) and lower in joint tissues compared to that of *la7*-complemented *B. burgdorferi* (*la7* Com, gray bars); * $P < 0.05$.

Fig. 4.

la7 is upregulated in *B. burgdorferi*-infected ticks. Mice were infected with *B. burgdorferi* and skin samples were collected after one to four weeks of infection and pooled together. A parallel group of *B. burgdorferi*-infected mice were fed on by larvae, which were collected at 66 hours of feeding, or 21 days after repletion. Molted infected nymphs were fed on naïve mice, and the tick gut and salivary glands were collected at the indicated time of feeding (48 and 66 hours). Total RNA was isolated from murine skin and tick samples and *la7* transcript level was measured using quantitative RT-PCR and presented as copies of *la7* transcript per copy of *flaB* transcript. Error bars represent the mean $\pm$ SEM from four qRT-PCR analyses of two independent murine-tick infection experiments.

Fig. 5. Identification of *B. burgdorferi* proteins that interact with La7. (A). Immunoprecipitation of borrelial proteins by La7 antibodies. Soluble *B. burgdorferi* lysates were incubated with either rabbit polyclonal antibodies against La7 or normal rabbit serum (NRS). Antigen-antibody complexes were immunoprecipitated using Protein G beads. After washing, the bound proteins were eluted with SDS-PAGE sample buffer, subjected to SDS-PAGE, and analyzed by Coomassie staining. (B) Immunoblot analysis. Immunoprecipitates (as shown in Fig. 5A) or spirochete lysates (*B. burgdorferi*) were probed using anti-*B. burgdorferi* antiserum. The bands in Fig. 5A (arrows), which were present only in La7 immunoprecipitated products but not in NRS, were processed for LC-MS/MS-based protein identification. (C) Identification of specific membrane proteins. Immunoprecipitates of anti-La7 antibodies but not that of NRS contained OspA and La7, as revealed by immunoblotting using corresponding antigen-specific antibodies. (D) Interaction of La7 and OspA *in vitro*. Recombinant La7 proteins were immobilized on the microtiter wells, probed with OspA or other control proteins, and detected by primary and secondary antibodies as detailed in the text. Binding of La7 to OspA is significantly higher than other control proteins (* $P < 0.05$). (E) Co-localization of La7 and OspA within the tick gut. The gut tissues from partially-fed nymphal *I. scapularis* were removed at 24 hours of feeding, and processed for confocal microscopy using anti-mouse OspA and anti-rabbit La7 antibodies, as detailed in the text. The tick tissue nuclei were labeled using DAPI. Arrows indicate spirochetes co-expressing La7 and OspA (scale bar = 10 μ M).

Table 1

The list of La7 antibody immunoprecipitated *B. burgdorferi* proteins as identified by Liquid Chromatography Mass spectrometry (LC-MS/MS)

Annotation (Common Name)	Molecular Weight (kDa)	Unique Peptides	Percentage sequence coverage	Predicted membrane localization
BBA15 (Outer Surface Protein A)	29	14	55.6%	Yes
BB0264 (Heat shock protein 70)	54	13	25.8%	No
BB0560 (Chaperone protein HtpG)	71	10	20%	No
<i>bb0518</i> (Molecular chaperone DnaK)	69	7	18.7%	No
<i>bb0116</i> (PTS system, maltose and glucose-specific IIA _{BC} component, MalX)	58	4	9.9%	No
<i>bb0337</i> (Phosphopyruvate hydratase)	47	3	10.4%	No
<i>bbb28</i> (Putative ankyrin repeat protein)	49	3	7.3%	No

Proteins are identified by LC-MS/MS analysis. Annotations are presented according to the JCVI database (<http://www.jcvi.org>). Signal peptide predictions are derived using PSORT server (<http://psort.ims.u-tokyo.ac.jp/form.html>)