The luxS Gene Is Not Required for Borrelia burgdorferi Tick Colonization, Transmission to a Mammalian Host, or Induction of Disease

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Borrelia burgdorferi, the causative agent of Lyme disease, persists in nature via a complex enzootic cycle that involves both warm-blooded mammalian hosts and Ixodes sp. arthropod (tick) vectors (24). Because B. burgdorferi must adapt to these two very diverse niches (11, 18, 22, 23), it may utilize any number of differential expression systems to regulate its virulence factors for each parasitic environment. One regulatory system operative in certain other bacterial pathogens is the LuxS/autoinducer-2 (AI-2) quorum-sensing system (9, 15). In selected bacteria, the product of the luxS gene is an enzyme involved in the generation of an AI (a furanosyl borate diester) (7) which, in turn, induces gene expression when the AI reaches a sufficiently high concentration. The increased local concentration of AI-2 is proportional to the increased density of the bacteria synthesizing AI-2, thereby allowing the AI to serve as a surrogate signal of population density (9, 15).

In B. burgdorferi, a homolog of luxS (BB0377) was identified from genome sequence information for strain B31 (10). Studies have since demonstrated that expression of borrelial luxS is enhanced during tick feeding or during the growth of spirochetes under in vitro conditions that mimic tick feeding (e.g., elevated temperature) (16, 18). These observations, combined with the finding that the expression of certain membrane lipoproteins of B. burgdorferi (e.g., OspC, Mlp’s, P35, and P7.5) is influenced by spirochete cell density (14, 21, 30), have led to the provocative notion that B. burgdorferi may utilize a LuxS/AI-2 quorum-sensing system to regulate differentially factors that sustain the organism in one or more phases of its complex life cycle in ticks and mammals (25, 26). Along these lines, it has been reported that the B. burgdorferi luxS ortholog can functionally complement a luxS deficiency in Escherichia coli (13, 25). However, thus far, AI-2 activity has not been detectable within B. burgdorferi culture supernatants or concentrated cell lysates (13, 25). Furthermore, infection of mice via needle inoculation revealed that a luxS mutant of B. burgdorferi strain 297 was fully infectious for susceptible animals at levels comparable to those of wild-type B. burgdorferi (13). Although the latter results are inconsistent with the notion that luxS plays an essential role in the infectious phenotype of B. burgdorferi, it remained possible that luxS might contribute at some other strategic phase in the natural transmission cycle of the spirochete, particularly one involving B. burgdorferi’s arthropod vector. For example, upon tick engorgement, resident spirochetes in the tick midgut undergo a replicative burst to higher cell density (19, 20), a condition ostensibly more conducive to quorum sensing. This population change may be critical for signaling alterations in borrelial gene expression, thereby prompting the spirochetes to migrate to the salivary glands of the tick (where they are subsequently transmitted into the mammalian host). The present study was thus undertaken to assess the potential role(s) of borrelial luxS in the ability of B. burgdorferi to (i) colonize ticks naturally, (ii) be tick transmitted to naive mice, and (iii) cause disease in the murine model of Lyme borreliosis.

Colonization of Ixodes scapularis with B. burgdorferi strains. Infectious, low-passage B. burgdorferi strain 297 (17) was the representative wild-type strain for this study. luxS mutants of strain 297, BbAH308 and BbAH309, are infectious clones isolated from ear punch biopsy-positive cultures from needle-inoculated mice (13). Previous characterization of these luxS mutants revealed that they express neither mRNA nor LuxS protein (13). B. burgdorferi strain 297 was cultured in BSK-H medium (Sigma) without selective agents, and luxS mutants were grown in BSK-H containing erythromycin (0.06 μg/ml). To obtain B. burgdorferi-labeled ticks, two independent experiments were carried out: one with strains 297 and BbAH308 and a second with strains 297 and BbAH309. Three- to five-week-old female C3H/HeJ mice (five per group) were first needle inoculated intradermally with 10^7 bacteria of either wild-type B. burgdorferi strain 297 or the luxS mutant (BbAH308 or BbAH309) as previously described (12). After 2 weeks, mice were confirmed to be infected by immunoblotting of their sera against borrelial whole-cell lysates and by culturing of ear punch biopsy specimens in BSK-H medium (12). Approximately 400 pathogen-free I. scapularis larvae, reared...
and generously provided by Thomas Mather (University of Rhode Island), were then placed on two infected mice per isolate and were allowed to feed to repletion. Engorged larvae were then collected over a 5- to 7-day period, transferred into 16-ml glass vials, and stored at 22°C over a saturated solution of potassium sulfate (to maintain a relative humidity of 97%) and within an incubator with a photoperiod of 16 h of light and 8 h of darkness. Engorged ticks were then allowed to molt to the nymphal stage.

PCR amplification of the *B. burgdorferi* *ospA* gene from chromosomal DNA extracted from these flat nymphs (20) revealed that at least 80% of the ticks were infected with either wild-type *B. burgdorferi* or the luxS mutants (data not shown). However, the number of spirochetes in flat ticks is relatively low (19, 20), thereby making determinations of infection efficiencies less than optimal. To obtain more precise PCR assessments of efficiencies of tick colonization by wild-type *B. burgdorferi* or the luxS mutants, we used advantage of the fact that spirochete populations markedly expand during tick feeding (19, 20). To accomplish this expansion, naïve 3-week-old female C3H/HeJ mice were each infested with five *I. scapularis* nymphs infected with either wild-type *B. burgdorferi* or one of the luxS mutants. Mice were housed individually in cages with wire bottoms above water and monitored until ticks fed to repletion and detached from the mice (ca. 72 h). Fed ticks were collected and stored individually at −70°C.

To isolate chromosomal DNA from fed nymphs, individual ticks were first surface disinfected using consecutive washes of sterile water, 0.5% sodium hypochlorite, 3% hydrogen peroxide, 70% ethanol, and sterile water. Ticks were then transferred into 1.5-ml tubes of a Kontes disposable homogenizer, frozen in liquid nitrogen, and pulverized using a fitted pestle. DNA was purified using the Invitrogen Easy-DNA kit according to the manufacturer’s instructions, and mussel glycogen was added to a concentration of 20 µg/ml at the precipitation step. Barrier tips were used during DNA extraction and preparation of PCR mixtures to protect against contamination of samples. The primer set used for detecting borrelial DNA in the extracted tick sample, OspA-2 (5′-GTTTTTGAATTTCTA ACTGCTGACC) and OspA-4 (5′-GTTTTGTAATTTCA AGGCACTT), generates a 156-bp amplicon specific for the *ospA* gene. As a control for contamination, an unfed naïve *I. scapularis* nymph was prepared with each set of *B. burgdorferi*-infected ticks. PCRs were carried out using Takara Ex-Taq, 1 µM (each) primers, and 0.5 mM MgCl2. The optimized reaction conditions required an initial 94°C denaturation for 3 min followed by 45 cycles of the following steps: 94°C for 15 s, 65°C for 15 s, and 72°C for 15 s.

PCR results showed that 100% of nymphs were infected with either wild-type *B. burgdorferi* strain 297 or the luxS mutant BbAH308 (Table 1). BbAH309 yielded similar tick colonization efficiencies (Table 1). These findings support the conclusion that luxS is not required for the initial colonization of *I. scapularis* larvae by *B. burgdorferi* or the spirochete’s persistence within the arthropod during subsequent maturation to the nymphal form.

**Infectivity of the luxS mutants for mice challenged via tick bite.** The presence of a luxS gene was not required for *B. burgdorferi* colonization of *I. scapularis* larvae or maintenance of spirochetes in nymphs. To examine whether the luxS mutant was deficient for subsequent transmission of *B. burgdorferi* into a mammalian host, naïve 3-week-old female C3H/HeJ mice were each infested with five *I. scapularis* nymphs infected with *B. burgdorferi* (the fewest number of ticks that has been demonstrated to result in consistent 100% infection by strain 297) (data not shown). Mice were housed individually in cages with wire bottoms above water. Three weeks after tick infestation, ear punch biopsy specimens from mice were cultured in BSK-H medium with 1× *Borrelia* antibiotic mixture (Sigma). One to two weeks later, dark-field microscopy was used to examine cultures for the presence of motile spirochetes. In the first set of experiments, motile spirochetes were observed in 100% of the cultures from mice infected with *B. burgdorferi* strain 297 (Table 1). Cultures from five (83%) of the six mice infected with BbAH308 via tick infestation were also positive for growth of *B. burgdorferi* (Table 1). Histopathological analysis of the single culture-negative BbAH308-infected mouse revealed pathology identical to that of mice infected with *B. burgdorferi* strain 297 (data not shown), suggesting that this mouse indeed was infected (raising the infection efficiency for BbAH308 to 100%). A second set of mouse infestation experiments, comparing *B. burgdorferi* strain 297 with BbAH309, showed 100% infection in both groups (Table 1). An aliquot of each ear punch biopsy culture from the above-described experiments was used to inoculate a larger volume of BSK-H medium without selection by erythromycin. Once these cultures reached an adequate cell density (ca. 10⁷ spirochetes/ml), chromosomal DNA was extracted and the genotypes of the recovered spirochetes were confirmed using a set of primers (priAH166, 5′-GTGACCGGTCAATGGGAAAAATTAGAT TTTTGATTGTTAAAGAAAAATAC, and priAH167, 5′-GAA AAGGTACCTTACTAAGGATATTTTCTTCTT TAATATTG) that flank the region of luxS where the erythromycin marker was inserted. In all cases, the cultures derived from mice infected with *B. burgdorferi* strain 297 via tick infestation yielded the wild-type 546-bp amplicon, whereas PCR amplification of DNA extracted from the cultures of the mice infected with the luxS mutants (with insertionally disrupted luxS genes) resulted in the larger 1.5-kb luxS::ermC PCR product (data not shown). The combined data suggest that the luxS mutants were fully capable of being transmitted from ticks to mice and, furthermore, were competent for establishing disseminated infection in the murine model of Lyme borreliosis.

**Histopathology in mice infected with the luxS mutants via tick bite.** Previous studies have established that borrelial infectivity and pathogenicity are dissociable, inasmuch as isolates of *B. burgdorferi* strain N40 can retain the ability to infect C3H/

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<td>1</td>
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* Number of fed nymphs testing positive by PCR for *B. burgdorferi* total number of nymphs tested.

**TABLE 1. Efficiencies of tick colonization by and mouse infectivity of wild-type *B. burgdorferi* strain 297 and derivative luxS mutants (BbAH308 and BbAH309)**
HeJ mice but are unable to induce arthritis or carditis (1, 27). To examine whether a deficiency in luxS perhaps allowed infectivity at the expense of pathogenicity, histological examinations were performed on heart and joint tissues from mice infected with either wild-type B. burgdorferi strain 297 or the luxS mutants. Three weeks post-tick infestation, the heart and right hind limb were harvested from each mouse, fixed in 10% neutralized formalin, and embedded in paraffin. A blind histological assessment was then performed with tissue sections stained with hematoxylin and eosin. Arthritis of the knee joint was evaluated for thickening of the tendon sheath, severity of inflammation, leukocyte infiltration, synovial proliferation, and involvement of adjacent bone or muscle tissue (3–5). Cardiac sections were examined for the severity of inflammation, leukocyte infiltration, and fibroblastic proliferation, as well as involvement of the aortic epithelium, the epicardium, and the endocardium (2, 4, 5).

Cardiac and knee tissues from mice infected with B. burgdorferi strain 297 (n = 8) or either of the luxS mutants (n = 8; four mice from each group) were virtually indistinguishable (Fig. 1). In all specimens from mice infected with either wild-type B. burgdorferi or the luxS mutants, there was significant inflammation within the myocardium at the base of the heart surrounding the aorta, pulmonary artery, and coronary arteries. The inflammatory response consisted of macrophages admixed with lymphocytes, plasma cells, and occasional polymorphonuclear cells. In more severely affected animals in all groups, inflammation extended into the media of the arteries, resulting in proliferation and inflammation of the overlying intima. Articular lesions consisted primarily of those of tenonitis. The tendon sheaths were infiltrated with macrophages and fibroblasts admixed with lymphocytes, plasma cells, and small numbers of degenerating neutrophils. There was mild proliferation of the synovial linings of the knee joints associated with infiltration of small numbers of neutrophils and lymphocytes.

**Summary and implications.** Increased recognition of the importance of LuxS/AI-2 quorum-sensing systems for bacterial gene regulation (7, 9, 15) has prompted the hypothesis that the luxS gene product of B. burgdorferi also may be involved in regulating borrelial gene expression in a LuxS/AI-2-dependent manner (25, 26). However, the relevance of observations implicating LuxS/AI-2 as a regulatory system in B. burgdorferi (25, 26) remains unclear. Furthermore, Winzer et al. (29) have cautioned strongly about the pitfalls of proposing AI-2-dependent quorum sensing as a regulatory system for any bacterium largely, if not solely, because of the mere presence of a luxS gene and/or the production of an AI-2-like molecule(s). To garner more direct evidence for the potential involvement of LuxS in the infectivity of B. burgdorferi for mammalian hosts, it was previously reported that a LuxS-deficient mutant of B. burgdorferi readily infects mice via intradermal (needle) inoculation (13); this finding suggests that LuxS function is not required for the mammalian phase of infection or, at the very least, adaptation of B. burgdorferi to the mammalian host. Herein we now show that the same luxS mutants are fully competent for (i) colonization of their natural arthropod (I. scapularis) vector, (ii) maintenance in I. scapularis throughout the molting process (from larval to nymphal forms), (iii) transfer from ticks to uninfected mice, and (iv) after dissemination in mice, elicitation of histopathology indistinguishable from that induced by wild-type B. burgdorferi. Of note, this is
not the first study to show that a bacterial luxS mutant can retain pathogenicity; luxS mutants of both Porphyromonas gingivalis and Shigella flexneri are not attenuated in their respective models (6, 8).

All studies by us to date have failed to garner support for the contention that B. burgdorferi requires a luxS gene product for any key regulatory aspect(s) of either its complex life cycle in ticks and mammals or its ability to produce disease in a susceptible mammalian host. Although the precise functional role of LuxS in B. burgdorferi remains unknown, until direct evidence indicates otherwise, it should be considered more plausible that the major, if not sole, function for LuxS is the conversion of S-ribosylhomocysteine to homocysteine and 4,5-dihydroxy-2,3-pentanedione. The latter product likely represents a dispensable by-product of methionine metabolism that ultimately is excreted to reduce toxicity and maintain a proper metabolic state (28, 29).

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REFERENCES