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Apparent Role for *Borrelia burgdorferi* LuxS during Mammalian Infection

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The Lyme disease spirochete, *Borrelia burgdorferi*, controls protein expression patterns during its tick-mammal infection cycle. Earlier studies demonstrated that *B. burgdorferi* synthesizes 4,5-dihydroxy-2,3-pentanedione (autoinducer-2 [AI-2]) and responds to AI-2 by measurably changing production of several infection-associated proteins. *luxS* mutants, which are unable to produce AI-2, exhibit altered production of several proteins. *B. burgdorferi* cannot utilize the other product of LuxS, homocysteine, indicating that phenotypes of *luxS* mutants are not due to the absence of that molecule. Although a previous study found that a *luxS* mutant was capable of infecting mice, a critical caveat to those results is that bacterial loads were not quantified. To more precisely determine whether LuxS serves a role in mammalian infection, mice were simultaneously inoculated with congenic wild-type and *luxS* strains, and bacterial numbers were assessed using quantitative PCR. The wild-type bacteria substantially outcompeted the mutants, suggesting that LuxS performs a significant function during mammalian infection. These data also provide further evidence that nonquantitative infection studies do not necessarily provide conclusive results and that regulatory factors may not make all-or-none, black-or-white contributions to infectivity.

Borrelia burgdorferi, the spirochetal agent of Lyme disease, encounters numerous environments during the transmission, dissemination, and colonization stages of its tick-mammal infectious cycle. To facilitate those host-pathogen interactions, the bacterium controls production of proteins and other factors throughout the cycle. Investigations into borrelial gene regulation have revealed several overlapping regulons, providing this bacterium with mechanisms to "fine-tune" the expression of genes and proteins to appropriate levels (1–3).

Bacteria use *S*-adenosylmethionine (SAM) as the methyl donor for methylation reactions (Fig. 1). In many species, including *B. burgdorferi*, the resulting by-product, *S*-adenosylhomocysteine (SAH), is detoxified by Pfs to *S*-ribosylhomocysteine (SRH). That product, in turn, is broken down by LuxS into homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD) (4–9). Although some bacterial species are able to recycle homocysteine into methionine, genetic and biochemical analyses demonstrated that *B. burgdorferi* lacks the necessary enzymes and thus cannot use homocysteine (8, 10). Several bacterial species, including the syphilis spirochete, *Treponema pallidum*, produce a Pfs enzyme but lack LuxS, indicating that SRH is not inhibitory to bacterial growth (8, 11). These observations beg the question of why *B. burgdorferi* possesses a LuxS enzyme.

The other product of the LuxS-catalyzed reaction, DPD, is also known as autoinducer-2 (AI-2). That molecule is used in an intercellular signaling mechanism by *Vibrio harveyi* to control bioluminescence (12–15). *Salmonella enterica* serovar Typhimurium also possesses a mechanism to detect and respond to AI-2 (12, 16–18). Phenotypic changes have been observed in *luxS* mutants of other bacterial species, although, since many of those bacteria possess a complete activated methyl cycle, it is not always clear whether the effects of *luxS* mutations are due to an inability to produce AI-2 or homocysteine (19).

As noted above, *B. burgdorferi* cannot use homocysteine, simplifying interpretations of data obtained from borrelial *luxS* mutants. Comparative analyses of wild-type and *luxS* mutant *B. burgdorferi* strains demonstrated differences in expression levels of numerous proteins (7, 20). Moreover, addition of AI-2 to cultured wild-type and *luxS* mutant *B. burgdorferi* strains measurably affected expression levels of several borrelial proteins. These included the VlsE, ErpA, and IpLA7 proteins, which are involved in mammalian infection (4, 7, 20). *B. burgdorferi* significantly increases *luxS* transcription during transmission from ticks to mammals (21).

A previous study examined whether a *B. burgdorferi luxS* mutant is able to infect mice (22). Animals were injected with *luxS* bacteria, and then tissues from those mice were incubated in borrelial culture medium. Quantification of bacteria in mouse tissues was not attempted. Despite the limitations of this nonquantitative approach, the authors interpreted outgrowth of bacteria as an indication that "a LuxS/AI-2 system is not involved in the overall mammalian infectious process, or, at the very least, in mammalian host adaptation by *B. burgdorferi*" (22). A subsequent study by the same group, again using nonquantitative methods, demonstrated that *luxS* mutant *B. burgdorferi* can also colonize ticks (23).

To reconcile the effects of AI-2 and *luxS* deletions on borrelial gene and protein expression patterns with the ability of *luxS*-deficient bacteria to infect mice, we used the same wild-type and *luxS* strains as in the earlier infection studies, but we performed quantitative PCR (qPCR) to measure bacterial loads in mouse tissues. Detailed analyses indicated that the *luxS* mutant was significantly

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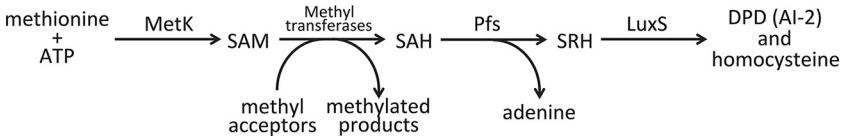

FIG 1 Activated methyl pathway of *B. burgdorferi*. MetK synthesizes *S*-adenosylmethionine (SAM) from ATP and methionine. SAM then acts as a methyl donor for many metabolic steps, also producing the by-product *S*-adenosylhomocysteine (SAH). SAH is toxic, so Pfs cleaves that molecule to produce adenine and *S*-ribosylhomocysteine (SRH). SRH is nontoxic, and some bacterial species, such as the spirochete *Treponema pallidum*, end the pathway at this step. *B. burgdorferi* instead uses LuxS to cleave SRH into homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD). Biochemical and genetic analyses demonstrated that *B. burgdorferi* lacks the ability to further metabolize homocysteine (7, 10). DPD, also known as autoinducer-2 (AI-2), is secreted by *B. burgdorferi* into the environment (7).

less infectious than the wild type. Thus, the data on the previously analyzed *luxS* mutant actually support the hypothesis that LuxS provides a significant advantage to *B. burgdorferi* during mammalian infection.

MATERIALS AND METHODS

Bacteria. The previously described *Borrelia burgdorferi* strain 297 and an isogenic *luxS* mutant, AH309, were provided by Michael Norgard (University of Texas Medical Center) (22, 23). *B. burgdorferi* cells were cultured in Barbour-Stoenner-Kelly II (BSK-II) broth at 35°C (24). Bacterial culture densities were determined by visual enumeration, using dark-field microscopy and a Petroff-Hausser counting chamber. There were no detectable differences in growth rate or other phenotypes between cultured strains 297 and AH309.

Mouse infections. All animal studies were performed under a protocol approved by the University of Kentucky Institutional Animal Care and Use Committee and in facilities of the University of Kentucky Division of Laboratory Animal Resources. BALB/cJ mice were used for all infection studies because they are as susceptible to *B. burgdorferi* infection as are other inbred strains, while being less likely to develop stressful arthritis than strains such as C3H/HeN (25, 26).

Mid-exponential-phase cultures (approximately 10^7 bacteria/ml) were adjusted to a density of 10^5 spirochetes/ml with sterile phosphate-buffered saline (PBS). For individual infection studies, cohorts of 8 BALB/cJ mice were injected subcutaneously with 100 μl of either *B. burgdorferi* 297 or AH309, resulting in a dose of 10^4 spirochetes. For competitive infection studies, 8 BALB/cJ mice were injected subcutaneously with 200 μl of a 1:1 mixture of 10^5 spirochetes of each strain/ml (i.e., 10^4 spirochetes of each strain per mouse). After 28 days, mice were euthanized, and hearts, urinary bladders, and ears were collected and snap-frozen at −80°C.

DNA isolation. For use in PCR specificity studies and as a reference for quantification studies, total bacterial DNAs were purified from mid-exponential-phase cultures (approximately 10^7 bacteria/ml) of strains 297 and AH309 by use of DNeasy blood and tissue kits (Qiagen, Germantown, MD). For analyses of bacterial loads in mouse tissues, total DNAs were isolated using a Mo Bio Ultraclean tissue and cell DNA isolation kit (Mo Bio Laboratories, Carlsbad, CA).

qPCR. Bacterial burdens in mouse tissues were assessed using Idaho Technologies/BioFire buffers (BioFire Diagnostics, Salt Lake City, UT) and Platinum *Taq* polymerase (Life Technologies, Grand Island, NY) with a CFX96 Touch real-time PCR detection platform (Bio-Rad, Hercules, CA). Oligonucleotide primers are listed in Table 1. Cycling was performed as follows: 94°C for 3 min and 40 cycles of 94°C for 10 s followed by 30 s at 60°C. Standard curves for each oligonucleotide pair were generated by diluting a known quantity of genomic DNA in a series of 10-fold serial dilutions. Threshold cycle (C_T) values obtained for experimental samples were then plotted against this curve to determine quantities of each target. Melting curve analyses were performed to assess the presence of single products. Results were analyzed using CFX Manager software (Bio-Rad). Data comparisons were analyzed by unpaired two-tailed *t* tests.

For analyses of tissues from mice infected with a single *B. burgdorferi* strain, quantities of the single-copy bacterial *flaB* gene were compared with quantities of the single-copy mouse *nidogen* gene, generating ratios of bacterial chromosomes/mouse chromosomes (27).

For the competition studies, oligonucleotide primer pairs were designed and validated to be specific for either the wild-type *luxS* gene of strain 297 or the inactivated *luxS* gene of AH309 (Table 1 and Fig. 2). For detection of the wild-type gene, the 3′ primer consisted of a sequence spanning the site of the insertion in the AH309 locus and therefore could not amplify DNA from the mutant strain AH309. For detection of the mutant locus, primers were used which amplified the inserted *ermC* gene. Quantities of both amplicons produced from each animal tissue were determined and compared.

Fold differences were calculated for competition studies by comparing the C_T values of the wild-type and mutant bacteria for each tissue specimen. A ΔC_T value of 1 is equivalent to a doubling of starting nucleic acid

TABLE 1 Oligonucleotide primers used for PCR

Purpose	Primer name	Primer sequence (5′ to 3′)[a]
Detection of mouse chromosomes	nido-F	CCAGCCACAGAATACCATCC
	nido-R	GGACATACTCTGCTGCCATC
Detection of *B. burgdorferi* chromosomes	flaB-F	GGAGCAAACCAAGATGAAGC
	flaB-R	TCCTGTTGAACACCCTCTTG
Detection of wild-type *luxS*	luxS-F	GAGCACATAGGAGCTACTTTACTT
	luxS-R	TGAGACTAAGTCAACAAGATC-TTTAC
Detection of AH309 mutant *luxS* locus	ermC-F	AAACGCTCATTGGCATTACTTT
	ermC-R	TGAGCTATTCACTTTAGGTTTAGGA

[a] The dash in the luxS-R sequence indicates the point of *luxS* into which the *ermC* gene was inserted to create mutant strain AH309. Due to the split of the luxS-R target sequence in AH309, that oligonucleotide cannot serve as a PCR primer for AH309 (see Fig. 2).

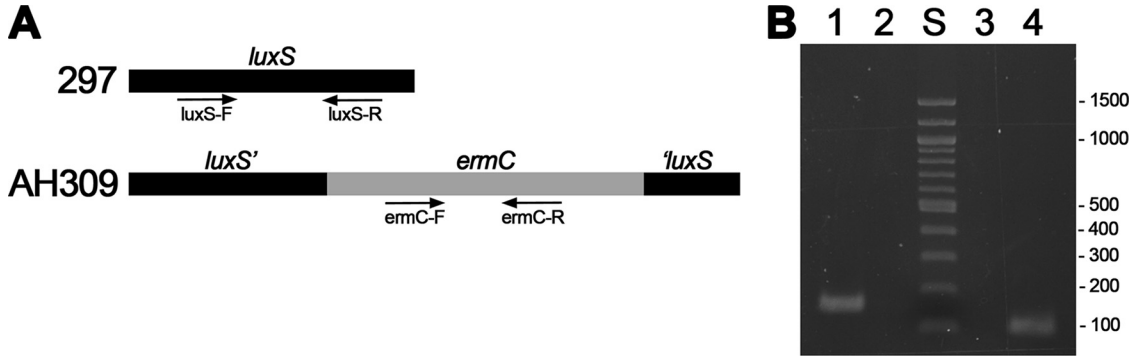

FIG 2 Specificity of PCR oligonucleotide primer pairs to detect wild-type (297) and *luxS* mutant (AH309) *B. burgdorferi* strains. (A) Schematic representation of locations of sequences complementary to PCR primers. Primers ermC-F and ermC-R both correspond to sequences within the *ermC* gene that is inserted into *luxS* of AH309 (22). Oligonucleotide luxS-R overlaps the *ermC* insertion site of the AH309 locus and thus cannot serve to prime PCR for that strain. (B) Purified genomic DNAs from strains 297 and AH309 were subjected to PCRs using each primer pair and then subjected to agarose gel electrophoresis and ethidium bromide staining. Lane 1, 297 with primers luxS-F and luxS-R; lane 2, 297 with primers ermC-F and ermC-R; lane S, molecular size markers; lane 3, AH309 with primers luxS-F and luxS-R; lane 4, AH309 with primers ermC-F and ermC-R. Sizes of markers are indicated to the right of the gel.

material. Therefore, $2^{\Delta CT}$ converts the difference in observed C_T to the fold difference for queried DNAs.

RESULTS

Infections with individual bacterial strains. Prior comparisons of wild-type and *luxS B. burgdorferi* infectivities examined whether or not mice became infected but did not quantify bacterial loads of the infected animals (22). To address that deficiency, cohorts of mice were inoculated with 10^4 bacteria of either the wild-type (297) or *luxS* mutant (AH309) strain. After 28 days, mice were euthanized and total DNA (bacteria and mouse) was purified from heart and ear tissues. Bacterial loads in each tissue were determined by ratios of bacterial genomes to the mouse genome, assessed by qPCR. Numbers of copies of the *B. burgdorferi flaB* locus served as proxies for numbers of bacterial chromosomes, and the mouse *nidogen* locus served as the target to determined numbers of mouse genomes. Mice infected with AH309 contained slightly larger numbers of bacteria in their hearts than did mice inoculated with 297, with the difference bordering on statistical significance ($P = 0.049$), but there were no significant differences in the bacterial loads of ears from mice infected with 297 or AH309 (Fig. 3). These data demonstrate that the *luxS* mutant does not have any metabolic deficiencies that inhibit mammalian infection.

Wild-type versus mutant competition infections. For a more sensitive analysis of the role of LuxS during mammalian infection, head-to-head competitions were undertaken. Mice were simultaneously inoculated with equal numbers of both wild-type and *luxS* mutant bacteria. The rationale for such studies is that if two strains are equally virulent, then mice will become infected with equal numbers of both strains, while differences in infectivity will be reflected by differences in relative bacterial loads.

To discriminate between the wild-type and *luxS* mutant strains, PCR primers were designed which specifically amplify only the wild-type or mutant *luxS* locus (Fig. 2). Control PCRs with purified DNA from each strain confirmed that the oligonucleotide pairs were equally efficient at priming PCRs from their respective templates.

The strain-specific primer pairs were then used for qPCR analyses of heart, ear, and urinary bladder tissues of eight doubly inoculated mice. The analyzed tissues were all distant from the site of inoculation and therefore measured the bacteria's ability to disseminate through mice and colonize three different types of tissues. Examination of distal tissues also ensured that detected bacteria had survived the processes of injection and dissemination. Comparisons of resultant data indicated that all tissues of all mice

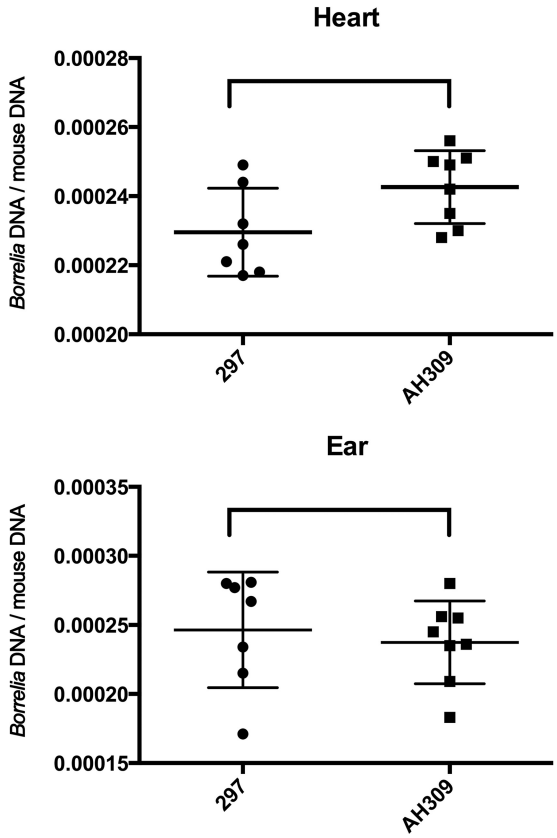

FIG 3 qPCR analyses of tissues from mice that were singly infected with either strain 297 or AH309. For each tissue, chromosomal loci of *B. burgdorferi* and mice (*flaB* and *nidogen*, respectively) were quantified, and data were plotted as numbers of *B. burgdorferi* genome equivalents per mouse genome equivalent. Differences in bacterial loads of heart tissues were equivocal ($P = 0.049$), while there were no significant differences in bacterial loads of ear tissues ($P > 0.05$).

TABLE 2 Results of dual-infection studies

Animal no.	Raw C_T value[a]											
	Heart				Bladder				Ear			
	297		AH309		297		AH309		297		AH309	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1	31.068	0.729	32.860	0.281	30.863	1.422	33.555	0.208	30.550	0.811	32.775	0.661
2	30.265	0.296	32.763	0.272	30.673	0.534	33.893	0.419	31.620	0.440	32.798	0.511
3	31.173	1.667	33.180	0.913	30.293	0.571	32.170	0.612	30.493	0.456	33.683	1.429
4	30.370	0.921	33.143	0.452	30.728	2.049	31.733	1.112	30.865	0.389	33.325	0.106
5	30.703	1.517	33.130	1.300	30.138	0.676	32.763	1.055	30.810	0.819	34.763	2.401
6	31.395	1.759	33.015	0.843	30.680	1.953	30.738	0.787	30.170	0.561	32.355	0.324
7	29.795	1.186	33.035	1.138	30.638	0.296	31.880	1.612	30.578	0.096	32.883	1.000
8	30.295	1.193	31.790	0.707	30.375	0.423	33.003	0.604	33.083	2.005	34.080	0.042

[a] Values represent the means for two separate trials, with two measurements per trial. Student's *t* test was performed, and C_T values obtained for target loci of strains 297 and AH309 were found to differ significantly across all tissues in all animals, with *P* values of <0.0001, 0.0015, and 0.0003 for the heart, bladder, and ear, respectively.

contained significantly larger numbers of wild-type than mutant bacteria (Table 2 and Fig. 4). Variations in wild-type/mutant ratios occurred between mice and between tissues of the same animals. The greatest degree of variation was found for the ears, with 3-fold to 18-fold more wild-type borreliae. Less variability was seen in the internal organs, i.e., the hearts and urinary bladders.

DISCUSSION

The two strains utilized in the current studies were previously used for nonquantitative analysis of infectivity, the results of which were published in this journal with statements such as "AH309 appeared to be fully infectious at wild-type levels" (22). The main purpose of the present studies was to determine whether or not the previous conclusions were accurate. Quantification of the bacterial loads in dually infected mice revealed that all three examined tissue types of all eight infected mice carried significantly greater loads of wild-type than *luxS* mutant *B. burgdorferi*. All examined tissues—ears, hearts, and urinary bladders—are distal from the midscapular injection site, which required bacteria to survive, disseminate, and colonize three different tissues. Although qPCR cannot discriminate live from dead bacteria, these analyses indicated that substantially larger numbers of wild-type bacteria migrated to all tissues, which in itself is a significant difference. Thus, it can be concluded that mouse infection studies with wild-type strain 297 and the congenic *luxS* mutant AH309 do not eliminate a role for LuxS during mammalian infection but instead provide support for the hypothesis that LuxS makes a significant contribution.

These results indicate that further studies of the function(s) of LuxS during mammalian infection are warranted. At present, there are no published methods to produce clean genetic complementation of *B. burgdorferi* mutants. Small differences in expression of regulatory factors can have large effect on targets, so complementation of the *luxS* mutation with the wild-type gene on a plasmid would not be useful. Likewise, restoration of wild-type *luxS* to its chromosomal locus by use of an adjacent selectable marker would also be questionable, since the marker's insertion would alter the structure of the chromosome at that locus. Until tools are available to examine the final molecular Koch's postulate on *B. burgdorferi* LuxS, the question of the enzyme's contribution to mammalian infection must remain unanswered, with the understanding that all available data indicate that LuxS and AI-2 affect the expression of numerous borrelial proteins and have a positive effect on mammalian infection.

Competition studies between mutant and wild-type bacteria can effectively identify bacterial factors that contribute to mammalian infection, because the question being asked is not whether the mutant is merely capable of infection but whether the infectivity of the mutant is equivalent to that of the wild-type pathogen. Previous studies demonstrated that competitive index studies magnify the negative impacts of mutations, making them more readily detectable (e.g., see references 28 and 29). Such analyses are particularly relevant to *B. burgdorferi*, since transmitting ticks are generally colonized with mixtures of borrelial variants (3). Thus, there is intense selective pressure on each Lyme disease spirochete to maximize its efficiency of infection and to avoid being outcompeted.

Although regulatory factors are often perceived as on/off switches, many function more like rheostats, incrementally adjusting transcription to meet specific needs of the organism. Prior analyses of the effects of AI-2 on *B. burgdorferi* indicated that this molecule affects borrelial protein levels in a rheostat manner (4, 7, 20). The previous (22, 23) and current studies indicate that the inability to produce LuxS and AI-2 does not render *B. burgdorferi* noninfectious, yet the impacts of LuxS/AI-2 on borrelial protein expression suggest an appreciable benefit of these factors (4, 7, 20). Combining results of these infection studies with previous studies of cultured borreliae, we hypothesize that AI-2 enables precise control of important host-interactive proteins.

Pathogens produce numerous substances that can be described as "virulence factors," or proteins and other molecules that contribute to infectivity. In addition to LuxS, the significance of at least three other *B. burgdorferi* factors became evident after quantitative infection assays. *B. burgdorferi* organisms that are unable to produce BBA03, a surface protein of unknown function, are capable of infecting mice yet were outcompeted in dual-infection studies with wild-type bacteria (30). Similar analyses indicated that the naturally occurring replicon lp28-3 encodes at least one factor that makes a significant contribution to mammalian infection (31). Mutant *B. burgdorferi* organisms that are unable to produce the surface-exposed fibronectin-binding protein BBK32 are capable of infecting mice (32). However, the 50% infective dose (ID_{50}) of a *bbk32* mutant was determined to be approximately 10-fold greater than that of the wild type (33). Subsequent studies

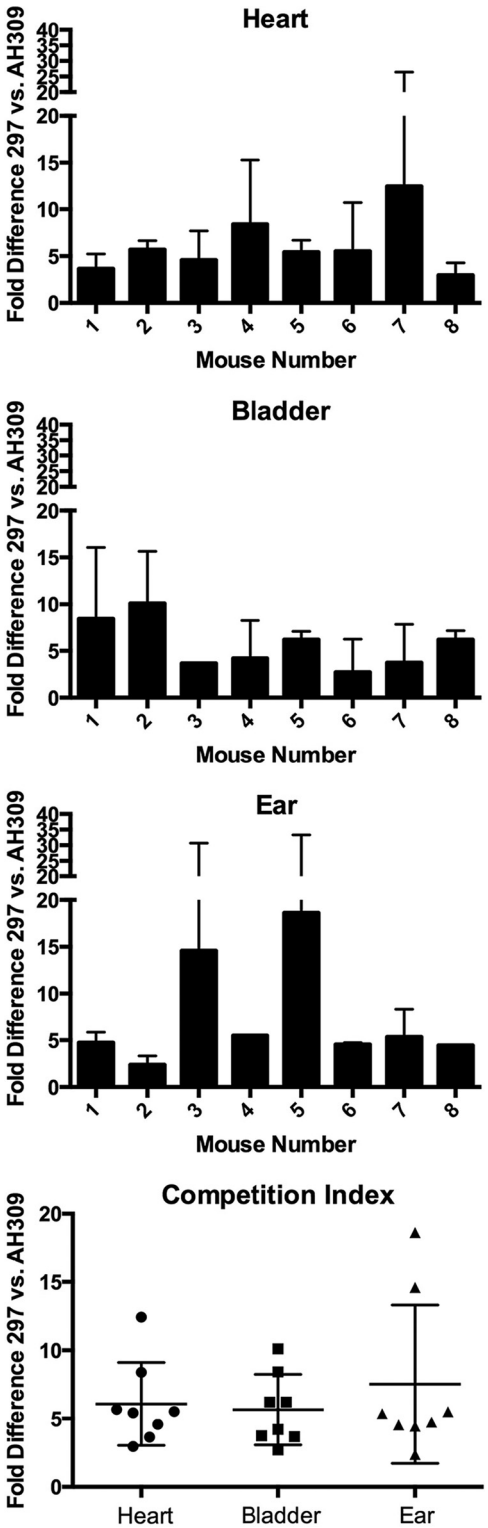

FIG 4 qPCR analyses of tissues from mice that were simultaneously injected with 10^4 bacteria (each) of both strains 297 and AH309. Total DNAs were extracted from the hearts, urinary bladders, and ear pinnae of eight mice. Ratios of wild-type (strain 297) to *luxS* mutant (strain AH309) bacteria were calculated from the ΔC_T values for each strain in each tissue. Competition indexes were calculated for every mouse tissue.

found that BBK32 plays a role in *B. burgdorferi* escape from the bloodstream (34).

B. burgdorferi secretes AI-2 into the culture medium and can respond to externally supplied AI-2, as do *V. harveyi* and *S.* Typhimurium (4, 7). The mechanism by which AI-2 influences *B. burgdorferi* protein expression is not yet known. *V. harveyi* and *S.* Typhimurium each use very different mechanisms to detect and respond to AI-2 (12, 14, 15). *V. harveyi* utilizes a two-component phosphorelay system that senses external AI-2 and sends an internal signal (15). *B. burgdorferi* possesses two two-component regulatory systems, the triggers for neither of which have been determined (3, 10). However, mutations in either of the two borrelial two-component systems have more drastic effects than does disruption of LuxS function, suggesting that they are probably not key to AI-2 signaling (35, 36). In contrast, *S.* Typhimurium uses an ABC transport system to internalize AI-2 (17, 18). DPD/AI-2 structurally resembles ribose, and the *S.* Typhimurium transporter is related to ribose transporters (13, 16–18). Along that line, *B. burgdorferi* cannot use ribose as a carbon source, yet the spirochete encodes a putative ABC transport system that resembles known ribose transporters (10, 37). *B. burgdorferi* also encodes two proteins that resemble pentose-sensing DNA-binding proteins, one of which has been named BadR and demonstrated to be a transcriptional regulator (10, 38). Alternatively, noting that two proteobacteria use radically different methods to respond to AI-2, it is possible that a distantly related spirochete may have evolved yet another mechanism.

As do other studied bacterial species, *B. burgdorferi* maximally produces DPD/AI-2 during periods of high metabolic activity (7, 39, 40). Thus, concentrations of AI-2 are reflective of the bacterial growth rate (40). In this way, AI-2 differs from "typical" quorum sensing molecules, which are thought to be indicative of bacterial density (41, 42). The rate at which *B. burgdorferi* grows varies during its tick-mammal infection cycle, with essentially no bacterial growth during colonization of the nutrient-poor environment in the unfed tick midgut but rapid growth during transmission, when the tick ingests nutritious blood, and during infection of tissues throughout the vertebrate host (1, 3, 43–49). Corresponding with the changes in growth rate, expression of *luxS* increases significantly during transmission of *B. burgdorferi* from ticks to mammals (21). The concomitant increase in AI-2 production may benefit *B. burgdorferi* at stages in which multiple bacteria are found in close proximity, such as during periods of cell division. Molecules that are secreted and have their concentrations sensed, such as AI-2, can also provide individual bacteria with information on the nature of their local environment: in an open environment, such as the bloodstream, AI-2 will rapidly diffuse away, whereas it will accumulate in a closed environment, such as the solid tissues that are preferred by *B. burgdorferi* for vertebrate colonization (3, 50, 51). This hypothesis can explain the observed variations in bacterial loads and differences in wild-type/mutant ratios possibly being affected by animal-to-animal differences in microvasculature structures or extracellular matrix distribution.

In conclusion, quantitative analyses of a previously examined *B. burgdorferi luxS* mutant demonstrated that the mutant is significantly impaired in the ability to infect mammals. These data disprove prior conclusions and suggest that LuxS does indeed perform a significant function(s) during mammalian infection. Previous studies indicated that AI-2 affects the expression levels of several proteins known to be involved in mammalian infection (4,

7, 20), suggesting that the defects of the *luxS* mutant are probably pleiotropic. Further studies should be undertaken to discover the mechanism by which the Lyme disease spirochete responds to AI-2. These results also serve as reminders that determination of whether or not a bacterial component is involved with infection cannot always be achieved through nonquantitative methods.

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