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Expression of a *luxS* Gene Is Not Required for *Borrelia burgdorferi* Infection of Mice via Needle Inoculation

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The *luxS* gene product is an integral component of LuxS/autoinducer-2 (AI-2) quorum-sensing systems in bacteria. A putative *luxS* gene was expressed at comparable levels by *Borrelia burgdorferi* strain 297 cultivated either in vitro or in dialysis membrane chambers implanted in rat peritoneal cavities. Although the borrelial *luxS* gene functionally complemented a LuxS deficiency in *Escherichia coli* DH5α, AI-2-like activity could not be detected within *B. burgdorferi* culture supernatants or concentrated cell lysates. Finally, a *luxS*-deficient mutant of *B. burgdorferi* was infectious at wild-type levels when it was intradermally needle inoculated into mice, indicating that expression of *luxS* probably is not required for infectivity but, at the very least, is not essential for mammalian host adaptation. Our findings also challenge the notion that a LuxS/AI-2 quorum-sensing system is operative in *B. burgdorferi*.

Quorum sensing is a cell-to-cell communication system that modulates a wide variety of bacterial phenotypes, including virulence expression (3, 11, 19). One of the quorum-sensing systems is the autoinducer-2 (AI-2)-dependent system, which utilizes a small cell-permeable signaling molecule (AI-2) whose synthesis is dependent on the product of a *luxS* gene. This AI-2-dependent quorum-sensing system is common among diverse bacteria, inasmuch as conserved LuxS orthologs exist in over 30 bacterial species (19). Genome analysis (20) previously revealed the presence of a *luxS* homolog (BB0377) in *Borrelia burgdorferi* strain B31. In addition, it has been shown that spirochete cell density influences the expression of a number of *B. burgdorferi* membrane lipoproteins, such as OspC, P7.5, Mlps, and P35 (9, 14, 25). These combined observations have led to the notion that *B. burgdorferi* may utilize quorum sensing as a means of adaptation during one or more phases of its complex life cycle. Stevenson and Babb (21) previously showed that *B. burgdorferi* open reading frame BB0377 was able to complement a *luxS* deficiency in *Escherichia coli* DH5α, thereby supporting a *luxS* designation for open reading frame BB0377. Furthermore, the addition of cell-free supernatants of *E. coli* DH5α harboring the *B. burgdorferi luxS* gene to *B. burgdorferi* cultures (at 23 or 34°C) induced the differential regulation of 25 to 30 borrelial proteins, ostensibly from the presence of AI-2 in the *E. coli* culture medium (21). However, AI-2-like activity was not detectable in high-density culture supernatants of *B. burgdorferi* strain B31, N40, or 297, nor was the expression of the *luxS* gene in *B. burgdorferi* evaluated (21). Nevertheless, it was concluded that the LuxS/AI-2 quorum-sensing system is operative in *B. burgdorferi* (21). The objectives of the present study were to examine directly the expression of *luxS* in *B. burgdorferi*, evaluate AI-2-like activity emanating from *luxS* expression, and determine whether LuxS was required for mammalian infectivity by the Lyme disease spirochete.

Generation of *luxS* mutants. Infectious *B. burgdorferi* strain 297 (12) and all other bacterial strains and plasmid constructs used in this study are listed in Table 1. The overall strategy for targeted gene disruption in *B. burgdorferi* strain 297 was described previously (8). All PCR primers were designed on the basis of strain B31 sequence information (5) (http://www.tigr.org), and their approximate positions are depicted in Fig. 1B. PCR was routinely performed with the Expand High Fidelity PCR System (Roche Diagnostics). First, a 5,014-bp region of the *luxS* gene with flanking sequences from strain 297 was PCR amplified by using primers priAH156 (5′-CTGCTAGTGCCT ATTCTGCTATC) and priAH159 (5′-GGTTCCTGCAAATG TTGTTCAGAG) and ligated into pGEMT-easy (Promega, Madison, Wis.) to yield pALH330 (Fig. 1A). The *luxS* gene was then disrupted by inserting an erythromycin resistance marker, *ermC* (8, 18), into the unique *Bgl*II site of pALH330 to yield the suicide plasmid pALH340 (Fig. 1B). To minimize polar effects, *ermC* was inserted in an orientation opposite to that of *luxS*; the orientation was confirmed by PCR (Fig. 1C) with primers internal to *ermC* (priAH102 and priAH104 [8]) in combination with priAH166 and priAH168 (see below).

Fifteen micrograms of purified pALH340 was used for electroporation of strain 297 (8), after which spirochetes were added to 2 ml of fresh BSK-H medium supplemented with antibiotic mixture for *Borrelia* (Sigma Chemical Co., St. Louis, Mo.). Following 36 h of recovery in the absence of erythromycin, the entire transformation mixture was added to 50 ml of prewarmed BSK-H medium (Sigma) containing erythromycin (0.06 μg/ml). Erythromycin-resistant spirochetes could easily be distinguished (via dark-field microscopy) from nonviable (nonmotile) cells after 10 to 14 days of incubation at 37°C. No viable spirochetes were detected in a control culture of BSK-H plus erythromycin inoculated with mock-electroporated wild-type bacteria, indicating that spirochetes were efficiently killed and that spontaneous erythromycin resistance did not occur among the population.

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TABLE 1. Strains and recombinant plasmids

Strain or plasmid	Description	Source and/or reference
Strains		
E. coli DH5α	F$^-$ ϕ80d*lac*ZΔM15 Δ(*lacZYA-argF*)U169 *endA1 recA1 hsdR17*(r_K^- m_K^+) *deoR thi-1 sup*E44 λ$^-$ *gyrA96 relA1*; does not express AI-2	GIBCO/Life Technologies
V. harveyi		
BB170	*luxN*::Tn*5*; AI-2 sensor strain	V. Sperandio (22)
BB152	Positive control, synthesizes AI-2	V. Sperandio (22)
B. burgdorferi		
297	Nonclonal, infectious human isolate	12
BbAH308	Clonal, infectious *lux*S mutant	This study
BbAH309	Clonal, infectious *lux*S mutant	This study
Plasmids		
pGEM-T	TA cloning vector, high-copy-number; Amp^r	Promega
pHAT20	Expression vector with His tag; Amp^r	Clontech
pALH133	pGEM-T, *erm*C from pJRS233; Amp^r Erm^r	8
pALH330	pGEM-T, 5,014-bp fragment (analogous B31 chromosome 385038–390052); Amp^r	This study
pALH340	pALH330, except *lux*S::*erm*C (*Bgl*II [1 kb]); Amp^r Erm^r	This study
pALH470	pHAT20Ω(*lux*S, *Age*I/*Kpn*I [560 bp]); Amp^r	This study

It was postulated that further selection and enrichment of transformed spirochetes under conditions that more closely mimic the mammalian host environment (as opposed to in vitro growth on solid medium) might assist with the recovery of *luxS* mutants that retained the ability to infect and replicate within mammalian tissues. To approach this, 10 ml of the erythromycin-resistant mutant pool or a mock-electroporated control pool (both diluted to 1,000 spirochetes per ml in fresh BSK-H plus erythromycin) were placed into sterile dialysis membrane chambers (DMCs), which were then implanted into rat peritoneal cavities (1). After 15 days, motile spirochetes were not detectable in DMCs inoculated with mock-electroporated wild-type bacteria, again indicating that initial erythromycin selection efficiently eliminated all wild-type *B. burgdorferi*. In contrast, *luxS*-disrupted, erythromycin-resistant spirochetes within DMCs replicated to normal levels (3.0×10^6 to 4.5×10^6 per ml), providing the first indication that *luxS* may not be required for the growth of *B. burgdorferi* during mammalian host-adapted conditions. Furthermore, PCR analysis of genomic contents from the pool of erythromycin-resistant spirochetes within DMCs did not reveal any amplicons representative of wild-type *luxS* (not shown), suggesting that erythromycin resistance correlated with the presence of *ermC* within *luxS*. As a preliminary assessment of whether the *luxS*-deficient mutants might be capable of infecting mice, approximately 10^5 spirochetes recovered from the DMCs were intradermally needle inoculated into each of five C3H/HeJ mice (6). Ear punch biopsies (6) were cultured in BSK-H plus erythromycin; 2 weeks postinfection, cultured biopsies from all five mice yielded motile spirochetes after 1 week in culture.

One of the resultant ear punch biopsy-positive cultures was then diluted in BSK-H and plated on solid selective medium (pBSK plus erythromycin) (17) to isolate single colonies. Two clonal isolates were randomly selected, designated BbAH308 and BbAH309, and subjected to PCR analysis to confirm *luxS* gene disruption (8). BbAH309 was chosen for further characterization because plasmid profiling (4) of this mutant (via PCR) indicated that it contained all plasmids except cp32-6 (not shown). Moreover, when the in vitro growth of BbAH309 in BSK-H medium was compared with that of wild-type 297 under various temperature (37°C) and pH (7.5 or 8.0) conditions over 5 days, starting inocula of 10^3 spirochetes grew at comparable rates and to comparable levels (ca. 2.5×10^7 cells per ml) (not shown).

Expression of *luxS* and its genetic organization in *B. burgdorferi*. Heretofore, expression of the putative *luxS* gene in *B. burgdorferi* had not been evaluated. Expression of *luxS* by wild-type 297 and mutant BbAH309 initially was assessed by reverse transcriptase PCR (RT-PCR) with the Titan one-step RT-PCR system (Roche Molecular Biochemicals, Indianapolis, Ind.) according to manufacturer's instructions. Spirochetes propagated at 37°C (pH 6.8) were harvested in the late-logarithmic phase of growth (ca. 2×10^7 cells per ml). Total RNA was extracted (15) and DNase treated, and 500-ng quantities of RNA were subjected to RT-PCR (26) with the primers priDTR506 (5′-GATTTCGGAAAAAAGCCATGAGAC) and priAH193 (5′-**ATG**GGAAAAATTAGATTTTGTAAAAAA AATAC [the start codon is in boldface]). For all RT-PCRs, a negative control lacking RT was included (Fig. 2, lanes 5 and 11). As shown in Fig. 2, wild-type 297 expressed *luxS* RNA when cultivated at 37°C (pH 6.8) (parameters analogous to the feeding-tick condition [15, 25]) (lane 6), whereas BbAH309 did not (lane 12). Next, we sought to determine whether mRNA detected by RT-PCR accounted for detectable levels of LuxS. For this purpose, we first constructed and expressed in *E. coli* a 6-histidine fusion protein of LuxS by standard protocols (6) and used the purified protein to generate polyclonal antisera in rats (10). LuxS antibodies in antisera were further affinity purified (HiTrap affinity columns; Amersham Biosciences, Piscataway, N.J.) to yield a monospecific antibody preparation. Wild-type 297 and *luxS*-deficient BbAH309 were cultivated in vitro under various conditions or in DMCs implanted in rat peritoneal cavities. As shown in Fig. 3, LuxS was detectable by immunoblotting at comparable levels within wild-type *B. burg-*

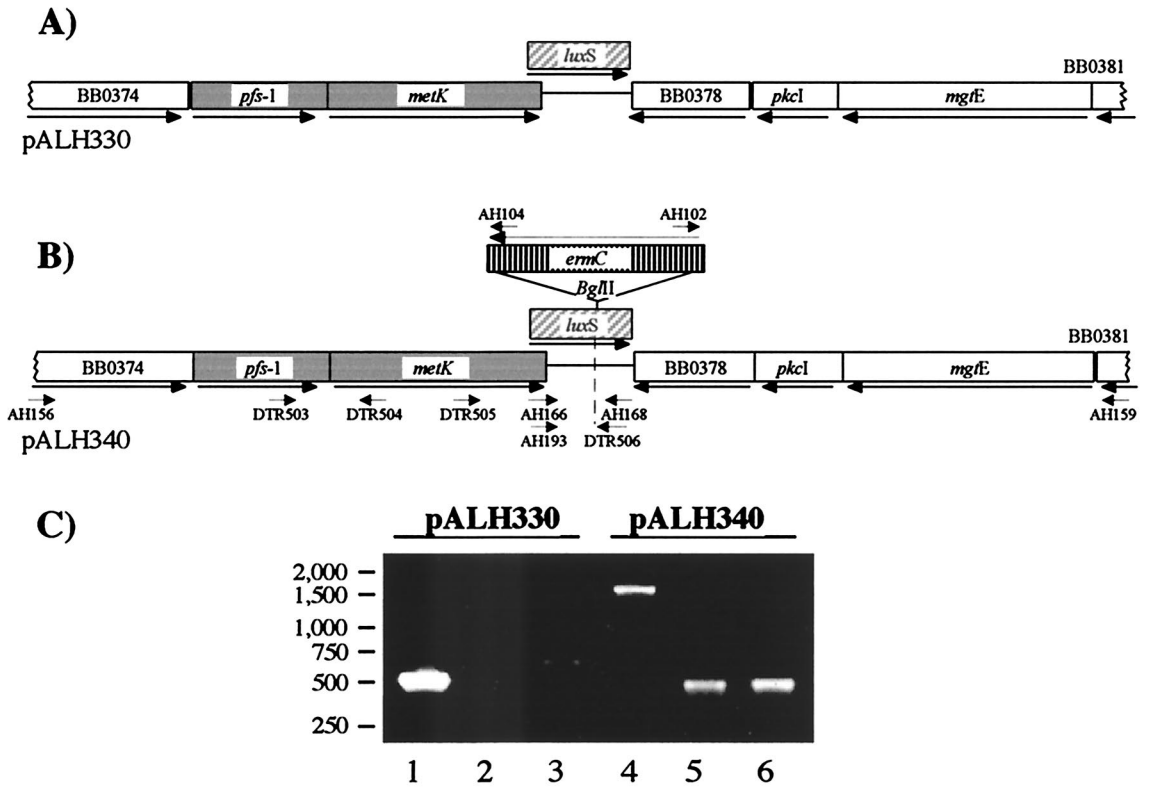

FIG. 1. Construction of the suicide plasmid (pALH340) for disruption of *B. burgdorferi luxS*. (A) pGEMT-easy-based pALH330 contains the cloned *B. burgdorferi luxS* (hatched box) and flanking sequences (only the relevant portion of the plasmid is shown). Two genes, *pfs-1* and *metK* (gray boxes), along with *luxS*, comprise a three-gene operon. (B) *luxS* was insertionally disrupted with *ermC* (vertical stripes), yielding pALH340. Short arrows indicate approximate primer locations. (C) Agarose gel electrophoresis patterns of amplicons for pALH330 (lanes 1 to 3) and pALH340 (lanes 4 to 6). The primers used to generate products were as follows: lanes 1 and 4, priAH166 and priAH168; lanes 2 and 5, priAH104 and priAH166; lanes 3 and 6, priAH102 and priAH168. PCR using combinations of *ermC*- and *luxS*-specific primers confirmed the reverse-transcriptional orientation of *ermC* relative to *luxS* in pALH340 (lanes 5 and 6).

dorferi cultivated in vitro (lanes 1 and 2) or in DMCs (lane 3). These results were consistent with those of previous gene array analyses (15), which also showed that the *luxS* gene of infectious *B. burgdorferi* strain B31 was expressed at similar levels when spirochetes were cultivated in vitro or in DMCs. As expected, LuxS was not detectable in BbAH309 grown either in vitro or in DMCs (Fig. 3, lanes 4 and 5). Of note, it was previously postulated that OspC expression by *B. burgdorferi* might be affected by LuxS-produced AI-2 when spirochetes replicate within a mammalian host (21). However, in the present study, OspC expression was unaffected in the *luxS*-deficient mutant cultivated within DMCs (Fig. 3, lane 5), suggesting that OspC expression is not influenced by a LuxS/AI-2 quorum-sensing system when replicating in a mammalian host-adapted state.

Stevenson and Babb (21) reported that the *B. burgdorferi luxS* gene cloned into *E. coli* functionally complemented the natural LuxS deficiency of *E. coli* DH5α (22). In the present study, we obtained similar results (not shown), implying that the product of the enzymatic reaction catalyzed by *B. burgdorferi* LuxS, when synthesized in *E. coli*, is functional in heterologous AI-2-dependent quorum-sensing systems. However, thus far we have been unable to detect the presence of any AI-2-like activity (via *Vibrio harveyi* bioluminescence) either in culture supernatants of *B. burgdorferi* 297 grown to a high density at 37°C (pH 6.8) or in cell lysates concentrated 100-fold (not shown). The simplest explanation of these findings is that even if an AI-2-like molecule is synthesized by the action of LuxS in *B. burgdorferi*, AI-2 may not achieve levels sufficient to function in a LuxS/AI-2-type quorum-sensing system.

Schauder et al. (20) first proposed that *B. burgdorferi luxS* might be in a three-gene operon with two other accessory genes, *pfs-1* and *metK*, involved in *S*-adenosylmethionine (SAM) utilization pathways. This hypothesized operonic organization of *pfs-1–metK–luxS* was assessed by RT-PCR with the primers priDTR503 (5′-GCTGCAATAGCTCAAGTAGCAC AC) and priDTR504 (5′-CTATTTATTTCTCTCCTGCTATT AC) (353-bp product for *pfs-1–metK*), as well as priDTR505 (5′-CTACCGGAGACACTGGGCTTACAG) and priDTR506 (see above; 770-bp product for *metK-luxS*). As shown in Fig. 2, wild-type 297 cultured at 37°C (pH 6.8) yielded RT-PCR products linking *pfs-1* to *metK* (lane 2) and *metK* to *luxS* (lane 4), indicating that *pfs-1*, *metK*, and *luxS* are transcriptionally linked. In mutant BbAH309, the *pfs-1* and *metK* genes are still transcribed together (lane 8), whereas primers priDTR505 and priDTR506 did not yield an RT-PCR product for *metK-luxS* due to disruption of the *luxS* gene (lane 10).

A *luxS*-deficient mutant of *B. burgdorferi* retains infectivity

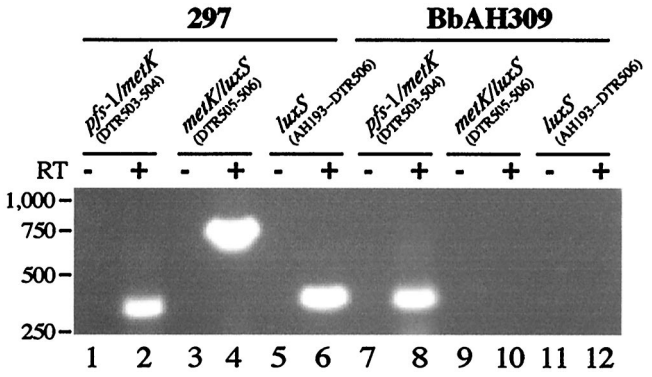

FIG. 2. RT-PCR analysis of *luxS* expression in wild-type *B. burgdorferi* 297 (lanes 1 to 6) and a *luxS* mutant (BbAH309) (lanes 7 to 12) cultured at 37°C (pH 6.8). + and −, PCRs with or without RT, respectively. Primer locations are depicted in Fig. 1B, and the primer pairs used are indicated above the pairs of the corresponding gel lanes. Lanes 2 and 4 show the operonic organization of *pfs-1*, *metK*, and *luxS* in wild-type 297. Lane 6 shows *luxS* expression in wild-type 297, whereas no similar RT-PCR product is detectable in the *luxS* mutant (lane 12). The absence of product in lane 10 is due to disruption of *luxS* by *ermC*.

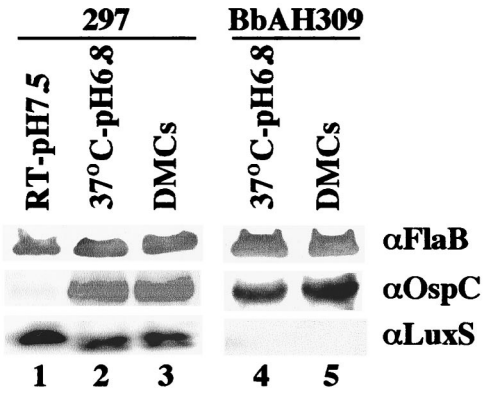

FIG. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting of wild-type (strain 297) and *luxS*-deficient (strain BbAH309) *B. burgdorferi*. For organisms cultivated in vitro at 37°C or at room temperature (RT) (lanes 1, 2, and 4), each strain was cultivated in BSK-H medium to the late-logarithmic stage (ca. 2×10^7 spirochetes per ml). To obtain spirochetes in a mammalian host-adapted state, *B. burgdorferi* strains were cultivated in DMCs (lanes 3 and 5) (1). A total of 5×10^7 spirochetes were loaded in each gel lane. To detect FlaB, conventional immunoblotting was performed with murine monoclonal antibody 8H3-33 (1). Detection of FlaB was used as a loading control for each gel lane. To detect OspC, rat polyclonal antiserum was used as a primary antibody (1). OspC expression was used, in part, as an indicator that in vitro-cultivated borreliae were suitably adapted to the various growth conditions; OspC expression was low in spirochetes cultivated in BSK-H medium (pH 7.5) at room temperature, as has been reported previously (25). To detect low-abundance LuxS, chemiluminescence (Western Lightening; Perkin-Elmer Life Sciences, Boston, Mass.) was employed with affinity-purified rat antibodies (see text).

for mice. The *luxS*-deficient mutant BbAH309 was used to verify retention of the mouse infectivity phenotype following its clonal selection. In these experiments, groups of five mice were inoculated intradermally with 100 spirochetes of either wild-type 297 or BbAH309. After 2 weeks, ear punch biopsies were obtained from all mice and were cultured in BSK-H medium under standard conditions (6). One week later, motile spirochetes were observed by dark-field microscopy in all cultures from both groups of mice. Given that five of five mice became infected by low levels of inocula, BbAH309 thus appeared to be fully infectious at wild-type levels. These findings suggest that even if a LuxS/AI-2 quorum-sensing system is operative at some stage(s) in the life cycle of *B. burgdorferi*, expression of the *luxS* gene is not required for mammalian host adaptation and probably is not required for infection. Finally, to our knowledge, this is the first report of a genetically manipulated *B. burgdorferi* mutant that has retained its ability to infect laboratory mice, although recently we also obtained evidence that a *csrA* (carbon storage regulator A) (16) mutant of *B. burgdorferi* 297 also is infectious for mice.

Summary and implications. Given recent attention to the importance of quorum sensing in bacterial virulence expression (3, 11, 19), it has been tempting to speculate that quorum sensing may be operative as a regulatory mechanism governing differential antigen expression in *B. burgdorferi*. However, based on results presented here, it appears 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*. This is consistent with the notion that the mammalian phase of Lyme borreliosis tends to be paucibacillary, and thus it is counterintuitive as to how quorum sensing would be beneficial under such conditions. Nonetheless, our results do not preclude that such a system might be operative when *B. burgdorferi* is harbored within tick midguts, where replicative bursts that accompany tick feeding culminate in higher spirochete densities (13). Also, our mouse infection experiments were performed with intradermal needle inoculation, a route which is not equivalent to natural tick infection (7). Therefore, further experiments examining the influence of LuxS on the tick transmission of *B. burgdorferi* are warranted.

Despite the initial report by Stevenson and Babb (21), there still is not direct evidence that *B. burgdorferi* exploits quorum sensing as a mechanism of gene regulation. In fact, Winzer et al. (24) have cautioned that the presence of a *luxS* gene product and the potential ability to synthesize AI-2 molecules should not be accepted as sole evidence for quorum sensing. Given the present paucity of additional supporting data, one therefore must continue to entertain the possibility that *B. burgdorferi* may not utilize a LuxS/AI-2 quorum-sensing system in any stage of its life cycle. This hypothesis also is consistent with our findings that AI-2-like activity was not detectable in either culture supernatants or 100-fold-concentrated cell lysates of *B. burgdorferi* 297. Therefore, AI-2 either may not be synthesized by *B. burgdorferi* or, alternatively, is a metabolic intermediate that is rapidly catabolized (23, 24). Regarding AI-2 synthesis in other bacteria, MetK is a SAM synthase. SAM subsequently is converted to *S*-adenosylhomocysteine, which is then acted upon by Pfs for conversion to *S*-ribosylhomocysteine (2, 23). LuxS cleaves *S*-ribosylhomocysteine to homocysteine and 4,5-dihydroxy-2,3-pentanedione, which then most likely cyclizes in the presence of borate to form AI-2 (a furanosyl borate diester) (2). Inasmuch as this pathway is common in bacteria, it has been proposed that AI-2 merely may be a dispensable by-product of methionine metabolism that leaves

the cell via simple diffusion (23) and thus serves as an autoinducer for quorum sensing in only some species of bacteria (24). In this regard, although we showed that *luxS* was expressed in *B. burgdorferi* and was capable of complementing the LuxS deficiency of *E. coli* DH5α, these findings may not be relevant to borrelial LuxS functioning in an AI-2-dependent quorum-sensing system, akin to what has been noted by Winzer et al. (24). It thus may not be coincidental that two other essential components of an AI-2-dependent quorum-sensing system, LuxP (periplasmic binding protein) and LuxQ (sensor kinase), thus far have not been identified in *B. burgdorferi*. Taken together, these findings indicate that *B. burgdorferi* may not have the molecular machinery requisite for sensing and responding to AI-2-like molecules, even if AI-2 is stably produced and excreted.

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