Synthesis of Autoinducer 2 by the Lyme Disease Spirochete, *Borrelia burgdorferi*  

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Received 30 November 2004/Accepted 27 January 2005  

Defining the metabolic capabilities and regulatory mechanisms controlling gene expression is a valuable step  
in understanding the pathogenic properties of infectious agents such as *Borrelia burgdorferi*. The present  
studies demonstrated that *B. burgdorferi* encodes functional PfS and LuxS enzymes for the breakdown of tox-  
ic products of methylation reactions. Consistent with those observations, *B. burgdorferi* was shown to synthesize  
the end product 4,5-dihydroxy-2,3-pentanedione (DPD) during laboratory cultivation. DPD undergoes sponta-  
neous rearrangements to produce a class of pheromones collectively named autoinducer 2 (Al-2). Addition  
of in vitro-synthesized DPD to cultured *B. burgdorferi* resulted in differential expression of a distinct subset of  
proteins, including the outer surface lipoprotein VlsE. Although many bacteria can utilize the other LuxS  
product, homocysteine, for regeneration of methionine, *B. burgdorferi* was found to lack such ability. It is  
hyposthesized that *B. burgdorferi* produces LuxS for the express purpose of synthesizing DPD and utilizes a  
form of that molecule as an Al-2 pheromone to control gene expression.

Most organisms live in dynamic environments, where survival often requires efficient use of resources or production of  
specific substances. Those demands necessitate that organisms sense conditions of their surroundings and respond appropriately to changes. *Borrelia burgdorferi*, which persists in nature by cycling between infection of vertebrates and infection of  
ticks, is no exception to that rule. As would be expected, this bacterium expresses a different repertoire of proteins at each  
step of its infectious cycle (1, 27, 46). Elucidating the regulatory networks that control expression of those proteins will  
provide insight into pathogenic properties of this bacterium, as well as direct development of novel therapies for the prevention  
and treatment of Lyme disease.  

To function properly, many proteins, nucleic acids, and other molecules need to be modified by chemical addition of  
methyl groups (13). Enzymes that catalyze these methylation reactions, other molecules need to be modified by chemical addition of  
methyl donor (Fig. 1). However, the by-product of such reactions, S-adenosylhomocysteine (SAH), is toxic, being a competitive inhibitor of the same methylation reactions by which it is produced (13, 21, 26, 38). Detoxification of SAH is accomplished by its breakdown to either S-ribosylhomocysteine (SRH) and adenosine (via PfS in many eubacteria) or homocysteine and adenosine (via SAH hydrolyse in eukaryotes and most other bacteria) (30, 63). Some bacteria further metabolize SRH using the enzyme LuxS to produce homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD) (17, 31, 43, 56, 63). However, SRH does not appear to be harmful to bacteria, as many organisms naturally possess homologs of PfS but lack LuxS (17, 19, 45, 52, 63). Bacteria containing luxS mutations are also generally viable (14, 17, 25, 47, 56, 67). Many organisms further metabolize homocysteine to produce methionine, which has led to suggestions that the primary role of LuxS in many bacteria is to facilitate reuse of homocysteine (64, 65). Yet some bacteria utilize the other product of LuxS, DPD, as a precursor of pheromones that influence protein expression patterns (67). DPD can spontaneously cyclize and/or interact with borate to form at least two different, interconvertible molecules collectively described as autoinducer 2 (AI-2) (12, 33). Although AI-2 was originally described as a quorum-sensing molecule for measuring cell density (56), it appears that a significant number of bacteria instead utilize AI-2 as a pheromone during the exponential growth phase to signal metabolic status and fitness (67).

Through complementation of an *Escherichia coli* luxS mutant, our laboratory previously demonstrated that *B. burgdorferi* encodes a functional LuxS enzyme (49). The complemented *E. coli* strain produced a molecule recognized as AI-2 by *Vibrio harveyi*. Significantly, cultivation of *B. burgdorferi* in the presence of culture supernatant from luxS-complemented *E. coli* detectably altered expression patterns of at least 50 *B. burgdorferi* proteins, including the factor H-binding outer surface lipoprotein ErpA/I/N (32, 49, 50). Supernatant from uncomplemented *E. coli* had no apparent effect on *B. burgdorferi* protein levels. We now present results of additional studies demonstrating that *B. burgdorferi* encodes all the enzymes required for synthesis of AI-2, that it synthesizes various levels of autoinducer depending upon bacterial fitness, and that addition of in vitro-synthesized AI-2 specifically alters expression of a subset of bacterial proteins.
FIG. 1. Metabolic pathways found in many organisms that lead to synthesis of AI-2 and recycling of homocysteine. Me-THF, 5-methyltetrahydrofolate. DPD can spontaneously cyclize and/or combine with borate to produce at least two different, interconvertible forms of AI-2 (12, 33, 43). Many characterized organisms are capable of regenerating methionine from homocysteine by use of one or more methionine synthase enzymes, such as MetE or MetH (53). The spirochetes Trepomenna pallidum and T. denticola both encode homologs of PfTs but lack homologs of LuxS or methionine synthase and are thus predicted to produce SRH as a waste product and not regenerate methionine. The spirochete Leptospira interrogans can apparently complete the entire cycle, as it contains homologs of SAH hydrolase and Me-THF-dependent methionine synthase. Studies described in this report indicate that B. burgdorferi produces DPD and homocysteine through PfTs and LuxS but lacks the ability to salvage homocysteine.

MATERIALS AND METHODS

Bacteria. B. burgdorferi strain B31 is the species type strain and was originally isolated from an infected tick collected on Shelter Island, New York (7). The complete genome sequence was determined for an infectious subculture of strain B31 (9, 18). Strain AH309, a luxS-deficient mutant of strain 297 (25), was obtained from Michael Norgard (University of Texas, Houston, Houston, Houston). All B. burgdorferi strains were cultured in Barbour-Stoenner-Kelly II (BSK-II) broth (2).

For analyses of AI-2 production by B. burgdorferi at differing stages of cultivation, cultures of strains 297 and AH309 were grown at 34°C to densities of approximately 10^8 bacteria per ml. Cultures were then plated at 32°C, which keeps the bacteria viable but greatly retards growth (51). Aliquots of the two initial cultures were diluted 1:1,000 into fresh medium on six subsequent days, with each secondary culture incubated at 34°C to allow optimal growth. On day 7, supernatant was removed from each secondary culture and assayed for AI-2 content. In this manner, cultures with essentially the same starting densities could be grown under the same conditions for 1 through 7 days and then assayed simultaneously. Bacterial density of each secondary culture was also determined at that time, using a Petroff-Hauser counting chamber and dark-field microscopy.

E. coli strain BL21(DE3)pLysE (Invitrogen, Carlsbad, CA) was used to overexpress recombinant proteins for purification. E. coli strains GS162 (wild type for both methionine synthase genes) and GS472 (metH and metE) were obtained from George Stauffer (University of Iowa) (60). Unless otherwise noted, E. coli was grown in LB medium (42). For attempted complementation of E. coli metE and metH, transformed GS472 was plated on M9 minimal salts agar supplemented with 1 μg/ml thiamine, with this same medium plus 100 μg/ml methionine serving as a positive control for growth (42, 60, 68). For use as a positive control for methionine synthase analyses, GS162 was cultured in M9 supplemented with 3.4 μM 1-hydroxyoaceticoin, 1 μg/ml thiamine, and 100 μg/ml phenylalanine (28, 60).

V. harveyi strains BB120 and BB170 (4) were obtained from Bonnie Bassler (Princeton University). V. harveyi was cultivated in modified autoinducer biosay (AB) medium (23) containing 40 μM sodium borate (pH 6.8).

FIG. 2. Diagram of the B. burgdorferi chromosomal region containing the pfs and luxS genes. Studies described in this report demonstrated that ORF BB0375 encodes a functional PfTs enzyme. The ORF between pfs and luxS has been shown to encode a functional S-adenosylmethionine synthase (MetK) enzyme (S. P. Riley and B. Stevenson, unpublished results). These three genes appear to form an operon with ORF BB0374, a gene lacking significant homology to any previously characterized ORF.

Leptospira interrogans serovar pomona type kennecwick strain JEN4 (34) was cultured at 30°C in Bovuminar PLM-5 medium (Intergen, Purchase, NY).

Recombinant proteins. B. burgdorferi encodes a potential PfTs homolog on its chromosome, annotated as open reading frame (ORF) BB0375 in the B. burgdorferi strain B31 genome (2). As the first step in producing B. burgdorferi AI-2 biosynthetic enzymes, a 3.7-kb fragment of the B. burgdorferi B31 chromosome that includes luxS and ORF BB0375 was amplified by PCR using oligonucleotide primers LUXS-14 (5'-ATATGATATACATGGTTAATAGAG-3') and ORF BB0375, using oligonucleotides B-PFS-3 (5'-ATGGTTTGAATAA TCAGCTATG-3') and B-PFS-4 (5'-ATATTGCATTAAGTTCTCGTC TGC-3'). In addition, the E. coli pfs gene (15) was amplified from genomic DNA by PCR using oligonucleotides EC-PFS-1 (5'-ATGAAAAATAACAAGCTTTA GAA-3') and EC-PFS-2 (5'-CCATGTGACAGGTTCTCGGACCAGT-3').

All amplification products were cloned into pBluescript KS- (Stratagene) and retransformed with pCPT-E (Invitrogen), according to the manufacturer's recommendations. Resulting plasmids encode polyhistidine-tagged recombinant B. burgdorferi LuxS, B. burgdorferi ORF BB0375, and E. coli Pfs proteins and were designated pRW3, pKV10, and pKV17, respectively. Inserts of these plasmids were completely sequenced on both strands to ensure that no mutations were introduced during the cloning processes. E. coli strain BL21(DE3)pLysE was transformed with each plasmid, and recombinant protein synthesis induced by addition of IPTG (isopropyl-β-D-thiogalactopyranoside) to culture media. Bacteria were harvested by centrifugation and lysed in B-PER II reagent (Pierce, Rockford, IL), and recombinant proteins purified using the MagneHis Protein Purification System (Promega, Madison, WI), according to the manufacturer's instructions.

New Zealand White rabbits were inoculated with recombinant B. burgdorferi Pfs protein to produce polyclonal antisera (Proteintech, Chicago, IL). Prior to use, antisera was preadsorbed with E. coli cellular lysate and passed through HiTrap Protein A columns (Amersham, Piscataway, NJ).

In vitro synthesis of AI-2. DPD was synthesized as previously described (43), using equimolar concentrations of recombinant B. burgdorferi LuxS paired with either recombinant E. coli Pfs or the B. burgdorferi ORF BB0375 (pfs) gene product. AI-2 synthesized using the B. burgdorferi enzymes was used for all analyses of the effects of autoinducer on B. burgdorferi protein expression.

LuxS and Pfs catalyze synthesis of equivalent amounts of DPD and homocysteine from SAH (Fig. 1). Hence, in vitro synthesis of DPD can be quantified biochemically by analyzing the concentration of free sulphydryl groups on the produced homocysteine (43). Briefly, in vitro AI-2 biosynthesis was carried out for 15 min and an aliquot was diluted 20-fold into 100 mM sodium phosphate buffer (pH 7.2), 0.1 mM EDTA. A 400-μl aliquot of the diluted assay mixture was then mixed with 200 μl of a 5 mM solution of Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid) (Sigma, St. Louis, MO) in the same sodium phosphate buffer. Control reactions were also performed using stock solutions having known concentrations of homocysteine. Absorption at 412 nm was used to determine concentrations of 2-nitro-5-thiobenzuate formed in the Ellman reaction, with 1 mole of 2-nitro-5-thiobenzenate being produced per mole of target sulphydryl group.

V. harveyi bioassay of AI-2. V. harveyi uses AI-2 and another pheromone, AI-1, to regulate bio luminescence. V. harveyi strain BB170 contains a mutation that renders it nonresponsive to AI-1, enabling its use in a bioassay specific for AI-2 (4). This bioassay was used to quantify synthesis of AI-2 both by recombinant proteins and by cultured B. burgdorferi. Bioassays were performed as previously described (49, 54), except that 40 μM borate was included in the V. harveyi bioassay.
were cultured to densities of approximately 10^7 bacteria per ml, pelleted by centrifugation and washed with phosphate-buffered saline. Total RNA was isolated using Trizol reagent (Invitrogen) per manufacturer's instructions and was solubilized in RNA Secure (Ambion, Austin, TX). cDNA was synthesized from 1 μg of total RNA using 1st Strand cDNA Synthesis Kit (Roche, Indianapolis, IN) and random hexamer primers with avian myeloblastosis virus reverse transcriptase (RT) enzyme at 42°C for 60 minutes. Control reactions with mixtures lacking RT were performed in parallel. Enzyme was inactivated by incubation at 94°C for 5 min. Using both cDNA and reaction mixtures that lacked RT as templates, luxS and ORF BB0175 (pfs) were amplified by PCR using the oligonucleotide primer pairs SRA13 (5'-AGAGCAATGATTTGAGTATTGTTCAACTCCG-3') and SRA14 (5'-GCTGCATCTAATGATTTGTTCAACTCCG-3') and primer pair SRA9 (5'-ACTACAGGAATGTTAAAAACTC-3') and SRA10 (5'-TTTGCGAATGGTTAAAACTC-3'), respectively. PCR was performed using Tag polymerase and 28 cycles of reaction conditions consisting of 94°C for 1 min, 50°C for 1 min, and 68°C for 2 min. Reaction products were subjected to 6% polyacrylamide gel electrophoresis and visualized by ethidium bromide staining. Additional mid-exponential-phase cultures were examined for Pfs expression by immunoblotting. One-dimensional SDS-polyacrylamide gel electrophoresis was used to resolve proteins. Separated proteins were then transferred to nitrocellulose membranes and incubated with polyclonal antiserum directed against B. burgdorferi Pfs, and bound antibody was detected with horseradish peroxidase-linked clonal antibody H9724, which recognizes the constitutively expressed FlaB clonal protein (3). To investigate whether this gene encodes enzymes for synthesis of AI-2, B. burgdorferi protein expression was assessed by two-dimensional gel electrophoresis (49). B. burgdorferi was cultured in either undiluted BSK-II medium or media containing various concentrations of in vitro-synthesized DPD/homocysteine, 2 μM homocysteine, or 100 μM SAH. Bacteria were cultured to densities of approximately 10^7 bacteria per ml, pelleted by centrifugation, washed twice with phosphate-buffered saline, and lysed by heating in a boiling water bath. One-mg aliquots of each lysate were subjected to SDS-PAGE and visualized by fluorography. As a positive control, recombinant E. coli Pfs protein was likewise synthesized and purified. E. coli Pfs was purified to homogeneity using a complete GenBank database at http://www.ncbi.nlm.nih.gov/BLAST/.

**RESULTS**

### B. burgdorferi encodes enzymes for synthesis of AI-2

To date, the complete genomes of four spirochetal species have been published, those of *B. burgdorferi*, *Treponema pallidum*, *T. denticola*, and *Leptospira interrogans* (9, 18, 36, 41, 45). Analyses of these genomes revealed that *B. burgdorferi* and the two treponemes encode possible homologs of Pfs: ORFs BB0375, TP0170, and TDE0105, respectively. *L. interrogans* appears to instead utilize the alternative pathway of SAH detoxification via an SAH hydrolase homolog, ORF LB106. Of these four spirochetes, only *B. burgdorferi* encodes a recognizable homolog of LuxS, which we demonstrated to be a functional enzyme (49). As noted previously (18, 43, 52), the likely *B. burgdorferi* pfs homolog, ORF BB0375, is located one gene away from luxS (Fig. 2). To investigate whether this gene encodes a functional Pfs enzyme, we sought to biochemically characterize the protein product of ORF BB0375.

### Recombinant forms of both the *B. burgdorferi* LuxS and ORF BB0375 product were expressed in *E. coli* and purified

As a positive control, recombinant *E. coli* Pfs was likewise synthesized and purified. Each identified spirochetal ORF was used to query all of the spirochete genomes, along with the complete GenBank database at http://www.ncbi.nlm.nih.gov/BLAST/

### Methionine synthase assay

5-Methyltetrahydrofolate-homocysteine 5-methylytransferase activity was determined essentially as described by Jarrett et al. (28). Briefly, bacterial cultures were harvested by centrifugation, resuspended in 1 M phosphate buffer (pH 7.2), and lyzed by sonication. Each cleared lysate was incubated with 5-[(4^-14)C]methyltetrahydrofolate (Amersham, Buckinghamshire, United Kingdom), 1-homocysteine, SAM, hydroxocobalamin, and dithiothreitol (all from Sigma) in ratios as previously described (28). Any ^14^-C-labeled methionine produced was separated from the reactants by passage through AG 1-X8 columns (Bio-Rad) and then measured using a scintillation counter. Both *E. coli* strain GS162 (wild type) and GS472 (metH metE) were analyzed as controls. All experiments also included a negative control that lacked bacterial lysate. Number of decays per minute in the column flowthrough of the negative control reaction was subtracted from each experimental value.

### TABLE 1. Functional analyses of recombinant LuxS and Pfs enzymes

<table>
<thead>
<tr>
<th>In vitro reaction combination</th>
<th>Calculated concn of homocysteine produced (μM)</th>
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<tbody>
<tr>
<td><em>B. burgdorferi</em> Pfs and LuxS</td>
<td>18</td>
</tr>
<tr>
<td>E. coli Pfs + <em>B. burgdorferi</em> LuxS</td>
<td>10</td>
</tr>
<tr>
<td>No added proteins</td>
<td>0</td>
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*Note that the catalyzed reactions produce equimolar concentrations of both DPD and homocysteine. Please refer to details of homocysteine assays. Since specific activities of enzymes varied between protein preparations, results of a representative set of parallel synthetic reactions are shown.*
Indeed encode a functional Pfs enzyme and will be referred to as such for the remainder of this report. These studies also demonstrated that, by encoding functional Pfs and LuxS enzymes, B. burgdorferi possesses the enzymatic capability to produce AI-2.

**B. burgdorferi synthesizes AI-2.** RT-PCR analysis of RNA purified from cultured B. burgdorferi detected transcripts of both pfs and luxS (Fig. 3). Control experiments lacking reverse transcriptase failed to amplify products, indicating absence of DNA contamination. Previous RT-PCR linkage studies also suggested expression of both pfs and luxS during laboratory cultivation (25). Furthermore, immunoblot analysis indicated production of Pfs protein by the cultured B. burgdorferi (Fig. 3). Despite these results, prior bioassays of cultured B. burgdorferi failed to detect synthesis of AI-2 (25, 49).

In the time since we performed those earlier bioassays, it has been discovered that the AI-2 molecule to which *V. harveyi* responds is a borate derivative of DPD (12). Addition of borate to bioassays stimulates formation of this derivative from DPD and, not surprisingly, yields greater bioluminescence levels from *V. harveyi* reporter bacteria (33). It has also been reported that many bacteria synthesize maximal levels of AI-2 during the exponential phase of growth and either repress its synthesis or degrade the autoinducer during late exponential and stationary phases (24, 55, 57, 58, 67). The pH of the culture medium during bioassays. With *B. burgdorferi* actively removes the molecule from the medium, as do several other studied bacteria (24, 55, 57, 58, 67). The pH of the *B. burgdorferi* media dropped only slightly during these experiments, from an initial 7.5 to a final pH of 6.8 after 3 days at stationary phase. Hence, it is unlikely that the pH of the tested *B. burgdorferi* culture media influenced *V. harveyi* bioluminescence (16).

*V. harveyi* bioluminescence is sensitive to cellular levels of cyclic AMP and is repressed by phosphoenolpyruvate-dependent phosphotransferase system (PTS) sugars such as glucose (11, 16). BSK-II medium contains glucose as its primary carbon source, in addition to other, essential PTS carbohydrates such as N-acetylglucosamine (2, 62). However, studies of carbohydrate utilization by *B. burgdorferi* revealed that the spirochete grows well only in media containing either PTS sugars or glycerol (62). Furthermore, it is capable of high growth rates for 3 to 4 days in media lacking any specifically added carbohydrate, due to energy provided by other medium components (62). Media capable of supporting growth of these fastidious spirochetes but lacking trace carbohydrates have yet to be developed. Due to the unavoidable effects of catabolite repression on *V. harveyi* reporter strain bioluminescence, it is possible...
that the AI-2 synthesis results described above for *B. burgdorferi* may be artificially low.

**DPD modulates *B. burgdorferi* protein expression.** Results of the above studies indicated that *B. burgdorferi* can and does synthesize AI-2. In a previous report, we expressed the *B. burgdorferi luxS* gene in a *luxS*-deficient *E. coli* strain and demonstrated that addition of sterile culture supernatant from the complemented bacteria influenced expression levels of more than 50 *B. burgdorferi* proteins (49). Culture supernatants from the uncomplemented *E. coli luxS* mutant had no detectable effects. A caveat to those studies is the possibility that AI-2 induced the *E. coli* to produce other molecules, which in turn were actually responsible for the observed effect on *B. burgdorferi* protein expression. For this reason, the direct precursor of AI-2, DPD, was analyzed for effects upon *B. burgdorferi* protein expression. Purified DPD is not commercially available but can be synthesized in vitro from SAH using recombinant Pfs and LuxS enzymes (43, 63). Reaction products were added to *B. burgdorferi* culture medium at final concentrations of 1 or 2 μM, concentrations of DPD previously determined to induce bioluminescent responses from *V. harveyi* (63). We observed that expression levels of a subset of *B. burgdorferi* proteins were measurably affected by addition of reaction products to cultures (Fig. 5B, C, and D and data not shown). Since in vitro-synthesized DPD contains equimolar concentrations of homocysteine, we examined the effect of homocysteine alone on *B. burgdorferi* protein expression and found none (Fig. 5E). The effects of adenine, the third product of the Pfs/LuxS reactions, were not examined, since *B. burgdorferi* is an adenine auxotroph (66), and culture medium already contains a substantial concentration of that nucleotide (2). In vitro synthesis of DPD uses SAH as the starting material, but that molecule alone had no detectable effect on *B. burgdorferi* protein expression (Fig. 5F). These control experiments demonstrated that the effects of adding reaction products to culture medium were due to DPD alone. The effect of DPD addition appeared to be dose dependent, with 2 μM DPD having a greater effect than did 1 μM (Fig. 5C and D). Some proteins were detected in lysates of uninduced strain 297 that were not visible in lysates of AH309 (Fig. 5G), suggesting that the amounts of DPD produced by 297 during laboratory cultivation were sufficient to cause appreciable effects on protein levels. Strain AH309 responded to addition of DPD in manners similar to strain 297 (Fig. 5H),

FIG. 5. Addition of in vitro-synthesized DPD affects protein expression profiles of cultured *B. burgdorferi*. Different isoelectric focusing and electrophoresis conditions reveal different portions of the *B. burgdorferi* proteome: illustrated are representative two-dimensional gels using nonlinear isoelectric focusing between pH 3 and 10. (A) A representative, complete two-dimensional gel. (B through H) Enlarged sections of two-dimensional gels corresponding with the boxed area shown in panel A. Cultures of strain 297 were incubated in plain medium (B) or in medium containing either 1 or 2 μM in vitro-synthesized DPD and homocysteine (Hcy) (C and D), Hcy alone (E), or SAH alone (F). Cultures of strain AH309 were grown in either plain medium (G) or medium containing 2 μM in vitro-synthesized DPD and Hcy (H). Signal strengths of all detected proteins were compared within each gel. Four representative proteins visible in these gels whose relative expression levels were increased by addition of DPD/ Hcy are indicated by arrows. Identities of these proteins have yet to be confirmed. As would be expected, relative mobilities of proteins during the first dimension of separation (isolectric focusing) occasionally varied somewhat between different gels. Numbers at left are molecular masses in kilodaltons.
of the proteins encoded by some bacteria (20, 37, 59). Both the betaine and methylmethionine, found in animals, or methylmethionine, found in plants and methionine synthase use as the methyl donor either betaine, have been identified (44). The two other identified types of methylcobalamin-dependent MetE of nine synthases, respectively (22), although variants such as the as cobalamin-independent and cobalamin-dependent methionine synthase (53). The MetE and MetH enzymes are commonly referred to be regulated by AI-2 levels. Several such proteins were previously characterized methionine synthase. The two major classes of this pathway, LuxS, is not needed for detoxification of SRH. It has also been hypothesized that B. burgdorferi and many other bacteria utilize LuxS for the sole purpose of producing homocysteine, which is then recycled to produce methionine (6, 25, 63–65). However, in the present studies, we demonstrated that the Lyme disease spirochete can detoxify this waste product to DPD and homocysteine via the enzymes Pfs and LuxS. V. harveyi bioassays indicated that these reactions occur during laboratory cultivation, as AI-2 was readily detected in used B. burgdorferi growth medium. The products of the Pfs-catalyzed reaction, SRH and adenine, appear not to be toxic, as many bacteria, including the spirochetes T. pallidum and T. denticola, naturally contain Pfs but lack LuxS. luxS mutants of many bacteria, B. burgdorferi included, do not exhibit detectable growth defects. Thus, it appears that the next enzyme of this pathway, LuxS, is not needed for detoxification of SRH. It has also been hypothesized that B. burgdorferi and many other bacteria utilize LuxS for the sole purpose of producing homocysteine, which is then recycled to produce methionine (6, 25, 63–65). However, in the present studies, we demonstrated that B. burgdorferi lacks a homolog of any known methionine synthase enzyme.

If B. burgdorferi does not require LuxS for regeneration of methionine or for detoxification of SRH, what purpose does this enzyme serve? Why does B. burgdorferi produce this enzyme, while other pathogenic spirochetes, such as the syphilis agent, survive well in its absence? We hypothesize that the other product of the LuxS reaction, DPD, functions as an AI-2 molecule known to exhibit pheromone-like activity in many species of bacteria. As we have herein demonstrated, addition of DPD to B. burgdorferi is accompanied by altered expression patterns of a discrete subset of bacterial proteins. Furthermore, this effect is dose dependent, as would be expected for a regulatory molecule. Addition of the other LuxS product, homocysteine, or the Pfs/LuxS substrate, SAH, had no detectable effects on B. burgdorferi protein expression patterns. These data indicate that DPD, or a derivative thereof, functions as an AI-2 molecule for B. burgdorferi. The nature of the B. burgdorferi AI-2 remains to be determined, as does the mechanism by which the pheromone affects protein expression. V. harveyi responds to a borate derivative of DPD via a two-component sensory mech-

### DISCUSSION

B. burgdorferi utilizes SAM as the methyl donor for many metabolic reactions, producing the toxic molecule SAH as a by-product. Through biochemical analyses, the present studies demonstrated that the Lyme disease spirochete can detoxify this waste product to DPD and homocysteine via the enzymes Pfs and LuxS. V. harveyi bioassays indicated that these reactions occur during laboratory cultivation, as AI-2 was readily detected in used B. burgdorferi growth medium. The products of the Pfs-catalyzed reaction, SRH and adenine, appear not to be toxic, as many bacteria, including the spirochetes T. pallidum and T. denticola, naturally contain Pfs but lack LuxS. luxS mutants of many bacteria, B. burgdorferi included, do not exhibit detectable growth defects. Thus, it appears that the next enzyme of this pathway, LuxS, is not needed for detoxification of SRH. It has also been hypothesized that B. burgdorferi and many other bacteria utilize LuxS for the sole purpose of producing homocysteine, which is then recycled to produce methionine (6, 25, 63–65). However, in the present studies, we demonstrated that B. burgdorferi lacks a homolog of any known methionine synthase enzyme.

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### FIG. 6. Effects of DPD/AI-2 on B. burgdorferi VlsE protein expression. Bacteria were cultured in the presence of indicated concentrations of in vitro-synthesized DPD/AI-2 and then analyzed by immunoblotting using VlsE-directed antiserum. As controls for equal loading, membranes were also analyzed using a monoclonal antibody directed against the constitutively expressed FlaB (flagellin) protein.

indicating that responses to that molecule can occur independently of LuxS.

We next undertook identification of proteins that appeared to be regulated by AI-2 levels. Several such proteins were extracted from polyacrylamide gels and subjected to proteomic analysis. One was tentatively identified as being VlsE, a well-characterized surface-exposed lipoprotein that is expressed during mammalian infection (69). Immunoblot analyses of B. burgdorferi lysates confirmed that preliminary identification, with VlsE protein levels increasing proportionally with addition of DPD (Fig. 6). Other tentatively identified proteins are less well characterized and include putative membrane proteins and enzymes, as well as proteins lacking homology with any other previously characterized protein. We are presently developing reagents to confirm the effects of DPD/AI-2 upon expression of those heretofore unexamined proteins.

B. burgdorferi lacks methionine synthase. Detoxification of SAH via either the Pfs/LuxS or the SAH hydrolase pathway yields homocysteine, which many organisms recycle into methionine (Fig. 1). For this reason, it has been suggested that the major, and possibly the only, function of LuxS is to produce homocysteine from SRH (6, 63–65). We therefore examined B. burgdorferi for evidence of an enzyme capable of methylating homocysteine to produce methionine.

First, the genome of the sequenced B. burgdorferi strain B31 was examined for an ORF homologous to a previously characterized methionine synthase. The two major classes of this enzyme use derivatives of 5-methyltetrahydrofolate as the methyl donor and are typified by the E. coli MetE and MetH enzymes. Proteins orthologous to MetE and/or MetH have been found in almost all examined prokaryotes and eukaryotes (53). The MetE and MetH enzymes are commonly referred to as cobalamin-independent and cobalamin-dependent methionine synthases, respectively (22), although variants such as the methylcobalamin-dependent MetE of M. thermoautotrophicum have been identified (44). The two other identified types of methionine synthase use as the methyl donor either betaine, found in animals, or methylmethionine, found in plants and some bacteria (20, 37, 59). Both the betaine and methylmethionine enzymes share recognizable sequence homology with MetH-type methionine synthases (20, 59). BLAST-P analyses of the proteins encoded by B. burgdorferi indicated that this bacterium does not encode a protein with homology to any known methionine synthase. T. pallidum and T. denticola likewise lack methionine synthase homologs. Alone among the four spirochete species examined, only L. interrogans encodes a potential homocysteine salvage enzyme, the ortholog of MetH encoded by ORF LB108.

Next, plasmid libraries of B. burgdorferi DNA were used in attempts to complement an E. coli metE metH mutant. This technique has previously been utilized to clone methionine synthase genes from organisms as different from E. coli as the potato (68). Our attempts were unsuccessful.

Finally, cellular extracts of virulent B. burgdorferi were examined for MetH activity, but no enzymatic activity was detected (data not shown). As anticipated, substantial methionine synthase activity was detected in lysates of L. interrogans (A. Verma and B. Stevenson, unpublished results).
anism, whereas *Salmonella enterica* serovar Typhimurium recognizes a borate-free derivative and transports that autoinducer into the cell via an ABC transporter (5, 12, 33, 57, 58). We are presently utilizing a combination of proteomic and genomic methods to identify additional *B. burgdorferi* genes and proteins affected by DPD and to elucidate mechanisms by which AI-2-dependent regulation occurs.

The finding that *B. burgdorferi* AI-2 is maximally produced during exponential growth is in line with results of studies of many other bacteria (67). DPD synthesis generally increases during rapid bacterial growth, presumably due to increased methylation reactions during such times, and is thereby thought to serve as a signal of bacterial fitness (67). Whether this functions to coordinate growth-related processes throughout a population, or as a form of positive feedback to the cell that produced it, is unknown for most bacteria. Since AI-2 synthesis frequently has little to do with bacterial culture density, it is perhaps more accurate to think of this molecule as a pheromone, rather than strictly as a “quorum-sensing” autoinducer. Viewed from that perspective, it is easier to understand how a bacterium such as *B. burgdorferi*, which achieves high densities only in the midguts of feeding ticks, might utilize AI-2 as a signal throughout its infectious cycle. Levels of *B. burgdorferi* luxS transcript increase when infected ticks feed on mammals (35), and we have previously hypothesized that the accompanying rise in AI-2 levels might help coordinate transmission of the bacteria from the tick vector to the vertebrate host (32, 49, 52). Production of AI-2 by growing bacteria within mammalian tissues may also serve to control gene expression via self-induction. Related to that hypothesis, it has also been suggested that isolated bacteria may utilize pheromones such as AI-2 to sense the permeability of their surroundings (29, 40).

Two recent publications reported that a luxS mutant of *B. burgdorferi* was capable of infecting both mice and ticks, leading those authors to suggest that neither LuxS nor AI-2 is involved in the spirochete’s infection processes (6, 25). However, a significant caveat to those experiments is the nature by which the luxS mutant was derived: bacteria were transformed by electroporation, cultured in liquid medium containing a selective antibiotic, placed in a dialysis bag implanted within the peritoneum of a rat for 15 days, removed from the dialysis bag, injected into a mouse, and then, after 2 weeks of infection, cultured from an ear punch biopsy specimen and finally plated in solid medium (25). Two resultant clones were then tested for infectious ability. Since this complicated scheme simultaneously selected for both luxS deletion and retention of infectivity, only bacteria capable of infecting mice could ever have been recovered. A more convincing argument could be made had luxS mutants been produced and cloned on selective medium first and then those bacteria subsequently tested for infectivity. At the present time, there is no way of knowing whether the examined bacteria contain only the introduced luxS lesion, or if spontaneous mutations arose at additional loci during the selection processes to compensate for the loss of luxS. Until that issue has been resolved, it is impossible to state definitively whether LuxS and AI-2-mediated gene regulation are essential for *B. burgdorferi* pathogenesis.

The conclusion that *B. burgdorferi* cannot recycle homocysteine to regenerate methionine is consistent with prior evidence that the spirochete is an auxotroph for all amino acids. Laboratory cultivation of this fastidious bacterium is possible only in rich media that contain amino acids, nucleotides, fatty acids, and many other nutrients (2, 39). Analysis of the strain B31 genome sequence revealed homologs of proteases and transporters of both polypeptides and individual amino acids but no amino acid biosynthetic enzymes (18). Presumably, *B. burgdorferi* parasitizes methionine from its hosts in quantities sufficient for protein synthesis, methylation reactions, and all other metabolic processes.

In conclusion, the present studies indicated that *B. burgdorferi* encodes the enzymes required for production of DPD/AI-2 and synthesizes that compound during growth in culture medium. Addition of in vitro-synthesized DPD and homocysteine to cultured bacteria resulted in altered expression of a distinct subset of *B. burgdorferi* proteins. Addition of either homocysteine alone or the Pfs substrate, SAH, had no detectable effects on protein levels, indicating that DPD was alone responsible for the observed alterations in protein expression. Through genomic and biochemical techniques it was demonstrated that *B. burgdorferi* lacks the ability to utilize homocysteine for regeneration of methionine. These results strongly suggest that the Lyme disease spirochete utilizes DPD or a derivative as an AI-2 pheromone to regulate gene expression and that this bacterium produces LuxS for the purpose of synthesizing AI-2. We are continuing to investigate the mechanism(s) by which *B. burgdorferi* controls gene expression through AI-2, to identify and characterize the proteins influenced by the pheromone, and to explore the effects of mutations in those genes and luxS on the borrelial infectious cycle. Such analyses will continue to provide insight into the regulation of gene expression by *B. burgdorferi* and the importance of such control on bacterial infectivity and pathogenicity.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grants R01-AI53101 and ST32-A149795.

All authors contributed equally to these studies. We thank Michael Norgard, Xiaofeng Yang, Bonnie Bassler, and George Stauder for providing bacterial strains; Tom Schwan for sharing unpublished results; Steven Norris and Jerrilyn Howell for providing anti-VisE antiserum; Bonnie Bassler for advice on in vitro synthesis of AI-2; Klaus Winzer for discussions on homocysteine metabolism and AI-2; Kenneth Cornell for discussions on Pfs; Sarah Wackerbarth for statistical analyses; and Sara Bair, Tomasz Bykowski, Sarah Kears, Natalie Mickelsen, Jennifer Miller, Ashutosh Verma, Michael Woodman, and Wolfram Zückert for assistance in this research and for helpful comments on the manuscript.

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