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

Kelly Babb,[1] Kate von Lackum,[1] Rachel L. Wattier,[1,2]† Sean P. Riley,[1] and Brian Stevenson[1]*

Department of Microbiology, Immunology, and Molecular Genetics, College of Medicine, University of Kentucky, Lexington, KY 40536,[1] and Agricultural Biotechnology Program, College of Agriculture, University of Kentucky, Lexington, KY 40546[2]

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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 toxic 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 spontaneous rearrangements to produce a class of pheromones collectively named autoinducer 2 (AI-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 hypothesized that *B. burgdorferi* produces LuxS for the express purpose of synthesizing DPD and utilizes a form of that molecule as an AI-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 frequently utilize *S*-adenosylmethionine (SAM) as the 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 adenine (via Pfs in many eubacteria) or homocysteine and adenosine (via SAH hydrolase 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.

* Corresponding author. Mailing address: Dept. of Microbiology, Immunology, and Molecular Genetics, University of Kentucky College of Medicine, MS 415 Chandler Medical Center, Lexington, KY 40536-0298. Phone: (859) 257-9358. Fax: (859) 257-8994. E-mail: bstev0@uky.edu.

† Current address: Yale University School of Medicine, 333 Cedar St., New Haven, CT 06510.

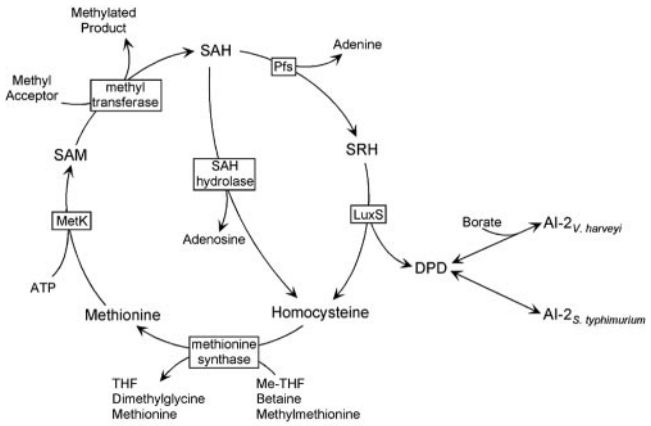

FIG. 1. Metabolic pathways found in many organisms that lead to synthesis of AI-2 and recycling of homocysteine. Me-THF, 5-methyltetrahydrofolate. DPD can spontaneously cyclize and/or combine with borate to produce at least two different, interconvertible forms of AI-2 (12, 33, 43). Many characterized 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 *Treponema pallidum* and *T. denticola* both encode homologs of Pfs 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 Pfs 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 297 was originally isolated from the cerebrospinal fluid of an infected girl in Westchester County, N.Y (48). Strain AH309, a *luxS*-deficient mutant of strain 297 (25), was obtained from Michael Norgard (University of Texas Southwestern, Dallas). 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 10^7 bacteria per ml. Cultures were then placed at 23°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-Hausser 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 μg/ml hydroxocobalamin, 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 bioassay (AB) medium (23) containing 40 μM sodium borate (pH 6.8).

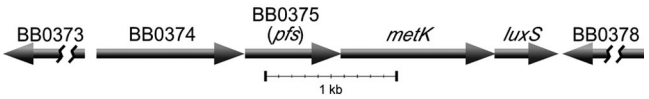

FIG. 2. Diagram of the *B. burgdorferi* chromosomal region containing the *pfs* and *luxS* genes. Studies described in this work demonstrated that ORF BB0375 encodes a functional Pfs enzyme. The ORF between *pfs* and *luxS* has been shown to encode a functional *S*-adenosylmethionine synthase (MetK) 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 kennewicki strain JEN4 (34) was cultured at 30°C in Bovuminar PLM-5 medium (Intergen, Purchase, NY).

Recombinant proteins. *B. burgdorferi* encodes a potential Pfs homolog on its chromosome, annotated as open reading frame (ORF) BB0375 in the *B. burgdorferi* strain B31 genome (Fig. 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-4 (5′-CTATTTTGTAAATTTTATGAGCTAAGG-3′) and LUXS-21 (5′-ATATGATATACATGGTTAATAGAG-3′). The resulting amplicon was cloned into the *E. coli* vector pCR2.1 (Invitrogen) to produce plasmid pRW1. This plasmid served as template for PCR amplification of both *luxS*, using oligonucleotides LUXS-13 (5′-ATGAAAAAAATAACAAGCTTTACA-3′) and LUXS-14 (5′-GGATATTTTAAATTTTCTTCTTTAATATTG-3′), and ORF BB0375, using oligonucleotides B-PFS-3 (5′-ATGATTTTGATAATATCAGCTATG-3′) and B-PFS-4 (5′-ATTAGCCTAATAAGTTCCTTTGTC-3′). In addition, the *E. coli pfs* gene (15) was amplified from genomic DNA by PCR using oligonucleotides EC-PFS-1 (5′-ATGAAAATCGGCATCATTGGTGCAATG-3′) and EC-PFS-2 (5′-CCATGTGCAAGTTTCTGCACCAGTG-3′). Each amplicon was separately cloned into pUni/V5-His-TOPO and recombined with pCRT7-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, pKVL10, and pKVL7, 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 antiserum (Proteintech, Chicago, IL). Prior to use, antiserum was preadsorbed with *E. coli* cellular lysate and passed through Hi-Trap 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 sulfhydryl 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-thiobenzuate being produced per mole of target sulfhydryl group.

***V. harveyi* bioassay of AI-2.** *V. harveyi* uses AI-2 and another pheromone, AI-1, to regulate bioluminescence. *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*

culture medium. Briefly, an overnight culture of *V. harveyi* BB170 was diluted 1:5,000 into fresh medium and a 1/10 volume of culture supernatant from either *B. burgdorferi* strain 297 or strain AH309. Bioassay cultures were incubated at 30°C with aeration, and aliquots removed at 1-h intervals from each bioassay tube and analyzed using a TopCount luminescence counter with 96-well format (Packard, Meriden, CT). AI-2 activities are reported as average luminescence values of each strain 297 assay minus the average luminescence of equivalent cultures of strain AH309. Studies were repeated at least three times using independent cultures.

Detection of *B. burgdorferi* mRNAs and proteins. Mid-exponential-phase (approximately 10^7 bacteria per ml) cultures of *B. burgdorferi* strain B31 were harvested 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). RNA was then treated with Turbo DNAfree (Ambion) to remove DNA contamination. cDNA was synthesized from 1 μg of total RNA using 1st Strand cDNA Synthesis (Roche, Indianapolis, IN) and random hexamer primers with avian myeloblastosis virus reverse transcriptase (RT) enzyme at 42° 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 BB0375 (*pfs*) were amplified by PCR using the oligonucleotide primer pairs SRA13 (5′-AGACATTAG AATCAAAGCTCCCAA-3′) and SRA14 (5′-CCAGTTCTGCATCCCATAGG GCCA-3′) and primer pair SRA9 (5′-ACTACAGGAATTGGAAAAGTTAAC-3′) and SRA10 (5′-TTGTGGCAAATTAGGGACTTGTCC-3′), respectively. PCR was performed using *Taq* 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 protein A (Amersham) and chemiluminescence (Pierce, Rockford, IL).

Effects of AI-2 on *B. burgdorferi* protein expression were 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 isoelectric focusing using precast IPG strips (Bio-Rad, Hercules, CA). Proteins were then separated in the second dimension by reducing SDS-polyacrylamide gel electrophoresis. Proteins were visualized by staining with blue silver (8). Apparently differentially expressed proteins were extracted from gels and analyzed by matrix-assisted laser desorption ionization (University of Louisville Mass Spectrometry Core Laboratory, Louisville, Ky.). Alternatively, proteins were separated by single-dimensional SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting as described above, using polyclonal antiserum directed against VlsE (Steven Norris, University of Texas, Houston) or monoclonal antibody H9724, which recognizes the constitutively expressed FlaB (flagellin) protein (3).

Genomic analyses. Genome databases of *B. burgdorferi* and three other spirochetal species were queried for the presence of specific gene homologs. Complete genome sequences of *B. burgdorferi* strain B31 (9, 18), *Treponema pallidum* Nichols strain (19), *Treponema denticola* ATCC 35405 (45), and *Leptospira interrogans* serovar Lai strain 56601 (41) were accessed through the Institute for Genomic Research microbial database at http://www.tigr.org. To search for homologs of enzymes involved in SAH metabolism, each genome was queried using BLAST-P with the amino acid sequences of the biochemically characterized *V. harveyi* and *B. burgdorferi* LuxS, *E. coli* Pfs, and *Rhodobacter capsulatus* SAH hydrolase proteins and the hypothetical SAH hydrolase of *L. interrogans* (GenBank accession numbers AF120098, AAC66762, U24438, M80630, and AAN51667, respectively). To search for homologs of enzymes catalyzing salvage of homocysteine to methionine, the spirochete genomes were queried with the MetE homologs of *E. coli*, *Mycobacterium tuberculosis*, the archaeon *Methanobacterium thermoautotrophicum*, and the plant *Solanum tuberosum* (potato); the MetH homologs of *E. coli*, *L. interrogans*, and the cyanobacterium *Synechococcus* sp. strain WH8102; the methylmethionine-dependent YagD enzyme of the *E. coli* CP4-6 prophage; and the human betaine-dependent methionine synthase (GenBank accession numbers M87625, AAK45422, X92082, AF082893, P13009, AAN51667, CAE07753, AAC73364, and U50929, respectively). Each identified spirochetal ORF was used to requery all of the spirochete genomes, along with the complete GenBank database at http://www.ncbi.nlm.nih.gov/BLAST/.

TABLE 1. Functional analyses of recombinant LuxS and Pfs enzymes[a]

In vitro reaction combination	Calculated concn of homocysteine produced (μM)
B. burgdorferi Pfs and LuxS	18
E. coli Pfs + *B. burgdorferi* LuxS	10
No added proteins	0

[a] Note that the catalyzed reactions produce equimolar concentrations of both DPD and homocysteine. Please see text for details of homocysteine assays. Since specific activities of enzymes varied between protein preparations, results of a representative set of parallel synthetic reactions are shown.

***B. burgdorferi* genomic library construction and analyses.** Total genomic DNA was purified using DNAeasy (Qiagen, Valencia, CA). DNA was digested with restriction endonuclease EcoRI or PstI for approximately 30 min at 37°C and then ligated with appropriately digested pUC118 (10, 61). Each ligation mixture was used to transform *E. coli* strain GS472. Bacteria were plated on solid minimal salts medium lacking methionine and incubated at 37°C.

Methionine synthase assay. 5-Methyltetrahydrofolate-homocysteine *S*-methyltransferase 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 lysed by sonication. Each cleared lysate was incubated with 5-[^{14}C]methyltetrahydrofolate (Amersham, Buckinghamshire, United Kingdom), L-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.

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, 19, 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. Equimolar combinations of LuxS plus either the *E. coli* Pfs protein or the *B. burgdorferi* ORF BB0375 product were incubated with SAH. As negative controls, reactions were also performed with reaction mixtures lacking either recombinant protein. Both pairs of recombinant proteins successfully converted SAH to DPD and homocysteine (Table 1). It was concluded that ORF BB0375 does

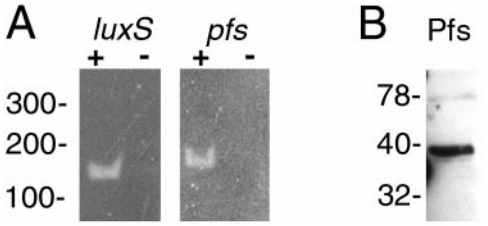

FIG. 3. Expression of (A) *luxS* and *pfs* genes and (B) Pfs protein by culture-grown *B. burgdorferi*. mRNAs were detected by RT-PCR either with (+) or without (−) added reverse transcriptase. Proteins were detected by immunoblotting using rabbit polyclonal antiserum raised against recombinant *B. burgdorferi* Pfs protein. For an as-yet-unknown reason, the native Pfs protein exhibits a lower mobility in polyacrylamide gel electrophoresis than is predicted by its molecular mass. Numbers on the left in panel A are sizes in base pairs, and those in panel B are molecular masses in kilodaltons.

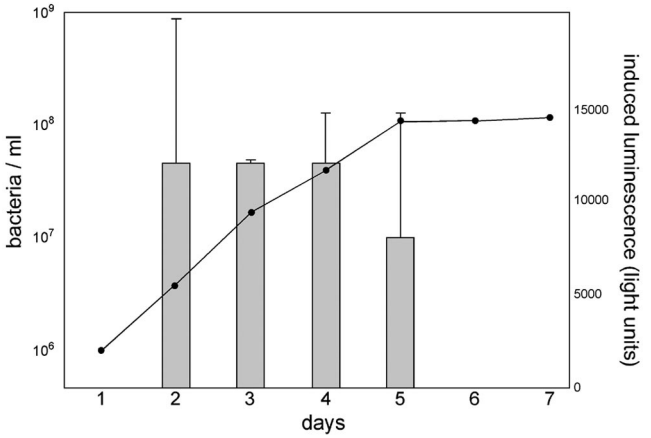

FIG. 4. Production of AI-2 by *B. burgdorferi*. Strains 297 (wild type) and AH309 (*luxS*) were diluted into fresh medium and grown for 1 to 7 days at 34°C. Culture densities were determined using a Petroff-Hausser counting chamber and are illustrated as a growth curve. *V. harveyi* reporter strain BB170 was cultured with each *B. burgdorferi* culture supernatant, and induced luminescence recorded. Values illustrated are those determined following 3 h of incubation. Luminescence values obtained for the negative control AH309 supernatants were subtracted from corresponding values obtained for strain 297. Statistically significant (>90% confidence interval by independent sample *t* test) mean luminescence values are illustrated as grey rectangles. Error bars represent standard deviations of two to five separate experiments.

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, 58, 67). Additionally, the AI-2 bioassay requires the incubation of *V. harveyi* with culture supernatant of the bacterium being tested. For that reason, unused growth medium is normally used as the negative control. In the case of *B. burgdorferi*, this is the rich, complex medium BSK-II. We noted that addition of unused BSK-II medium to *V. harveyi* medium generally resulted in greater rates of reporter strain growth than did addition of culture supernatant, presumably due to depleted nutrients and accumulated waste products in the used medium (data not shown). This phenomenon introduced the artifactual result of greater bioluminescence levels being attained from unused medium than from spent medium (data not shown).

Armed with the above information, we reexamined the ability of *B. burgdorferi* to synthesize AI-2 during laboratory cultivation. To avoid the artifact introduced by utilization of unused medium as the control, we instead compared bioassay results of wild-type strain 297 and an isogenic *luxS* mutant, AH309, using results from the mutant as background. Equivalent cultures of each strain were simultaneously examined during early through late exponential phases of growth, as well as after 2 to 3 days in stationary phase. Sodium borate was added to the *V. harveyi* culture medium during bioassays. With these modifications, we detected significant levels of AI-2 in the supernatants of cultured *B. burgdorferi* 297 (Fig. 4). AI-2 was readily detectable during exponential phase and then decreased as cultures entered stationary phase. The diminished levels of AI-2 in older cultures suggest that *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

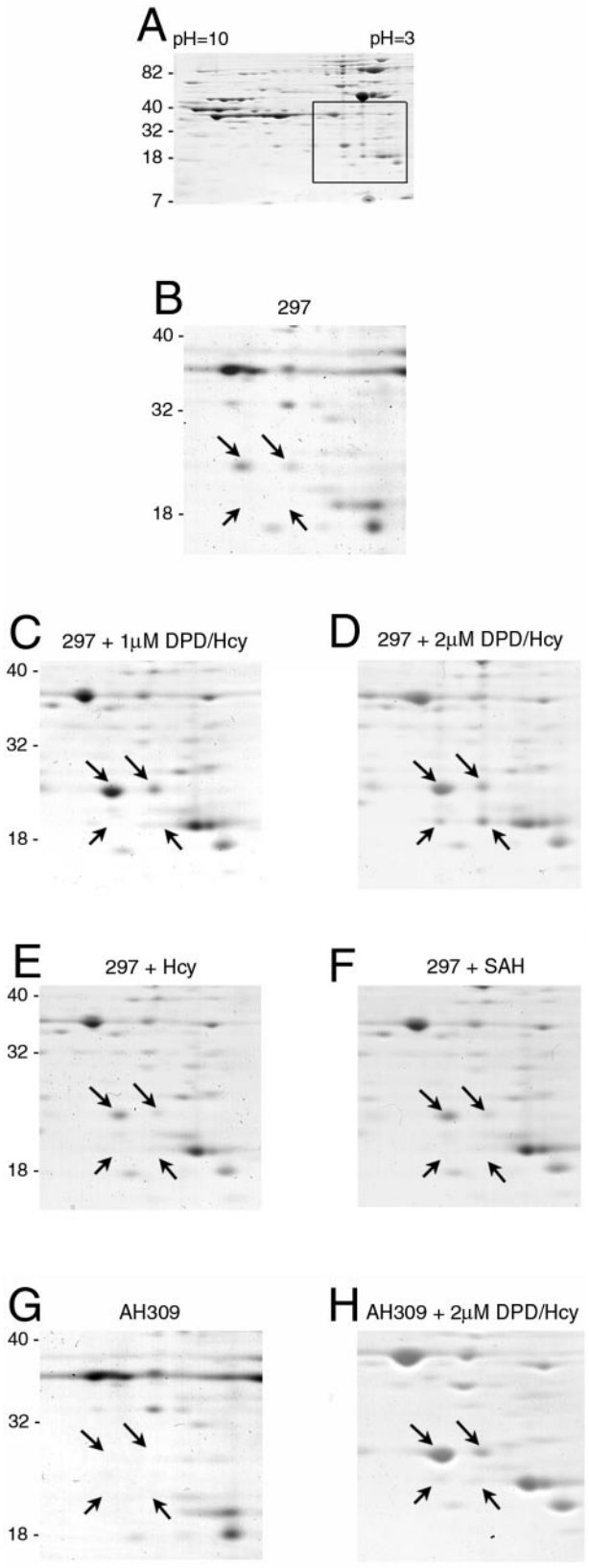

FIG. 5. Addition of in vitro-synthesized DPD affects protein expression profiles of cultured *B. burgdorferi*. Different isoelectric focusing and electrophoresis conditions reveal different portions of the *B. burgdorferi* proteome: illustrated are representative two-dimensional gels using nonlinear 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 (isoelectric focusing) occasionally varied somewhat between different gels. Numbers at left are molecular masses in kilodaltons.

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),

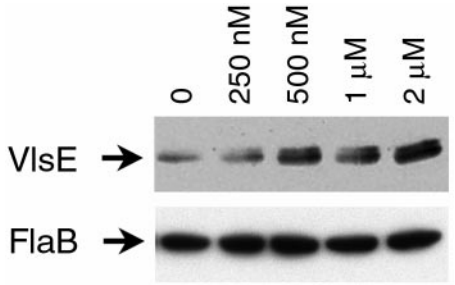

FIG. 6. Effects of DPD/AI-2 on *B. burgdorferi* VlsE protein expression. Bacteria were cultured in the presence of indicated concentrations of in vitro-synthesized DPD/AI-2 and then analyzed by immunoblotting using VlsE-directed antiserum. As controls for equal loading, membranes were also analyzed using 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).

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.

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, is the key to answering these questions. DPD can spontaneously cyclize to yield various forms of AI-2, a class of molecules 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-

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.

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All authors contributed equally to these studies.

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