Genetic and physiological characterization of the *Borrelia burgdorferi* ORF BB0374-*pfs-metK-luxS* operon

Sean P. Riley, Tomasz Bykowski, Kelly Babb,[†] Kate von Lackum[‡] and Brian Stevenson

Correspondence

Brian Stevenson

brian.stevenson@uky.edu

Department of Microbiology, Immunology and Molecular Genetics, University of Kentucky College of Medicine, MS415 Chandler Medical Center, Lexington, KY 40536-0298, USA

The Lyme disease spirochaete, *Borrelia burgdorferi*, produces the LuxS enzyme both *in vivo* and *in vitro*; this enzyme catalyses the synthesis of homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD) from a by-product of methylation reactions. Unlike most bacteria, *B. burgdorferi* is unable to utilize homocysteine. However, DPD levels alter expression levels of a subset of *B. burgdorferi* proteins. The present studies demonstrate that a single *B. burgdorferi* operon encodes both of the enzymes responsible for synthesis of DPD, as well as the enzyme for production of the Lyme spirochaete's only activated-methyl donor and a probable phosphohydrolase. Evidence was found for only a single transcriptional promoter, located 5' of the first gene, which uses the housekeeping σ^{70} subunit for RNA polymerase holoenzyme function. All four genes are co-expressed, and mRNA levels are growth-rate dependent, being produced during the exponential phase. Thus, high metabolic activity is accompanied by increased cellular levels of the only known borrelial methyl donor, enhanced detoxification of methylation by-products, and increased production of DPD. Therefore, production of DPD is directly correlated with cellular metabolism levels, and may thereby function as an extracellular and/or intracellular signal of bacterial health.

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INTRODUCTION

Lyme disease is an arthropod-borne zoonotic disease affecting humans and many domesticated and wild animals. The causative agent, *Borrelia burgdorferi*, is readily able to infect both *Ixodes* spp. ticks and a variety of vertebrate hosts (Steere, 2001). Throughout the processes of infecting these two distinct host types, and transmission between each, *B. burgdorferi* regulates expression of a large number of both metabolic and host-interface proteins. Deciphering the mechanisms by which *B. burgdorferi* controls gene and protein expression patterns is providing insights into both the physiology of this bacterium and its pathogenic abilities.

[†]Present address: BioVitesse, West Lafayette, IN 47906, USA.

[‡]Present address: Department of Oral Biology, University of Florida College of Dentistry, Gainesville, FL 32610, USA.

Abbreviations: AI-2, autoinducer-2; DPD, 4,5-dihydroxy-2,3-pentanedione; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; SRH, S-ribosylhomocysteine; RTase, reverse transcriptase; Q-RT-PCR, quantitative reverse transcription-PCR.

A supplementary figure showing the results of Q-RT-PCR analysis of *luxS* transcription by wild-type (*wt*), *rpoN* and *rpoS* *B. burgdorferi* and two supplementary tables of raw data for the Q-RT-PCR are available with the online version of this paper.

Some species of bacteria utilize the metabolic by-product 4,5-dihydroxy-2,3-pentanedione (DPD) in intercellular (and possibly intracellular) signalling. Several different, spontaneously derived forms of DPD have been identified that can function as signals, which are collectively termed 'autoinducer-2' (AI-2) (Chen *et al.*, 2002; Miller *et al.*, 2004). DPD/AI-2 is produced from the toxic end product of transmethylation reactions, S-adenosylhomocysteine (SAH), in a two-step reaction catalysed by the enzymes Pfs and LuxS. Some bacteria, such as the syphilis spirochaete *Treponema pallidum*, possess Pfs but lack LuxS, and therefore detoxify SAH only to the non-toxic S-ribosylhomocysteine (SRH) (Fraser *et al.*, 1998; von Lackum *et al.*, 2006). Other bacteria, *B. burgdorferi* included, further degrade SRH to DPD and homocysteine via the LuxS enzyme (Babb *et al.*, 2005; Stevenson & Babb, 2002; Stevenson *et al.*, 2003; Sun *et al.*, 2004; Xavier & Bassler, 2003). The majority of bacteria salvage homocysteine for regeneration of methionine, for use in additional transmethylation reactions or protein synthesis (Winzer *et al.*, 2002). *B. burgdorferi* is among the minority of bacteria which lack methionine synthase, and are therefore unable to utilize the homocysteine produced by LuxS (Babb *et al.*, 2005). Several lines of evidence indicate that *B. burgdorferi* instead uses the LuxS enzyme to

synthesize DPD/AI-2 as a means to control gene expression. Addition of either *in vitro*-synthesized DPD or supernatant from an *Escherichia coli* strain producing *B. burgdorferi* LuxS to cultured *B. burgdorferi* results in altered expression levels of a number of borrelial proteins (Babb *et al.*, 2005; Stevenson & Babb, 2002; von Lackum *et al.*, 2007). A *B. burgdorferi* *luxS* mutant, which is unable to synthesize DPD, likewise exhibits altered protein expression patterns as compared with its wild-type parent (Babb *et al.*, 2005; Stevenson & Babb, 2002; von Lackum *et al.*, 2007). *luxS* mutant *B. burgdorferi* are still able to infect mice and ticks, indicating that DPD synthesis is not absolutely essential for infection (Blevins *et al.*, 2004; Hübner *et al.*, 2003). Consistent with those results, *luxS* mutants can produce all of the proteins identified as being affected by DPD, although mutants produce different levels of those proteins than do wild-type *B. burgdorferi* (Babb *et al.*, 2005; von Lackum *et al.*, 2006, 2007). Taken together, these data suggest that *B. burgdorferi* uses DPD as a signal to fine tune gene expression levels, as opposed to a simple on/off switch.

Previous genetic and biochemical analyses identified the *pfs* and *luxS* genes of *B. burgdorferi* on the main chromosome, adjacent to two other genes that are also transcribed in the same direction (Fig. 1) (Babb *et al.*, 2005; Stevenson & Babb, 2002). The genes on either side of this four-gene cluster are transcribed in opposite directions. The gene flanked by *pfs* and *luxS*, open reading frame (ORF) BB0376 (ORF numbering system for *B. burgdorferi* strain B31 as used by Fraser *et al.*, 1997), bears homology to genes of other species that encode MetK [methionine adenosyl-transferase/S-adenosylmethionine (SAM) synthase], which catalyses the production of SAM from methionine and ATP (Fraser *et al.*, 1997). SAM serves as a methyl group donor for cellular methylation reactions, with substrates including DNA, tRNA and chemotaxis proteins (Lu, 2000). Due to widespread requirements for methylation reactions, cellular levels of activated methyl donors affect a broad

range of metabolic processes. As an example, the *metK* gene is indispensable for growth of *E. coli* (Greene *et al.*, 1973; Newman *et al.*, 1998; Wang *et al.*, 2005; Wei & Newman, 2002). The other uncharacterized gene, ORF BB0374, lacks any complete homologues outside the genus *Borrelia* (BLASTP and BLASTN searches of GenBank performed 4 November 2006). The encoded protein does, however, contain an amino acid sequence similar to that found in the HD-GYP subfamily of HD family protein domains (Galperin *et al.*, 1999). To date, all characterized HD domain-containing proteins have metal-dependent phosphohydrolase activity, with substrates that include cyclic di-GMP, guanosine tetraphosphate and tRNA (Aravind & Koonin, 1998; Galperin *et al.*, 2001; Ryan *et al.*, 2006a). For example, HD-GYP domain proteins of *Xanthomonas* spp. degrade the small signalling molecule cyclic di-GMP and are linked to both intercellular signalling and pathogenicity (Andrade *et al.*, 2006; Ryan *et al.*, 2006a, b, 2007; Slater *et al.*, 2000). A response regulator protein of *B. burgdorferi*, Rrp1, synthesizes cyclic di-GMP (Ryjenkov *et al.*, 2005), but the phosphohydrolase responsible for degrading cyclic di-GMP has yet to be identified.

Prior to the current study, nothing was known about the transcriptional organization of *pfs*, *luxS* and their neighbouring genes, other than that *pfs*, *luxS*, and the ORF located between them are encoded on the same mRNA (Hübner *et al.*, 2003). To better understand the genetics of the *B. burgdorferi* Pfs-LuxS system, we examined promoter location, transcriptional linkage and regulation of all four of the adjacent genes, in addition to biologically characterizing enzymic activity of the ORF BB0376 protein product.

METHODS

Bacterial strains and culture. Strains used are described in Table 1. *B. burgdorferi* were cultivated at 34 °C in modified Barbour–Stoenner–Kelly medium (BSK-II, Barbour, 1984). For growth related experiments, BSK-II was inoculated with a 1:500 dilution of

Fig. 1. (A) Diagram of the ORFs described in this work (to scale). Arrows indicating approximate location of oligonucleotides used in (B). (B) Transcriptional linkage analyses. PCR amplification of specific sequences from cDNA produced from total borrelial RNA. + indicates presence of RTase and cDNA production, and - indicates RTase-free RNA controls.

Table 1. Strains, plasmids and oligonucleotides used in this study

Designation	Relevant characteristics	Source or reference
<i>E. coli</i> strains		
MEW402	MEW1 <i>metK84</i> ; leucine-requiring	Newman <i>et al.</i> (1998)
SPRM1	MEW402(pBLS602)	This work
<i>B. burgdorferi</i> strains		
B31-MI-16	Clonal derivative of B31	Babb <i>et al.</i> (2005)
B31-A3	Clonal derivative of B31	Elias <i>et al.</i> (2002)
A3 <i>ntrA</i>	B31-A3 <i>ntrA</i> ::(P <i>flaB</i> :: <i>kan</i>)	Fisher <i>et al.</i> (2005)
A3 <i>rpoS</i>	B31-A3 <i>rpoS</i> ::(P <i>flaB</i> :: <i>kan</i>)	Elias <i>et al.</i> (2002); Fisher <i>et al.</i> (2005)
Plasmids		
pBLS602	pCR2.1:: <i>B. burgdorferi metK</i>	This work
pLuxS4-21	pCR2.1:: BB0374- <i>luxS</i>	Babb <i>et al.</i> (2005)
pSRBA	pCR2.1:: BB0374 promoter	This work
pSR1E3	pET100:: BB0374	This work
pSRE-HD	pET100:: BB0374-HD-GYP domain	This work
Oligonucleotides		
SRA1	5'-CTAGTGTTCATCTATAC-3'	
SRA2	5'-TTCTTCTTTGTTGTCAAG-3'	
SRA5	5'-CTTGCACTTATGGTCATT-3'	
SRA6	5'-GAGACATATATGCCAGGG-3'	
SRA7	5'-TAGGCGCTGCTAGTGCCTATTCTG-3'	
SRA8	5'-TTGGGTTAGACTCATCTATATCTA-3'	
SRA9	5'-ACTACAGGAATTGGAAAAGTTAAC-3'	
SRA10	5'-TTGTGGCAAATTAGGGACTTGTCC-3'	
SRA11	5'-TAGCTTGCGAGGTCATAATTGCAC-3'	
SRA12	5'-TTCCCTGATCCCCTGCTCCAAGGG-3'	
SRA13	5'-AGACATTAGAATCAAAGCTCCCAA-3'	
SRA14	5'-CCAGTTCTGCATCCCATAGGGCCA-3'	
SRA21	5'-GCTCAAGTAGCACACATA-3'	
SRA22C	5'-TATTCTTCTGATTCTTCTTG-3'	
SRB2	5'-CCTTGCTAAATATGATGAAT-3'	
SRB3	5'-GAAATCTTAAATTTGCCAGAA-3'	
SRB4	5'-TTCTTCTTTGTTGTCAAG-3'	
FLA3	5'-GGGTCTCAAGCGTCTTGG-3'	
FLA4	5'-GAACCGGTGCAGCCTGAG-3'	
SRE5	5'-CACCATGCAAAATTCTGAAAGCATT-3'	
SRE6B	5'-TTATATATAATATCTATTAAAGAATAT-3'	
OSPC7	5'-CAGGGAAAGATGCGAATACATCTGC-3'	
OSPC8	5'-TAAGCTAAAGCTAACAATGATCC-3'	
METK1	5'-TAGGAGGCTAATATGAATAAAATAATAG-3'	
METK2	5'-ATTTTTCATTTTTCATCGCTG-3'	
SRE-HD 1	5'-CACCCACATTATTCATTCGGTAAATACAGC-3'	
SRE-HD 2	5'-CTATTAGATAGCAGAATAGGCACTAGCGCC-3'	

mid-exponential-phase B31-MI-16 ($\sim 10^7$ bacteria ml^{-1}) and grown at 34 °C for 2 ($\sim 5 \times 10^6$ bacteria ml^{-1}), 4 ($\sim 1 \times 10^8$ bacteria ml^{-1}), 6 ($\sim 1.5 \times 10^8$ bacteria ml^{-1}) or 8 days ($\sim 2 \times 10^8$ bacteria ml^{-1}). In all other cases, bacteria were harvested when in mid-exponential phase.

***metK* genetic complementation.** *E. coli* MEW402 contains *lrp* and *metK84* mutations that make its production of MetK/SAM synthase acutely dependent on the presence of leucine in the culture medium (Newman *et al.*, 1998). A DNA segment of *B. burgdorferi* B31-MI-16, encompassing ORF BB0376 from the ribosome-binding site to 3' of the stop codon, was amplified by PCR using oligonucleotide primers METK-1 and METK-2 (Table 1). The resulting amplicon was cloned into pCR2.1 (Invitrogen). One recombinant plasmid, pBLS602, contains the complete ORF BB0376 under the transcriptional control

of the plasmid's *lac* promoter. The insert of pBLS602 was completely sequenced on both strands to ensure that errors had not been introduced during the cloning processes. *E. coli* MEW402 was transformed with pBLS602 to create *E. coli* SPRM1. Maintenance of pBLS602 was ensured by the addition of 50 μg kanamycin ml^{-1} to all cultures. MEW402 and SPRM1 were each inoculated into M9 medium supplemented with 0.2% glucose, 2 mg serine ml^{-1} and 200 μg glycine ml^{-1} , with or without 50 μg leucine ml^{-1} , and cultured at 37 °C (Newman *et al.*, 1998). Bacteria were diluted 1:100 daily into fresh media for three successive days. After the third passage, IPTG was added to a final concentration of 1 mM to both cultures, inducing transcription of the *B. burgdorferi* ORF BB0376 in SPRM1, and acting as a control for the parental strain. Wet mounts of bacteria from each culture were examined by darkfield microscopy

using a Nikon Optiphot, and were photographed at 40 × magnification using a Nikon Coolpix5700 digital camera with 8 × optical zoom.

Production of recombinant ORF BB0374 protein. The complete ORF BB0374 and the internal fragment encoding the HD-GYP domain were PCR amplified from pRW1 (Babb *et al.*, 2005) using primers SRE5+6B or SRE-HD1+2 respectively (Table 1), and cloned into pET100 (Invitrogen) to produce plasmids pSR1E3 and pSRE-HD. Plasmids were isolated from *E. coli* and inserts were sequenced. Plasmids were individually transformed into *E. coli* BL21star(DE3) (Invitrogen), cultured until early exponential phase (OD₆₀₀ ~0.5), then production of recombinant proteins was induced by addition of IPTG to 1 mM. Aliquots were taken hourly for OD₆₀₀ readings, then bacteria were pelleted by centrifugation for protein analysis. Induction of protein production was assessed by SDS-PAGE followed by either Coomassie brilliant blue staining or transfer and immunoblotting using anti-Xpress antibody (Invitrogen).

RNA isolation and cDNA preparation. Total RNA was extracted from each *B. burgdorferi* culture described above using TRIzol (Life Technologies), resuspended in RNase-free resuspension reagent (Ambion), and treated with DNase I (Ambion) to remove contaminating DNA. Single 1 µg aliquots of each DNA-free RNA preparation were reverse transcribed using a First Strand cDNA synthesis kit (Roche) with random hexamers and Avian Myeloblastosis Virus (AMV) reverse transcriptase enzyme (RTase) at 55 °C for 30 min. The RTase was inactivated for 10 min at 85 °C followed by 10 min at 4 °C.

Primer extension. RNA was isolated from a mid-exponential phase culture of *B. burgdorferi* strain B31-MI-16 as described above. Primer extensions were performed using the Primer Extension system/AMV Reverse Transcriptase system (Promega) with [γ -³²P]ATP. Briefly, oligonucleotide primers SRB2, SRA2, SRA22C and SRA6 (Table 1) were individually labelled using T4 polynucleotide kinase, then annealed to the RNA by heating to 58 °C followed by cooling to room temperature. Primer extension was performed with AMV RTase for 30 min at 42 °C, and the mixture was immediately heated to 70 °C with the addition of loading buffer (Promega). DNA markers for size determination were also labelled with [γ -³²P]ATP using T4 polynucleotide kinase. The pSRBA plasmid, which contains the DNA sequence 5' of ORF BB0374, was constructed by PCR amplification of *B. burgdorferi* DNA using the primer pair SRB3+4 and cloned into pCR2.1 (Invitrogen). DNA sequencing was performed on pSRBA (Table 1) using Sequenase version 2.0 (USB) and α -³³P-labelled nucleotides. Primer extension and sequencing products, and labelled size markers were simultaneously separated by urea-PAGE, and labelled DNAs were detected by autoradiography.

Linkage analysis. To examine transcriptional linkage, cDNA from mid-exponential-phase cultures of *B. burgdorferi* B31-MI-16 were subjected to PCR with primers SRA1+2, 5+6, 7+8, 9+10, 11+12, 13+14 or 21+22C, to amplify cDNA segments within and spanning the intergenic regions for each of the ORF BB0374, *pfs*, BB0376 (*metK*) and *luxS* open reading frames (Table 1 and Fig. 1). Reaction conditions were 94 °C for 30 s, 50 °C for 30 s, and 68 °C for 30 s for 35 cycles. A RTase-free RNA sample was examined using each primer set, to test for DNA contamination. PCR products were resolved in native 6% polyacrylamide gels stained with ethidium bromide, followed by observation under UV illumination. Each product was also cloned into pCR2.1, and plasmid inserts were sequenced to verify specific amplification.

Quantitative RT-PCR (Q-RT-PCR). cDNA samples were amplified in a LightCycler (Roche Applied Science) and data were analysed as described previously (Miller, 2006). Briefly, cDNA was added to a

master mix containing 1 × PCR buffer, enzyme diluent, dNTPs (Idaho Technology), Platinum Taq (Invitrogen), SYBR green (Molecular Probes), nuclease-free water, and oligonucleotide primers (0.4 µM final concentration). Primers used for amplification of *flaB*, ORF BB0374, *pfs*, ORF BB0376, and *luxS* were FLA3+4, SRA7+8, SRA9+10, SRA11+12 and SRA13+14, respectively (Table 1). Reaction conditions consisted of a 2 min initial 94 °C denaturation followed by 50 cycles of 94 °C for 5 s, 55 °C (FLA3+4, SRA11+12, 13+14) or 52 °C (SRA7+8, 9+10) for 10 s and 72 °C for 30 s. LightCycler software v. 3.5.3 was used for quantification and melting curve analysis (Gilmore *et al.*, 2001). All products were separated by electrophoresis through a 1% agarose gel and stained with ethidium bromide to verify predicted band size. The cDNA was produced from three independently grown cultures for each time point. Expression values obtained from quadruplicate runs of each cDNA sample for the four genes were calculated relative to the mean quadruplicate value for the *B. burgdorferi* housekeeping gene *flaB* from the same cDNA preparation.

To test sigma factor selectivity, DNA-free RNA samples from mid-exponential-phase cultures of *B. burgdorferi* A3, A3*ntrA*, A3*rhoS* were isolated and cDNAs were synthesized. Transcript levels for *luxS*, *flaB* and *ospC* were assessed by Q-RT-PCR as above. The same primers as above were used for *luxS* and *flaB*, while *ospC* was examined using OSPC7+8 (Table 1). Reaction conditions for *ospC* consisted of a 2 min initial 94 °C denaturation followed by 50 cycles of 94 °C for 5 s, 52 °C for 10 s, and 72 °C for 30 s. All analyses were performed a minimum of three times.

RESULTS

ORFs BB0374 and BB0376

Our previous studies demonstrated that *B. burgdorferi* *pfs* and *luxS* encode functional enzymes (Babb *et al.*, 2005; Stevenson & Babb, 2002). The previously uncharacterized ORF BB0376 is located between *pfs* and *luxS*, with all three genes overlapping. ORF BB0376 encodes a potential protein with a 49.4% identical and 67.8% similar amino acid sequence to *E. coli* K-12 MG1655 *metK* (data not shown). The product of MetK, SAM, serves as a methyl-group donor for biological processes. Upon methyl-group transfer to a substrate, SAM is converted to SAH, the substrate for the Pfs-catalysed reaction. If ORF BB0376 is indeed a *metK* homologue, it would play a role in both borrelial methylation reactions and production of DPD. Since it is inappropriate to assume that genes with similar sequences must necessarily encode enzymically identical proteins, we tested the functionality of the ORF BB0376-encoded protein.

Production of MetK is essential for normal growth and cell division of *E. coli* (Newman *et al.*, 1998; Wang *et al.*, 2005; Wei & Newman, 2002). *E. coli* strain MEW402 produces wild-type levels of MetK when grown in medium containing leucine (Fig. 2A). Depletion of leucine leads to inhibition of MetK synthesis, resulting in a septation defect and formation of long filaments (Fig. 2B) (Newman *et al.*, 1998; Wang *et al.*, 2005). Plasmid pBLS602, which contains *B. burgdorferi* ORF BB0376 under control of the *lac* promoter, was introduced into MEW402, producing strain SPRM1. Cultures of SPRM1 in media lacking leucine

Fig. 2. Genetic complementation of the *E. coli* MEW402 *metK84* mutation by *B. burgdorferi* ORF BB0376 (*metK*). All images are shown at the same magnification. (A) MEW402 cultured in minimal medium plus leucine. (B) Septation defect exhibited by MEW402 cultured in medium lacking leucine. (C) Septation defect of *E. coli* SPRM1 (MEW402 carrying pBLS602) when grown in medium lacking leucine. (D) Restoration of cell division in SPRM1 grown in leucine-deficient medium following IPTG induction of *B. burgdorferi* ORF BB0376. These results are comparable to those obtained previously when *metK84 E. coli* was complemented with the wild-type *E. coli metK* gene (Newman *et al.*, 1998).

formed filaments similar to those of the parent strain (Fig. 2C). Induction of the *lac* promoter by addition of IPTG restored normal cell division, and the filamentous phenotype was not observed (Fig. 2D). IPTG had no such effect on the parental strain MEW402 (data not shown). This complementation indicated that ORF BB0376 is indeed a *metK* homologue, and it will be referred to as such through the remainder of this report.

To attempt characterization of the ORF BB0374-encoded protein, constructs were made that directed production of either recombinant full-length protein or the HD-GYP domain only. However, *E. coli* carrying either of these constructs ceased growth immediately upon induction of recombinant protein synthesis. In contrast, induction of recombinant borrelial Pfs or LuxS production by *E. coli* using the same T7 polymerase/pET-based expression system had no apparent negative effects upon *E. coli* growth. Due to the apparent toxicity of the ORF BB0374 gene product to *E. coli*, we were unable to assess whether or not it is the borrelial cyclic di-GMP phosphohydrolase. We are continuing to pursue alternative approaches to expressing recombinant ORF BB0374 protein, to answer that question.

ORF BB0374, *pfs*, *metK* and *luxS* form an operon

The four genes in question are each transcribed in the same direction and are flanked by genes that are transcribed in opposite directions. There are very few noncoding nucleotides between the four genes: between ORF BB0374 and *pfs* there are 23 bp, and there are no bases between *pfs* and *metK* or *metK* and *luxS*. These observations suggest that all four genes are possibly co-transcribed. Indeed, a previous RT-PCR study indicated that *pfs* and *metK* are included on the same mRNA, as are *metK* and *luxS* (Hübner *et al.*, 2003). We extended that earlier work by demonstrating by RT-PCR that ORF BB0374 and *pfs* are also transcribed on the same mRNA (Fig. 1). The tight spacing of the four genes would prevent Rho-dependent termination prior to

the end of *luxS*. Examination of the sequence spanning ORF BB0374 through *luxS* did not reveal any inverted repeats that could serve as Rho-independent terminators. Northern blot analysis using probes derived from ORF BB0374 or *luxS* revealed only a broad RNA smear (data not shown).

Recognizing the possibility that this four-gene locus could have multiple promoters, we performed primer extension analysis on purified *B. burgdorferi* RNA. A product was obtained only from extensions originating within ORF BB0374 (data not shown). Control analyses without addition of RNA confirmed that the band observed was due to extension from mRNA. This transcriptional start site was finely mapped using high-resolution gels and simultaneous di-deoxy sequencing of a DNA template using the same primer. Two transcription start sites were detected, 23 and 25 bp 5' of the ORF BB0374 translation initiation codon (Fig. 3). Potential -10 and -35 sequences are located at appropriate distances 5' of the transcription start sites, as is also a consensus extended -10 sequence.

Growth rate dependent expression of the operon

A previous study indicated that cultivated *B. burgdorferi* secrete maximal levels of DPD during the exponential phase of growth, while that compound is largely undetectable in supernatants of stationary-phase cultures (Babb *et al.*, 2005). Q-RT-PCR analysis of each gene in the ORF BB0374-*pfs*-*metK*-*luxS* operon was performed to determine the connection between gene expression and production of DPD, as well as to examine for evidence of independent regulation. To that end, cultures were inoculated at low densities and harvested after 2 days ($\sim 1 \times 10^6$ cells ml $^{-1}$, early exponential phase, approximately four doublings), 4 days ($\sim 1 \times 10^8$ cells ml $^{-1}$ mid-exponential phase, approximately 8 doublings), 6 days ($\sim 1.5 \times 10^8$ cells ml $^{-1}$, early stationary phase), and 8 days ($\sim 2 \times 10^8$ cells ml $^{-1}$, well into stationary phase) (Babb *et al.*, 2005). Total RNA

Fig. 3. Fine mapping of the transcriptional start site upstream of ORF BB0374. Primer extension products were electrophoresed with the corresponding DNA sequences. The two specific primer extension products mapped to 23 and 25 bp 5' of the start codon for ORF BB0374 (asterisks). Potential -10 , extended -10 , and -35 sites are present 5' of these transcriptional initiation sites. The sequence CAT in bold type 5' of ORF BB0374 indicates the initial codon of the divergently transcribed ORF BB0373.

was extracted and transcript levels of each gene were determined (see Supplementary Table S1, available with the online version of this paper). Due to the constitutive expression of *flaB*, Q-RT-PCR results for ORF BB0374, *pfs*, *metK* and *luxS* were standardized relative to the *flaB* results. Similar relative levels of expression were observed for all four genes of the operon (Fig. 4). Moreover, expression levels of each gene were at least fivefold higher during the early exponential phase of growth than during the later stationary phase.

Sigma factor selectivity of the operon

B. burgdorferi encodes three RNA polymerase sigma subunits, the housekeeping σ^{70} and homologues of σ^S and σ^N . Several studies have implicated the importance of RNA polymerase subunits σ^S and σ^N in *B. burgdorferi* transitional events, including transmission from tick to mammal (Caimano *et al.*, 2004; Elias *et al.*, 2000; Yang *et al.*, 2000). Since the ORF BB0374-*pfs*-*metK*-*luxS* operon is induced during tick-to-mammal transmission

Fig. 4. Growth-phase-dependent expression of ORF BB0374, *pfs*, *metK* and *luxS*. Bacteria were harvested at densities of 6×10^6 , 1×10^8 , 1.5×10^8 and 2×10^8 bacteria ml^{-1} , corresponding to early exponential, mid-exponential, early stationary and late stationary phases, respectively (Babb *et al.*, 2005). Three independently grown cultures were analysed for each time point, and each cDNA was analysed in quadruplicate. Total RNA was extracted and relative mRNA levels analysed by Q-RT-PCR. All results were standardized relative to mRNA levels of the constitutively expressed *flaB* gene. Error bars indicate one standard deviation from the mean. Raw data used to generate graphs are presented in Supplementary Table S1.

(Narasimhan *et al.*, 2002), we examined whether either σ^S or σ^N played a role in regulation of operon transcription. The σ^S subunit is induced as cultures approach stationary phase, and σ^S levels are dependent on σ^N (Caimano *et al.*, 2004; Elias *et al.*, 2000; Yang *et al.*, 2000). *B. burgdorferi* with specific deletions of either *rpoS* (σ^S) or *rpoN* (σ^N) produced *luxS* transcripts at levels comparable to wild-type bacteria (Supplementary Fig. S1, Supplementary Table S2), indicating that neither alternative sigma factor is directly responsible for transcription of this operon. The control gene, *ospC*, was not transcribed by either mutant, consistent with previous analyses of that gene (Hübner *et al.*, 2001; Yang *et al.*, 2003). These data indicate that the housekeeping factor σ^{70} interacts with the RNA polymerase holoenzyme 5' of ORF BB0374 to direct transcription of this operon.

DISCUSSION

The present studies demonstrated that genes encoding the two enzymes responsible for synthesis of DPD from SAH, the enzyme that produces the activated-methyl donor SAM, and a putative phosphohydrolase are contained in a single operon. Evidence was found for only a single transcriptional promoter, located 5' of the first of the four genes, which uses the housekeeping σ^{70} RNA polymerase holoenzyme. All four genes were co-expressed, being produced during the exponential phase of growth, and repressed during the stationary phase. These data are

consistent with previous observations that *B. burgdorferi* produces maximal levels of DPD/AI-2 during exponential phase, and increased expression of *luxS* during the period of rapid growth accompanying transmission from feeding tick vectors to mammalian hosts (Babb *et al.*, 2005; Narasimhan *et al.*, 2002). Thus, high metabolic states are accompanied by increases in three enzymic activities: (1) MetK, which increases cellular levels of the only known borrelial methyl donor, SAM; (2) Pfs, which detoxifies the product of SAM-dependent methylation reactions; and (3) LuxS, which produces DPD and homocysteine. As described above, *B. burgdorferi* lacks the ability to use homocysteine; the Pfs product, SAH, is not toxic; and the addition of DPD to Lyme disease spirochaetes causes alterations in protein expression patterns. It is likely, therefore, that production of DPD is directly correlated with cellular fitness, and, reciprocally, that DPD may function as a signal of bacterial fitness (Xavier & Bassler, 2003). Such a signal may function extracellularly, to influence the entire population, intracellularly as an alarmone to modify expression levels of that bacterium's own genes, or as a combination of these two possibilities (Beeston & Surette, 2002; Camilli & Bassler, 2006; Redfield, 2002; Xavier & Bassler, 2003).

Since the product of the MetK-catalysed reaction is converted to the Pfs substrate by cellular methyltransferases, MetK makes a direct contribution to AI-2 synthesis, as well as to regulation of the total cellular methyl pool. In many bacteria, a second major methyl-group donor, methylenetetrahydrofolate (THF), is involved in nucleic and amino acid biosynthesis, but *B. burgdorferi* lacks homologues of most enzymes required for production of THF (Fraser *et al.*, 1997). *B. burgdorferi* encodes numerous postulated methyltransferases which have potential substrates including tRNA, rRNA, DNA and chemotaxis proteins, with SAM likely to be the only molecule capable of donating activated methyl groups. Therefore, we hypothesize that production of MetK is directly proportional to the rate of total methylation reactions in the cell.

In conclusion, the ORF BB0374-*pfs-metK-luxS* operon encodes three enzymic activities that directly connect cellular methylation and bacterial growth to production of the signalling molecule DPD. ORF BB0374 encodes a probable phosphohydrolase. The interrelatedness of pathways suggests that regulation of the BB0374-*pfs-metK-luxS* operon plays a role in coordinating physiological adaptations of *B. burgdorferi*. Elucidating the regulatory mechanisms controlling transcription of this operon and the effects of each gene product will illuminate further our understanding of the biology of *B. burgdorferi* and the mechanisms by which the Lyme disease spirochaete infects its diverse hosts.

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