JOURNAL OF BACTERIOLOGY, Apr. 2010, p. 1965–1974 Vol. 192, No. 7
0021-9193/10/$12.00 doi:10.1128/JB.01616-09

Analysis of the *dbpBA* Upstream Regulatory Region Controlled by RpoS in *Borrelia burgdorferi*[▽]

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Received 11 December 2009/Accepted 15 January 2010

Decorin-binding proteins B and A (DbpB and DbpA) are thought to play important roles in *Borrelia burgdorferi* pathogenesis by serving as adhesins for the extracellular matrix. It has been established that the expression of DbpBA is governed by the Rrp2-RpoN-RpoS regulatory pathway. However, the precise mechanism underlying the control of DbpBA expression has been unclear. In particular, it has been unknown whether RpoS influences DbpBA expression directly or indirectly (through an additional regulatory molecule[s]). Here, employing a wild-type *B. burgdorferi* strain and a *dbpBA*-deficient mutant, we analyzed the 5′ genetic elements of the *dbpBA* operon using deletion analysis, coupled with luciferase reporter assays, quantitative reverse transcription PCR, and immunoblot analyses. A minimal promoter, encompassed within 70 bp upstream of the ATG start codon of *dbpBA*, was identified and found to be necessary and sufficient to initiate *dbpBA* transcription. The minimal *dbpBA* promoter was responsive to environmental stimuli such as temperature, pH, and whole blood. Two *in silico*-identified inverted repeat elements were not involved in the response of *dbpBA* expression to *in vitro* stimulation by environmental factors. The expression of *dbpBA* from the minimal promoter was abolished when *rpoS* was inactivated. In addition, the targeted mutagenesis of a C at position −14 within the extended −10 region of *dbpBA*, which has been postulated to be strategic for $E\sigma^S$ binding in *Escherichia coli*, abolished *dbpBA* expression in *B. burgdorferi*. These combined data suggest that the Rrp2-RpoN-RpoS pathway controls *dbpBA* expression by the direct binding of RpoS to an RpoS-dependent promoter. However, given that there remains a distinct difference between the expression of DbpBA and other genes under the direct control of RpoS (e.g., OspC), our findings do not preclude the existence of another layer of gene regulation that may contribute to the modulation of DbpBA expression via an as-yet unknown mechanism.

Borrelia burgdorferi, the etiological agent of Lyme disease, is maintained in nature through a complex enzootic life cycle, which involves an arthropod vector (*Ixodes* tick) and a variety of mammalian hosts (10, 50). After *B. burgdorferi* is transmitted to humans through tick bites, spirochetes can disseminate and spread hematogenously to various target sites, such as heart, joints, and other distant locations, causing a broad spectrum of clinical manifestations, including carditis, arthritis, and neuroborreliosis (42, 49). It has long been presumed that the interactions between surface molecules such as the adhesins of *B. burgdorferi* and specific molecules in the mammalian hosts are critical for the pathogen to disseminate to and colonize specific niches (14, 16, 17). In this regard, *B. burgdorferi* expresses several adhesins, including the decorin-binding proteins (Dbp) DbpA and DbpB (22, 23), BBK32 (40), P66 (BB0603) (9, 15), and Bgp (*Borrelia*-GAG-binding protein BB0588) (37), which are thought to bind to integrin and mammalian extracellular matrix (ECM) components such as fibronectin, decorin, glycosaminoglycans (GAGs), and type I collagen. In addition, *B. burgdorferi* also expresses OspA, which binds to TROSPA expressed in tick midguts (32, 34, 35).

Among the putative adhesins expressed by the Lyme disease spirochete, DbpB and DbpA are encoded in a bicistronic operon, *dbpBA*, on linear plasmid lp54 in *B. burgdorferi* (20, 23, 24). These two surface-exposed proteins were first reported in a study to identify *B. burgdorferi* molecules that bind decorin, a collagen-binding proteoglycan produced in the connective tissues. Using gel overlay assays, Guo et al. (23) observed two decorin-binding proteins in *B. burgdorferi*, DbpB and DbpA, of 19 and 20 kDa, respectively. These two proteins are 56% similar, and both proteins contain conserved lysine residues critical for decorin recognition (7, 22, 38). Due to the propensity for the recombinant versions of DbpB and DbpA to bind to decorin and GAGs, DbpB and DbpA continue to be implicated as being important to the colonization and dissemination of *B. burgdorferi* within mammalian hosts. Furthermore, Brown et al. (8) reported that the ability of *B. burgdorferi* to disseminate, survive, and cause disease was impaired in decorin-deficient mice. More recently, data from three independent groups have revealed that the deletion of either *dbpB*, *dbpA*, or both genes in *B. burgdorferi* resulted in a dramatic decrease in the infectivity of needle-inoculated mice, suggesting that both DbpA and DbpB proteins contribute to *B. burgdorferi*'s infectivity (3, 46, 47, 54). However, Blevins et al. (3) reported that the deletion mutant lacking *dbpBA* still was able to infect naïve mice via tick bite (50% [1/2] and 67% [4/6] of mice became infected when challenged with 5 and 10 ticks containing the *dbpBA* mutant, respectively). These data suggest that although both DbpB and DbpA probably contribute to *Borrelia* virulence, their precise roles in *B. burgdorferi* pathogenesis and infectivity still are not fully understood.

Studies have shown that *B. burgdorferi* alters its expression of DbpBA in response to various environmental stimuli, such as temperature, pH, cell density, and dissolved CO_2 and O_2,

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▽ Published ahead of print on 29 January 2010.

changes that *B. burgdorferi* ostensibly encounters during its transition between the tick vector and mammalian hosts (28, 44, 45, 52, 56). Moreover, work from our laboratory and others has demonstrated that these signals influence *dbpBA* expression in *B. burgdorferi*, likely via the Rrp2-RpoN-RpoS regulatory pathway (5, 6, 11–13, 18, 27, 31, 33, 48, 58). In this pathway, under elevated temperature (e.g., 37°C) or low pH (pH 6.8), the putative response regulator Rrp2, along with the alternative sigma factor RpoN (σ^N), directly activates the expression of the central alternative sigma factor RpoS (σ^S) which, in turn, regulates the expression of a number of *B. burgdorferi* virulence-associated lipoproteins, such as DbpBA, OspC, and the Mlp family. There now are compelling data that the Rrp2-RpoN-RpoS pathway regulates the expression of OspC by the direct interaction of RpoS with the RpoS-dependent *ospC* promoter (1, 18, 59). However, it has remained unclear how the *dbpBA* operon is controlled by RpoS. This information gap is of particular interest in view of the fact that *dbpA* exhibits an expression pattern somewhat different from that of *ospC*. Although both *ospC* and *dbpA* are upregulated by a shift to elevated temperatures, only OspC expression is highly induced by the process of tick feeding (21, 26, 36). DbpA (and presumably DbpB) expression is absent in both flat and fed ticks (25), suggesting that the transient elevation at ambient temperature during tick feeding does not overcome a suppression mechanism involved in tempering *dbpBA* expression. Furthermore, DbpA likely is persistently expressed during the course of mammalian infection, whereas OspC expression diminishes in late phase or persistent infection in mammalian hosts (29, 30). In addition, relative to gene expression in *B. burgdorferi* cultivated under certain *in vitro* growth conditions, the transcription of both *rpoS* and *ospC* were upregulated when *B. burgdorferi* was cultured within intraperitoneal dialysis membrane chambers (DMCs), whereas *dbpA* transcription was downregulated (13). Nonetheless, the level of DbpA protein, together with the protein levels of RpoS and OspC, was increased when *B. burgdorferi* was cultured within DMCs (relative to *in vitro*-cultured *Borrelia*). This observation suggests that a suppression mechanism or posttranscriptional regulatory mechanism (probably involving signals from the mammalian host) influences *dbpBA* expression. Finally, Yang et al. (56) showed that o*spC* and *dbpA* respond differently to pH changes when *B. burgdorferi* is cultured in Barbour-Stoenner-Kelly (BSK) medium. These combined observations thus have prompted the hypothesis that, in addition to RpoS, some other regulatory molecule(s) is involved in the modulation of DbpBA expression. As a first step toward assessing the contribution of RpoS-mediated control over *dbpBA* expression, herein we focused on examining putative *cis* elements in the 5′ upstream regulatory region(s) of *dbpBA*, with emphasis on assessing whether *dbpBA* contains a minimal RpoS-dependent promoter. The use of luciferase reporter constructs (*luc* fused to the putative *dbpBA* promoter) within the native *Borrelia* background facilitated these studies.

MATERIALS AND METHODS

Bacterial strains and culture conditions. All strains and plasmids used in this study are described in Table 1. Barbour-Stoenner-Kelly II (BSK-II) medium or BSK-H medium (Sigma Chemical Co., St. Louis, MO) (39) supplemented with 6% rabbit serum (Pel-Freez Biologicals, Rogers, AR) was used routinely to grow *B. burgdorferi*. Spirochetes were enumerated using dark-field microscopy. To determine the effects of temperature and pH on gene expression, spirochetes were cultured under various environmental conditions, such as at 23 or 37°C or in BSK medium at pH 7.6 or 6.8, as described previously (33, 56). To determine the influence of blood supplementation on gene expression, various strains were inoculated into BSK-H medium at 1×10^3 spirochetes/ml. The culture was split into two groups when it reached a density of $\sim 1 \times 10^6$ to 5×10^6 cells/ml. To the test group, 6% fresh heparinized rabbit blood (from which the buffy coat was removed) was added, whereas 6% heparin-supplemented BSK-II (~14 USP/ml), instead of heparinized rabbit blood, was added to the control culture. Cultures were grown at 37°C for 48 h, with periodic mixing to prevent the settling of the red blood cells (RBCs). Cells from both groups were harvested for luciferase assays when the cell density reached late log phase (approximately 5×10^7 bacteria/ml). Immediately prior to harvest, 6% whole blood was added to the control culture. *Escherichia coli* strain TOP10 (Invitrogen, Carlsbad, CA) was used as the cloning host. When appropriate, kanamycin (Kan) or streptomycin (Strep) was added to BSK medium at a final concentration of 160 or 150 μg/ml, respectively.

Generation of luciferase reporter vectors and DbpBA expression complementation plasmids. The promoterless luciferase open reading frame (the luc_{Bb}^{+} ORF; referred to as *luc* hereafter) was excised from pJD48 (4) by digestion with BglII and HindIII and ligated into pJD54 (19, 41, 51) that was digested with the same enzymes; this generated pOY63. The *luc* gene was codon optimized for the optimal expression of luciferase in *B. burgdorferi* (4). DNA fragments containing various versions of the proposed *dbpBA* promoter (P*dbpBA*) were generated by PCR using Pfx50 DNA polymerase (Invitrogen). The primers for each insert are listed in Table 2. These DNA inserts then were digested using appropriate restriction enzymes (NcoI, BglII, and NdeI) and fused to the *luc* gene in pOY63 that was linearized by digestion with the same restriction enzymes. In particular, all inserts were amplified using different forward primers and the same reverse primer, ZM61 (CATATGCTTTTCCCGTGGCTTCTTTT). In the sequence complementary to primer ZM61, i.e., AAAAGAAGCCACGGGAAAAGCATATG, the ATG in the NdeI restriction enzyme site (CATATG) serves as the start codon for *luc* in all of the P*dbpBA-luc* reporter constructs, whereas AAGAAG serves as a ribosome-binding site (RBS). AAGAAG as an RBS has been employed in *E. coli* to express proteins efficiently. This strategy places the transcription of *luc* under the direct control of the cloned P*dbpBA*. All constructs were verified by restriction digestion and DNA sequence analysis. These constructs then were transformed into *B. burgdorferi* strain 297, and luciferase activity was assessed to monitor *dbpBA* transcription.

To validate data from luciferase reporter assays, various shuttle vectors harboring different versions of the *dbpBA* operon were generated. Briefly, various versions of *dbpBA* promoter were cloned into either pOY63 at the NcoI and NdeI sites or into pJD7 at the AscI site. These vectors were transformed into the *dbpBA* mutant, BbKH500 (3), and *dbpBA* expression in these strains was examined using quantitative RT-PCR (qRT-PCR) or immunoblot analyses.

***B. burgdorferi* transformation.** Plasmid DNA for electroporation was purified using a CompactPrep Plasmid Maxi kit (Qiagen, Valencia, CA). The transformation of *B. burgdorferi* was carried out as described previously (43, 59), with minor modifications. Briefly, 50 μl (~2×10^9 spirochetes) of electrocompetent *B. burgdorferi* suspension was transformed with 30 μg of plasmid DNA. After electroporation, cells were recovered using 25 ml of prewarmed BSK-II medium and incubated overnight at 37°C. Appropriate antibiotics then were added, and cultures were distributed into multiple 96-well tissue culture plates. Transformants were selected based on antibiotics resistance and verified using PCR amplification. To further confirm the presence of the shuttle vector in the transformants, genomic DNA was isolated from *Borrelia* clones using the Wizard Genomic DNA Purification kit (Promega Corp., Madison, WI) and transformed into *E. coli*. Plasmid DNA then was isolated from the resulting *E. coli* clones and verified by PCR, restriction digestion, and sequencing analysis.

Luciferase assays. Luciferase assays were performed using the Luciferase Assay System (Promega Corp.). Spirochetes were collected by centrifugation at $10{,}000 \times g$ for 10 min. Cell pellets then were lysed using 100 μl of cell culture lysis buffer containing 25 mM Tris-phosphate (pH 7.8), 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid, 10% glycerol, 1% Triton X-100, 1.25 mg/ml lysozyme, and 2.5 mg/ml bovine serum albumin. Relative luciferase units (RLU) were measured using a Centro LB 960 luminometer (Berthold Technologies, Oak Ridge, TN) as described previously (4). Results are presented as the RLU/1×10^6 spirochetes. At least three independent tests were performed, and the results were analyzed using a paired, two-tailed Student's *t* test, in which statistical significance was determined when $P < 0.05$.

Quantitative RT-PCR analysis. qRT-PCR was employed to examine *dbpBA* expression. Specific primers (Table 2) were designed by using PRIMEREXPRESS

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
B. burgdorferi		
297	Infectious, low-passage *B. burgdorferi*	27
BbAH206	297, *rpoS* mutant	27
BbKH500	297, *dbpBA* mutant, Kan^r	3
OY20	297 transformed with pOY63, Str^r	This study
OY16	297 transformed with pOY64, Str^r	This study
OY17	297 transformed with pOY66, Str^r	This study
OY21	297 transformed with pOY67, Str^r	This study
OY22	297 transformed with pOY68, Str^r	This study
OY23	297 transformed with pOY69, Str^r	This study
OY24	297 transformed with pOY70, Str^r	This study
OY25	297 transformed with pOY71, Str^r	This study
OY26	297 transformed with pOY72, Str^r	This study
OY27	297 transformed with pJSB165, Str^r	This study
OY28	297 transformed with pJSB175, Str^r	This study
OY45	BbKH500 transformed with pOY94, Kan^r, Str^r	This study
OY48	BbKH500 transformed with pOY98, Kan^r, Str^r	This study
OY49	297 transformed with pOY109, Str^r	This study
OY50	BbKH500 transformed with pOY107, Kan^r, Str^r	This study
OY51	BbKH500 transformed with pOY108, Kan^r, Str^r	This study
OY52	BbAH206 transformed with pOY69, Str^r	This study
E. coli		
TOP10	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*Δ*M15* Δ*lacX74 recA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL* (Str^r) *endA1 nupG*	Invitrogen
Plasmids		
pJD7	*B. burgdorferi*/*E. coli* shuttle vector with P*flgB-aadA*; Spc^r, Str^r	4
pJD44	*B. burgdorferi*/*E. coli* shuttle vector with aph[3′]-IIIa; Kan^r	41
pJD48	pJD44::promoterless luc_{Bb}+, Kan^r	4
pJD54	*B. burgdorferi*/*E. coli* shuttle vector with P*flgB-aadA*; Spc^r, Str^r	4, 41
pOY63	promoterless luc_{Bb}+ from pJD48 cloned into pJD54 at BglII and HindIII; Spc^r, Str^r	This study
pOY64	pOY63::WT P*dbpBA* (PCR product from primers ZM61 and ZM68); Spc^r, Str^r	This study
pOY66	pOY63::ΔIR1 P*dbpBA* (PCR product from primers ZM61 and ZM54); Spc^r, Str^r	This study
pOY67	pOY63::ΔIR1/2 P*dbpBA* (PCR product from primers ZM61 and ZM55); Spc^r, Str^r	This study
pOY68	pOY63::PCR product from primers ZM61 and ZM56; Spc^r, Str^r	This study
pOY69	pOY63::Min P*dbpBA* (PCR product from primers ZM61 and ZM57); Spc^r, Str^r	This study
pOY70	pOY63::Δ−35 P*dbpBA* (PCR product from primers ZM61 and ZM58); Spc^r, Str^r	This study
pOY71	pOY63::Δ−35/−10 P*dbpBA* (PCR product from primers ZM61 and ZM59); Spc^r, Str^r	This study
pOY72	pOY63::PCR product from primers ZM61 and ZM60; Spc^r, Str^r	This study
pOY94	pOY63::Min P*dbpBA-dbpBA* (PCR product from primers ZM102 and ZM104.3); Spc^r, Str^r	This study
pOY98	pOY63::Δ−35 P*dbpBA-dbpBA* (PCR product from primers ZM103 and ZM104.4); Spc^r, Str^r	This study
pOY107	pJD7::WT P*dbpBA-dbpBA* (PCR product from primers ZM109F and ZM110R); Spc^r, Str^r	This study
pOY108	pJD7::ΔIR1 P*dbpBA-dbpBA* (PCR product from primers ZM110F and ZM110R); Spc^r, Str^r	This study
pOY109	pOY63::Min P*dbpBA* with −14 C/A (PCR product from primers ZM61 and ZM57.2); Spc^r, Str^r	This study
pJSB165	pJD7::divergently oriented P*ospC-Bbluc*+; Spc^r, Str^r	4
pJSB175	pJD7::divergently oriented P*flaB-Bbluc*+; Spc^r, Str^r	4

software (Applied Biosystem, Foster City, CA) and validated as described previously (33). Spirochetes were grown in BSK-H medium at 37°C under 5% CO_2 and harvested when bacterial growth reached a density of 5×10^7 cells per ml. Total RNA was isolated using TRIzol (Invitrogen) according to the instructions. After genomic DNA was digested using RNase-free DNase I (GenHunter Corporation, Nashville, TN), RNA was further purified using an RNeasy Mini kit (Qiagen). cDNA was generated from 1 μg of RNA using the SuperScript III Platinum two-step qRT-PCR kit according to the manufacturer's protocol (Invitrogen). qPCR (in quadruplicate) using Platinum SYBR green qPCR Super Mix-UDG (Invitrogen) then was performed, and the relative quantification method (ΔΔ threshold cycle [$\Delta\Delta C_T$]) was used to calculate the variation in gene expression between *B. burgdorferi* strains. The *aadA* gene (encoding streptomycin-spectinomycin adenylyltransferases) carried by the cloning vectors (19) or the *Borrelia flaB* gene was used as the endogenous control to normalize all qRT-PCR data.

SDS-PAGE and immunoblot analysis. SDS-PAGE and immunoblot analysis were performed as previously described (57). Briefly, spirochetes were harvested and washed three times in 0.9% (wt/vol) NaCl. Cell pellets were resuspended in an appropriate volume of SDS sample buffer. A volume of whole-cell lysate equivalent to 4×10^7 bacteria was loaded per lane on a 12.5% acrylamide gel. Resolved proteins either were stained with Coomassie brilliant blue or transferred to nitrocellulose membranes for immunoblot analysis. An anti-DbpB polyclonal antibody, SS65, an anti-DbpA monoclonal antibody, 6B3, and a chicken IgY anti-FlaB antibody were used to detect DbpB, DbpA, and FlaB, respectively (48). Immunoblots were developed colorimetrically using 4-chloro-1-naphthol as the substrate.

RESULTS

***In silico* analysis of *dbpBA* promoter.** In a previous study (24), a strong transcriptional initiation site at nucleotide −28 (T) (asterisk in Fig. 1) upstream of the start codon of the *dbpB* ORF was identified using primer extension analysis. When analyzing the 5′ sequence upstream of the *dbpBA* operon using BPROM

TABLE 2. Oligonucleotide primers used in this study[a]

Name	5′–3′
ZM54	CCATGGCCTTTTAAGCCTGCCAATCC
ZM55	ATTTTAGATCTTTGATTCAATTTGC
ZM56	AGATCTTTGTAATTCCAAACAATGTTACTGC
ZM57	AGATCTATTTTATTTTATTTTTCATAAAGTG GGCTAAA
ZM57.2	TATCCATGGATTTTATTTTATTTTTCATAAA GTGGGATAAAATTTAAATTTAAC
ZM58	AGATCTCATAAAGTGGGCTAAAATTTAAAT
ZM59	AGATCTATGAAAATTGGAAAGCTAAAT TCAA
ZM60	GGCAGATCTACATCAACATACTAACTA
ZM61	CATATGCTTTTCCCGTGGCTTCTTTT
ZM68	TATCCATGGTGCTTTCTTCTGCCAGGTC
ZM102	TATCCATGGATTTTATTTTATTTTTCATAAA GTGGGCTAAA
ZM103	TATCCATGGCATAAAGTGGGCTAAAATTT AAAT
ZM104.3	GCCCATATGCTTTGGGTTAATTGCTTTAAC
ZM104.4	TATCATATGTTTAGATTCTAAAGTTTAGATA AAAATTGGTCGGG
ZM109F	TAATGGCGCGCCTGCTTTCTTCTGCCAGGTC
ZM110F	TAATGGCGCGCCTTTTAAGCCTGCCAATCC
ZM110R	TAATGGCGCGCCTTTAGATTCTAAAGTTTA GATAAAAATTGGTCGGG
bba25-F	TGGCTATGTTTGACTTAATGCTTGAG
bba25-R	GATTCCTCTAAAACACGGGCTTTT
bba24-F	GGGTAGTGGGGTATCAGAAAATC
bba24-R	GAGCTGTAGTTGGAGGATTCTC
flaB-F	ACTCTTAAAGTCCAAGACGCTTGAG
flaB-R	TTGGAATGCAGCCTGCAAA
aadA-F	TAAGGCTTGATGAAACAACGCGGC
aadA-R	CGTCGTGCACAACAATGGTGACTT

[a] Restriction enzymes sites are underlined. In ZM57.2, the A used to replace the −14 C in the *dbpBA* promoter is indicated in boldface.

(http://linux1.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb), a bacterial σ^{70} promoter recognition program, a typical bacterial σ^{70} promoter harboring canonical −10/−35 elements, was predicted in the upstream 5′ regulatory region of the *dbpBA* operon (Fig. 1), which is consistent with the −10/−35 elements previously predicted (13, 24) based on their relative proximity to the transcriptional start. Moreover, the putative *dbpBA* promoter (P*dbpBA*), or the upstream 5′ regulatory region of *dbpBA* operon, does not share significant sequence similarity with the consensus sequence of the σ^S-dependent promoter of the *Borrelia ospC* gene (1, 18, 59). Furthermore, possible upstream A/T-rich sequences (UP elements) for σ^S-specific promoters (53) were not predicted in the 5′ regulatory region of *dbpBA*. However, given that σ^S and σ^{70} promoters are very similar, it is difficult to discern whether P*dbpBA* is σ^{70} or σ^S specific based on sequence information alone (2, 53). In addition, using the Inverted Repeats Finder program (https://tandem.bu.edu/cgi-bin/irdb/irdb.exe?taskid=0), two sets of inverted repeats (IRs) were predicted in the upstream 5′ regulatory region of *dbpBA* (Fig. 1). Previously, four putative ORFs, including *bba26*, *bba27*, *bba28*, and *bba29*, were annotated in this region (20). However, given the fact that these four ORFs are extremely short (*bba26*, 132 bp; *bba27*, 120 bp; *bba28*, 126 bp; *bba29*, 126 bp), they likely do not encode functional proteins in *B. burgdorferi*. These considerations prompted the hypothesis that the IRs serve as potential binding sites for a putative transcriptional regulator(s) involved in the regulation of *dbpBA* expression.

Assessing *dbpBA* expression using luciferase reporter assays. A recently developed luciferase reporter assay (4) was employed to explore how the expression of the *dbpBA* operon is controlled in *B. burgdorferi*. Along these lines, initially we created one shuttle vector, pOY64, harboring 1,055 bp of DNA upstream of the *dbpB* start codon (including the putative P*dbpBA*) and 161 bp of *dbpB* ORF DNA (PCR product amplified using primers ZM68 and ZM61) (Fig. 2A). In addition, pOY71 was generated by cloning a 161-bp fragment of *dbpB* (PCR product amplified using primers ZM59 and ZM61) into the promoterless *luc* reporter vector pOY63. These constructs then were transformed into the low-passage, virulent, wild-type (WT) *B. burgdorferi* strain 297. As shown in Fig. 3, substantial luciferase activity was detected in the strain harboring pOY64 (WT P*dbpBA*, the longest construct) but not from the strains containing the promoterless *luc* constructs (pOY63 and pOY71). The observation that luciferase activity was not detected from pOY71 indicated that the 161 bp of *dbpB* ORF DNA cloned into the *luc* fusion constructs did not contain an occult promoter(s). On the other hand, the expression of luciferase from the P*dbpBA*-containing vector pOY64 indicated that the 5′ regulatory region of *dbpBA* cloned into pOY64 contains a functional promoter.

Minimal promoter for *dbpBA* transcription. As mentioned above, a functional P*dbpBA* was identified in the 5′ regulatory

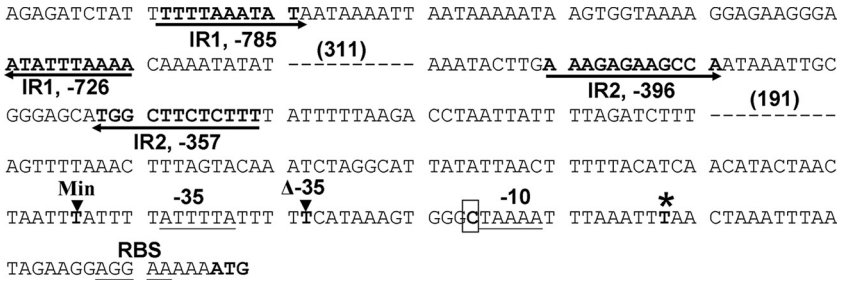

FIG. 1. 5′ Regulatory sequence of the *B. burgdorferi dbpBA* upstream region. Pairs of convergent arrows indicate the two putative inverted repeat elements (IR_1 and IR_2). The numbers in parentheses indicate numbers of nucleotides omitted (dash lines). The transcription start site (marked by the asterisk), the associated −35 and −10 elements (underlined), the ribosome-binding site (RBS) (underlined), and the ATG start codon (in boldface) are shown. Filled arrowheads denote the starting position of the minimal (Min) or the −35 deletion (Δ−35) promoter. The −14 C residue (boxed) within the extended −10 region was targeted for mutagenesis.

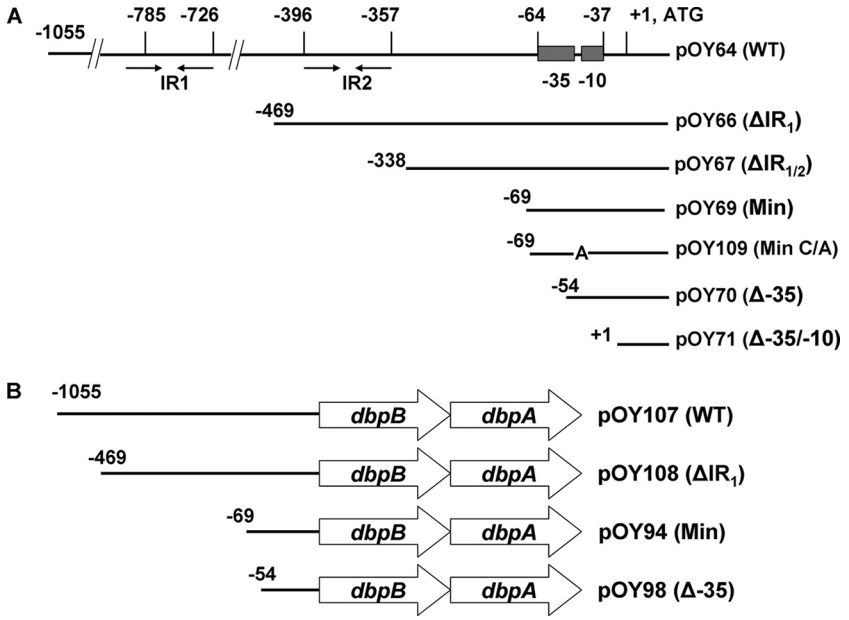

FIG. 2. Diagram of a series of *dbpBA* promoter (P*dbpBA*)-*luc* reporter constructs (A) and different constructs containing various versions of P*dbpBA* with the entire *dbpBA* operon to complement *dbpBA* expression in *trans* in the *dbpBA* deletion strain BbKH500 (B). Nucleotide positions are relative to the ATG start codon, where A is +1. WT, wild-type P*dbpBA*; ΔIR_1, deletion of IR_1; $\Delta IR_{1/2}$, deletion of both IR_1 and IR_2; Min, minimal P*dbpBA*; Min C/A, minimal P*dbpBA* with the −14 C mutated to A; Δ−35, deletion of −35 sequence; Δ−35/−10, deletion of both −35 sequence and −10 sequence.

sequence of *dbpBA*. To determine the minimal genetic element(s) required for P*dbpBA*, a series of P*dbpBA-luc* transcriptional deletion constructs was created (Fig. 2A) and introduced into *B. burgdorferi* 297. The expression of the *luc* gene from these constructs was compared to the expression of *luc* from the promoterless vector (pOY63) and the construct containing the WT P*dbpBA* (pOY64). As shown in Fig. 3, luciferase activity was readily detected in strains harboring the putative, intact P*dbpBA* (pOY64, pOY66, pOY67, and pOY69). In particular, pOY69, which contains the sequence from the −35 element of P*dbpBA* only (the sequence upstream of the −35 element was deleted), still expressed luciferase as efficiently as pOY64. In contrast, the construct pOY70, lacking the putative −35 element, displayed greatly diminished luciferase expression (~95% decrease compared to that of pOY69). In addition, when both the −35 and −10 elements of P*dbpBA* were deleted (pOY71), no luciferase activity was detected. These data indicate that pOY69 comprises a minimal sequence encompassed within 70 bp upstream of the ATG start codon of *dbpBA*, which is essential for P*dbpBA* to be functional.

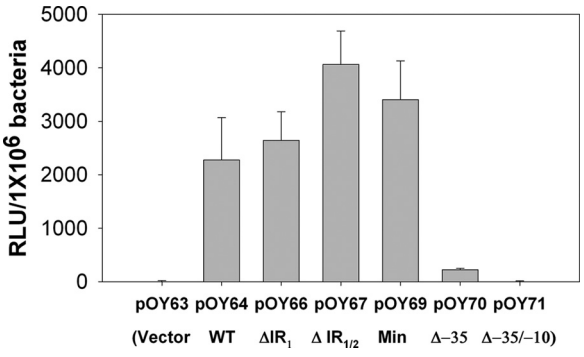

FIG. 3. Luciferase activity (denoted in RLU/10^6 bacteria) detected in *B. burgdorferi* strains transformed with various P*dbpBA-luc* constructs. Spirochetes were cultured in BSK-H medium at 37°C and harvested at late log phase. Results from three independent experiments are presented as the mean values ± standard errors of the means (SEM). pOY63, cloning vector containing a promoterless *luc*; pOY64, vector containing WT P*dbpBA*; pOY66, vector containing ΔIR_1 P*dbpBA*; pOY67, vector containing $\Delta IR_{1/2}$ P*dbpBA*; pOY69, vector containing minimal (Min) P*dbpBA*; pOY70, vector containing Δ−35 P*dbpBA*; and pOY71, vector containing Δ−35/−10 P*dbpBA*.

To further validate these data, two additional constructs, containing the entire *dbpBA* operon with either the minimal P*dbpBA* (same version of P*dbpBA* as that cloned in pOY69) (pOY94) or P*dbpBA* lacking the −35 element (Δ−35; the same version of P*dbpBA* as that cloned into pOY70) (pOY98), were created and introduced into the *dbpBA*-deficient mutant BbKH500 (3). The expression of DbpB and DbpA then was assessed using qRT-PCR and immunoblotting to determine whether these constructs could complement DbpBA expression in *trans*. As shown in Fig. 4, when *B. burgdorferi* was cultured in BSK-H medium at pH 7.6, both DbpB and DbpA were expressed (at both the RNA [Fig. 4A] and protein [Fig. 4B] levels) in strain OY45 (containing the minimal P*dbpBA*) but not from OY48 (containing the Δ−35 P*dbpBA*). Similar data were obtained when *Borrelia* was grown in BSK-H medium at pH 6.8 (data not shown). These data indicate that a minimal −35/−10 *dbpBA* promoter sequence is necessary and sufficient for *dbpBA* expression in *B. burgdorferi*.

IR elements in the 5′ *dbpBA* regulatory sequence. Two sets of IR elements, putative transcriptional regulator-binding sites, are present in the *dbpBA* 5′ regulatory sequence. To assess the potential roles of these two IRs in *dbpBA* regulation,

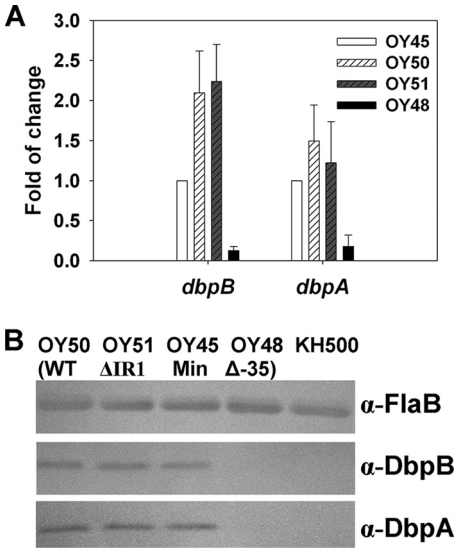

FIG. 4. Influence of upstream IRs on *dbpBA* expression. *B. burgdorferi dbpBA* deletion mutant BbKH500 transformed with different shuttle vectors were grown in BSK-H medium and harvested at late-log phase. The expression of DbpB and DbpA were assessed using qRT-PCR (A) or immunoblot analysis (B). (A) Results from three tests are presented as the mean fold changes (relative to the gene expression level in OY45) ± SEM. (B) Approximately 4×10^7 spirochetes were loaded onto each lane of an SDS-PAGE gel and transferred to a nitrocellulose membrane. FlaB, DbpB, and DbpA were detected using antibodies described in the Materials and Methods. α, anti; OY45, vector containing the minimal (Min) P*dbpBA*; OY50, vector containing the WT P*dbpBA*; OY51, vector containing ΔIR_1 P*dbpBA*; OY48, vector containing Δ−35 P*dbpBA*.

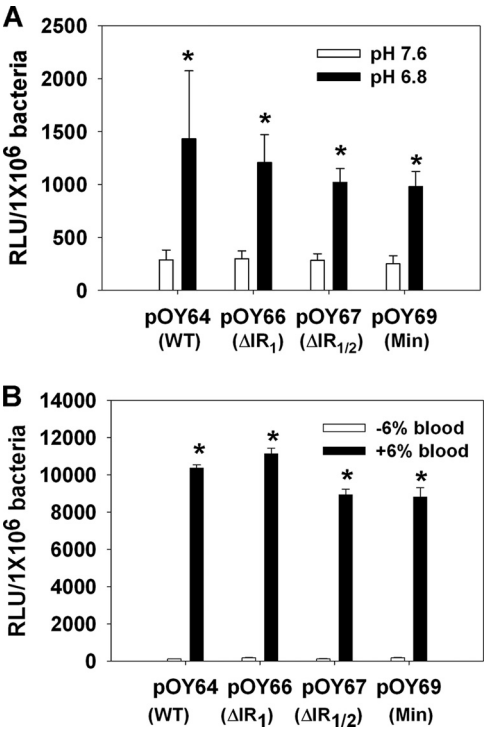

FIG. 5. Influence of culture pH (A) and whole blood (B) on luciferase expression from various P*dbpBA-luc* constructs. *Borrelia* organisms were cultivated in BSK-H medium and harvested when growth reached a cell density of $\sim 1 \times 10^6$ to 5×10^6 spirochetes/ml. The experiments were replicated thrice, and bars represent the mean measurements ± SEM. The asterisk indicates statistical significance using Student's *t* test ($P < 0.05$). pOY64, vector containing WT P*dbpBA*; pOY66, vector containing ΔIR_1 P*dbpBA*; pOY67, vector containing $\Delta IR_{1/2}$ P*dbpBA*; and pOY69, vector containing minimal (Min) P*dbpBA*.

a series of P*dbpBA* deletions and mutations fused to *luc* were created (Fig. 2A) and then introduced into strain 297. As shown in Fig. 3, when *Borrelia* was grown in BSK-H medium at pH 7.6 and harvested at late log phase, comparable luciferase activity was observed from constructs containing WT P*dbpBA* (pOY64), P*dbpBA* lacking either IR_1 (ΔIR_1) (pOY66) or both IR_1 and IR_2 ($\Delta IR_{1/2}$) (pOY67), or the minimal (Min) P*dbpBA* (pOY69), although a slightly higher level of luciferase expression was observed when both IRs were deleted. To substantiate these data, we also created constructs harboring various versions of P*dbpBA* and the entire *dbpBA* operon (Fig. 2B). We then introduced these constructs into BbKH500 and measured the expression of DbpB and DbpA RNA and protein using qRT-PCR and immunoblotting. As shown in Fig. 4A and B, similar levels of DbpB and DbpA expression were detected in OY50 (containing the WT P*dbpBA*), OY51 (containing the ΔIR_1 P*dbpBA*), and OY45 (containing the minimal P*dbpBA*), whereas the expression of DbpB and DbpA was dramatically reduced in OY48 (containing the Δ−35 P*dbpBA*). This was observed in *Borrelia* spirochetes grown at either pH 7.6 (Fig. 4) or 6.8 (data not shown). These data suggest that neither IR is involved in the primary regulation of *dbpBA*, at least not under the conditions tested.

***dbpBA* expression is influenced by various environmental parameters.** Previous studies have revealed that *dbpBA* expression is influenced by various environmental factors, such as temperature, pH, cell density, and whole blood (52, 56). As a first attempt to quantitatively determine the influence of environmental factors on *dbpBA* transcription, and to explore whether these factors impact *dbpBA* expression through the minimal *dbpBA* promoter or the IRs located upstream of the 5′ regulatory sequence of *dbpBA*, luciferase activities were measured in *B. burgdorferi* harboring different P*dbpBA* deletion constructs (including WT, ΔIR_1, $\Delta IR_{1/2}$, and minimal P*dbpBA*) under various environmental conditions. As shown in Fig. 5A, luciferase expression from all of these constructs was markedly induced in *Borrelia* grown at pH 6.8 (compared to that at pH 7.6). Moreover, when fresh whole rabbit blood was added to the culture, luciferase expression was dramatically increased (Fig. 5B). We also examined the effect of temperature on *dbpBA* expression using these *luc* constructs. Luciferase expression was barely detected in *Borrelia* grown at 23°C (data not shown) but was highly expressed in these *Borrelia* strains cultured at 37°C (Fig. 5), indicating that *dbpBA* expression is induced by elevated temperature, as previously reported (1, 11–13, 31, 52, 56). These data were further confirmed by probing for DbpB and DbpA in these strains using immunoblotting (data not shown). Of note, *luc* reporter constructs containing the *ospC* promoter (P*ospC*) or *flaB* promoter (P*flaB*) also were employed in this study as controls. As expected, P*ospC* displayed a response to the stimuli similar to that of P*dbpBA*, showing induction at elevated temperature, lower pH, or blood

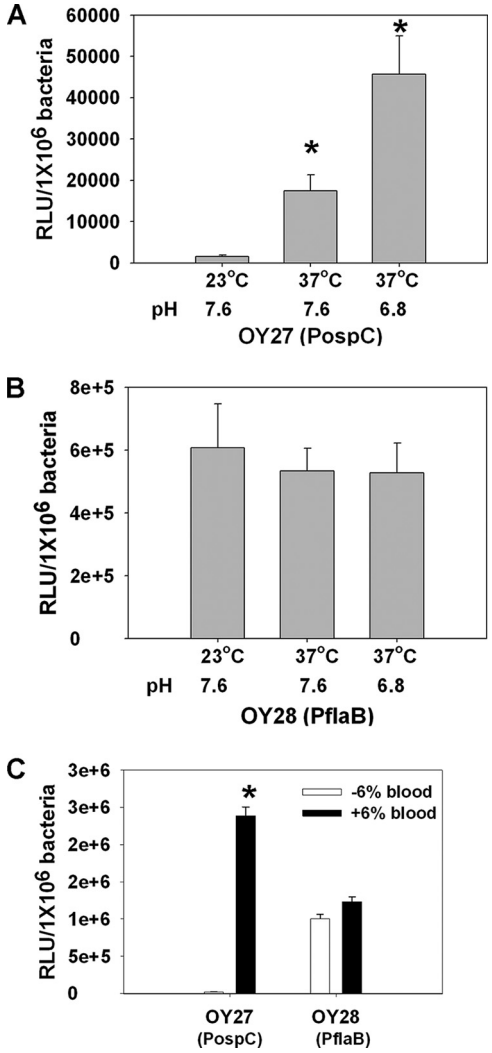

FIG. 6. Influence of temperature, pH (A and B), and whole blood (C) on luciferase expression driven by the *B. burgdorferi ospC* promoter (P*ospC*) or *flaB* promoter (P*flaB*). *Borrelia* organisms were cultivated in BSK-H medium and harvested when growth reached a cell density of $\sim 1 \times 10^6$ to 5×10^6 spirochetes/ml. The experiments were replicated thrice, and bars represent the mean measurements ± SEM. The asterisk indicates statistical significance using Student's *t* test ($P < 0.05$).

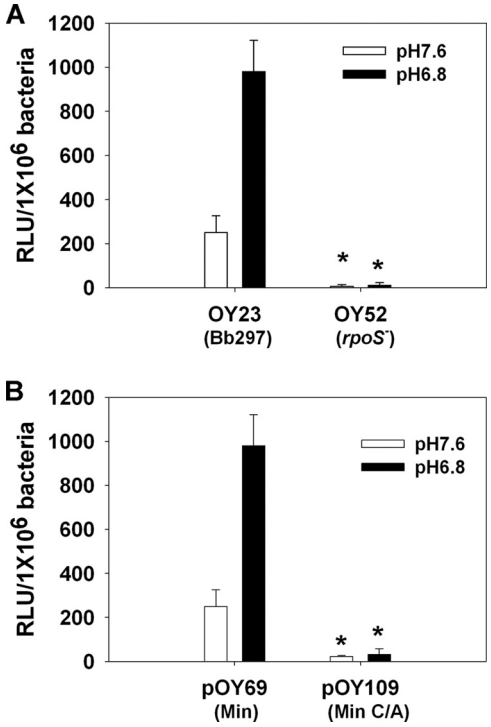

FIG. 7. *B. burgdorferi* P*dbpBA* is RpoS dependent. (A) Luciferase activity, driven from the minimal P*dbpBA*, was measured in *Borrelia* strain Bb297 or an *rpoS* mutant. (B) Luciferase was expressed from either the minimal P*dbpBA* (pOY69) or the minimal P*dbpBA* containing a mutated −14 residue (C replaced by A) (pOY109). Spirochetes were grown in BSK-H medium at a pH of either 7.6 (open bar) or 6.8 (black bar) and harvested at late log phase. Results from three tests are indicated as means ± SEM. The asterisk indicates statistical significance using Student's *t* test ($P < 0.05$).

addition, whereas P*flaB* showed no response to these stimuli (Fig. 6).

***dbpBA* and its minimal promoter are RpoS dependent.** To study how RpoS impacts *dbpBA* expression, the minimal P*dbpBA* construct, pOY69, was further introduced into an *rpoS*-deficient strain, BbAH206 (27). Consistently with previous reports that *dbpBA* expression is dependent on RpoS in *B. burgdorferi* (6, 11–13, 18, 27, 31, 33, 58), luciferase expression from pOY69 was abolished in BbAH206 (Fig. 7A). However, given that σ^S and σ^{70} are highly related and both σ^S and σ^{70} holoenzymes recognize very similar core promoter sequences, it is difficult to distinguish between σ^S and σ^{70} promoters based on sequence information alone or using gel shift assays. Nonetheless, in *E. coli*, studies have shown that a −13 C residue in the extended −10 region is essential for σ^S activity and is highly conserved in σ^S-dependent promoters, but not in σ^{70}-dependent promoters (2). Therefore, a C at position −13 introduces σ^S promoter selectivity and serves as a hallmark of σ^S-dependent promoters (53). An analogous situation has been verified in *B. burgdorferi*, in that a −15 C is critical for the activity of the RpoS-dependent *ospC* promoter (59), although the −15 C also was reported not to be essential for RpoS selectivity (13, 18). In P*dbpBA*, a −14 C (relative to the transcriptional start of *dbpBA* [defined as +1]) also is present in the extended −10 region (Fig. 1). Therefore, to garner evidence that RpoS controls *dbpBA* expression in *B. burgdorferi* via direct interaction with the minimal P*dbpBA*, one construct, pOY109, was created by cloning a PCR product amplified using ZM57.2 and ZM61 into pOY63. Thus, pOY109 encompassed a minimal P*dbpBA* with the −14 C mutated to adenine. Luciferase activity expressed from pOY109 was essentially abolished compared to that of the construct containing the minimal P*dbpBA*, regardless of whether *B. burgdorferi* was cultivated at pH 7.6 or 6.8 (Fig. 7B). These data suggest that RpoS governs *dbpBA* expression via direct interaction with the minimal promoter of *dbpBA*.

***E. coli* as a surrogate system for studying *B. burgdorferi* gene regulation.** Given the limited genetic tools available for *Borrelia* research, *E. coli* has been exploited as a surrogate system

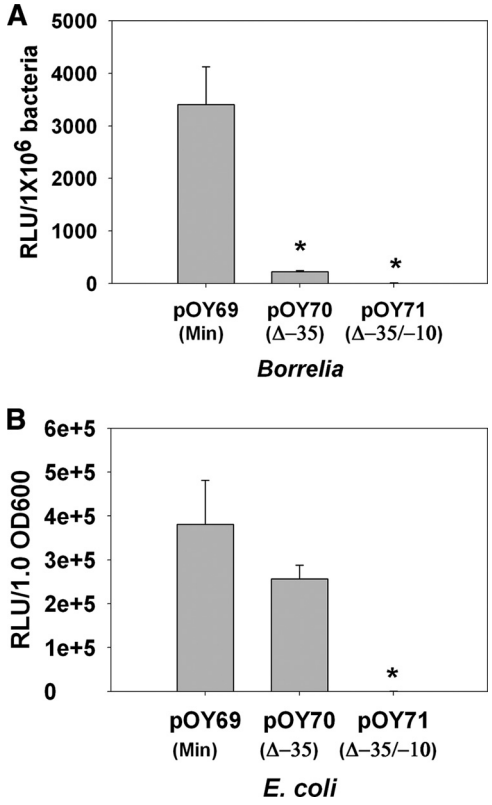

FIG. 8. Luciferase expressed from *B. burgdorferi* (A) or *E. coli* (B) containing various P*dbpBA-luc* constructs. *Borrelia* spirochetes were grown in BSK-H medium and collected at late log phase, whereas *E. coli* was grown in LB and harvested when growth reached an optical density at 600 nm of ~0.6. Results from three tests are indicated as means ± SEM. The asterisk indicates statistical significance using Student's *t* test ($P < 0.05$).

for the study of *B. burgdorferi* gene expression. However, there are a number of salient differences between *B. burgdorferi* and *E. coli* that warrant consideration. In addition to many obvious cellular differences, *B. burgdorferi* encodes very few predicted transcriptional regulators, despite a more complex overall genome consisting of a linear chromosome and numerous linear/circular plasmids. *Borrelia* also is dramatically different from *E. coli* in metal acquisition and metal homeostasis, systems typically under complex regulatory control. These and many other key differences call into question the suitability of *E. coli* for assessing *B. burgdorferi* gene regulation. To further examine the potential utility of *E. coli* as a surrogate system for assessing *B. burgdorferi* gene expression and regulation, we examined luciferase expression from the constructs comprising different versions of P*dbpBA*. As shown in Fig. 8A, pOY70, which harbors a Δ−35 P*dbpBA*, expressed very low levels of luciferase in *Borrelia*. However, when pOY70 was introduced into *E. coli*, it expressed a very high level of luciferase (Fig. 8B). These data indicate that the mutated P*dbpBA* cloned in pOY70 is capable of promoting gene transcription in *E. coli* but not in *Borrelia*, which in turn suggests that *E. coli* may not be an ideal surrogate system for studying *Borrelia* gene expression and regulation.

DISCUSSION

Using a sensitive luciferase reporter assay, we investigated the involvement of the 5′ regulatory sequence of the *dbpBA* operon in the regulation of *dbpBA* expression in *B. burgdorferi*. The luciferase reporter assay employed in our study had several advantages. First, this is a quantitative reporter assay. Moreover, the *luc* gene used to generate the reporter constructs is codon optimized, thereby rendering optimal luciferase expression in *B. burgdorferi* (4). In addition, the luciferase reporter assay is sensitive, convenient, and relatively simple. One potential problem for the luciferase reporter assay concerns the copy number of the shuttle vector used to generate the *luc* reporter constructs. Although it is unclear how many copies of these vectors are maintained in *Borrelia*, our qRT-PCR data revealed that similar numbers of the *aadA* gene (encoding streptomycin-spectinomycin adenylyltransferases in the shuttle vectors, including pOY63 and pJD7) were found in strains OY45, OY48, OY50, and OY51 (data not shown), suggesting that similar numbers of copies of plasmid constructs were present among the various strains. We further measured *dbpBA* expression using qRT-PCR or immunoblotting, which served as additional approaches to corroborate the data obtained from the luciferase reporter assays.

As an initial step for elucidating the molecular mechanism governing *dbpBA* expression, we created a series of luciferase reporter constructs by fusing various versions of deletion constructs of P*dbpBA* to the promoterless *luc* gene. We then examined whether the putative 5′ *cis* regulatory elements were involved in *dbpBA* expression. We identified a minimal promoter that is necessary and sufficient to drive *dbpBA* expression. Moreover, because the expression of *dbpBA* has been suggested to be influenced by several environmental factors, we also examined the effect of various factors on luciferase expression from these reporter constructs. Consistently with previous studies of *dbpA* expression (52, 56), our data revealed that luciferase expression driven from the minimal P*dbpBA* was induced by elevated temperature (37°C) or the supplementation of blood. However, luciferase expression driven from all functional P*dbpBA* constructs, including WT, IR-deleted, or minimal P*dbpBA*, was more induced at pH 6.8 than at pH 7.6, suggesting that *dbpBA* transcription also was induced by lower pH, which is disparate from a previous observation that the expression of DbpA was slightly repressed at pH 6.8 (56). The reason for this discrepancy currently remains unknown. It may emanate from different CO_2 levels used in these studies to grow the spirochetes. In the current study, spirochetes were grown at 5% CO_2, whereas 1% CO_2 was employed to grow *Borrelia* in the previous study (56). In accord with this possibility, DbpA expression was reported to be influenced by the CO_2 level (28). Alternatively, it also might be due to as-yet unknown subtle composition differences among BSK media.

Another goal of this study was to garner further evidence regarding whether RpoS controls *dbpBA* expression directly or indirectly. Based on *in silico* information, the *dbpBA* operon has been proposed to possess a typical −35/−10 σ^{70} promoter. However, because both σ^S and σ^{70} holoenzymes recognize the same core promoter elements, it is not feasible to discern whether the *dbpBA* promoter is σ^S or σ^{70} specific based on sequence information alone. Therefore, to garner direct evi-

dence for how σ^S (RpoS) influences *dbpBA* expression in *B. burgdorferi*, we examined the effect of RpoS on luciferase expression driven from the minimal P*dbpBA*. Subsequently, we found that (i) the minimal P*dbpBA* lost its ability to promote *luc* transcription in an *rpoS*-deficient mutant, and (ii) the minimal P*dbpBA* harbors an essential −14 C, similarly to the *E. coli* σ^S-dependent promoter and the *Borrelia ospC* promoter. These compelling data support the notion that RpoS controls *dbpBA* expression by direct interaction with the RpoS-dependent promoter in the 5′ regulatory region of the *dbpBA* operon.

Although the data described above that *dbpBA* expression was induced by elevated temperature, lower pH, or blood, and that RpoS controls *dbpBA* expression directly (similarly to *Borrelia ospC* expression), is compelling, there also is abundant evidence that *dbpA* has an expression pattern that is slightly different from that of *ospC* (13, 21, 25, 26, 29, 30, 36, 56). In particular, the expression of *ospC* (and *rpoS*) (13, 21, 26, 36), but not *dbpA* (25), has been observed in fed ticks, suggesting that, in addition to RpoS, another regulatory protein(s) (perhaps a repressor) is involved in the fine tuning of *dbpBA* expression. Relative to this hypothesis, two sets of conserved IRs in the 5′ regulatory sequence upstream of the P*dbpBA* potentially served as candidate binding sites for transcriptional regulators. However, the deletion of both IRs did not significantly alter the level of luciferase expression from the various reporter constructs in *Borrelia* (stimulated under various environmental conditions). All four versions of P*dbpBA* (including WT, IR_1 deleted, $IR_{1/2}$ deleted, and the minimal P*dbpBA*) displayed comparable (and not significantly different) abilities to promote *luc* transcription under all tested conditions. Similarly, using immunoblotting or qRT-PCR, the deletion of both IRs (from vectors harboring the entire *dbpBA* operon) also had no effect on *dbpBA* expression. These data suggest that the IRs are dispensable for the regulation of *dbpBA* expression, at least under the *in vitro* culture conditions tested. In the case of the *Borrelia ospC* promoter, a deletion of the IRs (*ospC* operator) located upstream of the *ospC* promoter also did not affect *ospC* expression when *Borrelia* was cultivated *in vitro* (59). However, the *ospC* operator assumes functional significance *in viv*o, wherein its presence is crucial for the repression of *in vivo ospC* expression in mammalian hosts and thus evasion from specific humoral immunity (55). As such, the *ospC* operator probably serves as a binding site for an unidentified DNA-binding protein that functions to suppress *ospC* during *in vivo* mammalian expression. Given what is now known about the *ospC* operator (18, 55, 59), it thus remains premature to exclude the possibility that these IRs contribute to the control of *dbpBA* expression in *B. burgdorferi* during its natural life cycle. The IRs may serve as binding sites for transcriptional regulator(s) involved in the regulation of *dbpBA* expression when *Borrelia* transits between its tick vector and mammalian hosts. Such a regulatory protein, potentially a repressor, may be expressed only when *B. burgdorferi* colonizes ticks and/or during tick feeding. Alternatively, a *dbpBA*-specific regulatory protein may be inactive when spirochetes are cultivated *in vitro*, perhaps first requiring some tick phase-specific cofactor(s) or ligands. Continued efforts are warranted to examine these possibilities by investigating the P*dbpBA* deletion construct strains in the tick and mammalian host phases of *B. burgdorferi*'s infectious life cycle.

ACKNOWLEDGMENTS

We thank Xiaofeng (Frank) Yang and Jon S. Blevins for many helpful discussions.

This work was supported by Public Health Service Grant AI-059062 from the National Institutes of Health.

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