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BpaB and EbfC DNA-Binding Proteins Regulate Production of the Lyme Disease Spirochete's Infection-Associated Erp Surface Proteins

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Vector-borne pathogens regulate their protein expression profiles, producing factors during host infection that differ from those produced during vector colonization. The Lyme disease agent, *Borrelia burgdorferi*, produces Erp surface proteins throughout mammalian infection and represses their synthesis during colonization of vector ticks. Known functions of Erp proteins include binding of host laminin, plasmin(ogen), and regulators of complement activation. A DNA region immediately 5′ of *erp* operons, the *erp* operator, is required for transcriptional regulation. The *B. burgdorferi* BpaB and EbfC proteins exhibit high *in vitro* affinities for *erp* operator DNA. In the present studies, chromatin immunoprecipitation (ChIP) demonstrated that both proteins bind *erp* operator DNA *in vivo*. Additionally, a combination of *in vivo* and *in vitro* methods demonstrated that BpaB functions as a repressor of *erp* transcription, while EbfC functions as an antirepressor.

Successful infection of a host requires that the invading pathogen control its production of virulence determinants. The infectious agent must sense its environment and respond by increasing production of appropriate factors and repressing production of unnecessary ones. These features are especially critical for vector-borne pathogens, which must not only efficiently infect two extremely different host types but also be transmitted back and forth between hosts. Deciphering the regulatory pathways used by pathogens to control production of infection-associated proteins provides significant insight into the infectious nature of those organisms. Moreover, regulatory factors are attractive candidates for development of novel preventative and curative therapies.

The spirochetal bacterium *Borrelia burgdorferi*, the agent of Lyme disease, is an excellent model organism for the study of gene regulation by a vector-borne pathogen. *B. burgdorferi* is genetically tractable, and its natural mammal-tick infectious cycle can be replicated in the laboratory. In addition, infection by *B. burgdorferi* is a significant cause of human morbidity, being the most commonly reported vector-borne disease in the United States and many other parts of the world (51, 55, 56).

B. burgdorferi Erp lipoproteins are produced throughout mammalian infection but are largely repressed during colonization of vector ticks (10, 31, 48, 49). Erp synthesis is greatly enhanced when *B. burgdorferi* is transmitted from a feeding tick into a warm-blooded host. Regulation of Erp protein production is controlled at the level of transcription (6). Erp proteins are located in the bacterial outer membrane and are exposed to the external environment (25, 32, 41). Known functions of Erp proteins include binding of host plasmin(ogen), laminin, and the complement regulators factor H and factor H-related proteins 1, 2, and 5 (2, 3, 11, 12, 34, 37, 40, 45, 59). These functions indicate roles for Erp proteins in host adherence, dissemination, and resistance to the alternative pathway of complement-mediated killing. Borrelial *erp* genes are located in mono- or bicistronic operons on extrachromosomal cp32 prophages, most of which replicate autonomously as circular episomes (24, 60, 63, 64, 72). Individual Lyme spirochetes naturally contain numerous different cp32 elements, each with a unique *erp* locus, and therefore produce multiple, distinct Erp surface proteins. A bacterium simultaneously expresses its entire repertoire of Erp proteins (26).

A highly conserved DNA region immediately 5′ of all *erp* promoters, the *erp* operator, is required for regulation of *erp* transcription (see Fig. 1) (6, 10, 64). Two *erp* operator-binding proteins have been identified, and their binding sites have been characterized: BpaB (borrelial plasmid ParB analogue) and EbfC (*erp*-binding factor, chromosomal) (4, 13, 52). BpaB binds with high affinity to a 5-bp sequence within the *erp* operator (13; C. A. Adams, unpublished). Binding of one BpaB protein to that sequence then facilitates binding of additional BpaB molecules along the DNA strand (13). EbfC binds a 4-bp broken palindromic sequence, with all *erp* operator elements containing 2 to 3 consensus EbfC binding sites adjacent to the BpaB high-affinity site (4, 13, 52). BpaB and EbfC compete with each other for binding to *erp* operator DNA (13). Like the *erp* genes, *ebfC* is poorly expressed in unfed ticks but significantly induced during tick feeding and during mammalian infection (44). For the present work, independent *in vivo* and *in vitro* studies were performed to determine the effects of these two proteins on Erp expression. Resulting data indicated that BpaB is a repressor of *erp* transcription, while EbfC functions as an antirepressor.

Received 18 October 2011 **Accepted** 1 December 2011

Published ahead of print 9 December 2011

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doi:10.1128/JB.06394-11

MATERIALS AND METHODS

Bacterial strains and culture conditions. All studies used derivatives of the *B. burgdorferi* type strain, B31, cultured in Barbour-Stoenner-Kelly II medium (73). Chromatin immunoprecipitation (ChIP) was performed using the clonal infectious derivative B31-MI-16, cultured at 34°C (48). For studies requiring *B. burgdorferi* carrying recombinant plasmids, the readily transformable clonal derivative B31-e2 was used (6, 18). Kanamycin was added to cultures of transformed bacteria at a final concentration of 200 μg/ml. The effects of culture temperature on expression levels of native borrelial proteins were assessed using infectious B31-MI-16, cultured at either 23 or 34°C (17, 29, 48, 62).

Recombinant proteins. Recombinant BpaB and EbfC were produced as previously described (4, 13). The allele of BpaB carried by the strain B31 lp56 plasmid was used for all studies (13). Recombinant proteins were produced using *Escherichia coli* strain Rosetta 2 (Novagen, Rockland, MA) and purified from cleared lysates using MagneHis Ni particles (Promega, Madison, WI). Purified proteins were dialyzed with DNA-binding buffer (100 nM dithiothreitol, 50 mM Tris [pH = 7.5], 10% [vol/vol] glycerol, 0.01% [vol/vol] Tween 20, and 0.1% [vol/vol] phenylmethanesulfonyl fluoride). Protein concentrations were determined by the Bradford assay (Bio-Rad, Hercules, CA). Purities were determined by SDS-PAGE and staining with Coomassie brilliant blue. Aliquots were stored at −80°C.

ChIP. Antiserum directed against BpaB (allele 56) was produced using BALB/c mice, as follows. Mice were injected subcutaneously with 10 μg recombinant BpaB in 80 μl 60% AlOH (m/vol), followed by 2 additional injections 2 weeks apart. One week after the final boost, mice were euthanized, and their blood was pooled and processed into serum. Antiserum was assessed for specificity by immunoblot and enzyme-linked immunosorbent assay (ELISA) with purified recombinant BpaB.

Antiserum directed against EbfC was produced in New Zealand White rabbits by NeoPeptide (Cambridge, MA) using the standard immunization protocol. The vaccinogen was a polypeptide derived from the EbfC sequence, MSSVKSNIDNIKKEM. Antibodies were affinity purified from serum using the vaccinogen polypeptide.

ChIP was performed as previously described (69, 70), with the following modifications. *B. burgdorferi* B31-MI-16 was cultured at 34°C to mid-exponential phase (approximately 5×10^7 bacteria/ml). Formaldehyde was added to a final concentration of 1%, followed by incubation for 8 min at room temperature while shaking. Cross-linking was stopped by addition of glycine to a final concentration of 0.3 M. Bacteria were pelleted by centrifugation and washed twice with Tris-buffered saline (20 mM Tris [pH 7.5] and 150 mM NaCl). Cell pellets were stored frozen at −80°C. As needed, bacterial pellets were thawed on ice and then resuspended in a 1:4 ratio of lysis buffer to IP buffer (lysis buffer is 10 mM Tris [pH 7.5], 20% sucrose, 50 mM NaCl, and 10 mM EDTA; IP buffer is 50 mM HEPES [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 0.1% SDS) (69, 70). Lysozyme was added to a final concentration of 5 mg/ml, and the mixture was incubated at 37°C for 30 min. To shear the bacterial DNA, lysates were sonicated using a Branson 102C sonicator (Branson Ultrasonics, Danbury, CT), with 6 pulses of 10 s each at 10% amplitude. Cellular debris was cleared by centrifugation at 12,000 × *g* for 10 min at 4°C.

Binding of antibodies to resin particles was performed using immunoprecipitation kit-protein A magnetic Dynabeads (Invitrogen, Carlsbad, CA), following the manufacturer's recommended protocol. Antibodies specific for either BpaB or EbfC were utilized. Donkey anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA) was used as a control for nonspecific antibody binding. Resin beads without added antibody were used in experiments as an additional control.

Borrelial supernatants (800 μl) were incubated with antibody-bead complexes or control beads alone for 20 min at room temperature. The supernatants were poured off, and beads were incubated a second time with a fresh aliquot of lysate. Bead complexes were washed 3 times with IP wash buffer (Invitrogen protein A Dynabead kit) and then resuspended in IP buffer. After transfer to clean microcentrifuge tubes, bead complexes were washed 4 times with IP buffer supplemented with 500 mM NaCl, followed by 2 washes with TE buffer (10 mM Tris and 1 mM EDTA). Beads were resuspended in TE and incubated at 65°C for 18 h. Eluted DNA was purified using DNeasy blood and tissue kits (Qiagen), and antigens were eluted from antibody using elution buffer (immunoprecipitation kit; Invitrogen). Immunoprecipitation was verified by Western blot analysis following antigen elution.

Eluted DNAs were assessed for *erp* operator DNA by PCR using oligonucleotide primer pairs complementary to conserved sequences flanking *erp* operators: 164F (5′-TGAGTAGAGCATTTGCAATGGAGAG-3′) and A50R (5′-AAATATATAATTTTGTTACATTTCAG-3′). As a control, oligonucleotide primers which are specific for the *B. burgdorferi flaB* gene, which does not contain either a BpaB or EbfC site (FLA-1, 5′-CACATATTCAGATGCAGACAGAGG-3′; FLA-2, 5′-CCGGTGCAG CCTGAGCAGTTTGAG-3′) were also used (46, 47). Amplicons were cloned into pCR2.1 (Invitrogen), and the inserts of 5 random clones were sequenced.

Control ChIP reactions that utilized beads alone or beads bound with nonspecific IgG were also subjected to PCR using the primer pairs listed above.

***erp*::*gfp* transcriptional fusions and flow cytometry.** A plasmid containing promoterless *gfp* (pBLS590), an operon fusion between the wild-type *erpA* operator/promoter and *gfp* (pBLS591), and a mutant thereof that lacks the entire *erp* operator (pBLS599) have been described previously (6). Plasmid pBLS672 was created from pBLS591 by removal of the 20-bp high-affinity BpaB-binding site through use of overlap extension PCR (36). Another derivative, pBLJ1, in which all EbfC-binding sites were mutated from the consensus GTnAC to TGATG, was produced. The ability or inability of BpaB and EbfC to bind each DNA construct was assessed by electrophoretic mobility shift assay (EMSA) with each recombinant protein and biotin-labeled DNA probes, as described previously (13, 52). EMSA band intensities were quantified using the Image J software program (http://rsbweb.nih.gov/ij).

All six plasmids were individually introduced into *B. burgdorferi* B31-e2. Cultures were incubated to mid-exponential phase (approximately 10^7 bacteria/ml) at either 23 or 34°C. Bacteria were harvested by centrifugation, washed in phosphate-buffered saline (PBS), and resuspended in PBS at approximately 10^6 cells/ml. Green fluorescent protein (GFP) fluorescence per bacterial cell was analyzed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA), with excitation at 488 nm and detection at 530 nm. Each experiment involved measuring a minimum of 50,000 individual bacteria.

***In vivo* induction of BpaB or EbfC from tetracycline-inducible plasmid constructs.** The previously described pCRW53 replicates autonomously in both *B. burgdorferi* and *E. coli* and contains both a constitutively expressed *tetR* gene and a TetR-repressible promoter, P*ost*, which drives transcription of *gfp* (71). By use of overlap extension PCR (36), the pCRW53 multiple cloning sites were deleted, and then the *gfp* gene was removed and replaced with single BamHI and PstI recognition sequences, producing pSZW53-4. The *B. burgdorferi* strain B31 lp56 *bpaB* (allele *bpaB56*) or *ebfC* gene was amplified by PCR from recombinant plasmid clones and individually ligated into pSZW53-4, producing pBLS705 and pBLS704, respectively. Those two chimeric plasmids and the parental empty vector pSZW53-4 were individually introduced into *B. burgdorferi* strain B31-e2 by electroporation. Transcription from the P*ost* promoter was induced by addition of anhydrotetracycline (AT), at a final concentration of 0.5 μg/ml, to early-exponential-phase cultures (approximately 10^5 bacteria/ml). After cultivation to final densities of approximately 10^7 bacteria/ml, bacteria were harvested by centrifugation and lysed, and proteins were separated by SDS-PAGE. Total proteins were detected by Coomassie brilliant blue staining. Individual proteins were identified by immunoblotting using monospecific antibodies (7, 13, 25, 26, 52) and analyzed densitometrically.

***In vitro* coupled transcription/translation.** A linear DNA fragment consisting of 471 bp of *erp* 5′ noncoding DNA fused to *gfp* was produced by PCR from template pBLS591 (6) using the oligonucleotide primers

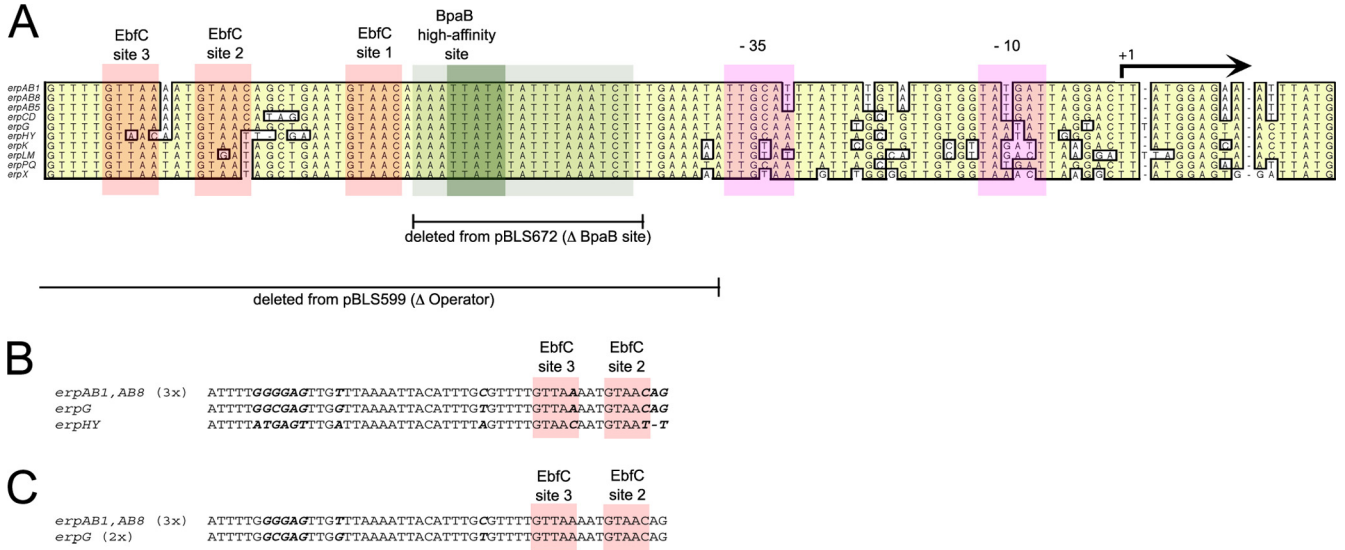

FIG 1 (A) Sequences of the 5′ noncoding DNA of the *B. burgdorferi* type strain B31, ending with the initiation codon (ATG) of the first *erp* gene of each locus. Identical nucleotides found in the majority of the 10 loci are boxed and shaded. All of the strain B31 *erp* loci contain at least 1 consensus EbfC-binding site (GTnAC), plus 1 or 2 additional half-sites (52). Each locus also contains a conserved BpaB-binding region, which consists of an initial binding site (TTATA) and a 19-bp flanking sequence that further stimulates BpaB binding (13; C. A. Adams, unpublished). Regions of noncoding DNA deleted from the mutant *erp*::*gfp* fusion constructs pBLS599 and pBLS672 are indicated. (B and C) PCR-amplified portions of DNA sequences bound by EbfC (A) or BpaB (C) in live *B. burgdorferi*, as assessed by ChIP followed by *erp*-specific PCR and cloning. Five clones were selected at random, and their inserts were sequenced. Polymorphisms that permit identification of specific *erp* loci are indicated with boldface italic type.

M13 Forward (5′-GTAAAACGACGGCCAG-3′) and M13 Reverse (5′-CAGGAAACAGCTATGAC-3′). As a control, a similarly sized DNA fragment, which consisted of the *B. burgdorferi ospAB* promoter and 5′ noncoding DNA fused to *gfp*, was also produced (16). Bovine serum albumin (BSA) was used for some control experiments. Protein concentrations were determined by Bradford assay (Bio-Rad). Reactions used the cell-free *E. coli* S30 extract transcription/translation system for linear templates (Promega). Each 75-μl reaction mixture contained 105 nM DNA template, 160 nM (each) protein (alone or together, as well as no added protein), 4 mM NaCl, 4 mM Tris-HCl, 80 μM $NaHPO_4$, and 0.75 nM dithiothreitol (DTT) in the following volumes of kit reagents: 30 μl S30 premix, 22.5 μl *E. coli* S30 extract, and 7.5 μl 1 mM amino acid mix. To ensure that experimental readouts were due to *gfp* transcription, rifampin was added to control reactions at a final concentration of 40 μg/ml. Additional control experiments replaced the DNA with 6 μl nuclease-free water. Reactions were lightly mixed and incubated at 37°C for 80 min. Reactions were stopped by incubation on ice for 15 min, and proteins were precipitated with acetone and then resuspended in 85 μl PBS.

For ELISA, 60 μl of resuspended products was added to 380 μl of ELISA coating buffer (50 mM Na_2CO_3, 500 mM $NaHCO_3$, pH 9.2). GFP product was measured using MACS molecular anti-GFP-horseradish peroxidase conjugate (Miltenyi Biotec, Auburn, CA) and Turbo tetramethyl benzidine (TMB) ELISA (Thermo-Fisher, Pittsburgh, PA). Reactions were stopped with 2 N H_2SO_4, and absorbance at 450 nm was measured with a Versamax tunable microplate reader. Each experiment was performed with four simultaneous identical reactions, and all experiment was replicated at least three times.

For immunoblotting, reaction products were subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Membranes were blocked with Sea Block buffer (Thermo-Fisher). GFP was detected by incubation with MACS molecular anti-GFP-horseradish peroxidase conjugate and SuperSignal West Pico chemiluminescent substrate (Thermo-Fisher).

Statistical analyses. Statistical significance between samples was determined by Student's *t* test, assuming unequal variance. Graphical representation of ELISA data reflects values that were normalized against the mean absorbance of a reaction which did not contain template DNA.

RESULTS

BpaB and EbfC bind to *erp* operators *in vivo*. Although we earlier found that BpaB and EbfC bind *erp* operator DNA under *in vitro* conditions (13, 52), protein-DNA interactions can only be considered biologically relevant if the protein binds DNA when inside the bacterial cell. To that end, we performed chromatin immunoprecipitation (ChIP) of each DNA-binding protein. Following cross-linking treatment of live *B. burgdorferi*, the cells were lysed, DNA was sheared by sonication, and immunoprecipitation was undertaken with antibodies specific for either BpaB or EbfC. Precipitated DNA fragments were subjected to PCR using oligonucleotide primers specific for *erp* operator and flanking DNAs. Appropriately sized amplicons were obtained for each experiment and cloned into pCR2.1. For each ChIP, 5 random clones were chosen and their plasmid inserts were sequenced. Control PCRs using oligonucleotide primers specific for the *flaB* gene, which does not contain either a BpaB or an EbfC site, all failed to generate amplicons, confirming the specificity of these analyses. Control ChIP reactions that used beads alone or beads coated with irrelevant antibody did not generate amplicons with either *erp*- or *flaB*-specific primers, further indicating that the BpaB- and EbfC-ChIP results were specific.

Sequencing of anti-BpaB and -EbfC precipitated DNAs revealed that both proteins had bound *in vivo* to *erp* operator DNA (Fig. 1B and C). Nucleotide polymorphisms occur among the 5′ noncoding regions of the different *erp* operons within a single bacterium. EbfC-ChIP yielded 3 clones derived from either *erpAB1* or *erpAB8* (both are identical in this region) and 1 clone each of *erpG* and *erpHY* (Fig. 1B). For BpaB ChIP, the 5 randomly selected PCR fragments consisted of 3 clones derived from *erpAB1* or *erpAB8* and 2 from *erpG* (Fig. 1C). Altogether, ChIP results confirmed that BpaB and EbfC bind to multiple *erp* loci in live *B. burgdorferi*.

***erp* operator/promoter-reporter fusions in *B. burgdorferi*.** *B.*

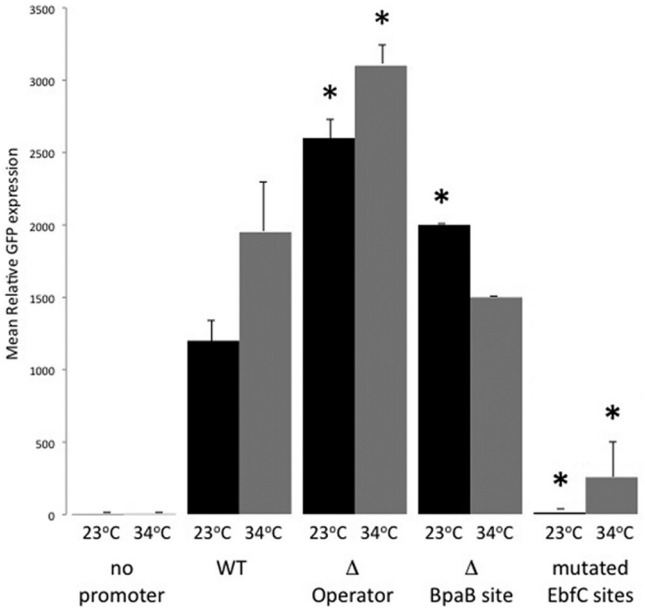

FIG 2 GFP production levels of *B. burgdorferi* containing *erp*::*gfp* transcriptional fusions. Constructs contained 471 bp of DNA immediately 5′ of *erpA* wild type (WT) and mutants thereof, consisting of deletion of all *erp* DNA 5′ of the promoter −35 sequence (Δ Operator), deletion of 20 bp within the operator that includes the high-affinity BpaB-binding site (Δ BpaB site), and all EbfC-binding sequences mutated to nonconsensus sequences (mutated EbfC sites). GFP was measured by flow cytometry with live *B. burgdorferi* cultures (6), and levels are reported as mean peak fluorescence values. Results for bacteria containing each mutant construct were compared with those for borreliae containing the WT plasmid. Statistically significant differences from WT results are indicated by asterisks ($P < 0.05$ by Student's *t* test).

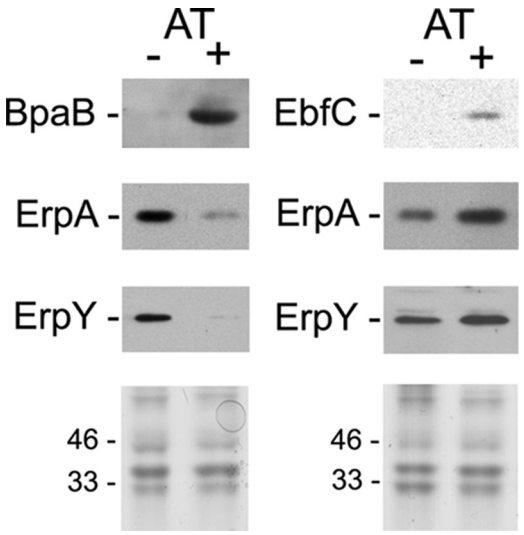

FIG 3 Effects of BpaB or EbfC overproduction by *B. burgdorferi*, using an anhydrotetracycline (AT)-inducible promoter system. Levels of BpaB, EbfC, ErpA, and ErpY in uninduced (−) and induced (+) bacteria were determined by immunoblotting, using specific antibodies, and densitometry. Lower panels illustrate SDS-PAGE of each bacterial lysate, stained with Coomassie brilliant blue, to confirm equal loading in each lane. Positions of molecular mass standards are shown to the left of each stained gel.

burgdorferi promoter activity can be assessed in the spirochete using transcriptional fusions between borrelial DNA and *gfp* (6, 16). Transcription of the reporter *gfp* gene results in production of GFP, which can be quantified by flow cytometry. This method was utilized to further define the effects of BpaB and EbfC on *erp* transcription. A promoterless *gfp* construct served as a negative control. A derived plasmid contains 471 bp of wild-type *B. burgdorferi* DNA, extending 5′ from the translation initiation codon of *erpA*, cloned such that the *erp* promoter drives transcription of *gfp*. Previous analyses determined that *B. burgdorferi* carrying this P*erpA*::*gfp* fusion plasmid regulated GFP production in response to environmental cues in a manner that directly correlates with native *erp* transcription and Erp protein production (6). The P*erpA*::*gfp* fusion plasmid was mutated as follows: (i) the entire *erp* operator and 5′ sequences were deleted, (ii) 20 bp that included the high-affinity BpaB-binding site were deleted from the *erp* operator, or (iii) all EbfC-binding sites were mutated to nonconsensus sequences. EMSAs using labeled probes derived from these constructs confirmed that each protein did not bind DNA that lacked its binding site or contained defective binding sites, while binding of the other protein was not affected (6, 13, 52; data not shown).

B. burgdorferi carrying the promoterless *gfp* construct did not produce GFP (Fig. 2) (6). *B. burgdorferi* produces significantly greater levels of Erp proteins when cultured at 34°C than when cultured at 23°C (58, 62). Likewise, borreliae carrying the wild-type operator-promoter-*gfp* construct produced greater amounts of GFP when grown at 34° than at 23°C (Fig. 2) (6). Deletion of the entire *erp* operator resulted in significantly greater expression at both culture temperatures, indicating that the operator is required for transcriptional repression (Fig. 2) (6). Removal of the high-affinity BpaB-binding site from the *erp* operator resulted in significantly greater production of GFP at 23°C compared with that of the wild-type promoter (Fig. 2). Changing the EbfC-binding sites to nonconsensus sequences significantly reduced GFP production by bacteria cultured at either temperature. Thus, transcription is enhanced if the *erp* operator cannot bind BpaB and is repressed greatly if EbfC cannot bind.

Overproduction of BpaB and EbfC in *B. burgdorferi*. Creation of *bpaB* and *ebfC* mutants in *B. burgdorferi* is proving to be very difficult. Repeated attempts to delete *ebfC* have failed, suggesting that it is an essential gene (52). Complete deletion of *bpaB* is complicated in that every known strain of *B. burgdorferi* contains genes for at least 3 cp32-encoded BpaB proteins, all of which bind to *erp* operator DNA (13). However, the recent development of an inducible expression system for *B. burgdorferi* allowed us to take an alternative approach to examine the roles of *erp* operator-binding proteins in *B. burgdorferi*. The previously constructed pCRW53 contains *tetR*, encoding the *tet* repressor, and a tetracycline-inducible promoter, P*ost*, that functions in *B. burgdorferi* (71). This plasmid was modified to place a *bpaB* or *ebfC* gene such that it is under the transcriptional control of P*ost*. The resulting constructs were transformed into *B. burgdorferi*, which was cultured in either the absence or presence of the nontoxic inducer molecule anhydrotetracycline (AT), and protein levels were examined by immunoblotting. Addition of AT to cultures of each transformed strain increased BpaB or EbfC to levels well above those of uninduced cultures (Fig. 3).

Two unlinked Erp proteins were examined: ErpA, encoded on cp32-1, and ErpY, encoded on cp32-4 (17, 63). Previous work

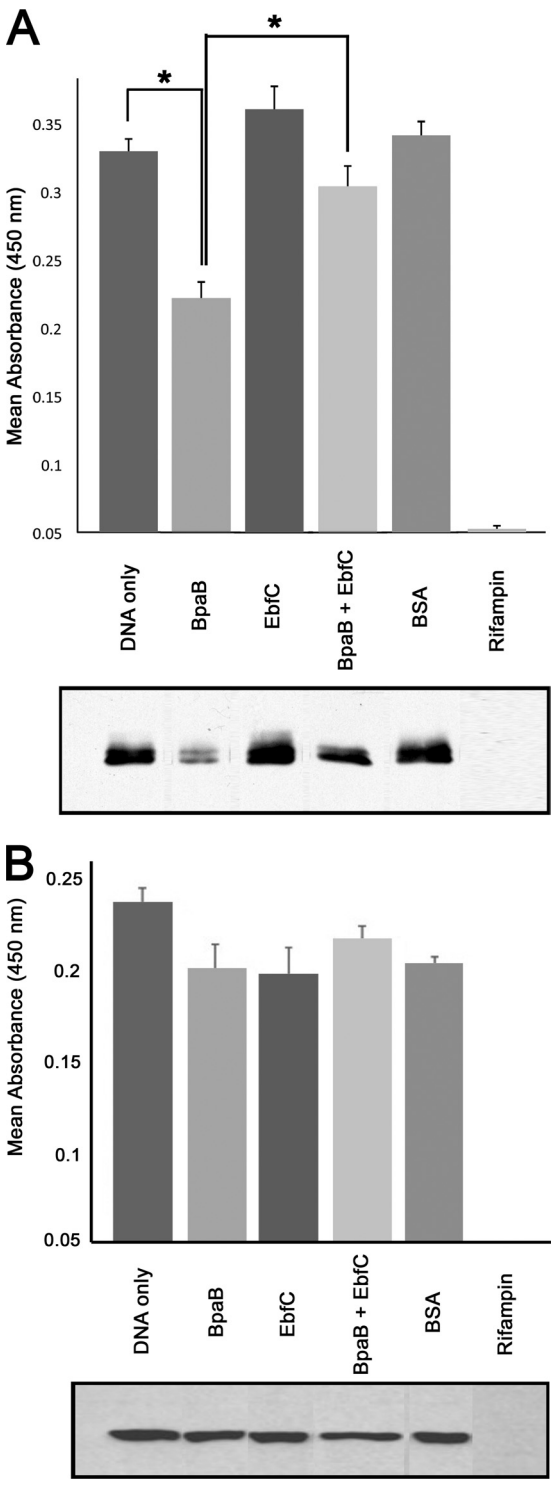

FIG 4 (A) Effects of purified BpaB and/or EbfC proteins on *erp* expression in a coupled *in vitro* transcription/translation system. In the upper panel, product levels were quantified by ELISA and are reported as mean absorbances for three independent experiments. Asterisks indicate statistically significant differences ($P < 0.001$ by Student's *t* test) between DNA only and BpaB added, or between BpaB alone and BpaB plus EbfC. The lower panel shows anti-GFP immunoblot analyses of one representative series of *in vitro* transcription/translation reactions. BpaB significantly repressed *erp* expression, while addition of EbfC counteracted the repressive effect of BpaB. BSA served as a control to confirm that results were specific for each protein. Addition of rifampin completely prevented product formation, demonstrating that results were dependent upon transcription. (B) Control studies using the *B. burgdorferi ospAB* promoter and 5′ noncoding DNA fused to *gfp*. The upper panel shows ELISA results. The lower panel shows anti-GFP immunoblotting results. No added protein or combination of proteins had a significant effect upon expression from the *ospAB* promoter.

indicated that BpaB proteins from all examined cp32 elements bind the same sequence of all *erp* operators (13). That was not a surprising conclusion, since the BpaB-binding site sequence of all *erp* operators is extremely well conserved (Fig. 1A) (1, 6, 10, 43, 60, 64). Further supporting that conclusion, overproduction of lp56-encoded BpaB greatly repressed production of the Erp protein encoded by other cp32 elements (Fig. 3). Those results also indicate that BpaB functions as an *erp* repressor in *trans*. Overproduction of EbfC resulted in increased levels of Erp proteins (Fig. 3). Thus, EbfC enhances *erp* expression *in vivo* as well as *in vitro*.

***In vitro* transcription-translation analyses.** Effects of the *erp* operator-binding proteins were directly assessed through use of a coupled *in vitro* transcription-translation system. The utilization of an *Escherichia coli* S30 extract for these studies conferred several advantages over use of a borrelial extract. Most significantly, *E. coli* does not produce proteins similar to BpaB, and while those bacteria do produce an EbfC-like protein, it does not specifically bind the same DNA sequence as does the *B. burgdorferi* protein (19). Thus, the effects of each borrelial protein could be examined in isolation, without complications due to their presence in *B. burgdorferi* extracts.

The *erpA* operator/promoter-*gfp* construct was utilized for these studies (6). Production of GFP was assayed by both ELISA and immunoblotting. For all these studies, each protein was added to the same final molar concentration. Control studies with added BSA demonstrated that experimental results were not due simply to inclusion of a protein (Fig. 4A and B). Addition of the RNA polymerase inhibitor rifampin completely eliminated readout signals (Fig. 4A and B), confirming that results were dependent on transcription of the P*erp*::*gfp* fusion.

Addition of BpaB to the coupled transcription-translation reaction significantly repressed production of GFP (Fig. 4A). EbfC did not exert a significant effect when added by itself. In contrast, simultaneous addition of EbfC and BpaB yielded GFP expression levels that did not differ from those observed when no borrelial protein was present. Those observations are consistent with results of previous studies which determined that EbfC and BpaB compete for binding to *erp* operator DNA (13). Noting that these studies were performed using nonreplicating template, the data also demonstrate that the effects of BpaB and EbfC on *erp* expression levels is not due to variations in the cp32 copy number per cell, but the proteins exert their effects directly on *erp* genes.

As a control, *in vitro* transcription-translation was also undertaken using a fusion between the *B. burgdorferi ospAB* promoter and *gfp* (16). Inclusion of BpaB and/or EbfC did not significantly affect expression from this promoter, indicating that the above-observed effect of BpaB on *erp* expression was specific to that operon and not a general, nonspecific effect (Fig. 4B).

Effects of growth conditions on BpaB and EbfC. The above-described studies demonstrated that BpaB and EbfC control *erp* expression. *B. burgdorferi* produces greater levels of *erp* transcripts and Erp proteins when cultured at 34°C than when grown at 23°C (58, 62). Therefore, the effects of culture temperature on cellular

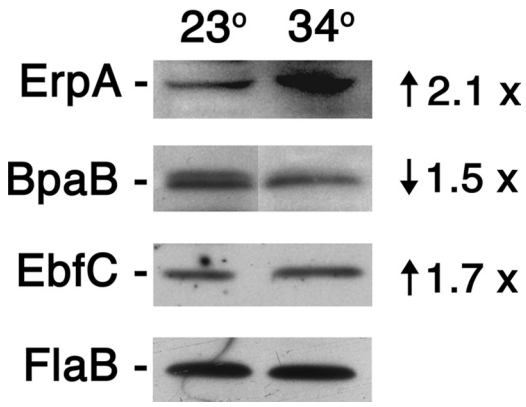

FIG 5 Representative immunoblots of protein levels in wild-type *Borrelia burgdorferi* cultured at either 23 or 34°C. *B. burgdorferi* produce appreciably greater levels of Erp proteins when cultured at 34°C compared with results at 23°C (58, 62). The same bacterial lysates were used for each immunoblot shown. The constitutively expressed FlaB (flagellin) protein was also examined, to serve as a control for equal loading of wells. Comparing results at 23°C versus those at 34°C for these cultures, ErpA levels increased 2.1-fold, BpaB decreased 1.5-fold, and EbfC increased 1.7-fold. This BpaB antiserum, raised against the allele carried by lp56, recognized two of the bacterium's BpaB proteins, both of which were affected by culture temperature in the same manner. The observed differential expression of the EbfC protein corresponds with results of previous studies which found the same effect on *ebfC* transcript levels (44).

levels of the *erp* operator-binding proteins were assessed (Fig. 5). *B. burgdorferi* cultured at 34°C contained appreciably lower levels of BpaB than did bacteria cultured at 23°C. In contrast, levels of EbfC were higher in bacteria grown at 34°C than they were in bacteria cultured at 23°C. The results for EbfC protein levels correlate with those of previous studies of *ebfC* mRNA levels (44).

DISCUSSION

As do other pathogenic bacteria, the Lyme disease spirochete regulates production of numerous proteins during infection processes (51). Research on the borrelial Erp outer surface proteins has revealed multiple properties associated with mammalian infection (2, 3, 11, 12, 34, 37, 40, 45, 59). Given what is known of their functions, it is not surprising that *B. burgdorferi* Erp proteins are produced during mammalian infection but repressed during colonization of tick vectors (10, 31, 48, 49). The *erp* operator is required for regulation of *erp* transcription (6), and the present studies demonstrated that both BpaB and EbfC bind to *erp* operator DNA in live *B. burgdorferi*. Results of the studies described in this report, along with previous data, permit the construction of a model of how BpaB and EbfC may control *erp* expression (Fig. 6). While the model is largely based on studies of cultured *B. burgdorferi*, such an approach can provide detailed insights into regulatory mechanisms, much as Jacob and Monod's seminal studies of cultured *E. coli* led to an understanding of *lac* operon regulation (38, 50).

BpaB initially binds *erp* operator DNA at a high-affinity site, and deletion of that site significantly reduces binding (13). The initial BpaB-DNA interaction facilitates binding of additional BpaB molecules to the DNA, apparently through protein-protein interactions that stabilize binding to less-desirable DNA sequences (13). Results presented in this report indicate that deletion of the BpaB high-affinity binding site increases *erp* promoter activity *in vivo*, and BpaB represses *erp* transcription *in vitro* and represses Erp protein levels when overproduced in *B. burgdorferi*. Altogether, these data indicate that BpaB is a repressor of *erp* transcription. We hypothesize that repression is due to the spreading of BpaB along *erp* DNA, which would occlude the promoter elements from recognition by RNA polymerase (Fig. 6A). Such a mechanism of transcriptional repression has been proposed for other bacterial DNA-binding proteins that similarly spread along DNA (8, 20, 28, 39, 42, 53, 54).

The lp56-carried allele of BpaB, expressed in *B. burgdorferi* from a recombinant plasmid, influenced the *erp* operons of the bacterium's native cp32-1 and cp32-4. Two important conclusions can be made from that result: (i) BpaB functions as an *erp* repressor in *trans*, and (ii) BpaB proteins encoded by one cp32 element will affect *erp* operons on other cp32s. Lyme spirochetes naturally carry several different cp32 elements, each carrying its own allele of BpaB and having its own *erp* locus. Our results indicate that the entire cohort of a spirochete's cp32-encoded BpaB proteins controls all of its *erp* genes. Such cross talk would facilitate the previously observed coexpression of Erp proteins (26, 32).

erp operons contain 2 to 3 consensus EbfC-binding sites adjacent to the BpaB high-affinity site (Fig. 1) (13, 52). EbfC and BpaB compete for binding to *erp* operator DNA (13). An earlier study observed that *B. burgdorferi* transcribes significantly higher levels of *ebfC* mRNA while within feeding ticks or during mammalian infection than while colonizing unfed ticks (44). This parallels the pattern of *erp* transcription during the borrelial mammal-tick in-

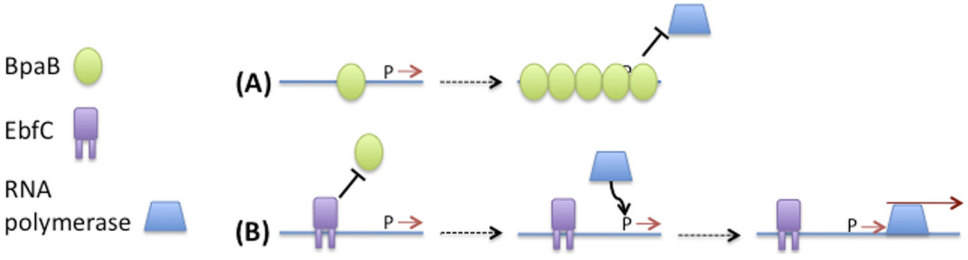

FIG 6 A model of the effects of BpaB and EbfC on *erp* transcription, which incorporates all current data. Both of these DNA-binding proteins serve additional functions in *B. burgdorferi*, and the borrelial genome contains additional binding sites for each, so effects on *erp* operons will be dependent upon levels of free BpaB and EbfC proteins in the cell. (A) When cellular levels of free BpaB exceed those of EbfC, a BpaB molecule will bind to the *erp* operator, which then facilitates binding of additional BpaB proteins to the DNA. BpaB spreading occludes the promoter region, preventing recognition by RNA polymerase and thereby repressing *erp* expression. (B) When levels of free EbfC exceed those of BpaB, EbfC preferentially binds to the *erp* operator and competes away BpaB, thereby allowing RNA polymerase to recognize the promoter and transcribe the *erp* genes.

fectious cycle (10, 31, 48, 49). The present studies indicate that EbfC counteracts repression by BpaB *in vitro*, and overexpression of EbfC in *B. burgdorferi* increases levels of Erp proteins. Moreover, mutation of the *erp* operator EbfC-binding sites to nonconsensus sequences resulted in very low transcription levels in *B. burgdorferi*. Together, these data indicate that EbfC is an antirepressor of *erp* transcription (Fig. 6B). EbfC by itself did not increase *erp* expression and therefore does not appear to be a transcriptional activator of *erp* operons.

Several lines of evidence indicate that both of these DNA-binding proteins carry out additional functions for *B. burgdorferi*. All relapsing-fever *Borrelia* species lack *erp* operons, yet they encode BpaB and EbfC proteins that are nearly identical to those of the Lyme disease spirochetes (61). BpaB plays a role in maintenance of the Erp-encoding cp32 prophages, possibly analogous to the ParB/SopB proteins of other bacterial replicons (9, 21, 65, 66). Related to that point, several such plasmid maintenance proteins also function as transcriptional repressors through mechanisms like that proposed for BpaB and the *erp* operons (8, 20, 39, 53, 54). Although the spirochete phylum diverged from the rest of the kingdom *Eubacteria* many millions of years ago, the retention of EbfC homologs by almost all members of the *Eubacteria* suggests that these proteins perform central, critical functions for many prokaryotes. Several characteristics of EbfC indicate that it is a type of nucleoid-associated ("histone-like") protein (52; our unpublished results). The *ebfC* gene is located on the *B. burgdorferi* chromosome, while *erp* operons are located on cp32 prophages. These bacteriophages have evidently commandeered a spirochetal protein for their own devices, joining the long list of viruses that utilize host factors as regulators.

It is notable that *erp* operon transcription is influenced by two distinct proteins, in that *erp* transcription will be dependent upon relative levels of each DNA-binding protein in the bacterial cell. Moreover, the additional functions of BpaB and EbfC indicate that their effects on *erp* transcription will be dependent upon cellular levels of free proteins, that is, protein that is not bound elsewhere in the genome. For example, high concentrations of free BpaB in the cytoplasm could result in either *erp* repression or derepression, depending upon whether free EbfC levels were low or high, respectively.

The conservation of BpaB- and EbfC-binding sites in all known *erp* operator elements indicates that both proteins will likely exert influence on transcription of all *erp* operons (1, 5, 6, 33, 43, 60, 64). Yet other factors can also affect levels of *erp* expression. As is evident from the DNA alignments illustrated in Fig. 1 and elsewhere (1, 5, 6, 33, 43, 60, 64), the −10 and −35 sequences of *erp* promoters exhibit considerable diversity, which can impact promoter activity and transcript levels (6, 23). Moreover, some *erp* promoter variants are poorly recognized by the borrelial housekeeping sigma factor RpoD but are better recognized by another sigma factor, RpoS (so called because its sequence resembles that of *E. coli* RpoS, although the *B. burgdorferi* RpoS is not associated with stress response) (14, 15, 22, 23, 27). Available data suggest that the *B. burgdorferi* RpoS-RNA polymerase holoenzyme recognizes the same promoter sequences as does the RpoD-containing enzyme and also variants thereof that are not recognized by RpoD, similar to the *E. coli* RpoS sigma factor (14, 15, 22, 23, 27, 30, 35, 67). It is not clear whether the variations in *erp* promoter sequences are due to degenerating transcriptional elements of unnecessary or defective genes, physiological benefits reaped from expressing some operons using both RpoD and RpoS but others using only RpoS, or combinations of those two causes. Very little is known about the mechanistic details of interactions between *B. burgdorferi* RpoS and *erp* promoters, since the majority of published studies purporting to be on that topic actually examined the distinct *E. coli* RpoS sigma factor (22, 23). However, a study of *erp* promoter-*gfp* fusions in *B. burgdorferi* strain 297 found that one of that strain's *erp* loci, named *ospF*, was not recognized by borrelial RpoD but was dependent upon borrelial RpoS, while another locus, named *ospE*, did utilize RpoD (23). Both of those strain 297 operons contain consensus BpaB- and EbfC-binding sites in their operators and are very likely to also be influenced by the two DNA-binding proteins. To argue otherwise requires that one invoke a mechanism by which BpaB and EbfC are excluded from a subset of *erp* operators, a complexity for which no evidence has been found. Intriguingly, a chimeric promoter consisting of the strain 297 *ospF* −35 and *ospE* −10 sequences was not functional in either wild-type or *rpoS B. burgdorferi* (23), indicating sequence defects in some promoters that, to the best of our knowledge, have yet to be explored. In addition, the metabolite 4,5-dihydroxy-2,3-pentanedione, also known as autoinducer 2 or AI-2, enhances expression of Erp proteins through an unknown mechanism (57, 68). It is worthwhile to note that neither RpoS nor AI-2 has been tested for its ability to control *erp* expression during actual mammal infection or tick colonization. Yet the effects of those factors on cultured *B. burgdorferi* strongly suggest that they, and BpaB and EbfC, do perform regulatory roles in nature.

Previous studies and our further ChIP analyses and global RNA sequencing indicate that both BpaB and EbfC bind sites throughout the borrelial genome, in addition to *erp* operator elements, and control production of numerous other bacterial genes (44; our unpublished results). Defining the mechanisms by which the Lyme disease spirochete controls the relative levels of the BpaB and EbfC DNA-binding proteins will provide further insight into how this pathogen senses and adapts to changes throughout its vertebrate-arthropod infectious cycle. In addition, the near ubiquity of EbfC proteins among diverse bacterial species implies that studies of the borrelial ortholog will have ramifications throughout the kingdom *Eubacteria*.

ACKNOWLEDGMENTS

This work was funded by National Institutes of Health grants R01-AI044254 to Brian Stevenson and R01-AI063261 to Wolfram Zückert.

We thank Greg Bowman and Jennifer Strange for assistance with flow cytometry analyses, Jay Carroll for providing the P*ospAB*::*gfp* fusion construct, and Kelly Babb, Gavin Ellis, Michael Fried, Jason Johnston, Jennifer Miller, Heather O'Daniel, and Brett Spear for their help and insightful comments on this work and the manuscript.

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