

## Molecular Characterization of *Borrelia burgdorferi* *erp* Promoter/Operator Elements

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Many *Borrelia burgdorferi* Erp outer surface proteins have been demonstrated to bind the host complement regulator factor H, which likely contributes to the ability of these organisms to evade the host innate immune system. *B. burgdorferi* controls Erp protein synthesis throughout the bacterial infectious cycle, producing the proteins during mammalian infections but repressing their synthesis during tick infections. Defining the mechanism by which *B. burgdorferi* regulates the expression of these virulence determinants will provide important insight into the biological and pathogenic properties of the Lyme disease spirochete. The present study demonstrates that two highly conserved DNA sequences located 5' of *erp* operons specifically bind bacterial proteins. Analyses with *B. burgdorferi* of transcriptional fusions between *erp* promoter/operator DNAs and the gene for green fluorescent protein indicated that the expression of these operons is regulated at the level of transcriptional initiation. These analyses also indicated significant differences in the promoter strengths of various *erp* operons, which likely accounts for reported variations in expression levels of different Erp proteins. Mutagenesis of promoter-*gfp* fusions demonstrated that at least one of the proteins which bind *erp* operator DNA functions as a repressor of transcription.

The spirochete *Borrelia burgdorferi* is maintained in nature through an infectious cycle involving warm-blooded vertebrates and *Ixodes* sp. ticks. These bacteria regulate the expression of a number of different proteins during the infectious cycle, among which are the Erp outer surface lipoproteins. Individual bacteria encode multiple members of the Erp family, with the type strain, B31, being known to carry 17 *erp* genes at 10 separate loci (12, 13, 50). Each locus occupies an allelic position on a different plasmid of the cp32 family. Although cp32 plasmids are largely identical in their sequences, many are compatible with each other, with 12 apparent incompatibility groups identified to date (16, 47). Many Erp proteins have been demonstrated to bind host factor H, a regulator of complement activation, and may thereby help protect the bacteria from the alternative pathway of complement-mediated killing (4, 5, 25–29, 34, 46). Consistent with that function, *B. burgdorferi* expresses Erp proteins during transmission between the tick vector and the vertebrate host, times when the bacterium is exposed to host serum and the innate immune system (22–24, 36, 37). For unknown reasons, *B. burgdorferi* down-regulates Erp expression during infections of ticks (22, 36).

A number of in vitro studies have provided insights into the regulatory mechanisms by which *B. burgdorferi* controls Erp synthesis. The culture temperature significantly impacts Erp expression levels, with bacteria grown at 34°C producing higher levels of Erp proteins than do bacteria cultivated at 23°C (24, 44, 48). These temperatures mimic those experienced by feeding and unfed ticks, respectively (40). The bacteria also respond to quorum sensing and to other, unidentified chemical

signals that potentially represent components of host blood or other tissues encountered during infection (1, 3, 8, 15, 23, 24, 41, 52, 53). The regulation of Erp expression appears to occur at the mRNA level (24, 44), although evidence has been presented suggesting that some *erp* operons are regulated by additional mechanisms (24).

Erp proteins have been given various other names by different researchers, including OspE, p21, Bbk2.10, pG, OspF, and Elp (50). The primary structures of Erp proteins may vary widely, with different proteins exhibiting anywhere between 18 and 100% identical amino acid sequences (2, 47, 50). These differences have led to the suggestion that the Erp family can be divided into three or more subfamilies (2). However, all *erp* genes hold a number of features in common, the most significant being that each *erp* operon is preceded by a highly conserved DNA sequence (Fig. 1) (2, 12, 13, 24, 32, 44, 49, 50). The transcriptional start site has been mapped to a point just 5' of the translational initiation codon (24). Electrophoretic mobility shift assays (EMSAs) have demonstrated that an unidentified *B. burgdorferi* protein(s) specifically binds to DNA immediately 5' of all tested *erp* operons (8). The expression of *erp* operons is not detectably affected by DNA supercoiling (6, 19), suggesting that the regulation of *erp* transcription is due to the binding of an activator and/or repressor protein(s) to operator sites.

To better understand the mechanisms by which *B. burgdorferi* regulates Erp expression, we have performed a detailed characterization of *erp* promoter/operator DNAs. EMSAs were utilized to precisely map protein-binding sites within these regions. Recent advances have enabled the use of transcriptional fusions with reporter genes for the study of the nature of promoter/operator elements in *B. burgdorferi* (11, 16). This technique was utilized to define the roles of 5' DNA in the transcriptional regulation of *erp* operons and to explore

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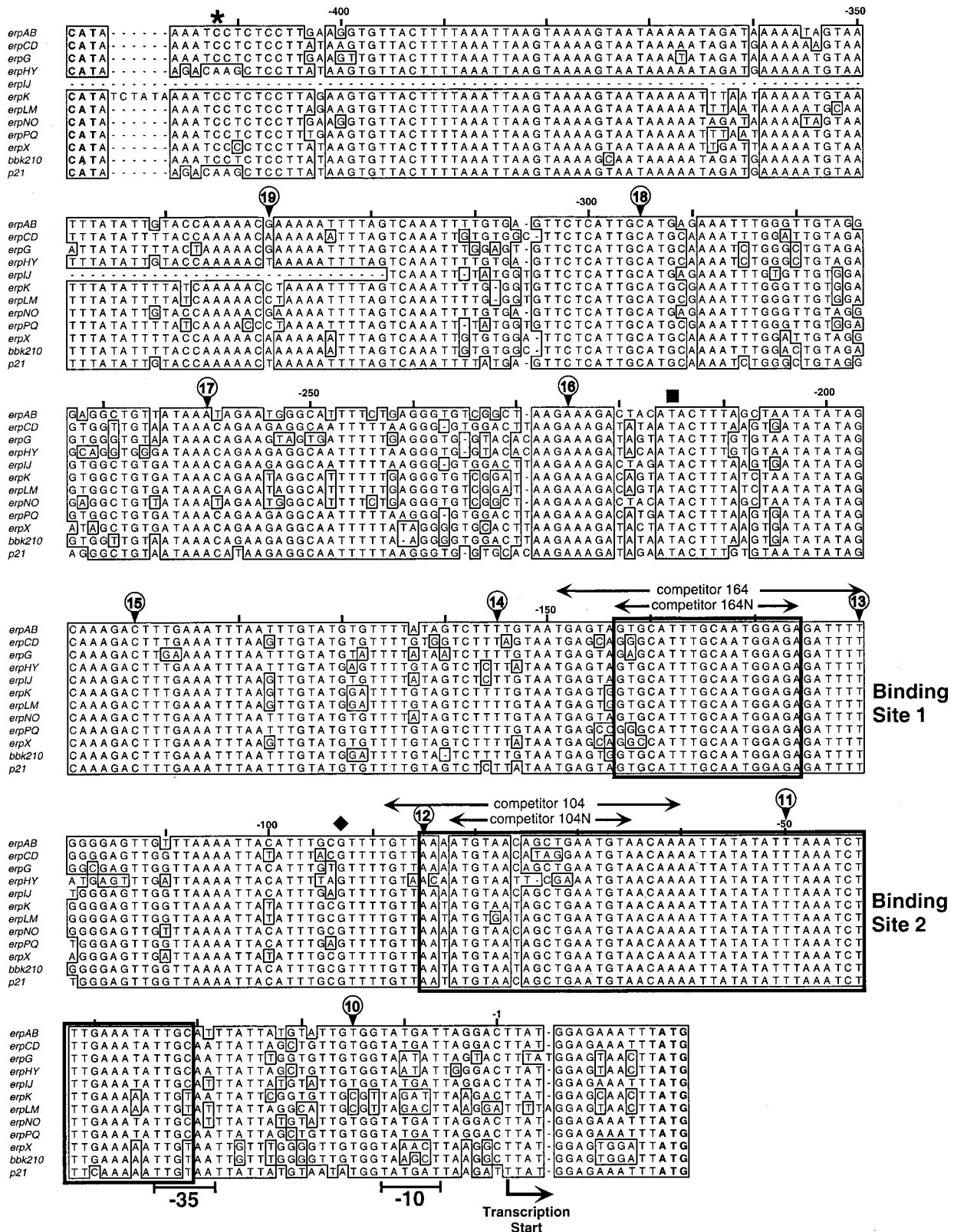


FIG. 1. Alignment of the DNA sequences located 5' of the 10 *erp* operons of strain B31, the *bbk2.10* operon of strain 297, and the *p21erp22* operon of strain N40. The complete sequence 5' of the B31 *erpII* operon has not been determined (12, 13). Identical nucleotides found in 50% or more of the sequences are boxed and shaded. Start codons (ATG) of the first gene of each *erp* operon are indicated in bold at the lower right. The start codon of each divergently transcribed *bppC* gene (CAT) is indicated in bold at the upper left. The identified start of *erp* transcription (24) and probable -10 and -35 sequences are indicated. Nucleotides are numbered above each line relative to the start of transcription. The

reasons for differences in promoter activity. In addition, promoter-reporter fusions were used to examine *erp* operons that were previously believed to possess regulatory mechanisms not found in other members of the *erp* family.

#### MATERIALS AND METHODS

**Bacteria.** The *B. burgdorferi* strains used for this work are listed in Table 1. All strains were grown in modified Barbour-Stoenner-Kelly medium (BSK-II) (9). Unless otherwise noted, *B. burgdorferi* strains were cultured at 34°C. By the use of PCR with previously described oligonucleotide primer pairs (35, 38, 42), strain B31-e2 was determined to contain only plasmids cp26, cp32-1, cp32-3, cp32-4, lp17, lp38, and lp54.

Temperature shift studies were performed as previously described (48). Briefly, cultures of *B. burgdorferi* were grown at 23°C until they reached mid-exponential phase (approximately 10<sup>7</sup> bacteria per ml). Aliquots of each culture were then diluted 1:100 into fresh medium and grown to mid-exponential phase at 34°C.

Erp protein expression by strain B31-e2 was examined by immunoblotting using monospecific rabbit polyclonal antisera (19). Aliquots of bacterial lysates containing equal amounts of total protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were then electrotransferred to nylon membranes and incubated with antisera, and bound antibodies were detected by enhanced chemiluminescence (Amersham Pharmacia, Piscataway, N.J.) (48).

**EMSAs.** Whole-cell lysates were prepared from *B. burgdorferi* B31-RML cultured at either 34 or 23°C as previously described (8). EMSAs were performed essentially as described previously (8), with the major change being the use of biotinylated DNA probes rather than <sup>32</sup>P-labeled DNA. Probes were produced by PCR using a biotinylated 5' primer and an unlabeled 3' primer (Integrated DNA Technologies, Coralville, Iowa), with previously cloned *erp* promoter DNAs as templates (49). Two different probes were utilized in these studies: one probe, created by a PCR using oligonucleotides Bio-E43 and E-8, extended from the start codon of *erpG* upstream to bp -330, and a shorter probe, created with oligonucleotides Bio-104 and R-8, spanned nucleotides -31 through -89 of *erpG* (Table 2). PCR products were purified through Centricon-100 microcentrifugers (Amicon, Beverly, Mass.) (18).

Competitor DNAs G-9 through G-18 were produced by PCRs using an *erpG* clone as a template, with oligonucleotide pairs G-9A plus G-9C, G-10A plus G-10C, etc. Three other competitor DNAs were also produced by PCRs using the same template, and they were named according to the distance of their 5' and 3' ends from the start of *erp* transcription. Competitor -149/-31 was produced with oligonucleotide primers 164-F and R-8, competitor -149/-79 was produced with oligonucleotides 164-F and R-350, and competitor -89/-31 was produced with oligonucleotides 104-F and R-8. An additional competitor, E-43/A-2, encompassed the entire *bppC-erpAB* intergenic region and was produced by a PCR using oligonucleotides E-43 and A-2. All PCR products were purified as described above. Other, shorter competitor DNAs were produced by annealing two complementary oligonucleotides to produce double-stranded DNAs. Competitor 104 was produced from oligonucleotides 104-F and 104-R, competitor 104N was produced from oligonucleotides 104N-F and 104N-R, competitor 164 was produced from oligonucleotides 164-F and 164-R, and competitor 164N was produced from oligonucleotides 164N-F and 164N-R.

A LightShift Chemiluminescent EMSA kit (Pierce Biotechnology) was utilized according to the manufacturer's recommendations. Briefly, 10-μg aliquots of bacterial extract were incubated either with or without 4 pmol of unlabeled specific competitor DNA in LightShift buffer containing 2 μg of poly(dI-dC) (nonspecific competitor). Samples were incubated for 5 min at room temperature to allow protein binding to poly(dI-dC) and/or the competitors. Next, 20-fmol aliquots of labeled DNA probe were added to each sample, such that each contained a 200-fold excess of competition. Samples were then subjected to electrophoresis through a 7% polyacrylamide gel (37.5:1 acrylamide-bisacrylamide). After electrophoresis, DNAs were transferred to a nylon membrane,

TABLE 1. Bacterial strains used for this work

Strain	Description
B31	Wild type, <i>B. burgdorferi</i> type strain
297	Wild type
N40	Wild type
B31-RML	Subculture of strain B31
B31-e2	Clone of strain B31
KS10	B31-e2 containing pBLS590 (pJAH2 containing promoter-less <i>gfp</i> )
KS11	B31-e2 containing pBLS591 (pBLS590 containing <i>PerpAB</i> from -442 to start)
KS12	B31-e2 containing pBLS592 (pBLS590 containing <i>PerpG</i> from -442 to start)
KS13	B31-e2 containing pBLS593 (pBLS590 containing <i>PerpAB</i> from -442 to -10 and <i>PerpG</i> from 3' of -10 to start)
KS15	B31-e2 containing pBLS594 (pBLS590 containing <i>PerpG</i> from -442 to 5' of -10 and <i>PerpAB</i> from -10 to start)
KS16	B31-e2 containing pEAP1 (pBLS590 containing <i>PerpAB</i> from -215 to start)
KS17	B31-e2 containing pEAP2 (pBLS590 containing <i>PerpAB</i> from -93 to start)
KS18	B31-e2 containing pBLS595 (pBLS590 containing <i>Pbbk2.10</i> from -442 to start)
KS19	B31-e2 containing pBLS598 (pBLS590 containing <i>PerpAB</i> from -442 to start, with <i>PerpG</i> -10 site)
KS20	B31-e2 containing pBLS599 (pBLS590 containing <i>PerpAB</i> from -34 to start)
KS21	B31-e2 containing pBLS600 (pBLS590 containing <i>Pp21erp22</i> from -442 to start)
KS23	B31-e2 containing pBLS603 (pBLS590 containing <i>erp</i> ribosome-binding site only)
GPB	B31-e2 containing pCJB1 (pBLS590 containing <i>PerpG</i> from -215 to start)
GPL	B31-e2 containing pCJB2 (pBLS590 containing <i>PerpG</i> from -93 to start)

cross-linked by UV irradiation, and then blocked for 15 min at room temperature with LightShift blocking buffer. The membrane was next incubated with streptavidin-horseradish peroxidase conjugate for 15 min, followed by a 5-min incubation in Luminol enhancer substrate solution. Labeled DNAs were visualized by exposure to X-ray film. The relative abundance of *erp* operator binding proteins was determined as described above, except the amounts of bacterial extract examined in each assay ranged between 0.7 and 5.1 μg.

***erp* promoter-*gfp* fusion plasmids.** With the recent development of independently replicating plasmid vectors suitable for transformation into *B. burgdorferi*, new avenues have been opened for dissecting the DNA elements involved in the regulation of *erp* gene expression. To that end, we constructed a series of plasmids that contain transcriptional fusions with *gfp* (Fig. 2). The inserts of all recombinant plasmids were sequenced on both strands to confirm that no mutations were introduced during the cloning process.

In the initial step of construction, the *B. burgdorferi*-*Escherichia coli* shuttle vector pBSV2 (51) was made smaller by the removal of 600 bp of nonessential DNA. To do so, we removed the gene encoding zeocin resistance by PCR with oligonucleotides BSV-G and BSV-H (Table 2), digestion with XhoI, and ligation with T4 DNA ligase. The resulting plasmid was designated pJAH2. The mutant 3 allele of *gfp* (14) was then amplified by PCR using oligonucleotides GFP-1 and GFP-2. The resultant *gfp* amplicon and pJAH2 were each digested with BamHI and HindIII and then were ligated.

With the oligonucleotides listed in Table 2, fragments of DNA were then amplified by PCRs from recombinant plasmid clones of the *erpAB* and *erpG* operons (49) or from uncloned PCR amplicons of the *bbk2.10* locus of strain 297 and the *p21erp22* locus of strain N40 (47). Each oligonucleotide pair contained

5'-most nucleotides of DNAs used in initial EMSA competition experiments are indicated by circled numbers as follows: the 5' end of G10-AC is labeled "10," the 5' end of G11-AC is marked "11," and so on. All of the competitors extended 3' beyond the sequences illustrated. Competitor G9-AC was composed of DNA sequences entirely within the *erpG* gene and therefore is not indicated in this figure. Heavily lined boxes indicate the boundaries of protein-binding sites 1 and 2. The asterisk above bp -412 indicates the 5' ends of full-length promoter/operator fragments used for the construction of most *Perp:gfp* fusions, the black square above bp -215 indicates the 5' ends of fragments used to produce pEAP1 and pGPB, and the diamond above bp -93 indicates the 5' ends of fragments used for the construction of pEAP2 and pGPL.

TABLE 2. Oligonucleotides used for this work Sequences of specific restriction endonuclease recognition sites are underlined

Name	Sequence (5' to 3') <sup>a</sup>
G9-A	.....TAAGAAAATGAAAAATTTAAT
G9-C	.....AATTCGTGAAATTCCTTCTA
G10-A	.....TGGTAATATTAGTACTTTATG
G10-C	.....TTTTTTCTTTTATCTATTTT
G11-A	.....TTAAATCTTTGAAATATTGCA
G11-C	.....GAATTCCTTTTCTTCTCTT
G12-A	.....AAAATGTAACAGCTGAATGTA
G12-C	.....TGACCACCATCTTTTGCTTGC
G13-A	.....TGGCGAGTTGGTTAAAATAC
G13-C	.....GGGTGTGTTATCGTGGGAACT
G14-A	.....TTGTAATGAGTAGAGCATTG
G14-C	.....GTGGTGGATTAACAGACTGT
G15-A	.....CTTGAAAATTAATTTGATG
G15-C	.....CCTTGCATTAACCTTTATCT
G16-A	.....AAAGATAGTATACTTTGTGTA
G16-C	.....TTTTCTTTACATTTTGT
G17-A	.....ACAGAAGTAGTGATTTTGGAG
G17-C	.....TCGCATCAATCTTGAAGAAA
G18-A	.....CATGCAAAATCTGGGCTGTAG
G18-C	.....ACTGCACAAATAATTAATTT
Bio-E43	.....biotin-AAAATTTAGTCAAATTTGGAGTG
Bio-104	.....biotin-TGTTAAAATGTAACAGCTGAATGTAACAAA
R-8	.....GCAATATTTCAAGATTTAAA
E-8	.....CATAAGTTACTCC
E-43	.....AAAATTTAGTCAAATTTGGAGTG
104-F	.....TGTTAAAATGTAACAGCTGAATGTAACAAA
104-R	.....TTTGTACATTCAGCTGTACATTTAACA
104N-F	.....ATGTAACAGCTGAATGTA
104N-R	.....TACATTCAGCTGTACAT
164-F	.....TGAGTAGAGCATTTGCAATGGAGAGATTTT
164-R	.....AAAATCTCTCCATTCGAAATGCTCTACTCA
164N-F	.....GAGCATTTGCAATGGAGA
164N-R	.....TCTCCATTGCAAAATGCTC
BSV-G	.....AAAAA <u>ACTCGAGT</u> GGCCGAGGAGCAGGACTGACAG
BSV-H	.....AAAAA <u>ACTCGAGT</u> CTGATTAGAAAACTCATCGAG
GFP-1	.....ATAATAGGATCCATACATATGAGTAAAGGAGAAG
GFP-2	.....ATAATA <u>AGCTT</u> ATTATTGTATAGTTCATCCATGCATG
G-1	.....AAAAAAGGTACCATATATAGCAAAG
G-2	.....TTTTTTGGATCCTCCATAAAAGTACTAATATTAC
G-2A	.....TTTTTTGGATCCTCCATAAAAGTACTAAT
G-3	.....AAAAAAGGTACCCTTTTGTAAAATGTAACAGCTG
A-1	.....AAAAAAGGTACCCTCTCCTTGAAGGTG
A-2	.....TTTTTTGGATCCTCCATAAAGTCCATAATCATA
A-4	.....ATAATGGATCCTCCATAAAGTCCATAATATTACCAC
A-10	.....ATAATAGGATCCCTTGCATTTATTATGTTATTGTGG
E-116	.....ATATAATTTGTTACATTCAG
BBK-2	.....CATAATGGATCCTCCATAAAGCCTTAAGCTTAC
21-1	.....ATAATAGGTACCACAAGCTCCTTATAAGTG
21-2	.....TTTTTTGGATCCTCCATAAATCTTAATCATA
PRO-1	.....CGCGCTTATGGAG
PRO-2	.....GATCCTCCATAAGCGCGGTAC

<sup>a</sup> Sequences of specific restriction endonuclease recognition sites are underlined.

a KpnI and a BamHI site in the 5' and 3' oligonucleotide, respectively. In addition, all 3' oligonucleotides contained an *erp* consensus ribosome binding site. Oligonucleotide pairs for the production of each promoter-reporter fusion plasmid were as follows: for pBLS591, A-1 plus A-2; for pBLS592, A-1 plus G-2; for pBLS593, A-1 plus G-2A; for pBLS594, A-1 plus A-2; for pBLS595, A-1 plus BBK-2; for pBLS598, A-1 plus A-4; for pBLS600, 21-1 plus 21-2; for pEAP1, G-1 plus A-2; for pEAP2, G-3 plus A-2; for pCJB1, G-1 plus G-2; and for pCJB2, G-3 plus G-2. Amplicons were digested with KpnI and BamHI and ligated between the KpnI and BamHI sites of pBLS590, such that the ribosome binding sites were located the same distance from the translational start codon as in most *erp* operons.

An additional plasmid was produced in which only the DNA 3' of the putative -35 sequence of *erpAB* was included in the fusion. To do so, we first subjected pBLS591 to PCR with oligonucleotides A-10 and E-116, which amplified in

opposite directions out from the *erpAB* promoter. This amplicon was then digested with KpnI and self-ligated.

Control plasmid pBLS603 contained only a ribosome binding site located 5' of *gfp*. This plasmid was constructed by annealing oligonucleotides PRO-1 and PRO-2 and then ligating the resulting double-stranded DNA between the KpnI and BamHI sites of pBLS590.

**Transformation of *B. burgdorferi*.** *B. burgdorferi* B31-e2 was transformed with recombinant plasmids under standard electroporation conditions (39). After an overnight incubation in BSK-II lacking antibiotics, bacterial cultures were plated in semisolid BSK-II containing 1.2% agarose (w/vol) and kanamycin at a concentration of 200 µg/ml (10, 30). Plates were incubated at 34°C in a 5% CO<sub>2</sub> atmosphere. Antibiotic-resistant colonies were screened for the presence of recombinant plasmids by PCRs using oligonucleotides GFP-1 and GFP-2. *B. burgdorferi* strains carrying pBLS590 and derivative plasmids are listed in Table 1.

To ascertain whether sequences of heterologous plasmids remained stable after passaging through *B. burgdorferi*, plasmids of several transformed strains were reisolated and sequenced. *B. burgdorferi* cultures (100 ml) were grown to mid-exponential phase, and total plasmids were purified by use of Qiagen Midiprep kits (Qiagen, Chatsworth, Calif.). Aliquots (approximately 1 µg) of each purified plasmid preparation were used to transform *E. coli*, and bacteria were plated on Luria-Bertani agar containing kanamycin at a concentration of 50 µg/ml. Plasmids of transformed *E. coli* were then purified and sequenced.

**Analysis of GFP expression.** The effects of the culture temperature on green fluorescent protein (GFP) expression from each promoter-reporter construct were assessed by both flow cytometry and immunoblot analysis. Cultures of each transformed *B. burgdorferi* strain were grown to mid-exponential phase in BSK-II broth either at a constant 23°C or with a shift from 23 to 34°C. Bacteria were collected by centrifugation, washed twice with phosphate-buffered saline (PBS), and then resuspended in PBS at concentrations of approximately 10<sup>6</sup> bacteria per ml. Aliquots of each bacterial suspension were analyzed in a FACScalibur flow cytometer (Becton Dickinson, San Jose, Calif.), with excitation and detection wavelengths of 488 and 530 nm, respectively. At least 70,000 events were measured for each flow cytometry analysis. GFP expression levels of transformed bacteria were further analyzed by immunoblotting. Additional aliquots of bacteria were lysed in a boiling water bath, and 1 µg of total protein from each was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were transferred to nylon membranes and incubated with rabbit anti-GFP polyclonal antisera (Oncogene, San Diego, Calif.), and bound antibodies were detected by enhanced chemiluminescence (Amersham Pharmacia).

## RESULTS

**Identification of protein-binding sites.** Members of our laboratory previously demonstrated that cultured *B. burgdorferi* produces at least one protein that specifically binds to regions 5' of all tested *erp* genes (8). In those studies, two DNA-protein complexes were observed when labeled DNA fragments were incubated with *B. burgdorferi* cell extracts, suggesting that proteins bound to two locations. To more precisely define the two protein-binding sites, we performed EMSAs with labeled *erpG* 5' DNA, with a variety of unlabeled DNA fragments as competitors for protein binding.

Initial studies used overlapping fragments of the *erpG* gene and its 5' noncoding DNA as competitors. Neither DNA fragment G9-AC, G10-AC, nor G11-AC competed for binding with protein-DNA complex 2, whereas fragments G12-AC through G18-AC all competed away protein binding to site 2 (Fig. 3A). Fragments G9-AC through G13-AC failed to compete for binding with site 1, while fragments G14-AC through G18-AC all competed for protein binding (Fig. 3B). These results indicate that the DNA component of complex 2 includes DNA located up through the 5' end of fragment G12-AC and that of complex 1 includes DNA up through the 5' end of fragment G14-AC (Fig. 1).

DNA fragment G14-AC contained a short sequence of the *erp* 5' noncoding region that was lacking from G13-AC (Fig. 1). EMSAs using a 30-bp DNA fragment with part of this se-

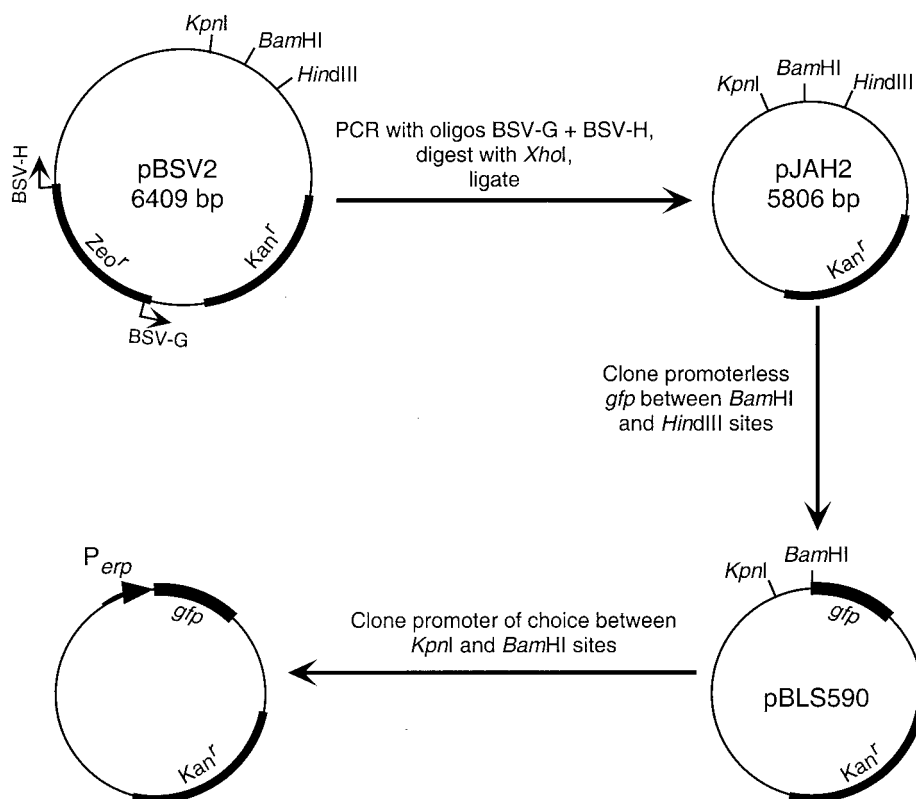


FIG. 2. Construction of *Perp::gfp* fusion plasmids. A nonessential region of the *B. burgdorferi*-*E. coli* shuttle vector pBSV2 was removed to produce pJAH2. The mutant 3 allele of *gfp* was then cloned into pJAH2 to produce pBLS590. To the 5' side of *gfp* are *KpnI* and *BamHI* sites that facilitate the easy cloning of any promoter element, with the GG bases of the *BamHI* site oriented to comprise the 3' end of any inserted ribosome binding site (consensus sequence, 5'-GGAGG-3').

quence as a competitor, called competitor 164, indicated efficient competition for protein binding with DNA site 1 (Fig. 3C). It was noted that this DNA sequence included a short inverted repeat, TTGCAA (Fig. 1). Competitor 164N, an 18-bp DNA segment centered on this inverted repeat, also competed with DNA site 1 for protein binding (Fig. 3C). This DNA sequence is almost completely conserved in all *erp* operon 5' regions (Fig. 1). Control studies showed that segments of DNA encompassing site 2 did not compete for protein binding to site 1, indicating that different proteins bind each site (Fig. 3C and data not shown).

The boundaries of binding site 2 proved to be much larger than those of site 1. Competition was observed only when the competitor DNA included the 59-bp segment found between nucleotides -31 and -89 (Fig. 3D). This region overlaps the putative -35 sequence of the promoter and is extremely well conserved in all *erp* operons (Fig. 1).

**Relative abundance of *erp* operator-binding proteins.** We hypothesized that at least one of the proteins binding to the *erp* 5' noncoding DNA influences *erp* transcriptional activity. If so, it was expected that the relative abundance of such a regulatory protein would be proportional to the abundance of Erp proteins. As noted above, Erp proteins are expressed at higher levels by bacteria cultured at 34°C than by those cultured at 23°C. EMSAs using various amounts of protein extract from *B. burgdorferi* indicated that bacteria grown at 23°C contained several times more of the protein that binds site 2 than did

bacteria cultured at 34°C (Fig. 4). The inverse relationship between the levels of DNA-binding protein and of Erp proteins suggests that the protein that binds site 2 may function as a repressor. No detectable differences in the level of the site 1 binding protein were observed (data not shown).

**Promoter-reporter fusion analyses of B31 *erp* operons.** To characterize the functions of the protein-binding sites for *erp* expression, we created a series of transcriptional fusions between *erp* 5' noncoding DNAs and *gfp* (Fig. 2; Table 2). Each plasmid was introduced into *B. burgdorferi* strain B31-e2, a subculture of type strain B31 that we were able to transform at a very high efficiency (data not shown). Importantly, B31-e2 bacteria demonstrate a temperature-dependent differential expression of Erp synthesis in patterns that are indistinguishable from those of low-passage-number, infectious bacteria (data not shown). Therefore, B31-e2 contains the mechanisms necessary to sense changes in environmental temperature and to control *erp* expression, and it is a suitable strain for the elucidation of those regulatory pathways.

Two concerns needed to be addressed prior to the use of pBSV2-derived plasmids for the analysis of *B. burgdorferi* promoter elements, namely the existence of other promoters in pBSV2 that might influence reporter gene expression and the genetic stability of heterologous DNA in *B. burgdorferi*. Due to the manner in which the parental pBSV2 plasmid was constructed, a putative *B. burgdorferi* promoter, that of *bppA* (paralog family 165), is located 5' of *gfp* in pBLS590 (12, 19, 51).

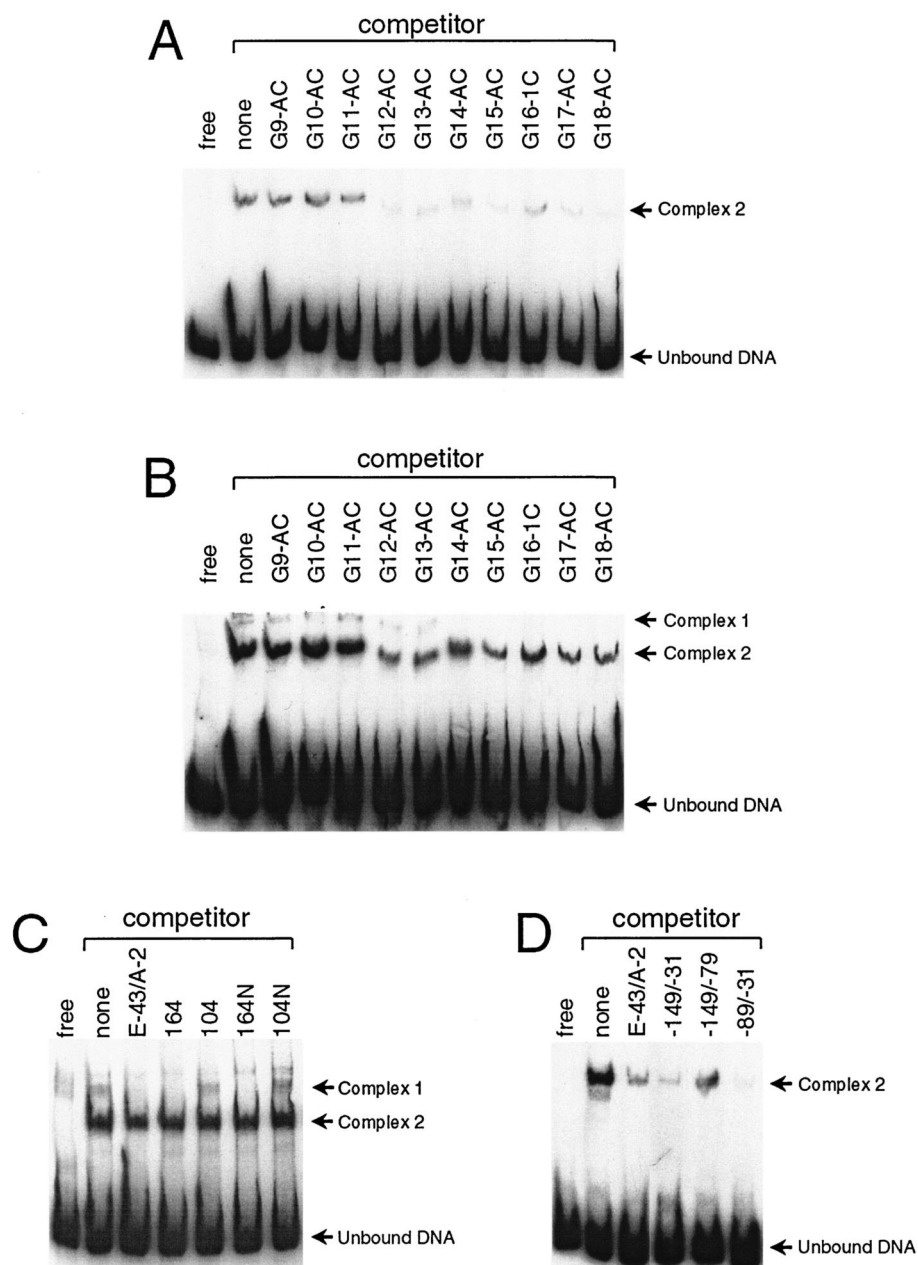


FIG. 3. EMSAs identifying the two protein-binding sites of *erp* operator DNAs. All assays used protein extracts of *B. burgdorferi* cultured at 34°C. Experimental data shown in panels A, B, and C were obtained with a labeled probe extending from +18 to -330, while those for panel D were obtained with a probe spanning -31 to -89. For all panels, lanes contained either free DNA or DNA, poly(dI-dC), protein extract, and the DNA competitor noted above the lane. (A and B) Short and long exposures of the same EMSA indicating DNA competition of protein binding to site 2 (A) and site 1 (B). (C) Identification of maximal boundary of site 1. (D) Identification of maximal boundary of site 2. As previously observed (8), complex 1 was less abundant than complex 2, and longer exposure times were required to detect complex 1 on X-ray film. As a result, signals from complex 2 often saturated films that were used to detect complex 1.

Derivatives of pBSV2 also contain an *E. coli lac* promoter that directs transcription towards the site in which the *gfp* gene of pBLS590 was inserted (51). However, *B. burgdorferi* strain KS10, which carries pBLS590 (Table 1), did not express GFP at levels that were detectable by either flow analysis or immunoblotting (Fig. 5 and data not shown). In addition, GFP expression was not detected for strain KS23, which carries control plasmid pBLS603, which has a consensus ribosome binding

site located 5' of *gfp*. We conclude that transcripts arising from the *bppA* promoter of pBLS590 and its derivatives terminate prior to reaching the *gfp* gene or that this promoter is inactive in these plasmids. These negative results also demonstrated that the *E. coli lac* promoter does not function in *B. burgdorferi*. During the course of this work, Carroll and coworkers reached the same conclusions from studies with similar pBSV2-derived promoter-*gfp* fusion plasmids (11). We addressed our second

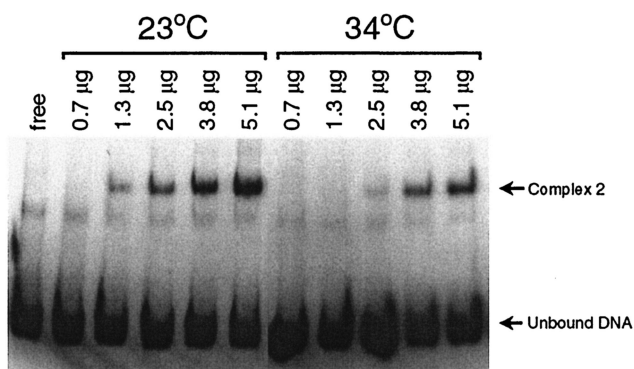


FIG. 4. Determination of relative abundance of the site 2 binding protein. Cell extracts from *B. burgdorferi* cultured at either 23 or 34°C were assayed by EMSA using labeled *erpG* promoter DNA. The total amounts of protein extract incubated with the DNA are indicated above each lane.

concern by reisolating and sequencing recombinant plasmids from several transformed *B. burgdorferi* strains. No differences were detected, indicating that heterologous DNA maintained by *B. burgdorferi* is genetically stable.

All *erp* loci are flanked at the 5' end by a divergently transcribed *bppC* gene, with the two genes being separated by approximately 430 bp of DNA (19, 49). Since the DNA sequences required for *erp* expression were unknown prior to this study, the entire sequences between the start codons of the B31 *erpAB* and *erpG* operons and their adjacent *bppC* genes were cloned to produce transcriptional fusions with *gfp* (Table 1; Fig. 1). The resultant plasmids, pBLS591 and pBLS592, were introduced into B31-e2 to produce strains KS11 and KS12, respectively (Table 1). Studies were then undertaken to deter-

mine whether the *Perp::gfp* fusion constructs exhibited temperature-dependent differential expression. KS11 and KS12 were cultured to mid-exponential phase at either 23 or 34°C, and GFP expression levels were examined by both flow cytometry and immunoblotting (Fig. 5 and data not shown). Expression of GFP was observed for both strains KS11 and KS12, with both strains producing higher levels of GFP when grown at 34°C than when cultivated at the lower temperature. These data indicate the regulation of *Erp* proteins at the level of transcriptional initiation and are consistent with earlier studies of *erp* genes and their encoded proteins (8, 24, 44, 48). Furthermore, these studies demonstrate that the temperature-dependent differential expression of *erp* genes is due to DNA elements located in the *bppC-erp* intergenic region.

To investigate the roles of 5' noncoding DNA sequences, we next constructed promoter-*gfp* fusions with deletions distal to the start of transcription. Plasmids pEAP1 and pCJB1 contain DNAs from bp -215 through the start of transcription of *PerpAB* and *PerpG*, respectively, and include both protein-binding sites (Table 1; Fig. 1). Plasmids pEAP2 and pCJB2 contain DNAs from bp -93 to the start of transcription of *PerpAB* and *PerpG*, respectively, and contain only the second protein-binding site. pBLS599 contains only the RNA polymerase contact sites of *PerpAB* and thus lacks both protein-binding sites. The deletion of protein-binding site 1 from either 5' DNA element had no detectable effect on GFP expression levels (Fig. 6), indicating that site 1 does not by itself greatly affect *erp* transcription in cultured bacteria. However, the deletion of both sites 1 and 2 resulted in elevated GFP expression, indicating that site 2 is involved in the repression of *erp* transcription.

Intriguingly, strain KS11 produced significantly higher levels

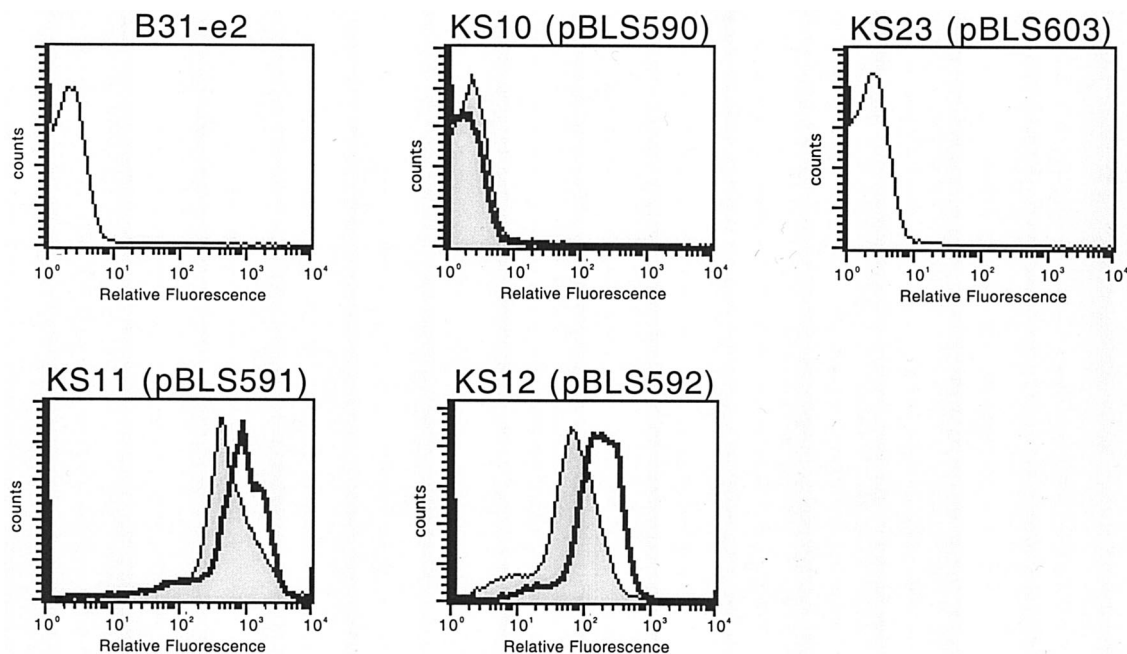


FIG. 5. Representative GFP expression by untransformed *B. burgdorferi* B31-e2 or bacteria carrying a promoterless *gfp* (pBLS590), *gfp* with a 5' ribosome-binding site only (pBLS603), *PerpAB::gfp* (pBLS591), or *PerpG::gfp* (pBLS592). Bacteria were either grown to mid-exponential phase at a constant 23°C (shaded) or were shifted from 23 to 34°C after reaching mid-exponential phase (unshaded).

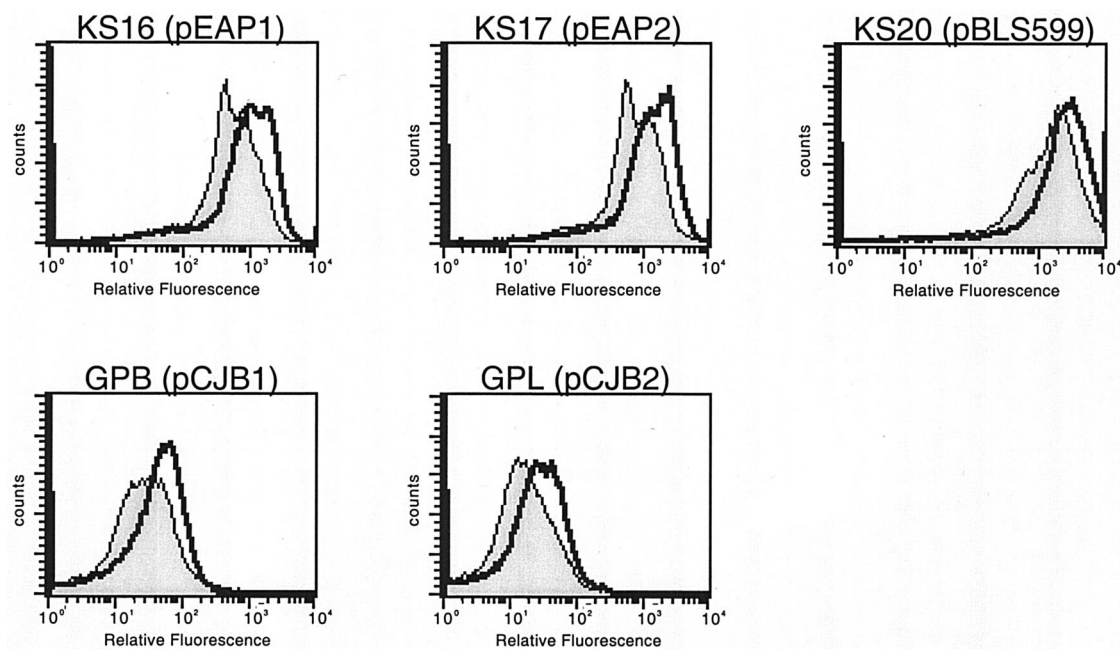


FIG. 6. Representative analyses of *B. burgdorferi* carrying deletions in the operators of *PerpAB::gfp* and *PerpG::gfp*. Bacteria were either grown to mid-exponential phase at a constant 23°C (shaded) or were shifted from 23 to 34°C after reaching mid-exponential phase (unshaded).

of GFP than did KS12, indicating that the *erpAB* promoter is stronger than that of *erpG*. Noting that these two promoters contain different  $-10$  sequences, we produced three chimeric promoter-*gfp* fusions (Fig. 7). The promoter element of pBLS593 is identical to that of *erpAB* but contains the sequence of *erpG* 3' of the  $-10$  site. pBLS594 contains the 5' element of *erpG* but has the  $-10$  site and 3' DNA of *erpAB*. pBLS598 contains the 5' DNA of *erpAB* and the  $-10$  site and 3' DNA of *erpG*. Strain B31-e2 was transformed with pBLS593, pBLS594, or pBLS598 to produce strain KS13, KS15, or KS19, respectively. The three chimeras exhibited GFP expression levels intermediate to those of the wild-type promoters (Fig. 7).

**Analyses of *erp* operons from other strains.** Characterizations of *B. burgdorferi* strains other than B31 have largely found identical temperature-dependent expression patterns of *erp* genes and proteins (1, 4, 23, 24, 28). However, some studies failed to detect expression of other strains' Erp proteins, leading to suggestions that a few *erp* operons are regulated through mechanisms that are different from that of most *erp* genes (1, 3, 15, 24, 52, 53). We therefore examined the promoter elements of those *erp* genes, using promoter-*gfp* fusion constructs.

Strain 297 encodes an Erp protein named Bbk2.10 (3). An initial characterization of cultured 297 bacteria did not detect Bbk2.10 by immunoblotting, and its mRNA was not detected by Northern blotting (1, 3). Subsequent reverse transcription-

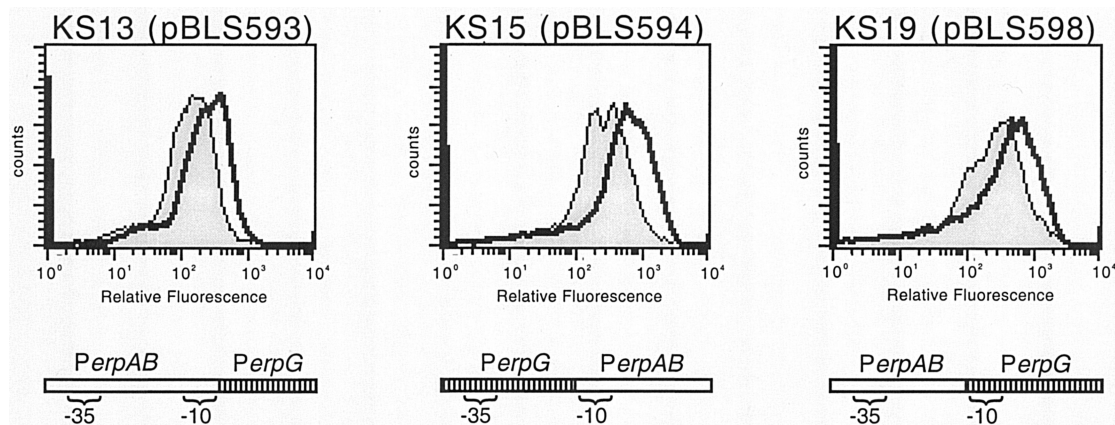


FIG. 7. Representative analyses of GFP expression by *B. burgdorferi* carrying chimeric *PerpAB/PerpG::gfp* transcriptional fusions. Bacteria were either grown to mid-exponential phase at a constant 23°C (shaded) or were shifted from 23 to 34°C after reaching mid-exponential phase (unshaded). Diagrams below each panel illustrate regions of the two *erp* promoter/operator regions contained in each construct.



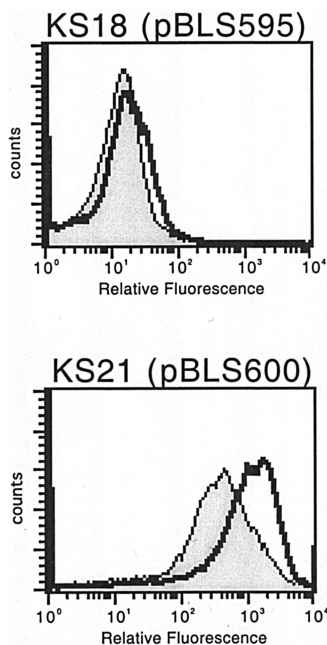


FIG. 8. Representative GFP analyses of *B. burgdorferi* carrying *Pbbk2.10::gfp* (pBLS595) or *Pp21erp22::gfp* (pBLS600). Bacteria were either grown to mid-exponential phase at a constant 23°C (shaded) or were shifted from 23 to 34°C after reaching mid-exponential phase (unshaded).

PCR (RT-PCR) analysis identified the *bbk2.10* transcript in cultured bacteria (24). We constructed a *Pbbk2.10::gfp* transcriptional fusion plasmid, pBLS595, and introduced it into B31-e2 to create strain KS18. GFP expression analysis of KS18 confirmed that *Pbbk2.10* is indeed functional in cultured bacteria, although it is a weak transcriptional promoter that yields very little protein product (Fig. 8).

Two *erp* operons, *ospEF* and *p21erp22*, of strain N40 have been well characterized (7, 15, 31, 37, 45, 47, 48, 52). *ospEF* mRNA and both OspE and OspF proteins have been detected in cultured organisms, and they exhibit a temperature-dependent expression pattern identical to that of other *erp* operons (15, 31, 37, 48). However, two studies failed to detect the *p21* transcript in cultured bacteria (15, 52), although *erp22* mRNA has been detected by RT-PCR (7) (referred to as “*erpD*” in that report). Plasmid pBLS600 was constructed to contain *Pp21erp22* fused with *gfp* and was introduced into B31-e2 to produce strain KS21. Flow cytometry indicated that *Pp21erp22* is a very active promoter in cultured bacteria and that it exhibits temperature-dependent differential expression similar to that from other *erp* promoters (Fig. 8).

## DISCUSSION

*B. burgdorferi* regulates Erp protein synthesis during the natural infectious cycle, producing these proteins during transmission between ticks and warm-blooded hosts but repressing their synthesis during persistent infection of the tick midgut (22, 23, 36, 37). Previous studies found that this regulation occurs at the mRNA level (24, 44). We have now demonstrated that *B. burgdorferi* proteins specifically bind to two DNA regions 5' of the *erp* transcription start site. Since there are no

obvious similarities between the two protein-binding sites and since neither DNA competed with the other for protein binding, we concluded that different proteins bind to each. The nature of the site 1 binding protein cannot yet be postulated, since the deletion of site 1 alone did not have appreciable effects on transcription in cultured bacteria. The deletion of site 2 resulted in elevated levels of transcription, indicating that the protein which binds that site is a transcriptional repressor. Also supporting that conclusion is the observation that bacteria cultured under conditions that repress Erp expression (i.e., 23°C) contained elevated levels of the site 2 binding protein. Since site 2 is immediately adjacent to and possibly overlaps the  $-35$  site of the *erp* promoter, it is highly probable that a repressor binding to site 2 physically inhibits interactions between RNA polymerase and the promoter DNA. Studies are currently under way in our laboratory to identify the proteins that bind *erp* operator DNA, with the intention of elucidating their roles in the regulation of *erp* expression, and ultimately, how *B. burgdorferi* controls their functions.

The present study indicated that different *erp* promoter elements differ in transcriptional activity. Both the B31 *erpAB* and the N40 *p21erp22* promoter/operator regions directed relatively high levels of transcription, while *PerpG* was a less active promoter and *Pbbk2.10* was weaker still. The DNA sequences of both sites 1 and 2 are nearly identical for all four of these operons, and both *PerpAB* and *PerpG* compete with each other for protein binding (8). However, it is not impossible that the few nucleotide differences between them change their affinities for the DNA-binding proteins and thereby alter promoter strengths. The four examined operons also differ in the sequences of their  $-10$  and  $-35$  sites, which may affect the binding of RNA polymerase and the formation of open complexes. *B. burgdorferi* encodes only three sigma factors, but it is currently unknown which one is used for *erp* transcription. While the consensus promoter sequence has not yet been determined for any *B. burgdorferi* RNA polymerase holoenzyme, the differences in promoter strength observed in our studies suggest that *PerpAB* and *Pp21erp22* are closer to the consensus than are *PerpG* or *Pbbk2.10*. An exchange of the  $-10$  sites of *PerpAB* and *PerpG* did not result in the reversal of their relative strengths, so other DNA sequences must also be responsible for the differences in their activities. Clearly, additional studies will be required to conclusively determine the roles of changes in the promoter and operator sequences in the different transcriptional activities of various *erp* operons.

It was previously noted that *B. burgdorferi* cultured at either 23 or 34°C synthesizes proteins that bind to *erp* promoter DNAs (8). Because at least one of the DNA-binding proteins probably functions as a transcriptional repressor, it appears that bacteria cultured at 34°C do not fully derepress *erp* expression. Erp proteins are expressed at significantly higher levels during mammalian infections than during cultivation at any temperature (1, 15, 24), suggesting that *B. burgdorferi* produces far fewer of the repressor proteins during mammalian infections than in culture. *B. burgdorferi* cells grown in artificial culture medium are apparently exposed to a confusing variety of external signals, since they produce both proteins that are generally synthesized during mammalian infections and others that are specific for tick infections. We propose that the culture medium provides a cue(s) to *B. burgdorferi* that causes it to

produce the repressor protein that binds site 2, resulting in the suboptimal expression of *erp* operons. In addition to being affected by temperature, the expression of Erp proteins is affected by unknown chemicals and by autoinducer 2 quorum sensing (1, 8, 24, 41), any of which may be involved in the inhibition of Erp synthesis during cultivation.

The observations that a repressor of *erp* transcription is produced during cultivation and that *erp* promoters vary significantly in strength may explain why certain Erp proteins have not been detected in cultured *B. burgdorferi* cells. The strain 297 *Pbbk2.10* is relatively very weak, being such a poor promoter that mRNAs from cultured bacteria can only be detected by the sensitive technique of RT-PCR (3, 24). It is quite possible that this low level of transcription results in the production of very little Bbk2.10 protein, so little that the protein could not be detected by immunoblotting or an immunofluorescence assay with the previously used antibody (1, 3, 23, 24). An initial characterization of the pG Erp protein of strain ZS7 also failed to detect that protein by immunoblotting of cell lysates, and pG mRNA could not be detected by Northern blotting either (53). However, pG is absolutely identical to *erpG* of strain B31, with both operons containing identical 5' and 3' flanking sequences (49, 53). Furthermore, it has been demonstrated in this work and elsewhere that *PerpG* is active during cultivation (17, 19, 44) and that ZS7 exhibits temperature-dependent expression of other *erp* operons (27, 28). Since the use of low-titer antisera can cause one to miss the detection of proteins (19), it is very likely that previous inability to detect the 297 Bbk2.10 and ZS7 pG proteins was due to the use of antibody preparations that were not of sufficiently high titers to detect the products of these poorly expressed genes.

It is not clear why previous studies failed to detect the production of strain N40 *p21* mRNA in cultured bacteria (7, 15, 52). The present study demonstrated that *Pp21erp22* is comparatively strong, being similar to B31 *PerpAB*. Consistent with this observation, mRNA for the second gene in the operon, *erp22* (previously referred to as N40 *erpD*), has been detected by RT-PCR of cultured organisms (7). Either bacteria must process *p21erp22* mRNA to rapidly degrade the first gene but retain the second or the RT-PCR oligonucleotides and/or reaction conditions used in earlier studies were suboptimal for detection of the *p21* message. Studies are currently on-going in our laboratory to identify which of these two possibilities is correct.

All examined Lyme disease spirochete isolates carry multiple, different *erp* loci (2, 13, 47, 50). Comparisons of *erp* promoter/operator regions have indicated extensive conservation, including protein-binding sites 1 and 2 (Fig. 1) (2, 3, 13, 32, 44, 47, 49). Furthermore, protein binding to one *erp* 5' noncoding region can be effectively competed away with the homologous region of another *erp* operon (this work; also see reference 8). These observations strongly suggest that when a bacterium produces the proteins that bind *erp* operator sites 1 and 2, those proteins bind to each of the bacterium's many *erp* loci. Thus, a bacterium will simultaneously regulate the expression of its entire repertoire of *erp* genes. Studies using double-labeling immunofluorescence analysis indicated that cells of strain B31 behaved exactly as predicted: all Erp proteins were coexpressed (19). While similar studies of strain 297 failed to detect the simultaneous coexpression of every Erp protein (23,

24), those results were quite likely due to an inability to detect weakly expressed proteins, as discussed above. However, it has been proposed that *B. burgdorferi* sequentially expresses different *erp* operons during an infection, requiring that the cells repress the transcription of some *erp* loci while activating the expression of others (33). That hypothesis was based on a study that observed the production of antibodies directed against some Erp proteins early in infection, while antibodies against other proteins were not detected until a few weeks later (33). Other studies have demonstrated that all examined Erp proteins are produced by *B. burgdorferi* at the time of transmission from a tick to a mammal but that the immune system responds to those proteins sometimes differ in their intensities (23, 24, 36). Those data, together with the results of the present study, make it highly probable that antibodies directed against Erp proteins are sometimes detected at different times of infection because promoter variations cause differences in Erp expression levels, rather than because some *erp* operons are expressed while other loci are completely silenced.

Both this and another study (11) observed that the *E. coli lac* promoter carried by pBSV2 and its derivatives is not functional in *B. burgdorferi*. Several other promoters that function in *E. coli* are also nonfunctional in the Lyme disease spirochete, to the end that most antibiotic resistance genes used as selectable markers of transformation in *B. burgdorferi* had to be constructed by the placement of a strong spirochetal promoter 5' of a resistance gene (10, 20, 21, 43). As noted above, none of the promoter consensus sequences for any *B. burgdorferi* RNA polymerase-sigma holoenzyme have been determined, but it certainly appears that the preferred sequences differ dramatically from those of *E. coli*. This highlights the importance of examining the expression of *B. burgdorferi* promoter elements in that bacterium only, rather than using unrelated organisms, such as *E. coli*, as proxies.

In conclusion, we have characterized the promoter/operator sequences of *erp* gene family members. At least two different proteins bind to DNA sites immediately 5' of *erp* promoters, one of which functions as a transcriptional repressor. Various *erp* promoters exhibit different relative promoter activities, which may account for the reported inability to detect certain Erp proteins in cultured bacteria as well as for differences in Erp-directed antibody titers during mammalian infections. The variations in promoter strengths appeared to be due to differences in the sequences of *erp* promoters and operators. These data strengthen our hypotheses that all *erp* operons are regulated through a common mechanism and that individual bacteria express their entire repertoire of Erp proteins simultaneously. Many Erp proteins have been demonstrated to bind host factor H, which probably helps protect bacteria from killing by the host innate immune system. Therefore, elucidating the regulatory mechanisms controlling Erp synthesis will be important for understanding the pathogenic properties of *B. burgdorferi* as well as for directing the development of novel antibacterial agents.

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