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lacZ Reporter System for Use in *Borrelia burgdorferi*▽†‡

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Regulation of gene expression is critical for the ability of *Borrelia burgdorferi* to adapt to different environments during its natural infectious cycle. Reporter genes have been used successfully to study gene regulation in multiple organisms. We have introduced a *lacZ* gene into *B. burgdorferi*, and we show that *B. burgdorferi* produces a protein with detectable β-galactosidase activity in both liquid and solid media when *lacZ* is expressed from a constitutive promoter. Furthermore, when *lacZ* is expressed from the *ospC* promoter, β-galactosidase activity is detected only in *B. burgdorferi* clones that express *ospC*, and it accurately monitors endogenous gene expression. The addition of *lacZ* to the repertoire of genetic tools available for use in *B. burgdorferi* should contribute to a better understanding of how *B. burgdorferi* gene expression is regulated during the infectious cycle.

Borrelia burgdorferi sensu lato, the pathogen that causes Lyme disease (7), alternates between two distinct environments, an arthropod vector and a vertebrate host. As *B. burgdorferi* moves from one milieu to the other, its ability to adapt and survive requires dramatic changes in gene expression. Many studies have shown that different *B. burgdorferi* gene products are upregulated or downregulated at specific times during the infectious cycle (19, 31) and in response to host and environmental signals (6, 8a, 15, 24, 25). Although it is clear that *B. burgdorferi* alters gene expression to adapt to different environments, the genetic tools for studying gene regulation in *B. burgdorferi* are limited.

Within the last 2 decades, the complete genomic sequence of *B. burgdorferi* strain B31 was published (10, 14) and techniques for basic genetic manipulation of *B. burgdorferi* became available (5, 11, 13, 27–29, 36). A chloramphenicol acetyltransferase (CAT) gene was the first reporter gene that was fused to *B. burgdorferi* promoters for analysis of promoter strength (33). The development of luciferase (4) and multiple fluorescent proteins (9, 11, 30) as reporter systems in *B. burgdorferi* followed. Although these systems have value, there are limitations with each. β-Galactosidase, encoded by *lacZ*, has been used extensively as a convenient reporter gene in *Escherichia coli* and is still applicable to a broad range of organisms, both prokaryotic and eukaryotic, but has not yet been used with *B. burgdorferi*. β-Galactosidase activity can be monitored easily and quickly by simple colorimetric assays in both liquid and solid media, neither of which require expensive or specialized equipment. Additionally, a wide variety of substrates for β-galactosidase allow for different levels of sensitivity in either *in vitro* or *in vivo* detection formats (17). Having *lacZ* available as a genetic tool for *B. burgdorferi* would enhance investigation of the complex regulatory events that are integral to the spirochete's infectious cycle. To this end, we developed *lacZ* as a reporter gene in *B. burgdorferi* and demonstrated its utility.

MATERIALS AND METHODS

All chemicals and materials were purchased from Sigma (St. Louis, MO) unless otherwise specified. Plasmids are listed in Table 1. Oligonucleotides are listed in Table 2.

Bacterial strains and growth conditions. Plasmids for cloning were transformed into electrocompetent or chemically competent Top10 *E. coli* (Invitrogen, Carlsbad, CA), and final constructs were transformed into Δ*lac E. coli* strain MC4100 *lamB-zjb*::Tn*10* (kindly provided by John Carlson, Rocky Mountain Laboratories, Hamilton, MT). *E. coli* cells were plated on LB agar with the appropriate antibiotic (spectinomycin at 300 μg/ml, kanamycin at 30 μg/ml, or gentamicin at 5 μg/ml). Liquid cultures were grown in LB broth supplemented with the appropriate antibiotic (spectinomycin or kanamycin at 100 μg/ml or gentamicin at 10 μg/ml).

BSK II medium (2) for culture of *B. burgdorferi* was made with CMRL 1060 lacking phenol red (US Biological, Swampscott, MA). *B. burgdorferi* strain B31 clones A34 (16) and B312 (26) were grown to mid-log phase (~5×10^7cells/ml). Spirochetes were enumerated using dark-field microscopy and a Petroff-Hausser counting chamber and prepared for electroporation as described previously (27). Transformed B31-A34 and B312 cells were selected using gentamicin at a concentration of 40 μg/ml. All *B. burgdorferi* cultures were grown at 35°C, and plates were incubated under 2.5% CO_2.

Plasmid construction. The *E. coli lacZ* gene ($lacZ_{Ec}$), the multiple cloning site (MCS), and the transcriptional terminator were amplified from pPBMB101 (8) using primers 1 and 2 (Table 2). The PCR product was cloned into the Gateway entrance vector pCR8/GW/TOPO (Invitrogen) and confirmed by direct sequencing with internal primers (Table 2, primers 1 through 10). pBSV2G_dvB2, kindly provided by James A. Carroll (Rocky Mountain Laboratories, Hamilton, MT), is an altered form of the *B. burgdorferi* shuttle vector pBSV2G, which has *attR1* and *attR2* sequences surrounding a chloramphenicol resistance cassette and the counterselectable gene *ccdB*, making it a suitable destination vector for Gateway cloning. The insert containing $lacZ_{Ec}$, the MCS, and the transcriptional terminator was transferred from pCR8 to pBSV2G_dvB2 by using Clonase II enzyme (Invitrogen), creating pBH-lacZEc (Fig. 1A).

The *flaB* promoter was amplified from *B. burgdorferi* genomic DNA using primers 11 and 12 (Table 2). The PCR fragment was cloned into pCR2.1 (Invitrogen) and sequenced to confirm the insert. The *flaB* promoter was excised with BamHI and XhoI and ligated into appropriately digested pBH-lacZEc, creating pBHflaBp-lacZEc (Fig. 1A). BamHI and XhoI were the enzymes used

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TABLE 1. Plasmids used in the study

Plasmid	Resistance[a]	Description (source or reference)
pPBMB101	Kan^r	Plasmid with an *E. coli lacZ* gene, used as template to amplify $lacZ_{Ec}$ (8)
pCR8/GW/TOPO	Spec^r	Gateway PCR entry vector (Invitrogen)
pCR2.1/TOPO	Kan^r, Amp^r	PCR cloning vector (Invitrogen)
pBSV2G	Gent^r	*B. burgdorferi* shuttle vector (13)
pBSV2G_dvB2	Gent^r	Altered pBSV2G, Gateway destination vector (J. A. Carroll, unpublished)
pBH-lacZEc	Gent^r	*E. coli* $lacZ_{Ec}$ cloned into pBSV2G_dvB2 (this work)
pBHflaBp-lacZEc	Gent^r	pBH-lacZEc with the *flaB* promoter cloned upstream of $lacZ_{Ec}$ (this work)
pBH-lacZBb	Gent^r	*B. burgdorferi* $lacZ_{Bb}$ cloned into pBSV2G (this work)
pBHflaBp-lacZBb	Gent^r	pBH-lacZBb with the *flaB* promoter cloned upstream of $lacZ_{Bb}$ (this work)
pBHospCp-lacZBb	Gent^r	pBH-lacZBb with the *ospC* promoter cloned upstream of $lacZ_{Bb}$ (this work)

[a] Kan, kanamycin; Spec, spectinomycin; Amp, ampicillin; Gent, gentamicin.

for creating promoter-*lacZ* fusions in pPBMB101, which has a ribosome binding site (RBS) between the XhoI site and the *lacZ* start codon (8).

pBH-lacZEc and pBHflaBp-lacZEc were transformed into electrocompetent *B. burgdorferi* cells (27). Spirochetes were allowed to recover overnight in BSK II medium without antibiotic selection and then plated in solid BSK medium supplemented with gentamicin. Individual colonies were screened by PCR for $lacZ_{Ec}$ and the gentamicin cassette.

***lacZ* optimization.** *E. coli lacZ* codon usage in *B. burgdorferi* was analyzed using the Graphical Codon Usage Analyser version 2.0 (www.gcua.schoedl.de). More than one-third of the codons in the *E. coli lacZ* gene, $lacZ_{Ec}$, are considered rare (used less than 20% of the time) in *B. burgdorferi.* A codon-optimized version of *lacZ*, $lacZ_{Bb}$, which uses less than 1% of *B. burgdorferi* rare codons, was synthesized by GenScipt Corporation (Piscataway, NJ). A sequence alignment of the codon-optimized $lacZ_{Bb}$ gene with the original $lacZ_{Ec}$ gene is provided in Fig. S1 of the supplemental material. The synthesized gene was preceded by the same MCS and RBS as the original $lacZ_{Ec}$. $lacZ_{Bb}$ (nucleotide sequence available upon request) was cloned into pBSV2G (13) with XbaI and KpnI, producing pBH-lacZBb (Fig. 1B). The *flaB* promoter was added as described above to create pBHflaBp-lacZBb (Fig. 1B). The intergenic region between *guaA* and *ospC*, which includes the *ospC* promoter and regulatory operator sequences up to the *ospC* start codon (12, 20, 21, 37–39), was amplified from *B. burgdorferi* genomic DNA with primers 13 and 14 (Table 2), cloned into pCR2.1, sequenced, and digested with BamHI and XhoI for ligation into pBH-lacZBb, creating pBHospCp-lacZBb. Constructs were transformed into electrocompetent *B. burgdorferi* (27) and Δ*lac E. coli* cells.

Screening for β-galactosidase activity. For β-galactosidase assays, aliquots of 1 ml of overnight *E. coli* cultures and 5 to 10 ml of mid-log *B. burgdorferi* cultures were washed twice in HEPES-NaCl (HN) buffer (50 mM each; pH 7.6). Bacterial pellets were resuspended in Z-buffer (36 mM NaH_2PO_4, 67 mM $NaHPO_4$, 0.1 mM $MgCl_2$, 2 mM $MgSO_4$, 2.7 ml/liter β-mercaptoethanol) at approximately 5×10^8 bacteria/ml before lysis with chloroform and SDS. β-Galactosidase assays were performed using a modified version of the Miller protocol (22). Briefly, aliquots (10 to 20 μl) of bacterial lysates were added to 96-well plates (Costar, Corning, NY) in triplicate. Z-buffer was added, increasing the volume to 160 μl. Fifty microliters of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) dissolved in Z-buffer (4 mg/ml) was added, and the plate was incubated at room temperature for 10 to 15 min. After incubation, 90 μl of stop buffer (1 M Na_2CO_3) was added, and the absorbance was measured at 405 nm (Labsystems Multiskan Plus; Fisher Scientific, Pittsburgh, PA). β-Galactosidase activity units, or Miller units (nmoles/minute), were calculated as described before and reported as units per mg of protein (23). Background activity (units/mg of protein) of bacteria lacking a shuttle vector was subtracted from reported values.

Both *E. coli* and *B. burgdorferi* were grown on plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; Roche Diagnostics, Indianapo-

TABLE 2. Primers used in the study

Primer no.[a]	Name	Sequence (5′–3′)[b]
1	Long lacZ 5′ (NotI)	AAGGAAAAAA**GCGGCCGC**TCTAGAGGATCCCGGGTCGACTAGTCTGCAGCTCGAGT
2	Long lacZ 3′ (NotI)	AAGGAAAAAA**GCGGCCGC**AGATCTTACTCAGGAGAGCGTTCACCGACAAACAACAG
3	lacZ 430	TGATGAAAGCTGGCTACAGGAAGG
4	lacZ 908	GTCACACTACGTCTGAACGTCGAA
5	lacZ 1525	GGCCACCGATATTATTTGCCCGAT
6	lacZ 2053	GGATGTCGCTCCACAAGGTAAACA
7	lacZ 2593	TATCAGCCGGAAAACCTACCGGAT
8	lacZ 3121	GCTGTTTTGGCGGATGAGAGAAGA
9	lacZ 1533 5′	ATATTATTTGCCCGATGTACGCGCGCGTGGATGAA
10	lacZ 1538 3′	TTCATCCACGCGCGCGTACATCGGGCAAAT
11	flaB promoter 5′ (BamHI)	CCCGC**GGATCC**CTGTCGCCTCTTGTGGCTTC
12	flaB promoter 3′ (XhoI)	CCCCG**CTCGAG**CATATATCATTCCTCCATGA
13	ospC promoter 5′ (BamHI)	CCCGC**GGATCC**AATTAAAACTTTTTTTATTAAAGTA
14	ospC promoter 3′ (XhoI)	CCCCG**CTCGAG**TAATTTGTGCCTCCTTTTTATTTAT
15	flaB forward	TCTTTTCTCTGGTGAGGGAGCT
16	flaB reverse	TCCTTCCTGTTGAACACCCTCT
17	flaB probe	AAACTGCTCAGGCTGCACCGGTTC
18	lacZBb forward	TTCTCTTGGAGGATTTGCTAAAT
19	lacZBb reverse	ATCCCAAACAAATCCACCTT
20	lacZBb probe	TGGCAAGCATTCAGACAATATCCAAG
21	ospC forward	ACGGATTCTAATGCGGTTTTACTT
22	ospC reverse	CAATAGCTTTAGCAGCAATTTCATCT
23	ospC probe	CTGTGAAAGAGGTTGAAGCGTTGCTGTCAT

[a] Primer no. 15 to 23 are TaqMan primers/probes.
[b] Sequence portions in bold denote recognition sites for restriction enzymes (which are indicated in parentheses after the oligonucleotide name).

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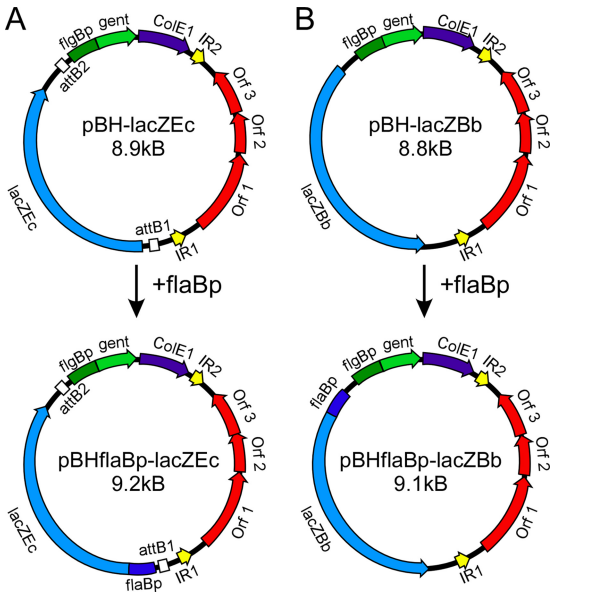

FIG. 1. Schematic diagram of the *lacZ* shuttle vectors. The *E. coli lacZ* gene was cloned into pBSV2G_dvB2, creating pBH-lacZEc (A, top). A *B. burgdorferi* codon-optimized *lacZ* gene, $lacZ_{Bb}$, was cloned directly into pBSV2G, creating pBH-lacZBb (B, top). The *flaB* promoter from *B. burgdorferi* (flaBp) was added to each construct, yielding pBHflaBp-lacZEc (A, bottom) and pBHflaBp-lacZBb (B, bottom), respectively.

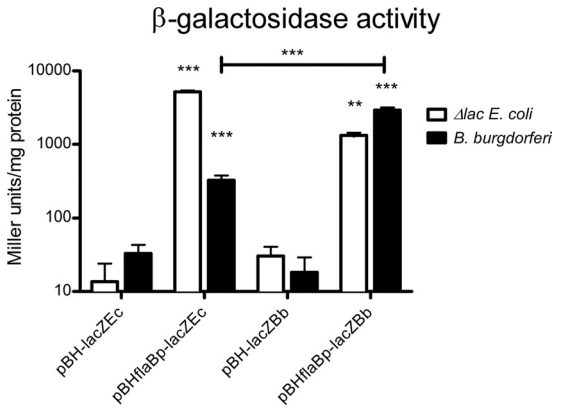

FIG. 2. β-Galactosidase activity in *E. coli* and *B. burgdorferi* lysates. Modified Miller ONPG assays (22) were performed on Δ*lac E. coli* and *B. burgdorferi* harboring pBH-lacZEc, pBHflaBp-lacZEc, pBH-lacZBb, and pBHflaBp-lacZBb. β-Galactosidase activity units (Miller units) are reported per mg of protein, and the levels represent means ± standard errors of the means. Background activity of bacteria without the shuttle vector was subtracted from reported values. ***, $P < 0.001$; **, $P < 0.01$ (compared to respective promoterless constructs or as indicated based on a Student two-tailed *t* test).

lis, IN) at 0.08 g/liter and gentamicin. Alternatively, ~0.5 ml X-Gal dissolved in dimethyl sulfoxide (DMSO; 20 mg/ml) was spread on *B. burgdorferi* plates after colony formation.

Protein assay. The concentration of protein in the bacterial lysates used for β-galactosidase assays was determined using the Bio-Rad protein assay (Hercules, CA) according to the manufacturer's protocol. Dilutions of bovine serum albumin (New England Biolabs [NEB], Ipswich, MA) were used as standards. Aliquots of 10 μl of the standards and 5 to 10 μl bacterial lysates were added to 96-well plates in triplicate. A standard curve was generated and used to determine protein concentrations of the bacterial lysates.

Quantitative RT-PCR. RNA was harvested from ~30 ml of a mid-log *B. burgdorferi* culture using a Masterpure RNA preparation kit (Epicentre Biotechnologies, Madison, WI) according to the manufacturer's protocol. Two to 5 μg of RNA was used as a template to synthesize cDNA using the high-capacity cDNA reverse transcriptase (RT) kit (Applied Biosystems, Life Technologies, Carlsbad, CA). Quantitative-PCR (q-PCR) was performed in triplicate on 100 ng cDNA using the TaqMan Universal PCR Mastermix (Applied Biosystems) and primer-probe combinations for $lacZ_{Bb}$, *flaB*, and *ospC* (Table 2). Real-time q-PCR was performed using the ABI Prism 7900 HT sequence detection system.

RESULTS AND DISCUSSION

***lacZ* gene constructs.** To investigate whether *lacZ* could be used as a simple reporter gene in *B. burgdorferi*, we constructed four shuttle vectors, pBH-lacZEc, pBHflaBp-lacZEc, pBH-lacZBb, and pBHflaBp-lacZBb (see Materials and Methods and Fig. 1). These constructs carry an *E. coli lacZ* gene ($lacZ_{Ec}$) or a *B. burgdorferi* codon-optimized *lacZ* gene ($lacZ_{Bb}$), with and without the constitutive *B. burgdorferi flaB* promoter. The $lacZ_{Ec}$ gene has 56% G+C base content, while the *B. burgdorferi* genome has only 28% G+C content (14). Previous studies have used codon optimization to express foreign genes in *B. burgdorferi* with much success (4, 13). Thus, we designed a synthetic *lacZ* gene, $lacZ_{Bb}$, with reduced G+C content to better reflect the *B. burgdorferi* codon preference in order to enhance β-galactosidase synthesis in *B. burgdorferi*. The nucleotide sequences of $lacZ_{Bb}$ and $lacZ_{Ec}$ are aligned for comparison in Fig. S1 of the supplemental material. The *flaB* promoter was chosen for initial experiments because it is constitutively expressed in *B. burgdorferi* and *E. coli* and has been used to drive expression of antibiotic resistance markers in *B. burgdorferi* (5, 13, 33). We transformed all constructs into electrocompetent *B. burgdorferi* B31-A34 and a Δ*lac E. coli* strain and assessed β-galactosidase activity in liquid and solid media.

β-Galactosidase activity in bacterial cultures. In order to assess the β-galactosidase activity in *B. burgdorferi* and *E. coli* transformants carrying the *lacZ* constructs, we adapted the well-established Miller ONPG assay (22) and normalized β-galactosidase activity to the protein content of the bacterial lysates as described by Nielsen et al. (23). Both *E. coli* and *B. burgdorferi* harboring pBHflaBp-lacZEc or pBHflaBp-lacZBb displayed significant activity compared to bacteria containing the promoterless *lacZ* constructs (Fig. 2). β-Galactosidase activity was significantly higher in *B. burgdorferi* lysates containing pBHflaBp-lacZBb than in lysates harboring pBHflaBp-lacZEc (Fig. 2). While we cannot rule out the possibility that flipping the orientation of *lacZ* in the vector influenced expression, the same plasmids used in *E. coli* gave the opposite pattern (higher activity in bacteria containing pBHflaBp-lacZEc versus pBHflaBp-lacZBb), suggesting that the codon preferences of the respective bacteria produced the discrepancies in activity levels. *B. burgdorferi* harboring either pBHflaBp-lacZBb or pBHflaBp-lacZEc produced detectable β-galactosidase protein when assayed by immunoblotting (data not shown). These results demonstrate that *lacZ* produces a functional β-galactosidase enzyme in *B. burgdorferi* when expressed using the constitutive *flaB* promoter.

β-Galactosidase activity on *B. burgdorferi* plates. Since β-galactosidase activity could be detected in *B. burgdorferi* lysates, we wanted to determine if *lacZ* could be used as a reporter

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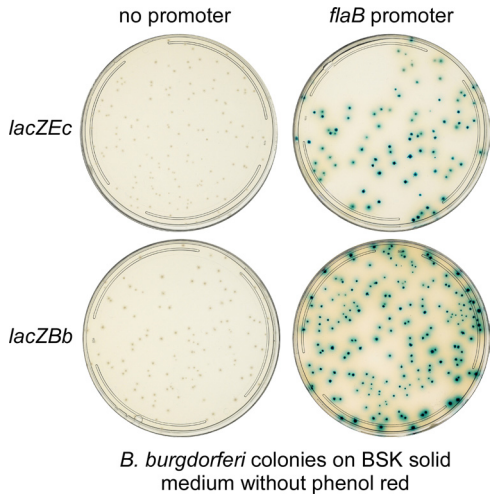

FIG. 3. *lacZ* for blue-white screening of *B. burgdorferi* colonies. BSK solid medium without phenol red was used to grow B31-A34 clones harboring pBH-lacZEc (top left), pBHflaBp-lacZEc (top right), pBH-lacZBb (bottom left), and pBHflaBp-lacZBb (bottom right). Approximately 0.5 ml of X-Gal in DMSO (20 mg/ml) was added to plates after colony formation, and plates were incubated overnight before photographs were taken.

gene for *B. burgdorferi* colonies in solid medium. Typically, addition of X-Gal to solid medium allows for the detection of bacterial colonies that possess an active β-galactosidase enzyme, which cleaves X-Gal into a blue product. Therefore, we used BSK solid medium without phenol red to easily assay for any color change. When X-Gal was included in BSK solid medium (0.08 g/liter), blue color development was observed 2 to 5 days after colony formation (data not shown) for *B. burgdorferi* colonies harboring either pBHflaBp-lacZEc or pBHflaBp-lacZBb. However, the incorporation of X-Gal into the medium considerably slowed colony growth. The production of β-galactosidase was not intrinsically toxic, since colonies that produced β-galactosidase grown without X-Gal were of normal size and morphology. Interestingly, this growth defect seemed to be caused by the cleavage of X-Gal, as X-Gal or solvent incorporation into the medium did not affect colony formation in *B. burgdorferi* lacking a *lacZ* gene. Still, when 2% X-Gal in DMSO was spread on *B. burgdorferi* plates after colony formation, color development in colonies harboring the *lacZ* gene driven by the *flaB* promoter was evident within 15 min and continued to increase overnight as the substrate diffused throughout the plates (Fig. 3). Importantly, colonies without a promoter to drive *lacZ* remained unchanged (white) even after overnight incubation (Fig. 3). Furthermore, live spirochetes were recovered from colonies 48 h after X-Gal treatment, indicating that X-Gal treatment did not hinder bacterial viability. Colonies of *B. burgdorferi* harboring the optimized *lacZ* gene, $lacZ_{Bb}$, when expressed by the *flaB* promoter developed a more uniform and greater color intensity than *B. burgdorferi* containing pBHflaBp-lacZEc, again suggesting that the optimized gene is better suited for *B. burgdorferi* (Fig. 3). These results demonstrated that *lacZ* can be used to monitor gene expression of *B. burgdorferi* colonies on plates. Furthermore, the codon-optimized *B. burgdorferi lacZ* gene ($lacZ_{Bb}$) increased β-galactosidase activity, presumably through increased protein production. Thus, we used only the *B. burgdorferi* optimized *lacZ* gene ($lacZ_{Bb}$) in further studies.

β-Galactosidase activity as a reporter for *ospC* expression. We demonstrated β-galactosidase protein production and activity in *B. burgdorferi* by expressing *lacZ* from a constitutive promoter, but we wished to confirm that *lacZ* expression reflects the expression of regulated genes in *B. burgdorferi* and therefore can be used as a reporter gene for transcriptional activity. For this objective, we used two strains of *B. burgdorferi* that differ in expression of the *ospC* gene: *B. burgdorferi* clones B31-A34 and B312. B31-A34 is derived from noninfectious clone B31-A (18) and does not produce OspC *in vitro*, while B312, a highly attenuated clone of B31 that lacks many plasmids, has been reported to produce abundant amounts of OspC *in vitro* (26). In order to determine *lacZ* expression driven by the *ospC* promoter in these two *B. burgdorferi* genetic backgrounds, we created pBHospCp-lacZBb. We transformed B31-A34 and B312 with pBHospCp-lacZBb and also introduced pBH-lacZBb and pBHflaBp-lacZBb into B312.

While performing these experiments, we noticed substantial variation in OspC synthesis among B312 transformants and between independent outgrowths of the same B312 derivative (data not shown). This observation has not been reported for B312 previously, although earlier studies did report variation in *ospC* expression on an individual cell basis in other *B. burgdorferi* backgrounds (12, 35). In order to use B312, we first confirmed OspC production in B312 transformants with immunoblotting before proceeding with further experiments on those clones (data not shown). Significant β-galactosidase activity was detected in B312 harboring the pBHospCp-lacZBb plasmid compared to clones with the promoterless construct (Fig. 4A). In contrast, β-galactosidase activity was not detected in B31-A34 when the *ospC* promoter was used to express $lacZ_{Bb}$ (Fig. 4A), consistent with the lack of OspC protein production in this strain. Notably, in B312 β-galactosidase activity controlled by the *flaB* promoter was 40-fold higher than activity from the *ospC* promoter (Fig. 4A). Thus, we investigated the transcript levels of *ospC* and $lacZ_{Bb}$ in B312, normalizing the levels to *flaB* transcript levels. Endogenous *ospC* transcript levels in B312 lacking a shuttle vector and B312 transformants were roughly half of *flaB* transcript levels (56 *ospC* transcripts for every 100 *flaB* transcripts) (Fig. 4B, solid blue bar). Similarly, $lacZ_{Bb}$ transcript levels in B312 were dramatically lower when controlled by the *ospC* promoter than by the *flaB* promoter (Fig. 4B, hatched blue bars). The increased activity and transcript levels from the *flaB* promoter compared to the *ospC* promoter are consistent with previous results from transient CAT assays in *B. burgdorferi* B31 (33) and demonstrate different strengths of the *flaB* and *ospC* promoters. Additionally, since FlaB and OspC protein levels were similar when assayed by immunoblotting or Coomassie blue staining, these results also suggest posttranscriptional regulation in *B. burgdorferi*; further studies are needed to investigate this possibility. Notably, the $lacZ_{Bb}$ transcript levels driven by either the *flaB* or *ospC* promoters were 5- to 10-fold higher than the endogenous gene transcripts, presumably reflecting the difference in copy number between the shuttle vector ($lacZ_{Bb}$) and genome (*flaB* or *ospC*) (3). This increase in gene expression from the shuttle vector may be beneficial when analyzing *lacZ* expression from weak *B. burgdorferi* promoters.

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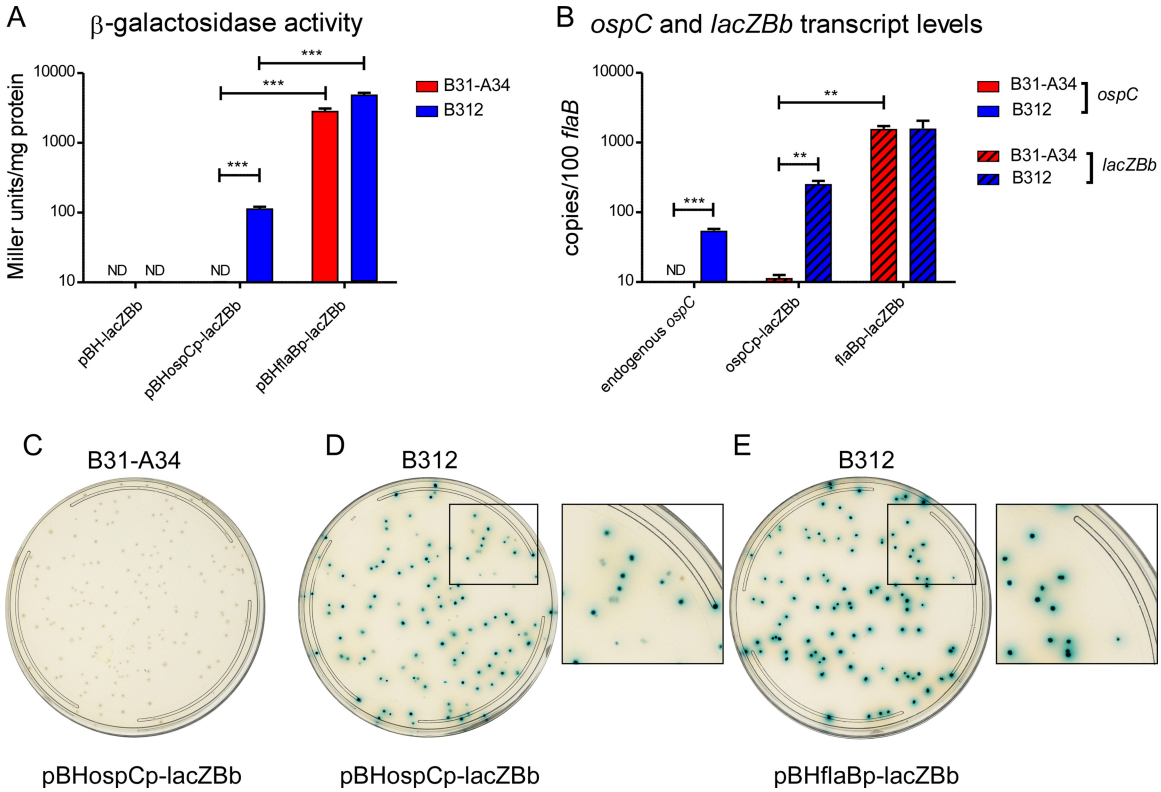

FIG. 4. *lacZ* expression and β-galactosidase activity as a reporter of *ospC* expression. (A) β-Galactosidase activity in *B. burgdorferi* B31-A34 and B312 harboring pBH-lacZBb, pBHospCp-lacZBb, and pBHflaBp-lacZBb, as indicated. Units are reported per mg of protein, and levels represent means ± standard errors of the means. The background activity of bacteria lacking a shuttle vector was subtracted from reported values. ND, no detectable activity above background (i.e., without plasmid) levels. ***, $P < 0.001$; **, $P < 0.01$ (compared to respective promoterless constructs or as indicated based on a Student two-tailed *t* test). (B) Transcript levels of *ospC* and $lacZ_{Bb}$ in B31-A34 and B312 clones harboring $lacZ_{Bb}$, as indicated, were measured by quantitative RT-PCR with primer/probe sets specific for *ospC* and $lacZ_{Bb}$, respectively, and normalized to the chromosomal *flaB* gene transcript. Values represent means ± standard errors of the means. cDNA from a RT-PCR mixture without reverse transcriptase was used to control for DNA contamination and was subtracted from reported values. (C to E) BSK solid medium without phenol red was used to grow B31-A34 harboring pBHospCp-lacZBb (C), B312 harboring pBHospCp-$lacZ_{Bb}$ (D), or B312 harboring pBHflaBp-lacZBb (E). X-Gal was added after colony formation, and plates were incubated overnight before photographs were taken. Magnified insets have been added to panels D and E to illustrate differences between B312 colonies harboring pBHospCp-lacZBb versus pBHflaBp-lacZBb.

We also assessed β-galactosidase activity controlled from the *ospC* promoter in *B. burgdorferi* colonies grown on BSK solid medium without phenol red. As expected, colonies of B31-A34 harboring pBHospCp-lacZBb remained white after overnight treatment with X-Gal (Fig. 4C), indicating these clones do not express *ospC* or $lacZ_{Bb}$ when under the control of the *ospC* promoter and thus did not synthesize the β-galactosidase protein. Even when OspC production was verified by immunoblotting and Coomassie blue staining (data not shown), not all of the B312 colonies harboring pBHospCp-lacZBb turned blue after X-Gal treatment, suggesting that *ospC* expression varied at the individual colony level (Fig. 4D). This variation in expression of β-galactosidase appeared to be due to stochastic differences in expression of the *ospC* promoter in this genetic background rather than an intrinsic property of the B312 strain, as B312 colonies harboring the constitutively expressed pBHflaBp-lacZBb plasmid did not vary in color intensity (Fig. 4E). Additionally, plasmid loss was not responsible for the variation seen with pBHospC-lacZBb, since at least 96% of colonies grown without antibiotic selection still contained the shuttle vector (data not shown). Together, these results demonstrate that β-galactosidase activity and $lacZ_{Bb}$ gene expression accurately reflect promoter strength and regulation of gene expression in *B. burgdorferi* (Fig. 4).

One advantage of using reporter gene fusions to assess the transcriptional activities of various promoters is that it eliminates differences in posttranscriptional regulation that may affect endogenous protein levels. The reporter gene transcript and encoded protein are the same irrespective of which promoter is used to express it. The *lacZ* gene is one of the most widely used reporter genes, and *B. burgdorferi* promoters have previously been fused to *lacZ*, but β-galactosidase activity was assessed in *E. coli*, not *B. burgdorferi* (8, 32). However, since many studies have detected significant differences in the expression profiles of *B. burgdorferi* and *E. coli* (1, 12, 33, 34), using *lacZ* as a reporter gene directly in *B. burgdorferi* will allow a more accurate analysis of native promoter activity. Additionally, there are many substrates for β-galactosidase, and this allows *lacZ* to be used in multiple applications, including simple *in vitro* assays to assess promoter activity and rapid screening for transformants on solid media. Thus, adding *lacZ* to the repertoire of genetic tools for *B. burgdorferi* should facilitate

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investigation of the regulatory mechanisms that allow *B. burgdorferi* to adapt to different environments.

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