Use of an Endogenous Plasmid Locus for Stable in trans Complementation in Borrelia burgdorferi

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Targeted mutagenesis and complementation are important tools for studying genes of unknown function in the Lyme disease spirochete Borrelia burgdorferi. A standard method of complementation is reintroduction of a wild-type copy of the targeted gene on a shuttle vector. However, shuttle vectors are present at higher copy numbers than B. burgdorferi plasmids and are potentially unstable in the absence of selection, thereby complicating analyses in the mouse-tick infectious cycle. B. burgdorferi has over 20 plasmids, with some, such as linear plasmid 25 (lp25), carrying genes required by the spirochete in vivo but relatively unstable during in vitro cultivation. We propose that complementation on an endogenous plasmid such as lp25 would overcome the copy number and in vivo stability issues of shuttle vectors. In addition, insertion of a selectable marker on lp25 could ensure its stable maintenance by spirochetes in culture. Here, we describe the construction of a multipurpose allelic-exchange vector containing a multiple-cloning site and either of two selectable markers. This suicide vector directs insertion of the complementing gene into the bbe02 locus, a site on lp25 that was previously shown to be nonessential during both in vitro and in vivo growth. We demonstrate the functional utility of this strategy by restoring infectivity to an ospC mutant through complementation at this site on lp25 and stable maintenance of the ospC gene throughout mouse infection. We conclude that this represents a convenient and widely applicable method for stable gene complementation in B. burgdorferi.

Lyme borreliosis is caused by Borrelia burgdorferi, which is transmitted through the bite of an infected ixodes tick (1), and remains the most prevalent vector-borne illness in North America. No Lyme disease vaccine is currently available for humans. However, genetic tools have been developed (2–11) to investigate molecular factors crucial for B. burgdorferi biological processes, such as gene regulation and host infectivity (12–24). The B. burgdorferi genome contains a linear chromosome and over 20 linear and circular plasmids (25, 26), some of which carry essential genes (13–18). More than half of all plasmid-borne genes have no homologs outside Borrelia (25), and many of the predicted open reading frames on the chromosome encode novel proteins (26). Therefore, the functions of a large set of genes in the B. burgdorferi genome remain to be determined.

Studies aimed at investigating gene function typically include inactivation of the gene of interest, assessment of phenotypic changes, and complementation of the mutation to determine if the wild-type (WT) phenotype is restored (27). Two methods are widely used for complementation in B. burgdorferi. The first is reintroduction of a wild-type copy of the mutated gene into the B. burgdorferi genome, either in trans at a chromosomal locus (22, 28) or in cis, adjacent to the original locus (9, 14, 29, 30). Complementation in cis should restore gene expression to a level comparable to that of the wild type, but potential complications include the impact on cotranscribed or closely clustered genes (27).

The second method involves the use of autonomously replicating shuttle vectors for in trans gene complementation (3, 4, 8, 9, 11, 31). Complications may arise with the use of shuttle vectors, however, including the effect of the vector copy number on the expression level of the gene being complemented (19, 31), the need to methylate shuttle vector DNA for successful transformation of wild-type strains (32, 33), and the possible loss of the shuttle vector carrying the complementing gene during the in vivo infectious cycle (19).

An improved strategy for stable complementation of genes on an essential plasmid of B. burgdorferi, such as lp25, has been suggested (27), but no studies to date have explored this method. Therefore, we examined the use of the bbe02 locus on lp25 as a target site for gene complementation through allelic exchange. The bbe02 locus encodes one of the restriction-modification enzymes that significantly inhibits shuttle vector transformation of wild-type B. burgdorferi (34, 35). Inactivation of bbe02 did not have an adverse effect on surrounding genes or cause any attenuation of B. burgdorferi in vivo (34, 36).

Here, we describe the construction of a multipurpose allelic-exchange vector (AEV) that contains the bbe02 flanking sequences linked to a multiple-cloning site (MCS) and either of two selectable markers. We determined that complementation of an ospC mutant with this AEV restored inducible OspC production and spirochete infectivity in a mouse model. Furthermore, we determined that lp25 and the ospC gene it carried were stably retained by B. burgdorferi during persistent mouse infection. These studies demonstrate the utility of targeting an endogenous plasmid for stable in trans gene complementation and present an additional tool for genetic studies of B. burgdorferi.

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ApaI site incorporated into the sequence of the transcriptional terminator of the *bmpB* and *ospC* genes, harboring K1/pBSV2G-<br>Used to confirm transformants<br>Colonies were propagated from frozen stocks in Barbour-Stoenner-Kelly II (BSK II) medium, pH 7.7, containing gelatin and 6% rabbit serum. Colonies were propagated in either NEB 5α/H11011 or Top10 (Life Technologies, Carlsbad, CA) cells. *E. coli* cultures were incubated at 37°C in LB broth/agar containing 5 µg/ml gentamicin, 50 µg/ml kanamycin, or 100 µg/ml spectinomycin as needed.<br><br>**MATERIALS AND METHODS**<br><br>*B. burgdorferi* strain and culture. The low-passage-number infectious clone B31-A3 of the type strain B31 (ATCC 35210) was used as the WT strain (37). The B31-A3 derivatives ospCK1, lacking the ospC gene, and ospCK1/pBSV2G-ospC, harboring ospC on a shuttle vector (19), were used as the ospC mutant and complemented strains, respectively. Bacteria were propagated from frozen stocks in Barbour-Stoenner-Kelly II (BSK II) medium, pH 7.7, containing gelatin and 6% rabbit serum. Colonies were isolated in solid BSK medium incubated at 35°C with 2.5% CO₂, as described previously (38). Antibiotic-resistant spirochetes were propagated in medium containing 200 µg/ml kanamycin, 40 µg/ml gentamicin, or 50 µg/ml streptomycin. To induce ospC expression by growth at lower pH (39), cultures were inoculated in BSK II medium, pH 7, at 10⁵ spirochetes per ml and harvested when they reached late log phase (10¹⁰ spirochetes/ml). Aliquots of each culture were processed for subsequent analysis of DNA, RNA, and protein contents.<br><br>*Escherichia coli* strains and culture. All plasmid constructs were propagated in either NEB 5α (New England BioLabs, Inc., Ipswich, MA) or Top10 (Life Technologies, Carlsbad, CA) cells. *E. coli* cultures were incubated at 37°C in LB broth/agar containing 5 µg/ml gentamicin, 50 µg/ml kanamycin, or 100 µg/ml spectinomycin as needed.<br><br>**Construction of a multiple-cloning site and antibiotic resistance cassette.** Two different antibiotic resistance genes, driven by either the flaBp or flaBp flagellar-gene promoter and flanked upstream by an MCS, were amplified by PCR and cloned into the pCR 2.1 TOPO vector (Life Technologies). Primers designed to amplify the promoters linked to the antibiotic resistance gene sequences contained 5 unique restriction endonuclease sites. TaqMan primers and probes were amplified from the pBBE02-Spec plasmid (33) using primers 1 and 2 (all the primers are listed in Table 1), and the flaBp-aphI cassette was amplified from the pBSV2 plasmid (11) using primers 3 and 4. The resulting PCR products, with 5’ Nhel and 3’ PacI sites flanking the MCS-antibiotic resistance cassette, were cloned into the pCR 2.1 TOPO vector (Life Technologies) to generate the pCR2.1-flaBp-aphI and pCR2.1-flaBp-adaA constructs.<br><br>**TABLE 1 Primers used in this study**<br><br| No. | Label | Sequence (5’→3’<sup>a</sup>) | Reference |
<table>
<thead>
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<td>1</td>
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<td>6</td>
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<tr>
<td>7</td>
<td>BmpBT_KpnI-3’</td>
<td>CCCCCGCGTAACAAGCTTGATTTTTTTTGGCGG</td>
<td>This work</td>
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</table>

<sup>a</sup> Sequences in boldface indicate restriction enzyme sites included in the oligonucleotide. MCS in oligonucleotides 1 and 2 represents the multiple-cloning site sequence GGTACC GGCGGCCGC. Oligonucleotides 7 and 8 indicate the Apal site incorporated into the sequence of the transcriptional terminator of the *bmbII* locus.
plasmids, conferring resistance to spectinomycin/streptomycin and kana-
mycin, respectively. The adaA gene confers resistance to both spectino-
mycin and streptomycin; the antibiotic used for selection (spectinomycin in E. coli and streptomycin in Borrelia) was chosen based on the relative level of background resistance. The fidelities of all plasmids and constructs generated in this study were confirmed by PCR, sequence analyses (prim-
ers 9 to 20 [Table 1]), and restriction enzyme digestion.

Construction of allelic-exchange vectors. A multipurpose AEV tar-
getting the bbe02 locus on lp25 was constructed in the pOK12 cloning vector (40) using previously characterized restriction sites in bbe02 for insertion of an antibiotic resistance cassette (34). A 2,596-bp internal fragment of the bbe02 gene on lp25 was amplified from WT B. burgdorferi genomic DNA, using primers 5 and 6, and cloned into the pcRII 1 TOPO vector (Life Technologies). This fragment was excised from the TOPO vector with flanking sites XhoI/NotI and cloned into the same sites in the pOK12 vector (40), creating the pOK-bbe02 plasmid. The pOK-bbe02 plasmid was digested with NheI/PacI (internal sites in ospC generated in this study were confirmed by PCR, sequence analyses (prim-
ers encompassing the WT pKBE-Strep and pKBE-Kan, confer resistance to spectinomycin/streptomycin and kanamycin, respectively. Two tandem Rho-independent transcriptional terminator sequences of the bmpB locus of B. burgdorferi (41, 42) were inserted using primers 7 and 8 (Table 1), as previously described (41), and cloned into the Nhel/KpnI sites of the MCS of each AEV. An Apal site was added at the 3’ end of the transcriptional terminator as an additional site in the MCS.

Construction of an AEV harboring wild-type ospC. A 1,015-bp fragment encompassing the WT ospC gene and its native promoter was cloned into the KpnI/BamHI sites of the MCS region of pKBE-Strep, creating pKBE-Strep/ospC”.

Transformation of B. burgdorferi with the AEV. Low-passage-num-
ber WT and ospCK1 strains were propagated from frozen stocks and used to prepare competent cells for transformation with the AEV, following established methodology (43, 44). Recombination between the AEV and the bbe02 locus of transformants was selected by growth under antibiotic selection and confirmed by PCR analysis (primers 9 to 14 and 17 to 20 [Table 1]). Transformant clones were assessed for full B. burgdorferi plas-
mid complement, as described previously (45). The pKBE-Strep/ospC” AEV was transformed into the ospCK1 strain by electroporation, and colon-
ones were screened for ospC by PCR (primers 13 and 14 [Table 1]). A positive transformant, ospCK1/ospC””, which was verified to carry the ospC gene at the bbe02 locus on lp25 (primers 15 and 16 [Table 1]) and contained the same plasmid profile as the parental strain, ospCK1, and the WT clone B31-A3 (lacking only circular plasmid 9 [pcp9]), was used in further experiments.

Southern blotting. The presence of a wild-type ospC gene on lp25 of the ospCK1/ospC” complemented strain was confirmed by Southern blotting. Approximately 0.15 µg of total genomic DNAs from WT, ospCK1, and ospCK1/ospC” cultures was separated on a 0.5% agarose gel under field inversion electrophoresis conditions and subjected to Southern blot analysis, as previously described (46). Digestogenin (DIG)-labeled probes (Roche, Indianapolis, IN) were amplified from WT genomic DNA using gene-specific primers for the ospC and pncA genes (primers 13 and 14 and primers 21 and 22 [Table 1]) following the manufacturer’s instructions and then sequentially hybridized with the blot as described previously (11).

Transcript level determination by quantitative RT-PCR. Total RNA was prepared from late-log-phase cultures of each strain cultivated in triplicate at pH 7.0 or 7.7, using the TRIzol reagent method and the man-
ufacturer’s instructions (Life Technologies). One microgram of total RNA was subjected to DNase treatment as outlined in the manufacturer’s pro-
tocol (Life Technologies) and used to synthesize cDNA using the High Capacity DNA Reverse Transcriptase (RT) kit (Life Technologies). A con-
trol reaction mixture lacking reverse transcriptase (no RT) was included for each cDNA synthesis reaction, and each sample was tested with three technical replicates by quantitative PCR (qPCR) using a 1:10 dilution of cDNA, the TaqMan Universal PCR Mastermix (Life Technologies), and ABI Vii7 software, as previously described (47). The constitutively expressed flaB gene was used as the PCR endogenous control to which ospC and pncA values were normalized. A standard curve using crude DNA samples prepared by the boiling method described below was run for each PCR primer-probe set (the primer sequences are listed in Table 1).

DNA copy number determination by quantitative PCR. In order to minimize variability in the purification of genomic DNA molecules with different structures and sizes, we used a simple but crude method of sam-
ple preparation to assess the gene copy number, as follows. A 0.3-ml sample of each late-log-phase culture, for which the spirochete density was determined by counting, was centrifuged at 9,000 rpm for 10 min, and the pellet was resuspended in RNase/DNase-free water at a final concent-
ration of 10^9 spirochetes/µl and boiled for 5 min. qPCR assays were run with 2 µl of each crude DNA sample diluted 1:10 or 1:30 and the TaqMan chemistry, as detailed previously (47). The chromosomal flaB gene was used as the endogenous control to which ospC and pncA values were normalized. The quantitative-PCR results for standard curves generated with crude DNA preparations from a defined number of organisms were identical to those generated with purified total genomic DNA (QiaGen) representing a defined number of genome equivalents as calculated from the DNA concentration and B. burgdorferi genome size.

Statistical analysis. The differences in gene copy numbers and tran-
script levels between B. burgdorferi strains and culture conditions were assessed using Graph Pad Prism software and a two-tailed, unequal Stu-
dent’s t test, with a P value of <0.01 as the cutoff for significant differences between groups.

OspC and Flab protein analysis. Spirochetes from the same log-phase cultures used for DNA and RNA analysis were enumerated with a Petroff-
Hausser chamber, washed twice with HN buffer (50 mM HEPES, 50 mM NaCl; pH 7.6), and resuspended in sample buffer at a final concent-
ration of 10^8 spirochetes/mL. Total cell lysates of each strain representing approx-
imately 10^7 spirochetes were separated by SDS-PAGE, transferred to ni-
trocellulose membranes, and analyzed with OspC polyclonal and Flab (flagellin) monoclonal antibodies, as described previously (47, 48).

Mouse infection. Animal experiments were performed according to the guidelines from the National Institutes of Health, with protocols approved by the Rocky Mountain Laboratories (RML) Animal Care and Use Committee. The Rocky Mountain Laboratories are accredited by the International Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). The B. burgdorferi WT, ospCK1, and ospCK1/ospC” strains were grown to mid-log phase, and 1 × 10^8 bacteria were inoculated (8 × 10^6 intraperitoneally and 2 × 10^5 subcutaneously) per 6-
to 8-week-old RML mouse. At 3 weeks postinoculation, the mice were retro-orbitally bled, and the reactivity of mouse sera against whole-cell B. burgdorferi was assessed by immunoblotting using a slot blot apparatus (Bio-Rad, Hercules, CA) and mouse sera diluted 1:200, as described pre-
viously (48). At 7 to 8 weeks postinoculation, the mice were euthanized, and the ear, bladder, and joint tissues were incubated in BSK II medium at 35°C for spirochete isolation. Spirochetes isolated from the ears and blad-
ers of mice inoculated with the ospCK1/ospC” strain were further propagated in liquid culture with and without streptomycin to assess retention of the ospC gene on lp25.

RESULTS AND DISCUSSION

Engineering allelic-exchange constructs. Shuttle vectors suitable for complementation of mutants in B. burgdorferi are available, but they have a higher copy number than endogenous plasmids and can be lost in the absence of selection, such as during in vivo infection (19). To circumvent these shortcomings, we examined the use of the nonessential bbe02 locus on the in vivo essential lp25 for complementation in B. burgdorferi. We constructed a multi-

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TABLE 2 Plasmids and B. burgdorferi strains used in this study

<table>
<thead>
<tr>
<th>Plasmid or strain</th>
<th>Resistancea</th>
<th>B. burgdorferi Description</th>
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<tbody>
<tr>
<td>Plasmids</td>
<td></td>
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<tr>
<td>pOK-bbe02</td>
<td>Kanamycin</td>
<td>Plasmid harboring partial bbe02 gene</td>
</tr>
<tr>
<td>pKBE-Strep</td>
<td>Spectinomycin and kanamycin</td>
<td>bbe02 allele-exchange vector, MCS, and flaBp-adaA</td>
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<tr>
<td>pKBE-Kan</td>
<td>Kanamycin</td>
<td>bbe02 allele-exchange vector, MCS, and flgBp-aphI</td>
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<tr>
<td>pKBE-Strep/ospC+</td>
<td>Spectinomycin and kanamycin</td>
<td>pKBE-Strep with native ospC cloned in the MCS</td>
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<td>B. burgdorferi strain</td>
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<td>B31-A3</td>
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<td>WT strain B31 infectious clone (3)</td>
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<tr>
<td>ospCK1</td>
<td>Kanamycin</td>
<td>B31-A3 derivative with deletion of ospC gene (19)</td>
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<tr>
<td>ospCK1/pBSV2G-ospC</td>
<td>Gentamicin and kanamycin</td>
<td>ospCK1 derivative with ospC on shuttle vector (19)</td>
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<tr>
<td>ospCK1/ospC+</td>
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<td>ospCK1 with deletion in bbe02 and insertion of ospC</td>
</tr>
<tr>
<td>A3 Δbbe02-Strep+</td>
<td>Streptomycin</td>
<td>B31-A3 with deletion in bbe02 and insertion of flgBp-aphI</td>
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<tr>
<td>A3 Δbbe02-Kan+</td>
<td>Kanamycin</td>
<td>B31-A3 with deletion in bbe02 and insertion of flgBp-aphI</td>
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</table>

* Antibiotic resistance conferred by plasmids in E. coli or B. burgdorferi or exhibited by the strain.

purpose AEV containing a multiple-cloning site and either of two different antibiotic resistance cassettes to generate the pKBE-Strep and pKBE-Kan plasmids, conferring streptomycin and kanamycin resistance, respectively (Table 2 and Fig. 1).

To test recombination of the AEV constructs with the bbe02 locus on lp25, we transformed the plasmids into WT B. burgdorferi by electroporation. Positive transformants confirmed by PCR (primers 9 and 10 and primers 11 and 12 [Table 1]), designated A3 Δbbe02-Strep+ and A3 Δbbe02-Kan+ strains (Table 2), were obtained from the pKBE-Strep and pKBE-Kan transformations, respectively. The A3 Δbbe02-Strep+ and A3 Δbbe02-Kan+ strains were shown to have the same plasmid content as B31-A3, lacking only cp9 (25).

**Complementation of an ospC mutant at the bbe02 locus.** To confirm the utility of the AEV for gene complementation in B. burgdorferi, a wild-type copy of ospC was cloned into pKBE-Strep. The resulting plasmid, pKBE-Strep/ospC+, was electroporated into the ospCK1 mutant strain to generate the ospCK1/ospC+ strain. We verified insertion of the wild-type ospC gene into the bbe02 locus on lp25 by PCR with primers listed in Table 1 (Fig. 2A, black arrowheads, and B) and Southern blot analysis (Fig. 3).

The streptomycin resistance cassette was amplified from only the ospCK1/ospC+ complemented strain, as expected (Fig. 2B, primers 9 and 10). The ospC gene was detectable only in the WT and ospCK1/ospC+ strains (primers 13 and 14), whereas the bbe02 region was present in the WT and ospCK1 strains but deleted in the ospCK1/ospC+ strain (primers 17 and 18). Finally, a PCR fragment connecting the ospC and bbe03 genes was obtained only when DNA was prepared from the complemented strain, but not from the WT or ospCK1 strain (primers 13 and 20), indicating allelic exchange with bbe02 on lp25. The ospC insertion in the complemented strain is expected to have no adverse effect on flanking genes, since insertion at this site was previously shown to have no impact on B. burgdorferi growth and mammalian infection (34). These results demonstrate that the DNA construct containing ospC recombined at the bbe02 locus of lp25 through a double-crossover event.

Southern blot analysis under field inversion electrophoresis conditions further confirmed insertion of the complementing ospC gene on lp25 (Fig. 3). In wild-type B. burgdorferi, ospC is located on cp26, and the pncA (bbe22) gene encoding nicotinamidase is located on lp25 (25, 26). As expected, the ospC probe hybridized to genomic DNA from the WT and ospCK1/ospC+ clones, but not to DNA from the ospCK1 clone (Fig. 3, left). Additionally, the ospC probe hybridized to different plasmids in the WT and ospCK1/ospC+ strains, representing forms of cp26...
and lp25, respectively. Conversely, the pncA probe hybridized to bands of the same size in the WT and ospC clones but to a larger DNA element in the ospC\(^+\)/ospC\(^+\)/H11001 clone, due to insertion of the 1,350-bp sequence of the ospC complementation construct into lp25 (Fig. 3, right). Hence, the results from Southern blot analysis are in agreement with the PCR analysis and show that the complementing ospC gene was inserted on lp25 in the ospC\(^+\)/ospC\(^+\)/H11001 strain.

Relative copy numbers and expression of the ospC gene at different genomic sites. Complementation in trans on a shuttle vector or elsewhere in the genome can alter the copy number and impact expression of the reintroduced gene relative to its native locus. *Borrelia* has both linear and circular replicons, and the form of the DNA on which a gene is located may also influence its expression. To address these potential concerns, we determined and compared basal and induced expression of the ospC gene from its native locus on the circular plasmid cp26 to that of the ospC gene introduced by allelic exchange on the linear plasmid lp25 or carried by the shuttle vector pBSV2G (19). We also assessed expression of pncA (bbe22) to determine if insertion of the ospC gene or a selectable marker on lp25 altered the expression of an

**FIG 2** Deletion in bbe02 and insertion of ospC on lp25 in the ospC\(^+\)/ospC\(^+\) strain. (A) Genome organization of the bbe02 locus on lp25 in the WT and ospC\(^+\) strains (i), with the region deleted (shown in black) and replaced with the ospC construct, an MCS (dark gray), and transcriptional terminators (TT) and a flaB-paadA selectable marker in the ospC\(^+\)/ospC\(^+\) complemented strain (ii). The flaB and ospC promoters are represented by divergent arrows, and the positions of the oligonucleotide primers used in panel B are indicated by arrowheads. (Not drawn to scale.) (B) Deletion in bbe02 and insertion of the ospC complementation construct as confirmed by PCR and agarose gel electrophoresis. The PCR primers used are identified by the numbers above the bracketed lanes and correspond to those shown in panel A and Table 1. The mobilities of the DNA size standards are shown on the left. WT, wild-type strain B31-A3; NTC, no-template control.

**FIG 3** Southern blot analysis of *B. burgdorferi* transformants. Equivalent amounts of genomic DNA from the WT, ospC\(^+\) (ospC mutant), and ospC\(^+\)/ospC\(^+\) (mutant complemented with ospC on lp25) strains were separated by field inversion gel electrophoresis, blotted, and hybridized with ospC and pncA probes, as indicated. The mobilities of the DNA size standards are shown on the left.
other gene on the plasmid. To do this, total RNA was prepared from WT, ospC mutant, and complemented strains grown in standard culture medium (pH 7.7) or at a lower pH (pH 7.0), which induces expression of ospC and other RpoS-dependent genes (29, 39, 47). Aliquots of each culture were processed for subsequent analysis of RNA, DNA, and protein. The replicon on which the ospC gene is located differs among the strains, as indicated in the key; the pncA (BBE22) gene is present on lp25 in all strains. (A and B) The transcript levels (A) and copy numbers (B) of the ospC and pncA genes relative to the chromosomally located and constitutively expressed flaB gene were determined by quantitative PCR from three independent biological replicates, with three technical replicates of each sample. (A) The gene transcript values represent the means plus standard deviations of all determinations for each strain under the indicated growth conditions (n = 9). (B) The gene copy numbers shown represent the means plus standard deviations of combined determinations for each strain under both growth conditions (n = 18). The asterisks denote a significant difference (P < 0.01, as determined by a two-tailed, unpaired Student t test) between the indicated strain and all other strains in the analysis; differences between unmarked strains were not significant. (C) The OspC and FlaB (flagellin) protein profiles of all strains were assessed by immunoblot analysis of whole-cell lysates with specific antisera; the results shown are representative examples of each strain grown under inducing conditions. OspC was not detected in any strains grown under noninducing conditions (not shown).

FIG 4 Relative expression and copy numbers of the ospC gene when carried by different replicons. Five different B. burgdorferi strains (identified in the key at the lower right) were grown in triplicate to late log phase under uninduced (pH 7.7) and ospC-induced (pH 7.0) culture conditions (39). Aliquots of each culture were processed for subsequent analysis of RNA, DNA, and protein. The replicon on which the ospC gene is located differs among the strains, as indicated in the key; the pncA (BBE22) gene is present on lp25 in all strains. (A and B) The transcript levels (A) and copy numbers (B) of the ospC and pncA genes relative to the chromosomally located and constitutively expressed flaB gene were determined by quantitative PCR from three independent biological replicates, with three technical replicates of each sample. (A) The gene transcript values represent the means plus standard deviations of all determinations for each strain under the indicated growth conditions (n = 9). (B) The gene copy numbers shown represent the means plus standard deviations of combined determinations for each strain under both growth conditions (n = 18). The asterisks denote a significant difference (P < 0.01, as determined by a two-tailed, unpaired Student t test) between the indicated strain and all other strains in the analysis; differences between unmarked strains were not significant. (C) The OspC and FlaB (flagellin) protein profiles of all strains were assessed by immunoblot analysis of whole-cell lysates with specific antisera; the results shown are representative examples of each strain grown under inducing conditions. OspC was not detected in any strains grown under noninducing conditions (not shown).
TABLE 3 Mouse infectivity of WT, ospCK1 mutant, and ospCK1/ospC\(^+\) strains of *B. burgdorferi*

<table>
<thead>
<tr>
<th>Clone</th>
<th>Seroconversion(^a)</th>
<th>Tissue isolate(^b)</th>
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<tbody>
<tr>
<td>B31-A3 (WT)</td>
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</tr>
<tr>
<td>ospCK1 (∆ospC)</td>
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<tr>
<td>ospCK1/ospC(^+)</td>
<td>5/5</td>
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</table>

\(^a\) Mice were inoculated with 1 \times 10^5 spirochetes, and seroreactivity with whole-cell *B. burgdorferi* protein lysate was determined by immunoblot analysis 3 weeks later.

\(^b\) Three tissues per animal (ear, bladder, and joint) were cultured 7 to 8 weeks postinoculation for spirochete isolation.

among strains (Fig. 4C and data not shown). Consistent with this, we have noted that spirochetes carrying a gfp reporter gene inserted on lp25 fluoresced less brightly than spirochetes carrying the same gene on a shuttle vector (data not shown). Allelic exchange at the telomeric *bbe02* locus did not alter either the copy number or the expression of the distal *pncA* locus on lp25, whereas the presence of the shuttle vector in the cell increased the copy number of lp25 approximately 2-fold (Fig. 4A and B, right). Additional experiments are needed to determine if this apparent in trans effect of the shuttle vector is reproducible and impacts the copy numbers of other endogenous plasmids and whether it results from *E. coli* or *Borrelia* sequences present on the shuttle vector.

**Utility of lp25 for stable in trans gene complementation.** Our next goal was to investigate whether lp25 could be used for stable complementation of *B. burgdorferi* mutants. OspC is required by *B. burgdorferi* for mammalian infection (14, 19, 49, 50). Complementation of an ospC mutant in trans with a shuttle vector restores mouse infectivity, but OspC is required by *B. burgdorferi* only during early mammalian infection (19) and the shuttle vector harboring the ospC gene is not maintained during persistent infection (19, 50). Therefore, we examined the phenotype of the *ospCK1/ospC\(^+\)* strain with respect to mouse infectivity, persistent infection, and stability of the wild-type copy of *ospC* on lp25.

To determine restoration of infectivity to the complemented strain, we inoculated mice with approximately 1 \times 10^4 WT, ospCK1, or ospCK1/ospC\(^+\) spirochetes. Three weeks later, we assessed seroreactivity with *B. burgdorferi* proteins. As expected, animals injected with the ospCK1 mutant were seronegative, while mice injected with WT spirochetes or *ospCK1/ospC\(^+\)* seroconverted (Table 3). These data indicate that the wild-type ospC gene inserted on lp25 is functional and restored infectivity to the ospCK1 mutant.

Next, we determined the ability of the *ospCK1/ospC\(^+\)* spirochetes to persist in mouse tissues. Mice infected with WT, ospCK1 mutant, and *ospCK1/ospC\(^+\)* strains were euthanized 7 to 8 weeks postinoculation, and spirochetes were isolated from the ear, bladder, and joint tissues. All mice injected with the complemented strain remained persistently infected, as did mice injected with the WT strain, but no spirochetes were isolated from any mice inoculated with the ospCK1 mutant (Table 3). Therefore, we conclude that OspC function was restored in *ospCK1/ospC\(^+\)* spirochetes and, furthermore, that these spirochetes were able to successfully colonize and persist in mouse tissues.

Persistent infection (which does not require OspC function) by *ospCK1/ospC\(^+\)* spirochetes indicated that lp25 was retained by the spirochetes, as some genes on the plasmid are essential for mouse infectivity (35, 51), but the presence of the ospC gene was not guaranteed. To confirm retention of both lp25 and ospC by *ospCK1/ospC\(^+\)* spirochetes in mice, the ear and bladder tissue isolates were passaged at 1:100 in BSK II medium with or without streptomycin supplementation. After 3 days, all tissue isolates had grown to high density, irrespective of the presence of the antibiotic, indicating that these spirochetes retained lp25 with the *flaBpaaA* cassette, as anticipated. Additionally, the presence of the linked ospC gene in these spirochetes was confirmed by PCR (data not shown).

There are advantages to using lp25 for general complementation of *B. burgdorferi* mutants. Since inactivation of *bbe02* significantly improves shuttle vector transformation of wild-type *B. burgdorferi* (33, 34) without any deleterious effect on surrounding genes or infectivity (34), it is possible to complement inactivated genes at this locus and subsequently perform efficient shuttle vector transformations, such as when introducing a reporter gene. A second advantage is the ensured retention of lp25 during *in vitro* propagation of complemented spirochetes (45, 52–55), with the insertion of the selectable marker into *bbe02*. Because lp25 carries genes essential for both tick and mammalian infectivity (13, 16, 18, 51), complementing an inactivated gene on lp25 ensures it will also be retained *in vivo*. In addition, lp25 can be used to complement mutations in genes that are lethal or deleterious when expressed from a higher-copy-number shuttle vector (9, 19, 31). Conveniently, complementation of mutants on lp25 using a multipurpose AEV eliminates the need for designing and constructing a new allelic-exchange plasmid for each experiment. Finally, complementation on lp25 does not alter the original mutation, as would be the case when performing in cis complementation (27).

We provide data validating the use of a multipurpose AEV for stable in trans complementation of an ospC mutant at a previously identified nonessential site on lp25. We conclude that using lp25, an endogenous and essential plasmid, provides an additional, convenient method for stable complementation of mutants in *B. burgdorferi*.

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