Experimental Assessment of the Roles of Linear Plasmids lp25 and lp28-1 of *Borrelia burgdorferi* throughout the Infectious Cycle

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***Borrelia burgdorferi*, which causes Lyme disease in humans, has an unusual genome composed of a linear chromosome and up to 21 extrachromosomal elements. Experimental data suggest that two of these elements, linear plasmids lp25 and lp28-1, play essential roles for infectivity in mice. In this study, we prove the essential natures of these two plasmids by selectively displacing lp25 or lp28-1 in an infectious wild-type clone with incompatible shuttle vectors derived from the native plasmids, rendering the respective transformants non-infectious to mice. Conversely, restoration of plasmid lp25 or lp28-1 in noninfectious clones that naturally lack the corresponding plasmid reestablished infectivity in mice. This approach establishes the ability to manipulate the plasmid content of strains by eliminating or introducing entire plasmids in *B. burgdorferi* and will be valuable in assessing the roles of plasmids even in unsequenced *B. burgdorferi* strains.**

Borrelia burgdorferi is maintained in nature in an enzootic cycle between *Ixodes* sp. ticks and small mammals, mainly rodents (9, 24). Humans develop Lyme disease when bitten by a tick infected with *B. burgdorferi* sensu lato (6, 39, 43). Several studies, culminating with the sequence of the type strain B31 (ATCC 35210), defined the unusual organization of the *B. burgdorferi* genome. The spirochete contains a linear chromosome and up to 21 circular and linear plasmids (3–5, 8, 15, 16, 18). The majority of the open reading frames (ORFs) on the plasmids encode proteins of unknown function without identified homologs in other organisms (8, 16). Plasmid analyses of several other *B. burgdorferi* sensu stricto isolates have revealed significant differences in both the plasmid content and protein-coding regions of different strains (7, 20, 31, 44, 50).

Previous studies of the relationship between a particular *B. burgdorferi* plasmid and a physiological or infectivity-related phenotype have relied upon *B. burgdorferi* B31 isolates that have spontaneously lost one or more plasmids (22, 23, 26, 32, 33, 38, 51). While continued growth and passage in artificial media can lead to loss of plasmids, demonstrating that these molecules are dispensable for in vitro growth (3, 17, 28, 36, 38, 42), certain plasmids have been shown to be important for infection of the mammalian host. Among these plasmids are the linear plasmids lp28-1 and lp25 (23, 33, 51). Loss of lp28-1 has been correlated with reduced infectivity in laboratory mice (23, 33, 52). Plasmid lp28-1 carries a VMP-like sequence (*vls*) locus, which undergoes antigenic variation during infection of the mammalian host and is presumably required to evade the host's humoral immune response and establish a persistent

infection (11, 19, 25, 29, 52). Clones lacking lp25 are unable to survive in the mammalian host (22, 23, 33). The BBE22 gene on this plasmid encodes a nicotinamidase and has been shown to be sufficient to restore infectivity in mice for clones lacking lp25 (32). To date, experimental manipulation of full-length plasmids to establish their roles in mouse or tick infectivity has not been accomplished.

In this study, we used a two-pronged approach to prove that lp28-1 and lp25 are essential for infectivity in mice. First, we exploited and extended recent studies demonstrating that specific genetic elements encoded on each *B. burgdorferi* plasmid determine incompatibility groups; i.e., two plasmids sharing those loci are incompatible (10, 45, 46). We used shuttle vectors derived from the B31 plasmids lp28-1 and lp25 to displace the endogenous plasmids from infectious strain 297 to broaden the generality of the infectivity and incompatibility studies. The results confirm the utility of using the defined B31 plasmid incompatibility regions to study the roles of plasmids in the physiology and virulence of both *B. burgdorferi* strain 297 and other unsequenced strains. Second, we used noninfectious B31 clones naturally lacking lp28-1 or lp25 as recipients for reintroduction of these plasmids and subsequent assessment of infectivity. We show that infectivity for mice can be reestablished by restoration of the missing plasmid. These findings prove the essential nature of lp28-1 and confirm the requirement for lp25 (32) for infectivity of *B. burgdorferi* in mice, thereby fulfilling molecular Koch's postulates. The described methods provide a powerful new genetic strategy for identifying novel plasmid-encoded virulence determinants and for studying the roles of specific plasmids in the life cycle of *B. burgdorferi*.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. The infectious *B. burgdorferi* strain 297 clone c155 (297-c155) used in this study (Table 1) was derived from a

TABLE 1. *B. burgdorferi* clones used in this study

Clone	Introduced DNA	Plasmid(s) missing	Reference or source
297-c155 (WT)	None	cp9, cp32-4	10
297/pBSV28-1	pBSV28-1	cp9, cp32-4, lp28-1	This study
297/pBSV25	pBSV25	cp9, cp32-4, lp25	This study
B31-A3 (WT)	None	cp9	14
B31-A3 lp28-1-Gm	<i>flgBp-aacC1</i>	cp9	This study
B31-A1	None	cp9, lp28-1	14
B31-A1 lp28-1-Gm	lp28-1:: <i>flgBp-aacC1</i>	cp9	This study
B31-A3 lp25-Gm	<i>flaBp-aacC1</i>	cp9	This study
B31-A44	None	cp9, lp25	This study
B31-A44 lp25-Gm	lp25:: <i>flaBp-aacC1</i>	cp9	This study

low-passage-number isolate (10, 43). Derivative clones 297/pBSV25 and 297/pBSV28-1 are described in Table 1 and below. The infectious strain *B. burgdorferi* B31 MI is the strain for which the genome sequence was determined (8, 16). For the restoration of plasmids, we used low-passage-number clones derived from B31 MI, which are described in Table 1 and below. All *B. burgdorferi* strains were grown in liquid Barbour-Stoenner-Kelly (BSK) II medium with gelatin and 6% rabbit serum (2) and plated in solid BSK medium as previously described (34, 37). For temperature shift experiments, clones were grown to a density of 1×10^7 to 5×10^7 cells/ml at 23°C, diluted to 1×10^4 cells/ml, and grown to 0.5×10^8 to 1×10^8 cells/ml at 37°C. For experiments using dialysis membrane chambers (DMCs), spirochetes were inoculated into dialysis membrane bags at 3×10^3 cells/ml in BSK-H medium (Sigma-Aldrich, St. Louis, Mo.) and implanted into the peritoneal cavities of rats as previously described (1).

Transformation of *B. burgdorferi*. *B. burgdorferi* clones were grown in 100 ml of liquid BSK II medium and harvested at late exponential phase (5×10^7 to 1×10^8 cells/ml). Electrocompetent cells were prepared and transformed as previously described (14, 37). Competent *B. burgdorferi* strain B31 or 297-c155 bacteria were transformed with 10 to 25 µg of plasmid DNA and were allowed to recover in BSK II medium at 35°C overnight. A dilution of the transformation culture was plated in solid BSK medium onto one plate without antibiotics; the remaining transformation was plated in solid medium containing the antibiotic kanamycin (200 µg/ml) or gentamicin (40 µg/ml), as appropriate. Strain 297 clones were screened for pBSV25 and pBSV28-1 (45) using primer 1 and either primer 2 or 3 (Table 2). Strain B31 colonies were screened by PCR for the presence of the antibiotic resistance marker with primers 10, 11, and 12 (Table 2). Transformants were further screened for their plasmid profiles as previously described (Table 2) (10, 14). Transformation frequency was calculated as the ratio of transformants relative to the total number of bacteria in the transformation, calculated from the number of colonies on plates without antibiotics.

Transformation efficiency was calculated as the number of transformants per microgram of transforming DNA.

Construction of p28-1::*flgBp-aacC1*. Plasmid p28-1::*flgBp-aacC1* consists of the vector pOK12 (47) and a 2-kb region of lp28-1, into which was inserted the gentamicin resistance gene *aacC1* fused to the *B. burgdorferi* *flgB* promoter (*flgBp*) (13). The plasmid was constructed as follows: a PCR product spanning nucleotides 15708 to 17377 of lp28-1 was amplified with primers 13 and 14 (Table 2) and cloned into the vector pCR2.1-TOPO (Invitrogen Life Technologies, Carlsbad, Calif.). Plasmid transformations were done by heat shock into chemically competent *Escherichia coli* TOP10 cells (Invitrogen Life Technologies). A NotI-KpnI fragment containing the lp28-1 fragment was excised and recloned into NotI-KpnI-digested pOK12 to yield the plasmid p28-1 (restriction enzymes and T4 DNA ligase were purchased from New England Biolabs, Beverly, Mass.). A fragment containing *flgBp-aacC1* was amplified from plasmid pTAGmG (13) with primers 10 and 11 (Table 2) and cloned into pCR2.1-TOPO. The *flgBp-aacC1* fusion was cut out from the resulting plasmid, pTA-GmG-HindIII, with HindIII and ligated into HindIII-cut p28-1. The resulting plasmid, p28-1::*flgBp-aacC1*, was verified by sequencing using an ABI 377 automated sequencer (Applied Biosystems, Foster City, Calif.). Plasmid preparations from *E. coli*, restriction digests, and ligations were performed as previously described (12).

Construction of p25::*flaBp-aacC1*. Plasmid p25::*flaBp-aacC1* has pOK12 as the vector backbone (47), with a 2-kb fragment of lp25 inserted, interrupted by the *aacC1* gentamicin resistance gene fused to the *B. burgdorferi* *flaB* promoter (*flaBp*). The plasmid was constructed as follows: a 2-kb fragment spanning nucleotides 4323 to 6261 of lp25 was amplified using primers 15 and 16 (Table 2) and cloned into pCR2.1-TOPO. The resulting plasmid was digested with KpnI and NotI; the product containing the lp25 fragment was subcloned into KpnI-NotI-digested pOK12, yielding p25. The gentamicin resistance cassette *flaBp-aacC1* was amplified from plasmid pTAGmA (13) using primers 17 and 18

TABLE 2. Oligonucleotide primers used in this study

Primer	Designation	Nucleotide sequence (5'→3') or reference	Use
1	Kan532-R	TAGATTGTCGCACCTGATTGCCCG	Detection of pBSV25 and pBSV28-1
2	BBE21-F	TTCTGCCTCCTCAACTTCGGATGG	Detection of pBSV25
3	BBF26-F	TGTAATGAACTTCGGTGGG	Detection of pBSV28-1
4	297-vlsE(+1)	GGAGCAATATTTGTTTTGTTAATTG	Detection of lp28-1 in 297, probe
5	297-vlsE(-1)	CTAATTTCTGCTATAGCACCTTGAC	Detection of lp28-1 in 297, probe
6	BBE16-5'	TTGCTGCCATTTCTCACTTGGTAA	Detection of lp25 in 297, probe
7	BBE16-3'	ATAAAAGCGACAGGTTATCGTGACG	Detection of lp25 in 297, probe
8	Bb-Fla-5'	10	Detection of the chromosome, probe
9	Bb-Fla-3'	10	Detection of the chromosome, probe
10	flgBaacC1-3+HindIIIB	AAGCTTATCTCGGCTTGAACGA	Construction of p28-1:: <i>flgBp-aacC1</i> , Probe, screening of transformants
11	flgBaacC1-5+HindIIIB	AATACCCAAGCTTCAAGGAAGATTTCTAT	Construction of p28-1:: <i>flgBp-aacC1</i> , probe
12	aacC1-5+NdeI	13	Screening of transformants, probe
13	lp28-1.15708F	TATTCTTACTAACTCAGACTGC	Construction of p28-1, probe
14	lp28-1.17377R	CATAAAAGGTGATGTTGG	Construction of p28-1, probe
15	lp25.4323F	ATTCATTTATCACTAGAGTTTG	Construction of p25, probe
16	lp25.6261R	AAGGTGTCGTAATAGGGC	Construction of p25, probe
17	PflaB-EcoRV	GATATCTGTCTGTGCGCTCTTGTGGCTTCCGG	Construction of p25:: <i>flaBp-aacC1</i>
18	aacC1-3+BstZ171	GTATACAAGCCGATCTCGGCTTGAACG	Construction of p25:: <i>flaBp-aacC1</i>

(Table 2) and cloned into pCR2.1-TOPO. The resulting plasmid was digested with EcoRV and BstZ17I, and the fragment containing *flaBp-aacC1* was ligated into SmaI-digested p25. The resulting plasmid, p25:*flaBp-aacC1*, was verified by PCR, sequencing, and restriction site mapping.

Southern blot analysis. For analysis of *B. burgdorferi* 297 derivatives, cultures of 297-c155 (wild type [WT]), 297/pBSV28-1, and 297/pBSV25 grown to a density of 5×10^7 cells/ml were pelleted, washed, and resuspended to a final concentration of 1×10^9 spirochetes/ml in 0.8% agarose in TN (10 mM Tris-Cl [pH 8.0], 1 mM EDTA, 100 mM NaCl). Plugs were incubated in a lysis solution (100 mM NaCl, 20 mM Tris-Cl [pH 8.0], 0.5% sodium dodecyl sulfate [SDS], 0.5 mg of proteinase K per ml, 100 mM EDTA) overnight at 37°C. Total DNA was resolved by contour-clamped homogenous electric field (CHEF) gel electrophoresis using a CHEF-DR III pulsed-field electrophoresis system (Bio-Rad, Hercules, Calif.). The parameters were set to resolve DNA molecules of 1 to 100 kb on a 0.8% agarose gel with a switch time of 0.1 to 10 s at 6 V/cm, as instructed by the manufacturer. After electrophoresis, the gel was transferred to a nylon membrane using the Turboblotter rapid downward transfer system (Schleicher & Schuell Bioscience, Keene, N.H.). Probes for the chromosome (*fla*), lp25 (BBE16), and lp28-1 (*vls*) were amplified by PCR using primers 4 through 9 (Table 2) and labeled with [α - 32 P]dATP using a random primer DNA labeling system (Invitrogen Life Technologies). Prehybridization and hybridization with probes were performed at 68°C as previously described (10).

For analysis of *B. burgdorferi* B31 derivatives, plasmid DNA was isolated (QIAGEN Maxi plasmid kit) and separated by electrophoresis through a 0.4% agarose gel. Equal amounts of DNA (400 ng) were loaded on the lanes. After visualization by ethidium bromide staining, the gel was bidirectionally blotted onto nylon membranes. Prehybridization and hybridization with [α - 32 P]dATP-labeled probes for lp28-1 (bp 15708 to 17377) and *flgBp-aacC1* or lp25 (bp 4323 to 6261) and *aacC1* without a promoter, as appropriate (primers listed in Table 2), were performed at 65°C as previously described (35).

Experimental mouse-tick infectious cycle. All animal experiments were performed using protocols approved by the institutions' Animal Care and Use Committees and according to the guidelines of the National Institutes of Health. Rocky Mountain Laboratories and the University of Connecticut Health Center are accredited by the International Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). To confirm the infectivity-associated phenotypes of the *B. burgdorferi* 297 plasmid-displaced clones, C3H/HeJ mice were infected by intradermal inoculation with 10^3 bacteria. Sera from mice at least 4 weeks postinoculation were diluted 1:1,000 and blotted against *B. burgdorferi* 297 lysates prepared from cultures grown at 37°C that had been transferred to nylon-supported nitrocellulose membranes. Blots were probed with a 1:50,000 dilution of horseradish peroxidase-conjugated goat anti-mouse antibody (Southern Biotechnology Associates, Birmingham, Ala.) and developed using the SuperSignal West Pico chemiluminescence substrate (Pierce, Rockford, Ill.).

For analysis of *B. burgdorferi* B31 clones, naive RML mice from a colony maintained at Rocky Mountain Laboratories (outbred Swiss Webster) were infected by intraperitoneal and subcutaneous inoculation with 5×10^3 bacteria as previously described (14, 17). Infection was assessed by seroconversion to P39 (41) and other *B. burgdorferi* antigens at 3 weeks postinoculation by xenodiagnosis using naive *Ixodes scapularis* larvae reared at Rocky Mountain Laboratories (40) and by reisolation from mouse tissues (14).

SDS-polyacrylamide gel electrophoretic analysis. *B. burgdorferi* cells cultured at 23 and 37°C or recovered from DMCs (1) were harvested by centrifugation at $8,500 \times g$, and the resulting pellets were washed twice with an equal volume of phosphate-buffered saline. Equivalent amounts of cells were resuspended, boiled in reducing Laemmli sample buffer (Bio-Rad), and separated through 2.4% stacking and 12.5% separating polyacrylamide mini-gels. Separated proteins were visualized by silver staining (27).

RESULTS

Selective displacement of plasmids lp28-1 and lp25 from *B. burgdorferi* clone 297-c155. To evaluate the roles of two infectivity-associated plasmids, lp25 and lp28-1, in strain 297, we chose to selectively displace these plasmids using a strategy based on the incompatibility of identical replicon regions (10, 45, 46). To eliminate lp25 or lp28-1 from WT 297-c155, cells were transformed with either pBSV25 (containing the replicon and compatibility region of lp25) or pBSV28-1 (containing the

replicon and compatibility region of lp28-1) (45) and selected in the presence of kanamycin.

Plasmid lp25 was displaced in all 297 transformants carrying pBSV25 (all 14 clones lost lp25, while 4 of 14 clones lost lp28-1), whereas lp28-1 was displaced in bacteria transformed with pBSV28-1 (all five clones lost lp28-1, and none of five clones lost lp25). The plasmid profiles of two clones, 297/pBSV28-1 and 297/pBSV25, were determined by PCR and compared to that of the parental 297-c155 (data not shown). We found that 297/pBSV25 no longer contained the native lp25, whereas lp28-1 was missing from 297/pBSV28-1 (Fig. 1A). A region specific to either pBSV25 (BBE21-*kan*) or pBSV28-1 (BBF26-*kan*) was amplified from the total DNA of clones 297/pBSV28-1 and 297/pBSV25 (Fig. 1A), providing further evidence that the elimination of the native plasmids was due to their incompatibility with the shuttle vectors. The plasmid contents of both of these transformants were otherwise unchanged from that of the parental clone, although the smallest linear plasmid, lp5, which can be lost spontaneously and is not relevant to infection in strain B31 (8), may have been lost in part of the 297/pBSV25 population (data not shown). Southern hybridization of total DNA with specific probes for these plasmids confirmed that lp25 and lp28-1 had been eliminated from 297/pBSV25 and 297/pBSV28-1, respectively (Fig. 1B).

Confirming the host-associated phenotypes of the *B. burgdorferi* 297 plasmid-displaced clones. In previous experiments, *B. burgdorferi* B31 mutants that had spontaneously lost lp25 were unable to adapt to and survive in the mammalian environment (22, 33), even when protected from the host immune system by cultivation within DMCs that had been implanted into the peritoneal cavities of rats (1, 32). Consistent with the results for B31, we found that the expression of *ospC* of the 297/pBSV25 (Δ lp25) mutant dramatically increased after a shift in temperature from 23 to 37°C in vitro, a hallmark of the *B. burgdorferi* temperature response, but the mutant did not grow in DMCs (Fig. 2) and was not able to infect mice (Table 3 and Fig. 3). Previous studies with strain B31 had found that naturally arising mutants lacking lp28-1 were able to survive in DMCs (32) but had attenuated infectivity in the mammalian host (22, 33). Correspondingly, we found that 297/pBSV28-1 (Δ lp28-1) was able to synthesize *OspC* in response to a temperature shift in vitro and able to grow in DMCs (Fig. 2). Furthermore, mice challenged with 297/pBSV28-1 mounted a response to several *B. burgdorferi* antigens before clearing the bacteria, suggesting that the loss of lp28-1 from strain 297 also resulted in attenuated infectivity (Table 3 and Fig. 3).

Restoration of plasmids lp28-1 and lp25 in *B. burgdorferi* B31. As demonstrated, loss of lp28-1 or lp25 from *B. burgdorferi*, whether naturally or experimentally, renders spirochetes unable to infect mice. Consequently, the reintroduction of these plasmids into *B. burgdorferi* clones that lack them should restore infectivity if plasmid loss were the only defect. To demonstrate this, we adopted the following strategy: an antibiotic resistance cassette was inserted into lp28-1 or lp25 in B31-A3, an infectious *B. burgdorferi* clone that contains both plasmids. The insertion was targeted to an innocuous site to avoid altering any critical genes. Marked plasmid DNA was prepared from these B31-A3 transformants and electroporated into noninfectious *B. burgdorferi* clones that only lacked either

FIG. 1. Displacement of native plasmids lp28-1 and lp25 in *B. burgdorferi* 297 by incompatible shuttle vectors. (A) PCR analysis of plasmid DNA from WT 297-c155 and transformants 297/pBSV25 and 297/pBSV28-1 using primer sets specific for shuttle vectors pBSV25 (BBE21-kan) (primers 1 and 2) and pBSV28-1 (BBF26-kan) (primers 1 and 3) or endogenous plasmids lp28-1 (*vls*) (primers 4 and 5) and lp25 (BBE16) (primers 6 and 7) (see Table 2 for primer information). Because of the redundancy in the *vlsE* region of strain 297, the *vls*-specific primers generate three products, all of which are within the *vls* locus and all of which are missing from 297/pBSV28-1. (B) Southern hybridization of total genomic DNA from *B. burgdorferi* 297 clones confirmed that the expected plasmid had been selectively eliminated from the transformants. Probes for the chromosome (*fla*) (primers 8 and 9), lp25 (BBE16), (primers 6 and 7), or lp28-1 (*vls*) (primers 4 and 5; all three amplicons were used) were radioactively labeled and hybridized to total genomic DNA from 297-c155, 297/pBSV25, and 297/pBSV28-1 resolved by pulsed-field gel electrophoresis. The migration positions of molecular size standards are indicated to the left of the gels.

lp28-1 or lp25. The resulting antibiotic resistant transformants were characterized for plasmid content and infectivity.

The insertion site for the gentamicin resistance cassette on the WT B31-A3 lp28-1 was within the ORF BBF29 (Fig. 4A), which carries a frameshift mutation (www.tigr.org) and probably is a pseudogene that does not encode a functional

polypeptide (8). Transformation of B31-A1 (lacking lp28-1) (14) with total plasmid DNA of clone B31-A3 lp28-1-Gm yielded transformant B31-A1 lp28-1-Gm. The average transformation frequency was 4.7×10^{-8} (transformants per transformed bacteria), with an average transformation efficiency of 0.55 (transformants per microgram of transforming DNA). PCR and Southern blot analyses confirmed that B31-A3 lp28-1-Gm and B31-A1 lp28-1-Gm contained lp28-1 with a gentamicin resistance cassette inserted into the plasmid (Fig. 5A).

To restore lp25 to clone B31-A44, we inserted a selectable marker conferring gentamicin resistance onto the WT B31-A3 lp25 in the intergenic region between ORFs BBE05 and BBE06 (Fig. 4B). Both ORFs are less than 150 nucleotides and are overlapped by a pseudogene, BBE05.1 (8); when analyzed in an array, BBE06 was expressed at neither 23 nor 35°C (30). We transformed B31-A44 (lacking lp25) with total plasmid DNA of B31-A3 lp25-Gm, resulting in transformant B31-A44 lp25-Gm. Plasmid DNA of A3 lp25-Gm transformed with a transformation frequency of 3.8×10^{-8} and a transformation efficiency of 0.23. The structure of lp25::*flaBp-aacC1* in the transformants was confirmed by PCR and Southern blot analyses (Fig. 5B).

Proficiency of *B. burgdorferi* B31 clones in the experimental mouse-tick infectious cycle. To demonstrate restoration of an infectious phenotype by reintroducing a plasmid essential for infectivity, we tested clones B31-A1 (lacking lp28-1), B31-A1 lp28-1-Gm, and B31-A3 lp28-1-Gm in mice by inoculation with a needle. We included B31-A3 lp28-1-Gm as a WT control to ensure that the marker was inserted into a nonessential site on lp28-1. Mice inoculated with all three clones seroconverted to P39 and other *B. burgdorferi* antigens, but only clones containing lp28-1 could be reisolated from mouse tissues and were acquired by feeding larval ticks (Table 4).

To confirm that clones containing lp28-1::*flaBp-aacC1* properly express *vlsE*, which is thought to be essential for infection, we tested mouse sera from needle-inoculated mice using a commercially available enzyme-linked immunosorbent assay (ELISA) kit specific for the C6 peptide of VlsE (Immunetics, Cambridge, Mass.). Sera from mice inoculated with B31-A1 (lacking lp28-1) were negative by ELISA, as expected, whereas sera from mice inoculated with B31-A1 lp28-1-Gm or B31-A3 lp28-1-Gm were positive (data not shown). Proficient infection of mice by clone B31-A3 lp28-1-Gm showed that the site chosen for insertion of the gentamicin resistance cassette represented a site nonessential for infectivity and that the insertion did not interfere with *vlsE* expression. The phenotypes of clones B31-A1 and B31-A1 lp28-1-Gm in mice demonstrated that the lack of lp28-1 rendered clone B31-A1 noninfectious and that restoration of lp28-1 fully restored infectivity.

We also tested clones B31-A44 (lacking lp25), B31-A44 lp25-Gm, and B31-A3 lp25-Gm for their proficiency in the mouse. Clone B31-A44 was unable to establish an infection in mice (Table 5). In contrast, mice inoculated with B31-A44 lp25-Gm or B31-A3 lp25-Gm seroconverted to *B. burgdorferi* antigens; spirochetes were acquired by larval ticks upon feeding and were isolated from mouse tissues (Table 5). This showed that the site used for insertion of *flaBp-aacC1* did not interfere with any essential functions of lp25, that the absence of lp25 rendered clone B31-A44 noninfectious, and that res-

FIG. 2. *B. burgdorferi* 297 clones grown in vitro at different temperatures or in DMCs. *B. burgdorferi* 297/pBSV25 (Δ lp25) and 297/pBSV28-1 (Δ lp28-1) were able to differentially express *ospC* in a temperature-inducible manner comparable to that of WT 297-c155, but only 297-c155 and 297/pBSV28-1 were able to grow in DMCs. Cell lysates from *B. burgdorferi* clones cultivated at 23 and 37°C after a temperature shift or clones grown in DMCs were separated by SDS-polyacrylamide gel electrophoresis and silver stained. The positions of OspA and OspC are indicated by arrows to the right of the gel. The migration positions of molecular mass standards are shown to the left of the gel.

toration of lp25 restored proficiency in mice and transmission to ticks.

DISCUSSION

The essential natures of linear plasmids lp28-1 and lp25 for infectivity of *B. burgdorferi* type strain B31 in mice have been recognized for several years (14, 22, 23, 32, 33). To date, the presumed roles of these plasmids were based largely on correlations between a B31 clone with a certain “naturally derived” plasmid profile and its in vivo phenotype. Plasmid lp28-1 carries the *vlsE* cassette, which undergoes antigenic variation and is presumably required for establishing a persistent infection in mice; furthermore, *B. burgdorferi* B31 lacking lp28-1 exhibits an attenuated infection in mice (14, 22, 23, 28, 33, 51, 52). *B. burgdorferi* B31 lacking lp25 was shown to be cleared from the mammalian host without eliciting an immune response (23, 33). Purser et al. (32) further determined that the BBE22 gene on lp25 encodes a nicotinamidase that is essential and sufficient for infectivity of *B. burgdorferi* lacking lp25 in mice. In this study, we have experimentally addressed the roles

of lp28-1 and lp25 of *B. burgdorferi* for infectivity in mice. We have extended the study of the roles of these plasmids to an additional strain, 297. We took a novel approach in that we displaced the endogenous plasmid in an infectious 297 clone by transformation with an incompatible shuttle vector and ana-

FIG. 3. Immunoblot analysis of representative mouse sera against *B. burgdorferi* 297 whole-cell lysates. Mice were challenged with either WT 297-c155, 297/pBSV25 (Δ lp25), or 297/pBSV28-1 (Δ lp28-1). Both plasmid-displaced clones were noninfectious to mice, although 297/pBSV28-1 elicited an immune response to a few *B. burgdorferi* antigens. The migration positions of molecular mass standards are indicated to the left of the gel.

TABLE 3. Infectivity of *B. burgdorferi* clones 297-c155, 297/pBSV25, and 297/pBSV28-1 in mice

Clone	No. of mice infected/no. analyzed	
	Seroconversion ^a	Reisolation
297-c155 (WT)	5/5	5/5
297/pBSV25 (Δ lp25)	0/5	0/5
297/pBSV28-1 (Δ lp28-1)	5/5 ^b	0/5

^a Seroconversion of mouse sera was assessed by immunoblot analysis with *B. burgdorferi* whole-cell lysate.

^b We observed a response to a small number (<4) of *B. burgdorferi* antigens in all mice challenged with this clone.

FIG. 4. Schematic diagram of *B. burgdorferi* B31 plasmid regions used for insertion of a selectable marker by allelic exchange. The gene identification numbers are shown in accordance with the database designation (16). The positions of primers used (Table 2) (arrowheads) and relevant restriction sites used for plasmid construction and Southern blot analysis (Fig. 5) are indicated. (A) Depiction of the marker insertion site on lp28-1 that creates lp28-1::*flgB_p*-*aacC1*. (B) Depiction of the marker insertion site on lp25 that results in lp25::*flaB_p*-*aacC1*.

lyzed the *in vivo* phenotypes of the resulting transformants. In parallel, we restored plasmids lp28-1 and lp25 in noninfectious B31 clones and tested them for infectivity in mice.

Stewart et al. (45, 46) and Eggers et al. (10) developed shuttle vectors carrying elements sufficient for stable replication in *B. burgdorferi* (and in *E. coli*). The *B. burgdorferi* maintenance and replication sequences are unique to each plasmid and confer incompatibility with the endogenous plasmid. The genes belonging to two paralogous gene families, 32 and 49, may function as pairs in determining plasmid incompatibility (8, 10, 44–46). Plasmid incompatibility makes the shuttle vectors valuable tools to directly assess the roles of plasmids, which we demonstrate here for the first time. We also show that plasmid maintenance and incompatibility functions, although unique for each plasmid, are functional across strains, which makes a shuttle vector derived from one strain incompatible with the cognate plasmid in another strain (10). Plasmid incompatibility is generally defined by the replication-partition region of a plasmid. Therefore, the gene content of the remainder of the plasmid does not seem to contribute to incompatibility and could vary significantly between strains. Hence, the techniques described in this paper can be used to confirm or identify the roles of plasmids even in unsequenced isolates.

In this study, we displaced *B. burgdorferi* plasmids by transforming an infectious strain 297 WT clone with shuttle vectors, pBSV28-1 and pBSV25, derived from plasmids in strain B31

(45). *B. burgdorferi* 297 clones in which lp28-1 was displaced elicited an immune reaction in mice but were unable to establish a persistent infection. Spirochetes lacking lp28-1 could grow in DMCs, protected from the host immune system. This result was consistent with previous data from experiments with *B. burgdorferi* B31 clones naturally lacking lp28-1, which were attenuated during infections in mice (14, 22, 23, 28, 33, 51, 52). The lp28-1 plasmids of B31 and 297 differ in at least several loci (20), so it was not a foregone conclusion that plasmid loss would lead to identical phenotypes. Additionally, the phenotypic similarities of the two lp28-1 mutants suggest a parallel function for the *vls* regions *in vivo*, despite the absence of direct repeats in the 297 *vls* locus (M. J. Caimano, C. H. Eggers, and J. D. Radolf, unpublished data), which means that the mechanism for the generation of sequence diversity in this isolate may be different from the predicted mechanism for the B31 *vls* locus (21, 52).

Clones of strain 297 lacking lp25 were not infectious to mice and were incapable of surviving in DMCs. These data were consistent with results obtained with *B. burgdorferi* B31 clones naturally lacking lp25 (14, 22, 23, 32, 33, 51, 52) and with the presence of the 297 BBE22 paralog encoded on that isolate's lp25 (data not shown). The concordance of the *in vivo* phenotypes exhibited by our engineered 297 mutants with those observed for the spontaneous B31 mutants further demonstrates the effectiveness and utility of introducing plasmid incompat-

FIG. 5. Southern blot analysis of *B. burgdorferi* B31 clones for the presence of plasmid lp28-1 or lp25. (A) Plasmid DNA was probed with PCR products from lp28-1 (primers 13 and 14, spanning BBF27 to BBF29) or *flgB_p-aacC1* (primers 10 and 11) (see Table 2 for primer information). The low intensity of the bands in B31-A3 lp28-1-Gm lanes 2 is due to the smaller amount of DNA loaded. (B) Plasmid DNA was probed with PCR products from lp25 (primers 15 and 16, spanning BBE03 through BBE07) or *aacC1* (primers 10 and 12) (see Table 2 for primer information). The additional band hybridizing with the lp25-specific probe is due to sequence homology of the BBE03-BBE07 probe fragment with *B. burgdorferi* plasmid lp17. The migration positions of molecular size standards are indicated to the left of the gel.

ibility determinants to generate desired plasmid-displaced mutants and assess infectivity.

Information about plasmids lp25 and lp28-1 of *B. burgdorferi* strain 297 is limited (10, 20, 48, 49). However, our partial sequence analysis of the regions of these 297 plasmids that determine incompatibility (encompassing members of the paralogous gene families 32 and 49) indicates that they are ~99% identical to the respective lp25 or lp28-1 genes from strain B31 present on the shuttle vectors (GenBank accession numbers AY675217 and AY675218). Consistent with conservation of sequences that confer incompatibility between anal-

TABLE 4. Infectivity of *B. burgdorferi* clones B31-A1, B31-A3 lp28-1-Gm, and B31-A1 lp28-1-Gm

Clone	No. of mice infected/no. analyzed		No. of ticks infected/no. analyzed
	Seroconversion ^a	Reisolation	
B31-A1 (lacking lp28-1)	4/4	0/4	0/9 ^b
B31-A3 lp28-1-Gm	1/1	1/1	3/3
B31-A1 lp28-1-Gm	4/4	4/4	12/12

^a Seroconversion of mouse sera was assessed by immunoblot analysis with *B. burgdorferi* whole-cell lysate and P39.

^b Ticks were allowed to feed on three mice.

ogous plasmids from different strains, lp25 or lp28-1 were displaced in all of the 297 transformants carrying pBSV25 or pBSV28-1, respectively, whereas most pBSV2 transformants retained both plasmids. In addition, the comparable in vivo phenotypes of our 297 displacement mutants and plasmid-deficient B31 clones strongly suggest that ORFs encoding similar essential functions occur on the same plasmid in both strains.

To demonstrate that restoring entire plasmids is feasible and to confirm that the noninfectious phenotype in plasmid-deficient clones was due to the lack of a specific plasmid, we complemented the plasmid-deficient genotype by transforming B31 clones lacking either lp28-1 or lp25 with the appropriate plasmid engineered to confer antibiotic resistance. Selectable markers were targeted to presumably innocuous sites to avoid deleterious effects on intact genes. This complementation restored the ability to persistently infect mice in previously noninfectious clones, proving the hypothesis that the absence of lp28-1 or lp25 rendered *B. burgdorferi* noninfectious to mice. Furthermore, this demonstrates that competent *B. burgdorferi* cells can be transformed with functional linear plasmids of up to 28 kb, thereby reestablishing a WT phenotype.

Plasmid DNA of both A3 lp28-1-Gm and A3 lp25-Gm transformed *B. burgdorferi* at a transformation frequency (fraction of bacteria surviving the electroporation that are transformed) and efficiency (transformants per microgram of transforming DNA) approximately 10-fold lower than those of shuttle vectors pBSV28-1 and pBSV25 (45). However, if the transformation efficiency with total borrelia plasmid DNA is normalized for the actual amount of relevant plasmid DNA, the *B. burgdorferi* transformation efficiencies of lp25-Gm and lp28-1-Gm were slightly higher than the efficiencies of the shuttle vectors.

Plasmids lp25 and lp28-1 are relatively unstable during in vitro propagation but are essential for mouse infectivity (3, 17,

TABLE 5. Infectivity of *B. burgdorferi* clones B31-A44, B31-A3 lp25-Gm, and B31-A44 lp25-Gm

Clone	No. of mice infected/no. analyzed		No. of ticks infected/no. analyzed
	Seroconversion ^a	Reisolation	
B31-A44 (lacking lp25)	0/4	0/4	ND ^b
B31-A3 lp25-Gm	4/4	4/4	12/12
B31-A44 lp25-Gm	3/6	3/6	9/9 ^c

^a Seroconversion of mouse sera was assessed by immunoblot analysis with *B. burgdorferi* whole-cell lysate and P39.

^b ND, not determined.

^c Ticks were allowed to feed on three seropositive mice.

22, 23, 28, 36, 38, 42, 51). This can be a problem during genetic manipulation of *B. burgdorferi* to generate mutants or complemented clones for in vivo analyses. The strains and techniques described in this study present a means to circumvent this shortcoming of the genetic system. Exploiting the unusual genomic architecture of *B. burgdorferi* to both displace and restore entire plasmids should provide important tools for elucidating the roles of the extrachromosomal elements of *B. burgdorferi* in its complex life cycle by fulfilling molecular Koch's postulates. We are currently using this technique to assess the roles of other endogenous plasmids in the infectious cycle of this spirochete.

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