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Conservation of Plasmid Maintenance Functions between Linear and Circular Plasmids in *Borrelia burgdorferi*

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The Lyme disease agent *Borrelia burgdorferi* maintains both linear and circular plasmids that appear to be essential for mammalian infection. Recent studies have characterized the circular plasmid regions that confer autonomous replication, but the genetic elements necessary for linear plasmid maintenance have not been experimentally identified. Two vectors derived from linear plasmids lp25 and lp28-1 were constructed and shown to replicate autonomously in *B. burgdorferi*. These vectors identify internal regions of linear plasmids necessary for autonomous replication in *B. burgdorferi*. Although derived from linear plasmids, the vectors are maintained in circular form in *B. burgdorferi*, indicating that plasmid maintenance functions are conserved, regardless of DNA form. Finally, derivatives of these vectors indicate that paralogous gene family 49 is apparently not required for either circular or linear plasmid replication.

Lyme disease is the leading arthropod-borne disease in the United States. The causative agent of Lyme disease, *Borrelia burgdorferi*, has an unusual genomic structure composed of a linear chromosome and the largest plasmid complement of all characterized bacteria (3, 11). The advantages conferred by a segmented genome and linear replicons are not clearly understood. However, this type of genomic structure appears to have occurred early and successfully in the evolution of the genus *Borrelia*, since relapsing fever spirochetes, such as *B. hermsii* and *B. turicatae*, also maintain linear and circular replicons (10, 18, 29, 42).

Abundant evidence indicates that the extrachromosomal elements of *Borrelia* species are essential to their life cycles. The relapsing fever spirochetes have elaborate genetic systems for antigenic variation encoded on linear plasmids, as does *B. burgdorferi*, albeit on a reduced scale (32, 47). The more extensively characterized plasmids of *B. burgdorferi* also have loci coding for sugar transporters, nucleotide synthesis, outer surface protein (Osp) A, B, C, and EF-related proteins (which have been implicated in evasion of the mammalian complement system), and collagen fiber adhesins (11, 14, 15, 17, 21, 24, 25, 35, 41). In addition, a correlation between the loss of certain plasmids and loss of infectivity has been observed, further supporting the requirement for some plasmids in vivo (22, 30, 37, 46).

The genome sequence of *B. burgdorferi* strain B31 includes 21 plasmids (3, 11). Due to the large number and various forms of plasmids, a systematic nomenclature has been developed. Circular plasmid names begin with cp, and linear plasmids begin with lp; plasmid designations end with a number denoting the approximate size in kilobases (e.g., cp9, lp25). Different plasmids that exist in the same size and form are further delineated by a hyphen followed by consecutive numbers (e.g., lp28-1, lp28-2).

Despite the large number of plasmids and their significance to the life cycle of *B. burgdorferi*, relatively little is known concerning plasmid maintenance in the borreliae. Hinnebusch and Barbour demonstrated that the plasmids of *B. burgdorferi* are maintained at a 1:1 ratio with the chromosome (16). Subsequently, Picardeau et al. used CG skew analysis to predict a bidirectional mode of replication from an internal origin for the plasmids, and they experimentally demonstrated this for the linear chromosome (27, 28). Although these initial studies indicated that *B. burgdorferi* maintains strict control of plasmid copy number and replication, the details of these mechanisms are unknown. Indeed, it was uncertain whether linear and circular plasmids utilize the same maintenance functions, although linear plasmid replication has been shown to use a telomere resolution step (4, 20).

The first experimental evidence identifying genetic elements involved in plasmid maintenance was the development of a *B. burgdorferi-Escherichia coli* shuttle vector designated pBSV2 (43). This shuttle vector utilizes a 3.3-kb region of the endogenous 9-kb circular plasmid (cp9) to produce a stable replicon in *B. burgdorferi*. Subsequently, Eggers et al. constructed another shuttle vector from the corresponding region of a cp32 and identified a locus that conferred incompatibility in *B. burgdorferi* (8). These two shuttle vectors contain members of large paralogous gene families (PF) widely distributed across the *B. burgdorferi* plasmids (3). Due to their ubiquity among the plasmids, these PF have been proposed to function in plasmid maintenance (7, 11, 39, 48). The two shuttle vectors experimentally confirmed that several members of these PF contribute to circular plasmid maintenance.

Both shuttle vectors were derived from circular plasmids, members of the cp32 plasmid family. Although most of the cp32 family members are approximately 32 kb in size and homologous to each other, two derivatives differ in size. In

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addition to the seven members of 32 kb size, another full copy of a cp32 has integrated into lp56, and cp9 is a smaller deletion derivative (3, 11). Hence, both shuttle vectors were derived from related circular plasmids that may be capable of sharing some functions in *trans*. However, elements necessary for linear plasmid maintenance have not been previously characterized.

To address specific issues of linear plasmid replication and to avoid the potential complications of studying plasmid maintenance genes in the highly similar cp32 family, we focused on linear plasmids lp25 and lp28-1 to construct circular vectors capable of autonomous replication in *B. burgdorferi*. Our data support an internal origin of replication for linear plasmids of *B. burgdorferi*. These results suggest that *B. burgdorferi* evolved a single system for linear and circular plasmid replication and segregation, and the main difference between them may lie in how replicated plasmid forms are resolved. Finally, these studies indicate that the proteins encoded by PF 49 do not appear to be required for either circular or linear plasmid replication.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *B. burgdorferi* strains were grown in liquid BSK-II at 35°C or in solid BSK medium incubated at 35°C under 1% CO_2 (33). Strain B31 (ATCC 35210) was originally isolated from a tick collected on Shelter Island, N.Y. (1). The genomic sequence of *B. burgdorferi* B31 culture MI has been determined (3, 11). Strain B31-A*chbC*72 is a culture-attenuated, noninfectious derivative of clone B31-A, and it lacks lp56 and lp25 as well as other plasmids (45). *B. burgdorferi* strain A3 is an infectious clone derived from B31 MI (9). TOP10 (Invitrogen, Carlsbad, Calif.) was the strain used in *E. coli* plasmid manipulations.

Construction of vectors. The strategy for vector construction is shown in Fig. 1 and is essentially the same as previously described (43). Briefly, regions putatively involved in plasmid maintenance were PCR amplified using the Expand Long Template PCR system (Roche Molecular Biochemicals, Indianapolis, Ind.). Templates for the PCR amplifications were either genomic DNA of *B. burgdorferi* or *E. coli* plasmid DNA. Oligonucleotide primers used in this study are presented in Table 1 and below in Fig. 4. Amplified fragments were cloned into pCR-XL-TOPO (Invitrogen). DNA fragments of interest were then isolated by digestion with the relevant restriction enzymes (Table 1), gel purified by electroelution, and subcloned into compatible sites of the vector pOZK (43). Large-scale plasmid DNA isolations were performed with the QIAfilter Plasmid Maxi kit (QIAGEN, Valencia, Calif.). Vector pE18::gnt was constructed by first deleting the multiple cloning site of pBSV25 (to remove the *Eco*RI site) and subcloning the gentamicin resistance cassette into the remaining *Eco*RI site present in *bbe18* (9). The *bbe18* gene is 579 bases long, and the *Eco*RI cleavage site is located at base 357.

DNA sequencing and analysis. Nucleotide sequences were determined with the ABI Big Dye Terminator cycle sequencing ready reaction kit (PE Applied Biosystems, Foster City, Calif.), using an ABI 3700 DNA sequencer. Nucleotide sequences were analyzed with DNAstar software (DNAstar, Madison, Wis.).

Electroporation of *B. burgdorferi* and identification of transformants. *B. burgdorferi* strains were transformed by electroporation as previously described (36, 43). *B. burgdorferi* colonies grown on selective medium were stabbed with a sterile toothpick and inoculated into 20-μl PCR mixtures to amplify the kanamycin resistance cassette as previously described (43). Colonies positive by PCR were aspirated from the agar plate, transferred to liquid BSK, and incubated at 35°C for ~5 days.

Total genomic DNA was isolated from 20-ml cultures of *B. burgdorferi* with the QIAGEN Genomic-tip 20/G kit (QIAGEN) or from 5-ml cultures by using the Wizard genomic DNA purification kit (Promega, Madison, Wis.). Genomic DNA was separated on a 0.3% agarose gel and visualized by ethidium bromide staining. Total genomic DNA from transformants was used to transform *E. coli* and recover vectors.

Vector incompatibility with endogenous *B. burgdorferi* plasmids. *B. burgdorferi* transformants were further characterized for plasmid content by PCR screening with plasmid-specific primers. Primers used were designed by Purser and Norris (30) or by D. Akins as described by Elias et al. (9). PCR cycling parameters were an initial denaturation at 94°C (5 min), followed by 30 cycles of 94°C (30 s), 50 or 55°C (30 s), and 72°C (1 min), with a final extension at 72°C (7 min).

Stability of vectors in *B. burgdorferi*. Stability of shuttle vectors was determined as previously described (43). Randomly chosen transformants of B31-A were cultured in 5 ml of liquid BSK at 34°C in the presence or absence of 200 μg of kanamycin/ml. Cultures were inoculated to a starting concentration of ~4×10^5 spirochetes/ml and grown to >10^8 spirochetes/ml. Cells were counted by dark-field microscopy with a Petroff-Hauser counting chamber. After 11 such serial passages (~90 generations), cultures were plated in the absence of selection and 20 colonies from each condition were PCR screened for the presence of the kanamycin resistance cassette. The stability of lp25 was assessed by selecting colonies PCR positive for lp25 and inoculating them into 5 ml of liquid BSK. Cultures were treated as described above, except no antibiotic selection was imposed. After 90 generations, cultures were plated and PCR screened for the presence of lp25.

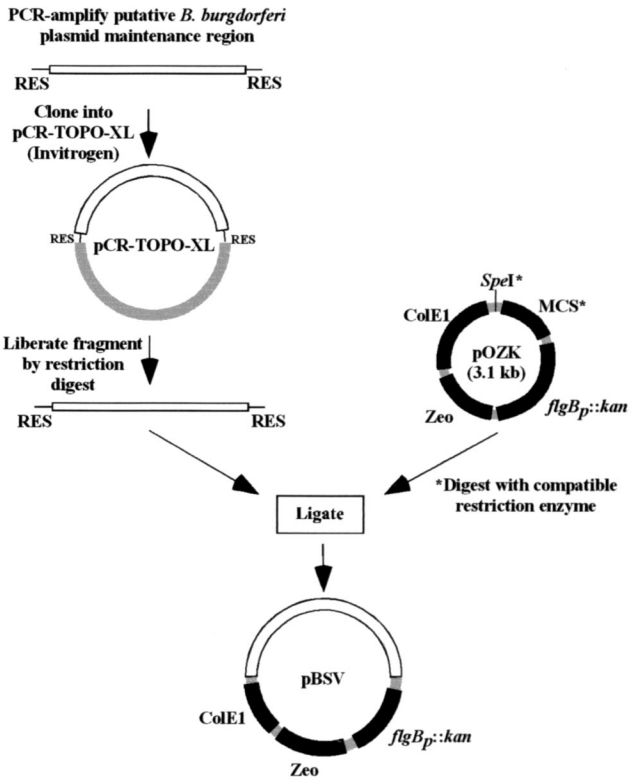

FIG. 1. Strategy for construction of vectors used in this study. *B. burgdorferi* plasmid regions putatively involved in plasmid maintenance were PCR amplified from genomic DNA or from previously constructed plasmids. Primers used for the PCR amplification were designed with restriction enzyme sites (RES) for subsequent cloning steps. Fragments were cloned into pCR-TOPO-XL, liberated by specific restriction enzyme digestion, and subcloned into compatible restriction enzyme sites of pOZK, either in the multiple cloning site (MCS) or into a unique *Spe*I site upstream of the MCS (43). Vector pOZK contains a kanamycin resistance cassette that functions in both *E. coli* and *B. burgdorferi*. Resulting vectors were tested for their ability to autonomously replicate in *B. burgdorferi*. ColE1, *E. coli* origin of replication; Zeo, zeocin resistance marker; $flgB_p$::*kan*, *B. burgdorferi* *flgB* promoter fused to the kanamycin resistance gene.

RESULTS

Construction and transformation of vectors. Members of five PF are present in various combinations on all *B. burgdorferi* plasmids and have been proposed to be involved in plasmid

TABLE 1. Oligonucleotides used in this study

Name[a]	Sequence[b]	Purpose
3′.ORF3.*Xba*I (A)	TCTAGAGCCCTATGGATTTAAGAACTG	Construction of pBSV2
ORF3.RC.*Hin*dIII (A1)	AAGCTTCCTAATCACTAAATTTCTTAC	Construction of pOZK-1–3
ORF3.*Xho*I.stop (B)	CTCGAGGCATCAT**TT**AACTAGTAAATTTGG	Construction of pOZK-3Δ
BBC02.*Hin*dIII (C)	AAGCTTTGCAACATTTTCCTTAATTCAT	Construction of pOZK-1–2
BBC01.*Xba*I (D)	TCTAGATTACGATCCAATATCAAGTAGC	Construction of pOZK-1–3 and pOZK-3Δ
9026.*Xba*I (E)	GTCTAGACTTGACTGCTTATTCCGGGTAATTTC	Construction of pOZK-1–2
1p25.15702.*Xba*I (F)	TCTAGAGTTGTATCAAGGGATATTGCC	Construction of pBSV25
1p25.9932.*Xba*I (G)	TCTAGACATCTGCACGATAACCTGTCG	Construction of pBSV25
1p28-1.15748.*Xba*I	CCTCTAGAGAGTCCTCTAGTGAGTTGTGC	Construction of pBSV28-1.Aut4
1p28-1.12165.*Xba*I	CGTCTAGAGCAAGGGTAAAATAAATTCAAG	Construction of pBSV28-1.Aut4
1p28-1.8292.*Xba*I	CCTCTAGAGCGAATTTCTTTTGATGAA	Construction of pBSV28-1.Aut3
1p28-1.5598.*Xba*I	GCTCTAGAGAATCGGAGAAAATGTTTACC	Construction of pBSV28-1.Aut3

[a] Letters in parentheses refer to oligonucleotides indicated in Fig. 4.
[b] Relevant restriction sites are underlined. Bolded letters indicate nucleotides that were altered to produce a stop codon.

maintenance (7, 11, 39, 48). However, only genetic elements involved in circular plasmid maintenance have been experimentally characterized (8, 43). To identify regions responsible for linear plasmid maintenance in *B. burgdorferi*, we focused on lp25 and lp28-1, both of which are associated with mammalian infectivity but not required for in vitro cultivation (22, 30, 37, 46).

The PF members putatively involved in plasmid maintenance are located in a single, contiguous region on lp25, whereas lp28-1 has two subsets of these gene families (Fig. 2). One region of lp28-1 consists of four open reading frames (ORFs; designated the Aut 4 region), and the other region consists of three ORFs (Aut 3 region). The Aut 3 region contains a member of PF 49, *bbf12*, that has a premature stop codon 40 amino acids from the amino terminus of the protein (Fig. 2), as reported by The Institute for Genomic Research (3, 11). A second start codon is present 46 nucleotides downstream from the premature stop codon that could result in a truncated protein of 140 amino acids. Vectors were constructed from these three regions potentially involved in plasmid maintenance (lp25, lp28-1 Aut 3, and lp28-1 Aut 4) (Fig. 2). Transformation of *B. burgdorferi* with these vectors demonstrated that the lp28-1 Aut 4 and lp25 regions conferred autonomous replication in *B. burgdorferi*, whereas the lp28-1 Aut 3 region did not (Fig. 3 and data not shown). Hence, all subsequent experiments were performed with the vectors derived from lp28-1 Aut 4 and lp25. These *Borrelia* shuttle vectors were designated pBSV25 and pBSV28-1, denoting the plasmid from which they were derived. The vectors, shown in Fig. 3A, are capable of autonomous replication in *E. coli* and *B. burgdorferi*

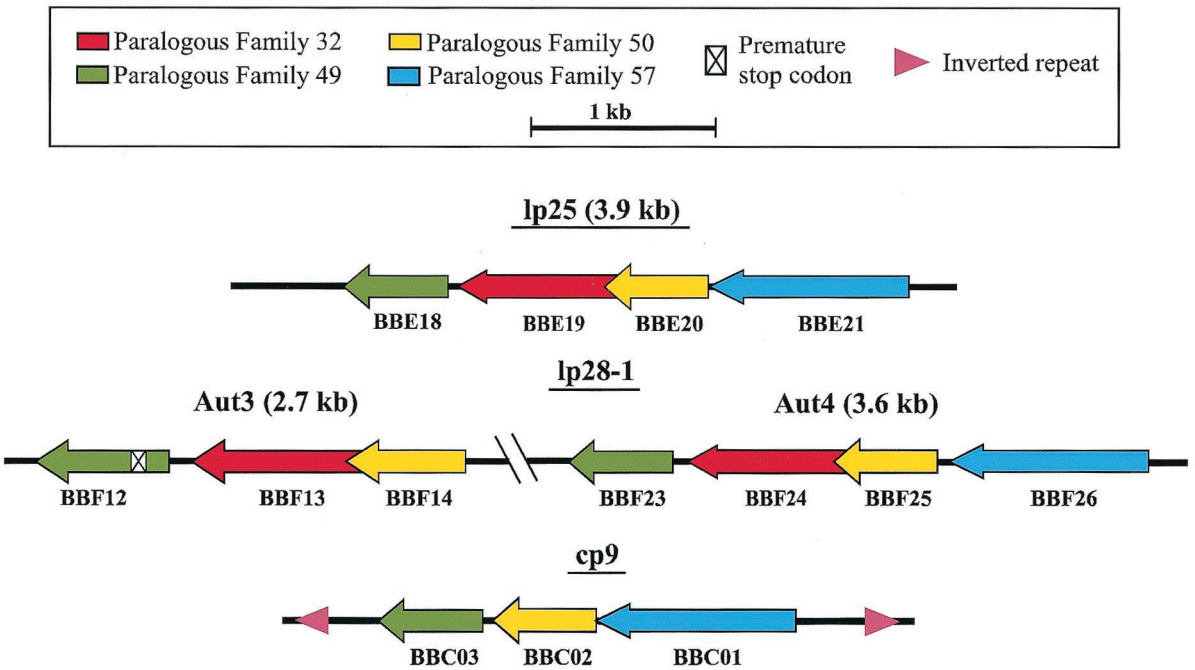

FIG. 2. PF from *B. burgdorferi* plasmids putatively involved in plasmid maintenance. These regions were tested for their ability to autonomously replicate in *B. burgdorferi*. Two sets of PF members are present on lp28-1. The region of cp9 conferring autonomous replication was previously characterized (43).

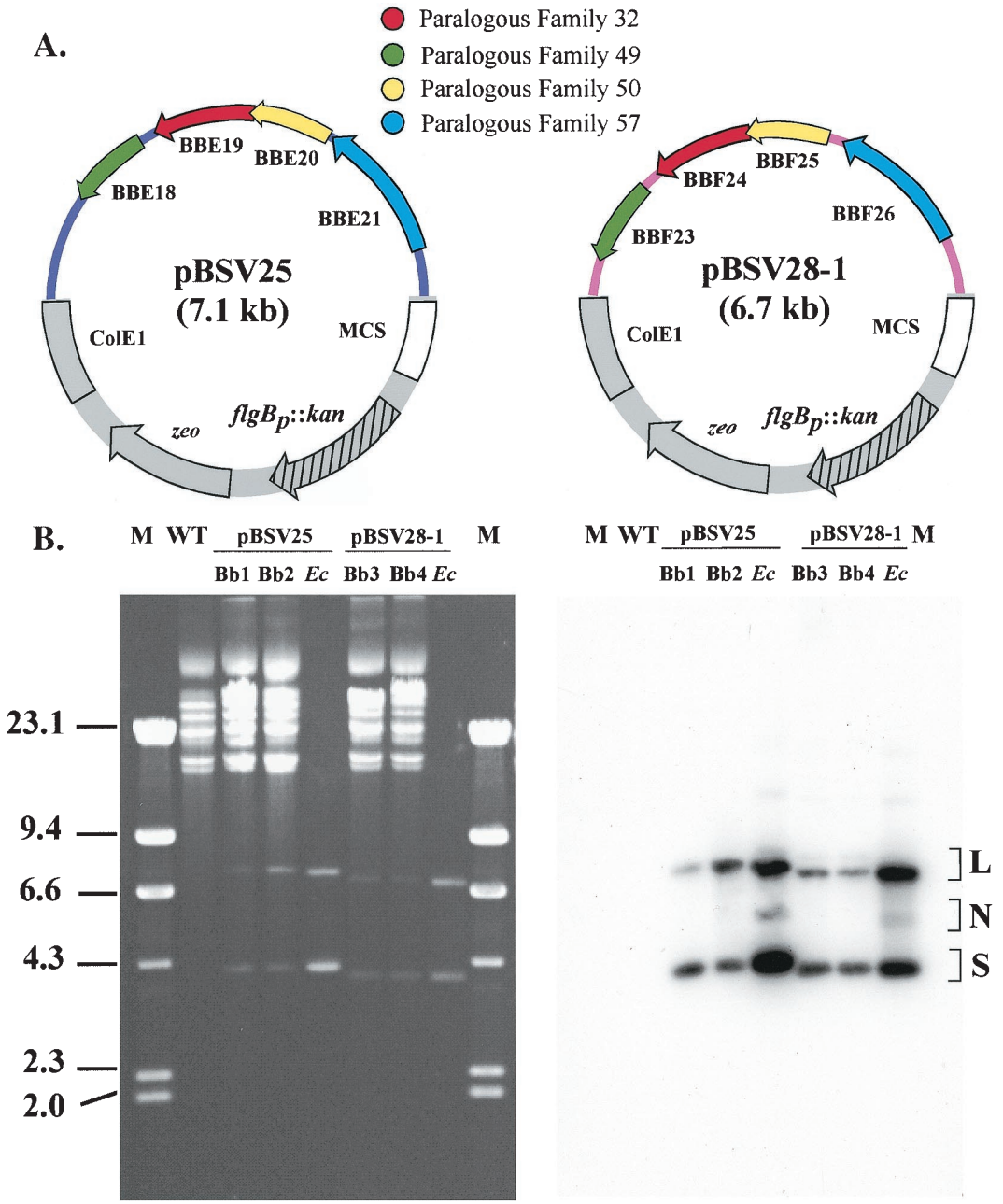

FIG. 3. Vectors derived from linear plasmids autonomously replicate in *B. burgdorferi*. (A) Plasmid maps of pBSV25 and pBSV28-1. ColE1, *E. coli* plasmid origin of replication; MCS, multiple cloning sites; $flgB_p$::*kan*, *B. burgdorferi flgB* promoter fused to the kanamycin resistance gene. (B) *B. burgdorferi* plasmid DNA separated on a 0.3% agarose gel and stained with ethidium bromide (left panel) and the corresponding Southern blot hybridized with the radiolabeled kanamycin gene (right panel). Molecular size standards (in kilobases) of *Hin*dIII-digested lambda DNA are given on the left; brackets on the right indicate the autonomously replicating vectors pBSV25 and pBSV28-1 and are consistent with linear (L), nicked (N), and supercoiled (S) plasmid forms. The parental A3 (WT), two transformants of each vector, and the corresponding plasmid DNA isolated from *E. coli* are shown.

(Fig. 3B and Table 2). Vectors could be recovered by transforming *E. coli* with total genomic DNA from *B. burgdorferi* transformants (data not shown). Both pBSV25 and pBSV28-1 had transformation frequencies and efficiencies similar to those of pBSV2 in noninfectious clone B31-A*chbC*72 and infectious clone B31 A3 (Table 2). Sequence analysis of pBSV25 identified a single nucleotide substitution in *bbe21*, relative to the published sequence, that resulted in the amino acid change I143T (11).

Plasmid incompatibility in *B. burgdorferi*. Incompatibility of *B. burgdorferi* plasmids carrying identical maintenance sequences was previously demonstrated for circular plasmids (8, 43). However, incompatibility functions on lp25 and lp28-1 are more difficult to assess due to the presence of a putative re-

TABLE 2. Plasmid stabilities and transformation frequencies and efficiencies in *B. burgdorferi* infectious clone A3 (9) and noninfectious clone B31-A*chbC*72 (45)

Plasmid	Transformation frequency[a]		Transformation efficiency[b]		Stability[c] (%)	
	A3	B31-A*chbC*72	A3	B31-A*chbC*72	+ Kan	− Kan
pBSV2	5.7×10^{-7}	2.8×10^{-4}	7	2,207	100	100
pBSV25	3.6×10^{-7}	9.7×10^{-5}	2	2,041	80	19
pBSV28-1	5.3×10^{-7}	3.0×10^{-4}	4	6,538	100	100
lp25	NA[d]	NA	NA	NA	NA	85

[a] Transformation frequency was calculated as the ratio of transformants relative to the total number of CFU on medium without kanamycin.
[b] Transformation efficiency was calculated as the number of transformants per microgram of DNA.
[c] Stability was measured as the percentage of total colonies that retain the vector after ~90 generations compared to the total number of colonies arising (40 colonies per culture condition were PCR screened for the presence of each vector).
[d] NA, not applicable.

striction-modification system encoded on lp25. Lawrenz and colleagues recently reported that all pBSV2 transformants examined lacked lp25 (23). Their results suggest that the associated loss of lp25 in pBSV2 transformants does not relate to incompatibility but to the presence of a putative restriction-modification system encoded on lp25. The proposed model speculates that shuttle vector transformation occurs in a preexisting subpopulation of cells lacking lp25. Therefore, cells retaining lp25 pose a barrier to shuttle vector transformation and make it difficult to demonstrate incompatibility functions from linear plasmids of *B. burgdorferi*.

Nevertheless, we assessed the presence of the parental plasmids in the shuttle vector transformants. Ten pBSV25 transformants were PCR screened, and all lacked lp25. Four of the 10 pBSV25 transformants had also lost lp28-1, most likely explained by the frequent loss of lp28-1 observed during in vitro cultivation of *B. burgdorferi* (22, 30, 46). Six B31-A3 transformants of pBSV28-1 were examined, and all lacked lp28-1. However, all six pBSV28-1 transformants also lacked lp25, reinforcing the observations of Lawrenz and colleagues (23).

Stability of vectors. To determine if the plasmid maintenance region of a linear plasmid was stable in a circular form, pBSV25 and pBSV28-1 transformants were continuously passaged with and without antibiotic selection. After 90 generations, 100% of the colonies tested retained pBSV28-1, in both the presence and absence of kanamycin (Table 2). In contrast, pBSV25 was much less stable, with only 19% of the colonies retaining the shuttle vector after ~90 generations in the absence of selection. Stability was greater in the presence of kanamycin, with 80% of the colonies tested retaining pBSV25 (Table 2). The parental plasmid, lp25, was more stable than the derivative pBSV25, with 85% of the colonies examined retaining lp25 (Table 2).

PF 49 is not required for circular or linear plasmid replication. Eggers et al. demonstrated that a cp32-based shuttle vector, containing only PF 57 and upstream regions (including an inverted repeat), replicated autonomously (8). Previously, we reported that three cp9 ORFs (*bbc01*-PF 57, *bbc02*-PF 50, and *bbc03*-PF 49) were the minimal elements necessary for autonomous replication, and the inverted repeats that flank these three ORFs were unnecessary (43). Subsequently, we constructed a derivative of pBSV2 containing inverted repeat A (IR-A) and upstream sequences, plus *bbc01* and *bbc02*. This vector, designated pOZK-1-2 (Fig. 4A), was capable of autonomous replication in *B. burgdorferi*. Stability assays (described above) showed pOZK-1-2 was present in 100% of the colonies examined after ~90 generations, with or without selection (data not shown). Therefore, a cp9-based vector requires either IR-A and upstream sequences or *bbc03* and downstream sequences for autonomous replication and stable maintenance.

To further delineate the genetic requirements of cp9 maintenance, a premature stop codon was engineered at a unique

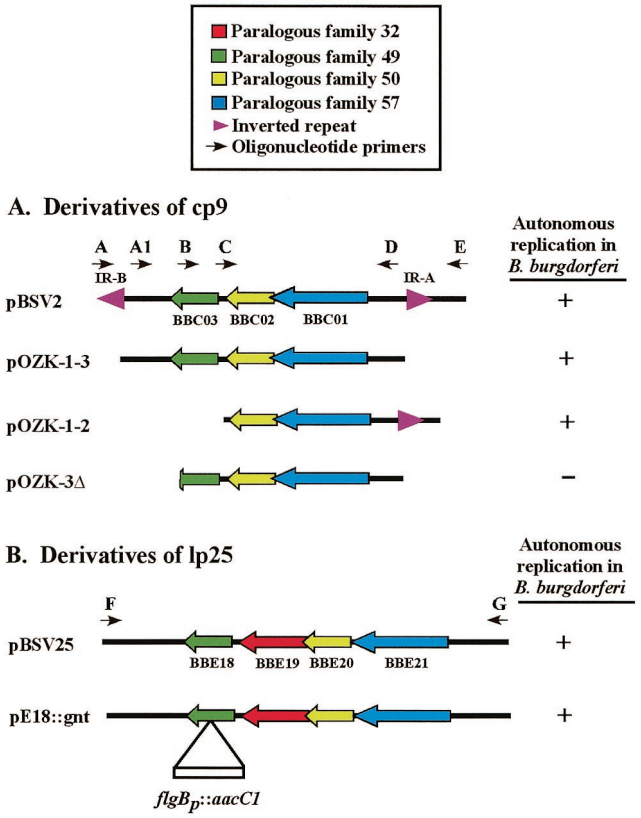

FIG. 4. Derivatives of *B. burgdorferi* plasmids and their ability to autonomously replicate. Regions shown were subcloned into pOZK and tested for their ability to confer autonomous replication functions in *B. burgdorferi* (shown on the right). Arrowheads with letters designate oligonucleotide primers used in the PCR amplification (see Table 1). (A) Regions derived from cp9; (B) regions derived from lp25. Inactivation of *bbe18* was produced by inserting the *flgB*$_p$*::aacC1* cassette, conferring gentamicin resistance (9).

*Spe*I site, resulting in a protein lacking the carboxy-terminal 27 amino acids. All sequences downstream of this new stop codon were deleted. This vector, pOZK-3Δ, lacked both IRs (Fig. 4A). Electroporation of pOZK-3Δ into *B. burgdorferi* cells did not result in any transformants, despite repeated attempts. Taken together, these results suggest that sequences downstream of the *Spe*I site are required for cp9 replication in the absence of the IR-A region.

The requirement for a PF 49 paralog in linear plasmid replication was assessed by inactivating *bbe18* by using a gentamicin resistance cassette (9) (Fig. 4B). This construct, designated pE18::gnt, was designed to determine whether the BBE18 protein was necessary (a *trans* requirement) or if a DNA sequence present within or downstream of *bbe18* was necessary (a *cis* requirement). Vector pE18::gnt autonomously replicated in *B. burgdorferi* (Fig. 4B), indicating that the BBE18 protein does not appear to be required for linear plasmid maintenance.

DISCUSSION

Why *B. burgdorferi* evolved a genome segmented between a chromosome and multiple plasmids, or minichromosomes, remains unknown. Although plasmids can be lost during in vitro cultivation, it seems likely that most *B. burgdorferi* plasmids either confer a selective advantage or are required for survival in vivo. As Casjens observed, infectious strains isolated from nature almost always maintain the full plasmid complement, and many functions presumed essential reside on the plasmids of *B. burgdorferi* (2). Several extrachromosomal elements appear to be ubiquitous, including cp26 and the cp32 plasmid family (2, 40, 44). Additionally, the importance of linear plasmids to mammalian infection, specifically lp25 and lp28-1, has been observed for over a decade (22, 30, 37, 46).

Because of their importance to mammalian infection, and to better understand linear plasmid maintenance in *B. burgdorferi*, derivatives of lp25 and lp28-1 were constructed and designated pBSV25 and pBSV28-1, respectively (Fig. 2 and 3). Recent evidence suggested *B. burgdorferi* linear plasmids use an internal origin of replication (4, 27). The data presented here confirm this prediction. The regions from linear plasmids lp25 and lp28-1 conferring autonomous replication are located internally and contain members of PF 49, PF 50, PF 32, and PF 57 (Fig. 2).

Vectors pBSV25 and pBSV28-1 were maintained in a circular form, despite being derivatives of linear replicons. This suggests that both linear and circular plasmids of *B. burgdorferi* can utilize the same mechanism for replication initiation, segregation, and compatibility, thereby minimizing the machinery necessary for plasmid maintenance. A major difference between linear plasmid replication and circular replication would be the ability to resolve the replicated form (a circular dimer in the case of linear plasmids) (2, 4, 19). Recently, the enzyme that carries out this function in *B. burgdorferi*, ResT, was identified, purified, and characterized (20).

Conservation of replication initiation functions for linear and circular plasmids is further supported by the conversion of a *B. burgdorferi* circular plasmid to a linear form by introduction of synthetic telomeres (4). The report by Ferdows et al. of the spontaneous conversion of a linear plasmid of *B. hermsii* to a stable circular form suggests a common replication initiation mechanism for both linear and circular plasmids (10). Finally, CG skew analysis predicted a common mode of replication for both linear and circular replicons in *B. burgdorferi* (27, 28). Together, these results reinforce the theory that plasmid maintenance in *B. burgdorferi* is largely conserved, regardless of DNA form. Similarly, linear plasmids with internal origins and bidirectional modes of replication have been observed in various actinomycetes and are capable of driving replication in a circular form (5, 6, 26, 31, 38). A general principal of bacterial linear plasmid replication appears to be the adaptation of preexisting replication and segregation functions.

Although both pBSV25 and pBSV28-1 were capable of autonomous replication, these vectors exhibited striking differences in stability after 90 generations in *B. burgdorferi* (Table 2). The pBSV25 shuttle vector was unstable, even in the presence of selection, whereas pBSV28-1 was completely stable in the presence or absence of selection (Table 2). Both lp25 and lp28-1, as well as cp9, are frequently lost during in vitro growth (22, 30), yet vectors derived from lp28-1 and cp9 are extremely stable (43). Since pBSV28-1 and pBSV2 are stable even in the absence of selection, the frequently noted loss of lp28-1 and cp9 with in vitro propagation does not appear to be due to a defect in their ability to autonomously replicate and segregate. Rather, lp28-1 and cp9 may not encode essential gene products for in vitro growth and may be lost from some cells at a low frequency and become fixed in the population. However, the instability of pBSV25 does not appear to relate to the stability of the parental plasmid lp25, which was lost in only 15% of the population over the same time period (Table 2). Possibly, lp25 may have dispersed genetic elements necessary for stable plasmid maintenance that pBSV25 does not include. The phage N15 is maintained as a linear plasmid within *E. coli* cells and has telomeric ends structurally similar to the linear plasmids of *B. burgdorferi* (34). Interestingly, N15 contains four separate centromeres dispersed across its length, with each centromere adding to N15 stability (13). Likewise, lp25 may have more than one region necessary for stable inheritance, and not all regions may have been incorporated into pBSV25. Alternatively, sequence analysis of pBSV25 identified a single nucleotide change resulting in an amino acid substitution in BBE21 (PF 57). The amino acid change, an isoleucine-to-threonine substitution, replaces a hydrophobic residue with a hydrophilic one. Eggers et al. demonstrated that the corresponding PF 57 member from a cp32 plasmid was the only coding region required for replication of shuttle vector pCE316 (8). This single amino acid change may be responsible for the instability of pBSV25.

Surprisingly, conversion of the circular shuttle vector pBSV2 to a linear form by addition of 35-bp telomeres to the multiple cloning site, as reported by Chaconas et al. (4), resulted in a highly unstable plasmid and reduced the copy number by fivefold (data not shown). Placement of the telomeres in the carboxy terminus of *bbc03* (PF 49) restored the copy number, but the vector remained unstable. This suggests that the DNA sequence near *bbc03* significantly impacts replication or partitioning functions of linear and circular plasmids.

Although the DNA sequence encompassing PF 49 members (such as *bbc03* and *bbe18*) appears to be necessary for both circular and linear plasmid maintenance, apparently the PF 49-encoded proteins are not. While constructing derivatives of

the cp9-based shuttle vector, pBSV2, we noted that an IR and upstream sequence could substitute for a member of PF 49 (BBC03) (Fig. 4A), suggesting that both the IR and the DNA sequence encompassing *bbc03* contain the same required binding site for plasmid maintenance. The IR plus upstream sequence of cp9 is less than 300 bp long and does not contain any ORFs of significant size. Eggers et al. reported similar results with a cp32-based shuttle vector and proposed candidate binding sites for the DnaA protein, which is required for initiation of plasmid replication in some systems (8). The requirement for a PF 49 member in linear plasmid replication was determined by inactivating *bbe18* (on pBSV25) with an antibiotic marker, creating the vector pE18::gnt (Fig. 4B). Our data support the view that a functional PF 49 protein is not required for circular or linear plasmid replication. However, the sequence, either within *bbe18* or downstream of it, appears to be necessary. Most likely, this region of DNA provides a binding site for a plasmid maintenance protein. PF 49 members are widely distributed among *B. burgdorferi* plasmids, are highly conserved, and are usually located near the origin of replication, suggesting a role in plasmid maintenance. Our results indicate that the PF 49 protein is not required for plasmid replication, but it may contribute to plasmid stability, perhaps as a functional component of the partitioning system (8, 12). However, the data do not exclude the possibility that a family 49 protein is supplied in *trans*.

The data presented here suggest that *B. burgdorferi* linear and circular plasmid maintenance functions are conserved, differing mainly in the resolution of replicated linear plasmids. Apparently, linear and circular plasmids in *B. burgdorferi* do not require a PF 49 protein for plasmid replication, but they do require the DNA sequence, perhaps serving as a protein binding site. Finally, the shuttle vectors reported here provide the tools necessary to dissect the components and functions of linear plasmid maintenance in *B. burgdorferi*.

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