Published in final edited form as:

Plasmid. 2012 July ; 68(1): 1–12. doi:10.1016/j.plasmid.2012.01.009.

Requirements for *Borrelia burgdorferi* plasmid maintenance

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Abstract

Borrelia burgdorferi has multiple linear and circular plasmids that are faithfully replicated and partitioned as the bacterium grows and divides. The low copy number of these replicons implies that active partitioning contributes to plasmid stability. Analyzing the requirements for plasmid replication and partition in *B. burgdorferi* is complicated by the complexity of the genome and the possibility that products may act in trans. Consequently, we have studied the replication-partition region (*bbb10-13*) of the *B. burgdorferi* 26 kb circular plasmid (cp26) in *Escherichia coli*, by fusion with a partition-defective miniF plasmid. Our analysis demonstrated that *bbb10*, *bbb11*, and *bbb13* are required for stable miniF maintenance, whereas *bbb12* is dispensable. To validate these results, we attempted to inactivate two of these genes in *B. burgdorferi*. *bbb12* mutants were obtained at a typical frequency, suggesting that the *bbb12* product is dispensable for cp26 maintenance as well. We could not directly measure cp26 stability in the *bbb12* mutant, because cp26 carries essential genes, and bacteria that have lost cp26 are inviable. Conversely, we were unable to inactivate *bbb10* on cp26 of *B. burgdorferi*. Our results suggest that *bbb12* is dispensable for cp26 maintenance, whereas *bbb10*, *bbb11*, and *bbb13* play crucial roles in that process.

Keywords

miniF; plasmid stability

1. Introduction

Stable maintenance of low copy number replicons in bacteria requires replication and active partitioning of plasmids to daughter cells (reviewed by Ebersbach and Gerdes, 2005). Both processes typically depend on plasmid-encoded products recognizing specific sites on the plasmid. In the case of replication, rep proteins bind to the plasmid origin (*ori*) at the initiation of replication, whereas partition depends on a centromere-like sequence (*parS*) being bound by partition proteins.

Borrelia burgdorferi, a Lyme disease agent (Burgdorfer et al., 1982; Donahue et al., 1987; Lane et al., 1991), has a complex genome composed of multiple linear and circular plasmids, plus a linear chromosome (Barbour and Garon, 1987; Baril et al., 1989; Casjens et al., 2000; Fraser et al., 1997; Stevenson et al., 1997). The plasmid copy numbers are

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approximately one per chromosome (Beaurepaire and Chaconas, 2007; Hinnebusch and Barbour, 1992), which is approximately one per cell (Morrison et al., 1999). The chromosome and at least several of the plasmids carry genes required for either bacterial survival or for the bacteria to fulfill the natural mouse-tick infectious cycle (Byram et al., 2004; Grimm et al., 2005; Grimm et al., 2004; Jewett et al., 2007b; Jewett et al., 2009; Kobryn and Chaconas, 2002; Labandeira-Rey and Skare, 2001; Purser et al., 2003; Revel et al., 2005; Stewart et al., 2006; Strother et al., 2005; Strother and de Silva, 2005; Tilly et al., 2007; Tilly et al., 2006). In order to ensure stable maintenance, the plasmids and chromosome likely carry genes and sites required for faithful partition and replication. On the basis of sequence conservation, several gene families have been implicated in plasmid replication and partition, including homologs of *parA* (called PF32, for paralogous family), encoding putative ATPases required for segregation of plasmids in other bacteria (Casjens et al., 2000; Zückert and Meyer, 1996). Other paralogous gene families implicated in plasmid maintenance include PF57 and PF62, thought to encode replication proteins, and PF49 and PF50, of unknown function (Casjens et al., 2000). One or several of these genes is present on each *B. burgdorferi* plasmid (Casjens et al., 2000). Several studies (described below) have addressed the requirements for plasmid replication and partition in *B. burgdorferi*, but no simple and clear picture has yet emerged.

Regions capable of conferring autonomous replication and partition in *B. burgdorferi* were isolated from circular plasmids cp9 (Stewart et al., 2001), cp26 (Byram et al., 2004), one of several cp32 plasmids (Eggers et al., 2002), and linear plasmids lp25, lp28-1 (Stewart et al., 2003), and lp38 (Dulebohn et al., 2011). Shuttle vectors carrying those regions were capable of maintenance in *B. burgdorferi*, and demonstrated incompatibility by displacing the resident plasmid, when possible. All such experiments, however, are complicated by the possibility that products required for replication or partition may be supplied in trans from another plasmid that is present in the spirochete. Cp9 appears to be related to the cp32 plasmids, but lacks a number of genes, including the PF32 member, encoding a potential ParA protein (Casjens et al., 2000; Dunn et al., 1994). Deletion analysis of this plasmid suggested that the product of cp9 PF49 is also not essential in cis for plasmid maintenance, although some or all of the gene sequence appears to perform an important function (Stewart et al., 2003; Stewart et al., 2001). The same authors found similar results for lp25, demonstrating that a shuttle vector carrying lp25 genes from paralogous families 32, 49, 50, and 57 conferred autonomous, although somewhat unstable, replication in *B. burgdorferi*, and that insertion of foreign sequences into the lp25 PF49 did not alter that ability (Stewart et al., 2003).

Further studies showed that the lp17 PF62 and cp32 PF57 were the only genes required for replication of their respective plasmids (Beaurepaire and Chaconas, 2005; Eggers et al., 2002) in *B. burgdorferi*. Again, interpretation of these results is complicated by the presence of other plasmids in *B. burgdorferi*, which may be capable of supplying replication or partition products in trans.

The 26 kb circular plasmid cp26 is unusual among the *B. burgdorferi* plasmids, since it carries genes whose products are essential for bacterial growth in all conditions, and others needed for survival in the mouse-tick cycle. The essential genes are *resT* (Byram et al., 2004), encoding the telomere resolvase required for linear DNA replication (Kobryn and Chaconas, 2002), along with *bbb26* and *bbb27*, whose products are of unknown and potentially overlapping function (Jewett et al., 2007b; Lawrence et al., 2009). Genes required during the natural life cycle of the spirochete include *ospC* (Grimm et al., 2004), which plays an essential role at the initiation of mammalian infection, and *guaA* and *guaB*, encoding purine biosynthesis enzymes (Margolis et al., 1994), which are required for mouse infectivity and contribute to growth within ticks (Jewett et al., 2009; Lawrence et al., 2009).

The presence of these genes on cp26 ensures that this plasmid is present in all bacteria during propagation in culture and found in all natural isolates. Presumably, plasmid stability is ensured by functional replication and partition functions, combined with the lethality of loss. We used the replication and partition region of cp26 as the starting point for our dissection of those functions. To facilitate analysis and avoid problems of potential trans-complementation with functions encoded by other *B. burgdorferi* plasmids, we assessed the effect of adding *B. burgdorferi* sequences to a derivative of the *E. coli* plasmid F (miniF) lacking the *sopABC* region, which is responsible for partitioning of this plasmid, using *E. coli* as a surrogate host. This plasmid (pDAG203; (Lemonnier et al., 2000) has been used to characterize partition functions from other bacteria, such as *Burkholderia cenocepacia* (Dubarry et al., 2006) and *Pseudomonas putida* (Godfrin-Estevenson et al., 2002).

We cloned the cp26 region capable of promoting stable plasmid replication and partitioning in *B. burgdorferi* (Byram et al., 2004) into pDAG203, generating plasmid miniF26. We then systematically deleted genes, generating miniF plasmids with various subsets of the genes and sites present in the *bbb10-13* region, and assessed their stability during growth in *E. coli* without selection. These studies demonstrate that *B. burgdorferi* plasmid maintenance functions can be analysed using a heterologous system in *E. coli*, which is a new and powerful tool for studying the mechanism of spirochete plasmid segregation.

2. Materials and Methods

2.1. Construction of miniF26 and deletion derivatives All oligonucleotides and TaqMan probes are described in Table 1

Plasmid pBSV26 (Byram et al., 2004) was digested with *SpeI* to release a 3417 bp fragment containing the cp26 genes *bbb10-13* (Fig. 1A). This DNA fragment was ligated with pDAG203 (Lemonnier et al., 2000) that had been linearized with *NheI*, yielding miniF26 (Fig. 1B). Derivatives of miniF26 with only *bbbl1-13* (miniF26Δ*bbb10*), with *bbb10*, *bbb12* and *bbb13* (miniF26Δ*bbbl1*), and just *bbbl2* and *bbbl3* (miniF26Δ*bbb10-11*) were constructed using PCR-mediated deletion (Fig. 2). Primers extending out from the translational start and stop sites of the genes (8 and 9 to remove *bbb10*, and 10 and 11 to remove *bbbl1*; Table 1) were used to amplify the remainder of pBSV26, thereby deleting the intervening coding sequences and adding *XhoI* sites. miniF26 with just *bbbl2* and *bbb13* (called miniF26Δ*bbb10-11*) was constructed by amplifying the remainder of pBSV26 with primers 8 and 11. In all cases, PCR products were digested with *XhoI* and *DpnI* (to remove the pBSV26 template) and self-ligated to recircularize the plasmid. The *B. burgdorferi* plasmid sequences were excised from the resulting plasmids with *SpeI*, purified from agarose gels using a MinElute kit (Qiagen, Valencia, CA), and ligated with pDAG203 that had been digested with *NheI* and treated with Antarctic Phosphatase (Fig. 2).

MiniF plasmids with *bbb10*, *bbb11*, and *bbbl3* (called miniF26Δ*bbbl2*) or *bbb10*, *bbb11*, and *bbbl2* (called miniF26Δ*bbbl3*) were made using restriction sites in miniF26 (Fig. 2). MiniF26 was digested with *MfeI* (for the *bbbl2* deletion) or *ScaI* (for the *bbbl3* deletion) and religated. A miniF derivative with just *bbbl3* (called miniF26Δ*bbb10-12*) was constructed starting with miniF26Δ*bbb10-11*, digesting with *MfeI*, and self-ligating. A miniF with no functional coding sequences derived from *B. burgdorferi*, but retaining the non-coding sequences, was derived from miniF26Δ*bbb10-12* by digesting with *ScaI* and religating (Fig. 2).

MiniF26Δ*bbbl1**, with replacement of a GAA codon at amino acid 15 of *bbbl1* with the stop codon TAA, was created by overlapping PCR. Products amplified with Vent polymerase (New England Biolabs) using primer pairs 1-15 and 14-2 were digested overnight with *DpnI*, and then used as substrate in a reaction with primer pair 1-2, also using Vent

polymerase. The resulting product was cloned into TopoXL. A *Bam*H1-*Eco*RV fragment including the *bbb10* and *bbb11** genes was substituted for the resident copies of those genes in *Bam*H1-*Eco*RV-digested pBSV26. The *bbb10-13* region was excised from the resulting plasmid with *Spe*I and ligated with *Nhe*I-digested pDAG203.

Allelic exchange vectors for attempting to make deletion-insertion mutations in the *bbb10* and *bbb12* loci in *B. burgdorferi* were constructed as follows. A plasmid for inactivating *bbb10* was derived from pBSV26Δ*bbb10* by digesting with *Xho*I and ligating with a *flgB_p-kan* cassette with *Xho*I sites on its ends (created by amplifying with primers 12 and 13), yielding plasmid pBSV26Δ*bbb10::flgB_p-kan*. To target *bbb12*, plasmid pBSV26 was digested with *Spe*I and *Nco*I and the *bbb10-13*-containing fragment eluted from a gel with the MinElute Kit (Qiagen). This fragment was cloned into a version of the pCR-XL-Topo plasmid (Invitrogen) that had been digested with *Eco*RI to remove an irrelevant insert, self-ligated, and digested with *Spe*I. To inactivate *bbb12*, the resulting plasmid (TopoXL-*bbb10-13*#3a) was digested with *Mfe*I and ligated with a *flgB_p-aacC1* cassette that had been digested out of pCR2.1 Topo with *Eco*RI, yielding plasmid TopoXL-Δ*bbb12::flgB_p-aacC1*. Versions of this plasmid with the *flgB_p-aacC1* cassette in either orientation were obtained.

Final constructs were confirmed by sequencing. Enzymes were purchased from New England Biolabs (Ipswich, MA). Plasmids were transformed into frozen competent HB101 (Promega, Madison, WI) or into other *E. coli* strains by CaCl₂-mediated transformation (Mandel and Higa, 1970).

2.2. Construction of an *E. coli* strain with *bbb10* and *bbb11* inserted in the chromosome

The *bbb10* and *bbb11* genes, along with the potential promoter upstream of *bbb10*, were amplified with Vent polymerase, using primers 1 and 2 and cloned into pZeroBluntTopo (Life Technologies, Carlsbad, CA). A clone with the correct sequence was digested with enzymes *Nsi*I and *Bam*HI and the resulting fragment was ligated with *Nsi*I-*Bam*HI-digested pUCR6KT, which contains a miniTn7 that will insert DNA into a unique site on the *E. coli* chromosome (Choi et al., 2005). Ligations were transformed into SM10(λpir). DNA was prepared from a colony with the correct plasmid and used to co-transform HB101, in which pUCR6KT cannot replicate, with pTNS2 (a helper plasmid encoding the Tn7 transposase). We selected for gentamicin-resistance conferred by miniTn7 insertion into its chromosomal insertion site, bringing with it the *bbb10* and *bbb11* genes. We then assessed the stability of various miniF26 derivatives in this strain.

2.3. Constructing a fusion between the promoter and *bbb13*

Integrated DNA Technologies (IDT, Coralville, IA) synthesized a fusion between the presumed *bbb10-13* promoter and the start codon of *bbb13*, using proprietary technology.

2.4. Plasmid stability assessment

Cultures grown overnight at 37° in L broth containing 30 μg/ml chloramphenicol (selecting for miniF plasmid retention) were diluted in duplicate into medium lacking antibiotic. At several time intervals, dilutions were plated on solid medium lacking antibiotic and cultures were further diluted so that they remained in logarithmic growth. Colonies were replica-plated to solid medium containing and lacking chloramphenicol to assess plasmid retention and doublings. All experiments were performed at least twice, and most were performed several times by two different people. Data sets were compared by Two-way ANOVA with a Bonferroni post-test using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA). For statistical analysis of data sets in which numbers of doublings for different strains were not identical, the average number of doublings was used.

2.5. TaqMan analysis of *bbb10* and *bbb12* transcription

We used TaqMan analysis to assess transcript levels for *bbb10* and *bbb12* in *E. coli* carrying various derivatives of miniF26. Transcript levels of the *E. coli* chromosomal gene *dxs* were used to normalize the qPCR. RNA was prepared from *E. coli* using a Nucleospin RNA II kit (Machery-Nagel, Bethlehem, PA) and cDNA generated with a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). Quantitative PCR was carried out using Universal PCR Master Mix (Applied Biosystems), primers and probes (Table 1; Sigma-Genosys), and an Applied Biosystems 7900 instrument.

2.6. Gene inactivation in *B. burgdorferi*

bbb12 of cp26 in *B. burgdorferi* strain B31-A34 (Jewett et al., 2007a) was inactivated by allelic exchange, following electroporation with 5 µg of plasmid TopoXLΔ*bbb12::flgB_p-aacC1* as previously described (Elias et al., 2003; Samuels, 1995). After recovery overnight in 5 ml BSKII medium, transformations were plated in solid BSKII medium containing 40 µg/ml gentamicin. Colonies arising after 5-10 days at 35°C with 2.5% CO₂ were screened for the presence of the *flgB_p-aacC1* fusion using primers 3 and 4. Transformants were obtained at a frequency of 1.5×10⁻⁶. Several of these colonies were picked into BSKII medium and grown to high density, at which time genomic DNA was prepared using a Wizard Genomic DNA Purification kit (Promega, Madison, WI). The structure of the *bbb12* mutation was confirmed using primer pairs 5-6 and 11-7, which demonstrated that at least 5 of 13 transformants analyzed had the appropriate deletion-insertion mutation at the *bbb12* locus (data not shown). These PCR reactions also confirmed the absence of a wild type *bbb12* locus. Further confirmation of the *bbb12* deletion-insertion mutation and lack of a wild type *bbb12* locus was obtained by Southern blot hybridization (data not shown).

We attempted to inactivate *bbb10* in *B. burgdorferi* by the same procedure used for *bbb12* inactivation, with selection on medium containing 200 µg/ml kanamycin. Upon analysis, all of the clones isolated retained the wild type locus. One clone had both wild type and mutant *bbb10* loci, yielding an allelic exchange frequency of 3×10⁻⁸. Transformation with linearized DNA, to eliminate the possibility of integration or autonomous replication of the entire transforming plasmid, did not yield any clones that had undergone allelic exchange events.

2.7. Copy number assessment

We prepared DNA for assessing copy number of various miniF-derivative plasmids using a modification of a published method (Beaurepaire and Chaconas, 2005) that does not include any nucleic acid precipitation or column binding steps. Briefly, we froze pelleted *E. coli* cultures, resuspended them in Tris-EDTA-NaCl (0.1M, pH8.2-10mM-30mM) with 1 mg/ml lysozyme, and incubated on ice for 45 min. We then added 200µl of 0.5%SDS-50mM Tris pH7.5-0.4M EDTA with 1 mg/ml proteinase (Life Technologies) and incubated for one hour at 55°C. We then extracted twice with phenol-CHCl₃ (1:1) and once with CHCl₃, before dialysing against 10mM Tris (pH 8) at room temperature for 1.5 hr. The DNA preparations were diluted to 50µg/ml, and relative copy number assessed in triplicate TaqMan analysis using miniF and *dxs* primer-probe sets (primers 16, 17, 22, and 23, and probes 1 and 4; Table 1). DNA extracted from HB101 carrying miniF26 was used for the standard curves. Genome copies per microgram of DNA were calculated based on the genome size of *E. coli*.

3. Results

3.1. Origin region from cp26 stabilizes miniF lacking partition functions

A region of cp26 containing the genes *bbb10-bbb13*, plus some flanking sequences (Fig. 1A), was previously shown to be sufficient to support replication and partition of an *E. coli* shuttle vector in *B. burgdorferi* (Byram et al., 2004). We cloned this region into pDAG203,

which is a miniF derivative lacking partition functions (Lemonnier et al., 2000). When the resulting plasmid, called miniF26 (Fig. 1B), was transformed into *E. coli* strain HB101, it was significantly more stable than pDAG203 when shifted from growth in the presence of chloramphenicol to antibiotic-free medium ($p < 0.01$ - 0.001 ; Fig. 3). Inserting an irrelevant segment of DNA into the miniF plasmid did not confer stabilization (Fig. 3). pDAG203 appeared to be surprisingly stable for a low copy number plasmid lacking a functional partition system, so we rescued the plasmid from HB101/pDAG203 and retransformed it into HB101. Stability was again assessed, taking care to minimize the time between transformation and stability measurement. In this case (Fig. 3), pDAG203 was lost much faster than in the original test. In contrast, miniF26 was similarly stable in HB101 when present in a fresh transformant or in HB101/miniF26 grown from a frozen stock (Fig. 3). These data suggest that growth of HB101/pDAG203 in the presence of chloramphenicol, which selects for the presence of the plasmid, exerts a powerful selection for stabilization by an unknown mechanism, which does not involve mutation of the plasmid DNA. Partial sequence analysis of the rescued DNA supported this idea, in that no differences between the original and rescued plasmids were detected.

3.2. Analysis of stabilizing region from cp26

The cp26 region that stabilizes miniF contains a number of interesting features. First, there are four open reading frames, oriented in the same direction, and preceded by a potential promoter (Figs. 1A and 1C). Although the predicted products of all four open reading frames belong to previously identified paralogous families associated with putative plasmid origins in *B. burgdorferi*, only BBB12, a ParA homolog, resembles a protein found in other bacteria (Casjens et al., 2000). BBB12 is predicted to start at a codon within the terminus of the BBB11 coding sequence, based on the position of nucleotides encoding a potential Walker box, involved in ATP hydrolysis by ParA proteins (Casjens et al., 2000; Zückert and Meyer, 1996). Although we have not mapped the promoter of the *bbb10-13* genes, we presume that it is located in the 243 bp preceding the start codon of *bbb10* that are located within the region used for constructing pBSV26 (Byram et al., 2004) and miniF26 (Fig. 1C). The ability of this region to confer stable maintenance on a *B. burgdorferi* shuttle vector (Byram et al., 2004; Jewett et al., 2007a) argues in favor of this hypothesis. Searching for a bacterial promoter using BPROM (Soffberry, Inc., Mt. Kisco, NY) predicted a promoter about 200 bp upstream of the *bbb10* start site (Fig. 1C), but *B. burgdorferi* promoter criteria have not been fully characterized, and several other potential -35 regions are located closer to the *bbb10* start site. TaqMan analysis (see Section 3.6) demonstrated that transcription of at least *bbb10* and *bbb12* was correlated, supporting the idea that the *bbb10-13* genes are co-transcribed.

Between *bbb12* and *bbb13* is a region that may comprise the *parS*, with complex inverted and direct repeats (Fig. 1D). We identified this site by analyzing the entire *bbb10-13* region with the GeneQuest program from the Lasergene suite (DNASTAR, Madison, WI) for direct or inverted repeats of at least 10 nucleotides. All identified repeats were compared to the potential centromere from the chromosome and to other *parS* homologs (Lin and Grossman, 1998; Livny et al., 2007). In the *bbb10-13* region, only the sequence between *bbb12* and *bbb13* contains an inverted repeat that is homologous to previously described *parS* sequences. Interestingly, the *B. burgdorferi* chromosomal *parS* is located between *gyrA*, encoding a gyrase subunit, and BB0434, which has been annotated as a *parB* homolog, involved in *parS* binding (Lin and Grossman, 1998; Livny et al., 2007). No other *B. burgdorferi* plasmid has a potential *parS* that was identified by this method.

Finally, downstream of *bbb13* is a potential origin of replication. This region contains multiple direct and inverted repeats and AT-rich regions (Fig. 1E). Also, this site is the

approximate location of the the minimum GC-skew (a characteristic often found at replication origins) (Picardeau et al., 2000).

3.3. Deletions removing genes from miniF26

We used two strategies to delete individual genes in the *bbb10-13* region of miniF26 (Fig. 2; see Materials and Methods for details). To remove *bbb10*, *bbb11*, or both genes, we used PCR-mediated deletion. For *bbb12* and *bbb13* deletions, we used restriction sites to remove large portions of the coding sequences of each gene. The deletions seemed unlikely to have polar effects on downstream genes, although altering the location of the potential promoter with respect to those genes may affect transcription.

We tested the stability of the miniF26 derivatives, and found that miniF26Δ*bbb10*, miniF26Δ*bbb11*, miniF26Δ*bbb10-bbb11* (Fig. 4A) and miniF26Δ*bbb13* (Fig. 4B) had greatly reduced stability compared to miniF26 ($p < 0.001$, except for two T_0 values). Surprisingly, miniF26Δ*bbb12* was as stable as miniF26, containing the entire *bbb10-13* region (Fig. 4C).

We constructed a chain termination mutation in *bbb11*, to see if stopping translation without removing the gene sequence had a similar effect on miniF stability. The stability of the plasmid with the point mutation (*bbb11*^{*}) was indistinguishable from that of miniF26Δ*bbb11* ($p > 0.05$; Fig. 4D), suggesting that the phenotype was caused by lack of the BBB11 product, not the absence of a nucleotide sequence within the deleted region.

We made two additional deletions, in an effort to determine if the instability of other miniF derivatives could be explained by polar effects, leading to decreased *bbb13* expression. The first derivative combined the *bbb10-bbb11* and *bbb12* deletions, yielding a miniF that has only the predicted promoter (up to the *bbb10* predicted ribosome binding site), potential *parS*, *bbb13*, and potential replication origin (Δ*bbb10-12*; Fig. 2). We predicted that linking the *bbb10* ribosome binding site to the start of the *bbb12* open reading frame, combined with the *bbb12* deletion, which has no effect on plasmid stability, would relieve any possible polarity resulting from the *bbb10* and *bbb11* deletions. The second miniF derivative combined all four deletions, creating a miniF that has only the potential promoter, *parS*, and replication origin (Δ*bbb10-13*; Fig. 2). These plasmids had the same phenotype as miniF26Δ*bbb10*, miniF26Δ*bbb11*, miniF26Δ*bbb10-11*, and miniF26Δ*bbb13* (Fig. 4), i.e., more than 90% plasmid loss by 30 doublings (data not shown), demonstrating that the sequences remaining do not confer any stability on pDAG203. These data support the idea that the phenotypes of the miniF bearing *bbb10* and *bbb11* deletions are not due to polar effects on the *bbb13* gene, but rather the consequences of loss of functions important for miniF stabilization.

3.4. Attempts to complement unstable miniF derivatives by supplying *B. burgdorferi* products in trans

In an endeavor to restore stable maintenance to the miniF derivatives lacking *bbb10*, *bbb11*, or both, we constructed an *E. coli* strain in which the *bbb10* and *bbb11* genes, including the 5'-flanking region, were integrated into the chromosome (Materials and Methods). Substantial transcription of *bbb10* from this strain was confirmed by TaqMan analysis of cDNA (data not shown). We introduced pDAG203 (no insert), miniF26 (*bbb10-13*), miniF26Δ*bbb10*, miniF26Δ*bbb11*, and miniF26Δ*bbb10-11* into that strain and assayed their stabilities. miniF26, with *bbb10-13*, remained stable in this strain. All other plasmids tested, including pDAG203, with no *B. burgdorferi* DNA, were extremely unstable (Fig. 5), as was seen for fresh transformants with pDAG203 (Fig. 2). We conclude that expressing *bbb10-11* from a chromosomal location did not stabilize miniF lacking one or both of those genes.

In an attempt to stabilize miniF26Δ*bbb13*, we created a version of *bbb13* in which the putative promoter 5' of *bbb10* and the start codon and open reading frame of *bbb13* were fused (Materials and Methods). The *bbb13* construct was cloned into the high copy plasmid pUC57 and transformed into a strain carrying miniF26Δ*bbb13*. This plasmid did not stabilize miniF26Δ*bbb13*, when both were contained within the same *E. coli* strain (data not shown). We were unable to confirm *bbb13* transcript levels (due to a lack of functional primer-probe sets for *bbb13*, despite testing several possible combinations), so it remains possible, although unlikely, that this plasmid does not provide adequate levels of BBB13. Several possibilities may explain the failure of these two attempts to restore the stability of miniF derivatives by supplying missing *B. burgdorferi* products in trans. Perhaps the expression ratios of some or all of the *bbb10-13* products are crucial to plasmid stability, these products act optimally in cis, on sequences proximal to their coding regions (Dong et al., 1988), or an important site has been deleted from the *bbb10-13* region in these miniF derivatives.

3.5. Analysis of *bbb10* and *bbb12* transcript levels in *E. coli* carrying various miniF derivatives

In an attempt to determine if the *bbb10-13* genes were co-transcribed and as an additional test for indirect effects of deletion of one gene on expression of another, we performed TaqMan quantitative PCR analysis on cDNA derived from *E. coli* carrying various miniF derivatives. Despite trying three primer-probe sets for both *bbb11* and *bbb13*, we were unsuccessful in amplifying either from DNA or cDNA. In contrast, our primer-probe sets for *bbb10* and *bbb13* amplified well, so we were able to assess transcript levels for those genes in *E. coli* containing miniF with no insert (negative control), with *bbb10-13*, and with all combinations of three of the genes (Fig. 6). As expected, no transcript was detectable for either gene when no *B. burgdorferi* genes were inserted in the miniF. Both transcripts were detectable when *bbb10-13* were present, but the *bbb10* transcript level was approximately 10 times greater than that of the *bbb12* transcript. When cDNA was derived from HB101 containing miniF plasmids lacking either *bbb10* or *bbb12*, transcript of the corresponding gene was not detected. Interestingly, miniF plasmids lacking any one of the four *B. burgdorferi* genes had somewhat reduced levels of both *bbb10* and *bbb12* transcripts, suggesting that the *bbb10-13* genes are co-transcribed and that altering the sequence of the mRNA affects transcript level but not necessarily plasmid stability.

3.7. *B. burgdorferi* *bbbl2* mutant

Since the *bbbl2* deletion did not alter the stability of miniF26, we decided to introduce this mutation into *B. burgdorferi* cp26, to extend our studies in *E. coli*. In order to facilitate allelic exchange, we made a deletion-insertion version of the mutation, in which a *B. burgdorferi* selectable marker (*flgB_p-aacC1*) was inserted in place of most of the *bbbl2* gene, using the *MfeI* sites used for the deletion in miniF26Δ*bbbl2* (Fig. 2).

miniF26Δ*bbbl2::flgB_p-aacC1* (with the *flgB_p-aacC1* cassette in either orientation) was used to transform B31-A34, a highly transformable strain lacking the restriction enzyme systems encoded by *lp25* and *lp56* (Jewett et al., 2007b; Kawabata et al., 2004; Rego et al., 2011). Since cp26 must be retained for *B. burgdorferi* viability, we used criteria other than plasmid stability to assess the contribution of *bbbl2*. First, the mutant was obtained at a normal frequency for allelic exchange in B31-A34 (1.5×10^{-6}), when the *flgB_p-aacC1* cassette was in the same orientation as the *bbb10-13* genes. Interestingly, we obtained very few transformants when the *flgB_p-aacC1* insertion was in the opposite orientation, in which transcription from the *flgB* promoter could potentially interfere with transcription of *bbb10* and *bbb11*. When analysed further, these few transformants retained wild type copies of the *bbb10-13* region, again suggesting that BBB10 and BBB11 play crucial roles in cp26

maintenance. Second, the $\Delta bbb12$ transformants had no altered colony morphology or reduced growth rate (data not shown), as would be expected if a plasmid carrying essential genes had reduced stability. Finally, PCR and Southern blot analysis of a subset of the transformants demonstrated that 5 of 13 tested had the expected insertion-deletion and all had approximately wild type cp26 copy number (data not shown). Among the other transformants were bacteria in which the allelic exchange vector appeared to have integrated in its entirety, and transformants with apparent cp26 dimers (data not shown), both events previously observed after attempted allelic exchange targeting cp26 genes (Bono et al., 1998; Byram et al., 2004; Stevenson et al., 1998; Tilly et al., 1998). We never observed autonomous replication of the transforming plasmid, which presumably could function as a shuttle vector, most likely because of incompatibility with the essential plasmid cp26. These data suggest that *bbb12* is not required for cp26 maintenance in *B. burgdorferi*, at least in the presence of several other plasmids, consistent with our studies of miniF26 derivatives in *E. coli*.

3.8. Failure to inactivate *bbb10* in *B. burgdorferi*

To further validate our use of miniF stabilization by *B. burgdorferi* genes in *E. coli* as a surrogate for gene inactivation in *B. burgdorferi*, we attempted to inactivate *bbb10* on cp26. Since miniF26 $\Delta bbb10$ was unstable and cp26 is an essential plasmid in *B. burgdorferi*, we predicted that inactivation of *bbb10* would not be possible because transformants would be inviable. To facilitate allelic exchange, we inserted a *flgB_p-kan* cassette in place of *bbb10* in pBSV26 (Materials and Methods) and used this DNA to transform *B. burgdorferi* B31-A34. Transformant colonies were obtained 10-fold less frequently than in the transformation inactivate *bbb12*. In contrast to our transformations with miniF26 $\Delta bbb12::flgB_p-aacC1$, we failed to isolate cp26 without an active *bbb10* gene. Instead, we obtained one clone that carried both wild type and mutant copies of *bbb10* (which would result from integration of the transforming plasmid, autonomous replication of the pBSV26 $\Delta bbb10::flgB_p-kan$, or allelic exchange into one copy of *bbb10* in a dimer of cp26). Transforming with linearized DNA, to eliminate the possibility of integration or autonomous replication, did not yield any transformants. Our inability to inactivate *bbb10* in *B. burgdorferi* is consistent with the profound instability of miniF26 lacking *bbb10* in *E. coli* and the previous assignment of PF62 as essential for replication in *B. burgdorferi*.

3.9 Copy number of miniF derivative plasmids

In an effort to determine if the *bbb10-13* genes increased miniF stability by increasing replication, partition, or both, we assessed the copy numbers of various miniF derivative plasmids relative to the *E. coli* chromosomal gene *dxs*. To minimize the effects of differential precipitation or column binding by chromosomal vs plasmid DNA, we used a DNA extraction method that does not include these steps (section 2.7). Copy number was assessed by TaqMan analysis, using *dxs* and miniF primer-probe sets (Table 1). Although we found an increased copy number of stable miniF derivatives (Fig. 7), the levels appeared to be low enough for active partitioning to be required for stable maintenance (see Discussion). Similar results were obtained by Southern blot analysis (data not shown). These data suggest that the *bbb10-13* products provide active partitioning functions, and may also increase replication of the miniF slightly.

4. Discussion

B. burgdorferi, with its complex genome composed of multiple linear and circular plasmids and a linear chromosome, faces unusual problems in DNA replication and partition. Although a number of studies have addressed the requirements for these processes, all were complicated by the possibility of trans-acting products contributed by other replicons present

in the bacterium. We have examined the replication-partition region of cp26 in isolation, by cloning portions of that region into a miniF plasmid defective in partitioning and studying plasmid stability in *E. coli*. These studies indicated that *bbb10*, *bbb11*, and *bbb13* were all required for stabilization of the miniF, whereas *bbb12* was dispensable. This result seems surprising, because *bbb12* (the PF32 member) is the only gene whose product resembles those found in other bacteria, being homologous to the essential partition protein ParA. However, similar results were obtained with other plasmids in *B. burgdorferi* (Beaurepaire and Chaconas, 2005; Eggers et al., 2002; Livny et al., 2007; Stewart et al., 2003; Stewart et al., 2001) and our findings are also consistent with the absence of genes from PF32 on cp9 and lp5. More recent biochemical experiments demonstrated that the PF32 member encoded on lp17, though not essential for replication, interacts with the protein required for replication (Deneke and Chaconas, 2008). These data suggest that the *bbb12* product could function as an auxiliary replication protein, in contrast to the ParA roe in other bacteria.

Another surprising aspect of these findings is that all three of the other products encoded in the region conferring autonomous replication are required for miniF stabilization. These include BBB10, which has been speculated to be the replication protein, based on a number of experiments and criteria described in the Introduction. Also, *bbb13* belongs to paralogous family 49, whose products were dispensable for stable shuttle vectors derived from cp9 and lp25 (Stewart et al., 2003; Stewart et al., 2001), so perhaps *bbb13* contains an essential site, rather than encoding an essential product. Our copy number data suggest that there is around one miniF26 plasmid copy per chromosome, a level that requires partition functions for stability (Nordstrom and Austin, 1989). Estimating absolute copy number from these data, however, requires the assumption that the miniF and *dxs* primer-probe sets amplify similarly, since HB101/miniF26 DNA was used for the standard curves. These data also demonstrate a three-fold lower copy number in unstable vs stable miniF derivatives (Fig. 7). Perhaps the BBB10-BBB13 products form a multi-protein complex that carries out both replication and partition functions.

Our analyses were unable to definitively exclude all poWbinty of effects of deleting one gene on the transcription or translation of another gene in the region. The fact that deleting any gene in the *bbb10-13* region affects transcript levels of both *bbb10* and *bbb12* suggests that all four genes may be co-transcribed. The 10-fold difference between gene transcript levels in bacteria containing miniF26 is consistent with transcription attenuation. Although we were unable to measure *bbb11* and *bbb13* transcript levels, several lines of reasoning suggest that the products of *bbb10*, *bbb11*, and *bbb13* are directly involved in stabilizing pDAG203. First, the stability of miniF26Δ*bbb12* means that expression of the other genes in bacteria carrying this plasmid is adequate for stable maintenance of miniF. Second, the reductions in *bbb10* transcript from the unstable Δ*bbb11* and Δ*bbb13* plasmids were similar to that found from the stable Δ*bbb12* (which presumably provides adequate BBB10 product), so these genes likely contribute essential stabilization functions also. Finally, the instability of the miniF26Δ*bbb13* plasmid is less likely to be due to indirect effects than any of the others, since *bbb13* is the last gene in the series.

The gene encoding the ATPase required for cp26 segregation remains unknown. In *B. burgdorferi*, *bbb12* may normally carry out this function, but the PF32 proteins encoded on other plasmids or the SpoOJ encoded on the chromosome (BB0434) may enable cp26 partition, when *bbb12* is mutant. This possibility is difficult to test.

Remaining questions include why was complementation unsuccessful? Improper ratios of the *B. burgdorferi* proteins is a possible explanation for the inability of chromosomally expressed *bbb10* and *bbb11* to compensate for their lack on the miniF. If this model were correct, however, miniF26 might be expected to be unstable in the strain with *bbb10* and

bbb11 expressed from the chromosome, because of the excess BBB10 and BBB11. Another possibility is that the proteins are preferentially cis-acting, as was found for other Rep proteins (Dong et al., 1988). Since transcription and translation are likely to be coupled, the concentration of the proteins in the region from which they are encoded would be higher, leading them to bind preferentially to sequences in that region. Such binding could lower the effective concentration within a bacterium, leading to inability to complement segregation of the miniF plasmid.

A second question is how *E. coli* stabilizes the miniF lacking partition functions. Although the mechanism may have no relevance to *B. burgdorferi* plasmid maintenance, it is relevant to the data presented here, since we used frozen aliquots of control strains to provide experiment-to-experiment reproducibility, and pDAG203 was more stable in the frozen strain than immediately after transformation. One possibility is that the miniF copy number increases, but we have seen no evidence of this by Southern blotting (data not shown).

Although these and other data demonstrate that *B. burgdorferi* plasmids carry partition systems, so far there is no evidence for the presence of other plasmid stability systems. Post-segregation killing systems, characterized by a stable toxin and an unstable antitoxin (Sengupta and Austin, 2011), would most likely render the plasmids more stable during bacterial growth in culture, when many are lost. The cp32 family, with several very similar plasmids that appear to be prophages, may possess such a system, but this question has not been addressed experimentally, since all isolates contain at least one cp32 and several genes are unique to this family. *B. burgdorferi* may have dimer resolution systems (Sengupta and Austin, 2011), but cp26 and lp54 dimers have been observed (Marconi et al., 1996; Tilly et al., 1998), and appear to be relatively stable during spirochete growth in culture. Maybe the existence of genes important for spirochete growth and survival at various stages of the infectious cycle, in combination with efficient partitioning mechanisms, is sufficient to guarantee that a substantial proportion of the bacterial population retains the plasmid throughout the natural life cycle of the spirochete. Also, the lack of any free-living stage in this cycle provides ongoing pressure for plasmid maintenance.

A number of aspects of *B. burgdorferi* plasmid maintenance remain areas for fruitful study. First, similar complementation and mutational analysis of the *rep-par* region of a plasmid that is dispensable for growth in culture, such as lp25, would facilitate confirmation of mutant phenotypes in *B. burgdorferi*. Second, there are hypothesized to be two classes of replication proteins, encoded by members of the paralogous families 57 and 62. Demonstrating that these products are required for replication initiation (or some other step in replication) has yet to be done. Purifying the BBB10-13 products, demonstrating DNA binding by BBB10, and ultimately, in vitro replication, would be useful. This biochemical approach might also confirm the location of the origin in the *bbb10-13* region. A similar approach could also delineate the *parS* region and which, if any, protein binds to that site. Our results lay the foundation for these more detailed biochemical studies by demonstrating that the *B. burgdorferi* proteins function in *E. coli* and identifying the crucial contributions of the BBB10, BBB11, and BBB13 products to miniF stability.

Acknowledgments

We thank Dr. David Lane (CNRS, Toulouse, France) for providing pDAG203 and bacterial strains. We thank Laura Cornelisse for help in characterizing the stability of the miniF26Δ*bbb12*. We thank Dan Sturdevant (Rocky Mountain Labs Research Technology Branch) for TaqMan primer-probe set design and Craig Martens (Rocky Mountain Labs Research Technology Branch) for constructing a phylogenetic tree with *bbb12* and related sequences. We are grateful to Jeff Skinner (Bioinformatics and Computational Biosciences Branch, NIAID) for advice on statistical analysis. We thank Paul Beare, John Carlson, Philip Stewart, Daniel Dulebohn, and Amit Sarkar for their helpful suggestions on the manuscript. Anita Mora and Austin Athman provided expert figure preparation. This research was supported by the Intramural Research Program of the NIH, NIAID.

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Highlights

- We assessed the ability of *B. burgdorferi* cp26 genes to stabilize defective miniF.
- Three genes in the region are required for miniF stabilization; one is dispensable.
- The dispensable gene was also inactivated on *B. burgdorferi* cp26.

Figure 1.

A. cp26 region capable of conferring autonomous replication in *B. burgdorferi*. Potential promoter upstream of *bbb10-13* denoted as P. Paralogous gene family (PF) names listed above. Potential functions, based on sequence homology (ParA) and ubiquitous presence on plasmids (Rep) indicated below. Putative *parS* is indicated by two arrowheads. Potential *ori* region downstream of *bbb13* indicated. B. Map of miniF26, in which the autonomous replication region from cp26 is cloned into the miniF plasmid pDAG203. *cam*, gene conferring chloramphenicol resistance. *ori2*, miniF replication origin. C. Region upstream of *bbb10-13* included in miniF26. Potential -35 and -10 regions of a promoter (predicted by the program BPPROM) in bold type. Other potential -35 regions underlined. *bbb10* potential

ribosome binding site (RBS) and start codon boxed in gray. D. Sequence of the putative *parS*. Direct and inverted repeats indicated by arrows. Stop codon of *bbb12* and start codon of *bbb13* are boxed in gray, as is the presumed RBS. Bases in bold type are identical to the *B. burgdorferi* chromosomal *parS* (see section 3.2). E. Potential replication origin downstream of *bbb13*. Direct and inverted repeats indicated by colored arrows. *Scal* site used in constructing *bbb13* deletion is boxed in gray, as is stop codon of *bbb13*.

Figure 2. miniF plasmids containing various portions of the *bbb10-13* region used in this study. Positions of deleted sequences are indicated (Δ). *Scal* and *MfeI* restriction enzyme sites used for mutant construction are indicated. P indicates position of putative promoter. Black triangles indicate repeats comprising the putative *parS*.

Figure 3.

Stability assessment of pDAG203 (no insert) versus miniF26 (*bbb10-13*) and pDAG203 with a control insert (*flgB_p*-*aacC1*). Cultures grown with chloramphenicol were diluted into medium lacking antibiotic and plated at several times after dilution. Plasmid retention was assessed by replica plating to medium with and without chloramphenicol. Cultures derived from frozen stocks of HB101/pDAG203 (no insert) or HB101 carrying miniF with the control insert often exhibited an intermediate stability phenotype, between those of HB101/miniF26 (*bbb10-13*) and that of a fresh transformant with pDAG203 (no insert, fresh transformant). In contrast, cultures derived from both fresh and frozen stocks of HB101/miniF26 exhibited similar stability.

Figure 4.

Stability assessment of various miniF26 derivatives compared to those of pDAG203 (no insert) and miniF26 (*bbb10-13*). A. Stability of plasmids miniF26 $\Delta bbb10$, miniF26 $\Delta bbb11$, and miniF26 $\Delta bbb10-11$, all of which are less stable than miniF26. B. Stability of miniF26 $\Delta bbb13$, which is less stable than miniF26. C. Stability of miniF26 $\Delta bbb12$, which exhibits similar stability to miniF26. D. Comparison of plasmid stability of miniF26 $\Delta bbb11$ and miniF26 with a chain termination mutation in *bbb11* (*bbb11**). Data shown are from single experiments with duplicate cultures; all stability tests were performed at least twice and usually three or more times, with comparable results. Numbers at late time points represent calculated upper limits of plasmid retention, when zero plasmid-containing cells were obtained in the presence of antibiotic and detection limits varied, depending on the number of colonies obtained in the absence of antibiotic.

Figure 5. Lack of stabilization of miniF derivative plasmids by *bbb10* and *bbb11* integrated into the *E. coli* chromosome. Stability of pDAG203 (no insert), miniF26 (*bbb10-13*), miniF26 $\Delta bbb10$, miniF26 $\Delta bbb11$, and miniF26 $\Delta bbb10-11$ in HB101 in which the *bbb10* and *bbb11* genes, with their own promoter region, were integrated into the *E. coli* chromosome.

Figure 6. Levels of *bbb10* and *bbb12* transcript in *E. coli* HB101 containing various miniF derivatives. Transcripts per 1000 copies of *dxs* (a chromosomal *E. coli* gene used as a normalization standard) detected in HB101 containing pDAG203 (no insert), miniF26 (*bbb10-13*), miniF26 $\Delta bbb10$, miniF26 $\Delta bbb11$, miniF26 $\Delta bbb12$, and miniF26 $\Delta bbb13$. Transcript levels were determined by TaqMan analysis of cDNA, with primer/probe sets specific for *bbb10* and *bbb12* (Table 1). Data shown are averages of two experiments with triplicate samples in each experiment. Omitting the reverse transcription reaction led to a reduction of approximately 70-1000-fold, depending on the sample and primer/probe set.

Fig. 7.

Assessing relative copy number of various miniF plasmids. Copy number was assessed by TaqMan analysis of total DNA preparations from HB101 containing pDAG203 (no insert), miniF26 (*bbb10-13*), miniF26Δ*bbb10*, miniF26Δ*bbb11*, miniF26Δ*bbb12*, and miniF26Δ*bbb13*. Each symbol represents the average of three assessments of miniF copies per *dxs* (*E. coli* chromosomal gene) copy. For pDAG203, miniF26, and miniF26Δ*bbb10*, three technical replicates of two biological replicates were performed, and for miniF26Δ*bbb11*, miniF26Δ*bbb12*, and miniF26Δ*bbb13*, three technical replicates were performed.

Table 1

Oligonucleotides

Number	name	sequence (5'→3') ¹
1	SpeF	ACTAGT CCTTACGGAGAAAAGGG
2	CC59	CTTTATATATATTGATATCTTTTGGACTAAGG
3	<i>flg5'</i> -AvrII	CCTAGG TAATACCCGAGCTTCAAGGAG
4	<i>aacC13'</i> -NheI	GCTAG CCCGATCTCGGCTTGAACG
5	<i>bbb11F</i>	ATGAAAAATAAAGAAACAAAAAAATTTTCA
6	<i>bbb12R</i>	TCATTAGAATTGAATCTTCTCTGTAAAAAATG
7	SpeRC	ACTAGT GGATTAGAAGATTAAAGC
8	CC51	CCG CTCGAG CTCCTATCGTTTGATCTTTATTGCATTAAGC
9	CC62	CCG CGCGAG ATGAAAAATAAAGAAACAAAAAAATTTTTCACAAAATTG
10	CC53	CCG CTCGAG ATTTTGTCTTACTTTTAAATTTTTTGTCT
11	CC54	CCG CTCGAG TTGGATAAAAAAACACAAAAATAATAACAATTGC
12	<i>flg5'</i> -XhoI	TAATA CTCGAG CTTCAAGGAAGATT
13	<i>kan3'</i> -XhoI	AT CTCGAG CTAGCGCCGTCCCGTCAA
14	CC60	CACACAAAAATCTTTAGCT <u>AAA</u> ATAAATAATTTTGAAGC ²
15	CC61	GCTTCAAAATTATTATT <u>AG</u> CTAAAGATTTTGTGTG ²
16	<i>dxs</i> for	GCGGCGTGAAACGAAGTG ³
17	<i>dxs</i> rev	AGTCCGGCAGGCCAATG ³
18	<i>bbb10</i> for	GCAATGTTGTAGCAATGACAAATCTATAT ³
19	<i>bbb10</i> rev	CCAAGTGGATTGAAATGACTTTTAAT ³
20	<i>bbb12</i> for	AAGCTTGCTCAATAATAGTTTAATCATCTC ³
21	<i>bbb12</i> rev	AAGATCATCAAGCCTGTTAATTATTAGGT ³
22	miniF for	TGTTTCGTTACCACACCGTAC ³
23	miniF rev	TGCGATTTCACCCCATCAG ³
Taqman probes (5'→3'; FAM-TAMRA labeled)		
1	<i>dxs</i>	CCCATCGTAAACCAGTACCCGTGCTG
2	<i>bbb10</i>	AAAGGATTCAAAAGGGTCACTAAAAGGACGATAC
3	<i>bbb12</i>	AATCATACCTTTGCCACAGACCAATGGG
4	miniF	TTAAAGACCGTAAGCAGGCTCGTTCC

¹ Added restriction sites in oligonucleotides indicated by bold type.

² Added stop codon in oligonucleotides indicated by underlining.

³ TaqMan primers