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Identification of loci critical for replication and compatibility of a *Borrelia burgdorferi* cp32 plasmid and use of a cp32-based shuttle vector for the expression of fluorescent reporters in the Lyme disease spirochaete

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Summary

The 32 kb circular plasmid (cp32) family of *Borrelia burgdorferi* has been the subject of intensive investigation because its members encode numerous differentially expressed lipoproteins. As many as nine different cp32s appear to be capable of stable replication within a single spirochaete. Here, we show that a construct (pCE310) containing a 4 kb fragment from the putative maintenance region of a *B. burgdorferi* CA-11.2A cp32 was capable of autonomous replication in both high-passage *B. burgdorferi* B31 and virulent *B. burgdorferi* 297. Deletion analysis revealed that only the member of paralogous family 57 and the adjacent non-coding segment were essential for replication. The PF32 ParA orthologue encoded by the pCE310 insert was almost identical to the PF32 orthologues encoded on the B31 and 297 cp32-3 plasmids. The finding that cp32-3 was selectively deleted in both B31 and 297 transformants carrying pCE310 demonstrated the importance of the PF32 protein for cp32 compatibility and confirmed the prediction that cp32 plasmids expressing identical PF32 paralogues are incompatible. A shuttle vector containing the CA-11.2A cp32 plasmid maintenance region was used to introduce green, yellow and cyan fluorescent protein reporters into *B. burgdorferi*. Flow cytometry revealed that the green fluorescent protein was well expressed by almost 90% of both avirulent and infectious transformants. In addition to enhancing our understanding of *B. burgdorferi* plasmid biology, our results further the development of genetic systems for dissecting pathogenic mechanisms in Lyme disease.

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Introduction

Borrelia burgdorferi, the causative agent of Lyme disease, the most common arthropod-borne disease in the United States, is maintained in an enzootic cycle involving both a tick vector from the genus *Ixodes* and a mammalian host, usually a small rodent (Lane *et al*., 1991; Centers for Disease Control and Prevention, 2001; Steere, 2001). These two hosts represent dramatically different nutritional, thermal and immunological environments for the bacterium (de Silva and Fikrig, 1997; Seshu and Skare, 2000). There is now a substantial body of evidence that the spirochaete's transition from arthropod to mammalian host is associated with striking changes in gene expression and antigenic composition (Schwan and Simpson, 1991; Akins *et al*., 1995; 1998; Schwan *et al*., 1995; Stevenson *et al*., 1995; 1998; Ryan *et al*., 1998; Carroll *et al*., 1999; Anguita *et al*., 2000; Indest *et al*., 2000; Schwan and Piesman, 2000; Yang *et al*., 2000; Hefty *et al*., 2001). Most of the differentially expressed borrelial genes identified to date are encoded on the bacterium's unusual complement of linear and circular plasmids (Champion *et al*., 1994; Akins *et al*., 1995; 1999; Schwan *et al*., 1995; Stevenson *et al*., 1995; 1998; Suk *et al*., 1995; Porcella *et al*., 1996; 2000; Lahdenne *et al*., 1997; Yang *et al*., 1999; Caimano *et al*., 2000; Carroll *et al*., 2000; Casjens *et al*., 2000). Evidence correlating the loss of specific plasmids with the attenuation of infectivity further underscores the importance of the extrachromosomal component of the genome as the repository for critical virulence determinants (Schwan *et al*., 1988; Norris *et al*., 1995; Xu *et al*.,

1996; Purser and Norris, 2000; Labandeira-Rey and Skare, 2001).

One group of *B. burgdorferi* plasmids, the 32 kb circular plasmids or cp32s, has been the source of intensive investigation because they encode multiple families of differentially expressed lipoproteins (Porcella *et al*., 1996; 2000; Stevenson *et al*., 1996; 2000; Akins *et al*., 1999; Yang *et al*., 1999; Caimano *et al*., 2000). Additionally, the cp32s are packaged by a lysogenic bacteriophage (ϕBB-1) capable of mediating the lateral exchange of these plasmids within a strain, as well as between strains (Eggers and Samuels, 1999; Eggers *et al*., 2001). The putative maintenance region is thought to be the key to understanding the remarkable stability and compatibility of the cp32 plasmids, up to nine of which can be faithfully maintained in a single *B. burgdorferi* isolate (Porcella *et al*., 1996; Stevenson *et al*., 1996; Casjens *et al*., 2000; Garcia-Lara *et al*., 2000). This region is composed of five open reading frames (ORFs), the paralogous family (PF) 57*,* PF50*,* PF32*,* PF49 and PF80 paralogues, flanked by two nearly identical inverted repeats, IR-A and IR-B (Fig. 1A) (Caimano *et al*., 2000; Casjens *et al*., 2000; Stevenson *et al*., 2000). The notion that this region is critical for cp32 maintenance is based on four lines of evidence: (i) analysis of the cumulative AT and GC skew of a number of *B. burgdorferi* cp32s indicates that the minimum cumulative skew, an indicator of the origin of replication, lies near their putative maintenance regions (Picardeau *et al*., 2000); (ii) four of the paralogous gene families, PF32, PF49, PF50 and PF57, are tightly clustered on a number of *B. burgdorferi* plasmids (Casjens *et al*., 2000); (iii) one of these genes, the PF32 paralogue, is an orthologue of *parA* and *sopA*, which play a role in the faithful partitioning of the low-copy-number plasmids P1 and F, respectively, in *Escherichia coli* (Helinski *et al*., 2000; Stevenson *et al*., 2000); and (iv) three members of the paralogous gene families PF49, PF50 and PF57 were shown recently to comprise the minimal replicon of cp9, the smallest circular plasmid of *B. burgdorferi* (Stewart *et al*., 2001).

The development of facile methodologies for manipulating *B. burgdorferi* genetically is a major objective of Lyme disease research (Tilly *et al*., 2000). Two strategies have been pursued to create the shuttle vectors necessary for gene complementation studies and the introduction of reporters for examining gene expression. The first is to use exogenous plasmids such as the Gram-positive, broad-host-range plasmid pGK12 (Saratokova *et al*., 2000). The second is to identify regions of *B. burgdorferi* plasmids capable of autonomous replication, as was accomplished recently for the cp9-based shuttle vector pBSV2 (Stewart *et al*., 2001). In line with the second approach, we have identified here the loci critical for replication and compatibility of a cp32 plasmid and demonstrated the feasibility of using cp32-based shuttle vectors for the expression of green fluorescent protein (GFP) reporters in *B. burgdorferi*. Interestingly, the seg-ment of DNA absolutely required for the replication of cp32 was found to be strikingly different from that required for the replication of cp9 (Stewart *et al*., 2001), a presumptive cp32 deletion derivative (Casjens *et al*., 2000). The results of our study broaden our understanding of the plasmid biology of an important human pathogen, as well as contributing to the further development of genetic systems for dissecting pathogenic mechanisms in Lyme disease.

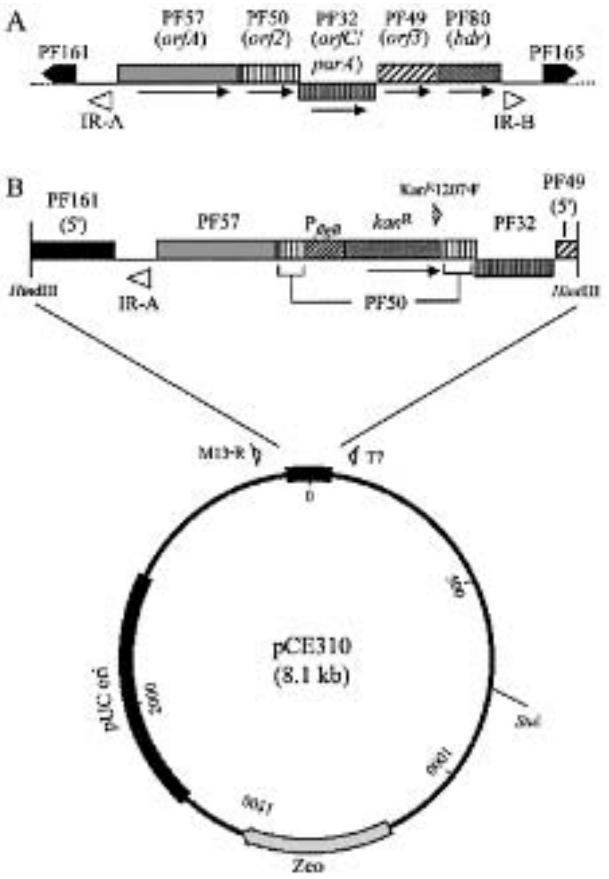


Fig. 1. Full-length and cloned cp32 maintenance regions.
A. Schematic showing the complete putative maintenance region of a cp32 plasmid. Published gene designations used before the completion of the *B. burgdorferi* B31 genomic sequence are shown in parentheses. The arrow below each ORF indicates the direction of transcription.
B. The portion of a CA-11.2A cp32 maintenance region cloned into pZErO-1 to create pCE310. Small arrowheads designate primers used to confirm the presence of pCE310 in transformants.

Results

Identification of a cp32 region sufficient for autonomous replication in B. burgdorferi

The starting point for the present study was the con-

struction of a pBluescript derivative, designated pCE210, containing an ≈ 4 kb fragment from a *B. burgdorferi* CA-11.2A cp32 as well as a 1.3 kb kanamycin resistance gene (*kan*^{R}) under the control of the *B. burgdorferi flgB* promoter (P_{flgB}) (Eggers *et al*., 2001). Sequence analysis revealed that the plasmid insert extended from the 5′ end of the PF161 gene to the 5′ end of the PF49 gene and that the *kan* cassette was inserted into the PF50 gene (Fig. 1B). The finding that the insert contained most of the putative cp32 plasmid maintenance region (Caimano *et al*., 2000; Casjens *et al*., 2000; Stevenson *et al*., 2000), suggested that it might be capable of supporting autonomous replication in *B. burgdorferi*. In order to examine this conjecture, we first transferred the pCE210 insert to a plasmid lacking the *amp*^{R} gene, thereby avoiding concerns about the introduction of beta-lactam resistance into *B. burgdorferi*. pZErO-1 was selected for this purpose, resulting in the creation of pCE310 (Fig. 1B).

pCE310 was electroporated into a high-passage *B. burgdorferi* B31 clone (B31-UM) or virulent *B. burgdorferi* 297 cells as described in *Experimental procedures*. Three lines of evidence confirmed that pCE310 was stably maintained in the kanamycin-resistant transformants. First, a polymerase chain reaction (PCR) product of the appropriate size was obtained from the B31 and 297 transformants using an internal *kan*^{R} primer and a pZErO-1 vector primer (Kan^{R}1207-F and T7 respectively; Table 1), whereas no products were obtained with Kan^{R}1207-F and a primer (cp32-3′cons-R; Table 1) directed against a highly conserved sequence in the PF80 paralogue that is not present in the pCE310 insert (Figs 1 and 2A). In contrast, a product was obtained with the Kan^{R}1207F/cp32-3′consR primer pair using DNA from *B. burgdorferi* B31/TR1, which contains the *kan* cassette integrated into the PF80 gene of a stably replicating cp32 transduced from strain CA-11.2A by ϕBB-1 (Fig. 2A) (Eggers *et al*., 2001). Secondly, using *kan*^{R} as a probe, a circular plasmid identical in size to pCE310 was detected by Southern hybridization of genomic DNA from the B31 and 297 transformants (Fig. 2B). When hybridized against *B. burgdorferi* B31/TR1, the same probe detected DNAs whose migration patterns were consistent with those previously associated with cp32s (Eggers *et al*., 2001). Lastly, a plasmid with a restriction profile identical to that of pCE310 was recovered from *E. coli* DH5α after transformation with DNA from the *B. burgdorferi* transformants (Fig. 2C), but not from *E. coli* transformed with DNA from the parental strains or B31/TR1 (data not shown). PCR analysis of the *E. coli* transformants using the Kan^{R}1207-F/T7 primer pair also confirmed the presence of pCE310 (data not shown). An analysis of the stability of pCE310 in a population of B31-UM transformants revealed that ≈ 75% of the cells passaged for 50 generations in the absence of antibiotic retained the shuttle vector, even in the presence of a competing endogenous plasmid (see below).

Table 1. Primers used in this study.

Primer		Sequence (5′→3′)
Screening for plasmids		
M13-R		CAGGAAACAGCTATGACCATG
T7		GTAATACGACTCACTATAGGGC
Kan^{R}1207-F	11[a]	ATTACGCTGACTTGACGGG
cp32-3′cons-R	12	TCATTATGAAGAAAAACAAAATCTATTGC
GFP601-R		GGTAATGGTTGTCTGGTAAAAGGACAGGGCC
BBS30-5′		ATGAAAATCATCAACATATTATTTT
BBS30-3′		CATTATTGCAGTTACTAACCGCTCC
PC-*osp*F-5′		CAGAACAAAATGTAAAAAAAACAGAGCAAG
PC-*osp*F-3′		CCCAAACTATTAGCACACTGCCAAG
Mapping of the cp32 replicon		
PF161 F[b]	1	CCCAATATTCTATACTCTTAAGCTCAG
PF161 KO-F	2	CCATATCCTTTGAGATTCTTATC
UpsPF57-F	3	CTCTGTTTGTATGTTATCCC
PF57-5′F	4	GCCACAACAAACACCACAC
PF57-5′R	5	GTGTGGTGTTTGTTGTGGC
MidPF57-R	6	GTTTTAAGATATTCATTGCTTCAATTTTCG
MidPF50-R	7	GCCAATGAACTTATCTCCTTC
PF50-3′R	8	CATTTGATTAACGGTCCTTGC
PF80-5′R	9	CTATTGCTTCTTCACTAAATCC
PF165-5′R	10	ATCTTTCAGCCCAGCACCTCCAAC
Promoter for reporter constructs		
Bb *flaB*-5′ prom (*Sph*I)[c]		GCATGCTGTCTGTCGCCTCTTGTGG
Bb *flaB*-3′ prom (*Sal*I)		GTCGACATATCATTCCTCCATGATAAAATTT

a. The numbers refer to the location of primers in Fig. 3B.
b. All construct primer sequences were derived from pCE310 or the original CA-11.2A cp32 and, unless mentioned in the text, the specificity to other paralogous genes is unknown.
c. Restriction sites underlined.

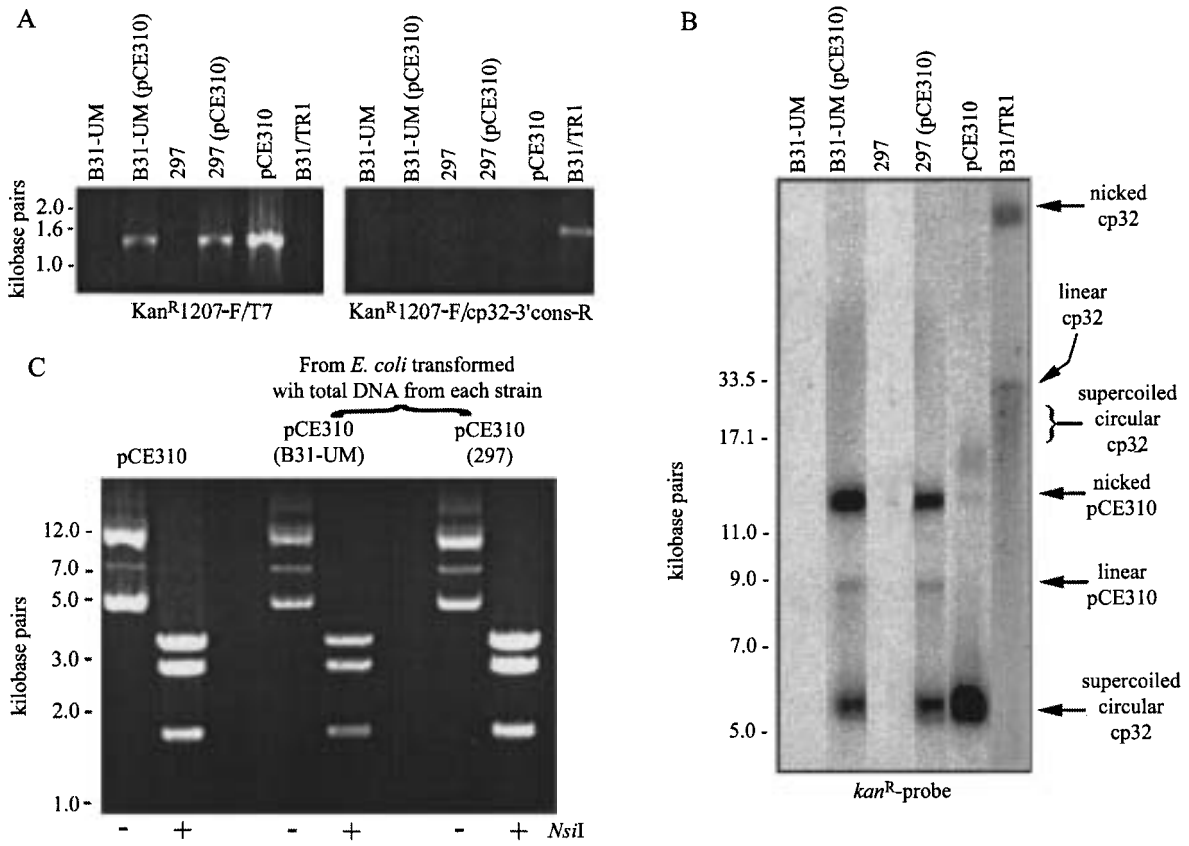
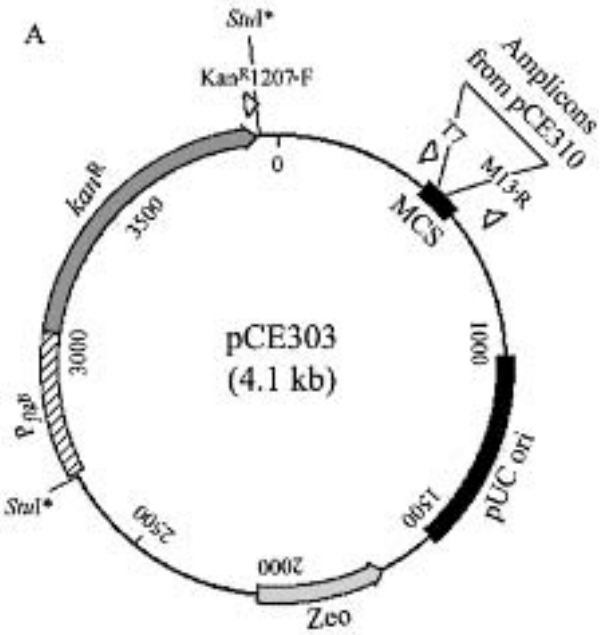


Fig. 2. Autonomous replication of pCE310 in *B. burgdorferi*.
A. DNAs from parental and transformed isolates of *B. burgdorferi* B31-UM and 297 were PCR amplified using primer sets consisting of an internal *kan*^{R} primer (Kan^{R}1207-F) and a vector primer (T7) or the internal *kan*^{R} primer and a primer directed against a conserved cp32 sequence not contained in the pCE310 insert (cp32-3′cons-R). B31/TR1 is a *B. burgdorferi* B31 clone containing a *kan* cassette integrated into a strain CA-11.2A cp32 transduced by ϕBB-1.
B. Southern hybridization of parental and transformant genomic DNAs using a *kan*^{R} probe. The nicked, linear and supercoiled circular forms of pCE310 and the B31/TR1 cp32 containing the *kan* cassette are indicated on the right.
C. Plasmid preparations of *E. coli* transformed with total genomic DNA from the B31-UM and 297 pCE310 transformants without (–) or with (+) digestion with *Nsi*I. Kilobase markers are shown on the left.

The transformation efficiency of the high-passage B31-UM clone with pCE310 was ≈ 80 colonies per 10 μg of DNA, whereas that of the low-passage 297 strain was ≥14 colonies per 10 μg of plasmid, with frequencies of 8.3×10^{-7} and 1.5×10^{-7} respectively. Although the value for the high-passage B31-UM clone is similar to that reported for another high-passage B31 clone, the transformation frequency of the infectious 297 isolate is approximately 10-fold greater than that reported for a virulent clone of *B. burgdorferi* N40 (Stewart *et al*., 2001). Consistent with previous reports that low-passage *B. burgdorferi* B31 MedImmune (B31-MI) is difficult to transform (Tilly *et al*., 2000; Stewart *et al*., 2001), we were unable to recover any transformants when this isolate was electroporated with pCE310.

Identification of the replicon in a strain CA-11.2A cp32

Having established that pCE310 can replicate in *B. burgdorferi*, we next wanted to define the site(s) critical for replication within the spirochaete. For these experiments, we took advantage of the serendipitous discovery of a high-passage B31 clone, B31-F, which has an ≈ 50-fold greater transformation efficiency than B31-UM. Interestingly, comparison of the plasmid profiles of B31-UM (see Fig. 6A) and B31-F (data not shown) isolates revealed that the latter is missing considerably more plasmids (including all the linear plasmids except lp17), suggesting an inverse relationship between plasmid content and transformability of these clones. Prospective replicons were PCR amplified and cloned into either pZErO-1 or pCE303 (Fig. 3A), as described in *Experimental procedures*. The ability of each construct to replicate in B31-F was assessed by its transformation frequency relative to that of pCE310; a summary of the data is presented in Fig. 3B. The minimum DNA required for replication was found to be the 2 kb insert in pCE316 that included all the PF57 gene and the entire intergenic



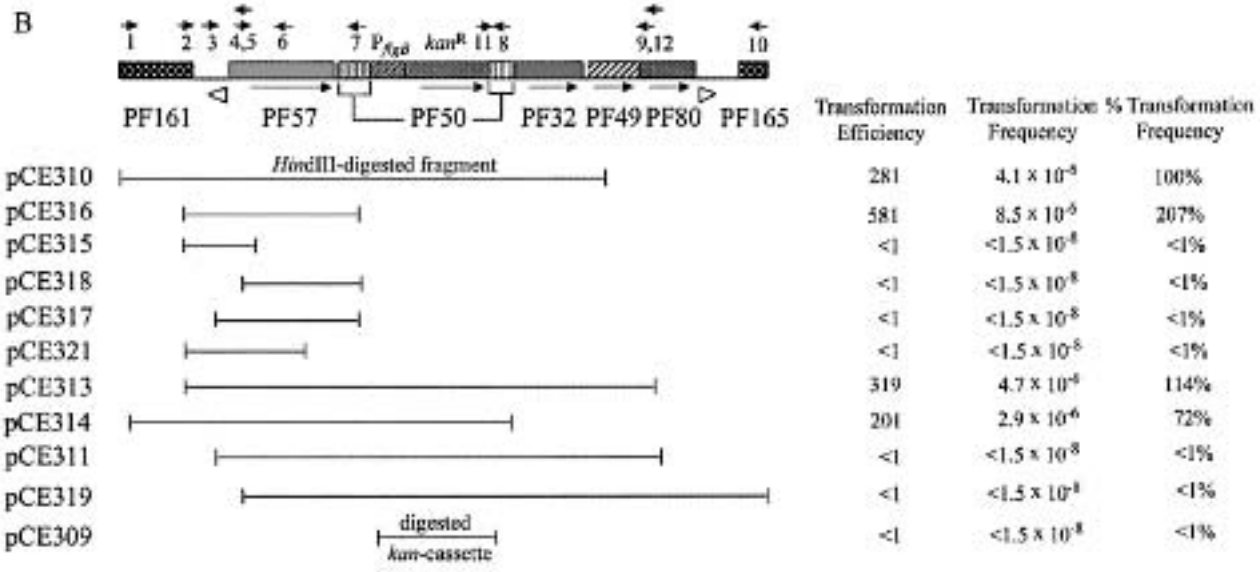


Fig. 3. Identification of the locus essential for replication from *B. burgdorferi* of a strain CA-11.2A cp32 plasmid.
A. Map depicting pCE303, the pZErO-1 derivative used to test the replication capacity of some of the fragments amplified from pCE310.
B. Diagrammatic representation of the maintenance region fragments used to identify the cp32 replicon; the numbered arrows indicate the primers used to generate constructs (see Table 1). Also shown are the transformation efficiencies and frequencies obtained for each construct, as well as the transformation frequency of each construct relative to that of pCE310 (indicated as a percentage). Values are based on three independent trials.

region between the PF57 and PF161 paralogues. An additional construct, pCE319, confirmed that the non-coding segments between the PF80 and the PF165 paralogues cannot substitute for the non-coding segment in pCE316, despite the presence of the PF57 gene. As expected, no transformants were recovered when B31-F was electroporated with pCE309, a vector containing the *kan* cassette alone.

The finding that the non-coding segment between the PF57 and PF161 paralogues is essential for replication prompted us to search for motifs that might function as binding sites for replication initiators. The relatively high AT content of this region (20% GC, whereas the average base composition of an entire cp32 is ≈ 29% GC) complicated the search for iterons, the imperfect AT-rich repeats that serve as binding sites for plasmid-encoded replication proteins (Helinski *et al*., 2000). However, using a relaxed sequence for the binding site of the DnaA initiation protein (Fuller *et al*., 1984; Moriya *et al*., 1988) and allowing for up to two mismatches per site (Picardeau *et al*., 1999), we were able to identify six possible DnaA boxes, as shown in Fig. 4. Boxes I–IV are located outside IR-A and represent sites deleted from the non-replicating constructs pCE311 and pCE317 (Fig. 3B), suggesting that one or more of these motifs is critical for plasmid replication. To garner additional evidence that DnaA-binding motifs are essential for replication, we examined the corresponding non-coding segments from *B. burgdorferi*

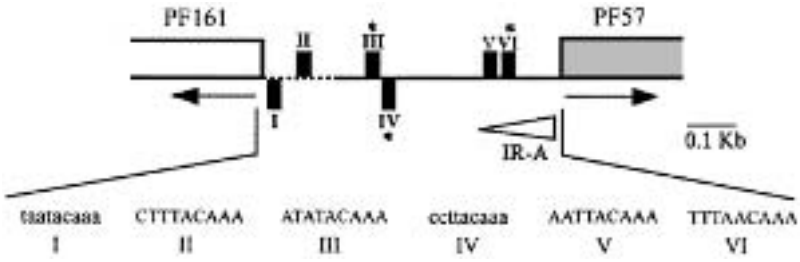


Fig. 4. Schematic showing the locations and sequences of the putative DnaA boxes in the pCE310 insert. Possible DnaA boxes were identified using the relaxed consensus sequence [T(C/T)(A/T)T(A/C)CA(A/C)A]. Boxes I and IV are shown below the line (schematic) and in lower case (sequences) to indicate that they are located on the opposite strand. Asterisks indicate boxes in conserved locations in the strain B31 and 297 cp32s. The dashed line indicates the portion of the strain CA-11.2A cp32 non-coding segment not found in any of the B31 or strain 297 cp32s.

B31-MI and 297 cp32s. Not surprisingly, a global alignment revealed that the intergenic sequence of the CA-11.2A cp32 represented by pCE310 and those of the strain B31 and 297 cp32s are highly similar (≈ 65–75% identity). Particularly noteworthy is that all the cp32s except for cp32-7 from *B. burgdorferi* 297 (which lacks box IV) contain two potential DnaA boxes in approximately the same location outside IR-A and a third identically placed potential binding site just within the repeat (Fig. 4).

pCE310 is incompatible with the cp32-3 plasmids of both B. burgdorferi *B31 and 297*

The differences among the PF32 ParA paralogues have been proposed to contribute to the compatibility of cp32 plasmids (Caimano *et al*., 2000; Casjens *et al*., 2000; Stevenson *et al*., 2000). Therefore, to determine whether pCE310 would be incompatible with a pre-existing cp32 in the *B. burgdorferi* transformants was of considerable interest. To examine this issue, we first assessed the phylogenetic relationships between the PF32 protein encoded on pCE310 and its B31-MI and 297 orthologues. The bootstrap values shown in the phylogram in Fig. 5 support a strong pairwise distribution of most of the B31-MI and strain 297 cp32 PF32 proteins, the exceptions being the orthologues from the strain 297 cp32-6 and cp32-7 plasmids, which lack B31-MI counterparts, and the orthologue from the cp32 integrated into lp56 of B31-MI, which lacks a 297 counterpart. The PF32 protein encoded by pCE310 is most closely related (96% amino acid identity) to the paralogues encoded on the B31 and 297 cp32-3 plasmids; the high bootstrap values generated during phylogenetic analysis strongly support placing the three plasmids into the same clade. We next assessed the plasmid contents of the B31-UM and 297 pCE310 transformants during serial passage in the presence or absence of kanamycin. Consistent with the phylograms, only cp32-3 was lost by either transformant (Fig. 6A and B). Interestingly, for the B31-UM transformant, the plasmid was lost only when kanamycin was present, whereas antibiotic pressure was not required for deletion of the plasmid from the 297 strain, which has a larger complement of cp32 plasmids. The importance of the PF32 paralogue for determining plasmid compatibility was underscored by the observation that cp32-3 was not deleted from B31-F transformants containing pCE314 or pCE316, both of which lack PF32 genes (Fig. 3B), even with prolonged passage under antibiotic pressure (Fig. 6C). Other investigators have noted that the cp32-3 can be lost spontaneously by the B31 strain during cloning (Purser and Norris, 2000). However, spontaneous loss of this plasmid is an unlikely explanation for our findings given that (i) it is extremely stable in the parental B31-UM, B31-F and 297 isolates used for these transformation experiments; and (ii) selective deletion of cp32-3 after transformation with pCE310 was highly reproducible.

pCE310 can be used for the expression of GFP reporters in B. burgdorferi

In order to be used for studying differential gene expression, a cp32-based shuttle vector must be able to serve as a platform for the maintenance and expression of reporters within the spirochaete. To assess the utility of the pCE310 insert for this purpose, we created a new plasmid, pCE320, in which the entire 4 kb fragment was moved to the *Stu*I site of pZErO-1, thereby liberating the MCS for the introduction of reporter genes (Fig. 7A). As described in *Experimental procedures*, we next cloned *gfp*, yellow fluorescent protein (*yfp*) or cyan fluorescent protein (*cfp*) genes (Miller *et al*., 2000) with or without an upstream *B. burgdorferi flaB* promoter (P_{flaB}) into the MCS of pCE320, creating pCE320(*gfp*), pCE320(*gfp*)-P_{flaB}, pCE320(*yfp*), pCE320(*yfp*)-P_{flaB}, pCE320(*cfp*) and

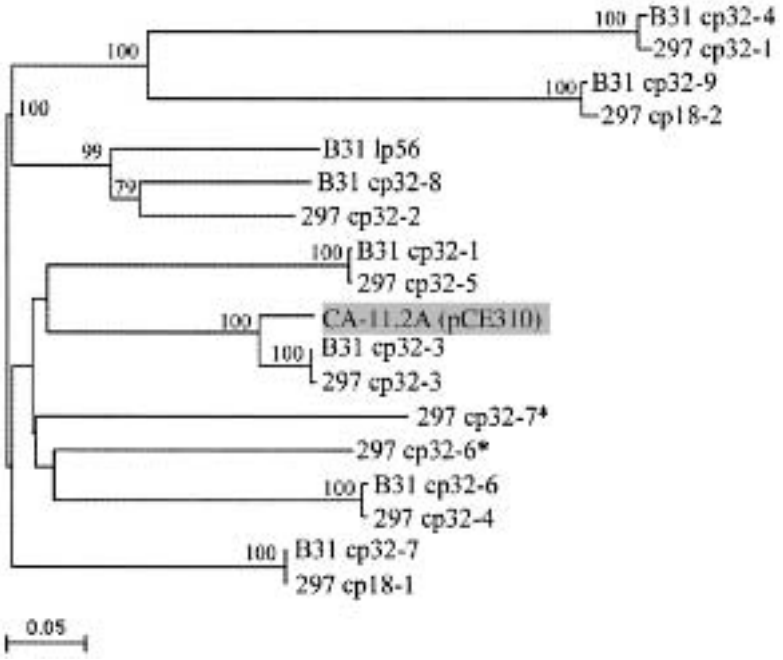


Fig. 5. Neighbour-joining phylogram of cp32 PF32 orthologues. Numbers represent bootstrap percentages (nodal support from 1000 pseudotrees randomly generated from the alignment). Higher bootstrap values represent stronger support for sequence associations. The number designations for the cp32s from strain 297 were assigned previously based on differences in the encoded lipoproteins (Yang *et al*., 1999; Caimano *et al*., 2000). Asterisks indicate the two strain 297 cp32s (cp32-6 and cp32-7) that lack B31 counterparts. The scale bar represents substitutions per site.

pCE320(*cfp*)-P_{flaB}. Examination of B31-F transformants by both darkfield and epifluorescence microscopy revealed that all three reporters were well expressed when driven by P_{flaB} (Fig. 7B and C) and that fluorescence was not enhanced by vigorous aeration of the cells (data not shown). In contrast, much lower levels of fluorescence were observed for spirochaetes transformed with the promoterless constructs (Fig. 7B; data not shown), and no fluorescence was observed for spirochaetes transformed with pCE320, demonstrating that the spirochaetes were not autofluorescent. Identical results were obtained with 297-c155, a virulent strain 297 clone, transformed with the *gfp*-containing plasmids (Fig. 7D). 297-c155, a well-characterized clone, was used to be sure that population heterogeneity with respect to GFP expression was not a reflection of lack of clonality

To complement the light microscopy studies, we next used multichannel flow cytometry to examine GFP expression patterns in populations of avirulent (B31-F) and virulent (297-c155) spirochaetes carrying the various plasmid constructs. Because of the unusual morphology of the spirochaetes, we first used the nucleic acid staining dye SYTO59 to determine whether events enumerated on the basis of forward and side light scatter actually represented organisms. Spirochaetes (i.e. SYTO59-positive events) were found to comprise between 97% and 99% of total events counted in mid-logarithmic phase cultures. We then gated on the SYTO59-positive events to assess levels of GFP expression as shown in Fig. 8. Consistent with the microscopy results, nearly 90% of the B31-F and 297-c155 organisms transformed with pCE320(*gfp*)-P_{flaB} were fluorescent as opposed to the markedly smaller proportions of fluorescent spirochaetes carrying the promoterless construct (Figs 7B and 8). Of particular importance, the mean fluorescence intensities (MFIs) of the populations containing pCE320(*gfp*)-P_{flaB} were between eight- and 10-fold greater than those containing pCE320(*gfp*).

We also sought to determine why some organisms within the transformant populations exhibited a non-fluorescent phenotype. Only 0.4% of the mid-logarithmic phase B31-F cells shown in Fig. 8 stained with propidium iodide, which labels only non-viable cells, thereby ruling out the possibility that the absence of fluorescence was related to a lack of cell viability. Analysis of B31-F transformants at different points in the growth curve revealed that the proportions of fluorescent and non-fluorescent organisms remained essentially constant (data not shown), indicating that lack of fluorescence was not a function of the stage of growth. Lastly, two lines of evidence argued that genetic rearrangements, such as loss of the P_{flaB} promoter and/or deletion of the entire reporter gene, had not occurred within a subpopulation of transformants. First, using the T7/GFP601-R and T7/M13-R primer pairs (Table 1), which amplify across the P_{flaB} promoter and the entire *gfp* allele, respectively, only single products were obtained from the B31-F and 297-c155 transformant populations. Secondly, we were repeatedly unable to isolate non-fluorescent colonies when transformant populations of both strains were cloned out onto

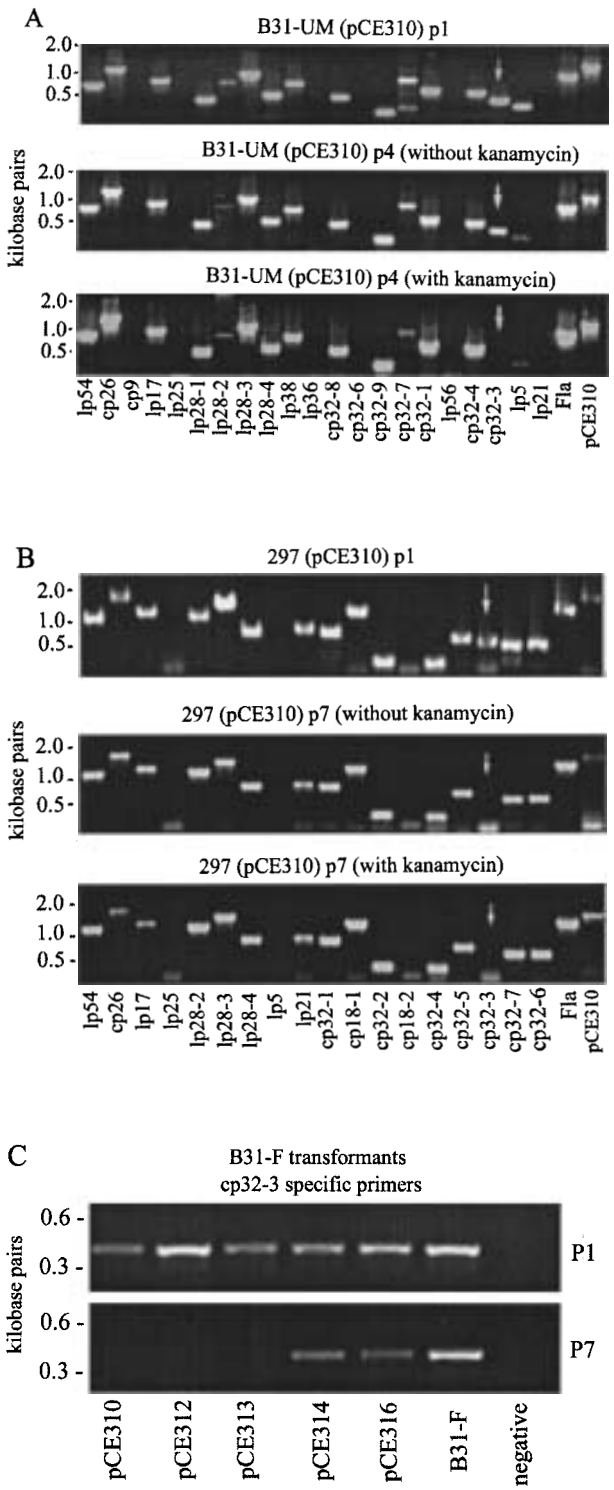


Fig. 6. pCE310 is incompatible with cp32-3 in *B. burgdorferi* B31 and 297. PCR using plasmid-specific primers was performed to analyse the plasmid contents of B31-UM (A) and strain 297 (B) transformants before and after passaging in the presence or absence of kanamycin. White arrows designate the PCR products derived from the cp32-3 plasmids.
C. PCR analysis demonstrating that transformation of B31-F with pCE314 and pCE316, both of which lack PF32 paralogues, fails to displace cp32-3.

solid medium. This latter finding also eliminated the possibility that the non-fluorescent cells were spontaneous kanamycin-resistant mutants.

Discussion

Here, we present the first experimental evidence supporting the prediction that the portion of the cp32s spanning from the PF57 to the PF80 paralogue encodes functions essential for plasmid maintenance (Caimano *et al*., 2000; Casjens *et al*., 2000; Garcia-Lara *et al*., 2000; Stevenson *et al*., 2000). Moreover, our studies have enabled us to extend these predictions by demonstrating that the replication and compatibility functions are spatially separated and dissociable. The flanking inverted repeats have been proposed to be insertion sequences that delimit the maintenance region (Carlyon *et al*., 1998; Stevenson *et al*., 2000). It was surprising to note therefore that the intergenic sequence upstream of IR-A was absolutely required for replication of pCE310 and presumably the cp32 of CA-11.2A, which was the original source of the pCE310 insert. Our analysis of this segment in a number of cp32s leads us to propose that it contains critical motifs for the binding of the DnaA initiator protein. Additionally, of the five genes that comprise the hypothesized maintenance machinery, only the PF57 paralogue was required *in cis* for replication. The high degree of conservation of cp32 PF57 proteins (75% identical with ≈ 80% sequence similarity) might lead one to predict that its function could be supplied *in trans* by another cp32-encoded paralogue. That this was not the case suggests either that sequence differences between these polypeptides confer plasmid specificity or that the ORF also contains unidentified binding sites for additional components of the replication machinery. In contrast, and contrary to predictions (Casjens *et al*., 2000), the PF50 gene was not required *in cis* for replication. Because of the lack of database matches, we presently cannot say whether the PF50 protein has no role in plasmid replication or whether its activity can be provided *in trans* by other paralogues. Regardless, the observation that the PF50 gene can be disrupted without a deleterious effect on plasmid replication is of utilitarian significance because the ORF provides a convenient site for the insertion of antibiotic resistance markers.

Stewart *et al*. (2000) recently described a 3.3 kb fragment from the small circular cp9 plasmid that enabled autonomous replication in *B. burgdorferi*. Because cp9 has been described as a cp32 deletion derivative (Stevenson *et al*., 1996; Zückert and Meyer, 1996; Casjens *et al*., 2000), a comparison of their findings with those presented here seems particularly instructive. The cp9 maintenance region consists of just the PF57, PF50 and PF49 genes flanked by IR-A- and IR-B-like inverted repeats (Dunn *et al*., 1994; Caimano *et al*., 2000; Casjens *et al*., 2000);

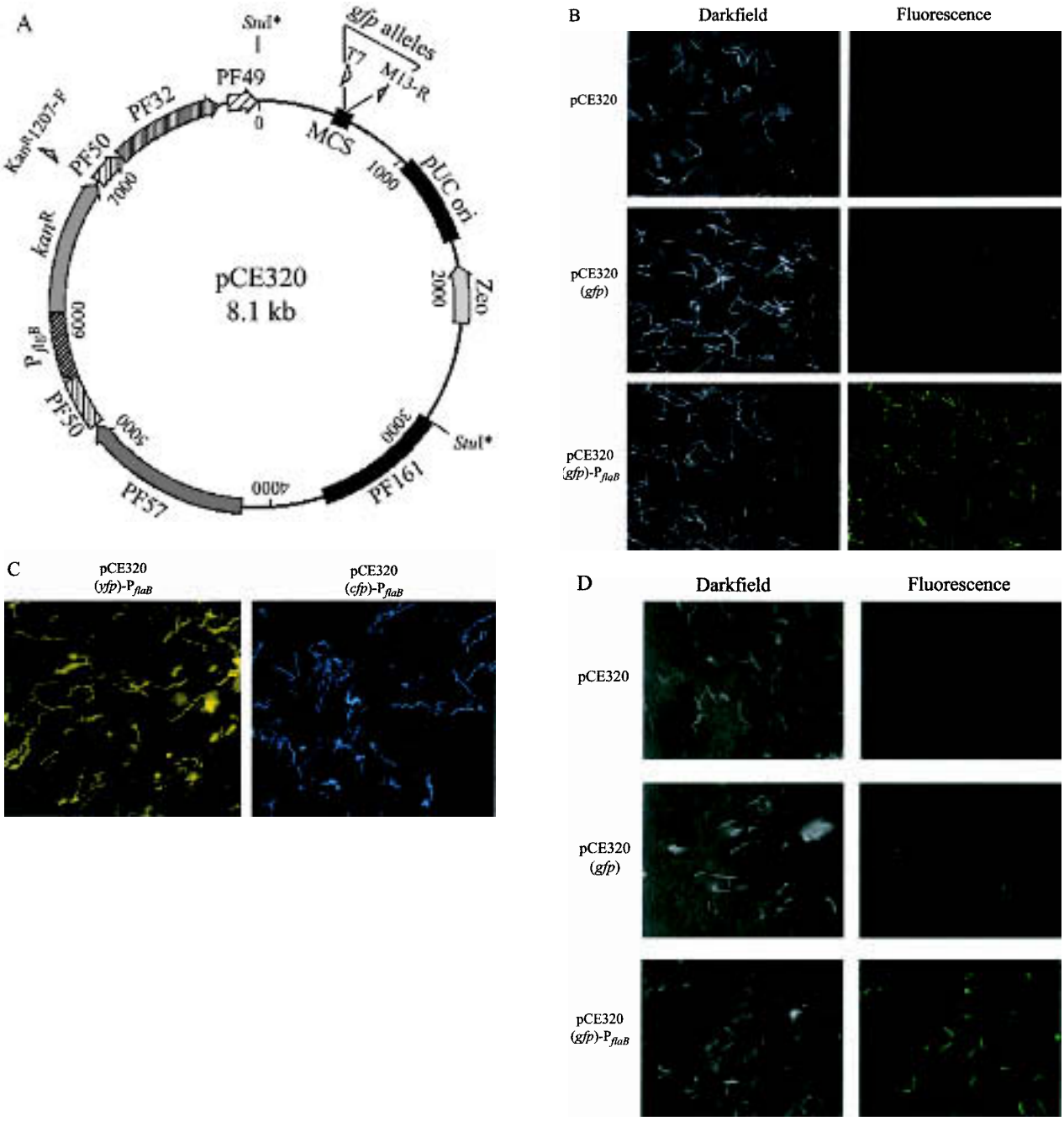


Fig. 7. Expression of GFP reporters in *B. burgdorferi*.
A. Map depicting pCE320, the shuttle vector used for the cloning of *gfp* alleles.
B. Darkfield and epifluorescence micrographs (400×) of *B. burgdorferi* B31-F transformed with pCE320, pCE320(*gfp*) and pCE320(*gfp*)-P_{flaB}.
C. *B. burgdorferi* B31-F carrying pCE320(*yfp*)-P_{flaB} and pCE320(*cfp*)-P_{flaB}, viewed under oil at 1000×.
D. Darkfield and epifluorescence micrographs (400×) of the virulent clone 297-c155 transformed with pCE320, pCE320(*gfp*) and pCE320(*gfp*)-P_{flaB}.

the plasmid lacks sizeable non-coding sequences outside the inverted repeats such as are present in the cp32s. In striking contrast to our findings, replication of a cp9-based shuttle vector in *B. burgdorferi* required all three maintenance region genes but neither inverted repeat. Thus, despite the ostensibly close evolutionary relationship between the cp9 and the cp32s, the two replicons differ markedly with respect to both the presence and the location of potential binding sites for replication initiators and the requirement for particular genes in the *cis* orientation.

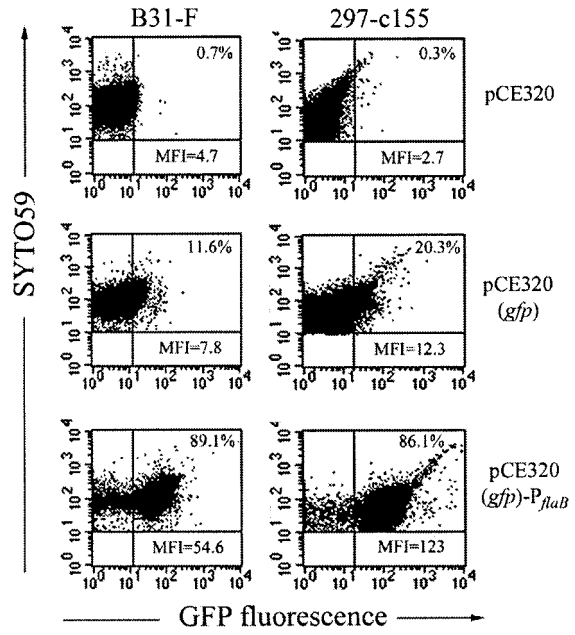


Fig. 8. Flow cytometric analysis of GFP expression in *B. burgdorferi* populations. The percentages in the upper right graph indicate the proportions of SYTO59-positive B31-F or 297-c155 organisms carrying pCE320, pCE320(*gfp*) or pCE320(*gfp*)-P_{flaB} that were positive for GFP expression. Also shown is the MFI for the total population. Approximately 25 000 events were analysed for each transformant.

It is tempting to speculate that the evolutionary forces driving the functional divergence between the cp9 and cp32 plasmids also involved the replication regions in order to eliminate competition between the replication machineries of the two types of plasmids. Another consequence of this divergence could be the relative instability of the cp9 plasmid, which is known to be readily lost upon repeated *in vitro* cultivation (Purser and Norris, 2000; Labandeira-Rey and Skare, 2001; McDowell *et al*., 2001).

Low-copy-number plasmids require a partitioning system to ensure stable transmission to daughter cells. Plasmids are incompatible when their partitioning apparatuses compete with each other, causing an unequal distribution of plasmids during successive divisions that culminates in plasmid loss (Helinski *et al*., 2000). The potential for plasmid instability would appear to be particularly severe among the cp32 family, the largest group of homologous genetic elements within the Lyme disease spirochaete. Consequently, any model for cp32 maintenance has to explain not only how the partitioning functions are carried out for individual plasmids, but also how this is accomplished without creating incompatibilities. It has been proposed previously, based upon sequence homology with the ParA Walker-type ATPase of the P1 plasmid, that the PF32 proteins comprise part of the cp32 partitioning apparatus and that sequence diversity among the paralogues plays a role in preventing plasmid incompatibilities (Casjens *et al*., 2000; Stevenson *et al*., 2000). In support of this, we found that the transformation of two different *B. burgdorferi* strains with pCE310 resulted in the selective deletion of the cp32 plasmid with the most closely related PF32 orthologue, as predicted by phylogenetic analysis. This result appears to be analogous to the observation that an excess of ParA or SopA will destabilize partitioning of the P1 and F plasmids respectively (Abeles *et al*., 1985; Lemonnier *et al*., 2000). The PF49 proteins also have significant sequence variability and have been proposed to fulfil a ParB function based on the location of the PF49 genes directly downstream of the PF32 paralogues on the cp32 plasmids (Gerdes *et al*., 2000). It is noteworthy that the PF49 proteins distribute phylogenetically in a pattern identical to that of the PF32 proteins (data not shown), suggesting a co-evolutionary relationship between these two paralogous families consistent with their proposed plasmid-specific functional interactions.

Our compatibility data, taken as a whole, have some provocative implications for future genetic studies. Thus far, correlations between plasmid content and borrelial virulence have been limited to those plasmids that are lost spontaneously (Purser and Norris, 2000; Labandeira-Rey and Skare, 2001; McDowell *et al*., 2001). Conceivably, the phylogenetic relationships among the partitioning components (in essence, establishing incompatibility groups) can be exploited to target specific cp32 plasmids for deletion and subsequent analysis of infectivity. Alternatively, one might use these groupings to avoid incompatibilities that could limit the use of cp32-based shuttle vectors for genetic manipulation of *B. burgdorferi* and analysis of virulence expression. As one obvious example, one could take advantage of the fact that there are two strain 297 cp32s that do not have closely matched B31-MI PF32 orthologues to create shuttle vectors that would be compatible with the full plasmid component of a B31-MI host.

To date, the use of reporters for studying gene expression in *B. burgdorferi* has mainly been limited to the introduction of chloramphenicol acetyl transferase (CAT) on non-replicative plasmids (Sohaskey *et al*., 1997; 1999). A major drawback with CAT, however, is that, being an enzymatic marker, it only provides information pertaining to mean levels of gene expression in the bacterial population under investigation. On the other hand, the use of an endogenous fluorescent label, such as GFP, enables one to monitor gene expression at the single-cell level and to obtain quantitative and statistically analysable data when combined with the use of flow cytometry (Valdivia and Falkow, 1998). When Saratokova *et al*. (2000) introduced enhanced *gfp* under the control of the *flaB* promoter into *B. burgdorferi* on a replicating broad-host-range plasmid,

they observed low levels of fluorescence, with only a fraction of the spirochaetes fluorescing intensely enough to be photographed. Using the same promoter but a different *gfp* allele (*gfpmut1*) (Cormack *et al*., 1996), however, we found that the large majority of both high-passage and virulent spirochaetes expressed easily detectable fluorescence. Lack of fluorescence by a small proportion of organisms was not a function of cell viability or phase of growth, nor was it the result of rearrangements within the reporter gene or the appearance of spontaneous kanamycin-resistant mutants. It is conceivable, therefore, that shuttle vector copy numbers within a transformant population follow a Poisson distribution and that fewer plasmids per cell are required for kanamycin resistance than for detectable fluorescence. Alternatively, the low fluorescence could result from the variation in the transcription of the *flaB* promoter at different stages in the cell cycle. Moreover, although we did observe some readthrough from promoter elements located elsewhere on the plasmid, the low level of fluorescence produced by the promoterless construct was easily distinguished from that produced by the constitutively expressed *flaB* promoter. Differential expression of *B. burgdorferi* lipoproteins often involves reciprocal and/or highly co-ordinated regulation of borrelial genes in response to changing environmental signals. The availability of several compatible shuttle vectors (Saratokova *et al*., 2000; Stewart *et al*., 2001), coupled with the ability to express *gfp* alleles with minimally overlapping emission spectra, should enable investigators in the near future to devise the genetic systems required to dissect these complex regulatory mechanisms at the single-cell level.

Experimental procedures

Borrelia burgdorferi *strains and culture conditions*

Borrelia burgdorferi strains were grown in liquid BSK II supplemented with 6% heat-inactivated normal rabbit serum (NRS) or solid BSK medium supplemented with 4% NRS at 34°C under a 4% CO_2 atmosphere (Barbour, 1984; Samuels, 1995). *B. burgdorferi* clones B31-UM and B31-F were picked as single colonies after plating high-passage B31 isolates in solid medium. Virulent (i.e. low-passage) *B. burgdorferi* 297 (Steere *et al*., 1983) was maintained as described previously (Akins *et al*., 1998). A virulent clone, designated 297-c155, was derived from low-passage *B. burgdorferi* 297 by two rounds of single-colony isolation on solid BSK medium followed by intradermal inoculation of a C3H/HeJ mouse with 1×10^3 organisms. An isolate obtained by ear punch (Sinsky and Piesman, 1989) was tested for infectivity by intradermal inoculation, recloned on solid medium and then re-evaluated for infectivity by intradermal inoculation.

Construction of plasmids

To create pCE310 (Fig. 1B), the insert of pCE210 (Eggers *et al*., 2001) was excised by digestion with *Hin*dIII and ligated into the corresponding site of pZErO-1 (Invitrogen Life Technologies). To create the intermediate vector pCE300, a 70 bp blunt-end fragment derived from *Hae*III-digested ϕX174 DNA was cloned into the *Eco*RV site of pZErO-1.

Plasmids used to map the CA-11.2A S plasmid replicon. Sequences for primer pairs used for the construction of the plasmids described below are shown in Table 1. The locations of the primers in the cp32 maintenance region are indicated in Fig. 3B. The *kan* cassette from pTAkanGn (Bono *et al*., 2000) (kindly provided by P. Rosa) was excised as a *Sac*I–*Not*I fragment, blunt-ended with mung bean nuclease (MBN; New England Biolabs) and ligated into the *Stu*I site of pCE300 to create pCE303 (Fig. 3A). PCR fragments amplified from pCE310 were cloned into pZErO-1 to create pCE314 or into pCE303 to create pCE315, pCE316, pCE317, pCE318 and pCE321. The entire maintenance region from the same strain CA-11.2A cp32 was PCR amplified from CA-11.2A TR-3 genomic DNA (Eggers *et al*., 2001) using the PF161-F/PF165-5′R primer pair and cloned into pZErO-1 to create pCE312. The inserts for plasmids pCE311, pCE313 and pCE319 were amplified from pCE312 using the indicated primer pairs and cloned into pZErO-1. pCE309 was created by cloning the *kan* cassette from pTAkanGn into *Sac*I–*Not*I-digested pZErO-1.

Plasmids used for the expression of GFP reporters in B. burgdorferi. The insert of pCE310 was excised by digestion with *Hin*dIII, blunt-ended with MBN and cloned into the *Stu*I site of pCE300 to create pCE320 (Fig. 7A). The *flaB* promoter (P_{flaB}) was amplified from *B. burgdorferi* B31-MI genomic DNA using the *flaB* prom-5′/*flaB* prom-3′ primer pair (Table 1) and cloned into *Sph*I–*Sal*I-digested pWM1015, pWM1018 and pWM1019, which contain the *gfpmut1*, *yfp* and *cfp* genes, respectively, flanked by upstream and downstream transcriptional terminators. pWM1015, pWM1018 and pWM1019 are identical to plasmids described by Miller *et al*. (2000), except that the downstream terminator in each has been replaced with one derived from phage λ. The *gfp*, *yfp* and *cfp* cassettes (± P_{flaB}) were excised with *Cla*I and *Bgl*II, ligated into *Bam*HI–*Cla*I-digested pBluescript II SK+ (Stratagene), excised from the pBluescript derivatives using *Spe*I and *Xho*I and then ligated into *Spe*I–*Xho*I-digested pCE320 to create pCE320(*gfp*), pCE320(*gfp*)-P_{flaB}, pCE320(*yfp*), pCE320(*yfp*)-P_{flaB}, pCE320(*cfp*) and pCE320(*cfp*)-P_{flaB}.

Transformation of B. burgdorferi *and screening of transformants*

Preparation of competent *B. burgdorferi* was performed as described previously (Samuels, 1995). Ten micrograms (B31-UM and 297) or 1 μg (B31-F) of circular plasmid DNA were used per electroporation of ≈ 1×10^9 cells. The cells were electroporated in a cuvette with a 0.1 cm electrode gap and a single exponential decay pulse of 1.25 kV, which produced time constants between 4.0 and 5.5 ms. The cells were allowed to recover for 16–20 h in BSK II without antibiotics and then plated in solid BSK medium supplemented with 400 μg ml^{-1} kanamycin. Colonies were picked with a sterile

Pasteur pipette and inoculated into 4 ml of BSK II containing 400 μg ml^{-1} kanamycin. After 2–3 days, DNA was extracted from an aliquot of culture using the Isoquick nucleic acid extraction kit (Orca). The presence of the plasmid of interest was confirmed by PCR. Transformation efficiencies were determined as the number of transformants recovered after electroporation. The transformation frequencies were determined as the number of transformants recovered after electroporation when selected with kanamycin divided by the total number of possible transformants recovered after plating in the absence of the antibiotic. The percentage transformation frequency for the replication constructs was determined as the transformation frequency of each construct divided by that of the cells transformed with 1 μg of pCE310. Values are based on three independent trials.

Southern hybridization

Total genomic DNA was extracted from 50 ml cultures of *B. burgdorferi* as described above. pCE310 was extracted from *E. coli* TOP10 using the Concert high-purity plasmid midiprep system (Invitrogen Life Technologies). Genomic DNA (300 ng per lane) was resolved in 0.5% GTG agarose gels in 0.5× TBE at 20 V for 30–40 h. Gels were stained, photographed and blotted to Immobilon-Ny^{+} nylon membrane (Millipore) using the Stratagene Posiblot 30-30 pressure blotter. The membranes were cross-linked in a Stratalinker 1800. The *kan*^{R} probe was derived from plasmid pOK12 (Vieira and Messing, 1991) and labelled using the Random Primers DNA labelling system (Invitrogen Life Technologies). Prehybridization, hybridization, washing and visualization were performed as described previously (Eggers and Samuels, 1999).

Restriction analysis of plasmid recovered from E. coli *transformed with total genomic DNA from* B. burgdorferi *B31(pCE310) and 297(pCE310)*

Total DNA was extracted from 5 ml of the B31-UM and 297 transformants as described above. DNA (100 ng) was transformed into chemically competent *E. coli* DH5α, prepared according to the $CaCl_2$ method (Ausubel *et al*., 1997). Transformants were selected on 50 μg ml^{-1} kanamycin LB plates. *E. coli* colonies were grown in LB broth supplemented with 50 μg ml^{-1} kanamycin, and plasmid DNA was extracted from 1.5 ml of culture using an alkaline lysis method (Ausubel *et al*., 1997). The recovered plasmid DNA was treated with RNase, and an aliquot was digested with *Nsi*I (New England Biolabs). The digested and undigested plasmids recovered from *E. coli* transformed with either pCE310 or total *B. burgdorferi* transformant DNA were resolved in a 0.8% agarose gel and stained with EtBr.

Comparative sequence analysis

DNA sequencing was performed with an Applied Biosystems model 377 automated DNA sequencer with the BigDye cycle sequencing kit. The complete sequence of the pCE310 insert was determined by primer walking along both strands from the T7 to M13-R primer sites of the pZErO-1 vector. All B31-MI sequences were obtained from the *B. burgdorferi* genome database website (http://www.tigr.org/tigrscripts/CMR2/GenomePage3.spl?database=gbb) (Fraser *et al*., 1997). The intergenic region sequences between the PF161 and the PF57 genes in *B. burgdorferi* 297 were determined directly from PCR amplicons generated using a conserved PF161 primer (PF161KO-F) and PF32 primers specific for strain 297 cp32s (see Table 1 and *Supplementary material*).

The *B. burgdorferi* 297 cp32 PF32 gene sequences were determined by sequencing TOPO-cloned fragments amplified from genomic DNA template using ml*p*-specific primers (Caimano *et al*., 2000) and a downstream conserved primer (cp32-3′-consR; Table 1). To produce a phylogenetic tree of the cp32 PF32 polypeptides from B31-MI, strain 297 and the CA-11.2A S plasmid, a multiple sequence alignment was generated using the CLUSTAL W (version 1.4) program within the MACVECTOR (version 7.0) software package (Accelrys Bioinformatics) and then analysed using the neighbour-joining tree-building method in BEST tree mode with uncorrected ('p') distance, mid-point rooting and proportionate gap distribution options. Bootstrap values were derived from 1000 pseudosamples.

The sequence of the pCE310 insert was submitted to GenBank under accession no. AF409199. The intergenic region sequences of the strain 297 cp32s were submitted to GenBank under accession nos AF409193 (cp32-4), AF409194 (cp32-6), AF09195 (cp18-1), AF409196 (cp32-7), AF409197 (cp32-5), AF427136 (cp32-1), AF427137 (cp32-2) and AF409198 (cp32-3). The PF32 sequences from the strain 297 cp32s were submitted to GenBank under accession nos AF410886 (cp32-1), AF410887 (cp32-2), AF410888 (cp32-3), AF410889 (cp32-4), AF410890 (cp32-5), AF410891 (cp32-6), AF410892 (cp32-7), AF410893 (cp18-1) and AF410894 (cp18-2).

Assessment of the compatibility of pCE310 with other B31-MI and strain 297 plasmids

The plasmid contents of cloned transformants were evaluated by PCR (25 ng per reaction, 40 cycles of 92°C for 15 s, 55°C for 15 s, 72°C for 90 s) using primer pairs specific for the *B. burgdorferi* B31-MI plasmids and the nine cp32s of *B. burgdorferi* 297 (see *Supplementary material*). Cultures were then split into two equal aliquots of 5 ml of BSK with and without kanamycin. Cultures were inoculated at $\approx 5 \times 10^5$ cells ml^{-1} and passaged when they reached 8×10^7 cells ml^{-1} (approximately seven generations). After each passage, DNA was extracted as above, and the presence or absence of cp32-3 was determined using primers BBS30-5′ and BBS30-3′ (B31) or PC-*ospF*-5′ and PC-*ospF*-3′ (297). Once the loss of cp32-3 had occurred, the entire plasmid content of the passaged isolate was determined by PCR as above. A 1 kb *flaB* fragment was amplified in parallel to ensure that comparable amounts of genomic DNA template were used for each set of reactions. Products were resolved on 1% agarose gels and stained with EtBr. The analysis of the presence of cp32-3 in the B31-F transformants was performed by PCR using the BBS30 primers and a 1:100 dilution of plasmid DNA extracted by alkaline lysis of 1.5 ml of culture.

The stability of pCE310 was determined by passaging the B31-UM transformant in the absence of antibiotic as above. After eight passages (approximately 50 generations), six plates of 100 cells each were plated on solid medium, three plates containing 400 $\mu g\,ml^{-1}$ kanamycin and three plates without. Total colonies were counted for each series, and the colony-forming units (cfus) in the presence of kanamycin were divided by the number of cfus in the absence of kanamycin to determine the percentage of cells that maintained pCE310. Additionally, 20 colonies from each series were picked and screened by PCR with Kan^R1207-F and T7 to verify the presence of the shuttle vector.

Expression and analysis of fluorescent reporters

Kanamycin-resistant transformants were picked from a plate using a sterile Pasteur pipette and placed in STE (100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA) on a microscope slide. GFP fluorescence was visualized with an Olympus Bx60 system microscope equipped with a mercury light source. Both darkfield and epifluorescence microscopy were performed at a magnification of 400×. Fluorescence was observed using a 470–490 nm excitation filter cube (Olympus). Digital images were captured using a Spot digital camera and the SPOT basic software (Diagnostic Instruments). Cells expressing *yfp* and *cfp* were observed under oil at 1000× using a Zeiss LSM 510 confocal microscope equipped with an argon laser with emissions at 458 and 514 nm respectively. PCR analysis of the transformed populations was performed using the T7/GFP601-R and M13-R/T7 primer pairs and pCE320, pCE320(*gfp*) and pCE320(*gfp*)-P_{flaB} as controls.

Flow cytometry

Aliquots containing $\approx 7 \times 10^7$ of the B31-F or 297-c155 transformants were incubated with SYTO59 (Molecular Probes) for 15 min according to the manufacturer's instructions. Cells were pelleted at 3000 g, resuspended in STE and then fixed with 1% paraformaldehyde in FA buffer (Difco). Samples were analysed on a Becton Dickson FACSCalibur flow cytometer with a 15 mW 488 nm air-cooled argon laser and an $\approx$ 635 nm red diode laser. For each sample, data were collected for 25 000 events. The spirochaete population was gated using SYTO59 fluorescence and then analysed for GFP fluorescence at 488 nm. In some samples, non-viable cells were stained with propidium iodide as instructed in the LIVE/DEAD *Bac*Light bacterial viability kit (Molecular Probes). Flow cytometry data were analysed using CELLQUEST version 3.3 (Becton Dickinson).

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Supplementary material

The following material is available from
http://www.blackwell-science.com/products/journals/suppmat/mole/mole2758/mmi2758sm.htm

Table S1. Primers used in this study.

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