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Characterization of cp18, a Naturally Truncated Member of the cp32 Family of *Borrelia burgdorferi* Plasmids

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We have mapped the genes encoding the antigenic lipoproteins OspE and OspF to an approximately 18-kb circular plasmid in *Borrelia burgdorferi* N40. Sequencing and restriction mapping have revealed that this plasmid, cp18, is homologous to an 18-kb region of the cp32 circular plasmids found in the Lyme disease spirochetes. Our data show that cp18 may have arisen from an ancestral cp32 plasmid by deletion of a 14-kb region of DNA, indicating that a significant portion of the cp32 plasmid is not essential in *cis* for plasmid maintenance. These findings suggest that a relatively small recombinant plasmid capable of being stably maintained in *B. burgdorferi* could be constructed from a cp32 plasmid.

The spirochete *Borrelia burgdorferi*, the causative agent of Lyme disease, is maintained in nature through an infectious cycle between mammals and certain *Ixodes* ticks (10, 40). *B. burgdorferi* isolates cultured from host animals generally carry large numbers of both circular and linear plasmids (4, 5, 11, 12, 15, 18, 21, 26, 27, 30, 32, 43, 47), and the functions of these plasmids and their gene products are major foci of studies of the biology of *B. burgdorferi* and the pathology of Lyme disease. A number of antigenic surface proteins are encoded on the extrachromosomal elements (2, 6, 11, 12, 19, 21, 22, 26, 30, 43, 45), and expression of at least some of these antigens appears to be specific to particular stages of the mammal-tick transmission-infection cycle (2, 8, 12, 14, 24, 35, 42, 44, 45). Long-term cultivation of *B. burgdorferi* in laboratory media is frequently accompanied by a loss of both extrachromosomal DNA and ability of the bacteria to infect mammals (4, 34, 36, 48), suggesting that there may be essential virulence factors encoded by these plasmids. Plasmid loss has also been associated with altered synthesis of bacterial proteins (30). All isolates of *B. burgdorferi* and nearly all isolates of related borreliae have been found to contain at least one member of the cp32 circular plasmid family (11, 37, 43), suggesting that these plasmids may play important roles in the life cycle of *B. burgdorferi.*

Within the first few weeks of infection, mammals produce antibodies that recognize a small number of *B. burgdorferi* antigens (1, 13, 16, 42, 46). Lam and colleagues (19) cloned a gene from *B. burgdorferi* N40 that encodes a membrane-bound lipoprotein, OspE, which is recognized by sera from infected humans and laboratory animals (19, 25, 42). The *ospE* gene is followed by the *ospF* gene, which encodes another lipoprotein (19), and these two genes may form an operon. The initial characterization of the *ospEF* locus reported it to be located on a 45-kb linear plasmid (19). *B. burgdorferi* B31 contains seven different loci, designated *erp*, that are related to the *B. burgdorferi* N40 *ospEF* locus (11, 43). Each *erp* locus is located on a separate, related 32-kb circular plasmid, called cp32-1 through cp32-7, all of which can apparently be maintained within a single *B. burgdorferi* bacterium (11, 43). *B. burgdorferi* N40 also contains several *erp-ospEF*-related genes, including an *ospE* homolog called *p21*, although the locations of these genes have not yet been characterized (2, 44). We have determined that the *ospEF* locus of *B. burgdorferi* N40 is located on an 18-kb circular plasmid that probably evolved from a cp32 plasmid through deletion of a 14-kb segment of DNA. This observation indicates that significant portions of the cp32 plasmids are not required in *cis* for plasmid maintenance.

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MATERIALS AND METHODS

Bacteria. *B. burgdorferi* N40, an infectious isolate collected from a tick in Westchester County, New York (9), was cloned by limiting dilution (7) and has since been grown in culture medium for fewer than five passages (obtained from Stephen Barthold, Yale University, New Haven, Conn.). *B. burgdorferi* B31 (ATCC 35210) was originally isolated from a tick collected on Shelter Island, New York (10). A clone of a high-passage, noninfectious culture of *B. burgdorferi* B31 (clone e1) (11) was used in this study. All *B. burgdorferi* were cultured at 34°C in BSK-H medium (Sigma, St. Louis, Mo.) supplemented with 6% rabbit serum (Sigma).

Gel electrophoresis and Southern blot analysis. *B. burgdorferi* N40 DNA was separated by one- or two-dimensional agarose gel electrophoresis and transferred to nylon membranes (ICN, Irvine, Calif.) as previously described (23, 43). DNA probes derived from *ospE*, *ospF*, and the gene located immediately 3′ of *p21* in *B. burgdorferi* N40 were produced from *Escherichia coli* plasmid clones of these genes (see below) by PCR amplification using oligonucleotides listed in Table 1. PCR conditions consisted of 20 cycles of 94°C for 1 min, 50°C for 30 s, and 72°C for 1 min. PCR products were diluted 1:100 in distilled water, and 1 μl was subjected to a second round of PCR under the same conditions. Amplifications were assayed for single products by agarose gel electrophoresis and staining with ethidium bromide. The final PCR products were purified by dilution in 2 ml of water and concentration through Centricon-100 microconcentrators (Amicon, Beverly, Mass.). Probes were radiolabeled with [α-^{32}P]dATP (du Pont, Boston, Mass.) by random priming (Life Technologies, Gaithersburg, Md.). The probes were individually hybridized with membranes at 55°C in 6× SSC (1× SSC contains 0.15 M NaCl, 0.015 M sodium citrate, and 0.1% sodium dodecyl sulfate), 0.5 g of nonfat dry milk per liter, 0.1% sodium dodecyl sulfate, and 1 mM sodium pyrophosphate and washed at 55°C in 0.2× SSC.

Cloning and sequencing of *B. burgdorferi* plasmid fragments. *B. burgdorferi* and *E. coli* plasmids were purified from 100-ml cultures (grown in BSK-H and terrific broth [31], respectively), using Qiagen (Chatsworth, Calif.) Midi plasmid purification systems as recommended by the manufacturer. Oligonucleotides used in PCR amplifications are listed in Table 1. Prior to sequencing, salts and oligonucleotides were removed from DNA fragments by dilution in water and concentration with Centricon-100 microconcentrators as described above. DNA sequencing was primarily performed with a model 370A automated DNA sequencer (Applied Biosystems, Foster City, Calif.). Some additional sequencing of *B. burgdorferi* DNA fragments cloned into *E. coli* plasmids was performed

TABLE 1. Oligonucleotides used in this work

Use for oligonucleotides	Our designation	Sequence (5′ to 3′)
DNA probe production		
ospE	E-1	ATGTAACAGCTGAATG
	E-2	CATATAAGCTTTCTCC
ospF	F-1	AGAAGTTGGAAGTATAGGAGAAAG
	F-2	AACAATAGTTTTTGGCATTTTCAC
3′ of *p21*	E-155	GATTTAAAACAAAATCCAGAAGGG
	E-182	GATCACCACTTTGTTCTGCTGATTTTG
B. burgdorferi B31 cp32-1 cloning		
*Pvu*II site to *orf3*	BBK-1	CACTAAAATCTATGGAAACAGCTG
	ORFD-2	AGTTCATCTAATAAAAATCCCGTG
*Pme*I site to *orf3*	BBK-21	CAAACATTTTTATAATTAAAATTGGCA(T/C)TGTAAAGG
	ORFD-2	AGTTCATCTAATAAAAATCCCGTG
B. burgdorferi N40 cp18 cloning		
ospF to *orf13*	F-1	AGAAGTTGGAAGTATAGGAGAAAG
	CP-0	GAAAAAGATAACATGCAAGATACG
ospE to *orf2*	E-2	CATATAAGCTTTCTCC
	ORFB-1	CAACAAAGTTTTATTTAGTATG
*Pvu*II site to *orfC*	BBK-1	CACTAAAATCTATGGAAACAGCTG
	E-312	CTGAATTATCAATTAATACATTCC
5′ of *orfC* to 3′ end of *ospF*	E-312	CTGAATTATCAATTAATACATTCC
	F-3	ATTAAAAAGATTGATGAAGAG
B. burgdorferi N40 cp32 cloning		
3′ of *p21* to *orf13*	E-155	GATTTAAAACAAAATCCAGAAGGG
	CP-0	GAAAAAGATAACATGCAAGATACG

using [α-^{35}S]dATP (Amersham, Cleveland, Ohio) and Sequenase 2.0 (U.S. Biochemical, Cleveland, Ohio).

The previously described (11) clone containing the 4-kb *Bam*HI-*Sal*I fragment of cp32-1 (Fig. 3A, fragment 1) from *B. burgdorferi* B31 clone e1 was used to characterize sequences of this plasmid located 3′ of the *erpAB* locus. The region of cp32-1 between the *Pvu*II site at position 16 kb and *orf3* (Fig. 3A, fragment 2) was PCR amplified from purified *B. burgdorferi* B31 clone e1 plasmids by using the oligonucleotides listed in Table 1 and an Extend PCR amplification system (Boehringer Mannheim, Indianapolis, Ind.). PCR conditions consisted of 10 cycles of 94°C for 10 s, 50°C for 30 s, and 68°C for 8 min, followed by 20 cycles, with successive extension steps increased by 20 s each. The region of cp32-1 between the *Pme*I site at position 12 kb and *orf3* (Fig. 3A, fragment 3) was PCR amplified from purified *B. burgdorferi* B31 clone e1 plasmids, using an Extend PCR amplification system as described above, but with extension times beginning at 15 min. Portions of the amplified DNA fragments were sequenced, without cloning into *E. coli* plasmids.

The regions of cp18 between *ospF* and the *Eco*RI site at position 18 kb (Fig. 3A, fragment 4) and between *ospE* and the *orf2* gene (Fig. 3A, fragment 5) were PCR amplified from purified *B. burgdorferi* N40 plasmids by 25 cycles of 94°C for 1 min, 50°C for 1 min, and 65°C for 5 min. The resultant PCR fragments were cloned into pCR2.1 (Invitrogen, San Diego, Calif.) and sequenced. The portion of cp18 extending from the *Pvu*II site at position 3 kb and *orfC* (Fig. 3A, fragment 6) and that of cp18 extending 5′ from *orfC* to the 3′ end of *ospF* (Fig. 3A, fragment 7) were PCR amplified from purified *B. burgdorferi* N40 plasmids, using the Extend PCR amplification system as described above, with 8 and 15 min starting extension times, respectively. Portions of these last two PCR fragments were sequenced, without cloning into an *E. coli* plasmid.

We have found two highly conserved open reading frames, *orf12* and *orf13*, located approximately 3 kb 3′ of all *erp* loci and the *ospEF* locus (Fig. 3B) (this work and references 11 and 41). The DNA between the *B. burgdorferi* N40 *p21* gene and this conserved region was amplified by PCR from purified *B. burgdorferi* N40 plasmids by 25 cycles of 94°C for 1 min, 50°C for 1 min, and 65°C for 5 min. The resultant DNA fragment was cloned into pCR2.1 and sequenced.

Restriction endonuclease mapping of cp18. Restriction endonuclease recognition sites on cp18 located 3′ of *orf2* and 5′ of *ospE* (Fig. 3A, fragment 5) were determined by examining the DNA sequence within this region. Restriction endonuclease cleavage sites within *ospE* and *ospF* were located by analyzing the published sequence of this locus (GenBank accession no. L13924 and L13925) (19).

The sites on cp18 located 5′ of *orf2* and 3′ of *ospF* (Fig. 3A, fragment 7) were determined by digesting the PCR amplification product for this region (see above). The resulting DNA fragments were separated by constant-field agarose gel electrophoresis and visualized by ethidium bromide staining. All restriction endonucleases were obtained from New England Biolabs (Beverly, Mass.).

Additionally, a total DNA preparation of *B. burgdorferi* N40 was digested in agarose blocks with restriction endonucleases, separated by contour-clamped homogeneous electric field pulsed-field electrophoresis, and transferred to Hybond-N$^+$ membranes (Amersham, Arlington Heights, Ill.) as previously described (11). The membrane was hybridized with a radiolabeled probe specific for *ospF* under previously described conditions (11).

Nucleotide sequence accession numbers. Additional sequences of cp18 located 5′ of the *B. burgdorferi* N40 *ospEF* locus have been added to GenBank accession no. U42599. The DNA sequence of cp18 encompassing the sequence at position 18/0 kb has been given accession no. U83899. Additional sequences of cp32-1 at position 30 kb have been added to accession no. U60963, and the sequence of cp32-1 at position 12 kb has been given accession no. U83898.

RESULTS

Localization of the *B. burgdorferi* N40 *ospEF* locus to an 18-kb circular plasmid. Lam et al. (19) reported that the *B. burgdorferi* N40 *ospEF* operon is located on a 45-kb linear plasmid. However, in a previous report, we demonstrated that a radiolabeled probe derived from the *B. burgdorferi* N40 *ospE* gene hybridized with circular plasmid DNA purified from this isolate (43). The technique that we used, two-dimensional agarose gel electrophoresis, results in strikingly different Southern blot hybridization patterns for circular and linear plasmids (43). A probe from a circular plasmid will hybridize with the supercoiled circular form that migrates on the circular DNA axis and the randomly sheared, linearized form that migrates on the linear DNA axis. By contrast, a probe from a linear plasmid will hybridize only with DNA that migrates on the linear DNA axis (43). To enhance the linearized form, we vortexed our plasmid preparations to introduce random nicks and breaks into the circular DNAs. We found that the *ospE* probe hybridized with circular DNA and two linear DNA species with approximate sizes of 32 and 18 kb (Fig. 1A) (43), indicating that there are *ospE*-related sequences on at least two plasmids of different sizes. By comparison, in *B. burgdorferi* B31, *erp* genes are located on 32-kb circular plasmids (11, 43), and probes derived from these genes hybridize with circular DNA and the 32-kb linearized form of the plasmid in two-dimensional gels (43). Faced with these differences, we decided

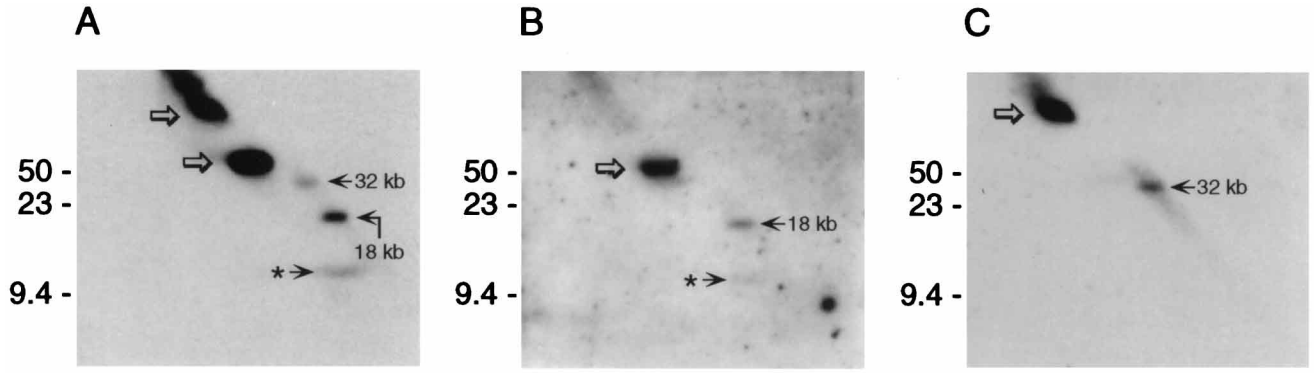

FIG. 1. Southern blot analysis of *B. burgdorferi* N40 plasmids separated by two-dimensional agarose gel electrophoresis. Circular DNAs that hybridized with these probes are indicated with open arrows, and linear DNAs are indicated with small arrows and their approximate sizes. (A) *ospE* probe; (B) *ospF* probe; (C) probe derived from the open reading frame located 3′ of the *p21* gene (44). The hybridization signals from the linearized forms of the circular plasmids are much weaker than those from the supercoiled circular forms due to greater abundance of supercoiled than of nicked plasmids in this preparation. Both the *ospE* and *ospF* probes hybridized with a very small circular DNA species (indicated by asterisks in panels A and B) that may indicate similar sequences located on a smaller, as yet uncharacterized circular plasmid. Sizes are indicated in kilobases.

to characterize both the location of the *B. burgdorferi* N40 *ospEF* operon and the 18-kb plasmid found in this isolate.

The two-dimensional Southern blot described above was stripped and rehybridized with a probe derived from the *B. burgdorferi* N40 *ospF* gene, which hybridized with circular DNA and the 18-kb linear DNA species (Fig. 1B). The *ospF* probe did not hybridize with the linear 32-kb fragment that was recognized by the *ospE* probe. The *ospF* hybridization pattern suggested that the *ospEF* operon of *B. burgdorferi* N40 is located on a circular plasmid that is approximately 18 kb in size. To confirm the size and circular nature of the *ospEF*-containing plasmid, we separated *B. burgdorferi* N40 plasmid DNAs by one-dimension pulsed-field agarose gel electrophoresis. The electrophoresis conditions that we used do not clearly resolve circular DNAs, which instead form broad bands (43). Southern blot analysis of uncut *B. burgdorferi* N40 plasmid DNA indicated that the *ospF* probe hybridized with DNA consistent with a circular plasmid (Fig. 2). The *ospF* sequence contains a *Bam*HI cleavage site near the middle the gene (19), and as the *ospF*-derived probe spans this *Bam*HI site, the probe should hybridize with DNA fragments on either side of the cleavage site in DNA cut with this enzyme. The *ospF* probe recognized only an 18-kb fragment in the plasmid DNA cut with *Bam*HI (Fig. 2), indicating that *ospEF* is located on an 18-kb circular plasmid that contains a single *Bam*HI site. If *ospEF* were located on a larger circular plasmid, we would have observed hybridization to either a larger DNA fragment (if the plasmid had a single *Bam*HI site) or two DNA fragments (if the plasmid contained more than one *Bam*HI site). Furthermore, *ospEF* cannot be located on a linear plasmid, as the *ospF* probe would then have hybridized with two DNA fragments. The 18-kb linear DNA that hybridized with the *ospF* probe in Fig. 1 must therefore be randomly linearized forms of the circular plasmid. We have designated the 18-kb circular plasmid that contains the *ospEF* locus as cp18.

We previously reported that a *B. burgdorferi* N40 *ospE*-derived probe hybridized with DNA molecules containing the *erpA* or *erpC* gene of *B. burgdorferi* B31; *erpA* and *erpC* share 85 and 88%, respectively, identical nucleotides with *ospE* (43). The *p21* gene of *B. burgdorferi* N40 contains large stretches of sequences that are identical to that of *ospE* (44), suggesting that the *ospE* probe may also cross-hybridize with DNA containing the *p21* gene. The *p21* gene is followed by an open reading frame that is distinct from *ospF* (44). A probe derived from this second open reading frame hybridized with circular DNA and the 32-kb linear species (Fig. 1C). These data indicate that the *p21* locus is located on a 32-kb circular plasmid, as are seven of the *erp* loci of *B. burgdorferi* B31 (11, 43). We have not determined whether *B. burgdorferi* N40 contains multiple 32-kb circular plasmids, although most other isolates of *B. burgdorferi* and related spirochetes contain multiple members of this plasmid family (11).

Hybridization of the two-dimensional Southern blot with the *ospE* and *ospF* probes indicated that *B. burgdorferi* N40 also contains a small circular DNA species containing sequences related to these two genes (Fig. 1A and B). Further analysis of *B. burgdorferi* N40 indicated that the small circular species did not contain other sequences found on cp32 and cp18 (data not shown), and we have not characterized this DNA any further.

cp18 contains extensive homology to the cp32 plasmid family. In a previous study (43), we found that at least four of the *erp* loci of *B. burgdorferi* B31 and the *ospEF* locus of *B. burgdorferi* N40 are preceded by nearly 2 kb of homologous DNA. Additional characterization of the known *B. burgdorferi* B31 cp32 plasmids has indicated that all seven of these plasmids

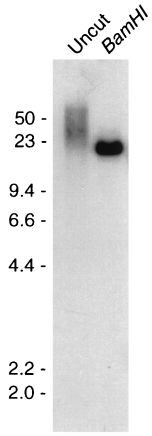

FIG. 2. Southern blot analysis of *B. burgdorferi* N40 plasmids separated by pulsed-field agarose gel electrophoresis. DNA was either uncut or cut with *Bam*HI, and the membrane was hybridized with the *ospF* probe. Sizes are indicated in kilobases.

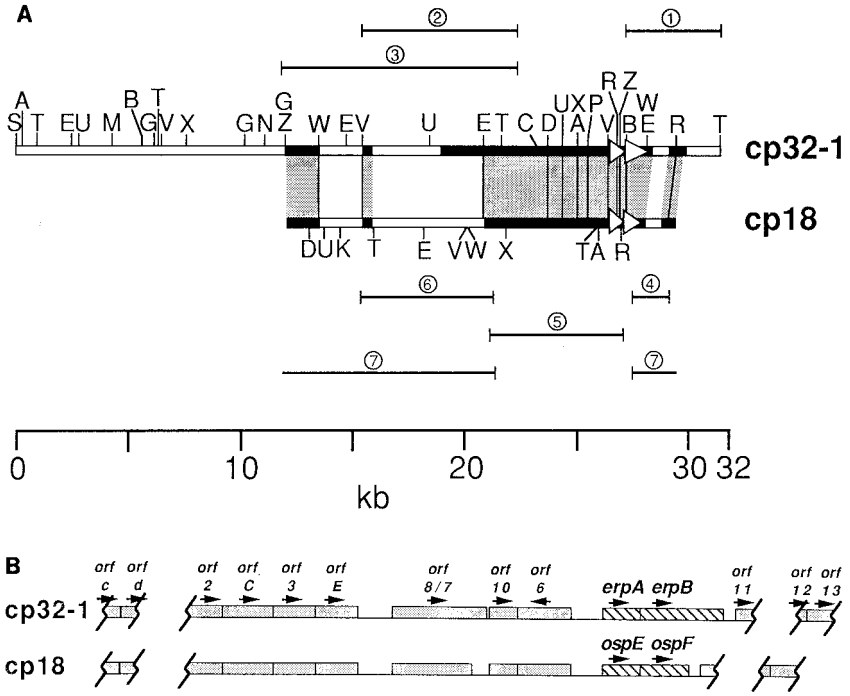

FIG. 3. (A) Alignment of linearized restriction maps of cp32-1 (11) and cp18. Abbreviations for restriction endonuclease cleavage sites: A, *Apa*LI; B, *Bam*HI; C, *Sac*II; D, *Sac*I; E, *Eco*109I; G, *Bgl*I, K, *Kpn*I; M, *Mlu*I; P, *Pml*I; R, *Eco*RI; S, *Sal*I; T, *Pst*I; U, *Stu*I; V, *Pvu*II; W, *Alw*NI; X, *Bst*XI; Z, *Pme*I. Sites found on both plasmids are indicated by long vertical lines. DNA fragments used in the characterization of these two plasmids are indicated by numbered horizontal lines located above and below the restriction maps of cp32-1 and cp18, respectively. Relevant portions of each plasmid that have been sequenced are indicated as filled bars. Triangles identify the locations of the *erpAB* (cp32-1) and *ospEF* (cp18) loci. Known homologous regions found on both plasmids are indicated by shaded areas between the two plasmids. The complete pattern of *Eco*RI recognition sites on cp32-1 was not determined. (B) Alignment of the open reading frames found on cp32-1 and cp18. Previously described open reading frames are named as before (11, 28, 50). Unsequenced gaps are indicated by jagged lines.

contain extensive regions of homology (11, 41). We next sought to further characterize the *ospEF*-containing cp18 plasmid to establish its relationship with the cp32 family.

Each of the cp32 plasmids of *B. burgdorferi* B31 contains sequences that are homologous to sequences first identified by Zückert et al. (11, 41, 49, 50). The *orf2* open reading frames of these regions are highly conserved in all seven of the characterized cp32 plasmids (41, 50). Oligonucleotides complementary to this conserved region and to *ospE* were used to PCR amplify *B. burgdorferi* N40 plasmid DNA. Sequencing of the resulting fragment (Fig. 3A, fragment 5) revealed that cp18 contains homologs of all the open reading frames found between *orf2* and the *erpAB* locus of cp32-1 (Fig. 3B).

Porcella et al. (28) identified several homologous DNA fragments from *B. burgdorferi* isolate 297 that hybridized with circular plasmids of approximately 32 and 18 kb. An oligonucleotide based on a highly conserved sequence within all of these homologous DNA fragments was used in conjunction with an oligonucleotide specific for cp32-1 or cp18 to PCR amplify DNA fragments from *B. burgdorferi* B31 or N40 plasmid DNA, respectively (Fig. 3A, fragments 2 and 6). The sequences of cp32-1 and cp18 located approximately 10 kb 5′ of the *erpAB* and *ospEF* loci, respectively, were found to be nearly identical to each other and to those found in *B. burgdorferi* 297 (*orfc* and *orfd* in Fig. 3B) (28).

We have also found that each of the cp32 plasmids contains homologs of two open reading frames, *orf12* and *orf13*, located 3′ of the *erp* loci (11). A portion of these genes has been proposed for use as a PCR amplification target site for identifying *B. burgdorferi* (3). *B. burgdorferi* N40 plasmid DNA was PCR amplified using oligonucleotides based on the sequences of *ospF* (19) and conserved bases within the *orf13* genes of the seven *B. burgdorferi* B31 cp32 plasmids (11). The sequence of the DNA fragment (Fig. 3A, fragment 4) indicated that cp18 also contains homologs of *orf12* and *orf13* (Fig. 3B). The sequence directly 3′ of the *ospEF* locus also contains an *orf11* homologous to that found immediately 3′ of the *erpAB* locus on cp32-1 (Fig. 3B) (41).

The sequenced regions of these plasmids indicate some differences between cp18 and cp32-1. While *ospE* and *erpA* are similar in sequence, *ospF* and *erpB* share less than 35% identical nucleotides (41, 43). The *orfC* and *orf3* genes of cp18 and cp32-1 share only 68 and 66% identical nucleotides, respectively, although all other open reading frames encoded in the portions of these plasmids that have been sequenced have greater than 90% identical nucleotides. The sequences of the *orfC* and *orf3* genes of the *B. burgdorferi* B31 cp32 plasmids are also variable (41, 50). In addition, the *orf8*/7 open reading frame of cp18 is shorter than that of cp32-1 due to a premature stop codon in the gene on cp18 (Fig. 3B). The presence of this mutation was confirmed by sequencing a second, independent PCR amplification product from this region.

Oligonucleotides designed to amplify in a 5′ direction from the *orfC* gene of cp18 and in a 3′ direction from *ospF* were used to PCR amplify an approximately 11.3-kb DNA fragment (Fig. 3A, fragment 7). This successful amplification confirmed that *ospEF* is located on a circular plasmid, since we have linked *ospEF* to *orfC* in both a 5′ and a 3′ direction, which would not be possible if *ospEF* were located on a linear plasmid. The amplified DNA fragment was digested with restriction endonucleases that were known to cleave the cp32 plasmids of *B. burgdorferi* B31 a few times (11). We also performed Southern blot analysis of digested total *B. burgdorferi* N40 DNA using the *ospF*-specific probe, obtaining banding patterns that could

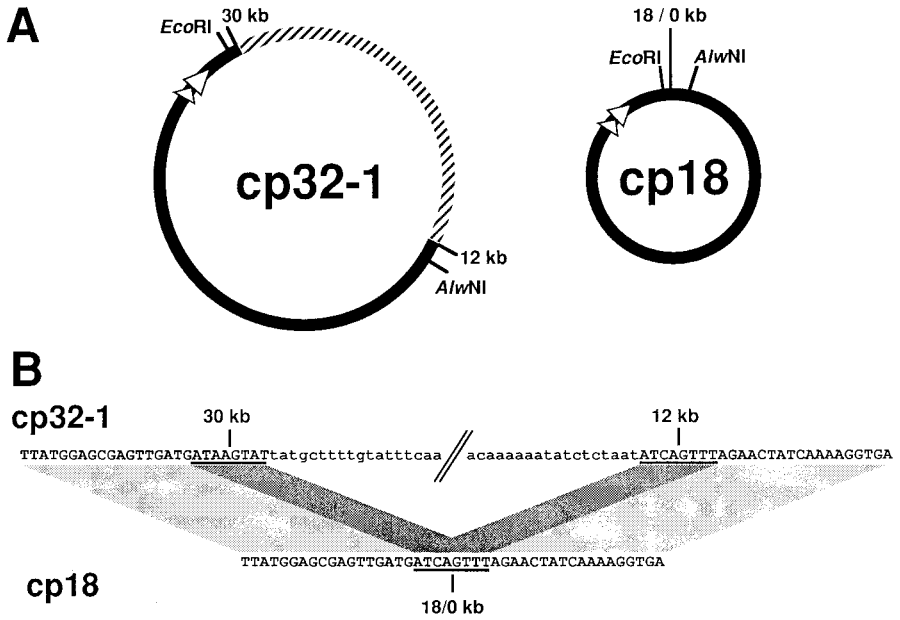

FIG. 4. Comparisons of the sequences found on both cp32-1 and cp18. (A) Circular maps of cp32-1 and cp18 indicating the relative positions of the *Eco*RI and *Alw*NI recognition sites and the relative locations of positions 12 and 30 kb on cp32-1 and position 18/0 kb on cp18. The locations of the *erpAB* (cp32-1) and *ospEF* (cp18) loci are indicated by triangles. The portion of cp32-1 not having a homolog on cp18 is indicated by the striped area. (B) Alignments of the sequences of cp32-1 at positions 30 and 12 kb with that of cp18 at position 18/0 kb. Capital letters indicate those bases that are homologous between both plasmids. Lowercase letters indicate nucleotides lacking significant similarity between the two plasmids. Underlined bases are those that are homologous at positions 30 and 12 kb on cp32-1 and position 18/0 kb on cp18.

be possible only if *ospEF* were located on an 18-kb circular plasmid. These methods, in conjunction with sequencing the region of cp18 between *orf2* and *ospE*, and the previously determined sequences of the *ospEF* locus (19), allowed us to construct a detailed restriction site map for cp18. Comparison of an arbitrarily linearized version of this map with that of cp32-1 (11) indicates that the two plasmids have numerous restriction sites in common across a contiguous 18-kb region of cp32-1 (Fig. 3A). These conserved sequences further indicate that much of cp18 is homologous to cp32-1 and other members of the cp32 family.

cp18 is a truncated member of the cp32 plasmid family. To more fully understand the relationship between cp18 and cp32-1, we sequenced the regions near positions 12 and 30 kb on cp32-1 and the homologous DNA of cp18 (Fig. 3A and 4A). The sequences of cp18 and cp32-1 clockwise of the *Eco*RI sites (Fig. 4A; rightward in Fig. 3A) are greater than 95% identical for 599 bp; the sequences abruptly diverged beyond this point (Fig. 4B). The sequences of cp18 and cp32-1 counterclockwise from the *Alw*NI sites (Fig. 4A; leftward in Fig. 3A) are also greater than 95% identical for 1,457 bp before abruptly diverging (Fig. 4B). Alignment of these sequences indicated that the sequence of cp18 appears to be a fusion of cp32-1 sequences from near position 30 kb to near position 12 kb, with a deletion of the intervening 14 kb (Fig. 4B). We have called the point on cp18 at which this change occurs position 18/0 kb (Fig. 4). There are no long repeated sequences in this region of either cp18 or cp32-1, although both positions of cp32-1 contain an 8-bp sequence that is homologous to position 18/0 kb on cp18. The simplest explanation for this observation is that cp18 arose from a 32-kb precursor plasmid by a deletion of approximately 14 kb (Fig. 4A). It is also theoretically possible that a 14-kb fragment of DNA recombined into a cp18 to give rise to a cp32 that then evolved into the numerous divergent cp32 plasmids. However, DNA hybridization studies using part of this 14-kb fragment as a probe indicated that these sequences are found only on plasmids of the cp32 family and not elsewhere in the genomes of *B. burgdorferi* N40 or related bacteria (11). Further characterization of these plasmids and their functions in *B. burgdorferi* may help resolve the history of their evolution.

DISCUSSION

We have shown that the *ospEF* locus of *B. burgdorferi* N40 is located on cp18, an 18-kb circular plasmid. In the initial description of *ospEF*, it was reported that this locus is located on a 45-kb linear plasmid (19). These researchers, however, used Southern blot analysis of DNA separated by one-dimensional agarose gel electrophoresis and found that an *ospF*-derived probe hybridized with two DNA species (19), which may have been two forms of cp18. Supercoiled circular plasmids are sensitive to nicking and shearing, and plasmid preparations often contain relaxed circular and linearized forms of these plasmids. While the bacteria that both Lam et al. (19) and we used were derived from the same clone of *B. burgdorferi* N40 (7), it is not impossible that the *ospEF* plasmid can exist in circular or linear forms in different bacterial cells. For example, a plasmid in the related bacterium *B. hermsii* has been identified in both circular and linear conformations (17). Nonetheless, our data suggest that one-dimensional gel electrophoresis with uncleaved DNA is not the best method for assigning size and linearity of *B. burgdorferi* plasmids.

Sequencing and restriction endonuclease recognition site mapping indicated that cp18 is homologous to an 18-kb portion of cp32-1, the best-characterized member of the cp32 family of *B. burgdorferi* plasmids. The conserved size of the multiple 32-kb circular plasmids suggests that they are under some constraint to maintain that particular size. One possibility is that they are prophages, with 32 kb being the packaging size of

the bacteriophage particle. An exception to this conservation of size, cp18 may be a defective prophage that, having lost a large region of the genome, is now maintained solely as a plasmid. Such a plasmid would no longer need to carry genes that were necessary for production of the phage particle and could further mutate some of those genes that had been retained. The truncated *orf8*/*7* gene of cp18 may represent one such mutation. Other deletions may also have resulted in the small circular plasmid of *B. burgdorferi* N40 (Fig. 1) or the cp8.3 plasmid found in *B. burgdorferi* sensu lato isolate Ip21 (15). These smaller plasmids may still, however, be capable of forming phage particles by using gene products of the larger prophages in a manner similar to that of the P2 and P4 bacteriophages of *E. coli* (20, 38). Another possibility is that the cp32s are not prophages at all but are under some unknown pressure to maintain plasmid size and that this restriction has somehow been removed for cp18 and the other smaller plasmids. Regardless of the actual origin of these plasmids, it is clear from this work that the size constraint on the cp32 plasmids can be overcome.

Our data indicate that cp18 may have evolved by the deletion of a 14-kb segment from a cp32 ancestor. *B. burgdorferi* N40 has been maintained for several passages in culture medium, indicating that cp18 can be stably maintained in these bacteria in the absence of the selective pressures of the natural state, i.e., the necessities of survival in both arthropod and vertebrate hosts and successful transmission between them. This observation indicates that cp18 contains all of the *cis* elements necessary for stable plasmid maintenance. *B. burgdorferi* N40 contains at least one cp32, so it is possible that essential replication and partition factors not encoded on cp18 are provided in *trans* by the 32-kb plasmid that carries *p21*.

Some other isolates of *B. burgdorferi* contain circular plasmids having sizes of approximately 18 kb (28, 34, 36, 37, 39), which may be homologs of cp18. Simpson et al. (37) found that several *B. burgdorferi* isolates contained circular plasmids of this size that hybridized with DNA fragments that include homologs of *erp* and *orf8*/*7* genes (41). Many other isolates of *B. burgdorferi*, including infectious cultures of *B. burgdorferi* B31, appear to lack 18-kb circular plasmids (30, 37, 39, 41, 43, 48), indicating that this plasmid probably does not carry unique genes that are required for bacterial survival or infectivity.

Previous work has demonstrated that DNA can be introduced into *B. burgdorferi* (29, 33), although the stable transformation of these bacteria with autonomously replicating DNA has not yet been accomplished due to the lack of a suitable plasmid vector. A number of features recommend the cp32/cp18 family of plasmids as possible starting blocks for the construction of practical cloning vectors for use in *B. burgdorferi*. To date, these plasmids have been found in nearly all isolates of Lyme disease spirochetes and their close relatives, indicating that they could be used in a broad range of these bacteria. Some of these plasmids appear to be extremely stable in *B. burgdorferi* in that they can still be reisolated after years of continual culturing in the laboratory. Many of the plasmids are compatible with each other, and so the presence of naturally occurring plasmids within a target *B. burgdorferi* isolate might not adversely effect the ability of the isolate to be transformed. The possibility that the cp32 plasmids are prophage genomes raises the possibility of their use as phagemid vectors or transducing phages. Finally, we have now demonstrated that a large portion of a cp32 plasmid is not required in *cis*, indicating that construction of a small, manageable vector based on a cp32 may be a reachable goal.

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