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Homology throughout the Multiple 32-Kilobase Circular Plasmids Present in Lyme Disease Spirochetes

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We have characterized seven different 32-kb circular plasmids carried by *Borrelia burgdorferi* isolate B31. Restriction endonuclease recognition site mapping and partial sequencing of these plasmids indicated that all seven are probably closely related to each other throughout their lengths and have substantial relationships to cp8.3, an 8.3-kb circular plasmid of *B. burgdorferi* sensu lato isolate Ip21. With the addition of the seven 32-kb plasmids, this bacterial strain is known to carry at least 10 linear and 9 circular plasmids. Variant cultures of *B. burgdorferi* B31 lacking one or more of the 32-kb circular plasmids are viable and, at least in some cases, infectious. We have examined a number of different natural isolates of Lyme disease borreliae and found that all of the *B. burgdorferi* sensu stricto isolates and most of the *B. burgdorferi* sensu lato isolates tested appear to carry multiple 32-kb circular plasmids related to those of *B. burgdorferi* B31. The ubiquity of these plasmids suggests that they may be important in the natural life cycle of these organisms. They may be highly conjugative plasmids or prophage genomes, which could prove to be useful in genetically manipulating *B. burgdorferi*.

Borrelia burgdorferi is the causative agent of Lyme disease, a multisystemic ailment of humans that is spread through the bite of certain species of *Ixodes* ticks (13, 61). As a spirochete, *B. burgdorferi* is a member of a morphologically and phylogenetically distinct order of eubacteria (11, 28, 42–44, 70). Although classically defined as gram-negative organisms (28), recent phylogenetic studies based on rRNA sequences indicated that the spirochetes are as distantly related to the gram-negative *Escherichia coli* as they are to gram-positive bacteria (42, 70).

One of the most striking differences between *B. burgdorferi* and other bacteria is its unusual genome, which includes a small (approximately 1 Mb) linear chromosome (12, 16, 19, 21, 23, 41) and numerous linear and circular plasmids, sometimes approaching 20 different plasmids in one bacterium (7, 8, 10, 22, 27, 30, 35, 57, 65, 71). A curious feature of these different plasmids is that they often appear to share regions of homologous DNA (58, 60, 65, 71, 73, 74). Homologs of DNA sequences mapped to circular plasmids have even been found on linear plasmids (74), although in a related bacterium, *Borrelia hermsii*, at least one plasmid exists in both linear and circular forms (24), blurring the distinction between these two forms of DNA. Several of the plasmids that these bacteria harbor appear to be present in all natural isolates; therefore, the term minichromosome may be a more apt description of their nature. For example, a 49- to 54-kb linear plasmid and a 26-kb circular plasmid, which carry the outer surface protein genes *ospAB* and *ospC*, respectively, are ubiquitous (7, 34, 37, 52, 54, 63, 71). It is not known whether members of either of these two plasmid families have similar overall gene orders in different bacterial isolates, but their nearly invariant sizes support the idea that they may have uniform structures.

We recently reported that high-passage *B. burgdorferi* B31 contains several loci capable of encoding homologs of the antigenic outer surface proteins OspE and OspF (64, 65). These four loci, *erpAB*, *erpCD*, *erpG*, and *erpH*, are encoded on separate 32-kb circular plasmids (cp32-1, cp32-2, cp32-3, and cp32-4, respectively). All four of these plasmids were shown to contain at least 2 kb of homologous DNA sequences, and all could be maintained within a single bacterium (65). We now present evidence that these four plasmids and three newly described B31 plasmids, cp32-5, cp32-6, and cp32-7, have homologies throughout their lengths. We also present additional sequences from these plasmids, which, together with the sequences presented by Zückert and Meyer (74), indicate that the cp32 plasmids contain homologs of almost the entire sequence of a *B. burgdorferi* sensu lato 8.3-kb circular plasmid (22). There have been several other reports of repeated *B. burgdorferi* DNA sequences that in Southern blot analyses hybridized with circular plasmids approximately 32 kb in size, and multiple *ospEF*-like loci have been cloned from other isolates of *B. burgdorferi* (1, 32, 37a, 45, 58, 66, 73, 74).

Genetic analysis of Lyme disease spirochetes has indicated that these bacteria are a heterogeneous group of related bacteria, which are referred to as *B. burgdorferi* sensu lato. Several distinct types have been identified within this group (3, 4, 16, 25, 46, 49, 72), and novel specific names have been given to five of these (*B. burgdorferi* sensu stricto, *B. afzelii*, *B. garinii*, *B. andersonii*, and *B. japonica*) (5, 14, 36, 47). Amouriaux et al. (2) noted a DNA sequence, located on an approximately 30-kb circular plasmid in *B. burgdorferi* B31, that could function as a conserved PCR target site for detecting Lyme disease spirochetes in patients. We have identified an identical sequence on cp32-1 and homologous sequences on the six other 32-kb circular plasmids of isolate B31 that were examined. In addition, we have examined a number of different isolates of *B. burgdorferi* sensu lato by Southern analysis and found that they also contain one or more circular plasmids related to the cp32s of isolate B31, indicating that multiple, related 32-kb circular plasmids are common in the Lyme disease spirochetes.

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TABLE 1. Bacterial strains used in this study

Isolate	Location of origin	Biological source	Passage no. in culture	Reference	Source
B. burgdorferi sensu stricto					
B31	New York	Tick isolate			
B31 T		Original tick isolate	4	13	A. Barbour
B31 MI		Passed through mouse	3 (post-mouse)		R. Lathigra
B31 low-passage		Mouse-tick cycles	4 (post-mouse)	56	T. Schwan
B31 clone 4a		Clone from low-passage B31	2 (postcloning)		This study
B31 high-passage		Cloned by limiting dilution	>>100		Our collection
B31 A3H.3		Clone from high-passage B31	2 (postcloning)	65	Our collection
B31 clones e1 & e2		Clones from high-passage B31	2 (postcloning)		This study
CA-2-87	California	Tick isolate	NA[a]	57	T. Schwan
CA-3-87	California	Tick isolate	12	57	T. Schwan
CA-11.2A	California	Tick isolate (cloned)	NA	50	Our collection
ECM-NY-86	New York	Tick isolate	NA	57	T. Schwan
HB-19	New York	Human isolate (cloned)	3 (postcloning)	16	A. Barbour
N40	New York	Tick isolate	NA	57	T. Schwan
NY-1-86	New York	Human isolate	NA	57	T. Schwan
Sh-2-82	New York	Tick isolate	>300	19	Our collection
WI91-23	Wisconsin	Bird isolate	2	16	R. Johnson
19535	New York	Mouse isolate	NA	49	T. Schwan
19678	New York	Mouse isolate	8	49	T. Schwan
20001	France	Tick isolate	NA	3	J. Anderson
21305	Connecticut	Mouse isolate	NA	49	T. Schwan
21645	Wisconsin	Tick isolate	6	49	J. Anderson
22921	New York	Mouse isolate	8	49	T. Schwan
26518	Connecticut	Chipmunk isolate	NA	49	J. Anderson
26816	Rhode Island	Vole isolate	NA	72	J. Anderson
27982	Connecticut	Tick isolate	6	72	J. Anderson
28359	Maryland	Tick isolate	4	72	J. Anderson
29968	Connecticut	Tick isolate	NA	72	J. Anderson
B. burgdorferi sensu lato					
B. afzelii RIP-3	Russia	Tick isolate (cloned)	2 (postcloning)	16	Our collection
B. garinii 20047	France	Tick isolate	NA	16	R. Marconi
B. garinii G2	Germany	Human isolate	NA	16	Our collection
B. japonica HO14	Japan	Tick isolate	<20	16	R. Johnson
B. andersonii 19857	New York	Rabbit isolate	10	16	J. Anderson
CA127	California	Tick isolate	7	16	R. Lane
CT-39	Illinois	Mouse isolate	NA	16	R. Marconi
CA55	California	Tick isolate	9	16	R. Lane
M63	Holland	Tick isolate	NA	40	L. Nohlmans
VS116	Switzerland	Tick isolate	11	46	R. Marconi
PotiB1	Portugal	Tick isolate	NA	46	D. Postic

[a] NA, information not available.

MATERIALS AND METHODS

Bacteria. The bacterial strains used in this study and their sources are listed in Table 1. *B. burgdorferi* B31 (ATCC 35210) was originally isolated from a tick and cloned by limiting dilution (9, 13). We will refer to the original uncloned tick isolate as B31 T in the present work. Low-passage B31 was maintained by infectious passage between ticks and mice (56) and has since been passaged fewer than five times in culture medium. *B. burgdorferi* B31 clone 4a was derived from a single colony of low-passage B31 that was plated on solid Barbour-Stoener-Kelly (BSK) medium (50), inoculated into a BALB/c mouse, and reisolated from mouse tissue 4 weeks postinoculation (55a, 57). High-passage B31 had been maintained continuously in culture medium for several years. *B. burgdorferi* B31 clones e1 and e2 were derived from individual colonies of high-passage B31 plated on solid BSK medium. B31 clone e1 contains cp32-1, -2, -3, and -4, while B31 clone e2 lacks cp32-2. The history of the B31 cultures used in this study is summarized in Fig. 1. *B. burgdorferi* were grown in BSK-H broth (Sigma, St. Louis, Mo.) supplemented with 6% rabbit serum (Sigma).

PCR analysis of *erp* loci in *B. burgdorferi* B31 cultures. The presence of *erpAB*, *erpCD*, *erpG*, and *erpH* loci within different *B. burgdorferi* cultures was analyzed by PCR amplification of either purified DNA (51) or a suspension of boiled bacteria by using the oligonucleotides listed in Table 2. PCR conditions consisted of 20 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min.

Subcloning from *B. burgdorferi* B31 plasmids. Sequencing of DNA was performed with Sequenase 2.0 (U.S. Biochemicals, Cleveland, Ohio) or a 373A automated DNA sequencer (Applied Biosystems, Foster City, Calif.). For the sake of consistency, we have named open reading frames (ORFs) that have homologs on cp8.3 according to the system of Dunn et al. (22). Thus, the names ORF-1, ORF-2, ORF-3, ORF-4, and ORF-8/7 (see below) have been assigned to the genes that Zückert and Meyer (74) named ORF-A, ORF-B, ORF-D, ORF-G, and ORF-F, respectively. Genes found on the cp32s that do not have homologs on cp8.3 have retained their original names (65, 74).

We have previously described the identification and analysis of *erp* locus-containing plasmid clones derived from a phage library (65). A 1.9-kb fragment of cp32-4, containing the region from approximately 5 to 7 kb of cp32-3 (shown below in Fig. 4), was isolated from this library as part of a chimeric insert attached to a fragment of cp32-3. Plasmids pOMB65, pOMB10, and pOMB14 (74), subclones of two approximately 30-kb circular plasmids and one approximately 50-kb linear plasmid, respectively, of *B. burgdorferi* B31, were obtained from W. Zückert (University of Pennsylvania, Philadelphia).

Additional DNA clones were obtained from *B. burgdorferi* B31 clone e1 grown to late-log phase in 100 ml of BSK-H. *B. burgdorferi* plasmid DNA was purified with a Qiagen plasmid purification kit (Qiagen, Chatsworth, Calif.) according to the manufacturer's directions. *B. burgdorferi* plasmid DNA and pUC118 (68) were digested with both *Sal*I and *Bam*HI or with *Eco*RI (New England Biolabs, Beverly, Mass.), ligated together with T4 DNA ligase (New England Biolabs), and transformed into *E. coli* InvαF′ (Invitrogen, San Diego, Calif.). Individual colonies were analyzed for plasmid inserts as follows. *E. coli* carrying recombinant plasmids containing the *B. burgdorferi* B31 *erp* loci were identified by PCR analysis of a suspension of boiled bacteria. Clones produced from digestion with *Sal*I and *Bam*HI were analyzed for *erpAB*, while clones produced from *Eco*RI digestion were analyzed for *erpCD*, *erpG*, or *erpH*. PCR conditions consisted of

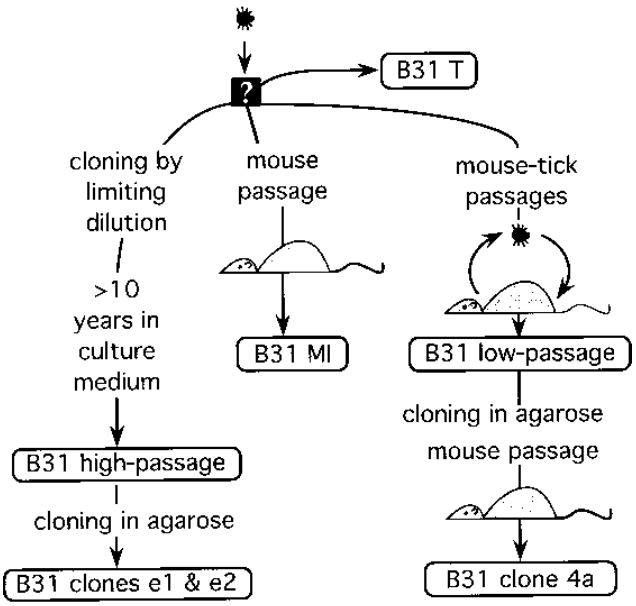

FIG. 1. A history of the different cultures of *B. burgdorferi* isolate B31 from an *Ixodes scapularis* tick (Shelter Island, N.Y., 1981) used in this study. Culture details are given in Table 1 and Materials and Methods. It is unclear when the different lineages of B31 were separated from each other or for exactly how many generations each was grown in culture medium, as indicated by the black box and question mark. Cloning in agarose: isolation through a single colony on an agarose plate according to the method of Rosa and Hogan (47a). It is clear from our analysis of 32-kb plasmid contents that B31 T is not simply ancestral to all of the other cultures of isolate B31 that we have studied.

25 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min. The oligonucleotides used in the analysis of the plasmid libraries are listed in Table 2. PCR products were analyzed by agarose gel electrophoresis and visualized by staining with ethidium bromide.

All four of the previously described 32-kb circular plasmids of *B. burgdorferi* B31 (65) were found to have a *Sal*I site separated by approximately 5 kb from a *Bam*HI site. Colonies from the *Sal*I-*Bam*HI library that did not contain the *erpAB* locus (see above) were screened by digestion of purified plasmid DNAs with both *Sal*I and *Bam*HI and were analyzed for a 5-kb insert by agarose gel electrophoresis. Recombinant plasmids were further analyzed by digestion with additional restriction endonucleases to confirm that the inserts were indeed fragments of the 32-kb circular plasmids.

A fragment of cp32-4 was produced by PCR amplification of *B. burgdorferi* B31 clone e1 plasmid DNA with oligonucleotides complementary to the region between *erpH* and ORF-4b and to a conserved region within the "PCR target site" (2) of cp32-1, cp32-2, and cp32-3 (determined from our sequencing of DNA clones described above). PCR conditions consisted of 25 cycles of 94°C for 1 min, 45°C for 1 min, and 65°C for 3 min. The PCR product was cloned directly into pCRII (Invitrogen) and transformed into *E. coli* InvaF′ (Invitrogen). Sequencing the ends of the cloned insert confirmed that this PCR product contained the ORF4b gene located 3′ of *erpH* on cp32-4 and a homolog of the PCR target site.

The *erpH* gene of low-passage, infectious *B. burgdorferi* B31 clone 4a was amplified from purified plasmids (Qiagen) by PCR with the oligonucleotides described above for PCR detection of this locus. The PCR product was cloned directly into pCRII (Invitrogen) and transformed into *E. coli* InvαF′ (Invitrogen).

The region of plasmid cp32-5 between ORF-3 and the *erpIJ* locus was PCR amplified from low-passage, infectious *B. burgdorferi* B31 clone 4a plasmid DNA by using oligonucleotides ORFD3 and ERP-116. PCR conditions consisted of 20 cycles of 94°C for 1 min, 55°C for 1 min, and 65°C for 6 min. Sequencing of the *erp* locus end of this DNA fragment revealed that it contained a nucleotide motif not found in either cp32-1, cp32-2, cp32-3, or cp32-4. This unique motif was incorporated into oligonucleotide ERP-501, and plasmid DNA from *B. burgdorferi* B31 clone 4a was PCR amplified by using oligonucleotides ERP-501 and CP-0 under the conditions described above. Serendipitously, this combination of oligonucleotide primers amplified the *erp* loci of not only cp32-5 but also cp32-6 and cp32-7. PCR products were cloned into pCRII (Invitrogen), transformed into *E. coli* INVαF′ (Invitrogen), and sequenced. A fragment of cp32-5 was also PCR amplified with oligonucleotides ORFD3 and CP-0 with an Expand PCR system (Boehringer Mannheim, Indianapolis, Ind.) according to the manufacturer's instruction and sequenced to determine that cp32-5 contains the *erpIJ* locus.

Southern blot analysis. Probe templates for use in Southern blotting were produced from purified plasmids containing the appropriate insert. Each plasmid was subjected to PCR amplification consisting 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 amplification. Amplifications were assayed for single products by agarose gel electrophoresis and visualization with ethidium bromide. Final PCR products were purified with Centricon-100 microconcentrators (Amicon, Beverly, Mass.).

Oligonucleotides used in the PCR amplification of DNA probe templates and the probes thus produced are listed in Tables 2 and 3, respectively. The locations of these probes are indicated below in Fig. 4.

DNA electrophoresis in agarose gels was carried out as previously described (19). Most restriction cleavage site mapping was carried out on contour-clamped homogeneous electric field (CHEF) pulsed-field electrophoresis gels, which were designed to optimally separate DNA fragments in the 3- to 50-kb range (19). All DNA preparations were digested in agarose blocks to avoid breakage. DNA preparation, electrophoresis conditions, passive transfer of DNA from electrophoresis gels to Hybond-N^+ membranes (Amersham, Arlington Heights, Ill.), and probing protocols have been described elsewhere (19). Probes were labeled by random priming with [α-^{32}P]dCTP by using a Pharmacia ReadyToGo labeling kit (Pharmacia, Piscataway, N.J.), and membranes were washed in three 10-min changes of 0.015 M NaCl–0.0017 M sodium citrate–0.1% sodium dodecyl sulfate (pH 7.3) at 54°C before exposure to X-ray film.

Nucleotide sequence accession numbers. Additional sequences surrounding the previously described *erp* loci of B31 (65) have been determined, and their GenBank submissions have been amended. The accession numbers for these regions of cp32-1, cp32-2, cp32-3, and cp32-4 are U44912, U44914, U42598, and U44913, respectively. The *erp* loci of cp32-5, cp32-6, and cp32-7 have been given the accession numbers U72996, U72997, and U72998, respectively. The PCR target site sequences of cp32-1, cp32-3, cp32-4, cp32-5, cp32-6, and cp32-7 have been given the accession numbers U60963, U60964, U60965, U72999, U73000, and U73001, respectively (U44914 includes the PCR target site of cp32-2). The sequence of cp32-4 from kb 5 to 7 (see Fig. 4 below) has also been submitted to GenBank and has been given the accession number U60642. The sequences of cp32-1, cp32-2, and cp32-3 at 5 kb have been given the accession numbers U60639, U60640, and U60641 (U60642 includes the homologous region of cp32-4).

RESULTS

Distribution of 32-kb circular plasmids and *erp* loci in cultures of *B. burgdorferi* B31 clone e1. We have previously characterized four *erp* loci from high-passage isolate B31 and found that some derivative cultures did not contain all of these loci (65). For example, one clone derived from high-passage B31 lacked the *erpCD* locus, which lies on the circular plasmid cp32-2 (65). We sought to characterize this and other derivative cultures of isolate B31 to determine whether loss of *erpCD* is the result of a deletion of this locus or of the entire cp32-2 plasmid. In order to simplify analysis of the *erp* locus content, we devised PCR conditions and oligonucleotide primer pairs that specifically amplify unique portions of each of the four *erp* loci (Fig. 2; oligonucleotides described in Table 2). We found that 32 of 74 clones of high-passage *B. burgdorferi* B31 contained *erpCD* (cp32-2), while 42 of 74 lacked this locus (data not shown). All 74 clones tested contained the *erpAB*, *erpG*, and *erpH* loci (cp32-1, cp32-3, and cp32-4, respectively) (data not shown). Restriction site mapping studies showed clear site polymorphisms among the four cp32s at scattered locations around the plasmids (described below). Cultures of strain B31 that lacked *erpCD* by PCR analysis also lacked all cp32-2-specific restriction fragments tested by Southern analysis (data not shown). Therefore, we conclude that loss of *erpCD* was the result of loss of cp32-2 rather than deletion of only *erpCD*. We selected one clone of each plasmid profile type for further characterization of the 32-kb circular plasmids; B31 clone e1 contains cp32-1, cp32-2, cp32-3, and cp32-4, and B31 clone e2 contains only cp32-1, cp32-3, and cp32-4.

We also analyzed the 32-kb plasmid content of B31 clone 4a, an infectious clone of low-passage *B. burgdorferi* B31, and determined that these bacteria did not contain cp32-2, since they lacked the *erpCD* locus (*erpCD* [Fig. 2, lane 2]). Six other

TABLE 2. Oligonucleotides used in this work

Use made of oligonucleotides	Our designation	Sequence (5′ to 3′)
PCR analysis of *erp* loci		
erpAB	ERP-1	ATGTAACAGCTGAATG
	ERP-108	CTTAAATTATGTCTAGTATCACTC
erpCD	ERP-401	AATATTGCAATTATTAGCTGTTG
	ERP-174	GTGTATACAAAATTTAGTTATATC
erpG	ERP-1	ATGTAACAGCTGAATG
	ERP-58	AGTCTAATCATATCCTCAGACAGG
erpH	ERP-401	AATATTGCAATTATTAGCTGTTG
	ERP-404	ATTCATTCTTAGGGTTTTCATATC
Library screen PCR		
erpAB	ERP-111	ACAATAAAGATGAATTAATGCAGG
	ERP-112	TCTATAACACCTTGTGCCCCC
erpCD	ERP-155	GATTTAAAACAAAATCCAGAAGGG
	ERP-168	CTGCTTTAGCCCTAGCTTC
erpG	ERP-33	TGCAAGATTGATGCG
	ERP-34	ATTTTGAGGCTCTGC
erpH	ERP-401	AATATTGCAATTATTAGCTGTTG
	ERP-404	ATTCATTCTTAGGGTTTTCATATC
cp32-4 cloning	ERP-403	ACCCCATGAACAATGTTTCAGAAA
	CP-0	GAAAAAGATAACATGCAAGATACG
cp32-5, cp32-6, and cp32-7 cloning	ORFD3	ACTGATAATGATGTTATGGTTAGG
	ERP-116	ATATAATTTTGTTACATTCAGCTG
	ERP-501	ATTGCATGAGAAATTTGTGTTGTG
	CP-0	GAAAAAGATAACATGCAAGATACG
DNA probe production		
1	ORFD-1	ACGATAGGGTAATATCAAAAAAGG
	ORFD-2	AGTTCATCTAATAAAAATCCCGTG
2	ERP-177	GAAAAGCCCATTAAAGATAGGTTG
	ERP-178	AAGTAACAACCCCATTTTTGTATCTCC
3	ERP-113	AGAATTATGCAATTAAAGATTTAG
	ERP-114	GATTCTTCTACTTTTTTCACTTTC
4	CP-4	AATACGTTGATCATGCGAAATGAC
	CP-5	TTACTTTCTACCATATGGGCTGCC
5	ERP-155	GATTTAAAACAAAATCCAGAAGGG
	ERP-168	CTGCTTTAGCCCTAGCTTC
6	ERP-33	TGCAAGATTGATGCG
	ERP-34	ATTTTGAGGCTCTGC
7	ERP-41	CAAAAGTAAGTTTAAAAGGGGTAG
	ERP-42	ATCAAAGTCTTTGGCGTTTAACTC
8	ERP-401	AATATTGCAATTATTAGCTGTTG
	ERP-404	ATTCATTCTTAGGGTTTTCATATC
9	ERP-403	ACCCCATGAACAATGTTTCAGAAA
	ERP-412	CCATTTTTAGAGCTGTTTTCATTG
10	ORFD-3	ACTGATAATGATGTTATGGTTAGG
	ORFD-4	TTTCTTAAGCTGAAATCTTAGGGG
11	ERP-505	GAGAAGTCGGATCCTAAAAGTG
	ERP-510	TCCAATTGCAGATTCAAC
12	ERP-702	CAACACCACCTCGGTTTTTAGACC
	ERP-703	AGGACTTTTAGAAATTCTAGAGAC
13	ORFD-4	TTTCTTAAGCTGAAATCTTAGGGG
	ORFD-5	TTACCAAAGAGGAGATATTTGCTC

infectious clones of low-passage B31 also lacked cp32-2 (data not shown). The culture from which these clones were derived contained some bacteria that carry cp32-2 (Fig. 2, lanes 3). All seven infectious clones of low-passage B31 contained cp32-1, cp32-3, and cp32-4 (Fig. 2, lanes 2, and data not shown). An independently maintained infectious culture of *B. burgdorferi* B31, B31 MI, was also found to contain cp32-1, cp32-3, and cp32-4 but to lack cp32-2 (data not shown). These results show that neither *erpCD* nor any other locus on cp32-2 is required to establish *B. burgdorferi* infection in laboratory mice, although it has not yet been determined whether bacteria that lack cp32-2 can infect ticks or be transmitted between hosts.

The *erpH* locus is apparently defective in low- as well as high-passage B31 clones. While the *erpA*, *-B*, *-C*, *-D*, and *-G* genes are all capable of encoding proteins of 19 to 39 kDa, the *erpH* gene of high-passage B31 encodes a protein that is only 33 amino acids long (65), which could have been caused by deletion of part of a longer *erpH* gene during laboratory

TABLE 3. DNA probes used in this study

Probe	Location[a]	Plasmid	Comment(s)
1	*orf-3*	cp32-1	cp32-1 specific
2	*orf-8/7*	cp32-1	Hybridizes to other cp32s[b]
3	*erpB*	cp32-1	Hybridizes with *erpB* on cp32-1 and *erpJ* on cp32-5
4	~5.5 kb on map	cp32-2	Hybridizes to other cp32s[b]
5	*erpD*	cp32-2	cp32-2 specific
6	*erpG*	cp32-3	cp32-3 specific
7	~7 kb on map	cp32-4	Hybridizes to other cp32s[b]
8	*erpH*	cp32-4	cp32-4 specific (also hybridizes weakly with the *erpG* gene)
9	*orf-4b*	cp32-4	cp32-4 specific (also hybridizes weakly to ORF-4 on at least cp32-1, -2, -3, -4)
10	*orf-3*	cp32-5	cp32-5 specific
11	*erpK*	cp32-6	cp32-6 specific
12	*erpL*	cp32-7	cp32-7 specific
13	*orf-3*	lp56	~56-kb linear plasmid specific

[a] The approximate locations of probes 1 through 12 are indicated in Fig. 4.
[b] Probe appears to hybridize to all B31 cp32s.

cultivation. PCR analysis of the infectious low-passage *B. burgdorferi* B31 and B31 clone 4a indicated that they both contain *erpH* loci with the same lengths as those found in high-passage B31 (Fig. 2). In addition, the DNA sequence of the *erpH* locus of B31 clone 4a is identical to that of the high-passage B31 (data not shown). We conclude that the *erpH* locus most likely existed in the short form in nature and that bacteria carrying it are infectious in laboratory mice.

Restriction endonuclease cleavage site maps of the 32-kb circular plasmids in high-passage B31 clone e1. We have previously found that the four 32-kb circular plasmids of *B. burgdorferi* B31 contain at least 2 kb of homologous sequences (65). DNA species that are homologous throughout should contain similar patterns of restriction endonuclease cleavage sites. Therefore, we constructed cleavage site maps for each plasmid using plasmid-specific probes derived from the *erpB*, *erpD*, *erpG*, and *erpH* genes, and a probe specific for the ORF-3 gene of cp32-1 (probes 3, 5, 6, 8, and 1, respectively; all probes are described in Table 3 and Fig. 4 below). These loci, although homologous, are not identical, and probes derived from each are specific for DNA fragments containing them (65, 74); however, note that probe 8 (*erpH*) does cross-hybridize weakly with *erpG* (cp32-3).

Initial map construction was done by Southern blot analysis of whole-cell B31 clone e1 DNA cut in single and double digests by approximately 40 restriction endonucleases. We found that *Bss*HII, *Eag*I, *Not*I, *Sfi*I, and *Sma*I did not cut any of the four 32-kb plasmids. A set of 20 restriction enzymes that generated large *erp* gene probe-reactive fragments was chosen for more detailed mapping of the cp32 plasmids. For example, the four different *erp* gene probes hybridized specifically with four differently sized fragments in *Sal*I-*Sac*I double digests of B31 clone e1 DNA (Fig. 3), allowing the measurement of *Sal*I-*Sac*I site distances specifically for each probe. From many similar experiments with the 20 restriction enzymes, we deduced the locations of a number of cleavage sites on the four plasmids by analyzing the DNA fragments that hybridized with the above probes. Sites known from sequencing the *erp* regions (65) allowed unambiguous location and orientation of the *erp* loci on the restriction maps (Fig. 4).

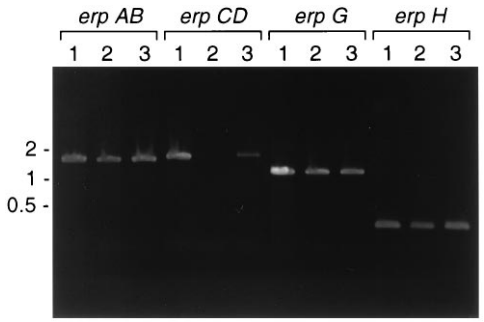

FIG. 2. DNA preparations of *B. burgdorferi* B31 variants subjected to PCR amplification with oligonucleotide primers specific for each *erp* locus. Amplified DNA was subjected to agarose gel electrophoresis and stained with ethidium bromide. Lanes: 1, B31 clone e1; 2, B31 clone 4a; 3, B31 low-passage (uncloned). Size markers are indicated in kilobases.

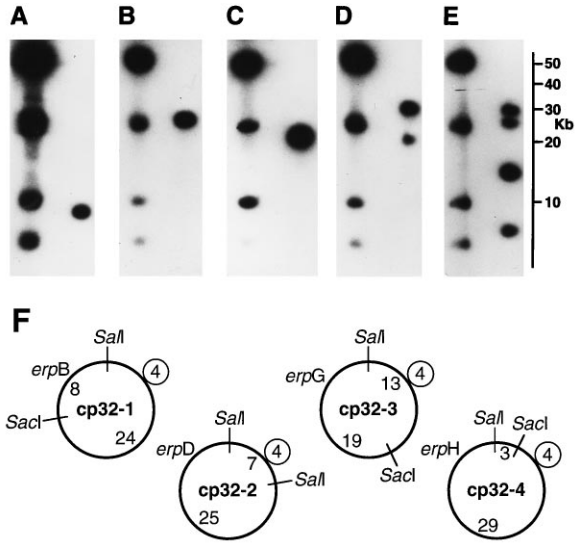

FIG. 3. Demonstration of different locations for each of the *erp* genes of *B. burgdorferi* B31 clone e1 DNA by Southern blot analysis. *B. burgdorferi* B31 clone e1 complete genomic DNA, prepared in agarose blocks, was cleaved with restriction endonucleases *Sac*I and *Sal*I and separated by agarose gel CHEF electrophoresis. DNA was transferred to a nylon membrane and probed as described in Materials and Methods. The probes used, described in Table 3, were as follows: probe 3 (*erpB*) (A), probe 5 (*erpD*) (B), probe 6 (*erpG*) (C), probe 8 (*erpH* [this probe also reacts weakly with *erpG*]) (D), probe 4 (hybridizes with all cp32 plasmids) (E). A phage λ probe was included in the hybridizations to show the locations of the *Hin*dIII-digested phage λ DNAs that were included in each left lane as size markers. (F) Locations of *Sac*I and *Sal*I cleavage sites on cp32-1, cp32-3, cp32-3, and cp32-4 and sources of the hybridizing bands in panels A through E. Outside of each map, the locations of the *erp* gene probes and probe 4 are shown. Inside each map, the lengths of the fragments are given in kilobases.

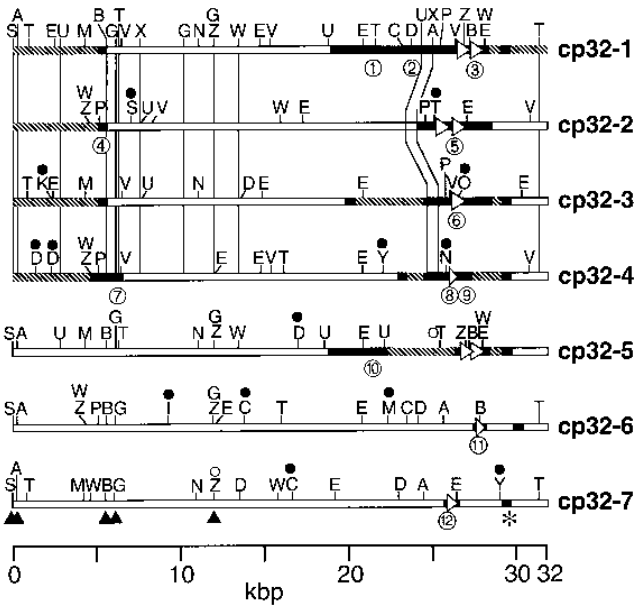

FIG. 4. Restriction maps of the 32-kb circular plasmids of *B. burgdorferi* B31. Abbreviations for restriction enzyme cleavage sites are as follows: A, *Apa*LI; B, *Bam*HI; C, *Sac*II; D, *Sac*I; E, *Eco*O109I; G, *Bgl*I; I, *Bsi*WI; K, *Kpn*I; M, *Mlu*I; N, *Nru*I; O, *Ngo*MI; P, *Pml*I; S, *Sal*I; T, *Pst*I; U, *Stu*I; V, *Pvu*II; W, *Alw*NI; X, *Bst*XI; Y, *Bsp*EI; and Z, *Pme*I. The conserved *Sal*I site found in all seven cp32s was arbitrarily assigned position 0/32 kb; the maps have been opened at this site to allow linear presentation. Long vertical lines indicate restriction sites present in cp32-1, -2, -3, and -4. The five sites that are known to be present in all seven cp32s are indicated by black triangles below the cp32-7 map. Regions of the 32-kb plasmids that have been cloned in *E. coli* are indicated by the cross-hatched regions of the horizontal bars representing the plasmid genomes, while those that have been sequenced are filled in. Open triangles, locations of the *erp* genes (see text and Fig. 5); circled numbers, locations on the map above them of probes used in this study (see Table 3); vertical gray rectangles, homologies with other cp32s among the sequenced regions. The locations of the PCR target sequences (indicated by asterisk) were determined by the lengths of specific PCR fragments that were amplified between these sequences and the *erp* genes. Black circles above restriction sites, restriction sites known to be present only in the indicated plasmid; open circles, positions of restriction sites that are present in six plasmids but known to be missing only in the indicated plasmid (e.g., missing *Bgl*I site at 12 kb in cp32-7). Restriction sites present in multiple plasmids are indicated by vertical lines. Enzymes used in map construction that did not cut particular cp32 plasmids are as follows: for cp32-1, I, K, O, and Y; for cp32-2, C, D, I, K, M, N, O, and Y; for cp32-3, C, I, and Y; for cp32-4, C, I, K, M, and O; for cp32-5, C, I, K, O, P, and Y; for cp32-6, K, N, O, and Y; and for cp32-7, I, K, O, and P. The cp32-5, -6, and -7 maps are less detailed than those of the other four plasmids; in these cases, U (except cp32-5), V, and X sites were not mapped. In most cases, there could be additional restriction sites outside the fragments that the probes react with, since the maps were constructed largely from Southern analysis.

Probes from several other locations on the 32-kb circular plasmids, such as probes 2, 4, and 7, hybridized with DNA fragments from all four plasmids (Fig. 3 and data not shown). These three probes were used to check the consistency of the maps generated with the plasmid-specific probes. Analysis of B31 clone e2 (which lacks cp32-2) was also used to clarify the origin of certain cp32-2 fragments.

These initial restriction maps made it possible to design strategies by which particular cp32 fragments could be cloned into an *Escherichia coli* vector. As an example, the conclusion that cp32-1 is cut by *Sal*I and *Bam*HI to produce a 4-kb fragment containing *erpB* led us to screen a library made from *Sal*I-*Bam*HI-digested B31 e1 plasmid DNA for a clone containing this region. In addition, 5.5-kb *Sal*I-*Bam*HI fragments derived from B31 e1 cp32-1, -2, -3, and -4 were cloned (Fig. 4), as were several other fragments derived from these plasmids. More accurate locations for the previously mapped restriction sites were determined from each of the new DNA clones, and probes 2, 4, and 7 were derived from these plasmids to help confirm and extend the cp32 restriction site maps.

The four cp32 maps are each about 32 kb in length and are clearly circular (Fig. 4). The latter fact is most simply demonstrated for cp32-3. The single *Ngo*MI site in cp32-3 lies within probe 6, so that DNA on both sides of this site hybridizes with the probe. The probe reacts only with a 32-kb band in an *Ngo*MI digest but reacts with two bands whose sizes add up to 32 kb in double digests with *Alw*NI, *Bam*HI, *Kpn*I, *Mlu*I, *Nru*I, *Pme*I, *Pml*I, and *Sal*I (all enzymes which by themselves created only a 32-kb fragment that reacted with the probe 6 [data not shown]). These data can only be reconciled with a 32-kbp circular DNA that each of the above enzymes cleaves once. Similar arguments can be made for all of the cp32s.

A majority of the mapped restriction enzyme cleavage sites were found in similar locations on two or more of the cp32 maps (Fig. 4). The 32-kb circular plasmids contain 15 mapped restriction sites that are present in cp32-1, -2, -3, and -4, and of 111 informative mapped restriction sites on the four plasmids, only 14 are present in only one of these four plasmids (Fig. 4). We considered sites to be conserved if they are located at indistinguishable positions at our resolution (about 1 kb). The conserved sites scattered throughout the four cp32 plasmids strongly indicate the presence of homologous sequences throughout these plasmids.

Nucleotide sequence homologies from the different 32-kb plasmids. To analyze the relationships of the above four previously observed 32-kb plasmids in more detail, we used the cloned DNA fragments of the 32-kb circular plasmids to gather sequence data from several sites around all four plasmids. We found that the four plasmids contain very similar sequences in each of the regions analyzed, further indicating that these plasmids are closely related.

The homologous cloned *Sal*I-*Bam*HI fragments from cp32-1, -2, -3, and -4 (positions 0 to 5.4 kb on Fig. 4) were partially sequenced. In sequencing over 300 bp from the *Bam*HI end of the fragments, we found that these four 32-kb plasmids share over 95% identical nucleotides at this location (data not shown). Our restriction mapping demonstrated that a *Pml*I site is located at position 5 kb only in cp32-2 and cp32-4 (Fig. 4). The sequencing of these homologous regions of the four cp32s showed that cp32-2 and cp32-4 contain the *Pml*I recognition site (CACGTG) at the correct location, while cp32-1 and cp32-3 have the sequence CACGCG (1 bp different from a *Pml*I recognition site) at that location. This suggests that other restriction endonuclease sites seen on only some of the 32-kb plasmids may have imperfect homologs on the other plasmids. Sequencing of nearly 2 kb of this region of cp32-4 indicated the presence of five potential ORFs (data not shown), none of which were homologous to any previously described sequence in GenBank.

We have previously speculated that an approximately 30-kb circular plasmid of *B. burgdorferi* B31 carrying a conserved PCR target site (2) may be the same as cp32-1, since both were cut twice by *Bam*HI to produce fragments of about 10 and 22 kb in size (2, 65). We found that cp32-1 contains a 333-bp sequence that is identical to that reported for the PCR target site (2) and that all of the other cp32s studied here contain homologs of this sequence in similar locations, although the sequences were not identical (Fig. 5).

Partial sequencing of *Eco*RI fragments containing *erpG* and *erpH* indicated that these genes are linked to homologs of the cp32-1 genes ORF-1 and ORF-E, respectively. Based on the sizes of these *Eco*RI fragments, we concluded that ORF-1 and ORF-E have locations similar to their homologs on cp32-1

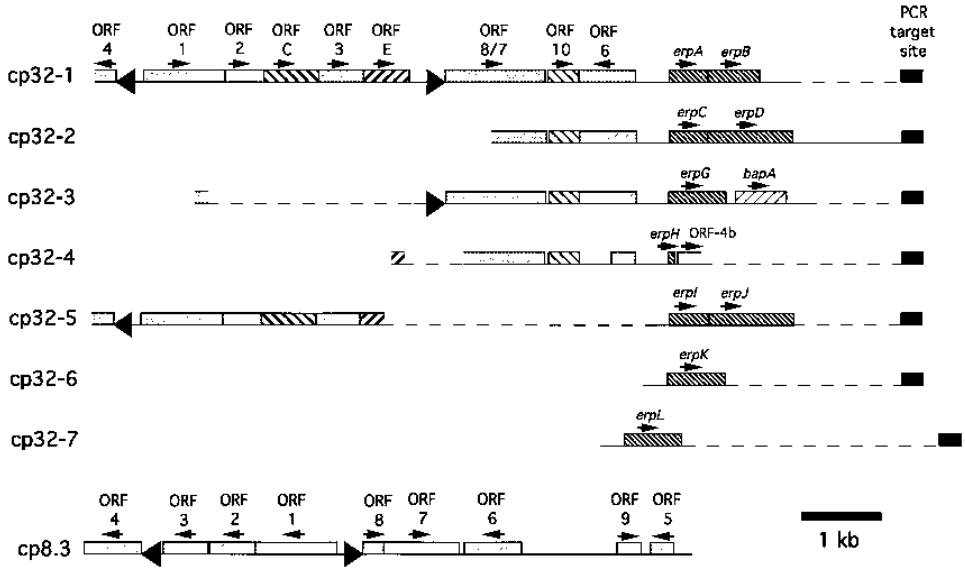

FIG. 5. ORFs in the sequenced regions of the seven characterized 32-kb circular plasmids of *B. burgdorferi* B31 and of cp8.3 of *B. burgdorferi* sensu lato Ip21 (22). Regions that have been PCR amplified but that have not been sequenced are indicated by dashed lines in the diagram. ORFs homologous to ORFs found on cp8.3 are stippled. The location of the PCR target site (2) of each 32-kb plasmid is also indicated by a black bar. Note that the spacing between *erpL* and the PCR target site of cp32-7 is approximately 1 kb longer than that of the other cp32s. Large triangles indicate the inverted repeat DNA sequences identified by Dunn *et al*. (22) in cp8.3.

(Fig. 4 and 5). We have not analyzed the other 32-kb plasmids to determine whether homologs are present.

Further evidence for homologous sequences present at similar locations on cp32-1, -2, -3, and -4 was revealed by hybridization of probes 2, 4, 7, and 9 to parallel locations in all four plasmids (data not shown; probe 9 hybridized weakly to sequences on each cp32 that align with the ORF-4 of cp32-1 and strongly to the ORF-4b gene at its unique location on cp32-4).

The cp32s also have significant differences. Restriction sites were identified that are unique in each of the plasmids except cp32-1 (Fig. 4). In addition, the regions located 3′ of each *erp* locus are different for each plasmid characterized; only cp32-3 contains *bapA*, and only cp32-4 contains ORF-4b. Each plasmid does, however, contain a homologous PCR target site within a few kilobases of the *erp* locus, indicating that these dissimilarities are not extensive. We cannot yet determine whether other regions of nonhomology exist elsewhere in these plasmids.

Additional 32-kb circular plasmids are present in other cultures of *B. burgdorferi* B31. Zückert et al. (73, 74) recently reported cloning and sequencing *B. burgdorferi* B31 DNA fragments related to the cp32 plasmids described here. They reported that two of the fragments (in *E. coli* plasmids pOMB10 and pOMB65) were derived from circular plasmids of approximately 30 kb in size (74).

A restriction map derived from their sequence of pOMB65 suggested that its DNA insert came from cp32-1. Additional sequencing has indicated that this plasmid carries *erpA* and ORF-6 genes that are identical to those that we identified in cp32-1 (65, 72a). In addition, probe 1, derived from the ORF-3 gene of pOMB65, recognized only DNA fragments from cp32-1 by Southern analysis (data not shown). We conclude therefore that the *B. burgdorferi* B31 plasmid called cp30.5 by Zückert et al. (74) is identical to the one that we have called cp32-1.

A restriction map derived from the sequence of the pOMB10 insert was not consistent with any of the maps of the four 32-kb plasmids of B31 clone e1. We found that a probe complementary to the ORF-3 gene of pOMB10 (probe 10) hybridized with unique DNA fragments present in low-passage B31 but not in B31 clone e1 (data not shown). These data indicate that low-passage B31 carries a fifth 32-kb circular plasmid, cp32-5, that is related to, but distinct from, the plasmids of B31 clone e1. Among the B31 cultures analyzed here (Table 1), only low-passage B31 and B31 clone 4a contain this plasmid. Probe 10 hybridized specifically to cp32-5 and was used to construct a rudimentary restriction site map of this plasmid (Fig. 4). Of the 10 sites tested that are present in all of the cp32-1, -2, -3, and -4 maps, two, *Stu*I and *Apa*LI sites at 24.5 and 25.5 kb, respectively, are not present in cp32-5. These differences and the presence of a *Sac*I site at 17 kb point out the uniqueness of cp32-5. This plasmid carries two *erp* gene homologs that were named *erpI* and *erpJ*. Although we have not yet sequenced these two genes in their entirety, the sequence that we have determined is identical to that of *erpAB* on cp32-1.

PCR spanning the *erpIJ* locus (see Materials and Methods) of cp32-5 fortuitously amplified two additional DNA fragments, which, when sequenced, each revealed a new monocistronic erp locus (named *erpK* and *erpL* [Fig. 5]). Southern blot analysis at high stringency showed that these two DNAs hybridized to unique restriction fragments. Again, rudimentary restriction maps were constructed from this Southern analysis, showing that *erpK* and *erpL* lie on two additional 32-kb circular plasmids, i.e., cp32-6 and cp32-7, respectively (Fig. 4). We also note that the promiscuous probes 2, 4, and 9 (Table 3) hybridize with a few restriction fragments from circular plasmid DNAs from low-passage B31, B31 MI, and B31 T that cannot be accounted for by the seven cp32s that we have characterized, so there could be additional cp32 related plasmid(s). Table 4 lists the seven characterized cp32s and related plasmids that are carried by each of the B31 cultures used in these studies.

Similarities between the 32-kb circular plasmids and other plasmids of *B. burgdorferi*. The cumulative sequences of the 32-kb plasmids indicate that these plasmids are homologous to

TABLE 4. cp32s and related plasmids present in *B. burgdorferi* B31 cultures used in this study

B. burgdorferi B31 culture	cp32-1	cp32-2	cp32-3	cp32-4	cp32-5	cp32-6	cp32-7	56-kb related linear plasmid	Additional related plasmid(s)[a]
High-passage B31									
Clone e1	+	+	+	+	−	−	−	−	−
Clone e2	+	−	+	+	−	−	−	−	−
Low-passage B31									
Uncloned	+	+[b]	+	+	+	+	+	ND[c]	+
Clone 4a	+	−	+	+	+	+	+	+	+
B31 MI	+	−	+	+	−	+	+	+	+
B31 T	+	−	+	+	−	+	+	−	+

[a] cp8.3 was not analyzed in this study, but a similar sized circular plasmid has been reported to be present in isolate B31 (19a, 22). Symbols in this column indicate the presence or absence of a larger uncharacterized circular DNA that hybridizes to our promiscuous probes 2, 4, and 7.

[b] In our low-passage cultures, cp32-2 is detectable by PCR with oligonucleotides ERP-174 and ERP-401, but not by Southern analysis with probe 5, indicating that a small fraction of the individuals in this culture carry cp32-2.

[c] ND, not determined.

most of cp8.3, a small circular plasmid of *B. burgdorferi* sensu lato Ip21 (22). Collective data (65, 74) show that cp32-1 contains homologs of seven of the nine ORFs identified on cp8.3 (Fig. 5). It is entirely possible that homologs of the remaining portions of cp8.3 may be located in the approximately 22 kb of cp32-1 that has yet to be sequenced. Our sequencing of cp32-1 and cp32-3 has revealed that both of these plasmids contain an ORF that is a fusion of cp8.3 ORF-8 and ORF-7 homologs (ORF-8/7). We have also found that the stop codon previously identified in ORF-8/7 of cp32-1 (74) was incorrect and that this gene could produce a protein of approximately 51 kDa (data not shown). As noted above, cp32-4 contains two ORF-4-like genes.

Zückert et al. (74) also cloned and sequenced a DNA fragment (in *E. coli* plasmid pOMB14) that was homologous to pOMB10 and pOMB65 but which hybridized to an approximately 50-kb linear plasmid from their *B. burgdorferi* B31 culture. Probe 13, derived from ORF-3 of lp56, reacted only with a 56-kb linear plasmid in B31 MI and 4a, but not in the other cultures of *B. burgdorferi* B31 studied here (data not shown). Ethidium bromide staining of CHEF gels of DNA from B31 MI and 4a showed one thick band in that region (data not shown). The *ospAB* operon is located on an approximately 54-kb linear plasmid in *B. burgdorferi* B31 (10, 19). Since this latter plasmid is present in all of our B31 cultures, it is likely that B31 MI and 4a and, therefore, the original B31 contained two different linear plasmids in this size range. This has been shown to be the case by restriction mapping with *ospAB* and number 13 probes (15).

cp32-like plasmids in other isolates of *B. burgdorferi* sensu stricto and related species. Since several studies (1, 32, 45, 66) suggest that other isolates of *B. burgdorferi* may also contain multiple 32-kb circular plasmids, we analyzed 19 additional isolates of *B. burgdorferi* sensu stricto (Table 1) to determine whether they carry plasmids related to the cp32s. In uncleaved DNAs, probe 4 (which hybridized with all of the cp32s of isolate B31) reacted only with several diffuse bands diagnostic of circular DNA in the 25- to 40-kb size range under our CHEF conditions (data not shown). Since the *Bgl*I digestion pattern is identical for the four 32-kb circular plasmids of *B. burgdorferi* B31 clone e1 (Fig. 4), we reasoned that these sites might also be conserved in any related plasmids found in other isolates. DNAs from all 19 isolates were digested with *Bgl*I and separated by CHEF electrophoresis, and Southern blot analysis was performed with probe 4 (Fig. 6A). The probe recognized a 24- to 25-kb fragment from 18 of the 19 isolates, which is the same size as that of the fragments in the four B31 clone e1 cp32s (isolate N40 was the exception; it has only a 32-kb probe 4-reactive band). The probe also hybridized with a 32-kb fragment from every isolate except CA-3-87, 26518, and B31 clone e1 (although B31 T, low-passage B31, and B31 MI did have a reactive 32-kb fragment derived from cp32-7), as well as additional bands between 9 and 22 kb in 13 of the isolates. The

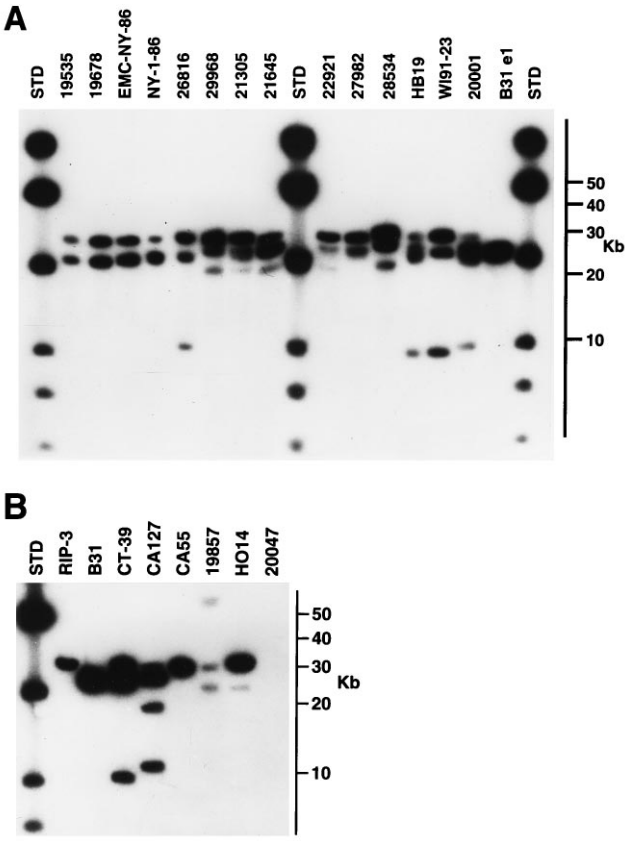

FIG. 6. Homologs of the cp32 plasmids in isolates of *B. burgdorferi* sensu stricto and related borreliae. (A) Isolates of *B. burgdorferi* sensu stricto. (B) Isolates of *B. burgdorferi* sensu lato. Total genomic DNAs from the indicated *B. burgdorferi* isolates (described in Table 1) were cleaved with restriction endonuclease *Bgl*I and separated by CHEF agarose gel electrophoresis. DNAs were transferred to a nylon membrane and probed with probe 4 as described in Materials and Methods. A phage λ probe was included to show the locations of the *Hin*dIII-digested phage λ DNA bands that were included as size markers.

probe did not hybridize with the chromosomes of any of the *B. burgdorferi* isolates tested. Only isolates B31 MI, 26816, and 21645 showed weak hybridization of probe 4 with a 50-kb DNA species (probable linear plasmids) in uncleaved DNA (data not shown). Very similar conclusions were drawn from experiments with probe 2, which hybridized with the same >23-kb bands as probe 4, but with different smaller ones (data not shown), suggesting that both the probe 2 and 4 regions are highly conserved in *B. burgdorferi* sensu stricto isolates. Among the 20 isolates studied here, 9 originated from New York, 3 from Connecticut, 3 from California, 2 from Wisconsin, and 1 each from Rhode Island, Maryland, and France (Table 1). These data suggest that at least 19 of the 20 isolates tested, which come from geographically diverse locations and a variety of biological sources, almost certainly carry multiple cp32 homologs.

Since *B. burgdorferi* sensu stricto is part of a diverse group of borreliae, we analyzed the DNAs of several other members of this group to determine whether they also contain plasmids related to the cp32s of B31. We used probes 2 and 4 in Southern blot analyses of *Bgl*I cleaved DNAs from 11 isolates of *B. burgdorferi* sensu lato collected around the world (Table 1). All of the isolates tested except 20047, G2, VS116, M63, and PotiB1 contain at least one *Bgl*I fragment that hybridized with both of these probes, and in no case did the probe react with the chromosome when uncleaved DNA was probed (Fig. 6B and data not shown). DNA from the *B. afzelii*, *B. andersonii*, and *B. japonica* isolates hybridized less strongly with both probes than did the other North American types. In addition, uncut CA55, 19587, and HO14 DNAs contained bands in the 50- to 100-kb range that hybridized weakly with probe 4 and could be linear plasmids. Again, the presence of 32- and 24-kb *Bgl*I bands that were recognized by both probes 2 and 4 suggests that most of these related borreliae carry cp32 homologs. The *B. burgdorferi* sensu lato isolates tested here represent all 10 known genomic types, including the VS116 and PotiB1 types that have recently been delineated by restriction site polymorphism analysis (see references 19, 47, and 67 and references therein). All members of the *B. garinii* (isolates 20047 and G2), VS116 (isolates VS116 and M63), and PotiB1 (isolate PotiB1) types tested failed to react with probes 2 and 4; thus, it is possible that these three types do not carry cp32s. It is also quite possible that they carry cp32 plasmids missing these sequences or contain very distantly related versions of them, since Amouriaux et al. were able to amplify the PCR target sequence from isolates of *B. garinii* (2).

DISCUSSION

We have found that cultures of *B. burgdorferi* isolate B31 with different culture histories contain as many as seven (and possibly more) different circular plasmids that are approximately 32 kb in size. We have also demonstrated through sequencing and restriction site mapping that these seven plasmids contain regions that are closely related to each other throughout their lengths. The B31 cultures used by Zückert and colleagues (73, 74), B31 MI and B31 clone 4a, also carry a linear 56-kb plasmid with a substantial region that is homologous to the 32-kb circular plasmids. With the 10 or more linear plasmids and the previously characterized circular plasmids of 26 and 9 kb present in isolate B31 (7, 15, 19a, 37, 52), there is now an amazing total of at least 19 known plasmids carried by the original B31 isolate. Surprisingly, B31 T, which is thought to have had the fewest in vitro passages from the original tick isolate (5a), lacks cp32-2, cp32-5, and the linear cp32 relative, so it is not possible to reconstruct a history of plasmid loss with the cultures presently available to us. It is possible that the original isolate may have been a mixture of closely related bacteria that had slightly different plasmid complements. St. Girons and Davidson (52a) reported that chromosomal DNAs from their high- and low-passage B31 cultures were not identical, which could have indicated that the original B31 was actually a mixture of strains and that a minority component took over in the high-passage culture. Our macrorestriction analysis (including the use of *Mlu*I, the enzyme that showed the difference reported above) of the chromosomes from all of our B31 cultures has shown no evidence for heterogeneity; all had patterns identical to that which we previously reported for high-passage B31 (16). It seems more likely that different methods of laboratory propagation may have favored the loss of different plasmids. In spite of this minor uncertainty as to whether all seven cp32s can be present in a single cell, we have shown here that at least six of them, cp32-1, -3, -4, -5, -6, and -7, coexisted in the founder cell of B31 clone 4a.

Other studies have demonstrated that *B. burgdorferi* is capable of recombining homologous DNA sequences (48, 50, 53), and the extended similarities among the 32-kb plasmids suggest that homologous recombination could possibly occur between them. If such events had occurred, predictable restriction fragment length changes would have been created. We did not observe any such predicted differences (even as weak bands present in addition to those derived from the eight characterized plasmids) among the cp32s in our different cultures of B31, indicating that recombinants among these plasmids have not accumulated in bacteria maintained in either culture medium or a tick-mouse cycle. It may be that there is enough sequence variation among the plasmids that recombination among them is unlikely to have occurred, although it has been demonstrated that *ospA* and *ospB*, which are not very similar in sequence, are capable of undergoing recombination (50). There may, therefore, be unique characteristics of circular DNAs in *B. burgdorferi* that influence homologous recombination or mechanisms to prevent establishment of recombinant plasmids. We have observed that transformation and recombination in *B. burgdorferi* are more efficient when the transforming DNA is in a linear form as opposed to circular (48).

B. burgdorferi is a fastidious organism, apparently incapable of de novo synthesis of many of the factors necessary for bacterial survival, since growth in the laboratory is possible only in complex, undefined media (6, 31). The chromosome of *B. burgdorferi* has been measured at less than 1 million bp (12, 16, 19, 21, 23, 41), indicating a paucity of genes relative to free-living bacteria such as *E. coli*. It is surprising, then, that this bacterium should contain such a large amount of plasmid DNA, much of it possibly redundant. The seven cp32s in isolate B31 add up to about 224 kb of DNA, nearly one-quarter the size of the chromosome. While it remains possible that each plasmid contains different genes that are essential for survival in the native environment, it seems surprising that this bacterium would require, for example, multiple copies of nearly identical ORF-10 genes (65).

Other researchers have also observed that *B. burgdorferi* cells contain numerous copies of repeated DNA sequences that can be related to the multiple 32-kb circular plasmids. Zückert and colleagues (73, 74) have cloned and sequenced three homologous DNA fragments, two of which originated from cp32-1 and cp32-5. Simpson et al. (58) isolated DNA fragments from *B. burgdorferi* isolate Sh-2-82 that by Southern blot analysis hybridized with circular DNAs with estimated sizes of 29, 26, 20, and 8.4 kb (58). Partial sequencing of two of these cloned fragments (pSPR13 and pSPR14) indicated that they each contain an *erp* locus and other sequences found on the cp32s of

isolate B31 (62). Porcella et al. (45) cloned seven different, related DNA fragments from *B. burgdorferi* isolate 297 that hybridized with circular DNA approximately 30 kb in size.

All isolates of *B. burgdorferi* sensu stricto and almost all of the related borreliae that we have examined contain at least one, and often multiple, plasmids that are related to the 32-kb circular plasmids. In some cases a multiplicity of plasmids may have been present that we failed to detect due to similar restriction fragment sizes or due to differences in DNA sequences that did not hybridize with our probes. The ubiquity of these plasmids may indicate that they encode something that is required for the bacteria to maintain their infectious cycle in nature. Erp homologs of B31 and other *B. burgdorferi* strains are recognized by the immune systems of infected humans and laboratory animals (1, 32, 39, 64–66, 69), although antigenicity does not necessarily indicate that these proteins play an essential role in infection. Not all of the *erp* alleles of B31 are required for infection of laboratory mice, since B31 clone 4a, a cp32-2-deficient variant, is capable of infection and dissemination in a laboratory mouse.

One possible explanation for the widespread distribution of these plasmids is that they are highly conjugative. All cultures of B31 that we examined contained cp32-1, cp32-3, and cp32-4. On the other hand, some cultures lack cp32-2, cp32-5, cp32-6, and/or cp32-7, which may be indicative of different stabilities or propensities of these plasmids to mobilize. If these plasmids are indeed conjugative, they may prove to be useful tools for genetic studies of *B. burgdorferi.*

Alternatively, the 32-kb circular plasmids may be ubiquitous genomes of lysogenic prophages. Several reports suggest that Erp protein homologs are selectively expressed in mammalian hosts (1, 66, 69), and there are many precedents for lysogenic bacteriophage-carried genes being important in the interactions of pathogenic bacteria with their hosts (20). The conserved 32-kb length of these plasmids is also suggestive of a bacteriophage family. Phage DNA size is constrained by the size of the phage capsid, so there is a strong selection for constant genome size within a family of phages with similar capsid proteins (17). The best understood family of bacteriophages is the lambdoid phages of the enteric bacteria, which all have essentially identical gene orders and transcription patterns (18). From our restriction mapping and sequencing data, it appears that the cp32s also have similar gene orders. Bacteriophages have occasionally been seen in cultures of *B. burgdorferi* (26, 38, 55), where their presence is most easily explained by prophage induction. The highly conserved size of the chromosomes of different *B. burgdorferi* sensu lato isolates from widely distributed locations around the world has led us to suggest that if prophages are present they probably exist not as chromosomally integrated DNAs, but as extrachromosomal elements (16). It even seems possible that the related plasmid cp8.3 might also be a prophage, perhaps dependent on larger 32-kb prophages for helper function in a relationship similar to the P2 and P4 phages of *E. coli* (33, 59). Bacteriophages have been used very effectively for moving genetic markers between bacteria, including spirochetes of the genus *Serpulina* (29), and may also prove useful in studying the genetics of *B. burgdorferi*. If the cp32s are prophages, the distinctive restriction endonuclease cleavage site maps that we have assembled will be helpful in their characterization and development as genetic tools.

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