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Correlation of Plasmids with Infectivity of *Borrelia burgdorferi* Sensu Stricto Type Strain B31

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The correlation of plasmid profiles with infectivity was investigated by using five clones of *Borrelia burgdorferi* sensu stricto strain B31 (ATCC 35210). Plasmid profiles were determined by pulsed-field and two-dimensional gel electrophoresis. The 50% infectious dose (ID_{50}) in hamsters was determined. The ID_{50} of the clone that possessed a full complement of eight linear and three circular plasmids was 10^3 cells. The loss of the 27.5- and 40-kb linear plasmids did not decrease the infectivity of these cells. Rather, the loss of the 27.5-kb linear plasmid was associated with a more disseminated infection. A moderate decrease of the ID_{50} from 10^3 to 10^5 cells correlated with the loss of the 9.0-kb circular plasmid and the 27.5-kb linear plasmid. A major loss of infectivity ($ID_{50} > 10^8$ cells) occurred with cells that lost the 24.7- and 27.5-kb linear plasmids and the 9.0-kb circular plasmid. A 3.0-kb *Hin*dIII fragment of the 24.7-kb linear plasmid was used as a probe to determine the presence of the homologous sequences in the three genospecies of Lyme disease spirochetes. An analysis of 21 infectious strains of *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii* revealed a consistent association of infectivity with strains possessing a linear plasmid (size range, 24 to 36 kb) that hybridized with the *Hin*dIII fragment. Western immunoblotting with hamster antisera against infectious B31 clone C-3 revealed two proteins with molecular masses of 28 and 43 kDa that were absent in the noninfectious B31 clone C-1. Additionally, a 14-kDa protein was absent in C-1 but present in infectious clone C-9 as shown by two-dimensional polyacrylamide gel electrophoresis.

Lyme disease is a multisystem disorder caused by three closely related *Borrelia* genospecies: *Borrelia burgdorferi* sensu stricto, *B. afzelii*, and *B. garinii* (1, 10, 12, 26, 37). *B. burgdorferi* sensu stricto will be referred to as *B. burgdorferi* hereafter in this work. The *Borrelia* genome is composed of one linear chromosome of about 950 kb with multiple linear and circular plasmids (3–5, 18, 24, 43, 45). Considerable diversity in plasmid profile among Lyme disease spirochetes obtained from different biological sources and geographical locations has been observed (3, 23, 45, 46). The function of plasmids in the virulence of *B. burgdorferi* is unknown. Genes specifying the outer surface lipoproteins (OspA, -B, -C, -D, -E, and -F) are located on plasmids. The genes *ospAB, ospEF*, and *ospD* are present on 49-, 45-, and 38-kb linear plasmids, respectively (7, 28, 32), while *ospC* is located on a 26- to 27-kb circular plasmid (19, 29, 38). Recently, the gene coding for exported plasmid protein A (EppA) was cloned from a 9-kb circular plasmid (15). The functions of these proteins in the virulence of *B. burgdorferi* remain to be elucidated.

Serial in vitro cultivation of *B. burgdorferi* results in plasmid profile changes which are accompanied by the loss of infectivity in experimental animals (25, 31, 42). Previous reports (25, 31, 42) suggested a potential role for plasmids in the infectivity of *B. burgdorferi*. Schwan et al. (42) and later Simpson et al. (44) reported that the losses of two related similar-sized circular plasmids (pBBC1 and pBBC2) and a 22-kb linear plasmid were accompanied by changes in protein profile and loss of infectivity in mice. However, pBBC1 and pBBC2 were not consistently correlated with infectivity, since some low-passage, infectious *B. burgdorferi* strains lacked these two plasmids. Norris et al. (33) examined the infectivity and the genetic and polypeptide content of clonal populations of in vitro-cultured *B. burgdorferi*. Only a few discernible differences in the plasmid and protein profiles of the high-infectivity and low-infectivity clones were observed. One difference noted was the apparent loss of a 22-kb plasmid in a low-infectivity clone (33). However, no clear correlation of the presence of this plasmid with virulence was established. Many species of pathogenic bacteria have extrachromosomal elements of DNA or plasmids that carry genes encoding properties which are responsible for virulence (8, 11, 35, 36). We investigated the possibility that virulence-associated determinants are also located on plasmids of *B. burgdorferi* by studying clonal populations derived from a common parent strain.

MATERIALS AND METHODS

Bacteria. The primary test organism was *Borrelia burgdorferi* low-passage strain B31 (ATCC 35210) which was isolated from an *Ixodes scapularis* tick in the state of New York (10). For a list of other Lyme disease spirochetes used in this study, see Table 3. Borreliae were grown in Barbour-Stoenner-Kelly medium (BSK) (2) with minor modifications (6). Cultures were incubated at 34°C and harvested when the concentration reached 10^7 to 10^8 cells per ml. *Escherichia coli* (*Epicurian coli*) XL1-blue MRF′ (Stratagene, La Jolla, Calif.) was cultured in Luria-Bertani medium (39) at 37°C. Low-passage strain B31 was subcultured by weekly passage of 0.3 ml (10^7 to 10^8 cells per ml) of culture into 7 ml of BSK medium at 34°C. Clones of *B. burgdorferi* were obtained by limiting dilution as described by Schwan et al. (43). Log phase cultures of each clone were aliquoted and stored at liquid nitrogen temperature in BSK medium containing 15% (vol/vol) glycerol. All cultures used for plasmid analysis, protein electrophoresis, and animal inoculation had been subcultured once or twice after retrieval from frozen stocks.

Plasmid analysis. Plasmid-enriched preparations of borreliae were prepared as described by Barbour (3). Plasmid profiles were analyzed by contour-clamped homogeneous electric field pulsed-field gel electrophoresis (PFGE) as previously described (46) and two-dimensional agarose gel electrophoresis (2D-AGE). The first dimension of the 2D-AGE was conducted by loading the plasmid-enriched preparation into 0.3% agarose gel and performing electrophoresis in 1× TAE buffer (40 mM Tris-acetate, 1 mM EDTA [pH 8.0]) at constant voltage of 0.3 to 0.4 V/cm for 20 to 24 h at room temperature. Following exposure to UV light irradiation for 6 to 8 min, the gel was rotated 90° and electrophoresis was performed as described above for 4 to 6 h. The DNA was stained with ethidium bromide and photographed.

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Alkaline agarose gel electrophoresis. To determine whether the telomeres of the linear plasmids had hairpin loops, alkaline agarose gel electrophoresis was carried out as described by Sambrook et al. (39). Briefly, the gel blocks containing the DNA fragments of interest were excised from a conventional agarose gel, incubated in 1× alkaline buffer (50 mM NaOH, 1 mM EDTA) for 60 min, and then loaded into an alkaline agarose gel (0.7% agarose in 1× alkaline buffer) and subjected to electrophoresis at a constant voltage of 0.2 V/cm for 48 h. The gel was washed twice in distilled H_2O, soaked in 0.25 N HCl for 10 min, washed twice in distilled H_2O and soaked in denaturing buffer (0.4 N NaOH, 1.0 M NaCl) for 45 min, and then the DNA was transferred to a nylon membrane and analyzed by Southern hybridization.

Single and double restriction enzyme digestion. The plasmids were separated by PFGE as previously described (46), and the gel blocks containing the plasmid of interest were excised and washed twice in TE buffer (10 mM Tris, 1 mM EDTA [pH 8.0]) at 4°C. After preincubation in reaction buffer for 20 min at 4°C, the gel blocks were treated with restriction enzymes (20 to 50 U per block) at 37°C for 6 to 8 h. The gel blocks were subjected to conventional electrophoresis in a 0.7 to 0.9% agarose gel, with 0.5× TBE buffer (45 mM Tris-borate, 1 mM EDTA [pH 8.0]) at a constant voltage of 1 to 3 V/cm. The gel was stained with ethidium bromide and photographed.

Southern hybridization. To prepare DNA probes, DNA fragments were separated in 0.7 to 0.9% agarose gel with 0.5× TBE buffer. The DNA fragments used for probes were excised from gels and purified with GeneClean II (Bio 101, La Jolla, Calif.) according to the manufacturer's instructions. The purified DNA fragments were digoxigenin labeled by the random-primed DNA labeling method (Genius System; Boehringer Mannheim, Indianapolis, Ind.) according to the manufacturer's instructions. The nucleotide hybridization was carried out at 60 to 62°C. Digoxigenin-labeled probes were detected with alkaline phosphatase-conjugated antibody to digoxigenin and the chemiluminescent substrate Lumi-Phos 530. Membranes were exposed to X-ray films for 0.5 to 2 h at room temperature. Membranes to be reprobed were stripped as described previously (46).

DNA cloning. The target plasmid of strain B31 used for cloning was excised from the preparative contour-clamped homogeneous electric field PFGE gel and digested in the gel with the restriction enzyme *Hin*dIII or *Bgl*II as described above. The digested DNA was then purified from the gel with GeneClean II (Bio 101) and ligated to the vector pBluescript II ks (+) (Stratagene). Electroporation was carried out with a field strength of 12.5 kV/cm in 0.2-cm-gap cuvettes for transfer of the recombinant DNAs into host cell *E. coli* XL-1 blue MRF′ (Stratagene). Transformants were screened on Luria-Bertani medium containing ampicillin (60 μg/ml), 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) and isopropylthio-β-D-galactoside (IPTG). The white colonies of transformants were further screened by Southern hybridization with plasmids of infectious B31. The source of the positive recombinant DNAs was determined by hybridization with the plasmids of infectious B31. The recombinant DNAs which hybridized with the target plasmid were determined to contain the plasmid DNA inserts. The cloned DNA fragments were subjected to restriction enzyme analysis and Southern hybridization to detect DNA rearrangement.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblotting. Proteins from the whole cell lysate were prepared as described previously (17, 22). Lysates of approximately 10^6 cells were loaded into each lane of the gels. The electrophoresis was carried out in 10 to 15% linear gradient gels with the discontinuous buffer system of Laemmli (27) at 8 mA until the dye front reached the bottom of the gels. Silver stain was used for visualizing proteins in the gels (39).

Western immunoblotting of the cell lysates was carried out as previously described (17, 23). Hamster antiserum was obtained from hamsters 14 days after intraperitoneal injection with 10^8 cells of infectious B31. The antisera from four hamsters were pooled and diluted 1:100 and used to probe the blot. Monoclonal antibodies (MAbs) were used to identify proteins in *B. burgdorferi* B31 clones separated by a 7.5 to 15% linear gradient polyacrylamide gel (17). The MAbs used in this study are listed in Table 5.

2D-PAGE. The 2D-PAGE, isoelectric focusing in the first dimension, was performed with 1.8% ampholytes of pH 5 to pH 7 and 0.2% ampholytes of pH 3 to pH 10 as described by O'Farrell (34). SDS-PAGE with a 10 to 15% acrylamide linear gradient gel as described above was used in the second dimension. Proteins were visualized by silver staining (39).

Infectivity assay in hamsters. Male golden Syrian hamsters, 5 to 7 weeks old (Harlan, Sprague, Dawley, Madison, Wis.), were injected intraperitoneally with various clones of *B. burgdorferi* B31. At 14 days postinjection, the hamsters were sacrificed and the bladder, spleen, and heart of each hamster were aseptically removed and homogenized in 5 ml of BSK medium in a stomacher bag with a Tekmar Stomacher Lab-Blender (Tekmar Co., Cincinnati, Ohio). After the tissue debris settled, a 1:10 dilution of the supernatant fluid was cultured in 6 ml of semisolid BSK medium at 32°C. Cultures were examined by dark-field microscopy at weekly intervals for up to 6 weeks for the growth of borreliae. The cultures were considered negative if borreliae were not seen after 6 weeks of incubation. The 50% infectious dose (ID_{50}) was determined by the Spearman-Karber method (9, 16), with positivity of any one of the three cultured organs being used as the criterion of infection for each hamster.

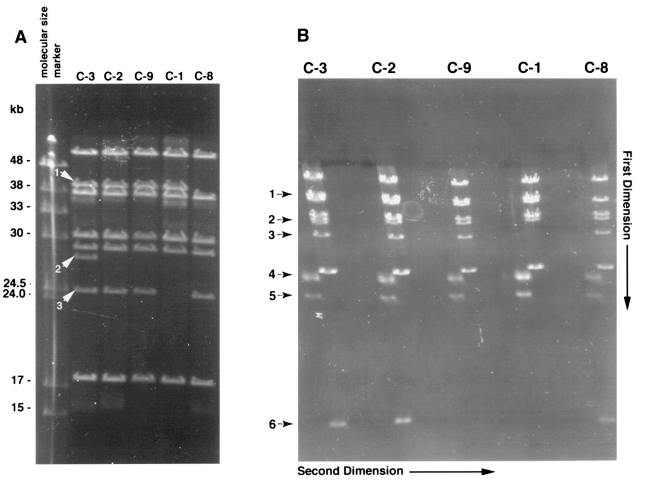

FIG. 1. Plasmid profiles of B31 clones. (A) PFGE; (B) 2D-AGE. The numbers with arrows indicate the linear plasmids which were lost among the B31 clones and the three circular plasmids observed in B31. Arrows: 1, 40-kb linear plasmid; 2, 27.5-kb linear plasmid; 3, 24.7-kb linear plasmid; 4, 30-kb circular plasmid; 5, 27-kb circular plasmid; 6, 9-kb circular plasmid. The molecular size marker is lambda DNA monocut and is shown in the leftmost lane of the PFGE gel (A). All eight of the plasmids shown in PFGE (A) were linear plasmids which migrated along the diagonal axis in 2D-AGE (B). Three plasmids in 2D-AGE which are not seen in PFGE migrated behind the diagonal axis in 2D-AGE, which indicated that their native conformation was circular.

RESULTS

Plasmid profiles of infectious low-passage B31. The plasmid profile of infectious low-passage B31 was assayed by PFGE and 2D-AGE. Eight distinct plasmid bands were observed in PFGE. When 2D-AGE was conducted to investigate the configuration of these plasmids, the eight plasmids observed in PFGE migrated along the diagonal axis and an additional three plasmids that migrated behind the diagonal axis were observed. The migration patterns of eight plasmids in PFGE and 2D-AGE were consistent with those of linear plasmids (14, 29), and the three plasmids with retarded migration in 2D-AGE were circular (29). The sizes (in kilobases) of the eight linear plasmids were approximately as follows (plasmid designations are given in parentheses): 52 (lp52), 40 (lp40), 38 (lp38), 30 (lp30), 28 (lp28), 27.5 (lp27.5), 24.7 (lp24.7), and 18 (lp18). The sizes of the three circular plasmids, based on hybridization with probes from the three circular plasmids reported to be present in strain B31 (15, 29, 38, 45, 47), were assumed to be about 30 (cp30), 27 (cp27), and 9 kb (cp9). The probes used were pOMB25, *ospC*, and *eppA* for the 30-, 27-, and 9-kb circular plasmids, respectively (15, 29, 38, 47).

Clones of B31. To obtain clones with different plasmid profiles, low-passage B31 was subcultured in vitro for 50 passages. Fifty clones were obtained from the 30th subculture by limiting dilution, and the plasmid profiles were analyzed by PFGE and 2D-AGE. Only five types of clones that differed in their plasmid profiles after 30 subcultures were found. The clones were designated C-1, C-2, C-3, C-8, and C-9 (Fig. 1). Compared with low-passage B31, C-1 had lost the 27.5- and 24.7-kb linear plasmids (lp27.5 and lp24.7, respectively) and the 9-kb circular plasmid (cp9), C-2 had lost the 27.5-kb linear plasmid (lp27.5), C-8 had lost the 40- and 27.5-kb linear plasmids (lp40 and lp27.5, respectively), and C-9 had lost the 27.5-kb linear plasmid (lp27.5) and the 9-kb circular plasmid (cp9), while C-3 had the same plasmid profile as that of low-passage B31 (Fig. 1; also see Table 2). lp27.5 was missing in four of the five clones

TABLE 1. ID_{50} determination and infectivity of clones of *B. burgdorferi* B31

Clone	Inoculum size (no. of cells)	No. of spirochete-positive organs				No. of infected hamsters ($n = 5$)	ID_{50} (no. of cells)
		Bladder ($n = 5$)	Spleen ($n = 5$)	Heart ($n = 5$)	All organs ($n = 15$)		
C-3	10^2	1	0	0	1	1	10^3
	10^3	2	0	1	3	2	10^3
	10^4	3	1	1	5	3	10^3
	10^5	5	0	3	8	5	10^3
	10^6	5	0	3	8	5	10^3
	10^7	5	5	4	14	5	10^3
	10^8	5	4	3	12	5	10^3
C-2	10^2	1	1	1	3	1	10^3
	10^3	2	2	2	6	2	10^3
	10^4	4	4	4	12	4	10^3
	10^5	5	5	5	15	5	10^3
	10^6	5	5	5	15	5	10^3
	10^7	5	5	5	15	5	10^3
	10^8	5	5	5	15	5	10^3
C-9	10^2	0	0	0	0	0	10^5
	10^3	0	0	0	0	0	10^5
	10^4	2	0	2	4	2	10^5
	10^5	2	0	2	4	2	10^5
	10^6	5	5	5	15	5	10^5
	10^7	5	3	4	14	5	10^5
	10^8	5	5	5	15	5	10^5
C-1	10^6	0	0	0	0	0	$>10^8$
	10^8	1	0	0	1	1	$>10^8$
C-8	10^2	1	1	1	3	1	10^3
	10^3	2	0	2	4	2	10^3
	10^4	3	1	2	6	3	10^3
	10^5	5	3	3	11	5	10^3
	10^6	5	4	4	13	5	10^3
	10^7	5	4	5	14	5	10^3
	10^8	5	4	5	14	5	10^3

(C-1, C-2, C-8, and C-9), and the circular plasmid cp9 was absent in two clones (C-1 and C-9). lp24.7 and lp40 were lost in clones C-1 and C-8, respectively. The presence of genes coding for OspD and the exported plasmid protein A, EppA, was investigated with specific probes described by Norris et al. (32) and Champion et al. (15). The plasmids lp40 and cp9 were found to contain the *ospD* and *eppA* genes, respectively (data not shown).

Infectivities of *B. burgdorferi* B31 clones. Hamsters were used to determine the ID_{50}s of the five clones of strain B31 (Table 1). An animal was considered infected if spirochetes could be cultured from either the bladder, spleen, or heart. Low-passage strain B31 had an ID_{50} of 10^3 cells. The plasmid content and infectivity in hamsters for each clone are summarized in Table 2. Clones C-3 (full complement of plasmids), C-2 (lp27.5 deletion), and C-8 (lp27.5 and lp40 deletions) each had the same ID_{50} as low-passage B31. The loss of cp9 in addition to lp27.5 in C-9 resulted in an ID_{50} of 10^5 cells, a moderate decrease in infectivity. A marked decrease in infectivity from 10^3 to $>10^8$ cells occurred in C-1. This decrease in infectivity was associated with the loss of a 24.7-kb linear plasmid (lp24.7) as well as cp9 and lp27.5. These results suggested that cp9 and especially lp24.7 might play an important role in the infectivity of *B. burgdorferi* B31. Although clones C-2, C-3, and C-8 had the same ID_{50}, i.e., 10^3 cells, the number of infected organs varied from 39 to 77% (Tables 1 and 2). These results suggested that plasmid content may influence dissemination of the borreliae in the host.

TABLE 2. Plasmid deletion and infectivity of *B. burgdorferi* B31 clones

Clone	Plasmid deletion(s)[a]	ID_{50} (no. of cells)	No. of positive organs/ total no. of organs (%)
C-3	None	10^3	41/105 (39)
C-2	lp27.5	10^3	81/105 (77)
C-9	lp27.5, cp9.0	10^5	52/105 (50)
C-1	lp27.5, cp9.0, lp24.7	$>10^8$	1/30 (3)
C-8	lp27.5, lp40	10^3	64/105 (61)

[a] lp, linear plasmid; cp, circular plasmid.

TABLE 3. Distribution of lp27.5, lp24.7, and cp8 in Lyme disease borreliae

Genospecies and strain	Infectivity[a]	Size (kb) of plasmid that hybridized with probe[b]		
		lp27.5 (probe 1/ probe 2)	lp24.7	cp9
B. burgdorferi				
B31	+	27.5/27.5	24.7	7.8
MMT1/59	+	27.9/27.9	24.4	6.9
MM1	+	29.2/29.2	32.7	6.8
NCH-1	+	28.4/28.4	24.5	Neg
LAX#7	+	28.9/28.9	24.6	Neg
297	+	28.7/28.7	24.6	Neg
10293	+	29.8/29.8	24	Neg
CT-1	+	27.9/27.9	24.9	7.6
IPS	+	28.9/28.9	24.9	Neg
ZS7	+	29.0/29.0	24.5	Neg
20001 P1	+	29.1/29.1	23.2	Neg
20004 P1	+	28.5/28.5	24.4	Neg
IRS	+	28.7/28.7	24.6	Neg
P/Ka1	−	Neg/Neg	Neg	Neg
IP1	−	Neg/Neg	Neg	Neg
IP2	−	Neg/Neg	Neg	Neg
H11	−	Neg/Neg	Neg	Neg
B31	−	Neg/Neg	Neg	Neg
297	−	Neg/Neg	24.6	Neg
IPS	−	Neg/Neg	Neg	Neg
B. garinii				
PD89	+	40.6/32.3	24.9	Neg
Fuji P1	+	Neg/Neg	24.3	Neg
Fuji P2	+	Neg/Neg	27.7	Neg
P/Bi	+	51.5/23.7	50	Neg
20047	+	43.4/Neg	32.8	Neg
IP89	+	Neg/Neg	26.6	Neg
IP553	−	Neg/Neg	26.2	Neg
Ir210	−	Neg/Neg	32.6	Neg
2226	−	Neg/Neg	26.4	Neg
2223	−	Neg/Neg	30	Neg
GerTick#3	−	ND	Neg	Neg
G25	−	Neg/Neg	32.7	Neg
VSBP	−	Neg/Neg	29.5	Neg
BITS	−	Neg/Neg	Neg	Neg
B. afzelii				
P/Gau	+	Neg/24.5	29.1	7.5
B023	+	24.0/25.0	36.8	7.5
M7	−	27.5/25.2	31.8	Neg
2246	−	44.8/26.4	35.1	Neg
IPF	−	27.7/Neg	27.8	Neg
ECM-1	−	24.0/26.1	Neg	7.5
VS461	−	24.3/Neg	27.7	7.5

[a] A strain was considered infectious (+) if hamsters could be infected with an inoculum size of 10^7 to 10^8 cells. −, noninfectious.
[b] Neg, lack of hybridization with probe; ND, not determined.

Plasmid content and infectivity of the Lyme disease borreliae. The association of plasmid content and infectivity was expanded to include additional strains of *B. burgdorferi* as well

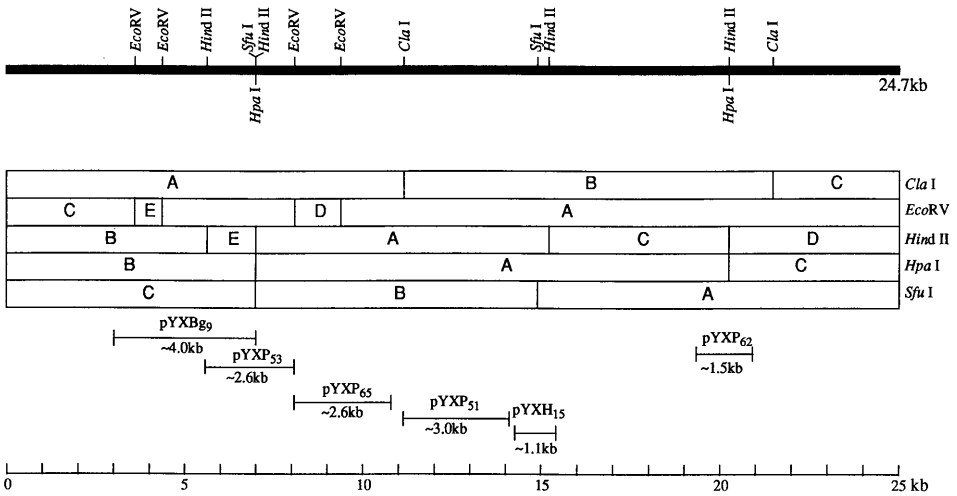

FIG. 2. Restriction map of lp24. The approximate locations of the cloned DNA fragments are shown below the map. pBg9 has a *Bgl*II fragment, and the other recombinant plasmids contain *Hin*dIII fragments of lp24.7. The numbers beneath the bars indicate DNA sizes in kilobases.

as strains of *B. garinii* and *B. afzelii*. The distribution of lp27.5, lp24.7, and cp9.0 in 21 infectious and 20 noninfectious strains of Lyme disease borreliae was investigated (Table 3). Two *Bam*HI DNA fragments from the 28.7-kb linear plasmid of low-passage *B. burgdorferi* 297 were previously shown to hybridize with linear plasmids of 27.5 to 29.8 kb that were present in infectious strains of this genospecies but not in the noninfectious strains (46) (Table 3). Since these probes hybridized with the 27.5-kb linear plasmid of low-passage strain B31, they were used to examine infectious and noninfectious strains of *B. garinii* and *B. afzelii*. In contrast to that of *B. burgdorferi* strains, the infectivity of strains of these two genospecies did not correlate with the presence of plasmids that hybridized with these probes. The DNA probe for the 9-kb circular plasmid that contains the gene for exported plasmid protein A (15) hybridized with only 8 of the 41 strains examined, and the presence of this small circular plasmid did not correlate with infectivity (Table 3). The 3.0-kb *Hin*dIII fragment pYXP51 (Fig. 2), cloned from the 24.7-kb linear plasmid of strain B31, hybridized with linear plasmids ranging in size from 23.2 to 36.8 kb. All infectious and some noninfectious strains of *B. burgdorferi, B. garinii*, and *B. afzelii* possessed a plasmid that hybridized with this probe (Table 3). These results suggested that this plasmid contained a gene or genes required for members of the three genospecies of the Lyme disease spirochetes to be infectious.

Restriction map of lp24.7 and telomere analysis. Sixteen restriction endonucleases were examined for their ability to cleave lp24.7. The restriction enzymes *Bam*HI, *Kpn*I, *Ksp*I, *Pst*I, *Pvu*II, *Sac*I, *Sal*I, and *Sph*I were unable to cleave lp24.7. *Eco*RI, *Eco*RV, *Bgl*II, *Hin*dII, *Hin*dIII, *Cla*I, *Hpa*I, and *Sfu*I did digest lp24.7. On the basis of the number and size of fragments generated, *Cla*I, *Eco*RV, *Hin*dII, *Hpa*I, and *Sfu*I were chosen to construct the physical map. The data derived from single and double enzyme digestions are summarized in Table 4. Utilizing this information, we constructed a restriction map of lp24.7 (Fig. 2). The confirmation of the proposed restriction map was accomplished with six recombinant plasmids containing DNA fragments of lp24.7 from our DNA library. The cloned DNA fragments hybridized with the appropriate DNA fragments of lp24.7 resulting from restriction endonuclease digestions, verifying that the proposed restriction map was correct (Fig. 2).

The telomeric structure of lp24.7 was investigated by alka-

TABLE 4. Restriction fragments of lp24.7

Restriction enzyme(s)[a]	No. of fragments	No. of digestion sites	Fragment size (kb)									Total size (kb)
			A	B	C	D	E	F	G	H	I	
*Eco*RI	>5	>4										
*Bgl*II	>6	>5										
*Hin*dIII	>7	>6										
*Hpa*I	3	2	13.38	6.96	4.43							24.77
*Sfu*I	3	2	9.96	7.91	6.91							24.78
*Cla*I	3	2	11.30	10.31	3.17							24.78
*Eco*RV	5	4	15.20	3.79	3.59	1.26	0.78					24.62
*Hin*dII	5	4	8.25	5.64	5.07	4.47	1.37					24.80
*Hpa*I + *Cla*I	5	4	9.01	6.91	4.35	3.17	1.30					24.74
*Hpa*I + *Sfu*I	4	4	7.91	6.91	5.51	4.40						24.73
*Cla*I + *Sfu*I	5	4	6.91	6.79	4.42	3.51	3.17					24.80
*Hpa*I + *Hin*dII	5	6	8.25	5.64	5.07	4.45	1.34					24.75
*Hpa*I + *Eco*RV	7	6	10.77	4.43	3.59	2.33	1.45	1.26	0.78			24.61
*Hin*dII + *Eco*RV	9	8	5.57	5.07	4.47	3.59	1.45	1.37	1.26	0.98	0.78	24.54

[a] lp24.7 was resistant to digestion by *Bam*HI, *Pst*I, *Pvu*I, *Sph*I, *Sal*I, *Sac*I, *Kpn*I, and *Ksp*I.

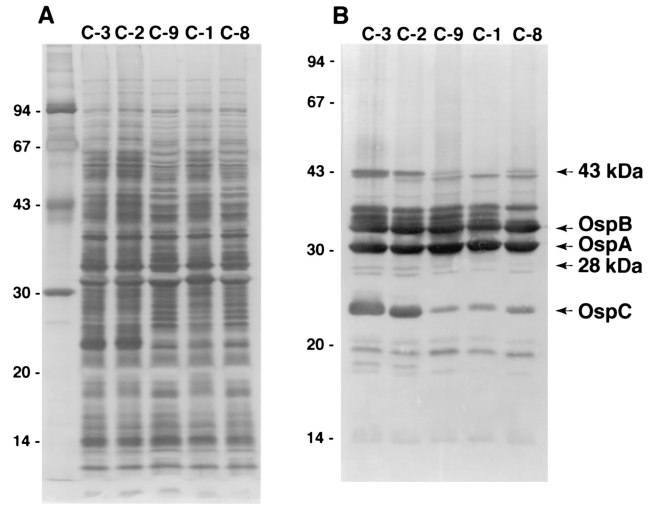

FIG. 3. (A) SDS-PAGE analysis of B31 clones. Proteins were visualized by silver stain. (B) Western immunoblot. Proteins from B31 clones were probed with hamster antisera raised against infectious clone C-3. Molecular mass markers (in kilodaltons) are shown on the left of each gel. The arrows on the right side of panel B indicate the location of OspA, -B, and -C and the 28- and 43-kDa proteins.

line agarose gel electrophoresis. The two *Sfu*I fragments examined were the 6.9-kb fragment C, corresponding to the left telomere, and the 7.9-kb fragment B, located in the middle area of the plasmid (Fig. 2). The visualization of the denatured forms of fragments B and C was accomplished by hybridization with the DNA clones pYXP53 and pYXBg9 (Fig. 2; hybridization data not shown). The migration rates of fragments B and C in the alkaline agarose gel electrophoresis were reversed. In the denatured form, fragment C migrated at a lower rate than fragment B. This retarded migration of the denatured form of fragment C suggests the presence of a hairpin loop.

Cloned telomeric sequences representing the right and left telomeres of the 16-kb linear plasmid of B31 were kindly provided by Alan Barbour (20, 21) and used as probes. Both probes hybridized with our 18-kb linear plasmid. The probe from the left telomere of the 16- to 18-kb plasmid hybridized with our 52-kb linear plasmid, which contains the *ospA* and *ospB* genes, as well as to a 28.9- to 30.3-kb linear plasmid which could be equivalent to the 25-kb linear plasmid described by Hinnebusch and coworkers (20, 21). Neither telomere probe hybridized with the telomeres of lp27.4.

Protein profiles and plasmid compositions. The effect of the plasmid profile on the protein composition of the five clones of strain B31 was examined by SDS-PAGE. As seen in Fig. 3A, the proteins present in the five clones are very similar. Major differences in the protein profiles among the five clones are difficult to discern and appear to be primarily quantitative. The protein composition of clone C-3 (full plasmid complement) and clone C-1 (lp24.7, lp27.5, and cp9 deletions) was also analyzed with a panel of 11 MAbs. The specificities of the MAbs are presented in Table 5. Proteins reactive with MAbs specific for polypeptides of the following molecular masses (in kilodaltons) were present in both of the two clones: 82 to 93, 72 (DNak), 58, 41 (fla), 39 (bmp), 34 (OspB), 31 (OspA), 24 (OspC), 22, and 19 (Table 5). The protein compositions of the five clones were further examined by Western immunoblotting using antisera from hamsters infected with cells from clone C-3 (Fig. 3B). The immunoblot revealed the absence of ~18-kDa reactive proteins in C-9 (lp27.5 and cp9.0 deletions), C-8 (lp27.5 and lp40.0 deletions), and C-1 (lp27.5, lp24.7, and cp9.0 deletions). In addition, C-1 appeared to be missing the 43- and 28-kDa proteins and C-8 appeared to be missing the 35- and 29-kDa proteins (Fig. 3B).

As described above, the loss of lp24.7 was associated with a marked decrease in infectivity. The protein profiles of C-1 and C-9, which differ from one another only by the loss of lp24.7, were compared. Two-dimensional electrophoresis was employed to achieve greater resolution of the proteins. The major difference in the protein profiles of C-1 and C-9 was the absence of a 14-kDa protein in the cells of C-1, which lack the 24.7-kb linear plasmid (Fig. 4).

DISCUSSION

We previously analyzed and compared the plasmid profiles of the genospecies *B. burgdorferi, B. garinii*, and *B. afzelii* (46). As a part of this study, the distribution of 27- to 29-kb linear plasmids was examined with two *Bam*HI DNA fragments from a 28.7-kb linear plasmid of low-passage *B. burgdorferi* 297. We found that strains of *B. burgdorferi* that possessed plasmids that hybridized with these probes were infectious for hamsters whereas those strains lacking this plasmid were noninfectious. When our study was expanded to include strains of *B. garinii* and *B. afzelii*, we were unable to demonstrate the association of infectivity with the presence of this plasmid. Similar results were obtained when we compared the plasmid profiles of the five clones of strain B31 with the infectivities of these clones. Paradoxically, the loss of the 27.5-kb linear plasmid from clones C-2 and C-8 of B31 was associated with increased dissemination of the borreliae in the hamster (Tables 1 and 2). This plasmid also appears to be easily lost since all clones of B31 which lost one or more plasmids always lost the 27.5-kb plasmid. We also analyzed the plasmid profiles of 200 clones from the 50th subculture of strain B31 and found that all the clones were lacking the 27.5-kb linear plasmid (data not shown).

Norris and coworkers (32) found that the 38-kb linear plasmid carrying the gene coding for OspD that was absent in the noninfective high-passage strain B31 was present in the low-passage, infectious B31. However, the association of the presence of the 38-kb plasmid with infectivity was not consistently observed when additional strains of *B. burgdorferi* were examined. Marconi et al. (30) conducted an extensive analysis of the distribution of the *ospD* gene among the Lyme disease borreliae. They found that the presence of this gene was not universal among these spirochetes. The gene was carried by 90, 50,

TABLE 5. MAbs reactive with proteins present in clones C-1 and C-3

MAb	MAb specificity[a]
D4	82–93
CB312	72 (DNaK)
062a	58
H9724	41 (Fla)
P39	39 (bmp)
H5TS	34 (OspB)
H5332	31 (OspA)
L22 1F8	24 (OspC)
CB625	22
P20a	22
CB49	19

[a] Values are protein sizes (in kilodaltons). Data in parentheses are protein designations. Sources of MAbs are provided in reference 17.

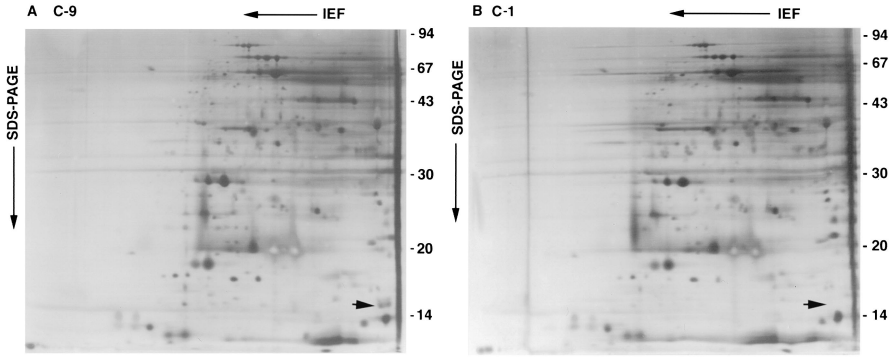

FIG. 4. 2D-PAGE of B31 clones. (A) Infectious clone C-9; (B) noninfectious clone C-1. The arrows on the gels identify the location of a protein with a molecular mass of about 14 kDa that is present in infectious C-9 and absent in noninfectious C-1. Molecular mass markers (in kilodaltons) are indicated at the right side of each gel. IEF, isoelectric focusing.

and 24% of *B. garinii, B. afzelii*, and *B. burgdorferi* strains, respectively. Our results also demonstrated that the *ospD* gene is not necessary for infectivity. Utilizing an *ospD* probe, we established that our 40-kb linear plasmid was the same as the *ospD*-containing 38-kb linear plasmid reported by Norris et al. (32). We found that our clone C-8 lacks the 27.5- and 40-kb linear plasmids but has the same ID_{50} as C-3, which possesses the full complement of plasmids (Table 2). Thus, it can be concluded from the above studies that the 38- to 40-kb linear plasmid containing the *ospD* gene does not play a significant role in the infectivity of the Lyme disease spirochetes.

Champion et al. (15) reported that a 9.0-kb circular plasmid present in low-passage B31 contained the *eppA* gene coding for exported plasmid protein A (EppA). They suggested that EppA, which is only expressed in vivo, may be a virulence factor. Our results also suggest that the 9.0-kb circular plasmid may be involved in the infectivity of strain B31. We found that in clone C-9 the loss of cp9.0, which contains *eppA* in addition to lp27.5, resulted in a decrease in ID_{50} from 10^3 to 10^5 cells. However, it appears that the *eppA* gene is not consistently associated with infectivity (Table 3). Only 31% (4 of 13) of the infectious strains of *B. burgdorferi* examined contained a plasmid that hybridized with the *eppA* probe. Also, this gene was absent from the six infectious strains of *B. garinii* analyzed. However, the two infectious strains of *B. afzelii* available for testing each contained a 7.5-kb circular plasmid that hybridized with the *eppA* probe. Additional infectious strains of *B. afzelii* need to be examined to determine the relationship of the *eppA* gene to the infectivity of this genospecies.

Carroll and Gherardini (13) investigated membrane protein variations associated with in vitro passage of *B. burgdorferi* B31. Using cross-absorbed human serum, they detected five proteins, with relative molecular masses of 78, 58, 34, 28, and 20 kDa, that can be expressed during human infection. Norris et al. (32) identified low-passage-associated proteins (35, 28, 24, and 20 kDa) that are not expressed or poorly expressed in high-passage noninfectious *B. burgdorferi* B31. We found that the loss of plasmids in *B. burgdorferi* B31 appeared to be associated with the lack of expression of proteins with relative molecular masses of 43, 35, 29, 28, 18, and 14 kDa. The absence of three of these proteins (43, 28, and 14 kDa) was associated with the loss of infectivity in clone C-1. Since the conditions for electrophoresis can vary among laboratories, it is difficult to compare the low-passage-associated proteins described above without the appropriate MAbs. For example, Norris et al. (32) identified OspD as a 28-kDa protein whereas it migrated as a 29-kDa protein in our electrophoresis.

A strong relationship between infectivity of the three genospecies of Lyme disease borreliae and the presence of a 24.7- to 36.8-kb linear plasmid was observed. All infectious strains contained a plasmid that hybridized with a probe derived from the 24.7-kb linear plasmid of *B. burgdorferi* B31 (Table 3). The ID_{50} of clone C-9 (lp27.5 and cp9.0 deletions) was 10^5 cells, whereas the ID_{50} of clone C-1 (lp27.5, cp9.0, and lp24.7 deletions) was greater than 10^8 cells. This observation suggested a strong correlation between the loss of infectivity of strain B31 and the loss of the 24.7-kb linear plasmid. The importance of the loss of the 24.7-kb linear plasmid and the decreased infectivity of C-1 is difficult to ascertain because the 9.0-kb circular plasmid is also lost. We examined the plasmid profiles of 50 clones of the 30th subculture of B31 for a clone that maintained the 9.0-kb circular plasmid but lost the 24.7-kb linear plasmid. All of the clones examined had plasmid profiles that corresponded to one of the five clones described above.

The results of this study implicate the 24.7-kb linear plasmid or an equivalent linear plasmid as playing an important role in the infectivity of the three genospecies of the Lyme disease spirochetes. All infectious strains examined contained a linear plasmid that hybridized with a probe derived from lp24.7 of low-passage B31. The successful transformation of wild-type *B. burgdorferi* B31 to a coumermycin-resistant genotype by Samuels et al. (40, 41) suggests that gene transfer techniques can be applied to study the functional role of plasmids, and this will be necessary to confirm the role of the 24.7-kb plasmid in infectivity. The 24.7-kb linear plasmid of strain B31 is being sequenced, and genetic transfer techniques will be applied to the study of this plasmid.

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