Analysis of Mechanisms Associated with Loss of Infectivity of Clonal Populations of *Borrelia burgdorferi* B31MI

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Numerous studies have provided suggestive evidence that the loss of plasmids correlates with the loss of infectivity of the Lyme disease spirochetes. In this study we have further investigated this correlation. Clonal populations were obtained from the skin of a mouse infected for 3 months with a clonal population of *Borrelia burgdorferi* B31MI. The complete plasmid compositions of these populations were determined using a combination of PCR and Southern hybridization. The infectivities of clones differing in plasmid composition were tested using the C3H-HeJ murine model for Lyme disease. While several clones were found to be noninfectious, a correlation between the loss of a specific plasmid and loss of infectivity in the clones analyzed in this report was not observed. While it is clear from recent studies that the loss of some specific plasmids results in attenuated virulence, this study demonstrates that additional mechanisms also contribute to the loss of infectivity.

Infection with pathogenic species of the *Borrelia burgdorferi* sensu lato complex can lead to Lyme disease, an infection characterized by highly variable multisystem clinical manifestations (30, 31). Early studies suggested that plasmid-encoded proteins play an important role in *Borrelia* pathogenesis (26, 28), and in recent years it has been demonstrated that they also play an important role in immune evasion (37, 43). In addition, several plasmid-borne genes have been demonstrated to be up-regulated during infection, supporting a functional role for the proteins in the mammalian environment (1, 11, 39). The ability to define a correlation between specific plasmids and infectivity has until recently been complicated by the inherent instability of the *Borrelia* plasmids and the variation in plasmid content among isolates (3, 9, 17, 18, 20, 25, 28, 29, 40, 41). Prior to the determination of the complete genome sequence and plasmid content of *B. burgdorferi*, these features made it difficult to interpret and compare much of the earlier data. Determination of the genome sequence of *B. burgdorferi* B31MI and other analyses revealed the existence of both linear and circular comigrating plasmids of between 24 and 56 kb that could not be distinguished by agarose gel electrophoresis (5, 10, 15, 35). There are also several distinct yet closely related circular plasmids (designated cp) of approximately 32 kb (cp32) (33, 35) and multiple linear plasmids (designated lp) of approximately 28 kb (lp28s). The cp32s have extended regions of homology, while the lp28s have significantly less identity. In addition to comigrating plasmids, the genome also contains an extraordinary number of plasmid-carried paralogous gene families ($n = 175$). This genetic redundancy, which could allow for functional complementation of plasmid-encoded proteins, coupled with the inherent instability of the *Borrelia* plasmids has made it difficult to pinpoint one or more specific plasmids as essential for infectivity (4, 9, 17, 28, 37, 38, 43). However, two recent independent studies by Labandeira-Rey and Skare and by Purser and Norris have provided evidence that lp25 and lp28-1 may be necessary for full virulence (16, 22). These studies clearly demonstrate that the loss of specific plasmids is one mechanism by which decreased virulence can occur. It should also be noted that in a recent study by Siebers et al. (27) it was demonstrated that clonal variations in pathogenicity arise after infection and subsequent subsurface plating of the Lyme disease spirochetes. However, Siebers et al. utilized *B. burgdorferi* ZS7, an isolate of undefined genomic composition, and did not determine the plasmid contents of the clones with different pathogenicities. Hence, the basis for the loss of infectivity in the clones analyzed was not determined.

In this report, we have continued to assess putative correlations between plasmid content and infectivity and have employed clonal populations of defined genetic composition to achieve this goal. By defining the plasmid compositions of a series of clones derived from an infectious clone of *B. burgdorferi* B31MI and then testing the infectivities of these clones in C3H-HeJ mice, we demonstrate that loss of infectivity can occur in isolates that still retain lp25 and lp28-1. While lp25 and lp28-1 appear to be important for infectivity, the data presented here demonstrate that mechanisms in addition to the loss of these plasmids can also lead to the loss of infectivity.

MATERIALS AND METHODS

Generation of postinfection isogeneic clones of *B. burgdorferi* B31MI. All analyses were conducted using the cloned isolate *B. burgdorferi* B31MI (kindly provided by Mark Hanson at MedImmune Inc., Gaithersburg, Md.), designated the parental clone (pc), and clonal populations derived from it. This isolate was selected for analysis because the sequence of its entire genome is now available (http://www.tigr.org) (15). Upon receipt of the isolate, its infectivity was con-

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TABLE 1. Probe and primer sequences and target sites

Probe or primer	Sequence (5′ to 3′)	Target site
uhb(+)	GTTGGTTAAAATTACATTTGCG	Binds within the UHB element (19) of plasmids cp32-3, cp32-9, cp32-6, cp32-5, cp32-7, cp32-1, cp32-2, and cp32-8
uhb2(+)	CTTTGAAATATTGCAATTAT	Binds within the UHB element of cp32-9, cp32-4 and cp32-3, cp32-5 and cp32-2
bbc10F	GAACTATTTATAATAAAAAGGAGAGC	Positions 6742 to 6717 of cp9
bbc10R	ATCTTCTTCAAGATATTTATTATAC	Positions 6284 to 6309 of cp9
bbb19F	AATAATTCAGGGAAAGATGGG	Positions 16960 to 16980 of cp26
bbb19R	AGGTTTTTTTGGACTTTCTGCC	Position 17511 to 17532 of cp26
lp5F	CTTGCTTTAAGCCCTATTTCAC	Positions 1921 to 1942 of lp5
lp5R	GCACACTACCCATTTTTGAATC	Positions 2543 to 2565 of lp5
bbd10F	CAAACTTATCAAATAGCTTATG	Positions 5935 to 5957 of lp17
bbd10R	ACTGCCACCAAGTAATTTAAC	Positions 6432 to 6454 of lp17
bbu21F	AGTAAAGGAGTTCTGCAAAAATT	Positions 15242 to 15264 of lp21
bbu21R	GTTGTCACCTCGTGTAATATG	Positions 15787 to 15818 of lp21
bbe16F	ATGGGTAAAATATTATTTTTTGGG	Positions 10163 to 10187 of lp25
bbe16R	AAGATTGTATTTTGGCAAAAAATTTTC	Positions 9570 to 9594 of lp25
bbf20F	ATGAACAAAAATTTTCTATTTC	Positions 10969 to 10991 of lp28-1
bbf20R	GTTGCTTTTGCAATATGAATAGG	Positions 10701 to 10723 of lp28-1
bbg02F	TCCCTAGTTCTAGTATCTACTAGACCG	Positions 1116 to 1142 of lp28-2
bbg02R	TTTTTTTTGTATGCCAATTGTATAATG	Positions 1899 to 1925 of lp28-2
bbh06F	GATGTTAGTAGATTAAATCAG	Positions 2320 to 2340 of lp28-3
bbh06R	TAATAAAGTTTGCTTAATAGC	Positions 2950 to 2970 of lp28-3
bbh08	CCTGAGGACCTTTTATGGAGAC	Positions 3657 to 3677 of lp28-3
bbi16F	AGGCCGGATTTTAATATCGA	Positions 7231 to 7253 of lp28-4
bbi16R	TTTATATTTTGACACTATAAG	Positions 8511 to 8534 of lp28-4
bbk19F	AAGTTTATGTTTATTATTGC	Positions 12625 to 12644 of lp36
bbk19R	ATTGTTAGGTTTTTCTTTTCC	Positions 13234 to 13214 of lp36
bbj34F	AAATTCTATGGAAGTGATG	Positions 26350 to 26332 of lp38
bbj34R	TTTATCTTTATTTTTAGGC	Positions 25340 to 25358 of lp38
bba16F	GCACAAAAGGTGCTGAG	Positions 10272 to 10289 of lp54
bba16R	TTTTAAAGCGTTTTTAAGC	Positions 11111 to 11093 of lp54
bbq47F	AAGATTGATGCAACTGGTAAAG	Positions 29997 to 30018 of lp56
bbq47R	CTGACTGTAACTGATGTATCC	Positions 30951 to 30971 of lp56
D645(−)	CTCCTATGATAGTTTGACCT	Targets the *erpD* gene on cp32-2
D701(−)	CCAAGTTGTACGTATAGAGC	Targets the *erpD* gene on cp32-2
K647(−)	TTTCTTTAGCTATATCGCTAG	Targets within BBM38 on cp32-6
M527(−)	AGTCGTTCTTCTCTTTCTC	Targets within BBO40 on cp32-7
bbn39F	AATAGAAAGTAGGAATGGTGCCGAAC	Positions 26644 to 26669 on cp32-9
bbn39R	GTATACGCAAATTTAGTTACACC	Positions 27848 to 27870 of cp32-9
C241(−)	TGCGAATGTATCAGAGTCTCC	Positions 241 to 261 of *erpC* on cp32-2
PG(+)	CCCACGATATCTCCCCGTAT	Targets BBS41 carried on cp32-3
BBL	TCCAGATTCTGGACTTTTTTGAA	Positions 21501 to 21523 of cp32-8
BBP	AATTATGGACGTGGGAATAAA	Positions 21557 to 21777 of cp32-1

firmed using the C3H-HeJ murine model for Lyme disease as previously described (37). In brief, 10^3 spirochetes were needle inoculated intradermally between the shoulder blades of three 6-week-old C3H-HeJ mice. After 4 weeks, 1-mm-diameter ear punch biopsy specimens were collected and placed in BSK-H complete medium (Sigma) containing antibiotics (phosphomycin, 20 μg ml^{-1}; rifampin, 50 μg ml^{-1}; amphotericin, 2.5 μg ml^{-1}; Sigma). After the infection was confirmed, it was allowed to persist for a total of 3 months. At this point, ear punch biopsy specimens were again collected and placed in medium for cultivation as described above. The spirochetes were maintained in this medium until growth became evident as determined by dark-field microscopy and were then subsurface plated (37). Colonies were evident after approximately 2 weeks, and well-isolated colonies were selected for analysis. These populations are henceforth referred to as postinfection clonal populations.

PCR analyses. PCR analyses were performed using isolated DNA or DNA released from lysed cells as a template. When lysed cells were used as a template, 1 ml of culture (~2.0×10^8 cells) was pelleted ($14,500 \times g$; 5 min), and the cells were resuspended in phosphate-buffered saline (pH 7.4) and enumerated by dark-field microscopy. The cells were pelleted, resuspended in sterile water (5×10^6 cells/μl), and boiled for 5 min; the cellular debris was removed by centrifugation, and the supernatant was transferred to a new tube for use as the template in PCR analyses.

PCR was performed using DNA from 5×10^6 cells, 300 to 400 pg of each primer pair (Table 1), Supermix (Gibco-BRL), and 0.4 U of recombinant *Taq* in a reaction volume of 20 μl. The reaction mixtures were overlaid with mineral oil, and PCR was performed as follows: 1 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 45°C, and 2 min at 72°C. A final extension step of 72°C for 6 min was performed. The amplicons were analyzed in 1% agarose gels containing 0.5 μg of ethidium bromide/ml. Positive controls included a low-passage, infectious, wild-type clonal isolate derived from B31 (MSK5) known to contain most of the known *B. burgdorferi* plasmids (16) and *B. burgdorferi* B31MIpc, for which the entire plasmid content and genome sequence are known. The absence of specific plasmids, inferred from a negative PCR result, was confirmed through Southern hybridization analyses as described below.

Southern hybridization analyses. Isolated genomic DNA from each clonal population, obtained as previously described (18), was digested with *Hae*III (standard conditions) and fractionated by electrophoresis in 0.8% agarose gels. The DNA was stained with ethidium bromide and transferred onto Hybond N membranes by vacuum blotting, using the VacuGene system (Pharmacia) and protocols provided by the manufacturer. The DNA was fixed to the membrane by UV irradiation using the Bio-Rad GS Gene Linker. Hybridization conditions, washes, and oligonucleotide probe labeling were as previously described (6). All oligonucleotide probes and primers are described in Table 1.

Analysis of the infectivity of *B. burgdorferi* clones in mice. The infectivities of *B. burgdorferi* B31MI clones were tested using the murine model for Lyme disease infection. The infective potential of *B. burgdorferi* B31MIpc has been previously established, and we have experienced a 100% success rate in the

TABLE 2. Summary of plasmid profiles and infective potentials of postinfection clonal populations of *B. burgdorferi* B31MI[a]

Infectivity and plasmid[b]	Value and presence									
	MSK5	pc	c9	c14	c17	c24	c29	c36	c37	c53
Infectivity	ND	7/7	0/6	0/6	0/6	0/6	4/4	4/4	4/4	7/7
lp5 (T)	+	+	+/−	−	+/−	+/−	−	+	−	+/−
cp9 (C)	+	+	+/−	−	+/−	+/−	−	−	−	+/−
cp26 (B)	+	+	+	+	+	+	+	+	+	+
lp17 (D)	+	+	+	+	+	+	+	+	+	+
lp21 (U)	+	+	+	+	+	+	+	+	+	+
lp25 (E)	+	+	+/−	+	+	+	+	+	+	+
lp28-1 (F)	+	+	+	+	+	+	+	+	+	+
lp28-2 (G)	+	+	+	+	+	+/−	−	−	+	+
lp28-3 (H)	+	+	−	−	−	−	−	−	−	−
lp28-4 (I)	+	+	+	+	+	+	+/−	+	+	+
lp36 (K)	+	+	+	+	+	+	+	+	+	+
lp38 (J)	+	+	+	+	+	+	+/−	+	+	+
lp54 (A)	+	+	+	+	+	+	+	+	+	+
lp56 (Q)	+	+	+	−*	+	+	+	+	+	+
cp32-1 (P)	+	+	+	+	+	+	+	+	+	+
cp32-3 (S)	+	+	+	+	+	+	+	+	+	+
cp32-4 (R)	+	+	+	+	+	+	+	+	+	+
cp32-6 (M)	+	+	−	+	+	−	+	+	+	+
cp32-7 (O)	+	+	+	+	+	+	−	+	+	+
cp32-8 (L)	+	+	+	+	+	+	+	+	+	+
cp32-9 (N)	−	+	−	+	−	−	+	−	−	+

[a] The presence or absence of each plasmid was determined by PCR. Negative PCR results were confirmed by Southern hybridization. Infectivity data are presented as the total number of culture-positive mice over the total number tested. The pluses and minuses indicate that product (of the predicted size) was or wasn't obtained, respectively; +/− implies weak amplification. The asterisks indicates that an amplification product was obtained but differed in size from that predicted by the *B. burgdorferi* B31MI genome sequence (15). ND, not determined.
[b] The letters in parentheses are designations assigned by The Institute for Genomic Research.

recovery of spirochetes from experimentally infected C3H-HeJ mice (*n* = 30) by cultivation of ear punch biopsy specimens (37). In the first round of experiments designed to test for infectivity, an inoculum of ~500 spirochetes was used. In the second round of experiments, three mice were inoculated with 10^7 spirochetes each. Ear punch biopsy specimens were collected at 4 and 12 weeks postinoculation and placed in BSK-H complete medium containing antibiotics. After 12 weeks, the mice were sacrificed and the spleens, kidneys, and blood were collected and placed individually in BSK-H complete medium.

RESULTS

Analysis of the cp32 plasmid family in pre- and postinfection clonal populations derived from *B. burgdorferi* B31MIpc. We began our assessment of a possible correlation between specific plasmids and infectivity by determining the cp32 plasmid compositions of several clonal populations derived from *B. burgdorferi* B31MIpc. We initially focused on the cp32a, since these plasmids carry several gene families suggested to contribute to pathogenicity (21, 32, 36, 37, 42). C3H-HeJ mice were infected for 3 months, and then postinfection clonal populations were collected (*n* = 100). The composition of the cp32 plasmid family was determined for several clones by using a hybridization approach. The preinfection clonal population was confirmed to harbor seven cp32s (Table 2). All oligonucleotide probes used in this report were designed based on the *B. burgdorferi* B31MI genome sequence (9). The uhb(+) oligonucleotide targets a segment of the cp32s that is referred to as the upstream homology box (UHB) element (19). This conserved element resides upstream of three different lipoprotein-encoding gene families (*ospE, ospF,* and family 163) that are carried by the cp32s (and lp56) (2, 4, 15, 19, 35, 38). Note that the designation "family 163" was devised by The Institute for Genomic Research (15). Using the known cp32 sequences for

B. burgdorferi B31MI, the sizes of *Hae*III restriction fragments derived from each plasmid that carry target sites for each probe could be deduced (Table 3), thereby allowing the determination of which cp32s were present or absent. All clones were found to carry several uhb(+)-hybridizing fragments, with 7 of 20 clones exhibiting hybridization profiles identical to that of *B. burgdorferi* B31MIpc (Fig. 1 shows representative data). cp32-4 and cp32-3 were present in all clones, while

TABLE 3. Predicted sizes of the *Hae*III restriction fragments targeted by various probes

Plasmid[b]	Probe	Hybridization target size (bp)[a]
cp32-1 (P)	uhb(+)	2,048
cp32-1 (P)	BBP	2,301
cp32-2	uhb2(+)	1,863
cp32-3 (S)	uhb(+)	7,821
cp32-4 (R)	ospF	3,978
cp32-5	uhb(+)	ND
cp32-6 (M)	uhb(+)	3,360
cp32-7 (O)	uhb(+)	2,684
cp32-8 (L)	uhb(+)	2,048
cp32-8 (L)	BBL	3,744
cp32-9 (N)	uhb(+)	3,518
lp56 (Q)	uhb(+)	869

[a] Predicted *Hae*III restriction fragment sizes were determined based on the *B. burgdorferi* B31MI genome sequence available at http://www.tigr.org. The size of the uhb(+)-targeted *Hae*III restriction fragment derived from plasmid cp32-5 could not be determined (ND), since the sequence of this plasmid is not known. The size of the fragment derived from cp32-2 was determined based on limited sequence information available in the databases for this plasmid. As indicated in the text, neither cp32-2 nor cp32-5 was found to be present in the genome of *B. burgdorferi* B31MI.
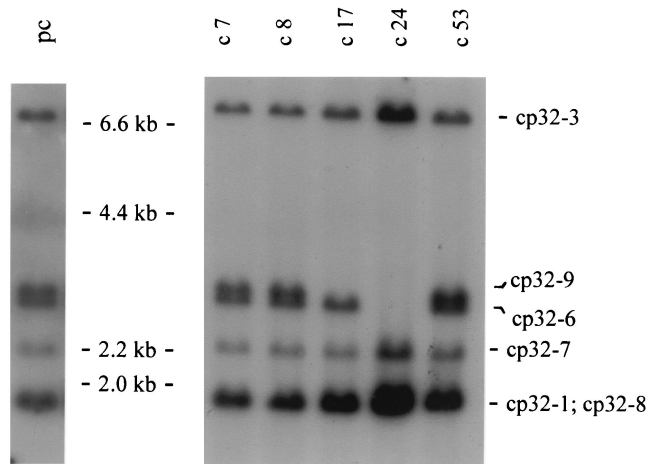[b] The letters in parentheses are designations assigned by The Institute for Genomic Research.

FIG. 1. Restriction fragment length polymorphism pattern analysis of the cp32 family of plasmids. DNA was isolated from each clone, digested with *Hae*III, fractionated by agarose gel electrophoresis, transferred onto Hybond N membrane by vacuum blotting, and hybridized with the ^{32}P-labeled uhb(+) oligonucleotide as described in the text. The clones analyzed are indicated above each lane, and the plasmid from which each hybridizing restriction fragment was derived is indicated on the right. The migration positions for the size standards are indicated between the panels. pc indicates DNA from the parental clone, B31MI.

others lacked cp32-6, cp32-9, and/or cp32-7. The loss of these plasmids was confirmed through additional hybridization analyses using oligonucleotides targeting other regions of the plasmids. For example, the loss of cp32-6 was confirmed using a probe targeting the BBM38 gene (Fig. 2). In all other cases, these additional analyses corroborated the absence of these plasmids from certain clones. In some additional cases, plasmid-specific probes were required to ascertain the presence or absence of specific plasmids. For example, both cp32-8 and cp32-1 are predicted to yield *Hae*III restriction fragments of 2,048 bp that possess binding sites for the uhb(+) probe; hence, to differentiate between these plasmids, additional probes were required. A plasmid-specific probe was required for cp32-4 as well. The BBL, BBP, and ospF-R oligonucleotides are specific for plasmids cp32-8, cp32-1, and cp32-4, respectively. Hybridization analyses revealed that these plasmids were carried by all clones. The results of the hybridization analyses are summarized in Table 2.

The linear plasmid lp56 carries an integrated cp32 (9) and is predicted to yield a uhb(+)-hybridizing *Hae*III restriction fragment of 869 bp. An appropriately sized hybridizing restriction fragment was detected in all clones analyzed (data not shown).
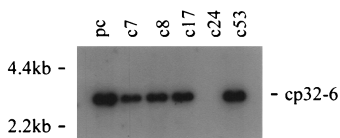

FIG. 2. Confirmation through Southern hybridization analyses of the loss of cp32-6 from clones. *Hae*III-digested DNA from each clone (indicated above each lane) was fractionated and blotted as described in the legend to Fig. 1 and then hybridized with the BBM38-targeting cp32-6 probe. pc indicates DNA from the parental clone, B31MI.

This hybridization band is not visible in Fig. 1, as it was necessary to run it off the gel in order to obtain maximal separation of other similarly sized UHB-carrying restriction fragments.

Analysis of infectivities of clonal populations derived from *B. burgdorferi* B31MIpc that differ in their cp32 plasmid compositions. To determine if the observed variation in cp32 composition influences the abilities of clonal populations to establish infection in mice, clones lacking one or more cp32s were needle inoculated into C3H-HeJ mice. Initial experiments were performed using an inoculum of 10^3 spirochetes for the parental clone and clones 17, 24, and 53. At 1 and 3 months postinoculation, ear punch biopsy specimens and blood were recovered and placed in complete BSK-H medium to allow cultivation of the spirochetes. Positive cultures were obtained for clones 53 and pc from both the 1- and 3-month time points, while all other cultures were negative. To allow for the possibility that the kinetics of growth might be slower for some clones, the cultures were allowed to persist for 2 months. However, even after this time only clones 53 and pc were culture positive. The experiment was repeated using three mice per clone, an inoculum of 10^7 spirochetes, and a broader range of clones (clones pc, 9, 14, 24, 29, 36, 37, and 53). At 1 month postinoculation, positive cultures were obtained for clones pc, 29, 36, 37, and 53 but not clones 9, 14, 17, and 24. At 3 months postinoculation, the mice were sacrificed, and ear punch biopsy specimens, blood, spleens, and kidneys were collected. Clones pc 29, 36, 37, and 53 were culture positive, whereas clones 9, 14, 17, and 24 were culture negative for all tissues tested.

Complete analysis of the plasmid compositions of infectious and noninfectious clonal populations derived from *B. burgdorferi* B31 MI. To determine if there were additional differences in the linear- and non-cp32 circular-plasmid profiles in the infectious and noninfectious clonal isolates described above, a PCR strategy was employed to test for each plasmid. *B. burgdorferi* B31MIpc and *B. burgdorferi* MSK5 served as positive controls to verify that the primers were specific. Amplification was scored as follows: −, no amplification; +/−, weak amplification; and +, strong amplification. Weak amplification may indicate that certain plasmids have been lost from the majority of the population. Upon PCR, all primer sets yielded amplicons of the expected sizes. Representative PCR results are shown in Fig. 3, and the data are summarized in Table 2. Several clones were found to lack one or more different plasmids. The plasmids found to be lost from one or more clones included lp5, lp28-2, lp28-3, cp9, cp32-6, or cp32-9. In addition, as previously reported, we did not detect plasmids cp32-2 and cp32-5 in *B. burgdorferi* B31 MIpc or (not surprisingly) in any of its clonal derivatives. Several clones were found to have lost as many as four different plasmids relative to the parental population.

To confirm the negative PCR results obtained for some plasmids, Southern hybridization analyses were performed (the hybridization analyses of the cp32s are described separately). For example, using an oligonucleotide specific for lp28-3 (BBH08) as a probe, we confirmed that the plasmid had been lost from all postinfection clonal populations (Fig. 4).
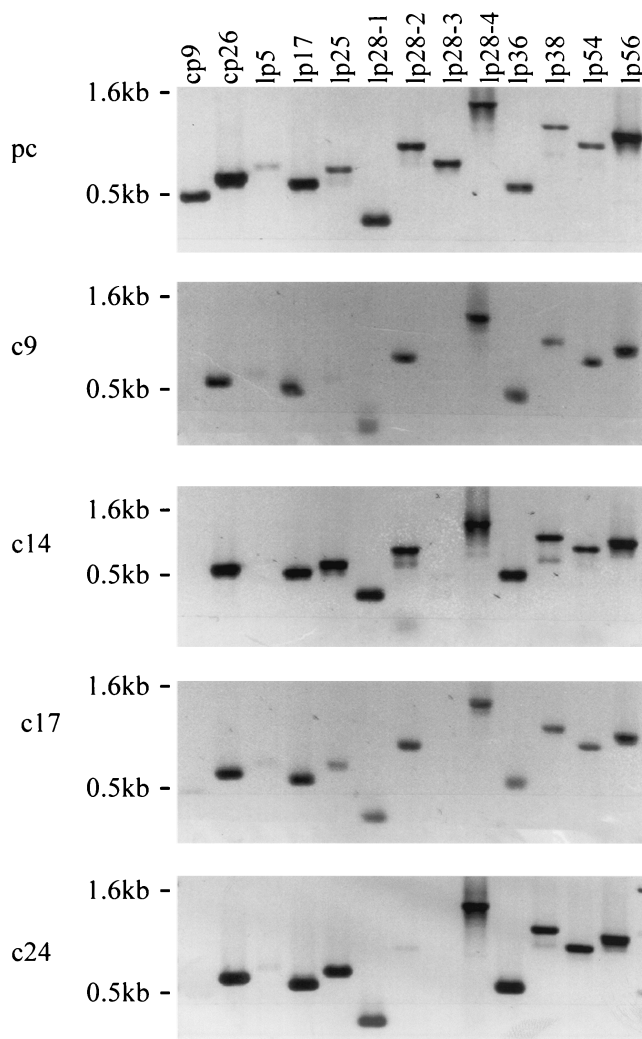
FIG. 3. Determination of the plasmid compositions of *B. burgdorferi* B31MI clones by using a PCR approach. Isolated genomic DNA served as a template for PCR analyses using plasmid-specific primer sets. Amplification products were fractionated on a 1% agarose gel and stained with ethidium bromide. The gel was then scanned to generate the figure. All methods were as described in the text. On the left, the *B. burgdorferi* B31MI clones analyzed and the migration positions of the size standards are indicated. The plasmid tested for is indicated above each lane.

DISCUSSION

Several studies have demonstrated that loss of infectivity is associated with plasmid loss in the Lyme disease spirochetes (26, 41) and that this is the primary mechanism associated with the phenomenon. One of the first studies to suggest that such a correlation exists was conducted by Schwan and colleagues (26), where the loss of cp7.6 and lp22 was concomitant with the loss of infectivity. It is unclear if *B. burgdorferi* B31MI carries homologs of these plasmids, since the sequences for these plasmids were not determined. It is likely that cp7.6 is analogous to cp9.0 (13, 15). Analysis of cp9.0 revealed that it does not appear to encode proteins with homology to proteins known to participate in virulence. In this report, we demonstrate that several clones that have lost this plasmid remain infective. A similar observation has recently been reported by Purser and Norris and by Labandeira-Rey and Skare (16, 22). However, the absence of a strict requirement for cp9.0 should not be interpreted to suggest that cp9.0-encoded proteins are not potentially important in *Borrelia* pathogenesis. Most of the open reading frames present on cp9.0 are actually members of plasmid-carried paralogous gene families (5, 15), so paralogs encoded by other plasmids might complement lost functions. The identification of a homolog of lp22, a second plasmid linked to virulence by Schwan et al. (26), in *B. burgdorferi* B31MI is complicated by the presence of numerous linear plasmids in this isolate, ranging from 17 to 30 kb. Other linear plasmids in this size range have also been implicated as necessary for virulence. The loss of lp28.7 coincides with an increase in the 50% infective dose (ID_{50}), (40, 41). This plasmid is likely a homolog of one of the four lp28s present in the *B. burgdorferi* B31MI genome (8). In general, the complete plasmid contents of the isolates analyzed in these earlier studies were largely unknown, making it difficult to interpret the data and to compare it with the data obtained from other studies that used different isolates. Hence, while a correlation between plasmids and infectivity appeared to exist, the specific plasmids associated with the phenomenon remained elusive.

In this study, we have focused our analyses on clonal populations derived from *B. burgdorferi* B31MI. The genome sequence for this isolate has recently been determined, allowing these studies to be conducted with an isolate of known genetic composition (15). Other recent studies of this topic have also exploited the genome sequence in their analyses of plasmid composition and infectivity (16, 22). The studies described here began with a particular interest in the possible role of the cp32 plasmid family in infectivity and virulence. Analyses conducted by our laboratory and others have demonstrated that several of the genes carried by these plasmids are differentially expressed during infection (1, 12, 36, 39; J. V. McDowell, S.-Y. Sung, G. Price, and R. T. Marconi, submitted for publication) and that some may play a role in immune evasion (37). An initial assessment of cp32 compositions in different B31MI-derived clones led to the identification of several with differing cp32 restriction fragment length polymorphism patterns. To determine if these variants were infective, we employed the C3H-HeJ mouse model. Initial analyses utilized low inoculating doses of ~500 spirochetes (intradermal inoculation), and infectivity was assessed by the ability to cultivate the bacteria from ear punch biopsy specimens. We found that several of the clones were not infective. One possibility was that these particular clones might simply have altered ID_{50}s. To maximize the potential establishment of infection, we used a high inoculating dose of ~10^7 spirochetes. As before, the same results were obtained for each clone. In addition, cultures of other tissues and organs from the mice that were culture negative with the ear punch biopsy specimens were also culture negative. Hence, the phenomenon that we observed was that the clones were either infectious or noninfectious. The use of two different inoculum sizes (500 and 10^7 spirochetes) that are at opposite ends of the scale but yielded the same results demonstrates that the molecular events that occurred in these clones did not simply alter the ID_{50}. It is important to note that the 10^7-spirochete inoculating dose had no discernible negative impact on any of the inoculated mice. In assessing these data,
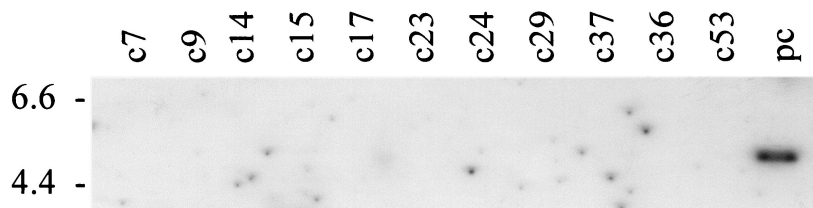
FIG. 4. Southern hybridization analysis of lp28-3. *Hae*III-digested DNA from each clone (indicated above each lane) was fractionated and blotted as described in the legend to Fig. 1. The blot was hybridized with the lp28-3 targeting probe, BBH08. Molecular standards are indicated on the left. pc, indicates DNA from the parental clone, B31MI.

a clear-cut correlation between the loss of any one specific cp32 and the loss of infectivity was not discernible. Recent studies conducted by others have reached similar conclusions (16, 22). From this, we conclude that a complete set of all seven cp32s is not required for infectivity, maintenance of infection, or in vitro cultivation. The apparent lack of a requirement for a complete set of cp32s is consistent with the extensive variation in composition that has been demonstrated for this plasmid family in a wide range of isolates (19, 38). However, in view of the extensive genetic redundancy among the cp32s, it is perhaps not surprising that the loss of a subset of these genetic elements would have miminal impact on infectivity, virulence, or the ability to grow in vitro, since the proteins encoded by the multiple cp32s may complement each other. It should be noted that lp56 also contains an integrated cp32 and could serve to complement functions lost through the loss of cp32s (8). Interestingly, all Lyme disease isolates analyzed, as well as relapsing fever isolates, carry at least some cp32s (6, 7, 19, 23, 24, 34, 38). This observation suggests that maintenance of at least a subset of these plasmids is a necessary biological feature at the genus-wide level. It is important to note that the clones analyzed in this report were only briefly cultivated postinfection, suggesting that the changes in cp32 plasmids occurred during infection. Alternatively, the plasmids could have been lost during the postinfection cultivation period. Several observations argue against this. We have found that the cp32s of *Borrelia garinii* Pbi were retained after 311 in vitro passages (~4 years of continuous cultivation) (R. T. Marconi, unpublished data). Furthermore, to our knowledge, the loss of cp32s specifically during in vitro cultivation has not been reported. Hence, the cp32s may actually be less stable during infection than they are during in vitro cultivation. Studies by Eggers and Samuels suggest that the cp32s may actually encode prophage (14). It is possible that the mammalian environment stimulates bacteriophage transduction or plasmid transfer during infection, leading to changes in cp32 composition.

To determine if there might be a correlation between the loss of other plasmids and infectivity, we determined the complete plasmid compositions of several clones that were found in the analyses above to be either infectious or noninfectious. lp28-3 was the only plasmid absent from all noninfectious clones. lp28 is one of four similarly sized linear plasmids carried by *B. burgdorferi* B31MI (15). The specific absence of this plasmid was confirmed by both PCR and Southern hybridization. While we initially interpreted these data to suggest a correlation between lp28-3 and infectivity, additional analyses of other infectious clones revealed that some of them also

lacked lp28-3. Analysis of clones lacking this plasmid revealed that they were infective even when an inoculum of ~500 spirochetes was used. Hence, it is evident that lp28-3 is not strictly required for infectivity and that its loss does not affect the ID_{50}. Two of the lp28-3-minus infectious clones also lacked lp28-2. In a previous analysis, it was demonstrated that the loss of an ~28-kb linear plasmid correlated with an increase in ID_{50} from 10^3 to 10^5 in the hamster model (41). However, in that study it was not specifically determined which of the four lp28s was lost. In an analysis of linear plasmids, Palmer et al. demonstrated that lp28-1, lp28-3, lp28-4, lp36, and lp54 were carried by all 15 Lyme disease spirochete isolates analyzed. They interpreted this to mean that these plasmids are biologically necessary (20). However, the data presented here demonstrate that the loss of lp28-3 and lp28-2 does not abrogate the ability of B31MI clones to establish and maintain infection. Purser and Norris also reported that lp28-2 (and lp28-4) is not required for infectivity (22). In that analysis, the authors did not test clones lacking lp28-3, since all of the clones in their collection carried the plasmid. It is possible that the decrease in ID_{50} reported by Xu et al. resulted from the loss of lp28-1 (41).

Evidence has recently been presented which demonstrates that the loss of lp25 and lp28-1 is coincident with a low-infectivity phenotype (16, 22). However, none of the noninfectious clones analyzed as part of our study had lost either of these plasmids. We do not believe this to represent a discrepancy between these reports and the data presented here. Specific plasmids may very well play critical roles in infectivity or virulence, and the data presented by others suggest that lp25 and lp28-1 are in this category. What is striking is that in the three recent reports that have assessed the possible link between specific plasmids and infectivity, the specific plasmids lost by the clonal populations differed widely. We found that most of the clones analyzed in this report had lost lp28-3, while this plasmid was universally present in the clones analyzed by Purser and Norris and Labandeira-Rey and Skare. A second notable difference was in the cp32s. Purser and Norris found that all of the cp32s, with the exception of cp32-3, which was lost from only a single clone (*B. burgdorferi* 5A3), were retained (note that Labandeira and Skare did not determine the complete composition of the cp32 plasmid family in all clones analyzed). In contrast, we found that six of the eight clones derived from *B. burgdorferi* B31MI pc had lost one or more cp32s. The data presented here suggest that undefined mechanisms independent of the loss of a specific plasmid can also lead to the loss of infectivity. In addition, it is also evident from the variation noted in this and other recent studies that the

dynamics of plasmid loss can vary widely under different experimental conditions (16, 22).

The central point of the analyses presented here is that the loss of infectivity does not in all cases correlate with the loss of a specific plasmid. However, this is not to say that such a correlation does not exist. It remains possible that there is an essential plasmid or combination of plasmids that are required for infectivity and virulence. In view of the genetic redundancy among the plasmids and thus the potential for complementation of function, it would seem most likely that this putative essential plasmid would be one of those that carries genes not harbored by other elements of the genome so that complementation could not occur. Perhaps a possible biological role for the redundancy in the Lyme disease spirochete genome is to shield the bacteria from the potentially adverse consequences associated with the inherent instability of its plasmid-based genome. Studies by others suggest that lp25 and lp28-1 are possible essential plasmids (16, 22). Nonetheless, even though certain plasmids may prove essential, the data presented here demonstrate that other mechanisms independent of plasmid loss appear to also be at play. These may include small-scale mutations or rearrangements in essential genes and/or differences in gene expression patterns among clonal populations. The analyses presented here provide further insight into the fluid nature of the *Borrelia* genome and the mechanisms associated with loss of infectivity. Furthermore, this study illustrates the complexity of assessing the contribution of the plasmid component of the *Borrelia* genome to the biology and pathogenesis of the Lyme disease spirochetes.

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