

## Distribution of Twelve Linear Extrachromosomal DNAs in Natural Isolates of Lyme Disease Spirochetes

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**We have analyzed a panel of independent North American isolates of the Lyme disease agent spirochete, *Borrelia burgdorferi* (sensu stricto), for the presence of linear plasmids with sequence similarities to the 12 linear plasmids present in the *B. burgdorferi* type strain, isolate B31. The frequency of similarities to probes from each of the 12 B31 plasmids varied from 13 to 100% in the strain panel examined, and these similarities usually reside on plasmids similar in size to the cognate B31 plasmid. Sequences similar to 5 of the 12 B31 plasmids were found in all of the isolates examined, and >66% of the panel members hybridized to probes from 4 other plasmids. Sequences similar to most of the *B. burgdorferi* B31 plasmid-derived DNA probes used were also found on linear plasmids in the related Eurasian Lyme agents *Borrelia garinii* and *Borrelia afzelii*; however, some of these plasmids had uniform but substantially different sizes from their *B. burgdorferi* counterparts.**

The spirochetes that cause Lyme disease, members of the *Borrelia burgdorferi* (sensu lato) group of species, are known to harbor numerous extrachromosomal DNA elements. For ease of discussion we will refer to these elements as plasmids, although some may be present in all natural isolates, and some may carry essential genes, so they should perhaps more correctly be called “mini-chromosomes” (2). All natural isolates examined carry multiple linear plasmids in the 5 to 110 kbp size range and multiple circular plasmids in the 9 to 70 kbp range. Different isolates have similar but nonidentical linear plasmid band patterns in electrophoresis gels (e.g., those seen in references 3, 4, 5, and 33). Circular plasmid contents are more difficult to display, but in the isolates that have been analyzed, multiple, related plasmid types are always present (e.g., those seen in references 9, 22, 27, 31, and 36). A number of studies have shown that plasmid loss correlates with loss of infectivity in mice (15, 25, 34), so it is of interest to understand whether these plasmids have uniform structures in the wild and to understand the distribution of these plasmids among natural isolates.

Only one *Borrelia* isolate, *B. burgdorferi* B31, has been the subject of a comprehensive study that unequivocally identified all of its plasmids. The analyzed culture of this strain, B31 MI, carries 12 linear and 9 circular plasmids, and the nucleotide sequence of each is known (8, 12). Over 90% of the genes on the characterized Lyme agent plasmids have no known homologs outside of the *Borrelia* genus (8), and a number of these genes encode outer surface proteins that are antigenic during infection of mammals (10, 13, 17, 21, 23, 29, 32, 35). We report here an analysis of plasmids that are related to the 12 known linear B31 plasmids in a panel of Lyme disease borreliae.

### MATERIALS AND METHODS

The *B. burgdorferi* strains used were previously described (7); *Borrelia garinii* and *Borrelia afzelii* strains and sources are listed (see Table 4). Contour-clamped homogeneous electric field (CHEF) electrophoresis and Southern analysis (28) were performed as previously described (6, 9). Southern probes were prepared

with [<sup>32</sup>P]dCTP (Amersham) and Pharmacia ReadyToGo random priming kits. The DNA templates for random priming were either whole *Escherichia coli* plasmid DNA clones (cloned DNA fragments in plasmid pUC18 [12]) or DNA inserts from such plasmids amplified by PCR using opposing primers outside of the DNA insertion site. DNA transfer, hybridization, and wash conditions were

TABLE 1. DNA probes used in this study

<i>Borrelia</i> plasmid source	DNA probe <sup>a</sup>	Location <sup>b</sup>	ORFs in probe <sup>b</sup>
lp5	CB63 <sup>c</sup>	3413–5195	T05–T06
lp17	CL47	63–2418	D01–D04
	D11 <sup>d</sup>	6949–7679	D11
lp21	CZ32	6788–8697	63-bp repeat region
	DF29 <sup>c</sup>	16772–18682	U10–U12
lp25	DE21	13151–14265	E20–E21
	CG18	19568–21587	E29
lp28-1	DD60	5149–6980	F11–F13
	EN57 <sup>c</sup>	24797–26510	<i>vsE</i> cassettes
lp28-2	DK44	1–442	G01
	EI58	6700–8688	G09–G10
lp28-3	CT53 <sup>c</sup>	3659–4392	H08–H09
	QO68	12620–14584	H18–H20
lp28-4	EE50	2526–3627	I06–I07
	CV69	4726–6439	I11–I14
	EL95	17047–19017	I27–I29
lp36	DK60	2334–4214	K02–K04
	FI78	7569–9698	K12–K15
	CQ33 <sup>c</sup>	12657–15072	K19–K22
	GI88	26201–26513	K41
	EH86	31975–33745	K49–K50
lp38	BA19 <sup>c</sup>	22–1483	J001
	DH08	3958–6033	J07–J08
	CQ63	13730–16123	J20–J22
	DH46	21666–23409	J28–J30
	FA84	26057–28222	J34–J36
	CT79 <sup>c</sup>	35768–37561	J48–J50
lp54	CM64	427–2449	A01–A04
	A24/25 <sup>d</sup>	~15800–17300	A24–A25 ( <i>dbpAB</i> )
lp56	BL05	3022–3666	Q05–Q06
	EK58	42201–44134	Q67–Q69

<sup>a</sup> DNA clone numbers from genome sequencing project (8, 12).

<sup>b</sup> Base pair location and open reading frame (ORF) nomenclature are as in Fraser et al. (12) and Casjens et al. (8).

<sup>c</sup> Showed some weak hybridization to other plasmids when probing B31 MI DNA.

<sup>d</sup> Uncloned PCR product made with whole *B. burgdorferi* B31 MI DNA as template.

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as previously described (6), except that membranes were washed with two final 15-min posthybridization washes of  $0.1 \times$  SSC–0.1% sodium dodecyl sulfate ( $1 \times$  SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 54°C for high-stringency analysis of *B. burgdorferi* (*sensu stricto*) strains; for other *Borrelia* species the washes were done at 24°C to maximize hybridization there. All membranes were only probed once.

## RESULTS AND DISCUSSION

**B31-like linear plasmids in North American *B. burgdorferi* (*sensu stricto*) isolates.** We first screened linear plasmid DNA clones from the genome sequencing project (8, 12) to identify ones whose insert DNAs hybridized uniquely or nearly so to only one of the strain B31 linear plasmids when used as probes in Southern analyses; substantially more than half of the plasmids tested were found to be unsuitable. Table 1 indicates where each of the chosen 31 DNA probes lies on the linear plasmids. These DNAs were then used as probes in Southern analyses (28) of CHEF electrophoresis gels of whole cellular DNAs from a panel of 15 geographically diverse North American isolates of *B. burgdorferi* (*sensu stricto*) (data not shown). Table 2 presents the results of probing this panel with the above probes, and Table 3 summarizes the findings (the B31 linear plasmids are named according to their approximate DNA content in kilobase pairs [8, 12]).

These data allow a number of conclusions to be drawn. (i) Of the 465 probe-strain combinations tested, 322 (69%) showed clear high-stringency hybridization to apparently linear plasmids in members of the strain panel. Only 16 probe-strain combinations (3.4%) showed clear hybridization to the chromosome as well as to a plasmid, and two reacted only with the chromosome. (ii) When their hybridization targets are present, probes from strain B31 linear plasmids nearly always hybridize with linear plasmids of similar, but often not identical, size in other isolates; only 47 of the 322 reactive probe-strain combinations (15%) hybridized only to a linear plasmid not within 15% of the size of the cognate B31 plasmid. (iii) In most cases (for example, see lp38), all probes from a given B31 MI plasmid hybridize to the same plasmid (identically sized DNAs) within individual reactive panel members. It appears that ongoing exchange of DNA sequences among linear plasmids of different size is not extremely rapid, although it does occur. (iv) In 47 of 322 reactive probe-strain combinations (15%), multiple linear plasmids clearly hybridized to the probe, indicating the presence of probe-like sequences on two or more linear plasmids in those isolates. This suggests that the paralogies on plasmids in these strains are at least partly different from those in the B31 plasmids (8). (v) B31 plasmid lp36 is unusual in that probes derived from it often hybridize to targets of substantially different size from the B31 MI 36-kbp plasmid. Although similarity to at least one of the five B31 lp36 probes used is present in all of the 15 isolates, in 10 of the 15 isolates tested they are present on linear plasmids in the 23 to 30 kbp size range, and five isolates carry them on a 35- to 38-kbp plasmid. (vi) Plasmids apparently cognate to a number of the B31 MI linear plasmids are present in all or nearly all members of our strain panel. Five of the B31 plasmids, lp28-1, lp28-3, lp28-4, lp36, and lp54, appear to have similarly sized counterparts in all 15 members of the panel (the lp36 counterparts are more variable in size than the other four [above]); lp25 and lp28-2 have counterparts in 14 of the 15 isolates; and lp17 and lp38 have counterparts in 10 of the isolates. Plasmids cognate to the lp5, lp21, and lp56 probes are less common, being present in 3, 2, and 3 of the 15 isolates, respectively. Our definition of cognate or corresponding plasmid simply means a plasmid that hybridizes to the B31 plasmid probe; it does not imply that the plasmids must have completely similar structures.

The presence of corresponding plasmids in most natural isolates tested strongly suggests that they are important in the natural life of *B. burgdorferi*. Our methods could underestimate the plasmids present for the following reasons: some linear

TABLE 2. Similar sequences among linear plasmids of independent isolates of *B. burgdorferi*<sup>a</sup>

Strain (origen)	Hybridization of strain B31 probes to independently isolated strains																														
	lp5	lp17	lp21	lp25	lp28-1	lp28-2	lp28-3	lp28-4	lp36	lp38	lp54	lp56																			
19678 (NY)	—	—	—	(24)	(30)	30, (27)	—	—	—	—	—	—																			
21645 (WI)	—	(17)	—	(24)	—	(25)	29	29	29	29	—	—																			
21721 (MI)	—	—	—	(24)	26	(21, 37)	30	30	30	30	—	—																			
27579 (CT)	(15)	—	—	30	—	21	28	28	28	28	—	—																			
27982 (PA)	—	—	—	(24)	26	(25, 29)	29	29	29	29	—	—																			
27985 (CT)	—	17, (22)	17, 22	—	24	(24, 54)	28	28	28	28	—	—																			
28534 (MD)	—	—	—	—	—	23, (28)	26	26	26	26	—	—																			
29592 (CT)	—	(17)	—	—	—	(26, 29)	27	27	27	27	—	—																			
29805 (CT)	—	—	—	—	—	30, (36)	29	29	29	29	—	—																			
30757 (CT)	—	—	—	—	—	(29)	29	29	29	29	—	—																			
CA-3-87 (CA)	—	(17)	—	—	—	28, (45, 54)	29	29	29	29	—	—																			
WI91-23 (WI)	5	—	—	—	—	33, (27)	27	27	27	27	—	—																			
N40 (NY)	(5)	—	—	—	—	29	29	29	29	29	—	—																			
HB19 (CT)	—	—	—	—	—	26, (37)	24, 27	27, 29	28, (54)	27	—	—																			
B31 (NY)	5, (20)	17	17	20	20	24, (54)	28	28	28, (36, 54)	29	29	29																			

<sup>a</sup> The United States state from which each of the strains was isolated is indicated in parentheses next to the strain name. Probes were picked from *E. coli* pUC18 plasmid clones used in the genome sequencing project (6, 12). Values in table are sizes in kilobase pairs for plasmids that hybridize to the indicated DNA probes (accurate to about  $\pm 0.5$ ,  $\pm 1.5$ , and  $\pm 3$  kbp for 5 to 10, 10 to 30, and 30 to 60 kbp ranges, respectively). —, no hybridization was seen. Values in parentheses indicate relatively weak hybridization.

TABLE 3. Summary of B31 counterpart linear plasmids present in natural isolates of *B. burgdorferi*<sup>a</sup>

Strain (origin)	Hybridization of DNA probes to <i>B. burgdorferi</i> isolates											
	lp5	lp17	lp21	lp25	lp28-1	lp28-2	lp28-3	lp28-4	lp36	lp38	lp54	lp56
19678 (NY)	-	--	--	++	++	++	++	+++	+--+	++++	--	--
21645 (WI)	-	-+	--	-+	-+	++	++	+++	--+	-----	++	--
21721 (WI)	-	--	--	-+	+⊕	-+	++	+++	--+	++++	++	--
27579 (CT)	-	+	--	-+	-+	++	++	+++	-+	++++	++	--
27982 (PA)	-	++	--	++	++	++	++	+++	-+	-----	--	--
27985 (CT)	-	--	--	++	++	++	++	+++	+---	++++	++	++
28534 (MD)	-	-+	--	--	++	++	++	+++	-+	-----	++	--
29592 (CT)	-	++	--	++	++	++	++	+++	⊕+	++++	++	--
29805 (CT)	-	⊕+	--	++	-+	++	++	+++	-+	++++	++	--
30757 (CT)	-	-+	++	++	++	++	++	+++	+---	-----	++	++
CA-3-87 (CA)	-	--	--	+⊕	-+	--	++	-+	-+	-----	++	--
WI91-23 (WI)	+	++	--	++	-+	++	++	+++	-+	++++	-+	--
N40 (NY)	+	++	--	++	⊕+	++	-+	+++	⊕+	++++	++	--
HB19 (CT)	-	--	--	++	++	++	++	+++	-+	++++	++	--
B31 MI (NY)	+	++	++	++	++	++	++	+++	++++	++++	++	++

<sup>a</sup> Strain names are followed by the name of the United States state (in parentheses) in which they were isolated. Each of the remaining columns represents a different DNA probe from Table 1, going from left to right across each plasmid map (orientation as in references 8 and 12). -, no hybridization; +, hybridization occurred with a plasmid similar in size (<15% different) to the B31 plasmid from which the probe was derived (hybridization to other plasmids may also have occurred); + and ⊕, hybridization to *only* a plasmid(s) >15% different in size from the B31 plasmid from which the probe was derived; ⊕, hybridization in that strain *only* to a plasmid(s) of a size >10% different from the plasmid(s) with which other probes from that B31 plasmid react.

plasmids tend to be lost quite rapidly with passage in culture (15, 25, 34), our rather stringent hybridization conditions would cause us to miss divergent but orthologous sequences, and a probe's target could be missing from an otherwise cognate plasmid. We attempted to minimize the first problem by using low-passage cultures; 14 of the 15 cultures used had been passaged fewer than 12 times since isolation. In only a few of the tested strains did ethidium bromide-stained electrophoresis gels show a clear linear plasmid band that did not hybridize to the probes used; these were ~5-kbp apparently linear plasmids in isolates 21579, 21721, 28534, and CA-3-82, which did not react with the B31 lp5 probe. DNAs that were <7 kbp were not analyzed with the other probes, so it is not known if these plasmids are related to other probes used in this study. The above data do not imply that no additional types of linear plasmids are present in *B. burgdorferi*, but it does suggest that there may not be a large number of other, as yet unknown, common linear plasmid types.

#### Linear plasmids in non-burgdorferi Lyme agent spirochetes.

We also analyzed a geographically diverse panel of 12 *B. garinii* and 8 *B. afzelii* isolates with a subset of the probes listed in Table 1. The results of these experiments are given in Table 4. A majority (73%) of the *B. garinii* and *B. afzelii* probe-strain combinations showed some sequence similarity to B31 on apparently linear plasmids. In some cases, the plasmids may be quite similar in the three species. For example, B31 plasmid lp54 clone CM64 hybridizes to 53- to 56-kbp plasmids in all three species. This agrees with the observations that the *ospAB* and *P27* genes have been found on a plasmid of this size in members of these species that have been analyzed (5, 18, 23, 24). The hybridization target of lp17 probe CL47 is universally present on 21- to 28-kbp plasmids in *B. garinii* and *B. afzelii*, but is present in less than half of the *B. burgdorferi* isolates we examined. In other cases, the sizes of the plasmids harboring the hybridization targets in the other species are either variable (e.g., lp28-4 and lp28-2) or are systematically very different from *B. burgdorferi*. For example, the lp28-3 target is present on a 54-kbp plasmid in all of the *B. garinii* and *B. afzelii* isolates tested, the lp36 target is usually present on a 21- to 23-kbp plasmid in *B. garinii* but is rarely present in *B. afzelii*, and the lp38 probe DH46 target is usually present on a 22-kbp plasmid

in *B. garinii* and a 25-kbp plasmid in *B. afzelii*. A large fraction of B31 plasmid sequences have similar sequences on plasmids in these other species; however, there are substantial differences in plasmid structure among the three species.

Plasmid lp56 appears to have formed by the relatively recent integration of a member of the 32-kbp circular plasmid (cp32) family into a 20- to 25-kbp linear plasmid (8). The probe from the non-cp32-like portion of lp56 hybridized only to ~55-kbp linear plasmids in our panel of *B. burgdorferi* isolates, suggesting that the putative linear progenitor of lp56 is not common in this species. However, most *B. garinii* and *B. afzelii* isolates carry ~20- and ~25-kbp linear plasmids, respectively, that do hybridize with the EK58 probe. It thus appears possible that the linear progenitor of *B. burgdorferi* lp56 (without the integrated cp32) could be one of these more common 20- or 25-kbp *B. garinii* or *B. afzelii* plasmids.

**Conclusions.** This is the first study to systematically analyze the linear plasmid contents of *Borrelia* isolates from the perspective gained from knowledge of the complete plasmid content of *B. burgdorferi* B31 MI. We find that at least one of the sequences tested from 5 of the 12 B31 linear plasmids are present in all 15 of the *B. burgdorferi* (sensu stricto) isolates examined, and at least one of the sequences from two additional plasmids was present in 14 of 15 isolates. Two B31 linear plasmids had relatives in 10 of the 15 isolates, and only three plasmids appear to have cognates in fewer than 25% of the isolates examined. Previous, single-probe studies on B31 lp17, lp28-1, lp25, lp38, and lp54 generally agree with the above conclusions (1, 3, 5, 14, 16, 19–21, 24, 33). Circular plasmids similar to B31 cp9, cp26, and multiple cp32s have also been found to be present in nearly all isolates that have been carefully examined (9, 10, 11, 15, 22, 26, 27, 30, 31). In summary, the B31 linear plasmid sequences are usually present in other *B. burgdorferi* isolates, and when present they are highly likely to be located on a plasmid of similar size. Thus, there appears to be a substantial uniformity of linear plasmid sequence content among various independent *B. burgdorferi* isolates. Most probes from the B31 linear plasmids also hybridized with linear plasmids from *B. garinii* and *B. afzelii*, but in a number of cases they have substantially different sizes from the cognate B31

TABLE 4. *B. garinii* and *B. afzelii* linear plasmid hybridization targets<sup>a</sup>

Strain [passage no.] (location)	Source	Results of hybridization with strain B31 plasmid probes									
		lp17 (CL47)	lp25 (DE21)	lp28-1 (DD60)	lp28-2 (EI58)	lp28-3 (CQ68)	lp28-4 (CV69)	lp36 (CQ33)	lp38 (DH46)	lp54 (CM64)	lp56 (EK58)
<i>B. garinii</i>											
I53 [11] (FR)	R. Marconi	22, (34)	(37)	—	34, 48	35, (54)	48	—	22	54	34
FujiP2 (JA)	R. Johnson	23, (28)	25	—	28	(54)	29, (23)	22	30	54, (20)	23, 31
G1 (GE)	T. Schwan	21, (30)	(30)	—	—	(54)	—	(21)	(22)	(54)	(20)
G25 [8] (SW)	R. Marconi	21, (30)	30	—	—	(54)	—	21	(22)	54	20
IP89 (RU)	R. Johnson	22	(30)	—	29	ND	(29)	22	30	56	31
IP90 (RU)	T. Schwan	23, (35, 37, 42)	(36.5)	—	42	35, 54	34	23	23	54	—
IR210 (RU)	I. Schwartz	22, 30, (38)	(30, 38)	—	—	(54)	36	21	36, 38, (22)	54	20, 37
Las (AU)	I. Schwartz	22, (35)	—	—	—	(54)	—	21	(22)	54	20
NBS23a (SW)	I. Schwartz	23	(38)	—	—	(54)	(40)	—	22	53	—
NBS23b (SW)	I. Schwartz	23	—	—	—	54	(32)	—	22	(53)	—
PBi (GE)	T. Schwan	22, 31	30	—	—	54	(25)	21	(22)	54	20
VSBP [10] (ST)	R. Marconi	(21, 30)	27.5	—	—	(54)	36.5	(21)	36, (22)	55	(20)
<i>B. afzelii</i>											
AO1 (NL)	R. Marconi	26	56	—	—	(54)	ND	—	—	(56)	—
EMC1 (SW)	R. Marconi	25, (34)	—	—	26, 29	(54)	34	—	(25)	54, (24)	(25)
HT10 (JA)	R. Marconi	23, (34)	30	—	25	(54)	34, (25)	—	(25)	54, (26)	(25)
IP21 (RU)	R. Marconi	25, (28)	—	—	28	(54)	32, (25)	—	(25)	53, (28)	(25)
PGau (GE)	T. Schwan	25	27	—	29	(54)	(34)	—	(25)	54, (24)	—
PKo [5] (GE)	R. Marconi	26	27	—	27	(54)	(34)	—	(26)	54, (24)	(32)
UM01 (SW)	R. Marconi	25, (30)	27	—	30	(54)	(25)	—	(25.5)	54, (24, 30)	(25)
UO1 (SW)	R. Marconi	23, (34, 37, 41)	24 (37)	—	42	35, 54	34	23.5	24	54	—

<sup>a</sup> All cultures were low passage, exact passage number is given where it is known, and country where the strain was isolated is given in parentheses (AU, Austria; FR, France; GE, Germany; JA, Japan; NL, The Netherlands; RU, Russia; SW, Sweden; ST, Switzerland). Values are sizes (in kilobase pairs) of linear plasmids that hybridized to the probes (listed in Table 1) given at the top of each column; values in parentheses indicate less intense hybridization. ND, not determined; —, no hybridization.

plasmid. Such systematic differences suggest that there may not be free exchange of these plasmids between species.

How similar are the overall structures of corresponding plasmids of similar size in different isolates? In general, we do not yet know the answer to this question; however, the lp54 plasmids from the four natural Lyme agent isolates examined have similar gene orders and restriction maps (18; R. van Vugt and S. Casjens, unpublished observations), and the circular cp9's and cp26's have been shown to each have similar structures in different isolates (11, 12, 31). We have found that multiple probes from individual B31 linear plasmids nearly always hybridize to the same plasmid in other isolates, suggesting that other plasmids may also have generally conserved genetic structures. Curiously, in spite of this evidence of uniformity, examination of linear plasmid sequences has shown considerable evidence of recent, rather massive genetic instability on the strain B31 linear plasmids in the form of many past duplicative rearrangements (8). In addition, observations made here that unique B31 linear plasmid-derived probes sometimes hybridize to multiple plasmids or to plasmids of different sizes in other strains supports the idea that the genetic information on these plasmids is not completely constant (unlike the *B. burgdorferi* chromosome, which appears to be very stable with the exception of the extreme right few kilobase pairs [5, 8]). How can the apparent overall uniformity in plasmid size observed here exist in the face of evidence for apparently frequent plasmid rearrangement events? A more detailed analysis of plasmids present in other strains will be required to resolve this paradox.

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