Correlation between plasmid content and infectivity in *Borrelia burgdorferi*

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Infectivity-associated plasmids were identified in *Borrelia burgdorferi* B31 by using PCR to detect each of the plasmids in a panel of 19 clonal isolates. The clones exhibited high-, low-, and intermediate-infectivity phenotypes based on their frequency of isolation from needle-inoculated C3H/HeN mice. Presence or absence of 21 of the 22 plasmids was determined in each of the clones by using PCR primers specific for regions unique to each plasmid, as identified in the recently available genome sequence. Southern blot hybridization results were used to confirm the PCR results in some cases. Plasmid lp25 exhibited a direct correlation with infectivity in that it was consistently present in all clones of high or intermediate infectivity and was absent in all low-infectivity clones. lp28–1, containing the *vmp*-like sequence locus, also correlated with infectivity; all clones that lacked lp28–1 but contained lp25 had an intermediate infectivity phenotype, in which infection was primarily restricted to the joints. Plasmids cp9, cp32–3, lp21, lp28–2, lp28–4, and lp56 apparently are not required for infection in this model, because clones lacking these plasmids exhibited a high-infectivity phenotype. Plasmids cp26, cp32–1, cp32–2 and/or cp32–7, cp32–4, cp32–6, cp32–8, cp32–9, lp17, lp28–3, lp36, lp38, and lp54 were consistently present in all clones examined. On the basis of these results, lp25 and lp28–1 appear to encode virulence factors important in the pathogenesis of *B. burgdorferi* B31.

Borrelia burgdorferi strain B31 (*Bb*), one of a group of closely related spirochetes that cause Lyme disease (1), has a genome consisting of a linear chromosome of 910,724 base pairs (2) and 21 linear and circular plasmids containing over 610,694 base pairs (plus unsequenced telomere regions) (3). The chromosome contains 853 predicted genes, of which 500 (59%) have predicted functions based on amino acid sequence similarity with orthologous gene products (2). The 535 putative gene products encoded by the *B. burgdorferi* plasmids are predominantly of unknown function and include many paralogous gene families (2, 3). Comparison of the plasmid profiles of Lyme disease spirochetes, including *B. burgdorferi*, *Borrelia afzelii*, and *Borrelia garinii*, demonstrates that a high degree of heterogeneity and plasticity exists in terms of plasmid content (4).

Considerable evidence indicates that *Bb* plasmids are important in pathogenesis. *In vitro* passage of *Bb* is associated with loss of plasmids (5). Plasmid loss after 10–17 passages is also coupled with decreased infectivity in mice and changes in spirochetal protein expression (6–9). High- and low-infectivity phenotypes of *Bb* coexist in an uncloned population, but the proportion of high-infectivity phenotypes decreases with serial *in vitro* passage (10). Plasmid profiles vary among certain *Bb* sensu lato strains (including *Bb* sensu stricto, *B. garinii,* and *B. afzelii*) as well as within high- and low-passaged cultures; this variance is associated with infectivity of *Bb* in mice (5, 9–13).

Before the availability of the *Bb* genome sequence, methods such as pulsed-field agarose gel electrophoresis (PFGE), electron microscopy, two-dimensional agarose gel electrophoresis, and Southern blotting were used to identify *Borrelia* plasmids associated with infectivity in mammals. Schwan *et al.* (9) discovered a correlation between infectivity in white-footed mice and presence of the 7.6-kb circular and 22-kb linear plasmids by using agarose gel electrophoresis and electron microscopy to identify the plasmids. Xu *et al.* (12) found a relationship between infectivity in hamsters and presence of the 24.7- and 28.1-kb linear plasmids by using PFGE and two-dimensional agarose gel electrophoresis for plasmid detection. The infectivity of 10 *Bb* B31 clonal isolates and an equivalent number of Sh2–82 clones correlated with the presence of lp28–1 by using PFGE and Southern blotting (13). In this study, the *Bb* B31 genome sequence information in combination with PCR and Southern blot hybridization provided a comprehensive assessment of plasmid content and its correlation with infectivity in a panel of 19 *Bb* B31-derived clonal isolates.

Materials and Methods

Bacteria. Infectious *Bb* B31 were originally obtained from Alan Barbour (University of California at Irvine College of Medicine) and had undergone three *in vitro* passages. Clones were isolated by the subsurface agarose plating method described previously and thereby had undergone a total of five *in vitro* passages (10, 14). The B31 clones (B31–5A1 through B31–5A19) were maintained as frozen stocks, as described previously (10), and were subsequently subjected to a maximum of two additional *in vitro* passages in Barbour/Stoenner/Kelly (BSK) II medium (15) during this study.

Infectivity of *Bb* Clones. Infectivity for each *Bb* B31 clonal isolate was determined as previously described (10). Two weeks after s.c. injection with 10^5 spirochetes, animals were killed, and bladder, heart, joint, and ear samples were aseptically transferred to 6 ml of BSK II medium. After 2 and 4 weeks of incubation, cultures were examined for the presence or absence of spirochetes by using dark-field microscopy.

Identification of Unique Regions and Design of PCR Primers. The nucleotide sequences for each of the plasmids were accessed on The Institute for Genomic Research (TIGR) website (http://www.tigr.org/tdb/CMR/gbb/htmls/Span.html). In most cases, the complete nucleotide sequence for each plasmid was tested for homology with other *Bb* entries by using Basic Local Alignment Search Tool (BLASTN) (16) at the National Center for Biotechnology Information. Regions with little or no sequence identity with other *Bb* genome sites were designated unique regions and were targeted for design of PCR primers by using the program PRIMER3 (17) (http://www-genome.wi.mit.edu/genome_software/other/primer3.html). Amplicons of varying sizes were created so that several plasmid sequences could

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Abbreviations: *Borrelia burgdorferi* B31, *Bb*; PFGE, pulsed-field agarose gel electrophoresis.

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Table 1. Infectivity phenotypes of 19 *Bb* B31 clonal isolates

Bb B31 clone	No. of cultures positive/total no.					No. of mice positive/ total no.
	Bladder	Heart	Joint	Ear	All sites	
High-infectivity phenotype						
5A1	3/3	3/3	3/3	3/3	12/12	3/3
5A3	3/3	3/3	3/3	3/3	12/12	3/3
5A4	3/3	3/3	3/3	3/3	12/12	3/3
5A6	3/3	3/3	3/3	3/3	12/12	3/3
5A9	3/3	3/3	3/3	3/3	12/12	3/3
5A11	3/3	3/3	3/3	3/3	12/12	3/3
5A15	3/3	3/3	3/3	3/3	12/12	3/3
5A16	3/3	3/3	3/3	3/3	12/12	3/3
5A18	3/3	3/3	3/3	3/3	12/12	3/3
5A19	3/3	3/3	3/3	3/3	12/12	3/3
Intermediate-infectivity phenotype						
5A2	0/3	0/3	2/3	0/3	2/12	2/3
5A8	0/3	0/3	3/3	1/3	4/12	3/3
Low-infectivity phenotype						
5A5	0/3	0/3	0/3	0/3	0/12	0/3
5A7	0/3	0/3	0/3	0/3	0/12	0/3
5A10	0/3	0/3	0/3	0/3	0/12	0/3
5A12	0/3	0/3	0/3	0/3	0/12	0/3
5A13	0/3	0/3	0/3	0/3	0/12	0/3
5A14	0/3	0/3	0/3	0/3	0/12	0/3
5A17	0/3	0/3	0/3	0/3	0/12	0/3
Negative control						
—	0/3	0/3	0/3	0/3	0/12	0/3

potentially be amplified in one PCR reaction. The cp32–1 PCR primers, originally named ORFD-1 and ORFD-2, were designed by Casjens *et al*. (18). cp32–2 and/or cp32–7 were detected by using primers originally named E-311 and E-328, respectively, designed by Stevenson *et al*. (19); because both plasmids have identical *orfC-orf3* loci, the primers could not differentiate between cp32–2 and cp32–7. Cp32–6 (CP6–1 and CP6–2), cp32–8 (CP8–1 and CP8–2), and cp32–9 (CP9–1 and CP9–2) were detected by using previously reported oligonucleotides (19). All other primers were designed in this study. Two unique PCR target sites were identified in the plasmids lp25 and lp28–1, whereas single sites were determined for the other plasmids. Thus far, we have been unable to obtain plasmid-specific amplification for lp5; it therefore was not included in this study. Primer sequences were analyzed for potential false priming on the Institute for Genomic Research website (http://www.tigr.org/tdb/CMR/gbb/htmls/SeqSearch.html) by using GRASTA, a modified form of the FASTA program (20). The oligonucleotide primers selected for this study are published as supplemental data on the PNAS web site, www.pnas.org.

PCR Detection of Plasmids. PCR (total volume = 50 μl) was performed by using the Amresco Tbr polymerase kit according to the manufacturer's conditions (Euclid, OH). Two microliters of frozen stock cultures (in Barbour/Stoenner/Kelly II medium plus 15% glycerol) for each clone was used as template rather than purified plasmid DNA. PCR primers (Sigma–Genosys, The Woodlands, TX, and Integrated DNA Technologies, Coralville, IA) were used at a final concentration of 1.25 μM. Reaction solutions were overlaid with 20 μl of ChillOut wax (MJ Research, Cambridge, MA) and performed in an MJ Research Minicycler. An annealing temperature of 55°C and 35 cycles were used for most reactions. In cases where the T_m of either primer was below 55°C, the annealing temperature was adjusted to the lowest primer T_m minus 2°C. Ten-microliter samples of each reaction were electrophoresed in 1.5% agarose gels and visualized by ethidium bromide staining (21).

Southern Blotting. Purified plasmid DNA was isolated from 100-ml logarithmic phase cultures of *Bb* B31 clones by using Qiagen (Chatsworth, CA) Plasmid Midi kits, following a modified Qiagen protocol for isolation of *Borrelia* plasmid DNA. Approximately 200 ng of *Bb* B31 plasmid DNA for each of the 19 clones was electrophoresed in 0.6%, 12 × 14-cm SeaKem gold agarose gels (FMC) run overnight in 1× TBE buffer (21) for approximately 788 direct-current volt hours. The DNA was transferred to HyBond N+ nylon membranes (Amersham Pharmacia Biotech) under neutral conditions (21), denatured, and crosslinked to the membranes by using UV light. Probes from primary and secondary sites on plasmids lp25 and lp28–1 (see supplemental data) were amplified by using PCR as described above and purified by using the Wizard PCR Prep kit (Promega). The purified denatured DNA was labeled by using the Amersham Gene Images Random Prime Labeling and Detection System (Amersham Pharmacia Biotech). Southern blot hybridization and detection were performed according to the manufacturer's instructions.

Statistical Analysis. A 2 × 2 contingency table and χ^2 goodness-of-fit test were used to test the null hypothesis, "Infectivity is independent of presence of the plasmid." To eliminate bias that can occur in χ^2 goodness-of-fit testing when expected frequencies are less than 6 (the mean frequency for this data set was 4.75), the log-likelihood ratio was applied to the contingency table data and the G statistic calculated (22).

Results

Infectivity Phenotypes of 19 *Bb* B31 Clones. A test of infectivity was used for *Bb* B31 clones 5A1 through 5A19, in which 10^5 spirochetes were injected s.c. into groups of three 6-week-old female C3H/HeN mice. This high dose of organisms was used to permit clear differentiation of infectivity phenotypes (23). Three infectivity phenotypes were identified among the clones (Table 1). In the high-infectivity phenotype, all tissue samples tested were culture positive; in the low-infectivity clones, all tissue

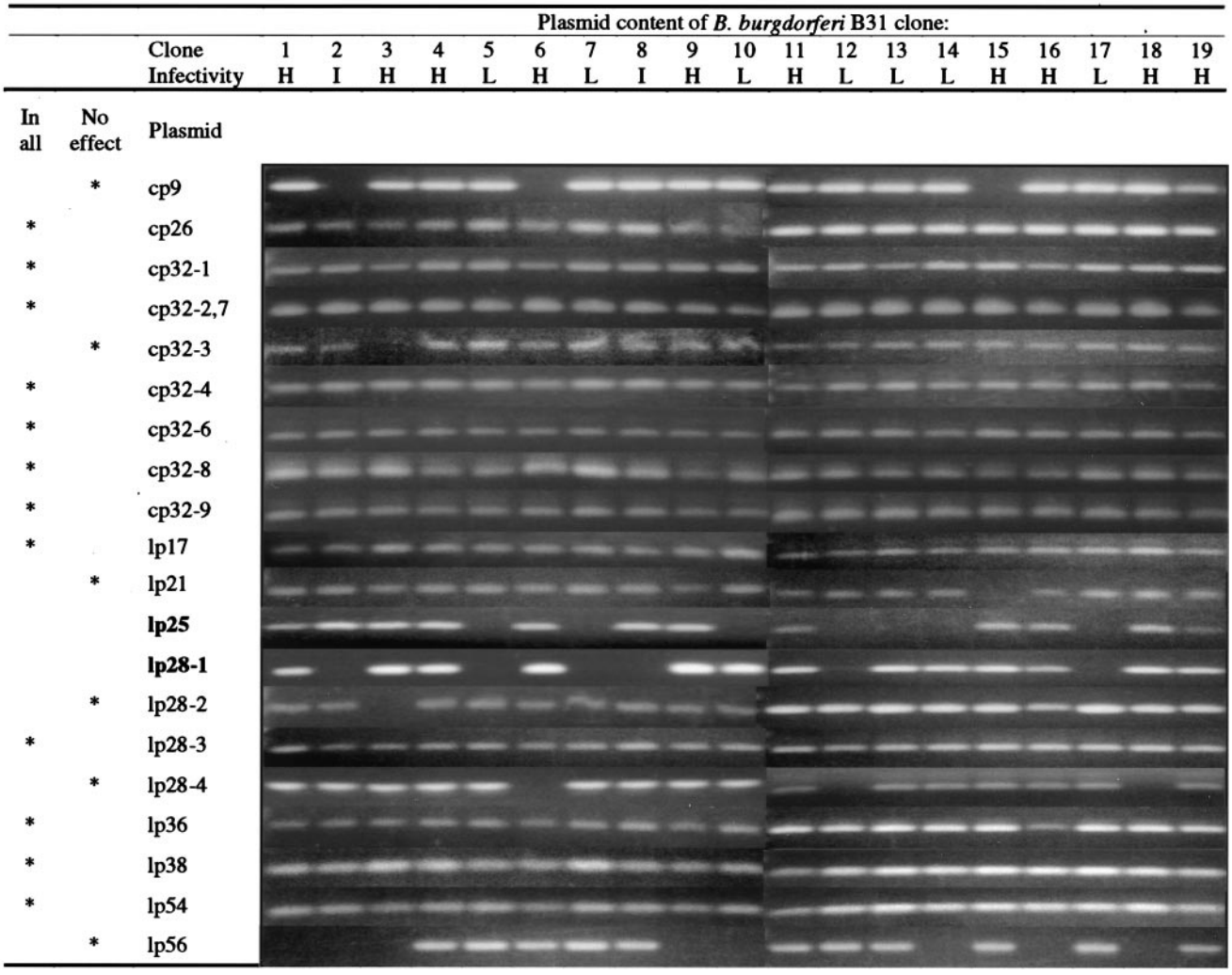

Fig. 1. Detection of plasmid-specific regions in 19 *B*. B31 clones by PCR. PCRs were performed under standard conditions, with annealing temperatures at 2°C below the lowest primer T_m (see supplemental data). Ten-microliter samples of each reaction were electrophoresed in 1.5% agarose gels and visualized by ethidium bromide staining (21). H, I, L, high, intermediate, and low infectivity, respectively. In all, present in all 19 *Bb* B31 clones. No effect, PCR detection of plasmid had no apparent correlation with infectivity in mice under the conditions used in this study.

samples tested were culture negative. Clones 5A2 and 5A8 were primarily culture negative, except for cultures of tibiotarsal joints, which were predominantly culture positive. These were designated as intermediate-infectivity clones. In other studies, it was determined that the intermediate-infectivity phenotype of B31 5A2 was retained after serial infection of mice; therefore this phenotype was not the result of the outgrowth of high-infectivity subpopulations within a low-infectivity background (J.-R. Zhang and S.J.N., unpublished data).

Plasmid Content of 19 *Bb* B31 Clones. Plasmid content of each of the 19 *Bb* B31 clones was determined before mouse inoculation. PCR primers were selected for specific detection of each plasmid in the Institute for Genomic Research *Bb* genome sequence database. Primer sequences, amplicon coordinates, and T_m information are included in the supplemental data. PCR amplification resulted in clear differentiation of B31 clones that contained or lacked the corresponding plasmids, as exemplified by the amplicons shown in Fig. 1. The interpretation of these results is presented in Table 2. On the basis of the PCR data, plasmid lp25 was present in all high- and intermediate-infectivity clones but was absent in all low-infectivity clones tested. PCR positivity for plasmid lp28–1 was absent in all of the intermediate-infectivity clones and in some of the low-infectivity clones but was never absent in a high-infectivity clone. In addition, all clones that lacked lp28–1 but contained lp25 expressed an intermediate-infectivity phenotype, in which infection was primarily restricted to the joints. Plasmids cp9, cp32–3, lp21, lp28–2, lp28–4, and lp56 were detected in some of the 19 clones; however, each was absent in at least one high-infectivity clone, indicating they are not required for infectivity in this model. Plasmids cp26, cp32–1, cp32–2,7 (see Table 2), cp32–4, cp32–6, cp32–8, cp32–9, lp17, lp28–3, lp36, lp38, and lp54 were present in all clones examined. Therefore an association between these plasmids and infectivity could not be determined in this study.

cp32–2 and cp32–7 are closely related, and the primer set used in this study for detection of these plasmids (19) could not differentiate between them. Amplification was consistently observed in each of the 19 B31 clones when this primer set was used, indicating that either one or both of these plasmids was consistently present. Sequences for cp32–5 were unavailable at the time of this study. Primers useful for the specific amplification of lp5 were not identified.

Table 2. Correlation of plasmid content with infectivity in *Bb* B31

	Plasmid content*																			
	Infectivity associated		Present in all clones												Not infectivity associated					
Clone†	lp25	lp28-1	cp26	cp32-1	cp32-2,7	cp32-4	cp32-6	cp32-8	cp32-9	lp17	lp28-3	lp36	lp38	lp54	cp9	cp32-3	lp21	lp28-2	lp28-4	lp56
High-infectivity phenotype																				
5A1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−
5A3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	−	+	−
5A4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5A6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	−	+
5A9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−
5A11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5A15	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	−	+	+	+
5A16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−
5A18	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−
5A19	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Intermediate-infectivity phenotype																				
5A2	+	−	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	−
5A8	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Low-infectivity phenotype																				
5A5	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5A7	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5A10	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−
5A12	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+
5A13	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5A14	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−
5A17	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

*As determined by PCR amplification (Fig. 1) and confirmed by Southern hybridization for lp25 and lp28-1 (Fig. 2).
†Infectivity phenotype determined by needle inoculation of C3H/HeN mice (Table 1).

The possibility existed that some of the negative PCR results were because of deletions or recombinations in the amplified region. To test this possibility, a second set of PCR primers was designed for amplification of lp25 and lp28–1 at sites distant from the initial amplification site (see supplemental data). In each of the 19 clones, the results obtained with the primary and secondary PCR reactions were identical (data not shown), supporting the interpretation that PCR positivity or negativity corresponded to the presence or absence of the targeted plasmid.

Southern Blot Hybridization. Southern blot analysis of plasmid DNA from *Bb* B31 clones 5A1 through 5A19 were performed by using probes of PCR-amplified primary and secondary unique regions from plasmids lp25 and lp28–1. Fig. 2 demonstrates that the Southern blot results for each plasmid agreed with the PCR results.

Statistical Analysis. A 2×2 contingency table was used to test the null hypothesis, ''Infectivity is independent of presence of

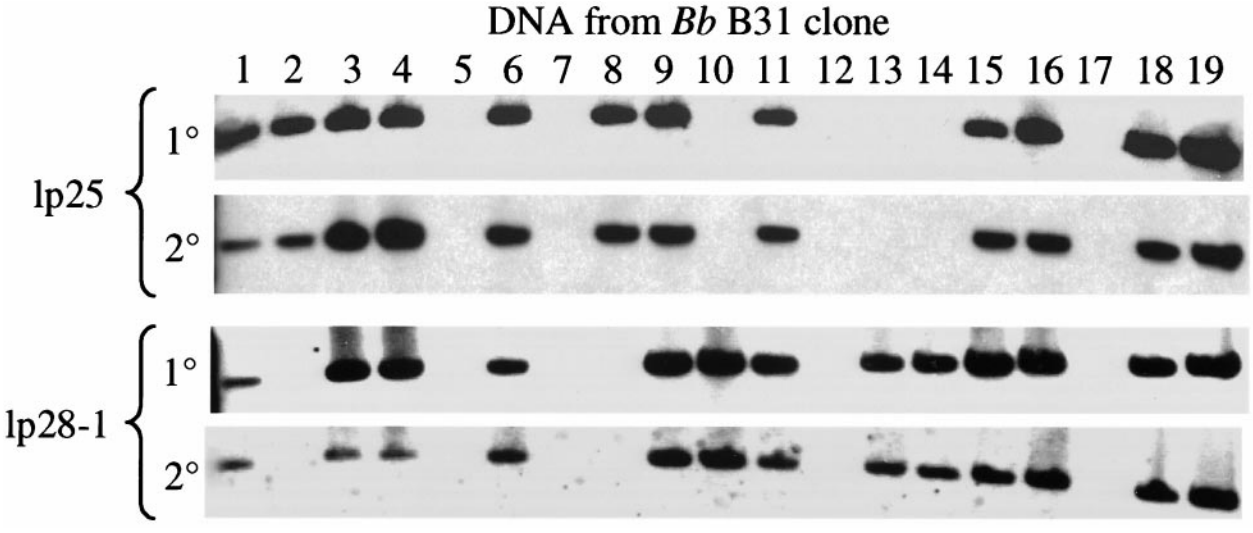

Fig. 2. Confirmation of lp25 and lp28–1 plasmid content in 19 *B. burgdorferi* B31 clones by Southern blot analysis. PCR-amplified probes from two different regions of each plasmid (marked 1° and 2°) were labeled and hybridized to Southern blots of agarose gel-electrophoresed plasmid preparations from each of the 19 clones. The positions of the hybridizing bands corresponded precisely with lp25 and lp28–1, respectively (data not shown).

lp25." The calculated χ^2 of 14.95 was greater than $\chi_2^{0.05,1}$ of 3.841, so the null hypothesis was rejected ($P < 0.001$). Bias can occur in χ^2 goodness-of-fit testing when expected frequencies are less than 6. Because the mean frequency for this data set was 4.75, the logarithm/likelihood ratio was applied to the contingency table data and G statistic calculated as a second test of goodness of fit. The calculated G of 25.043 was greater than $\chi_2^{0.05,1}$of 3.841. Again, the null hypothesis was rejected, $P < 0.001$ (22). Similar results were obtained by using the χ^2 test for lp28–1 ($\chi^2 = 70.4$, $\chi_2^{0.05,1} = 3.841$, $P < 0.001$). However, the association of lp28–1 with infectivity was not statistically significant by using the G-statistic analysis ($0.10 < P < 0.05$) (22).

Discussion

In this study, we took advantage of the natural occurrence of genetic change (plasmid loss) in combination with the recently available genome sequence data to determine the association between individual plasmids and infectivity. Examination of *Bb* B31 clones isolated after 5 *in vitro* passages revealed that only 3 of 19 clones exhibited a full complement of the 21 plasmids examined. The PCR and Southern blot data indicated that plasmid lp25 is required for infectivity in mice. lp28–1 was also associated with infectivity; the two clones that lacked lp28–1 but contained lp25 expressed an intermediate-infectivity phenotype. Plasmids cp9, cp32–3, lp21, lp28–2, lp28–4, and lp56 were absent in high-infectivity clones; they are therefore not required for infectivity in this model system. Plasmids cp26, cp32–1, cp32–2 and/or cp32–7, cp32–4, cp32–6, cp32–8, cp32–9, lp17, lp28–3, lp36, lp38, and lp54 were present in all 19 clones, so it is not known whether they are essential for infectivity.

The *Bb* B31 clones analyzed in this study exhibited high-, intermediate-, and low-infectivity phenotypes (Table 1). The intermediate phenotype was not recognized in previous studies (23) because of the small number of mice used. In the intermediate-infectivity clones 5A2 and 5A8, tissue samples were primarily culture negative except for tibiotarsal joints, which were predominantly culture positive. Needle inoculation of mice was used in this analysis; it is possible that plasmid-content variants could exhibit different phenotypes (both in terms of survival in ticks and infection of the mammalian host) during tick transmission to mice and other animals.

Palmer *et al.* (4) recently used the *Bb* B31 genome sequence information to demonstrate the distribution of homologous plasmid sequences in different borrelial isolates by using hybridization with plasmid-specific probes. Our study utilized the genome sequence to analyze the relationship between infectivity and plasmid content. BLASTN searches of entire plasmids against the *Bb* B31 sequence database were performed to identify unique regions and to design PCR primers specific for each plasmid (see supplemental data). However, one PCR-negative result does not show definitively that a clone lacks a particular plasmid; an alternative possibility is that the plasmid is still present, but that recombination has either deleted or modified the targeted region. Conversely, a plasmid could be truncated or otherwise modified while still preserving the region amplified by using a particular set of PCR primers. The amplicons were not sequenced, so base substitutions could also be present either within or outside the amplified regions.

To determine the validity of our initial PCR results, the presence or absence of the plasmids lp25 and lp28–1 was determined by using a second distant PCR site. In addition, Southern blot analysis was performed by using the primary and secondary amplicons as probes (Fig. 2). There was an exact correspondence between the initial PCR findings, the PCR results by using a second amplification site, and the Southern blot hybridizations by using either the primary or secondary site amplicons. In addition, the band migration of lp25 and lp28–1 as detected by hybridization did not vary among the clones containing these plasmids, indicating the lack of detectable recombination in the regions outside the amplified segments. Taken together, these results indicate that PCR was a reliable indicator of plasmid presence or loss in this study. Definitive identification of plasmids or genes required for infectivity will require additional studies, including recovery of full infectivity on complementation with the candidate plasmids or genes.

The results strongly suggest that lp25 is associated with infectivity in mice and provide independent confirmation of the previous findings of Xu *et al.* (12). In their studies, contour-clamped homogeneous electric field PFGE and two-dimensional agarose gel electrophoresis followed by ethidium bromide staining were used to determine plasmid profiles of infectious low-passage *Bb* B31 clones. Their *Bb* B31 clone C-2 lacked only a 27.5-kb linear plasmid; no change in the ID_{50} in hamsters was observed. Clone C-9 lacked both the 27.5 linear plasmid and a 9.0-kb circular plasmid; there was a moderate increase in ID_{50} from 10^3 to 10^5 cells. However clone C-1, which lacked 24.7-kb and 27.5-kb linear plasmids and the 9.0-kb circular plasmid, had an increased ID_{50} of $>10^8$ cells. The detailed restriction map of the 24.7-kb linear plasmid provided by Xu *et al.* (12) corresponds precisely with that of the lp25 sequence (2).

lp25 contains 31 predicted ORFs, many of which are either small (<200 bp) or closely related to paralogous sequences in other plasmids. BBE22 lacks paralogs in *Bb* but has sequence similarity to genes encoding nicotinamidases and pyrazinamidases, including *pncA*, which confers pyrazinamide resistance in *Mycobacterium tuberculosis* (24). Its function in *Bb* is currently unknown. The putative gene product of BBE31 is similar to the differentially expressed protein p35 (25, 26) and has a strong signal peptidase I recognition site, suggesting that it is a lipoprotein. The predicted BBE09 gene product is similar to previously identified *Bb* strain 297 protein p23 (GenBank accession no. AAA22961). BBE18, BBE19, BBE20, and BBE21 correspond to highly conserved paralogous plasmid gene families 49, 32, 50, and 57, respectively, and are putative replication and partition genes (3). The large predicted product of BBE02 (1,277 amino acids) has extensive protein homology with BBH09 of lp28–3; portions also share homology with multiple genes on lp36. The predicted genes of lp25 need to be examined more thoroughly to determine which are transcriptionally and translationally active and encode possible virulence factors.

Plasmid lp28–1 is associated with reduced infectivity; every *Bb* B31 clone that lacked lp28–1 was either low or intermediate in infectivity. The two intermediate-infectivity clones that lacked lp28–1 but contained lp25 could be isolated from the tibiotarsal joints of most mice but only rarely from other sites. The joints may represent a preferred site for colonization and survival, where the relatively small numbers of the surviving attenuated borrelia can be detected. Alternatively, lack of lp28–1 may reduce the ability of *Bb* to colonize organs other than the joints. lp28–1 contains the *vmp*-like sequence (*vls)* locus; in this system, antigenic variation results from gene conversion events whereby segments of 15 silent cassettes recombine into the central cassette region of the expressed *vlsE* gene (13, 27, 28). The decreased infectivity observed with the absence of lp28–1 may be related to the loss of the *vls* system, although other genes present on the plasmid could account for this effect. Xu and Johnson (11) noted that the loss of a 27.5-kb linear plasmid from clones C-2 and C-8 of B31 was associated with decreased dissemination of the borreliae in hamsters. They also found that the plasmid appeared to be easily lost. In our study, lp28–1 was absent in 6 of the 19 clones (Fig. 1, Table 2). There is currently insufficient information to determine whether the 27.5-kb linear plasmid described by Xu and Johnson is the same as lp28–1.

Plasmid-localized genes encoding virulence factors have been identified in several bacterial pathogens. These include toxin genes (29–37), capsule synthesis operons (38–43), adhesins

(44–51), and invasion factors (51–63). Pathogenesis of *Bb* in humans is poorly understood. Several genes potentially important during host adaptation, dissemination, infection, and persistence of *Bb* are plasmid localized. As *Bb* enters the mammalian host, expression of the temperature-regulated cp26-localized outer surface protein C gene (*ospC*) increases (64). *Bb* binds plasminogen and plasminogen-activator urokinase via OspA, which generates bioactive plasmin at the spirochetal surface, which in turn may aid in degradation of extracellular matrix components and dissemination (50, 51, 54, 65–68). However, expression of OspA is down-regulated during the process of tick feeding and *Bb* transmission (64, 69). Because purine levels in mammalian blood are low, two cp26-localized purine biosynthesis genes, *guaB* and *guaA,* could enable *Bb* to adapt to the mammalian environment (70). As *Bb* migrate to the connective tissues, gene products of lp54-localized *dbpA* and *dbpB* may confer binding to decorin and mediate the adherence of *Bb* to collagen fibers in skin and other tissues (46, 71). Persistent treatment-resistant Lyme arthritis may be related to host immune reactivity to OspA (72). Chronic infection and immune evasion may involve VlsE, a surface-localized lipoprotein that undergoes antigenic variation (13, 23, 28). An increased understanding of the above putative virulence factors and identification of additional virulence-associated genes are necessary to elucidate the mechanisms of pathogenesis of Lyme disease borrelia.

Identifying infectivity-associated plasmids is a first step toward identifying new virulence factors in *Bb* and understanding the molecular basis of pathogenicity of this organism. The availability of sequence information of the complete genome (2, 3) and recent improvements in the methods for genetic manipulation (73, 74) should facilitate the characterization of potential virulence factors and their contribution to the disease process.

Note Added in Proof. M. Labandeira-Rey and J. T. Skare (75) have also demonstrated a correlation between decreased infectivity and loss of lp25 and lp28-1, whereas a study by R. T. Marconi (personal communication) indicates that genetic changes other than plasmid loss may contribute to loss of infectivity.

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