JOURNAL OF BACTERIOLOGY, Aug. 1992, p. 5251–5257
0021-9193/92/165251-07$02.00/0

Vol. 174, No. 16

# Linear- and Circular-Plasmid Copy Numbers in *Borrelia burgdorferi*

J. HINNEBUSCH† AND A. G. BARBOUR*

*Departments of Microbiology and Medicine, University of Texas Health Science Center, San Antonio, Texas 78284-7758*

Received 19 March 1992/Accepted 10 June 1992

***Borrelia burgdorferi*, the Lyme disease agent, and other members of the spirochetal genus *Borrelia* have double-stranded linear plasmids in addition to supercoiled circular plasmids. The copy number relative to the chromosome was determined for 49- and 16-kb linear plasmids and a 27-kb circular plasmid of the type strain, B31, of *B. burgdorferi*. All three plasmids were present in low copy number, about one per chromosome equivalent, as determined by relative hybridizations of replicon-specific DNA probes. The low copy number of *Borrelia* plasmids suggests that initiation of DNA replication and partitioning are carefully controlled during the cell division cycle. The copy numbers of these three plasmids of strain B31 were unchanged after approximately 7,000 generations in continuous in vitro culture. A clone of *B. burgdorferi* B31 that did not contain the 16-kb linear plasmid was obtained after exposure of a culture to novobiocin, a DNA gyrase inhibitor. The plasmid-cured strain contains only one linear plasmid, the 49-kb plasmid, and thus has the smallest genome reported to date for *B. burgdorferi*.**

Spirochetes of the genus *Borrelia* have a genomic configuration that is different from those of other prokaryotic genomes that have been described. Of all *Borrelia* genomes, that of *Borrelia burgdorferi*, the cause of Lyme disease, has been examined in the most detail. All isolates of this species appear to contain linear plasmids, which range in size from 5 to 55 kb (3, 36). In addition, the *B. burgdorferi* chromosome appears to be a linear molecule of 1 Mb (7, 16). Only a minority of species of DNA in the cell are circular (26, 35), the usual form of prokaryotic replicons. The genomes of other *Borrelia* species which cause relapsing fever also have these features, and linear plasmids as large as 200 kb in some species have been noted (17, 20). This segmentation of the *Borrelia* genome into predominantly linear pieces has led to the suggestion that the relatively small linear chromosome and the linear plasmids actually constitute a set of linear minichromosomes (18, 20).

The linear plasmids of *B. burgdorferi* are double-stranded linear DNA molecules that have terminal hairpin loops and short inverted terminal repeats (21). Genes for the major outer surface proteins of *B. burgdorferi* and *B. hermsii* are found on linear plasmids (5, 32), but the genetic character of the majority of the linear plasmids as well as the circular plasmids is uncharted. In *B. burgdorferi*, some linear and circular plasmids are spontaneously lost from cells that are serially passaged in culture media, whereas others are more stably maintained (3, 34). For example, B31, the type strain of *B. burgdorferi*, originally contained at least nine linear and supercoiled plasmids, but after 9 years of continuous in vitro culture in our laboratory, only two linear plasmids, 49 and 16 kb long, and two prominent supercoiled circular plasmids of approximately 27 and 30 kb remained (3, 17).

A basic attribute of a plasmid is its copy number relative to the chromosome. Knowing this parameter, one may gain insight into the control of plasmid DNA replication and segregation. In the present study, we determined the copy numbers of two linear plasmids of 49 and 16 kb and a 27-kb circular supercoiled plasmid with respect to the chromosome of *B. burgdorferi* B31. To indicate the relative concentrations of each replicon, hybridizations of chromosome- and plasmid-specific probes with total cellular DNA were compared. The results indicate that the plasmids are stably maintained at about one copy per chromosome in a population of cells.

## MATERIALS AND METHODS

***Borrelia* isolates and broth cultivation.** The *B. burgdorferi* type strain, B31 (ATCC 35210), was grown in BSK II liquid medium (2). The low-passage isolate had been passaged in BSK II medium three times after its original isolation (12). The high-passage isolate of B31 had been cultured continuously in vitro since 1982 and had been cloned by limiting dilution twice. For plasmid curing experiments, cells of the high-passage isolate were serially cultured two to four times in medium containing 64 μg of novobiocin (Sigma Chemical Co., St. Louis, Mo.) per ml, 1 μg of acridine orange (Allied Chemical, New York, N.Y.) per ml, or 1 μg of ethidium bromide (Sigma) per ml.

**Solid medium cultivation.** Clones were isolated as discrete colonies growing in semisolid medium overlaying solid medium. The bottom agarose was prepared by a modification of a previously described formula (11, 27). Briefly, a 3% aqueous solution of LE agarose (SeaKem; FMC Bioproducts, Rockland, Maine) was autoclaved and cooled to 40°C, and an equal volume of prewarmed (37°C) 2× BSK II medium was added to the molten agarose and mixed. Ten-milliliter portions were dispensed immediately onto polystyrene petri plates (60 by 15 mm); the plates were incubated overnight at room temperature in a candle jar.

The semisolid overlay containing spirochetes was prepared in the following way. Borrelias in BSK II broth were counted in a Petroff-Hausser chamber under phase-contrast microscopy. The culture was diluted in broth to yield 600 cells per ml; 0.1 ml of this cell suspension was added to 1.5

* Corresponding author. Electronic mail address: barbour@uthscsa.bitnet or barbour@uthscsa.edu.

† Present address: Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, Hamilton, MT 59840.

TABLE 1. Plasmids and probes used in this study

| Plasmid | Insert fragment[a] | Probe fragment | Reference(s) |
|---|---|---|---|
| pHMB35 | 10.7-kb partial *Sau*3AI fragment of chromosome in pBR322 | 920-bp *Sph*I-*Mlu*I subfragment | 16, 26 |
| pTRH46 | 1.1-kb *Hpa*I-*Eco*RI fragment of 49-kb linear plasmid in pUC9 (contains *ospB* gene) | 874-bp *Eco*RI-*Pst*I subfragment | 25 |
| pBHB63 | 3.4-kb *Bgl*II fragment of 16-kb linear plasmid in pBR322 | 793-bp *Eco*RI subfragment | 10, this study |
| pBC27a | 1.2-kb partial *Hin*dIII fragment of 27-kb circular plasmid in pUC13 | 940-bp *Hin*dIII subfragment | This study |

[a] All plasmid inserts are from *B. burgdorferi* B31 DNA.

ml of molten 37°C top agarose. Top agarose was constituted immediately before use by mixing equal volumes of prewarmed (37°C) 2× BSK II medium and 2% low-gelling-temperature agarose (SeaPlaque; FMC) that had been sterilized by filtration through a 0.2-μm-pore-size membrane. The top agarose cell suspension was gently mixed and poured onto the surface of a bottom agarose plate. Inoculated plates were incubated at 34°C in a candle jar for 7 days. The efficiency of plating was determined by comparing the number of colonies with the estimated number of cells plated. Isolated colonies were cultivated in BSK II broth and replated in BSK II agarose to obtain a second round of single colony clones.

**DNA preparation and techniques.** Total DNA was extracted from broth-cultivated cells by a previously described procedure (30). Briefly, washed spirochetes were resuspended in 50 mM Tris (pH 8.0)–40 mM EDTA–25% sucrose and incubated with lysozyme at 2 mg/ml. The lysate was next incubated with 0.3 mg of proteinase K per ml in the presence of 1% sodium dodecyl sulfate (SDS) for 1 h at 37°C and extracted with phenol and chloroform. Nucleic acids were precipitated with ethanol, and the precipitate was dissolved in 10 mM Tris (pH 8.0)–1 mM EDTA. This suspension was sequentially treated with RNase A at 20 μg/ml, proteinase K-SDS, and phenol-chloroform before a second ethanol precipitation. The concentration of the DNA preparation was measured with a fluorometer (model TKO 100; Hoefer Scientific Instruments, San Francisco, Calif.). DNA was digested with restriction endonucleases under conditions recommended by the manufacturer (Boehringer Mannheim, Indianapolis, Ind.). The buffer for agarose gel electrophoresis of DNA was 90 mM Tris (pH 8)–90 mM borate–2 mM EDTA. DNA in dried 0.2% agarose gels was hybridized in situ by a previously described procedure (3).

**Preparation and characterization of probes.** The recombinant plasmids used as sources of probes are listed in Table 1. The original derivation of the recombinant plasmid pHMB35, which expresses a 60-kDa polypeptide, was described previously (24). The cloned insert of pHMB35 has been physically mapped to the megabase-sized chromosome of *B. burgdorferi* (15). pTRH46, which contains a fragment from the 49-kb linear plasmid of the B31 strain, has also been described elsewhere (23). Clone pBHB63 contains a fragment from the 16-kb linear plasmid (9). To prepare a probe specific for the 27-kb supercoiled circular plasmid, this plasmid was isolated from 0.2% agarose gels of fractionated *B. burgdorferi* B31 total DNA with a model UEA electroelutor (International Biotechnologies, Inc., New Haven, Conn.). The DNA was digested with *Hin*dIII, and the fragments were ligated with the *Hin*dIII-digested plasmid cloning vector pUC13. The products of the ligation were used to transform *Escherichia coli* DH5α (19). One of the recombinant plasmids recovered, pBC27a, contained a 1.2-kb *Hin*dIII fragment of the 27-kb circular plasmid.

Partial restriction enzyme maps of the inserts of the recombinant plasmids are shown in Fig. 1. The *Borrelia* chromosome-specific and plasmid-specific probes were obtained by first digesting the plasmids with the indicated restriction enzymes and then separating the resulting fragments in 1.2% agarose gels. The desired fragments were electroeluted from the gels and quantitated. Probes for dot blots were prepared by radiolabelling 1.0 μg of each fragment with [α-$^{32}$P]dATP by nick translation with a commercial kit (Bethesda Research Laboratories, Gaithersburg, Md.). Probes for Southern blots were prepared with a random primer labelling kit (Boehringer Mannheim) and 0.5 μg of each fragment. After removal of unincorporated nucleotides on a Sephadex G-50 column (Pharmacia, Piscataway, N.J.), the activity of each probe was measured.

The fragments of pHMB35 and pBC27a that were used as probes were partially sequenced to provide an estimate of their G+C contents. pHMB35 was first digested with *Bgl*II, and a 1.0-kb insert fragment was subcloned into *Bam*HI-digested pUC13. The nucleotide sequences of the insert of this subclone, as well as the insert of pBC27a, were determined by using the pUC forward and reverse sequencing primers and a Sequenase kit (U.S. Biochemicals, Cleveland, Ohio) as previously described (22).

**Copy number determination by dot blotting.** Relative concentrations of individual plasmids and chromosomal DNA were determined by a modification of the dot blot procedure of Bresser et al. (10, 13, 33). Borrelias were grown in broth to the stationary phase, at which the cell density was $1 \times 10^8$ to $2 \times 10^8$/ml. Ten-milliliter cultures were centrifuged at 10,000 × *g* for 20 min to harvest the cells. The cells were washed in phosphate-buffered saline containing 5 mM $MgCl_2$ and recentrifuged. The cells were resuspended in 2.5 ml of 10 mM Tris (pH 7.4)–50 mM EDTA–2 mg of lysozyme per ml. Portions (450 μl) of the cell suspension were immediately placed in 2-ml sterile screw-cap polypropylene tubes (Sarstedt Inc., Newton, N.C.) and incubated at room temperature for 15 min. An equal volume of 40 mM Tris (pH 8.0)–50 mM EDTA–1% SDS–200 μg of proteinase K per ml was added, and the tubes were incubated at 37°C for 1 h. To enhance lysis, the suspensions were subjected to three cycles of freezing at −70°C in an ethanol bath and thawing at 37°C in a water bath. The lysates were then sequentially extracted with phenol, a 50:50 mixture of phenol and chloroform, and chloroform. Two volumes of 10 M NaI preheated to 95°C was mixed with the lysates. Each tube was heated in a boiling water bath for 10 min, and replicate 200-, 100-, 50-, and 25-μl portions were immediately vacuum filtered onto nitrocellulose (BA 85; Schleicher & Schuell, Keene, N.H.) that had been soaked in 6× SSC (1× SSC is 150 mM NaCl–15 mM sodium citrate) and placed on a plastic manifold (Bethesda Research Laboratories). After blotting, the filters were washed three times in 70% ethanol for 5 min, washed once in 100 mM triethanolamine containing 0.25%

(vol/vol) acetic anhydride for 10 min, and air dried prior to hybridization.

Prehybridization and hybridization solutions contained 50% formamide, 6× SSC, 5× Denhardt's solution, 0.5% SDS, and 0.1 mg of denatured, sheared salmon sperm DNA per ml. Identical filters containing four replicate dot blots of each volume of cell lysate blotted were separately hybridized with one of the probes described above. After a 4-h prehybridization, a volume of each probe was added such that all hybridizations contained approximately $5 \times 10^6$ cpm/ml. The hybridization reaction mixtures were incubated at 37°C for 16 h. The hybridization fluid was removed, and the filters were washed at 62°C in 0.1× SSC–0.1% SDS–2 mM EDTA. Individual circular areas containing each hybridization reaction mixture were cut from the filters and put into a scintillation vial. Ten milliliters of scintillation fluid (ReadyGel; Beckman Instruments Inc., Fullerton, Calif.) was added to each vial, and the bound radioactivity in each sample was determined on a Beckman LS 7800 scintillation counter.

**Copy number determination by Southern blotting.** A known amount of total DNA was digested to completion with *Hin*dIII, and portions were applied to a 0.9% agarose gel. Three wells were loaded for each determination. After electrophoresis, the fragments were transferred to a nylon membrane (0.2-μm pore size; Nytran; Schleicher & Schuell) by capillary action for 14 h (28). The membrane was then dried at 85°C for 2 h, prehybridized, and hybridized as described above with a mixture containing approximately $5 \times 10^6$ cpm of each of the four probes per ml. Before autoradiography, the blot was washed at 62°C as described above. By using the autoradiograph as a template, each of the four bands in each lane which hybridized to the probe mix was excised from the blot, placed into a separate vial containing 10 ml of scintillation fluid, and counted.

**Calculation of plasmid copy number.** The average counts per minute (cpm) of three identical blots and the standard error of the mean (SEM) were calculated for each type of sample. To determine the relative copy number of each plasmid, the ratio of the average counts per minute from blots hybridized with the chromosomal probe to the average counts per minute from blots hybridized with each of the plasmid probes was calculated. The standard error of each ratio was determined by the formula $\{[\text{SEM (chromosome)}/\text{mean cpm (chromosome)}]^2 + [\text{SEM (plasmid)}/\text{mean cpm (plasmid)}]^2\}^{0.5}$ (37).

## RESULTS AND DISCUSSION

**Preparation and characterization of replicon-specific probes.** Inasmuch as the linear plasmid and chromosomal DNA are not separable by density gradient centrifugation or by other methods based on a circular plasmid's supercoiled conformation (4), hybridization methods were used to determine relative copy numbers. Total DNA from a population of cells was hybridized with a chromosome-specific probe and with individual plasmid-specific probes. The resulting hybridization signals were quantitated and compared to indicate relative copy numbers. The accuracy of these methods depended on attaining the following: (i) replicon-specific probes that were present in only one copy per replicon, (ii) comparable labelling of each probe, (iii) equivalent hybridization of each probe with a given amount of target sequence, and (iv) stoichiometric release of each DNA species from lysed cells and blotting of the DNA in a single-stranded state. The first three specifications concern the replicon-specific probes and are addressed in turn below.

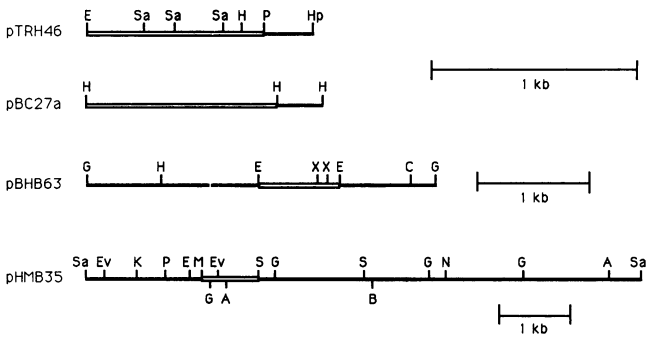


FIG. 1. Partial restriction endonuclease maps of the inserts of the plasmids listed in Table 1. White bars indicate the subfragments that were isolated for use as probes in the copy number determinations. The inserts of pTRH46 and pBC27a are drawn to the uppermost of the three scales indicated. Relative to pTRH46 and pBC27a, the map of pBHB63 is drawn one-half scale, and the map of pHMB35 is drawn one-fourth scale. A, *Acc*I; B, *Bam*HI; C, *Cla*I; E, *Eco*RI; Ev, *Eco*RV; G, *Bgl*II; H, *Hin*dIII; Hp, *Hpa*I; K, *Kpn*I; M, *Mlu*I; N, *Nde*I; P, *Pst*I; S, *Sph*I; Sa, *Sau*3AI; X, *Xba*I.

The probes were derived from recombinant plasmids containing cloned DNA fragments of the chromosome, 49-kb linear plasmid, 16-kb linear plasmid, and 27-kb circular supercoiled plasmid of *B. burgdorferi* B31 (Table 1). To verify that each DNA segment used as probe hybridized only with a specific *Borrelia* replicon, 0.2% agarose gels of undigested *B. burgdorferi* B31 DNA were probed with each of the four recombinant plasmids listed in Table 1. These analyses confirmed that pHMB35 hybridized only to the chromosomal band, pTRH46 hybridized only to the 49-kb linear plasmid band, pBHB63 hybridized only to the 16-kb linear plasmid band, and pBC27a hybridized only to the 27-kb circular plasmid band (data not shown).

The segments of the four plasmids indicated in Fig. 1 were isolated and used as replicon-specific probes in all copy number determinations. Southern blots of B31 DNA digested with several restriction endonucleases were probed with each of the four fragments. As was the case for the *ospA* and *ospB* genes (5), the hybridization patterns confirmed that the fragments used as probes were not present in more than one copy per replicon (data not shown).

The four probes were matched in size, quantity, and specific activity to ensure uniform hybridization conditions. The lengths of the probes were similar, ranging from 793 to 940 bp (Table 1). When 1.0 μg of each fragment was radiolabelled by nick translation, the total activities that resulted ranged from $0.6 \times 10^8$ to $1.1 \times 10^8$ cpm. Activities of the four probes ranged from $1.3 \times 10^8$ to $1.9 \times 10^8$ cpm when 0.5 μg of each was labelled with random primers. Thus, the specific activities of each of the four probes produced by the radiolabelling procedures were equal or nearly equal. The 20-ml hybridization reaction mixtures contained $5 \times 10^6$ cpm/ml, a $10^4$-fold molar excess of probe.

Because of the influence of G+C content on hybridization equilibria, the nucleotide sequences of the four probe fragments were analyzed. The 49- and 16-kb linear plasmid fragments used as probes have been completely sequenced (8, 9). The 49-kb linear plasmid probe is 32% G+C, and the 16-kb plasmid probe is 25% G+C. We next determined the sequence of 685 nucleotides of a 1.0-kb *Bgl*II insert fragment of pHMB35, the source of the chromosome-specific probe,

TABLE 2. Relative copy numbers of plasmids determined by dot blotting of low-passage *B. burgdorferi* B31

| Vol of cell lysate blotted[a] (μl) | cpm (mean ± SE)[b] with probe specific for: | | | |
|---|---|---|---|---|
| | Chromosome | 49-kb linear plasmid | 16-kb linear plasmid | 27-kb circular plasmid |
| 200 | 12,903 ± 730 (1.0) | 12,165 ± 1,050 (0.9) | 3,431 ± 294 (0.3) | 18,660 ± 668 (1.5) |
| 100 | 6,419 ± 278 (1.0) | 4,721 ± 370 (0.7) | 2,050 ± 84 (0.3) | 8,921 ± 277 (1.4) |
| 50 | 3,251 ± 76 (1.0) | 2,486 ± 54 (0.8) | 1,047 ± 28 (0.3) | 3,964 ± 65 (1.2) |
| 25 | 1,687 ± 75 (1.0) | 1,329 ± 20 (0.8) | 588 ± 40 (0.3) | 1,782 ± 45 (1.1) |
| Avg plasmid-to-chromosome ratio (95% CI) | 1.0 | 0.8 (0.5–1.1) | 0.3 (0.1–0.5) | 1.3 (1.1–1.5) |

[a] Cell lysate contained approximately $10^8$ lysed *B. burgdorferi* cells per ml.
[b] Means are from three blots. Numbers in parentheses are ratios of counts per minute with plasmid-specific probe to counts per minute with chromosome-specific probe.

and 351 nucleotides of the 1.2-kb insert of pBC27a, the source of the 27-kb circular plasmid-specific probe. The G+C contents were 27 and 32%, respectively. The overall G+C content of *B. burgdorferi* genomic DNA has been estimated to be 27 to 30% (25). Thus, the probes were generally representative of *B. burgdorferi* DNA and were used in subsequent experiments.

The substrate for the replicon-specific probes was phenol- and chloroform-extracted total DNA from lysed *B. burgdorferi* cells. To preserve the stoichiometry of the chromosome and plasmids, lysis conditions were chosen to maximize liberation of the DNA from cellular components and to shear the chromosomal DNA prior to extraction. Furthermore, amphiphilic material at the interface was included with the aqueous phase during phenol and chloroform extractions to minimize loss of DNA (30).

**Copy numbers of linear and circular plasmids.** The relative copy numbers of the 49- and 16-kb linear plasmids and the 27-kb circular plasmid compared with the chromosome were determined for both low- and high-passage *B. burgdorferi* B31.

In the dot blot method, total DNA released from cells was heated to 100°C in concentrated NaI and blotted onto nitrocellulose while denatured. This process degrades RNA but not DNA (13, 33). To verify this, 3 μg of borrelia RNA in 5 M NaI was heated at 100°C for 10 min. The RNA was completely degraded, as determined by agarose gel electrophoresis and ethidium bromide staining. An RNA sample in unheated 5 M NaI was not degraded (data not shown). Identical dot blot membranes containing a series of increasing amounts of DNA were hybridized with one of the replicon-specific probes. The membranes were washed at high stringency following hybridization, and bound counts were measured. The ratio of counts from blots hybridized with the chromosomal probe to counts from blots hybridized with each of the plasmid probes was calculated to determine the relative copy number of each plasmid per chromosome equivalent. The results for the low- and high-passage strains were very similar (Tables 2 and 3), and the 95% confidence intervals (CI) for both sets of data were calculated. The copy numbers of the 49-kb linear plasmid and the 27-kb circular plasmid were 0.3 to 1.1 and 0.7 to 1.5, respectively, per chromosome equivalent. By this method, the 95% CI for the copy number of the 16-kb linear plasmid was 0.1 to 0.5.

Copy numbers of the plasmids in the high-passage B31 strain were determined by a second, independent method. A Southern blot of an agarose gel containing two quantities of *Hin*dIII-digested total DNA was hybridized with a mixture of the four probes (Fig. 2). *Hin*dIII yielded well-separated, singly hybridizing fragments of about 1 to 3 kb. The chromosome probe hybridized to a 2.4-kb *Hin*dIII fragment, the 16-kb linear plasmid probe hybridized to a 2.9-kb fragment, and the 27-kb circular plasmid probe hybridized to a 0.9-kb fragment (data not shown). The cloned segment of the 49-kb linear plasmid used as probe contains a *Hin*dIII site, so we expected that it would hybridize with two bands of *Hin*dIII-digested B31 DNA, a 1.2-kb fragment predicted from the sequence of Bergström et al. (8) and one of unknown size. Only the 1.2-kb band was evident (Fig. 2). The second, undetected fragment may have been small enough to migrate off the agarose gel. Alternatively, because the probe fragment overlaps this fragment by only 0.1 kb, hybridization to this fragment might have been comparatively weak under the stringent conditions. In any case, the effect on probe hybridization to the fragment of interest would not have been substantial.

TABLE 3. Relative copy numbers of plasmids determined by dot blotting of high-passage *B. burgdorferi* B31

| Vol of cell lysate blotted[a] (μl) | cpm (mean ± SE)[b] with probe specific for: | | | |
|---|---|---|---|---|
| | Chromosome | 49-kb linear plasmid | 16-kb linear plasmid | 27-kb circular plasmid |
| 200 | 24,297 ± 1,290 (1.0) | 15,583 ± 1,819 (0.6) | 6,347 ± 113 (0.3) | 27,583 ± 1,697 (1.1) |
| 100 | 12,744 ± 592 (1.0) | 8,943 ± 392 (0.7) | 3,726 ± 368 (0.3) | 13,673 ± 960 (1.1) |
| 50 | 6,085 ± 388 (1.0) | 4,255 ± 57 (0.7) | 1,687 ± 125 (0.3) | 6,233 ± 294 (1.0) |
| 25 | 3,102 ± 247 (1.0) | 2,414 ± 120 (0.7) | 826 ± 51 (0.3) | 3,185 ± 190 (1.0) |
| Avg plasmid-to-chromosome ratio (95% CI) | 1.0 | 0.7 (0.3–1.1) | 0.3 (0.1–0.5) | 1.1 (0.7–1.4) |

[a] Cell lysate contained approximately $1.8 \times 10^8$ lysed *B. burgdorferi* cells per ml.
[b] Means are from three blots. Numbers in parentheses are ratios of counts per minute with plasmid-specific probe to counts per minute with chromosome-specific probe.

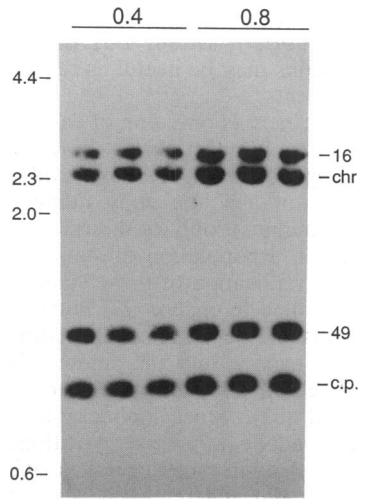


FIG. 2. Autoradiograph of a Southern blot used to determine plasmid copy numbers in high-passage *B. burgdorferi* B31. A 0.9% agarose gel in which 0.4 or 0.8 μg of *Hin*dIII-digested total DNA in triplicate had been separated was blotted and hybridized with a mixture of the four replicon-specific probes described in the text. The bands corresponding to *Hin*dIII fragments hybridized by the chromosome-specific probe (chr), the 49-kb linear plasmid-specific probe, the 16-kb linear plasmid-specific probe, and the 27-kb circular plasmid-specific probe (c.p.) are indicated. The migration of *Hin*dIII-digested λ DNA fragments (in kilobases) used as size standards is indicated at the left.

The four bands represented in the Southern blot of Fig. 2 were excised from the membrane, and their bound radioactivity was determined. The results are given in Table 4. By this method, the copy numbers of the 49-kb linear plasmid and the 27-kb circular plasmid were estimated to be 0.8 to 1.4 and 0.9 to 1.5, respectively, per chromosome equivalent. Whereas these values were similar to those determined by the dot blot method, the copy number of the 16-kb linear plasmid determined by the Southern blot method was twice the estimate obtained by the dot blot method. By the Southern blot method, the 95% CI of the copy number for the 16-kb plasmid was 0.5 to 1.1 per chromosome equivalent, a value similar to those determined for the other two plasmids by both methods.

The dot blot method may underestimate the copy number of the smaller linear plasmid because of the "snap-back" characteristics of intact linear plasmids (4). Although the DNA was blotted quickly while denatured, these plasmids' cross-linked ends would have facilitated rapid reannealment once denaturing conditions were alleviated (4, 22). Consequently, reannealing may have occurred in at least some of the plasmids during filtration. The reannealing effect would have been predicted to be more pronounced in the 16-kb linear plasmid than the 49-kb linear plasmid, and this is what we observed.

Reannealment would not be expected to be a factor for the *Hin*dIII-digested DNA used in the Southern blot method, unless the target fragment contained the telomeric hairpin. This was not the case for the linear plasmid-specific *Hin*dIII fragments (5, 8, 9) or the chromosomal sequence represented by pHMB35 (15). The relative copy numbers of the linear plasmids as determined by Southern blotting were slightly higher than those determined by dot blotting. The relative copy number estimates for the circular plasmid, which would not have any fragments with cross-linked ends, were the same by both methods. We concluded from these data that the linear plasmids were present at a low copy number, estimated to be about one per chromosome equivalent. The circular plasmid examined also had a low copy number of one, or at most two, relative to the chromosome. The plasmid copy numbers had not changed after several years of continuous in vitro culture, during which time our strain had undergone an estimated 7,000 generations and been cloned twice by limiting dilution.

**Plasmid curing and maintenance.** Another explanation of the slightly lower copy number of the 16-kb linear plasmid by the dot blot method was that some cells lacked this plasmid. To assess this possibility, and to further study plasmid stability, clones derived from single cells of the high-passage B31 strain were examined for plasmid content. This isolate does not autoaggregate in broth medium (2), and thus the colonies that grew on plates were assumed to represent clonal populations derived from a single cell. A known number of cells in broth medium were plated on solid BSK II medium. After 7 days, the colonies were easily visualized and were well demarcated from the surrounding medium. When the semisolid overlay method was used, the efficiency of plating of strain B31 was 95 to 100%. Twenty-five isolated colonies were randomly selected and recultured in BSK II broth medium. Total DNAs were extracted from the clones and compared by electrophoresis. All 25 clones were identical to the parent population in having 16- and 49-kb linear plasmids and 237- and 30-kb circular plasmids (data not shown). If only 70% of individuals in the population contained the 16-kb linear plasmid (plasmid copy number ≈ 0.7 and chromosome copy number ≈ 1), then a random sampling of 25 clones would be expected to include 6 to 10 that lacked the plasmid.

Although the efficiency of plating was near or equal to 100%, it was possible that cells putatively lacking the 16-kb linear plasmid could not grow on solid medium. To deter-

TABLE 4. Relative copy numbers of plasmids determined by Southern blotting of high-passage *B. burgdorferi* B31

| Amt of DNA[a] (μg) | cpm (mean ± SE)[b] with probe specific for: | | | |
|---|---|---|---|---|
| | Chromosome | 49-kb linear plasmid | 16-kb linear plasmid | 27-kb circular plasmid |
| 0.8 | 2,111 ± 239 (1.0) | 2,292 ± 158 (1.1) | 1,574 ± 80 (0.7) | 2,367 ± 155 (1.1) |
| 0.4 | 1,201 ± 45 (1.0) | 1,343 ± 118 (1.1) | 918 ± 148 (0.8) | 1,454 ± 38 (1.2) |
| Avg plasmid-to-chromosome ratio (95% CI) | 1.0 | 1.1 (0.8–1.4) | 0.8 (0.5–1.1) | 1.2 (0.9–1.5) |

[a] Amount of *Hin*dIII-digested total *B. burgdorferi* DNA per lane of Southern blot.
[b] Means are from three blots. Numbers in parentheses are ratios of counts per minute with plasmid-specific probe to counts per minute with chromosome-specific probe.

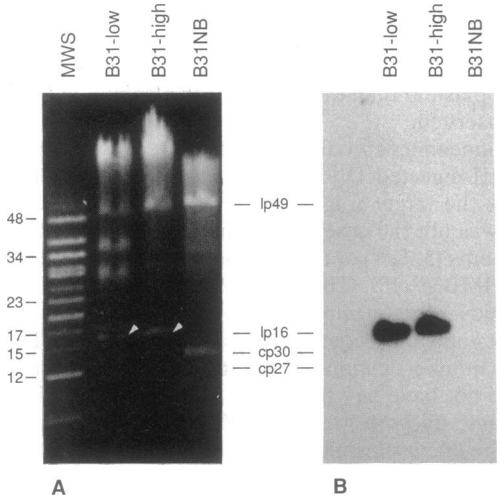


FIG. 3. Loss of 16-kb linear plasmid from *B. burgdorferi* B31. (A) Ethidium bromide-stained 0.2% agarose gel of total DNA from low-passage isolate B31 (B31-low), high-passage isolate B31 (B31-high), and novobiocin-treated isolate B31NB. (B) Autoradiograph of the gel after direct hybridization with radiolabelled pBHB63, which contains a 3.4-kb insert of the 16-kb linear plasmid of *B. burgdorferi* B31. The positions of the 49-kb linear plasmid (lp49), 16-kb linear plasmid (lp16), 30-kb circular plasmid (cp30), and 27-kb circular plasmid (cp27) are indicated. The arrowheads point to the position of the 16-kb linear plasmid. MWS, 8.3- to 48.5-kb linear DNA size standards (Bethesda Research Laboratories).

mine whether high-passage *B. burgdorferi* B31 lacking one or more of the four remaining plasmids could grow in vitro under these conditions, we attempted to cure this borrelia of plasmids (14, 29). Borrelias were grown for 15 to 30 generations in broth medium containing 64 μg of novobiocin per ml, 1 μg of acridine orange per ml, or 1 μg of ethidium bromide per ml. These levels were half the MICs that had been established for each agent in preliminary experiments. After cultivation in the presence of the agent, a culture was then plated on solid medium in its absence for selection of single-colony clones. Total DNA was extracted from 12 clones representing each curing agent and then examined for plasmid content. Cultures that appeared to have lost a plasmid were recloned by a second round of single-colony plating.

One of 12 clones derived from the novobiocin-treated culture had lost the 16-kb linear plasmid (Fig. 3). The growth rates in broth of the plasmid-cured isolate, designated B31NB, and the parent, high-passage B31, were indistinguishable. None of 12 acridine orange-treated or ethidium bromide-treated clones were different from the parent B31 strain with respect to discernible plasmid content. Figure 3 also demonstrates that total DNA from the plasmid-cured B31NB strain was not hybridized with radiolabelled pBHB63, which contains a 3.4-kb fragment of the 16-kb linear plasmid (Table 1). This result was evidence that in this isolate the 16-kb plasmid had not transposed to or recombined with other replicons, including the chromosome. The 16-kb linear plasmid and other plasmids that are spontaneously lost during passage in culture medium, though nonessential for in vitro growth, may be required for infectivity or persistence of the spirochetes in their natural arthropod or vertebrate hosts (3, 34). The B31NB strain has only one linear plasmid, of 49 kb, which contains the major surface protein genes *ospA* and *ospB* (4, 5), and two similarly sized circular plasmids. It has the smallest genome reported to date for *B. burgdorferi*. Strain B31NB and other such plasmid-cured strains may be useful as recipients in genetic exchange experiments.

The finding that there is only one of each of the plasmids per chromosome has implications for borrelial plasmid replication and segregation. The methods we used did not allow the determination of absolute copy number per cell but allowed the determination only of the relative copy number per chromosome in a population of stationary-phase cells. Nevertheless, the replication of these stable plasmids must be carefully controlled to ensure that initiation occurs only once per chromosome replication. In addition, unit-copy-number plasmids cannot rely on random distribution between daughter cells. If they did, cells frequently would not inherit a plasmid (31). Most low-copy-number plasmids studied to date encode a precise partitioning system to ensure that each daughter cell receives one copy of the plasmid (31). How *Borrelia* replicons segregate during cell division is not known. The long, thin shape of borrelias, which are up to 100 times longer than they are wide (6), suggests that if the various replicons occur randomly throughout the cytoplasm, daughter molecules must be positioned at the incipient cell division site.

Bacterial plasmids with similar origins of replication and partition systems are not stably maintained in the cell but tend to segregate away from each other (1). This would imply that all of the different linear and circular plasmids of *B. burgdorferi* have different origins of replication and partition systems. On the other hand, the linear "plasmids" may in fact be minichromosomes. In that case, the major chromosome of *B. burgdorferi*, which has characteristics of a linear DNA (7, 16), and its smaller linear replicons would likely have the same replication and segregation systems. Partitioning might then be analogous to mitotic segregation of eukaryotic chromosomes.

## ACKNOWLEDGMENTS

We thank C. Garon for advice and D. LeBlanc for comments on the manuscript.

This research was supported by Public Health Service grant AI29731 from the National Institute of Allergy and Infectious Diseases.

## REFERENCES

1. **Austin, S., and K. Nordström.** 1990. Partition-mediated incompatibility of bacterial plasmids. Cell **60**:351–354.
2. **Barbour, A. G.** 1984. Isolation and cultivation of Lyme disease spirochetes. Yale J. Biol. Med. **57**:521–525.
3. **Barbour, A. G.** 1988. Plasmid analysis of *Borrelia burgdorferi*, the Lyme disease agent. J. Clin. Microbiol. **26**:475–478.
4. **Barbour, A. G., and C. F. Garon.** 1987. Linear plasmids of the bacterium *Borrelia burgdorferi* have covalently closed ends. Science **237**:409–411.
5. **Barbour, A. G., and C. F. Garon.** 1987. The genes encoding major surface proteins of *Borrelia burgdorferi* are located on a plasmid. Ann. N.Y. Acad. Sci. **539**:144–153.
6. **Barbour, A. G., and S. F. Hayes.** 1986. Biology of *Borrelia* species. Microbiol. Rev. **50**:381–400.
7. **Baril, C., C. Richaud, G. Baranton, and I. Saint Girons.** 1989. Linear chromosome of *Borrelia burgdorferi*. Res. Microbiol. **140**:507–516.
8. **Bergström, S., V. G. Bundoc, and A. G. Barbour.** 1989. Molecular analysis of linear plasmid-encoded surface proteins, OspA and OspB, of the Lyme disease spirochete *Borrelia burgdorferi*. Mol. Microbiol. **3**:479–486.
9. **Bosworth, C., J. Hinnebusch, and A. G. Barbour.** Unpublished data.

10. **Bresser, J., J. Doering, and D. Gillespie.** 1983. Quick-blot: selective mRNA or DNA immobilization from whole cells. DNA **2**:243–254.
11. **Bundoc, V. G., and A. G. Barbour.** 1989. Clonal polymorphisms of outer membrane protein OspB of *Borrelia burgdorferi*. Infect. Immun. **57**:2733–2741.
12. **Burgdorfer, W., A. G. Barbour, S. F. Hayes, J. L. Benach, E. Grunwaldt, and J. P. Davis.** 1982. Lyme disease: a tick-borne spirochetosis? Science **216**:1317–1319.
13. **Costanzi, C., and D. Gillespie.** 1987. Fast blots: immobilization of DNA and RNA from cells. Methods Enzymol. **152**:582–587.
14. **Cozzarelli, N. R.** 1977. The mechanism of action of inhibitors of DNA synthesis. Annu. Rev. Biochem. **46**:641–648.
15. **Davidson, B., and I. Saint Girons (Institut Pasteur).** 1991. Personal communication.
16. **Ferdows, M. S., and A. G. Barbour.** 1989. Megabase-sized linear DNA in the bacterium *Borrelia burgdorferi*, the Lyme disease agent. Proc. Natl. Acad. Sci. USA **86**:5969–5973.
17. **Ferdows, M. S., and A. G. Barbour.** Unpublished data.
18. **Garon, C. F., D. W. Dorward, and M. D. Corwin.** 1989. Structural features of *Borrelia burgdorferi*, the Lyme disease spirochete: silver staining for nucleic acids. Scanning Microsc. **S3**:109–115.
19. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166**:557–580.
20. **Hayes, L. J., D. J. M. Wright, and L. C. Archard.** 1988. Segmented arrangement of *Borrelia duttoni* DNA and location of variant surface antigen genes. J. Gen. Microbiol. **134**:1785–1793.
21. **Hinnebusch, J., and A. G. Barbour.** 1991. Linear plasmids of *Borrelia burgdorferi* have a telomeric structure and sequence similar to those of a eukaryotic virus. J. Bacteriol. **173**:7233–7239.
22. **Hinnebusch, J., S. Bergström, and A. G. Barbour.** 1990. Cloning and sequence analysis of linear plasmid telomeres of the bacterium *Borrelia burgdorferi*. Mol. Microbiol. **4**:811–820.
23. **Howe, T. R., F. W. LaQuier, and A. G. Barbour.** 1986. Organization of genes encoding two outer membrane proteins of the Lyme disease agent *Borrelia burgdorferi* within a single transcriptional unit. Infect. Immun. **54**:207–212.
24. **Howe, T. R., L. W. Mayer, and A. G. Barbour.** 1985. A single recombinant plasmid expressing two major outer surface proteins of the Lyme disease spirochete. Science **227**:645–646.
25. **Hyde, F. W., and R. C. Johnson.** 1984. Genetic relationship of Lyme disease spirochetes to *Borrelia*, *Treponema*, and *Leptospira* spp. J. Clin. Microbiol. **20**:151–154.
26. **Hyde, F. W., and R. C. Johnson.** 1988. Characterization of a circular plasmid from *Borrelia burgdorferi*, etiologic agent of Lyme disease. J. Clin. Microbiol. **26**:2203–2205.
27. **Kurtti, T. J., U. G. Munderloh, R. C. Johnson, and G. G. Ahlstrand.** 1987. Colony formation and morphology in *Borrelia burgdorferi*. J. Clin. Microbiol. **25**:2054–2058.
28. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
29. **McHugh, G. L., and M. N. Swartz.** 1977. Elimination of plasmids from several bacterial species by novobiocin. Antimicrob. Agents Chemother. **12**:423–426.
30. **Meier, J. T., M. I. Simon, and A. G. Barbour.** 1985. Antigenic variation is associated with DNA rearrangements in a relapsing fever spirochete. Cell **41**:403–409.
31. **Nordström, K., and S. J. Austin.** 1989. Mechanisms that contribute to the stable segregation of plasmids. Annu. Rev. Genet. **23**:37–69.
32. **Plasterk, R. H. A., M. I. Simon, and A. G. Barbour.** 1985. Transposition of structural genes to an expression sequence on a linear plasmid causes antigenic variation in the bacterium *Borrelia hermsii*. Nature (London) **318**:257–263.
33. **Porteus, D.** 1985. Rapid and quantitative detection of unique sequence donor DNA in extracts of cultured mammalian cells: an aid to chromosome mapping. Somatic Cell Mol. Genet. **11**:445–454.
34. **Schwan, T. G., W. Burgdorfer, and C. F. Garon.** 1988. Changes in infectivity and plasmid profile of the Lyme disease spirochete, *Borrelia burgdorferi*, as a result of in vitro cultivation. Infect. Immun. **56**:1831–1836.
35. **Simpson, W. J., C. F. Garon, and T. G. Schwan.** 1990. Analysis of supercoiled circular plasmids in infectious and non-infectious *Borrelia burgdorferi*. Microb. Pathog. **8**:109–118.
36. **Stålhammar-Carlemalm, M., E. Jenny, L. Gern, A. Aeschlimann, and J. Meyer.** 1990. Plasmid analysis and restriction length polymorphisms of chromosomal DNA allow a distinction between *Borrelia burgdorferi* strains. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. **274**:28–39.
37. **Taylor, J. R.** 1982. An introduction to error analysis. The study of uncertainties in physical measurements. University Science Books, Mill Valley, Calif.