The Relapsing Fever Agent *Borrelia hermsii* Has Multiple Copies of Its Chromosome and Linear Plasmids

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ABSTRACT

Borrelia hermsii, a spirochete which causes relapsing fever in humans and other mammals, eludes the immune response by antigenic variation of the "Vmp" proteins. This occurs by replacement of an expressed *vmp* gene with a copy of a silent *vmp* gene. Silent and expressed *vmp* genes are located on separate linear plasmids. To further characterize *vmp* recombination, copy numbers were determined for two linear plasmids and for the 1-megabase chromosome by comparing hybridization of probes to native DNA with hybridization to recombinant plasmids containing borrelial DNA. Plasmid copy numbers were also estimated by ethidium bromide fluorescence. Total cellular DNA content was determined by spectrophotometry. For borrelias grown in mice, copy numbers and 95% confidence intervals were 14 (12–17) for an expression plasmid, 8 (7–9) for a silent plasmid, and 16 (13–18) for the chromosome. Borrelias grown in broth medium had one-fourth to one-half this number of plasmids and chromosomes. Staining of cells with 4′,6-diamidino-2-phenylindole revealed DNA to be distributed throughout most of the spirochete's length. These findings indicate that borrelias organize their total cellular DNA into several complete genomes and that cells undergoing serotype switches do one or more of the following: (1) coexpress Vmps from switched and unswitched expression plasmids for at least three to five generations, (2) suppress transcription from some expression plasmid copies, or (3) partition expression plasmids nonrandomly. The lower copy number of the silent plasmid indicates that nonreciprocal Vmp gene recombination may result from loss of recombinant silent plasmids by segregation.

BORRELIAS comprise a genus within the eubacterial phylum of spirochetes (PASTER *et al.* 1991). Included among borrelias are the etiologic agents of relapsing fever (BARBOUR and HAYES 1986). *Borrelia hermsii* is the best studied of these agents. Relapsing fever borrelias evade their mammalian host's immune response through multiphasic antigenic variation (BARBOUR 1990). Twenty-five different variants or serotypes have been identified from the progeny of a single cell of the HS1 strain of *B. hermsii* (STOENNER, DODD and LARSEN 1982). Serotype switching occurs at an estimated frequency of 10^{-3} to 10^{-4}/cell per generation in broth medium or in mice (STOENNER, DODD and LARSEN 1982). Serotype specificity is determined by an outer membrane protein known as the variable major protein, or Vmp (BARBOUR, TESSIER and STOENNER 1982). Each Vmp is apparently encoded by a separate and complete structural gene, the *vmp* gene (BURMAN *et al.* 1990; MEIER, SIMON and BARBOUR 1985). The *vmp* genes may be found either in a common expression site or in unique storage sites where they are transcriptionally silent (BARBOUR *et al.* 1991a; PLASTERK, SIMON and BARBOUR 1985). All *vmp*s examined to date reside on double-stranded, linear DNA plasmids (KITTEN and BARBOUR 1990; PLASTERK, SIMON and BARBOUR 1985). Antigen switching results from a nonreciprocal recombination event, in which a *vmp* gene on an expression plasmid is replaced by a copy of a formerly silent *vmp* gene (PLASTERK, SIMON and BARBOUR 1985). The expressed *vmp* gene is adjacent to a promoter upstream (BARBOUR *et al.* 1991a) and usually adjacent to a telomere downstream (KITTEN and BARBOUR 1990). Silent genes that have been examined lack both upstream promoters and a telomeric location (BARBOUR *et al.* 1991a; KITTEN and BARBOUR 1990).

A plausible explanation for these data is that expression of a *vmp* gene results solely from its placement downstream from the common promoter (BARBOUR 1990; BARBOUR *et al.* 1991a). In a cell possessing one copy of the expression plasmid, recombination would be followed immediately by loss of transcription of the old *vmp* and nascent transcription of a new *vmp* gene. A challenge for this model would be the occurrence of the expression plasmid in multiple copies. Replacement of the expressed gene in only one copy would result in transcription of both new and old *vmp* genes. Cells coexpressing new and old *vmp* genes would not be expected to escape the immune response directed against the old Vmp. To minimize coexpression with multiple expression plasmids, the cell would need either a mechanism for achieving rapid segre-

gation of expression plasmids carrying different *vmps* or a mechanism for suppressing expression from some of the plasmids. An alternative model to account for suppression of one expression-linked gene in favor of another has not been established, but there is precedence for such a process in trypanosomes, which activate one expression site and deactivate another by an unknown mechanism (CROSS 1990).

A second unknown aspect of this phenomenon is the nature of the recombination causing antigen switching. Recombination between the silent *vmp21* gene and expressed *vmp7* gene appears nonreciprocal (PLASTERK, SIMON and BARBOUR 1985). That is, when serotype 7 cells switched to serotype 21, the expressed *vmp7* gene was replaced by silent *vmp21* but replacement of silent *vmp21* by expressed *vmp7* was not detected. This may happen by a gene conversion mechanism, such as double strand-break repair (SZOSTAK, ORR-WEAVER and ROTHSTEIN 1983), or by reciprocal recombination with loss of one of the products–namely, the silent plasmid containing the formerly expressed *vmp* gene. If the silent plasmids are present at high copy numbers, the recombinant silent plasmid might be lost through segregation. If copy numbers are low, loss of silent genes from the population could occur.

We assessed the copy number of borrelial replicons to address the issues listed above. The copy number of the expression plasmid was determined to better understand the control of *vmp* gene expression. We ascertained the copy number of a silent plasmid for clues to the type of recombination responsible for Vmp switching. Finally, the total number per cell of the 1-megabase *B. hermsii* chromosome which, like the *Borrelia burgdorferi* chromosome, appears to be linear (FERDOWS and BARBOUR 1989; DAVIDSON, MACDOUGALL and SAINT GIRONS 1992), was also determined.

MATERIALS AND METHODS

Bacterial strains and recombinant plasmids: Serotypes 7 and C (BARBOUR, TESSIER and STOENNER 1982; STOENNER, DODD and LARSEN 1982) of *B. hermsii* strain HS1 (ATCC 35209) were studied. *Escherichia coli* examined by fluorescence microscopy were of strain ATCC 25922. The recombinant plasmids p7.12 and p7.16, propagated in *E. coli* DH5, have been described previously (PLASTERK, SIMON and BARBOUR 1985). Other recombinant plasmids were created by ligation of borrelial DNA into dephosphorylated vectors by standard techniques (MANIATIS, FRITSCH and SAMBROOK 1982). Transformation of *E. coli* DH5 or DH5α was by the method of HANAHAN (1983). Probes for colony blots were obtained by the polymerase chain reaction using conditions described previously (BARBOUR *et al.* 1991b), and radiolabeled with a random primer labeling kit (Boehringer Mannheim).

Growth of borrelias in mice and culture: Female BALB/c mice 7–8 weeks of age were irradiated with 900 rad from a ^{137}Cs source and inoculated intraperitoneally with serotype 7 borrelias from a frozen stock. Spirochetemia was monitored by daily microscopic examination (STOENNER, DODD and LARSEN 1982), and when it reached its peak, blood was collected from the mice. In some experiments, serotype 7 cells were grown to late log phase (~5 × 10^7 cells/ml) in BSK II medium (BARBOUR 1984), collected by centrifugation and frozen in 20% glycerol at −80° until use. Serotype C cells, which are adapted to growth in culture, were also grown in BSK II medium and processed immediately after harvest. Borrelias were counted in a Petroff-Hausser chamber viewed by phase contrast microscopy (KOCH 1981).

Preparation and quantification of DNA standards: Lyophilized *E. coli* and *Clostridium perfringens* genomic DNA (Sigma) was resuspended in TE (10 mM Tris-Cl, pH 8.0–1 mM EDTA). DNA concentrations were determined by measuring optical densities at 260 nm (OD_{260}). Both samples had $OD_{260/280}$ ratios of 2.0. The concentration of bacteriophage λ DNA in TE (BRL) was verified by microfluorometry (Hoefer) with *E. coli* genomic DNA as a standard. The λ DNA was then digested with *Apa*I, mixed with gel-loading buffer (MANIATIS, FRITSCH and SAMBROOK 1982), aliquoted, and frozen at −20 ° until use in fluorescence experiments.

Plasmid DNA for use in hybridization experiments was extracted from *E. coli* by the alkaline lysis method (MANIATIS, FRITSCH and SAMBROOK 1982) and purified by $CsCl_2$ density centrifugation. The concentration of one of the recombinant plasmids was determined from its OD at 260 nm. The concentrations of the other two recombinant plasmids were determined relative to the first by comparing the ethidium bromide fluorescence of restriction fragments from each plasmid in agarose gels.

Hybridization analysis: Borrelias grown in broth medium were harvested by centrifugation (2 min at 15,000 × *g*), washed twice with 50 mM Tris-Cl, pH 8.0, 150 mM NaCl (TN), and then resuspended in TN. Cells were counted by microscopy. Cells at 10^7 per μl were mixed with 3 volumes molten 1% agarose (SeaKem Incert; FMC, Rockland, Maine). Borrelias obtained directly from mice were collected from plasma by centrifugation for 4 min at 8000 × *g* and examined by indirect immunofluorescence microscopy (STOENNER, DODD and LARSEN 1982) to confirm serotypic identity. Vmp-specific monoclonal antibodies used were H12915 (αVmp7), H10022 (αVmp21) and H79.9E (αVmpC) (BARSTAD *et al.* 1985; T. KITTEN, A. V. BARERRA and A. G. BARBOUR, submitted for publication; A. G. BARBOUR, C. J. CARTER and C. S. FREITAG, manuscript in preparation). Monoclonal antibodies directed against the borrelial flagellin (H9724) (BARBOUR *et al.* 1986) and OspA of *B. burgdorferi* (H5332; BARBOUR, TESSIER and TODD 1983) were used as positive and negative controls, respectively. The remaining borrelias were imbedded in agarose as described above, except that 250 mM NaF was included in all washes to halt glycolysis and, thus, render the cells nonmotile for counting (FULTON and SMITH 1960).

Aliquots of 20–40 μl were pipeted into wells of an acrylic mold. Solidified plugs were incubated in lysis buffer which was 50 mM Tris-Cl (pH 8.0), 50 mM EDTA, 1% sodium dodecyl sulfate (SDS), 2 mg/ml proteinase K (FERDOWS and BARBOUR 1989), at 22 ° for 16 hr, and then sequentially soaked in TE for 30 min, TE with 1 mM phenylmethylsulfonyl fluoride for 60 min, three times in TE for 30 min each, and finally for 30 min in the appropriate restriction enzyme reaction buffer. Then plugs were individually melted for 5 min at 65°, and the DNA was digested with 15 units of restriction enzyme for 1–3 hr at 37°. Gel-loading buffer was added and the molten plugs were either loaded onto 0.5% agarose gels (SeaKem GTG, FMC) for electrophoresis or stored at 4° for later use. DNA within the plugs

was stable over the duration of storage. To estimate loss of DNA from agarose plugs, the lysis solution from some experiments was cooled on ice to precipitate SDS, clarified by centrifugation and ethanol precipitated in the presence of 20 μg/ml oyster glycogen. The DNA was resuspended, reprecipitated, digested with restriction enzymes and electrophoresed alongside digested plugs prepared from the same lysis solution. Recombinant plasmids, which served as standards, were digested with restriction enzymes, mixed with 3 volumes molten agarose and 0.5–1 μg *E. coli* genomic DNA, and loaded onto adjacent lanes of the gel. Electrophoresis was at 5 V/cm for 4–5 hr in 40 mM Tris acetate (pH 8.0), 1 mM EDTA containing 200 ng/ml ethidium bromide. After electrophoresis, gels were alkali treated, neutralized with Tris-Cl (pH 7.5), and dried (BARBOUR and GARON 1987). Oligonucleotide probes were radiolabeled with [γ-^{32}P]ATP and T_4 polynucleotide kinase (MANIATIS, FRITSCH and SAMBROOK 1982), and purified by passage through Nensorb 20 cartridges (Du Pont, Boston). Radiolabeled probes were hybridized with dried gels in 4 × SSC (SSC is 0.15 M NaCl, 0.01 M sodium citrate), 5 × Denhardt's solution (MANIATIS, FRITSCH and SAMBROOK 1982), 0.5% SDS, 0.1% sodium pyrophosphate, 100 μg/ml salmon sperm DNA at 50° overnight. The gels were washed at high stringency (60° in 6 × SSC, 0.1% SDS) and exposed to X-ray film (Hyperfilm; Amersham; Arlington Heights, Illinois) without intensifying screen.

For some experiments, genomic DNA was obtained by lysis in agarose plugs and separated by transverse alternating field electrophoresis (GeneLine system; Beckman). Conditions for electrophoresis were those of FERDOWS and BARBOUR (1989) except that the pulse time was changed to 25 sec. *Saccharomyces cerevisiae* chromosomal DNA (strain 334; Beckman) was used as a molecular weight standard. Gels were dried and hybridized as described above.

Determination of total cellular DNA content: Total DNA was extracted from borrelias by a liquid lysis procedure (MEIER, SIMON and BARBOUR 1985) and measured by spectrophotometry and microfluorometry. Inasmuch as binding of the dye used for microfluorometry (Hoechst #33258) is highly dependent on A + T content (DAXHELET *et al.* 1989), *C. perfringens* DNA, which has an A + T content of 73.5%, was used as a standard for measuring the *B. hermsii* DNA, whose A + T content is 70% (HYDE and JOHNSON 1984).

To estimate DNA loss during extraction, ^{32}P-labeled borrelial DNA was first either diluted in TE or added to lysed cells, then measured by scintillation counting when the extraction was complete. Efficiency of extraction was calculated as cpm of the extracted DNA divided by the cpm of the TE-diluted DNA.

Fluorescence analysis of gels: Borrelias were imbedded in agarose to yield plugs containing 1.0–2.5 × 10^7 cells. After the cells were lysed, the plugs were washed three times for 30 min each in TE, melted at 65°, mixed with *Apa*I-digested λ DNA and gel-loading buffer, and loaded onto 1% agarose gels for field inversion gel electrophoresis. A PPI-100 power inverter (MJ Devices, Waltham, Massachusetts) generated a run cycle with a forward duration of 0.5 sec and a reverse duration that initially was 0.15 sec and increased by increments of 0.1 sec through three steps. Electrophoresis was at 8 V/cm. The electrophoresis buffer (45 mM Tris, 45 mM borate, 1 mM EDTA) was recirculated and maintained at 18°. Linear plasmid sizes were estimated by comparison with migrations of linear duplex DNA markers 8–48 kb in size (BRL). Gels were stained with 500 ng/ml ethidium bromide for 40–60 min and destained for 60 min in distilled water.

Analysis of images: Autoradiograms and photographs were digitized with an 8-bit, 300-dot per inch flatbed scanner (Microtek, MSF-300G; Torrance, California) interfaced with an Apple Macintosh II computer. Images were scanned at a resolution of 75 pixels per inch. The scanner was controlled and calibrated with Digital Darkroom 2.0 software (Silicon Beach Software, San Diego, California). The gel plotting routine (O'NEILL *et al.* 1989) of Image software (v. 1.38, by WAYNE RASBAND, National Institutes of Health) was used to quantify the intensity of bands in the images. A selection rectangle was drawn to include all the bands that lay in a given row. This selection was equivalent to the path traveled by the light beam of a scanning densitometer. A plot was generated in which each point represented the density of a single column of pixels within the selection rectangle. For autoradiograms, a second selection rectangle was drawn below or above the bands to measure the background density of the film. The plot generated from this measurement served as the baseline for the sample peaks. Photographs of gels used to quantify recombinant plasmids had negligible background density. For photographs of field inversion gels, background fluorescence in the sample lanes was high. Therefore, each lane was scanned lengthwise, and the baselines were manually drawn. The Image software was used as supplied for densitometry and peak area calculation except that the maximum and minimum values allowed for the density plots were first set to the value of the highest and lowest densities that would be measured in a given image. This change increased the size of the peaks and, thus, the accuracy of measurements.

Density measurements obtained for standards were plotted against their concentrations, expressed as copies per cell, to generate calibration curves. Curves were approximated with third-degree polynomial equations generated by the least squares method using DeltaGraph 1.5c software (DeltaPoint, Inc., Monterey, California). Density measurements of test samples were entered into the appropriate least squares equations to obtain results in terms of copies per cell. This procedure was repeated for each image. Two images, created from exposures of different durations, were analyzed for each experiment. The numerical values from all experiments were adjusted to correct for loss of DNA from agarose plugs during cell lysis.

DNA staining and fluorescence microscopy: *E. coli* in L broth were incubated with 50 μg/ml cephalexin for 90 min and 200 μg/ml chloramphenicol for 10 min. Borrelias and *E. coli* were harvested from culture media by centrifugation, washed once, resuspended in TN (pH 7.5), then spread on slides to dry. In some experiments, slides were fixed with methanol for 5 min and dried again. 4′,6-Diamidino-2-phenylindole (DAPI; Sigma) was added to 1–5 μg/ml, coverslips were positioned and slides were viewed by epifluorescence and phase microscopy (HIRAGA *et al.* 1989) using a Leitz microscope fitted with appropriate filters. Photographs were made with Kodak Tmax 3200 film.

Error analysis and statistics: Three sources of error contributed to uncertainty in determining plasmid and chromosome copy number. The first was the uncertainty in the concentration of the DNA standards. For λ DNA and one recombinant plasmid, uncertainty was calculated from the variation in two or three replicate microfluorometry or spectrophotometry measurements, and calculated as the standard error of the mean (SEM). For two other recombinant plasmids, 12–15 comparisons were made between the ethidium bromide fluorescence of each of these plasmids and that of the first plasmid. The variation in these measurements was combined with the uncertainty from the measurement of the first. Uncertainty in counting cells, which

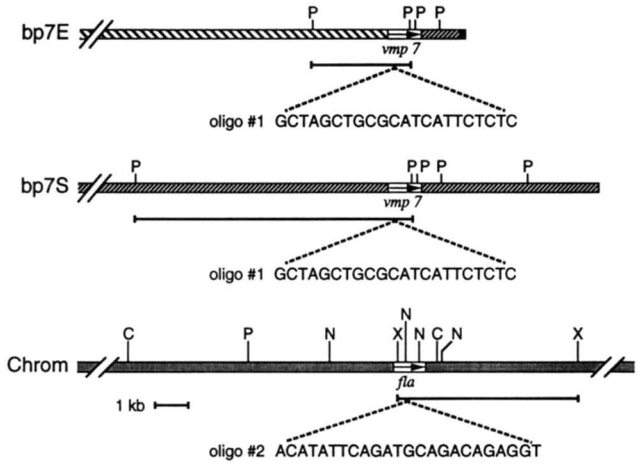

FIGURE 1.—Restriction maps of linear plasmid and chromosomal loci examined by hybridization analysis. The right ends of linear plasmids bearing silent *vmp7* (bp7S) and expressed *vmp7* (bp7E) are shown, along with the chromosomal *fla* locus. Like fill patterns indicate sequence identity. Arrows indicate the positions of the *vmp7* and *fla* genes. Horizontal lines below maps indicate restriction fragments examined by hybridization analysis. The sequence and binding sites of the two oligonucleotides used as probes in Southern blots are indicated. Recognition sites are shown for the restriction enzymes *Pst*I (P), *Cla*I (C), *Nde*I (N), and *Xba*I (X). *Nde*I sites are not shown for bp7S and bp7E; *Pst*I sites on these plasmids are from PLASTERK, SIMON and BARBOUR (1985) and KITTEN and BARBOUR (1990).

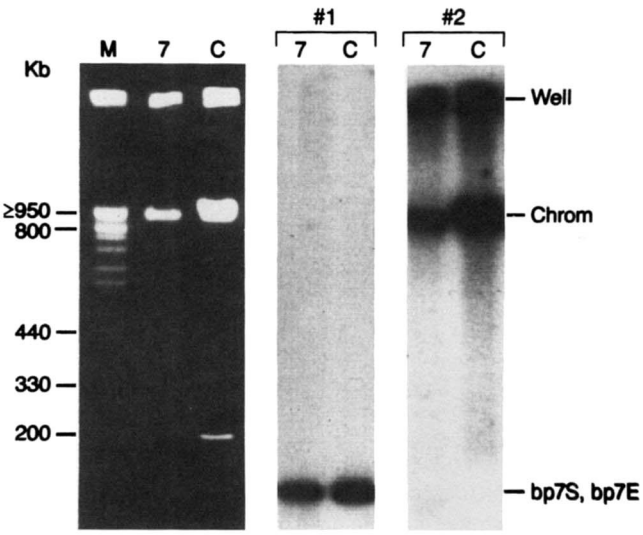

FIGURE 2.—Binding of *vmp7*-specific and *fla*-specific oligonucleotide probes to *B. hermsii* DNA separated by pulsed field electrophoresis. Lanes are *S. cerevisiae* chromosomal markers (M), DNA from serotype 7 borrelias (7), and DNA from culture serotype borrelias (C). (Left panel) Ethidium bromide-stained gel showing ~950-kb chromosome and linear plasmids. Migration of selected *S. cerevisiae* chromosomes are indicated to the left. (Center panel) Dried gel hybridized with oligo #1, specific for *vmp7* (see Figure 1). (Right panel) Dried gel stripped of bound probe and hybridized with oligo #2, specific for the *fla* gene (see Figure 1).

was determined as the higher of the Poisson error (KOCH 1981) or the variation in results from duplicate measurements, was combined with the other sources of error.

The second source of error was variation between replicate samples in a given photograph or autoradiogram. For hybridization experiments, two to three replicates were present on each autoradiogram. For fluorescence experiments, four to seven replicates were present on each photograph.

The third possible source of error was introduced during the analysis of the photographs and autoradiograms. Uncertainty was estimated from the variation obtained when the same image was digitized and quantified twice. Error in calibration curves resulting from lack of fit of the least squares line was evaluated and found to be negligible in all cases.

Each of the above sources of error was then converted to a coefficient of variation, defined as the product of the mean and the SEM. These coefficients were combined by taking the square root of the sum of their squares, a method of error propagation that assumes individual errors are random and independent (TAYLOR 1982). The calculated combined error for each experiment was then converted back to an SEM value. The 95% confidence intervals (CI) were obtained by doubling the SEM and adding or subtracting from the mean (SNEDECOR and COCHRAN 1967). Results and errors from separate experiments were combined by taking weighted averages (TAYLOR 1982).

RESULTS

Selection and construction of probes for hybridization analysis: We used several approaches to determine absolute copy numbers of replicons, as well as their number relative to one another. One method was hybridization of oligonucleotides to DNA from borrelias and to known amounts of recombinant plasmids.

As a plasmid probe, we made an oligonucleotide specific for the *vmp7* gene, which is carried in expressed form in serotype 7 cells on a 28-kb linear plasmid and in silent form in all serotypes on a 32-kb linear plasmid (see Figure 1, oligo #1). This oligonucleotide was predicted to be specific for *vmp7* because it was not found among the sequences of 6 other *vmp* genes examined (our unpublished results). As a chromosomal probe, we made an oligonucleotide specific for the flagellin (*fla*) gene of *B. hermsii*. The DNA sequence and chromosomal siting of this gene were established by L. NOPPA, A. SADZIENE, A. G. BARBOUR and S. BERGSTRÖM (submitted for publication). The sequence and location of this oligonucleotide, designated oligo #2, are indicated in Figure 1. The specificity of these probes was confirmed by hybridization to total borrelial DNA. Figure 2 (left) shows an ethidium bromide-stained gel in which the chromosome and linear plasmids from serotype 7 or C cells have been separated by pulsed-field electrophoresis. The gel was dried and DNA in it hybridized without transfer to oligo #1. Figure 1 (middle panel) shows that this oligo binds to linear plasmids. When electrophoretic conditions were altered to enhance separation of the linear plasmids, oligo #1 hybridized with plasmids 32 kb (bp7S) and 28 kb (bp7E) in size in serotype 7 cells (data not shown). The third panel shows that

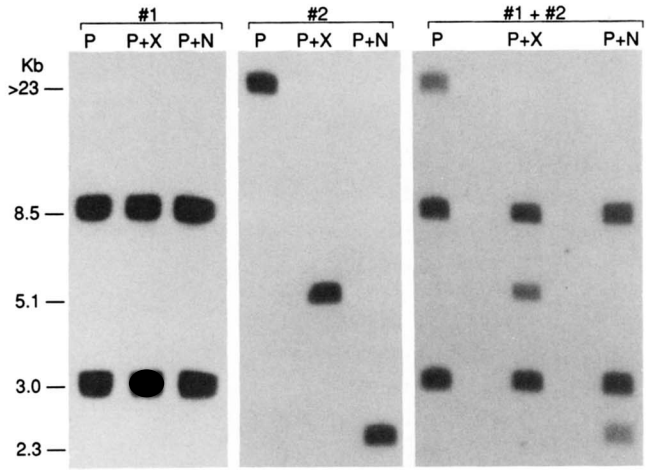

FIGURE 3.—Binding of *vmp7*-specific and *fla*-specific oligonucleotide probes to restriction fragments of *B. hermsii* DNA. (Left panel) Binding of oligo #1, specific for *vmp7*, to a dried gel containing DNA digested with *Pst*I (P), *Pst*I + *Xba*I (P+X), or *Pst*I + *Nde*I (P+N). (Center panel) Binding of oligo #2, specific for the *fla* gene, to DNA digested with the same enzymes. (Right panel) Binding of both oligo #1 and oligo #2 to restriction enzyme-digested DNA.

oligo #2 hybridizes only with the linear chromosome that is about 950 kb in size and with DNA remaining in the well. This experiment confirmed that the oligo #1 was specific for linear plasmids, and that oligo #2 probe was specific for the chromosome. Although we cannot be certain that there are not additional species of (950 kb) chromosomes to which oligo #2 does not bind, this seems unlikely because *B. burgdorferi*, which is 44–59% homologous to *B. hermsii* (HYDE and JOHNSON 1984), has a single species of 950-kb chromosome (BARIL *et al.* 1989; DAVIDSON, MACDOUGALL and SAINT GIRONS 1992; FERDOWS and BARBOUR 1989).

The next step was identification of hybridizing restriction fragments from each replicon that were nontelomeric and separable from one another by electrophoresis. Telomeric restriction fragments were avoided because in preliminary studies they bound probes with decreased efficiency, owing to their rapid renaturation (BARBOUR and GARON 1987; KITTEN and BARBOUR 1990).

Figure 3 shows autoradiograms of dried gels containing DNA from serotype 7 cells cut with *Pst*I, *Pst*I + *Xba*I, and *Pst*I + *Nde*I, electrophoretically separated, and hybridized with radiolabeled probes. In the leftmost panel, the probe is oligo #1. *Pst*I fragments of 8.5 and 3.0 kb hybridize in each lane. The 8.5-kb fragment contains most of the silent *vmp7* gene; the 3.0-kb band contains most of expressed *vmp7* (Figure 1) (PLASTERK, SIMON and BARBOUR 1985). Neither fragment is telomeric (KITTEN and BARBOUR 1990; PLASTERK, SIMON and BARBOUR 1985). The middle panel shows a duplicate gel probed with oligo #2. Bands of >23, 5.1 and 2.3 kb are bound in lanes 1–3, respectively. The rightmost panel shows the results of including both probes in the hybridization mix. This experiment confirmed that both probes were specific. DNA was not detected in the wells by ethidium bromide staining or by hybridization with either probe (not shown). The 8.5- and 3.0-kb fragments are contained, respectively, on the recombinant plasmids p7.12 and p7.16 (PLASTERK, SIMON and BARBOUR 1985), and were thus ideal candidates for use as hybridization standards in subsequent experiments. The location of these fragments on the borrelial plasmids is indicated in Figure 1 by horizontal lines.

The data in Figure 3 suggested that the 5.1-kb fragment bound by oligo #2 would be a suitable hybridization standard for the chromosome. After it was determined that the 5.1-kb fragment was produced by *Xba*I alone, an *Xba*I digest of *B. hermsii* DNA was separated by electrophoresis, and restriction fragments 4.5–6.5 kb were ligated with pUC18. *E. coli* were transformed with the ligation mix. Colonies containing *fla* sequences were identified by hybridization to a fragment of the *fla* gene amplified by the polymerase chain reaction. Amplification primers were oligo #2 (see Figure 1) and an oligonucleotide with sequence 5′-GCAATCATAGCCATTGCAGATTGT-3′ located 600 bp downstream (L. NOPPA, A. SADZIENE, A. G. BARBOUR and S. BERGSTRÖM, submitted for publication). The four colonies that hybridized to the probe contained plasmids with 5.1-kb inserts that had identical restriction maps. The recombinant plasmid recovered from one colony was designated pFX and served thereafter as the chromosomal hybridization standard. The location of the 5.1-kb *Xba*I fragment is shown in Figure 1.

Because some bacteria contain tandemly repeated genes for the major flagellar structural proteins (GUERRY *et al.* 1990; PLEIER and SCHMITT 1989), we first determined whether the *fla* gene sequence was present in multiple copies in *B. hermsii*. Figure 3 suggested that if additional copies of the *fla* gene were present, they were located near one another, within the same *Pst*I, *Nde*I and *Xba*I restriction fragments. Southern blots were performed on borrelial DNA and on pFX, both cut with various restriction enzymes and probed with oligo #2. A partial restriction map of the *fla* locus is shown in Figure 1. There was no indication of additional copies of the *fla* gene within the pFX insert. Because pFX contains no DNA upstream from the *fla* gene, the 9.5-kb *Cla*I fragment overlapping the pFX insert (see Figure 1) was also cloned and examined. Additional *fla* genes were not detected in this fragment.

Hybridization analysis of serotype C cells: Studies were initially performed with serotype C borrelias. Cells of this serotype are more easily studied than others because they do not switch serotypes at a detectable frequency (STOENNER, DODD and LARSEN 1982). Furthermore, serotype C cells are adapted to

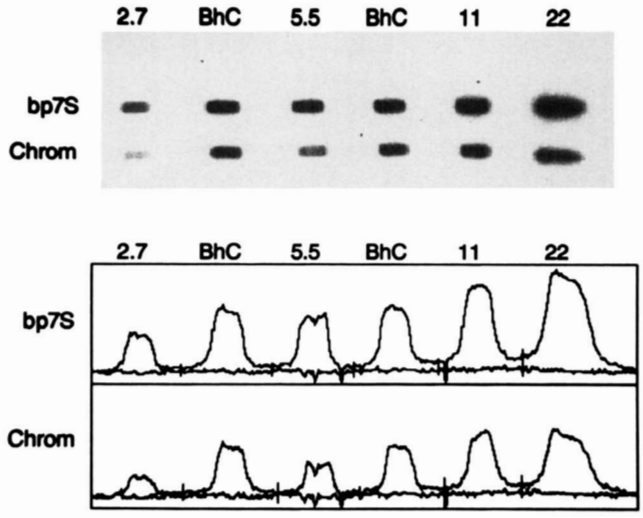

FIGURE 4.—Hybridization analysis of serotype C cells grown in broth medium. Autoradiogram of oligo #1 and oligo #2 hybridized to dried gels containing serotype C DNA (BhC) and recombinant plasmids with cloned genes from bp7S and the chromosome. Recombinant plasmids were present in numbers equal to 2.7, 5.5, 11 and 22 copies per serotype C cell. DNA was digested with *Pst*I and *Xba*I. The positions of the 8.5-kb *Pst*I fragment from bp7S that contains silent *vmp7* and the 5.1-kb *Xba*I fragment containing the chromosomal *fla* gene are shown to the left. A densitometric analysis of the digitized autoradiographic image is shown below the autoradiogram.

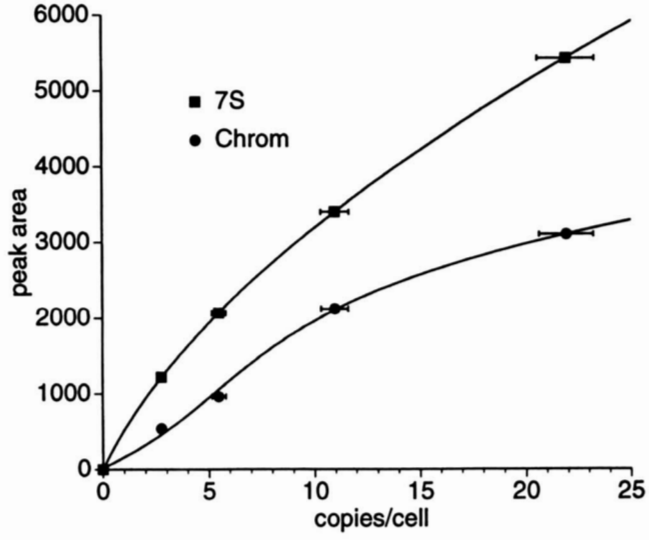

FIGURE 5.—Calibration curves for hybridization analysis of serotype C cells. Curves were created by plotting the copy numbers of recombinant plasmid standards against the areas calculated from density peaks shown in Figure 4. Error bars indicate 1 SEM and are a measure of uncertainty in the copy numbers of the standards.

growth in broth medium. Under these conditions, they exhibit doubling times approaching those of borrelias grown in mice. We reasoned that serotype C chromosome and silent plasmid copy numbers might also resemble those of cells growing in mice. In one experiment, cells grown in broth medium to a density of 3.0×10^8 cells/ml were harvested and equally divided. One portion was imbedded and lysed in agarose plugs. Some of these plugs were digested with *Xba*I and *Pst*I and electrophoresed alongside digested recombinant plasmid standards. Molten agarose and *Pst*I-digested *E. coli* DNA were added to the standards so that the concentrations of agarose and total DNA in the standards and the test samples would be equal. An autoradiogram of the dried gel probed with oligos #1 and #2 is shown in Figure 4. Lanes containing DNA from serotype C borrelias are marked "BhC." Other lanes contain recombinant plasmid standards in amounts equal to 2.7, 5.1, 11 and 22 copies of bp7S and the chromosome per cell. The larger (8.5-kb) band is from bp7S or the recombinant plasmid derived from bp7S and the smaller (5.1-kb) band is from the chromosome or pFX.

A representative densitometric analysis is shown beneath the autoradiogram. The area under each peak is a measure of the density of one band on the autoradiogram. The peak areas for the standards were plotted against their copy numbers. These points were connected with best fit lines to generate calibration curves, shown in Figure 5. Uncertainty in concentration of the standards is shown by SEM error bars. Peak areas for bp7S and the chromosome were entered into best fit equations to determine copy numbers. Results are given in Table 1, experiment 1. A correction was made for loss of borrelial DNA from agarose plugs into the lysis solution. Loss was determined by including ethanol-precipitated DNA from lysis solutions in experiments such as this one, and was estimated to be 10.1% for bp7S and 3.4% for the chromosome (not shown).

The 95% confidence intervals in Table 1 reflect uncertainty in determining the concentration of the standards, variation in the results obtained from the replicate serotype C samples, and uncertainty in analysis of the autoradiograms. As an illustration, the sources of bp7S copy number uncertainty are listed below. Errors are given as coefficients of variation, or CVs. Uncertainty in concentration of the bp7S standard, p7.12, had the following three components. Uncertainty in measurement of p7.12 relative to pFX was 1.6%, uncertainty in determining the concentration of pFX was 5.1%, and uncertainty in cell counting was 3.1% as estimated from the Poisson error. (If estimated from duplicate counts, cell counting error would have been 0.2%.) The variation in the duplicate serotype C samples was 1.1%. Variation in results obtained from analysis of separate autoradiograms of the same gel was 4.5%. These errors were added by taking the square root of the sum of their squares; their total was 7.7%. To correct for loss of DNA into the lysis solution, the estimated copy number was multiplied by 1.1. This correction factor carried a CV of 4.1%. Adding this error as before gave a final CV of 8.7%. Multiplying by the corrected mean copy

TABLE 1

Copy numbers of plasmids and chromosome

Exp.	Method[a]	Serotype	Source	Copies/cell		
				Chromosome	bp7S	bp7E
1	H	C	Culture	8.4 (6.7–10.0)[b]	7.3 (6.0–8.5)	
2	F	C	Culture		9.5 (8.0–11.0)	
3	H	7	Culture	3.8 (2.7–4.8)	4.0 (3.1–4.9)	6.6 (4.9–8.4)
4	H	7	Culture	5.0 (3.1–6.8)	4.5 (2.9–6.1)	6.4 (4.6–8.3)
5	H	7	Culture	6.2 (3.8–8.6)	4.0 (3.0–5.1)	5.5 (3.4–7.5)
6	F	7	Culture		8.1 (6.5–9.7)	
7	H	7	Mouse	15.8 (12.7–18.9)	6.8 (5.4–8.2)	13.5 (9.8–17.3)
8	H	7	Mouse	15.3 (9.8–20.9)	8.4 (7.0–9.7)	14.6 (11.4–17.8)

[a] Hybridization (H) or fluorescence (F).
[b] (95% confidence interval).

number of 7.3 gave an SEM of 0.635. Adding and subtracting 2 × the SEM from the mean gave the upper and lower values for the 95% confidence interval: 6.0 and 8.5.

Determination of total DNA content of serotype C cells: The total DNA content of each cell was estimated from the above results. If the contributions of the chromosome and bp7S are added, the content is calculated as (8.4 × 950 kb) + (7.3 × 32 kb), or 8,214 kb. If it is assumed that the other linear plasmids (with sizes of 180, 70, 38, 28, 28 and 15 kb) and the two 28-kb supercoiled plasmids detected by electrophoresis (M. S. FERDOWS and A. G. BARBOUR, manuscript in preparation; our unpublished results) are also present at 7.3 copies per cell, the total expected DNA content rises to 11,244 kb per cell.

To test this prediction, we extracted DNA from the second portion of serotype C cells by a liquid lysis procedure (MEIER, SIMON and BARBOUR 1985). The concentration of recovered DNA was measured by microfluorometry and spectrophotometry. A small amount of nick-translated DNA was added to the cells immediately after lysis. When the extraction was complete, scintillation counting was used to measure the efficiency of the procedure. After adjusting for DNA loss during the extraction procedure, we estimated that the DNA present in 3.3×10^9 cells was 40 μg. From these figures, we calculated that there was 12,100 kb of DNA per cell, with a 95% CI of 10,300–13,900. The predicted value of 11,244 kb lies within these limits. The results obtained from the hybridization technique were thus consistent with the measured DNA content of serotype C cells.

Hybridization analysis of serotype 7 cells: Cells of an infectious serotype were also examined. Hybridization experiments were performed with serotype 7 as described above for serotype C with the exception that p7.16 was included in these experiments as a hybridization standard for bp7E. Cells were grown in broth medium to late log phase, confirmed to be at least 90% serotype 7 by indirect immunofluorescence with monoclonal antibodies, and frozen at −80°. Aliquots were removed and assayed in separate experiments. The results are given in Table 1 as experiments 3 to 5. The 95% confidence intervals from the 3 experiments overlap, indicating that the results are reproducible. Combining results and errors from the three experiments gives a mean copy number of 4.3 for the chromosome, with a 95% CI of 3.5–5.2. For bp7S, the combined copy number is 4.1 with a 95% CI of 3.5–4.7; for bp7E, the copy number is 6.3 with 5.2–7.3 as the 95% CI.

Fluorescence analysis of the silent plasmid bp7S: To confirm the results of these experiments, serotype 7 and C cells were also analyzed by a method that measures the fluorescence of an intercalating dye. DNA from cells lysed in agarose was mixed with *Apa*I-digested bacteriophage λ DNA, then separated by field inversion gel electrophoresis. Gels were then stained with ethidium bromide, placed on a UV transilluminator and photographed. Photographs were digitized, and scanning densitometry was performed on the images. The λ DNA served as a calibration control. Comparisons were made between the intensity of staining of the 32-kb bp7S and the 38-kb *Apa*I fragment of λ. Because ethidium bromide fluorescence is a measure of DNA mass, these comparisons indicate relative mass rather than relative copy number. The mass of 38-kb fragment added to each lane was therefore adjusted to equal the mass of a given number of copies of bp7S. Figure 6 shows the ethidium bromide-stained gel and densitometric analysis from one experiment. The first seven lanes contain DNA prepared from the same batch of serotype 7 cells analyzed above. Lanes 1–8 contain amounts (in terms of mass) of the 38-kb *Apa* I fragment equivalent to 0, 3.2, 6.5 or 9.7 copies of bp7S per cell, as indicated above each lane. The boundary of the area analyzed by densitometry is indicated, and the analysis is shown beneath the gel. Values obtained for this analysis are shown in Table 1.

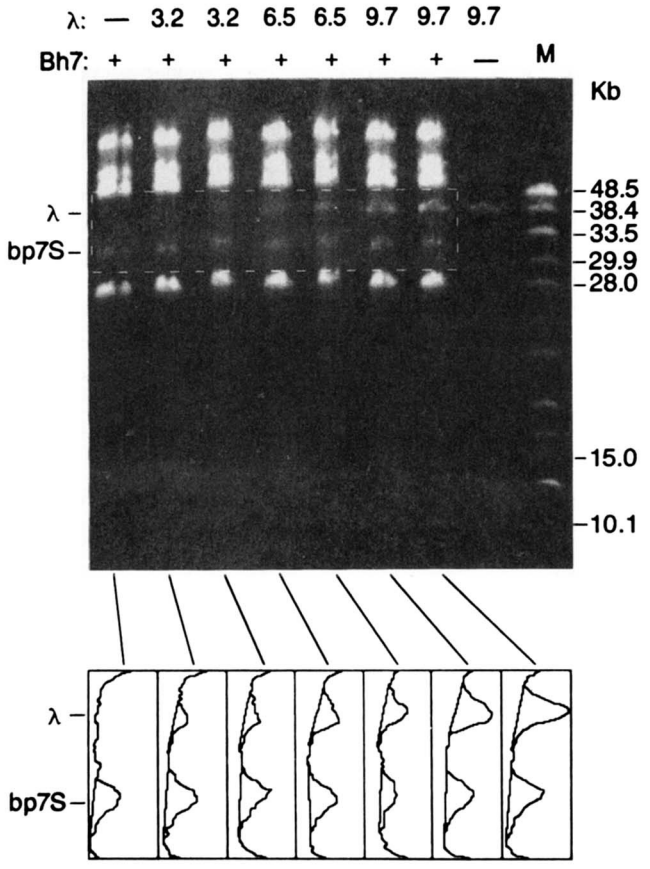

FIGURE 6.—Fluorescence analysis of the silent plasmid bp7S in serotype 7 cells. Lanes marked (+) were loaded with equal amounts of serotype 7 (Bh7) DNA. λ DNA (λ) was included in amounts equal to 3.2, 6.5 or 9.7 copies of bp7S per cell. The location of a 38-kb *Apa*I fragment of λ and the silent plasmid bp7S are indicated. Sizes of selected high molecular weight markers are shown to the right. The dashed rectangle indicates the region of the gel analyzed to create the density plot shown beneath. Lines connect density plots to the lanes from which they were made. The positions of the λ and bp7S peaks are indicated to the left of the density plots.

The megabase-sized chromosome could not be analyzed by this technique because some of it remains in the wells (FERDOWS and BARBOUR 1989) or is sheared and migrates below the main band. It was possible that the 32-kb linear plasmid bp7S also remained in part at the gel's origin or was in part degraded. Accordingly, an agarose gel similar to the one shown in Fig. 6 was dried and hybridized with oligo #1. The results were identical to those shown for the gel in Figure 1, that is, hybridization to DNA remaining in the well, or to DNA smaller than 28 kb was negligible compared to hybridization with 28 and 32-kb plasmids (data not shown). Although we were not able to analyze bp7E, the serotype 7 expression plasmid, by the fluorescence technique because it comigrates with two other linear plasmids common to serotype 7 and C cells, we could ensure that there were no plasmids comigrating with bp7S. Field inversion gels containing serotype 7 DNA were run as above, and the 32-kb band was electroeluted. Digestion with the restriction enzyme *Bam*HI produced fragments 28.0 and 3.4 kb in size; digestion with *Cla*I produced fragments of 20.2, 4.1, 4.0, 2.2 and 1.6 kb (not shown). The sum of these fragments is 31.4 kb for *Bam* HI and 32.1 kb for *Cla*I, indicating that the 32-kb band contains only bp7S.

A similar fluorescence analysis was performed with serotype C cells from the same batch. The analysis was complicated by the comigration of the 38-kb λ fragment and a 38-kb plasmid unique to serotype C cells of this lineage (A. G. BARBOUR, C. J. CARTER and C. S. FREITAG, manuscript in preparation). To correct for the presence of the 38-kb plasmid, its fluorescence was quantified by densitometry of a lane containing no λ DNA and then subtracted from the values obtained for the λ standards. The results (Table 1, experiment 2) are consistent with those obtained from the other methods.

Determination of copy number under different growth conditions: Because culture conditions are known to have profound effects on copy numbers of bacterial plasmids (ENGBERG and NORDSTRÖM 1975; KONTOMICHALOU, MITANI and CLOWES 1970; ROWND 1969) and chromosomes (BREMER and DENNIS 1987), we examined serotype 7 cells under different growth conditions. Cells grown in mice were compared with cells grown in broth medium. BALB/c mice were first irradiated to prevent the generation of a neutralizing immune response and then infected. Borrelias were harvested from the infected mice, counted, and imbedded in agarose plugs. The recovered cells were at least 95% serotype 7 by immunoassay. Table 1 lists the results from an analysis performed with borrelias taken from a single mouse (experiment 7) as well as borrelias isolated from the pooled sera of 4 mice (experiment 8). The two experiments yielded similar results. Combining the results and errors of experiments 7 and 8 yields mean copy numbers and 95% CIs of 15.7 and 13.0–18.4 for the chromosome, 7.6 and 6.6–8.6 for bp7S, and finally 14.1 and 11.7–16.6 for bp7E. All replicons tested were present in higher numbers than in the cultured serotype 7 cells. Interestingly, the chromosome and bp7E were present at significantly higher numbers than bp7S ($P < 0.01$, by Dunnett t test).

To determine the effect of cell density on copy number, serotype C cells were examined by hybridization analysis after growth in broth medium to densities of 0.4, 0.9 or 2.5×10^8 cells/ml. Average doubling times determined for the three cultures (KOCH 1981) were 7.5, 8.7 and 9.7 hr, respectively. Cells were harvested and imbedded in agarose in parallel, then added to lanes of the same gel for electrophoresis and subsequent analysis. Cells from cultures with 0.4 or 0.9×10^8 cells/ml had 15 copies of bp7S and 11 copies of the chromosome per cell. Cells grown to a

density of 2.5×10^8 cells/ml had 11 copies of both the chromosome and bp7S per cell (data not shown). These values are higher than those reported in experiment 1 (Table 1), for serotype C cells grown to a density of 3.0×10^8 cells/ml. These results suggest that copy numbers decrease as cells enter the stationary phase of growth. These results also indicate that copy number is inversely related to cell length, since cells in dense cultures are longer than cells in log phase cultures or cells taken from infected mice (BARBOUR and HAYES 1986).

Assessment of cell counting accuracy: We were concerned that the validity of the three methods was dependent upon the accuracy of cell counts in a Petroff-Hausser chamber. Systematic error in this step could have resulted in consistent but incorrect results in each experiment. To assess the accuracy of this step, serotype C cells were counted and serially diluted into tubes of a broth medium (BSK II) that supports the growth of single organisms (BARBOUR 1984). In tubes that were expected to contain 2.4, 0.24 and 0.024 organisms, growth was observed in 9, 1 and 0 of 11 tubes, respectively. This result is not significantly different from the values of 10, 2.3 and 0.3 of 11 tubes predicted from the Poisson distribution.

Fluorescent microscopy of stained DNA: We wondered how the multiple copies of the borrelial genome were distributed within the cell and, accordingly, employed the DNA-specific fluorochrome, DAPI. As a control for a filamentous organism, *E. coli* was treated with cephalexin, which inhibits cell division but not chromosome replication (HEDGE and SPRATT 1985), and with chloramphenicol, which increases nucleoid condensation (DONACHIE and BEGG 1989). The filamentous bacterial cells were dried onto slides, stained with DAPI, and visualized by mixed phase and fluorescent microscopy (Figure 7a). As reported previously for cell division mutants (DAI and LUTKENHAUS 1991; RICARD and HIROTA 1973), multiple discrete nucleoids are apparent.

Figure 7, b and c, shows untreated serotype C borrelias dried on slides, stained with DAPI and viewed as for *E. coli*. In cultures grown to densities of $2–3 \times 10^8$ cells ml^{-1}, 40–60% of fluorescing cells exhibited irregular and discrete staining, as shown in Figure 7b. Relatively homogeneous staining, which appears as a bright halo by this method of preparation and viewing (Figure 7c, demarcated with arrows), occurred in the remainder. When serotype C cultures grown to lesser densities or serotype 7 cells grown in mice were examined, 95–99% of cells stained homogeneously, with the remainder staining discretely. The majority of cells 7–11 μm in length stained homogeneously, fluorescence being absent only from cell termini. In most longer cells (12–60 μm in length), which were more common in dense cultures, staining was also broken by one or more interior gaps (Figure 7c). These gapped regions were often thinner than adjacent staining regions, suggesting that the gaps occurred at predivision sites. Chloramphenicol treatment of borrelias had no effect on distribution of DNA.

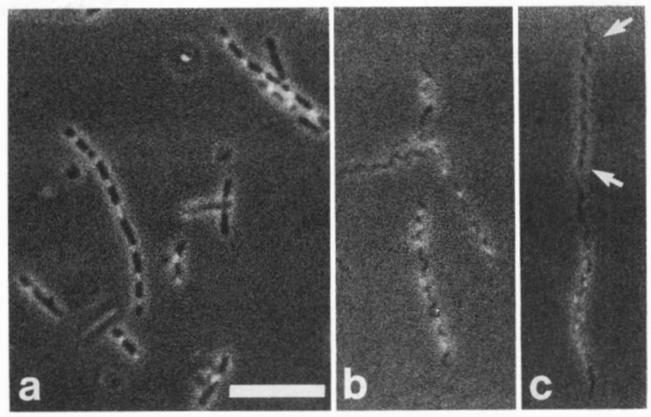

FIGURE 7.—Fluorescence microscopy of *E. coli* and *B. hermsii* cells stained with DAPI. Cells were dried onto slides, stained, and viewed by epifluorescence and phase microscopy. (a) *E. coli* grown in the presence of cephalexin and choramphenicol. Nucleoids appear white. (b) Discrete staining of *B. hermsii* serotype C cells. (c) Homogeneous staining of a cell from the same culture as (b). Arrows indicate the boundaries of staining in the upper half of the cell. Bar equals 10 μm.

Borrelias grown in mice and to different densities in culture were also examined after fixation on slides with methanol. Fixation increased the frequency of staining from approximately 50% to greater than 95% and had no effect on staining patterns; however, fixed cells retained insufficient contrast for simultaneous observation by phase and fluorescence microscopy (data not shown). Staining was judged to be DNA-dependent because treatment of fixed cells with 1 mg/ml DNase I for 1 hr greatly diminished fluorescence while 1 mg/ml RNase A had no effect. DAPI staining of borrelias that had been incubated in 1 M NaCl (AURAN, JOHNSON and RITZI 1972), which causes the outer sheath to partially separate from the protoplasmic cylinder, indicated that fluorescence was mostly associated with the protoplasmic cylinder (data not shown).

DISCUSSION

The hybridization method employed here derived from existing copy number determination procedures. NAGPAL *et al.* (1989) hybridized nick translated probes to chromosomal DNA and to cloned fragments of chromosomal DNA in Southern blots. PRENTKI, CHANDLER and CARO (1977) described the use of calibration curves and O'NEILL *et al.* (1989) demonstrated Image software. Innovations for this study include lysis of cells in agarose plugs rather than in liquid to avoid shearing and incomplete recovery of

the chromosome. To preclude loss of smaller restriction fragments by diffusion (FRITZ and MUSICH 1990), plugs were melted rather than suspended in buffer for restriction enzyme digestion. Finally, dried gels instead of Southern blots were probed because oligonucleotide probes bind more efficiently to DNA in dried gels than in blots (MEINKOTH and WAHL 1984), and because DNA fragmentation (WAHL, STERN and STARK 1979) and transfer steps are eliminated.

The hybridization method allowed for the simultaneous analysis of three replicons. Use of calibration curves constructed with standards of known copy number allowed for determination of absolute rather than relative copy number. Comparison of restriction fragments of identical size and sequence is expected to have eliminated inequalities in gel retention, denaturation, or probe-binding between standards and genomic DNA. Although calibration curves (Figure 5) were not linear, the number of data points from which they were constructed was sufficient to determine the shape of the curves. The curve flattening likely resulted from saturation of the X-ray film, inasmuch as a linear response was observed when bands were cut from dried gels and measured by scintillation counting (our unpublished findings). The chromosomal calibration curve in Figure 5 is more shallow than the 7S curve, probably because of less efficient labeling of the chromosomal probe. The range of copy numbers that can be measured by this method is limited but could be increased by making a separate exposure of a different duration for each replicon. Another approach would be to measure the radioactivity of each band by scintillation counting rather than by densitometric analysis of an autoradiogram. This method was not adopted because of the difficulty of locating, excising, and dissolving the sample bands.

One shortcoming of the hybridization method is that values obtained from it vary based on the proximity of a hybridization probe to a replicon's origin of replication. Loci replicated early will have higher copy numbers than loci replicated later. Origins of replication have not been identified in any of the replicons; their distance from our probes is thus unknown. However, we can examine the extreme possibilities, that is, the probes bound to either origins or termini of replication. The ratio of origins to termini may be calculated as $2^{(C/\tau)}$, where C is the time required to replicate a given replicon and τ is the doubling time of the cell (BREMER and DENNIS 1987). For the 28–32-kb silent and expression plasmids, C is likely to be small relative to τ (which was at least 4 hr in our experiments). Therefore, the proximity of the *vmp7* gene to the plasmid origins is likely of little consequence. We have no estimate of the time needed to replicate the 950-kb chromosome. Unless its replication time is longer than the cell's generation time, however, the chromosomal origin would have no more than twice the copy number of the terminus.

Measurement of total DNA content, our second procedure, is used commonly for copy number determination (HANSEN 1978; POSTGATE *et al.* 1984; SADOFF, SHIMEL and ELLIS 1979). Because this method does not yield exact copy numbers when plasmids are present, we used the results to confirm copy numbers determined by the hybridization method. The finding that the total DNA content of *B. hermsii* was near that expected from the copy number of the *vmp7* and *fla* genes (Table 1) suggests that, at least under one set of growth conditions, the copy number of these genes reflected the average copy number of the plasmids and chromosome. This result was most telling for the chromosome's copy number, because inaccuracy in its measurement (due for instance to the location of the *fla* gene, as discussed above) would have had a larger impact on the cell's expected DNA content than inaccuracy in plasmid copy number.

Several studies have indicated that the fluorescence of DNA-intercalated ethidium bromide can be used as a measure of replicon copy number (FREEMAN, LARCOM and THOMPSON 1990; PROJAN, CARLETON and NOVICK 1983; RIBEIRO, LARCOM and MILLER 1989). Requirements for this procedure include incorporation of internal calibration standards and use of appropriate background subtraction controls (RIBEIRO, LARCOM and MILLER 1989). The first requirement was fulfilled by mixing λ DNA with the borrelial DNA. Our choice of a standard was based on three considerations: (1) For comparison with the linear silent plasmid, a linear standard was required because binding of ethidium bromide is dependent upon DNA conformation (LEPECQ and PAOLETTI 1967). (2) It was preferable to use a standard near in size to that of the silent plasmid because the rate of DNA loss from agarose gels by diffusion is size-dependent (FRITZ and MUSICH 1990). (3) The DNA could have any A + T content because binding of ethidium bromide to DNA is not influenced by this property (LEPECQ and PAOLETTI 1967). A calibration curve was created by densitometry of the λ DNA. As with the hybridization experiments, the calibration curve was nonlinear but the number of standards used adequately described its shape (our unpublished findings).

The second requirement, subtraction of background fluorescence, is met by drawing baselines for density peaks (see Figure 6). Background fluorescence was determined in regions of the gel containing the λ standard and the silent plasmid. Densitometry of the first lane in Figure 6, which contains no λ DNA, suggested that the contribution of background fluorescence could be subtracted from the λ peak in other lanes by connecting points immediately above and below each peak with a straight line. Background

fluorescence in the region of the silent plasmid could not be evaluated in this way inasmuch as we did not have a *B. hermsii* strain lacking bp7S. However, isolation and digestion of the 32-kb band with restriction enzymes suggested the absence of comigrating plasmids (not shown). We therefore drew baselines for the bp7S peaks as we did for the λ peaks. Error in estimation of background density may account for the higher copy numbers obtained by the fluorescence method compared to the hybridization method.

It was possible that extracellular DNA contributed to our measurements. Borrelias produce extracellular membrane blebs (BARBOUR and HAYES 1986) which, in *B. burgdorferi*, have been shown to contain DNA (GARON, DORWARD and CORWIN 1989). However, it seems unlikely that blebs would have been included in our analysis. Whereas the procedure developed for their collection calls for centrifugation at 208,000 × *g* for 90 min (GARON, DORWARD and CORWIN 1989), we centrifuged borrelias at no more than 15,000 × *g* for 4 min in these experiments. DAPI staining of cells in which the protoplasmic cylinder was partially separated from the outer sheath suggested that most of the DNA in our cells was within the protoplasmic cylinder (data not shown.)

We determined that *B. hermsii* possesses multiple copies of two linear plasmids and the chromosome. The results of previous molecular cloning experiments suggested to us (PLASTERK, SIMON and BARBOUR 1985) that the *vmp* genes of *B. hermsii* were contained on plasmids with high copy numbers relative to the chromosome. The present results indicate that the two *vmp*7-bearing plasmids have, in fact, copy numbers equivalent to if not lower than that of the chromosome. The earlier assumption was in part based on the supposition that *B. hermsii*'s chromosome was equal in size and complexity to that of *E. coli*. The genome size of borrelias, as estimated from pulsed field electrophoresis (Figures 2 and 6) (our unpublished results), is only one fourth that of *E. coli*.

The expression plasmid's copy number has implications for the mechanism of antigenic variation. Serotype switching results from a recombination event that replaces the *vmp* gene residing on an expression plasmid with a copy of a formerly silent *vmp* gene. A switched expression plasmid apparently differs from its parent only at the *vmp* expression site (KITTEN and BARBOUR 1990; PLASTERK, SIMON and BARBOUR 1985). Unless the *vmp* gene contains the origin of plasmid replication, a partition locus, or another stability determinant, switched and unswitched expression plasmids would by definition be incompatible (NORDSTRÖM and AUSTIN 1989). Descendants of the switching cell would therefore eventually lose one or the other of the two plasmid types through segregation. If a theoretical analysis of plasmid incompatibility (NORDSTRÖM, MOLIN and AAGAARD-HANSEN 1981) is applied to plasmids of mouse-grown borrelias, we arrive at a minimum of five generations between a gene switch and the appearance of a cell lacking an unswitched expression plasmid.

Coexpression could be shortened by quicker segregation of switched from unswitched expression plasmids. In the model applied above, plasmids are chosen randomly from a common cytoplasmic pool for replication and partitioning. We could imagine another model in which there are multiple nucleoids, each containing a complement of plasmids and a chromosome which replicate and segregate together. Mouse-grown cells would then require only three generations to completely segregate switched and unswitched expression plasmids. Figure 7 suggests that borrelial DNA is contained within multiple nucleoids. If so, the only difference between cells staining homogeneously and those staining discretely may be the degree of nucleoid condensation. It is difficult to estimate the concentration of DNA within borrelias because of their irregular shape and slight width. However, it is plausible that uncondensed nucleoids would occupy most of the borrelia's length, as occurs in *E. coli* untreated with chloramphenicol (our unpublished findings). At the very least, Figure 7 shows that DNA within borrelias is distributed through most of the cell's length rather than contained within a single central nucleoid and that segregation of cellular DNA into at least two compartments occurs well in advance of cell division. These findings indicate that a given plasmid would not have an equal probability of segregating to either daughter cell upon cell division.

Coexpression could be eliminated entirely if borrelias possess a mechanism for preventing the simultaneous expression of two *vmp* genes. African trypanosomes undergo antigenic variation that is similar in many respects to that of *B. hermsii* (BORST 1991; CROSS 1990). However, trypanosomes differ from *B. hermsii* in stably maintaining several different telomeric expression sites, which are probably present in only one or two copies each (VAN DER PLOEG *et al.* 1989). Expression usually occurs at only one of these sites at any given time. Antigen genes may be activated either by placement into an active expression site or by a process termed *in situ* activation, in which one expression site is deactivated and another activated without any detectable recombination. Borrelias may also transcribe *vmp*s from only one or a few of their many copies of expression plasmids. Inactive copies might be heritably silenced without DNA sequence alteration. This phenomenon, called imprinting (HOLLIDAY 1990), is a common mechanism by which eukaryotic cells inactivate a sequence or entire chromosome when a duplicate sequence or chromosome occurs in the same cell. Imprinting has recently been described for

prokaryotic cells as well (THALER, ROTH and HIRSCHBEIN 1990).

Perhaps coexpression of Vmps does occur for extended periods. If coexpressing cells are eliminated by the immune system as efficiently as the original serotype, this would imply a recombination rate that is much higher than the rate of switching to a uniformly different serotype. Applying an analysis of NEWCOMBE (1948) to this case, we would calculate the rate of *vmp* gene switching to be 2^m times the observed rate of serotype switching, where m is the number of generations during which coexpression occurs. If $m = 3$ generations, the rate of recombination would be 8 times the estimated rate of 1×10^{-3} to 1×10^{-4} switches per cell per generation (STOENNER, DODD and LARSEN 1982). For $m = 5$, the recombination rate would be multiplied by 32, implying that *vmp* gene replacement would occur in one of every 30–300 cells. The finding that recombination does not always produce variants with antigenically distinct Vmps (T. KITTEN, A. V. BARERRA and A. G. BARBOUR, manuscript in preparation) raises the possibility that recombination is even more frequent. This would suggest that antigen switching is a directed process initiated by site-specific enzymes rather than a consequence of incidental homologous recombination (BORST and GREAVES 1987).

The copy number of the silent plasmid bp7S was determined in part to distinguish between gene conversion and reciprocal recombination with loss of one of the products as more likely mechanisms for *vmp* switching. The reciprocal recombination product that has not been detected is a silent plasmid bearing a formerly expressed *vmp* gene in place of a silent *vmp* gene. If the copy number of silent plasmids were one or two, random partitioning following recombination would frequently give rise to descendents possessing the recombinant silent plasmid. With a mean copy number of about 8 per cell, we cannot exclude the possibility that failure to detect a recombinant silent plasmid results from its loss by segregation.

Chromosomal copy number was estimated at 16 copies per cell for serotype 7 borrelias grown in mice. This is much higher than copy numbers measured for the bacteria *E. coli* and *Salmonella typhimurium*. Chromosomal copy number increases with increased growth rate in these cells, but only to the degree necessary to ensure that each daughter cell receives a single chromosome (BREMER and DENNIS 1987). *E. coli* growing at its near maximal rate, with a doubling time of 24 min, has on average 6.5 chromosomal origins and 1.9 termini per cell (BREMER and DENNIS 1987). Chromosomal copy numbers similar to those of *B. hermsii* have been reported for several unrelated bacteria. *Micrococcus radiodurans* reportedly contains no fewer than four chromosomes per cell under conditions of slow growth and up to 8 copies under more favorable growth conditions (HANSEN 1978). *Desulfovibrio gigas* contains 9 genomes per cell when grown in a chemostat under conditions of ammonia limitation and 17 genomes per cell when grown in standard batch culture (POSTGATE *et al.* 1984). The copy number of the *Azotobacter vinlandii* chromosome was estimated to be 40 copies/cell or greater under one set of growth conditions (NAGPAL *et al.* 1989).

For these cell populations, copy number generally correlated with growth rate. Serotype 7 cells grown in broth culture have doubling times of 10–12 hr in logarithmic growth (our unpublished findings). In contrast, serotype C cells, which are selected for growth in culture, have doubling time of 6–8 hr and serotype 7 cells grown in mice have doubling times of 4–6 hr (BARBOUR and STOENNER 1985; STOENNER, DODD and LARSEN 1982). Although the copy number of bacterial chromosomes is expected to increase at increased growth rates (as discussed above), multiple-copy plasmids such as R1, R6K and NR1, more commonly exhibit the opposite response (ENGBERG and NORDSTRÖM 1975; KONTOMICHALOU, MITANI and CLOWES 1970; ROWND 1969). In this respect, the linear plasmids of *B. hermsii* more closely resemble the F and P1 plasmids of *E. coli*. These plasmids have copy numbers equal to or less than that of the chromosome and must therefore increase their copy number at higher growth rates to ensure their inheritance (KEASLING, PALSSON and COOPER 1991; PRENTKI, CHANDLER and CARO 1977). It is also interesting to note that, unlike *E. coli*, borrelias decrease in size at increased growth rates (BARBOUR and HAYES 1986). Thus, smaller cells generally contain more DNA per unit volume than larger cells.

As described above, copy numbers of the replicons changed both absolutely and relative to one another under different growth conditions. All three examined replicons were present in similar numbers in serotype 7 cells grown in broth medium. Under these conditions, copy numbers were lower for each replicon in these cells than in serotype C cells in medium and serotype 7 cells growing in mice. The latter cells contained significantly fewer silent (bp7S) plasmids than either expressed plasmids or chromosomes. The higher copy number of the chromosome and expression plasmid compared to that of the silent plasmid may be driven by a requirement for increased dosage of genes encoded by these replicons. The chromosome presumably carries most of the cell's structural genes, and the most abundant outer membrane protein of the cell, the Vmp, is transcribed from the expression plasmid (BARBOUR *et al.* 1991a). Silent plasmids may function solely as repositories for silent *vmp* genes, the dosage of which would presumably be less important.

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LITERATURE CITED

AURAN, N. E., R. C. JOHNSON and D. M. RITZI, 1972 Isolation of the outer sheath of *Leptospira* and its immunogenic properties in hamsters. Infect. Immunity **5:** 968–975.

BARBOUR, A. G., 1984 Isolation and cultivation of Lyme disease spirochetes. Yale J. Biol. Med. **78:** 521–525.

BARBOUR, A. G., 1990 Antigenic variation of a relapsing fever *Borrelia* species. Annu. Rev. Microbiol. **44:** 155–171.

BARBOUR, A. G., and C. F. GARON, 1987 Linear plasmids of the bacterium *Borrelia burgdorferi* have covalently closed ends. Science **237:** 409–411.

BARBOUR, A. G., and S. F. HAYES, 1986 Biology of *Borrelia* species. Microbiol Rev **50:** 381–400.

BARBOUR, A. G., and H. G. STOENNER, 1985 Antigenic variation of *Borrelia hermsii*, pp. 123–135 in *Genome Rearrangement*, edited by I. HERSKOWITZ and M. I. SIMON. Alan R. Liss, New York.

BARBOUR, A. G., S. L. TESSIER and H. G. STOENNER, 1982 Variable major proteins of *Borrelia hermsii*. J. Exp. Med. **156:** 1312–1324.

BARBOUR, A. G., S. L. TESSIER and W. J. TODD, 1983 Lyme disease spirochetes and ixodid tick spirochetes share a common surface antigenic determinant defined by a monoclonal antibody. Infect. Immun. **41:** 795–804.

BARBOUR, A. G., S. F. HAYES, R. A. HEILAND, M. E. SCHRUMPF and S. L. TESSIER, 1986 A *Borrelia*-specific monoclonal antibody binds to a flegellar epitope. Infect. Immun. **52:** 549–554.

BARBOUR, A. G., N. BURMAN, C. J. CARTER, T. KITTEN and S. BERGSTRÖM, 1991a Variable antigen genes of the relapsing fever agent *Borrelia hermsii* are activated by promoter addition. Mol. Microbiol. **5:** 489–493.

BARBOUR, A. G., C. J. CARTER, N. BURMAN, C. S. FREITAG, C. F. GARON and S. BERGSTRÖM, 1991b Tandem insertion sequence-like elements define the expression site for variable antigen genes of *Borrelia hermsii*. Infect. Immun. **59:** 390–397.

BARIL, C., C. RICHAUD, G. BARANTON and I. SAINT GIRONS, 1989 Linear chromosome of *Borrelia burgdorferi*. Res. Microbiol. **140:** 507–516.

BARSTAD, P. A., J. E. COLIGAN, M. G. RAUM and A. G. BARBOUR, 1985 Variable major proteins of *Borrelia hermsii*. Epitope mapping and partial sequence analysis of CNBr peptides. J. Exp. Med. **161:** 1302–1314.

BORST, P., 1991 Molecular genetics of antigenic variation. Immunol. Today **12:** A29-A33.

BORST, P., and D. R. GREAVES, 1987 Programmed gene rearrangements altering gene expression. Science **235:** 658–667.

BREMER, H., and P. P. DENNIS, 1987 Modulation of chemical composition and other parameters of the cell by growth rate, pp. 1527–1542 in *Escherichia coli and Salmonella typhimurium*, edited by F. C. NEIDHARDT. American Society for Microbiology, Washington, D.C.

BURMAN, N., S. BERGSTRÖM, B. I. RESTREPO and A. G. BARBOUR, 1990 The variable antigens Vmp7 and Vmp21 of the relapsing fever bacterium *Borrelia hermsii* are structurally analogous to the VSG proteins of the African trypanosome. Mol. Microbiol. **4:** 1715–1726.

CROSS, G. A. M., 1990 Cellular and genetic aspects of antigenic variation in trypanosomes. Annu. Rev. Immunol. **8:** 83–110.

DAI, K., and J. LUTKENHAUS, 1991 *ftsZ* is an essential cell division gene in *Escherichia coli*. J. Bacteriol. **173:** 3500–3506.

DAVIDSON, B. E., J. MACDOUGALL and I. SAINT GIRONS, 1992 Physical map of the linear chromosome of the bacterium *Borrelia burgdorferi* 212, a causative agent of Lyme disease, and localization of rRNA genes. J. Bacteriol. **174:** 3766–3774.

DAXHELET, G. A., M. M. COENE, P. P. HOET and C. G. COCITO, 1989 Spectrofluoromotry of dyes with DNAs of different base composition and conformation. Anal. Biochem. **179:** 401–403.

DONACHIE, W. D., and K. J. BEGG, 1989 Cell length, nucleoid separation, and cell division of rod-shaped and spherical cells of *Escherichia coli*. J. Bacteriol. **171:** 4633–4639.

ENGBERG, B., and K. NORDSTRÖM, 1975 Replication of R-factor R1 in *Escherichia coli* K-12 at different growth rates. J. Bacteriol. **123:** 179–186.

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.

FREEMAN, S. E., L. L. LARCOM and B. D. THOMPSON, 1990 Electrophoretic separation of nucleic acids: evaluation by video and photographic densitometry. Electrophoresis **11:** 425–431.

FRITZ, R. B., and P. R. MUSICH, 1990 Unexpected loss of genomic DNA from agarose gel plugs. BioTechniques **9:** 542–550.

FULTON, J. P., and P. J. C. SMITH, 1960 Carbohydrate metabolism in *Spirochaeta recurrentis*. I. The metabolism of spirochaetes *in vivo* and *in vitro*. Biochem. J. **76:** 491–499.

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. Suppl. **3:** 109–115.

GUERRY, P., S. M. LOGAN, S. THORNTON and T. J. TRUST, 1990 Genomic organization and expression of *Campylobacter* flagellin genes. J. Bacteriol. **172:** 1853–1860.

HANAHAN, D., 1983 Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:** 557–580.

HANSEN, M. T., 1978 Multiplicity of genome equivalents in the radiation-resistant bacterium *Micrococcus radiodurans*. J. Bacteriol. **134:** 71–75.

HEDGE, P. J., and B. G. SPRATT, 1985 Amino acid substitutions that reduce the affinity of penicillin-binding protein 3 of *Escherichia coli* for cephalexin. Eur. J. Biochem. **151:** 111–121.

HIRAGA, S., H. NIKI, T. OGURA, C. ICHINOSE, H. MORI, B. EZAKI and A. JAFFE, 1989 Chromosome partitioning in *Escherichia coli*: novel mutants producing anucleate cells. J. Bacteriol. **171:** 1496–1505.

HOLLIDAY, R., 1990 Genomic imprinting and allelic exclusion. Development 3 (Suppl.): 125–129.

HYDE, F. W., and R. C. JOHNSON, 1984 Genetic relationship of Lyme disease spirochetes to *Borrelia*, *Treponema*, and *Leptospira*. J. Clin. Microbiol. **20:** 151–154.

KEASLING, J. D., B. O. PALSSON and S. COOPER, 1991 Cell-cycle specific F plasmid replication: regulation by cell size control of initiation. J. Bacteriol. **173:** 2673–2680.

KITTEN, T., and A. G. BARBOUR, 1990 Juxtaposition of expressed variable antigen genes with a conserved telomere in the bacterium *Borrelia hermsii*. Proc. Natl. Acad. Sci. USA **87:** 6077–6081.

KOCH, A. L., 1981 Growth measurement, pp. 179–207 in *Manual of Methods for General Bacteriology*, edited by P. GERHARDT. American Society for Microbiology, Washington, D.C.

KONTOMICHALOU, P., M. MITANI and R. C. CLOWES, 1970 Circular R-factor molecules controlling penicillinase synthesis, replicating in *Escherichia coli* under either relaxed or stringent control. J. Bacteriol. **104:** 34–44.

LEPECQ, J.-B., and C. PAOLETTI, 1967 A fluorescent complex

between ethidium bromide and nucleic acids: physical-chemical characterization. J. Mol. Biol. **27:** 87–106.

MANIATIS, T., E. F. FRITSCH and J. SAMBROOK, 1982 *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

MEIER, J. T., M. I. SIMON and A. G. BARBOUR, 1985 Antigenic variation is associated with DNA rearrangements in a relapsing fever Borrelia. Cell **41:** 403–409.

MEINKOTH, J., and G. WAHL, 1984 Hybridization of nucleic acids immobilized on solid supports. Anal. Biochem. **138:** 267–284.

NAGPAL, P., S. JAFRI, M. A. REDDY and H. K. DAS, 1989 Multiple chromosomes of *Azotobacter vinelandii.* J. Bacteriol. **171:** 3133–3138.

NEWCOMBE, H. B., 1948 Delayed phenotypic expression of spontaneous mutations in *Escherichia coli.* Genetics **33:** 447–476.

NORDSTRÖM, K., and S. J. AUSTIN, 1989 Mechanisms that contribute to the stable segregation of plasmids. Annu. Rev. Genet. **23:** 37–69.

NORDSTRÖM, K., S. MOLIN and H. AAGAARD-HANSEN, 1981 Plasmid R1 incompatibility, pp. 291–301 in *Molecular Biology, Pathogenicity, and Ecology of Bacterial Plasmids,* edited by S. B. LEVY, R. CLOWES and E. L. KOENIG. Plenum, New York.

O'NEILL, R. R., L. G. MITCHELL, C. R. MERRIL and W. S. RASBAND, 1989 Use of image analysis to quantitate changes in form of mitochondrial DNA after x-irradiation. Appl. Theor. Electrophor. **1:** 163–167.

PASTER, B. J., F. E. DEWHIRST, W. G. WEISBURG, L. A. TORDOFF, G. J. FRASER, R. B. HESPELL, T. B. STANTON, L. ZABLEN, L. MANDELCO and C. R. WOESE, 1991 Phylogenetic analysis of the spirochetes. J. Bacteriol. **173:** 6101–6109.

PLASTERK, R. H., 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 **318:** 257–263.

PLEIER, E., and R. SCHMITT, 1989 Identification and sequence analysis of two related flagellin genes in *Rhizobium meliloti.* J. Bacteriol. **171:** 1467–1475.

POSTGATE, J. R., H. M. KENT, R. L. ROBSON and J. A. CHESSHYRE, 1984 The genomes of *Desulfovibrio gigas* and *D. vulgaris.* J. Gen. Microbiol. **130:** 1597–1601.

PRENTKI, P., M. CHANDLER and L. CARO, 1977 Replication of the prophage P1 during the cell cycle of *Escherichia coli.* Mol. Gen. Genet. **152:** 71–76.

PROJAN, S. J., S. CARLETON and R. P. NOVICK, 1983 Determination of plasmid copy number by fluorescence densitometry. Plasmid **9:** 182–190.

RIBEIRO, E. A., L. L. LARCOM and D. P. MILLER, 1989 Quantitative fluorescence of DNA-intercalated ethidium bromide on agarose gels. Anal. Biochem. **181:** 197–208.

RICARD, M., and Y. HIROTA, 1973 Process of cellular division in *Escherichia coli*: physiological study on thermosensitive mutants defective in cell division. J. Bacteriol. **116:** 314–322.

ROWND, R., 1969 Replication of a bacterial episome under relaxed control. J. Mol. Biol. **44:** 387–402.

SADOFF, H. L., B. SHIMEL and S. ELLIS, 1979 Characterization of *Azotobacter vinelandii* deoxyribonucleic acid and folded chromosomes. J. Bacteriol. **138:** 871–877.

SNEDECOR, G. W., and W. G. COCHRAN, 1967 *Statistical Methods.* The Iowa State University Press, Ames.

STOENNER, H. G., T. DODD and C. LARSEN, 1982 Antigenic variation of *Borrelia hermsii.* J. Exp. Med. **156:** 1297–1311.

SZOSTAK, J. W., T. L. ORR-WEAVER and R. J. ROTHSTEIN, 1983 The double-strand-break repair model for recombination. Cell **33:** 25–35.

TAYLOR, J. R., 1982 *An Introduction to Error Analysis: The Study of Uncertainties in Physical Measurements.* University Science Books, Mill Valley, Calif.

THALER, D. S., J. R. ROTH and L. HIRSCHBEIN, 1990 Imprinting as a mechanism for control of gene expression, pp. 445–456 in *The Bacterial Chromosome,* edited by K. DRLICA and M. RILEY. American Society for Microbiology, Washington, D.C.

VAN DER PLOEG, L. H. T., C. L. SMITH, R. I. POLVERE and K. M. GOTTESDIENER, 1989 Improved separation of chromosome-sized DNA from *Trypanosoma brucei,* stock 427-60. Nucleic Acids Res. **17:** 3217–3227.

WAHL, G. M., M. STERN and G. R. STARK, 1979 Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization by using dextran sulfate. Proc. Natl. Acad. Sci. USA **76:** 3683–3687.

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