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The Bacterial Nucleoid Visualized by Fluorescence Microscopy of Cells Lysed within Agarose: Comparison of *Escherichia coli* and Spirochetes of the Genus *Borrelia*

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The nucleoids of *Escherichia coli* and the spirochetes *Borrelia burgdorferi* and *Borrelia hermsii*, agents of Lyme disease and relapsing fever, were examined by epifluorescence microscopy of bacterial cells embedded in agarose and lysed in situ with detergent and protease. The typical *E. coli* nucleoid was a rosette in which 20 to 50 long loops of DNA emanated from a dense node of DNA. The percentages of cells in a population having nucleoids with zero, one, two, and three nodes varied with growth rate and growth phase. The borrelia nucleoid, in contrast, was a loose network of DNA strands devoid of nodes. This nucleoid structure difference correlates with the unusual genome of *Borrelia* species, which consists primarily of linear replicons, including a 950-kb linear chromosome and linear plasmids. This method provides a simple, direct means to analyze the structure of the bacterial nucleoid.

Advances in DNA technology have led to an era of genomic sequencing. The entire nucleotide sequences of four bacterial genomes have been determined, and others, including that of *Escherichia coli*, will soon follow (1). Thus, the primary structure of the bacterial genome is becoming known in complete detail. By comparison, higher-order structures of the chromosome and how these structures accommodate transcription, replication, and partition during the bacterial cell cycle remain poorly understood (41). Because of a relative paucity of visual and cytologic clues, the structure of the prokaryotic nucleoid is also poorly understood in comparison with the structure of the eukaryotic chromosome (35).

Based primarily on the *E. coli* model, the bacterial chromosome has long been envisaged as a single large circular molecule. The *E. coli* chromosome consists of 4.7 Mb of DNA which, when extended, is about 1.5 mm long. Its being packaged into a cell only 2 μm long mandates a highly ordered structure. Studies of the *E. coli* nucleoid over many years have revealed an irregularly shaped, compact structure confined to less than half of the intracellular space (23, 29). During periods of rapid growth, new rounds of chromosomal replication initiate before segregation of daughter chromosomes, and nucleoid size and shape change with growth conditions (17, 32, 41). The DNA is negatively supercoiled in vivo, and a number of abundant small, basic nucleoid-associated proteins (sometimes referred to as histone-like proteins) are thought to be involved in DNA packaging, with additional roles in transcription, recombination, and replication (23, 29). Worcel and Burgi first showed that the *E. coli* chromosome is organized into independent supercoiled domains (42), a model later supported by electron microscopy and other methods (22, 23, 29, 30, 37).

Recently it has become apparent that the *E. coli* paradigm does not apply to all bacteria (21). Spirochetes of the genus *Borrelia*, for example, have a predominantly linear genome that is segmented among a 950-kb linear chromosome and several linear plasmids of 15 to 200 kb (3, 34). Circular supercoiled plasmids, the form typical of plasmids of other bacteria, are also present in borreliae. The linear plasmids, and likely the chromosome, have single-stranded hairpin loops connecting the two DNA strands at each end (4, 19). The presence in relapsing fever borreliae of linear plasmids that are 20% of the size of the chromosome blurs the distinction between the two, and it has been suggested that the linear DNA of *Borrelia* spp. represents a collection of minichromosomes (3).

Chromosome structure and size are not the only characteristics in which *Borrelia* and *E. coli* genomes differ. *Borrelia hermsii* has been determined to be polyploid in the sense that cells cultured in vitro contain 8 to 11 copies each of the chromosome and plasmids and contain up to 16 copies when grown in mice (24). Cytological examination revealed that about half of the spirochetes in a stationary-phase population contained a series of discretely staining bodies of DNA, suggestive of multiple nucleoids, but the remainder exhibited a homogeneous DNA staining pattern along the length of the protoplasmic cylinder. During exponential phase, the homogeneous staining pattern was seen in 95 to 99% of cells (24). These findings suggested that the DNA content of a borrelia is not contained within a central condensed nucleoid, as it is for *E. coli*, but instead is distributed throughout the approximately 20-μm length of the spirochete during most stages of growth.

Previous analyses of the bacterial nucleoid have usually relied on subjecting disrupted cells to various chemical and physical treatments to isolate nucleoids. In this study we used fluorescence microscopy to directly visualize intact chromosomes released from individual agarose-embedded bacteria lysed in situ (7). This method allowed a facile examination of the nucleoids of individual *E. coli* and *Borrelia* cells under different growth conditions. The results support the previously described domain substructure model of the *E. coli* nucleoid. Variations in nucleoid appearance were observed, however, which correlated with different growth conditions. The borrelia nucleoid exhibited a structural organization clearly different from that of *E. coli*.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* N99 (*galK strA*), a plasmidless K-12 strain, was cultivated at 37°C with shaking at 250 rpm in a rich M63 liquid medium (27) supplemented with 0.5% Casamino Acids and 0.2% glucose or in a minimal M63 medium containing 0.2% sodium acetate. Cell doubling times in the two media were 30 min and 3 h, respectively (18). *Borrelia burgdorferi* B31, a high-passage isolate containing linear plasmids of 49, 29, and 17 kb and circular plasmids of 27 and 32 kb, and *B. hermsii* HS1, containing at least seven linear plasmids of 15 to 180 kb and two small circular plasmids, were cultured in BSK II liquid medium at 34°C as previously described (2). The cell doubling time under these conditions is 8 to 10 h, the maximum in vitro growth rate of *Borrelia* spp. (5).

Bacteria from exponential-phase, stationary-phase, and chloramphenicol (CAM)-treated cultures were examined. For rapidly growing *E. coli*, supplemented M63 medium was inoculated with 0.01 volume of an overnight culture and incubated for 3 h to obtain exponential-phase cells. Cell density as determined by using a Petroff-Hausser counting chamber was 9×10^7 cells per ml. Stationary-phase cells were from a 24-h culture containing 4×10^9 cells per ml. To determine the effects of inhibition of protein synthesis on the nucleoid, CAM was added to an exponential-phase culture at a concentration of 170 μg/ml, and incubation was continued for an additional 24 h. Slow-growing *E. coli* cells in exponential phase (7×10^7 cells per ml) were collected after 33 h of incubation in M63-acetate minimal medium, and those in stationary phase (8.0×10^8 cells per ml) were collected from another culture after 54 h of incubation. CAM treatment was the same as for rapidly growing cells. Exponential-phase *B. burgdorferi* and *B. hermsii* were from 24-h BSK II cultures and contained 4×10^6 to 7×10^6 spirochetes per ml. Stationary-phase cultures had been incubated for 72 h and contained approximately 1.5×10^8 spirochetes per ml. CAM-treated cells were from a log-phase culture to which 170 μg of the antibiotic per ml had been added and incubated for an additional 48 h. The MIC of CAM for *B. burgdorferi* is 2 μg/ml (14).

Preparation of DNA and PFGE. *E. coli* and *Borrelia* cells from exponential-phase, stationary-phase, and CAM-treated cultures were embedded in agarose and lysed in situ by the method of Ferdows and Barbour (16). Cells were washed twice with 50 mM Tris (pH 8)–150 mM NaCl (TN) and resuspended in TN to a concentration of 5×10^6 cells per ml. Four serial 10-fold dilutions of this suspension were made in TN. High-density suspensions containing approximately 5×10^9 cells per ml of TN were also prepared. An equal volume of molten (37°C) 1% InCert low-melting-temperature agarose (FMC BioProducts, Rockland, Maine) was mixed with the cell suspensions, and 0.1-ml aliquots were transferred to a mold and allowed to gel in the form of agarose blocks. Cells were lysed in situ by incubating the blocks for 16 h at 45°C in 50 mM Tris (pH 8)–50 mM EDTA-1% sodium dodecyl sulfate (SDS) containing 1 mg of proteinase K per ml. The agarose blocks were washed three times for 1 h with 10 mM Tris (pH 8)–1mM EDTA (TE) and stored at 8°C in a small volume of TE until analysis. Exposure to lysozyme and RNase prior to addition of the lysis buffer did not alter the appearance of the nucleoids by fluorescence microscopy (see below) for *E. coli* (7).

DNA in slices of the treated agarose blocks was subjected to pulsed-field gel electrophoresis (PFGE) in a 1.5% low-melting-point agarose gel (Gibco BRL, Gaithersburg, Md.) in 0.5× TBE (1× TBE is 90 mM Tris, 90 mM boric acid, and 2 mM EDTA, pH 8). Electrophoresis was for 24 h at 5 V/cm, during which a pulsed field was achieved by rotation of the circular gel 105° in the buffer tank every 120 s. After electrophoresis, DNA in the gel was stained for 1 h in 0.5 μg of ethidium bromide per ml and destained in three changes of 0.25× TBE during a 2-h period.

Fluorescence microscopy. Isolated linear chromosome and 49-kb linear plasmid molecules of *B. burgdorferi* B31 were obtained by removing 5- to 10-μl plugs from the appropriate areas of an ethidium bromide-stained PFGE gel. *Borrelia* and *E. coli* DNA molecules that did not enter the gels were sampled from the well area of the gels after PFGE. The agarose plugs were equilibrated for 20 min in two changes of 3% β-mercaptoethanol–0.1 μg of ethidium bromide per ml in 0.5× TBE (BET). A single plug was placed on a glass microscope slide warmed to 58°C, and 10 to 15 μl of molten (37°C) BET containing 1% low-melting-point agarose (ABET) was placed next to it. A glass coverslip was carefully lowered until it contacted the agarose plug, and gentle pressure was applied until the plug melted, mixed with the ABET, and spread evenly under the coverslip. The slide was then cooled to allow the agarose to regel. The top and bottom edges of the coverslip were sealed with clear nail polish, and molten ABET was applied along the two side edges. For visualization of intact genomic DNA from individual cells, agarose blocks containing 2.5×10^5 or 2.5×10^6 cells per ml that had been lysed but not subjected to electrophoresis were stained by soaking in BET. Gel slices with volumes of about 15 to 20 μl were melted on a glass slide under a coverslip as described above.

Preparations were viewed with a Nikon Microphot-FX microscope with an epifluorescence attachment and a 60× oil immersion objective as described by Smith et al. (39). An image intensifier and a charge-coupled device camera were used to view images on a television monitor and to record them on S-VHS videotape. For some images an additional 2× magnifier was employed. An electric field was also applied in some cases by placing fine platinum wires next to the left and right edges of the coverslip and applying 0.5× TBE to them to complete the circuit. This technique allowed observation of individual DNA molecules undergoing electrophoresis in the gel layer under the coverslip (39). A 10-fold dilution series of agarose plugs was examined. Plugs with 2.5×10^5 cells per ml resulted in fewer than one nucleoid per 10 fields of view, indicating that each example encountered derived from a single cell. Images used for figures were made by still photography of the video monitor screen while the VCR tape was running, using a shutter speed of 1/15 s and Kodak T-MAX 400 film. Size estimates for individual plasmid DNA molecules were based on previous measurements of molecules of known size (8). Minimal size estimates for regions of nucleoids were based on 3 kb of B-form DNA per μm and 0.315 μm/mm of videoscreen image (or 0.158 μm/mm when the supplemental 2× magnifier was used) (8).

Differential counts of *E. coli* nucleoids. Nucleoids from individual *E. coli* cells were categorized according to the number of dense, brightly staining nodes that they contained. Slides prepared from agarose plugs not subjected to electrophoresis that contained 2.5×10^6 cells per ml were examined, and 61 to 104 individual nucleoids encountered while scanning the slide were scored as 0, 1, 2, or 3 depending on the number of nodes present within the nucleoid. An estimated 70 to 90% of the nucleoids on a given slide were scored; the remainder were insufficiently spread to score confidently but clearly resembled those scored in that arms or fibers of DNA extended from nodes. Replicate counts from rapidly growing cells were from separate slides prepared and counted by each author.

[^{3}H]thymidine incorporation by *B. burgdorferi*. To determine the effect of inhibition of protein synthesis on borrelial DNA replication, 0.6 mCi of aqueous [*methyl*-^{3}H]thymidine (DuPont NEN, Boston, Mass.) was added to 24 ml of an exponential-phase BSK II culture of *B. burgdorferi* B31. The culture was immediately split into two 12-ml aliquots. To one aliquot 60 μl of a 34-mg/ml stock solution of CAM dissolved in ethanol was added (final concentration, 170 μg/ml), and to the other aliquot 60 μl of ethanol was added. After 0, 0.5, 1, 2, 4, 8, 12, 24, 30, and 36 h of incubation at 34°C, triplicate 0.1-ml samples were removed from each culture and filtered onto individual 1-in.-diameter Hybond-N membranes (Amersham, Cleveland, Ohio) by using a vacuum filter apparatus. The membranes were immediately washed three times with 10 ml of H_2O, three times with 10 ml of 10% trichloroacetic acid, three times with 10 ml of 5% trichloroacetic acid, and twice with 10 ml of 95% ethanol. After the membranes dried, thymidine incorporation into acid-precipitable DNA was measured by using a Beckman LS 3800 scintillation counter.

RESULTS

We used epifluorescence microscopy to directly visualize the nucleoid structures of two disparate eubacterial groups represented by three species: the well-studied *E. coli*, a member of the *Protobacteriaceae* which has a (presumably) circular chromosome of 4.7 Mb, and the spirochetes *B. burgdorferi* and *B. hermsii*, which have a 950-kb linear chromosome. Bacterial cells were embedded in agarose and lysed in situ. The liberated DNA was stained with ethidium bromide while still contained within the protective gel matrix. The agarose was then melted when it was placed on a warmed glass slide and gentle pressure was applied with a coverslip. During melting and subsequent regelling of the agarose beneath the coverslip, the compact nucleoid was spread out, but hydrodynamic shear forces were sufficiently low that intact chromosome-sized molecules could be examined by fluorescence microscopy (7).

Properties of the *E. coli* nucleoid. During the cell cycle, the chromosome is in a dynamic state as replication proceeds and sister chromosomes are resolved and partitioned. We therefore examined the *E. coli* nucleoid from exponential-phase, stationary-phase, and CAM-treated cells grown in either a rich medium or a minimal medium. Several examples of individual *E. coli* chromosomes are shown in Fig. 1. In general, they displayed rosette structures in which an estimated 20 to 50 long arms of DNA emanated from one or more brightly stained dense spots or nodes. The extended arms of DNA did not appear to have free ends but instead were long loops of DNA (9). As reported previously (7), the loops at the ends of the DNA arms (arrowheads in Fig. 1C) were obvious only when they extended into a thin liquid layer directly underneath the coverslip and "opened up." This picture conforms to the domain substructure model of the *E. coli* nucleoid in which the nucleoid unfolds into approximately 50 independently super-

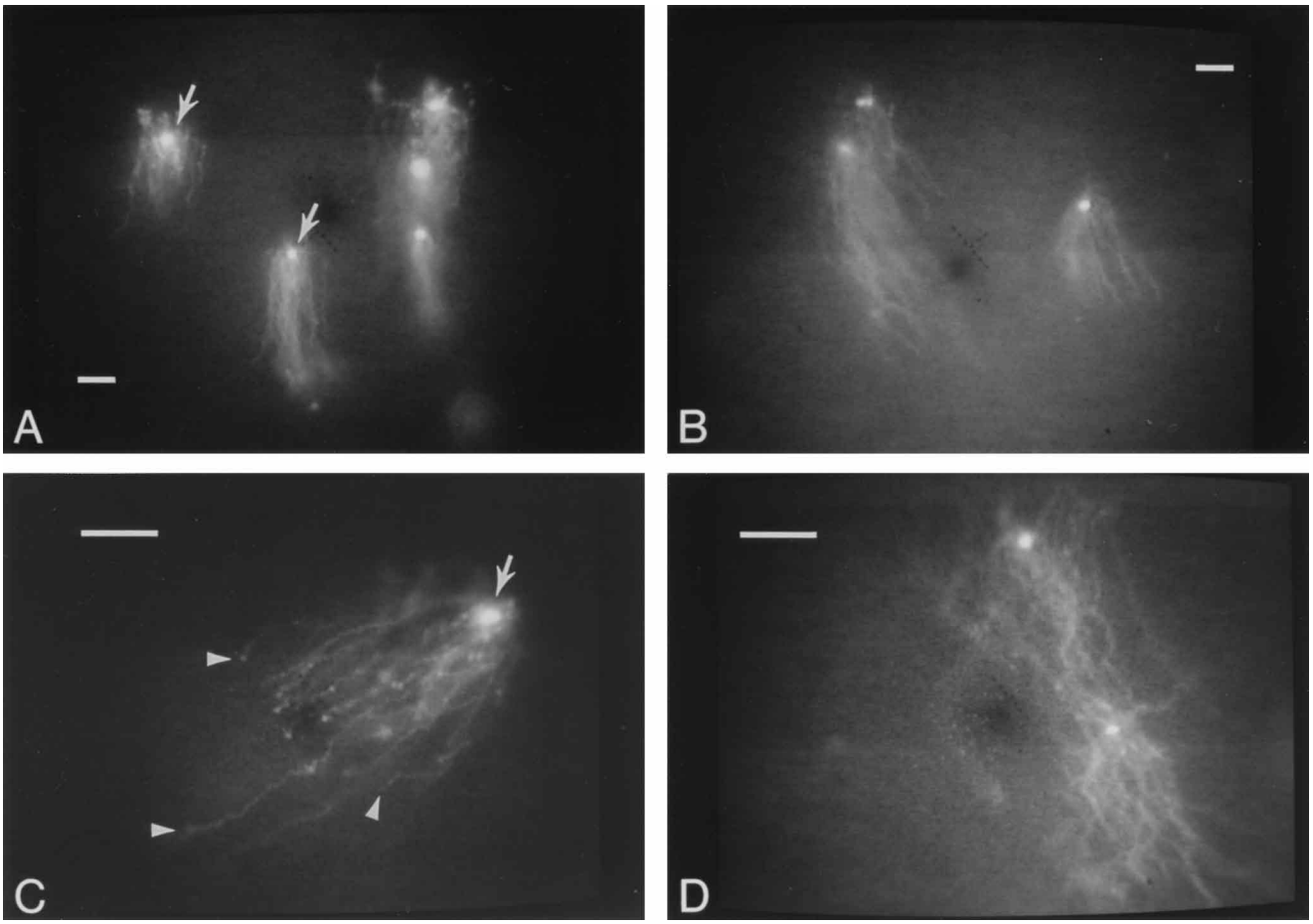

FIG. 1. Fluorescence microscopy of genomic DNA from *E. coli* cells lysed in situ in agarose. (A) Two nucleoids with a single dense, brightly staining node (arrows) from which long arms of DNA emanate and one nucleoid with three nodes and a greater amount of total DNA. (B) Examples with one and two nodes. (C) Higher magnification of a typical nucleoid showing the "rosette of loops" or "Medusa head" appearance with a single condensed node (arrow) and about 30 arms of DNA of various lengths. The DNA arms appear to terminate in loops (arrowheads), which are not resolved in these images (see text). (D) Higher magnification of an example with two nodes. (E and F) Single-node nucleoids in which spreading forces during preparation resulted in highly stretched arms of DNA extending unidirectionally from the node (E) and in a radial distribution of arms around the central node (F). (G and H) Examples showing a more diffuse staining pattern lacking a dense node. The magnification for panels A and B is one-half of that for panels C to H. Bars, 20 μm. Nucleoids from exponential-phase cells (B and D), stationary-phase cells (G and H), and CAM-treated cells (A, C, E, and F) are shown. All cells were grown in rich medium with a doubling time of 30 min.

coiled loops after treatment to remove nucleoid-associated proteins (29, 30, 32, 37, 42). The structure and number of the long arms of DNA seen in the examples in Fig. 1 thus are consistent with the domain loop model. Most of the DNA was contained in these arms, and nearly every rosette appeared to contain at least one genome equivalent of DNA. For example, Fig. 1E shows a nucleoid in which about 30 DNA arms (counted while focusing up and down) were spread unidirectionally from a node during preparation. At the magnification used, a strand of B-form DNA extending from the top to bottom of the picture would be 65 μm or 195 kb (see Materials and Methods) (8). With this estimate, the DNA arms in Fig. 1E vary in length from about 50 to 200 kb and, assuming they are loops, represent an aggregate of 4 to 6 Mb. Accurate determination of the size of large molecules is difficult because of local areas of DNA strand compaction and stretching during preparation (8). Measurements of these long molecules are underestimates, because the DNA arms rarely appeared fully stretched. Large relative differences (two- to fourfold) in the amount of DNA per nucleoid among individual examples in the same preparation were scored by inspection of the total density of the stained DNA strands.

A feature of *E. coli* nucleoid structure that varied with growth conditions was the brightly staining condensed node of DNA. Most nucleoids exhibited a single node from which the long arms of DNA extended (Fig. 1A, C, E, and F). In some nucleoids, however, two or three nodes were evident (Fig. 1A, B, and D). Other nucleoids did not show a rosette structure but instead exhibited a more dispersed organization, indicated by a more diffuse staining pattern with no large brightly staining condensed node (Fig. 1G and H). The proportion of cells in a population containing nucleoids with zero, one, two, or three condensed nodes varied with growth condition. During rapid growth in rich medium (doubling time, 30 min), exponential-phase cells had a higher percentage of multiple-node nucleoids than did stationary-phase cells or exponential-phase cells treated with CAM for 24 h to inhibit protein synthesis and initiation of new rounds of replication (Table 1). Nucleoids from slow-growing cells in acetate minimal medium (doubling time, 3 h) were more likely to show the diffuse staining pattern that lacked a local condensed node (Table 2). Nucleoids with the diffuse pattern were assigned to the zero-node category in the differential counts (Tables 1 and 2), but often they contained three or more smaller, less intensely stained spots (Fig.

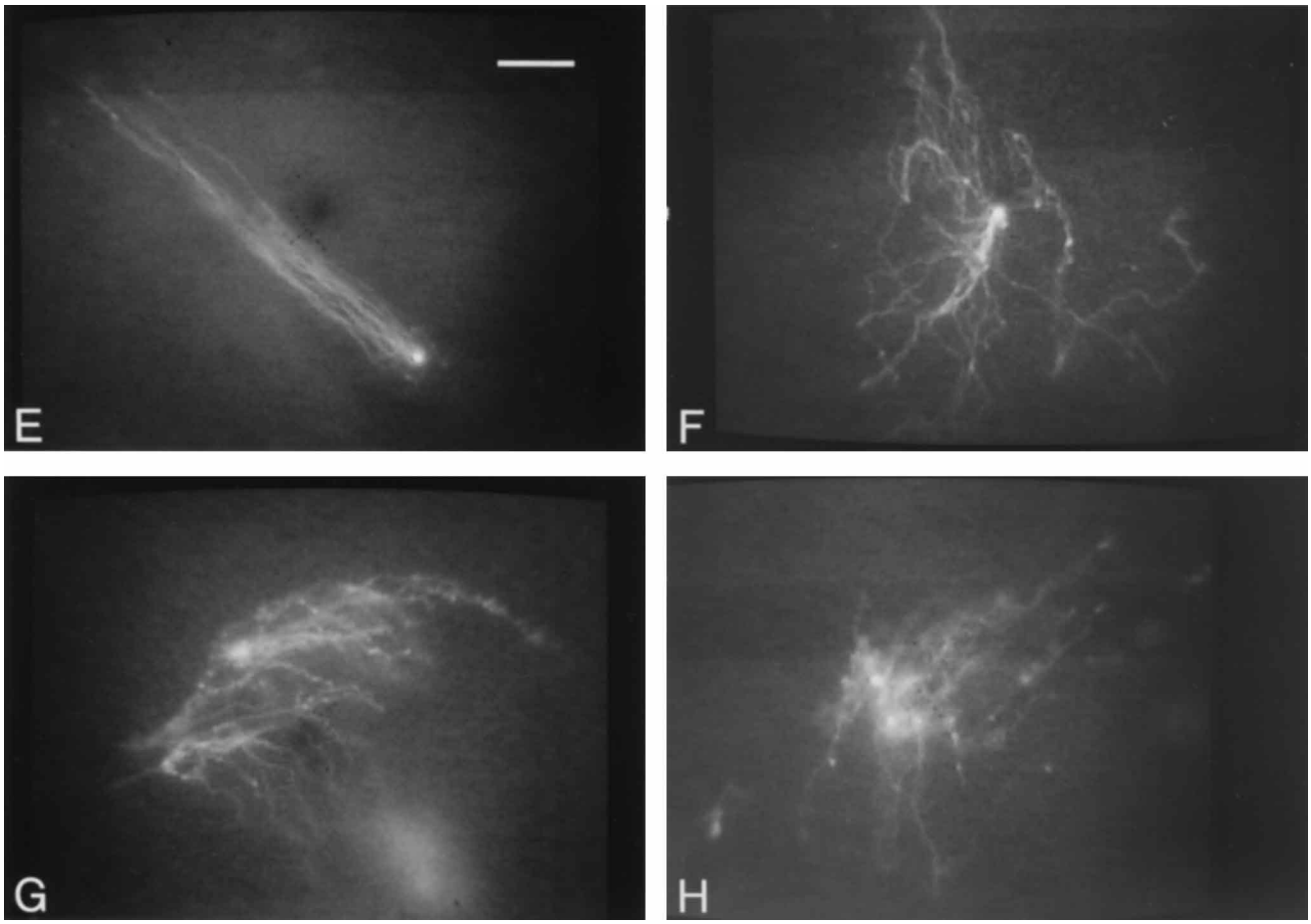

FIG. 1—*Continued.*

1G and H), making it difficult to place them unambiguously in the zero- or the three-node category. This difficulty is reflected in the greater variation between replicate counts for the zero-node category.

An advantage of this methodology is that the cell density of the agarose preparations can be adjusted so that each example encountered during microscopic examination could be confidently attributed to the DNA content of an individual cell. The total amount of DNA per cell appeared to vary approximately fourfold, by inspection, among the more than 1,000 individual examples examined. The apparent amount of total DNA correlated with the number of nodes (see, e.g., Fig. 1A). Since multinode nucleoids were more common in rapidly growing

TABLE 1. DNA nodes in *E. coli* N99 cells grown in M63-glucose-Casamino Acids medium with a 30-min doubling time

State of cells and count	% of nucleoids with the following no. of nodes:				No. of cells counted
	0	1	2	3	
Exponential phase					
A	1	64	30	5	61
B	0	73	25	2	100
Stationary phase					
A	19	71	9	1	100
B	11	81	8	0	100
C	2	86	12	0	101
D	3	87	8	1	100
CAM treated					
A	0	86	12	1	73
B	1	82	15	2	100

TABLE 2. DNA nodes in *E. coli* N99 cells grown in M63-acetate minimal medium with a 3-h doubling time

State of cells and count	% of nucleoids with the following no. of nodes:				No. of cells counted
	0	1	2	3	
Exponential phase					
A	21	68	9	3	102
B	10	69	20	1	101
Stationary phase					
A	58	40	2	1	104
B	41	54	5	0	100
CAM treated					
A	13	72	15	0	100
B	18	72	10	0	103

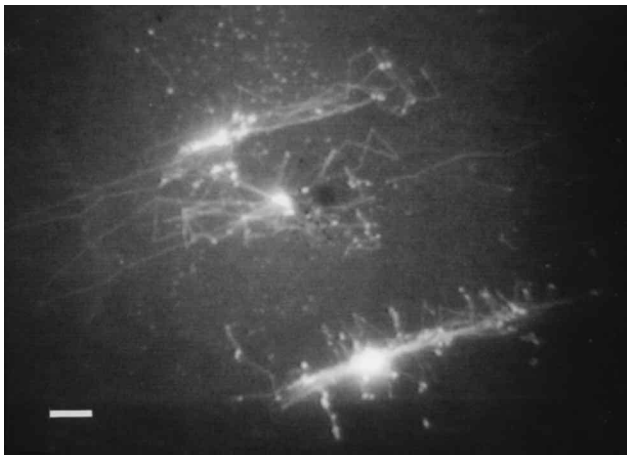

FIG. 2. *E. coli* DNA complexes from the well area of an agarose gel after PFGE. These forms are larger and more complex than most forms of DNA that were observed. Cells were from an exponential-phase culture in rich medium. Bar, 20 μm.

exponential-phase cells, they may represent separating chromosomes in a cell about to divide. Although we did not systematically monitor nucleoid size as a function of growth conditions, rosettes occasionally appeared to contain less than one genome equivalent of DNA, for example, in stationary-phase cells grown in rich medium.

We also examined in-gel-prepared DNA that had been subjected to 24 h of PFGE. The *E. coli* chromosome does not enter the gel under these conditions, but we reasoned that electrophoresis pressure might extend the arms of DNA and provide a somewhat different picture. Plugs of agarose that were taken from the well area of the gel after electrophoresis and staining were melted and spread for microscopy. A variety of structures was seen in these preparations, from typical rosettes to large, multinode complexes (Fig. 2). Notably, the DNA strands sometimes formed geometric shapes with straight-line segments and sharp vertices. These may be due to loops of extended DNA becoming impaled on agarose fibers, as occurs for large circular plasmid (40), chloroplast (10), and mitochondrial (8, 28) DNA molecules.

Properties of the borrelia nucleoid. Unlike molecules such as the *E. coli* chromosome, large linear molecules are able to migrate through PFGE gels (16, 36), and such gels can therefore be used to separate the linear replicons of *Borrelia* spp. PFGE of agarose blocks containing four different amounts of *B. burgdorferi* DNA is shown in Fig. 3. This strain contains the linear chromosome and three linear plasmids of 17 to 49 kb which migrate closely together under the electrophoresis conditions used. After electrophoresis and ethidium bromide staining, plugs of agarose were removed for examination from the areas of the gel containing the 950-kb linear chromosome and the 49-kb linear plasmid. Long linear molecules with an estimated size of 1 Mb were seen in the chromosomal sample (Fig. 4A). Analysis of the 1-Mb chromosomal band of *Saccharomyces cerevisiae* YNN295 revealed simple linear molecules of similar size (not shown). For both the *B. burgdorferi* and *S. cerevisiae* preparations, smaller molecules that probably represented chromosomes broken during the melting and spreading of the agarose plugs were also seen. Pictures of the *B. burgdorferi* 49-kb linear plasmid in an electric field (Fig. 4B and C) show several molecules stretched to various degrees during migration through the agarose on the microscope slide. These images are similar to those observed for bacteriophage lambda DNA (8).

To investigate how the genome is organized within a spirochete, we next examined the DNA content of cells lysed within agarose blocks that had not been subjected to electrophoresis. Examples from individual cells are shown in Fig. 5A to D. Comparison of individual 1-Mb genome-sized molecules (Fig. 4A) with the nucleoids in Fig. 5 indicates that the total amount of DNA per spirochete appears to be much greater than 1 Mb. As for *E. coli*, the total DNA content varied approximately fourfold, by inspection, among individual spirochetes, but even the examples containing the least amount of DNA appeared to consist of more than 1 Mb of DNA. This observation is consistent with the previous finding that *B. hermsii* contains 8 to 11 copies of its genome per cell (24). The structures of the nucleoids of both *B. burgdorferi* and *B. hermsii* were different from that of the *E. coli* nucleoid. Instead of a rosette of loops emanating from a central bright node, the borrelia nucleoid appeared to be composed of a loose network of strands with no obvious central organizing node. Arms of DNA extended from the network or mesh of DNA but were shorter than those seen for *E. coli*. The DNA strands of the borrelia nucleoid also had a more beaded or clot-like appearance than those of *E. coli* (see, e.g., Fig. 5D). During viewing, strands of borrelia DNA occasionally appeared to break, a phenomenon not observed for the DNA arms of *E. coli*. Thus, the DNA of *Borrelia* spp. had a more fragile appearance. Such breakage was described previously and attributed to cleavage at single-stranded regions under the intense irradiation used to excite the ethidium fluor (8, 28).

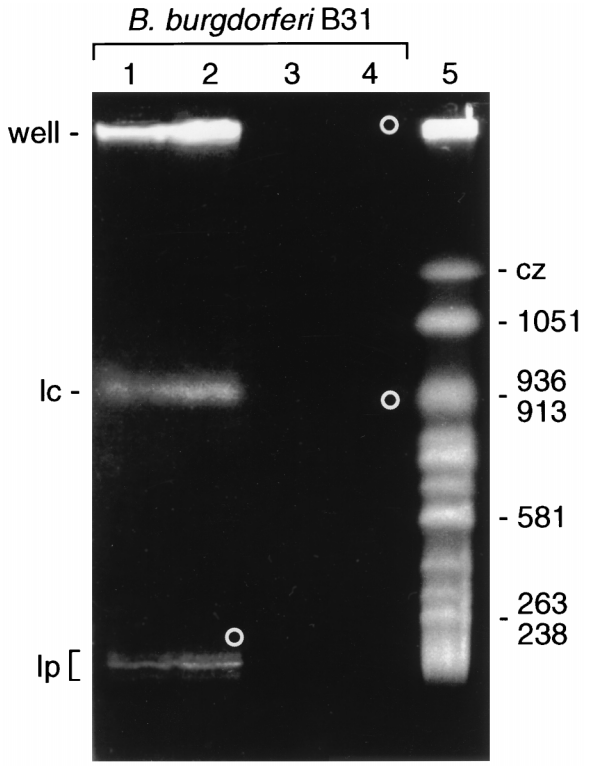

FIG. 3. PFGE gel of *B. burgdorferi* B31 genomic DNA. Agarose blocks prepared with 2.5×10^9 (lanes 1 and 2) or 5×10^5 (lanes 3 and 4) cells per ml were subjected to PFGE and stained with ethidium bromide. The sample volume used for lanes 1 and 3 was one-half of that used for lanes 2 and 4, respectively. Numbers on the right refer to sizes (in kilobases) of selected *S. cereviseae* YNN295 chromosomes (lane 5). The locations of the compression zone (cz), the linear chromosome (lc), and the linear plasmids (lp) are indicated. White rings indicate areas where plugs of agarose were removed for microscopy.

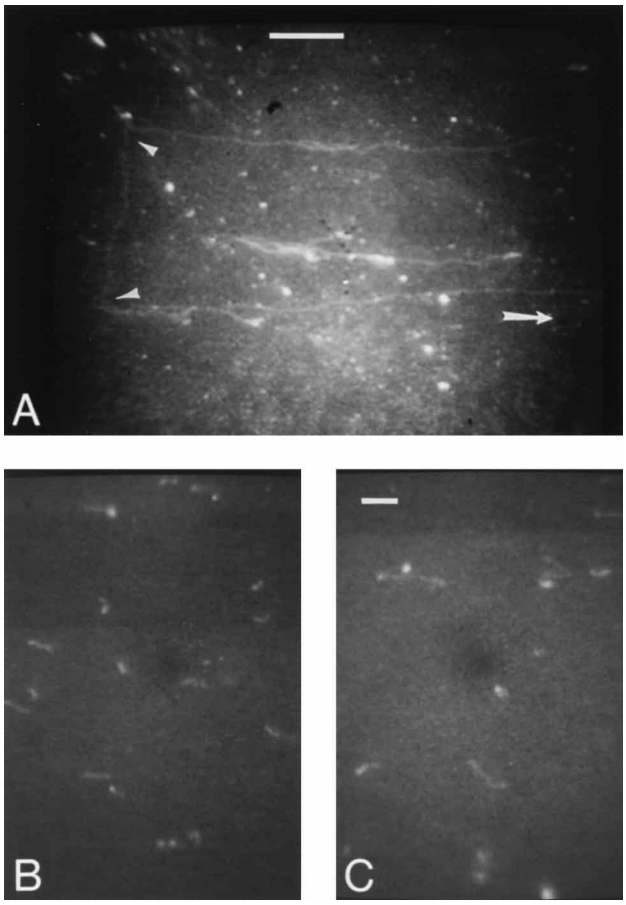

FIG. 4. (A) Fluorescence microscopy of a linear chromosome molecule of *B. burgdorferi* B31 during electrophoresis. The C-shaped molecule appeared to be draped around two impediments (probably agarose fibers [arrowheads]) with one end of the molecule visible in the upper right and the other end just beyond the field of view in the lower right. When this image was recorded, the lower end of the molecule was moving in the direction of the arrow, toward the anode. The trailing end snaked around the two agarose fibers and rapidly caught up with the leading end until this molecule resembled the bunched-up molecule in the center of this image. Both molecules subsequently migrated downfield in a manner typical for linear yeast chromosomes of about 1 Mb, and they appeared to be about that size. (B and C) Fluorescence microscopy of the 49-kb linear plasmid of *B. burgdorferi* B31. Several individual molecules are shown during electrophoresis from left to right and migrated as do lambda DNA molecules. The magnification of panel A is one-half of that of panels B and C. Bars, 20 μm.

The nucleoids of *Borrelia* spp. had a more constant appearance than was seen for *E. coli*. Nucleoids of both *B. burgdorferi* and *B. hermsii* cells from either exponential-phase, stationary-phase, or CAM-treated cultures were indistinguishable. CAM treatment had no effect on the cytological appearance of the *B. hermsii* nucleoid (24), and we also found no effect of CAM on nucleoid structure. CAM inhibits initiation of new rounds of DNA replication and induces marked changes in nucleoid shape in *E. coli* (26, 41). Because of its apparent lack of effect on nucleoid appearance in *Borrelia* spp., we tested the effect of CAM on replication. The antibiotic proved to inhibit DNA replication in *B. burgdorferi* within one generation time (Fig. 6).

It was not possible to determine if the complex meshes of DNA that typified the borrelia nucleoid were composed of more than one molecule (for example, a collection of several individual chromosomes and plasmids) or if they represented complex branched structures. Attempts to tease apart individual molecules from the complex networks by application of an electric field were unsuccessful, suggesting that the strands in the network were bonded rather than merely touching. Small linear pieces occasionally were released, but it was uncertain whether they were present originally or derived from strand breakage. Large circular plasmids, such as those of 20 to 35 kb present in these *Borrelia* species, would not be expected to migrate from the complexes because they would be nicked during observation and rendered immobile (8). We were also unable to determine whether the short arms of DNA extending from the loose meshes were actually loops, as for *E. coli*, or had free ends.

Although the simplest model of the *B. burgdorferi* genome is a collection of linear molecules and small circular plasmids, most *B. burgdorferi* DNA did not enter the PFGE gel (Fig. 3). We investigated the relation of this well-bound material to the linear 950-kb molecule that did enter the gel and to the nucleoid structure before electrophoresis. Agarose plugs were removed from the well area of the gel and examined (Fig. 5E to H). These samples revealed complex mesh-like structures that resembled those seen in individual cells, but they showed greater variation in the amount of DNA. Some examples had long arms of up to 500 kb of DNA that extended from the main complex (Fig. 5G), as if the electrophoresis had partially extricated a 1-Mb linear chromosome molecule. Unlike the analogous samples of *E. coli* (Fig. 2), these long arms never generated open forms indicative of a loop structure.

DISCUSSION

In this study fluorescence microscopy was used to obtain high-resolution pictures of nucleoids of *E. coli* and the spirochetes *B. burgdorferi* and *B. hermsii*. In keeping with the chromosomal domain model, the *E. coli* nucleoid resembled a rosette of loops in which long arms of DNA extended from a dense core or node. A single node was most commonly seen, but in a population of cells the percentages of nucleoids exhibiting zero, one, two, or three nodes varied with growth conditions. The nucleoid of *Borrelia* spp. presented a different picture, consisting of a loose meshwork of DNA strands that lacked a central organizing node. We will discuss how the observed nucleoid structures relate to what is known about the genomics and DNA replication for these two bacteria.

Structure and replication of the *E. coli* nucleoid. The most notable feature of the nucleoid of *E. coli* is the presence of nodes. Their intense ethidium bromide fluorescence implies that DNA is more densely packed in a node than in the arms (DNA loops) (7) that emanate from them. We will consider the possible relationship between nodes and DNA replication, and the structure and composition of DNA at a node, although as yet we have no definitive model.

A node may have nothing to do with DNA replication but merely be a locus at which DNA is gathered when 1.5 mm of DNA is compacted into a 2 μm cell. Our data, however, suggest that node structure is dynamic and thus may reflect changes in DNA topography associated with replication or segregation. The zero-node class is more frequent in slow-growing cells (Table 2) than in fast-growing cells (Table 1) for each physiological state. The zero-node class may represent cells in which rounds of replication have been completed but reinitiation and cell division have not occurred. Such cells were identified by Chai and Lark by thymine autoradiography and cytology (12). Conversely, multinode cells are more frequent during rapid growth when reinitiation occurs before completion of a round of DNA replication. When reinitiation in rapidly growing cells is blocked by CAM, the frequency of multi-

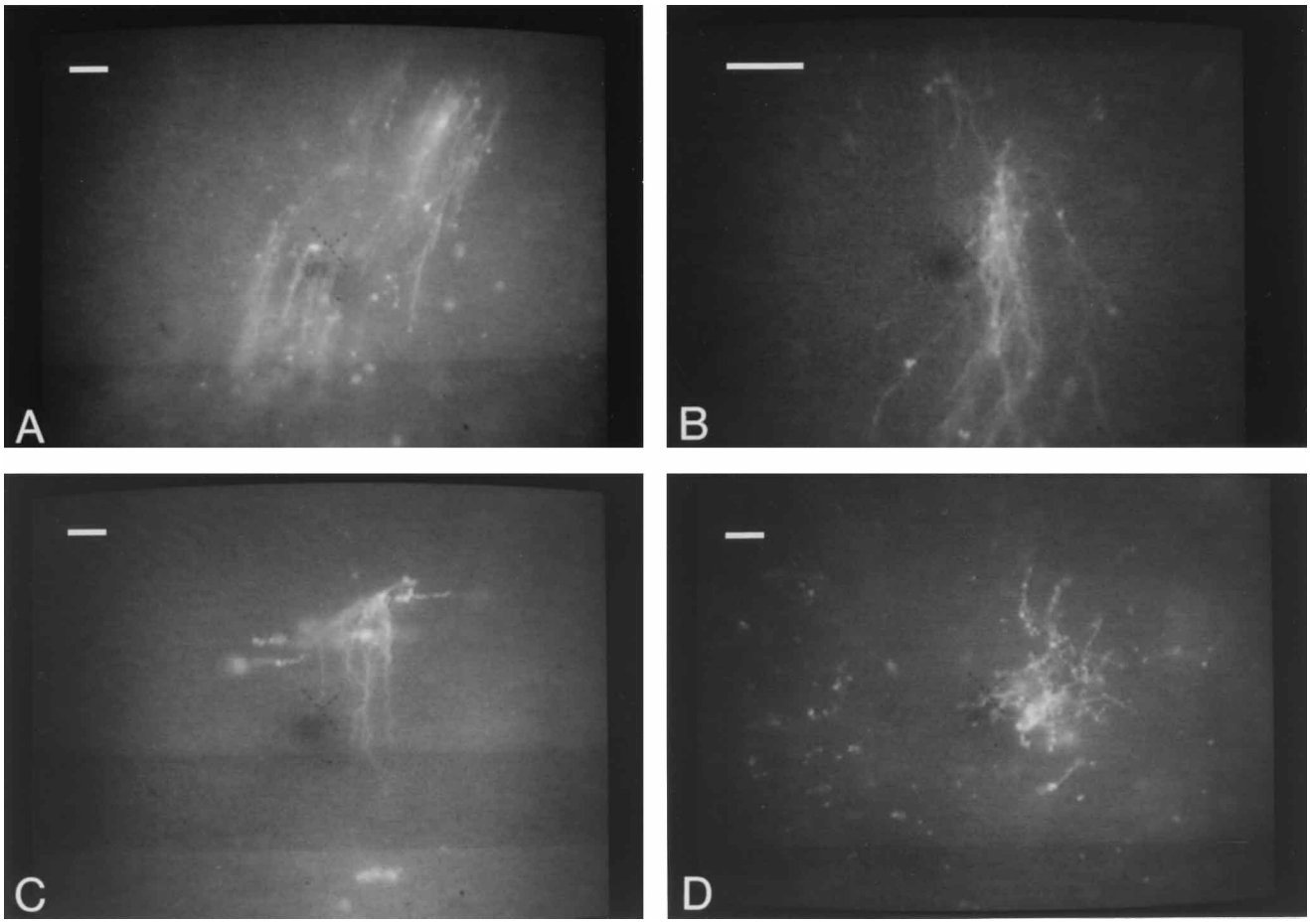

FIG. 5. Fluorescence microscopy of genomic DNA from *Borrelia* cells lysed in situ in agarose (A to D) and from the well of an agarose gel after PFGE (E to H). The nucleoid in panel C had been exposed to an electric field on the slide before this image was recorded. (A and H) Nucleoids from stationary-phase cells of *B. hermsii*; (B and G) nucleoids from exponential-phase cells of *B. hermsii*; (C, E, and G) nucleoids from exponential-phase cells of *B. burgdorferi*; (D) nucleoids from CAM-treated cells of *B. burgdorferi*. The magnification in panels A, C, D, and G is one-half of that in the other panels. Bars, 20 μm.

node cells is half of that for untreated cells. Changes in local structure at the origin or terminus of replication during the cell cycle may create a node. In either case a node would contain a particular region of the genome, such as *oriC* or a *ter* locus. Alternatively, a node could occur at the site of a replication fork. In this case any DNA sequence could be found in a node. If the replication apparatus is fixed within the cell, as proposed by Cook for the eukaryotic nucleus (13), bidirectional replication would proceed as DNA is reeled through the apparatus holding both forks in close proximity, creating pairs of loops.

A major question concerns the nature of the bonds holding the loops of *E. coli* DNA in a rosette structure. In a current model of the *E. coli* genome, the loops are held by a protein(s) at the center of the rosette (29). It is possible that residual proteins not removed by our SDS-proteinase K treatment are responsible for the node structures we observe with in-gel preparations. Sedimentation analysis indicated that treatment with SDS or trypsin or heating at 60 to 70°C could maximally unfold isolated *E. coli* chromosomes (15), although their structures were not visualized. We previously found that the rosette forms for both *Agrobacterium tumefaciens* and *E. coli* were indistinguishable and resisted RNase-Sarkosyl-proteinase K treatment, whereas no DNA arms or fibers were released from *E. coli* or *Pseudomonas aeruginosa* spheroplasts produced by treatment with lysozyme without detergent or protease (7). Typical rosette forms were also observed after Sarkosyl-proteinase K treatment of *Streptomyces lividans* and the thermophilic archaebacterium *Pyrococcus* strain ES4 (6, 31). Any putative residual organizing protein must be resistant to detergent-protease treatment for all of these bacteria.

An all-DNA alternative can be considered for the nodes of densely packed DNA. One possibility is that the loops of DNA are catenated or knotted such that the node represents the region at which many strands overlap. It is not clear, however, why the many overlap regions would form a node rather than be distributed around the circumference of each loop. Another possibility is that sequence-dependent associations exist at the base of each loop and that such pairings among loops create the node. There are about 100 copies per genome of a repetitive DNA element that binds the integration host factor of *E. coli* (11), and these, or any repetitive sequences, could serve as sites of synapsis to establish a node. A synaptic process should involve recombination functions, and this possibility can be tested by examining nucleoids from strains defective in such functions.

Nucleoid structure in *Borrelia*, a bacterium with a linear genome. The unusual genome structure and cytology of *Borrelia* spp. is reflected in a nucleoid structure that is distinct from that of *E. coli* or any of the other bacteria analyzed. Nucleoids of *B. burgdorferi* and *B. hermsii* were loose meshes that lacked

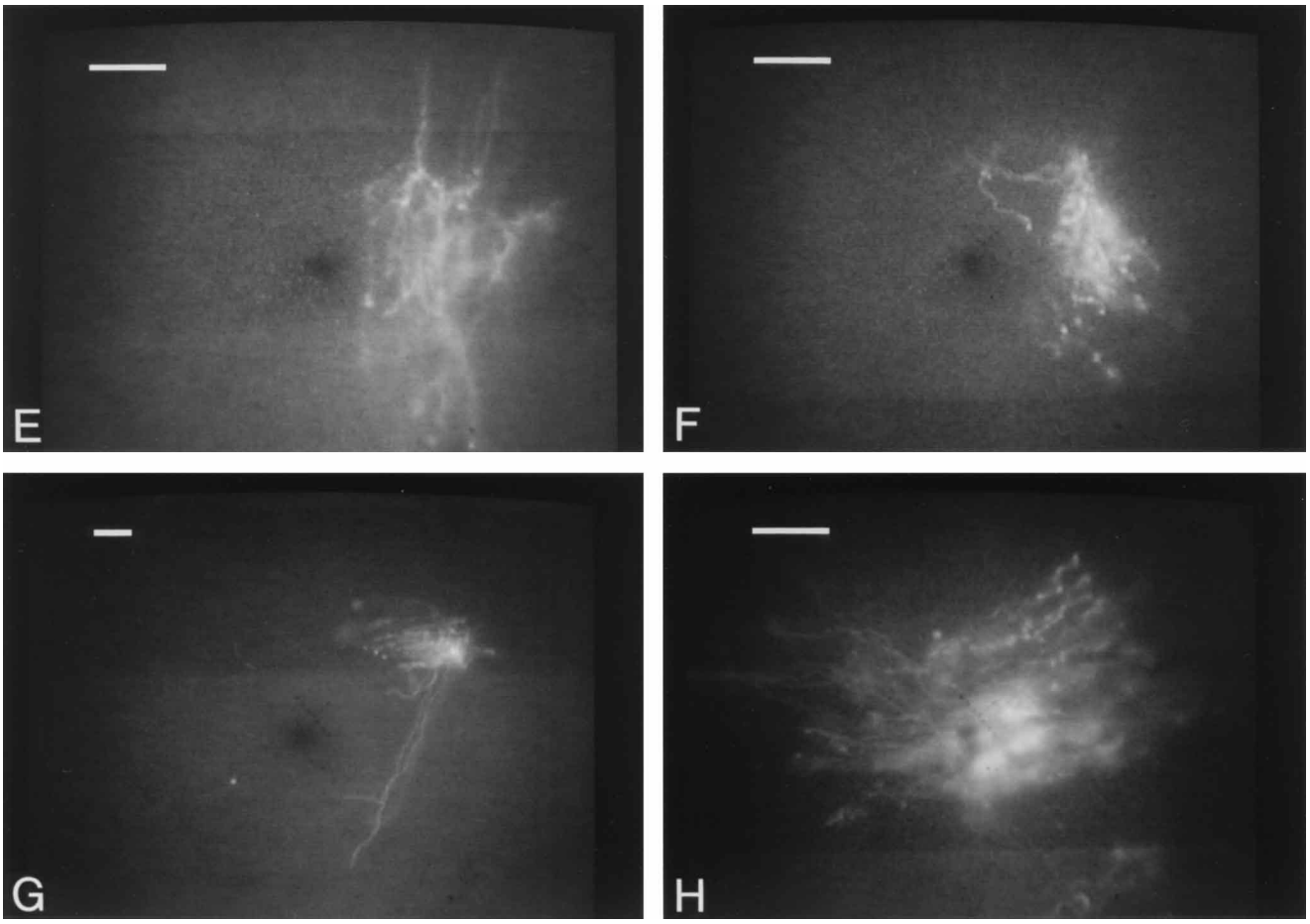

FIG. 5—*Continued.*

an obvious node. As for *E. coli*, the alternatives of a residual protease-resistant protein(s) or all DNA can be considered for the maintenance of the compact mesh form. Since no prominent nodes occurred, any catenates, knots, or synaptic junctions may be distributed more frequently throughout the borrelia genome than for *E. coli*. The DNA arms extending peripherally from the mesh were shorter and more fragile than the DNA loops of *E. coli*. The nucleoids contained much more than 1 Mb of DNA, the approximate unit genome size. The previous finding (24) that cultured *B. hermsii* cells contain 8 to 11 copies of the chromosome and linear plasmids could account for this. Our results suggest that *B. burgdorferi* may also be polyploid, because nucleoids of the two species appeared to contain equal amounts of DNA.

Kitten and Barbour (24) demonstrated two patterns of nucleoid staining in intact *B. hermsii*. Homogeneous DNA staining throughout the long, thin cells was seen in 95 to 99% of exponential-phase cells, but in 40 to 60% of stationary-phase cells a series of discrete stained bodies was evident, suggestive of multiple nucleoids. During examination of at least 200 nucleoids from isolated *B. hermsii* and *B. burgdorferi* cells, we did not detect a structural difference associated with growth phase. Upon lysis of the cell membrane, however, nucleoids exhibiting either of the two in vivo staining patterns may have assumed the same conformation. Furthermore, the spreading required for preparation of samples for microscopy would likely have scattered discrete nucleoids originally present in a single spirochete.

The simplest model of the borrelia nucleoid is a congregation of many independent DNA molecules: linear chromosomes, linear plasmids, and circular plasmids. Upon cell lysis and spreading, however, a collection of independent replicons would be unlikely to consistently yield the compact mesh structure that we invariably saw. The nucleoid structure we observed indicates a more complex situation and may reflect genomic replication and segregation mechanisms. The network of strands that typified the DNA in all cells during all stages of growth could represent a branched replication intermediate, with the single-stranded regions inferred from fragility under irradiation suggesting multiple replication forks. Accordingly, much of a borrelia's DNA would exist in a replication complex throughout the cell cycle, even though replication of its small genome theoretically requires only a fraction of the 8-h cell division time. Individual replicons could also be connected in these complexes, perhaps via a recombination-dependent replication mechanism as is known for T4 phage DNA (25). The nucleoid would then be a network of individual chromosomes and plasmids that is distributed along the protoplasmic cylinder. Upon cell lysis and spreading, a nucleoid would adopt the amorphous mesh form (Fig. 5), which appears to contain several genome equivalents of DNA. Such a nucleoid structure would account for the large amount of DNA that does not enter PFGE gels (Fig. 3). This well-bound fraction is often ignored or is assumed to consist of circular plasmids and linear chromosome molecules that do not migrate because of trap-

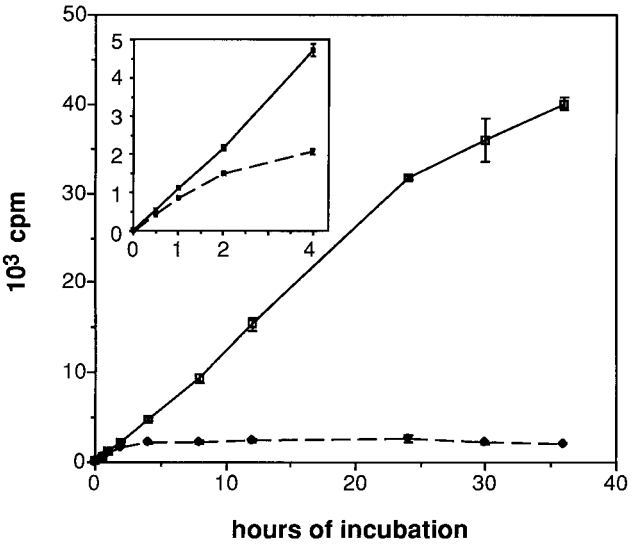

FIG. 6. [^{3}H]thymidine incorporation by *B. burgdorferi* B31 in the presence (dashed line) and absence (solid line) of CAM. The inset shows the data for the first 4 h only. The cell doubling time for *B. burgdorferi* is 8 h, which is the maximum in vitro growth rate.

ping in the agarose (38). The complex networks of DNA observed for individual cells would not be predicted to migrate, however, and these structures were in fact recovered from the well after PFGE (Fig. 5).

Our pictures, along with the previous cytological data (24), suggest that individual borrelia replicons are not independently partitioned to daughter cells but that multigenomic complexes are the units of segregation. This model provides a mechanism for the stable inheritance of the many linear and circular plasmids, which are in unit copy number relative to the chromosome (20, 24). If individual genomes are interconnected to form nucleoids, the connections may be labile or exist only during a certain portion of the replication cycle, because many individual linear chromosome and plasmid molecules are resolved by PFGE of genomic DNA from a population of spirochetes, and clones of *B. burgdorferi* cured of individual linear plasmids have been isolated (33).

The images presented here demonstrate the utility of this method for comparing the nucleoid structures of different bacteria. High-resolution pictures are obtained from samples prepared with a minimum of physical manipulations and without fixation and hydration artifacts associated with electron microscopy. Many nucleoids from a single preparation can be examined, and regions of DNA within a nucleoid can be moved electrophoretically to reveal additional structural information. The method is thus well suited to assess the effects of different growth conditions, biochemical treatments, and genetic backgrounds on nucleoid structure.

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