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Changes in Infectivity and Plasmid Profile of the Lyme Disease Spirochete, *Borrelia burgdorferi*, as a Result of In Vitro Cultivation

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In vitro cultivation of *Borrelia burgdorferi*, the etiologic agent of Lyme spirochetosis, allows for the isolation and growth of this bacterium from infected tissues. However, continuous cultivation in modified Kelly medium causes a reduction in the number of detectable plasmids and the loss of infectivity in the white-footed mouse, *Peromyscus leucopus*. In an unpassaged culture of *B. burgdorferi*, nine plasmids were present, including seven linear plasmids ranging in size from 49 to 16 kilobases (kb) and two circular plasmids of 27 and 7.6 kb. The 7.6-kb circular and 22-kb linear plasmids were no longer detectable in spirochetes noninfective in white-footed mice, suggesting that a gene(s) encoding for factors responsible for infection may be present on one or more of these extrachromosomal elements. Furthermore, changes in spirochetal proteins and lipopolysaccharide-like material were observed also during early cultivation and may be related to loss of infectivity.

Many species of pathogenic bacteria have extrachromosomal elements of DNA or plasmids that contain genes encoding for properties responsible for their pathogenicity and virulence in mammalian hosts (12, 24). Plasmid DNA has been reported in seven species of *Borrelia* (18, 19, 26). *Borrelia burgdorferi*, the causative agent of Lyme disease and of a variety of related human disorders (10, 21, 30), is unusual in that various strains have both linear and circular plasmids (3, 4, 17). Genes encoding for two outer surface proteins designated OspA and OspB have been identified on the largest plasmid, a 49-kilobase (kb) linear duplex with covalently closed ends (4, 17). No other biological properties of *B. burgdorferi* are yet known to be associated with plasmids.

Cultivation of *B. burgdorferi* in modified Kelly medium (2, 22, 32) permits isolation and growth of this organism from infected tissues. However, several independent studies have demonstrated changes in surface proteins (8, 28, 35), infectivity (20), and plasmid profile of *B. burgdorferi* associated with in vitro cultivation (3, 19). Johnson et al. (20) observed that *B. burgdorferi* lost infectivity for Syrian hamsters (*Mesocricetus auratus*) sometime between 3 and 30 passages in modified Kelly medium when passaged once a week and incubated at 30°C. In our laboratory, one of us (W.B.) observed that *B. burgdorferi* cultivated in modified Kelly medium failed to infect New Zealand White rabbits (*Oryctolagus cuniculi*), whereas previous studies had demonstrated this host to be susceptible to infection via bites of infected ticks (9, 23). Evidence for changes in the number of plasmids include an isolate recovered from a tick in Wisconsin that contained a 9.2-kb plasmid which was not found in other strains that had been maintained in culture for longer unspecified lengths of time (19). Recently, it has been observed that a cultured strain of *B. burgdorferi* lost two plasmids at unknown times during 4 years of cultivation (3). Therefore, we investigated the influence of in vitro cultivation on the infectiousness and plasmid profile of this spirochete to determine whether there was an association between the loss of specific plasmids and infectivity. Herein we present these results and characterize the plasmids found in a low-passaged strain of *B. burgdorferi*. Detailed structural analyses of these extrachromosomal elements will be described elsewhere.

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MATERIALS AND METHODS

Strains of *B. burgdorferi* and in vitro cultivation. *B. burgdorferi* strains Sh-2-82, CA-2-87, and ECM-NY-86 used in this study were described previously (29). Briefly, Sh-2-82 originated from naturally infected adult *Ixodes dammini* ticks collected on Shelter Island, N.Y. CA-2-87 originated from naturally infected adult *Ixodes pacificus* ticks from California. ECM-NY-86 was isolated from a skin biopsy from an erythema chronicum migrans lesion of a human patient from New York. Strain B-31 (ATCC 35210), the prototype strain of *B. burgdorferi*, has been maintained in our laboratory for several years.

Strain Sh-2-82 was used to examine changes associated with serial passage in culture. Its cultivation began by isolating it from an *I. dammini* tick that had been experimentally infected by allowing it to feed on a spirochetemic white-footed mouse (*Peromyscus leucopus*) as described previously (29). This spirochete culture was designated P-0; aliquots of spirochetes in culture medium were frozen in final concentrations of 20% glycerol and 20% rabbit serum while 0.3 ml was used to initiate the serial in vitro passage. Every 3.5 days, 0.3 ml of the fully grown spirochete culture was passaged into a new 15-ml tube containing 9 ml of modified Kelly medium (also known as BSK-II) (2) and incubated at 34°C. After every fifth culture had fully grown, aliquots were inoculated into white-footed mice and frozen with glycerol and rabbit serum for subsequent analysis of their plasmid and protein profiles.

Animal inoculation and reisolation of spirochetes. White-footed mice were from a uninfected laboratory colony at Rocky Mountain Laboratories established from progeny of specimens obtained from J. Benach and E. Bosler, State of New York Department of Health, State University of New York, Stony Brook.

The initial culture of strain Sh-2-82 (P-0), and every fifth passage thereafter up to 25 passages (P-5, P-10, P-15, P-20, and P-25, respectively), and one culture each of B-31, ECM-NY-86, and CA-2-87 were tested for infectivity in

white-footed mice. All inoculations contained freshly grown spirochetes, except P-0 which had been frozen at −70°C. When each fifth passage was available, four adult white-footed mice of either sex between 2 and 5 months old were each inoculated intraperitonealy with approximately 3×10^7 to 7.5×10^7 *B. burgdorferi*. Spirochetes were first removed from modified Kelly medium by centrifugation (9,000 × *g* for 15 min) and then suspended in 0.5 ml of phosphate-buffered saline–5 mM $MgCl_2$ (pH 7.4). Numbers of spirochetes in cultures were determined by the counting method of Stoenner (31). White-footed mice were caged separately to prevent the possibility of contact transmission (11). Two of the four mice were examined at 2 weeks postinoculation and two more mice were examined at 3 weeks postinoculation. They were anesthetized, bled by cardiac puncture, and killed with ether. The bladder, spleen, and left kidney were removed, and each was triturated in 1 ml of modified Kelly medium in a glass tissue grinder. Two aliquots of approximately 0.5 ml of each suspension were inoculated into two 15-ml tubes containing 9 ml of modified Kelly medium, containing the antibiotics phosphomycin (100 μg/ml) and rifampin (50 μg/ml). Whole blood (0.1 ml) was also inoculated into modified Kelly medium with and without antibiotics. Culture tubes were incubated at 34°C and examined by dark-field microscopy every 2 to 4 days for 1 month. The presence of spirochetes in culture confirmed infection of the tissues tested.

DNA purification and characterization. Plasmid DNA was purified from all strains and all six passages of Sh-2-82 (P-0 to P-25), using a technique described previously (3, 4) but without cesium chloride-ethidium bromide ultracentrifugation. DNA concentration and purity were determined by UV absorption at wavelengths of 260 and 280 nm (25). DNA preparations were examined by agarose gel electrophoresis, using a Mini-Sub DNA Cell (Bio-Rad Laboratories, Richmond, Calif.). Equal quantities of DNA were electrophoresed in 0.2% agarose gels with TBE buffer (90 mM Tris, 90 mM boric acid, 20 mM EDTA). Gels were run at 50 V for 5 min and then 12 V for 16 h, followed by staining with ethidium bromide. Two gels were required to resolve all plasmids. The larger bands were distinguishable by using 250 ng of total DNA per well, while 1,500 ng of total DNA was necessary to detect the 7.6-kb circular plasmid.

The various classes of plasmid DNA as demonstrated by agarose gel electrophoresis were recovered by elution and examined by electron microscopy. Electroelution was done in a model UEA electroeluter (International Biotechnologies, Inc., New Haven, Conn.) run at 100 V for 30 min to 2 h, using a buffer of 20 mM Tris (pH 8.0)–5 mM NaCl–0.2 mM EDTA. DNA was trapped in a salt sink containing 3 M sodium acetate. To determine the structure and size of the plasmids, DNA was mounted for electron microscopy, using the Kleinschmidt aqueous technique as described previously (13, 14). Adenovirus 2 DNA (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) was the standard used to calibrate contour lengths. Grids were examined in a JEOL 100B electron microscope at 40-kV accelerating voltage. Electron micrographs were taken on Electron Image plates (Eastman Kodak Co., Rochester, N.Y.) at a magnification of ×7,000. The magnification was calibrated for each set of plates with a grating replica (E. F. Fullam, Schenectady, N.Y.), and contour lengths were measured with a Numonics Graphics calculator interfaced with a Tektronix 4052A computer. Contour length measurements included 20 to 50 molecules in each category.

SDS-PAGE. Whole-cell lysates of spirochetes were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using procedures previously described (6). Proteinase K digestion of whole-cell lysates was done as previously described (16). However, spirochetal concentrations were standardized by adjusting suspensions to give an A_{600} of 0.2, using a Spectronic 21 spectrophotometer (Bausch & Lomb, Inc., Rochester, N.Y.). Silver staining was done by the method of Tsai and Frasch (34) as modified by Hitchcock and Brown (16).

TABLE 1. Evidence that *B. burgdorferi* Sh-2-82 loses infectivity in white-footed mice after 11 to 15 passages (5.5 to 7.5 weeks) of in vitro cultivation[a]

Passage	No. of tissues infected				No. of mice infected
	Blood	Spleen	Kidney	Bladder	
P-0	0	4	3	4	4
P-5	0	3	3	4	4
P-10	2	4	3	4	4
P-15	0	0	0	0	0
P-20	0	0	0	0	0
P-25	0	0	0	0	0

[a] Four mice were inoculated intraperitoneally with each passage; two mice each were examined after 2 and 3 weeks postinoculation.

RESULTS

Serial cultivation of spirochetes and infectivity. Inoculations of culture passages P-0, P-5, and P-10 caused infections in white-footed mice detectable by reisolation of spirochetes from two or more of the tissues tested (Table 1). Inoculations with cultures of 15 or more passages (P-15, P-20, and P-25) and B-31 resulted in no reisolations from any mice. Therefore, using our regimen of in vitro cultivation, *B. burgdorferi* Sh-2-82 lost infectivity after 11 to 15 passages corresponding to 5.5 to 7.5 weeks in culture. Strains CA-2-87 and ECM-NY-86, passaged once and five times, respectively, both infected white-footed mice.

Serial cultivation and plasmid profile of Sh-2-82. With agarose gel electrophoresis of a total extrachromosomal fraction, 10 distinct bands were detectable in plasmid purifications of the early-passaged spirochete cultures P-0 and P-5. These bands, numbered 1 to 10, correspond to the slowest to fastest migrating bands (Fig. 1). After serial cultivation, some of the bands present in the earlier passages were no longer detectable. Band 2 could not be detected after passage P-5 and bands 6 and 10 were lost during passages P-11 to P-15, corresponding to the loss of infectivity. We use the word lost to denote the disappearance of a particular plasmid size class. Whether these sequences were in fact lost from the spirochetes or were recombined into other plasmids or chromosomal DNA remains to be determined.

Band 2 was no longer detectable in P-10; however, this culture was infective in mice. If the plasmid represented by this band was essential for spirochete infectivity, then there may have been a selection during the infection for only those relatively few spirochetes that might still have contained this particular plasmid. Therefore, we examined the purified plasmid DNA of the spirochetes reisolated from the urinary bladder of a white-footed mouse that had been infected with culture P-10. The reisolate, like the infective inoculum, also lacked band 2 (Fig. 2), suggesting that this particular change had no apparent effect on the infectiousness of the spirochetes in our assay system.

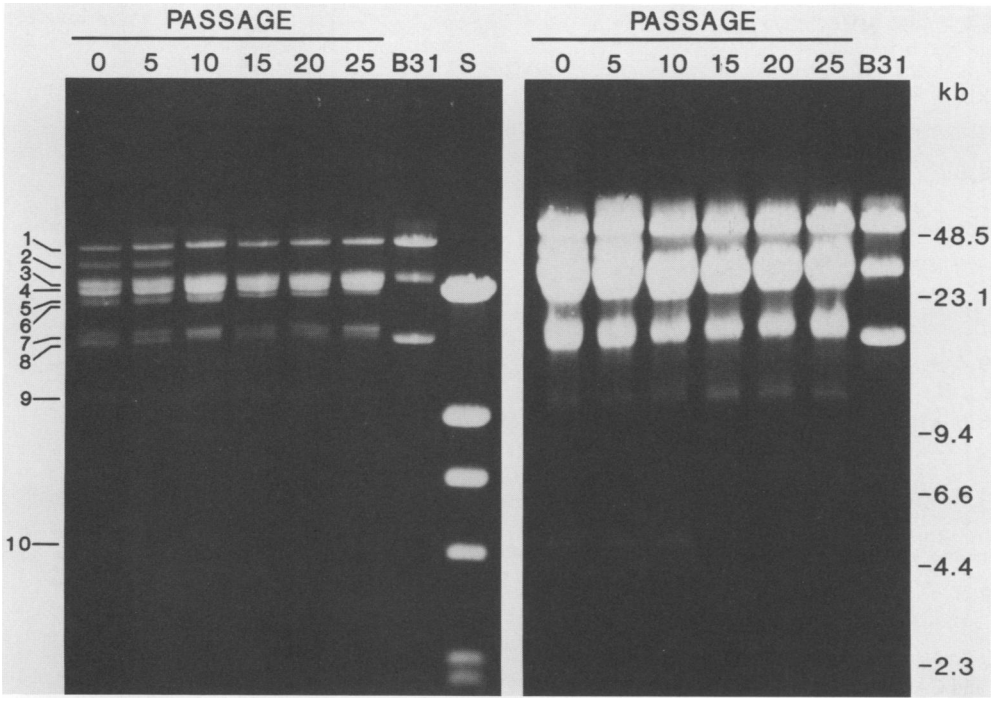

FIG. 1. Agarose gel analysis of plasmid profiles of *B. burgdorferi* Sh-2-82 serially passaged in modified Kelly medium and the longtime cultured strain B-31. Large plasmids were resolved with 250 ng of total DNA per well (left), while detection of the smallest plasmid required 1,500 ng of total DNA per well (right). Molecular size standards (lane S and given on the right) are lambda DNA (48.5 kb) and lambda DNA/*Hin*dIII digest fragments. Bands numbered 1 to 10 are discussed in the text. Bands 6 and 10 were lost by P-15, when spirochetes were no longer infective for white-footed mice.

Plasmid characterization. The individual bands 1, 2, 7, 8, 9, and 10 and the closely spaced bands 3 to 6 were electroeluted and examined by electron microscopy. Bands 1 to 7 all appeared linear in structure. Band 1 comigrated with the 49-kb linear plasmid described previously (4). Bands 2 and 7 measured 33 and 16 kb, respectively, by both agarose gel electrophoresis and contour length measurement. The four closely migrating bands, labeled 3 to 6 in Fig. 1, ranged in size from 25 to 22 kb. What appeared as the three bands 8, 9, and 10 actually contained only two circular plasmids of different sizes and conformations. Band 8, which migrated near the position of a 16-kb linear molecule, contained the relaxed form of a circular plasmid measuring 26.8 ± 0.4 kb. Band 9, while appearing to be a single species of approximately 12 kb, actually contained both 27-kb supercoiled molecules and smaller relaxed circles measuring 7.66 ± 0.13 kb. Band 10, migrating near the position of a 4.4-kb marker, contained 7.6-kb supercoiled molecules. Therefore, the 10 bands detectable by agarose gel analysis were actually composed of seven linear and two circular plasmids. The 22-kb linear and 7.6-kb circular plasmids were no longer detectable when the spirochetes lost infectivity in white-footed mice.

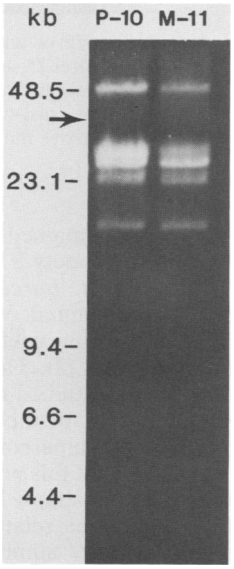

FIG. 2. Agarose gel analysis of plasmid profiles of *B. burgdorferi* Sh-2-82 passage P-10 and the resulting reisolate M-11 from a white-footed mouse. Band 2 in Fig. 1 is absent from both profiles (arrow). Molecular size standards are lambda DNA (48.5 kb) and lambda DNA/*Hin*dIII digest fragments.

Total plasmid DNA was purified from strains CA-2-87 and ECM-NY-86 and examined by agarose gel electrophoresis and electron microscopy. Both strains had mixed populations of linear and circular plasmids that differed in their migration profiles (Fig. 3). However, both strains had circular plasmids of similar size. CA-2-87 contained opened circles approximately 7.6 and 25.2 kb; ECM-NY-86 contained opened circles approximately 8.2 and 24.5 kb. Therefore, both strains were infective in white-footed mice and contained a small circular plasmid nearly identical in size to the 7.6-kb plasmid lost with infectivity from the serially passaged strain. Whether these various small circular plasmids share sequences or not remains to be determined.

SDS-PAGE and proteinase K digestion of spirochetes. As we reported elsewhere (28), SDS-PAGE of whole-cell ly-

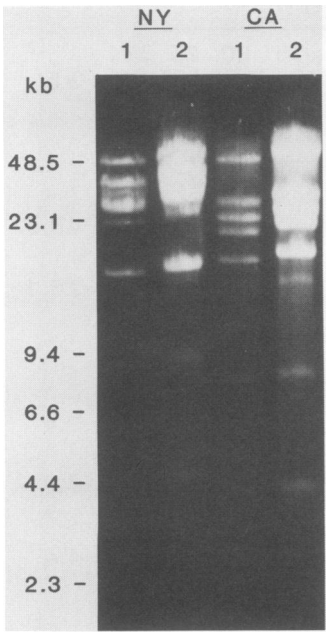

FIG. 3. Agarose gel analysis of plasmid profiles of *B. burgdorferi* ECM-NY-86 (NY) and CA-2-87 (CA). Total DNA used per well was as follows: 170 and 1,000 ng for NY 1 and 2, respectively; 250 and 1,500 ng for CA 1 and 2, respectively. Molecular size standards are lambda DNA (48.5 kb) and lambda DNA/*Hin*dIII digest fragments. The bands migrating near the 4.4-kb standard are supercoiled plasmids of approximately 7.6 to 8 kb.

sates and Western blot (immunoblot) analysis of the six passage levels (P-0, 5, 10, 15, 20, and 25) demonstrated that OspB, an outer surface protein with an apparent molecular mass of 34 kilodaltons (kDa), was lost after 11 to 15 passages, correlating to when infectivity was lost in mice. Besides the loss of OspB, there was a relative increase in a lower-molecular-mass protein with an apparent size of 20 kDa (Fig. 4). Furthermore, proteinase K digestion revealed an apparent change in "lipopolysaccharide (LPS)-like" material (Fig. 5) which occurred during the 25 serial passages. Proteinase-K-resistant material as visualized solely by modified silver stain had a higher apparent molecular weight in P-25 than in the unpassaged culture (P-0). This material was of spirochetal origin as it was absent in lanes of previous gels containing only proteinase K for controls.

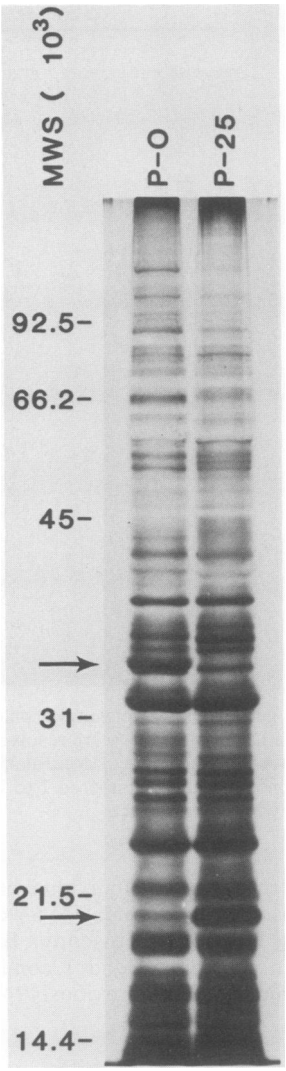

FIG. 4. SDS-PAGE and silver stain of whole-cell lysates of *B. burgdorferi* Sh-2-82 before (P-0) and after 25 serial passages (P-25) in modified Kelly medium. Molecular weight standards (MWS; Bio-Rad) are given on the left. The upper arrow indicates OspB lost during cultivation, and the lower arrow indicates an increase in 20-kDa protein.

DISCUSSION

Although large numbers of *B. burgdorferi* were inoculated into the highly susceptible white-footed mouse, these spirochetes were no longer infective after 11 to 15 passages (5.5 to 7.5 weeks) in culture medium. The reduction in the number of detectable plasmids with the loss of infectivity raises the possibility that a gene(s) which encodes products important in maintaining infectivity may be present on one or more of these extrachromosomal elements. The 7.6-kb supercoiled and 22-kb linear plasmids are the most promising candidates as both were no longer discernible when strain Sh-2-82 became noninfective. The 33-kb linear plasmid (band 2) was no longer detectable after only 6 to 10 passages (3 to 5 weeks), although spirochetes remained infective. The other isolates of *B. burgdorferi* examined, one from *I. pacificus* from California and the other from a human erythema chronicum migrans lesion from New York, both infected white-footed mice and both contained a relatively small supercoiled plasmid of approximately 8 kb. Recently, plasmid profiles of 13 isolates of *B. burgdorferi* from North America and Europe were presented, with the suggestion that agarose gel analysis might prove useful in typing isolates from different geographical areas (3). The apparent number of plasmid bands in the isolates studied ranged from four to seven. No bands were detected in the region of the gels which corresponded to a 7.6-kb supercoiled plasmid. Given the relatively low concentration of this plasmid, it is possible that this plasmid was present but simply below the detection limits of the system. Whether the relative scarcity of this small plasmid is due to low copy number or because it is selectively discarded during the purification process is not known. However, we observed nine distinct plasmids in the unpassaged culture, three of which were no longer detectable after only 11 to 15 passages. Our initial culture (P-0) was not cloned; therefore, one must consider the possibility that

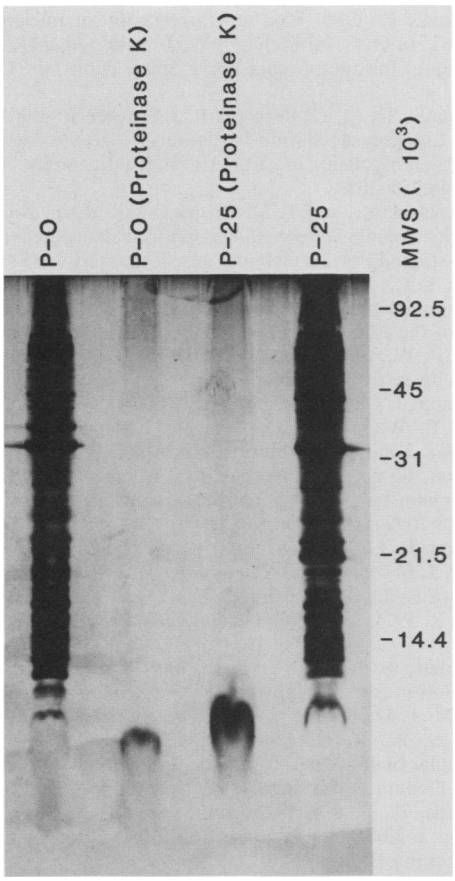

FIG. 5. SDS-PAGE and silver stain of whole-cell lysates of *B. burgdorferi* Sh-2-82, with and without proteinase K digestion, before (P-0) and after 25 serial passages (P-25). Molecular weight standards (MWS; Bio-Rad) are given on the right.

the population of spirochetes we started with varied in plasmid profiles. The reduction in the apparent number of plasmids could, in theory, have been due to an in vitro selection for those variants containing fewer plasmids rather than to a loss of plasmids from a specific strain, although the latter has been observed (3).

The purification of individual bands in agarose gels stained with ethidium bromide, followed by electron microscopic examination and contour-length measurements of the DNA, demonstrates the danger of characterizing isolates by interpreting migration profiles of mixed plasmid populations in agarose gels alone. Opened circles and supercoiled conformations of circular plasmids of the same size will migrate separately, the supercoil faster than the open circle, and neither will have an accurate apparent size compared with linear standards. Furthermore, the relative proportion of supercoiled, relaxed, and even linear forms of the same plasmid may be a consequence of how well the sample is protected from nuclease and mechanical shear damage. While the actual size of linear plasmids can be estimated by their migration in agarose gels, the size estimate is dependent on knowing that the plasmid is in fact linear. At present, this cannot be determined simply by band migration in a gel. Our band 9 exemplifies the problem. By distance migrated in the gel, the band appeared as a single plasmid with an apparent size of approximately 12 kb. In fact, this band contained circular plasmids measuring 7.6 and 27 kb. Such problems in gel interpretation and the relative instability of some plasmids with their subsequent loss from even low-passaged strains could complicate attempts to type isolates based on their plasmid profiles in agarose gels.

We observed other biological changes in *B. burgdorferi* associated with its serial cultivation that may relate to infection. As we reported elsewhere (28), the same serially passaged strain (Sh-2-82) lost OspB, an outer surface protein with an apparent molecular mass of 34 kDa (7), between the 11th and 15th passages in culture. Also, we observed changing reactivities of OspB to the monoclonal antibody H6831 during the first 10 passages, demonstrating antigenic changes in OspB in the early passages (28). The loss of OspB during the same period of cultivation when infectivity was lost raises the possibility that this outer surface protein might have some role in the infection of *B. burgdorferi* in its mammalian host. Strain B-31, which has been maintained in serial cultivation in our laboratory for approximately 6 years, was not infective in white-footed mice but still contained an OspB. However, there is considerable heterogeneity in the 34-kDa range proteins of *B. burgdorferi* (5), and clearly not all 34-kDa proteins in this spirochete are the same. Previous studies including *B. burgdorferi* HB-19, a strain isolated from human blood in Connecticut (6, 30), demonstrated that this spirochete contained an OspB (6). Yet, this same strain has also been maintained by serial cultivation in our laboratory for approximately 4 more years, and it presently does not have an OspB (T. G. Schwan, unpublished data). The possibility of one 34-kDa protein being specifically involved in infection deserves further investigation, as does the mechanism responsible for the expression of the OspB group of proteins. *Escherichia coli* harboring a recombinant plasmid containing the *ospA* and *ospB* genes of *B. burgdorferi* will express these two proteins (17), suggesting that sequences on other plasmids or the chromosome are not required for their expression. However, transposable genetic elements may exist which can insert into the *ospB* gene to prevent expression of this protein. Other types of DNA rearrangements, such as that responsible for antigenic variation in *B. hermsii* (26), or other "*trans*-acting" elements may be involved.

Currently, there are conflicting reports regarding the exact chemical composition of *B. burgdorferi* LPS, including whether or not lipid A is present (7, 33). The apparent increase in molecular weight of the proteinase-K-resistant material during the first 25 serial passages of *B. burgdorferi* suggests that an LPS-like substance changes during early cultivation. However, the change we observed is opposite to that seen for other bacteria in which the conversion is to lower-molecular-weight LPS due to the loss of sugars in the O side chains (27). Whether this modification in *B. burgdorferi* has any relationship to loss of infectivity remains to be seen. However, one should not ignore this observation in view of the relationship known for other pathogenic bacteria between in vitro cultivation and loss of virulence, changes in colony formation, and conversion from smooth to rough LPS (1, 14, 27).

In this report we demonstrated several biological changes in *B. burgdorferi* occurring together early during in vitro cultivation, including (i) the loss of infectivity, (ii) a reduction in the number of detectable plasmids, (iii) protein changes in whole-cell lysates, and (iv) an apparent increase in molecular weight of the LPS-like component. While the evidence is circumstantial, genes encoding important infectivity factors may reside on certain extrachromosomal elements and may be lost early during cultivation. We are presently preparing nucleotide probes containing sequences

from the 7.6-kb circular and 22-kb linear plasmids to determine whether, in fact, these plasmids were lost, recombined, or integrated into the chromosome. If these sequences are absent, then reintroducing them into a noninfective, high-passaged strain might be expected to restore infectivity, and then fine mapping of important genes can begin. Finally, if these plasmids are lost and do have some role in infectivity, their sequences could be used as probes to identify those strains of *B. burgdorferi* in cultivation that are still infective.

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