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Genetic Stability of *Borrelia burgdorferi* Recovered from Chronically Infected Immunocompetent Mice

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Persistent infection with *Borrelia burgdorferi* in the presence of a vigorous host immune response has been demonstrated in humans and in animal models of Lyme disease. Long-term persistence of *B. burgdorferi* was documented recently in our studies of BALB/c and C3H mice infected with cloned and uncloned strains of *B. burgdorferi*. From mice inoculated with the cloned strain, 11 isolates were recovered from the skin, bladder, and blood after 1 year of infection. Analysis of the genes encoding the major outer surface proteins (OspA and OspB) by restriction digestion and DNA sequencing showed no evidence of point mutations or other small genetic alterations after 1 year. Genomic macrorestriction analysis of whole *B. burgdorferi* showed no loss or gross alterations of the plasmids encoding OspA, OspB, or OspC. However, in two isolates, loss of a 38-kb plasmid encoding outer surface protein D was noted. Our studies suggest that loss or alteration of the genes encoding OspA and OspB is not a common occurrence during persistent spirochetal infection and that other possible mechanisms, including invasion of immunologically privileged sites, should be actively explored.

Lyme disease is a multisystem disorder with musculoskeletal, neurological, and cardiac features that are often of greater severity early in the course of disease. Although the symptoms of Lyme disease may subside or resolve completely with or without therapy (41), in most infected individuals, the symptoms do not resolve spontaneously and recur intermittently. Studies of patients with chronic Lyme arthritis have demonstrated persistent spirochetal infection, as determined by PCR, for up to 7 years after the onset of arthritis (28, 31). Persistent infection has also been demonstrated in patients with acrodermatitis chronica atrophicans, from whom spirochetes can be recovered by culture after nearly a decade of infection (2). Indeed, persistent infection of reservoir hosts is likely to be an important component of the enzootic transmission cycle of *B. burgdorferi* (40).

In recent years, several mechanisms for host avoidance of *B. burgdorferi* have been explored. One possible mechanism is the rearrangement or alteration of genes encoding outer surface proteins, in a manner similar to that described for the major outer membrane proteins of *Borrelia hermsii* (4, 38). Recently, Rosa and colleagues described intragenic recombination of the genes encoding the two major outer surface proteins (OspA and OspB) of *B. burgdorferi* (34); such a process might allow escape of immunodominant epitopes within either protein from immune recognition. Fikrig et al. (15) demonstrated the presence of a naturally occurring *B. burgdorferi* variant of strain N40 that harbors a nonsense mutation in the OspB open reading frame (ORF); this mutation, which eliminates an immunodominant portion of the OspB protein, allows escape from immune protection in OspB-immunized hosts (14, 15). *B. burgdorferi* variants that harbor alterations in OspA and OspB (13, 35) or lack these ORFs entirely (36) have been generated by exploiting in vitro growth inhibition of antibodies to outer surface proteins (12, 36, 37). However, although it is possible that such mechanisms play a role in persistent infection, no in vivo studies have been performed to address this issue directly.

An alternative and equally plausible model for persistence of *B. burgdorferi* involves dissemination into privileged sites that are relatively free of immune surveillance, such as an intracellular compartment or the central nervous system. Intracellular compartmentalization of spirochetes has been observed (17, 27), and the persistence of spirochetes in the skin, other tissues, and central nervous system has been observed in experimental systems (1, 5, 8, 9, 18, 41). Long-term persistence of *B. burgdorferi* was documented recently in our studies of BALB/c and C3H mice infected for up to 1 year after inoculation with cloned and uncloned strains of *B. burgdorferi* (8). This study demonstrated an intermittent disease course in mice that is similar to chronic human disease; spirochetes were consistently isolated from skin, and PCR detection of OspA targets could be demonstrated in all of the culture-positive specimens. The consistent detection of the gene encoding OspA in these specimens suggested that loss or significant alteration of this gene was not a major mechanism of persistence, at least in skin specimens.

Although these conceptually different mechanisms are not mutually exclusive, the "compartmentalization model" which was postulated in our previous studies might not pose a stringent requirement for antigenic variation, since less selective pressure is likely to be encountered in an immunologically privileged site. A requirement for antigenic variation might be more likely for organisms in direct contact with circulating antibody, as is the case for *B. hermsii* in the bloodstream (4).

In our studies of persistent infection in laboratory mice, 11 isolates of *B. burgdorferi* were recovered from the skin, bladder, and blood of animals infected for 1 year (8). In this article, genetic analysis of these spirochetes is described.

MATERIALS AND METHODS

Cultivation and maintenance of *B. burgdorferi*. The N40 strain of *B. burgdorferi* is a tick isolate that has undergone only three in vitro passages and has proven infectivity and patho-

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genicity in mice. A virulent clone of N40 was produced by three in vitro passages of terminal dilutions; all isolates analyzed in this study were derived from this N40 clone (cN40).

The conditions for cultivation and recovery of *B. burgdorferi* from tissues were as described previously (3, 8). Briefly, spirochetes were grown in modified Barbour-Stoenner-Kelly (BSKII) medium at 33°C without antibiotics. Samples of blood (2 drops), ear skin (1.5-mm-diameter ear punch), and all or part of the urinary bladder were collected aseptically and cultured in 8-ml glass screwtop tubes containing 7.0 ml of medium. Cultures were incubated for 2 weeks and then examined for the presence of spirochetes by dark-field microscopy. Spirochetal inocula were grown to log phase, quantified with a blood counting chamber, and diluted to the desired concentration with BSKII medium. A total of 11 isolates were recovered and analyzed (3 from bladder tissue, 3 from blood, and 5 from skin). Details of the protein analysis of these isolates by polyacrylamide gel electrophoresis were reported elsewhere (8).

Mouse strains. Random-sex, virus-antibody-free C3H/HeJ and BALB/c/cByJ mice were purchased from The Jackson Laboratory, Bar Harbor, Maine, and C3H/HeNCRLBR (C3H-N) mouse strains were purchased from Charles River Laboratories, Portage, Mich. Mice were shipped and maintained as described before (6–8).

Pulsed-field gel electrophoresis. *B. burgdorferi* isolates were grown to ca. 10^9/ml in 20 ml of BSK-H medium (Sigma) plus 6% rabbit serum and then harvested by centrifugation at 9,000 × *g* for 15 min. The cell pellet was resuspended in 300 to 500 μl of EET (100 mM EDTA, 10 mM EGTA [ethylene glycol tetraacetic acid], 10 mM Tris [pH 8.0]) and warmed to 42°C. Agarose plugs were made by diluting 60°C 1.6% SeaPlaque agarose (FMC Bioproducts, Rockland, Maine) in EET with an equal volume of resuspended cells for a final concentration of 0.8%. With a 1-ml syringe with a 1-inch (ca. 2.5-cm), 19-gauge blunt-end needle, the agarose-cell mixture was aliquoted into a Bio-Rad plug mold. After hardening, the plugs were removed and submerged overnight at 55°C in a lysis solution containing EET, 1% sodium dodecyl sulfate (SDS), and 1 mg of proteinase K per ml. After overnight incubation, the plugs were washed by gentle shaking in 40 ml of TE (10 mM Tris [pH 8.0], 1 mM EDTA) for 30 min, four times. Agarose plugs thus prepared could be stored in TE at 4°C until further analysis.

Approximately one-fifth of the Bio-Rad mold-formed plug (50 μl) was soaked in 500 μl of 1 × *Mlu*I restriction buffer for 15 min, at which time 400 μl was removed. *Mlu*I (3 μl at 10 to 12 U/μl) was then added, and the plug was incubated overnight at 37°C. A 1.2% Fastlane agarose gel (FMC Bioproducts) in 0.5× TAE was prepared (0.75-mm comb). When the gel hardened, a pulse marker (50 to 1,000 or 0.1 to 200 kb ladder; Sigma) was added to wells at both ends of the gel.

Restriction enzyme and buffer were removed after digestion, and the plug was washed in 1 ml of 0.5× TAE for 10 min or longer. The TAE was then removed, and the plug was melted at 65°C for 5 to 10 min. From 20 to 30 μl of melted plug was gently loaded into a well and left to harden. The gel was then placed in a Bio-Rad CHEF-DRII electrophoresis chamber containing precooled (11 to 14°C) 0.5× TAE and prerun (no voltage) for 1 h. The conditions for running the gel were as follows: initial switch time of 1 s, linear ramp with a final switch time of 10 s, and 200 V (6 V/cm) for 16 to 17 h. The pulsed-field gel was stained with ethidium bromide, and the DNA was visualized by UV transillumination.

Sequence analysis of the OspA-B operon. The OspA-B coding region of *B. burgdorferi* was amplified by PCR with 50 pmol each of the primers OspA5NC1 and OspB3 (see Table 1), 10% glycerol, a 200 μM concentration of each deoxynucleoside triphosphate, 1× PCR buffer (10 mM Tris [pH 8.3], 50 mM KCl, 0.001% bovine serum albumin [BSA]), 1.5 mM $MgCl_2$, and 1.25 U of *Taq* DNA polymerase. Target DNA consisted of *B. burgdorferi* DNA extracted with the IsoQuick kit (Microprobe Corporation, Garden Grove, Calif.). Genomic N40 DNA was used as a positive control. Reaction mixtures were incubated at 94°C for 4 min before cycling and then at 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min for a total of 50 cycles, followed by 72°C incubation for 5 min. A 10-μl aliquot of amplified DNA was electrophoresed in a 1% Seakem GTG agarose gel (FMC Bioproducts) to ensure that amplification had occurred, and the reaction products were subjected to either restriction fragment length polymorphism analysis or DNA sequencing as described below. For all of the PCR procedures used in this study, recommended measures for preventing PCR product carryover were observed.

TABLE 1. Primers used for generation of probes

Primer and position (bp)	Sequence
OspA6 sense (15→40)	5′ ATT GGG AAT AGG TCT AAT ATT AGC CT
OspA3NCI antisense (850→820)	5′ GAG GGA TCC TAT TAA TAA TCT CAT AAA TTC TCC TTA
OspC1 sense (1→24)	5′ ATG AAA AAG AAT ACA TTA AGT GCG
OspC2 antisense (633→610)	5′ TTA AGG TTT TTT TGG ACT TTC TGC
OspD1 sense (−25→−7)	5′ GAC ATT ATA TTT AAG GAG
OspD792 antisense (770→751)	5′ GTA TTT AAC AAG GCC ACA AC
1.8-kb OspA-B primers	
OspA5NCI sense (−30→3)	5′ CAG GAA TTC AGT TAT ATT AAT ATA AAA GGA GAA TAT ATT ATG
OspB3 antisense (1719→1693)	5′ TCT GGA TCC TTT TAA AGC GTT TTT AAG CTC TGA AAG

Restriction fragment length polymorphism analysis of the *osp* operon. Approximately 1 μg of the intact 1.8-kb *ospA-ospB* amplification product was subjected to restriction digestion with *Sau*3A and *Alu*I as described before (31a). The digestion products were resolved on a 1% SeaKem–3% NuSieve (FMC Bioproducts) agarose gel, stained, and visualized by UV transillumination.

DNA sequencing. The nucleotide sequence of the *ospA-ospB* operon was determined by cycle sequencing (Gibco-BRL, Life Technologies, Gaithersburg, Md.) with 500 fmol of template DNA and 1 pmol of ^{32}P-labeled internal sequencing primer. A total of six internal sequencing primers were used to sequence the 1.8-kb amplification product from isolates. Sequencing reaction mixes were incubated at 95°C for 5 min before thermal cycling for 20 cycles at 95°C for 30 s, 55°C for 30 s, and 70°C for 1 min, followed by 10 cycles at 95°C for 30 s and 70°C for 1 min. The termination products were resolved on a 6% denaturing polyacrylamide gel (acrylamide-bisacrylamide, 19:1) containing 7 M urea. Autoradiography was performed with Kodak X-OMAT AR diagnostic film (Eastman Kodak, Rochester, N.Y.).

Southern blot analysis of pulsed-field gel electrophoresis

blots. Pulsed-field gel electrophoresis gels were transferred to nylon membranes after depurination for 10 min in 0.25 N HCl. Blots were UV cross-linked and prehybridized and then hybridized with chemiluminescent probes prepared to the *ospA-B* operon, *ospC*, and *ospD*. The probes were prepared as follows. Coding regions for OspA, OspC, and OspD were amplified by PCR with the primers listed in Table 1. Under standard PCR amplification conditions (Perkin-Elmer, Norwalk, Conn.), probes were constructed from N40 genomic DNA in a 50-μl PCR mix containing 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% BSA, 200 μM each of the four deoxynucleotide triphosphates, 10% glycerol, 0.5% Tween 20, 1.25 U of *Taq* polymerase, and 50 pmol each of two primers (Table 1). Thermal cycling was performed as follows: 1 min of denaturation at 94°C, 1 min of annealing at 45°C, and 1 to 3 min of extension at 72°C for 40 to 45 cycles. The PCR product was purified by either gel extraction or Centricon-100 column ultrafiltration (Amicon, Beverly, Mass.). Labeling of the prepared probe was carried out according to the manufacturer's instructions for the enhanced chemiluminescence gene detection system (Amersham, Arlington Heights, Ill.) (19).

RESULTS

Two types of experiments were performed to detect possible genetic differences between the original cloned *B. burgdorferi* input strain (strain cN40) and isolates obtained from these mice after 1 year of infection. Since genetic alterations in the *ospA-ospB* operon have already been documented in vitro, we focused initially on the genetic fine-structure analysis of this region. A second set of experiments examined the isolates by genomic macrorestriction analysis and Southern blotting to identify additional genetic rearrangements or other gross alterations in genomic or plasmid DNA that might have developed during chronic infection.

Amplification of the *ospA-ospB* bicistronic region and restriction fragment length polymorphism analysis. In our previous study of mice inoculated with an uncloned strain of *B. burgdorferi*, substantial differences in the electrophoretic migration of OspB and other lower-molecular-weight proteins were observed among isolates recovered 1 year after inoculation (8). However, this was probably due to strain heterogeneity in the original inoculum (41); when a cloned strain of the same isolate of *B. burgdorferi* was used as the inoculum, such variation was not apparent. In order to analyze the OspA and OspB coding regions for the presence of missense mutations or other minor genetic alterations, we recovered the genes encoding the OspA and OspB proteins from 11 *B. burgdorferi* isolates cultured from mouse tissues after 1 year of infection (Table 2). PCR was used to amplify the conjoined *ospA-ospB* ORFs.

All 11 isolates gave rise to a 1,766-bp amplification product predicted to contain the entire *ospA* and *ospB* ORFs (11) joined by a short intergenic spacer region. Restriction fragment length polymorphism analysis was then performed on this fragment by using restriction enzymes *Sau*3A and *Alu*I. Eight *Sau*3A sites (representing 32 nucleotides) and 13 *Alu*I sites (representing 54 nucleotides) were predicted to be present in the fragment. A representation of one such analysis is shown in Fig. 1. Cleavage of the 1,766-bp fragment by *Sau*3A and *Alu*I alone and in combination yielded restriction fragments that were indistinguishable for all 11 isolates. Under the conditions used, it is usually possible to detect insertions or deletions of greater than 10 nucleotides within a 300-bp or smaller fragment; all of the cleavage products shown in Fig. 1 are less than 280 nucleotides in length. From this analysis, we concluded that no substantial genetic alterations (i.e., insertions or deletions of more than 10 nucleotides) were present in the products and that no detectable alterations were present at the 86 nucleotide positions tested.

TABLE 2. Analysis of OspA and OspB coding regions for minor genetic alterations

Isolate	Source	RFLP[a] analysis[b]	Sequence analysis[b]	Reaction with:		
				OspA probe	OspC probe	OspD probe
77633	Bladder	NCD	ND	+	+	+
77639	Bladder	NCD	NCD	+	+	−
77643	Bladder	NCD	NCD	+	+	+
77636	Blood	NCD	NCD	+	+	−
77643	Blood	NCD	ND	+	+	+
77644	Blood	NCD	ND	+	+	+
77632	Ear	NCD	ND	+	+	+
77633	Ear	NCD	NCD	+	+	+
77639	Ear	NCD	NCD	+	+	+
77643	Ear	NCD	ND	+	+	+
77644	Ear	NCD	ND	+	+	+

[a] RFLP, restriction fragment length polymorphism.
[b] NCD, no change detected; ND, not done.

Nucleotide sequence analysis of the *ospA-ospB* operon. To further examine the OspA and OspB coding regions for genetic alterations that might have occurred below the level of detection by restriction fragment length polymorphism analysis, we determined the complete nucleotide sequences of the *ospA-ospB* 1,766-bp amplification product recovered from five of the 1-year isolates (Table 2). The cognate region from cN40, the starting strain, was also sequenced. To reduce the effect of *Taq* incorporation errors, which occur at a frequency of approximately 10^{-4} to 10^{-5} per cycle, bulk amplification products were sequenced directly by using a cycle sequencing protocol. Previous studies in our laboratory and those of others

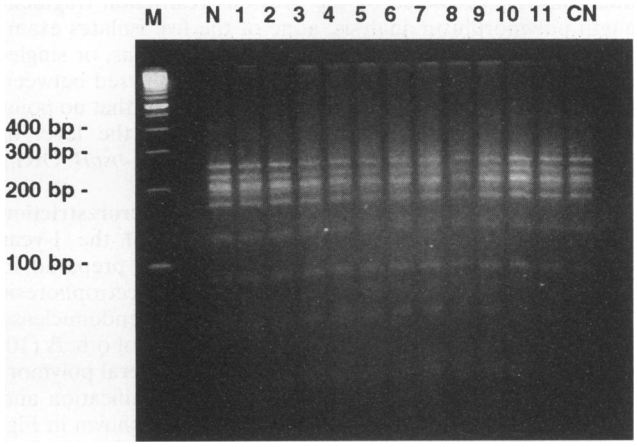

FIG. 1. *Alu*I and *Sau*3A digestion of amplification products derived from the *ospA* and *ospB* ORFs of 1-year isolates. The *ospA* and *ospB* ORFs recovered by PCR from cloned inoculum strain cN40 and 11 1-year isolates were digested by *Sau*3A and *Alu*I. Eight *Sau*3A sites (representing 32 nucleotides) and 13 *Alu*I sites (representing 54 nucleotides) were present in the 1,766-bp fragment. Lanes contained a 100-bp ladder (M); N40 (N); bladder isolate 77633 (lane 1), 77639 (lane 2), or 77643 (lane 3); blood isolate 77636 (lane 4), 77643 (lane 5), or 77644 (lane 6); ear skin isolate 77632 (lane 7), 77633 (lane 8), 77639 (lane 9), 77643 (lane 10), or 77644 (lane 11); and cloned N40 (CN).

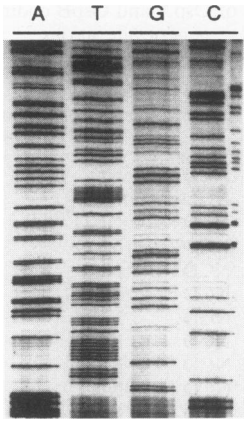

FIG. 2. Nucleotide sequence analysis of the *ospA-ospB* ORFs. The nucleotide sequences of the 1,766-bp amplification product comprising the *ospA* and *ospB* ORFs were determined by dideoxy chain termination sequencing followed by electrophoretic separation of the termination products with a zebra gel loading format. Five of the 1-year isolates and the inoculum strain cN40 were analyzed. In this format, termination mixes for the same dideoxynucleotides were loaded adjacent to each other to allow rapid visual detection of single-base polymorphisms. A sequence of the *ospA* ORF from nucleotides 451 to 625 is represented here with N40, cloned N40, and bladder isolates 77633, 77639, and 77643, using the sequencing primer OspA3 antisense (5′ GCC ATT TGA GTC GTA TTG TTG TAC TG).

have shown this method to be more accurate and less cumbersome than sequencing of subcloned PCR products (30).

A representative nucleotide sequence analysis of the OspA-OspB coding region is shown in Fig. 2. In this analysis, the sequencing reaction mixtures were loaded onto the electrophoretic sequencing gel so that termination mixes for a given nucleotide were located adjacent to each other. This so-called "zebra gel" format allows rapid scanning of multiple sequences for the presence of minor genetic alterations and single-base mutations. As predicted by the previous restriction fragment length polymorphism analysis, none of the five isolates examined contained any detectable insertions, deletions, or single-base polymorphisms in the 1,721-bp region analyzed between the PCR primers. We conclude from this analysis that no point mutations or other genetic alterations below the level of detection of the analysis occurred in the *ospA-ospB* ORFs recovered from these five isolates.

Analysis of 1-year isolates by genomic macrorestriction digestion. Whole genomic DNA from eight of the 1-year isolates as well as the parent strain cN40 was prepared in agarose plugs and subjected to pulsed-field gel electrophoresis analysis with and without digestion by restriction endonuclease *Mlu*I. Recent studies in our laboratory and those of others (10, 22) have shown that *Mlu*I digestion produces several polymorphic fragments that can be used for strain identification and characterization. The results of this analysis are shown in Fig. 3 and 4. None of the isolates showed obvious alterations in *Mlu*I genomic macrorestriction fragments compared with the starting strain cN40 (Fig. 3). All of the 11 isolates demonstrated the presence of a ca. 54-kb band that hybridized to an *ospA*-specific probe; this band was thus identified as the linear plasmid species that encodes OspA and OspB (Fig. 4). All 11 isolates hybridized with a probe for detection of the *ospC* ORF, which resides on a 28-kb supercoiled plasmid in *B. burgdorferi* (16) (data not shown). No change in mobility for these two plasmid species was observed in the cut versus the uncut pulsed-field gel electrophoresis preparations. Thus, neither of these plasmid species contained *Mlu*I restriction sites.

Analysis of 1-year isolates lacking a 38-kb plasmid species. Pulsed-field gel analysis of the uncut genome and *Mlu*I genomic macrorestriction analysis demonstrated in 2 of the 11 1-year isolates the absence of a DNA fragment migrating at 38 kb (Fig. 3, lanes 2 and 4). The presence of this band in uncut preparations of cN40 and the fact that its migration was unaffected by *Mlu*I digestion (Fig. 3B) suggested that this species was a plasmid. Recently, the gene encoding a minor outer surface protein (OspD) was mapped to a 38-kb linear plasmid in *B. burgdorferi* B31. Accordingly, we determined whether the gene encoding OspD could be detected in strain cN40 or in the 1-year isolates. An *ospD*-specific chemiluminescent probe was prepared by PCR amplification of the sequence of *ospD* which was described recently (29); the probe was labeled by glutaraldehyde cross-linking to horseradish peroxidase and used in a chemiluminescent detection protocol as described above. The results are shown in Fig. 4B. Both of the isolates that demonstrated loss of the presumptive 38-kb plasmid species (Fig. 3, lanes 2 and 4) also failed to hybridize to the *ospD* probe under conditions in which all of the other isolates, including the cloned N40 input strain, demonstrated *ospD* probe hybridization (Fig. 4B). These results are consistent with the loss of a 38-kb plasmid containing the ORF encoding OspD (29) in 2 of the 11 1-year isolates. A summary of the findings of this and the other analyses is provided in Table 2.

DISCUSSION

Several recent studies have pointed toward possible genetic mechanisms of host avoidance by *B. burgdorferi*. These include intragenic recombination between the genes encoding OspA and OspB (34), development of point mutations within the *ospA* or *ospB* ORFs resulting in truncated or antigenically altered proteins (13–15, 35), and loss of plasmids encoding major outer surface proteins with or without immunologic counterselection (36, 39). However, all studies of this type to date have been performed in vitro, and hence it is not known whether these phenomena are relevant to long-term survival of *B. burgdorferi* in vivo. An in vivo model for the study of the pathogenetic mechanisms of *B. burgdorferi* infection imposes a unique set of conditions and requirements that are not usually operative in an in vitro system, such as relatively higher incubation temperatures (38°C core body temperature in mice versus 32°C for optimal growth in culture), tissue attachment and invasion, availability of immunologically privileged sites, and influence of cellular and humoral immune responses. In an in vivo model, successful spirochetal escape variants would need to strike a balance between the loss or alteration of genes encoding outer surface proteins and other virulence factors and the possible requirement of those genes or gene products for persistence or continued infectivity.

Of possible relevance to the question of the requirement for OspA and/or OspB for virulence is the observation that *ospA* and *ospB* DNA sequences are consistently detected by PCR in late Lyme disease; indeed, these sequences were detected more frequently than potentially more stable genomic targets, such as the genes encoding 16S rRNA and flagellin (31, 42). Our studies recently demonstrated *ospA* DNA sequences in synovial fluid specimens from 96% of untreated chronic Lyme disease patients with up to 7-year histories of Lyme arthritis (28). These observations are consistent with those of the present study, which demonstrate that loss of OspA-OspB-encoding plasmids or drastic alteration of the *ospA-ospB* ORF, while demonstrable in vitro, is not a predominant mechanism

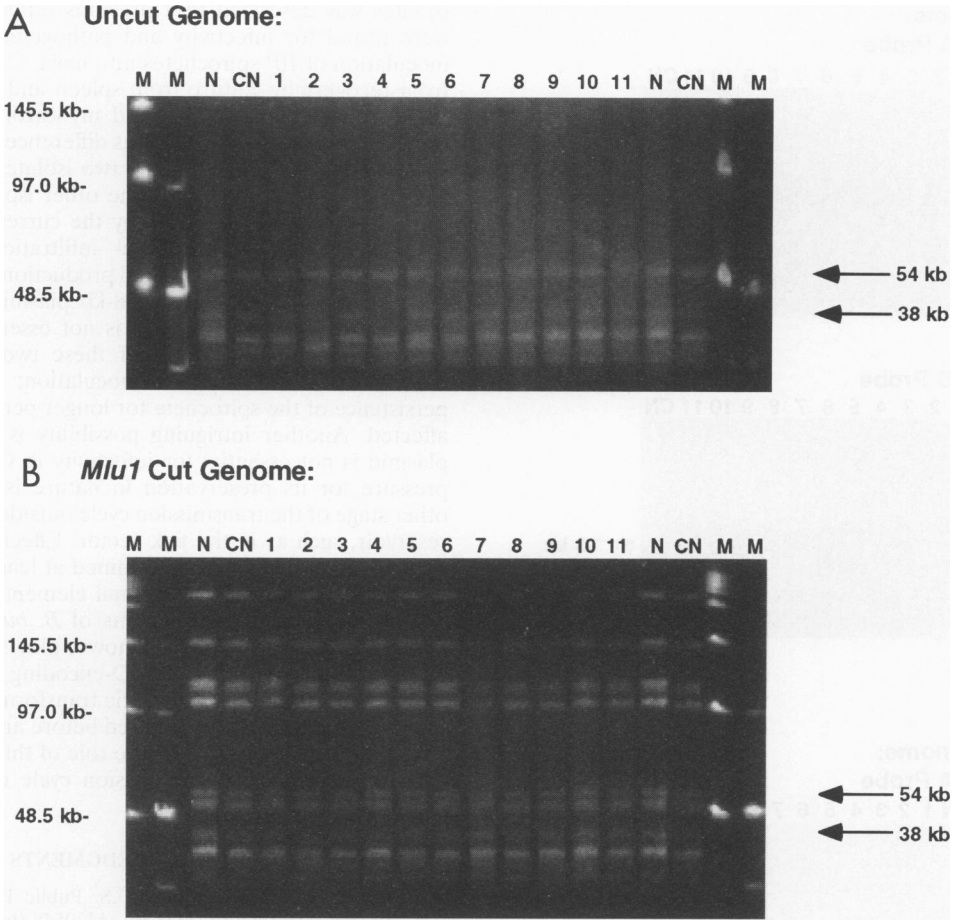

FIG. 3. Analysis of 1-year isolates by pulsed-field gel electrophoresis and genomic macrorestriction digestion. In situ-lysed DNA samples were separated at 200 V with an initial switch time of 1 s and a linear ramp with a final switch time of 10 s for 16 to 17 h. The DNA was not cut (A) or cut with *Mlu*I (B). Lambda DNA concatemers (first lane M) and a mixture of lambda DNA *Hin*dIII fragments, lambda DNA, and lambda concatemers (second lane M) were loaded as pulse markers. The subsequent lanes contained N40 (lane N); cloned N40 (lane CN); bladder isolate 77633 (lane 1), 77639 (lane 2), or 77643 (lane 3); blood isolate 77636 (lane 4), 77643 (lane 5), or 77644 (lane 6); and ear skin isolate 77632 (lane 7), 77633 (lane 8), 77639 (lane 9), 77643 (lane 10), or 77644 (lane 11).

of host avoidance in vivo. Indeed, our results suggest that even minor genetic variation in these ORFs, as demonstrated by PCR-restriction fragment length polymorphism analysis and DNA sequencing of five of the isolates, is not a requirement for persistence of infection in an immunocompetent and seroreactive vertebrate host. Although we cannot rule out the possibility that such changes occur infrequently, even among the six isolates not analyzed by DNA sequencing, the consistent expression of apparently full-length OspA and OspB proteins in these isolates and the reactivity of the OspA and OspB of the isolates with autologous antisera argue against the frequent development of such mutations.

The antigenic stability of the long-term isolates can also be inferred on immunological grounds. The results of the present study are consistent with the recent findings of one of us (S.W.B.) (6); in that study, mice cured of *B. burgdorferi* infection after several time intervals were protected against needle challenge by autologous late isolates just as well as against the original cloned inoculum. These studies suggest that if antigenic variation of *B. burgdorferi* occurs at all, it is not sufficient to ameliorate immunologic recognition of the 1-year isolates in animals that had developed protective immunity during infection. Taken together, the data suggest that *B. burgdorferi* remains genetically and antigenically stable relative to its phylogenetic cousin, *B. hermsii*, during the course of persistent infection. In light of these findings, other mechanisms of host avoidance, such as escape into immunologically privileged sites, should be actively explored.

The current studies do not address whether escape variation in *B. burgdorferi* might occur at the transcriptional level. Several studies have demonstrated significant interisolate variation in outer surface protein expression (42). Recently, Margolis and Rosa demonstrated decreased OspA and OspB expression and a concomitant increase in OspC expression in an unusual tick isolate of *B. burgdorferi* (25). When experimental mice are inoculated with low doses of spirochetes, only a few proteins (particularly the 22-, 39-, and 41-kDa proteins) are recognized early in the course of infection (7). In contrast, immunologic responses to the 31- and 34-kDa OspA and OspB proteins are often delayed. Similar patterns of the humoral immune response appear to occur in human Lyme disease. Thus, it is conceivable that "antigenic variation" of some *B. burgdorferi* proteins occurs at the level of transcription. Further studies examining the expression of *B. burgdorferi* genes in vivo will be necessary to determine whether decreased expression of genes encoding immunodominant antigens can explain the

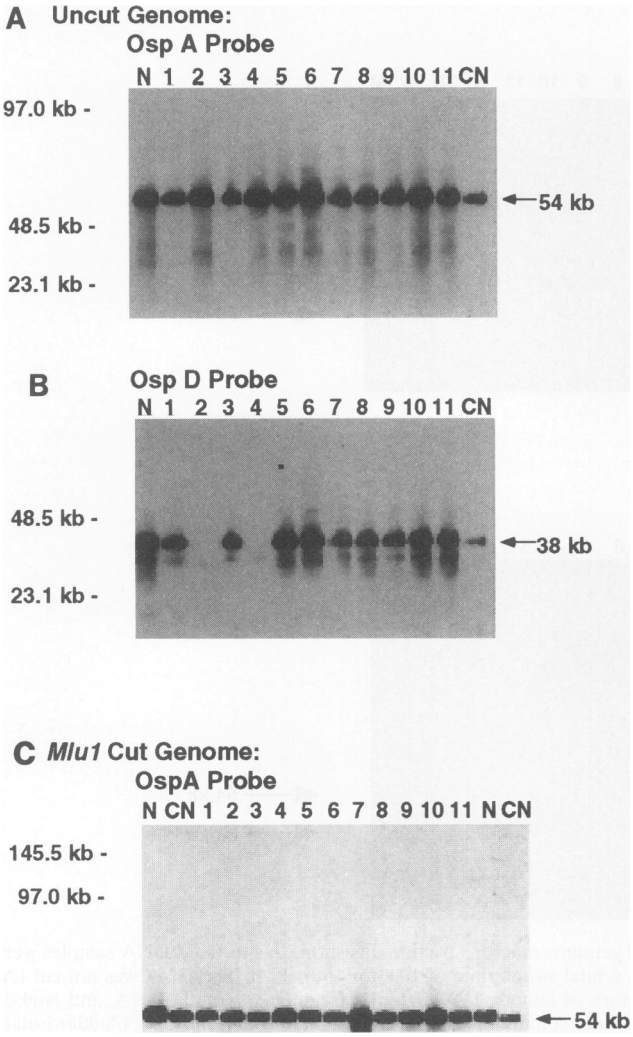

FIG. 4. Probe analysis of pulsed-field electrophoresis gels and genomic macrorestriction digestion products. Autoradiographs of Southern blots of gels (see Fig. 3) hybridized with *ospA* (54 kb) and *ospD* (38 kb) chemiluminescent probes. Probe DNA was amplified from genomic N40 *B. burgdorferi* with *ospA* and *ospD* primers in a PCR. (A and B) Lanes contained N40 (lane N); bladder isolate 77633 (lane 1), 77639 (lane 2), or 77643 (lane 3); blood isolate 77636 (lane 4), 77643 (lane 5), or 77644 (lane 6); ear skin isolate 77632 (lane 7), 77633 (lane 8), 77639 (lane 9), 77643 (lane 10), or 77644 (lane 11); and cloned N40 (lane CN). (C) Lanes contained N40 (lane N); cloned N40 (lane CN); bladder isolate 77633 (lane 1), 77639 (lane 2), or 77643 (lane 3); blood isolate 77636 (lane 4), 77643 (lane 5), or 77644 (lane 6); or ear skin isolate 77632 (lane 7), 77633 (lane 8), 77639 (lane 9), 77643 (lane 10), or 77644 (lane 11).

paradoxical coexistence of live infectious *B. burgdorferi* spirochetes alongside an immune response that is protective against rechallenge by the same spirochetes. In addition to studies of gene expression in vivo, sensitive methods for identifying subtle genetic differences between two closely related genomes, such as representational difference analysis (22a), may be required to identify genetic alterations below the level of detection of the methods used in our analysis that may have occurred.

The significance of the loss of the 38-kb plasmid encoding OspD is not known at present. The infectivity of all 11 1-year isolates was described in a previous report (8). The isolates were tested for infectivity and pathogenicity by intradermal inoculation of 10^4 spirochetes into naive C3H mice. As judged from recovery by culture from spleen and bladder and histologic examination of joints and myocardium, all 11 isolates were pathogenic, and no obvious differences in disease severity could be demonstrated for the two isolates lacking the 38-kb plasmid compared with the nine other isolates or the cloned N40 inoculum strain. Judged by the current criteria used for the determination of virulence—infiltration of target organs such as the bladder and skin and production of inflammation in joints and myocardium—the 38-kb plasmid, and presumably the function(s) encoded by it, is not essential for infectivity. However, the pathogenicity of these two isolates was only determined at 14 days postinoculation; it is possible that persistence of the spirochete for longer periods is in some way affected. Another intriguing possibility is that although this plasmid is not essential for infectivity in C3H mice, selective pressure for its preservation in nature is supplied by some other stage of the transmission cycle outside of the mammalian reservoir, such as in the tick vector. Effective transmission of *Yersinia pestis* by fleas is determined at least in part by a gene encoded by an extrachromosomal element (26). It is possible that extrachromosomal elements of *B. burgdorferi*, the functions of which are largely unknown, play similar roles. Additional studies of the 38-kb OspD-encoding plasmid by recently developed techniques for genetic transformation and targeted mutagenesis (32) will be required before any definitive conclusions can be made regarding the role of this plasmid and/or its gene products in the transmission cycle or pathogenicity of Lyme disease.

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