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# Clonal Polymorphisms of Outer Membrane Protein OspB of *Borrelia burgdorferi*

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**The outer membrane protein OspB of *Borrelia burgdorferi*, the Lyme borreliosis agent, differs in relative molecular weight ($M_r$) among strains. To determine whether antigenic variation occurs in *B. burgdorferi*, a cell population of the human isolate HB19 was cloned first by being diluted in broth and then by being plated on agar medium. Several clones were obtained and characterized by polyacrylamide gel electrophoresis, in situ protease treatment, and Western (immunoblot), Southern, and Northern (RNA) blot analyses. Variants featuring OspB proteins that differed in $M_r$s and in reactivities with monoclonal antibodies were found. One variant made increased amounts of a 21,000-molecular-weight protein (21K protein) in addition to normal amounts of a 33K OspB protein. Another variant did not produce an OspB protein at all but did express an 18.5K protein. Both the 18.5K and 21K proteins were susceptible in situ to trypsin and were bound by a monoclonal antibody directed against the OspB of strain HB19. There were no differences in the Southern and Northern blot analyses of the different variants. The results led to the following conclusions. (i) Clonal polymorphisms in the surface protein OspB occurred in *B. burgdorferi*. (ii) Hitherto uncharacterized 18.5K and 21K proteins were protease susceptible, antigenically related to OspB, and apparently produced in greater amounts when an OspB either was not produced or was altered in structure. (iii) The OspB variations, including its absence from cells, were not accounted for by major DNA rearrangements or failure of transcription of the *ospB* gene.**

Lyme borreliosis is a common tick-transmitted zoonosis of temperate latitudes of North America, Europe, and Asia (reviewed in reference 6). Infection with the spirochete *Borrelia burgdorferi* is the proximate cause of the disorder (12, 14, 27). Some infected persons recover uneventfully after an acute illness, while others suffer a disorder that may progress through three stages in a course measured in years (26, 28). Patients with late disease present with chronic arthritis or disabling dysfunctions of the nervous system. Despite vigorous humoral and cellular immune responses by the host to the agent, the spirochete is able to persist for years in these patients (26). The mechanisms by which *B. burgdorferi* avoids elimination by the immune system of the host are unknown.

Immunological and biochemical analyses have revealed differences between *B. burgdorferi* isolates from North America and Europe (7, 30). In large measure, serotypic distinctions are attributable to differences in abundant outer surface proteins that have been designated Osp proteins. The type strain B31 and the majority of other North American isolates of *B. burgdorferi* have two Osp proteins, A and B, with relative molecular weights ($M_r$) of approximately 31,000 and 34,000 (31K and 34K proteins), respectively (9, 11). In a collection of North American isolates, OspB proteins were more heterogeneous than OspA with respect to size and reactivity with monoclonal antibodies (7–9). In strain B31, *ospA* and *ospB*, the genes for the two Osp proteins, are tandemly arrayed on linear plasmids and cotranscribed (13, 18, 19).

One explanation for the persistence of bacteria in the host is antigenic variation by the pathogen. This strategy is used by the closely related borrelia, *B. hermsii*, an agent of relapsing fever (10). Antigenic differences between strains in *B. burgdorferi* were documented as reviewed above (7–9, 11, 30). There was also evidence of antigenic variation within a strain of *B. burgdorferi*. During infections in rabbits, cotton rats, gerbils, and mice, spirochete numbers in the blood of the animals fluctuated (15, 16, 25). There were alternating periods during which borreliae were or were not cultivable from the blood of cotton rats (K. Gage, W. Burgdorfer, and A. G. Barbour, unpublished observations). During in vitro cultivation of *B. burgdorferi*, changes in the presence, apparent size, and antibody reactivity of the OspB protein were noted (23, 24). In a study of human disease, Craft et al. found that some patients with Lyme borreliosis exhibited the new appearance of OspB-specific immunoglobulin M (IgM) antibodies a year or more after infection (17).

The foregoing reports indicated that the OspB protein may play an important role in evasion of the immune response by the pathogen. To determine whether true antigenic variation in this protein occurs and to characterize any changes in OspB, we examined the progeny of single organisms grown in vitro. Previous studies had employed what were possibly mixed populations of *B. burgdorferi* (15, 16, 23–25). The experiments described herein demonstrate that *B. burgdorferi* is polymorphic with respect to the OspB protein.

## MATERIALS AND METHODS

**Spirochete strains.** *B. burgdorferi* strains were B31 (ATCC 35210), the type strain, and HB19, a blood isolate from a patient with Lyme disease in Connecticut (9, 27). Both strains had been cloned twice by limiting dilution (4). The two strains were distinguished by their reactivities with monoclonal antibodies (8, 9) and by their plasmid profiles (2).

**Cultivation of borreliae.** The borreliae were cultivated in BSK II broth as previously described (1, 4); the medium supports the growth of *B. burgdorferi* cultures from single cells. Cells were harvested for studies by centrifugation

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(12,000 × *g* for 12 min at 20°C) when there were approximately $10^8$ spirochetes per ml.

A solid medium based upon BSK II was also prepared by using a modification of the method of Kurtii et al. (20). To 400 ml of deionized water was added the following: 100 ml of 10× CMRL 1066 concentrate without glutamine (GIBCO Laboratories, Grand Island, N.Y.)–50 g of bovine serum albumin (Pentex Fraction V; Miles Diagnostics, Kankakee, Ill.)–5 g of Neopeptone (Difco Laboratories, Detroit, Mich.)–6 g of HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Research Organics, Cleveland, Ohio)–0.7 g of sodium citrate–5.0 g of glucose–0.8 g of sodium pyruvate–0.4 g of *N*-acetylglucosamine (Sigma Chemical Co., St. Louis, Mo.)–2.2 g of sodium bicarbonate. The pH was adjusted to 7.6, and 100 ml of 14% gelatin (Difco) was added before the mixture was filtered through nitrocellulose membranes (0.22 μm pore size; Millipore Corp., Bedford, Mass.). Rabbit serum (Pel-Freez, Rogers, Ark.), rifampin, and phosphomycin (Sigma) were added to give concentrations of 12% (vol/vol) 100 μg/ml, and 200 μg/ml, respectively, in the 2× concentrate. The medium was warmed to 50°C in a water bath. An equal volume of 3% agarose (SeaKem LE; FMC Bioproducts, Rockland, Maine), which had been autoclaved and kept molten at 65°C, was added to the concentrated medium. After the mixture was inverted several times, 8.5 ml was dispensed to polystyrene Petri dishes (60 by 15 mm). The plates were kept in candle jars at 37°C overnight before use. BSK plates were spread with 100 μl of the spirochete suspension in liquid BSK II medium, sealed with Parafilm (American Can Co., Greenwich, Conn.), and incubated at 34°C in a candle jar. After 2 weeks of incubation, the plates were examined with a dissecting microscope for the presence of colonies.

**Variant isolation.** For cloning by limiting dilution, late log phase broth cultures ($10^8$ spirochetes per ml) were serially diluted 10-fold from $10^{-2}$ to $10^{-10}$. One 10-ml tube was used for dilutions of $10^{-2}$ to $10^{-6}$, two were used for dilutions of $10^{-7}$, and ten were used for dilutions of $10^{-8}$ to $10^{-10}$. The tubes of the $10^{-8}$ to $10^{-10}$ dilutions were examined after 2, 3, and 4 weeks of incubation. For cloning by single colony formation, well-isolated colonies on BSK plates were picked up with sterile Pasteur pipettes and inoculated directly into BSK II broth supplemented with rifampin and phosphomycin.

**PAGE and Western immunoblot analysis.** Whole-cell lysates of the spirochetes were subjected to polyacrylamide gel electrophoresis (PAGE) and Western blot analysis essentially as described previously (10, 11). TNE (50 mM Tris [pH 7.5], 150 mM NaCl, 5 mM EDTA) was used for all antibody and radioiodinated protein A incubations. The blocking solution was 2% bovine serum albumin in TNE. Tween 20 was added to TNE for a final concentration of 0.05% for the washing steps. When a second antibody was needed for monoclonal antibody detection, the membrane was incubated in a 1:400 dilution of the IgG fraction of rabbit anti-mouse immunoglobulin (Miles) in TNE for 1 h before incubation with protein A (9).

**Monoclonal antibodies.** The OspB-specific monoclonal antibody H6831 had been derived from BALB/c mice immunized with strain B31 organisms (9); H6831 does not bind to the OspB of HB19 (7–9). H5TS was the product of immunization of a New Zealand black mouse infected with strain HB19; H5TS binds to the OspB of HB19 but not to that of B31 (9). Additional monoclonal antibodies directed against HB19 were produced for this study. Harvested borreliae were washed with phosphate-buffered saline (PBS), fixed in 1% formaldehyde in PBS for 30 min on ice, washed again with PBS, and suspended in PBS. Adult BALB/c mice were inoculated intraperitoneally on day 1 with a suspension of $10^8$ cells emulsified with an equal volume of Freund complete adjuvant. On day 28, the mice received $10^8$ formaldehyde-fixed cells intravenously, and spleens for fusion were taken on day 32. Hybridoma supernatant fluids were screened by immunofluorescence assays performed on formaldehyde-fixed HB19 organisms (10, 11). The hybridoma supernatant fluids were diluted 1:10, and the specificities of the monoclonal antibodies for the OspB protein of HB19 were confirmed by Western blot analysis (16). Cloning of hybridomas, PAGE analysis of light chains for confirmation of antibody purity, and isotype determinations were carried out as described previously (9–11).

**Protease treatment.** Intact spirochetes were treated with trypsin essentially as described previously (9). In brief, freshly harvested cells were washed once with PBS with 5 mM $MgCl_2$ and suspended in this buffer at a concentration of $2 \times 10^9$ cells per ml. To 950 μl of the cell suspension in a microcentrifuge tube was added 50 μl of 1 mM HCl with or without trypsin (1 mg/ml; Sigma). After the cells were incubated in trypsin or control solutions for 40 min at 20°C, 50 μg of phenylmethylsulfonyl fluoride (Sigma) was added to all tubes. The cells were centrifuged at 12,000 × *g* for 12 min and washed twice with PBS with 5 mM $MgCl_2$. Afterward, the cells were centrifuged and the pellet was suspended in PAGE sample buffer containing 50 μg of phenylmethylsulfonyl fluoride per ml.

**DNA probes.** The probes used for these studies were the following: (i) pTRH46, a recombinant plasmid containing the entire *ospB* gene (888 bases), 183 bases at the 3′ end of the *ospA* gene, and the 12-base intervening sequence between the end of *ospA* and start of *ospB* of strain B31 (13, 18, 19); (ii) plasmid pBBU34, which was constructed for this study from the vector pUC8 and contained the terminal 317 bases of the *ospB* gene of B31 (13); and (iii) plasmid pBBU31, which was constructed for this study from the vector pUC8 and contained the first 177 bases of the protein coding sequence and 151 bases upstream of the start codon in the *ospA* gene of B31 (13). Preliminary studies showed that the plasmid vectors carrying the inserts did not hybridize to *B. burgdorferi* DNA or RNA in Southern and Northern blots. The probes were labeled with [$^{32}$P]ATP by nick translation, using a commercial kit (Bethesda Research Laboratories, Inc., Gaithersburg, Md.).

**DNA extraction and Southern blot analysis.** Extraction of borrelia plasmid DNA was as previously described, with some modifications (5). A pellet of $10^9$ cells was washed once with 1 ml of PBS with Mg and suspended in 250 μl TES (50 mM Tris [pH 8.0], 50 mM EDTA, 15% sucrose). The suspension was cooled on ice for 10 min before adding 250 μl of 1% sodium deoxycholate (Sigma) in TES and 6 μl of diethylpyrocarbonate (Sigma). After the sample had been shaken for 10 min, 250 μl of 7.5 M ammonium acetate was added and the samples were centrifuged at 12,000 × *g* for 10 min. The plasmid DNA was precipitated from the supernatant fluid by the addition of an equal volume of cold isopropanol. The precipitate was recovered by centrifugation (12,000 × *g* for 25 min) and dissolved in 10 mM Tris (pH 8.0)–1 mM EDTA.

The procedure for Southern blot analysis was essentially as described previously (7). *B. burgdorferi* DNA was cleaved with restriction endonucleases, which were obtained from Boehringer Mannheim Biochemicals (Indianapolis, Ind.), and used according to the recommendations of the

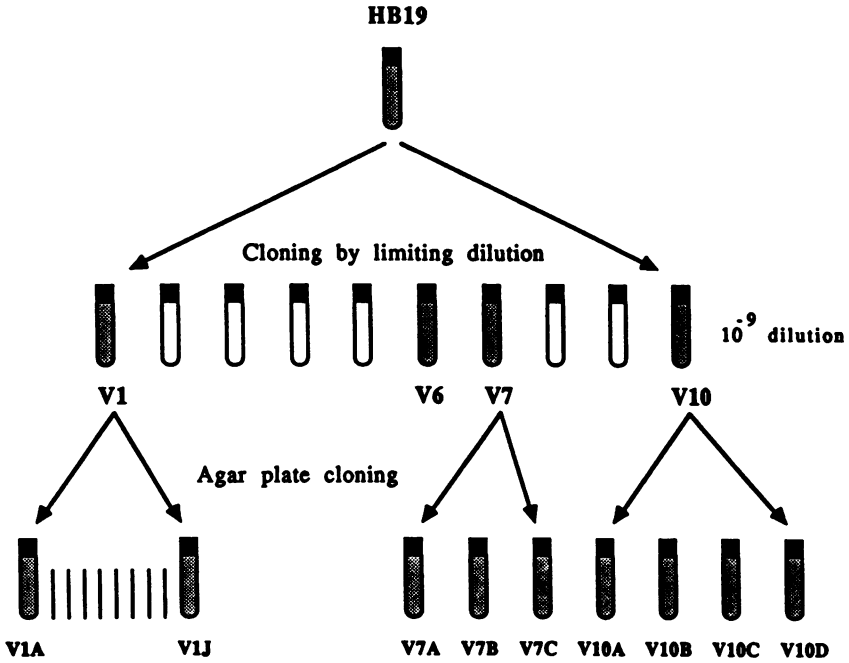


FIG. 1. Pedigrees of variants obtained by cloning a population of *B. burgdorferi* HB19 spirochetes first by limiting dilution in broth and then by plating on agar medium.

supplier. Fragments were separated in a 1% agarose gel and then were transferred to nylon membranes (Biotrans; 1.2-μm pore; ICN Biomedicals, Irvine, Calif.). The prehybridization and hybridization solutions were composed of 50% formamide–5× Denhardt–6× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate)–5 mM EDTA–0.5% sodium dodecyl sulfate–0.1% sodium pyrophosphate–0.01% salmon sperm DNA (5). Hybridization procedures were performed at 37°C, and washes with 0.1× SSC–0.1% sodium dodecyl sulfate–1 mM EDTA were carried out at 64°C. The filters were placed on X-ray film with an intensifying screen at −79°C.

**RNA extraction and Northern (RNA) blot analysis.** Total RNA was extracted from freshly harvested borreliae and subjected to Northern blot analysis as described previously (18, 29). The cell lysing solution was 4 M guanidine thiocyanate–25 mM sodium citrate (pH 7.0)–2% 2-mercaptoethanol–2% sodium lauryl sarcosinate. RNA was stored in 70% ethanol at −20°C until electrophoresis, at which time it was recovered by centrifugation, dried, and suspended in diethylpyrocarbonate-treated water and sample buffer (18). The electrophoresis buffer for 1.2% agarose gels was 2.2 M formaldehyde–200 mM HEPES–sodium salt (pH 7.0)–50 mM sodium acetate–10 mM EDTA. Electrophoresis was at 2 V/cm for 15 h. The gel was stained with acridine orange to visualize the 23S, 16S, and 5S rRNA bands (21). The fractionated RNA was transferred to a nylon membrane. The prehybridization, hybridization, and washing procedures were the same as described for Southern blot analysis.

## RESULTS

**Cloning by limiting dilution.** Strain HB19 of *B. burgdorferi* was originally recovered from the blood of a patient with first-stage Lyme borreliosis in 1982 (9, 27). It had been passed in broth medium continuously for 3 years and had been cloned by limiting dilution twice during that period. HB19 was chosen for study of possible antigenic variation because it had been observed to vary in the $M_r$ of its OspB protein during the course of serial passage (unpublished observations). In addition, HB19 did not aggregate; 95% or more of the cells examined by phase-contrast microscopy occurred singly. Thus, cloning by limiting dilution was a feasible technique for isolating clonal populations of *B. burgdorferi*. Monoclonal antibody H5TS was known to recognize the OspB protein of HB19 (8, 9). To further define possible antigenic changes, additional murine monoclonal antibodies to the OspB protein of HB19 were sought. Three different IgG antibodies were found to bind to the OspB protein of HB19 in Western blots and were designated H68, H614, and H63.

A culture of HB19 that had been passaged continuously for 3 months (or approximately 600 generations) after the last cloning by limiting dilution was examined by PAGE. We noted that the band corresponding to the OspB protein appeared to be a doublet (data not shown). This finding suggested the presence of OspB variants in this clonally derived population. To test this hypothesis, a third round of cloning by limiting dilution was carried out. Spirochetes were serially diluted in 10 tubes at each dilution (Fig. 1). After up to 4 weeks of cultivation, all tubes at the $10^{-8}$ dilution, 4 of 10 tubes at the $10^{-9}$ dilution (numbers 1, 6, 7, and 10), and no tubes at the $10^{-10}$ dilution had growth. The cultures in the four $10^{-9}$ dilution tubes were designated V1, V6, V7, and V10.

**Characterization of HB19 clones.** Cultures of clones V1 and V6 exhibited cell clumping that was visible to the unaided eye. Free-floating flocculent material, 0.5 to 3 mm in diameter, appeared by day 3 of culture. In contrast, cells of variants V7 and V10, as well as those of strain B31, did not aggregate.

Proteins in total cell lysates of the clones were examined by PAGE (Fig. 2). The only differences noted among isolates were in the migrations of bands corresponding to the OspB

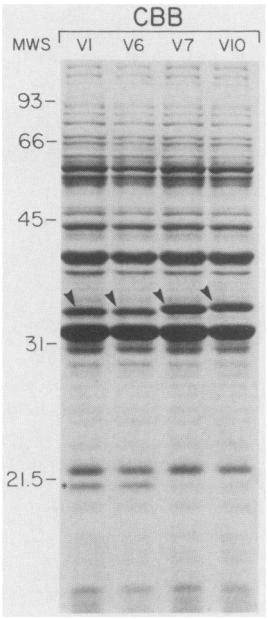


FIG. 2. PAGE of whole-cell lysates of clones V1, V6, V7, and V10 from *B. burgdorferi* HB19. The gel contained 12.5% acrylamide and was stained with Coomassie brilliant blue (CBB). The molecular weight standards (MWS), whose $M_r$s ($\times 10^3$) are listed on the left, were: phosphorylase B, 93; bovine serum albumin, 66; ovalbumin, 45; carbonic anhydrase, 31; and soybean trypsin inhibitor, 21.5 (Bio-Rad Laboratories, Richmond, Calif.). The arrowheads indicate the OspB of each isolate; the asterisk identifies the 21K protein in variants V1 and V6.

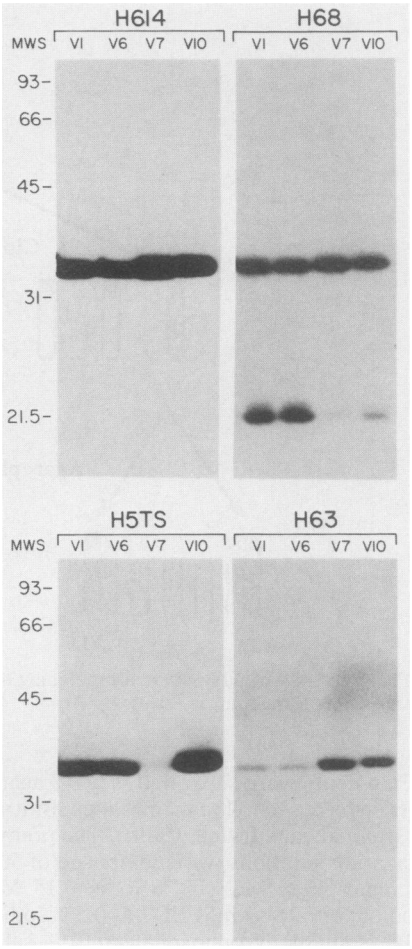


FIG. 3. Western blot analyses of lysates of clones V1, V6, V7, and V10 of *B. burgdorferi* HB19. The electrophoretically separated proteins were transferred to nitrocellulose and incubated with monoclonal antibody H614, H5TS, H63, or H68. Bound antibody was detected with $^{125}$I-labeled protein A. The blot was exposed to film for 3 h in the cold with an intensifying screen. A second antibody, rabbit anti-mouse IgG, was used for the H5TS blot before the protein A incubation. The molecular weight standards (MWS) are as described in the legend to Fig. 2.

proteins and in the presence or absence of a 21K protein. The $M_r$s of the OspB of V1, V6, V7, and V10 were 33,000, 33,000, 33,500, and 33,700, respectively. Both V1 and V6 produced the 21K protein. V1 and V6 also appeared to have less OspB protein than did V7 or V10.

Proteins separated by PAGE were transferred to membranes and probed with different monoclonal antibodies to OspB in Western blot analyses (Fig. 3). Although both H614 and H63 bound to the OspB of all clones, antibody H63 consistently bound less well in Western blots to the OspB of V1 and V6 than to the OspB of V7 and V10. Antibody H5TS recognized the OspB of V1, V6, and V10 but not that of V7. H68 bound to the OspB of all HB19 derivatives and, in addition, to the 21K protein of V1 and V6. A small amount of the 21K protein was also detectable in the cells of V7 and V10. Antibody H6831 bound, as expected, to the OspB of B31 but not to any of the HB19 derivatives (data not shown).

After noting the antigenic relationship between the 21K protein and OspB, we investigated whether the 21K protein, like OspB, was cleaved from the cells by proteases and, thus, probably at the cell surface (9, 11). Intact spirochetes treated with trypsin were examined by PAGE and Western blot (Fig. 4). Trypsin cleaved the 21K protein as well as the various OspB proteins from the cells; these protein bands were not detectable with strains or antibodies in lysates of trypsin-treated cells. These findings indicated that the 21K protein like the OspB protein is at the surface of the spirochete. The stained gel in Fig. 4 also confirmed that V1 and V6 produced lesser amounts of their OspB than did V7 and V10.

Because V1 and V6 had the same phenotype by criteria of aggregation, OspB size, and monoclonal antibody reactivities, we assumed V1 and V6 to be identical clones. Thereafter, we used only V1 for studies.

**Southern blot analyses of limiting dilution variants.** Antigenic variation in the related *Borrelia* species, *B. hermsii*, is the consequence of DNA rearrangements that were easily detected as restriction fragment length polymorphisms (21, 22). To determine whether major DNA rearrangements are associated with OspB variation, Southern blot analyses were carried out.

The *osp* genes had been shown to reside on a 49-kilobase (kb) linear plasmid (5), and, therefore, total plasmid DNA was extracted from the clonal variants for further analysis. Clone V10 had the OspB phenotype of the original HB19 isolate and represented the parental population. The digested plasmid DNA samples were probed with the *ospB* gene-bearing plasmid pTRH46 (Fig. 5).

Strain B31 was included in the Southern blots because restriction fragments incorporating all or part of the *ospB* gene could be predicted from the DNA sequence (13). Eight restriction endonucleases (*Eco*RI, *Sca*I, *Dra*I, *Rsa*I, *Alu*I,

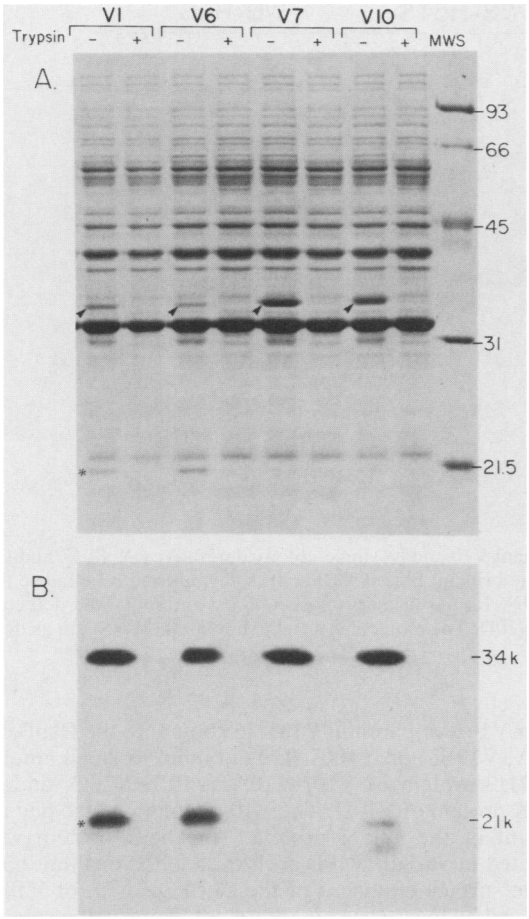


FIG. 4. PAGE and Western blot analysis of trypsin-treated clones V1, V6, V7, and V10 of *B. burgdorferi* HB19. Intact spirochete cells were incubated with (+) or without (−) trypsin. Total cell lysates were fractionated on a 12.5% acrylamide gel and were either stained with Coomassie brilliant blue (A) or transferred to nitrocellulose for Western blot analysis (B). The blot was probed with monoclonal antibody H68. The arrowheads indicate the positions of the OspB proteins, and the asterisk indicates the 21K protein in the lanes of untreated cells. (B) The apparent sizes of the two trypsin-susceptible proteins that were bound by H68 are shown. The molecular weight standards (MWS) are as described in the legend to Fig. 2.

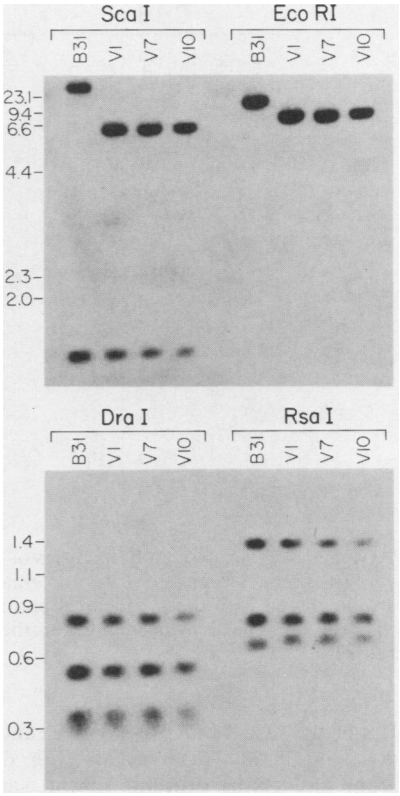


FIG. 5. Southern blot analyses of total plasmid DNA from *B. burgdorferi* B31 and variants V1, V7, and V10 of strain HB19. *Sca*I and *Eco*RI digests were separated on a 1.0% agarose gel, and *Dra*I and *Rsa*I digests were separated on a 1.8% gel. The nylon membrane blots were probed with $^{32}$P-labeled plasmid pTRH46. The size standards (in kilobases) shown on the left were either *Hin*dIII fragments of bacteriophage lambda DNA (upper panels) or *Hae*III fragments of bacteriophage ϕX174 (lower panels).

*Nla*IV, *Hin*fI, and *Sau*3A) that had recognition sites within *ospB* were selected for use. In B31, there is a *Sca*I site in *ospB* and an *Eco*RI site in *ospA* but not in *ospB* (13). We noted two *Sca*I bands and one *Eco*RI band on Southern blots of B31 and the HB19 derivatives (Fig. 5). The HB19 clones differed from B31 in the sizes of their *Eco*RI fragments and the larger of their two *Sca*I fragments. The predicted internal *Dra*I fragments of 528 and 322 bases of *ospB* were seen in digests of B31 as well as in V1, V7, and V10. The third *Dra*I fragment, which had an estimated size of 810 bases, was assumed to be the terminus of the *ospB* gene and downstream sequence. The predicted 721-base *Rsa*I fragment, which includes the *ospA* sequence, was seen in the digest of B31. The larger *Rsa*I fragment of 760 bases in V1, V7, and V10 was evidence that the gene sequences of HB19 and B31 in this region are not identical. Blots of *Alu*I, *Nla*IV, *Hin*fI, and *Sau*3A digests showed no differences among B31, V1, V7, and V10 (data not shown).

These studies, which were sensitive enough to detect restriction fragment length polymorphisms between B31 and HB19, did not reveal differences in *ospB* organization among the three HB19 clones examined. The findings suggested that DNA rearrangements involving large segments of *ospB* were not responsible for the observed OspB variation in these clones.

**Characterization of single-colony clones.** Variants V1, V7, and V10 were plated on a solid medium to isolate clones as single colonies. Colonies visible to the eye formed within 2 to 3 weeks of incubation, but the efficiencies of plating were not greater than 1%. Different colony types were noted on the plates, but there was no apparent correlation between colony phenotype and PAGE protein profile in preliminary studies. Therefore, several colonies from each variant population were picked at random and examined as to their OspB phenotypes with PAGE and monoclonal antibodies. The colonies were inoculated into broth tubes and passaged once before examination. The single-colony clones were designated with the number of the parental variant and a letter, e.g., V7B for clone B from population V7.

Single-colony clones from V1 (V1A through V1J) or V7 (V7A through V7C) had OspB proteins with the same electrophoretic migrations as the OspB proteins of V1 or V7, respectively (data not shown). Four single-colony clones of V10 were examined and were designated V10A, V10B, V10C, and V10D. Two of these V10 derivatives had phenotypes not seen among other HB19 progeny studied up to that

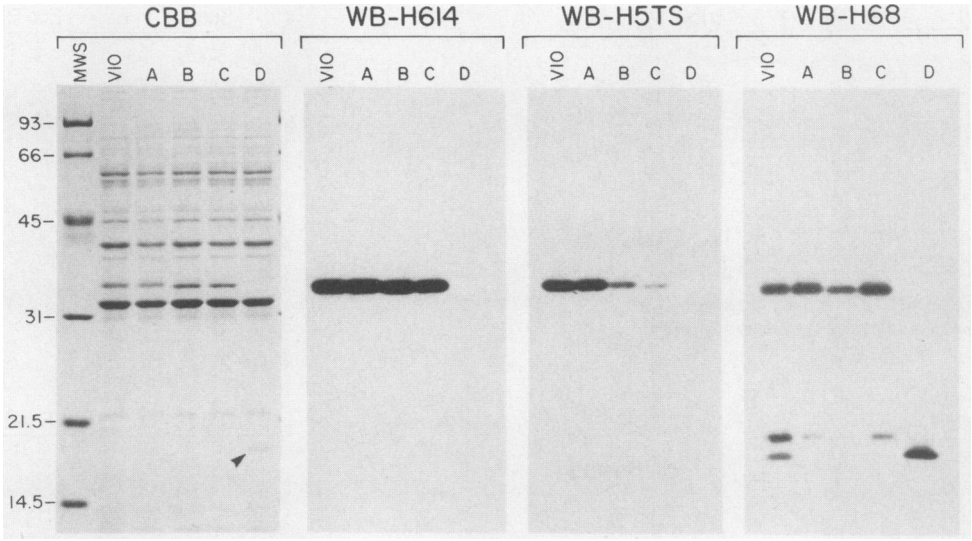


FIG. 6. PAGE and Western blot (WB) analyses of *B. burgdorferi* HB19 variant V10 and its single-colony derivatives A, B, C, and D. Total cell lysates were fractionated on a 12.5% gel and either stained with Coomassie brilliant blue (CBB) or transferred to nitrocellulose for WB. The blots were incubated with the monoclonal antibody H614, H5TS, or H68. Bound primary or secondary antibody was detected with radioiodinated protein A. The arrowhead indicates the 18.5K protein of clone V10D. The molecular weight standards (MWS) are as described in the legend to Fig. 2.

time. The $M_r$s of the OspB of V10A, V10B, and V10C were 33,700, 33,500, and 33,500, respectively (Fig. 6). V10A and the V10 parent had OspB proteins of the same apparent sizes. Unlike the other variants examined, V10D did not appear to produce any detectable OspB. V10D did, however, have a new protein with an $M_r$ of 18,500.

V10A through V10D were subjected to Western blot analyses with the OspB-reactive monoclonal antibodies (Fig. 6). H614 and H5TS bound the OspB proteins of V10 and V10A through V10C. H5TS consistently bound less well in repeat blots to the OspB of V10B or V10C than to that of either V10A or parental V10. H68 bound to the OspB of V10, V10A, V10B, and V10C. It also bound to small amounts of the 21K protein in V10, V10A, V10B, V10C, and to the 18.5K protein of V10D. The V10 population also had a small amount of the 18.5K protein. An OspB protein was not detected in variant V10D with the monoclonal antibodies.

The surface exposure of the 18.5K protein of V10D was assessed by in situ trypsin treatment. Total cell lysates of the trypsinized cells were examined by PAGE, and Western blot analysis with the monoclonal antibody H68 was carried out (Fig. 7). The parental V10 population had small amounts of

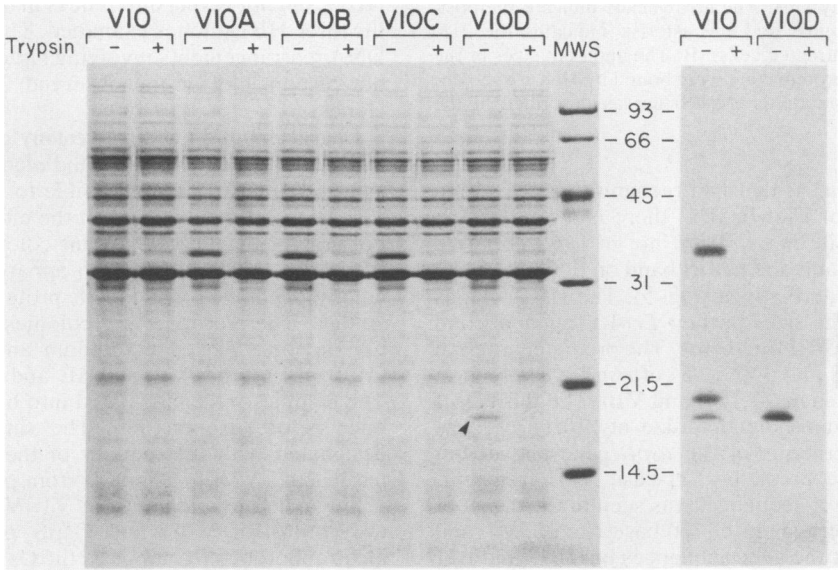


FIG. 7. PAGE and Western blot analyses of trypsin-treated *B. burgdorferi* HB19 variant V10 and its derivatives A, B, C, and D. Total cell lysates, which were prepared from cells that had been incubated with (+) or without (−) trypsin, were fractionated on a 15% gel and stained with Coomassie brilliant blue (left) or transferred to nitrocellulose for Western blot analysis with monoclonal antibody H68 (right). The arrowhead indicates the 18.5K protein of clone V10D. The molecular weight standards (MWS) were as described in the legend to Fig. 2.

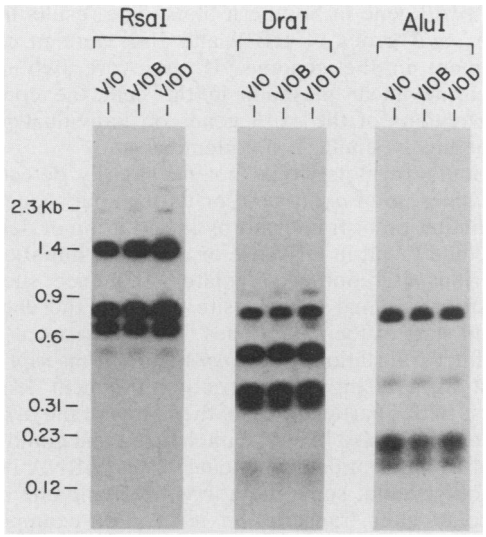


FIG. 8. Southern blot analyses of *Rsa*I, *Dra*I, and *Alu*I digests of total plasmid DNA from *B. burgdorferi* HB19 clones V10, V10B, and V10D. The digests were separated on a 1.8% agarose gel and blotted into a nylon membrane. The size standards were *Hin*dIII fragments of lambda and *Hae*III fragments of φX174 as described in the legend to Fig. 5. The blots were probed with $^{32}$P-labeled plasmid pTRH46.

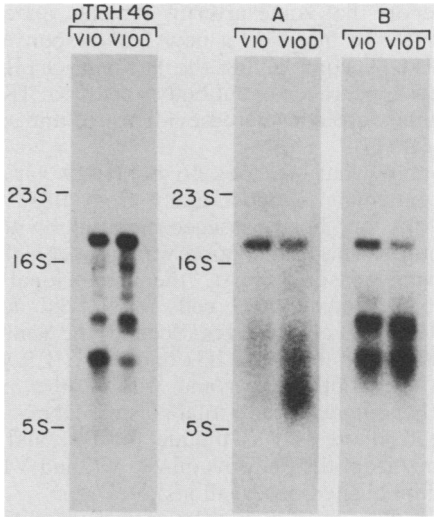


FIG. 9. Northern blot analyses of mRNA from *B. burgdorferi* HB19 clones V10 and V10D. Total RNA was separated in a 1.2% agarose gel under denaturing conditions, blotted to a nylon membrane, and probed with $^{32}$P-labeled plasmid pTRH46, pBBU31 (A), or pBBU34 (B). The positions of 5S, 16S, and 23S rRNA bands in the gel, which were revealed by staining with acridine orange, are indicated on the left.

the 21K and 18.5K proteins. The 18.5K protein of V10D, as well as the OspB of each of the four other clones, was not detectable in the lanes of trypsinized cells by either protein staining or Western blot. This finding indicated that the 18.5K protein was exposed on the surface of the borrelia.

To look for gene rearrangements or deletions in the second group of variants, plasmid DNA from V10, V10B, and V10D was digested with *Rsa*I, *Dra*I, and *Alu*I and probed with pTRH46 (Fig. 8). Restriction fragment length differences among these variants were not found. If a deletion had occurred in the *ospB* gene of V10D, it was not detected by Southern blot with these enzymes.

**Northern blot analyses.** Northern blots were used to determine whether the absence of OspB in V10D was the result of failure of *ospB* transcription. If nonexpression of OspB was due to premature termination of transcription of the *osp* operon, we expected to find that V10D had a hybridizable transcript that was shorter in length than the corresponding mRNA of the OspB-producing isolates.

For one blot we used as a probe pTRH46, which contains an entire *ospB* gene and the 3′ end of the *ospA* gene (13, 18). Both V10 and V10D had a major transcript of about 2.2 kb (Fig. 9, left). In this blot, we noted two major and two minor hybridizing bands of shorter lengths in V10 and V10D (Fig. 9, left). These additional bands were also present in extracts of the original isolate of HB19 and all other HB19 variants (data not shown).

To determine whether the shorter bands represented transcripts of *ospA* or *ospB* or both, the subclones pBBU31 and pBBU34, which contained the 5′ end of *ospA* and the 3′ end of *ospB*, respectively, were used as probes in other Northern blots. There is no sequence similarity between these different regions of the *osp* genes (13), and, thus, cross-hybridization would not occur. The right side of Fig. 9 shows that both *ospA* (pBBU31) and *ospB* (pBBU34) probes hybridized to RNA of V10D and V10. The *ospA* probe hybridized to the 2.2-kb transcript and some degraded material in both extracts but not to the two other major mRNA species hybridized by the pTRH46 probe. This and two repeat experiments showed more degraded material in V10D than V10; the significance of this finding is not known.

The *ospB*-specific probe, pBBU34, prominently hybridized to the two smaller major mRNAs in addition to the 2.2-kb transcript. In the three Northern blots with the pBBU34 probe of separate RNA preparations, there were differences between V10 and V10D in the relative amounts of each of the three types of hybridizable mRNA that were detected in each blot. However, these quantitative differences were slight and not consistent, and, as Fig. 9 also demonstrates, both V10 and V10D produced full-length 2.2-kb transcripts.

## DISCUSSION

Clonal polymorphisms of a major surface antigen of *B. burgdorferi* were observed and characterized. Variants of strain HB19 cloned by either limiting dilution or by plating on solid medium featured OspB proteins that differed in $M_r$s and in their reactivities with monoclonal antibodies. One variant did not detectably produce any OspB. Furthermore, some variants made either of two smaller, protease-susceptible proteins that had $M_r$s of 21,000 and 18,000. These smaller proteins shared at least one epitope with OspB.

Previous studies showed that *B. burgdorferi* isolates from North America and Europe differed in the $M_r$s and antibody reactivities of their OspA and OspB proteins (8, 9, 30). Some isolates were found to entirely lack an OspB protein (9, 30). Schwan and colleagues demonstrated that some populations of *B. burgdorferi* exhibited changes in OspB proteins during serial passage in vitro (23, 24). The original spirochete inocula for their experiments were obtained directly from ticks, however, and possibly were mixtures of strains.

Western blot studies of serial sera from patients with Lyme borreliosis who develop chronic arthritis showed that antibody to some *B. burgdorferi* antigens appeared only after periods of months (3, 17). Of relevance to the present study

was the report that some arthritis patients unaccountably developed late in infection a new IgM response to OspB proteins (17). Accompanying the late anti-OspB response was the new appearance of antibody to 18K to 21K proteins. These results were considered evidence of antigenic variation of OspB (17).

Conceivably what we took to be HB19 variants were actually contaminants, most likely from B31, the strain which had for long been passaged in parallel with HB19 in our laboratory. This explanation was unlikely, though, for the following reasons. First, the monoclonal antibody H6831, which binds to B31 cells (8, 9), did not bind to parental HB19 or its derivatives. Second, the Southern blots showed differences between B31 and the HB19 variants in restriction sites both within and without the *ospB* gene. Third, the banding pattern of total plasmid DNA as revealed by gel electrophoresis was unique for B31 and could be distinguished from the patterns of V1, V7, and V10 plasmid DNA (2; unpublished observations).

In this study, we did not determine the frequency of variation. The variants appeared in the population of HB19 organisms during the approximately 600 generations that passed from the last date of cloning by limiting dilution. At the start of the present study, there undoubtedly was a mixed population of HB19 variants. The variants included in this and subsequent generations were isolated by the two methods of cloning. Preliminary studies suggest that the OspB mutation rate is less than $10^{-2}$ per cell per generation in broth (unpublished observations).

Plating of bacteria on solid medium can provide an estimate of the rate of phase variation in colony type. Initial studies showing colony phenotype differences encouraged us to pursue this line. However, we did not find the correlation between colony phenotype and OspB phenotype to be sufficiently high for colony phenotype to be useful as a marker for OspB variation rate studies. Moreover, such studies would be difficult to interpret given an efficiency of plating that was at most 1%.

The antigenic relatedness between OspB and the 21K and 18.5K proteins was demonstrated with a monoclonal antibody. Increased production of an approximately 20K protein in isolates without OspB had been shown previously (9, 23, 24), but antigenic cross-reactivity between OspB and the smaller proteins had not been appreciated. The small amounts of 21K and 18.5K proteins noted in Western blots of variants other than V1 and V10D may represent heterogeneity of the population or low-level production of these proteins by each cell. Among the variants studied here, increased production of the 21K or 18.5K protein was associated with decreased or halted production of OspB itself.

In trying to understand the mechanism behind OspB antigenic variation, we first looked for DNA rearrangements. In the related *Borrelia* species, *B. hermsii*, which switches surface proteins to avoid the immune response of the host during relapsing fever, changes in serotype-specifying proteins are the consequence of the translocation of an antigen-encoding gene from a silent to an expression locus (21, 22; T. Kitten and A. G. Barbour, submitted for publication). Translocations in *B. hermsii* were detected in Southern blots; the different serotypes had unique restriction fragments revealed by DNA probes. DNA rearrangements involving the *ospB* gene may also have been the basis for OspB variation in *B. burgdorferi*. For this reason, samples of DNA of the HB19 variants were digested with enzymes that had one or more sites in the *ospB* gene and then were probed with an *ospB* gene in Southern blots. The results indicated that the *ospB* genes of HB19 and B31 were in only one environment in the genome. If there are two or more *ospB*-bearing linear plasmids in the cell, the copies and flanking regions of the *ospB* genes on individual plasmids would be highly similar if not identical.

The Southern blots would not necessarily detect single-base changes in an *ospB* gene or its flanking sequences. A base substitution or frameshift by the addition or deletion of a base could result in missense or nonsense mutations or in a deleterious alteration in a regulatory sequence, such as the consensus ribosomal binding site between the end of the *ospA* and start of the *ospB* genes (13). These local changes could affect translation of an *ospA-ospB* transcript not yet manifest as a change in restriction fragment lengths or numbers. In this particular case, the length of the *ospA-ospB* transcript would also likely be unchanged and could only be detected by nucleotide sequencing of the mRNA or DNA. On the other hand, some mutations involving one or a few bases could alter transcription itself. For example, base substitutions bringing a greater potential for formation of a hairpin in mRNA could result in the stalling of transcription before the RNA polymerase complex had completed the *ospB* gene. In this situation, we would expect the *osp* transcript to be shorter in variants lacking OspB than in isolates producing that protein.

To detect the latter types of mutations, we performed Northern blot analyses. These studies did not reveal qualitative differences among the mRNAs of the different variants. The OspB$^-$ variant V10D had a full-length *ospA-ospB* transcript. Thus, the failure to express OspB by V10D could not be explained by premature termination of transcription of the *ospA-ospB* tandem. The significance of the shorter transcripts that were hybridized by the *ospB* probe but not by the *ospA* probe is not known. This finding suggests that there is either another transcriptional start in the operon, presumably within the *ospA* gene, or that degradation occurs first of the 5′, i.e., *ospA*, end of the transcript. The smaller hybridizing mRNAs may encode the 18.5K and 21K proteins. However, the amounts of each of these shorter mRNAs were more or less the same regardless of whether a shorter protein was produced or not.

Another model contends that the shorter proteins are the products of action by an endogenous protease. Changes in the nucleotide sequence may have yielded altered OspB proteins that were more susceptible to proteolytic cleavage. Although the primary change is at the level of the gene, expression of the phenotype, e.g., OspB$^-$, is dependent on posttranslational events. A proteolysis mechanism might explain what seems to be an inverse relationship between production of wild-type OspB and production of a smaller, cross-reactive protein. In any case, the 21K and 18.5K proteins have retained enough sequence for them to be successfully translocated to the surface of the spirochete.

The present study furthers the definition of antigenic variation in *B. burgdorferi* in terms of biology, biochemistry, and molecular genetics. The role of this type of antigenic variation in disease pathogenesis is suggested but not established at this point, and, thus, additional investigations of its occurrence in animal models and human infections should follow. Determinations of the rates of variation and whether variation is reversible are needed. Another important analysis will be of the nucleotide sequences of the *ospB* genes from different variants, especially V1, V7, and V10D. Sequence determinations may reveal single-base changes that affect translation or protein structure.

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