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# High- and Low-Infectivity Phenotypes of Clonal Populations of In Vitro-Cultured *Borrelia burgdorferi*

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**Borrelias that cause Lyme disease lose the ability to infect and cause disease in laboratory animals following 10 to 16 passages of in vitro culture. In this study, clonal populations of the Sh-2-82 (Sh2) and B31 strains of *Borrelia burgdorferi* were isolated by subsurface plating on BSK-II agar plates and examined for infectivity in the C3H/HeN mouse model. Mice were injected intradermally with $10^5$ *B. burgdorferi* organisms, and the tibiotarsal joint, heart, and bladder were cultured 2 to 4 weeks postinfection to determine whether viable organisms were present. Clones exhibited either a high-infectivity phenotype, in which cultures were consistently positive at all organ sites, or a low-infectivity phenotype, in which a low proportion of cultures were positive (5 of 40 in a representative experiment). In an Sh2 population that had undergone five in vitro passages, 7 of 10 clones were of the high-infectivity phenotype, and the remaining clones were of the low-infectivity phenotype. The proportion of high-infectivity clones decreased with continued in vitro passage, with only 1 of 10 clones exhibiting the high-infectivity phenotype after 10 passages and 0 of 10 clones yielding positive cultures after 20 passages. Representative high- and low-infectivity clones from passage 5 Sh2 cultures had 50% infectious doses of $1.8 \times 10^2$ and $1 \times 10^5$, respectively. Subclones consistently reflected the same infectivity phenotypes as those of the parent clones. The protein profiles and plasmid contents of the high- and low-infectivity clones were compared and exhibited few discernible differences. On the basis of these results, the loss of infectivity during in vitro culture results from the outgrowth of low-infectivity clones and begins to occur within the first five in vitro passages. Further examination of clonal populations may lead to the identification of genetic and protein factors important in the virulence and pathogenicity of Lyme disease borrelias.**

Infection by *Borrelia burgdorferi* or other Lyme disease borreliae (including *Borellia garinii* and *Borrelia afzelii* [group VS461] [1]) results in erythema migrans, a localized, erythematous rash, in the region of the bit of an infected *Ixodes* tick or other vector (3, 26). Dissemination occurs during this early stage of infection (10, 12, 23) and can result in additional neurologic, cardiologic, arthritic, and dermatologic manifestations. Without treatment, the spirochetes can persist in the host for months to years despite a demonstrable immune response and the presence of borreliacidal antibodies. At present, the pathogenetic mechanisms of *B. burgdorferi* in Lyme disease are not well understood. Motility, the adherence to and penetration of mammalian tissues, persistence in extracellular matrix and possibly within host cells, induction of cytokines, cellular responses, and other elements of the host inflammatory response, and the activation of autoimmune reactions may all potentially be involved (20).

One approach to the study of infectivity and virulence of *B. burgdorferi* is through the comparison of virulent and avirulent strains. Strains of *B. burgdorferi* lose their ability to infect laboratory animals following 10 to 17 passages of in vitro culture (11, 14, 21). Analyses of the uncloned Sh-2-82 (Sh2) (21, 24) and B31 (2) strains demonstrated the loss of some circular and linear plasmids and changes in protein profiles following prolonged in vitro passage. A 38-kb linear plasmid encoding a major lipoprotein, outer surface protein D (OspD), was present in low-passage B31 but absent from high-passage B31 (15). The infectious, low-passage form of the Sh2 strain contained two related 8.4- and 8.8-kb supercoiled plasmids, pBBC1 and pBBC2, but these plasmids were not detectable in noninfectious Sh2 acquired by in vitro culture for more than 15 passages. Sh2 cultured for 25 passages also lost expression of the 34-kDa outer surface protein B (OspB), which was replaced by a 20-kDa protein (21). However, no clear correlation between plasmid content and virulence has been established. For example, the OspD-encoding 38-kb plasmid is present in some high-passage, avirulent strains but is absent from low-passage, infectious Sh2 (13, 15). Also, some low-passage, infectious strains of *B. burgdorferi* lack the plasmids pBBC1 and pBBC2 (24), and many infectious European isolates exhibit diminished or altered expression of the major outer surface proteins OspA and OspB (28, 29). The inherent heterogeneity and plasticity of Lyme disease borrelia strains thus complicates the identification of plasmids, genes, and gene products related to virulence.

Examination of the infectivity and the genetic and polypeptide content of clonal populations of *B. burgdorferi* derived from a common parent strain may decrease this background variability and permit a more direct correlation of these properties. In this study, clones of the Sh2 and B31 strains of *B. burgdorferi* were analyzed for infectivity in the C3H mouse model (5) and for polypeptide and plasmid content.

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## MATERIALS AND METHODS

**Bacteria.** The low-passage B31 and Sh2 strains of *B. burgdorferi* were originally isolated from *Ixodes scapularis* ticks in the state of New York (8, 22) and had undergone five in vitro passages at the initiation of these studies. The B31 isolate used in this study differs from *B. burgdorferi* ATCC 35210 in that the latter is a nonvirulent, high-passage strain. All liquid cultures were incubated in BSK-II medium without gelatin and with 50 μg of rifampin per ml and 100 μg of phosphomycin per ml at 34°C in an anaerobic chamber (Coy Laboratories, Ann Arbor, Mich.) adapted to maintain 1.5 to 3.0% $O_2$–5% $CO_2$–balance $N_2$ (16). To obtain higher-passage numbers, *B. burgdorferi* was subcultured by weekly passage of 0.2 ml of culture into 6 ml of BSK-II medium. Approximately 30-fold multiplication was obtained per passage (see Fig. 1), corresponding to about five generations. Late-log-phase cultures were frozen at −70°C or liquid nitrogen temperatures in BSK-II medium with 15% (vol/vol) glycerol. To minimize variation, frozen stocks were never thawed; rather, the frozen cultures were kept on dry ice when transported from the freezer. A small amount of culture was scraped from the surface with a sterile inoculating loop, inoculated into 2 ml of BSK-II medium, and incubated for 2 to 4 days as described above. All cultures utilized for cloning, animal inoculation, protein electrophoresis, and plasmid analysis in this study had undergone a maximum of two in vitro passages after recovery from frozen stocks. In growth rate studies, *B. burgdorferi* was inoculated into 6 ml of BSK-II medium and incubated statically at 34°C in an atmosphere of 1.5% $O_2$–5% $CO_2$–balance $N_2$. Cultures were mixed gently by inversion prior to sampling. Concentrations of borrelia were determined by dark-field microscopy. Ten-microliter samples were placed under no. 1 glass coverslips (22 by 22 mm), and the numbers of organisms per field were determined for a minimum of 50 fields (45× objective lens) or 100 organisms per slide. Triplicate counts were averaged and divided by a correction factor based on the calculated volume of medium per field. The concentration was adjusted with fresh BSK-II medium as needed for animal or culture inoculation.

Clones of *B. burgdorferi* were obtained by the subsurface agarose plating method described by Dever et al. (9). Briefly, dilutions of cultures in 1 ml of BSK-II medium with 1% Seaplaque low-melting-point agarose (FMC, Rockland, Maine) were overlaid onto petri dishes (60 by 15 mm) containing 8 ml of BSK-II medium with 1.5% Seakem LE agarose (FMC). Plates were sealed in a humidified chamber and incubated at 34°C in the microaerobic chamber. Translucent colonies were apparent 7 to 14 days postinoculation. Well-isolated colonies were removed with sterile Pasteur pipettes, and each colony was inoculated into two screw-capped, polypropylene freezer vials containing 2 ml of BSK-II medium. High concentrations of organisms were obtained 2 to 4 days postinoculation. Samples were removed for immediate animal inoculation, and the remaining cultures were frozen and retrieved for further study as described above.

**Animal studies.** Three-week-old, female C3H-HeN mice (Harlan Sprague-Dawley, Houston, Tex.) and CB-17 severe combined immunodeficient (SCID) mice (Charles River Laboratories, Wilmington, Mass.) were maintained in microisolator cages with ad libitum access to antibiotic-free food and water. *B. burgdorferi* cultures were diluted to the desired concentration in BSK-II medium, and 0.1 ml was injected intradermally into each animal at the base of the tail. Two to four weeks later, mice were anesthetized with methoxyflurane (Metofane; Pitman-Moore, Mundelein, Ill.), bled by cardiac puncture, and sacrificed. The tibiotarsal joints, heart, and bladder were aseptically removed; one-half of the heart and bladder tissue and one tibiotarsal joint (with skin removed) were inoculated into separate tubes containing 6 ml of BSK-II medium. The remaining heart and bladder tissue and one hind limb were fixed in 10% buffered formalin for histologic studies. Cultures were examined weekly for up to 6 weeks for the growth of borreliae. The 50% infectious dose ($ID_{50}$) was determined by the method of Reed and Münch (18), with positivity of a single culture being used as the criterion of infection for each mouse. Tissues for histologic examination were embedded in paraffin, processed by standard procedures, and stained with hematoxylin and eosin.

**Protein electrophoresis.** *B. burgdorferi* polypeptides were characterized by two-dimensional gel electrophoresis (2DGE) and silver staining as described previously (15). Nonequilibrium pH gradient electrophoresis (17) was used in the first dimension, while sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 8 to 20% acrylamide gradient gels was performed in the second dimension. Culture supernatants containing the anti-P39 monoclonal antibody H11-F3 (18a) were used at a 1:20 dilution for the Western blot (immunoblot) analysis of 2DGE patterns as described previously (15). Gel patterns were analyzed with an ImageMaster system (Pharmacia, Uppsala, Sweden) and associated software.

**Plasmid analysis.** The plasmid content of *B. burgdorferi* was examined by continuous electric field electrophoresis of purified plasmids in 0.3% agarose as described previously (2). In addition, total *B. burgdorferi* DNA was prepared in agarose inserts and subjected to contour-clamped homogeneous electric field (CHEF) electrophoresis. Approximately $10^9$ organisms of each *B. burgdorferi* isolate examined were washed twice in 1 M NaCl–10 mM Tris-HCl (pH 7.6), resuspended in 100 μl of the same buffer, mixed with an equal volume of molten 1.6% Incert agarose (FMC), and placed in an insert mold (Bio-Rad Laboratories, Richmond, Calif.). Agarose inserts were then processed for the purification of intact genomic and plasmid DNA as described previously (27). Portions of the inserts were subjected to CHEF electrophoresis with a Bio-Rad CHEF-DR III unit. Gels (1% Bio-Rad ultrapure DNA-grade agarose) and the electrophoresis chamber contained 0.5× TBE buffer (1× TBE buffer is 90 mM Tris HCl, 90 mM boric acid, 1 mM EDTA). CHEF electrophoresis conditions consisted of a ramped pulse time of 1 to 10 s at 200 V for 20 h. Plasmid bands were visualized by ethidium bromide staining.

TABLE 1. Infectivity screening of clonal populations derived from *B. burgdorferi* Sh2 (passages 5, 10, and 20) and B31 (passage 5)

| Strain and passage | Tissue | Culture results for *B. burgdorferi* clone[a]: | | | | | | | | | | No. of clones positive[b]/total no. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | |
| Sh2, passage 5 | Joint | − | − | + | + | + | + | − | + | + | + | 7/10 |
| | Heart | − | − | + | + | + | + | − | + | + | + | 7/10 |
| Sh2, passage 10 | Joint | − | − | − | − | − | + | − | − | − | − | 1/10 |
| | Heart | − | − | − | − | − | + | − | − | − | − | 1/10 |
| Sh2, passage 20 | Joint | − | − | − | − | − | − | − | − | − | − | 0/10 |
| | Heart | − | − | − | − | − | − | − | − | − | − | 0/10 |
| B31, passage 5 | Joint | + | + | + | + | − | + | − | + | + | − | 7/10 |
| | Heart | + | − | + | + | − | + | − | − | + | − | 5/10 |
| | Blood | + | − | + | − | − | + | − | − | + | − | 4/10 |

[a] Cultures from mice infected with each clone ($10^5$ organisms per mouse) were scored as positive (+) or negative (−) for growth of *B. burgdorferi*. Sh2 passage 5 clones were designated Sh2-5A1 through Sh2-5A10 in subsequent experiments.
[b] Number of clones positive for that culture site (e.g., joint).

## RESULTS

**Infectivity screening of clonal populations.** The Sh2 strain of *B. burgdorferi* was cloned by colony formation following 5, 10, and 20 in vitro passages, corresponding to an estimated 25, 50, and 100 in vitro generations, respectively. Colonies were uniformly translucent and white and varied from 3 to 7 mm in diameter; the size variation was the same in all passage numbers and seemed to be related to the vertical location of colonies in the agarose overlay. Ten colonies from each of these passages were selected randomly, inoculated into 2-ml cultures, and cultured for 2 to 4 days. Portions of these cultures were used in mouse inoculation studies, and the remaining stock cultures were frozen for use in future studies. Clones of the B31 strain, which had undergone five in vitro passages, were obtained in a similar manner. The clones did not differ appreciably in their in vitro growth rates or other culture characteristics.

Sh2 and B31 clonal populations were subjected to an infectivity screening assay to determine their ability to infect C3H/HeN mice. In this assay, an individual mouse was infected with $10^5$ organisms of a given clone. The mouse was sacrificed 2 to 4 weeks postinoculation, and tibiotarsal joint, heart, and (in some experiments) bladder and blood specimens were cultured in vitro to assay the presence of viable borreliae. This approach permitted the assessment of a large number of clones while minimizing the number of animals and the expense.

The results of such an assay are shown in Table 1. It was found that the clones exhibited two different patterns, described here as high-infectivity and low-infectivity phenotypes. In the high-infectivity phenotype, joint, heart, and bladder cultures were consistently positive, with the majority of cultures containing $\geq 10^7$ borreliae per ml when first examined after 2 weeks of culture. Clones expressing the low-infectivity phenotype yielded negative cultures at most sites, with occasional joint or heart cultures being positive; in representative experiments, 5 of 40 joint, heart, and bladder cultures were positive (Table 2). In addition, the positive cultures from low-infectivity clones generally contained fewer organisms after 2 weeks of culture ($\leq 10^6$/ml). In the Sh2 population cloned at in

TABLE 2. Verification of high- and low-infectivity phenotypes of *B. burgdorferi* Sh2 clones[a]

| Clone | No. of cultures positive/total no. | | | | No. of mice positive/ total no. |
|---|---|---|---|---|---|
| | Joint | Heart | Bladder | All Sites | |
| High-infectivity phenotype | | | | | |
| Sh2-5A3 | 5/5 | 5/5 | 5/5 | 15/15 | 5/5 |
| Sh2-5A4 | 5/5 | 5/5 | 5/5 | 15/15 | 5/5 |
| Sh2-5A5 | 5/5 | 5/5 | ND[b] | 10/10 | 5/5 |
| Low-infectivity phenotype | | | | | |
| Sh2-5A1 | 3/5 | 0/5 | ND | 3/10 | 3/5 |
| Sh2-5A2 | 2/5 | 0/5 | 0/5 | 2/15 | 2/5 |
| Sh2-5A7 | 0/5 | 0/5 | 0/5 | 0/15 | 0/5 |

[a] Groups of five C3H/HeN mice were inoculated with each clone ($10^5$ organisms per mouse); after 2 weeks, the mice were sacrificed and the organs were cultured for detection of viable *B. burgdorferi* organisms.
[b] ND, not determined.

vitro passage 5, seven clones exhibited the high-infectivity phenotype, whereas the remaining three clones had the low-infectivity phenotype (Table 1). The proportion of high-infectivity clones decreased with increasing passage number, with 1 of 10 and 0 of 10 clones being of the high-infectivity phenotype following 10 and 20 in vitro passages, respectively. Thus, outgrowth of low-infectivity clones appears to be responsible for the loss of infectivity and virulence observed in uncloned, high-passage strains. Seven of the 10 clones obtained from passage 5 of the B31 strain yielded positive joint, heart, or blood cultures, whereas the other 3 clones were culture negative at all sites tested (Table 1). With some clones, only the joint cultures were positive. Blood cultures were positive less consistently than either the joint or heart cultures.

**Verification of infectivity screening results.** To confirm the accuracy of the infectivity screening results, representative high-infectivity and low-infectivity clones were inoculated into groups of five mice each. As shown in Table 2, the results mirrored those obtained in the screening assay. All joint, heart, and bladder cultures were positive when mice were inoculated with the high-infectivity clones Sh2-5A3, -5A4, and -5A5. Borreliae were recovered sporadically from joint cultures of some of the low-infectivity clones (Table 2); in other experiments, heart cultures were also positive, but at a lower frequency than that of joint cultures (data not shown). Thus, the low-infectivity clones obtained from low-passage *B. burgdorferi* may yield positive cultures, albeit at a lower frequency than that of the high-infectivity clones.

**Infectivity of high-infectivity and low-infectivity subclones.** Subclones of the high-infectivity clone Sh2-5A5 and the low-infectivity clone Sh2-5A1 were isolated by colony formation and examined for infectivity in the C3H/HeN mouse model (data not shown). All joint and heart cultures from mice inoculated with the subclones derived from Sh2-5A5 were positive. In contrast, all heart cultures and 8 of 10 joint cultures from mice inoculated with Sh2-5A1 subclones were culture negative; the two positive joint cultures most likely reflect the ability of low-infectivity clones to yield occasional positive cultures.

**$ID_{50}$ determinations.** All studies described up to this point used a high dose of *B. burgdorferi* ($10^5$ organisms per mouse) to yield consistent results for infectivity screening and other studies. To determine the minimum dose required to yield culture positivity in 50% of mice, groups of three C3H/HeN mice were inoculated with 10-fold dilutions of Sh2-5A5 (a representative high-infectivity clone) and Sh2-5A1 (a low-infectivity clone) (Tables 3 and 4). All cultures from mice injected with Sh2-5A5

TABLE 3. $ID_{50}$ determination for high-infectivity clone Sh2-5A5[a]

| Inoculum | No. of cultures positive/total no. | | | | No. of mice positive/total no. |
|---|---|---|---|---|---|
| | Joint | Heart | Bladder | All Sites | |
| Sh2-5A5 | | | | | |
| $10^5$ | 3/3 | 3/3 | 3/3 | 9/9 | 3/3 |
| $10^4$ | 2/3 | 3/3 | 3/3 | 8/9 | 3/3 |
| $10^3$ | 3/3 | 3/3 | 3/3 | 9/9 | 3/3 |
| $10^2$ | 1/3 | 1/3 | 1/3 | 3/9 | 1/3 |
| $10^1$ | 0/3 | 0/3 | 0/3 | 0/9 | 0/3 |
| BSK-II medium | 0/3 | 0/3 | 0/3 | 0/9 | 0/3 |

[a] The calculated $ID_{50}$ was $1.8 \times 10^2$ organisms.

were positive for inocula of $10^3$, $10^4$, and $10^5$ borreliae and were positive in three of nine cultures at $10^2$ organisms (Table 3). The calculated $ID_{50}$ for this clone was $1.8 \times 10^2$ organisms. In contrast, none of the mice inoculated with $10^5$ Sh2-5A1 organisms were culture positive at any of the three tissue sites, and only two of nine cultures were positive for mice receiving inocula of $10^4$ or $10^3$ organisms (Table 4). The $ID_{50}$ calculated for the Sh2-5A1 clone was $10^5$ organisms. The $ID_{50}$ studies reinforced the consistent versus sporadic culture positivity results observed previously for high-infectivity and low-infectivity clones, respectively.

The observed differences in culture positivity could be due to differences in growth rates. However, the in vitro growth rates of Sh2-5A1 and Sh2-5A5 were found to be essentially equivalent (Fig. 1), with doubling times of 13.6 and 16.5 h, respectively.

**Infectivity in SCID mice.** The poor persistence of low-infectivity clones also could be due to an increased susceptibility to elimination by the adaptive immune system (i.e., B- and T-cell activities). To examine this possibility, groups of five CB-17 mice bearing the SCID defect were inoculated with either Sh2-5A1 or Sh2-5A5 ($10^5$ organisms per mouse); the same inocula were used for groups of five C3H/HeN mice. Essentially equivalent results were obtained in the SCID mouse group and in the immunocompetent C3H/HeN controls (data not shown). Neither the SCID mice nor the normal mice inoculated with $10^5$ Sh2-5A1 organisms yielded any positive cultures, whereas four of five mice from each group yielded positive joint, heart, or bladder cultures when injected with $10^5$ Sh2-5A5 organisms. These results indicate that the poor survival of Sh2-5A1 in mice is not due to activities of the adaptive immune system.

**Comparison of protein profiles.** The 2DGE protein profiles of clones Sh2-5A1 through Sh2-5A10 were compared to determine if any differences between the high- and low-infectivity clones could be detected. The polypeptide patterns of these

TABLE 4. $ID_{50}$ determination for low-infectivity clone Sh2-5A1[a]

| Inoculum | No. of cultures positive/total no. | | | | No. of mice positive/total no. |
|---|---|---|---|---|---|
| | Joint | Heart | Bladder | All sites | |
| Sh2-5A1 | | | | | |
| $10^5$ | 0/3 | 0/3 | 0/3 | 0/9 | 0/3 |
| $10^4$ | 2/3 | 0/3 | 0/3 | 2/9 | 2/3 |
| $10^3$ | 1/3 | 1/3 | 0/3 | 2/9 | 1/3 |
| $10^2$ | 0/3 | 0/3 | 0/3 | 0/9 | 0/3 |
| $10^1$ | 0/3 | 0/3 | 0/3 | 0/9 | 0/3 |
| BSK-II medium | 0/3 | 0/3 | 0/3 | 0/9 | 0/3 |

[a] The calculated $ID_{50}$ was $10^5$ organisms.

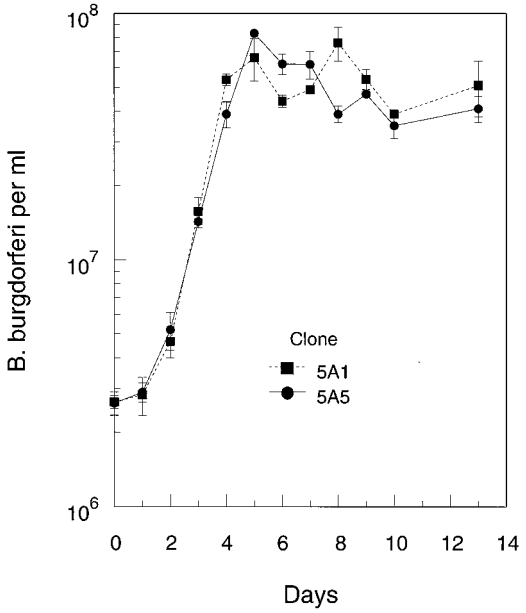


FIG. 1. In vitro growth curves for the high-infectivity clone Sh2-5A5 and the low-infectivity clone Sh2-5A1. Mean ± standard error values are shown for daily determinations of triplicate cultures.

clones were remarkably similar, as exemplified by the comparison of clones Sh2-5A1 (low infectivity) and Sh2-5A5 (high infectivity) in Fig. 2. The only difference observed was that some of the 2DGE patterns of high-infectivity clones contained a 38.8-kDa polypeptide spot that migrated just below the 41-kDa flagellin. The 38.8-kDa spot is distinct from the P39 protein described by Simpson et al. (25), as demonstrated by the fact that the anti-P39 monoclonal antibody H11-F3 reacted with a more acidic spot. However, the 38.8-kDa spot was not present reproducibly in high-infectivity clones.

**Plasmid content of high-infectivity and low-infectivity clones.** The plasmid content of clones Sh2-1A1 through Sh2-1A10 was examined by both CHEF electrophoresis and continuous field electrophoresis in 0.3% agarose gels (Fig. 3) (1). There were few discernible differences in the plasmid electrophoresis patterns. Some variation occurred in the presence and intensity of ethidium bromide-stained bands in the range of 22 to 23 kb. In particular, the low-infectivity clone Sh2-5A7 appeared to lack a 22-kb band present in the other plasmid profiles (Fig. 3).

## DISCUSSION

This study is part of an ongoing effort to identify virulence factors of Lyme disease borreliae through the comparison of

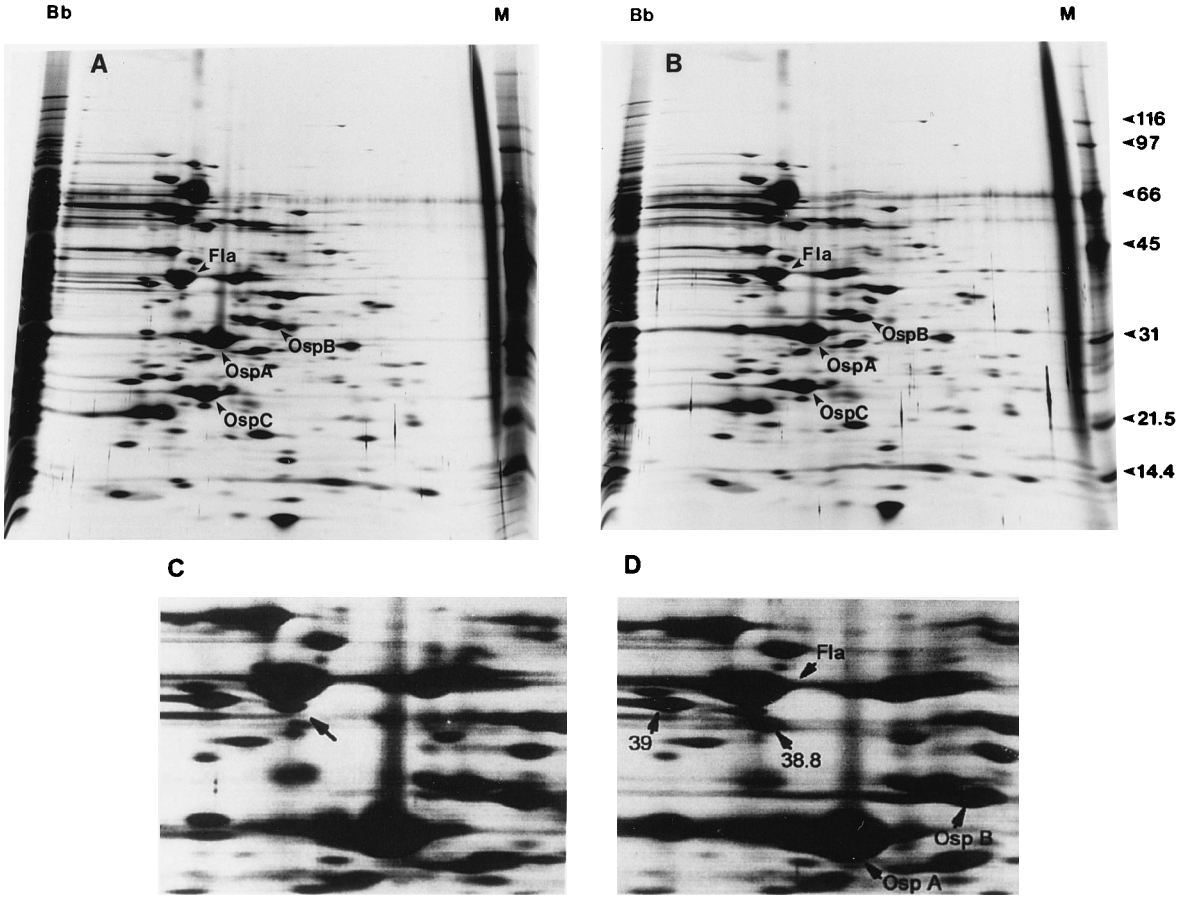


FIG. 2. Analysis of protein content of high- and low-infectivity clones of *B. burgdorferi* strain Sh2. The complete 2DGE profiles of low-infectivity clone Sh2-5A1 (A) and high-infectivity clone Sh2-5A5 (B) are shown. The 2DGE patterns are oriented with the acid end toward the left side and are flanked by single-dimension SDS-PAGE patterns of *B. burgdorferi* Sh2-5A5 (Bb) and Bio-Rad high- and low-molecular-weight standards (M). Locations of the flagellin protein (Fla) and outer surface proteins OspA, OspB, and OspC are indicated. Digitized gel images of Sh2-5A1 (C) and Sh2-5A5 (D) in the region of Fla show an apparent difference in the presence of a 38.8-kDa spot (arrow). Relative locations of Fla, OspA, OspB, and P39 (39) proteins are marked.

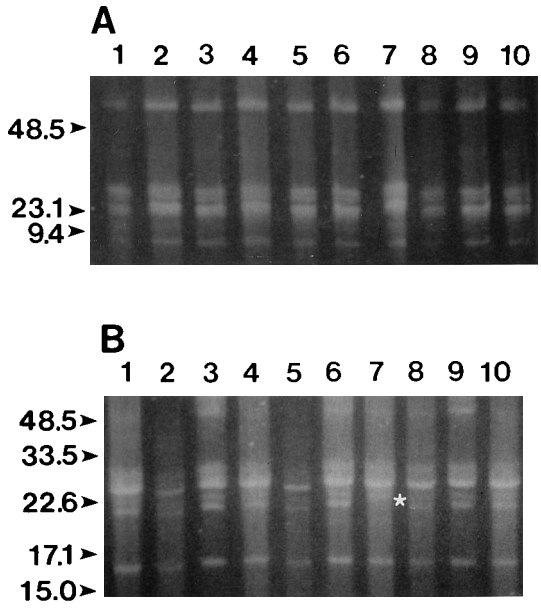


FIG. 3. Plasmid electrophoresis profiles of Sh2 clones 5A1 through 5A10 (lanes 1 through 10, respectively) by use of CHEF electrophoresis (A) or continuous-field electrophoresis in 0.3% agarose (B). Locations of molecular size standards (in kilobases) are indicated on the left side; note that the migration of plasmids relative to the molecular size standards is different for the two electrophoresis procedures. Differences in the content of plasmids migrating at 22 to 23 kb were detectable in some Sh2 clones, such as Sh2-5A7 (star, lane 7). The varied intensity of plasmid bands migrating at 49 kb was apparently due to quantitative differences in the recovery of DNA in plasmid preparations rather than in the plasmid content of the *B. burgdorferi* cells, on the basis of the results obtained with CHEF electrophoresis of *B. burgdorferi* DNA processed in agarose inserts.

high-infectivity and low-infectivity variants of *B. burgdorferi*. Comparison of clonal populations from a single, low-passage Sh2 strain decreased the number of confounding variations unrelated to infectivity, as discussed below. Recovery of viable organisms from infected mice by culture was used as the principal criterion for distinguishing clones. Culture positivity is a measure of dissemination, colonization, growth, and persistence in tissue but is not indicative of other aspects of virulence, such as the induction of inflammation and pathologic changes; therefore, the clones were rated in terms of infectivity, not virulence. High-infectivity clones were easily cultured from multiple sites, and one clone examined in detail had a low $ID_{50}$. Low-infectivity clones were recovered only sporadically from the organs of infected mice and had a high $ID_{50}$ in the one clone tested. This clonal variation in infectivity underscores the need to maintain culture stability by carefully preserving frozen stocks of Lyme disease borreliae and minimizing the number of in vitro passages prior to use. Strain variation occurring during in vitro culture could cause complications in the standardization of antigen for serodiagnostic tests, as well as in research studies.

The results of this study confirm that the loss of virulence by *B. burgdorferi* during in vitro culture is due to the outgrowth of low-infectivity clonal populations. Outgrowth is a more accurate term than selection in this case, since it is not known whether selection of low-infectivity clones occurs. Variants containing a low- or high-frequency, irreversible genetic change will eventually replace the original population, regardless of whether positive selection is present. High-infectivity clones were present in the highest proportion in the low-passage (passage 5) Sh2 strain. Mice infected with high-infectivity clones consistently yielded positive cultures from the tibiotarsal joint, heart, and urinary bladder; numerous organisms were present after 2 weeks of culture. Sh2 (passage 5) clones of the low-infectivity phenotype yielded a much lower proportion of positive cultures, usually from the joints of infected mice; in addition, those cultures that were positive required a longer period until borreliae were detectable, and fewer organisms were present. A logical conclusion based on these results is that high numbers of borreliae were consistently present in the organs of mice infected with high-infectivity clones, whereas only occasional organisms were able to persist at some sites in mice inoculated with the low-infectivity clones.

The loss of infectivity does not appear to be an all-or-none phenomenon. The low-infectivity clones obtained from passage 5 Sh2 cultures were still able to colonize the mouse host at low frequency, whereas high-passage organisms are not recovered from inoculated mice (14, 14a, 21). Thus, the loss of infectivity and virulence does not seem to be a single-step process achieved through a single alteration of a genetic element. Rather, it is likely that multiple genetic elements encoding virulence factors are involved. However, the 500-fold difference in $ID_{50}$s between high- and low-infectivity clones Sh2-5A5 and Sh2-5A1 indicates that genetic events occurring within the first five in vitro passages can cause a significant decrease in infectivity.

Initial infectivity screening studies demonstrated that only 7 of 10 clones were of the high-infectivity phenotype in passage 5 of Sh2, and this proportion decreased in 1 in 10 and 0 in 10 after 10 and 20 in vitro passages, respectively (Table 1). The infectivity properties of three high-infectivity and three low-infectivity clones were verified by studies with groups of five mice (Table 2). Thus, the results of the infectivity screening assay appear to be dependable. The short-term stability of these phenotypes was demonstrated by infectivity screening of subclones of Sh2-5A5 and Sh2-5A1.

Careful analysis of the frequency of conversion from high-infectivity to low-infectivity phenotypes will require examination of the progeny of a known high-infectivity clone after different numbers of generations. However, assuming that (i) 100% of the cells were of the high-infectivity phenotype in the initial (passage 0) Sh2 population and (ii) there is no positive or negative selection, the data presented in Table 1 are consistent with a conversion frequency of $\geq 10^{-3}$ per generation.

What is the nature of these conversion events? The apparent irreversibility and relatively high rate of conversion from high to low infectivity are consistent with the loss of one or more plasmids and their gene products. Previous comparisons of uncloned low-passage and high-passage *B. burgdorferi* strains had indicated that changes in both plasmid content and protein content coincided with loss of virulence. Studies by Schwan et al. (21) and Simpson et al. (24) showed that two related, supercoiled plasmids, pBBC1 (8.4 kb) and pBBC2 (8.8 kb), were present in low-passage Sh2 but were not detectable in noninfectious Sh2 strains acquired following 20 or 202 in vitro passages. These changes were accompanied by loss of OspB expression (encoded on a 49-kb linear plasmid [6]) and a concomitant increase in a 20-kDa protein (21), which may represent a truncated form of OspB (4, 7); increased quantities of a proteinase K-resistant, lipopolysaccharide-like material were also present in the high-passage strain (21). A previous study by our group (15) had shown that the noninfective, high-passage B31 strain lacked a 28-kDa lipoprotein, OspD, that was present in low-passage, infectious B31; the 38-kb linear plasmid encoding OspD was also absent from the high-passage strain. However, these differences were not found to correlate consistently with infectivity. Simpson et al. (24) showed that some infectious strains of *B. burgdorferi* lacked detectable

pBBC1 and pBBC2, and Norris et al. (15) found that OspD and the encoding 38-kb plasmid were absent from infectious strains (including low-passage Sh2) and present in some high-passage, noninfectious strains. Marconi et al. (13) conducted an extensive survey of the distribution of the *ospD* gene in Lyme disease borreliae. This study revealed that only 24, 90, and 50% of the low-passage *B. burgdorferi*, *B. garinii*, and *B. afzelii* strains, respectively, tested contained the *ospD* gene. Thus, many genetic differences unrelated to virulence may exist among Lyme disease isolates, further complicating the identification of proteins and genetic elements involved in infectivity.

The results of this study indicate that the changes causing decreased infectivity may be more subtle than previously thought. High-infectivity and low-infectivity clones from a single low-passage strain would presumably have fewer differences unrelated to the decrease in virulence. Indeed, the protein and plasmid profiles of high-infectivity and low-infectivity clones were found to be remarkably similar. The migration characteristics and abundance of the major polypeptides of Sh2 clones were virtually identical as determined by 2DGE (Fig. 2), despite marked differences in infectivity. The plasmid profiles, as revealed by the relatively sensitive technique of ethidium bromide staining, were also very similar. Some differences were observed in the 22- to 23-kb range, particularly in clone Sh2-5A7 (Fig. 3). These results will have to be confirmed by other means, such as hybridization with plasmid-specific probes. The different infectivity phenotypes may be due to (i) subtle differences in plasmid and/or polypeptide content or (ii) genetic changes caused by mutations, DNA rearrangements, or other mechanisms that would not alter the plasmid profile or polypeptide content. The latter could involve the chromosome, plasmids, or as-yet-undescribed genetic elements (e.g., transposons or phages).

From the results obtained with SCID mice, the low-infectivity phenotype is not due to an increased susceptibility to the adaptive immune system. In this experiment, the low-infectivity Sh2-5A1 clone was not recovered from either immunologically normal C3H/HeN mice or B- and T-cell-deficient SCID mice. On the other hand, the high-infectivity Sh2-5A5 clone was cultured from multiple organs in both mouse strains. Thus, the low-infectivity clone must be deficient in the ability of Lyme disease borreliae to flourish and persist in the mammalian host. In other words, these organisms must be defective in at least one of the following functions: colonization of local and distant sites, dissemination through tissue invasion and hematogenous spread, acquisition of nutrients necessary for growth, and evasion of the innate and adaptive arms of the immune system. Preliminary histologic studies indicate that some inflammation can be present in the joint and heart tissue of mice infected with low-infectivity clones (unpublished data). These organisms are apparently capable of some dissemination and colonization of distant sites but do not persist as well as do wild-type organisms at those sites.

Further analysis of the biologic and immunologic properties of high- and low-infectivity isolates may permit correlation of protein expression, genetic content, and pathogenesis. However, the implementation of genetic transfer procedures and the conversion of low-infectivity clones to the high-infectivity phenotype will be needed to verify the role of genetic factors and gene products in the virulence of *B. burgdorferi*. Samuels et al. (19) recently reported the successful transformation of wild-type *B. burgdorferi* B31 to a coumermycin-resistant genotype by electroporation and homologous recombination. On the basis of these results, the application of genetic transfer techniques to the study of factors involved in Lyme disease pathogenesis appears to be feasible.

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## REFERENCES

1. **Baranton, G., D. Postic, I. Saint Girons, P. Boerlin, J. C. Piffaretti, M. Assous, and P. A. D. Grimont.** 1992. Delineation of *Borrelia burgdorferi* sensu stricto, *Borrelia garinii* sp. nov., and group VS461 associated with Lyme borreliosis. Int. J. Syst. Bacteriol. **42:**378–383.
2. **Barbour, A. G.** 1988. Plasmid analysis of *Borrelia burgdorferi*, the Lyme disease agent. J. Clin. Microbiol. **26:**475–478.
3. **Barbour, A. G., and D. Fish.** 1993. The biological and social phenomenon of Lyme disease. Science **260:**1610–1616.
4. **Barbour, A. G., S. L. Tessier, and S. F. Haye.** 1984. Variation in a major protein of Lyme disease spirochetes. Infect. Immun. **45:**94–100.
5. **Barthold, S. W., M. deSouza, E. Fikrig, and D. H. Persing.** 1992. Lyme borreliosis in the laboratory mouse, p. 223–242. *In* S. E. Schutzer (ed.), Lyme disease: molecular and immunologic approaches. Cold Spring Harbor Laboratory Press, Plainview, N.Y.
6. **Bergström, S., V. G. Bundoc, and A. G. Barbour.** 1989. Molecular analysis of linear plasmid-encoded major surface proteins, OspA and OspB, of the Lyme disease spirochaete, *Borrelia burgdorferi*. Mol. Microbiol. **3:**479–486.
7. **Bundoc, V. G., and A. G. Barbour.** 1989. Clonal polymorphisms of outer membrane protein OspB of *Borrelia burgdorferi*. Infect. Immun. **57:**2733–2741.
8. **Burgdorfer, W., A. G. Barbour, S. F. Hayes, J. L. Benach, E. Grunwaldt, and J. P. Davis.** 1982. Lyme disease: a tick-borne spirochetosis? Science **216:** 1317–1319.
9. **Dever, L. L., J. H. Jorgensen, and A. G. Barbour.** 1992. In vitro antimicrobial susceptibility testing of *Borrelia burgdorferi*: a microdilution MIC method and time-kill studies. J. Clin. Microbiol. **30:**2692–2697.
10. **Garcia-Monco, J., B. F. Villar, J. C. Alen, and J. L. Benach.** 1990. *Borrelia burgdorferi* in the central nervous system: experimental and clinical evidence for early invasion. J. Infect. Dis. **161:**1187–1193.
11. **Johnson, R. C., N. Marek, and C. Kodner.** 1984. Infection of Syrian hamsters with Lyme disease spirochetes. J. Clin. Microbiol. **20:**1099–1101.
12. **Luft, B. J., C. R. Steinman, H. C. Neimark, B. Murlidhar, T. Rush, M. F. Finkel, M. Kunkel, and R. J. Dattwyler.** 1992. Invasion of the central nervous system by *Borrelia burgdorferi* in acute disseminated infection. JAMA **267:** 1364–1367.
13. **Marconi, R. T., D. S. Samuels, R. K. Landry, and C. F. Garon.** 1994. Analysis of the distribution and molecular heterogeneity of the *ospD* gene among the Lyme disease spirochetes: evidence for lateral gene exchange. J. Bacteriol. **176:**4572–4582.
14. **Moody, K. D., S. Barthold, and G. A. Tergwilliger.** 1990. Lyme borreliosis in laboratory animals: effect of host species and in vitro passage of *Borrelia burgdorferi*. Am. J. Trop. Med. Hyg. **43:**87–92.

14a. **Norris, S. J.** Unpublished data.

15. **Norris, S. J., C. J. Carter, J. K. Howell, and A. G. Barbour.** 1992. Low-passage-associated proteins of *Borrelia burgdorferi* B31: characterization and molecular cloning of OspD, a surface-exposed, plasmid-encoded lipoprotein. Infect. Immun. **60:**4662–4672.
16. **Norris, S. J., and D. G. Edmondson.** 1986. Serum requirement for the multiplication of *Treponema pallidum* in a tissue culture system: association of growth-promoting activity with the protein fraction. Sex. Transm. Dis. **13:**207–213.
17. **O'Farrell, P. Z., H. M. Goodman, and P. H. O'Farrell.** 1977. High-resolution two-dimensional electrophoresis of basic as well as acidic proteins. Cell **12:**1133–1142.
18. **Reed, L. J., and H. Münch.** 1938. A simple method of estimating fifty per cent endpoints. Am. J. Hyg. **27:**493–497.

18a. **Sadziene, A., and A. G. Barbour.** Unpublished data.

19. **Samuels, D. S., K. E. Mach, and C. F. Garon.** 1994. Genetic transformation of the Lyme disease agent *Borrelia burgdorferi* with coumarin-resistant *gyrB*. J. Bacteriol. **176:**6045–6049.
20. **Schutzer, S. E. (ed.).** 1992. Lyme disease: molecular and immunologic approaches, Cold Spring Harbor Laboratory Press, Plainview, N.Y.
21. **Schwan, T. G., W. Burgdorfer, and C. F. Garon.** 1988. Changes in infectivity and plasmid profile of the Lyme disease spirochete, *Borrelia burgdorferi*, as a result of in vitro cultivation. Infect. Immun. **56:**1831–1836.
22. **Schwan, T. G., W. Burgdorfer, M. E. Schrumpf, and R. H. Karstens.** 1988. The urinary bladder, a consistent source of *Borrelia burgdorferi* in experimentally infected white-footed mice (*Peromyscus leucopus*). J. Clin. Microbiol. **26:**893–895.
23. **Shih, C.-M., S. R. Telford III, R. J. Pollack, and A. Spielman.** 1993. Rapid

dissemination by the agent of Lyme disease in hosts that permit fulminating infection. Infect. Immun. **61:**2396–2399.
24. **Simpson, W. J., C. F. Garon, and T. G. Schwan.** 1990. Analysis of supercoiled circular plasmids in infectious and non-infectious *Borrelia burgdorferi*. Microb. Pathog. **8:**109–118.
25. **Simpson, W. J., M. E. Schrumpf, and T. G. Schwan.** 1990. Reactivity of human Lyme borreliosis sera with a 39-kilodalton antigen specific to *Borrelia burgdorferi*. J. Clin. Microbiol. **28:**1329–1337.
26. **Steere, A. C.** 1989. Lyme disease. N. Engl. J. Med. **321:**586–596.
27. **Walker, E. M., J. K. Arnett, J. D. Heath, and S. J. Norris.** 1991. *Treponema pallidum* subspecies *pallidum* has a single circular chromosome with a size of ~900 kilobase pairs. Infect. Immun. **59:**2476–2479.
28. **Wilske, B., A. G. Barbour, S. Bergström, N. Burman, B. I. Restrepo, P. A. Rosa, T. Schwan, E. Soutschek, and R. Wallich.** 1992. Antigenic variation and strain heterogeneity in *Borrelia* spp. Res. Microbiol. **143:**583–596.
29. **Wilske, B., V. Preac-Mursik, G. Schierz, and K. V. Busch.** 1986. Immunochemical and immunological analysis of European *Borrelia burgdorferi* strains. Zentralbl. Bakteriol. Mikrobiol. Hyg. Ser. A **263:**92–102.