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Plasmid Analysis of *Borrelia burgdorferi*, the Lyme Disease Agent

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A simple procedure for extraction of plasmid-enriched DNA from borreliae was used in a plasmid analysis of 13 strains of the Lyme disease agent, *Borrelia burgdorferi*. The extracted DNA was subjected to low-percentage agarose gel electrophoresis and examined either directly by ethidium bromide staining or after hybridization of the plasmids in situ with a DNA probe for the gene encoding the major outer membrane protein OspA. Each isolate had four to seven discernible plasmids of various sizes. Only 2 of the 13 strains had the same plasmid profile. The *ospA* gene probe hybridized to large plasmids to strains from both North America and Europe. A strain which had been passaged many times was found to have lost two of the six plasmids originally present. These findings indicate the potential usefulness of plasmid analysis as a strain-typing procedure and for identifying possible plasmid-conferred virulence factors.

Borrelia burgdorferi, the agent of Lyme disease, has been isolated from humans, small mammals, birds, and arthropods in North America and Europe (reviewed in references 4 and 6). Different isolates have been distinguished on the basis of their reactivities with monoclonal antibodies and the apparent weights of their major surface proteins (5, 6, 16). In general, North American strains have shown less heterogeneity in these traits than have European isolates. For example, almost all U.S. isolates have an abundant outer membrane protein, called OspA, with an apparent molecular weight in polyacrylamide electrophoresis gels of 31×10^3 (31 K protein); this 31K protein is recognized by certain monoclonal antibodies (5–7). Many European strains also have a surface protein with the characteristic size and antibody reactivities of the North American OspA protein (5, 6, 16). However, as many, if not more, European isolates have major proteins with either higher or lower molecular weights and with antibody reactivity patterns different from that of the 31K OspAs.

A feature that can be used to characterize and group bacteria within a given species is the plasmid content of an isolate. *B. burgdorferi* has supercoiled circular plasmids (10, 11). In addition, this species and the related spirochete, *B. hermsii*, uniquely among all eubacteria, have linear plasmids (3, 14). The linear plasmids account for most of the extrachromosomal DNA in *B. burgdorferi* cells (3). The structure of the linear DNA resembles that of the DNAs of some animal viruses, such as vaccinia virus, in having covalently closed ends (3). In the *B. burgdorferi* B31, a 49-kilobase (kb) linear plasmid carries the genes for the OspA protein, as well as for the other major outer membrane protein in that strain, OspB; the size of this linear molecule was established by contour length measurements (3).

Plasmid profiles were envisioned to be another useful typing tool in the classification of *B. burgdorferi* isolates. Plasmid analysis would, moreover, allow direct examination of those discrete pieces of the borrelia genome that contain the genes for major serotype-specific antigens. In this way, correlations between two different typing systems, i.e., outer membrane proteins and plasmids, might be made. On the basis of these considerations, I looked at the plasmid profiles of several isolates of *B. burgdorferi*. The aims in this study were the following: (i) to analyze total plasmid contents by a method that would be suitable, in ease of performance, for typing a large number of isolates; (ii) to assess whether the plasmid profile of a strain might change with time as the isolate was passed in culture; and (iii) to determine whether sequences homologous to the OspA-specifying gene (*ospA*) of strain B31 are located on a plasmid. A previous study had shown that sequences homologous to *ospA* were present in the total DNAs of European and North American strains (5).

MATERIALS AND METHODS

Strains. All isolates of *B. burgdorferi* were grown in BSKII medium and harvested by methods previously described (1, 7). One culture of strain B31 (2, 8; ATCC 35210) had been passed continuously from 1982 to 1986 and had been cloned three times by limiting dilution; this particular isolate was designated B31-86. A pure culture of strain B31 that had been frozen in 1982 after cloning by limiting dilution was thawed and cultivated again; this passage of the strain was called B31-82. All other isolates had been passed in culture, from the date of original isolation, no more than four times; this represented a maximum of 30 bacterial generations. Strains A to F and their origins were described under these designations in reference 5. Strains 3028, 297, ECMIV, HB4, PBi, and FI were described in reference 6; FI and F are synonymous (5, 6). Isolate DN127, recovered from an *Ixodes pacificus* tick in Del Norte, Calif., was kindly provided by M. Bisset and W. Hill, California State Department of Health (7a).

DNA extractions. The plasmid enrichment method described previously (3) was modified for work with smaller cell harvests; the step of density gradient ultracentrifugation was eliminated. A harvest of 10^9 borreliae (10 to 20 ml of a stationary-phase culture) was suspended in 240 μl of TES (50 mM Tris [pH 8.0], 50 mM EDTA, 15% [wt/vol] sucrose) in a 2-ml microcentrifuge tube. To the suspension was added 60 μl of lysozyme solution (2 mg/ml). After 15 min of incubation on ice, the following reagents were added to the sample: 300 μl of 1% sodium deoxycholate in TES and 7 μl of diethyl pyrocarbonate (DEP; Sigma Chemical Co., St. Louis, Mo.). The tube was shaken in an Eppendorf 5432 mixer (Brinkmann Instruments, Inc., Westbury, N.Y.) at room temperature for 10 min. To the lysate was added 250 μl of 7.5 M

ammonium acetate, and the tube was centrifuged in the horizontal position at 11,000 × *g* for 10 min at room temperature in a microcentrifuge. The supernatant was transferred to another tube, and the nucleic acids were precipitated by addition of 850 μl of isopropanol and placement of the tube on ice for 20 min. The precipitate was recovered by centrifugation as described above, with the exceptions of a temperature of 4°C and a duration of 25 min. The precipitate was rinsed with 70% ethanol, dried in a Speed-Vac (Savant Instruments, Inc., Hicksville, N.Y.), suspended in 300 μl of TE (10 mM Tris [pH 7.8], 1 mM EDTA), and then treated with RNase A at a final concentration of 0.1 mg/ml (37°C for 30 min). DEP (2 μl) was added, and the mixture was shaken for 5 min. Residual proteins were removed by precipitation with 7.5 M ammonium acetate (150 μl), followed by centrifugation in a microcentrifuge for 5 min at room temperature. DNA in the supernatant was recovered by addition of 1.3 ml of ethanol, incubation on ice for 20 min, and centrifugation at 4°C for 25 min. The precipitate was rinsed with 70% ethanol, dried, and suspended in 50 μl of TE.

Low-percentage agarose gel electrophoresis. DNA samples (approximately 2 μg each) were applied to 9-cm-long, 0.2% agarose gels (Seakem GTG; FMC Corp., Marine Colloids Div., Rockland, Maine). The electrophoresis was run with Tris (90 mM)–borate (90 mM)–EDTA (2 mM) buffer at 5.5 V/cm for 30 min and 1.4 V/cm for another 15 h (or until the bromphenol blue marker reached the end of the gel). The DNA in the gel was then stained with ethidium bromide. Size markers in the gels were whole lambda bacteriophage DNA (47 kb) and the 23- and 9.6-kb *Hin*dIII restriction fragments of lambda.

Direct gel hybridization. After being destained in water, the gel was placed first in 0.5 N NaOH–0.15 M NaCl for 30 min at room temperature and then in 0.5 M Tris (pH 7.6) for 30 min at 4°C (3). The gel was subsequently dried under vacuum onto a nylon membrane (1.2-μm pore size; Biotrans; ICN Radiochemicals, Irvine, Calif.) at 60°C. The dried gel was removed from the membrane and then prehybridized at 37°C for 4 h in the following solution: 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate; see reference 13)–0.1% sodium dodecyl sulfate (SDS)–5 mM EDTA–10× Denhardt solution (13)–100 μg of sonicated and denatured herring sperm DNA per ml. The DNA probe was a 1-kb *Eco*RI-*Hae*III fragment of pTRH43 (9); this fragment contains the entire *ospA* gene of B31 and 25 bases of the vector pBR322. (Preliminary studies had shown that pBR322 does not hybridize to *B. burgdorferi* DNA.) The probe was radiolabeled with ^{32}P with a nick translation kit (Bethesda Research Laboratories, Inc., Gaithersburg, Md.), denatured, and incubated with the gel in the hybridization solution described above at 65°C for 15 h. The hybridized gel was washed first with 1× SSC–0.1% SDS–1 mM EDTA at 65°C and then 0.1× SSC–0.1% SDS–1 mM EDTA at 37°C. The gel was placed between sheets of Saran Wrap and exposed to X-ray film.

RESULTS

Laboratory cultures of strain B31. Our previous studies of the DNA of strain B31 had shown that there were at least three types of linear plasmids and at least two forms of circular plasmids in the isolate that had been passed continuously, i.e., B31-86. The three linear plasmids of B31-86 are the major bands in Fig. 1; the apparent sizes of these plasmids are 49, 29, and 16 kb. The minor band, with an apparent size of 24 kb, is the supercoiled form of the predominant circular plasmid of this isolate; its actual contour length is 28 kb (3). (For the purposes of this study, the plasmid DNA was not fractionated on the basis of density in ethidium bromide-CsCl gradients. Thus, plasmids of the other strains examined could not be categorized as linear molecules, open circles, or supercoiled circles. Sizes are given only as points of reference in the gels and, for the circular molecules, would not be the true molecular lengths.)

Examination of the plasmid profile of cells from a culture of many fewer passages than B31-86, i.e., B31-82, showed two additional bands in the gel: a prominent plasmid with a size of 38 kb and a less-abundant plasmid with an apparent size of 24 kb in the gel. In the course of continuous laboratory cultivation, the DNAs of these plasmids were either lost completely from the cell or took another form, such as insertion into the chromosome. Because of this plasmid instability, the other isolates of *B. burgdorferi* were examined after only a few passages after initial isolation.

Plasmid profiles. The first strains examined were the six isolates, A to F, that had been analyzed with polyacrylamide gel electrophoresis, monoclonal antibodies, and Southern blots previously (5). Strains A and C were from the United States; strains B, D, E, and F were from Europe. Strain B was one of the central European strains that resembled typical North American strains in having a 31K OspA protein. Isolates D, E, and F, from Sweden, had either larger (D and E) or smaller (F) OspA-like major proteins; these strains reacted incompletely (D and E) or not at all (F) with a battery of monoclonal antibodies specific for the 31K OspA protein (5).

No two of the six strains A to F had the same plasmid

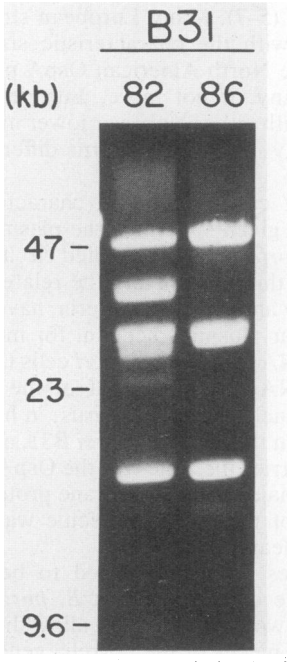

FIG. 1. Low-percentage agarose gel electrophoresis of plasmid-enriched DNA fractions of *B. burgdorferi* B31 of different passages. Strain B31 has been passed continuously since 1981; different passages of this strain have been stored frozen for later evaluation. In this study, passages from 1982 (82) and 1986 (86) were examined. Duplex linear DNA size standards were bacteriophage lambda DNA (47 kb) and the 23- and 9.6-kb *Hin*dIII fragments of lambda DNA.

profile (Fig. 2a). Strains A to C, typical of North American isolates, resembled one another in having large plasmids of approximately 48 and 38 kb; the smaller extrachromosomal elements of these strains showed more variety. (In another gel, the largest plasmid of strain A was found to have electrophoretic migration identical to that of the 49-kb plasmid of strain B31 [data not shown].) The European isolates D to F, which had major proteins of a size other than 31K, had plasmid profiles completely different from those of strains A to C. Strains D to F, in common with one another, carried a plasmid that was about 4 kb larger than the largest plasmid of strains A to C.

When the plasmid-enriched DNAs of an additional eight strains were examined in this way, a similar result was obtained (Fig. 2b). That is, the North American isolates, DN127 from California, 3028 from Texas, 297 from Connecticut, and HB4 from New York, had a large plasmid of a size identical to that of the 49-kb plasmid of strain B31-86, and the European isolates ECM-IV and PBi, with major proteins that were different from the 31K OspA protein (5, 6, 13), had large plasmids of sizes larger than those of the U.S. isolates. In this gel, strain F, here given its full name of FI, served as a size control for the plasmids of the European isolates.

Each strain had from four (B31-86 and 3028) to seven (E) discernible plasmids. Most strains had five plasmids.

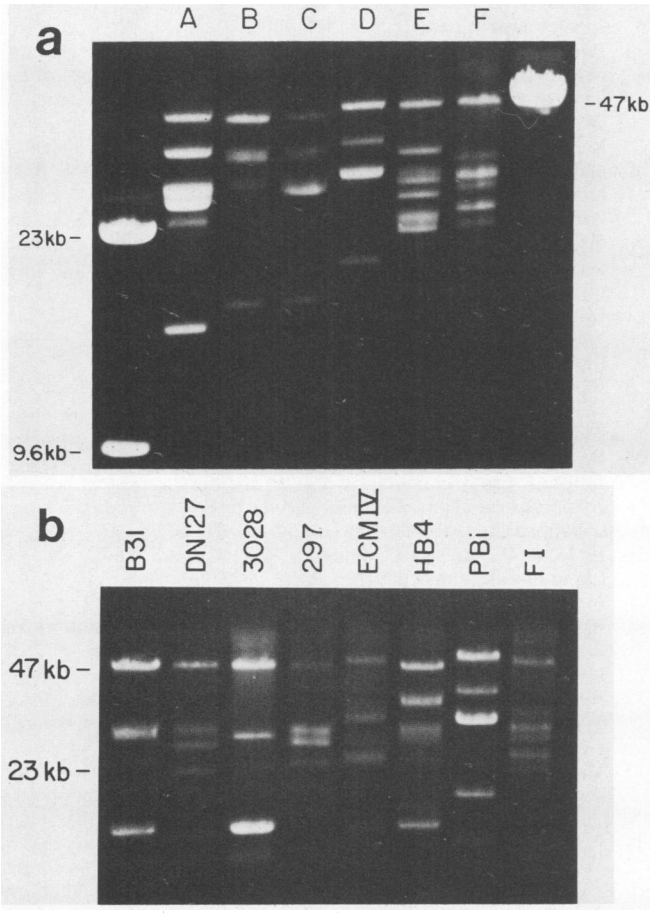

FIG. 2. Low-percentage agarose gel electrophoresis of plasmid-enriched DNA fractions of *B. burgdorferi* isolates from North America and Europe. (a) Strains A to F (described in the text and in reference 5). (b) Strains B31-86, DN127, 3028, 297, ECMIV, HB4, PBi, and FI (described in the text and in reference 6). The size standards are given in the legend to Fig. 1.

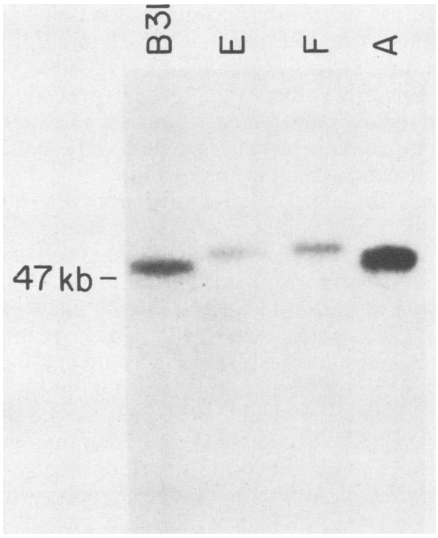

FIG. 3. Direct gel hybridization of *B. burgdorferi* B31, E, F, and A. Plasmid-enriched DNA was separated on a low-percentage agarose gel and then hybridized in situ with a radiolabeled *ospA* gene probe. The migration of the duplex linear size standard, lambda (47 kb), is shown.

If, within the broad categories of North American strain-like and European strain-like isolates, there are similarly sized large plasmids, then Fig. 2 also shows that there is heterogeneity among the smaller plasmids within each group. Interestingly, the plasmid profile of strain 3028, which was in its third passage from original isolation, was the same as that of B31-86. The absence of the 38-kb plasmid from 3028, as well as from B31-86, suggests that this plasmid may not be essential for survival in either animal hosts or culture medium.

Direct gel hybridization. If there is a correlation between the size of the largest plasmid in a profile and the outer membrane protein a strain displays, is there evidence of a direct relationship between these two characteristics? Inasmuch as a previous study had shown that European strains, such as D, E, and F, which had major proteins with either no or only partial antigenic relatedness to the 31K OspA protein, had DNA sequences that were homologous to the B31 *ospA* gene, an *ospA* probe was used in a direct gel hybridization of the separated plasmids of strains B31-86, E, F, and A. As expected, the probe hybridized to the 49-kb plasmid of strain B31 (Fig. 3). It also strongly hybridized to the similarly sized large plasmid of the North American strain A, which has a 31K OspA protein very similar, if not identical, to that of B31 (5, 6). As in a Southern blot analysis of restriction fragments (5), the *ospA* gene probe weakly hybridized to sequences in strains E and F. These partially homologous sequences were contained within a plasmid of about 53 kb in these European strains. There was no detectable hybridization of the probe to the smaller plasmids of these strains.

DISCUSSION

The DNA extraction method used in these studies greatly enriches for the plasmids of *B. burgdorferi*; very little chromosomal DNA is found in the gels (3). The procedure takes comparatively little time, can be carried out on small-volume cultures, and does not require ultracentrifugation.

These features make this plasmid extraction and analysis method ideally suited for use as a typing tool in *B. burgdorferi* isolate surveys and epidemiological studies. The present report demonstrates the utility of the procedure for this purpose and shows that there is considerable heterogeneity among strains in their plasmid profiles. Only 2 of 13 isolates examined had the same plasmid profile.

There was an area of congruity between the plasmid profiles within the two major groups of strains, i.e., strains typical of North American isolates and those more representative of isolates from northern Europe. Whereas the North American strain-like isolates examined each had a large plasmid of approximately 49 kb, European strain-like isolates each had a distinctly larger plasmid in this area of the gel. A previous study had shown that the 49-kb plasmid of strain B31 is a linear molecule. This may be the structure of the similarly sized plasmids of the other strains, but plasmid structure was not examined in the present study.

The hybridization of the *ospA* DNA probe to both the 49- and 53-kb plasmids indicates the large plasmids of other strains besides B31 likely contain the genes for outer membrane proteins. The hybridization of the strain B31 *ospA* gene probe to what is presumably an *ospA*-like gene is not surprising. Although there are size and antigenic differences between the major outer membrane proteins of various isolates, there are conserved epitopes between the 31K OspA proteins typical of North American strains and the 32,000-to 33,000-molecular-weight proteins of some European strains (5, 6). Thus, one might expect to find some degree of DNA homology in the genes for these analogous proteins, even under the stringency of the hybridization and Southern blot washing conditions used in this study.

The results of monoclonal antibody testing and polyacrylamide gel analyses can be related to the plasmid profiles. Whereas strains with approximately 31,000-molecular-weight outer membrane proteins that were bound by monoclonal antibody H3TS (5, 6) had a 49-kb plasmid, strains with a major outer membrane protein of a molecular weight other than 31,000 and not recognized by antibody H3TS had a plasmid of about 53 kb. Additional isolates from different parts of the world and from different sources now need to be examined by these three techniques to test further the correlation between these types of analysis.

The heterogeneity in plasmid contents should be useful for distinguishing strains. Indeed, plasmid profiles may be the easiest, as well as the most discriminating, method to differentiate one isolate from another. Nevertheless, the very distinctiveness of strains in their plasmid contents may be a reflection of the instability of these types of DNA molecules in *B. burgdorferi*. Although this instability was not noted if isolates were examined within a few passages of initial isolation, loss or rearrangement of plasmids upon continued in vitro cultivation was seen in this investigation, as well as in a previous study of the plasmids of *B. hermsii* (14). The latter report showed the appearance of a new species of plasmid—apparently through rearrangement of other plasmids—in an isolate that had been long-passaged in vitro. In the present study, strain B31 lost 24- and 38-kb plasmids at some time during continuous cultivation; presumably, cells with one or two fewer plasmids had a selective advantage in the culture medium over cells with their full complement of plasmids. At what point this happened is unknown. If it happened within 10 to 20 passages (or 70 to 140 generations), then plasmid loss or rearrangement may have been the cause of diminished virulence of a strain of *B. burgdorferi* for hamsters, as seen by Johnson et al., after 30 passages of in vitro cultivation (12). Plasmid loss or rearrangement may also have had a role in the change in OspB proteins noted by Schwan and Burgdorfer during early serial passage of a *B. burgdorferi* isolate (15). Plasmid analysis of various subcultures of strains of known virulence may be a way to identify plasmid-conferred virulence factors.

In summary, the present study has demonstrated the usefulness of plasmid profile analysis for characterizing strains of *B. burgdorferi* and has shown that there are correlations between the results of other serotyping schemes and the plasmid analyses.

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