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DNA Analysis of *Borrelia burgdorferi* NCH-1, the First Northcentral U.S. Human Lyme Disease Isolate

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The DNA of the first northcentral United States human Lyme disease isolate, *Borrelia burgdorferi* NCH-1, was characterized and compared with the DNAs of nine other *B. burgdorferi* isolates. Strain NCH-1 was isolated in August 1989 from a human skin biopsy specimen. DNA was analyzed by pulsed-field gel electrophoresis and restriction endonuclease analysis. Contour-clamped homogeneous electric field pulsed-field gel electrophoresis of in situ-lysed cells was performed to compare the plasmid profiles of the various isolates. The plasmid profile of isolate NCH-1, which included five plasmids of approximately 69, 42, 38, 32, and 23 kb, could be distinguished from those of the other isolates examined. The DNA profile of NCH-1 was most similar to those of strain 297 (human cerebrospinal fluid isolate, Connecticut) and strain PAL (human erythema migrans isolate, New York) and most dissimilar from those of strain P/Gau (human erythema migrans isolate, Germany) and strain IPF (*Ixodes persulcatus* tick isolate, Japan). These results indicate that genetic diversity exists among *B. burgdorferi* strains isolated from different geographical areas.

Lyme disease is the most common arthropod-borne infection in the United States. Since Lyme disease was first described by Steere et al. (35), it has been reported in all but four states (Arizona, Montana, Nebraska, and Arkansas; 20). Endemic areas in the United States include the northeast (Connecticut, Massachusetts, New Jersey, New York, Pennsylvania, and Rhode Island), the northcentral United States (Minnesota and Wisconsin), and the Pacific west (California, Oregon, and Washington). Lyme disease has also been reported frequently in Europe, the USSR, and Asia. The causative agent of the disease is the spirochete *Borrelia burgdorferi* (15). Lyme disease spirochetes have been recovered from wild and domestic animals, including rodents, raccoons, dogs, horses, cattle, and birds. *B. burgdorferi* has also been isolated from *Ixodes* sp. ticks (see reference 4 for a review).

Although Lyme disease spirochetes have been isolated from blood (7, 21, 33), cerebrospinal fluid (22, 34), synovial fluid (26), and biopsy specimens of skin lesions of erythema migrans and acrodermatitis chronicum atrophicans (2, 8, 23, 33), the frequency of recovery of *B. burgdorferi* from Lyme disease patient specimens is low. To date, most human isolates in the United States have been recovered from patients in the northeast, where the disease is most prevalent. Although *B. burgdorferi* has been isolated from animals and ticks in the northcentral United States, the isolation of *B. burgdorferi* from a human specimen has not been reported. This report characterizes the DNA of the first northcentral U.S. human Lyme disease isolate, *B. burgdorferi* NCH-1.

MATERIALS AND METHODS

Isolation of NCH-1. NCH-1 was isolated in our laboratory in August 1989 from a human skin biopsy specimen obtained from a patient in Wisconsin. The patient had presented with a 1-week history of multiple erythema migrans lesions over the upper torso, manifested as macular patches with central clearing and peripheral erythema; a dull, occipital headache; a mild stiff neck; general fatigue; and fever. The patient had no history of recent travel outside the northcentral United States. At the time, serological analysis for Lyme disease revealed 275 fluorescence signal units, where greater than 75 units is considered positive (FIAX test; Whittaker Bioproducts, Inc., Walkersville, Md.). Under sterile conditions, a 4-mm punch biopsy specimen from the peripheral margin of an erythema migrans lesion was obtained for culturing in Barbour-Stoenner-Kelly medium (3) at 30°C. The patient was treated with doxycycline (100 mg twice a day for 21 days), the rash resolved, and the patient's general health and well-being returned to normal.

The spirochete was isolated from the biopsy culture and determined to be virulent in our hamster model (14). The organism was also examined by an immunofluorescence assay with a panel of murine monoclonal antibodies (10). These monoclonal antibodies included NYS-11G1 (provided by the New York Department of Health, Stony Brook), which recognizes OspA protein; H5332 and H3TS, which recognize OspA protein; and H9724, which recognizes the flagellin protein (the last three antibodies were provided by Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, Hamilton, Mont.).

Other strains and cultivation. The biographical and geographical origins of the strains used in this study are shown in Table 1. *B. burgdorferi* strains were cultured in Barbour-Stoenner-Kelly medium at 30°C. Strains were cultured no more than 10 times from the original isolation, with the exception of strains B31 and 297, which were maintained from stock cultures.

DNA isolation. Total (genomic) DNA was isolated as previously described (11).

Moles percent G+C content. Moles percent G+C content analysis by the method of Marmur and Doty (19) was done on DNA isolated from selected *B. burgdorferi* strains. Thermal denaturation curves were generated by use of a Gilford Response spectrophotometer with thermal programming (Ciba-Corning Diagnostics Corp., Medfield, Mass.), and the denaturation temperature (T_m) was determined for each strain from the results of at least three separate experiments

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TABLE 1. Isolates used in this study

Strain	Source	Location
NCH-1	Human skin	Wisconsin
B31 (IDS, ATCC 35210)	*Ixodes dammini*	New York
297	Human spinal fluid	Connecticut
PAL	Human skin	New York
MMT1	*I. dammini*	Minnesota
CT-1	*I. dammini*	Wisconsin
IPS	*Ixodes pacificus*	California
IPF	*Ixodes persulcatus*	Japan
P/Gau	Human skin	Germany
P/Bi	Human spinal fluid	Germany

for each strain. From these determinations, the moles percent G+C content was calculated.

Restriction endonuclease analysis. Isolated DNA was digested with the restriction endonucleases *Bam*HI, *Eco*RI, *Hin*dIII, *Dpn*I, *Mbo*I, and *Sau*3AI in accordance with the manufacturers' directions (Boehringer Mannheim Corp., Biochemical Products, Indianapolis, Ind., and GIBCO BRL, Life Technologies, Inc., Gaithersburg, Md.). Following digestion, the DNA fragments were separated by electrophoresis in agarose gels. Lambda DNAs digested with *Hin*dIII served as size markers. The gels were stained with ethidium bromide, illuminated with UV light, and photographed.

PFGE. An LKB 2015 Pulsaphor system (Pharmacia LKB Biotechnology, Uppsala, Sweden) equipped with a contour-clamped homogeneous electric field (CHEF) electrode was used in pulsed-field gel electrophoresis (PFGE) experiments. DNA from *B. burgdorferi* was prepared by the gel insert method (30). In brief, *B. burgdorferi* grown in Barbour-Stoenner-Kelly medium to the stationary phase (approximately 1 week) was collected by centrifugation and washed in 10 mM Tris-HCl (pH 7.6)–1 M NaCl. The cell pellet was thoroughly resuspended in 10 mM Tris-HCl (pH 7.6)–1 M NaCl at a concentration of about 10^9 cells per ml, and the suspension was warmed to 37°C, mixed with an equal volume of 1% low-gelling-temperature agarose, and distributed to insert molds. The molds were cooled at −20°C for 5 min to allow the agarose to solidify, and the inserts were pushed out of the molds with a bent glass Pasteur pipet into lysis solution (6 mM Tris-HCl [pH 7.6], 1 M NaCl, 100 mM EDTA, 0.5% Brij-58, 0.2% deoxycholate, 0.5% Sarkosyl, 20 μg of DNase-free RNase per ml, 1 mg of lysozyme per ml). The samples were incubated overnight at 37°C with gentle shaking. The solution was changed to 0.5 M EDTA (pH 9 to 9.5)–1% sodium lauroyl sarcosine–2 mg of proteinase K per ml, and the inserts were incubated for an additional 2 days at 50°C with gentle shaking. The inserts were washed exhaustively with TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) and stored at 4°C in TE.

The inserts were placed (1/4 of a block per sample) in the wells of 1.2% agarose gels. The electrophoresis system was buffered with 45 mM Tris-borate (pH 8.0)–1 mM EDTA. The gels were run at a constant voltage of 200 V at 9°C with varying pulse parameters. Molecular weight markers consisted of lambda phage DNA concatamers (FMC BioProducts, Rockland, Maine) and lambda DNAs digested with *Hin*dIII. The gels were stained with ethidium bromide, illuminated with UV light, and photographed.

The DNA was transferred from the agarose gels to a membrane support (GeneScreen Plus; NEN Research Products, Boston, Mass.) by the method of Southern (31). NCH-1 genomic DNA radiolabeled by nick translation (24) with [α-^{32}P]dATP was used as the probe. Hybridization and subsequent washings were done with a Hybrid-Ease chamber (PR800; Hoefer Scientific Instruments, San Francisco, Calif.) in accordance with the manufacturer's recommendations. In brief, the filter was washed in 2× SSPE (1× SSPE is 150 mM NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.4])–1% sodium dodecyl sulfate (SDS) for 30 min at 60°C, twice in 0.5× SSPE–1% SDS for 15 min each time at 60°C, and twice in 0.1× SSPE–1% SDS for 15 min each time at room temperature. The blot was dried and exposed to X-ray film.

TABLE 2. Moles percent G+C contents of selected *B. burgdorferi* isolates

Strain	T_m (°C)	Mol% G+C content[a]
NCH-1	81.36 ± 0.16	29.41
B31	81.34 ± 0.07	29.37
297	81.41 ± 0.13	29.54
PAL	81.54 ± 0.37	29.85
MMT1	81.48 ± 0.24	29.71
CT-1	81.54 ± 0.46	29.85
IPS	81.44 ± 0.36	29.61
IPF	81.21 ± 0.06	29.05
P/Gau	81.14 ± 0.06	28.88
P/Bi	81.39 ± 0.46	29.49

[a] Results are the averages of three experiments and were determined with the method of Marmur and Doty (19), where T_m = 69.3 + [0.41(mol% G+C content)] when the solvent is 0.2 M Na^+.

RESULTS

Identification of NCH-1. The spirochete was isolated from the skin biopsy culture and examined by an immunofluorescence assay with a panel of monoclonal antibodies. The organism was reactive with three monoclonal antibodies which recognize the OspA protein of *B. burgdorferi*: NYS-11G1, H5332, and H3TS. In addition, the organism was reactive with monoclonal antibody H9724, which recognizes the flagellin protein of *Borrelia* species. On the basis of these results, we determined that this isolate could be referred to as *B. burgdorferi* NCH-1, the first human Lyme disease isolate from the northcentral United States.

Characterization of NCH-1 DNA. The DNA of *B. burgdorferi* NCH-1 was compared with those of other known *B. burgdorferi* isolates from various sources and geographical locations by a number of different techniques.

Total (genomic) DNAs were isolated from the 10 test strains, and the moles percent G+C contents were determined (Table 2). The T_m for NCH-1 was determined to be 81.36°C. The T_ms for the other strains were not found to be significantly different. On the basis of these determinations, the moles percent G+C content of NCH-1 was calculated to be 29.4, similar to those calculated for the other strains studied. These results are in concordance with those for other *B. burgdorferi* strains (13).

The DNAs from these strains were also examined for the presence of methylated adenine residues. The DNAs were incubated with restriction endonucleases *Dpn*I, *Mbo*I, and *Sau*3AI. The DNA of strain NCH-1 was not digested with *Dpn*I, which recognizes GATC sequences with methylated adenine residues, suggesting that this strain lacks an adenine methylation system (Table 3). This observation was verified by the digestion of the DNA with *Mbo*I, which is active on unmethylated GATC sequences. The DNA was also di-

TABLE 3. DNA methylation in selected *B. burgdorferi* isolates

Strain	Genomic DNA digestion by[a]:		
	*Dpn*I	*Mbo*I	*Sau*3AI
NCH-1	−	+	+
B31	−	+	+
297	−	+	+
PAL	+	+	+
MMT1	−	+	+
CT-1	−	+	+
IPS	−	+	+
IPF	+	+	+
P/Gau	+	+	+
P/Bi	−	+	+

[a] −, no digestion; +, digestion.

TABLE 4. Plasmid profiles of *B. burgdorferi* NCH-1 and other selected isolates

Strain	Plasmid size (kb)[a]
NCH-1	69, 42.1, 38.7, 32.8, 23.6
B31	96.1, 69, 38.7, 22.6
297	96.1, 69, 40.3, 37.1, 32.8, 23.6
PAL	69, 53.9, 45.7, 38.7, 32.8, 21.7
MMT1	63.6, 37.1, 35.6, 32.8, 23.6
CT-1	63.6, 35.6, 30.2, 25.6, 21.7
IPS	69, 38.7, 35.6, 32.8, 30.2, 23.6
IPF	69, 49.6, 34.2, 32.8, 28.9
P/Gau	75, 51.7, 45.7, 35.6, 31.5
P/Bi	69, 63.6, 35.6, 30.2, 25.6

[a] Approximated following CHEF PFGE.

gested with *Sau*3AI, which recognizes both methylated and unmethylated GATC sequences. B31, the type strain of the species, and strains 297, MMT1, CT-1, IPS, and P/Bi also lacked methylated adenine residues. In contrast, strains PAL (New York, skin), IPF (Japan, tick), and P/Gau (Germany, skin) were found to contain methylated DNA.

In situ-lysed DNA samples from each strain were analyzed by CHEF PFGE to determine plasmid content (Fig. 1). Strain NCH-1 contained five plasmids of approximately 69, 42, 39, 33, and 24 kb (Table 4). The 69-kb plasmid (or the 63.6-kb plasmid in strains MMT1 and CT-1) appears to correspond to the linear plasmid that is 49 kb in size in conventional agarose gel electrophoresis and that encodes the OspA and OspB proteins of *B. burgdorferi*. The plasmid profiles of the other strains are also indicated in Table 4. Each strain had from four (B31) to six (297, PAL, and IPS) discernible plasmids, with most strains containing five plasmids. The plasmids ranged in size from 96 to 20 kb, with a majority of the plasmids in the 20- to 40-kb range.

The DNAs of the 10 strains were separated by CHEF PFGE, transferred to a membrane support, and probed with radiolabeled NCH-1 genomic DNA. Examination of an overexposed autoradiograph of a Southern blot indicated that DNA homology exists among these strains (Fig. 2). The NCH-1 genomic DNA probe recognized all NCH-1 plasmids

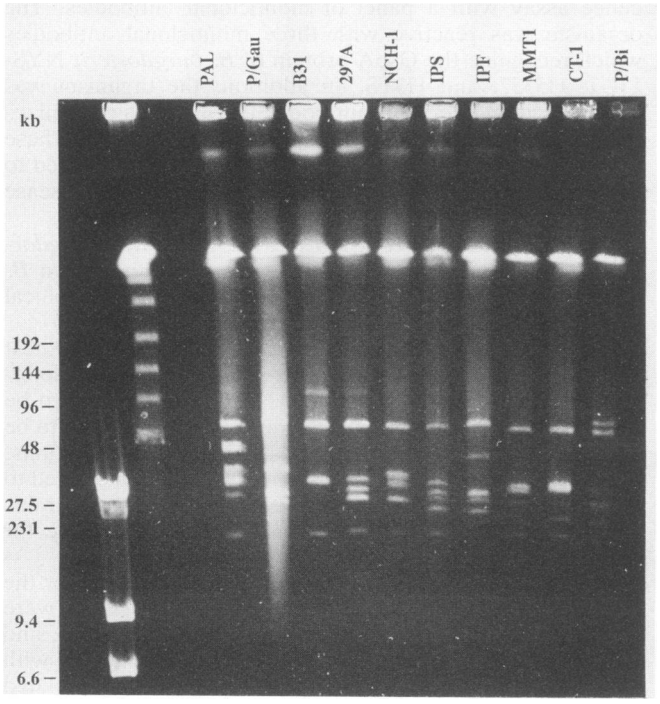

FIG. 1. CHEF PFGE of *B. burgdorferi* DNA. In situ-lysed DNA samples were separated at 200 V with 15-s pulses for 10 h and then with 1-s pulses for 10 h. Lambda DNA concatamers and lambda DNAs digested with *Hin*dIII were loaded as standards.

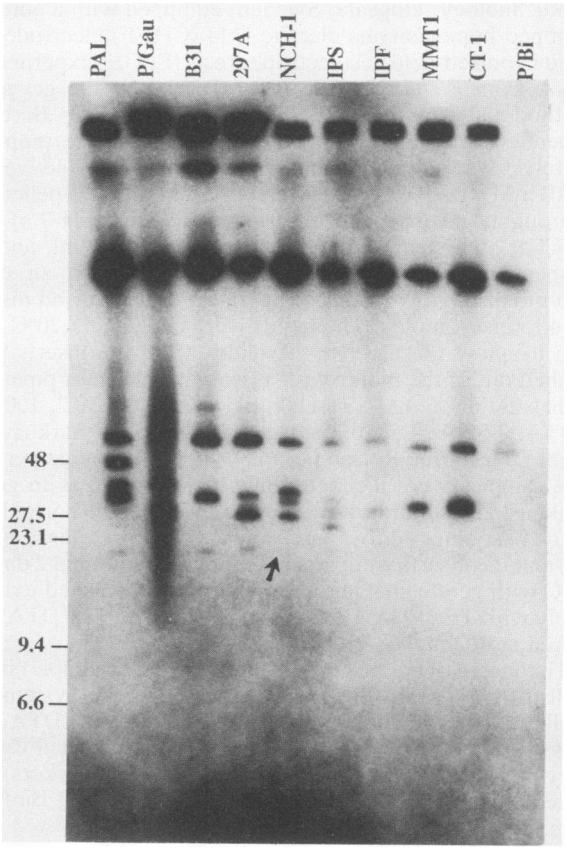

FIG. 2. Autoradiograph (overexposed) of the Southern blot of the pulsed-field gel in Fig. 1, which contained in situ-lysed DNA samples from *B. burgdorferi* isolates. Genomic DNA from NCH-1 was radiolabeled and used as a probe. The arrow indicates the position of the 23.6-kb plasmid of NCH-1. Numbers at left are in kilobases.

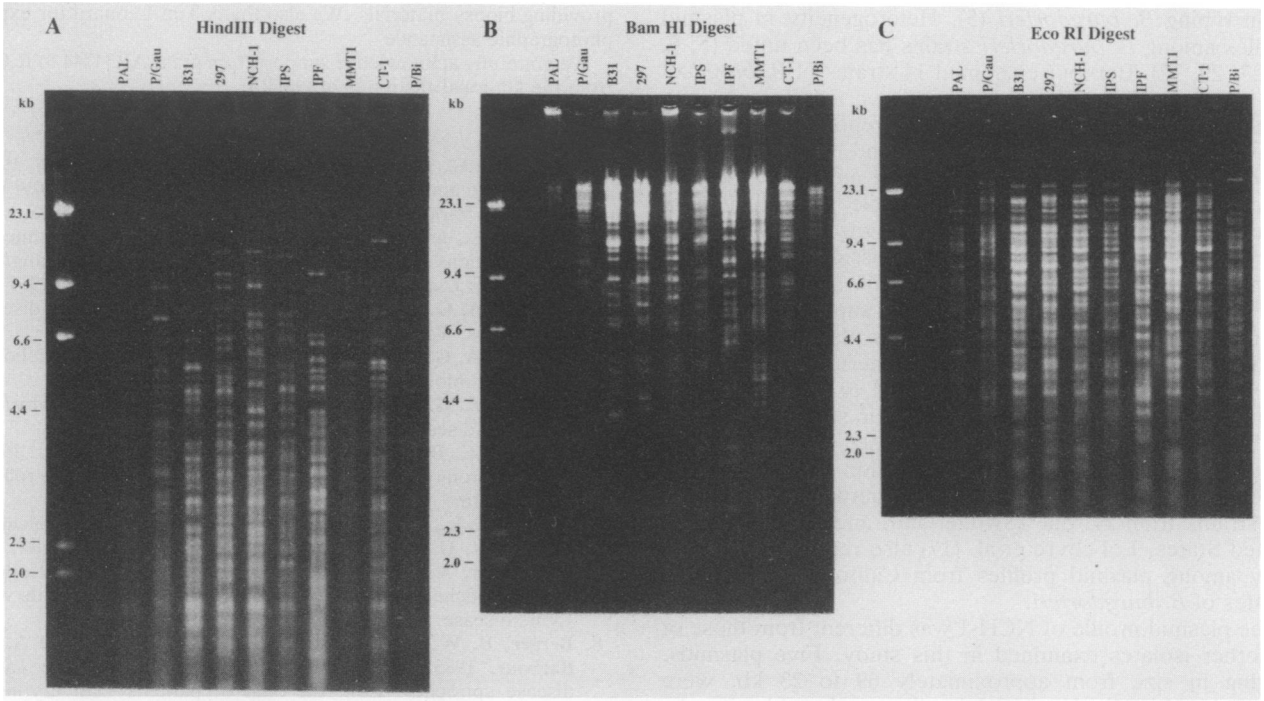

FIG. 3. Restriction endonuclease digests of DNAs isolated from *B. burgdorferi* isolate NCH-1 and other selected isolates. (A) *Hin*dIII digest. (B) *Bam*HI digest. (C) *Eco*RI digest. DNA fragments were separated on 0.7% agarose gels and stained with ethidium bromide under UV illumination.

in addition to all of the plasmids in strains B31, 297, PAL, and IPS; four of five plasmids in strains MMT1, CT-1, P/Gau, and P/Bi; and three of five plasmids in strain IPF. The 23.6-kb plasmid of NCH-1, which was in proportionately lower quantity than the other plasmids (Fig. 1), was recognized by the NCH-1 genomic DNA probe (Fig. 2, arrow). The NCH-1 genomic DNA probe did not appear to hybridize with the 36-kb plasmid of P/Bi or the 33-kb plasmid of IPF and only weakly hybridized under these conditions with the 52-kb plasmid of P/Gau, the 50-kb plasmid of IPF, the 33-kb plasmid of MMT1, and the 30-kb plasmid of CT-1.

DNAs isolated from these strains were also compared by restriction endonuclease analysis (Fig. 3). The electrophoretic pattern following *Hin*dIII digestion is shown in Fig. 3A. Similarities were observed among the patterns of NCH-1, 297, B31, and IPS, especially in the lower portions of the gel, in the 4.4- to 2.0-kb range. The results of *Bam*HI digestion are shown in Fig. 3B. Similarities among the NCH-1 pattern and those of MMT1 and CT-1, two other northcentral U.S. strains, were evident, especially in the 9.4- to 4.4-kb range. The *Bam*HI digestion patterns of P/Gau and P/Bi (Germany) and IPF (Japan) were very different from that of NCH-1. Finally, when NCH-1 DNA was digested with *Eco*RI, the resulting pattern was very similar to the *Eco*RI digestion patterns of the other U.S. strains: 297, B31, IPS, MMT1, CT-1, and PAL (Fig. 3C). The *Eco*RI digestion patterns of the European strains, P/Gau and P/Bi, appeared to be similar to each other but were distantly related to those of strain NCH-1 and the other U.S. strains. The *Eco*RI banding pattern of the Japanese strain, IPF, was distinctly different from those of strain NCH-1 and the other strains compared in this study.

DISCUSSION

This study was undertaken to characterize and compare the DNA of the first northcentral U.S. human Lyme disease isolate with those of nine other *B. burgdorferi* isolates. NCH-1 was isolated from a biopsy specimen obtained from the peripheral margin of a lesion from a patient who had presented with multiple erythema migrans lesions. NCH-1 was identified as *B. burgdorferi* on the basis of its reactivity in an immunofluorescence assay with a panel of monoclonal antibodies.

The DNA of isolate NCH-1 was found to have a moles percent G+C content of 29.4. This content is similar to those of the other isolates studied and also agrees with previously published values for other *B. burgdorferi* isolates (12, 13, 25). The method of Marmur and Doty [moles percent G+C content = $(T_m - 69.3)/0.41$; 19] was used in this study and previously in our laboratory (12, 13), whereas the method of De Ley [moles percent G+C content = $2.44(T_m - 69.4)$; 9] was used by Schmid et al. (25). The moles percent G+C contents generated by the two methods differed by only 0.24%.

The DNA of isolate NCH-1 was also found to lack methylated adenine residues. Although the relapsing fever borreliae (*B. hermsii*, *B. duttonii*, *B. crocidurae*, *B. turicatae*, and *B. parkeri*) are known to contain methylated adenine residues, most *B. burgdorferi* isolates are unmethylated (11, 16). The other northcentral U.S. isolates examined for methylated DNA (MMT1 and CT-1) lacked methylated adenine residues. In contrast, human isolates from the northeastern United States (PAL) and Germany (P/Gau) contained methylated DNA. The role of methylated DNA in *Borrelia* species remains undefined.

Barbour has suggested plasmid analysis as a method of

strain typing *B. burgdorferi* (5). Heterogeneity in plasmid profiles among *B. burgdorferi* strains has been noted (5, 6, 17, 27, 29, 32). Barbour examined 14 strains of *B. burgdorferi* and found that plasmid contents varied from four to seven in number (5). Schwan et al. (27) reported that in vitro cultivation of *B. burgdorferi* was found to influence the plasmid profile and the ability to infect animals. A loss of infectivity and a reduction in detectable plasmid number were observed after 11 to 15 passages in vitro. They reported the presence of nine plasmids in an unpassaged culture of *B. burgdorferi*. The plasmid profile of this same strain was further analyzed and reported to contain supercoiled circular plasmids in addition to numerous linear plasmids (29). The genomes of selected European and American strains were analyzed by Baril et al. (6) and found to contain several circular and linear plasmids that ranged in size from 15 to 60 kb. Stalhammar-Carlemalm et al. (32) used plasmid analysis to supplement their studies on restriction fragment length polymorphisms of chromosomal DNA between *B. burgdorferi* strains from Europe (Switzerland) and those from the United States. LeFebvre et al. (17) also reported heterogeneity among plasmid profiles from California ixodid tick isolates of *B. burgdorferi*.

The plasmid profile of NCH-1 was different from those of the other isolates examined in this study. Five plasmids, ranging in size from approximately 69 to 23 kb, were detected by PFGE when NCH-1 cells were lysed in situ. An advantage to the in situ lysis and PFGE separation methods used in this study is that the DNA remains in as near an intact state as possible (28). Shear damage and breakage due to handling and isolation procedures are minimized. LeFebvre et al. used encapsulation of cells in agarose beads and PFGE to analyze and compare the DNAs of isolates of *B. burgdorferi* from California with the DNA of the type strain, B31 (17). Others have determined plasmid profiles for isolates of *B. burgdorferi*; however, their methods involved isolation and purification of DNA followed by separation of DNA components by low-percentage-agarose gel electrophoresis (5, 27, 29), CHEF PFGE (6), or field inversion gel electrophoresis (32). In studies which compared strain B31 with other strains (5, 17, 27, 32), B31 was observed to contain four plasmids. We were also able to discern four plasmids in in situ-lysed preparations of strain B31 separated by PFGE. However, direct comparisons with previously published results were difficult, as the sizes of the plasmids varied with the methods used for their preparation, separation, and characterization. Nonetheless, plasmid profiles are useful in the characterization of *Borrelia* DNA, especially when determined following in situ lysis and PFGE.

The results of PFGE and hybridization with NCH-1 DNA demonstrate that homology exists in both the chromosomes and some of the plasmids of the isolates studied. The hybridization results together with the restriction endonuclease analysis results indicate that strain NCH-1 appears to be more closely related genetically to strains B31, 297, and IPS (U.S. isolates) than to the European strains, P/Gau and P/Bi, or the Asian strain, IPF. The restriction endonuclease patterns observed support the usefulness of restriction endonuclease analysis in the classification of *B. burgdorferi* isolates (18). These results confirm that genetic diversity exists among *B. burgdorferi* strains isolated from different geographical areas, as reported by others (1, 5, 17, 18).

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