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Characterization of a Circular Plasmid from *Borrelia burgdorferi*, Etiologic Agent of Lyme Disease

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***Borrelia burgdorferi*, the etiologic agent of Lyme disease, was recently shown to contain plasmid DNA. Two plasmid species have been described in strain CT1, a Wisconsin tick isolate: a 9.2-kilobase entity; and a larger, 70-kilobase entity. Characterization of the 9.2-kilobase entity by using DNase I and restriction endonucleases demonstrated that the plasmid is supercoiled and exists as a stable dimer in this strain. The role played by the plasmid in *B. burgdorferi* is unknown.**

Lyme disease is a malady characterized by a unique skin lesion, erythema chronicum migrans (11), and transmitted primarily by ticks of the genus *Ixodes* (4). The disease has been reported in 32 states, with primary endemic foci being the northeastern United States, the Midwest (Minnesota and Wisconsin), and the Pacific Northwest. The disease has widespread distribution in Europe (11). Left untreated, the disease may progress to such sequelae as myocarditis, neuropathy, and arthritis. The etiologic agent, a spirochete, was shown to be a new species of *Borrelia*, *Borrelia burgdorferi* (5).

During investigations into the genetic relationships between the Lyme disease spirochete and *Treponema*, *Leptospira*, and *Borrelia* spp., plasmid DNA was observed in all species of *Borrelia* examined, including *B. burgdorferi* (5). Plasmid DNA has also been reported to be in three species of *Treponema*, *Treponema pallidum* (8), the etiologic agent of syphilis, and two swine pathogens, *T. hyodysenteriae* and *T. innocens* (L. A. Joens, A. B. Margolin, and M. J. Hewlett, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, H-173, p. 156).

Several plasmid species are present in *B. burgdorferi*: an 8.5 to 9.5-kilobase (kb) entity that has only been detected in fresh North American tick and some fresh animal isolates, and a 70-kb entity which is apparently common to all strains of the Lyme disease spirochete. Recently, Plasterk et al. reported the presence of linear plasmid DNA in relapsing fever *Borrelia* (9); these plasmids encode the structural genes for the major outer surface proteins of the spirochetes. In this report, we describe the characterization of the 9.2-kb plasmid from *B. burgdorferi* strain CT1.

B. burgdorferi CT1, isolated from *Ixodes dammini* collected in northern Wisconsin, was cultivated in BSK II medium (1) until maximal cell density (10^8/ml) was reached. Cells were harvested by centrifugation and washed three times in GTE buffer (50 mM glucose, 10 mM Tris hydrochloride, pH 7.4, 1 mM EDTA). The cell pellets were suspended in GTE buffer and divided into equal portions for total DNA purification and plasmid extraction.

Whole spirochetal DNA was harvested as described previously (6). The bacteria were lysed by the addition of sodium dodecyl sulfate to a final concentration of 1% and then gently mixed to complete lysis of the cells. The mixture was extracted twice with STE-saturated phenol (STE is 100 mM sodium chloride, 10 mM Tris hydrochloride, pH 8.0, 1 mM EDTA), followed by a single extraction with chloroform. The nucleic acids were precipitated with ethanol. The DNA or RNA was suspended in TE buffer (10 mM Tris hydrochloride, pH 8.0, 1 mM EDTA), and RNase was added to a final concentration of 500 μg/ml; incubation was at 37°C for 1 h. The extractions with phenol and chloroform were repeated, and the DNA was precipitated with ethanol. The DNA was suspended in TE buffer for use in nucleic acid profile analysis. Electrophoresis of all DNA samples was performed in 0.7% agarose gels in Tris acetate-EDTA buffer (0.04 M Tris acetate, pH 8.0, 1 mM EDTA [6]) at 0.4 V/cm overnight. The gels were stained with 1 μg of ethidium bromide per ml and photographed on Polaroid type 47 film.

Plasmid DNA from strain CT1 was extracted by using the alkaline lysis method of Birnboim and Doly (3). The spirochetal cell suspension was treated with lysis buffer (0.2 N NaOH, 1% sodium dodecyl sulfate) to disrupt the cells. Chromosomal DNA and cell debris were removed from the mixture by the addition of 5 M potassium acetate (pH 4.8) followed by centrifugation at 14,500 rpm for 15 min. The supernatant was collected, and the nucleic acids were precipitated with isopropanol. The nucleic acids were collected by centrifugation, and plasmid DNA was further purified by cesium chloride density gradient centrifugation at 39,000 rpm for 72 h (10). The plasmid band was collected from the gradient and precipitated with ethanol. The purified plasmid was suspended in TE buffer for further characterization.

The total DNA of strain CT1 is shown in Fig. 1. Several forms of extrachromosomal DNA are seen. The two lowermost bands on the gel correspond to, respectively, open circular and supercoiled forms of the small plasmid. The other bands on the gel correspond to the chromosomal DNA and the large plasmid species, which may in fact be the linear plasmids described by other investigators (2, 9).

The small plasmid from strain CT1 (designated pCT1) was separated from the other DNA species by cesium chloride density gradient centrifugation; the larger plasmids and chromosomal DNA were likely sheared in the alkaline lysis extraction procedure and migrated through the gradient with the linearized DNA.

DNase I digestions were performed with 5 μg of pCT1 DNA and 160 pg of the enzyme in a timed reaction in TM buffer (10 mM Tris, pH 7.4, 10 mM $MgCl_2$) at 37°C. Samples were removed from the reaction at 0, 1, 2, 5, 10, 20, 40, and 60 min. Reactions were terminated by adding 5 μl of 300 mM

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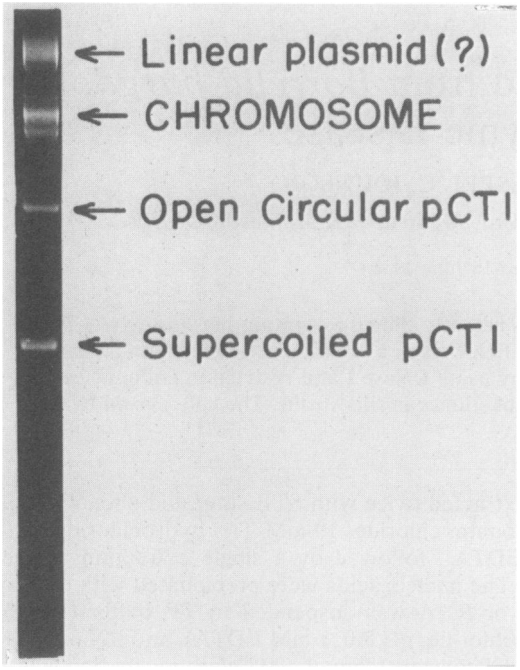

FIG. 1. Plasmid profile of *B. burgdorferi* CT1. The nucleic acids of the spirochete were electrophoresed on 0.7% agarose gels and strained with ethidium bromide. The two major extrachromosomal entities and chromosomal DNA are shown. The uppermost band on the gel may correspond to linear plasmids observed in the spirochete.

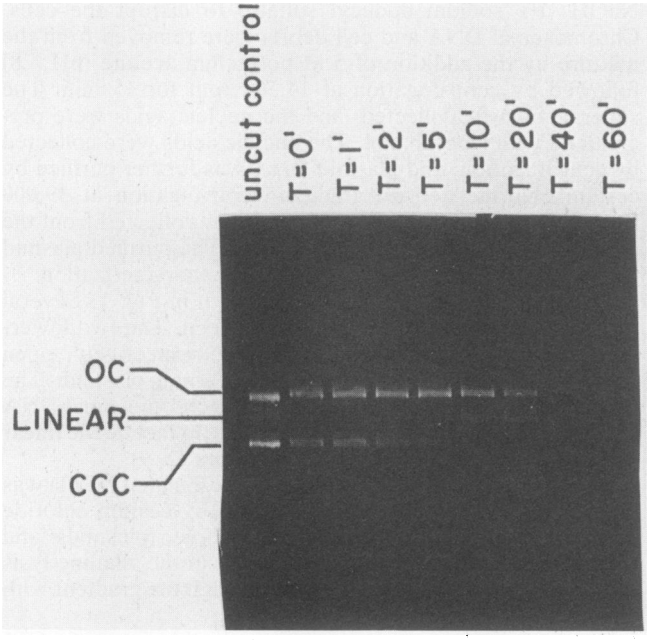

FIG. 2. Timed DNase I digestion of the small plasmid from strain CT1 (pCT1). The covalently closed supercoiled (CCC) species (lowest band) disappears as time progresses, proceeding to the open circular (OC) (top band) and linear (middle band) forms. DNAs were separated by electrophoresis as described in the text.

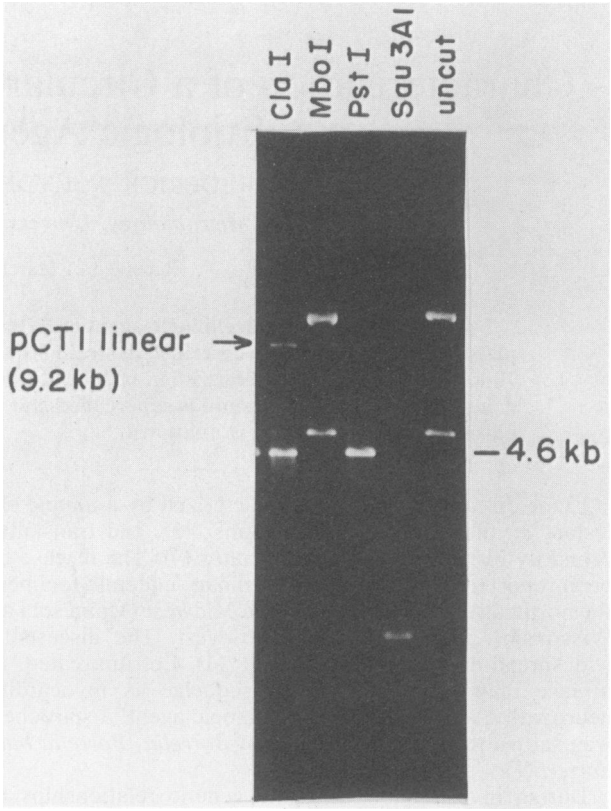

FIG. 3. Restriction enzyme digestion analysis of pCT1, using endonucleases *Pst*I, *Cla*I, *Mbo*I, and *Sau*3A1. Digestion of pCT1 with *Cla*I and *Pst*I yielded a single band at 4.6 kb. This band migrated through the gel faster than did the supercoiled form of the uncut plasmid, suggesting that pCT1 exists as a multimer.

EDTA. Undigested and digested DNAs were analyzed by agarose gel electrophoresis in 0.7% agarose gels at 0.4 V/cm overnight. This procedure was deemed necessary to differentiate pCT1 from the previously described linear plasmids.

DNase I digestion of pCT1 revealed it to be a closed circular entity of 9.2 kb. The molecular weight of the linearized plasmid fragments were determined by gel electrophoresis, using phage lambda DNA digested with the restriction enzyme *Hin*dIII as size markers. As digestion of pCT1 with DNase I progressed, the supercoiled form was progressively converted to the open circular and linear forms (Fig. 2).

The plasmid was further characterized with restriction endonucleases *Cla*I, *Pst*I, *Mbo*I, and *Sau*3A1. Digestion of the plasmid with *Pst*I yielded a single fragment of 4.6 kb (Fig. 3). This band migrated through the gel farther than the supercoiled plasmid (lane 5, Fig. 3), suggesting that the plasmid exists as a multimer in the spirochete. The digestion of pCT1 with *Cla*I is incomplete, and the profile of the uncut plasmid may be seen along with the final digestion product, a single fragment of 4.6 kb. Digestion of pCT1 with *Mbo*I yielded no digestion products. This enzyme is unable to cut DNA which has methylated adenosine residues. The digestion of pCT1 with the isoschizomer enzyme *Sau*3A1 (which can digest methylated DNA) yielded several fragments, the largest being about 1.5 kb. *Mbo*I yielded the appropriate fragments when used to digest phage lambda unmethylated DNA as a digestion control. These data suggest the presence

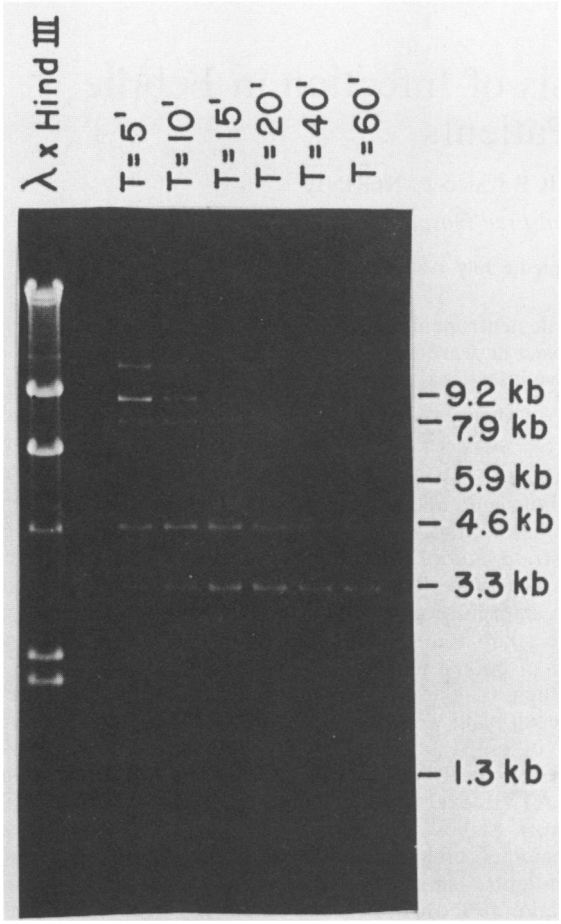

FIG. 4. Timed restriction enzyme digestion of pCT1, using *Bam*HI and *Pst*I. As time progressed, pCT1 was degraded into fragments of 9.2, 7.9, 5.9, 4.6, 3.3, and 1.3 kb, evidence that the plasmid is a dimer.

of a DNA methylation system in *B. burgdorferi*. A similar phenomenon was observed by Meier et al. in the relapsing fever spirochete *Borrelia hermsii* (7).

Complete digestion of pCT1 with *Bam*HI and *Pst*I yielded fragments of 3.3 and 1.3 kb. To determine whether pCT1 is indeed a multimer, a timed reaction was conducted, using 0.1 U of each enzyme. Samples were removed from the reaction mixture at 0, 5, 10, 15, 20, 40, and 60 min. The reactions were terminated by adding 5 μl of stop buffer. The samples were applied to a 0.7% agarose gel and separated by electrophoresis (Fig. 4). As digestion time progressed, pCT1 was degraded into fragments of 9.2, 7.9, 5.9, 4.6, 3.3, and 1.3 kb. These data suggest that the plasmid exists as a tandem repeat in the spirochete. If pCT1 existed as an inverted repeat, fragments of 6.6 and 2.6 kb would have been observed. A partial restriction map is shown in Fig. 5.

Plasmid DNA is ubiquitous in microorganisms and may encode a variety of traits which are beneficial to the bacterium. *B. burgdorferi* strains from North America and Europe share an extrachromosomal element of approximately 70 kb (F. W. Hyde and R. C. Johnson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, H-150, p. 116). This element may actually be a linear plasmid, similar to those observed by Barbour, Plasterk, and other investigators (2, 9).

A smaller plasmid of 9.2 kb was observed and characterized from strain CT1, a fresh tick isolate from northern

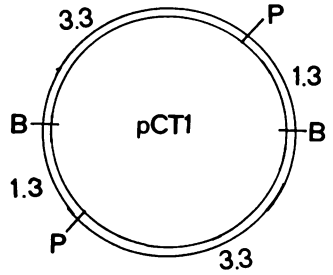

FIG. 5. Restriction map of tandem repeat of pCTI. *Bam*HI and *Pst*I sites are shown. Fragment sizes are shown in kilobases.

Wisconsin. Plasmids of similar molecular weight have been observed in other fresh tick isolates and in some fresh animal isolates from North America. It has not been observed in any human isolates or in any European isolates screened to date. Preliminary data suggest a role for this plasmid in the infectivity of the spirochete, but further experiments are necessary to substantiate this finding. Cultivation of *B. burgdorferi* B31 over many months resulted in the loss of a plasmid with a molecular weight similar to that of pCT1 (F. Hyde, Ph.D. thesis, University of Minnesota, Minneapolis, 1985). Further work is necessary to determine the role played by this plasmid in the biology and possibly the virulence of *B. burgdorferi*.

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