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Physical map of chromosomal and plasmid DNA comprising the genome of *Leptospira interrogans*

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ABSTRACT

The size and physical structure of the *Leptospira interrogans* genome was characterized using contour-clamped homogenous electric field (CHEF) gel electrophoresis. The *L. interrogans* genome is approximately 4750 kb in size and is composed of two molecular species of DNA: a 4400 kb chromosome; and a 350 kb plasmid, pLIN1. A physical map of the chromosome was constructed with the restriction enzymes *Not*I and *Sfi*I. A physical map of pLIN1 was constructed with *Apa*I, *Not*I, *Sse*8387I, *Sgr*AI, and *Sma*I. Both the *L. interrogans* chromosome and pLIN1 are circular.

INTRODUCTION

Leptospirosis, caused by *Leptospira interrogans*, is a common disease in livestock and an important zoonotic disease in humans. Little is known about the molecular basis for pathogenicity in *L. interrogans* infections. There are no known mechanisms for genetic exchange among leptospires. Thus, little is known about the structure or organization of the leptospiral genome. The genetics of *Leptospira* are of interest since these bacteria (along with other spirochetes) are thought to occupy a unique position in bacterial evolution (1,2). Several recent studies show that spirochete genomes possess several unusual features such as: linear plasmids (3); linear chromosomes (4,5); and significant amounts of repetitive sequence DNA (6,7). Initial descriptions of the *L. interrogans* genome using pulsed-field gel electrophoresis (PFGE) conflict and describe the genome as composed of either: (1) a circular 5000 kb chromosome (8); or (2) three distinct molecular species, including a linear plasmid, with a cumulative size of 3100 kb (9).

In the present study, the PFGE technique of contour clamped homogenous-electric field (CHEF) gel electrophoresis (10) was used with DNA blot analysis to construct a physical map of the *L. interrogans* serovar *pomona* type kennewicki genome. This serovar of *L. interrogans* was selected since it is one of the most commonly encountered pathogenic leptospires. The results demonstrate that the *L. interrogans* genome contains a circular 4400 kb chromosome and a circular 350 kb plasmid. This is the first physical map of any spirochete genome.

MATERIAL AND METHODS

DNA preparation and gel electrophoresis

Leptospira interrogans serovar *pomona* type kennewicki strain RZ11 was isolated from an aborted swine fetus, colony purified, and propagated as usual (6). *L. interrogans* in logarithmic phase growth were harvested and genomic DNA packaged in agarose beads as described by Kauc and Goodgal (12). Bacterial cells from 100 ml cultures were encapsulated into 4 ml of beads. DNA containing beads (25 μl) were digested with 10 units of restriction enzyme for 2–4 hr at 37°C. To obtain partially digested *L. interrogans* DNA, 50 μl bead encapsulated DNA was incubated with diluted *Not*I or *Sse*8387I (0.25 to 2 units) for 1 hr at 37°C. DNA samples were separated by CHEF electrophoresis at 200V through 1% agarose gels buffered with 0.5×TBE (1×TBE is 89 mM Tris, 89 mM boric acid, 2 mM EDTA). The gel temperature was maintained at 10°C by buffer circulation through a refrigerated water bath. Pulse times were programmed using a CHEF DR II drive module (BioRad) as described in the figure legends. Ethidium bromide stained gels were visualized with UV light. Fragment lengths were calculated by comparison to linear DNA standards including: a 1 Kb ladder (0.5 to 12 kb, BRL); *Saccharomyces cereviseae* (210 to 1600 kb, BRL) and *Schizosaccharomyces pombe* chromosomes (3 to 5.7 Mb, BioRad); concatemers of λc1857 (monomer = 48.5 kb, New England Biolabs); and concatemers of λDASH (monomer = 40.5 kb, Stratagene). λDASH concatemers were prepared by ligation of purified phage DNA. Circular DNA standards were pBR322 (4.5 kb), and recombinant plasmids pRS1 (11 kb), pRS16 (15 kb), and pRS18 (18 kb). A scanning laser densitometer (LKB) quantitated fluorescence of DNA fragments.

Two dimensional gel electrophoresis was done by separating undigested, *Not*I-, and *Sse*8387I-digested DNA by CHEF as described above. The appropriate lanes were excised from the first dimensional gel and cast in a 1% agarose gel buffered with 0.5×TBE. The DNA was separated by constant-field gel electrophoresis for 20 hrs at 50 V using a submerged horizontal gel electrophoresis apparatus. The direction of electrophoresis in the second dimension was at 90° with respect to the first dimension.

DNA blot analysis

DNA was fragmented in the gel by UV irradiation then transferred to nylon membranes as described previously (6). DNA hybridization analysis was done as described previously (6).

Radioactive probes were made with either [^{32}P]dATP or [^{32}P]UTP (ICN) by nick-translation or run-off transcription, respectively (6). Probe templates were either purified restriction fragments isolated from agarose gels using Agarase (New England BioLabs) or random clones from a plasmid-based *Bam*HI genomic library of RZ11 DNA constructed in pKSII+ (Stratagene).

RESULTS

Analysis and sizing of the *L. interrogans* genome

The *L. interrogans* genome has a base content between 35 and 40% G+C (13). Enzymes expected to recognize few sites in the *L. interrogans* genome (ie. those which recognize GC rich sequences and/or sites >6 bp in size) were used to digest intact genomic DNA. The enzymes tested were *Apa*I, *Bgl*I, *Bss*HII, *Ecl*XI, *Kpn*I, *Mlu*I, *Nae*I, *Nar*I, *Not*I, *Pac*I, *Rsr*II, *Sac*I, *Sac*II, *Sal*I, *Sgr*AI, *Sph*I, *Sse*8387I, *Sma*I, *Xba*I, and *Xho*I. *Not*I and *Sfi*I digested the genome into 11 and 4 fragments, respectively, making these two enzymes useful for sizing and constructing a physical map of the genome. The other enzymes tested digested genomic DNA into many similar sized fragments which were not resolved. However, several large resolvable fragments were generated when genomic DNA was digested with *Apa*I, *Bss*HII, *Pac*I, *Sgr*AI, *Sse*8387I, or *Sma*I. These large fragments were useful for aligning *Not*I fragments. Typical restriction endonuclease digestion patterns with several of these enzymes are shown in Fig. 1. Little undigested DNA migrated from the origin (Fig. 1C). The size of the *Not*I and *Sfi*I fragments were calculated by comparison to standards of known size (Table 1). Conventional gel electrophoresis showed that neither enzyme generated fragments smaller than those listed (data not shown). The average size of the *L. interrogans* genome is approximately 4750 kb.

Attempts to resolve intact *L. interrogans* chromosomes by CHEF electrophoresis using pulse times >240 sec were unsuccessful (data not shown). Under these same electrophoresis conditions each of the three *S. pombe* chromosomes (3 to 5.7 Mb in length) were resolved.

Physical map of the *L. interrogans* chromosome

Initial studies showed that *L. interrogans* contained a 4400 kb chromosome and a 350 kb plasmid (pLIN1) (see below). A physical map of the chromosome was constructed with the enzymes *Sfi*I and *Not*I (Fig. 2). The *Not*I site between the *Not*I A and B fragments is arbitrarily set as the first nucleotide on the map.

Three *Sfi*I sites were mapped on the chromosome by analysis of single and double enzyme digestion data using *Not*I and *Sfi*I (Table 1). The *Sfi*I C fragment mapped within the *Not*I A fragment, and was located 40 kb and 1100 kb from the flanking *Not*I sites. The third chromosomal *Sfi*I site was mapped close to the *Not*I site joining the *Not*I C and K fragments by DNA hybridization analysis. Cloned fragments specific for the *Not*I

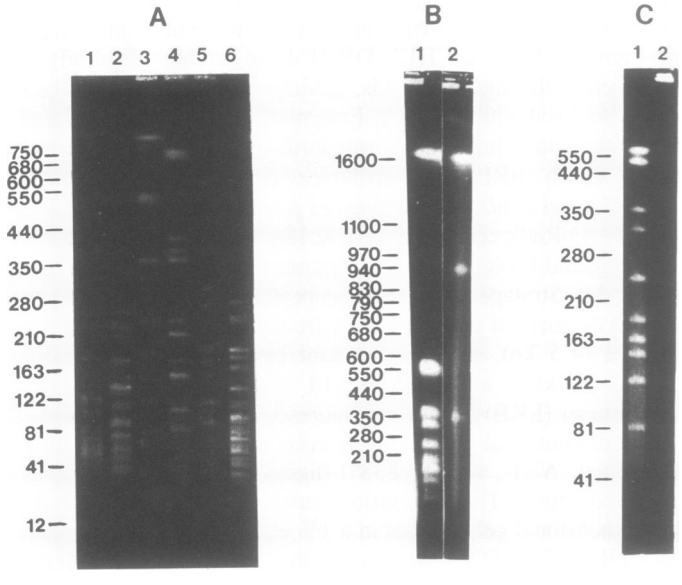

Figure 1. CHEF electrophoresis of restriction endonuclease digested *L. interrogans* DNA. (A)Intact agarose-encapsulated genomic DNA was digested with *Apa*I (lane 1), *Bss*HII (lane 2), *Not*I (lane 3), *Sse*8387I (lane 4), *Sgr*AI (lane 5), and *Sma*I (lane 6) followed by CHEF electrophoresis (5-s to 50-s pulse for 22 hr followed by 70-s to 90-s pulse for 2 hr at 200 V). (B)CHEF electrophoresis of *Not*I (lane 1) and *Sfi*I (lane 2) digested DNA (10-s to 70-s pulse for 24 hr at 200V). (C)Comparison of *Not*I (lane 1) and undigested (lane 2) *L. interrogans* DNA (3-s to 30-s pulse for 24 hr at 200 V). Positions of size standards (in kb) are shown to the left.

Table 1. Genome size of *L. interrogans* as determined by *Not*I and *Sfi*I digestion.

*Not*I		*Not*I+*Sfi*I		*Sfi*I	
Band	Size(kb)	Band	Size(kb)	Band	Size(kb)
A	2100	A	1100	A[b]	1800
B	550	B	940	B[b]	1700
C	515	C	550	C	940
D	350	D	515	D	350
E	310	E	350		
F	230	F	310		
G	180	G	230		
H	155	H	180		
I	145	I	155		
J	120	J	145		
K	80	K	120		
		L	80		
		M	40		
		N[a]	20		
Total:	4735		4735		4790

a: Fragment identified by DNA hybridization.
b: A and B fragments poorly resolved.

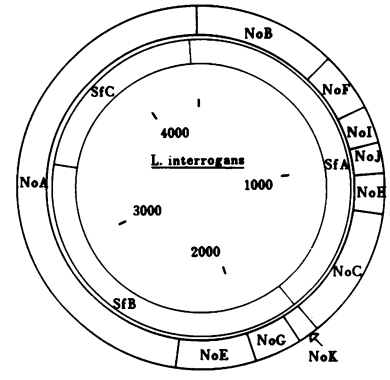

Figure 2. Physical map of the *L. interrogans* chromosome. Physical location of *Not*I (No) and *Sfi*I (Sf) restriction fragments are shown. Tick marks at 1000 kb intervals are shown inside the map.

C fragment were used to hybridize with *L. interrogans* DNA digested with *Sfi*I and either *Bss*HII, *Sgr*AI, or *Sse*I to localize this *Sfi*I site (data not shown).

The chromosomal *Not*I sites were mapped relative to each other using DNA hybridization analysis. Random cloned DNA fragments were screened for hybridization with specific *Not*I fragments. Probes hybridizing with each specific *Not*I fragment were used to identify *Apa*I, *Bss*HII, *Pac*I, *Sgr*AI, *Sma*I, or *Sse*8387I fragments which overlapped adjacent *Not*I sites. Since the *Sfi*I A and B fragments were difficult to resolve, these were not used to establish specific *Not*I linkages. Overlapping fragments (Table 2) were identified for each *Not*I site showing that the chromosome is circular.

Analysis of the *L. interrogans* plasmid

Several lines of evidence showed that *L. interrogans* contained a 350 kb plasmid, termed pLIN1, and that the *Not*I D fragment represented a linearized form of pLIN1. A cloned restriction fragment which hybridized specifically with sequences on the *Not*I D fragment (pLIN1) also hybridized with a 350 kb band in undigested genomic DNA (see below) and with 350 kb fragments generated with *Sfi*I or *Sse*8387I. Attempts to link this 350 kb fragment to other parts of the genome failed, including analysis of genomic DNA partially digested with *Not*I or *Sse*8387I and hybridized with the *Not*I D fragment (pLIN1) specific clone (Fig. 3). The *Not*I D fragment was gel purified, radiolabeled by

Table 2. Size of restriction fragments which span *Not*I sites in the *L. interrogans* genome.

*Not*I junction	Size (kb) and enzyme used to establish junction
A/B	740 kb *Sse*8387I
B/E	240 kb *Bss*HII
	740 kb *Sse*8387I
E/I	200 kb *Pac*I
I/J	45 kb *Bss*HII
J/H	290 kb *Sgr*AI
H/C	240 kb *Apa*I
C/K	175 kb *Bss*HII
	400 kb *Sgr*AI
K/G	175 kb *Bss*HII
G/E	170 kb *Sgr*AI
	175 kb *Sma*I
	155 kb *Sse*8387I
E/A	600 kb *Sgr*AI
	705 kb *Sse*8387I

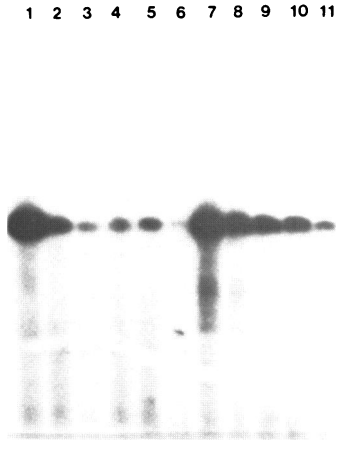

Figure 3. Autoradiogram of *L. interrogans* DNA partially digested with *Not*I-, or *Sse*8387I and hybridized with cloned DNA specific for pLIN1. Genomic *L. interrogans* DNA (25 μl) was digested with *Not*I (lanes 1–5) or *Sse*8387I (lanes 7–11) for 1 hr at 37°C followed by CHEF electrophoresis (as described in Fig. 1A). DNA was digested with 10 units enzyme (lanes 1 and 7), 2 units enzyme (lanes 2 and 8), 1 unit enzyme (lanes 3 and 9), 0.5 unit enzyme (lanes 4 and 10), or 0.25 unit enzyme (lanes 5 and 11). Undigested DNA is in lane 6.

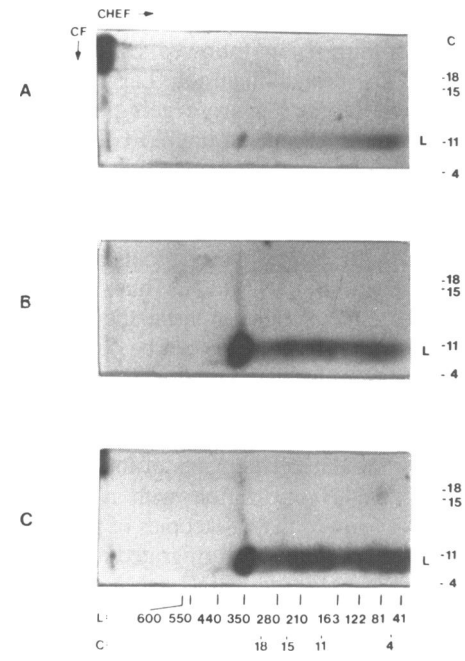

Figure 4. Two dimensional gel electrophoresis of *L. interrogans* DNA. Samples of undigested (A), *Not*I (B), and *Sse*8387I (C) digested DNA were separated by CHEF electrophoresis (as described in Fig. 1A) in the first dimension. The lanes were excised, cast in a new gel, and separated by constant field (CF) gel electrophoresis (50V for 24 hr) in the second dimension. The direction of DNA migration in the second dimension was perpendicular to the first dimension. DNA was transferred to nylon membranes and hybridized with a cloned fragment specific for sequences contained on pLIN1. The resulting autoradiogram is shown. The migration of linear (L) and circular (C) size standards (in kb) during CHEF is shown at the bottom. The migration of size standards during CF is shown on the right.

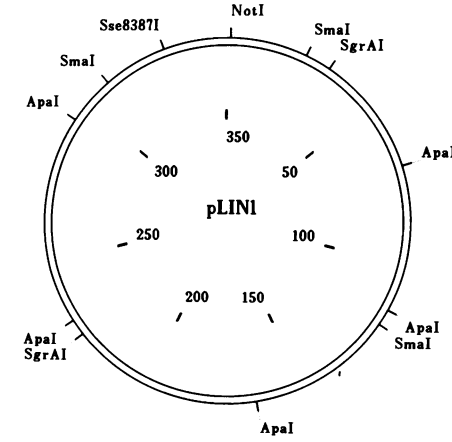

Figure 5. Physical map of pLIN1. Physical location of restriction sites for *Apa*I, *Not*I, *Sse*8387I, and *Sma*I on pLIN1 are shown. Tick marks at 50 kb are shown inside the map.

nick-translation, and hybridized with genomic DNA digested with *Apa*I, *Not*I, *Sse*8387I, *Sgr*AI, or *Sma*I. The sum total of hybridizing fragments equalled 350 kb (not shown). Based on these results it was concluded that the *Not*I D fragment comprised a plasmid, pLIN1. Since pLIN1 may be homologous to a previously described 350 kb *L. interrogans* 'linear' plasmid (9), experiments were done to establish its structure.

A two dimensional gel electrophoresis protocol was used to determine if pLIN1 was a circular or linear plasmid. This experiment used CHEF to separate pLIN1 from chromosomal DNA in the first dimension followed by constant field gel electrophoresis in the second dimension. Large open circular and supercoiled molecules enter agarose gels poorly and migrate slower than linear molecules under both PFGE and constant field strength conditions (14,15,16). The DNA was not treated with ethidium bromide or UV light until after the completion of electrophoresis through both dimensions. Hence, the rate of DNA migration under both electrophoretic conditions reflects the linearity or circularity the DNA (17). Thus, by comparing the mobility of pLIN1 to the mobility of linear and circular molecules in both dimensions, its structure should be obvious. In this two dimensional gel system linear (L) fragments > 12kb which were separated by CHEF in the first dimension migrated at the same rate in the second dimension (Fig. 4). Circular DNA molecules migrated slower than linear fragments of the same size in both dimensions (Fig. 4). Hybridization with a cloned fragment specific for pLIN1 showed that few copies of pLIN1 in a sample of undigested *L. interrogans* DNA migrated in either dimension (Fig. 4A). In this sample, the pLIN1 DNA which entered the gel migrated as linear molecules (Fig. 4A). In contrast, most copies of pLIN1 migrated as linear molecules when *L. interrogans* DNA was digested with either *Not*I or *Sse*8387I (which each cut pLIN1 once) (Fig. 4B and 4C). Lack of electrophoretic mobility of pLIN1 in both dimensions is consistant with a circular structure.

A physical map of pLIN1 (Fig. 5) was constructed by analysis of single and double enzyme digests of genomic DNA hybridized with gel purified pLIN1 DNA and with a cloned fragment specific for pLIN1. The unique *Not*I site is arbitrarily set as the first nucleotide. Quantitative scanning laser densitometry was used to determine the intensity of ethidium bromide fluorescence of pLIN1 as compared to similar sized *Not*I and *Sse*8385I fragments. Based on these results, pLIN1 is in 1−2 copies per genome.

DISCUSSION

This study reports the first physical map of any spirochete genome, and shows that *L. interrogans* contains a circular 4400 kb chromosome and a circular 350 kb plasmid, pLIN1. The size of the *L. interrogans* genome (4750 kb) determined in this report is in good agreement with an earlier estimate of 5000 kb (8), but it is significantly larger than another estimate of 3100 kb (9). The differences in size estimates for the *L. interrogans* genome may reflect differences between serovars, similar to differences in restriction endonuclease digestion patterns reported previously (7). Variation in size or organization between serovars may result from recombination between repetitive sequences in the genome (7). The size and structure of the *L. interrogans* chromosome reported here is similar to many other free-living bacteria. However, the *L. interrogans* chromosome is much larger than the chromosome of two other spirochetes: *Borrelia burgdorferi* (4,5) and *Treponema pallidum* (18), both of which have chromosomes approximately 1 Mb in length.

In *L. interrogans* the chromosome is circular, which is unlike *B. burgdorferi* which appears to contain a linear chromosome (4,5). Evidence for the circular structure of the *L. interrogans* chromosome is provided by identification of junction fragments which span each of the *Not*I sites in the chromosome. Also, the *L. interrogans* chromosome failed to enter agarose gels under conditions which separated *S. pombe* chromosomes of comparable size.

The *L. interrogans* plasmid, pLIN1, described in this report may be homologous to a 'linear' plasmid described previously (9). However, both the physical map and electrophoretic mobility of pLIN1 show that most copies of pLIN1 in intact genomic DNA are circular. Based on comparison of ethidium bromide fluorescence, pLIN1 has a copy number of 1 to 2 copies per chromosome. Although no genes have yet been located on pLIN1, its presence in *L. interrogans* may prove useful for studying genetic exchange among leptospires.

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