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Pulsed-Field Gel Electrophoretic Analysis of Leptospiral DNA

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The genomic structures of spirochete species are not well characterized, and genetic studies on these organisms have been hampered by lack of a genetic exchange mechanism in these bacteria. In view of these observations, pulsed-field gel electrophoresis was used to examine the genomes of *Leptospira* species. Live cells, prepared in agarose plugs, were lysed in situ, and the DNA was analyzed under different electrophoretic conditions. Pulsed-field gel electrophoresis of DNA digested with infrequently cutting restriction enzymes showed that the genome of *Leptospira interrogans* serovar canicola is approximately 3.1 Mb, while that of the saprophytic *L. biflexa* serovar patoc I is 3.5 Mb. DNA forms of approximately 2,000 and 350 kb which were present in samples from *L. interrogans* serovars were not readily detected in nonpathogenic serovars. Three distinct populations, designated type α, β, and γ, of *L. interrogans* DNA molecules were further analyzed with two-dimensional gel electrophoresis. Evidence suggested that two of these DNA forms, type α and γ, were linear structures. Pulsed-field gel electrophoresis has proven to be a valuable tool with which to size bacterial genomes and to take the first steps toward characterization of a form of leptospiral DNA which behaves as a linear molecule and which may be related to the virulence of *L. interrogans*.

Leptospirosis, caused by *Leptospira interrogans*, is an infection of domestic and wild animals. Humans can become infected via contact with contaminated water or infected animals (12, 13, 15). In spite of the worldwide distribution of leptospirosis and its significant economic impact (29), little is known about the pathogenesis of the disease or the molecular biology of these bacteria. The spirochetes, of which *Leptospira* species are members, occupy among eubacteria a unique position, exemplified by their unusual architecture and distinctive rRNA sequences (5, 10, 19). There is no known mechanism for genetic transfer among these bacteria. This lack has significantly hampered genetic studies and, therefore, our understanding of spirochete molecular biology.

Recently, however, several groups have utilized pulsed-field gel electrophoresis (PFGE) to conduct molecular analyses of various bacterial genomes (3, 7, 8, 16, 21, 25, 26, 28). This technique can be used to separate large DNA molecules or fragments up to approximately 10 Mbp in size (6, 24, 31). Restriction digestion by rarely cutting restriction endonucleases coupled with PFGE has allowed the characterization of whole bacterial genomes (18). This approach has been utilized to successfully construct physical maps of bacterial genomes (24, 25, 28) and has made possible the accurate calculation of chromosome size in a variety of organisms (7, 8, 16, 21).

In particular, Ferdows and Barbour (7) and Baril et al. (3) have recently employed PFGE to determine the size and physical configuration of the chromosome of another pathogenic spirochete, *Borrelia burgdorferi*. These studies indicated that *B. burgdorferi* possesses a chromosome with an estimated size of 950 kb, which behaves in PFGE as a linear molecule (3, 7). In this paper, we describe experiments performed to characterize the size and structure of the *L. interrogans* serovar canicola and *L. biflexa* serovar patoc I genomes. While members of *L. interrogans* are pathogens, members of *L. biflexa* are nonpathogenic, saprophytic organisms. Through the use of infrequently cutting endonucleases, the size of the *L. interrogans* genome was estimated to be 3.1 Mb whereas the *L. biflexa* genome was calculated to be 3.5 Mb. Analysis revealed three distinct populations of DNA molecules present in the genomes of virulent species of leptospires, while a single population of DNA was observed in nonpathogenic, saprophytic leptospires. Further experiments also provided preliminary evidence that two of the DNA species in the pathogenic *Leptospira* genome behave as linear molecules.

MATERIALS AND METHODS

Bacteria. Virulent *L. interrogans* serovars canicola, pomona, and grippotyphosa were obtained from the American Type Culture Collection, Rockville, Md., as was the saprophytic *L. biflexa* serovar patoc I. *L. interrogans* serovar icterohaemorrhagiae was kindly provided by Russell Johnson, University of Minnesota. *L. biflexa* serovars waz P-438, wa reiden, and semaranga were furnished by David Miller, National Veterinary Service Laboratories, and Nyles Charon, University of West Virginia, made available *L. biflexa* serovar Sao paulo. Organisms were grown at 32 to 34°C in PLM leptospira medium (Intergen, Kankakee, Ill.) and were used when the log phase of growth was reached. Pathogenic leptospires were used at passage 10 or lower and were hamster infective. *B. burgdorferi* HB19 (27) was cultured in BSK II medium, as previously described (1).

Preparation of DNA plugs. Agarose blocks containing leptospiral DNA were prepared by a modification of the method of Ferdows and Barbour (7). Spirochetes, at a density of 10^8 cells per ml, were collected by centrifugation at 17,000 × *g* for 20 min at 25°C, washed with 50 mM Tris containing 150 mM NaCl (TN) buffer, pH 8.0, and were resuspended in TN buffer at 37°C to a density of 2×10^{10} to 4×10^{10}/ml. An equal volume of molten 1% low-melting-point agarose (SeaPlaque GTG; FMC Bioproducts, Rockland, Maine) in TN buffer was added to the cell suspension, making the final agarose concentration in the plug equal to 0.5%. The agarose-leptospire suspension was poured into acrylic casting wells and was allowed to harden for 15 min at

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4°C. Solidified blocks (containing 5×10^9 to 1×10^{10} organisms) were immersed in a digestion solution of 50 mM Tris–50 mM EDTA–1% sodium dodecyl sulfate (SDS; pH 8.0)–2 mg of proteinase K (Boehringer Mannheim) per ml and incubated at 56°C for 24 h. Agarose blocks were stored at 4°C in 50 mM EDTA for up to 4 weeks.

PFGE. DNA plugs were washed three times with TE buffer and were loaded on 1% agarose (SeaKem; FMC) gels in 0.5× Tris–borate–EDTA buffer. Except where noted in the text, pulsed-field experiments were performed in a clamped homogeneous field (CHEF) electrophoresis apparatus (CHEF-DR II; Bio-Rad Laboratories, Richmond, Calif.) at 14°C with buffer recirculation. Unless otherwise noted, runs were performed on 1% agarose gels at a pulse time of 90 s for 17 h followed by a switch to a pulse time of 135 s for 9 h. A constant voltage of 175 V was maintained throughout each run. *Saccharomyces cerevisiae* chromosomes, *Schizosaccharomyces pombe* chromosomes, λ ladder (Bio-Rad), λ *Hin*dIII markers (Promega Corp., Madison, Wis.), and high-molecular-weight DNA markers (catalog number 5618SA; Bethesda Research Laboratories Life Technologies, Inc., Gaithersburg, Md.) were used as molecular weight standards. Gels were stained with ethidium bromide at a concentration of 200 ng/ml.

Nuclease treatments. Restriction enzymes were obtained from Promega. The DNA-containing plugs were equilibrated in TE buffer on ice for 30 min and then incubated in 1× enzyme buffer (Promega) on ice for 30 min. Next, the enzyme and bovine serum albumin (0.05 mg/ml) were added, and reaction mixtures were incubated for an additional 30 min on ice prior to digestion at the temperature specified by the manufacturer. Digestions were carried out for 16 h with 100 to 200 U of enzyme per ml. Plugs were also subjected to DNase and RNase treatments as follows. Plugs were equilibrated in TE buffer and 1× DNase buffer for 30 min on ice. Plugs to be treated with RNase were equilibrated in TE alone. DNase was used at final concentrations of 20 and 200 ng/ml. RNase digestions were carried out with final concentrations of 50 and 100 μg/ml. Plugs were digested for 2 h at 37°C prior to equilibration and electrophoresis.

Two-dimensional agarose gel electrophoresis. Analysis of leptospiral DNA by electrophoresis in two dimensions was performed in order to examine the structure of the migrating DNA forms. CHEF electrophoresis was run in the first dimension by using the parameters described above. For the second dimension, the gel was removed from the CHEF unit and placed in a constant-field horizontal submarine apparatus (Maxi-cell; Bio-Rad), with the anode and cathode perpendicular to the first dimension electrodes. Constant-field strength electrophoresis was then conducted at 100 V in 0.5× Tris–borate–EDTA buffer for various time intervals prior to staining with ethidium bromide.

RESULTS

CHEF analysis of leptospiral DNA. DNA from pathogenic and saprophytic leptospires was analyzed by CHEF PFGE. Leptospires were lysed in agarose plugs by incubation in buffer containing SDS and protease. The best resolution of molecules larger than 800 kb was obtained at a pulse time of 90 s for 17 h followed by a pulse time of 135 s for 9 h. Initial experiments determined that increasing the incubation time for protease digestion or treating the DNA plugs with additional detergent, proteinase K, or lysozyme did not cause more DNA to enter the gel. A low level of background degradation was observed in the DNA samples regardless of the plug protocol used. In an effort to reduce degradation, shorter digestion incubations were tested. However, such shorter incubations resulted in reduced resolution of the leptospiral bands. Therefore, a plug preparation protocol which gave maximal resolution of the bands while yielding a minimal amount of degradation was chosen. On the basis of ethidium bromide staining, approximately 50% of the total DNA loaded migrated into the gel, while the rest of the sample remained in the wells following PFGE. Ethidium-staining material remaining in the wells is a common occurrence in PFGE studies, and this material consists mainly of degraded DNAs caught up in cellular debris (23). Size estimates were made with *Saccharomyces* chromosomes and λ ladder standards (Bio-Rad). *B. burgdorferi* DNA samples were included in gel runs in order to compare the migration of *Leptospira* DNA to molecules which have been shown to behave as linear or supercoiled entities in PFGE (7). *L. interrogans* samples of the four different serovars tested contained three populations of large DNAs that migrated into the gel. These forms were designated α, β, and γ. Types α and β migrated as a doublet with an average apparent size of approximately 2,000 kb. Type γ migrated with an apparent size of about 350 kb (Fig. 1). The nonpathogenic *L. biflexa* samples each gave rise to only one major band, which migrated at approximately 2,500 kb. Occasionally, a diffuse, poorly stained band of about 300 kb appeared in *L. biflexa* samples; this finding, however, was not consistently reproducible. Only a small amount of sheared DNA, observed as a faint smear at the bottom of the gels, resulted from the plug preparation method used.

Samples of *L. interrogans* serovar canicola and *L. biflexa* serovar patoc I were also examined by using transverse-alternating-field electrophoresis (9). The results of this experiment gave rise to a migration pattern identical to that observed by using CHEF electrophoresis (data not shown).

In order to confirm that the bands observed under these conditions were in fact composed of DNA and were free of contaminating RNA, plugs were treated with RNase A or DNase I. Bands visualized in the CHEF gel with ethidium bromide were not significantly affected by treatment of the plugs with RNase A but were digested when treated with DNase 1 (Fig. 2). Ethidium-staining material remaining in the wells was also digested by DNase I, indicating that some form of DNA remained at the origin. Identical results were obtained when greater concentrations of DNase and RNase were used.

Estimation of leptospiral genome size. A total of 10 restriction endonucleases were used to fragment the total genomic DNA of both *L. interrogans* serovar canicola and *L. biflexa* serovar patoc I (Fig. 3 and Table 1). The restriction enzymes *Not*I, *Sma*I, *Apa*I, and *Sac*II gave the most consistent and reproducible restriction patterns for both species (Fig. 3 and Table 2), and these enzymes were subsequently used to size the genomes of these serovars. Restriction digests were run at several different parameters in order to resolve all restriction fragments. To ensure the best estimate of genome size, restriction fragments observed at various pulse parameters were monitored for changes in band intensity or number (presence of multiple fragments). Figure 3 represents the best average parameters to illustrate the range of restriction fragments generated by the enzymes used. On the basis of these restriction patterns, the genome size of serovar canicola was estimated to be 3.1 Mb and the size of serovar patoc I was found to be approximately 3.5 Mb.

L. interrogans and *L. biflexa* were also subjected to PFGE by using parameters specified by the manufacturers in order to resolve the chromosomes of *S. pombe*, which range in size from 3.5 to 5.7 Mb (30). The results showed that the

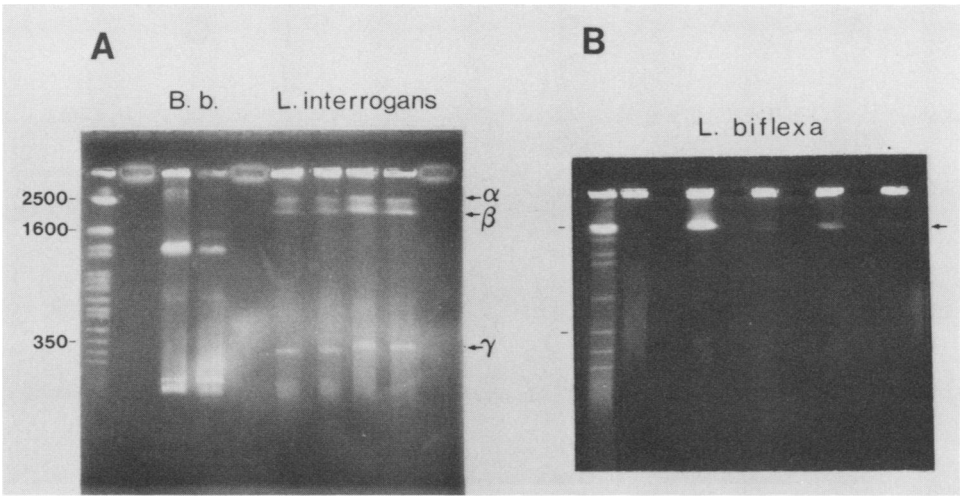

FIG. 1. CHEF electrophoresis of *Leptospira* DNA. Organisms lysed in agarose plugs were subjected to CHEF at 175 V with a 90-s pulse for 17 h followed by a 135-s pulse for 9 h. Linear duplex DNA standards were *S. cerevisiae* chromosomes (leftmost lanes [lanes 1]). (A) Lanes 6 to 9, *L. interrogans* serovars canicola, icterohaemorrhagiae, pomona, and grippotyphosa, respectively; lanes 3 and 4, samples of *B. burgdorferi* HB 19 run as controls. (Lanes 2, 5, and 10 were not loaded with sample plugs and were empty.) (B) Lanes 2, 4, 6, 8, and 10, *L. biflexa* serovars Sao paulo, patoc I, semaranga, waz P-438, and wa reiden, respectively. (Lanes 3, 5, 7, and 9 were not loaded with sample plugs and were empty.) Arrows indicate the migration of type α, β, and γ DNA in *L. interrogans* samples. Numbers on the left indicate sizes in kilobases of some of the *S. cerevisiae* chromosomes seen in lanes 1.

differences in migration between the two species were maintained at these parameters. Figure 4 shows that *L. biflexa* DNA migrated at approximately 3.5 Mb, which is consistent with the size estimate obtained from restriction analysis. Though somewhat compressed, a doublet was still maintained in the *L. interrogans* samples, migrating with an average size of 2.0 Mb. This observation is consistent with the migration of *L. interrogans* DNA at standard parameters.

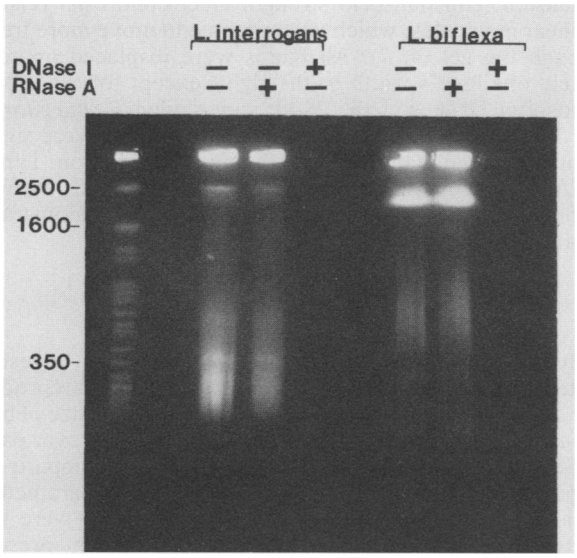

FIG. 2. CHEF electrophoresis of *L. interrogans* serovar canicola and *L. biflexa* serovar patoc I following nuclease treatment. Agarose plugs containing *L. interrogans* serovar canicola or *L. biflexa* serovar patoc I were incubated in 1× DNase buffer solution containing DNase I (20 ng/ml) or in TE buffer containing RNase A (50 μg/ml). Numbers on the left indicate the sizes in kilobases of some of the *S. cerevisiae* chromosomes seen in the leftmost lane (lane 1). *L. interrogans* DNA was undigested, treated with RNase A, or treated with DNase 1 (lanes 3 to 5, respectively). DNA from *L. biflexa* was undigested, treated with RNase A, or treated with DNase 1 (lanes 7 to 9, respectively). Lanes 2, 6, and 10 were not loaded with sample plugs and were empty.

Structure of leptospiral DNA. Because both linear molecules and circular plasmids have been described in *Borrelia* species (2, 7), determination of the structure of the bands present in leptospiral DNA preparations was of interest. To establish whether the bands resulted from random shearing of the chromosome or compression of an unresolved population of DNA species, DNA plugs were subjected to CHEF electrophoresis (pulse time of 30 s for 14 h followed by a pulse time of 45 s for 7 h). Then, following staining of the gel with ethidium bromide and treatment with UV light, constant-field-strength electrophoresis (100 V for 1.5 h) was performed (Fig. 5). The parameters used in this gel were not sufficient to resolve the leptospiral α and β doublet well, and the high-molecular-weight leptospiral and borrelial molecules appeared to be the same size (approximately 1,000 kb). Upon expanding these parameters (pulse time of 90 s for 17 h followed by pulse time of 135 s for 9 h), it was apparent that this area had been compressed by using the initial pulse parameters, since under optimal conditions the megabase-sized borrelial molecule migrated at a lower molecular weight than the leptospiral doublet. If any DNA band represented a compression of heterogeneous fragments, high-voltage constant-field electrophoresis following ethidium bromide staining of the gel would bring about the separation of such fragments. The leptospire bands did not change in appearance following the constant field run, indicating that these bands probably represent a homogeneous population of molecules (Fig. 5). Following ethidium bromide staining and exposure to UV light, however, two additional bands appeared below the supercoiled plasmid of *B. burgdorferi*. These bands are, most likely, nicked and linearized forms of the plasmid.

Supercoiled or circular molecules are more susceptible to intercalation of ethidium bromide and subsequent nicking by UV light than are linear species (4, 7). Following this

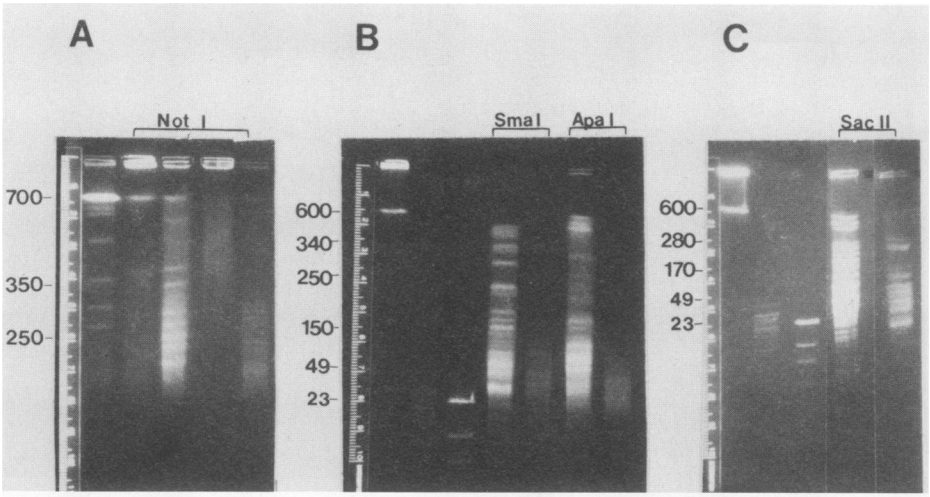

FIG. 3. Restriction enzyme fragments generated from *L. interrogans* serovar canicola and *L. biflexa* serovar patoc total DNA and separated with CHEF electrophoresis. (A) *S. cerevisiae* standards (leftmost lane [lane 1]), agarose plugs containing undigested canicola and patoc DNAs (lanes 2 and 4, respectively), and DNA digested with *Not*I in the same order (lanes 3 and 5). (B) Lambda ladder DNA, high-molecular-weight size standards, and λ *Hin*dIII markers (lanes 1 to 3, respectively), canicola and patoc DNA plugs digested with *Sma*I (lanes 4 and 5, respectively), and *Apa*I digests of the same serovars (lanes 6 and 7, respectively). (C) Standards described for panel B (lanes 1 to 3, respectively) and results of digesting canicola and patoc DNA plugs with *Sac*II (lanes 4 and 5, respectively). Numbers to the left indicate sizes in kilobases of some of the size standards.

treatment, the mobility of supercoiled molecules tends to be increased. We tested whether the migration of any of the leptospiral DNA forms would be altered by such treatment. CHEF electrophoresis was run at the standard parameters, stained with 200 ng of ethidium bromide per ml, and treated with UV light for 5 min. When PFGE was continued for 10 additional h at the same parameters, results indicated that the mobility of *L. interrogans* type α and γ DNA, as well as the mobility of the linear forms of *B. burgdorferi* DNA, was not affected by this treatment (data not shown). This relative insensitivity to ethidium bromide-UV treatment suggests that type α and γ DNAs are linear. The calculated relative mobility (total migration distance of the band from the wells, as compared with linear standards) of type β DNA, however, was increased from approximately 0.6 to 0.8. In addition, two new bands, which may be nicked or linearized forms of a circular or supercoiled molecule, appeared directly beneath the β band (data not shown). Changes in mobility and the generation of additional bands under these conditions are characteristic of nonlinear molecules (4).

Two-dimensional electrophoresis was performed in order to further investigate the structure of the *Leptospira* DNA molecules. It has been shown that the migration of supercoiled or open circular molecules is retarded in the second dimension (constant-field strength electrophoresis) relative to linear molecules, which will continue to move more freely through the gel (4, 7). All bands were displaced approximately one lane's width to the right, except for the type β leptospiral DNA and the 28-kb supercoiled *B. burgdorferi* HB 19 plasmid (Fig. 6). These two DNA forms were significantly retarded in their second dimension migration. Type β DNA exhibited a migration pattern which is consistent with a nonlinear species, while type α and γ DNAs demonstrated migrations characteristic of linear molecules.

TABLE 1. Restriction fragment analysis of *L. interrogans* serovar canicola and *L. biflexa* serovar patoc I[a]

Restriction endonuclease	No. of fragments detected	
	L. interrogans	*L. biflexa*
*Not*I	10	12
*Sma*I	22	24
*Apa*I	20	23
*Sac*II	22	19
*Xba*I	>30	>30
*Kpn*I	17	NR[b]
*Bgl*I	18	NR
*Sal*I	NR	15
*Mlu*I	27	16
*Pvu*I	NR	>30

[a] DNA-containing plugs were equilibrated in the appropriate restriction buffers and then digested according to the manufacturer's instructions with the indicated enzymes for 16 h. Plugs were then subjected to CHEF electrophoresis at pulse parameters sufficient to separate all fragments generated.

[b] NR, Not resolved.

DISCUSSION

By using a panel of restriction endonucleases, digestion patterns were obtained which were consistent and reproducible and which were used to estimate the genome size of both *L. interrogans* serovar canicola and *L. biflexa* serovar patoc I. Several enzymes were used in order to compare the migration patterns obtained at different pulse parameters. Bands which showed particularly high intensity were suspected of having more than one size of fragment present. Pulse parameters were then adjusted, and changes in band intensity or the appearance of new bands was monitored. By using this protocol, we were able to obtain consistent and reproducible results, indicating that the genome size of *L. interrogans* serotype canicola was 3.1 Mb and that of *L. biflexa* serotype patoc was approximately 3.5 Mb.

To provide supporting evidence for the sizes obtained with restriction endonuclease digestions, PFGE experiments were performed by using parameters designed to separate the chromosomes of *S. pombe*, which range in size from 3.0 to 5.7 Mb (31). Larger molecules should resolve better under

TABLE 2. Leptospiral genome size estimates derived from restriction endonuclease digestions[a]

Mean fragment size ± SD (kb) derived with:					
*Not*I		*Sma*I		*Apa*I	
L. interrogans	*L. biflexa*	*L. interrogans*	*L. biflexa*	*L. interrogans*	*L. biflexa*
72 ± 3	57 ± 5	24 ± 1	10 ± 1	22 ± 2	12 ± 1
144 ± 4	105 ± 10	49 ± 1	11 ± 1	27 ± 4	17 ± 2
182 ± 9	122 ± 4	54 ± 2	12 ± 1	31 ± 5	19 ± 1
224 ± 14	136 ± 8	63 ± 4	15 ± 1	45 ± 4	24 ± 3
271 ± 1	170 ± 2	68 ± 4	19 ± 2	53 ± 2	37 ± 4
292 ± 2	218 ± 11	70 ± 4	24 ± 2	62 ± 2	45 ± 2
337 ± 5	250 ± 9	74 ± 5	29 ± 2	64 ± 4	47 ± 1
386 ± 12	287 ± 4	83 ± 4	33 ± 3	68 ± 5	50 ± 1
535 ± 6	436 ± 6	94 ± 1	38 ± 2	79 ± 6	54 ± 2
717 ± 36	494 ± 11	98 ± 1	49 ± 1	94 ± 7	57 ± 3
	595 ± 14	101 ± 1	65 ± 4	99 ± 7	63 ± 3
	646 ± 19	121 ± 5	75 ± 4	172 ± 9	85 ± 5
		127 ± 7	95 ± 11	194 ± 7	106 ± 8
		189 ± 6	132 ± 6	231 ± 4	141 ± 11
		202 ± 4	147 ± 3	240 ± 2	175 ± 5
		205 ± 4	171 ± 4	247 ± 2	186 ± 2
		213 ± 3	187 ± 7	257 ± 6	196 ± 3
		218 ± 3	217 ± 10	340 ± 7	223 ± 13
		223 ± 6	246 ± 9	355 ± 20	289 ± 8
		258 ± 4	295 ± 14	378 ± 21	347 ± 8
		302 ± 11	339 ± 14		373 ± 5
		312 ± 13	370 ± 11		401 ± 20
			405 ± 6		587 ± 6
			535 ± 7		

[a] Values represent fragment sizes (kilobases) obtained from *L. interrogans* and *L. biflexa* genomic DNA plug samples digested with the restriction endonucleases *Not*I, *Sma*I, and *Apa*I. Fragments sizes are expressed as the average of three separate digestions ± the standard deviation. Estimated genome sizes (kilobases) for *L. interrogans* and *L. biflexa* were determined by the sum of fragments generated from each restriction enzyme as follows: 3,121 ± 67 (*L. interrogans*; *Not*I); 3,516 ± 71, (*L. biflexa*; *Not*I); 3,146 ± 70 (*L. interrogans*; *Sma*I); 3,519 ± 111 (*L. biflexa*; *Sma*I); 3,058 ± 106 (*L. interrogans*; *Apa*I); and 3,534 ± 88 (*L. biflexa*; *Apa*I).

these conditions. Thus, for leptospiral DNA species which might be compressed at the top of the gel during standard runs (90-s pulse for 17 h followed by a pulse of 135 s for 9 h), greater resolution would be expected. In addition, another comparison of the migration of native leptospiral molecules relative to larger linear standards could be made by this method. The results (Fig. 4) showed that under these conditions, as well as with our standard parameters, differences in migration between *L. interrogans* and *L. biflexa* DNA forms do exist. The major population of *L. biflexa* DNA migrated at approximately 3.5 Mb, which was consistent with the size estimates obtained from restriction analysis.

Three major DNA bands, designated α, β, and γ, were observed in *L. interrogans* serovars. Type α and β composed a doublet with an apparent molecular size of about 2,000 kb, while type γ migrated at approximately 350 kb. In contrast, only one major band was observed in *L. biflexa* samples. The apparent size of this band was approximately 2,500 kb. The results of transverse-alternating-field electrophoresis experiments also confirmed the presence of these forms and their apparent sizes with respect to linear standards.

Two-dimensional electrophoresis was performed in order to further investigate the possibility that some leptospiral bands were of linear configuration. It has been shown that linear molecules will continue to move through the gel in the second dimension (constant field strength), while migration of supercoiled or circular molecules is retarded (4, 7). Only the supercoiled *B. burgdorferi* HB 19 plasmid and the leptospiral type β DNA were retarded in the second dimension (Fig. 6). These data, along with the data from the ethidium bromide-UV migration studies (Fig. 5), suggest that

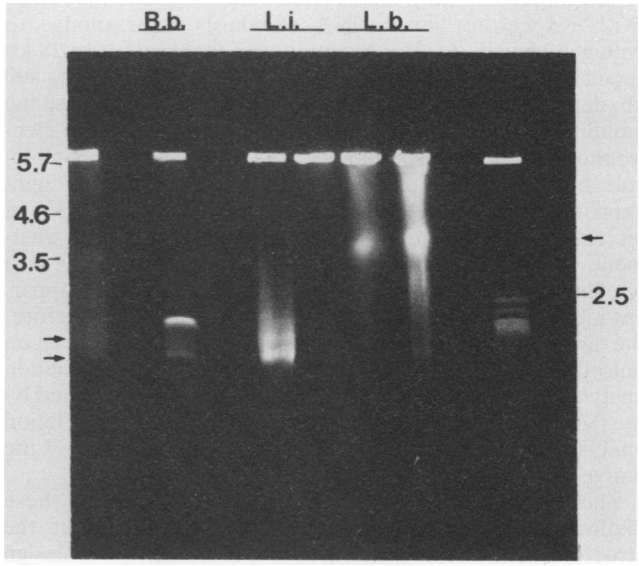

FIG. 4. CHEF electrophoresis of *L. interrogans* serovar canicola and *L. biflexa* serovar patoc I at high pulse intervals. Serovar canicola and serovar patoc I DNAs were subjected to PFGE at a pulse of 25 min for 48 h followed by a pulse of 35 min for 24 h. Migration of *S. pombe* and *S. cerevisiae* chromosome standards are indicated in the leftmost (lane 1) and rightmost (lane 10) lanes, respectively. Arrows indicate the migration of the doublet in canicola (lanes 5 and 6) and of the major band found in patoc I (lanes 7 and 8). *B. burgdorferi* was run in lane 3 for comparison. Lanes 2, 4, and 9 were not loaded with sample plugs and were empty. Numbers in the left and right margins indicate sizes in megabases of some *Saccharomyces* chromosomes.

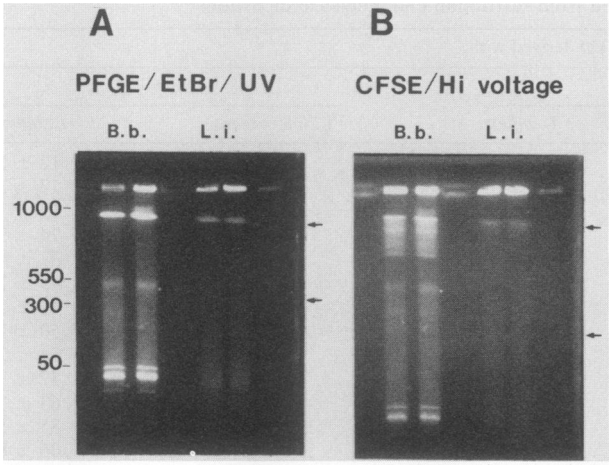

FIG. 5. Constant-field-strength electrophoretic migration of *L. interrogans* serovar canicola and *L. biflexa* serovar patoc I DNA molecules subjected first to CHEF electrophoresis. (A) CHEF was carried out for 21 h. Arrows indicate the positions of *L. interrogans* doublet DNA (types α and β) and γ DNA following CHEF electrophoresis, staining with ethidium bromide, and exposure to UV light (lanes 5 and 6). Lane 7 contains *L. biflexa* DNA. Lanes 2 and 3 contain *B. burgdorferi* DNA. (B) Next, the same gel was subjected to constant field electrophoresis for 1.5 h. Arrows indicate the migration distances of the *L. interrogans* doublet and type γ DNA bands following constant-field-strength electrophoresis. Numbers on the left indicate sizes in kilobases of some *S. cerevisiae* chromosomes.

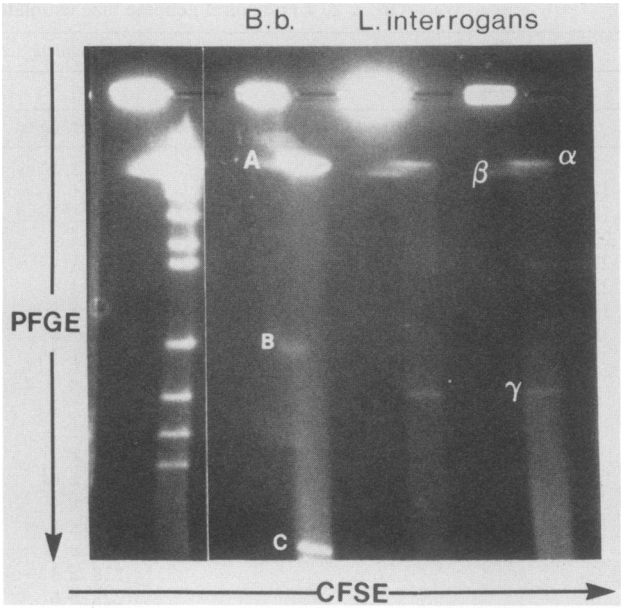

FIG. 6. Two-dimensional electrophoresis of *L. interrogans* DNA. *S. cerevisiae* chromosomes (lane 1), *B. burgdorferi* DNA (lane 3), and *L. interrogans* serovar canicola (lanes 5 and 7) were subjected to CHEF electrophoresis in the first dimension and constant field electrophoresis in the second dimension (large arrows). Migrations of α, β, and γ DNAs are indicated. A, B, and C indicate the migration of *B. burgdorferi* chromosome, supercoiled plasmid, and linear plasmids, respectively.

the type α and γ DNAs behave as linear molecules, while type β DNA may differ in shape or conformation. Supercoiled molecules may display anomalous PFGE mobilities (with respect to linear DNA standards) in response to voltage changes (6). For example, the *B. burgdorferi* 28-kb supercoiled plasmid migrates at a point between 500 and 800 kb, depending on the pulse parameters used (7). Because the mobility of the type β DNA varied depending on the electrophoretic conditions and because new forms of this molecule became visible after ethidium bromide and UV light exposure, type β DNA may represent circular or supercoiled molecules which become nicked or linearized by UV treatment of stained DNA. Type β DNA may represent a population of supercoiled molecules, migrating at an apparent molecular weight higher than its true weight. Therefore, the discrepancy between the apparent sizes of bands seen on pulsed field gels and the genome size obtained by restriction analysis can be resolved. This interpretation is supported by the fact that the genome size estimate obtained by restriction analysis is smaller than the sum of the apparent sizes of the native bands observed on PFGE.

These results raise questions as to the origin of these distinct populations. Because little is known about the structure of the leptospiral genome, it is difficult to assign chromosomal or extrachromosomal origins to the bands consistently observed in our experiments. On the basis of the findings reported for *B. burgdorferi*, it is possible that the relatively small γ DNA molecule represents a linear plasmid whereas type α and β may represent two distinct megabase-sized chromosomes which possess different structural conformations. The presence of two unique chromosomes has been reported for the bacterium *Rhodobacter sphaeroides* (27). Indeed, there is increasing evidence that a procaryotic genome may consist of a group of molecules, varying in size, which together comprise the chromosomal information. The concept of multiple chromosomes or minichromosomes has also been reported for yeast (17, 20), protozoan (22), and bacterial (7, 11) species. In particular, Ferdows and Barbour (7) suggested that the megabase-sized linear molecule and several smaller linear plasmids observed in *B. burgdorferi* might represent minichromosomes, and Hayes et al. (11) proposed a segmented genome for *Borrelia duttonii*. The molecules observed in *Leptospira* samples may be a further example of this type of genomic diversity.

It is also possible that the lower bands arise from specific excision events dependent on particular sequences found in the *Leptospira* chromosome or that all three bands are extrachromosomal, since it appears that the type α and γ bands behave as linear molecules while the type β DNA of the doublet does not. The possibility that all three populations of molecules arise from a circular chromosome which has been sheared by the electrophoretic manipulations has been considered. Therefore, the material which remains in the well during PFGE runs may represent intact chromosomal molecules. This hypothesis, however, seems unlikely in view of the fact that (i) these bands are consistently found in multiple *L. interrogans* serovars, (ii) the migration of these bands has remained constant despite a large variation in pulse parameters, and (iii) the β-type DNA behaves as a nonlinear molecule. We do not understand the relationship of these DNA bands to each other or to the chromosomes of the leptospires examined. Further experiments will be needed to understand these relationships.

Because type γ DNA behaves as a linear molecule and is not observed in samples from nonpathogens, it is possible that this molecule is an extrachromosomal element that is unique to pathogenic *Leptospira* serovars. A 49-kb linear plasmid in *B. burgdorferi* strains encodes the major outer membrane proteins Osp A and Osp B (2). These proteins

have been implicated in adherence of the organisms to host tissues (30). Large linear plasmids carrying genes for antibiotic synthesis have been observed in several *Streptomyces* species (14, 22). These plasmids, ranging in size from 300 to 500 kb, were not isolated until the advent of PFGE technology (14). These giant linear plasmids (22) have also been referred to as minichromosomes because of their size and potential for genetic expression and diversity. The type γ DNA observed in *L. interrogans* species may be a representation of this class of molecule.

On the basis of findings for other pathogenic bacteria, it is possible that virulence-related genes are carried on plasmids in leptospires as well. Further investigations are necessary to elucidate the exact nature of these three distinct DNA forms. Because we do not fully understand the origin of these bands or their relationships to each other, hybridization studies are under way to address these questions. More extensive studies are being directed at determining whether type γ DNA may represent a linear plasmid which plays a role in the virulence of *L. interrogans*.

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ADDENDUM IN PROOF

Since this paper was submitted, another group has also reported sizing the *L. interrogans* and *L. biflexa* genomes by pulsed-field electrophoresis (C. Baril and I. Saint-Girons, FEMS Microbiol. Lett. **71**:95, 1990).

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