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Comparison of Genetic Maps for Two *Leptospira interrogans* Serovars Provides Evidence for Two Chromosomes and Intraspecies Heterogeneity

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Genetic maps were constructed for *Leptospira interrogans* serovars icterohaemorrhagiae and pomona. Previously we independently constructed physical maps of the genomes for these two serovars. The genomes of both serovars consist of a large replicon (4.4 to 4.6 Mb) and a small replicon (350 kb). Genes were localized on the physical maps by using Southern blot analysis with specific probes. Among the probes used were genes encoding a variety of essential enzymes and genes usually found near bacterial chromosomal replication origins. Most of the essential genes are on the larger replicon of each serovar. However, the smaller replicons of both serovars contain the *asd* gene. The *asd* gene encodes aspartate β-semialdehyde dehydrogenase, an enzyme essential in amino acid and cell wall biosyntheses. The finding that both *L. interrogans* replicons contain essential genes suggests that both replicons are chromosomes. Comparison of the genetic maps of the larger replicons of the two serovars showed evidence of large rearrangements. These data show that there is considerable intraspecies heterogeneity in *L. interrogans*.

Leptospirosis is an anthropozoonose, a disease common among most animal species, including humans. Leptospirosis is caused by pathogenic members of the genus *Leptospira*. Until recently, all pathogenic leptospires were classified within the same species, *Leptospira interrogans* (sensu lato). Recent DNA homology studies have resulted in reclassification of *L. interrogans* with the formation of seven species: *L. borgpetersenii*, *L. inadai*, *L. interrogans* (sensu stricto), *L. kirschnerii*, *L. noguchii*, *L. santarosai*, and *L. weilii* (34, 44). Because of extensive antigenic heterogeneity, each species is further divided according to antigenic composition into serogroups and serovars. Some serovars occur in more than one of the newly formed species (e.g., serovar hardjo is found in *L. interrogans* [sensu stricto] and *L. borgpetersenii* [34]). Comparison of different serovars shows there is considerable genetic heterogeneity within each of the newly formed species. With few exceptions, each serovar has a unique restriction endonuclease digestion pattern, as shown by continuous-field gel electrophoresis (40, 47) or by pulsed-field gel electrophoresis (PFGE) (18, 19). In spite of genetic diversity between pathogenic leptospires, the clinical signs of leptospirosis are similar.

Leptospirosis manifests itself in one of two typical ways: a chronic infection with little or no clinical symptoms in the normal maintenance host or an acute infection of an incidental host, often with obvious clinical symptoms (17). Little is known about the molecular basis of disease caused by *L. interrogans* (sensu lato). Assessment of the pathogenic potential of *L. interrogans* (sensu lato) is complicated by lack of any known mechanism for genetic exchange among leptospires. Thus, classical genetic techniques which have been useful in understanding pathogenic mechanisms in other bacterial genera have not been applied to leptospires. The genetics of *Leptospira* spp. and of other spirochetes are of particular interest since these bacteria occupy one of the deepest branches in eubacterial evolution (30).

PFGE is a powerful tool for characterizing spirochete genomes, which have several unusual features (36). Spirochete genomes vary in size and structural organization. Among spirochetes are one of the smallest circular bacterial chromosomes reported (42), a small linear chromosome (2, 9, 12), linear plasmids (32), and significant levels of repetitive DNA (29, 37, 41, 43, 49). Recently our two laboratories independently developed physical maps of two *L. interrogans* (sensu stricto) serovars (icterohaemorrhagiae and pomona) (1, 45). The genomes of these two serovars consist of a large replicon (4.4 to 4.6 Mb) and a small replicon (350 kb). Both replicons are circular (45). Although the physical maps of these two genomes are different, we expected that their genetic organizations would be similar. However, our initial results showed that rRNA gene organizations in these two serovars were different.

We undertook this study to develop a better understanding of the structural and function relationships of both replicons and to begin assessing the level of genetic diversity within *L. interrogans* (sensu stricto). DNA homology experiments show that these two serovars are genetically similar (34, 44). However, these serovars have distinct antigenic profiles and are maintained in different animal host species (17). We are interested in identifying and characterizing genetic loci encoding virulence determinants, including those affecting host species adaptation. We are using differences in genetic organization between these two serovars as a starting point for understanding the basis and extent of intraspecies diversity in *L. interrogans* (sensu stricto).

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TABLE 1. Gene probes used for this study

Gene	Functions	Species of origin	Reference(s)
aroD	3-Dehydroquinate dehydratase	*L. interrogans*	3
asd	Aspartate β-semialdehyde dehydrogenase	*L. interrogans*	3
leuB	β-Isopropyl malate dehydrogenase	*L. interrogans*	10, 35
proAB	γ-Glutamyl phosphate reductase and γ-glutamyl kinase	*L. interrogans*	35
rrf[a]	5S rRNA	*L. interrogans*	14
rrs[a]	16S rRNA	*L. interrogans*	15
rrl[a]	23S rRNA	*L. interrogans*	13
rpoC	RNA polymerase β′ subunit	*L. biflexa*	50
rpsLG	Ribosomal protein	*L. biflexa*	51
fus	EF-G	*L. biflexa*	47
fus tuf	EF-G and EF-Tu	*T. thermophilus*	25
dnaA	Replication protein	*E. coli*	27
gidA	Glucose-inhibited division protein	*B. burgdorferi*	28
gyrBA	DNA gyrase	*B. burgdorferi*	28
gyrA	DNA gyrase	*L. interrogans*	20
IS*1533*	IS-like element	*L. borgpetersenii*	49
IS	IS-like element	*L. interrogans*	35a

[a] The probe was prepared by PCR amplification (see Materials and Methods).

MATERIALS AND METHODS

DNA preparation and gel electrophoresis. *L. interrogans* serovar icterohaemorrhagiae strain Verdun and serovar pomona type kennewicki strain RZ11 were described previously (1, 45). Bacteria were grown in EMJH medium at 30°C (11, 22). Exponential-phase cells were mixed with molten low-melting-temperature agarose, which was allowed to solidify in plugs or beads as described previously (1, 45). Except when noted in the text, PFGE experiments were done as described previously (1, 45). Most PFGE experiments used either a Pharmacia-LKB apparatus (Uppsala, Sweden) or a contour-clamped homogeneous-field apparatus (CHEF DR-II; Bio-Rad Laboratories, Richmond, Calif.). Restriction enzymes were used as recommended by the suppliers.

DNA blot analysis. DNA was fragmented in the gel by UV irradiation or by depurination and then transferred to nylon membranes as described previously (1, 45). Probes were prepared as described previously (1, 45) with [α-^{32}P]dATP (ICN Pharmaceuticals, Irvine, Calif.) by nick translation using a commercially available kit (GIBCO-BRL, Gaithersburg, Md.), with [α-^{32}P]UTP (ICN Pharmaceuticals) by runoff transcription with phage T3 or T7 RNA polymerase, or with digoxigenin-11-dUTP by random hexanucleotide priming using a commercially available kit (Boehringer Mannheim). DNA hybridization analysis was done as described previously (1, 45). Probes used in this study are listed in Table 1. Hybridizations using homologous probes were done at 56 to 65°C, and blots were washed with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 sodium citrate–0.1% sodium dodecyl sulfate [SDS]) at the hybridization temperature. Alternatively, blots were washed with 2× SSC–0.1% SDS and then with 0.1× to 0.2× SSC–0.1% SDS at the hybridization temperature. Hybridizations with heterologous probes at 50°C were followed by washing with either 4× SSC–0.1% SDS or 6× SSC–0.1% SDS at 50°C. Alternatively, blots hybridized with heterologous probes were washed with 2× SSC–0.1% SDS at room temperature. Blots were either exposed directly to film (for ^{32}P-labeled probes) or first reacted with antidigoxigenin antibody conjugated with alkaline phosphatase and then subjected to chemiluminescent or colorimetric reactions.

PCR. Polymerase chain reactions (PCR) were used to prepare probes for the *L. interrogans* 5S, 16S, and 23S rRNA genes. Standard reaction mixtures were prepared as recommended by the manufacturer (Perkin-Elmer, Norwalk, Conn.). The right and left PCR primer sets used for rRNA gene amplification were 5S (5′-ATGCAGCGAACCACAT AGTA-3′ and 5′-CAACGGGAGAAGATTGAATC-3′) (amplifying between nucleotides 110 and 300 [14]), 16S (5′-ACCATGCAGCACCTGTGAAG-3′ and 5′-TGCAAGTCA AGCGGAGTAGC-3′) (amplifying between nucleotides 56 and 1025 [15]), and 23S (5′-GAGAGAACCAGCTATCGC CT-3′ and 5′-TGTTGTAGGACTGGCAGGAG-3′) (amplifying between nucleotides 296 and 884 [13]). Amplification conditions were 94°C for 1 min, 50°C for 1 min, and 72°C for 3 min (increased by 10 s per cycle after six cycles) for 25 cycles in a Perkin-Elmer thermal cycler. Unincorporated nucleotides were removed by precipitation with ethanol, and the products were labeled as described above.

RESULTS

Comparison of physical maps of the serovar icterohaemorrhagiae and serovar pomona genomes. Previously, we independently defined the physical structures for the genomes of *L. interrogans* (sensu stricto) serovars icterohaemorrhagiae (1) and pomona (45). There are two circular replicons in each genome, 4.4 to 4.6 Mb and 350 kb in size. However, the physical locations of restriction sites in these two genomes are different. In this study, the resolution of the serovar pomona map was improved by including the positions for several of the largest fragments generated with *Asc*I, *Srf*I, *Sgr*AI, and *Sse*8387I (Fig. 1).

Localization of rRNA genes. rRNA gene organization in *L. interrogans* (sensu lato) is unusual (1, 16). The *rrf*, *rrl*, and *rrs* genes, which encode the 5S, 23S, and 16S rRNAs, respectively, are not transcriptionally linked as they are in most other bacteria (16). The positions of these genes in the serovar icterohaemorrhagiae genome are known (1). As a starting point for comparing genetic organization between serovars icterohaemorrhagiae and pomona, we localized these genes in the serovar pomona genome. Hybridization with specific probes showed there is one *rrf* locus and two loci each for *rrl* (*rrlA* and *rrlB*) and *rrs* (*rrsA* and *rrsB*). All rRNA genes are located on the large serovar pomona replicon (Fig. 1). The positions of the *rrl* and *rrs* loci on the large

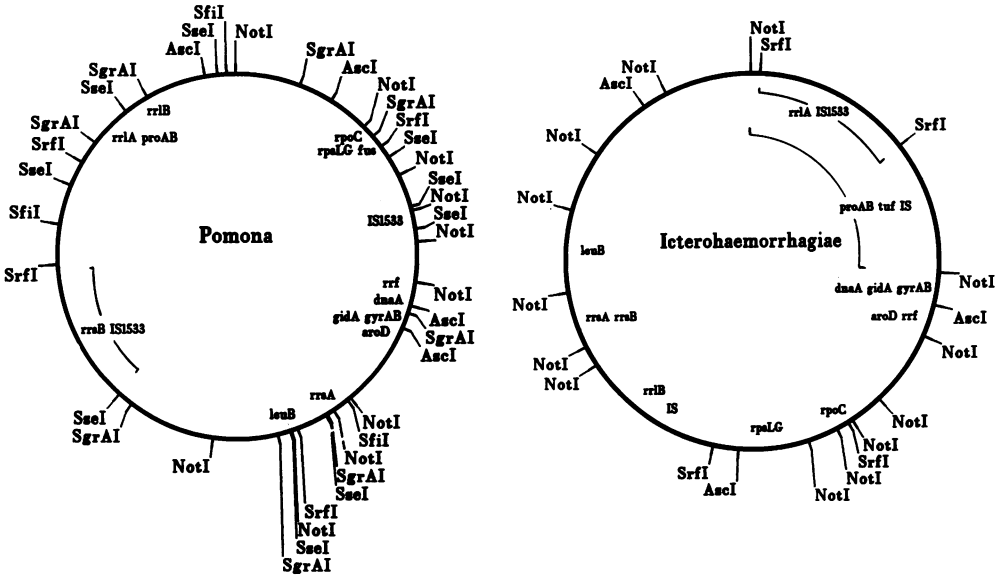

FIG. 1. Physical and genetic maps of *L. interrogans* serovars icterohaemorrhagiae and pomona. The physical maps are oriented as published previously (1, 45). Starting at the top, the order of *Not*I fragments in the large serovar icterohaemorrhagiae replicon is NtA-NtF2-NtE-NtH1-NtJ-NtH2-NtB-NtI-NtG-NtD-NtF1-NtC2-NtC1 (1). The order of *Not*I fragments in the large serovar pomona replicon is NtB-NtF-NtI-NtJ-NtH-NtC-NtK-NtG-NtE-NtA (45). Locations of genes on the physical maps are shown. Approximate gene placements are indicated by lines spanning the areas in which they are located.

replicons of serovars icterohaemorrhagiae and pomona are different. In serovar icterohaemorrhagiae, *rrsA* and *rrsB* are located on the same 85-kb *Apa*I fragment of the *Not*I E (NtE) fragment (data not shown), while *rrlA* and *rrlB* are on opposite sides of the large replicon (Fig. 1). In serovar *pomona*, *rrsA* and *rrsB* are found on different parts of the large replicon, while *rrlA* and *rrlB* are located on the same 366-kb *Sgr*AI fragment within the NtA and *Sfi*I C fragments (Fig. 1).

Chromosomal replication genes. In most bacteria, the *gidA*, *gyrBA*, and *dnaA* genes are located near the chromosomal replication origin, *oriC* (23, 26, 38). Using probes to the *Borrelia burgdorferi* 212 *gyrBA* and *gidA* genes (28), the *L. interrogans gyrA* gene (20), and the *Escherichia coli dnaA* gene (27), we localized the corresponding genes in serovars icterohaemorrhagiae and pomona. These three genes are found together on the large replicons of both serovars. In serovar icterohaemorrhagiae, the *gyrBA*, *gidA*, and *dnaA* genes are on a 160-kb *Asc*I-*Not*I fragment within NtF2 (Fig. 1). In serovar pomona, the *dnaA*, *gyrBA*, and *gidA* genes are located on NtC. The serovar pomona gyrBA and *gidA* genes are on the 90-kb *Asc*I fragment (Fig. 1). Weak hybridization signals from the *dnaA* hybridization probe prevented more precise localization of this gene. No hybridization was seen between the *gidA*, *gyrBA*, or *dnaA* probe and the small replicon from either serovar.

Amino acid biosynthetic genes. Several amino acid biosynthetic genes have been cloned from *L. interrogans* (sensu stricto) (3, 10, 35). These genes were localized on the physical maps of both serovars. Surprisingly, the *asd* gene, which encodes aspartate β-semialdehyde dehydrogenase (3, 7), is located on the small replicons (350 kb) of both serovars. The presence of the *asd* gene on the linearized 350-kb small replicon of serovar icterohaemorrhagiae is shown in Fig. 2A. There are no *Asc*I sites in the small replicon of serovar icterohaemorrhagiae. However, variable amounts of the small replicon appear linear in most DNA preparations as a result of degradation and can be resolved from the large replicon in uncut preparations (Fig. 2A) (45). In serovar pomona, the small replicon is cut once with *Not*I, *Sse*8387I, and *Srf*I, cut twice by *Sgr*AI, and cut into several fragments by *Sma*I (45). There are no *Asc*I or *Sfi*I sites in the small replicon of serovar pomona (45). Hybridization of the *asd* probe to restriction endonuclease-digested, PFGE-separated serovar pomona DNA shows that the *asd* gene is on 350-kb fragments generated with *Not*I, *Sse*8387I, and *Srf*I, the 190-kb *Sgr*AI fragment, and the 100-kb *Sma*I fragment (Fig. 2B). Most of the hybridization signal was at the gel origin in the lanes containing *Asc*I- or *Sfi*I-digested DNA, but weak signals were seen at 350 kb. This result is consistent with hybridization with the intact small replicon (at the origin) and a small amount of the linearized form of the small replicon (at 350 kb). The data in Fig. 2B allowed the *asd* gene to be localized to a region on the small replicon (Fig. 2C). Because of comigrating fragments from the large replicon, a physical map of the small replicon in icterohaemorrhagiae has not been constructed (1). The other amino acid biosynthetic genes (*aroD*, *leuB*, and *proAB*) are located on the large replicon (Fig. 1).

Transcription- and translation-related genes. Two *L. biflexa* ribosomal protein genes (*rpsL* and *rpsG*) have been cloned and characterized (51). In *L. biflexa*, *rpsL* and *rpsG* are closely linked and are immediately downstream from a gene which complements *E. coli* mutants in *argE*. However, that gene encodes a protein homologous to the second-largest subunit of RNA polymerase (a product of the *rpoC* gene) (50). Although the actual function of this gene in *L. biflexa* is not known, we assume that it is the *rpoC* gene. In serovar pomona, the *rpoC*, *rpsL*, and *rpsG* genes are found on the NtF fragment of the large replicon. *rpoC* spans a *Sma*I site located between 150- and 50-kb *Sma*I fragments. Both *rpsL* and *rpsG* are located on the 50-kb *Sma*I fragment. In serovar icterohaemorrhagiae, the *rpoC*, *rpsL*, and *rpsG* genes are located on the *Asc*I B fragment. However, *rpoC* is

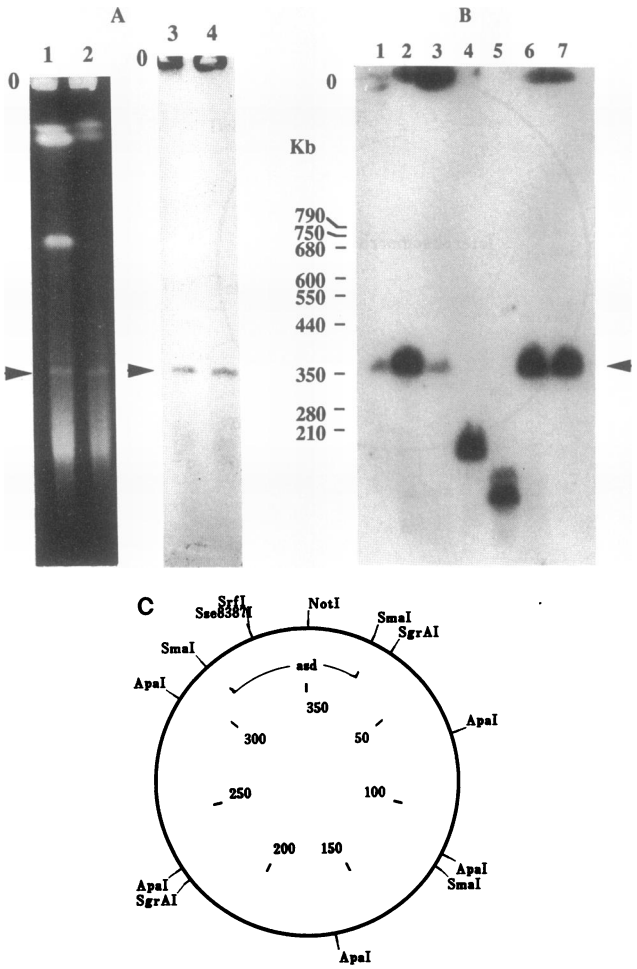

FIG. 2. Localization of the *asd* gene to the small replicon. (A) Serovar icterohaemorrhagiae DNA cut with *Asc*I (lanes 1 and 3) and uncut (lanes 2 and 4) and separated by PFGE. Lanes 1 and 2 show the ethidium bromide-stained gel; lanes 3 and 4 are the nylon membrane blot of the PFGE gel after hybridization with the *asd* gene. (B) Serovar pomona DNA cut with *Asc*I (lane 1), *Not*I (lane 2), *Sfi*I (lane 3), *Sgr*AI (lane 4), *Sma*I (lane 5), *Srf*I (lane 6), and *Sse*8387I (lane 7), then separated by PFGE, blotted to a nylon membrane, and hybridized with the *asd* probe. The resulting autoradiograph is shown. Migration of molecular size markers is shown on the left. Arrowheads in panels A and B indicate the linearized small replicon of each serovar; O indicates gel origin. (C) Restriction map of the small serovar pomona replicon with the location of the *asd* gene.

on the NtJ fragment, while *rpsLG* is on NtB. Thus, *rpoC* is separated from *rpsLG* by at least 130 kb (Fig. 1).

Two elongation factor (EF) probes were used in this study (Table 1). Immediately downstream from the *L. biflexa rpsL* and *rpsG* genes is a gene with homology to the *E. coli fusA* gene, which encodes EF-G (47). The corresponding gene in serovar pomona is located on NtF. No hybridization was seen between the second EF probe (for EF-G and EF-Tu from *Thermus thermophilus* [25]) and serovar pomona. In serovar icterohaemorrhagiae, the *T. thermophilus* probe hybridized with the NtA fragment (35a) (Fig. 1).

IS-like elements. We previously cloned and sequenced an insertion sequence (IS)-like element (designated IS*1533*) from *L. borgpetersenii* serovar hardjo (48, 49) and an IS*3*-like element from *L. interrogans* (sensu stricto) serovar icterohaemorrhagiae (35a). These cloned sequences were used to localize IS-like elements in serovars icterohaemorrhagiae and pomona. There are at least four copies of IS*1533* in the large replicon of serovar pomona (data not shown) clustered in two different regions and at least one copy in serovar icterohaemorrhagiae (Fig. 1). The IS*3*-like element hybridized with three *Not*I fragments, NtA, NtB, and NtF, in serovar icterohaemorrhagiae. The locations for two of these IS*3*-like elements (on NtA and NtB) are shown in Fig. 1. Because NtF comigrates with another fragment, the position of the third IS*3*-like element was not determined.

DISCUSSION

This study shows that the *L. interrogans* genome is unusually variable, with several large inversions and insertions occurring throughout the large replicon. We also provide evidence suggesting that both *L. interrogans* replicons may function as chromosomes.

Genetic mapping of *L. interrogans* serovars icterohaemorrhagiae and pomona showed that most of the essential genes are located on the larger of the two replicons. These data show that the large replicon is both structurally and functionally similar to chromosomes of most other bacteria. Our results with the *asd* probe were surprising and unexpected. The *asd* gene encodes aspartate β-semialdehyde dehydrogenase, an enzyme essential for the synthesis of threonine, methionine, lysine, and diaminopimelate (7). The latter compound is found in the cell walls of leptospires (21). Since aspartate β-semialdehyde dehydrogenase is essential for amino acid and cell wall syntheses, and since *asd* is present in one copy within the *L. interrogans* genome, we suggest that the small replicons of both serovars should also be considered chromosomes. Several other lines of evidence suggest that *L. interrogans* has two chromosomes. Both the large and small replicons are seen in all serovars examined to date. This finding shows that the small replicon is a uniform feature of *L. interrogans* (sensu lato) genomes. The small replicons are also exceptionally stable, since they are found both in isolates obtained fresh from clinical material and in isolates from type strains kept in continuous culture for over 80 years (17a, 47).

The suggestion that *L. interrogans* has two chromosomes is provocative, since few other bacteria are thought to have more than one chromosomal replicon. *Rhodobacter sphaeroides* (39) and *Brucella* spp. (24) appear to contain two chromosomal replicons but belong to a different branch of bacterial evolution (the α-photosynthetic bacteria) from the leptospires. Genetic evidence for the presence of essential genes on the smaller *R. sphaeroides* chromosomal replicon has been reported (39a). However, lack of suitable genetic methods for specifically inactivating leptospiral genes prevents us from conclusively showing that the *asd* gene on the small replicon is essential. Genes on the large replicon may complement mutations in the *asd* gene, perhaps by using an alternate biosynthetic pathway. Leptospires possess an alternate isoleucine biosynthetic pathway (6), and additional alternate pathways may also occur. Lack of suitable genetic manipulation techniques has also prevented obtaining genetic data supporting the conclusion that *Brucella* spp. contain two chromosomes.

The areas surrounding the replication origins of both replicons are organized differently. In *L. interrogans*, the *dnaA*, *gidA*, and *gyrBA* genes are clustered together on the large replicon. This result is similar to the organization of

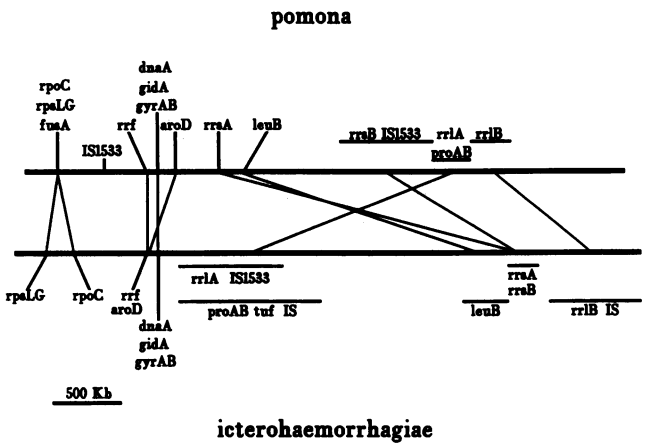

FIG. 3. Alignment of genetic maps of *L. interrogans* serovars icterohaemorrhagiae and pomona. Genetic maps of the large replicons from both serovars are aligned at the *rrf*, *dnaA*, *gidA*, and *gyrBA* loci. Lines show regions of rearrangements which differentiate the two chromosomes.

oriC of many bacterial genera (23, 26, 38), and we assume that this is the likely position for the replication origin for the large *L. interrogans* replicon. Two possible chromosomal replication origins of another spirochete, *B. burgdorferi*, have been described: one next to *gyrBA* and *dnaA*, and one near *gidA* (28). The *dnaA*, *gyrBA*, and *gidA* genes did not hybridize with the small replicon of either serovar, showing that the genetic organizations of the two replication origins are different. The mechanism controlling replication of these two replicons is unknown but must be tightly coordinated since they are present in equimolar amounts (45). Since the *dnaA* gene product is *trans* acting, one copy of *dnaA* (on the large replicon) may be sufficient to coordinate replication of both replicons. Tight control of minichromosome replication also appears to occur in *R. sphaeroides*. Mobilization of the small *R. sphaeroides* chromosome to another strain results in loss of the resident minichromosome (39a).

Ribosomal gene organization in leptospires is unusual (1, 16). In most bacteria, rRNA genes are organized in operons (*rrn*) in the order *rrs-rrl-rrf*, encoding the 16S, 23S, and 5S rRNAs, respectively (23). In contrast, the *L. interrogans* (sensu lato) rRNA genes are not closely linked, and there is only one copy of *rrf* but two copies each of *rrs* and *rrl* (1, 16). In both serovars, *rrf* is located near the *dnaA*, *gyrBA*, and *gidA* genes of both serovars, while the other rRNA genes are scattered around the large replicon. Placement of *rrf* near the presumed replication origin may help compensate for a lower copy number of this gene compared with *rrs* and *rrl*. Genes located near *oriC* are transiently amplified compared with genes located elsewhere in the genome because synthesis of nascent chromosomes and passage of replication forks generate additional copies (23).

Comparison of the genetic maps of these two serovars provides insight into the variability among *L. interrogans*. Although DNA homology results show that *L. interrogans* serovars icterohaemorrhagiae and pomona are similar (34, 44), these serovars have different physical maps (1, 45) and genetic organizations. There are several rearrangements which differentiate the large replicons of serovar icterohaemorrhagiae and serovar pomona. Figure 3 highlights the difference in genetic organization between these two serovars. Inversions in the large replicons are evident by comparing the positions of *rrsA-leuB* and *rrlA-proAB* (Fig. 3) of each serovar. An inversion also appears to have occurred around *rrsA* and *rrsB*. An inversion may also be present near the presumed *oriC*, for in serovar pomona, *rrf* and *aroD* are split by *dnaA*, *gyrBA*, and *gidA*, but in serovar icterohaemorrhagiae, *rrf* and *aroD* are adjacent. A possible insertion may also be present between the serovar icterohaemorrhagiae *rpoC* and *rpsLG* genes. These genes map together in both serovar pomona and *L. biflexa* serovar patoc (50, 51) but are separated by at least 130 kb in serovar icterohaemorrhagiae.

Few intraspecies comparisons of bacterial genetic organization have been done. Studies on *Clostridium perfringens* (4), *E. coli* (8), and *Mycoplasma mycoides* (33) show that genetic organization within these species is generally well conserved, with only a few deletions or inversions detected. In contrast, genetic organization within *Bacillus cereus* is quite variable, with several chromosomal rearrangements (5). Variability in genetic organization also occurs in *L. interrogans* (Fig. 3).

Repetitive DNA sequences which are common in leptospiral genomes (29, 41, 43, 49) are probably involved in generating some of the rearrangements detected in this study. Homologous recombination between repeated DNA sequences is a common mechanism in generating rearrangements (31). Chromosomal rearrangements have also been detected in *L. borgpetersenii*, and these appear to involve recombination between different copies of IS*1533* (52). While IS*1533* is present in only a few copies in *L. interrogans* (sensu stricto), other repetitive DNA sequences are present in higher numbers (1, 46) and may be involved in rearrangements. Studies of other bacterial genera show that chromosomal rearrangements can have dramatic effects on phenotype (23). Since leptospires are phenotypically diverse, genetic rearrangements may be important in adapting to differing environments and generating this diversity.

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