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Linear Chromosomes of Lyme Disease Agent Spirochetes: Genetic Diversity and Conservation of Gene Order

SHERWOOD CASJENS,[1]* MICHAEL DELANGE,[1] HERBERT L. LEY III,[1] PATRICIA ROSA,[2] AND WAI MUN HUANG[1]

Department of Oncological Sciences, Division of Molecular Biology and Genetics, University of Utah Medical Center, Salt Lake City, Utah 84132,[1] and Laboratory of Microbial Structure and Function, Rocky Mountain Laboratory, National Institute of Allergy and Infectious Diseases, Hamilton, Montana 59840[2]

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We have constructed physical and genetic maps of the chromosomes of 21 Lyme disease agent spirochetes from geographically diverse locations. All have linear chromosomes whose lengths range from 935 to 955 kbp, and all contain multiple linear plasmids in the 16- to 175-kbp size range. The locations of 11 gene clusters on the chromosomes of these different isolates are indistinguishable at the resolution achieved in this study, indicating that the members of this related group of species have highly conserved chromosomal gene orders. However, chromosomal restriction endonuclease cleavage site maps are unique for nearly all isolates. The 22 chromosomal maps currently available define eight classes of Lyme disease agents. Four of these correspond to the previously proposed species *Borrelia burgdorferi*, *Borrelia garinii*, *Borrelia afzelii*, and *Borrelia japonica*. In addition, the North American isolates 21038, DN127 cl9-2, 25015, and CA55 typify four additional chromosomal types that are as phylogenetically distinct as the species listed above. These findings support the idea that comparison of restriction maps is currently the most robust and definitive method for determining overall chromosomal relationships among closely related bacteria. In the course of this work, we located on the chromosome the previously unmapped outer surface protein-encoding LA7 gene and genes homologous to the *Escherichia coli priA*, *plsC*, *parE*, and *parC* genes, and we have substantially refined the locations of the *recA*, *fla*, p22A, and *flgE* genes.

Lyme disease is caused by spirochetes of the genus *Borrelia* that are carried by hard-bodied (ixodid) ticks. The various Lyme disease agent bacterial isolates have been reported to be heterogeneous by a number of criteria as follows: (i) nucleotide sequences of chromosomal and plasmid genes, (ii) the ability of particular oligonucleotide primers to function in PCR with *Borrelia* DNA, (iii) arbitrary primer PCR-generated DNA fragment patterns, (iv) restriction fragment length polymorphisms (RFLPs), (v) multienzyme electrophoretic analysis, (vi) DNA-DNA hybridization, (vii) surface protein properties, (viii) seroprotection, (ix) fatty acid profiles, and (x) polyacrylamide gel electrophoresis of whole-cell proteins (see the following references and citations therein: 10, 13, 24, 28, 36–38, 53, and 74). Understanding the nature and extent of these genetic differences will be useful in understanding possible geographic variations in Lyme disease symptoms and has important implications in detection of the disease organism and in vaccine development (7, 68, 69). The above studies support the idea that there are at least four closely related species of *Borrelia* spirochetes that cause Lyme disease. In this report, we refer to this group of species as Lyme disease agents (also called *Borrelia burgdorferi* sensu lato), even though many isolates have not been shown directly to cause Lyme disease. The four characterized types of Lyme agent spirochetes are currently known by the following names: (i) *B. burgdorferi*, which is largely North American but which is also found in Eurasia (represented by isolate B31 [11]; until recently, all Lyme disease agent spirochetes were categorized as this species); (ii) *Borrelia garinii*, which is found in Eurasia (represented by isolate 20047 [4]); (iii) *Borrelia afzelii*, which is found in Eurasia (represented by isolate VS461 [13]); and (iv) *Borrelia japonica*, which is found in Japan (represented by isolate HO14 [28]).

Borrelia spirochetes have small linear chromosomes that are approximately 950 kbp in length (15, 18, 22). In addition, they harbor numerous linear and circular plasmids (e.g., see reference 57), which can compose up to one-third of the genetic information carried by these organisms. In a number of the studies referred to above, the differences observed among isolates may be due, at least in part, to variation in the plasmids present. Comparison of scattered chromosomal restriction endonuclease cleavage site locations in closely related bacteria is a valuable way to obtain information on the overall extent of genetic variation within a species and between closely related species (e.g., see references 12, 14, 16, 17, 33, 34, and 71). This approach is different from comparison of nucleotide sequences from single loci (35, 45), since it samples short nucleotide sequences (the enzyme recognition sites) across the length of the chromosome. In order to more clearly define the genetic variation among and within the different types of Lyme disease agent bacteria, we present and compare restriction endonuclease cleavage site maps of the chromosomes of the following 22 isolates: 7 geographically diverse *B. burgdorferi*, 3 *B. garinii*, 3 *B. afzelii*, 2 *B. japonica*, and 7 other isolates whose relationships to the four previously defined types were ambiguous. We find the last group to consist of four additional Lyme disease agent types.

MATERIALS AND METHODS

Bacterial strains. The *Borrelia* isolates used in this study or discussed in this report are listed in Table 1. Most of these have not been overtly isolated as cloned populations that are derived from a single ancestor cell; however, CA-11 2A (41) was propagated from a single colony of CA-11-90 (63), DN127 cl9-2 is a clone derived from DN127 (32), R-IP3 was cloned by us for this study, and HB19 was cloned by A. Barbour and A. Sadziene (6a). Isolate IPF was designated J1 by Rosa et al. (56) and Marconi and Garon (38, 39), R-IP3 was

* Corresponding author. Phone: (801) 581-5980. Fax: (801) 581-3607.

TABLE 1. *Borrelia* isolates used in this study

Isolate	Location (yr)	Biological source	References	Passage in culture	Source
B. burgdorferi (sensu stricto)					
Sh-2-82	Shelter Island, N.Y. (1982)	*I. scapularis*	62	p6, p165, and p320	T. Schwan, J. Weis, and our collection
B31	Shelter Island, N.Y. (1981)	*I. scapularis*	11	High passage	Our collection
JD1	Ipswich, Mass.	*I. scapularis*	54	p16	T. Schwan
CA-11 2A	Sacramento County, Calif.	*I. pacificus*	41, 63	Cloned in solid agarose from low-passage culture	Our collection
WI91-23	St. Croix River Valley, Wis.	Song sparrow blood	44	p3	R. Johnson
HB19	Connecticut	Human blood	8, 69	Cloned in solid agarose from low-passage culture	A. Barbour
1352	See Materials and Methods	See Materials and Methods	38, 56	Passage information not available	R. Marconi
212	France	*I. ricinus*	18	Low passage	
B. garinii					
20047	Brittany, France	*I. ricinus*	2	Passage information not available	R. Marconi
G2	Würzburg, Germany	Human cerebrospinal fluid	27	p40 to 60	Our collection
FujiP1	Mt. Fuji, Shizuoka, Japan	*I. persulcatus*	41a	≤p9	I. Schwartz
B. afzelii					
VS461	Vouvry, Valais, Switzerland	*I. ricinus*	52	p10	R. Marconi
R-IP3	St. Petersberg, Russia	*I. persulcatus*	31	Cloned in solid agarose for this study	T. Schwan
IPF	Furano, Hokkaido, Japan	*I. persulcatus*	46	High passage	R. Marconi
B. japonica					
HO14	Hokkaido, Japan	*I. ovatus*	42	≤p20	R. Johnson
IKA2	Ikawa, Shizuoka, Japan	*I. ovatus*	43	≤p10	R. Johnson
DN127 type					
DN127 cl9-2	Del Norte County, Calif.	*I. pacificus*	9, 32	Cloned by limiting dilution from low-passage culture	Our collection
CA127	Mendocino County, Calif.	*I. neotomae*	33a	p7	R. Lane
21038 type					
19857	Millbrook, N.Y.	Cottontail rabbit kidney	3	p10	J. Anderson
21038	Millbrook, N.Y.	*I. dentatus* larva	3	p6	J. Anderson
25015 type					
25015	Dutchess County, N.Y.	*I. scapularis* larva	1	p7	R. Marconi
CT-39	Cook County, Ill.	White-footed mouse ear punch	47a	Passage information not available	R. Marconi
CA55 type					
CA55	Mendocino County, Calif.	*I. neotomae*	33a	p9	R. Lane

designated IP3 by some investigators, CT-39 has also been designated Illinois-1 (40, 60), and HB19 was originally designated 272 by Steere et al. (69).

We also analyzed *B. burgdorferi* isolate 1352, which was reported to have been isolated from an *Amblyomma americanum* tick in Texas (38–40, 56). We found this isolate to be identical to high-passage strain B31 in its plasmid pattern and in its restriction site cleavage map. Although the possibility exists that this is a natural isolate that is virtually identical to B31, since all other isolates that we have analyzed are different from one another in at least one of these properties, we believe that caution should be exercised regarding interpretation of the origin of this strain.

DNA preparation and electrophoresis. *Borrelia* cultures were grown in BSK-H medium (Sigma) (5) at 32 to 35°C under microaerobic conditions. Bacteria were harvested, and DNA was prepared in agarose blocks as described previously (15). Restriction endonuclease cleavage of these DNA preparations and contour-clamped homogeneous electric field (CHEF) electrophoresis in agarose gels were carried out as previously described (15). To prepare probes for Southern hybridizations, regions within each of the indicated genes were PCR amplified by using specific oligonucleotides as primers and either whole-cell or cloned *Borrelia* DNA fragments as templates. ^{32}P-labeled probes were prepared from these PCR products by random priming according to the recommendations of the kit manufacturer (Bethesda Research Laboratories). Templates for probe production have been previously described (15), except for the *priA*, LA7, *parC*, and *parE* gene probes. The last four were prepared as PCR-generated 500- to 1,000-bp DNA fragments from within the indicated gene.

RESULTS

Physical maps of the chromosomes of several *B. burgdorferi* (sensu stricto) isolates. We have previously reported a detailed physical and genetic map of the chromosome of *B. burgdorferi* isolate Sh-2-82 and have shown that this map has not changed with propagation of the bacteria in culture for up to 320 passages (>2,100 generations) (15). A map of the chromosome of the European *B. burgdorferi* isolate 212 has also been reported elsewhere (18). Comparison of these two maps indicated the presence of a number of chromosomal restriction site polymorphisms (RSPs) that distinguish them (15).

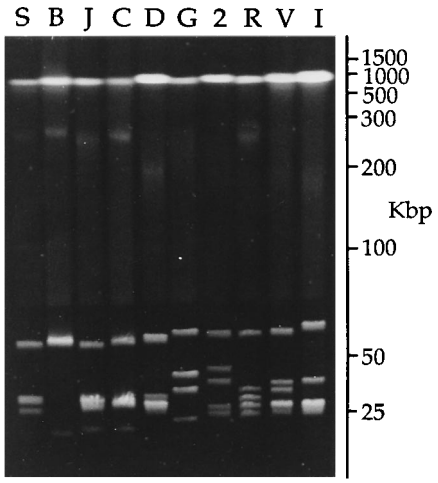

FIG. 1. CHEF electrophoresis of whole Lyme disease agent DNAs. DNAs from Lyme disease agent isolates were prepared in agarose blocks, subjected to CHEF electrophoresis in 1% agarose, and stained with ethidium bromide as previously described (15). Different isolates are indicated in the figure as follows: S, Sh-2-82; B, B31; J, JD1; C, CA-11 2A; D, DN127 cl9-2; G, G2; 2, 20047; R, R-IP3; V, VS461; I, IPF. Note that DNA mobility in CHEF electrophoresis gels is inversely related to the amount of DNA loaded; we found the largest B31 and Sh-2-82 linear plasmids to be very nearly the same size when smaller amounts of B31 DNA were used. The same is true for the largest IPF and VS461 linear plasmids.

To begin to understand chromosomal genetic diversity within the *B. burgdorferi* species, we analyzed isolates B31, HB19, and JD1 from the northeastern United States, CA-11 2A from California, and WI91-23 from Wisconsin (Table 1). These isolates belong to the *B. burgdorferi* (sensu stricto) species by several criteria (44, 56, 63). Figure 1 shows that the chromosomes of isolates B31, JD1, and CA-11 2A enter CHEF agarose electrophoresis gels, indicating that they are linear (22). The chromosomes of isolates HB19 and WI91-23 also enter such gels (data not shown). These chromosomes have similar sizes (of approximately 1 Mbp) by this analysis (more-accurately determined sizes are reported below). In addition, the gel in Fig. 1 displays the linear plasmid DNAs present in some of these isolates.

Lyme disease agent spirochete DNAs have an A+T content of approximately 70% (61). Therefore, restriction endonucleases with mostly G−C recognition give relatively simple restriction fragment patterns. Ten enzymes that cleave the previously characterized *B. burgdorferi* Sh-2-82 chromosome seven or fewer times (*Bss*HII, *Eag*I, *Mlu*I, *Not*I, *Rsr*II, *Sac*II, *Sfi*I, *Sgr*AI, *Srf*I, and *Sse*8387I) were chosen for this comparison. Fragments derived from the chromosomes were identified by cutting agarose blocks containing uncleaved chromosomes from CHEF electrophoresis gels, cleaving them with the enzymes listed above, and separating the resulting fragments by CHEF electrophoresis (data not shown). Chromosomal restriction maps were constructed using methods we have previously described (15). Complete cleavage site maps for these *B. burgdorferi* isolates are shown in Fig. 2 for each of the 10 enzymes. The sizes of the restriction fragments in the maps in this report are not given but can be obtained from the corresponding author (S. Casjens). Figure 2 also includes the cleavage sites reported by Casjens and Huang (15) for isolate Sh-2-82 and, when possible, those reported by Davidson et al. (18) for the French *B. burgdorferi* isolate 212. The linear chromosomes of the seven *B. burgdorferi* (sensu stricto) isolates examined have lengths in the 935- to 950-kbp range (Table 2), and there are a number of RSPs among the isolates. A quantitative discussion of the RSP differences is given below.

Two apparent DNA fragment length differences were also observed. One is in the B31 *Eag*I F fragment (9 kbp), which is about 300 bp longer than the cognate fragment from the other isolates (data not shown); this observation could be explained by an *Eag*I site that creates an unobserved fragment of several hundred base pairs in the other isolates (we analyzed only fragments of ≥2 kbp). A second apparent length variation is located near the right end. CA-11 2A and B31 right-end fragments are about 8 kbp shorter than the cognate fragments in Sh-2-82 and JD1. In addition, the right-end fragments of isolates WI91-23 and HB19 are about 7 kbp shorter than those of CA-11 2A or B31 (data not shown). These length differences lie to the right of the rightmost mapped cleavage site (the highly conserved *Sgr*AI no. 2, which is 32 kbp from the right end in Sh-2-82), and these strains have no extra right-end *Sgr*AI restriction sites relative to JD1 and Sh-2-82 that could explain the fragment length differences (46a). Data reported by Davidson et al. (18) indicate that isolate 212 may have a right-end *Sgr*AI fragment length that is intermediate between the lengths of Sh-2-82 and B31. No other convincing length differences were found, and we estimate that length differences of larger than 5 kbp would have been observed.

Physical maps of the chromosomes of *B. garinii*, *B. afzelii*, and *B. japonica*. Three *B. garinii* isolates, 20047 (France), FujiP1 (Japan), and G2 (Germany); three *B. afzelii* isolates, VS461 (Switzerland), R-IP3 (Russia), and IPF (Japan); and two *B. japonica* isolates, HO14 (Japan) and IKA2 (Japan), were chosen for analysis (Table 1). Isolate 20047 is the type strain for *B. garinii* (4), and FujiP1 and G2 belong to this group according to the analyses of I. Schwartz (63a) and T. Masuzawa (41a) and Marconi and Garon (38, 39), respectively. VS461 typifies *B. afzelii* (13). IPF belongs to this group by the analysis of Marconi and Garon (38), and R-IP3 is included in this group by the analyses of Marconi and Garon (38), Schwartz et al. (64), and Baranton et al. (4). HO14 is the type strain for *B. japonica*, and IKA2 is included in this group by the analysis of Kawabata et al. (28). CHEF electrophoresis of these whole-cell DNAs indicates that these isolates all contain apparently linear chromosomes similar in size to those of *B. burgdorferi* (Fig. 1; data not shown).

Restriction endonuclease cleavage site physical maps for each of these eight isolates were constructed as described by Casjens and Huang (15) (data not shown). These maps, which are linear, are shown in Fig. 3, 4, and 5. The locations of restriction enzyme cleavage sites on the physical maps of these three groups of isolates are quite different from one another, as they are from those of *B. burgdorferi* (sensu stricto). Among the 22 Lyme disease agent isolates examined to date, only 20047 and FujiP1 have chromosomes with identical cleavage patterns by the 10 enzymes used here. When comparisons are made within each of these groups, only a few RSPs are found, showing that there is limited chromosomal sequence variation within each type. A more quantitative analysis of the map relationships is presented below. After this study was completed, physical maps of the chromosomes of isolates 20047 and VS461 were reported by Ojaimi et al. (49). The *Mlu*I, *Sgr*AI, *Eag*I, and *Bss*HII sites reported by them are in general agreement with the locations reported here, except that sites *Sgr*AI no. 1 and *Eag*I no. 4 in VS461 reported here are not reported in their map.

Isolate R-IP3 *Sgr*AI cleavage site no. 2 and HO14-IKA2 *Rsr*II site no. 2 were consistently incompletely cut under our digestion conditions, even when larger amounts of restriction

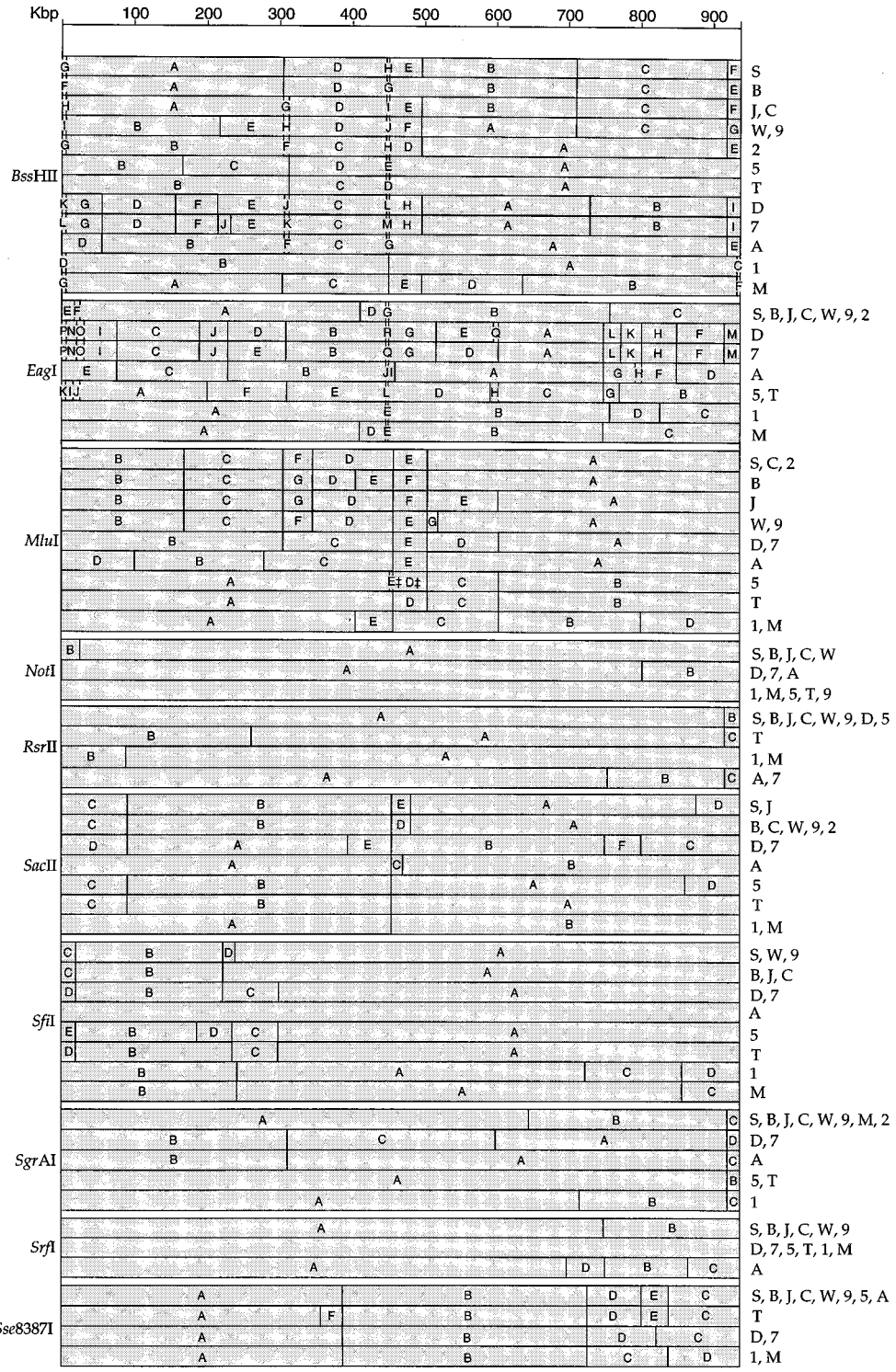

FIG. 2. Physical maps of the linear chromosomes of *B. burgdorferi* (sensu stricto) and other North American Lyme disease agent isolates. Vertical lines indicate restriction endonuclease cleavage sites. Letters designate the restriction fragments, with the largest designated A and successively smaller chromosomal fragments designated in alphabetical order. Fragment length differences described in the text are not shown. Restriction enzymes are indicated on the left, and restriction sites are designated by numbering from left to right. To the right of each map, the following symbols indicate the chromosomes with that restriction map: S, Sh-2-82; B, B31; J, JD1; C, CA-11 2A; 9, HB19; 2, 212; D, DN127 cl9-2; 7, CA127; 1, 19857; M, 21038; 5, 25015; T, CT-39; A, CA55. The Sh-2-82 and 212 maps were constructed by Casjens and Huang (15) and Davidson et al. (18), respectively, and are included here for comparison with the isolates studied in this report.

enzyme were used; all other restriction sites in this analysis were completely cut. To test the possibility that the R-IP3 culture in reality contains a mixture of two strains, one with and one without this *Sgr*AI site, we obtained five different R-IP3 clones by single-colony isolation according to the method described by Rosa and Hogan (55). DNA from each of these clonal cultures was found to have a restriction map identical to DNA from the uncloned culture and to be incompletely

TABLE 2. Linear plasmid and chromosome sizes for Lyme agent isolates used in this study

Isolate	Size (kbp)	
	Chromosome	Plasmid
B. burgdorferi		
Sh-2-82 (p6)	950	53,[a] 30, 29, 28, 27, 25, and 18
B31	940	53,[a] and 18
JD1[d]	950	53,[a] 32, 31, 29.5, 28,[b] 26, and 19
CA-11 2A	940	53,[a] 30, 29,[b] 27, and 19
WI91-23	935	53,[a] 40, 29, 27.5, 26,[c] and 17
HB19	935	53,[a] 40, 29, 28.5, 27.5, 26,[c] 25, and 17
DN127		
DN127 cl9-2[e]	935	53,[a] 31.5, 29,[c] 27.5,[b] 26, and 24
CA127	935	53,[a] 32, 31, 28,[b] 27,[b] and 25
21038		
19857	935	175,[c] 53,[a] 35, 19, and 17
21038	935	53,[a] 44, 36,[b] 35, and 17
CA55	935	53,[a] 45, 43, 32, 31.5, 26, 22, and 19
25015		
25015	935	53,[a] 47,[c] 40,[c] 35,[a] 29, 24,[c] and 19
CT-39	935	53,[a] 32,[b] 29,[c] 27.5, and 25[c]
B. garinii		
20047	935	58,[a] 44, 41, 28, and 23
G2	935	58,[a] 42, 37,[b] 27.5, 27,[c] and 23.5
FujiP1	935	58,[a] 40, 34, 32, 25, and 23
B. afzelii		
R-IP3	935	58,[a] 46,[c] 36, 31, 27,[b] and 24
VS461	935	58,[a] 36, 33, 28, 26,[b] and 24.5
IPF	935	58,[a] 37, 29,[b] 28, 26, and 24
B. japonica		
HO14	935	103,[a,c] 40,[c] 35, 30,[b] and 22
IKA2	950	140,[a,c] 95,[a,c] 47,[a,c] 36, 30,[b] and 21

[a] Contains *ospA* gene by our Southern analyses.
[b] Appears as band of molar intensity more than three times that of chromosomal bands, suggesting that there could be multiple plasmids of similar sizes present.
[c] Plasmid band that appears weaker than chromosomal band.
[d] Contains an apparently circular 60-kbp plasmid that is linearized by *Rsr*II and *Mlu*I. We have not systematically searched for other circular plasmids in this isolate.
[e] Has an apparently circular 65-kbp plasmid that is linearized by *Mlu*I. We have not systematically searched for other circular plasmids in this isolate.

cut at *Sgr*AI no. 2, which is similar to that of the parent culture, suggesting that the nucleotide sequence surrounding this particular site affects its cleavage. Thus, we found no indication that any of the isolates used in this study was heterogeneous.

We determined the *B. garinii*, *B. afzelii*, and *B. japonica* (HO14) chromosomes all to be 935 ± 10 kbp in length by summing several sets of contiguous restriction fragments across the chromosome (all <250 kbp in size). These values are consistent with those determined for uncut chromosomes. No variation in length was found among the *B. afzelii* and *B. garinii* isolates. On the other hand, we observed that left-end fragments from *B. japonica* IKA2 were 15 to 20 kbp longer than cognate left-end fragments from *B. japonica* HO14, indicating a length difference within 140 kbp of the left end.

Chromosomes of seven noncanonical North American Lyme disease agent isolates. (i) California isolates. The arbitrary primer PCR data described by Welsh et al. (76) suggested that the Northern California *Borrelia* isolate DN127 might not belong to any of the above four Lyme disease agent species. We analyzed the chromosomal DNA of DN127 cl9-2, a cloned derivative of DN127 (9, 32). Two other previously uncharacterized California isolates, CA55 and CA127 (33a), were also analyzed. Maps were constructed of the linear chromosomes (935 ± 10 kbp) of these three isolates (Fig. 2). The DN127 cl9-2 and CA127 chromosome maps are very similar and strikingly different from the four map types observed above. We conclude that these two isolates constitute a fifth chromosomal type and that by the same criteria CA55 represents a sixth type.

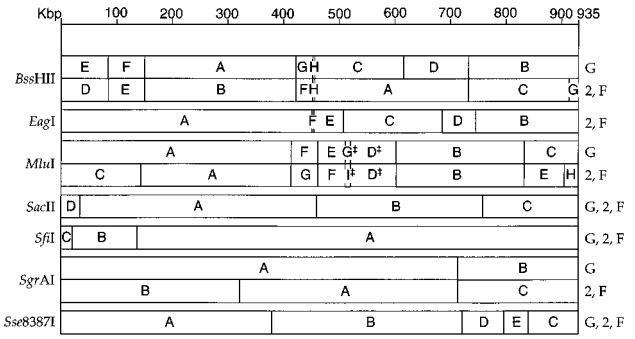

FIG. 3. Physical maps of the linear chromosomes of *B. garinii* isolates. Vertical lines indicate restriction endonuclease cleavage sites. At the right of each map, the isolates with that map are indicated. Restriction fragments and sites are designated as indicated in the legend to Fig. 2. To the right of each map, the following symbols indicate the chromosomes with that restriction map: 2, 20047; F, FujiP1; G, G2. ‡, adjacent fragments that have not been ordered. *Not*I, *Rsr*II, and *Srf*I did not cut the G2, 20047, or FujiP1 chromosome.

(ii) Eastern North American isolates. Isolate 25015 (New York) was reported to have unusual outer surface proteins (OspA and OspB), chromosomal gene sequences, and infectious properties (1, 21, 39), and CT-39 (Illinois) was reported to have an unusual rRNA sequence (39). Similarly, it had been noted that isolates 19857 and 21038 (Millbrook, New York), from a cottontail rabbit kidney and an *Ixodes dentatus* larva on the same rabbit, respectively, did not appear to belong to one of the four previously defined Lyme disease agent types by

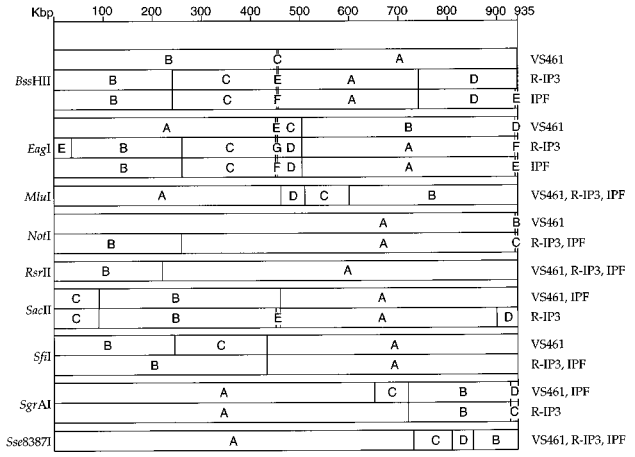

FIG. 4. Physical maps of the linear chromosomes of *B. afzelii* isolates. Vertical lines indicate restriction endonuclease cleavage sites. At the right of each map, the isolates with that map are indicated. Restriction fragments and sites are designated as indicated in the legend to Fig. 2. *Srf*I did not cut the chromosomes of isolates VS461, R-IP3, and IPF.

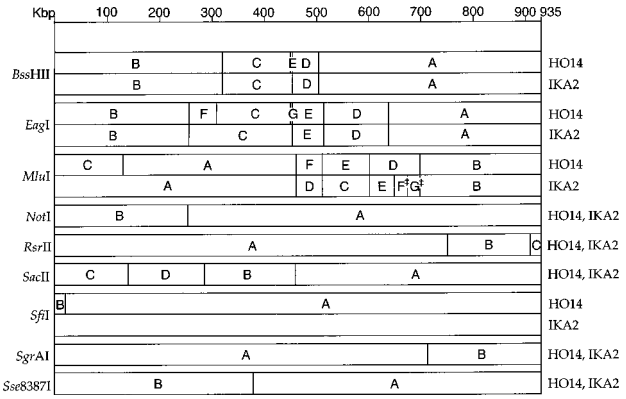

FIG. 5. Physical maps of the linear chromosomes of *B. japonica* isolates. Vertical lines indicate restriction endonuclease cleavage sites. At the right of each map, the isolates with that map are indicated. The fragment length differences described in the text are not shown. Restriction fragments and sites are designated as indicated in the legend to Fig. 2. ‡, adjacent fragments that have not been ordered. *Srf*I did not cut the chromosomes of isolates HO14 and IKA2.

several criteria (21, 74). We constructed restriction enzyme cleavage site maps of the chromosomes of these four isolates as described above. These are shown in Fig. 2. Isolates 19857 and 21038 have very similar physical maps with only a few RSPs; therefore, their linear chromosomes, which are 935 ± 10 kbp long, are quite closely related to one another. *I. dentatus* rarely feeds on humans; thus it is uncertain whether isolates 19857 and 21038 are in fact human Lyme disease agents. Nonetheless, we find enough chromosomal similarities with the other Lyme disease agent types to suggest that this possibility should be considered. The linear maps (935 ± 10 kbp) of the 25015 and CT-39 chromosomes are also different from all others and are very similar to one another. The differences between these two types of maps and those of the six types described above suggest that isolates 21038 and 25015 typify a seventh and an eighth type of Lyme disease agent spirochete (see below).

Genetic maps of Lyme disease agent chromosomes. Eleven genes, which are distributed across the chromosome, were positioned on the chromosomes of all 20 Lyme disease agent isolates used in this study: the *recA* gene (whose product functions in homologous DNA recombination [26a]), the *fla* gene (flagellin [53]), the *rho* gene (transcription termination factor [72]), the *flgE* gene (flagellar hook protein (35a), the p22A gene (periplasmic protein with an unknown function [66]), the *gyrA* gene (DNA gyrase [26a]), the *dnaK* gene (hsp70 chaparonin [73]), the *groEL* gene (hsp60 chaperonin [74]), the p83 gene (outer surface protein with an unknown function [51]), the *priA* gene (DNA primase subunit [71a]), and the rRNA gene cluster (18, 64, 65). In addition, the LA7 gene (outer surface protein [75]) and a cluster of genes homologous to the *Escherichia coli plsC*, *parE*, and *parC* genes (26a) were mapped on a subset of the strains used here. All but the *par* cluster, *priA*, and LA7 genes have been previously mapped on the chromosome of isolate Sh-2-82 (15). The locations of these loci were determined by Southern (67) analysis using fragments of the genes listed above as probes as described by Casjens and Huang (15) and are shown in Fig. 6. The data are compatible with the idea that these genes lie in identical positions in all types of Lyme disease agent chromosome.

Previously analyzed Lyme disease agent bacteria carry one 16S rRNA gene and two 23S identical rRNA genes clustered near the center of the chromosome (15, 18, 23, 64, 65). We find here that isolates 19857 and 21038 lack the *Bss*HII site that is present in the left (transcriptionally downstream) member of the two tandem 23S rRNA genes. Southern analyses using a probe that contains the 5′ portion of the 23S rRNA gene that is transcriptionally upstream of the *Bss*HII site showed that this portion of the 23S rRNA gene is present in two copies (the probe hybridizes with *Bss*HII fragments A and B in 19857 and fragments C and E in 21038 and *Eag*I fragments B and E in both isolates; data not shown). In 19857 and 21038, *Eag*I sites are present at both of the locations expected for two 23S rRNA genes. In these two isolates, (i) at least the 5′ portion of the left 23S gene is present twice and (ii) there has not been a detectable (>100-bp) deletion between the two *Eag*I sites relative to other Lyme disease agent isolates. We have not characterized this unusual 23S rRNA gene further; however, the simplest explanation of our data is that the sequences of the two 23S rRNA genes are not identical at the *Bss*HII site in these isolates. In eubacterial species with multiple rRNA genes, the nucleotide sequences of the different loci are not always identical (e.g., in *E. coli*, the *rrnB*- and *rrnG*-encoded 16S rRNAs differ by six nucleotides [25]).

I. Schwartz (63a) has found that isolate IKA2 has only one copy of the 23S rRNA gene. Our results support this observation in that there are only one *Eag*I site and one *Bss*HII site in the rRNA operon in DNA from this isolate (Fig. 2). We find duplicated *Eag*I and *Bss*HII sites 3.2 kbp apart in isolate HO14 (Fig. 5), indicating that the typical two tandem 23S rRNA genes are probably present there. Thus, a single 23S rRNA gene is not a universal property of *B. japonica* Lyme disease agent isolates, and two 23S rRNA genes are not a universal property of the Lyme disease agent bacteria.

Linear plasmids. We also characterized the linear plasmids present in the isolates used in this study. We assume that all sharp, nonchromosomal CHEF electrophoresis bands in uncut Lyme disease agent DNAs are linear plasmids (15). Our detailed analysis of the *ospC*-containing 26-kbp circular plasmids, as well as several other circular plasmids in the 23- to 30-kbp size range, shows that they do not run as sharp bands under our electrophoresis conditions (14a); however, the possibility remains that some of the plasmids referred to below are in fact circular. Table 2 lists the sizes, estimated from CHEF gels, of the apparently linear plasmids present in the isolates analyzed in this study. In most cases, the copy numbers of the electrophoretically well-resolved linear plasmids are between one and three per chromosome, as determined by their ethidium bromide staining intensities relative to those of chromosomal fragments with similar sizes. Similar copy numbers have been reported for *B. burgdorferi* isolate B31 by Hinnebusch and Barbour (26), for isolate Sh-2-82 by Casjens and Huang (15), and for the non-Lyme disease agent *B. hermsii* by Kitten and Barbour (29). The more-intense bands (indicated in Table 2) represent either multiple plasmids of similar size or higher-copy-number plasmids. We believe the first explanation to be true at least in part, since in CHEF electrophoresis gels of JD1 and DN127 cl9-2 DNAs that expand the 20- to 50-kbp size range, the more-intense bands of Fig. 1 are partially resolved into multiple, closely spaced bands.

Most isolates studied here have been previously shown to produce OspA and/or OspB proteins (Sh-2-82 [62]; B31 [8]; JD1 [54]; CA-11 2A [41]; WI91-23 [44]; HB19 [8]; 20047 [74]; G2 [58]; VS461 [52]; R-IP3 [31]; IPF [46]; DN127 cl9-2 [63]; 19857 and 21038 [3, 74]; 25015 [1]; CT-39 [40]; HO14 [42]). OspA-OspB production has not been analyzed for FujiP1, IKA2, CA55, or CA127. Southern (67) analysis using Sh-2-82 *ospA-ospB* operon DNA as a probe showed that sequences from this operon are present on the 53- to 58-kbp linear plasmid in all isolates studied here except HO14 and IKA2 (Table

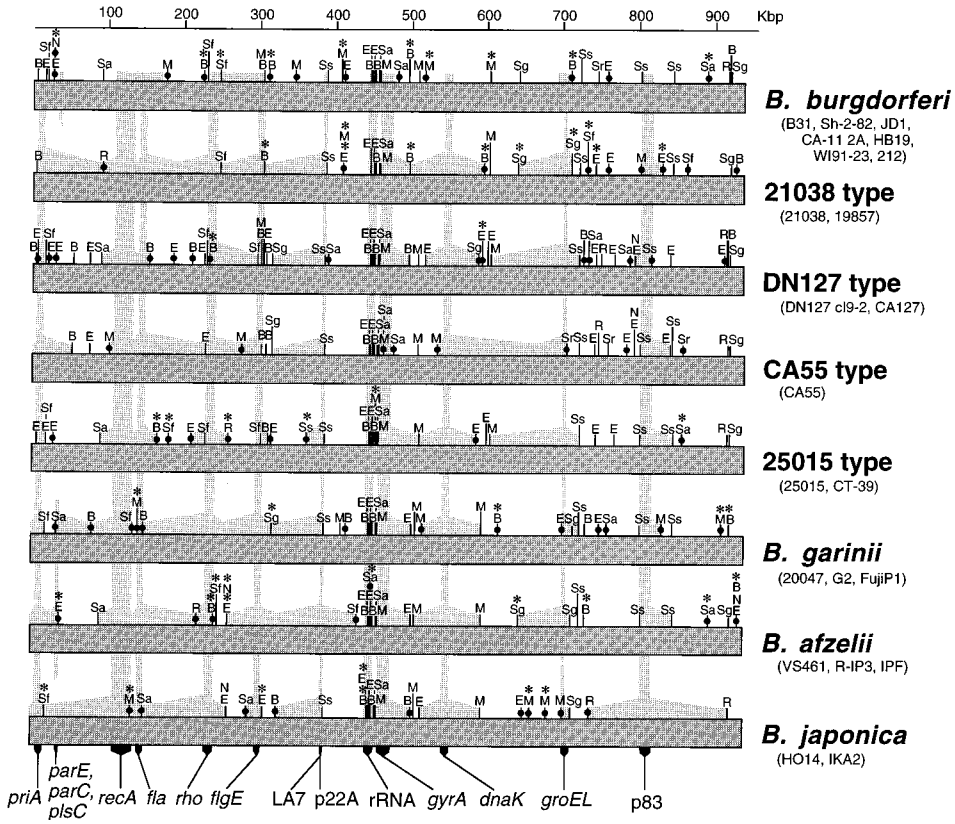

FIG. 6. Comparison of the physical and genetic maps of the chromosomes of the eight types of Lyme disease agent spirochetes. The eight horizontal bars represent the chromosomes of the eight types of Lyme disease agent chromosomes, with a kilobase pair scale above. Short vertical lines indicate the positions of restriction sites (B, *Bss*HII; E, *Eag*I; M, *Mlu*I; N, *Not*I; R, *Rsr*II; Sa, *Sac*II; Sf, *Sfi*I; Sg, *Sgr*AI; Sr, *Srf*I; Ss, *Sse*8387I). An asterisk indicates that a site is not present in all of the analyzed members of that type. A black circle indicates that a site is unique to that chromosomal type. Below, the positions of the 13 gene clusters mapped on these chromosomes are indicated. The shaded areas immediately above each map indicate the intervals in which the genes below are located; when one of the indicated interval boundaries is an unconserved site within a type (e.g., right boundary of the *priA* interval in the *B. afzelii* map), the shaded area indicates the smallest map interval for that gene among the members of that type. The widths of the vertical shaded bars connecting the maps represent the smallest intervals that might contain the relevant genes in the combination of all types. The indicated *B. burgdorferi* gene locations are smaller than the intervals used in the present study; these are the intervals in which the genes are located on the more detailed map of isolate Sh-2-82 (Casjens and Huang [15]; in the *B. burgdorferi* isolates studied here, all of the genes mapped to intervals that include the smaller Sh-2-82 intervals).

2). In our CHEF electrophoresis gels, the *ospA-ospB*-carrying plasmid appears to be reproducibly about 5 kbp larger in *B. garinii* and *B. afzelii* isolates (58 kbp) than in North American Lyme disease agents isolates (53 kbp) (Fig. 1 and data not shown). A similar difference was previously noted by Barbour (6) for several untyped European isolates and independently by Samuels et al. (60) for better-characterized isolates.

The *ospA-ospB* probe hybridized with a 103-kbp linear plasmid in HO14 and with 47-, 90-, and 140-kbp plasmids in IKA2 (data not shown). All four of these plasmids are low copy number; comparison with nearby chromosomal restriction fragments suggests one 47-, 90-, or 103-kbp plasmid for each one to two chromosomes (data not shown). The 140-kbp plasmid is even less abundant. The sizes of the three IKA2 plasmids are consistent with a monomer, dimer, and trimer of the 47-kbp plasmid, and the size of the HO14 plasmid suggests a possible dimerization of a now lost 52-kbp plasmid. A more detailed analysis of these plasmids will be reported separately (14a).

Quantitative comparison and phylogenetic relationships of the Lyme disease agent chromosome physical maps. Figure 6 summarizes the 131 restriction enzyme cleavage site locations for the 10 enzymes used here on the chromosomes of the eight types of Lyme disease agent spirochetes. Although many restriction sites are type specific, some appear to be present in more than one type. The extent of sequence difference between a pair of chromosomes can be estimated from the number of restriction site differences. Two important assumptions are made in this calculation. (i) If the evolutionary conservation of these restriction sites is representative of the conservation of the chromosomal sequence as a whole, then the calculated values can be considered to be estimates of overall diversity. (ii) Restriction sites at indistinguishable locations are in fact at homologous positions. We considered a restriction site to be conserved between two isolates if its position is constant with respect to either the rRNA operon or the chromosomal ends (except in cases of end length polymorphism [see above]). It is not possible, without information about the nucleotide sequence surrounding these sites, to unequivocally prove that these are homologous sites. However, since there are relatively few sites for each enzyme, and since the nucleotide sequences of specific chromosomal genes that have been determined for more than one Lyme disease agent isolate are all less than 10% different (see below), we believe that sites present at indistinguishable locations in different isolates are very likely homologous. The sites we considered to be homologous between chromosomal types can be deduced from Fig. 6. Calculations assuming one nucleotide difference at each RSP

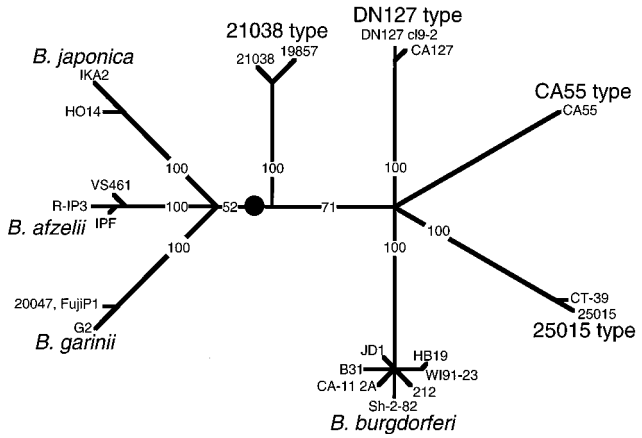

FIG. 7. Unrooted Lyme disease agent phylogenetic tree derived from chromosomal RSPs. Phylogenetic tree calculated by Dollo parsimony analysis and the branch-and-bound algorithm with the computer program PAUP implemented for the Macintosh computer (70). The tree is a consensus tree with all branches drawn to scale; in the primary trees, the *B. garinii* branch is attached to the *B. afzelii* branch near its base, and the CA55 branch is attached near the base of the DN127 cl9-2 branch. Several equally parsimonious primary trees which differ in the order of the short branches of the *B. burgdorferi* arm are found. The numbers on the branches indicate the percentage of bootstrap trees in which those branches appear (calculated from a parsimony analysis using all of the Lyme disease agent isolates listed in the figure except HB19, B31, and 212).

(which is realistic for sequence differences of less than about 5%) show that among the seven canonical *B. burgdorferi* isolates studied, pairwise nucleotide sequence differences estimated by this method range from 1.1 to 2.6%. The *B. garinii* isolates examined are estimated to be 2.4% different, the three *B. afzelii* isolates ranged from 3.0 to 4.8% different, the two *B. japonica* isolates are 4.6% different, the two 21038 type isolates are 5.1% different, the two 25015 type isolates are 2.3% different, and the two DN127 type isolates are 1.8% different. We thus estimate that there can be as much as approximately 5% variation in overall chromosomal nucleotide sequence among isolates within the different classes of Lyme disease agent. Calculation methods which compensate for possible multiple nucleotide sequence differences at RSPs (10, 47) do not significantly affect these intratype difference estimates.

Intertype sequence difference estimates from the RSP data by the method described by Brown et al. (10) and Nei and Li (47), which takes into account the probability of multiple nucleotide changes at RSPs, range from 13% (DN127 cl9-2 versus CA55) to 29% (DN127 cl9-2 versus R-IP3). Since the rRNA genes are very highly conserved and have a higher-than-average G+C content in Lyme disease agents (15, 18, 65), it might be argued that the cleavage sites of the restriction enzymes used in the present study are inordinately highly represented in rRNA genes. Thus, chromosomal divergences calculated from the observed RSPs would be underestimates of the values for non-rRNA genes, which constitute the bulk of the chromosome. If the six cleavage sites in the rRNA genes are removed from the calculations, the intertype sequence difference estimates increase by a factor of 1.4 ± 0.2.

Figure 7 shows an unrooted phylogenetic tree derived from parsimony analysis using the 131 mapped restriction sites as characters in the tree building computer program PAUP (70). This type of analysis assumes that all locations which do not have a particular restriction site are identical at that site, which is not necessarily true. One effect of this assumption is a tendency to move isolates with fewer restriction sites closer together; for example, since DN127 cl9-2 and CA127 have more restriction sites than the other isolates this assumption may make their branch length artificially long. In an effort to at least partially overcome this difficulty, we used Dollo parsimony analysis (each character can arise only once in the tree) as recommended by DeBry and Slade (19) and Swofford (70); however, very similar trees were found when Dollo parsimony analysis was not used. The tree in Fig. 7 strongly supports the idea that the DN127, 21038, 25015, and CA55 type isolates are as divergent from one another and from the other four Lyme disease agent types (*B. burgdorferi*, *B. garinii*, *B. afzelii*, and *B. japonica*) as those four previously described types are divergent from one another. This analysis clearly demonstrates the phylogenetic clustering of the various isolates, and it is tempting to speculate that the root of the tree might lie at the point between the largely North American and Eurasian types (indicated by a black circle in Fig. 7), thus giving it a geographic sense, in that the Lyme disease agents found mainly on each of these land masses would form two supertypes.

DISCUSSION

We have compared the chromosomal organizations of 22 Lyme disease agent (*B. burgdorferi* sensu lato) isolates in order to assess the genetic relationships among them. These spirochetes, which were chosen to encompass most of the previously observed diversity among the known Lyme disease agent spirochetes, were isolated from seven different tick species or from infected humans, rabbits, mice, or birds and have geographically diverse origins.

The physical and genetic maps of the Lyme disease agent bacteria are highly conserved. We found that all of these bacteria have linear chromosomes with similar sizes. No evidence was found for major chromosomal rearrangements since their evolutionary divergence from a common ancestor. Thus, the Lyme disease agents form a group of species whose chromosomes have significantly different nucleotide sequences but virtually identical gene orders, which is not unlike, for example, the *E. coli*, *Shigella flexnerii*, and *Salmonella typhimurium* group (30). It is not yet known if the conserved Lyme disease agent gene order extends to the rest of the *Borrelia* genus; however, we have observed linear chromosomes of about 950 kbp in *B. anserina*, *B. hermsii*, *B. coriaceae*, *B. turicatae*, and *B. parkeri* (14a), which suggests that it may.

The 13 gene clusters used in this study lie at indistinguishable positions on all eight types of Lyme disease agent chromosomes (Fig. 6), showing that chromosomal gene order is highly conserved among the various types of Lyme disease agent spirochetes. Several additions and improvements to the published genetic map (15, 50) were made during this study; the *priA* gene, LA7 gene, and the *plsC*, *parE*, and *parC* gene cluster were located on the chromosome, and the *fla*, *recA*, *flgE*, and p22A genes were located more accurately than was previously known (the p22A and LA7 genes lie within 3 kbp of *Sse*83871 no. 1, and the *par* cluster includes *Xho*I no. 3 of Sh-2-82 [14a]).

The high chromosomal similarity among the Lyme disease agents and the apparently high variability in the numbers and sizes of the plasmids present suggest that the genetic determinants for the basic cellular functions required for propagation reside on the chromosome. This agrees with the previous observation that high-passage B31 has been shown to be viable in culture when all of its linear plasmids are lost (6a, 26, 59).

Chromosomal variation among Lyme disease agent spirochetes. In spite of this overall similarity of the Lyme disease agent chromosomes, we find evidence for significant sequence divergence among the chromosomes that we analyzed. The 22

TABLE 3. Lyme disease agent sequence divergence at known chromosomal locations[a]

Isolate type	% Differences in sequence at the following loci:								
	fla			*orfX*	16S rRNA		*groEL*	*dnaK*	*dnaJ*
	BB	BG	BA	BB	BB	BG	BB	BB	BB
BB	1.3			1.0 ± 0.3	0.035 ± 0.03		ND	0.8 ± 0.6	0.6
BG	2.35 ± 0.65	≤0.5		8.35 ± 0.25	0.85 ± 0.15	0.02	ND	ND	ND
BA	2.0 ± 0.4	2.6 ± 0.1	0	ND	1.15 ± 0.05	1.2	4.8	ND	ND

[a] Values are percent differences in nucleotide sequences between Lyme disease agent isolates at the following chromosomal loci: *fla* (53), *orfX* (56), 16S rRNA (38, 39); *groEL* (74), *dnaK* (73), and *dnaJ* (73). ND, values not yet determined. The ranges of observed values in comparing sequences among more than two members of a group are indicated when appropriate. BB, BG, and BA, *B. burgdorferi*, *B. garinii*, and *B. afzelii*, respectively.

known restriction maps define eight types of Lyme disease agent chromosome. Four of these types correspond to the four previously proposed species of Lyme disease agent, *B. burgdorferi*, *B. garinii*, *B. afzelii*, and *B. japonica*. In addition, we find that isolates, DN127 cl9-2, 21038, 25015, and CA55 define four new chromosomal types. Since several of the last four types contain only a small number of isolates, it seems possible that additional undiscovered Lyme disease agent chromosomal types exist in nature. Within each of the eight chromosomal types, the maps are quite similar, with 0 to 27% of the restriction sites that were analyzed differing between the various isolate pairs. However, 60 to 85% of the cleavage sites are at different locations when pairwise comparisons are made between the different types. Thus, although they are usually not identical, members of each type are considerably more like one another than they are like the other types (Fig. 7).

We have found no evidence for integration of whole plasmids into the chromosome. Some of the observed chromosomal length differences could be due to such events if the plasmids involved were ≤20 kbp; however, the observed chromosome length differences are shorter than those of most known linear and circular plasmids. Similarly, if Lyme disease agent chromosomes harbor integrated prophage DNAs, the phages must have much smaller genomes than lysogenic phages known from other bacterial systems, or any prophages present must be present at the same location in all of the isolates we examined. We favor the idea that integrated prophages are not present, because of the diverse sources of the isolates examined.

(i) Sequence variation within chromosomal types. Among the Lyme disease agent isolates that we studied, only *B. garinii* isolates 20047 and FujiP1 have identical chromosomal macrorestriction maps. In the seven canonical *B. burgdorferi* isolates that have been examined (Sh-2-82, B31, JD1, CA-11 2A, HB19, WI91-32, and 212), 39 different cleavage sites are known for the panel of 10 restriction enzymes. Of these, 10 sites are absent in at least one isolate (Fig. 6). These polymorphic sites are scattered, apparently randomly, across the chromosome. Similar frequencies of RSPs are seen within the other types of Lyme disease agent chromosomes analyzed. Quantitative intratype comparisons of the Lyme disease agent restriction site differences estimate that there is ≤5.1% sequence difference between isolates (see Results). This is not an unusual level of intraspecies variability for bacterial chromosomal DNA (30, 45, 48).

(ii) Relationships among chromosomal types. A minority of chromosomal restriction enzyme cleavage sites analyzed appear to be conserved among all or most of the Lyme disease agents studied (Fig. 6). Of note is the cluster of highly conserved sites near the center of the chromosome. The *Eag*I no. 4 and 5, *Bss*HII no. 3 and 4, *Mlu*I no. 4, and *Sac*II no. 2 sites (Sh-2-82 sites numbered from the left) lie within the rRNA gene cluster (15, 18, 23, 64). Each of the two 23S rRNA genes contains one *Eag*I site and one *Bss*HII site in previously analyzed isolates. Exceptional in this regard are isolates 21039 and 19857, in which one of the two 23S rRNA genes is missing the usually conserved *Bss*HII site, and isolate IKA2, which apparently has only one 23S rRNA gene (see Results). It is apparent from Fig. 6 that the rRNA locus is the most highly conserved region of the *Borrelia* chromosome that is seen by this type of analysis (among loci with G+C contents high enough to contain sites for the panel of enzymes used). This is expected, since the rRNA genes are thought to be among the most highly conserved genes in bacteria (77).

In addition to those sites in the rRNA genes, various other cleavage sites appear to lie at the same locations on the chromosomes of more than one genomic type (Fig. 6). Calculations using these similarities among the maps indicate 22% ± 8% pairwise sequence differences among the chromosomes of the eight Lyme disease agent types (see Results). These values are within the range of previously reported values (10 to 50%) for intertype Lyme disease agent DNA sequence differences from hybridization and heteroduplex melting temperature measurements (e.g., see references 4 and 28). But are these values consistent with those from comparisons of known DNA sequences from these organisms? In Table 3, we compare reported nucleic acid sequences within and among Lyme disease agent chromosomal types; the conclusions are similar to those previously reached with amino acid sequences, in that for a given gene, intratype differences are always less than intertype differences.

It is curious that, unlike comparisons among other closely related bacterial species (e.g., see reference 48), Lyme disease agent genes often have a higher percent identity than the proteins they encode. This must reflect an unusually large fraction of the variation giving rise to nonsynonymous codon differences. It seems that either these genes are under evolutionary pressure to diversify (which may well be the case for the outer surface proteins) or codon usage rules are exceptionally strict. The various Lyme disease agent chromosomal loci are not equally diverged among types. The known intertype nucleotide sequence differences vary from 1.0% ± 0.25% at the 16S rRNA gene to 8.35% ± 0.25% at *orfX*. (Known intratype differences vary from 0.35% ± 0.30% in 16S rRNA genes to 0.7% ± 0.6% in non-rRNA genes.) The overall sequence difference values that we estimate here and that others have measured are higher than the largest reported intertype chromosomal sequence variation (in *orfX*, a randomly chosen chromosomal molecular clone [56]) and considerably higher than the variation reported for the 16S rRNA, *fla* or *groEL* genes. Either these genes are more highly conserved than average chromosomal DNA, or the restriction sites that we used in this

study are less conserved. The *groEL* gene is known to encode an exceptionally highly conserved heat shock protein, and the flagellins are also known to be highly conserved. On the other hand, restriction sites are not constrained to be within genes, which are in general more highly conserved than intergenic regions; thus, both explanations may be true in part.

Geographic locations and host tick species. Lyme disease agent chromosomal types are not restricted to particular ixodid tick species. Two different chromosomal Lyme disease agent types came from *I. ricinus*, *I. persulcatus*, *I. neotomae*, and *I. pacificus*, and we have found no evidence for higher-than-average chromosomal similarity between Lyme disease agents of a given type isolated from the same host tick species. For example, the *B. burgdorferi* isolates studied from *I. scapularis* are not significantly more like one another than they are like the isolate from *I. pacificus*. We also found no systematic variation with geographic location within each type. For example, the chromosome of *B. burgdorferi* isolate 212 from France is not significantly more different from the North American *B. burgdorferi* isolates than the North American *B. burgdorferi* isolates are different from one another. In addition, the B31 and Sh-2-82 isolates, from *I. scapularis* ticks found on Shelter Island, N.Y., in 1981 and 1982, respectively, were not found to be particularly closely related to one another. Conversely, the two most closely related *B. burgdorferi* isolates, HB19 and WI91-23, are from rather distant locations, Connecticut and Wisconsin, respectively. In addition, the most closely related isolates that we analyzed, *B. garinii* 20047 and FujiP1, which have identical macrorestriction patterns, are from France and Japan, respectively. Although these observations do not disagree with most previous analyses (which typically measured only one or a few loci [e.g., see reference 74]), our identification of very similar isolates from distant locations may indicate that in nature the rate of Lyme disease agent bacterial migration is greater than previously supposed (21).

Lyme disease agent types or species? (i) Multiple Lyme disease agent species? Our studies clearly indicate that most (and quite possibly all) Lyme disease agent spirochetes have similar chromosome structures and gene arrangements. This constancy in cultured strains suggests that rearrangements do not occur at significant frequencies during propagation in culture (see also reference 15). In spite of this overall similarity, the eight Lyme disease agent types are easily distinguishable by the positions of particular restriction endonuclease cleavage sites in their chromosomes, and we have found no convincing clusters of restriction sites characteristic of one type embedded in a chromosome of another type. There does not appear to have been recent, widespread recombination between chromosomes of the different types.

Since plasmids and individual chromosomal loci can in theory be exchanged among related bacterial species, and since even single base pair differences can significantly alter the outcome of some of the tests designed to distinguish among them (e.g., particular protein epitopes or PCR targets), overall chromosomal sequence similarity would seem to be the best genetic criterion for determining the overall phylogenetic relationships among closely related bacterial groups. Restriction site maps are particularly useful in this regard, since they contain small stretches of sequence information at many locations scattered across the chromosome. This strategy for determining phylogenetic relationships is therefore likely to be more robust than methods comparing DNA sequences, RFLPs, antigenic properties (for example) at a single genetic locus. In addition, it is more precise than solution DNA-DNA hybridization and, unlike methods such as arbitrary primer PCR, is not compromised by the presence of more variable plasmid DNAs, which often make up a significant fraction of Lyme disease agent DNA.

Identification of bacterial species, subspecies, and serovars by their macrorestriction fragment patterns is often possible and useful; however, determination of the chromosomal type of Lyme disease agent isolates by simple CHEF electrophoretic display of the chromosomal DNA fragments created by a particular restriction endonuclease may often be insufficient for unambiguous identification, since there is enough diversity within chromosomal types for any given isolate to have RSPs relative to the known isolates (for example, the differences between the *Bss*HII fragments of the chromosomes of isolates G2 and 20047 or of VS461 and R-IP3). Identification of Lyme disease agent isolates by this method thus minimally requires cleavage and electrophoretic analysis of the DNA from the isolate in question by a battery of enzymes and then a decision as to which type is most similar to the patterns observed. Although we believe identification by restriction mapping to be the most definitive available, it is much too laborious to be practical for screening large numbers of isolates. Indeed, our analysis confirms that most previously reported tests involving one or a few loci are sufficient to tentatively classify new isolates.

(ii) Lack of plasmid exchange among Lyme disease agent types. Numerous studies have shown that the linear plasmid-carried *ospA-ospB* operon also falls into multiple nucleotide sequence or antigenic types (e.g., see references 21 and 74). Although these studies have not analyzed members of all eight chromosomal Lyme disease agent types defined here, the related groups derived from linear plasmid-specific properties are almost universally consistent with those groups defined by the chromosomal phylogenetic tree derived here. Thus, there appears not to have been widespread, recent exchange of *ospA-ospB*-carrying plasmids among the Lyme disease agent chromosomal types. In addition, our observation of *ospA-ospB* plasmid size differences among *B. burgdorferi* (53 kbp), *B. garinii-B. afzelii* (58 kbp), and *B. japonica* (90 to 105 kbp) Lyme disease agent types suggests a lack of plasmid exchange among these three groups. Dykhuizen et al. (21) have independently reached a similar conclusion with a substantially different set of 15 Lyme disease agent isolates and different chromosomal typing methods.

Although it is presently not possible to pass objective judgment on the question of whether the different Lyme disease agent types should be classified as separate species or as subgroups within an encompassing species (see references 20 and 21), our data strongly support the existence of at least eight distinct and recognizable types of Lyme disease agent chromosomes. We suggest that if *B. burgdorferi*, *B. garinii*, *B. afzelii*, and *B. japonica* deserve separate species status, then the additional types exemplified by isolates DN127 cl9-2, 21038, 25015, and CA55 should have a similar status.

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