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Physical Map of the Linear Chromosome of the Bacterium *Borrelia burgdorferi* 212, a Causative Agent of Lyme Disease, and Localization of rRNA Genes

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The spirochete *Borrelia burgdorferi*, which causes Lyme disease, and other members of the *Borrelia* genus are unique among characterized bacteria in having a linear chromosome. A restriction map of the chromosome of *B. burgdorferi* 212 was constructed by making extensive use of digestions in agarose blocks of restriction endonuclease fragments or chromosomal DNA that had been purified by pulsed-field gel electrophoresis. A total of 47 digestion sites for the enzymes *Sgr*AI, *Sac*II, *Mlu*I, *Bss*HII, *Eag*I, *Sma*I, *Nae*I, and *Apa*I were located. In most regions of the map, the gap between sites is 50 kbp or less, and 122 kbp is the largest distance between adjacent sites. The mapping data were consistent with previous conclusions that the *B. burgdorferi* chromosome is linear. The total size of the *B. burgdorferi* 212 chromosome was determined to be 946 kbp from the sums of the sizes of *Sac*II, *Mlu*I, *Bss*HII, and *Sma*I fragments, making it one of the smallest known bacterial chromosomes. The rRNA genes were found to be located near the center of the chromosome. One copy of the 16S rRNA gene (*rrs*) and two copies of the 23S rRNA gene (designated *rrlA* and *rrlB*), the latter pair in a tandem repeat, were detected. This particular complement of these two genes has not been reported for another bacterium.

Bacterial genomes can be arranged in several ways. The best-studied example, that of *Escherichia coli* K-12, contains one circular chromosome as determined by genetic mapping and confirmed by physical mapping (17, 26, 29). The presence of one circular chromosome appears to be a property shared by most bacteria (18).

Two exceptions to this arrangement have been reported. One is for *Rhodobacter sphaeroides*, which has two, unique circular chromosomes of quite different sizes (28), and the other is found in the spirochetes, of which members of the genus *Borrelia*, such as *B. burgdorferi*, appear to have a linear chromosome (5, 11).

B. burgdorferi causes Lyme disease (8), a disease of worldwide distribution that is transmitted among vertebrates, including man, usually by ticks of the genus *Ixodes* (8). Lyme disease often manifests itself initially by the skin lesion erythema chronicum migrans (1), followed by a systemic illness involving the nervous system, heart, and joints (13, 27). Genetic studies of this medically important bacterium have been hampered by the absence of genetic tools such as transduction, transformation, or conjugation. To circumvent these deficiencies, we have commenced the elucidation of the genetic map of the *B. burgdorferi* chromosome by other means. Using pulsed-field gel electrophoresis (PFGE), we have determined a restriction map of the chromosome for *Sgr*AI, *Sac*II, *Mlu*I, *Bss*HII, *Eag*I, *Sma*I, *Nae*I, and *Apa*I. These enzymes digest *B. burgdorferi* DNA infrequently. For this determination, extensive use was made of a sequential digestion approach whereby PFGE was used to separate large DNA fragments generated by one endonuclease prior to digestion with a different enzyme and analysis by a second PFGE step.

The availability of the physical map enabled us, by hybridization with specific probes, to locate the position of rRNA genes on the *B. burgdorferi* chromosome, thereby commencing the construction of a genetic map for this organism. The data suggest a novel arrangement in this species of the genes encoding the 16S and 23S rRNA species.

MATERIALS AND METHODS

Bacterial strains and plasmids. *B. burgdorferi* 212 was a low-passage strain recently isolated from *Ixodes ricinus* and kindly given to us by F. Milward. This is a European isolate of the same genospecies as the type strain B31 (2). Because it was still infectious, its chromosome was considered to be wild type for genes essential for infectivity. Plasmid vectors used for cloning were pNEB193 (New England Biolabs, Beverly, Mass.), which is pUC19 (31) with a single site for *Asc*I between the *Bam*HI and *Sma*I sites, and pKNot, which carries the *Sac*I-*Pst*I polylinker fragment from pBluescript (Stratagene, La Jolla, Calif.) in pK19 (21).

PFGE. PFGE was performed in Pharmacia LKB apparatuses (Uppsala, Sweden) with a hexagonal electrode array (Pulsaphor 2015). Unless otherwise specified, 1.2% (wt/vol) agarose gels in 0.5× TBE (1× TBE is 89 mM Tris, 89 mM borate, and 2 mM EDTA [pH 8.0]) were used. Programs of different pulse times were used to optimize band resolution, according to the sizes of the fragments being separated. Fragment sizes were measured by comparison of band mobilities with those of molecular size standards. The standards were multimers of the DNA from a 44.3-kbp bacteriophage λ derivative (9) and *Hin*dIII digests of λcI857 DNA. The technique for two-dimensional PFGE involving sequential restriction endonuclease digestions and electrophoretic separations was carried out as described previously (6, 30).

Preparation of high-molecular-weight genomic DNA. *B. burgdorferi* 212 was grown in BSKII medium (3) in 50-ml flasks containing 45 ml of medium. The flasks were incu-

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TABLE 1. Restriction endonuclease fragments of the chromosome of *B. burgdorferi* 212[a]

Fragment	Mean (SEM) size (kbp) of fragment generated by:															
	*Sgr*AI		*Sac*II		*Mlu*I		*Bss*HII		*Eag*I		*Sma*I		*Nae*I		*Apa*I	
	Observed	Adj[b]	Observed	Adj	Observed	Adj	Observed	Adj	Observed	Adj	Observed	Adj	Observed	Adj	Observed	Adj
A	654 (6)	644	460 (1.1)	458	426 (0.9)	435	459 (1.7)	468	374 (0.5)	379	198 (0.5)	195	299 (1.0)	299	110 (0.6)	110
B	286 (1.2)	275	363 (1.3)	364	164 (0.6)	162	303 (1.0)	298	299 (1.0)	317	134 (0.3)	133	172 (0.9)	172	104 (ND)[c]	
C	27.1 (0.3)	27	93 (0.2)	93	146 (0.4)	145	135 (0.6)	133	181 (0.8)	177	115 (0.3)	114	165 (0.6)	165	96 (ND)	
D			30.7 (0.4)	31	112 (0.2)	111	26.7 (0.2)	27	38.6 (0.7)	39	111 (0.3)	110	153 (0.3)	153	85[d] (0.3)	89
E					54 (0.3)	53	9.7 (0.1)	10	21.6 (0.3)	21	88 (0.3)	87	78 (1.3)	78	69 (ND)	
F					39.8 (0.1)	40	6.9 (0.1)	7	9.7 (0.2)	10	82 (0.2)	81	51 (0.3)	51	59 (ND)	
G							3.3 (0.01)	3	3.3 (ND)	3	52 (0.1)	52	28 (0.5)	28	55 (0.1)	55
H											50 (0.1)	50			51 (ND)	
I											48 (0.1)	48			43 (0.1)	43
J											27.1 (0.1)	27			34[d] (0.2)	39
K											20.6 (0.1)	20			18 (ND)	
L											14.0 (0.1)	14			10.5 (0.02)	11
M											12.2 (0.1)	12				
N											3.3 (0.02)	3				
Total	967		946		942		943		927		955		946		938[d]	

[a] Fragment nomenclature is defined in Materials and Methods.

[b] Adjusted values (Adj) are the observed values after corrections to give a value of 946 for the sum of fragment sizes for each enzyme, except *Apa*I, and after minor adjustments necessary to fit fragments into the map.

[c] ND, not determined; one measurement only.

[d] Relative intensities of ethidium bromide staining suggested the presence of three *Apa*I fragments of 85 kbp (ApD1, ApD2, and ApD3) and two fragments of 34 kbp (ApJ1 and ApJ2). These fragment sizes are included in the total.

bated at 30°C for 5 to 10 days until growth reached 2×10^8 to 4×10^8 bacteria per ml. Cells were harvested, washed in phosphate-buffered saline (8 mM Na_2PO_4, 1.5 mM KH_2PO_4, 0.02% [wt/vol] KCl, 0.8% [wt/vol] NaCl [pH 7.2]), and then incorporated into blocks (2 by 5 by 10 mm) of low-melting-temperature agarose (FMC Corp., Rockland, Maine) for in situ preparation of high-molecular-weight genomic DNA as reported previously (5).

Purification of high-molecular-weight chromosomal DNA. Portions (2 by 5 by 3 mm) of agarose blocks containing high-molecular-weight genomic DNA were subjected to PFGE in 0.5× TPE (1× TPE is 90 mM Tris-phosphate and 2 mM EDTA [pH 8.0]) through 1% (wt/vol) Molecular Biology-Certified agarose (Bio-Rad Laboratories, Richmond, Calif.) for 18 h with a pulse time of 10 s. Gel strips were stained with ethidium bromide to reveal the location of the chromosomal DNA so that blocks of agarose containing unstained chromosomal DNA could be excised from neighboring lanes. The blocks were washed two times with TE (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) for 1 h at 37°C before use or storage at 4°C in TE.

Purification of DNA restriction fragments. Digests of DNA were subjected to PFGE in 0.5× TPE through 1% (wt/vol) Molecular Biology-Certified agarose. The locations of bands of interest were established by staining a second sample of the digest run in the adjacent gel lane. Blocks of agarose were excised and washed as described above.

Digestions of DNA in agarose and fragment nomenclature. Restriction endonucleases *Sgr*AI, *Sac*II (*Ksp*I), *Nae*I, and *Apa*I were from Boehringer (Mannheim, Germany), *Mlu*I and *Sma*I were from Pharmacia, and *Bss*HII and *Eag*I were from New England Biolabs. Genomic DNA, in low-melting-temperature agarose, was digested with 5 to 20 U of restriction endonuclease for 3 to 20 h in 200 μl of the buffer recommended by the supplier. Chromosomal DNA and individual restriction fragments, after purification by PFGE, were digested in excised agarose blocks with the restriction endonuclease (10 U/100 μl of buffer) for 16 to 24 h in the recommended buffer (100 μl/60 μl of agarose block). Fragments produced by digestion with a single restriction endonuclease have been designated Sg, Ml, Sc, Bs, Ea, Sm, Na, or Ap to identify the enzyme (*Sgr*AI, *Mlu*I, *Sac*II, *Bss*HII, *Eag*I, *Sma*I, *Nae*I, and *Apa*I, respectively) with a capital letter suffix, A or B, etc., in the order of decreasing fragment size (Table 1). For fragments indistinguishable in size, the suffix is numbered 1 or 2, etc., e.g., ApJ1 and ApJ2. Fragments produced by digestion with two restriction endonucleases have been designated by the two single-digest fragments from which they were derived; e.g., MlA-SmE in Table 2 contains DNA from the overlapping region of MlA and SmE.

DNA hybridizations. Southern blots of PFGE agarose gels were prepared on Hybond N membranes (Amersham, Amersham, United Kingdom) by vacuum blotting with 0.4 M NaOH as the eluant (22). ^{32}P- and ^{35}S-labelled probes were prepared by random priming (10) with a commercial kit (Boehringer). Hybridizations with *E. coli* rRNA probes were carried out at 55°C for 18 h in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [24]) containing 2.5% (wt/vol) skim milk powder and 10 mg of herring sperm DNA per ml denatured for 30 min at 100°C in 10 mM Tris-HCl (pH 7.5)–10 mM EDTA–5 mM NaOH. Final washing of the membrane was done at 55°C with 2× SSC containing 0.1% (wt/vol) sodium dodecyl sulfate. The probes used were pKK3535 (7), which contains the entire *E. coli rrnB* operon plus 1.5 kbp of flanking DNA on each side in pBR322, and pS1 (23), which contains a 1.8-kbp *Sst*I-*Sac*II fragment from the 23S rRNA gene of pKK3535 in pKNot.

Cloning and analysis of BsG. *Bss*HII digests of *B. burgdorferi* 212 genomic DNA were resolved by constant field gel electrophoresis in low-melting-temperature agarose, and pure BsG was obtained by Qiaex (Qiagen, Studio City, Calif.) extraction of excised agarose blocks. A sample of this DNA was cloned directly into the *Asc*I site of the vector

TABLE 2. Products of digestion of *Mlu*I and *Sma*I fragments of *B. burgdorferi* 212 DNA by *Sma*I and *Mlu*I, respectively

Fragment (size [kbp])[a] after first digestion with:	Products after second digestion[b]
*Mlu*I	
MlA (435)	201(SmA)*a*, 52(SmG)*f2*, 50(SmH)*g*, 48(SmI)*h*, 33(MlA-SmE)*j*, 27(SmJ)*l*, 20(SmK)*m*, 14(SmL)*n2*
MlB (162)	81(SmF)*d*, 68(MlB-SmD)*e*, 12(SmM)*o*
MlC (145)	106(MlC-SmB)*b*, 40(MlC-SmD)*i*
MlD (111)	101(MlD-SmC)*c*, 5.4(MlD-SmE)*p*, 3.2(SmN)
MlE (53)	51(MlE)*f1*
MlF (40)	29(MlF-SmB)*k*, 14(MlF-SmC)*n1*
*Sma*I	
SmA (195)	201(SmA)*a*
SmB (133)	106(MlC-SmB)*b*, 29(MlF-SmB)*k*
SmC (114)	101(MlD-SmC)*c*, 14(MlF-SmC)*n1*
SmD (110)	68(MlB-SmD)*e*, 40(MlC-SmD)*i*
SmE (87)	51(MlE)*f1*, 33(MlA-SmE)*j*, 5.4(MlD-SmE)*p*
SmF (81)	81(SmF)*d*
SmG (52)	52(SmG)*f2*
SmH (50)	50(SmH)*g*
SmI (48)	48(SmI)*h*
SmJ (27)	27(SmJ)*l*
SmK (20)	20(SmK)*m*
SmL (14)	14(SmL)*n2*
SmM (12)	12(SmM)*o*
SmN (3)	3.2(SmN)

[a] Fragments were purified in agarose by PFGE and then digested in situ with the second enzyme. Adjusted map sizes are given in parentheses.

[b] The number indicates the observed size (in kilobase pairs) of the product, its identity is given in parentheses, and the lowercase italic suffix indicates its location in Fig. 2B.

pNEB193. Dideoxy sequencing on a resulting clone, pBsG, was carried out on plasmid DNA by standard procedures.

RESULTS

Restriction digestion patterns of genomic and chromosomal DNA from *B. burgdorferi*. PFGE patterns of undigested high-molecular-weight genomic DNA from *B. burgdorferi* 212 contained a strongly staining band, corresponding to a molecular size of approximately 1 Mbp, and a number of more weakly staining bands running in the 20- to 80-kbp region of the gel (Fig. 1A). Little DNA remained at the origin. This pattern is similar to those reported previously for other isolates of *B. burgdorferi* and is consistent with the conclusion that *B. burgdorferi* has a linear chromosome of approximately 1 Mbp (5, 11) and a number of linear and circular plasmids (4, 16).

Interpretation of the PFGE patterns of digests of this genomic DNA by rare-cutting enzymes such as *Mlu*I, *Nae*I, or *Sgr*AI (Fig. 1A) was made difficult by the presence of the plasmids. Thus, it was not possible to establish readily whether DNA bands of less than 80 kbp were intact plasmids, plasmid fragments, or chromosome fragments. To avoid this problem, high-molecular-weight chromosomal DNA was separated from the remainder of the genomic DNA by PFGE through ultrapure agarose and excised from the gel in blocks of agarose (see Materials and Methods). Subsequently, restriction endonuclease digests of the chromosomal DNA, carried out in the blocks, gave clear PFGE patterns which could be readily interpreted (Fig. 1B).

Because the *B. burgdorferi* genome has a G+C content of 27 to 30% (16), restriction endonucleases with GC-rich recognition sequences were tested for their suitability in the construction of a physical map of the chromosome. *Not*I and *Rsr*II did not cleave the chromosome, and *Nru*I yielded a complex pattern that was difficult to interpret. *Sgr*AI, *Sac*II, *Mlu*I, *Bss*HII, and *Sma*I each yielded between 3 and 14 fragments and were used to prepare a physical map. Subse-

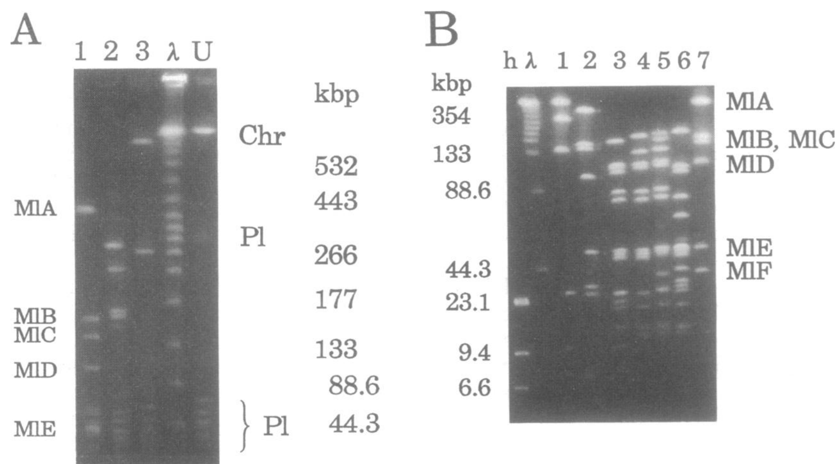

FIG. 1. (A) PFGE of restriction endonuclease digests of *B. burgdorferi* 212 genomic DNA. The lanes contain *B. burgdorferi* genomic DNA digested with *Mlu*I (lane 1), *Nae*I (lane 2), and *Sgr*AI (lane 3), λ DNA concatamers (lane λ), and undigested *B. burgdorferi* 212 genomic DNA (lane U). The locations of *Mlu*I fragments are shown on the left, and the locations of bands containing undigested chromosome (Chr) and plasmid DNA (Pl) are shown on the right. The numbers on the right show the positions of bands for the λ DNA markers of the sizes indicated. The pulse times were 10 s for 10 h and then 35 s for 12 h. (B) PFGE of restriction endonuclease digests of *B. burgdorferi* 212 chromosomal DNA. The lanes contain a *Hin*dIII digest of λ DNA (lane h), λ DNA concatamers (lane λ), and *B. burgdorferi* 212 chromosomal DNA digested with *Bss*HII (lane 1), *Bss*HII + *Mlu*I (lane 2), *Bss*HII + *Sma*I (lane 3), *Sma*I (lane 4), *Nae*I + *Sma*I (lane 5), *Mlu*I + *Sma*I (lane 6), and *Mlu*I (lane 7). The numbers on the left show the positions of bands for the λ DNA markers of the sizes indicated. The locations of *Mlu*I fragments are shown on the right. The pulse times were 3 s for 9.5 h, 5 s for 8.5 h, and 25 s for 4 h.

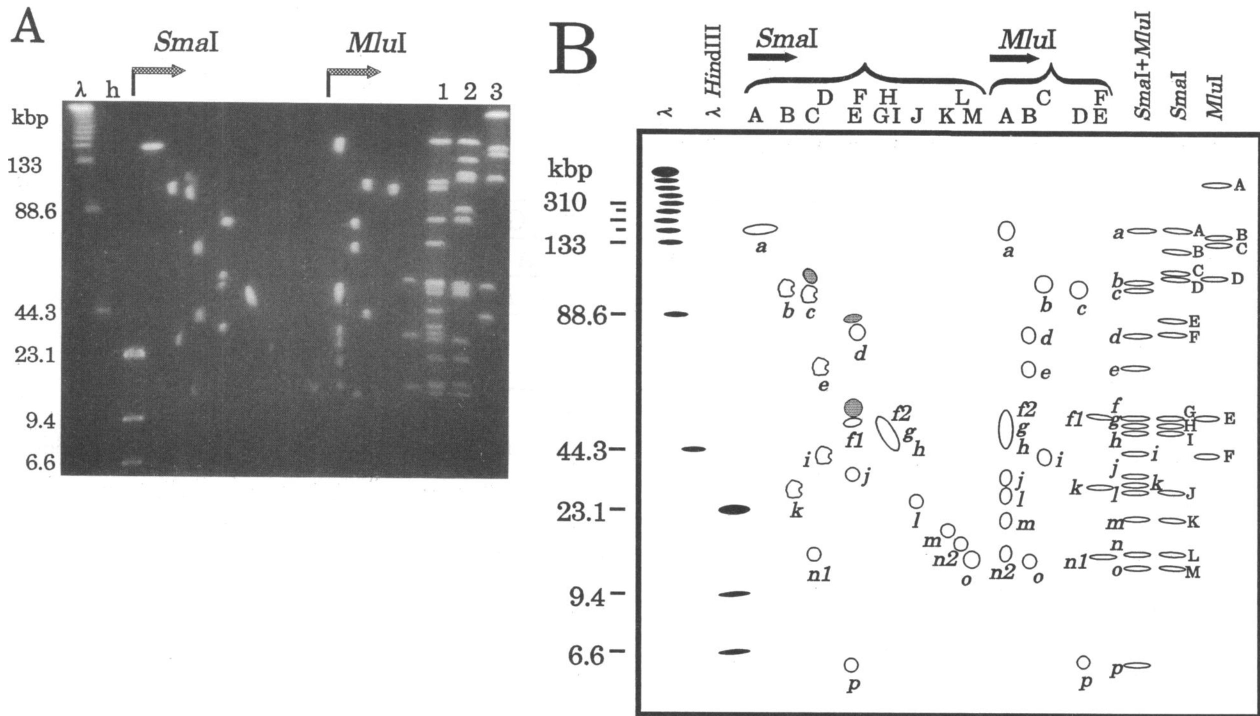

FIG. 2. (A) Two-dimensional PFGE separation of the DNA fragments generated by *Sma*I-*Mlu*I and *Mlu*I-*Sma*I double digests of *B. burgdorferi* 212 chromosomal DNA. The left half of the gel shows the products from *Sma*I fragments, separated by PFGE in the first dimension in the direction of the arrow, and then digested with *Mlu*I; the right half shows the products from *Mlu*I fragments, separated by PFGE in the first dimension in the direction of the arrow, and then digested with *Sma*I. The individual lanes contain λ DNA concatamers (lane λ), a *Hin*dIII digest of λ DNA (lane h), and *B. burgdorferi* 212 chromosomal DNA digested with *Mlu*I + *Sma*I (lane 1), *Sma*I (lane 2), and *Mlu*I (lane 3). The pulse times for the second dimension were 3 s for 10 h, 5 s for 9 h, and 25 s for 3 h. (B) Schematic representation of the results shown in panel A. The uppercase letters alongside the open fragments and across the top show the locations of the *Sma*I and *Mlu*I single-digest fragments, and the italic lowercase letters show the locations of the double-digest fragments. The italic lowercase letters have been used in Table 2 to identify the double-digest fragments. Stippled spots indicate partial digestion products, and closed bands indicate λ DNA size markers. Numbers on the left are the sizes of the size markers at the positions shown by the bars.

quently, the locations on this map of the *Eag*I and *Nae*I sites and some of the sites for *Apa*I were also determined.

The sizes of the fragments produced by each of these enzymes were determined by PFGE (fragments larger than 10 kbp) and constant-field gel electrophoresis (fragments between 1.5 and 10 kbp) and are listed in Table 1. Fragments less than approximately 1.5 kbp would probably not have been detected by ethidium bromide staining. A noteworthy feature of the list of fragment sizes is the presence of a fragment of 3.3 kbp in the digests produced by three different enzymes. This unusual occurrence suggested the presence of a 3.3-kbp tandem repeat in a GC-rich segment of the chromosome.

The sums of the fragment sizes for *Sac*II, *Mlu*I, *Bss*HII, and *Sma*I were in close agreement, with a mean of 946 kbp. Since the size of each fragment generated by these enzymes was measured at least six times, we have used a value of 946 kbp for the molecular size of the *B. burgdorferi* 212 chromosome. When the restriction map for the chromosome was constructed, individual fragment sizes were adjusted proportionately so that the sum of the fragment sizes for each enzyme was 946 kbp.

Mapping of *Mlu*I and *Sma*I sites by two-dimensional separation of reciprocal double digests. The two-dimensional PFGE procedure (6, 30) was used to analyze reciprocal double digests by *Mlu*I and *Sma*I of *B. burgdorferi* 212 chromosomal DNA (Fig. 2A). The single-lane separations showed that there were 6 and 13 fragments larger than 6 kbp in the *Mlu*I and *Sma*I digests, respectively, and 18 fragments in the double digest, counting the double-digest bands *f* and *n* (Fig. 2B) as doublets. The smallest *Sma*I fragment detected in this study, SmN (Table 1), was too small to be retained on this gel. Analyses with constant-field gel electrophoresis indicated that SmN was not digested by *Mlu*I and that it was generated by *Sma*I digestion of MlD. No other fragments of less than 6 kbp were present in *Mlu*I-*Sma*I double digests (data not shown). For a linear DNA molecule, these fragment numbers were consistent with the presence of 5 *Mlu*I and 13 *Sma*I sites. The correspondence in Fig. 2B between the digestion products in the two-dimensional segment of the gel and those in the lane carrying the *Sma*I-*Mlu*I double digest is indicated by the lowercase italic lettering. This correspondence was established from the relative mobilities of the fragments in the different sections of the gel rather than from their absolute mobilities because of uncertainties in the precise location of the origin in such gels. The sizes of these products and the identity of the *Mlu*I and *Sma*I single-digestion fragments from which each was derived are shown in Table 2.

Because each of the *Sma*I fragments contained less than three *Mlu*I sites, it was possible to use these data to deduce unambiguously the following order for the six *Mlu*I fragments: B-C-F-D-E-A (Fig. 3). Concomitantly, the order of the *Sma*I fragments was deduced as (F,M)-D-B-C-N-E-(A,

FIG. 3. Physical map of the chromosome of *B. burgdorferi* 212. Fragment nomenclature is defined in Materials and Methods.

G, H, I, J, K, L). The availability of a map for the *Mlu*I sites and a partial map for the *Sma*I sites provided a basis for mapping the digestion sites of the other endonucleases.

Mapping of the *Sac*II sites. Samples of ScA, ScB, ScC, and ScD in excised agarose blocks were purified by PFGE of *Sac*II digests of *B. burgdorferi* 212 DNA and digested with *Sgr*AI, *Mlu*I, or *Sma*I, and then the final products were separated and sized by further PFGE. The products of *Sac*II digests of purified samples of *Sgr*AI, *Mlu*I, and *Sma*I fragments were also analyzed by PFGE. The resulting data showed that ScA contained MlA and was, therefore, at the right end of the map (Fig. 3 and Table 3). ScC was placed at the left end because it contained SmM and was derived by *Sac*II digestion of MlB; ScB was placed alongside ScC because it yielded MlC, MlD, SmD, SmB, and SmC; and the location of ScD was established because it was produced from MlE.

Mapping of the *Bss*HII sites. Reciprocal *Bss*HII and *Sma*I double digests analyzed by the two-dimensional PFGE procedure indicated that BsF was located at the left end of the map, since it was the only intact *Bss*HII fragment generated from MlB and SmM, and revealed the locations of BsB, BsC, and BsA. BsD was produced from MlA, and since the left boundary of MlA is within BsA (Fig. 3), BsD must be to the right of BsA. BsG was produced from MlD and therefore lies between BsC and BsA, while BsE is located between BsB and BsC because it was generated from SmB and digested by *Mlu*I.

Mapping of the *Sgr*AI sites. Digestion of samples of SgA in excised agarose blocks with either *Sac*II, *Mlu*I, *Bss*HII, or *Sma*I established that SgA is at the left end of the chromosome, since, for example, it yielded all the *Mlu*I fragments except MlA. Because SgB was not digested by *Bss*HII to give BsD, and BsA did not yield SgC, it was concluded that SgB abuts SgA and that SgC is at the right end of the map.

Mapping of the *Sma*I sites and some *Apa*I sites. The relative order of (F,M)-D-B-C-N-E-(A, G, H, I, J, K, L) for the *Sma*I fragments was determined as described above from the data shown in Fig. 2. Subsequently, the digestion of SmM by *Bss*HII to yield BsF indicated that SmM was at the left end of the map and resolved the ambiguity in that region, while the presence of SgC and BsD in *Sgr*AI and *Bss*HII digests of SmA enabled the identification of SmA as the other-end *Sma*I fragment. Both SmG and SmH were placed in undefined order to the right of SmE because both were observed in *Sma*I digests of SgA. SmJ could be positioned across the SgrAI site between SgA and SgB because of a small (1-kbp) reduction in the size of SmJ in *Sgr*AI + *Sma*I double digests. The relative positioning of SmG and SmH and of SmK, SmI, and SmL required the use of an additional enzyme because they were all undigested by *Sgr*AI, *Sac*II, *Mlu*I, or *Bss*HII. The enzyme chosen was *Apa*I, after preliminary studies showed *Apa*I sites in SmH and SmI.

PFGE analyses of *Apa*I digests of *B. burgdorferi* 212 chromosomal DNA revealed 12 bands containing fragments larger than 4 kbp. One of these bands, ApC, apparently contained three fragments, and another band, ApJ, apparently contained two fragments, giving a total of 15 fragments (Table 1). To concentrate on the region of uncertainty in the *Sma*I map, that between 540 and 751 kbp, both SgB and SgA-MlA were purified in excised agarose blocks and subjected to two-dimensional PFGE analyses after reciprocal *Sma*I and *Apa*I digests. In addition, the products of *Apa*I and *Apa*I + *Sma*I digests of MlA excised in agarose were also analyzed by PFGE. The location of ApG at the right end of the map was deduced from its presence in SmA and absence from SgB, and the partial *Apa*I map shown in Fig. 3 was deduced from the data. These data also enabled the relative positions of SmG and SmH and of SmK, SmI, and SmL to be established, thereby completing the elucidation of the *Sma*I map.

Mapping of the *Nae*I sites. Complete digestion of *B. burgdorferi* 212 DNA by *Nae*I was difficult to achieve, with a significant amount of partially digested material present in most digests. An example of this can be seen in Fig. 1A in which the 230-kbp *Nae*I fragment was a partially digested product that contained both NaD and NaE (designated NaDE). NaD itself was the weak band of 153 kbp. The relative yields of different fragments varied significantly from one another, but the variation was reproducible in different digests, indicating that there were considerable differences in the rates of *Nae*I digestion at different sites. Seven *Nae*I fragments were consistently observed in highest yield (Table 1). Two additional fragments, of 22 and 7 kbp, respectively, were seen once, after an extensive *Nae*I digestion of chromosomal DNA. These two fragments were not included in the map in Fig. 3 because the sum of the sizes of the seven other *Nae*I fragments, 946 kbp, was identical to the size previously determined for the chromosome, suggesting that the 22- and 7-kbp fragments were part of either NaA, NaB, NaC, NaD, NaE, NaF, or NaG.

The presence of SmM and SmF in NaD placed NaD at the

TABLE 3. Locations of restriction endonuclease sites in the *B. burgdorferi* 212 chromosome[a]

Restriction endonuclease	Map coordinate of site (kbp)	Fragment	
		Left	Right
*Bss*HII	7	BsF	BsB
*Sma*I	12	SmM	SmF
*Eag*I	22	EaE	EaF
*Eag*I	32	EaF	EaA
*Sac*II	93	ScC	ScB
*Sma*I	93	SmF	SmD
*Nae*I	153	NaD	NaE
*Mlu*I	164	MlB	MlC
*Sma*I	203	SmD	SmB
*Nae*I	231	NaE	NaB
*Bss*HII	305	BsB	BsE
*Mlu*I	307	MlC	MlF
*Bss*HII	315	BsE	BsC
*Sma*I	336	SmB	SmC
*Mlu*I	347	MlF	MlD
*Nae*I	403	NaB	NaA
*Eag*I	410	EaA	EaD
*Eag*I	449	EaD	EaG
*Bss*HII	449	BsC	BsG
*Sma*I	450	SmC	SmN
*Eag*I	452	EaG	EaB
*Bss*HII	452	BsG	BsA
*Sma*I	453	SmN	SmE
*Sac*II	457	ScB	ScD
*Mlu*I	458	MlD	MlE
*Sac*II	488	ScD	ScA
*Mlu*I	511	MlE	MlA
*Apa*I	512	ND[b]	ApL
*Apa*I	523	ApL	ApA
*Sma*I	540	SmE	SmG
*Sma*I	592	SmG	SmH
*Apa*I	633	ApA	ApD2
*Sma*I	642	SmH	SmJ
*Sgr*AI	644	SgA	SgB
*Sma*I	669	SmJ	SmK
*Sma*I	689	SmK	SmI
*Nae*I	702	NaA	NaF
*Apa*I	722	ApD2	ApJ1
*Sma*I	737	SmI	SmL
*Sma*I	751	SmL	SmA
*Nae*I	753	NaF	NaC
*Eag*I	761	EaB	EaC
*Apa*I	761	ApJ1	ND
*Apa*I	891	ND	ApG
*Nae*I	918	NaC	NaG
*Sgr*AI	919	SgB	SgC
*Bss*HII	921	BsA	BsD

[a] The map coordinates are based on measurements of the sizes of single-digestion products (Table 1) and double-digestion products (data not shown). Minor adjustments to fragment sizes (see Table 1) have been made to obtain the best fit of the map to the available data.
[b] ND, not determined.

left end of the map. NaDE also contained these fragments as well as SmD, indicating that it was a partial-digestion product of NaD and the adjacent fragment. This fragment was concluded to be NaE because its size was that of the size difference between NaDE and NaD and because of the absence of another map location for NaE. NaB, NaA, and NaF were placed on the basis of their *Sma*I digestion products, with confirmatory data from *Sgr*AI and *Bss*HII digestions. Since NaC was not digested by *Sma*I, it was placed in SmA, which is the only *Sma*I fragment big enough to contain it. NaG was generated by *Nae*I digestion from

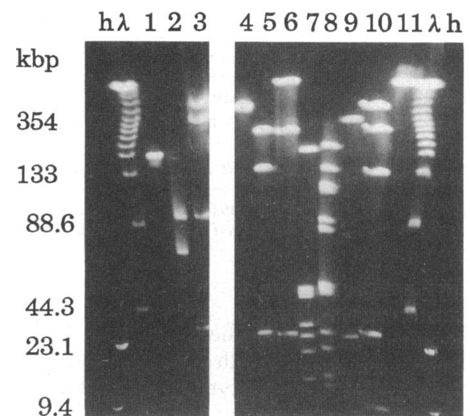

FIG. 4. PFGE patterns of restriction digests of end *Mlu*I fragments from the *B. burgdorferi* 212 chromosome. The lanes contain a *Hin*dIII digest of λ DNA (lanes h), λ DNA concatamers (lanes λ), undigested MlB (lane 1), a *Sac*II digest of MlB (lane 2), a *Sac*II digest of chromosome (lane 3), undigested MlA (lane 4), an *Sgr*AI digest of MlA (lane 5), an *Sgr*AI digest of chromosome (lane 6), an *Sma*I digest of MlA (lane 7), an *Sma*I digest of chromosome (lane 8), a *Bss*HII digest of MlA (lane 9), a *Bss*HII digest of chromosome (lane 10), and undigested chromosome (lane 11). The pulse times were 3 s for 8 h, 5 s for 8 h, and 30 s for 6 h. The numbers on the left show the positions of bands for the λ DNA markers of the sizes indicated.

SmA but not from SgB and must therefore be at the right end of the map. The different yields of partial and limit products in *Nae*I digests indicated that the sites at 153 and 918 kbp were the slowest to digest, with that at 153 kbp being the most refractory.

Mapping of the *Eag*I sites. Samples of the seven *Eag*I fragments (Table 1), with the exception of EaG, were purified in agarose by PFGE and redigested with *Sma*I. Analysis of the sizes of the products enabled the relative order of the five largest fragments to be deduced as EaE, EaA, EaD, EaB, and EaC. To determine the locations of EaF and EaG and to confirm the above order, the products of *Eag*I digests of SmA, SmC, SmD, SmE, SmF, BsD, and MlD samples in agarose were analyzed by PFGE. The results showed that EaF was produced from SmF, placing it between EaE and EaA, and that EaG was produced from MlD but not from SmC, placing it between EaD and EaB.

Linearity of the *B. burgdorferi* 212 chromosome. Previous studies led to the conclusion that the *B. burgdorferi* chromosome is linear (5, 11). Since there are no other known examples of linear bacterial chromosomes, we carried out a simple experiment to see whether this property could be confirmed by another approach. The *Mlu*I fragments MlA and MlB are at the right and left ends, respectively, of the physical map of the *B. burgdorferi* 212 chromosome (Fig. 3). Pure samples of MlA or MlB in excised agarose blocks were obtained from PFGE analyses of *Mlu*I digests of high-molecular-weight genomic DNA. These fragments were digested by either *Sac*II, *Sgr*AI, *Sma*I, or *Bss*HII, and the resulting products were separated by PFGE (Fig. 4). For comparison, a digest by the second endonuclease of the intact chromosome was electrophoresed in the adjacent lane. With each of *Sac*II, *Sgr*AI, *Sma*I, or *Bss*HII, digestion of the *Mlu*I fragment generated only one product that was not present in the digest of the chromosome (Fig. 4). For example, with *Sac*II this product was the faster moving of the two bands, and with *Bss*HII it was the more slowly

TABLE 4. *B. burgdorferi* 212 chromosome fragments that hybridized with rRNA probes[a]

Probe	Gene product	Reference	Hybridizing fragments	Map location
pKK3535	*E. coli rrnB* cluster containing 5S, 16S, and 23S rRNA and $tRNA_2^{Glu}$ genes	7	MlD, MlD-BsC, MlD-BsA, MlD-SmE, BsA, BsC, SmE, SmC	347–458 kbp
pS1	Portion of *E. coli* 23S rRNA gene	23	MlD, MlD-BsA, MlD-SmC, SmC	347–453 kbp

[a] Southern blots of PFGE separations (lower size limit, 5.5 kbp) of the following digests of *B. burgdorferi* chromosomal DNA were probed: *Mlu*I, *Bss*HII, *Sma*I, *Mlu*I + *Bss*HII, *Mlu*I + *Sma*I, and *Bss*HII + *Sma*I. The fragments BsG and SmN (3.3 kbp) were too small to be present in these blots.

moving band. By definition, these products must have been double-digestion products with one *Mlu*I end and one end generated by the second enzyme. Each of these products mapped to the internal end of MlA or MlB for the *Sac*II fragment (Fig. 3). Normally, digestion of a restriction fragment by a second endonuclease produces either no or two such double-digestion products, as exemplified by the *Sma*I products of MlC, MlD, MlE, and MlF (Table 2). The failure to observe the second fragment in each case could result from the presence of a cluster of sites for *Sac*II, *Sgr*AI, *Sma*I, *Bss*HII, and *Mlu*I within a 1.5-kbp region of DNA at the right end of MlA. Given the low G+C content of *B. burgdorferi* DNA, this possibility is most unlikely, although it cannot be excluded absolutely. A more likely explanation is that the double-digestion products containing the other end of MlA or MlB (those from the extremities of the map) were present in the chromosomal digests generated by *Sac*II, *Sgr*AI, *Sma*I, and *Bss*HII and did not require the action of *Mlu*I for their production. This could have occurred only if the chromosome were a linear molecule and if both MlA and MlB each had one *Mlu*I end and one telomere or chromosome end.

Mapping of *B. burgdorferi* genes for rRNA. In Southern blots of strains of *B. burgdorferi* sensu stricto, a single *Eco*RI fragment of 9 kbp hybridizes with *E. coli* 16S and 23S rRNA (20), indicating that the rRNA genes in these strains are contained within a 9-kbp segment of the genome. The approximate map location of the rRNA genes in *B. burgdorferi* 212, which is also a member of the *B. burgdorferi* sensu stricto genomic group (2), was determined by hybridizing Southern blots of PFGE gels with *E. coli* rRNA gene probes. The results indicated a location between 347 and 458 kbp (Table 4).

The nucleotide sequence of *B. burgdorferi* 16S rRNA (18) contains a *Sac*II site and an *Mlu*I site separated by 410 bp but no sites for any of the other enzymes mapped by us in this study. From the hybridization data, it was concluded that these were the sites at 457 and 458 kbp, respectively, on the physical map (Fig. 3 and Table 3) and that they were spanned by a 16S rRNA gene. In the 8 kbp to the left of these sites, we noted a cluster of GC-rich restriction endonuclease sites in a region of the chromosome that contained a number of 3.3-kbp restriction fragments (Fig. 3 and Table 1). Since GC-rich stem and loop structures are a normal feature of rRNA molecules, it seemed possible that this cluster was within genes encoding rRNA species. To investigate this idea further, we cloned one of these 3.3-kbp fragments, BsG, in the vector pNEB193, determined the nucleotide sequence at each end of the fragment, and mapped its *Eag*I and *Sma*I sites.

Comparison of the nucleotide sequences with the GenBank data base (version 69.0) revealed considerable homology with the sequence of 23S rRNA, as shown in Fig. 5 in which the *B. burgdorferi* sequence has been aligned with that of *Bacillus subtilis* 23S rRNA. An unusual feature of the sequence data was that one end of BsG exhibited homology with the nucleotide sequence 3′ to bp 1689 in *B. subtilis* 23S rRNA, whereas the other end was homologous with the sequence 5′ to the same base pair (Fig. 5). The simplest explanation for this observation is that the *B. burgdorferi* 212 chromosome contains tandem copies of the 23S rRNA gene (designated *rrlA* and *rrlB*) and that BsG contains a portion of each (Fig. 6). The presence of *Sma*I and *Eag*I sites in the tandemly repeated region explained the previously noted observation of 3.3-kbp fragments in digests by these enzymes. The sizes of these fragments gave the length of the tandem repeat, assuming that each enzyme digested only once within a repeat. The precise locations of *rrlA* and *rrlB* on the *B. burgdorferi* 212 physical map (Fig. 6) were obtained from the size of the MlD-EaB fragment, by using the *Eag*I site in the sequenced region of BsG as a reference point (Fig. 5).

DISCUSSION

The structure of the *Borrelia* chromosome is of particular interest since it is the only bacterial chromosome thought to be linear. This unusual property raises interesting questions about its evolutionary origin and the mechanism of replication of its telomeres. Answers to these questions require knowledge of the structure and genetic organization of the chromosome. The absence of genetic transfer techniques for the genus *Borrelia* caused us to investigate the chromosome structure by other methods. The physical map reported above provides a basis for the derivation of a genetic map and the elucidation of the chemical structure of the telomeres.

The large complement of plasmids in *B. burgdorferi* made direct analysis of digestion mixtures of genomic DNA impossible, because of uncertainty as to whether individual components were chromosomal fragments, undigested plasmids, or plasmid fragments. Purification of the chromosome in agarose avoided this problem. The mapping procedure made extensive use of the digestion in agarose blocks of this purified chromosomal DNA or of separate restriction endonuclease fragments.

A total of 47 restriction sites were mapped by using eight different restriction endonucleases. In most regions of the map, the gap between sites is 50 kbp or less, which should enable good resolution in the mapping of genetic loci by hybridizing Southern blots with specific gene probes. The largest distance between adjacent sites on the map is 122 kbp, and this region contains one unmapped *Apa*I site. Measurements of the *Sac*II, *Mlu*I, *Bss*HII, and *Sma*I digestion products yielded four independent values for the size of the *B. burgdorferi* 212 chromosome. These values were in close agreement, and the resulting average of 946 kbp provides a reliable estimate for this size. This value is close to those of 1,000 and 950 kbp reported previously for the size of the *B. burgdorferi* B31 chromosome (5, 11). It is hoped that the relatively small size of this chromosome (18) and the availability of the restriction map will provide a stimulus to the elucidation of its complete nucleotide sequence.

```
Bbu  AAATCCGTTA---AGAGAGCTAAGATGTGATGATGAGTGCTATTTAGGTAGCATGAAATG   57
     *********    * *  *** ** *******  *** *  ** ** *****  *** *
Bsu  AAATCCGTTTCCCATAAGGCTGAGCTGTGATGGCGAGCGAAATATA-GTAGC--GAAGTT 1594

Bbu  TAGGTAGTCAAGGTGCCAAGAAATAGCTTCTAAGGTTAGGCTATAAGGGACCGTACCGCA  117
        *    **   ********** *** ****  *  ***  * * * * **********
Bsu  CCTGATTCCACACTGCCAAGAAA-AGCCTCTAGCG--AGGTGAGAGGTGCCCGTACCGCA 1651

                                         rp←‖→up
Bbu  AACCGACACAGGTAGGTGGGATGAAAATTCTAAGGCGCGCGAGAGAATCCACGTTAAGGA  177
     ***** ********** * *  ** *** ****** *  ********  * *********
Bsu  AACCGTCACAGGTAGGCGAGGAGAGAATCCTAAGGTGATCGAGAGAACTCTCGTTAAGGA 1711

Bbu  ACTCTGCAAAATACGTACGTAACTTCGGGATAAGTACGACCTAAGCAATTAGGTA-----  232
     **** *******     ************* ***       *   *  *****
Bsu  ACTCGGCAAAATGACCCCGTAACTTCGGGAGAAGGGG----TGCTCTGTTAGGGTGCAAG 1767

Bbu  ------------GCATAAAAATGGTCCAAACGACTGTTTACCAAAAACACAGGTCTCTGC  280
                 ***   **  ** ***  ********** *******************
Bsu  CCCGAGAGAGCCGCAGTGAATAGGCCCAGGCGACTGTTTAGCAAAAACACAGGTCTCTGC 1827

Bbu  AAATCTGTAAAGAGAAGTATAGGGACTGACACCTGCCCGGTGCTGGAAGGTTAAGAGGAG  340
      ** * **** * *********** ***** *****************************
Bsu  GAAGCCGTAAGGCGAAGTATAGGGGCTGACGCCTGCCCGGTGCTGGAAGGTTAAGAGGAG 1887

Bbu  ATGTTAGTTTATGCGAAGCATTGAATT-AAGCCCCAGTAAACGGCGGCCGTAACTATAA   398
        ****  ** **** *    ***** *******************************
Bsu  CGCTTAGCGTAAGCGAAGGTGCGAATTGAAGCCCCAGTAAACGGCGGCCGTAACTATAA  1946
```

FIG. 5. Comparison of portions of the 23S rRNA sequences of *B. burgdorferi* 212 and *B. subtilis*. The nucleotide sequence of the ends of BsG were determined by using the universal sequencing primer (up) and the reverse primer (rp) for the complementary strand. The *B. subtilis* sequence is from Green et al. (14) and is numbered from the 5′ end. Sequences were aligned by the Dayhoff ALIGN program, with a gap penalty of 5. The *Bss*HII site (GCGCGC) used for cloning and the *Eag*I site (CGGCCG) near 452 kbp (Fig. 6) are underlined.

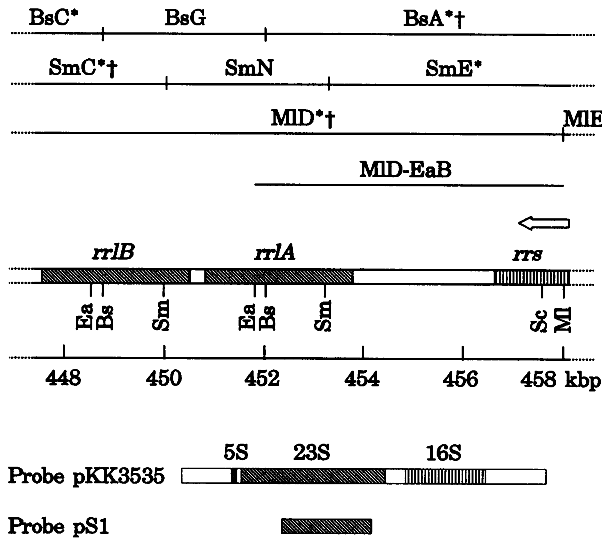

FIG. 6. Map of the region of the *B. burgdorferi* 212 chromosome containing rRNA genes. The locations of individual restriction fragments are shown in the top portion, the map of the *B. burgdorferi* 212 chromosome is shown in the middle, and maps showing the regions of the *E. coli rrnB* operon that were used as probes to locate the *B. burgdorferi* rRNA genes are at the bottom. Asterisks and daggers indicate fragments that hybridized with pKK3535 and pS1, respectively. The 3.3-kbp fragments BsG, SmN, and EaG were not tested for hybridization with either probe. In addition to the points described in the text, the following conditions were used in the map construction. The limits of *rrlA* and *rrlB* with respect to the *Bss*HII sites were defined by assuming homology throughout with *B. subtilis* 23S rRNA; the position of the *Sma*I site was established from digests of cloned BsG; and the direction of transcription of the rRNA genes, shown by the arrow, was deduced from the relative locations of the *Mlu*I and *Sac*II sites (for *rrs*) and from the nucleotide sequence data for BsG (for *rrlA* and *rrlB*).

The linearity of the *B. burgdorferi* chromosome was deduced from the results of initial PFGE analyses of the total genome (5, 11). All the experimental data presented in this paper are consistent with this conclusion, that is, no fragments were observed that circularized the ends of the linear map. To confirm linearity specifically, we subjected purified *Mlu*I fragments (MlA or MlB) that had been placed at the map extremities to separate digestions with four of the other enzymes. In each case, the digestion products contained only one fragment that was not present in a digest of intact chromosome by the corresponding enzyme. Normally, two new fragments would be expected under these circumstances. The observation of only one fragment was consistent with linearity of the chromosome.

The alternative explanation that the chromosome is circular and contains *Sac*II, *Sgr*AI, *Sma*I, *Bss*HII, and *Mlu*I sites clustered within 1.5 kbp is unlikely, given the low G+C content of the *B. burgdorferi* genome and the paucity of sites for all of these enzymes elsewhere in the chromosome. Final proof of the linearity of the chromosome may be achieved by structural analysis of the ends, as has been attained for some of the *B. burgdorferi* linear plasmids (4, 15). Depending on the chemical structure of the ends, this approach could eliminate the unlikely possibility that the linearity was generated during the preparation of high-molecular-weight genomic DNA. Our observation of a small *Bss*HII fragment at one end of the chromosome, and presumably carrying a telomere, has provided a handle for such a structural analysis.

By using *E. coli* rRNA gene probes, it was possible to map the chromosomal location of *B. burgdorferi* rRNA genes. To our knowledge, these are the first chromosomal genetic loci to be mapped for members of the family *Spirochaetaceae*. Using restriction mapping data, in conjunction with the reported sequence of the 16S rRNA (19) and some nucleotide sequence which we determined for the 3.3-kbp fragment BsG, we concluded that there are two tandemly located genes (*rrlA* and *rrlB*) encoding 23S rRNA. The total length of DNA containing *rrlA*, *rrlB*, and *rrs* was calculated to be 10.6

kbp (Fig. 6), a value consistent with the previously reported observation that 16S and 23S rRNA probes hybridize with a 9-kbp *Eco*RI fragment of the *B. burgdorferi* 212 genome (2, 20). The presence of a second tandem copy of *rrs* was ruled out because it would require the total length of the DNA containing *rrl* and *rrs* genes to be 12.1 kbp, significantly larger than the 9-kbp *Eco*RI fragment shown to carry these genes (2, 20). A similar conclusion concerning the number and arrangement of *rrl* and *rrs* genes was reached independently by Schwartz et al. from the results of a series of elegant experiments involving PCR amplification of DNA from several *B. burgdorferi* strains (25). In addition, Schwartz et al. observed two 5S rRNA genes, each 22 bp downstream of the 3′ end of a 23S rRNA gene.

This juxtaposition of rRNA genes contrasts sharply with the situation found for most other prokaryotes in which rRNA genes are present in transcriptionally linked operons in the order 16S, 23S, and 5S. A few exceptions to this arrangement have been reported, including an example of another spirochete *Leptospira interrogans*, which has unlinked rRNA genes with two copies of 23S, two copies of 16S, and only one copy of 5S (12). However, we are unaware of any reports of a bacterium that possesses two 23S rRNA genes but only one 16S rRNA gene. The three genes in *B. burgdorferi* have the same orientation and may be transcribed as a single transcriptional unit, even though there is no evidence on this point. Given the requirement of the cell to synthesize equivalent molar amounts of 23S and 16S RNA, the twofold difference in gene dosage presents an interesting problem in genetic regulation, assuming that both 23S genes are expressed.

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