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Physical and Genetic Map of the *Serpulina hyodysenteriae* B78^T Chromosome

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A combined physical and genetic map of the *Serpulina hyodysenteriae* B78^T genome was constructed by using pulsed-field gel electrophoresis and DNA blot hybridizations. The *S. hyodysenteriae* genome is a single circular chromosome about 3.2 Mb in size. The physical map of the chromosome was constructed with the restriction enzymes *Bss*HII, *Ecl*XI, *Not*I, *Sal*I, and *Sma*I. The physical map was used to construct a linkage map for genes encoding rRNA, flagellum subunit proteins, DNA gyrase, NADH oxidase, and three distinct hemolysins. Several *flaB2*-related loci, encoding core flagellum subunit proteins, were detected and are dispersed around the chromosome. The rRNA gene organization in *S. hyodysenteriae* is unusual. *S. hyodysenteriae* has one gene each for 5S (*rrf*), 16S (*rrs*), and 23S (*rrl*) rRNAs. The *rrf* and *rrl* genes are closely linked (within 5 kb), while the *rrs* gene is about 860 kb from the other two rRNA genes. Using a probe for the *S. hyodysenteriae gyrA* gene, we identified a possible location for the chromosomal replication origin. The size and genetic organization of the *S. hyodysenteriae* chromosome are different from those of previously characterized spirochetes.

Serpulina hyodysenteriae causes swine dysentery, an intestinal disease of pigs. The genus *Serpulina* is a distinct line of descent in spirochete evolution (26, 33) and has two recognized species: *S. hyodysenteriae* and *S. innocens*, a nonpathogen (26, 30, 33).

Little is known about the genetics or molecular biology of *Serpulina* spp., including *S. hyodysenteriae*. Genetic exchange between *S. hyodysenteriae* cells has not been reported, and only recently has this bacterium been transformed (34). Therefore, classical genetic studies, which have often proved useful for studying the genetics of pathogenicity in many bacteria, have not been applied to *S. hyodysenteriae*. Because spirochetes constitute a unique but diverse branch in eubacterial evolution (26, 37), the genetics of these bacteria are of particular interest.

A few spirochetal genomes have been characterized by pulsed-field gel electrophoresis (PFGE). The results of those studies show that the sizes and structural organizations of spirochete genomes are varied. Unusual features among *Borrelia* spp. include linear chromosomes (4, 7) and linear plasmids (27). Evidence suggesting that *Leptospira interrogans* has two circular chromosomal replicons has recently been presented (40). Spirochete genomes range in size from 1 Mb in *Treponema pallidum* (28, 36) to 4.75 to 5 Mb in *L. interrogans* (1, 38).

Genetic maps have been constructed for only two spirochetes, *Borrelia burgdorferii* (4, 7, 25) and *L. interrogans* (1, 40). The limited comparison that is possible between these two genetic maps indicates that the genetic organizations of these two spirochetes are different. Further, there is considerable heterogeneity in genomic organization within *L. interrogans* (40), making intragenic comparisons difficult. Additional studies show that some spirochetal genes are organized in uncommon or unique patterns (e.g., the rRNA genes [1, 9, 10, 29, 40]).

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To establish a foundation for further genetic analysis, we used PFGE and Southern blot analysis to construct a physical map of the *S. hyodysenteriae* B78^T genome. The positions of genes encoding rRNA, DNA gyrase, flagellar proteins, hemolysins, and NADH oxidase were also located on this map.

(Preliminary reports of this research were presented at the Conference of Research Workers in Animal Disease [33a] and at the 93rd General Meeting of the American Society for Microbiology [33b]).

MATERIALS AND METHODS

DNA preparation and gel electrophoresis. *S. hyodysenteriae* B78^T, the type strain for *S. hyodysenteriae* (30, 33), was used for all studies. Bacteria were cultured in brain heart infusion broth containing 10% fetal bovine serum beneath an atmosphere of 99% N_2 and 1% O_2 (33). Cells in the exponential growth phase were harvested, and genomic DNA was encapsulated in agarose beads as described previously (38). DNA samples were digested with restriction enzymes and then separated by clamped homogeneous electric field gel electrophoresis as described previously (38).

After electrophoresis, DNA was stained with ethidium bromide and visualized with UV light. Restriction fragment lengths were calculated by comparing the migrations of the fragments through gels with those of linear DNA standards. These standards included a 1-kb ladder (0.5 to 12 kb; Gibco-BRL, Gaithersburg, Md.), digests of λc1857 (2 to 23 kb), concatemers of λc1857 (monomer = 48.5 kb; New England Biolabs, Beverly, Mass.), and *Saccharomyces cerevisiae* (210 to 2,500 kb; New England Biolabs), *Hansenula wingeli* (1 to 3.3 Mb; Gibco-BRL), and *Schizosaccharomyces pombe* (3 to 5.7 Mb; Gibco-BRL) chromosomes.

Southern blot analysis. Following gel electrophoresis, genomic DNA was fragmented in the gel by UV irradiation, denatured, and transferred to nylon membranes (Hybond-N; Amersham Corp., Arlington Heights, Ill.) (38). DNA hybridization analysis was done as described previously (39, 40). Radioactive probes were synthesized either with [α-^{32}P]dATP (ca. 3,000 Ci/mmol) by nick translation or with [α-^{32}P]UTP (ca.

650 Ci/mmol) (ICN Pharmaceuticals, Irvine, Calif.) by runoff transcription (39). Probes were synthesized from either genomic restriction fragments isolated from agarose gels with Gelase (Epicenter Technologies, Madison, Wis.), specific cloned DNA, or PCR products.

PCRs. Probe templates for rRNA and flagellum genes were prepared by PCR amplification of genomic DNA. Genomic DNA was prepared as described previously (35). The *L. interrogans rrf*, *rrs*, and *rrl* gene sequences were amplified by PCR as described previously (40). Probes for the *S. hyodysenteriae flaA* and *flaB2* genes were synthesized from the following primer sets: 5′-GCGGTGCTGATGGTACTAA-3′ and 5′-TAGCAGCAGCACCTTGATC-3′, amplifying sequences between bp 309 and 857 of the *flaA* coding region (17), and 5′-TATGGCTGCTCTTTCTTCTG-3′ and 5′-GCTCTAATC TGTTTTGGTAA-3′, amplifying between bp 77 and 671 of the *flaB2* coding sequence (15). PCRs were done according to the recommendations of the manufacturer (Perkin-Elmer, Norwalk, Conn.) with the following conditions: 1.2 min at 95°C, 1 min at 51°C, and 3 min at 72°C. Amplified DNA products were ethanol precipitated and then labeled directly.

RESULTS

Estimation of the size of the *S. hyodysenteriae* genome. As a first step in characterizing the *S. hyodysenteriae* genome, it was necessary to identify restriction enzymes which digest genomic DNA into only a few fragments. Several enzymes were tested which were expected to recognize only a few sites in the *S. hyodysenteriae* genome due to its low (26 to 28%) G+C content (21). The enzymes *Asc*I, *Sfi*I, *Sgr*AI, and *Srf*I did not digest *S. hyodysenteriae* DNA. Several enzymes (*Apa*I, *Pvu*II, *Sac*II, *Spe*I, *Sph*I, and *Xba*I) generated too many fragments which were not resolved. Although the enzymes *Bgl*I, *Mlu*I, *Nar*I, *Spl*I, and *Xho*I produced many fragments which were difficult to resolve, each of these enzymes produced some fragments larger than 250 kb, some of which helped in constructing the physical map. The best digestion results were obtained with *Bss*HII (two sites), *Ecl*XI (seven sites), *Not*I (one site), *Sal*I (six sites), and *Sma*I (six sites) (Fig. 1). These enzymes were used to determine the size and to construct a physical map of the *S. hyodysenteriae* genome.

By summing the sizes of fragments generated with enzymes *Bss*HII, *Ecl*XI, *Sal*I, and *Sma*I, the size of the *S. hyodysenteriae* genome was determined to be about 3.2 Mb. The results of this analysis are summarized in Table 1. Most undigested *S. hyodysenteriae* genomic DNA remained at the gel origin, even under conditions in which linear fragments of >3 Mb were well resolved (data not shown). This result suggests that the genome is circular and contains a single replicon.

Construction of a physical linkage map of the *S. hyodysenteriae* genome. A physical map of the *S. hyodysenteriae* genome was constructed with *Bss*HII, *Ecl*XI, *Not*I, *Sal*I, and *Sma*I (Fig. 2). The unique *Not*I site in the chromosome was used as a reference point and is placed at the top of the map (Fig. 2).

Initial restriction fragment linkages were determined by analysis of PFGE-separated restriction fragments generated with one or two enzymes. The unique *Not*I site is located within the *Bss*HII A (BsA), *Sal*I A (SaA), and *Sma*I B (SmB) fragments (Fig. 1 and Table 1). These results show that the BsA, SaA, and SmB fragments overlap. Digestion of genomic DNA with different combinations of enzymes showed the presence of additional overlapping fragments (Table 1).

Construction of the physical map of the *S. hyodysenteriae* genome was completed by Southern blot analysis using PFGE-separated genomic restriction fragments purified from agarose gels and several gene probes. The data from these experiments are summarized in Table 2 and established the linkage groups BsA-B, EcA-E-D-B-F-C-G, SaA-F-C-B-E-D, and SmA-B-F-D-E-C. Because each restriction fragment is contiguous with two other fragments generated with the same enzyme and because the single *Not*I site is within the BsA, SaA, and SmA fragments, we conclude that the *S. hyodysenteriae* genome consists of a single circular replicon (Fig. 2).

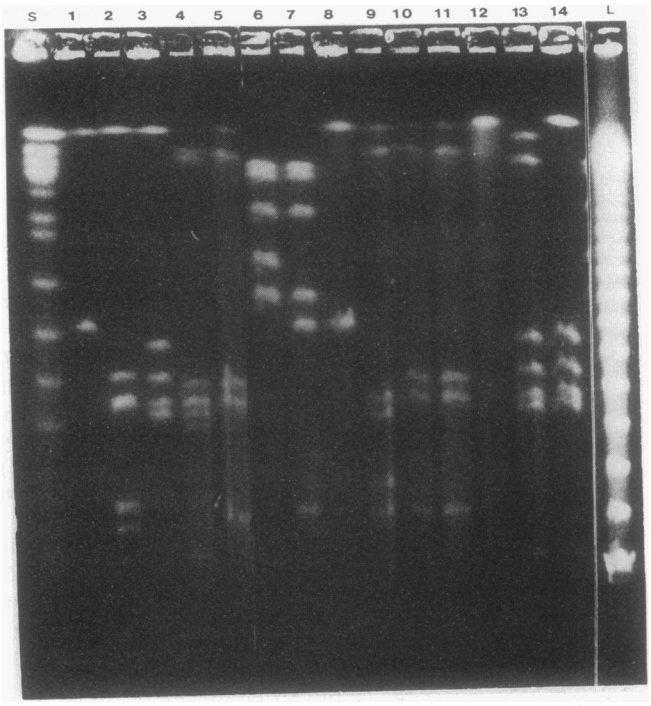

FIG. 1. Separation of *S. hyodysenteriae* DNA by PFGE. Agarose-encapsulated genomic DNA was digested with *Bss*HII (lane 1), *Bss*HII and *Sal*I (lane 2), *Sal*I (lane 3), *Sal*I and *Sma*I (lane 4), *Sma*I (lane 5), *Ecl*XI (lane 6), *Ecl*XI and *Bss*HII (lane 7), *Bss*HII (lane 8), *Bss*HII and *Sma*I (lane 9), *Sma*I (lane 10), *Sma*I and *Not*I (lane 11), *Not*I (lane 12), *Not*I and *Sal*I (lane 13), and *Sal*I (lane 14) and separated by PFGE at 200 V for 20 h (pulse times, 5 to 50 s) and 2 h (pulse times, 70 to 90 s). Migrations of size markers are shown in lanes S (*S. cerevisiae* chromosomes) and L (λcI857 multimers).

Construction of a genetic map. The physical map was used as a guide for localizing specific genes (Table 3) on the *S. hyodysenteriae* chromosome. Hybridization results with several of these probes were also useful in improving the resolution of the physical map.

rRNA genes. Southern blot analysis of small (<12-kb) fragments showed that *S. hyodysenteriae* possesses one gene each for the 16S (*rrs*), 23S (*rrl*), and 5S (*rrf*) rRNAs (data not shown). Hybridization analysis of PFGE-separated fragments showed that the *rrf* and *rrl* genes are both on the SmA fragment (Table 2). Further, the *rrf* and *rrl* genes are on different, but adjacent, *Sal*I and *Ecl*XI fragments (Table 2). Analysis of small restriction fragments (<12 kb) showed that the *rrf* and *rrl* probes hybridized with several small restriction fragments in common (data not shown), placing these genes within 5 kb on the physical map. Hybridization with the *rrs* probe showed that this gene is about 860 kb from *rrl* or *rrf* (Fig. 2).

Hemolysin genes. Three *S. hyodysenteriae* genes conferring hemolytic activity to transformed *Escherichia coli* have been cloned (23, 24). One of these genes was further characterized and designated *tly* (34). For convenience these genes will be

TABLE 1. Genome size of *S. hyodysenteriae*

Fragment	Size (kb) with the following restriction enzyme(s):								
	*Bss*HII	*Bss*HII, *Not*I	*Ecl*XI	*Bss*HII, *Sal*I	*Sal*I	*Not*I, *Sal*I	*Sma*I	*Not*I, *Sma*I	*Sal*I, *Sma*I
A	2,900	1,500	870	2,100	2,100	1,300	1,600	1,600	1,050
B	350	1,400	810	285	320	800	960	850	940
C		350	600	240	285	320	280	280	280
D			490	225	240	285	250	250	250
E			420	115	230	240	100	110	230
F			55	110	65	230	20	100	215
G			25	95		65		20	80
H				65					60
I									45
J									25
K									20
Total	3,250	3,250	3,270	3,235	3,240	3,240	3,210	3,210	3,195

referred to as *tlyA* (clone pJBA), *tlyB* (clone pSH1), and *tlyC* (clone pS10). Both *tlyA* and *tlyB* are located on the EcC and SmB fragments. The *tlyC* gene is on the EcB and SaD fragments (Fig. 2).

NADH oxidase gene. The *S. hyodysenteriae nox* gene encodes NADH oxidase, an enzyme which is thought to enable the bacteria to survive in an oxygen-containing environment (32). The *nox* gene is located on the EcA fragment (Fig. 2).

Flagellum genes. *S. hyodysenteriae* flagella are complex structures composed of several distinct proteins (14, 16, 22).

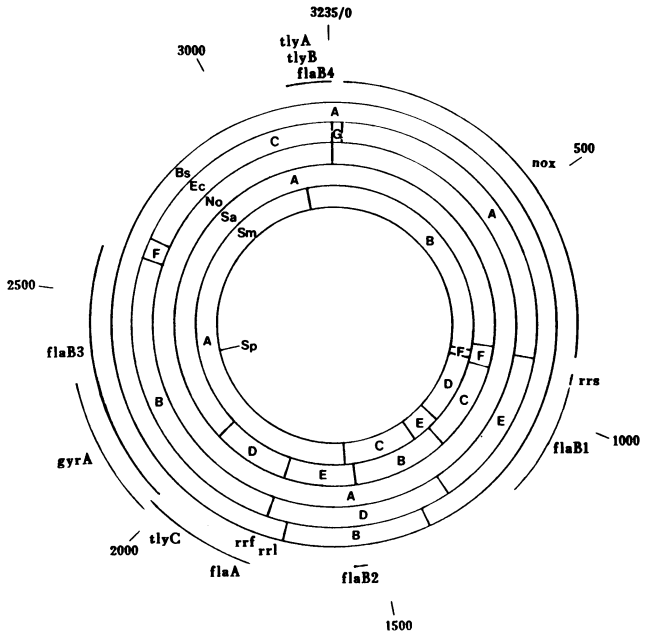

FIG. 2. Combined physical and genetic map of *S. hyodysenteriae* B78. The physical map is positioned with the *Not*I site at the top. The positions of sites for the restriction enzymes *Bss*HII (Bs), *Ecl*XI (Ec), *Not*I (No), *Sal*I (Sa), and *Sma*I (Sm) are indicated in concentric circles, starting from the outer circle. Fragments are labeled as in Table 1. The approximate position of the *Spl*I (Sp) site used to localize the *gyrA* gene is shown on the inner circle. Reference marks showing 500-kb segments of the chromosome are indicated on the outside of the map. The locations of genes on the physical map are shown. Approximate positions for genes are indicated by lines spanning the area where each gene is located.

Two *S. hyodysenteriae* flagellum genes, *flaA* and *flaB2*, have been cloned (15, 17). The *flaA* gene encodes one of two sheath proteins (17). The *flaB2* gene encodes one of several core flagellum proteins (15). Under low-stringency hybridization conditions, the *flaB2* probe hybridizes with multiple fragments, suggesting the presence of several *flaB*-related genes in the *S. hyodysenteriae* genome (15). We used low-stringency hybridization conditions with both probes to detect *flaA*- and *flaB2*-related sequences. The *flaA* probe hybridized to a single site in the *S. hyodysenteriae* genome (Fig. 2). Under the lowest stringency conditions used (approximately 40% base mismatch), four regions of the *S. hyodysenteriae* genome hybridized with the *flaB2* probe (Fig. 3A). The results of washing the blot under more stringent conditions (allowing for approximately 29% [data not shown] and 8% [Fig. 3B] base mismatch), were similar and three regions of the *S. hyodysenteriae* genome were detected under these conditions. One of the *flaB*-hybridizing regions (designated *flaB2* [see Discussion]) produced a stronger hybridization signal than the other regions (which we designate *flaB1*, *flaB3*, and *flaB4*). The *flaB*-hybridizing regions are not adjacent on the chromosome, nor are they close to the *flaA* gene (Fig. 2).

Gyrase gene. The *gyrA* gene is located near the chromosomal origin of replication (*oriC*) of all bacteria studied to date (18). Therefore, we used a probe for the *S. hyodysenteriae gyrA* gene (12) to identify a possible location for *oriC*. This probe hybridized with the EcB, SaA, and SmA fragments. The *gyrA* gene also hybridized to a 290-kb *Spl*I fragment in common with *tlyC*, further narrowing the area where this gene could be located.

DISCUSSION

This paper reports the first characterization of the *S. hyodysenteriae* genome by PFGE. Data from this analysis have led to construction of a physical map of the *S. hyodysenteriae* B78^T genome. Only a small number of *S. hyodysenteriae* genes have been cloned, and many of these genes (Table 3) were used to construct a genetic linkage map (Fig. 2). By comparing the physical and genetic organizations of *S. hyodysenteriae* with those of other spirochetes, we provide further data showing variation between spirochete genomes.

The *S. hyodysenteriae* genome is about 3.2 Mb in size. The *S. hyodysenteriae* genome is similar in size to genomes of many free-living bacteria and is of an intermediate size compared with those of other spirochetes. *B. burgdorferi* (4, 7) and *T.*

TABLE 2. Probes used to construct a physical map of the *S. hyodysenteriae* genome

Probe	Restriction fragment(s) hybridized[a]				
	*Bss*HII	*Bss*HII-*Not*I	*Ecl*XI	*Sal*I	*Sma*I
*Bss*HII B	B	ND[b]	D	B/E	A/C
*Ecl*XI E	A	ND	E	C/F	D/E
*Ecl*XI F	A	A	F	A	A
*Ecl*XI G	A	ND	G	A	B
*Sal*I B	A/B	ND	D/E	B	C/E
*Sal*I C	A (B)	B (A, C)	E (A, B, C, D)	C (A)	D/E (A, B)
*Sal*I F	A (B)	ND	A/E (C, D)	F (A, E)	B/D/F (A)
rrl	A	ND	D	E	A
rrf	A	ND	B	D	A

[a] /, hybridization with more than one restriction fragment due to overlapping fragments. Fragments in parentheses gave substantially weaker hybridization signals and are not necessarily adjacent. These weak signals are thought to arise from the presence of repeated sequences contained in the genomic restriction fragment.
[b] ND, not determined.

pallidum (28, 36) have genomes approximately 1 Mb in size, while *L. interrogans* has a genome 4.75 to 5 Mb in size (1, 38).

The *S. hyodysenteriae* genome consists of a single circular chromosome. Electrophoretic analysis showed that undigested genomic DNA failed to migrate from the gel origin, suggesting the presence of large circular molecules. Furthermore, physical mapping experiments established that this genome contains a single circular linkage group. Although most bacteria are thought to have single circular chromosomes, the structural organizations of two spirochetal genomes are different. The *B. burgdorferi* chromosome is linear (4, 7), while *L. interrogans* may have two circular chromosomal replicons (40).

No extrachromosomal DNA was seen in *S. hyodysenteriae* B78^T, although extrachromosomal DNA is present in other spirochete genera. Restriction fragments from different regions of the *S. hyodysenteriae* genome hybridized with the SaC and SaF fragments (Table 2). These results suggest that *S. hyodysenteriae*, like other spirochetes, may contain repetitive DNA.

Several genes were located on the physical map of the *S. hyodysenteriae* genome by using specific probes. To identify a possible location for the chromosomal replication origin, we used a probe for the *S. hyodysenteriae gyrA* gene. Because the *gyrA* gene is near the chromosomal replication origin (*oriC*) of all bacteria studied to date (18), it is often used as a marker for *oriC*. We speculate that the region of the chromosome near the *S. hyodysenteriae gyrA* gene functions as the replication origin. However, more experiments are needed to determine whether this region of the *S. hyodysenteriae* chromosome functions in replication initiation. Although potential replication origins have been identified in *B. burgdorferi* (4, 25) and *L. interrogans* (40), demonstration of replication initiation at these sites is also needed.

S. hyodysenteriae has one copy for each of the rRNA genes *rrs* (16S), *rrl* (23S), and *rrf* (5S). In contrast, most bacterial genera studied to date possess multiple copies of each rRNA gene (18). In *S. hyodysenteriae* the rRNA genes are organized in an unusual way. Instead of the more common *rrn* operon structure (*rrs-rrl-rrf*) (18), the *S. hyodysenteriae rrf* and *rrl* genes are approximately 860 kb from *rrs* (Fig. 2). Three other bacteria with a similar rRNA gene organization are known: *Mycoplasma gallisepticum* (6), *Pirellula marina* (19), and *Thermus thermophilus* (2, 11).

Although spirochetes have a monophyletic origin (26, 37), the copy number and organization of rRNA genes differ

TABLE 3. Gene probes used for this study

Gene	Function	Species of origin	Reference
flaA[a]	Flagellum sheath	*S. hyodysenteriae*	17
flaB2[a]	Flagellum core	*S. hyodysenteriae*	15
gyrA	DNA gyrase	*S. hyodysenteriae*	12
nox	NADH oxidase	*S. hyodysenteriae*	31
pJBA (*tlyA*)	Hemolysin	*S. hyodysenteriae*	24
pSH1 (*tlyB*)	Hemolysin	*S. hyodysenteriae*	23
pS10 (*tlyC*)	Hemolysin	*S. hyodysenteriae*	23
rrf[a]	5S rRNA	*L. interrogans*	40
rrs[a]	16S rRNA	*L. interrogans*	40
rrl[a]	23S rRNA	*L. interrogans*	40

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

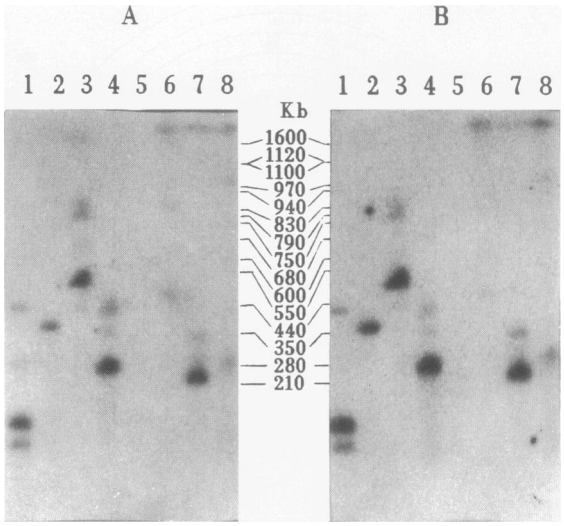

FIG. 3. Hybridization analysis with a *flaB2* probe. *S. hyodysenteriae* DNA was cut with *Bgl*I (lanes 1), *Bss*HII (lanes 2), *Ecl*XI (lanes 3), *Mlu*I (lanes 4), *Nar*I (lanes 5), *Rsr*II (lanes 6), *Sal*I (lanes 7), and *Sma*I (lanes 8), separated by PFGE, transferred to Hybond-N, and hybridized with the *flaB*$_2$ probe at 50°C. The membrane was washed under conditions of low (4× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate–0.1% sodium dodecyl sulfate [SDS] at 50°C), medium (2× SSC–0.1% SDS at 56°C), and high (0.2× SSC–0.1% SDS at 60°C) stringency, with film exposures performed between different wash conditions. The resulting autoradiographs are shown for low (A)- and high (B)-stringency washes. The migrations of size markers are shown in the center.

between different spirochete genera. In *Treponema phagedenis* and *T. pallidum* the rRNA genes appear to be arranged in two typical *rrn* operons (8). A single rRNA locus is found in most *Borrelia* spp., with *rrs* separated from *rrl* and *rrf* by a small segment of DNA (~4 kb) (10, 29). In *B. burgdorferi* the *rrf-rrl* cluster is duplicated and immediately adjacent to the *rrs-rrl-rrf* cluster (9, 29). Pathogenic *Leptospira* spp. possess two copies each of *rrs* and *rrl* and one copy of *rrf* (1, 7a, 40). Nonpathogenic leptospires contain two copies of each rRNA gene (7a). The rRNA genes of *Leptospira* spp. are dispersed around the genome (1, 40). Different patterns in rRNA gene organization are seen among various branches of spirochete evolution, as depicted by Paster et al. (26). Further study is needed to determine whether rRNA gene organization reflects phylogenetic relationships among spirochetes.

Four genes presumed to be important in interactions between *S. hyodysenteriae* and its host were mapped on the chromosome. Three of these genes confer hemolytic activity to *E. coli* (23, 24). Inactivation of one of these hemolysin genes (*tlyA*) appears to decrease *S. hyodysenteriae* virulence in mice (34). The roles of the other two hemolysin genes in virulence are unknown. A fourth gene, *nox*, encodes NADH oxidase (31). This enzyme is thought to enhance the ability of *S. hyodysenteriae* to survive in a microaerobic environment such as the intestinal mucosa (32). Two of the hemolysin genes (*tlyA* and *tlyB*) are near the *Not*I site and are within 110 kb of each other. The other hemolysin gene (*tlyC*) is near *rrl-rrf*.

The *S. hyodysenteriae* periplasmic flagella are complex structures consisting of a core filament surrounded by a sheath (14, 16, 22). Several proteins compose the core and sheath structures (14, 16, 22). Genes encoding spirochete core proteins (the *flaB* family) often have regions with similar nucleotide sequences (5, 20). Several copies of *flaB*-related sequences were detected in blots of PFGE- (Fig. 3) and continuous-field gel electrophoresis-separated fragments (reference 15 and data not shown) under low-stringency hybridization conditions. We designate these loci *flaB1*, *flaB2*, *flaB3*, and *flaB4* on the basis of their locations relative to the *Not*I site in the chromosome. A stronger hybridization signal was seen with the *flaB2* locus (Fig. 3), suggesting that this locus either is the homologous *flaB2* gene or may contain multiple *flaB*-related genes. Two of the *T. pallidum flaB* genes (*flaB1* and *flaB3*) are located together (5). Multiple *flaB*-related genes have also been observed in *T. phagedenis* (20). One *flaA*-hybridizing sequence (a sheath protein gene) was detected in the *S. hyodysenteriae* genome under low-stringency hybridization conditions. This result is similar to hybridization results obtained by using cloned *flaA* genes from *Spirochaeta aurantia* (3) and *T. pallidum* (13).

We expect that this genetic map will be a useful tool for further genetic analysis of *S. hyodysenteriae*. Because there are no known mechanisms for genetic exchange among *S. hyodysenteriae* cells, this map can be used to establish additional genetic linkages and provide a basis for comparing different members of this species.

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