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Physical Map of the *Treponema denticola* Circular Chromosome

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A physical map of the *Treponema denticola* ATCC 33520 genome was constructed by pulsed-field gel electrophoresis and DNA hybridization. The organism possesses a single, circular chromosome of approximately 3.0 Mbp and a 2.6-kbp circular plasmid, pTD1. The physical map of the A+T-rich genome was constructed with the rare-cutting restriction enzymes *Asc*I, *Not*I, and *Srf*I, which have 8-bp G+C-rich recognition sites. The genes *flgE*, *tdpA*, and *prtB* encoding the flagellar hook protein, a 53-kDa immunogenic protein, and chymotrypsinlike protease, respectively, were located on the map. This treponeme was found to have two copies of each of the rRNA genes, as has been found to be the case for both *Treponema phagedenis* and *Treponema pallidum*.

The oral spirochete *Treponema denticola* is frequently implicated in both the initiation and the progression of periodontal diseases (20, 32). However, the complexity of the oral flora and the fastidious nature of this particular organism make its precise role difficult to ascertain. Many features of this bacterium are indicative of its potential to destroy host tissue and evade the host immune response. These include motility (6), the production of proteolytic enzymes (25, 36), the synthesis of a factor that inhibits polymorphonuclear leukocyte function (6), and the ability to attach to host epithelial cells (16, 27).

Although some genes from *T. denticola* have been cloned and expressed in *Escherichia coli* (1, 16, 18, 22), genetic studies of this species, in common with those of other pathogenic spirochetes, have been limited. A cryptic plasmid of 2.6 kb, pTD1, has been found to replicate stably in strains of *T. denticola* (17). Sequence analysis of pTD1 has shown that it has strong homology with a group of plasmids common to gram-positive organisms, the single-stranded DNA plasmids (21). This was unexpected in view of the considerable evolutionary distance of spirochetes from either gram-positive or gram-negative bacteria. Despite the presence of plasmids in several spirochete species, there are only two reports of successful electroporation in spirochetes. The first resulted in insertional inactivation of the hemolysin *tly* gene from *Serpulina hyodysenteriae* (35) and, more recently, site-directed mutations into the *gyrB* gene of *Borrelia burgdorferi* (31). Therefore, at this moment in time, there is no consistently reproducible and effective means of genetic transfer which would facilitate investigation of potential virulence factors by methods such as reverse genetics and site-directed mutagenesis.

Therefore, in order to increase our knowledge of the genetic makeup of *T. denticola*, we undertook to construct the physical map of the chromosome of strain ATCC 33520 by pulsed-field gel electrophoresis (PFGE). This technique has been applied to the analysis of chromosomal DNA from many bacteria (11), including (from the order *Spirochaetales*) the agent of Lyme disease, *B. burgdorferi*, which, unusually, has a linear chromosome (3, 9, 12, 13), and *Leptospira interrogans*, which possesses two circular chromosomes (2, 40, 41). A preliminary study of the chromosome of *Treponema pallidum* subsp. *pallidum*, the syphilitic agent, showed the presence of a single, circular chromosome of 1 Mbp (29, 38). A detailed physical map of *T. denticola* might, therefore, highlight novel features of the genus *Treponema*. It may also provide a basis for future genetic studies such as the localization of cloned genes (as demonstrated here), analysis of the genomic distribution of loci encoding virulence factors, and characterization of deletion and transposon mutants.

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MATERIALS AND METHODS

Bacterial strains and plasmids. *T. denticola* ATCC 33520 was maintained anaerobically as described previously (17). The plasmid vector used for cloning was pNEB193 (New England Biolabs, Beverly, Mass.), which is pUC19 (39) with alterations to the polylinker region to include a single site for *Asc*I between the *Bam*HI and *Sma*I sites. *E. coli* XL-1 Blue (Pharmacia, Uppsala, Sweden), used as a host for molecular cloning, was grown in Luria broth or Luria broth supplemented with 1.5% agar for solid media (30).

Preparation of genomic DNA. Spirochetes were harvested from 50-ml cultures after 2 to 3 days of growth when the cell density had reached between 2×10^8 and 4×10^8 bacteria per ml. Cells were washed twice with 1× TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.5), by centrifugation at 3,000 rpm for 15 min at 4°C in a Sigma 3K20 bench top centrifuge. Cells were resuspended at a concentration of either 2×10^9 or 6×10^9 cells per ml and incorporated into 100-μl blocks of low-melting-temperature agarose (FMC Corp., Rockland, Maine). In situ preparation of the genomic DNA was carried out as reported previously (3).

Digestions of DNA in agarose and fragment nomenclature. DNA in agarose plugs was digested with 5 to 20 U of the restriction endonucleases *Asc*I, *Bss*HII, *Not*I, and *Srf*I for 3 to 20 h under the conditions specified by the suppliers (New England Biolabs, Pharmacia, and Stratagene [San Diego, Calif.]). Sequential digestions were performed by the method for two-dimensional PFGE as described by Bautsch (5). The correspondence between fragments generated by double and single digests was established from their relative mobilities 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. Fragments produced by digestion with a single restriction endonuclease have been designated As (*Asc*I), Bs (*Bss*HII), No (*Not*I), and Sr (*Srf*I) to identify the enzyme, followed by a capital letter, A or B, etc., denoting the order by decreasing size (see Tables 1 and 4). For fragments of indistinguishable size, the suffix is numbered 1 or 2, etc., e.g., NoB1 and NoB2.

To achieve maximum efficiency with the enzyme I-*Ceu* I, one-third of a plug was digested for 2 to 4 h at 37°C with 0.4 U of I-*Ceu* I (New England Biolabs) in a 100-μl reaction mixture.

PFGE. PFGE was performed in either a CHEF-DRII apparatus (Bio-Rad Laboratories, Richmond, Calif.) or a Pharmacia-LKB apparatus, with a hexagonal electrode (Pulsaphor 2015), or in a ZIFE apparatus (Q-Life, Kingston, Ontario, Canada). Agarose gels (1 to 1.2% [wt/vol]) in 0.5× TBE buffer (1× TBE is 89 mM Tris-borate, 89 mM boric acid, and 2 mM EDTA, pH 8) were used for the two former apparatuses. For the ZIFE, gels were 0.8% agarose in 1× TBE. The fragment sizes were determined by comparison of band mobility with those of known molecular weight from bacteriophage lambda DNA multimers (monomer = 48.5 kb) and *Saccharomyces cerevisiae* chromosomes (Bio-Rad): 2,200, 1,600, 1,125, 1,020, 945, 850, 770, 700, 630, 580, 460, 370, 290, and 245 kbp.

TABLE 1. Restriction endonuclease fragments of the chromosome of *T. denticola* ATCC 33520

Fragment	Mean size in kbp (identification) of fragments generated by:					
	*Asc*I	*Srf*I	*Not*I	*Asc*I-*Not*I	*Asc*I-*Srf*I	*Srf*I-*Not*I
A	1,650	1,055	350	350 (NoA)	740	310 (NoB1)
B	1,155	600	310[a]	310[a] (NoB1,B2)	600 (SrB)	250[a] (SrE, NoC)
C	150	345	250	250 (NoC)	345 (SrC)	220[b] (NoD1, 2, SrF)
D	75	270	220[a]	220 (NoD1)	250 (SrE)	200
E		250	210	180 (NoF)	240	180 (NoF)
F		200	180	145 (NoG)	200 (SrF)	135 (SrH)
G		165	145	135 (NoH)	165 (SrG)	125 (NoI)
H		135	135	125 (NoI)	150 (AsC)	120 (NoJ)
I		20	125	120 (NoJ)	135 (SrH)	115
J			120	115[a]	110	100 (NoL)
K			110	100 (NoL)	75 (AsD)	90
L			100	95	20 (SrI)	70
M			60	80		60[a] (NoM)
N			55	75 (AsD)		55 (NoN)
O			50	60 (NoM)		50 (NoO)
P			35	55 (NoN)		35 (NoP)
Q			17	50 (NoO)		26
R			14	35[a] (NoP)		20
S			12	26		17 (NoQ)
				17 (NoQ)		14 (NoR)
				14 (NoR)		12 (NoS)
				10 (NoS)		5
Total	3,030	3,040	3,028	3,030	3,027	2,959

[a] Two fragments migrating with the same mobility within a band.
[b] Three fragments migrating with the same mobility within a band.

For estimation of the size of larger DNA bands, *Schizosaccharomyces pombe* chromosomes (Bio-Rad) were used: 5.7, 4.6, and 3.5 Mbp.

Construction of *Asc*I linking clones. *Asc*I-*Hin*dIII fragments were cloned to construct the *Asc*I physical map. These were obtained by digestion of DNA in agarose with *Asc*I and then *Hin*dIII. The digested DNA was purified from the agarose by Qiaex extraction (Diagen GmbH) and ligated to *Asc*I-*Hin*dIII-linearized pNEB193. All subsequent DNA manipulations were carried out by standard procedures (30).

Mapping of restriction fragments by reciprocal hybridization. Portions of low-melting-temperature agarose gels containing bands generated by restriction endonuclease digestion and separated by PFGE were excised and washed in 1 ml of water (1 h, 20°C). The agarose blocks, in 300 μl of water, were heated at 100°C for 10 min. The tubes were placed on ice, and 25 μl of the DNA-agarose mixture was labelled overnight at 37°C with digoxigenin-11-dUTP in a scaled-up random prime reaction (50 μl) (Boehringer, Mannheim, Germany).

Probes. Probes used to map the rRNA genes were the plasmid pK3535, which encodes the whole *E. coli rrnB* ribosomal operon (7), two derivatives of pK3535 containing *rrsB* (16S)-*rrlB* (23S) and *rrlB* alone, and *L. interrogans rrf* (5S), which had been synthesized by PCR (2).

The number of copies of the rRNA genes was determined by hybridization with the *rrsB-rrlB*, *rrlB*, and *rrf* rRNA probes to Southern blots of chromosomal DNA, previously digested with a panel of different restriction enzymes. Restriction of the chromosomal DNA with the intron-encoded I-*Ceu* I (15) was also used to extend and confirm these data.

Digoxigenin probes were made from recombinant plasmids containing genes cloned from *T. denticola*. These were a 522-bp fragment of the *flgE* gene, which encodes the hook protein (10), and the *prtB* gene for the chymotrypsinlike protease (1). The probe for the gene *tdpA* (24) was generated by PCR with primers internal to the given sequence. Primer 1, 5′-TGGAAGGCGGTAGAG CCCGC-3′, annealed to bp 786 to 805 of the published sequence, and primer 2, 5′-AACGTACAGCCGTCATCGGC-3′, annealed to bp 1908 to 1889. The resultant 1.1-kb fragment was excised from a TAE agarose gel (1× TAE is 0.04 M Tris-acetate and 100 mM EDTA) and purified by Qiaex extraction, prior to labelling with digoxigenin.

DNA-DNA hybridizations. Following PFGE, the DNA was transferred to a Hybond-N nylon membrane (Amersham International, Les Ulis, France) for Southern blot analysis (33). For homologous probes, hybridization was performed at 65°C overnight in 5× SSC (1× SSC is 0.15 M NaCl and 0.015 M trisodium citrate, pH 7)–0.1% (wt/vol) *N*-sodium lauryl sarcosinate–0.02% (wt/vol) sodium dodecyl sulfate (SDS)–2% (wt/vol) blocking agent (Boehringer Mannheim). When heterologous probes were used, the hybridization temperature was varied between 50 and 55°C and all subsequent washes were performed at ambient temperature. The hybridized membranes were washed with 2× SSC–0.01% (wt/vol) SDS and then 0.1% SSC–0.01% (wt/vol) SDS. Detection of the digoxigenin-labelled probe was with anti-digoxigenin-alkaline phosphatase.

RESULTS

The strategy for the construction of the physical map of the *T. denticola* chromosome involved (i) determination of chromosome size by digestion with restriction endonucleases (*Asc*I, *Not*I, and *Srf*I) that cut infrequently and resulted in fragments that could be separated and sized accurately by PFGE; (ii) the construction of linking clones containing sites for the enzyme *Asc*I to ascertain the contiguity of such fragments; (iii) the use of two-dimensional PFGE to map the products from the double digestion of the above fragments; (iv) the labelling of individual restriction fragments to position, by hybridization, the fragments resulting from *Asc*I, *Not*I, and *Srf*I digestion; and (v) labelling of restriction fragments from *Bss*HII digestion to position, by hybridization, the *Not*I and *Srf*I unresolved by strategy iv.

Determination of chromosome size. The G+C content of *T. denticola* DNA has been reported to be in the range of 35 to 37% (8). Rare-cutting endonucleases with GC-rich recognition sequences were therefore considered to be the most appropriate for the construction of the physical map of the chromosome. Restriction digestion with the enzyme *Asc*I (GG ↓ CG CGCC) produced 4 fragments, 21 were obtained with *Not*I (GC ↓ GGCCGC), and 9 were obtained from digestion with *Srf*I (GCCC ↓ GGGC). The sums of the fragment sizes generated from these digestions were in close agreement (Table 1), giving a total chromosome size of 3.0 Mbp.

Large, circular molecules such as intact chromosomes do not migrate from the origin under the conditions imposed by PFGE. DNA in plugs from *T. denticola* which had not been subjected to restriction by endonucleases remained in the wells of the agarose gels, although smaller DNA structures, corresponding to plasmid pTD1 (on hybridization) (17) were visible in the matrix (data not shown). This suggested that, in common with the majority of bacteria, *T. denticola* possesses a circular chromosome.

TABLE 2. *Asc*I-*Hin*dIII linking clones in the chromosome of *T. denticola* ATCC 33520

Linking clone	Linking clone size (kbp)	Hybridizing fragment:	
		*Hin*dIII size (kbp)	Corresponding *Asc*I band
AH11	1.9	6.0	B
AH10	4.0	6.0	C
AH2	1.4	5.0	A
AH6	3.2	5.0	D
AH19	0.9	2.3	D
AH22	1.5	2.3	B

Identification of linking clones containing *Asc*I sites. One stratagem for joining the fragments of the *T. denticola* chromosome generated by *Asc*I restriction digest involved the identification of linking clones with a site for this rare-cutting enzyme at one extremity and a *Hin*dIII site at the other. Clones varying in size between 0.9 and 4.0 kbp were obtained (Table 2). These clones were hybridized by Southern blot analysis to restriction patterns generated by either *Asc*I or *Hin*dIII. Hybridization of two different recombinant plasmids carrying an *Asc*I-*Hin*dIII fragment to the same *Asc*I fragment indicated that they were both from that *Asc*I fragment. Hybridization of individual clones to the same *Hin*dIII fragment (determined by size) permitted the identification of complementary *Asc*I-*Hin*dIII fragments within each *Hin*dIII fragment. This technique was used successfully to position the four *Asc*I fragments in the contiguous arrangement A-D-B-C. However, as only six bona fide linking clones from the predicted eight were obtained, it proved impossible to confirm the circular nature of the chromosome by this method.

Mapping of *Asc*I and *Srf*I fragments by two-dimensional PFGE. The two-dimensional PFGE procedure was used to analyze reciprocal double digests by *Asc*I and *Srf*I of the *T. denticola* chromosome. By comparison of the single separations and the data from the two-dimensional gels, it was possible to map the *Asc*I-generated fragments within those from digestion with *Srf*I.

Of the nine fragments generated by digestion with *Srf*I, only SrA and SrD contained restriction sites for *Asc*I (Table 1). SrA (1,055 kbp) was digested to give three fragments, corresponding to two sites for *Asc*I. Of these, one had the same electrophoretic mobility as AsD (75 kbp) and the other two, with mobilities of 240 and 740 kbp, were from AsA and AsB. The order of the *Asc*I fragments A-D-B, demonstrated by the linking clones, was therefore confirmed by these data. By the same method, the fragment SrD (270 kbp) also was shown to contain two *Asc*I sites, incorporating the 150-kbp AsC fragment. However, in gels of this two-dimensional digestion, only one other fragment of 110 kbp was visible, although the presence of a third fragment of a size of less than 10 kbp was calculated to be present. This was confirmed by conventional agarose gel electrophoresis.

The electrophoretic mobility of the digestion products of fragments AsA and AsB with *Srf*I showed that AsA contained SrB (600 kbp), SrC (345 kbp), SrF (200 kbp), SrH (135 kbp), and SrI (20 kbp). The 240-kbp band present in this digest corresponded to that described above from SrA, and a remaining fragment of 110 kbp was deduced to be from SrD. Fragment AsB (1,155 kbp) contained bands corresponding to SrE (250 kbp) and SrG (165 kbp) and one of 740 kbp which was from SrA. Although it was not possible to order the *Srf*I fragments, these data enabled the accurate size determinations of AsA and AsB (1,650 and 1,155 kbp, respectively) to be made and confirmed the expected linkage of AsA to AsC and hence the circularity of the chromosome.

Both the circular nature and size of this chromosome, obtained from this macrorestriction study, were confirmed by the technique of gamma irradiation (38) by Eldon Walker, Houston, Tex. (37).

Mapping of *Srf*I and *Not*I fragments by two-dimensional PFGE. Digestion with the enzyme *Not*I was utilized to gain additional information as to the order of the *Srf*I fragments. Double digestion with these two enzymes resulted in a total of 25 fragments discernible by electrophoresis (Table 1 and Fig. 1), ranging in size from 310 to 5 kbp. The *Srf*I-generated fragments SrA, SrB, SrC, SrD, SrG, and SrI all contained sites for *Not*I (Table 1 and Fig. 1). Of the *Not*I fragments, NoA, NoB2, NoE, NoG, NoH, and NoK were digested by *Srf*I.

It was possible to place the following *Not*I fragments within each *Srf*I band. SrA (1,055 kbp) contained NoC (250 kbp), NoD1, NoD2 (220 kbp), NoI (125 kbp), NoJ (120 kbp), and NoM (60 kbp); SrB (600 kbp) contained NoB1 (310 kbp), NoQ (17 kbp), and parts of NoG (105 kbp) and NoH (115 kbp); SrC (345 kbp) contained NoF (200 kbp), NoL (180 kbp), 40 kbp of NoG, and approximately 20 kbp of NoA; SrD contained NoP (35 kbp), 190 kbp of NoE, and 45 kbp of NoK; and SrG (165 kbp) contained NoN (55 kbp), NoO (50 kbp), and approximately 60 kbp of NoK (Fig. 1). However, because of the presence of many small fragments of similar size, it was possible to deduce contiguous arrangements for restriction fragments only where unique and therefore easily identified overlapping fragments existed, e.g., the 105 and 40 kbp of NoG joining SrC-SrB. Likewise, contiguous arrangements were ascertained for SrH-SrF, SrG-SrD, NoF-NoL, NoG-NoL-NoF, and NoK-NoP-NoE.

Reciprocal hybridization with fragments generated by restriction digest. Digoxigenin-labelled probes, made by using purified restriction fragments, were hybridized with Southern blots of gels in which the products of the three single and three double digests had been separated. This approach enabled further positioning of the *Not*I and *Srf*I fragments, with respect to one another and to the four *Asc*I fragments (Table 3), by confirmation and extension of the restriction data above. An example of this type of hybridization is shown in Fig. 2.

Several *Not*I fragments, containing sites for neither *Srf*I nor *Asc*I, still remained to be positioned definitively after the reciprocal hybridizations had been performed. Therefore, a further rare-cutting enzyme, *Bss*HII, was employed. The largest fragments obtained from digestion with this enzyme were used as probes on Southern blots of *Not*I and *Srf*I gels. This information, confirmed by restriction data, enabled the satisfactory placing of all but the smallest *Not*I fragments (of 14 and 10 kbp) on the physical map (Table 4).

The final restriction map of the *T. denticola* chromosome, obtained from these various approaches, is as shown in Fig. 4.

Positioning and numeration of the rRNA genes on the physical map. Use of the heterologous probes for *rrlB* (23S rRNA) and *rrf* (5S rRNA) resulted in hybridization with restriction fragments NoF, NoH, SrB, SrC, and AsA. A similar result was obtained when the probe for the whole *E. coli rrnB* operon was used as with the probes derived from portions of the operon. This mapped the rRNA genes to one region of the chromosome encompassed by the largest *Asc*I fragment, AsA (see Fig. 4).

To determine accurately the number of copies of the rRNA genes present, we used a strategy similar to that described by Michel and Cossart (23) in which the DNA was digested with enzymes known to cut frequently in the chromosome. As the

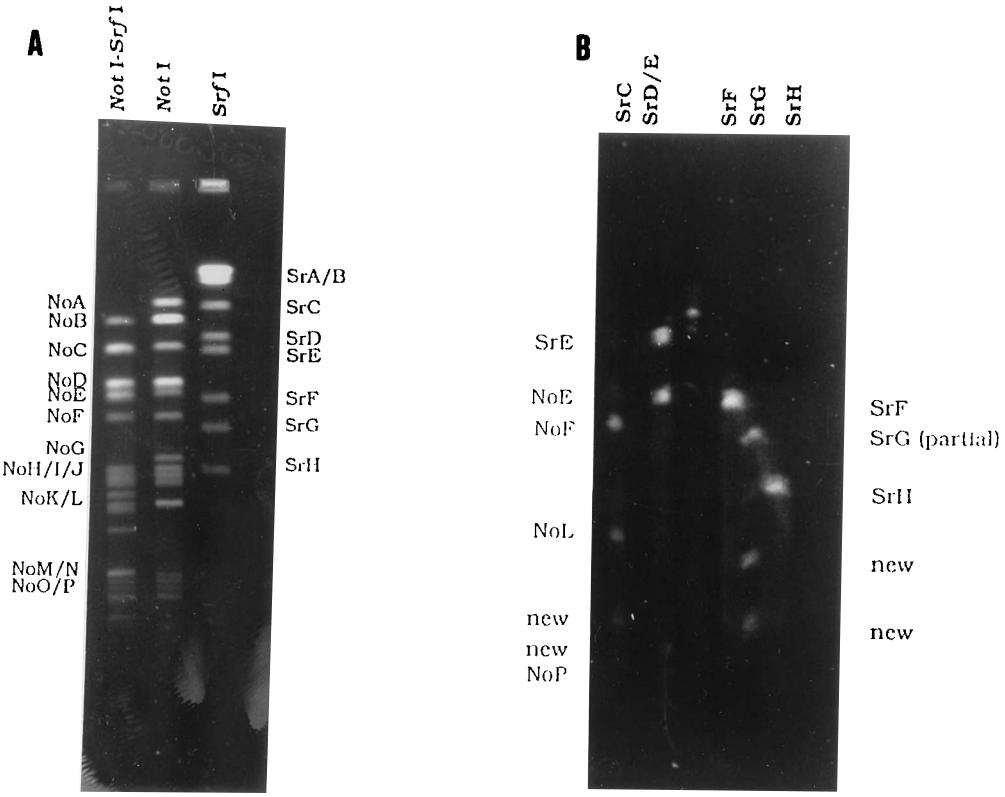

FIG. 1. (A) Two-dimensional PFGE gel of *T. denticola* ATCC 33520 DNA digested with the enzymes *Srf*I and *Not*I both separately and as a double digestion. (B) The bands from the *Srf*I track, cut from the gel and redigested with *Not*I. The 1% agarose gel was run in the CHEF (Bio-Rad) apparatus with a ramping of 4 to 24 s, at 200 V for 24 h. Bands smaller than 35 kbp are not visible under these conditions.

sequence for the *T. denticola* 16S RNA gene is known (28), some of the enzymes chosen were those with a unique site in the *rrs*, e.g., *Mlu*I, *Sph*I, *Eco*RI, *Sgr*AI, and *Kpn*I. Additionally, hybridization patterns were obtained from enzymes that did not cut in the *rrs* but cut frequently elsewhere in the chromosome, e.g., *Hin*dIII, *Bam*HI, and *Apa*I. With each of this latter group of enzymes, two major bands hybridized regardless of which of the three probes was used. This result suggested the presence of two copies of each of the rRNA genes.

The enzyme I-*Ceu*I is encoded by a mobile intron in the chloroplast 23S rRNA gene (*rrl*) of *Chlamydomonas eugametos* (15). It cuts a 26-bp site in the *rrl* gene and at no other sites in

TABLE 3. Reciprocal hybridization of restriction fragments generated by digestion with *Asc*I, *Not*I, and *Srf*I

Probe	*Asc*I profile	*Not*I profile	*Srf*I profile	Probe	*Asc*I profile	*Not*I profile	*Srf*I profile
AsC	AsC	NoE, NoK, NoP	SrD	SrA	AsA, AsD, AsB	B2, C, D2, D2, I, J, M	SrA
AsD	AsD	NoD2	SrA	SrB	AsA	NoB1, NoG, NoH, NoQ	SrB
NoA	AsA	NoA	SrF, SrH	SrC	AsA	NoF, NoL, NoG	SrC
NoB1	AsA	NoB1	SrB	SrD	AsA, AsC, AsB	NoE, NoK, NoP	SrD
NoB2	AsB	NoB2	SrE, SrA	SrE	AsB	NoB2	SrE
NoC	AsB	NoC	SrA	SrF	AsA	NoA	SrF
NoD1	AsB	NoD1	SrA	SrG	AsB	NoK, NoO, NoN	SrG
NoD2	AsD, AsB, AsA	NoD2	SrA	SrH	AsA	NoA	SrH
NoE	AsC, AsA	NoE	SrD, SrI	SrI	ND[a]	ND	ND
NoF	AsA	NoF	SrC				
NoG	AsA	NoG	SrB, SrC				
NoH	AsA	NoH	SrB, SrI				
NoI	AsA	NoI	SrA				
NoJ	AsB	NoJ	SrA				
NoK	AsC, AsB	NoK	SrD, SrG				
NoL	AsA	NoL	SrC				
NoM	AsB	NoM	SrA				
NoN	AsB	NoN	SrG				
NoO	AsB	NoN	SrG				
NoP	AsC	NoP	SrD				

[a] ND, not done.

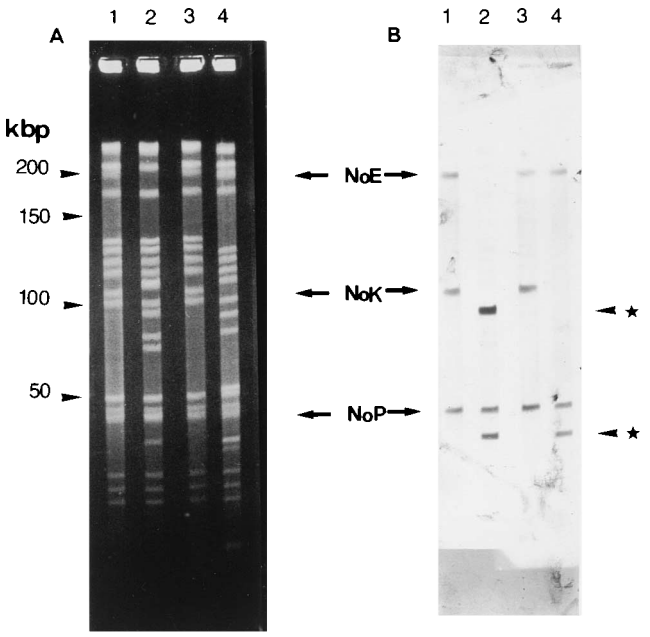

FIG. 2. (A) Reciprocal hybridization with the band AsC as a probe on the following restriction enzyme digests of *T. denticola* DNA: lane 1, *Not*I; lane 2, *Asc*I-*Not*I; lane 3, *Not*I; lane 4, *Srf*I-*Not*I. (B) Corresponding digoxigenin blot, which confirms that NoE (lane 2) is cut only by *Asc*I although NoK (lanes 2 and 4) has sites for both *Asc*I and *Srf*I. NoP, all four lanes, is also encompassed by AsC but is uncut by any of the enzymes. New bands resulting from the digestion of NoE and NoK are marked by stars. The gel was 0.8% agarose and was run in a ZIFE apparatus preprogrammed for the resolution of fragments in the size range of 50 to 150 kbp.

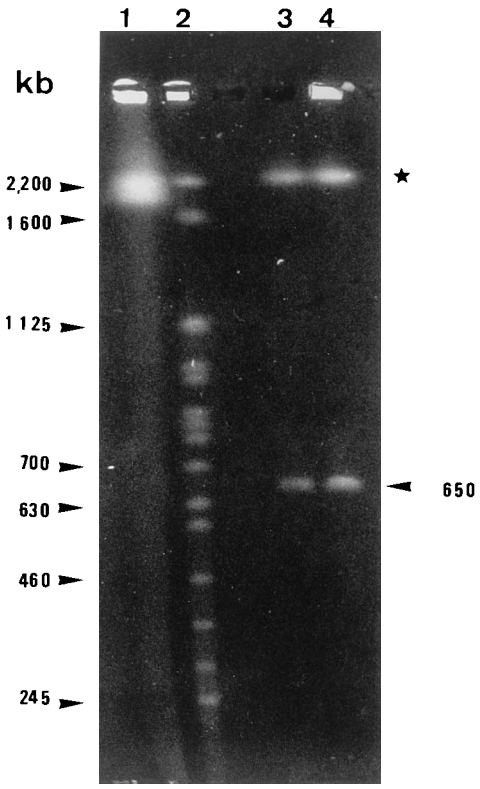

FIG. 3. Digestion of the *T. denticola* ATCC 33520 chromosome with the intron-encoded I-*Ceu* I. Lanes: 1, *S. pombe* chromosomes; 2, *S. cerevisiae* chromosomes; 3 and 4, *T. denticola* chromosome digested with I-*Ceu* I to release a fragment of 650 kbp from the remainder of the chromosome (marked by a star). The gel was run in the CHEF apparatus, 1% agarose with a ramping of 20 to 120 s, at 200 V for 22 h.

the chromosomes of all the bacteria so far tested (19). Therefore, the generation of two fragments from the *T. denticola* chromosome confirmed the presence of two *rrl* genes, and as a 650-kbp fragment was released, this provided the distance between the copies (as shown in Fig. 3). Their mapping on fragments NoF and NoH is shown in Fig. 4.

Generation of a preliminary genetic map. Hybridization with probes encoding the *tdpA* (immunogenic protein), *prtB* (chymotrypsinlike protease), and *flgE* (hook protein) genes from *T. denticola* enabled the initiation of a preliminary genetic map as shown in Fig. 4. The *tdpA* gene could be mapped to the region on NoE just adjacent to the SrI fragment. The other two probes hybridized to the opposite side of the chromosome.

TABLE 4. Restriction fragments of a *Bss*HII digest of the *T. denticola* chromosome, used as reciprocal probes

Fragment	Size (kbp)	Restriction by:		Hybridization with:	
		*Not*I	*Srf*I	*Not*I fragments	*Srf*I fragments
BsA	290	−	+	NoB2	SrA, SrE
BsB	250	+	+	NoA, NoF	SrC, SrH
BsC	190	+	−	NoB1, NoH, NoQ	SrB
BsD	145[a]	+	−	NoD2, NoI	SrA
		+	+	NoH, NoE	SrB, SrD, SrI
BsG	80	+	−	NoC, NoD1, NoM	SrA

[a] Two fragments of indistinguishable size, migrating as a doublet.

DISCUSSION

In the absence of suitable techniques for genetic manipulation of members of the order *Spirochaetales*, there has been considerable interest in the use of PFGE to learn about the chromosomal arrangement of these bacteria. The genome size for several spirochetes is known and appears to reflect their distribution in nature. That of the free-living, aerobic *L. interrogans* is, at 4.6 Mbp, of magnitude comparable to that of the genome of *E. coli* (2, 11, 40). The recent report suggesting the presence of an additional, circular chromosome of 350 kbp in this organism is suggestive of the unconventionality of the order (41). In contrast, the chromosome of the host-associated, anaerobic *T. pallidum* is only 1 Mbp (29, 38). In this study, the *T. denticola* chromosome was found to be circular and approximately 3.0 Mbp in size, as determined from the measurement of restriction fragment sizes by PFGE. The presence of one small extrachromosomal element corresponding to the plasmid pTD1 was confirmed by hybridization. *T. denticola* is also a host-associated anaerobe, but, unlike *T. pallidum*, can be maintained successfully in artificial media if supplemented with 10% serum–thymine PP_i–cysteine (17). Presumably, the additional chromosomal DNA provides the capacity to encode at least some necessary biosynthetic genes.

The apparent diversity of members of the genus *Treponema* has long been the topic of phylogenetic discussions as DNA homology studies have shown little similarity amongst members. Indeed, *Treponema hyodysenteriae* and the closely associated, nonpathogenic *Treponema innocens* have now been

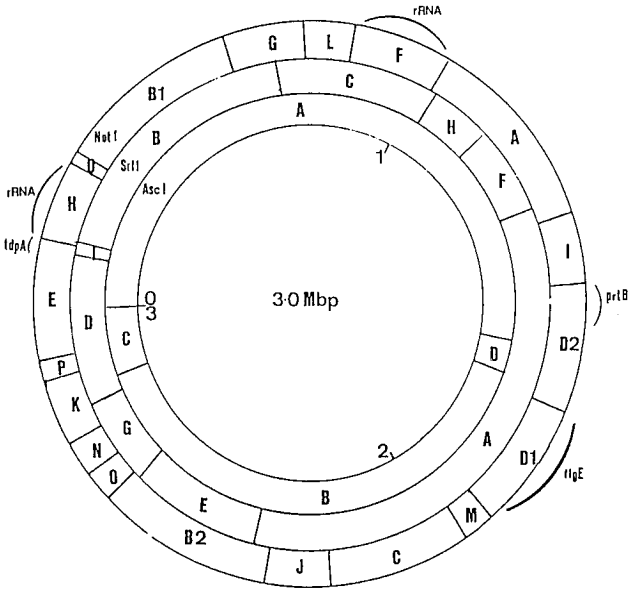

FIG. 4. Physical map of the chromosome of *T. denticola* ATCC 33520. The *Not*I, *Srf*I, and *Asc*I fragments are indicated by a letter, from the outer to the inner circle. The order of the *Not*I fragments NoO and NoN is presumptive. Locations of rRNA genes and homologous *T. denticola* genes are as indicated.

reclassified as species of the genus *Serpulina* (34). The relationship between *T. pallidum* and *T. denticola* was shown convincingly by the extensive 16S homology studies of Paster et al. (28) although the G+C content of the two organisms is widely different (that of *T. pallidum* is 50% [8]). The physical map of the *T. denticola* chromosome represents the first for this genus. Already, the chromosome shows a marked difference from that of *T. pallidum* in being three times its size (38), but coincidentally, almost the same size as that of *S. hyodysenteriae* (42). Such an intragenus variation in size is remarkable. Studies on different species of the genus *Borrelia* (12, 29) have shown both the linear character and the size of the chromosomes to be similar, as indeed is the genetic arrangement (26). In the different *Leptospira* strains studied (4, 40), two circular chromosomes of 4.6 Mbp and 350 kbp have been shown to exist. However, differences in physical and genetic makeup are apparent on more detailed analysis and comparison of these chromosomes (40, 41), despite the bacteria in question being of the same species. In this vein, it is possible that speculatively, future genetic comparisons between *T. denticola* and *T. pallidum* may provide evidence for the evolution of the latter, an obligate parasite, by large deletions of what became redundant portions of the chromosome as the organism found a perfect niche for survival and transmission.

From our data, it can be derived that there are two copies of each of the rRNA genes on the chromosome of *T. denticola*. The hybridization patterns suggest that the genes may be closely linked, which is in agreement with the results found for *Treponema phagedenis* (14). A fragment cloned from this organism showed the arrangement to be *rrs-rrl-rrf*. Hybridization studies showed two copies of each gene to be present (and closely linked) in both *T. phagedenis* and *T. pallidum*. On the basis of 16S RNA sequence homology, *T. denticola* and *T. phagedenis* have been shown to be closely related (less than 5% difference) (28). From our results, it can be predicted that an arrangement of the rRNA genes similar to that in the other two treponemes exists in *T. denticola*. Amongst members of the *Spirochaetales*, it would appear that only the treponemes have this expected arrangement as scattering of the RNA genes occurs in *Leptospira* spp. (2), the *rrs* gene is separated from the *rrl* and *rrf* genes in *S. hyodysenteriae* (42), and widespread DNA polymorphism occurs in the intragenic region between the *rrs* and *rrl* genes in the different species of the Lyme disease spirochetes *B. burgdorferi*, *Borrelia garinii*, and *Borrelia afzelii* (26).

Few genes from *T. denticola* have yet been cloned and characterized. In this study, we mapped genes for an antigen, a flagellar component, and a potential virulence factor. The two latter genes could be mapped within a 440-kbp region of the chromosome, directly opposite one copy of the rRNA genes. This positioning is probably entirely coincidental and is indicative of a region rich in G+C rather than the presence of a virulence operon such as those found in *Bacillus subtilis* or *Clostridium perfringens.*

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