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Physical Map of the Genome of *Treponema pallidum* subsp. *pallidum* (Nichols)

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A physical map of the chromosome of *Treponema pallidum* subsp. *pallidum* (Nichols), the causative agent of syphilis, was constructed from restriction fragments produced by *Not*I, *Sfi*I, and *Srf*I. These rare-cutting restriction endonucleases cleaved the *T. pallidum* genome into 16, 8, and 15 fragments, respectively. Summation of the physical lengths of the fragments indicates that the chromosome of *T. pallidum* subsp. *pallidum* is approximately 1,030 to 1,080 kbp in size. The physical map was constructed by hybridizing a variety of probes to Southern blots of single and double digests of *T. pallidum* genomic DNA separated by contour-clamped homogeneous electric field electrophoresis. Probes included cosmid clones constructed from *T. pallidum* subsp. *pallidum* genomic DNA, restriction fragments excised from gels, and selected genes. Physical mapping confirmed that the chromosome of *T. pallidum* subsp. *pallidum* is circular, as the *Sfi*I and *Srf*I maps formed complete circles. A total of 13 genes, including those encoding five membrane lipoproteins (*tpn47*, *tpn41*, *tpn29-35*, *tpn17*, and *tpn15*), a putative outer membrane porin (*tpn50*), the flagellar sheath and hook proteins (*flaA* and *flgE*), the cytoplasmic filament protein (*cfpA*), 16S rRNA (*rrnA*), a major sigma factor (*rpoD*), and a homolog of cysteinyl-tRNA synthetase (*cysS*), have been localized in the physical map as a first step toward studying the genetic organization of this noncultivable pathogen.

Treponema pallidum subsp. *pallidum*, a spirochete of the family *Spirochaetaceae*, is the etiologic agent of venereal syphilis. Genetic analysis of the members of the *Spirochaetaceae* has been limited by a lack of techniques for the introduction of DNA into these organisms. Recent reports of successful transformation of *Serpulina hyodysenteriae* (47) and *Borrelia burgdorferi* (42) indicate that experimental introduction of DNA into pathogenic spirochetes is possible. However, studies of the biology and genetics of *T. pallidum* are further complicated by the lack of a system for continuous in vitro culture of the organism. Because clonal populations of *T. pallidum* cannot be propagated, standard genetic approaches such as mutation, conjugation, transduction, and transformation are not feasible.

Previously, we used contour-clamped homogeneous electric field (CHEF) electrophoresis, a type of pulsed-field gel electrophoresis (PFGE), to determine the size and conformation of the *T. pallidum* chromosome (49). This study indicated that *T. pallidum* has a single, circular chromosome with a size of approximately 10^6 bp. The ability of PFGE to resolve large DNA fragments has been exploited in the construction of physical maps of bacterial chromosomes (19, 26). Because physical mapping does not require the use of genetic transfer systems, it provides a previously unavailable means to study the genetic organization of *T. pallidum*.

In this study, we report the construction of a physical macrorestriction map of the chromosome of *T. pallidum*. This map was generated by CHEF electrophoresis of the products of single and double digestions of the intact *T. pallidum* chromosome with the restriction endonucleases *Not*I, *Sfi*I, *Srf*I, and *Spe*I and by hybridization of Southern blots of these gels with probes prepared from a *T. pallidum* cosmid library, restriction fragments eluted from CHEF gels, or DNA segments encoding certain *T. pallidum* genes. This map represents a first step in studying the arrangement and linkage of genes on the *T. pallidum* chromosome.

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MATERIALS AND METHODS

Preparation of DNA inserts. The Nichols strain of *T. pallidum* was extracted from infected rabbit testes as described previously (13) and purified by sodium diatrizoate density gradient centrifugation (4). The organisms were washed and resuspended in TE buffer (10 mM Tris, 0.1 mM EDTA [pH 8.0]), mixed with an equal volume of 1.6% low-melting-point agarose (Incert agarose; FMC BioProducts, Rockland, Maine) at 45°C, and dispensed in 200-μl portions into molds (2×10^9 to 4×10^9 *T. pallidum* cells per insert). The inserts were incubated overnight at 37°C in TE-sodium dodecyl sulfate (SDS) (TE buffer containing 0.5% SDS) and then treated for 2 days at 55°C with the same buffer containing 100 μg of proteinase K per ml (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). The inserts were washed at ambient temperature for 1 h with three changes of TE buffer and stored at 4°C.

Restriction endonuclease treatment. Restriction endonuclease digestions were carried out in agarose inserts as described previously (49). *Asc*I, *Nhe*I, *Not*I, and *Spe*I (New England BioLabs, Inc., Beverly, Mass.) digests were incubated for 4 h at 37°C, and *Srf*I (Stratagene, La Jolla, Calif.) digests were incubated for 12 h at 37°C. *Sfi*I (New England BioLabs) digests were incubated for 16 h at 50°C. *Not*I and *Spe*I double digests were done simultaneously in the buffer supplied by the manufacturer for *Not*I; in other double digests, inserts were equilibrated in the second buffer after digestion by the first restriction endonuclease and incubated under the conditions appropriate for the second enzyme.

PFGE. Gels to be used for Southern blotting were prepared by loading restriction endonuclease-treated inserts into wells in 1% Fastlane agarose (FMC) prepared in 0.25× TBE buffer (1× TBE is 90 mM Tris, 90 mM boric acid, and 1 mM EDTA [pH 8.3]). CHEF electrophoresis (CHEF-DR II Pulsed-Field Electrophoresis System; Bio-Rad Laboratories, Richmond, Calif.) was performed in 0.25× TBE at 15°C for 30 h at 200 V with a linearly increasing switching time of 2 to 20 s. Other pulse- and run-time parameters were used as necessary to achieve optimal separation of particular fragment size ranges. In addition, to aid in the resolution of the widest possible range of restriction fragments, the CHEF unit was modified to accept large gels of 24 by 22 cm. Lambda concatamers (multiples of 48.5 kbp; FMC) and lambda *Hin*dIII fragments (23.1 to 0.125 kbp; Bio-Rad) were used as markers.

To obtain restriction fragments for elution and probe preparation, the frag-

TABLE 1. *T. pallidum* subsp. *pallidum* (Nichols) gene probes used in this study

Gene[a]	Probe	Description	Reference(s)
tpn50	p4A2HC2	Candidate porin protein with homology to OmpA	15
tpn47	pNC81	Membrane-associated 47-kDa lipoprotein with carboxypeptidase properties	22, 31, 51, 52
tpn41	pB7	41-kDa homolog of galactose-glucose-binding protein (MglB)	5, 18
tpn29-35	pMN20	Membrane-associated 29- to 35-kDa lipoprotein	44–46
tpn24(c)	pPH21	Minor 24-kDa polypeptide	23
tpn17	pMN7	Membrane-associated 17-kDa lipoprotein	1
tpn15	pMN406	Membrane-associated 15-kDa lipoprotein	38
cfpA	pTA6	83-kDa cytoplasmic filament subunit	33, 56
flaA	pRI18	37-kDa flagellar sheath protein	24, 25
flgE	PCR product	Flagellar hook protein	27–29
rpoD	PCR product	Open reading frame with homology to the major sigma factors of *E. coli* and *B. subtilis*	21
rrnA	PCR product	16S rRNA gene from *T. pallidum* subsp. *pertenue* (Gauthier)	11, 35, 36
cysS	pRAN18	Open reading frame with sequence homology to *E. coli* cysteinyl-tRNA synthetase	50

[a] The *tpn* prefix is a nomenclature for *T. pallidum* subsp. *pallidum* genes as described in reference 33.

ments generated by restriction endonuclease treatment of *T. pallidum* DNA inserts were separated by CHEF electrophoresis in 1% SeaKem Gold (FMC) agarose prepared in 0.5× TAE buffer (1× TAE is 40 mM Tris-acetate and 1.0 mM EDTA [pH 8.3]). CHEF electrophoresis was performed in 0.5× TAE at a variety of pulse and run times to allow optimal resolution of the desired fragments.

Southern blotting and hybridization. CHEF gels used for Southern blotting were stained with 2 μg of ethidium bromide per ml, photographed, and UV irradiated (254-nm source) for 5 min to facilitate DNA transfer. The DNA was transferred overnight to a Hybond-N^+ nylon membrane (Amersham Corporation, Arlington Heights, Ill.) with 0.4 N NaOH. Following transfer, the membranes were neutralized with 0.5 M Tris (pH 7.2) for 5 min and then rinsed briefly in 2× SSC (1× SSC is 0.15 M NaCl and 0.015 M trisodium citrate [pH 7.0]) and air dried.

Prehybridization was performed for ≥1 h at 65°C in a buffer containing 50 mM Tris (pH 8.0), 1 M NaCl, 1% SDS, and 10% dextran sulfate supplemented with 200 μg of salmon sperm DNA per ml. Lambda DNA and *T. pallidum*-specific DNA probes were labelled with [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham) with the Multiprime kit (Amersham). Labelled DNA (25 to 50 ng) was added directly to the prehybridization buffer. After ~16 h of hybridization at 65°C, the membranes were washed (15 min per wash) once in 2× SSC with 0.5% SDS (at 65°C), twice in 1× SSC with 0.5% SDS (at 65°C), and twice in 0.1× SSC with 0.5% SDS (at ambient temperature). Autoradiography was performed with the use of X-Omat AR film (Eastman Kodak, Rochester, N.Y.) and intensifying screens.

Elution of restriction fragments from gels. Prior to elution of the restriction fragments, the gels were stained with 2 μg of ethidium bromide per ml and the bands were excised with a clean scalpel under UV illumination. The DNA was eluted from the agarose gel slices with the Geneclean II kit (Bio 101 Inc., La Jolla, Calif.).

Cosmid and other recombinant DNA constructs. Approximately 10^{11} treponemes were incubated at 37°C for 4 h in TE-SDS and then incubated for an additional 6 h at 56°C in TE-SDS containing 100 μg of proteinase K per ml. DNA was extracted twice with an equal volume of phenol-chloroform-isoamyl acetate (25:24:1) and once with chloroform-isoamyl acetate (24:1) and precipitated by addition of 2 volumes of ethanol. Prior to use, the DNA was stored at 4°C in TE buffer. For cosmid cloning, partial digests of *T. pallidum* were prepared with *Bam*HI or *Pst*I. Briefly, 50-μg portions (seven total, 250 μl each) of *T. pallidum* DNA were digested for 1 h with twofold-decreasing amounts of restriction endonuclease, beginning with 50 U (1 U/μg of DNA) of enzyme. After 1 h of digestion at 37°C, the reactions were stopped by adding EDTA to 13 mM. The portions were pooled and fractionated in a 10 to 40% sucrose density gradient. Fractions containing DNA in the size range of 18 to 28 kbp were pooled and dialyzed against TE buffer. *T. pallidum* DNA was ligated into the cosmid vector pLAFRx (30, 53) at an insert-to-vector molar ratio of 8:1. The products of ligation were packaged into bacteriophage lambda with the Packagene Extract System (Promega Corporation, Madison, Wis.); the resultant assembled phage particles were used to introduce the cosmids into *Escherichia coli* LE 392 (41). Transformants were selected on Luria-Bertani agar containing 12.5 μg of tetracycline per ml.

Cosmid DNA was obtained from tetracycline-resistant clones with the Magic Minipreps kit (Promega). The cosmid DNA was cleaved with the appropriate restriction endonuclease and assayed for the presence of *T. pallidum* insert DNA by continuous-field agarose gel electrophoresis.

The gene probes used in this study are summarized in Table 1. Recombinant clones previously characterized are identified by using the format proposed by Norris and the *Treponema pallidum* Polypeptide Research Group (33). Also used in this study were several genes identified recently in our laboratory. These included putative genes for the cytoplasmic filament protein subunit (56), the sigma 70 major transcription initiation factor of *T. pallidum* (21), and cysteinyl-tRNA synthetase (50). These genes will be described in greater detail elsewhere. Briefly, amino acid sequences of the N terminus and a CNBr cleavage fragment of the cytoplasmic filament protein subunit were obtained from material excised from two-dimensional polyacrylamide gels; these sequences were used to construct primers for PCR. The resultant PCR product was cloned into the plasmid pCR according to the manufacturer's instructions (Invitrogen, San Diego, Calif.). Several clones containing the gene were identified by hybridization with the PCR probe derived from the N-terminal amino acid sequence. The *rpoD* gene was obtained by PCR amplification with consensus primers (48). The PCR product was cloned with PCRII (Invitrogen). A gene with homology to cysteinyl-tRNA synthetase was identified by random sequencing of a library of *T. pallidum* DNA that had been partially digested with *Hin*dIII and cloned into pBluescript KS^+ (Stratagene). In each case, homology of the amplified fragment was confirmed by sequence analysis. The sequence of the *Treponema pallidum* subsp. *pertenue* (Gauthier) 16S genes coding for rRNA differs from that of *T. pallidum* subsp. *pallidum* (Nichols) at only two base pairs (35, 36). Therefore, the Gauthier recombinant clone was effective as a hybridization probe when used with Nichols strain blots.

RESULTS

Restriction fragments and genome size estimation. Intact *T. pallidum* DNA was subjected to digestion with *Not*I, *Sfi*I, *Spe*I, and *Srf*I for both size determination and mapping purposes (Fig. 1). The physical lengths of the fragments generated by digestion with these enzymes are shown in Table 2. The fragment sizes represent average values calculated from a minimum of three separate determinations on gels run under standard conditions (2- to 20-s pulse time for 30 h) (Fig. 1C). Fragments were given letter designations, with A being the largest; two or more fragments comigrating as a single band were provided with alphanumeric designations (e.g., G1 and G2).

*Sfi*I produced a total of eight fragments ranging in size from 301 to 55 kbp, with the latter representing two comigrating fragments. The size of band *Sfi*I-A (301 kbp) was confirmed on gels run for 36 h with a ramped 5- to 35-s pulse time (Fig. 1B). Band *Sfi*I-G, which appeared as a doublet on the basis of ethidium bromide staining intensity (Fig. 1B and C), was resolved into two closely spaced fragments (G1 and G2) on CHEF gels with a 1- to 10-s ramped pulse time for 12 h (data not shown). Digestion with *Srf*I yielded 15 fragments of 170 to 6 kbp. Band *Srf*I-C was found to contain a pair of comigrating 117-kbp fragments (*Srf*I-C1 and -C2) on the basis of greater

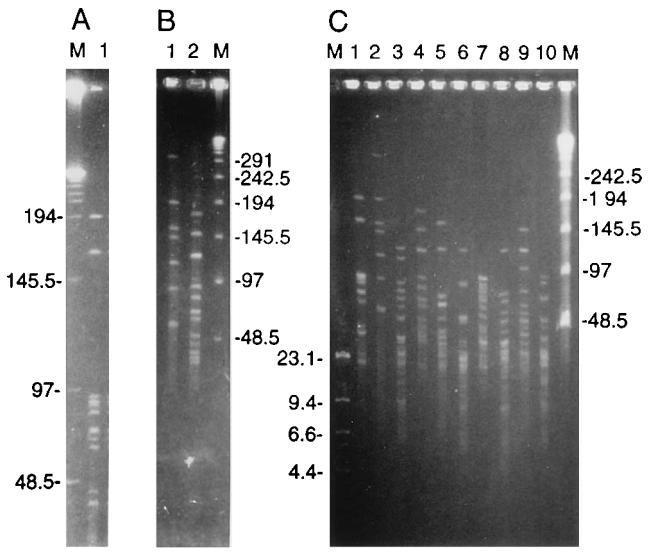

FIG. 1. Fragments of the *T. pallidum* subsp. *pallidum* (Nichols) genome obtained by treatment with *Not*I, *Sfi*I, and *Srf*I. (A) Ethidium bromide-stained CHEF gel of intact *T. pallidum* DNA digested with *Not*I (lane 1) and lambda concatamers (lane M). The gel was run for 36 h at 200 V with a constant pulse time of 6 s to optimize resolution in the 50- to 100-kbp range. The bands between the 48.5-kbp and 97-kbp markers correspond to fragments *Not*I-C, -D1, -D2, -E, -F1, -F2, and -G. An additional band of ~65 kbp between F and G is seen in this gel pattern but has not been observed consistently and has not been localized in the physical map. (B) Ethidium bromide-stained CHEF gel of intact *T. pallidum* DNA digested with *Sfi*I (lane 1) or *Srf*I (lane 2); lambda concatamers are also shown (lane M). The gel was run for 36 h at 200 V with a pulse time of 5 to 35 s to improve resolution in the 100- to 300-kbp range. (C) Ethidium bromide-stained CHEF gel of intact *T. pallidum* DNA digested with *Not*I, *Sfi*I, *Spe*I, and *Srf*I and all possible double digest combinations of these enzymes. The gel was run for 30 h at 200 V with a pulse time of 2 to 20 s. The lanes contain *T. pallidum* DNA digested with *Not*I (lane 1), *Sfi*I (lane 2), *Spe*I (lane 3), *Srf*I (lane 4), *Not*I-*Spe*I (lane 5), *Not*I-*Sfi*I (lane 6), *Not*I-*Srf*I (lane 7), *Spe*I-*Sfi*I (lane 8), *Spe*I-*Srf*I (lane 9), and *Sfi*I-*Srf*I (lane 10). Lanes labelled M contain lambda *Hin*dIII fragments (left) and lambda concatamers (right). DNA standard sizes are indicated in kilobase pairs.

TABLE 2. Physical lengths of fragments produced by digestion of total intact genomic *T. pallidum* DNA with restriction endonucleases *Not*I, *Sfi*I, *Srf*I, and *Spe*I

Band	Size of fragment (kbp)			
	*Not*I	*Sfi*I	*Srf*I	*Spe*I
A	188	301	170	124
B	154	188	145	110.5
C	87	153	117 (2)[a]	85
D	85 (2)	139	84	76
E	78	109	75	68
F	69 (2)	83	63	60.5
G	60	55 (2)	58	51.5
H	39		46	48
I	34 (2)		39	30 (2)
J	20		34	28
K	17 (2)		31	26
L	6		28	24
M			17	19 (5)
N			6	16 (2)
O				15
P				13
Q				11
R				9.1
S				8.3
T				7.3 (2)
U				5.6
V				4.6
Total	1,042	1,083	1,030	969.7[b]

[a] Number in parentheses = number of comigrating fragments identified within band.
[b] Not all *Spe*I fragments have been identified.

ethidium bromide staining intensity relative to neighboring bands and subsequent hybridization data indicating two separate map locations.

The sizes of the *Not*I restriction fragments (Table 2) have been modified from those described previously (49), on the basis of the results obtained on large-format (24 by 22 cm) CHEF gels. Evaluation of the *Not*I digestion pattern on large-format gels run for 48 h at a constant pulse time of 6 s showed that the *Not*I-D band contained two closely spaced fragments (Fig. 1A). The *Not*I-F and *Not*I-I bands are also doublets on the basis of ethidium bromide staining intensity and hybridization results. Thus, the sum of the *Not*I fragment sizes was 1,042 kbp (Table 2), rather than 900 kbp as reported previously (49). The 6-kbp *Not*I-L fragment described previously (49) has not been observed consistently on either pulsed-field or constant-field electrophoresis gels, and it has not been localized in the physical map of the *T. pallidum* chromosome through hybridization with radiolabelled probes. Therefore, its existence is uncertain at this time. The *Spe*I pattern (49) contains many comigrating fragments and thus is less useful for size determination and physical mapping.

The estimated size of the *T. pallidum* genome, as calculated by summation of restriction fragments produced by digestion with *Not*I, *Sfi*I, or *Srf*I, is in the range of 1,030 to 1,083 kbp (Table 2).

Hybridization results. The macrorestriction map of the *T. pallidum* chromosome was constructed by hybridizing radiolabelled probes to Southern blots of pulsed-field gels containing *T. pallidum* DNA treated with *Not*I, *Sfi*I, *Spe*I, and *Srf*I and all possible combinations of two enzymes. Probes consisted of 56 cosmid clones, 18 restriction fragments excised from CHEF gels, and 13 gene segments. Seventeen cosmid clones representative of all *Not*I-*Sfi*I hybridization patterns obtained were hybridized with the combined *Not*I-*Sfi*I-*Srf*I Southern blots. The remaining 39 cosmids were hybridized with blots containing *Not*I-*Spe*I single and double digests, but their precise positions in the *Sfi*I and *Srf*I restriction maps were not determined; hence, not all cosmid clones are shown in the physical map.

Hybridization of cosmid clones with blots of PFGE gels was the initial phase of our mapping strategy. A cosmid library of random clones from a partial *Bam*HI digest of *T. pallidum* DNA was constructed because of the small number of genetic probes otherwise available for the *T. pallidum* chromosome. pLAFRx, a cosmid based on the broad-host-range plasmid RK2 that maintains a relatively low copy number (four to eight copies per cell) (9, 30, 53), was selected for this purpose. Use of a low-copy-number plasmid should mitigate the problem of *T. pallidum* gene toxicity in *E. coli* that has been observed previously (25, 26, 55). The cosmid also accepts a relatively large insert (18 to 28 kbp) and has been used successfully in gene complementation, as demonstrated by complementation of *E. coli* auxotrophs with *Enterococcus faecalis* DNA (30). Size selection of DNA in sucrose gradients was employed to reduce the chance of ligation of two or more noncontiguous fragments that would be of appropriate size for ligation into the cosmid and subsequent packaging in lambda phage heads.

An example of a hybridization pattern obtained with a cosmid clone is shown in Fig. 2. In this case, cosmid BA28 hy-

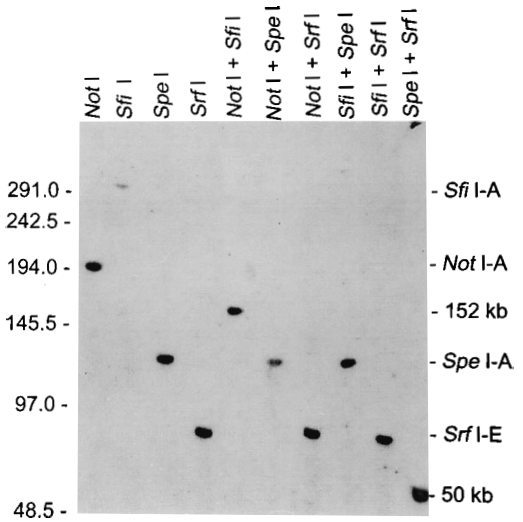

FIG. 2. Hybridization of cosmid clone BA28 with *T. pallidum* genomic DNA digested with *Not*I, *Sfi*I, *Spe*I, *Srf*I, and combinations thereof. *T. pallidum* DNA was treated with the enzymes indicated at the top of the gel pattern and subjected to PFGE. Restriction fragments hybridizing with BA28 are indicated on the right side of the autoradiograph, and the migration of DNA size standards (in kilobase pairs) is shown on the left side.

bridized with fragments *Not*I-A, *Sfi*I-A, *Spe*I-A, and *Srf*I-E. When *T. pallidum* DNA was digested with *Not*I-*Spe*I or *Sfi*I-*Spe*I, the fragment hybridizing with BA28 was the same size as *Spe*I-A. This result indicated that *Spe*I-A was located within fragments *Not*I-A and *Sfi*I-A and thus had no internal *Not*I or *Sfi*I sites. Fragment *Srf*I-E was also localized within *Not*I-A and *Sfi*I-A. Fragments *Sfi*I-A and *Not*I-A were found to overlap by 152 kb, as demonstrated by hybridization of BA28 with a 152-kb fragment in the *Not*I-*Sfi*I digest. Similarly, *Spe*I-A and *Srf*I-E were found to overlap by 50 kb as determined by the *Spe*I-*Srf*I double digest result. The results obtained with BA28 were combined with the other hybridization results to construct a physical map of this region.

In some cases, a cosmid hybridized with more than one fragment in a single-enzyme digest, establishing a linkage between these fragments. Examples of linking clones identified by hybridization with *Not*I and *Spe*I digests are provided in Fig. 3. A total of 18 such linkages among *Not*I, *Sfi*I, *Spe*I, and *Srf*I restriction fragments were identified through hybridizations with 20 cosmid clones.

Restriction fragments from *T. pallidum* genomic DNA were isolated from pulsed-field gels, radiolabelled, and used as probes as another means of establishing linkages. *Sfi*I, *Not*I, and *Srf*I fragments were supplemented with large fragments produced by *Asc*I and *Nhe*I, restriction enzymes that were otherwise not useful for physical mapping because of the many small fragments they produced (data not shown). Where restriction fragments overlapped the locations of cosmid clones, the restriction fragment hybridizations confirmed the linkages and alignments obtained with the cosmid probes. For example, hybridization with *Sfi*I-A showed that fragments *Srf*I-C2, -E, -H, and -I were linked and contained entirely within *Sfi*I-A. Hybridization with the largest fragment produced by digestion of *T. pallidum* DNA with *Nhe*I (124 kbp) showed the linkage between *Not*I fragments A and F2 (data not shown).

Hybridization of cloned or PCR-amplified genes of *T. pallidum* not only provided additional information for the physical map but also began the process of generating a combined physical-genetic map of the *T. pallidum* genome. Thus far, 13 *T. pallidum* genes have been localized on the physical map by hybridizing radiolabelled probes to PFGE blots. The genes used are listed in Table 1. Most genes were localized within 30 to 60 kbp on the basis of hybridization within overlapping regions of restriction fragments. In some cases, it was possible to localize genes within a much narrower range. For example, *tpn47*, which encodes a major 47-kDa lipoprotein, was found to be located on a 6-kbp *Srf*I fragment (*Srf*I-N), which was itself contained entirely within the 6.7-kbp overlap of *Sfi*I-C and *Not*I-J (Fig. 4). A total of five lipoprotein genes were localized: *tpn47*, *tpn41*, *tpn29-35*, *tpn17*, and *tpn15*. These genes hybridized to different fragments and were found to be scattered throughout the map. Other genes localized to date include those encoding an Mg1B homolog (*tpn41*), a porin-like protein (*tpn50*), the flagellar sheath and hook proteins (*flaA* and *flgE*, respectively), the 83-kDa cytoplasmic filament protein (*cfpA*), a minor 24-kDa antigenic polypeptide (*tpn24*), a putative transcriptional sigma factor (*rpoD*), and a deduced sequence homologous to the cysteinyl-tRNA synthetase of *E. coli* (*cysS*) (Table 1).

Physical map. Combination of the hybridization data resulted in the circular and linearized forms of the physical map of the *T. pallidum* chromosome shown in Fig. 5 and 6, respectively. The circular nature of the *T. pallidum* chromosome (49)

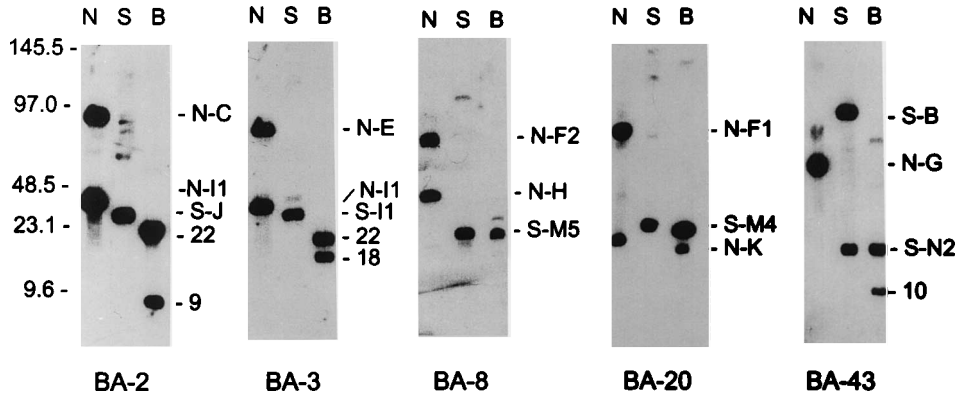

FIG. 3. Demonstration of the linkage of *Not*I or *Spe*I restriction fragments by cohybridization with *T. pallidum* DNA cosmid clones. Genomic DNA was treated with *Not*I (N), *Spe*I (S), or both enzymes (B), separated by CHEF, and transferred to nylon membranes. The resulting blots were hybridized with the cosmid clones indicated at the bottom of the figure. Restriction fragments hybridizing with each clone are identified on the right side of each autoradiograph; migration of DNA size standards (in kilobase pairs) is shown at the far left side.

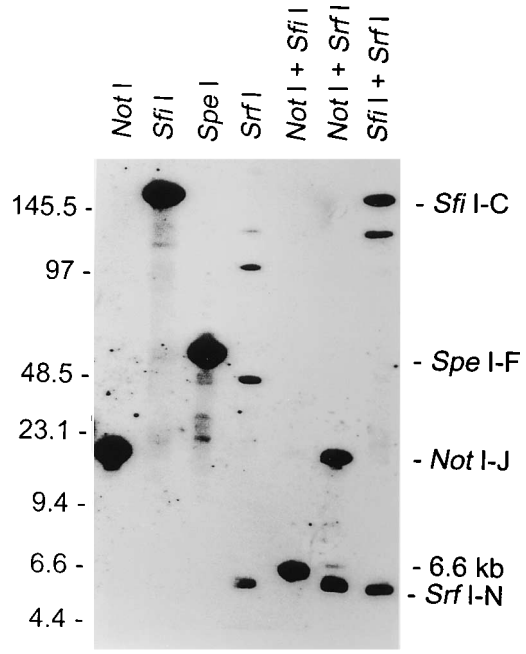

FIG. 4. Localization of the gene *tpn47* within a 6-kbp region of the *T. pallidum* genome. A Southern blot of *T. pallidum* genomic DNA was prepared with the restriction enzymes shown; bands hybridizing with radiolabelled recombinant plasmid pNC81 (31) are indicated on the right side. Hybridizing bands greater than 9 kbp in size in the *Srf*I, *Not*I-*Srf*I, and *Sfi*I-*Srf*I lanes represent partial digestion products. Relative migration of DNA standards (in kilobase pairs) is shown on the left side.

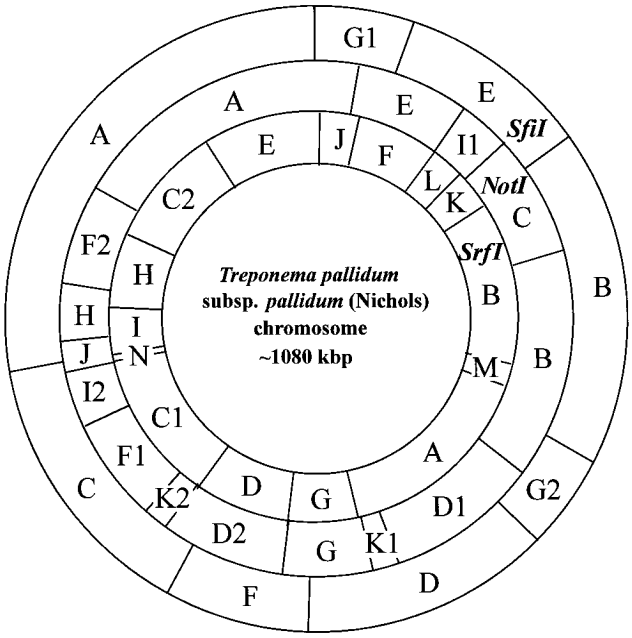

FIG. 5. Circular map of the chromosome of *T. pallidum* showing the relative arrangements of the restriction fragments generated by digestion with *Not*I, *Sfi*I, and *Srf*I. A more detailed version of the *T. pallidum* subsp. *pallidum* (Nichols) physical map with numerical coordinates can be obtained by contacting the corresponding author.

was confirmed by the physical mapping data. The *Sfi*I, *Not*I, and *Srf*I maps form closed circles as shown in Fig. 3, with the exception that the 6-kbp *Not*I-L fragment described previously has not been localized. The total genome size of 1,030 and 1,042 kbp obtained by adding the *Srf*I and *Not*I fragments, respectively, is smaller than the 1,083-kbp size obtained from summation of the *Sfi*I fragments (Table 2). This difference may be due to inaccuracies in the fragment size estimates (particularly for the larger DNA fragments) or to the presence of fragments not detected in this study. A faint, 65-kbp band has been observed in some gel patterns between the *Not*I-F and *Not*I-G bands (Fig. 1A), but this band has not been localized in the physical map by hybridization. The *Spe*I map is incomplete because of the presence of multiple small fragments which have not been localized.

DISCUSSION

In this report, we describe the construction of a physical map of the chromosome of *T. pallidum* subsp. *pallidum* (Nichols) based on the restriction fragments produced by *Not*I, *Sfi*I, and *Srf*I. The resultant macrorestriction map confirms that the genome of this bacterium consists of a single circular chromosome. This finding had been inferred previously from the migration of *T. pallidum* DNA in PFGE gels following exposure to gamma irradiation (49). Untreated, intact DNA did not migrate into the agarose matrix (indicative of its circular nature) whereas DNA linearized by treatment with 4 kilorads of gamma irradiation migrated as a single, 1-Mbp band (49).

Although most bacteria have single, circular chromosomes (26), this conformation is not universal among spirochetes (40). *B. burgdorferi*, a Lyme disease agent, has a 1-Mbp linear chromosome and multiple linear and circular plasmids (3, 6, 8, 10). In addition, *Leptospira interrogans* serovars icterohaemorrhagiae and pomona were shown by physical mapping to contain a large, circular chromosome of 4,400 to 4,600 kbp and a smaller, circular replicon of 350 kbp (2, 57, 58). *S. hyodysenteriae* has a single, circular chromosome with no extrachromosomal DNA detectable by PFGE (59), similar to *T. pallidum*. The chromosome of *S. hyodysenteriae*, at 3,200 kbp (59), is approximately three times larger than that of *T. pallidum*.

The approximate genome size of 1,030 to 1,083 kbp derived from this study is larger than the ~900-kbp size reported previously (49). The estimated genome size may require further revision as a more detailed physical map is developed. The difference from the previously reported genome size reflects the resolution of *Not*I doublets and new data provided by the digestion with the restriction endonucleases *Sfi*I and *Srf*I.

The localization of 13 genes in the physical map represents an early step in the construction of a genetic map of the *T. pallidum* chromosome. Thus far, approximately 30 *T. pallidum* genes have been identified (33). Genes for which functions are known include those that encode the endoflagellar subunits (7, 24, 25, 34), a gene for a chaperonin (GroEL) protein (16, 17, 20), 1-pyrroline-5-carboxylate reductase (12), a homolog of the galactose-glucose-binding protein (MglB) (5, 18), and the rRNA genes (11, 36). Recently, Weigel et al. (52) have provided evidence that the major 47-kDa lipoprotein of *T. pallidum* (22, 31, 51) functions as a carboxypeptidase involved in cell wall synthesis. In addition, several major lipoproteins of unknown function have been characterized (1, 14, 33, 37, 43, 45, 46, 53). Our laboratory has also cloned putative *T. pallidum* genes or gene fragments encoding the sigma 70 transcription initiation protein RpoD (21), a tRNA synthetase (50), and the protein subunit of the cytoplasmic filaments, ribbonlike structures unique to treponemes (56).

At present, little is known regarding the organization or regulation of genes within the *T. pallidum* chromosome. The

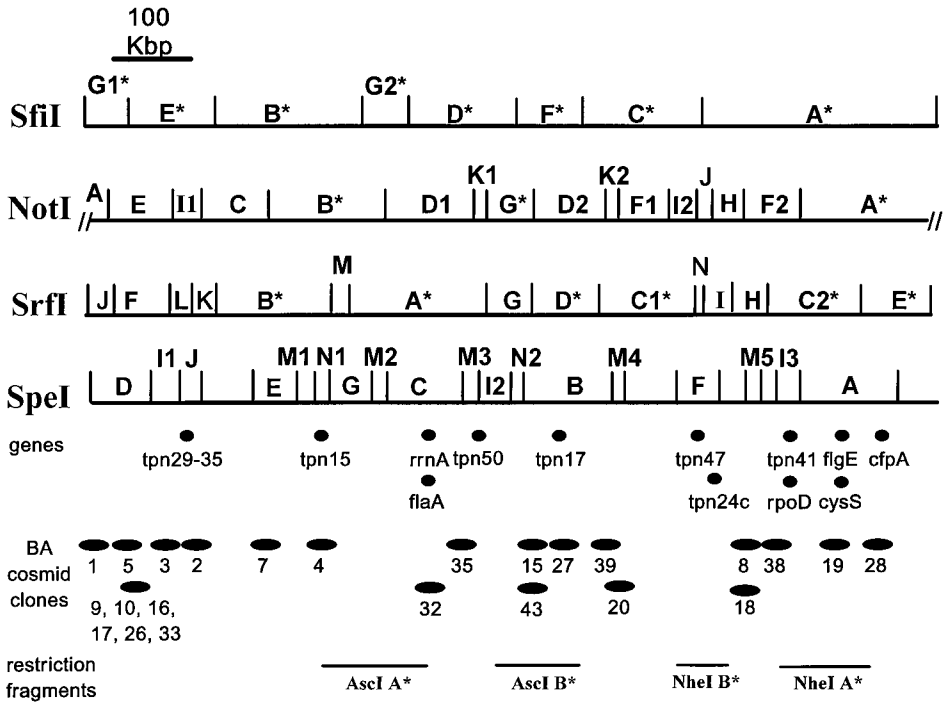

FIG. 6. Linear representation of the circular *T. pallidum* chromosome showing the relative locations of the restriction fragments produced by *Not*I, *Sfi*I, *Srf*I, and *Spe*I and the locations of genes, cosmid clones, and *Nhe*I and *Asc*I fragments as determined by hybridization. Restriction fragments used as probes for hybridization to genomic PFGE Southern blots are indicated by asterisks.

results of this study indicate that the *T. pallidum* lipoprotein genes *tpn47*, *tpn41*, *tpn29-35*, *tpn17*, and *tpn15* are scattered throughout the map. This finding demonstrates that these genes are not arranged in operons or gene clusters but does not preclude the possibility that they are members of regulons. Most *T. pallidum* genes characterized to date appear to be monocistronic, although three putative polycistronic operons have been identified. The membrane proteins TmpA and TmpB (TpN44.5a and TpN36 [33]) and their homologs in *Treponema phagedenis* are apparently translated from a single mRNA. The encoding regions of the two genes overlap by 1 nucleotide, and Northern (RNA) blot analysis of *T. phagedenis* mRNA indicates that both gene sequences are localized on a 2.5-kb message (14, 55). The *T. pallidum* genes encoding the flagellar core proteins FlaB1 and FlaB3 are located within 277 bp of one another, and the only recognizable promoter region consists of sequences resembling *Bacillus subtilis* −35 and −10 sigma 28 consensus sequences upstream of the initiation codon of *flaB1* (7); identical sequences are present upstream of the separate *flaB2* gene (34). Recently, Limberger and coworkers (27, 28) have located homologs of the motility genes *flgE*, *motA*, and *fliM* within a 6-kbp sequenced region of the *T. pallidum* genome; these genes appear to be part of a large, polycistronic operon under control of a class 2 (sigma 28-related) motility promoter.

Fukunaga et al. (11) reported that the 16S, 23S, and 5S rRNA genes of *T. pallidum* and *T. phagedenis* may be arranged into at least two sets on their chromosomes. Probes for each of the three rRNA genes hybridized with two fragments in *Bam*HI, *Bgl*II, *Eco*RV, and *Pst*I digests of genomic DNA. In this study, a gene probe coding for 16S rRNA (*rrnA*) obtained by PCR amplification of *T. pallidum* subsp. *pertenue* (Gauthier) DNA (35) hybridized with a single, 65-kbp region of the *T. pallidum* subsp. *pallidum* (Nichols) chromosome (Fig. 6). Further analysis will be necessary to determine (a) if there are multiple copies of the rRNA genes in the *T. pallidum* genome within the 65-kbp region and (b) how the rRNA genes are arranged. It is of interest that the 16S rRNA sequences of the closely related Nichols and Gauthier strains differ by 2 nucleotides (35, 36), providing a possible basis for distinguishing between syphilis and yaws isolates; this finding will be examined further in a separate publication.

The regulation of *T. pallidum* genes is a largely unexplored topic. *T. pallidum* lacks a detectable heat shock response (32, 43). Consistent with this finding, a sequence resembling the consensus sigma 70 promoter was found upstream of the *groEL* homolog of *T. pallidum* (16), rather than the sigma 32 promoter sequence typically associated with heat shock genes. Thus, gene regulation in *T. pallidum* may be simplified in some ways relative to that of other bacteria. It is interesting that other bacteria (particularly *Bacillus* species) lack sigma 32 promoter sequences for *dnaK*, *groEL*, and other heat shock genes and yet exhibit a heat shock response (54).

We have hypothesized that a correlation may exist between the highly fastidious nature of this organism and its small genome size (49). At approximately 10^6 bp, the genome is one of the smallest among the prokaryotes. Only members of the class *Mollicutes* are known to have a smaller genome size (as small as 570 kbp) (26, 37), and the genome of *B. burgdorferi* also is in the range of 10^6 bp (3, 6, 8, 10). These obligate parasites thus appear to be approaching the minimum genome coding potential required for prokaryotes. Thus far, too few genes have been localized on the *T. pallidum* genome to compare the gene content and organization of these organisms.

T. pallidum and other obligate parasites have undoubtedly evolved from a free-living bacterium with greater and more diverse genetic capabilities. All primordial bacteria were initially free living, and parasitism and dependence upon animal

hosts could only have occurred after these hosts themselves evolved. In the process of adaptation to the animal host, genes that provided selective advantages for survival and growth in water, soil, and other environments may have been lost by deletion through recombination or other means. Large-scale random sequence analysis of 100,993 nucleotides of the *Mycoplasma genitalium* genome was conducted recently by Peterson et al. (37). Their data indicated that a large proportion of the open reading frames with sequence homology to database entries encoded gene products required for basic housekeeping metabolic processes. For example, 51 of 106 (48%) putative *M. genitalium* gene products identified were involved in macromolecular synthesis (translation, transcription, or DNA synthesis), and none were involved in biosynthesis of small molecules (e.g., amino acids) (37). In contrast, only 24% of 1,720 *E. coli* gene products of known function surveyed by Riley (39) were involved in macromolecular synthesis, and 19% were enzymes involved in small-molecule biosynthesis. This information is fragmentary at this time; it is also subject to bias based on the types of genes currently in the databases and the evolutionary conservation (and hence preservation of homology) among important genes. However, it indicates that obligate parasites such as *M. genitalium* and *T. pallidum*, in the process of adapting to the relatively stable, nutrient-rich host environment, have lost biosynthetic capabilities and other adaptive properties. The resulting reduction in genome size may provide a selective advantage by decreasing the amount of energy expended on genome replication and maintenance. On the other hand, deletion as an irreversible process would not require positive selection. Merely the absence of positive selection for the DNA segment(s) undergoing deletion would be sufficient to reinforce the evolutionary trend toward a smaller genome size.

Further investigation of the genome sequences and functional capabilities of *T. pallidum* and other small-genome pathogens will not only provide information on the minimum genetic complement for metabolic requirements but will also identify genes required for survival, growth, and pathogenesis in a host that has itself evolved many mechanisms to prevent infection. Physical maps such as the one derived in this study will provide a basis for the derivation of contiguous DNA libraries and for continued analysis of the genetic content and organization of the *T. pallidum* genome.

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