INFECTION AND IMMUNITY, July 1991, p. 2476–2479
0019-9567/91/072476-04$02.00/0

Vol. 59, No. 7

Treponema pallidum subsp. *pallidum* Has a Single, Circular Chromosome with a Size of ~900 Kilobase Pairs

ELDON M. WALKER,[1]* JERRILYN K. ARNETT,[1] JOE DON HEATH,[2] AND STEVEN J. NORRIS[1]

Department of Pathology and Laboratory Medicine[1] and Department of Biochemistry and Molecular Biology,[2] University of Texas Medical School at Houston, Houston, Texas 77030

Received 21 December 1990/Accepted 18 April 1991

The genome size and chromosome conformation of *Treponema pallidum* subsp. *pallidum*, Nichols strain, were determined by contour-clamped homogeneous electric field electrophoresis, a pulsed-field gel electrophoresis technique. Digestion of *T. pallidum* subsp. *pallidum* DNA with the restriction endonucleases *Not*I and *Spe*I produced 12 and 26 fragments, respectively. Summation of the physical lengths of the fragments produced by *Not*I and *Spe*I cleavage yielded average sizes of 900 and 913 kbp, respectively, for the genome of *T. pallidum* subsp. *pallidum*. Contour-clamped homogeneous electric field electrophoresis of *T. pallidum* subsp. *pallidum* DNA exposed to 4 krads of gamma irradiation resolved a single band of 800 to 1,000 kbp; treatment of the DNA with 16 krads of gamma irradiation resulted in the production of smaller fragments, whereas untreated DNA did not migrate into the gels. The gamma irradiation results indicate that *T. pallidum* subsp. *pallidum* has a single, circular chromosome that was linearized at a dosage of 4 krads of gamma irradiation. The size estimate provided by restriction endonuclease digestion with *Not*I and *Spe*I shows that the genome of *T. pallidum* subsp. *pallidum*, at approximately 900 kbp, is considerably smaller than the 13,700-kbp genome size calculated from renaturation kinetics.

Genetic analysis of *Treponema pallidum* subsp. *pallidum* (*T. pallidum*), the causative agent of venereal syphilis, has been hampered by the lack of a system for continuous in vitro cultivation of the organism. Because clonal populations of *T. pallidum* cannot be propagated, genetic studies with standard methods such as conjugation, transduction, and transformation are not feasible.

Pulsed-field gel electrophoresis (PFGE) allows separation of linear DNA molecules in excess of 1,000 kbp; in contrast, circular DNA molecules in the size range of bacterial chromosomes do not migrate into agarose gels in response to pulsed-field electrophoresis techniques (16, 17). Purification and manipulation of DNA within a supportive agarose matrix makes it possible to generate a limited set of large fragments from intact chromosomal DNA, typically by digestion with rare-cutting restriction enzymes (15–17), although other methods can be used to achieve infrequent DNA cleavage (5, 16). Because PFGE can resolve such limited sets of large DNA fragments, it allows accurate determination of genome size and facilitates genetic characterization of prokaryotic and eukaryotic genomes by physical mapping (11, 16, 17).

As a first step in a comprehensive genetic study, we used a type of PFGE, contour-clamped homogeneous electric field (CHEF) electrophoresis (7), to determine the genome size and chromosome conformation of *T. pallidum*.

MATERIALS AND METHODS

Preparation of DNA inserts. The Nichols strain of *T. pallidum* was extracted from infected rabbit testes and purified by Percoll density gradient centrifugation as described previously (9). *T. pallidum* DNA was prepared by the method of Smith and Cantor (15). Briefly, *T. pallidum* was washed and resuspended in 1 M NaCl–10 mM Tris-HCl (pH 7.6), mixed with an equal volume of 1.6% low-melting-point agarose at 45°C (Incert Agarose; FMC BioProducts, Rockland, Maine), 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 EC lysis solution (6 mM Tris-HCl [pH 7.6], 1 M NaCl, 100 mM EDTA [pH 7.5], 0.5% polyoxyethylene 20 cetyl ether [Brij-58; Sigma Chemical Co., St. Louis, Mo.], 0.2% sodium deoxycholate [Sigma], 0.5% *N*-lauroylsarcosine [Sigma], RNase [20 μg/ml] [Sigma], and lysozyme [1 mg/ml] [Sigma]). The inserts were then treated for 2 days at 55°C with ESP solution (0.5 M EDTA [pH 9.3], 1% *N*-lauroylsarcosine, and proteinase K [50 μg/ml] [Sigma]). To remove proteinase K activity, the inserts were washed briefly at room temperature with TE buffer (10 mM Tris, 1 mM EDTA [pH 7.6]) and incubated for 1 h with 2 changes of TE buffer containing 1 mM phenylmethylsulfonyl fluoride (Sigma). The DNA inserts were rinsed thoroughly with TE and stored up to 2 weeks at 4°C prior to use.

Inserts containing *Escherichia coli* K-12 wild-type strain MG 1655 DNA (a strain that lacks plasmids) were generously provided by George Weinstock (Department of Biochemistry and Molecular Biology, University of Texas Medical School at Houston, Houston, Tex.) and were prepared as described for *T. pallidum*. Inserts containing *Rhodobacter sphaeroides* 2.4.1 DNA were the kind gift of Antonius Suwanto and Samuel Kaplan, Department of Microbiology, University of Texas Medical School at Houston, Houston, Tex., and were prepared as described previously (17).

Restriction endonuclease treatment. Prior to experimental manipulations, insert slices of approximately 30 μl in volume (equivalent to approximately 3×10^8 *T. pallidum* cells) were cut with a clean glass coverslip. For restriction endonuclease treatment, insert slices were equilibrated for 15 min at 4°C with 150 μl of the restriction buffer provided by the manufacturer. The buffer was replaced with 150 μl of fresh buffer containing 10 units of either *Not*I (Promega Corp., Madison, Wis.) or *Spe*I (New England BioLabs, Inc., Beverly, Mass.). The reaction mixtures were incubated at 37°C for 3 h, after

* Corresponding author.

which the inserts were rinsed for 30 min at room temperature with TE buffer.

Gamma irradiation studies. For gamma irradiation, insert slices containing *T. pallidum*, *E. coli*, or *R. sphaeroides* DNA were immersed in 1 ml of TE buffer in a 1.5-ml microcentrifuge tube. The inserts were exposed to increasing dosages of gamma irradiation from a ^{137}Cs source (model 143-45 irradiator; J. L. Shepherd and Associates, San Fernando, Calif.) that produced 288 rads/min, as calibrated by the manufacturer.

Pulsed-field gel electrophoresis. Restriction endonuclease-treated inserts were loaded 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). CHEF electrophoresis (CHEF II-DR Pulsed-Field Electrophoresis System; Bio-Rad Laboratories, Richmond, Calif.) for separation of restriction fragments was performed in 0.25× TBE at 15°C for 12 h at 200 V with a linearly increasing switching time of 1 to 10 s. These standard conditions provided optimal resolution of restriction fragments from 4 to 200 kbp. To confirm results obtained with our standard conditions, other pulse- and run-time parameters were also used to optimize the migration of bands in specific size ranges. Inserts treated with gamma irradiation were loaded into wells in 1% Seaplaque agarose (FMC) prepared in 0.5× TBE. The products of gamma irradiation were fractionated in 0.5 × TBE at 15°C for 96 h at 70 V with a switching time of 900 s. Lambda concatamers (multiples of 48.5 kbp; FMC), lambda *Hin*dIII fragments (0.125 to 23.1 kbp; Bio-Rad), and *Saccharomyces cerevisiae* chromosomes (245 to 2,500 kbp; Bio-Rad) were used as size standards. Following electrophoresis, gels were stained with ethidium bromide (2 μg/ml), and DNA bands were visualized with a UV transilluminator.

RESULTS

The results obtained from CHEF electrophoresis of the fragments produced by the cleavage of *T. pallidum* DNA with the restriction endonucleases *Not*I and *Spe*I are shown in Fig. 1 and tabulated in Table 1. Digestion with *Not*I produced 12 bands on CHEF gels (Fig. 1, lane 1); each band appears to represent a unique fragment species. In gels subjected to our standard CHEF electrophoresis parameters, bands C, D, and E (approximately 97, 93, and 87 kbp, respectively) were clearly resolved in lanes loaded with smaller quantities of *Not*I-cleaved DNA, conditions that did not allow visualization of the lower-molecular-weight bands (not shown). Gels run for 16 h at 200 V with a 6-s pulse time also afforded better resolution of fragments in the 70- to 140-kbp size range (not shown). Digestion of *T. pallidum* DNA with *Spe*I produced 26 fragments (Fig. 1, lane 2). Bands I, M, N, and T (approximately 30, 19, 16, and 7 kbp, respectively) appear to be doublets, as judged by ethidium bromide staining intensity. The molecular sizes of the fragments obtained from cleavage of *T. pallidum* DNA with *Not*I and *Spe*I are tabulated in Table 1 and represent the results of four gels for each restriction endonuclease. Summation of the molecular sizes of the *Not*I and *Spe*I fragments gave values of 900 ± 7 and 913 ± 14 kbp, respectively, for the total genome size of *T. pallidum*.

Gamma irradiation of circular double-stranded DNA at the appropriate dosage introduces a single double-stranded break per molecule (5); these linearized molecules can be resolved by PFGE. The results of a representative gamma irradiation experiment are shown in Fig. 2. When untreated DNA from either *T. pallidum* or *E. coli* (Fig. 2A, lanes 1 and 5, respectively) was subjected to CHEF electrophoresis, the DNA remained in the sample well, behavior typical of circular bacterial chromosomal DNA (16, 17). Exposure of *T. pallidum* DNA to 4 krads resulted in a strong band (Fig. 2A, lane 3) that migrated with linear DNA standards in the 800- to 1,000-kbp range; more precise sizing was difficult because of compression of the markers under the electro-

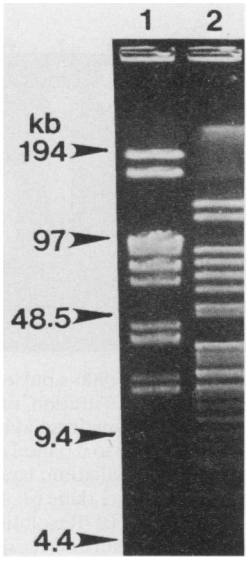

FIG. 1. Electrophoretic banding patterns of *Not*I-digested (lane 1) and *Spe*I-digested (lane 2) digested *T. pallidum* DNA after CHEF electrophoresis for 12 h at 200 V with a linearly increasing pulse time of 1 to 10 s. Positions of selected markers are indicated (arrowheads).

TABLE 1. Genome size of *T. pallidum* determined by *Not*I and *Spe*I restriction endonuclease digestion

*Spe*I fragment		*Not*I fragment	
Band	Size (kbp)	Band	Size (kbp)
A	124 ± 4.3	A	194 ± 0
B	110.5 ± 1.5	B	166 ± 2.5
C	85 ± 1.2	C	97 ± 0
D	76 ± 1.7	D	93 ± 1.0
E	68 ± 1.8	E	86.5 ± 1.9
F	60.5 ± 1.0	F	76.5 ± 1.9
G	51.5 ± 1.3	G	66 ± 0.8
H	48 ± 1.3	H	42.5 ± 1.3
I[a]	30 ± 1.7	I	36 ± 1.7
J	28 ± 2.2	J	20 ± 0.9
K	26 ± 2.5	K	16.5 ± 0.7
L	24 ± 2.6	L	5.9 ± 0.2
M[a]	19 ± 1.7		
N[a]	16 ± 1.6	Total	899.9 ± 7.1
O	15 ± 1.3		
P	13 ± 1.1		
Q	11 ± 0.3		
R	9.1 ± 0.1		
S	8.3 ± 0.1		
T[a]	7.3 ± 0.2		
U	5.6 ± 0.4		
V	4.6 ± 0.3		
Total	912.7 ± 13.9		

[a] Doublets.

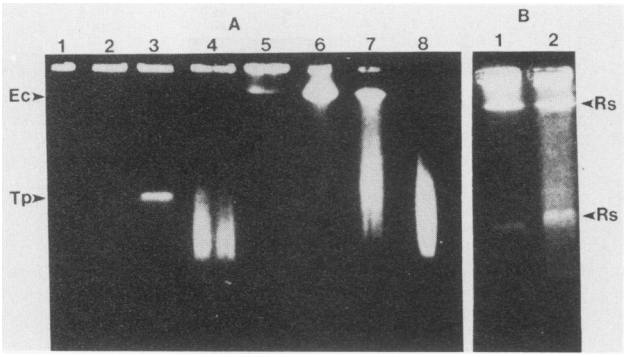

FIG. 2. CHEF electrophoresis (900-s pulse time for 96 h at 70 V) of gamma irradiated DNA. (A) Titration of the response of *T. pallidum* and *E. coli* DNA to gamma irradiation. Lanes 1 through 4 contain *T. pallidum* DNA exposed to 0 (lane 1), 1 (lane 2), 4 (lane 3), and 16 (lane 4) krads of gamma irradiation; lanes 5 through 8 contain *E. coli* DNA exposed to 0 (lane 5), 1 (lane 6), 4 (lane 7), and 16 (lane 8) krads of gamma irradiation. (B) Resolution of the 3,140- and 914-kbp chromosomes after exposure of *R. sphaeroides* DNA to 1 (lane 1) and 4 (lane 2) krads of gamma irradiation. The positions of the *T. pallidum* (Tp), *E. coli* (Ec), and *R. sphaeroides* (Rs) chromosomes are indicated (arrowheads). The position of the *T. pallidum* chromosome corresponds to the migration distance of a compressed group of *S. cerevisiae* linear chromosome markers in the 800- to 1,000-kbp range.

phoresis conditions used. Exposure to 1 krad produced a fainter band at the same position (Fig. 2A, lane 2), whereas 16 krads resulted in a smear of smaller fragments (Fig. 2A, lane 4), indicating multiple, random double-stranded breaks per molecule. These results indicate that a single population of linear DNA molecules was produced after exposure of *T. pallidum* DNA to 4 krads of gamma irradiation and are consistent with the interpretation that the organism has a single circular chromosome with a physical length in the range of the genome size determined by restriction endonuclease digestion (approximately 900 kbp). Treatment of *E. coli* with 1 krad yielded the expected large DNA molecule (Fig. 2A, lane 6), consistent with linearization of its 4,600-kbp chromosome (17), while 4 (Fig. 2A, lane 7) and 16 krads (Fig. 2A, lane 8) of gamma irradiation caused progressive fragmentation of the DNA. Exposure of *R. sphaeroides* DNA to 1 and 4 krads of gamma irradiation confirmed the presence of two circular chromosomes (Fig. 2B, lanes 1 and 2, respectively), as determined previously by physical mapping techniques (18, 19).

DISCUSSION

The results of our restriction fragment analysis show that the genome of *T. pallidum* is approximately 900 kbp in length. Previously, the genome of *T. pallidum* was calculated on the basis of renaturation kinetics to be 13,700 kbp (9.05×10^9 Da [13]). Unlike indirect methods of calculating genome molecular weight, PFGE allows visualization of large DNA fragments and measurement of the lengths of these fragments, thus providing a direct and accurate means of determining the physical sizes of bacterial genomes (11, 17). Given the agreement between the genome size estimates obtained by digestion of *T. pallidum* DNA with *Not*I and *Spe*I, the *T. pallidum* genome size reported in this study appears accurate and corrects the previous size estimate (13). The reason for the high estimated size of the *T. pallidum* genome calculated from renaturation kinetics is not clear. *T. pallidum* has a G+C base content of approximately 53% (13); therefore, explanations based on low G+C base composition (6, 12) are not applicable to the disparity between the genome size estimates obtained for *T. pallidum* from renaturation kinetics and PFGE.

The results of our gamma irradiation experiments indicate that *T. pallidum* has a single, circular chromosome; definitive confirmation of this result will require construction of a physical map of the chromosome of *T. pallidum*. In the initial description by Beverley (5), relatively small DNA molecules (a 33-kbp plasmid and an 85-kbp circular DNA molecule from *Leishmania major*) were used to demonstrate the efficacy of gamma irradiation and PFGE for estimating circular DNA size. Our report indicates that this method can be applied to the determination of the approximate size, circularity, and number of bacterial chromosomes, including those in the size range of *E. coli* (4,600 kbp).

Norgard and Miller (14) reported the presence of a small, circular plasmid of approximately 11.4 kbp (7.5×10^6 Da) in preparations of *T. pallidum* DNA, thereby suggesting that extrachromosomal DNA may contribute to the DNA content of *T. pallidum*. We were unable to detect by CHEF electrophoresis the presence of bands corresponding to plasmids in our *T. pallidum* DNA preparations. Although open circular DNA molecules larger than 30 kbp do not appear to enter pulsed-field gels, supercoiled circular molecules (up to at least 80 kbp) as well as small open circular molecules can enter pulsed-field gels and migrate in a pulse-time-independent fashion (4, 10). Untreated *T. pallidum* DNA remained almost entirely in the well under the standard CHEF electrophoresis conditions that we used for the separation of restriction fragments; no bands corresponding to the expected size for plasmids were visible. In contrast, control recombinant plasmids (12 and 19 kbp), as well as plasmids from preparations of total *R. sphaeroides* DNA, migrated to form discrete bands corresponding to supercoiled, relaxed, and linear forms when subjected to the same electrophoresis conditions (20). Resolving the question of the existence of *T. pallidum* extrachromosomal DNA will require further studies.

The size of the *T. pallidum* genome derived from this study places it among the smallest known bacterial genomes (11). *Borrelia burgdorferi*, the spirochete that causes Lyme disease, and *Mycoplasma* spp. and *Ureaplasma urealyticum*, bacteria that lack cell walls, also have been reported to have genomes in the range of 1,000 kbp or less (2, 8, 11). The small sizes of the genomes of *T. pallidum* and *B. burgdorferi* are consistent with the highly fastidious nature of these spirochetes; *T. pallidum* cannot be propagated continuously in vitro, while *B. burgdorferi* requires an enriched growth medium (1). On the other hand, the chromosome size of the spirochetes *Leptospira interrogans* and *Leptospira biflexa* (pathogenic and saprophytic species, respectively) was shown by CHEF electrophoresis to be 5,000 kbp, which correlates with the ability of these spirochetes to grow in a defined medium and survive for prolonged periods in water and other environmental locations (3).

This report represents the first application of PFGE technology to the study of the DNA of *T. pallidum*. Identification of restriction enzymes that cleave total DNA of *T. pallidum* into a small number of fragments establishes a basis for the physical mapping of the genome of the organism. Because physical mapping does not require genetic manipulations, it provides a previously unavailable means for studying the genetics of *T. pallidum*. Construction of a physical map will

allow us to investigate the genetic organization of *T. pallidum*, with the goal of gaining insight into the physiological properties and virulence mechanisms of this unique pathogen.

ACKNOWLEDGMENTS

We thank George Weinstock for providing us with equipment and materials essential to this study. We also thank Samuel Kaplan, Antonius Suwanto, and Page Nelson for technical assistance and helpful discussions.

This work was supported in part by Public Health Service grant RO1 AI 20006 from the National Institute of Allergy and Infectious Diseases.

REFERENCES

1. **Barbour, A. G.** 1984. Isolation and cultivation of Lyme disease spirochetes. Yale J. Biol. Med. **42**:634–638.
2. **Baril, C., C. Richaud, G. Baranton, and I. Saint Girons.** 1989. Linear chromosome of *Borrelia burgdorferi*. Res. Microbiol. **140**:507–516.
3. **Baril, C., and I. Saint Girons.** 1990. Sizing of the *Leptospira* genome by pulsed field agarose gel electrophoresis. FEMS Microbiol. Lett. **71**:95–100.
4. **Beverley, S. M.** 1988. Characterization of the unusual mobility of large circular DNAs in pulsed-field gradient electrophoresis. Nucleic Acids Res. **16**:925–938.
5. **Beverley, S. M.** 1989. Estimation of circular DNA size using gamma-irradiation and pulsed-field electrophoresis. Anal. Biochem. **177**:110–114.
6. **Chang, N., and D. E. Taylor.** 1990. Use of pulsed-field agarose gel electrophoresis to size genomes of *Campylobacter jejuni* UA580. J. Bacteriol. **172**:5211–5217.
7. **Chu, G., D. Vollrath, and R. W. Davis.** 1986. Separation of large DNA molecules by contour-clamped homogeneous electric fields. Science **234**:1582–1585.
8. **Ferdows, M. S., and A. G. Barbour.** 1989. Megabase-sized linear DNA in the bacterium *Borrelia burgdorferi*, the Lyme disease agent. Proc. Natl. Acad. Sci. USA **86**:5969–5973.
9. **Hanff, P. A., S. J. Norris, M. A. Lovett, and J. N. Miller.** 1984. Purification of *Treponema pallidum*, Nichols strain, by Percoll density gradient centrifugation. Sex. Transm. Dis. **11**:275–286.
10. **Hightower, R. C., D. W. Metge, and D. V. Santi.** 1987. Plasmid migration using orthogonal-field-alternation electrophoresis. Nucleic Acids Res. **15**:8387–8398.
11. **Krawiec, S., and M. Riley.** 1990. Organization of the bacterial chromosome. Microbiol. Rev. **54**:502–539.
12. **Maniloff, J.** 1989. Anomalous values of *Mycoplasma* genomes sizes determined by pulse-field gel electrophoresis. Nucleic Acids Res. **17**:1268.
13. **Miao, R. M., and A. H. Fieldsteel.** 1978. Genetics of *Treponema*: relationship between *Treponema pallidum* and five cultivable treponemes. J. Bacteriol. **133**:101–107.
14. **Norgard, M. V., and J. N. Miller.** 1981. Plasmid DNA in *Treponema pallidum* (Nichols): potential for antibiotic resistance by syphilis bacteria. Science **213**:553–555.
15. **Smith, C. L., and C. R. Cantor.** 1987. Purification, specific fragmentation and separation of large DNA molecules. Methods Enzymol. **155**:449–467.
16. **Smith, C. L., and G. Condemine.** 1990. New approaches for physical mapping of small genomes. J. Bacteriol. **172**:1167–1172.
17. **Smith, C. L., J. Econome, A. Schutt, S. Klco, and C. R. Cantor.** 1987. A physical map of the *E. coli* K12 genome. Science **236**:4481–4490.
18. **Suwanto, A., and S. Kaplan.** 1989. Physical and genetic mapping of the *Rhodobacter sphaeroides* 2.4.1 genome: genome size, fragment identification, and gene localization. J. Bacteriol. **171**:5840–5849.
19. **Suwanto, A., and S. Kaplan.** 1989. Physical and genetic mapping of the *Rhodobacter sphaeroides* 2.4.1 genome: presence of two unique circular chromosomes. J. Bacteriol. **171**:5850–5859.
20. **Walker, E. M., and S. J. Norris.** Unpublished data.