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Size of the *Streptococcus mutans* GS-5 Chromosome as Determined by Pulsed-Field Gel Electrophoresis

JOHN J. TUDOR,[1] LAURA MARRI,[2] PATRICK J. PIGGOT,[3] AND LOLITA DANEO-MOORE[3]*

Biology Department, Saint Joseph's University, Philadelphia, Pennsylvania 19131[1]; Istituto di Microbiologia, Università di Siena, Siena, Italy[2]; and Department of Microbiology and Immunology, Temple University School of Medicine, Philadelphia, Pennsylvania 19140[3]

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Rare cutting restriction endonucleases were used to cut the *Streptococcus mutans* chromosome into large fragments. Restriction enzymes utilizing recognition sites containing 6-, 7-, or 8-base-pair sequences with only G and C nucleotides produced few fragments, most of which were >100 kilobase pairs in size. Addition of the fragments from digests of *Sma*I, *Not*I, *Apa*I, *Rsr*II, and *Eag*I yielded a molecular size for the *S. mutans* GS-5 genome of 2,819 ± 60 kilobase pairs.

Pulsed-field gel electrophoresis can be used for the separation of large DNA fragments up to >10 megabase pairs in size (3, 12). Combining this technique with controlled restriction digestion by rare cutting restriction endonucleases permits restriction analysis of whole bacterial genomes. This approach has recently been used successfully for several bacterial species, yielding accurate calculation of chromosomal size (2, 5, 7) and construction of restriction maps for the entire genome (1, 6, 13). Obtaining restriction patterns of the whole chromosome is central to the construction of a physical map of the genome. In an ongoing effort to construct a complete physical map of the *Streptococcus mutans* genome, we have been able to identify a number of restriction endonucleases which yield large DNA fragments, five of which produced 19 or fewer fragments, the sizes of which were determined by field inversion gel electrophoresis (FIGE; 3) or contour-clamped homogeneous electric field (CHEF; 4) electrophoresis or both. Addition of these fragments was used to determine the molecular size of the *S. mutans* GS-5 chromosome.

S. mutans GS-5 (10) was routinely cultured in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) supplemented with 2% glucose and 0.1% L-cysteine (THG) at 37°C. Cultures were stored frozen at −70°C as 10× concentrates in THG containing 10% glycerol. High-molecular-weight DNA was prepared from bacteria embedded in agarose beads by a modification of the method of Piggot and Curtis (11). One milliliter of a 10×-frozen glycerol culture was added to 30 ml of fresh prewarmed THG and incubated at 37°C. The optical density of the cultures was monitored until the culture reached the mid-log phase of growth (approximately 5×10^7 to 1×10^8 cells per ml). The cells were pelleted by centrifugation and suspended in 1 ml of TE (10 mM Tris hydrochloride, 1 mM EDTA) buffer (pH 8.0), transferred to a 50-ml round-bottom flask, and incubated at 40°C for 30 min. A 0.3-ml amount of molten 2.5% low-melting-point agarose (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) solution at 40°C was added, and the solution was mixed vigorously for 1 min followed by the addition of 2.5 ml of light mineral oil and, again, vigorous mixing for 90 s. The flask was immediately chilled in ice-cold water for at least 10 min to allow for the formation of agarose beads. The beads were washed three times in T100E (10 mM Tris hydrochloride, 100 mM EDTA buffer, pH 7.5) and suspended in 1 ml of T100E, and the cells were digested with 4 mg of lysozyme (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and 50 U of mutanolysin (Sigma Chemical Co., St. Louis, Mo.) for 30 min at 40°C. Sarkosyl (final concentration, 1.5%) and 4 mg of pronase (Calbiochem-Behring, La Jolla, Calif.) were added; incubation was at room temperature for up to 1 h. The digested beads were then washed in T100E and suspended in T100E to be stored at 4°C for up to 3 weeks until use.

Agarose beads were washed five times in TE buffer, pH 8.0, followed by three washes in the appropriate restriction buffer. Restriction endonucleases were added (5 to 8 U per 50 μl), and digestion was carried out for 2 h. All reaction mixtures were incubated at 37°C except *Sma*I digests, which were incubated at 25°C, and *Bss*HII and *Sfi*I digests, which were incubated at 50°C. Agarose (1%) gels were loaded with 25 to 30 μl of the digested beads. Lambda oligomers (14) were routinely used as molecular size standards in all experiments. Gels were electrophoresed by using either FIGE (Bio-Rad Pulsewave 760; Bio-Rad Laboratories, Richmond, Calif.) or CHEF electrophoresis (LKB Pulsaphor Plus; Pharmacia-LKB Biotechnology, Piscataway, N.J.) with a variety of running conditions. Generally, the CHEF electrophoresis was run at 4°C at 180 V with switching time of 20 or 30 s for 24 h or at 120 V with a 30-s switching time for 72 h. FIGE gels were electrophoresed at 10°C for 24 or 40 h, using a program to give a forward pulse of 2.25 to 22.5 s and a reverse pulse of 0.75 to 7.5 s. Electrophoresis was carried out in 1% agarose (SeaKem LE; FMC BioProducts, Rockland, Maine) with 0.5× TAE (0.04 M Tris acetate, 0.002 M EDTA) buffer, pH 8.0. Following electrophoresis, the gels were stained with ethidium bromide and viewed with a UV transilluminator to locate the separated fragments.

Figure 1 illustrates typical CHEF (panel a) and FIGE (panel b) digests, using various restriction enzymes. Additional electrophoresis conditions were used to resolve material poorly resolved in any one gel. As would be expected (9), due to the low G+C content (36%) of the *S. mutans* GS-5 genome, restriction enzymes which recognize sites containing only G and C residues resulted in the fewest number of bands and the largest number >100 kilobase pairs (kbp) in size. Table 1 lists the restriction enzymes we have tested, their recognition sequences, and the number of produced fragments >100 kbp. Restriction endonucleases *Bgl*I, *Bst*XI,

* Corresponding author.

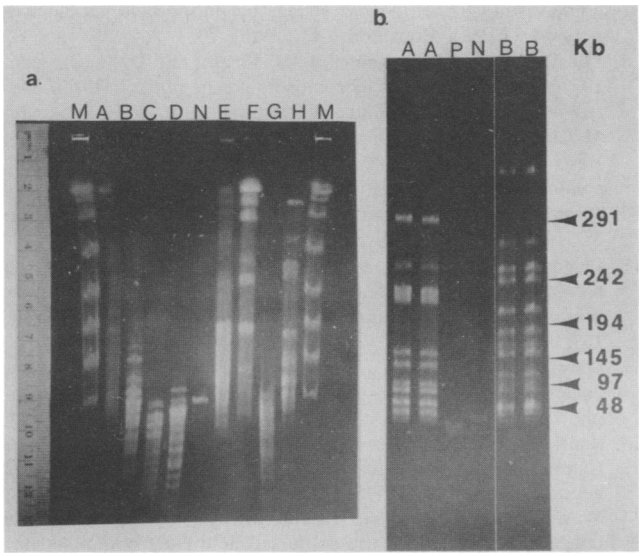

FIG. 1. Restriction enzyme digests of *S. mutans* GS-5 chromosomal DNA. The bands in lane M of the lambda ladder represent lambda oligomers corresponding to, reading upwards, 48.5, 97, 145.5 kbp, etc. (a) CHEF electrophoresis. Lanes: A, *Apa*I; B, *Bgl*I; C, *Bst*XI; D, *Eco*NI; E, *Nar*I; F, *Not*I; G, *Pfl*MI; H, *Sma*I. Lane M contains lambda ladder, and lane N contains lambda. Running conditions: 185 V for 24 h at 4°C, with a switching time of 18 s. (b) FIGE. Lanes: A, *Sma*I; B, *Apa*I; N, lambda; P, lambda *Hin*dIII digest. Size of lambda oligomers is indicated. The oligomers are faintly visible only on the negative of the photograph. Running conditions: 150 V at room temperature, with an initial forward pulse of 2.2 s, increasing over 24 h to a final forward pulse of 22.5 s; the ratio of forward to reverse pulse was 3:0.

*Eco*NI, *Pfl*MI, and *Bss*HII all produced a large number of fragments, most of which were smaller than 100 kbp. *Bss*HII did, however, produce a number of larger fragments ranging from 100 to 200 kbp which could be useful as probes in subsequent mapping experiments. The other enzymes used produced at least 10 fragments >100 kbp. All of these endonucleases should be valuable in the construction of a restriction map of the *S. mutans* genome.

Five restriction endonucleases, *Sma*I, *Not*I, *Apa*I, *Rsr*II, and *Eag*I, consistently produced fewer than 20 fragments which could be separated by using the pulsed-field conditions described above. The number of fragments and their sizes are listed in Table 2. The distance each fragment traveled in the gel from the origin was measured, and sizes of DNA fragments obtained were calibrated by using a lambda DNA ladder and lambda *Hin*dIII digests as molecular size markers. Addition of all sizes of fragments generated by these enzymes was used to determine the molecular size of the *S. mutans* GS-5 chromosome. The size determinations ranged from 2,710 kbp, *for Rsr*II digests, to 2,855 kbp, for *Apa*I digests, with a mean value of 2,819 kbp. This size is in reasonable agreement with the genome size of *S. mutans* GS-5 which has been estimated previously by using reassociation kinetics without correction for base composition (8) to be 1.79×10^9 daltons, corresponding to 2,725 kbp. Visualization and size measurement of restriction fragments directly by pulsed-field gel electrophoresis provide a more accurate means of determining genome size.

The identification of several rare cutting restriction endonucleases which produce a number of large fragments will be useful in the further analysis of the *S. mutans* GS-5 genome. Known *S. mutans* genes which have been cloned can be localized on specific fragments by Southern hybridization. Also, the large fragments generated by one restriction endonuclease can be used as labels to probe restriction digests produced by other restriction enzymes. The use of such a procedure would identify adjoining fragments that hybridize with the same probe and would provide a means to construct a physical map of the entire *S. mutans* GS-5 chromosome.

TABLE 1. Number of fragments >100 kbp in size generated by various restriction endonucleases

Restriction endonuclease	Recognition site	No. of fragments >100 kbp
*Apa*I	GGGCCC	12
*Bgl*I	GCCNNNNNGGC	3
*Bss*HII	GCGCGC	7
*Bst*XI	CCANNNNNNTGG	0
*Eag*I	CGGCCG	10
*Eco*NI	CCTNNNNNAGG	0
*Nae*I	GCCGGC	10
*Nar*I	GGCGCC	11
*Not*I	GCGGCCGC	10
*Pfl*MI	CCANNNNNTGG	4
*Rsr*II	CGGACCG	11
*Sac*II	CCGCGG	—[a]
*Sfi*I	GGCCNNNNNGGCC	—[b]
*Sma*I	CCCGGG	11

[a] *Sac*II digests consistently appeared as smears on the gels, without distinct bands being produced.
[b] *Sfi*I apparently does not cut the *S. mutans* GS-5 chromosomal DNA.

TABLE 2. Restriction analysis of the *S. mutans* chromosomal DNA[a]

Fragment no.	Fragment size (kbp)[b]				
	*Sma*I	*Not*I	*Apa*I	*Rsr*II	*Eag*I
1	320 ± 12	435 ± 20	415 ± 30	390 ± 5	510
2	300 ± 7	385 ± 22	350 ± 7	330 ± 19	465
3	285 ± 8	330 ± 14	285 ± 10	300 ± 16	345
4	240 ± 6	300 ± 13	255 ± 8	245 ± 12	275
5	225 ± 6	275 ± 9	245 ± 10	230 ± 9	245
6	205 ± 3	235 ± 7	210 ± 6	190 ± 5	195
7	195 ± 5	215 ± 4	190 ± 7	180 ± 8	135
8	170 ± 3	175 ± 15	175 ± 5	150 ± 4	120
9	145 ± 7[c]	140 ± 5	160 ± 4	130 ± 3	110
10	125 ± 6	125 ± 8	135 ± 8	120 ± 2	100
11	100 ± 5	82 ± 4	120 ± 7	105 ± 5	90
12	90 ± 3	70 ± 4	100 ± 5	85 ± 4	80
13	80 ± 2	35 ± 4	85 ± 5	75 ± 5	60
14	70 ± 4	25 ± 2	70 ± 3	60 ± 2	50
15	55 ± 3	5 ± 0.5	60 ± 4	50 ± 2	40
16	45 ± 3			40 ± 1	30
17	35 ± 4			30 ± 3	
18	15 ± 2				
19	5 ± 2				
Total bp, 10^6	2.850	2.830	2.855	2.710	2.850

[a] Calculated genome size was $2.819 \pm 0.06 \times 10^6$ bp.
[b] Each fragment listed represents the mean of at least five different gels for fragments >100 kbp and at least four gels for fragments <100 kbp, with the exception of the *Eag*I fragments, which represent only two gels. Conditions in addition to those listed in the legend to Fig. 1 were used to resolve fragments that were poorly resolved.
[c] Two fragments are thought to comigrate in this position.

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