Construction of a *NotI* restriction map of the *Streptococcus mutans* genome

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Streptococcus mutans and *Streptococcus sobrinus* are the major causative organisms of human dental caries. Pulsed-field gel electrophoresis (PFG) showed that the restriction enzyme *NotI* produced ten and six DNA fragments from the genomes of *S. mutans* strain MT8148 and *S. sobrinus* strain 6715, respectively. The sizes of the chromosomes of *S. mutans* and *S. sobrinus* were each estimated to be about 2200 kb. The *NotI* restriction map of *S. mutans* MT8148 genome was constructed by Southern blot analysis with probes that overlapped two adjacent *NotI* fragments. Several virulence-associated genes of *S. mutans* were placed on the *NotI* restriction map. In addition, unique 'fingerprints' of *S. mutans* chromosomal DNA digested with *NotI* were produced by PFG, and these may be useful for epidemiological studies.

Introduction

Among the mutans streptococci, *Streptococcus mutans* and *Streptococcus sobrinus* play a significant role in the development of human dental caries (Hamada & Slade, 1980; Loesche, 1986). These organisms possess several characteristic activities which are associated with their cariogenicity: they produce a large amount of lactic acid and other acids from various sugars, synthesize water-insoluble glucan from sucrose, and colonize human tooth surfaces in either the absence or presence of sucrose (Hamada & Slade, 1980).

Genetic approaches have been recently introduced to analyse virulence factors of *S. mutans* and *S. sobrinus* (Curtiss, 1986; Kuramitsu, 1987). Several genes potentially involved in their cariogenicity have been cloned and characterized (Abiko *et al.*, 1989; Barrett *et al.*, 1987; Ferretti *et al.*, 1987, 1988, 1989; Hanada & Kuramitsu, 1989; Lee *et al.*, 1988; Lunsford & Macrina, 1986; Ogundipe & Holt, 1989; Okahashi *et al.*, 1989a, b; Sato & Kuramitsu, 1988; Shiroza & Kuramitsu, 1988; Shiroza *et al.*, 1987; Sommer *et al.*, 1987; Takahashi *et al.*, 1989; Ueda *et al.*, 1988). However, little is known of the overall genetic organization of these bacteria.

Recently, pulsed-field gel electrophoresis (PFG) was developed to separate large DNA fragments (Schwartz & Cantor, 1984). Physical maps of several bacterial genomes have been constructed by using PFG (Bautsch, 1988; Canard & Cole, 1989; Ely & Gerardot, 1988; Kauc *et al.*, 1989; Lee *et al.*, 1989; Smith *et al.*, 1987). Here we present a physical map of the genome of *S. mutans* MT8148 (serotype *c*) constructed by Southern blot analysis of restriction fragments separated by PFG.

Methods

Bacterial strains. *S. mutans* MT8148 (serotype *c*) and GS-5 (serotype *c*) (Ohta *et al.*, 1989), and *S. sobrinus* 6715 (serotype *g*) were mainly used. In some experiments, *S. mutans* strains MT6801, MT6861, MT6879, Xc and Xc100L (Koga *et al.*, 1989; Okahashi *et al.*, 1984) were used. These strains were selected from the stock culture collection in the Department of Dental Research, National Institute of Health, Tokyo, Japan.

Preparation of DNA in agarose blocks. Chromosomal DNA of *S. mutans* and *S. sobrinus* was prepared as described by Smith & Cantor (1987). An overnight cell culture was diluted 1:50 into Todd-Hewitt broth (Difco) supplemented with 20 mM-DL-threonine, and was grown to a density of about 2×10^9 cells ml⁻¹. The cells were centrifuged, washed with 10 mM-Tris/HCl (pH 8.0) containing 1 M-NaCl, and suspended in one-fifth of the original volume in the same buffer. The bacterial suspension was mixed with an equal volume of 1% (w/v) low-gelling/melting temperature agarose (InCert agarose; FMC). The mixture was allowed to solidify in a 100 µl mould to form blocks. The agarose block was put into a lysis solution [1 M-NaCl, 0.1 M-EDTA,

Abbreviations: PFG, pulsed-field gel electrophoresis; OFGE, orthogonal field alternation gel electrophoresis; CHEF, contour-clamped homogeneous field electrophoresis.

10 mM-Tris/HCl (pH 8.0), 0.5% (w/v) Brij 58, 0.2% (w/v) sodium deoxycholate, 0.5% (w/v) Sarkosyl (Ciba-Geigy), lysozyme (1 mg ml⁻¹) (Smith & Cantor, 1987) supplemented with *N*-acetylmuramidase SG (0.1 mg ml⁻¹; Seikagaku Kogyo). After overnight incubation at 37 °C, the agarose block was incubated in a solution of 0.25 M-EDTA (pH 8.0) containing 1% (w/v) Sarkosyl and proteinase K (0.1 mg ml⁻¹; Boehringer Mannheim) overnight at 50 °C. The block was then incubated for 4 h in TE buffer [10 mM-Tris/HCl (pH 8.0), 1 mM-EDTA] containing 1 mM-phenylmethylsulphonyl fluoride, and washed four times with TE buffer.

Restriction enzyme digestion. The agarose block of DNA (100 µl) was incubated for 18 h with 20 to 40 units of *Not*I, *Sfi*I, *Apa*I, *Nae*I, *Sma*I or *Sac*II (Toyobo) in a total volume of 400 µl, following the recommendations of the manufacturer. For *Not*I partial digestion, the agarose block (100 µl) was incubated at 37 °C for 18 h in the presence of 3 units of *Not*I in a total volume of 400 µl.

PFG. Both orthogonal field alternation gel electrophoresis (OFAGE; Carle & Olson, 1984) and contour-clamped homogeneous field electrophoresis (CHEF; Chu *et al.*, 1986) systems (Pulsaphor Plus; Pharmacia LKB Biotechnology) were used. In both systems, 1.0% (w/v) agarose gels in 1 × TBE buffer [0.89 M-Tris, 0.89 M-boric acid, 2 mM-EDTA (pH 8.3)] were used at 10 °C. OFAGE gels were run at 330 V for 24 to 40 h with pulse times ranging from 2 to 60 s, depending on the resolution range desired. CHEF gels were run at 170 V for 40 h with pulse times ranging from 10 to 60 s. λ DNA concatemers (Clontech) were used as the size standards.

Construction of *Not*I linking clones. Chromosomal DNA from *S. mutans* MT8148, prepared as described previously (Okahashi *et al.*, 1989a), was digested with *Eco*RI, *Hind*III, *Pst*I or *Bgl*II, and self-ligated with T4 DNA ligase (Takara Shuzo). The ligated fragments which contained *Not*I restriction sites were then linearized with *Not*I, and ligated to the *Not*I-digested alkaline-phosphatase-treated plasmid vector pBlueScript II (Stratagene). *Escherichia coli* NM522 (Stratagene) was then transformed with the plasmid and plated on LB agar (Maniatis *et al.*, 1982) containing ampicillin (50 µg ml⁻¹), 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside (40 µg ml⁻¹) and isopropyl β -D-thiogalactopyranoside (0.2 mM). Plasmids were isolated from the *Lac*⁻ transformants, digested with *Not*I, *Eco*RI, *Hind*III, *Pst*I or *Bgl*II, subjected to agarose gel electrophoresis to establish fragment size, and used as the *Not*I linking clones.

Southern hybridization. DNA fragments separated by CHEF were transferred to nitrocellulose membranes (Maniatis *et al.*, 1982). DNA fragments containing a *Not*I restriction site were isolated from an agarose gel, and labelled by the random-oligonucleotide priming method (Feinberg & Vogelstein, 1983) using deoxycytidine 5'- α -[³²P]triphosphate ([³²P]dCTP, Amersham). Hybridization on nitrocellulose membranes was as described previously (Maniatis *et al.*, 1982; Okahashi *et al.*, 1989a).

DNA probes of the virulence-associated genes of *S. mutans*. The 1.5 kb *Pst*I fragment of the *pac* gene for the high-molecular-mass protein antigen (Pac) of serotype *c* *S. mutans* cloned by Okahashi *et al.* (1989a) was used as a probe. Probes for the *gtfB* gene coding for glucosyltransferase B (Shiroza *et al.*, 1987), the *ftf* gene coding for fructosyltransferase (Shiroza & Kuramitsu, 1988), the *scrB* gene coding for sucrose-6-phosphate hydrolase (Sato & Kuramitsu, 1988) and the *wapA* gene coding for wall-associated protein antigen A (Ferretti *et al.*, 1989) were amplified by the polymerase chain reaction (PCR; Saiki *et al.*, 1985). The primers used in the PCR were as follows: *gtfB* [primer I, 5'-GAATTCCTTTTGCCCAAC; primer II, 5'-GGATCCCCCTTAG-CAGCTT; (positions 1921 to 2131; Shiroza *et al.*, 1987)], *ftf* [primer I, 5'-CTCGAGCGACCCAATGTG; primer II, 5'-GAATTCCTTACCT-TTACCTG; (positions 2178 to 2509; Shiroza & Kuramitsu,

1988)], *scrB* [primer I, 5'-ACAGGTACAGTCCTTTA; primer II, 5'-ACGATTGACATACTTTG; (positions 499 to 946; Sato & Kuramitsu, 1988)], *wapA* [primer I, 5'-GGTACAGCACTTTCTAA; primer II, 5'-CAACATCAGTCAAAGTC; (positions 500 to 1016; Ferretti *et al.*, 1989)]. These probes were radiolabelled as described above.

Results

OFAGE separation of *Not*I fragments

For constructing a physical map of the *S. mutans* genome, restriction enzymes that yielded a small number of fragments were sought. Since the G+C content of *S. mutans* is 36–38% (Hamada & Slade, 1980), enzymes with GC-rich recognition sites such as *Not*I (GCGGCCGC), *Sfi*I (GGCCN₅GGCC), *Apa*I (GGGCCC), *Nae*I (GCCGGC), *Sma*I (CCCGGG) and *Sac*II (CCGCGG) were tested. *Not*I produced 10 fragments from the *S. mutans* MT8148 genome, *Apa*I more than 15 fragments, and *Nae*I and *Sac*II more than 30 fragments. The sizes of almost all of the fragments ranged from 50 to 100 kb. *Sfi*I and *Sma*I produced no clear fragments (data not shown). *Not*I was used for further studies since it resulted in the smallest number of fragments.

Fig. 1 shows a representative result of OFAGE separation of *Not*I fragments of genomes of *S. mutans* MT8148, *S. mutans* GS-5 and *S. sobrinus* 6715. OFAGE was carried out at various pulse times to determine the sizes of the *Not*I fragments. To check for the existence of fragments smaller than 20 kb, nonpulsed 0.7% agarose gels of the digests (10 µg of DNA) were also run (data not shown). A summary diagram of the gel patterns is shown in Fig. 2. The *Not*I fragments of *S. mutans* MT8148 genome were named A through J in order of size (Fig. 2). From summation of lengths of the *Not*I fragments, the sizes of the *S. mutans* and *S. sobrinus* chromosomes were estimated to be about 2200 kb (Table 1).

Construction of a *Not*I restriction map

To generate a physical map of the *S. mutans* MT8148 genome, we used two procedures: (i) Southern blotting with *Not*I linking probes that were selected to prove the contiguity of two fragments (Smith *et al.*, 1987); and (ii) partial *Not*I digestion (Smith *et al.*, 1987).

To isolate *Not*I linking clones, the chromosomal DNA of *S. mutans* MT8148 was digested with *Eco*RI, *Hind*III, *Pst*I or *Bgl*II, and self-ligated. The ligated fragments were digested with *Not*I, and ligated to the *Not*I-digested plasmid vector pBlueScript II. The *Not*I restriction sites

Fig. 1. Banding pattern after OFAGE of the *NotI* digests of *S. mutans* and *S. sobrinus* genomes. The pulse times were 10 s for 10 h, 30 s for 22 h, and then 50 s for 10 h. Lanes: λ , λ ladder; 1, *S. mutans* MT8148; 2, *S. mutans* GS-5; 3, *S. sobrinus* 6715.

on the cloned fragments were determined by mapping with *NotI*, *EcoRI*, *HindIII*, *PstI* and *BglII* (data not shown). An example of Southern blot analysis probed with the linking clones is shown in Fig. 3. The linking analysis by using many *NotI* linking clones revealed that the *NotI* fragments of the *S. mutans* MT8148 genome were arranged in the following order: F-A-G-H-J-C-B-D-I.

In this study, clones linking fragment E could not be obtained, although this fragment was expected to be located between F and I from the above results. To confirm the location of fragment E, partial *NotI* digests were analysed by hybridization with cloned probes. A D-specific probe and an I-specific probe were prepared from a linking clone that hybridized both fragments D and I. The Southern blotting showed that the fragments were arranged in the expected order: -D-I-E-F- (Fig. 4). This finding was also confirmed by Southern blot analysis with an F-specific probe (data not shown). These results positioned ten *NotI* fragments of the *S. mutans* MT8148 genome as shown in Fig. 5.

Fig. 2. Idealized gel band plot of the *NotI* restriction fragments. Lanes: 1, *S. mutans* MT8148; 2, *S. mutans* GS-5; 3, *S. sobrinus* 6715.

Table 1. Sizes of *NotI* restriction fragments of *S. mutans* MT8148, *S. mutans* GS-5 and *S. sobrinus* 6715

Fragment	Fragment size (kb)		
	<i>S. mutans</i> MT8148	<i>S. mutans</i> GS-5	<i>S. sobrinus</i> 6715
A	550	450	750
B	430	450	490
C	330	330	450
D	220	300	340
E	220	220	100
F	150	150	100
G	130	140	
H	90	90	
I	25	25	
J	20	20	
Total	2165	2175	2230

Placement of genetic markers on the *NotI* map

We used Southern blotting to map five virulence-associated genes on the *NotI* restriction map. Probes for the *pac*, *gtfB*, *ftf*, *scrB* and *wapA* genes hybridized to the G, C, E, E and C fragments, respectively, of the *S. mutans* MT8148 genome (Table 2).

Comparison of *NotI* restriction patterns of various strains of *S. mutans*

As shown in Fig. 1, the fragment patterns of the *NotI* digests of the *S. mutans* MT8148 and GS-5 genomes were

Fig. 3. An example of Southern blot analysis of the *S. mutans* MT8148 genome using the *NotI* linking clones. Fragments from *NotI* linking clones were radiolabelled and used as probes in blot hybridization experiments on CHEF-separated *NotI* digests. Lanes: 1, probed with a J-C linking clone; 2, probed with a C-B linking clone; 3, probed with a B-D linking clone. Positions of the *NotI* fragments of the *S. mutans* genome are indicated on the left. The results indicate the fragment order J-C-B-D.

not identical, suggesting that OFAGE may be useful for chromosomal DNA fingerprinting. Therefore, chromosomal DNAs of various strains of serotype *c* *S. mutans* were digested with *NotI*, and analysed by OFAGE. *S. mutans* strains MT6801, MT6861 and MT6879, which form large mucoid colonies on mitis-salivarius agar, were isolated from a mother and her two daughters. Their unique colony morphology has been thought to be evidence of the intrafamilial transmission of *S. mutans* (Okahashi *et al.*, 1984). The *NotI* restriction patterns of strains MT6801, MT6861 and MT6879 were identical (Fig. 6). On the other hand, *S. mutans* Xc100L was isolated from a 100-fold subculture of strain Xc, a freshly isolated strain of serotype *c* *S. mutans* (Koga *et al.*, 1989). The parent strain Xc is hydrophobic, and strain Xc100L was a relatively hydrophilic variant (Koga *et al.*, 1989). The *NotI* restriction patterns of strain Xc and Xc100L were essentially identical (Fig. 6). However, the patterns of MT8148, Xc and MT6801 (and GS-5: see Fig. 1) were different from one another.

Fig. 4. Southern blot analysis of complete and partial digests of the *S. mutans* MT8148 genome with *NotI*. Blots of complete (lane 1) and partial (lane 2) *NotI* digests of the *S. mutans* MT8148 genome were probed with (A) D-specific probe, and (B) I-specific probe. The interpretation of the digestion pattern is shown at the bottom.

Table 2. *Placing of genetic markers on the restriction map of the S. mutans* MT8148 genome

Markers were mapped by Southern hybridization.

Marker	<i>NotI</i> fragment	Reference
<i>pac</i>	G	Okahashi <i>et al.</i> (1989a)
<i>gtfB</i>	C	Shiroza <i>et al.</i> (1987)
<i>ftf</i>	E	Shiroza & Kuramitsu (1988)
<i>scrB</i>	E	Sato & Kuramitsu (1988)
<i>wapA</i>	C	Ferretti <i>et al.</i> (1989)

Discussion

In the present study, the restriction enzyme *NotI* was found to produce distributions of DNA fragment size

Fig. 5. *NotI* restriction map of the *S. mutans* MT8148 genome.

Fig. 6. OFAGE analysis of *NotI* digests of DNAs from *S. mutans* strains. Lanes: λ , λ ladder; 1, *S. mutans* MT8148; 2, *S. mutans* Xc; 3, *S. mutans* Xc100L; 4, *S. mutans* MT6801; 5, *S. mutans* MT6861; 6, *S. mutans* MT6879. Running conditions were as in Fig. 1.

useful for mapping of the genomes of *S. mutans* and *S. sobrinus* by PFG. Although we may have missed some very small fragments, since ethidium bromide searches alone are not likely to reveal all such fragments, the genome sizes of *S. mutans* MT8148 and GS-5 and *S. sobrinus* 6715 determined by PFG analysis were estimated to be about 2200 kb. Results of PFG of *NotI* digests of *S. mutans* strains MT6801 and Xc suggested

that the genome sizes of these strains were also around 2200 kb (Fig. 6). In addition, summation of lengths of *ApaI* fragments of *S. mutans* MT8148 genome supported our conclusion (data not shown). These sizes are very similar to the genome sizes (1800–2500 kb) of *Lactococcus* and *Streptococcus* species reported by Bourgeois *et al.* (1989). These results suggest that the genome sizes of streptococci are smaller than that of *E. coli* (4700 kb) (Smith *et al.*, 1987). On the other hand, the genome of *Haemophilus influenzae* has been reported to be of the same magnitude as that of *S. mutans* (Kauc *et al.*, 1989; Lee *et al.*, 1989). Recently, Tudor *et al.* (1990) reported that the size of the *S. mutans* GS-5 genome is 2800 kb. Their size estimate is larger than that obtained in this study. This discrepancy might result from divergence or deletion in the *S. mutans* genome on prolonged laboratory culture.

The restriction map of *S. mutans* MT8148 constructed here is one of a few complete bacterial restriction maps (Bautsch, 1988; Canard & Cole, 1989; Ely & Gerardot, 1988; Kauc *et al.*, 1989; Lee *et al.*, 1989; Smith *et al.*, 1987) demonstrated by PFG. Although only one restriction enzyme, *NotI*, was used for mapping, in the present study, this map will be useful for both physical and genetic analyses of the *S. mutans* genome.

Perry & Kuramitsu (1989) recently demonstrated that the *gtfA* (a gene coding for glucosyltransferase A), *scrB* and *ftf* genes are clustered on the *S. mutans* chromosome, with *ftf* located between *gtfA* and *scrB*. They showed that the cotransfer system used in their study is efficient for mapping closely linked markers (Perry *et al.*, 1985). However, the system seems to be unsuitable for large-scale mapping. In the present study, the *pac*, *gtfB*, *ftf*, *scrB* and *wapA* genes were placed on the *NotI* restriction map. The *ftf* and *scrB* genes were located on the same *NotI* fragment, E (Table 2); this result supports the finding of Perry & Kuramitsu (1989) that the two genes are closely linked. It will also be possible to locate other genes on the restriction map by Southern hybridization. Procino *et al.* (1988) have shown that transposon Tn916 can be used to mutagenize *S. mutans* by insertional inactivation and have isolated a variety of different amino acid auxotrophs. This method will also be useful in constructing the *S. mutans* genome map. In addition, our preliminary study showed that the restriction enzyme *ApaI* produced relatively few fragments (more than 15). We are presently attempting to complete the map of *ApaI* fragments and to develop a set of mapped cosmid clones covering the entire genome.

Molecular biological methods have recently been applied to type *S. mutans* strains for epidemiological investigations. One of the general and reliable methods is agarose gel electrophoresis analysis of genomic DNA fragments produced by restriction endonucleases

(Caufield & Walker, 1989; Kulkarni *et al.*, 1989). However, the commonly used endonucleases produce too many fragments in agarose gel electrophoresis. In contrast, PFG showed relatively simple electrophoretic patterns of genomic DNA digested with *NotI* (see Figs 1 and 6). This technique will make it easier to compare numerous *S. mutans* strains from various origins.

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