Chromosome organization of *Streptococcus mutans* GS-5

MICHAEL J. HANTMAN, SHUZHONG SUN, PATRICK J. PIGGOT and LOLITA DANEOMOORE*

Department of Microbiology and Immunology, Temple University School of Medicine, Philadelphia, Pennsylvania 19140, USA

(Received 5 May 1992; revised 24 September 1992; accepted 6 October 1992)

Twenty-eight genetic loci have been physically mapped to specific large restriction fragments of the *Streptococcus mutans* GS-5 chromosome by hybridization with probes of cloned genes or, for transposon-generated amino acid auxotrophs, with probes for Tn916. In addition, restriction fragments generated by one low-frequency-cleavage enzyme were used as probes to identify overlapping fragments generated by other restriction enzymes. The approach allowed construction of a low resolution physical map of the *S. mutans* GS-5 genome using restriction enzymes *Apa*I (5'-GGGCC/C), *Sma*I (5'-CCC/GGG), and *Not*I (5'-GC/GGCCGC).

Introduction

Streptococcus mutans is considered the principal causative agent of human dental caries, and strains producing the serotype c surface polysaccharide are the predominant human isolates (Loesche, 1986). GS-5 is a representative serotype c strain which has been extensively used in genetic and biochemical studies of virulence characteristics involving sucrose metabolism and extracellular polysaccharide synthesis. Genetic mapping of the *S. mutans* chromosome has involved linkage analysis by measuring the cotransfer of genetic markers into competent cells. Perry *et al.* (1985) first demonstrated that *gtfA* was cotransformable with a methionine marker and then showed that *gtfA* was closely linked to *fru-1*, *ftf*, *scrB*, and *gbp* (Perry & Kuramitsu, 1989, 1990). In a limited number of instances, direct analysis of cloned fragments of the *S. mutans* chromosome has established physical linkage of genes. Sequence analysis of cloned DNA fragments has revealed *gtfC* to be adjacent to *gtfB* (Shiroza *et al.*, 1987) and *scrA* to be adjacent to *scrB* (Sato *et al.*, 1989). Burne *et al.* (1986) cloned a 6 kb fragment of *S. mutans* DNA containing the *gtfA* gene and found that it also encodes a dextranase gene, *dexB*. Recently, Russell and co-workers (Aduse-Opoku *et al.*, 1991; Russell & Ferretti, 1990; Russell *et al.*, 1991) sequenced a 12 kb region of the *S. mutans* chromosome and confirmed the linkage of *gtfA* to *dexB* as well as to alpha-galactosidase (*aga*) and

five other genes involved in the utilization of melibiose and isomaltosaccharides. A cluster of galactose utilization genes has also been found on a cloned fragment of *S. mutans* DNA (Smorawinska *et al.*, 1983). Nevertheless, information about the *S. mutans* genetic and physical map remains fragmentary.

The application of pulsed field gel electrophoresis (PFGE) (Carle & Olson, 1984; Schwartz & Cantor, 1984) to the separation of very large DNA molecules has made it possible to construct a low resolution *Not*I physical map of the entire *E. coli* chromosome (Smith *et al.*, 1987) and an *Sfi*I map of the *Bacillus subtilis* 168 chromosome (Amjad *et al.*, 1990; Itaya & Tanaka, 1991), for which many genetic mapping data are available, and macrorestriction maps for other less well studied chromosomes (e.g. Chang & Taylor, 1990; Krause & Mawn, 1990). A physical map of the *S. mutans* chromosome will make it possible to study the relationships of genes or gene clusters with each other and to locate cloned genes on the chromosome. Okahashi *et al.* (1990) have used these methods to construct a *Not*I restriction map of *S. mutans* MT8148 DNA. We report here the location of twenty-eight genetic loci on the *Not*I map of *S. mutans* GS-5. We also extend the genomic restriction mapping by aligning *Apa*I and *Sma*I restriction fragments with the *Not*I map.

Methods

Bacterial strains. *Streptococcus mutans* GS-5, serotype c (Bratthall, 1970) and transposon-generated auxotrophs of *S. mutans* GS-5 (Procino *et al.*, 1988) were grown in Todd-Hewitt broth (Difco)

* Author for correspondence. Tel. 215 221 3229; fax 215 221 7788.

Table 1. Cloned genes used as hybridization probes, the activities they encode, and restriction fragment location

Gene†	Activity	Restriction fragment		
		<i>Apa</i> I	<i>Not</i> I	<i>Sma</i> I
<i>aga</i>	α -Galactosidase	B	C	H/I*
<i>asd</i>	Aspartate-semialdehyde dehydrogenase	B	C	B
<i>dexB</i>	Dextran glucosidase	B	C	H/I*
<i>ftf</i>	Fructosyltransferase	E	A	G
<i>gal</i>	Galactose metabolism	A	B	Q/R/S*
<i>gbp</i>	Glucan binding protein	E + L†	F	G + Q/R/S*†
<i>gtfA</i>	Sucrose phosphorylase	B	C	H/I*
<i>gtfB</i>	Glucosyltransferase	C	C	B
<i>gtfC</i>	Glucosyltransferase	C	C	B
<i>gtfD</i>	Glucosyltransferase	B	C	B
<i>ldh</i>	Lactate dehydrogenase	C	C	B
<i>pac</i>	Surface protein antigen	D	G	A
<i>scrA</i>	Enzyme II ^{scr} , sucrose PTS	F	D	K/L/M*
<i>scrB</i>	Sucrose-6-phosphate hydrolase	F	D	K/L/M*
<i>wapA</i>	Surface protein antigen	B	C	B

* Fragments could not be resolved in Southern blots.

† Probe hybridized to more than one fragment.

‡ References: *aga*, Aduse-Opoku *et al.* (1991); *asd*, Jagusztyn-Krynicka *et al.* (1982); *dexB*, Russell & Ferretti (1990); *ftf*, Sato & Kuramitsu (1986); *gal*, Smorawinska *et al.* (1983); *gbp*, Russell *et al.* (1985); *gtfA*, Robeson *et al.* (1983); *gtfB*, Aoki *et al.* (1986); *gtfC*, Hanada & Kuramitsu (1988); *gtfD*, Hanada & Kuramitsu (1989); *ldh*, Hillman *et al.* (1990); *pac*, Lee *et al.* (1988), Okahashi *et al.* (1989); *scrA*, Sato *et al.* (1989); *scrB*, Hayakawa *et al.* (1986), Lunsford & Macrina, (1986); *wapA*, Ferretti *et al.* (1989).

supplemented with 2% (w/v) glucose and 0.1% (w/v) L-cysteine (THG) at 37 °C.

DNA isolation. *S. mutans* chromosomal DNA was isolated from bacteria embedded and lysed in agarose beads using methods described in the literature (Cook, 1984; Piggot & Curtis, 1987; Reider & Macrina, 1976; Smith & Cantor, 1987), with some modifications as follows. Bacteria (0.2 ml frozen stock) were inoculated into 20 ml THG, and the cultures were incubated overnight at 37 °C. Prewarmed THG (60 ml) was added and incubation was continued for 60 min. Glycine was added to 3.75% (w/v) and incubation continued for 60 min. The cultures were chilled on ice and bacteria harvested by centrifugation at 4 °C were washed with solution A (1 M-NaCl, 0.01 M-Tris/HCl, pH 8.0). Washed bacteria were harvested, re-suspended in 2.5 ml solution A, and equilibrated at 42 °C in a 50 ml round-bottom flask. Low-melting-temperature agarose [2.5 ml molten 1% (w/v) solution, equilibrated at 42 °C [Seaplaque GTG (FMC)]] was added. The mixture was mixed vigorously for 1 min, 10 ml paraffin oil (42 °C) was added and swirling continued for 2 min. The solution was mixed with 4 vols ice-cold solution A, stirred for 5 min and centrifuged at low speed (3000 g) for 10 min at 4 °C. The supernatant was removed with a pipette and the agarose beads were suspended in 15 ml solution B (1 M-NaCl, 0.1 M-EDTA, 0.01 M-Tris/HCl, pH 8.0, 0.5% Brij 58, 0.2% deoxycholate, 0.5% *N*-lauroylsarcosine) with hen egg white lysozyme (Boehringer Mannheim) added to a final concentration of 2 mg ml⁻¹. The beads were mechanically shaken at 130 r.p.m. for 18 h at 37 °C. Following low-speed centrifugation (3000 g) the supernatant was removed and the beads were suspended in 15 ml solution C [0.4 M-EDTA, 1% (w/v) *N*-lauroylsarcosine, 200 µg proteinase K ml⁻¹ (Boehringer Mannheim)]. The beads were incubated at 50 °C for 8 h and centrifuged, and the supernatant was replaced with fresh solution C, continuing the 50 °C incubation for 18 h. The beads were then harvested by centrifugation (3000 g) and washed twice in 10 vols 1 mM-PMSF (in TE, 10 mM-Tris/HCl, pH 8.0, 1 mM-EDTA) followed by two

washes in 10 mM-Tris/HCl, pH 8.0, 100 mM-EDTA. The beads were stored in this buffer at 4 °C.

Restriction enzyme digests. Agarose beads containing DNA were equilibrated twice in 12 vols TE and twice in 5 vols restriction enzyme buffer and finally suspended in enzyme buffer with the appropriate restriction enzyme. All restriction enzymes were purchased from Promega or New England Biolabs and hydrolysis conditions used were those specified by the supplier.

Electrophoresis. Contour-clamped homogeneous electric field (CHEF) gel electrophoresis (Chu *et al.*, 1986) was performed using an LKB 2015 Pulsaphor apparatus with a hexagonal electrode array (LKB) or a similar noncommercial system. Electrophoresis was performed in a 1% (w/v) agarose gel (SeaKem LE; FMC) using 20 mM-Tris-acetate/1 mM-EDTA buffer (0.5 × TAE, pH 8.5) or 45 mM-Tris-borate/1 mM-EDTA buffer (0.5 × TBE, pH 8.5) at 5–10 °C. Each gel contained size standards (Mathew *et al.*, 1988) which included concatemers of λ DNA (*ctf857 ind1 Sam7*, monomer size 48.5 kb), fragments of λ DNA generated by restriction digests, or intact *Saccharomyces cerevisiae* YPH80 chromosomes (size range 225–2500 kb), purchased from New England Biolabs. Following electrophoresis, gels were stained with ethidium bromide and photographed, and the DNA was transferred to supported nitrocellulose (Nitroplus 2000; MSI) essentially by the method of Southern (1975) using a vacuum transfer device (Millipore).

Gene probes. Table 1 lists the cloned genes used as probes. *dexB*, *aga*, *wapA*, *pac*, and *gbp* were bacteriophage λ L47-1 recombinants with inserts of *S. mutans* Ingbritt DNA and contained the cloned genes as well as flanking sequences (Morrissey *et al.*, 1985). They were provided by R. R. B. Russell (London Hospital Medical College, London E1 2AD, UK). The *ftf* probe consisted of part of the *ftf* gene and 3' flanking sequences from *S. mutans* GS-5 (Shiroza & Kuramitsu, 1988). *scrA* and *scrB* were carried on pMH613 (Hayakawa *et al.*, 1986) and

gtfD was on a plasmid clone isolated from strain NH5 (Hanada & Kuramitsu, 1989). These four probes were provided by H. K. Kuramitsu (University of Texas Health Science Center, San Antonio, TX, USA). The *asd* and *gal* probes from *S. mutans* PS14 were isolated from pYA574 (Jagusztyn-Krynicka *et al.*, 1982) and pYA501 (Smorawska *et al.*, 1983), respectively, using low-melt agarose (Seaplaque; FMC) electrophoresis following restriction digests. The *gtfA* probe was from a plasmid clone pYA601 (Robeson *et al.*, 1983). These three probes were provided by R. Curtiss III (Washington University, St. Louis, MO, USA). *gtfB* and *gtfC* share extensive homology (Ueda *et al.*, 1988) and one probe was used from plasmid pKS5, provided by Robert Burne (University of Rochester School of Medicine and Dentistry, Rochester, NY, USA). The *ldh* gene probe was a plasmid clone, p10-5 (Hillman *et al.*, 1990), provided by M. J. Duncan (Forsyth Dental Center, Boston, MA, USA). Plasmid pAM150 (Gawron-Burke & Clewell, 1984) which contained transposon Tn916 was used as a probe for all transposon-generated amino acid auxotrophs (Procino *et al.*, 1988).

Fragment probes. To isolate restriction fragments for use as probes, *ApaI* or *NotI* digests were fractionated by pulsed field electrophoresis in a 1% (w/v) agarose gel (SeaKem GTG; FMC), visualized by ethidium bromide staining, and the appropriate band was cut from the gel. DNA was extracted from the agarose using the GeneClean system (Bio101). Probes were labelled with ³²P by random hexanucleotide primer labelling (Feinberg & Vogelstein, 1983) (Prime-a-Gene; Promega).

Results

Restriction fragments and genome size

In order to generate a limited number of large fragments, restriction enzymes were tested that recognize sites containing predominately G and C nucleotides, since the GC content of serotype c *S. mutans* is 36–38% (Coykendall, 1974). Tudor *et al.* (1990) demonstrated the utility of using restriction enzymes *ApaI* (5'-GGGCCC), *EagI* (5'-CGGCCG), *NaeI* (5'-GCCGGC), *NotI* (5'-GCGGCCGC), *RsrII* (5'-CGG^ACCG), and *SmaI* (5'-CCCGGG) in digesting the *S. mutans* GS-5 genome into a small number of large fragments which can be analyzed by pulsed-field gel electrophoresis. A new 8-base recognition site restriction enzyme, *AscI* (5'-GGCGCGCC), also proved useful as it generated only five fragments with DNA from strain GS-5.

The patterns obtained with *ApaI*, *EagI*, *NaeI*, *NotI*, and *RsrII*, following fractionation by CHEF gel electrophoresis are illustrated in Fig. 1(a) (lanes 3, 4, 5, 7 and 8, respectively). Intermediate-sized fragments are well resolved using a pulse time of 12 s. These conditions produced good resolution between 50 and 200 kb and allowed us to resolve two similarly sized fragments, *NotI*-F (141 kb) and *NotI*-G (134 kb) (Fig. 1a, lane 7). In this gel resolution decreased dramatically above 200 kb and below 50 kb, therefore with other gels it was necessary to vary the pulse times from 2 s to 60 s to resolve fragments ranging from 5 kb to 920 kb. As the resolution in a particular size range increased, the accuracy in deter-

mining fragment size and number increased. The enzymes *EagI* and *NaeI* generated many fragments under 100 kb (Fig. 1a, lanes 4 and 5, respectively). *EagI* also consistently produced many incompletely digested fragments which appeared as faint bands in ethidium-bromide-stained gels. We did not analyse *EagI* or *NaeI* digests further. Other restriction enzyme digests occasionally produced faint bands resulting from incomplete hydrolysis. Partial-digest experiments have been used by others to identify adjacent fragments with hybridization probes (Smith *et al.*, 1987) and using this type of analysis we have determined some linked fragments but, in general, we have avoided partial restriction enzyme digests.

Our updated estimates for the size and number of DNA fragments generated by *SmaI*, *NotI*, *ApaI*, *RsrII*, and *AscI* and fractionated by PFGE (Hantman *et al.*, 1991) are given in Table 2. Fragment sizes were determined by comparison to size standards of phage λ chromosome oligomers and λ DNA fragments (Mathew *et al.*, 1988). We consider the sizes for fragments between 500 kb and 1000 kb to be accurate to 50 kb, between 300 kb and 500 kb to be accurate to 20 kb, between 100 kb and 300 kb to be accurate to 10 kb, between 50 kb and 100 kb to be accurate to 5 kb and between 5 kb and 50 kb to be accurate to 3 kb. Some of the bands [for example, *ApaI* fragments L and M (51 kb) or N and O (38 kb); Table 2] were judged to contain comigrating fragments because of increased ethidium bromide fluorescence. By summing the individual fragments for each restriction digest the genome size range was estimated to be between 2079 and 2162 kb (Table 2). For mapping we have assumed a genome size of 2125 (± 100) kb.

Localization of cloned genes by hybridization

Southern analysis has been used to place several cloned genes on specific large restriction fragments generated by low-frequency-cleavage restriction enzymes. The genes used and the fragments they hybridized to are listed in Table 1. Each cloned gene probe was hybridized to a group of filters containing DNA fractionated at several different pulse times. Often, filters were hybridized with one probe, washed, and reprobbed with another gene. Fig. 1(b–d) show the hybridization patterns of the *scrB*, *gfp*, and *ftf* probes, respectively, on the CHEF gel shown in Fig. 1(a). The probe for *scrA* and *scrB* (Fig. 1b) hybridized to the *ApaI*-F (145 kb) fragment (lane 3; Table 1) and probes for *gfp* (Fig. 1c) and *ftf* (Fig. 1d) both hybridized to the *ApaI*-E (177 kb) fragment (lane 3; Table 1). An *ApaI* site separates these two pairs of genes. The *gfp* probe also hybridized to the *ApaI*-L/M (51 kb) fragment (Fig. 1c, lane 3). *ApaI*-L and -M comigrated, so

Table 2. Sizes of restriction fragments of *S. mutans* GS-5 DNA

Fragment	Fragment size (kb)				
	<i>Sma</i> I	<i>Not</i> I	<i>Apa</i> I	<i>Rsr</i> II	<i>Asc</i> I
A	327	433	450	238	920
B	240	433	249	190	720
C	230	332	223	170	450
D	218	287	202	168	29
E	135	223	177	147	24
F	132	141	145	139	
G	103	134	117	135	
H	91	85	103	133	
I	91	30	65	125	
J	77	25	61	90	
K	61		59	80	
L	59		51	80	
M	57		51	75	
N	46		38	75	
O	46		38	60	
P	44		30	55	
Q	39		13	55	
R	38		7	50	
S	36			35	
T	31			15	
U	28			5	
V	15				
W	13				
X	5				
Total	2162	2123	2079	2120	2143

we have designated the 51 kb fragment containing *gbp* as L. The *gbp* probe DNA contained a restriction site for *Apa*I and thus was a linking clone establishing that *Apa*I-E and *Apa*I-L were contiguous. The *gbp* probe was also a linking clone for *Sma*I, joining *Sma*I-G and *Sma*I-Q/R/S (data not shown). Although the published sequence for *gbp* (Banas *et al.*, 1990) does not show an *Apa*I or a *Sma*I site, flanking sequences included in the probe contain these recognition sites (data not shown).

The *gbp* probe hybridizes to the *Not*I-F fragment (Fig. 1c, lane 7) while *scrB* and *ftf* hybridize to poorly resolved *Not*I fragments above 200 kb (Fig. 1b,d, lane 7). Other Southern blots demonstrated the *ftf* probe hybridized to *Sma*I-G and *Not*I-A/B, while the probe for *scrB* hybridized to *Sma*I-K/L/M and to *Not*I-D. The *gbp* probe hybridized to *Sma*I-G and *Sma*I-Q/R/S. This information allows us to order these genes *ftf*-*gbp*-*scrB* in this GS-5 strain (Fig. 2).

Localization of transposon insertions

DNA from 13 amino acid auxotrophs (Table 3), generated by insertion of transposon Tn916 (Procino *et al.*, 1988), were analysed by CHEF gel electrophoresis. The transposon serves to mark the gene of interest so that hybridization of a labelled probe for Tn916 to auxotroph DNA identifies the restriction fragment that contains the gene. In separate experiments we found that *Sma*I, *Not*I, and *Apa*I do not cut Tn916 (data not shown). DNA from three methionine auxotrophs, containing mutations *met-5*, *met-6*, and *met-7*, was digested with *Sma*I and fractionated using a pulse time which maximizes resolution below 100 kb (Fig. 3a). Southern hybridization using a probe for Tn916 is shown in Fig. 3b. In addition to marking the inserted gene for hybridization, the 16.4 kb Tn916 increases the size of the restriction fragment containing the transposon insertion. The position of the insert, detected by hybridization, can be related to fragments from the parent strain with no insert. Thus for the strain containing *met-5* (Fig. 3a and b, lanes 1 and 2) the mobility of the *Sma*I-J fragment (77 kb) was altered and the transposon was detected on a fragment of approximately 93 kb. Similarly, in strains containing *met-6* (Fig. 3a and b, lanes 3 and 4) and *met-7* (Fig. 3a and b, lanes 5 and 6) the transposon was found on a fragment of approximately 61 kb which would relate to poorly resolved fragments N, O or P in the parent strain. The locations of the other insertions examined are shown in Table 3 and Fig. 4. Since Tn916 increases the size of a fragment, it can be useful in separating the two comigrating bands of a doublet. The *Not*I-A and B fragments were thought to be a comigrating doublet by ethidium bromide fluorescence. Fig. 5 shows a *Not*I digest of DNA from a transposon-generated auxotroph containing mutation *met-1*. Using a pulse time of 57 s and a run time of 50 h it was possible to resolve the 433 kb fragment (now designated *Not*I-A) from the 450 kb fragment containing Tn916 (now designated *Not*I-B) (Fig. 5, lanes 7-9).

Map

Okahashi *et al.* (1990) have published a *Not*I restriction map for *S. mutans* MT8148 and have included *Not*I restriction fragment data, but no map, for *S. mutans* GS-5. For GS-5, the mobility patterns we have obtained

Fig. 1. Fractionation by CHEF gel electrophoresis (a) and Southern blot analysis (b) of *S. mutans* GS-5 DNA digested with low-frequency-cleavage restriction enzymes. Electrophoresis was done in 0.5 × TAE buffer, using 150 V and a constant pulse time of 12 s for 40 h at 4 °C. Size standards consisted of bacteriophage λ DNA (*cl857 ind1 Sam7*) monomer (lane 1, 48.5 kb) or concatemers (lanes 2 and 6). The enzymes used were *Apa*I (lane 3), *Eag*I (lane 4), *Nae*I (lane 5), *Not*I (lane 7), and *Rsr*II (lane 8). (a) Gel photographed using UV irradiation following ethidium bromide staining. (b, c, d) Hybridization with probes for cloned *S. mutans* genes *scrB* (b), *gbp* (c), and *ftf* (d). Probes were labelled with 32 P and hybridized individually to a blot of the CHEF gel shown (a). A labelled λ probe was included.

Fig. 2. Gene order in the *NotI*-A-F-D region of *S. mutans* GS-5. The *NotI*, *ApaI*, and *SmaI* fragments shown are vertically aligned.

Table 3. *Tn916*-inactivated auxotrophic mutants (Procino *et al.*, 1988) and restriction fragment location

Insertion	Restriction fragment		
	<i>ApaI</i>	<i>NotI</i>	<i>SmaI</i>
<i>met-1</i>	C	B	E
<i>met-2</i>	D	G	A
<i>met-3</i>	D	H	A
<i>met-4</i>	D	A	A
<i>met-5</i>	G	A	J
<i>met-6</i>	G	A	N/O/P*
<i>met-7</i>	G	A	N/O/P*
<i>lys-1</i>	C	C	E
<i>thr-1</i>	D	H	A
<i>his-1</i>	A	B	C
<i>phe-1</i>	A	B	C
<i>ser-1</i>	D	H	A
<i>pro-1</i>	M	D	F

* Fragments could not be resolved in Southern blots.

are in agreement and the fragment sizes differ by approximately 3%. Since most of the *NotI* fragments from MT8148 and GS-5 comigrated and the number of fragments were the same, it was possible that the overall organization of their genomes were similar.

Using large restriction fragments obtained by *ApaI* digestion (*ApaI*-A, -B, -C, -D, -E, -F, -G, -H; Table 2) as hybridization probes to overlapping fragments generated by *NotI*, we aligned the *ApaI* fragments with the *NotI* fragments (Fig. 4). The hybridization patterns indicating *NotI* fragments adjacent (or close) to each other in GS-5 were consistent with the pattern presented for MT8148 (Okahashi *et al.*, 1990). The main differences in mobility between the two strains were with *NotI* fragments A, B, D, and E. In MT8148, *NotI* fragments D and E comigrate and in GS-5, *NotI* fragments A and B comigrate.

Since GS-5 *NotI*-A and -B comigrate, we have defined the two fragments by using the information provided by gene localization. The mutation *met-4* is located on *ApaI*-D and can mark *NotI*-A. *ApaI*-D hybridized to

NotI-H, -G, and one fragment of the A/B doublet. *met-1* is located on *ApaI*-C and can mark *NotI*-B. *ApaI*-C hybridized to *NotI*-C and one fragment of the A/B doublet. The *gal* probe hybridized to *NotI*-A/B and *ApaI*-A. The same probe hybridized to an *ApaI* partial-digest fragment of 675 kb which suggested *ApaI*-A (450 kb) and *ApaI*-C (223 kb) were adjacent. We concluded that one fragment of the *NotI*-A/B doublet was hybridized to by *ApaI*-A and -C and contained *met-1* and *gal* and is adjacent to *NotI*-C. We designated this fragment *NotI*-B. The other fragment of the A/B doublet is hybridized to by *ApaI*-D, contains *met-4*, and is adjacent to *NotI*-G. We designated this fragment *NotI*-A.

The *NotI* fragments E (223 kb) and D (287 kb) were placed as follows. *ApaI*-A hybridized only to *NotI*-A/B and *NotI*-E in complete digests. It also hybridized to a *NotI* partial-digest fragment slightly larger than *NotI*-E which suggested that *NotI*-I (30 kb) and -E were contiguous. [The similarly sized *NotI*-J (25 kb) was located on the other side of the chromosome.] *ApaI*-F hybridized to *NotI*-E, -I, and -D. *NotI*-D was not linked to either *NotI*-A or -B. This information allowed us to place *NotI*-E between *NotI*-B and -I (Fig. 4).

The *ApaI* fragment probes were also hybridized to *SmaI* digests allowing alignment of many *SmaI* fragments on the map (Fig. 4). The genetic markers, located by hybridization, provided confirmation for the placement of some of the fragments. One example is the placement of one *SmaI*-Q/R/S fragment. The 450 kb *ApaI*-A fragment hybridized to multiple *SmaI* fragments including Q/R/S and D. The *gal* probe hybridized to the *NotI*-B, *ApaI*-A, and *SmaI*-Q/R/S fragments, which limits the alignment of *SmaI*-Q/R/S between the *NotI*-E-B junction and the *ApaI*-A-C border, approximately 325 kb. Partial-digest patterns with the *gal* probe also suggested that *SmaI*-Q/R/S and *SmaI*-C were contiguous.

While all of the *NotI* fragments are represented in Fig. 4, the *ApaI* fragments shown represent 85% of the

Fig. 3. CHEF gel electrophoresis (a) and Southern blot analysis (b) of *Sma*I digests of DNA from *Tn916*-generated amino acid auxotrophs of *S. mutans* GS-5. Electrophoresis was performed with a constant pulse time of 3 s at 140 V for 40 h in $0.5 \times$ TAE. Two concentrations of each auxotroph DNA sample were loaded. (a) Lanes 1 and 2, *met5*; lanes 3 and 4, *met6*; lanes 5 and 6, *met7*; lane 7, λ DNA concatemer (monomer size, 48.5 kb); lane 8, λ DNA restriction fragments of 48.5, 38.4, 33.5, 30.0, 24.5, 24.0, 17.1, 15.0, 10.1, and 1.5 kb; lane 9, *Hind*III digest of λ DNA; lane 10, *Sma*I digest of GS-5 DNA. The large arrows (1 and 2) indicate the position of the fragment containing the transposon insertion as determined by hybridization; 1, *met5*; 2, *met6* and *met7*. The small arrows indicate the position of the corresponding fragment in the parent strain without a transposon insertion. (b) DNA transferred to nitroplus 2000 (MSI) and hybridized to ^{32}P -labelled pAM150 containing *Tn916*. Labelled λ DNA was included.

Fig. 4. Genome map of *S. mutans* GS-5. *Sma*I and *Apa*I restriction fragments aligned with a *Not*I restriction map by hybridization of isolated fragments from one restriction enzyme to fragments generated by other restriction enzymes, hybridization of cloned DNA fragments to fractionated restriction fragments, and hybridization of a probe for Tn916 to transposon generated auxotroph DNA restriction fragments. The outer circle represents *Not*I fragments, the middle circle *Apa*I fragments, and the inner circle *Sma*I fragments. Spaces between fragments indicate contiguity has not been established. *Not*I fragment contiguity for strain GS-5 has been inferred from linking clone data provided for strain MT8148 (Okahashi *et al.*, 1990). The exact locations of the indicated genes have not yet been determined, but their relative positions on the chromosome have been established. The relative positions of the genes within a group have not been established.

entire genome and the *Sma*I fragments represent 89%. Although the approach used here can identify restriction fragments which are close together on the chromosome, it does not establish that the fragments are contiguous. It also does not precisely locate the ends of each large fragment. Determination of the order of genes linked together on a single large restriction fragment was not always possible, but the use of several enzymes often helped. For example (Fig. 6), both *asd* and *gtfB* hybridized to the *Not*I-C fragment and the *Sma*I-B fragment, indicating linkage over a maximum of 240 kb but not the order of the two genes. *Apa*I digestion separated the two genes and hybridization located *asd* to *Apa*I-B and *gtfB* to *Apa*I-C so the position on the map

could be ordered. Therefore, the gene order in this region of the chromosome is *gal*-*gtfB*-*asd*-*pac*.

Discussion

The use of low-frequency-cleavage restriction enzymes and PFGE has allowed us to construct a physical map of the genome of *S. mutans* GS-5. The genome size of 2125 (± 100) kb presented here is essentially in agreement with the *S. mutans* MT8148 genome size of 2165 kb (Okahashi *et al.*, 1990). The size previously reported for GS-5 (Tudor *et al.*, 1990) was overestimated due, at least in part, to the inclusion of fragments resulting from partial digestion of the chromosome. The *S. pneumoniae*

Fig. 5. CHEF gel of a *NotI* digest of DNA from GS-5 and a transposon generated amino acid auxotroph containing mutation *met-1*. Electrophoresis was performed in $0.5 \times$ TBE buffer with a constant pulse time of 57 s using 160 V for 50 h. Lanes 1 through 4, increasing concentrations of GS-5 DNA; lanes 7 through 9, decreasing concentrations of *met-1* DNA; lane 5, λ concatemers (monomer size, 48.5 kb); lane 6, intact *Saccharomyces cerevisiae* (YPH80) chromosomes. The 433 *NotI* A/B fragments are indicated by an arrow.

Fig. 6. Ordering of the genes between *gal* and *pac* on the GS-5 genome. The *NotI*, *ApaI*, and *SmaI* fragments shown are vertically aligned.

chromosome, which also has a low GC content, was estimated to have a size of 2270 kb (Gasc *et al.*, 1991), and lactococcal genomes have been reported to have genome sizes in a range from 1750 to 2700 kb (Le-Bourgeois *et al.*, 1989; Tanskanen *et al.*, 1990).

S. mutans strains GS-5 and MT8148, both serotype c, clearly have restriction-fragment-length polymorphisms, although their genome size is similar (Okahashi *et al.*, 1990). Much of the GS-5 physical map presented here does appear to correspond to the MT8148 map.

Chromosome differences in closely related strains of *S. mutans* have been demonstrated by others. Caulfield & Walker (1989), using frequent-cutting restriction enzymes, demonstrated restriction fragment polymorphism among randomly selected biotype 1 strains of *S. mutans* including prototype serotype c strains 10449 and GS-5. Polymorphism in specific regions of the chromosome has been demonstrated within the glucosyltransferase genes (Chia *et al.*, 1991) and the *spaP* gene (Brady *et al.*, 1991) among serotype c strains. The sequences flanking the *gtfA* gene also demonstrated heterogeneity between serotype c strains (Pucci & Macrina, 1986).

These differences as well as possible chromosome rearrangements may account for discrepancies between genetic and physical mapping data from different laboratories. Burne *et al.* (1986) showed that the *gtfA* and *dexB* genes were located on a single 6 kb fragment, while Perry *et al.* (1985) did not find genetic linkage between *gtfA* and a *dex* gene. It is not clear if multiple *dex* genes are present. Perry & Kuramitsu (1989, 1990) determined several sucrose utilization genes from *S. mutans* to be linked by interrupting the cloned genes with antibiotic resistance determinants and introducing them into the chromosome. The frequency of cotransfer for these marked genes indicated linkage and a gene order of *gtfA*, *fru-1*, *ftf*, *scrB*, and *gbp* (Perry & Kuramitsu, 1990). In the present study, hybridization probes for *ftf*, *scrB*, and *gbp* also demonstrated clustering of these genes on the *S. mutans* genome. However, we have found an *ApaI* site which separates *scrB* from *ftf* and *gbp* (Fig. 2). This indicates that the proposed gene order in our GS-5 strain does not correspond to the order proposed by Perry & Kuramitsu (1990).

We had previously placed *gtfA* in the region containing *ftf* and *scrB*, but further analysis of the hybridization patterns obtained from multiple blots with DNA fractionated at greater resolution has prompted a relocation of some of the genes previously reported (Hantman *et al.*, 1991). Using the cloned *gtfA* as well as the *aga* and *dexB* genes, which have been cloned on one 12 kb fragment (Russell *et al.*, 1991), we have placed these genes on the *NotI*-C, *ApaI*-B, and *SmaI*-H/I fragments (Fig. 4). Genetic linkage has been reported for *gtfA*, *fru-1*, *ftf*, *scrB*, and *gbp* (Perry & Kuramitsu, 1990).

but the distance between *gtfA* and the *ftf-gbp-scrB* cluster in this strain makes genetic linkage unlikely. Other sucrose utilization genes also clustered in one region of the chromosome. Probes for *gtfB*, *gtfC*, and *gtfD* localized these genes on one 240 kb *Sma*I-B fragment within the *Not*I-C fragment (Fig. 4). Thus, the sucrose utilization genes are not grouped in one region of the chromosome.

Some of the methionine auxotrophs, created by transposon insertion, also demonstrated clustering in one region of the chromosome. The sites of the Tn916 insertions *met-3*, *met-2*, and *met-4* are located on the *Apal*-D fragment of 202 kb and the insertions in *met-2*, *met-4*, *met-5*, *met-6*, and *met-7* are all located on the *Not*I-A fragment (433 kb). From the map shown in Fig. 4, *met-2* and *met-4* are probably within a 50 kb region and *met-6*, *met-7* and *met-5* are within a 110 kb region. Cross-feeding patterns suggested to Procino *et al.* (1988) that the transposon-generated methionine auxotrophs could be divided into six classes, with mutations *met-6* and *met-7* in the same class and probably the same gene. These classes form a biochemical pathway with the mutations ordered *met-2*→*met-4*→*met-6/met-7*→*met-5*→*met-3*→*met-1*. None of these markers correspond to the methionine mutation described by Perry *et al.* (1985) as linked to *gtfA* (J. Procino, personal communication). Possibly, groups of these genes are close to each other to enable the coordinate regulation of their expression.

The physical map constructed with *Not*I, *Apal*, and *Sma*I will aid in physical and genetic analysis of the *S. mutans* genome. Fig. 4 contains the most extensive map of genetic markers on the *S. mutans* genome to date.

We thank G. D. Shockman for criticism and R. R. B. Russell, H. K. Kuramitsu, R. Curtiss III, R. A. Burne, M. J. Duncan, D. B. Clewell, and F. L. Macrina for providing strains and probes. This work was supported by public health service grant DE08942 from the National Institutes of Health. M.J.H. was supported in part by training grant 5 T32 AI07101.

References

- ADUSE-OPOKU, J., TAO, L., FERRETTI, J. J. & RUSSELL, R. R. B. (1991). Biochemical and genetic analysis of *Streptococcus mutans* α -galactosidase. *Journal of General Microbiology* **137**, 757–764.
- AMJAD, M., CASTRO, J. M., SANDOVAL, H., WU, J.-J., HENNER, D. J. & PIGGOT, P. J. (1990). An *Sfi*I restriction map of the *Bacillus subtilis* 168 genome. *Gene* **101**, 15–21.
- AOKI, H., SHIROZA, T., HAYAKAWA, M., SATO, S. & KURAMITSU, H. K. (1986). Cloning of a *Streptococcus mutans* glucosyltransferase gene coding for insoluble glucan synthesis. *Infection and Immunity* **53**, 587–594.
- BANAS, J. A., RUSSELL, R. R. B. & FERRETTI, J. J. (1990). Sequence analysis of the gene for the glucan-binding protein of *Streptococcus mutans* Ingbritt. *Infection and Immunity* **58**, 667–673.
- BRADY, L. J., CROWLEY, P. J., MA, J. K.-C., KELLY, C., LEE, S. F., LEHNER, T. & BLEIWEIS, A. S. (1991). Restriction fragment length polymorphisms and sequence variation within the *spaP* gene of *Streptococcus mutans* serotype c isolates. *Infection and Immunity* **59**, 1803–1810.
- BRATTHALL, D. (1970). Demonstration of five serological groups of streptococcal strains resembling *Streptococcus mutans*. *Odontologisk Revy* **21**, 143–152.
- BURNE, R. A., RUBINFELD, B., BOWEN, W. H. & YASBIN, R. E. (1986). Tight genetic linkage of a glucosyltransferase and dextranase of *Streptococcus mutans* GS-5. *Journal of Dental Research* **65**, 1392–1401.
- CARLE, G. F. & OLSON, M. V. (1984). Separation of chromosomal DNA molecules from yeast by orthogonal field alternation gel electrophoresis. *Nucleic Acids Research* **12**, 5647–5664.
- CAUFIELD, P. W. & WALKER, T. M. (1989). Genetic diversity within *Streptococcus mutans* evident from chromosomal DNA restriction fragment polymorphisms. *Journal of Clinical Microbiology* **27**, 274–278.
- CHANG, N. & TAYLOR, D. E. (1990). Use of pulsed field agarose gel electrophoresis to size genomes of *Campylobacter jejuni* UA580. *Journal of Bacteriology* **172**, 5211–5217.
- CHIA, J.-S., HSU, T.-Y., TENG, L.-J., CHEN, J.-Y., HAHN, L.-J. & YANG, C.-S. (1991). Glucosyltransferase gene polymorphism among *Streptococcus mutans* strains. *Infection and Immunity* **59**, 1656–1660.
- CHU, G., VOLLRATH, D. & DAVIS, R. W. (1986). Separation of large DNA molecules by contour-clamped homogenous electric fields. *Science* **234**, 1582–1585.
- COOK, P. R. (1984). A general method for preparing intact nuclear DNA. *EMBO Journal* **3**, 1837–1842.
- COYKENDALL, A. L. (1974). Four types of *Streptococcus mutans* based on their genetic, antigenic, and biochemical characteristics. *Journal of General Microbiology* **83**, 327–338.
- FEINBERG, A. P. & VOGELSTEIN, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Analytical Biochemistry* **132**, 6–13.
- FERRETTI, J. J., RUSSELL, R. R. B. & DAO, M. L. (1989). Sequence analysis of the wall-associated protein precursor of *Streptococcus mutans* antigen A. *Molecular Microbiology* **3**, 469–478.
- GASC, A. M., KAUC, L., BARRAILLÉ, P., SICARD, M. & GOODGAL, S. H. (1991). Gene localization, size, and physical map of the chromosome of *Streptococcus pneumoniae*. *Journal of Bacteriology* **173**, 7361–7367.
- GAWRON-BURKE, C. & CLEWELL, D. B. (1984). Regeneration of insertionally inactivated streptococcal DNA fragments after excision of transposon Tn916 in *Escherichia coli*: strategy for targeting and cloning of genes from gram-positive bacteria. *Journal of Bacteriology* **159**, 214–221.
- HANADA, N. & KURAMITSU, H. K. (1988). Isolation and characterization of the *Streptococcus mutans gtfC* gene, coding for synthesis of both soluble and insoluble glucans. *Infection and Immunity* **56**, 1999–2005.
- HANADA, N. & KURAMITSU, H. K. (1989). Isolation and characterization of the *Streptococcus mutans gtfD* gene, coding for primer-dependent soluble glucan synthesis. *Infection and Immunity* **57**, 2079–2085.
- HANTMAN, M. J., TUDOR, J. J., SUN, S., MARRI, L., PIGGOT, P. J. & DANE-MOORE, L. (1991). Physical and genetic mapping of the *Streptococcus mutans* GS-5 genome. In *Genetics and Molecular Biology of Streptococci, Lactococci, and Enterococci*, p. 289. Edited by G. M. Dunne, P. P. Cleary & L. L. McKay. Washington, DC: American Society for Microbiology.
- HAYAKAWA, M., AOKI, H. & KURAMITSU, H. K. (1986). Isolation and characterization of the sucrose 6-phosphate hydrolase gene from *Streptococcus mutans*. *Infection and Immunity* **53**, 582–586.
- HILLMAN, J. D., DUNCAN, M. J. & STASHENKO, K. P. (1990). Cloning and expression of the gene encoding the fructose-1,6-diphosphate-dependent L-(+)-lactate dehydrogenase of *Streptococcus mutans*. *Infection and Immunity* **58**, 1290–1295.
- ITAYA, M. & TANAKA, T. (1991). Complete physical map of the *Bacillus subtilis* 168 chromosome constructed by a gene-directed mutagenesis method. *Journal of Molecular Biology* **220**, 631–648.
- JAGUSZTYN-KRYNICKA, E. K., SMORAWSKA, M. & CURTISS, R., III. (1982). Expression of *Streptococcus mutans* aspartate-semialdehyde dehydrogenase gene cloned into plasmid pBR322. *Journal of General Microbiology* **128**, 1135–1145.
- KRAUSE, D. C. & MAWN, C. B. (1990). Physical analysis and mapping

- of the *Mycoplasma pneumoniae* chromosome. *Journal of Bacteriology* **172**, 4790–4797.
- LEBOURGEOIS, P., MATA, M. & RITZENTHALER, P. (1989). Genome comparison of *Lactococcus* strains by pulsed field gel electrophoresis. *FEMS Microbiology Letters* **59**, 65–70.
- LEE, S. F., PROGULSKE-FOX, A. & BLEIWEIS, A. S. (1988). Molecular cloning and expression of a *Streptococcus mutans* major surface protein antigen, P1 (I/II), in *Escherichia coli*. *Infection and Immunity* **56**, 2114–2119.
- LOESCHE, W. J. (1986). Role of *Streptococcus mutans* in human dental decay. *Microbiol. Rev.* **50**, 353–380.
- LUNSFORD, R. D. & MACRINA, F. L. (1986). Molecular cloning and characterization of *scrB*, the structural gene for the *Streptococcus mutans* phosphoenolpyruvate-dependent sucrose phosphotransferase system sucrose-6-phosphate hydrolase. *Journal of Bacteriology* **166**, 426–434.
- MATHEW, M. K., SMITH, C. L. & CANTOR, C. R. (1988). High-resolution separation and accurate size determination in pulsed field gel electrophoresis of DNA. 1. DNA size standards and the effect of agarose and temperature. *Biochemistry* **27**, 9204–9210.
- MORRISSEY, P., DOUGAN, G., RUSSELL, R. R. B. & GILPIN, M. (1985). Cloning of *Streptococcus mutans* antigens in *Escherichia coli* as an aid to the development of a dental caries vaccine. In *Vaccines 85*, pp. 117–120. Edited by R. A. Lerner, R. M. Chanock & F. Brown. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- OKAHASHI, N., SASAKAWA, C., YOSHIKAWA, M., HAMADA, S. & KOGA, T. (1989). Cloning of a surface protein antigen gene from serotype c *Streptococcus mutans*. *Molecular Microbiology* **3**, 221–228.
- OKAHASHI, N., SASAKAWA, C., OKADA, N., YAMADA, M., YOSHIKAWA, M., TOKUDA, M., TAKAHASHI, I. & KOGA, T. (1990). Construction of a *NotI* restriction map of the *Streptococcus mutans* genome. *Journal of General Microbiology* **136**, 2217–2223.
- PERRY, D. & KURAMITSU, H. K. (1989). Genetic linkage among cloned genes of *Streptococcus mutans*. *Infection and Immunity* **57**, 805–809.
- PERRY, D. & KURAMITSU, H. K. (1990). Linkage of sucrose metabolizing genes in *Streptococcus mutans*. *Infection and Immunity* **58**, 3462–3464.
- PERRY, D., NILSEN, L. J. & KURAMITSU, H. K. (1985). Mapping of a cloned glucosyltransferase gene in *Streptococcus mutans*. *Infection and Immunity* **50**, 130–135.
- PIGGOT, P. J. & CURTIS, C. A. M. (1987). Analysis of the regulation of gene expression during *Bacillus subtilis* sporulation by manipulation of the copy number of *spo-lacZ* fusions. *Journal of Bacteriology* **169**, 1260–1266.
- PROCINO, J. K., MARRI, L., SHOCKMAN, G. D. & DANEOMOORE, L. (1988). Tn916 insertional inactivation of multiple genes on the chromosome of *Streptococcus mutans* GS-5. *Infection and Immunity* **56**, 2866–2870.
- PUCCI, M. J. & MACRINA, F. L. (1986). Molecular organization and expression of the *gtfA* gene of *Streptococcus mutans* LM7. *Infection and Immunity* **54**, 77–84.
- REIDER, J. L. & MACRINA, F. L. (1976). Plasmid DNA isolation in *Streptococcus mutans*: glycine enhanced cell lysis. In *Proceedings: Microbial Aspects of Dental Caries (a special supplement to Microbiology, abstracts)*, vol. 3, pp. 725–736. Edited by H. M. Stiles, W. J. Loesche & T. C. O'Brien. Washington, DC: Information Retrieval.
- ROBESON, J. P., BARLETTA, R. G. & CURTISS, R., III. (1983). Expression of a *Streptococcus mutans* glucosyltransferase gene in *Escherichia coli*. *Journal of Bacteriology* **153**, 211–221.
- RUSSELL, R. R. B. & FERRETTI, J. J. (1990). Nucleotide sequence of the dextran glucosidase (*dexB*) gene of *Streptococcus mutans*. *Journal of General Microbiology* **136**, 803–810.
- RUSSELL, R. R. B., COLEMAN, D. & DOUGAN, G. (1985). Expression of a gene for glucan-binding protein from *Streptococcus mutans* in *Escherichia coli*. *Journal of General Microbiology* **131**, 295–299.
- RUSSELL, R. R. B., ADUSE-OPOKU, J., TAO, L. & FERRETTI, J. J. (1991). Binding protein-dependent transport system in *Streptococcus mutans*. In *Genetics and Molecular Biology of Streptococci, Lactococci, and Enterococci*, pp. 244–247. Edited by G. M. Dunny, P. P. Cleary & L. L. McKay. Washington, DC: American Society for Microbiology.
- SATO, S. & KURAMITSU, H. K. (1986). Isolation and characterization of a fructosyltransferase gene from *Streptococcus mutans* GS-5. *Infection and Immunity* **52**, 166–170.
- SATO, Y., POY, F., JACOBSON, G. R. & KURAMITSU, H. K. (1989). Characterization and sequence analysis of the *scrA* gene encoding enzyme II^{scr} of the *Streptococcus mutans* phosphoenolpyruvate-dependent sucrose phosphotransferase system. *Journal of Bacteriology* **171**, 263–271.
- SCHWARTZ, D. C. & CANTOR, C. R. (1984). Separation of yeast chromosome-sized DNAs by pulsed field gradient gel electrophoresis. *Cell* **37**, 67–75.
- SHIROZA, T. & KURAMITSU, H. K. (1988). Sequence analysis of the *Streptococcus mutans* fructosyltransferase gene and flanking regions. *Journal of Bacteriology* **170**, 810–816.
- SHIROZA, T., UEDA, S. & KURAMITSU, H. K. (1987). Sequence analysis of the *gtfB* gene from *Streptococcus mutans*. *Journal of Bacteriology* **169**, 4263–4270.
- SMITH, C. L. & CANTOR, C. R. (1987). Purification, specific fragmentation, and separation of large DNA molecules. *Methods in Enzymology* **155**, 449–467.
- SMITH, C. L., ECONOME, J. G., SCHUTT, A., KLCO, S. & CANTOR, C. F. (1987). A physical map of the *Escherichia coli* K12 genome. *Science* **235**, 1448–1453.
- SMORAWINSKA, M., HSU, J. C., HANSEN, J. B., JAGUSZTYN-KRYNICKA, E. K., ABIKO, Y. & CURTISS, R., III. (1983). Clustered genes for galactose metabolism from *Streptococcus* cloned in *E. coli*. *Journal of Bacteriology* **153**, 1095–1097.
- SOUTHERN, E. M. (1975). Detection of sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology* **98**, 503–507.
- TANSKANEN, E. I., TULLOCH, D. L., HILLIER, A. J. & DAVIDSON, B. E. (1990). Pulsed field gel electrophoresis of *SmaI* digests of lactococcal genomic DNA, a novel method of strain identification. *Applied and Environmental Microbiology* **56**, 3105–3111.
- TUDOR, J. J., MARRI, L., PIGGOT, P. J. & DANEOMOORE, L. (1990). Size of the *Streptococcus mutans* GS-5 chromosome as determined by pulsed field gel electrophoresis. *Infection and Immunity* **58**, 838–840.
- UEDA, S., SHIROZA, T. & KURAMITSU, H. K. (1988). Sequence analysis of the *gtfC* gene from *Streptococcus mutans* GS-5. *Gene* **69**, 101–109.