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Characterization of Glucosyltransferase-Deficient, Plasmid-Containing Mutants of *Streptococcus mutans* LM-7

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The possibility that glucosyltransferase (GT)-mediated insoluble-glucan synthesis from sucrose is controlled by the 3-megadalton plasmid pAM7 in *Streptococcus mutans* LM-7 has been examined. A low-sucrose agar medium was developed to readily detect and quantitate presumptive GT-negative mutants. Such mutants were isolated from Todd-Hewitt broth cultures grown either with or without sodium dodecyl sulfate (10 μg/ml) or acriflavine (0.5 μg/ml) at frequencies ranging from about 0.01 to 1%. Independently isolated mutants had the following characteristics: (i) cells were virtually devoid of cell-associated GT and did not aggregate upon addition of sucrose; (ii) cell-free culture fluids synthesized 10× less insoluble glucan than those of the parent; and (iii) cultures grown with sucrose did not form adherent deposits on the wall of the culture tube, as is typical of *S. mutans*. Both parent and mutants formed relatively little soluble glucan in 1-h assays. Three independently isolated mutants and the parent were found to contain similar amounts of plasmid DNA. Analysis by sucrose density gradient centrifugation and agarose gel electrophoresis did not reveal a size difference between the plasmids from parent and mutants. These results show that (i) *S. mutans* LM-7 generates GT-deficient mutants at relatively high frequency that still contain a 3-megadalton plasmid; (ii) both cell-associated and extracellular GT levels are depressed in the mutants, which suggests that these activities are directly or indirectly controlled by the same gene or by genes that segregate as a unit.

About 10 years ago, several investigators demonstrated that the smooth-surface cariogenicity of *Streptococcus mutans* in animal model systems is associated with the formation of an adherent and water-insoluble glucan containing primarily α-1-6- and α-1,3-linkages (16, 19). This concept has since been amply confirmed. The glucosyltransferase (GT) activity responsible for insoluble-glucan synthesis from sucrose has been relatively refractile to biochemical and genetic characterization. This appears to be at least partly due to its tendency to form complexes with itself and other sucrose- and dextran-metabolizing enzymes with apparent molecular weights greater than 10^6 (3, 14, 26, 31). However, at least three major and several minor forms of the enzyme have been isolated from strain OMZ-176 (18), and electrophoretically distinct activities with molecular weights ranging from about 160,000 to 225,000 have since been reported from the genetically and biochemically related strain 6715 (3). It is not known whether the observed enzyme multiplicity is due to multiple GT genes or to posttranscriptional modification of a single GT gene product. The location of the gene(s) controlling insoluble-glucan synthesis is also open to question. Whereas it was first proposed that this trait is associated with an inducible prophage (17, 24), later evidence suggested that GT synthesis is controlled by a plasmid because plasmid-curing agents induced mutants at high frequency that synthesized less insoluble glucan (21) and were devoid of the plasmid present in the parent strains PK-1 and JC-2 (20). However, other investigators have been unable to find a plasmid in these strains (4, 33). More recently, it was reported that the mutants derived from strain PK-1 had also lost the inducible prophage carried by the parent. Moreover, the ability to adhere with sucrose could be introduced into these mutants with phage and also with free phage DNA (22). These data did not show whether the loss of plasmid and prophage are associated. To answer this question and to establish whether insoluble-glucan synthesis is controlled by a prophage or a plasmid, we used plasmid-curing techniques to obtain GT mutants of the plasmid-carrying strain LM-7 (12, 15). In this paper we describe that such mutants arise at a relatively high frequency, but that

they still contain plasmid DNA that appears identical in size and amount to that present in the parent.

MATERIALS AND METHODS

Bacterial strains. *S. mutans* LM-7 (15) was kindly provided by L. A. Thomson, who had received it from D. A. Bratthall. This *e* serotype (1) strain appears identical to the LM-7 strain that carries about 16 copies of a 3-megadalton plasmid pAM7 per genome equivalent (12). The origin of the GT mutants derived from LM-7 is given in Table 1. All cultures were maintained as described previously (11) and cultivated at 35 to 37°C. They were streaked on mitis salivarius or Columbia horse blood agar to check purity. These plates were incubated anaerobically (GasPak, Baltimore Biological Laboratory) at 37°C for 18 to 24 h and then aerobically at 20 to 25°C for further inspection.

Detection of GT mutants. Initially, cultures were plated on mitis salivarius agar, and, after 2 days of anaerobic incubation, individual colonies were inoculated into 5 ml of Trypticase soy broth (BBL) with 1% sucrose added. The tubes were incubated in a slanted position and inspected daily for the absence of adherence to the wall of the tube. Two nonadhering mutants were detected in this manner. Because these mutants had lost about 90% of their GT activity, they were used to develop a screening agar. Different amounts of sucrose (final concentration, 0.2 to 20%) and agar (1.5%) were dissolved in Jordan broth (11) without glucose, and the media were sterilized. Plates were poured and left to dry at 22°C for 18 to 40 h. A known mixture of parent and mutant cells was then spread on these plates and incubated anaerobically for 2 days. Inspection by eye or with a dissecting microscope showed that mutants could be recognized readily by their smooth colonial morphology (see below) on the 0.2 and 0.5% sucrose media. Parent and mutant colonies appeared alike on media with more than 1% sucrose. The 0.5% sucrose agar (LS agar) was subsequently used to estimate mutation frequencies.

Mutation experiments. The parent strain was streaked out on LS agar and a typical "rough" (see below) colony was incubated in Todd-Hewitt broth (Difco Laboratories). After growth had occurred, 0.1 ml was inoculated into 5 ml of Todd-Hewitt broth containing either 10 μg of sodium dodecyl sulfate (SDS) per ml or 0.5 μg of acriflavine (AF) per ml. After overnight incubation at 35 to 37°C, the cultures were diluted in phosphate-buffered 0.85% saline (pH 6.5), exposed to ultrasonic oscillations from a Kontes cell disrupter (power setting, 8; 30 s) to break chains of streptococci, and plated on LS agar at a density of about 500 colony-forming units per plate. Presumptive GT mutants were transferred to fluid thioglycolate medium (11) for maintenance and further identification.

Fermentation tests. After overnight growth in purple broth base (Difco) with 1% glucose, each culture was diluted 100-fold, and 0.1 ml was inoculated into 5 ml of purple broth base with 1% of the compound to be tested. Tests were conducted at 37°C for 1 week. Typically, the parent rapidly fermented glucose, sucrose, inulin, trehalose, sorbitol, mannitol, and salicin. Raffinose was fermented more slowly, and glycerol and melibiose were not fermented.

Antibiotic susceptibility tests. Sensitivity disks containing cephalothin (30 μg), clindamycin (2 μg), erythromycin (15 μg), gentamycin (10 μg), lincomycin (2 μg), neomycin (30 μg), penicillin G (10 U), rifampin (5 μg), streptomycin (10 μg), and tetracycline (30 μg) were used, and the tests were conducted as described by Matsen and Barry (29), except that brain heart infusion agar was used. The plates were incubated anaerobically for 16 to 24 h before measurement of inhibition zones.

Adherence assay. Sucrose-dependent adherence to glass was quantitated in triplicate essentially as described by Olson et al. (32), except that the culture volume was 5 ml and the cultures were incubated in screw-capped tubes for 20 h.

Enzyme assays. Cultures were grown overnight in Jordan broth with 5 mM glucose (11) or in the chemically defined FMC medium of Terleckyj et al. (38) with 0.2% glucose, 0.2% Na_2CO_3, and 0.05% (vol/vol) Tween 80 (39, 40). After centrifugation at 15,000 × *g*, supernatant fluids were adjusted to pH 6.5 and assayed for insoluble- and soluble-glucan synthesis and fructosyltransferase (FT) with specifically labeled sucrose as described previously (28). Because the linkages in the polymers synthesized by these enzymes have not been characterized, we prefer not to identify the LM-7 GT and FT activities as dextransucrase (EC 2.4.1.5) and levansucrase (EC 2.4.1.10), respectively (40). The amount of product formed was proportional to the amount of supernatant fluid added. Assay results were converted to international units (28). For cell-associated GT assays, cells were washed twice with 0.9% NaCl, resuspended in 0.05 M potassium phosphate buffer (pH 6.5) at an apparent absorbance (600 nm) of 1.0, and assayed for insoluble-glucan synthesis (28) in the presence of 10 mM NaF. To study sucrose-induced agglutination of the washed cells, sucrose and sodium azide were added to final concentrations of 1 and 0.04%, respectively, and the suspensions were inspected visually for agglutination during incubation at 35°C.

To measure insoluble-glucan synthesis turbidimetrically (16), 10 ml of cell-free culture fluid, obtained after overnight growth in Jordan broth, was adjusted to pH 6.5, concentrated under reduced pressure to 1.0 ml in a collodion bag (no. 100; Schleicher & Schuell), and equilibrated in a cuvette at 37°C. After addition of 0.1 ml of 10% sucrose, the apparent absorbance was recorded at 400 nm in a recording spectrophotometer. The reference cuvette contained sterile medium instead of culture fluid.

Specific growth rates. Doubling times were measured at 37°C in Todd-Hewitt broth in the absence and presence of SDS. About 15 ml of medium in a screw-capped tube was inoculated with a logarithmically growing Todd-Hewitt broth culture. Every 30 to 60 min each culture was gently mixed, and the growth was measured turbidimetrically as apparent absorbance at 600 nm. If necessary, the sample was diluted with phosphate-buffered saline (pH 6.5) so that the reading would be proportional to the biomass. The results were plotted on semilogarithmic graph paper, and the data from the linear part of the graph were used to compute doubling time by least-squares analysis.

Labeling of DNA and preparation of cell ly-

sates. A 2-ml amount of a culture that had been grown for 8 h in brain heart infusion (Difco) was inoculated into 100 ml of brain heart infusion supplemented with 20 mM glucose, 0.5 mCi of [*methyl*-^{3}H]thymidine (40 to 60 Ci/mmol; Schwarz/Mann), 10 mM L-threonine (or 20 mM DL-threonine), and 25 mM potassium phosphate, pH 7.5. After incubation at 37°C for 16 h, the labeled cells were collected by centrifugation at 4°C (16,000 × *g*, 15 min), resuspended in 0.02 M tris(hydroxymethyl)aminomethane-hydrochloride, pH 8.2 (T8.2), and recentrifuged. After suspension of the washed cells in 12.5 ml of T8.2, 25 ml of 24% (wt/vol) polyethylene glycol 20,000 (Fisher Scientific Co.) and 400 mg of lysozyme (grade I, Sigma; dissolved in 12.5 ml of T8.2) were added sequentially. After incubation at 37°C for 1 h, microscopic inspection showed primarily agglutinated spheroplasts. These were collected by centrifugation at 4°C (16,000 × *g*, 15 min), drained well, and resuspended in 9 ml of 0.05 M tris(hydroxymethyl)aminomethane–5 mM ethylenediaminetetraacetate buffer, pH 9.0 (TE9). Lysis was affected by addition of 1 ml of 10% SDS and 4 mg of Pronase (Calbiochem, nuclease-free; self-digested at 37°C for 2 h and heated at 80°C for 5 min), and the lysate was incubated at 37°C for 30 min.

Enrichment of plasmid DNA. The covalently closed circular (CCC) plasmid DNA in the lysate was purified essentially as described by Currier and Nester (8). The redissolved DNA from the ethanol precipitation step was dialyzed overnight against 0.01 M tris(hydroxymethyl)aminomethane–0.01 M NaCl–1 mM ethylenediaminetetraacetate, pH 8.0 (TES8) buffer.

Equilibrium density gradient centrifugation. CCC plasmid DNA was separated from linear and nicked circular DNA by isopycnic centrifugation (44,000 rpm, 44 h) in the presence of ethidium bromide and CsCl essentially as described previously (8). Fractions of 200 μl were collected, and the refractive index was determined for calculation of apparent buoyant density. Portions, usually 10 μl, were spotted on Whatman GF/B glass fiber disks dampened with 10% trichloroacetic acid. The disks were dried, washed with 5% trichloroacetic acid and then with acetone, dried again, and counted in 5 ml of Econofluor (New England Nuclear Corp.). Fractions containing CCC DNA were mixed with an equal volume of Dowex-50W-X12 (Na^+ form) to remove ethidium bromide, dialyzed against TES8 buffer, and subjected to sucrose gradient centrifugation, electron microscopy, and agarose gel electrophoresis.

Velocity density gradient analysis. Linear 5 to 20% sucrose gradients in 0.01 M tris(hydroxymethyl)aminomethane–0.01 M ethylenediaminetetraacetate–1 M NaCl buffer, pH 7.5, were layered over a 0.6-ml cushion of 40% sucrose to give a volume of 11.6 ml per tube. Up to 200 μl of sample was layered on the gradient, and the tube was centrifuged at 22,000 rpm for 17 h at 10°C in a Beckman SW41 rotor. [^{14}C]DNA from simian virus 40 (Bethesda Research Laboratories) was included as an internal and external marker. The sedimentation coefficients in neutral sucrose of the open (form II) and closed circle (form I) forms of simian virus 40 DNA are 16 and 21S, respectively. Fractions of 200 μl were collected, and the ^{3}H and ^{14}C radioactivities were determined.

Electron microscopy. The modification described by Davis et al. (9) of the Kleinschmidt technique was used.

Agarose gel electrophoresis. CCC and relaxed circular plasmid DNA were separated by electrophoresis in 0.7% agarose slab gels as described by Meyers et al. (30). For molecular weight determination, plasmids pBR317 (5.8 megadaltons; Bethesda Research Laboratories) and ColE1 (4.2 megadaltons; Boehringer Mannheim Corp.) and DNA from bacteriophage PM2 (6.3 megadaltons; Boehringer Mannheim Corp.) were used as markers.

RESULTS

Screening agar. To test the thesis that a plasmid controls GT synthesis (20, 21), strain LM-7, which contains about 16 copies of a 3-megadalton plasmid (12), was subjected to plasmid-curing agents to induce GT-negative mutants. Whereas such mutants from other strains have been detected as colonial morphology mutants on media containing 5% sucrose (10, 13, 23, 25), this method did not appear suitable for LM-7 because its high FT level (2) induces the formation of mucoid colonies, independent of GT activity. The low-sucrose (LS) screening agar that was developed (see above) allowed rapid screening of large numbers of LM-7 clones for GT-deficient mutants. Whereas parent colonies appear convex papillate and undulate ("rough") after a 2-day anaerobic incubation on LS agar, mutant colonies are convex and entire ("smooth") and can be recognized readily.

Induction of GT mutants. Because both SDS and AF have been used to cure plasmids in gram-positive organisms (6, 36), *S. mutans* LM-7 was grown in the presence of these agents to induce GT-negative mutants. Plating on LS agar showed that the great majority of the colonies were either distinctly rough or smooth. However, the AF-treated cultures occasionally gave rise to intermediate, and sometimes unstable, colonial morphologies; these are not considered in this paper. Table 1 summarizes the results of three independent experiments and shows that

TABLE 1. *Summary of mutation experiments*

Expt	Addition to Todd-Hewitt broth	Colonies screened	GT⁻ mutants found	GT⁻ mutants (%)	Typical mutant
1[a]	None	3,500	1	0.03	BT20
	0.001% SDS	700	6	0.86	A17
	0.5 μg of AF per ml	2,600	6	0.23	AF19
2[b]	None	3,200	0	≤0.03	
	0.001% SDS	1,740	1	0.06	A22
3[b]	None	2,500	1	0.04	BT30
	0.001% SDS	1,800	0	<0.06	

[a] Experiment inoculated with a culture originally derived from a single colony but transferred many times.

[b] Experiment inoculated with a culture directly derived from a new single colony.

mutants were found at relatively high frequencies. Whereas the first experiment suggested that growth in the presence of SDS or AF substantially increased the percentage of mutants, the two subsequent experiments did not show this trend. Whereas variability in plasmid curing rates between replicate experiments is common, the higher yield of mutants in the first experiment also could have originated from a higher amount of contaminating sucrose in the Todd-Hewitt broth used in that experiment. Sucrose causes the parent to aggregate (see below), which leads to an apparent enrichment of mutants because they grow in a more dispersed form. The possibility that SDS enriched for mutants already present in the inoculum (36) was also examined. Measurement of specific growth rates showed that the two mutants examined grew about 20% faster than the parent in the presence of 0.001% SDS (Table 2). There was no consistent difference between parent and mutants in the lag periods, and they did not interfere with each other when grown together in a reconstruction experiment (data not shown). Thus, whereas SDS may have favored growth of the mutants to a certain extent, the effect was not as pronounced as that observed with two strains of *Staphylococcus aureus* carrying a penicillinase plasmid (36). In addition, it can be concluded that there was no bacteriocin-like killing of mutants by the parent.

Identity of mutants. All presumptive GT-negative mutants isolated from the LS agar plates had the same fermentation pattern and the same colonial morphology on blood agar as the parent. After 2 days of incubation on mitis salivarius agar, parent and mutant colonies appeared alike due to synthesis of transparent exudate, presumably fructan, which gave all colonies a glossy and smooth appearance. To see whether the presumptive loss of GT was accompanied by the loss of an antibiotic resistance marker, antibiotic sensitivity patterns were determined. The tests showed that parent and mutants were resistant to gentamycin, neomycin, and streptomycin, but sensitive to the other seven antibiotics tested.

TABLE 2. *Effect of SDS on doubling time of parent and GT mutants*

Strain	Doubling time (min)[a]	
	No SDS	+0.001% SDS
LM-7 parent	63	110
A17 mutant	64	77
A22 mutant	66	90

[a] In Todd-Hewitt broth; measured turbidimetrically.

Sucrose metabolism. To characterize the smooth colonial morphology mutants, they were assayed for sucrose-dependent adherence to glass and extracellular and cell-associated GT activities. Table 3 shows that these properties were lost simultaneously in all of the independently isolated mutants. In agreement with these findings, parent cells visibly agglutinated within 1 h after addition of sucrose; mutant cells did not do so within 21 h. The data also show that parent and mutants had identical extracellular FT activity (Table 3), which indicates that the loss of GT did not result from a general defect in protein secretion from the cell.

The radioisotope assays for insoluble-glucan synthesis consistently showed that independently isolated mutants had about 9% of the extracellular activity of the parent (Table 3). This residual activity was not due to revertants because no rough colonies were detected among the 1,000 colonies inspected on the LS agar plates from the assayed cultures. These findings do not necessarily rule out that GT is controlled by a plasmid, because the residual activity could be due to a chromosomal determinant (34). Turbidimetric assays proved that the residual GT activity of the mutants was not due to an artifact of the radioisotope assay procedure (Fig. 1). In addition, these assays confirmed the nonlinear kinetics observed with the radioisotope assay.

Whereas Table 3 shows that the mutants have much less cell-bound and extracellular GT, it does not prove that native cell-associated and extracellular GT are controlled by the same gene, because the cell-bound activity could conceivably have originated from extracellular GT due to traces of sucrose or dextran (37) in the complex culture medium. However, cultures

TABLE 3. *Comparison of sucrose metabolism between parent and mutants*

Strain	Adherence (%)	Glucan synthesis[a]			FT[a] extracellular
		Extracellular, insoluble	Extracellular, soluble	Cell-bound, insoluble	
LM-7 parent	88	1,064 ± 55	71 ± 16	280 ± 57	2,156 ± 132
A17, A22, AF19, BT20, BT30 mutants[b]	≤1	92 ± 10	94 ± 16	15 ±7	2,170 ± 114

[a] Results expressed as counts per minute average ± standard error; 1,000 cpm corresponds to 0.0055 IU of GT or FT per ml of culture (Jordan broth).

[b] All mutants were assayed; average values are given.

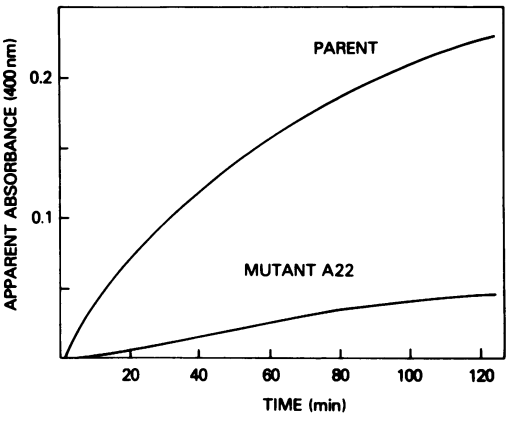

FIG. 1. *Turbidimetric GT assays of S. mutans LM-7 and mutant A22 cell-free culture fluids.*

grown on a defined medium, free of sucrose and dextran, gave similar results (Table 4). In agreement with these findings, parent cells grown on this medium readily agglutinated upon addition of sucrose, whereas mutant cells did not agglutinate (Table 4). These results suggest that the extracellular and cell-associated activities are directly or indirectly controlled by the same gene or by genes that segregate as a unit.

Soluble-glucan synthesis. Insoluble-glucan-synthesis-defective mutants from various other strains of *S. mutans* have been reported to produce more soluble glucan than their respective parent strains (13, 21, 23). However, examination of strain LM-7 and the mutants showed that both synthesized relatively little soluble glucan in 1 h (Table 3) and that the difference was not significant (t test; $P > 0.1$). In support of this, enzyme activity stains after gel electrophoresis revealed that, unlike strain 6715, LM-7 does not secrete a GT that specifically catalyzes soluble-glucan synthesis (manuscript in preparation).

Plasmid content. The facile loss of the ability to synthesize insoluble glucan, which occurred both in the absence and presence of plasmid-curing agents (Table 1), suggested that this property might be controlled by the plasmid present in strain LM-7. To study this possibility, mutant and parent cells were examined for the presence of CCC DNA by ethidium bromide-CsCl equilibrium density gradient centrifugation. Figure 2 shows that all three independently isolated mutants had CCC DNA. The LS agar plates inoculated just before cell lysis did not show any rough colonies among the more than 100 examined. Figure 2 also shows that the yield after centrifugation of mutant CCC DNA, which varied between 0.1% (BT30) and 0.4% (A17) of the total DNA in the culture, was not significantly different from that of the parent (0.2%). Thus, the possibility that the low residual GT activity in the mutants is due to a major decrease in the number of plasmid copies per cell is excluded. Electron microscopic examination of the molecules present in the CCC DNA peak of mutant A17 showed supercoiled and relaxed circular forms and replicating intermediates similar to those observed in preparations from the parent (data not shown).

The possibility that the relatively high-frequency loss of GT is due to a spontaneous deletion from the plasmid, similar to the 2.8-megadalton deletion from the α-plasmid that caused loss of tetracycline resistance in *S. faecalis* DS-5 (5), was also examined. Analysis of plasmid preparations by agarose gel electrophoresis showed that the plasmids from all the mutants and the parent were very similar in size. Figure 3 shows that all six preparations had a faster-moving band, representing CCC plasmid DNA, and a slower-moving band representing nicked circular DNA. Based on the mobility of the reference plasmids, it can be estimated that a deletion from the parent plasmid of at least 500,000 daltons would have been detected. Velocity sedimentation analysis of mutant plasmid confirmed these results. Centrifugation through a neutral linear 5 to 20% sucrose gradient showed that the CCC DNA from mutant A17 sedimented with an S value of 22, slightly faster than the internal 21S simian virus 40 marker (data not shown). This is within experimental error of the 21.5S value reported for the LM-7 plasmid (12).

DISCUSSION

The results show that mutants of *S. mutans* LM-7 that have little residual GT activity, yet still retain supercoiled plasmid DNA, can be isolated at relatively high frequency (Tables 1 and 3, Fig. 2 and 3). Thus, it is clear that these mutants are different from the plasmid-negative,

TABLE 4. *Insoluble-glucan synthesis by cell-free culture fluids and washed cells after growth in a chemically defined medium*[a]

Strain	Glucan synthesis[b]		Cellular aggregation	
	Cell-free culture fluid	Washed cells	+Sucrose	−Sucrose
LM-7 parent	900	250	+1[c]	−18[c]
A17 mutant	10	10	−18	−18

[a] FMC medium (38). For modifications, see text.
[b] Expressed as counts per minute; 1,000 cpm corresponds to 0.0055 IU of GT activity per ml of culture.
[c] +1, Positive within 1 h; −18, negative after 18 h.

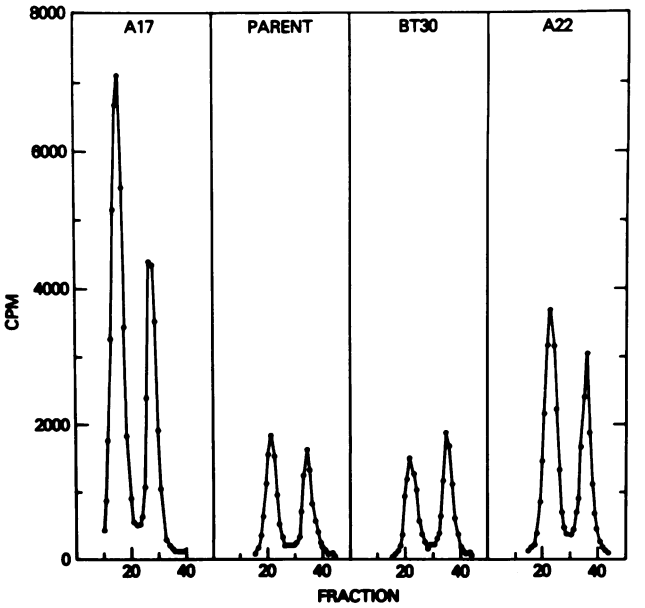

FIG. 2. *Ethidium bromide-cesium chloride buoyant density gradient analysis of partially purified plasmid preparations from S. mutans LM-7 and three GT-deficient mutants. The density increases from right to left in each panel. The average apparent buoyant density was 1.582 (standard deviation, 0.003) for the CCC DNA and 1.542 (standard deviation, 0.002) for the non-CCC DNA.*

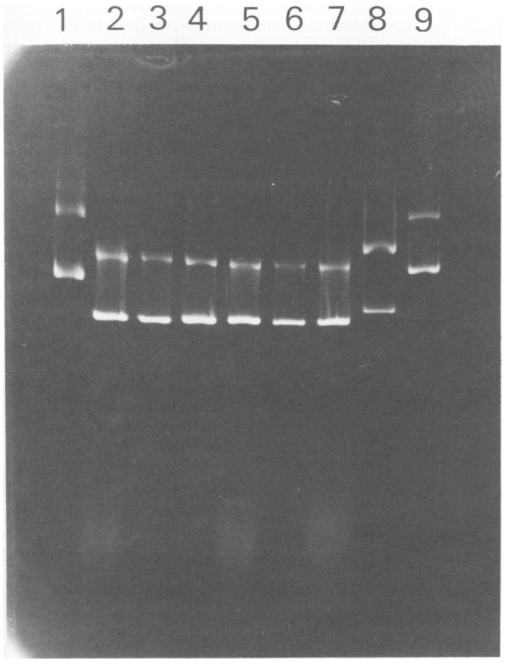

FIG. 3. *Electrophoresis of parent and mutant plasmid preparations in an 0.7% agarose gel slab. The following preparations were applied (from left to right): pBR317 (5.8 megadaltons), A17, A22, AF19, parent LM-7, BT20, BT30, ColE1 (4.2 megadaltons), and DNA from bacteriophage PM2 (6.3 megadaltons). Electrophoresis was conducted at 30 mA for 2.5 h. The anode was at the bottom.*

GT-deficient mutants obtained from strains PK-1 and JC-2 by Higuchi et al. (20). This difference could be due to the high concentration of plasmid-curing agents used in the derivation of the PK-1 and JC-2 mutants, which could have caused plasmid curing independent of the GT mutation (20), or to inherent differences between the strains examined.

Higuchi et al. also noted that parent strains lysed more readily with mitomycin C than GT mutants obtained by plasmid-curing techniques (21) and subsequently reported an association between GT and a lysogenic phage in strain PK-1 (22). Based on studies with other strains, Klein and Frank had previously arrived at the same conclusion (24). These findings suggested that the facile loss of GT in strain LM-7 might be due to the loss of a prophage and that the effect of mitomycin C on parent and mutant LM-7 cultures should be examined. However, we have so far been unable to show a difference between the mitomycin C-induced lysis of parent and mutant LM-7 cultures (data not shown). Whether this is due to a strain difference or to other factors remains to be determined.

The mutants described in this paper are also different from the GT mutants described by others because (i) they do not synthesize significantly more soluble glucan than their parent (13, 21, 23) and (ii) they do not adhere to glass when grown with sucrose (26). The first difference appears to be due to the fact that LM-7

lacks a GT that specifically catalyzes soluble-glucan synthesis like the one present in strain 6715 (3). The second difference could be due to a lower residual GT activity of the LM-7 mutants.

Two different assays (Table 3, Fig. 1) convincingly demonstrated that the LM-7 mutants had about 10% residual GT activity. Whether this remaining activity results from the expression of a second, independent GT gene or is the result of a much decreased expression of a single GT gene remains to be established. In this context, it should be noted that mutants obtained by other investigators after plasmid-curing techniques also had residual activity (20–22, 25). A third possibility is that the GT deficiency resulted from a mutation affecting the secretion of GT from the cell. Such a mutation would have to have a certain specificity because the extracellular FT level was not affected (Table 3). Little is known about the secretion of GT, except that surfactants like Tween 80 stimulate GT synthesis (39, 40) and affect the fatty acid composition of the cellular lipids (39). However, the extracellular GT level of the mutants was not stimulated by including 0.05% Tween 80 in the growth medium (data not shown).

The relatively high frequency of spontaneous loss of GT could still be due to the loss of a plasmid if LM-7 harbors a second, much more labile, plasmid that controls GT and that has not been detected so far by the methods used. A second possibility is that LM-7 harbors two functionally distinct plasmids of equal size. If one of these plasmids controls GT and if its copy number is much smaller than that of the other plasmid, then its loss might not have been detected by the methods used. Restriction endonuclease mapping of parent and mutant plasmids, currently in progress, should provide evidence for or against this hypothesis. Both sucrose gradient and electrophoretic analyses of the plasmids (Fig. 3) show that the mutant phenotype is not due to the deletion of a relatively large part of the plasmid (5). However, the possibility of a small deletion or insertion in a chromosomal or extrachromosomal determinant for GT has not been excluded and should be considered.

It is not necessary to infer the existence of a plasmid to explain the relatively high frequency loss of GT because other studies have shown that the high-frequency loss of a virulence determinant is not necessarily associated with the loss of a plasmid (for instance, see references 27 and 35). In fact, it has become clear that a higher-than-normal rate of spontaneous mutation can have a variety of origins (7), and it is evident that further study is required to define the nature of the GT loss in *S. mutans* LM-7.

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