Genetic Transformation of Putative Cariogenic Properties in *Streptococcus mutans*

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Rough colonial morphology and bacteriocin production, two properties which may be associated with the cariogenicity of *Streptococcus mutans*, were transformed into several strain GS-5 mutants defective in each respective property. Transformation was determined by observing the frequency of cotransfer of these properties with different reference markers. The rough colonial transformants were identical to the parental GS-5 strain with respect to ability to synthesize water-insoluble glucans and undergo in vitro sucrose-dependent colonization of glass surfaces. Alterations in the growth medium and the concentration of the initial cell inoculum resulted in an approximate 10-fold increase in the frequency of transformation of strain GS-5 compared to previous observations.

Considerable evidence indicates that *streptococcus mutans* plays a significant role in the initiation and development of dental caries (3, 4). Several recent investigations have been concerned with the development of a genetic transfer system in this organism to determine the genetic basis for its cariogenicity. LeBlanc et al. (9) reported the transfer of the pAMβ1 plasmid, which codes for erythromycin and lincomycin resistance, from a group F *Streptococcus* species to *S. mutans* by a process resembling conjugation. More recently, this laboratory demonstrated the transformation of three strains of *S. mutans* to streptomycin resistance by homologous and heterologous chromosomal DNA (12). In addition, we have also shown that these strains, as well as other *S. mutans* strains (8), can be transformed to erythromycin resistance with streptococcal plasmids. Cariogenic strains of *S. mutans* possess the ability to tenaciously colonize smooth surfaces, a property which is primarily dependent on the synthesis of insoluble glucan mediated by glucosyltransferase (GTF) (10). Many of these strains produce bacteriocins, and it is possible that these antibacterial substances may also play a role in colonization (13). The present study describes the transformation of several potential caries-associated properties to mutant strains with chromosomal DNA from cariogenic *S. mutans* GS-5. Furthermore, conditions resulting in enhanced frequencies of transformation of this strain are also described.

MATERIALS AND METHODS

Bacterial strains. The bacterial cultures used in this study were all derivatives of human cariogenic strain GS-5 (serotype c). This strain, which exhibits sucrose-dependent colonization of glass surfaces, characteristically exhibits GTF activity, produces insoluble glucan, and forms rough colonies on mitis salivarius (MS) agar. A mutant doubly resistant to DL-*p*-fluorophenylalanine and streptomycin (Pfa^r Str^r) was obtained by spontaneous isolation from a chemically defined medium (14) containing 25 μg of DL-*p*-fluorophenylalanine per ml followed by insertion of the Str^r marker by transformation with GS-5 Str^r DNA (12). The tryptophan-requiring (Trp$^-$) and isoleucine-valine-requiring (Ilv$^-$) auxotrophs were isolated from chemically defined minimal medium after mutagenesis of strain GS-5 cells with ethyl methane sulfonate (Sigma Chemical Co.) followed by enrichment with streptozotocin (A. Bleiweis, personal communication). A bacteriocin-negative mutant (B211, Bac$^-$) was isolated after mutagenesis of strain GS-5 cells with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Sigma). Appropriate dilutions of the mutagenized cells were spread onto Trypticase soy agar plates containing 0.2% yeast extract (TSAY) and incubated for 18 h at 37°C in an anaerobic GasPak system (BBL Microbiology systems). The plates were then overlaid with 5 ml of 0.7% agar containing approximately 10^7 indicator cells (group C *Streptococcus* strain 26RP66). After 18 h of incubation, the plates were examined for the absence of zones of inhibition surrounding the *S. mutans* GS-5 colonies. The Bac$^-$ mutant was purified from the indicator cells by streaking on blood agar plates. Smooth colonial mutants of GS-5 were obtained after streaking the appropriate strain onto MS agar. Mutants Sp1 and Sp2 were spontaneously derived from strain GS-5 and mutant S3

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TABLE 1. Effect of different growth media on the transformation of strain GS-5 to streptomycin resistance[a]

Transformation medium[b]	Transformants per ml ($\times 10^2$)	CFU/ml ($\times 10^7$)	Transformants per 10^7 CFU
PYG	72	12.2	590
PYG + 0.5% glucose	80	13.2	606
THB	492	36.6	1,340
Brain heart infusion	137	15.7	873
Trypticase soy broth	54	10.3	524

[a] Cells were grown for 18 h in brain heart infusion broth, diluted 1:200 in the different media, and incubated for 5 h at 37°C. *S. mutans* GS-5 Str[r] DNA was then added to a concentration of 10 µg/ml, and incubation was continued for 2 h. Transformants were scored on brain heart infusion agar containing 200 µg of streptomycin per ml, and the total CFU were determined on Trypticase soy agar.

[b] All media contained 5% heat-inactivated horse serum.

Trp[−] was similarly obtained from strain GS-5 Trp[−]. The SS1 Str[r] mutant was isolated after mutagenesis of strain GS-5 Str[r] with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Maintenance of cultures and the preparation of DNA were performed as previously described (12).

GTF activity and in vitro colonization of glass surfaces. Extracellular total and insoluble GTF activities were determined by a radioisotope method as described previously (7). One GTF unit was defined as 1,000 cpm of [^{14}C]sucrose incorporated into glucan per Klett unit in the standard assay system. To determine sucrose-dependent colonization, we grew cells in tubes containing Todd-Hewitt broth (THB) plus 2% sucrose for 18 h at a 30° angle. The culture fluids were decanted, and adherent cells were washed gently with water and visually examined.

Gel-filtration chromatography. Each organism was grown in 500 ml of chemically defined glucose medium (5), and the culture fluids were harvested after centrifugation and concentrated on PM-10 ultrafilters (Amicon). The sample (5 ml) then was applied to a Biogel A-0.5 column (2.5 by 30 cm) and eluted with 0.01 M potassium phosphate buffer (pH 6.0) at 4°C. Total GTF activity of each fraction was measured as previously described in the presence of dextran T10.

Transformation. Cells were grown for 18 h in THB containing 5% heat-inactivated (60°C, 30 min) horse serum (THBS), diluted 1:40 into fresh THBS, and incubated at 37°C until an optical density of 0.18 to 0.23 (at 540 nm) was attained (0.5 × 10^8 to 1.0 × 10^8 CFU/ml; 3.0 to 3.5 h). Competent cultures were then exposed to DNA for 30 min, diluted 1:2 into fresh THBS, and incubated for 90 min more. Before plating, the cells were centrifuged at 4°C for 15 min at 754 × *g*, washed once with cold saline, and sonicated for 30 s (0.6 A) in a Sonic Dismembrator (model 300; Fisher Scientific Co.). This treatment resulted in disruption of the streptococcal chains (8 to 12 cells per chain), mostly into single and double cells. The transformants were scored on the appropriate media after incubating

the plates at 37°C for 48 h in an anaerobic GasPak system.

RESULTS

Optimal conditions for transformation of strain GS-5. Since the initial demonstration of transformation in *S. mutans* (12), continued efforts have been aimed at defining the optimal conditions for the system. Consequently, such studies have resulted in a substantial increase in the frequency of transformation in strain GS-5. In previous studies, 0.002 to 0.006% transformants (Str[r]) were obtained when this strain was grown in a proteose peptone-yeast extract-glucose medium (PYG) (12). When strain GS-5 cells were grown in either THB or brain heart infusion broth, however, a significant increase in the number of transformants was observed (Table 1). The development of competence in THB consistently resulted in the highest number of transformants of all the media tested. The number of transformants resulting from growth in Trypticase soy broth was approximately the same as for cells grown in PYG. In this experiment, strain GS-5 cells grown for 18 h were diluted 1:200 in the different transformation media and grown for 5 h before adding the DNA. These conditions were originally designed for cells grown in PYG and might not have been optimal for cells grown in THB. Therefore, the effect of the initial inoculum on the time for optimal competence of cells grown in THB was examined. The time for optimal competence was inversely proportional to the size of the initial inoculum (Table 2). However, the number of transformants reached a maximum when the cells were diluted 1:40 and gradually declined upon further dilution. This procedure consistently yielded 0.02 to 0.05% transformants (Str[r]), which was approximately 10-fold higher than our initial observations (12).

Transformation of potential caries-associated

TABLE 2. Effect of initial cell concentration on the time of appearance of optimal competence and the number of transformants in strain GS-5 cells grown in THB[a]

Initial cell dilution	Time of optimal competence (h)	Transformants per 10^7 CFU
1:20	2	2,600
1:40	3	3,500
1:100	4	2,100
1:200	5	1,400

[a] Cells were grown for 18 h in THBS and diluted in the same medium as indicated. At different times during incubation, portions from each dilution were removed and exposed to 10 µg of *S. mutans* GS-5 Str[r] DNA per ml, and incubation was continued for 2 h. Transformants were scored as described in Table 1, footnote *a*.
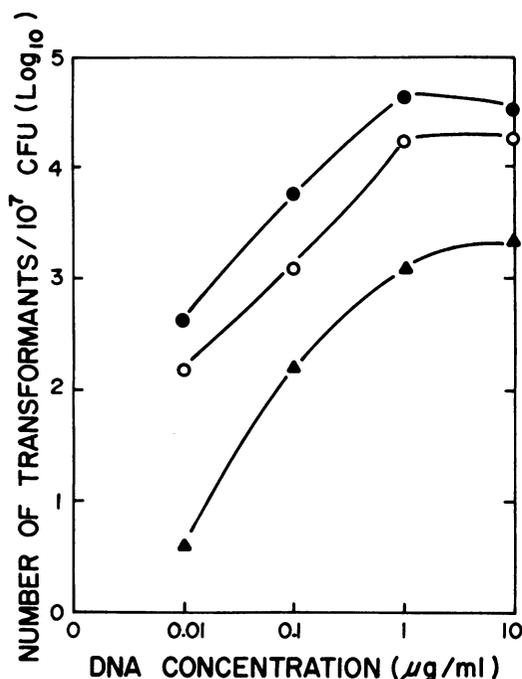
FIG. 1. Frequency of transformation of strain GS-5 Ilv$^-$ as a function of GS-5 Pfa^r Str^r DNA concentration. Symbols: ●, Ilv$^+$; ○, Pfa^r; ▲, Str^r.

properties. The transformation of potential caries-associated properties (rough colonial morphology, bacteriocin production) was determined by observing the frequency of cotransfer of these properties with different reference markers. This procedure was necessary due to the relatively low frequencies of transformation of the strain GS-5 mutants and the lack of suitable media for the direct selection of transformants. Figure 1 shows the results obtained when three reference markers were transformed into a GS-5 Ilv$^-$ strain. The number of transformants for all three markers increased with increasing concentrations of DNA up to about 1 μg/ml when saturation occurred. At this concentration, the levels of Ilv$^+$, Pfa^r, and Str^r transformants were approximately 0.4, 0.2, and 0.01%, respectively. This relationship was observed even when different mutants were employed as DNA recipients, thus suggesting differences in the efficiency of marker integration in strain GS-5.

Since sucrose-dependent colonization and rough colonial morphology are associated with the cariogenic properties of *S. mutans*, it was important to determine whether these properties were transformable. Therefore, three strain GS-5 spontaneous mutants (Sp1, Sp2, and S3 Trp$^-$),

which exhibited smooth colonies on MS agar plates, were isolated as previously described. These mutants failed to colonize glass surfaces and did not synthesize detectable insoluble glucan (Table 3). The results in Table 4 show that Sp1, Sp2, and S3 Trp$^-$ were readily transformed to Pfa^r and Str^r, and the latter strain also transformed to Trp$^+$. These transformants were subsequently replica plated onto MS agar to determine the number that also had acquired the rough colonial trait. Indeed, it could be seen that a small percentage (0.3 to 1.1%) of the transformants exhibited the rough colonial morphology on MS agar plates. For controls, each mutant was also transformed with DNA from the smooth colonial mutant SS1 Str^r. However, when the resulting transformants (Str^r Trp$^+$) were screened on MS agar plates, rough colonies were not observed (Table 4). Reasons for the decreased levels of Str^r transformants induced by SS1 Str^r DNA are presently unknown. Saturating concentrations of DNA (10 μg/ml) were used in all of these experiments to ensure the occurrence of double transformants. Transformants possessing the rough colonial morphology were not observed when limiting concentrations of DNA (0.2 μg/ml) were employed (data not shown). The number of double transformants was dependent on DNA concentration, and therefore no linkage existed between the rough colonial character and the other markers. Colonial morphology was also transformed in the opposite direction (rough to smooth), al-

TABLE 3. GTF activities and in vitro colonization of strain GS-5 smooth colonial mutants and their rough transformants

Bacterial strain	Extracellular GTF activity (U per 100 Klett units)		Sucrose-dependent colonization
	Total	Insoluble	
Sp1	0.17	0	−
Sp1 Pfa^r R^a	0.41	0.26	+
Sp1 Str^r R	0.38	0.21	+
Sp2	0.06	0	−
Sp2 Pfa^r R	0.37	0.20	+
Sp2 Str^r R	0.41	0.27	+
S3 Trp$^-$	0.18	0	−
S3 Pfa^r R	0.46	0.36	+
S3 Str^r R	ND^b	ND	+
S3 Trp$^+$ R	0.52	0.35	+
SS1 Str^r	0.24	0.04	−
SS1 Str^r Pfa^r R	0.47	0.26	+
GS-5 Trp$^-$	0.43	0.17	+
GS-5	0.41	0.28	+

^{a} Bacterial strains with the suffix R (rough) represent double transformants of the respective smooth colonial mutant.
^{b} ND, Not done.

TABLE 4. Transformation of smooth colonial mutants of strain GS-5

DNA recipient[a]	DNA	Transformants per 10^7 CFU					
		Pfa^r	Pfa^{r}R[b]	Str^r	Str^{r}R	Trp$^+$	Trp$^+$R
Sp1	Pfa^r Str^r	4,360	28 (0.6)[c]	788	3 (0.4)		
	SS1 Str^r			119	0		
Sp2	Pfa^r Str^r	2,270	25 (1.1)	986	11 (1.1)		
	SS1 Str^r			121	0		
S3 Trp$^-$	Pfa^r Str^r	1,050	5 (0.5)	705	3 (0.4)	5,160	16 (0.3)
	SS1 Str^r			60	0	3,930	0

[a] Recipient cells were exposed to 10 µg of DNA per ml and plated in duplicate on the appropriate selective medium. Cells were diluted such that at least 400 to 600 transformant CFU appeared on each plate. The rates of spontaneous mutation of control cells (no DNA) ranged from 0 to 9 per 10^7 CFU.

[b] Rough (R) double transformants were detected by replica plating the single transformant colonies (Pfa^r, Str^r, Trp$^+$) onto MS agar plates.

[c] Numbers in parentheses represent percent single transformants that also possessed the R (rough) marker.

though these results were interpreted with caution since rough strains spontaneously produce smooth variants at a fairly high rate (0.1 to 0.3%). In contrast, the spontaneous conversion from smooth to rough colonial morphology was never observed among the several thousand colonies screened during these investigations.

GTF activity and colonization of mutants and transformants. The smooth colonial mutants and several of their rough transformants (Pfa^{r}R, Str^{r}R, Trp$^+$R) were also examined for GTF activity and ability to colonize glass surfaces. As shown in Table 3, the rough colonial trait is associated with the synthesis of insoluble glucan and sucrose-dependent colonization of glass surfaces. The levels of insoluble glucan synthesized by the transformants were similar to the amounts synthesized by the two parental rough colonial strains, GS-5 and GS-5 Trp$^-$. None of the spontaneous smooth colonial strains synthesized detectable amounts of insoluble glucan or colonized glass surfaces in the presence of sucrose. Identical results were observed with Sp1, Sp2, S3 Trp$^-$, and their rough colonial transformants.

Gel filtration chromatography of the culture fluids of mutants and transformants. To further examine the GTF activities of a representative smooth colonial mutant (SS1) and its rough transformant (SS1R), gel filtration chromatography of the extracellular fluids of the cultures was performed (Fig. 2). The GTF activity eluting in the void volume (fractions 8 through 14) was enriched for insoluble glucan synthesis as previously demonstrated for parental strain GS-5 enzymes (7). This peak was much reduced in activity in culture fluids derived from the smooth colonial strain. The retarded GTF activity (fractions 20 through 24), which is enriched for soluble glucan synthesis, was also reduced for mutant SS1 relative to SS1R. The pattern of the rough colonial transformant was similar to

that of the GS-5 parent strain grown in chemically defined glucose media (Wondrack and Kuramitsu, unpublished data).

Transformation of bacteriocin production. The colonization of tooth surfaces by *S. mutans* is not only dependent upon its specific properties but may also be influenced by other factors. In this regard, several investigations have demonstrated that bacteriocins, which are produced by *S. mutans* cells and other oral organisms, may be involved in colonization (13). Therefore, it was of interest to determine whether the ability to produce bacteriocin was a transformable trait. For reasons described previously, cotransformation rather than direct selection was used to detect Bac$^+$ transformants. Mutant B211 Bac$^-$ was thus exposed to different concentrations of
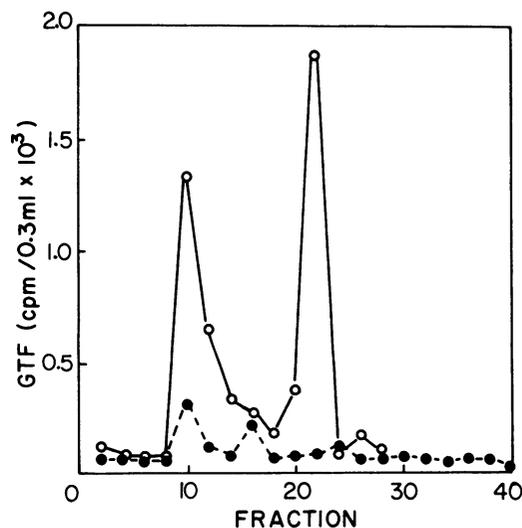

FIG. 2. Biogel A-0.5 gel filtration chromatography of the extracellular fluids of mutant SS1 (●) and transformant SS1R (rough colony) (○).

strain GS-5 Pfa^r Str^r DNA and the resulting Pfa^r and Str^r transformants were selected on the appropriate media (Fig. 3). Again, the number of transformants was directly proportional to the DNA concentration as previously demonstrated in mutant GS-5 Ilv$^-$ (Fig. 1). However, the level of competency of mutant B211 Bac$^-$ was decreased when compared to mutant GS-5 Ilv$^-$, since the number of transformants was lower at all DNA concentrations (especially the Pfa^r marker). Transformant colonies were subsequently replica plated onto TSAY and after 18 h of incubation overlaid with soft agar containing the indicator strain to determine the number that were also Bac$^+$. Among the Str^r and Pfa^r transformants, a low percentage (1 to 6%) of the Bac$^+$ colonies were detected. Double transformants (Pfa^r Bac$^+$, Str^r Bac$^+$) increased with increasing concentrations of DNA, indicating that the Bac$^+$ marker was not linked to either the Pfa^r or Str^r marker.

DISCUSSION

Our efforts to further define the optimal conditions for transformation of S. mutans GS-5 have resulted in a substantial increase in the frequency of transfer of certain markers in this strain. The improved procedure has yielded as high as 0.05% Str^r transformants, which is approximately 10-fold greater than previous observations (12). Even greater frequencies (0.4%) were obtained when the strain GS-5 Ilv$^-$ mutant was transformed to prototrophy (Fig. 1). Similar observations were made when a Trp$^+$ marker was employed (data not shown). It should be mentioned that the smooth colonial mutants and the Bac$^-$ mutant used in these studies were less competent (Table 4 and Fig. 1) than their corresponding parental strains and therefore lower frequencies of transformation were observed in these mutants. Nevertheless, it would appear that the frequencies of transformation in the S. mutans GS-5 system are comparable to other systems and should be quite suitable for studying the cariogenic properties of S. mutans.

Previous investigations showed that the rough colonial morphology and the ability to colonize smooth surfaces were transferred between strains of Streptococcus sanguis by transformation (15). However, similar experiments with DNA from a rough colonial strain of S. mutans were unsuccessful. The present studies have demonstrated that not only this property but also the ability to produce bacteriocin were transformed in strains of S. mutans. Since cariogenic transformants did not possess any selective advantage over nontransformants on the media used in these experiments, the relatively low frequencies of transformation of the smooth colonial mutants (0.007 to 0.05%) would have

made the detection of these transformants difficult. Therefore, the transformation of potential caries-associated markers was performed by determining their frequency of cotransfer with other markers which were obtainable by positive selection procedures. The double transformants observed in these studies obviously arose by random coincidence since their frequencies were dependent on DNA concentration. This approach, however, should prove useful for determining the degree of linkage between cariogenic markers and a variety of reference markers. In this regard, several additional S. mutans GS-5 auxotrophic mutants (His$^-$, Leu$^-$, and Trp$^-$), which were readily isolated, are currently being tested for frequency of cotransfer with the cariogenic markers. Ultimately, it should be possible to determine the relative position of caries-related genes on the S. mutans chromosome.

It is of interest that all of the smooth colonial mutants examined in the present investigation displayed little or no insoluble glucan synthetic activity and also lower soluble-glucan-producing activities relative to the parental GS-5 strain (Table 3). Therefore, regardless of whether these mutants were isolated after mutagenesis (SS1) or as spontaneous mutants (Sp1, Sp2, and S3 Trp$^-$), each displayed much lower extracellular GTF (soluble and insoluble activities) compared to the parental organism. These latter mutants most likely represent single-point muta-
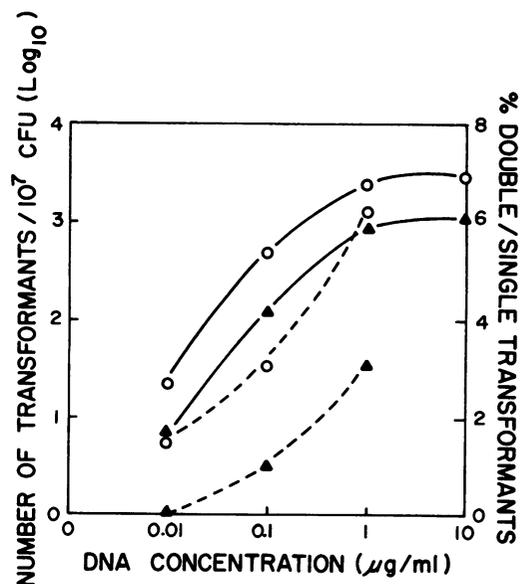

FIG. 3. Frequency of transformation of B211 Bac$^-$ as a function of strain GS-5 Pfa^r Str^r DNA concentration. Symbols; O—O, Pfa^r; ▲—▲, Str^r; O--O, Pfa^r Bac$^+$; ▲--▲, Str^r Bac$^+$.

tions in the strain GS-5 chromosome. Measurement of the cell-associated activities of these mutants indicated that the mutants also displayed reduced cell-associated GTF activities (data not shown). In contrast, another mutant of strain GS-5 has been isolated (6), which appears to synthesize somewhat elevated levels of soluble glucan and decreased insoluble glucan relative to strain GS-5. Furthermore, smooth colonial mutants of *S. mutans* 6715 (serotype g) also display elevated soluble glucan synthetic activity and a reduced capacity to synthesize insoluble glucans (1). Since recent evidence suggests that strain 6715 synthesizes two distinct GTF enzymes (2), it is likely that a defect in the enzyme responsible for synthesizing α-1,3-linked glucans (mutansynthetase) results in increased α-1,6-rich soluble glucans synthesized by the dextransucrase (EC 2.4.1.5) of the organism. No such behavior was exhibited by any of the strain GS-5 spontaneous smooth colonial mutants isolated in our laboratory. It is possible, therefore, that only a single GTF enzyme is produced by strain GS-5. Consequently, a mutation in the gene for this enzyme results in the reduction of both soluble and insoluble glucan synthesis (Fig. 2). In this regard, highly purified dextransucrase preparations from serotype c organisms have been demonstrated to synthesize insoluble glucans under aggregating conditions (11; H. Kuramitsu and L. Wondrack, in press). However, conclusive evidence for the existence of only a single GTF gene in strain GS-5 is still lacking.

An alternative explanation for the properties of the *S. mutans* GS-5 smooth colonial mutants would involve mutations in a regulatory gene. Such a gene could control the expression of putative mutansynthetase and dextransucrase activities, each of which is coded by distinct structural genes. This explanation appears less likely since the extracellular protein patterns of the smooth colonial mutants (Sp1, Sp2, SS1) and parental strain GS-5 displayed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels are identical (data not shown). Nevertheless, further biochemical and genetic analyses of these mutants will be required to determine the molecular basis for their properties. The development of a genetic mapping system for *S. mutans* GS-5, utilizing transformation, appears to be a reasonable approach toward accomplishing this goal.

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