

Inactivation of the *gbpA* Gene of *Streptococcus mutans* Increases Virulence and Promotes In Vivo Accumulation of Recombinations between the Glucosyltransferase B and C Genes

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Glucan-binding protein A (GbpA) of *Streptococcus mutans* has been hypothesized to promote sucrose-dependent adherence and the cohesiveness of plaque and therefore to contribute to caries formation. We have analyzed the adherence properties and virulence of isogenic *gbpA* mutants relative to those of wild-type *S. mutans*. Contrary to expectations, the *gbpA* mutant strains displayed enhanced sucrose-dependent adherence in vitro and enhanced cariogenicity in vivo. In vitro, *S. mutans* was grown in the presence of [³H]thymidine and sucrose within glass vials. When grown with constant rotation, significantly higher levels of *gbpA* mutant organisms than of wild type remained adherent to the vial walls. Postgrowth vortexing of rotated cultures significantly decreased adherence of wild-type organisms, whereas the adherence of *gbpA* mutant organisms was unaffected. In the gnotobiotic rat model, the *gbpA* mutant strain was hypercariogenic though the colonization levels were not significantly different from those of the wild type. The *gbpA* mutant strain became enriched in vivo with organisms that had undergone a recombination involving the *gtfB* and *gtfC* genes. The incidence of *gtfBC* recombinant organisms increased as a function of dietary sucrose availability and was inversely correlated with caries development. We propose that the absence of GbpA elevates the cariogenic potential of *S. mutans* by altering the structure of plaque. However, the hypercariogenic plaque generated by *gbpA* mutant organisms may be suboptimal for *S. mutans*, leading to the accumulation of *gtfBC* recombinants whose reduced glucosyltransferase activity restores a less cariogenic plaque structure.

The primary virulence traits of *Streptococcus mutans* are sucrose-dependent adherence, aciduricity, and acidogenicity (7). Sucrose-dependent adherence is mediated by glucans, the products of the enzymatic glucosyltransferases (GTFs) (5). GTF-I, GTF-SI, and GTF-S, encoded by the *gtfB*, *gtfC*, and *gtfD* genes, respectively, are extracellular enzymes which polymerize the glucose moieties of sucrose into glucans. The capacity of the GTFs to bind glucans is prerequisite for their enzymatic activity (18). The importance of the GTFs in cariogenicity, particularly of the products of the *gtfB* and *gtfC* genes, has been established by many laboratories (11, 25, 26).

S. mutans also synthesizes three nonenzymatic glucan-binding proteins (GBPs) whose contribution to virulence is uncertain. These are GBP₇₄, GBP₅₉, and GbpC. Although no comprehensive survey has been reported, work from several laboratories suggests that these GBPs are common to many, if not all, strains of *S. mutans* (3, 14, 16, 19). GBP₇₄ was identified by Russell (14) and was so designated based on an apparent size of 74 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). However, the deduced size of the mature protein following DNA sequencing was 59 kDa (2), which is similar to the sizes subsequently reported by Smith et al. for GBP₅₉ (19) and by Sato et al. for GbpC (16). To avoid confusion and to follow the initiative of Sato (16), we propose to designate the GBPs in the order of their discovery as GbpA (GBP₇₄), GbpB (GBP₅₉), and GbpC.

Like the GTFs, GbpA is a secreted protein found both in

association with the cell surface and in the extracellular medium. The carboxyl-terminal three-quarters of GbpA has homology to the carboxyl-terminal repeat domains of the GTFs (2). Functionally, GbpA has been proposed to contribute to *S. mutans* adherence and to the cohesiveness of plaque based on experiments which demonstrated that antisera raised against a preparation of GbpA inhibited sucrose-dependent adherence (4) and on the observation that a chemically generated *S. mutans* GbpA mutant formed a softer, more brittle plaque in vitro (15). GbpA is antigenically distinct from both GbpB and GbpC. Although immunization with GbpB has been reported to be protective against caries (20), the actual function of GbpB is unknown, and the gene encoding it has yet to be cloned. GbpC appears to be anchored to the cell wall, is partially similar to the Spa family of oral streptococcal proteins, and is involved in dextran-dependent aggregation under defined, stressful growth conditions (16). The need for the multiplicity, and potentially the balance, of the three GTFs and three GBPs in plaque formation and structure is unknown. In this report, we compare properties of wild-type (wt) *S. mutans* with those of isogenic *gbpA* mutants. We show that GbpA⁻ mutants are elevated in sucrose-dependent adherence in vitro, are hypercariogenic in vivo, and accumulate in vivo recombinations between the *gtfB* and *gtfC* genes. The data presented here are the first demonstration of a GBP affecting the virulence of *S. mutans*.

MATERIALS AND METHODS

Bacterial strains. Construction of the *gbpA* mutant strains of *S. mutans* 3209 and UA130 was achieved by allelic replacement of the *gbpA* gene with an erythromycin resistance gene flanked by the 5' and 3' portions of the *gbpA* gene as previously described (1). Southern and Western blotting analyses were used to confirm strain constructions.

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S. mutans 3209 and UA130 (both serotype c) and their respective *gbpA* isogenic mutant strains were routinely grown anaerobically at 37°C in either of the chemically defined media FMC (21) and CDM (JRH Biosciences, Lexena, Kans.). Growth on Todd-Hewitt agar (Difco Laboratories, Grand Island, N.Y.) and mitis salivarius (MS) agar (Difco Laboratories) plates was used to confirm culture purity and colony morphology, respectively. The *gbpA* mutant strains were maintained in vitro with 25 µg of erythromycin per ml.

In vitro adherence assays. Overnight cultures of *S. mutans* 3209 wt and *gbpA* mutant were diluted in FMC and grown to a cell density of 0.05 at 540 nm. One milliliter of each culture was placed in 15-by-45-mm, 3.7-ml flat-bottomed cylindrical glass vials (Fisher) to which sterile sucrose and [³H]thymidine (New England Nuclear) were added to 1% and 0.30 µCi/ml, respectively. The vials were sealed and incubated at 37°C either standing upright or rotating on a variable-angle hematological rotator at a rotation speed of 10 rpm and an angle with the vial top 30° greater than horizontal. After 5 h of incubation, the vials were either gently rinsed twice with 3 ml of phosphate-buffered saline (PBS), pH 7.2, delivered to the vial walls via peristaltic pump (2 ml/min), or vortexed (Fisher Vortex Genie 2) at top speed for 30 s prior to rinsing. Supernatants were gently decanted along the edge of a toothpick held perpendicular to the mouth of the vial. Adherence was quantified by scintillation counting.

The preparation of hydroxyapatite-coated microtiter plates has been previously described (17). Overnight cultures of rough and smooth *S. mutans* 3209 wt and *gbpA* mutant were diluted in fresh CDM and grown to a cell density of 0.35 at 540 nm. A total of 25 µl of 50% sucrose (final concentration, 5%) and 225 µl of each culture were placed in the wells of a hydroxyapatite-coated microtiter plate which was incubated at 37°C while rotating, as described above. After 3 h of incubation, the contents of the wells were aspirated and the wells were rinsed with 300 µl of PBS and stained with a crystal violet solution (Becton Dickinson, Cockeysville, Md.) for 2 min. The contents of the wells were aspirated, and the wells rinsed twice with 300 µl of PBS prior to release of retained crystal violet. Following the addition of 300 µl of Gram stain decolorizer (ethanol/acetone ratio of 3:1), the absorbance at 610 nm was measured by a plate reader. The signal which was derived from non-sucrose-containing wells was considered background and was subtracted.

Virulence testing of *S. mutans* in germfree rats. The cariogenic potentials of *S. mutans* UA130 and UA130 *gbpA* mutant were determined in gnotobiotic Fischer rats as previously described (9). Specifically, 21-day-old weanling rats were provided the caries-promoting diet 305 (5% sucrose) ad libitum (24 h/day) for 7 days. On days 1, 2, and 3 of this period, the rats were orally challenged with saturated swabs of UA130 or UA130 *gbpA* mutant (2×10^8 CFU/ml). On day 8, the rats were either switched to a 6-h/day feeding schedule or continued on the ad libitum diet. Colonization was assessed on days 8, 16, and 36 by culturing oral swabs on MS and blood agar plates. The rats were sacrificed on day 36, and plaque microbiology (10) and caries scores were determined by the method of Keyes (6). Colony morphology of postmortem cultures from infected animals was assessed by plating cultures on MS agar with erythromycin where appropriate.

Determination of GTF activity. To visualize GTF activity of *S. mutans*, cell-associated proteins were resolved by SDS-PAGE followed by incubation with Triton X-100 (Fisher Biotech) and sucrose (13). Specifically, overnight CDM broth cultures of UA130 and *gbpA* mutant were diluted into 20 ml of fresh CDM, grown to an optical density at 540 nm of 1.0, and harvested by centrifugation. Cells were resuspended in 150 µl of 2× cracking buffer (0.038 M Tris HCl [pH 6.8], 1% SDS, 2.5% β-mercaptoethanol, 15% glycerol) and incubated at 37°C for 1 h. Twenty microliters of cell-free supernatant was resolved by SDS-PAGE. Following electrophoresis, SDS was eluted from the gel by two 30-min washes with PBS-2% Triton X-100. In situ GTF activity was visualized by incubation of the gel for 16 h in PBS (pH 6.5) containing 1% sucrose and 0.05% 11,000-molecular-weight dextran (Sigma). The gels were briefly rinsed with PBS and incubated in a 45% methanol-10% acetic acid solution for 10 min to enhance the glucan bands. Gels were photographed against a black background.

To quantify GTF activity, cell-associated proteins from rough and smooth colonies of UA130 wt were resolved by SDS-PAGE. The 84-kDa molecular mass standard was used to separate the gel into the top half, which was developed for GTF activity as described above, and the bottom half, which was analyzed for GbpA content by Western blotting. Scanning densitometry was used to quantify the glucan and GbpA signals, and GTF activity was normalized to GbpA content.

Isolation of chromosomal DNA. *S. mutans* chromosomal DNA was isolated by a modification of the method of Marmur (8). Specifically, 20 ml of *S. mutans* overnight broth culture was harvested by centrifugation, resuspended in 100 µl of 10 mM Tris HCl (pH 8) containing 5 mg of lysozyme and 50 U of mutanolysin, and incubated with agitation at 37°C for 1 h. Proteinase K (200 µg) was added, and incubation was continued for 1 h. Cell lysis was achieved by the addition of SDS to 2.5% and gentle mixing by inversion. The lysed-cell solution was phenol extracted, and the aqueous phase was subjected to four rounds of organic-phase extraction with phenol-chloroform (1:1), phenol-chloroform-isoamyl alcohol (125:24:1), chloroform-isoamyl alcohol (24:1), and finally chloroform. Two volumes of 95% ethanol was added to the aqueous phase, mixed by gentle inversion, and incubated overnight at -20°C. Nucleic acids were gently pelleted by brief (25-s) centrifugation (5,800 × g), rinsed with 70% ethanol, and resuspended in 300 µl of sterile 10 mM Tris HCl (pH 8) containing 2 µg of RNase A.

Long-range PCR. Amplification of the *gtfB-gtfC* region was achieved with the primers *gtfB*.Prom.F (5'-GGCTTGTGCTGGAATCAATGC-3') and *gtfC*-R

TABLE 1. In vitro sucrose-dependent adherence of *S. mutans*^a

Genotype	Adherent cpm for culture			
	Rotating		Stationary	
	Rinsed	Vortexed	Rinsed	Vortexed
<i>gbpA</i> mutant	4,882 ± 584	4,867 ± 1,009	15,013 ± 1,659	2,995 ± 329
wt	3,151 ^b ± 292	2,031 ^b ± 545	13,804 ± 1,598	3,233 ± 487

^a *S. mutans* 3209 cultures, either wt or *gbpA* mutant, were allowed to grow, stationary or while rotating, for 5 h in the presence of sucrose and [³H]thymidine, after which the vials were either rinsed or vortexed prior to rinsing. Adherent bacteria were quantified by scintillation counting and reported here as counts per minute ± standard deviations. Boldface numbers are statistically higher ($P < 0.05$) than others in the same column ($n = 9$).

^b Numbers bearing the superscript *b* are significantly different ($P < 0.05$) from each other.

(5'-TTCTTCTTTGAAAACGGGTACG-3') (Life Technologies) with the Boehringer Mannheim Expand Long Template PCR system as per the manufacturer's instructions with a slight modification. Specifically, the use of buffer 3 and Hot-Start tubes (Molecular Bio-Products) increased yield and specificity. Amplification conditions were 3 min at 94°C followed by 10 cycles of 94°C for 30 s, 55°C for 90 s, and 68°C for 8 min, followed by 20 cycles of 94°C for 30 s, 55°C for 90 s, and 68°C for 8 min 20 s plus 20 s/cycle, followed by a final extension at 68°C for 7 min. The MJ Research MiniCycler thermal cycler was used for all amplification reactions. PCR products were resolved by gel electrophoresis through 0.7% agarose and visualized by ethidium bromide staining.

Statistical analysis. All numerical data presented here are expressed as the means ± the standard deviations. Data analysis and determination of significance were performed by the unpaired, two-tailed Student *t* test or, if appropriate, the unpaired, two-tailed nonparametric Mann-Whitney test. Differences were considered significant when a value of $P \leq 0.05$ was obtained.

RESULTS

In vitro adherence. To ensure that growth rate differences would not be an additional factor in the interpretation of in vitro adherence assay data, [³H]thymidine uptake and culture optical density increase of wt and *gbpA* mutant *S. mutans* 3209 were measured. No differences in growth rate were found under any of the culture conditions employed in the in vitro adherence assays.

To investigate the contribution of GBP to sucrose-dependent adherence, cultures of *S. mutans* 3209, either wt or *gbpA* mutant, were allowed to grow in a stationary, upright position or with rotation for 5 h in the presence of sucrose and [³H]thymidine, after which the unincorporated label and nonadherent bacteria were removed. The plaque produced by the stationary cultures was heavily deposited on the bottom of the vials, whereas the plaque produced by the cultures grown with rotation was evenly dispersed over the bottom and the wall of the vials and covered four times more surface area than did the stationary-growth plaques. Visual inspection of the vials prior to the addition of scintillation cocktail revealed that markedly higher levels of adherent *gbpA* mutant plaque than of wt plaque were retained in the rotated cultures. Adherent bacteria were quantified by scintillation counting, and the results are shown in Table 1. The effect of GbpA on sucrose-dependent adherence was clearly seen in the rotated cultures. No effect was apparent in the stationary cultures. Among the rotated cultures, the adherent counts per minute were significantly higher for the *gbpA* mutant group ($P < 0.05$). These differences are significant at the 95% confidence interval according to the Student *t* test. Also as shown in Table 1, the adherence of rotated wt *S. mutans* decreased significantly in response to the increased mechanical agitation of vortexing whereas the adherence of rotated *gbpA* mutant *S. mutans* was unaffected,

suggesting that plaque developed in the absence of GbpA may be structurally different from wt *S. mutans* plaque.

Virulence in germfree rats. Table 2 contains the pooled data from two separate determinations of the cariogenicity of *S. mutans* UA130 wt and *gbpA* mutant in the gnotobiotic rat model. The levels of colonization of both the wt and *gbpA* mutant groups were significantly higher in the animals fed ad libitum (24 h) than in the animals on a 6-h feeding schedule. GbpA plays no apparent role in colonization as evident by the similar colonization levels of the wt and *gbpA* mutant groups within each dietary regimen.

It had been predicted that GbpA was a virulence factor whose inactivation would result in a reduction of caries. In contrast, Table 2 shows that under a restricted feeding regimen the *gbpA* mutant strain had an increased virulence that reached statistical significance on the buccal surfaces. The sulcal caries scores tended to be higher for the *gbpA* mutant strain, but these differences were not statistically significant according to the Student *t* test. With the exception of enamel involvement of the proximal surfaces, in which the caries score of the *gbpA* mutant group was significantly lower than that of the wt, no significant differences in cariogenicity were observed when the animals had unrestricted access to the caries-promoting diet. A likely explanation for the different results obtained from the restricted versus the unrestricted diets will be addressed below.

Postinfection colony morphology of *S. mutans*. At the termination of the cariogenicity experiment, oral flora of the rats was plated on MS agar for enumeration. Examination of colony morphology revealed that a significant portion of the colonies recovered from the *gbpA* mutant-infected rats displayed a smooth morphology (Fig. 1), whereas the incidence of recoverable smooth colonies from the wt-infected rats was less than 1%. Retrospective analysis of the wt and *gbpA* mutant cultures from which the initial rat inocula were generated revealed negligible levels of smooth colonies in either population, suggesting that the accumulation of the smooth colonies was an in vivo event. To confirm this hypothesis, the cariogenicity experiment was repeated with inocula generated from a single isolated rough colony of either UA130 *gbpA* mutant or wt. Examination of postinfection colony morphology again revealed that smooth colonies accumulated in vivo, specifically in the *gbpA* mutant populations. This phenomenon was observed in both the restricted and the unrestricted feeding groups, and the differences between wt and *gbpA* mutant smooth colony frequencies were statistically significant (Table 2). Repeated streaking of these smooth colonies on MS agar revealed that the smooth phenotype was stable, with no smooth-to-rough reversions observed.

Molecular basis of the smooth colony phenotype. We compared the relative contributions of GbpA absence and the smooth phenotype to sucrose-dependent adherence in a modification of the hydroxyapatite-coated microtiter plate adherence assay described by Schilling et al. (17). As was found with adherence to glass (Table 1, data for rinsed, rotated group), the rough wt strain displayed 68% of the adherence of the rough *gbpA* mutant strain. No significant differences in adherence between the smooth *gbpA* mutant strain and the smooth wt strain were found, suggesting that the effect of GbpA absence is dependent upon the presence of the rough phenotype. The smooth strains of *S. mutans* wt and *gbpA* mutant were reduced in sucrose-dependent adherence by 72 and 78%, respectively, relative to their rough counterparts.

Investigation by quantitative activity gel electrophoresis revealed smooth colony variants to have 15 to 20% of the GTF activity of their rough counterparts. This decrease in enzyme activity correlated with a specific decrease of GTF protein

TABLE 2. Virulence of *S. mutans* UA130 wt and *gbpA* mutant in monoinfected germfree rats^a

Diet and genotype	Cell recovery ^c		Mean caries score \pm SD ^b											
	CFU/ml	% Smooth ^d	Buccal				Sulcal				Proximal			
			E	Ds	Dm	Dx	E	Ds	Dm	Dx	E	Ds	Dm	Dx
6 h														
<i>gbpA</i> mutant	$1.3 \times 10^6 \pm 2.7 \times 10^6$	14.9 \pm 12.9	16.08 \pm 2.7	12.50 \pm 2.1	8.83 \pm 2.2	6.08 \pm 1.5	20.17 \pm 3.1	17.50 \pm 2.7	10.33 \pm 2.7	5.67 \pm 3.1	4.17 \pm 3.2	1.50 \pm 2.0	0	0
wt	$5.4 \times 10^6 \pm 1.6 \times 10^7$	0.3 \pm 0.9	13.42 \pm 1.4	10.33 \pm 1.4	6.58 \pm 1.2	5.33 \pm 0.9	19.50 \pm 2.1	15.08 \pm 2.7	9.92 \pm 2.2	4.00 \pm 2.3	4.50 \pm 2.4	1.63 \pm 1.7	0	0
24 h														
<i>gbpA</i> mutant	$1.6 \times 10^9 \pm 2.2 \times 10^9$	31.6 \pm 17.7	16.27 \pm 1.7	12.73 \pm 1.8	8.91 \pm 1.9	5.73 \pm 2.2	19.72 \pm 1.6	16.36 \pm 1.9	10.73 \pm 1.9	6.73 \pm 1.3	4.18 \pm 1.4	1.50 \pm 1.4	0.50 \pm 0.8	0
wt	$9.6 \times 10^8 \pm 1.4 \times 10^9$	<0.1	15.64 \pm 1.2	13.09 \pm 1.3	9.73 \pm 1.5	6.73 \pm 1.5	21.09 \pm 2.0	17.18 \pm 2.2	10.82 \pm 2.6	6.73 \pm 1.9	6.36 \pm 1.5	2.57 \pm 1.4	0.17 \pm 0.4	0

^a For each experiment, groups of six rats were tested for each strain within each diet group (animals fed for 6 h [6 h] or ad libitum [24 h]). Data shown here are the culmination of two separate experiments. Boldface numbers are statistically higher ($P < 0.05$) than those of the isogenic counterpart in the same diet group.

^b Caries scores as determined by the method of Keyes (6) indicate the relative depths of the carious lesions and the relative areas of the molar surfaces affected by the lesions (numerical score). Abbreviations: E, enamel involvement; Ds, slight dental involvement; Dm, moderate dental involvement; Dx, extensive dental involvement.

^c *S. mutans* recovered from rat molar surfaces prior to caries scoring.

^d Percentage of *S. mutans* organisms recovered from rat molar surfaces displaying a smooth morphology on MS agar.

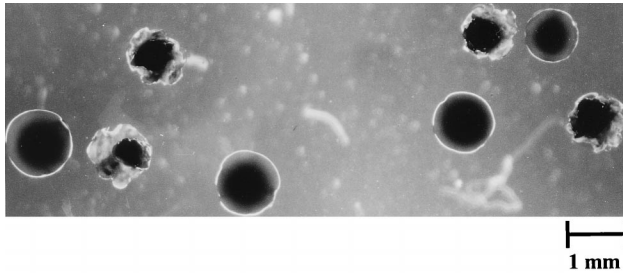


FIG. 1. Colony morphology of *gbpA* mutant *S. mutans* UA130 recovered 36 days postinfection from monoinfected gnotobiotic rats fed ad libitum.

levels as revealed by Coomassie blue staining of SDS-PAGE gels (data not shown). Figure 2 shows the GTF activity of *gbpA* mutant *S. mutans* UA130 rough and smooth colonies. Two bands of GTF activity (GtfB and GtfC) were found in cell-associated proteins of rough colonies, whereas one GTF activity band was found in smooth colony cell-associated protein preparations. Because the enzymatic activity of GtfB produces only insoluble glucan, whereas the activity of GtfC produces both soluble and insoluble glucan (23), the GtfC glucan band is fainter than that of GtfB. No differences in fructosyltransferase activity were observed between rough and smooth colonies. To date, all smooth colony variants of a given strain appear to possess similar levels of GTF activity.

Kuramitsu and colleagues have reported that smooth, colonization-defective colonies arise *in vitro* at a frequency of 1×10^{-3} to 3×10^{-3} as the result of recombination events involving the highly homologous, tandemly arranged *gtfB* and *gtfC* genes (12, 22, 25). On the basis of these reports, we examined the *gtfBC* region of our rough and smooth colonies by long-range PCR. Specific primers were designed to hybridize upstream of *gtfB* and directly downstream of *gtfC* with a predicted PCR product of 10 kb from an intact *gtfB-gtfC* template. Primer design was based on the published sequence of the *gtfB* and *gtfC* genes of *S. mutans* GS-5 (18, 23). Two PCR products, one of 10 and one of 5.3 kb, were amplified from chromosomal DNA isolated from rough colonies, whereas one product of 5.3 kb was amplified from the chromosomal DNA of smooth colonies (Fig. 3). Increasing the annealing temperature did not reduce the yield of the 5.3-kb product, suggesting that the 5.3-kb product was not the result of false priming. Both the 10- and the 5.3-kb products hybridized with a *gtfB*-derived probe when subjected to Southern hybridization analysis (data not shown). Southern analysis of *SphI*-digested chromosomal DNAs probed with a *gtfB*-derived probe revealed a 5.8-kb band in DNA from smooth colonies and a 10.8-kb band present in DNA from rough colonies (data not shown). From these results, we con-

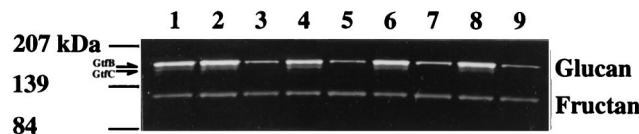


FIG. 2. GTF activities of *gbpA* mutant *S. mutans* UA130 rough and smooth colonies. Equivalent levels of cell-associated proteins were resolved by SDS-PAGE and incubated at 37°C in PBS, pH 6.5, containing sucrose and Triton X-100. Gels were photographed against a black background. Positions of SDS-PAGE molecular mass standards and GTF activities are indicated in the left margin. Positions of glucan and fructan are indicated in the right margin. Lane 1, preinoculum UA130. Lanes 2 to 9, colonies recovered from four rats (A2 to A5) 36 days postinoculation; 2, A2-1 rough; 3, A2-1 smooth; 4, A3-1 rough; 5, A3-1 smooth; 6, A4-1 rough; 7, A4-1 smooth; 8, A5-1 rough; 9, A5-1 smooth.

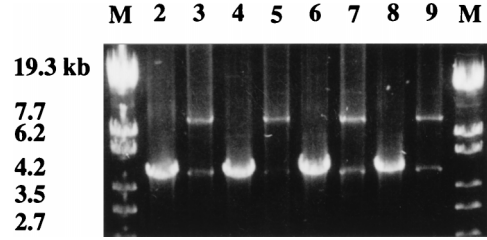


FIG. 3. Amplification of the *gtfB-gtfC* region of *gbpA* mutant *S. mutans* UA130 rough and smooth colonies. Long-range PCR and electrophoresis were carried out as described in the text. The sizes (in kilobases) of *StyI*-digested lambda DNA (lanes M) are indicated in the left margin. Lanes 2 to 9, colonies recovered from four rats (A2 to A5) 36 days postinoculation; 2, A2-1 smooth; 3, A2-1 rough; 4, A3-1 smooth; 5, A3-1 rough; 6, A4-1 smooth; 7, A4-1 rough; 8, A5-1 smooth; 9, A5-1 rough.

cluded that the 10-kb product was amplified from an intact *gtfB-gtfC* template and the 5.3-kb product was amplified from the recombinant fusion gene *gtfBC*. With 0.1 to 0.3% of the *S. mutans* population being *gtfBC* recombinant (12), it seemed logical that, with the inherent preferential amplification of smaller PCR products, the 5.3-kb PCR product would be found even in a population arising from a single isolated rough colony. Significantly, the 10-kb product has not been amplified from the chromosomal DNA of smooth colonies even under the least stringent conditions tested. All smooth colony isolates to date have displayed the reduced GTF phenotype and the recombinant *gtfBC* genotype.

Ueda and Kuramitsu have reported that, within *Escherichia coli*, recombination involving plasmid-borne *gtfB* and *gtfC* genes was mediated by homology (RecA dependent) and yielded *gtfBC* gene fusions with a variety of recombination sites (22). Preliminary work in our laboratory suggests that several independently isolated *gtfBC* fusion genes from various *S. mutans* strains and genetic backgrounds (including *recA*) are homogeneous.

***gtfBC* recombination and the absence of GbpA have opposing effects.** The caries data in Table 2 suggested that the absence of GbpA promoted caries only when sucrose intake was restricted. Since *gtfBC* recombinant organisms have been shown to be less cariogenic (25), it seemed likely that the absence of GbpA potentiated caries development in both diets but that this effect was mitigated in the 24-h diet by the increased frequency of *gtfBC* recombinants. To investigate whether a high incidence of *gtfBC* recombination could suppress the cariogenic potential of *gbpA* mutant organisms, we reexamined the data from *gbpA* mutant-infected rats on the restricted diet. Reevaluation of this data set was possible because the incidence of recombination ranged from less than 1% to 35%. By segregating the data at the mean level of recombination for this data set (14.9% [Table 2]), it was possible to examine the 6-h *gbpA* mutant data set as a subgroup with high levels (mean, 22.33%) and a subgroup with low levels (mean, 0.17%) of recombination. A similar dissection of the data from the unrestricted diet groups was not possible because too few of the *gbpA* mutant groups had low levels of *gtfBC* recombination. Table 3 shows the caries scores of restricted-diet-fed rats infected with UA130 wt, highly recombinant *gbpA* mutant, and *gbpA* mutant with a wt level of recombination. No significant differences in colonization were found among these groups. In the absence of significant levels of *gtfBC* recombination, *gbpA* mutant *S. mutans* was significantly more cariogenic than wt *S. mutans* on buccal and sulcal surfaces. As expected, a high incidence of *gtfBC* recombinants within the *gbpA* mutant group

TABLE 3. Virulence of *S. mutans* UA130 as a function of GbpA and *gtfBC* recombinant incidence^a

Strain	Cell recovery ^c (mean ± SD)		Mean caries score ± SD ^b											
	% Smooth	CFU/ml	Buccal				Sulcal				Proximal			
			E	Ds	Dm	Dx	E	Ds	Dm	Dx	E	Ds	Dm	Dx
wt	0.3 ± 0.9	5.4 × 10 ⁶ ± 1.6 × 10 ⁷	13.42 ± 1.4	10.33 ± 1.4	6.58 ^d ± 1.2	5.33 ± 0.9	19.50 ± 2.1	15.08 ± 2.7	9.92 ± 2.2	4.00 ± 2.3	4.50 ^e ± 2.4	1.63 ± 1.7	0	0
<i>gfpA</i> mutant														
High <i>gtfBC</i>	22.33 ± 8.3	5.7 × 10 ⁵ ± 2.9 × 10 ⁵	14.50 ± 2.1	11.17 ± 1.5	7.83 ± 1.9	5.83 ± 0.8	17.83 ± 2.6	15.67 ± 2.4	8.67 ^d ± 2.6	3.33 ± 1.9	1.67 ^{d,e} ± 2.6	0	0	0
Low <i>gtfBC</i>	0.17 ± 0.3	4.0 × 10 ⁶ ± 5.0 × 10 ⁶	17.67 ± 2.3	13.83 ± 1.7	9.83 ^d ± 2.1	6.3 ± 2.0	22.50 ± 0.8	19.33 ± 1.3	12.00 ^d ± 1.5	8.00 ± 2.1	6.67 ^d ± 1.0	0	0	0

^a Data from the *S. mutans* UA130 wt- and *gfpA* mutant-infected rats from the restricted diet group. Data from the *gfpA* mutant-infected rats were divided at the mean level of recombination (14.9%) into a group with a high proportion of *gtfBC* recombinants (High *gtfBC*) (*n* = 6) and a group with a low incidence of recombinants (Low *gtfBC*) (*n* = 6). Boldface numbers are statistically higher (*P* < 0.05) than all others in the same column.

^b Caries scores as determined by the method of Keyes (6) indicate the relative depths of the carious lesions and the relative areas of the molar surfaces affected by the lesions (numerical score). Abbreviations: E, enamel involvement; Ds, slight dental involvement; Dm, moderate dental involvement; Dx, extensive dental involvement.

^c *S. mutans* organisms recovered from rat molar surfaces prior to caries scoring.

^d Scores within the same column bearing the superscript *d* are statistically different from each other (*P* < 0.05).

^e Scores within the same column bearing the superscript *e* are statistically different from each other (*P* < 0.05).

reduced cariogenicity to near-wt levels. With the exception of enamel involvement of the proximal surfaces, the caries scores for the wt and *gfpA* mutant highly recombinant groups are not statistically different. It appears that the absence of GbpA increases cariogenicity and that *gfpA* mutant plaque accumulates *gtfBC* recombinant organisms which have reduced cariogenic potential.

DISCUSSION

The nonenzymatic GBPs of *S. mutans* have been shown to have properties presumed to be associated with cariogenicity; however, there has been no experimental verification of a role in virulence. To our knowledge, this report is the first direct demonstration of a role for a nonenzymatic GBP in the cariogenicity of *S. mutans*.

Based on the fact that the C terminus of GbpA bears homology to the C termini of the GTFs and shares the capacity to bind glucan, it has been hypothesized that GbpA may promote sucrose-dependent adherence and the cohesiveness of plaque and therefore contribute to caries formation. Several reports have provided evidence for these hypotheses. Anti-GbpA serum was found to reduce sucrose-dependent adherence of *S. mutans* (4), and a chemically generated GbpA mutant which displayed a smooth phenotype was reduced in sucrose-dependent adherence (15). On the basis of these observations, it was expected that a *gfpA* mutant strain of *S. mutans* would be reduced in cariogenicity. Surprisingly, our data show that the absence of GbpA enhanced virulence.

We propose that the enhanced virulence of *gfpA* mutant *S. mutans* results from a change in plaque structure. Several observations from this and other laboratories are consistent with this hypothesis. It has previously been observed that the sucrose-induced aggregation of a *gfpA* mutant results in a fluffier, less densely packed plaque (1). Preliminary results suggest that overexpression of GbpA in a *gtfBC* recombinant background restores the rough colony morphology phenotype (unpublished results). These observations suggest that GbpA contributes to the development of cohesive, densely packed plaque. The observation that the sucrose-dependent adherence of rotationally grown wt *S. mutans* is susceptible to the increased mechanical stress of vortexing whereas the adherence of rotationally grown *gfpA* mutant *S. mutans* is resistant (Table 1) suggests that GbpA affects the mechanical integrity of plaque. We believe that wt organisms were more susceptible to the shearing forces of rotation and vortexing due to the formation of larger and more cohesive aggregates and that organisms associated with a less cohesive *gfpA* mutant plaque were not as prone to dislodgment by these shearing forces, because smaller, less cohesive aggregates could break away without affecting adjacent bacteria. The fact that the *gfpA* mutant strain colonized the rat molars to a similar degree as the wt but was more cariogenic (Tables 2 and 3) suggests that the *gfpA* mutant strain is hypercariogenic. We have found no differences in the acidogenicities of *gfpA* mutant and wt *S. mutans* in batch culture, suggesting that it is the *gfpA* mutant plaque and not the *gfpA* mutant cell that imparts hypercariogenicity.

On the basis of these data, we believe that GbpA contributes to the cell density and cohesiveness of *S. mutans* plaque. We suspect that the hypercariogenicity of *gfpA* mutant plaque is a result of greater plaque porosity which facilitates more rapid and sustainable nutrient influx to deeper levels of the plaque biofilm, allowing for sustained generation of an acidic, cariogenic microenvironment. Work by Van Houte et al. (24) has shown both in vitro and in vivo that a decrease in *S. mutans* cell

density resulted in an increased porosity which was associated with an increase in the acidogenicity and demineralization capacity of the *S. mutans* cell mass. Assays to directly measure the acidogenic potential of wt and *gbpA* mutant plaque are being carried out.

Perhaps the most striking result presented in this work was the propensity of *gbpA* mutant plaque to become enriched in vivo with organisms which had undergone recombination of the *gtfB* and *gtfC* genes. For us to be absolutely confident of this result, the cariogenicity studies were repeated with inocula specifically generated from single isolated rough (nonrecombinant) colonies. Both trials of the animal studies revealed an enrichment of *gtfBC* recombinant organisms specifically in *gbpA* mutant *S. mutans*. The increased incidence of recombinant organisms recovered from *gbpA* mutant plaque could have resulted from either an increased frequency of recombination or a selection for recombinant organisms. Analysis of the recombination frequencies of *recA* mutant, *gbpA* mutant, and wt *S. mutans* in batch culture and in a plaque model is in progress. In either case, the *gbpA* mutant plaque favored the accumulation of *gtfBC* organisms whereas the incidence of recombinant organisms recovered from the wt plaque was in agreement with the previously reported in vitro spontaneous recombination frequency of 0.1 to 0.3% (12, 22). The present report is the first to describe an in vivo accumulation of the virulence-attenuating recombinations involving the *gtfB* and *gtfC* genes. Although at this time we cannot rule out other unknown functions of GbpA, whose absence may have favored the accumulation of recombinant organisms, we hypothesize that recombinant organisms accumulated in response to the altered plaque deposited by *gbpA* mutant *S. mutans*. Perhaps the hypercariogenic conditions of the *gbpA* mutant plaque (putatively a sustained lower pH) were also detrimental for the growth and metabolism of *S. mutans* such that organisms with reduced association with the *gbpA* mutant plaque were selected for. Alternately, an optimal balance between glucan and GBPs may exist, such that loss of a GBP favors accumulation of organisms producing reduced levels of glucan. Obviously, many questions about the causes, mechanisms, and effects of recombinant organism generation-selection remain, yet the intriguing fact that *S. mutans gbpA* mutant populations containing significant proportions of recoverable recombinant organisms were not decreased in colonization (Tables 2 and 3) suggests the possibility that recombinant organisms may be more than molecular mistakes and may in fact fill an ecological niche in *S. mutans* colonization.

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