Inactivation of the *gbpA* Gene of *Streptococcus mutans* Alters Structural and Functional Aspects of Plaque Biofilm Which Are Compensated by Recombination of the *gtfB* and *gtfC* Genes

KARSTEN R. O. HAZLETT,† JOSEPH E. MAZURKIEWICZ, AND JEFFREY A. BANAS*

*Department of Microbiology, Immunology, and Molecular Genetics,
Albany Medical College, Albany, New York 12208*

Inactivation of the *gbpA* gene of *Streptococcus mutans* increases virulence in a gnotobiotic rat model and also promotes in vivo accumulation of organisms in which *gtfB* and *gtfC* have recombined to reduce virulence (K. R. O. Hazlett, S. M. Michalek, and J. A. Banas, Infect. Immun. 66:2180–2185, 1998). These changes in virulence were hypothesized to result from changes in plaque structure. We have utilized an in vitro plaque model to test the hypothesis that the absence of GbpA alters *S. mutans* plaque structure and that the presence of *gtfBC* recombinant organisms within a *gbpA* background restores a wild-type (wt)-like plaque structure. When grown in the presence of sucrose within hydroxyapatite-coated wells, the wt *S. mutans* plaque consisted primarily of large aggregates which did not completely coat the hydroxyapatite surface, whereas the *gbpA* mutant plaque consisted of a uniform layer of smaller aggregates which almost entirely coated the hydroxyapatite. If 25% of the *gbpA* mutants used as inoculum were also *gtfBC* recombinants (*gbpA/25%gtfBC*), a wt-like plaque was formed. These changes in plaque structure correlated with differences in susceptibility to ampicillin; *gbpA* plaque organisms were more susceptible than organisms in either the wt or *gbpA/25%gtfBC* plaques. These data allow the conclusion that GbpA contributes to *S. mutans* plaque biofilm development. Since the changes in plaque structure detailed in this report correlate well with previously observed changes in virulence, it seems likely that *S. mutans* biofilm structure influences virulence. A potential model for this influence, which can account for the *gtfBC* recombination compensating *gbpA* inactivation, is that the ratio of glucan to glucan-binding protein is a critical factor in plaque development.

Streptococcus mutans is the primary etiologic agent of smooth-surface tooth decay in humans (14). The primary virulence traits of *S. mutans* are sucrose-dependent adherence, aciduricity, and acidogenicity (14). Sucrose-dependent adherence is mediated by glucans, the products of the extracellular glucosyltransferase (GTF) enzymes, which have glucan-binding properties (8). GTF-I, GTF-SI, and GTF-S, encoded by the *gtfB*, *gtfC*, and *gtfD* genes, respectively, polymerize the glucose moieties of sucrose into glucans. The importance of the GTFs, particularly the products of the *gtfB* and *gtfC* genes, in cariogenicity has been established by many labs (16, 26, 27). Ueda and Kuramitsu found that within *S. mutans* the tandemly arranged, highly homologous *gtfB* and *gtfC* genes could spontaneously recombine at a frequency of 10^{-3} to form a single hybrid *gtfBC* gene (24). This recombination resulted in a dramatic decrease in the synthesis of water-insoluble glucan (24) and a reduction in virulence (26). When the intact *gtfB* and *gtfC* genes were cloned in *Escherichia coli*, it was found that recombination of these genes was RecA dependent and resulted in recombinant *gtfBC* genes with markedly dissimilar sites of recombination (24). It was later reported that *gtfBC* recombination within *S. mutans* was not RecA dependent and that a variety of in vivo-generated *gtfBC* recombinants had similar sites of recombination (11).

S. mutans also synthesizes three glucan-binding proteins with no known enzymatic activities, GbpA, GbpB, and GbpC, whose contributions to virulence, or properties associated with virulence, have begun to be explored. GbpC appears to be anchored to the cell wall, is partially similar to members of the Spa family of oral streptococcal proteins, and is involved in rapid dextran-dependent aggregation under defined, stressful growth conditions (19). The contribution of GbpC to virulence has yet to be documented. Immunization with GbpB has been reported to be caries protective (23), although the actual function of GbpB is unknown and the gene encoding it has yet to be cloned.

Like the GTFs, GbpA is a secreted protein found in association with the cell surface and in the extracellular medium. The carboxyl-terminal three-quarters of GbpA, which has homology to the carboxyl-terminal repeat domains of the GTFs (1), mediates binding to α-1,6 glucosidic linkages (10, 18) present in water-soluble and, to a lesser extent, water-insoluble glucans and undergoes a conformational shift upon binding to dextran (9). Analysis of *gbpA* transcriptional regulation, as determined with a *gbpA::cat* reporter construct, indicated that *gbpA* was maximally expressed under anaerobic and neutral-pH conditions but that sucrose did not induce *gbpA* expression (3).

Previously we utilized wild-type (wt) and *gbpA*-isogenic strains to test the hypothesis that GbpA contributes to the virulence of *S. mutans* (11). Contrary to expectations, the *gbpA* strain was hypercariogenic in the gnotobiotic rat model. Since the colonization levels of *gbpA* mutant and wt *S. mutans* were not significantly different, the increased virulence of the *gbpA* strain was not due to an increase in the number of adherent organisms. In vitro, the *gbpA* mutant plaque was more resistant to mechanical stress than that of the wt, suggesting that they were structurally different (11). On the basis of these findings, we hypothesized that *gbpA S. mutans* was hypercariogenic due to an altered plaque structure which modified either acid pro-

---

* Corresponding author. Mailing address: Department of Microbiology, Immunology, and Molecular Genetics, Albany Medical College, Albany, NY 12208. Phone: (518) 262-6286. Fax: (518) 262-5748. E-mail: Jeff_Banas@ccgateway.amc.edu.
† Present address: Center for Microbial Pathogenesis, University of Connecticut Health Center, Farmington, CT 06032.

duction or diffusion. After 35 days in vivo, the *gbpA* strain had become enriched to various degrees with organisms with reduced GTF activity due to recombination involving the highly homologous, contiguous *gtfB* and *gtfC* genes. The incidence of *gtfBC* recombinant organisms within the *gbpA* background was inversely correlated with caries development, such that *gbpA* *S. mutans* containing 22.33% *gtfBC* recombinant organisms was not significantly different from wt *S. mutans* in cariogenicity or colonization levels. These results suggested the possibility that the reduced GTF activity of *gtfBC* recombinant organisms restored a less cariogenic, more wt-like plaque structure.

The purpose of the present work was to test the hypothesis that the absence of GbpA alters *S. mutans* plaque structure and that the presence of *gtfBC* recombinant organisms within a *gbpA* background restores a wt-like plaque structure. We show that the loss of GbpA dramatically alters the structure of *S. mutans* plaque biofilm and that *gtfBC* recombination within a *gbpA* background partially restores a wt-like plaque structure. We also show that these changes in plaque structure both have functional consequences and correlate with changes in virulence. Our findings have implications both for further understanding *S. mutans* cariogenicity and for the study of biofilms. To our knowledge, this work, in conjunction with our previous findings, represents the first experimental evidence that changes in biofilm structure influence virulence.

(Part of this work was conducted by K. R. O. Hazlett in partial fulfillment of the requirements for a Ph.D. from Albany Medical College, Albany, N.Y.)

MATERIALS AND METHODS

Bacteria and their cultivation. Construction of the *gbpA* strains of *S. mutans* UA130 (serotype c) has been described previously (2). When grown on mitis salivarius (MS) agar, nonrecombinant (GTF-wt) *S. mutans* produces rough colonies whereas *gtfBC* recombinant *S. mutans* produces smooth colonies. The laboratory *gbpA gtfBC* strain used in this work was a spontaneous mutant isolated by streaking *S. mutans* UA130 *gbpA* on MS agar (Difco Laboratories, Grand Island, N.Y.) plates and picking a smooth colony. This isolate was phenotypically indistinguishable from *gtfBC* recombinant organisms previously recovered from *gbpA* mutant-infected rats. PCR amplification of the *gtfB-gtfC* region (11) confirmed that this isolate was a *gtfBC* recombinant. The clinical *gbpA gtfBC* strains were recovered from *gbpA S. mutans*-infected gnotobiotic rats (11). Broth cultures of *gbpA gtfBC* strains were individually mixed with broth cultures of the nonrecombinant *gbpA* strain such that the resulting mixture (termed *gbpA/25%gtfBC*) contained 25% *gtfBC* recombinant organisms. These mixtures were immediately used to generate frozen glycerol stocks. By plating the glycerol stocks of the *gbpA/25%gtfBC* mixtures on MS agar and enumerating the smooth and rough colonies, we confirmed that inoculation broths generated from the glycerol stocks contained the appropriate ratio of recombinants and nonrecombinants. This ratio of nonrecombinants to recombinants was used to mimic the previously observed level of in vivo accumulation of *gtfBC* organisms by *gbpA S. mutans* (11). Glycerol stocks of *S. mutans* UA130 wt, *gbpA*, and the *gbpA/25%gtfBC* mixtures were stored at −70°C and used to generate overnight chemically defined medium (CDM) broth cultures. *S. mutans* strains were routinely grown anaerobically at 37°C in CDM (JRH Biosciences, Lenexa, Kansas). Growth on Todd-Hewitt (Difco Laboratories) and MS agar plates was used to confirm culture purity and colony morphology, respectively. The *gbpA* and *gbpA/25%gtfBC* strains were maintained in vitro with erythromycin at 25 μg/ml.

In vitro *S. mutans* plaques. The method described by Schilling et al. (20) was used in the preparation of hydroxyapatite-coated plates. Briefly, 333 μl (96-well plates [product no. 25860; Corning, Corning, N.Y.] and 16-well Nunc Lab-Tek Chamber slides [Fisher Scientific, Pittsburgh, Pa.]) or 2.5 ml (24-well plates [product no. 3524; Costar, Cambridge, Mass.]) of a 2.5 mM CaCl$_2$ · H$_2$O–7.5 mM KH$_2$PO$_4$–250 mM triethanolamine solution (pH 7.3) was added to each of the wells of tissue culture plates. The plates were incubated at 75°C without lids for 90 min. Following the incubation, the supernatants were carefully aspirated, the plates were allowed to dry, and the process was repeated three times. Hydroxyapatite-coated plates and lids were sterilized prior to use by exposure to 2 kJ of UV (254-nm) radiation.

To generate *S. mutans* plaques on deposited hydroxyapatite, overnight CDM cultures were diluted with fresh CDM to an optical density at 540 nm (OD$_{540}$) of 1.5. Either 280 μl (96-well plates) or 1.5 ml (240-well plates) of CDM containing 5% sucrose was added to each of the sterile, hydroxyapatite-coated wells. Either 20 μl (96-well plates) or 100 μl (24-well plates) of CDM broth culture (OD$_{540}$ = 1.5) was added to each of the medium-containing wells. The plates were incu-

bated overnight on a hematological rotator, within a 37°C CO$_2$ incubator, at a rotation speed of 10 rpm and an angle of 60°C less than horizontal. Following overnight incubation, the supernatants were aspirated, fresh CDM containing 5% sucrose was added to the wells, and the plates were incubated overnight as described above. Unless stated otherwise, data presented here were derived from 4-day-old plaques.

Light microscopy. *S. mutans* plaques grown within the wells of hydroxyapatite-coated 24-well plates were photographed unmagnified against a black background with a Polaroid MP-4 Land camera and at low magnification with a 35-mm camera coupled to an Olympus IM inverted microscope with a 4× objective.

Confocal microscopy. *S. mutans* plaques for use in confocal microscopy were generated within the hydroxyapatite-coated wells of 16-well Nunc Lab-Tek Chamber slides (Fisher Scientific). Following 4 days of growth, plaques were rinsed twice with 300 μl of TKS buffer (10 mM Tris-HCl, 50 mM KCl, 5% sucrose; pH 7.0)/well, stained for 15 min in the dark with 200 μl of the LIVE *Bac*light Bacterial Gram Stain fluorescent dye mixture (5 μM SYTO 9, 7 μM hexidium iodide, and 0.3% dimethyl sulfoxide in TKS buffer) (Molecular Probes, Eugene, Oreg.)/well, and rinsed once with 300 μl of TKS buffer/well. The well walls were gently removed, 50 μl of TKS was deposited on each plaque, and the plaques were covered with a 22-mm by 50-mm coverslip which was secured with superglue. Because the silicone sealing gasket (which previously connected the well walls to the slide) was left intact on the slide, the coverslip did not disturb the *S. mutans* plaques.

Plaques were examined by confocal microscopy with a Noran OZ confocal laser imaging system (Noran Instruments, Madison, Wis.) on a Nikon Diaphot 200 inverted microscope equipped with a 20× 0.75 N.A. objective lens and a Kr/Ar laser. Optical sections were collected at 1-μm steps through a sample depth of ~130 μm. Three-dimensional volumes were rendered by using the Noran InterVision 3D Analysis software. Maximum-intensity projection images were constructed, and TIFF images of *y-z* slices were made randomly through the rendered volumes. Peak-to-base heights of individual aggregates were measured on the TIFF images by using the Sigma ScanPro 4 program (Jandel Scientific, San Rafael, Calif.). Solid volumes were also rendered to reveal the surface morphology of the aggregates in each sample.

Determination of GTF activity. To visualize water-insoluble GTF activity of *S. mutans*, cell-associated proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by incubation with Triton X-100 (Fisher Biotech) and sucrose (18). Specifically, overnight CDM broth cultures were diluted into 20 ml of Todd-Hewitt broth, grown to an OD$_{540}$ of 1.3, 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% 2-mercaptoethanol 15% glycerol) and incubated at 25°C for 2 h. A 25-μl volume of cell-free supernatant was resolved by SDS-PAGE. Following electrophoresis, the SDS was eluted from the gel by two 30-min washes with 50 mM Tris buffer, pH 7.5. In situ water-insoluble GTF activity was visualized by incubation of the gel for 16 h in phosphate-buffered saline (PBS; pH 6.5) containing 2% sucrose, 2% Triton X-100, and 0.05% 11,000-molecular-weight dextran (Sigma, St. Louis, Mo.). The gels were briefly rinsed with PBS and incubated in a 45% methanol–10% acetic acid solution for 20 min to enhance the glucan bands. Gels were photographed against a black background. Scanning densitometry was used to quantify water-insoluble GTF activity. The glucan signals were normalized to the fructan signals; the normalized glucan signals were divided by the normalized wt glucan signal and presented as a percentage of wt GTF activity.

Sequencing of *gtfBC* recombination junctions. A combination of restriction digestions, Southern blotting analysis, and PCR analyses indicated that the recombination junctions of all *gtfBC* gene fusions were contained on 1.8-kb HindIII fragments of chromosomal DNA. Cloning of this region was achieved by PCR amplification of *gtfBC* gene fusions (11), digestion of the 5.3-kb PCR products with HindIII (Promega, Madison, Wis.), and ligation of the 1.8-kb fragments to HindIII-digested, alkaline phosphatase (Promega) treated pUC19. INV$_\alpha$F′ One Shot competent cells (Invitrogen, Carlsbad, Calif.) were transformed with ligation mixtures as per the manufacturer's instructions and plated on 2× yeast extract-tryptone agar plates containing 100 μg of ampicillin (Sigma)/ml and 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside Gold Biotechnology, St. Louis, Mo.)/ml. Clones containing a 1.8-kb HindIII insert were sequenced in both directions with universal M13 primers and primers gtfB@1639 (5′-GCAA CTATTCAAGCAAAAATTG) and gtfC@2107 (GAAAGTGCCGTATTATA GTG), using a Perkin-Elmer ABI Prism 310 Genetic Analyzer. The published sequences of the *gtfB* (21) and *gtfC* (25) genes and the DNA analysis program MacDNASIS Pro version 3.5 (Hitachi Software, San Bruno, Calif.) were used for sequence alignment and analysis.

Antibiotic sensitivity. *S. mutans* plaques were generated within the wells of hydroxyapatite-coated 96-well plates as described above. On the 4th day of growth, plaques were incubated with fresh CDM containing 0 or 100 μg of ampicillin (Sigma)/ml for 2 h while rotating within a 37°C CO$_2$ incubator. Following ampicillin challenge, the plaques were washed three times with 300 μl of CDM and incubated with 250 μl of CDM containing 5% sucrose and 1 μCi of [^{3}H]thymidine/ml for 16 h while rotating at 37°C. Labeled plaques were subsequently rinsed twice with 300 μl of PBS, digested with 250 μl of 1 M NaOH–10 mM EDTA for 2 h at 37°C, and subjected to scintillation counting. The percentage of plaque organisms killed was calculated as follows: $1.0 − $ (mean counts per minute of the challenged plaque organisms/mean counts per minute of the unchallenged plaque organisms of the respective genotype).

RESULTS

The contribution of GbpA and GtfBC to *S. mutans* plaque structure. We previously hypothesized that the enhanced virulence of *gbpA S. mutans* in the gnotobiotic rat model resulted from a change in plaque structure and that the accumulation of recombinant *gtfBC* genes by *gbpA S. mutans* attenuated virulence by restoring a wt-like plaque structure (11). To test the hypothesis that inactivation of *gbpA* and recombination of the *gtfB* and *gtfC* genes within a *gbpA* background reciprocally alter plaque structure, we used an in vitro plaque model in which *S. mutans* wt, *gbpA*, and *gbpA*/25%*gtfBC* plaques were grown within hydroxyapatite-coated wells of microtiter dishes and examined by light and confocal microscopy. The *gbpA* mutant mixture containing 25% *gbpA gtfBC* recombinant organisms was used to mimic the level of in vivo accumulation of *gtfBC* organisms (22.33%) by *gbpA S. mutans* in the gnotobiotic rat model (11).

Following overnight plaque deposition and aspiration of supernatants, gross differences were noted among these plaques. Figure 1A shows *S. mutans* wt, *gbpA*, and *gbpA*/25%*gtfBC* plaques after 4 days of growth in hydroxyapatite-coated wells of a 24-well plate. While the plaque deposited by wt *S. mutans* appeared as an opaque, granular, thick layer, the plaque deposited by *gbpA S. mutans* appeared as a translucent, amorphous, thinner layer. As the percentage of *gtfBC* organisms within the *gbpA* inocula was increased from 0 to 25%, the opaque and granular patterns were partially restored. Restoration of these patterns was characteristic of spontaneous laboratory *gtfBC* recombinants as well as *gtfBC* recombinants recovered from *gbpA* mutant-infected gnotobiotic rats (clinical recombinants), suggesting that this is a general phenomenon of *gbpA gtfBC* recombinant organisms. It should be noted that for recombinants with higher-than-average recombinant levels of GTF activity (C5-2 and A6-1 [see Fig. 4]), a recombinant proportion of greater than 25% of the inoculum was required to restore plaque structure. The plaque deposited by nonrecombinant laboratory *gbpA* strains was indistinguishable from the plaque produced by nonrecombinant clinical *gbpA* strains (data not shown). Both laboratory and clinical *gbpA* strains produced wt-like plaques when *gtfBC* recombinant (either spontaneous or clinical) organisms were included in the inoculum. Plaques produced by *gbpA*/100%*gtfBC S. mutans* were as opaque and granular as wt plaques but appeared thinner than the other *S. mutans* plaques, perhaps due to the marked decrease in GTF activity. Quantification of plaque organisms in this model by the crystal violet release assay (11) revealed no differences in cell number among these plaques (data not shown).

Examination of these plaques by light microscopy offered insight into the possible mechanisms underlying the differences in plaque appearance. Figure 1B shows 4-day-old *S. mutans* wt, *gbpA*, and *gbpA*/25%*gtfBC* plaques deposited in hydroxyapatite-coated wells, as viewed with an inverted microscope. While the wt plaque consisted of many large aggregates adherent to smaller aggregates, which were themselves adherent to the hydroxyapatite, the *S. mutans gbpA* plaque consisted of primarily smaller aggregates adherent to the hydroxyapatite. Notably, the smaller aggregates of the *gbpA* plaque more completely covered the hydroxyapatite, whereas the mixture of smaller and larger aggregates of the wt plaque left larger areas of the hydroxyapatite uncovered. As the proportion of *gtfBC* organisms
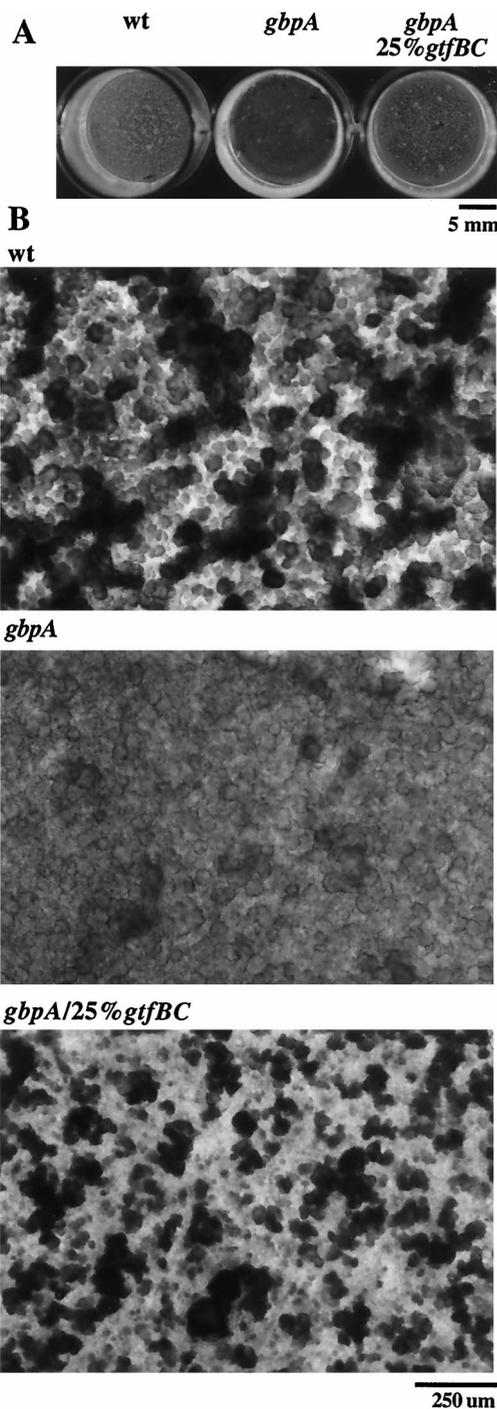

FIG. 1. *S. mutans* plaques deposited in hydroxyapatite-coated wells of 24-well plates. *S. mutans* strains were grown in hydroxyapatite-coated wells on a rotator for 4 days in CDM with 5% sucrose as described in the text. Supernatants were aspirated, and the plaques were photographed. (A) Unmagnified hydroxyapatite wells containing *S. mutans* plaques photographed against a black background. (B) *S. mutans* plaques at low magnification, viewed with an inverted microscope with a 4× objective. With the exception of the focal plane, light micrographs of *S. mutans* plaques were taken under identical photographic conditions (magnification, lighting, exposure time, etc.).

within the *gbpA* inocula was increased from 0 to 25%, the capacity of *gbpA S. mutans* to form large aggregates was restored.

To extend the above-described observations and to perform quantitative analysis, confocal laser scanning micros-
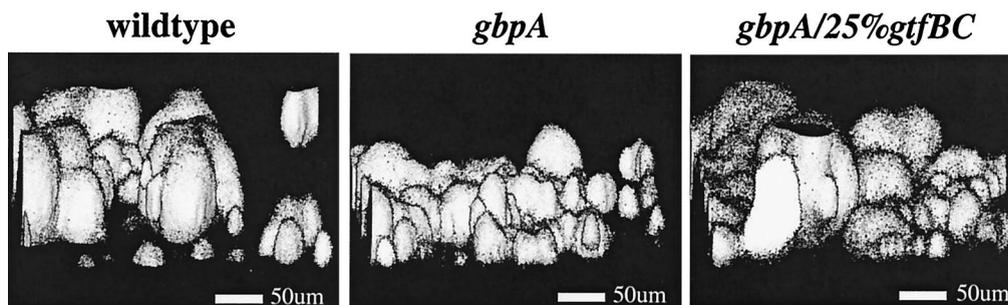
wildtype gbpA gbpA/25%gtfBC

FIG. 2. Tilted saggital solid renderings of *S. mutans* plaques acquired by CLSM. *S. mutans* strains were grown in hydroxyapatite-coated wells of a Nunc Lab-Tek Chamber slide on a rotator for 4 days in CDM with 5% sucrose as described in the text. Fluorescently stained plaques were optically sectioned at 1-μm increments by CLSM; saggital views of solid renderings were tilted ($x = -75, y = -5, z = 0$) by using the InterVision 3-D Analysis program, converted to gray scale by using Adobe Photoshop 3.0.4 (Adobe Systems Inc., Mountain View, Calif.), and assembled by using Canvas 5.02 (Deneba Software, Miami, Fla.). All parameters of data collection, analysis, and presentation were applied identically to each of the *S. mutans* plaques.

copy (CLSM) was used to examine *S. mutans* plaques. Figure 2 shows solid renderings of data collected by CLSM of wt, *gbpA*, and *gbpA*/25%*gtfBC S. mutans* plaques. The renderings in Fig. 2 have been tilted ($x = -75, y = -5, z = 0$) to give the impression of viewing a terrain from above and slightly to one side. As was seen by light microscopy, wt and *gbpA*/25%*gtfBC* plaques were composed primarily of large aggregates, while the *gbpA* plaque consisted of uniformly smaller aggregates. To quantify the differences in plaque depth, the peak-to-base distance of individual aggregates was measured. As shown in Figure 3A, the median aggregate heights of the wt and *gbpA*/ 25%*gtfBC* plaques were both significantly greater than that of the *gbpA* plaque ($P < 0.0001$) but were not statistically different from each other.

Characterization of *gtfBC* recombinant organisms. It thus appears that the presence of *gtfBC* recombinants within a *gbpA* population can restore a wt-like plaque structure whether the *gbpA gtfBC* recombinants were obtained as spontaneous laboratory isolates or recovered from *gbpA* mutant-infected gnotobiotic rats. Since recombinants could potentially vary based on the point of recombination and the active site retained in the hybrid enzyme, the genotypic and phenotypic characteristics of several *gbpA gtfBC* recombinants were examined for insoluble GTF activity and the site(s) of recombination. An early analysis (11) provided evidence that the recombinants recovered from infected rats all recombined near the same region. There existed

the possibility that selection for certain recombinants occurred even if multiple sites of recombination were possible.

Cell-associated proteins from wt, *gbpA*, and representative *gbpA gtfBC* isolates were extracted, resolved by SDS-PAGE, and developed for determination of insoluble GTF activity. Since inactivation of *gbpA* does not influence Gtf-S or Gtf-I activity, as determined by the radiolabeled-sucrose assay (unpublished data), and since the genes affected by *gtfBC* recombination encode primarily Gtf-I activity, we focused on the Gtf-I activity of the strains used in this work. As with the radioactive-sucrose method, no differences in insoluble GTF activity were found between wt and *gbpA S. mutans* by the activity gel method (Fig. 4). All *gbpA gtfBC* isolates exhibited a marked reduction (50 to 90%) in insoluble glucan synthesis relative to that of nonrecombinant *S. mutans*, although differences in insoluble GTF activity among the isolates were apparent (Fig. 4). The degree of GTF activity reduction likely influenced the extent to which particular recombinants accumulated within individual animals. Among *gbpA* mutant-infected rats which experienced wt levels of caries, a general trend was noted: the greater the accumulation of a recombinant within a *gbpA* mutant-infected rat, the greater the GTF activity of the recombinant. Similarly, *gbpA* mutant-infected rats which experienced elevated levels of caries harbored low levels of recombinants which retained higher-than-average levels of recombinant GTF activity (26.7% of wt GTF activity).
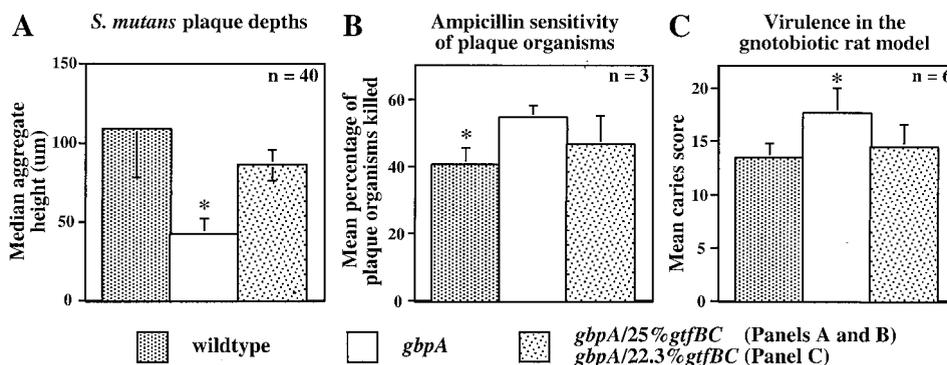

FIG. 3. Quantitative analysis of structural and functional aspects of *S. mutans* plaques. Asterisks indicate significant differences between adjacent columns. (A) Median heights (in micrometers) with upper and lower 95% confidence intervals of aggregates comprising *S. mutans* plaques ($P < 0.0001$). (B) Sensitivity of *S. mutans* plaque organisms to ampicillin challenge. Four-day-old *S. mutans* plaques were treated for 2 h with fresh CDM medium containing 0 or 100 μg of ampicillin/ml, washed three times, and incubated with CDM containing 5% sucrose and 1 μCi of [³H]thymidine/ml. The percentage of plaque organisms killed was calculated as follows: $1.0 -$ (mean counts per minute of the challenged plaque organisms/mean counts per minute unchallenged plaque organisms of the respective genotype) ($P < 0.02$). (C) Virulence of *S. mutans* in the gnotobiotic rat model, as previously reported in the form of tabular data (Table 3 in reference 11 [enamel involvement, buccal surfaces]), represented here in graphical form to facilitate comparison with results from the in vitro plaque model ($P < 0.03$).
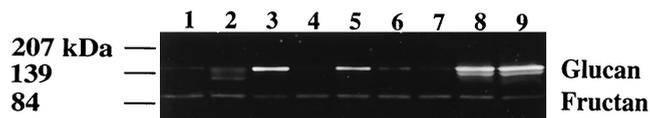
FIG. 4. GTF activity of *S. mutans* UA130 *gbpA gtfBC*, *gbpA*, and wt. Equivalent levels of cell-associated proteins were resolved by SDS-PAGE and incubated at 37°C in PBS (pH 6.5) containing sucrose and Triton X-100. Gels were photographed against a black background. The positions of SDS-PAGE molecular mass standards are indicated in the left margin. The positions of water-insoluble glucan and fructan are indicated in the right margin. Lanes 1 to 6: contain *gbpA gtfBC* isolates recovered from six individual *gbpA* mutant-infected gnotobiotic rats from two separate determinations of cariogenicity. Lanes: 1, B3-B; 2, D2-A; 3, C5-2; 4, C3-2; 5, A6-1; 6, A2-1; 7 to 9, laboratory strains (7, *gbpA* spontaneous *gtfBC*; 8, *gbpA*; 9, wt).

These observations make sense since it appears that a certain level of insoluble glucan reduction by *gbpA S. mutans* is necessary for restoration of plaque structure. When both the levels of recombinant GTF activity and the degree of accumulation of the recombinants were considered, it appeared that in order to restore plaque structure and cariogenicity, *gbpA S. mutans* accumulated *gtfBC* recombinant organisms to a degree such that the total insoluble glucan production of the mixed population was approximately 80% of wt levels.

The regions which contained the *gtfB gtfC* recombination junctions of 11 recombinant isolates were cloned and sequenced. Among these were seven UA130 *gbpA gtfBC* isolates recovered from various animals representing both animal experiments, the UA130 *gbpA* spontaneous *gtfBC* recombinant described here, the previously described strain GS-5 spontaneous recombinant SP2 (24), an independent GS-5 spontaneous recombinant, and a strain 3209 *recA* spontaneous recombinant. Using the Higgins-Sharp algorithm of the MacDNASIS program, the sequences of these isolates were multiply aligned along with the published sequences of the GS-5 *gtfB* and *gtfC* genes (21, 25). Ten of these isolates had recombination junctions within a region 270 bp downstream of the catalytic site of *gtfB* and spanning approximately 200 bp. Only the recombination junction of SP2 was in a markedly different region, approximately 580 bp upstream of the *gtfB* catalytic site. Nucleotide differences existed among several *gtfBC* genes, and multiple recombinations could not be ruled out. While an intact *recA* gene was not required for the recombination (11), our results suggested that *gtfBC* recombination within the mammalian host was mediated by the homology exhibited by these genes, as has been documented for in vitro-cultivated *S. mutans* (24, 26).

These observations, combined with our finding that *gbpA S. mutans* does not have a higher basal level of spontaneous *gtfBC* recombination in vitro (data not shown), favor a role for the selection of recombinants within a *gbpA* plaque, rather than an increased frequency of recombination, as the mechanism of recombinant accumulation within gnotobiotic rats.

The contribution of GbpA and GtfBC to *S. mutans* plaque function. Dental plaque, being composed of adherent aggregates of cells imbedded in a matrix of extracellular polysaccharide surrounded by fluid-filled spaces, exhibits features common to biofilms (13). A characteristic of biofilm organisms is decreased susceptibility to biocide agents compared to that of their planktonic counterparts (17). It has recently been reported that *Pseudomonas aeruginosa* organisms within a thin, unstructured, mutant biofilm were more susceptible to a biocidal agent than were organisms within the wt biofilm (6). Since the plaque deposited by *gbpA S. mutans* was thinner and less structured than wt and *gbpA/25%gtfBC* plaques, we hypothesized that *gbpA* plaque organisms would be more susceptible to a biocidal agent. To test this hypothesis, 4-day-old *S. mutans* plaques

were challenged for 2 h with a dose of ampicillin lethal to planktonic cells and incubated overnight in the presence of [^{3}H]thymidine. As shown in Fig. 3B, *gbpA* plaque organisms were significantly ($P < 0.02$) more susceptible to ampicillin challenge than wt plaque organisms. As the percentage of *gtfBC* organisms within the *gbpA* inocula was increased, the sensitivity of plaque organisms to ampicillin decreased. No differences in ampicillin sensitivity were found among these strains when planktonic cultures were tested (data not shown). These results indicate that the observed changes in plaque structure have functional consequences.

DISCUSSION

Having observed that inactivation of the *gbpA* gene of *S. mutans* increased virulence and promoted accumulation of virulence-attenuating *gtfBC* recombinant organisms in vivo, we hypothesized that these findings might have resulted from changes in plaque structure (11). Here we report the results of experiments designed to test the hypothesis that the absence of GbpA alters plaque structure and that the presence of *gtfBC* recombinant organisms within a *gbpA* background compensates for this alteration. Our findings have implications for further understanding *S. mutans* cariogenicity as well as for the study of biofilms and the relationship between biofilm structure and virulence.

Several previous observations have suggested that the absence of GbpA may have altered the structure of *S. mutans* plaque. An analysis of virulence in the gnotobiotic rat model revealed that while levels of colonization of *gbpA* and wt *S. mutans* were not significantly different, *gbpA S. mutans* was hypercariogenic (11). In vitro, *gbpA* mutant plaque was more resistant to mechanical stress than wt plaque (11). Since levels of acid production in batch cultures of wt and *gbpA S. mutans* were not different (11), we hypothesized that the hypercariogenicity of *gbpA S. mutans* in vivo resulted from a change in plaque structure which either increased acid production or decreased the diffusion of acid away from the tooth enamel. We have subsequently found no differences in the cell numbers of (as was found in vivo) or in the rates of acid production by wt, *gbpA*, or *gbpA/25%gtfBC S. mutans* in our plaque model (unpublished data). In light of this and the finding that *gbpA* mutant plaque is composed of smaller aggregates which more completely coat the underlying substratum, we hypothesize that GbpA mediates aggregation and that *gbpA S. mutans* is hypercariogenic in vivo due to the altered plaque structure, which, we speculate, forms a tighter barrier between the tooth surface and the saliva. We propose that a tighter barrier would allow for sustained exposure of the tooth enamel to demineralizing conditions by decreasing both the influx of salivary buffering capacity and the efflux of bacterially derived acids. We are presently developing techniques to directly test this barrier hypothesis.

Several observations led us to believe that data derived from our in vitro plaque model are relevant to our previous findings concerning cariogenicity. First, in both the gnotobiotic rat model and our in vitro plaque model, wt and *gbpA/25%gtfBC S. mutans* strains gave results which were highly similar to each other yet distinct from those of *gbpA S. mutans*. In the rats, this pattern was observed in measurements of cariogenicity, and in our in vitro model, this pattern was repeated with respect to plaque structure, aggregate size, and ampicillin sensitivity (Fig. 3). Second, in our plaque model, clinical isolates (those recovered from *gbpA* mutant-infected rats) produced plaques which were grossly indistinguishable from their laboratory counterparts. This finding also supports the hypothesis that *gtfBC* recombination was the change (as opposed to some other in

vivo-selected adaptation) which attenuated the hypercariogenicity of *gbpA S. mutans* in the gnotobiotic rat model.

The ability of *gtfBC* recombination to compensate for *gbpA* inactivation in terms of plaque structure, aggregate size, ampicillin sensitivity, and cariogenicity led us to consider the possibility that the ratio of glucan to glucan-binding protein may be an important parameter in *S. mutans* plaque development. This hypothesis predicts that inactivation of other GBP-encoding genes would impart both an altered plaque structure phenotype and a propensity toward in vivo accumulation of organisms with reduced GTF activity. Since GbpA is the quantitatively predominant *S. mutans* nonenzymatic glucan-binding protein (22), it is possible that these effects would be most marked in the *gbpA* background. Obviously, many questions would have to be addressed in order to validate the glucan–glucan-binding protein hypothesis, yet the fact that a 20% reduction in GTF activity within a *gbpA* background restored every parameter measured (cariogenicity, plaque structure, etc.) to near-wt levels suggests that this hypothesis warrants further investigation.

While the objective of this investigation was to elucidate the contribution of GbpA and *gtfBC* recombination to *S. mutans* plaque development and cariogenicity, we feel that our results have important implications to the study of biofilms. Biofilms are communities of bacteria which develop on solid surfaces as pillar-like structures separated by fluid-filled spaces (5). These structures are composed of bacteria embedded in a matrix of extracellular polysaccharide. While the universal characteristics of biofilm structure—copious extracellular polysaccharide production and decreased antibiotic susceptibility—have been well documented, the analysis of specific gene products in biofilm formation has just recently begun (12). Davies et al. have shown that *P. aeruginosa* mutants defective in acylated homoserine lactone production form a thin, flat, unstructured biofilm (6). By screening a *Pseudomonas fluorescens* transposon mutagenesis library, O'Toole and Kolter isolated mutants defective in biofilm initiation. Of 24 mutants isolated, 21 contained transposon insertions in genes of unknown function (17). Burne et al. have found that *S. mutans* polysaccharide synthesis genes are differentially regulated in biofilms (4); Mack et al. have reported that defects in production of a unique polysaccharide (PIA) by *Staphylococcus epidermidis* impair biofilm formation (15). Given the association of biofilms with extracellular polysaccharides, it might seem elementary that inactivation of a gene encoding a nonenzymatic polysaccharide-binding protein would alter biofilm structure, yet to our knowledge this communication represents the first report to this effect and, in conjunction with our previous findings, presents the first experimental evidence that changes in biofilm structure influence virulence. It has been suggested that interfering with the ability to form mature biofilms by disrupting cell-to-cell communication may be a novel method for attenuating the negative impact of biofilms in medicine and industry (6, 7). Our findings suggest that disruption of mature biofilm structure can have unanticipated, undesirable ramifications.

ACKNOWLEDGMENTS

REFERENCES

1. **Banas, J. A., R. R. B. Russell, and J. J. Ferretti.** 1990. Sequence analysis of the gene for the glucan-binding protein of *Streptococcus mutans* Ingbritt. Infect. Immun. **58:**667–673.
2. **Banas, J. A., and K. S. Gilmore.** 1991. Analysis of *Streptococcus mutans* and *Streptococcus downei* mutants insertionally inactivated in the *gbp* and *gtfS* genes, p. 281–283. *In* G. M. Dunny, P. P. Cleary, and L. L. McKay (ed.), Genetics and molecular biology of streptococci, lactococci, and enterococci. American Society for Microbiology, Washington, D.C.
3. **Banas, J. A., H. C. Potvin, and R. N. Singh.** 1997. The regulation of *Streptococcus mutans* glucan-binding protein A expression. FEMS Microbiol. **154:**289–292.
4. **Burne, R. A., Y. Y. Chen, and J. E. Penders.** 1997. Analysis of gene expression in *Streptococcus mutans* in biofilms in vitro. Adv. Dent. Res. **11:**100–109.
5. **Costerton, J. R., Z. Lewandowski, D. E. Caldwell, D. R. Korber, and H. M. Lappin-Scott.** 1995. Microbial biofilms. Annu. Rev. Microbiol. **49:**711–745.
6. **Davies, D. G., M. R. Parsek, J. P. Pearson, B. H. Iglewski, J. W. Costerton, and E. P. Greenberg.** 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science **280:**295–298.
7. **Dixon, B.** 1998. Biofilms: cultural diversity in action. ASM News **64:**484–485.
8. **Gibbons, R. L., and J. van Houte.** 1975. Dental caries. Annu. Rev. Med. **26:**121–136.
9. **Haas, W., R. MacColl, and J. A. Banas.** 1998. Circular dichroism analysis of the glucan binding domain of *Streptococcus mutans* glucan binding protein-A. Biochim. Biophys. Acta **1384:**112–120.
10. **Haas, W., and J. A. Banas.** 1997. The glucan-binding domain of the *Streptococcus mutans* glucan-binding protein. Adv. Exp. Med. Biol. **418:**707–708.
11. **Hazlett, K. R. O., S. M. Michalek, and J. A. Banas.** 1998. Inactivation of the *gbpA* gene of *Streptococcus mutans* increases virulence and promotes in vivo accumulation of recombinations between the glucosyltransferase B and C genes. Infect. Immun. **66:**2180–2185.
12. **Kolter, R., and R. Losick.** 1998. One for all and all for one. Science **280:**226–227.
13. **Larsen, T., and N. E. Fiehn.** 1995. Development of a flow method for susceptibility testing of oral biofilms in vitro. APMIS **103:**339–344.
14. **Loesche, W. J.** 1986. Role of *Streptococcus mutans* in human dental decay. Microbiol. Rev. **50:**353–380.
15. **Mack, D., M. Nedelmann, A. Krokotsch, A. Schwarzkopf, J. Heesemann, and R. Laufs.** 1994. Characterization of transposon mutants of biofilm-producing *Staphylococcus epidermidis* impaired in the accumulative phase of biofilm production: genetic identification of a hexosamine-containing polysaccharide intercellular adhesin. Infect. Immun. **62:**3244–3253.
16. **Munro, C., S. M. Michalek, and F. L. Macrina.** 1991. Cariogenicity of *Streptococcus mutans* V403 glucosyltransferase and fructosyltransferase mutants constructed by allelic exchange. Infect. Immun. **59:**2316–2323.
17. **O'Toole, G. A., and R. Kolter.** 1998. Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signaling pathways: a genetic analysis. Mol. Microbiol. **28:**449–461.
18. **Russell, R. R. B.** 1979. Glucan-binding proteins of *Streptococcus mutans* serotype c. J. Gen. Microbiol. **112:**197–201.
19. **Sato, Y., Y. Yamamoto, and H. Kizaki.** 1997. Cloning and sequence analysis of the *gbpC* gene encoding a novel glucan-binding protein of *Streptococcus mutans*. Infect. Immun. **65:**668–675.
20. **Schilling, K. M., R. G. Carson, C. A. Bosko, G. D. Golikeri, A. Bruinooge, K. Hoyberg, A. M. Waller, and N. P. Hughes.** 1994. A microassay for bacterial adherence to hydroxyapatite. Colloids Surf. **3:**31–38.
21. **Shiroza, T., S. Udea, and H. K. Kuramitsu.** 1987. Sequence analysis of the *gtfB* gene from *Streptococcus mutans*. J. Bacteriol. **169:**4263–4270.
22. **Smith, D. J., H. Akita, W. F. King, and M. A. Taubman.** 1994. Purification and antigenicity of a novel glucan-binding protein of *Streptococcus mutans*. Infect. Immun. **62:**2545–2552.
23. **Smith, D. J., and M. A. Taubman.** 1996. Experimental immunization of rats with a *Streptococcus mutans* 59-kilodalton glucan-binding protein protects against dental caries. Infect. Immun. **64:**3069–3073.
24. **Ueda, S., and H. K. Kuramitsu.** 1988. Molecular basis for the spontaneous generation of colonization-defective mutants of *Streptococcus mutans*. Mol. Microbiol. **2:**135–140.
25. **Ueda, S., T. Shiroza, and H. K. Kuramitsu.** 1988. Sequence analysis of the *gtfC* gene from *Streptococcus mutans* GS-5. Gene **69:**101–109.
26. **Yamashita, Y., W. H. Bowen, and H. K. Kuramitsu.** 1992. Molecular analysis of a *Streptococcus mutans* strain exhibiting polymorphism in the tandem *gtfB* and *gtfC* genes. Infect. Immun. **60:**1618–1624.
27. **Yamashita, Y., W. H. Bowen, R. A. Burne, and H. K. Kuramitsu.** 1993. Role of the *Streptococcus mutans* gtf genes in caries induction in the specific-pathogen-free rat model. Infect. Immun. **61:**3811–3817.

Editor: V. A. Fischetti