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Cariogenicity of *Streptococcus mutans* V403 Glucosyltransferase and Fructosyltransferase Mutants Constructed by Allelic Exchange

CINDY MUNRO,[1] SUZANNE M. MICHALEK,[2] AND FRANCIS L. MACRINA[1]*

Department of Microbiology and Immunology, Virginia Commonwealth University, Richmond, Virginia 23298-0678,[1] and Department of Microbiology, University of Alabama at Birmingham, Birmingham, Alabama 35294[2]

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Streptococcus mutans **produces several enzymes which metabolize sucrose. Three glucosyltransferase genes (*gtfB*, *gtfC*, and *gtfD*) and a single fructosyltransferase gene (*ftf*) encode enzymes which are important in formation of exopolysaccharides. Mutants of *S. mutans* V403 carrying single and multiple mutations of the *gtfB*, *gtfC*, *gtfD*, and *ftf* genes recently have been constructed by allelic exchange in our laboratory. Using selected strains from this panel of mutants, we examined the importance of water-insoluble glucan, water-soluble glucan, and fructan production in cariogenicity while controlling for the effects of strain and species variability. Genetic and biochemical characterization of mutants and assays of glucosyltransferase and fructosyltransferase activities were performed to ensure that the phenotypes of strains coincided with deficiencies predicted by genotype. The young gnotobiotic rat model of cariogenicity was used to assess virulence of the wild-type strain and isogenic mutants. Mutant strains were less virulent than the wild type in almost every location examined for caries on tooth surfaces and level of involvement of lesions (depth and severity). Inactivation of either *gtfB* and *gtfC* or *ftf* dramatically reduced virulence; the subsequent inactivation of *gtfD* did not enhance the effect of reduced virulence.**

In the human oral cavity, *Streptococcus mutans* utilizes dietary sucrose as a substrate for extracellular polymer synthesis (3). The role of exopolysaccharides in the development of tooth decay has been the subject of intensive research, and many studies have implicated insoluble glucan polymers in tenacious attachment to and accumulation on the tooth surface (3, 10). However, the relative importance of particular exopolysaccharides in cariogenicity using isogenic strains in an animal model has not been demonstrated.

S. mutans V403 produces several enzymes which metabolize sucrose. Glucosyltransferases (Gtf enzymes, EC 2.4.1.5) and fructosyltransferase (Ftf enzyme, EC 2.4.1.10) are important in the formation of exopolysaccharides. Table 1 summarizes some of the characteristics of these enzymes. The glucosyltransferase B (*gtfB*) gene directs production of an enzyme which converts sucrose to an insoluble glucan polymer and fructose (25). The product of the glucosyltransferase C gene (*gtfC*) converts sucrose to insoluble glucan, soluble glucan, and fructose (5). The glucosyltransferase D (*gtfD*) gene product converts sucrose to a soluble glucan polymer and fructose (6). Fructosyltransferase produces fructan polymer and glucose from sucrose (24). All four genes have been sequenced previously (7, 24, 25, 27), and the three glucosyltransferase genes exhibit high levels of nucleotide and amino acid sequence homology. *gtfB* and *gtfC* are tightly linked, but neither *gtfD* nor *ftf* is linked to the other known genes involved in exopolysaccharide production (18).

Isogenic strains of *S. mutans* V403, which carry single and multiple mutations of the *gtfB*, *gtfC*, *gtfD*, and *ftf* genes, recently have been constructed by allelic exchange in our laboratory. With this panel of strains, it became possible to examine the importance of water-soluble glucan, water-insoluble glucan, and fructan production in virulence while controlling for the effects of strain and species variability. Genetic and biochemical characterization and assays of glucosyltransferase and fructosyltransferase activity have been performed, and the ability of these organisms to cause caries in the rat model has been tested. Mutant strains were less virulent than the wild type in almost every location and level of involvement. Inactivation of either *gtfB* and *gtfC* or *ftf* dramatically reduced virulence; the subsequent inactivation of *gtfD* did not enhance the effect of reduced virulence.

MATERIALS AND METHODS

Bacterial strains and media. The strains used in this work are summarized in Table 2. *S. mutans* strains were grown in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.) anaerobically at 37°C; Todd Hewitt broth (Difco) supplemented with 20 mM DM-threonine (Sigma Chemical Co., St. Louis, Mo.) was used in the preparation of genetically competent cells. Agar (Difco) was added to a final concentration of 1.5% in the preparation of solid media. *Escherichia coli* DB11 cells were grown aerobically at 37°C in Lennox broth (GIBCO, Madison, Wis.). Genetic transformation of *S. mutans* cells was performed as described by Lindler and Macrina (12) with purified plasmid or chromosomal DNA. Transformants which had acquired antibiotic resistance genes were selected by growth on solid media containing tetracycline (final concentration, 5 μg/ml), kanamycin (500 μg/ml), erythromycin (5 μg/ml), or a combination of these antibiotics. Colonial morphology was examined on mitis salivarius (MS) agar (Difco).

DNA isolation and characterization. *S. mutans* chromosomal DNA was isolated by the method of Marmur (15) with modification as described by Schroeder et al. (22). *E. coli* plasmid DNA was isolated by the method of Clewell and Helinski (2). The plasmid used in insertional inactivation of the *gtfD* gene, pNH5, and *S. mutans* GS5-DD1, which carried a *gtfD* gene which had been inactivated via allelic exchange, were kindly provided by Howard Kuramitsu, University of Texas, San Antonio. Digestion of chromo-

* Corresponding author.

TABLE 1. Characteristics of *S. mutans* exopolysaccharide-producing enzymes

Enzyme (reference[s])	Products from sucrose	Gene size (bp)	No. of amino acids	Gene product size (kDa)		Estimated pI	pH optimum	K_m for sucrose (mM)
				ORF[a]	Mature[b]			
GtfB (25)	Insoluble glucan + free fructose	4,424	1,475	166	162	6.4	6.0	2.2
GtfC (5, 19)	Insoluble and soluble glucan + free fructose	4,218	1,375	153	149	5.1	6.5	3.9
GtfD (7)	Soluble glucan + free fructose	4,293	1,430	159	155	6.5	5.5–6.0	2.1
Ftf (24)	Fructan + free glucose	2,391	797	88	84	5.7		4

[a] Gene product size deduced from open reading frame (ORF).
[b] Predicted size of gene product following removal of the signal sequence.

somal and plasmid DNA with restriction endonucleases was performed according to the manufacturer's instructions (GIBCO/BRL, Gaithersburg, Md.). Restriction endonuclease digests were analyzed by agarose gel electrophoresis (21) and transferred bidirectionally to nitrocellulose (26). Southern hybridization was performed as previously described (21) with probe DNA which was ^{32}P labeled in vitro by nick translation by using a commercially available system according to the manufacturer's instructions (GIBCO/BRL).

Preparation of extracellular protein fractions from *S. mutans*. Strains were grown overnight in BHI broth at 37°C. Following addition of phenylmethylsulfonyl fluoride (Sigma) to a final concentration of 1 mM and EDTA (Sigma) to a final concentration of 1 mM, culture supernatants were harvested by centrifugation at 7,000 × *g* for 15 min. Acetone precipitation of the supernatants was carried out at 4°C as described by Scopes (23), with the addition of acetone to 50%, assuming additive volumes. The precipitated material was recovered by centrifugation at 7,000 × *g* at 4°C for 15 min. The pellet was washed with cold 50% acetone, centrifuged at 12,000 × *g* at 4°C for 10 min, suspended in 10 mM imidazole (pH 6.5) with 1 mM phenylmethylsulfonyl fluoride and 1 mM EDTA, and dialyzed overnight against 10 mM imidazole at 4°C. Protein concentration was determined by the Lowry method (13), by comparison of samples with a standard curve prepared with bovine serum albumin. Samples were stored at −20°C in small volumes. Separate samples were thawed for each experiment, and no samples were refrozen or used in more than one experiment.

Demonstration of exopolysaccharide formation in polyacrylamide gels. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 10% acrylamide gels according to the method of Hames and Rickwood (4). Each set of samples was loaded in duplicate on a gel. Following electrophoresis, one set of samples from each gel was stained with Coomassie brilliant blue to detect protein bands, and the sizes of the bands were determined by comparison with commercially obtained protein size standards (Bio-Rad Laboratories, Richmond, Calif.). The half of each SDS-PAGE gel carrying lanes identical to those stained with Coomassie brilliant blue was treated to reveal polysaccharide formation as previously described (8, 16, 22). Briefly, the gel was washed for 24 h in 50 mM imidazole at room temperature to permit renaturation of enzymes in the gels, incubated in sucrose buffer (5% sucrose, 50 mM sodium acetate, 1% Triton X-100, 0.5% acetic acid, pH 6.5) for 24 h at 37°C, washed extensively in distilled water, and then incubated with 1% periodic acid for 30 min. Following washing in distilled water, the gel was incubated with Schiff's reagent (Sigma) at 4°C until red bands developed.

In vitro exopolysaccharide synthesis assay. Gtf and Ftf activity assays (glucan and fructan synthesis assays) as described by Koga et al. (10) and Pucci and Macrina (20) were used to obtain quantitative information about Gtf and Ftf enzymatic activities in the mutants. Assays were performed with ^{14}C-sucrose labeled in the glucosyl portion of the molecule (NEN Research Products, Boston, Mass.) to determine glucan synthesis and with ^{14}C-sucrose labeled in the fructosyl moiety (NEN Research Products) to determine fructan synthesis. The reaction mixture, containing 80 μl of unlabeled substrate buffer (10 mM imidazole, 10 mM sucrose, pH 6.5), 5 μl of dextran T-10 primer (Pharmacia), and 5 μl of radiolabeled sucrose (0.5 μCi; 261 mCi/mmol for glucose-labeled sucrose or 267 mCi/mmol for fructose-labeled sucrose) was warmed to 37°C prior to addition of the enzyme preparation. Reaction mixtures were incubated for 18 h at 37°C following addition of the extracellular protein products from the strain under study. The polymer formed was collected by methanol precipitation on glass fiber filters; triplicate filters were prepared for each sample. To determine the level of glucan synthesis, two identical groups of triplicate filters were prepared for each reaction. Filters then were split into two groups for parallel analysis; one set was washed in methanol (in order to determine the total level of water-insoluble and water-soluble glucan synthesis), and the second set of filters was washed in distilled water (in order to quantify water-insoluble glucan synthesis). The amount of radiolabeled substrate incorporated into newly formed polymer was determined by measuring the radioactivity of the filters by liquid scintillation spectrometry. Averages of the triplicate filter values were recorded for each reaction, the value of the negative control (reaction mixture without

TABLE 2. Glucan and fructan production by isogenic strains[a]

Strain	Genotype[b]				Polysaccharide production[c] of:		
	gtfB	*gtfC*	*gtfD*	*ftf*	Total glucan	Water-insoluble glucan	Fructan
V403	+	+	+	+	1,340 ± 154	269 ± 156	1,042 ± 416
V1741	+	+	+	−	2,472 ± 402	69 ± 35	7 ± 7
V1786	−	−	+	+	76 ± 16	20 ± 14	867 ± 76
V1789	−	−	+	−	24 ± 15	3 ± 4	3 ± 5
V1995	−	−	−	+	2 ± 2	0	825 ± 272
V1996	−	−	−	−	2 ± 3	0	7 ± 5

[a] As determined by 18-h assays of exopolysaccharide synthesis using material from a single protein preparation of each strain, incubated at 37°C with radiolabeled sucrose as described in text.
[b] +, wild type; −, mutant.
[c] Counts per minute per microgram of protein (adjusted for negative control; mean from six assays [three replicates per assay]) ± standard deviation.

protein product) for the experiment was subtracted, and levels of radiolabeled glucose or fructose incorporated into polymer on the filters were expressed as counts per minute per microgram of protein. Through comparison of the counts per minute of methanol-washed and distilled-water-washed disks in assays using sucrose labeled in the glucosyl moiety, the relative production of soluble and insoluble glucan was inferred.

Sucrose hydrolysis assay. The ability to hydrolyze sucrose was tested by a photometric method with copper and arsenomolybdate reagents as described by Nelson (17). The reaction mixture, containing 1, 10, or 50 μg of protein preparation, 850 μl of substrate buffer (10 mM imidazole, 10 mM sucrose, pH 6.5), and 50 μl of dextran T-10 primer, was incubated for 1 h at 37°C. Copper and arsenomolybdate reagents were prepared as described by Nelson (17), and 1 ml of copper reagent was added to each reaction mixture, boiled for 20 min, and cooled to room temperature. One milliliter of arsenomolybdate color regent was added to each reaction mixture, and the optical density of each sample was read at a wavelength of 520 nm. The amount of reducing sugar liberated by hydrolysis of sucrose in the samples was determined by comparison of samples with a standard curve prepared with equimolar amounts of glucose and fructose.

Adherence assay. Adherence of strains to glass was demonstrated by the technique described by Schroeder et al. (22). Pyrex borosilicate glass culture tubes (13 by 100 mm) containing 8 ml of BHI broth alone or supplemented with 2% sucrose were inoculated with 50 μl of an overnight culture, and cultures were grown for 24 h at 37°C. Culture fluid was poured out, and the tubes were rinsed with 5 ml of fresh BHI broth three times. Tubes were filled with 10% aqueous solution of crystal violet and allowed to stand for 1 min to stain adherent cells. Tubes were rinsed three times with distilled water following decanting of the crystal violet solution.

Virulence testing. The young gnotobiotic rat model which was used to assess the virulence of wild-type and isogenic mutants in exopolysaccharide production has been previously described (1, 22). In general, germ-free Fischer rat pups, 19 days of age, were inoculated with an overnight culture of the test strain (approximately 2×10^8 cells per ml). Rats were fed diet 305 which contained 5% sucrose ad libitum. After 35 days, the animals were sacrificed and their mandibles were removed for microbiologic and caries analysis. In this study, one mandible was used for microbiologic analysis (see below) and then both were stained, hemisectioned, and examined for caries by the method of Keyes (9). For microbiologic analysis, one mandible from each rat was placed in a 3-ml volume of phosphate buffer, and following sonication, serial dilutions were plated on MS agar, BHI agar containing appropriate antibiotics (kanamycin [500 μg/ml], tetracycline [5 μg/ml], and erythromycin [5 μg/ml] alone or in combinations), and BHI agar to test for contaminating colonies. Following incubation for 2 days at 37°C in an atmosphere of 95% nitrogen and 5% carbon dioxide, the number of *S. mutans* CFU per mandible was determined and averaged for each group of animals corresponding to a specific test strain. Representative colonies from individual analyses were examined to verify that cells isolated from rats were the original test strains. Chromosomal DNA was prepared and examined by Southern hybridization as described above. DNA probes from the drug resistance genes used to inactivate Gtf and/or Ftf determinants were used to confirm the identities of representative strains recovered from animals. Extracellular protein fractions were prepared from cultures of the representative strains and assayed for in vitro exopolysaccharide synthesis as described above.

RESULTS

Mutant construction and characterization. By the method of allelic exchange (1, 14, 22), mutants carrying single and multiple mutations of all combinations of the *gtfB*, *gtfC*, *gtfD*, and *ftf* genes have been made. Insertional inactivation of *ftf* in V403 has been previously described (22). Simultaneous insertional inactivation of *gtfB* and *gtfC* was accomplished as summarized in Fig. 1. The 8-kb *Eco*RI fragment which was isolated from a lambda library of *S. mutans* LM7 chromosomal DNA and contained a portion of the *gtfC* gene and the ligation of a 3-kb portion of this fragment defined by *Eco*RI and *Sst*I sites to pOP203 in the construction of pVA1392 have been previously described (19). A 300-bp *Hin*dIII-*Hin*dIII segment internal to the *gtfC* coding region was deleted from pVA1392 in *E. coli*, and the resultant plasmid was designated pVA1394. The *Cla*I fragment of pVA1394 was then ligated to a pACYC184 vector (which had its unique *Hin*dIII site deleted and thus did not confer tetracycline resistance) at the vector's *Cla*I site in order to provide the plasmid with a unique *Sal*I site; this construct, pVA1396, now had a single *Hin*dIII site, and ligation with the *Hin*dIII fragment of pDL406 (11) inserted a kanamycin resistance gene from *Enterococcus faecalis* into the *Hin*dIII site internal to the *gtfC* sequence. The *Hin*dIII fragment of pDL406 also carried a streptomycin resistance determinant, but this marker was not used in these studies. The final construct, pVA1444, had four important features. First, it contained a portion of the *gtfC* sequence which provided an area of homology to the *gtfB* and *gtfC* genes on the V403 chromosome necessary for allelic exchange to occur. A single reciprocal recombination event could therefore result in the exchange of the kanamycin resistance determinant for both *gtfB* and *gtfC* in cases in which one flanking *gtfC* sequence of pVA1444 recombines with the chromosomal copy of *gtfB* and the other flanking *gtfC* sequence of pVA1444 recombines with *gtfC* on the chromosome. Second, the *gtfC* sequence on pVA1444 was insertionally interrupted by a kanamycin resistance marker selectable in *S. mutans*. Third, the plasmid contained an origin of replication which was functional in *E. coli* but nonfunctional in *S. mutans*. Fourth, a unique *Sal*I restriction site was present, permitting linearization of the plasmid prior to transformation. Kanamycin-resistant transformants could arise by only a reciprocal recombination event that resulted in the exchange of the wild-type gene for the defective one. pVA1444 was linearized with *Sal*I and transformed into *S. mutans* V403 or V1741 (which was an *ftf* mutant), and transformants were selected on plates containing kanamycin. Although transformants which carried a defective copy of only *gtfC* (V1785 and V1788) or only *gtfB* (V1787 and V1804) were obtained, we focused on strains which had either both genes intact (V403 and V1741) or both genes insertionally inactivated (V1786, V1789, V1995, and V1996).

Insertional inactivation of the *gtfD* in test strains also was accomplished by allelic exchange. In this case, V403 was transformed with chromosomal DNA from *S. mutans* GS5-DD1, which had a tetracycline resistance cassette inserted into *gtfD* (6). Chromosomal DNA from tetracycline-resistant colonies obtained from this primary transformant then was used to transform V403, V1786, and V1789, and transformants were again selected on agar containing appropriate antibiotics.

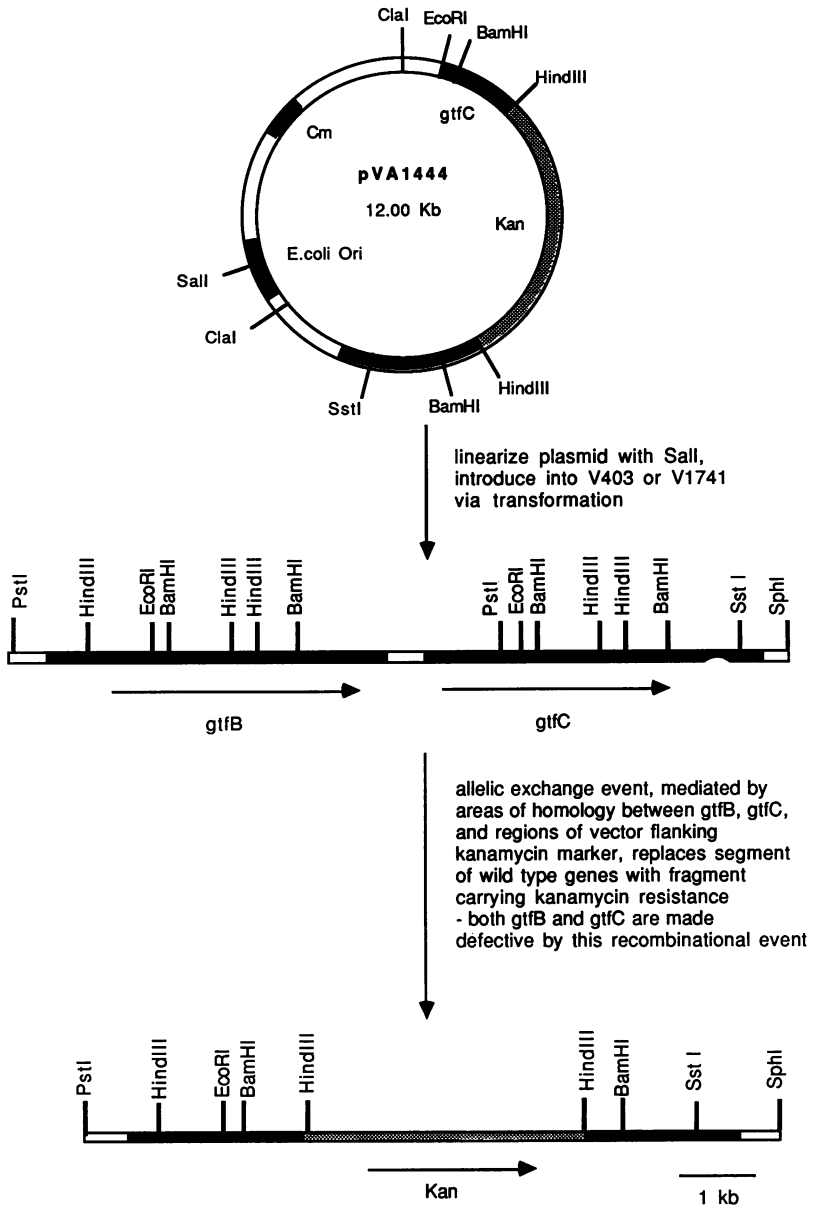

FIG. 1. Specific mutant construction by allelic exchange. pVA1444 contained portions of the *gtfC* gene interrupted by a kanamycin resistance determinant which was expressed in *S. mutans*. The plasmid was linearized with *SalI* (a unique restriction site in pVA1444) and introduced into *S. mutans* V403 or V1741 via transformation as described in Materials and Methods. *gtfB* and *gtfC* have high sequence homology (18, 27). A reciprocal recombination event between areas of homology between *gtfB* on the target cell's chromosome and the 5′ portion of the *gtfC* region of pVA1444 and between the chromosomal *gtfC* and the 3′ portion of the *gtfC* region of pVA1444 replaced the segment of chromosome containing *gtfB* and *gtfC* with the fragment of pVA1444 containing the kanamycin resistance gene.

Analysis of Southern blots confirmed that the expected allelic-exchange events had occurred (see Fig. 2). In order to demonstrate the presence or absence of wild-type *gtfB* and *gtfC*, chromosomal DNA from V403 and mutant strains was digested with *Eco*RI and Southern blotted with the 300-bp *Hin*dIII-*Hin*dIII fragment deleted from the internal portion of the *gtfC* region in construction of pVA1394 labeled with ^{32}P as a probe (Fig. 2, panel 1). Two bands, corresponding to *gtfB* and *gtfC*, were present in the lane containing V403. In lanes containing V1986 and V1989, both bands were absent. *Xba*I digests of DNA from the strains were Southern blotted with ^{32}P-labeled pNH5 as a probe (Fig. 2, panel 2). In V403, V1986, and V1989, two bands corresponding to the predicted fragments of the wild-type *gtfD* were present. In V1995 and V1996, two bands were present, but the larger band demonstrated a shift in mobility consistent with the insertion of the 5.4-kb tetracycline resistance determinant. DNA from V403, V1986, and V1989 was digested with *Eco*RI and Southern blotted with the ^{32}P-labeled *ermAM* portion of pVA1668 as a probe (Fig. 2, panel 3). A single band was present in V1989, while no bands were detected in V403 or V1986.

The mutant strains did not differ from the wild type in growth rates in BHI broth, colonial morphology on BHI agar plates, or microscopic appearance when Gram stained. On

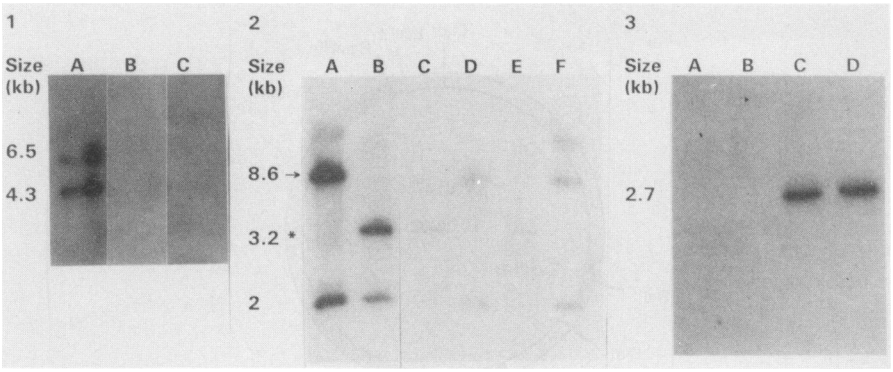

FIG. 2. Analysis of allelic exchange events. Total cellular DNA from *S. mutans* strains was cleaved with restriction endonucleases, electrophoresed through 1.0% agarose, and bidirectionally transferred to nitrocellulose. Probes were prepared by nick translation with [^{32}P] dCTP. Photographs of autoradiographs are shown. (Panel 1) DNA cleaved with *Eco*RI, probed with the 300-bp *Hin*dIII-*Hin*dIII fragment deleted from the internal portion of *gtfC* region in construction of pVA1394. The probe hybridizes to a fragment containing the wild-type *gtfC* which migrates at the same rate as a 4.3-kb lambda *Hin*dIII marker fragment and to a highly homologous portion of a 6.5-kb fragment containing the wild-type *gtfB*. Lane A, V403; lane B, V1786, lane C, V1789. (Panel 2) DNA cleaved with *Xba*I, probed with pNH5. The *gtfD* gene was divided into two fragments by *Xba*I digestion. The smaller fragment appeared constant in all lanes, while the larger fragment (3.2 kb) increased in size consistent with insertion of the 5.4-kb tetracycline resistance gene in mutants with insertional inactivation of *gtfD*. The larger wild-type fragment is indicated by an arrow, while the corresponding band in *gtfD* mutants is indicated by an asterisk. Lane A, GS5-DD1; lane B, V403; lane C, V1786; lane D, V1995; lane E, V1789; lane F, V1996. (Panel 3) DNA cleaved with *Eco*RI, probed with *ermAM* determinant from pVA1668. A 2.7-kb fragment was detected in strains which contain the *ftf* gene inactivated with *ermAM*. Lane A, V403; lane B, V1786; lane C, V1741; lane D, V1789.

MS agar, V403 exhibited the characteristic gumdrop appearance associated with hyperfructan production noted by Schroeder et al. (22). V1786 and V1995 also had a gumdrop colonial morphology on MS agar. V1741, V1789, and V1996 (all of which were *ftf* mutants) colonies grown on MS agar were small and crystalline in appearance.

Analysis of enzymatic activity of extracellular preparations of mutant strains. Analysis of extracellular protein preparations from the wild-type and mutant strains was performed with SDS-PAGE activity gels. Results of treatment of sucrose-incubated gels with periodic acid and Schiff's reagent were as follows (Fig. 3). In the lanes containing extracellular material from V403, stained material was present at the 150- and the 90-kDa levels, corresponding to Gtf and Ftf activity, respectively. In the *ftf* mutant, V1741, stained components were present at the 150-kDa level, but no activity signal was seen at 90 kDa. In the lane containing V1786 (GtfB$^-$ GtfC$^-$ GtfD$^+$ Ftf$^+$), activity signals were observed at the 150- and 90-kDa levels, but the signal at 150 kDa was fainter in intensity than the corresponding signal in the wild type (indicating the presence of GtfD activity alone). A faint activity signal was also detected at 150 kDa in V1789 (GtfB$^-$ GtfC$^-$ GtfD$^+$ FtfD$^-$), but no signal was seen at 90 kDa. An activity signal was present at the 90-kDa level in V1995 (GtfB$^-$ GtfC$^-$ GtfD$^-$ Ftf$^+$), but no signals were observed at the 150-kDa level. V1996 (GtfB$^-$ GtfC$^-$ GtfD$^-$ Ftf$^-$) did not have any stained material detectable by this technique.

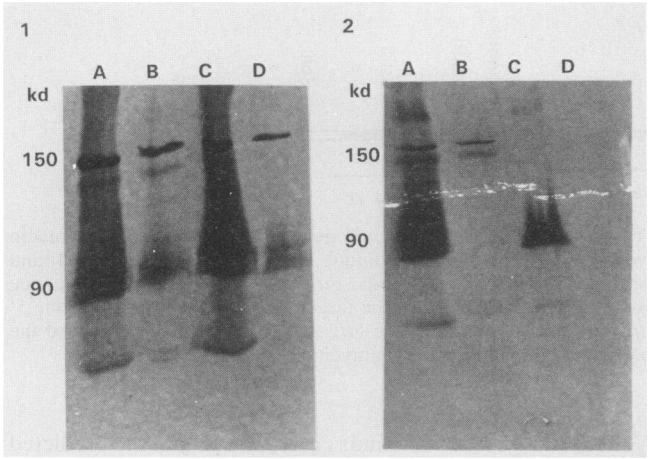

FIG. 3. Analysis of Gtf and Ftf activity of extracellular protein products of *S. mutans* strains. For SDS-PAGE (10% acrylamide concentration) of proteins obtained from acetone precipitation of culture supernatants, soaked in sucrose buffer, and treated with periodic acid-Schiff's reagent, equivalent amounts of protein from each strain were loaded. The molecular masses in kilodaltons were obtained by comparison of positions of standard markers and bands on duplicate halves of the same gel stained with Coomassie brilliant blue. (Panel 1) Lane A, V403; lane B, V1741; lane C, V1786; lane D, V1789. (Panel 2) Lane A, V403; lane B, V1741; lane C, V1995; lane D, V1996. Some background staining is present in panel 1 (compare lanes B).

Assays of glucosyltransferase and fructosyltransferase activity which used radiolabeled sucrose as a substrate for in vitro polymer formation were performed to provide quantitative information about the enzymatic activity of the strains. The results, summarized in Table 2, indicated that extracellular protein products from mutant strains differed from those of the wild type in production of glucan and fructan. Interestingly, the Ftf$^-$ mutant V1741 (Table 2) displayed higher activity than the wild type in assays using glucose-labeled sucrose; this Ftf$^-$ strain also had a larger ratio of water-soluble to water-insoluble glucan. These results confirmed earlier studies which indicated that the Ftf$^-$ mutant with intact glucosyltransferase genes produced larger quantities of glucan than did the wild-type organism (22). In V1786 and V1789 (Table 2), which carried an intact *gtfD* gene, activity in assays using glucose-labeled sucrose was detectable but was considerably less than wild-type activity. Assays of V1995 and V1996 (Table 2) with glucose-labeled sucrose did not demonstrate detectable activity, although V403 material was active in the same experiments. While V1786 and V1995 had activities comparable to that of V403

TABLE 3. Virulence of *S. mutans* V403 and isogenic mutants in monoinfected gnotobiotic rats

Strain	Genotype[a]				No. of rats	Mean ± SE caries score[b]							
						Buccal			Sulcal			Proximal	
	gtfB	*gtfC*	*gtfD*	*ftf*		E	DS	DM	E	DS	DM	E	DS
V403	+	+	+	+	12	12.2 ± 0.5	8.4 ± 0.6	5.2 ± 0.3	16.8 ± 0.5	11.5 ± 0.6	3.3 ± 0.5	2.8 ± 0.3	0
V1741[c]	+	+	+	−	14	9.9 ± 0.5	6.0 ± 0.3	3.7 ± 0.4	15.3 ± 0.5	8.7 ± 0.6	2.5 ± 0.3	1.1 ± 0.3	0
V403	+	+	+	+	9	12.3 ± 0.4	9.4 ± 0.4	6.1 ± 0.4	17.8 ± 0.6	14.1 ± 0.8	6.9 ± 1	3.6 ± 0.6	0.7 ± 0.3
V1786	−	−	+	+	15	9.7* ± 0.4	6.9* ± 0.5	4.1* ± 0.4	16.7 ± 0.6	11.9** ± 0.6	4.5** ± 0.5	1.9** ± 0.4	0.1 ± 0.1
V403	+	+	+	+	5	11.6 ± 0.2	8.6 ± 0.2	5.8 ± 0.4	17.6 ± 0.2	13.0 ± 0.7	5.6 ± 0.4	2.6 ± 0.8	0.6 ± 0.5
V1789	−	−	+	−	7	8.3* ± 0.4	6.4* ± 0.4	2.7* ± 0.5	14.6* ± 0.6	8.7* ± 0.6	2.6* ± 0.4	0*	0
V403	+	+	+	+	6	11.5 ± 0.7	9.8 ± 0.8	6.7 ± 0.7	16.3 ± 0.7	12.7 ± 0.6	6.0 ± 0.5	1.3 ± 0.4	0.2 ± 0.2
V1995	−	−	−	+	6	8.3** ± 1	5.0* ± 0.8	2.0* ± 0.8	14.5** ± 0.3	9.2* ± 0.5	2.8* ± 0.7	0.3 ± 0.3	0
V1996	−	−	−	−	6	8.9 ± 1.1	6.7** ± 1	3.3* ± 0.7	13.3** ± 0.8	7.8* ± 1.2	2.8* ± 0.5	0*	0

[a] +, wild type; −, mutant.

[b] Caries scores were determined by the Keyes procedure (9). Abbreviations: E, enamel involvement; DS, slight dentinal involvement; DM, moderate dentinal involvement. Significance of difference (experimental strain score versus V403 score); *, $P < 0.01$; **, $P < 0.05$.

[c] Values of V403 versus V1741 enamel and moderate dentinal involvement were previously published by Schroeder et al. (22) and are provided here for purposes of comparison.

in assays in which fructose-labeled sucrose was used as a substrate, V1741, V1789, and V1996 had low levels of radioactivity which were similar to those of negative controls in the same experiments.

The abilities of extracellular preparations from V403 and V1996 to hydrolyze sucrose were compared. In a 1-h assay, the reaction mixture containing V403 liberated 46.9 μg of reducing sugar per μg of protein; reaction mixtures prepared with V1996 liberated less than 0.1 μg of reducing sugar per μg of protein.

Adherence to glass. The ability of strains to adhere to glass was tested. Parallel cultures were grown in BHI broth alone or BHI broth supplemented with 2% sucrose. After 24 h of growth, the culture fluids were decanted, the flasks were rinsed three times with fresh BHI broth, and the adherent cells remaining on the glass interior surface of the culture vessel were stained with a 10% aqueous solution of crystal violet. V403 adhered well in the presence of sucrose, but minimal adherence was noted when the wild-type strain was grown in BHI broth. Strains which contained lesions in the glucosyltransferase genes did not demonstrate adherence whether sucrose was present or not.

Virulence in the young gnotobiotic rat model. The ability of mutant strains to cause caries lesions was compared with that of the wild-type V403 in a germ-free rat model system. Thirty-five days following infection with either V403 or one of its isogenic mutants, rats were sacrificed and their molar teeth were scored for caries lesions as described by Keyes (9). The results of these experiments are summarized in Table 3. The mean caries scores of lesions in rats infected with mutant strains were significantly lower than the scores of rats infected with V403 on buccal, sulcal, and proximal surfaces with few exceptions in specific levels of involvement. Although proximal enamel involvement (Table 3) in rats infected with V1786, V1789, and V1996 was significantly different from that in rats infected with V403, no differences in proximal dentinal involvement were noted. Buccal and sulcal scores were significantly different at all levels of involvement, with the exception of buccal enamel lesions in V1996-infected rats and sulcal enamel lesions in V1786-infected rats.

The number of CFU recovered from rat mandibles was determined. One mandible from each rat was placed in a 3-ml volume of phosphate buffer and sonicated, and serial dilutions were plated on MS agar, BHI agar, and BHI agar containing appropriate antibiotics (kanamycin [500 μg/ml], tetracycline [5 μg/ml], and/or erythromycin [5 μg/ml]). The average number of CFU differed for groups of animals inoculated with specific test strains. The CFU counts on MS agar for animals inoculated with V403 in these experiments ranged from 1.35 (±0.4) × 10^6/ml to 2.34 (±0.95) × 10^8/ml. Mandibles of animals inoculated with V1786 yielded 6.65 (±2.5) × 10^7 CFU/ml, and those from animals inoculated with V1789 yielded 4.1 (±0.96) × 10^7 CFU/ml. The mean number of CFU recovered from animals inoculated with V1995 was 8.8 (±1) × 10^5/ml, and 3.0 (±1.8) × 10^5 CFU/ml were recovered from animals inoculated with V1996.

In order to verify that strains retained the appropriate genotype and exopolysaccharide production phenotype following passage in the rat, isolates from the animals were characterized genetically and biochemically. Isolates were identified only by the alphanumeric code used in rat experiments (rather than strain number) until analysis was complete. Chromosomal DNA was prepared from all isolates for Southern blots. *Eco*RI-digested DNA was probed with pVA1444 in order to demonstrate the *gtfB* and *gtfC* genes and probed with pVA1668 to examine *ftf*. *Xba*I-digested chromosomal DNA was probed with pNH5 in order to demonstrate insertion into the *gtfD* region. In each case, the pattern of bands observed from the rat isolate was the same as that of the original infecting strain (data not shown).

The phenotypes of isolates on BHI plates containing kanamycin, tetracycline, erythromycin, or a combination of these drugs were consistent in each case with the phenotype of the strain inoculated into the rat from which the isolate was obtained. Three isolates which differed from each other in growth on antibiotic plates and Southern blot band patterns were selected for further analysis. Following analysis, the identities of these isolates were determined to be V403, V1995, and V1996. Extracellular proteins from these three isolates were prepared by acetone precipitation of culture supernatants and assayed for exopolysaccharide production in vitro with radiolabeled sucrose as a substrate as described above. Results are reported in Table 4 and confirmed that the

TABLE 4. Phenotype of selected isolates after passage in the rat

Strain	Growth on agar[a]				Exopolymer production in vitro[b] of:		
	BHI	BHI-kanamycin-tetracycline	BHI-kanamycin-tetracycline-erythromycin	MS	Total glucan	Water-insoluble glucan	Fructan
V403	+	−	−	+	220	92	869
1995	+	+	−	+	0	0	1,031
1996	+	+	+	+	0	0	0

[a] Agar containing antibiotics prepared as described in Materials and Methods. +, growth; −, no growth.

[b] Mean counts per minute per microgram of protein for three replicates from single assay.

strains isolated from these rats at the conclusion of the experiment were indistinguishable from the inoculated strain in antibiotic resistance phenotype and pattern of exopolysaccharide production.

DISCUSSION

S. mutans has the ability to make insoluble glucans because of the polymer-forming enzymes which are produced by *gtfB* and *gtfC*. Soluble-glucan production is accomplished by the enzymes encoded by *gtfC* and *gtfD*. Fructan is produced by the *ftf* product. The construction of these strains, which differed genetically only in the regions coding for selected enzymes of sucrose metabolism, provided an excellent opportunity to explore the contribution of production of particular exopolymers to virulence in the rat caries model.

Although insertional inactivation by allelic exchange results in a well-defined lesion, effects on the expression of genes downstream from the lesion are possible. No obvious changes in phenotypes of mutant strains other than the expected growth on antibiotics and colonial morphology changes on MS agar were detected. The mutant strains did not differ from the wild type in growth rate in BHI broth or colonial morphology on BHI agar. Furthermore, we consider the hypothesis that alteration in downstream genes contributes to mutant phenotypes to be unlikely in the case of these mutants, since sequence data indicate that inverted repeat sequences characteristic of rho-independent termination sequences are located 200 bp downstream from the *gtfD* stop codon (7), 78 bp downstream from the *ftf* stop codon (24), and 621 bp downstream (following a 185-bp open reading frame) from the *gtfC* stop codon (27).

Protein preparations from those strains in which both *gtfB* and *gtfC* were inactivated lost the ability to produce water-insoluble glucan in the in vitro assay of glucosyltransferase activity. In strains in which all three glucosyltransferase enzymes were insertionally inactivated (V1995 and V1996), the ability to make water-soluble and water-insoluble glucan was lost. Strains derived from V1741 (V1789 and V1996), which had an insertional inactivation of *ftf*, lost the ability to form fructan in the in vitro fructosyltransferase activity assay. When tested for the ability to liberate reducing sugars from sucrose, V1996 demonstrated a loss of the ability to hydrolyze sucrose. These phenotypes were consistent with the appearance of the extracellular protein preparations of the strains on SDS-PAGE treated with periodic acid-Schiff's reagent and remained stable following passage in the rat.

The virulence of these isogenic strains was examined in the rat caries model. V1996, which had all four exopolysaccharide genes insertionally inactivated (Table 3), was less cariogenic than the wild type in all areas and levels of involvement except buccal enamel lesions. V1789 differed from V1996 only in that V1789 carried an intact *gtfD* gene. V1789 produced caries scores which were significantly ($P < 0.01$) lower than those associated with the wild type (Table 3), and differences between V1789 and V403 had a higher level of statistical significance than did the differences between V1996 and V403 (Table 3). This suggests that the product of the *gtfD* gene does not contribute greatly to virulence in a *gtfB* mutant, *gtfC* mutant, *ftf* mutant background.

In cases in which *ftf* was intact, the role of *gtfD* was less clear. V1995 was less cariogenic than the wild type in all sites except proximal (Table 3). V1786 was also less virulent than the wild type, except in sulcal enamel and proximal dentinal lesions (Table 3). Neither strain appeared to achieve scores which had higher statistical significance than the other in comparison with the wild type.

All four strains in which *gtfB* and *gtfC* had been insertionally inactivated (V1786, V1789, V1995, and V1996) were less virulent than the wild type (Table 3). All four strains were also less adherent to glass culture tubes than the wild type when incubated in media containing sucrose. Since neither V1786 nor V1789 was able to adhere to glass, GtfD alone cannot mediate adherence in the glass culture tubes. This clearly supports the hypothesis that water-insoluble glucan, necessary for adherence to glass in vitro, is an important aspect of virulence in the animal model.

Schroeder et al. (22) reported evidence that the Ftf-deficient V1741 was less cariogenic in the rat than its wild-type parent, V403 (provided for purposes of comparison in Table 3). Interestingly, it appeared that in one set of mutants (V1786 and V1789, in which *gtfD* remained intact) the rats inoculated with the Ftf-deficient mutant had somewhat lower caries scores than did rats inoculated with an otherwise similar mutant (Table 3). Differences between V403 and V1789 were all significant at $P < 0.01$ (Table 3); differences between V403 and V1786 were significant at $P < 0.01$ in buccal lesions and $P < 0.05$ or less in sulcal and proximal lesions (Table 3). Since the results for V1741, V1786, and V1789 were each obtained from different experiments, no statistical conclusions are drawn in this case. However, no similar clear pattern was seen in the caries scores for V1995 and V1996 (which were both obtained from the same experiment). In some locations and levels of involvement, V1995 was associated with higher caries scores than V1996; in other locations and levels of involvement, V1996 had higher scores than did V1995.

A decrease in the cariogenicity of glycosyltransferase-deficient strains may be related to a decreased adherence to the tooth surface. This hypothesis is supported by the evidence that strains with lesions in *gtfB* and *gtfC* were less adherent to glass surfaces than the wild-type organism when grown in sucrose. Also, fewer organisms were recovered from the animals infected with glucosyltransferase mutants, indicating that they were not as efficient in colonization of rats as the wild-type organism even though they grew equally well in culture. However, decreased ability to colonize the tooth surface may not be the only explanation for reduced cariogenicity of mutants, since V1741, which adheres comparably to the wild-type strain, demonstrated reduced cariogenicity in previous work (22). Schroeder et al. (22) suggest that fructan may serve as an extracellular reserve of carbohydrate, enabling the organism to survive

periods of nutrient deprivation; the exopolymer thus would provide a survival advantage to the wild-type organism which *ftf*-deficient mutants would lack. The use of isogenic mutants with well-defined lesions of glucosyltransferase and fructosyltransferase genes to explore cariogenicity of *S. mutans* has never before been reported. The results we have obtained, using mutants constructed by allelic exchange, confirm the importance of water-insoluble glucan, water-soluble glucan, and fructan to cariogenicity (3, 14).

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