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Biochemical Characterization and Evaluation of Virulence of a Fructosyltransferase-Deficient Mutant of *Streptococcus mutans* V403

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The *Streptococcus mutans* extracellular fructosyltransferase (FTF) enzyme may play a role in the formation of dental caries by synthesizing a fructan polymer that serves as an extracellular storage polysaccharide. We sought to determine if an FTF-deficient strain of *S. mutans* was less virulent than wild-type cells in a rat animal model system. Cloned *ftf* gene sequences from *S. mutans* GS5 were used to generate a defective copy of the *ftf* gene by inserting into the *ftf* coding region a DNA fragment which encoded erythromycin resistance. The plasmid which carried the defective *ftf* construct was introduced into *S. mutans* V403 by using genetic transformation. This defective construct replaced, by allelic exchange, the wild-type copy of the *ftf* gene carried on the V403 chromosome. FTF activity assays indicated that the recombinant strain, V1741, was deficient in fructan synthesis. However, extracellular protein preparations from this strain displayed an increased ability to generate glucose polymers (glucans) compared with V403 preparations. Levels of adherence to glass and rat tooth surfaces by strain V1741 were similar to those of the V403 strain. Both strains caused moderate decay on rat tooth surfaces; however, the FTF-deficient strain was less pathogenic compared with the wild-type strain. These results suggest that FTF activity contributes to the pathogenicity of *S. mutans* V403, possibly by generating extracellular fructans which serve as storage compounds.

Streptococcus mutans is recognized as the primary etiological agent of dental caries in humans (14, 18, 26). Studies with human and animal model systems suggest that dietary sucrose is required for high levels of caries-forming activity (16, 26, 44). In the absence of sucrose, *S. mutans* cells reversibly attach to smooth tooth surfaces through interactions between the acquired enamel pellicle and bacterial cell surface components (39). However, a more tenacious attachment is achieved through synthesis of adherent extracellular glucose polymers (glucans). Extracellular glucosyltransferase (GTF) enzymes of *S. mutans* polymerize the glucose moiety of sucrose into a heterogeneous group of extracellular water-soluble and water-insoluble glucan polymers, releasing fructose as a by-product (18, 26). Studies with animal model systems indicate that water-insoluble glucan synthesis is a virulence factor required for efficient tooth colonization (17, 45).

In addition to glucan synthesis, most human isolates of *S. mutans* synthesize one or more antigenically distinct fructosyltransferase (FTF) (EC 2.4.1.10) enzymes which direct the synthesis of fructose polymers (fructans) from sucrose, releasing free glucose (5, 15, 33). Fructans synthesized by *S. mutans* consist primarily of inulin-like β(2->1) linkages with occasional branching at the 6 position of the fructose moiety (3, 6, 40). Fructan may serve as an extracellular carbon reserve under conditions of nutrient starvation. This hypothesis is based on the demonstration of an extracellular fructan hydrolase activity (exo-β-D-fructosidase; 4) from *S. mutans* and other oral bacteria which is able to degrade inulin and levan-type fructans into fructose monomers (43, 48, 49). In theory, these monosaccharides would then be transported into the cell and would be used as a substrate for glycolysis (48).

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A gene coding for the *S. mutans* GS5 FTF has been cloned and its nucleotide sequence determined (40, 41). In this communication, we report the use of this cloned DNA sequence to generate an FTF-deficient strain of *S. mutans* V403. This mutant strain was devoid of FTF activity and displayed an increased ability to synthesize glucan polymers from sucrose, suggesting some type of interaction between FTF and GTF in this strain. The FTF-deficient mutant was less virulent than its wild-type parent in an animal model system, suggesting that FTF activity plays a role in caries pathogenesis.

MATERIALS AND METHODS

Bacterial strains and media. *Escherichia coli* DB11 was obtained from Julian Davies (Institute Pasteur; 31). *E. coli* cells were grown aerobically at 37°C in Lennox broth (GIBCO Diagnostics, Madison, Wis.) supplemented with 0.5% glucose. Erythromycin or tetracycline was added to Lennox broth agar to a final concentration of 10 μg/ml for selection of *E. coli* transformants. *Streptococcus mutans* LM7 (Bratthall serotype e) was used as a control in this study. *S. mutans* V403 (Bratthall serotype c) was originally obtained from R. R. Facklam (Centers for Disease Control, Atlanta, Ga.) and has been previously described (31). *S. mutans* cells used for enzyme assays, adherence assays, and polyacrylamide gel electrophoresis were grown in chemically defined FMC medium (47) under 10% nitrogen at 37°C. *S. mutans* cells grown for purposes of DNA isolation were cultivated in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) supplemented with 20 mM DL-threonine (Sigma Chemical Co., St. Louis, Mo.). Erythromycin (5 μg/ml) was added to Todd-Hewitt agar to select for *S. mutans* transformants. Mitis salivarius agar (Difco Laboratories) was used to examine the morphology of *S. mutans* colonies.

Plasmid and chromosomal DNA isolation and characteriza-

tion. *S. mutans* chromosomal DNA was isolated by the method of Marmur (34), with modification. Briefly, a 250-ml brain heart infusion broth culture (supplemented with 20 mM DL-threonine; Sigma) was grown to a final optical density (660 nm) of 0.8. Cells were harvested by centrifugation and washed twice in 50 ml of ice-cold water. Washed cells were suspended in 40 ml of prewarmed (37°C) osmotic buffer (0.4 M sucrose, 75 mM Tris base [pH 7.0], 2 mM $MgSO_4$, 2,000 U of mutanolysin [Sigma], 0.5 g of lysozyme [Sigma]). Cell suspensions were sonicated for two 15-s pulses at 250 watts (Branson 450 sonicator; Branson Sonic Power Co., Danbury, Conn.). A 160-ml portion of additional osmotic buffer then was added to the suspension. This cell suspension was incubated for 2 h at 37°C. After incubation, cells were pelleted and suspended in 30 ml of TE buffer (Tris base, 10 mM [pH 7.4]; EDTA, 1 mM). Two milliliters of 20% sodium dodecyl sulfate (SDS) was added dropwise to the cell suspension to promote cell lysis. Cell lysates were extracted twice with an equal volume of chloroform isoamyl alcohol (24:1). DNA was precipitated by adding 2 volumes of ice-cold ethanol and harvested by being spooled around a glass rod. Precipitated DNA was dissolved in 4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [Sigma]) and incubated with 0.2 mg of RNase per ml (Promega Biotec, Madison, Wis.) for 30 min. The DNA was extracted a second time with chloroform-isoamyl alcohol, harvested by ethanol precipitation, dissolved in 5 ml of 1× SSC and dialyzed against the same buffer at 4°C for 24 h.

E. coli plasmid DNA was isolated by the method of Clewell and Helinski (8). Isolation of *S. mutans* pVA403 was carried out as previously described (29). Plasmid pSS22, which was used in site-specific insertional mutagenesis experiments, was kindly provided by Howard Kuramitsu, Northwestern University (40).

Restriction endonuclease digests of plasmid and chromosomal DNA were carried out according to the instructions of the manufacturer and were analyzed by agarose gel electrophoresis. Size standards were linear bacteriophage lambda DNA digested with *Hin*dIII (Bethesda Research Laboratories). Southern hybridization analysis of DNA was performed as previously described (32, 42).

DNA enzymology. Klenow fragment (International Biotechnologies, Inc., New Haven, Conn.) was used according to the instructions described by the manufacturer to fill in recessed ends of restriction-digested plasmid DNA. DNA samples were incubated with Klenow fragment for 30 min at 25°C. Blunt-end DNA ligation reactions were performed with T4 ligase (International Biotechnologies) at 4°C for 18 h by using reagents supplied by the manufacturer. Restriction enzymes *Eco*RI and *Hin*dIII were purchased from Bethesda Research Laboratories.

Bacterial transformation. *E. coli* DB11 was transformed by the $CaCl_2$ method (32), with mid-log-phase cells harvested after reaching an optical density of 0.2 to 0.3 at 660 nm. *E. coli* transformants were tested for plasmid content by growth on antibiotic-containing medium (10 μg of erythromycin per ml). *S. mutans* was transformed by the method of Kuramitsu and co-workers (37, 38). *S. mutans* transformants were selected for by growth on media containing 5 μg of erythromycin per ml.

Preparation of *S. mutans* extracellular protein fractions. An overnight 25-ml culture of *S. mutans* cells in FMC broth was added to one liter of prewarmed (37°C) FMC medium to give an initial optical density at 660 nm of 0.05. Cultures were incubated at 37°C until they reached late log phase (12 to 15 h; optical density at 660 nm = 0.8). Cells then were pelleted by centrifugation (5,000 × *g*, 15 min, 4°C). Culture supernatants were precipitated with ammonium sulfate (70%, 10°C). The precipitate was harvested after a 24-h incubation at 4°C by centrifugation (12,000 × *g*, 30 min), dissolved in 15 ml of 10 mM phosphate buffer (8.0 mM Na_2HPO_4, 2.0 mM KH_2PO_4, pH 6.5) and dialyzed overnight against the same buffer at 4°C. Protein preparations were stored at −70°C for up to 2 weeks. Protein concentrations were determined by the method of Lowry by using bovine serum albumin to construct a standard curve (28).

Exopolysaccharide synthesis assay. Synthesis of total glucan or fructan exopolysaccharides by streptococcal extracellular supernatants was determined by the method of Germaine et al. (13). Water-insoluble glucan synthesis was determined by the method of Chassy et al. (7). Levels of fructan and glucan were determined by measuring the radioactive counts per minute emitted from both water-insoluble and methanol-insoluble reaction products. Levels of water-soluble glucan were inferred as the difference between total and water-insoluble glucan values. Assays were carried out in a 10 mM phosphate buffer. Glucan synthesis was measured by using [^{14}C]sucrose radiolabeled in the glucose position (Dupont, NEN Research Products, Boston, Mass.; 0.5 μCi at 313 mCi/mmole), while fructan synthesis was determined by using [^{14}C]sucrose radiolabeled in the fructose position (Dupont, NEN Research; 0.5 μCi at 316 mCi/mmole). Four micrograms of extracellular streptococcal protein was used for exopolysaccharide synthesis assays except where stated otherwise. Negative controls for enzyme assays consisted of heat-inactivated protein samples which were obtained by immersion of material in boiling water for 3 min. Exopolysaccharide synthesis reactions were carried out for 18 h at 37°C. Reactions were stopped by immersing samples in boiling water for 3 min. Assays were performed in triplicate, and averages were reported. Levels of glucose or fructose incorporated into polymer were expressed as counts per minute per milligram of protein.

Glucan synthesis inhibition assays. The extracellular protein preparation from *S. mutans* V403 was used to test its effect on glucan synthesis by extracellular proteins of the FTF-deficient strain, V1741. In these experiments, 2 μg of V403 extracellular protein was added to V1741 glucan synthesis assay reaction mixtures. Levels of glucan synthesis in the presence of V403 proteins were determined as described above. *S. mutans* GS5 extracellular proteins served as a negative control. A 2-μg sample of heat-inactivated V403 protein was used as an additional negative control. Similarly, 2 μg of trypsin- or chymotrypsin-digested V403 protein was added to glucan synthesis assay mixtures of V1741 extracellular protein. These trypsin- and chymotrypsin-treated samples of *S. mutans* V403 extracellular protein were generated by digesting 2 μg of streptococcal protein with 25 μg of the protease for 2 h at 25°C. Proteases were inhibited after digestion by adding phenylmethylsulfonyl fluoride (Sigma) to a final concentration of 10 mM. Strains V403 and V1741 were tested for dextranase activity by using previously described methods (2).

Adherence assays. Glass screw-cap tubes (16 by 75 mm) containing 20 ml of FMC medium supplemented with either 2% glucose or 2% sucrose were inoculated with 50 μl of an overnight culture and were grown for 18 h at 37°C. Culture fluids were poured off and the tubes were rinsed three times with 5 ml of fresh FMC medium. Adherent cells were stained by filling the tubes with a 10% aqueous solution of crystal violet (Difco Laboratories) and allowing them to stand for 1

min. The dye was decanted, and the tubes were rinsed three times with distilled water.

Gel electrophoresis and in situ exopolysaccharide synthesis assays. Electrophoresis of SDS–7.5% polyacrylamide gels was carried out by the method of Hames and Rickwood (19). After electrophoresis, gels were stained in Coomassie blue dye to detect protein bands. Identical tracks of the gel were stained by the periodate-Schiff base stain to identify protein bands which could generate polysaccharide in the presence of sucrose. These gels were soaked in a solution of 50 mM sodium acetate buffer [pH 6.5]–2% sucrose–0.5% Triton X-100 for 20 h at 37°C (21) to allow for the formation of glucan and fructan. The gels were washed twice in 7.5% acetic acid for 30 min to remove nonpolymerized substrate. The gel was transferred to a solution of 0.2% periodic acid for 1 h at 4°C. After incubation, gels were soaked in Schiff reagent (Sigma) for 2 h at 4°C. Gels were destained by using a solution of 1 N HCl–0.5% potassium metabisulfate at 4°C. Polysaccharides which were generated by GTFs or FTFs were identified as red-staining bands. These components were compared with protein bands stained by the Coomassie blue dye and size standards (High-molecular-weight standards; Bio-Rad Laboratories, Richmond, Calif.) to determine the apparent molecular masses (in kilodaltons [kDa]) of these enzymes.

Virulence testing. The young gnotobiotic rat model used to assess the virulence of V403 and V1741 has been described (1, 36). 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/ml). Rats were fed diet 305 ad libitum, which contained 5% sucrose. After 35 days, the animals were sacrificed and 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 (23, 24, 25). For microbiologic analysis, one mandible from each rat was placed in a 3-ml volume of phosphate buffer and, after sonication, serial dilutions were plated on mitis salivarius agar, mitis salivarius plates containing 10 μg of erythromycin per ml, and brain heart infusion agar to test for contaminating colonies. After incubation for 2 days at 37°C in an atmosphere of 95% nitrogen and 5% CO_2, the number of *S. mutans* CFU per mandible was determined and averaged for each group of animals corresponding to a specific test strain. In addition, representative colonies from individual animals were examined by colony blot analysis by using plasmid pVA403, a cryptic plasmid present in the strains used, as a probe to verify that cells isolated from the rats were the original test strains. Colony blot analysis was carried out as follows. Isolated cell colonies were transferred to nitrocellulose by placing the nitrocellulose paper directly on the agar plate. Blotted colonies were incubated on filter paper (Whatman 3MM) saturated with lysozyme buffer solution (0.4 M sucrose, 5 mM Tris base, 2 mM $MgSO_4$, 20 mg of lysozyme per ml [Sigma]) for 1 h at 37°C. Blots were transferred to a second filter paper saturated with a solution of 10% SDS (5 min, 25°C) to promote cell lysis. Each blot next was transferred to a third filter that was saturated with a DNA denaturing solution (1.5 M NaCl, 0.5 M NaOH; 5 min, 25°C). Finally, blots were soaked on a filter containing a neutralizing buffer (1.5 M NaCl, 0.5 M Tris base; 5 min, 25°C). Analysis of DNA from the lysed cells was carried out as described by Maniatis et al. (32) by using ^{32}P-labeled pVA403 DNA as a probe.

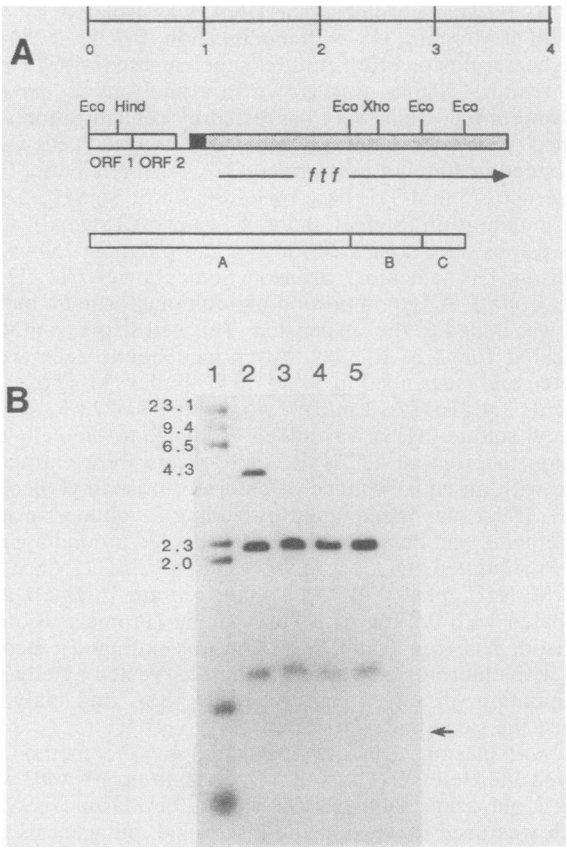

FIG. 1. Restriction endonuclease map of the *S. mutans* GS5 FTF gene and Southern blot hybridization of pSS22 to *S. mutans* chromosomal DNA. (A) A restriction endonuclease map of the *S. mutans* GS5 *ftf* gene is illustrated at the top of the figure. The plasmid pSS22 carries an insert containing three *Eco*RI restriction endonuclease fragments of 2.3, 0.7, and 0.33 kb, designated by the open rectangles A, B, and C indicated below the restriction map. Abbreviations of restriction endonucleases: Eco, *Eco*RI; Hind, *Hin*dIII; Xho, *Xho*I. The coding region of the *ftf* gene is represented by the stippled rectangle. The putative *ftf* promoter region is indicated by the black square. (B) Plasmid pSS22 was nick translated and used as a probe against plasmid and chromosomal *Eco*RI digests. Lanes: 1, lambda *Hin*dIII-digested size standards; 2, pSS22 digested with *Eco*RI; 3, *S. mutans* LM7 digested with *Eco*RI; 4, *S. mutans* GS5 digested with *Eco*RI; 5, *S. mutans* V403 digested with *Eco*RI. The arrow on the right indicates the position of a band representing the 0.33-kilobase *Eco*RI fragment which is not visible in this photographic exposure.

RESULTS

Insertional inactivation of *ftf* in *S. mutans* V403. A restriction endonuclease map of the *S. mutans* GS5 FTF gene (*ftf*), as deduced by DNA nucleotide sequence analysis, is illustrated in Fig. 1A (40, 41). Plasmid pSS22 contains the three *Eco*RI fragments, designated by the open rectangles A, B, and C (2.4, 0.7, and 0.33 kilobases [kb], respectively) shown below the map. The plasmid pSS22 was used as a probe to identify homologous *ftf* sequences in an *Eco*RI digest of *S. mutans* LM7, GS5, and V403 chromosomal DNA (Fig. 1B). The *Eco*RI digest of *S. mutans* LM7 chromosomal DNA was included in this experiment to serve as an alternate test strain for V403 in the event that the *ftf* gene from strain V403 did not share homology with the *ftf* from *S. mutans* GS5. *Eco*RI DNA digests of GS5 chromosomal DNA and plasmid

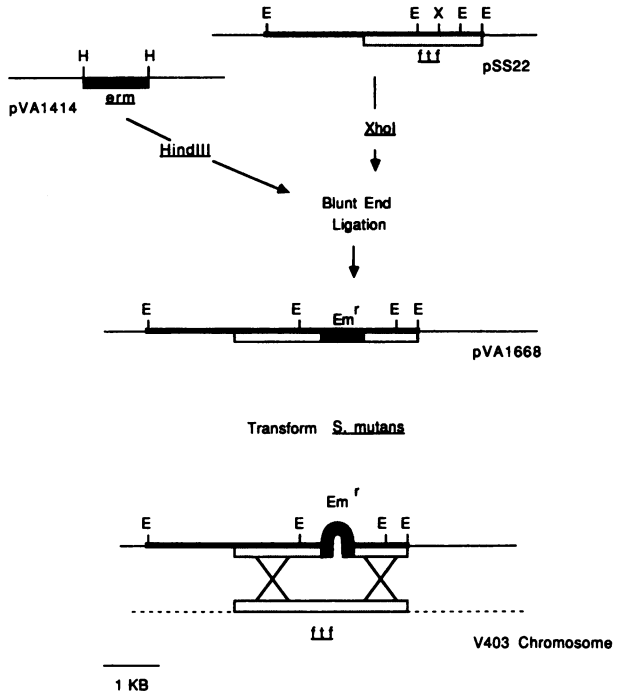

FIG. 2. Insertional inactivation scheme for the *S. mutans* V403 *ftf* gene. Restriction endonucleases: E, *Eco*RI; H, *Hin*dIII; X, *Xho*I. Symbols: ▨, *ftf* gene; ■, *ermAM* gene. DNA flanking the *ftf* gene in pSS22 and pVA1668 is designated by a thick line. The thin lines on pSS22, pVA1414, and pVA1668 represent vector sequences. The dashed lines represent sequences flanking the chromosomal copy of the *ftf* gene. Each x represents an area of predicted genetic crossover.

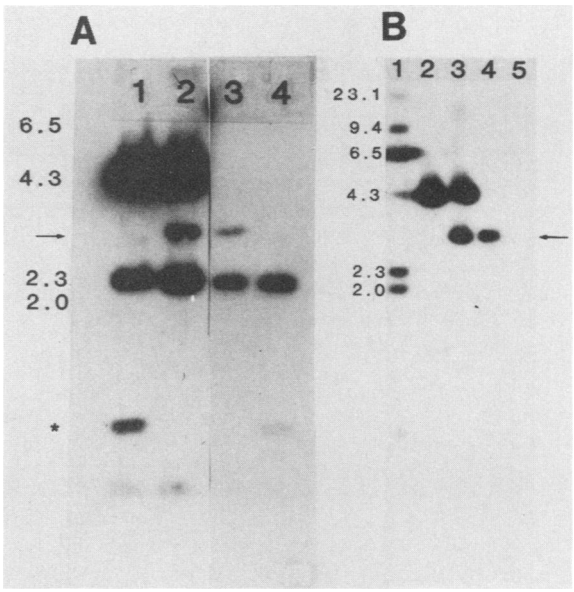

FIG. 3. Southern blot DNA hybridization of pSS22 and pVA891 to *S. mutans* chromosomal DNA. (A) Plasmid pSS22 was used as a probe. Lanes: 1, pSS22 digested with *Eco*RI; 2, pVA1668 digested with *Eco*RI; 3, *S. mutans* V1741 digested with *Eco*RI; 4, V403 digested with *Eco*RI. Migration of ethidium bromide-stained lambda *Hin*dIII-digested size markers is designated on the left. (B) Plasmid pVA891 was used as a probe. Lanes: 1, radiolabeled lambda *Hin*dIII digest molecular weight standards; 2, pSS22 digested with *Eco*RI; 3, pVA1668 digested with *Eco*RI; 4, V1741 digested with *Eco*RI; 5, V403 digested with *Eco*RI. pSS22 and pVA891 are both pACYC184 derivatives, and this accounts for the 4.3-kb hybridization signal seen in lanes 2 and 3.

pSS22 served as controls. The plasmid probe pSS22 hybridized with three DNA fragments of 2.4, 0.7, and 0.33 kb (marked by the arrow) in *S. mutans* V403 and LM7 DNA digests. These fragments corresponded in size to those cloned from *S. mutans* GS5. This hybridization to the V403 and LM7 chromosomes suggested that the cloned GS5 *ftf* gene could be used for in vitro modification of the *ftf* gene in either strain; however, strain V403 was used for the remainder of this study.

Insertional mutagenesis of the *ftf* gene of *S. mutans* V403 was carried out as illustrated in Fig. 2. Nucleotide sequence data (41) revealed a unique *Xho*I DNA restriction endonuclease site within the coding sequence of the GS5 *ftf* gene. This site was used to make a defective copy of the gene by inserting into it a DNA fragment which carried an erythromycin resistance (*ermAM*; 11, 28) determinant.

The plasmid vector, pSS22, was linearized with *Xho*I. Plasmid pVA1414 was digested with *Hin*dIII, liberating a fragment of 2 kb in size that contained the *ermAM* gene. Both DNA samples were treated with Klenow fragment to produce blunt ends. The resulting fragments were mixed and ligated together by using T4 DNA ligase. This ligation mixture was transformed into *E. coli* DB11, and transformants were selected by growth on erythromycin. Analysis of the erythromycin-resistant colonies confirmed the presence of the plasmid carrying the appropriate insert of 2 kb. This plasmid was designated pVA1668.

The recombinant plasmid, pVA1668, was linearized at a unique *Sal*I site contained within the vector sequence and was transformed into *S. mutans* V403. Because pVA1668 lacked a streptococcal origin of replication, erythromycin resistance was expected to be expressed only if the DNA sequences become integrated into the streptococcal chromosome. This would be the result if the defective copy of the gene exchanged with wild-type *ftf* sequences on the V403 chromosome. Southern blot analysis of erythromycin-resistant colonies was used to confirm this predicted recombination event. Radiolabeled plasmids pSS22 (carrying the *ftf* sequences) and pVA891 (which carried the same *ermAM* gene on a pACYC184 *E. coli* vector [30]) were used as probes against *Eco*RI DNA digests of V403 and a typical recombinant (designated V1741). *Eco*RI was chosen because the *Xho*I cloning site was carried on a 0.7-kb *Eco*RI fragment within the *ftf* coding region and was the site of insertion for the 2-kb *ermAM*-containing DNA fragment. Accordingly, we expected that the 0.7-kb *Eco*RI DNA fragment of the wild-type strain would appear larger in the insertional mutant strain. The 0.7-kb *Eco*RI fragment (marked by the asterisk in Fig. 3A) is seen in the plasmid pSS22 digest (Fig. 3A, lane 1) and in the wild-type *S. mutans* V403 (lane 4). This fragment was no longer present in the mutant V1741 strain (lane 3) or the mutant plasmid construct pVA1668 (lane 2). Instead, a new fragment of 2.7 kb appeared (indicated by the arrow at the left of the photograph), consistent with the predicted insertion into the 0.7-kb *Eco*RI fragment. This new 2.7-kb fragment also hybridized with the radiolabeled *ermAM* probe (Fig. 3B, lanes 3 and 4, marked by the arrow at the right of the photograph), confirming that the *ermAM* sequence was inserted into the original 0.7-kb *Eco*RI fragment, thus interrupting the *ftf* reading frame.

Phenotype properties of *S. mutans* V1741. Colonial mor-

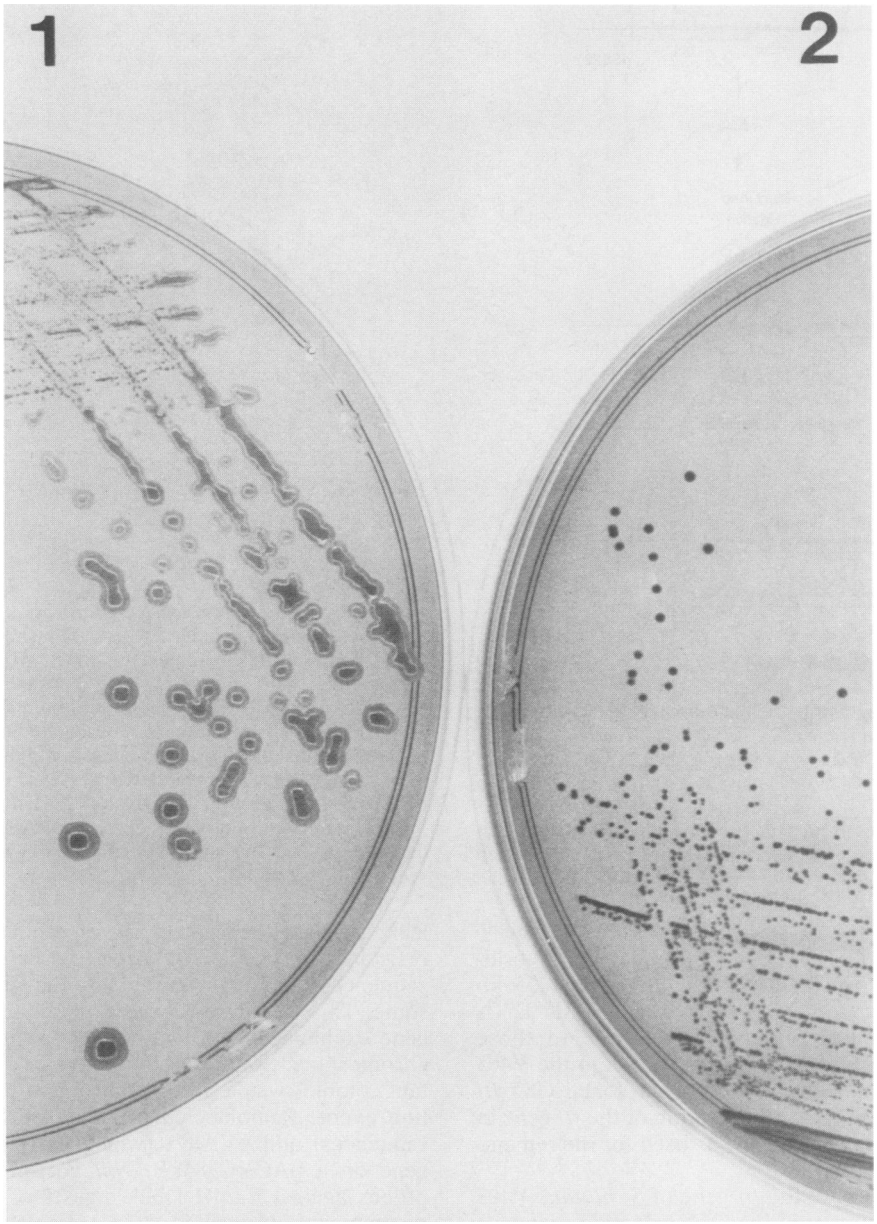

FIG. 4. Colonial morphology of *S. mutans* V403 and V1741 on mitis salivarius agar. 1, V403 colonies; 2, V1741 colonies.

phology of strain V1741 was examined by plating cells on mitis salivarius agar (Fig. 4). The wild-type V403 grew as large, glossy, gumdroplike colonies. In contrast, V1741 colonies were small and crystalline in appearance.

Strain V1741 was tested for the ability to colonize the surface of glass. Figure 5 is a photograph of tubes which contained cultures of either V403 (tubes 1 and 3) or V1741 (tubes 2 and 4). Sucrose (tubes 1 and 2) or glucose (tubes 3 and 4) was supplied as a sole carbon source for the growing cells. In the presence of sucrose, both wild-type and mutant strains adhered to the sides and bottoms of the glass tubes. In the presence of glucose, adherence by either strain was minimal.

SDS-polyacrylamide gel electrophoresis of extracellular proteins from strains V403 and V1741. Polyacrylamide gel electrophoresis of concentrated total extracellular proteins from strains V403 and V1741 was used to identify a protein band that corresponded to the FTF protein from wild-type strain V403 (Fig. 6). A protein component with an apparent molecular mass of 97 kDa was identified in V403 preparations which displayed polymer-forming activity (lanes 1 and 3). A corresponding protein was absent in lanes containing V1741 material (lanes 2 and 4). A component of approximately 82 kDa (lane 2) in V1741 preparations was detected by Coomassie blue stain, but this polypeptide lacked the ability to catalyze the synthesis of polysaccharides. Additional protein components exhibiting polymer-forming activity were in the size range of GTF activities reported for other serotype c *S. mutans* isolates (6, 21).

Extracellular polysaccharide synthesis in *S. mutans* V403 and V1741. Extracellular proteins of *S. mutans* V1741 were tested for the ability to synthesize fructan in the presence of

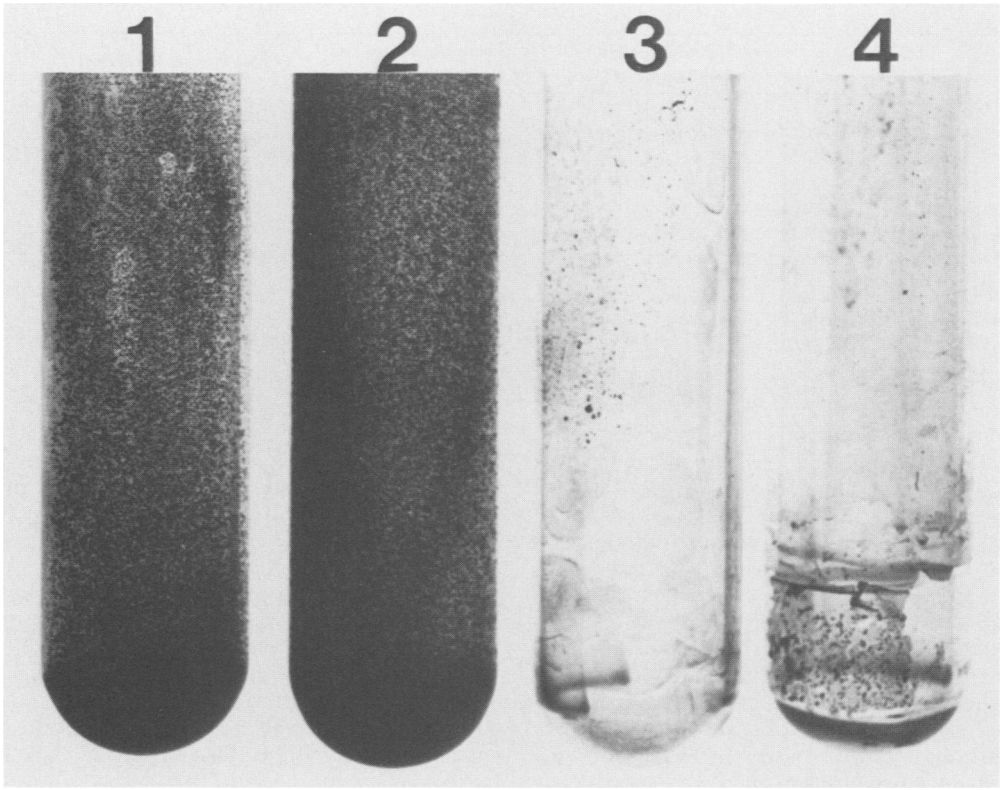

FIG. 5. Adherence of *S. mutans* V403 and V1741 to glass. Tubes: 1, V403 cells grown in sucrose; 2, V1741 cells grown in sucrose; 3, V403 cells grown in glucose; 4, V1741 cells grown in glucose. Adherent cells that remained after decanting of the culture and washing of the tubes were stained with crystal violet as described in Materials and Methods.

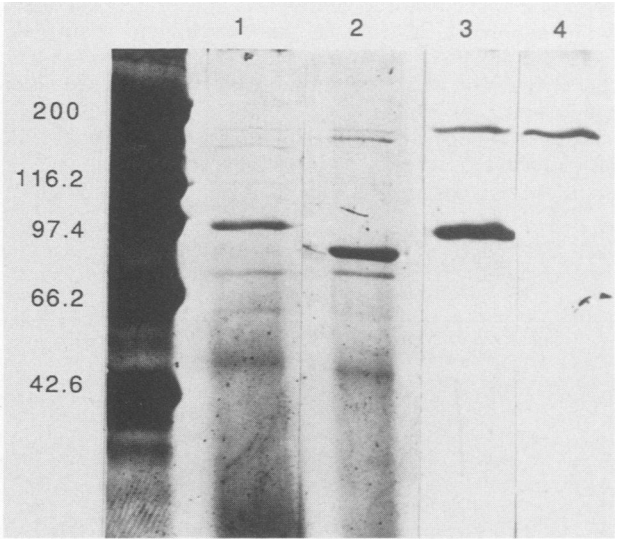

FIG. 6. SDS-polyacrylamide gel electrophoresis analysis of *S. mutans* V403 and V1741 total extracellular proteins. Lanes: 1, V403 protein (25 μg) stained with Coomassie blue dye; 2, V1741 protein (25 μg) stained with Coomassie blue dye; 3, V403 protein (25 μg) stained by the periodate-Schiff base stain procedure; 4, V1741 protein (25 μg) stained by the periodate-Schiff base stain procedure. The sizes of the molecular mass standards (kDa) are designated on the left. The acrylamide concentration was 7.5%.

sucrose (Table 1). Extracellular protein preparations from *S. mutans* GS5 and V403 were used as positive controls for fructan synthesis. For comparative purposes, polysaccharide synthesis by the wild-type strain V403 was assigned a relative activity value of 1.0 (Table 1, line 1). Strain V1741 was found to be deficient in fructan synthesis and had a relative activity of <0.0001 (Table 1, line 2). Also included in Table 1 are levels of glucan synthesis for strains V403 and V1741 (lines 3 and 4, total glucan; lines 5 and 6, water-insoluble glucan). These results indicated that the mutant strain, V1741, generated approximately two- to threefold higher levels of both total glucan synthesis and water-insoluble glucan synthesis compared with the wild-type strain.

On the basis of these findings, it was of interest to determine whether V403 extracellular protein preparations contained a GTF inhibitory component or whether dextranase activity was higher in strain V403 than in V1741. To test for an inhibitory activity, 2 μg of V403 extracellular protein was added to V1741 and GS5 reaction mixtures. V1741 reaction mixtures containing only buffer were used as a control, and the level of glucan synthesis in these reactions was given a relative activity value of 1.0. The amount of V403 protein added to V1741 glucan synthesis reaction mixtures was set at 2 μg, on the basis of the ability of this amount to mediate an arbitrary 80% decrease in the relative glucan synthesis activity by V1741 extracellular proteins (Table 2, line 2). In the presence of 2 μg of V403 protein, glucan synthesis by GS5 extracellular preparations was decreased by approximately 70% (Table 2, line 4). Similarly, a 2-μg sample of GS5 extracellular protein was added to

TABLE 1. Analysis of extracellular polymer-forming activity in *S. mutans* V403 and V1741[a]

Strain	Substrate	Methanol-insoluble ^{14}C cpm/mg of protein	Water-insoluble ^{14}C cpm/mg of protein	Presumed product	Relative activity
V403	[U-^{14}C-fructose]sucrose	$(1.82 \pm 0.03) \times 10^{8}$[b]	ND[c]	Total fructan	1.0
V1741	[U-^{14}C-fructose]sucrose	<100	ND	Total fructan	<0.0001
V403	[U-^{14}C-glucose]sucrose	$(1.2 \pm 0.15) \times 10^{7}$	ND	Total glucan	1.0
V1741	[U-^{14}C-glucose]sucrose	$(4.3 \pm 0.21) \times 10^{7}$	ND	Total glucan	3.6
V403	[U-^{14}C-glucose]sucrose	ND	$(5.6 \pm 0.37) \times 10^{5}$	Water-insoluble glucan	1.0
V1741	[U-^{14}C-glucose]sucrose	ND	$(9.7 \pm 0.22) \times 10^{5}$	Water-insoluble glucan	1.7

[a] Reactions were carried out for 18 h at 37°C as described in the text.
[b] The data shown represent averages of three determinations ± the standard deviations.
[c] ND, Not determined.

V1741 reaction mixtures; however, glucan synthesis was not significantly affected (Table 2, line 3).

The addition of trypsin- or chymotrypsin-digested samples of V403 extracellular protein did not significantly alter glucan synthesis by V1741 proteins (Table 3, lines 3 and 4). Similarly, heat-inactivated samples of V403 had no inhibitory effect (Table 3, line 5). No significant difference in dextranase activity was observed between V403 and V1741 preparations (data not shown).

In vivo gnotobiotic rat studies. Strain V1741 was tested in a germ free rat model system to determine whether FTF activity contributed to virulence. Rats infected with V403 or V1741 were sacrificed after 35 days, and molars were examined for caries activity. The levels of caries activity in rats infected with V403 and V1741 are listed in Table 4.

Both strains were able to cause a moderate extent of decay on all tooth surfaces examined, which included buccal, sulcal, and proximal surfaces. However, the level of caries activity on all molar surfaces (except sulcal DX and proximal DM) of rats infected with V1741 was lower than that observed in animals infected with V403.

Colony blot analysis of cells recovered from individual mandibles (data not shown) indicated that the cells were representative of the original test strain and that no contamination by other bacterial species had occurred. Furthermore, V1741 isolates maintained erythromycin resistance as well as their characteristic colonial morphology. Levels of CFU isolated from mandibles were similar for both strains and averaged 6.0×10^{7} cells/ml.

DISCUSSION

The ability of *Streptococcus mutans* to cause tooth decay is dependent upon complex interactions among several virulence determinants. Important virulence factors include GTFs, which probably facilitate adherence and accumulation on the tooth surface, acid tolerance, bacteriocin production, and synthesis of organic acids from metabolism of dietary carbohydrate or intracellular storage polysaccharides (12, 18, 20, 46). Fructans generated by the extracellular FTF of *S. mutans* have been hypothesized to act as an extracellular carbon source when dietary sources of carbohydrate are limited (33). Therefore, the generation of fructans may represent a virulence determinant in caries pathogenesis.

To evaluate the contribution of fructan in the disease process, we sought to construct an *ftf*-deficient strain of *S. mutans* V403 which could be tested for virulence in an animal model system. Plasmid pSS22, which contained the cloned *S. mutans* GS5 *ftf* gene (40, 41), was used as a probe against Southern blots of *S. mutans* V403 chromosomal digests. The plasmid probe identified three *Eco*RI fragments which corresponded in size to those cloned from GS5 (Fig. 1). This indicated that the *ftf* genes of both GS5 and V403 were related; therefore, we used the cloned *ftf* gene to insertionally inactivate the V403 chromosomal copy of the gene. Our strategy was to generate a defective copy of the cloned GS5 *ftf* gene by inserting DNA coding for erythromycin resistance into the coding region of the *ftf* gene (Fig. 2). The modified GS5 gene was transformed into strain V403, where the defective construct was exchanged for the wild-type copy of the *ftf* gene by a double-crossover recombinational event. This strain was designated V1741.

The wild-type strain V403 exhibited a characteristic colonial morphology when grown on mitis salivarius agar. The colonies were large and gumdroplike in appearance. In

TABLE 2. The effect of extracellular proteins from *S. mutans* V403 on total glucan-forming ability by *S. mutans* V1741 and GS5 extracellular protein preparations[a]

Origin of extracellular protein preparation		Methanol-insoluble ^{14}C cpm/mg of protein	Relative activity
Primary reaction component[b]	Addition[c]		
V1741	Buffer	$(2.6 \pm 0.66) \times 10^{6}$	1.0
V1741	V403	$(5.0 \pm 0.58) \times 10^{5}$	0.2
V1741	GS5	$(2.4 \pm 0.72) \times 10^{6}$	0.9
GS5	Buffer	$(5.8 \pm 0.73) \times 10^{5}$	1.0
GS5	V403	$(1.5 \pm 0.82) \times 10^{5}$	0.3

[a] [U-^{14}C-glucose]sucrose was used as a substrate in all reactions. Reactions were carried out for 18 h at 37°C as described in the text. Data shown represent averages of three determinations ± the standard deviations.
[b] Four micrograms of extracellular material was used in each reaction.
[c] Two micrograms of extracellular material was used for each reaction.

TABLE 3. Effects of heat and trypsin or chymotrypsin digestion on the glucan polymer formation-inhibiting activity of V403 extracellular protein[a]

Origin of extracellular protein preparation		Methanol-insoluble ^{14}C cpm/mg of protein	Relative activity
Primary reaction component[b]	Addition[c]		
V1741	Buffer	$(2.0 \pm 0.3) \times 10^{7}$	1.0
V1741	V403, native	$(2.6 \pm 0.3) \times 10^{6}$	0.13
V1741	Trypsin-treated V403	$(1.8 \pm 0.2) \times 10^{7}$	0.90
V1741	Chymotrypsin-treated V403	$(2.1 \pm 0.4) \times 10^{7}$	1.05
V1741	Heat-treated V403	$(2.0 \pm 0.1) \times 10^{7}$	1.0

[a] [U-^{14}C-glucose]sucrose was used as a substrate in all reactions. Reactions were carried out for 18 h at 37°C, as described in the text. Data shown represent averages of three determinations ± the standard deviations.
[b] Four micrograms of extracellular material was used in each reaction.
[c] Two micrograms of extracellular material was used in each reaction.

TABLE 4. Virulence of *S. mutans* V403 and V1741 in monoinfected gnotobiotic rats

Strain	No. of rats	Mean ± SE caries score[a]					
		Buccal		Sulcal		Proximal	
		E	DM	DS	DX	E	DM
V403	12	12.2 ± 0.5	5.2 ± 0.3	11.5 ± 0.6	0.8 ± 0.2	2.8 ± 0.3	0.0
V1741	14	9.9 ± 0.5	3.7 ± 0.4	8.7 ± 0.6	0.5 ± 0.1	1.1 ± 0.3	0.0

[a] Caries scores were determined by the Keyes procedure. Abbreviations: E, enamel involvement; DS, slight dentinal involvement; DM, moderate dentinal involvement; DX, extensive dentinal involvement.

contrast, colonies of strain V1741 were small and crystalline. We believe that FTF activity is responsible for the mucoid appearance of the wild-type colonies because of high levels of fructan synthesis. Wild-type colonies of *S. mutans* GS5 grown on mitis salivarius agar do not resemble those of V403; instead, these colonies are similar to those of strain V1741 in appearance (data not shown). This finding is consistent with fructan synthesis assays which indicate that extracellular preparations of V403 synthesize sevenfold-higher levels of fructan than the GS5 preparations in comparable assay systems. Although fructan has not been implicated in the process of adherence to tooth surfaces, we initially hypothesized that strains which produced high levels of fructan may more easily adhere to surfaces if cells become trapped within thick layers of fructan. We tested V1741 cells for the ability to adhere to glass compared with the wild-type strain, V403, during growth on sucrose. The results from this experiment indicated that the *ftf*-deficient strain was able to adhere to glass at levels similar to those of the wild type (Fig. 5). Furthermore, *S. mutans* GS5 exhibited levels of adherence that were similar to those of V403 and V1741 (data not shown). These results taken together with our in vivo animal data (below) suggest that fructan does not seem to play a role in adherence to smooth surfaces.

Extracellular proteins from strain V1741 were characterized by polyacrylamide gel electrophoresis (Fig. 6). A protein component in V403 extracellular preparations that had an apparent molecular mass of 97 kDa and was able to synthesize polysaccharide after incubation of the gel in sucrose was identified. A corresponding protein component was absent in V1741 extracellular preparations. This component is believed to represent the FTF protein of strain V403. The molecular mass of the GS5 FTF was reported as 82 kDa, as deduced from DNA sequencing analysis data and by SDS-polyacrylamide gel electrophoresis analysis. This apparent size difference between the V403 and the GS5 FTF has not been resolved. An 82-kDa band was identified in lanes containing V1741 extracellular proteins; however, it lacked the ability to synthesize polysaccharide in the presence of sucrose. We believe this polypeptide represents a truncated FTF, since the size of this protein corresponds to the predicted size of the gene product made from the *ftf* gene containing an insert at the *Xho*I site of the *ftf* gene.

Total extracellular proteins of the wild-type strain, V403, and V1741 were tested for fructan synthesis by using sucrose which was radiolabeled in the fructose position (Table 1). As a point of reference, wild-type levels of fructan synthesis were given a relative activity of 1.0 and fructan synthesis by the mutant strain was compared with this value. The mutant strain was deficient in fructan synthesis, with a relative activity of 0.0001. Radioactivity measured in the assays equaled background counts of negative controls which were presumably caused by nonhydrolyzed sucrose remaining on the filter. Taken together with the Southern blot analysis (Fig. 3), these results suggest that *S. mutans* V403 contains a single *ftf* gene which expresses FTF activity.

Of interest was the finding that V1741 extracellular proteins exhibited higher glucan-synthesizing activity than did those of the wild-type strain, V403 (Table 1). We hypothesized that a component of the V403 extracellular protein preparation was responsible for this apparent glucan synthesis-inhibitory effect. In an attempt to demonstrate an inhibitory agent, we carried out mixing experiments in which a 2-μg sample of V403 extracellular protein was added to V1741 or GS5 GTF activity assay reaction mixtures (Table 2). The addition of V403 protein led to an 80% decrease in the relative total glucan synthesis activity for strain V1741 and a 70% decrease in extracellular preparations from GS5. In a similar fashion, GS5 extracellular proteins were added to V1741 glucan synthesis reaction mixtures; however, there was no significant decrease in total glucan synthesis. Heat-inactivated V403 did not cause a reduction of glucan synthesis by V1741 extracellular proteins (Table 3). Samples of trypsin- or chymotrypsin-treated V403 protein had no effect on glucan synthesis by V1741 (Table 3). The heat lability and the trypsin and chymotrypsin sensitivity of glucan synthesis inhibitory activity suggest that the effect was caused by a protein. We tested the possibility that the dextranase enzyme was expressed at higher levels in V403. If glucan was degraded into small methanol-soluble oligomers, such polymers might not be detected by the glucan assay. Instead, it would appear as if GTF activity was lower in the V403 strain. Levels of dextranase activity were, however, similar for both strains (data not shown). Experiments in which the substrate concentrations were elevated to 20 mM failed to reveal a decrease in the inhibition of glucan formation. Therefore, it is not likely that simple competition for sucrose by FTF was responsible for inhibition. Alternatively, molecular complexes which decrease GTF activity may form between FTFs and GTFs. Finally, it is possible that, the insertion of DNA into the V403 *ftf* gene may have effected expression of flanking coding regions, one of which may include a regulator of GTF activity.

To evaluate the in vivo virulence of the FTF-deficient strain, 19-day-old gnotobiotic rats were infected with either strain V403 or strain V1741. After 35 days, these animals were sacrificed and the level of decay was evaluated and averaged for each test group. On the basis of caries scores of the FTF-deficient strain, compared with the wild-type strain, the results showed decreased cariogenicity on smooth (buccal and proximal) and sulcal surfaces. These results support the hypothesis that FTF activity contributes to virulence; however, strain V1741 remained cariogenic, suggesting that additional virulence determinants contribute to the disease process. Because test animals were fed ad libitum, the role of fructan may have been minimized, since fructan hydrolysis may be more important during extended periods of

nutrient deprivation. This hypothesis could be tested by repeating these experiments but limiting access to food to defined feeding periods. Animal model cariogenicity studies of intracellular polysaccharide storage mutants also showed decreased virulence on smooth and fissure surfaces, with the greatest decrease in virulence occurring on smooth tooth surfaces (46).

As described earlier, fructan may serve as an extracellular carbon reserve. Fructans produced by oral bacteria have a high molecular masses (10^6 to 10^7 daltons) and a predicted low diffusability, suggesting that fructan would be available as a carbon source, providing that plaque also contains fructan hydrolase enzymes (3, 10). Several species of oral bacteria known to synthesize fructan also produce a fructan hydrolase activity, including *S. mutans*, *Streptococcus salivarius*, *Streptococcus sanguis*, and the actinomyces (22, 35, 43, 49). Existing data suggest that synthesis of fructan hydrolase by *S. mutans* is induced by the presence of fructan and low growth rates but is repressed by glucose (49). This type of regulation would benefit colonization of host tissue, since fructan accumulates rapidly after sucrose intake and would be available when carbon sources are limited (48, 50). Oral plaque samples have been shown to generate metabolic acid from fructan. Moreover, levels of fructan decrease in fasting plaque samples (9, 19, 33, 45). Utilization of either intracellular storage polysaccharides or fructan for glycolysis could result in prolonged exposure of teeth to metabolic organic acids (33, 46).

The decrease in cariogenicity of strain V1741 is not likely due to a failure to colonize teeth, since recovery of cells from rat molar surfaces and levels of adherence to glass were comparable to those of the wild-type strain. It is our hypothesis that the decreased cariogenicity of strain V1741 reflects lower levels of metabolic acid production in the absence of fructan. This can be further explored by evaluating the contribution of fructan hydrolase to cariogenicity in a fashion similar to the approach we have discussed in this paper. Specifically, inability to degrade fructan should be the catabolic equivalent to lack of synthesis of fructan polymer. Alternatively, the contribution of fructan production to virulence may be linked to other aspects of intra- or extracellular metabolism which are not readily apparent at this time. It also should be noted that variations in exopolysaccharide-synthesizing enzymes of oral streptococci are well documented (7, 21). Generalizations on the role of fructans in virulence need to be tempered with this knowledge. Extension of our findings to other strains of *S. mutans* should be experimentally supported.

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