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Characteristics and Cariogenicity of a Fructanase-Defective *Streptococcus mutans* Strain

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Polymers of D-fructose produced by a variety of oral bacteria are believed to function as extracellular carbohydrate reserves. Degradation of these polysaccharides in plaque following exhaustion of dietary carbohydrates is thought to contribute to the extent and duration of the acid challenge to the tooth surface and thus to the initiation and progression of dental caries. *Streptococcus mutans* produces a fructanase, the product of the *fruA* gene, which is capable of degrading β(2,6)- and β(2,1)-linked fructans that are commonly synthesized by dental plaque microorganisms. To evaluate the role of the FruA protein in exopolysaccharide metabolism and to assess the contribution of this enzyme to the pathogenic potential of *S. mutans*, a fructanase-deficient strain of *S. mutans* was constructed. Inactivation of a cloned *fruA* gene was accomplished in *Escherichia coli* by using a mini-Mu dE transposon, and then an isogenic mutant of *S. mutans* UA159 was constructed by allelic exchange. Successful inactivation of *fruA* was confirmed through the use of biochemical assays, Western blotting (immunoblotting) with anti-recombinant FruA antisera, and Southern hybridization. The data indicated that FruA was the only fructan hydrolase produced by *S. mutans* UA159. Inactivation of *fruA* had no significant effects on glucosyltransferase or fructosyltransferase activity. In the rat caries model using animals fed a high-sucrose diet ad libitum, there were no significant differences in the number or severity of smooth surface, sulcal, or root caries elicited by the *fruA* mutant and the wild-type organism.

The ability to enzymatically hydrolyze polysaccharide reserves is a possible virulence property of cariogenic organisms (5, 19). Many oral bacteria, such as *Streptococcus salivarius* (1, 14, 15), *Streptococcus mutans* (1, 14), and *Actinomyces viscosus* (50), produce polymers of D-fructose extracellularly via the action of fructosyltransferases (FTF) on sucrose. *A. viscosus* and *S. salivarius* produce fructans of mainly β(2,6)-linked fructosyl units, or levan-type polysaccharide (1, 14, 15, 50), whereas *S. mutans* produces an inulin-type fructan with predominantly β(2,1) linkages (1, 14). On the basis of their large mass (>10^6 daltons) and solution viscosity properties (1, 15), it has been proposed that fructans would not diffuse from the dental plaque matrix and thus may serve as storage polysaccharides (29). Support for this hypothesis has been obtained by demonstrating the rapid accumulation of fructans in dental plaque following the consumption of sucrose by human volunteers (18). Subsequent to the sucrose challenge, levels of plaque fructans declined slowly (18). The ability to enzymatically hydrolyze both inulin- and levan-type fructans by human plaque samples has been demonstrated by several investigators (29, 45, 52). Furthermore, pure cultures of oral streptococci and actinomycetes have also been demonstrated to hydrolyze fructans (45).

The *S. mutans* enzyme responsible for the hydrolysis of fructans is the product of the *fruA* gene, fructanase. Fructanase is a single polypeptide with exo-β-D-fructofuranosidase activity capable of hydrolyzing both levans and inulins, although levan is the preferred substrate (10). The enzyme can release the fructosyl moiety from the trisaccharide raffinose and can cleave sucrose to produce equal amounts of glucose and fructose (10). On the basis of this latter property, it has been postulated that FruA could also function as an extracellular invertase (10). Such an enzyme has been described for *S. mutans* (13) but never purified to homogeneity. The model for fructan utilization by *S. mutans* proposes that, during sucrose intake, fructanase expression may be repressed by multiple variables, including glucose concentration and growth rate, allowing fructosyltransferase to form fructan polymers from sucrose. Following exhaustion of dietary carbohydrates, fructanase could then be derepressed and induced by fructose or by its substrate, fructan. The fructose released from enzymatic fructan hydrolysis by FruA can be transported by one of two phosphoenolpyruvate-dependent phosphotransferase systems (16), enter the glycolytic cycle, and be metabolized to lactic acid and other acids, which can damage the enamel surface of the tooth (10, 26). The utilization of plaque fructans could have the net effect of prolonging both the extent and the duration of the acid challenge to the tooth surface, since the efficiency of utilization of sucrose should be greater, and the period over which carbohydrate metabolism occurred would be extended (5, 29). This postulated mechanism for fructan utilization by *S. mutans* is consistent with existing in vitro and in vivo data.

This investigation focuses on the fructanase of *S. mutans* UA159 (Brathall serotype *c*). To determine the role of the enzyme in polysaccharide metabolism and to assess the contribution of FruA to virulence, a *fruA* strain was engineered. A discrete number of relevant biochemical and physiologic properties of this mutant were evaluated. Furthermore, to assess the contribution of FruA to virulence, the abilities of otherwise isogenic fructanase-proficient and fructanase-deficient strains to cause dental caries in rats fed a high-sucrose diet were compared.

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MATERIALS AND METHODS

Bacterial strains and media. *S. mutans* GS-5 and UA159 (both Bratthall serotype *c*) were maintained on brain heart infusion agar (BHI) (Difco Laboratories, Detroit, Mich.). For preparation of genetically competent *S. mutans*, overnight cultures in BHI were diluted (1:20) into fresh BHI supplemented with 5% horse serum. Transformants were selected on BHI supplemented with erythromycin (10 μg/ml). *S. mutans* UA159 and its derivatives, when used for preparation of proteins from supernatants for Western blotting (immunoblotting), chromosomal DNA isolation, and enzymatic assays, were grown in a tryptone-yeast extract medium (Difco) (10) supplemented with 1% fructose and, when appropriate, erythromycin (7 μg/ml). *Escherichia coli* M8820 (11) and HK730B (23) were maintained on Luria agar (L agar) and on L agar supplemented with kanamycin (50 μg/ml), respectively. *E. coli* strains were grown in Luria broth with vigorous aeration. *E. coli* transformants were selected on L agar containing kanamycin (50 μg/ml) and chloramphenicol (10 μg/ml). *E. coli* transductants were selected on MacConkey agar (Difco) supplemented with kanamycin (50 μg/ml) and chloramphenicol (10 μg/ml). Sucrase-positive colonies of *E. coli* were selected on M9 minimal medium containing 1% sucrose, proline (50 μg/ml) thiamine hydrochloride (10 μg/ml), and leucine (50 μg/ml) (8). For infection of rats, *S. mutans* strains were grown in tryptone-yeast extract broth containing 29 mM potassium phosphate buffer (pH 7.35), 4 mM magnesium sulfate, 16 mM glucose, and 5.5 mM fructose. For microbiological assessment of flora in rats, the lower left mandible was aseptically dissected and sonicated in sterile saline and dilutions were plated on sheep blood agar for counts of total flora and on mitis salivarius agar with bacitracin (1 μg/ml) (MSB) (Difco) or mitis salivarius agar plus erythromycin (10 μg/ml) (MSE) for counts of mutans streptococci. Cultures were incubated for 48 h at 37°C in a 5% CO_2 aerobic atmosphere.

Enzymes and chemicals. All restriction enzymes were obtained from Bethesda Research Laboratories (Gaithersburg, Md.) and were used as recommended by the supplier. Levan from *Aerobacter levanicum* and inulin from chicory root were obtained from Sigma Chemical Co., St. Louis, Mo. The radiolabelled nucleotide [α-^{32}P]dATP was purchased from Amersham Corporation (Arlington Heights, Ill.). Radiolabelled sugars, [fructose-1-^{3}H(N)]-sucrose and [glucose-^{14}C(U)]-sucrose, were obtained from New England Nuclear, Boston, Mass.

Preparation of enzymes from *S. mutans*. One liter of tryptone-yeast extract broth containing 1% fructose (TYF) was inoculated with 5 ml of a TYF starter culture of wild-type and FruA-defective *S. mutans* strains, and the mixture was incubated at 37°C for 18 h. The cells were collected by centrifugation at 5,000 × *g* for 20 min at 4°C. The cell pellet was used for DNA isolation or for determination of activity of cell-associated enzymes (see below). The precipitate from an ammonium sulfate fractionation (70% saturation) of the supernatant was collected after centrifugation at 5,800 × *g* for 20 min at 4°C. After suspension in 10 mM potassium phosphate buffer, pH 6.0, with 1 mM *n*-caproic acid (Sigma), this material was dialyzed against the same buffer overnight to remove residual fructose. The dialysate was clarified by centrifugation at 10,000 × *g* for 10 min at 4°C, and the supernatant was used in Western blots and for enzyme assays (see below). As an alternative to ammonium sulfate precipitation, culture supernatants were concentrated by ultrafiltration in a stirred-cell ultrafiltration device (Amicon model 8400) through a membrane with a 30,000-molecular-weight cutoff (Amicon model YM30) (38).

The method described by Walker et al. (49) was used for the production of cell-associated enzyme preparations. Briefly, cell pellets were washed in 0.1 M citrate buffer (pH 5.5) twice and then suspended to one-seventh the original volume. A one-third volume of glass beads (average size, 0.2 mm; Baxter Scientific) was added, and the suspension was homogenized at 4,000 rpm with CO_2 cooling for 2 min. The samples were centrifuged at 15,000 × *g* for 30 min, and the supernatant was used in fructanase, GTF, and FTF assays.

Protein electrophoresis and blotting. Proteins from supernatants of cultures of wild-type and fructanase-deficient strains were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions, according to the method of Towbin et al. (48). High-molecular-weight, prestained protein standards were from Bethesda Research Laboratories. Proteins were transferred to Immobilon-P membranes (Millipore Corporation, Bedford, Mass.), and then nonspecific protein binding was blocked with 5% nonfat dry milk in Tris-buffered saline (TBS; 10 mM Tris hydrochloride, pH 7.4, 0.9% NaCl). Rabbit antiserum prepared against the purified recombinant fructanase (10) was utilized at a dilution of 1:200 in TBS. The serum was adsorbed with a sonic lysate of stationary-phase *E. coli* JM83 cells to remove antibodies to *E. coli* components that are cross-reactive with streptococci (10). Secondary antibody (goat anti-rabbit immunoglobulin G, Fc portion) conjugated to horseradish peroxidase (Cappel Research Products, Durham, N.C.) was diluted in TBS (1:1500) and used to detect immune reactivity with 4-chloro-1-naphthol as the substrate (28).

DNA manipulations. *E. coli* was transformed by using the $CaCl_2$-heat shock method as described previously (11, 28). *E. coli* plasmid content was examined by using a rapid boiling method (28). Restriction endonuclease analysis of plasmid DNA was conducted by electrophoresis in a 0.8% agarose gel in Tris-borate buffer (28). *Hin*dIII-digested bacteriophage lambda DNA fragments (Bethesda Research Laboratories) were used as molecular weight standards.

S. mutans was transformed by the method of Perry and Kuramitsu (35). *E. coli* plasmid DNA was digested to completion with *Eco*RI and added to competent *S. mutans* cultures, and DNA uptake was allowed to occur. After 1 h at 37°C, erythromycin was added to a final concentration of 75 ng/ml and the cultures were incubated for 1 h more. The cells were collected by centrifugation at 12,000 × *g* for 2 min, resuspended in 100 μl of BHI, and plated on appropriate medium. Transformants were observed after incubation for 48 h at 37°C in a 5% CO_2 aerobic atmosphere.

DNA was prepared from *S. mutans* by modifying the method of Chassy (12) as described by Burne et al. (8). Briefly, cells from a 1-liter culture were resuspended in 25 ml of 50 mM Tris (pH 8.0)–10 mM EDTA–3% glycine. After stirring for 1 h at room temperature and centrifugation, pellets were resuspended in 20 ml of 50 mM Tris (pH 8.0)–10 mM EDTA–10% polyethylene glycol 8000. Lysozyme (1 mg/ml) and mutanolysin (500 U) were added, and the mixture was incubated at 37°C for 1 h. Cells were lysed by using 1% sodium dodecyl sulfate, and the NaCl concentration of the mixture was adjusted to 1 M by addition of 5 M NaCl. DNA was purified by two extractions with phenol-chloroform-isoamyl alcohol (25:24:1), followed by one chloroform-isoamyl alcohol (24:1) extraction. RNase was added to a

concentration of 1 μg/ml, and the mixture was incubated at 65°C for 15 min. The DNA was precipitated with ethanol and resuspended at a concentration of 1 mg/ml in 10 mM Tris (pH 7.4)–1 mM EDTA.

Southern hybridization was carried out by the method of Southern (44), as outlined by Maniatis et al. (28). *S. mutans* chromosomal DNA was digested with *Hin*cII, and the fragments were separated by electrophoresis through a 0.8% agarose gel. Transfer of nucleic acids to nylon membranes (Hoefer Scientific, San Francisco, Calif.) was accomplished through the use of a vacuum blotter (Trans-Vac TE 80; Hoefer Scientific), and the nucleic acids were cross-linked by UV light to the membrane. Plasmid pFRU1 DNA (10), labelled with [α-^{32}P]dATP and the Random Primers Kit (Bethesda Research Laboratories), was utilized as a probe.

Enzymatic assay. Determination of fructanase activity was accomplished by measuring the release of reducing sugar from levan or inulin via the dinitrosalicylic acid method of Luchsinger and Cornesky (27) as described previously (8). Protein preparations were incubated with levan or inulin (1 mg/ml) in potassium citrate buffer (0.1 M, pH 5.5) for 4.5 h at 37°C. Samples were boiled for 10 min in the presence of the dinitrosalicylic acid reagent. Activity was expressed in units (U), defined as the amount of enzyme needed to produce 1 μmol of reducing equivalent from levan or inulin per h. Fructose was used as the standard in the assay. Protein concentration was determined by the method of Bradford (4) by using a commercially available dye reagent concentrate (Bio-Rad), with bovine serum albumin as the standard.

Glucosyltransferase (GTF) and FTF activities were assayed by a modification of the method of Robrish et al. (37) and Germaine et al. (17). Briefly, for FTF, the assay measured the incorporation of the fructose moiety from [fructose-1-^{3}H(N)]-sucrose into non-methanol-soluble polysaccharides. Enzyme preparations were incubated with an equal volume of substrate mixture containing 0.01 M potassium phosphate buffer, pH 6.0, 0.02% sodium azide, sucrose (200 mM), and 100 mM [fructose-1-^{3}H(N)]-sucrose. After incubation for 4 h at 37°C, 1 ml of ice-cold methanol was added. The precipitated polysaccharides were washed with ice-cold methanol in a filter manifold (Millipore) onto a 2.5-cm glass fiber filter (Whatman). The filters were air dried and immersed in scintillation fluid (Ecoscint A; National Diagnostics, Manville, N.J.). The amount of radiolabelled sugar incorporated into polysaccharide was measured by scintillation counting. GTF assays were performed essentially in the same manner as FTF assays except that [glucose-^{14}C(U)]-sucrose, 40 μM dextran 9000, and 0.04 M imidazole-HCl buffer (pH 6.5) were used in the substrate mixture instead of [fructose-1-^{3}H(N)]-sucrose and 0.01 M phosphate buffer. One unit of GTF or FTF activity was defined as the amount of enzyme necessary to incorporate 1 μmol of glucose or fructose, respectively, into non-methanol-soluble material in 1 min.

Animal studies. Dams of six litters of specific-pathogen-free female Sprague-Dawley rats were obtained from Charles River Breeding Laboratories. Prior to infection, dams were screened for mutans streptococci by plating from oral swabs onto MSE and MSB. None of the animals was found to harbor detectable levels of these organisms. Pups were weaned at 20 to 21 days of age and infected by oral swabbing with cultures of *S. mutans* grown to late exponential phase. Successful infection of the animals was confirmed after 5 to 7 days by plating oral swabs from each animal on appropriate media. Animals were fed Diet 2000, which contained 56% sucrose (22) (Zeigler Bros., Gardners, Pa.), and were given sweetened drinking water (distilled H_2O plus 10% sucrose) ad libitum. The experiment was continued for 5 weeks, at which point the animals were sacrificed by decapitation after carbon dioxide asphyxiation. The jaws were removed, and molars of all four quadrants were scored for caries activity. The extent of dental caries was assessed by the Keyes method (21). Root exposure and root caries were scored by the method of Rosen et al. (39). Microbiological assessment was conducted by using the lower left mandible. The mandible was dissected aseptically, placed in sterile saline (5 ml), and sonicated at 320 W for 30 s at 10-s intervals. Dilutions in sterile saline were plated on sheep blood agar for determination of total cultivable flora and on MSB or MSE for mutans streptococcus counts. Counts (CFU) on MSB and MSE were compared for the *fruA* strain to assess the stability of the erythromycin marker. In addition, DNA prepared from strains recovered from infected rats was examined by Southern hybridization (44) to verify the stability of the fructanase locus. Statistical analyses of caries scores, root surface area, and microbiological composition were computed by using one-factor analysis of variance (ANOVA), followed by the Fisher protected least significant difference (PLSD) test when ANOVA resulted in a significant F test ($P \leq 0.05$).

RESULTS

Inactivation of the *S. mutans fruA* gene. The strategy for insertional inactivation of the fructanase gene (*fruA*) is depicted in Fig. 1. *E. coli* HK730B, which contains a chromosomal copy of a mini-Mu dE transposon (23) expressing kanamycin resistance (Km^r; 50 μg/ml) and erythromycin resistance (Em^r; 500 μg/ml) in *E. coli* and Em^r (10 μg/ml) in the oral streptococci, was transformed with a *fruA*-containing plasmid with a chloramphenicol resistance (Cm^r) marker selectable in *E. coli* (Fig. 1). A phage lysate preparation from this strain was used to transduce *E. coli* M8820 (11). Transductants (Km^r Cm^r) were selected at random and gridded onto L agar and minimal sucrose medium supplemented with appropriate antibiotics (8). Isolates which failed to grow on minimal sucrose medium were chosen because previous results (9) indicated that only fructanase-positive *E. coli* cells were able to grow with sucrose as the sole carbohydrate source. *Hin*dIII digestion of recombinant plasmids confirmed the presence of the 9.2-kb mini-Mu dE transposon inserted into the *fru* gene (data not shown). Plasmid DNA was isolated from these recombinant *E. coli* cells and digested to completion with *Eco*RI in order to linearize the plasmid. There are no *Eco*RI sites in the transposon, and on the basis of the insertion point of the mini-Mu dE transposon, sufficient *fruA* sequences would be flanking the transposon for a double crossover recombination event to occur. *S. mutans* was transformed with the linearized plasmid DNA, and two Em^r strains from a single transformation, *S. mutans* DW-A and DW-E, were utilized for further study.

Southern hybridization to *Hin*cII-digested chromosomal DNA from *S. mutans* DW-A and DW-E was utilized to determine that the transposon had inserted into the *S. mutans fruA* locus (Fig. 2). The data indicated that the transposon had inserted into the 811-bp *Hin*cII fragment representing nucleotides 723 to 1534 of the *fruA* structural gene. This *Hin*cII fragment is known to harbor a region of the *fruA* gene which has strong homology to the active sites of other β-fructosidases (6).

Proteins from culture supernatants of the putative fructanase-deficient mutants, DW-A and DW-E, and from that of

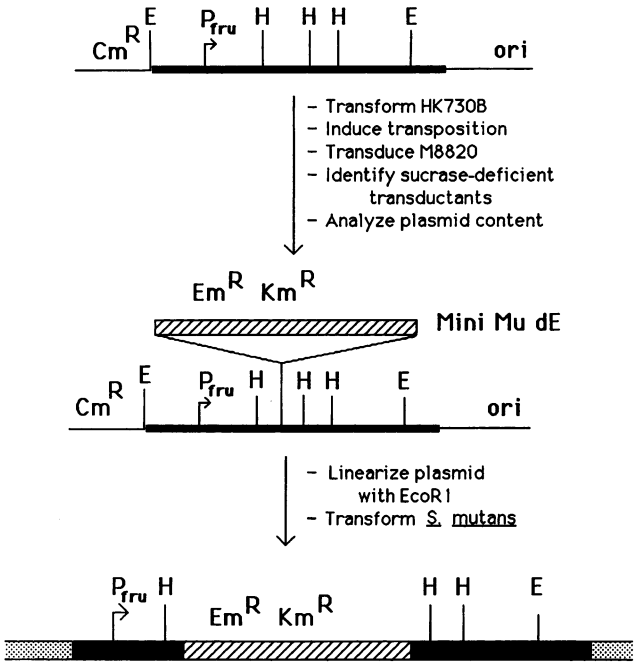

FIG. 1. Strategy for inactivation of the *fruA* gene of *S. mutans*. A recombinant plasmid carrying *fruA* DNA sequences, an *E. coli* replicon, and a Cm^r marker which was selectable in *E. coli* was introduced into *E. coli* HK730B. Following induction of the transposon (11, 23), the transducing lysate was used to infect *E. coli* M8820. Transductants (Km^r Cm^r) were selected on MacConkey agar supplemented as outlined in Materials and Methods. Clones were picked to a master plate and to a minimal-sucrose-containing medium. Clones which failed to grow on the minimal medium were presumed to be sucrase negative and thus lacked the $fruA^+$ phenotype. These clones were analyzed by restriction enzyme digestion and gel electrophoresis. Recombinants with insertions in the *fruA* gene were identified, linearized with *Eco*RI, and utilized to transform *S. mutans* UA159. Em^r transformants were analyzed as described in the text to confirm the fructanase-deficient phenotype. E, *Eco*RI; H, *Hin*cII; P_{fru}, the proposed *fruA* promoter; ori, *E. coli* plasmid origin of replication.

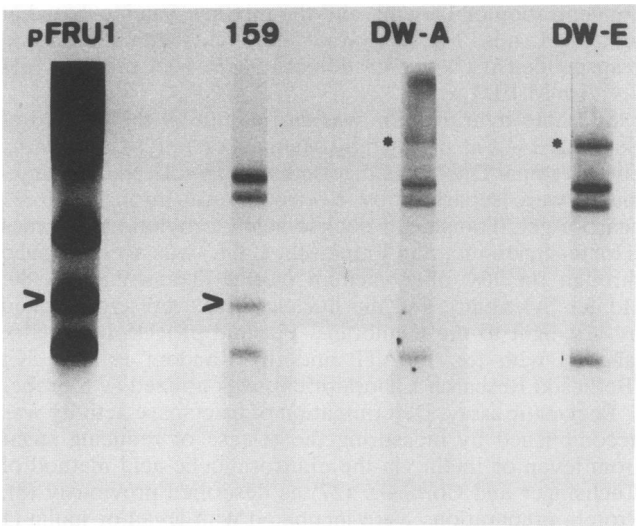

FIG. 2. Southern hybridization of pFRU1 with chromosomal DNA of UA159 wild type and fructanase mutants. Lane pFRU1, pFRU1 DNA digested with *Hin*cII; lane 159, UA159 (wild type) digested with *Hin*cII; lane DW-A, DW-A digested with *Hin*cII; lane DW-E, DW-E digested with *Hin*cII. Insertion of mini-Mu dE in DW-A and DW-E strains resulted in altered migration of the 811-bp *Hin*cII fragment of the wild type (>), resulting in the appearance of a higher-molecular-weight homologous fragment (*).

S. mutans UA159 were compared by Western blotting using antiserum prepared to a purified, recombinant FruA protein. The mutant strains no longer synthesized the 140-kDa FruA protein found in the parent supernatants (Fig. 3). The presence of an immunologically reactive 40-kDa protein in DW-A could potentially represent a truncated form of FruA. A protein of this size would be consistent with the insertion point of mini-Mu dE within the *fruA* gene, but the relationship between this species and FruA has not been determined.

Biochemical characteristics of fructanase-deficient strains. In order to confirm inactivation of fructanase activity in the mutants and to ascertain whether other fructan hydrolases were being produced by *S. mutans*, the abilities of culture supernatants and cell-associated enzymes of the wild-type and mutant strains to release reducing sugar from levan and inulin were compared. FruA is known to attack fructans exohydrolytically, but the presence of an enzyme(s) which attacked endohydrolytically should also have been detected in such an assay, unless this additional fructan hydrolase was highly specific for sugars found at β2,6 and β2,1 branch points. Table 1 shows specific activities of batch-grown *S. mutans* UA159 and fructanase-defective derivatives. The greater activity with levan as the substrate is consistent with previous reports (10). Approximately 15% of the total fructanase activity was found to be associated with the cells in batch culture, differing from results obtained with chemostat-cultured organisms (10), which could reflect an influence of culture conditions on the localization of FruA. There was no detectable fructan hydrolase activity in the mutant strains, as assessed by measuring fructose released from levan or inulin. To test for possible changes in localization of FruA enzyme activity in the mutant strains, the cell-associ-

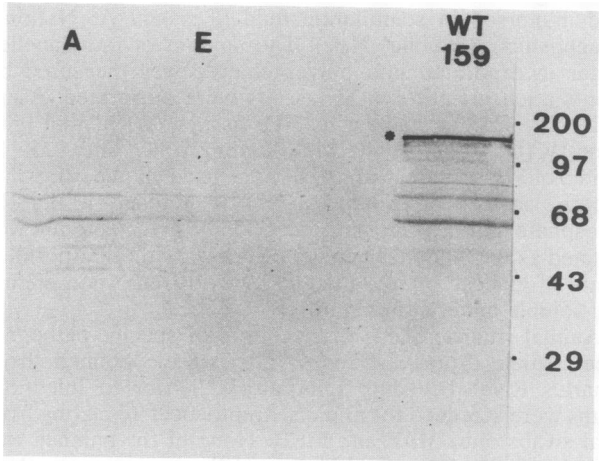

FIG. 3. Western blotting. Dialyzed supernatants of *S. mutans* UA159 wild type and mutant strains, probed with fructanase antiserum as described in the text. Lane A, DW-A; lane E, DW-E; lane WT 159, UA159 wild type. *, 140-kDa FruA protein. Lower-molecular-weight species may represent either cross-reacting extracellular proteins from *S. mutans* culture supernatants or breakdown products of FruA.

TABLE 1. Analysis of fructanase activity of the wild type and *fruA* mutants of *S. mutans* UA159

S. mutans strain	Enzyme activity[a]	Sp act (U/mg of protein)		
		Concentrated supernatant	Ammonium sulfate precipitation	Cell associated
UA159 (wild type)	Levanase	13.47	7.31	12.51
	Inulinase	2.07	4.34	3.61
	GTF	0.19	ND[b]	ND
	FTF	0.10	ND	ND
DW-A	Levanase	0[c]	0	0
	Inulinase	0[c]	0	0
	GTF	0.18	ND	ND
	FTF	0.07	ND	ND
DW-E	Levanase	0	0	0
	Inulinase	0	0	0
	GTF	0.15	ND	ND
	FTF	0.12	ND	ND

[a] Levanase, inulinase, GTF, and FTF were assayed as described in Materials and Methods.
[b] ND, not determined.
[c] Levanase and inulinase activity were measured, and less than 25 μg of reducing sugar was released in a 21-h assay.

ated fraction was assayed (Table 1). No assayable fructan hydrolase activity was associated with the DW-A or DW-E strain. Supernatants from these strains were also concentrated by ultrafiltration and assayed for fructan hydrolase. No activity was detected in the mutants.

Because of the genetic linkage of *fruA* and the *ftf* gene (36), there was a possibility that the disruption of overlapping regulatory circuits by *fruA* inactivation would perturb normal fructan synthesis potential. Measurements of FTF activity were performed (Table 1). Likewise, because of the potential for perturbation of other extracellular pathways for sucrose and exopolysaccharide metabolism as a result of inactivation of *fruA*, GTF activity was also quantitated. There were no significant differences detected in GTF or FTF activity in culture supernatants or in cell-associated activity between the wild-type *S. mutans* and the fructanase-deficient mutants. Likewise, no differences were found between mutant and wild-type strains when supernatants were concentrated by ultrafiltration and assayed.

Rat caries studies. DW-A and DW-E arose from transformation of *S. mutans* UA159 with a single recombinant construction from *E. coli*. Furthermore, DW-A and DW-E were indistinguishable in all biochemical and genetic properties. Therefore, DW-E was used in the rat study as a representative fructanase-deficient mutant. There were 12 rats in each of three groups: UA159, DW-E, and no inoculation. All rats in the DW-E and UA159 groups became infected. The rats were maintained and fed as described in Materials and Methods. No significant differences in weight change during the experiment between the animal groups were noted. Virulence of the strains, as measured by caries scoring, is shown in Table 2. There were no statistically significant differences between the infected groups in numbers or severity of smooth surface caries or in sulcal caries limited to the tooth enamel or dentin. Nor were there any statistically significant differences between any of the groups with respect to sulcal moderate dentinal involvement caries or sulcal extensive dentinal involvement caries. When root exposure and numbers of carious root surfaces were analyzed, no statistically significant differences ($P \leq 0.05$) were detected between any infected groups or between infected groups and uninfected control groups.

TABLE 3. Total CFU and total mutans streptococci recovered from rats infected with *S. mutans* UA159 or *S. mutans* DW-E

S. mutans strain	CFU/mandible (10^7) on:		% Mutans streptococci
	SBA[a]	Mitis salivarius agar	
UA159	2.54 ± 0.61	0.84 ± 0.22[b]	47.9 ± 8.9
DW-E	4.46 ± 0.57[c]	3.00 ± 0.7[c,d]	64.7 ± 8.1

[a] Mean CFU on 5% sheep blood agar.
[b] Mean number of mutans streptococci enumerated on mitis salivarius agar with bacitracin.
[c] Statistically significant within columns at $P \leq 0.05$.
[d] Mean number of mutans streptococci enumerated on mitis salivarius agar with erythromycin (10 μg/ml). There were no statistically significant differences between the counts of DW-E on MSB and those on MSE.

The total cultivable flora in rats infected with the DW-E mutant strain was significantly greater than that in rats infected with the wild type, UA159 (Table 3). Total numbers of mutans streptococci isolated from plaque, as measured by growth on MSE and MSB, were also significantly greater for DW-E than for UA159. However, when taken as a percentage of the total cultivable flora, there were no significant differences among the wild type and mutant strains in the proportion of the total microbial population which was recoverable as mutans streptococci (Table 3).

The genetic stability of the fructanase locus was assessed at the end of the animal study, because deletion of the selectable marker (Em^r) or intramolecular recombination to restore a functional FruA could have confounded the results. First, to determine whether the erythromycin determinant

TABLE 2. Cariogenicity of *S. mutans* UA159 and *S. mutans* DW-E in Sprague-Dawley rats

Group[a]	Caries score (mean ± SEM)[b]						
	Smooth surface	Sulcal				Root exposure	Root caries
		E	DS	DM	DX		
S. mutans UA159	4.20 ± 2.5	31.75 ± 1.96	10.17 ± 1.82	3.75 ± 1.04	0.92 ± 0.58	176.08 ± 2.96	0.83 ± 0.46
S. mutans DW-E	4.58 ± 1.87	32.30 ± 1.49	12.50 ± 1.77	5.58 ± 1.47	0.33 ± 0.26	174.25 ± 3.74	0.417 ± 0.34
No infection	0.33 ± 0.23[c]	30.33 ± 0.88	7.08 ± 0.95	4.0 ± 0.63	0.17 ± 0.11	179.42 ± 3.25	0.08 ± 0.08[d]

[a] All groups were composed of 12 animals.
[b] Caries scores were obtained and subjected to statistical analysis as described in Materials and Methods. E, enamel involvement; DS, slight dentinal involvement; DM, moderate dentinal involvement; DX, extensive dentinal involvement.
[c] $P \leq 0.05$ by Fisher PLSD test.
[d] Only one animal in the uninfected group developed root caries, and this animal had only a single lesion. In the infected group, many animals developed more root caries lesions. However, when these data were analyzed, there were no statistically significant differences between infected and uninfected animals in root caries scores.

was stable in *S. mutans* DW-E, the total population of organisms recovered on MSB was compared with that recovered on MSE plates. There were no statistically significant differences in these numbers, suggesting that the marker was not lost at an appreciable frequency. Random isolates of UA159 and DW-E which were recovered from the rats were further characterized by Southern hybridization using pFRU1 as a probe, in order to detect any chromosomal rearrangement associated with the *fruA* locus. The migration of *fruA*-related sequences following digestion of chromosomal DNAs with *Hin*cII, electrophoresis, and Southern hybridization was identical to those of the parent strains which were implanted into the rat, indicating that the *fruA* locus of the mutant, as well as of the parent, did not undergo gross restructuring during the in vivo experiments (data not shown).

DISCUSSION

Synthesis and metabolism of fructans are thought to be important in plaque ecology and disease, and yet very little is known about the polysaccharides and enzymes participating in these processes. It has been assumed that these polysaccharides are not critical structural determinants of dental plaque, primarily because of their short half-life. Nevertheless, fructans are detectable in plaque after long periods of fasting (18), in spite of the presence of fructan hydrolase activity (29, 45, 52), suggesting that these polymers could be integrated as a semipermanent component of the polysaccharide superstructure of dental plaque. Also, little data exist regarding the ability of plaque levans and inulins to function in adherent interactions with oral microbes. In addition, there is the possibility that fructan synthesis indirectly affects plaque composition and architecture. Polymer synthesis in plaque is a complex, multienzyme process. The FTFs and fructanase may influence glucan structure and properties indirectly through competition with the GTFs for a common substrate, sucrose. Likewise, the presence of high-molecular-weight fructans in plaque may influence the final polysaccharide network in plaque. Knowledge of the impact of the interactions between glucan and fructan metabolic machineries on plaque structure, composition, and ecology is clearly lacking.

This report described the insertional inactivation of the *S. mutans fruA* gene of a Bratthall serotype *c S. mutans*. *S. mutans* UA159 was chosen over strain GS-5, from which the gene was originally isolated (10), for two principal reasons. First, in our experience with the rat caries model, *S. mutans* GS-5 is not as strongly cariogenic as UA159. This may be due to a number of surface adherence properties which decrease this strain's ability to effectively colonize a host (33, 40). *S. mutans* UA159, on the other hand, has been used to elicit high numbers of smooth surface and sulcal caries (32). Secondly, there were no detectable differences between the chromosomal homologs of *fruA* from GS-5 and UA159 as determined by Southern hybridization (7) (Fig. 2).

Biochemical and immunologic data obtained previously had indirectly suggested that FruA was the sole fructan hydrolase of *S. mutans* GS-5 (10). Also, use of a *fruA*-specific DNA probe has not revealed additional homologous genes in *S. mutans* GS-5 or UA159 (7). Inactivation of *fruA* in *S. mutans* appears to ablate all fructan hydrolase activity in strain UA159 grown in batch culture, indicating that FruA is at least the major fructan hydrolase produced by this strain. Nevertheless, these data do not eliminate the possibility that a second fructan hydrolase is cotranscribed with *fruA*. Preliminary results with Northern (RNA) blotting (51) of *fruA* mRNA indicated that the transcript consists of approximately 4,500 bp. This would be a sufficiently large transcript to encode for FruA but probably no additional gene products, suggesting that the expression of multiple fructanases from the *fruA* promoter is unlikely. Data derived from the DNA sequence of the regions 3′ of *fruA* (6) also did not suggest the presence of additional gene products transcribed from the *fruA* promoter. The fact that insertional inactivation of *fruA* eliminates fructanase activity also does not rule out that *S. mutans* can synthesize a second fructanase under appropriate culture conditions; however, under the conditions utilized in this study and in previous experiments (10), this activity was not expressed. Possession of a second fructan hydrolase by plaque microbes is not unusual. It is known that certain strains of *A. viscosus* (31) and *S. salivarius* (45) produce multiple fructan hydrolases. On the other hand, *Bacillus subtilis* produces a single gene product (SacC), which shares significant homology with FruA (>60% at the nucleotide sequence level with *fruA*), that encodes for a levanase with properties similar to those of the FruA protein (30). It is important to note that a second fructan-hydrolyzing enzyme in *S. mutans* would be redundant since FruA can degrade all of the types of fructans identified in plaque (10).

Using transformational mapping techniques, Perry and Kuramitsu demonstrated that *fruA* and *ftf* are tightly linked in the chromosome (36). In light of the tight genetic linkage, it is conceivable that *ftf* and *fruA* are coordinately regulated. Overlapping regulation of these genes would be logical also from the standpoint that repression of FruA during periods of fructan synthesis would facilitate increased accumulation of these polysaccharides in plaque. Insertional inactivation of *fruA* appeared to have no effect on the fructan synthesis capabilities of *S. mutans* UA159. Although it may seem that ablation of FruA would result in an apparent increase in fructan synthesis capacities, the rates of breakdown of fructans by batch-cultured *S. mutans* are markedly lower than that of the rate of synthesis in vitro. Consequently, during the FTF reaction in vitro only a negligible amount of fructan is hydrolyzed by FruA. FruA-defective mutants of *S. mutans* are also not impaired in their abilities to synthesize glucans from sucrose. The lack of perturbation of normal glucan synthesis capabilities was an important consideration for the animal experimentation, since altered glucan synthesis could have impacted on the results of the animal caries experiments, particularly those involving smooth surface caries (19, 46).

An extracellular invertase of *S. mutans* has been neither purified to homogeneity nor isolated by using recombinant DNA technology. As reported previously (9), FruA has biochemical and physical properties very similar to those of the extracellular invertase described by others (13). The use of the *fruA* strains of *S. mutans* and a *fruA/ftf* double mutant, which we have recently constructed (7), should be particularly helpful in determining whether there are other β-fructosidases produced in culture supernatants or as surface-associated enzymes of *S. mutans*. A rigorous examination of the fractionated culture supernatants of *fruA*-deficient strains would be a reasonable starting point for detection of a putative extracellular invertase.

By using the rat caries model, there were no detectable differences in the cariogenic potential between the wild type and fructanase-deficient mutants of *S. mutans* in rats fed Diet 2000. This result suggests that fructan degradation is not an essential determinant for smooth surface or sulcal caries.

It is critical to note, however, that the animals were fed a high-sucrose diet and allowed to drink sucrose-sweetened distilled H_2O ad libitum. It is known that eating patterns can dramatically influence the caries process (3). The choice to use animals fed ad libitum was based on the standard rat caries protocols utilized in numerous laboratories. Our results with rat animal study, therefore, can be validly interpreted to indicate that FruA is not essential for smooth surface caries initiation, in contrast to insoluble glucan synthesis (19, 46). This is consistent with the finding that *ftf* inactivation also does not severely impair the cariogenic potential of *S. mutans* (24, 41). Nevertheless, it is critical to emphasize that the postulated role of FruA, the degradation of storage compounds, is such that the major contribution to the caries process could be during fasting periods.

Studies with chemically derived mutants of *S. mutans* which were defective in the synthesis of iodophilic polysaccharide (IPS) indicated that such strains were partially impaired in their abilities to elicit both smooth surface and sulcal caries (47). More recently, studies by Harris (20) indicated that IPS-defective mutants constructed by allelic exchange were also less cariogenic than wild-type strains. And although the kinetics of fructan utilization and IPS metabolism are likely distinct in vivo, the decreased cariogenicity of IPS-defective strains indicates a clear contribution of storage polysaccharide metabolism to the caries process. Interestingly, the differences in the cariogenic potentials of the wild type and IPS-deficient strains of *S. mutans* were further augmented when a restricted feeding regimen was imposed on the animals, suggesting that in fact the utilization of storage compounds during fasting periods is a contributing factor in the caries process (47). Thus, for defects in fructan metabolism to be fully manifested in decreased caries activity in the rat model, it may be necessary to feed the animals at programmed intervals. Toward the verification of this hypothesis, experiments with the *fruA*-defective strains described here and a *fruA/ftf* double mutant of *S. mutans* UA159 will be conducted by using a programmed feeding regimen.

There is another possible explanation as to why there were no differences between the wild type and mutant strains of *S. mutans*. Larrimore et al. (25) have previously reported two mutants which had impaired abilities to elicit smooth surface caries because of different genetic defects. However, when implanted together in the rat these strains were able to complement one another in vivo and restore the levels of caries production to that of the wild-type strain. These data indicate that in vivo complementation of exoenzyme deficiencies can occur between strains of oral bacteria and raise the question as to whether oral bacteria such as *A. viscosus* or *S. salivarius* were able to supply the capacity to enzymatically hydrolyze fructans to the FruA mutants in the plaque of the experimental animals in this study. Although not measured in this study, *A. viscosus* is capable of colonizing the oral cavity of rats fed Diet 2000 at high levels (34). It is also known that these species possess a high capacity to produce and degrade fructans (31, 50). Relatively few *S. salivarius* cells are recoverable from rats fed a high-sucrose diet (2), and yet these organisms synthesize a large quantity of fructanase when compared with *S. mutans* (45). So, other plaque bacteria may have synthesized sufficient fructan hydrolase enzymes to mask the FruA defect of *S. mutans* DW-E. Gnotobiotic animals could be utilized to test this hypothesis. Alternatively, if complementation could occur, and if FTF from *S. mutans* is the principal source of plaque fructans, then one would predict that a mutant defective in both *fruA* and *ftf* would have reduced cariogenic potential. Again, the utilization of a *fruA/ftf* double mutant constructed in this laboratory, in conjunction with the programmed feeding experiments described above, should more definitively assess the role of fructans in plaque ecology and cariogenesis.

It is of interest that both the total recoverable microbial populations and the total counts of mutans streptococci were elevated in rats which were infected with the fructanase mutant. However, it cannot be readily determined whether such an increase was an artifact of the methods utilized to assess microbial populations, i.e., sonication, dilution, and plating. In this study, higher recoveries of the DW-E strain may have arisen due to the fact that plaques from animals infected with this strain were more readily dissociated by sonication, due to some intrinsic defect in the mutant or to altered dental plaque carbohydrate or plaque microbial composition. On the other hand, if the differences are real, the most straightforward explanation is that there were increased levels of plaque fructans on the teeth of the rats colonized with the mutant strain, although this would be contrary to the aforementioned proposal of in vivo complementation of exopolysaccharide metabolism in plaque. Plaque fructans may have acted to trap bacteria at the surface of the tooth. This may have allowed bacteria to become established on the tooth surface by offsetting the normal mechanical debridement of plaque which occurs as a function of saliva and soft tissue interactions with the tooth. Alternatively, plaque fructans could have acted as a receptor in dental plaque for oral bacteria. Specific stereochemical interactions, or bridging mechanisms mediated by lipoteichoic acid, salivary or bacterial products (e.g., proteins) (38), or host immunoglobulins, since levans are known to be antigenic (42), may have acted to mediate initial or secondary bacterial interactions at the tooth surface. The enhanced recovery of bacteria from plaques of animals colonized with the mutant strain may be the result of an increase in adherence mediated by an increase in plaque fructans.

It is also of interest that both the root exposure and the root caries scores were indistinguishable between rats infected with the mutants and the wild-type *S. mutans*. On the basis of existing data, one may have predicted a possible increase in root exposure, and perhaps increased root caries, due to an inflammatory response to increased plaque levans accompanied by gingival recession in rats infected with the mutant strain. However, no such increase occurred. And the fact that greater numbers of *S. mutans* were present in the rats infected with the DW-E strain implies that these strains were less cariogenic, because the larger numbers of *S. mutans* present in plaque did not increase caries incidence. A conclusion regarding this cannot be drawn from the results presented here. Nevertheless, it is clear that *fruA* ablation had a clear effect on plaque ecology. Experiments to further define the role of fructans in the caries process and plaque ecology are under way.

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