Isolation and Characterization of a Fructosyltransferase Gene from *Streptococcus mutans* GS-5

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A fructosyltransferase (FTF) gene from *Streptococcus mutans* GS-5 has been isolated from a λL47.1 clone bank. The gene was contained on an 11.7-kilobase GS-5 DNA fragment and was initially subcloned into plasmid pACYC184 as a 5.4-kilobase HindIII fragment. However, further analysis revealed that transcription of the FTF gene was initiated at the P1 promoter contained on the vector. It was possible to subclone the FTF gene with its presumed promoter as a 3.4-kilobase EcoRI fragment to produce the chimeric plasmid pSS22 expressing FTF activity. The cloned enzyme was purified to apparent homogeneity after ammonium sulfate precipitation, gel filtration, and DEAE-Bio-Gel-A chromatography followed by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The purified enzyme displayed a lower molecular weight (63,000) compared with the multiple activities detected in the culture fluids of strain GS-5. In addition, storage of the purified enzyme resulted in the formation of even lower-molecular-weight enzymatically active species. These results suggested that proteolytic degradation of the FTF occurs both in *S. mutans* and in *Escherichia coli*. In addition, a comparison of the properties of the cloned enzyme with those previously characterized from another serotype c *S. mutans* strain suggests that multiple FTF genes may be present in these organisms.

The important role of organisms resembling *Streptococcus mutans* in the development of human dental caries has been well established (5). Several properties of these organisms appear to contribute to their cariogenicity; (i) the ability of the organisms to synthesize water-insoluble glucan from dietary sucrose and colonize tooth surfaces, (ii) their ability to rapidly convert sucrose to lactic acid, and (iii) the relative ability of these organisms to tolerate acidic environments (8).

Although a variety of strains resembling *S. mutans* have been identified, most of those isolated from the human oral cavity belong to serotype c (3). One characteristic which distinguishes these human oral isolates from many rodent isolates is the ability of the former organisms to produce fructans from sucrose. The enzyme(s) responsible for this conversion, fructosyltransferases (FTF; EC 2.4.1.10), have been demonstrated in many serotype c organisms but have not been detected in strains belonging to serotype a, d, or g (8). However, since the rodent strains are also cariogenic in animal model systems it is likely that the synthesis of fructans is not required for cariogenicity. Nevertheless, it has been proposed that the fructans synthesized by the *S. mutans* strains, as well as by several other oral microorganisms (15), may serve as a reserve polysaccharide. A number of organisms isolated from human dental plaque, including *S. mutans* strains, produce fructanases which could degrade the polymers to metabolizable saccharides (23). Nevertheless, the role of fructan formation in either enhancing or decreasing the cariogenicity of *S. mutans* has not been adequately investigated up to now.

One approach toward examining the role of fructan formation in cariogenicity involves the utilization of specific FTF mutants in animal model systems. However, such mutants have not been isolated up to the present. Such mutants could be isolated if it were possible to isolate FTF genes from *S. mutans* serotype c strains and reintroduce mutated forms of the genes back into the organisms by transformation (16). The present communication describes the isolation and characterization of an FTF gene from *S. mutans* GS-5.

**MATERIALS AND METHODS**

**Bacterial strains.** *Escherichia coli* C600 was kindly provided by R. R. B. Russell (Royal College of Surgeons, Downe, England), and *E. coli* DH1 was provided by T. Laffler (Northwestern University, Chicago, Ill.). The *E. coli* strains were maintained on LB agar plates and routinely grown in LB medium (13). *S. mutans* GS-5 was maintained and grown in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) as previously described (14).

**Screening for FTF clones.** The *S. mutans* GS-5 clone bank was prepared as recently described (H. Aoki, T. Shiroza, M. Hayakawa, S. Sato, and H. K. Kuramitsu, submitted for publication). Briefly, GS-5 chromosomal DNA was partially cleaved with Sau3A1 and ligated to BamHI-cut λL47.1 DNA. After in vitro packaging, the clone bank was generated after infection of C600 (P2) indicator cells. For detecting sucrase-positive clones, the procedure recently described was utilized (6). Samples (10 μl) of the clone bank were infected into C600 indicator cells, mixed with soft agar containing M9 medium (13) plus sucrose (1%), and overlaid onto M9 agar plates. Sucrase-positive clones were identified as plaques surrounded by a white zone of C600 growth. After isolation of individual sucrase-positive clones, the recombinant phage were infected into C600 cells and grown in NZCYM broth (13) at 37°C for 12 h with vigorous shaking. The resultant phage lysates were utilized to assay directly for FTF activity.

**Subcloning of the FTF gene.** One of the FTF clones, λF3, was purified, and DNA was extracted as previously described (13). λF3 DNA was digested to completion with HindIII or partially with EcoRI and ligated with pACYC184 linearized with HindIII or EcoRI, respectively. The ligation mixture was utilized to transform either DH1 or C600 cells by the CaCl₂ procedure (13). Transformants were selected on LB agar plates containing the appropriate antibiotics.
chloramphenicol (34 μg/ml) or tetracycline (12.5 μg/ml). After replicate plating, transformants containing inserts were detected as either tetracycline-resistant (Tc) and chloramphenicol-susceptible (Cm') (EcoRI cleavage) or Cm' Tc' (HindIII cleavage) colonies. Each colony was purified and grown in LB, toluidined (17), and assayed directly for FTF activity.

**Localization of FTF activity.** *E. coli* C600 (control) and F22 (containing the FTF-coding plasmid pSS22) were grown in LB broth (10 ml) and harvested by centrifugation at 3,000 × g for 5 min, and subcellular fractions were isolated as previously described (9). The periplasmic fraction was obtained as the cold osmotic shock fluid. After osmotic shock, the cells were washed twice with 10 mM Tris hydrochloride buffer (pH 7.0), suspended in the same buffer with glass beads (14), and disrupted in a Mickel shaker (H. Mickie Ltd., Gomshall, Surrey, England) for 15 min at 4°C. After centrifugation at 13,000 × g for 10 min, the supernatant fluid was utilized as the cytoplasmic fraction, and the resuspended washed residue was utilized as the membrane fraction. The activities of FTF, the cytoplasmic marker β-galactosidase (9), and the periplasmic marker alkaline phosphatase (9) were determined in each fraction.

**Enzyme assays.** Sucrase activity was determined by the Somogyi-Nelson procedure as previously outlined (21). FTF activity was determined by measuring the incorporation of [3H]fructose-sucrose (New England Nuclear Corp., Boston, Mass.; 1.8 μCi/μmol) into methanol-precipitable polysaccharide as previously described (12). One unit of FTF activity is defined as the amount of enzyme required to incorporate 1.0 μmol of sucrose into fructan per min under standard assay conditions.

Protein concentrations were determined with a Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, Calif.) (2).

**DNA manipulations.** DNA from recombinant phage was purified from cleared lysates as recently described (13). Plasmids were isolated by the alkaline-lysis procedure (1) for rapid screening of transformants and purified after CsCl ultracentrifugation (13) from large-scale cultures (1.0 liter). Restriction digestions were carried out as recommended by the supplier (Bethesda Research Laboratories, Gaithersburg, Md.). Ligations were routinely performed utilizing T4 DNA ligase (Bethesda Research Laboratories) for 16 h at 4°C.

**Electrophoretic analysis.** Proteins were analyzed on 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels essentially as previously described by utilizing Coomassie blue staining (19). FTF activity was detected on the gels by the procedure previously described by Russell (19) utilizing either sucrose or raffinose as substrates followed by periodic acid-Schiff staining (24).

DNA was analyzed on 0.7% agarose gels with a Tris-borate-EDTA buffer (13). The gels were incubated in ethidium bromide (1.0 μg/ml) for 1 h, washed with water for an additional hour, and photographed with a Polaroid MP-3 camera equipped with a Wratten orange filter.

**Purification of the cloned FTF.** Subclone F2 was harvested by centrifugation at 9,000 × g for 5 min in a Sorvall RC-2B refrigerated centrifuge after growth in LB (6 liters). The cells were disrupted in a Mickel shaker (14), and the supernatant fluid was treated with saturated ammonium sulfate (to 75% saturation) for 2 h at 4°C. The precipitated proteins were dissolved in Tris hydrochloride buffer (pH 7.5), dialyzed against the same buffer for 6 h, and applied (8 ml) to an Ultrogel AcA34 (LKB Instruments, Inc., Rockville, Md.) gel filtration column (2.5 by 65 cm). The column was developed with 10 mM Tris hydrochloride buffer (pH 7.5), and 7.5-ml fractions were collected and assayed for FTF activity. The active fractions were pooled and applied to a DEAE-Bio-Gel-A (Bio-Rad) column (1.5 by 25 cm) and eluted with a 300-ml linear gradient from 0 to 0.2 M KCl in the same Tris buffer. The active fractions were pooled and applied (2.5 ml) to a 6% preparative SDS-PAGE slab gel. After electrophoresis at 4°C for 10 h, the FTF bands (identified after activity staining of one lane) were excised from the gel. The gel fragments were macerated and extracted with 10 mM Tris hydrochloride buffer (pH 7.5) for 18 h at 4°C. The mixture was then centrifuged at 12,000 × g for 10 min, and the supernatant fluid served as the purified enzyme fraction.

**RESULTS**

**Identification of an FTF clone.** The phage library constructed from λA47.1 and *S. mutans* GS-5 DNA was infected into *E. coli* C600 and grown on sucrose-containing M9 agar plates (13). Clones which expressed sucrase activity demonstrated the formation of a white ring (*E. coli* indicator cells growing in the presence of glucose and fructose released from sucrose) surrounding individual plaques (Fig. 1). Each positive plaque was harvested and propagated, and phage lysates were prepared and assayed directly for sucrase and FTF activities. Two of the sucrase-positive clones expressed FTF activity when assayed with [3H]fructose-sucrose in the standard FTF assay. In addition, the clones synthesized polysaccharide from both sucrose and raffinose. However, the frequency of isolation of the FTF gene from the clone bank (approximately 1 out of 2,000 clones) is somewhat lower than predicted from the average size insert for the λA47.1 system (13). The clone expressing the strongest FTF activity, λF3, was selected for further investigation and was utilized to characterize the FTF gene and its enzyme product.

FTF activity present in λF3 lysates was demonstrated to result from the activity of two proteins of 91 and 84

![FIG. 1. Detection of sucrase-positive clones. Samples (10 μl) of the clone bank lysates were mixed with *E. coli* C600 indicator cells and added to M9 agar plates as soft agar overlays containing sucrose. Arrows indicate sucrase-positive clones surrounded by zones of C600 growth.](http://iai.asm.org/Downloadedfrom)
kilodaltons (kDa) after SDS-PAGE and activity staining (Fig. 2). These results suggested that the FTF gene was at least 2.6 kilobases (kb) in length and that proteolysis in the lysates resulted in multiple active species. Alternatively, more than one FTF gene may reside on the GS-5 insert. Therefore, it was of interest to clarify both the number and localization of the FTF gene on λF3 DNA.

Characterization of λF3 DNA. The DNA of λF3 was purified from lysates of λF3 after cesium chloride ultracentrifugation as previously described (13). The DNA was digested by various restriction enzymes, and a restriction map was generated (Fig. 3). The resultant map indicated that λF3 DNA was approximately 45.7 kb in length and contained an 11.7-kb insert from strain GS-5.

Subcloning of the FTF gene. The DNA of λF3 was completely digested with HindIII and ligated with vector pACYC184 that was also digested with the same enzyme. The ligation mixture was transformed into E. coli DH1, and recombinant transformants were selected as Cm'-Tc' colonies. Of 13 resultant transformants, only 1 expressed FTF activity when tolueneized cells of each were assayed. The chimeric plasmid pSS7 from the positive subclone F7 was isolated and subjected to restriction analysis (Fig. 4). pSS7 contained a 5.4-kb fragment from the GS-5 insert of AF3. However, the promoter for the FTF gene did not appear to reside on the 5.4-kb insert since a subclone, F12, containing the fragment inserted in the opposite orientation relative to the vector did not express FTF activity. It is likely that the transcription of the FTF gene on pSS7 was initiated at the nearby P1 promoter of pACYC184, which is known to transcribe heterologous genes inserted into the HindIII site in the opposite direction relative to the transcription of the Tc' gene (22).

To obtain the complete FTF gene (including its promoter and putative signal sequence), AF3 DNA was partially digested with EcoRI, ligated to linearized pACYC184, and transformed into strain C600. Of 22 Cm'-Tc' transformants, two expressed FTF activity. One of these, F22, was shown to contain a chimeric plasmid, pSS22, composed of pACYC184 and a 3.4-kb GS-5 fragment (Fig. 4). The GS-5 fragment was composed of three EcoRI fragments from λF3 of 0.33, 0.7, and 2.4 kb (Fig. 5). The other positive subclone, F2, was shown to contain the same 3.4-kb EcoRI fragment inserted into the opposite orientation relative to the vector. Therefore, the 3.4-kb GS-5 insert in pSS22 and pSS2 appears to contain the promoter for the FTF gene.

Localization of FTF in subclone F22. Since the FTF activity is expressed as an extracellular enzyme by S. mutans GS-5, it was of interest to determine the cellular location of the
TABLE 1. Localization of FTF activity in E. coli F22a

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Relative enzyme activity (%) in fraction:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Periplasmic</td>
</tr>
<tr>
<td>FTF</td>
<td>43</td>
</tr>
<tr>
<td>β-Galactosidase</td>
<td>1</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>62</td>
</tr>
</tbody>
</table>

* F22 cells were fractionated into three subcellular fractions, and FTF, β-galactosidase (cytoplasmic marker), and alkaline phosphatase (periplasmic marker) were assayed in each fraction as described in the text.

The cloned enzyme in E. coli. The cellular distribution of FTF in E. coli F22 was determined by assaying FTF activity in the cytoplasm, membrane fraction, and periplasmic space of the subclone (Table 1). FTF activity was almost equally distributed between the periplasmic space (43%) and the cytoplasm (54%). In comparison, the cytoplasmic marker β-galactosidase was found almost exclusively in the cytoplasm, whereas the majority of the periplasmic marker alkaline phosphatase was observed in the periplasmic space. However, since a significant portion of the latter enzyme (35%) was detected in the cytoplasm under the extraction conditions utilized, it is likely that an even higher portion, greater than 43%, of the FTF of subclone F22 is localized in the periplasmic space. Therefore, these results suggest that the signal sequence of FTF is present on pSS22 and allows a large portion of the cloned enzyme to be transported through the E. coli cytoplasmic membrane.

**Purification of the cloned FTF.** The FTFs from subclones F7, F22, and F2 as well as from λF3 were detected on SDS-PAGE gels as multiple protein bands ranging from 59 to 91 kDa (Fig. 2). In addition, FTF activity in the culture fluids of strain GS-5 could also be observed as proteins with multiple molecular weights. The multiple bands displayed in the clones as well as in strain GS-5 appeared to result from proteolytic degradation. Storage of the enzyme preparations at 4°C indicated less degradation compared with frozen and thawed preparations (data not shown).

The FTF activity from subclone F22 was purified to near homogeneity after ammonium sulfate precipitation, gel filtration, and anion-exchange chromatography and subsequent preparative SDS-PAGE (Table 2). The purified FTF exhibited only one protein band and a single corresponding activity band of 63 kDa after SDS-PAGE (Fig. 6). However, storage of the purified enzyme resulted in degradation of the enzyme to lower-molecular-weight active species as small as 59 kDa.

The purified enzyme did not bind to insoluble glucan (prepared from S. mutans 6715) or react with antisera against the serotype c glucan-binding protein (supplied by R. R. B. Russell). Therefore, the cloned FTF gene does not correspond to the recently described FTF activity displaying glucan-binding activity (20). The purified FTF also synthesized an inulolike fructan since its polysaccharide product did not react with concanavalin A (7). In addition, no primer was required for FTF activity since the addition of exogenous Streptococcus salivarius fructan (prepared from culture fluids of that organism) had no effect on the activity of the cloned enzyme.

**DISCUSSION**

Previously, several genes have been isolated from cariogenic S. mutans strains by utilizing plasmid or cosmid vectors (10, 18). However, the genes coding for extracellular sucrose-metabolizing genes were not detected in these gene banks. The present communication has demonstrated the isolation of an FTF gene from strain GS-5 by utilizing a bacteriophage λ47.1 vector system. This latter system has also been exploited to isolate several other genes from serotype c S. mutans strains (6, 20; Aoki et al., submitted for publication; M. Hayakawa, H. Aoki, and H. K. Kuramitsu, submitted for publication). It is of interest that the frequency of identifying dextransucrase clones from the same GS-5 clone bank is even lower (Aoki et al., submitted for publication), as is that of the streptolysin O gene cloned in this system (11). Thus, identification of genes coding for extracellular proteins appears to be somewhat more difficult than predicted in this vector system.

Since FTF subclone plasmid pSS22 contained a 3.4-kb GS-5 insert and the largest FTF species (91 kDa) observed in the F22 extract would require a 2.5-kb gene, it is clear that more than 70% of the insert is composed of FTF sequences. Furthermore, the observation that almost one half of the FTF activity detected in subclone F22 is found in the periplasmic space (Table 2) suggests that the signal sequence is also present in the insert.

Since multiple FTF bands are detected in subclone F22 extracts after SDS-PAGE (Fig. 2) and the GS-5 insert is large enough to code for only a single FTF gene, it is likely that proteolytic processing of FTF occurs in the extracts. The demonstration that the purified FTF is isolated as a 63-kDa protein (Fig. 6) indicates that such processing also occurs during purification of the enzyme. Subsequent storage of the enzyme also leads to further proteolysis, suggesting that trace amounts of a putative protease are present in the purified enzyme preparation.

Multiple FTF bands are also observed after SDS-PAGE of

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**TABLE 2. Purification of FTF from E. coli F22a**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Sp act (U/mg)</th>
<th>Recovery (%)</th>
<th>Puriﬁcation (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell-free extract</td>
<td>9.5</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Ultragel AcA34</td>
<td>20.7</td>
<td>61</td>
<td>2</td>
</tr>
<tr>
<td>DEAE–Bio-Gel-A</td>
<td>125.5</td>
<td>62</td>
<td>13</td>
</tr>
<tr>
<td>Preparative SDS-PAGE</td>
<td>4,045.2</td>
<td>30</td>
<td>426</td>
</tr>
</tbody>
</table>

* Activity was determined with the FTF radioactive assay. Protein concentrations were determined as described in the text.
GS-5 culture fluids (Fig. 2). These species may represent the products of multiple FTF genes or may correspond to processed products from a single gene. Russell et al. (R. R. B. Russell, E. Abdulla, M. L. Gilpin, and K. Smith, Proceedings of the 2nd International Conference on Cellular, Molecular, and Clinical Aspects of Streptococcus mutans, in press) have recently speculated that more than one FTF gene may be present on the chromosome of S. mutans serotype c strains. One of these appears to display glucan-binding properties and apparently synthesizes an inulinlike product from sucrose. However, the FTF isolated from LF3, although also synthesizing an inulinlike product, does not bind to insoluble glucan. In addition, the enzyme does not react with antibody prepared against the glucan-binding protein suggested to possess FTF activity from another serotype c strain, Ingbritt (provided by R. R. B. Russell). Therefore, these results favor the existence of at least two distinct FTF genes in serotype c organisms.

Confirmation of this hypothesis will be sought after in vitro mutagenesis of the cloned FTF gene and subsequent transformation of the mutated gene back into strain GS-5 to produce specific FTF mutants. In this manner, it will be possible to discriminate between a single or multiple FTF genes in serotype c strains.

The cloned FTF gene will also be mapped on the chromosome of strain GS-5 by a procedure recently utilized to map the position of the gtfA gene of strain GS-5 (16). It will be of interest to determine whether the FTF gene is closely linked to other sucrose-metabolizing genes recently cloned in this laboratory (gtfA, sucrose-6-phosphate hydrolase, dextransucrase). It is possible that several genes coding for sucrose-metabolizing enzymes may be clustered on the S. mutans chromosome. In addition, it will be of interest to determine the nucleotide sequence of the GS-5 FTF gene for comparison with the sequence recently generated for the FTF of Bacillus subtilis (4).

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LITERATURE CITED