INFECTION AND IMMUNITY, Oct. 1991, p. 3434–3438
0019-9567/91/103434-05$02.00/0

Vol. 59, No. 10

Cloning of a *Streptococcus sobrinus gtf* Gene That Encodes a Glucosyltransferase Which Produces a High-Molecular-Weight Water-Soluble Glucan

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Received 12 March 1991/Accepted 10 July 1991

The *gtf* gene coding for glucosyltransferase (GTF), which produces a water-soluble glucan, was cloned from *Streptococcus sobrinus* OMZ176 (serotype d) into plasmid vector pBR322. This gene was expressed in *Escherichia coli*, and the product was purified to near homogeneity. The antigenicity of recombinant GTF (rGTF) was examined with the antisera raised against purified GTF P1, P2, P3, and P4 obtained from *S. sobrinus* AHT (serotype g). The rGTF reacted only with anti-GTF P1 serum in a Western blot (immunoblot) analysis. The rGTF closely resembled GTF P1 in its molecular mass, K_m value for sucrose, optimal pH, primer dependency, and immunological properties. The high-molecular-weight, water-soluble glucan produced by the rGTF also resembled that of GTF P1, which is the most efficient primer donor for primer-dependent, water-insoluble glucan synthesis. Properties of the rGTF were also compared with those of rGTFS, which was purified from *E. coli* carrying the *gtfS* gene isolated from *Streptococcus downei* (previously *S. sobrinus* serotype h) MFe28. Both rGTF and rGTFS synthesized water-soluble glucan from sucrose without primer dextran, but their characteristics in K_m values for sucrose, optimal pHs, and polymer sizes of the glucan were different. Furthermore, the *gtf* gene did not hybridize with the *gtfS* gene in a Southern blot analysis. These results showed that rGTF is similar to *S. sobrinus* AHT GTF P1 but distinct from rGTFS that has been previously purified from *E. coli* carrying the *gtfS* gene.

Mutans streptococci are essential to the etiology of dental caries (12). This group of bacteria consists of seven species (23). These organisms produce extracellular glucosyltransferases (GTFs; EC 2.4.1.5) which catalyze glucan synthesis from dietary sucrose. These glucans are believed to be important in the pathogenicity of these bacteria (12). Three kinds of *gtf* genes (*gtfB*, *gtfC*, and *gtfD*) which code for GTFs have been isolated from *Streptococcus mutans* GS5 chromosomal DNA (1, 7, 8). Several immunological studies have showed that the GTF enzymes of *S. mutans* are distinct from those of *Streptococcus sobrinus* and *Streptococcus cricetus* (21, 22, 25).

Many workers have reported the purification of GTF enzymes from *S. sobrinus* and *S. cricetus* (3, 14). Recently, four GTFs (GTF P1, P2, P3, and P4) were purified from the culture supernatant of *S. sobrinus* AHT (24, 25). These four GTFs are different in their enzymatic characteristics and immunoreactivities. These results suggest that four *gtf* genes must exist in *S. sobrinus* AHT.

Up to now, two *gtf* genes coding for GTFS and GTFI which were similar to those of *S. sobrinus* and *S. cricetus* (15, 16) have been isolated from *S. downei* (previously *S. sobrinus* serotype h) MFe28 (6, 15, 16, 23) and their nucleotide sequences have been reported (4, 5). In this report, we describe the properties of the recombinant GTF (rGTF) purified from *Escherichia coli* carrying the *gtf* gene isolated from *S. sobrinus* OMZ176 compared with GTF P1 from *S. sobrinus* AHT.

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MATERIALS AND METHODS

Strains, plasmid, and culture conditions. The bacterial strains used in this study were as follows: *S. sobrinus* OMZ176 (serotype d), *S. sobrinus* AHT (serotype g), *S. downei* MFe28 (serotype h), *E. coli* C600, and *E. coli* HB101. Streptococci were grown anaerobically in brain heart infusion broth at 37°C for 18 h. *E. coli* strains were grown aerobically in Luria-Bertani (LB) broth (17) at 37°C. The plasmid pMLG60 was kindly provided by R. R. B. Russell, Hunterian Dental Research Unit, London Hospital Medical College, London, England.

DNA manipulations and cloning methods. DNA isolation, endonuclease restriction, ligation, and transformation of competent *E. coli* cells were carried out as recently described (17). An *S. sobrinus* OMZ176 DNA library was constructed by *Pst*I digesting chromosomal DNA, the ligation mixture was transformed into *E. coli* C600, and transformants harboring chimeric plasmids were selected on LB agar plates containing tetracycline (20 μg/ml). Then the colonies grown on LB-tetracycline agar plates were blotted onto nitrocellulose membrane disks. After bacterial lysis by chloroform vapor (ca. 2,000 transformants), the disks were screened for colonies that expressed antigens which reacted with antibody directed against GTF fractions of *S. sobrinus* OMZ176. *E. coli* C600 carrying a chimeric plasmid pGT72 was selected by this immunoscreening method.

Enzyme and protein assays. Sucrose hydrolase activity was determined by the Somogyi procedure as originally described (18). GTF activity was determined as previously described (9). One unit of GTF activity is defined as the amount of enzyme catalyzing the incorporation of 1.0 μmol of glucose from sucrose into glucan per min under standard

assay conditions. The amount of protein was estimated by the method of Bradford (2). The effects of exogenous dextran T10 on water-soluble and -insoluble glucan syntheses, the optimum pH, and the K_m value of the enzyme were determined as previously described (9).

Purification of rGTF. *E. coli* HB101(pGT72) was harvested by centrifugation at 5,000 × *g* for 5 min after growth in LB-tetracycline (8 liters) at 37°C. The cells were resuspended with 20 mM Tris hydrochloride buffer (pH 7.5) containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF). The cells were disrupted with glass beads (0.2 g/ml) in the cell mill for 30 min. After removal of the beads, the suspension was ultracentrifuged at 100,000 × *g* in a Hitachi 55P-72 centrifuge with an RP40-958 rotor for 60 min. The supernatant fluid was concentrated by precipitation with ammonium sulfate (50% saturation). After centrifugation at 10,000 × *g* for 30 min, the pellet was redissolved in 20 mM Tris hydrochloride buffer (pH 7.5) and dialyzed against the same buffer. This fraction was applied to a DE52-cellulose (Whatman International, Maidstone, United Kingdom) column. The GTF-active fractions were pooled and applied to a Butyl-Toyopearl (TOSOH, Tokyo, Japan) hydrophobic interaction column. Adsorbed material was eluted with a linear gradient of increasing ethylene glycol concentrations to 60% in 50 mM sodium acetate buffer (pH 6.0). The GTF-active fractions were dialyzed and applied to an AF-Blue Toyopearl (TOSOH) column which was equilibrated with 50 mM sodium acetate buffer (pH 6.0) containing 60% ethylene glycol. The column was washed with the same buffer, and the enzyme was then eluted with a linear gradient of 0 to 0.5 M NaCl in the same buffer. Fractions containing the GTF activity were pooled and dialyzed against 5 mM phosphate buffer (pH 7.0). The enzyme was applied to the hydroxylapatite column equilibrated with the same buffer. Elution was carried out with a linear gradient from 5 to 500 mM phosphate buffer (pH 7.0).

Gel electrophoresis. Proteins were analyzed by sodium dodecyl sulfate–7% polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (1). DNA fragments were analyzed on a 0.5, 0.7, or 1.2% agarose gel with Tris-borate-EDTA buffer (17).

Western and Southern blot analyses. Western blot analysis was carried out as described by Towbin et al. (20). Because the four types of antisera raised against the *S. sobrinus* OMZ176 GTFs were not yet available, we used previously described *S. sobrinus* AHT antisera (15). Before use, the antisera were absorbed with heat-killed cells of *E. coli* HB101 carrying pBR322. Southern blot analysis utilizing biotin-labeled probes was performed as recently described (7), according to the instructions of the supplier of the DNA detection system (Bethesda Research Laboratories, Gaithersburg, Md.).

Localization of rGTF in *E. coli*. Subcellular fractions of *E. coli* HB101(pGT72) were isolated as described by Heppel (10). After cold osmotic shock, the cell suspension was centrifuged. The supernatant fluid was utilized as the periplasmic fraction. Then the cells were washed twice with 10 mM Tris hydrochloride buffer (pH 7.0), suspended in the same buffer with glass beads, and disrupted in the cell mill for 15 min at 4°C. After centrifugation, the supernatant fluid was utilized as the cytoplasmic fraction and the resuspended, washed residue was utilized as the membrane fraction.

Preparation of the *S. sobrinus* GTF antisera. *S. sobrinus* AHT (g) secretes four kinds of GTFs (19). Each GTF (100 μg) was mixed with an equal volume of Freund's incomplete adjuvant and injected subcutaneously into rabbits three times at 1-week intervals, and antisera were collected from ear veins 10 days after the final injection.

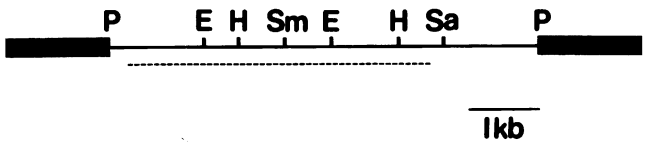

FIG. 1. Restriction enzyme cleavage map of recombinant pGT72. The *gtf* gene is located in a 6.1-kb *Pst*I fragment of *S. sobrinus* OMZ176 chromosomal DNA. Vector pBR322 (thick line) and the approximate location of the *gtf* gene (dashed line) are shown. E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; Sa, *Sal*I; Sm, *Sma*I.

Glucan analysis. The *gtf* gene product (rGTF) was extracted from the soluble fraction of disrupted *E. coli* HB101(pGT72). Glucan was synthesized by rGTF in 50 mM sodium acetate buffer (pH 6.0) containing 5% sucrose and 5 mM NaF and then precipitated with 70% ethanol. The precipitated glucan was collected and washed twice with 70% ethanol by centrifugation (15 min at 8,000 × *g*). Although this glucan was a water-soluble type, the glucan precipitated with ethanol could not be solubilized in distilled water. Thus, the glucan was suspended in 1 M NaOH, neutralized with 1 M HCl, and then dialyzed against distilled water. The resulting solutions were filtered through a column guard (pore size, 0.22 μm; Millipore, Bedford, Mass.) and subjected to high-pressure liquid chromatography (HPLC) using a TSK-gel G6000PW column (TOSOH) at a flow rate of 1 ml/min. The carbohydrates were detected with a refractometer (RI-8; TOSOH). Commercial dextrans (T10 and T2000) were purchased from Pharmacia-LKB Biotechnology (Uppsala, Sweden) for molecular weight determinations.

RESULTS

Isolation of the *gtf* gene and subcellular distribution of the gene product. Chromosomal DNA of *S. sobrinus* OMZ176 was digested with *Pst*I and ligated into *Pst*I-cleaved plasmid vector pBR322. The ligation mixtures were transformed into *E. coli* C600, and recombinant clones were screened with an antibody directed against the GTF enzyme prepared from *S. sobrinus* OMZ176. Five immunoscreening-positive transformant cells were selected from about 2,000 transformants. One positive clone carried plasmid pGT72, consisting of an insert of a 6.1-kb OMZ176 chromosomal DNA. An endonuclease cleavage map is shown in Fig. 1. Further attempts to reduce the size of the insert revealed that the presence of the 1.3-kb *Pst*I-*Sal*I fragment did not affect the properties of GTF produced in recombinant *E. coli* strains. Thus, the *gtf* gene must be located on the 4.8-kb *Sal*I-*Pst*I fragment (the approximate location of the *gtf* gene is indicated by the dashed line in Fig. 1). Fractionation of crude extracts from *E. coli* HB101 carrying pGT72 revealed that most (67%) of GTF activity was found in the soluble cytoplasmic fraction, compared with 26% in the periplasmic fraction and only 7% in the membrane-associated fraction. In comparison, the cytoplasmic marker β-galactosidase was found almost exclusively in the cytoplasm (93%), and most of the periplasmic marker alkaline phosphatase was observed in the periplasmic space (63%).

Purification and characterization of rGTF. rGTF was purified 631-fold from *E. coli* HB101(pGT72), as shown in

TABLE 1. Purification of the *gtf* gene product (rGTF)

Fraction	Total activity (mU)	Total protein (mg)	Sp act (mU/mg)	Purification (fold)	Yield (%)
Crude enzyme	5,477	1,215	4.5	1	100
DE52-cellulose	3,429	9.98	343.6	76	63
Butyl-Toyopearl	1,318	2.38	553.8	123	24
AF-Blue Toyopearl	1,131	0.96	1,178.1	262	21
Hydroxylapatite	369	0.13	2,838.5	631	7

Table 1. The purified enzyme preparation yielded a single protein band following Coomassie blue (Fig. 2A) and activity staining (Fig. 2B) of SDS-PAGE gels. The estimated molecular size of the enzyme in the crude extracts of the *E. coli* clone was approximately 160 kDa. The purified enzyme exhibited a molecular mass of approximately 132 kDa and synthesized water-soluble glucan exclusively. rGTF synthesized water-soluble glucan from sucrose without primer dextran; however, the quantity of glucan synthesized in the presence of dextran T10 was increased twofold. Since primer-dependent GTFs were stimulated extraordinarily in the presence of primer dextran (13, 25), rGTF must be a primer-independent GTF. Water-insoluble glucan was not detected on the electrophoresis gel after a 24-h incubation period with sucrose.

Molecular sizes of the glucans produced by rGTF. The molecular sizes of the glucans produced by rGTF from *E. coli* HB101(pGT72) and sucrose were determined by high-pressure liquid chromatography analysis (Fig. 3). The major peak of the glucans was located near the void volume of the column ($>2 \times 10^6$), and the minor peak was found in the flanking region of the elution point of dextran T10 ($>10^4$). In contrast to the oligoisomaltosaccharide production of rGTFS (15), rGTF produced high-molecular-mass glucan from sucrose. The high-pressure liquid chromatography elution profile of this glucan resembled that of the glucan produced by the *S. sobrinus* AHT GTF P1 enzyme (19, 25). rGTF and GTF P1 were similar in molecular masses, K_m values for sucrose, optimal pHs, primer dependencies, and polymer sizes of the glucan (Table 2).

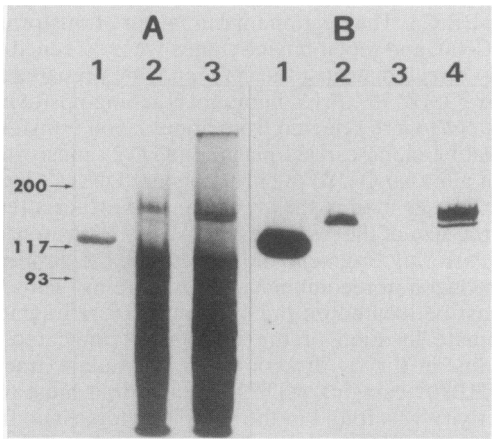

FIG. 2. SDS-PAGE analysis of the *gtf* gene product. (A) Protein staining. (B) Glucan staining. After SDS-PAGE, the gel was incubated in the presence of 5% sucrose plus 1% Triton X-100. Then synthesized glucan was stained with periodic acid-Schiff reagent. Lanes: 1, purified rGTF from *E. coli* HB101(pGT72); 2, crude rGTF; 3, *E. coli* HB101; 4, culture supernatant of *S. sobrinus* AHT. Numbers in the left margin indicate molecular mass markers in kilodaltons.

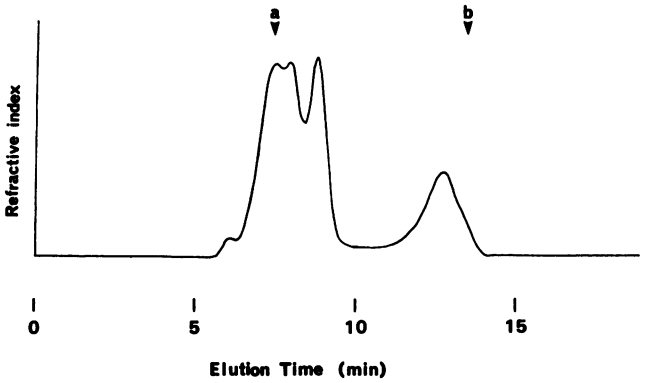

FIG. 3. High-pressure liquid chromatography analysis of glucan synthesized from sucrose by the rGTF of *E. coli* carrying pGT72. The glucan was analyzed by HPLC with a TSK-gel G6000PW column. Arrowheads a and b indicate the elution times of dextran T2000 (molecular weight, 2,000,000) and dextran T10 (molecular weight, 10,000), respectively.

DNA and protein homology between the *gtf* (rGTF) and *gtfS* (rGTFS) genes. Probe pGT72 hybridized with two *Pst*I fragments of pGT72 (Fig. 4B, lane 1) and the vector pACYC184 of pMLG60 (Fig. 4B, lane 2). Another weak band observed in Fig. 4B, lane 2, may have been due to incomplete digestion of the plasmid. Probe pMLG60 hybridized with three *Eco*RI fragments of pMLG60 and the only vector pBR322 of pGT72 (Fig. 4C).

Western blot analysis indicated that rGTF in the crude cell extract from *E. coli* HB101(pGT72) reacted with anti-GTF P1 serum in the region of 130 to 150 kDa (Fig. 5A). The other antiserum raised against GTF P2, P3, or P4 did not react with the rGTF (Fig. 5B to D). The diffused, positive bands in the ~60-kDa regions in the nitrocellulose membrane were also found in *E. coli* HB101(pBR322), which was used as a negative control (data not shown).

DISCUSSION

In our previous studies, four kinds of purified GTFs were prepared from *S. sobrinus* AHT (19, 25). They were composed of two primer-dependent GTFs and two primer-independent GTFs. Of the primer-dependent GTFs, one was a water-insoluble glucan synthase (GTF P3) and the other was a water-soluble glucan synthase (GTF P4); both of the

TABLE 2. Physical and kinetic properties of rGTF and GTF P1

Characteristic	rGTF	GTF P1[a]
M_r	132,000	135,000, 130,000
K_m value (mM) for sucrose	8	3.9
pI	ND[b]	5.8
Optimal pH	4.7	4.7
Primer dependency	Independent	Independent
Glucan	Water soluble	Water soluble
Extent of glucose polymerization	Extremely high molecular mass	Extremely high molecular mass

[a] Data were described by Hanada et al. (9).
[b] ND, not determined.

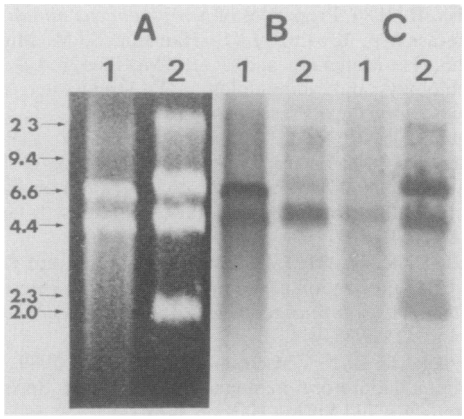

FIG. 4. Southern hybridization between *S. sobrinus gtf* (pGT72) and *S. downei gtfS* (pMLG60). (A) Agarose gel electrophoresis of plasmids pGT72 and pMLG60; (B) hybridization with probe pGT72 bearing *gtf*; (C) hybridization with probe pMLG60 bearing *gtfS*. Lanes: 1, pGT72 DNA cleaved with *Pst*I; 2, pMLG60 DNA cleaved with *Eco*RI. The numbers in the left margin of the figure indicate reference markers (kilobases) for molecular weight determinations.

primer-independent GTFs (GTF P1 and P2) were water-soluble glucan synthases. Previous data indicated that these four GTFs were also separated from the *S. sobrinus* OMZ176 culture supernatant (11).

The present experiments described the results of isolating the *gtf* gene from *S. sobrinus* OMZ176 chromosomal DNA. The *gtf* gene in plasmid pGT72 was distinct from the previously isolated *S. downei* MFe28 *gtfS* gene in pMLG60 (5, 6, 15), which codes for a protein similar to *S. sobrinus* AHT GTF P2 (24, 25), as judged by several criteria. The restriction enzyme map of the *gtf* gene (Fig. 1) is distinct from that of the *gtfS* gene.

The *S. downei* MFe28 rGTFS expressed in *E. coli* synthesizes oligoisomaltosaccharides from sucrose (15). However, the rGTF synthesizes high-molecular-weight, water-soluble glucans (Fig. 3). Southern blot analysis (Fig. 4) indicates that the *gtf* gene of pGT72 differs from the *gtfS* gene of pMLG60 in this stringency of hybridization.

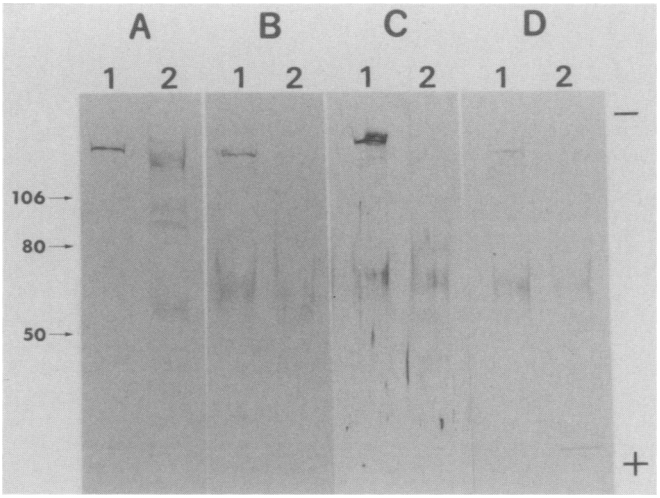

FIG. 5. Western blot analysis of the *gtf* gene product (rGTF). Antiserum was raised against purified GTF (GTF P1, P2, P3, or P4) from *S. sobrinus* AHT. (A) Anti-GTF P1 serum; (B) anti-GTF P2 serum; (C) anti-GTF P3 serum; (D) anti-GTF P4 serum. Lanes: 1, *S. sobrinus* AHT culture supernatant; 2, crude rGTF from *E. coli* HB101(pGT72).

Antisera raised against four kinds of GTFs from strain AHT were used in this study. Although four GTF peaks of strain OMZ176 appeared from the chromatofocusing column (11), antisera against four kinds of GTFs are not yet available from strain OMZ176. In the previous study (15), antisera derived from *S. sobrinus* AHT were tested. The anti-GTF P2 serum, which reacted with rGTFS extracted from *E. coli* (pMLG60) in the previous study, did not react with the rGTF extracted from *E. coli*(pGT72). The anti-GTF P1 serum, which did not react with rGTFS, reacted with rGTF (Fig. 5). These facts suggested that the amino acid sequences of these two GTFs are distinct.

It is easy to presume that the signal peptide of *S. sobrinus* does not work well in *E. coli*. In this experiment, 26% of the GTF activity was found in the periplasmic fraction and most (67%) of the GTF activity was in the cytoplasmic fraction. The fractionation of crude extracts from *E. coli* HB101 carrying pMLG60 (15) revealed that most (73%) of the GTF activity was found in the membrane fraction, compared with only 23% in the cytoplasmic fraction and only 5% in the periplasmic fraction. Since both the *gtf* and *gtfS* genes code for extracellular GTFs, we are interested in their differences in secretion.

Previous results (11) have indicated that the size of the purified GTF (GTase-S) of strain OMZ176 was 150 kDa. In this study, the estimated molecular size of the crude enzyme of the *E. coli* clone was approximately 160 kDa. However, it was apparent that extensive proteolysis of rGTF occurred despite the addition of the protease inhibitor PMSF (Fig. 2B and 5A). The same phenomenon was observed during the purification of the *S. mutans* GS5 *gtfD* gene product (8).

Properties of rGTF were different from those of rGTFS from *E. coli*(pMLG60) in (i) K_m values for sucrose (8 mM for rGTF versus 154 mM for rGTFS), (ii) optimal pHs (4.7 versus 6.0), and (iii) polymer sizes of the glucans (with an extremely high molecular mass, ca. the mass of 27 glucose molecules). On the other hand, properties of rGTF were similar to those of the GTF P1 from *S. sobrinus* AHT in (i) molecular weights of the enzyme, (ii) K_m values for sucrose, (iii) optimal pHs, (iv) primer dependencies, and (v) extents of glucose polymerization (Table 2) and antigenicity (Fig. 5A). Therefore, the results of this study suggested that the *S. sobrinus* OMZ176 *gtf* gene codes for a protein similar to *S. sobrinus* AHT GTF P1.

The priming effectiveness of the water-soluble glucans on the activities of primer-dependent GTFs (GTF P3 and P4) were determined (13, 25). GTF P1 is a good donor of primer for primer-dependent GTF P3 and P4. Therefore, GTF P1 may initially supply water-soluble primer dextrans from sucrose and then primer-dependent GTFs synthesize adhesive water-insoluble glucan on tooth surfaces.

There are probably four distinct *gtf* genes in strain OMZ176. This assumption was based primarily on the observation that *S. sobrinus* OMZ176 GTFs showed four separated peaks upon chromatofocusing (11). rGTFS closely resembles *S. sobrinus* AHT GTF P2 (15). Recently, we isolated the *gtfI* (*gtfP3*) gene from *S. sobrinus* OMZ176 (unpublished results). This recombinant GTF produced a water-insoluble glucan. We are now searching for the fourth *gtf* gene of *S. sobrinus* corresponding to GTF P4, which is the primer-dependent, water-soluble glucan synthase.

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