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Expression of *Streptococcus mutans gtf* Genes in *Streptococcus milleri*

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The *Streptococcus mutans* glucosyltransferase (GTF) genes *gtfB* and *gtfC* were ligated into *Escherichia coli*-streptococcus shuttle plasmids and introduced into *Streptococcus milleri*. *gtfB* transformant KSB8 formed an *S. mutans*-like rough colony on mitis salivarius agar and expressed an extracellular GTF-I, of 158 kDa, and two cell-bound GTF-Is, of 158 and 135 kDa. *gtfC* transformant KSC43 formed a semirough colony on mitis salivarius agar and expressed primarily an extracellular GTF-SI, of 146 kDa, and two cell-bound GTF-SIs, of 146 and 152 kDa. The extracellular GTFs from KSB8 and KSC43 were purified and characterized. The two types of GTF also reacted specifically with monoclonal antibodies directed against each enzyme. Both enzymes synthesized significant amounts of oligosaccharides, consisting primarily of α-1,6-glucosidic linkages, as well as water-insoluble glucans, containing α-1,3-glucosidic linkages. Insoluble-glucan-synthesizing activities of both enzymes were stimulated (three- to sixfold) by the addition of dextran T10 and were inhibited in the presence of 1.5 M ammonium sulfate. The K_ms for sucrose and the optimal pHs were also similar for both enzymes. However, when the transformants were grown in Todd-Hewitt broth supplemented with sucrose, KSC43 cells, expressing GTF-SI activity, adhered to glass surfaces in vitro, while KSB8 cells, expressing GTF-I activity, did not. These results are discussed relative to the potential role of the *gtfB* and *gtfC* genes in *S. mutans* cariogenicity.

The mutans streptococci have been implicated as the principal causative agents in the development of human dental caries (20). One important pathogenic property of these organisms is their ability to colonize tooth surfaces in the presence of dietary sucrose. This phenotype is primarily dependent on the synthesis of water-insoluble adhesive glucans, which is mediated by glucosyltransferases (GTFs; EC 2.4.1.5) (20). Previous results have indicated that the glucans involved in colonization are synthesized by the cooperative action of at least two distinct GTFs: GTF-I, producing primarily water-insoluble α-1,3-linked glucans, and GTF-S, producing water-soluble α-1,6-linked glucans (7, 14, 19). Although the important role of GTFs in sucrose-dependent colonization has been elucidated by biochemical approaches (16, 20), it is not clear how many distinct GTFs are involved in colonization and how the expression of each enzyme is regulated.

The recent introduction of molecular genetic approaches for studying the mutans streptococci (4) has now made it possible to answer some of these still-unresolved questions. Two *gtf* genes, coding for GTF-I and GTF-S activities in *Streptococcus downei* MFe28 (serotype h), have been isolated recently in *Escherichia coli* and characterized (6, 9). Two tandemly arranged genes (*gtfB* and *gtfC*) of *Streptococcus mutans* GS-5 (serotype c) have been isolated (1, 11) in our laboratory, and their nucleotide sequences have been determined (31, 35). These results indicate that the *gtfB* gene codes for the GTF-I enzyme, while the *gtfC* gene codes for the GTF-SI enzyme. Both enzymes synthesize primarily insoluble glucans but are distinguishable because the GTF-SI enzyme synthesizes more soluble glucan than the GTF-I enzyme. In addition, the *gtfD* gene, coding for GTF-S activity, has been isolated more recently from strain GS-5 (12), and its nucleotide sequence has been determined (13).

One approach using these genes to assess their possible roles in cariogenicity involves insertional inactivation of the genes and reintroduction of the altered genes back into *S. mutans*. With this approach, recent results have indicated that both the *gtfB* and the *gtfC* genes are required for sucrose-dependent colonization by *S. mutans* (1, 11). In addition, the cloned genes in question can be introduced into heterologous oral streptococci to detect the appearance of phenotypic properties corresponding to those of cariogenic *S. mutans* strains. This approach could also prove useful in defining the minimum molecular requirements for a specific cariogenic property in *S. mutans*. For the latter approach, *Streptococcus milleri* appears to be a suitable recipient organism, because it is a noncariogenic oral streptococcus that cannot synthesize extracellular glucans (15).

The present communication describes the introduction of the *gtfB* and *gtfC* genes from *S. mutans* GS-5 into transformable *S. milleri* Is57 (NCTC 10707) and the characterization of the transformants.

MATERIALS AND METHODS

Strains and culture conditions. Streptococcal strains were maintained and grown in Todd-Hewitt broth (Difco) as previously described (18). *E. coli* strains were maintained on LB agar plates and routinely grown in L broth (1).

Transformation. *E. coli* transformation was carried out as outlined previously (23), and transformants were selected on the appropriate LB agar plates containing ampicillin (40 μg/ml), chloramphenicol (30 μg/ml), or erythromycin (200 μg/ml). *S. milleri* Is57 was transformed by the procedure described for *S. mutans* and *Streptococcus sanguis* strains (27). Following growth of the transformants on mitis salivar-

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ius agar (MS agar) containing erythromycin (10 μg/ml), individual colonies were isolated and analyzed.

DNA manipulations. Plasmids were isolated from *E. coli* strains by the alkaline sodium dodecyl sulfate (SDS) procedure (23). Streptococcal plasmids were isolated by the procedure of Macrina et al. (22). Conditions for restriction endonuclease cleavage and ligation were those recommended by the suppliers of the restriction endonucleases.

Enzyme and protein assays. GTF activity was determined by a standard radioactive assay as previously described (17), except for the use of membrane filters (0.45-μm pore size). Sucrase activity was determined with a reaction mixture containing 100 mM sodium acetate buffer (AAb) (pH 6.0), 50 mM sucrose, 0.01% sodium azide, 33 μM dextran T10 or distilled water, and enzyme solution in a total volume of 0.3 ml. After incubation at 37°C for 30 min, reducing sugars formed during the reaction were determined by the Somogyi method (32) with glucose as the standard. One unit of enzyme activity was defined as the amount of enzyme catalyzing the formation of 1.0 μmol of glucose from sucrose per min. Protein was estimated by the method of Bradford (3) with bovine serum albumin as the standard protein.

Insoluble glucan synthesis detected following SDS-PAGE. Cells from 100-ml cultures were suspended in 2 ml of phosphate-buffered saline, and the culture fluids were concentrated to 2 ml by ammonium sulfate precipitation. These samples were treated with an equal volume of 2% SDS sample buffer and centrifuged at 10,000 × *g* for 5 min, and each 10 μl of the supernatants was subjected to SDS-polyacrylamide gel electrophoresis (PAGE). After electrophoresis, the gel was incubated at 37°C for 15 h or 3 days in sucrose–Triton X-100 solution (28) to detect insoluble glucan synthesis.

Purification of GTFs expressed in *S. milleri*. Extracellular GTF-I and GTF-SI were purified from the culture fluids of KSB8 and KSC43 cells grown overnight anaerobically at 37°C in Todd-Hewitt broth (2 liters) containing erythromycin (10 μg/ml) and 10 μM *p*-aminophenylmethylsulfonyl fluoride (p-APMSF; Wako, Osaka, Japan). Unless otherwise noted, all purification procedures were carried out at 4°C and all buffers used during purification were supplemented with 10 μM p-APMSF and 0.01% sodium azide. Both crude enzymes were obtained following 60% saturation ammonium sulfate precipitation and dialysis against 10 mM potassium phosphate buffer (KPb) (pH 6.0). Crude GTF-I was applied to a hydroxylapatite column (1.2 by 10 cm) equilibrated with 50 mM KPb (pH 7.5) containing 0.1% Triton X-100. After the column was washed with the equilibrating buffer, bound proteins were eluted with 300 mM KPb (pH 7.5) containing 0.1% Triton X-100. The eluate was applied to a Toyopearl HW-55 (Toso, Tokyo, Japan) gel filtration column (1.5 by 80 cm) equilibrated with 20 mM KPb (pH 7.5) containing 2 M urea and 0.1% Triton X-100. Elution was carried out with the equilibrating buffer at a flow rate of 10 ml/h. The active fractions eluting immediately following the void volume were pooled, dialyzed against 10 mM KPb (pH 6.0) containing 0.1% Triton X-100, and used as the purified GTF-I preparation.

Crude GTF-SI was supplemented with 15% ammonium sulfate and applied to a Toyopearl HW-65 column (1.2 by 8 cm) equilibrated with 15% ammonium sulfate in 10 mM KPb (pH 6.0). After the column was washed with the equilibrating buffer, bound proteins were eluted with a reverse linear gradient of 15 to 0% ammonium sulfate. Fractions possessing GTF activity were pooled and concentrated by dialysis against solid polyethylene glycol. The concentrated sample was applied to a Toyopearl HW-55 column and chromatographed under the same conditions as those used for GTF-I purification. The active fractions were pooled, dialyzed, and used as the purified GTF-SI preparation.

Gel electrophoresis. DNA fragments were analyzed following electrophoresis in agarose gels (23). SDS-PAGE analysis of the GTFs was carried out as previously described (1), and insoluble-glucan-synthesizing activity on the gels was detected as described by Russell (28), except that the gel with the *gtfC* gene product was incubated at 37°C for 3 days.

Western blot (immunoblot) and Southern blot analyses. For immunoblotting, proteins were transferred by electrophoresis from SDS-polyacrylamide gels to nitrocellulose paper as described by Towbin et al. (33) and developed with monoclonal antibodies specific for GTF-I (P#18 and P#72) and for GTF-SI (P#12 and P#32). Hybridomas secreting these antibodies were generated by fusing P3U1 myeloma cells with splenocytes from mice immunized with a partially purified GTF-I preparation from *S. mutans* PS14 (serotype c). The details of the production and characterization of the antibodies will be reported elsewhere. Southern hybridization was carried out as previously described (34) with a biotinylated probe and *Bam*HI digests of chromosomal DNA from *S. milleri* transformants.

Isolation and analysis of glucans. Isolation and chemical analysis of the glucans synthesized by purified GTF-I and GTF-SI were carried out as follows. Reaction mixtures (0.9 ml) consisting of 100 mM AAb (pH 6.0), 50 mM sucrose, 33 μM dextran T10 or distilled water, 0.01% sodium azide, and enzyme solution (each approximately 60 mU) were incubated at 37°C for 15 h. After incubation, insoluble material was collected by centrifugation at 10,000 × *g* for 5 min, washed twice with distilled water, and dissolved in 1 N NaOH. The soluble glucans in the supernatant fluids were isolated by the addition of 9 volumes of cold absolute ethanol, overnight storage at 4°C, centrifugation at 10,000 × *g* for 10 min, two washes with 90% ethanol, and dissolving of the precipitates in distilled water. A fraction of the soluble glucans was further dialyzed against tap water. Total amounts of insoluble glucans, soluble glucans, and nondialyzable soluble glucans were measured by the phenol-sulfuric acid method (5) with glucose as the standard.

Glucan samples for linkage analysis were prepared in a similar manner, except that 9 ml of the reaction mixture was incubated for 24 h in the absence of dextran T10 and the washed glucans were lyophilized. Methylation analysis of the insoluble glucans was performed as described by Shimamura et al. (29) with a Shimazu GC-9A gas-liquid chromatograph. The soluble glucans and the dextranase digests were analyzed by high-pressure liquid chromatography (HPLC) with a carbohydrate analysis column (Waters, Milford, Mass.) and a Waters 410 differential refractometer. Molecular size markers for oligo-isomaltosaccharides were from Seikagaku Kogyo (Tokyo, Japan). Endodextranase (1,6-α-D-glucan-6 glucanohydrolase; EC 3.2.1.11) from a *Penicillium* sp. (Sigma), glucodextranase (1,6-α-D-glucan glucohydrolase; EC 3.2.1.70) from *Arthrobacter globiformis* (Funakosi, Tokyo, Japan), and isomaltodextranase (1,6-α-D-glucan isomaltohydrolase; EC 3.2.1.94) from *A. globiformis* (Wako) were used for enzyme digestion of the soluble glucans.

In vitro sucrose-dependent colonization. Sucrose-dependent colonization of the transformants on glass surfaces was achieved as previously described (18), except that the adherent cells were vortexed (10 s) during washing, stained with 1% methylene blue, and photographed. The number of

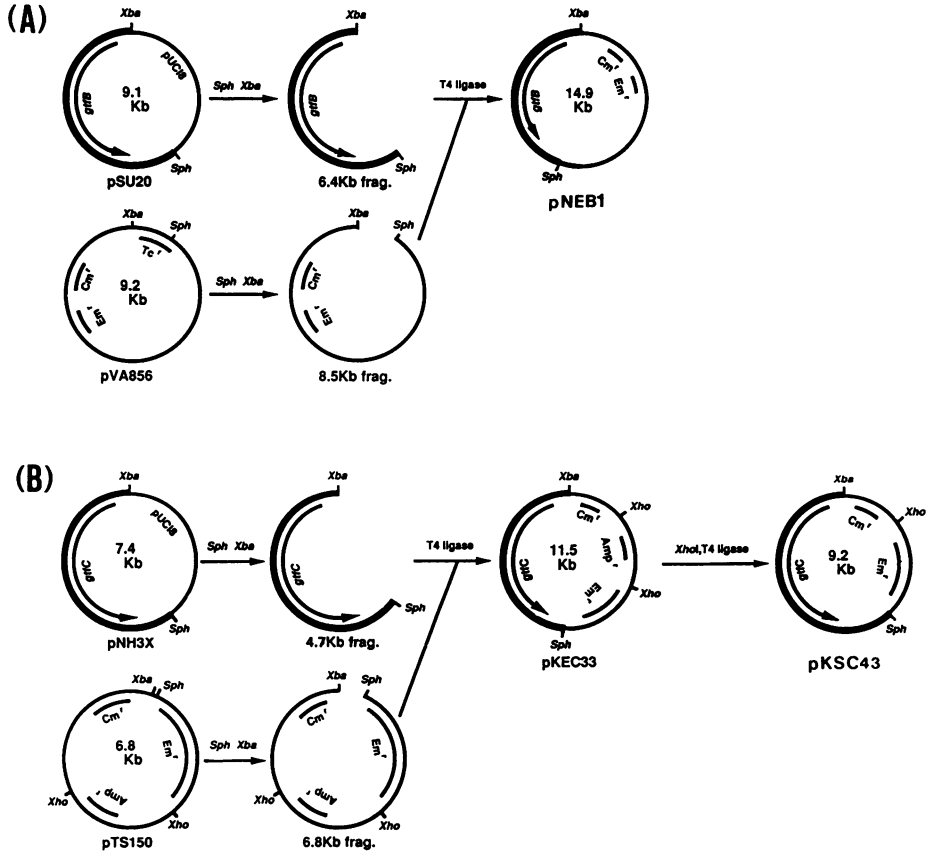

FIG. 1. Construction of *E. coli*-streptococcus shuttle plasmids. (A) Construction of *gtfB*-containing shuttle plasmids. (B) Construction of *gtfC*-containing shuttle plasmids. Heavy black bars represent *S. mutans* GS-5 inserts. Arrows represent the approximate location and direction of the transcription of each gene. Restriction enzymes: *Sph*, *Sph*I; *Xba*, *Xba*I; *Xho*, *Xho*I. frag., fragment.

adherent cells was estimated by determining the turbidity at 550 nm after sonication (50 W, 10 s) of the attached cells.

RESULTS

Construction of GTF-I- and GTF-SI-expressing shuttle vectors. To transfer the *gtfB* and *gtfC* genes into transformable oral streptococci, we found it necessary initially to insert DNA fragments containing these genes into appropriate *E. coli*-streptococcus shuttle vectors. For this purpose, the intact *gtfB* gene contained in pSU20 (the *Pst*I *gtfB* fragment in the orientation opposite from that in pSU5; Shiroza et al. [31]) was cleaved with restriction enzymes *Xba*I and *Sph*I and ligated into shuttle plasmid pVA856 (21), which had been cut with the same enzymes (Fig. 1A). *E. coli* transformants expressing both Cm^r and Em^r were selected and assayed directly for GTF activity. Ten transformants were initially screened, and all of them displayed GTF activity. One of these transformants, NEB1, displayed the structure predicted (Fig. 1A) following restriction endonuclease analysis of the isolated plasmid and was used as the GTF-I shuttle vector in subsequent experiments.

The GTF-SI shuttle vector was constructed by use of a new *E. coli*-streptococcus shuttle vector, pTS150 (30a). Initially, the single *Sma*I site in plasmid pNH3 (11) was converted into an *Xba*I site by use of an *Xba*I linker to produce plasmid pNH3X. The *gtfC* gene in pNH3X was cleaved with *Xba*I and *Sph*I and ligated into shuttle vector pTS150, which had been cleaved with the same enzymes (Fig. 1B). *E. coli* transformants expressing both Em^r and Ap^r were selected and assayed directly for GTF activity. One transformant expressing GTF activity, KEC33, displayed the structure predicted following restriction endonuclease analysis of the isolated plasmid.

Expression of the *gtf* genes in *S. milleri*. Oral isolates of *S. milleri*, including strain Is57, do not express GTF activity (Table 1). Therefore, the GTF-I shuttle plasmid, pNEB1, was transformed into *S. milleri* Is57, and Em^r transformants were identified on MS agar plates. However, very few Em^r transformants were obtained, and only 26 transformants were obtained after repeated attempts. Almost all of these

TABLE 1. Extracellular GTF activities of *S. milleri* transformants

Enzyme (μg of protein)[a]	GTF activity[b] (cpm/16 h)			
	Insoluble glucans		Soluble glucans	
	With dextran T10	Without dextran T10	With dextran T10	Without dextran T10
Is57 (29)	0	0	0	0
KSB8 (35)	7,494	2,105	1,273	30
KSC43 (33)	3,447	1,255	883	51

[a] Sixty percent saturation ammonium sulfate precipitation fractions of culture fluids.

[b] GTF activity was determined by a radioactive assay as described in the text.

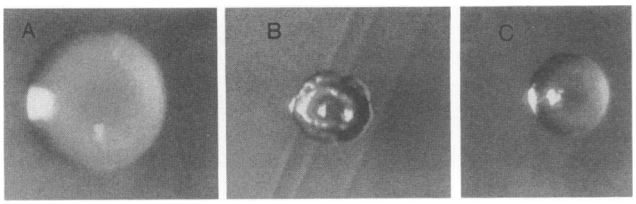

FIG. 2. Colony morphologies of the *gtfB* and *gtfC S. milleri* transformants. KSB8 (B) and KSC43 (C) cells were grown anaerobically for 24 h and incubated aerobically for 48 h on MS agar plates containing erythromycin (10 μg/ml). The recipient cells (Is57) (A) were grown on MS agar plates.

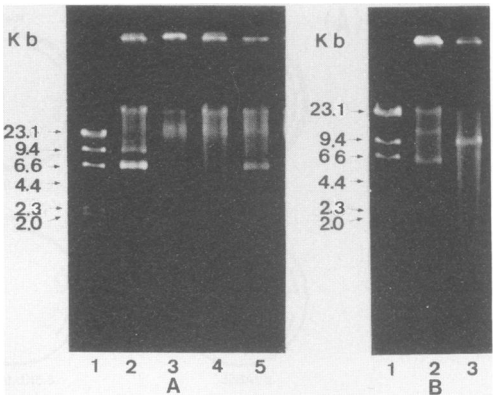

FIG. 3. Plasmid analysis of the *S. milleri* transformants. (A) Detection of plasmids in the transformants. Lanes: 1, *Hin*dIII digest of lambda DNA; 2 to 5, plasmid fractions from Is57(pVA838) (this transformant was used as a positive control for plasmid isolation), KSB7, KSB8, and KSC43 transformants, respectively. (B) *Xho*I digestion of the plasmid fraction (pKSC43) from the KSC43 transformant. Lanes: 1, *Hin*dIII digest of lambda DNA; 2, nondigested pKSC43; 3, *Xho*I-digested pKSC43.

transformants expressed negligible GTF activity and displayed the same smooth colony morphology as that of the recipient cells on MS agar plates. Only two Em^r transformants, KSB7 and KSB8, which were isolated from different plates in the same transformation experiment, expressed significant GTF activity and formed an *S. mutans*-like rough colony on MS agar plates (Fig. 2). One of these, selected for further investigation, KSB8, synthesized primarily water-insoluble glucans in the presence or absence of dextran T10 (Table 1). However, the addition of the primer led to significant amounts of soluble glucan formation.

The GTF-SI shuttle plasmid, pKEC33, contains an Ap^r gene, which was removed following *Xho*I digestion and self-ligation with T4 ligase, and the resultant plasmid, pKSC43, was transformed into *S. milleri* Is57 (Fig. 1B). In contrast to the results obtained with the GTF-I shuttle plasmid, numerous Em^r transformants were obtained. Seven of 10 randomly isolated transformants expressed significant GTF activity. One of these, KSC43, synthesized primarily water-insoluble glucans (Table 1) and formed a semirough colony in which the center was raised on MS agar plates (Fig. 2).

Detection and analysis of plasmids in the *S. milleri* transformants. Extraction of plasmids from the transformants indicated that KSB7 and KSB8 harbored no detectable plasmids, while KSC43 exhibited distinct plasmid bands on agarose gels (Fig. 3A). When the plasmid from KSC43 cells was cleaved with *Xho*I, one fragment of approximately 9.2 kb was identified (Fig. 3B). Cleavage with *Sph*I or *Xba*I also produced a fragment of the same size (data not shown). On the other hand, when chromosomal DNAs from these transformants were digested with *Bam*HI and analyzed by Southern blotting, a probe from the *gtfB* gene hybridized with a 1.6-kb fragment from KSB8 cells but not KSC43 cells (data not shown). These results suggest that plasmid pNEB1 was integrated into the *S. milleri* chromosome, while plasmid pKSC43 was maintained intact in the heterologous recipient.

Characterization of the GTF-I enzyme expressed by transformant KSB8. The insoluble-glucan-synthesizing abilities of the transformants and relevant streptococci were compared by SDS-PAGE analysis (Fig. 4). The KSB8 transformant produced an extracellular 158-kDa GTF-I that was similar in size to the cell-associated GTF-I of *S. mutans* GS-5. In addition, KSB8 cells also produced large amounts of cell-associated GTF-I consisting of at least two components, of 158 and 135 kDa.

The extracellular GTF-I from strain KSB8 was purified following hydroxylapatite column chromatography and Toyopearl HW-55 gel filtration chromatography. The specific activity and yield of the final preparation were 11.4 U mg^{-1} and 26%, respectively. The results of SDS-PAGE analysis and subsequent immunoblotting analysis of this preparation indicated that a single major protein band of approximately 158 kDa displayed GTF activity and reacted with a GTF-I-specific monoclonal antibody, P#18, but not with a GTF-SI-specific monoclonal antibody, P#32 (Fig. 5). The purified enzyme synthesized, in addition to water-insoluble glucans, significant amounts (over 65% of the total glucans synthesized) of dialyzable water-soluble glucans that were insoluble in 90% ethanol (Table 2). These latter products were not detected by the standard GTF assay (Table 1), which detects only high-molecular-mass soluble glucans. As with the crude enzyme, insoluble glucan synthesis by the purified enzyme was stimulated approximately threefold by the addition of dextran T10. The presence of 1.5 M ammonium sulfate resulted in 45% inhibition of insoluble glucan synthesis. The pH optimum for the insoluble-glucan-synthesizing activity was 6.2 to 7.2, while the K_m for sucrose was estimated to be 7.2 mM at pH 6.5.

Characterization of the GTF-SI enzyme expressed by trans-

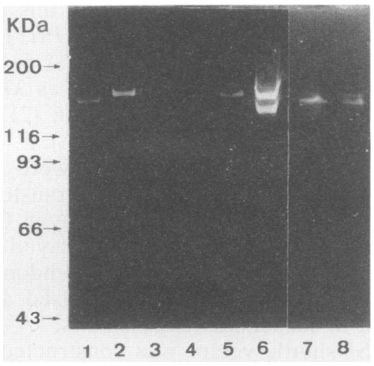

FIG. 4. Expression of water-insoluble-glucan-synthesizing activities by the *gtfB* and *gtfC* transformants. Lanes: 1, 3, 5, and 7, culture fluids of *S. mutans* GS-5, *S. milleri* Is57, and transformants KSB8 and KSC43, respectively; 2, 4, 6, and 8, cell-associated activities of *S. mutans* GS-5, *S. milleri* Is57, and transformants KSB8 and KSC43, respectively.

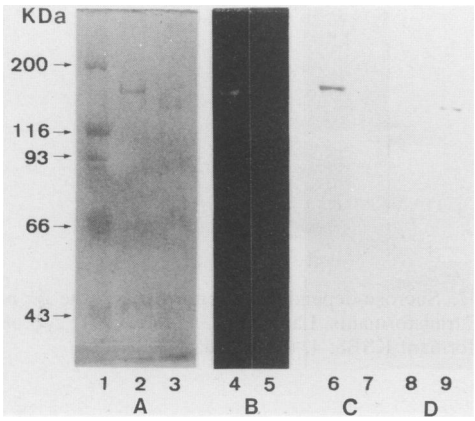

FIG. 5. SDS-PAGE and Western blot analyses of purified GTF-I and GTF-SI. (A) Coomassie blue staining. (B) Activity displayed after incubation with sucrose. (C) Immunoblotting with a GTF-I-specific monoclonal antibody, P#18. (D) Immunoblotting with a GTF-SI-specific monoclonal antibody, P#32. Lanes: 1, protein standards; 2, 4, 6, and 8, GTF-I enzyme; 3, 5, 7, and 9, GTF-SI enzyme.

formant KSC43. The KSC43 transformant produced primarily an extracellular GTF-SI, of 146 kDa, and two cell-associated GTF-SIs, of 146 and 152 kDa (Fig. 4). Although the ratios of extracellular to cell-bound enzyme activities were altered by culture conditions, over 60% of the insoluble-glucan-synthesizing activity was observed generally in the culture fluids. As with strain KSB8, the crude enzyme from strain KSC43 also synthesized exclusively water-insoluble glucans in the absence of dextran T10, while the addition of the primer stimulated insoluble glucan synthesis (approximately threefold) and led to significant soluble glucan synthesis (Table 1).

It was of interest to purify GTF-SI from strain KSC43, because this enzyme has not been isolated from *S. mutans* cultures. By means of hydrophobic and gel filtration chromatographies, a purified GTF-SI fraction with a specific activity of 4.7 U mg^{-1} was obtained, with a 13% yield. The results of SDS-PAGE analysis and subsequent immunoblotting of this preparation indicated that a single major protein band of approximately 140 kDa displayed GTF activity and reacted with a GTF-SI-specific monoclonal antibody, P#32, but not with a GTF-I-specific antibody, P#18 (Fig. 5). When the reaction products of the purified enzyme were isolated and quantitated colorimetrically, it was observed that over 85% of the glucans synthesized in the absence of dextran T10 represented the dialyzable low-molecular-weight water-soluble products, while the remainder represented water-insoluble glucans (Table 2). As with GTF-I, insoluble glucan synthesis by this enzyme was stimulated approximately sixfold in the presence of dextran T10 and strongly inhibited (>80% inhibition) by the addition of 1.5 M ammonium sulfate. The pH optimum for the insoluble-glucan-synthesizing activity was 6.4 to 6.6, while the K_m for sucrose was estimated to be 11.2 mM at pH 6.5.

TABLE 2. Glucans synthesized by GTF-I and GTF-SI

Enzyme (μg of protein)[a]	Glucan formed in μg[b]			
	Insoluble		Soluble[c]	
	With dextran T10	Without dextran T10	With dextran T10	Without dextran T10
GTF-I (5.3)	2,270	770	ND	1,510 (24)
GTF-SI (12.8)	1,550	260	ND	1,870 (33)

[a] Purified preparations.
[b] Isolation and determination of glucans were carried out as described in the text.
[c] Low-molecular-mass glucans (precipitated by 90% ethanol). ND, not determined. Values in parentheses are those for nondialyzable water-soluble glucans.

Structural analysis of the glucans synthesized by the GTFs. To detect possible differences in the products of the purified GTF-I and GTF-SI enzymes, we isolated and subjected both the water-insoluble and the soluble glucans synthesized in the absence of dextran T10 to methylation analysis and HPLC analysis, respectively. The results of these structural analyses indicated that the insoluble and soluble products synthesized by both cloned GTFs were very similar. The water-insoluble glucans synthesized by GTF-I contained 1,3-α-D-glucans (87.5%), with 6.7% 1,6-α- and 5.5% 1,3,6-α-glucosidic linkages, and the GTF-SI products contained 1,3-α-D-glucans (85.6%), with 7.8% 1,6-α- and 7.0% 1,3,6-α-glucosidic linkages. On the other hand, the soluble glucans synthesized by both cloned GTF-I and cloned GTF-SI consisted primarily of penta- to decasaccharides (Fig. 6B and C). These oligosaccharides were degraded to isomaltose or glucose following digestion with endodextranase, isomaltodextranase, or glucodextranase (Fig. 6D and E).

Sucrose-dependent adherence to smooth surfaces by the *S. milleri* transformants. It was of interest to compare the *S. milleri* transformants with *S. mutans* GS-5 in terms of their ability to adhere tightly to smooth surfaces in vitro in the presence of sucrose. As shown in Fig. 7, KSB8 cells, expressing GTF-I activity, as well as *S. milleri* Is57 cells, could not adhere to glass surfaces in the presence of sucrose. However, KSB8 cells rapidly aggregated in the presence of sucrose, and this action may have prevented attachment of the cells to the sides of the glass tubes. In contrast, KSC43 cells, expressing GTF-SI activity, could adhere to these surfaces, although the extent of colonization was smaller than that of *S. mutans* GS-5. When the percentages of adherent cells among total cells were estimated turbidimetrically, the percentages for GS-5, Is57, KSB8, and KSC43 cells were approximately 50, <1, 1, and 12%, respectively.

DISCUSSION

The present investigation was initiated to examine the expression of *S. mutans* virulence-associated genes in *S. milleri* and to identify the minimum number of *gtf* gene products required for sucrose-dependent colonization. The *gtfB* and *gtfC* genes from *S. mutans* GS-5 were introduced into streptococcus-*E. coli* shuttle plasmids and transformed into competent *S. milleri* cells. The *gtfC* gene could be recovered on a plasmid in the transformants, while the *gtfB* gene could not be recovered on plasmid pVA856 (Fig. 3A). The results of Southern blot hybridization further suggested that the *gtfB* gene in transformant KSB8 was integrated into the host chromosome. When *S. milleri* cells were transformed with shuttle vector pTS150, containing the *gtfB* gene, many transformants expressing GTF activity were initially obtained. However, these transformants were extremely unstable and lost GTF activity rapidly (data not shown). These results suggest that *S. milleri* cannot tolerate high-level expression of the *gtfB* gene. Because the expression of the *gtfC* gene appears to be under the control of a relatively

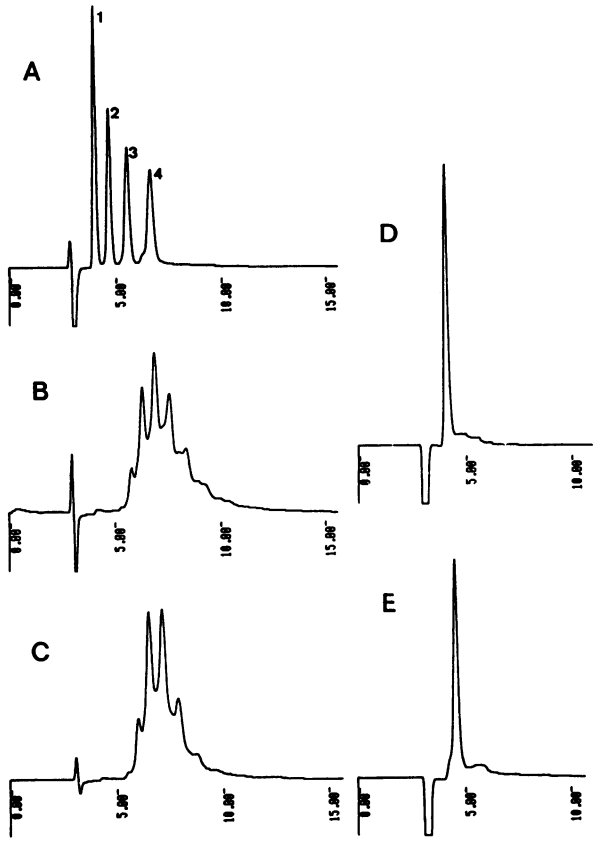

FIG. 6. HPLC analysis of water-soluble glucans and dextranase digests. Glucans were analyzed by HPLC with a carbohydrate analysis column. Elutions were carried out at 37°C, with a mobile phase of 50% CH_3CN–50% H_2O and at a flow rate of 1 ml/min. One milligram of each glucan was digested with 1 U of glucodextranase or isomaltodextranase (at 37°C, for 20 h, in pH 6.0 AAb), and the digests were analyzed identically. (A) Saccharide standards: 1, glucose; 2, isomaltotriose; 3, isomaltopentaose; 4, isomaltoheptaose. (B) Water-soluble glucans synthesized by the GTF-I enzyme. (C) Water-soluble glucans synthesized by the GTF-SI enzyme. (D) Glucodextranase digests of water-soluble glucans synthesized by the GTF-I enzyme. (E) Isomaltodextranase digests of water-soluble glucans synthesized by the GTF-I enzyme.

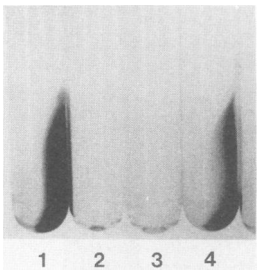

FIG. 7. Sucrose-dependent colonization of the *S. milleri gtfB* and *gtfC* transformants. Lanes: 1, *S. mutans* GS-5; 2, *S. milleri* Is57; 3, transformant KSB8; 4, transformant KSC43.

weak promoter (35) and GTF activity in transformant KSC43 is lower than that from the presumed single copy of the *gtfB* gene in KSB8, the *gtfC* gene can be expressed from a multicopy plasmid in *S. milleri*. It will be of interest to examine the molecular basis for this differential property of *S. milleri*.

The introduction of the *gtfB* gene from *S. mutans* GS-5 into *S. milleri* Is57 resulted in a change from large smooth to small rough colony morphology as well as the expression of the GTF-I enzyme (Fig. 2 and 4). This finding suggests that rough colony formation on MS agar, which is one of the specific characteristics of *S. mutans*, could be correlated with the expression of the *gtfB* gene. On the other hand, the introduction of the *gtfC* gene into the same organism resulted in the acquisition of the ability to adhere to glass surfaces in the presence of sucrose (Fig. 7). In addition, the expression of the GTF-SI enzyme could be correlated with the distinct morphology of *S. mutans* relative to *S. milleri* Is57 and KSB8 (Fig. 2). Furthermore, these findings suggest that the ability of *S. mutans* to adhere tightly to tooth surfaces might be, in part, dependent on the expression of the *gtfC* gene. This suggestion is compatible with previous results demonstrating that *S. mutans* GS-5 *gtfC* mutants did not display sucrose-dependent adherence to smooth surfaces in vitro (11). Since GS-5 *gtfB* mutants also did not adhere to glass surfaces in the presence of sucrose (1), it was surprising that the *S. milleri* transformant expressing GTF-I activity did not adhere to glass surfaces (Fig. 7). However, the aggregation of the latter strain in the presence of sucrose may have precluded its ability to colonize under the in vitro assay conditions used. It will be of interest to examine the caries-promoting abilities of these transformants in rats fed diets containing high levels of sucrose.

The fact that the transformant harboring only the *gtfC* gene could adhere to hard surfaces in the presence of sucrose suggests that the sucrose-dependent adherence of *S. mutans* could be mediated by a single GTF enzyme, GTF-SI. This suggestion would be analogous to previous results suggesting that the adherence to glass surfaces of heat-killed *Streptococcus sobrinus* cells was enhanced by the addition of only the GTF-I enzyme from the same organism as well as by the cooperative action of the GTF-I and GTF-S enzymes (8). In the case of *S. mutans*, however, the role of the GTF-S enzyme in colonization still remains to be determined.

The isolation of GTFs synthesizing water-insoluble glucans from *S. mutans* cultures has been described by several laboratories. Kuramitsu and Wondrack isolated a highly purified GTF-I-like enzyme of 155 kDa in addition to a homogeneous GTF-S of 140 kDa from the culture fluids of strain GS-5 (19). Furthermore, Mukasa et al. also purified an insoluble-glucan-synthesizing GTF of 99 kDa from the culture fluids of strain Ingbritt and suggested that it may be a partially degraded form of GTF-I (26). More recently, Hamada et al. (10) and Mukasa et al. (25) purified a cell-associated GTF of 156 to 158 kDa from cell extracts of strains MT8148 and Ingbritt, respectively. Although biochemical and immunological approaches suggest that all of these purified GTF-I enzymes are free of GTF-S enzymes, the presence or absence of the similar GTF-SI enzyme in these preparation has not yet been determined. Polyclonal antibodies against GTF-I cannot distinguish between GTF-I and GTF-SI, because both share extensive amino acid homology (35). In this regard, almost two-thirds of the monoclonal antibodies prepared from mice immunized with a partially purified GTF-I from strain PS14 recognized both cloned GTF-I and cloned GTF-SI (6a). Because of its recent identification (11), the GTF-SI enzyme has not yet been detected in culture fluids of *S. mutans*. In this study, the identification of *gtf* gene products was carried out by use of specific monoclonal antibodies that distinguish between

GTF-I and GTF-SI. These monoclonal antibodies will be very useful in identifying the GTF proteins produced by *S. mutans*. Using these monoclonal antibodies, we have found that most strains of *S. mutans* (reference strains and clinical isolates) produce variable amounts of extracellular and cell-associated GTF-SI in addition to GTF-I and GTF-S (6a).

The use of *E. coli* or *S. milleri* transformants harboring either the *gtfB* or the *gtfC* gene appears to be ideal for the preparation of GTF-I and GTF-SI enzymes free of contamination with each other. In this regard, both cloned GTF-I and cloned GTF-SI have been purified from cell extracts of *E. coli* MH76 and NH3 transformants, respectively (11, 12a). However, the purification and characterization of the *E. coli*-expressed enzymes are complicated by their tendency to aggregate and to be proteolytically modified. Thus, the purification of these extracellular GTFs from *S. milleri* transformants was carried out in this study. Although both cloned GTFs were purified in relatively high yields (26 and 16%, respectively) from the culture fluids of KSB8 and KSC43 cells, the final preparations were still contaminated with some low-molecular-mass components (Fig. 5). Purification in the absence of Triton X-100 resulted in a pronounced decrease in yield, suggesting that the extracellular GTFs expressed in the *S. milleri* transformants also had the tendency to aggregate. The molecular size of the purified GTF-SI enzyme, 140 kDa, was somewhat lower than that of the enzyme in the crude preparation and was identical to that of the purified enzyme from *E. coli* transformants (11). By contrast, the size of the GTF-I enzyme (155 kDa) did not change during purification (Fig. 4 and 5).

The present results also indicated that both purified GTF-I and GTF-SI synthesized large amounts of low-molecular-mass water-soluble glucans, consisting primarily of α-1,6-glucosidic linkages, in addition to water-insoluble glucans. The synthesis of dialyzable water-soluble glucans by the GTF-SI enzyme has been reported with the enzyme purified from an *E. coli* clone (11). However, the present investigation also indicated that the *gtfB* gene product also synthesized similar water-soluble glucans. The low-molecular-mass water-soluble glucans synthesized by these cloned GTF enzymes are obviously different from the high-molecular-mass water-soluble glucans synthesized by GTF-S enzymes from *S. mutans* (2, 19) and other streptococci (24, 30). These glucans are also distinct in the degree of glucose polymerization from the products of an oligo-isomaltosaccharide synthase purified from *S. sobrinus* culture fluids (36). The role of these low-molecular-mass glucans in adhesive glucan synthesis is currently under investigation. The GTF-I enzyme purified from the culture fluids of *S. mutans* GS-5 (19) and extracellular GTF-I and GTF-SI from the *S. milleri* transformants were activated severalfold in the presence of dextran T10 (Tables 1 and 2). In contrast, water-insoluble glucan synthesis by GTF-I and GTF-SI from *E. coli* is not significantly enhanced by the addition of dextran T10 (1, 11). This difference in the primer dependence of the GTF-I enzymes may result from the earlier utilization of a *gtfB* gene lacking several of the carboxyl-terminal direct repeat sequences in the *E. coli* clones (1). In addition, differential modification of the enzymes in *E. coli* relative to *S. milleri* may also be involved in these properties. Therefore, it will be of interest to determine the molecular basis for some of the differences displayed by each enzyme expressed in either *E. coli* or *S. milleri*. Such information may be relevant to the mechanism of glucan synthesis by the GTFs of mutans streptococci.

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