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Isolation and Characterization of the *Streptococcus mutans gtfC* Gene, Coding for Synthesis of Both Soluble and Insoluble Glucans

NOBUHIRO HANADA AND HOWARD K. KURAMITSU*

Department of Microbiology-Immunology, Northwestern University Medical-Dental Schools, Chicago, Illinois 60611

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The intact *gtfC* gene from *Streptococcus mutans* GS-5 was isolated in *Escherichia coli* in plasmid vector pUC18. The glucosyltransferase activity expressed by the gene synthesized both low-molecular-weight water-soluble glucan and insoluble glucan in a primer-independent manner. Purification of the enzyme by procedures that minimize proteolytic digestion yielded a purified preparation with a molecular weight of 140,000. Insertional inactivation of the *gtfC* gene with a streptococcal erythromycin resistance gene fragment followed by transformation of strain GS-5 suggested that the *gtfC* gene product was required for sucrose-dependent colonization in vitro. In addition, evidence for the presence of a third *gtf* gene coding for soluble glucan synthesis was obtained following the construction of mutants containing deletions of both the *gtfB* and *gtfC* genes.

The role of insoluble glucan synthesis in the cariogenicity of the mutans streptococci has been well documented (12). Biochemical approaches have suggested that at least two distinct glucosyltransferases (GTFs) are involved in the formation of the adhesive polymer: GTF-S, incorporating glucose in α-1,6-linkages into water-soluble glucan products, and GTF-I, synthesizing α-1,3-linked insoluble glucans (12). For certain *Streptococcus sobrinus* strains, evidence for three (21) and four (24) distinct GTFs has been reported. However, so far only two distinct enzymes have been isolated from *S. mutans* strains (8, 11, 14, 15).

For clarification of the number of *gtf* genes present in individual strains of *S. mutans* as well as their respective roles in sucrose-dependent colonization of tooth surfaces, several genes expressing GTF activity have been isolated by recombinant DNA techniques (1, 7). The genes coding for GTF-I activity in *S. mutans* GS-5 (*gtfB*) (22) and *S. sobrinus* MFe28 (6) have been isolated, and their nucleotide sequences have been determined. More recently, a gene coding for GTF-S activity in the latter strain has also been isolated (R. R. B. Russell, personal communication). In addition, a DNA fragment coding for GTF-S activity has been isolated from *S. mutans* LM-7, although neither the intact gene nor the enzyme product has been characterized (19). It was of interest that this fragment appeared to contain two partially homologous *gtf* genes in tandem.

The present communication reports the initial isolation of the intact *gtfC* gene from *S. mutans* GS-5. The gene was isolated by use of a strategy suggested by recent sequencing data (22) indicating that a distinct *gtf* gene was located immediately downstream from the previously cloned *gtfB* gene. The enzyme product was purified, and its properties were compared with those previously reported for the GTFs isolated from strain GS-5 (11). The implications of the tandem arrangement of the *gtfB* and *gtfC* genes are discussed relative to the cariogenic properties of *S. mutans*.

MATERIALS AND METHODS

Microorganisms. *S. mutans* GS-5 and *Escherichia coli* MM294 (2) and JM83 (26) were maintained and grown routinely as previously described (1).

DNA manipulations. DNA isolation, endonuclease restriction, ligation, and transformation of competent *E. coli* cells were carried out as recently described (1). *S. mutans* transformations were carried out as originally described (18), and transformants were isolated on mitis salivarius agar plates containing erythromycin (10 μg/ml). Construction of the *Pst*I clone bank was recently described (22). Clones containing the *gtfC* gene were initially identified following colony hybridization with a biotinylated 1.6-kilobase (kb) *Bam*HI fragment from the *gtfB* gene (1).

Localization of GTF activity. The subcellular distribution of the *gtfC* gene product in *E. coli* clones was determined as recently described (20).

Enzyme and protein assays. Sucrase activity was determined by the Somogyi-Nelson procedure as previously described (23). GTF activity was determined as previously described (9) with [^{14}C]glucose-sucrose. Low-molecular-weight glucans were assayed by the phenol-sulfuric acid method (5) following 90% (vol/vol) ethanol precipitation of the reaction mixtures. One unit of enzyme activity was defined as the amount of enzyme catalyzing the incorporation of 1.0 μmol of glucose from sucrose into glucan per minute under standard assay conditions. Protein estimation was carried out by the method of Bradford (3) with bovine serum albumin as the standard protein.

The optimum pH and K_m values of the enzyme were determined as previously described (9).

In vitro sucrose-dependent colonization. Sucrose-dependent colonization of glass surfaces by *S. mutans* cells was carried out as originally described (17), except that the adherent cells were mildly vortexed during washing.

Gel electrophoresis. Proteins were analyzed by sodium dodecyl sulfate–7% polyacrylamide gel electrophoresis (SDS-PAGE) essentially as recently described (1). DNA fragments were analyzed on 0.5 or 0.7% agarose gels with Tris-EDTA-borate buffer (13).

Purification of the *gtfC* gene product. The *gtfC* gene product was extracted from *E. coli* MM294(pNH3) cells which had been grown overnight in Luria broth containing 1.0 mM phenylmethylsulfonyl fluoride (PMSF) and ampicillin (40 μg/ml). The cells were harvested and suspended in a quantity of extraction buffer (20 mM Tris hydrochloride buffer [pH 8.3] containing 1.0 mM PMSF and 2.5 mM EDTA equivalent to

* Corresponding author.

1% of the original culture volume. The cells were disrupted for 10 min in a Mickle disintegrator (H. Mickle Ltd., Surrey, England) with glass beads (0.2 g/ml). After centrifugation at 12,000 × *g* for 15 min, the supernatant fluids were retained. The resultant pellet was suspended in the same buffer containing 4% Triton X-100 and extracted twice. Following centrifugation, the pellet was suspended in extraction buffer and treated for 2 min with alkali (1 N KOH [pH 11.7]). The suspension was then immediately neutralized with 4 N H_2SO_4 and centrifuged. The supernatant fluid was pooled with the other extracts and constituted fraction I.

Solid ammonium sulfate was added to fraction I up to 35% saturation, and the mixture was stirred gently for 3 h at 4°C and centrifuged at 12,000 × *g* for 30 min. The pellet was dissolved in 20 mM Tris hydrochloride buffer (pH 8.3) containing 1.0 mM PMSF, 2.5 mM EDTA, and 1% Triton X-100 (fraction II). This fraction was then loaded onto a Bio-Gel A-5m (Bio-Rad Laboratories, Richmond, Calif.) gel filtration column (1.6 by 100 cm) which had been equilibrated with the same buffer. The column was developed with the equilibration buffer, and 7.0-ml fractions were collected. The active fractions were pooled and concentrated (fraction III) through an Amicon PM10 membrane (Amicon Corp., Danvers, Mass.).

Isoelectric focusing on an LKB 8102 electrofocusing column (Pharmacia LKB Biotech, Inc., Piscataway, N.J.) with a column volume of 440 ml was carried out essentially as previously described (15). The enzyme (30 ml of fraction III) was mixed with 0.18 ml of Ampholine (pH, 3 to 10) and added near the middle of the gradient. After focusing was done, the gradient was pumped from the bottom of the column. The fractions containing GTF activity were pooled and centrifuged at 12,000 × *g* for 15 min. All of the GTF activity was found in the pellet, which was dissolved in 20 mM Tris hydrochloride (pH 7.5) containing 1.0 mM PMSF, 2.5 mM EDTA, and 1% Triton X-100 (fraction IV).

Triton X-100 was added to fraction IV to produce a 4% solution. The aggregated enzyme was vigorously shaken (10 min) in a Mickle disintegrator without glass beads. The resultant suspension was then briefly treated with alkali (1 N KOH [pH 10.5]) and immediately loaded onto a glycerol density gradient (4). The step gradient of glycerol was formed from solutions containing 10 and 30% glycerol. The enzyme mixture was added near the middle of the gradient and centrifuged at 200,000 × *g* for 20 h in a Spinco model L5-50 ultracentrifuge (Beckman Instruments, Inc., Fullerton, Calif.). The gradient was then removed from the bottom of the tube, and the fractions were assayed for GTF activity. Activity was detected both at the top (disaggregated enzyme in the Triton X-100 layer) and bottom (aggregated enzyme) of the tube. The disaggregated enzyme was dialyzed against 20 mM Tris hydrochloride (pH 7.5) (fraction V).

In vitro inactivation of the *gtfC* gene. The chimeric plasmid containing all of the *gtfC* gene except for the amino-terminal sequences, pNH2, was digested with *Pst*I, and the 7.3-kb DNA fragment was isolated from an agarose gel (13). This fragment was ligated to *Pst*I-cleaved pUC9dE (pUC9 with the *Eco*RI site deleted), and the resultant plasmid, pNH2dE, was used to isolate a 6.6-kb *Eco*RI-*Sph*I fragment containing the *gtfC* gene. This fragment was then ligated to an *Eco*RI-*Sph*I fragment containing an erythromycin resistance gene isolated from plasmid pTS19E (1). Following ligation and transformation into strain JM83, plasmid pNH2EM was isolated from ampicillin- and erythromycin-resistant transformants. A *Bgl*I fragment containing the inactivated *gtfC* gene was then isolated from the latter plasmid following agarose gel electrophoresis and used to transform *S. mutans* GS-5 cells.

Southern blot analysis. Southern blot analysis was carried out as recently described (25) with biotinylated probes. The probes were constructed following nick translation as recommended by the supplier of biotin-dUTP (Bethesda Research Laboratories, Gaithersburg, Inc., Md.).

RESULTS

Isolation of the *gtfC* gene. Based on recent nucleotide sequence data (22), it was suggested that a gene sharing extensive homology with the *gtfB* gene was positioned immediately downstream from that gene. Furthermore, Southern blot analysis suggested that most of this homologous gene was contained within a 7.3-kb *Pst*I fragment (1). Since this gene was also likely to code for GTF activity, an attempt was made to isolate it from a size-fractionated *Pst*I clone bank of *S. mutans* GS-5 DNA constructed in vector pUC18. Screening of the clone bank with a probe containing a 1.6-kb *Bam*HI fragment from the *gtfB* (1) gene revealed that 7 of approximately 600 clones reacted positively with the probe. One of these harbored a plasmid with the *gtfB* gene contained on a 6.4-kb *Pst*I fragment. Restriction enzyme analysis of plasmids from two additional positive clones indicated that the plasmids each contained a 7.3-kb *Pst*I fragment, indicating the presence of the homologous gene arranged in both orientations relative to the vector. One of the plasmids, pNH2 (Fig. 1), was used to isolate the intact homologous gene designated *gtfC*. *E. coli* strains harboring pNH2 were devoid of GTF activity.

Construction of the intact *gtfC* gene. Nucleotide sequence data (22) suggested that plasmid pSU5 (Fig. 1), harboring the intact *gtfB* gene, also contained the amino-terminal sequences of the downstream homologous gene *gtfC*. Therefore, to construct the intact *gtfC* gene, we isolated a DNA fragment known to contain the amino-terminal sequences of the gene from M13-16N, an M13 chimeric bacteriophage isolated during the sequencing of the *gtfB* gene and flanking regions (22). A 1.1-kb *Sma*I-*Pst*I fragment containing this region was isolated from an agarose gel and ligated to *Sma*I-*Pst*I-digested plasmid pNH2dSP (pNH2 with an *Sph*I fragment deleted) (Fig. 1). Following transformation, a clone expressing GTF activity was identified and shown to harbor plasmid pNH3. The enzyme appeared to synthesize significant amounts of both water-soluble and insoluble glucan when assayed in the standard assay system (Table 1). However, the presence of primer dextran was not required for enzymatic activity.

Expression of the *gtfC* gene in *E. coli*. The *gtfC* gene appeared to be expressed from its own promoter, since the addition of isopropyl-β-D-thiogalactopyranoside to *E. coli* transformants harboring plasmid pNH3 did not increase GTF activity (data not shown). In addition, when the *gtfC* gene was isolated on a 4.7-kb *Sph*I-*Sma*I fragment from pNH3 and introduced into vector pUC19 (the gene was now oriented in the opposite direction relative to the *lac* promoter), GTF activity was still expressed. These results suggested that the *gtfC* gene fragment contained a promoter sequence which functioned in *E. coli*.

It was also of interest to determine the localization of the GTF activity expressed from the *gtfC* gene in *E. coli*, since the nucleotide sequence of the amino-terminal portion of the protein (22) suggested that GTF was an extracellular protein. When *E. coli* cells containing plasmid pNH3 were fractionated into different cellular compartments, it was observed

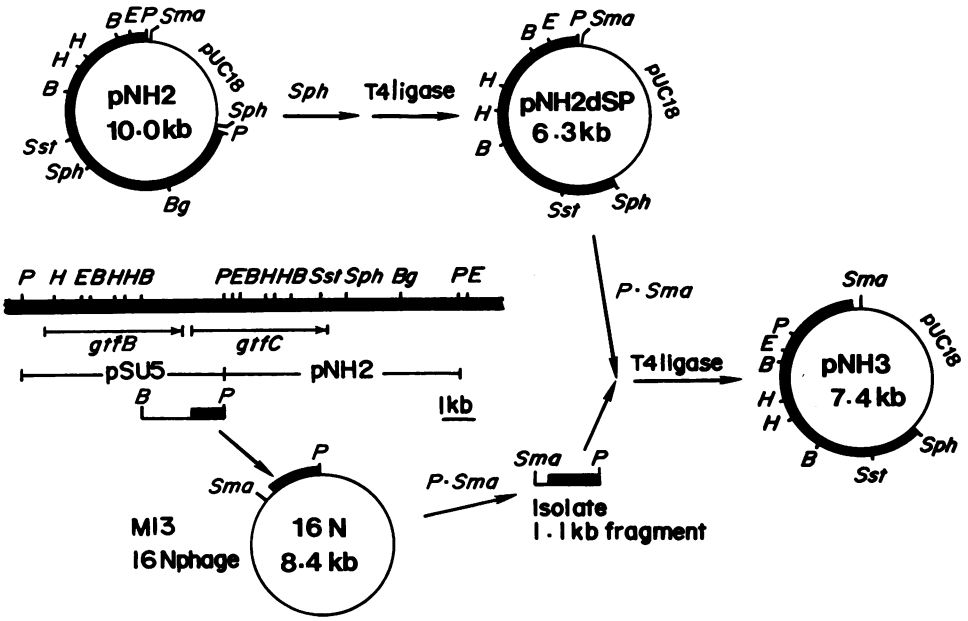

FIG. 1. Isolation of the *gtfC* gene. Thin lines represent plasmid vectors; black bars represent GS-5 chromosomal DNA. The relevant restriction sites are as follows: B, *Bam*HI; Bg, *Bgl*I; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; Sma, *Sma*I; Sph, *Sph*I; and Sst, *Sst*I.

that most of the GTF activity (74%) was associated with the cell membrane, and the remainder was found in the cytoplasm. No GTF activity was detected in the periplasmic fraction, indicating that none of the enzyme was secreted through the cytoplasmic membrane.

Purification of cloned GTF. Initial purification of the *gtfC* gene product following gel filtration, ion-exchange chromatography, and chromatofocusing resulted in highly purified enzyme preparations exhibiting molecular weights of 99,000 and 109,000 on SDS-PAGE gels (data not shown). However, since crude extracts run on the same gels contained enzyme preparations with molecular weights near 155,000 (Fig. 2), it was apparent that extensive proteolysis of GTF occurred during purification. To minimize proteolysis during enzyme isolation, we carried out subsequent purification steps with Lon$^-$ *E. coli* MM294 containing plasmid pNH3 in the presence of known inhibitors of proteolysis. Since most of the enzymatic activity was found associated with the cytoplasmic membrane, it was necessary to extract the enzyme in the presence of the nonionic detergent Triton X-100. GTF was purified following ammonium sulfate precipitation, gel filtration chromatography, preparative isoelectric focusing (Fig. 3), and glycerol density ultracentrifugation (Table 2). The final enzyme preparation, fraction V, represented an approximate 25-fold purification relative to the crude extract. One major protein band of GTF activity with a molecular weight of approximately 140,000 was observed in this fraction, along with two minor lower-molecular-weight protein bands (Fig. 2). However, the final yield of enzyme was extremely low, 0.5%, owing to the highly aggregated nature of the enzyme. Such aggregation resulted in major losses of enzymatic activity during several of the purification steps. Attempts to diaggregate the enzyme complex with detergents during purification did not increase GTF recovery.

Characterization of purified GTF. Like the crude enzyme fraction, purified GTF (fraction V) synthesized both soluble and insoluble glucans (Table 1) in a primer-independent manner. Significant amounts of insoluble glucan could be detected following incubation of the SDS-PAGE-resolved enzyme in the presence of sucrose (Fig. 2B). The purified enzyme had a pH optimum of approximately 6.5 and a K_m for sucrose of 3.9 mM. The pI of the enzyme was estimated to be pH 5.1 following isoelectric focusing (Fig. 3).

TABLE 1. Soluble and insoluble glucan synthesis catalyzed by the *gtfC* gene product

Assay	Fraction	Amt of glucan synthesized			
		Soluble		Insoluble	
		Without dextran T10	With dextran T10	Without dextran T10	With dextran T10
Standard radioactive[a]	Crude extract (fraction I, 55 μg of protein)	521	468	902	853
	Purified enzyme (fraction V, 1.6 μg of protein)	148	207	260	288
Phenol-sulfuric acid[b]	Purified enzyme (fraction IV, 43 μg of protein)	1,622	ND[c]	250	ND

[a] GTF activity was measured with [^{14}C] glucose-sucrose. Data are given in counts per minute.
[b] Water-soluble glucan synthesized by the *gtfC* gene product was assayed by the phenol-sulfuric acid method following 90% (vol/vol) ethanol precipitation of the supernatant fluids derived from the removal of insoluble glucan. Data are given in micrograms.
[c] ND, Not determined.

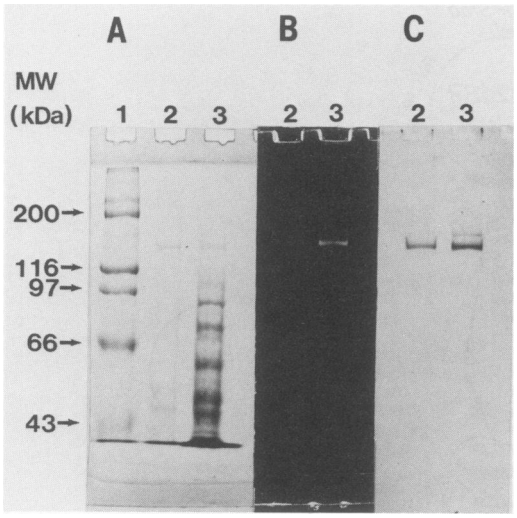

FIG. 2. SDS-PAGE analysis of the *gtfC* gene product. (A) Coomassie blue staining after SDS-PAGE. (B) Detection of insoluble glucan synthesis after SDS-PAGE. (C) Staining for periodic acid-Schiff-sensitive glucan synthesis. Lanes: 1, protein standards; 2, purified *gtfC* gene product (fraction V); 3, crude extract from *E. coli* MM294(pNH3). MW, Molecular mass; kDa, kilodaltons.

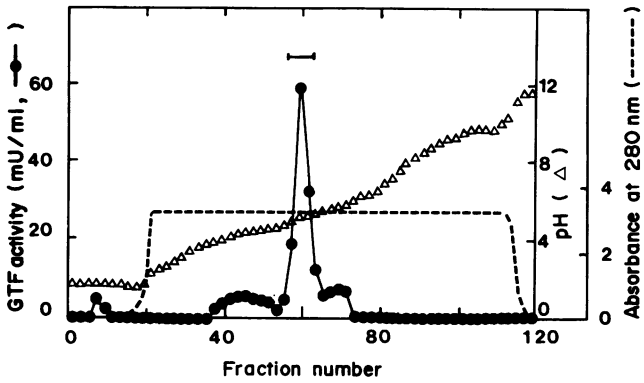

FIG. 3. Preparative isoelectric focusing of the Bio-Gel A-5m fraction from the *gtfC* clone. Fractions indicated by the horizontal line were pooled.

As with the GTF-S activities previously characterized in *S. mutans* serotype c strains (11), insoluble glucan synthesis by the purified enzyme was stimulated in the presence of ammonium sulfate. Such activity was increased almost 2.5-fold at 1.5 M ammonium sulfate. Likewise, it was observed that the enzyme synthesized increasing amounts of insoluble glucan as the enzyme concentration was increased (data not shown). Therefore, it appeared that the purified enzyme synthesized increasing levels of insoluble glucan under conditions of enzyme aggregation.

When glucan synthesis by the purified enzyme was determined with a chemical procedure (phenol-sulfuric acid) and when high concentrations of ethanol (90%) were used to precipitate low-molecular-weight glucan (Table 1), it was observed that over 87% of the glucan synthesized was water soluble. Preliminary analysis of this soluble glucan indicated that it was dextranase sensitive and had a relatively low molecular weight (data not shown). Therefore, the standard radioactive assay (Table 1) had underestimated the amount of water-soluble glucan produced by the enzyme, since its relatively small size prevented its detection during the former assay.

Insertional inactivation of the *gtfC* gene. To determine the role of the *gtfC* gene in the cariogenicity of *S. mutans*, we devised a strategy to replace most of the gene with a heterologous DNA fragment coding for erythromycin resistance (Em^r) (Fig. 4). This approach was used to avoid introduction of the gene fragment into the homologous *gtfB* gene and was also based on recent nucleotide sequence information indicating that the *gtfC* gene terminated between the *Sst*I and *Sph*I sites of pNH3 (S. Ueda and H. K. Kuramitsu, unpublished results). The 5.2-kb *Bgl*I fragment containing the inactivated *gtfC* gene was then transformed into strain GS-5, and Em^r transformants were identified on mitis salivarius agar plates. Since such transformants could only arise following recombination of the fragment into the GS-5 chromosome by means of flanking homologous regions and since the carboxyl-terminal sequences of the *gtfC* gene were missing from the fragment (Fig. 4), inactivation of only the *gtfC* gene should have occurred. Approximately 99% of the resulting transformants had a unique rough colony morphology on mitis salivarius agar plates. These colonies were similar to but readily distinguishable from the wild-type GS-5 colonies. Less than 1% of the transformants had the smooth colony morphology characteristic of insoluble-glucan-defective mutants (19). Such transformants could have resulted from a relatively rare recombination event involving homologous regions at the 5′ end of the *gtfB* gene and downstream from the *gtfC* gene (Fig. 4).

One of the rough transformants, NHR1, displayed wild-type levels of soluble glucan synthesis and 63% of normal insoluble glucan synthesis (Table 3). However, this transformant had a negligible ability to colonize smooth surfaces in the presence of sucrose. A typical smooth Em^r transformant, NHS1, displayed nearly normal soluble glucan synthesis but did not synthesize insoluble glucan. As would be predicted from such activity, this strain could not carry out sucrose-dependent colonization of smooth surfaces. To confirm the integration of the Em^r fragment into the *gtfC* gene, we carried out Southern blot analysis of the transformants (Fig.

TABLE 2. Purification of the *gtfC* gene product[a]

Fraction	Total protein (mg)	Total activity (mU)	Sp act (mU/mg)	Purification (fold)	Recovery (%)
I (crude extract)	268.50	1,068	3.98	1.0	100.0
II (ammonium sulfate)	88.80	532	5.99	1.5	49.8
III (Bio-Gel A-5m)	25.80	224	8.68	2.2	21.0
IV (preparative isoelectric focusing)	0.85	25	29.41	7.4	2.3
V (ultracentrifugation)	0.05	5	100.00	25.1	0.5

[a] Activity was determined with the GTF radioactive assay. Protein concentrations were determined as described in the text.

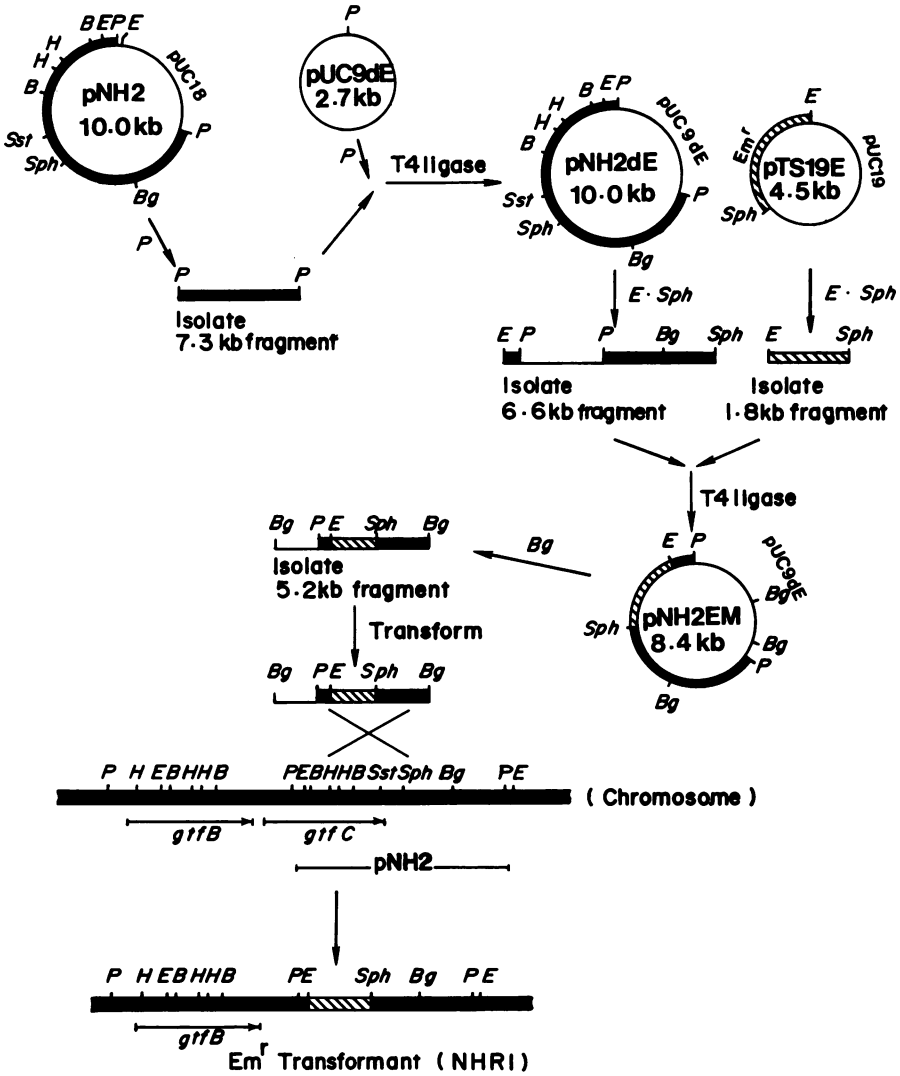

FIG. 4. Insertional inactivation of the *gtfC* gene. Thin lines represent plasmid vectors; black bars represent GS-5 chromosomal DNA; hatched bars represent the Em^r gene from plasmid pTS19E. The relevant restriction sites are described in the legend to Fig. 1.

5). Following *Eco*RI digestion of the chromosomal DNAs from the transformants, a 1.6-kb *Bam*HI fragment which is common to both the *gtfB* and *gtfC* genes was used as a probe to detect the presence of the genes. Strain NHR1 yielded a positive band indicative of the intact *gtfB* gene but no band corresponding to the *gtfC* gene. In contrast, strain NHS1 yielded no positive bands corresponding to either gene. In addition, an Em^r probe also indicated that the gene was

TABLE 3. Insertional inactivation of the *gtfC* gene

Strain	Synthesis (cpm) by GTF of[a]:		Colonization[b]
	Soluble glucan	Insoluble glucan	
GS-5	2,434	333	+
NHR1 (rough)	2,262	212	−
NHS1 (smooth)	1,931	0	−

[a] Each strain was grown to the mid-log phase in 5.0 ml of Todd-Hewitt broth. After centrifugation, GTF activities were determined by the standard radioactive assay.

[b] Sucrose-dependent colonization of glass surfaces was carried out as described in the text. +, Colonization; −, no detectable colonization.

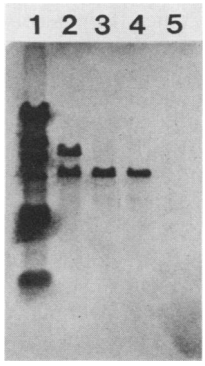

FIG. 5. Southern blot analysis of insertionally inactivated *gtfC* mutants. Chromosomal DNA was cleaved with *Eco*RI. Hybridization was done with a 1.6-kb *Bam*HI probe from the *gtfB* gene. Lanes: 1, *Hin*dIII digest of lambda DNA (23, 9.4, 6.6, 4.4, 2.3, 2.0, and 0.6 kb); 2, *S. mutans* GS-5; 3, rough-type transformant (NHR1); 4, rough-type transformant (NHR2); 5, smooth-type transformant (NHS1).

integrated into the predicted locations in both strains (data not shown). Therefore, these results confirmed the predictions of the integration strategy that the Em^r gene would replace most of the *gtfC* gene in the rough transformants and both of the *gtf* genes in the few smooth transformants. Furthermore, these results suggested that the *gtfC* gene product was required for sucrose-dependent colonization in vitro.

DISCUSSION

The present results have demonstrated that the *gtfC* gene located immediately downstream from the *gtfB* gene on the *S. mutans* GS-5 chromosome codes for GTF activity. The construction of the intact *gtfC* gene in plasmid pNH3 (Fig. 1) was based on the detection of a DNA fragment sharing homology with the *gtfB* gene (1) as well as recent nucleotide sequencing of the region immediately downstream from the latter gene (22). Verification of the structure of the *gtfC* gene has been recently obtained both by the isolation from GS-5 of a single 10.5-kb *Sph*I fragment containing both the *gtfB* and *gtfC* genes (25) and by nucleotide sequencing of the entire *gtfC* gene (S. Ueda, T. Shiroza, and H. K. Kuramitsu, Gene, in press).

The present results also indicate that the product of the *gtfC* gene synthesizes significant amounts of both water-soluble and insoluble glucans (Table 1). Like the GTF-I enzyme expressed from the *gtfB* gene (1), the *gtfC* gene product synthesizes glucans in the absence of exogenous primer dextran. Primer-independent GTFs have also been demonstrated in other *S. mutans* strains (21). When procedures designed to detect low-molecular-weight soluble glucans are used, it appears that most of the product is water soluble. However, significant amounts of insoluble glucan are synthesized by this enzyme, since the insoluble product is visible following SDS-PAGE analysis of the purified enzyme (Fig. 2). Therefore, this enzyme differs from GTF-S previously isolated from strain GS-5 (11), since no insoluble glucan synthesis can be detected for GTF-S following SDS-PAGE. The product of the *gtfC* gene is also distinct from GTF-I coded for by the *gtfB* gene, since the latter enzyme synthesizes almost 90% insoluble glucan (10), while the former enzyme synthesizes 64% insoluble polymer under identical assay conditions (Table 1). Therefore, by analogy with the other GTFs produced by strain GS-5, the enzyme coded for by the *gtfC* gene is designated as GTF-SI.

Like the GTF-S enzymes from serotype c strains (11, 16), the GTF-SI enzyme appears to synthesize increasing amounts of insoluble glucan under conditions of enzyme aggregation. However, the stimulation of insoluble glucan synthesis detected in the presence of ammonium sulfate (2.5-fold) is much lower than that observed with GTF-S from strain GS-5 (11). The molecular weight of the purified GTF-SI enzyme of 140,000 is identical to that of GTF-S purified from strain GS-5 (11) and somewhat lower than that of the *gtfB* gene product, 150,000 (1). However, because of possible differences in posttranslational modification of the GTFs in *E. coli* and *S. mutans*, such comparisons must be made with some caution. In this regard, recent sequence analysis of the *gtfC* gene suggests a molecular weight of approximately 159,000 for the enzyme cleaved at a signal peptide recognition site (Ueda et al., in press).

The pI purified GTF-SI of 5.1 resembles that of GTF-I from strain GS-5 (11) and is clearly distinct from the alkaline pI of GTF-S. Therefore, based on a comparison of the enzymatic properties of the GTFs isolated from strain GS-5, it is apparent that GTF-SI is distinct from the GTF-I and GTF-S proteins.

It is also of interest that the GTF-SI protein expressed in *E. coli* is found primarily associated with the cytoplasmic membrane, and no activity is secreted into the periplasmic space. However, monoclonal antibodies specific for the GTF-SI protein react with an extracellular protein secreted from strain GS-5 (K. Fukushima and H. K. Kuramitsu, unpublished results). Likewise, the extracellular GTF-I protein expressed from the *gtfB* gene is not secreted into the periplasmic space of *E. coli* strains harboring this gene (1). In contrast, the *S. mutans* fructosyltransferase expressed from the cloned *ftf* gene in *E. coli* is efficiently transported through the cytoplasmic membrane into the periplasmic space (20). One possible explanation for these observations may be that both the cloned GTF-SI and GTF-I proteins expressed in *E. coli* appear to be highly aggregated. Such aggregation may prevent the passage of the proteins through the cytoplasmic membrane of *E. coli*. Further investigation will be required to determine the molecular basis for the inability of *E. coli* to transport both the GTF-SI and GTF-I proteins through the cytoplasmic membrane.

Expression of GTF-SI activity in both plasmid vectors pUC18 and pUC19 suggests that the GS-5 fragment contains a promoter sequence active in *E. coli*. Recent nucleotide sequencing of the region immediately upstream from the *gtfC* gene did not reveal the presence of a strong promoter sequence (22). Therefore, it is not clear whether the *gtfC* gene is expressed from its own promoter in *S. mutans* or from the strong *gtfB* promoter. mRNA analysis will be required to determine whether both genes are expressed from the same polycistronic message.

Since replacement of most of the *gtfC* gene by the heterologous Em^r gene fragment resulted in the inability of the transformants to undergo sucrose-dependent colonization in vitro, it is likely that the GTF-SI enzyme is required for this process in vivo. However, it is not clear whether it is the soluble or insoluble glucan product (or both) which is required for colonization. Therefore, these results as well as recent observations in our laboratory (1) indicate that both the *gtfB* and *gtfC* genes are required for sucrose-dependent colonization in vitro. For these reasons, it will be of interest to test these mutants in rodent models.

It was also of interest that transformants lacking most of the *gtfB* and *gtfC* genes were still capable of synthesizing wild-type levels of soluble glucan. Such transformants may have been produced either by recombination between the homologous regions of the *Bgl*I fragment (Fig. 5) and the amino-terminal regions of the *gtfB* gene and regions downstream from the *gtfC* gene or following integration of the fragment into a hybrid *gtf* gene produced as a result of spontaneous recombination between the tandem *gtfB* and *gtfC* genes (25). The high level of soluble glucan synthesis in these rare transformants suggests that a third *gtf* gene coding for GTF-S activity must be present on the GS-5 chromosome. Attempts to isolate this gene are currently in progress, and the isolation and characterization of this putative gene will be required to thoroughly comprehend the mechanism of glucan synthesis by *S. mutans* strains.

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