INFECTION AND IMMUNITY, July 1989, p. 2079–2085
0019-9567/89/072079-07$02.00/0

Vol. 57, No. 7

Isolation and Characterization of the *Streptococcus mutans gtfD* Gene, Coding for Primer-Dependent Soluble Glucan Synthesis

NOBUHIRO HANADA AND HOWARD K. KURAMITSU*

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

Received 27 January 1989/Accepted 10 April 1989

Two glucosyltransferase genes from *Streptococcus mutans* GS-5, *gtfB* and *gtfC*, have been previously isolated and sequenced in this laboratory. In the present communication a third *gtf* gene, *gtfD*, was isolated and characterized. Isolation of the gene involved a novel procedure utilizing the integration plasmid pVA891. A peptide expressed by the 1.7-kilobase DNA fragment from strain NHS1 (containing deletions in both the *gtfB* and *gtfC* genes) was initially identified in a pUC18 clone bank with antiglucosyltransferase antibodies. This fragment was integrated into the GS-5 chromosome following ligation into pVA891 and transformation, yielding strain DP2. The vector together with one complete and one incomplete copy of the *gtfD* gene was removed from the chromosome of strain DP2 following *Eco*RI digestion, religation, and transformation of *E. coli* HB101. The resultant plasmid, pNH4, expressed glucosyltransferase S (GTF-S) activity. The enzyme was purified to near homogeneity and was shown to synthesize water-soluble glucan exclusively in a primer-dependent manner. The molecular mass (155 kilodaltons) and the kinetic parameters of the purified enzyme were similar to those observed for the GTF-S enzyme previously purified from culture fluids of strain GS-5. Insertional inactivation of the *gtfD* gene indicated that this gene is not required for in vitro sucrose-dependent adherence to smooth surfaces. Furthermore, inactivation of the *gtfD* gene in a *gtfC gtfB* mutant indicated that three distinct *gtf* genes involved in glucan formation are present on the *S. mutans* GS-5 chromosome. Southern blot analysis further suggested that the *gtfD* gene does not share demonstrable homology with the *gtf* genes from *Streptococcus sanguis* or *Streptococcus sobrinus*.

Streptococcus mutans has been recognized as the principal causative agent of human dental caries (11). This organism produces extracellular glucosyltransferases (GTFs; EC 2.4.1.5) which catalyze the synthesis of glucans from sucrose. Biochemical approaches have suggested that *S. mutans* strains secrete at least two distinct GTFs which cooperatively synthesize insoluble glucans involved in the colonization of tooth surfaces (11). One of these, GTF-S, synthesizes primarily water-soluble α-1,6-linked glucans, while the other, GTF-I, is responsible for the formation of insoluble glucans containing primarily α-1,3-glucose linkages (11).

Enzyme purification and immunological comparisons have suggested that some strains of mutans streptococci may secrete three (19) or four (22) distinct GTFs. However, molecular genetic verification of these suggestions has not yet been reported. Two *gtf* genes coding for GTF-I and GTF-S activities have been recently isolated from *Streptococcus sobrinus* MFe28 (4), and the nucleotide sequence of the former gene has been determined (3). In our laboratory two tandemly arranged *gtf* genes from *S. mutans* GS-5 have been isolated (1, 6) and their nucleotide sequences have been determined (20, 24). One of these, the *gtfB* gene, codes for GTF-I activity, while the other gene, *gtfC*, codes for a GTF-SI enzyme which synthesizes predominantly insoluble glucan and significant amounts of soluble glucan. In addition, a GS-5 mutant which lacks all of the *gtfC* gene and most of the *gtfB* gene synthesized wild-type levels of water-soluble glucan (6). Therefore, the phenotype of this mutant suggested that a third *gtf* gene coding for GTF-S activity must reside on the strain GS-5 chromosome.

The present communication describes the isolation and characterization of a *S. mutans* GS-5 gene, designated *gtfD*, which codes for GTF-S activity. The cloned enzyme exhibits properties similar to the GTF-S activity previously purified in this laboratory from culture fluids of strain GS-5 (10).

* Corresponding author.

MATERIALS AND METHODS

Microorganisms. *S. mutans* human oral isolates GS-5 and UA101 (from R. Curtiss III, Washington University, St. Louis, Mo.), *S. sobrinus* 6715, *Streptococcus sanguis* Challis, and *Escherichia coli* JM83 and HB101 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 previously described (1). *S. mutans* transformations were carried out as originally described (15), and transformants were isolated on mitis salivarius agar plates containing erythromycin (10 μg/ml) or tetracycline (4 μg/ml). A *S. mutans* DNA library was constructed by partially digesting chromosomal DNA with *Sau*3AI and ligating the DNA fragments to *Bam*HI-cleaved vector pUC18 which was treated with alkaline phosphatase. The ligation mixture was transformed into *E. coli* JM83, and transformants harboring chimeric plasmids were selected on Luria-Bertani agar plates containing ampicillin (50 μg/ml) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). White colonies grown on Luria-Bertani agar plates were blotted to nitrocellulose membrane disks. After bacterial lysis with chloroform vapor (7), the disks were screened for colonies that expressed antigens which reacted with antibody directed against a partially purified GS-5 GTF fraction, GTF-B (9).

Plasmids. The plasmid encoding the tetracycline resistance (Tc^r) gene from transposon Tn*916*, pLN2, was recently described (14). The construction of plasmid pVA891 was described previously (12).

Enzyme and protein assays. Sucrase activity was deter-

mined by the Somogyi-Nelson procedure as originally described (21). GTF activity was determined as previously described (8) with [^{14}C]glucose-sucrose. One unit of enzyme activity is 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 (2) with bovine serum albumin as the standard protein. The effects of exogenous dextran T10 on soluble and insoluble glucan synthesis, the optimum pH, and K_m values for the enzyme were determined as previously described (8). The effects of $(NH_4)_2SO_4$ on insoluble glucan synthesis were evaluated as described previously (10).

Purification of cloned GTF-S activity. *E. coli* HB101 (pNH5) was harvested by centrifugation at 4,000 × *g* for 5 min after growth in Luria-Bertani broth (2 liters) at 30°C. The cell pellet was suspended with 20 mM Tris hydrochloride buffer (pH 7.5) containing 0.1 mM phenylmethylsulfonyl fluoride. After addition of Ballotini beads (0.2 g/ml) the cells were disrupted in a Mickle disintegrator (H. Mickle, Gomshall-Surrey, England) for 15 min. The disrupted cell suspension, approximately 40 ml, was incubated with 16 mg of lysozyme, 10 mg of RNase, and 0.5 mg of DNase at room temperature for 30 min. After removal of the Ballotini beads, the suspension was centrifuged at 17,000 × *g* for 30 min. The supernatant fluid was dialyzed against 20 mM Tris hydrochloride (pH 7.5)–0.1 mM phenylmethylsulfonyl fluoride and used as the crude enzyme solution (fraction I). The crude enzyme solution (60 ml) was next applied to a column (2.6 by 8 cm) of DE52 cellulose (Whatman International, Kent, United Kingdom) previously equilibrated with 20 mM Tris hydrochloride (pH 7.5). Proteins were eluted with a linear gradient of 0 to 0.4 M NaCl in the same buffer (500 ml), and fractions (8 ml) were collected at a flow rate of 50 ml/h. Sucrase-active fractions were pooled and concentrated through ultrafiltration membranes (PM10, XM100A, and Centricon 10; Amicon Corp., Danvers, Mass.) (fraction II). After centrifugation at 39,000 × *g* for 30 min, the supernatant fluid (500 μl) was applied onto a fast-protein liquid chromatography MonoQ column (Pharmacia LKB Biotech, Inc., Piscataway, N.J.) equilibrated with 20 mM Tris hydrochloride buffer (pH 7.5). The enzyme was eluted with a linear 0 to 0.5 M NaCl gradient in the same buffer. Fractions (2 ml) were collected at a flow rate of 0.5 ml/min. Sucrase-active fractions were pooled and concentrated through Centricon 10 Ultrafilters (fraction III). A sample (200 μl) of the concentrated sucrase-active fractions was then subjected to fast protein liquid-gel permeation chromatography over a TSK-G3000SW column. The column was equilibrated with 50 mM potassium phosphate buffer (pH 6.0) containing 0.5 M NaCl, and fractions were eluted with the same buffer. Fractions (2 ml) were collected at a flow rate of 0.5 ml/min. Sucrase-active fractions were then pooled and concentrated through Centricon 10 Ultrafilters (fraction IV).

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

Western and Southern blot analysis. Western blot (immunoblot) analysis was carried out as previously described (23). Southern blot analysis was performed as previously described (18), utilizing biotin-labeled probes according to the instructions of the supplier of the DNA detection system (Bethesda Research Laboratories, Inc., Gaithersburg, Md.).

In vitro inactivation of the *gtfD* gene. A 5.4-kilobase (kb) *Bam*HI fragment coding for the Tet^r gene was isolated from plasmid pLN2 and inserted into *Bgl*II-cleaved pNH5 (one *Bgl*II site exists within the *gtfD* gene). The resultant plasmid, pNH5TET, was isolated and used as the source of the in vitro-inactivated *gtfD* gene. The 11.9-kb fragment was then isolated from agarose gels after *Sal*I and *Sst*I cleavage of plasmid pNH5TET. This fragment was used for the transformation of *S. mutans*.

In vitro sucrose-dependent adherence. Sucrose-dependent adherence to glass surfaces by *S. mutans* cells was carried out as originally described (8) except that the adherent cells were mildly vortexed during washing.

RESULTS

Isolation of the *gtfD* gene. Chromosomal DNA from *S. mutans* NHS1, lacking most of the *gtfB* and *gtfC* genes (6), was partially digested with *Sau*3AI and ligated into *Bam*HI-cleaved pUC18. The ligation mixtures were transformed into *E. coli* JM83, and recombinant clones were screened with antibody directed against the GTF-S enzyme prepared from strain GS-5. One positive clone was identified after screening of approximately 3,500 recombinant clones. This clone designated 4888, also reacted with a monoclonal antibody (MAb4) directed against the GTF-S purified from another *S. mutans* strain, PS-14 (provided by K. Fukushima, Nihon University Dental School, Matsudo, Japan). This antibody also reacted strongly with the GTF-S from strain GS-5 (data not shown). Following restriction mapping of plasmid pNH4888 isolated from the clone, a 1.7-kb GS-5 chromosomal DNA insert was identified. Western blot analysis indicated that the clone expressed a 66-kilodalton (kDa) protein which reacted with the monoclonal antibody (data not shown). However, this clone exhibited neither GTF nor sucrase activities.

Since previous results have indicated that the sizes of the GTFs of strain GS-5 are approximately 150 kDa (10) and clone 4888 appeared to express only a portion of the GTF-S protein from strain GS-5, a strategy was devised to utilize the 1.7-kb gene fragment to isolate the intact *gtfD* gene. This approach was based on the utilization of the integration vector pVA891 (12). Since it was probable that the 1.7-kb GS-5 fragment contained the 5′ end of the *gtfD* gene, integration of pVA891 containing this fragment into the GS-5 chromosome would result in the generation of two copies of the *gtfD* gene (one intact and one incomplete gene copy; Fig. 1). Therefore, the 1.7-kb fragment was isolated and ligated into pVA891 and the mixtures were transformed into strain GS-5. Several Em-resistant (Em^r) transformants were isolated, and one of these, strain DP2, was selected for further investigation. Analysis of the culture fluids of this strain following SDS-PAGE and Western blotting with MAb4 revealed that this transformant secreted two extracellular proteins (155 and 55 kDa) which reacted with MAb4 (data not shown). These results suggested that the 1.7-kb DNA fragment did indeed contain the 5′ end of the *gtfD* gene since an internal fragment would not have yielded transformants expressing a full-size GTF protein (155 kDa).

In order to isolate the intact *gtfD* gene, chromosomal DNA from strain DP2 was purified, cleaved with *Eco*RI, and self-ligated (Southern blot analysis with plasmid pNH4888 had indicated that the 1.7-kb insert hybridized with a 9.0-kb *Eco*RI fragment of GS-5 chromosomal DNA). The ligation mixtures were transformed into *E. coli* HB101, and Em^r (200 μg/ml) transformants were screened for GTF activity. One of these, NH4, was shown to express GTF activity and was

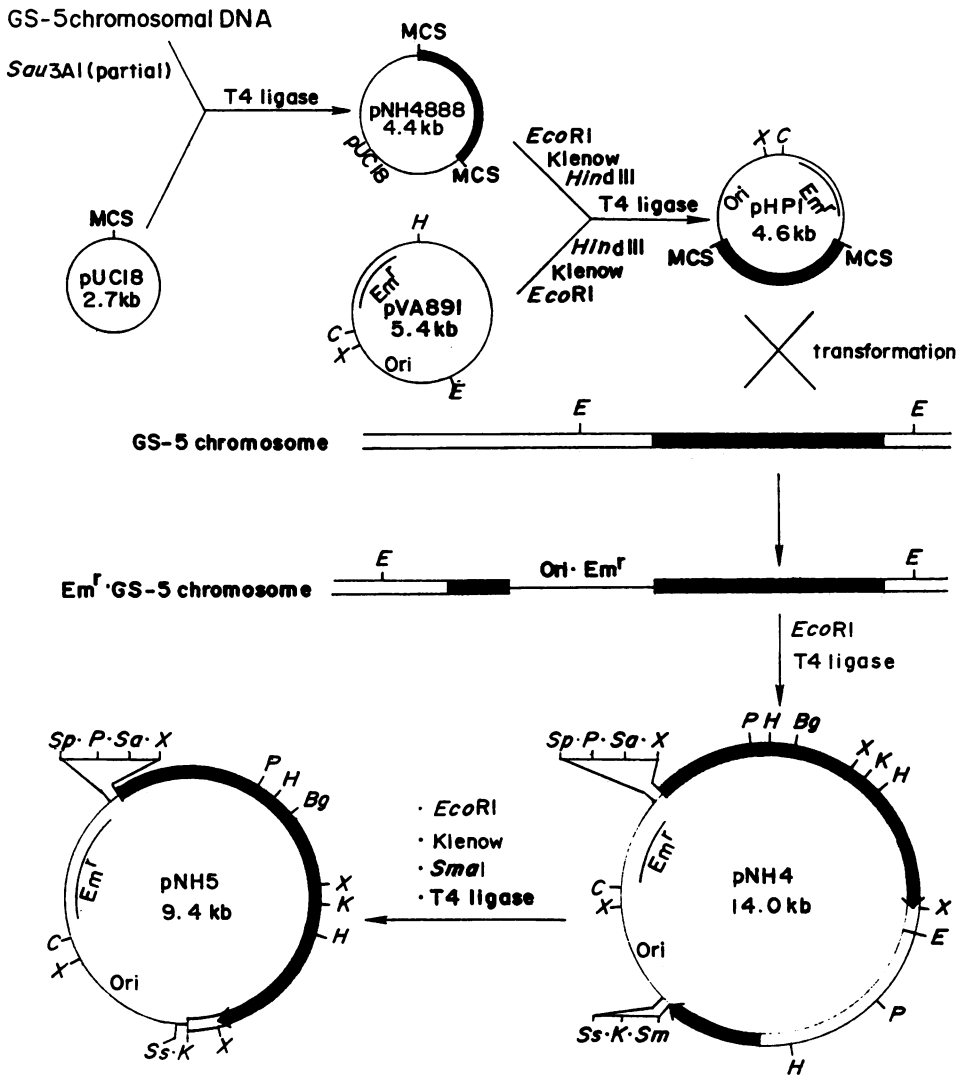

FIG. 1. Isolation of the *gtfD* gene. ——, Plasmid vectors; □, GS-5 chromosomal DNA; ■, the *gtfD* gene. The approximate size of the *gtfD* gene and the direction of its transcription (indicated by the arrowheads) were deduced from the molecular weight of the gene product and the expression of two GTF-S proteins by strain DP2, respectively. Relevant restriction sites: *Bg*, *Bgl*II; *C*, *Cla*I; *E*, *Eco*RI; *H*, *Hin*dIII; *K*, *Kpn*I; *P*, *Pst*I; *Sa*, *Sal*I; *Sm*, *Sma*I; *Sp*, *Sph*I; *Ss*, *Sst*I; *X*, *Xba*I. MCS, Multiple cloning sites.

selected for further characterization. Restriction mapping of the plasmid, pNH4, isolated from this strain indicated a structure which was compatible with the existence of one complete and one partial *gtfD* gene (Fig. 1). The putative incomplete gene was removed from plasmid pNH4 after cleavage with *Eco*RI, filling in with the Klenow fragment, and *Sma*I digestion, followed by self-ligation. The resultant plasmid, pNH5, still expressed high GTF activity (Table 1).

Expression of the *gtfD* gene. The activity expressed by strain NH5 resembled the previously characterized GTF-S enzyme purified from strain GS-5 (10), since the clone synthesized primarily water-soluble glucan in a primer-dependent manner (Table 1). Staining following SDS-PAGE of crude extracts from strain NH5 revealed that a 155-kDa protein synthesized soluble (Fig. 2) but not insoluble (data not shown) glucan. Confirmation of the identity of the *gtfD* gene product was provided by the observation that the 155-kDa GTF band also reacted with MAb4. The antibody also detected a somewhat smaller protein band of approximately 140 kDa.

Fractionation of crude extracts from strain NH5 revealed that the majority of the GTF activity was found in the cytoplasm (68%), with somewhat lower amounts associated with the cytoplasmic membrane fraction (22%) and in the periplasmic space (10%). The synthesis of water-soluble

TABLE 1. Soluble and insoluble glucan synthesis catalyzed by the *gtfD* gene product from *E. coli* HB101(pNH5)[a]

Fraction	Glucan synthesis (cpm)			
	Soluble		Insoluble	
	−Dextran T10	+Dextran T10	−Dextran T10	+Dextran T10
Crude extract (fraction I) (15.6 μg of protein)	966	6,799	187	92
Purified enzyme (fraction IV) (0.34 μg of protein)	173	2,104	0	6

[a] GTF activity was measured with [^{14}C]glucose-sucrose as described in the text.

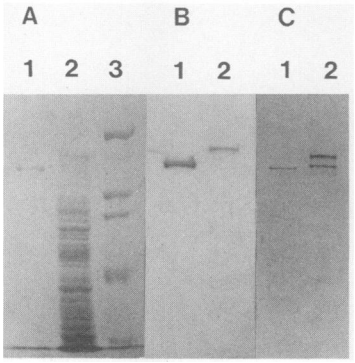

FIG. 2. SDS-PAGE analysis of the *gtfD* gene product. (A) Coomassie blue staining after SDS-PAGE; (B) staining for periodic acid-Schiff-sensitive glucan synthesis; (C) Western blot analysis with MAb4. Lanes: 1, purified *gtfD* gene product from *E. coli* HB101(pNH5) (fraction IV); 2, crude extract from *E. coli* HB101(pNH5) (fraction I); 3, molecular mass marker proteins (from top to bottom: myosin, 200,000 Da; *E. coli* β-galactosidase, 116,250 Da; rabbit muscle phosphorylase *b*, 97,400 Da; bovine serum albumin, 66,200 Da; hen egg white ovalbumin, 42,699 Da).

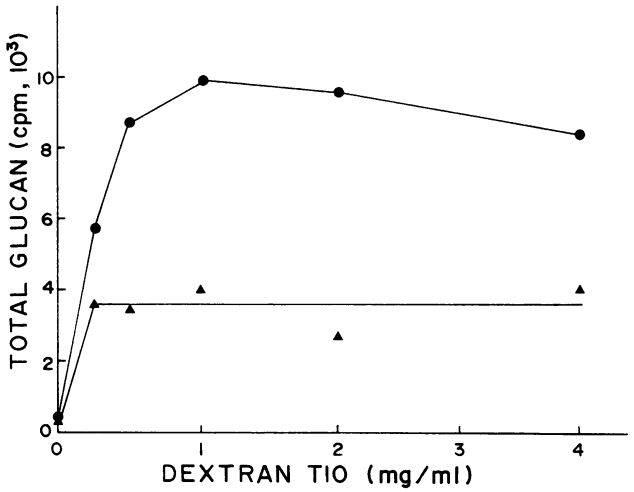

FIG. 3. Effects of dextran T10 on purified and crude GTF-S activities. The indicated amounts of dextran T10 were added to the reaction mixtures, and GTF activities were determined following 2-h incubations. Symbols: ●, purified GTF (fraction IV); ▲, crude GTF (fraction I).

glucans by strain NH5 appeared to be lethal to *E. coli*, since this strain did not grow on Luria-Bertani agar plates containing 1% sucrose.

Purification of the *gtfD* gene product. The GTF-S activity expressed by clone NH5 was purified to near homogeneity following DE52 cellulose chromatography, MonoQ ion exchange, and TSK-G3000SW gel-filtration chromatography (Table 2). GTF activity eluted as a single activity peak following DE52 cellulose ion-exchange chromatography and was not retained by the MonoQ chromatography column (data not shown). Final passage of the enzyme through the TSK column resulted in a single activity peak. The final enzyme preparation represented a 35-fold purification, with an approximately 3.7% yield relative to the initial crude extract.

The purified enzyme preparation yielded a single protein band following Coomassie blue staining of SDS-PAGE gels (Fig. 2). The purified enzyme exhibited a molecular mass of approximately 140 kDa and synthesized water-soluble glucan exclusively.

Characterization of the purified GTF-S enzyme. Like the crude enzyme fraction, the purified GTF-S (fraction IV) synthesized soluble glucan in a primer-dependent manner (Table 1). No detectable insoluble glucan was detected on SDS-PAGE gels following 24-h incubations in the presence of sucrose. Very little enzyme activity was detected in the absence of dextran T10, and the purified enzyme was stimulated approximately 22-fold in the presence of saturating amounts of the primer (Fig. 3). The purified enzyme exhibited a pH optimum of 5.5 to 6.0 and a K_m for sucrose of approximately 2.1 mM. As with the GTF-S activity purified from strain GS-5 (10), insoluble glucan synthesis by the purified enzyme was stimulated in the presence of high concentrations of ammonium sulfate (data not shown).

TABLE 2. Purification of the *gtfD* gene product from *E. coli* HB101(pNH5)

Fraction	Total protein (mg)[a]	Total activity (mU)[b]	Sp act (mU/mg)[b]	Purification (fold)	Recovery (%)
I (crude)	468.0	30,120	64	1.0	100.0
II (DE52)	13.7	6,732	491	7.7	22.4
III (MonoQ)	6.4	4,203	657	10.3	14.0
IV (TSK-G3000SW)	0.5	1,113	2,226	34.8	3.7

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

Insertional inactivation of the *gtfD* gene. In order to determine the role of the *gtfD* gene in cariogenicity, strain GS-5 mutants defective in this gene were constructed. A DNA fragment coding for Tc^r from transposon Tn*916* (14) was isolated and inserted within the *gtfD* gene (Fig. 4). The 11.9-kb insertionally inactivated *gtfD* gene fragment was then isolated and transformed into strain GS-5. The resultant Tc^r transformants exhibited the typical rough colonial morphology of *S. mutans* on mitis salivarius agar plates. The culture fluids from a representative transformant (GS-5DD) exhibited relatively low water-soluble-glucan synthesizing activity relative to the wild-type GS-5 strain (Table 3). Mutant GS-5DD also exhibited reduced insoluble-glucan synthesis activity when assayed in the absence of the primer dextran T10. Nevertheless, this mutant exhibited significant sucrose-dependent adherence to glass surfaces, like the wild-type organism. Southern blot analysis of chromosomal DNA digests of strains GS-5 and GS-5DD confirmed the insertional inactivation of the *gtfD* gene in the transformants (Fig. 5), since the *Eco*RI fragment harboring the *gtfD* gene increased in size in these mutants.

The insertionally inactivated *gtfD* gene fragment was also transformed into strain NHS1, which is unable to synthesize water-insoluble glucan but produces normal amounts of soluble glucan (6). The resulting transformants, typified by strain NHS1DD, exhibited negligible GTF activity (Table 3).

Homology of the *gtfD* gene with other streptococcal *gtf* genes. Since other oral streptococci are also capable of synthesizing water-soluble glucans (5), it was of interest to determine whether the *S. mutans gtfD* gene shared significant homology with the *gtf* genes from these other organisms. A biotin-labeled pNH5 probe was utilized to analyze Southern blots of *Eco*RI-digested chromosomal DNA from *S. mutans* UA101, *S. sobrinus* 6715, and *S. sanguis* Challis

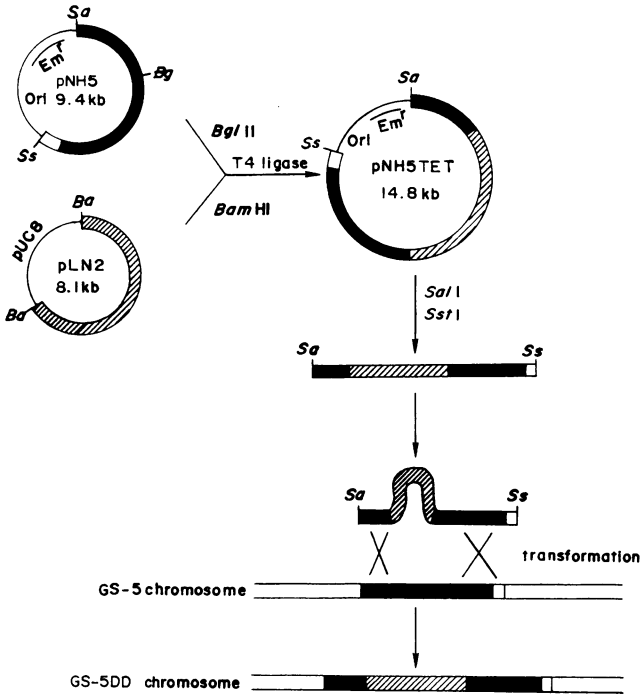

FIG. 4. Insertional inactivation of the *gtfD* gene. ——, Plasmid vectors; □, GS-5 chromosomal DNA; ■, the *gtfD* gene; ▨, the Tc^r gene. Indicated restriction sites: *Ba*, *Bam*HI; *Bg*, *Bgl*II; *Sa*, *Sal*I; *Ss*, *Sst*I.

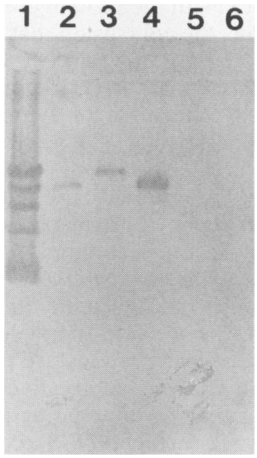

FIG. 5. Southern blot analysis with the *gtfD* gene probe. Chromosomal DNA was cleaved with *Eco*RI, and hybridization was carried out with a *gtfD* (pNH5) probe. Lanes: 1, *Hin*dIII digest of lambda DNA (23, 9.4, 6.6, 4.4, 2.3, 2.0, and 0.6 kb), using biotinylated lambda DNA as a probe; 2, *S. mutans* GS-5; 3, GS-5 Tet^r transformant (GS-5DD); 4, *S. mutans* UAB101; 5, *S. sobrinus* 6715; 6, *S. sanguis* Challis.

(Fig. 5). Strain UA101 hybridized readily with the pNH5 probe and exhibited an *Eco*RI fragment of approximately the same size as that from strain GS-5. In contrast, no positive bands were detected for the *S. sobrinus* and *S. sanguis* strains.

DISCUSSION

The present report describes the isolation of the *gtfD* gene from *S. mutans* GS-5 by a novel cloning procedure. Initially, attempts to identify a clone which expressed GTF-S activity in a lambda 47.1 gene library were unsuccessful despite the screening of sufficient plaques to detect such activity (1). It is not clear why this approach did not yield at least one plaque containing enough of the *gtfD* gene to express GTF-S activity. However, the identification of clone 4888 in a plasmid gene library by utilizing MAb4 allowed the isolation of a GS-5 gene fragment containing the 5′ end of the *gtfD* gene. Insertion of this gene fragment into the streptococcal insertion vector pVA891 followed by transformation of GS-5 resulted in integration of the vector between two copies (one complete and the other partial, Fig. 1) of the *gtfD* gene in transformant DP2. Such a chromosomal arrangement was confirmed by the identification of two extracellular proteins of 155 and 55 kDa which reacted with MAb4 and represented the products of the complete and partial *gtfD* genes, respectively. It was then possible to isolate the intact gene copy along with pVA891 following *Eco*RI digestion and ligation.

TABLE 3. Insertional inactivation of the *gtfD* gene

Strain	Glucan synthesis (cpm)[a]				Adherence[b]
	Soluble glucan		Insoluble glucan		
	−Dextran T10	+Dextran T10	−Dextran T10	+Dextran T10	
GS-5 (rough)[c]	535	6,688	1,822	2,073	+
GS-5DD (rough) (*gtfD*)	402	398	448	1,708	+
NHS1 (smooth) (*gtfB gtfC*)	101	5,670	21	67	−
NHS1DD (smooth) (*gtfB gtfC gtfD*)	0	1	53	39	−

[a] Each strain was grown to the mid-log phase in 5.0 ml of Todd-Hewitt broth. After centrifugation, the GTF activities of the culture fluids were determined for similar quantities of cells by the standard radioactivity assay.
[b] Sucrose-dependent adherence to glass surfaces was determined as described in the text. +, Adherence; −, no detectable adherence.
[c] Colonial morphology on mitis salivarius agar plated is given in parentheses.

The *gtf* gene contained on plasmid pNH4 was clearly distinct from the previously isolated *gtfB* and *gtfC* genes by several criteria. The restriction map of the *gtfD* gene (Fig. 1) is distinct from those of the other two genes (1, 6). Southern blot analysis of strain GS-5 DNA with a *gtfD* gene probe resulted in only a single positive band of approximately 9.0 kb (Fig. 5). Since previous results (1) have indicated that the *gtfB* and *gtfC* genes are contained on 4.6- and 7.0-kb *Eco*RI fragments, respectively, it is clear that the *gtfD* gene does not share extensive homology with the other two *gtf* genes. More significantly, the *gtfB* and *gtfC* gene products synthesize primarily water-insoluble glucans (1, 6). In contrast, the enzyme coded for by the *gtfD* gene synthesizes soluble glucans. In addition, the latter activity is strongly dependent upon the presence of the primer dextran T10 (Fig. 3), while the activities of the GTF-I and GTF-SI proteins coded by the other two *gtf* genes are primer independent (1, 6). Moreover, insertional inactivation of the *gtfD* gene resulted in a marked decrease in soluble glucan synthesis (Table 3). Recent results from this laboratory (K. Fukushima and H. K. Kuramitsu, unpublished results) have also indicated that MAb4 reacts with *S. mutans* GTF-S enzymes but not with the products of the *gtfB* and *gtfC* genes. The reaction of this

monoclonal antibody with the protein coded for by the *gtfD* gene further confirms the conclusion that the product of this gene is the GTF-S activity of strain GS-5.

It was also of interest that the GTF-S protein expressed in *E. coli* did not appear to traverse the cytoplasmic membrane, since less than 10% of the total GTF activity was found in the periplasmic space. Previous results (1, 6) have also indicated that the products of the *gtfB* and *gtfC* genes expressed in *E. coli* were found in the cytoplasm or associated with the membrane fraction. Therefore, although all three genes code for extracellular proteins in *S. mutans*, none of the three gene products is transported efficiently through the cytoplasmic membrane in *E. coli*. It is possible that these three streptococcal extracellular proteins are not correctly processed for export in *E. coli*. Alternatively, since all three protein products appear to undergo aggregation when expressed in *E. coli* (1, 6; the GTF-S protein did not pass through the TSK-G3000SW column and was eluted only with high-ionic-strength salts), aggregation of the proteins might prevent passage through the cytoplasmic membrane of *E. coli*. Such aggregation may not occur in the cytoplasm of *S. mutans* if the proteins are processed differently than in *E. coli*. The secretion of the *S. mutans* GTFs in *E. coli* is currently under investigation in this laboratory.

The approximate molecular mass (155 kDa) of the GTF-S expressed in *E. coli* is similar to those of the GTF-I and GTF-SI enzymes (1, 6). However, following purification of the cloned GTF-S protein, a molecular mass of 140 kDa was observed. This suggests that some proteolysis of the enzyme took place during purification. It is of interest that this molecular size is identical to that observed for the GTF-S enzyme purified from the culture fluids of strain GS-5 (10). Nevertheless, differential processing of the enzyme may occur in the two organisms.

The purified GTF-S enzyme from *E. coli* also exhibited a number of properties similar to those of the homologous enzyme from strain GS-5: (i) pH optimum, (ii) K_m for sucrose, (iii) primer dependence, (iv) stimulation of insoluble glucan synthesis in the presence of ammonium sulfate, and (v) relatively alkaline pI (8, 10). Therefore, the results of the present communication clearly support the suggestion that the *gtfD* gene codes for the GTF-S activity of strain GS-5. In addition, the observation that inactivation of the *gtfB*, *gtfC*, and *gtfD* genes results in a GS-5 mutant which expresses negligible GTF activity (Table 3) strongly supports the conclusion that only three genes are involved in glucan synthesis in this strain. However, similar investigations of other strains will be required before these results can be generalized to all strains of *S. mutans*.

GS-5DD (*gtfD* mutant) was still capable of colonizing smooth surfaces in the presence of sucrose in vitro (Table 3). However, these results do not necessarily prove that the *gtfD* gene is not involved in the colonization of tooth surfaces. It will be necessary to compare the colonizing ability of this mutant with that of the parental organism in an animal model before such a conclusion is warranted.

It was of interest that the *gtfD* gene probe detected no positive bands after Southern blot analysis of selected strains of *S. sobrinus* and *S. sanguis* (Fig. 5). Since these two species are capable of synthesizing soluble glucans (5), it is likely that the *gtf* genes coding for GTF-S activity in the three species are not highly related. However, these genes could still share significant homology, since recent results have indicated more than 50% homology between the *S. mutans* and *S. sobrinus* genes coding for GTF-I activities (17), although the genes exhibit only weak hybridization following Southern blot analysis (16).

The isolation of the *gtfD* gene from *S. mutans* makes it possible to determine both the nucleotide sequence of the gene and the amino acid sequence of the GTF-S protein. This information will be compared with comparable data for the *gtfB* and *gtfC* genes in order to identify the amino acid sequences involved in the synthesis of both water-soluble and insoluble glucans.

ACKNOWLEDGMENTS

We thank Dennis Perry for assistance in the construction of strain DP2.

This investigation was supported in part by Public Health Service grant DE 06082 from the National Institutes of Health.

LITERATURE CITED

1. **Aoki, H., T. Shiroza, M. Hayakawa, S. Sato, and H. K. Kuramitsu.** 1986. Cloning of a *Streptococcus mutans* gene coding for insoluble glucan synthesis. Infect. Immun. **53:**587–594.
2. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
3. **Ferretti, J. J., M. L. Gilpin, and R. R. B. Russell.** 1987. Nucleotide sequence of a glucosyltransferase gene from *Streptococcus sobrinus* MFe28. J. Bacteriol. **169:**4271–4278.
4. **Gilpin, M. L., R. R. B. Russell, and P. Morrissey.** 1985. Cloning and expression of two *Streptococcus mutans* glucosyltransferases in *Escherichia coli* K-12. Infect. Immun. **49:**414–416.
5. **Hamada, S., and H. D. Slade.** 1980. Biology, immunology, and cariogenicity of *Streptococcus mutans*. Microbiol. Rev. **44:** 331–384.
6. **Hanada, N., and H. K. Kuramitsu.** 1988. Isolation and characterization of the *Streptococcus mutans gtfC* gene, coding for synthesis of both soluble and insoluble glucans. Infect. Immun. **56:**1999–2005.
7. **Helfman, D. M., J. R. Feramisco, J. C. Fiddes, G. P. Thomas, and S. H. Hughes.** 1983. Identification of clones that encode chicken tropomyosin by direct immunological screening of a cDNA expression library. Proc. Natl. Acad. Sci. USA **80:**31–35.
8. **Kuramitsu, H. K.** 1975. Characterization of extracellular glucosyltransferase activity of *Streptococcus mutans*. Infect. Immun. **12:**738–749.
9. **Kuramitsu, H. K., and L. Ingersoll.** 1976. Immunological relationships between glucosyltransferases from *Streptococcus mutans* serotypes. Infect. Immun. **14:**636–644.
10. **Kuramitsu, H. K., and L. Wondrack.** 1983. Insoluble glucan synthesis by *Streptococcus mutans* serotype c strains. Infect. Immun. **42:**763–770.
11. **Loesche, W. J.** 1986. Role of *Streptococcus mutans* in human dental decay. Microbiol. Rev. **50:**353–380.
12. **Macrina, F. L., K. R. Jones, and P. H. Wood.** 1980. Chimeric streptococcal plasmids and their use as molecular cloning vehicles in *Streptococcus sanguis* (Challis). J. Bacteriol. **143:**1425–1435.
13. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
14. **Perry, D., and H. K. Kuramitsu.** 1989. Genetic linkage among cloned genes of *Streptococcus mutans*. Infect. Immun. **57:** 805–809.
15. **Perry, D., L. M. Wondrack, and H. K. Kuramitsu.** 1983. Genetic transformation of putative cariogenic properties in *Streptococcus mutans*. Infect. Immun. **41:**722–727.
16. **Russell, R. R. B., M. L. Gilpin, H. Mukasa, and G. Dougan.** 1987. Characterization of glucosyltransferase expressed from a *Streptococcus sobrinus* gene cloned in *Escherichia coli*. J. Gen. Microbiol. **133:**935–944.
17. **Russell, R. R. B., T. Shiroza, H. K. Kuramitsu, and J. J. Ferretti.** 1988. Homology of glucosyltransferase gene and pro-

tein sequences from *Streptococcus sobrinus* and *Streptococcus mutans*. J. Dent. Res. **67**:543–547.
18. **Sato, S., S. Ueda, and H. K. Kuramitsu.** 1987. Construction of *Streptococcus mutans* glucosyltransferase mutants utilizing a cloned gene fragment. FEMS Microbiol. Lett. **48**:207–210.
19. **Shimamura, A., H. Tsumori, and H. Mukasa.** 1983. Three kinds of glucosyltransferases from *Streptococcus mutans* 6715 (serotype g). FEBS Lett. **157**:79–84.
20. **Shiroza, T., S. Ueda, and H. K. Kuramitsu.** 1987. Sequence analysis of the *gtfB* gene from *Streptococcus mutans*. J. Bacteriol. **169**:4263–4270.
21. **Somogyi, M.** 1945. A new reagent for the determination of sugars. J. Biol. Chem. **160**:61–68.
22. **Takehara, T., N. Hanada, and E. Saeki.** 1984. Interaction of glucosyltransferase isozymes on glucan synthesis by *Streptococcus mutans* AHT (serotype g). Microbios Lett. **27**:113–120.
23. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
24. **Ueda, S., T. Shiroza, and H. K. Kuramitsu.** 1988. Sequence analysis of the *gtfC* gene from *Streptococcus mutans* GS-5. Gene **69**:101–109.