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Mechanism of *Streptococcus mutans* Glucosyltransferases: Hybrid-Enzyme Analysis

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Streptococcus mutans **GS5 expresses three glucosyltransferases (GTFs): GTF-I and GTF-SI, which synthesize water-insoluble glucans in a primer-independent manner, and GTF-S, which is responsible for the formation of primer-dependent soluble glucan. The amino acid sequences of the GTF-I and GTF-S enzymes exhibit approximately 50% sequence identity. Various hybrid genes were constructed from the structural genes for the enzymes, and their products were analyzed. Three different approaches were used to construct the hybrid enzymes: (i) ligation of DNA fragments containing compatible endonuclease restriction sites of the two genes at homologous positions; (ii) in vivo recombination between the homologous regions of each gene; and (iii) random fusion of DNA fragments from each gene generated following exonuclease III digestion of tandemly arranged fragments corresponding to the two functional domains of each enzyme. Hybrid GTFs composed of the sucrose-binding domain of one enzyme (GTF-I or GTF-S) with the glucan-binding domain of the other synthesized insoluble glucan exclusively in the absence of primer dextran. Insoluble glucan synthesis by some, but not all, of the GTF-S:GTF-I chimeric enzymes was stimulated by primer dextran T10 addition. In addition, glucan binding by the former but not latter group of hybrid GTFs was demonstrated. These results suggest that the glucan-binding domain alone does not solely determine primer dependence or independence or the structure of the resulting glucan product, although this carboxyl-terminal domain containing direct repeating units does appear to play a significant role in primer dependence.**

Streptococcus mutans has been strongly implicated as the principal etiological agent in human dental caries (18). One of the important virulence properties of these organisms is their ability to tenaciously colonize teeth in the presence of dietary sucrose. This process is dependent on the synthesis of water-insoluble glucans by the glucosyltransferases (GTFs; EC.2.4.1.5) elaborated by *S. mutans* strains. Both genetic and biochemical characterizations of the GTFs produced by the mutans group streptococci (principally *S. mutans* and *Streptococcus sobrinus*) have suggested two different classes of these enzymes: GTF-I, which catalyzes the formation of α-1,3-linked water-insoluble glucans, and GTF-S, which synthesizes water-soluble glucans containing primarily α-1,6-glucose linkages (18). Recent results have suggested that *S. mutans* strains contain three *gtf* genes (2, 10, 11). Two of these, the *gtfB* and *gtfC* genes, are tandemly located on the chromosome of strain GS5 and express the GTF-I and GTF-SI enzymes, respectively, which synthesize primarily insoluble glucans. In addition, the *gtfD* gene codes for the GTF-S enzyme responsible for soluble glucan synthesis. Furthermore, the activity of the latter enzyme is strongly stimulated in the presence of exogenous soluble glucans (primer dependent), while the former two enzymes are primer independent (2, 10, 11).

Nucleotide sequencing of the *gtfB* and *gtfD* genes has indicated that the GTF-I and GTF-S enzymes from strain GS5 exhibit approximately 50% amino acid sequence identity and almost 64% similarity when conservative amino acid replacements are considered (12, 26). Likewise, like all other GTFs sequenced (1, 6, 7, 8, 32), the GTF-I and GTF-S enzymes contain multiple highly homologous direct repeating units containing approximately 65 amino acids at their carboxyl termini. Recent results have suggested that these regions of the enzymes constitute a glucan-binding domain (GBD) which is required for GTF but not sucrase activity (14). However, the isolated GBD exhibits neither sucrase nor GTF activity. Therefore, it has been proposed that the GTFs are composed of two functional domains: the amino-terminal sucrose-binding domain (SBD), capable of binding and hydrolyzing sucrose but not of synthesizing glucan, and the carboxyl-terminal GBD. For the GTF-I enzyme, the former would correspond to amino acid residues 1 to 1095, while the latter would involve residues 1096 to 1475 (14).

To further investigate the mechanism of GTF action, it is important to investigate the relative roles of the two proposed functional domains in determining the nature of the glucan product (soluble versus insoluble glucan) as well as primer dependence or independence. Therefore, in this investigation we used two different strategies to generate hybrid GTF-I:GTF-S enzymes: in vitro fusions utilizing appropriate restriction sites, and in vivo-generated hybrids following homologous recombination. The latter strategy has also been used to generate chimeric proteins in other microorganisms (21, 24, 30, 34). A comparison of the enzymatic properties of the hybrid GTF molecules is discussed relative to the mechanisms of action of these enzymes.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and relevant plasmids used are listed in Table 1. *Escherichia coli* strains were grown in Luria-Bertani medium at 30°C as previously described (2). When required, the antibiotics ampicillin (50 μg/ml), chloramphenicol (20 μg/ml), kanamycin (50 μg/ml), and erythromycin (200 μg/ml) were added to the medium. Isopropyl-β-D-thiogalactoside (IPTG) and 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-Gal) were each added at 40 μg/ml where indicated.

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TABLE 1. *E. coli* strains and plasmids used

Strain or plasmid	Relevant characteristics[a]	Reference or source
E. coli		
C600	*supE44 hsdR thi-1 thr-1 leuB6 lacY1 tonA21*	22
HB101	*supE44 hsdS3*(r_B^- m_B^-)*recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*	22
JM109	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ(*lac-proAB*) F′ (*traD36 proAB*$^+$ *lacI*^{q} *lacZ* M15)	35
Plasmids		
pACYC184	Cm^r, Tc^r, (M)	4
pUC118,119	Ap^r, phagemid cloning vector, (H)	33
pBluescript KS,SK	Ap^r, phagemid cloning vector, (H)	27
pGD103	Km^r, (L)	5
pMCL18,19	Cm^r, medium-copy-number vector containing MCS and *lacZ* of pUC18 and -19, respectively	This study
pMCL20,21	Cm^r, medium-copy-number vector containing MCS and *lacZ* of pBluescript KS and SK, respectively	This study
pTSU5	Ap^r, a pUC119 derivative containing the intact *gtfB* gene, GTF$^+$, (H)	14
pCK41	Ap^r, 3.73-kb *Pst*I-*Bam*HI fragment from pTSU5 in pUC119, GTF$^-$, (H)	14
pNH5	Em^r, a pACYC184 derivative containing the intact *gtfD* gene, GTF$^+$, (M)	11
pYND7	Em^r, pNH5 lacking the 0.92-kb *Dra*I-*Sac*I fragment downstream from *gtfD*, (M) (Fig. 1)	This study
pYNDB1	Em^r, in vitro hybrid *gtfDB*, GTF$^+$, (M) (Fig. 1)	This study
pYNDB2	Em^r, a precursor plasmid for in vivo recombination, GTF$^-$, (M)	This study
pYNBD1	Ap^r, a pBluescript KS derivative containing in vitro hybrid *gtfBD*, GTF$^-$, (H)	This study
pYNBD2	Ap^r, a pUC119 derivative containing in vitro hybrid *gtfBD*, a precursor plasmid for in vivo recombination GTF$^-$, (H) (Fig. 1)	This study
pYNBD5	Km^r, a pGD103 derivative containing in vitro hybrid *gtfBD*, GTF$^-$, (L)	This study
pYNBD7	Cm^r, a pMCL20 derivative containing in vitro hybrid *gtfBD*, a precursor plasmid for in vivo recombination, GTF$^+$, (M) (Fig. 1)	This study
pYNBD10	Cm^r, inserted fragment from pYNBD1 into pMCL21, GTF$^-$, (M)	This study
pYNBD20	Cm^r, inserted fragment from pYNBD2 into pMCL20, GTF$^-$, (M)	This study
pYNBD50	Cm^r, inserted fragment from pYNBD5 into pMCL20, GTF$^-$, (M)	This study
pYNBD201	Ap^r, an in vivo recombinant generated from pYNBD2, GTF$^+$, (H)	This study
pYNBD701	Cm^r, an in vivo recombinant generated from pYNBD7, GTF$^+$, (M)	This study
pYNBD725	Cm^r, an in vivo recombinant generated from pYNBD7, GTF$^+$, (M)	This study
pYNBD742	Cm^r, an in vivo recombinant generated from pYNBD7, GTF$^+$, (M)	This study

[a] (H), high copy number; (M), medium copy number; (L), low copy number; MCS, multiple cloning site; Cm, chloramphenicol; Tc, tetracycline; Ap, ampicillin; Km, kanamycin; Em, erythromycin.

In vitro construction of GTF hybrid enzymes. Plasmid pYNDB1 was constructed as indicated (Fig. 1) following fusion of the GBD of GTF-I with the SBD of GTF-S. A similar general strategy was used to construct other hybrid GTFs in vitro. Plasmid pYNBD1 (structure not shown) resulted from initial transfer of the GBD of GTF-S as a 2.0-kb *Hin*dIII-*Kpn*I fragment into pBluescript KS. The *Bgl*II-*Sca*I fragment from pTSU5 (Fig. 2) was then inserted into the *Bam*HI-*Eco*RV site of the resultant plasmid to produce an in-frame fusion of the *gtfB* and *gtfD* genes. Plasmid pYNBD2 (Fig. 2) was constructed following insertion of the 1.6-kb *Sac*I-*Kpn*I fragment from pYND7 (containing the intact *gtfD* gene) into the corresponding site in plasmid pCK41, which contains the *gtfB* gene lacking sequences corresponding to its carboxyl-terminal GBD. To construct plasmid pYNBD5 (structure not shown), a derivative of the low-copy-number plasmid pGD103 (5) was modified by replacing the multiple cloning site from pUC8 with that from pUC18 to produce plasmid pGD103Xb. A *Pst*I-*Sac*I fragment from pCK41 was then subcloned into the corresponding sites of pGD103Xb. The resulting plasmid was digested with *Sac*I and *Kpn*I, and the multiple cloning site from pBluescript KS was ligated to the linearized plasmid to generate plasmid pYNB2. The GBD of the GTF-S enzyme coded by the *Xba*I-*Sac*I fragment from pYND7 (Fig. 2) was introduced into pYNB2 following digestion of the latter plasmid with *Xba*I and *Sac*I and subsequent ligation. The resulting plasmid, pYNBD5, contains an in-frame fusion of the SBD of GTF-I with the GBD of GTF-S.

An additional GTF-I:GTF-S hybrid protein was expressed from a construction beginning with plasmid pTSU5. In this procedure, pTSU5 was digested with *Acy*I, the resulting overhangs were filled in with the Klenow fragment, and the linearized plasmid was digested with *Pst*I. The 4.2-kb fragment containing the SBD and one direct repeating unit of the GBD from GTF-I was then inserted into the *Pst*I-*Hin*cII sites of pUC118. The inserted fragment was then reisolated following digestion with *Pst*I and *Xba*I and inserted into the *Pst*I-*Xba*I sites of pMCL20 (Fig. 2). The resultant plasmid was next digested with *Bam*HI and *Xba*I and ligated to the *Bam*HI-*Xba*I fragment coding for the GBD of GTF-S from pYND7. This latter construction, pYNBD7 (Fig. 2), resulted in an in-frame fusion of the GTF-I and GTF-S enzymes.

Plasmids pYNBD1 and pYNBD2 appeared to be very unstable when maintained in *E. coli* cells. Therefore, the hybrid *gtfB* and *gtfD* genes were transferred to the medium-copy-number plasmid pMCL20 to produce plasmids pYNBD10 and pYNBD20, respectively. The latter plasmids were demonstrated to be quite stable when grown at 30°C.

Construction of hybrid GTF enzymes following in vivo recombination. To generate hybrid GTF-S:GTF-I enzymes, plasmid pYNDB2 (Fig. 2) was initially constructed. Plasmid pTSU5 was digested with *Eco*RI and *Spe*I, and the resulting DNA fragment (containing the 3′ end of the *gtfB* gene) was ligated into pBluescript SK. The resulting plasmid was digested with *Kpn*I and *Sac*I and ligated with similarly digested pNH5 (containing the intact *gtfD* gene). Plasmid pYNDB2 contains the 5′ region corresponding to the SBD of

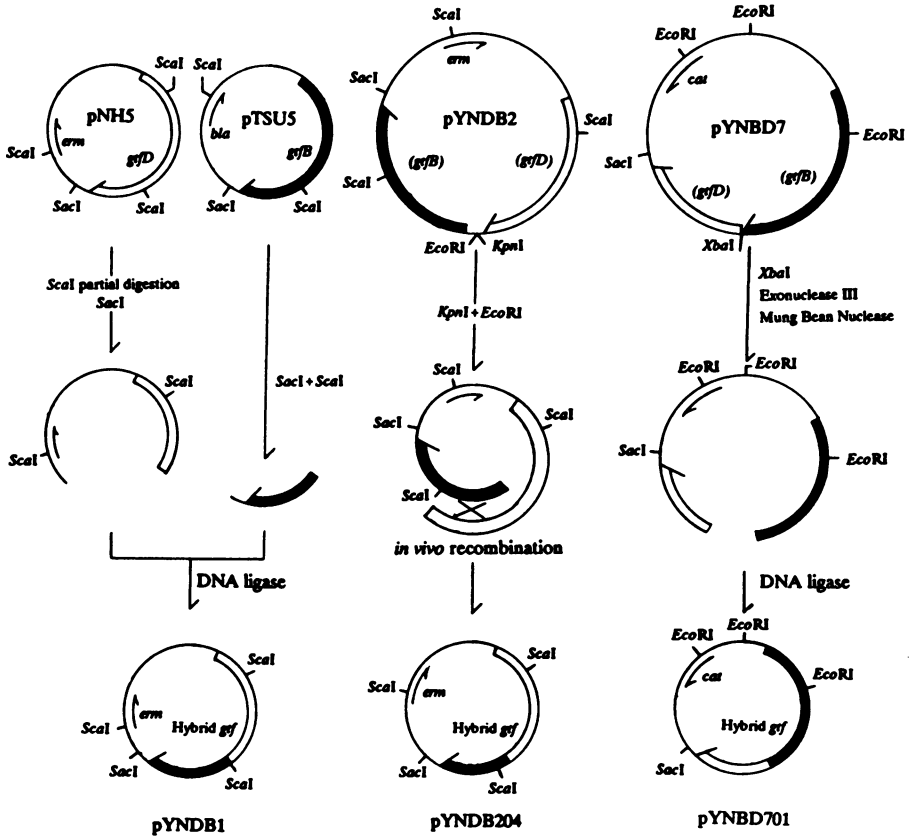


FIG. 1. Scheme for construction of the hybrid *gtf* genes. Closed and open bars indicate DNA fragments derived from the *gtfB* and *gtfD* genes, respectively. pYNDB1 is a typical in vitro hybrid. DB204 is one of the in vivo recombinant hybrid GTFs. pYNBD201 was generated from pYNBD2 by a similar procedure. pYNBD7 is a precursor plasmid for random fusion of fragments from each gene generated following exonuclease III digestion. pYNBD701, pYNBD725, and pYNBD742 are derivatives from pYNBD7.

the *gtfD* gene separated from the 3′ end of the *gtfB* gene coding for the GBD by a short sequence containing unique *Kpn*I, *Apa*I, *Xho*I, and *Eco*RI restriction sites. Cleavage of pYNDB2 with any combination of these latter restriction enzymes resulted in a linearized plasmid with a low probability of religation. These linear DNA fragments (1 to 5 μg) were then transformed in *E. coli* C600 by standard procedures (22). Erythromycin-resistant transformants were selected and screened for the presence of plasmids as well as sucrase activity. Intact plasmid pYNDB2 does not express sucrase or GTF activity. Any transformant expressing GTF activity would contain a plasmid harboring a hybrid *gtfDB* gene produced following recombination of overlapping homologous sequences from the two genes (Fig. 2). A similar strategy was used to construct hybrid *gtfBD* genes from pYNBD7 (Fig. 2).

Enzyme assays. Sucrase activity was determined by the Somogyi-Nelson procedure as previously described (28). GTF activity was measured as routinely carried out in this laboratory (15), using [U-^{14}C]sucrose as a substrate. Protein was determined as previously described (3), and insoluble glucan formation on polyacrylamide gels was assayed as described earlier (9).

PAGE and Western immunoblot analysis. Proteins were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (6% polyacrylamide gel) essentially as previously described (17). Western blot analysis utilizing anti-GTF-I (16) or anti-GTF-S (obtained from K. Fukushima, Nihon University, Matsudo, Japan) serum was carried out as recently described (31).

Glucan binding determinations. The abilities of various GTFs to bind glucans were assessed as recently described (1) except that Sephadex G-200 (Pharmacia-LKB) beads were used. Crude extracts (10 μl) from *E. coli* cells containing plasmids expressing GTF activity were incubated with 100 μl of Sephadex G-200 beads in 50 mM sodium acetate buffer (pH 6.0) for 20 min at room temperature. The beads were then washed three times with the same buffer, using Ultrafree-MC filters (Millipore). The proteins bound to the beads were eluted twice with 10 μl of 2× SDS gel-loading buffer containing 100 mM Tris-HCl (pH 6.8), 10% 2-mercaptoethanol, 4% SDS, 0.2% bromophenol blue, and 20% glycerol and analyzed following SDS-PAGE and Western blotting.

DNA sequence analysis. The nucleotide sequences around the fusion regions were determined by the dideoxy method (23) with a Sequenase kit (Stratagene).

RESULTS

Construction of intermediate-copy-number vectors. To investigate the roles of the SBD and GBD of the GTFs in determining the nature of the glucan product and dependence of the enzymes on exogenous primer, hybrid GTFs were constructed from the *gtfB* and *gtfD* genes. Initially, each of the genes was transferred to pUC-based plasmid

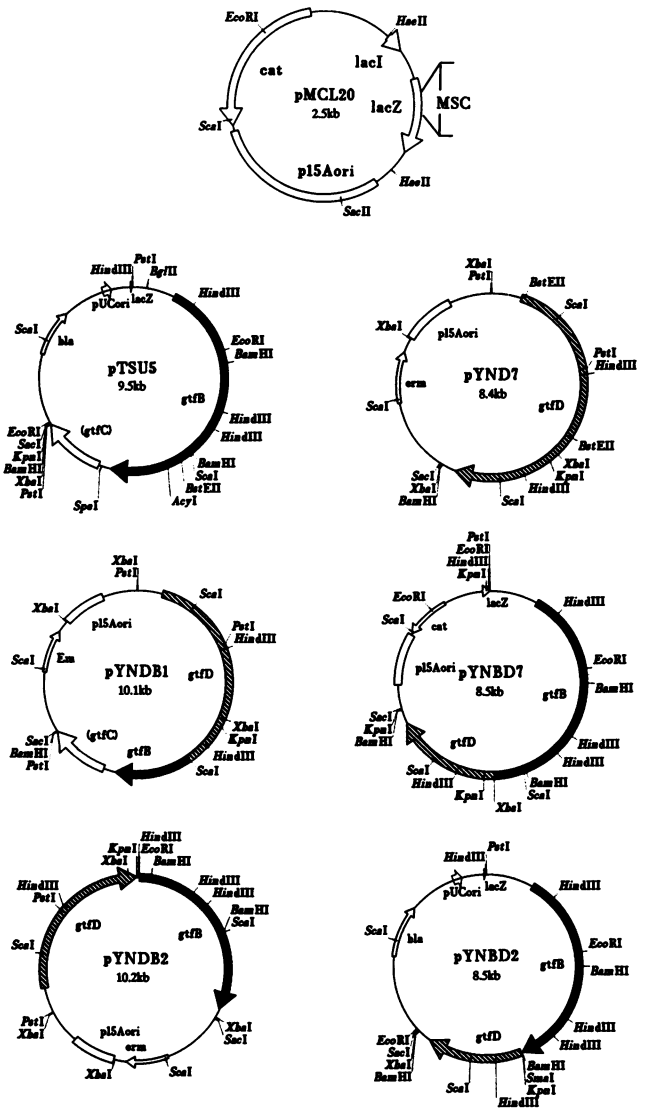


FIG. 2. Structures of plasmids used in this study. pMCL20 is a new medium-copy-number vector containing *lacZ* and the multiple cloning sites (MCS) of pBluescript KS (24). Closed arrows or bars indicate the intact (pTSU5) portion of the *gtfB* gene, and cross-hatched arrows or bars indicate the intact (pYND7) portion of the *gtfD* gene.

vectors, but it was not possible to construct stable high-copy-number plasmid vectors containing multiple cloning sites for subsequent manipulation of the *gtfD* gene.

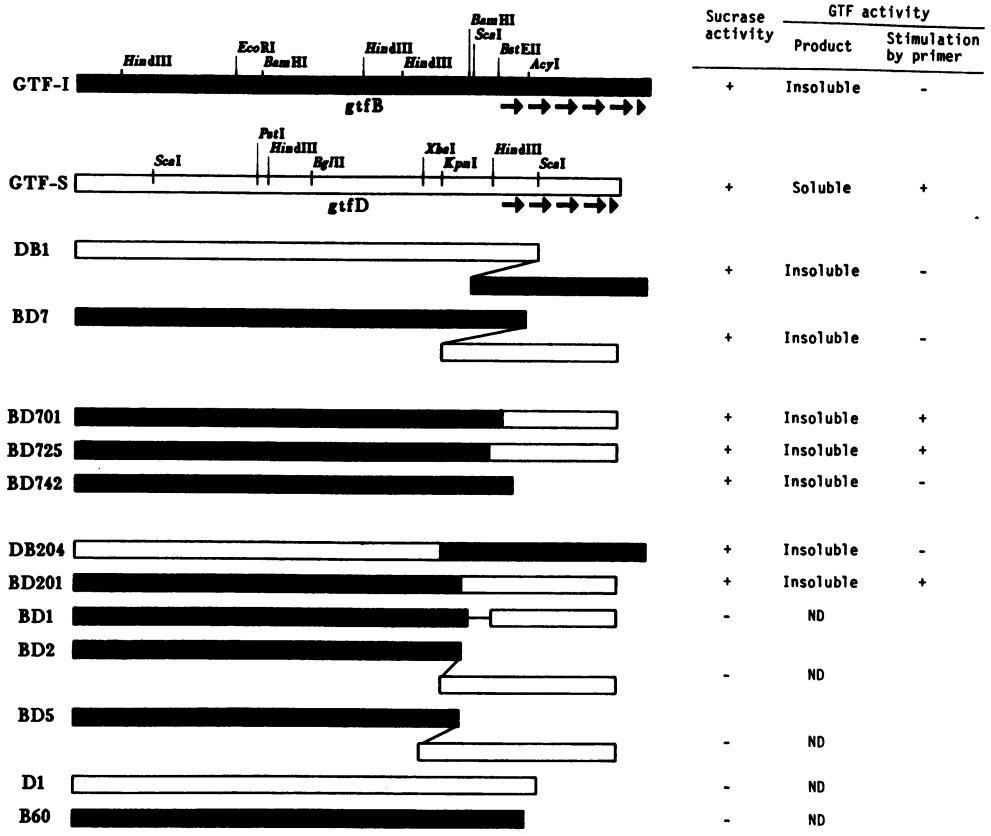
The medium-copy-number plasmid vector pACYC184 (4) contains the p15A replicon and exists in 10 to 12 copies per *E. coli* cell (22). To introduce multiple cloning sites into this plasmid, the vector was digested with *Xba*I and *Ban*I, blunt ended with the Klenow fragment, and religated. The resulting plasmid was further digested with *Hae*II, and the 640-bp *Hae*II fragment from pBluescript SK or KS encoding the multiple cloning site (27) was ligated into the linearized plasmid. Following transformation into *E. coli* JM109, colonies containing the correct constructs were identified, and the corresponding plasmids were designated pMCL20 and pMCL21, respectively (Fig. 2). The intact *gtfD* gene was introduced into the former plasmid for use in generating several of the hybrid *gtf* genes utilized in this investigation. The 440-bp *Hae*II fragment containing the multiple cloning site from pUC18 and pUC19 was similarly ligated to modified pACYC184 to produce plasmids pMCL18 and pMCL19, which were used in sequencing the hybrid *gtf* genes. The sequence of the restriction sites in the multiple cloning sites in the latter plasmids was more convenient for isolating the required DNA fragments.

In vitro construction of *gtfBD* and *gtfDB* hybrid genes. From the known nucleotide sequences of the *gtfB* and *gtfD* genes (12, 26), it was possible to use several existing restriction sites of the two genes to construct in-frame hybrid enzymes (Fig. 1 and 3). By this strategy, one DB hybrid (the first letter corresponds to the amino-terminal SBD from either the *gtfB* or *gtfD* gene, and the second letter corresponds to the carboxyl-terminal GBD), DB1, and four BD hybrid GTFs were constructed. However, only DB1 and BD7 expressed GTF activity; the others (BD1, BD2, and BD5) were enzymatically inactive. Assuming that the constructions did not alter the predicted nucleotide sequences of the hybrids, it is likely that the resulting hybrid proteins of the latter three constructs existed in protein conformations which were incompatible with enzymatic activity. The gene corresponding to the enzymatically inactive hybrid protein BD5 was initially constructed in the low-copy-number vector pGD103 (5). However, transfer of the hybrid genes into the medium-copy-number vector pMCL20 also did not result in detectable sucrase activities.

To generate potential BD hybrid proteins, plasmid pYNBD7 (Fig. 2) was initially constructed in vitro and displayed significant GTF activity (Fig. 3). Cleavage of the plasmid near the fusion junction between the *gtfB* and *gtfD* domains, followed by controlled exonuclease digestion and religation, would be expected to generate additional novel hybrid proteins. Therefore, the plasmid was digested with *Xba*I, incubated with exonuclease III for various time periods, and then treated with mung bean nuclease and Klenow fragment. The linearized plasmid fragments were then religated and transformed into *E. coli* HB101. Of 78 transformants containing plasmid, three, pYNBD701 (Fig. 1), pYNBD725, and pYNBD742, expressed sucrase activity. However, nucleotide sequencing of these plasmids indicated that all three were generated following homologous recombination of the overlapping *gtfB* and *gtfD* sequences (see below). The majority of the plasmids contained deletions in the *gtf* hybrid genes, leading to inactive GTFs.

In vivo generation of GTF hybrid enzymes. An alternate strategy for generating hybrid GTFs was based on previous results indicating that *gtfB* and *gtfD* shared more than 50% nucleotide and amino acid sequence similarities (12, 26). Therefore, transformation of *E. coli* with a plasmid containing overlapping tandem DNA fragments from each of the two genes linearized in the overlapping regions should generate circularized recombinants following homologous recombination (Fig. 1). Plasmid pYNDB2 (Fig. 2) was initially constructed to generate DB hybrid proteins. The plasmid was cleaved at the unique *Kpn*I and *Eco*RI sites, and the linearized plasmid was transformed into *E. coli* C600 (*recA*$^+$); 70 erythromycin-resistant colonies were screened for GTF activity. Only one transformant containing plasmid pYNDB204 expressed detectable sucrase and GTF activities.

To isolate potential BD hybrid proteins, plasmid pYNBD2 (Fig. 1), which was constructed following in vitro ligation and expressed an enzymatically inactive GTF, was linearized with *Kpn*I and *Sma*I and transformed into strain C600. Screening of 75 transformants yielded 1 sucrase-positive transformant containing plasmid pYNBD201.



	Sucrase activity	GTF activity: Product	GTF activity: Stimulation by primer
GTF-I	+	Insoluble	-
GTF-S	+	Soluble	+
DB1	+	Insoluble	-
BD7	+	Insoluble	-
BD701	+	Insoluble	+
BD725	+	Insoluble	+
BD742	+	Insoluble	-
DB204	+	Insoluble	-
BD201	+	Insoluble	+
BD1	-	ND	
BD2	-	ND	
BD5	-	ND	
D1	-	ND	
B60	-	ND	

FIG. 3. Structures of the parental and hybrid genes and their enzymatic properties. The restriction maps of *gtfB* and *gtfD* are shown. Closed and open bars indicate DNA fragments derived from the *gtfB* and *gtfD* genes, respectively. Arrows under the bars denote the direct repeating units. ND, not determined.

Expression of the hybrid GTF enzymes. To ensure that expression of the hybrid *gtf* genes was not altered during the in vitro or in vivo constructions, Western blot analysis of the hybrid proteins was carried out (Fig. 4B and C). The approximate molecular sizes of the hybrid GTFs corresponded well with the predicted sizes based on the known amino acid sequences of the GTF-I and GTF-S enzymes. All of the hybrid *gtf* genes expressed similar levels of enzyme except for the hybrid enzyme coded by plasmid pYNBD201. For unexplained reasons, this plasmid expressed very high levels of the hybrid GTF. Several of the enzymatically inactive GTFs (BD2 and BD5) were detectable with anti-GTF-I serum and exhibited sizes compatible with the predicted sizes of the hybrid proteins (data not shown). Therefore, the lack of enzymatic activity displayed by these latter hybrid proteins was not the result of premature truncation of the enzymes.

Enzymatic properties of the hybrid GTFs. To determine whether GTF domain shuffling significantly altered the properties of the hybrid enzymes, both the solubility of the glucan products and the dependence of the enzymes on exogenous primer dextran were analyzed (Table 2). Although the specific activities of the hybrid GTFs were much lower than those of the GTF-I and GTF-S enzymes, most of these activities were relatively strong and reproducible. When the GBD of the GTF-I enzyme was fused to the SBD of the GTF-S protein (proteins DB1 and DB201), the resulting enzymes behaved identically with the GTF-I enzyme, producing water-insoluble glucan in a primer-independent manner. Furthermore, like the GTF-I enzyme, the hybrid enzymes produced water-soluble glucan only in the presence of exogenous dextran T10. These results initially suggested that the GBD of the GTFs alone determines the nature of the glucan product. To further test this hypothesis, the BD fusions constructed both in vitro and in vivo were analyzed (Table 2). In all cases, fusion of the GBD from the GTF-S enzyme to the SBD of the GTF-I enzyme resulted in an enzyme which produced insoluble glucan in the absence of primer. Therefore, the GBD of the GTFs cannot be the sole determinant of the nature of the glucan product synthesized. Furthermore, in contrast to the GTF-I enzyme, insoluble glucan synthesis by several of the hybrid GTFs (BD701, BD725, and BD201) was significantly stimulated in the presence of exogenous dextran T10. However, insoluble glucan synthesis by hybrid BD7 was not affected by dextran T10. Likewise, soluble glucan formation by the former enzymes, but not BD7, was also stimulated in the presence of the acceptor molecule. These results suggested that the GBDs of the GTFs are important but not the sole determinants of whether or not glucan synthesis will be stimulated in the presence of soluble glucan acceptors.

Additional evidence for the formation of insoluble glucans by the hybrid GTFs was demonstrated following SDS-PAGE analysis (Fig. 5). Direct demonstration of water-insoluble glucan synthesis by GTFs can be obtained after washing of the gels with detergents and incubation with sucrose (9). In addition to the GTF-I enzyme, the hybrid enzymes BD701

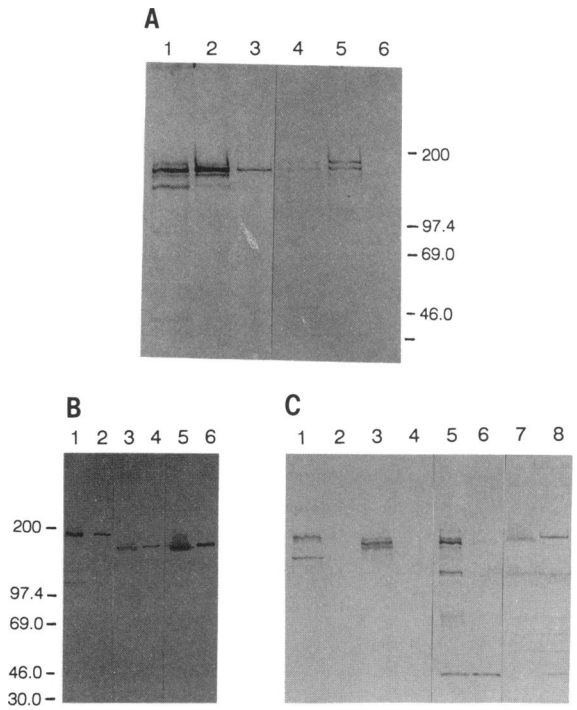


FIG. 4. Western blot analysis of gene expression and binding of GTFs to glucan. Procedures are described in Materials and Methods. (A) Glucan binding of GTF-S (lanes 1 to 3) and GTF-I (lanes 4 to 6) was detected with anti-GTF-S and anti-GTF-I sera, respectively. Lanes: 1, crude extract of cells containing pYND7 (10 μl); 4, crude extract of cells containing pTSU5 (10 μl); 2 and 5, glucan-bound protein when 100 μl of crude extracts was used; 3 and 6, glucan-bound protein when 10 μl of crude extracts was used. (B) Gene expression (lanes 1, 3, and 5) and glucan binding (lanes 2, 4, and 6) of BD7 (lanes 1 and 2) and its derivatives BD701 (lanes 3 and 4) and BD725 (lanes 5 and 6). GTF proteins were detected with anti-GTF-S serum. (C) Gene expression (lanes 1, 3, 5, and 7) and glucan binding (lanes 2, 4, 6, and 8) of DB1 (lanes 1 and 2), DB204 (lanes 3 and 4), BD201 (lanes 5 and 6), and BD1 (lanes 7 and 8). GTF proteins were detected with anti-GTF-S serum. Ten microliters of crude extract was used in panels B and C. Protein concentrations of the crude extracts were 12 to 15 mg/ml except for BD201 (1.2 mg/ml).

and BD725 demonstrated insoluble glucan bands after SDS-PAGE. However, although the hybrid enzyme DB204 synthesized significant amounts of insoluble glucan (Table 2), a similar insoluble glucan band could not be detected on the gels. This apparent contradiction was explained by the subsequent observation that the DB204 enzyme, unlike GTF-I and BD7, was irreversibly inactivated in the presence of 1% SDS.

Glucan binding by the hybrid GTFs. Since recent results have suggested that the *S. mutans* GS5 GTF-S displays higher affinity for α-1,6-linked glucans than does GTF-I (18) and that this binding is mediated by the GBD of the GTFs (6, 14), it was of interest to assess the ability of the hybrid GTFs to bind soluble glucans. Representative BD and DB hybrids as well as the GTF-I and GTF-S enzymes were examined for their relative abilities to bind to Sephadex G-200 (containing α-1,6-linked glucan) by using anti-GTF-I and anti-GTF-S antibodies (Fig. 4). Under the conditions used, binding of GTF-S to Sephadex was much more apparent than binding of GTF-I (Fig. 4A). However, since the reaction of GTF-I with anti-GTF-I was much weaker than that of GTF-S with anti-GTF-S, it is difficult to compare the relative affinities of the two enzymes for Sephadex on the basis of these results. However, except for hybrid BD201, when the GBD of GTF-S was fused with the SBD of GTF-I, the anti-GTF-I antibody detected significantly more hybrid GTF binding to Sephadex than for GTF-I. This was the case even when the BD hybrid, BD1, was enzymatically inactive. By contrast, hybrids DB1 and DB204, containing the GBD of GTF-I, did not bind to Sephadex. These results support the hypothesis that the carboxy-terminal GBD of the GTFs plays a significant role in determining the glucan-binding capacity of the enzymes.

TABLE 2. Insoluble and soluble glucan synthesis catalyzed by the hybrid *gtf* gene products

Enzyme	Glucan synthesis (10^3 cpm/mg of protein)			
	Insoluble glucan		Soluble glucan	
	Without DT10	With DT10	Without DT10	With DT10
GTF-I	155	159	0	24
GTF-S	5	0	54	422
DB1	1.77	1.72	0	0.58
DB204	2.99	2.98	0	1.02
BD7	1.12	1.08	0	0
BD701	5.94	11.1	0	3.63
BD725	1.00	1.34	<0.1	0.13
BD742	0.52	0.34	0	<0.1
BD201	<0.1	0.34	<0.1	0.22

Analysis of the fusion regions of the GTF hybrids. In-frame fusions of the hybrid proteins generated by using restriction sites should lead to predictable fusion points. However, the fusions generated by recombination had unknown fusion junctions. To determine the junctions for the in vivo fusion BD201, it was necessary to determine the nucleotide sequence of the junction region. Following sequencing, it was confirmed that this fusion appeared to result from homologous recombination between sequences of the *gtfB* and *gtfD* genes which were similarly positioned in the genes (Fig. 6). Likewise, sequencing of BD204 indicated that homologous recombination within a 5-bp identical sequence resulted in an in-frame hybrid GTF. Surprisingly, nucleotide sequencing of the plasmids generated by the exonuclease III-religation strategy (Fig. 1) revealed that all three GTF-positive constructs (pYNBD701, pYNBD725, and pYNBD742) were also generated following homologous recombination between *gtfB* and *gtfD* identical sequences (Fig. 6). However, recombination in plasmid pYNBD742 resulted between iden-

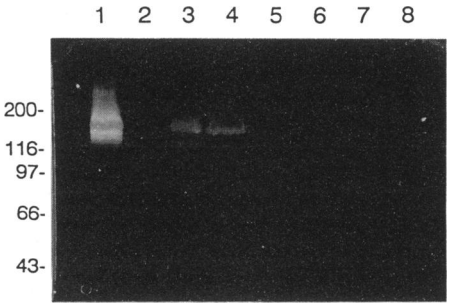


FIG. 5. Assay for insoluble glucan synthesis following SDS-PAGE. Lanes: 1, GTF-I; 2, BD7; 3, BD701; 4, BD725; 5, BD201; 6, DB1; 7, DB204; 8, negative control (pACYC184).

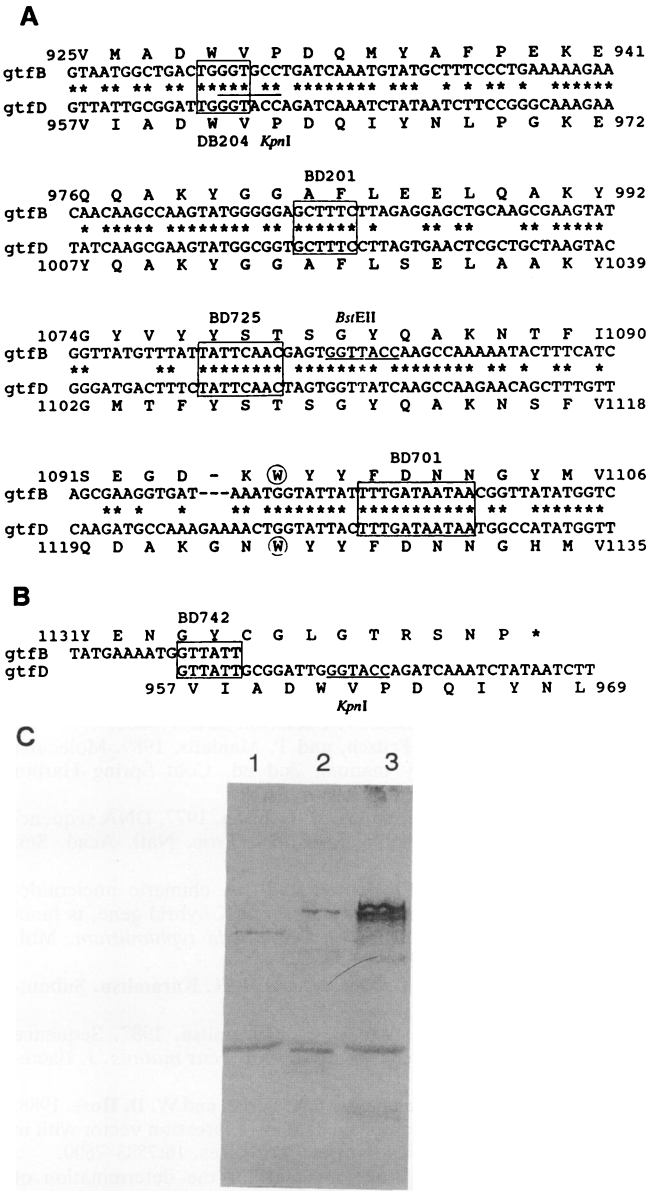


FIG. 6. (A and B) Nucleotide sequences around the fusion sites in the *gtfBD* and *gtfDB* hybrid genes. The corresponding amino acid sequences are shown above and below. The regions in which in vivo recombination took place in the hybrid genes are boxed. The repeating units begin at the circled tryptophan. Residues are numbered from the amino termini of the proteins. (C) Western blot analysis of BD742. Lanes: 1, BD742; 2, BD201; 3, GTF-I. Proteins in crude extracts were detected with anti-GTF-I serum.

tical 6-bp sequences, which did not occur in regions of the two genes that were similarly aligned. This latter recombination event resulted in an out-of-frame fusion whose translation was prematurely terminated 10 amino acids from the recombination region. Western blot analysis of hybrid BD742 confirmed the smaller molecular size of the GTF relative to GTF-I and BD201 (Fig. 6C). In addition, this truncated GTF displayed lower activity than did BD701 and BD725, and the activity was not stimulated by dextran T10 (Table 2).

DISCUSSION

Previous results have demonstrated that the GTF-I and GTF-S enzymes from *S. mutans* GS5 exhibit approximately 50% amino acid sequence identity (12, 26). Nevertheless, the GTF-I enzyme synthesizes a water-insoluble glucan product containing approximately 77% α-1,3 glucose linkages in the absence of primer (20). By contrast, the GTF-S enzyme synthesizes a soluble glucan containing 89% α-1,6 glucose linkages and is highly primer dependent (11, 20). The present study using hybrid enzymes has examined the relative importance of the two functional domains of the GTFs, SBD and GBD, in determining the structure of the glucan products as well as their dependence on exogenous primer dextrans.

Two different strategies were successfully used to generate hybrid GTFs: in vitro construction utilizing appropriate restriction sites, and in vivo generation of hybrid proteins following homologous recombination. The third strategy attempted (ligation following exonuclease III treatment of tandemly positioned *gtfB* and *gtfD* fragments; Fig. 1) actually generated hybrids following homologous recombination (Fig. 6). As previously reported (13), the homologous recombination events were not dependent on *recA* function, since hybrid formation occurred in *E. coli* HB101. The recombination event generating hybrid BD742 was demonstrated to result from homology of a 6-bp sequence in both the *gtfB* and *gtfD* genes (Fig. 6). This finding is consistent with a previous suggestion that a 5-bp homologous sequence is sufficient to generate recombination between single-stranded plasmid DNA fragments (29). Hybrid proteins BD701, BD725, and BD742 were apparently generated following homologous recombination of linearized plasmid molecules which were not ligated prior to transformation.

The in vivo hybrid constructions used plasmid pYNDB2 (Fig. 1), which contained 1.57 kb of overlapping sequences between the *gtfB* and *gtfD* genes that had apparently generated many hybrid genes. However, only one recombinant, pYNDB204, expressed significant GTF activity (Table 2). Some of the inactive hybrid GTFs may have been out of frame, while others may have been in frame but yielded inactive enzymes. Enzymatically active hybrid BD proteins (BD701, BD725, and BD742) were unexpectedly generated in vivo from plasmid pYNBD7 (Fig. 2). With the exception of BD742, all of the hybrid proteins resulted from fusions of the large carboxyl-terminal domain (equivalent to the GBD) of one protein with the even larger amino-terminal domain (equivalent to the SBD) of the other GTF. These results are consistent with previous suggestions (1, 6, 14) that GTFs are composed of two functional domains, SBD and GBD. BD742 was constructed from a fusion of a DNA fragment equivalent to the SBD of GTF-I together with a small portion of its GBD (containing one of the six direct repeating units) and a small fragment equivalent to no more than 11 amino acids from the GBD of the GTF-S enzyme. The weak GTF activity of this hybrid enzyme containing essentially only one direct repeating unit, together with its lack of stimulation by exogenous dextran T10 (Table 2), is consistent with recent suggestions that at least two of the direct repeating units are required for significant GTF activity (14) and primer stimulation (25).

These results also confirm a recent suggestion that the GTF-S enzyme from strain GS5 binds α-1,6-linked glucans better than the GTF-I enzyme does (19). Fusion of the GBD of GTF-S to the SBD of GTF-I generally resulted in significant binding of the hybrid proteins to Sephadex (Fig. 4). By contrast, replacement of the GBD of GTF-S with the comparable domain from GTF-I yielded no detectable binding of

the hybrid proteins despite the fact that intact GTF-S binds very well to Sephadex. However, one of the BD hybrids, BD201, bound very weakly to Sephadex. This finding suggests that the conformation of the enzymes also plays a significant role in modulating enzyme binding to glucan. That the overall conformation of the enzymes is important was also illustrated by the observation that the GTF activity of hybrid BD7, although binding glucan very well (Fig. 4), was not stimulated by exogenous dextran T10 (Table 2).

These results suggest that the GBD alone does not solely determine primer dependence or independence or the structure of the resulting glucan product. However, this carboxyl-terminal domain and its direct repeating units do appear to play a significant role in these enzymatic properties. These results taken together provide additional information to support the concept of two functional domains in the GTFs. Additional investigations altering individual amino acids will be required to further define the structural determinants of GTF specificity. In addition, it will be of interest to examine the chemical structures of the glucans synthesized by the hybrid GTFs to determine whether unique water-insoluble glucans are produced by these enzymes and whether these novel enzymes also modulate sucrose-enhanced colonization of teeth (18).

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REFERENCES

1. **Abo, H., T. Matsumura, T. Kodama, H. Ohta, K. Fukui, K. Kato, and H. Kagawa.** 1991. Peptide sequences for sucrose splitting and glucan binding within *Streptococcus sobrinus* glucosyltransferase (water-insoluble glucan synthetase). J. Bacteriol. **173:**989–996.
2. **Aoki, H., T. Shiroza, M. Hayakawa, S. Sato, and H. K. Kuramitsu.** 1986. Cloning of a *Streptococcus mutans* glucosyltransferase gene coding for insoluble glucan synthesis. Infect. Immun. **53:**587–594.
3. **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.
4. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the p15A cryptic miniplasmid. J. Bacteriol. **134:** 1141–1156.
5. **Deich, R. A., B. J. Metcalf, C. W. Finn, J. E. Farley, and B. A. Green.** 1988. Cloning of genes encoding a 15,000-dalton peptidoglycan-associated outer membrane lipoprotein and an antigenically related 15,000-dalton protein from *Haemophilus influenzae*. J. Bacteriol. **170:**489–498.
6. **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.
7. **Giffard, P. M., C. L. Simpson, C. P. Milward, and N. A. Jacques.** 1991. Molecular characterization of a cluster of at least two glucosyltransferase genes in *Streptococcus salivarius* ATCC 25975. J. Gen. Microbiol. **137:**2577–2593.
8. **Gilmore, K. S., R. R. B. Russell, and J. J. Ferretti.** 1990. Analysis of the *Streptococcus downei gtfS* gene, which specifies a glucosyltransferase that synthesizes soluble glucans. Infect. Immun. **58:**2452–2458.
9. **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.
10. **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.
11. **Hanada, N., and H. K. Kuramitsu.** 1989. Isolation and characterization of the *Streptococcus mutans gtfD* gene, coding for primer-dependent soluble glucan synthesis. Infect. Immun. **57:** 2079–2085.
12. **Honda, O., C. Kato, and H. K. Kuramitsu.** 1990. Nucleotide sequence of the *Streptococcus mutans gtfD* gene encoding the glucosyltransferase-S enzyme. J. Gen. Microbiol. **136:**2099–2105.
13. **Jones, D. H., and B. H. Howard.** 1991. A rapid method for recombination and site-specific mutagenesis by placing homologous ends on DNA using polymerase chain reaction. BioTechniques **10:**62–66.
14. **Kato, C., and H. K. Kuramitsu.** 1990. Carboxyl-terminal deletion analysis of the *Streptococcus mutans* glucosyltransferase-I enzyme. FEMS Microbiol. Lett. **72:**299–302.
15. **Kuramitsu, H. K.** 1975. Characterization of extracellular glucosyltransferase activity of *Streptococcus mutans*. Infect. Immun. **12:**738–749.
16. **Kuramitsu, H. K., and L. Ingersoll.** 1976. Immunological relationships between glucosyltransferases from *Streptococcus mutans* serotypes. Infect. Immun. **14:**636–644.
17. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
18. **Loesche, W. J.** 1986. Role of *Streptococcus mutans* in human dental decay. Microbiol. Rev. **50:**353–380.
19. **Mooser, G.** Personal communication.
20. **Mukasa, H.** Personal communication.
21. **Nogami, T., T. Mizuno, and S. Mizushima.** 1985. Construction of a series of *ompF-ompC* chimeric genes by in vivo homologous recombination in *Escherichia coli* and characterization of the translational products. J. Bacteriol. **164:**797–801.
22. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
24. **Schneider, E., and C. Walter.** 1991. A chimeric nucleotide-binding protein, encoded by a *hisP-malK* hybrid gene, is functional in maltose transport in *Salmonella typhimurium*. Mol. Microbiol. **5:**1375–1383.
25. **Shiroza, T., C. Kato, G. Mooser, and H. K. Kuramitsu.** Submitted for publication.
26. **Shiroza, T., S. Ueda, and H. K. Kuramitsu.** 1987. Sequence analysis of the *gtfB* gene from *Streptococcus mutans*. J. Bacteriol. **169:**4263–4270.
27. **Short, J. M., J. M. Fernandez, J. A. Sorge, and W. D. Huse.** 1988. Lambda ZAP: a bacteriophage lambda expression vector with in vivo excision properties. Nucleic Acids Res. **16:**7583–7600.
28. **Somogyi, M.** 1945. A new reagent for the determination of sugars. J. Biol. Chem. **160:**61–68.
29. **Sung, W. L., and D. M. Zahab.** 1987. Site-specific recombination directed by single-stranded crossover linkers: specific deletion of the amino-terminal region of the β-galactosidase gene in pUC plasmids. DNA **6:**373–379.
30. **Tommassen, J., P. van der Ley, M. van Zeijl, and M. Agtergerg.** 1985. Localization of functional domains in *E. coli* K-12 outer membrane porins. EMBO J. **4:**1583–1587.
31. **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.
32. **Ueda, S., T. Shiroza, and H. K. Kuramitsu.** 1988. Sequence analysis of the *gtfC* gene from *Streptococcus mutans* GS-5. Gene **69:**101–109.
33. **Vieira, J., and J. Messing.** 1987. Production of single-stranded plasmid DNA. Method Enzymol. **153:**3–11.
34. **Weber, H., and C. Weissmann.** 1983. Formation of genes coding for hybrid proteins by recombination between related, cloned genes in *E. coli*. Nucleic Acids Res. **11:**5661–5669.
35. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.