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Construction of a Model Secretion System for Oral Streptococci

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A DNA fragment corresponding to the secretory domain from the *Streptococcus mutans* GS-5 *gtfB* gene, which encodes the putative 38-amino-acid signal peptide of the glucosyltransferase I (GTF-I) enzyme product, has been constructed. This fragment was fused with the α-amylase structural gene from alkalophilic *Bacillus* sp. strain 707. This hybrid gene as well as the intact amylase gene were introduced into an *Escherichia coli*-streptococcus shuttle vector consisting of three components: the *E. coli* replicon p15Aori from pACYC177, an erythromycin resistance gene from pAMβ-1, and the streptococcal replicon from pVA838. Transformation of the oral noncariogenic bacterium *Streptococcus gordonii* with the chimeric plasmid harboring the hybrid amylase gene resulted in strong extracellular amylase production. By contrast, transformants containing the intact amylase gene exhibited only trace amounts of amylase activity in culture fluids. Since the two signal peptide structures of the GTF-I enzyme and the *Bacillus* amylase are distinct from each other, these differences might result from the inability of *S. gordonii* to correctly process the *Bacillus* signal peptide. Furthermore, culture fluids from transformants of *S. mutans* as well as *Streptococcus milleri* harboring the hybrid amylase gene showed only weak amylase activity. Deletion of the *gtfB*, *gtfC*, or *ftf* gene from *S. mutans* GS-5 did not increase amylase secretion following transformation with the hybrid amylase gene. These results suggest that in contrast to *S. gordonii*, the inability of *S. mutans* and *S. milleri* to secrete hybrid amylase molecules could result from incorrect interaction of the secretory components of these organisms with amylase precursor molecules.

The oral gram-positive bacterium *Streptococcus mutans* has the ability to secrete several extracellular enzymes involved in polysaccharide metabolism, such as glucosyltransferases (GTFs) and fructosyltransferase (FTF) (16). Among these, the GTF-I and GTF-SI enzymes, which produce water-insoluble glucan from dietary sucrose, have been recognized as potential virulence factors in human dental decay (1, 7). Therefore, the incidence of dental caries might be reduced if oral noncariogenic bacteria could be genetically engineered to express antigens which induce immunity to the GTF enzymes or surface proteins which participate in attachment to smooth tooth surfaces. Alternatively, oral bacteria could be constructed to secrete enzymes, such as dextranase or invertase, which inhibit glucan synthesis. Therefore, it was of interest to construct a model secretion system in oral gram-positive bacteria.

In *Bacillus subtilis*, an industrially important gram-positive organism which secretes amylolytic and proteolytic enzymes into the surrounding media, secretion vector systems have been constructed, and secretion of mouse beta interferon (IFN-β) as well as human alpha interferon has been reported (24, 34). These systems were initially constructed by isolating the secretory domain of the α-amylase gene from either *B. subtilis* or *Bacillus amyloliquefaciens* and the structural gene for the β-lactamase from *Escherichia coli* plasmid pBR322. These genes were fused, and model secretion systems were generated following transformation of *B. subtilis* (21, 25). In the *B. subtilis* construct, a recombination-deficient strain was used because its chromosome and the secretion plasmid both contain portions of the α-amylase gene.

S. mutans GS-5 contains three *gtf* genes, *gtfB*, *gtfC*, and *gtfD*, and one *ftf* gene coding for extracellular enzymes, all of which have been cloned and sequenced in our laboratory (9, 33, 35, 40). Since the *gtfB* gene promoter appeared to be more active than promoters of the other genes (4), this gene was used for the isolation of the promoter and signal sequence coding regions in this study. Subsequently, it was necessary to select a passenger gene which would satisfy the following three criteria: (i) the gene encodes a bacterial extracellular protein, (ii) transformants are positively selected, and (iii) the gene product is readily detected.

The structural gene for the β-lactamase could not be used as passenger DNA, since expression of this gene in streptococci is prohibited. Therefore, we have selected the α-amylase gene from alkalophilic *Bacillus* sp. strain 707. This organism secretes several enzymes possessing amylase activity, and two genes expressing this activity in both *E. coli* and *B. subtilis* cells have been cloned and sequenced (11, 38). One gene encodes the maltohexaose-producing amylase, or G6 amylase, and the other codes for the maltooligosaccharide-forming enzyme (Gn amylase). These two genes share high sequence homology at both the nucleic acid and amino acid levels, and both enzymes cross-react with anti-G6 amylase serum. The structural gene for the latter enzyme was isolated as passenger DNA and fused to the promoter and signal sequence coding regions of the *gtfB* gene. In this report, we describe the preparation of these two DNA fragments, the construction of a secretion shuttle vector, and the production of α-amylase in oral streptococci.

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MATERIALS AND METHODS

Bacterial strains and transformation. *S. mutans* GS-5, in vivo recombinant mutant *S. mutans* SP2 (39), mutant derivatives of SP2, *Streptococcus gordonii* Challis, and *Streptococcus milleri* Is57 were maintained and grown in Todd-Hewitt broth (Difco, Detroit, Mich.) as previously described (13). Transformation of these bacteria was carried out by using heat-inactivated horse serum (Sigma, St. Louis, Mo.) (26), and transformants were selected on tryptic soy broth (Difco) agar plates containing erythromycin (10 μg/ml), spectinomycin (200 μg/ml), or kanamycin (250 μg/ml). *E. coli* JM109 was transformed by a standard $CaCl_2$ method (30), and transformants were screened on LB plates supplemented with ampicillin (50 μg/ml), kanamycin (50 μg/ml), spectinomycin (50 μg/ml), or erythromycin (200 μg/ml).

DNA manipulations. DNA isolation, endonuclease restriction, ligation, and nucleotide sequencing were carried out as previously described (35). Southern blot analysis was carried out as described earlier (1) except that nylon membranes and biotinylated probes were used. DNA fragments used in this study are listed in Table 1; a detailed preparation protocol for each fragment will be published elsewhere. Construction of cloning vectors by using the DNA fragments listed in Table 1 is outlined in Table 2.

Detection of recombinant clones possessing amylase activity on selective agar plates. LB or tryptic soy broth plates containing 1% soluble starch (EM Science, Gibbstown, N.J.) were stored at 4°C for 40 to 60 days. During storage, solubilized starch forms micelles which result in opaque agar plates. Appropriate antibiotics were added to the plates before competent cells were spread, and recombinant clones possessing amylase activity were identified as those with clear halos surrounding the colonies.

Preparation of enzyme samples from culture fluids of streptococcal transformants. Streptococcal transformants were grown with 4 ml of Todd-Hewitt broth containing erythromycin (10 μg/ml) in the presence or absence of phenylmethylsulfonyl fluoride (PMSF; 0.02%) at 37°C for 18 h. Cells were removed by centrifugation, supernatant fluids were treated with 2 volumes of cold acetone, and the resulting precipitates were recovered following centrifugation. The precipitates were dissolved in 100 μl of Tris-EDTA (TE) buffer, and insoluble material was removed by centrifugation (40-fold-concentrated enzyme samples). Samples of 5 or 10 μl were used for activity staining or Western blot (immunoblot) analysis, respectively, following sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE).

Gel electrophoresis. Each of the 40-fold-concentrated enzyme preparations was analyzed following SDS-PAGE and subjected to either activity staining or Western blot analysis. For activity staining, samples were loaded on 7% gels containing 1% soluble starch. Following electrophoresis, the gel was incubated in TE buffer containing 1% Triton X-100 at 37°C for 2 h. The active bands were visualized by soaking the gel in an iodine solution (0.002 N iodine in 2% potassium iodide; Sigma). For Western blot analysis, samples were resolved following SDS-PAGE and amylase molecules were identified with anti-G6 amylase serum following electroblotting onto nitrocellulose membranes.

GTF assay. GTF activity was determined by a standard radioactivity assay as previously described (12).

RNA extraction. Overnight cultures (18 h) of both *S. mutans* and *S. gordonii* derivatives in 5 ml of Todd-Hewitt broth were centrifuged, and the cells were suspended with 200 μl of TE containing 5 mg of lysozyme per ml. After

TABLE 1. DNA fragments used in this study

Name	Source	Reference	Domain[a]	Fragment	Size (kb)
Amp^r	pUC18	44	*Eco*O109 (2674)-Amp^r-*Dra*I (1528)	*Bam*HI-Amp^r-*Bam*HI	1.1
Em^r	pAMβ1	2	*Dde*I (150)-Em^r-*Dra*I (1168)	*Bam*HI-Em^r-*Bam*HI	1.0
Km^{r}903	pBGS8	22, 36	*Hae*II (830)-Km^{r}903-*Sau*3AI (2076)	*Bam*HI-Km^{r}903-*Bam*HI	1.3
Prepro[b]	pUB110	18, 19	1212-Prepro-1118	*Bam*HI-Prepro-*Xho*I	0.1
Km^{r}Δpro[c]	pUB110	18, 19	*Ase*I (3044)-Km^{r}Δpro-*Ase*I (2052)	*Sal*I-Km^{r}Δpro-*Bam*HI	1.0
Km^{r}110[d]	pUB110	This study	*Bam*HI-Prepro-*Xho*/*Sal*-Km^{r}Δpro-*Bam*HI	*Bam*HI-Km^{r}110-*Bam*HI	1.1
Spec^r	pDL269	15a	*Pst*I-Spec^r-*Nde*I	*Bam*HI-Spec^r-*Bam*HI	1.2
p15Aori	pACYC177	28	*Bsp*HI (713)-p15Aori-*Sac*II (1338)	*Bgl*II-p15Aori-*Pvu*II-*Bgl*II	0.65
				*Bgl*II-p15Aori-*Not*I-*Bgl*II	0.65
pUCori	pUC18	44	*Dra*I (1563)-pUCori-*Nde*I (183)	*Xho*I-pUCori-*Xho*I	1.4
pVA380-1 replicon	pVA838	15, 17	*Ava*I-pVA380-1 replicon-*Hin*dIII	*Bgl*II-pVA380-1 replicon-*Hin*dIII	3.1
MCS		This study	*Not-Bgl-Eco-Sst-Kpn-Sma-Bam-Xba-Sal-Pst-Sph-Hin*d-*Bcl-Not*		
gtfB[e]	pTS20	35	*Bgl*II (236)-*gtfB-Sal*I	*Sal*I-*gtfB-Sal*I	4.5
ftf[f]	pTS102	33	*Eco*RV (619)-*ftf-Bgl*II (3187)	*Bam*HI-*ftf-Bgl*II	2.6
Gn amylase	pYI10	11	*Pst*I-Gn amylase-*Hin*dIII	*Bam*HI-Gn amylase-*Bam*HI	3.0

[a] Numbers in parentheses denote positions of restriction endpoints for which sequence data are available.

[b] The gram-positive plasmid pUB110 contains the open reading frame encoding the putative protein homologous to the protein (Pre) involved in plasmid recombination (27). The promoter region of this gene (*pre*) was isolated by independently digesting each end with a combination of exonuclease III and mung bean nuclease following appropriate restriction digestions, and the 5′ and 3′ ends (positions 1212 and 1118) were then ligated with *Bam*HI and *Xho*I linker DNAs, respectively.

[c] The promoterless Km^r gene (Km^{r}Δpro) was isolated by digesting plasmid pUB110 with *Ase*I, and 5′ and 3′ termini were ligated with *Sal*I and *Bam*HI linker DNAs, respectively.

[d] The promoter region of the putative *pre* gene was fused with the promoterless Km^r gene to prepare the active Km^{r}110 gene.

[e] Used as a probe DNA for Southern blot analysis (Fig. 4).

[f] Used as a probe DNA for Southern blot analysis (Fig. 5).

TABLE 2. Cloning vectors used in this study

DNA fragment	Intermediate		Plasmid	Size (kb)
Bam-Amp^r-*Bam* *Bgl*-p15Aori-*Pvu-Bgl*	pResAmpPvu[a]	*Pvu*II *Hin*dIII linker	pResAmpHind	1.6
Bam-Em^r-*Bam* *Bgl*-p15Aori-*Not-Bgl*	pResEmNot[b]	*Not*I MCS	pResEmMCS10 pResEmMCS11	1.6 1.6
Bam-Km^{r}903-*Bam* *Xho*-pUCori-*Xho*	KmOZ′ 18[c]	*Pvu*II 320-bp *Pvu*II fragment	KmOZ′ 19	2.7

[a] The Amp^r gene (1.1-kb *Bam*HI fragment) was fused with 0.65-kb p15Aori (*Bgl*II fragment containing a *Pvu*II site), and pResAmpPvu was constructed. *Hin*dIII linker DNA was introduced into this plasmid to construct pResAmpHind, which was used for cloning of the *ftf* gene (Fig. 5).

[b] Plasmid pResEmNot was initially constructed by ligating the Em^r gene (1.1-kb *Bam*HI fragment) with 0.65-kb p15Aori (*Bgl*II fragment having a *Not*I site), and the MCS was then added in both orientations, resulting in pResEmMCS10 and -11. The latter was used for construction of the *E. coli*-streptococcus shuttle vector, pResEm749 (Fig. 2), following introduction of the pVA380-1 replicon (3.1-kb *Bgl*II-*Hin*dIII fragment).

[c] Each of the DNA fragments was ligated following filling in with the Klenow fragment of DNA polymerase I, and the pUC-type Km^r plasmid, KmOZ′ 18, was constructed. The 320-bp *Pvu*II fragment containing the pUC18-type MCS was replaced with the *Pvu*II fragment from pUC19 to construct KmOZ′ 19. These vectors contain no *Xmn*I recognition sequences and were used for preparation of the Gn amylase structural gene (Fig. 1B).

standing for 20 to 30 min on ice, cell suspensions were treated with 20 μl of 10% SDS and boiled for 2 min. Cellular debris was removed by centrifugation, and the supernatants were extracted with 100 μl of water-saturated phenol and subsequently 100 μl of chloroform. After ethanol precipitation by addition of 20 μl of 3 M sodium acetate and 500 μl of ethanol, RNA was dissolved with 100 μl of TE and 5 μl of each sample was subjected to Northern (RNA) blot analysis (30). Since these RNA fractions contained significant amounts of plasmid and chromosomal DNAs, aliquots were treated with RNase A and proteinase K, and DNA fractions (plasmids and contaminating chromosome) were dissolved with 100 μl of TE following polyethylene glycol precipitation. Aliquots (5 μl) of these samples were used as negative controls for Northern blot analysis.

RESULTS

Preparation of the secretory domain from the *gtfB* gene and the amylase passenger DNA for construction of a model secretion system. The *gtfB* gene of *S. mutans* GS-5 resides on a 6.4-kb *Pst*I chromosomal DNA fragment which has been cloned and sequenced (35); its restriction map is shown at the top of Fig. 1A. Preparation of the promoter and signal sequence coding regions which specify the putative 38-amino-acid residue signal peptide was carried out essentially as previously described (31). Since the 6.4-kb *Pst*I fragment was originally cloned into the multiple cloning site (MCS) of a pUC vector, a *Hin*dIII site was present upstream from the 5′ end of the *Pst*I fragment in pTS20 (35). Therefore, it was possible to isolate the 5′ end of the *gtfB* gene and its promoter region by isolating the 1.0-kb *Hin*dIII fragment from the plasmid, which was next cloned into the *Hin*dIII site of *E. coli* phage M13mp18. Single-stranded DNA, which contains the antisense strand of the cloned fragment, was purified, and the synthetic oligonucleotide 3′-AAAGCC CACCGAACCAATTTC-5′ was then annealed. This complex was partially filled in with the Klenow fragment of DNA polymerase I in the presence of deoxynucleoside triphosphates (dNTPs). A commercially available *Hin*dIII linker (8-mer; 5′-CAAGCTTG-3′) was ligated following the removal of the single-stranded moiety with mung bean nuclease. The 800-bp functional *Hin*dIII fragment, whose 3′-terminal structure is shown at the bottom of Fig. 1A, was gel purified and cloned into the *Hin*dIII site of pUC19.

The termini of the Gn amylase gene from alkalophilic *Bacillus* sp. strain 707, initially isolated on a 3-kb *Pst*I-*Hin*dIII fragment, were converted to *Bam*HI sites following linker ligation and subcloned into a pUC-type Km^r plasmid, KmOZ′ 19 (2.7 kb) (Fig. 1B). Since the vector used has no *Xmn*I recognition sequence, the *Xmn*I site present close to the region corresponding to the signal peptide processing site (Fig. 1B, downward arrowhead) is unique. This chimeric plasmid was digested with *Xmn*I, a *Hin*dIII linker was ligated, and the plasmid was redigested with *Hin*dIII. Since one *Hin*dIII site existed at the 5′ end of MCS in this vector, this strategy eliminated the 700-bp DNA fragment that contains the promoter and signal sequence coding regions of the amylase gene. Several amylase-positive clones were identified on LB-kanamycin plates containing 1% soluble starch in the presence of mostly amylase-negative colonies following recircularization and transformation of *E. coli* JM109. The restriction enzyme *Xmn*I recognizes an interrupted palindromic sequence, -GAANNNNTTC-, which contains four undefined bases. Therefore, it was likely that *Xmn*I cleavage might not be as stringent as cleavage with other restriction enzymes recognizing completely defined sequences. As a result, *Xmn*I digestion yields blunt ends, -GAANN NNTTC- (Fig. 1B, upward large arrowhead), while only a small fraction of molecules would be cut asymmetrically (for example, -GAANNN NTTC-; upward small arrowhead). Insertion of the 8-mer *Hin*dIII linker DNAs into only the latter cleavage site would allow functional expression of the Gn amylase structural gene. Sequencing of the DNA fragments from the amylase-positive clones confirmed the in-frame fusion of the *lacZ′* and the Gn amylase structural genes at the asymmetric site (Fig. 1B, bottom). The Gn amylase structural gene (2.3-kb *Hin*dIII-*Bam*HI fragment) was thus recovered following gel purification of *Hin*dIII and *Bam*HI digests of the plasmid.

Since both the 3′ and 5′ ends of the relevant fragments containing each gene are present in the same reading frame, it was possible to express the Gn amylase structural gene under the control of the *gtfB* secretory domain. The fusion of the two genes was accomplished by initially cloning the Gn amylase structural gene into *Hin*dIII-*Bam*HI-cleaved pBR322. The *gtfB* secretory domain (800-bp *Hin*dIII fragment) was then introduced into the single *Hin*dIII site of the resultant plasmid in both orientations. The correct plasmid

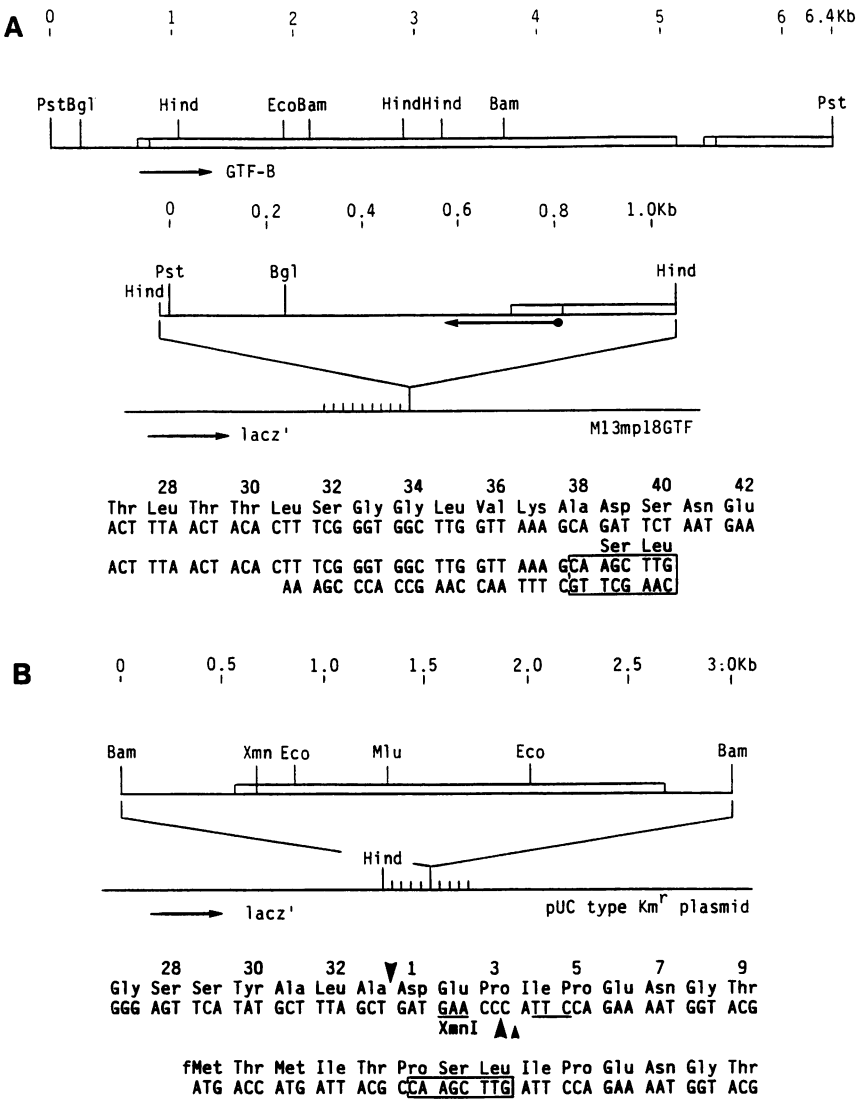

FIG. 1. Preparation of the secretory domain of the *S. mutans* GS-5 *gtfB* gene (A) and the passenger DNA of the Gn amylase gene from alkalophilic *Bacillus* sp. strain 707 (B). (A) Top, restriction map of the chromosomal 6.4-kb *Pst*I fragment of *S. mutans* GS-5 containing the *gtfB* gene. Middle, single-stranded DNA of M13mp18GTF containing the antisense strand of the 5' end of the 1-kb *gtfB* gene *Pst*I-*Hin*dIII fragment. This single-stranded DNA was annealed with the 21-mer oligonucleotide (shown as the dot) and partially filled in with the Klenow fragment of DNA polymerase I and dNTPs. Bottom, nucleotide and deduced amino acid sequences of the *gtfB* gene and the 3'-terminal structure of the secretory domain. (B) Top, restriction map of the Gn amylase gene cloned into the *Bam*HI site of the pUC-type Km^r plasmid. Bottom, nucleotide and deduced amino acid sequences of the Gn amylase gene. The downward arrowhead shows the signal peptide processing site; the large and small upward arrowheads represent the major and minor digestion sites, respectively, of the restriction enzyme *Xmn*I. Boxed sequences in both panels represent the *Hin*dIII linker DNA.

was purified from amylase-positive *E. coli* transformants identified on LB-ampicillin plates containing 1% soluble starch. The hybrid gene (2.9-kb *Bgl*II-*Bam*HI fragment) was subsequently gel purified following *Bgl*II and *Bam*HI digestion of the resultant plasmid.

Transformation of oral streptococci with the Gn amylase genes. Since the ultimate goal of this strategy was to construct secretion vectors active in noncariogenic oral streptococci, we initially attempted to secrete the Gn amylase in *S. gordonii*. *S. gordonii* Challis can be readily transformed either with linear DNA fragments which share homology with the host chromosome or with gram-positive plasmids such as pVA380-1 derivatives (15, 17). To ensure high-level expression of the amylase gene in oral streptococci, it was necessary to initially construct shuttle vectors capable of replication in both *E. coli* and streptococci. We constructed four shuttle vectors by combining the DNA fragments containing gram-positive replication sequences from plasmids pUB110 (18, 19), pC194 (10), pLS5 (14), and pVA838 (17) with the 1.6-kb plasmid pResEmMCS11 (see Materials and Methods). Transformation of both *E. coli* and oral streptococci with each shuttle vector indicated that (i) streptococcal transformants harboring the pUB110 replicon grew very poorly on selective agar plates, (ii) the hybrid Gn amylase gene could not be cloned into the pC194-based shuttle plasmid, and (iii) although the pLS5 derivative, and all other

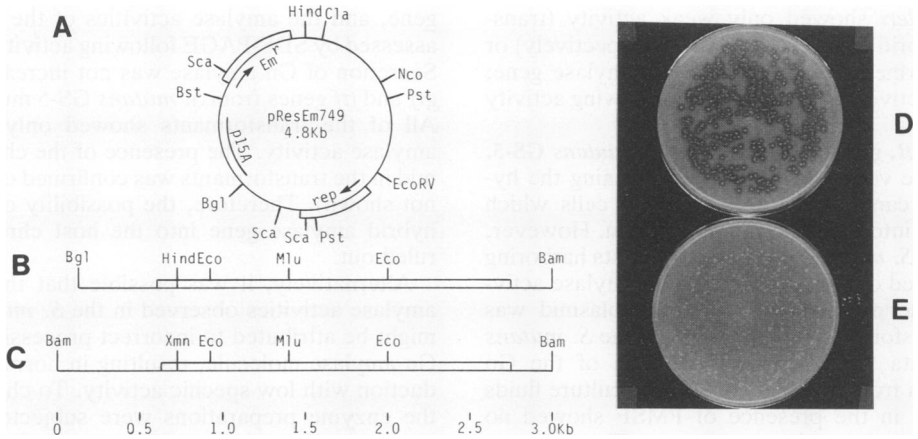

FIG. 2. (A to C) Restriction maps of the *E. coli*-streptococcus shuttle vector pResEm749 (A), the hybrid Gn amylase (B), and the intact Gn amylse genes (C). (D and E) Tryptic soy broth-erythromycin agar plates containing 1% soluble starch following transformation of *S. gordonii* with the hybrid Gn amylase gene (D) and the intact Gn amylase gene (E).

shuttle vectors constructed, contained p15Aori, this plasmid was able to transform only *E. coli rec*$^+$ strains (such as JM83), suggesting that the Rec function is essential (data not shown). On the other hand, plasmid pResEm749 (4.8 kb; Fig. 2A) exhibited high transformation efficiency and was stably maintained in streptococcal cells; this plasmid was used for further study.

S. gordonii competent cells were transformed with the chimeric shuttle vector containing either the hybrid amylase gene or the intact Gn amylase gene inserted into the unique *Bgl*II site (Fig. 2B and C, respectively), and amylase-positive transformants were detected on tryptic soy broth-erythromycin plates containing 1% soluble starch (Fig. 2D and E). *S. gordonii* transformants harboring the hybrid amylase gene exhibited large clear halos around the colonies (Fig. 2D), while those with the intact Gn amylase gene exhibited only weak clearing (Fig. 2E). These results suggested that secretion of the Gn amylase molecule is strongly dependent upon the structure of the signal peptide sequence and/or the promoters present on the two constructs.

It was also of interest to examine the secretion of Gn amylase in two other oral streptococci, *S. mutans* and *S. milleri*. *S. mutans* GS-5 has three *gtf* genes and one *ftf* gene, expressing extracellular proteins which have similar signal peptide structures. On the other hand, *S. milleri* has neither *gtf* nor *ftf* genes. Therefore, it was anticipated that in *S. mutans* GS-5, high-level expression of Gn amylase from the chimeric plasmid containing the hybrid Gn amylase gene would be detected. However, both *S. mutans* GS-5 and *S. milleri* transformants showed the same weak halos of amylase activity observed with *S. gordonii* transformants harboring the intact Gn amylase gene (data not shown).

Secretion of the Gn amylase from oral streptococcal transformants. To confirm the secretion of the Gn amylase molecule from *S. gordonii* transformants and to estimate the molecular weight of this protein, activity staining was carried out following SDS-PAGE (Fig. 3A). As shown in lane 4, culture fluids containing PMSF from *S. gordonii* harboring the hybrid amylase gene exhibited strong amylase activity of approximately 70 kDa. Cultivation of this transformant in the absence of PMSF severely reduced amylase activity (lane 3), indicating that the host cells secrete a serine-type protease(s) that degrades the Gn amylase. Culture fluids from the strain expressing the intact Gn amylase gene showed only trace amounts of amylase activity even in the presence of PMSF (lane 6).

The amylase activity of the culture fluids treated with PMSF from the transformants of *S. mutans* GS-5 as well as *S. milleri* were examined and compared with those from the *S. gordonii* transformants (Fig. 3B). Again, *S. gordonii* transformed with either the hybrid or the intact Gn amylase gene exhibited strong or weak amylase activity, respectively (lanes 2 and 5), while the enzyme preparations from *S.*

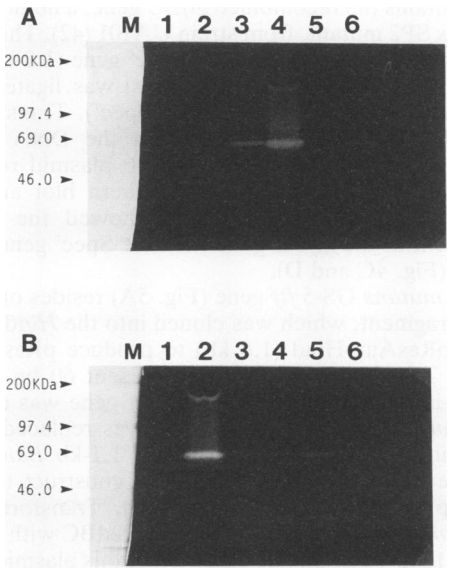

FIG. 3. Activity staining of culture fluids from *S. gordonii* transformants (A) and from oral streptococci (B) following SDS-PAGE. (A) Lanes: 1, tryptic soy broth without bacteria; 2, *S. gordonii* harboring pResEm749; 3 and 4, *S. gordonii* harboring the hybrid Gn amylase gene without and with PMSF, respectively; 5 and 6, *S. gordonii* harboring the intact Gn amylase gene without and with PMSF, respectively. (B) Lanes: 1 to 3, *S. mutans* GS-5, *S. gordonii*, and *S. milleri* harboring the hybrid Gn amylase gene; 4 to 6, *S. mutans* GS-5, *S. gordonii*, and *S. milleri* harboring the intact Gn amylase gene. In panel B, all transformants were cultured in the presence of PMSF.

mutans and *S. milleri* showed only weak activity (transformed with the hybrid gene; lanes 1 and 3, respectively) or no activity (transformed with the intact Gn amylase gene; lanes 4 and 6, respectively) on SDS-PAGE following activity staining.

Deletion of the *gtfB*, *gtfC*, and *ftf* genes in *S. mutans* GS-5. The chimeric shuttle vector pResEm749 containing the hybrid amylase gene can transform *S. gordonii* cells which secrete Gn amylase into the surrounding medium. However, the culture fluids of *S. mutans* GS-5 transformants harboring this plasmid exhibited only trace amounts of amylase activity even though the presence of the intact plasmid was confirmed by retransforming *S. gordonii* with the *S. mutans* plasmid isolate (data not shown). Incubation of the Gn amylase preparation from *S. gordonii* with the culture fluids of *S. mutans* GS-5 in the presence of PMSF showed no decrease of amylase activity (data not shown). Therefore, no prominent protease activity affecting the amylase molecule appears to be present in *S. mutans* GS-5 culture fluids. A plausible explanation for the poor secretion of amylase molecules in *S. mutans* GS-5 transformants harboring the hybrid amylase gene might be that the amylase molecules would have to compete with multiple endogenous extracellular proteins (including GTFs and FTF) for the cell secretory apparatus. To assess this possibility, *S. mutans* GS-5 mutants containing deletions of the *gtfB*, *gtfC*, and *ftf* genes were constructed and used as host cells for expression of the hybrid Gn amylase gene.

The *S. mutans* GS-5 chromosome contains three *gtf* genes, two of which, *gtfB* and *gtfC*, share high homology and are tandemly arranged. In vivo recombination of these two genes produced mutant strain SP2 (Fig. 4A), which exhibits a smooth colony morphology on mitis salivarius agar plates (39). Plasmid pS01 (13.6 kb) harbors a 9.0-kb *Pst*I fragment which contains the recombined *gtfBC* gene, a homolog of the *S. mutans* SP2 mutant, from strain UA101 (42). This plasmid was digested with *Bgl*II, and the Spec^r gene (1.2-kb *Bam*HI fragment; see Materials and Methods) was ligated to construct the 6.5-kb plasmid (pS01dBglSpec^r). Transformation of the *S. mutans* SP2 mutant with the DNA fragment following *Pst*I digestion of this 6.5-kb plasmid resulted in Spec^r transformants (SP2dBC). Southern blot analysis of chromosomal *Pst*I digests clearly showed the complete replacement of the *gtfBC* gene with the Spec^r gene in strain SP2dBC (Fig. 4C and D).

The *S. mutans* GS-5 *ftf* gene (Fig. 5A) resides on a 4.3-kb *Hin*dIII fragment, which was cloned into the *Hin*dIII site of plasmid pResAmpHind (1.6 kb) to produce pResAmpFTF (5.9 kb). The unique *Eco*RV site present 60 bp upstream from the initiator Met codon of the *ftf* gene was converted into a *Bam*HI site, and the *ftf* gene was replaced with the kanamycin resistance gene (Km^{r}110; 1.1-kb *Bam*HI fragment; see Materials and Methods) to construct the 4.4-kb plasmid pResAmpdFTFKm^r (Fig. 5B). Transformation of either *S. mutans* GS-5 or *S. mutans* SP2dBC with the DNA fragment following *Hin*dIII digestion of this plasmid resulted in the isolation of Km^r strains. Southern blot analysis of chromosomal *Hin*dIII digests (Fig. 5C and D) indicated the elimination of the *ftf* gene in the Km^r transformants. In addition, the presence of an additional unexpected 1.7-kb *Hin*dIII fragment hybridizing with the Km^r gene was detected (Fig. 5D, lanes 3 and 4).

Transformation of *S. mutans gtfB*, *gtfC*, and *ftf* deletion derivatives with the hybrid Gn amylase gene. *S. mutans* GS-5 mutants SP2, SP2dBC, and SP2dBCF were transformed with the chimeric plasmid containing the hybrid Gn amylase gene, and the amylase activities of the culture fluids were assessed by SDS-PAGE following activity staining (Fig. 6A). Secretion of Gn amylase was not increased by deleting the *gtf* and *ftf* genes from *S. mutans* GS-5 mutants (lanes 1 to 4). All of the transformants showed only trace amounts of amylase activity. The presence of the chimeric shuttle plasmid in the transformants was confirmed on agarose gels (data not shown). Therefore, the possibility of integration of the hybrid amylase gene into the host chromosome could be ruled out.

Alternatively, it was possible that the low extracellular amylase activities observed in the *S. mutans* transformants might be attributed to incorrect processing of the precursor Gn amylase molecule, resulting in normal Gn amylase production with low specific activity. To check this possibility, the enzyme preparations were subjected to Western blot analysis using anti-G6 amylase serum. Faint immunoreactive bands with mobility similar to that of the amylase bands visualized following activity staining were detected for all four enzyme preparations from the *S. mutans* Em^r transformants. However, this band was also detected in the culture fluid of the nontransformed *S. mutans* GS-5 wild-type strain, indicating that this protein probably represents a nonamylase molecule which cross-reacts with anti-G6 amylase serum. By contrast, a single strong immunopositive band was detected in the enzyme preparation of *S. gordonii* Em^r transformants (lane 6), corresponding to a 98-kDa protein. No comparable band was observed in any of the samples from either the *S. mutans* GS-5 or the *S. gordonii* wild-type strain.

Extracellular GTF-S enzyme production in the *S. mutans* SP2dBCF mutant and *S. gordonii*. Both *S. mutans* SP2dBCF and *S. gordonii* cells secrete only one GTF species, GTF-S, into culture fluids. Therefore, if the observed differences in relative secretion of amylase by *S. mutans* compared with *S. gordonii* resulted from differential competition for the secretory apparatus in the two organisms, the presence of the chimeric plasmid might be expected to affect GTF-S secretion in the two organisms to different degrees. Therefore, the extracellular GTF-S enzyme activities of these strains as well as those of Em^r transformants were determined in an assay in which each of the concentrated enzyme samples (5 μl) was used for the measurement of total glucan synthesis activity in culture fluids in the presence of 1 mg of dextran T10 per ml. GTF activities (counts per minute) were as follows: *S. mutans* SP2dBCF, 71,000; *S. mutans* SP2dBCF Amy$^+$, 84,000; *S. gordonii*, 78,000; and *S. gordonii* Amy$^+$, 61,000. Expression of the amylase gene from the chimeric plasmid did not reduce secretion of GTF-S from either *S. mutans* or *S. gordonii*. Therefore, it is unlikely that the poor secretion of Gn amylase by *S. mutans* relative to *S. gordonii* results from the inability of the former organism to carry out normal extracellular protein secretion in the presence of the hybrid Gn amylase gene resident on the chimeric plasmid.

Northern blot analysis. To further characterize the inability of the *S. mutans* derivative SP2dBCF to efficiently secrete the amylase molecule, Northern blot analysis was performed. For the extraction of RNA molecules from *S. mutans* and its derivatives, we observed that boiling cells without any lysozyme treatment in the presence of 1% SDS yielded considerable amounts of RNA following centrifugation. Furthermore, these molecules were more easily isolated from *S. gordonii* and its derivatives by a simple 10-min boiling of cells suspended with only TE buffer. In general, recovery of RNA molecules from *S. gordonii* cells is 1.5 to 2 times higher than recovery from *S. mutans* cells (data not

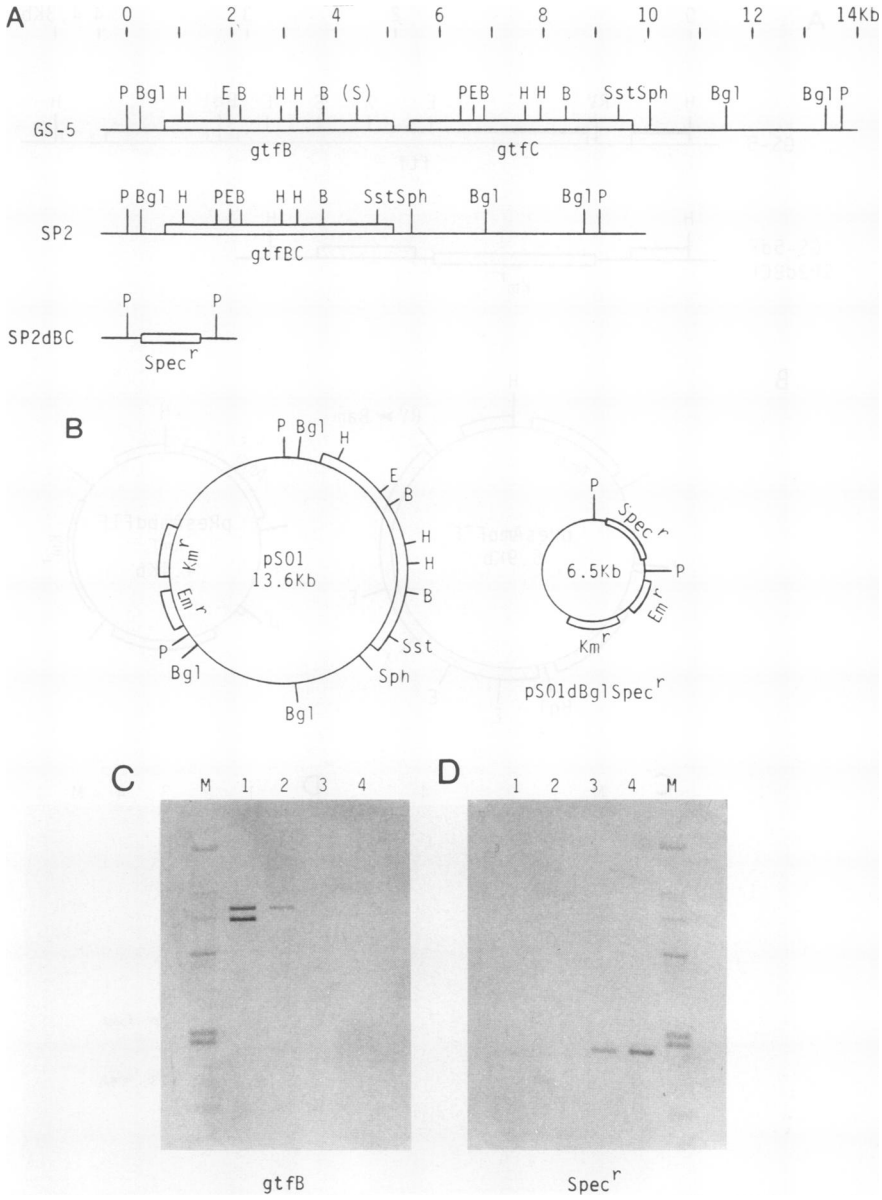

FIG. 4. Deletion of the *gtfBC* gene in *S. mutans* SP2. (A) Chromosomal restriction maps of *S. mutans* GS-5 and mutant SP2 encompassing the *gtfB* and *gtfC* genes. (B) Structures of plasmids pS01 (42) and pS01dBglSpec^r. (C and D) Southern blot analysis of the chromosomal *Pst*I digests of *S. mutans* GS-5 (lanes 1), *S. mutans* SP2 (lanes 2), and *S. mutans* Spec^r transformants SP2dBC 1 and 2 (lanes 3 and 4, respectively). Lanes M, molecular size markers (λ DNA *Hin*dIII digest). Probes used were the *gtfB* (C; see Table 2) and Spec^r (D) genes.

shown). RNA molecules were extracted from streptococci as described in Materials and Methods and subjected to Northern blot analysis using the biotinylated intact amylase gene as the probe DNA (Fig. 7B). No amylase mRNA was detected in the total RNA from the two host cells, *S. mutans* SP2dBCF and *S. gordonii* (lanes 1 and 3). However, mRNA molecules migrating slightly faster than 23S RNA could be visualized from each of Em^r transformants containing the chimeric plasmids (lanes 2 and 4). Since the latter bands were absent in the RNase A-treated blots (lanes 6 and 8), these two molecules should correspond to the amylase mRNA. The relative levels of amylase mRNA appeared to be similar in *S. mutans* and *S. gordonii*. Therefore, the poor secretion of amylase in *S. mutans* is not the result of inefficient transcription of the hybrid gene in this organism.

DISCUSSION

The genetic engineering approach is a powerful tool for the construction of recombinant organisms which produce physiologically important polypeptides or industrially useful proteins. These latter molecules have been produced from recombinant *E. coli* or *B. subtilis* strains. The former organism accumulates the gene products inside the cells, and it was necessary to solubilize these products before purification (41). By contrast, the gram-positive bacterium *B. subtilis* has the ability to directly secrete gene products into culture fluids, making this organism an ideal host cell for the extracellular production of proteins (34). However, these host cells are precluded from our strategy for controlling human dental caries by secreting anticariogenic enzymes or

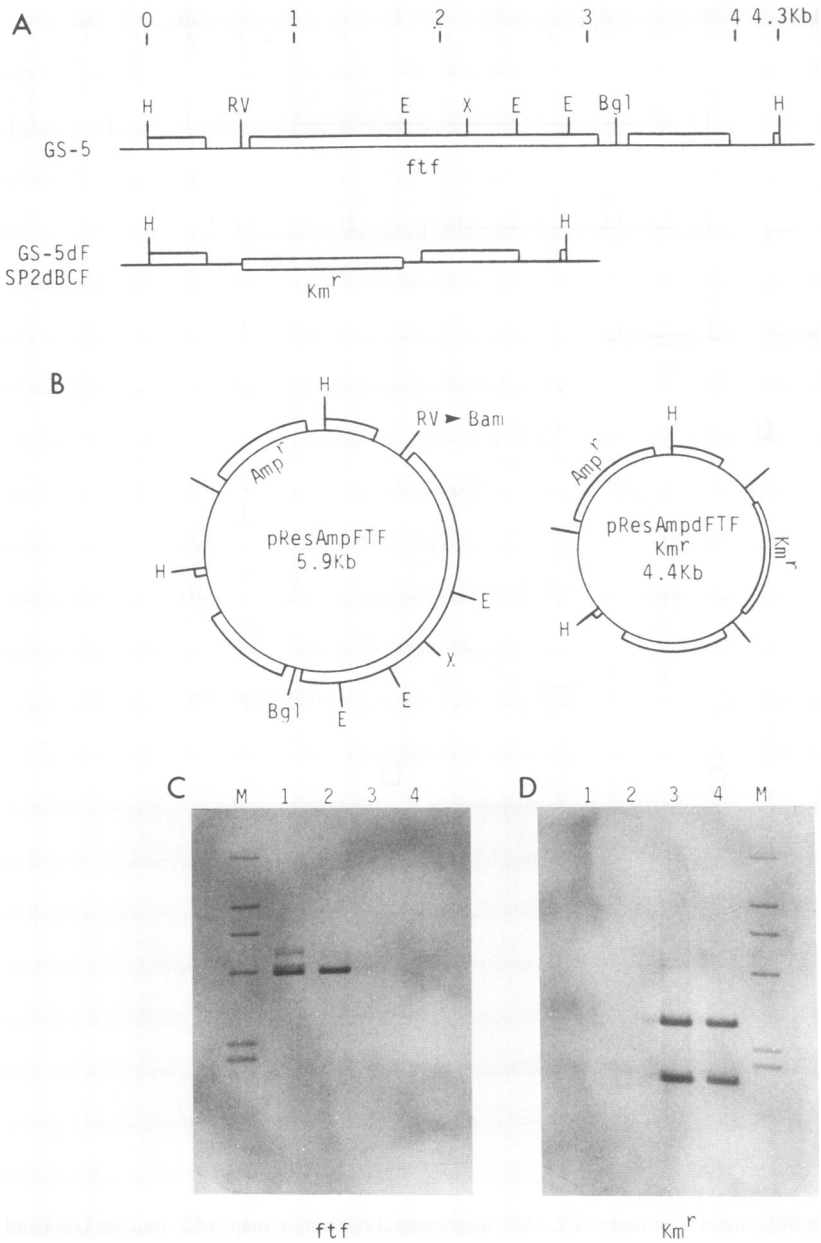

FIG. 5. Deletion of the *ftf* gene in *S. mutans* GS-5. (A) Chromosomal structure of the *ftf* gene in *S. mutans* GS-5. (B) Restriction maps of pResAmpFTF and pResAmpdFTFKm^r. (C and D) Southern blot analysis of the chromosomal *Hin*dIII digests of *S. mutans* GS-5 (lanes 1), *S. mutans* SP2 (lanes 2), Km^r transformants of *S. mutans* GS-5 dF (lanes 3), and *S. mutans* SP2dBCF (lanes 4). Probes used were the *ftf* (C; see Table 2) and Km^{r}110 (D) genes.

proteins from recombinant clones, since these organisms do not normally colonize the oral cavity. This prompted us to construct a secretion system in noncariogenic oral streptococci such as *S. gordonii*.

For the construction of a model system, it was initially necessary to prepare a secretory domain functional in *S. gordonii*. Since strain Challis is recombination proficient and high-level production of gene products from a multicopy plasmid rather than from single-copy integrants was desirable, the *S. mutans* GS-5 *gtfB* gene was used as the source of the functional secretory domain carried on a plasmid. Likewise, since recent results have suggested that the length of the signal peptide of the GTF-I enzyme is 38 amino acids (3, 23), we designed a 21-mer oligonucleotide for subsequent gene fusion so that the secretory domain encodes these 38 residues. This domain prepared from single-stranded DNA and the oligonucleotide complex were fused with the Gn amylase structural gene from alkalophilic *Bacillus* sp. strain 707. This hybrid gene as well as the intact Gn amylase gene were successfully cloned into the *E. coli*-streptococcus shuttle plasmid pResEm749, a derivative of pVA380-1. It appeared that the culture fluids of Em^r transformants harboring the hybrid amylase gene showed strong amylase activity in the presence of PMSF, while those containing the intact Gn amylase gene exhibited only weak amylase activity following SDS-PAGE and activity staining. Western blot

Sample	Strain	Number of GTFs	FTF	Amylase Act.
0	S.mutans GS-5 wild	3 (B,C,D)	1	-
1	S.mutans GS-5 Amy$^+$	3 (B,C,D)	1	±
2	S.mutans SP2 Amy$^+$	2 (BC,D)	1	±
3	S.mutans SP2 dBC Amy$^+$	1 (D)	1	±
4	S.mutans SP2 dBCF Amy$^+$	1 (D)	0	±
5	S.gordonii wild	1 (G)	0	-
6	S.gordonii Amy$^+$	1 (G)	0	+++

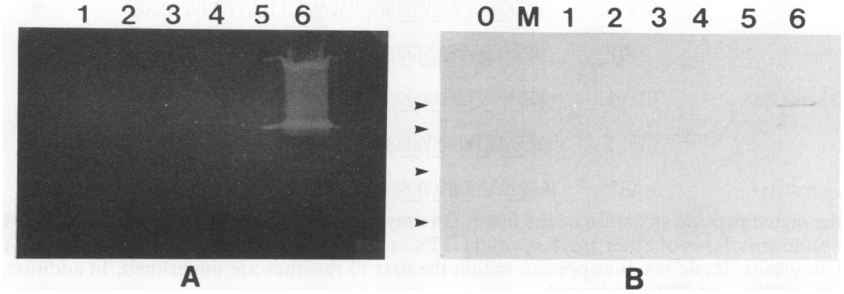

FIG. 6. Activity staining (A) and Western blot analysis (B) of culture fluids of the *S. mutans* GS-5 deletion derivatives harboring the hybrid amylase gene. Arrowheads represent molecular size markers (lane m; from top to bottom, 97.4, 69.0, 46.0, and 30.0 kDa). The amylase activity indicated is scored on the basis of the activity staining shown in panel A.

analysis of these two culture fluids with anti-G6 amylase serum confirmed the presence of immunoreactive protein bands only in the former enzyme samples (data not shown). These results rule out the possibility of normal secretion of the extracellular Gn molecules with low specific activity resulting from incorrect processing of the *Bacillus* signal peptide structure in the Em^r transformants. The signal peptide structures of the two genes are quite distinct from each other (Fig. 8). The Gn amylase enzyme contains only 2 basic amino acid residues, while the GTF-I protein contains 9 basic residues within the first 15 amino acids. In addition, several strictly conserved residues, such as Glu-2, Trp-16, and Val-17, could be detected among the GTF and FTF signal peptides. Therefore, it may be possible that the weak amylase activity observed in the culture fluids of Em^r transformants harboring the intact Gn amylase gene results from the inability of *S. gordonii* to process the *Bacillus* signal peptide structure correctly, leading to poor secretion of the Gn amylase molecules. Our Northern blot analysis of the two *S. gordonii* Em^r transformants revealed that the transcriptional level of the intact Gn amylase gene was considerably decreased relative to that of the hybrid Gn amylase gene (data not shown). However, significant levels of mRNA were still detected in the former cells, which suggests that secretion of the intact α-amylase may also be defective.

Since *S. mutans* GS-5 actively secretes GTF and FTF enzymes, it was anticipated that considerable Gn amylase secretion would be detected following transformation of this organism with the plasmid containing the hybrid Gn amylase gene. However, only weak amylase activity could be detected in culture fluids from the transformants of *S. mutans* GS-5 as well as the deletion mutants lacking the *gtfB*, *gtfC*, or *ftf* gene. These results were apparently not due to the presence of proteases that degrade Gn amylase in the *S. mutans* GS-5 culture fluids, the integration of the chimeric plasmid into host chromosomes, or competition for the secretion apparatus by endogenous proteins that have similar signal peptide structures.

Northern blot analysis of the *S. mutans* SP2dBCF harboring the hybrid Gn amylase gene clearly exhibited the presence of the transcript of the amylase gene (Fig. 7B, lane 2), although its level is lower than that of *S. gordonii* Em^r transformants (lane 4). We have found that the extraction of RNA molecules from *S. gordonii* cells is more efficient than that from *S. mutans* cells. Therefore, it is probable that the level of transcription of the hybrid Gn amylase gene in *S. mutans* SP2dBCF is almost the same as that in *S. gordonii* cells. These observations rule out the possibility of low-level transcription of the hybrid Gn amylase gene attributable to poor secretion of the amylase molecules in the former strain.

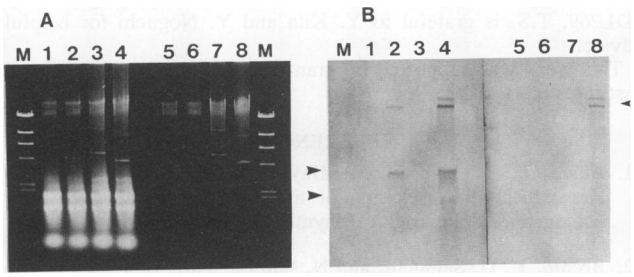

FIG. 7. Agarose gel electrophoresis (A) and Northern blot analysis (B) of the extracted RNA molecules. Lanes: 1 and 5, *S. mutans* SP2dBCF; 2 and 6, *S. mutans* SP2dBCF harboring the hybrid Gn amylase gene; 3 and 7, *S. gordonii*; 4 and 8, *S. gordonii* harboring the hybrid Gn amylase gene. Samples 1 to 4 and 5 to 8 were prepared before and after RNase A treatment, respectively. Large arrowheads mark positions of 23S and 16S RNAs; the small arrowhead represents the plasmids in panel B.

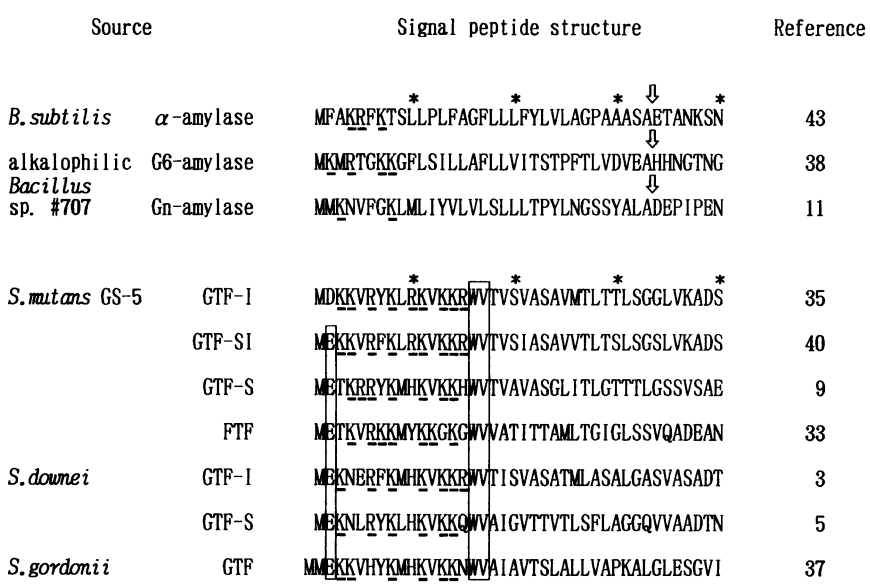

FIG. 8. Comparison of the signal peptide structure of the intact Gn amylase enzyme with that of the *S. mutans* GS-5 GTF-I protein (*gtfB* gene product). The signal peptide structures of other amylases and GTFs as well as FTF are also compared. Arrows represent processing sites of the signal peptides in the amylases. Basic residues present within the first 15 residues are underlined. In addition, highly conserved amino acid residues found among the GTFs and FTF are boxed.

Several genes involved in protein secretion, including the *secA*, *secB*, *secE*, and *secY* genes, have been isolated from *E. coli* (8), and homologs of these genes have been identified in other organisms, including *S. mutans* (20, 29, 32). Among these, the *secB* gene product, or chaperone, functions as an antifolding factor to maintain precursor molecules in a secretionally active form. The precursor molecules of both the GTF-S and the hybrid Gn amylase enzymes should have very similar signal peptide structures. However, the *S. mutans* SP2dBCF mutant could effectively secrete only the former protein. Therefore, the observed differences in the efficiency of amylase secretion in the oral streptococci examined in this investigation could result from differential activity of any of these *sec* gene products. Furthermore, it may be possible to identify the active *S. gordonii sec* gene component by screening for amylase-positive *S. mutans* GS-5 integrants harboring the hybrid Gn amylase gene following transformation with an *S. gordonii* clone bank.

Recently, Fukushima et al. have reported the extracellular production of *S. mutans* GS-5 *gtfB* and *gtfC* gene products in *S. milleri* (4), suggesting that this organism correctly processes extracellular proteins with the *gtfB* secretory domain. Therefore, the inability to secrete the Gn amylase protein in *S. milleri* Em^r transformants harboring the hybrid Gn amylase gene may also be due to the same secretory deficiency as exhibited by *S. mutans* GS-5.

The molecular weight of the Gn amylase molecules on SDS-PAGE following either activity staining or Western blotting resulted in estimates of 70 and 98 kDa, respectively. The former gels contain 1% soluble starch. We found that when starch-containing gels were used for Western blot analysis, only small amounts of the Gn amylase molecules could be electrotransferred onto nitrocellulose membranes, while the protein markers were completely transferred following SDS-PAGE. Furthermore, activity staining of the gel after electrotransfer revealed strong residual amylase activity indistinguishable from that of the enzyme samples before transfer (data not shown). Therefore, the observed discrepancy in molecular weights might result from the effects of soluble starch in the gel which could interact with the Gn amylase molecules and affect enzyme migration during electrophoresis.

This report describes the initial construction of a model secretion system using noncariogenic oral streptococci. However, it is likely that not all heterologous proteins are secreted by each organism. Nevertheless, the demonstration that one of the major colonizers of human teeth, *S. gordonii* (6), is able to process and secrete significant amounts of α-amylase suggests that this approach may be feasible. Subsequent experiments will need to address the important questions of plasmid stability and protection of secreted proteins against dental plaque proteases prior to testing of the genetically engineered organisms in animal models.

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