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Immunologic Characteristics of a *Streptococcus mutans* Glucosyltransferase B Sucrose-Binding Site Peptide–Cholera Toxin B-Subunit Chimeric Protein

PATRICK LALOI,[1] CINDY L. MUNRO,[2] KEVIN R. JONES,[3] AND FRANCIS L. MACRINA[3]*

Centre de Génetique Moléculaire et Cellulaire, UMR CNRS 106, Université Claude Bernard, 69622 Villeurbanne Cedex, France[1]; Department of Adult Health Nursing, School of Nursing, Virginia Commonwealth University, Richmond, Virginia 23298-0567[2]; and Department of Microbiology and Immunology, Virginia Commonwealth University, Richmond, Virginia 23298-0678[3]

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Glucosyltransferases (Gtfs) produced by the mutans streptococci are recognized as virulence factors in dental caries, and the inhibition of Gtfs by secretory immunoglobulin A is predicted to provide protection against this disease. The basis of such mucosal immunity is linked to the ability to reliably stimulate production of secretory immunoglobulin A against Gtfs. In this regard, we are exploring the immungenicities of various Gtf peptides genetically fused to the B subunit of cholera toxin (CTB), a known mucosal adjuvant. In this work, we have created a gene fusion linking the GtfB active-site (AS) peptide DANFDSIRVDAVDNVDADLLQIA to the amino terminus of CTB. This sequence, deduced from the nucleotide sequence of *gtfB* from *Streptococcus mutans* GS5, has been found to be strongly conserved in Gtfs from several mutans streptococci. We have purified this recombinant protein (AS:CTB) from *Escherichia coli* carrying the fusion gene under the control of the lactose operon promoter. This protein was immunogenic in rabbits and produced specific serum antibodies against both the Gtf peptide and the CTB moiety. The antiserum was tested for its ability to inhibit GtfB activity obtained from a mutant of *S. mutans* able to make only this enzyme and none of the other usual Gtfs or fructosyltransferase. Approximately 50% of the GtfB activity was inhibited in such assays. These results suggest that the AS of this enzyme is accessible to antibody binding and that this region of the protein may be considered a vulnerable target for vaccine design and development. The AS:CTB was able to bind GM_1 ganglioside in enzyme-linked immunosorbent assays, indicating that the recombinant protein retained this property, which is thought to be critical to the mucosal immunoadjuvant properties of CTB. Thus, this protein may be promising as a candidate anticaries vaccinogen alone or in combination with other Gtf peptides or conjugates.

Glucosyltransferases (Gtfs) that synthesize glucan polymers from sucrose are recognized as virulence factors in smooth-surface dental decay caused by *Streptococcus mutans*. This was initially postulated because carious lesions were observed in experimental animals only when sucrose was included in their diets (25). The definitive role of Gtfs as virulence factors in cariogenicity was demonstrated with Gtf-deficient mutants that were created by allelic exchange (31, 48). Such mutants were reduced in cariogenicity in rat models compared with their wild-type progenitors. Typical strains of *S. mutans* appear to carry a repertoire of three Gtf genes, all of which contribute to maximal cariogenicity in rats (5, 7, 32, 49). In *S. mutans* GS5, the product of the *gtfB* gene synthesizes primarily a water-insoluble glucan polymer rich in α-1,3-linked glucose molecules. The *gtfD* gene product, on the other hand, forms a water-soluble glucan composed of α-1,6-linked glucose molecules. The third gene, *gtfC*, encodes an enzyme that is able to synthesize both water-insoluble and water-soluble glucan polymers. In many wild-type strains, *gtfB* and *gtfC* are tandemly arranged and tightly linked (36, 45). The amino acid sequences of Gtf enzymes from *S. mutans* and a variety of other oral streptococci show striking similarity to one another, suggesting that the various genes have shared a common ancestor. For example, the GtfB and GtfD enzymes have an overall sequence identity of 52% (19). Central regions of GtfB, -C, and -D, constituting approximately one-third of these proteins, display 84 to 95% similarity. Molecular genetic and physical biochemical studies have revealed that the Gtfs have at least two domains: a catalytic region involved in sucrose hydrolysis and a glucan binding domain involved in synthesis of the nascent polymer (13, 14, 22, 23, 33). The amino acid sequences of the catalytic domain are conserved among the Gtfs of the oral streptococci. The glucan binding domain is similar among these enzymes, consisting of a few to several copies of a repeated amino acid motif (13, 36, 37, 45). Mooser et al. (30) originally identified a Gtf active-site (AS) peptide sequence and localized the glucosyl residue to an aspartate residue occurring at position 6 in a nine-residue sequence. Recently, Shimamura et al. (35) were able to alter the catalytic properties of the *gtfB* gene product by creating site-directed mutations in the AS peptide sequence. These investigators found that AS amino acid replacements could change the GtfB enzyme to one that synthesized primarily water-soluble, instead of water-insoluble, glucan.

The role of Gtfs in cariogenicity makes them rational targets

* Corresponding author. Mailing address: Box 980678 MCV, Virginia Commonwealth University, Richmond, VA 23298-0678. Phone: (804) 828-9728. Fax: (804) 828-9946. Electronic mail address: Macrina @GEMS.VCU.EDU.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description[a]	Reference
Strains		
S. mutans		
GS5	Bratthall serotype c	17
V2158	V403 background carrying mutations in *gtfC*, *gtfD*, and *ftf* (all genes inactivated by insertion of antibiotic resistance cassettes)	32
E. coli		
HB101	Δ(*gpt-proA*)*62 leuB6 thi-1 lacY1 hsdS*$_B$*20 recA rpaL20 ara-14 galK2 xyl-5 mtl-1 supE44 mcrB*$_B$	4
TB1	F$^-$ *ara* Δ(*lac-proAB*) *rpsL* ϕ80d*lacZ*Δ*M15 hsdR17* (r_K^- m_K^+)	
BL21(DE3)	*hsdS20 gal* [λ-T7RNA pol]	41
V1542	Host HB101 carrying pVA1542	9
V2143	Host TB1 carrying pVA2143	This study
V2145	Host TB1 carrying pVA2145	This study
V2147	Host BL21(DE3) carrying pVA2145	This study
V2149	Host HB101 carrying pT7-7	22
V2151	Host HB101 Carrying pTSU5	This study
Plasmids		
pVA 1542	Carries a truncated *ctxB* gene; Ap^r Tc^r	9
pVA 2143	See Fig. 2; Ap^r Tc^r	This study
pVA 2145	See Fig. 2; Ap^r	This study
pT7-7	Expression vector containing a T7 promoter and used for expressing gene with the T7 RNA polymerase; Ap^r	43
pTSU5	pUC119-based vector with the cloned *gtfB* gene from *S. mutans* GS5; Ap^r	22

[a] Ap^r, ampicillin resistance; Tc^r, tetracycline resistance.

for an anticaries vaccine strategy. We have prepared fusion proteins consisting of GtfB peptides and the B subunit of cholera toxin (CTB), and our biochemical and immunologic studies of one such recombinant protein have provided encouraging results (9, 10). This CTB fusion protein carried a 15-amino-acid peptide designated GtfB.1 that was located 94 amino acids from the AS of the enzyme (12, 30, 35). The selection of this sequence was based on its hydrophilic properties and predicted random-coil structure, two good predictors of antigenicity. A synthetic oligonucleotide encoding GtfB.1 was fused to the N-terminal end of the gene encoding CTB, and a fusion protein was produced and purified from *Escherichia coli*. Rabbit antibodies to this protein inhibited glucan production in vitro, reducing soluble-glucan synthesis by 50% and insoluble-glucan synthesis by 90% (10). When the GtfB.1 chimeric protein was administered as an oral vaccine to rats, modest protection against caries was observed (29).

Here we report on the design and construction of a fusion gene which encodes the sucrose-binding peptide (AS peptide) of GtfB linked to the amino terminus of CTB. This protein has been overexpressed, purified, and characterized. Rabbit antisera raised against the AS:CTB protein were able to inhibit insoluble glucan synthesis in vitro. This recombinant protein may be useful as a component of an experimental anticaries vaccine when administered in concert with other native or fusion proteins.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used are listed in Table 1.

Media and reagents. Todd-Hewitt broth was purchased from Difco Laboratories (Detroit, Mich.). [U-^{14}C]sucrose was obtained from Dupont, NEN Research Products (Boston, Mass.). CTB and pooled normal rabbit serum were purchased from Sigma Chemical Co. (St. Louis, Mo.). T10 dextran was supplied by Pharmacia, Inc. (Piscataway, N.J.) Goat anti-CTB was obtained from Calbiochem-Behring (La Jolla, Calif.). Phosphatase-labeled second antibodies and 5-bromo-4-chloro-indolylphosphate–nitroblue tetrazolium (BCIP-NBT) substrate were purchased from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, Md.). Restriction endonucleases were supplied by Bethesda Research Laboratories, Inc. (Gaithersburg, Md.). Enzymatic reactions were performed according to the manufacturer's directions.

Genetic construction. Complementary synthetic oligonucleotides corresponding to the AS of *S. mutans* GS5 *gtfB* were constructed on an Applied Biosystems model 380A synthesizer and run in a 12% polyacrylamide gel containing 8 M urea. Following elution from the gel, the oligonucleotides were purified with a NACS Prepac column (Bethesda Research Laboratories) according to the manufacturer's instructions. The oligonucleotide sequences were 5′ AATTCCGGATGCTAACTTTGATTCCATTCGTGTTGATGCGGTAGATAATGTGGATGCTGACTTGCTCCAAATTGCA 3′ and 5′ TATGCAATTTGGAGCAAGTCAGCATCCACATTATCTACCGCATCAACACGAATGGAATCAAAGTTAGCATCCGG 3′. When these two oligonucleotides were annealed, the underlined bases created single-stranded, cohesive termini compatible with *Eco*RI (AATT) and *Nde*I (TA).

The cloning strategy used to construct a gene fusion of the *gtfB* AS and *ctxB* is shown in Fig. 1. Plasmid pVA1542 contained a truncated version of *ctxB* from the El Tor strain of *Vibrio cholerae* (21). The 5′ end of *ctxB* was deleted to remove the ribosomal binding site and the DNA encoding the leader sequence of the protein. The oligonucleotides were inserted at the 5′ end of *ctxB* after annealing, phosphorylation, and ligation according to the procedures described by Stover et al. (40). The chimeric *as:ctxB* gene was transferred to the expression vector pT7-7 (43). Proper insertion of the chimeric gene was monitored by restriction enzyme digestion of plasmid DNA prepared from minilysates (26). The nucleotide sequence of the construct presenting the expected restriction enzyme digestion pattern was confirmed by sequencing. Sequencing reactions were performed by the Sanger dideoxy chain termination method (Sequenase Kit 2.0; U.S. Biochemicals) according to the manufacturer's directions.

Enzyme preparation. Extracellular proteins of *S. mutans* V2158 were prepared as described by Dertzbaugh and Macrina (10). *E. coli* containing pTSU5 was grown at 37°C overnight in 2× TY medium, centrifuged, washed, and sonically disrupted (29). Partially purified Gtfs were prepared from *S. mutans* GS5 as described by Wong et al. (47) by affinity chromatography. T10 dextran was linked to epoxy-activated Sepharose 6B (Pharmacia), and this dextran-agarose resin was equilibrated in 0.01 M sodium phosphate buffer (pH 7.0). The GS5 extracellular protein extract then was applied to the resin, washed with 2.5 M guanidine-HCl, and eluted with 4 M guanidine-HCl in the same phosphate buffer. The purified preparation then was dialyzed against water and concentrated.

Electrophoretic analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with the buffer system described by Laemmli and Favre (24) and 15% acrylamide slab gels. The gels were run at 13 mA per slab gel until the bromophenol blue tracking dye had moved to the bottom of the gel. Proteins were stained with Coomassie brilliant blue R250 (Sigma) diluted in methanol-acetic acid-water (50:10:40, vol/vol/vol). Excess dye was eliminated by successive washings in methanol-acetic acid-water (5:10:85, vol/vol/vol). Rainbow molecular weight markers from Amersham (Bucks, United Kingdom) were used.

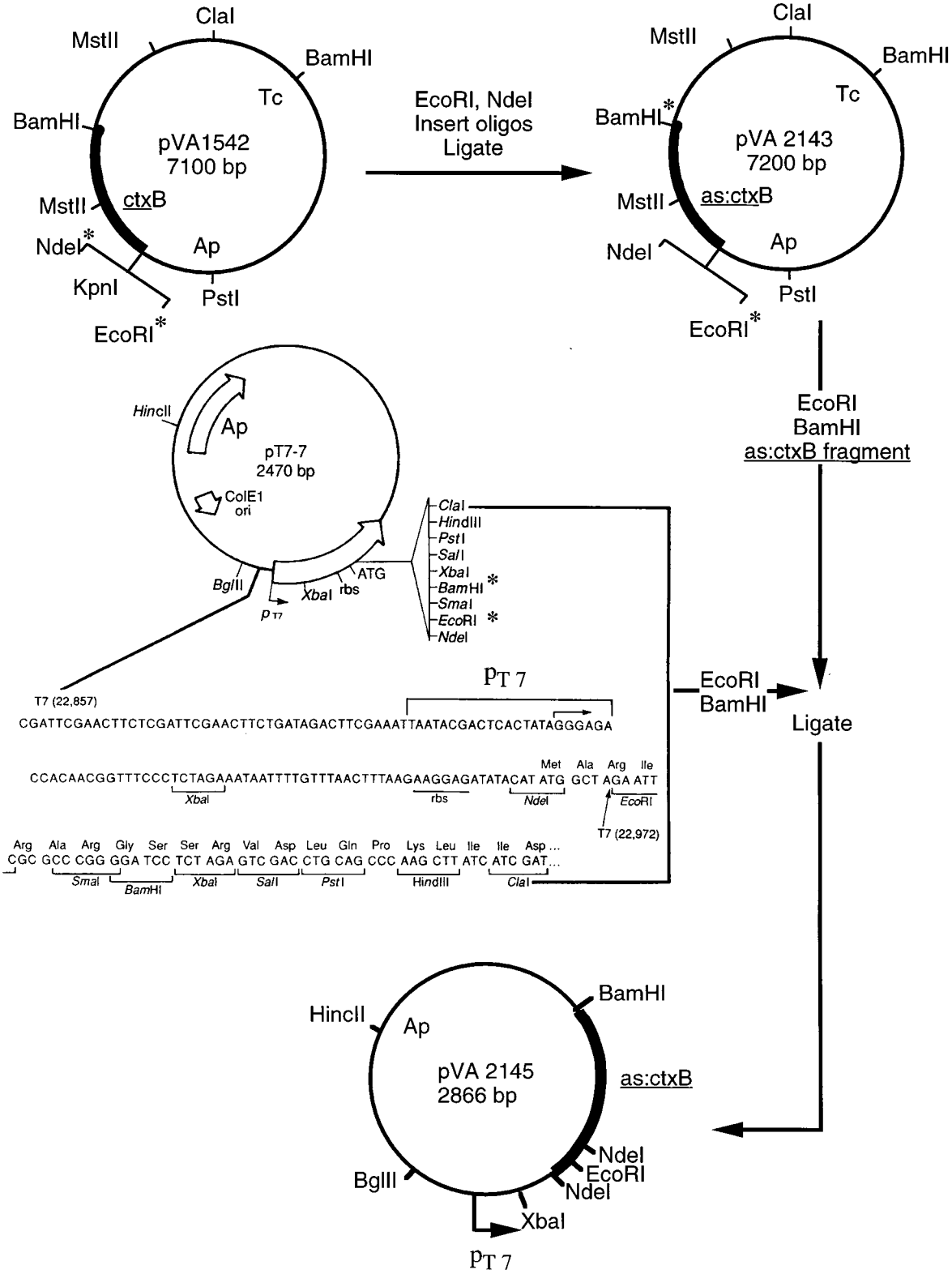

FIG. 1. Genetic fusion of the AS peptide to CTB. The plasmid pVA1542 contained a promoterless version of *ctxB* gene of *V. cholerae* that lacked the sequence coding for first 17 amino acids of the leader sequence of the protein. The synthetic oligonucleotide encoding the AS was inserted into pVA1542 as an *Eco*RI-*Nde*I fragment to create pVA2143. The resulting *as:ctxB* gene fusion was inserted as an *Eco*RI-*Bam*HI fragment into the expression vector pT7-7. This expression vector contained DNA encoding the first amino acid from protein 10 of phage T7. The gene fusion is under the control of the phage T7 promoter. The gene coding for the T7 RNA polymerase present in the strain BL21(DE3) is under the control of the inducible *lac* promoter.

Immunoblotting analysis. Protein samples were separated by SDS-PAGE and then electrophoretically transferred to nitrocellulose sheets (44). Nonspecific binding of antibody was prevented by blocking the sheets for 30 min at 25°C with a 1% bovine serum albumin (BSA) solution in Tris-buffered saline (TBS) (20 mM Tris, 500 mM NaCl [pH 7.5]). The sheets were incubated for 1 h in antiserum diluted 1:2,000 in TBS with 1% BSA. The sheets were washed three times for 10 min each in TBS with 0.05% Tween 20 and then incubated for 1 h in enzyme-labeled second antibody diluted 1:2,000 in TBS. The sheets were washed three times in TBS–0.05% Tween 20 and once in water and then developed with BCIP-NBT substrate.

Enzyme assays. Gtf activity and inhibition assays were performed as described by Dertzbaugh and Macrina (10). Data were analyzed with a mixed linear statistical model (34, 46). In these experiments, the mixed linear model is ideally suited for comparing the means of Gtf activities obtained in the presence of rabbit serum. The mixed linear model has two advantages over analysis by analysis of variance. First, the mixed linear model allows the inclusion of different days on which experiments were performed as a random effect. Second, the mixed linear model does not assume independent observations; for our data, all observations obtained with a given antiserum are correlated rather than independent. All statistical analyses were done with the SAS system by using PROC MIXED (46).

Purification of the chimeric protein. A 20-liter culture of *E. coli* V2145 was grown in M9 medium supplemented with 10 g of Bacto Tryptone per liter and 1% glucose. The bacteria were grown to an optical density at 660 nm of 0.2, and then 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was added to induce the *lac* promoter. The culture was grown for an additional 5 h at 37°C, and cells were harvested by centrifugation (6,000 × *g*, 15 min). The pellet was suspended in 2 liters of 0.1 M Tris-HCl (pH 8.0), and cells were disrupted with a French press (20,000 lb/in^2 at 20°C; Aminco, Urbana, Ill.). Following centrifugation (27,500 × *g*, 1 h, 4°C), the pellet was washed with 2 liters of water and centrifuged (27,500 × *g*, 1 h, 4°C). The pellet was suspended in 500 ml of 10 mM Tris-HCl–5 M urea (pH 8.0) and sonicated (5 min, 50 W, 4°C). This extract was incubated overnight at 4°C and centrifuged (27,500 × *g*, 30 min). The supernatant was collected, and the pellet was discarded. Sodium metaphosphate (2.5 g/liter) was added to the supernatant, the pH was adjusted to 4.5 with HCl, and the solution was stirred for 2 h at 4°C. After centrifugation (10,000 × *g*, 15 min), the pellet was dissolved in 25 ml of 20 mM Tris-HCl–5 M urea (pH 8.0) and sonicated. The concentrated material was mixed with ampholytes (LKB; pH 5 to 8) to 1%, and 100 ml of the mixture was loaded into the focusing chamber of the isoelectrofocusing cell (RF3 unit; Rainin Instrument Co., Woburn, Mass.). Focusing was carried out at 100 W. After 1.5 h, the voltage stabilized at 1,600 V and samples were collected. Fractions in the range of pH 6 to 7 were centrifuged (5,000 × *g*, 15 min), and the pellet was suspended in 10 ml of 20 mM Tris-HCl–5 M urea (pH 8.0). These fractions were subjected to ion-exchange chromatography on a Sepharose Q column (16 by 10 cm; Pharmacia) equilibrated with 20 mM Tris-HCl–5 M urea (pH 8.0). Electrofocused extract corresponding to 13 mg of protein was applied to the column. The column was washed with 30 ml of the equilibration buffer. The protein was eluted with a 100-ml linear gradient of 0.0 to 0.4 M NaCl in 20 mM Tris-HCl–5 M urea (pH 8.0) buffer. The flow rate was 2 ml/min, and 2-ml fractions were collected. The fractions containing the chimeric protein were pooled (8 ml), dialyzed against water, and then lyophilized.

Analysis of the protein. The N-terminal sequence of the monomeric protein was determined by using an Applied Biosystems model 470A sequencer, with in-line phenythiohydantoin-amino acid analysis.

Antiserum. Antiserum to the purified chimeric protein was raised in male New Zealand White rabbits by the method of Dertzbaugh and Macrina (10). The protocol was approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University (Richmond, Va.). Following collection, the serum was divided and stored at −20°C.

CTB-specific antibodies were removed from the polyclonal rabbit serum in the following manner. Purified CTB (Sigma) was linked to a solid matrix by its free amino groups. The protein was linked to a glutaraldehyde-activated affinity adsorbent (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) by a protocol described by Jones and Fischetti (20). Anti-purified chimeric protein rabbit serum (1 ml) was adsorbed to the CTB-linked glutaraldehyde affinity column by rotating the mixture for 4 h at 4°C. The eluate was collected, the column was washed with 20 ml of phosphate-buffered saline (PBS) (pH 7.4), and the eluent was added to the previous fractions. The antibodies adsorbed to the resin were eluted with 0.1 M glycine (pH 2.5), neutralized immediately, and dialyzed against PBS.

The polyclonal anti-GtfB sera use in this work were provided by Mark Dertzbaugh (U.S. Army Research Institute for Infectious Diseases, Frederick, Md.) and Susanne Michalek (University of Alabama, Birmingham).

RESULTS

Construction and expression of the GtfB AS:CTB fusion protein. We have previously demonstrated that peptides of GtfB fused to CTB can be used as immunogens to raise antibodies which may inhibit Gtf activity (10, 11). Mooser et al. (30) described the structure and the location of the *Streptococcus sobrinus* Gtf sucrose binding site and demonstrated that an aspartic acid within this site covalently binds sucrose. These workers found that the sucrose binding site for the GtfI (which makes primarily water-insoluble glucans) and GtfS (which makes water-soluble glucans) enzymes differed by only two of nine amino acid residues. This AS peptide is conserved in the glucosyltransferases from a variety of oral streptococci (Fig. 2). Specifically comparing *S. mutans* GS5 with *S. sobrinus*, the AS peptide identified from GtfI was found intact in the amino acid sequence of GtfB and -C, and the AS peptide from GtfS was highly conserved in GtfD. The 23 amino acids located around the sucrose-binding aspartic acid are highly conserved in all of the Gtfs. Thus, we decided to make a fusion peptide between this 23-amino-acid sequence and CTB to explore the immunogenicity of this peptide and to use antibodies against it to probe enzyme activity.

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                    * * * * * * * * *
          442                                                 464
GtfB      D A N F D S I R V D A V D N V D A D L L Q I A      (1475)
          467                                                 489
GtfC      D A N F D S I R V D A V D N V D A D L L Q I A      (1375)
          455                                                 477
GtfD      E A N F D G V R V D A V D N V N A D L L Q I A      (1430)
          438                                                 460
GtfI      D A N F D S I R V D A E D N V D A D Q L Q I S      (1593)
          443                                                 465
GtfI*     D A N F D S I R V D A V D N V D A D L L Q I S      (1559)
          428                                                 450
GtfS      D A N F D G V R V D A V D N V N A D L L Q I Q      (1365)
          441                                                 463
GtfT      N A N F D G I R V D A V D N V N A D T L Q L Y      (1467)
          461                                                 483
GtfJ      D A N F D G I R V D A V D N V D A D M L Q L Y      (1517)
          453                                                 475
GtfK      D A H F D G I R V D A V D N V S V D M L Q L V      (1597)
          496                                                 518
GtfL      D E N F D G V R V D A V D N V N A D L L Q I A      (1448)
          548                                                 570
GtfM      S A N F D G V R I D A V D N V D A D L L Q I A      (1576)

AS        D A N F D S I R V D A V D N V D A D L L Q I A
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FIG. 2. Comparison of the Gtf sequences from mutans and related streptococci. The amino acid sequence encompassing the AS is shown in the standard single-letter code. The asterisks above the subpeptide sequence designate the AS peptide as defined by Mooser et al. (30). The aspartic acid (D) residue implicated or proven to participate as general base catalyst in glycosyl cleavage or transfer is indicated by the boldface asterisk. The total number of amino acids (beginning with the N-terminal methionine) in the Gtf molecules is given in parentheses to the right of each sequence. The number of the amino acid residue beginning and ending the sequence is shown above each line. The conserved peptide (AS peptide) selected for use in this study is shown in boldface at the bottom. Sequence information may be found in the following references: GtfB, 36; GtfC, 45; GtfD, 19; GtfI, 1; GtfI*, 13; GtfS, 16; GtfT, 18; GtfJ, 15; GtfK, 14; and GtfL and -M, 37.

A complementary set of oligonucleotides which encoded the amino acid sequence of this peptide was synthesized. Following oligonucleotide annealing, the resultant double-stranded fragment was genetically fused to the promoterless *ctxB* gene in pVA1542 (Fig. 1). This new chimeric gene, designated *as*:*ctxB*, was cloned into the pT7-7 vector. The expression vector pT7-7 contained a T7 promoter, and this plasmid also had a strong ribosome binding site and a start codon (ATG) upstream of the polylinker sequence. *E. coli* BL21(DE3), which contained the gene for the T7 RNA polymerase on the *E. coli* chromosome under the control of the *lac* promoter, was used as the host for expressing the *as*:*ctxB* fusion construct (42). The nucleotide sequence of our construct was determined and confirmed. Figure 3 presents the sequence of the fusion joints of the chimeric protein. We added a nucleotide sequence encoding a proline residue (underlined in Fig. 3) between the first amino acids encoded by the vector and the AS. This created an acid-sensitive Pro-Asp dipeptide that could be used for further

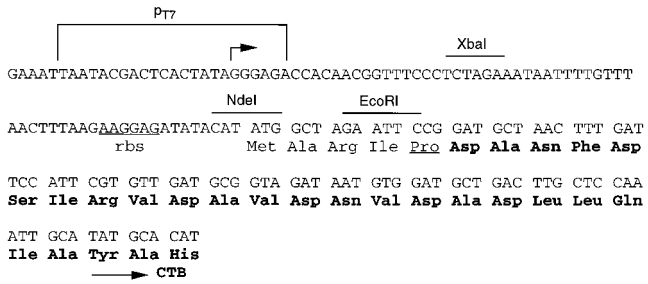

FIG. 3. Sequence of the chimeric protein. The region encoding amino acids 442 to 463, corresponding to a conserved peptide around the AS, is in boldface. A 3-base sequence encoding the underlined proline (immediately 3′ to the *Eco*RI site) was introduced, creating an acid-sensitive Pro-Asp dipeptide. This site was not used in the purification of the chimeric protein, however, pT7 represents the T7 promoter, with the origin of transcription indicated by the arrow.

chimeric protein purification if needed. The expected molecular mass of the chimeric protein was 15 kDa. Immunoblotting of whole-cell *E. coli* lysates subjected to SDS-PAGE demonstrated a 15-kDa protein which was immunoreactive with the antiserum to the cholera toxin (Fig. 4). This immunoreactive species was present only in cell lysates which had been induced by 1 mM IPTG (Fig. 4B, lane 2), indicating the expected participation by the *lac* operon control elements built into the T7 expression system. After IPTG induction, the bacteria carrying the *as*:*ctxB* fusion construct (pVA2145) produced inclusion bodies that could be observed by light microscopy of Gram-stained cells (data not shown).

Purification of the chimeric protein. After the French pressure cell treatment, the chimeric protein was collected by centrifugation along with the total cell protein. The chimeric protein was insoluble in water (Fig. 5, lanes 1 and 2) but could be solubilized with 5 M urea (lane 3). By electrofocusing we were able to precipitate the protein at its expected pI of 6.1 (Fig. 5, lanes 4 and 5). Ion-exchange chromatography gave an electrophoretically homogeneous protein (Fig. 5, lane 6). Forty milligrams of the AS:CTB chimeric protein was produced by our purification procedure.

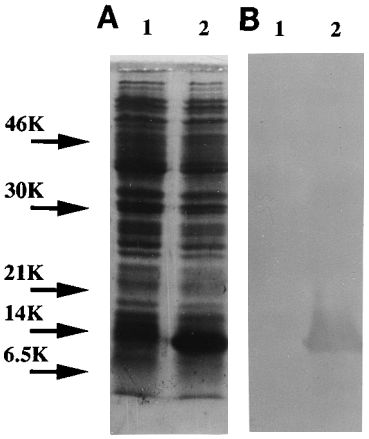

FIG. 4. Expression of the chimeric protein. *E. coli* V2149 containing the expression vector or V2147 containing the chimeric gene (see Fig. 3) was grown in M9 medium supplemented with Bacto Tryptone and glucose and induced with 1 mM IPTG. The cells were allowed to incubate for 3 h after addition of the IPTG. The cells then were harvested by centrifugation, suspended in SDS-PAGE loading buffer, and heated at 100°C for 5 min. (A) Samples subjected to SDS–15% PAGE and stained with Coomassie blue. (B) Duplicate gel blotted to nitrocellulose and probed with antiserum to CTB by Western blot technology as described in Materials and Methods. Lanes 1, V2149; lanes 2, V2147.

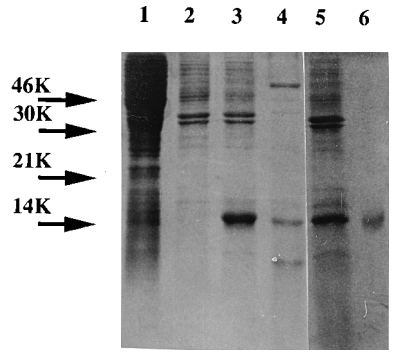

FIG. 5. Purification of the chimeric protein. All samples were fractionated by electrophoresis through SDS–15% PAGE. The gel was stained with Coomassie blue in order to visualize protein components. Lane 1, supernatant obtained from cells disrupted by French pressure cell treatment; lane 2, supernatant obtained after washing of the pellet from French pressure cell treatment; lane 3, pellet obtained after the French pressure cell treatment, suspended in 5 M urea; lane 4, supernatant obtained after centrifugation of the fractions subjected to isoelectric focusing; lane 5, pellet obtained from the fractions subjected to isoelectric focusing; lane 6, purified chimeric protein.

A sample of the purified chimeric protein was fractionated by gel filtration chromatography. By using native molecular mass standards, the purified protein was estimated to be 75,000 Da (data not shown). The purified protein was transferred from the SDS-polyacrylamide gel to a polyvinylidenedifluoride membrane and subjected to multiple cycles of Edman degradation. The amino acid sequence of the amino-terminal end was found to be Ala-Arg-Ile-Pro-Asp, which is identical to that predicted by the construction of such a fusion protein; this result was consistent with the removal of the amino-terminal Met residue associated with deformylation.

Properties of the anti-chimeric protein antiserum. To investigate if antibodies were produced against the GtfB moiety of the chimeric protein, the antisera raised against the AS:CTB fusion protein were adsorbed with CTB. The different fractions obtained from the adsorption were assayed for their abilities to bind to the purified chimeric protein. The fraction without antibodies against CTB was still able to bind the chimeric protein (Fig. 6B, lane 2), but it did not bind to CTB (Fig. 6B, lane 1). These data demonstrated production of antibody to

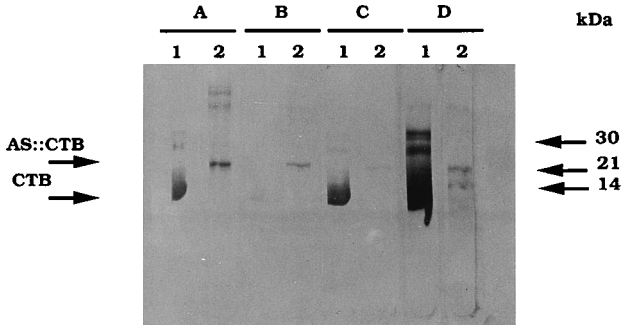

FIG. 6. Immunogenicity of the AS:CTB chimeric protein. Protein samples were fractionated by SDS–20% PAGE and then electrophoretically transferred to nitrocellulose. Lanes 1, CTB (25 μg); lanes 2, AS:CTB (25 μg). The blot was probed with total antiserum to the purified AS:CTB (A), with the total antiserum adsorbed to CTB (B), with the anti-CTB antibodies purified from the total antiserum (C), or with antiserum to CTB (D). Molecular sizes are indicated by the arrows to the right.

TABLE 2. Inhibition of Gtf activity by rabbit antisera

Antiserum	Mean Gtf activity (SE)[a]	Test *t* statistic[b]	*P*[b]	% Inhibition of Gtf activity
Preimmune	925.21 (96.32)			
Anti-GtfB	458.19 (98.89)	4.00	0.0001	51
Anti-GtfB.1:CTB	319.05 (48.24)	5.20	<0.0001	66
Anti-AS:CTB	511.48 (99.49)	3.55	0.0006	45

[a] Expressed as counts per minute per microgram of protein; 36 assays (three replicates per assay) were performed for each antiserum.
[b] Compared with results for preimmune serum in a mixed linear model.

the peptide corresponding to the AS. The affinity-purified antibodies to CTB were eluted from the column and reacted with the cholera toxin and the chimeric protein (Fig. 6C).

Antiserum prepared against partially purified Gtfs from *S. mutans* GS5 did not give a signal in Western blot (immunoblot) analysis when reacted with the peptide corresponding to the GtfB AS of the chimeric protein (data not shown). However, this polyclonal antiserum reacted appropriately with purified GtfB as well as with extracellular proteins from *S. mutans* GS5. The serum raised against the purified AS:CTB chimeric protein recognized the AS:CTB chimeric protein, but it failed to recognize purified Gtf or Gtfs present in the protein extract from *S. mutans* (data not shown).

Antisera raised against AS:CTB, GtfB.1:CTB (9), or GtfB were evaluated for the ability to inhibit Gtf activity of extracellular-protein preparations from *S. mutans* V2158 (32). This strain contained a *gtfB* gene and produced water-insoluble glucan. However, the *gtfC*, *gtfD*, and *ftf* genes in V2158 have been inactivated by allelic exchange mutagenesis. V2158 thus allows the examination of antibody effects on enzyme activity in a defined in vitro system. Since the chimeric proteins contained GtfB amino acid sequences, this afforded specific measurement of any inhibitory effects of antibodies on enzyme activity. Thus, the potential effects of other polymer-synthesizing enzymes, e.g., GtfC or GtfD, on GtfB activity are eliminated in this system. Compared with the mean activity levels of the extracellular-protein preparations in the presence of preimmune serum (Table 2), all antisera significantly inhibited the production of total glucan ($P < 0.001$ in all three cases). The inhibition levels of antisera to GtfB, GtfB.1:CTB, and AS:CTB were not statistically different from each other (Table 2).

DISCUSSION

Our interest in Gtfs as virulence factors is related to their possible use as vaccinogens. An anticaries vaccine would have to primarily involve the mucosal immune system, specifically, a secretory immunoglobulin A response (28). Secretory immunoglobulin A antibodies able to bind to Gtfs, thereby inhibiting their enzyme activity, would effect qualitative and quantitative changes in the polymer components of the dental plaque. On the basis of evaluations of Gtf-deficient mutants in in vivo virulence studies in animals, this would likely reduce cariogenicity in humans. Such a vaccine would modify the behavior of a pathogenic microorganism without eliminating the pathogen. In our approach to an anticaries vaccine, we have focused on the use of CTB as a mucosal adjuvant (9, 11). When CTB was conjugated to antigens that were weakly immunogenic when administered orally, dramatic increases in their immunogenicities were observed (3, 27). We sought to achieve a similar effect by constructing recombinant plasmids which contained nucleotide sequences from *gtfB* fused in frame to the 5′ terminus of the CTB gene (*ctxB*) (9, 11). One such chimeric protein produced by this method consisted of a 15-amino-acid peptide (GtfB.1) linked to CTB. The GtfB.1 peptide was approximately 100 amino acids upstream of the AS peptide. This GtfB.1:CTB protein was immunogenic in rabbits, and rabbit antisera raised to this protein significantly inhibited glucan synthesis in vitro (9). The GtfB.1:CTB protein was packaged into liposomes and fed to rats, which were subsequently challenged with cariogenic *S. mutans*. This vaccine preparation elicited a secretory immunoglobulin A response which correlated with a modest protection against caries (29).

In this work we have successfully created another CTB fusion protein containing the AS region of the GtfB enzyme. This catalytic site is highly conserved among the Gtfs of the mutans streptococci (Fig. 2). We selected this region and its flanking amino acids for two reasons. First, the AS of an enzyme, if accessible to antibodies, should be an excellent target for blocking or inhibiting catalytic activity. Second, the conserved nature of this peptide in many different Gtfs would serve as the basis of broad immunologic protection against caries. Purification and analysis of the chimeric peptide consisting of the 23-amino-acid sequence encompassing the GtfB AS and CTB revealed it to have the predicted molecular size of 15 kDa. Analysis by gel filtration chromatography demonstrated a native protein structure of 75 kDa. This result suggested that the AS:CTB chimeric protein formed a pentamer in fashion similar to that of native CTB. Enzyme-linked immunosorbent assays to detect the chimeric protein were also performed. These were dependent on the protein binding to GM_1-coated microdilution wells, a feature characteristic of pentameric CTB but not monomeric CTB. The AS:CTB chimeric protein was readily detected by this method, indicating that this protein was behaving similarly to native CTB with respect to binding to GM_1 gangliosides (data not shown). This physical property is important to such fusion proteins intended for use as mucosal vaccines, as the CTB adjuvant effects appear to be linked to pentamerization (8). Immunologic analysis of the chimeric peptide clearly indicated that the chimeric protein was able to elicit production of antibodies to both of its component sequences (Fig. 6). We noted that our antisera raised to the AS:CTB protein did not react with intact GtfB enzyme from *S. mutans* GS5. It is possible that the antibodies against the GtfB AS sequences in this antiserum are binding to the GtfB protein but that the limited number of immunoglobulin molecules per enzyme molecule may not be enough to create a detectable signal in the Western blot assay.

The results of our antibody inhibition studies with the anti-AS:CTB serum differ somewhat from those of similar studies carried out with antisera against the GtfB.1:CTB peptide (9). Inhibition of insoluble-glucan synthesis by the latter antisera was approximately 90%, while the anti-AS:CTB serum inhibited enzyme activity approximately 66%. We believe that this discrepancy can be traced to differences in the elements of the assay systems. In our previous work with GtfB.1:CTB (9), our construct used the *S. mutans* GS5 GtfB amino acid sequence, and the enzyme preparations used in our inhibition studies were from the same strain. These preparations included GtfB, -C, and -D as well as Ftf enzymes. In the present work we again used the *S. mutans* GS5 GtfB AS sequence, but our inhibition studies involved enzyme preparations from a different strain, *S. mutans* V403. Nucleotide sequence determination of the AS region of the V403 *gtfB* gene has revealed different amino acid residues at two positions, and this could account for the reduced inhibition (66 versus 90%). The V403 GtfB C-terminal octapeptide was deduced to be DAYVLQIA instead of the DADLLQIA sequence found in the GS5 enzyme (see bottom of Fig. 2 for the complete AS sequence). Moreover, we used a

TABLE 3. Antibody inhibition of Gtfs from mutans streptococci

Strain of enzyme origin	Enzyme (product)	No. of amino acids in enzyme[a]	Coordinates of subsequence targets (designation)	Comments	Immunogenic reagent	Assay	Enzyme inhibition (%)	Reference
S. mutans GS5	GtfB (insoluble glucan)	1,475	345–359 (GtfB.1)	Located 94 amino acids on amino-terminus side of AS; 15-amino-acid peptide genetically fused to amino terminus of CTB	Rabbit polyclonal antibody	Total glucan	50	9
						Insoluble glucan	90	
S. sobrinus	GtfI (insoluble glucan)	1,559	1303–1324 (GLU)	Repeated-region motif comprising glucan binding domain; synthesized as MAP[b] containing four copies linked to a lysine backbone	Rat polyclonal antibody	Soluble glucan (primer dependent)	30	38
S. mutans GS5	GtfC (soluble and insoluble glucan)	1,375	435–453	Located 18 amino acids on amino-terminus side of AS; 19-amino-acid peptide conjugated to BSA (sequence identical to GtfB residues 410–428)	Mouse monoclonal antibody	Total glucan synthesis	25–30	6
						Insoluble-glucan synthesis	50	
	GtfB (insoluble glucan)	1,475	442–462 (CAT)	21-amino-acid sequence encompassing AS synthesized as MAP containing four copies linked to a lysine backbone; identical sequences found in GtfC (487–507) and GtfI (444–464)	Mouse monoclonal antibody	Insoluble glucan	84	39
					Rat polyclonal antibody	Insoluble glucan	17	
					Mouse monoclonal antibody	Soluble glucan	13–35	
			442–463	23-amino-acid sequence encompassing AS genetically fused to amino terminus of CTB	Rabbit polyclonal antibody	Insoluble glucan	45	This paper

[a] Numbering of amino acid residues begins with the amino-terminal methionine.
[b] MAP, multiply antigenic peptide.

mutant of V403 (V2158) which was missing all extracellular polymer-forming enzymes except GtfB. Accordingly, our assay technique used methanol precipitation to capture newly synthesized polymer, because all such material is the result of catalysis by a single gene product, GtfB. In our studies with GS5 we had to use water insolubility to differentiate GtfB-catalyzed polymers from water-soluble ones. This difference in the assay systems may have slightly affected their sensitivities, thus contributing to the observed antibody inhibition levels. The fact that GtfB does produce some soluble glucans (2) may have affected our results as well. Finally, different batches of antisera, raised to partially purified GtfB from *S. mutans* GS5, were used in these two separate studies. The compositions of the enzyme preparations used as immunogens, along with the variation in the rabbit antibody response, could also contribute to our observed differences.

Three laboratories now have used antibodies to probe the function of Gtf enzymes from mutans streptococci. Such studies are beginning to shed light on sites that may be useful vaccine targets. These results, which include our present findings, are summarized in Table 3. As can be seen, there is variation in the result depending on the location of the Gtf subsequence peptide selected as a target, the type of antibody used to inhibit activity, and the chemical nature of the immunogen used to raise the antisera. However, taken together, the results suggest that the development of Gtf subunit vaccines is plausible. Further work is needed to better define the optimal target or target combination. Such work must progress simultaneously with the exploration of delivery systems to stimulate a mucosal antibody response.

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