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Expression of a *Streptococcus mutans* Glucosyltransferase Gene in *Escherichia coli*

JAMES P. ROBESON,† RAÚL G. BARLETTA, AND ROY CURTISS III*

Department of Microbiology and Institute of Dental Research, University of Alabama in Birmingham, Birmingham, Alabama 35294

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Chromosomal DNA from *Streptococcus mutans* strain UAB90 (serotype *c*) was cloned into *Escherichia coli* K-12. The clone bank was screened for any sucrose-hydrolyzing activity by selection for growth on raffinose in the presence of isopropyl-β-D-thiogalactoside. A clone expressing an *S. mutans* glucosyltransferase was identified. The *S. mutans* DNA encoding this enzyme is a 1.73-kilobase fragment cloned into the *Hin*dIII site of plasmid pBR322. We designated the gene *gtfA*. The plasmid-encoded *gtfA* enzyme, a 55,000-molecular-weight protein, is synthesized at 40% the level of pBR322-encoded β-lactamase in *E. coli* minicells. Using sucrose as substrate, the *gtfA* enzyme catalyzes the formation of fructose and a glucan with an apparent molecular weight of 1,500. We detected the *gtfA* protein in *S. mutans* cells with antibody raised against the cloned *gtfA* enzyme. Immunologically identical *gtfA* protein appears to be present in *S. mutans* cells of serotypes *c*, *e*, and *f*, and a cross-reacting protein was made by serotype *b* cells. Proteins from serotype *a*, *g*, and *d S. mutans* cells did not react with antibody to *gtfA* enzyme. The *gtfA* activity was present in the periplasmic space of *E. coli* clones, since 15% of the total *gtfA* activity was released by cold osmotic shock and the clones were able to grow on sucrose as sole carbon source.

Streptococcus mutans is a principal agent of dental caries whose virulence derives from the ability (i) to colonize the tooth surface and (ii) to produce, by fermentation of carbohydrates, lactic acid which causes demineralization of the tooth, leading to caries formation (42). These two virulence factors are intimately related to an efficient metabolism of sucrose by *S. mutans*, and caries development by this microorganism is enhanced by the presence of sucrose in the diet of experimental animals (33). Although the precise mechanisms underlying *S. mutans* cariogenicity are not fully understood, particularly those regarding the establishment of *S. mutans* in dental plaque, it is nevertheless recognized that synthesis of water-insoluble glucans from sucrose is essential for *S. mutans* to adhere to and aggregate on the tooth surface (35).

S. mutans produces glucosyltransferases (GTF; EC 2.4.1.5) that catalyze glucan formation (9, 26, 41). However, the precise mechanism of glucan formation remains unknown (10, 15). The number of enzyme proteins responsible for the synthesis of soluble and insoluble glucans, as well as the putative precursor-product relationship between the different glucan molecules, is a question which has not been answered. Part of the difficulty has arisen from the complications in obtaining enzyme preparations purified to homogeneity from *S. mutans*, due to the formation of heteromolecular complexes among the different proteins and glucan species involved.

One way to overcome some of these problems is to use recombinant DNA technology to clone the *S. mutans* genes involved in glucan formation in another bacterial species such as *Escherichia coli*, which does not have any sucrose-cleaving activities. Thus, a single enzyme can be obtained in high yield and thoroughly analyzed. The ease of manipulating genes in *E. coli* and the possibility of studying expression of gram-positive genes in a gram-negative background also prompted us to undertake this approach. Here, we describe an *S. mutans* GTF gene that is cloned and expressed in *E. coli*. We have also been successful in applying this technology to the study of other *S. mutans* potential virulence determinants, such as the major surface protein antigen (7, 20).

MATERIALS AND METHODS

Bacterial strains and growth media. Bacterial strains used are listed in Table 1. For *E. coli* strains grown in liquid or solid (1.5% agar) minimal media (6), nutritional supplements (Sigma Chemical Co.) were used in the following concentrations (micrograms per milliliter) as

† Present address: Laboratorio de Microbiología, Universidad Católica de Valparaíso, Casilla 4059, Valparaíso, Chile.

TABLE 1. Bacterial strains

Strain	Description	Source or reference
E. coli K-12		
χ1274	F⁻ *minA1 tsx-63 purE41 glnU42 pdxC3 minB2 recA1 metC65 rpsL97 xyl-14 ilv-277 cycB2 cycA1*	This laboratory
χ1849	F⁻ *tonA53 dapD8 minA1 purE41 glnU42 Δ(gal-uvrB)47* λ⁻ *minB2 hsd-53 nalA25 metC65 oms-1* T3ʳ *Δ(bioH-asd)29 ilv-277 cycB2 cycA1 hsdR2*	16
BW490.9	F⁻ *rpsL envZ*	14, 46
BW490.12	F⁻ *rpsL*	14, 46
HB101	F⁻ *ara-14 leuB6 proA2 lacY1 glnU44 galK2* λ⁻ *recA13 rpsL20 xyl-5 mtl-1 thi-1 hsdS20*	2
S. mutans		
UAB62	Serotype *c*, strain PS14; Strʳ Rifʳ	36
UAB66	Serotype *g*, strain 6715; Strʳ Spcʳ	36
UAB90	Derivative of UAB62 with a defective cell wall	37

needed: adenine, 40; L-histidine, 22; L-isoleucine, 20; L-lysine, 88; L-*meso*-diaminopimelic acid, 50; DL-methionine, 20; DL-threonine, 80; DL-valine, 40; D-biotin, 0.05; pyridoxine, 2; thiamine, 2. Sucrose (ultrapure; Schwarz/Mann), raffinose (Pfansteihl), and other carbohydrates (Sigma) were used at 0.5 and 1% or at 25 mM.

The complex media used were L broth or L agar (29) and MacConkey base agar (Difco Laboratories) supplemented with 1% carbohydrate as a fermentation indicator medium. Antibiotics were used in the following concentrations: ampicillin, sodium salt (Lederle Laboratories), 25 μg/ml; tetracycline (Lederle), 20 μg/ml. Buffered saline with gelatin was used as diluent (6).

S. mutans strains were stored on brain heart infusion (Difco) agar slants and grown in brain heart infusion broth, tryptic soy broth (Difco), or modified FMC medium (36). Small cultures were incubated anaerobically as described before (16); large cultures for batch production were incubated as standing cultures at 37°C.

Preparation of DNA. Overnight cultures of *S. mutans* strain UAB90 grown in brain heart infusion broth were diluted 100-fold into 250 ml of the same medium and incubated for 20 h at 37°C without aeration. Cells were harvested by centrifugation for 10 min at 10,000 rpm and 4°C in a Sorvall SS34 rotor. Cells were washed twice with 2 M NaCl and twice with ice-cold water. The final cell pellet was suspended in 3 ml of 20% (wt/vol) glucose in 25 mM sodium phosphate buffer, pH 6.5, and *S. mutans* cells were lysed with mutanolysin (47), a gift from Kanae Yokogawa (Dainippon Pharmaceutical Co. Ltd., Osaka, Japan), as described by Jagusztyn-Krynicka et al. (21). The lysate was treated with RNase (Sigma) and proteinase K (Merck & Co., Inc.) as described by Zasloff et al. (48), and the DNA was purified by two successive CsCl (1.1 g/ml of lysate) density gradient centrifugations. DNA was dialyzed against and stored at 4°C in 10 mM Tris-hydrochloride (pH 8.0)–1 mM EDTA.

Construction and detection of clones. *S. mutans* DNA was partially digested at 37°C with *Hin*dIII (New England Biolabs) in 6 mM Tris-hydrochloride (pH 7.4)–6 mM $MgCl_2$–50 mM NaCl and ligated by using T4 DNA ligase (New England BioLabs) with pBR322 vector DNA, which was digested to completion with *Hin*dIII. Total DNA concentration was 80 μg/ml, with a 3:1 ratio of *S. mutans*/vector DNA. *E. coli* χ1849 was transformed with the ligation mixture by a modification of the procedure of Cohen et al. (4; J. P. Robeson, Ph.D. thesis, University of Alabama in Birmingham, 1981). Ampicillin-resistant transformants were selected, and raffinose-fermenting phenotypes were detected by replica plating onto MacConkey base agar containing 1% raffinose, 0.1 mM isopropyl-β-D-thiogalactoside (IPTG; Sigma), and appropriate nutritional supplements. Colonies that gave a fermentation reaction were purified and analyzed further.

Preparation of minicells and analysis of plasmid-encoded proteins. Minicells were prepared as described previously (40), with the exception that instead of linear sucrose gradients, step gradients (5, 12.5, and 25% [wt/vol] sucrose in buffered saline in gelatin) were used in purifying minicells. Minicells obtained from a 50-ml culture grown to an optical density at 600 nm of 0.5 to 0.6 were finally suspended in 0.6 ml of glucose minimal medium and preincubated for 20 min at 37°C. Proteins were then labeled by addition of 2 μCi of ^{14}C-labeled amino acid mixture (New England Nuclear) followed by 20-min incubation at 37°C. Minicells were lysed by 2 min of boiling in sample buffer (16), and proteins were resolved in a 10% sodium dodecyl sulfate-polyacrylamide gel (28). The gel was treated with scintillation fluid (Enhance; New England Nuclear) and dried. Labeled proteins were detected by fluorography, using Kodak X-Omat XS-5 film.

Purification of *S. mutans* GTF from *E. coli* clones. χ1274(pYA601) cells (which provide a *recA* background to ensure stability of the recombinant plasmid) were grown in L broth with aeration to an optical density at 600 nm of 0.8 to 1.0. All further operations were carried out at 4°C. The cells were harvested and washed once with 50 mM Tris-hydrochloride, pH 7.3 (T buffer). The cell paste (60 g, wet weight) was

disrupted by grinding with 120 g of alumina (type 305; Sigma). After the addition of 120 ml of T buffer, the mixture was centrifuged to separate alumina and coarse debris (12,000 × *g*, 30 min). The supernatant fluid was centrifuged in a Beckman type 60 Ti rotor at 35,000 rpm for 1 h. To the resulting supernatant fluid, $MgCl_2$ and DNase (Worthington Diagnostics) were added to 10 mM and 5 μg/ml, respectively. After incubation for 2 h on ice, the crude extract was dialyzed overnight against 4 liters of T buffer. The dialysate was then applied to a DEAE-cellulose (DE52; Whatman Ltd.) column equilibrated with T buffer and eluted with a 0 to 0.5 M NaCl linear gradient. Fractions with enzyme activity (detected by reducing sugar formation; see below) were pooled, concentrated by ultrafiltration (PM10; Amicon Corp.), and chromatographed through Ultrogel AcA44. Fractions with enzyme activity were again pooled, and the remaining protein contaminants were precipitated with ammonium sulfate at 33% saturation. The supernatant fluid was dialyzed against sterile water, lyophilized (VirTis Research Equipment), and suspended in phosphate-buffered saline, pH 7.2. About 3 mg of *gtfA* enzyme was recovered, representing a 17% yield. Subsequent improvements in this purification protocol gave substantially higher yields.

Paper chromatography. Reaction components from the incubation of radiolabeled sucrose with the cloned GTF were analyzed by descending paper chromatography. The chromatography was performed on Whatman 3 MM strips (47 cm in length) run for 18 to 20 h at room temperature with an ethyl acetate-pyridine-water (12:5:4) solvent system. Radioactive reaction components were detected by scanning the paper strips in a radiochromatogram scanner (Packard model 7201). Quantitation of various reaction components was performed by measuring the areas under peaks with an electronic graphics calculator (Numonics Corp. model 250-117). Radiolabeled substrates used were [*U*-^{14}C]sucrose, [*glucose-U*-^{14}C]sucrose (New England Nuclear), and [*fructose-U*-^{14}C]sucrose, a gift from Bruce Chassy (National Institute of Dental Research).

Purification and molecular weight determination of the glucan synthesized by the cloned GTF. The radioactive glucan produced upon incubation of the cloned GTF in 50 mM sodium phosphate buffer with [*U*-^{14}C]sucrose as substrate was purified by direct elution with water from the chromatographed paper strips. The molecular weight of the glucan was estimated by gel filtration chromatography on a Bio-Gel P-10 (Bio-Rad Laboratories) column equilibrated and eluted with 0.05 M NH_4HCO_3, pH 7.9. Polysaccharide size standards were a gift of James Christner (University of Alabama in Birmingham).

Preparation of *S. mutans* protein fractions. *S. mutans* strains were grown overnight at 37°C anaerobically in tryptic soy broth, diluted 50- to 100-fold into 0.5, 1, or 2 liters (as needed) of tryptic soy broth or modified FMC medium, and incubated at 37°C as standing cultures until dense growth was achieved (20 to 90 h). Cells were harvested by centrifugation at 5,000 rpm for 30 min in a Sorvall HG-4L rotor.

(i) Cells grown in FMC medium. To determine the localization in *S. mutans* cells and culture supernatant fluids of the GTF activity expressed by *E. coli* recombinant clones, the parental *S. mutans* PS14 strain (UAB62) was used. Cells were grown in modified FMC medium, and three protein fractions were prepared.

(a) Fraction 1, culture supernatant fraction. *S. mutans* cells were harvested and washed once at 4°C with 5 ml of 50 mM sodium phosphate buffer, pH 7.0, and the fluid wash was pooled with the culture supernatant fluid; this pool was then filtered through a 0.45-μm membrane (Millipore Corp.), concentrated by ultrafiltration to a final protein concentration of 15 to 30 mg/ml, and dialyzed against 50 mM sodium phosphate buffer.

(b) Fraction 2, NaCl fraction. The remaining cells were then washed twice with 5 ml of 1 M NaCl for 15 min at 4°C with shaking to remove cell-associated proteins (25); both NaCl washes were then pooled and treated as described for fraction 1.

(c) Fraction 3, cellular fraction. The washed cells (2.6 g, wet weight) were resuspended in 10 ml of 50 mM sodium phosphate buffer and sonicated at 0°C with a model W185D cell disruptor (Hot Systems-Ultrasonics, Inc.), using a microtip at maximum output and applying 14 intermittent 30-s pulses. The sonic extract was then treated as described for fraction 1.

(ii) Cells grown in tryptic soy broth. A shorter procedure was used to prepare *S. mutans* protein fractions for studying the distribution of the cloned GTF among *S. mutans* serotypes. *S. mutans* strains were grown in tryptic soy broth and two protein fractions were prepared.

(a) Fraction A, culture supernatant fraction. *S. mutans* cells were harvested and washed twice with sodium phosphate buffer, and the supernatant fluids from these two washes were pooled with the culture supernatant fluid. The combined pool was precipitated overnight with ammonium sulfate at 70% saturation at 4°C. The precipitates were suspended in the minimal volume of sterile water and dialyzed against water. After removal of insoluble materials by centrifugation (12,000 × *g*, 20 min), the supernatant fluid was lyophilized and the lyophilized material was finally resuspended in a minimal volume of water. Any remaining insoluble material was removed by centrifugation (Eppendorf microfuge; 4°C for 5 min). The clear supernatant fluid was stored at −20°C until used.

(b) Fraction B, cellular fraction. The cell pellet was suspended in 50 mM sodium phosphate buffer and sonicated as described above. The sonic extract was then filtered through a 0.45-μm membrane, dialyzed against water, and lyophilized.

Preparation of periplasmic and cellular protein fractions from *E. coli*. Three *E. coli* fractions were prepared. Total cell protein fraction was prepared from cells disrupted by sonication at 0°C, using a microtip at 70 to 100% of maximal output and applying three intermittent 30-s pulses. Periplasmic protein fraction from *E. coli* cells was prepared by cold osmotic shock as described by Hazelbauer and Harayama (18). Cellular protein fraction was prepared by sonication of the osmotically shocked cells. Fractions were then made cell-free by filtration through a 0.45-μm membrane, and the filtrates were centrifuged (Eppendorf microfuge; 2 min) at 4°C to remove any insoluble material. Cellular fractions from entire or shocked cells may include not only cytoplasmic proteins, but also some proteins attached to membranes that could have been released by sonication.

Enzyme assays. Assays of α-galactosidase (43), β-

galactosidase (34), and β-lactamase (39) were as described previously; *o*-nitrophenyl-D-galactosides were from Sigma, and the chromogenic cephalosporin substrate was a gift from Barry Gray (University of Alabama in Birmingham). The generation of reducing sugars by GTF from sucrose was measured by the method of Nelson (38). Clones were analyzed for the ability to generate reducing sugar in cells or in crude cell-free extracts. When the reducing sugar activity was assayed in nondenaturing polyacrylamide gels, the gel system of Laemmli and Favre (28) was used except that sodium dodecyl sulfate was omitted. The gels were incubated for 1 to 2 h at 37°C in 0.1 M sucrose in 0.2 M sodium phosphate buffer, pH 7.0, and the reducing sugar activity was located as described before (11). Protein concentration was measured by the method of Lowry et al. (32), using bovine serum albumin (Sigma) as the standard.

Immunological procedures. Antiserum against the purified cloned GTF (anti-GTF antiserum) was raised in female Norwood rabbits as described previously (17); each injection used a suspension of 200 μg of purified enzyme in 1.0 ml of phosphate-buffered saline and 1.0 ml of complete Freund adjuvant (Difco). Immunoprecipitation with *Staphylococcus aureus* Cowan I cells (24) (a gift of James Bradac, University of Alabama in Birmingham), immunodiffusion in agarose gels (12), and rocket electrophoresis (12) were performed as described before.

RESULTS

Detection and characterization of the *gtfA* clone. Total *S. mutans* UAB90 chromosomal DNA partially digested with *Hin*dIII was cloned into the *Hin*dIII site of plasmid pBR322 and transformed into *E. coli* strain χ1849. These recombinant clones were then selected for their ability to ferment raffinose in the presence of IPTG. Raffinose is composed of galactose, glucose, and fructose moieties and can be cleaved to yield sucrose and galactose or melibiose and fructose. IPTG induces the *lacY* gene product, the galactoside permease, which transports raffinose inside *E. coli* (1). Once inside *E. coli* raffinose induces the *melA* gene product (44), which splits raffinose into sucrose and galactose. Since *E. coli* does not have any sucrose-hydrolyzing activities and *E. coli* strain χ1849 has a deletion of galactose utilization genes, χ1849 cannot ferment raffinose in MacConkey indicator plates containing IPTG. Hence, selection for raffinose fermentation would detect clones able to hydrolyze sucrose or the sucrose moiety of raffinose. In this way, we obtained 16 clones (approximately 1 in 4,000 transformants) with a sucrose-hydrolyzing activity. All 16 clones lacked the ability to ferment galactose and contained plasmid DNA larger in molecular size than the cloning vector pBR322.

We chose one recombinant clone, designated χ1849(pYA601), for further studies. Toluenization of χ1849(pYA601) cells and incubation with 0.1 M sucrose resulted in rapid production of reducing sugars (7). As we will show below, this sucrose-hydrolyzing activity is due to an *S. mutans* GTF gene cloned on recombinant plasmid pYA601 and expressed in *E. coli*. We will term the gene *gtfA* and the GTF enzyme encoded by that gene as *gtfA* enzyme. We adopt the nomenclature at this point to facilitate the presentation of the rest of our data.

Restriction endonuclease analysis of recombinant plasmid pYA601 showed the absence of *Bam*HI, *Bgl*I, *Eco*RI, *Sal*I, or *Pst*I sites in the *S. mutans* DNA insert. We found an internal *Hin*dIII site that splits the 1.73-kilobase (kb) insert into fragments of 1.36 and 0.37 kb in size (Fig. 1A, lane 7). These fragments are *S. mutans* UAB90 DNA since they hybridized to UAB90 chromosomal DNA but not to *E. coli* chromosomal DNA (data not shown). The internal *Hin*dIII site was closer to the *Pst*I site of the pBR322

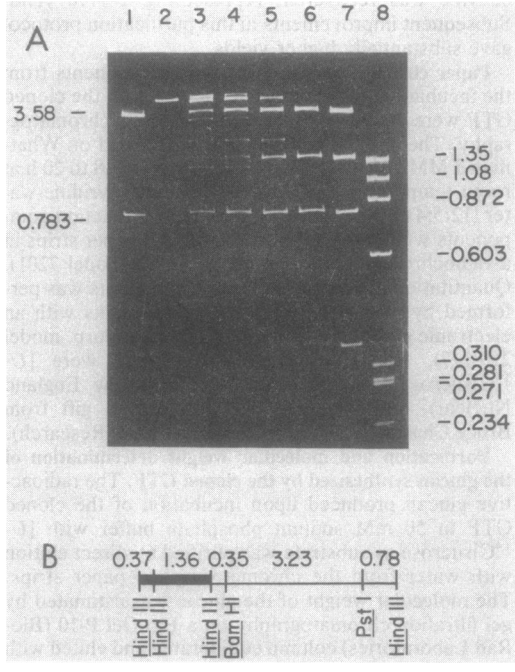

FIG. 1. Restriction endonuclease analysis (A) and schematic representation (B) of pYA601. (A) DNA fragments were electrophoresed in a 5% polyacrylamide gel in 40 mM Tris-hydrochloride (pH 8.0)–20 mM sodium acetate–1 mM EDTA. Lane 1, pBR322 digested to completion with *Pst*I and *Hin*dIII; lane 2, pYA601 digested with *Pst*I; lanes 3 to 6, progressive *Hin*dIII partial digests of pYA601 previously digested to completion with *Pst*I; lane 7, pYA601 digested to completion with *Pst*I and *Hin*dIII; Lane 8, ΦX174 RF digested with *Hae*III. Fragment lengths are in kilobases. The appearance of a 1.15-kb fragment is discussed in the text. (B) The thin line corresponds to pBR322 DNA. The thick line represents the *S. mutans* UAB90 DNA coding for the *gtfA* enzyme.

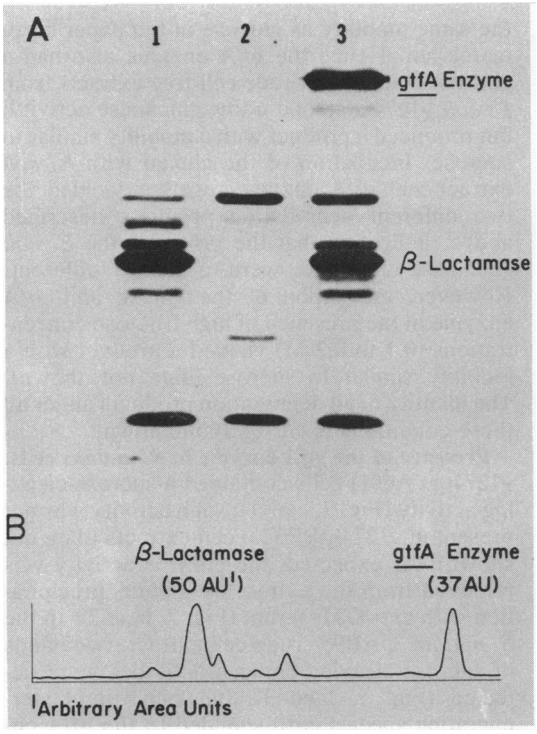

FIG. 2. Proteins synthesized by *E. coli* minicells. (A) Preparation of minicells and protein labeling were carried out as described in the text. Lane 1, Proteins from χ1849(pBR322) minicells; lane 2, proteins from χ1849 minicells; lane 3, proteins from χ1849(pYA601) minicells. (B) Density scan of autoradiogram from lane 3 to quantitate the amount of proteins encoded for by pYA601. Autoradiogram was scanned with a soft-laser density scanner (LKB Instruments).

vector, since a complete digestion with *Pst*I (Fig. 1A, lane 2) followed by progressive digestion with *Hin*dIII (Fig. 1A, lanes 3 to 6) yielded a 1.15- kb fragment which was ultimately cleaved to 0.37- and 0.78-kb fragments (Fig. 1A, lane 7). A schematic representation of pYA601 is shown in Fig. 1B. Neither the 0.37- nor the 1.36-kb *Hin*dIII fragments subcloned into the *Hin*dIII site of pBR322 allowed for growth on minimal medium containing raffinose plus IPTG. Four other recombinant plasmids specifying a sucrose-hydrolyzing activity were analyzed, and they all gave the same *Hin*dIII restriction pattern as pYA601.

Expression of the *gtfA* gene in *E. coli* minicells. Protein synthesis directed by pYA601 was studied in χ1849(pYA601) minicells to compare the level of expression of the *S. mutans gtfA* gene with that of the pBR322 *bla* locus coding for β-lactamase. Figure 2A shows the production of a 55,000-subunit-molecular weight protein corresponding to the *gtfA* enzyme in χ1849(pYA601) minicells (lane 3); plasmidless (lane 2) and pBR322-containing (lane 1) χ1849 minicells did not produce a polypeptide of the same size. The number of *gtfA* enzyme molecules produced was 40% the level of β-lactamase, as determined by the molecular weight of the two proteins and the areas under the corresponding peaks in the scan of the autoradiogram (Fig. 2B). It is likely that the *gtfA* gene was transcribed from an internal promoter since the *Hin*dIII fragment (1.35 kb) of the *S. mutans* DNA insert bound *E. coli* RNA polymerase in a filter-binding assay. This binding was resistant to 0.2 M KCl (Fig. 3, lanes 4 and 5), indicating the presence of promoter-like sequences. At lower KCl concentrations (0.05 M) the binding occurred with less specificity (Fig. 3, lanes 6 and 7).

Reaction catalyzed by the *gtfA* enzyme. The *gtfA* enzyme purified to homogeneity from *E. coli* had a K_m of 1.25 mM for sucrose, a turnover number of 27 s^{-1}, an activation energy of 7 kcal (29,301 J)/mol, and an apparent molecular weight of 55,000 in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The enzyme was active over a wide pH range, giving the maximum activity at pH 6.5. The *gtfA* enzyme was moderately heat stable since about 30% of the activity still remained after 20 min of incubation at 50°C (Fig. 4).

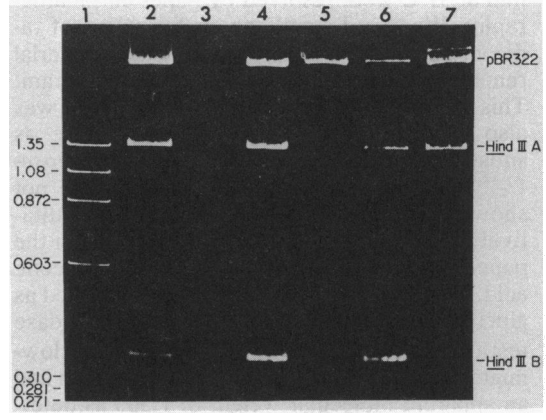

FIG. 3. *E. coli* RNA polymerase binding by *S. mutans* DNA in pYA601 digested with *Hin*dIII. RNA polymerase binding assay was carried out as described previously (23). Lane 1, ΦX174 RF digested with *Hae*III; lane 2, filtrate of pYA601 DNA through nitrocellulose filter when no RNA polymerase was added; lane 3, absence of DNA retained in filter when no RNA polymerase was added; lane 4, filtrate of pYA601 DNA eluted through filter in presence of RNA polymerase at 0.2 M KCl; lane 5, DNA retained in filter in presence of RNA polymerase at 0.2 M KCl; lane 6, filtrate of pYA601 DNA through filter in presence of RNA polymerase at 0.05 M KCl; lane 7, DNA retained in filter in presence of RNA polymerase at 0.05 M KCl. Fragment lengths are in kilobases. DNA fragments were resolved in a 5% polyacrylamide gel.

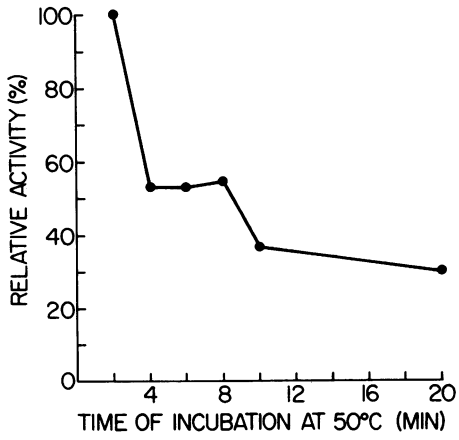

FIG. 4. Effect of preincubation at 50°C on the activity of the *gtfA* enzyme from *E. coli*. Different samples containing 0.075 μg of the purified enzyme were preincubated for various times in 0.5 ml of 100 mM sodium phosphate buffer, pH 7.0. Tubes were then placed at 37°C, and 0.5 ml of 0.2 M sucrose (in water) was added. The reaction was measured and expressed as a percentage of the activity of an unheated control.

The products of the reaction of the *gtfA* enzyme incubated with [*U*-^{14}C]sucrose for 30 min at 37°C were resolved by paper chromatography. We found simultaneous formation of radioactive fructose and a radioactive material remaining near the origin of the chromatogram. This slowly migrating radioactive material was also detected with [*glucose-U*-^{14}C]sucrose as substrate, but it was not found when [*fructose-U*-^{14}C]sucrose was used as substrate (data not shown). The radioactive material was quantitatively recovered by elution with water from the paper strips and hydrolyzed with trifluoroacetic acid. The product of hydrolysis was identified as glucose (7) and gave a positive glucose oxidase test (Robeson, Ph.D. thesis). Hence, the slow-migrating material was a glucan. This glucan had an apparent molecular weight of 1,500 as determined by gel filtration chromatography (Fig. 5). The *gtfA* GTF activity did not require an α(1→6)-glucan primer, since addition of dextran T10 did not alter the rate of glucan formation by the *gtfA* enzyme (7).

With [*U*-^{14}C]sucrose as substrate, we found an equimolar incorporation of ^{14}C into fructose and glucan (Fig. 6), indicating that the *gtfA* enzyme catalyzed a GTF reaction. The incorporation of ^{14}C into the glucose polymer reached a maximum at 30 min, and upon prolonged incubation (24 h) we observed release of glucose, with a concomitant decrease of about 40% in the amount of glucan formed (7). Furthermore, purified *gtfA* enzyme was observed to slowly degrade the glucan, producing a product that had the same mobility as glucose in the paper chromatogram. Hence the *gtfA* enzyme also had a glucanase activity. Crude cell-free extracts from *E. coli* χ1274 also had a low glucanase activity, but produced a product with a mobility similar to sucrose. Incubation of the glucan with *E. coli* extract and *gtfA* enzyme together yielded the two different degradation products described above, indicating that the *gtfA* and the *E. coli* glucanase activities were probably different. However, incubation of the glucan and *gtfA* enzyme in the presence of high fructose concentrations (0.1 to 0.2 M) yielded a product with a mobility similar to sucrose (data not shown). The identity of all degradation products under all these conditions is currently unknown.

Presence of the *gtfA* enzyme in *S. mutans* cells. χ1274(pYA601) cells contained a sucrose-cleaving activity (Fig. 7, lane 1); such activity was not present in χ1274(pBR322) cell extracts (data not shown). As expected, the enzyme activity was removed from the extract by immunoprecipitation with anti-GTF serum (Fig. 7, lane 2). In the *S. mutans* UAB90 crude cell extract, two bands of sucrose-cleaving enzyme activities were detected (Fig. 7, lane 3), but only the faster-migrating species corresponded to the *gtfA* enzyme in χ1274(pYA601); it was immunoprecipitated from the extract by anti-GTF serum (Fig. 7, lane 4). We were unable to detect any individual band of sucrose-cleaving activity in culture supernatant fluids of *S. mutans* UAB90 cells on nondenaturing gels, since detectable activity remained at the top of the gels. However, we could detect by rocket immunoelectrophoresis the *gtfA* enzyme in supernatant fluids

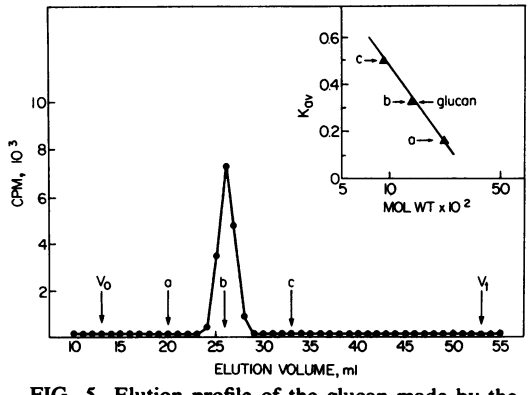

FIG. 5. Elution profile of the glucan made by the *gtfA* enzyme. Purified ^{14}C-labeled glucan (10,000 cpm) was chromatographed on a Bio-Gel P-10 column as described in the text. V_0, Void volume; V_t, total bed volume. Standards were: (a) dodecasaccharide of hyaluronic acid; (b) hexasaccharide of chondroitin-4-sulfate; (c) tetrasaccharide of chondroitin-4-sulfate. (Inset) Apparent molecular weight of the glucan. K_{av}, Partition coefficient.

of UAB62 and UAB90 cells grown in modified FMC medium. Using this methodology, we determined the amount of *gtfA* protein and analyzed its distribution in *S. mutans* UAB62 cultures grown in modified FMC medium to stationary phase. Fifteen percent of the *gtfA* protein was present in the supernatant fluids and 5% was released by two 1 M NaCl washes, whereas the rest of the *gtfA* protein activity was released from the washed cells by sonic disruption. Since sonic disruption is not very effective in lysing *S. mutans* cells, we could have overestimated the *gtfA* activity in culture supernatant fluids and NaCl washes. Furthermore, the *gtfA* activity released by sonication might have been, at least in part, associated with membranes.

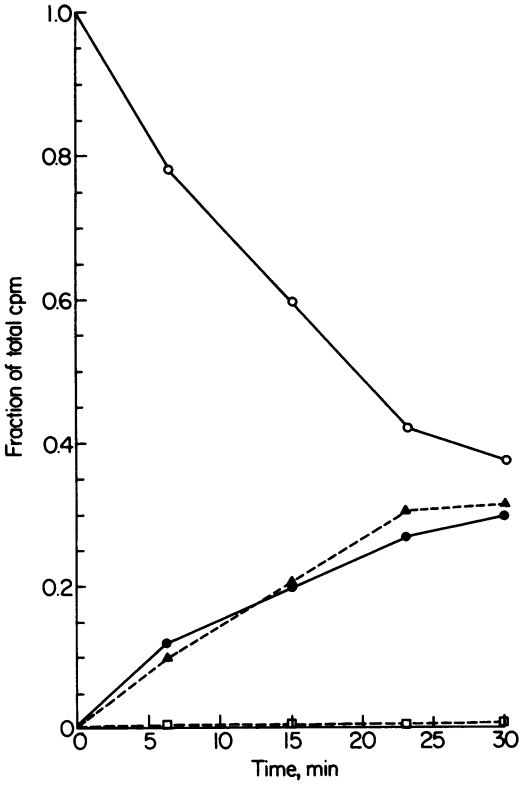

FIG. 6. Kinetics of the reaction catalyzed by the *gtfA* enzyme with sucrose as substrate. Reactions (0.5-ml volume) were carried out at 37°C in a mixture containing 50 mM sucrose–50 mM sodium phosphate buffer (pH 6.8), 30 μg of purified *gtfA* enzyme (from *E. coli*), and 20 μCi of [*U*-^{14}C]sucrose (3.6 Ci/mol). Samples were withdrawn at different times, and the reactions were stopped by heating at 100°C for 1 min. Radioactive reaction components were resolved by paper chromatography and identified as described in the text. Conversion of sucrose to glucan is shown by the proportion of each component at the times indicated. Symbols: ○, sucrose; ●, glucan; ▲, fructose; □, glucose.

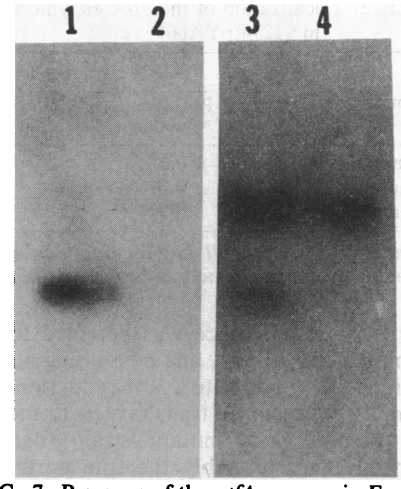

FIG. 7. Presence of the *gtfA* enzyme in *E. coli* and *S. mutans* cells. Sucrose-cleaving enzyme activities (dark regions) were detected in 10% nondenaturing polyacrylamide gels as described in the text. Lane 1, *gtfA* enzyme activity present in *E. coli* χ1274(pYA601) crude cell extracts; lane 2, absence of *gtfA* enzyme activity in χ1274(pYA601) crude cell extract after immunoprecipitation with anti-GTF; lane 3, enzyme activities in *S. mutans* UAB90 crude cell extract; lane 4, sucrose-cleaving enzyme activity remaining in *S. mutans* UAB90 crude cell extract after immunoprecipitation with anti-GTF.

Immunodiffusion in agarose gels showed that the same *gtfA* protein (no spur detected) was present in *E. coli* clones and cellular extracts (fraction B; see Materials and Methods) of *S. mutans* serotypes *c* (strains GS5, Ingbritt, and PS14), *e* (strain LM7), and *f* (strain OMZ175). A cross-reacting protein (bands of identity and nonidentity detected) was present in serotype *b* (strain BHT) but not in serotypes *a*, *d*, and *g* (R. Curtiss III, R. G. Holt, R. G. Barletta, J. P. Robeson, and S. Saito, Ann. N.Y. Acad. Sci., in press). No cross-reacting material was detected in any *S. mutans* culture supernatant extract (fraction A) from cells grown in tryptic soy broth (data not shown).

Presence of *gtfA* in the periplasmic space of *E. coli* and absence of processing. The cellular distribution of the *gtfA* enzyme in *E. coli* clones was determined by measuring the amount of *gtfA* protein released by cold osmotic shock compared with the amount remaining in the shocked cells (Table 2); β-galactosidase (cytoplasmic marker) and β-lactamase (periplasmic marker) were assayed to assess the degree of contamination of one fraction with the other. The *gtfA* enzyme was present in both the periplasmic fraction and the shocked-cell fraction. However, only 15% of the total *gtfA* enzyme was released from the periplasm by cold osmotic shock. No variation in the amount of *gtfA* protein present

TABLE 2. Localization of the *gtfA* enzyme activity in χ1274(pYA601) cells[a]

Enzyme	Relative enzyme activity	
	Periplasmic fraction	Shocked cells
gtfA enzyme	0.15	0.85
β-Galactosidase	0.01	0.99
β-Lactamase	0.95	0.05

[a] Preparation of *E. coli* protein fractions and enzyme assays were performed as described in the text.

in the periplasmic space was detected for two isogenic *E. coli* strains, one possessing an *envZ* mutation (14, 46), despite a 30% reduction in the amount of β-lactamase translocated to the periplasm of the *envZ* mutant strain (data not shown). Hence, probably the transport of the *gtfA* protein into the periplasmic space of *E. coli* does not follow the pathway of a typical *E. coli* exported protein such as β-lactamase. Alternatively, the *gtfA* enzyme could be bound to the cytoplasmic membrane facing the periplasmic space; that would also explain the release of some *gtfA* enzyme by cold osmotic shock.

In accordance with the above results, both χ1849(pYA601) and χ1274(pYA601) could grow on sucrose as sole carbon source. Since sucrose can enter the periplasm but not the cytoplasm, at least in the absence of the lactose carrier (19), part of the *gtfA* enzyme should have been present in the periplasmic space of *E. coli* recombinant clones. Figure 8 shows growth of χ1274 containing pYA601 or pBR322 on various carbon sources. χ1274(pYA601) grew better with lactose plus IPTG and raffinose plus IPTG than with sucrose, which should be hydrolyzed in the periplasm with the subsequent transport of the resulting monosaccharides. χ1274(pBR322) grew slowly with raffinose plus IPTG, since it is Gal^+, and thus could use the galactose split from raffinose by the *E. coli* α-galactosidase (χ1849 used in the original cloning, which is Gal^-, cannot grow on raffinose plus IPTG). Indeed, the *gtfA* enzyme seems to catalyze the same reaction in the periplasmic space in the in vivo system as the reaction catalyzed in vitro. Incubation of *E. coli* clones with [*U*-^{14}C]sucrose and analysis by paper chromatography of the reaction products released by cold osmotic shock showed that a glucan was also synthesized in the periplasmic space (data not shown).

DISCUSSION

S. mutans genes involved in sucrose metabolism can be cloned and expressed in *E. coli* K-12. Specifically, an *S. mutans* gene coding for a sucrose-hydrolyzing enzyme with GTF activity was cloned. Clones were selected for the ability to utilize sucrose generated inside *E. coli* cells by cleavage of raffinose by α-galactosidase. Direct hydrolysis of raffinose was ruled out since it was not a substrate for the purified *gtfA* enzyme (data not shown).

The high level of expression of the *gtfA* gene in *E. coli* minicells (Fig. 2B), along with RNA polymerase-binding data (Fig. 3), showed that *E. coli* cells could express the *S. mutans* genetic information as it does genes of other gram-positive microorganisms (3, 13). The complete

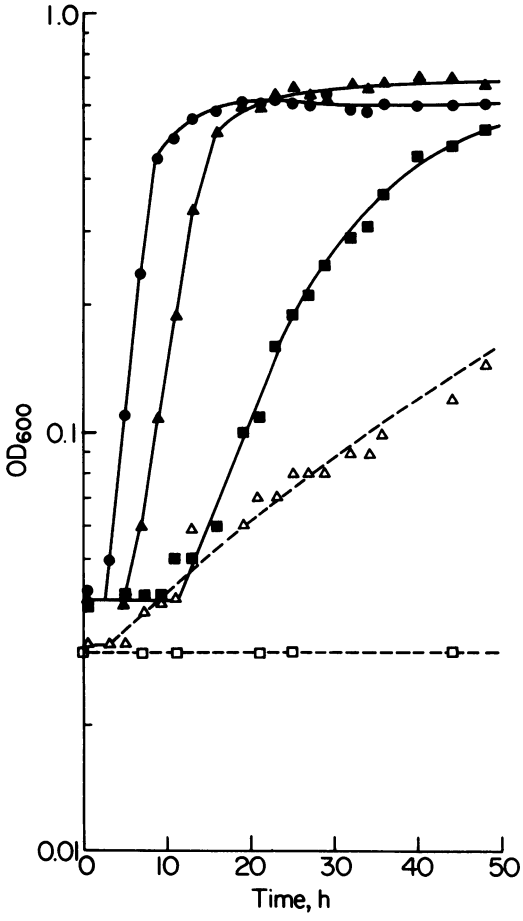

FIG. 8. Growth of *E. coli* χ1274 cells containing pYA601 or pBR322 in liquid minimal medium with various carbon sources. Cultures were grown overnight in minimal medium containing 1% glycerol. Cells were harvested, washed two times in buffered saline with gelatin, and then inoculated 1:20 into prewarmed minimal medium containing the indicated carbon source at 25 mM, with IPTG, when present, at 0.1 mM. Growth at 37°C with aeration was monitored by increase in optical density measured at 600 nm (OD_{600}). Symbols: ●, χ1274(pYA601) in lactose-IPTG; ▲, χ1274(pYA601) in raffinose-IPTG; ■, χ1274(pYA601) in sucrose; △, χ1274(pBR322) in raffinose-IPTG; □, χ1274(pBR322) in sucrose.

coding sequence for the *gtfA* protein was contained in the *S. mutans* UAB90 DNA cloned in pYA601. This conclusion is supported by analysis of a *S. mutans* GS-5 recombinant clone obtained from a cosmid library (Robeson, Ph.D. thesis). Cell extracts from this recombinant clone (which carried a larger *S. mutans* DNA insert than in pYA601) showed the presence of a sucrose-hydrolyzing activity and the same migration as the *gtfA* enzyme in denaturing and nondenaturing polyacrylamide gels. In addition, the *gtfA* protein from *E. coli* clones and *S. mutans* cells had the same mobility in nondenaturing polyacrylamide gels (Fig. 7).

We found that the *gtfA* enzyme had a GTF activity since it produced a small water-soluble glucan by hydrolysis of sucrose. The K_m for sucrose as substrate is similar to the one reported by Fukui et al. (9) for a serotype *a S. mutans* GTF. The glucan produced by the *gtfA* enzyme was fairly homogeneous in size, since it gave a sharp peak in gel filtration chromatography (Fig. 5). We have not yet determined the nature of the glycosidic linkages in the glucan. This information will be of importance in determining the putative role of the *gtfA* enzyme, if any, in the formation of water-soluble glucans, which are preponderantly $\alpha(1\rightarrow6)$, or water-insoluble glucans, which are preponderantly $\alpha(1\rightarrow3)$ (8, 15). The small size of the glucan synthesized by the *gtfA* enzyme suggests that this glucan could be a primer for the synthesis of larger glucan products. This hypothesis is currently being tested. The role of the glucanase activity associated with the *gtfA* enzyme has not been elucidated since we have not yet determined the optimal conditions to measure this degradative activity. Indeed, the *gtfA* enzyme catalyzed a very slow hydrolysis of its own synthetic product. Nevertheless, it is attractive to hypothesize that the glucanase activity plays a role in the overall process of glucan formation by *S. mutans*.

The faster-migrating enzyme species of the *S. mutans* UAB90 cell extract corresponded to the *gtfA* enzyme in *E. coli* clones (Fig. 7). This sucrose-hydrolyzing activity in *S. mutans* cells, like the cloned gene product, was also heat stable (7). Evidently, the slow-migrating (heat-labile) activity in *S. mutans* cells corresponds to a different enzyme(s).

According to the distribution of the *gtfA* protein between cells and culture supernatant fluids of *S. mutans*, the *gtfA* enzyme seems to be tightly associated with *S. mutans* cells. The *gtfA* protein was not efficiently released by two 1 M NaCl washes, a treatment that was previously shown to remove most of the cell-associated GTF activity in *S. mutans* (22, 25). We have not yet studied the effect of cultural conditions and growth cycle upon the amount or localization or both of the *gtfA* enzyme in *S. mutans* cells and supernatant fluids. It has been shown that these parameters have an effect on the total *S. mutans* GTF activity (22), which is probably due to several enzymes. The distribution of the *gtfA* enzyme in various *S. mutans* serotypes was in perfect agreement with previous studies on antigenic relatedness of GTF enzymes from *S. mutans* (27, 45) and strongly supports the subdivision into genospecies proposed by Coykendall (5).

The *gtfA* enzyme seems to be either translocated into the periplasmic space or bound to the cytoplasmic membrane facing the periplasmic space in *E. coli*. If part of the *gtfA* enzyme is translocated across the cytoplasmic membrane, this transport would be in the absence of N-terminal modification. Had the putative cytoplasmic *gtfA* protein been cleaved into a small leader polypeptide and a secreted product during translocation into the periplasmic space, we would have observed a double or an asymmetric *gtfA* peak in the autoradiogram from χ1849(pYA601) minicells (Fig. 2B). Instead, the existence of an internal hydrophobic sequence (31) or the existence of an N-terminal signal sequence not recognized by the *E. coli* signal peptidase (30) is possible. Besides, that the *envZ* mutation which affects transport of several *E. coli* periplasmic and cell envelope proteins (14, 46) did not have any effect on the transport of this GTF suggests the existence of a different mechanism for the translocation of proteins in gram-positive as opposed to gram-negative microorganisms. Alternatively, the tight association of the *gtfA* enzyme with *S. mutans* cells in conjunction with the absence of N-terminal modifications and the low level of release by cold osmotic shock in *E. coli* are compatible with the idea that this protein is imbedded in the cytoplasmic membrane in both *S. mutans* and *E. coli* but with the catalytic site to the exterior.

Our approach illustrates the validity of the recombinant DNA approach to the study of *S. mutans* genes involved in sucrose metabolism. These *S. mutans* gene products, like the *gtfA* enzyme, could be easily detected and purified from *E. coli* cells. Eventually, the cloned *S. mutans gtfA* gene can be mutated in vitro and transformed into *S. mutans* strains to obtain isogenic strains of mutants to assess the role of the *gtfA* enzyme in *S. mutans* cariogenicity.

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