Cloned gtfA Gene of Streptococcus mutans LM7 Alters Glucan Synthesis in Streptococcus sanguis

MICHAEL J. PUCCI AND FRANCIS L. MACRINA

Department of Microbiology and Immunology, Virginia Commonwealth University, Richmond, Virginia 23298

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Streptococcus mutans LM7 (Bratthall serotype e) chromosomal DNA was partially digested with EcoRI and ligated into the positive-selection plasmid vector pOP203(A2+). The ligation mixture was transformed into Escherichia coli, and transformants were selected for tetracycline resistance. Recombinant-bearing clones were screened for their ability to ferment raffinose, using the procedure of Robeson et al. (J. Bacteriol. 153:211-221, 1983). One raffinose-fermenting clone was isolated and found to contain a plasmid with an insert consisting of four EcoRI fragments totalling approximately 10.3 kilobases (kb). This strain was capable of growth on defined medium plus raffinose or sucrose and generated reducing sugars from a sucrose substrate. Southern hybridization analysis of the four EcoRI fragments revealed homology not only to S. mutans LM7 chromosomal DNA but also to S. mutans serotypes b, c, and f. Subcloning of this fragment array into a streptococcal E. coli shuttle vector indicated that a 2.4-kb EcoRI fragment was essential for sucrose activity. E. coli minicell experiments revealed a gene product of 55 kilodaltons. These data along with restriction endonuclease analysis and Southern hybridizations suggested that the cloned S. mutans LM7 gene was closely related to the gtfA gene cloned by Robeson et al. from S. mutans PS13 (Bratthall serotype c). The shuttle plasmid containing the 2.4-kb fragment was transformed into Streptococcus sanguis, which subsequently displayed increased sucrose activity in both intracellular and extracellular fractions. Elevated levels of synthesis of alcohol-insoluble and water-insoluble glucans were observed with crude extracellular fractions of the S. sanguis strain bearing the 2.4-kb fragment. An isolate cured of the shuttle plasmid plus the 2.4-kb fragment displayed wild-type S. sanguis glucan synthesis. In S. sanguis, this gtfA allele may play a role in glucan synthesis by interacting with extant high-molecular-weight glucosyltransferases.

Streptococcus mutans is recognized as a primary causative agent of dental caries (11, 24). Two characteristics responsible for the pathogenicity of this organism are (i) its ability to colonize the hard tooth surface (12, 14) and (ii) its ability to produce large amounts of lactic acid from fermentable carbohydrate (9, 39). Although strains of S. mutans are phenotypically homogeneous, they display significant heterogeneity by various genetic, biochemical, and serological criteria (15). Bratthall (1) reported the presence of five serotypes, a, b, c, d, and e, which were found to be due to unique cell wall-associated carbohydrates. Subsequently, two more serotypes, f and g, have been discovered (33). Serotype c is the most frequently isolated serotype of S. mutans from human plaque, with serotypes d, e, f, and g being occasionally isolated (11, 33). S. mutans has also been divided into five species: mutans (serotypes c, e, and f), ratti (serotype b), cricetus (serotype a), sobrinus (serotypes d and g), and ferus (serotype c) (7). For convenience in this report, we will refer to the strains by their serotype designation.

From both animal models and human dietary studies, it is apparent that cariogenicity is sucrose dependent (13, 37). There is compelling evidence that water-insoluble glucans produced by extracellular glucosyltransferases, which use sucrose as a substrate, contribute to virulence. Mutants deficient in synthesis of these polysaccharides demonstrated reduced cariogenicity in animals (38). Several other enzymes are involved in sucrose metabolism in S. mutans, but the roles of these remain unclear, as does the exact mechanism(s) of virulence. One approach to the study of these questions involves the use of recombinant DNA methodology to isolate, produce, and further characterize these enzymes.

Cloning S. mutans genes in Escherichia coli offers the advantage of a sucrose nonutilizing host and the choice of a wider variety of cloning vectors. One group of vectors becoming increasingly more popular are termed positive-selection vectors (4, 34, 42). One such vector, pOP203(A2+) (42), was used as a cloning vehicle in the work described here. These vectors contain genes that are lethal to the host bacterial cell and that contain unique restriction endonuclease sites. When DNA fragments are inserted at one of these sites, expression of the lethal gene is disrupted, and the transformed bacterium survives entry and replication of the plasmid. Hence, recombinant plasmids are directly selected by using these vectors.

This paper describes the cloning of a sucrose gene from S. mutans LM7 (serotype e) into E. coli by using the pOP203(A2+) plasmid. Southern hybridization analyses investigated the occurrence of the gene and flanking DNA sequences among the various serotypes of S. mutans. This gene was found to be homologous to the gtfA gene cloned by Robeson et al. (35) from an S. mutans serotype c strain into E. coli. Subcloning of this gene into a transgeneric shuttle vector, pVA856 (26), and transformation into Streptococcus sanguis Challis revealed stimulatory effects upon endogenous polysaccharide synthesis and sucrose activity in the resulting S. sanguis transformant.

MATERIALS AND METHODS

Bacterial strains and media. The strains used or constructed in this work are described in Table 1. Streptococcal strains were grown in brain heart infusion broth (Difco Laboratories, Detroit, Mich.). This broth containing 10% heat-inactivated (56°C for 30 min) horse serum was used to grow streptococcal cells to competence for genetic transform-
tion. Streptococci were also grown in the chemically defined FMC medium (40). *E. coli* strains were cultivated in LB broth (8) or minimal salts (29) medium supplemented with glucose, sucrose, or raffinose (1%). MacConkey base agar (Difco) was supplemented with 1% carbohydrate. Erythromycin or tetracycline (Sigma Chemical Co., St. Louis, Mo.) was added to media at a concentration of 10 μg/ml. Isopropyl-
β-D-thiogalactoside (IPTG; Sigma) was added to the media for a final concentration of 0.5 mM where indicated.

Plasmid and chromosomal DNA isolation. Covalently closed circular plasmid was isolated from *E. coli* by the method of Clewell and Helinski (6). *E. coli* strains were screened rapidly for plasmid DNA as previously described (28). Streptococcal strains were screened for plasmid DNA by the same method with minor modification (28). Chromosomal DNA was obtained by using the method of Marmur (30). Purified DNA and restriction endonuclease digestions were analyzed by agarose gel electrophoresis or polyacrylamide gel electrophoresis. Size standards were linear phage lambda DNA digested with *Hind*III (Bethesda Research Laboratories, Gaithersburg, Md.) or *φX174* DNA digested with *Hae*III (Bethesda Research Laboratories).

**DNA enzymology.** Restriction endonuclease digestions were performed in low-, medium-, or high-salt buffers as recommended by Maniatis et al. (29). Partial DNA digestions were carried out with an appropriate enzyme dilution at 37°C for 1 h (29). DNA ligation reactions were performed with T4 DNA ligase (Bethesda Research Laboratories) at 4°C for 18 h. Restriction enzymes were obtained from Boehringer Mannheim Biochemicals, Indianapolis, Ind.

**Bacterial transformations.** *E. coli* was transformed by the CaCl$_2$-heat shock method (8), with cells harvested at an absorbance of 0.2 to 0.3 (660 nm). Competent *E. coli* V871 cells also were cryogenically stored at −70°C and used in

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**TABLE 1. Bacterial strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype, or phenotype or Bratthall serotype*</th>
<th>LM7 EcoRI fragment insert(s)</th>
<th>Plasmid and designation</th>
<th>Plasmid size (kb)</th>
<th>Comments (reference)</th>
</tr>
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<tbody>
<tr>
<td><em>E. coli</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V871</td>
<td>hsdR4 Gal$^+$</td>
<td>None</td>
<td>pOP203(A$_2^+$) Tc$^+$</td>
<td>7.0</td>
<td>Sk1592 from S. Kushner (21), University of Georgia D1204 from L. Gold and R. Winter (42), University of Colorado</td>
</tr>
<tr>
<td>V875</td>
<td>lacP$^{ab}$</td>
<td></td>
<td>pBH20 Tc$^+$ Ap$^f$</td>
<td>4.4</td>
<td>From H. Henyeker (16), Genentech, Inc. M2141 from F. Neidhardt, University of Michigan</td>
</tr>
<tr>
<td>V880</td>
<td>Δ(pro-lac) Str$^+$ minA-minB</td>
<td>None</td>
<td>pYA601 Ap$^f$ gtfA</td>
<td>6.1</td>
<td>∇1849(pYA601) from R. Curtiss III (35), Washington University</td>
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<tr>
<td>V900</td>
<td></td>
<td></td>
<td>pVA1009$^c$</td>
<td>17.3</td>
<td>This paper: pOP203 (A$_2^+$)::EcoRI fragments A-D</td>
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<td>V1148</td>
<td>hsdR2 dapD8</td>
<td>pVA856::EcoRI</td>
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<td></td>
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<td>pVA380-1</td>
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<td>(27)</td>
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<tr>
<td>V685</td>
<td>Chalis background</td>
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<tr>
<td>V857</td>
<td>Em$^c$ Cm$^f$</td>
<td>pVA856$^c$</td>
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<td>9.2</td>
<td>Shuttle plasmid (26)</td>
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<td>V1129</td>
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<td>pVA1120$^c$</td>
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<td>V1213</td>
<td>Tc$^c$</td>
<td>pVA868$^c$</td>
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<td>13.5</td>
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<td>V1214</td>
<td>Em$^c$</td>
<td>pVA1120$^c$</td>
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<td>11.6</td>
<td>Isolate equivalent to V1129</td>
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<tr>
<td>H56</td>
<td>a</td>
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<td>FA-1</td>
<td>b</td>
<td></td>
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<td></td>
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<td>(18)</td>
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<td>V403</td>
<td>?</td>
<td></td>
<td></td>
<td></td>
<td>Clinical isolate</td>
</tr>
<tr>
<td>V825</td>
<td>?</td>
<td></td>
<td></td>
<td></td>
<td>Clinical isolate; Tc$^c$ (41)</td>
</tr>
</tbody>
</table>

* Ra$t^c$, raffinose fermenting; Gal$^+$, galactose nonfermenting; hsdR, host-specific restriction deficient; Δ(pro-lac), deletion encompassing proline and lactose loci; minA minB, minicell producer; dap, diaminopimelic acid requiring; Tc$^c$, growth on media containing >10 μg of tetracycline per ml; Ap$^f$, growth on media containing >25 μg of ampicillin per ml; Em$^c$, growth on media containing >10 μg of erythromycin per ml; Cm$^f$, growth on media containing >10 μg of chloramphenicol per ml; Str$^f$, growth on media containing >100 μg of streptomycin per ml. Bratthall serotypes of *S. mutans* are described in the text.

* Hyperlactose repressor-producing mutation carried by *F lac* exogenote.

* Recombinants.
706  PUCCI AND MACRINA

in 1% per ml. Growth was at 37°C with aeration, and increase in optical density at 600 nm (OD600) was measured. Symbols: ●, growth in 1% glucose; △, growth in 1% raffinose; ○, growth in 1% sucrose.

transformation, identified by their reddish color, were picked for further characterization.

Construction of S. sanguis V1213 by plasmid incompatibility-induced curing. S. sanguis V1129(pVA1120 EmR gtfA+) was transformed to TcR with pVA868 (41). pVA1120 and pVA868 were incompatible. Transformants selected on medium containing 10 μg of tetracycline per ml were transferred to medium containing 10 μg of erythromycin per ml and checked for erythromycin sensitivity. TcR EmR colonies were examined for plasmid content by agarose gel electrophoresis. Frequency of curing of the pVA1120 plasmid under such conditions was about 25%.

Preparation of minicells and electrophoresis analysis of plasmid-encoded proteins. E. coli minicells (strain M2141) were purified and labeled essentially as described by Kennedy et al. (17), except that before labeling, cycloserine was added to the minicell preparation at a final concentration of 40 μg/ml for 90 min at 37°C. Labeling of proteins was performed by incubation with 20 μCi of [35S]methionine (New England Nuclear Corp., Boston, Mass.) in methionine assay media (Difco). Minicell lysates were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (22) on a 10% polyacrylamide slab gel with a 3% stacking gel. Molecular weight standards were purchased from Pharmacia Fine Chemicals, Piscataway, N.J. Gels were dried, and autoradiography was carried out for 24 h or longer at −70°C with Kodak K-Omat R film.

Preparation of streptococcal protein fractions. S. mutans or S. sanguis strains were grown in 10-ml volumes overnight at 37°C aerobically in chemically defined medium (39; glucose as the carbon source) supplemented with 10 mM sodium bicarbonate. These cultures then were used to inoculate 1 liter of the same medium. Aerobic growth of the cultures at 37°C for 24 to 36 h was allowed to proceed. Cells were harvested by centrifugation at 9,000 × g for 20 min in a Sorvall GSA rotor.

(i) Cellular fractions. The washed cells were resuspended in 10 ml of 10 mM sodium phosphate buffer (pH 6.5) and

FIG. 1. Restriction endonuclease site map of pOP203(A2+). pOP203(A2+) is 7.0 kb and contains a tetracycline resistance marker in the location indicated. The Q8 phage maturation protein, A2, gene follows the lactose promoter-operator. Unique restriction endonuclease sites within the A2 gene that may be used for insertional inactivation include: Xhol, EcoRI, SrfI, and BglII. The construction and characterization of this vector has been published by Winter and Gold (42).

FIG. 2. Growth of E. coli V871 and V1009 in minimal medium. Cultures were grown overnight in broth. Cells were harvested, washed with buffered saline, and inoculated 1:50 into prewarmed minimal medium containing 1% carbohydrate, 0.5 mM IPTG, and 10 μg of thiamine hydrochloride per ml. Growth was at 37°C with aeration, and increase in optical density at 600 nm (OD600) was measured. Symbols: ●, growth in 1% glucose; △, growth in 1% raffinose; ○, growth in 1% sucrose.
FIG. 3. Southern blot hybridization of pVA1009 EcoRI fragments to S. mutans chromosomal DNA. (A) Ethidium bromide-stained 0.7% agarose gel of S. mutans chromosomal DNA cleaved with EcoRI. The lane designations are as for panel B below. (B) S. mutans chromosomal DNA cleaved with EcoRI with 32P-labeled pVA1009 as a probe. Lanes (Brathall serotype): A, HS6 (a); B, FA-1 (b); C1, GS5 (c); C2, V403 (c); C3, V825 (c); D, 0-1 (d); E, LM7 (e); F, OMZ175 (f); G, 6715 (g); P, pVA1009 probe hybridized to itself.

were disrupted in a French pressure cell (American Instrument Co., Silver Spring, Md.). This was followed by centrifugation of unbroken cells and cell debris at 3,000 × g and retention of the supernatant.

(ii) Extracellular fractions. Streptococcal extracellular fractions consisted of ammonium sulfate (60% saturated) precipitations of culture supernatants at 4°C. The precipitates were suspended in 10 ml of 10 mM sodium phosphate buffer (pH 6.5) and dialyzed against the same buffer. Further concentration was achieved by lyophilization and resuspended in 1 ml of sodium phosphate buffer. After dialysis, any remaining insoluble material was removed by centrifugation at 12,000 × g in an Eppendorf microcentrifuge (Brinkmann Instruments, Westbury, N.Y.) for 2 min.

Enzyme assays. All protein concentrations were determined by the method of Lowry et al. (25), using bovine serum albumin (J. T. Baker Chemical Co., Phillipsburg, N.J.) as the standard. Assays were done by using crude cellular and extracellular extracts as described above. Generation of reducing sugars from sucrose at 37°C was measured by the method of Nelson (32). Cultures (10 ml) of E. coli were grown overnight, pelleted, washed twice with phosphate-buffered saline and resuspended in 10 ml of saline. Toluene (0.5%, vol/vol) was added, and the tubes were shaken mechanically for 10 min at 37°C. Toluenized cells (1 ml) were mixed with 1.0 ml of 0.2 M sucrose in phosphate-buffered saline and incubated at 37°C. Samples (0.1 ml) were taken every 30 min and assayed as described by Nelson (32). Streptococcal reducing sugar assays were done with 0.1 ml of intracellular or extracellular preparations in 1.0 ml of 0.1 M sucrose in phosphate-buffered saline. Synthesis of total exopolysaccharides at 37°C from streptococcal extracellular fractions was determined by the method of Germaine et al. (10). The preparation was added (10 μl) to reaction tubes containing 10 μl of sucrose, 50 μg of dextran, and [U-14C]sucrose (1 to 5 mCi/mmol) at approximately 5 μCi/ml in a 100-μl total volume of 50 mM sodium acetate buffer (pH 5.5). Tubes were incubated at 37°C, and 10-μl samples were taken every 10 min. The samples were spotted on Whatman GF/A glass fiber filters, immediately submerged in methanol, and washed three times for 15 min each time. The filters were air dried, immersed in scintillation fluid (Amersham, Arlington Heights, Ill.), and insoluble radioactivity was determined by scintillation counting. Water-insoluble polysaccharide was measured by the procedure of Chassy et al. (3). This assay was similar to the that with methanol-insoluble polysaccharide except that filters with samples were placed in a Millipore filtration apparatus and washed three times with phosphate-buffered saline before being air dried and counted. [glucose-U-14C]sucrose or [fructose-U-14C]sucrose (0.042 Ci/mol) (New England Nuclear Corp.) was used, and samples were taken at 10-min intervals. Standardization was achieved by dividing counts per minute by the amount of protein present in the extracellular fraction aliquots used in the assays.

RESULTS

Construction and screening of S. mutans LM7 genomic library. Plasmid pOP203(A2~<sup>+</sup>), a pMB9 Tc<sup>+</sup> derivative, contains the lactose promoter-operator fused to the Qβ phage A<sub>2</sub> gene (Fig. 1), which is lethal to an E. coli host when it is expressed (42). This gene contains several unique restriction endonuclease sites, including EcoRI. When DNA fragments are inserted at these sites, expression of the A<sub>2</sub> gene is insertionally inactivated, and cells containing such recombinant plasmids can survive. This allows for positive selection of tetracycline-resistant recombinants in the presence of IPTG, an inducer of the lactose operon. Chromosomal DNA was isolated from S. mutans LM7, partially digested with EcoRI, and cloned into the EcoRI site of pOP203(A2~<sup>+</sup>). The ligation mixture then was transformed into E. coli V871. Clones then were screened by the procedure of Robeson et al. (35). Colonies were tested for their ability to ferment raffinose in the presence of IPTG. Raffinose is a trisaccharide which can be cleaved to yield galactose and sucrase or melibiose and fructose. Since E. coli V871 did not hydrolyze sucrose and was galactose nonutilizing, any raffinose fermenters should be capable of sucrose hydrolysis. Two raffinose-fermenting clones were detected after screening the equivalent of a genomic library as defined by Clarke and Carbon (5). One such clone, designated V1009, was selected for further study. Figure 2 shows that strain V1009 was capable of limited growth in both minimal sucrose and minimal raffinose media. A toluenized culture of strain V1009 was assayed for the production of reducing
sugars, using 0.1 M sucrose as the substrate via the method of Nelson (32). Increasing production of reducing sugars over time by V1009 compared to a control V871 culture verified that this strain was capable of hydrolyzing sucrose (data not shown).

Occurrence of cloned sequence among *S. mutans* serotypes.

Restriction endonuclease analysis of plasmid pVA1009 (17.3 kilobases [kb]) revealed that four *S. mutans* EcoRI chromosomal fragments had been cloned into the pOP203(As') vector (7.0 kb). These four fragments were 4.4, 2.4, 2.2, and 1.3 kb. In situ DNA hybridization experiments, using the method of Southern (36), revealed that all four of these EcoRI fragments hybridized to EcoRI-cleaved chromosomal DNA of serotypes c (strains GS5 and V403) and e (Fig. 3B). The 2.4-, 2.2-, and 1.3-kb fragments hybridized to corresponding fragments in serotype f chromosomal DNA and homology was also seen with a fourth higher-molecular-weight fragment. The 2.2- and 1.3-kb fragments hybridized to chromosomal DNA from serotype b along with at least two other fragments. Serotypes a, d, and g as well as *S. sanguis* Challis (data not shown) showed no hybridization under the same conditions. The pVA1009 EcoRI fragments hybridized only to a 2.4-kb EcoRI fragment (Fig. 3B) of strain V825, a serotype c clinical isolate. A "fingerprint" comparison of chromosomal DNA from serotypes a to g was performed by complete EcoRI digestion. Figure 3A shows that although serotypes c, e, and f have been reported to be related by biochemical and physical criteria (15), many differences are apparent in the digestion patterns of representative strains.

Subcloning of sucrase gene and analysis of protein products

![Diagram of restriction endonuclease site maps of pBH20 and pVA856](http://iai.asm.org)

**FIG. 4.** Restriction endonuclease site maps of pBH20 and pVA856. (A) pBH20 is 4.4 kb and contains ampicillin resistance (Ap') and tetracycline resistance (Tc') markers at the indicated locations. The lactose promoter-operator is followed by the β-galactosidase gene containing a unique EcoRI site. This vector has been described by Itakura et al. (16). (B) pVA856 is a 9.2-kb shuttle plasmid vector with chloramphenicol resistance (Cm'), Tc', and erythromycin resistance (Em') markers as indicated. This plasmid is further discussed by Macrina et al. (26).
DNA digested with EcoRI contained pBH20. These minicells were used to obtain unique fragments to MacConkey-raffinose-IPTG was transformed and examined using 2.4-kb containing raffinose-fermenting colonies were used to prepare the products of the clones were synthesized in this fashion. The results of these assays are summarized in Table 3. First, neither S. sanguis V857 nor V1129 produced fructan. V1129 showed an approximate twofold increase in total glucan synthesis, and as illustrated, it appeared that almost all of this increase was in the form of water-insoluble glucan. A second independently derived isolate, S. sanguis V1214, displayed the same properties as did V1129 (Fig. 7A). In addition, S. sanguis V1129 was cured of the pVA1120 plasmid by transforming with an incomparable plasmid, pVA868 Tc', into the strain, selecting for Tc', and screening for the Em' phenotype. Both pVA1120 and pVA868 cannot simultaneously exist in the same cell since both plasmids replicate with the pVA380-1 replicon (26, 41). A strain (V1213) missing the pVA1120 plasmid was constructed in this fashion. V1213 displayed in vitro exopolysaccharide synthesis levels almost identical to those of S. sanguis V857 (Fig. 7A).

**DISCUSSION**

A sucrose-hydrolyzing gene from S. mutans was cloned into and expressed in E. coli. This was accomplished through cloning of gtfA. The gene was used as a probe, it hybridized to pVA601 and the 2.4-kb fragment in the same serotypes (data not shown).

**Effects on sucrose activity and polysaccharide synthesis in S. sanguis.** The shuttle plasmid pVA1120 was transformed into S. sanguis Challis strain V685. When assayed for in vitro production of reducing sugars, V1129 showed increases in activity both intracellularly and extracellularly when compared with S. sanguis V857 (data not shown). The plasmid pVA856. However, sucrose activity levels in both fractions were far below those of S. mutans LM7 (Table 2). Strain V1129 also showed elevated in vitro exopolysaccharide synthesis when compared with S. sanguis V857. With [14C]sucrose as a substrate, 2 × 10^4 methyl-insoluble polysaccharides appeared, using V1129 crude extracellular fraction when compared with V857, as measured by counts per minute incorporated into polymer per milligram of protein present (Fig. 7A). Again, however, these levels were well below those of S. mutans LM7 (data not shown). These methyl-insoluble polysaccharides include both water-soluble and water-insoluble polymers. When the assay was repeated using only in vitro water-insoluble polysaccharide formation, amounts produced by V1129 were considerably elevated when compared with V857 but again less than S. mutans LM7 (Fig. 7B). Since S. mutans synthesized both glucans and fructans, the preceding assays were repeated with [14C]sucrose labeled in either the glucose or fructose portion to determine whether one or both were elevated in S. sanguis V1129. The results of these assays are summarized in Table 3. First, neither S. sanguis V857 nor V1129 produced fructan. V1129 showed an approximate twofold increase in total glucan synthesis, and as illustrated, it appeared that almost all of this increase was in the form of water-insoluble glucan. A second independently derived isolate, S. sanguis V1214, displayed the same properties as did V1129 (Fig. 7A).

**TABLE 2. Reducing sugar activity**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Location*</th>
<th>Avg μmol of reducing sugar†</th>
<th>Avg enzyme U°</th>
<th>Protein concn (mg)</th>
<th>Avg sp act</th>
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<tr>
<td>LM7</td>
<td>Intracellular</td>
<td>0.082</td>
<td>0.0024</td>
<td>0.0016</td>
<td>1.49 ± 0.37</td>
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<tr>
<td>V857</td>
<td>Intracellular</td>
<td>0.059</td>
<td>0.0028</td>
<td>0.200</td>
<td>0.012 ± 0.008</td>
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<tr>
<td>V1129</td>
<td>Intracellular</td>
<td>0.017</td>
<td>0.0053</td>
<td>0.009</td>
<td>0.060 ± 0.03</td>
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<tr>
<td>LM7</td>
<td>Extracellular</td>
<td>0.176</td>
<td>0.0061</td>
<td>0.0016</td>
<td>3.96 ± 0.36</td>
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<tr>
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<td>0.050</td>
<td>0.04 ± 0.011</td>
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<tr>
<td>V1129</td>
<td>Extracellular</td>
<td>0.050</td>
<td>0.0011</td>
<td>0.0035</td>
<td>0.32 ± 0.12</td>
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</table>

* Intracellular and extracellular fractions were obtained as described in the text.

† Determined by the method of Nelson (32).

° One unit is the amount of enzyme that produces 1 μmol of reducing sugar per min.

In minicells, the four EcoRI fragments of pVA1009 then were subcloned in an attempt to determine which fragment(s) contained the sucrose-hydrolyzing gene. This plasmid was digested with EcoRI and mixed with EcoRI-cleaved pVA856 or pBH20. These vectors are shown in Fig. 4. Plasmid pBH20 carries the lactose promoter-operator in front of a unique EcoRI site and a Tc' determinant (16). Plasmid pVA856 was constructed as a shuttle vector capable of replication in both E. coli and streptococci (26). The pVA1009 fragments were mixed with and allowed to ligate to these vectors. The resulting recombinants were used to transform V871 to tetracycline resistance. Colonies then were screened on MacConkey-raffinose-IPTG medium, and acid-producing clones were examined for plasmid content. All strains containing raffinose-fermenting recombinant plasmids shared a common 2.4-kb EcoRI fragment insert.

Plasmid DNA was isolated from both V1118 and V1120 and transformed into an E. coli minicell-producing strain. By using [35S]methionine to label newly synthesized proteins, we examined the products of plasmid-directed minicell protein synthesis by polyacrylamide gel electrophoresis. Figure 5 demonstrates that a 55-kilodalton (kdal) polypeptide was synthesized by both pVA1120 and pVA1118 (lanes B and C, respectively). Lanes A displays the proteins encoded by the pVA856 vector itself. Because the EcoRI site lies in the chloramphenicol acetyl transferase gene (approximately 25 kdal), a truncated product was expected in the case of the pVA1120-containing minicells. Such a truncated protein (approximately 17 kdal) appears in the pVA1120 lane (Fig. 5, lane B).

**Relationship of S. mutans LM7 sucrose gene to gtfA.** Since the 55-kdal polypeptide was similar in size to the gtfA protein of Robeson et al. (35) cloned from S. mutans PS14 (Bratthall serotype c), we next wished to determine whether these genes were related. Restriction endonuclease mapping of pVA1118 indicated that the 2.4-kb EcoRI fragment contained two HindIII fragments identical in size to those found in plasmid pYA601 which contains the gtfA gene (data not shown). In situ filter hybridization was performed by using pYA601 as a probe against plasmid pVA1120 and chromosomal DNA from S. mutans serotypes a to g digested with EcoRI. pYA601 showed homology to pVA1120 and 2.4-kb fragments in serotypes c, e, and f (Fig. 6). When pVA1120 was used as a probe, it hybridized to pVA601 and the 2.4-kb fragment in the same serotypes (data not shown).
the use of pOP203(A2 +), a positive selection vector (42). Use of such a vector eliminates the need for alkaline phosphatase treatment of plasmid DNA as 75 to 95% of the recombinant plasmids contained inserts. DNA fragments as large as 30 kb can be inserted into this vector (9a). Using the described screening procedure of Robeson et al. (35), we examined the equivalent of a genomic library of S. mutans LM7 yielding two raffinose-fermenting clones. No other raffinose-hydrolyzing activities were detected by using this screening method, and we suggest that other sucrose-hydrolyzing activities (e.g., invertases, glucosyltransferases, etc.) may not be able to use raffinose as a substrate. It is possible that EcoRI cleavage within another gene(s) is inactivating that gene(s); however, the use of partially digested chromosomal DNA should have eliminated this problem. Strain V1009 was selected for further study (V1008 was found to be a derivative of V1009). V1009 contained four EcoRI fragments totalling 10.3 kb. This strain was capable of growth in minimal sucrose and minimal raffinose media and hydrolyzed sucrose as determined by the generation of reducing sugar (32).

The four cloned EcoRI fragments were further analyzed to determine their occurrence in the other S. mutans Bratthall serotypes. We believe that the four EcoRI fragments of pVA1009 are adjacent to each other (i.e., represent a partially digested fragment) on the S. mutans chromosome for the following reasons. First, similar fragment patterns were present in serotype c and f strains as well as in the serotype e LM7 strain (Fig. 3). These similarities occur despite the obvious differences in the EcoRI fingerprint patterns of these serotypes (Fig. 3A). It is for this reason that we chose to use the serotype rather than the subspecies division of S. mutans as there are some differences apparent within subspecies at the DNA level. Second, another independently isolated raffinose-fermenting clone, V1008, contained fragments b, c, and d but not fragment a. Third, several raffinose-fermenting clones have been isolated from V403, a serotype c clinical isolate, and they also show either all of the EcoRI fragments or some combination of them (data not shown). These data argue against a random association of EcoRI fragments during the ligation reaction. The data in Fig. 3 enable us to argue that the sucrose-hydrolyzing gene has been conserved in serotypes c, e, and f along with some flanking DNA. The reason(s) for the conservation in these regions is not clear but may possibly reflect regulatory regions or clustering of

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**TABLE 3. Specificity of exopolysaccharides synthesized**

<table>
<thead>
<tr>
<th>Strain</th>
<th>dpm/mg of protein (10^4)</th>
<th>Substrate</th>
<th>Polysaccharide measured</th>
</tr>
</thead>
<tbody>
<tr>
<td>V857</td>
<td>387</td>
<td>[glucose-U-14C]sucrose</td>
<td>Total ethanol-insoluble glucan</td>
</tr>
<tr>
<td></td>
<td>63</td>
<td>[fructose-U-14C]sucrose</td>
<td>Total ethanol-insoluble fructan</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>[glucose-U-14C]sucrose</td>
<td>Water-insoluble glucan</td>
</tr>
<tr>
<td>V1129</td>
<td>773</td>
<td>[glucose-U-14C]sucrose</td>
<td>Total ethanol-insoluble glucan</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>[fructose-U-14C]sucrose</td>
<td>Total ethanol-insoluble fructan</td>
</tr>
<tr>
<td></td>
<td>325</td>
<td>[glucose-U-14C]sucrose</td>
<td>Water-insoluble glucan</td>
</tr>
</tbody>
</table>

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*See text for experimental details.*
genes involved in sucrose metabolism or catabolism. It is interesting to note that *S. mutans* V825, a serotype C clinical isolate, displayed hybridization only to the 2.4-kb fragment bearing the sucrase gene. The putative flanking sequences were not apparent in this strain as measured by DNA-DNA hybridization. We do not know yet whether there is a physiological difference between this strain and other serotype C strains.

Subcloning experiments revealed that the sucrose gene resided within a 2.4-kb EcoRI fragment. This fragment was cloned into plasmids pBH20 and pV8A56 as described above, the latter plasmid being capable of replication in both *E. coli* and streptococci (26). Restriction endonuclease analysis and minicell protein synthesis analyses yielded data consistent with those obtained with *E. coli* χ1849(pYA601), the strain described by Robeson et al. (35). A Southern hybridization experiment with pYA601 as a probe against plasmid pVA1120 and chromosomal DNA from *S. mutans* serotypes 7A). These results indicated homology between the strain LM7 sucrose gene and the gtfA gene under conditions of high stringency. We believe that we have cloned the ·· allele of the serotype C *gtfA* gene. The shuttle plasmid pVA1120 containing the *gtfA* gene was transformed into *S. sanguis* Challis. This strain showed increased activities of both intracellular and extracellular levels of reducing sugar activity when compared with the control strain *S. sanguis* V857 (which contains the shuttle plasmid pVA856 without gtfA). Similarly, V1129 showed increased glucan synthesis and, upon further study, increased water-insoluble glucan synthesis. This is significant in that *S. sanguis* normally produces little detectable water-insoluble glucan (3). An independently obtained second transformant of *S. sanguis* yielded results similar to those obtained with *S. sanguis* V1129. Curing pVA1120 from V1129 yielded wild-type *S. sanguis* exopolysaccharide synthesis levels (Fig. 7A). These data compellingly associate the glucan synthesis alterations seen in *S. sanguis* V1129 with the strain LM7 *gtfA* gene.

Several possibilities can account for these findings. The increased activities can be the result of direct expression of the cloned *S. mutans* *gtfA* gene. Alternatively, the cloned gene product might be indirectly affecting expression of a gene(s) already present in *S. sanguis*. These possibilities are not mutually exclusive. We favor the former notion. Since *S. sanguis* produces water-soluble glucans (3), the *S. mutans* cloned gene product may play a role in a branching reaction forming α-1,3 glucosidic linkages. The enzyme might work in association with other glucosyltransferases which have been previously reported (15, 19, 20; R. Curtiss III, personal communication). This could account for the production of water-insoluble glucan in the *S. sanguis* host. It is interesting to note that the cloned *gtfA* gene of *S. mutans* PS14 could be demonstrated to synthesize small (MW15000) glucans in *E. coli* (35). This observation, taken together with our data, leads to an attractive hypothesis: the *gtfA* gene product may be specifying the synthesis of a primer glucan that specific glucosyltransferases use in manufacturing water-insoluble glucan.

Since synthesis of water-insoluble glucan seems to be important in virulence, it would be interesting to examine whether *S. sanguis* V1129 might be pathogenic in an *in vivo* system. We are proceeding to dissect the biochemistry of the altered glucan synthesis of *S. sanguis* V1129 by a variety of approaches. We also plan to search for other glucosyltransferase activities in *S. mutans* LM7 using a lambda genomic library followed by immunological screening of the library with antibodies raised to extracellular antigenic determinants. Clearly, the application of recombinant DNA methodology to the study of *S. mutans* should afford us the opportunity to study all of the genetic components of glucan synthesis and help us better understand the nature of this established virulence trait.

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