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Isolation and Characterization of the Sucrose 6-Phosphate Hydrolase Gene from *Streptococcus mutans*

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The *Streptococcus mutans* GS-5 gene, *scrB*, coding for sucrose 6-phosphate hydrolase activity has been cloned into *Escherichia coli* utilizing the bacteriophage replacement vector λL47.1. DNA sequences containing the gene were initially subcloned into the moderate-copy-number plasmid vector pLG339 to yield active subclones. However, due to the instability of the resultant chimeric plasmids, the gene was subsequently subcloned into the low-copy-number vector pOU61 to yield the stable hybrid plasmid pMH613. Both plasmids contain a 6.6-kilobase *Eco*RI fragment from strain GS-5 and express both hydrolase and sucrase activities. The relative position of the gene in the insert has been determined after Tn*5* mutagenesis and deletion analysis. The cloned enzyme was purified to near homogeneity after gel filtration and anion-exchange chromatography, chromatofocusing, and preparative polyacrylamide gel electrophoresis. The purified enzyme displayed a molecular mass of 58 kilodaltons, which is significantly higher than the 48-kilodalton enzyme previously purified from *S. mutans* GS-5. These results suggest that processing of the hydrolase occurs in *S. mutans*.

A variety of experimental approaches have indicated that oral bacteria resembling *Streptococcus mutans* are involved in human dental caries formation (7). These microorganisms are able to synthesize water-insoluble glucans from dietary sucrose, which enhances tooth colonization (8). In addition, these organisms are able to rapidly convert sucrose to lactic acid, leading to tooth demineralization (17). Thus, the ability of these organisms to metabolize sucrose appears to be an important cariogenic property.

Although sucrose can be converted to extracellular polymers by various strains of *S. mutans*, the majority of the sucrose molecules metabolized by these organisms are transported into the cells and converted to acid end products (30). Recent results have suggested that these organisms may possess two different systems for transporting sucrose: a sucrose phosphotransferase system (26) as well as a nonphosphotransferase system (25). The former system would involve the formation and subsequent metabolism of the intermediate sucrose 6-phosphate (Suc-6-P). Earlier results indicated that several strains of *S. mutans* possessed intracellular invertase (EC 3.2.1.26) activity (11, 16, 29). However, more recent information indicated that these enzymes should be more accurately termed sucrose-6-phosphate hydrolases (Suc-6-PH), since both enzymes from strain 6715 copurify (3). In addition, the K_m for Suc-6-P is several orders of magnitude lower for these enzymes compared with sucrose. Since the intracellular invertases of serotype g (6715) and c (GS-5) are immunologically related (15), it is likely that the GS-5 enzyme also expresses Suc-6-PH activity, as previously suggested (3).

One approach toward further analyzing the mechanism of intracellular sucrose metabolism in *S. mutans* would be to isolate each of the genes involved through recombinant DNA techniques. Until recently, only the *gtfA* gene of *S. mutans* has been isolated and characterized (22). In addition, a 59-kilodalton (kDa) sucrase gene from *S. mutans* Ingbritt was recently identified in a phage library (18). However, neither the nature of the gene nor its product was further characterized. Utilizing a λ L47.1 cloning vector, several sucrose-metabolizing genes (*scrB*, *gtfA*, and genes encoding fructosyltransferase and glucosyltransferase activities) from *S. mutans* GS-5 (serotype c) have been isolated in this laboratory. The present communication describes the isolation and characterization of the *scrB* gene coding for Suc-6-PH activity.

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MATERIALS AND METHODS

Bacterial strains. *S. mutans* GS-5 was grown and maintained as previously described (11). *Escherichia coli* C600 was maintained on LB (1% Difco tryptone, 0.5% Difco yeast extract, 0.5% NaCl) agar plates and routinely grown at 37°C in LB medium. *E. coli* C600(pLG339) was kindly provided by N. Fairweather (Wellcome Research Laboratories, Beckenham, England), and strain C600(pOU61) was obtained from S. Molin (Odense University, Odense, Denmark). Antibiotics were added to growth media as indicated at the following concentrations: kanamycin, 50 μg/ml.

Preparation of *E. coli* cell fractions. To determine the cellular localization of cloned enzymes, cell fractions of strain C600 were isolated as previously described (9). By this procedure, the periplasmic and cytoplasmic membrane fractions were isolated. β-Galactosidase was assayed as a cytoplasmic marker, whereas alkaline phosphatase was utilized as a periplasmic marker.

Enzyme assays. Sucrase activity was determined by quantitating reducing sugar generation from sucrose as previously described (11). One international unit (IU) is defined as the amount of enzyme required to hydrolyze 1.0 μmol of sucrose per min under standard assay conditions. When intact cells were utilized, the cells were initially toluenized as described earlier (21). Suc-6-PH activity was measured by the coupled spectrophotometric assay as recently described (3). The substrate, Suc-6-P, was kindly provided by B. Chassy (National Institute of Dental Research, Bethesda, Md.).

Protein estimation was carried out by the method of Bradford (2) with bovine serum albumin as the standard protein.

Electrophoretic analysis. Polyacrylamide gel electrophoresis (PAGE) was carried out essentially as previously described (6) in 9% gels. Enzymatic activity in the gels was

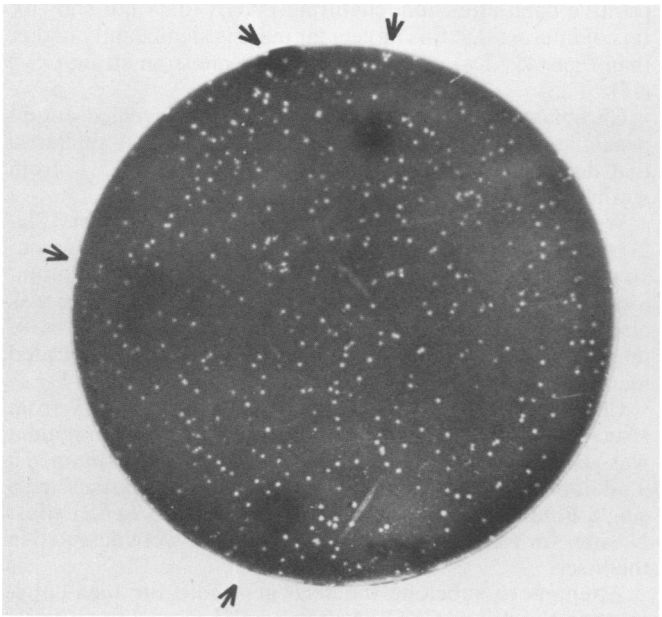

FIG. 1. Detection of sucrase-positive clones. Samples of the clone bank (10 μl) were mixed with *E. coli* C600 indicator cells and applied to LB agar plates containing sucrose and triphenyltetrazolium chloride in a soft agar overlay as described in the text. The arrows indicate sucrase-positive plaques.

detected by the triphenyltetrazolium chloride staining procedure after incubation of the gel at 37°C for 1 h in buffer containing 1% sucrose (15). Sodium dodecyl sulfate (SDS)-PAGE was performed by the method of Studier (28) in 7 or 9% gels.

DNA fragments were analyzed on 0.5 or 0.7% agarose gels with a Tris-EDTA-borate buffer (14).

Western blot analysis. For immunoblotting, proteins were transferred by electrophoresis from SDS-PAGE gels to nitrocellulose filters as described by Towbin et al. (31) and developed with anti-invertase (1:200) followed by chloronapthol staining.

Preparation of the GS-5 clone bank. The *S. mutans* GS-5 clone bank was prepared with the bacteriophage λL47.1 as recently described (H. Aoki, T. Shiroza, S. Sato, M. Hayakawa, and H. K. Kuramitsu, Infect. Immun., in press). Briefly, purified *S. mutans* GS-5 chromosomal DNA was partially cleaved with *Sau*3A1 and ligated to *Bam*HI-cleaved λL47.1. After in vitro packaging, the clone bank containing GS-5 inserts was harvested after infection of *E. coli* C600 (P2) indicator cells.

Screening for sucrase-positive clones. The GS-5 clone bank was screened for clones exhibiting sucrase activity essentially as previously described (18). Samples (10 μl) of the clone bank were incubated with C600 indicator cells (0.20 ml) for 15 min at 37°C, mixed with 3 ml of soft agar containing 1% sucrose plus 0.1% triphenyltetrazolium chloride and overlaid onto LB agar plates. After incubation for 16 h at 37°C, individual plaques exhibiting sucrase activity were detected by the presence of a reddish zone surrounding positive plaques. Individual plaques were removed with sterile Pasteur pipettes, suspended in λdil buffer (14), and amplified in *E. coli* C600 for further examination.

Subclones expressing sucrase activity were detected by plating cells on MacConkey agar base (GIBCO Diagnostics, Madison, Wis.) containing 1% sucrose at 30°C for 16 h. Sucrase-positive clones were detected by the appearance of red colonies.

DNA manipulations. DNA was purified from phage lysates as previously described (14). Plasmid DNA was routinely extracted from the appropriate cells propagated in LB medium containing antibiotics by the alkaline lysis procedure (1). For large-scale purification, plasmid DNA was further purified by ultracentrifugation in cesium chloride gradients containing ethidium bromide (14).

Restriction enzyme digestions were carried out according to the directions of the supplier (Bethesda Research Laboratories, Gaithersburg, Md.). Ligation reactions were carried out at 12°C for 16 h with T4 DNA ligase (Bethesda Research Laboratories).

Transformation of the *E. coli* recipients was carried out by the $CaCl_2$ proccedure cited previously (14). For Tn*5* mutagenesis, the procedure of DeBrujin and Lupski (4) was carried out. The λ Tn*5* derivative was kindly provided by C. Plate (Northwestern University).

Purification of the cloned Suc-6-PH. Cleared lysates of clone λSuc-6 were prepared from 1.0 liter cultures as described recently (14). Ammonium sulfate was dissolved in the pooled cleared lysate fluids to 70% saturation. After 16 h at 4°C with stirring, the precipitate was recovered by centrifugation at 16,000 × *g* for 20 min and dissolved in 20 ml of 10 mM Tris hydrochloride (pH 7.5). A small amount of insoluble residue was removed by centrifugation (20,000 × *g* for 20 min), and the resultant supernatant fluid (fraction 1, Table 1) was fractionated on a 2.5- by 45 cm Sephadex G-150 (Pharmacia Fine Chemicals, Piscataway, N.J.) column. The column was developed with 10 mM Tris hydrochloride buffer (pH 7.5), and 10-ml fractions were collected and assayed for sucrase activity. The active fractions (fraction 2) were pooled and applied to a DEAE–Bio-Gel A (Bio-Rad Laboratories, Richmond, Calif.) column (1.5 by 25 cm) and eluted with a 300-ml linear gradient from 0 to 0.5 M KCl in the same buffer. The active fractions (20 ml) were then pooled and precipitated with 70% ammonium sulfate.

The concentrated enzyme (fraction 3) was then dissolved in 2.0 ml of 0.025 M histidine hydrochloride buffer (pH 6.2) and dialyzed against the same buffer. The enzyme was applied to a chromatofocusing column (previously equilibrated with the same buffer) and eluted with Polybuffer 74 (pH 4.0). The active fractions were pooled (15 ml) and concentrated as described above. The enzyme was next dissolved in 1.0 ml of 0.14 M Tris hydrochloride buffer (pH 6.8) and dialyzed against the same buffer.

The sample (fraction 4) was applied to a 7% polyacrylamide preparative disc for PAGE (1 by 5 cm) and electrophoresed at 4°C, and the gel was sliced into 25 fragments. Each fragment was extracted with 1.0 ml of 0.1 M Tris hydrochloride buffer (pH 7.5) for 16 h at 4°C. The superna-

TABLE 1. Purification of Suc-6-PH from *E. coli* MH613[a]

Fraction	Total protein (mg)	Sp act (IU/mg)	Total activity (IU)	Recovery (%)	Purification (fold)
1. Ammonium sulfate	211	1.9	401	100	1
2. Sephadex G-150	65.7	3.6	237	61	2
3. DEAE–Bio-Gel A	9.0	16.9	152	39	9
4. Chromatofocusing	1.8	38.9	70	18	21
5. Preparative PAGE	0.5	86.0	43	11	46

[a] Suc-6-PH purification was carried out as described in the text by utilizing the sucrase activity to monitor each step.

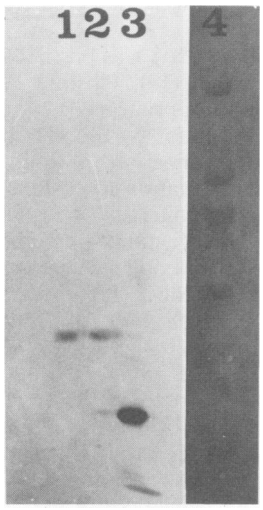

FIG. 2. Western blot analysis of Suc-6-PH activities. The enzyme fractions were subjected to 7% SDS–PAGE, transferred to nitrocellulose, and immunoblotted with anti-intracellular invertase. Lanes 1, crude extract of MH613; 2, purified Suc-6-PH (fraction 5); 3, partially purified *S. mutans* GS-5 invertase; 4, protein standards.

tant fluids were assayed for sucrase activity, and the active fractions served as the purified Suc-6-PH (fraction 5).

RESULTS

Isolation of a Suc-6-PH clone. A gene bank of *S. mutans* GS-5 DNA constructed in *E. coli* with the bacteriophage replacement vector λL47.1 was screened for sucrase activity. With the sucrose-tetrazolium agar plate procedure (Materials and Methods), more than 30 sucrase-positive clones were detected out of approximately 5,000 plaques (Fig. 1). All of the positive clones appeared to code for the same sucrase activity. One of the sucrase-positive clones, λSuc-6, was selected and purified for further investigation. This clone did not exhibit glucosyltransferase or fructosyltransferase activities when assayed directly. However, crude lysates of λSuc-6 displayed significant Suc-6-PH activity when assayed by the coupled assay procedure (3).

In addition, when λSuc-6 lysates were analyzed on PAGE followed by sucrase activity staining, a single sucrase activity migrating near the position of the Suc-6-PH (intracellular invertase) of strain GS-5 crude extracts was detected (data not shown). An additional sucrase band detected in the GS-5 extracts corresponded to GtfA activity (22), identified by utilizing authentic GtfA (supplied by R. Curtiss, Washington University, St. Louis, Mo.). Therefore, these results indicated that λSuc-6 contains the *S. mutans* GS-5 gene coding for Suc-6-PH activity.

Purification of the cloned Suc-6-PH. The Suc-6-PH of λSuc-6 lysates was purified to apparent homogeneity after gel filtration and DEAE-anion-exchange chromatography, chromatofocusing, and preparative PAGE (Table 1). Overall, Suc-6-PH was purified approximately 45-fold, with a recovery of 11%. To verify that the purified enzyme expressed the same activity detected in crude lysates, the kinetics of Suc-6-P hydrolysis were determined with the purified preparation by utilizing the standard assay system (3). Like the crude lysates, the purified enzyme also displayed strong hydrolyzing activity (data not shown).

The purified enzyme displayed a single protein band of approximately 58 kDa on SDS-PAGE and a single sucrase-positive band after nondenaturing PAGE (data not shown). It is of interest that this molecular mass is significantly higher than that (48 kDa) of the purified enzyme from strain GS-5 (15).

Chromatofocusing of the cloned enzyme revealed an approximate pI of 4.9 (data not shown). This value is similar to that determined for the homologous enzyme, pI 5.1, from strain GS-5 (15).

Western blot analysis of the purified cloned enzyme (Fig. 2) with anti-GS-5 intracellular invertase (15) also demonstrated a single major antigenic band of 58 kDa. In addition, a minor band (48 kDa) that reacted with the antibody was also detected in the purified enzyme preparation. It is of interest that this lower-molecular-weight species migrated identically with the invertase purified from strain GS-5.

Characterization of the Suc-6-PH gene. The DNA from λSuc-6 was purified, and a restriction map of the fragment was generated (Fig. 3a). The recombinant phage contained a 9.4-kilobase (kb) fragment of cloned GS-5 DNA possessing a single *Bam*HI site, two *Hin*dIII sites, and two *Eco*RI sites. No sites for *Cla*I, *Sal*I, *Xba*I, *Sph*I, or *Kpn*I were detected in the insert.

Attempts to subclone the *scrB* gene into the high-copy-number plasmid pACYC184 after *Bam*HI, *Eco*RI, or *Hin*dIII cleavage failed to yield active subclones. However, it was possible to subclone a 6.6-kb *Eco*RI fragment from λSuc-6 into the moderate-copy-number plasmid pLG339 (27) and to isolate sucrase-positive subclones. It was of interest that sucrase-positive subclones could be readily detected on MacConkey-sucrose agar plates despite the fact that *E. coli* cells are not known to transport sucrose into the cytoplasm. Subsequent analysis revealed that the chimeric plasmid pMH3395, containing the *scrB* gene, was unstable, since sucrase-negative subclones (containing deletions in the plasmid) were observed frequently after subculture.

Stable subclones containing the *scrB* gene were subsequently isolated by utilizing the low-copy-number, temperature-sensitive vector pOU61 (12). The 6.6-kb *Eco*RI fragment was ligated into the single *Eco*RI site of the vector to produce stable transformants coding for sucrase activity. One of these, MH613, was chosen for further investigation. The chimeric plasmid coding for Suc-6-PH activity, pMH613, was shown to harbor the 6.6-kb *Eco*RI fragment of λSuc-6 (Fig. 3b) and is stably maintained in the *E. coli* host cells below 37°C.

The putative promoter for initiating transcription of the *scrB* gene appeared to be located on the 6.6-kb *Eco*RI insert

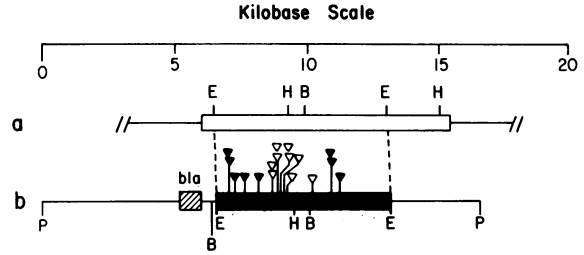

FIG. 3. Restriction maps of λ Suc-6 and pMH613 DNA. (a) λ Suc-6 DNA. Symbols: ——, λ L47.1; ▭, *S. mutans* GS-5 insert. (b) pMH613 DNA. Symbols: ——, pOU61; ▬, *S. mutans* GS-5 insert. Tn*5* insertion mutagenesis yielded (▼) sucrase-positive subclones and (▽) sucrase-negative subclones. The insertion sites were determined after *Bam*HI digestion of the plasmids (4). The restriction enzymes utilized included the following: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII.

TABLE 2. Localization of Suc-6-PH activity in *E. coli* MH613[a]

Enzyme	Relative enzyme activity (%) in:		
	Periplasm	Cytoplasm	Membrane
Suc-6-PH	0.4	95	4.6
β-Galactosidase	1.0	91	8
Alkaline phosphatase	62	35	3

[a] *E. coli* MH613 cells were fractionated into the three subcellular fractions (see text), and sucrase, β-galactosidase (cytoplasmic marker), and alkaline phosphatase (periplasmic marker) activities were assayed in each fraction.

of pMH613, since recloning of the insert into the vector in both orientations yielded sucrase-positive subclones (data not shown). In addition, further subcloning of the *scrB* gene from pMH613 indicated that the gene spans the single *Bam*HI site of the insert since it was possible to subclone the 3.1- and 3.5-kb *Eco*RI-*Bam*HI fragments of the insert into pOU61 individually and isolate subclones containing each fragment. However, both types of transformants were sucrase negative. Therefore, either the scrB gene or regulatory regions for the gene or both must span the *Bam*HI site on the 6.6-kb GS-5 insert.

To further define the location of the *scrB* gene, Tn*5* mutagenesis of pMH613 was carried out. A number of sucrase-positive and -negative transformants harboring Tn*5* insertions into the chimeric plasmid were isolated and mapped after agarose gel analysis of restriction fragments (Fig. 3b). The position of a number of Tn*5* insertions which inactivate the gene confirm the location of the *scrB* gene near the *Bam*HI site. Insertions resulting in the sucrase-negative phenotype spanned approximately a 2-kb region of the *Eco*RI insert. Since the 58-kD *scrB* gene would require a 1.6-kb fragment of DNA, it appears that the Tn*5* insertions covered the entire coding sequence for the gene and may extend into adjacent regulatory regions.

Localization of the subcloned Suc-6-PH. To determine the cellular localization of the Suc-6-PH in MH613, the cells were fractionated into the periplasmic, cytoplasmic, and membrane fractions (Table 2). The two marker enzymes β-galactosidase and alkaline phosphatase were found predominantly in the cytoplasm and periplasmic space, respectively. However, essentially all of the Suc-6-PH activity was detected in the cytoplasmic fraction (95%).

DISCUSSION

The data presented in this communication demonstrates that the sequence encoding the *scrB* gene of *S. mutans* GS-5 has been cloned in *E. coli* on a λL47.1 replacement vector. The gene has been subsequently subcloned into low-copy-number plasmid vectors for stable expression. Recombinant phage expressing this activity were detected at a relatively high frequency of approximately 1 in 100 to 200 in the strain GS-5 gene bank. In contrast, the same screening procedure for sucrase-positive clones led to the identification of only two fructosyltransferase clones (frequency of 1 in 1,000 to 2,000; S. Sato and H. K. Kuramitsu, submitted for publication) and no glucosyltransferase clones (Aoki et al., in press). It is likely that the *scrB* gene contains a very strong promoter in *E. coli* which may be responsible for the ease of detection of these clones. The instability of high-copy-number plasmids containing this gene in *E. coli* is compatible with such an explanation. It is also of interest that another *S. mutans* gene previously cloned, aspartate-semialdehyde dehydrogenase, also appears to express strong promoter activity in *E. coli* (10).

The low-copy-number hybrid plasmid pMH613 containing the cloned *scrB* gene was stably maintained in *E. coli* C600. Plasmids containing the 6.6-kb *Eco*RI insert in either orientation relative to the vector also expressed strong enzymatic activity. Therefore, hybrid plasmids containing this insert also possess the putative promoter for the *scrB* gene. The instability of C600 cells harboring moderate- or high-copy-number hybrid plasmids expressing Suc-6-PH activity may result from the relatively high frequency of Suc-6-PH expression or from a toxic product coded by another gene on the 6.6-kb insert. It will be necessary to test the former possibility directly by utilizing a fragment of the Suc-6-PH coding sequence containing the promoter ligated into promoter probe plasmids (23).

The major *scrB* gene product in λSuc-6 lysates and subclone extracts with a molecular mass of 58 kDa was detected by immunoblotting with anti-invertase after SDS-PAGE (Fig. 2). However, the purified GS-5 intracellular invertase (Suc-6-PH) displays a molecular mass of 48 kDa (15). Therefore, the size of the cloned gene product is significantly greater than that of the putative homologous enzyme in GS-5. Many secretory proteins are known to be synthesized as precursors containing signal sequences which are removed during the secretory process (5). However, the Suc-6-PH enzyme appears to be a cytoplasmic enzyme both in *S. mutans* (11) and in *E. coli* (Table 2). These results suggest that the *scrB* gene product is subjected to posttranslational processing (degradation) by a cytoplasmic or membrane-associated protease in *S. mutans*. However, no 58-kDa protein which reacted with anti-invertase could be detected in crude extracts of strain GS-5 (data not shown). In addition, since a minor protein band of 48 kDa could be detected in the purified clone enzyme preparation after immunoblotting (Fig. 2), similar modification at a reduced level may also occur in *E. coli* extracts.

Furthermore, it is unlikely that the 58-kDa protein resulted from fusion of the 48-kDa protein with an extraneous polypeptide during the cloning procedure, since Southern blot analysis indicated that the *scrB* gene is located on a single 6.6-kb *Eco*RI fragment of the GS-5 chromosome (Shiroza and Kuramitsu, unpublished results). Comparison of this result with the restriction map of the *scrB* gene fragment (Fig. 3) indicated that gene scrambling had not occurred in *E. coli* during isolation of the *scrB* gene. However, the direct demonstration of a processing mechanism will require the development of an in vitro system utilizing *S. mutans* extracts.

Although the Suc-6-PH expressed in *E. coli* migrates in SDS-PAGE as a larger protein than the homologous enzyme purfied from strain GS-5, it is of interest that both proteins exhibit similar mobilities on nondenaturing gels as well as near identical pIs (15). Thus, under the conditions utilized for the nondenaturing gels, the net charge of the proteins rather than their molecular size has a major influence on their relative migration rates.

Utilizing the same λL47.1 cloning vector, Morrissey et al. (18) previously described a *S. mutans* Ingbritt clone expressing a 59-kDa sucrase activity. Recent results suggest that this clone also expresses Suc-6-PH activity, since the cloned enzyme reacts strongly with anti-GS-5 intracellular invertase sera (24). In addition, Macrina et al. (13) have also isolated the same gene from strain GS-5 in *E. coli* utilizing a shuttle plasmid cloning strategy. Thus, the strong expression of the *scrB* gene in *E. coli* has made detection of the gene product relatively straightforward.

The availability of the *S. mutans* GS-5 *scrB* gene will now

make it possible to map the location of the gene on the GS-5 chromosome relative to other sucrose-metabolizing genes by a procedure recently described (19). In addition, in vitro mutagenesis of the gene followed by introduction of the altered gene back into strain GS-5 by transformation (20) will result in the isolation of mutants specifically altered in Suc-6-PH activity. Such mutants could prove useful in determining the number of intracellular sucrase genes expressed in strain GS-5 as well as in further analyzing the mechanism of sucrose metabolism in these cariogenic organisms. For example, since the accumulation of the substrate Suc-6-P is toxic to *S. mutans* cells (26), the Suc-6-PH mutants could be used to isolate mutants altered in the sucrose phosphotransferase system.

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