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 pL47.1. 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 pOU6l to yield the stable hybrid plasmid pMH613. Both plasmids contain a 6.6-kilobase EcoRI 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 Tn5 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 *Km* 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 *gfaA* gene of *S. mutans* has been isolated and characterized (22). In addition, a 59-kilodalton (kDa) sucrose 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, gfaA*, 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.

**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(pOU6l) 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 toluidized 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...
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 CaCl2 procedure cited previously (14). For Tn5 mutagenesis, the procedure of DeBrujin and Lupski (4) was carried out. The λ Tn5 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 2.5-45 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 KC1 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-

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**FIG. 1.** Detection of sucrose-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 sucrose-positive plaques.

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

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

*Suc-6-PH purification was carried out as described in the text by utilizing the sucrose activity to monitor each step.*
RESULTS

Isolation of a Suc-6-PH clone. A gene bank of S. mutans GS-5 DNA constructed in E. coli with the bacteriophage λ-exchange vector λL47.1 was screened for sucrase activity. With the sucrose-tetrazolium agar plate procedure (Materials and Methods), more than 30 sucrose-positive clones were detected out of approximately 5,000 plaques (Fig. 1). All of the positive clones appeared to code for the same sucrose activity. One of the sucrose-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 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-PH 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 sucrose-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. 3). The recombinant phage contained a 9.4-kilobase (kb) fragment of cloned GS-5 DNA possessing a single BamHI site, two HindIII sites, and two EcoRI sites. No sites for ClaI, SalI, XbaI, SphI, or KpnI were detected in the insert.

Attempts to subclone the scrB gene into the high-copy-number plasmid pACYC184 after BamHI, EcoRI, or HindIII cleavage failed to yield active subclones. However, it was possible to subclone a 6.6-kb EcoRI fragment from λSuc-6 into the moderate-copy-number plasmid pLG339 (27) and to isolate sucrose-positive subclones. The clone corresponding to the sclb gene was unstable, since sucrose-negative subclones (containing deletions in the plasmid) were observed frequently after subculture.

Stable subclones containing the sclb gene were subsequently isolated by utilizing the low-copy-number, temperature-sensitive vector pOU61 (12). The 6.6-kb EcoRI fragment was ligated into the single EcoRI site of the vector to produce stable transformants coding for sucrose 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 EcoRI 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 sclb gene appeared to be located on the 6.6-kb EcoRI insert.
TABLE 2. Localization of Suc-6-PH activity in E. coli MH613a

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Relative enzyme activity (%)</th>
<th>Periplasm</th>
<th>Cytoplasm</th>
<th>Membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suc-6-PH</td>
<td></td>
<td>0.4</td>
<td>95</td>
<td>4.6</td>
</tr>
<tr>
<td>β-Galactosidase</td>
<td></td>
<td>1.0</td>
<td>91</td>
<td>8</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td></td>
<td>62</td>
<td>35</td>
<td>3</td>
</tr>
</tbody>
</table>

a E. coli MH613 cells were fractionated into the three subcellular fractions (see text), and sucrase, β-galactosidase (cytoplasmatic marker), and alkaline phosphatase (periplasmatic 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 BamHI site of the insert since it was possible to subclone the 3.1- and 3.5-kb EcoRI-BamHI 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 BamHI site on the 6.6-kb GS-5 insert.

To further define the location of the scrB gene, Tn5 mutagenesis of pMH613 was carried out. A number of sucrose-positive and -negative transformants harboring Tn5 insertions into the chimeric plasmid were isolated and mapped after agarose gel analysis of restriction fragments (Fig. 3b). The position of a number of Tn5 insertions which inactivate the gene confirm the location of the scrB gene near the BamHI site. Insertions resulting in the sucrose-negative phenotype spanned approximately a 2-kb region of the EcoRI insert. Since the 58-kD scrB gene would require a 1.6-kb fragment of DNA, it appears that the Tn5 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 XL71.1 replacement vector. The gene has been subsequently subcloned into low-copy-number plasmid vectors for stable expression. Reconstituted 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 sucrose-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 EcoRI 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 S-Suc-6 lyases 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 EcoRI 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 purified 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 XL71.1 cloning vector, Morrissey et al. (18) previously described a S. mutans Ingbrit clone expressing a 59-kDa sucrose 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 sucrose 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-P mutants could be used to isolate mutants altered in the sucrose phosphotransferase system.

ACKNOWLEDGMENTS

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LITERATURE CITED


