Isolation of DNA Encoding Sucrase Genes from *Streptococcus salivarius* and Partial Characterization of the Enzymes Expressed in *Escherichia coli*

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Restriction enzyme fragments containing two sucrase genes have been isolated from a cosmid library of *Streptococcus salivarius* DNA. The genes were expressed in *Escherichia coli* cells, and the properties of both enzymes were studied in partially purified protein extracts from *E. coli*. One gene encoding an invertase-type sucrase was subcloned on a 2.4-kilobase-pair fragment. The sucrase enzyme had a $K_m$ for sucrose of 48 mM and a pH optimum of 6.5. The *S. salivarius* sucrase clone showed no detectable hybridization to a yeast invertase clone. Two overlapping subclones which had 1 kilobase pair of DNA in common were used to localize a fructosyltransferase gene. The fructosyltransferase had a $K_m$ of 93 mM and a pH optimum of 7.0. The product of the fructosyltransferase was a levan. A fructosyltransferase clone from *Bacillus subtilis* did not hybridize to *S. salivarius* DNA. The properties of the enzymes were compared with those of previously characterized sucrases.

Sucrose-metabolizing enzymes are of considerable interest to workers in plant science because of the central role of sucrose in carbon translocation in plants. Genes encoding various sucrose-metabolizing enzymes will be valuable in the genetic engineering of altered carbon metabolism in higher plants. With the ability to express a single gene encoding a sucrose-metabolizing enzyme, the course of sucrose metabolism and, perhaps, partitioning might be altered. The linkage of the glucose-fructose bond in sucrose has sufficient free energy (~6.6 kcal/mol) (14) to make the hydrolysis reaction irreversible or to supply the energy for polymerization of either hexose moiety. There are many sources of genes for sucrose catabolism. For example, invertases from various species have been widely studied (43), and a single gene encoding both the intracellular and extracellular forms of yeast invertase (EC 3.2.1.26) has been cloned and sequenced (6, 39). In addition, both an invertase-type sucrase (16) and a levansucrase (β-2,6-fructan:α-glucose-1-fructosyl transferase, EC 2.4.1.10) (19) from *Bacillus subtilis* have recently been cloned and sequenced (17, 37).

The sucrose-metabolizing activities of bacteria in the genus *Streptococcus* have been widely studied because of their proposed role in the development of dental caries (22, 41). *Streptococcus* species contain glucosyltransferase (EC 2.4.1.5) and fructosyltransferase activities as well as an invertase-type activity which has more recently been shown to be a sucrose-6-phosphate hydrolase (7, 9). Genes encoding glucosyltransferase activities have been isolated by several groups from various *Streptococcus mutans* serotypes (21, 29–31, 33), and there appear to be at least three or four different glucosyltransferase enzymes. In addition, a sucrose-6-phosphate hydrolase gene has been isolated from several *S. mutans* strains (23, 26, 33). Streptococci have at least two types of fructosyltransferase activity which differ in the structure of the product, producing either an inulin (β2 → 1 fructan) or a levan (β2 → 6 fructan). An inulin-producing fructosyltransferase gene has been isolated from *S. mutans* GS-5 (34). A glucan-binding protein gene has been isolated from the *S. mutans* strain Ingbert. This protein shows fructosyltransferase activity when isolated from *S. mutans*, but the cloned gene expressed in *Escherichia coli* shows no fructosyltransferase activity (32).

Several groups have identified clones encoding sucrose-metabolizing genes by expressing them in *E. coli* (1, 21, 23, 26, 30, 31, 33, 34). In this report we have used a variation of the method of Robeson et al. (31) to isolate DNA fragments from *Streptococcus salivarius* that contain two sucrases, an invertase-type gene that hydrolyzes sucrose and a fructosyltransferase gene which produces a levan. These genes were expressed in *E. coli*, and the properties of the enzymes produced by *E. coli* cultures have been determined.

MATERIALS AND METHODS

**Bacterial strains and media.** *S. salivarius* ATCC 13419 was obtained from the American Type Culture Collection and grown on BHI plates (Difco Laboratories) or in BHI liquid medium in standing cultures at 37°C. *E. coli* SK1592 (3) or JM109 (44) was grown on L broth supplemented when appropriate with kanamycin (50 μg/ml), tetracycline (10 μg/ml), or penicillin (300 μg/ml).

Indicator plates to identify *E. coli* colonies able to ferment raffinose had a MacConkey base (Difco) and contained 1% (wt/vol) raffinose, 50 μg of kanamycin per ml, 0.1 mM isopropylthiogalactoside (IPTG), and 0.01% (wt/vol) cresol red or phenol red. Recombinants were grown overnight in microtiter dishes containing L broth with kanamycin (50 μg/ml) and replica plated onto indicator plates. Indicator plates containing 1% galactose were used to distinguish galactose-metabolizing revertants from sucrose-utilizing strains.

**DNA isolation and cloning techniques.** *S. salivarius* DNA was prepared from 2-liter standing cultures essentially as described by Robeson et al. (31). Standard procedures for DNA manipulation and cloning were used as described by Maniatis et al. (27).
Construction of *S. salivarius* cosmid library. Total chromosomal DNA of *S. salivarius* was isolated and partially digested with *Sall*. The DNA was then size fractionated on a 10 to 40% (wt/vol) sucrose gradient (27), and fragments of 18 to 24 kilobases (kb) were selected for cloning into the *Sall* site of the cosmids vector pVK102 (25). Ligated DNA was packaged in vitro (27) with packaging extracts which were a gift from L. Comai. The recombinant phage were used to infect *E. coli* SK1592. Transformants were selected on kanamycin plates, and recombinants were identified by replica plating onto medium containing tetracycline.

Protein isolation. For screening clones, 250-ml cultures were grown overnight in a shaker at 37°C in L broth containing the appropriate antibiotic (see above) and harvested by centrifugation at 7,000 × g for 5 min. The cells were washed in McIlvaine buffer, pH 5.0 (15), repelleted, and suspended in 1 ml of McIlvaine buffer. Lysozyme was added to a final concentration of 5 mg/ml. The cells were kept at 4°C for 1 h. DNase I (Sigma Chemical Co.) and MgCl₂ were added to final concentrations of 0.4 mg/ml and 8 mM, respectively. The cells were frozen in liquid N₂ and thawed at 37°C five times. The crude cell lysate was clarified by spinning in an Eppendorf Microfuge for 5 min.

For characterizing the cloned fructosyltransferase, nine 1-liter cultures were grown overnight, harvested, and lysed as described above. In addition, 1 liter of the broth was concentrated by filtration to 25 ml. Lysates and broth concentrates were further fractionated by ammonium sulfate precipitation. Protein was determined by the method of Bradford (5).

Sucrose hydrolysis assay. Sucrose-hydrolyzing reactions were run as described by Shiomi and Izawa (35). Equal volumes of 0.5 M McIlvaine buffer (pH 5.0, containing 0.8 M sucrose) and enzyme were mixed and incubated at 37°C. The reaction was stopped and deproteinized with 0.5 M ZnSO₄ and neutralized with 0.3 M BaOH (36). The supernatant was used for analysis of products by the methods detailed below.

TLC assays. Thin-layer chromatographic (TLC) separation of sucrose hydrolysis products was performed as described by De Stefanis and Ponte (13) with 0.25-mm-thick silica gel chromatography plates (SilG). Ascending chromatography was run for 24 h with the aid of a wick composed of a pleated sheet of Whatman 3MM chromatography paper attached to the top edge of each plate. Carbohydrates were visualized by spraying the plates with 1% (wt/vol) orcinol in 50% H₂SO₄ and heating for 2 to 5 min at 90°C.

Glucose assay for screening colonies. The technique of Trinder (40) was modified and used to detect the release of glucose from sucrose by *E. coli* cell lysates (see above). The reaction mixture contained 165 μmol of sodium phosphate, pH 7.5, 0.375 μmol of aminoantipyrine, 1.6 μmol of p-hydroxybenzoate, 6.4 U of glucose oxidase (Sigma), and 0.2 U of peroxidase (Sigma) in a total volume of 490 μl. The reaction was started by adding 375 μl of lysate. To distinguish substrate-specific reactions from nonspecific background, the reactions were monitored (A₅₅₀) in real time rather than being allowed to go to completion. The initial slope was proportional to the amount of glucose released, and rates as low as 5 nmol/h were detectable.

Glucose and fructose assays. Glucose and fructose were assayed enzymatically by the procedure of Bergmeyer et al. (2). Following glucose determination, phosphoglucose isomerase was added to convert fructose-6-phosphate to glucose-6-phosphate. Glucose was again assayed to determine fructose levels by difference. Comparison of the amounts of glucose and fructose produced by hydrolysis of sucrose was used to confirm enzyme function.

Polysaccharide analysis. The product of the fructosyltransferase was confirmed by running the sucrose hydrolysis reaction for 19 h in the presence of 14C-labeled fructosyl sucrose and determining the amount of label incorporated into methanol-insoluble products (20). Reaction mixtures were dried on filter papers, which were washed with methanol prior to liquid scintillation counting.

The nature of the fructan linkage was determined by examining its reactivity with concanavalin A (ConA) in the double-diffusion gel technique of Corrigan and Robyt (11). Levan (linked β2 → 6) from *Aerobacter levunicum* and inulin (linked β2 → 1) from chicory root were used as standards. Both standards were obtained from Sigma Chemical Co.

RESULTS

A library of *S. salivarius* DNA was constructed in the wide-host-range cosmids cloning vector pVK102 (25). The library contained approximately 1,300 recombinant colonies, each bearing inserts of approximately 20 kb. The library was propagated in *E. coli* SK1592 to take advantage of a screening procedure described by Robeson et al. (31), in which recombinant bacteria containing a galactose mutation are grown on fermentation indicator plates containing IPTG and raffinose. *E. coli* do not ferment sucrose, but in the presence of IPTG they take up and cleave raffinose to yield galactose and sucrose. Thus, recombinants which are found to ferment raffinose either (i) are galactose revertants, (ii) contain a gene from *S. salivarius* which complements the galactose mutation, or (iii) contain a gene from *S. salivarius* which encodes a sucrose-metabolizing enzyme. Events of the first two types can be distinguished from the third by simply testing the ability of the strain to ferment galactose on indicator plates.

Of the 1,300 recombinants tested on raffinose indicator plates, 6 gave strong color reactions with both the phenol red and cresol red indicators. When these were rescreened on galactose indicator plates, five of the six were found to ferment galactose. In addition to these strong reactions, 68 colonies gave weak color reactions on either the phenol red or cresol red raffinose plates.

Extracts of the colony which gave the most intense color reaction on the raffinose indicator plate and no reaction on the galactose plate exhibited an activity that hydrolyzed sucrose to yield glucose and fructose (Fig. 1). Assay of the culture broth showed no detectable activity. The S.
S. salivarius cell pellet fraction contained sucrose-metabolizing activities which produced hexoses and compounds with lower rfs (presumably carbohydrate polymers produced by glucosyltransferases and fructosyltransferases) (Fig. 1). No significant amount of these low-rf carbohydrates were produced by the E. coli cell lysate or in the E. coli culture broth, indicating that the sucrose-metabolizing activity was a hydrolase rather than a fructosyltransferase or glucosyltransferase.

The plasmid which encoded this invertase-type activity was named pIN1. The S. salivarius insert was a single Sall fragment approximately 19.7 kb in length (Fig. 2). Two additional clones were later identified from the original colony screen and found to contain the same 19.7-kb Sall fragment as pIN1. Several subclones of the pIN1 insert were constructed in pUC18 (44) (Fig. 2) and assayed for sucrose hydrolysis activity by the endpoint spectrophotometric assay described in Materials and Methods. The results (Table 1) indicate that the gene was localized on a 2.4-kb fragment (pIN29, Fig. 2).

The sucrose encoded by pIN1 showed activity over a fairly broad pH range, with the maximum activity at approximately pH 6.5 (Fig. 3). The pH optimum was unaffected by buffer composition. The Km of the enzyme for sucrose was 48 mM.

We compared the pIN1 clone with a clone of yeast invertase, pKB56 (6), by Southern hybridization. Hybridization was performed at low stringency (50°C in 0.9 M NaCl) with the yeast invertase plasmid as a probe to the S. salivarius pIN1 DNA on a nitrocellulose filter and the S. salivarius pIN1 DNA as a probe to the yeast invertase pKB56 DNA on a separate filter. The filters were washed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (0.33 M NaCl at 50°C). In neither case was any hybridization observed (data not shown), indicating that the genes encoding these two enzymes are not closely related.

Under these hybridization conditions one would expect sequences with approximately 40% mismatch to cross-hybridize (4). As positive controls, pIN1 plasmid DNA and total S. salivarius DNA did hybridize with a pIN1 probe under both the nonstringent conditions (above) and normal stringency hybridization conditions (27).

The 68 clones described above which showed weaker reactions on the raffinose indicator plates showed no sucrose-metabolizing activity by the TLC method. When the more sensitive real-time glucose assay described in Materials and Methods was used, however, three additional sucrose-metabolizing clones were identified. Two were the sucrose isolates mentioned above, and the third, pLE43, was later shown to encode a fructosyltransferase (see below).

Because of the low activity found in the E. coli extracts, it was necessary to partially purify the putative fructosyltransferase protein from 9 liters of cells (Materials and Methods). Sucrose-metabolizing activity found in the 20 to 40% saturated ammonium sulfate fraction was used for all subsequent enzymological studies. The Km of the enzyme for sucrose

![FIG. 2. Restriction map of pIN1 cosmids clone and invertase-type sucrose subclones. The upper line shows a restriction map of the 19.7-kb Sall insert of S. salivarius DNA found in the pIN1 cosmids clone. Restriction enzymes: S, Sall; B, BglII; K, KpnI; E, EcoRI; X, XhoI. pIN2 (second line) contains the 6.3-kb XhoI-Sall fragment cloned into the Sall site of pUC18. pIN29 (third line) contains the 2.6-kb KpnI-Sall fragment cloned into pUC18 from the KpnI to Sall sites. All three clones encoded invertase-type sucrose activity (see text).](http://jb.asm.org/)

![FIG. 3. Effect of pH on sucrose-hydrolyzing activity. (A) pIN1 cell lysate; (B) pLE43, 20 to 40% (NH4)2SO4 precipitate of cell lysate. The soluble cell-associated activities were determined as a function of pH in different buffer systems. Buffers were 0.5 M Mcllvaine (C), 0.1 M sodium succinate (D), or 0.1 M HEPES hydrochloride (E).](http://jb.asm.org/)
The enzyme showed maximal activity at pH 7.0 (Fig. 3). In contrast to results with the invertase-type sucrase, the level of activity of the fructosyltransferase was affected by the buffer composition (Fig. 3).

We showed that the sucrose-metabolizing activity was a fructosyltransferase by using radiolabeled sucrose as a substrate and demonstrating that the fructosyl moiety was incorporated into a methanol-insoluble compound. $^{14}$C-labeled sucrose was incorporated into a methanol-insoluble form by the enzyme in a 19-h reaction as described in Materials and Methods. Uniformly labeled sucrose (0.0711 Ci/mol) and fructosyl-labeled sucrose (0.0143 Ci/mol) gave 25,617 and 16,600 cpm incorporation, respectively. Gluco-syl-labeled sucrose (0.0143 Ci/mol) gave no methanol-insoluble counts over background levels (determined by binding $^{14}$C-labeled sucrose without enzyme treatment to filter papers). Further analysis of the reaction product was done by challenging the product with ConA in an Ouchterloney double-diffusion test. It has been shown previously that levan (B2 $\rightarrow$ 6 linkage) precipitates with ConA but inulin (B2 $\rightarrow$ 1 linkage) does not (28). The fructan formed by the pLE43 enzyme was found to be a levan (B2 $\rightarrow$ 6 linkage) by comparing its reaction with that of commercial preparations of levan and inulin (data not shown).

A map of the pLE43 plasmid is shown in Fig. 4. The gene was found on the 13.3-kb cosmid insert which had lost one of the bordering SauI sites. Two overlapping subclones, pLE44 and pLE45, were constructed in pUC18 (Fig. 4). Extracts of E. coli containing each of the three plasmids were assayed for the presence of the levansucrase by the glucose assay. The culture broth was also assayed, since the enzyme is excreted from S. salivarius. All three of the clones showed levansucrase activity (Table 2), indicating that the gene is probably localized in the 1-kb region common to all three clones (Fig. 4). In addition, pLE45 showed less activity in the broth, which may indicate that a portion of the sequence directing the product to the outside of the cell is missing in this subclone.

A levansucrase clone from B. subtilis (pLS8 [19]) was used to determine whether the S. salivarius fructosyltransferase gene was related to the B. subtilis gene by DNA hybridization. A Southern filter containing total S. salivarius genomic DNA was probed with $^{32}$P-labeled DNA from pLS8 under relatively low stringency conditions (50°C in 0.9 M Na$^+$ with filter washes at 50°C in 0.33 M Na$^+$). No hybridization was detected to the S. salivarius DNA under conditions which should have allowed sequences differing by approximately 40% to hybridize, indicating that the B. subtilis levansucrase gene is not closely related to the S. salivarius levansucrase gene.

### DISCUSSION

We have isolated DNA fragments containing two sacruse genes from S. salivarius. The enzymes encoded by these clones were identified by various assays for sucrose-metabolizing activity conferred on E. coli cells or cell extracts by expression of the S. salivarius genes. Although the data do not exclude the possibility that the S. salivarius genes are expressed from E. coli plasmid promoters, it is possible that the S. salivarius promoters are recognized by E. coli, as is the case for many other genes from gram-positive organisms (see, for example, references 10 and 31).

Three invertase activities have been reported for S. salivarius cultures: an extracellular activity, an intracellular soluble activity, and an activity associated with a particulate fraction (7, 8). Invertases of another bacterium in the same genus, S. mutans, have been studied more extensively. S. mutans has an extracellular invertase with a relatively low $K_m$ for sucrose and an intracellular activity with a much higher $K_m$ for sucrose (30 to 140 mM) (8). The latter enzyme, which has been cloned (23), is a sucrose-6-phosphate hydrolase with a $K_m$ for sucrose 6-phosphate of 0.21 mM (9). The clone we isolated from S. salivarius, when expressed in E. coli, encoded a sacrase that had a $K_m$ for sucrose of 48 mM and did not appear to be excreted into the culture broth. The $K_m$ was measured in 0.2 M phosphate (McIlvaine buffer). However, the activity was unaltered when measured in the absence of phosphate (succinate buffer) at the pH optimum (Fig. 3), suggesting that the S. salivarius enzyme does not have the type of phosphate regulation of activity that has been reported for invertase-type sucrases from some, but not all, serotypes of S. mutans (38).

Finally, in at least one study (26) in which DNA-DNA hybridization was attempted, no sucrose-6-phosphate hydrolase was detected in an S. salivarius strain under stringent hybridization conditions.

The nucleotide sequence of the B. subtilis sucrase gene (sacA) has recently been determined (17) and was compared with the sequence of the yeast invertase gene (39). The two sequences show significant amino acid homology in two different regions, but as no DNA-DNA hybridization was reported, we do not know whether homology of this type could have been detected by hybridization under the nonstringent conditions we used. Invertases from various sources which have been characterized appear to have widely varying properties (see, for example, reference 43), so it will be interesting to see what structural homology

### TABLE 2. Location of levansucrase in partially purified extracts of E. coli

<table>
<thead>
<tr>
<th>Clone</th>
<th>Vector</th>
<th>Total activity (U)*</th>
<th>Ratio, L/B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lysate (L)</td>
<td>Broth (B)</td>
<td></td>
</tr>
<tr>
<td>pLE43</td>
<td>pVK102</td>
<td>1.28</td>
<td>0.54</td>
</tr>
<tr>
<td>pLE44</td>
<td>pUC18</td>
<td>0.45</td>
<td>0.055</td>
</tr>
<tr>
<td>pLE45</td>
<td>pUC18</td>
<td>0.13</td>
<td>0.081</td>
</tr>
</tbody>
</table>

* One unit of activity is defined as 1 μmol of glucose released per h. Total activity in 9 liters of culture was determined by measuring glucose released from sucrose by the technique of Bergmeyer et al. (2).
differently sucrose hydrolases may share as they are further characterized by sequence analysis.

When our S. salivarius levansucrase gene was expressed in E. coli, a significant fraction of the levansucrase activity was found in the culture broth. This result is consistent with reports that fructosyltransferase activity is usually found in the extracellular fraction of S. salivarius cultures, the amount varying with the culture conditions (7). The K_m measured for the cloned levansucrase was high (93 mM) compared with a published value of 17 mM (42). There are several possible explanations for this discrepancy. The previous group (42) used a different S. salivarius strain (SS2). Our measurement was made with a crude enzyme preparation (see Materials and Methods). In addition, Whitaker and Edwards (42) demonstrated an enhancement of activity with Mg^{2+} following treatment with EDTA. No Mg^{2+} was added to our reaction mixtures. Finally, it is possible that the S. salivarius enzyme is similar to the B. subtilis enzyme, which hydrolyzes sucrose to fructose and glucose when it is incubated in the absence of a levan primer (12). In the absence of primer, we detected free fructose amounting to only about 10% of the sucrose metabolized (data not shown).

We found the pH optimum of the S. salivarius levansucrase to be 7.0 when measured in 0.5 M McIlvaine buffer (Fig. 3). The curve was fairly broad, and the optimum was higher than the pH 5.6 reported by Garzczynski and Edwards (18). No attempt was made in that report to distinguish hydrolytic activity from that of levansucrase, since only glucose was measured. In contrast, Jacques and Wittenberger (24) measured fructan synthesized by an S. salivarius enzyme and reported a pH optimum of 6.5 to 7.0, which agrees more closely with our data.

The effect of buffer composition on levansucrase activity is curious. At lower ionic strength, in 100 mM succinate or HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), much lower activities were observed than in 0.5 M McIlvaine buffer. This has been shown to inhibit the enzyme (18), but since the activity was lower in the presence of either succinate or HEPES, inhibition by the buffering compound is unlikely. A possible explanation could be stimulation by high osmotic potential, as demonstrated by Dedonder (12) with polyethylene glycol 4000 for the B. subtilis enzyme, or a specific effect of phosphate as discussed above.

It is interesting that another bacterial levansucrase clone (pLS8 from B. subtilis) did not have significant homology to the S. salivarius levansucrase by DNA-DNA hybridization. Further characterization of the structure of the enzyme and its activity as well as sequencing of the gene encoding it would be needed to determine whether the bacterial levansucrases are at all related. It would also be interesting to compare the structure of the S. salivarius levansucrase with the recently isolated S. mutans inulinocasucrase (34).

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LITERATURE CITED