Characterization of a Sucrase Gene from
Staphylococcus xylosus

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The Staphylococcus xylosus gene scrB, encoding a sucrase, has been isolated from a genomic library of S. xylosus constructed in Escherichia coli. The gene was detected by its ability to confer utilization of the glucose and fructose residues of raffinose in an E. coli strain that is not able to metabolize galactose. It was found to reside within a 1.8-kb DNA fragment, the nucleotide sequence of which was determined. One large open reading frame, which is preceded by a ribosome binding site, is encoded on the fragment. Its deduced amino acid sequence yields a protein with a molecular mass of 57,377 kDa which shows significant homology with bacterial sucrose-6-phosphate hydrolases and sucrases. Overexpression of scrB in E. coli by the bacteriophage T7 polymerase promoter system resulted in the production of a protein with an apparent molecular mass of 58 kDa. Disruption of the scrB gene in the S. xylosus genome rendered S. xylosus unable to utilize sucrose. Thus, the ScrB sucrase is essential for sucrose metabolism in S. xylosus.

The disaccharide sucrose can serve as a carbon source for a wide variety of bacteria. Sucrose can be cleaved by sucrases (invertases) or sucrose-6-phosphate hydrolases after uptake into the bacterial cytoplasm (22, 52). In many bacteria, sucrose is transported across cytoplasmic membranes by means of the phosphoenolpyruvate-dependent carbohydrate phosphotransferase system (PTS) (31). By this mechanism, sucrose enters the cell as sucrose-6-phosphate, which is then hydrolyzed to glucose-6-phosphate and fructose. Sucrose can also be split in the culture medium by extracellular enzymes such as levansucrase, levanase, and glucosyl- and fructosyltransferases (26, 29, 49). In some bacteria, more than one sucrose-hydrolyzing activity is present (24, 29).

At the molecular level, genes encoding secreted sucrose-cleaving enzymes of Bacillus subtilis and Streptococcus mutans (20, 26, 44, 45, 49, 53) and systems mediating sucrose uptake and metabolism of B. subtilis, S. mutans, Streptococcus sobrinus, Lactococcus lactis, Salmonella typhimurium, Klebsiella pneumoniae, and Vibrio alginolyticus have been characterized (4, 8, 10, 12, 13, 17, 32, 36, 37, 39, 42, 47, 55). These sucrose utilization systems belong to the PTS, and their genes are clustered on the chromosome, on conjugal transposons, or on plasmids. Genes for sucrose-specific transport enzymes (enzyme IISucr) and sucrose hydrolases are essential components of all these gene clusters. On the S. typhimurium plasmid pUR400 and in the genome of K. pneumoniae, genes for a sucrose-specific outer membrane porin and an ATP-dependent fructokinase are found (3, 18, 40). The latter is also encoded in the V. alginolyticus system (4). The gram-negative sucrose PTS genes constitute operons that are controlled by repressors (5, 39). In B. subtilis, the sucrose PTS operon is regulated by an antiternination mechanism which is part of a complex regulatory network controlling sucrose metabolism (1, 48).

To get an insight into the molecular organization and regulation of the sucrose utilization system in another gram-positive species, we cloned and characterized scrB, the gene encoding a sucrase of Staphylococcus xylosus which is essential for sucrose utilization in this bacterium.

MATERIALS AND METHODS

Bacterial strains, plasmids, and phage DNAs. S. xylosus C2a was used for the isolation of chromosomal DNA. It is a derivative of S. xylosus DSM20267 (38) that had been cured of its endogenous plasmid pSX267 (15). A spontaneous lac+ (lacY1) revertant of Escherichia coli HB101 [supE44 hsdS20 (rK- mK-) recA13 ara-14 proA2 leuB6 rpsL20 xylS met-l galK2 lacY1], designated HB101lac+, was used to construct the S. xylosus library and for scrB subcloning experiments. In E. coli TG1 [supE hsdS ΔlacIq ΔlacCMX74 thl (lac-proAB) F‘ (traD36 proAB lacIq lacZAM15)], M13 cloning for sequencing was performed. T7 polymerase-directed overexpression experiments were carried out in E. coli K38 (51). The S. xylosus library was constructed with pBR322 (50), and scrB subcloning was carried out with the shuttle vector pBR473, a derivative of pBR373 (7) conferring chloramphenicol (CM) instead of kanamycin (KM) resistance. CM resistance is a more efficient selectable marker for Staphylococcus protoplast transformation than KM resistance. Subcloning for sequencing was done by using M13mp18 or M13mp19 (56). For sucrose overexpression in E. coli, plasmids pT7-5 and pGPI-2 were applied (51). To carry out gene disruption experiments in S. xylosus, a shuttle vector (pBT2) was constructed by joining the PsiI-BamHI fragment of pBR322 containing the β-lactamase gene and the ColE1 origin with the PsiI-BamHI fragment of pTV1ts (57), which had been blunt ended at the PsiI site. The pTV1ts fragment contains the CM resistance gene of pC194 (cat) and the temperature-sensitive replicon of PE194 (repTs). An erythromycin (EM) resistance cassette (ermB) flanked by various restriction sites was obtained on plasmid pEH after insertion of the HpaI-BglII fragment of transposon Tn511 (30) into pUC19 (56) cleaved with BamHI and HincII.

Growth media, DNA manipulations, transformation, and transfection. DNA manipulations, plasmid DNA isolation, transformation of E. coli, and preparation of media and agar plates for bacterial growth were done by standard procedures (34). Plasmids were introduced into S. xylosus by...
protoplast transformation (14). M13 cloning and single-stranded-DNA preparation were carried out by the method of Messing (27). Southern blot analysis was performed with a vacuum blotting device (Pharmacia) and a digoxigenin DNA detection kit (Boehringer Mannheim, GmbH) as recommended by the suppliers. DNA sequencing was done by the dideoxy chain termination method (35) with a Sequenase sequencing kit (U.S. Biochemicals).

**Genomic library construction and detection of sucrase activity in E. coli.** Chromosomal S. xylosus DNA was prepared by the method of Marmur (25). Genomic DNA was partially digested with Sau3AI, and fragments of 5 to 20 kb were isolated by preparative agarose gel electrophoresis and electrodialution into dialysis bags. A 1-μg amount of pBR322 was cleaved with BamHI and dephosphorylated by calf intestine alkaline phosphatase. Ligation was carried out with a 1:4 molar ratio of vector to chromosomal fragments in a volume of 100 μl. E. coli HB101(lac-), which had been made competent by CaCl2, was transformed with aliquots of the ligation mix, yielding approximately 1,000 transformants which were then plated on McConkey agar plates containing AP (100 μg/ml), raffinose (1%), and isopropyl-β-D-thiogalactoside (IPTG). Transformants were grown at 30°C. Colonies with sucrase activity appear dark red according to the rifinone fermentation scheme of Robeson et al. (33).

**Overexpression of scrB in E. coli by the T7 RNA polymerase promoter system.** Expression of scrB under control of the T7 promoter was carried out by the method of Tabor and Richardson (51), using plasmids pT7-5 and pGP1-2. E. coli cells harboring pTSL, a scrB-containing derivative of pT7-5, and pPG1-2 were grown in 2X TY medium (100 μg of AP per ml, 40 μg of KM per ml) at 30°C to an optical density at 578 nm (OD578) of 1.5. The culture was shifted to 42°C for 25 min, rifampin was added to a final concentration of 100 μg/ml, and the cells were incubated for 2 h at 37°C and then harvested. Cell extracts for sodium dodecyl sulfate-polyacrylaldehyde gel electrophoresis (SDS-PAGE) (23) were prepared by lysozyme treatment and boiling in SDS-PAGE sample application buffer. Aliquots of the extracts were separated on 10% polyacrylamide gels, and proteins were visualized by Coomassie blue staining.

**Determination of sucrase activity.** Sucrase activity in bacterial cell extracts was quantified by measuring the amount of glucose liberated after hydrolysis of sucrose (41). Crude extracts of S. xylosus or E. coli were prepared by opening the cells with glass beads. The amount of glucose produced by sucrase action was determined by means of a commercially available glucose test kit (Boehringer Mannheim, GmbH). Protein concentrations in cell extracts were determined by the method of Bradford (6). Sucrase activities are expressed in nanomoles of glucose per minute per milligram of protein.

**Insertional inactivation of scrB.** For gene disruption experiments, S. xylosus was transformed with the temperature-sensitive shuttle plasmid pBT2 containing a scrB fragment and ermB (Tn551) cloned into scrB. Transformants were selected with CM (20 μg/ml) at 30°C. One transformant was grown in Luria-Benzoni (LB) medium (20 μg of CM per ml) at 30°C overnight. The culture was diluted into 500 ml of fresh LB medium containing 5 μg of EM per ml and grown at 40°C to an OD578 of about 3. Appropriate dilutions were plated onto LB agar plates with 5 μg of EM per ml and incubated at 40°C for 36 h. Colonies were patched onto LB plates with either CM (20 μg/ml) or EM (5 μg/ml). Successful transfer of ermB to the S. xylosus chromosome is indicated by the appearance of colonies that are EM resistant and CM sensitive. These were then tested for sucrase utilization.

Since there is no minimal medium available to test growth of S. xylosus depending on a specific carbon source, acid production on sucrose fermentation test plates (28) was taken as an indication of sucrase utilization. Integration of ermB into the scrB locus was subsequently verified by Southern blot analysis.

**Nucleotide sequence accession number.** The nucleotide sequence of scrB (see Fig. 2) has been submitted to the EMBL Data Library under accession no. X67744.

**RESULTS**

**Cloning of the scrB gene.** In a genomic library of S. xylosus DNA in Staphylococcus carnosus (46), which seemed to be representative for the S. xylosus genome, transformants that were able to utilize sucrose could not be detected. In S. carnosus, which cannot metabolize sucrose, a Scr+ phenotype is dependent on the concomitant cloning of genes for sucrose transport and hydrolysis. To bypass the need for sucrose transport to detect sucrose activity, a genomic library of S. xylosus DNA in E. coli HB101(lac+) was constructed and screened for sucrose activity on McConkey agar plates containing AP, raffinose, and IPTG. Utilization of raffinose in E. coli gal strains is dependent on the hydrolysis of sucrose. Raffinose is internalized by the LacY permease and cleaved by the α-galactosidase to yield galactose and sucrose. Therefore, clones producing sucrase activity can be detected on McConkey agar plates with raffinose. Among about 1,000 transformants, three dark red colonies appeared. The plasmids of the respective clones were isolated and reintroduced into HB101(lac+). Transformants turned uniformly red on McConkey raffinose plates, indicating that the observed phenotype was plasmid associated. Testing the transformants also for galactose and sucrose fermentation revealed a Gal+ phenotype mediated by one of the plasmids. Obviously, a gene that could complement the galK2 mutation of HB101(lac+) had been cloned. None of the clones could metabolize sucrose. Colonies harboring plasmids isolated from the other two red colonies, designated pRA2 and pRA7, were raffinose fermenting and therefore sucrose+ and Gal-. By restriction analysis, the sizes of the insertions in pRA2 and pRA7 turned out to be about 8 and 7.5 kb, respectively. It was confirmed by Southern blot analysis that the inserted DNA originated from the S. xylosus genome. Comparison of restriction patterns of the plasmids indicated a common region of about 4 kb (Fig. 1). Fragments of pRA2 obtained by restriction
FIG. 2. Nucleotide sequence of the sucrose gene. The nucleotide sequence of the shortest, Bal 31-generated scrB fragment and the deduced amino acid sequence of ScrB are shown. Nucleotides constituting the potential scrB promoter and the Shine-Dalgarno sequence (SD) are underlined. Stretches of amino acids representing highly conserved regions in sucrases are underlined and labeled a through f according to the system of Gunasekaran et al. (16). An additional well-conserved region is designated g. An inverted repeat is indicated by broken arrows. Relevant single restriction sites are shown.

endonuclease or exonuclease Bal 31 digested were cloned in the shuttle vector pRB473, and the respective clones were tested for raffinose fermentation. The sucrose gene scrB (41) was localized on a 1.8-kb Bal 31 fragment (Fig. 1). The plasmid containing this 1.8-kb scrB fragment was designated pRA200. Subsequently, the nucleotide sequence of its insertion was determined.

**Nucleotide sequence of scrB.** The nucleotide sequence of the scrB fragment was determined for both strands. It comprises 1,801 bp and is shown in Fig. 2. The sequence contains one large open reading frame of 1,511 nucleotides. At positions 166 to 171, a Shine-Dalgarno sequence (43) followed by an ATG 9 nucleotides away is found (Fig. 2). Taking this ATG as the most likely start codon, a polypeptide of 494 amino acids with a calculated molecular mass of 57,377 kDa is encoded in the region. Comparison of this deduced amino acid sequence revealed significant similarities to bacterial sucrases. Thus, the open reading frame is scrB, the gene coding for the sucrase of S. xylosus. Regions showing homology with sucrose hydrolases are found throughout the ScrB protein. The following percentages of identical amino acids were found in comparison with the
indicated other sucrases: sucrose-6-phosphate hydrolase (Suc-6-PH) Scrb of V. alginolyticus, 41% (42); Suc-6-PH SacA of B. subtilis, 39% (13); Suc-6-PH Scrb of S. mutans, 36% (36); Suc-6-PH Scrb of E. coli, 35% (21); Suc-6-PH Scrb of L. lactis, 35% (9); sucrose SacA of Zymomonas mobilis, 33% (16); and sucrase RafD of E. coli, 33% (2). The S. xylosus sucrase shares slightly more identical residues with enzymes whose natural substrate is sucrose-6-phosphate than with sucrases that normally cleave sucrose.

Expression of scrb in E. coli and S. carnosus. The initial deletion analysis of the scrb locus suggested that the gene spans the KpnI and PstI restriction sites (Fig. 2). Deletion of the DNA upstream of the KpnI site (pRK1) or downstream of the PstI site (pRP1) resulted in E. coli clones that were not able to metabolize raffinose. It can be deduced from the scrb sequence (Fig. 2) that almost 60 amino acids at the carboxy terminus of the sucrase were removed on plasmid pRP1. Obviously, the truncated Scrb protein is no longer active. However, the entire scrb coding region is contained in plasmid pRK1. In the vector for the deletion experiments (pRB473), insertions in the multiple cloning site are protected from transcriptional readthrough initiated at vector promoters by two terminators. Thus, fragments mediating sucrase activity (pRA200, Table 1) should also contain the scrb promoter. On pRK1 it is most likely deleted. This indicates that the scrb promoter is located at the KpnI restriction site or upstream of it.

Plasmid pRA200 was also able to direct sucrase activity in S. carnosus, which lacks an endogenous sucrose (Table 1). As for E. coli, plasmid pRK1 did not confer detectable sucrase activity upon S. carnosus. This also suggests that the sucrose gene had been cloned with its own promoter.

In order to confirm the scrb open reading frame, protein extracts of E. coli cells harboring pRA2 and pRA200 were prepared and compared by SDS-PAGE with extracts of E. coli containing the vector without the insertion. After Coomassie blue staining, no additional protein band could be detected in extracts of pRA2- or pRA200-containing cells.

To increase sucrose production in E. coli, the T7 polymerase promoter system was applied. The scrb structural gene without its own promoter, present as a KpnI-blunt-end (generated by Bal 31) fragment in the multiple cloning site of plasmid pRK1, was isolated as a HindIII-SrfI fragment and cloned into the T7 expression vector pT7-7. In the resulting plasmid, pTS1, scrb expression is driven by the strong T7 promoter on pT7-5 when T7 RNA polymerase is provided. SDS-PAGE analysis of protein extracts of E. coli cells harboring pTS1 revealed a prominent protein band of 58 kDa, the synthesis of which is dependent on the induction of T7 polymerase production (Fig. 3). The molecular mass of 58 kDa agrees perfectly with the calculated value of 57.377 kDa for the Scrb sucrase.

The identification of the 58-kDa protein as the Scrb sucrase is supported by the determination of sucrase activity in TS1-containing E. coli cells. In parallel to the appearance of the 58-kDa protein, an increase of sucrase activity is observed upon induction of T7 polymerase production (Table 1). Hence, the 58-kDa protein is the S. xylosus sucrase.

In contrast to the rather high level of T7 promoter-directed Scrb protein production that is detected on protein gels, the increase in sucrase activity is moderate (Table 1). Sucrase activity in pTS1-containing cells is only eightfold higher than in extracts of cells containing pRA200 (Table 1), in which the Scrb protein was not detectable on gels. It seems that only a portion of the Scrb protein synthesized in the T7 system is active. Because of its overproduction, it may be trapped in a denatured form as inclusion bodies.

Insertional inactivation of scrb in S. xylosus. To demonstrate the role of the Scrb sucrase in sucrose metabolism in S. xylosus, the scrb gene was inactivated in the chromosome. For that purpose, the PvuI-scrb fragment of pRA7 (Fig. 1) was cloned into plasmid pBT2. Subsequently, the KpnI-PstI fragment was replaced by the ermB resistance cassette of pEH. In the resulting plasmid, pBTSE2, most of the scrb gene, including the ribosome binding site and the start codon, is deleted. By this disruption, scrb expression is
completely abolished. *S. xylosus* C2a was transformed with plasmid pBTSE2, and the transformants were maintained at 30°C. By raising the temperature to 40°C, colonies that contained *ermB* transferred to the *S. xylosus* genome were isolated. One colony was chosen for further characterization. Correct integration of *ermB* into *scrB* was confirmed by Southern blot. The new *S. xylosus* strain (*scrB::ermB*), designated TX50, was tested for sucrose utilization on sucrose fermentation test plates and found to be *Scr*−. Accordingly, the level of sucrose activity in *S. xylosus* TX50 is clearly reduced compared with that of wild-type strain C2a (Table 1). However, a residual level of activity was observed. Obviously, this remaining sucrose activity is not sufficient to confer a *Scr*+ phenotype on sucrose fermentation test plates. In addition to the *Scr*− phenotype, *S. xylosus* TX50 showed a slightly reduced growth rate in complex media containing sucrose. In contrast to *S. mutans*, disruption of *scrB* did not lead to a sucrose-sensitive phenotype in *S. xylosus*. This different behavior of *scrB* mutants of the two bacterial species is possibly due to the additional sucrose-hydrolyzing activity found in *S. xylosus*.

By introduction of plasmid pRA200 into *scr* mutant TX50, the Scr+ phenotype could be recovered (Table 1). The complementation of the mutation by pRA200 is consistent with the deletion mapping in *E. coli* and the sucrose expression in *S. carnosus*, which indicated that *scrB* was contained with its own promoter in pRA200. The results of the *scrB* gene disruption demonstrate that the gene is essential for sucrose metabolism in *S. xylosus*.

**DISCUSSION**

The ScrB protein has a size similar to that of the sucrose of *Z. mobilis* (58 kDa) but is larger than most of the other bacterial sucrases, the sizes of which range from 52 to 56 kDa. The amino acid sequence of the ScrB protein shows strong homology with other bacterial sucrases. Homology is found throughout the *S. xylosus* enzyme, and five highly conserved regions in sucrases, as pointed out by Guneskareran et al. (16), are clearly detectable (Fig. 2). Within the last 60 amino acids at the C terminus of the ScrB sucrose, we noticed a seventh stretch of residues that is conserved in all other bacterial sucrases (region g in Fig. 2). Interestingly, this part of the enzyme is removed by deletion of *scrB* DNA downstream of the *PstI* restriction site (Fig. 2), which resulted in complete loss of enzymatic activity. The ScrB sucrose shares slightly more identical residues with enzymes whose role is to cleave sucrose-6-phosphate than with enzymes whose natural substrate is sucrose. This could indicate that the *S. xylosus* ScrB enzyme normally hydrolyzes sucrose-6-phosphate rather than sucrose. Thus, we expected to find a sucrose-specific PTS permease in *S. xylosus* and, analogous to the genetic organization of other PTS sucrose utilization systems, its gene close to *scrB*.

Testing the *E. coli* clones harboring *scrB* plasmids pRA2 and pRA7 for sucrose utilization yielded no indication that a sucrose transport function is mediated by the cloned DNA. Since carbohydrate-specific PTS enzymes of gram-positive bacteria may interact poorly with nonspecific PTS components of *E. coli* (11), the pRA2 and pRA7 insertions were also tested in the gram-positive bacterium *S. carnosus*. *S. carnosus* is not able to transport sucrose, as determined by sucrose uptake studies, and consequently exhibits a *Scr*− phenotype. Again, no sucrose transport could be detected, but sucrose activity was present. The cloned *S. xylosus* *scrB* DNA is extended by about 4 and 6 kb on either side of *scrB* (Fig. 1). Its coding capacity should be sufficient for additional *scr* genes. It seems that *scrB* is located on the *S. xylosus* chromosome without a sucrose permease gene in its vicinity. Further evidence for the dispersed localization of *scr* genes in *S. xylosus* is provided by the analysis of transposon-induced sucrose utilization mutants. A new *scr* locus, distinct from *scrB*, was identified and found to contain a gene encoding a sucrose-specific enzyme II of the PTS (54). DNA from this new *scr* locus did not hybridize to the DNA cloned in pRA2 and pRA7. However, cloned *scrB* DNA hybridized to a locus where the transposon insertion resulted in a strong reduction of sucrose activity (54). The existence of a PTS sucrose permease gene in *S. xylosus* suggests that sucrose enters the cells as sucrose-6-phosphate, which should then be cleaved by the ScrB sucrose. The essential function of the ScrB sucrose in sucrose metabolism in *S. xylosus* is corroborated by the *Scr*− phenotype of the *scrB* disruption mutant. The residual, low-level sucrose activity that is present in the mutant must be due to another enzyme. Currently, it is unclear what type of enzyme, other than the ScrB sucrose, can also cleave sucrose in *S. xylosus*.

From *scrB* expression in *E. coli*, *S. carnosus*, and *S. xylosus*, we concluded that the promoter is contained on the 1.8-kb fragment on pRA200 at or beyond the *KpnI* restriction site (Fig. 2). Inspection of the respective sequence revealed a potential Eo70-like promoter sequence about 90 bp upstream of the *scrB* start codon (Fig. 2). The −35 and −10 regions would each differ by one nucleotide from the *E. coli* consensus sequence (19) and would have appropriate spacing. At the *KpnI* restriction site, a 16-bp palindromic sequence that could possibly serve as an operator sequence for control of *scrB* expression is present. In gram-negative PTS *scr* operons, *scr* gene expression is regulated by repressors, whereas it is controlled positively by antitermination in *B. subtilis*. In *S. xylosus*, *scrB* expression seems to be inducible by the addition of sucrose to the culture medium (data not shown). It remains to be determined by which mechanism *scr* expression is regulated in *S. xylosus*.

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