The *Bacillus stearothermophilus* NUB36 *surA* gene encodes a thermophilic sucrose related to *Bacillus subtilis* SacA

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The complete nucleotide sequence of the *surA* gene, encoding a sucrose from *Bacillus stearothermophilus* NUB36, was determined. *surA* was composed of 1338 bp and encoded 445 amino acid residues. The deduced polypeptide of *M_r* 51 519 showed strong sequence similarity to sucrose and sucrose phosphate hydrolases from *Bacillus subtilis*, *Klebsiella pneumoniae* and *Vibrio alginolyticus*, and contained the 'sucrose box' residues thought to be important for catalysis of the transfer of fructose from sucrose. The enzyme was partially purified using affinity chromatography from extracts of *Escherichia coli* containing the cloned *surA*. SurA displayed an optimum temperature for sucrose hydrolysis of 55 °C and high stability. The *M_r* of SurA determined by gel filtration was 105 000, which suggested that the active form of the enzyme is a dimer. SurA exhibited an apparent *K_m* of 40 mM for sucrose but, unlike the homologous *B. subtilis* enzyme, had no detectable sucrose phosphate hydrolase activity.

Keywords: invertase, sucrose utilization, sucrose-phosphate hydrolase, *Bacillus stearothermophilus*

INTRODUCTION

Little is known about the genetics of sucrose metabolism in *Bacillus stearothermophilus*. In contrast, bacterial sucrose metabolism has been intensively studied in *Bacillus subtilis* (Lepesant, 1976; Fouet *et al.*, 1986), *Streptococcus mutans* (St Martin & Wittenberger, 1979; Robeson *et al.*, 1983; Russell *et al.*, 1985; Lunsford & Macrina, 1986), and *Escherichia coli* harbouring the conjugative sucrose-plasmid pUR400, which was originally isolated in *Salmonella typhimurium* (Schmid *et al.*, 1982, 1988; Ebner & Lengeler, 1988). In all the above-studied organisms, sucrose is transported through the cell membrane via the phosphoenolpyruvate-dependent phosphotransferase system (PTS) and phosphorylated into sucrose 6-phosphate which is then cleaved by a sucrose-6-phosphate hydrolase yielding glucose 6-phosphate and fructose as cleavage products. Nucleotide sequences of sucrose hydrolase genes have been reported for *B. subtilis* (Fouet *et al.*, 1986), *Vibrio alginolyticus* (Scholle *et al.*, 1989), *Strep. mutans* (Sato & Kuramitsu, 1988), *Zymomonas mobilis* (Gunasekaran *et al.*, 1990), and the pUR400-encoded system from *S.*

typhimurium (Schmid *et al.*, 1988). The deduced amino acid sequences of these enzymes show strong homology, especially in several conserved regions suggested to be important in the transfer of fructose from sucrose.

Here we present the nucleotide sequence of the *B. stearothermophilus* NUB36 *surA* gene (1338 bp), which encodes a thermostable sucrose (SurA). Comparison of the deduced amino acid sequence of *surA* with the sucrose hydrolases of *B. subtilis* (sucrose-6-phosphate hydrolase), *Klebsiella pneumoniae* (sucrose-6-phosphate hydrolase) and *V. alginolyticus* (sucrose) revealed strong homology in the putative enzymically functional regions. High expression of the gene was achieved in *E. coli*, which permitted partial purification of the enzyme and characterization of its activity.

METHODS

Bacterial strains, plasmids, and culture conditions. *B. stearothermophilus* NUB36 was the source of chromosomal DNA (Welker, 1978; Chen *et al.*, 1986). *Escherichia coli* NM522 *supE thi* $\Delta(lac-proAB) \Delta hsd-5(r^- m^-)$ [F' *proAB lacI*^qZ Δ M15] was used as a host strain for subcloning and analysis of sucrose-hydrolysing activity. *E. coli* LE392 F⁺ *hsdR574(r^- m^+)* *supE44 supF58 lacY1* or $\Delta(lacIZY)6 galK2 galT22 metB1 trpR55$ was used to host the recombinant plasmid pAM2001, which contains a 4.5 kb insert

The GenBank accession number for the sequence reported in this paper is U34872.

of *B. stearothermophilus* NUB36 DNA in pBR325. A minicell-producing strain of *E. coli*, DE3 *endA1 recA1 gyrA96 thi, hsdR17(r_k m_k) relA1 supE44 Δ(lac-proAB)* [F' *traD36 proAB lacI^qΔM15*] λ(DE3), was used to study the expression of the cloned gene encoding the sucrose-hydrolysing enzyme. The cloning vectors pT7T3 (Pharmacia) and pGEM-7Zf(+) (Promega) were used to obtain nested deletions of inserted DNA. Ampicillin (100 µg ml⁻¹) was used to maintain plasmids.

E. coli transformants were propagated on both Luria-Bertani (LB) medium (10 g polypeptone, 5 g yeast extract, and 5 g NaCl l⁻¹, pH 7.2) and EMB plates (10 g bacto tryptone, 1 g yeast extract, 5 g NaCl, 2 g KH₂PO₄, 0.04% eosin Y, 0.0065% methylene blue, 1% sucrose and 15 g agar per litre).

Plasmid constructions and subcloning of the *surA* gene.

Plasmid pAM2001 was isolated by E. Liong during construction of a gene bank of *B. stearothermophilus* NUB36 DNA in pBR325 (Liong, 1995). A 4.5 kb *Bam*HI–*Sph*I-digested fragment from pAM2001 was isolated and ligated to *Bam*HI–*Sph*I-digested pT7T3 to produce pAM2002. The 4.7 kb *Hind*III–*Sac*I fragment of pAM2002 (including some vector DNA) was subcloned into *Hind*III–*Sac*I-digested pGEM-7Zf(+) to create pAM2003. The ligation mixtures were transformed into *E. coli* NM522 using the standard competent-cells method (Maniatis *et al.*, 1982), and transformants were selected on LB agar plates containing X-Gal and ampicillin (100 µg ml⁻¹). Transformants containing the intact *surA* gene were identified on EMB plates containing 4% sucrose. The resultant plasmids, pAM2002 and pAM2003, which contained the *surA* fragment in the two orientations relative to the vectors, were used to subclone and sequence the gene.

Progressive deletions of both orientations were generated by unidirectionally digesting *Sac*I–*Bam*HI-digested pAM2002 and *Aat*II–*Hind*III-digested pAM2003 with exonuclease III (Promega). The end-points of the deletions were separated by about 200 bp. The fragments were ligated with T4 ligase and transformed into *E. coli* NM522, in which the plasmids were amplified and purified. The resulting constructs, whose insert end-points are shown in Fig. 1, served as the source of DNA for nucleotide sequencing and sucrase activity determinations.

In order to increase the yield of the sucrase expressed in *E. coli*, the *surA* gene was subcloned into the high-expression vector pTrc99A (Pharmacia) by double-digesting pAM2008 with *Nco*I and *Sac*I. The 2.45 kb digested DNA fragment was recovered and ligated with *Nco*I and *Sac*I double-digested pTrc99A. The resultant clone, pAM2020, was chosen for further purification of sucrase (SurA protein).

DNA sequencing. Nucleotide sequences were determined by the dideoxy chain-termination method (Sanger *et al.*, 1975) using plasmid DNA as the template, *Taq* DNA polymerase (Promega), and fluorescent-dye-coupled primers. The reaction products were analysed with a model ABI370A automated DNA sequencer (Applied Biosystems). Sequencing was performed for the entire length of both strands. The sequence data obtained were analysed using the Australian National Genomic Information Service (ANGIS) at Sydney University.

Expression of SurA. The *E. coli* strain DE3 was used to analyse the protein encoded by the recombinant plasmid. Purification of bacteria and L-[³⁵S]methionine incorporation experiments were performed according to the method of Tabor & Richardson (1985).

Cellular localization of sucrase in *E. coli*. Periplasmic and cytoplasmic cell fractions were prepared by the osmotic shock method (Kellermann & Ferenci, 1982). Cell extract was prepared using the method described previously (Francis *et al.*,

1991). β-Galactosidase was assayed as a cytoplasmic marker, and β-lactamase as a periplasmic marker.

Purification of SurA. *E. coli* NM522 harbouring pAM2020 was grown overnight with shaking at 37 °C in 50 ml nutrient broth supplemented with 0.4% sucrose and 100 µg ampicillin ml⁻¹. A 40 ml volume of overnight culture was inoculated into 4 l nutrient broth supplemented with 0.4% sucrose and 100 µg ampicillin ml⁻¹ and shaken at 37 °C for about 3 h until the OD₅₉₀ reached 0.5. IPTG was added (0.2 mM final concentration) and shaking continued at 37 °C for another 2.5 h. Cells were harvested by centrifugation and washed with 400 ml 20 mM HEPES buffer (pH 7.5), containing 1 mM DTT. Bacteria were collected by centrifugation and resuspended in 50 ml 20 mM HEPES buffer (pH 7.5), containing 1 mM DTT, 2 mM PMSF and RNase/DNase mixture (each 1 mg ml⁻¹ final concentration). Bacteria were broken using a French press [12000 p.s.i. (82.8 MPa)] and MgSO₄ (1 mM final concentration) was added to the broken cells. The suspension was centrifuged (15 min, 4000 g). The supernatant was collected and centrifuged (60 min, 48400 g) for 1 h and the supernatant dialysed overnight against 3 l 20 mM HEPES buffer (pH 7.5), containing 1 mM DTT.

The dialysed crude extract was loaded onto a 6.5 × 2.5 cm affinity column prepared following the method of Ferenci & Lee (1982), except that 50 mg sucrose ml⁻¹ was coupled to the oxirane-activated Sepharose. The affinity column was eluted with 35 ml 20 mM HEPES buffer (pH 7.5) followed by 20 ml 20 mM HEPES buffer (pH 7.5) containing 0.1 M glycine. Fractions containing sucrase activity were eluted between 40 and 55 ml; these were pooled and applied to a 2.5 × 12 cm column of DEAE-Sephacel previously equilibrated with 20 mM HEPES buffer (pH 7.5), containing 1 mM DTT. A linear 400 ml gradient of 80 mM to 0.4 M KCl was applied over 15 h elution. Fractions (eluted after 230–350 ml) with high sucrase activity were combined and concentrated by freeze-drying. The concentrated sample was applied at a flow rate of 5.2 ml h⁻¹ to a 1.5 × 80 cm column of Sephacryl-300 (Pharmacia) equilibrated with 20 mM HEPES buffer (pH 7.5), containing 1 mM DTT. Fractions eluted between 60 and 80 ml containing sucrase activity were pooled and concentrated for further enzymic studies. A summary of the purification is shown in Table 1.

Sucrase assay. *E. coli* was grown at 37 °C overnight in 10 ml nutrient broth supplemented with 0.4% sucrose and 100 µg ampicillin ml⁻¹ and harvested by centrifugation. In assays of clones, the bacteria were washed twice with 100 mM sodium phosphate buffer (pH 6.6) and adjusted to OD₄₉₀ 2.0. Then 20 µl toluene was added to 1 ml bacterial suspension followed by incubation at room temperature for 10 min. Each 1 ml decryptified cell suspension or broken cell extract then received 100 µl 500 mM sucrose followed by incubation for 30 min at 55 °C. The assay mixture was heated in a boiling water bath for 1 min to stop the reaction. The samples were centrifuged for 2 min and the supernatant was collected for determination of glucose concentrations. Glucose concentration was measured using the Sigma Glucose Trinder Reagent according to the manufacturer's instructions. Sucrase activity of toluene-treated bacteria was expressed as nmol glucose released min⁻¹ by 10⁸ bacteria at 55 °C. Sucrase activity in French press extracts and in fractions during purification was expressed as nmol glucose released min⁻¹ (mg protein)⁻¹ at 55 °C.

Sucrose-6-phosphate hydrolase assay. *E. coli* was grown at 37 °C overnight in 100 ml nutrient broth supplemented with 0.4% sucrose and 100 µg ampicillin ml⁻¹. The cultures were harvested by centrifugation (10 min at 4000 g at 4 °C). The bacteria were washed with 100 ml 10 mM sodium phosphate

buffer (pH 6.8) and resuspended in 10 ml 10 mM sodium phosphate buffer (pH 6.8) containing PMSF (2 mM), RNase and DNase (each at 1 mg ml⁻¹). Bacteria were lysed by passing through a French press at 12000 p.s.i. and 1 mM MgSO₄ was added. The mixture was centrifuged to remove unbroken bacteria and recentrifuged (60 min at 48400 *g* at 4 °C). The sucrose-6-phosphate hydrolase activity of the supernatant was assayed by measuring the release of glucose 6-phosphate after 30 min at 55 °C. Each 1 ml reaction mix contained 100 mM MES buffer pH 7.1, 1 mM sucrose 6-phosphate (Sigma) and 100–200 µl extract. The glucose 6-phosphate was measured in a spectrophotometric coupled assay in the presence of glucose-6-phosphate dehydrogenase and NADP (Chassy & Porter, 1979). Each 1 ml reaction mix contained 100 mM MES buffer (pH 7.1), 0.5 mM NADP, 3 units glucose-6-phosphate dehydrogenase and 100–200 µl extract at 30 °C.

RESULTS

Subcloning and localization of the sucrase gene (*surA*)

Plasmid pAM2001 contained an insert of chromosomal DNA from *B. stearothermophilus* NUB36 and conferred on *E. coli* K-12 the ability to utilize sucrose, as tested on fermentation indicator plates. The presence of the plasmid also permitted growth of *E. coli* on sucrose-minimal media. Assays of extracts indicated the presence of an additional sucrase activity in *E. coli* containing the pAM2001-derived plasmid pAM2003, as indicated in Fig. 1. Subclones of the 4.5 kb DNA insert and deletion derivatives generated using exonuclease III were used to identify the coding region for sucrose utilization. As also shown in Fig. 1, deletions from the left end of the clone decreased sucrose utilization, but sucrase activity was retained in a subclone containing only 2 kb of insert (pAM2008). In contrast, deletion of 0.2 kb from the right (*Dra*I) side led to the complete loss of sucrose-hydrolysing activity. Therefore, the likely location of the sucrose-hydrolysing enzyme was between the *Nde*I and *Dra*I sites (Fig. 1).

Nucleotide sequence of *surA*

The nucleotide sequence of *surA* was determined in the subclone (pAM2008) retaining sucrase activity (Fig. 1). The sequence (not shown; GenBank accession number U34872) indicated one open reading frame starting with an ATG codon at position 630 and ending with TAA codon at position 1965. This gene is composed of 1338 nucleotides and codes for a 445-amino-acid protein with a calculated *M_r* of 51 519. The G + C content of the *surA* gene was 41.8 mol%. The coding region of *surA* is preceded by a putative translational start signal, ...GAGGGGGGAG..., 7 bp upstream of the start codon. No potential -35 and -10 consensus sequence was found upstream of the *surA* gene, and a coding region is apparent before an intergene region of 152 bp. Another feature of the DNA sequence was an inverted repeat ($\Delta G - 77 \text{ kcal mol}^{-1}$; -322 kJ mol^{-1}) downstream from the *surA* termination codon (98 nt after the *surA* stop codon), which might act as a transcription terminator for the gene.

Deduced amino acid sequence of SurA

The deduced amino acid sequence of the *B. stearothermophilus* NUB36 *surA* gene product was compared with those of other related protein sequences (Fig. 2). The SurA protein exhibited significant amino acid identity to the sucrose-6-phosphate hydrolase (SacA) of *B. subtilis* (55.3% identity in a 421-amino-acid overlap) and to the sucrose-6-phosphate hydrolases (ScrB) of *K. pneumoniae* and *Strep. mutans* (47.3% identity in a 357-amino-acid overlap and 38.1% identity in a 181-amino-acid overlap, respectively). SurA also showed a high level of identity to the sucrase (SacA) of *Z. mobilis* (36.8% identity in a 418-amino-acid overlap), the sucrase (ScrB) of *V. alginolyticus* (42.9% identity in a 354-amino-acid overlap), the levanase (SacC) of *B. subtilis* (31% identity in a 384-amino-acid

Fig. 1. Linear map of deletion derivatives of pAM2003. The cloned 4.5 kb DNA fragment of *B. stearothermophilus* NUB36 is shown in the black box. +, Positive fermentation phenotype on sucrose-EMB plates; -, lack of sucrose fermentation; ±, partial positive phenotype. The sucrase activity of each clone (nmol glucose released min⁻¹ by 10⁸ bacteria) is also shown.

SurA	MSKETEREK-----IEQAYRE---IEKYR
bacsaca_1	MTAH-DQEL-----RRAYEE---VEKYE
kpnscriab_3	MSLPS---R-----LPAILQA---VMQGO
scrib_zymmo	MESPSYKNIKAEDAQKK---AGKRLLS-----SEW---
scrib_strmu	M-----NLQNIYRR-YQDW-----
bacsacc-1	VFSYINNSDSETEENCQKRTSLRTSLFKCSNMKGAKQMKKRLIQVMIMTLLLTMAF
SurA	DKVEKD-----YRLQ YHLMPPVGLMNDPNG LIHWN GVIH
bacsaca_1	PIANSD-----PHRQH FHIMPPVGLMNDPNG VIYWK GSYH
kpnscriab_3	POALAD-----SHYPQ WHLAPVNGLLNDPNG FCQVA GRVH
scrib_zymmo	-----Y-----PG FHVPLTSMNDPNG LIPFK GEYH
scrib_strmu	--TEEEKSIKNNVALS-----PWHTT-----YHIEPKTCLLNDPNG FSYFN GKFN
bacsacc-1	SADAADSSYYDED-----YRPQ YHFTPEANWMNDPNG MVIYA GEYH
SurA	VFYQWMP FHTGHGAKF WGHYTSCLIHMKQEKIALAPSE WYD KDCYSGSAIDHDGVLTL
bacsaca_1	VFFQWQP FQTGHGAKF WGHYTTQDVVNMKREELAPSD WFD KNGCYSGSAVTKDDRLLYL
kpnscriab_3	LFYQWNP LACDHTYKC WGHWSADLLHWRHEPIALMPDE EYD RNCYSGSAVEFEFEGALTL
scrib_zymmo	LFYQYYP FAPVWGPMPH WGHAKSRDLVHWETLPVALAPGD SFD RDGCFSGCAVDNNGVLT
scrib_strmu	LFYQWNP FGAAHGLKS WHTSEDLVHFKEGTGTLYPDT SHD SHGAYSGSAYEIGDQLFL
bacsacc-1	LFYQYYP YGLQWGPMPH WGHAVSKDLVTWEHLPLVALYPDE --- KGTIFSGSAVVDKNNSTG
SurA	FYTG NVKDE-H---GNRQTY--QCMAMSKNGIDFEKKGIWVT-LPDGY-TAHFRDPKVV-
bacsaca_1	FYTG NVRDQ-D---GNRETY--QCLAVSDGLSFEEKGVVAR-LPEAILTAHFSRSEVW-
kpnscriab_3	CYTG NVKFP-D---GGRTAW--QCLATENADGTFKRLGPVLP-LPEGY-TGHVRDPKVV-
scrib_zymmo	IYTG HVLND---SPDAIREVQCMATSIDGIHFQKEGIVLEKAPMPQV-AHFRDPKVV-
scrib_strmu	FYTG NVR---D---ENWVRHPLQIGAFMDKKGNIQKFTDVLIKQPN-D-VTEHFRDPQIF-
bacsacc-1	FQGTG KEKPLVAIYTDREGHQVQSIAYSNKGRWTWKYAGNPVNP-GKKDFRDPKVVW
SurA	-KKGDKWYMIWGAQSESLD---GKAVLFRSQNLRDWEHLGPITGNSR-OL GKFGYMW
bacsaca_1	-EHEGTWYMIWGAQTEENL---GQAVLFASDNLTEWFLGPITGAGFN-GL DDFGYMW
kpnscriab_3	-RQDGRWYMIWGAQDVQQR---GKVLFTASDLREWRVGEIAGHDVN-GL ANAGYMW
scrib_zymmo	-KENDHWFMYVGYRTDDKKHQGIGHVALYRSENLDWIFVKTLLGDSNLPL GKRAFMW
scrib_strmu	-NYKGQFYAIVGAQSLD-----RILYTSKNLQWITYASEFGQDQ-----GSHGGVW
bacsacc-1	YEKEKWMVLAAGD-----RILYTSKNLQWITYASEFGQDQ-----GSHGGVW
SurA	CPD LFEL--DGQEVLIQVCPGLEAEMRYQNVHQSIFYV-GRLDYEPQFLHGP-FAEL-
bacsaca_1	CPD LFSL--QGSVDLIVSPQGLEADGFRYQNVYQSGIFYV-GRLDYKPKELKHGE-PTL-
kpnscriab_3	CPD LFPL--ADTHLLICCPGLAREAQRFNTYPAVWMA-GRFDAERGIFDHGP-LHEL-
scrib_zymmo	CPD FFSL--GNRSVLMFSPQGLKASGYKRNLFNGYIL-GKWQA-PQFTPETS-FOEL-
scrib_strmu	CPN LVFI--NEQPLVLYSPQGLSKSELDYHNIYPTNYKQCSFDTKEKPAVDASEIQNL-
bacsacc-1	CPD LFELPVDGNPNQKKWQVSVGNGAVSGSGSQMYFVG---DFDGFHKNEYPNPKVL
SurA	--DRGFEFYAP QT---MLDAKGR---ILAWMGVPPQDEDKHPTITHRWVHALTLPR
bacsaca_1	--DQGFDFYAP QT---LEDDQGR---ILFAMWVPPQDEGSHPTIDCHWIHCMTLPR
kpnscriab_3	--DSGFDFYAP QT---MQADDGR---LLVGMVPPQDEMHQPTRAQGWIHQMTCVRE
scrib_zymmo	--DYGHDFYAA QR---LKLKDGQRNINCMVMKSKPSQRD-----GWAGCMTLPRK
scrib_strmu	--DFGFECYAT QA---FNAPDGRV---YAVSWIGLP---DIDYPSDSYDYGALSIVKE
bacsacc-1	WT DYGRDFYAA VSWSDIPSTDSRR---LWLGMNSNQYANDVPTSPWRSATSIPE
SurA	LKLKPD---RKLYQT PVEELQMLR KDKIAYSNVTIANEEMILEGISG-DVIEL--NLNI
bacsaca_1	LTLSG---QKLIQ PLPELKAMR RNEKKI-HNMHGSGGALPVEKP-ERTEL--LLEDI
kpnscriab_3	LEWQA---GTLVOR PLRELVALR GEAGWCGQTPLAPMELAFDLS-PDSTL--GLD-
scrib_zymmo	LDLID---NKIVMT PVREMEILR QSEKIESVVTLSDAEHFFTMDSPLQEIETLIDLEKS
scrib_strmu	LSLKH---GKLYQY PVEAVRSLR SEKEAVTYKPTNNTYELTEFDSSSVNELLFADN
bacsacc-1	LKLKAPTEGVRVQT PVKELETIR GTSKKWKNLTISPASHNVLAGQSGDAYEINAEFKVS

Fig. 2. Comparison of the deduced amino acid sequence of *B. stearothermophilus* NUB36 SurA with sucrose hydrolases from other organisms. The proteins compared are the products of the following genes: *bacsaca_1*, *B. subtilis* *sacA* gene encoding sucrose-6-phosphate hydrolase; *kpnscriab_3*, *Klebsiella pneumoniae* *scrB* gene encoding sucrose-6-phosphate hydrolase; *scrib_zymmo*, *Zymomonas mobilis* *sacA* gene; *scrib_strmu*, *Streptococcus mutans* *scrB* gene encoding sucrose 6-phosphate hydrolase; *bacsacc-1*, *B. subtilis* *sacC* gene encoding levansucrase. Asterisks indicate identity across all sequences; dots indicate conservative substitutions. The last 65 residues of SurA, less obviously alignable with the other proteins, are not shown.

overlap) and Suc1, the yeast invertase (26.6% identity in a 338-amino-acid overlap).

Alignment of the amino acid sequence of SurA with five published amino acid sequences of enzymes involved in sucrose hydrolysis revealed strong homology in seven areas (Fig. 2). The so called 'sucrose box' (area D) identified by Sato & Kuramitsu (1988) and suggested to be important for enzymes catalysing the transfer of fructose from sucrose was conserved in all six aligned sequences. The amino acid sequence G-Y-M-W-E-C-P-D around cysteine 222 (area E) in the *surA*-encoded protein was also highly conserved in all the sequences. The importance of this region, as postulated by Martin *et al.* (1987), is that it may contain a catalytic thiol group. Five amino acid residues, N-D-P-N-G, in area A were identical in all six sugar hydrolases, suggesting the importance of this region to sucrose hydrolytic activity. The amino acid residues were particularly conserved in the NH₂-terminus of all the above compared enzymes (areas A–D), suggesting the importance of this region in the formation of the enzyme active site. The amino acid sequence of SurA and *B. subtilis* levansucrase show < 18% identity, as was

the case (< 12%) between the *B. subtilis* sucrose and levansucrase (Fouet *et al.*, 1986).

Analysis of the products of *surA* in *E. coli*

The *B. stearothermophilus* NUB36 *surA* gene was expressed in *E. coli* DE3 transformed by pAM2008 (containing *surA* only). After the incorporation of [³⁵S]methionine, the protein bands were analysed by SDS-PAGE and autoradiography. One major protein band of *M_r* around 57 000 was observed (Fig. 3). This value agrees reasonably with that (51 519) deduced from the nucleotide sequence of *surA*. No other major products were seen, even with the full-length (4.5 kb) clone.

Localization and characterization of SurA expressed in *E. coli*

Most (90%) of the sucrose activity in *E. coli* NM522 harbouring pAM2008 was recovered in the soluble cell fraction (cytoplasm and periplasm); no sucrose activity was found in the culture supernatant. To further identify the distribution of the sucrose (SurA) between cytoplasm

Fig. 3. Expression of the SurA protein in *E. coli*. The figure shows an autoradiograph of SDS-PAGE analysis of the [35 S]methionine-labelled proteins extracted from *E. coli* DE3 harbouring the following plasmids: lanes 1 and 2, pAM2003 (containing the entire cloned 4.5 kb DNA fragment); lanes 3 and 4, pAM2006 (containing 3.25 kb of the cloned 4.5 kb fragment; see Fig. 1); lanes 5 and 6, pAM2008 (containing close to 2 kb of the cloned 4.5 kb fragment; see Fig. 1); lanes 7 and 8, the cloning vector pGEM-7Zf(+). Odd-numbered lanes, IPTG-induced cells; even-numbered lanes, uninduced cells. The arrowheads point to the band migrating at M_r 57000 present in all sucrose-expressing clones.

and periplasm, osmotic shock analyses were carried out. In two experiments, 64–80% of the total sucrose-hydrolysing activity was retained in the cytoplasm and 9–19% was recovered from the osmotic shock fluid, which suggested that the *E. coli*-expressed SurA was a soluble, cytoplasmic protein. Indeed, the distribution of sucrose was closer to the cytoplasmic marker enzyme β -galactosidase (88–92% in the cytoplasm) than the periplasmic marker β -lactamase (11–17% in the cytoplasm) in the same fractionation.

Partial purification of SurA

To improve the yield of SurA for purification, *surA* was subcloned into the high-expression vector pTrc99A, resulting in clone pAM2020. Comparison of the sucrose-hydrolysing activity of *E. coli* JM105 harbouring pAM2008, pAM2006 or pAM2020 showed that about five times greater activity was achieved by subcloning the *surA* into pTrc99A. The sucrose hydrolase (SurA) was

partially purified after sucrose affinity chromatography, DEAE-anion-exchange and gel-filtration chromatography. Overall, sucrose hydrolase was purified approximately 22-fold, with a recovery of 26% (Table 1). The protein was not entirely pure, as four other minor bands were present in the preparation analysed by SDS-PAGE (not shown).

Properties of the expressed SurA

The optimum temperature for the activity of the sucrose was determined by incubating the partially purified enzyme with sucrose in phosphate buffer at different temperatures. Activity was maximal at 55 °C, but rapidly dropped to one-quarter of this activity at 60 °C. Almost no sucrose-hydrolysing activity was detected at 70 °C, which is above the 65 °C growth optimum of the organism (Fig. 4a).

The stability of the sucrose at different temperatures was tested by incubating the partially purified enzyme at 55, 60 and 70 °C, and assaying samples taken at different time points for sucrose-hydrolysing activity. The sucrose was stable at 55 °C for at least 1 h but gradually lost activity at 60 °C. The enzyme activity dropped to almost zero after 5 min at 70 °C (Fig. 4b). The enzyme was very stable at room temperature (enzyme activity remained the same after 3 d at room temperature).

The activity and affinity of the *B. stearothermophilus* sucrose for sucrose and sucrose 6-phosphate were studied using the partially purified enzyme. Using a sucrose concentration range from 1 to 200 mM, the rates of sucrose hydrolysis were measured by calculating the rate of glucose released from the slopes of the initial velocity curves. The apparent K_m value of the sucrose was found to be 40 mM from a linear double-reciprocal plot.

As sucrose activity is associated with a sucrose-6-phosphate hydrolase in *B. subtilis* (Kunst *et al.*, 1974), this second activity was also assayed, using two different methods (Chassy & Porter, 1979; St Martin & Wittenberger, 1979). Despite strong sucrose-hydrolysing activity being detected in the cell extract of *E. coli* NM522 harbouring pAM2008, no cleavage of sucrose 6-phosphate to glucose 6-phosphate was detected. This was surprising considering the strong sequence homology with the sucrose-6-phosphate hydrolases mentioned above, and it is possible that SurA may have the ability to hydrolyse sucrose 6-phosphate under assay conditions not tested by us.

The M_r of the native form of sucrose (SurA) was determined using gel-filtration chromatography (Sephacryl 300) and calibration with standard markers [thyroglobulin (670 000), bovine gamma globulin (158 000), chicken ovalbumin (44 000), equine myoglobin (17 000) and vitamin B₁₂ (1350)]. An M_r of 105 000 was calculated from a linear calibration curve. The M_r of SurA of *B. stearothermophilus* deduced from the DNA sequence was 51 519, so, the native form of the enzyme is probably a dimer.

Table 1. Partial purification of the cloned *B. stearotherophilus* NUB36 sucrose from *E. coli* extracts

Sucrase was assayed in the extract and subsequent column fractions as described in Methods. The conditions for sucrose-Sepharose affinity chromatography, ion-exchange chromatography and gel filtration are also defined in Methods.

Step	Total activity (units)	Total protein (mg)	Specific activity (units mg ⁻¹)	Recovery (%)
Crude extract	1193	260	4.6	100
Affinity chromatography	462	21	22	39
DEAE-Sepharose chromatography	384	5.8	66.8	32
Sephacryl-300 chromatography	312	3.0	103	26

Fig. 4. (a) Temperature-activity profile for the partially purified *B. stearotherophilus* NUB36 sucrase. (b) Stability of the sucrase at 55 °C (◆), 60 °C (■) and 70 °C (▲).

DISCUSSION

Comparison of the amino acid sequence of the *surA*-encoded protein with other functionally similar proteins showed that it shares 55.3%, 38.1% and 47.3% amino acid identity with the sucrose-6-phosphate hydrolases of *B. subtilis* (SacA), *Strep. mutans* (ScrB) and *K. pneumoniae* (ScrB), respectively. Amino acid identities of 42.9% and 36.8% were found by comparison with the sucrases of *V. alginolyticus* (ScrB) and *Z. mobilis* (SacA), respectively. Further sequence analysis showed that the sucrose box, a consensus sequence containing nine amino acids (Sato &

Kuramitsu, 1988) was conserved in *B. stearotherophilus* NUB36 sucrase. This result supports the suggestion (Sato & Kuramitsu, 1988) that the sucrose box is important for enzymes catalysing the hydrolysis of sucrose into glucose and fructose.

The strong homology with both sucrose-6-phosphate hydrolases and sucrases suggested that the *surA*-encoded protein of *B. stearotherophilus* NUB36 would be able to hydrolyse both sucrose 6-phosphate and sucrose, with different affinities, as does the *scrB*-encoded sucrose-6-phosphate hydrolase of *Strep. mutans* expressed in *E. coli*, which showed a K_m of 180 mM for sucrose and 0.08 mM for sucrose 6-phosphate (Lunsford & Macrina, 1986). However, the partially purified *B. stearotherophilus* SurA demonstrated an apparent K_m of 40 mM for sucrose but no sucrose 6-phosphate hydrolase activity was observed.

Sequence analysis showed that the *surA*-encoded sucrase had no secretory signal sequence, and further experiments indicated that the *E. coli*-expressed SurA was located in the cytoplasmic fraction of the cell extract. This suggests that the enzyme is not secreted and that the substrate has to be accumulated in the cytoplasm by one or more sucrose transport systems. It is likely that the truncated open reading frame upstream of *surA* is involved in sucrose transport, as deletion of this upstream region reduced sucrose fermentation (Fig. 1). The sequence of the upstream gene (submitted to GenBank with accession number U34873) resembled the sucrose enzyme II of *B. subtilis* (SacP), and the absence of a promoter in front of *surA* makes it likely that *surA* is coregulated with the upstream gene(s). In *B. subtilis*, the *sacP* sucrose phosphotransferase gene is upstream of the *surA* homologue (*sacA*) but it remains to be shown whether there is a functional sucrose enzyme II present in *B. stearotherophilus*. It appears paradoxical that SurA operates in sucrose hydrolysis but not in sucrose phosphate cleavage, given that the gene sequence and gene organization resemble those responsible for sucrose-phosphotransferase-mediated metabolism in *B. subtilis*.

The optimum temperature for the activity of SurA was 55 °C and the enzyme was highly stable at room temperature. The stability of SurA resembles that of the *Strep. mutans* sucrose-6-phosphate hydrolase, which was stable for several days at room temperature (Chassy & Porter, 1979). The M_r of the SurA (expressed and partially purified from *E. coli*) determined by gel filtration was 105000, indicating that the active form of the enzyme is probably a dimer. The M_r of *B. subtilis* SacA determined by chromatography was 40000 (Pascal *et al.*, 1971), and to our knowledge no report about the dimerization of sucrose-hydrolytic enzymes has previously been published. The availability of the cloned *surA* gene will permit more detailed future characterization of a thermophilic sucrase.

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REFERENCES

- Chassy, B. M. & Porter, E. V. (1979). Initial characterization of sucrose 6-phosphate hydrolase from *Streptococcus mutans* and its apparent identity with intracellular invertase. *Biochem Biophys Res Commun* **89**, 307–314.
- Chen, Z. F., Wojcik, S. F. & Welker, N. E. (1986). Genetic analysis of *Bacillus stearothermophilus* by protoplast fusion. *J Bacteriol* **165**, 994–1001.
- Ebner, R. & Lengeler, J. W. (1988). DNA sequence of the gene *scrA* encoding the sucrose transport protein EnzymeII^{scr} of the phosphotransferase system from enteric bacteria: homology of the EnzymeII^{scr} and EnzymeII^{Bgl} proteins. *Mol Microbiol* **2**, 9–17.
- Ferenci, T. & Lee, K. S. (1982). Directed evolution of the lambda receptor of *Escherichia coli* through affinity chromatographic selection. *J Mol Biol* **160**, 431–444.
- Fouet, A., Klier, A. & Rapoport, G. (1986). Nucleotide sequence of the sucrase gene of *Bacillus subtilis*. *Gene* **45**, 221–225.
- Francis, G., Brennan, L. & Ferenci, T. (1991). Affinity-chromatographic purification of sixteen cysteine-substituted maltoporin variants: thiol reactivity and cross-linking in an outer membrane protein of *Escherichia coli*. *Biochim Biophys Acta* **1067**, 89–96.
- Gunasekaran, P., Karunakaran, T., Cami, B., Mukundan, A. G., Preziosi, L. & Baratti, J. (1990). Cloning and sequencing of the *sacA* gene: characterization of a sucrase from *Zymomonas mobilis*. *J Bacteriol* **172**, 6727–6735.
- Kellermann, O. K. & Ferenci, T. (1982). Maltose-binding protein from *Escherichia coli*. *Methods Enzymol* **90**, 459–463.
- Kunst, F., Pascal, M., Lepesant, J. A., Walle, J. & Dedonder, R. (1974). Purification and some properties of an endocellular sucrase from a constitutive mutant of *Bacillus subtilis* Marburg 168. *Eur J Biochem* **42**, 611–620.
- Lepesant, J. A. (1976). Specific and pleiotropic regulatory mechanisms in the sucrose system of *Bacillus subtilis* 168. In *Microbiology – 1976*, pp. 58–69. Edited by D. Schlessinger. Washington, DC: American Society for Microbiology.
- Liong, E. C. (1995). *Maltose genes in Bacillus stearothermophilus ATCC7953*. PhD thesis, University of Sydney.
- Lunsford, R. D. & Macrina, F. L. (1986). Molecular cloning and characterization of *scrB*: the structural gene for the *Streptococcus mutans* PTS sucrose 6-phosphate hydrolase. *J Bacteriol* **166**, 426–434.
- Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Martin, I., Debarbouille, M., Ferranri, E., Klier, A. & Rapoport, G. (1987). Characterisation of the levanase gene of *Bacillus subtilis* which shows homology to yeast invertase. *Mol Gen Genet* **208**, 177–184.
- Pascal, M., Kunst, F., Lepesant, J. A. & Dedonder, R. (1971). Characterisation of two sucrase activities in *Bacillus subtilis* Marburg. *Biochimie* **53**, 1059–1066.
- Robeson, J. P., Barletta, R. G. & Curtiss, R. (1983). Expression of a *Streptococcus mutans* glucosyltransferase gene in *Escherichia coli*. *J Bacteriol* **153**, 211–221.
- Russell, R. R. B., Morrissey, P. & Dougan, G. (1985). Cloning a sucrase gene from *Streptococcus mutans* in bacteriophage lambda. *FEMS Microbiol Lett* **30**, 37–41.
- St Martin, E. J. & Wittenberger, C. L. (1979). Regulation and function of sucrose 6-phosphate hydrolase in *Streptococcus mutans*. *Infect Immun* **26**, 487–491.
- Sanger, F., Nicklen, S. & Coulson, A. R. (1975). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74**, 5463–5467.
- Sato, Y. & Kuramitsu, H. K. (1988). Sequence analysis of the *Streptococcus mutans scrB* gene. *Infect Immun* **56**, 1956–1960.
- Schmid, K., Schupfner, M. & Schmitt, R. (1982). Plasmid-mediated uptake and metabolism of sucrose by *Escherichia coli* K12. *J Bacteriol* **151**, 68–76.
- Schmid, K., Ebner, R., Altenbuchner, J., Schmitt, R. & Lengeler, J. W. (1988). Plasmid-mediated sucrose metabolism in *Escherichia coli* K12: mapping of the *scr* genes of pUR400. *Mol Microbiol* **2**, 1–8.
- Scholle, R. R., Robb, S. M., Robb, F. T. & Woods, D. R. (1989). Nucleotide sequence and analysis of the *Vibrio alginolyticus* sucrose gene (*scrB*). *Gene* **80**, 49–56.
- Sharp, R. J., Bown, K. J. & Atkinson, T. (1980). Phenotypic and genotypic characterisation of some thermophilic species of *Bacillus*. *J Gen Microbiol* **117**, 201–210.
- Tabor, S. & Richardson, C. (1985). A bacteriophage T7 RNA polymerase promoter system for controlled exclusive expression of specific genes. *Proc Natl Acad Sci USA* **82**, 1074–1078.
- Welker, N. E. (1978). Physiological and genetic factors affecting transfection and transformation in *Bacillus stearothermophilus*. In *Biochemistry of Thermophily*, pp. 127–147. Edited by S. M. Friedman. New York: Academic Press.

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