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SHORT CONTRIBUTION

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Production of trehalose synthase from a basidiomycete, *Grifola frondosa*, in *Escherichia coli*

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Abstract The genomic DNA and cDNA for a gene encoding a novel trehalose synthase (TSase) catalyzing trehalose synthesis from α-D-glucose 1-phosphate and D-glucose were cloned from a basidiomycete, *Grifola frondosa*. Nucleotide sequencing showed that the 732-amino-acid TSase-encoding region was separated by eight introns. Consistent with the novelty of TSase, there were no homologous proteins registered in the databases. Recombinant TSase with a histidine tag at the NH_2-terminal end, produced in *Escherichia coli*, showed enzyme activity similar to that purified from the original *G. frondosa* strain. Incubation of α-D-glucose 1-phosphate and D-glucose in the presence of recombinant TSase generated trehalose, in agreement with the enzymatic property of TSase that the equilibrium lay far in the direction of trehalose synthesis.

Introduction

Trehalose (1-α-D-glucopyranosyl α-D-glucopyranoside) is a disaccharide with an α,α-1,1 glycosidic linkage and is widely distributed in plants, insects, fungi, yeast, and bacteria (Elbein 1974). In trehalose-producing organisms, it supposedly serves as a storage form of energy, a protectant from stresses, such as desiccation and freezing, and a stabilizer of proteins (Rudolph and Crowe 1985; Carpenter and Crowe 1988; van Laere 1989; Wiemken 1990). Trehalose is highly resistant to heat, pH, and Maillard's reaction owing to the absence of reducing ends (Ohtani and Usui 1994). It has therefore been expected that trehalose would become a useful compound as a sweetener and a stabilizer in foods and as an additive in cosmetics and pharmaceuticals (Colaco et al. 1992; Roser 1991).

We have recently discovered a trehalose synthase (TSase) catalyzing the synthesis of trehalose from D-glucose and α-glucose 1-phosphate from a basidiomycete, *Grifola frondosa*, during a screening program (unpublished results). There has been no report on the purification or enzymatic characterization of TSases that use α-D-glucose 1-phosphate as one of their substrates, although the presence of such activity was detected in yeast, *Pichia fermentans* (Schick et al. 1995), and a basidiomycete, *Flammulina velutipes* (Kitamoto et al. 1988). The TSase equilibrium of *G. frondosa* lies far in the direction of trehalose synthesis. The enzyme shows strict substrate specificity; only trehalose is phosphorolyzed among various disaccharides and only α-glucose 1-phosphate serves as a donor substrate to glucose as the acceptor in trehalose synthesis. Since α-D-glucose 1-phosphate is liberated by the action of sucrose phosphorylases from an abundant and inexpensive disaccharide, sucrose, the combination of the TSase and a sucrose phosphorylase would generate trehalose from sucrose by an enzymatic process. In fact, this enzymatic process has turned out to be useful in trehalose synthesis and generated trehalose from sucrose at a rate of 18 mmol/h with a final yield of 90 mol% (manuscript in preparation). We then started to reveal the amino acid sequence of this novel enzyme by cloning and nucleotide sequencing of the cDNA and to produce the enzyme by recombinant DNA techniques, which would lead to production and supply of the TSase in a large quantity without purification from fruiting bodies of the basidiomycete through several steps of column chromatography. This communication describes cloning and characterization of the TSase gene as the first steps

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towards establishing an efficient enzymatic system for trehalose synthesis.

Materials and methods

Microorganisms and plasmids

G. frondosa CM-236 was a stock culture from the Nishiki Research Laboratories, Kureha Chemical Industry Co. Ltd., Fukushima. This strain has been deposited at the National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology, Japan, with the accession number FERM BP-35. *Escherichia coli* strains DH5α and JM109 were used as the hosts for most of DNA manipulations with pUC18 (Yanisch-Perron et al. 1985). The *E. coli* BL21 (DE3) used for production of TSase was purchased from Promega Co. (Madison, Wis.). Plasmid pET-16b (Novagen, Madison) was used for production of TSase in *E. coli* BL21 (DE3).

Amino acid sequencing

TSase was purified to homogeneity from fruiting bodies of *G. frondosa* by ammonium sulfate fractionation and six steps of column chromatography (unpublished results). The amino acid sequence of the NH_2-terminal region was determined by Edman degradation (Edman and Begg 1967) on a Shimazu PSQ-1 protein sequencer. For determination of inner amino acid sequences, the purified TSase was digested by lysyl endopeptidase (Wako Pure Chemicals, Tokyo) and several cleaved fragments were prepared by HPLC column chromatography. The amino acid sequences of their NH_2-terminal regions were similarly determined.

Cloning of the genomic TSase gene

Mycelium from *G. frondosa* was frozen with liquid nitrogen and disrupted by grinding in a mortar. From the disrupted mycelium, genomic DNA and total RNA were prepared by the standard procedures. DNA manipulations were described by Maniatis et al. (1982). The following two primers were synthesized and used for amplification of part of the TSase gene with the genomic DNA as the template: 5′-GGG-TAC-ACC-AGC-*CTG-CAG*-CCC-ATG-TGG-GC-3′ (corresponding to part of the NH_2-terminal amino acid sequence, Gly-Tyr-Thr-Ser-Leu-Thr-Pro-Met-Trp-Ala; italic letters indicate a *Pst*I site) and 5′-GGC-*AAG-CTT*-GAT-GAC-CGA-GTT-CCA-AGT-3′ (corresponding to the NH_2-terminal amino acid sequence of one of the lysyl-endopeptidase-generated fragments, Thr-Trp-Asn-Ser-Val-Ile-Lys; italic letters indicate a *Hin*dIII site). The polymerase chain reaction (PCR) under the standard conditions yielded a 800-bp fragment, which was then cloned in pUC18 by making use of the *Pst*I and *Hin*dIII sites at the ends. Southern hybridization with the 800 bp as probe against the genomic DNA revealed the presence of a 4.5-kb signal in the *Sac*I digest. Because cloning and nucleotide sequencing of the 4.5-kb fragment suggested that the 3′ portion of the TSase gene was absent, we further cloned that region by the standard gene-walking techniques including colony hybridization. A genomic DNA in a total of 7.5 kb was cloned in this way. The nucleotide sequence of the whole TSase gene was determined by the M13 dideoxynucleotide method (Sanger et al. 1977) with M13mp18 and M13mp19 (Yanisch-Perron et al. 1985).

Cloning of cDNA

Comparison of the amino acid sequences and the nucleotide sequence of the cloned genomic DNA predicted the initiation and termination codons. cDNA for the TSase gene was cloned by reverse transcription (RT) PCR with the following primers (see Fig. 1): 5′-GCC*GGATCC*CC**ATG**GCTCCTCCCCACCAGTTCC-3′ (bold letters indicate the initiation codon and italic letters indicate a *Bam*HI site) and 5′-GCC*GAATTC***TCA**TCCCTGCACATGCAGTTCG-CC-3′ (bold letters indicate the termination codon and italic letters indicate an *Eco*RI site). cDNA was amplified with the Takara RT-PCR kit according to the protocol. The *Bam*HI and *Eco*RI sites facilitated cloning of the cDNA in pUC18.

Expression of the TSase gene in *E. coli*

The recombinant pUC18 containing the TSase gene was first cut with *Eco*RI and filled-in with Klenow fragment. The linear DNA was then cut with *Bam*HI to generate a DNA fragment with a *Bam*HI end at one end and a blunt end at the other. Plasmid pET-16b was cut with *Bpu*1102I and blunt-ended with Klenow fragment. The linear pET vector was then cut with *Bam*HI, into which the TSase gene was inserted. TSase, if produced under the control of the T7 promoter in pET, would be produced as a fusion protein with an additional 26 amino acids in front of the NH_2-terminal amino acid, methionine. The additional region contained 10 histidine residues, which facilitated purification of the protein with a nickel column.

A portion (10 ml) of seed culture of *E. coli* BL21 (DE3) harboring the recombinant pET plasmid was inoculated in 1 l of Luria-Bertani (LB) broth supplemented with 50 μg/ml ampicillin and grown at 30 °C overnight. The cells were washed once with 10 mM TRIS/HCl (pH 7.5) containing 150 mM NaCl and suspended in 20 mM potassium phosphate buffer (pH 7.0) containing 20% (v/v) glycerol, and 1 μM each of pepstatin A (Wako Pure Chemicals) and 4-(2-aminoethyl)-benzosulfonylfluoride hydrochloride (pefabloc SC; Merck). The cells were disrupted by sonication and insoluble materials were removed by centrifugation. The supernatant was filtered through a 0.45-μm filter and applied to a nickel column according to the pET system manual. Proteins were eluted with a linear gradient of imidazol (60–500 mM) in 20 mM potassium phosphate containing 500 mM KCl. Each fraction was analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli 1970). Fractions containing TSase were pooled and dialyzed against 20 mM potassium phosphate buffer containing 20% glycerol.

Enzyme assay

TSase was measured by the phosphorolysis of trehalose into glucose and α-glucose 1-phosphate. The amount of the α-glucose 1-phosphate produced was determined by using phosphoglucono-mutase (PGM; α-D-glucose 1-phosphate → α-D-glucose 6-phosphate) and glucose-6-phosphate dehydrogenase (G6PDH; α-D-glucose 6-phosphate + $NADP^+$ → 6-phospho-D-gluconolactone + NADPH + H^+). PGM and G6PDH were purchased from Boehringer Mannheim. TSase activity was thus monitored by the change in absorbance at 340 nm, because the rate of NADPH generation was proportional to the TSase activity. The reaction mixture contained 200 mM trehalose, 40 mM potassium phosphate buffer (pH 7.0), 10 mM glutathione, 0.16 mM EDTA, 1 mM $NADP^+$, 1.3 mM $MgCl_2 \cdot 6H_2O$, 0.067 mM glucose 1,6-bisphosphate, 1.55 U/ml PGM, 1.75 U/ml G6PDH, and an enzyme sample in a total volume of 2 ml. During incubation, the change in absorbance at 340 nm was followed with an automated spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol α-glucose 1-phosphate/min under the assay conditions.

Synthesis of trehalose

The reaction mixture, containing 100 mM HEPES buffer (pH 7.0), 225 mM glucose, 300 mM α-glucose 1-phosphate, and 0.3 U TSase in a total volume of 250 μl, was incubated at 35 °C for 24 h. The reaction was stopped by boiling for 5 min. The reaction mixture

was then subjected to thin-layer (silica gel 60; Merk no. 5715) chromatography with a solvent system of 2-propanol/acetone/0.1 M lactate (2:2:1) and the trehalose generated was detected with sulfuric acid.

Nucleotide sequence accession numbers

The genomic and cDNA sequences reported here have been deposited in the DDBJ, EMBL, and GenBank databases under the accession numbers AB010104 and AB010105 respectively.

Results

Cloning of genomic DNA covering the TSase gene

SDS-PAGE and gel-filtration analyses of purified TSase showed that it was composed of two identical subunits, each of 60 kDa (unpublished results). For cloning of the genomic DNA covering the TSase gene, the NH_2-terminal amino acid sequence of the purified enzyme was determined by the Edman degradation procedure. These experiments established the sequence of 21 amino acids from lysine to alanine (Fig. 1). We also determined inner amino acid sequences by using lysyl-endopeptidase-generated fragments that had been isolated by HPLC. Nine fragments recovered by HPLC gave the respective NH_2-terminal amino acid sequences, as shown in Fig. 1.

We synthesized an oligonucleotide corresponding to one of the inner amino acid sequences in order to use it as one primer to amplify the corresponding genomic sequence by PCR, with the other primer corresponding to the NH_2-terminal amino acid sequence of the mature enzyme, as described in Materials and methods. This PCR yielded an 800-bp DNA fragment. Nucleotide sequencing of the amplified DNA showed that it encoded two of the inner amino acid sequences, and also suggested that it included introns. We therefore cloned cDNA to produce TSase in *E. coli*, as described below. Gene-walking techniques on the basis of the 800-bp fragment yielded a 7.5-kb genomic DNA covering the whole TSase gene. Figure 1 shows the nucleotide sequence of the region covering the TSase gene.

Cloning of cDNA for TSase

Because the nucleotide sequence of the genomic DNA showed the presence of introns, we cloned cDNA to identify the open reading frame and the exact location of introns. Analysis of the nucleotide sequence on the basis of the NH_2-terminal and inner amino acid sequences and the molecular mass (60 kDa) of the mature enzyme predicted that the ATG at nucleotide positions 1–3 in Fig. 1 and the TGA at positions 2744–2746 served as the initiation and termination codons respectively. We therefore synthesized two primers, one of which included the putative initiation codon and the other the termination codon, as described in Materials and methods. RT-PCR with these two primers yielded a 2.2-kb DNA fragment.

The nucleotide sequence of cDNA identified the exact location of eight introns (Fig. 1). All the introns had GT at their 5′ ends and AG at their 3′ ends, consistent with the consensus sequence of introns in eukaryotes. It was thus apparent that TSase was primarily produced as a protein of 732 amino acids and the NH_2-terminal 25 amino acids were processed during maturation. The calculated molecular mass of the TSase (Lys^{26}–Gly^{732}) was 78.8 kDa, whereas the mature enzyme purified from *G. frondosa* was 60 kDa, as determined by SDS-PAGE. The discrepancy may be due to unusual electrophoretic behavior of TSase or to possible processing at the COOH-terminal region. Consistent with the novelty of a TSase of this type, no proteins homologous with this TSase were found in the databases. In front of the putative ATG codon was a pyrimidine-rich sequence (nucleotide positions −43 to −12), as observed for many genes in fungi (Gurr et al. 1987). A TATA box, important for the initiation of transcription (Ballance 1986), was also present at nucleotide position −58. Downstream from the termination codon, a TATATA sequence, similar to the consensus polyadenylation signal sequence TAYRTA in *Saccharomyces cerevisiae* (Irniger and Brans 1994), was present.

Expression of the TSase gene in *E. coli*

The NH_2-terminal 25 amino acids that might be processed during maturation were unlikely to serve as a signal sequence for secretion. We therefore constructed an expression plasmid, pET-TS2, by which the whole TSase protein (Met^1–Gly^{732}) would be produced fused to a 26-amino-acid peptide with 10 histidines derived from pET-16b (Fig. 2A), in order to determine whether the cloned TSase gene actually specifies the trehalose synthase activity. *E. coli* harboring pET-TS2 produced almost the same amount of a protein of approximately 90 kDa in the presence and absence of isopropyl β-D-thiogalactoside (IPTG), as determined by SDS-PAGE (data not shown). The calculated molecular mass of the fusion protein was 84.7 kDa. We therefore grew the recombinant *E. coli* cells in the absence of IPTG and disrupted the cells by sonication. SDS-PAGE of the lysate is shown in Fig. 2B. More than half of the 90-kDa protein was produced in the insoluble fraction, probably as inclusion bodies, and less appeared in the soluble fraction. We applied the soluble fraction to a nickel column to purify the histidine-tagged protein. The purified TSase gave a single band on SDS-PAGE (Fig. 2B).

The histidine-tagged TSase protein was measured by its trehalose-phosphorolyzing activity, as described in Materials and methods. The specific activity was determined to be 3.4 U. Mature TSase purified from *G. frondosa* had a specific activity of 4.2 U (unpublished data). All of these data showed that the fusion protein actually had trehalose phosphorolysis, and probably

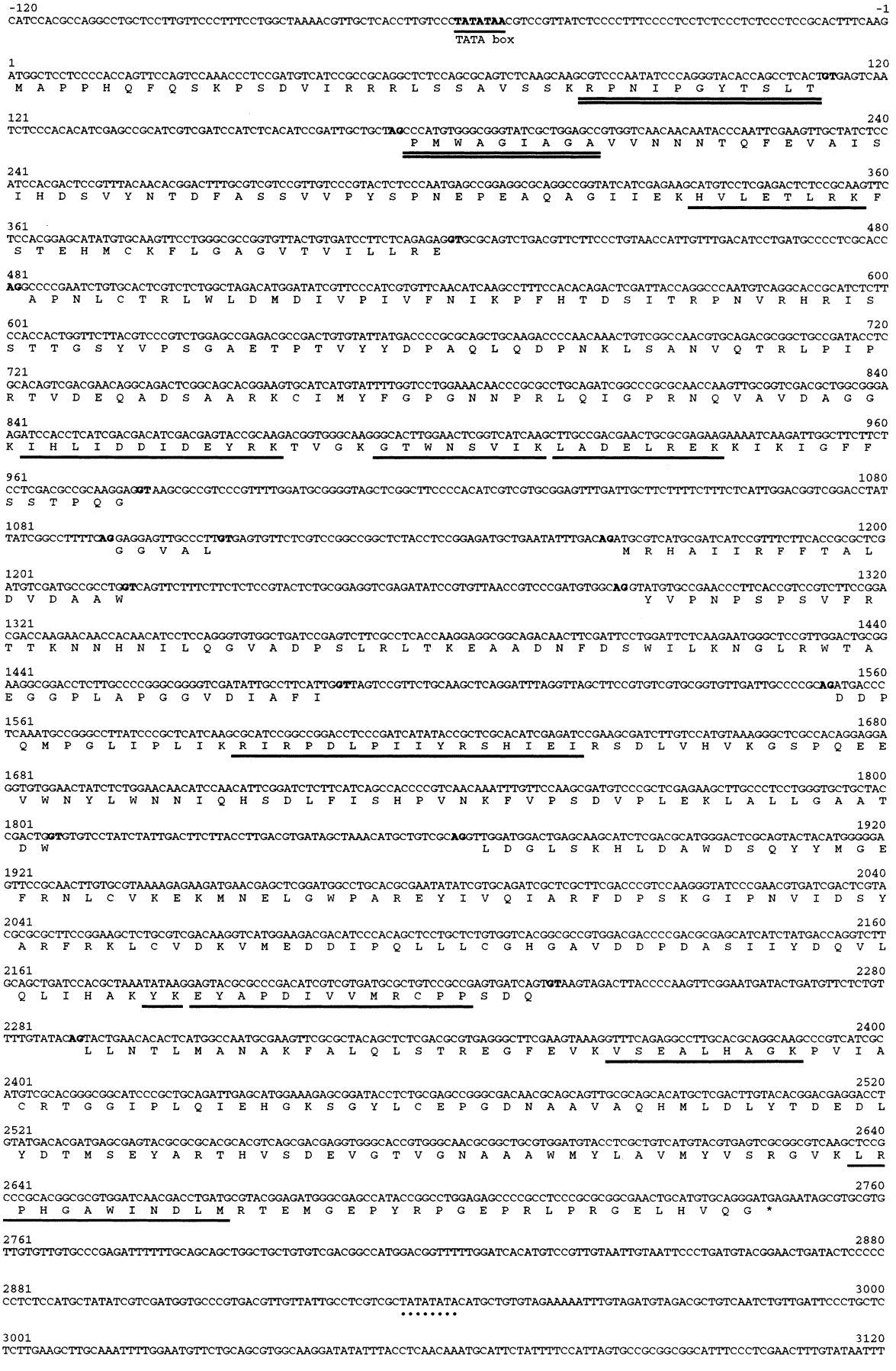
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Fig. 1 Nucleotide sequence of the genomic DNA covering the trehalose synthase (TSase) gene from *Grifola frondosa*. The amino acid sequence deduced from comparison of the genome and cDNA is shown. The NH_2-terminal amino acid sequence of mature TSase purified from *G. frondosa* is shown by a double underline. Inner amino acid sequences determined with the purified TSase are also shown by underlines. Eight introns, all of which have the consensus sequence, 5′-GT/AG-3′ (*in bold*), are indicated. A probable TATA box and a poly(A) signal (···) are also indicated

synthesis, activity. In addition, it was apparent that the presence of the NH_2-terminal 25 amino acids in mature TSase had almost no effect on enzyme activity.

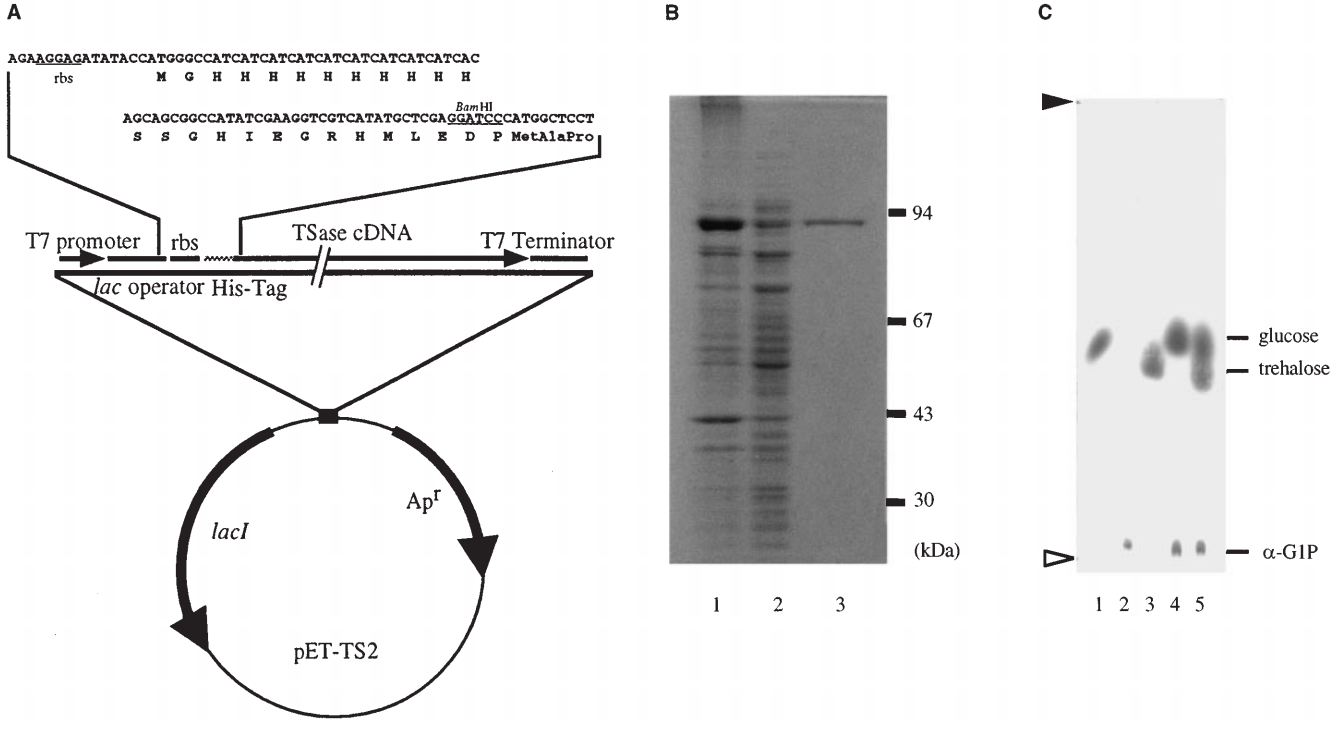
Fig. 2A–C Expression of the TSase gene in *Escherichia coli* and production of a histidine-tagged TSase. **A** Structure of the expression plasmid derived from pET-16b. The 26-amino-acid peptide containing 10 histidines is attached to the entire TSase-coding region. Ap^r ampicillin-resistance determinant; *rbs* ribosome-binding sequence. **B** Sodium dodecyl sulfate/polyacrylamide gel electrophoresis of recombinant TSase produced in *E. coli*. The sonicate from *E. coli* harboring the expression plasmid was centrifuged to obtain insoluble (*lane 1*) and soluble (*lane 2*) fractions. Passage of the lysate through a nickel column gave almost homogeneous TSase (*lane 3*). **C** Thin-layer chromatography of the reaction mixture. After incubation of α-D-glucose 1-phosphate and D-glucose in the presence of recombinant TSase for 24 h, a portion of the reaction mixture, together with references, was developed on a silica gel plate. The origin (▷) and the solvent top (▶) are indicated. Lanes: *1* D-glucose, *2* α-D-glucose 1-phosphate, *3* trehalose, *4* a reaction mixture without TSase (a negative control), *5* the reaction mixture after incubation. Densitometric analysis of the trehalose produced showed that about 35% of glucose in the mixture was converted into trehalose

Synthesis of trehalose

Since the equilibrium of TSase from *G. frondosa* lay far in the direction of trehalose synthesis from α-D-glucose 1-phosphate and D-glucose, we next used the fusion protein for trehalose synthesis. Incubation of the two substrates in the presence of the fusion protein for 24 h generated trehalose in approximately 35 mol% yield (Fig. 2C). TSase from *G. frondosa* converted the substrates into trehalose at 65 mol% under the optimum conditions (unpublished data). We assume that the yield of trehalose might be improved by optimizing the reaction conditions.

Discussion

We cloned the cDNA for TSase, which was useful in enzymatic trehalose synthesis from sucrose. The structure of the TSase gene was also characterized by comparison with the sequences of the genome and cDNA. This is the first step towards establishing a more efficient system for enzymatic synthesis of trehalose from sucrose. For practical purposes, we will have to (i) determine the most efficient fusion by changing the peptide to be fused and the part of the TSase sequence, to obtain high enzyme activity, (ii) improve culture conditions of the recombinant *E. coli* cells or use some other hosts to avoid formation of inactive inclusion bodies, and (iii) determine the optimum reaction conditions for the recombinant TSase.



In addition to the practical use of TSase, the present work will shed light on trehalose synthesis in basidiomycetes and fungi. Similar trehalose synthesis activity has been found to be distributed in a wide variety of these lower eukaryotes (manuscript in preparation). Although trehalose is synthesized mainly from trehalose 6-phosphate by the dephosphorylating activity of trehalose-6-phosphate phosphatase (Matula et al. 1971; Lapp et al. 1972), TSases catalyzing trehalose synthesis from α-D-glucose 1-phosphate and D-glucose may have a physiological role. The TSase gene will also facilitate studies of the mechanism of catalysis by means of protein engineering.

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