Trehalose 6-phosphate phosphorylase is part of a novel metabolic pathway for trehalose utilization in *Lactococcus lactis*

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Summary

*Lactococcus lactis* splits phosphorylated trehalose by the action of inorganic phosphate-dependent trehalose 6-phosphate phosphorylase (TrePP) in a novel catabolic pathway. TrePP was found to catalyze the reversible conversion of trehalose 6-phosphate into β-glucose 1-phosphate and glucose 6-phosphate by measuring intermediate sugar phosphates in cell extracts from trehalose-cultivated lactococci. According to native PAGE and SDS-PAGE, TrePP was shown to be a monomeric enzyme with a molecular mass of 94 kDa. Reaction kinetics suggested that the enzyme follows a ternary-complex mechanism with optimal phosphorolysis at 35 °C and pH 6.3. The equilibrium constants were found to be 0.026 and 0.032 at pH 6.3 and 7.0, respectively, favoring the formation of trehalose 6-phosphate. The Michaelis-Menten constants of TrePP for trehalose 6-phosphate, inorganic phosphate, β-glucose 1-phosphate and glucose 6-phosphate were determined to be 6 mM, 32 mM, 0.9 mM, and 4 mM, respectively. The TrePP-encoding gene, designated *trePP*, was localized in a putative trehalose operon of *L. lactis*. This operon includes the gene encoding β-phosphoglucomutase in addition to three open reading frames believed to encode a transcriptional regulator and two trehalose-specific phosphotransferase system components. The identity of *trePP* was confirmed by determining the N-terminal amino acid sequence of TrePP and by its overexpression in *Escherichia coli* and *L. lactis*, as well as the construction of a lactococcal *trePP* knockout mutant. Furthermore, both TrePP and β-phosphoglucomutase activity were detected in *Enterococcus faecalis* cell extract, indicating that this bacterium exhibits the same trehalose assimilation route as *L. lactis*.
Introduction

Trehalose is abundant in nature and serves as an important carbon and energy source to many organisms, including the lactic acid bacterium *Lactococcus lactis*, which is found on plant material (1). The metabolism of trehalose has been studied extensively in many microorganisms and there are a number of reports in the literature concerning alternative catabolic pathways of this disaccharide (Fig. 1). Trehalose may be transported across the cytoplasmic membrane either by a permease or by a phosphotransferase system (PTS), leaving trehalose unmodified or phosphorylated as trehalose 6-phosphate (T6P) inside the cell, respectively (2,3). The further degradation of trehalose or T6P may involve a hydrolyzing enzyme such as trehalase (4), trehalose 6-phosphate hydrolase (5), phospho-α-(1-1)-glucosidase (6) or phosphotrehalase (7). Trehalose phosphorylase may also split trehalose by exerting a phosphate attack on the bond joining the glucose moieties (8).

In *L. lactis*, it was recently demonstrated that the enzyme, β-phosphoglucomutase (β-PGM), which catalyzes the reversible conversion of β-glucose 1-phosphate (β-G1P) to glucose 6-phosphate (G6P), is essential in the catabolism of both maltose and trehalose (9). Indication of a novel degradation pathway for trehalose, involving phosphorylation and cleavage of T6P into G6P and β-G1P, was observed by measuring the intracellular accumulation of sugar phosphates in a β-PGM mutant of *L. lactis*. Furthermore, it has previously been proved that the gene encoding β-PGM, *pgmB*, in *L. lactis* is induced by the presence of either maltose or trehalose in the growth medium, while the presence of glucose or lactose mediates gene repression (10,11). According to the genome sequence of *L. lactis*, *pgmB* is part of a putative trehalose operon (12). The predicted gene products of this operon are a transcriptional regulator (12) and two trehalose-specific components of a PTS (13). In the present study, we
show that *L. lactis* contains a novel enzyme for trehalose assimilation, designated trehalose 6-phosphate phosphorylase (TrePP). TrePP was purified and characterized, and the locus of its corresponding gene, *trePP*, was determined. In addition, the existence of TrePP in other bacteria is discussed as well as the physiological role of metabolic reactions involving the β-isomer of G1P.

**Experimental procedures**

**Bacterial strains, bacteriophages, plasmids and culture conditions**

The bacterial strains, bacteriophages and plasmids used in this study are represented in Table 1. *Escherichia coli* LE392 was cultivated in Luria-Bertani medium containing 0.2 % (wt/vol) maltose and 10 mM MgSO₄. The cell cultures were grown according to a Wizard™ Lambda Preps DNA Purification System kit (Promega). *E. coli* JM 83 was grown in LB medium supplemented with 100 μg/ml ampicillin, 32 μg/ml isopropyl-β-D-thiogalactoside (IPTG) and 32μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) when required. Cultivation was performed in shaking water baths at 37 °C. All lactococcal strains were cultivated in M17 medium (Oxoid) as standing batch cultures at 30 °C. Carbohydrates were autoclaved and added separately to a final concentration of 10 g/l. For the selection of certain strains, erythromycin was used at a final concentration of 2 μg/ml. Parent cultures were grown overnight under the same conditions as the experimental cultures, washed twice and resuspended in fresh medium before being used as inoculum, (1-2 % (vol/vol)). For the purification of TrePP a 2-liter standing batch culture of *Lactococcus lactis* ssp. *lactis* 19435 was prepared using trehalose as the sole carbon source. Cell growth was monitored by measuring the optical density (OD) at 620 nm on a Hitachi U-2000 spectrophotometer (Hitachi Ltd., Tokyo, Japan).
**Cell extract preparation and protein determination**

Both lactococcal and *E. coli* cells were harvested in the stationary growth phase by centrifugation. The cells were washed twice and resuspended in 20 mM triethanolamine (TEA) buffer, pH 7.2 containing 0.5 mM EDTA, 0.5 mM dithiothreitol (DTT), and protease inhibitors (Complete™ Protease Inhibitor Cocktail Tablets, Roche Molecular Biochemicals, Roche Diagnostics Scandinavia AB). Disintegration of the cells was achieved using an X-press (Biox, Gothenburg, Sweden). Cell debris was removed by centrifugation at 19, 500 g, at 2 °C for 10 minutes. Cell extracts were stored at -80 °C until used. The protein concentration was determined according to the method of Bradford (14). Bovine serum albumin was used as a standard.

**Enzyme assays**

Two different assays were applied in order to detect TP in cell extracts of *L. lactis* cultivated on trehalose. The first assay was performed according to the assay of MP (13), except that maltose was replaced by trehalose. Another assay for TP activity detection was performed using a glucose oxidase-peroxidase method to determine the amount of D-glucose released, as previously described (8,15). To detect the presence of TH in cell extracts the first method of TP measurement was performed, omitting phosphate in the assay mixture. All measurements of TP and TH were performed on a Hitachi U-2000 spectrophotometer (Hitachi Ltd., Tokyo, Japan). Measurements of TrePP activity were conducted on a Cobas Mira autoanalyzer (ABX Diagnostics, France). The measurement of TrePP activity was coupled to the formation of NADPH and the absorbance was determined at 340 nm. The assay mixture (total volume 150 µl) contained 0.1 M potassium phosphate buffer, pH 7.0, 3.75 U/ml glucose 6-phosphate dehydrogenase, 0.8 mM NADP⁺ and 0.67 mM T6P. T6P was used as the starting reagent. The
above conditions for the TrePP activity measurements were employed to follow the
purification of the enzyme and as the starting point for the kinetic studies of the enzyme.

Measurement of substrate consumption and product formation in TrePP catalysis

In order to investigate the presence of a trehalose metabolic enzyme in cell extract from
trehalose-cultivated *L. lactis*, the cell extract was incubated with either trehalose or T6P. The
consumption of substrate as well as product formation, resulting from any possible catalysis,
was determined using high-performance anion-exchange chromatography (HPAEC) on a
Carbopac PA-1 column with a precolumn (Dionex, Sunnyvale, CA, USA). Sugar phosphates
and mono- and disaccharides were separated at room temperature using a 120 mM NaOH
mobile phase at a flow rate of 1.0 ml/min. A linear sodium acetate gradient from 100-350 mM
was applied from 0 to 15 minutes after sample injection. The injection volume was set to 25
µl and the compounds were quantified by pulsed amperometric detection with an ED40
detector (Dionex, Sunnyvale, CA, USA). The assay mixture (0.5 ml) containing 0.1 M
potassium phosphate buffer, pH 7.0, cell extract (50 µg/ml protein) and 2 mM substrate, was
prepared and an aliquot was directly withdrawn, diluted 1:10, and kept on ice until analyzed.
The rest of the mixture was incubated at 35 °C for 150 minutes and then kept on ice until
analyzed.
**Purification procedure**

The cell extract was pretreated with MgCl₂ at a final concentration of 10 mM to prevent the inhibitory effect of EDTA. The cell extract was then treated with DNaseI (Appligene Oncor, France) to reduce the viscosity resulting from the presence of DNA. DNaseI was added to a final concentration of 1 mg/ml and the cell extract was incubated at 16 °C for 1 h. Degraded DNA was removed by centrifugation at 19, 500 g, at 2 °C for 10 minutes. Solid ammonium sulfate was added to the supernatant and the precipitate was collected at 60 % ammonium sulfate saturation. The precipitate was further dissolved in and dialyzed against 20 mM TEA buffer, pH 7.2, containing 30 mM KCl, 5 % (wt/vol) glycerol, 0.5 mM EDTA and 0.5 mM DTT (Buffer A).

All procedures for the purification of TrePP were carried out at 8 °C. The chromatography procedures were performed on a fast protein liquid chromatography (FPLC) system (Pharmacia Biotech, Sweden) containing two P-500 high-precision pumps, a model LCC-501 plus liquid chromatography controller, two motor valves (MV-7 and MV-8) and an REC 102 recorder. Protein elution was monitored at 280 nm with a UV-M II control unit, and fractions were collected with a FRAC-200 fraction collector. Gel filtration chromatography was carried out on a Hiload 16/60 Superdex 200 column that had been equilibrated with Buffer A. Proteins were eluted using the same buffer at a flow rate of 1.0 ml/min. Fractions showing the highest TrePP activities were pooled and further applied to a Pharmacia MonoQ HR 5/5 anion-exchange column (5×0.5 cm) equilibrated with Buffer A. Proteins were eluted using Buffer A and Buffer B, which had the same composition and pH as Buffer A, but contained 500 mM KCl instead of 30 mM. A gradient elution of proteins was started using of 40 % Buffer B and continued until an elution volume of 7 ml was reached. Between 7 ml and 27 ml elution volume the concentration of Buffer B was increased linearly to a final value of 75 %.
The flow rate was set to 1.0 ml/min. TrePP-active fractions were pooled, concentrated ten-
fold and dialyzed against Buffer A using a centrifugal filter device with a \( M_w \) cut-off of 30
kDa (Microcon, Amicon BioSeparations). The resulting TrePP was checked for purity using
sodium dodecyl sulfate-polyacrylamide gel-electrophoresis (SDS-PAGE).

**SDS-PAGE, native PAGE and molecular mass determination**

All reagents used for SDS-PAGE and precast native gels were purchased form Bio-Rad. SDS-
PAGE was performed according to the Laemmli method (16), using an acrylamide
concentration of 10 %. The acrylamide concentration gradient was 4-15 % in the native gels.
Cell extract and pools from the purification procedure of TrePP were denatured by heating
with an SDS-buffer containing 2-mercaptoethanol and then separated using SDS-PAGE. The
protein bands in the gels were visualized by staining with Comassie brilliant blue R-250 or
by silver staining (Silver Stain Plus kit, Bio-Rad). In native PAGE the samples were not
denatured but separated directly and proteins were detected by either method used for SDS-
PAGE gels. Native PAGE was carried out at 8 ºC, using precooled gels and running buffer.

Molecular masses were determined using SDS-PAGE and native PAGE. A low-molecular-
weight standard (Pharmacia Biotech, Sweden) containing phosphorylase b (94.0 kDa), BSA
(67.0 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.1
kDa), and \( \alpha \)-lactalbumin (14.4 kDa) was used for SDS-PAGE. For the native PAGE a high-
molecular mass standard (Pharmacia Biotech, Sweden) including thyroglobulin (669 kDa),
ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa), and BSA (67 kDa)
was used.
N-terminal sequencing

Purified TrePP was denatured and subjected to SDS-PAGE. The protein was transferred to a polyvinylfluoride (PVDF) membrane (Sequi-Blot™ PVDF Membrane, 0.2 µm, Bio-Rad) using a Trans-Blot Semi-Dry transfer cell (Bio-Rad), according to the manufacturer’s instructions. The protein band was cut out from the membrane and the N-terminal amino acid sequence was determined by Edman degradation at the Department of Plant Biology (The Swedish University of Agricultural Science, Uppsala, Sweden).

Determination of optimal conditions and the kinetics of TrePP catalysis

Investigations of optimal conditions for the enzymatic activity of TrePP were carried out using the standard assay on a Cobas Mira autoanalyzer (ABX Diagnostics, France) as described above, with varying parameters. For determination of the pH optimum, the pH of the potassium phosphate buffer was varied in the range from 5.5 to 8.0. In the investigations of divalent cation requirement, MgSO₄ was added to the TrePP assay at concentrations of 0-20 mM.

For the determination of the kinetic constants of the TrePP enzymatic reaction, one substrate was varied while the other substrate was kept constant at various excess concentrations. The reaction mixtures were incubated at 35 °C for 15 minutes, (diluted 1:20) and the product formation was analyzed using HPAEC. In all reaction mixtures less than 5 % of the reactants had been converted and thus the reactions were assumed to follow initial-rate kinetics. The $K_m$ values were determined from Lineweaver-Burk plots and the $K_m$ values of the enzyme for the reactants of the phosphorolysis reaction were also confirmed using the spectrophotometric assay (see above). To distinguish between sequential (ternary-complex) and non-sequential (ping-pong) kinetic mechanisms, initial-velocity measurements were carried out in the...
direction of phosphorolysis, with one substrate being varied at several constant concentrations of the second substrate. Reciprocal initial velocities were plotted against reciprocal substrate concentrations and the kinetic pattern was identified from these plots (17). The equilibrium constant of TrePP interconversion of T6P and phosphate and βG1P and G6P was determined by incubating pure TrePP with 2 mM each of T6P and inorganic phosphate or with 2 mM each of βG1P and G6P at 35 °C for 4 hours, using 50 mM citrate buffer, pH 6.3 or 50 mM TEA buffer, pH 7.0. The reaction mixtures were diluted 20-fold and the concentrations of substrates and products were determined using HPAEC. The concentration of inorganic phosphate was assumed to be the same as the measured T6P concentration.

Genetic techniques

Southern blotting and colony hybridization were performed as described by Sambrook et al., 1989 (18). Probe labeling was conducted using the Random Primed DNA-Labeling kit (Amersham Life Science). Plasmid DNA was purified from E. coli using a Bio-Rad Quantum Miniprep kit (Bio-Rad Laboratories). Digestion using restriction enzymes, ligations and agarose gel electrophoresis was performed according to Sambrook et al., 1989 (18), Current Protocols (19) or to the manufacturer’s instructions. All DNA-modifying enzymes were purchased from Roche Molecular Biochemicals (Roche Diagnostics Scandinavia AB). DNA was extracted from gel fragments obtained by agarose gel electrophoresis either by digestion with AgarACE™ (Promega) and then conventional ethanol precipitation, or with the aid of a Qiaquick Gel Extraction kit (Qiagen). Competent E. coli cells were prepared and transformed according to Sambrook et al., 1989 (18). PCR was used for automatic sequencing using the reaction conditions described in the protocol of the DyeDeoxy Terminator Cycle Sequencing kit (ABI, Applied Biosystems, Perkin Elmer) and construct pTMB2010 as a template. The sequencing was performed on a 373A DNA Sequencing System (Applied Biosystems, Perkin
The 15-20-mer oligonucleotides used as primers were synthesized in the Biomolecular Laboratory at Lund University, Sweden. The nucleotide sequence was determined from both strands.

Cloning protocol and construction of plasmids

A genomic library of *L. lactis* 19435 DNA, partially digested with *Sau3A*, was prepared in *E. coli* LE392 using the λEMBL3 Arms Cloning System and Packagene System (Promega). The genomic bank was screened for the *trePP* DNA sequence by performing Southern blots of DNA recovered from bacteriophage λ plaques. A DNA probe including the DNA sequence of *pgmB*, the gene directly upstream of *trePP*, was synthesized with PCR and used in the Southern blots. According to the results of the Southern blots five λ clones were selected and recombinant bacteriophage λ DNA was extracted and purified according to the Wizard™ Lambda Preps DNA Purification System kit (Promega). The λ DNA was digested by *HindIII* and cloned into pUC19 according to conventional methods described in Sambrook *et al.*, 1989 (18). pUC19 clones were propagated in *E. coli* JM83 and single transformants were transferred to a fresh selection plate. Colony hybridization was performed on these colonies using a 1.2 kb DNA intergenic sequence of *trePP* and *pgmB* as a probe (GenBank accession no. Z70730). *E. coli* harboring a construct including *trePP* and *pgmB* sequences were cultivated and plasmid extraction was performed. The resulting construct chosen for further applications was termed pTMB2010. For the preparation of a construct including only the *trePP* gene, pTMB2010 was digested by *HindIII* and *NsiI* and the resulting 2.6 kb restriction fragment was ligated into pUC18 for propagation in *E. coli*. This construct was called pTMB2011. In order to create a construct to be used for expression in *L. lactis*, the gene encoding TrePP was amplified from pTMB2010 with PCR using the primers 5’-ggcgtcgacaggcagtgctgataaat-3’ (forward) containing a *SalI* restriction site and 5’-ggcgtcgacaggcagtgctgataaat-3’ (reverse).
ggctgcagtaagcaagtacct-3′ (reverse) containing a PstI restriction site. The 2.4 kb PCR product was ligated into the lactococcal expression vector pMG36e (20) and the resulting construct was named pTMB5011. For insertional inactivation of the trePP gene, a 1.2 kb internal DNA sequence of the gene was removed from pTMB2010 using the restriction enzymes Sau3A and XbaI and ligated into vector pFL20 (9), unable to replicate in *L. lactis*. The construct, pTMB5012, was transformed into *L. lactis* 19435 and homologous recombination was screened for on erythromycin-selective plates and confirmed by PCR.

**Bioinformatic tools**

For sequence similarity searches BLAST programs (21), found under the National Center for Biotechnology Information (NCBI) home page, were used. Both standard protein-protein blast searches and blast with microbial genomes (completed and uncompleted) were performed. Multiple sequence alignments were made using the ClustalX program (22). Phylogenetic trees were constructed using the Neighbor Joining method (23) and visualized using the TreeView program (24).

**Results**

**Enzyme characterization**

Cell extracts from *L. lactis* grown on trehalose were analyzed in various spectrophotometric assays specifically detecting trehalase, trehalose phosphorylase and trehalose 6-phosphate hydrolase activity. However, none of these enzyme activities could be detected. Instead, lactococcal cell extracts were incubated with trehalose or T6P and quantification and separation of sugars and phosphorylated sugars were carried out using HPAEC. Incubation of cell extracts with trehalose did not lead to any production of phosphorylated sugar, as would be expected if trehalose phosphorylase were present in the lactococcal cell extract. Neither could any glucose be
detected, which would be produced by a trehalase. However, when the cell extracts were
incubated with T6P there was considerable formation of G6P and βG1P (Fig. 2A).

The enzyme, TrePP, responsible for converting T6P into G6P and β-G1P, was purified using
ammonium sulfate ((NH₄)₂SO₄) precipitation, size-exclusion and anion-exchange chromatography
(Table 2). One explanation of the remarkable changes in TrePP activity along the purification
could be the presence of inhibitors, which were subsequently separated from the fractions
containing TrePP. The final fraction of purified TrePP showed one band in SDS-PAGE
corresponding to a molecular mass of 94 kDa under denaturing conditions (Fig. 3). According to
native PAGE, TrePP is a monomeric enzyme, since a band could be observed at 95 kDa. The
purified pool of TrePP was used for the determination of the optimal conditions of its catalytic
action (Fig. 4). The temperature optimum of the phosphorolysis was estimated to be 35 °C, and
the highest activity of TrePP was obtained when the pH of the phosphate buffer was set to 6.3. No
requirement of a divalent cation in the TrePP catalysis could be found (data not shown). The
Michaelis-Menten constants of TrePP for inorganic phosphate (Pᵢ) and T6P were determined to be
32 mM and 6 mM, respectively, and for the reverse reaction the constants were determined to be
0.9 mM and 4 mM for β-G1P and G6P, respectively. When incubating pure TrePP with equal
concentrations of either T6P and Pᵢ or β-G1P and G6P at 35 °C, the equilibrium constants were
estimated to be 0.026 at pH 6.3 and 0.032 at pH 7.0. These results were obtained in both
directions of the catalysis (Fig. 2B). According to double-reciprocal plots, TrePP catalysis was
indicated to follow a ternary-complex mechanism (Fig. 5) (17).

Localization of the TrePP gene in L. lactis

By determining the N-terminal amino acid sequence of the purified peptide of TrePP, the
respective nucleotide sequence was found in L. lactis (GenBank accession no. Y18267). The
first 8 amino acids obtained from the Edman degradation, (T E K D W I I Q), were identical to the
deduced first amino acids of the open reading frame (ORF) upstream of \textit{pgmB}.

When the TrePP-encoding gene, designated \textit{trePP}, was expressed in \textit{E. coli} and in \textit{L. lactis}
significant increases in TrePP activity were seen (Table 3). The high activities obtained in the \textit{E.}
coli cell extracts are probably due to the presence of trehalose 6-phosphate hydrolase (5), which
also contributes to G6P formation in its catalysis. Furthermore, the TrePP activity was found to be
twenty times higher in trehalose-grown lactococci than in glucose-grown. By insertional
inactivation of \textit{trePP}, resulting in the lactococcal strain TMB5012, it was demonstrated that
TrePP is essential for trehalose utilization, since the mutant was unable to grow on trehalose. Cell
extracts from TMB5012 were checked for substrate consumption and product formation in the
catalysis of TrePP (Fig. 2). In this investigation a \textit{pgmB}-deficient strain, \textit{L. lactis} TMB5002, was
also included (9). The chromatogram from the HPAEC method showed that the concentration of
T6P decreased when using a cell extract of \textit{L. lactis} TMB5002, while the substrate was not
converted in strain TMB5012. These results underline the connections between \(\beta\)-PGM and
trehalose metabolism, by the action of TrePP as well as the identity of \textit{trePP}.

\textbf{Discussion}

A novel route for trehalose catabolism was found in \textit{L. lactis}. The key enzyme, TrePP,
established for trehalose utilization in this bacterium, was shown to act according to a ternary-
complex kinetic mechanism. This sequential mechanism has also been determined for other
trehalose-interacting enzymes, such as trehalose phosphorylase of \textit{Schizophyllum commune},
to which enzyme the substrates P, and \(\alpha\)-\(\alpha\)-trehalose bind by an ordered Bi Bi kinetic
mechanism (25,26). Furthermore, the equilibrium constant of the TrePP catalysis was
estimated to be 0.026 using the optimal reaction conditions, demonstrating that the reaction is
directed against T6P synthesis rather than its phosphorolysis. This explains the lower rate of
phosphorolysis in the β-PGM mutant, *L. lactis* TMB 5002, in which the conversion of β-G1P
to G6P was blocked (Fig. 2). Furthermore, recent results showed that *L. lactis* TMB5002,
cultivated on maltose, also accumulated T6P (9). Maltose metabolism in lactococci is
performed by the concerted action of maltose phosphorylase, splitting the disaccharide into
glucose and β-G1P (13), and β-PGM, catalyzing the interconversion of β-G1P and G6P
(10,11,13). Thus, we may conclude that a build-up of β-G1P in trehalose- or maltose-
cultivated *L. lactis* TMB5002 promotes the synthesis of T6P due to the favored direction of
the TrePP catalysis.

Several organisms may utilize trehalose as an osmoprotectant or in cell wall construction and
therefore possess one or more biosynthetic pathway(s) for this disaccharide (27). Contrarily,
in *L. lactis* there were indications that trehalose is not synthesized (Fig. 2), but exclusively
utilized for catabolism. In addition, according to the genome sequence of *L. lactis*, no
sequence can be found corresponding to amino acid sequences of known trehalose 6-
phosphate phosphatase enzymes (GenBank accession nos. P31678, S72829). Thus, TrePP
seems to be the only enzyme acting upon T6P in *L. lactis*.

By determining the N-terminal sequence of the purified TrePP, its corresponding gene,
designated *trePP*, could be cloned and its genetic locus was identified in a putative trehalose
operon, including the β-PGM-encoding gene, *pgmB* in *L. lactis* (GenBank accession nos.
Y18267, Z70730). When comparing TrePP activities in lactococcal cells grown on trehalose
or glucose there was an indication that the TrePP gene was regulated by carbon catabolite
repression. In earlier studies *pgmB* was shown to be subject to the glucose effect (11). Since
*trePP* and *pgmB* are highly likely to be located in the same operon, these genes are probably regulated similarly.

When searching for similar gene sequences of the TrePP gene in the database we observed that the amino acid sequence of TrePP shows 42\% similarity to the previously characterized maltose phosphorylase of *Lactobacillus sanfranciscensis* (28) and 44\% similarity to a putative maltose phosphorylase of *Neisseria meningitidis* (Fig. 6). This may be explained by the fact that maltose phosphorylase enzymes and TrePP have similar catalytic activity with regard to their phosphorolytic action. However, no significant sequence similarity could be found when the amino acid sequence of TrePP was compared with those of fungal trehalose phosphorylases (GenBank accession nos. BAA31349, Q9UV63), even if their catalytic actions seem to be similar. Furthermore, no trehalose 6-phosphate phosphatase or trehalose 6-phosphate synthase enzymes (GenBank accession nos. P31678, S72829, S48761, T05453, CAC17748, P31677, AAD30578, BAB54790) showed significant amino acid sequence similarity to that of TrePP, even if all kinds of enzymes act upon the same compound.

Interestingly, we concluded that some microorganisms are highly likely to harbor an enzyme of the same activity as TrePP. In the genome of *Enterococcus faecalis* a DNA sequence was found, whose resembling amino acid sequence showed 57\% similarity to that of TrePP (Fig. 6). According to the phylogenetic tree, *E. faecalis* and *L. lactis* are grouped in the same cluster as other microorganisms harboring hypothetical proteins similar to TrePP, different from the cluster constituting the maltose phosphorylases of some microorganisms. In *L. lactis* the location of *pgmB* is directly downstream of the TrePP gene (13), which was also predicted for *E. faecalis*, since an ORF downstream of the putative TrePP gene (contig 10497, TIGR) shows amino acid sequence homology of 78\% with the β-PGM of *L. lactis* (GenBank accession no. CAA94734). By the use of a cell extract of *E. faecalis* cultivated on trehalose,
we were able to confirm both TrePP and β-PGM activity in this bacterium (data not shown).

The organization of TrePP and β-PGM genes in close proximity was also found in *Clostridium difficile* (contig 1496, Sanger Centre, UK). Regarding trehalose transport systems in *E. faecalis* and *C. difficile*, it is possible that these microorganisms take up this disaccharide by a PTS, as predicted for *L. lactis* (13) and found in *E. coli* (29) and *Streptococcus mutans* (30), since sequences similar to the putative trehalose-specific PTS component of *L. lactis* were found in their genomes (contig 10495, TIGR, contig 1496, Sanger Centre, UK).

Interestingly, in the genomes of two *E. coli* strains, ORFs likely to encode T6P-degrading enzymes, different from the characterized TPH, were observed (Fig. 6). Furthermore, adjacent to these putative TrePP encoding genes, ORFs showing 62 % similarity to the amino acid sequence of β-PGM were detected (GenBank accession nos. AAG56485, AAC74399). It is tempting to believe that this introduces a new aspect of trehalose metabolism in *E. coli* similar to that in *L. lactis*.

We may conclude from the present study that lactococci harbor a novel route of trehalose metabolism. TrePP is an enzyme, which specifically catalyzes the interconversion between T6P and P₆, and β-G1P and G6P. The gene encoding TrePP is located in a putative trehalose operon in *L. lactis*, including the genes probably encoding (in the following order) a regulator of the trehalose operon, trehalose-specific PTS components, TrePP and β-PGM. In the future, it would be interesting to investigate other bacteria, especially members of the different genera of lactic acid bacteria, for possession of TrePP and its connections to β-PGM activity.
Acknowledgements

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1 References

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Footnotes

1 U/mg protein is defined as 1 µmol of T6P converted per min per mg protein added under standard assay conditions.
Figure 1. (suggested position in manuscript: introduction section) A summary adapted from currently known trehalose catabolic pathways in *E. coli* (5,29,31), *B. subtilis* (2,32), *Euglena gracilis* (26), *Catellatospora ferruginea* (33), *Bacillus popilliae* (7), *Streptomyces coelicolor* (34), and this study. The left part of the figure shows a simplified model of trehalose catabolism in Gram-positive bacteria, fungi, and algae, while the right part shows a model for *E. coli*, representing Gram-negative bacteria. Enzymes are denoted in bold face.


Figure 2. (suggested position in manuscript: results section) HPAEC chromatograms. A. Comparison of cell extracts from *L. lactis* strains 19435, TMB5002 and TMB5012 cultivated on trehalose. The chromatograms demonstrate the separation of consumed and produced phosphorylated sugars using the HPAEC method after incubation of cell extracts with trehalose 6-phosphate. B. Chromatograms showing the equilibrium of the TrePP catalysis. The top chromatogram shows a standard with 100 µM each of trehalose 6-phosphate (T6P), β-glucose 1-phosphate (β-G1P) and glucose 6-phosphate (G6P), the second shows T6P phosphorolysis after incubation of T6P and inorganic phosphate (Pi) with pure TrePP, and the lower shows T6P synthesis after incubation of β-G1P and G6P with pure TrePP.

Figure 3. (suggested position in manuscript: results section) SDS-PAGE and native PAGE of trehalose 6-phosphate phosphorylase (TrePP) from *L. lactis*. To the left a gel representing...
SDS-PAGE is shown. A low-molecular-mass standard was loaded in the first lane and in the second pure TrePP. In the right part of the figure results from a native PAGE gel are shown. Pure TrePP was loaded in the first lane and in the second a high-molecular-mass standard. The arrows indicate the protein bands representing denatured and native TrePP.

**Figure 4.** *(suggested position in manuscript: results section)* Estimation of optimal reaction conditions for TrePP phosphorolysis. A. pH. B. Temperature.

**Figure 5.** *(suggested position in manuscript: results section)* Interpretation of the kinetic mechanism of TrePP catalysis. A. Inorganic phosphate (Pi) was kept at various excess concentrations. ●: 100 mM, ○: 33 mM, ▼: 20 mM, ▽: 6.7 mM. B. Trehalose 6-phosphate (T6P) was kept at various excess concentrations. ●: 2 mM, ○: 1.33 mM, ▼: 1 mM, ▽: 0.67 mM, ■: 0.5 mM.

**Figure 6.** *(suggested position in manuscript: discussion section)* Phylogenetic relationship based on amino acid sequence similarities to the deduced amino acid sequence of the *trePP* gene. The reliability of the branching was assessed using bootstrapping analysis. The branch lengths are scaled in proportion to the extent of change per position as indicated by the scale bar. No comments about protein identity state that it is a hypothetical protein with an amino acid sequence deduced from its DNA sequence. GenBank accession numbers are given in parentheses. In cases of uncompleted genomes the center responsible for the sequencing project is given. TIGR: The Institute for Genomic Research, USA, GTC: Genome Therapeutics Corporation, USA, TreC: trehalose 6-phosphate hydrolase, TP: trehalose phosphorylase, TreA: periplasmic trehalase, TreF: cytoplasmic trehalase, TrePP: trehalose 6-phosphate phosphorylase, MP: maltose phosphorylase, GT: glycosyl transferase.
Table 1. Bacterial strains, bacteriophages and plasmids used in this study.

<table>
<thead>
<tr>
<th>Strains, phages and plasmids</th>
<th>Characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lactococcal strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. lactis</em> ssp. lactis 19435</td>
<td>Lac⁺</td>
<td>ATCC</td>
</tr>
<tr>
<td><em>L. lactis</em> ssp. lactis TMB5011</td>
<td><em>L. lactis</em> ssp. lactis 19435 carrying pTMB5011, EmR</td>
<td>This study</td>
</tr>
<tr>
<td><em>L. lactis</em> ssp. lactis TMB5012</td>
<td><em>L. lactis</em> ssp. lactis 19435 with pTMB5012 integrated into the genome, EmR</td>
<td>This study</td>
</tr>
<tr>
<td><em>L. lactis</em> ssp. lactis TMB5002</td>
<td><em>L. lactis</em> ssp. lactis 19435 double crossover pgmB mutant</td>
<td>(9)</td>
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<tr>
<td><strong>Escherichia coli strains</strong></td>
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<td></td>
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<tr>
<td><em>E. coli</em> LE392</td>
<td>supE44 supF58 hsdR514 glaK2 galT22 metB1 trpR55 lacY1</td>
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<tr>
<td><em>E. coli</em> JM83</td>
<td>ara (lac-proAB) lacZΔM15 rpsLΔ80</td>
<td></td>
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<tr>
<td><em>E. coli</em> TMB2010</td>
<td><em>E. coli</em> JM83 carrying pTMB2010</td>
<td>This study</td>
</tr>
<tr>
<td><em>E. coli</em> TMB2011</td>
<td><em>E. coli</em> JM83 carrying pTMB2011</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Bacteriophages</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>λGEM12</td>
<td>Cloning vector</td>
<td></td>
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<tr>
<td>λBA1</td>
<td>λGEM12 with a 15-20 kb Sau 3A chromosomal fragment of <em>L. lactis</em> ssp. lactis 19435</td>
<td>This study</td>
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</table>
### Table 1, continued.

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Description</th>
<th>Source</th>
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</thead>
<tbody>
<tr>
<td>pUC18/19</td>
<td>Cloning vectors, Ap&lt;sup&gt;+&lt;/sup&gt;, lac&lt;sup&gt;+&lt;/sup&gt;</td>
<td>(35)</td>
</tr>
<tr>
<td>pNQ3</td>
<td>pUC19 with a 3.8 kb Sau3A-EcoRI chromosomal fragment</td>
<td>(11)</td>
</tr>
<tr>
<td>L. lactis ssp. lactis 19435</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pTMB2010</td>
<td>pUC19 with an 8 kb HindIII L. lactis ssp. lactis 19435 chromosomal fragment</td>
<td>This study</td>
</tr>
<tr>
<td>pTMB2011</td>
<td>pUC18 with a 2.6 kb HindIII-NsiI fragment of pTMB2010, including trePP</td>
<td>This study</td>
</tr>
<tr>
<td>pMG36e</td>
<td>Lactococcal expression vector, Em&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(20)</td>
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<tr>
<td>pTMB5011</td>
<td>pMG36e with a 2.4 kb PCR product, containing trePP, from pTMB2010, Em&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pFL20</td>
<td>Insertional inactivation vector, pUC18 derivative, Em&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(9)</td>
</tr>
<tr>
<td>pTMB5012</td>
<td>pFL20 containing 1.2 kb Sau 3A-XbaI internal trePP DNA sequence</td>
<td>This study</td>
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Table 2. Purification of trehalose 6-phosphate phosphorylase.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume cell extract (ml)</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity (U/mg protein)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>15</td>
<td>28</td>
<td>1.23</td>
<td>0.04</td>
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<tr>
<td>(NH₄)₂SO₄ precipitation</td>
<td>6</td>
<td>7</td>
<td>0.023</td>
<td>0.003</td>
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<tr>
<td>Size-exclusion chromatography</td>
<td>12</td>
<td>0.35</td>
<td>1.65</td>
<td>4.71</td>
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<tr>
<td>Anion-exchange chromatography</td>
<td>3</td>
<td>0.04</td>
<td>1.29</td>
<td>32.4</td>
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</table>
Table 3. *trePP* expression in *E. coli* and *L. lactis*.

<table>
<thead>
<tr>
<th>Cell extract</th>
<th>TrePP specific activity (mU/mg protein)</th>
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</thead>
<tbody>
<tr>
<td><em>E. coli</em> JM83</td>
<td>153</td>
</tr>
<tr>
<td><em>E. coli</em> TMB2011</td>
<td>420</td>
</tr>
<tr>
<td><em>L. lactis</em> 19435 (glucose)</td>
<td>2</td>
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<tr>
<td><em>L. lactis</em> 19435 (trehalose)</td>
<td>35</td>
</tr>
<tr>
<td><em>L. lactis</em> TMB5011 (glucose)</td>
<td>24</td>
</tr>
<tr>
<td><em>L. lactis</em> TMB5011 (trehalose)</td>
<td>44</td>
</tr>
</tbody>
</table>
Fig. 1
Fig. 2
A.  

![Graph showing pH vs. % activity.](image1)

B.  

![Graph showing Temperature (°C) vs. % activity.](image2)

Fig. 4
Fig. 5
Fig. 6
Trehalose 6-phosphate phosphorylase is part of a novel metabolic pathway for trehalose utilization in Lactococcus lactis
Ulrika Andersson, Fredrik Levander and Peter Rådström

J. Biol. Chem. published online September 11, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M108279200

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