Characterization of Trehalose Phosphorylase from *Bacillus stearothermophilus* SK-1 and Nucleotide Sequence of the Corresponding Gene

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A bacterial trehalose phosphorylase (TPase; EC 2.4.1.64) was purified from the culture supernatant of *Bacillus stearothermophilus* SK-1 to apparent homogeneity, and some properties were investigated. Furthermore, a gene from SK-1 responsible for the TPase was cloned by Southern hybridization with a degenerate oligonucleotide probe synthesized on the basis of the N-terminal sequence of the purified enzyme. The $M_r$ of the enzyme was estimated to be 150,000 by gel filtration and 83,000 by SDS-PAGE, so the enzyme is likely to be a homodimer. The enzyme had optimum activity at pH 7.0–8.0 or nearby and the optimum temperature was about 75°C. The deduced amino acid sequence of the SK-1 TPase encodes a theoretical protein with a $M_r$ of 87,950. Alignment of amino acid sequences with a maltose phosphorylase from *Lactobacillus brevis* the crystal structure and active site of which had been analyzed suggested that these two phosphorylases evolved from a common ancestor. The *Escherichia coli* cells harboring the plasmid containing the cloned TPase gene had about 100 times the activity of SK-1.

Key words: trehalose phosphorylase; trehalose phosphorylase gene; *Bacillus stearothermophilus* 

Trehalose phosphorylase (TPase; $\alpha,\alpha$-trehalose: orthophosphate $\beta$-d-glucosyltransferase, EC 2.4.1.64) catalyzes the reversible phosphorylisis and synthesis of trehalose as follows:

\[
\text{Trehalose} + \text{orthophosphate} \rightarrow \beta(\alpha)-d\text{-glucose 1-phosphate} + d\text{-glucose}
\]

This enzyme has been found in various organisms: *Thermoanaerobium brockii* ATCC 35047, a *Plesiomonas* sp., *Catellatospora ferruginea*, *Micrococcus varians*, *Euglena gracilis*, *Flammulina velutipes*, *Schizopyllum commune*, *Agaricus bisporus*, *Grifola frondosa*, *Pichia fermentans*, *Bradyrhizobium japonicum*, and a cyanobacterial *Scytonema* species. However, there are very few reports about the genes and amino acid sequences of this enzyme.

We screened for microorganisms producing TPase, and isolated *Bacillus stearothermophilus* SK-1. This enzyme might be useful in some applications, such as the enzymatic synthesis of trehalose from maltose coupled with maltose phosphorylase, because it was produced and secreted by an aerobic bacterium and had some advantageous characteristics for commercial use. In this paper, we describe the isolation and characterization of a TPase produced by *Bacillus stearothermophilus* SK-1, and the cloning and sequence of its gene.

Materials and Methods

**Chemicals.** Isomaltose was purchased from Seikagaku Kogyo Co., Ltd., Japan. $p$-Nitrophenyl $\alpha$-$\alpha$-glucopyranoside, $p$-nitrophenyl $\beta$-$\beta$-glucopyranoside, trehalose, and $\beta$-$\beta$-glucose 1-phosphate ($\beta$-G1P) were purchased from Sigma-Aldrich, St. Louis, MO. DEAE-Toyopearl 650M and phenyl-Toyopearl 650M were products of Tosoh Corp., Tokyo, Japan. Superdex 200 pg 16/60, MonoP HR 5/20, and Polybuffer 74 were products of Amersham Pharmacia Biotech, Uppsala, Sweden. Pentax GH-0810M was a product of Asahi Optical Co., Ltd., Tokyo. Other chemicals were purchased from Wako Pure Chemical Industries, Osaka, Japan.

**Medium.** The test media used in screening were as follows. The selection medium contained 0.2% trehalose, 0.05% yeast extract, 0.2% (NH$_4$)$_2$SO$_4$, 0.1% sodium citrate, 0.02% MgSO$_4$·7H$_2$O, 0.35% KH$_2$PO$_4$, and 1.5% agar, pH 7.0. The standard medium contained 0.5% peptone (Difco Laboratories, Detroit, MI), 0.25% yeast extract, 0.1% glucose, and 1.5% agar, pH 7.0. The medium YPT contained 1% yeast extract, 2% Polypepton (Nihon Seiyaku Corp., Tokyo, Japan), and 2% trehalose, pH 7.0.

**Screening method.** Soils from various areas in Japan were used. A small amount (about 2 g) of soil was suspended in 5 ml of sterilized saline. Then the
suspension (0.2 ml) was spread on agar plates of selection medium. Colonies that appeared on the plates were used to inoculate 3 ml of the YPT medium in test tubes, which were shaken during cultivation at 55°C for 2 d. One milliliter portions of each culture broth was homogenized with glass beads (60.3 mm). The homogenate was centrifuged at 10,000 × g for 15 min to remove cell debris, and the precipitate that formed at 80% saturation with ammonium sulfate was collected by centrifugation at 15,000 × g for 10 min. The precipitate was washed with 1 ml of 5 mM potassium phosphate-citrate buffer, pH 6.0, containing 80% ammonium sulfate, and dissolved in 0.2 ml of the same buffer without ammonium sulfate.

The trehalose phosphorolytic activity of this enzyme solution was tested under standard assay conditions. The trehalose synthetic activity also was tested under the following conditions. The enzyme solution was dialyzed against 5 mM acetate buffer, pH 6.0. The reaction mixture contained 0.03 ml of the enzyme solution, 0.02 ml of 0.2 M glucose, 0.02 ml of 0.2 M β-GIP, 0.008 ml of 0.5 M acetate buffer, pH 6.0, and water, in a total volume of 0.1 ml. The mixture was incubated at 55°C for 1 d and then boiled for 10 min to stop the reaction. The detection of trehalose and phosphate was done by HPLC.

A strain capable of producing TPase was selected as the enzyme producer. Identification of the isolated bacterium was done by Japan Food Research Laboratories (Tokyo) by the methods described in "Bergey's Manual of Systematic Bacteriology" and "The Genus Bacillus".

Assay of TPase activity. TPase activity was assayed by the measurement of the glucose liberated from trehalose in the presence of phosphate. The standard assay system contained 0.6 ml of 2% trehalose, 0.06 ml of 0.5 M potassium phosphate-citrate buffer, pH 6.0, an enzyme solution, and water in a total volume of 1.2 ml. The mixture was incubated for 10 min at 60°C and then boiled for 10 min to stop the reaction. The amount of glucose liberated was measured by the mutarotase-glucose oxidase method with a Glucose CII-Test kit (Wako). One unit of enzyme activity was defined as the amount of the enzyme that liberates 1 micromole of glucose per minute under these conditions. The specific activity was expressed by the enzyme activity per milligram of protein.

Assay of protein. In the purification by column chromatography, the protein elution pattern was monitored spectrophotometrically as the absorbance at 280 nm. Protein concentrations were measured by the method of Lowry et al. with bovine serum albumin as the standard.

Purification of TPase. The purification of TPase was monitored by assays of TPase activity, and all procedures were done at 10°C.

SK-1 was cultured aerobically for 3 d by shaking at 55°C in 31 of YPT medium. Solid ammonium sulfate was added to the culture supernatant after the cells had been removed by centrifugation at 10,000 × g for 10 min, and the precipitate that formed at between 40 and 60% saturation with ammonium sulfate was collected by centrifugation at 15,000 × g for 20 min. The precipitate was dissolved in a small volume of 5 mM potassium phosphate-citrate buffer, pH 6.0 (buffer A), and then dialyzed against the same buffer. After removal of the insoluble materials formed during the dialysis by centrifugation, the dialyzed sample was put on a column (2.5 × 20 cm) of DEAE-Toyopearl 650M equilibrated with buffer A. After the column was washed with buffer A, the enzyme was eluted with a linear gradient of NaCl from 0 to 0.5 M in buffer A at the flow rate of 4 ml/min. The eluate was fractionated into 8-ml portions and the active fractions were pooled. This step was repeated under the same conditions except that the elution was done with a linear gradient of NaCl from 0 to 0.25 M in buffer A. The active fractions were pooled and concentrated to a small volume by ultrafiltration with a Centriprep-10 apparatus (Millipore Corp., Bedford, MA). Solid ammonium sulfate was dissolved in this enzyme solution to a final concentration of 40%, and this solution was put on a column (2.5 × 20 cm) of phenyl-Toyopearl 650M equilibrated with buffer A containing 40% ammonium sulfate. After the column was washed with buffer A containing 40% ammonium sulfate, elution was done with a linear gradient of ammonium sulfate from 40 to 0% in buffer A at the flow rate of 4 ml/min. The eluate was fractionated into 8-ml portions and the active fractions were pooled. The enzyme solution was concentrated to a small volume by ultrafiltration with a Centriprep-10 and put on a Superdex 200 pg 16/60 column equilibrated with buffer A containing 0.15 M NaCl. The enzyme was eluted with the same buffer at the flow rate of 0.5 ml/min and the active fractions were pooled. The enzyme solution was put on a hydroxyapatite column, Pentax GH-0810M equilibrated with 1 mM potassium phosphate-citrate buffer, pH 6.0, containing 0.3 mM CaCl₂. After the column was washed with the same buffer, elution was done with a linear gradient of potassium phosphate-citrate buffer, pH 6.0, from 1 to 200 mM at the flow rate of 0.5 ml/min. The eluate was fractionated into 1-ml portions and the active fractions were pooled as the purified TPase.

Amino-terminal amino acid sequence of TPase. The amino-terminal amino acid sequence of purified TPase was analyzed by Edman degradation with a gas-phase protein sequencer (ABI model 477A). The
sample for sequence analysis was prepared by the method of Matsudaïra.\(^{18}\)

**Electrophoresis.** SDS-PAGE of the purified TPase was done by the method of Laemmli\(^{17}\) with a Phast System (Pharmacia, Uppsala, Sweden) and a PhastGel Gradient 10–15 (10–15% polyacrylamide gel; Pharmacia). An LMW electrophoresis calibration kit (Pharmacia) was used for standard proteins. Isoelectric focusing was done with a Phast System and PhastGel IEF 4–6.5 (Pharmacia). Protein bands were stained with Coomassie Brilliant Blue R-250.

**Molecular weight (M) measurement.** A Superdex 200 pg 16/60 column was used to measure the \(M_s\) of the purified TPase. The analysis was done in 10 mm acetate buffer, pH 6.0, containing 0.15 m NaCl at 4°C at the flow rate of 0.5 ml/min. A \(M_s\) marker kit (Boehringer, Mannheim, Germany) was used for standard proteins.

**HPLC for identification of sugars and phosphate.** HPLC conditions for measurement of trehalose and glucose were as follows: TSK gel Amido 80 (4.6 \times 250 mm, Tosoh); mobile phase, acetonitrile:H\(_2\)O = 76:24 (w/w); flow rate, 0.8 ml/min; column temp., 80°C and detector, refractive index detector (RI). HPLC conditions for measurement of \(\beta\)-GIP and phosphate were as follows: TSK gel SAX (6.0 \times 150 mm, Tosoh); mobile phase, 0.1 m potassium acetate (pH 5.0, adjusted with HCl); flow rate, 1.0 ml/min; column temp., 30°C, and detector, RI.

**Preparation of DNAs.** B. stearothermophilus SK-1 chromosomal DNA was prepared by the method of Saito and Miura\(^{19}\) except that achromopeptidase from Achromobacter lycicus (Wako) was used as a lytic enzyme. Plasmid DNA was prepared by the method of Birnboim and Doly.\(^{19}\)

**Cloning of the TPase gene.** On the basis of the entire nucleotide sequence of the TPase from SK-1, two oligonucleotide primers, 5'-AAACTGCAGTTGAAACAAATCAGTTCA-A3' (5' of the TPase gene) and 5'-AAACTGCAGTTAATCAACACGCCGTTAT3' (3' of the TPase gene), including PstI restriction sites (underlined), were synthesized (Espec Oligo Service) and used together with total DNA from SK-1 to amplify the TPase gene. With Taq DNA polymerase (Takara), a 2448-bp fragment was amplified. The PCR fragment was cloned into the pCR2.1 vector and used to transform E. coli INVHoF\(^{18}\) with an Original TA Cloning Kit (Invitrogen Corp., Carlsbad, CA) by the manufacturer's instructions. Clones were analyzed for the correct insert by DNA sequencing as described above. The resulting plasmid was designated pSTP1.

**Results**

**Screening for TPase-producing bacteria.** From among about 200 isolates that could grow at 55°C in a medium containing trehalose as the sole carbon source, 11 isolates with trehalose degradation activity were chosen and their trehalose phosphoro-
lytic activity was tested. Only one isolate was obtained and confirmed to have trehalose synthetic activity. The isolate was identified as *Bacillus stearothermophilus* and named SK-1.

**Purification and amino-terminal amino acid sequence analysis of TPase**

SK-1 produced TPase almost completely extracellularly. Most of the TPase activity was detected in the culture supernatant after cultivation for 1 to 3 d with shaking at 55°C in YPT medium. Therefore, we purified TPase from the culture supernatant of SK-1. The purification procedure resulted in 5.53% recovery of the enzyme activity and a 72-fold enrichment of TPase compared with the culture supernatant of a 5-liter culture (Table 1). SDS-PAGE of this purified TPase gave a single protein band (data not shown). The first 19 residues of the amino-terminal sequence of the purified TPase, identified chemically, were SWSISSNQLNIEHNLHEES.

**M, and pl**

The apparent *M*<sub>c</sub> of the purified enzyme was estimated to be 150,000 by gel filtration on a Superdex 200 pg column. SDS-PAGE of the purified enzyme gave a single protein band with an apparent *M*<sub>c</sub> of 83,000 (data not shown). These results suggested that the enzyme consisted of two identical subunits. The *pl* of the purified enzyme was estimated to be 5.0 by isoelectric focusing (data not shown).

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**Table 1. Purification of TPase from *Bacillus stearothermophilus* SK-1**

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Total activity (units)</th>
<th>Specific activity (units/mg)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture supernatant&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2845</td>
<td>945</td>
<td>0.33</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Ammonium sulfate (40-60%)</td>
<td>1845</td>
<td>864</td>
<td>0.47</td>
<td>91.4</td>
<td>1.4</td>
</tr>
<tr>
<td>DEAE-Toyopearl 650M (first)</td>
<td>253</td>
<td>389</td>
<td>1.54</td>
<td>41.2</td>
<td>4.7</td>
</tr>
<tr>
<td>DEAE-Toyopearl 650M (second)</td>
<td>73.8</td>
<td>279</td>
<td>3.78</td>
<td>29.5</td>
<td>11.5</td>
</tr>
<tr>
<td>Phenyl-Toyopearl 650M</td>
<td>15.2</td>
<td>117</td>
<td>7.70</td>
<td>12.4</td>
<td>23.3</td>
</tr>
<tr>
<td>Superdex 200 pg</td>
<td>3.7</td>
<td>81.6</td>
<td>22.1</td>
<td>8.63</td>
<td>67.0</td>
</tr>
<tr>
<td>Pentax GH-0810M</td>
<td>2.2</td>
<td>52.3</td>
<td>23.8</td>
<td>5.53</td>
<td>72.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Corresponding to 5 liters of the culture broth.

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**Table 2. Substrate Specificity of Phosphorolysis of TPase**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trehalose</td>
<td>100</td>
</tr>
<tr>
<td>Isomaltose</td>
<td>3</td>
</tr>
<tr>
<td>Maltose</td>
<td>1</td>
</tr>
<tr>
<td>Sucrose</td>
<td>1</td>
</tr>
<tr>
<td>Neotrehalose</td>
<td>1</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>2</td>
</tr>
<tr>
<td><em>p</em>-Nitrophenyl α-D-glucopyranoside</td>
<td>2</td>
</tr>
<tr>
<td><em>p</em>-Nitrophenyl β-D-glucopyranoside</td>
<td>2</td>
</tr>
</tbody>
</table>

The enzyme activity was assayed as described in the text in the presence of 1% substrate, except that incubation was for 20 min. The activity with trehalose was taken to be 100%.

**Fig. 1. Effects of pH (A) and Temperature (B) on the TPase Activity.**

(A) To examine the optimum pH, we measured TPase activities in 25 mM buffer solutions at various pHs. The buffer systems used were: ○, potassium phosphate-citrate; △, Tris-HCl (+25 mM K<sub>2</sub>HPO<sub>4</sub>). For examination of pH stability, the enzyme solution was kept at 60°C for 24 h in 25 mM buffer solutions at various pHs and the activity remaining was measured. The buffer systems used were: ●, potassium phosphate-citrate; ▲, Tris-HCl.

(B) To examine the optimum temperature, we measured TPase activities at various temperatures in 25 mM potassium phosphate-citrate buffer (pH 6.0) (○). For examination of thermal stability, the enzyme solution was kept at various temperatures for 15 min in 10 mM acetate buffer (pH 6.0) and the activity remaining was measured (●).

**Substrate specificity**

The specificity of phosphorolysis of various disaccharides and related compounds by the purified enzyme was examined (Table 2). All substrates except trehalose were nearly non-reactive with TPase. These results suggested that this enzyme had high substrate specificity for the α, 1-1 glycosidic linkage between D-glucose and D-glucose.

**Effects of pH and temperature**

The activities were measured in the range of pH 4.0 to 9.0 with 25 mM potassium phosphate-citrate buffer (pH 4.0-7.5) and 25 mM Tris-HCl buffer (pH 7.5-9.0). When Tris-HCl buffer was used, 25 mM K<sub>2</sub>HPO<sub>4</sub> was added to the reaction mixture, and the pH after mixing in the latter compound is indicated. The enzyme had an optimum pH range from 7.0 to 8.0 (Fig. 1(A)). The pH stability was assessed by treatment at 60°C for 24 h from pH 4.0 to 9.0 with 25 mM potassium phosphate-citrate buffer (pH 4.0-8.0) and 25 mM Tris-HCl buffer (pH 7.5-9.0).
The TPase was stable at pHs between 6.0 and 8.0 under these conditions.

The effects of temperature on activity were examined. The enzyme activities were measured at various temperatures under the standard assay conditions. The maximum activity was at 75°C (Fig. 1(B)). The thermal stabilities of the enzyme were assessed in 25 mM potassium phosphate-citrate buffer, pH 6.0, after incubation at various temperatures for 15 min. The purified TPase was completely stable up to 60°C.

**Kinetics**

When the reciprocals of the initial velocities were plotted against those of the initial concentrations of trehalose at several fixed concentrations of Pn, the lines intersected in the left quadrant, so the kinetic mechanism was sequential (data not shown). The Km for trehalose and Pn were 4.1 and 1.1 mM, respectively.

**DNA sequence of the TPase gene**

The DNA sequence of the TPase gene, consisting of 2796 bp, was identified (Fig. 2). The open reading frame of 765 codons, from positions 389 to 2683, encoded a theoretical protein with a M, of 87,950. This agreed with the results of SDS-PAGE of purified TPase from SK-1 (data not shown). The amino acid sequence deduced from the DNA sequence contained the amino-terminal amino acid sequence of the purified TPase identified chemically (amino acid residues 2 through 20 [Fig. 2]). This confirmed that the open reading frame was the coding sequence for TPase. That the amino-terminal amino acid sequence contained no signal sequence is indicated that it was synthesized in the cytoplasm. It is not known why TPase is found in the culture supernatant of SK-1. Analysis of the DNA sequences upstream of the TPase gene showed that there was a potential -35 sequence, -10 sequence, and a ribosomal binding site at nt 257 to 262, 280 to 285, and 376 to 380, respectively.

**Expression of the protein encoded by TPase gene in E. coli**

The expression of the recombinant TPase encoded by pSTP1 was checked by an assay of the TPase activity. E. coli cells carrying pSTP1, designated STP1, were cultured aerobically for 1 d with shaking at 37°C in Luria-Bertani medium containing ampicillin (50 μg/ml). The culture broth was homogenized with glass beads (φ0.3 mm), followed by centrifugation at 10,000 × g for 15 min to remove cell debris, and the supernatant was assayed for TPase activity. STP1 produced about 20,000 units per liter culture of TPase, about 100 times that produced by SK-1.

**Discussion**

We isolated *Bacillus stearothermophilus* SK-1 producing TPase, and purified and characterized the enzyme. We have cloned its gene and expressed it in *E. coli*. This is the first report of the production of TPase, not to mention its gene, by *Bacillus* bacteria.

TPase has been found in various organisms and some properties are compared in Table 3. Generally speaking, bacterial TPases are similar to each other in many points, to judge from known properties. One difference is the diversity of the number of subunits, although the M, of the subunits is not very different. TPase from SK-1 was one of the most thermostable ones. From the viewpoint of large-scale fermentation, together with the extracellular production described below, high thermostability is advantageous when the enzyme is to be prepared for commercial use.

The metabolism of trehalose has been studied in many organisms, and TPase may be located in the cell to degrade trehalose transported into the cell from the culture medium. Also, with TPase from SK-1, low K, for trehalose and Pn suggest that the TPase from SK-1 can proceed in the direction of phosphorylase under physiological conditions. In this study, most of the TPase activity was detected in the culture supernatant of SK-1, but the DNA sequence of the TPase gene showed that there was no secretion signal peptide, and analysis of the amino-terminal residues of the TPase produced in SK-1 suggested that it is processed by a methionine aminopeptidase. One possibility is that this observation arose because of autolysis of the cells, but the reason why SK-1 produces TPase extracellularly remains unknown.

The deduced amino acid sequence of the TPase gene had 46% similarity to the TPase from *Thermoaerobium brockii* ATCC 35047 (Table 4). Some highly conserved regions were observed, suggesting them to be crucial for the expression of its activity. On the other hand, two TPase genes of basidiomycetes, *Grifola frondosa* (AB010104) and *Pleurotus sajor-caju* (AF149777) were less than 20% similar to those of SK-1 and *T. brockii* ATCC 35047, and there were no conserved regions. These observations may suggest that TPase evolved independently in eukaryotes and prokaryotes. We could not find any other similar genes in the databases of the DDBJ including phosphorylases, glucosyltransferases, and trehalases.

TPases have been classified in family 65 of the glycoside hydrolases, which includes sequences coding for maltose phosphorylases, trehalases, and uncharacterized proteins. During this work, the crystal structure of the maltose phosphorylase from *Lactobacillus brevis* (Lb-MP) was published and the catalytic residue of Glu487 was identified.24) The TPase from SK-1 had only 32% similarity to Lb-MP as a whole, but residues that form the putative active-site pocket of Lb-MP agreed well with those of the TPase. Especially, the hypothetic catalytic acid and...
residues contacting the phosphate coincided completely (Fig. 3). TPase resembles maltose phosphorylase in its reaction mechanism, that is, both enzymes catalyze reversible phosphorylase of a disaccharide with β-GIP as a glucosyl donor and with inversion of the anomeric configuration. These observations suggest that TPase and MPase could have evolved from a common ancestor.

An overexpression system for the efficient production of TPase is under investigation. The DNA sequence in this report will appear in the DDBJ, EMBL, and GenBank databases with the accession number of AB079610.

Fig. 2. Nucleotide and Deduced Amino Acid Sequence of the TPase Gene of SK-1.

Numbers on the right indicate nucleotide positions. The putative –35 sequence, –10 sequence, and ribosomal binding site are shown. The restriction enzyme sites of HindIII and MspI are shown. The stop codon is indicated by an asterisk. The deduced amino acid sequences are shown below the nucleotide sequence. The amino-terminal amino acid sequence of TPase, identified chemically, is underlined. The sequences corresponding to those of primers used in the text are also underlined (TR and TL).
Table 3. Properties of Trehalose Phosphorylases from Various Origins

<table>
<thead>
<tr>
<th>Origin</th>
<th>B. stearo&lt;sup&gt;a&lt;/sup&gt;</th>
<th>T. brockii&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Plesiomonas&lt;sup&gt;c&lt;/sup&gt;</th>
<th>C. ferruginea&lt;sup&gt;d&lt;/sup&gt;</th>
<th>M. varians&lt;sup&gt;e&lt;/sup&gt;</th>
<th>E. gracilis&lt;sup&gt;f&lt;/sup&gt;</th>
<th>F. velutipes&lt;sup&gt;g&lt;/sup&gt;</th>
<th>S. commune&lt;sup&gt;h&lt;/sup&gt;</th>
<th>A. bisporus&lt;sup&gt;i&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>$M_*$</td>
<td>150,000</td>
<td>190,000</td>
<td>200,000</td>
<td>400,000</td>
<td>570,000</td>
<td>344,000</td>
<td>ND</td>
<td>61,000</td>
<td>240,000</td>
</tr>
<tr>
<td>$M_*$ of subunit</td>
<td>87,950</td>
<td>88,000</td>
<td>88,000</td>
<td>98,000</td>
<td>105,000</td>
<td>344,000</td>
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<tr>
<td>No. of subunits</td>
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<td>2</td>
<td>2</td>
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<td>6</td>
<td>ND</td>
<td>ND</td>
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<td>ND</td>
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<td>7.0</td>
<td>ND</td>
<td>6.0-7.5</td>
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<td>6.0-9.0</td>
<td>6.0-9.0</td>
<td>6.0-7.0</td>
<td>5.5-7.5</td>
<td>ND</td>
<td>ND</td>
<td>6.0-7.5</td>
<td>6.0-7.5</td>
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<tr>
<td>Optimum temperature&lt;sup&gt;1&lt;/sup&gt;</td>
<td>(60°C for 24 h)</td>
<td>(4°C for 24 h)</td>
<td>(50°C for 15 min)</td>
<td>(4°C for 24 h)</td>
<td>(4°C for 24 h)</td>
<td>(30°C for 60 min)</td>
<td>(pH 6.0 for 15 min)</td>
<td>(pH 7.0 for 60 min)</td>
<td>(pH 6.0 for 15 min)</td>
</tr>
<tr>
<td>Heat stability&lt;sup&gt;1&lt;/sup&gt;</td>
<td>75°C</td>
<td>70°C</td>
<td>50°C</td>
<td>45°C</td>
<td>32°C</td>
<td>ND</td>
<td>ND</td>
<td>30°C</td>
<td>25°C</td>
</tr>
<tr>
<td>Apparent $K_m$ (mM)</td>
<td>(pH 6.0 for 15 min)</td>
<td>(pH 7.0 for 60 min)</td>
<td>(pH 6.0 for 15 min)</td>
<td>(pH 6.5 for 60 min)</td>
<td>(pH 6.0 for 15 min)</td>
<td>(pH 7.0 for 60 min)</td>
<td>(pH 6.0 for 15 min)</td>
<td>(pH 6.0 for 15 min)</td>
<td>(pH 6.0 for 15 min)</td>
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<td>Phosphorylisis</td>
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<td>Trehalose</td>
<td>4.1</td>
<td>0.94</td>
<td>ND</td>
<td>12.5</td>
<td>10</td>
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<td>ND</td>
<td>6.0</td>
<td>3.1</td>
<td>9.4</td>
<td>5.0</td>
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<td>4.7</td>
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<td>$\beta$-G1P</td>
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<td>Acceptor (substrate)</td>
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<td>23</td>
<td>32</td>
<td>6.0</td>
<td>47</td>
<td>2.8</td>
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<td>ND</td>
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<td>Sequential</td>
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<td>ND</td>
<td>Sequential</td>
<td>ND</td>
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<td>Walden inversion</td>
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<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
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<sup>a</sup> Bacillus steatorophilus SK-1 (this study).
<sup>b</sup> Thermoanaerobium brockii ATCC 35047<sup>2</sup>.
<sup>c</sup> Plesiomonas sp.<sup>3</sup>.
<sup>d</sup> Catellatospora ferruginea.<sup>2</sup>.
<sup>e</sup> Micrococcus varians.<sup>4</sup>.
<sup>f</sup> Euglena gracilis.<sup>5</sup>.
<sup>g</sup> Flammulina velutipes.<sup>6</sup>.
<sup>h</sup> Schizosaccharomyces pombe.<sup>7</sup>.
<sup>i</sup> Agaricus bisporus.<sup>8</sup>.

1. Indicated values are in the case of a phosphorolytic reaction.
2. The incubation temperature and time are given in parentheses.
3. The incubation pH and time are given in parentheses.
4. The enzyme was in an extremely high concentration (800 mM) of potassium phosphate buffer.
5. $\alpha$-G1P; $\alpha$-glucose 1-phosphate, $\beta$-G1P, $\beta$-glucose 1-phosphate; ND, not determined.
Fig. 3. Alignment of the Amino Acid Sequences of the TPase from SK-1 and Maltose Phosphorylases.

The alignment includes sequences of the TPase of *Bacillus steaetherophilus* SK-1 (Bs-TP), the maltose phosphorylases of *Lactobacillus brevis* (Lb-MP), *Lactobacillus sanfranciscensis* (Ls-MP), and *Neisseria meningitidis* (Nm-MP); of the putative maltose phosphorylases of *Mycobacterium tuberculosis* (Mt-MP), *Mycobacterium leprae* (Mi-MP), and *Bacillus subtilis* (Bs-MP). The residues that form the active-site pocket of Lb-MP are marked (▲). The hypothetical catalytic acid and residues contacting the phosphate are shaded.

| Table 4. Similarities of Amino Acid Sequences of Various TPases |
|------------------|------------------|------------------|------------------|
|                  | 1    | 2    | 3    | 4    |
| **1Bs-SK-1**     | 100.0| 46.0 | 17.6 | 18.3 |
| **2Tb (ATCC 35047)** | 46.0 | 18.3 | 17.6 | 18.3 |
| **3Gf**          | 17.6 | 18.3 | 100.0| 73.7 |
| **4Ps**          | 18.1 | 17.5 | 73.7 | 100.0|

Amino acid sequences were aligned with Genetyx Mac software Ver. 10.1, and the alignment scores (%) are shown. 1. *B. steaetherophilus* SK-1 (AB079610); 2. *B. steaetherophilus* SK-1 (AB089176); 3. *G. fujita* f. *fujita* (AB010104 and AB010105); 4. *P. aeruginosa* ssp. *audax* (AF149977).

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