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A highly thermostable trehalase from the thermophilic bacterium *Rhodothermus marinus*

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Abstract Trehalases play a central role in the metabolism of trehalose and can be found in a wide variety of organisms. A periplasmic trehalase (α,α-trehalose glucohydrolase, EC 3.2.1.28) from the thermophilic bacterium *Rhodothermus marinus* was purified and the respective encoding gene was identified, cloned and overexpressed in *Escherichia coli*. The recombinant trehalase is a monomeric protein with a molecular mass of 59 kDa. Maximum activity was observed at 88°C and pH 6.5. The recombinant trehalase exhibited a K_m of 0.16 mM and a V_{max} of 81 μmol of trehalose $(min)^{-1}$ $(mg\ of\ protein)^{-1}$ at the optimal temperature for growth of *R. marinus* (65°C) and pH 6.5. The enzyme was highly specific for trehalose and was inhibited by glucose with a K_i of 7 mM. This is the most thermostable trehalase ever characterized. Moreover, this is the first report on the identification and characterization of a trehalase from a thermophilic bacterium.

Keywords *Rhodothermus marinus* · Periplasmic trehalase · Glycosidase · Thermostability

Introduction

Trehalose (α-D-glucopyranosyl (1-1)-α-D-glucopyranoside) is a nonreducing disaccharide of glucose that is widespread throughout the biological world. This sugar plays multiple physiological roles, namely as an energy and carbon source, as a stabilizer and protector of proteins and cellular membranes against a variety of environmental stresses, as a sensing and regulator compound, and as a structural component of the bacterial cell wall (Elbein et al. 2003).

α,α-Trehalases (α,α-trehalose glucohydrolase, EC 3.2.1.28) specifically hydrolyze the α-1,1 bond of trehalose, but there are other glycosidases able to catalyze the hydrolysis of this disaccharide. Based on the amino acid sequences of known and hypothetical trehalases, two groups of α,α-trehalases have been proposed: one belongs to the glycoside hydrolase family 37 and the other to the family 65 (Henrissat and Bairoch 1993; CAZy, http://www.afmb.cnrs-mrs.fr/CAZY). Trehalases are extremely important in trehalose metabolism, as they are either directly involved in the assimilation of exogenous trehalose, or play a regulatory function in controlling the level of this osmolyte in the cell. These enzymes are found in a wide variety of organisms including bacteria, filamentous fungi, yeast, plants, insects and mammals (Elbein 1974). The function and properties of trehalases from eukaryotic cells have been amply illustrated (Parrou et al. 2005; Jorge et al. 1997), in contrast with the paucity of information available for bacterial trehalases. Among the bacterial sources, only the trehalases from *Acidobacterium capsulatum* (Inagaki et al. 2001), *Escherichia coli* (Boos et al. 1987; Horlacher et al. 1996), *Frankia* ArI3 (Lopez and Torrey 1985), and *Ectothiorhodospira halochloris* (Herzog et al. 1990) have been isolated and characterized.

Most yeast and fungi have two types of trehalases, usually designated "acid" and "neutral" trehalases to reflect their optimal pH for activity. These two groups exhibit characteristic primary structure, subcellular localization, and biochemical and regulatory properties (Parrou et al. 2005; Jorge et al. 1997; de Aquino et al. 2005). Based on the current knowledge on the molecular characterization of a large number of trehalases, Parrou et al. (2005) proposed to rename "acid" and

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"neutral" trehalases as "extracellular" and "cytosolic", respectively.

The biochemical properties of trehalases are highly variable, with optimal temperatures ranging from 30 to 65°C, optimal pH from 2.5 to 7.8, and apparent K_m values for trehalose ranging from 130 μM to 55 mM (Mansure et al. 1992; Kadowaki et al. 1996; Inagaki et al. 2001; Herzog et al. 1990; Kalf and Rieder 1958; Van Assche and Carlier 1975).

Rhodothermus marinus, an aerobic marine thermophilic bacterium, with optimal growth temperature of 65°C, produces a number of highly thermostable polysaccharide hydrolysing enzymes, such as cellulase (Hreggvidsson et al. 1996), xylanase (Dahlberg et al. 1993; Manelius et al. 1994), β-mannanase (Gomes and Steiner 1998), α-L-arabinofuranosidase (Gomes et al. 2000); α-galactosidase (Blücher et al. 2000), amylase and pullulanase (Gomes et al. 2003). Some of these enzymes have half-lives for thermal denaturation at 90°C in the order of 10 h. A recent review on the physiology, molecular biology and enzymology of *R. marinus* is available (Bjornsdottir et al. 2006).

In the course of our studies on the trehalose transport in *R. marinus*, the presence of a highly active trehalase became apparent. In this work, the trehalase from *R. marinus* was identified and purified; the respective encoding gene was cloned and overexpressed in *E. coli*, and the biochemical properties of the recombinant enzyme were characterized in detail. This enzyme is the most thermostable trehalase so far reported.

Materials and methods

Bacterial strain and growth conditions

Rhodothermus marinus strain DSM 4252^T was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany. Cultures of *R. marinus* were grown in fermentors at 65°C, on Degryse 162 medium, as modified by Nunes et al. (1992), containing per liter, 2.5 g of tryptone, 2.5 g of yeast extract, and 10 g of NaCl. Cell growth was monitored by measuring the turbidity at 600 nm.

Preparation of *R. marinus* cell extracts

Cells were collected during the late-exponential phase of growth, centrifuged and the resulting pellet frozen at −80°C. The cell sediment was thawed and suspended in 20 mM Tris–HCl, pH 7.6, containing 5 mM $MgCl_2$ and DNase I (10 μg/ml). The cells were disrupted in a French press apparatus, and the supernatant obtained after centrifugation was used for trehalase purification. The protein content was estimated by the method of Bradford (1976) using bovine serum albumin as standard.

Purification of native trehalase

The native trehalase was purified by fast protein liquid chromatography (Amersham Biosciences). *R. marinus* cell-free extract was fractionated with solid $(NH_4)_2SO_4$, and trehalase activity was found in the precipitate from 30 to 60% $(NH_4)_2SO_4$ saturation. The protein sediment was dissolved in Tris–HCl (50 mM, pH 7.6), and the resulting solution dialyzed against the same buffer. The dialyzate was applied on a DEAE-Sepharose column (20 mM Tris–HCl, pH 7.6) and eluted with a linear gradient of NaCl (0–1 M) in the same buffer. Trehalase activity was found in the fractions eluted between 510 and 570 mM NaCl. These fractions were pooled, dialyzed against Tris–HCl (50 mM, pH 7.6), loaded on a Q-Sepharose column (20 mM Tris–HCl, pH 7.6) and elution was carried out with a linear gradient of NaCl (0–1 M) in the same buffer. Trehalase activity was detected in the fractions eluted between 460 and 520 mM NaCl; active fractions were pooled and dialyzed against KPi (20 mM, pH 7.6) buffer. The sample was applied to a hydroxyapatite column, and trehalase activity was detected in the flow-through.

The flow-through of the previous step was dialyzed against Tris–HCl (20 mM, pH 7.6). Solid ammonium sulfate was added to the dialyzate to a final concentration of 1 M. This sample was loaded to a phenyl-Sepharose column, and elution was carried out with a decreasing linear gradient of ammonium sulfate (1–0 M). Samples containing trehalase activity were eluted between 350 and 270 mM $(NH_4)_2SO_4$. Active fractions were pooled and dialyzed against Tris–HCl (20 mM, pH 7.6) buffer. The resulting sample was applied to a Resource Q column (20 mM Tris–HCl, pH 7.6), and elution was performed with increasing concentrations of NaCl. Trehalase activity eluted at about 260 mM NaCl. A second similar step was performed.

Electrophoresis and activity staining

The purity of the final protein preparation was evaluated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, proteins were stained with Coomassie Blue. For in situ detection of trehalase activity, proteins in the gel were transferred to a polyvinylidene fluoride membrane (PVDF, Millipore) at 150 mA, for 1 h at 4°C, and stained as described by Jahagirdar and Seligy (1992). In brief, the PVDF membrane was incubated in a solution containing trehalose (20 mg/ml), glucose oxidase (40 U/ml), nitroblue tetrazolium chloride (0.24 mg/ml), and phenazine methosulfate (0.06 mg/ml) in 20 mM sodium phosphate buffer, pH 6.5. Only one band showed trehalase activity.

The amino acid sequences of three internal peptides were determined by Edman degradation (Edman and Begg 1967), after digestion of the native trehalase with trypsin and separation of the peptides by micro-HPLC

(Microchemical Facility, Emory University School of Medicine, GA, USA).

Qualitative trehalase assays

To detect trehalase activity during protein purification, 20 µl of sample was incubated with 10 mM trehalose, at 80°C for 1 h. The glucose production was analysed by thin-layer chromatography (TLC). Chromatograms were run on silica gel plates (Silica 60; Merck) with a solvent system composed of butanol, ethanol, water (5:3:2 v/v). Sugars were visualized by spraying the TLC plates with α-naphtol-sulfuric acid solution, followed by charring at 120°C.

Quantitative trehalase assays

Biochemical properties of the recombinant trehalase were assessed in reaction mixtures (100 µl) that contained precise quantities of enzyme and 10 mM trehalose in 50 mM Bis/Tris-propane buffer. Mixtures were preheated for 2 min at the assay temperature, and the reaction was started by addition of recombinant enzyme. The reaction was stopped at different times by boiling the mixture for 5 min and then cooling it on ice. The released glucose was quantified using a glucose assay kit (Merck) according to the supplier's instruction. A radiolabelling method (^{14}C) was used to determine the kinetic parameters of the recombinant enzyme at 88 and 65°C, in the presence or absence of glucose. Recombinant trehalase was incubated with a final concentration of 0.13 μCi of [U-^{14}C]-trehalose (Trenzyme) plus different concentrations of cold trehalose, in the presence or absence of 25 mM glucose. Appropriate volumes (35–50 µl) of reaction mixtures were spotted on the TLC plates, and the radiolabelled glucose formed was quantified on the TLC plates using a Molecular Dynamics PhosphorImager (Laser Scanner).

Identification, cloning, and expression of the trehalase gene

Several degenerated primers were designed based on alignments between DNA sequences of known trehalases and the three internal amino acid sequences (YYDL GEGPAPEVVAG; YLWDSER and TYYLTR) of the native trehalase. Chromosomal DNA of *R. marinus* was purified by the method of Marmur (1961) and used as a template to amplify the trehalase gene by PCR. Amplification products were purified from agarose gels and ligated to pGEM-T Easy vector (Promega), which allowed in obtaining a 408 bp fragment of the trehalase gene. The full gene was finally obtained by designing appropriate primers based on the N- and C-terminus regions of two truncated trehalase gene sequences identified in the genome of a different *R. marinus* strain (ITI-378) that had been partially sequenced by Prokaria Ltd, Reykjavik, Iceland. The complete gene, including the signalling peptide, was amplified from the genomic DNA of *R. marinus* by PCR, using the forward primer 5′-GAATTCCATATGCGATTTCGAGCCGTCTGG-3′ containing the additional *Nde*I recognition sequence (underlined), and the reverse primer 5′-CCGCTCGAGATCCATCTGGGCCAGCAGTTC-3′ with an additional *Xho*I recognition sequence (underlined). The PCR product was digested with *Nde*I and *Xho*I and purified using the Quiaquick PCR purification kit (Qiagen), after which it was ligated to pET-23a(+) expression vector (Novagen), yielding pTre1.

Cells of *E. coli* DH5α (Amersham Biosciences) harbouring pTre1 were grown on Luria–Bertani (LB) medium, containing ampicilin (0.1 mg/ml), to a OD_{600} = 0.6, and trehalase synthesis was induced by addition of 1 mM of isopropylthiogalactopyranoside (IPTG, Roche Molecular Biochemicals). After induction, growth was allowed to continue for further 4 h.

Purification of recombinant trehalase

Escherichia coli cells were harvested by centrifugation, suspended in binding buffer (20 mM phosphate buffer pH 7.6/20 mM imidazole/0.5 M NaCl) and disrupted twice in a French press. After removal of the cell debris by centrifugation (20,000*g*; 30 min; 4°C), cell-free extracts were loaded on a His-Trap chelating HP column (Amersham-Pharmacia Biotech). Chromatography was performed according to the supplier's protocol. Active fractions were pooled and desalted by dialysis against Tris–HCl (20 mM, pH 7.6) buffer. The resulting sample was applied to a Resource Q column previously equilibrated with the same buffer and elution was carried out with a linear NaCl concentration gradient (0–1 M). Trehalase activity was detected by TLC, and the purity of the active fractions evaluated by SDS-PAGE. Pure recombinant trehalase fractions were dialyzed against Tris–HCl (20 mM, pH 7.6) buffer and concentrated in a 10 kDa cutoff centricon (Amicon). The N-terminus amino acid sequence of the recombinant protein was determined to confirm the correct protein synthesis (Edman and Begg 1967).

Biochemical characterization of recombinant trehalase

The temperature profile, pH dependence, cation effects, kinetic parameters, substrate specificity and thermostability of the recombinant trehalase, carrying a histidine tag at the C-terminus were determined. The temperature profile for trehalase activity was studied in the range of 50–97°C, in 50 mM Bis/Tris/propane–HCl buffer (this buffer will be referred as buffer A) (pH 8.1 at 25°C). The effect of pH on trehalase activity was investigated at 88°C using 50 mM acetate buffer (pH 4.0–4.5), 50 mM MES (pH 5.5–6.0) and buffer A (pH 6.5–8.0). For each buffer system, the pH values were measured at room

temperature (25°C); pH values at a given temperature were calculated by using the conversion factor $\Delta pK_a/\Delta T$ (°C) = −0.011 for MES and −0.015 for Bis/Tris/propane–HCl. The effect of divalent cations and EDTA on trehalase activity was evaluated by incubating the enzyme at 80°C with 5 mM trehalose, in the presence of 5 mM of different chloride salts or EDTA in buffer A (pH 7.3). Kinetic parameters (K_m and V_{max}) were determined at 65°C in reaction mixtures containing 0.1 to 5 mM trehalose in buffer A (pH 6.5). The K_m of the recombinant protein was also determined at 88°C, by the radioactive method, in reaction mixtures with final concentrations of 0.05 to 10 mM trehalose in buffer A (pH 6.5). The inhibition of glucose (25 mM) on trehalase activity was measured at 65°C (radioactive method) in reaction mixtures with final concentrations of 0.05 to 2.5 mM trehalose in buffer A (pH 6.5). Substrate specificity was assessed by incubating the recombinant trehalase with several sugars (at 10 mM concentration), namely, trehalose, trehalose-6-phosphate, maltose, sucrose, lactose, cellobiose, dextrin, starch, palatinose, α-methyl glucopyranoside and *p*-nitrophenyl α-D-glucopyranoside, using buffer A (pH 6.5). Reaction products obtained from these substrates were visualized by TLC, with the exception of *p*-nitrophenyl α-D-glucopyranoside. The release of *p*-nitrophenol from the latter substrate was followed spectrophotometrically. The reaction mixture contained 10 mM *p*-nitrophenyl α-D-glucopyranoside, buffer A (pH 6.5), and appropriate concentration of enzyme solution. After incubation at 88°C for 1 h, the reaction was stopped by adding 700 µl of ice-cold borate buffer (0.2 M, pH 9.8) and 300 µl of buffer A (pH 6.5). The colour developed was measured at 405 nm.

Enzyme thermostability was evaluated at 70 and 80°C by incubating the enzyme in 20 mM Tris–HCl (pH 7.6). At appropriate times, samples were withdrawn and immediately examined for residual trehalase activity at the optimal temperature for activity, 88°C.

The molecular mass of the recombinant trehalase was estimated by gel filtration on Superose 12 column (Amersham Pharmacia Biotech). Molecular mass standards included cytochrome C (12 kDa), carbonic anhydrase (29 kDa), bovine serum albumin (67 kDa), alcohol dehydrogenase (150 kDa) and *β*-amylase (200 kDa).

Nucleotide sequence accession number

The nucleotide sequence of trehalase from *R. marinus* has been deposited in GenBank under accession number DQ328334.

Results

Purification of native trehalase and gene identification

The trehalase activity was purified 325-fold from cell-free extracts, from an initial activity of 40 nmol of trehalose $(min)^{-1}$ $(mg\ of\ protein)^{-1}$ to a final activity of 13 µmol of trehalose $(min)^{-1}$ $(mg\ of\ protein)^{-1}$. The final preparation showed three proteins on SDS-PAGE (Fig. 1). The band corresponding to trehalase was identified by its activity on the gel. Only one band, corresponding to a molecular mass of around 60 kDa, stained positively for trehalase activity. All the attempts to determine the amino acid sequence of the N-terminus of the protein were unsuccessful; however, the sequences of three internal fragments were obtained. Based on this information it was possible to obtain 408 bp of the trehalase gene. The complete sequence of the gene was obtained using sequence information obtained by Prokaria Ltd, Reykjavik, Iceland, for another *R. marinus* strain, ITI-378.

Purification of recombinant trehalase

Recombinant trehalase was purified from the cell-free extract of *E. coli* DH5α harbouring pTre1 plasmid. On SDS-PAGE the recombinant protein migrated with an apparent molecular mass of 60 kDa (Fig. 1). From analysis of the amino acid sequence of the N-terminus (QDRVACQVPL) of the purified trehalase and DNA sequences, it was concluded that the mature recombinant trehalase was composed of 516 amino acids (including the 6 extra histidines at the C-terminus), which yields a protein with a deduced molecular mass of 59,269 Da. On gel filtration, the recombinant trehalase behaved as a protein with a molecular mass of around 77 kDa, leading to the conclusion that the protein is monomeric.

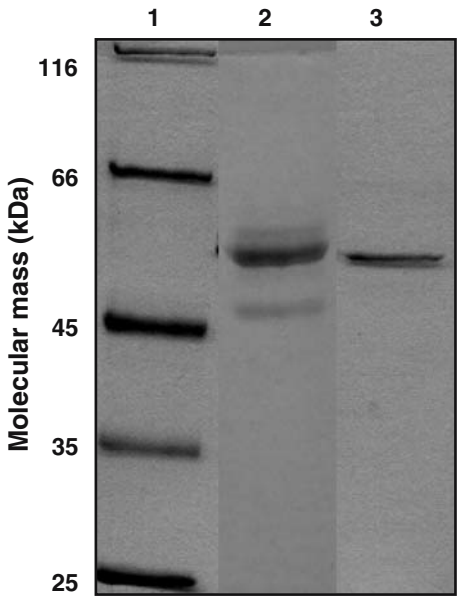

Fig. 1 SDS-PAGE of purified native and recombinant trehalase. Coomassie blue staining was used for visualization. *Lane 1* molecular mass markers; *lane 2* native trehalase; *lane 3* recombinant trehalase carrying a histidine tag at the C-terminus

Biochemical characterization of recombinant trehalase

The activity of the recombinant trehalase was investigated at different temperature and pH values. Maximal activity of the enzyme was observed at 88°C and pH 6.5 (Fig. 2). The effect of the presence of several divalent cations and EDTA on trehalase activity was investigated. Trehalase activity was strongly inhibited by Co^{2+} (71%) and Zn^{2+} (95%); moderate inhibitory levels were observed with Mg^{2+}, Mn^{2+}, or EDTA, whereas Ca^{2+} had no significant effect.

The kinetic parameters of pure recombinant trehalase were determined at 65°C, in the absence of divalent cations. The enzyme exhibited typical Michaelis Menten kinetics with a K_m of 0.16 mM and a V_{max} of 81 μmol of trehalose $(min)^{-1}$ (mg of protein)$^{-1}$. The K_m was also determined at 88°C, and a value of 0.27 mM was obtained. Glucose was an inhibitor of trehalase activity. To establish the type of inhibition, the kinetic parameters of the enzyme were determined by the radioactivity assay, in the presence or absence of glucose, at 65°C. A K_m of 0.15 mM was found in the absence of glucose, which is in good agreement with the K_m previously calculated by quantification of glucose using the enzymatic assay. Using the radioactive method, it was also observed that in the presence of 25 mM glucose, the apparent K_m increased from 0.15 to 0.7 mM, indicating that glucose behaved as a competitive inhibitor of trehalase activity (Fig. 3). The K_i value determined for glucose was 7 mM. The recombinant trehalase was highly specific for trehalose and it did not hydrolyze any of the 10 alternative substrates examined.

Trehalase thermostability was studied at 70 and 80°C (Fig. 4). The enzyme exhibited approximately a half-life of 6 h at 70°C, and of 2.5 h at 80°C. Incubation of 10 min, at 87°C resulted in 70% loss of the enzyme activity and after 10 min at 90°C, no residual activity was detected (Fig. 5).

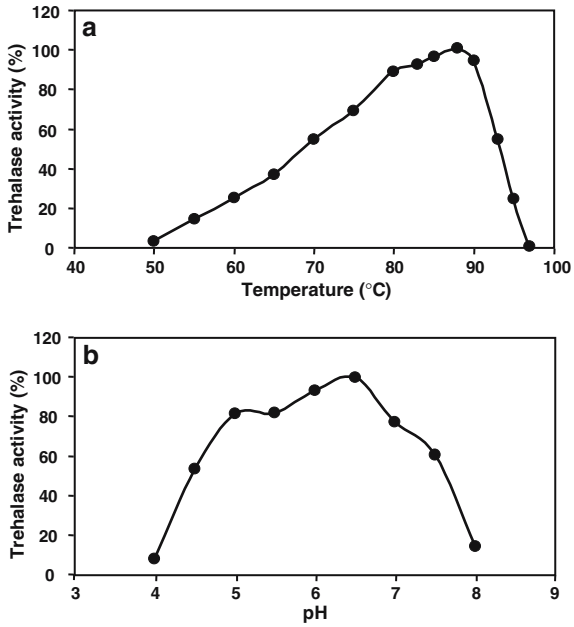

Fig. 2 Temperarure profile (**a**) and pH dependence (**b**) of the activity of recombinant trehalase. Maximal trehalase activity corresponded to 65 μmol of trehalose $(min)^{-1}$ (mg of protein)$^{-1}$. The data are the mean values of two independent experiments. The experimental conditions are described under "Materials and methods"

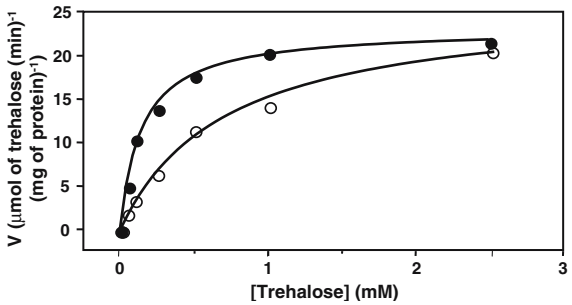

Fig. 3 Determination of the kinetic parameters (K_m, V_{max} and K_i) of recombinant trehalase. Rate dependence on trehalose concentration in the presence (*open circles*) or absence (*solid circles*) of 25 mM glucose

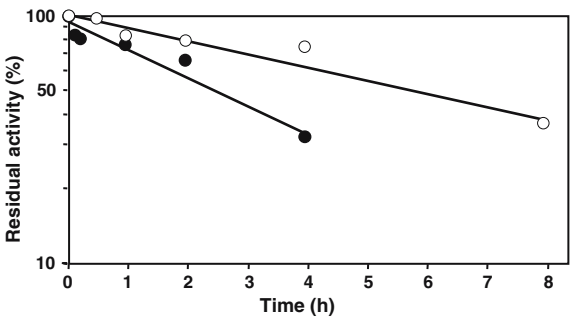

Fig. 4 Thermostability of the recombinant trehalase at 70°C (*open circles*) or 80°C (*solid circles*). Samples were withdrawn at different incubation times and examined for residual activity as described under "Materials and methods". One hundred percent trehalase activity corresponds to 215 μmol of trehalose $(min)^{-1}$ (mg of protein)$^{-1}$

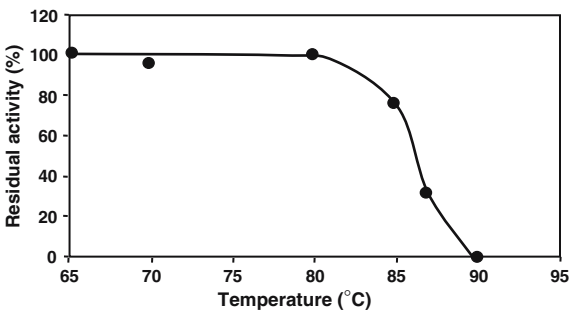

Fig. 5 Thermostability of the recombinant trehalase at different temperatures. Residual activity was measured at 65°C after 10 min of enzyme incubation at given temperatures as described under "Materials and methods". Trehalase activity of 100% corresponded to 21 μmol of trehalose $(min)^{-1}$ (mg of protein)$^{-1}$

Moving the histidine tag from the C-terminus to the N-terminus in the trehalase sequence did not improve thermostability. In fact, this modification induced a notable decrease of stability: the recombinant trehalase with a histidine tag at the N-terminus lost 50% of the activity after 10 min incubation at 80°C (data not shown).

Homology with trehalases from other microorganisms

A search for homology between the sequence of trehalase from *R. marinus* and trehalase sequences from other organisms was performed by using the available protein databases and the BLAST algorithm. The trehalase from *R. marinus* showed high similarity with several trehalases already described. *R. marinus* trehalase had 60% amino acid identity with the putative trehalase from *Solibacter usitatus*, 59% with the putative trehalase from *Nostoc* sp. (strain PCC 7120), 47% with the putative trehalase from *Legionella pneumophila* (strain Paris), 40% with the trehalase from *Neurospora crassa*, and 39% with the trehalase from *Kluyveromyces lactis*. The *R. marinus* trehalase also contains the two conserved regions (PGGRFXEXYXWDXY and QWD XPX(G/A)W(P/A/S)P) that are characteristic of the glycoside hydrolase family 37; therefore this enzyme should be considered as a new member of this family (Henrissat and Bairoch 1993).

Discussion

A large number of trehalase encoding genes have been putatively assigned in a wide variety of organisms by sequence homology in the rapidly expanding DNA sequence databases. However, in most cases neither the function nor the properties of the proteins have been investigated. This work describes the identification and characterisation of a trehalase from the thermophilic bacterium *R. marinus*.

An unrooted phylogenetic tree constructed on the basis of the alignment of the amino acid sequences of known or putative trehalases predicted the existence of five different clusters: two clusters of fungal trehalases, two clusters of bacterial trehalases and one cluster of trehalases from higher eukaryotes (Fig. 6). *R. marinus* trehalase is included in the bacterial cluster presently comprising the putative bacterial trehalases from *Legionella pneumophila*, *Nostoc* sp. and *Solibacter usitatus* with which it shares a high sequence identity (47 to 60%). Curiously, this group shows greater proximity with the fungal cytosolic trehalases than with the second cluster of bacterial trehalases that comprises the homologous proteins of the model organism, *E. coli*.

The model bacterium, *E. coli*, possesses both periplasmic and cytoplasmic trehalases (Boos et al. 1987; Horlacher et al. 1996). Our work shows that *R. marinus* has a single trehalase located in the periplasm as evidenced by the presence of a peptide sequence on the N-terminus region exhibiting the characteristic features of a bacterial signal sequence (Hiller et al. 2004). Analysis of the full-length trehalase sequence by SignalP 3.0 software (http://www.cbs.dtu.dk/services/SignalP) predicted the existence of a signal sequence composed by 21 amino acids. The predicted cleavage site was confirmed by the N-terminal sequence of the recombinant protein, showing that this cleavage site was recognized by *E. coli*. Probably, this site is also recognized by *R. marinus*, but a definite proof is missing since we failed to determine the N-terminus sequence of the native protein.

From previous studies, it is known that *R. marinus* accumulates mannosylglycerate and mannosylglyceramide as major compatible solutes in response to osmotic and/or heat stress (Silva et al. 1999). Trehalose is a minor intracellular solute present in the early phases of growth but depleted at the end of the exponential phase. These observations indicate that trehalose plays a minor role in osmo- or thermo-adaptation of this organism and probably is used primarily as a carbon source. In fact, *R. marinus* was able to grow on minimal medium supplemented with trehalose (results not shown). The presence of a periplasmic trehalase is likely to assist the uptake of trehalose via transport of the resulting glucose, as indicated by kinetic data on the transport of trehalose and glucose in *R. marinus* (our unpublished data).

The most remarkable feature of the recombinant trehalase of *R. marinus* is its high thermostability. Indeed, this is the most thermostable trehalase reported thus far, exhibiting a half-life of 2.5 h for loss of activity at 80°C. Trehalases thus far isolated from thermophilic fungi are less thermostable. For instance, the cytosolic trehalase from *Scytalidium thermophilum* has a half-life of 4.5 min at 65°C, and the extracellular enzyme is even less stable: half-life of 3 min at 60°C (Kadowaki et al. 1996). Considerably the extracellular trehalase of the thermophilic fungus *Humicola lanuginosa* is more heat-resistant (half-life of 1.5 h at 60 °C), but comparison with the *R. marinus* trehalase is clearly unfavourable for the fungal enzyme (Prasad and Maheshwari 1978).

As trehalose can be hydrolyzed by enzymes other than trehalase, i.e., α-glucosidases (EC 3.2.1.20) and glucan 1,4-α-glucosidases (EC 3.2.1.3), it is pertinent to include them in this comparison (Manjunath et al. 1983; Nakao et al. 1994; Murakami et al. 1998; Berthelot and Delmotte 1999; Saha and Bothast 1993; Im and Henson 1995). Data on thermostability are available only for α-glucosidases isolated from *Aureobasidium pullulans* (Saha and Bothast 1993), *Bacillus* sp. SAM1606 (Nakao et al. 1994) and *Bacillus flavocaldarius* KP1228 (Murakami et al. 1998). Only the α-glucosidase from *B. flavocaldarius* was more thermostable than *R. marinus* trehalase, exhibiting a half-life of 10 min at 90°C.

The molecular determinants of protein stability are far from being elucidated. In the past, the comparison between the three-dimensional structures of proteins from psychrophilic to mesophilic and hyperthermophilic sources provided some clues, but a convincing, general

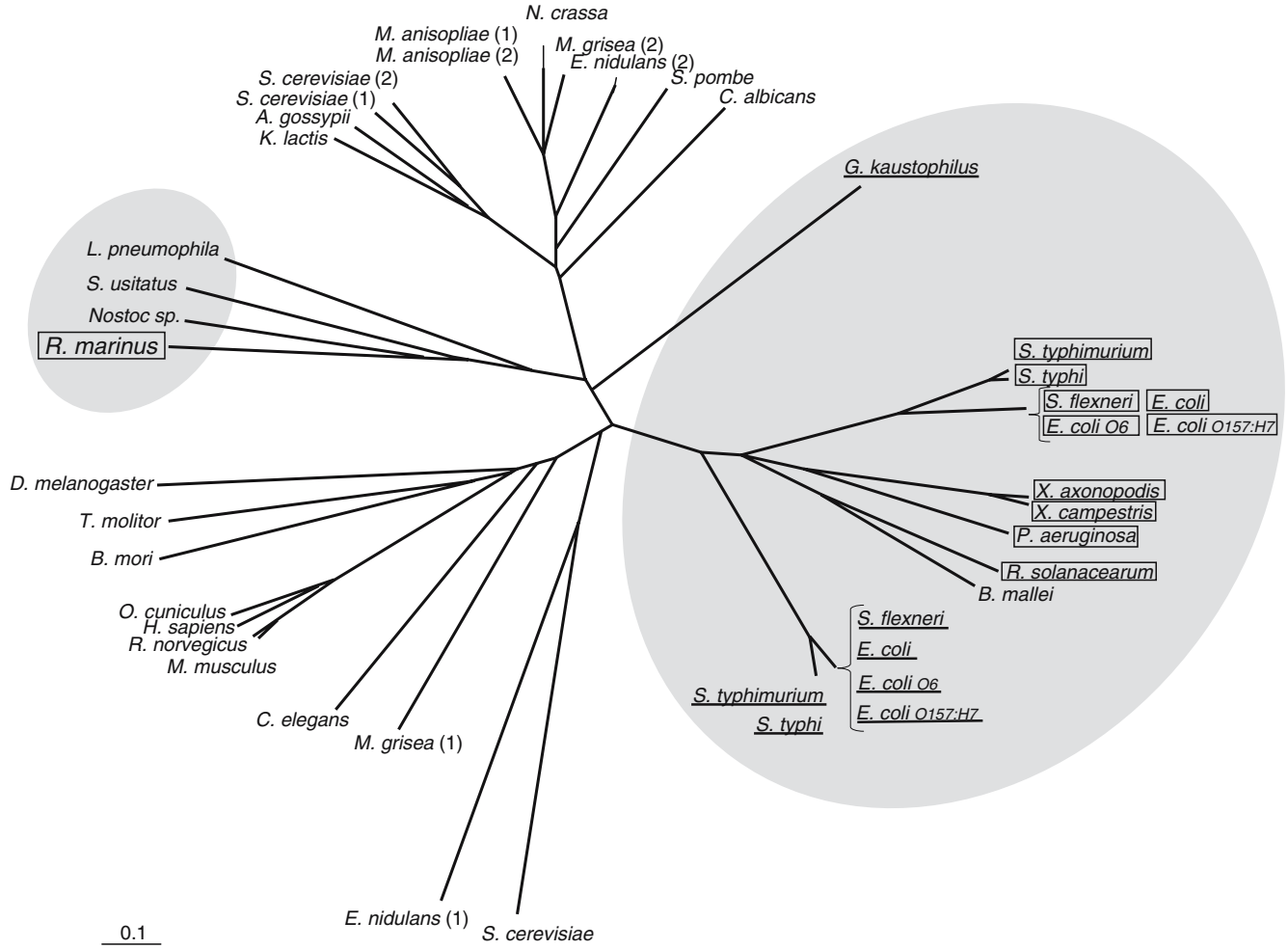

Fig. 6 Unrooted phylogenetic tree based on available amino acid sequences of known or hypothetical trehalases. The CLUSTALX program (Thompson et al. 1997) was used for sequence alignments and to generate the phylogenetic tree. The bacterial branches are highlighted with a *gray background*; periplasmic trehalases are designated by *boxes* around the names of the organisms; cytoplasmic trehalases are highlighted with *underlining*. *R. marinus* (GenPept accession number DQ328334); *E. coli* O6 (Q8CW46); *E. coli O157:H7* (Q8XDH7); *E. coli* (P13482); *S. flexneri* (Q83RP6); *S. typhimurium* (Q8ZP20); *S. typhi* (P59765); *R. solanacearum* (Q8XT38); *P. aeruginosa* (Q9I165); *X. campestris* (pv. campestris) (Q8P519); *X. axonopodis* (pv. citri) (Q8PPT1). *E. coli* O6 (Q8FCI4); *E. coli O157:H7* (P62602); *E. coli* (P62601); *S. flexneri* (Q83PS8); *S. typhimurium* (Q8ZLC8); *S. typhi* (Q8Z277); *G. kaustophilus* (Q5KZC3). Putative bacterial trehalases: *B. mallei* (Q62BW3); *L. pneumophila* (CR628336); *S. usitatus* (ZP00523643); *Nostoc* sp. PCC 7120 (Q8Z0D2). Animal trehalases: *D. melanogaster* (Q9W2M2); *T. molitor* (P32359); *B. mori* (P32358); *O. cuniculus* (P19813); *H. sapiens* (O43280); *R. norvegicus* (O70282); *M. musculus* (Q9JLT2); *C. elegans* (Q27463). Extracellular trehalases from fungi: *E. nidulans* (1) (P78617); *S. cerevisiae* (P48016). Fungal cytosolic trehalases: *K. lactis* (P49381); *A. gossypii* (Q757L1); *S. cerevisiae* (1) (P32356; NTH1); *S. cerevisiae* (2) (P35172; NTH2); *M. anisopliae var. anisopliae* (1) (Q9HDE9); *M. anisopliae var. acridium* (2) (Q6Q5X7); *N. crassa* (O42783); *M. grisea* (2) (Q875L8; NTH1); *E. nidulans* (2) (O42777); *S. pombe* (O42893); *C. albicans* (P52494); putative fungal trehalase: *M. grisea* (1) (Q8J0H8)

mechanism for protein stability has not yet been put forward. It appears that subtle differences in the structure, compactness, or the number of hydrogen bonds or ionic bridges can have dramatic impact on protein thermostability (Robinson-Rechavi et al. 2006). As no three-dimensional structure of a trehalase has been reported it is impossible to elaborate on the molecular properties responsible for the greater stability of *R. marinus* trehalase. It is interesting to note, however, that a search of the databases for trehalase counterparts did not find any hits from extreme thermophiles or hyperthermophiles, indicating that the occurrence of a trehalase more stable than that of the *R. marinus* may be unlikely.

In conclusion, herein we report the characterization of a highly thermostable trehalase from *R. marinus*. Attempts to fully elucidate the physiological relevance of this periplasmic enzyme are in progress.

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