Biochemical and genetic characterization of the pathways for trehalose metabolism in *Propionibacterium freudenreichii*, and their role in stress response

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Propionibacterium freudenreichii accumulates high levels of trehalose, especially in response to stress. The pathways for trehalose metabolism were characterized, and their roles in response to osmotic, oxidative and acid stress were studied. Two pathways were identified: the trehalose-6-phosphate synthase/phosphatase (OtsA–OtsB) pathway, and the trehalose synthase (TreS) pathway. The former was used for trehalose synthesis, whereas the latter is proposed to operate in trehalose degradation. The activities of OtsA, OtsB and TreS were detected in cell extracts; the corresponding genes were identified, and the recombinant proteins were characterized in detail. In crude extracts of *P. freudenreichii*, OtsA was specific for ADP-glucose, in contrast to the pure recombinant OtsA, which used UDP-, GDP- and TDP-glucose, in addition to ADP-glucose. Moreover, the substrate specificity of OtsA in cell extracts was lost during purification, and the recombinant OtsA became specific to ADP-glucose upon incubation with a dialysed cell extract. The level of OtsA was enhanced (approximately twofold) by osmotic, oxidative and acid stress, whereas the level of TreS remained constant, or it decreased, under identical stress conditions. Therefore, the OtsA–OtsB pathway plays an important role in the synthesis of trehalose in response to stress. It is most likely that trehalose degradation proceeds via TreS to yield maltose, which is subsequently catabolized via amylomaltase activity. Hydrolytic activities that are potentially involved in trehalose degradation (trehalase, trehalose phosphorylase, trehalose-6-phosphate phosphorylase and trehalose-6-phosphate hydrolase) were not present. The role of trehalose as a common response to three distinct stresses is discussed.

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INTRODUCTION

Trehalose is widely distributed in living cells, where it plays a variety of roles generally associated with protection against stress (Argüelles, 2000; Elbein *et al.*, 2003). This disaccharide is frequently found in yeast, fungi and plants, but it also occurs in many bacteria, and several halophilic or hyperthermophilic archaea (da Costa *et al.*, 1998; Santos & da Costa, 2001; Elbein *et al.*, 2003).

At least four pathways for the synthesis of trehalose have been reported thus far: (i) the OtsA–OtsB pathway, the most common route, involves the transfer of glucose from UDP-glucose to glucose 6-phosphate to yield trehalose 6-phosphate, which is subsequently converted to trehalose (Brüggemann *et al.*, 2004); (ii) the TreS pathway, a reversible

pathway involving the intramolecular rearrangement of the α -(1 $\rightarrow$ 4) glycosidic bond of maltose into an α -(1 $\rightarrow$ 1) glycosidic bond to yield trehalose (Nishimoto *et al.*, 1996); (iii) the TreY–TreZ pathway, in which the terminal α -(1 $\rightarrow$ 4) bond of an α -(1 $\rightarrow$ 4)-glucan polymer is converted into an α -(1 $\rightarrow$ 1) bond via transglycosylation, and subsequently the terminal disaccharide is cleaved to release trehalose (Nakada *et al.*, 1995); and (iv) a single-step pathway involving trehalose glycosyltransferring synthase, a recently discovered enzyme that catalyses the reversible conversion of glucose and NDP-glucose into trehalose (Qu *et al.*, 2004).

In many organisms, trehalose catabolism proceeds via trehalase, resulting in the formation of two glucose molecules (Horlacher *et al.*, 1996; Elbein *et al.*, 2003). Other enzymes for hydrolysis of trehalose or trehalose 6-phosphate have been described: trehalose phosphorylase catalyses the phosphorolysis of trehalose producing β -glucose 1-phosphate and glucose; trehalose-6-phosphate

The GenBank/EMBL/DDBJ accession numbers for the nucleotide sequence of the DNA fragment containing the *otsA* and *otsB* genes, and the sequence containing the *treS* gene, are DQ356268 and DQ356269, respectively.

phosphorylase converts trehalose 6-phosphate into β -glucose 1-phosphate and glucose 6-phosphate; and, finally, trehalose-6-phosphate hydrolase produces glucose and glucose 6-phosphate from trehalose 6-phosphate (Belocopitow & Maréchal, 1970; Rimmele & Boos, 1994; Andersson *et al.*, 2001).

Dairy propionibacteria are important organisms in cheese-making, and in the production of other dairy products. In addition, the potential probiotic properties of some species (*Propionibacterium acidipropionici*, *Propionibacterium freudenreichii*, *Propionibacterium jensenii* and *Propionibacterium thoenii*) have been suggested in recent years (Jan *et al.*, 2002; Huang & Adams, 2004). The presence of trehalose in propionibacteria has been known for nearly five decades (Stjernholm, 1958); however, trehalose metabolism has not been studied in any member of the genus *Propionibacterium*.

A few years ago, we showed that trehalose is widespread within the genus *Propionibacterium* (Cardoso *et al.*, 2004). In particular, *P. freudenreichii* subsp. *shermanii* strain NIZO B365 accumulates trehalose to remarkable levels, and the trehalose content increases considerably in response to osmotic, oxidative and acid stress [up to 40 % (w/w) of the cell protein]. These findings triggered our interest in elucidating trehalose metabolism and regulation in *P. freudenreichii* under different stressful conditions. Given the importance of trehalose as a preservative of food flavour, and in cell protection against different stresses, knowledge of trehalose metabolism could be of importance in directing strain manipulation: for example, in improving acid resistance, and allowing extended fermentation.

In the present work, the pathways for synthesis and degradation of trehalose were investigated. The OtsA–OtsB and TreS pathways were genetically and biochemically characterized. Additionally, the levels of OtsA and TreS were examined under different growth conditions by immunoassays to study the role of the two pathways, and their regulatory properties under osmotic, oxidative and acid stress.

METHODS

***P. freudenreichii* and growth conditions.** *P. freudenreichii* subsp. *shermanii* strain NIZO B365 [Netherlands Institute for Dairy Research (NIZO), Ede, The Netherlands] was grown in medium buffered with potassium phosphate, in 2 l fermenters, under controlled conditions of pH (7.0) and temperature (30 °C), as previously described (Cardoso *et al.*, 2004). For enzymic assays, and for purification of native OtsA, cells were grown under osmotic stress [1.5 % (w/v) NaCl] at pH 5.5.

Preparation of *P. freudenreichii* cell extracts. Cells were harvested during the exponential phase of growth, and suspended in PIPES buffer (50 mM, pH 6.5), containing 2 mM DTT and a mixture of protease inhibitors: 100 μ M PMSF, 10 μ M leupeptin, and 10 μ M antipain. The cells were disrupted in a French press, and cell debris was removed by centrifugation. For enzymic assays, low-molecular-mass compounds were removed in a PD-10 column (Amersham Biosciences); for the purification of native OtsA, the

extract was dialysed twice (membrane cut-off 12–14 kDa) against Tris/HCl buffer (20 mM, pH 7.6). The amount of protein was estimated by using the BCA protein assay kit (Pierce).

Enzyme assays. Activity of OtsA was determined in a reaction mixture containing acetate buffer (50 mM, pH 5.5), 10 mM MgCl₂, 10 mM glucose 6-phosphate, and 5 mM NDP-glucose (in which N represents A, U, G or T). The activity of OtsA using CDP-glucose was not studied because this substrate is not commercially available. The reaction was initiated with the enzyme (0.8 μ g), then incubated at 37 °C for different periods of time, and stopped by incubation in boiling water for 5 min. Afterwards, the reaction mixture was incubated for 30 min at 37 °C with 1 μ g recombinant OtsB. The Pi released was determined using the spectrophotometric method described by Ames (1966), after confirming that OtsB was specific for trehalose 6-phosphate. In cell extracts, or during the purification of native OtsA, trehalose formation was detected by TLC after treatment with alkaline phosphatase. The OtsB activity was assayed in a reaction mixture containing MES buffer (50 mM, pH 6.5), 10 mM MgCl₂, and 5 mM trehalose 6-phosphate. The reaction was initiated with the enzyme (0.3 μ g), then incubated at 37 °C for different periods of time, and stopped by freezing in liquid nitrogen. Pi was determined as described above.

The biochemical characterization of TreS was performed in a reaction mixture containing phosphate buffer (50 mM, pH 5.2) and 5 mM maltose. The reaction was started with the enzyme (1 μ g), then incubated at 37 °C for different periods of time, and stopped by incubation in boiling water for 5 min. The same conditions were used to study the reverse reaction, except that 5 mM trehalose was used instead of maltose. The reaction product (trehalose or maltose) was incubated for 1 h at 50 °C with 30 μ g trehalase (isolated from *Rhodothermus marinus*) or 0.1 U maltase (Sigma), respectively, for 1 h at 37 °C. Commercially available trehalase was not suitable because it contains α -glucosidase as a contaminant. The formation of glucose was determined enzymically (D-glucose kit; Boehringer Mannheim). In cell extracts, the samples were incubated for 1 h at 37 °C, and the formation of trehalose or maltose was evaluated by TLC. Whenever necessary, the assignment of spots was confirmed by treatment with trehalase or maltase; moreover, the reaction products were analysed in separate runs using two solvents in order to optimize spot identification.

The activity of amylomaltase was investigated in reaction mixtures containing sodium phosphate buffer (25 mM, pH 6.5), 5 mM maltoheptaose, maltopentaose, maltotetraose or maltotriose (Sigma), and an appropriate amount of enzyme. The reaction mixtures were incubated at 37 °C for 0–24 h, and the reactions were stopped by incubation in boiling water for 5 min. The products were analysed by TLC.

The activities of the TreY–TreZ pathway were investigated as described by Maruta *et al.* (1995). Trehalose phosphorylase was examined as described by Maréchal & Belocopitow (1972). The activity of trehalose glycosyltransferase was tested in both directions, as reported by Qu *et al.* (2004). Assays to investigate the presence of trehalase in cell extracts were performed as described by Uhland *et al.* (2000). The activity of trehalose-6-phosphate phosphorylase and trehalose-6-phosphate hydrolase was assessed by the methods described by Andersson *et al.* (2001) and Rimmele & Boos (1994), respectively. In all cases, incubations were performed at 37 °C for 1 h, and end-product formation was analysed by TLC.

TLC conditions. TLC was performed on silica gel 60 plates (Merck). Butanol/pyridine/water (7:3:1, by vol.) or butanol/ethanol/water (5:3:2, by vol.) was used as the solvent system. The former solvent system distinguishes trehalose from maltose, while

the latter has the advantage of separating phosphorylated compounds. Spots were visualized by spraying with α -naphtholsulfuric acid solution, followed by charring at 120 °C.

Identification of *otsA*, *otsB* and *treS* genes. Chromosomal DNA from *P. freudenreichii* was isolated by standard procedures (Sambrook *et al.*, 1989). Degenerate primers, based on two conserved regions (FFLHIPFP and GMNLVAKE) of known OtsA proteins, and on two conserved regions (AVFYEV and GDEIGM) of known TreS proteins, were designed to amplify *otsA* and *treS*, respectively. Single PCR products of 595 bp (for *otsA*) and 1111 bp (for *treS*) were amplified, cloned into pGEM-T, and sequenced (AGOWA, Berlin, Germany). The complete sequences of *otsA* and *treS* were obtained by inverse PCR. For this, *P. freudenreichii* genomic DNA was partially digested with *SacI*, recircularized, and used to perform inverse PCR. Single PCR products of 4 kb (for *otsA*) and 2.5 kb (for *treS*) were obtained, cloned into pGEM-T, and sequenced (STAB-VIDA, Oeiras, Portugal).

Cloning and overexpression of *otsA*, *otsB* and *treS* genes.

The *otsA* and *otsB* genes were amplified by PCR using the GC-Rich PCR System Kit (Amersham Biosciences), and cloned separately in the pGEX-4T-2 plasmid between the *XmaI*–*XhoI* sites and the *BamHI*–*EcoRI* sites, respectively. The *treS* gene was cloned in the pET-19b plasmid between the *NdeI*–*NdeI* sites. The resulting plasmids were designated pOtsA, pOtsB and pTreS. Cloning methodology followed standard protocols (Sambrook *et al.*, 1989). The plasmids pOtsA and pOtsB were separately introduced into *Escherichia coli* JM107, and the plasmid pTreS was introduced into *E. coli* BL21(DE3). *E. coli* strains were grown at 37 °C in LB medium, supplemented with ampicillin (100 µg ml⁻¹), to an OD₆₀₀ of 0.6, and induced with 1 mM IPTG for 3 h. No inducer was added for *otsB* expression.

Purification of recombinant proteins. *E. coli* cells were collected by centrifugation, and suspended in PBS buffer pH 7.3 (for *E. coli* pOtsA and pOtsB), or in His-Trap binding buffer (for *E. coli* pTreS). Cells were disrupted in a French press, and cell debris was removed by centrifugation. OtsA and OtsB were purified in two chromatographic steps using a GST Prep FF column and a Mono Q column, after digesting the fusion proteins with thrombin (12 h at 22 °C). TreS was purified in a His-Trap column, and the histidine tag was cleaved with enterokinase (18 h at 37 °C). Single bands of 55, 30 and 69 kDa were observed in SDS-PAGE gel, corresponding to OtsA, OtsB and TreS, respectively.

Purification of native trehalose-6-phosphate synthase

Native OtsA was partially purified by FPLC (Amersham Biosciences).

Ammonium sulfate precipitation. The cell extract was treated with ammonium sulfate (50 %) at 4 °C. The precipitate was dissolved in 20 mM Tris/HCl (pH 7.6) containing 1 mM EDTA and 5 mM β -mercaptoethanol, and dialysed overnight.

Ion-exchange chromatography. The dialysed sample was applied to a DEAE-Sepharose column (20 mM Tris/HCl, pH 7.6), and eluted with a linear gradient 0–1 M NaCl. OtsA activity was detected at around 0.75 M NaCl. After desalting, active fractions were loaded onto a Q-Sepharose column (20 mM Tris/HCl, pH 7.6). The enzyme was eluted around 0.5 M NaCl. The dialysed active fractions were applied onto a Resource Q column (20 mM Tris/HCl, pH 7.0). OtsA activity was eluted at 0.3 M NaCl.

Hydrophobic chromatography. Solid ammonium sulfate was added slowly to the active fractions to a final concentration of 0.9 M. The sample was applied to a phenyl-Sepharose column, and eluted with a linear gradient of ammonium sulfate (0.9–0 M). OtsA activity was detected at 0.15 M ammonium sulfate.

Ion-exchange chromatography. After dialysis, active fractions were applied onto a Mono Q column (20 mM Tris/HCl, pH 7.0), and the OtsA activity was detected at 0.3 M NaCl.

Characterization of recombinant enzymes. OtsA substrate specificity was studied by combining a sugar acceptor unit (glucose 6-phosphate, trehalose 6-phosphate, fructose 6-phosphate, mannose 6-phosphate, galactose 6-phosphate, glucosamine 6-phosphate, ribose 5-phosphate, nitrophenyl phosphate, glucose 1-phosphate, fructose 1-phosphate, mannose 1-phosphate, galactose 1-phosphate, glucosamine 1-phosphate, glucose, fructose, maltose, lactose or galactose) with one of the four glucosyl donor substrates (ADP-, UDP-, GDP- or TDP-glucose). All the phosphate sugars mentioned above were also examined as possible substrates for OtsB. The temperature profiles for activity of OtsA, TreS and OtsB were studied between 30 and 70 °C at pH 5.2 (OtsA and TreS) or pH 6.5 (OtsB). The pH profiles of OtsA and OtsB were determined at 37 °C using 50 mM acetate buffer in the pH range 4.5–5.5, 50 mM MES buffer in the pH range 5.5–7.0, and 50 mM BisTris propane/HCl buffer in the pH range 6.5–8.5. The effect of pH on TreS was determined at 37 °C using 50 mM phosphate buffer in the pH range 5.0–7.5, and 50 mM acetate buffer in the pH range 4.0–5.5. Kinetic parameters ($V_{\max}$ and K_m) were determined under optimum conditions, using Lineweaver–Burk plots. The effect of Mg²⁺, Mn²⁺, Ca²⁺, Co²⁺, Cu²⁺ (10 mM) and NaCl (100 mM) was tested on OtsA, OtsB and TreS activities. The effect of KCl (100 mM), heparin (20 ng µl⁻¹), pure recombinant OtsB (0.02 µg protein µl⁻¹), and a dialysed extract of *P. freudenreichii* (1 µg total protein µl⁻¹), on OtsA activity was also examined. Moreover, the effect of NaF (10 mM) and EDTA (10 mM) on the activity of OtsB was also studied. The effect of EDTA (10 mM) and Tris/HCl buffer (10 mM) on TreS activity was evaluated. All the reactions were done at least in duplicate, and most were done in triplicate.

Levels of enzymes and trehalose produced in response to osmotic, oxidative and acid stress.

P. freudenreichii cells were grown under controlled conditions of pH and temperature. The medium was gassed with argon during 1 h preceding inoculation, and the cells were allowed to grow until the mid-exponential phase. Osmotic, oxidative and acid stress were imposed in separate experiments by adding a NaCl solution to obtain a final concentration of 2 % (w/v) NaCl, bubbling air through the culture to a final oxygen concentration of 0.115 mM, and shifting the pH to 5.0 with 5 M HCl, respectively. The cultures were allowed to grow further for 10 h under stress conditions. Samples were withdrawn at 0, 2, 5 and 10 h, and used to determine protein levels (OtsA and TreS) by immunoblotting, and intracellular trehalose by ¹H-NMR (Cardoso *et al.*, 2004).

Western blot analyses. *P. freudenreichii* (5 µg of total protein) was suspended in potassium phosphate buffer (50 mM, pH 7.0) containing 2 % (w/v) SDS. Cells were disrupted with glass beads (<106 µm diameter), and the cell debris was removed by centrifugation. The rabbit primary antibodies (anti-OtsA and anti-TreS) were produced at Eurogentec (Belgium), and further purified for the immunoassays (Smith & Fisher, 1984). The resulting anti-OtsA and anti-TreS specifically recognized the OtsA and TreS in *P. freudenreichii* cell extracts. After SDS-PAGE, the proteins were transferred onto PVDF membranes (Millipore), which were incubated overnight with primary antibodies. Binding was detected with the enhanced chemiluminescence system (Amersham Biosciences) after treatment with anti-rabbit antibodies conjugated with horseradish peroxidase (Sigma). The signals were detected with X-ray film, and scanned and quantified by using a program written in MatLab (MathWorks). The experiments were performed at least in triplicate.

RESULTS

Enzyme activities involved in trehalose metabolism

The genome sequence of *P. freudenreichii* is not available; hence, the enzyme activities implicated in known pathways for the synthesis or degradation of trehalose were sought in dialysed extracts of *P. freudenreichii*. OtsA activity was assayed using glucose 6-phosphate, and ADP-, GDP-, TDP- or UDP-glucose, as the substrates. ADP-glucose was the only glucose donor used to synthesize trehalose 6-phosphate. The identification of this metabolite was confirmed by the detection of trehalose upon treatment of the final products with alkaline phosphatase (Fig. 1a). Phosphatase activity able to dephosphorylate trehalose 6-phosphate was also detected in cell extracts.

TreS activity was detected in both directions using TLC for visualization of substrate consumption and product formation (Fig. 1b). The weak spot of glucose detected when maltose was the substrate was attributed to the presence of a low activity of α -glucosidase (Fig. 1b). Activities potentially involved in maltose catabolism were also examined. We looked for maltooligosaccharide hydrolytic activities. Amylomaltase was detected: 1 h incubation of G7 with extracts of *P. freudenreichii* led to the formation of G8 and G6 (weak bands), and G4 (data not shown). For longer periods of incubation, G8, G6, G5, G4, G3, G2 and glucose were clearly detected (Fig. 1c). Spots due to formation of multiple maltooligosaccharides were also observed with G5, G4 and G3, but not with maltose. However, when maltose was incubated with trace amounts of G3 or G5, longer oligosaccharides, as well as glucose, were detected (data not shown). The activities of other enzymes potentially involved in trehalose metabolism, such as

maltooligosyl trehalose synthase and trehalohydrolase, trehalose glycosyltransferring synthase, trehalase, trehalose-6-phosphate hydrolase and trehalose-6-phosphate phosphorylase, were also sought in dialysed cell extracts of *P. freudenreichii*. No evidence was found for the presence of these enzymes.

Identification of *otsA*, *otsB* and *treS* genes

PCR products of 4 and 2.5 kb were amplified by inverse PCR using primers based on *otsA* and *treS* genes, respectively, as described in Methods. The nucleotide sequence of the 4 kb fragment revealed an ORF encoding a protein with 493 aa that had high identity with known OtsA proteins (Table 2). At 152 bp upstream of the *otsA* gene, a second ORF was found encoding a protein with 288 aa, with considerable identity with known OtsB proteins (Table 2), and downstream of the *otsA* gene, a fragment (518 bp) of a third ORF was encountered, which corresponds to part of the gene *cbiB* encoding cobinamide synthase (Fig. 2a), an enzyme involved in the synthesis of vitamin B₁₂ (Roessner *et al.*, 2002). Sequencing of the 2.5 kb fragment showed an ORF encoding a protein of 605 aa, with at least 58 % identity with known TreS proteins (Table 2, Fig. 2b). Downstream of the *treS* gene there was an ORF with 37 % identity with a hypothetical protein involved in trehalose synthesis in *Propionibacterium acnes* (YP_055818), and 28 % identity with maltokinase from *Actinoplanes missouriensis* (AAQ_01690). Another ORF located upstream of the *treS* gene was found to have high homology (49 %) with α -amylase from *P. acnes* (YP_055820).

Characterization of recombinant enzymes

We deemed it interesting to characterize the enzymes involved in trehalose metabolism of *P. freudenreichii*. For

Fig. 1. Identification of enzymic activities in cell extracts by TLC analysis of reaction products. (a) Cell extract incubated with ADP-glucose and glucose 6-phosphate before (lane 1) and after (lane 2) treatment with alkaline phosphatase (solvent system butanol/ethanol/water); (b) cell extract incubated with maltose (lane 3) and trehalose (lane 4) (solvent system butanol/pyridine/water); (c) cell extract incubated with maltopentaose (G5) for 0–8 h (lanes 5 and 6) and maltoheptaose (G7) (lanes 7 and 8) (solvent system butanol/pyridine/water). M1, M2 and M3 are standards; G, glucose; T, trehalose; M, maltose; G2–G7, maltooligosaccharides.

Fig. 2. Genomic organization and flanking regions of the *otsA-otsB* operon-like structure (a), and the *treS* gene (b). *cbiB*, gene encoding cobinamide synthase; *α-am*, gene encoding α-amylase; *orf1*, gene encoding a hypothetical protein. The nucleotide sequences identified in this work are indicated in grey. The sequence of 732 bp upstream of *otsB* was also obtained.

this purpose, the *otsA*, *otsB* and *treS* genes were separately cloned and overexpressed in *E. coli*, and the respective proteins were purified.

Trehalose-6-phosphate synthase. The recombinant OtsA showed an absolute specificity for glucose 6-phosphate as a glucosyl acceptor. In contrast, the enzyme used ADP-glucose, UDP-glucose, GDP-glucose or TDP-glucose as glucosyl donor substrates. OtsA was not strictly dependent on Mg²⁺, but the presence of 10 mM Mg²⁺ was required for maximal activity. Co²⁺ had no effect on the activity, while other divalent cations had an inhibitory effect (Table 1). An increase in the concentration of NaCl from 0 to 100 mM led to a decrease in the OtsA activity by more than 35 % when ADP-glucose or TDP-glucose was the substrate. In contrast, when we used UDP-glucose or GDP-glucose, the OtsA activity was increased by 10–30 % (Table 1). Upon an increase in the KCl concentration from 0 to 100 mM, the OtsA activity decreased by more than 40 %, whereas it was not significantly affected

when UDP or GDP-glucose was used as the substrate (Table 1). A strong activation of OtsA (62 %) was observed with heparin when UDP-glucose was the substrate, but no significant activation was observed with the other glucosyl donors examined (data not shown).

The maximum activity of OtsA was detected at pH 5.5 for GDP-glucose, and pH 5.25 for ADP-glucose, UDP-glucose and TDP-glucose (Fig. 3). In this range of pH, maximal specific activity of OtsA was determined with TDP-glucose (30.0 μmol min⁻¹ mg⁻¹), followed by UDP-glucose (16.8 μmol min⁻¹ mg⁻¹), ADP-glucose (14.2 μmol min⁻¹ mg⁻¹) and GDP-glucose (13.6 μmol min⁻¹ mg⁻¹). The temperature profiles for activity were very similar for the four glucosyl donors, and maximal activity was observed at 45 °C (data not shown). A typical Michaelis–Menten behaviour was observed for all the NDP-glucose substrates. OtsA had a higher affinity for UDP-glucose (*K_m* value of 0.3 mM), but the maximal specific activity was reached when TDP-glucose was used (Table 1).

Table 1. Kinetic parameters and biochemical properties of recombinant OtsA, OtsB and TreS from *P. freudenreichii*

Activities in the presence of divalent cations, and NaCl and KCl, are expressed as percentages of the maximum activity; the values are the means of at least two independent measurements, and the deviation was not greater than 6 %. Tre6P, Trehalose 6-phosphate; glc, glucose; G6P, glucose 6-phosphate.

Kinetic parameter/biochemical property	Concn (mM)	OtsA				OtsB Tre6P	TreS	
		UDP-glc	ADP-glc	GDP-glc	TDP-glc		Maltose	Trehalose
<i>K_m</i> (mM)	–	0.3 ± 0.05	0.6 ± 0.15	0.4 ± 0.1	2.0 ± 0.4	0.7 ± 0.1	6.4 ± 0.3	58.9 ± 3.0
<i>K_m</i> (mM) for G6P	–	3.0 ± 0.4	4.2 ± 0.7	7.1 ± 1.6	5.3 ± 0.8	–	–	–
<i>V_{max}</i> [U (mg protein) ⁻¹]	–	16.8 ± 0.7	14.2 ± 0.8	13.6 ± 0.8	30.0 ± 1.2	133.1 ± 4.1	47.9 ± 0.8	157.0 ± 2.7
Mg ²⁺	0	84	85	84	83	3	100	–
Mg ²⁺	10	100	100	100	100	100	88	–
Mn ²⁺	10	54	69	63	58	5	90	–
Ca ²⁺	10	54	63	51	41	0	100	–
Co ²⁺	10	83	81	73	78	4	77	–
Cu ²⁺	10	0	0	0	1	0	0	–
NaCl*	100	128	65	110	59	93	89	–
KCl*	100	99	56	90	45	–	–	–

*The enzymic assay contained 10 mM Mg²⁺.

Table 2. Amino acid sequence identity of OtsA, OtsB and TreS from *P. freudenreichii* (Pf) compared with known protein counterparts

The GenPept nos for OtsA, OtsB and TreS, respectively, are: YP_055672, YP_055673 and YP_055819 for *P. acnes*; NP_218007, NP_217889 and NP_214640 for *M. tuberculosis*; NP_601825, NP_601827 and NP_601502 for *C. glutamicum*; NP_825113, NP_825114 and NP_823979 for *Streptomyces avermitilis*; NP_302467 (OtsA) and NP_301392 (OtsB) for *Mycobacterium lepra*; NP_959507 (OtsA) and NP_962412 (OtsB) for *Mycobacterium avium* subsp. *paratuberculosis*; ZP_00659535 (OtsA) and ZP_00659540 (OtsB) for *Nocardioide* sp. JS614 and NP_739118 (OtsA) and NP_739120 (OtsB) for *Corynebacterium efficiens*.

Organism	Sequence identity (%)		
	Pf OtsA	Pf OtsB	Pf TreS
<i>P. acnes</i>	60	37	65
<i>Nocardioide</i> sp. JS614	56	40	–
<i>M. avium</i> subsp. <i>paratuberculosis</i>	53	20	–
<i>M. leprae</i>	52	31	–
<i>C. efficiens</i>	51	23	–
<i>S. avermitilis</i>	33	42	58
<i>M. tuberculosis</i>	52	32	60
<i>C. glutamicum</i>	48	23	58

Trehalose-6-phosphate phosphatase. The recombinant OtsB showed absolute substrate specificity for trehalose 6-phosphate. OtsB activity was strictly dependent on Mg^{2+} , and 50 % of the maximum activity was reached with 0.1 mM Mg^{2+} ; the activity was abolished by addition of EDTA (10 mM). The phosphatase activity was very low, or not detected, when Mg^{2+} was replaced by Mn^{2+} , Ca^{2+} , Co^{2+} or Cu^{2+} (Table 1). NaCl had a slightly negative effect on OtsB activity in the range of 0–250 mM, but was clearly inhibitory (over 30 %) at higher concentrations. The activity was strongly inhibited (70 %) by 10 mM NaF, a well-known phosphatase inhibitor. OtsB had maximal activity at 50 °C and pH 6.5 (Fig. 3). A hyperbolic dependence of the activity on the concentration of trehalose 6-phosphate was found. The kinetic parameters for OtsB are shown in Table 1.

Trehalose synthase. The presence of Ca^{2+} , Mg^{2+} , Mn^{2+} or Co^{2+} did not affect, or only slightly inhibited, TreS activity, while Cu^{2+} had a strong inhibitory effect (Table 1). Furthermore, EDTA (10 mM) or NaCl (100 mM) did not affect TreS activity, whereas Tris/HCl (10 mM) had an inhibitory effect of 64 %. The temperature profile of TreS activity was investigated in the range of 30–60 °C using maltose as the substrate; maximal activity was observed at 45 °C, and the optimal pH was 5.2 (Fig. 3). The K_m for maltose was 6.4 mM, which was nine times lower than that for trehalose (Table 1).

Fig. 3. Effect of pH on the activity of OtsA, OtsB and TreS. The OtsA activity was determined with TDP-glucose and glucose 6-phosphate as substrates; the activity of TreS was determined using maltose. Error bars indicate SD.

Modulation of the substrate specificity of trehalose-6-phosphate synthase by component(s) of the cell extract

OtsA activity in *P. freudenreichii* extracts was observed only when ADP-glucose was the glucosyl donor substrate, whereas recombinant OtsA showed activity with ADP-, GDP-, UDP- and TDP-glucose. To investigate this discrepant behaviour, the native OtsA was partially purified. After the third purification step, the initial specificity of OtsA for ADP-glucose was lost (Fig. 4). Surprisingly, the recombinant protein became specific for ADP-glucose upon incubation with a dialysed *P. freudenreichii* extract (Fig. 4). It appeared that the cell extract contained one or more components that interacted with the protein, and induced a

Fig. 4. Differential substrate specificity of OtsA in cell extracts and purified preparations. TLC analysis of reaction products: (a) native OtsA in a dialysed cell extract, and (b) partially purified OtsA with different glucosyl donors; and recombinant OtsA without (c) and with (d) addition of a dialysed extract of *P. freudenreichii*. Details of the reaction mixtures and incubations are described in Methods.

clear change in the substrate specificity of OtsA. To check whether OtsB could be responsible for this effect, we examined the substrate specificity of recombinant OtsA in the presence of pure recombinant OtsB (OtsA : OtsB, molar ratio 1.8 : 1). The substrate preference of OtsA was not affected by the presence of OtsB (data not shown).

Levels of OtsA and TreS in response to acid, oxidative and osmotic stress

To obtain insight into the physiological role of the two pathways identified in *P. freudenreichii* for trehalose metabolism, immunoblotting assays were used to assess the levels of OtsA and TreS during acid stress (pH downshift from 7 to 5), oxidative stress (oxygen concentration 0.115 mM) and osmotic stress [increase of NaCl in the medium from 0 to 2 % (w/v)]. Stress submission started at a time designated zero. Constitutive levels of OtsA and TreS were detected under optimal growth conditions (Fig. 5). The amount of OtsA increased approximately twofold when any of the three stresses were imposed from 2 to 10 h. In contrast, TreS decreased to about one-quarter of the initial level (acid or oxidative stress, from 0 to 5 h), or did not change significantly (osmotic stress) (Fig. 5). The effect of these stresses on the intracellular level of trehalose was assessed in the same experiments. Trehalose content increased notably (around sevenfold), from a basal level of 30 mg trehalose (g protein)⁻¹ to approximately 200 mg trehalose (g protein)⁻¹ during the initial 5 h period in response to oxidative stress and osmotic stress. In cells subjected to acid stress, the level of trehalose increased 2.3-fold only, to around 70 mg trehalose (g protein)⁻¹ (data not shown).

DISCUSSION

In the present work, the pathways for trehalose metabolism in *P. freudenreichii* were characterized, and their roles in response to osmotic, oxidative and acid stress were examined. As the genome sequence of this organism is not available (*P. acnes* is the only species of the genus *Propionibacterium* whose genome sequence is known),

relevant activities were sought in cell extracts, and the respective genes obtained using degenerate primers and inverse PCR. Two pathways for trehalose metabolism were found in *P. freudenreichii*: the OtsA–OtsB pathway, used for

Fig. 5. Immunoblotting assays of OtsA and TreS in *P. freudenreichii* cells subjected to oxidative, osmotic and acid stress. Cells grown at optimal temperature and pH were subjected to oxidative stress (oxygen concentration 0.115 mM), osmotic stress [2 % (w/v) NaCl] and acid stress (pH shift to 5). Cell samples were withdrawn at the times indicated, and the levels of OtsA and TreS were assessed by immunoblotting. Time zero corresponds to cells immediately before stress imposition. Each lane contains 5 µg total protein. Each experiment was done at least in triplicate with samples from a single growth for each condition.

Fig. 6. Proposed scheme of trehalose metabolism in *P. freudenreichii*. 1, Glucokinase; 2, trehalose-6-phosphate synthase; 3, trehalose-6-phosphate phosphatase; 4, trehalase synthase; 5, amylomaltase; PolyP, polyphosphate; G, glucose.

that accumulate high levels of this disaccharide as a compatible solute or carbon reserve.

BLAST searches of the protein databases with the sequences of *P. freudenreichii* TreS and OtsA revealed an enormous disparity in the number of hits for the two enzymes, highlighting the frequent occurrence of OtsA, and the scarce distribution of TreS. The common OtsA–OtsB pathway is generally assumed to be involved in the synthesis of trehalose, but, for example, in *C. glutamicum* it has been shown that the mutant with a deletion of *otsA* accumulates levels of trehalose comparable with those of the wild-type under identical osmotic stress conditions, with the synthesis proceeding via the TreY–TreZ pathway (Wolf *et al.*, 2003). In *R. sphaeroides*, the OtsA–OtsB pathway is the major route for trehalose synthesis under osmotic stress, and the second

pathway, TreY–TreZ, contributes to a minor extent (Makihara *et al.*, 2005).

The regulation of the multiple pathways in response to stress other than osmotic stress has not been reported, but herein we show that the enhancement of the OtsA–OtsB pathway in *P. freudenreichii* is a response common to all the three stresses imposed. Trehalose accumulation was stimulated in response to osmotic, oxidative and acid stress, suggesting a role of this solute in cell protection against these diverse stresses. Trehalose is a canonical osmolyte, accumulating to high levels to preserve cell turgor in environments of elevated osmolarity (Argüelles, 2000; Elbein *et al.*, 2003). Recent studies, however, have demonstrated the ability of this disaccharide to protect cells against other types of aggressions, namely oxidative stress and heat stress. The role of trehalose as a scavenger of oxygen radicals has been shown in *S. cerevisiae* (Benaroudj *et al.*, 2001). On the other hand, the accumulation of trehalose in response to elevated temperature has been reported in several mesophilic organisms, such as yeast, *E. coli* and *Salmonella enterica* (Strom & Kaasen, 1993; De Virgilio *et al.*, 1994; Cánovas *et al.*, 2001). Protection against acid stress has not been reported; however, it is interesting that genes for the synthesis of trehalose are present in the genome of *Picrophilus torridus*, an extreme acidophile with optimal pH for growth of 0.7 (Fütterer *et al.*, 2004). It is conceivable that trehalose plays a role in the stabilization of cell membranes, which must withstand a strong pH gradient in such acidic environments.

Herein, a global picture of trehalose metabolism in *P. freudenreichii* was obtained regarding adaptation to osmotic, oxidative and acid stress. Under all types of stress examined, the expression of the OtsA–OtsB pathway for trehalose synthesis was clearly enhanced. The experimental data presented here strongly suggest the involvement of the TreS pathway in trehalose catabolism; final proof, however, was not obtained due to lack of mutants. These findings represent an important contribution to our understanding of stress responses in propionibacteria with respect to trehalose synthesis, and this knowledge is essential to the design of dairy strains optimized for the *in situ* production of trehalose, an efficient flavour preservative of fermented foods. However, further progress in this area of research must await the development of genetic tools for the manipulation of *P. freudenreichii*.

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