Engineering trehalose synthesis in *Lactococcus lactis* for improved stress tolerance

Ana Lúcia Carvalho\(^{1}\), Filipa S. Cardoso\(^{1}\), Andreas Bohn\(^{1}\), Ana Rute Neves\(^{1}\), and Helena Santos\(^{1*}\)

\(^{1}\)Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Av. da República – EAN, 2780-157 Oeiras, Portugal.

**Running title**: Engineering trehalose synthesis in *Lactococcus lactis*

*Corresponding author*: Helena Santos

E-Mail: santos@itqb.unl.pt; Tel. +351-214469541; Fax. +351-214469543.

Copyright © 2011, American Society for Microbiology and/or the Listed Authors/Institutions. All Rights Reserved.
Abstract

Trehalose accumulation is a common cell defense strategy against a variety of stressful conditions. In particular, our team detected high levels of trehalose in Propionibacterium freudenreichii in response to acid stress, a result that led to the idea that endowing Lactococcus lactis with the capacity to synthesize trehalose could improve the acid tolerance of this organism. To this end, we took advantage of the endogenous genes involved in the trehalose catabolic pathway of L. lactis, i.e., trePP and pgmB encoding, respectively, trehalose 6-phosphate phosphorylase and β-phosphoglucomutase, which enabled the synthesis of trehalose 6-phosphate. Given that L. lactis lacks trehalose 6-phosphate phosphatase, the respective gene, otsB, from the food-grade organism, P. freudenreichii, was used to provide the required activity. The trehalose yield was approx. 15% in resting cells and in mid-exponential cells grown without pH control. The intracellular concentration of trehalose reached maximal values of approximately 170 mM, but at least 67% of the trehalose produced was found in the growth medium. The viability of mutant and control strains was examined after exposure to heat, cold or acid shock, and freeze-drying. The trehalose-producing strains showed improved tolerance (5 to 10-fold higher survivability) to acid (pH 3) and cold shock (4 ºC); there was also a strong improvement in cell survival to heat shock (45 ºC) and no protection was rendered against dehydration. The insight provided by this work may help the design of food-grade strains optimized for the dairy industry as well as for oral drug delivery.
INTRODUCTION

*Lactococcus lactis* is a mesophilic homofermentative lactic acid bacterium used worldwide as a starter culture in food fermentations. In the dairy industry its primary function is the conversion of lactose to lactic acid, which provides an effective preservation of the fermented product. In addition, this organism contributes to the organoleptic and nutritional properties of the fermented foods. A wealth of information on lactococcal physiology has accumulated during the last decades and a battery of tools for genetic manipulation is now available (reviewed in 14, 31). Hence, it is not surprising that the potential of this microorganism as a cell factory for the production of flavours, texturizers, and nutraceuticals has been explored to a great extent (27). *L. lactis* is also an excellent host for the production of heterologous proteins (37). Indeed, the ease of gene expression combined with the GRAS (generally regarded as safe) status of *L. lactis* prompted the use of this bacterium as a live vehicle for delivery of antigens (live vaccines) or therapeutic proteins to mucosal surfaces (4, 60).

The use of *L. lactis* strains in starter cultures depends on functional properties (flavour and texture development) as well as growth performance and robustness. *L. lactis* grows optimally at pH values in the range 6.3 to 6.9, but as a consequence of its metabolic activity, lactic acid accumulates, causing acidification of the growth medium, and ultimately growth arrest at pH around 4.3 (56). Acid stress has detrimental effects on the physiology of *L. lactis*, including cell membrane damage and inhibition of metabolic reactions (35). Moreover, the usefulness of *L. lactis* as a live vehicle for oral delivery of pharmaceuticals depends to a large extent on the ability of cells to endure the harsh acidic conditions in the upper gastrointestinal tract. Furthermore, during culture handling, storage and product processing, lactic acid bacteria have to cope with dehydration (freeze-drying), elevated temperatures (≥ 41 ºC, e.g., in cheese processing), cold stress (2-6 ºC), among other stresses (56). Although lactococcal
growth occurs in the range 10 °C to 40 °C, beyond these limits cell viability is severely affected (61). In this context, it is clear that a good performance in clinical and industrial applications largely depends on the ability of \textit{L. lactis} to withstand various stresses, and in particular acid stress.

Trehalose is a non-reducing disaccharide ubiquitously distributed in nature, well-known for its role in protecting cells against a variety of stresses (3, 28, 30, 49). Our team observed a substantial increase in the intracellular content of trehalose in \textit{Propionibacterium freudenreichii} in response to acid stress (9). Inspired by this observation we anticipated that accumulation of trehalose could be a good strategy to improve \textit{L. lactis} survival against acid stress. Therefore, we set out to engineer trehalose production in \textit{L. lactis} by de novo introduction of the \textit{P. freudenreichii} trehalose biosynthetic pathway. In this organism trehalose is synthesized in two steps via the TpS-TpP pathway, the most widely used route for the synthesis of this disaccharide (8). Firstly, glucose is transferred from NDP-glucose to glucose 6-phosphate to yield trehalose 6-phosphate (Tre6P), in a reaction catalyzed by Tre6P synthase (TPS); subsequently, Tre6P is dephosphorylated to trehalose by the action of TPP, \textit{i.e.}, Tre6P phosphatase (8). Unfortunately, despite several attempts, functional expression in \textit{L. lactis} of the gene encoding \textit{P. freudenreichii} TPS was not achieved (10). Concurrently, while characterizing glucose metabolism in an \textit{L. lactis} CcpA (carbon catabolite protein A) deletion mutant we observed a transient accumulation of Tre6P. In view of previous work on trehalose catabolism in \textit{L. lactis} (2), we concluded that the synthesis of Tre6P occurred via the action of the Tre6P phosphorylase (TrePP) and \(\beta\)-phosphoglucomutase (\(\beta\)-PGM), which catalyze reversible steps (Fig. 1). Based on these results, we envisaged to produce trehalose in \textit{L. lactis} using genes exclusively from food grade organisms: overexpression of the endogenous \textit{trePP} and \textit{pgmB} genes in addition to \textit{otsB} from the dairy \textit{P. freudenreichii} (Fig. 1). Meanwhile, a report appeared in the literature on the production of trehalose by a \textit{L. lactis}
construct overexpressing the *Escherichia coli* *otsBA* operon (53); therefore, this strain was also constructed and used for comparison.

Herein we report the outcome of engineering *L. lactis* for trehalose synthesis. The intracellular and extracellular trehalose content was assessed in the engineered strains; moreover, their ability to survive stress associated with acid, cold, heat and dehydration was examined.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** Strains and plasmids used in this study are listed in Table 1. For molecular biology procedures, *L. lactis* strains were grown as batch cultures (flasks) in static conditions on M17 (Difco™) with 0.5% (w/v) glucose at 30 ºC. *Propionibacterium freudenreichii* was grown as described by Cardoso et al. 2004 (9). *E. coli* was grown aerobically at 37 ºC in LB medium. For physiological studies, *L. lactis* strains were grown in chemically defined medium (CDM) (44) with 1% glucose at 30 ºC in static conditions and without pH control (initial pH 6.5) or in a 2-liter fermenter with pH controlled at 6.5. In the fermenter pH was kept constant by automatic addition of NaOH. Plasmid selection was achieved by addition of chloramphenicol at a final concentration of 5 mg l⁻¹. Nisin (2 µg l⁻¹) was added when the optical density at 600 nm (OD₆₀₀) reached approximately 0.4. Growth was monitored by measuring the OD₆₀₀. Specific growth rates (µ) were calculated through linear regressions of the plots of ln(OD₆₀₀) versus time during the exponential growth phase.

**DNA techniques.** General molecular techniques were performed essentially as described elsewhere (48). Chromosomal and plasmid DNA were isolated by the method of Johansen and Kibenic (29) and Birnboim (5), respectively. *L. lactis* was transformed with plasmid DNA by electroporation, as described by Holo and Nes (25). Restriction enzymes
and T4 DNA ligase were obtained from New England Biolabs (Ipswich, MA, USA); Pwo polymerase and Taq polymerase were obtained from Roche Applied Science (Mannheim, Germany); all were used according to the supplier’s instructions. Polymerase chain reactions were performed in a MyCycler™ thermal cycler (Bio-Rad, Hercules, CA, USA). The primers used are listed in Table S1 (supplementary material) and were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

Construction of strains and plasmids. Deletion of ccpA was performed in L. lactis NZ9000 using a two-step homologous recombination method as described by Zomer et al., (33, 65). The coding region otsB\textsuperscript{Pf} from P. freudenreichii B365 was amplified by PCR using primer pairs tpp1-fw/tpp1-rev. The 0.89 kb BamHI/EcoRI fragment was digested with the indicated enzymes and cloned into BamHI/EcoRI-digested pNZ8020, yielding construct pNZotsB. The resulting construct was transformed into L. lactis strain NZ9000\Delta ccpA. The adjacent lactococcal trePP and pgmB genes (trehalose operon) and the gene otsB from P. freudenreichii were cloned and overexpressed in L. lactis NZ9000 as follows: the coding region of trePP-pgmB was amplified by PCR using primer pairs trePPpgmB-fw/trePPpgmB-rev. The 3.14 kb SpeI/SacI fragment was digested with the indicated enzymes and cloned into SpeI/SacI-digested pNZ8020, yielding construct pNZ8020-trePPpgmB. The coding region of otsB was amplified by PCR using primer pairs tpp-fw/tpp-rev. The 0.89 kb SacI/XbaI fragment was digested with the indicated enzymes and cloned into SacI/XbaI-digested pNZ8020-trePPpgmB, yielding constructs pNZ8020-trePPpgmB\textsubscript{otsB}, hereafter denominated pNZtpo. The resulting construct was transformed into L. lactis strains NZ9000 and NZ9000\Delta ccpA. L. lactis MG1363 DNA and P. freudenreichii B365 DNA were used as template for PCR amplification of trePP-pgmB and otsB inserts, respectively.

The coding region otsBA from E. coli was amplified by PCR using primer pairs otsBA-fw/otsBA-rev. The 3.811 kb NcoI/XbaI fragment was digested with the indicated enzymes
and cloned into NcoI/XbaI-digested pNZ8048, yielding construct pNZotsBA. The resulting construct was transformed into *L. lactis* strain NZ9000. *E. coli* DH5α DNA was used as template for PCR amplification of *otsBA* insert. The primer sequences used in this work are listed in Table S1.

**Quantification of fermentation products during growth.** Culture samples (2 ml) were taken at different growth stages and centrifuged (2,000 g, 5 min, 4°C); the supernatant solutions were stored at -20°C until analyzed by high-performance liquid chromatography (HPLC). Glucose, trehalose, acetate, ethanol and lactate were quantified in a DIONEX apparatus equipped with a refractive index detector (Shodex RI-101, Showa Denko K.K., Oita, Japan) using an HPX-87H anion-exchange column (Bio-Rad Laboratories, Inc., Richmond, Calif.) at 60°C, with 5 mM H₂SO₄ as the elution fluid and a flow rate of 0.5 ml min⁻¹.

**Enzyme activity measurements.** Cells were harvested during exponential phase, washed twice and suspended in 50 mM MES buffer, pH 6.5. To measure β-PGM (EC 5.4.2.6) and TrePP (EC 2.4.1.216), cells were disrupted using 0.5 g glass beads (⌀ 50-105 µm, Fischer Scientific BV, Den Bosch, The Netherlands), using a Mini-BeadBeater-8 (Biospec products, Inc., Bartlesville, OK) with two 1-min pulses separated by 1-min of cooling down. TPP (EC 3.1.3.12) and TPS (EC 2.4.1.15) were assayed after mechanical disruption of the cell suspension by passage through a French press (twice at 120 MPa). After cell disruption, cell debris was pelleted and activities assayed at 30 °C. One unit of enzyme activity is the amount of enzyme catalyzing the conversion of 1 µmol of substrate per minute under the experimental conditions used. Protein concentrations were determined by the method of Bradford (6).

The activity of β-PGM was measured as described by Qian *et al.* (45). The assay mixture (1 ml) contained 50 mM potassium phosphate buffer (KP₂) pH 7, 0.5 mM MgCl₂,
1.75 U glucose 6-phosphate dehydrogenase, 0.5 mM NADP⁺, and 50 µM glucose 1,6-bisphosphate. Reactions were started by the addition of 1.5 mM β-glucose 1-phosphate.

The TrePP activity was measured according to Andersson et al. (2). The assay mixture (1 ml) contained 100 mM KP₄ buffer pH 7.0, 3.75 U glucose 6-phosphate dehydrogenase, and 0.8 mM NADP⁺. Tre6P (0.67 mM) was used to start the reaction.

The TPP activity was assayed in a reaction mixture containing MES buffer (50 mM, pH 6.5), and 10 mM MgCl₂. The reaction was initiated by the addition of 5 mM Tre6P, incubated at 30 ºC for different time periods, stopped by adding phosphate reagent (1 part of a 10% ascorbic acid solution and 6 parts of 0.42% ammonium molybdate in 1N H₂SO₄). The A₈₂₀ was proportional to the phosphate concentrations (1).

TPS activity was determined in a reaction mixture containing MES buffer (50 mM, pH 6.5), 10 mM MgCl₂, 15 mM glucose 6-phosphate, and 5% of ²H₂O. The reaction was initiated by adding 7.5 mM UDP-glucose and followed online at 30 ºC by ³¹P-NMR spectroscopy. Spectra were acquired on a Bruker AVANCE II 500 MHz spectrometer (Bruker BioSpin GmbH, Karlsruhe, Germany) with a selective 5-mm-diameter probe head (SEX-P) by employing a pulse width of 8.7 µs (flip angle, 75°) and a recycle delay of 2.3 s. Chemical shifts are referenced to the resonance of external 85% H₃PO₄, designated at 0 ppm.

Reverse transcription assays: semiquantitative RT-PCR. Strains NZ9000[pNZ8020], NZ9000[pNZotsBA] and NZ9000[pNZtpo] were grown as described above. Total RNA was isolated from cells at the mid-exponential phase of growth using the SV total RNA isolation system (Promega, Woods Hollow Road, MA, USA), with the following modifications: incubation with lysozyme (5 mg ml⁻¹, 20 min, 37 ºC) preceded the first step of the kit protocol, and an additional incubation step with the kit DNaseI (1.5 h, 23 ºC) was required to remove chromosomal DNA. Total RNA (1 µg), dNTPs (final concentration of 0.5 mM) and random oligonucleotides (12 µg ml⁻¹) (Invitrogen, Carlsbad,
CA, USA) were heated to 65 °C for 5 min and chilled on ice. Dithiothreitol (final concentration 5 mM), First-strand RT buffer and Superscript III (1/20; v/v) (Invitrogen, Carlsbad, CA, USA) were added and samples were incubated for 5 min at 25 °C, 60 min at 50 °C, and 15 min at 70 °C for enzyme inactivation. A parallel sample was treated in the same way, except for the addition of enzyme. cDNA was subsequently used 1/30 (v/v) in standard PCR reactions. To test for contamination of RNA with DNA, the RNA samples without reverse transcriptase were used as negative controls for all conditions tested. Chromosomal DNA from strains NZ9000[pNZ8020], NZ9000[pNZotsBA] and NZ9000[pNZtpo] were used as positive control for the PCR reaction. The following primer pairs were designed to amplify internal fragments of *dnaK* (dnaK_fwd/dnaK_rev), *groEL* (groEL_fwd/groEL_rev), *recA* (recA_fwd/recA_rev), *clpP* (clpP_fwd/clpP_rev) and *tufA* (tuf_fwd/tuf_rev). *L. lactis* *tufA*, a housekeeping gene coding for the elongation factor TU required for continued translation of mRNA, was used as control. RT-PCR was performed with RNA isolated from two or three independent cultures.

**Extraction and quantification of intra and extracellular trehalose during growth.**

*L. lactis* strains were grown as described above, harvested at the exponential phase of growth and rapidly pelleted by centrifugation (2,000 g, 5 min, 4 °C). The resulting supernatants were lyophilized, and the residues suspended in $^2$H$_2$O for further quantification of extracellular trehalose. Ethanol cell extracts for quantification of intracellular trehalose were prepared as described elsewhere (46). In brief, the cell pellets were suspended in 70% ethanol and extraction was performed for 30 min with vigorous agitation in an ice-bath. Debris was removed, ethanol was evaporated and the residue was freeze-dried. The dried extracts were dissolved in $^2$H$_2$O. Trehalose in supernatants or cell extracts was quantified by $^1$H-NMR spectroscopy. Formate was added as an internal concentration standard. $^1$H-spectra were acquired on a Bruker AVANCE II 500 MHz spectrometer (Bruker BioSpin GmbH,
Karlsruhe, Germany) with a broadband 5-mm-diameter probe head with reverse detection employing a pulse width of 8 µs (flip angle, 90°) and a recycle delay of 2.5 s. The water resonance was suppressed with a pre-saturation pulse.

**In vivo NMR studies with resting cells.** Cells were grown in CDM containing 1% glucose (w/v) and suspensions were prepared and made anaerobic as described elsewhere (39). In vivo NMR experiments were performed using an on-line system and glucose specifically labeled with $^{13}$C on carbon one (40 mM) as substrate (38, 39). In vivo $^{13}$C-NMR spectra were acquired at 125.77 MHz using a quadruple nuclei probe head at 30°C on a Bruker AVANCE II 500 MHz spectrometer (Bruker BioSpin GmbH, Karlsruhe, Germany) as described before (39). Lactate was quantified in the NMR-sample extract by $^1$H-NMR in a Bruker AMX300 (Bruker BioSpin GmbH). The concentration of other metabolites was determined in fully relaxed $^{13}$C spectra of the NMR-sample extracts as previously described (40). Each experiment was repeated at least twice and the results were highly reproducible.

**Quantification of trehalose 6-phosphate and trehalose in resting cells.** For quantification of intracellular and extracellular pools of trehalose and trehalose 6-phosphate cell suspensions were prepared as described for in vivo NMR studies (see above). After addition of [1-$^{13}$C]glucose (40 mM), 1 ml and 4 ml samples were taken independently for supernatants and total cell extracts, respectively, at different time points as follows: for strain NZ[pNZtpo] samples were taken after 2.5, 5, 10, 15, 20 and 40 min, whereas for strain NZ[pNZotsBA] samples were collected at 2.5, 5, 7.5, 10, 20 and 40 min after glucose addition. For quantification of extracellular trehalose, 1-ml samples were centrifuged (20 s, 4 °C, 13,000 g) and supernatants stored at -20 °C until further analysis. For the determination of total trehalose and intracellular trehalose 6-phosphate a cold solution of perchloric acid (final concentration 0.6M) was immediately added to the 4 ml samples. After stirring on ice for 20 min, the pH of the samples was adjusted to neutrality with 5 M KOH and centrifuged...
(20 min, 4 ºC, 30,000 g). The resulting cell extracts were used for quantification of trehalose and Tre6P. 13C-NMR spectra of supernatants and cell extracts were acquired with a 5 mm selective probe head using a pulse width corresponding to 70º flip angle and a recycle delay of 1.5 s. Correction factors to take into account incomplete relaxation of resonances were calculated by comparison with spectra acquired under fully relaxed conditions (recycle delay, 60.5 s). Chemical shifts are referenced to the resonance of methanol in a glass capillary, designated at 49.3 ppm.

Cell viability assays. L. lactis cells were grown in CDM at 30 ºC without pH control (initial pH 6.5) until exponential phase (OD600 about 1.3). After exposure to each stress, cell suspensions were adequately diluted in 50 mM KP, pH 6.5 and the serial dilutions plated in M17-agar (1.5%) supplemented with glucose 0.5% (w/v) and 5 mg l⁻¹ chloramphenicol, and incubated for approximately 36 h at 30 ºC for CFU counting. The viability was calculated as the ratio of CFU mg prot⁻¹ of the sample exposed to stress for a given time period, over the value determined at time 0. Values are averages of four to seven independent experiments and are given as percentage.

Acid shock. Cultures (1 ml) were centrifuged (5 min, 25 ºC, 2,000 g) and suspended in the same volume of 50 mM KP, pH 3 (acidified with HCl) or pH 6.5 (control condition). Suspensions were incubated for defined time intervals (0, 10, 20, 30 min) at 30 ºC, rapidly centrifuged (0.5 min, 25 ºC, 2000 g) and suspended in 50 mM KP, pH 6.5 prior to plating.

Freeze-drying. Cell samples (1 ml) were quickly frozen in liquid nitrogen and subsequently freeze-dried for 24 h. After lyophilization, the dried cells were reconstituted in 50 mM KP, pH 6.5 and plated as described above.

Cold stress. Cell cultures (40 ml) were placed in an ice bath during 5 min for rapid cooling down. Viability was assessed after 0, 1, 4, 8 and 14 days at 4 ºC. Day 0 (control condition) corresponds to cells plated immediately after the cooling down step.
Heat shock. Cell samples (1 ml) were transferred to a water bath at 45 °C and incubated for different periods of time (0, 10, and 30 min).

**Statistical analysis.** Statistical analyses of cell viability were performed using the R Language for Statistical Computing, Version 2.10.1 (R Development Core Team 2009). Prior to subjecting the data to 2-way ANOVA for factors *Time* and *Strain*, we applied the Levene test for equality of variances. A rank transformation was applied in the case of inequality. For all stress conditions, a significant interaction between both factors was observed (p=0.018 or lower for all stresses), indicating that the temporal development of viability was significantly different for each strain. We therefore proceeded with multiple testing for differences in the mean viabilities against the level at time 0 (Viability = 100%), as well as the mean viabilities between the strains, using the Welch t-test and the Holm correction for multiple testing.

**RESULTS**

**Detection of trehalose 6-phosphate accumulation in *L. lactis* ΔccpA.** *L. lactis* subsp *cremoris* NZ9000 can not synthesize trehalose, but it possesses the enzymatic machinery to catabolize this disaccharide (2). The trehalose catabolic genes, *llmg*-*0453, llmg*-*0454, trePP* and *pgmB* encoding the trehalose-PTS PTS<sup>Tre</sup>, Tre6P phosphorylase, and β-phosphoglucomutase, are under negative control of the carbon catabolite protein A (CcpA) (59, 65). Curiously, an *in vivo* NMR study of glucose metabolism in resting cells of strain NZ9000ΔccpA revealed the transient accumulation of Tre6P (Fig. 2A). Based on these data we designed the synthesis of trehalose in *L. lactis* by expressing an exogenous Tre6P phosphatase in the *ccpA* mutant. The *otsB* gene from *P. freudenreichii* (*otsB<sup>Pf</sup>) was cloned under a P<sub>nisa</sub> promoter, and the resulting plasmid pNZotsB introduced in NZ9000ΔccpA.

We resorted to <sup>13</sup>C-NMR analysis of cell extracts obtained during the metabolism of [1-<sup>13</sup>C]glucose by resting cells to monitor trehalose and Tre6P. A resonance at 93.70 ppm
assigned to the C1/C1′ atoms of trehalose was detected in \( ^{13}\text{C} \)-spectra of strain NZ9000∆ccpA[pNZotsB]; in contrast, in spectra of strain NZ9000∆ccpA the resonances (at 93.75 and 93.85 ppm), due to the two anomic carbon atoms of Tre6P were observed, while the resonance due to trehalose was absent (Fig. 2B).

**L. lactis strains engineered for trehalose synthesis.** Capitalizing on our previous data, we devised a strategy to obtain an *L. lactis* trehalose producer using genes only from GRAS organisms by over-expressing simultaneously the lactococcal *trePP* and *pgmB* together with *otsB\(^{PF}\) from *P. freudenreichii*. Genes *trePP* and *pgmB* were cloned under *PnisA* promoter in pNZ8020, originating plasmid pNZ8020-trePPpgmB. Cloning of *otsB\(^{PF}\)* downstream of *pgmB* rendered plasmid pNZtpo in the host strain NZ9000, herein denominated NZ9000[pNZtpo]; pNZtpo was subsequently transformed into NZ9000∆ccpA. While this work was in progress, Termont *et al.* (53) reported the production of trehalose in an engineered *L. lactis* strain overexpressing the *otsBA* operon (TPP\(^{EC}\) and TPS\(^{EC}\)) from *E. coli*. Therefore, we decided to construct a similar strain and compare the two constructs. The *E. coli* *otsBA* genes were cloned under the control of *PnisA*, resulting in plasmid pNZotsBA, yielding strain NZ9000[pNZotsBA]. The NZ9000 strain harboring the pNZ8020, NZ9000[pNZ8020], was used as control.

To evaluate the functional expression of the cloned gene products, cell-free extracts were obtained from mid-exponential phase of nisin-induced cultures (2 µg l \(^{-1}\)) and the relevant activities assayd. In NZ9000[pNZtpo], activity of TrePP was 0.30±0.01 µmol min\(^{-1}\) mg protein\(^{-1}\), 298-fold higher than in the control strain NZ9000[pNZ8020], β-PGM was 22-times overexpressed, showing a specific activity of 1.11±0.19 µmol min\(^{-1}\) mg protein\(^{-1}\) (about 0.05 µmol min\(^{-1}\) mg protein\(^{-1}\) in the control) and the activity of the heterologous TPP\(^{PF}\) was 0.027±0.001 µmol min\(^{-1}\) mg protein\(^{-1}\). In strain NZ9000[pNZotsBA], the heterologous activity of TPP\(^{EC}\) was 0.26 µmol min\(^{-1}\) mg protein\(^{-1}\) and the TPS activity was about tenfold
lower. A similar activity profile has been previously reported for the expression of the *E. coli* *otsBA* genes in *Corynebacterium glutamicum* (42).

**Trehalose produced by engineered strains grown with pH control.** All strains were grown in CDM supplemented with 1% glucose and pH controlled at 6.5 under anaerobic conditions and induced with nisin (2 µg l⁻¹) at OD₆₀₀ of 0.5. During mid-exponential (OD₆₀₀ of 2.2) and stationary (OD₆₀₀ approx. 5) phases of growth, samples were collected and rapidly centrifuged to remove the growth medium. The cell pellets were subjected to ethanol extraction for quantification of intracellular trehalose, while extracellular trehalose was measured in the supernatant solutions (growth medium) (Table 2).

The three engineered strains excreted trehalose to the medium. In strain NZ9000ΔccpA[pNZtpo] the level of intracellular and extracellular trehalose decreased by about 30- and 3-fold, respectively, from mid-exponential to stationary phase of growth (Table 2). These results show that this strain utilizes efficiently the produced trehalose, most likely due to derepression of the trehalose operon triggered by *ccpA* deletion. In view of this non-desired feature, strain NZ9000ΔccpA[pNZtpo] was not considered further in this study.

In contrast, in strains NZ9000[pNZtpo] and NZ9000[pNZotsBA] the levels of extracellular trehalose were higher during the stationary phase and intracellular trehalose was only slightly reduced.

**Growth profiles of recombinant and control strains without pH control.** Strains NZ9000[pNZtpo], NZ9000[pNZotsBA] and control (NZ9000[pNZ8020]) were grown in CDM without pH control (initial pH 6.5) and induced with 2 µg l⁻¹ nisin at OD₆₀₀ of 0.4 (Fig. 3). Maximal biomass was identical for all strains, but the specific growth rate of NZ9000[pNZtpo] was only 70% of that of the control strain, while NZ9000[pNZotsBA] exhibited a growth profile identical to that of the control (Fig. 3).
Despite trehalose production, the major end-product of NZ9000[pNZtpo] was lactate accounting for 83.5% of the glucose consumed and a similar fermentation pattern was observed for strain NZ9000[pNZotsBA]. As expected, the control strain was fully homolactic and unable to produce trehalose (Table 3).

In the mid-exponential phase of growth (OD_{600} 1.3) the concentration of trehalose inside the cells was determined by NMR as described in Materials and Methods (Table 3). Strain NZ9000[pNZtpo] accumulated 150±7 mM trehalose, while NZ9000[pNZotsBA] accumulated 92±2 mM. Taking into consideration the total amount of trehalose produced (intracellular plus extracellular trehalose), we estimated that 15.8±1.4% of glucose supplied was converted to trehalose in NZ9000[pNZtpo], whereas only 8.5±0.2% of glucose was directed to trehalose production in NZ9000[pNZotsBA] (Table 4).

Dynamics of trehalose and trehalose 6-phosphate in resting cells of engineered strains. The metabolism of [1-{\textsuperscript{13}}C]glucose (40 mM) in strains NZ9000[pNZtpo] and NZ9000[pNZotsBA] was studied by \textit{in vivo} \textsuperscript{13}C-NMR in suspensions of non-growing cells under an argon atmosphere and at a constant pH of 6.5 (Fig. 4). Maximal glucose consumption rates of 0.33±0.01 and 0.39±0.01 \(\mu\)mol min\(^{-1}\) mg protein\(^{-1}\) were determined for strains NZ9000[pNZtpo] and NZ9000[pNZotsBA], respectively. These values should be compared with 0.37±0.01 \(\mu\)mol min\(^{-1}\) mg protein\(^{-1}\) determined for the control strain (not shown). Both recombinant strains produced as major end-product lactate, which accounted for approx. 83% of the supplied glucose. In the engineered strains, the profile of accumulation of fructose 1,6-bisphosphate (FBP) resembled that of wild-type and control strains (38, 47); in brief, FBP accumulated transiently and started to decline at the onset of glucose depletion (Fig. 4A and 4B). Trehalose was detected immediately after glucose addition; in strain NZ9000[pNZtpo] the build-up of total trehalose plus Tre6P was very fast during the first two minutes, and continued at a slower rate reaching a maximal level of 83
mM (calculated on the basis that all trehalose was inside the cells). Therefore, approx. 15% of the glucose supplied was channeled to trehalose synthesis. Once glucose was exhausted, trehalose was consumed at a low rate \((0.04 \mu\text{mol min}^{-1} \text{ mg protein}^{-1})\). In strain NZ9000[pNZotsBA] trehalose increased steadily while glucose was available, leveling off at 68 mM (calculated as though total trehalose was intracellular). In this strain 10.7% of the glucose supplied was directed to trehalose synthesis. It is impossible to distinguish between the NMR signals of trehalose and Tre6P in spectra of living cells due to extensive line broadening and overlapping. Moreover, extracellular and intracellular trehalose can not be distinguished by NMR. To quantify intra and extracellular trehalose and discriminate between trehalose and Tre6P we analyzed by \(^{13}\text{C-NMR} \) the cell extracts and supernatant solutions derived from samples taken during the metabolism of glucose (Fig. 4C and D). Trehalose 6-phosphate was detected in strain NZ9000[pNZtpo] (up to 4 mM), but not in strain NZ9000[pNZotsBA]. In strain NZ9000[pNZtpo] the concentration of trehalose increased concomitantly with glucose consumption, in both intra and extracellular compartments. Upon glucose depletion the concentration of intracellular trehalose decreased while the extracellular trehalose moderately increased. It is curious that, while considerable amounts of glucose 6-phosphate (G6P) accumulated in strain NZ9000[pNZotsBA] (similar to wild-type strains, 38), only traces were detected in NZ9000[pNZtpo] (not shown). The lack of accumulation of the intermediate metabolite G6P is probably related with the lower glycolytic flux in this strain (Fig. 4A).

Data on the percentage of trehalose that was secreted to the external medium as well as the trehalose yield are summarized in Table 4 for the various experimental conditions examined.

**Synthesis of trehalose improves acid tolerance of *L. lactis*.** The survival of engineered and control strains when exposed to pH 3 for time periods up to 30 min was...
evaluated. To perform these tests, cell suspensions were harvested, centrifuged and the supernatants discarded to remove extracellular trehalose. Cells were then suspended in 50 mM KP₄ acidified with HCl to pH 3 or in 50 mM KP₄ at pH 6.5 (reference condition). After incubation, cells were quickly centrifuged to remove acid and suspended in buffer with optimal pH (KP₄ at pH 6.5). For each strain examined, the survival of cells in KP₄ at pH 6.5 (reference condition) was similar for all the incubation times examined. The number of viable cells per mg of protein was $2.83\pm 0.81 \times 10^{10}$ for the control strain, $3.14\pm 0.42 \times 10^{8}$ for NZ9000[pNZtpo], and $2.50\pm 0.55 \times 10^{9}$ for NZ9000[pNZotsBA]. In contrast, exposure to pH 3.0 reduced considerably the viability of the control strain in a time-dependent manner (Fig. 5). Noteworthy is the fast decay (about 72% reduction, $p=0.012$) within the first 10 min. Conversely, the trehalose producer NZ9000[pNZtpo] showed no significant loss of viability ($p=0.296$) during the first 20 min of exposure to pH 3.0. Only after 30 min exposure a significant decrease of about 45% in survival ($p=0.011$) was observed. However, this survival rate (~55%) was significantly higher ($p=0.002$) than that of the control strain (~8%). The performance of strain NZ9000[pNZotsBA] at low pH was comparable to that of NZ9000[pNZtpo] as differences were not significant at any time point ($p > 0.5$ for all times).

**Trehalose protects *L. lactis* against cold shock.** The experimental design consisted in rapidly transferring and incubating the cultures on ice for 5 min; subsequently the cultures were moved to a chamber at 4 °C. One day at 4 °C sufficed to significantly reduce the viability of the control strain to survival values around 12% ($p=0.022$, Fig. 5). In contrast, the viability of strain NZ9000[pNZtpo] after eight days at 4 °C was approx. 80% and was significantly reduced ($p=0.001$) only after 14 days at this temperature. Strain NZ9000[pNZotsBA] showed apparently higher survival rates than NZ9000[pNZtpo] upon cold exposure, but the differences were not significant ($p=0.146$ or higher for all times).
Trehalose confers tolerance to heat shock in *L. lactis*. In this study, the viability of strain NZ9000[pNZtpo] was not significantly affected after 10 or 30 min incubation at 45 °C (p=0.495 and p=0.307, respectively), whereas the percentage of survival of the control strain significantly decreased to 40% after 30 min at 45 °C (p=0.033, Fig. 5). Although no significant differences were found between the survival of strains NZ9000[pNZtpo] and NZ9000[pNZotsBA] when exposed to 45 °C, (p=0.161 and p=0.092 at times 10 and 30 min, respectively), it is noteworthy that the latter strain consistently presented higher survival rates.

Effect of trehalose accumulation on cell survival to freeze-drying. The survival of the trehalose producers NZ9000[pNZtpo] and NZ9000[pNZotsBA], and that of the control strain was assessed after one cycle of freeze-drying. The viability of the control strain was reduced by 75%, while strains NZ9000[pNZtpo] and NZ9000[pNZotsBA] showed viability reductions of nearly 50 and 60%, respectively (Fig. 5). The differences with respect to the control strain, however, were not significant (p=0.17 and p=0.20), hence trehalose did not protect *L. lactis* against the stress imposed by freezing and dehydration.

Stress genes are not induced by heterologous expression of biosynthetic genes. To confirm that the beneficial effects of trehalose synthesis on the stress resistance of engineered strains were not caused by unintended overexpression of stress genes we performed semiquantitative RT-PCR assays. The transcription levels of four genes known to be involved in stress response of *L. lactis* were assessed. Genes *dnaK* and *groEL* encode a chaperone and a chaperonin, respectively, both overproduced in response to acid and heat stress conditions (7, 61, 63). Gene *recA* mediates the stress response upon DNA damage and its involvement in the acid shock response has been reported (7). Gene *clpP* encodes a heat shock protein also involved in the acid shock response (19).
The results showed that these stress response genes are equally transcribed in the control strain NZ9000[pNZ8020] and in the trehalose-producing strains, NZ9000[pNZotsBA] and NZ9000[pNZtpo] (Fig. S1). We conclude that the observed improved stress resistance of the engineered strains is directly associated with the presence of trehalose.

**DISCUSSION**

*Lactococcus lactis* is an important industrial organism widely used in dairy fermentations. In addition to this traditional use *L. lactis* has recently been proposed as an efficient producer of heterologous proteins and vehicle for drug delivery. The commercial importance of these applications underlies the demand for the development of robust strains, able to perform well even under adverse bioprocess conditions such as extremes of temperature or pH, high concentrations of weak acids, and dehydration. In view of the use of *L. lactis* in the food industry as well as for oral drug delivery we envisaged the construction of a food-grade strain with improved tolerance to stress, in particular to acid stress. To this end, the synthesis of trehalose, a solute widespread in stress response, was engineered in *L. lactis* using genes derived exclusively from food-grade organisms.

The growth rate of strain NZ9000[pNZtpo] was approximately 30% lower than those of the control and the mutant overexpressing *otsBA* and a similar trend was observed for the specific glucose consumption rate and the cell viability in the absence of stress. We noticed that the two recombinant strains were clearly different in respect to Tre6P accumulation: in resting cells of NZ9000[pNZtpo] the concentration of Tre6P was 3 mM, whereas in NZ9000[pNZotsBA] the level of this metabolite was below the detection level of the NMR technique (Fig. 4C). Furthermore, Tre6P was also detected (thin layer chromatography assays) in cell extracts derived from mid-exponential growing cultures of NZ9000[pNZtpo], but not in *L. lactis* overexpressing *otsBA* (data not shown). In view of these results it is
tempting to suggest the involvement of Tre6P in the mechanisms leading to impairment of the growth rate, glucose consumption and viability of NZ9000[pNZtpo]. The toxicity ascribed to sugar-phosphate accumulation is often invoked to justify impaired or arrested growth, but a comprehensive explanation remains elusive in many cases (17, 58). However, it is known that accumulation of Tre6P in *S. cerevisiae* causes a strong reduction in the glycolytic flux because Tre6P inhibits hexokinase in this organism (54). Also, the deleterious effect of Tre6P accumulation on cell viability has been demonstrated in a TPP-deficient mutant of *S. cerevisiae* (16). To our knowledge the effect of Tre6P on the regulation of the glycolytic enzymes of *L. lactis* has not been studied, and our data on metabolite dynamics during glucose metabolism (Fig. 4) do not provide a clue for a putative glycolytic target. Therefore, the hypothetical toxic effect of Tre6P in *L. lactis* requires further investigation.

In the present work, production of trehalose was achieved by overexpressing the genes *trePP* and *pgmB* from *L. lactis* (trehalose operon) and the gene *otsB* from *P. freudenreichii*. Growing cultures and resting cells of the resulting trehalose-producing strain, NZ9000[pNZtpo], converted a maximum of 16.8% of the glucose supplied into trehalose, a value far from the theoretical maximum of 66.7%. It is conceivable that the low activity of TPPPr (0.027 µmol min⁻¹ mg protein⁻¹) could limit the synthesis of trehalose. In fact, this view is strongly supported by the accumulation in this strain of Tre6P, the substrate of TPP (Fig. 4). Therefore, the enhancement of the TPP activity should be a primary goal in future strategies aimed at improving the yield of trehalose in *L. lactis*.

Curiously, the strain engineered with the trehalose pathway of *E. coli*, NZ9000[pNZotsBA], showed even lower yields of trehalose production despite a 10-fold higher TPP activity (0.26 µmol min⁻¹ mg protein⁻¹). In this case the metabolic bottleneck for trehalose synthesis is probably at the level of the reaction catalyzed by TPS, whose activity was very low (0.02 µmol min⁻¹ mg protein⁻¹). Likewise, a defective TPS activity has been...
evoked as the main reason for the poor trehalose production in *Corynebacterium glutamicum* engineered for trehalose overproduction by expression of *otsBA* from *E. coli* (43).

Altogether, the results with the two recombinant strains strongly indicate that a “pull-strategy” should be followed to drive G6P away from glycolysis and direct it to the synthesis of trehalose; this implies high activity of the enzymes that use G6P (TrePP or TPS), combined with TPP activity of a similar magnitude. This type of approach has proven highly effective in the optimization of mannitol production in *L. lactis* (21). Combining high activities of mannitol 1-phosphate dehydrogenase and mannitol 1-phosphate phosphatase led to efficient channeling of fructose 6-phosphate and a mannitol yield close to the theoretical maximum.

In *L. lactis* trehalose was detected not only inside the cells but also in the extracellular medium, both during growth and in non-growing conditions. In fact, when mid-exponential cultures of NZ9000[pNZtpo] were analyzed for trehalose production, at least 67% of the total trehalose was found in the extracellular medium. In resting cells, however, only around 20% of the trehalose produced was exported to the medium (Table 4). Fairly similar results were observed with strain NZ9000[pNZotsBA]. The ability of many organisms to synthesize and accumulate trehalose, and other compatible solutes, in response to osmotic stress has been extensively documented (49, 64). Regrettably, the assessment of solute excretion is rarely performed, probably because it seems counterintuitive that a protecting compound is synthesized and then lost to the medium, apparently without a sound physiological reason. The excretion of trehalose is well known in *C. glutamicum* and *E. coli* (23, 52). In the latter bacterium, the cytoplasmic trehalose level is regulated by a futile cycle involving overproduction, excretion and reutilization of this sugar (52), and a similar excretion/reutilization cycle has been proposed for ectoine in *Halomonas elongata* (24); in fact, this hypothesis was validated by disruption of the uptake system in *H. elongata*, which
led to a beneficial 20% increase in ectoine productivity. In view of these reports, the
excretion of trehalose observed in the two **L. lactis** trehalose-producing strains was not
unexpected. It is known that lactic acid bacteria in general, and **L. lactis** in particular, have a
limited capacity to synthesize compatible solutes, but are able to import glycine betaine,
carnitine or proline to counterbalance the external osmotic pressure (36, 62). Therefore, it is
conceivable that trehalose, a compatible solute extraneous to **L. lactis**, is released via
mechanosensitive channels that respond to an increase in cell turgor pressure (18).
Subsequently, the excreted trehalose can be taken up via the specific phosphotransferase
system described in the literature (2) and further metabolized. This convoluted pathway,
involving excretion and uptake of trehalose, probably provides the only route to catabolize
intracellular trehalose in the engineered trehalose-producing **L. lactis**, since trehalase or
trehalose phosphorylase activities have not been detected in this organism (2).

The trehalose-producing strains engineered in this work showed a remarkable tolerance
to acid, cold and heat shocks. In fact, the survival of strains NZ9000[pNZtop] and
NZ9000[pNZotsBA] was not significantly affected by severe insults such as up to 20 min
exposure to pH 3, eight days at 4 °C or 30 min at 45 °C (Fig. 5). The role of trehalose in the
protection of **L. lactis** during stress emerges from the sharp contrast between the high
tolerance of trehalose-accumulating cells and the poor performance of the control strain.
However, the possible contribution of stress proteins should be considered. As many other
organisms, **L. lactis** develops adaptation strategies when the environmental conditions are
shifted far away from the optimal parameters. This is called the stress response and involves
induction of the synthesis of several proteins whose role is to prevent cell death by
counteracting the damage provoked by harsh conditions. The response of **L. lactis** to acid,
osmotic, cold, and heat stress has been extensively investigated and many stress proteins
were identified (7, 19, 61, 63). The primary role of trehalose in the protection of **L. lactis**
against acid and cold stress is clearly demonstrated by our results since the strain lacking trehalose was severely affected by these stresses in contrast with the excellent performance of the trehalose-producing strains. Therefore the intrinsic stress response alone did not provide sufficient protection. However, there is an apparent synergism between the actions of trehalose and the heat stress response, specially evident in strain NZ9000[pNZotsBA] that exhibits survival rates that are consistently higher than in the absence of heat shock.

The engineering strategy used in this work involved the overexpression in L. lactis of heterologous genes encoding trehalose biosynthetic enzymes. Therefore, one could argue that the observed increased stress resistance could, at least in part, result from the induction of stress proteins. This hypothesis was ruled out by semi-quantitative RT-PCR experiments; the transcript levels of four selected genes encoding stress proteins, i.e., dnaK, recA, groEL, and clpP were assessed and the results showed no evidence for alterations in the expression of these genes (Fig. S1). We conclude that the stress resistance phenotype derives primarily from the presence of trehalose.

The mechanisms underlying the protecting effect of trehalose are not clearly understood and it is especially intriguing that this compound can protect cells against a variety of stresses (cold, heat, low pH, high osmolarity, free radicals, and desiccation). We reported the involvement of trehalose in the acid stress response (9), and a similar behavior has recently been found in Rhizopus oryzae (55). On the other hand, the protecting role of trehalose against cold stress has been extensively studied in plants and the model organisms E. coli and S. cerevisiae (20, 30, 50). Interestingly, trehalose also increases the life-span of the nematode C. elegans as demonstrated recently (26). Additionally, the ability of trehalose to stabilize protein structure against heat denaturation and to prevent concomitant protein aggregation has been extensively documented both in vitro and in vivo (28, 51). Trehalose also acts as a scavenger of reactive oxygen species, which accumulate under several stressful conditions,
thereby protecting proteins and other cell components from damage caused by strong oxidizing agents (3).

Despite the limited knowledge on the mode of action of trehalose, or indeed of any other protecting osmolyte, a potential stabilizing effect of trehalose on cell membranes has frequently been proposed (30, 34, 57). At low temperature, trehalose would counteract the decrease in membrane fluidity (12). At low pH, the extra-stabilization conferred by trehalose would result in decreased permeability to hydrogen ions, a highly beneficial trait when membranes are subjected to strong pH gradients. This latter view stemmed mainly from the huge trehalose levels observed in *Sulfolobus solfataricus*, a thermoacidophilic archaeon whose cell membranes can stand gradients as high as 5 pH units (41).

Trehalose-producing *L. lactis* showed no significant improved survival when exposed to a cycle of freeze-drying, despite the high level of intracellular trehalose (in the 100 mM range). This result is not surprising as protection against dehydration is largely provided by exogenous trehalose though this effect is amplified by the presence of intracellular trehalose (15). Upon addition of 0.1 to 0.5 M trehalose, enhanced survival to freeze-drying was observed in different bacterial cultures, *e. g.*, *E. coli, Bacillus thuringiensis, Lactobacillus acidophilus* (11, 34). Herein, the concentration of trehalose typically found in the external medium of *L. lactis* cultures (around 1 mM) was too low to provide effective protection. Our results contrast with those of Termont *et al.* (53) that report 100% retention of cell viability after freeze-drying of NZ9000[pNZotsBA]. The reasons for this discrepancy probably arise from the different conditions used by those authors for induction of trehalose synthesis, namely highly toxic levels of nisin (400 µg l⁻¹) and aerobic conditions.

In summary, we demonstrated that trehalose, a compound unrelated to wild-type *L. lactis* strains, plays a definite role in the protection of this bacterium against damage caused by acid, cold or heat shock. Moreover, this work represents a proof-of-concept for the
development of robust, food-grade *L. lactis* strains able to perform under demanding working conditions.

Acknowledgements

We would like to thank Prof. Oscar Kuipers (Groningen University) for providing plasmid pORI280::ΔccpA. ALC and FSC held fellowships SFRH/BD/30419/2006 and SFRH/BD/5080/2001 from Fundação para a Ciência e a Tecnologia (FCT). We thank Rui Neves for the construction of strain NZ9000ΔccpA. The NMR spectrometers are part of the National NMR Network (REDE/1517/RMN/2005), supported by "Programa Operacional Ciência e Inovação (POCTI) 2010” and FCT.

REFERENCES


Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant properties</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MG1363</td>
<td>Plasmid-free derivative of SH4109</td>
<td>(22)</td>
</tr>
<tr>
<td>NZ9000</td>
<td>MG1363; pepNxisBnsK</td>
<td>(32)</td>
</tr>
<tr>
<td>NZ9000[pNZ8020]</td>
<td>Derivative of NZ9000 carrying pNZ8020</td>
<td>This work</td>
</tr>
<tr>
<td>NZ9000ΔccpA</td>
<td>NZ9000 chromosomal deletion of ccpA</td>
<td>This work</td>
</tr>
<tr>
<td>NZ9000ΔccpA[pNZotsB]</td>
<td>Derivative of NZ9000ΔccpA carrying pNZotsB</td>
<td>This work</td>
</tr>
<tr>
<td>NZ9000ΔccpA[pNZipo]</td>
<td>Derivative of NZ9000ΔccpA carrying pNZipo</td>
<td>This work</td>
</tr>
<tr>
<td>NZ9000[pNZipo]</td>
<td>Derivative of NZ9000 carrying pNZipo</td>
<td>This work</td>
</tr>
<tr>
<td>NZ9000[pNZotsBA]</td>
<td>Derivative of NZ9000 carrying pNZotsBA</td>
<td>This work</td>
</tr>
<tr>
<td>Propionibacterium freudenreichii subsp. shermanii strain NIZO B365</td>
<td></td>
<td>(9)</td>
</tr>
<tr>
<td>E. coli DH5α</td>
<td>Amersham Biosciences</td>
<td></td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pORI280::ΔccpA</td>
<td>pORI280 derivative carrying ccpA up- and downstream regions</td>
<td>(65)</td>
</tr>
<tr>
<td>pNZ8020</td>
<td>Cm^R, nisin inducible PaniA</td>
<td>(13)</td>
</tr>
<tr>
<td>pNZotsB</td>
<td>pNZ8020 with P. freudenreichii otsB cloned in the BamHI / EcoRI site, Cm^R</td>
<td>This work</td>
</tr>
<tr>
<td>pNZtrePPpgmB</td>
<td>pNZ8020 with the lactococcal trePP and pgmB cloned in the SpeI / SacI site, Cm^R</td>
<td>This work</td>
</tr>
<tr>
<td>pNZipo</td>
<td>pNZtrePPpgmB with P. freudenreichii otsB cloned in the SacI / Xbal site, Cm^R</td>
<td>This work</td>
</tr>
<tr>
<td>pNZotsBA</td>
<td>pNZ8048 with E. coli otsB and otsA cloned in the Ncol / Xbal site; Cm^R</td>
<td>This work</td>
</tr>
</tbody>
</table>

* Cm^R, resistance to chloramphenicol.
Table 2. Trehalose content in ethanol extracts (intracellular trehalose) or in the growth medium (extracellular trehalose) of *L. lactis* cells collected during mid-exponential (Exp) and stationary (Stat) phases of growth. Cultures were grown with pH controlled at 6.5. Trehalose was quantified by proton NMR.

<table>
<thead>
<tr>
<th></th>
<th>Extracellular trehalose (mM)</th>
<th>Intracellular trehalose (mM)</th>
<th>Total trehalose (µmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exp</td>
<td>Stat</td>
<td>Exp</td>
</tr>
<tr>
<td>NZ9000ΔccpA[pNZtpo]</td>
<td>1.2*</td>
<td>0.4*</td>
<td>170.8*</td>
</tr>
<tr>
<td>NZ9000[pNZtpo]</td>
<td>0.8±0.1</td>
<td>2.1±0.1</td>
<td>167.0±9.2</td>
</tr>
<tr>
<td>NZ9000[pNZotsBA]</td>
<td>0.4±0.1</td>
<td>1.2±0.04</td>
<td>79.2±18.1</td>
</tr>
</tbody>
</table>

* A single experiment was performed.
Table 3. Major end-products (mM) from the metabolism of glucose in *L. lactis* strains NZ9000[pNZtpo], NZ9000[pNZotsBA] and control (NZ9000[pNZ8020]) during growth without pH control. Nisin (2 µg l⁻¹) was added at OD₆₀₀ of 0.4. Metabolites were measured by HPLC in supernatant solutions of culture samples collected at different time points during growth.

<table>
<thead>
<tr>
<th>OD₆₀₀ of sampling</th>
<th>NZ9000[pNZtpo]</th>
<th>NZ9000[pNZotsBA]</th>
<th>NZ9000[pNZ8020]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lactate</td>
<td>Trehalose</td>
<td>Lactate</td>
</tr>
<tr>
<td>0.4 (Induction)</td>
<td>12.2</td>
<td>nd</td>
<td>9.0</td>
</tr>
<tr>
<td>1.3 (Mid-exp)</td>
<td>25.2</td>
<td>0.3</td>
<td>20.8</td>
</tr>
<tr>
<td>2.0 – 2.2 (Late-exp)</td>
<td>47.2</td>
<td>1.1</td>
<td>47.7</td>
</tr>
<tr>
<td>&gt; 2.4 (Stationary)</td>
<td>75.1</td>
<td>2.1</td>
<td>77.6</td>
</tr>
<tr>
<td>% from Glucose</td>
<td>83.5</td>
<td>16.8</td>
<td>82.9</td>
</tr>
</tbody>
</table>

Initial and final glucose concentrations were 65.6 and 20.6 mM for NZ9000[pNZtpo], 63.0 and 16.2 mM for NZ9000[pNZotsBA] and 62.9 and 18.1 mM for control strain, respectively. Values for a representative growth out of two experiments. Induction means the time of nisin addition; Mid-exp, mid-exponential phase; Late-exp, late-exponential; nd, below detection limit. *Values calculated for total trehalose (intracellular plus extracellular) determined by NMR in cells collected during the mid-exponential phase. nd, below detection level.
Table 4. Yield of trehalose and percentage of trehalose excreted in strains NZ9000[pNZtpo] and NZ9000[pNZotsBA]. Trehalose produced and glucose consumed were quantified by NMR (see Materials and Methods). In growing cells, intracellular and extracellular trehalose was determined, respectively, in cell extracts and supernatants derived from cultures harvested during mid-exponential (Exp) and stationary (Stat) phases of growth. Unless stated otherwise the values are averages of at least two independent experiments.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Resting cells at pH 6.5</th>
<th>Growing cells</th>
<th>Trehalose yield (%)</th>
<th>Trehalose excreted (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Growing cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>pH controlled</td>
<td>Without pH control</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>at 6.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NZ9000[pNZtpo]</td>
<td>14.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.5 ± 2.0&lt;sup&gt;Exp&lt;/sup&gt;</td>
<td>15.8 ± 1.4&lt;sup&gt;Exp&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>11.4 ± 4.0&lt;sup&gt;Stat&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NZ9000[pNZotsBA]</td>
<td>10.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.4&lt;sup&gt;Exp*&lt;/sup&gt;</td>
<td>8.5 ± 0.2&lt;sup&gt;Exp&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.2&lt;sup&gt;Stat*&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NZ9000[pNZtpo]</td>
<td>26.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>78.0 ± 3.9&lt;sup&gt;Exp&lt;/sup&gt;</td>
<td>66.7 ± 8.0&lt;sup&gt;Exp&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>86.9 ± 0.6&lt;sup&gt;Stat&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NZ9000[pNZotsBA]</td>
<td>21.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>80.5 ± 1.3&lt;sup&gt;Exp&lt;/sup&gt;</td>
<td>80.7 ± 1.2&lt;sup&gt;Exp&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>84.2 ± 0.3&lt;sup&gt;Stat&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Values refer to the experiment shown in Figure 4. *A single experiment was performed.
Figure Captions

**Fig. 1.** Scheme of the strategy followed to engineer *Lactococcus lactis* for the synthesis of trehalose. Symbols: PTS, phosphotransferase system; TrePP, trehalose 6-phosphate phosphorylase; β-PGM, β-phosphoglucomutase; TPP<sub>Pf</sub>, trehalose 6-phosphate phosphatase from *Propionibacterium freudenreichii*. Tre6P, trehalose 6-phosphate; Glc6P, glucose 6-phosphate; Glc1P, glucose 1-phosphate; Glc, glucose; Tre, trehalose; Lac, lactate. The genes encoding TrePP, β-PGM, and TPP<sub>Pf</sub> were overexpressed in *L. lactis* NZ9000 with the purpose of channeling Glc6P for the synthesis of trehalose.

**Fig. 2.** (A) Glucose metabolism in non-growing cell suspensions of *L. lactis* strains with a deletion of the *ccpA* gene. Kinetics of [1-<sup>13</sup>C]glucose (20 mM) consumption and pools of intracellular metabolites in resting cells of *L. lactis* NZ9000Δ*ccpA* at 30 °C under anaerobic conditions with pH controlled at 6.5. The maximal glucose consumption rate is 0.31 µmol min<sup>-1</sup> mg protein<sup>-1</sup>. Symbols: (closed diamond), glucose; (closed triangle), fructose-1,6-bisphosphate; (open circle), 3-phosphoglycerate; (open square), trehalose 6-phosphate. The lines drawn in the graph are simple interpolations. (B) <sup>13</sup>C-NMR spectra of perchloric acid extracts of non-growing cell suspensions metabolizing [1-<sup>13</sup>C]glucose. Strain NZ9000Δ*ccpA*[pNZotsB<sub>Pf</sub>] (upper spectrum) and strain NZ9000Δ*ccpA* (lower spectrum). The resonance labeled with an asterisk is due to glucose 6-phosphate. Symbols: Glc, glucose; FBP, fructose-1,6-bisphosphate; 3-PGA, 3-phosphoglycerate; Tre6P, trehalose 6-phosphate.

**Fig. 3.** Growth profiles of *L. lactis* strains engineered for trehalose synthesis. Growth was performed in CDM with 1% glucose (w/v) at 30 °C, without pH control (initial pH 6.5). Growth rate (µ): control strain, 0.69±0.006 h<sup>-1</sup>; NZ9000[pNZtpo], 0.49±0.006 h<sup>-1</sup>; NZ9000[pNZotsBA], 0.70±0.026 h<sup>-1</sup>. Symbols: Diamonds, control strain; squares,
Fig. 4. Kinetics of [1-13C]glucose (40 mM) consumption and pools of metabolites in resting cells of *L. lactis* strains engineered for the synthesis of trehalose: NZ9000[pNZtpo] (A) and NZ9000[pNZotsBA] (B). The experiments were monitored online by *in vivo* 13C-NMR and carried out at 30 ºC, under anaerobic conditions and pH controlled at 6.5. Maximal glucose consumption rates (µmol min⁻¹ mg protein⁻¹) are 0.33 (A) and 0.39 (B). Symbols: (closed diamond), glucose; (closed triangle), fructose 1,6-bisphosphate; (open circle), 3-phosphoglycerate; (open square), total trehalose plus trehalose 6-phosphate expressed as intracellular concentration. Parallel experiments were run to study the kinetics of trehalose 6-phosphate and trehalose (intracellular and extracellular pools) in NZ9000[pNZtpo] (C) and NZ9000[pNZotsBA] (D). The extracellular trehalose was determined in cell supernatants, while intracellular trehalose and trehalose 6-phosphate were determined in perchloric acid extracts. These metabolites were quantified by proton NMR. Symbols: (closed diamond), extracellular trehalose; (open diamond), intracellular trehalose; (open triangle), total trehalose expressed as an intracellular concentration; (open square), trehalose 6-phosphate. Each type of experiment was performed twice with good reproducibility.

Fig. 5. Effect of different stresses on the survival of *L. lactis* strains engineered for trehalose synthesis: control strain (black bars); NZ9000[pNZtpo] (grey bars); NZ9000[pNZotsBA] (dashed bars). Viability was calculated as the number of CFU mg protein⁻¹ of cells exposed to stress as a percentage of the number of CFU mg protein⁻¹ of non-stressed cells (for details see Materials and Methods). Acid stress: cells harvested during mid-exponential phase were exposed at pH 3.0 (50 mM KP₅ acidified with HCl) for defined time periods (10, 20 and 30
Values are the mean of, at least, five independent experiments. Cold stress: survival at 4 °C after 1, 4, 8 and 14 days. Values are the mean of at least four independent experiments. Heat stress: survival upon exposure to 45 °C for different time periods. Values are the mean of at least four independent experiments. Freeze-drying cells: survival of cells subjected to one-cycle freeze-drying for 24 hours. Values are the mean of at least five independent experiments. Error bars indicate standard deviations. The asterisk designates statistically significant differences (p < 0.05) between the survival of the engineered strain and the control strain. The sharp designates significant differences (p<0.05) to the viability = 100% level.
Carvalho et al. – Fig. 3
Carvalho et al. – Fig. 4
Viability (%) vs Time (min) for various shock and freeze-drying conditions:

- **Acid Shock**: Viability decreases with time, marked with asterisks (*) indicating significant differences.
- **Cold Shock**: Viability remains consistent over time, marked with hashtags (#) suggesting no significant changes.
- **Heat Shock**: Viability increases slightly with time, marked with an asterisk (*) indicating a trend.
- **Freeze-Drying**: Initial viability is relatively high, with a slight decrease over time, marked with an asterisk (*) indicating a trend.

Carvalho et al. – Fig. 5