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Hosts, environment, stress, phages

Lactobacillus delbrueckii ssp. *bulgaricus* thermotolerance

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Abstract — *Lactobacillus delbrueckii* ssp. *bulgaricus* is a lactic acid bacterium widely used in the dairy food industry. Since the industrial processes are a succession of constraints, it is essential to understand the behaviour of *L. bulgaricus* when facing usual stresses. The influence of heat stress was investigated on the viability of *L. bulgaricus* cells grown in a chemically defined medium. The susceptibility of cells to heat-shock was obvious only above 55 °C. We investigated the acquisition of thermotolerance as a result of exposure to a moderate heat-shock, and the acquisition of a cross-stress-tolerance by exposure to a mild osmotic stress. When cells were submitted, before lethal temperature challenge (65 °C), to a heat pre-treatment at 50 °C or to a hyper-osmotic pre-treatment, the viability of cells increased. For the industrial strain RD 546, the addition of glycine betaine (GB) in 0.4 mol·L⁻¹ NaCl during the pre-treatment decreased the acquired thermotolerance, while GB alone enhanced cell viability. The thermotolerance of the type strain was not influenced by GB. We demonstrated that the stress tolerance induced by a moderate heat-shock was dependent on protein synthesis, while the effect of GB on RD 546 thermotolerance was independent of such biosynthesis. Thermotolerance acquired in presence of GB depends on a strain-dependant mechanism that differs from the mechanism involved after a moderate heat-shock.

heat-shock response / cross-protection / osmotic stress / betaine / thermoadaptation

Résumé — **Stress thermique et thermotolérance chez *Lactobacillus delbrueckii* ssp. *bulgaricus*.** *Lactobacillus delbrueckii* ssp. *bulgaricus* est une bactérie lactique largement utilisée en industrie alimentaire. Les procédés industriels étant une succession de contraintes, il est essentiel de connaître le comportement de *L. bulgaricus* face aux stress rencontrés. L'influence du stress thermique sur la viabilité de 2 souches de *L. bulgaricus*, cultivées en milieu chimiquement défini, a été étudiée. La viabilité des cellules n'est affectée qu'au-delà de 55 °C. Les cellules acquièrent une thermotolérance vis-à-vis d'un choc thermique à 65 °C durant 10 min, c'est-à-dire une viabilité accrue, après exposition à un prétraitement thermique modéré à 50 °C ou un prétraitement hyperosmotique. Pour la

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souche industrielle RD 546, l'addition de glycine bêtaïne (GB) dans le milieu de prétraitement contenant $0,4 \text{ mol}\cdot\text{L}^{-1}$ NaCl diminue la thermotolérance acquise, tandis que la glycine bêtaïne seule en tant que prétraitement avant le choc léthal augmente la viabilité cellulaire. La thermotolérance induite par un prétraitement thermique modéré est dépendante de la synthèse protéique, tandis que l'effet de la GB sur la thermotolérance est indépendant de biosynthèse. La thermotolérance acquise en présence de GB nécessite un mécanisme souche-dépendant distinct par rapport à celui impliqué lors d'un prétraitement thermique.

réponse au stress thermique / protection croisée / stress osmotique / glycine bêtaïne / thermotolérance

1. INTRODUCTION

Lactobacillus delbrueckii ssp. *bulgaricus* is a widely used lactic acid bacterium in the dairy food industry, especially in yoghurt manufacturing. The knowledge of physiological adaptation by *L. bulgaricus* to different stresses is essential for the understanding of bacterial behaviour when facing dairy processes and during starter elaboration. One of the industrial processes that causes a large loss of viability is bacterial conservation, by freezing, freeze-drying or by spray-drying. During these processes, bacteria are subjected to adverse conditions, one of the most encountered and drastic stresses being the heat stress. The best-described effects induced by high temperatures concern the protein denaturation [28], but membranes, nucleic acids and certain enzymes have been equally identified as cellular sites of heat injury [25]. Heat stress is also responsible for a disturbance of the transmembrane proton gradient, leading to a decrease of the intracellular pH [21, 22, 29]. Most of the studies about heat stress in Gram-positive bacteria describe the synthesis of a protein family (HSP), a phenomenon attributed to a universal response after a heat-shock [11]. The HSP, mainly chaperones and proteases, are responsible for refolding or degrading. The expression of the corresponding genes is positively or negatively controlled at the transcriptional level. The positive regulation is based on the tran-

scription modulation by alternative sigma factors, whereas the negative regulation requires the intervention of repressor dependent mechanisms. For *Bacillus subtilis*, two major regulation systems are used for the heat-shock response [11, 18]. One implies a repressor encoded by *hrcA* acting on a conserved motif (CIRCE) in some thermoinducible genes promoters as found in *groE* and *dnaK* operons. Nevertheless many heat-shock genes are positively controlled in *B. subtilis* by a general stress σ factor, σ^B . The σ^B factor is involved in the induction of heat-shock response by different stimuli [31].

Thermotolerance represents the ability of treated cells to exhibit increased survival after a severe heat-shock [19]. The heat-inducible thermotolerance allows bacteria, after a non-lethal heat-shock, to tolerate a second heat stress higher in intensity [2]. Teixeira et al. [24] demonstrated that *Lactobacillus bulgaricus* NCFB1489 shows an inducible thermotolerance when pre-treated at 52°C before being stressed at 64°C .

Cells' survival to a determined stress can be improved by pre-treatment of cells by a mild shock (heterologous pre-treatment) like low pH, moderate temperature, oxidative agent, UV irradiation or moderate osmolarity [4, 8, 10, 20, 30]. The beneficial effect of a mild osmotic pre-treatment on thermotolerance reported for several bacteria has been poorly correlated to the known osmoregulation phenomena. On the other hand, the

effect of the well-known osmoprotectant glycine betaine (GB) on bacterial growth under high osmolarity has been widely described in numerous bacteria [5]. The growth of many osmotically stressed bacteria is protected by less than 1 mmol·L⁻¹ GB.

We describe in this study the physiological effect of heat stress and the induction of a thermotolerance by pre-exposure to different mild stresses on *L. bulgaricus* cells. Cells harvested during stationary phase have the higher thermotolerance. If cells are pre-treated at 50 °C or with 0.4 mol·L⁻¹ NaCl, an acquired thermotolerance was observed. We tested the incidence of a mild osmotic pre-treatment coupled with the presence of GB. An effect on viability after a heat challenge was observed for only one strain. Relationships between osmotic and heat stress in *L. bulgaricus* are discussed.

2. MATERIALS AND METHODS

2.1. Bacterial strains and media

The *Lactobacillus delbrueckii* ssp. *bulgaricus* type strain ATTC11842 and the strain RD 546 isolated from industrial yoghurt were used. The rich medium was MRS (Difco Laboratories, Detroit, Michigan, USA) [6], and defined medium was the minimal medium MPL [16] with 5 g·L⁻¹ D-glucose as carbon source. Liquid cultures were performed at 37 °C without stirring, MRS agar (MRSA) plates were incubated anaerobically (Anaerocult® A, Merck, 64271 Darmstadt, Germany) at 37 °C. Cells from single colonies were inoculated into MRS medium and grown for 16 h at 37 °C. They were then subcultured at 1:50 (v/v) dilution into MPL medium supplemented with 5 g·L⁻¹ D-glucose and incubated for 10 h at 37 °C without stirring. This MPL pre-culture was used to inoculate MPL working cultures. Growth of cell cultures was determined by OD₅₇₀ measurements (model DU 7400 spectrophotometer, Beckman, 93220 Gagny, France).

2.2. Heat-shock treatment: challenge

Cells were harvested in exponential or stationary phase by centrifugation (16 000 g, 10 min; Biofuge 15, Heraeus, Sepatech, 3360 Osterode, Germany) at room temperature. The pellet was resuspended in MPL medium at room temperature to reach an OD₅₇₀ of 1 unit, which corresponds to a population of 2 × 10⁸ CFU·mL⁻¹. Then 200 µL aliquots were transferred into a water bath at the challenge temperature for 10 min. At intervals indicated, samples were immediately diluted in a 1 g·L⁻¹ peptone, 8.5 g·L⁻¹ NaCl, pH 7.0 solution at room temperature and poured in duplicate on MRSA plates. The viable cell numbers in these samples were determined after 24 h of an anaerobic incubation at 37 °C. The data is expressed as the percent of colony-forming ability after exposure to the challenge temperature, with the colony-forming units at 0 min set to 100%.

2.3. Adaptation conditions: pre-treatment

Pre-treatments were performed on exponential cells resuspended at OD₅₇₀ of 1 unit in MPL medium. Cells were exposed to a given stress agent in non-lethal conditions before exposure to a heat challenge. Different pre-treatments (adaptation conditions) were applied for 30 min. Cells were incubated at several temperatures (50 °C, on ice) or at room temperature in MPL media containing different mild stressing agents (0.4 mol·L⁻¹ NaCl, 0.4 mol·L⁻¹ NaCl plus 10 mmol·L⁻¹ GB, 0.6 mol·L⁻¹ trehalose, 0.66 mol·L⁻¹ glycine betaine, 0.48 mol·L⁻¹ sucrose or 0.64 mol·L⁻¹ glycerol). Then samples were submitted to the heat challenge, 65 °C for 10 min. Viable cell number was determined on MRSA plates.

For experiments testing the effect of the inhibition of protein synthesis on thermoadaptation, chloramphenicol (Cm) was used at 10 µg·mL⁻¹. Exponential cells were

resuspended at OD_{570} of 1 unit in MPL medium containing $10 \mu\text{g}\cdot\text{mL}^{-1}$ Cm and were incubated at room temperature for 20 min before pre-treatment and challenge at 65°C for 10 min.

3. RESULTS

3.1. Effect of heat-shock on the survival of *Lactobacillus delbrueckii* ssp. *bulgaricus*

The survival of the two *L. bulgaricus* strains, the ATTC11842 and RD 546 strains, from cultures grown to exponential phase, was tested at increasing temperatures. As shown in Figure 1, viability of cells was not altered for heat challenge between 37°C to 55°C . The strains became heat sensitive for a challenge temperature above 55°C . The percentage of survivors dropped sharply for a 10 min heat-shock at temperatures above

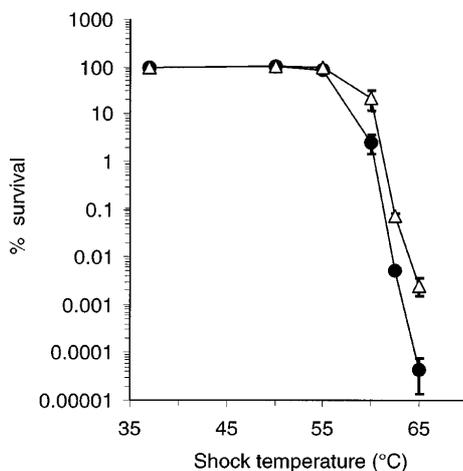


Figure 1. Effect of increasing temperatures on *L. bulgaricus* survival in MPL medium. Exponential cells were centrifuged, resuspended in MPL medium and incubated 10 min at indicated temperature. ATTC11842 (Δ); RD 546 (\bullet). Each point is the average of at least five independent experiments. Standard deviation is shown by bars.

55°C . A temperature of 65°C for 10 min was then chosen as the fixed challenge condition. Compared with exponential-phase cells, stationary-phase cells of *L. bulgaricus* were significantly more resistant to heat at 65°C for 10 min (results not shown). In an attempt to study thermoadaptation in a homogenous context, log phase cells (OD_{570} below 0.6) were used for the measurement of the thermotolerance in subsequent experiments.

3.2. Induction of a thermotolerance by a heat pre-treatment

In this study, we investigated the adaptive heat-shock response of *L. bulgaricus* strains. The non-lethal temperature selected for the pre-treatment was 50°C as *L. bulgaricus* strains were not affected by a heat challenge at this temperature (Fig. 1).

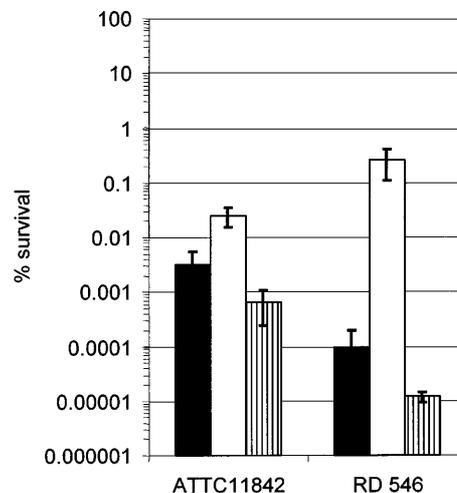


Figure 2. Effect of heat pre-treatment on *L. bulgaricus* thermotolerance. Exponential cells were centrifuged, resuspended in MPL medium and incubated 10 min at 65°C without pre-treatment (\blacksquare), or after an incubation at 50°C for 30 min (\square), or after 30 min on ice (\square with vertical lines). Each point is the average of at least five independent experiments. Standard deviation is shown by bars.

Figure 2 shows the influence of heat pre-treatment on *L. bulgaricus* survival after a heat-shock challenge at 65 °C for 10 min. 30 min incubation at 50 °C resulted in increased viability of tested strains. ATTC11842 showed a ten-fold increase of thermotolerance compared to viability under non-adapted conditions. The RD 546 strain, extremely heat sensitive without pre-treatment, exhibited a three-log increase of viability.

A common lab. practice is to keep bacterial samples on ice until experimentation, as cooled samples are thought to stay at the original physiological state. The influence of ice incubation as a pre-treatment on ATTC11842 thermotolerance after a heat challenge was minor, but resulted in a reduction of cells' viability (Fig. 2). The effect was obvious for the strain RD 546 as the pre-treatment at reduced temperature caused a survival reduction of 1 log after heat challenge. The effect of ice incubation on viability of the control cells (unstressed cells) was weak, but deleterious, for the ATTC11842 strain. For the strain RD 546, the ice pre-treatment led to a loss of 30% of viability of control cells in absence of challenge (results not shown).

3.3. Effect of osmotic pre-treatment on thermotolerance

We tested the effect of several osmotica, used at concentrations generating the same osmotic strength as 0.4 mol·L⁻¹ NaCl (1 050 mOs·kg⁻¹ H₂O), on *L. bulgaricus* thermotolerance (Fig. 3). After pre-treatment of 30 min in 0.4 mol·L⁻¹ NaCl, cells were more resistant to a heat challenge (65 °C, 10 min). The effect of this salt pre-treatment was then compared to the effect of non-electrolyte osmotica (0.66 mol·L⁻¹ trehalose, 0.48 mol·L⁻¹ sucrose) in order to differentiate salt response from osmotic response. The type strain ATTC11842, when pre-treated for 30 min in presence of 0.66 mol·L⁻¹ trehalose or 0.48 mol·L⁻¹

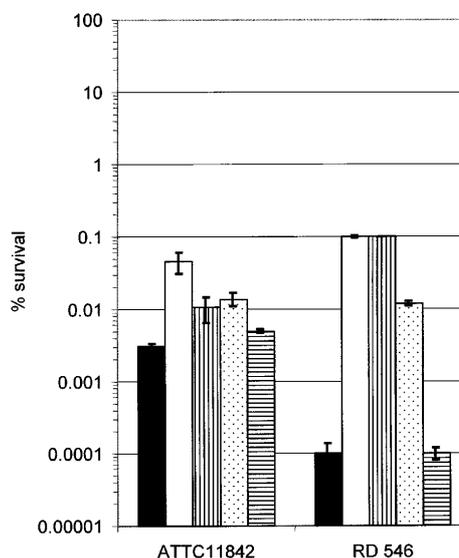


Figure 3. Effect of osmotic pre-treatment on *L. bulgaricus* survival submitted to a challenge at 65 °C. Exponential cells were centrifuged, resuspended in MPL medium and incubated 10 min at 65 °C without pre-treatment (■), or after incubation in 0.4 mol·L⁻¹ NaCl (□), in 0.66 mol·L⁻¹ trehalose (▨), in 0.48 mol·L⁻¹ sucrose (▩) or in 0.64 mol·L⁻¹ glycerol (▧). Each point is the average of at least five independent experiments. Standard deviation is shown by bars.

sucrose, showed a weak protection towards heat stress compared to the NaCl effect (Fig. 3). The thermotolerance was increased compared to the control by a three-fold and a four-fold factor for trehalose and sucrose, respectively. For the RD 546 strain, the effect of 0.66 mol·L⁻¹ trehalose was comparable to a pre-treatment in 0.4 mol·L⁻¹ NaCl, while 0.48 mol·L⁻¹ sucrose was less efficient on thermoprotection. However, the pre-treatment in sucrose did give rise to a two log increase in RD 546 survival compared to the control.

These results show that thermoprotection of *L. bulgaricus* can be achieved by ionic and non-ionic pre-treatments, that induce a moderate increase of osmolarity. Then we tested the effect of a permeant

solute, glycerol on *L. bulgaricus* growth. The solute used at $0.64 \text{ mol}\cdot\text{L}^{-1}$ induced only a slight decrease in growth rate and was not used as a carbon source (results not shown). Treatment in $0.64 \text{ mol}\cdot\text{L}^{-1}$ glycerol on *L. bulgaricus* thermotolerance prior to a heat challenge at 65°C (Fig. 3) had no effect on RD 546 and ATTC11842 viability.

3.4. Effect of glycine betaine on *L. bulgaricus* thermotolerance

Effects of the osmoprotectant, GB, on thermotolerance was tested at $10 \text{ mmol}\cdot\text{L}^{-1}$ added to $0.4 \text{ mol}\cdot\text{L}^{-1}$ NaCl and alone at

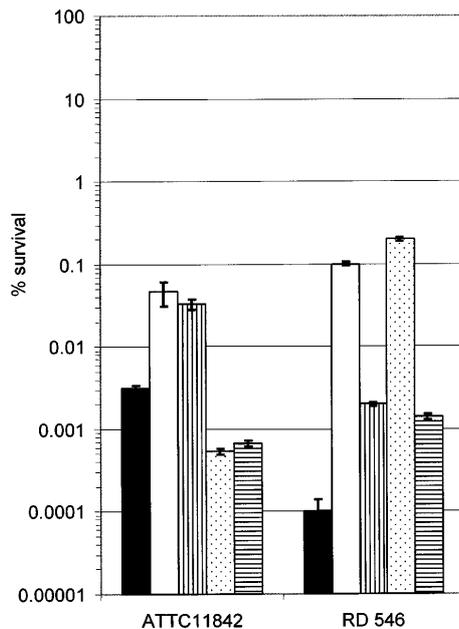


Figure 4. Effect of glycine betaine on *L. bulgaricus* survival after a heat challenge. Exponential cells were centrifuged, resuspended in MPL medium and incubated 10 min at 65°C without pre-treatment (■), or after incubation in $0.4 \text{ mol}\cdot\text{L}^{-1}$ NaCl (□), in $0.4 \text{ mol}\cdot\text{L}^{-1}$ NaCl + $10 \text{ mmol}\cdot\text{L}^{-1}$ GB (▨), in $0.6 \text{ mol}\cdot\text{L}^{-1}$ GB (▩) or in $10 \text{ mmol}\cdot\text{L}^{-1}$ GB (▧). Each point is the average of at least five independent experiments. Standard deviation is shown by bars.

$0.6 \text{ mol}\cdot\text{L}^{-1}$ (concentration for which it develops an osmolarity equivalent to $0.4 \text{ mol}\cdot\text{L}^{-1}$ NaCl) (Fig. 4). For the RD 546 strain, GB partially reversed the thermotolerance induced by the medium osmolarity. On the other hand, the solute had no influence on ATTC11842 NaCl-induced thermotolerance. When the osmoprotectant was used as an osmoticum at $0.6 \text{ mol}\cdot\text{L}^{-1}$, it induced thermoprotection for RD 546, but was inefficient on ATTC11842 thermotolerance. Thus, the universal osmoprotectant GB clearly triggers different responses in the two strains of *L. bulgaricus*.

3.5. Influence of protein synthesis on thermotolerance

We investigated the involvement of protein neosynthesis on the thermotolerance response of strain RD 546; this strain exhibited the greater response. Addition of chloramphenicol ($10 \mu\text{g}\cdot\text{mL}^{-1}$) caused a growth arrest by protein synthesis inhibition. Cells were incubated in the presence of chloramphenicol 20 min before pre-treatment and heat challenge. When chloramphenicol-treated RD 546 cells were submitted to a mild heat pre-treatment before the heat challenge, the acquired thermotolerance was abolished (Fig. 5). However when chloramphenicol treated cells were pre-treated by $10 \text{ mmol}\cdot\text{L}^{-1}$ GB for 30 min, the acquired thermotolerance observed after the heat challenge is preserved, allowing cells to reach the same viability as measured in protein synthesis-permissive conditions.

4. DISCUSSION

This study investigated the heat-shock response and cross-protection in two strains of *L. bulgaricus*. We used a chemically defined medium for culture and challenge media. Previous studies used the rich medium MRS. Such a medium contains lots of nutrients and solutes, of which the

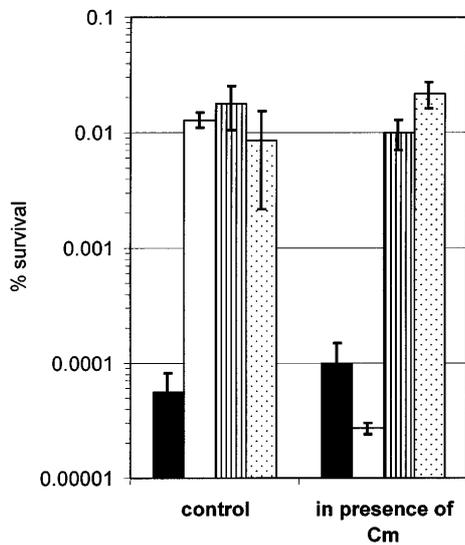


Figure 5. Effect of blocked protein synthesis on *L. bulgaricus* RD 546 acquired thermotolerance. Exponential cells were centrifuged, resuspended in MPL medium and incubated 20 min at room temperature in presence of 10 $\mu\text{g}\cdot\text{mL}^{-1}$ chloramphenicol and then submitted to a heat challenge of 10 min at 65 °C without pre-treatment (■), after an incubation at 50 °C for 30 min (□), after an incubation at 50 °C for 30 min in presence of 10 $\text{mmol}\cdot\text{L}^{-1}$ GB (▨), after incubation in 10 $\text{mmol}\cdot\text{L}^{-1}$ GB (▩). Each point is the average of at least five independent experiments. Standard deviation is shown by bars.

effects on cell adaptation response are unknown. A chemically defined medium allowed us to determine conditions and to test the real effect of added solutes on thermotolerance. The use of the MPL medium explained, at least partly, the fluctuations observed between published results and ours.

We observed a beneficial effect of stationary phase on thermotolerance of cells grown in chemically defined medium. Teixeira et al. [24] have already demonstrated that *Lactobacillus bulgaricus* NCFB1489, when grown in the rich medium MRS, shows a better thermotolerance in stationary phase than in exponential phase.

This effect of growth phase on cell resistance has been reported for several micro-organisms after environmental stresses including high temperature, oxidising agents, osmotic shock and acid pH [10, 12, 13, 17]. To minimise thermotolerance of *L. bulgaricus* related to growth stage, all experiments were subsequently performed on exponential-phase cells.

A heat pre-treatment of *L. bulgaricus* cells grown in MRS has been reported to improve the resistance of cells submitted to a lethal temperature challenge [24]. The heat-inducible thermotolerance is a widespread phenomenon already described in several micro-organisms [19]. The enhanced capability of bacteria to survive after exposure to a lethal temperature has been associated with the production of heat-shock proteins [15]. Indeed, we demonstrated in this study that acquired thermotolerance in *L. bulgaricus* disappeared when protein synthesis was blocked. We also showed that incubation on ice can be deleterious for cell survival. Our results indicate that putting cultures on ice could be a cause of artefact in experiments.

Adaptation of cells by a moderate salt pre-treatment is known to increase the resistance of cells to a sudden exposure to high temperature challenge [7, 8, 26], although the mechanism by which this occurs is unclear. The thermotolerance was stimulated by the osmotic pressure for the two *L. bulgaricus* strains, with a slighter protective effect for the sucrose in the case of the RD 546 strain. It has been demonstrated that sugar and salt did not induce the same cellular response. Indeed, Glaasker et al. [9] have shown that sucrose-stressed and NaCl-stressed *Lactobacillus plantarum* cells did not accumulate the same compounds in response to osmotic stress. Sugar-stressed cells contain sugar and sugar-derived compounds while salt-stressed cells do not. In the case of *L. bulgaricus* strains, the decrease in turgor pressure produced by the addition of sucrose or NaCl should be compared in terms of cytoplasmic accumulation.

Owing to its diffusion behaviour, the glycerol does not modify the intracellular pressure, it limits the cell volume decrease usually observed during an osmotic shock [23]. As behaviour of the two *L. bulgaricus* strains was not modified in presence of glycerol in response to heat-shock, we concluded that the acquired thermotolerance induced by osmotica was a consequence of the increased osmolarity rather than a reduction of water activity.

GB had an influence on thermoprotection only for the RD 546 strain, while, when added in presence of NaCl, GB induced a decrease of the acquired thermotolerance. In *Salmonella typhimurium*, addition of 1 mmol·L⁻¹ GB reversed completely the ability of NaCl to enhance viability at high temperature [8]. From these authors, the thermotolerance can be quantified by two types of data: growth rate or survival. They demonstrated that these two phenomena are controlled by two different regulatory mechanisms. Indeed, while GB acted on NaCl-induced thermotolerance, the osmoprotectant did not have any influence on growth rate in the presence of NaCl at high temperature. In conclusion, GB can act independently on different mechanism(s) in cells. Its effect on thermoprotection in an osmotic context can be explained through its osmoprotective properties by which GB decrease the deleterious effect of osmotic stress and so decrease the osmotic signal, inducing a cellular response of thermoprotection. Caldas et al. [3] have demonstrated that thermoprotection of *Escherichia coli* is enhanced by GB when cells were stressed at 42 °C. Although a physiological overlap between osmotolerance and thermotolerance has been demonstrated [14, 27], the effect of the osmoprotectant is not clear. From Caldas et al.'s [3] hypothesis, GB could act as a chemical chaperone at an intracellular concentration as low as 50 mmol·L⁻¹. GB also acts as a thermoprotectant in plants, indeed the tolerance of *Arabidopsis* to high temperatures is enhanced by increasing the synthesis of GB [1]. It thus appears that GB is involved in thermoprotection through a

mechanism controlling survival of cells facing heat stress, although no mechanism of action has so far been proposed. Here we demonstrate that the effect of GB on thermotolerance may not require protein synthesis. Moreover our results show that the effect of GB on thermoprotection is strain-dependant, suggesting that an extensive and comparative study of ATTC11842 and RD 546 strains would give us some clues about the implicated mechanism.

To summarise, mild starvation, or a readily performed heat pre-treatment are simple conditions that can be used to obtain better survival of *L. bulgaricus* cells upon heat-shock, as occurs during industrial processes. Osmotic pre-treatment is also efficient but the presence of salt in the medium could be a problem during the preservation process and/or subsequent cell uses. As this species is rather delicate, such pre-treatments could be used during starter elaboration to improve survival rate. We demonstrated in this study that thermotolerance acquired after a mild heat-shock depends on protein synthesis. The characterisation of the implicated proteins is in progress using whole cell protein 2-D electrophoresis separation and mass spectrometry analysis. Identification of the mechanisms involved in thermoprotection will provide useful information leading to a better control of bacterial behaviour when facing dairy processes and during starter elaboration.

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