Inducible thermotolerance in *Lactobacillus bulgaricus*

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P. TEIXEIRA, H. CASTRO AND R. KIRBY. 1994. The effect of a sublethal heat challenge on the subsequent thermotolerance of *Lactobacillus bulgaricus* at different stages of growth was investigated along with the effect of heating menstrum on survival.

The response of the cells to heat stress was shown to be dependent upon both cell age and heating menstrum. Heat-inducible thermotolerance could be provoked in cells which had been growing exponentially when they were subjected to the sublethal heat stress: pre-incubation at 10°C above the optimum growth temperature. The same effect could not, however, be reproduced in cells taken from the stationary phase.

Cells from the stationary phase were shown to always be more thermotolerant as compared to exponential phase cells. Cells showed a greater thermotolerance when heated in milk as compared to buffer.

INTRODUCTION

Heat-inducible thermotolerance was first described by Ritossa (1962) in *Drosophila melanogaster* and was called the heat shock (HS) response. The first publications referring to the response in bacteria (Yamamori *et al.* 1978) suggested that it had a possible role in improving the survival/adaptation capabilities of the organisms. The HS response represents a pronounced alteration in metabolism, involving a transient global adjustment towards new environmental conditions. One physiological consequence of heat shock is an elevated resistance to subsequent heat challenge which has been reported in a range of cultured mammalian cells, bacteria, yeast and other fungi (Mackey and Derrick 1986). Although the response in terms of control and regulation is becoming well established, the mechanism by which heat shock causes the apparent increase in thermoresistance remains poorly understood (Boutibones *et al.* 1992). It has been reported that protein synthesis occurs during the period of shock (Yamamori and Yura 1982; Mackey and Derrick 1986; Whitaker and Batt 1991). These proteins have been shown to be involved in the regulation of the response. The response is dependent on the function of a single Effector-Gene, *htpr* (Neidhardt and van Bogelen 1987).

Lactic acid bacteria are involved in the manufacture of fermented products from raw agricultural materials such as milk, meat, vegetables and cereals. The degree of resistance conferred on lactic acid bacteria by heat shock has not been

examined in detail; however, previous reports (Whitaker and Batt 1991) have demonstrated induced thermotolerance in *Lactococcus lactis*. Studies with other organisms have shown that factors such as previous growth history of the cells including medium and culture age, stress and shock media, all affect the manifestations of the response (Hogg 1989).

Lactobacillus bulgaricus is important for a number of industrial applications and it is frequently used as a starter culture for dairy fermentation. Production of commercial starter cultures often involves treatments, including heat, that stress the bacteria in such a way that they lose some of their original activity (Busta 1976). The purpose of this paper was to determine the degree of thermotolerance conferred on *Lact. bulgaricus* by the HS response and to study the effects with regard to culture age and heating menstrum composition.

MATERIALS AND METHODS

Organism

Lactobacillus delbrueckii subsp. *bulgaricus* NCFB 1489 was used. Reference cultures were maintained in cryogenic storage at -80°C on glass beads. Working cultures were grown on De Man, Rogosa, Sharp (MRS) plus 1.5% agar (MRSA) slopes (37°C for 24 h). Slopes were stored at 4°C and subcultured every month. New working cultures were prepared from the original reference culture every 3 months.

Preparation of cultures

MRS broth was inoculated from the MRSA slopes and incubated for 24 h at 42°C. This broth was then used to inoculate a second MRS broth (1% v/v). The cultures were incubated at 42°C for 6 h and 24 h according to the experiment and agitated using magnetic stirrer bars.

Preparation of media

Unless otherwise stated, all media were prepared using deionized water and sterilized by autoclaving for 15 min at 121°C.

Diluent. Phosphate buffer was prepared by mixing 0.01 mol l⁻¹ K₂HPO₄ (Merck) and 0.01 mol l⁻¹ KH₂PO₄ (Merck), both dissolved in a solution of 0.15 mol l⁻¹ NaCl (Merck), adjusted to pH 7.0 ± 0.1 and sterilized.

Growth medium. The medium used was MRS broth (Lab M). It was prepared and sterilized according to the manufacturer's instructions.

Recovery media. The medium used for plate count enumeration was MRSA. MRS was prepared according to the manufacturer's instructions and 15 g l⁻¹ of technical agar number 1 (lab M) was then added. The media was sterilized, then cooled to 55°C before distribution into Petri dishes or bottles.

Heat-treatment in phosphate buffer

Two ml of cultures grown to exponential phase (6 h) and late stationary phase (24 h) were transferred to 18 ml of phosphate buffer containing 0.0017 g ml⁻¹ of glucose (filter sterilized) and maintained at 52°C (test cells) and 42°C (control cells) for 20 min, under constant agitation, before being submitted to the stress temperatures. One ml of cells was transferred to 49 ml of buffer previously equilibrated at the stress temperature.

Heat-treatment in skim milk (Lab M)

Two ml of cultures grown to exponential phase (6 h) and late stationary phase (24 h) were transferred to 18 ml of skim milk 11% solids and maintained at 52°C (test cells) and 42°C (control cells) for 20 min, under constant agitation,

before being submitted to the stress temperatures. One ml of cells was transferred to 49 ml of skim milk previously equilibrated at the stress temperature.

Enumeration of survivors

At appropriate intervals, samples were taken from the heating menstrum and added to 9 ml of phosphate buffer. Survivors were enumerated on MRSA by the drop count technique (Miles and Misra 1938). Plates were examined after incubation at 37°C for 48 h.

D-Value determination

D-values were determined by plotting the log₁₀ of the number of survivors against time at a specific temperature. The best straight line was obtained by regression analysis to derive a regression equation of the type $y = a + bx$. The D-value corresponds to the slope of the best straight line (b) inverted and with the sign changed from negative to positive.

RESULTS

Table 1 illustrates the effects of age and temperature on survival of *Lact. bulgaricus* in skim milk. At each test temperature, cells at the stationary phase showed a higher D-value. It was also demonstrated that the heat resistance is influenced by the heating menstrum. Both stationary and exponential phase cells were more thermotolerant when heated in reconstituted skim milk powder than in phosphate buffer (Table 2).

The effect of heat shock on the increase in thermotolerance was evaluated as a function of the age of the cells. As shown in Fig. 1 and Fig. 2, heat shock was responsible for an increase in the heat resistance of cells in the exponential phase but not in the stationary phase. Table 3 shows that cells from exponential phase, even after heat shock, were not as resistant to the standard heat treatment as cells from the stationary phase.

DISCUSSION

The results relating to growth phase and heat resistance are in agreement with many previous articles (Beuchat and Lechowich 1968; Kenis and Morita 1968; Griffiths and

Table 1 Effects of age and temperature on survival of *Lactobacillus bulgaricus* in skim milk

Phase of growth	D _{62°C} (min)	D _{64°C} (min)	D _{67°C} (min)
Stationary (24 h; 42°C)	18.52	10.87	1.01
Exponential (6 h; 42°C)	2.42	0.47	<0.1

Table 2 Effects of heating menstium on survival of *Lactobacillus bulgaricus* at 64°C

Phase of growth	$D_{64^\circ\text{C}}$ (min)	
	Phosphate buffer	Skim milk
Stationary (24 h; 42°C)	1.59	10.87
Exponential (6 h; 42°C)	0.17	0.47

Haigh 1973; Hurst *et al.* 1974). In this case, however, it is not clear if the increase in resistance can be wholly accounted for by the explanations proposed regarding changes in cell physiology such as the reduced amount of replicating DNA (Mirhabibollahi 1988), or whether the exposure to gradual increases in acidity conferred an increased resistance to cells incubated for 24 h. The phenomenon of acid shock-induced thermotolerance has previously been reported in *Listeria monocytogenes* (Farber and Pagotto 1992). In the experiments reported here, pH was not controlled in the culture medium prior to stress.

Significant differences were found in the heat resistance when the heating menstium was skim milk or phosphate buffer. Results show that cells are more resistant when heated in the complex medium than in buffer. These results are in agreement with those of Dabbah and Moats (1969) who showed that more complex heating media stabilized *Pseudomonas* sp. and favoured recovery. It is well known that proteins in the heating menstium have a protective effect on micro-organisms. The mechanism of protein protection of microbial cells is unknown but it has been known for many years that proteins may increase the stability of enzymes and other proteins (Hansen and

Fig. 1 Effect of heat shock on the survival of *Lactobacillus bulgaricus* at exponential phase heated at 64°C in skim milk. ○, Control cells; △, heat-shocked cells **Fig. 2** Effect of heat shock on the survival of *Lactobacillus bulgaricus* at stationary phase heated at 67°C in skim milk. ○, Control cells; △, heat-shocked cells

Riemann 1963). It is unlikely that lactose is affording protection since Fay (1934) reported no increase in heat resistance when cells were heated in lactose solutions.

Results presented here show that *Lact. bulgaricus* can be made more thermotolerant by a brief temperature shift before subsequent exposure to lethal temperatures. The same increases in heat resistance after a heat-shock have been reported for other organisms including *Salmonella typhimurium* (Mackey and Derrick 1986; Hogg 1989), *Saccharomyces cerevisiae* (McAlister and Finkelstein 1980), *Neurospora crassa* (Plesofsky-Vig and Bramb 1985), *Escherichia coli* (Yamamori and Yura 1982) and *Lactococcus lactis* (Whitaker and Batt 1991). The mechanism remains unknown. The ion-coded protease counteracts part of the SOS response, and might be involved in the degradation of stress-derived, abnormal proteins. Regulatory systems, other than htpR but induced in parallel, might play a major role in increased thermotolerance (Hogg 1989). Working with *E. coli*, Mackey and Derrick (1986) found that degradation of heat-denatured proteins could be a possible process contributing to thermotolerance.

Results show that the manifestation of the HS response was dependent on the age of the cells. The enhancement of

Table 3 Effects of heat shock on the heat resistance, in skim milk, of *Lactobacillus bulgaricus* cells at exponential phase as compared to heat resistance of non-heat-shocked stationary phase cells

Phase of growth	Heat shock	$D_{62^\circ\text{C}}$ (min)	$D_{64^\circ\text{C}}$ (min)
Stationary (24 h; 42°C)	No	18.52	10.87
Exponential (6 h; 42°C)	Yes	5.51	1.21

thermotolerance was more pronounced in younger cells. A possible explanation is that cells in late stationary phase have already activated mechanisms of resistance in such a way as to act as an HS response. Sanchez *et al.* (1992) have found that a yeast protein (Hsp104) was strongly induced following a heat shock and that the expression of this protein was also activated when cells entered the stationary phase. Regarding how thermoresistance is activated in the stationary phase, intracellular pH has been suggested to play an essential part in triggering the response, since the acquisition of thermotolerance is enhanced in cells having an acidic external environment (pH 4.0) as compared with a neutral one (Coote *et al.* 1991).

The stage of growth, the type of medium in which the bacteria are heated and the previous history of the cells were all found to affect heat resistance. This indicates that to obtain accurate data on the heat resistance of previously unstudied bacteria, or on previously studied bacteria under new conditions, empirical studies may still be necessary.

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