Enhancement of apparent resistance to ethanol in Lactobacillus hilgardii

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The survival of Lactobacillus hilgardii, a highly ethanol-tolerant organism, after an ethanol challenge at 25% (v/v) for 10 min, increased by several log cycles when cells, grown in the absence of ethanol, were pre-treated with 10% (v/v) ethanol, 15% (v/v) methanol or 2% (v/v) butanol for 4 h. A temperature upshift (25 to 40°C) before ethanol challenge demonstrated a similar enhancement of apparent resistance to ethanol. Ethanol shock enhanced apparent resistance to methanol, butanol and heat challenges. The addition of chloramphenicol to cells prior to any pre-treatment did not significantly diminish the increase in ethanol tolerance, suggesting that de novo protein synthesis is not required for induced tolerance in this organism.

Introduction

Certain strains of *Lactobacillus* are the most ethanol tolerant of all free living vegetative cells (Ingram and Buttke, 1984; Gold *et al.*, 1992). An example is *Lact. hilgardii*, an heterofermentative species, strains of which are capable of growing in fortified wines (18 to 20% v/v ethanol, pH 3–4) influencing the quality of the final product either by conducting the malolactic fermentation and/or causing wine spoilage. The capability for survival or growth under what would normally be considered extremely adverse conditions requires specific cellular strategies which are of fundamental importance for microbial life in varied environments (Hecker and Völker, 1990).

Stress proteins are apparently involved in the adaptation of bacteria to certain growth-limiting conditions (Hecker and Völker, 1990; Völker *et al.*, 1992). A protective function was established for heat shock proteins (HSPs) of *Escherichia coli* and some other species enabling bacteria to survive otherwise lethal temperatures (Gottesman, 1984; Neidhardt *et al.*, 1984; LaRossa and Van Dick, 1991). HSPs can also be induced by a variety of stress agents and conditions; ethanol, heavy metals, starvation of carbon source, high salt concentration and anoxia being among the most commonly cited (Lindquist and Craig, 1988; Borkovich *et al.*, 1989; Völker and Hecker, 1992).

Groat and Matin (1986) noted that, in *E. coli*, ethanol elicits the synthesis of a subset of the HSPs; ethanol-treated and glucose-starved cells synthesised a number of proteins among which the *dna*K and *gro*EL gene products were found. These two proteins were also synthesised by *Enterococcus faecalis* in response to the addition of 2% and 4% (v/v) ethanol (Boutibonnes *et al.*, 1993). In response to ethanol, the yeast *Candida albicans* displays an altered pattern of protein synthesis (Zeuthen *et al.*, 1988). Six proteins were predominantly synthesised during exposure to 7% (v/v) ethanol, four of them being similar in size to the HSPs synthesised by cells shifted from 23 to 37°C. Some experiments on thermotolerance have shown, however, that a block in the protein synthesis does not prevent the induction of tolerance by mild heat treatments (Lindquist and Craig, 1988).

In this work the enhancement of resistance of *Lact. hilgardii* to a challenge of ethanol at 25% (v/v) after pre-treatment of cells with this and other alcohols and heat shock was studied. Cross-protection between these factors and the involvement of stress proteins are also discussed.

Materials and methods

Lact. hilgardii 5 (see Couto and Hogg, 1994) and *Lact. hilgardii* NCFB 264 (from the National Collection of Food Bacteria, Food Research Institute, Reading, UK) were grown unagitated at 25°C in MRS broth (Lab M) with pH adjusted to 4.5 (modified MRS broth) to a density of about 5×10^8 cells/ml. Bacterial counts were carried out on plates of modified MRS broth + 2% agar (no. 1, Lab M) after appropriate 10-fold dilutions in sterile modified MRS broth. For alcohol tolerance experiments cells were grown at 25°C in the absence of alcohol. The culture was then divided into 5 ml aliquots, each receiving one of the following additions: ethanol, to give 5, 10, 15 or 18% (v/v); methanol

to give 5, 10 or 15% (v/v); and butanol to give 0.5 or 2% (v/v). These shock cultures were held at 25°C during 4 h before being challenged at 25% (v/v) ethanol [modified MRS broth + ethanol (25%)]. For heat shock treatments, aliquots were shifted from 25 to 30, 35 and 40°C for the same period of time (4 h) before ethanol stress. At the time points 0, 5 and 10 min samples were removed, appropriately diluted in sterile modified MRS broth and plated in triplicate for determination of viability. Counts were made after incubation at 25°C aerobically for 4–5 days. Survival at any given time point was determined as the ratio colony forming units (c.f.u.) after treatment to the number of c.f.u. at the zero time point.

To study apparent resistance to environmental challenges, untreated cells, grown in the absence of ethanol at 25°C, and treated cells, exposed to 5, 10 and 15% ethanol for 4 h at 25°C before stress, were challenged at 30% (v/v) methanol, 5% (v/v) butanol and at 55°C.

Results

Figures represent average values of three separate experiments. Standard deviation of c.f.u. never varied more than 18% of the mean value in each time point.

The ability of *Lact. hilgardii* 5 and NCFB 264 to survive to 25% ethanol challenge increased when sub-lethal concentrations of ethanol were added to exponentially growing cells before stress (Fig. 1). 25% ethanol was used as a stress challenge, having been determined as a concentration which, at 25°C, gives a reduction in survival of the type strain of *Lact. hilgardii* of 4 log cycles over a 5 min exposure, permitting the determination of apparent inactivation kinetics over a relatively short period of time. When a culture of strain 5 was treated with sub-lethal concentrations of ethanol, its ability to tolerate an otherwise lethal challenge of ethanol after 10 min increased 10-fold following pre-treatment with 5% ethanol and about 10,000-fold with 10–18% for 4 h (Fig. 1A). The reason for such a long period of shock is that the strains used in this work, in comparison to other organisms growing in appropriate environments, show low growth rates under the conditions employed. Similar results were obtained for *Lact. hilgardii* NCFB 264 except that the 5% shock did not increase survival (Fig. 1B).

Pre-treatment of cultures of strain 5 with sub-lethal concentrations of other alcohols (methanol and butanol) also induced considerable tolerance to ethanol challenge. D-values were calculated from semilogarithmic survival curves (Table 1). 15% methanol and

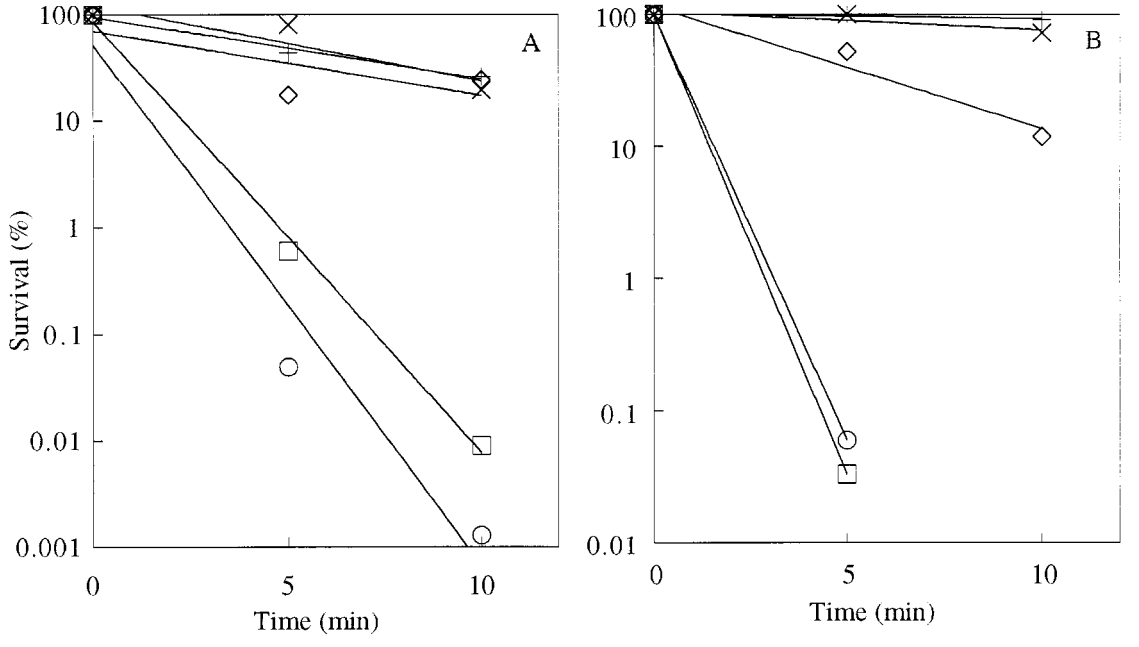

Figure 1 Effect of ethanol stress (25%) on the survival of (A) Lact. hilgardii 5 and (B) Lact. hilgardii NCFB 264. Prior to exposure, cultures were either maintained in the absence of ethanol (control) (○), or pre-treated with 5% (□), 10% (◇), 15% (X) and 18% (+) ethanol for 4 h at 25°C.

Table 1 Effect of ethanol stress (at 25% for min) on the survival of Lact. hilgardii 5 (D-values). D-value is used here to represent the time taken for a given challenge to effect a 10-fold reduction in c.f.u. Prior to exposure cultures were either maintained in the absence of any alcohol at 25°C (control) or pre-treated with methanol at 5%, 10% and 15%; pre-treated with butanol at 0.5% and 2%; and shifted to 30°C, 35°C and 40°C for 4 h.

	Treatment								
		+ Methanol (% v/v)			+ Butanol (% v/v)		Heat (°C)		
	Control	5	10	15	0.5	2	30	35	40
D-value (min)	2.2	1.3	2.8	7.1	3.7	15.6	3.9	4.3	14.1

2% butanol shocks increased the D-value three and seven times respectively. Similar enhanced survival was observed when a culture was heat shocked (25 to 40°C) for 4 h before exposure to 25% ethanol (Table 1). This increase in ethanol tolerance was acquired to a lesser extent when cells were heat shocked from 25 to 30°C or from 25 to 35°C. To attain an effect similar to 5% ethanol on *Lact. hilgardii* 5 with methanol (4 h shock), a concentration of 10% was needed, while 5% proved to be ineffective in enhancing survival (Table 1). Pre-treatment of cells with a concentration of butanol ten times lower than this (0.5%) produced an almost 70% increase in the D-value (Table 1).

Other experiments with strain 5 demonstrated cross-protection between the factors studied. Cells were pre-treated with ethanol at 5, 10 and 15% (v/v) and induction of tolerance was determined exposing cells to 30% (v/v) methanol, 5% (v/v) butanol and 55°C for 10 min (these stress challenges were determined as having similar apparent inactivation kinetics as 25% ethanol). As seen in Table 2, pre-adaptation by 4 h shocks at 10% and 15% ethanol markedly increased the D-values in all subsequent challenges. 5% ethanol was less effective and did not confer any protection against 5% butanol.

Table 2 D-values for Lact. hilgardii 5 when exposed to methanol, butanol and heat stress for 10 min. Prior to exposure cultures were either maintained in the absence of ethanol (control) or pre-treated with ethanol at 5%, 10% and 15% for 4 h at 25°C

	D-value (min)		
Ethanol (% v/v)	Methanol (30%)	Butanol (5%)	Heat (55°C)
0	2.1	1.9	3.0
5	5.9	1.1	5.0
10	10.9	9.8	8.5
15	26.2	no data	11.9

To determine whether protein synthesis *per se* is a necessary condition for the acquisition of this apparent ethanol tolerance, cells were incubated in 20 μg chloramphenicol/ml [2 × Minimum Inhibitory Concentration (MIC, previously determined as the lowest dilution of antibiotic, in a doubling dilution series, in which growth is inhibited)] during the pre-treatments before exposure to the 25% ethanol challenge. From Table 3 it can be seen that these cells also acquired tolerance to this stress and a clear difference was not found in the survival between chloramphenicol treated and untreated cells. Similar results were obtained incubating cells with 40 μg chloramphenicol/ml and with 20 μg rifampicin/ml (data not shown).

Table 3 Effect of ethanol stress (at 25% for 10 min) on the survival of Lact. hilgardii 5. Cells were either submitted or not to chloramphenicol (20 μg/ml) added to cultures 1 h before pre-treatment (shock) of cells with ethanol, methanol, butanol and heat for 4 h.

	Survival (%)								
		Ethanol (15%)		Methanol (15%)		Butanol (2%)		Heat (40°C)	
Time (min)	Control[a]	–Cm[b]	+Cm[c]	–Cm	+Cm	–Cm	+Cm	–Cm	+Cm
0	100	100	100	100	100	100	100	100	100
5	0.01	38.4	12.7	5.7	16.0	30.5	25.5	1.3	10.
10	0.003	39.2	12.8	3.5	4.5	8.7	9.1	0.09	0.05

[a] Control, cells not submitted to any pre-treatment

Discussion

This study involved organisms demonstrating intrinsic resistance to ethanol and in response to challenge concentrations of ethanol which are high for most vegetative cells (Ingram and Buttke, 1984). The data showed an increase in ethanol tolerance, as a reduction in the rate of inactivation at 25% (v/v), in *Lact. hilgardii* following ethanol shock (5, 10, 15 and 18%; 4 h).

Sub-lethal pre-treatments of *Lact. hilgardii* 5 with ethanol also induced tolerance to stress temperatures and sub-lethal heat treatments induced tolerance to otherwise lethal challenges of ethanol. This strain cannot be considered as having elevated intrinsic resistance to heat when compared to other lactobacilli (Adams and Moss, 1995). The results also show that shock exposure to sub-lethal concentrations of methanol and butanol give protection against ethanol challenge and that pre-treatments with sub-lethal concentrations of ethanol enhanced apparent resistance to methanol and butanol challenges. Cross-protection suggests that a common mechanism is induced or activated by these stimuli, thus providing a non-specific protection to cells under adverse environmental conditions.

Ethanol-induced synthesis of stress proteins has been reported and suggestions have been made that these elements are associated with enhanced ethanol tolerance in yeasts (Zeuthen, 1988; Sanchez *et al.*, 1992) and in bacteria (Arnosti *et al.*, 1986; Boutibonnes *et al.*, 1993). The experiments presented here show that induction of ethanol tolerance in *Lact. hilgardii* is obtained in the absence of stress protein synthesis. Other examples from the literature also conflict with the causal role of HSP in the acquisition of tolerance to heat. It has been found in yeasts (Hall, 1983; Watson *et al.*, 1984) and in bacteria (McCallum and Iniss, 1990; Boutibonnes *et al.*, 1992) that protein synthesis might not be required for the induction of thermotolerance.

For many organisms and other biological systems, the potency of alcohols in perturbing them can be directly correlated to their chain length and hydrophobicity (Ingram, 1986; Ingram and Buttke, 1984). This was confirmed here; the effectiveness of the pre-treatment was found to be dependent on the chain length of the alcohol, thus most resistance to stress, being induced by butanol and the least by methanol. These adaptations may be related to alcohol induced changes in membrane composition.

The possibility of pre-adapting cells to otherwise lethal concentrations of ethanol is of practical interest. Pre-treatments of *Leuconostoc oenos* at 42°C was found to improve the survival of this organism in wine and its ability to carry out the malolactic fermentation (Guzzo *et al.,* 1994).

Acknowledgements

J.A. Couto was supported by a JNICT (Junta Nacional de Investigação Científica e Tecnológica, Portugal) post-graduate studentship. This work was made possible by the generous support of the EC-AIR-CT94 2468 project on the deterioration of fortified wine.

References

Adams M.R. and Moss M.O. (1995). The microbiology of food preservation. In *Food Microbiology*. The Royal Society of Chemistry, Cambridge UK, pp 55–100.

Arnosti S.N., Singer V.L. and Chamberlin M. (1986). *J. Bacteriol.* 168, 1243–1249.

Borkovich C.A., Farrely F.W., Finkelstein D.B., Taulien J. and Lindquist S. (1989). *Mol. Cel. Biol.* 9, 3919–3930.

Boutibonnes P., Tranchard C., Hartke A., Thammavongs B. and Auffray Y. (1992). *Int. J. Food Microbiol.* 16, 227–236.

Boutibonnes P., Giard J.C., Hartke A., Thamavongs B. and Auffray Y. (1993) *Antonie van Leeuwenhoek* 64, 47–55.

Couto J.A. and Hogg T. (1994). *J. Appl. Bacteriol.* 76, 487–491.

Gold R.S., Meagher M.M., Hutkins R. and Conway T. (1992). *J. Ind. Microbiol.* 10, 45–54.

Groat R.G. and Matin A. (1986). *J. Ind. Microbiol.* 1, 169–173.

Gottesman S. (1984). *Annu. Rev. Genetics.* 18, 415–441.

Guzzo J., Cavin J.F. and Divies C. (1994). *Biotechnol. Letters* 16, 1189–1194.

Hall B.G. (1983) *J. Bacteriol.* 156, 1363–1365.

Hecker M. and Völker U. (1990). *FEMS Microbiol. Ecol.* 74, 197–214.

Ingram L. (1986). *Trends Biotechnol.* 4, 40–44.

Ingram L. and Buttke T. (1984). *Adv. Microbial Physiol.* 25, 253–300.

LaRossa A. and Van Dyk T.K. (1991). *Mol. Microbiol.* 5, 529–534.

Lindquist S. and Craig E.A. (1998). *Annu. Rev. Genetics.* 22, 631–677.

McCallum K.L. and Iniss W.E. (1990). *Arch. Microbiol.* 153, 585–590.

Neidhardt F.C., VanBogelen R.A. and Vaughn V. (1984). *Annu. Rev. Genetics.* 18, 295–329.

Sanchez Y., Taulien J., Borkovich K.A. and Lindquist S. (1992). *The EMBO Journal* 11, 2357–2364.

Völker U., Mach H., Schmid R. and Hecker M. (1992). *J. Gen. Microbiol.* 138, 2125–2135.

Watson K., Dunlop G. and Cavicchioli R. (1984). *FEBS Letters.* 169, 267–173.

Zeuthen M.L., Dabrowa N., Aniebo C.M. and Howard D.H. (1988). *J. Gen. Microbiol.* 134, 1375–1384.