Biotechnology Letters Vol 5 No 10 683-688 (1983)

ACQUISITION OF ETHANOL TOLERANCE IN YEAST CELLS BY HEAT SHOCK

Kenneth Watson* and Rick Cavicchioli

Department of Chemistry and Biochemistry
James Cook University of North Queensland
Townsville, Australia, Q4811

SUMMARY. The experiments reported here show that heat shock treatment of a number of different strains of *Saccharomyces* results in a marked increase in ethanol tolerance as compared to control cells. Our observations thus imply a relationship between the synthesis of heat shock proteins and the acquisition of ethanol tolerance in yeast.

INTRODUCTION

Strains of *Saccharomyces cerevisiae* are recognized as being among the most ethanol tolerant of microorganisms. However, the physiological basis for high ethanol tolerance is not known (Rose,1980). The plasma membrane lipid composition has been shown to influence the inhibitory effect of ethanol on the transport of amino acids and sugars into yeast (Thomas and Rose, 1979) and the viability of cells in media containing ethanol (Thomas *et al.*, 1978). Furthermore, lipid enhanced ethanol production has been observed in *Saccharomyces* (Hayashida and Ohta, 1981) and *Kluveromyces lactis* (Janssens *et al.*, 1983). More specifically, we have reported (Watson, 1982) that unsaturated fatty acids in membrane phospholipids are essential for the production of high ethanol concentrations, up to 15.5% w/v, by *Saccharomyces*.

Studies on ethanol enhanced thermal death in yeast have also led to the suggestion that membrane lipids are the target sites for ethanol inhibition (Leao and van Uden, 1982). It is generally concluded that the higher the fermentation temperature the greater is the inhibitory effect of both exogenous and endogenous ethanol (Nagodawithana and Steinkraus, 1976; Navarro and Durand, 1978).

A clue as to the involvement of protein moieties in ethanol tolerance comes from studies on a causative relationship between heat shock protein synthesis and development of thermotolerance (Li *et al.*, 1980; Li, 1983).

These studies have shown that agents, apart from heat, known to induce thermotolerance induce the synthesis of heat shock proteins and conversely, agents known to induce synthesis of heat shock proteins also induce thermotolerance. One of these agents is ethanol. The present studies report that heat shock treatment of *Saccharomyces* results in a marked increase in ethanol tolerance as compared to control cells.

MATERIALS AND METHODS

Yeast cultures. *Saccharomyces cerevisiae* CBS 1171,1242 and 1237 were obtained from the Centraalbureau voor Schimmelcultures, Delft, The Netherlands. *S.sake* ATCC 26422 was obtained from the American Type Culture Collection, Rockville, USA. Cells were grown to early log phase in 1% yeast extract, 0.5% peptone and salts (Watson, 1982) containing 10% w/v glucose (YE media). Cultures were grown at 23°C on an orbital shaker (180 rpm). Heat shock treatment of cells was as described in legends to the figures.

Viability. Cell viability was determined by staining with methylene blue (0.01% w/v) and by standard dilution plate counts on YE media containing 2% glucose and 2% agar. Colonies were counted after growth at 23°C for 3-5 days. There was good agreement between the staining and dilution plate count methods for the determination of cell viability.

RESULTS

Exponentially growing yeast cells are much more sensitive to heat killing than stationary phase cells (Schenberg-Frascino and Moustacchi, 1972). The previous growth temperature of the exponential cells, subject to heat stress, is an important factor contributing to cell survival. Cells grown exponentially for a period at 36-37° are relatively resistant to heat stress (52°C) in comparison to cells grown exponentially at 23°C (McAlister and Finkelstein, 1980; Walton and Pringle, 1980). We have exploited this acquired thermotolerance to examine the relationship between the synthesis of heat shock proteins and ethanol tolerance in yeast.

Fig. 1 illustrates the effect of exogenous ethanol on the viability of exponential cells grown at 23°C and cells heat shocked at 37°C and 52°C. Heat stressed (52°C, 5 min) cells were most susceptible to ethanol and 100% cell death occurred after 6-8 h exposure to high ethanol concentrations (14% w/v). Control cells initially showed a marked decrease in viability,

down to 40% viable after 6 h, followed by a gradual decrease in viability and finally 100% cell death at around 24-32 h. By contrast, cells grown at 23°C, heat shocked at 37°C for 30 min, and then exposed to exogenous ethanol retained their viability for prolonged periods. After an initial 10-20% cell death after 5 h exposure to ethanol, cells remained 60% viable after 24 h before decreasing to about 40% viable after 36 h. Thereafter viability gradually decreased to less than 10% after 3-4 days exposure to ethanol (results not shown). The retention of cell viability in high ethanol concentrations was also marked in experiments in which heat shock at 37°C for 30-60 min was followed by heat stress at 52°C for 5 min (Fig. 1).

Control or heat shocked cells exposed to high exogenous ethanol concentrations did not undergo significant cell division during the course of the experiments and remained in a dormant state. However, viable cells, as tested by methylene blue or rose bengal staining, when transferred to fresh growth media lacking ethanol, immediately resumed normal cell growth.

Control cells did not resume normal cell growth after exposure to exogenous ethanol for 32 h, at which time the majority of cells were dead (Fig. 1). However, even after 72 h exposure to exogenous ethanol, heat shocked cells, which remained viable, resumed normal cell growth after transfer to fresh growth media.

The ability of yeast strains to withstand heat stress and ethanol differs from strain to strain and it was important to establish that heat shock induction of ethanol tolerance is a general phenomenom in yeast. In addition to ATCC 26422 (Fig. 1) and CBS 1242 (Fig. 2) strains CBS 1171 and CBS 1237 were also used in our experiments and showed similar responses to heat shock.

As shown in Fig. 2, *S.cerevisiae* CBS 1242, responds to heat shock in a similar way to ATCC 26422 (Fig. 1). However, in this case, heat stress at 52°C/5 min resulted in 40-60% cell death before the addition of ethanol. A prior heat shock at 37°C for 30-60 min provided protection against a subsequent heat stress at 52°C/5 min and to cell death induced by exogenous ethanol. Heat shocked cells, either 37°C/30 min or 37°C/30 min followed by 52°C/5 min, remained 70% viable after 36 h in 14% w/v

ethanol. On the other hand, rapid cell death occurred after 4-6 h and 24 h in heat stressed and control cells respectively.

DISCUSSION

In *S.cerevisiae*, Plesset *et al.*(1982) have shown that preincubation of cells in the presence of ethanol (5-7% w/v) induces the synthesis of the same set of proteins that is motivated by heat shock. Protection against heat killing, although not as effective as a preincubation at 37°C, was also observed after ethanol pretreatment. Li and co-workers (Li *et al.*, 1980; Li, 1983) have examined the induction of thermotolerance and enhanced heat shock protein synthesis in Chinese hamster fibroblasts by ethanol and other agents. These studies have shown that heat shock provides partial protection against ethanol. It was concluded that a causal relationship exists between the synthesis of heat shock proteins and the survival of cells subjected to heat shock and other environmental stresses.

We report now that heat shock of yeast cells, under conditions which are known to induce synthesis of heat shock proteins, results in a marked tolerance to ethanol. It is noteworthy that, in our experiments, cells were subject to two environmental insults namely, heat shock followed by ethanol. It would be tempting to speculate that there is a class of heat shock or heat stress proteins associated with the acquisition of ethanol tolerance. If this was the case, our observations would suggest, for the first time, the possibility of cloning ethanol tolerant genes into micro-organisms.

ACKNOWLEDGEMENTS

This work was supported by grants from the Australian Research Grants Scheme, the Robert Logan Memorial Grants and Special Research Grants from the James Cook University of North Queensland.

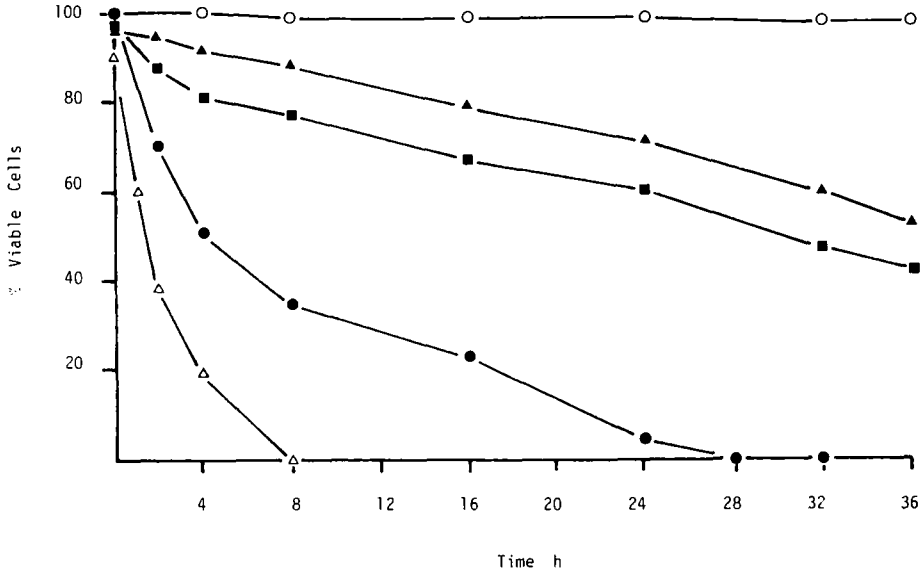

Fig. 1 Effect of exogenous ethanol on control, heat shocked and heat stressed cells of *Saccharomyces cerevisiae* ATCC 26422, a saké yeast. The final ethanol concentration was 14 W/v. (O) control cells maintained at 23°C without ethanol; (●) control cells maintained at 23°C with exogenous ethanol; (■) heat shocked cells: control cells incubated at 37°C for 30 min, cooled to 23°C and ethanol added: (▲) heat stressed cells: control cells incubated at 37°C for 30 min followed immediately by heat stress at 52°C for 5 min. Cells were cooled to 23°C and ethanol added; (Δ) heat stressed cells: control cells heat stressed at 52°C for 5 min, cooled to 23°C and ethanol added. The experiment illustrated here is typical of the results obtained on ten independent experiments.

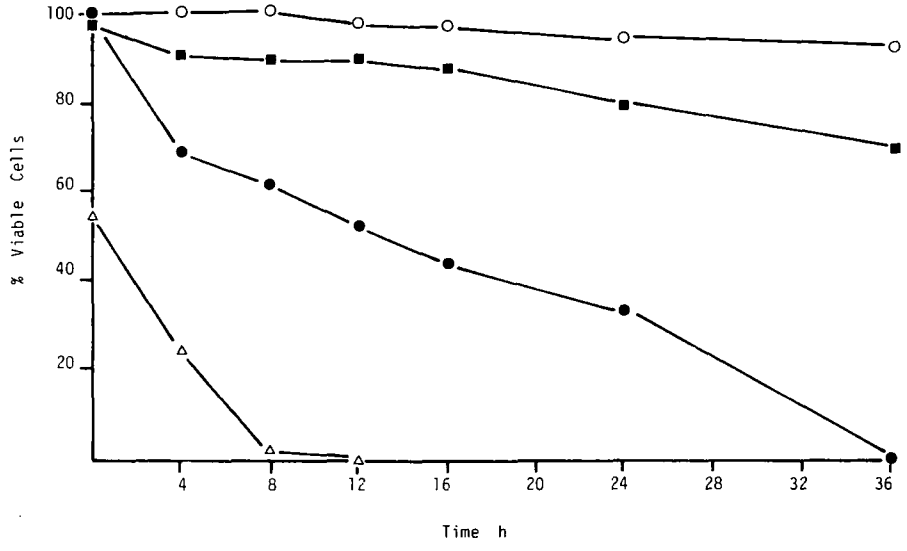

Fig. 2 Effect of exogenous ethanol on control, heat shocked and heat stressed cells of *S. cerevisiae* CBS 1242. The final ethanol concentration was 14% W/v. (O) control cells maintained at 23°C; (●) control cells maintained at 23°C with exogenous ethanol; (■) heat shocked cells: control cells heat shocked at 37°C for 30 min, cooled to 23°C and ethanol added; (Δ) heat stressed cells: control cells heat stressed at 52°C for 5 min, cooled to 23°C and ethanol added. The experiment illustrated here is typical of the results obtained on four independent experiments.

REFERENCES

Hayashida, S. and Ohta, K. (1981) *J. Inst. Brew. Lond.* 87, 42-44.

Janssens, J.H., Burris, N., Woodward, A., and Bailey, R.B. (1983) *Appl. Environ. Microbiol.* 45, 598-602.

Leao, C. and van Uden, N. (1982) *Biotechnol. Bioeng.* 24, 1581-1590.

Li, G.(1983) *J.Cellular Physiol.* 115, 116-122.

Li, G.C., Shiu, E.C. and Hahn, G.M. (1980) *Radiat. Res.* 82, 257-268.

McAlister, L. and Finkelstein, D.B. (1980) *Biochem. Biophys. Res. Commun.* 93, 819-824.

Nagodawithana, T.W. and Steinkraus, K.H. (1976) *Appl. Environ. Microbiol.* 31, 158-162.

Navarro, J.M. and Durand, G. (1978) *Ann. Microbiol. (Inst. Pasteur)* 129B, 215-221.

Plesset, J., Palm, C. and McLaughlin, C.S. (1982) *Biochem. Biophys. Res. Commun.* 108, 1340-1345.

Rose, A.H. (1980) in *Biology and Activities of Yeasts* (eds. Skinner, F.A., Passmore, S.M. and Davenport, R.R.) 103-121. London: Academic Press.

Schenberg-Frascino, A. and Moustacchi, E. (1972) *Molec. Gen. Genet.* 115, 243-257.

Thomas, D.S. and Rose, A.H. (1979) *Arch. Microbiol.* 122, 49-55.

Thomas, D.S., Hossack, J. and Rose, A.H. (1978) *Arch. Microbiol.* 117, 239-245.

Walton, E.F. and Pringle, J.R. (1980) *Arch. Microbiol.* 124, 285-287.

Watson, K. (1982) *Biotechnol. Lett.* 4, 397-402.