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Effect of NaCl on Heat Tolerance of *Enterococcus faecium* and *Enterococcus faecalis*

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Abstract: The effect of NaCl on heat tolerance of *Enterococcus faecium* BAR_1 and *Enterococcus faecalis* MI_2 was determined at 55, 60 and 62.5 °C for half an hour. Cells of both isolates were grown in Brain Heart Infusion broth containing 6.5% NaCl. Exponential phase cells were used as inoculants. Cells of both isolates were found resistant to heat treatment as compared with cells grown in BHI broth without presence of salt. The decimal reduction times (D-values) were determined by 1 log fall in viable count. Both isolates showed similar results at 55 °C but at 60 °C *E. faecalis* MI_2 showed higher D-value than *E. faecium* BAR1. At 62.5 °C *E. Faecium* BAR1 was found more tolerant to heat treatment than *E. faecalis* MI_2. Experiments showed that the presence of salt in the growth medium induces more heat tolerance in enterococci than the cells grown in normal medium.

Key words: Enterococci, NaCl, D-value, heat tolerance

Introduction

The enterococci are of great interest due to their characteristics of being the most thermoresistant among the non-sporulated microorganisms (Perez *et al.*, 1982). The enterococci are the major group of heat resistant organism that survive the processing of meat products (Gordon and Ahmad, 1991). Both *Enterococcus faecium* and *Enterococcus faecalis* have been implicated in the spoilage of meat products (Bell and DeLacy, 1984; Magnus *et al.*, 1986). The enterococci are widely distributed in foods (Perez *et al.*, 1982) and they may be etiologic agents of food poisoning (Gordon and Ahmad, 1991). The enterococci can survive in media at 60 °C for 30 minutes. They can grow in media that contain 6.5% NaCl (Kaye, 1982). The presence of high concentrations of NaCl causes many organisms difficulties in regulating and maintaining cell turgor (Cummings and Gilmour, 1995).

The 6.5% NaCl tolerance test is commonly used for presumptive identification of enterococci (Chang, 1991). Sodium chloride treatment was found to be very effective in the induction of general stress proteins in *Bacillus subtilis*: the additions of NaCl to exponentially growing cells enhance the synthesis of general stress proteins, whereas specific heat shock proteins are not induced (Hecker *et al.*, 1988). General stress proteins are synthesized with very high intensity soon after the addition of NaCl (6% w/v). Many general stress proteins have a low molecular mass (Völker *et al.*, 1992). A mild heat stress appears to be very effective in the induction of tolerance against lethal salt concentrations (Völker *et al.*, 1992).

It was shown that *Lactobacillus plantarum*, the strain not producing bacteriocin was more tolerant to salt than the strain producing bacteriocin, because the bacteriocin producing strain was unable to use lactate which is accumulated during glucose metabolism. The oxygen-dependent utilization of lactate is important as it provides an ATP yield from pyruvate oxidase and acetate kinase enzymes when other substrates are exhausted. This energy yield mechanism is absent in the strain producing bacteriocin (Montano *et al.*, 1993). Salt affects the growth and metabolism of *Lactobacilli* associated in food fermentation. It has been shown that NaCl reduces the acetate production by *Lactobacillus plantarum* (Montano *et al.*, 1993). The physiology of the bacterial cell can be affected by large increases in external salinity. Increasing levels of salt usually change the composition of the cell membrane and give rise to the production or accumulation of organic compounds to maintain positive turgor pressure. Many halotolerant bacteria require low levels of NaCl and utilize the Na^+ ion to produce a gradient across the cytoplasmic membrane. The Na^+ gradients are important in the process of solute transport. (Cummings and Gilmour, 1995). In this study the effect of NaCl on heat tolerance of barley isolate *E. faecium* BAR_1 and hospital isolate *E. faecalis* MI_2 was investigated.

Materials and Methods

Organisms and growth conditions: *E. faecium* BAR_1 was isolated from malted barely seeds provided by Dr. David G. Smith Department of Biology University College London U.K. *E. faecalis* MI_2 was obtained from Microbiology Laboratory University College Hospital U.K. Both isolates were identified by API 20 STREP kits. Stock cultures were maintained in Microbank cryovials and stored at -70 °C. Subcultures required for experimental work were kept in refrigerator. Fresh subcultures were used in each experiment. Both isolates were grown in Brain Heart Infusion (BHI, Oxoid CM 225) broth containing 6.5% NaCl to exponential phase with shaking at 37 °C.

Determination for heat tolerance: Cultures for heat tolerance determinations were grown in BHI broth containing 6.5% NaCl. A 0.1 mL sample of exponential phase cells was diluted in 25mL Maximum Recovery Diluent (MRD, Oxoid CM 733) placed at 55 °C, 60 and 62.5°C. One mL sample was removed from recovery diluent after 5 minutes interval for half an hour and diluted into 9 mL MRD at room temperature and further dilutions were made by factors of 10 upto 6 dilutions. A 20μl sample was removed from each dilution and spotted on Brain Heart Infusion (BHI, Oxoid CM 375) agar. When plates became dry, all the plates were incubated at 37 °C for 24 h. The colonies were counted and the number of survivors was plotted on a logarithmic scale against time of heat treatment. For control experiment, cells were grown to exponential phase in BHI broth at 37 °C under shaking conditions without addition of salt and treated at 62.5 °C. From the survival curves the D-value was determined. The D-value is the time taken at a particular temperature for a 1 log fall in viable count.

Results and Discussion

In this study barley isolate *E. faecium* BAR_1 and hospital isolate *E. faecalis* MI_2 were selected to investigate the heat tolerance of exponential phase cells grown in BHI broth containing 6.5% NaCl. The heat tolerance was determined at 55, 60 and 62.5°C. It was found that the cells of both isolates were resistant to heat treatments as compared with the control experiment in which the cells were grown to exponential phase at 37 °C without addition of salt and treated at 62.5 °C. At 55, 60 and 62.5°C, the cells of *E. faecium* BAR_1 survived for more than half an hour (Fig. 1). At 60 °C *E. faecium* BAR_1 was found less tolerant to heat (D-value = 9.5 min) than *E. faecalis* MI2 (D-value = 12.0 min). At 62.5 °C *E. faecium* BAR_1 was found more tolerant to heat (D-value = 7.5 min)

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Table 1: D-values (minutes) for heat treatment exponential phase cells of enterococci grown in BHI broth containing 6.5% NaCl

	D-values (min.)			
	Temperature (°C)			
Strains	55	60	62.5	Control
E. faecium BAR_1	>30.0	9.5	7.5	2.5
E. faecalis MI_2	>30.0	12.0	2.75	1.75

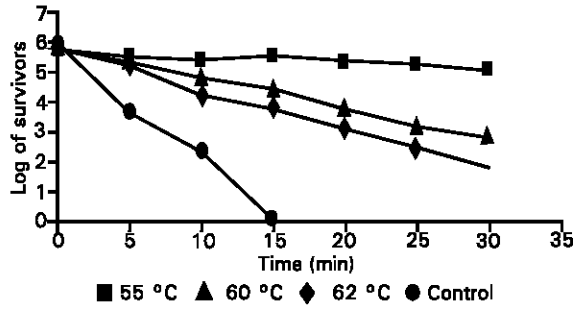

Fig.1: Heat tolerance of exponential cells of barley isolate *E. faecium* BAR_1 grown at 37 °C in BHI broth containing 6.5% NaCl. Heat tolerance was determined at 55, 60 and 62.5 °C for half an hour. In control experiment exponentially grown cells at 37°C were treated at 62.5°C.

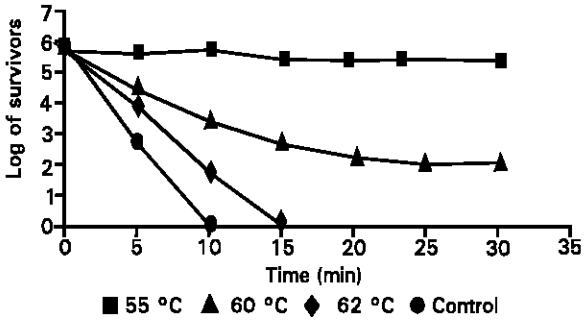

Fig. 2: Heat tolerance of exponential cells of hospital isolate *E. faecalis* MI_2 grown at 37 °C in BHI broth containing 6.5% NaCl. Heat tolerance was determined at 55, 60 and 62.5 °C for half an hour. In control experiment exponentially grown cells at 37°C were treated at 62.5°C.

than hospital isolate *E. faecalis* MI_2 (D-value = 2.75 min). *E. faecalis* MI_2 survived at 55 and 60°C for more than half an hour. At 60°C *E. faecalis* MI_2 showed higher D-value (Table 1), but at 62.5°C *E. faecalis* MI_2 survived for 10 min only (D-value = 2.75 min) Fig. 2). There was no significant difference between cells of both isolates when treated at 55°C but at 60°C *E. faecalis* MI_2 was found more tolerant to heat than *E. faecium* BAR_1. At 62.5°C *E. faecium* BAR_1 was found more tolerant to heat treatment than *E. faecalis* MI_2.

The thermoduric nature of enterococci and the mechanisms by which they suffer heat injury and subsequently recover have been investigated by several researchers (Clark *et al.*, 1968; White, 1953). When cells of *E. faecalis* R_{57} were exposed to sub lethal heat treatment, they showed a temporary change in the salt tolerance and growth of the organisms. After sub lethal heat treatment the cells were found to be unable to reproduce on media containing 6% NaCl when these heat injured cells (at 60°C for 15 min) were placed on medium, they showed an increased lag phase. It was found that during the prolonged lag phase the cells recovered their salt tolerance and they started to grow like the untreated cells (Clark *et al.*, 1968).

The 6.5% NaCl tolerance test is used to differentiate the enterococci and streptococci (Chang, 1991). Salt tolerance induces salt specific proteins as well as general stress proteins (Völker *et al.*, 1992). Hecker *et al.* (1988) reported that salt stress enhances the synthesis of general stress proteins in bacteria. The induction of stress proteins is the response of the cells to growth limiting conditions (Völker *et al.*, 1994). In the case of *Bacillus subtilis* cells pre-treated with lower salt concentrations for 30 min are able to survive the lethal salt concentrations. The stress proteins protect the cells from damage by lethal salt stress. Salt stressed cells are killed more slowly if they are treated at lethal temperatures (Völker *et al.*, 1992). *B. subtilis* is a soil living bacterium and it has to cope with adverse conditions such as desiccation and hypersalinity. It is necessary for soil living bacterial like *B. subtilis* to possess an adaptation system which protects them against high salinity in the environment (Kunst and Rapoport, 1995). *Halomonas* species are able to tolerate high-levels of NaCl concentrations. Many moderate halophiles require NaCl in the growth media. *Halomonas* species require up to 75mM NaCl (Cummings and Gilmour, 1995).

When salt shock induces stress tolerance, thermotolerance is found to be increased in the presence or absence of protein synthesis. Salt shock did not induce synthesis of proteins which are induced by heat shock. Cells with or without protein synthesis show same level of thermotolerance. It indicates that heat shock protein synthesis is not necessary for salt shock induced thermotolerance (Lewise *et al.*, 1995).

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