Effect of temperature on the role of Hsp104 and trehalose in barotolerance of *Saccharomyces cerevisiae*

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Abstract We have studied the effect of temperature on the contribution of Hsp104 and trehalose to barotolerance using mutants deficient in Hsp104 and trehalose synthesis. When compared with a corresponding wild type strain, mutants of Hsp104 did not show temperature dependent barotolerance when the incubation temperature during the hydrostatic pressure treatment was increased. However, a mutant deficient in trehalose synthesis showed features similar to a wild type strain. Furthermore, the Hsp104 level was low in the insoluble fraction of the wild type strain after pressure treatment at 35°C but not at 4°C, and the protein profiles in the insoluble fraction were different between 35°C and 4°C. In contrast to the Hsp104 deficient mutants, the protein profile of the wild type after pressure treatment at 35°C favors the role of Hsp104 as a disaggregator of proteins during hydrostatic pressure stress. These results suggest that the role of Hsp104 in barotolerance is temperature dependent in contrast to trehalose.

Key words: Barotolerance; Hsp104; Trehalose; Hydrostatic pressure; *Saccharomyces cerevisiae*

1. Introduction

When yeast cells are exposed to a temperature slightly higher than optimum, or when they enter into the stationary phase of growth, the synthesis of heat shock proteins (Hsps) and trehalose is enhanced, and the cells acquire the ability to survive under conditions of a much higher than optimum temperature [1,2]. Hsps are highly conserved from bacteria to mammalian cells and are directly involved in the biogenesis of proteins [2]. In the yeast *Saccharomyces cerevisiae*, targeted hsp gene disruption studies have shown the importance of Hsp104 on thermotolerance and only minor contributions by the other Hsps [1,3]. Parsell et al. [4] suggested that Hsp104 belongs to the family of *ClpA* and *ClpB* proteins of *Escherichia coli* which are believed to be involved in protein degradation [5]. Hsp104 forms an oligomeric structure in the presence of ATP and this is also very similar to that of the *ClpA* protein [6]. Thus, Hsp104 may regulate proteases or be involved in preventing or resolving the aggregation of vital cellular proteins [4,6,7].

Trehalose and neutral trehalase are also believed to be protectants when yeast cells are exposed to temperature extremes. It is observed that trehalose prevents protein denaturation [8] and stabilizes membrane fluidity [9,10]. In addition, neutral trehalase is induced by heat shock treatment and plays an important role in thermotolerance [11–13]. Thus, the function of trehalose in yeast is proposed to be a protectant of the macromolecular structures and an energy source for repair of cellular damage by heat [1,12].

On the other hand, hydrostatic pressure affects almost all physiological activities in living cells. Bett and Cappi [14] studied the viscosity of water as a function of pressure up to 10000 kg/cm^2. They found that relative and absolute viscosities decrease with pressure increases from zero to 2000 kg/cm^2 at ambient temperature [14]. Decreased viscosity due to high pressure results in the destruction of hydrogen bonding, and this has been reported to be a consequence of increased temperature [14]. This suggests that high temperature and high hydrostatic pressure are physically analogous. Thus high temperature and high hydrostatic pressure may cause similar damage to organisms.

Using *S. cerevisiae*, we have been studying the analogy between the biological effects of hydrostatic pressure and temperature with a view to understanding their mechanisms of action. Recently, we showed that trehalose and Hsp104 contribute to barotolerance (resistance to hydrostatic pressure) as well as thermotolerance [15]. In comparison to thermotolerance, we speculated that the low incubation temperature (4–30°C) during the hydrostatic pressure treatment was not optimal for the Hsp104 function [15] as Hsp104 showed less contribution to barotolerance than to thermotolerance. In this report, we have studied the effect of temperature on the functions of Hsp104 and trehalose toward barotolerance using mutants deficient in Hsp104 and trehalose synthesis. The results suggest that the contribution of Hsp104 to barotolerance is temperature dependent, while that of trehalose is not. We also present evidence for the differential function of Hsp104 at 35°C and 4°C.

2. Materials and methods

2.1. Strains and growing conditions

The *S. cerevisiae* strains used in this study are as follows: CWG13 (wild type, *MATa ade2 his(3 and/or 4) leu2 ura3*), CWG14 (trehalose deficient, *MATa ade2 his(3 and/or 4) leu2 ura3*), CWG15 (*MATa ade2 his(3 and/or 4) leu2 ura3 Δhsp104::LEU2*$^+$), and CWG12 (double mutant, *MATa ade2 his(3 and/or 4) leu2 ura3 Δhsp104::LEU2*$^+$) were segregants obtained from the mating between Δhsp104LEU+ (*MATa can1 ade2 his3 leu2 trp1 ura3 Δhsp104::LEU2*$^+$) provided by S. Lindquist and 224A-12D (*Matα ura3 leu2 his4* trehalose$^-$) provided by De Virgilio [15]. The trehalose and Hsp104 deficiencies were confirmed as previously described [15].

Cells were grown at 30°C on YPD medium (2% polypeptone, 1% yeast extract, and 2% glucose) or with SD medium (0.67% (w/v) Bact-yeast nitrogen base without amino acids, 2% (w/v) glucose, 50 mg amino acids or 25 mg nucleic acids were added to 1 l). One tenth of the volume of preculture grown for 2 days was inoculated into the fresh YPD medium and incubated for 4 h for logarithmic phase cells. After this period the cells were immediately cooled in ice. Heat

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shocked cells were obtained by incubating the logarithmic phase cells (without cooling) at 43°C for 1 h and immediately cooled in ice.

2.2. Hydrostatic pressure conditions

Yeast cell cultures in cooled YPD medium were poured into a 3.15 ml glass syringe (Disetronic Medical Systems AG, Switzerland), and these syringes were put into four stainless steel vessels in ice cold water. The hydrostatic pressure in the four vessels was increased in 2 min using a pressure generating system (Type wt 30000, High Pressure Equipment Co., USA). These vessels were connected to each other with a stainless flexible needle so that the hydrostatic pressure was equivalent in each vessel. After pressurization, each vessel was transferred to a different water bath at the required temperature and incubated for 80 min. Before depressurization, each vessel was transferred to an ice cold water bath and cooled for 5 min, then the vessels were depressurized for 2 min. Barotolerance was expressed as % cfu ((cfu after pressure/cfu before pressure)×100) of treated cells relative to the untreated control and is shown as the mean value from four to six independent experiments [15].

2.3. Electrophoresis and detection of Hsp104

After hydrostatic pressure treatment, cells were collected by centrifugation (2000×*g* for 5 min), washed and resuspended in 3.15 ml of Tris-HCl buffer, pH 7.4. Cells were broken with glass beads (425–600 μm, Sigma Chemical Co., USA) by vortexing 10 times for 30 s each, with 3 min intervals on ice. Broken cells were centrifuged (2000×*g* for 5 min), and the supernatant was further ultracentrifuged (200 000×*g* for 30 min). The pellets were dissolved in 50 μl of SDS lysis buffer (625 mM Tris-HCl, pH 6.8, 2.3% SDS, and 10% glycerol) or 50 μl of IEF lysis buffer (8 M urea, 2% Nonidet P-40, 2% ampholine (pH 3.5–10, Bio-Rad, Japan), 5% 2-mercaptoethanol). Hsp104 in 10 μl of SDS lysis buffer was analyzed by immunoblots, and proteins in 20 μl of IEF buffer were analyzed by 2-dimensional electrophoresis as previously described [16]. Proteins in the gels were visualized using a silver stain kit (Pharmacia, Sweden).

3. Results

3.1. Barotolerance in lower temperature region

As a follow-up of our hypothesis that low temperature during hydrostatic pressure treatment is not optimal for Hsp104 function, we studied the effect of Hsp104 on barotolerance in the temperature region of 4–30°C.

Fig. 1A shows the relative barotolerance (relative to barotolerance at 4°C) of logarithmic phase cells of CWG13, 14, 15 and 12 in various lower temperatures regions (4–30°C) under 180 MPa. The relative barotolerance is shown as relative % cfu after treatment at 180 MPa from 4°C to 30°C using the barotolerance at 4°C as factor 1. With logarithmic phase cells, the wild type (CWG13) and trehalose deficient strains (CWG14) showed a dramatic increase in relative barotolerance when temperature was increased. On the other hand, the Hsp104 deficient strain (CWG15) and the double mutant (CWG12) showed only a slight increase when compared to the wild type and trehalose deficient strains. As the wild type Hsp104 increased relative barotolerance contrary to the mutants, increased barotolerance with increased temperature up to 20°C was possibly dependent on the function of Hsp104.

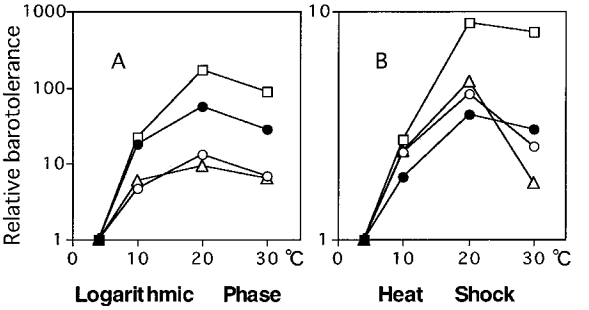

Fig. 1. Relative barotolerance of the Hsp104 and trehalose deficient mutants in the lower temperature region. The wild type strain (CWG13, ●), the trehalose deficient strain (CWG14, □), the Hsp104 deficient strain (CWG15, ○), and the double mutant strain (CWG12, △) were pressurized under 180 MPa in the lower temperature region for 80 min as described in the text. The relative barotolerance is shown as the relative % cfu after treatment at 180 MPa from 4°C to 30°C using % cfu at 4°C as factor 1.

With heat shocked cells, the trehalose deficient strain increased relative barotolerance as the temperature increased from 4°C to 20°C (Fig. 1B). However, other strains including the wild type strain did not show a significant increase in relative barotolerance compared to the trehalose deficient strain. Heat shock treatment increased the baseline of barotolerance (ex. wild type, from 4.1×10^{-2}% to 4.2%, Table 1) and this is the reason why the scales of Figs. 1A and 1B are different. Table 1 shows % cfu after hydrostatic pressure treatment at 180 MPa and at various temperatures. The wild type strain shows high barotolerance after heat shock treatment under the hydrostatic pressure conditions at 4°C (4.2%). This may be the reason why the wild type strain did not show a significant increase in relative barotolerance as the relative barotolerance was calculated relative to the barotolerance at 4°C. It should be noted that the wild type strain increased barotolerance more than 10% from 4°C to 20°C (from 4.2% to 15%), and this value is higher than any values with other strains.

3.2. Barotolerance in higher temperature region

It is known that the cellular damage by hydrostatic pressure will be dramatically increased when the incubation temperature is increased to more than room temperature [17]. Thus, barotolerance will be decreased with increasing incuba-

Table 1
Barotolerance of the Hsp104 and trehalose deficient mutants at various temperatures

Strain	Growth phase	Barotolerance (% cfu) at 180 MPa and		
		4°C	20°C	35°C
CWG13	L[a]	4.1×10^{-2}	5.6	
(wild type)	H	4.2	15	6.2
CWG14	L	9.3×10^{-3}	1.6	
(trehalose deficient)	H	2.2×10^{-1}	2.0	5.0×10^{-1}
CWG15	L	1.2×10^{-2}	1.7×10^{-1}	
(Hsp104 deficient)	H	3.9×10^{-1}	1.7	1.9×10^{-2}
CWG12	L	5.7×10^{-5}	5.4×10^{-4}	
(double mutant)	H	3.6×10^{-3}	1.8×10^{-2}	3.3×10^{-4}

[a]L, logarithmic phase cells; H, heat shocked cells.

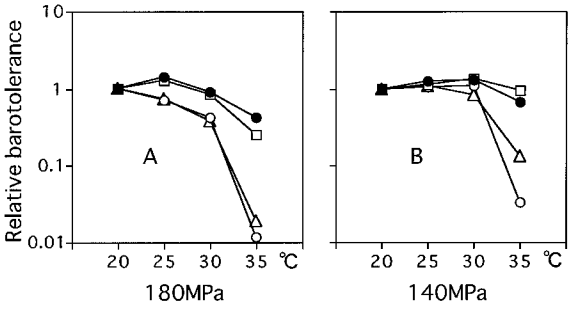

Fig. 2. Relative barotolerance of the Hsp104 and trehalose deficient mutants in the higher temperature region. The wild type strain (CWG13, ●), the trehalose deficient strain (CWG14, □), the Hsp104 deficient strain (CWG15, ○), and the double mutant strain (CWG12, △) were pressurized under 180 MPa and 140 MPa in the higher tempwerature region for 80 min as described in the text. The relative barotolerance is shown as the relative % cfu after treatment at 180 MPa and 140 MPa from 20°C to 35°C using % cfu at 20°C as factor 1.

tion temperature in the higher temperature region, in contrast to barotolerance in the lower temperature region. If the contribution of Hsp104 to barotolerance is dependent on temperature, the wild type strains would be expected to show better barotolerance than the Hsp104 deficient strains in the higher temperature region. To confirm this possibility, we examined the barotolerance of the four mutants in the higher temperature region.

As shown in Fig. 2A, the Hsp104 deficient and the double mutants dramatically decreased *relative* barotolerance as the temperature increased. In contrast, the wild type and the trehalose deficient strains maintained nearly the same degree of barotolerance with a slight increase at 25°C and decrease at 35°C under 180 MPa. At 140 MPa (Fig. 2B), all the strains showed a higher barotolerance than at 180 MPa. However, the decrease in barotolerance with the Hsp104 deficient strain and the double mutants was still obvious. The highest barotolerance of the wild type strain and the trehalose deficient strain was observed at 30°C. These results also suggest that the function of Hsp104 in barotolerance is temperature dependent while that of trehalose is not.

3.3. *Levels of Hsp104 in insoluble aggregates at low and high temperature under high hydrostatic pressure conditions*

It has been reported that Hsp104 disaggregates the temperature sensitive luciferase-fusion protein from insoluble aggregates [7]. This indicates that Hsp104 binds to insoluble aggregates for playing the role of disaggregation. If Hsp104 functions at higher temperature and does not function at lower temperature, there must be some unique feature of Hsp104 in insoluble aggregate after hydrostatic pressure treatment. Thus we determined Hsp104 in the insoluble aggregates after high hydrostatic pressure treatment (Fig. 3). The amounts of Hsp104 in the insoluble aggregates were lower after the treatment at 35°C and 140 MPa (Fig. 3, lanes 3 and 6 from the left) than after the treatment at 4°C and 140 MPa (Fig. 3, lanes 2 and 4 from the left). The same results were observed with the cells without heat shock treatment (Fig. 3, lanes 1, 2 and 3 from the left) and after heat shock treatment (Fig. 3, lanes 4, 5 and 6 from the left). This suggest that Hsp104 disaggregated from the insoluble fractions at 35°C and 140 MPa but not at 4°C and 140 MPa. It is possible that the decrease in Hsp104 is due to proteolytic digestion because increased temperature induces some enzyme activities or physiological changes. However, it is true that the differential features of Hsp104 between low temperature and high temperature are in agreement with the result that the Hsp104 deficient strain could not increase barotolerance as the temperature increased.

3.4. *Differential protein patterns at low and high temperatures under high hydrostatic pressure conditions*

Under high hydrostatic pressure conditions, Hsp104 seems to function at 35°C but not at 4°C. Thus proteins, which are substrates of Hsp104 in insoluble aggregates at 35°C, might be different from that at 4°C. Fig. 4 shows the 2D gel electrophoresis analysis of insoluble aggregates after hydrostatic pressure treatment (140 MPa) at 35°C and 4°C. Fig. 4A shows the profile of CWG13 (wild type) after treatment of 140 MPa and 4°C, Fig. 4B shows the profile of CWG13 after treatment at 140 MPa and 35°C, and Fig. 4C shows the profile of CWG15 (Hsp104 deficient) after treatment at 140 MPa and 35°C. These pressure treatments were carried out after the induction of Hsp104 by the heat shock treatment. The protein profiles in the 2D gel are different from each other. This suggests that reactions in the cells during the hydrostatic pressure treatments are different during these conditions. Some proteins could be detected in the sample of the wild type strain at 4°C as well as in the Hsp104 deficient strain at 35°C, however, they could not be detected in the samples of the wild type strain at 35°C (Fig. 4, shown in circles). These are the candidate proteins disaggregated by Hsp104 during the hydrostatic pressure and high temperature treatment.

4. Discussion

Our results show the temperature dependence of Hsp104 on the contribution to barotolerance. The Hsp104 deficient mutants could not increase (Fig. 1) and maintain (Fig. 2) their relative barotolerance when the incubation temperature was

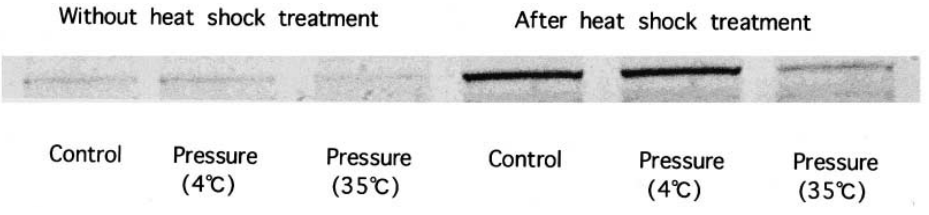

Fig. 3. Detection of Hsp104 in insoluble fractions after high hydrostatic pressure treatment. Wild type cells of logarithmic phase (lanes 1–3 from the left) and heat shocked (lanes 4–6 from the left) were pressurized at 4°C (lanes 2 and 5 from the left) and 35°C (lanes 3 and 6 from the left) under 140 MPa for 80 min. Hsp104 in insoluble fractions was visualized by immunobloting methods as described in the text. Lanes 1 and 4 from the left (control) were samples without hydrostatic pressure treatment.

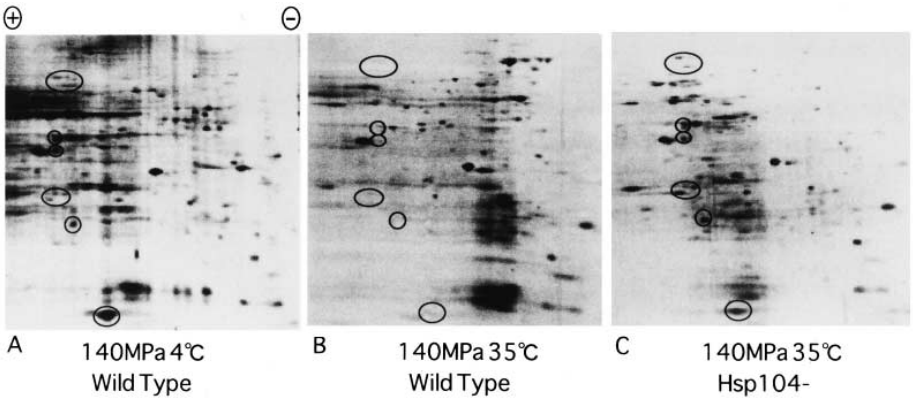

Fig. 4. 2D protein profiles of insoluble fractions obtained after high hydrostatic pressure treatment. Heat shocked cells were pressurized, broken, fractionated, and analysed by 2D gel electrophoresis as described in the text. Circles show the proteins which could be detected in 2D gels of the wild type strain (CWG13) at 4°C (A) and the Hsp104 deficient strain (CWG15) at 35°C (C) but not of the wild type strain at 35°C (B).

increased under high hydrostatic pressure conditions. The temperature dependence of Hsp104 suggests that Hsp104 is an enzyme and that the proposed function of Hsp104 as a disaggregator [7] may be a catalytic function.

In a previous report, we showed that trehalose is more important for barotolerance than for thermotolerance [15]. The reason why trehalose is more important for barotolerance is possibly due to lower activity of Hsp104 during hydrostatic pressure treatment (barotolerance) than during heat shock treatment (thermotolerance). The differential profiles of Hsp104 (Fig. 3) and proteins (Fig. 4) in the aggregated fractions support these considerations. Generally, thermotolerance is determined at around 50°C and barotolerance at 0–30°C. This different temperature possibly decreases the importance of Hsp104 and increases the importance of trehalose for barotolerance.

The substrates, which are disaggregated by Hsp104, could be detected by comparison among 2D gels of the wild type strain at 4°C, the Hsp104 deficient strain at 35°C, and the wild type strain at 35°C (Fig. 4, shown in circles). These substrates could be among the critical cellular targets protected by Hsp104 [18] during exposure to high hydrostatic pressure and high temperature. Experiments to identify these substrates are under way.

The trehalose deficient strain showed similar features of temperature dependence as the wild type strain (Figs. 1 and 2). Thus it seems that trehalose contributes to barotolerance in a temperature independent manner when compared to Hsp104. It is also true that the Hsp104 deficient strains slightly increased barotolerance. This observation may indicate that the trehalose function is also temperature dependent. However, the temperature dependence of trehalose is not as significant as that of Hsp104. It is general to understand this observation as a function of other heat shock proteins like neutral trehalase [12].

It is widely accepted that hydrostatic pressure sterilization is more effective at low temperatures (around 0°C) than at room temperature. For example, *Lactobacillus casei* is killed by hydrostatic pressure (300 MPa) with the death rate of 0.32 at 0°C, 0.1 at 20°C, and 0.32 at 60°C [17]. This phenomenon is widely understood as a mechanism similar to denaturation of enzymes under hydrostatic pressure. For example, chymotrypsinogen is denatured by hydrostatic pressure (4000 atm) with a denaturation ΔG (approximately) of -4 kcal/mol at 0°C, 0 kcal/mol at 30°C, and -4 kcal/mol at 50°C [19]. It is true that the two phase diagrams for *L. casei* and chymotrypsinogen are very similar but similarity does not always mean the same mechanisms. We postulate that effective hydrostatic pressure sterilization at low temperature is because of the low activity of Hsps, not because of an event similar to chymotrypsinogen. For hydrostatic pressure process sterilization, Hsps will be one of the most important factors under lower hydrostatic pressure and higher temperature conditions and trehalose will be important under higher hydrostatic pressure and lower temperature conditions.

Our findings in this report suggest that hydrostatic pressure could be a useful factor to analyze the mechanisms of heat shock proteins, as we can control the activity of the heat shock proteins without changing the cellular damage (from 0°C to 30°C).

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