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A stress protein is induced in the deep-sea barophilic hyperthermophile *Thermococcus barophilus* when grown under atmospheric pressure

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Abstract The whole-cell protein inventory of the deep-sea barophilic hyperthermophile *Thermococcus barophilus* was examined by one-dimensional SDS gradient gel electrophoresis when grown under different pressure conditions at 85°C (T_{opt}). One protein (P60) with a molecular mass of approximately 60 kDa was prominent at low pressures (0.3 MPa hydrostatic pressure and 0.1 MPa atmospheric pressure) but not at deep-sea pressures (10, 30, and 40 MPa). About 17 amino acids were sequenced from the N-terminal end of the protein. Sequence homology analysis in the GenBank database showed that P60 most closely resembled heat-shock proteins in some sulfur-metabolizing Archaea. A high degree of amino acid identity (81%–93%) to thermosome subunits in *Thermococcales* strains was found. Another protein (P35) with molecular mass of approximately 35.5 kDa was induced at 40 MPa hydrostatic pressure but not under low-pressure conditions. No amino acid sequence homology was found for this protein when the 40 amino acids from the N-terminal end were compared with homologous regions of proteins from databases. A *PTk* diagram was generated for *T. barophilus*. The results suggest that $P_{habitat}$ is about 35 MPa, which corresponds to the in situ pressure where the strain was obtained.

Key words Heat-shock protein · Barophile · *Thermococcus barophilus* · Pressure · Hyperthermophile

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Introduction

Bacteria have evolved abilities to sense and respond to a variety of physical signals including temperature, osmolarity, pH, and viscosity (Alibadi et al. 1988; Booth and Higgins 1990; McCarter et al. 1988; VanBogelen and Neidhart 1990). In addition to this suite of environmental parameters, deep-sea bacteria appear to be able to acclimatize to alterations of hydrostatic pressure (Prieur and Marteinsson 1998). Such alterations have been found to affect gene expressions, protein synthesis, and the extent of lipid saturation in deep-sea bacteria (Bartlett et al. 1989; DeLong and Yayanos 1985; Holden and Baross 1995; Jaenicke et al. 1988; Marteinsson et al. 1997). Even microorganisms that were not isolated from high-pressure environments may respond to high pressure by inducing the synthesis of certain "stress" proteins (PIPs), which have been identified either as heat shock, cold shock, or ribosomal proteins, while others corresponded to proteins of unknown function (for a review, see Bartlett et al. 1995).

Hydrostatic pressure was found to influence the abundance of cell-surface proteins in deep-sea bacteria adapted to low or high pressure. The moderately barophilic deep-sea bacterium *Photobacterium* sp. strain SS9 modulated the abundance of several outer membrane proteins (OMP) in response to hydrostatic pressures (Bartlett et al. 1989; Chi and Bartlett 1993). One outer membrane protein, designated OmpH, was found to be induced 10- to 100 fold in cells grown at optimum pressure conditions (28 MPa), compared with cells grown at atmospheric pressure. A second OMP, designated OmpL, was repressed at elevated pressure while a third OMP, designated OmpI, was induced at pressures above the pressure optima of SS9 and was most abundant at 40 MPa. The function of these proteins is not clear, but it has been suggested that the OmpH protein is probably a nonspecific porin protein, facilitating the uptake of substrates larger than 400 daltons (Bartlett et al. 1995).

One of the most extensively studied stress responses is the heat-shock response (Lindquist 1992), and recently this

response and its related proteins were described in the Archaea (Baross and Holden 1996; Kagawa et al. 1995; Trent et al. 1990). Some of these Archaea were obtained from deep-sea hydrothermal vents at depths greater than 2000 m, and therefore they offer an opportunity to study the adaptive responses to high temperatures and pressures. A number of thermophilic Archaea produce heat-shock proteins in response to superoptimal growth temperatures or nutritional stress (Holden and Baross 1993; Peeples and Kelly 1995), and pressure-induced alterations in protein patterns have been observed in one thermophilic species, although this organism originated from a shallow marine environment (Jaenicke et al. 1988).

The present study focused on the protein composition of two deep-sea hyperthermophilic species, *Thermococcus barophilus* and *Pyrococcus abyssi*, grown under experimentally reproduced deep-sea and shallow hydrothermal vent conditions.

Materials and methods

Cultures and media

Two deep-sea hyperthermophilic microorganisms originally isolated from hydrothermal vents were used in this study: *Thermococcus barophilus*, a barophilic strain that was isolated under in situ (35 MPa) hydrostatic pressure (Marteinsson et al. 1999), and *Pyrococcus abyssi*, a moderate barophile isolated under atmospheric pressure (Erauso et al. 1993). Cells were grown anaerobically as described by Balch and Wolfe (1976). Standard YPS medium buffered with PIPES buffer (7.0 g l^{-1} at pH 6.8) (Sigma, St. Quentin Fallavier, France) was used for growing cells under high- and low-pressure conditions (Marteinsson et al. 1999). All cultures were performed anaerobically in an anaerobic chamber with $N_2/H_2/CO_2$ (90%/5%/5%) as the gas phase in the chamber. Serum flasks (50 ml) were used for cultures grown at atmospheric pressure, incubated in ventilated ovens (Memmert). Atmospheric pressure was generated with the gas in the headspace of the serum flasks (50%). Cells were grown under high and low hydrostatic pressures in sterile gas-tight glass syringes (Ultrafit; Heinke-Sass-Wolf, GMBH, Germany) as described by Marteinsson et al. (1997). The syringes were sealed with needles plunged in rubber stoppers before medium was dispensed in the syringes containing about 0.1 g sulfur, which were then inoculated with 1%–2% exponentially growing cells. The cut pistons were put in place and the gas phase was expelled before tightening the seal on each syringe. Finally, the syringes were transferred into the high-pressure, high-temperature incubation system for cultivation. The temperature and pressure of each pressure vessel can be controlled independently, or the pressure can be equilibrated between two vessels. Cultures were stopped by cooling the pressurized vessels under a continuous flow of cool water (~2 min for cooling to 50°C).

The *PTk* diagram

A high-pressure growth curve or *PTk* diagram for *T. barophilus* was generated from published and unpublished determination of growth parameters under hydrostatic pressures (Marteinsson et al. 1999). Each line is drawn at a constant growth rate. The highest contour level surrounds the values of Pk_{max} and Tk_{max}. Duplicates were pressurized to the test pressure before heating the vessel ovens. Heating started at 22°C; approximately 30 min were required to obtain stable test temperatures (75°, 80°, 85°, 90°, 95°, and 98°C). Each temperature was tested at high (40 MPa) and low pressure (0.3 MPa) at the same time. Pk_{max} was determined under 30 MPa and 50 MPa at 85°C.

Cellular protein extraction and PAGE

Proteins were extracted, as described by Marteinsson et al. (1995), from cells grown at different pressure conditions to late exponential growth phase (approximately 5×10^8 cells/ml) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970). Equal amounts of protein (protein concentrations, 40 μg) were estimated by the Bradford method (1976) using the Bio-Rad assay kit (Bio-Rad, Richmond, CA, USA), were loaded onto 5%–20% one-dimensional gradient polyacrylamide gels (5% stacking gels). The following molecular weight standards were loaded onto each gel: rabbit muscle phosphorylase b (97400), bovine serum albumin (66200), hen eggwhite ovalbumin (45000), bovine carbonic anhydrase (31000), soybean trypsin inhibitor (21500), and hen egg white lysozyme (14400). Coomassie brilliant blue R-250 was used to stain the gels, which were then dried with a gel drying kit (Promega, Madison, WI, USA). The gels were scanned (Hewlett Packard Scan Jet 3C) and a densitogram was produced from each lane with the NIH image 1.54 program (Ohlendorf Research, Ottawa, Canada). Protein profiles were determined for cells grown to midexponential, late exponential, and stationary growth phases.

N-terminal sequence analysis

Cultures grown in YPS-20 medium at 85°C under 40 MPa and atmospheric pressures were run on SDS-PAGE gradient gel (5%–20%) (Hoefer electrophoresis apparatus; San Francisco, CA, USA). Proteins were transferred onto PVDF (Bio-Rad, Ivry Sur Seine, France) membrane in a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad) in 50 mM Tris and 50 mM boric acid. After an overnight transfer and a wash in double-distilled water, the membrane was stained with 0.1% (v/v) Amido Black [in 45% (v/v) methanol and 1% (v/v) acetic acid], before excising the pressure-induced proteins from the air-dried membrane. The protein sequence reaction was performed in the laboratory for protein microsequences of the Institute of Pasteur (Paris, France).

Results

The *PTk* diagram

The *PTk* diagram illustrates and quantifies several aspects of bacterial growth with respect to temperature and pressure (Fig. 1). The highest contour level surrounds the values of Pk_{max} and Tk_{max}. The Pk_{max} of *T. barophilus* varies from 35 MPa to at least 50 MPa, or $Pk_{max} = P_{habitat}$. The relationship between Tk_{max} and $T_{habitat}$ is difficult to determine because of the steep temperature gradients at vents and consequently is unknown.

Cellular protein profiles

The responses of *T. barophilus* and *P. abyssi* to growth at high pressure were surveyed by comparing proteins synthesized at high and low hydrostatic pressure. Identical protein profiles were produced in *P. abyssi* when grown at low and high hydrostatic pressures (data not shown), but two proteins were induced under different growth conditions in *T. barophilus* (Fig. 2). One protein, P35 (approximately 35.5 kDa), was significantly induced under 40 MPa at 85°C (T° opt), whereas no apparent induction occurred under low-pressure conditions or 0.1 MPa atmospheric and 0.3 MPa hydrostatic pressure (T° opt). The second protein (P60) of approximately 60 kDa is present at high pressures; however, it is significantly induced at atmospheric pressure or under low hydrostatic pressure (0.3 MPa) at 85°C (Fig. 2) and at 75°, 80°, and 90°C (data not shown). The protein is not induced at 85°C under 10 or 30 MPa, but it is slightly induced at 98°C under 40 MPa (data not shown). Comparison of extracts from cells harvested at various times during logarithmic and stationary growth phases indicated that the pressure-induced proteins obtained after SDS-PAGE did not exhibit visible significant changes (data not shown).

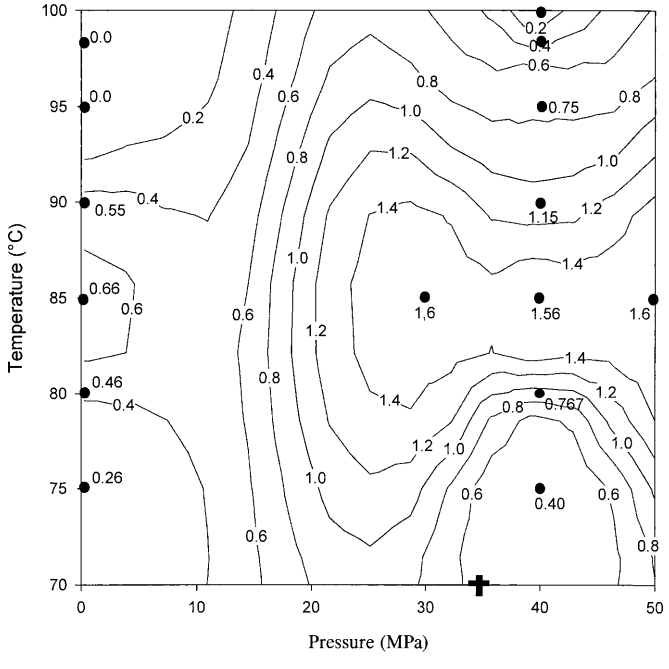

Fig. 1. A PT_k diagram as described by Yayanos (1995) of the growth response of *Thermococcus barophilus* under different hydrostatic pressures. The diagram was generated from published (Marteinsson et al. 1999) and unpublished data. The numbers next to the *black dots* are the growth rate-constant values used to generate the contours. The *large cross marks* are the pressure conditions where the chimney sample was retrieved and from which the strain was later isolated

N-terminal sequence analysis and homology

From the N-terminal end of proteins P35 and P60, 40 and 17 amino acids, respectively, were sequenced. No amino acid sequence homology was found in the GenBank database for protein P35. However, P60 closely resembled the hypothetical thermophilic factor (549 amino acid residues) in *Pyrococcus horikoshii* with 93% amino acid identity and 93% positives; heat-shock protein HHSP (M_r = 59.137 or 546 amino acid residues), a thermosome subunit in *Pyrococcus* sp. strain KOD1 with 81% identity and 93% positives; thermosome β-subunit in *Thermococcus* sp. strain KS-1 (546 amino acid residues) and KS-8 (545 amino acid residues) with 81% identity and 93% positives; thermosome α-subunit in *Thermococcus* sp. strain KS-1 (548 amino acid residues) and KS-8 (549 amino acid residues) with 75% identity and 93% positives; thermosome β-subunit (545 amino acid residues) in *Desulfurococcus* with 81% identity and 93% positives (Kagawa et al. 1995); and thermosome β-subunit (543 amino acid residues) in *Thermoplasma acidophilus* with 73% identity and 80% positives (Waldmann et al. 1995) (Fig. 3).

Discussion

We have shown that pressure-sensitive proteins are expressed in the barophilic hyperthermophile (piezohyperthermophile) *Thermococcus barophilus*, originating from a hydrothermal vent at depth of 3500 m, and confirmed that such proteins are not expressed in the other barophilic hyperthermophile *Pyrococcus abyssi* (Marteinsson et al. 1997), which was isolated from a vent at a depth of 2000 m (Erauso et al. 1993).

Two different protein responses were observed in this study. One protein (P60) was induced at low pressures (0.3 MPa), but not at the in situ pressure of the strain (40 MPa). Production of a second protein (P35) was increased under 40 MPa, but not at low pressure. Both proteins are expressed at optimal temperature (85°C) for both high- and low-pressure conditions, respectively. The variation in P60 abundance was not significantly altered at other temperatures tested under atmospheric pressure. This result indicates that protein synthesis did not change with growth rate and that protein synthesis is reduced at high hydrostatic pressure.

Comparisons with the GenBank database indicate that P60 most closely resembled the stress or heat-shock

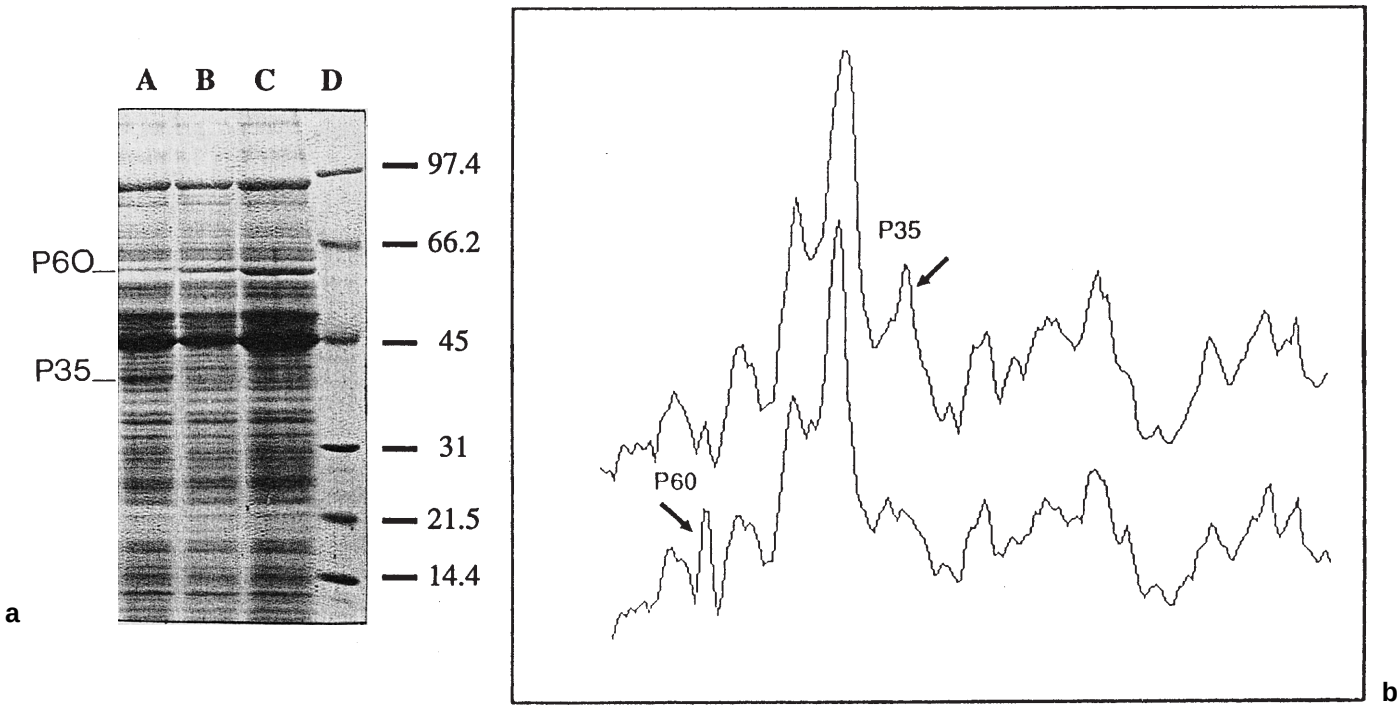

Fig. 2. **a** Cellular protein profiles of *T. barophilus* grown under different pressures. *Lanes A, B*: total cellular proteins from cells (late exponential growth phase) grown at 85°C under 40 or 0.3 MPa; cultures were prepared in syringes in an anaerobic chamber with $N_2/H_2/CO_2$ (90%/5%/5%) as the gas phase in the chamber, and the gas phase was expelled before tightening the seal on each syringe. *Lane C*: total cellular proteins from cells (late exponential growth phase) grown at 0.1 MP; cultures were grown in bottles with the anaerobic chamber gas as head space. *Lane D*: molecular weight standards as described in text. Cells grown to midexponential, late exponential, and stationary growth phases under high and low pressures were used as controls (data not shown). *Bars* indicate the induced stress protein P60 in *lanes B* and *C* and the pressure-induced protein P35 in *lane A*. **b** Densitograms of *lanes A* and *B* using Hewlett Packard Scan Jet 3C and NIH image 1.54. *Arrows* indicate the peaks for the pressure-sensitive proteins P60 and P35

Thermococcus barophilus	hypothetical HSP, P60	1:		-	Q	L	A	G	Q	P	I	L	I	L	P	E	G	T	T	R	17	100%	100%
Pyrococcus horikoshii	hypoth. thermophilic factor	1:	-	-	Q	L	A	G	Q	P	I	L	I	L	P	E	G	T	Q	R	18	93%	93%
Pyrococcus sp. strain KOD1	thermosome subunit	1:	-	-	Q	L	A	G	Q	P	V	V	I	L	P	E	G	T	Q	R	18	81%	93%
Desulfurococcus mobilis	thermosome subunit	1:	-	-	Q	L	A	G	Q	P	V	V	I	L	P	E	G	T	Q	R	18	81%	93%
Thermococcus sp. strain KS-1	thermosome, ß subunit	1:	-	-	Q	L	A	G	Q	P	V	V	I	L	P	E	G	T	Q	R	18	81%	93%
Thermococcus sp. strain KS-8	thermosome, ß subunit	1:	-	-	Q	L	A	G	Q	P	V	V	I	L	P	E	G	T	Q	R	18	81%	93%
Thermococcus sp. strain KS-1	thermosome, æ subunit	1:	-	-	Q	L	A	G	Q	P	V	V	I	L	P	E	G	T	Q	R	18	75%	93%
Thermococcus sp. strain KS-8	thermosome, æ subunit	1:	-	-	Q	L	A	G	Q	P	V	V	I	L	P	E	G	T	Q	R	18	75%	93%
Thermoplasma acidophilus	thermosome, ß subunit	1:		-	Q	I	A	G	Q	P	I	F	I	L	K	E	G	T	K	R	16	73%	80%

Fig. 3. Comparison of the N-terminal amino acid sequence of the induced stress protein P60 from *Thermococcus barophilus* with heat-shock proteins in some sulfur-metabolizing Archaea. Amino acid residues that are not identical to the stress protein P60 are *boxed*. Percentage (%) shows the degree of amino acid identity and positives, respectively

proteins. However, no amino acid sequence homology was detected for P35, suggesting that it is a novel protein that requires pressure for expression.

The expression of P60 may be repressed under pressure as the OmpL protein of the *Photobacterium* mutant strain SS9 (Chi and Bartlett 1993). However, this is unlikely because the protein P60 is not induced at 10 MPa or under higher hydrostatic pressures, while the abundance of OmpL decreased with pressure. Moreover, the P60 protein is induced both under low-pressure conditions (less favorable for growth) and under 40 MPa at 98°C (the extended temperature limit for growth of this organism under pressure),

thus indicating a more general protein expression response, such as a stress or heat-shock response. This explanation is further supported because the N-terminal sequence of the protein P60 is most homologous to stress or heat-shock proteins. P60 most closely resembled (75%–93%) several heat-shock proteins belonging to the GroEL/chaperonin family (thermosome β-subunit chaperonin) that are found in some sulfur-metabolizing thermophilic Archaea. Although we have only sequenced 17 amino acids of the P60 protein, comparisons of complete protein sequence of two heat-shock proteins closely related to our protein exhibited a very high degree of similarity throughout the entire protein sequence. Therefore, comparison of the N-terminal ends should be sufficient to obtain an estimation of the degree of relatedness between the proteins. The results also suggest that these proteins are well conserved within sulfur-metabolizing Archaea.

The function of heat-shock proteins in stabilizing proteins has been well studied with bacterial GroES/GroEL systems (Gibbons and Horowitz 1996; Schmidt et al. 1994; Weissman et al. 1996). One such chaperonin, the β-subunit of the chaperonin from *Pyrococcus* sp. strain KOD1, shows a high degree of amino acid identity to P60. It has been reported that this heat-shock protein has both stabilization and solubilization effects on proteins (Yan et al. 1997; Yoshida et al. 1997). The *T. barophilus* protein may have a similar role. Both pressure and heat influence the fluidity in cells and promote liquid or gel transitions on cells. At superoptimal growth temperature, the fluidity of cellular components is increased while pressure causes loss of fluidity (the entropy decreases). The chaperonin P60 is slightly induced in *T. barophilus* when it grows at superoptimal growth temperatures, which suggests it may have a stabilizing effect on denatured proteins. Moreover, the protein P60 could also have a solubilization effect on proteins when the strain is grown at optimal temperature without in situ hydrostatic pressure (unfavorable conditions). *T. barophilus* could have a mechanism similar to processes involving a solubilization effect on cellular components in cells exposed to lethal temperatures at atmospheric pressure whereby the fluidity increases. The P60 protein may have a solubilization effect on cellular components when *T. barophilus* is grown at low-pressure conditions as the fluidity increases. This process provides a mechanism for organisms that are adapted to deep-sea environments to survive shallow conditions if ocean currents transport them away from their deep-sea niche. Additionally, closely related members of the order *Thermococcales* were found in hydrothermal vents at many different depths, from the deep sea to terrestrial environments (Prieur et al. 1995), and the protein responses reported here may support theories on the dispersal and evolution of *Thermococcales*. Support for this hypothesis is provided by P60, which is very similar (93% similarity) to a another stress protein found in a *Thermococcales* species (*Pyrococcus*, KOD1) isolated from a solfatara at a wharf on Kodakara Island (Hoaki et al. 1994).

Furthermore, *T. barophilus* is adapted to deep-sea pressures. The *PTk* diagram shows how temperature and pressure affect the growth rate of a bacterium and facilitates identifying relationships among bacteria from habitats differing in temperature and pressure (Yayanos 1995). The diagram suggests that the $P_{habitat}$ for *T. barophilus* is around 35 MPa, which correlates with the pressure conditions where the chimney sample was retrieved and from which the strain was later isolated. However, the strain also grows well at higher pressures, similar to those that would be expected in the deep subsurface. If deep-sea hydrothermal vents are "windows into the subsurface biosphere" (Deming and Baross 1993), then *T. barophilus* may serve as a model organism that may be encountered in such a subsurface environment.

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