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# A review of acquired thermotolerance, heat shock proteins, and molecular chaperones in archaea

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## Abstract

Acquired thermotolerance, the associated synthesis of heat-shock proteins (HSPs) under stress conditions, and the role of HSPs as molecular chaperones under normal growth conditions have been studied extensively in eukaryotes and bacteria, whereas research in these areas in archaea is only beginning. All organisms have evolved a variety of strategies for coping with high-temperature stress, and among these strategies is the increased synthesis of HSPs. The facts that both high temperatures and chemical stresses induce the HSPs and that some of the HSPs recognize and bind to unfolded proteins in vitro have led to the theory that the function of HSPs is to prevent protein aggregation in vivo. The facts that some HSPs are abundant under normal growth conditions and that they assist in protein folding in vitro have led to the theory that they assist protein folding in vivo; in this role, they are referred to as molecular chaperones. The limited research on acquired thermotolerance, HSPs, and molecular chaperones in archaea, particularly the hyperthermophilic archaea, suggests that these extremophiles provide a new perspective in these areas of research, both because they are members of a separate phylogenetic domain and because they have evolved to live under extreme conditions.

## Introduction

Acquired thermotolerance refers to the enhanced survival of organisms exposed to lethal temperatures after being exposed to a near-lethal temperatures for a short time [1,2]. During exposure to the near-lethal temperature, referred to as heat shock, organisms stop synthesizing their normal diverse array of proteins and focus on synthesizing a limited number of heat-shock proteins or HSPs [2-5]. The HSPs generally fall into five groups on the basis of their molecular masses: the "small HSPs" (less than 40 kDa); the HSP60s

(60 ± 5 kDa); the HSP70s (73 ± 5 kDa); the HSP90s (approximately 90 kDa); and the HSP100s (105 ± 5 kDa) [6]. Although the classification by molecular mass is somewhat arbitrary, in some cases, particularly the HSP60s and HSP70s, it reflects a clear evolutionary relationship [7,8].

The fact that heat shock and chemical agents that induce HSP synthesis also effect protein conformation (i.e., cause proteins to unfold and aggregate) led to the conclusion that the role of HSPs in vivo is to help cells cope with unfolded proteins, either by binding to them to prevent aggregation, marking them for proteolysis, or assisting in their refolding [6,9-11]. The specific roles of individual HSPs in preventing unfolded proteins from aggregating or disaggregating or in recycling denatured proteins is still a matter of active research [12-14]. In vitro experiments indicate that two of the most abundant HSPs, HSP60 and HSP70, recognize and bind unfolded proteins, thereby preventing aggregation; and through this interaction, the HSPs help proteins regain their functional conformation [15-17]. These in vitro observations, as well as the abundance of HSP60 and HSP70 under normal growth conditions, have led to the hypothesis that these proteins may be assisting in the initial folding of newly synthesized proteins in vivo. In this role, HSPs have been referred to as "molecular chaperones" [18-20]. As molecular chaperones, these proteins are believed to play an essential role in the final stages of the protein folding in the cell under normal conditions [15], and their role as HSPs in acquired thermotolerance is an extension of their normal function [10,21].

The correlation between acquired thermotolerance and HSP synthesis led to the hypothesis that the two phenomena are related, and subsequent research generally supports this hypothesis [3,22-25]. Acquired thermotolerance and HSPs are reported in all organisms, although at least one species of hydra (phylum: Coelenterata) does not acquire thermotolerance or synthesize HSPs [26]; however, most studies do support the claim of universality of acquired thermotolerance and HSP synthesis [27,28]. In different species, different HSPs may be focal; and in some species,

macromolecules besides HSPs are also important [29-33]. In the yeast *Saccharomyces cerevisiae,* for example, thermotolerance depends on the synthesis of HSP104 under some conditions [24,34]; but under other stress conditions, other HSPs, proteins, or even carbohydrates are involved [32,35,36]. In the fruit fly, *Drosophila melanogaster,* no HSP104 is detected, and HSP70s are believed to be critical [37,38]. In mammalian cells, a variety of HSPs, especially the small HSPs, appear to be important [30,39-41]; although under some conditions, thermotolerance is acquired without HSP synthesis; and carbohydrates, steroids, or other proteins have been implicated [42,43]. In *Escherichia coli*, the role of specific HSPs or groups of HSPs in thermotolerance is not yet clear, and some evidence suggests that it may be independent of HSP synthesis [44]. Mutations in HSP60 (GroEL) and HSP70 (DnaK), however, produce temperature-sensitive phenotypes [16,45,46].

Relatively little research has been done in acquired thermotolerance and the associated HSPs in archaea; however the work that has been done has provided some interesting insights. This may be due to the distinct phylogenetic position of the archaea [47,48] and the extreme environmental conditions to which many archaeal species are adapted. In this paper, I critically review the published research on archaeal thermotolerance, HSPs, and molecular chaperones, to indicate the status of our knowledge in these areas and to stimulate further research.

## Acquired thermotolerance in archaea

Three reports of acquired thermotolerance in archaea describe the responses of two species, one from each of the major archaeal groups [49-51]. One species, *Sulfolobus shibatae,* is a crenarchaeon that grows between 60 and 86 °C [52]; and the other, ES4, is a euryarchaeon that grows between 66 and 110 °C [53]. The reports about *S. shibatae* clearly demonstrate that a one-hour heat shock at 88 °C significantly increases the ability of cells cultured at 70 °C to survive exposures to the lethal temperature of 92 °C [49]. Shorter heat-shock treatments (15 and 30 minutes) did not provide adequate

time for the cells to prepare themselves for survival at the lethal temperature, and longer treatments (two and four hours) did not significantly improve survival compared with the one-hour treatment. The heat-shocked cells survived for approximately two hours longer than control cells (shifted directly from 70 to 92 °C) and then died at the same rates, suggesting that whatever stabilizing factors were produced during heat shock lost their efficacy with time. This report correlates the observed acquired thermotolerance with significant changes in protein synthesis, suggesting that during heat shock, only a single, very abundant protein is synthesized. (A later report [54] shows that what appeared to be a single protein on polyacrylamide gels was in fact two closely related proteins--see below). The link between thermotolerance and HSP synthesis in *S. shibatae* was corroborated by heat shock experiments using 4-azetidine-2-carboxylic acid (azetidine) [51]. Azetidine is an amino acid analog that is known to disrupt functional protein synthesis. Its presence during heat shock of *S. shibatae*, which was presumed to eliminate the accumulation of functional HSPs, also eliminated thermotolerance.

The report of acquired thermotolerance in ES4 is difficult to interpret but suggestive. Cultures were grown at 95 °C, were heat-shocked at 102 °C for 90 minutes, and were challenged at the lethal temperature of 105 °C [50]. (This suggests that the reported upper temperature limit of 110 °C for ES4 may be somewhat exaggerated). Indications of thermotolerance were based on death curves of heat-shocked and non-heat-shocked cells at 105 °C, determined by direct counts of DAPI-stained cells. The authors recognized that DAPI (4,6-diamidino-2-phenylindole·2HCl), which is a DNA-specific fluorescent dye, does not distinguish between living and dead cells and therefore calibrated their DAPI counts by comparing them with viable cell numbers determined by most probable number techniques. The fact that these two procedures gave similar results suggests that under their experimental conditions, the DNA from ES4 was extracted from lysed cells and that the majority of intact cells (retaining their DNA) remained viable. In this report, thermotolerance was not correlated

with protein synthesis, but minor changes were observed in the protein pattern indicated by silver staining of total proteins on denaturing polyacrylamide gels.

All three of these studies suggest that hyperthermophilic archaea acquire thermotolerance after heat shock. Like the typical thermotolerance experiments with mesophiles, a part of a culture grown at normal temperatures is heat-shocked for a prescribed period of time, and then the survival of non-heat-shocked and heat-shocked cells at a lethal temperature was compared. Unlike experiments with mesophiles, in which viability is usually assayed by monitoring the number of cells that appear on plates after a recovery period under optimal growth conditions [55], determining the viability of hyperthermophilic archaea required alternative assays. While plating techniques are available for hyperthermophiles [56], these procedures are not quantitative, and therefore viability was assayed by either a dilution technique for *S. shibatae* [49,51] or by "viable counting" for ES4 [50]. The difficulty with dilution techniques is that the method is labor intensive; and results are only approximate, depending on the dilutions used. The problem with viable counting is that it must be correlated with viability measured independently, and this result depends on the labeling dye used and the response of the organism. Improvements in plating procedures for hyperthermophilic archaea or experiments with archaea that can be quantitatively plated, such as halophiles and some methanogens, may provide additional information about acquired thermotolerance. Alternatively, procedures measuring the rate of recovery of normal protein synthesis after heat shock, such as those described for HeLa cells [41], which use incorporation of radiolabeled amino acids or mixture of amino acids into newly synthesized proteins to indicate the condition of cells, may be used with archaea, provided defined media are available. This procedure also provides information on archaeal HSPs.

## Heat shock proteins in archaea

Two of the three studies of thermotolerance in hyperthermophilic archaea reported changes in protein synthesis associated with heat shock and the acquisition of thermotolerance [49,51]. Five other studies have reported on HSPs in archaea, including halophiles [57], methanogens [58], and hyperthermophiles [54,59,60]. In all of these studies, cells were pulse-labeled at normal and heat-shock temperatures using radiolabeled amino acids (usually L-{$^{35}$S}methionine); total cellular proteins were extracted and separated by polyacrylamide gel electrophoresis (PAGE); and the HSPs were identified in autoradiographs as protein bands that were significantly more prominent after heat-shock treatment than during normal growth. Such experiments revealed that euryarchaea (halophiles and methanogens) that have been studied synthesize a diversity of HSPs under stress, while the crenarchaea synthesize a very limited number.

In an early report showing the stress response of seven halophilic strains (*Halobacterium volcanii, H. trapanicium, H. marismortui, H. halobium, H. salinarium*, R-4, and Y-27), cells pulse-labeled at normal (37 °C) or heat-shock (60 °C) temperatures induced between four and six protein bands, ranging in molecular mass from 75-105, 44-45, and 21-28 kDa [57]. It is tempting to relate these HSPs to those with corresponding molecular masses described from bacteria and eukaryotes, but in most cases no sequence data or immunological cross-reactivities support this relationship. Daniels and coworkers [57] reported that unpublished Southern analyses indicated a gene with homology to the bacterial HSP70 may be present in *H. volcanii.* An HSP70 gene was later cloned and sequenced from *Halobacterium marismortui* [7], suggesting that HSP70 may be among the pulse-labeled protein bands seen in other halophiles. Evidence that the HSP70 gene is heat inducible and sequence information from other HSPs in halophiles remain to be determined.

For methanogens, a heat-shock response has been reported in *Methanococcus voltae* (a mesophilic species) pulse-labeled at a normal growth temperature of 30 °C and heat-shocked at 40-50 °C

[58]. In this species, autoradiographs after one-dimensional PAGE showed 11 heat-inducible bands ranging in molecular mass from 18 to 89 kDa. Most of these same proteins were also shown to be induced by stresses other than heat (i.e., ethanol, hydrogen peroxide, and osmolarity), which are stresses known to stimulate HSP synthesis in bacteria and eukaryotes [28]. Attempts to identify two of the most conserved HSPs in bacteria and eukaryotes (HSP70 or HSP60) in *M. voltae* were unsuccessful using immunoassays and heterologous Southern hybridizations. Negative results provide no definitive proof that these proteins or genes are not present; however, they clearly indicate that further research is required to identify these and other putative HSPs in this organism.

Both HSP70 and HSP60 have been positively identified in other methanogens. Southern hybridizations indicated that an HSP70-related gene is present in *Methanosarcina barkeri* [61], and an HSP70 gene was later cloned and sequenced from *M. mazei* [62]. Immunoassays suggest the presence of HSP60-related proteins in *M. barkeri,* as well as *Methanobacterium thermoautotrophicum* [63], and a partial sequence of an hsp60-related gene was found in *Methanococcus jannaschii* (G. Olsen, personal communication). The entire genome of *M. jannaschii* is now being sequenced, which will make it possible to identify the full range of HSPs in this organism, either by analyzing conserved HSP sequences or by matching partial amino acid sequences determined from the HSPs themselves with the complete sequence information.

Knowledge of the heat-shock response in crenarchaea, indicated by pulse-labeling experiments, is limited to two species of *Sulfolobus*. *Sulfolobus acidocaldarius* grown at the normal temperature of 70 °C and heat-shocked at 85 °C significantly increased the synthesis of two protein bands (64-66 kDa) and slightly increased the synthesis of three others [64]. *Sulfolobus shibatae* grown at 70 °C and heat-shocked at 88 °C showed a similar response, significantly increasing the synthesis of what appeared to be a single major protein band (55 kDa) and slightly increasing four others of 27, 32, 49, and 82 kDa [49,51]. In both species, nearly all

normal protein synthesis was inhibited during heat shock. In *S. shibatae,* the amount of isotope associated with the 55-kDa band increased nearly eight-fold during heat shock relative to its synthesis at 70 °C [60]. Contrary to reports that a single 55-kDa protein, referred to as TF55, was associated with this band in polyacrylamide gels [65], the 55-kDa band consists of two closely related proteins of 59 kDa each [54]. The genes for both of these proteins were cloned and sequenced [54,65], and their deduced amino acid sequences have 54% homology [54]. Both gene products are heat inducible and appear to be coregulated so that both transcription and translation are significantly increased by heat and to about the same level. Kagawa and coworkers [54] report similarities in their promoter regions that may be regulatory, but further work is needed to determine the mechanism for the coordination of these two genes.

Both electron microscopy and non-denaturing PAGE indicate that the two *S. shibatae* HSP60s are associated with a double-ring complex superficially resembling the bacterial HSP60 double-ring complexes known as molecular chaperones [65].

It should be noted that while HSP70 is clearly present in the euryarchaea that have been studied, it is conspicuously missing in both species of *Sulfolobus* and perhaps other crenarchaea. Efforts to identify the HSP70 gene in Southern hybridizations with total DNA from many species of crenarchaea have been unsuccess (Trent, unpublished), as have attempts to amplify the HSP70 gene from genomic DNA of *S. shibatae* using PCR (Trent and Osipiuk, unpublished). Considering the diversity of biological functions that have been ascribed to HSP70 [69], it will be interesting to explore what other proteins fulfill these functions in the crenarchaea if this protein is indeed absent.

## The archaeal HSP60s as chaperonins

The bacterial HSP60s are abundant proteins under normal growth conditions and can be isolated from cells in a high-molecular-mass complex consisting of 14 subunits, two rings of seven subunits each [66-68]. These double-ring structures are believed to function

as chaperonins [15,20], binding newly synthesized proteins and assisting in their proper folding and assembly through an iterative process that involves other chaperones and ATP hydrolysis [69-72]. The current model suggests that the nascent polypeptide 'hides' from the chemically complex intracellular milieu within the hollow core of the chaperonin rings and in this context it attempts to find its functional conformation [20,73,74].

The archaeal HSP60s are also abundant proteins that can be isolated as double-ring complexes; although rather than 14 subunits, they contain 16 or 18 subunits, arranged in rings of eight or nine subunits [65,75-77]. On the basis of symmetry considerations, it was suggested that the two rings of the *S. shibatae* chaperonin are homo-oligomeric, consisting of one or the other HSP60 proteins [54]; but this suggestion has yet to be substantiated. The double-ring complex itself, which has a rosette appearance in electron micrographs (Fig. 1), has been referred to as a "rosettasome." It was later referred to as an "archaeosome" [78], but the name rosettasome takes precedence in the literature. Double-ring structures isolated from both *Pyrodictium occultum* [75,79] and *Thermoplasma acidophilum* [80] have been referred to as "thermosomes." There is evidence that all of these proteins are closely related. Polyclonal antibodies against both the *S. shibatae* rosettasome and the *P. occultum* thermosome cross-react with each other, as well as a diversity of approximately 60-kDa proteins in immunoblots of total proteins from 18 out of 22 archaeal species tested (Table 1). Electron microscopic studies indicate that 60-kDa proteins are associated with double-rings complexes in five archaeal species (Table 2); and sequence information, which is complete for five archaeal HSP60s and is partial for four others, confirms that these archaeal proteins are closely related; in fact, they are highly conserved (>50% amino acid identities) between species (see [54]). Some evidence exists that these archaeal double-ring structures have functional features similar to those of the bacterial chaperonins. Such features include ATPase activity, the ability to recognize and bind unfolded polypeptides, and an involvement in protein folding, at least in vitro.

Table 3 lists the reports on four archaeal species that have been shown to have chaperonin complexes (cpn) by electron microscopy or native gel electrophoresis and to have at least one of the functional features of chaperonins [81,82]. Table 3 also lists the reports on species that are known to have acquired thermotolerance and that produce HSPs.

Structurally and functionally, the archaeal HSP60s appear to be related to the bacterial chaperonins, at least superficially; however sequence information reveals that the archaeal HSP60s are only distantly related to the bacterial HSP60s and are remarkably closely related to a family of eukaryotic proteins known as TCP1s [54,65]. The TCP1s are not HSPs, but are ubiquitous among eukaryotes, are primarily cytosolic, and form double-ring complexes referred to as TRiC (TCP1 ring complex) or CCT (chaperonin-containing TCP1). Since the discovery of the relationship between the *S. shibatae* HSP60 and TCP1 [65], a significant effort has been made to demonstrate that TRiC functions as a chaperonin in the eukaryotic cytosol (for review see: [71,83]; however, despite these efforts, there is only limited evidence that TCP1s have any chaperonin functions [84-86], and a growing number of studies suggest that this protein family has other important functions in the cell [87-91].

## Concluding remarks

Limited research efforts have focused on studies of acquired thermotolerance, HSPs, and molecular chaperones in archaea, and relatively few papers have been published in these areas; however, it is clear that hyperthermophilic archaea, living near the upper temperature limits for life, acquire thermotolerance after a short heat shock, and during this heat shock, they primarily synthesize 60-kDa proteins that share structural and functional features with bacterial chaperonins. The discovery of the relationship between the archaeal HSP60s and the cytosolic proteins in eukaryotes known as TCP1s was hailed as added proof that the chaperonin model for in vivo protein folding could be generalized to include eukaryotes [92]; however, as research progresses with both the archaeal HSP60s and

the TCP1s, it is becoming increasingly evident that the chaperonin model, based primarily on in vitro observations from bacterial chaperonins, may not be generally valid. Structural studies continue to reinforce the homology between the archaeal HSP60s and the eukaryotic TCP1s (proteins in organisms that evolutionarily diverged billions of years ago and inhabit mutually exclusive environments) and insist that a functional link must exist between them; however, functional studies continue to confound their putative role as chaperonins and suggest alternative functions exist for these proteins in vivo.

While efforts to understand thermotolerance, HSPs and chaperonins in archaea have been limited to relatively few laboratories, these extremophiles are clearly providing an interesting perspective.

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Figure Caption

**Fig. 1:** Electron micrograph of the *Sulfolobus shibatae* chaperonin known as a "rosettasome." Photographed using a Philips EM420T with LAB6 filaments at 80 kV and processed using Adobe Photoshop on a PowerMac computer. The diameter of the ring is approximately 16 nm.

**Table 1:** Immunological cross-reactivity between archaeal HSP60s using polyclonal antibodies against *Sulfolobus shibatae* rosettasome (Rsm) and the *Pyrodictium occultum* thermosome (Tsm). (Number of pluses indicates strength of reaction, minuses indicate no reaction, and "NR" means not reported.)

| Organism | Names | Cross-Reactivity | | |
|---|---|---|---|---|
| | | anti-Rsm* | anti-Tsm* | no. of bands |
| **Crenarchaeota** | | | | |
| *Sulfolobus shibatae* | α&β rosettasome | +++ | NR | 2 |
| *S. acidocaldarius* | | +++ | +++ | 2 |
| *S. solfataricus* | TF55 α&β Ssocpn | +++ | NR | 2 |
| *Acidianus brierleyi* | | +++ | ++ | 2 |
| *Desulfurolobus ambivalens* | | +++ | NR | 3 |
| *Hyperthermus butylicus* | | +++ | NR | 2 |
| *Pyrodictium occultum* | α&β thermosome | ++ | +++ | 2 |
| *P. abyssum* | | ++ | +++ | 2 |
| *Thermoproteus tenax* | | ++ | NR | 1 |
| *Staphylothermus marinus* | | ++ | ++ | 2 |
| *Pyrobaculum islandicum* | | NR | + | |
| **Euryarchaeota** | | | | |
| *Pyrococcus furiosus* | | ++ | (+) | 1 |
| *Thermococcus litoralis* | | ++ | (+) | 1 |
| ES4 (*Thermococcus sp.*) | | ++ | NR | 1 |
| *Archaeoglobus fulgidus* | | +/- | ++ | 1 |
| *Thermoplasma volcanium* | | +/- | NR | 1 |
| *T. acidophilum* | α&β thermosome | NR | +++ | 2 |
| *Methanothermus fervidus* | | +/- | ++ | 1 |
| *Methanopyrus kandler* | | - | - | |
| *Methanobacterium thermoautotrophicum* | | - | NR | |
| *Methanococcus thermolithotrophicus* | | - | - | |
| *Mc. igneus* | | - | - | |

* Rsm data from Kagawa et al. [54]; Tsm data from Phipps et al. [79].

**Table 2:** Summary of structural information about archaeal HSP60 double rings, including the number of subunits per ring from electron microscopy (EM) studies and the number of amino acids (aa) sequenced for the α and β subunits. (numbers in "parentheses" are partial sequences, and "NR" means not reported.)

| Organism | EM subunits/ring | Reference | Sequence subunit & no. aa | Reference |
|---|---|---|---|---|
| **Crenarchaeota** | | | | |
| *Sulfolobus shibatae* | 9 | Trent et al 1991 [65]<br>Marco et al. 1994 [76] | α **560**<br>β **552** | Trent et al 1991 [65]<br>Kagawa et al. 1995 [54] |
| *S. solfataricus* | 9 | Knapp et al. 1994 [77] | α **(10)**<br>β **(10)** | Knapp et al. 1994 [77] |
| *Pyrodictium occultum* | 8 | Phipps et al. 1991[79] | α<br>β **NR** | |
| **Euryarchaeota** | | | | |
| *Pyrococcus furiosus* | ? | Trent, unpubl. | **NR** | |
| *P.* sp. strain KOD1 | NR | | **560** | Kogita et al. unpublished data |
| *Thermococcus litoralis* | 8 | | **(55)** | Trent & Joachimiak unpublished data |
| ES4 (*Thermococcus* sp.) | ? | Shen, Adams,<br>& Trent, unpubl. | **NR** | |
| *Thermoplasma acidophilum* | 8 | Waldmann et al. 1995 [80] | α **554**<br>β **553** | Waldmann et al. 1995 |
| *Methanococcus jannaschii* | NR | | **(26)** | Olsen, unpublished data |

**Table 3**: Papers reporting acquired thermotolerance, HSP, a double-ring chaperonin structure (cpn) and chaperonin functions (ATPase, peptide binding, and protein folding) in the two major branches of archaea.
(Numbers refer to references listed below; "NR" means not reported).

| | acquired thermotol. | HSP | cpn | ATPase | peptide binding | protein folding |
|---|---|---|---|---|---|---|
| **Crenarchaeota** | | | | | | |
| *Sulfolobus shibatae* | 1, 2 | 1-3 | 3,4 | 4 | 4 | NR |
| *S. acidocaldarius* | NR | 5 | NR | NR | NR | NR |
| *S. solfataricus* | NR | NR | 7 | 6 | 6 | 6 |
| *Pyrodictium occultum* | NR | NR | 8 | 8 | NR | NR |
| **Euryarchaeota** | | | | | | |
| *ES4 (Thermococcus* sp.*)* | 9 | NR | NR | NR | NR | NR |
| *Thermoplasma acidophilum* | NR | NR | 10 | 10 | 10 | NR |
| *Methanococcus voltae* | NR | 11 | NR | NR | NR | NR |
| *Halobacterium volcanii* | NR | 12 | NR | NR | NR | NR |


**References:**

1. Trent *et al.* 1990 [49]
2. Trent *et al.* 1994 [51]
3. Kagawa *et al.* 1995 [54]
4. Trent *et al.* 1991 [65]
5. Jerez 1988 [59]
6. Guagliardi *et al.* 1994 [81]; 1995 [82]
7. Knapp *et al.* 1994 [77]
8. Phipps *et al.* 1991[79]
9. Holden *et al.* 1993 [50]
10. Waldmann *et al.* 1995 [80]
11. Herbert *et al.* 1991 [58]
12. Daniels *et al.* 1984 [57]