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THE MOLECULAR CHAPERONE SYSTEM AND OTHER ANTI-STRESS MECHANISMS IN ARCHAEA

Alberto J. L. Macario, and Everly Conway de Macario

Wadsworth Center, New York State Department of Health; and Department of Biomedical Sciences, School of Public Health, The University at Albany (SUNY); Albany, New York, USA

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1. ABSTRACT

This article presents a brief review of stressors, their cellular and intracellular targets, stress proteins, molecular chaperones, and other anti-stress mechanisms. New data are reported on cochaperones and multicellular structures in archaea. The molecular chaperoning systems of bacteria and eukaryotes have been studied for many years and are relatively well known in terms of their components and mechanisms of action, although many details remain to be elucidated and almost certainly other components will be discovered in the future. By comparison, the molecular chaperoning system of archaea is still unexplored. Since archaea have some molecular genetic and physiologic features similar to those of bacteria and some resembling those of eukaryotes, extrapolation from what is known of organisms from these two phylogenetic domains to archaeal species is unwarranted. For example, the components of the molecular chaperone machine, Hsp70(DnaK), Hsp40(DnaJ), and GrpE, in the archaeal species that have it, are closely related to bacterial counterparts, whereas the archaeal chaperonins are like the eukaryotic equivalents. Furthermore, many archaeal species lack the chaperone machine, in contrast to bacteria and eukaryotes that have it without any known exception. A search for the cochaperones trigger factor, Hop, Hip, BAG-1, and NAC in archaeal genomes demonstrated no conserved equivalents, but two families of archaeal molecules were identified that might be related to NAC and Hop, respectively. Multicellular structures with a single species such as packet and lamina are formed by *Methanosarcina* species, among which the best studied is *M. mazeii*. Multispecies multicellular structures are formed by a variety of archaeal organisms, which are either flat (biofilm) or globular (granule) and constitute a functional association or consortium. Details of morphology, formation, and internal organization are described for representative examples of multicellular structures. These

may be seen as the result of primitive histogenesis reflecting primeval mechanisms of differentiation-development that might have evolved driven by environmental stressors. Cells in these complex threedimensional arrangements are not only positioned so they can interact with each other for more efficient functioning as in a tissue or organ, but are also protected from stressors. Single cells lacking the protective shield of other cells packed together with intercellular connective material, which is typical of multicellular structures, are directly exposed to environmental stressors and, thus, are at a disadvantage from the evolutionary standpoint. It seems reasonable to argue that differentiation-development leading to histogenesis might have arisen in primeval times as a consequence of the harsh conditions that primitive life forms had to endure, and that the ability to form tissue-like structures was a primary characteristic that ensured positive selection.

2. INTRODUCTION

Living cells need a set of proteins in their native configuration within certain levels of concentration to perform their functions and to survive emergencies. Protein synthesis, maturation (*e.g.*, post-translational modifications), transport, assembly into polymers in some cases, and degradation are the main steps towards maintaining a protein balance adequate for life and survival. The cell has evolved means to achieve and maintain protein balance even under stressful conditions. Among these means the chaperoning system is crucial. Its relevance is more manifest when a cell encounters a stressor and has to mount a stress response. The major players in the stress response are the stress proteins, among which are the molecular chaperones. The cell also has other ways to cope with stress. An example of an anti-stress mechanism different from the chaperoning system is the formation of multicellular structures. The ability to build multicellular structures reflects differentiation and development, which increase in complexity with the degree of complexity of the organism. It is possible that formation of multicellular structures in simple organisms represents a primitive form of differentiation and development, namely, a manifestation of primeval histogenesis. It is clear that some simple organisms respond to stressors by generating multicellular structures. How much did stressors contribute to the appearance and subsequent development of organismal complexity during evolution? Were primitive, very simple organisms driven towards the development of multicellular structures lest they perish in the harsh environments in which they were thrown by Nature in primeval times?

This article deals with the chaperoning system of archaeal organisms and it also presents examples of multicellular structures formed by these organisms. Since the molecular chaperone machine and the chaperonins of archaea have already been reviewed elsewhere, they will not be treated to any significant extent here. Rather, new data will be reported on chaperone cofactors as they pertain to the Archaea.

3. STRESSORS AND THEIR TARGETS

Stressors are varied in nature, table 1 (1); see also contributions by Cannio *et al.*, Conway de Macario & Macario, and Roberts in this Special Issue). They all cause a set of effects on every cell. A subset of these effects is common to all stressors while other subsets are each characteristic of a given stressor or family of similar stressors. Moreover, the type of cell stressed also contributes to determine the type and magnitude of the effects a stressor may cause. Examples of the cell's attributes that can be affected by stressors are listed in table 2 (1).

4. ANTI-STRESS MECHANISMS

Survival during eons of evolution, and during every day's routine, depends to a considerable extent on anti-stress mechanisms. Living cells, whether organisms in themselves (single-celled organisms) or part of multi-celled animals, plants, and fungi, are exposed to stressors, probably quite often throughout nature. Consequently, cells have evolved anti-stress mechanisms, some of which are listed in table 3; see also article by Conway de Macario & Macario in this Special Issue. The various components of the cell, prokaryotic and eukaryotic, are endowed with anti-stress mechanisms. These mechanisms, one must assume, work together in coordination to counteract the effects of stressors. A sample of the pro-life consequences of the anti-stress mechanisms is shown in table 3. Detailed descriptions of anti-stress molecules and mechanisms are provided in the other chapters of this Special Issue, as indicated in table 3.

5. STRESS PROTEINS

Anti-stress mechanisms are mediated by molecules. These are proteins and non-protein compounds and structures that in one way or another impede or delay protein denaturation caused by stressors, restore partially denatured molecules to the native configuration, or eliminate proteins damaged beyond repair. Examples of stress proteins are given in table 4 (1). Group 1 includes the molecular chaperones and the chaperonins. Some of these molecules are considered the landmarks of the cell's response to stress, named the heat-shock or stress response. The reasons why the terms heat-shock response, heat-shock gene, and heat-shock protein (Hsp) were coined and are still in use are given in the Preface of this Special Issue. Hsp means heat-shock or stress protein(s), although there are distinctions to be made in this regard. A stress protein is the product of a stress gene whose distinctive characteristic is that it is induced by a stressor or stressors (these are listed in table 1). More specifically, Hsp refers to a protein from a gene that is induced by the stressor heat (heat shock). Nowadays one tends to consider as stress proteins all those that play a prominent role during the stress response and in the post-stress phase during which the cell recovers and returns to normality. They are all named Hsp or stress proteins, interchangeably, regardless of the stressor that induces their genes, heat shock or any other.

Table 1. Cell stressors[a]

Type	Name. Description
Physical	Heat (including fever); cold; several types of irradiation, including ultraviolet light.
Oxygen	Oxygen-derived free radicals (ROS); hydrogen peroxide; anaerobiosis to aerobiosis shift (*e.g.*, reperfusion); hypoxia-anoxia (ischemia)
pH	Alkalosis; acidosis; pH shift
Biological	Infection; inflammation; fever
Psychological	Emotion; hormonal imbalance due to HPA axis dysfunction
Osmotic	Changes in the concentration of salt, sugars, other osmolytes (hyper- or hypo-osmotic shock)
Nutritional	Starvation: multiple; specific (carbon, glucose, nitrogen, phosphate, nitrate)
Antibiotics	Puromycin; tetracycline; nalidixic acid
Alcohols	Ethanol; methanol; butanol; propanol; octanol
Metals	Cadmium; copper; chromium; zinc; tin; aluminum; mercury; lead; nickel
Insecticides, pesticides	Lindane; diazinon; paraquat; thiram; tributyltin
Mechanical	Compression; shearing
Other	Benzene and derivatives; phenol and derivatives; teratogens; carcinogens; mutagens; arsenite; arsenate; amino-acid analogs; nicotine; anesthetics; desiccation

[a]Reproduced from reference (1) with permission from the copyright owner. Abbreviations are: ROS, reactive oxygen species; HPA axis, hypothalamic-pituitary-adrenal axis. These agents cause stress in cells from the three phylogenetic domains, Bacteria, Archaea (both prokaryotes), and Eucarya (eukaryotes).

Table 2. Stressor targets and methods for study[a]

Target	Method
Viability; growth	Cell counts
Morphology (size; shape; formation of multicellular structures, or spores)	Light and electron microscopies. Biochemical and immunochemical analyses of cell envelopes and intercellular connective materials. Histochemistry. Immunohistochemistry
Proteins contents	One-D and two-D electrophoresis
Synthesis	
• General	Incorporation of radioisotope-labeled amino acids followed by quantification of radioactive fractions
• Specific	Separation-quantification of stress proteins using specific antibodies (immunoprecipitation, Western blotting). Identification of protein complexes by electron microscopy-computerized imaging combined with immunochemistry and functional assays. Microsequencing. Mass spectrometry
Genes[b]	Measurement of stress genes' transcripts: quantity, length, transcription-initiation and termination sites (Northern blotting, primer extension, S-1 nuclease protection, reverse PCR)

[a]Reproduced from reference (1) with permission from the copyright owner. [b]Presence or absence of genes is determined by: cloning and sequencing; genome sequencing; Southern, Northern, and Western blottings.

6. CHAPERONES AND CHAPERONINS

Several stress proteins are molecular chaperones or chaperonins that assist protein folding, refolding, and translocation (2-6). Other stress proteins are neither chaperones nor chaperonins, and vice versa there are chaperones that are not stress proteins, since their genes are not stress inducible. Chaperones and chaperonins are instrumental in maintaining cellular proteins at physiologic levels; they are a central component of the mechanism that maintains in an active configuration and within the normal range of concentrations the array of proteins necessary for all physiologic functions. An alteration in the native configuration of a protein will most likely induce a stress response, and will call into action the Hsp, including the chaperones and chaperonins, and also protein-degrading enzymes (Group 3 in table 1; see contribution by Maupin-Furlow *et al.* in this Special Issue).

7. STRESS PROTEINS IN PROKARYOTES

A sample of the known stress proteins--mostly chaperones and chaperonins--of bacteria and archaea is displayed in table 5 (7)--those present in eukaryotes have been reviewed elsewhere (8). These proteins are classified into families according to their molecular mass. Those belonging to the 70 kDa family are considered the typical chaperones, whereas those of the 60 kDa family are the chaperonins. Hsp70 (also named DnaK) exercises its chaperone functions by teaming up with Hsp40 (also termed DnaJ) and GrpE--in the cytosol of the eukaryotic

Table 3. Examples of anti-stress mechanisms: cell locale and effects

Main locale	
Intracellular	
Chaperones and chaperonins (see tables 4 and 5)	
Other stress proteins (Hsp) from stress-inducible genes (see table 4)	
Other proteins from non-stress-inducible genes (see tables 4 and 5)	
Non-protein molecules (see table 4, Group 6)	
Peri- and extra-cellular	
Cell membrane as a whole; membrane lipids and proteins	
Cell envelope (periplasm, sheath, capsule)	
Intercellular-connective material of multicellular structures	
Main effect	**See, in this Special Issue, article by:**
Protein stability	Scandurra *et al.*
Osmoadaptation; resistance to osmotic stress	Roberts
Cell membrane resistance	Albers *et al.*
Protein folding and re-folding, and formation of multicellular structures	Macario & Conway de Macario (this article)
Accelerate formation of *cis* peptide bonds preceding proline residues (protein folding) with participation of peptidyl-prolyl *cis-trans* isomerases (PPIases or rotamases)	Maruyama & Furutani
Protein degradation and recycling	Maupin-Furlow *et al.*
Anti-oxidant	Cannio *et al.*
Cold acclimation; resistance to cold stress	Conway de Macario & Macario

Table 4. Stress proteins[a]

Group	**Examples**
1	Molecular chaperones and chaperonins in organisms of the three phylogenetic domains: Bacteria, Archaea, and Eucarya. Prokaryotic representatives are listed in table 5
2	Regulators of stress (heat-shock) genes: positive regulators or activators (*e.g.*, heat-shock factor or HSF, in eukaryotes); negative regulators (*e.g.*, HSF4 in eukaryotes; HrcA (and CIRCE), HspR, and CtsR in bacteria); sigma factors (*e.g.*, Φ^{32}, Φ^{E}, Φ^{B}) in bacteria
3	Proteasome components and ubiquitin in archaea and eukaryotes. Other proteases: Clp family and FtsH(HflB), Lon/La and HB8, and HtrA(DegP), in bacteria
4	Proteins in the intercellular connective material of multicellular structures formed as a response to stress (*e.g.*, in the archaeon *Methanosarcina mazeii*)
5	Transport proteins (*e.g.*, TrkA in the archaeon *Methanosarcina mazeii*)
6	Enzymes and co-factors involved in the synthesis of the proteins listed in 1-5, above. Enzymes and cofactors responsible for the synthesis and/or accumulation of thermoprotectants ("chemical" chaperones, osmolytes, compatible solutes), for example trehalose in the yeast *Saccharomyces cerevisiae*, di-myo-inositol phosphate and cyclic diphosphoglycerate in some archaea, and compatible solutes in bacteria and archaea
7	All proteins, enzymes, co-factors, and gene-regulatory factors involved in sporulation *(e.g.*, in the bacterium *Bacillus subtilis*)

[a]Modified from reference (1), with permission from the copyright owner. For additional references see (8).

cell GrpE is replaced by other cochaperones or cofactors. Hsp70(DnaK), Hsp40(DnaJ), and GrpE form the molecular chaperone machine in bacteria, and also in some archaeal species.

Hsp70(DnaK) is important for life, particularly under stressful conditions, and is therefore highly conserved in occurrence and sequence in bacteria and eukaryotes. Strikingly, however, several archaeal species do not have Hsp70(DnaK) or the other components of the chaperone machine, Hsp40(DnaJ) and GrpE (9). This biological and evolutionary puzzle has been discussed elsewhere (10). It is difficult to accept the fact that a cell will survive stress without the molecular chaperone machine. The immediate question is: What replaces the machine, so those species that do not have it, can still produce the complete physiologic array of proteins, correctly folded and translocated to their place of action in the cell? How do these Hsp70(DnaK)-less cells survive stress? How do these cells cope with stress-induced protein denaturation?

The archaea with documented molecular chaperone machine genes are listed in table 6 (11). Others will most likely be found as the number of sequenced archaeal genomes increases. In the case of archaea that possess the molecular chaperone machine genes the question is: Does the archaeal chaperone machine work like

Table 5. Stress proteins and chaperones in prokaryotes[a]

Family		Examples	
Name(s)	**Mass (kDa)**	**Bacteria**	**Archaea**
Heavy, High M.W.,Hsp100	100 or higher	ClpE	No[b]
Hsp90	81-99	HtpG	No
Hsp70, DnaK, Chaperones	65-80	DnaK; Hsc66	Hsp70(DnaK)
Hsp60, Chaperonins	55-64	GroEL	TF55; Chaperonin subunits (thermosome subunits)
Hsp40, DnaJ	35-54	DnaJ; Trigger Factor (TF)	Hsp40(DnaJ)
Small Hsp, sHsp	34 or lower	GroES; Hsc20; GrpE; PPIase; PDIase (DsbA-D, G); IbpA and IbpB; SecB; Hsp15; other	GrpE; PPIase; PDIase (?); Prefoldin (GimC)

[a]Modified from reference (7) with permission from the copyright owner. For additional references see (11). [b]No, not yet investigated, or investigated but not yet found, or found but incompletely characterized.

Table 6. The molecular chaperone-machine genes found in archaea

Gene	Organism	OTG °C[a]
hsp70(dnaK)	*Methanosarcina mazeii* S-6	37
	• *thermophila* TM-1	50
	Methanobacterium thermoautotrophicum ΔH	65
	Haloarcula marismortui	45
	Halobacterium cutirubrum	45
	Thermoplasma acidophilum	55
hsp40(dnaJ)	*Methanosarcina mazeii* S-6	37
	• *thermophila* TM-1	50
	Methanobacterium thermoautotrophicum ΔH	65
	Halobacterium cutirubrum	45
grpE	*Methanosarcina mazeii* S-6	37
	• *thermophila* TM-1	50
	Methanobacterium thermoautotrophicum ΔH	65

[a]OTG, optimal temperature for growth.

that of bacteria or differently? This question is pertinent because archaea have other components of the chaperoning system that are different from those found in bacteria, and that are supposed to cooperate or interact with the chaperone machine. In other words, how can a chaperone machine made up of molecules of the bacterial type work in the archaeal cell whose other components (*e.g.*, chaperonins that are supposed to interact with the chaperone machine) are of eukaryotic type? Moreover, the genes *hsp70(dnaK)*, *hsp40(dnaJ)*, and *grpE* that encode the components of the molecular chaperone machine have apparently been established in archaea by lateral transfer from bacteria (10). If that is so, how are these bacterial-like genes regulated in a cell that has transcription-initiation mechanisms of eucaryal type, substantially different from those of bacteria? These, and other similar questions are awaiting answers, which will clarify important aspects of the evolution of stress-gene transcription and regulation, and of how lateral gene transfer modifies the host cell or vice versa in terms of gene regulation.

8. RECENT ADVANCES: CHAPERONE COFACTORS AND THE RIBOSOME

The whole range of molecules that participate in protein folding in archaea has not yet been elucidated, and to achieve this goal years of research are certainly going to be needed. Probably, some chaperones and their functions and mechanisms of action will turn out to be very similar to those of bacteria. Little is known about the interaction, if any, between the chaperone machine and the chaperonin complex in archaea, just to give an example of the information voids that must be filled.

Protein folding is a complex event that involves several molecules (2-6). The central player is the polypeptide to be folded or refolded. The target for the mechanism of folding or refolding is either a nascent polypeptide that has to achieve the native configuration so it turns into a mature, functional protein, or a partially unfolded (partially denatured) protein that has to regain its native configuration. The complexity of protein folding depends on the organism, prokaryote or eukaryote, and on the protein (2-6). For example, in bacteria some proteins do not seem to need assistance from other molecules such as chaperones to attain a functional configuration, while others do. If a polypeptide is long with more than one functional domain, it will most likely require assistance from the chaperoning system. This assistance must be provided very early in the polypeptide's life to avoid misfolding during translation and, thus, there is co-translational chaperoning. Consequently, the role of the ribosome and ribosome-associated proteins in the folding

Table 7. Distribution of molecular chaperones and chaperonins in the three phylogenetic domains[a]

Bacteria	Archaea	Eucarya
GroEL (Hsp60)	No[b]	mt, chl: Hsp60[c] (Rubisco subunit binding protein) ct, ER: No
GroES (Hsp10)	No	mt, chl: Yes ct, ER: No
No	TF55; thermosome subunits	ct: TRiC (CCT; TCP-1) subunits mt, chl, ER: No
G+, DnaK[d] (Hsp70)	Hsp70(DnaK)[e] No-hyp.[f]	No
G-, DnaK[d] (Hsp70)	No	mt, chl: Hsp70 ct, ER: Hsp70 para.[g]
DnaJ (Hsp40)	Hsp40(DnaJ)[e] No-hyp.	ct, mt, chl, ER: Yes
GrpE	GrpE No-hyp	mt, chl: Yes ct, ER: No
G-, HptG	No	ct, ER: Hsp90 para. mt, chl: No

[a]Reproduced from reference (8) with permission from the copyright owner. [b]No, not yet investigated or not well characterized, or investigated but not yet found. [c]Abbreviations are: mt, mitochondria; chl, chloroplast; ct, cytosol; ER, endoplasmic reticulum. [d]G+ and G-, Gram positive and negative bacterial type of DnaK, respectively. [e]Protein similar to Gram positive bacterial homologs but transcription initiation mechanism similar to that of eucarya. [f]No-hyp., not yet investigated, or investigated but not yet found in hyperthermophiles. [g]para., paralogous

of nascent polypeptides is currently under scrutiny (2,6,12-17; see also trigger factor and NAC, below).

The chaperoning system in prokaryotes includes the molecular chaperone machine, described above, and the chaperonin complex formed by GroEL and GroES (11). In addition other factors have been implicated, such as trigger factor (TF) (18-22). In eukaryotes the chaperoning systems vary with the cell compartment: cytosol, mitochondrion, chloroplast, or endoplasmic reticulum (ER). In the cytosol the chaperone machine is composed of Hsp70, Hsp40, and other proteins such as prefoldin (GimC) (23-24), Hop (4, 25-32), Hip (4, 33-40), BAG-1 (41-47), and NAC (2, 48-57), which have been implicated in one way or another in the process of protein folding. In addition, there is a chaperonin complex called CCT or TRiC (58,59). In the other compartments of the eukaryotic cell, however, things are different. For instance, the mitochondrion contains a chaperone machine and a chaperonin complex similar to the bacterial counterparts (60-68). The molecular chaperone machine and the chaperonins and their evolutionary relationships in organisms of the three phylogenetic domains have been discussed elsewhere and are summarized in table 7 (8,10,11).

9. THE CHAPERONING SYSTEM IN ARCHAEA

Some archaeal species have the molecular chaperone machine Hsp70(DnaK), Hsp40(DnaJ), and GrpE, which is similar to that of bacteria, particularly Gram positives (9-11,64,65). All archaeal species investigated thus far have a chaperonin complex that is similar to TRiC, namely the eukaryotic chaperonin, but no GroEL/S complex (11). This is just one of the many examples showing that archaea share some properties with eukaryotes and some with the other prokaryotes, the bacteria.

Recent work (69) suggests that archaea have prefoldin, or GimC, a system discovered in eukaryotes (23,24), and found to have homologs in archaea but not in bacteria. It is not yet known from experimental data whether archaea have other chaperoning molecules that would be similar to TF, or Hop, Hip, or any of the others found in eukaryotes.

10. PREFOLDIN

This is a multimeric complex also named GimC that might assist protein folding in the cytosol of eukaryotic cells (23,24). Homologs of some of the eukaryotic subunits have been detected in archaea, including methanogens (69,70). Six subunits have been identified in eukaryotes, but only two have been found in archaea.

The functions of prefoldin and its role in the stress response are incompletely understood. The subunit-encoding genes are not activated by stressors, and in this regard prefoldin may not be considered a genuine stress protein system. However, preliminary data suggest that prefoldin assists protein folding *in vivo.* It is quite likely that ongoing and future experiments will contribute to a better understanding of this chaperoning complex, including its probable interactions with the chaperone machine, the chaperonins, and other Hsp. We should soon learn about the role of prefoldin in survival during stress,

and/or in recovery after stress. These two processes are essential for all cells and in preventing disease and injuries affecting the integrity of tissues and organs of higher organisms.

An archaeal prefoldin system, that from the thermophilic methanogen *Methanobacterium thermoautotrophicum*, has been studied in some detail and named MtGimC (69). The complex is a hexamer formed by two different subunits, alpha and beta, with a total mass of 87 kDa. The stoichiometry is two alpha and four beta subunits per hexamer. From the evolutionary standpoint, molecular comparisons suggest that the archaeal alpha subunit is the equivalent of the eukaryotic subunits Gim2 and Gim5, while beta is the homolog of the eukaryotic Gim 1, 3, 4, and 6 subunits.

The archaeal complex from *M. thermoautotrophicum* was tested *in vitro* to assess possible chaperone functions. MtGimC was found to: (*i*) Form a complex with unfolded actin, and bind this substrate with relatively low affinity; (*ii*) Suppress aggregation of unfolded hen lysozyme (a relatively small polypeptide with a molecular mass of 14 kDa); (*iii*) Prevent aggregation of chemically unfolded bovine mitochondrial rhodanese (30 kDa) and glucose dehydrogenase (39 kDa); (*iv*) Form complexes with non-native dihydrofolate reductase (DHFR; 23 kDa) and firefly luciferase (62 kDa); (*v*) Stabilize non-native actin for at least 15 min; this allowed transfer of actin to TRiC (the eukaryotic chaperonin complex) for its folding in an ATP-dependent manner; and (*vi*) Prevent aggregation of unfolded rhodanese (as mentioned in point *iii*, above) and allow its folding by the bacterial chaperonin GroEL.

It is important to point out that archaea have a chaperonin complex evolutionarily closer to the eukaryotic homolog TRiC than to the bacterial chaperonin GroEL/S. Thus the above observations must be reviewed with caution, particularly concerning the interaction of MtGimC with GroEL. Another cautionary note stems from the fact that the above observations were obtained *in vitro* under experimental conditions that match only partially those of real life, *in vivo*.

More experimentation *in vitro* and data from *in vivo* assays are needed before we can be certain about the role of the archaeal prefoldin system in protein folding, refolding, and perhaps translocation, too. The mechanism of action of archaeal prefoldin, preferred substrates inside the cell, and activity (or lack thereof) during stress and role in preventing and/or reversing the effects of stress, all remain to be elucidated. Knowledge on these topics might help in the development of strategies and methods to fortify cells and make them more resistant to stressors, and thus provide the grounds for medical applications.

11. TRIGGER FACTOR

In *Escherichia coli*, the nascent polypeptide is met by trigger factor (TF) as it emerges from the ribosome (2,6,18-22). TF is a protein of 45-50 kDa with three domains. The N-terminal domain is the ribosome-binding segment, while the middle domain has PPIase activity. The chaperoning ability attributed to TF is probably a function of the C-terminal domain in conjunction with the other two. These conclusions come from results obtained *in vitro*. The extent to which TF acts as a chaperone *in vivo* has not yet been determined, neither has it been established whether it plays a role in the stress response as a anti-stress mechanism. Nevertheless, we asked the question: Is there a gene in the sequenced archaeal genomes that encodes a protein similar to the bacterial TF? We searched the genomes of *Methanobacterium thermoautotrophicum*, *Archaeoglobus fulgidus*, *Methanococcus jannaschii*, *Aeropyrum pernix*, and *Pyrococcus horikoshii* using as probes TF from *E. coli*, *Haemophilus influenzae*, *Azospiririllum brasilense*, and *Haemophilus actinomycetecomitans*. No significant hits were detected (see possible exception below). While these results do not rule out the occurrence of TF in archaea, they indicate that if a TF homolog does occur it is not similar in sequence to the bacterial TF, or at least not similar enough to be detected by standard genome-searching methods.

The TF molecule from *A. brasilense* (af094574; 444 aa long) produced a hit with a considerably shorter protein (268 aa) encoded in the *A. pernix* genome. This shorter molecule had been annotated as a PPIase on account of its similarity, relatively low, with a 255-aa long protein from *A. fulgidus* that had also been annotated as a PPIase. These observations parallel those discussed in the previous paragraph. They indicate that if archaea possess a TF homolog it is different in structure from the bacterial TF to an extent that makes its detection using bacterial probes very difficult.

12. HOP

Hop (Hsc70-Hsp90-organizing protein) is also called STI1 (stress-inducible 1) protein, STI1 mediator of *hsp70* genes, p60, and IEP SSP 3521 (4,25-32). It has on the average 555 aa and a molecular mass of 60 kDa. Hop is a cofactor or cochaperone that interacts with chaperones in several cellular processes involving protein folding. For example, Hop participates in the folding of glucocorticoid receptor along with Hsc70, Hsp90, Hip (see following section) and PPIases (see contribution by Maruyama & Furutani in this Special Issue).

Hsp90 binds to the hormone-binding domain of the hormone receptor in the cytosol. The hormone receptor complexed with Hsp90 has high affinity for its ligand, the glucocorticoid hormone. Dissociation of Hsp90 from the receptor-glucocorticoid complex results in the loss of the affinity of the receptor for the steroid hormone. Hop stabilizes the Hsp90-receptor complex, and thus contributes to the receptor's high affinity for the hormone. Upon dissociation of the Hsp90-Hop-receptor complex, the receptor is able to bind DNA after it has been translocated from the cytosol into the nucleus. The binding occurs at the glucocorticoid response elements in the promoter regions of glucocorticoid-inducible genes.

Table 8. Search for Hop in archaeal genomes

Probe (eucaryal protein)				Archaeal genome[a]				
Name	**Amino acids**	**Organism**	**Accession number**	***M.t.***	***A.f.***	***M.j.***	***A.p.***	***P.h.***
STI1	589	*Saccharomyces cerevisiae* (budding yeast)	m28486	Yes[b]	No	No	No	Yes
id.	386	*Saccharomyces cerevisiae* (budding yeast)	s59774	Yes	No	No	No	No
mSTI1	544	*Mus musculus* (mouse)	u27830	Yes	No	Yes	No	Yes
LmSTI1	546	*Leishmania major* (protozoan)	u73845	Yes	No	Yes	No	No
TcSTI1	565	*Trypanosoma cruzi* (protozoan)	af107772	Yes	No	No	No	No
IEF SSP 3521	543	*Homo sapiens* (human)	m86752	Yes	No	Yes	No	Yes

[a]Abbreviations: *M.t., Methanobacterium thermoautotrophicum; A.f., Archaeoglobus fulgidus; M.j., Methanococcus jannaschii; A.p., Aeropyrum pernix, P.h., Pyrococcus horikoshii*. [b]Yes, significant hit, and No, no hit using FASTA for *M.t.*, GRASTA for *A.f.* and *M.j*., and BLASTA for *A.p.* and *P.h.*

The five archaeal genomes mentioned in the TF section, above, were screened in search of genes that would encode Hop homologs. Initially, the screening probes were the Hop proteins from five eukaryotic species and hits were observed in four of the five archaeal genomes (table 8). Subsequently, the hit proteins found in the archaeal genomes were compared one-to-one with the eucaryal probes and the identity and similarity percentages from Gap alignments are displayed in table 9. The *M. jannaschii* protein MJ1345 showed the highest percent identity with an eucaryal probe and was, therefore, selected to re-screen the archaeal genomes. The proteins detected with this archaeal probe, MJ1345, were in turn compared with each other. In this way a group of related proteins was identified (table 10). One of these proteins, mt0072 from *M. thermoautotrophicum*, was used as a probe to screen again the archaeal genomes; it produced hits with MJ1345 as expected, and with another protein in *M. jannaschii* and with a protein in *P. horikoshii* (table 11). These proteins share identities of about the same order of magnitude as eucaryal Hops (compare data in tables 10 and 11 with data in table 12). The results indicate that there are proteins in the archaeal genomes that are related to each other and have no established function but might be Hop equivalents.

13. HIP

Hip stands for Hsc70-interacting protein and designates a cytosolic molecule that participates in the folding and maturation of some newly-made polypeptides, for example the progesterone receptor (4,33-40).

In the cytosol of the eukaryotic cell, polypeptide folding involves the chaperones Hsp70(DnaK), its cognate equivalent Hsc70, and Hsp40(DnaJ). In addition, as mentioned in preceding Sections, there are cofactors or co-chaperones that help the chaperones in their chaperoning activity and/or modulate their action. Among these cofactors is Hip, which like Hop, has tetratricopeptide repeats (26,29,30,32,35,39,71). These repeats are directly involved in the interaction of Hip and Hop with Hsc70.

In bacteria, a major cofactor for hsp70(DnaK) and Hsp40(DnaJ) is GrpE (72-76). These three proteins form the molecular chaperone machine. Hsp70(DnaK)-ATP has low affinity for the substrate (*i.e.*, the polypeptide in need of folding or refolding), whereas Hsp70(DnaK)-ADP has high affinity for substrate and releases it slowly if at all. Hsp40(DnaJ) binds Hsp70(DnaK)-ATP and accelerates ATP hydrolysis by activating the ATPase function of Hsp70(DnaK). The resulting Hsp70(DnaK)-ADP complex has, as mentioned above, high affinity for the polypeptide substrate and would not release it after folding. At this point, GrpE, also called nucleotide exchange factor or cofactor, joins the complex and causes release of ADP and binding of ATP to Hsp70(DnaK). The low affinity of Hsp70(DnaK)-ATP allows substrate release. Another cycle will begin with the binding of Hsp40(DnaJ) to Hsp70(DnaK)-ATP and the process will continue until all polypeptide molecules are correctly folded.

In the cytosol of eukaryotes there seems to be no GrpE, and the chaperoning cycle is somewhat different from that of bacteria. Hip stabilizes the ADP status of Hsc70, which thus has high affinity for the substrate. Hip also contributes, as a chaperone in its own right, to the interaction between Hsc70 and substrate. BAG-1 (see Section on BAG-1, below) blocks the action of Hip on Hsc70.

We conducted a search for Hip homologs in the five archaeal genomes mentioned in the previous Sections using as probes Hip sequences from three eucaryal species and detected hit proteins (table 13). However, when these hit proteins were compared one on one with the eucaryal probes, no significant similarity was found (table 14). It was concluded that archaea do not have detectable Hip homologs.

14. BAG-1

Programmed cell death or apoptosis is under intense investigation nowadays. It seems to play a critical

Table 9. Comparison of eucaryal Hop with potential archaeal homologs

Probe (eucaryal protein)				Potential archaeal Hop homolog					
Name	**aa**[a]	**Organism**	**Accession number**	**Name**	**Accession number**	**Organism**	**aa**	**Percent**	
								Iden.[b]	**sim.**[b]
STI1	589	*Saccharomyces cerevisiae* (budding yeast)	m28486	c. p.	mt0083	*M.t.*	379	27.1	39.4
					mt0072		403	27.0	35.4
					mt0068		228	27.1	39.4
				u. p.	PH1836	*P.h.*	338	23.2	33.0
id.	386	*Saccharomyces cerevisiae* (budding yeast)	s59774	c. p.	mt0072	*M.t.*	403	27.0	37.1
					mt1585		351	26.6	41.3
					mt0083		379	22.0	31.6
mSTI1	544	*Mus musculus*	u27830	c. p.	mt0083	*M.t.*	379	25.0	39.0
					mt0072		403	25.3	41.5
					mt0068		228	24.5	35.0
					MJ1345	*M.j.*	314	30.4	41.2
				c. h. p.	MJ0940		318	25.7	36.0
				h. p.	MJ0941		338	27.4	38.4
LmSTI1	546	*Leishmania major* (protozoan)	u73845	u. p.	PH1836	*P.h.*	338	24.8	34.5
				c. p.	mt0083	*M.t.*	379	24.5	38.0
					mt0068		228	25.2	41.0
					mt0072		403	24.4	35.7
					mt1585		351	21.5	34.7
				h. p.	MJ0941	*M.j.*	338	20.0	31.1
				c. h. p.	MJ1345		314	24.7	38.6
TcSTI1	565	*Trypanosoma cruzi* (protozoan)	af107772	c. p.	mt0072	*M.t.*	403	24.9	36.0
					mt0083		379	24.7	33.2
					mt0068		228	21.8	33.6
					mt1585		351	23.5	36.1
					mt0083		379	25.5	39.2
IEF SSP 3521	543	*Homo sapiens* (human)	m86752	c. p.	mt0072	*M.t.*	403	25.3	41.5
					mt0068		228	25.1	35.6
				c. h. p.	MJ1345	*M.j.*	314	27.7	40.5
					MJ0940		318	26.3	36.0
				h. p.	MJ0941		338	28.8	39.7
				u. p.	PH1836	*P.h.*	338	23.9	32.7

[a]Abbreviations are: aa, amino acids; c. p., conserved protein; u. p., unnamed protein; c. h. p., conserved hypothetical protein; h. p., hypothetical protein; *M.t., Methanobacterium thermoautotrophicum*; *P.h., Pyrococcus horikoshii*. [b]Identity (iden.) and similarity (sim.; identitities plus conservative substitutions) determined by pairwise alignment using the Gap program (GCG). Identity and similarity between any two eucaryal Hop proteins are shown in table 12.

Table 10. Similarity of potential archaeal Hop homologs detected with the archaeal probe MJ1345

Sequence	**Name**	**Organism**	**aa**	**mt0083 (*M.t.*)**[a]	**mt0072 (*M.t.*)**	**MJ1345 (*M.j.*)**	**APE1272 (*A.p.*)**	**PH0026 (*P.h.*)**
mt0083	c. p.	*M.t.*	379		68.1[b]	35.4	21.4	26.9
mt0072	c. p.	*M.t.*	403	74.1		33.4	25.2	31.9
MJ1345	c. h. p.	*M.j.*	314	45.9	44.6		27.6	24.8
APE1272	u. p.	*A.p.*	142	30.5	34.8	34.1		N. a.[c]
PH0026	u. p.	*P.h.*	338	36.1	45.0	37.1	N. a.[c]	

[a]The abbreviations for the names of the organisms are explained in table 8. Other abbreviations are: aa, amino acids; c. p., conserved protein; c. h. p., conserved hypothetical protein; u. p., unnamed protein. [b]Percent identity and percent similarity (identities plus conservative substitutions) above and below, respectively, the diagonal array of blank spaces, determined by the Gap program (GCG). [c]Not alignable.

Table 11. Similarity of potential archaeal Hop homologs detected with the archaeal probe mt0072

Sequence	Name	Organism	aa	mt0072 (*M.t.*)[a]	MJ1345 (*M.j.*)	PH0026 (*P.h.*)	MJ0941 (*M.j.*)	AF1970 (*A.f.*)
mt0072	c. p.	*M.t.*	403		33.4[b]	31.9	31.5	25.3
MJ1345	c. h. p.	*M.j.*	314	44.6		24.8	33.1	29.0
PH0026	u. p.	*P.h.*	338	45.0	37.1		27.2	27.6
MJ0941	u. p.	*M.j.*	338	40.7	46.1	39.1		26.2
AF1970	u. p.	*A.f.*	313	36.9	40.7	39.7	40.3	

[a]The abbreviation for the names of the organisms are explained in table 8. mt0072 was identified in the genome of *Methanobacterium thermoautotrophicum* using as probes eucaryal Hops (see tables 8 and 9). Other abbreviations are: aa, amino acids; c. p., conserved protein; c. h. p., conserved hypothetical protein; u. p., unnamed protein. [b]Percent identity and percent similarity (identities plus conservative substitutions) above and below, respectively, the diagonal array of blank spaces, determined by the Gap program (GCG). mt0083 is 68.1, 35.4, and 26.9 % identical to mt0072, MJ1345, and PH0026, respectively (table 10).

Table 12. Similarity of eucaryal Hop molecules

Sequence	Name	Organism	aa[a]	m86752 (*H.s.*)	u27830 (*M.m.*)	af107772 (*T.c.*)	u73845 (*L.m.*)	m28486 (*S.c.*)	s59774 (*S.c.*)
m86752	IEF SSP 3521	*H.s.*	543		97.4[b]	37.4	40.2	41.9	24.6
u27830	mSTI1	*M.m.*	544	98.3		37.4	40.4	43.0	25.3
af10772	TcSTI1	*T.c.*	565	48.0	48.0		59.6	35.3	22.8
u73845	LmSTI1	*L.m.*	546	50.3	50.7	70.0		39.2	25.9
m28486	STI1	*S.c.*	589	52.4	53.3	43.8	48.4		27.8
s59774	STI1	*S.c.*	386	33.8	33.5	34.8	39.6	38.1	

[a]Abbreviations are: aa, amino acids*; H.s.*, *Homo sapiens*; *M.m.*, *Mus musculus*; *T.c.*, *Trypanosome cruzi*; *L.m.*, *Leishmania major*; *S.c.*, *Saccharomyces cerevisiae*; [b]Percent identity and percent similarity (identities plus conservative substitutions) above and below, respectively, the diagonal array of blank spaces, determined by the Gap program (GCG).

Table 13. Search for Hip in archaeal genomes

Probe (eucaryal protein)				Archaeal genome[a]				
Name	**Amino acids**	**Organism**	**Accession number**	***M.t.***	***A.f.***	***M.j.***	***A.p.***	***P.h.***
h. p. r. p48 p.	369	*Homo sapiens* (human)	u28918	Yes[b]	No	No	No	Yes
Hip	368	*Rattus norvegicus* (rat)	x82021	Yes	No	No	No	Yes
h. s. r. p.	376	*Plasmodium berghei* (protozoan)	l04508	Yes	No	No	No	No

[a]Abbreviations are: *M.t., Methanobacterium thermoautotrophicum; A.f., Archaeoglobus fulgidus; M.j., Methanococcus jannaschii; A.p., Aeropyrum pernix; P.h., Pyrococcus horikoshii*; h. p. r. p48 p., human progesterone receptor-associated p48 protein; h. s. r. p., heat-shock related protein. [b]Yes, significant hit, and No, no hit using FASTA for *M.t.*, GRASTA for *A.f.* and *M.j.*, and BLASTA for *A.p.* and *P.h.*

Table 14. Comparison of eucaryal Hip with potential archaeal homologs

Probe (eucaryal protein)				Potential archaeal Hip homolog					
Name	**aa**[a]	**Organism**	**Accession number**	**Name**	**Accession number**	**Organism**	**aa**	**Percent iden.**[b]	**sim.**[b]
h. p. r. p48 p.	369	*Homo sapiens* (human)	u28918	c. p.	mt0072	*M.t.*	403	25.3	39.2
				t. c. f.	mt1585		351	23.5	36.7
				c. p.	mt0068		228	20.0	32.4
				u. p.	PH0553	*P.h.*	399	23.3	35.3
					PH1987		324	21.5[c]	32.3
Hip	368	*Rattus norvegicus* (rat)	x82021	t. c. f.	mt1585	*M.t.*	351	26.8	39.6
				c. p.	mt0072		403	25.3	39.8
				u. p.	mt0674		966	25.0[c]	25.0
				c. p.	mt0068		228	19.3	33.6
					PH0553	*P.h.*	399	19.4	30.8
h. s. r. p.	376	*Plasmodium berghei* (protozoan)	l04508	c. p.	mt0072	*M.t.*	402	28.4	42.8
					mt0068		228	19.0	30.8

[a]Abbreviations are: aa, amino acids; h. p. r. p48 p., human progesterone receptor-associated p48 protein; c. p., conserved protein; t. c. f., transcription control factor; u. p., unnamed protein; h. s. r. p., heat-shock related protein*; M.t., Methanobacterium thermoautotrophicum*; *P.h., Pyrococcus horikoshii*;. [b]Identity (iden.) and similarity (sim.; identities plus conservative substitutions) determined by pairwise alignment using the Gap program (GCG). Identity between human and rat proteins was 91.6%, human and plasmodium is 39.6%, and rat and plasmodium was 39.7 percent.

role in several physiologic and pathologic events in eukaryotic organisms with participation of Hsp70 and perhaps other Hsp as well (77-79). A similar phenomenon has been observed in bacteria (80). A series of genes has been identified that are involved in apoptosis, some producing repressors and others inducers of it. One of these genes blocks cell death and is somehow involved in the t[14;18] chromosomal translocation observed in most cases of non-Hodgkin's B-cell lymphomas. This gene's name is *bcl-2* for B-cell lymphoma 2, and produces the protein Bcl-2. BAG-1 stands for Bcl-2-associated athanogene 1, where athanogene is a derivative from the Greek word athanos that refers to anti-death (45).

BAG-1 is known to be conserved from unicellular fungi (*e.g., S. cerevisiae*) and worms (*e.g., Caenorhabditis elegans*) to humans. It has several isoforms, some of which are generated by alternative translation initiation.

In the cytosol of mammalian cells, BAG-1 regulates the molecular chaperone machine via interaction with Hsc70 (41-43,46). BAG-1 binds the ATPase domain of Hsc70, and stimulates ATP hydrolysis. It also accelerates the release of ADP from Hsc70, an effect that mimics that of GrpE in bacteria--these organisms do not possess BAG-1 homologs. As mentioned above (see section on Hip), BAG-1 competes with Hip for the ATPase domain of Hsc70; the two cofactors seem to have opposite effects on the action of the molecular chaperone machine. While Hip stabilizes the Hsc70-ADP complex, BAG-1 induces release of ADP and binding of ATP.

The five archaeal genomes were screened with eucaryal BAG-1 molecules as probes and hits were detected in one genome (table 15), but when the hit proteins were compared one-to-one with the eucaryal BAG-1 molecules no similarity was found (table 16). These data strongly suggest that archaea do not have BAG-1 homologs, since this protein is highly conserved in eukaryotes: for example, human and mouse BAG-1 sequences are over 70 percent identical and one would have expected to find a conserved homolog also in archaea considering this degree of conservation within the domain Eucarya.

15. NAC

The nascent polypeptide-associated complex (NAC) is a heterodimer formed by the alpha-NAC and the beta-NAC subunits of approximately 33 and 21 kDa, respectively (48-57). The complex binds *in vitro* to nascent polypeptide chains at the ribosome and controls the specificity of the signal peptide-SRP interaction, which ensures translation accuracy. Beta-NAC in yeast is also named Egd1p, and its human counterpart is the BTF3b protein, where BTF stands for basic transcription factor (55-57). The yeast alpha-NAC is known also as Egd2p, and is the homolog of the human alpha NAC (48-54).

The same five archaeal genomes listed in previous Sections were screened in search of beta-NAC homologs using as probes eucaryal beta subunits, but no hits were observed (table 17). It was therefore concluded that archaea do not have a gene that encodes a protein significantly related to the eukaryotic beta-NAC molecule. In contrast, the search carried out with eukaryotic alpha-NAC probes did produce hits with three of the five genomes (table 18). The hit proteins were then compared one-to-one with the eucaryal molecules and found to share

Table 15. Search for BAG-1 in archaeal genomes

Probe (eucaryal protein)				**Archaeal genome**[a]				
Name	**Amino acids**	**Organism**	**Accession number**	***M.t.***	***A.f.***	***M.j.***	***A.p.***	***P.h.***
BAG-1	345	*Homo sapiens* (human)	af022224	No[b]	No	No	No	Yes
hBAG-1	274	*Homo sapiens* (human)	u46917	No	No	No	No	Yes
BAG-1	355	*Mus musculus* (mouse)	af022223	No	No	No	No	Yes

[a]Abbreviations are: *M.t., Methanobacterium thermoautotrophicum; A.f., Archaeoglobus fulgidus; M.j., Methanococcus jannaschii; A.p., Aeropyrum pernix; P.h., Pyrococcus horikoshii*. [b]No, no hit, and Yes, significant hit, using FASTA for *M.t.*, GRASTA for *A.f.* and *M.j*., and BLASTA for *A.p.* and *P.h.*

Table 16. Comparison of eucaryal BAG-1 with potential archaeal homologs

Probe (eucaryal protein)				**Potential archaeal BAG-1 homolog**					
Name	**aa**[a]	**Organism**	**Accession number**	**Name**	**Accession number**	**Organism**	**aa**	**Percent**	
								iden.[b]	**sim.**[b]
BAG-1	345	*Homo sapiens* (human)	af022224	u. p.	PH0799	*P.h.*	267	24.3	36.8
hBAG-1	274	*Homo sapiens* (human)	u46917	u. p.	PH0799	*P.h.*	267	22.6	30.5
BAG-1	355	*Mus musculus* (mouse)	af022223	u. p.	PH0799	*P.h.*	267	20.2	29.0

[a]Abbreviations are: aa, amino acids; u. p., unnamed protein; *P.h., Pyrococcus horikoshii*. [b]Identity (iden.) and similarity (sim.; identities plus conservative substitutions) determined by pairwise alignment using the Gap program (GCG). Identity and similarity between any two eucaryal BAG-1 molecules was over 70 percent.

Table 17. Search for beta-NAC in archaeal genomes

Probe (eucaryal protein)				**Archaeal genome**[a]				
Name	**Amino acids**	**Organism**	**Accession number**	***M.t.***	***A.f.***	***M.j.***	***A.p.***	***P.h.***
BTF3	162	*Homo sapiens* (human)	x53281	No[b]	No	No	No	No
Beta-NAC	169	*Drosophila melanogaster* (fruit fly)	af151116	No	No	No	No	No
EGD1	145	*Saccharomyces cerevisiae* (budding yeast)	s49596	No	No	No	No	No
EGD1 (BTF3)	157	*id*.	x78725	No	No	No	No	No

[a]Abbreviations are: *M.t., Methanobacterium thermoautotrophicum; A.f., Archaeoglobus fulgidus; M.j., Methanococcus jannaschii; A.p., Aeropyrum pernix; P.h., Pyrococcus horikoshii*. [b]No, no hit using FASTA for *M.t.*, GRASTA for *A.f.* and *M.j*., and BLASTA for *A.p.* and *P.h.*

Table 18. Search for alpha-NAC in archaeal genomes

Probe (eucaryal protein)				Archaeal genome[a]				
Name	**Amino acids**	**Organism**	**Accession number**	***M.t.***	***A.f.***	***M.j.***	***A.p.***	***P.h.***
Alpha-NAC	217	*Drosophila melanogaster* (fruit fly)	af017783	Yes[b]	No	No	No	No
id.	174	*Saccharomyces cerevisiae* (budding yeast)	u17134	Yes	No	No	Yes	Yes
id.	215	*Homo sapiens*[c] (human)	x80909	Yes	No	No	No	Yes
id.	215	*Homo sapiens*[c] (human)	af054187	Yes	No	No	No	Yes
id.	215	*Mus musculus* (mouse)	u22151	Yes	No	No	No	Yes

[a]Abbreviations are: *M.t., Methanobacterium thermoautotrophicum; A.f., Archaeoglobus fulgidus; M.j., Methanococcus jannaschii; A.p., Aeropyrum pernix; P.h., Pyrococcus horikoshii.* [b]Yes, significant hit, and No, no hit using FASTA for *M.t.*, GRASTA for *A.f.* and *M.j.*, and BLASTA for *A.p.* and *P.h.* [c]The two *H. sapiens* protein sequences (x80909 and af054187) are 100 % identical (see table 20).

23-29 percent identity with them (table 19). The highest percent identity was with the protein MT0177 from M. thermoautotrophicum. This protein was subsequently used to screen the five archaeal genomes and significant hits were again obtained with the same proteins that had been detected with the eucaryal probes. In addition, the archaeal probe MT0177 detected other molecules in the genomes of A. fulgidus and M. thermoautotrophicum. These archaeal alpha-NAC homolog candidates detected in all five archaeal genomes were then compared with each other, and found to share 38-56 percent identity (table 20).

The screening data with the eucaryal and archaeal probes, the comparative analyses of the proteins that gave significant hits with each other and with the probes, and the results of sequence alignments all concur to suggest that archaea have a gene that encodes a protein similar to the eucaryal alpha-NAC subunit. The archaeal protein is shorter (109-137 aa) than the eucaryal molelcule (174-217 aa). The alignment data suggest that the archaeal protein lacks approximately the main portion of the N-terminal half of the eucaryal molecule.

16. OTHER ANTI-STRESS MECHANIMS

Cells and whole organisms possess a number of means to deal with stressors and avoid or minimize their impact (see also Chapter by Conway de Macario & Macario in this Special Issue). The main instrument is the stress response that entails activation of many genes and molecules and down-regulation of other genes and pathways. A synthesis of anti-stress mechanisms may be derived from information in tables 1-5. Many of them have been described in more or less detail, or alluded to, in the various articles in this Special Issue, as indicated in table 3. Here, we will focus on the formation of multicellular structures as a way of protecting individual cells. Some archaeal species form multicellular structures, such as the packets characteristic of *Methanosarcina* species (81-85). We have studied *Methanosarcina mazeii* in some detail (81,84,86), and will summarize the main observations. One of the objectives is to direct attention to this archaeon as a convenient model to study primitive histogenesis and to explore whether formation of multicellular structures (*e.g.*, tissues and organs in higher organisms) is an evolutionary result generated at least in part by stress.

17. MULTICELLULAR STRUCTURES: STRESS *VS.* DIFFERENTIATION-DEVELOPMENT

Archaea are known to form, and be part of, different types of multicellular structures. They can be single- or multi-species structures if one considers the identity of the components. Both single- and multi-species structures can be subdivided into subtypes according to their gross and microscopic anatomies and also considering the probable mechanism of formation and functions. Here we will focus on methanosarcinas, specifically *M. mazeii*. Therefore, we will describe the types of structures in which this species participates.

18. SINGLE-SPECIES MULTICELLULAR STRUCTURES

18.1. Packet and lamina

M. mazeii undergoes morphologic changes during growth that depend on the environmental conditions, *e. g.*, culture conditions in the laboratory. These changes include three distinct morphotypes, two of which are multicellular: packet and lamina (figure 1) (84). It is not yet clear whether these multicellular morphotypes are stress-resistant forms that arose during evolution as a consequence of changing environments involving stressful situations. We know, though, that packets and laminae are considerably resistant to stressors of various kinds: mechanical, osmotic, physical (temperature elevation), biological (antibiotics), and chemical (pH), particularly in comparison with the single-cell morphotype (unpublished observations). In this regard packets are very resistant; the cells inside the structure are not affected by stressors that would rapidly destroy single cells.

Table 19. Comparison of eucaryal alpha-NAC with potential archaeal homologs

Probe (eucaryal protein)				Potential archaeal NAC homolog					
Name	**aa**[a]	**Organism**	**Accession number**	**Name**	**Accession number**	**Organism**	**aa**	**Percent iden.**[b]	**sim.**[b]
Alpha-NAC	217	*Drosophila melanogaster* (fruit fly)	af017783	c. p.	mt0177	*M.t.*	117	28.0	34.6
id.	174	*Saccharomyces cerevisiae* (budding yeast)	u17134	c. p.	mt0177	*M.t.*	117	29.0	38.3
				u. p.	PH1524	*P.h.*	115	23.6	32.7
				u. p.	APE0198	*A.p.*	137	23.3	38.0
id.	215	*Homo sapiens* (human)	x80909	c. p.	mt0177	*M.t.*	117	29.0	34.6
				u. p.	PH1524	*P.h*	115	26.1	32.4
id.	215	*Homo sapiens* (human)	af054187	c. p.	mt0177	*M.t.*	117	29.0	34.6
				u. p.	PH1524	*P.h.*	115	26.1	32.4
id.	215	*Mus musculus* (mouse)	u22151	c. p.	mt0177	*M.t.*	117	29.0	34.6
				u. p.	PH1524	*P.h.*	115	26.1	32.4

[a]Abbreviations are: aa, amino acids; c. p., conserved protein; u. p., unnamed protein; *M.t., Methanobacterium thermoautotrophicum*; *P.h., Pyrococcus horikoshii*; *A.p., Aeropyrum pernix*. [b]Identity (iden.) and similarity (sim.; identities plus conservative substitutions) determined by pairwise alignment using the Gap program (GCG). Percent identity between any two eucaryal proteins was: 100 for the two human sequences, 99.1 for human *vs.* mouse, 62.4 for human *vs.* fruit fly, 43.4 for the latter *vs.* yeast, and 41.4 for yeast *vs.* human.

Table 20. Similarity of potential archaeal NAC homologs

Sequence[a]	**Name**	**Organism**	**aa**	**mt0177 (*M.t.*)**	**PH1524 (*P.h.*)**	**AF0215 (*A.f.*)**	**MJ0280 (*M.j.*)**	**APE0198 (*A.p.*)**
mt0177	c. p.	*M.t.*	117		55.5[b]	46.3	45.2	37.7
PH1524	u. p.	*P.h.*	115	67.3		55.1	44.6	42.5
AF0215	u. p.	*A.f.*	109	59.3	72.9		46.8	45.0
MJ0280	c. p.	*M.j.*	128	61.7	57.1	58.7		43.8
APE0198	u. p.	*A.p.*	137	52.3	54.9	63.3	50.4	

[a]The sequences are from the archaeal organisms whose full names are given in table 8. mt0177, PH1524, and APE0198 were identified in the archaeal genomes using as probes ecuaryal alpha-NAC proteins (see tables 18 and 19). They were subsequently confirmed using as probe the archaeal protein mt0177 from *M. thermoautotrophicum*, which in addition identified AF0215 and MJ0280, and produced a highly significant hit (methano.3.12627) with the genome of *Methanosarcina mazeii*. Abbreviations are: aa, amino acids; c. p., conserved protein; u. p., unnamed protein. [b]Percent identity and percent similarity (identities plus conservative substitutions) above and below, respectively, the diagonal array of blank spaces, determined by the Gap program (GCG).

Crucial questions are whether stress during evolution selected species that were able to form multicellular structures and thus shield cells inside resistant envelopes, and whether this is the origin of histogenesis and differentiation-development. Was stress the driving force that selected species capable of not only changing their metabolism and/or their surface, but also generating an intra-species diversity that would allow the organism to form multicellular structures and subsequently different tissues and, ultimately, organs? This is a major question in biology and evolution impacting disciplines pertinent to the three phylogenetic domains, Archaea, Bacteria, and Eucarya. Organisms of the domains Bacteria (87-90) and Eucarya--lower (91-96) and higher (97-99) species--can form multicellular structures and undergo differentiation-development during which stress genes are differentially regulated. How about archaeal organisms? *M. mazeii* is a suitable model to seek an answer to the question.

We have standardized the conditions that induce the passage of one morphotype of *M. mazeii* to another (figure 2). Modifications of the culture medium precede morphotype changes. One may assume, as a working hypothesis, that the changes are partly due to the stress caused by the brusque alteration of the environment (*i.e.*, culture medium modifications), and partly as a metabolic adaptation to the variation in nutrients available.

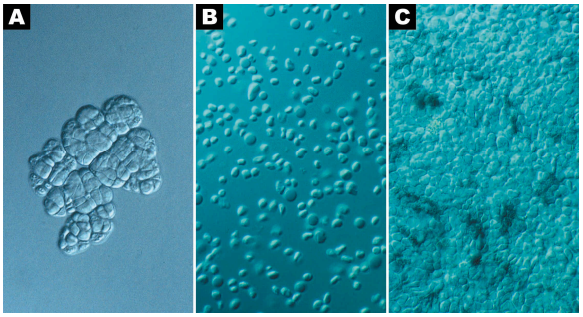

Figure 1. The three characterized morphologic stages (morphotypes) of *M. mazeii*: packets (**A**), single cells (**B**), and lamina (**C**). Wet preparations of living cultures photographed under Nomarski illumination. The diameter of single cells in (**B**) is 3-5 µm, and the three panels are shown with the same magnification.

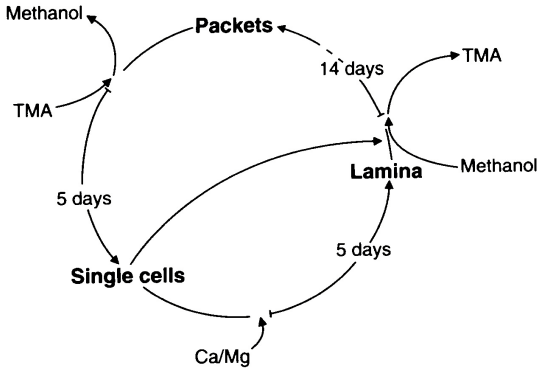

Figure 2. Schematic representation of morphotype conversions showing the main medium changes that precede the conversions. Packets grow on methanol and convert to single cells when methanol is replaced by trimethylamine (TMA), the concentrations of $CaCl_2$ and $MgCl_2$ are raised to 3.4 and 4.9 mM, respectively, and the cultures are thoroughly mixed periodically. Single cells convert to lamina if the sum of the concentrations of $CaCl_2$ and $MgCl_2$ reaches 15 mM, and the cultures are not mixed. Single cells and laminae revert to packets when methanol is provided as substrate instead of TMA, and the divalent cation concentrations are lowered to their initial values. The three morphotypes can be maintained indefinitely by successive passages into new medium. Single cells require passaging more frequently than the other two morphotypes, which represents more labor and more consumption of medium. Therefore, when not immediately needed, single cells are reverted to packets by appropriately changing the culture conditions (internal arrow) for long-term culturing, or for frozen storage.

18.2. Morphologic heterogeneity

Histological thin sections of *M. mazeii* packets and laminae have shown that the cells in these structures are diverse in size, the way in which they are grouped, and distribution (unpublished studies). These findings confirm the same type of observations made by examining fresh packets and lamina using DIC or phase-contrast optics (figures 1 and 3). The meaning of this morphologic heterogeneity has not been elucidated, but it suggests a certain degree of differentiation and, therefore, specialization. One may envisage this heterogeneity as the image of a very primitive biologic complex made of an association of different "tissues". These "tissues" would be groups of cells that are together in separate conglomerates, each with a distinct function (the same for all the components of any given conglomerate). In this way, cells in each group would work more efficiently, being close together and isolated from other, different groups with different functions.

18.3. Laminogenesis

Lamina formation in *M. mazeii* is accompanied by a series of anatomic changes that suggest a differentiation process in the sense that the final structure is different in several ways from the preceding stages. Typically, morphologic changes are paralleled by changes at the genetic (some genes are upregulated, whereas others are downregulated) and molecular levels. This, for example, is manifested in *M. mazeii* by the variations in quantity and distribution of antigenic molecules, which can be monitored using specific antibodies and immunologic, immunocyto-, and immunohistochemical methods, during the formation of each morphotype. Illustrative data pertaining to laminogenesis in M. mazeii are displayed in figure 4 (L. E. Mayerhofer, E. Conway de Macario & A. J. L. Macario, unpublished results). The time-course variations in the distribution and density of a protein (termed Ma) were mapped in successive samples taken at various stages of laminogenesis, from the beginning of lamina formation until the structure reached its final stage. The series of images in figure 4 were obtained by immunofluorescence with a monoclonal antibody made against purified Ma on thin histologic sections. These images picture the topography and in situ quantitative variations of Ma at representative stages of laminogenesis. In parallel, the overall quantitative changes of Ma were measured by a quantitative immunoenzymatic assay using the same monoclonal antibody (data not shown).

18.4. Production of extracellular material

Another sign of specialization is the synthesis of a distinctive product by a cell group, which is not produced by other cell groups. Packet and lamina formation involves the synthesis of molecules for the intercellular connective material that keeps the cells tied together and that also encloses the entire conglomerate. The genes involved in this synthesis, and their direct (protein) and indirect (e.g., carbohydrates) products are considered stress genes and molecules, respectively (table 4), because the resulting multicellular structures are more resistant to stressors than single cells. However, these genes might also be involved in the process of differentiation and development, regardless of whether or not stress is the inducer.

Lamina is an illustrative source of images suggesting that some cells produce and secrete extracellular material, which extends between cells and groups of cells, and displays morphologic heterogeneity of its own (figures 3 and 5); extracellular material also forms films on top of mature lamina (figure 6) (Macario & Conway de Macario, unpublished results).

19. MULTISPECIES MULTICELLULAR STRUCTURES

19.1. Types

Archaea, methanogens in particular, associate with other organisms, mostly bacteria and more rarely

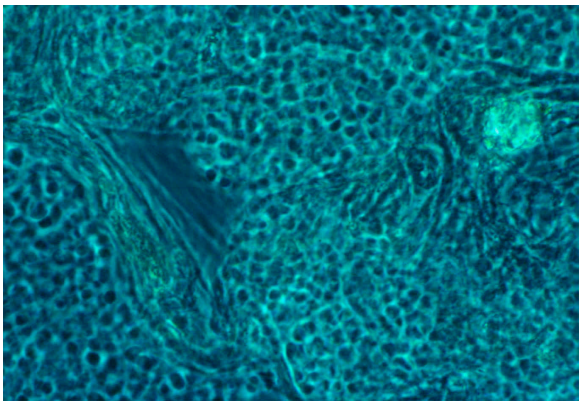

Figure 3. Microanatomic heterogeneity of lamina. Cell groups are discernable that differ in size and location with regard to one another, and to the fibrilar structures characteristic of the lamina. The latter fibrous material is also heterogeneous in appearance and distribution. The photograph was taken with phase contrast optics under the same conditions described for figure 1.

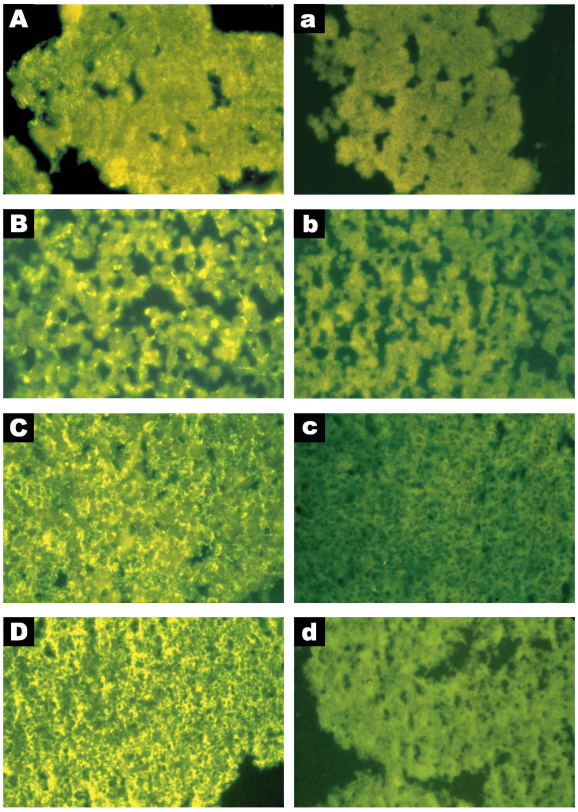

Figure 4. Topography of the Ma protein at different stages of laminogenesis in *M. mazeii*. **A**, **B**, **C**, and **D**: early (*e.g.*, day 1), "young" (*e.g.*, day 4),"mature" (*e.g.*, day 8), and stable (*e.g.*, day 11) lamina, respectively (**a**, **b**, **c**, and **d**, respective negative controls). Indirect immunofluorescence with a anti-Ma monoclonal antibody on thin histologic sections of fixed samples. Photographs were taken with 100X objective and 10X eyepiece.

single-celled eukaryotes, in consortia with different morphology. These consortia can be divided considering their anatomy into biofilms and granules. As these names suggest, the former are flat, while the latter are globular. In both cases three or more different species belonging to the domains Bacteria and Archaea coexist and work together in biofilms and granules. Furthermore, several species of methanogens have been shown to co-exist in the same granule or biofilm (100-105).

19.2. Biofilm

An illustrative example of biofilm is given in figure 7 (106). This biofilm was made up of methanogens, including a species very closely related to *M. mazeii*, and bacteria. The implications of biofilm formation and its advantages in terms of survival and function in a bioreactor for waste treatment have been mentioned above. These bioreactors are a changing environment where the microbes are submitted to severe mechanical forces and physical (*e.g.*, high temperature) and chemical (*e.g.*, low pH) stressors. Thus, association to each other and to a solid surface (without too much piling up to avoid restriction of nutrients to the deeper cells) represents a survival mechanism that enhances stability and metabolic efficiency. No doubt the cells in a biofilm gain in resistance to stressors, even if perhaps this gain occurs at the expense of mobility and ability to propagate and conquer distant ecosystems.

19.3. Granule

The *M. mazeii* packet described above is a representative example of a globular multicellular structure, in which all diameters of the structure amount to several individual cell diameters. Thus, the structure overall reminds the sphere. Packet is a single-species structure. There are also spheroidal multicellular structures composed of more than one species. Those that form methanogenic consortia in anaerobic bioreactors are called granules. The cells in the granules are, like those of packets, considerably more resistant to stressors than individual counterparts in suspension, for example. Isolated cells in suspension, or on a surface by themselves, are devoid of the protective surroundings provided by the crowd of cells and the intercellular connective material typical of granules.

The microanatomy of the granules is quite complex, as revealed by microscopy (100,107,108). Histologic thin sections in combination with histo- and immunohisto-chemical methods demonstrate a complex organization of microbes being arranged into distinct groups, figure 8, panel A. The identity of the microbes can be ascertained using specific antibodies. For instance, in figure 8, panel B, the occurrence of methanosarcina packets and laminae is shown by a specific antibody and immunofluorescence microscopy.

One potential problem with the globular structure one may envisage is that cells in the deep interior do not have proper access to nutrients and cannot get rid of catabolites. However, granules have a net of microtubules, which most likely are passageways for nutrients and catabolites (107,108). These tubules can be demonstrated in thin histologic sections of granules (figure 9).

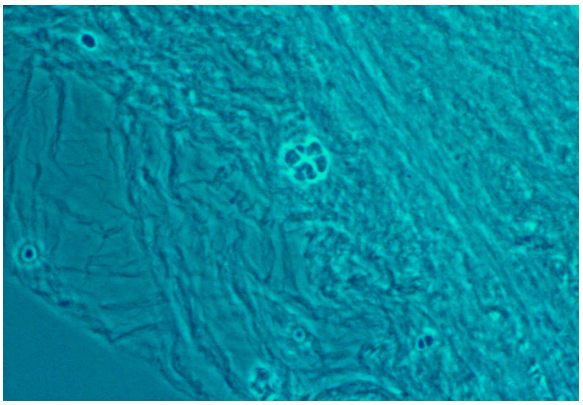

Figure 5. Extracellular material in lamina. A cell cluster and isolated cells (one of them possibly in the last stages of division) are seen lodged on the layer of fibrous material (filaments) in the periphery of a growing lamina (not seen in this microscopic field). The extracellular material would seem to provide a substratum for cell multiplication and migration during laminogenesis. The photograph was taken with phase contrast optics under the same conditions described for figure 1.

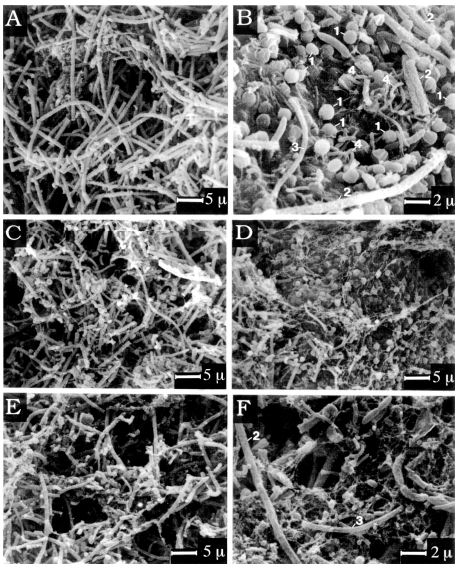

Figure 7. Example of a microbial consortium in the form of biofilm made of methanogens and associated, syntrophic bacteria visualized by scanning electron microscopy (SEM). The biofilm was attached to the substratum (curler-type polypropylene) in a fixed-bed anaerobic methanogenic bioreactor processing synthetic waste-water containing acetate, propionate, and butyrate, at 35 °C. The samples were collected from the top (**A** and **B**), middle (**C** and **D**) and bottom (**E** and **F**) of the bioreactor 57 days after its inoculation with sludge from another digestor treating municipal sewage. Discernable are cells that were identified as related to *M. mazeii* (single cells, 1), *Methanosaeta (Methanothrix) soehngenii* (2), *Methanospirillum hungatei* (3), and *Desulfovibrio* sp. (4). *M. mazeii* occurred as single cells (best visible in **B**) and as laminae. The fibrous intercellular connective material in the laminae appeared as filaments in **D** (see also figures 5 and 6). Scale bars (in :m) are shown at the right-bottom corner of each panel. Reproduced from reference 106 with permission from the copyright owner.

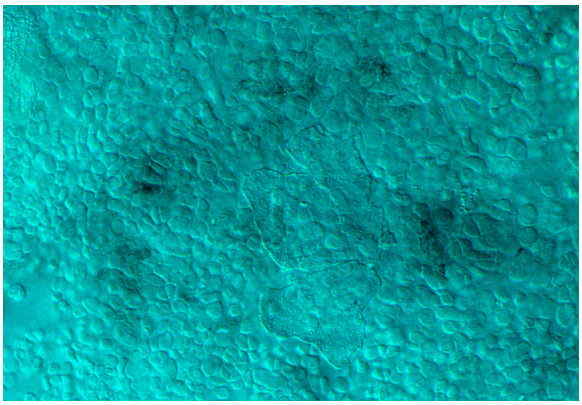

Figure 6. Extracellular material in lamina. A thin layer of extracellular material is discernable in the center lying on the surface of a mature lamina. The photograph was taken with Nomarski optics under the same conditions described for figure 1.

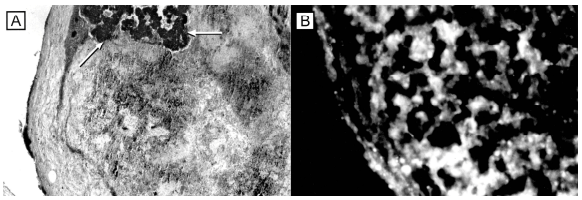

Figure 8. Spheroidal multicellular structure (granular consortium, or granule) formed by methanogens associated with bacteria in a thermophilic (50 °C), anaerobic, methanogenic bioreactor, as seen in a thin histological section. (**A**) Cross section of the granule showing the cortex and medulla (107,108) and a large island of methanosarcina packets (arrows). Hematoxylin-eosin (magnification 800 X). (**B**) Another section of the same granule in which the presence of *Methanosarcina thermophila* TM-1 (optimal temperature for growth, 50 °C) is demonstrated with a antibody probe for TM-1 by immunofluorescence. The methanosarcina cells are arranged mostly in laminae. Magnification, 4,000 X. Reproduced from reference 11 with permission from the copyright owner.

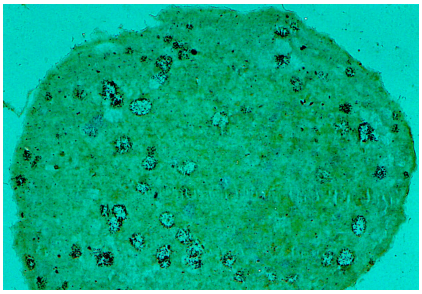

Figure 9. Superficial, histologic thin section of a granule like the one shown in figure 8, passing through the cortex. Visible are circular openings that are cross sections of the microtubules that crisscross the granule, possibly communicating different zones of it between themselves and with the immediate surroundings of the granule (107,108). Hematoxylin-eosin (magnification 800 X). Reproduced from reference 11 with permission from the copyright owner.

It would then appear that while formation of granules might tax heavily the cell's resources for building such complex structures, it also provides protection. Furthermore, it provides stability in the face of current (typical of bioreactors), and functional linkage for interaction with adjacent cells in foodwebs. The methanogenic consortia are indeed the anatomical substratum for a foodweb that ends up with generation of methane by methanogenic archaea.

Stressors and stress are likely to have played a significant role in selecting cells endowed with the capability of aggregation in a functionally efficient manner, as suggested by studies of multicelluar structures formed by *M. mazeii*, and by the microbes that carry out methanogenic conversion in bioreactors.

20. ACKNOWLEDGEMENTS

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Send correspondence to: Dr Alberto J. L. Macario, Wadsworth Center, Room B-749, Division of Molecular Medicine, New York State Department of Health, Empire State Plaza, P.O. Box 509, Albany, New York 12201-0509, USA, Courier address: Wadsworth Center; Room B-749, P-1, South Dock J3, Empire State Plaza, Albany, New York 12201, USA, Tel: 518-474-2781, Fax: 518-474-1213;-8590; -7992, E-mail**:** macario@wadsworth.org