

## OSMOADAPTATION AND OSMOREGULATION IN ARCHAEA

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### 1. ABSTRACT

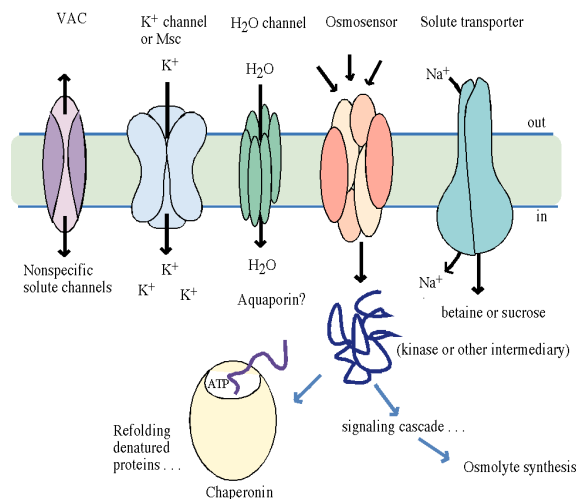
The response of archaea to changes in external NaCl is reviewed and compared to what is known about osmoadaptation and osmoregulation in bacteria and eukaryotes. Cells placed in altered external NaCl exhibit short term and long term responses. The earliest events are likely to be water movement through aquaporin-like channels (efflux if external NaCl has been increased, influx into the cell if the external NaCl has been decreased) and ion movement (e.g.,  $K^+$  moving in the direction opposite to water flow) through channels sensitive to osmotic pressure. Accumulation of organic solutes, either by uptake from the medium or *de novo* synthesis, is triggered after these initial changes. Archaea have some unique organic solutes (osmolytes) that are not used by other organisms. These as well as other more common solutes have a role in stabilizing macromolecules from denaturation. Many osmolytes are distinguished by their stability in the cell and their lack of strong interactions with cellular components. A cell may respond by accumulating one or more temporary osmolytes, then over time readjust the intracellular solute distribution to what is optimal for cell growth under the new conditions. Coupled with the movement and accumulation of solutes is the induction of stress proteins (e.g., chaperonins) and, in some cases, transcriptional regulation of key enzymes. The response to NaCl stress of *Methanococcus thermolithotrophicus* is presented as an example of how one particular archaeon responds and adapts to altered osmotic pressure. Clearly, the detailed response of other archaea to osmotic stress will be needed in order to identify features (aside from some of the organic osmolytes) unique to the organisms in this kingdom.

### 2. INTRODUCTION

Cells have evolved in niches covering a wide range of salinities from low ionic strength to nearly saturated NaCl. The osmotic pressure of the growth medium, which is related to the water activity of the solution (and hence the concentration of solutes), is a critical parameter for cell growth. Most plant and bacterial cells maintain an osmotic pressure in the cytoplasm that is higher than that of the surrounding environment ( $P_{\text{cyto}} - P_{\text{ext}} > 0$ ). This outward-directed pressure, turgor, is necessary for cell division and growth and must be maintained (1). Thus, the ability of an organism to adapt to changes in the external osmotic pressure (osmoadaptation) and the development of mechanisms to achieve this (osmoregulation) are fundamental to its survival of (2-4).

Changes in the environmental osmolarity, most often created by alterations in the external  $Na^+$  concentration, will initially trigger the flux of water across the cytoplasmic membrane leading to changes in cell volume. This alters the concentrations of intracellular metabolites and can lead to the inhibition of a variety of cellular processes (2,5); in the case of reduced external  $Na^+$ , the influx of water can lead to cell lysis. To avoid lysis under low-osmolarity or dehydration under high-osmolarity growth conditions (net flux of water from the cell to the cytoplasm), cells must possess active mechanisms that permit rapid and efficient adaptation to changes in environmental osmolarity. The response to altered external osmotic pressure has three components (figure 1): (i) recognition of osmotic imbalance by an osmosensor, (ii) accumulation of solutes (known as osmolytes) in response to the imposed pressure difference, and (iii)

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**Figure 1.** Schematic of the different components of osmotic stress mechanisms (based on what is known about bacteria and eukaryotes) that may be operational in archaea.

stabilization of macromolecules under the new intracellular conditions (often this can involve chaperonins, heat shock proteins or other mechanisms for ensuring correct folding of proteins). Cells must possess ‘osmosensors’ located in their membranes that undergo conformational transitions in response to changes in extracellular water activity (for a recent review see (6)). Direct osmosensing could be achieved by a membrane component responding to changes in its interactions with particular solutes, hydration changes, or macromolecular crowding. Alternatively, the membrane-sensor may detect and respond to changes in membrane structure. These conformational changes must then be coupled to a longer time-scale adaptation. In most cells, the solution to increased external osmotic pressure is the accumulation of low molecular weight solutes or ‘osmolytes.’ The solutes, which may be present at very high concentrations, must not inhibit cellular processes, hence are termed “compatible solutes” (7). Solute accumulation upon hyperosmotic shock is a ubiquitous response observed throughout all three kingdoms. The usual response to decreased external osmotic pressure (hypoosmotic shock) is to reduce, either by efflux or catabolism, osmolyte concentrations. Since changes in water activity can also have a profound effect on protein stability and folding, cells also may respond to changes in osmotic pressure with mechanisms to promote correct protein folding. This may involve increasing the levels of chaperonins and other macromolecular complexes that aid in protein refolding.

Archaea, often found in high salt as well as high temperature environments, display many of the same characteristics of bacteria and eukaryotes in their response to osmotic stress. They also synthesize and accumulate several unique solutes for use as osmolytes. The response to high-osmolarity environments has been studied in detail in bacteria such as *Escherichia coli* and *Bacillus subtilis*.

However, much less is known about the archaeal stress response. This review will summarize what is known about osmoadaptation and osmoregulation in archaea in the context of how bacteria and eukaryotes respond to salt stress. A case history, *Methanococcus thermolithotrophicus*, will be presented. One might ask why should detailed studies of osmoadaptation and osmoregulation be undertaken in archaea? Given the novel nature of some of the organic solutes used by these organisms, their moderately small genomes, and their potential usefulness in generating interesting reagents (either small molecules or heat stable enzymes) or in bioremediation, they represent intriguing targets for investigating stress responses. It has also been proposed that organisms that live and adapt to saline environments can provide insights into extraterrestrial life. Halophilic and halotolerant anaerobic archaea, in particular, may be important models for life on early Mars or for life that might exist today in sub-glacial oceans that are believed to exist on the Jovian moon, Europa (8).

### 3. WATER EFFLUX AND OSMOSENSORS

An immediate response of cells to osmotic imbalance is for water to rush from the higher activity compartment to the lower activity one (e.g., from low solute concentrations to high solute concentrations) until the solvent activity is the same on both sides of the membrane. Water can cross the cell membrane by diffusing through the lipid bilayer. However, water fluxes have also been shown in mammalian and plant cells to occur through defined channels in cells. A hyperosmotic external environment would dehydrate cells while a hypoosmotic environment would increase the cytoplasmic volume. Large changes in cell volume are not tolerable so that there must be sensors in the cell membrane that detect such pressure differentials either directly or indirectly and cause the accumulation (in the case of hyperosmotic shock) or release (hypoosmotic shock) of solutes to redress the imbalance.

#### 3.1. Water Movement

Aquaporins are a large family of membrane channels involved in osmoregulation. They were first identified in mammalian cells and have now been shown to exist in bacteria. In *E. coli* the channel formed by the aquaporin gene (*aqpZ*), has been shown to mediate large water fluxes in response to sudden changes in extracellular osmolarity (9). The *E. coli* aquaporin channel is composed of monomers with six membrane spanning alpha-helices (as predicted by the sequence). The surface of aquaporin Z has been imaged with atomic force microscopy (10). A loop at the extracellular surface is thought to undergo a reversible force-induced conformational change. Although no aquaporin has been tentatively identified in archaea, it is possible that a homologue exists in most of these cells and is involved in mediating water fluxes.

#### 3.2. Msc’s and VAC’s

Solute release or uptake in response to an applied osmotic gradient is mediated through gated transmembrane channels that open when there is a pressure differential.

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Two types of these channels exist in bacteria and eukaryotes. Msc are a family of mechansensitive ion channels that are gated by membrane tension. They are thought to be primary biosensors for osmoregulation in bacteria (11). Such channels could lead to internalization of  $K^+$  upon hyperosmotic shock. Another class of transmembrane proteins, the volume-activated channels (VAC's), share characteristics of anion channels and are likely to play a critical role in the hypoosmotic response. When cells swell because of a hypoosmotic shock, the release of cytoplasmic solutes can induce an efflux of water and a return to the original cell volume. VAC's, the class of channels that respond to these volume / pressure differentials (12), appear to serve as the conduit for expulsion of a wide variety of osmolytes (e.g., amino acids, polyols). Whether these relatively nonspecific VAC's are also important components of solute uptake in response to hyperosmotic stress is less clear. In bacteria, solute uptake in response to hyperosmotic shock appears to occur by more specific transporters (a few examples of these will be discussed in 3.3). Archaea may well possess Msc's and VAC's although there are no putative candidates in any of the sequenced genomes and no careful evidence that solute expulsion occurs immediately in cells subjected to hypoosmotic stress. However, this represents an area ripe for investigation.

### 3.3 Exogenous Osmolyte Transport

Many cells have transport mechanisms that can selectively internalize solutes from the medium when placed under hyperosmotic stress. Glycine betaine, an osmolyte found in all three kingdoms, is rarely synthesized *de novo* by cells. Rather, it is actively transported into cells and often replaces the pool of synthesized osmolytes. The mechanism of the betaine transporter has been studied in a variety of bacteria. In *E. coli*, the transporter ProP (a member of the major facilitator superfamily) mediates osmoprotective proline or glycine betaine accumulation by bacteria exposed to high osmolarity environments (13). The transporter has a relatively broad specificity and can internalize ectoine, pipercolate, proline betaine, N,N-dimethylglycine, carnitine and 1-carboxymethylpyridinium solutes (choline, a cation and a structural analogue of glycine betaine, is a low affinity inhibitor but not a substrate for internalization). ProP mediates cotransport of  $H^+$  and proline; the pH gradient provides the energy for accumulating betaine. It has also been shown that exogenous proline or glycine betaine elicits  $K^+$  release from osmo-adapting *E. coli* cells. Presumably in this way, the cells sense the availability of betaine, a zwitterion, and use it to replace a  $K^+$ -salt osmolyte pair, although ProP is unlikely to mediate  $K^+$  efflux directly. Betaine uptake systems have been studied in other bacteria as well. The betaine uptake system (BetP) of *Corynebacterium glutamicum* has been expressed in *E. coli* (14). BetP is regulated by the external osmolality and is the only element needed to sense and accumulate betaine. Membrane properties, both protein/membrane interactions and the physical state of the membrane, as well as external ions regulate betaine uptake. Therefore, osmosensor action is likely to be mediated through changes in membrane

properties that alter BetP activity. Some bacteria such as *Listeria monocytogenes* contain two transport systems for this osmolyte (15): (i) a  $Na^+$ -glycine betaine symport, and (ii) a second transporter that is osmotically activated but does not require a high concentration of  $Na^+$  for activity.

Homology searches using *E. coli* ProP have not been able to identify a putative betaine transporter in any of the archaeal genomes available thus far. However, a number of methanogens, notably *Methanogenium cariaci* (16), *Methanosarcina thermophila* (17), and *Methanohalophilus portucalensis* (18), have been shown to actively accumulate exogenous betaine in response to osmotic stress. Betaine uptake suppresses the *de novo* synthesis of osmolytes in these cells. In *M. thermophila* and *M. portucalensis*, the betaine transporter is very specific for glycine betaine (glycine, choline, sarcosine and N,N-dimethylglycine do not compete effectively with betaine for transport into the cell). This is in contrast to the broader specificity of *E. coli* ProP. Betaine transport in the two methanogens could be abolished by protonophores and ionophores, implying that the archaeal transporter is energized by ion gradients.

*Natronococcus* sp. have been shown to accumulate sucrose from the medium (19). As little as 0.1 mM external sucrose will dramatically suppress synthesis of endogenous osmolytes. The transporter prefers sucrose compared to other disaccharides (cellobiose was not internalized and only a small amount of maltose, neither effectively suppressing endogenous osmolyte synthesis). The energy source for sucrose transport is likely to be an ion gradient, although there is no definite evidence for that at this time.

### 3.4. Relay of Osmosensing Via Signal Transduction

How is the osmotic stress detected by a membrane-localized sensor communicated to intracellular components if solute transport is not available to relieve the stress? Loomis et al. (20) have shown that eukaryotic microorganisms use signal transduction pathways that employ phosphorelay from histidine kinases through an intermediate transfer protein (H2) to response regulators. Several of these pathways are linked to mitogen-activated protein kinase (MAP kinase) cascades. These networks control different physiological responses including osmoregulation. In *E. coli*, there is a sensor kinase, KdpD, that responds to ProP and MscL (mechanosensitive large conductance channel). This sensor kinase is involved in regulating cellular energetics, cytoplasmic ionic strength, and ion composition as well as on cytoplasmic osmolality.

Are similar osmotic stress sensors and signal transduction pathways operational in archaea? For most archaea, appropriate compatible solutes are not available in the medium for transport into the cell so that osmolytes must be synthesized *de novo*. Nothing is known about how synthesis and accumulation of solutes are regulated in these cells. In fact, for many of the compatible solutes examined the biosynthetic pathway is unknown. Thus in order to identify osmosensors one will first need to identify biosynthetic pathways and key enzymes.

### 4. COMPATIBLE SOLUTES

Accumulation of solutes in response to osmotic stress is a response conserved through all three kingdoms. Interestingly, there are only two organic solutes common to organisms from the three kingdoms: L-alpha-glutamate and betaine. The solutes can be broken down into inorganic ions (with appropriate organic counterions) and organic solutes – both charged (usually anionic) and neutral (either zwitterions or nonionic) species. For recent reviews of organic osmolytes in archaea see da Costa et al. (21) and Martin et al. (22).

#### 4.1. Inorganic ions: Intracellular $K^+$ , $Mg^{2+}$ , and $Na^+$

In contrast to bacteria, most of the archaea examined have high intracellular concentrations of inorganic cations, primarily  $K^+$ , under optimal growth conditions (see Table 1 in (22)). Even in nonhalophilic archaea, intracellular  $K^+$  is relatively high ( $>0.5$  M), suggesting that under normal conditions these cells exist with high turgor pressure if the intracellular  $K^+$  is free and not tightly complexed to macromolecules (23). The intracellular  $K^+$  is presumably maintained by a potassium channel (possibly an Msc?). In general, accumulation of these cations is not the optimal response since at high concentrations monovalent ions can inhibit various enzymes. However, halophiles with extremely high intracellular  $K^+$  (24) have evolved more acidic enzymes that require  $K^+$  for optimal activity (the ions are thought to be critical for compact folding of the macromolecule). The increased acidity of proteins has been documented in various nonhalophilic methanogens, (25). High ion concentrations can also affect functional group pKa's (for macromolecules, altered pKa's could affect self-assembly and ligand binding (26)). Thus for most organisms, utilization of inorganic ions as primary osmolytes appears to be a suboptimal adaptation. In bacteria, the intracellular  $K^+$  pool is often sensitive to external  $Na^+$ , however  $K^+$  is usually not the main osmolyte. In archaea,  $K^+$  is likely to be a much larger portion of the intracellular solutes. Of the archaea examined to date, organisms fall into two classes – those that accumulate  $K^+$  in amounts that vary with external NaCl (e.g., *M. thermolithotrophicus*, *M. portucalensis*) and those with relatively invariant internal  $K^+$  (e.g., *M. thermoautotrophicum*).

Only a few organisms use inorganic ions almost exclusively to balance external osmotic pressure. Growing marine bacterioplankton have an internal environment where  $Mg^{2+}$  is the dominant cation and, along with chloride, acts as the major component of cell turgor (27). The internal concentrations of  $Na^+$  in the bacterioplankton are 50 to 180 mM, and the  $[K^+]/[Na^+]$  ratio is in the range of 0.1 to 0.5. Extreme halophilic archaea such as *Halobacterium* and *Haloferax* are the archaeal examples that use inorganic ions exclusively as osmolytes. They use  $Na^+$  and  $K^+$  (rather than  $Mg^{2+}$ ) for osmotic balance and do not appear to accumulate small soluble organic anions as the counterions (24).

#### 4.2. Organic Solutes

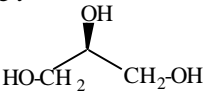
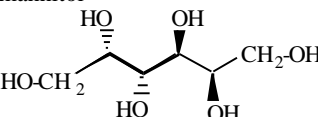
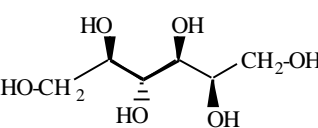
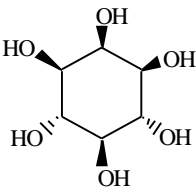
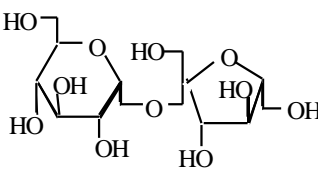
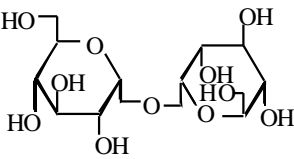
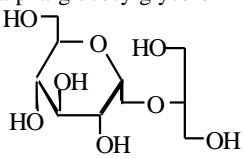
The distribution of organic osmolytes found in archaea falls into the same major classes as for bacteria and

eukaryotes: (i) zwitterions (amino acids and derivatives including betaine), (ii) neutral solutes (sugars and polyhydric alcohols), and (iii) anionic solutes where the negative charge is supplied by a carboxylate, phosphate or sulfate. Archaea also accumulate some very unusual solutes that have no obvious bacterial or eukaryote counterpart (e.g., cyclic-2,3-diphosphoglycerate or cDPG (28), the most prominent solute in the hyperthermophilic *Methanopyrus kandleri* (29), and 1,3,4,6-hexanetetra-carboxylic acid (30)), all polyanions).

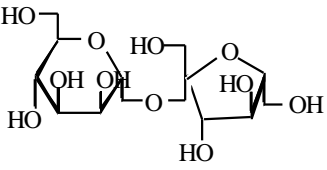
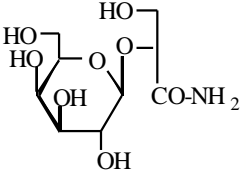
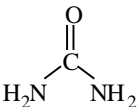

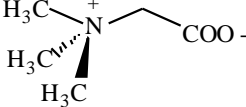
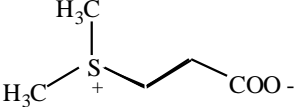
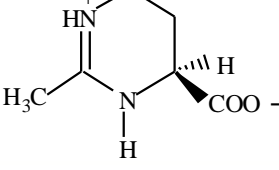
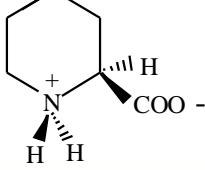
The amino acid class contains the two osmolytes common to all kingdoms: anionic L-alpha-glutamate and zwitterionic glycine betaine. Several common zwitterions found in bacteria and eukaryotes, notably taurine (used as an osmolyte in mammalian tissues (31) and ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidine carboxylic acid (32)), have not been detected in archaea. Alpha-glutamate is found as a counterion to the high intracellular  $K^+$  in a wide range of archaea. However, it is rarely the major compatible solute. Archaea also contain D-amino acids (D-enantiomers of serine, alanine, proline, glutamate (glutamine) and aspartate (asparagine) have been found in *Pyrobaculum islandicum*, *Methanosarcina barkeri* and *Halobacterium salinarium*), but at low concentrations that would not contribute to osmotic balance (33). Instead, methanogenic archaea have developed a novel strategy to produce amino acid-like molecules that are unlikely to interact with any metabolic or biosynthetic machinery in the cells. These anaerobic organisms synthesize and accumulate several beta-amino acids (the anion beta-glutamate, and the two zwitterions beta-glutamine and N-epsilon-acetyl-beta-lysine) to balance external osmotic stress (17, 18, 34-38). The distribution among anionic and zwitterionic or noncharged solutes is correlated with intracellular  $K^+$  and external  $Na^+$  in archaea (17, 18, 37-40). Typically these organisms use beta-glutamate (or alpha-glutamate) as an osmolyte at low external salt but accumulate N-epsilon-acetyl-beta-lysine at external NaCl  $> 0.5$  M (38, 40, 41). Another zwitterion unique to methanogens is beta-glutamine (18). This solute has only been detected in the halophile *M. portucalensis* and represents a significant fraction of the osmolyte pool at the highest external NaCl levels. It is synthesized from beta-glutamate by an unusual glutamine synthetase (42). What regulates this glutamine synthetase to begin to generate beta-glutamine at high external NaCl is unclear. Betaine is a compatible solute that, due to convergent evolution, is ubiquitous in all kingdoms of life. It is actively transported into several archaea (as it is in many bacteria and eukaryotes) and used in preference to de novo synthesis of osmolytes (16, 38, 42, 43). However, there is at least one archaeon, *M. portucalensis*, which is capable of synthesizing betaine from glycine (41, 42). This reductive pathway for betaine synthesis is relatively uncommon. Methylation of glycine is carried out with S-adenosylmethionine as the methyl donor (43), and the extent of methylation (whether sarcosine or betaine is produced as the major product) depends on the concentration of  $K^+$ . Below 0.4 M  $K^+$ , sarcosine is the major product; above 0.4 M  $K^+$ , betaine

## Osmoregulation in Archaea

**Table 1.** Structure and occurrence of organic osmolytes in diverse organisms.

Osmolyte	Occurrence		
	Eukarya	Bacteria	Archaea
<b>Nonionic Solutes:</b>			
Polyols:			
glycerol 	algae ( <i>Dunaliella</i> sp.) fungi	Cyanobacteria <i>Saccharomyces cerevisiae</i>	-
mannitol 	yeast	?	-
sorbitol 	yeast	?	-
<i>myo</i> -inositol 	plants mammalian brain	?	-
<b>Carbohydrates:</b>			
sucrose 	plants and animals	cyanobacteria: <i>Anabaena</i> sp. <i>Phormidium autumnale</i> , <i>Chroococidiopsis</i> sp.	+ (by transport)
trehalose 	plants and animals	cyanobacteria: <i>Anabaena</i> sp. <i>Phormidium autumnale</i> , <i>Chroococidiopsis</i> sp. <i>Rhodothermus marinus</i>	<i>Natronobacterium magadii</i> <i>Sulfolobus solfataricus</i> <i>S. ambivalens</i>
alpha-glucosylglycerol 	-	marine & freshwater cyanobacteria: <i>Synechocystis</i> sp. <i>Microcystis firma</i> phototrophic bacteria: <i>Rhodobacter sulfidophilus</i> <i>Pseudomonas mendocina</i> <i>P. pseudoalcaligenes</i>	-

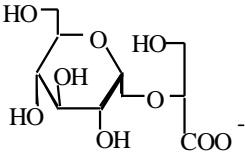
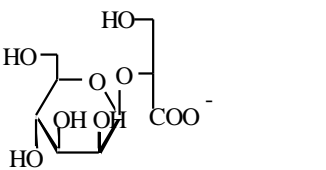
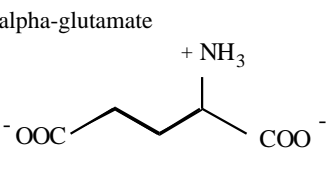
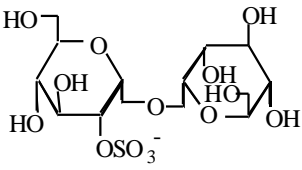
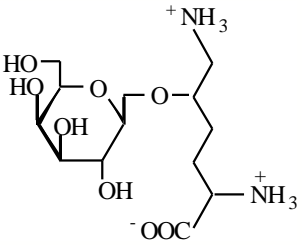
## Osmoregulation in Archaea

mannosucrose	-	-	<i>Thermoplasma acidophilum</i> <i>Pyrobaculum aerophilum</i> <i>Thermoproteus tenax</i> <i>Sulfolobus solfataricus</i> <i>Acidianus ambivalens</i>
			
alpha-mannosylglyceramide	-	<i>Rhodothermus marinus</i> <i>R. obamensis</i>	-
			
<b>Other:</b>			
urea	cartilagenous fish <i>Drosophila melanogaster</i>	-	-
			
N-acetylglutaminylglutamine amide	-	<i>Sinorhizobium meliloti</i> <i>Rhizobium leguminosarum</i>	-
			
Zwitterionic Solutes:			
glycine betaine	+	Transported by <i>E. coli</i> , <i>Corynebacterium glutamicum</i> <i>Listeria monocytogenes</i>	<i>Methanohalophilus portucalensis</i> <i>Methanosarcina thermophila</i>
			
dimethylsulfoniopropionate	-	+	-
			
ectoine	plants and algae	<i>Ectothiorhodospira</i> sp. <i>Brevibacterium linens</i> <i>Bacillus</i> sp. Proteobacteria <i>Vibrio costicola</i> <i>Micrococcus</i> sp. (aerobic, halophiles from Mono Lake)	-
			
proline	?	+	-
			
beta-glutamine	-	-	<i>Methanohalophilus portucalensis</i>

## Osmoregulation in Archaea

$\text{H}_2\text{N}-\text{C}(\text{O})-\text{CH}_2-\text{CH}(\text{NH}_3^+)-\text{CH}_2-\text{COO}^-$	-	-	
<p>N-epsilon-acetyl-beta-lysine</p> $\text{CH}_3-\text{C}(=\text{O})-\text{NH}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{COO}^-$	-	-	<p><i>Methanosarcina thermophila</i>  <i>Methanigenium cariaci</i>  <i>Methanohalophilus</i> sp.  <i>Methanococcus thermolithotrophicus</i></p>
<p>Trimethylamine oxide (TMAO)</p> $\text{H}_3\text{C}-\text{N}^+(\text{CH}_3)_3-\text{O}^-$	Elasmobranchs deep-living invertebrates teleost fishes	?	-
<b>Anionic Solutes:</b>			
<b>Phosphates:</b>			
<p>alpha-diglycerol phosphate</p> $\text{HO}-\text{CH}_2-\text{CH}(\text{OH})-\text{CH}_2-\text{O}-\text{P}(\text{O})(\text{O}^-)-\text{O}-\text{CH}_2-\text{CH}(\text{OH})-\text{CH}_2-\text{OH}$	-	-	<i>Archaeoglobus fulgidus</i>
<p>di-myo-1,1'-inositol phosphate (DIP)</p> $\text{HO}-\text{C}_6\text{H}_8\text{O}_5-\text{O}-\text{P}(\text{O})(\text{O}^-)-\text{O}-\text{C}_6\text{H}_8\text{O}_5-\text{OH}$	-	<i>Thermotoga maritima</i>	<p>hyperthermophilic archaea:  <i>Methanococcus igneus</i>  <i>Pyrococcus furiosus</i>  <i>P. woesei</i>  <i>Thermatoga maritima</i>  <i>Pyrodictium occultus</i></p>
<p>cyclic-2,3-diphospho-glycerate (cDPG)</p> $\text{C}_3\text{H}_5\text{O}_7\text{P}_2$	-	-	<p><i>Methanobacterium thermoautotrophicum</i>  <i>Methanopyrus kandleri</i>  <i>Methanothermus fervidus</i></p>
<b>Carboxylates:</b>			
<p>1,3,4,6-hexanetetracarboxylic acid (HTC)</p> $\text{OOC}-\text{CH}_2-\text{CH}(\text{COO}^-)-\text{CH}_2-\text{CH}_2-\text{COO}^-$	-	-	<p><i>Methanobacterium thermoautotrophicum</i>  Marburg and delta H</p>
<p>alpha-glucosylglycerate</p> $\text{C}_6\text{H}_{11}\text{O}_7$	-	marine heterotrophs	<i>Methanohalophilus portucalensis</i>

## Osmoregulation in Archaea

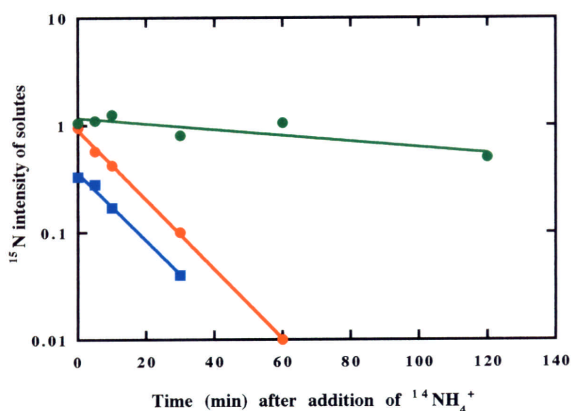
	-		<i>Rhodothermus marinus</i> <i>R. obamensis</i>	<i>Archaeoglobus fulgidus</i> <i>Thermus thermophilus</i> <i>Rhodothermus marinus</i> (alpha-isomer)
	mammals plants yeast		<i>E. coli</i>	<i>Halomonas elongata</i> <i>Methanococcus</i> sp. <i>Methanobacterium thermoautotrophicum</i> <i>Natronococcus occultus</i>
	-	+		<i>Methanococcus thermolithotrophicus</i> <i>M. igneus</i> <i>M. jannaschii</i> (other methanogens)
Sulfate: sulfotrehalose	-	-		<i>Natronobacterium</i> sp. <i>Natronococcus occultus</i>
				
Cationic Solute: beta-galactopyranosyl-5-hydroxylysine	-	-		<i>Thermococcus litoralis</i>
				

dominates (43). Clearly, if intracellular  $K^+$  changes, the intracellular concentration of betaine is likely to change as well.

Nonionic osmolytes are very common in bacteria and eukaryotes. Carbohydrates (sucrose, trehalose, mannosucrose) and polyols (glycerol and inositol) are the most common solutes in this class; they have been shown to protect cells from dehydration. For example, in mammalian cells (L929), sorbitol, sucrose, and trehalose

were shown to protect isolated chromatin both from dehydration and from disrupting effects of NaCl (44). Other nonionic solutes include the dipeptide N-acetylglutaminylglutamine amide (found in *Sinorhizobium meliloti* and *Rhizobium leguminosarum* (45)) and urea. *Drosophila melanogaster* and a number of fish accumulate urea (46) but need to accumulate 'urea-counteracting' solutes to mitigate the toxic effects of urea. All of these noncharged osmolytes are relatively rare (and in most cases not found) in archaea, although as mentioned above *N.*





**Figure 2.** Intensity of  $^{15}\text{N}$  labeling of osmolytes (red circle, alpha-glutamate; green circle, beta-glutamate; blue square, aspartate) in *M. thermolithotrophicus* grown at 4% NaCl in media with 2 mM  $^{15}\text{NH}_4\text{Cl}$  then diluted with  $^{14}\text{NH}_4\text{Cl}$  (to 40 mM total  $\text{NH}_4^+$ ).

*occultus* internalizes sucrose from the medium and uses this nonionic solute to balance osmotic stress (19).

The major class of osmolytes in archaea are anionic solutes. Most of these solutes represent the addition of a negatively charged group to carbohydrates and polyols that are used as osmolytes by bacteria and eukarya. The negative charge is provided by carboxylate, phosphate and sulfate groups; these modified solutes serve as counterions for  $\text{K}^+$ . As shown in table 1, these solutes include glucosylglycerate (37), beta-mannosylglycerate (47), alpha-diglycerol phosphate (39), di-*myo*-inositol-1-phosphate (DIP) (48, 49), and sulfotrehalose (19). Anionic carbohydrates are much less common in bacteria, although alpha-mannosylglycerate has been detected in *Rhodothermus* sp. (50). DIP is a particularly interesting solute that is accumulated in archaea and one bacterium (*Thermotoga maritima*) grown at supraoptimum temperatures (51). This is a complex, chiral solute for which two related biosynthetic pathways have been proposed (52, 53). In several of the archaea in which DIP is synthesized, there is no other known use of inositol (e.g., no inositol containing lipids), so that production of L-inositol-1-phosphate from D-glucose-6-phosphate (via inositol-1-phosphate synthase (IPS)) directs carbon resources to DIP only. This suggests that regulation of IPS could directly regulate the accumulation of DIP. Aside from these carbohydrates and polyols, the only other anionic solutes used as osmolytes in archaea are the alpha- and beta-glutamates.

#### 4.3. Stability of osmolytes *in vivo*

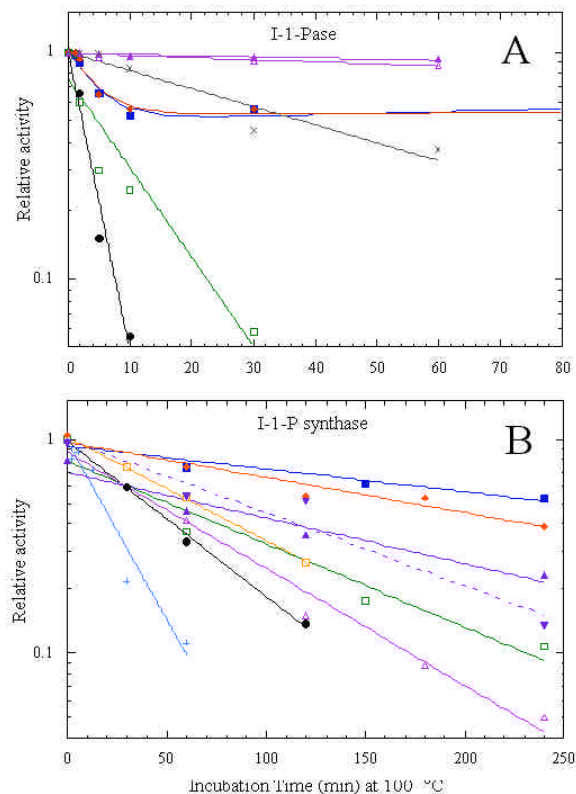
One of the properties attributed early on to a compatible solute is that it is relatively inert in terms of chemical reactivity in the cell (7). Studies in the early 90's using pulse labeling with stable isotopes detectable by NMR ( $^{13}\text{C}$ ,  $^{15}\text{N}$ ) followed by chasing with non-NMR active isotopes ( $^{12}\text{C}$ ,  $^{14}\text{N}$ ) have shown that many of the unique

osmolytes in archaea are, in fact, quite stable in cells adapted for growth at a particular NaCl concentration (37, 40, 54). Beta-amino acids such as beta-glutamate lose isotopic label on a much longer time scale than the L-alpha-glutamate which is also used by the cell for protein and cell wall biosynthesis. An example of this is shown in figure 2 where *M. thermolithotrophicus* cells labeled with  $^{15}\text{NH}_4^+$  were diluted with medium containing  $^{14}\text{NH}_4^+$ ; note the rapid decrease in  $^{15}\text{N}$  intensity of alpha-glutamate and alpha-aspartate (another minor component of the solute pool that is also incorporated into proteins) compared to beta-glutamate. Anionic carbohydrates such as DIP also turnover slowly compared to alpha-glutamate or other L-alpha-amino acids (49). However, osmolyte pools must have some dynamic character so that they can be altered when the external NaCl changes. *M. thermolithotrophicus* cells exposed to hypoosmotic shock show increased turnover of osmolyte pools that suggest cells have some mechanism to remove them if the external osmotic pressure drops (54).

#### 4.4. Effect of solutes on protein stability

Compatible solutes are accumulated in response to osmotic stress because of the lack of strong, specific interactions with proteins (55) and because less specific (generic) interactions with protein surfaces can replace water and maintain water at the surface where it is most needed. There are currently two explanations for how osmolytes stabilize proteins. The first of these postulates that solutes have a solvophobic effect on the protein (56, 57). Protein unfolding results in an increase of total protein surface area. Osmolytes destabilize the unfolded form of proteins compared to the folded structure to a much greater extent than water. Hence, proteins will want to assume a compact folded structure in the presence of osmolytes. The second recognizes a difference in properties (i.e., density) of bulk water and water adjacent to the macromolecule surface (58). Compatible solutes are thought to maintain equilibrium between these two water phases by accumulating at the interface regions. There is experimental evidence that supports the preferential accumulation of solutes at protein interfaces. For example, the preferential hydration of ribonuclease is 600 mol water per mol protein, but in the presence of sarcosine (a common osmolyte in eukaryotic organisms) the preferential hydration is reduced to 70 mole water / mol protein (59). That osmolytes do not interact with the macromolecule directly but alter water interactions with the protein has been examined by detailed NMR studies (measuring amide hydrogen exchange rates) of the effect of a model osmolyte, glycine, on the stabilization of chymotrypsin inhibitor 2 and horse heart cytochrome c (60). A high concentration of glycine was accompanied by a large reduction of the exchange rate constants of most slowly exchanging amide protons, although the changes in exchange rates were not uniform but varied over 2 orders of magnitude. This effect occurs without significant changes in the three-dimensional structure of the protein and is consistent with changing water density (fewer pathways for exchange since there is less water in the vicinity of the protein) in the presence of the compatible solutes.

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**Figure 3.** Stability of *A. fulgidus* (A) inositol-1-phosphate (I-1-P) phosphatase (I-1-Pase) and (B) I-1-P synthase (IPS) at 95°C as a function of added solutes: black circle, control (no added solutes); green square, glycerol; black square, inositol; blue square, sucrose; red diamond, trehalose; X, NaCl; hollow triangle, KCl; filled triangle, alpha-glutamate; upside-down triangle, beta-glutamate; +, -

+ glycine betaine. All solutes were present at 0.45 M except for the two glutamate isomers which were present at 0.3 M.

If solutes increase the energy of the denatured compared to the folded state, they should shift the denaturation equilibrium toward a higher  $T_m$  (61). A number of osmolytes common to bacteria and eukaryotes have been shown to increase the  $T_m$  of proteins. For example, betaine and related osmolytes provide an extraordinary degree of protection for hen egg white lysozyme. These interactions stabilize proteins by raising the chemical potential of the denatured protein. This leads to contraction of the random coil to a folded structure (62-64). Bolen and associates have also validated this hypothesis by showing that glycine-based osmolytes (glycine, sarcosine, and betaine) increased the  $T_m$  values for RNase and lysozyme (e.g., 8.2 M sarcosine increased the  $T_m$  for RNase by 22° (65)). Detailed studies with another zwitterion, trimethylamine oxide (TMAO), showed that it increased the population of native state of RNase relative to denatured structures by nearly five orders of magnitude (62). The same osmolyte was also shown to protect muscle-type lactate dehydrogenase homologues from trypsinolysis and pressure denaturation (66). Glycine, in contrast, showed no ability to reduce pressure denaturation and little or no ability to reduce the rate of proteolysis of these enzymes.

The ability of osmolytes, and most critically the ubiquitous  $K^+$ -salts to stabilize archaeal proteins to thermal denaturation is more anecdotal and less quantitative to date. There appears to be a trade-off in stabilizing proteins and partially inhibiting enzyme activities at high  $K^+$  concentrations. There are a number of examples where  $K^+$  salts (which can be as simple as KCl or  $K^+$ -phosphate) can stimulate the activity at low concentrations but inhibit enzymes at high concentrations. For example, *in vitro* nitrogenase enzyme assays using the *M. barkeri* enzyme showed that 100 mM  $K^+$ -alpha-glutamate enhanced activity but higher concentrations inhibited activity (50% inhibition occurred at 400 mM  $K^+$ -alpha-glutamate (41)). Two enzymes involved in methane formation from carbon dioxide and dihydrogen in *Methanopyrus kandleri*, cyclohydrolase and formyltransferase, were shown to be stabilized by 1 M  $K^+$ -cDPG (29). The protective effect was not, however, specific to cDPG, since  $K^+$ -phosphate and  $K^+$ -2,3-bisphosphoglycerate, the biosynthetic precursor of cDPG, could also activate and stabilize these enzymes. Two extremely thermophilic glutamate dehydrogenases (GDHs) from *P. furiosus*, the native GDH and a recombinant GDH mutant containing an extra tetrapeptide at the C-terminus, have been shown to be stabilized by added glycerol (67).

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Inositol monophosphatase (I-1-Pase) from several archaea and *T. maritima* has been cloned, overexpressed in *E. coli*, and examined for its stability at 95°C (68-70). The enzyme from *Archaeoglobus fulgidus* is the least stable of them with a half-life ( $t_{1/2}$ ) of 2-3 minutes at 95°C (70). Thermal denaturation is irreversible. Of the selection of known osmolytes examined for protection, only the K<sup>+</sup>-salts (a selection of these are shown in figure 3A) are extremely effective at stabilizing the protein for extended periods of time in boiling water. There was no apparent difference in effects of alpha- versus beta-glutamate. Interestingly, the zwitterions that were effective in stabilizing both RNase and lysozyme (glycine betaine, TMAO, proline) had little effect on the thermal stability of this archaeal protein. Thermal stability of another enzyme from the same organism (figure 3B), inositol-1-phosphate synthase (IPS), was also not enhanced by zwitterionic solutes (70). However, IPS was only marginally stabilized by K<sup>+</sup>-glutamate. For *A. fulgidus* IPS, the best thermal stabilization was achieved by the nonionic solutes sucrose or trehalose. Thus far there is no unique pattern associated with osmolyte stabilization of all archaeal proteins. However, the efficiency of K<sup>+</sup>-salts in enhancing the thermostability of many archaeal enzymes may be a relatively common observation. Where there is solute specificity in protecting proteins, we will need to understand how the different solutes interact with both water phases and the protein surface.

### 5. INDUCTION OF STRESS PROTEINS AND TRANSCRIPTIONAL REGULATION

There are several early responses involving transcription and translation that might occur when cells are osmotically shocked. (i) Key regulator molecules might be synthesized (as a result of interactions of osmosensors) that in turn effect osmolyte accumulation (by affecting signaling pathways, etc.). (ii) The proteins needed for osmolyte synthesis and/or accumulation might be synthesized. (iii) Protein machinery analogous to heat shock proteins might be synthesized in order to refold proteins whose conformation was adversely affected by the altered intracellular environment. For each of these regulation at the level of transcription may be critical.

#### 5.1 Yeast

Studies in yeast have provided the most detailed definition of the molecular nature of solute induction by external osmotic shock. Osmotolerance in yeast is regulated by at least two distinct mechanisms. The acquired or adapted response following long-term exposure to hypertonic medium is the accumulation of glycerol as an osmolyte. This requires the induction of the HOG-MAP (high-osmolarity glycerol mitogen-activated protein) kinase cascade. The more immediate or 'acute' response to external high Na<sup>+</sup> appears to be dependent on normal vacuole function and relies on the yeast endosomal/prevacuolar Na<sup>+</sup>/H<sup>+</sup> exchanger Nhx1 9 (71). The transcription of the GDP1 and GDP2 genes, which encode enzymes involved in glycerol biosynthesis, are activated by increased osmolarity (72). Heat shock protein

genes are also transcribed upon osmotic stress (e.g., HSP12). The mechanism involves an array of stress protective gene products (Msn1p, Msn2p, Msn4p, and Hot1p). Upon hyperosmotic shift, *S. cerevisiae* cells respond by transiently inducing the expression of stress-protective genes Msn2p and Msn4p. There are two transcription factors that determine the extent of this response (73). Two structurally related nuclear factors, Msn1p and Hot1p (for high-osmolarity-induced transcription), are also involved in osmotic stress-induced transcription. Hot1 single mutants are defective in the transient induction of GDP1 and GDP2 and exhibit delayed glycerol accumulation upon osmotic stress. Cells lacking the four stress protective gene products are almost devoid of the short-term transcriptional response of the genes GDP1, GDP2, CTT1, and HSP12 to osmotic stress. Such cells also show a distinct reduction in the nuclear residence of the mitogen-activated protein kinase Hog1p upon osmotic stress. Thus, Hot1p and Msn1p may define an additional tier of transcriptional regulators that control responses to high-osmolarity stress.

#### 5.2. Bacteria and Archaea

The level of understanding of how increased external NaCl is translated to solute accumulation is much less well understood in bacteria and archaea. In *E. coli*, there are several proline transport systems regulated by salt (74). In addition, there is a signaling system that responds to changes in salinity and controls expression of genes in porin formation (75). Expression of heat shock proteins in bacteria such as *E. coli* is often induced with stress, classically thermal stress but also osmotic stress. Presumably, the same behavior is exhibited by archaea. There may also be specific stress proteins that are synthesized in these methanogens. Chaperonins are synthesized in many archaeal cells; their role has usually been defined as refolding misfolded proteins when the incubation temperature is increased (76-78). There is a single study of protein synthesis in an archaeon, *Haloflex volcanii* (79, 80), that was osmotically shocked. Specific bands at 98 and 21 kDa were radiolabeled and detected by SDS-PAGE. The same two proteins appeared to be induced by salt and heat shock in that organism, supporting the theory that some HSPs can serve as general stress proteins.

### 6. CASE HISTORY: METHANOCOCCUS THERMOLITHOTROPHICUS

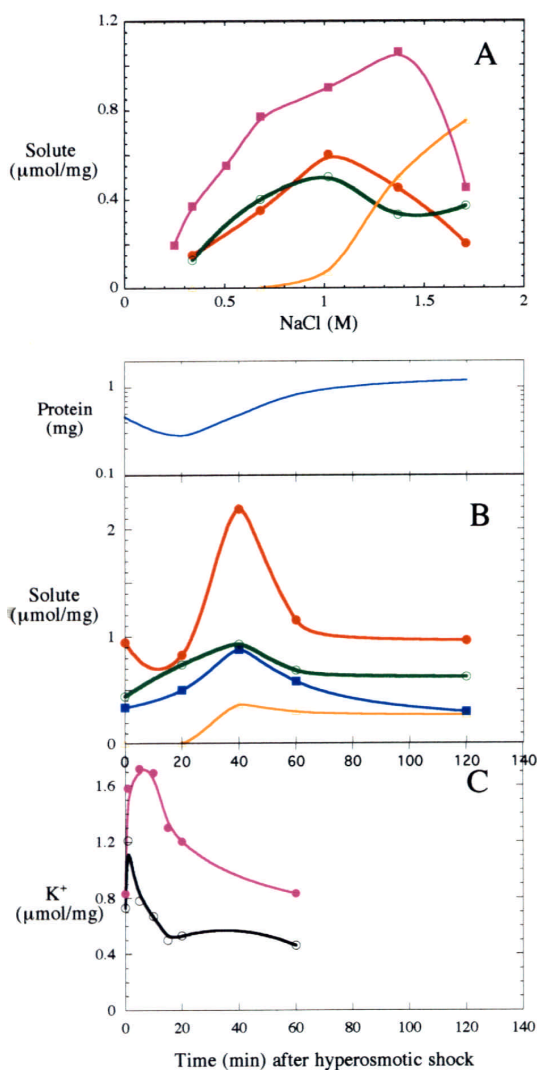
The adapted response of cells to a specific NaCl concentration (in terms of compatible solutes accumulated) may be different from what happens immediately upon altering the NaCl in the media. It has been shown (81) that intracellular water in *E. coli* is reduced rapidly upon increasing external NaCl. Within a few minutes, intracellular K<sup>+</sup> rises, soon followed by the synthesis and accumulation of alpha-glutamate (unless an appropriate osmolyte such as betaine is available in the medium and can be transported into the cell). K<sup>+</sup>-alpha-glutamate represents a temporary osmolyte pair (81) that is replaced by trehalose over a longer time course. Thus,

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osmoregulation requires an immediate response and a longer term adapted one. Very little is known about immediate versus adaptive responses of archaea to osmotic stress.

*Methanococcus thermolithotrophicus* is a halotolerant, thermophilic methanogen that accumulates the  $K^+$ -salts of alpha- and beta-glutamate when adapted to growth below 1 M NaCl. Adapted to growth at  $>1$  M NaCl, the organism accumulates the zwitterion N-epsilon-acetyl-beta-lysine (40) and it rapidly becomes the major solute (figure 4A). Given the switch in compatible solutes upon adaptation, it is of interest to examine the immediate response of the cells to hyperosmotic stress. As shown in figure 4B, when external NaCl in the medium of *M. thermolithotrophicus* cells is increased from 4 to 6% (0.67 to 1.0 M), the major organic solute that the cells accumulate is alpha-glutamate with much smaller increases in beta-glutamate, aspartate, and very little N-epsilon-acetyl-beta-lysine. Alpha-glutamate is the major solute during this period; the lower relative glutamate concentration and increased N-epsilon-acetyl-beta-lysine associated with adapted cells take at least one or two generations to be established (82). Also of interest is the time scale of osmolyte accumulation upon osmotic stress. There is a lag time in cell growth immediately after the hyperosmotic shock (figure 4B, top). The duration of the lag time depends on the magnitude of the shock - for the increase shown a 15-20 min lag is observed. Organic solute synthesis and accumulation occurs at the end of this

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**Figure 4.** (A) Distribution of osmolytes in *M. thermolithotrophicus* as a function of concentration of NaCl in the medium: red circle, L-alpha-glutamate; green circle, beta-glutamate; orange triangle, N-epsilon-acetyl-beta-lysine; purple square,  $\text{K}^+$ . (B) Organic osmolyte concentrations as a function of time after hyperosmotic shock (4 to 6 % NaCl); the symbols are the same as in (A) with the addition of aspartate (blue square). At the top is shown how growth, as represented by cell protein, is affected after the osmotic shock. (C) Intracellular  $\text{K}^+$  (filled circle) as a function of time after the 4 to 6% NaCl osmotic shock. The control, cells grown in 4% NaCl and diluted into the same concentration of NaCl (open circle), is also shown.

lag period. What happens during the lag period? It is assumed that the first response of the cells is a very rapid efflux of water. Rather than organic solutes,  $\text{K}^+$  is the first solute accumulated after hyperosmotic shock (figure 4C). This occurs rapidly within minutes after placing the cells in the higher NaCl medium.  $\text{K}^+$  overshoots what would be expected (based on it acting as a counterion to compatible

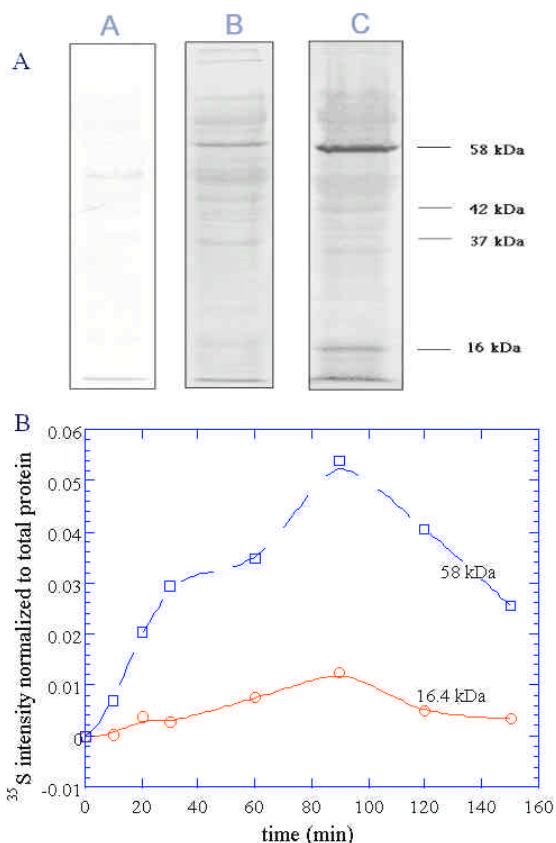
solute such as alpha- or beta-glutamate) to balance the external osmotic pressure. Within 10-15 min, it decreases to a new steady-state. It is at this stage that alpha-glutamate is accumulated. Thus, there is a hierarchy in the solutes accumulated in response to external osmotic stress. At 1.0 M NaCl, N-epsilon-acetyl-beta-lysine represents only about 6% of the total organic osmolyte pool. When the cells are grown in 8% (1.37 M) NaCl, N-epsilon-acetyl-beta-lysine represents 40% of the total pool. Nonetheless, when *M. thermolithotrophicus* cells grown in 4% NaCl are shifted to medium containing 8% NaCl, the response resembles that shown in figure 4 (rapid increase in  $\text{K}^+$  and reduction to a new steady-state, then the accumulation of alpha-glutamate), except that a longer lag is observed. Amounts of N-epsilon-acetyl-beta-lysine comparable to what would occur with steady-state growth in 8% NaCl are not synthesized. Like *E. coli*, this archaeon uses  $\text{K}^+$ -alpha-glutamate as a temporary osmolyte pair.

How else does *M. thermolithotrophicus* deal with increased external NaCl? Specific proteins may be synthesized to either generate solutes or to enhance refolding of proteins that become misfolded upon osmotic stress (misfolding may occur when intracellular water is lost and intracellular solute concentrations increased). The addition of  $^{35}\text{S}$ -methionine with increased NaCl and 1D SDS-PAGE or 2D IEF/SDS PAGE analyses can identify protein subunits whose synthesis is increased upon changing the external NaCl (83). Preliminary experiments with *M. thermolithotrophicus* (figure 5) show rapid  $^{35}\text{S}$ -labeling of two bands: one at  $58 \pm 2$  kDa and one at  $16 \pm 2$  kDa (figure 5A). The labeling of both these bands at a given time point after hyperosmotic shock increases with the magnitude of the shock (figure 5B). A recombinant chaperonin from *M. thermolithotrophicus* (84) has been identified and has about the right MW and pI for the 58 kDa band. The lower molecular weight protein is consistent with a recently cloned peptidyl prolyl cis-trans isomerase (85). Thus, it would appear that an important component of the cell's response to hyperosmotic stress is the production of protein machinery to aid in refolding proteins. What about generation of enzymes involved in glutamate biosynthesis? Since other  $^{35}\text{S}$ -labeled proteins are not detected, these may be constitutive (or occur at much lower numbers) and possibly regulated directly by  $\text{K}^+$ .

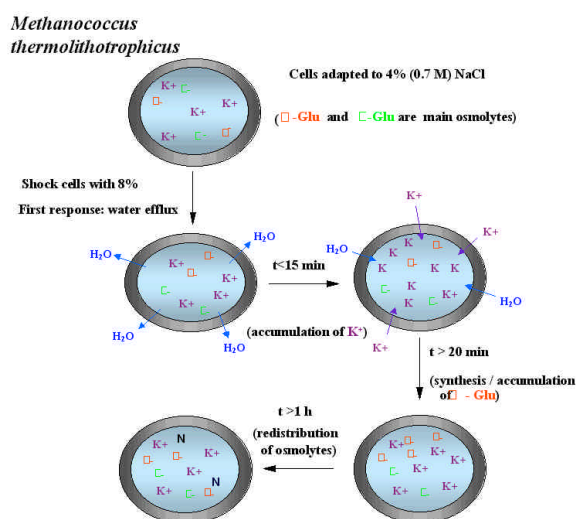
These analyses of *M. thermolithotrophicus* solutes and protein synthesis outline a temporal ordering in how the cells respond to changes in external NaCl. This is schematically indicated in figure 6. Immediately after hyperosmotic shock, water is released from the cell and  $\text{K}^+$  is internalized possibly by a mechanosensitive channel. As  $\text{K}^+$  re-enters the cell, water is again internalized (perhaps by an aquaporin?). On a longer time-scale, intracellular  $\text{K}^+$  decreases to a new steady-state that is higher than what was observed for cells grown in the lower NaCl-containing medium. This is coupled to synthesis and accumulation of alpha-glutamate (with smaller amounts of other solutes synthesized as well). At the same time, chaperonins and other refolding proteins are synthesized in response



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**Figure 5.** (A) SDS-PAGE autoradiogram of aliquots of *M. thermolithotrophicus* cultures taken 30 minutes after addition of <sup>35</sup>S-trans label along with dilution into new medium: (a) 4 to 4% NaCl control; (b) 4 to 6% NaCl hyperosmotic shock; (c) 4 to 8% NaCl hyperosmotic shock. (B) The increase in <sup>35</sup>S labeling of the 58±2 (blue square) and 16±2 (red circle) kDa bands as a function of time after increasing the external NaCl from 4 to 8%.



**Figure 6.** Schematic outlining the response of *M. thermolithotrophicus* to an increase in external NaCl (from

4 to 8% NaCl). A = alpha-glutamate, B = beta-glutamate, N = N-epsilon-acetyl-beta-lysine.

to the increased NaCl. As alpha-glutamate is increased, the cells begin to grow and over one or more generation times the intracellular organic solutes are readjusted to 'adapted' levels (i.e., with a higher proportion of N-epsilon-acetyl-beta-lysine compared to alpha-glutamate). Similar behavior may be exhibited by other archaea. *M. jannaschii*, which accumulates beta-glutamate as its primary osmolyte, also synthesizes alpha-glutamate immediately upon hyperosmotic stress (86). Since the genome sequence is known for this organism, it may be possible to identify other proteins induced upon NaCl shock.

## 7. PERSPECTIVE – KEY PROBLEMS IN ARCHAEA

At the present time, we have a detailed and rather comprehensive view of the identity of unusual organic solutes used by archaea for osmotic balance. However, for many of these biosynthetic pathways have not been elucidated. Identification of the enzymes involved is necessary to understand what steps control osmolyte accumulation. As an example, is the reaction that adds sulfate to the trehalose to produce sulfotrehalose in *N. occultus* a step regulated by external salt or is trehalose synthesis regulated? For anions with phosphate groups (e.g., DIP, alpha-diglycerol phosphate, cDPG), which enzymes in their biosynthetic pathways are osmotically regulated (presumably indirectly via some signaling cascade)? Another area of interest in osmoadapted cells is to understand why some organisms use multiple organic osmolytes (either anionic or zwitterionic) rather than a single one. *Methanococcus igneus* uses three anions (beta-glutamate, alpha-glutamate, and DIP) as osmolytes. When the external NaCl is increased, all three are increased. Similarly, for *M. portucalensis*, the three major zwitterionic osmolytes in these cells are glycine betaine, N-epsilon-acetyl-beta-lysine, and beta-glutamine. In both cases, at least two of the solutes are relatively inert (slow turnover when grown at a given NaCl concentration). What is there about the mixture of all three that either stabilizes the cellular milieu or macromolecules better than individual osmolytes?

The temporal response of archaea to hyperosmotic stress is an area ripe for investigation. It is likely that these organisms share features of bacterial stress responses, i.e., osmosensors and a signal transduction system. A major thrust for the future should be identification of sensors and identification of the appropriate signaling pathways. Tied in with these studies should be experiments to define the mechanisms that control osmolyte biosynthesis and accumulation. There is anecdotal evidence that supraoptimal growth temperatures enhance DIP accumulation, but how this is achieved on a molecular level is unknown. Most of the published work with archaea has also stressed hyperosmotic shock. Since many archaea possess rigid cell walls, it is possible that they will be able to withstand hypoosmotic shock to a greater degree than other types of cells. If solutes are released from the cells, are there specific VAC's or Msc's

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that mediate this? There may be archaeal homologues to the osmosensing transmembrane channels in eukaryote and bacterial systems. If solutes are not released, how is the cell growth affected?

Once we understand what controls the accumulation of unusual osmolytes in archaea and how these solutes stabilize macromolecules, we may use this information in the bioengineering arena. The ultimate hope is that one can engineer organisms with enhanced osmolyte biosynthesis capability. This is currently being done in plants (87) with the expression of genes encoding critical steps in the synthesis of osmoprotectant compounds. These transgenic plants generally accumulate low levels of osmoprotectants and have increased stress tolerance.

### 8. ACKNOWLEDGEMENT

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