Minireview

Compatible solutes of organisms that live in hot saline environments

Helena Santos[1] and Milton S. da Costa[2]

[1]*Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Apartado 127, 2780–156 Oeiras, Portugal.*
[2]*Departamento de Bioquímica, Universidade de Coimbra, 3001–401 Coimbra, Portugal.*

Summary

The accumulation of organic solutes is a prerequisite for osmotic adjustment of all microorganisms. Thermophilic and hyperthermophilic organisms generally accumulate very unusual compatible solutes namely, di-*myo*-inositol-phosphate, di-mannosyl-di-*myo*-inositol-phosphate, di-glycerol-phosphate, mannosylglycerate and mannosylglyceramide, which have not been identified in bacteria or archaea that grow at low and moderate temperatures. There is also a growing awareness that some of these compatible solutes may have a role in the protection of cell components against thermal denaturation. Mannosylglycerate and di-glycerol-phosphate have been shown to protect enzymes and proteins from thermal denaturation *in vitro* as well, or better, than compatible solutes from mesophiles. The pathways leading to the synthesis of some of these compatible solutes from thermophiles and hyperthermophiles have been elucidated. However, large numbers of questions remain unanswered. Fundamental and applied interest in compatible solutes and osmotic adjustment in these organisms, drives research that, will, in the near future, allow us to understand the role of compatible solutes in osmotic protection and thermoprotection of some of the most fascinating organisms known on Earth.

Slightly halophilic thermophiles and hyperthermophiles

Thermophiles and hyperthermophiles have been isolated from a large variety of thermal environments. These organisms belong to the *Bacteria* and to the *Archaea*, although the latter domain includes a larger variety of organisms living at higher temperatures than the former. The term thermophile is often used to define organisms that have optimum growth temperatures between 65°C and 80°C, while hyperthermophilic organisms are those with optimum growth temperatures above 80°C. (Blöchl *et al.*, 1995).

Most thermophilic organisms have been isolated from continental geothermal or artificial thermal environments, but some thermophiles have been isolated from marine hydrothermal environments, the best known of which are *Rhodothermus marinus* and *Thermus thermophilus* (Alfredsson *et al.*, 1988; da Costa *et al.*, 2001). The water venting from continental hot springs is generally low in sodium and the isolates, being fresh water organisms, rarely grow in media containing more than 1.0% NaCl (w/v). Some organisms isolated from continental fresh water hot springs are, however, halotolerant with optimum growth in media without added NaCl but, like the strains of *Thermus thermophilus,* are able to grow in media containing 4.0–6.0% NaCl (da Costa *et al.*, 2001).

On the other hand, hyperthermophilic organisms, with a few notable exceptions such as the species of the Order *Sulfolobales*, *Pyrobaculum islandicum* and *Thermococcus zilligii*, originate from shallow or abyssal marine geothermal areas (Blöchl *et al.*, 1995). The water in shallow marine or abyssal environments is generally saline but, varies from low salinity to that of seawater. The organisms that originate from these environments are, as would be expected, generally slightly halophilic, requiring sodium for growth. Optimum growth occurs in media containing 0.5–2.0% NaCl. Few organisms, if any, grow at salinities higher than 6.0–8.0% NaCl.

Moderate halophiles are defined as organisms having higher growth rates in media containing between 0.5 M and 2.5 M NaCl, while extreme halophiles have higher growth rates in media containing over 2.5 M NaCl (Ventosa *et al.*, 1998). Moderate or extremely halophilic organisms simultaneously capable of growth at very high temperatures have not yet been identified. The reason for our inability to isolate such organisms possibly stems from the fact that appropriate environments may be very rare or may not even exist on Earth. There are continental

springs with salinities higher than seawater but, the water temperature is usually fairly low. Deep, hot brines in the Red Sea reach temperatures of about 60°C, although higher temperatures have been estimated but not actually measured (Hartmann *et al.*, 1998). The latter environments could, in the future, be the source of truly halophilic hyperthermophiles. For the time being the most thermophilic and halophilic organisms known include *Halothermothrix orenii* isolated from sediments of a salt lake, with an optimum growth temperature of 60°C and a salt range for growth of 4–20% (Cayol *et al.*, 1994), and *Thermohalobacter berrensis* isolated from solar salterns, with an optimum growth temperature of 65°C and a salt range between 2 and 15% (Cayol *et al.*, 2000). Scientists interested in osmotic adjustment in (hyper)thermophiles are, for now, only able to examine halotolerant and slightly halophilic organisms.

Roles of compatible solutes

Hyperthermophiles are, like all organisms living in aqueous environments, faced with alterations in the water activity due to fluctuations in the levels of dissolved salts or sugars. To adjust to lower water activities of the environment and the resulting decrease in cytoplasmic water, microorganisms must accumulate intracellular ions or organic solutes to re-establish the cell turgor pressure and/or cell volume and, at the same time, preserve enzyme activity (Brown, 1990).

Microorganisms have developed two main strategies for osmotic adjustment. One strategy relies on the selective influx of K^+ from the environment to, sometimes extremely, high levels and is known as the 'salt-in-the-cytoplasm' type of osmotic adaptation (Galinski, 1995; da Costa *et al.*, 1998; Roeßler and Müller, 2001). This type of osmotic adjustment occurs in the extremely halophilic archaea of the family *Halobacteriaceae*, the anaerobic moderately halophilic bacteria of the Order *Halanaerobiales* (Oren, 1999) and the extremely halophilic bacterium *Salinibacter ruber* (Anton *et al.*, 2002; Oren and Mana, 2002). The presence of the saline type of osmotic adaptation in three distinct lineages of organisms implies independent development of this strategy, it being difficult to envision as an ancient characteristic retained in a few scattered groups of organisms or a characteristic occurring through massive lateral gene transfer.

The majority of microorganisms have not, however, undergone extensive genetic alterations as a prerequisite for adaptation to a saline environment and, the intracellular macromolecules are generally sensitive to high levels of inorganic ions. These organisms favour the accumulation of specific small-molecular weight compounds, known as compatible solutes or osmolytes (Brown, 1976, Brown, 1990; Ventosa *et al.*, 1998). A large variety of microorganisms, ranging from archaea, bacteria, yeast, filamentous fungi and algae, rely exclusively on the accumulation of compatible solutes for osmoadaptation, indicating that this strategy is very successful. Compatible solutes can also be taken up from the environment if present or, they can be synthesized *de novo*. The most common compatible solutes of microorganisms are neutral or zwitterionic and, include amino acids and amino acid derivatives, sugars, sugar derivatives (heterosides) and polyols, betaines and the ectoines (da Costa *et al.*, 1998). Some are widespread in microorganisms, namely trehalose, glycine betaine and α-glutamate, while others are restricted to a few organisms. Polyols, for example, are widespread among fungi and algae but are very rare in bacteria and unknown in archaea. Ectoine and hydroxyectoine are examples of compatible solutes found only in bacteria.

Many slightly and moderately halophilic methanogens possess a mixed type of osmoadaptation where K^+ accumulates to high levels along with neutral and negatively charged organic solutes (Martin *et al.*, 1999).

A. D. Brown originally defined compatible solutes as small organic compounds used for osmotic adjustment that do not interfere with cell function (Brown, 1976). It is also implicit by this definition that compatible solutes protect proteins and other cell components from osmotic-induced dehydration. However, the role of compatible solutes goes beyond osmotic adjustment alone, to the protection of cells and cell components from freezing, desiccation, high temperature and oxygen radicals (da Costa *et al.*, 1998; Argüelles, 2000; Welsh, 2000; Benaroudj *et al.*, 2001; Santos and da Costa, 2001). In view of these properties, many compatible solutes can, in fact, be regarded as stress protectants. The protective role of trehalose against several stress conditions has been amply demonstrated (Singer and Lindquist, 1998; Simola *et al.*, 2000), and the accumulation of glycerol, the canonical osmolyte of yeast, has also been correlated with the acquisition of thermotolerance (Siderius *et al.*, 2000). Some hyperthermophiles accumulate very high levels of di-*myo*-inositol-1,1′-phosphate (DIP) primarily during growth at supraoptimum temperatures, which may have a role in thermoprotection of these organisms (Martins and Santos, 1995; Martins *et al.*, 1997; L. Gonçalves, R. Huber, M. S. da Costa and H. Santos, unpublished results).

Compatible solutes of thermophiles and hyperthermophiles

Thermophiles and hyperthermophiles accumulate compatible solutes that have not been found, or are rarely encountered in mesophilic organisms, leading to the view that the compatible solutes of (hyper)thermophiles are specifically associated with life at high temperatures.

These compatible solutes are generally negatively charged, as are many of those of archaea, while other microorganisms generally accumulate neutral or zwitterionic compatible solutes. In this respect, the negatively charged compatible solutes of thermophilic and hyperthermophilic bacteria resemble those of the archaea and, not those of mesophilic bacteria (Fig. 1). Inevitably there are exceptions, as some thermophilic bacteria, e.g. *Thermus thermophilus,* and hyperthermophilic archaea, namely *Pyrobaculum aerophilum*, accumulate primarily the neutral compatible solute trehalose (Nunes *et al.*, 1995; Martins *et al.*, 1997).

Thus far, DIP is the most widespread small-molecular weight solute of hyperthermophilic archaea and has never been found in mesophilic organisms. DIP was initially identified in *Pyrococcus woesei* (Scholz *et al.*, 1992) and *Methanococcus igneus* (Ciulla *et al.*, 1994), and was later detected in other hyperthermophilic archaea, namely in *Pyrodictium occultum*, *Pyrodictium furiosus*, *Archaeoglobus fulgidus*, *Themococcus celer*, *Themococcus stetteri*, *Themococcus litoralis* and *Pyrolobus fumarii* (T. Faria, R. Huber and H. Santos, unpublished results; Martins *et al.*, 1997; Lamosa *et al.*, 1998). In most of these organisms, large increases in the levels of DIP are observed at growth temperatures above the optimum, reaching 20-fold in *P. furiosus* at 101°C (Martins and Santos, 1995). With respect to DIP, the strict definition of a compatible solute

in osmotic adjustment is not applicable but, as discussed above, this is one of many roles presently assigned to compatible solutes in stress protection and DIP could have a role associated with thermoprotection.

The hyperthermophilic bacteria *Thermotoga neapolitana* and *Thermotoga maritima* accumulate DIP, di-*myo*-inositol-1,3′-phosphate and di-mannosyl-di-*myo*-inositol-phosphate. These *myo*-inositol derivatives increase dramatically at temperatures above the optimum for growth, although DIP is the major organic solute during salt stress (Martins *et al.*, 1996).

α-Mannosylglycerate (MG) is another widely distributed compatible solute among thermophilic and hyperthermophilic organisms. This compound has otherwise only been encountered in the red algae of the order *Ceramiales* (Bouveng *et al.*, 1955), although these organisms, like many other algae, accumulate primarily mannitol for osmotic adjustment (Karsten *et al.*, 1994). α-Mannosylglycerate together with the corresponding amide derivative, α-mannosylglyceramide, was identified in the thermophilic bacterium *Rhodothermus marinus* (Nunes *et al.*, 1995; Silva *et al.*, 1999). The negatively charged form (MG) has also been detected in several thermophilic bacteria such as *Thermus thermophilus*, *Rubrobacter xylanophilus*, euryarchaeotes of the genera *Pyrococcus* and *Thermococcus*, *Methanothermus fervidus*, *Archaeoglobus venificus*, *Archaeoglobus profundus* and, more
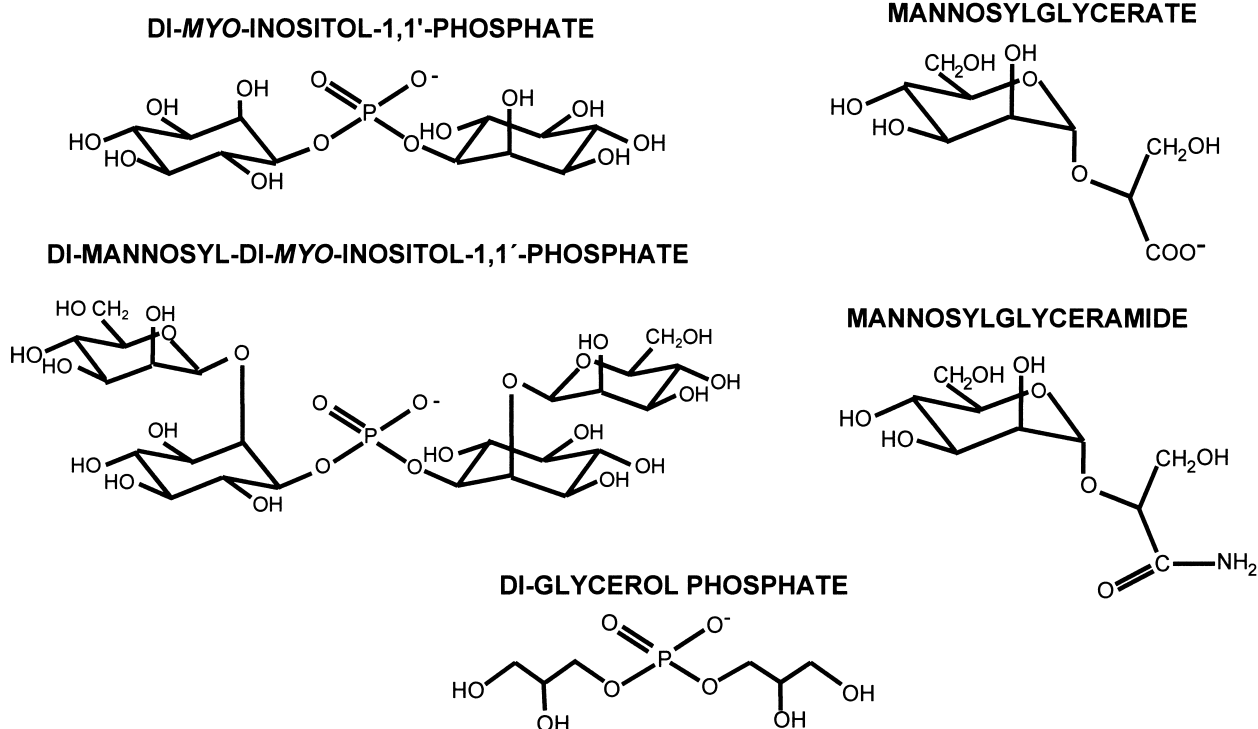

Fig. 1. Compatible solutes restricted to thermophilic and hyperthermophilic bacteria and archaea.

recently, in the crenarcheaote *Aeropyrum pernix* (Martins *et al.*, 1997; Santos and da Costa, 2001; L. Gonçalves, R. Huber, M. S. da Costa and H. Santos, unpublished results). In contrast to DIP, the concentration of MG increases concomitantly with the NaCl concentration of the medium and serves, therefore, as a compatible solute under salt stress. The neutral MG-derivative, mannosylglyceramide, has only been found, in addition to MG, in a few strains of *R. marinus*. In these strains, MG accumulates during salt stress at supraoptimal growth temperatures, while mannosylglyceramide accumulates preferentially during salt stress at lower growth temperatures (Silva *et al.*, 1999). Although *T. thermophilus* accumulates MG, trehalose is the most abundant organic solute at high salinity.

The very rare di-glycerol-phosphate (DGP) has only been found in *Archaeoglobus fulgidus*, where it is by far the major compatible solute during salt stress (Martins *et al.*, 1997; L. Gonçalves, R. Huber, M. S. da Costa and H. Santos, unpublished results). However, DIP also accumulates in this organism and becomes the major solute when the temperature is raised above the optimum for growth. Recently, and contrary to expectations, MG was also detected in *A. veneficus* and *A. profundus*. From the complete genome sequence of *A. fulgidus* we know that putative genes for the synthesis of MG are not present in the type strain of *A. fulgidus*, but it is now obvious that the other strains of *Archaeoglobus* synthesize MG. It appears therefore that the genes for the synthesis of MG were lost during the evolution of the type strain of *A. fulgidus*, and that this organism was able to develop an alternative solute, DGP, to cope with osmotic stress. (L. Gonçalves, R. Huber, M. S. da Costa and H. Santos, unpublished results).

Almost all halotolerant or slightly halophilic thermophiles and hyperthermophiles examined, accumulate α-glutamate during growth in media containing low levels of NaCl, but this amino acid is replaced by other compatible solutes as the salinity of the medium increases (da Costa *et al.*, 1998; Santos and da Costa, 2001). The rare amino acid, β-glutamate, also accumulates in the hyperthermophilic and slightly halophilic bacterium *Thermotoga neapolitana* but, is never the dominant compatible solute (Martins *et al.*, 1996).

Trehalose, one of the most common compatible solutes of mesophilic organisms, is also frequently encountered in (hyper)thermophilic organisms. In some, namely in *T. thermophilus*, trehalose is the major compatible solute during salt stress in media containing yeast extract (Nunes *et al.*, 1995). Trehalose has recently been shown to be a *de facto* compatible solute in one strain of this species, as mutants in which the genes encoding trehalose phosphate synthase and trehalose phosphate phosphatase had been deleted, were unable to grow in defined medium (lacking yeast extract as a source of trehalose)

containing over 2% NaCl. Nevertheless, growth was restored at higher salt concentrations by the addition of trehalose to the medium (Z. Silva, S. Alarico, A. L. Nobre, R. Horlacher, W. Boos, H. Santos and M. S. da Costa, unpublished results).

Low level accumulation of trehalose in *Pyrococcus horikoshii* and *P. furiosus*, and high levels of trehalose in *Thermococcus litoralis*, appears to derive exclusively from yeast extract in the medium. In these organisms, trehalose appears to be taken up from yeast extract through a high affinity ABC maltose/trehalose transporter (Horlacher *et al.*, 1998; Koning *et al.*, 2002). The hyperthermophilic crenarchaeote *Pyrobaculum aerophilum,* accumulates trehalose exclusively at its optimum growth temperature and salinity, unlike other hyperthermophilic archaea. Trehalose also accumulates in the archaea of the Order *Sulfolobales*, but its function is unclear as these organisms do not grow in medium containing NaCl above 1.0% (Nicolaus *et al.*, 1988).

Another compatible solute, restricted to methanogens, that deserves mention is cyclic 2,3-bisphosphoglycerate (cBPG). This solute has been detected in some mesophilic as well as (hyper)thermophilic methanogens (Martin *et al.*, 1999). In most cases, there seems to be a positive correlation between the levels of intracellular K^+, cBPG and higher growth temperatures of the organisms, culminating in the accumulation of very large concentrations of cBPG in *Methanopyrus kandleri*. This observation led to the hypothesis that this solute plays a role in thermoprotection of macromolecules and is supported by *in vitro* studies showing that cBPG protects selected enzymes from *M. kandleri* against thermal denaturation (Shima *et al.*, 1998). However, this solute also contributes to osmotic adjustment by virtue of its intracellular concentration in media containing NaCl.

Regulation of compatible solute accumulation in (hyper)thermophilic organisms

Most thermophiles and hyperthermophiles accumulate unusual compatible solutes by *de novo* synthesis during salt stress even when potential compatible solutes are present in the growth medium. Yeast extract is generally added to these growth media, and it is well known that it is a source of compatible solutes such as trehalose and glycine betaine. However, *Pyrococcus* spp. accumulate primarily MG and DIP, while *R. marinus* accumulates MG and mannosylglyceramide, excluding trehalose and glycine betaine from the compatible solute pool. Low levels of glycine betaine accumulate in a few strains of *Thermus thermophilus*, but this compatible solute does not appear to accumulate in the other thermophilic organisms examined (Nunes *et al.*, 1995; Martins *et al.*, 1997). Moreover,

glycine betaine is common in mesophilic methanogens but has not been detected in thermophilic or hyperthermophilic methanogens (Martin *et al.*, 1999).

The absence of glycine betaine and ectoine, among other common compatible solutes of mesophiles, in organisms that live at extremely high temperature supports the hypothesis that common compatible solutes of mesophiles are not used by these organisms. These compatible solutes do not meet the requirements of thermophiles and hyperthermophiles for osmotic adjustment or other forms of stress protection, perhaps because they are not taken up from the environment through lack of the appropriate transporters or, are unstable at high growth temperatures. No general answer may be appropriate for all (hyper)thermophilic organisms because of their diverse phylogenetic relationships but some preliminary conclusions are possible. It is, for example, unlikely that the absence of uptake systems is the primary reason for the inability of (hyper)thermophilic organisms to accumulate common compatible solutes. Glycine betaine is one of the most widespread compatible solutes of mesophilic bacteria and methanogens, which generally take it up from the medium. It is therefore likely, that some thermophiles could have transport systems for glycine betaine. Moreover, *T. thermophilus* chooses trehalose and MG over glycine betaine for osmotic adjustment despite its ability to accumulate the latter compatible solute (Nunes *et al.*, 1995).

In contrast to many (hyper)thermophiles which synthesize specific compatible solutes, *T. litoralis* uptakes a panoply of solutes, some of them very unusual, from peptone and yeast extract of the medium, namely aspartate, β-galactopyranosyl-5-hydroxylysine, hydroxyproline and trehalose, while the canonical MG and DIP are moderate constituents of the compatible solute pool (Lamosa *et al.*, 1998). Growth of *T. litoralis* in media containing tryptone instead of peptone, and lacking yeast extract, leads to the accumulation of high levels of MG and DIP, while the other solutes are absent or are minor components. In contrast to this species, *T. celer* and *T. stetteri* accumulate primarily MG and DIP in medium containing yeast extract and peptone. Thus, *T. litoralis*, unlike many other hyperthermophiles examined, takes advantage of compatible solute uptake as a mechanism for osmotic adjustment, although glycine betaine is not one of the compatible solutes.

The role of hypersolutes in thermoprotection of enzymes

Compatible solutes from thermophiles have been shown to contribute, at least *in vitro*, to the thermostability of enzymes, and we are sometimes tempted to extrapolate these results to *in vivo* systems. However, the majority of intracellular enzymes from thermophiles and hyperther-

mophiles are intrinsically stable to heat and compatible solutes should not be necessary for 'gross' stabilization of cell components. It is, nevertheless, tempting to note that compatible solutes may have an auxiliary function and improve protein stability further. One major difficulty with the view that osmolytes have an important role in stabilization of cell components of thermophiles and hyperthermophiles at supraoptimal growth temperatures, is raised by the observation that compatible solutes have not been encountered, with the exception of species of the *Sulfolobales*, in (hyper)thermophiles, such as *Thermotoga thermarum*, *Hydrogenobacter islandicum*, *Thermococcus zilligii* and *Pyrobaculum islandicum* that grow in media without additional NaCl (Martins *et al.*, 1996; Martins *et al.*, 1997) but have growth temperature ranges similar to other species that are slightly halophilic and accumulate compatible solutes. These observations indicate that compatible solutes accumulate to cope with osmotic stress and, that cell components of (hyper)thermophiles do not require them for thermoprotection. It is, nevertheless, impossible not to interpret the massive accumulation of organic solutes, such as DIP, at temperatures above the optimum for growth as a strategy to cope with heat stress. At this stage of our knowledge, we should refrain from premature conclusions on the relative importance of compatible solutes from (hyper)thermophiles on *in vivo* thermostabilization of cell components, although some, notably MG and DGP, have a profound stabilizing effect on proteins *in vitro*. Mannosylglycerate has also been shown to have a profound effect on thermoprotection and protection against desiccation on enzymes of mesophilic, thermophilic and hyperthermophilic origin (Ramos *et al.*, 1997).

An extensive comparative study using trehalose, ectoine, hydroxyectoine, DIP, DGP, MG and mannosylglyceramide on rabbit muscle lactate dehydrogenase showed that MG and hydroxyectoine were the most efficient stabilizers of this enzyme from heat inactivation (Fig. 2) (Borges *et al.*, 2002; Lamosa *et al.*, 2000). Glucosylglycerol, a compatible solute of cyanobacteria and some algae, was also very efficient in protecting lactate dehydrogenase, while glycerol had no significant effect. Ectoine conferred no protection, and DIP had a strong destabilizing effect (Borges *et al.*, 2002). The last observation is difficult to reconcile with the results that show that DIP accumulates to high levels during supraoptimal growth temperatures. The role of DIP in the thermostabilization of enzymes remains questionable, although it has been shown to have a positive effect on the stabilization of glyceraldehyde-3-phosphate dehydrogenase of *P. woesei*, but did not increase the stability of hydrogenase and pyruvate ferredoxin oxidoreductase of *Thermotoga maritima* (Scholz *et al.*, 1992; Ramakrishnan *et al.*, 1997). The stabilizing effect of cBPG was also recently demonstrated on two
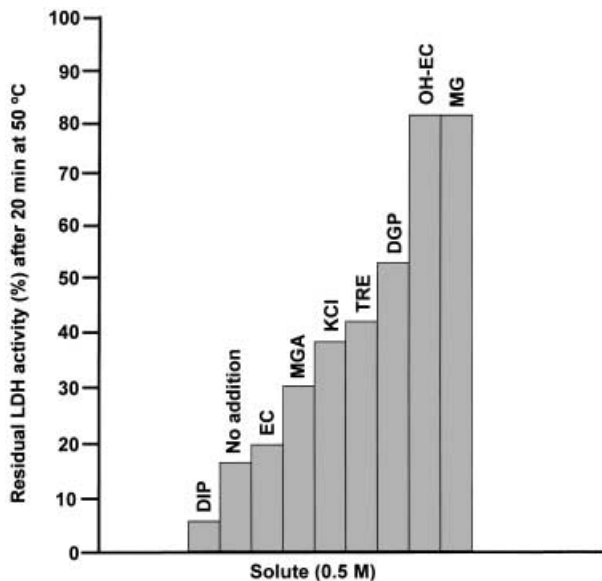
Fig. 2. Effect of compatible solutes (0.5 M) on the thermostability of rabbit muscle lactate dehydrogenase (50 µg ml^{-1}) at 50°C for 20 min. DIP, di-*myo*-inositol-1,1′-phosphate; EC, ectoine; MGA, mannosylglyceramide; KCl, potassium chloride; TRE, trehalose; DGP, di-glycerol-phosphate; OH-EC, hydroxyectoine; MG, mannosylglycerate. (Data from Borges *et al.*, 2002).

enzymes involved in methane formation in *Methanopyrus kandleri* (Shima *et al.*, 1998).

It should be mentioned that salts, such as ammonium sulphate and potassium phosphate, have a thermoprotective effect on enzymes that may equal the effect of organic solutes *in vitro*. However, it is unlikely that these salts could play a role *in vivo*, as the large concentrations required for protection would interfere with enzyme activity and metabolic regulation, and have never been found *in vivo*.

Genes and enzymes for the synthesis of compatible solutes from hyperthermophiles

Basic knowledge of the biosynthesis of specific compatible solutes is needed to understand the mechanisms underlying the events leading to salt and thermal tolerance, from water stress sensing to maintenance of the appropriate intracellular levels of compatible solutes. The pathways for the synthesis of cBPG (Lechmacher *et al.*, 1990; Gorkovenko and Roberts, 1993; Matussek *et al.*, 1998) and DIP (Chen *et al.*, 1998; Scholz *et al.*, 1998) have already been studied in some detail, and we will restrict ourselves to discussing the synthesis of mannosylglycerate in *R. marinus* and *Pyrococcus* spp.

The biosynthetic route for the synthesis of mannosylglycerate has been examined in *R. marinus* (Fig. 3) and *P. horikoshii*. The synthesis of MG proceeds via two alter-

nate routes in *R. marinus*. In one pathway, GDP-mannose is condensed with D-glycerate to produce MG in a single reaction catalyzed by mannosylglycerate synthase (Martins *et al.*, 1999), while in the other pathway, mannosylphosphoglycerate synthase (MPGS) catalyzes the conversion of GDP-mannose and D-3-phosphoglycerate into mannosylphosphoglycerate, which is subsequently converted to MG by the action of mannosylphosphoglycerate phosphatase (MPGP).

In contrast to *R. marinus*, the synthesis of MG in *Pyrococcus* proceeds via only a two-step pathway involving the phosphorylated intermediate (Empadinhas *et al.*, 2001). The genes encoding the enzymes that convert fructose-6-phosphate into MG are clustered together, in an operon-like structure, in the genome of *P. horikoshii*. (Fig. 4) The genes clustered upstream of *mpgs* and *mpgp*, encoding putative phosphomannose mutase and bifunctional phosphomannose isomerase/mannose-1-phosphate guanynyl transferase, may be committed to MG synthesis, as other candidate mannose-1-phosphate guanynyl transferase and phosphomannose mutase genes are found in the *P. horikoshii* genome. This gene structure is also found in *P. furiosus* and *P. abyssi*, but only adjacent MGPS and MPGP genes are found in the genome of the crenarchaeote *Aeropyrum pernix*, while phosphomannose mutase and bifunctional phosphomannose isomerase/mannose-1-phosphate guanylyl transferase are found elsewhere in the genome (Fig. 4). We have evidence that *T. thermophilus* only possesses the two-step pathway leading to the synthesis of MG (N. Empadinhas, L. Pinto, A. Enke, H. Santos and M. S. da Costa, unpublished results). This two-step pathway is similar to those described for the synthesis of osmolytes like trehalose (Giæver *et al.*, 1988), glucosylglycerol (Hagemann and Erdmann, 1994) and galactosylglycerol (Yokoyama *et al.*, 1987), as all proceed via a phosphorylated intermediate. The existence of a single pathway for the synthesis of MG
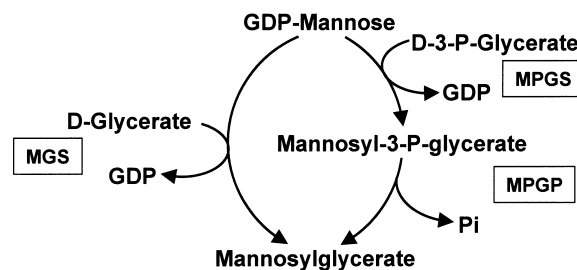

Fig. 3. Pathways for the synthesis of mannosylglycerate in *Rhodothermus marinus* and *Pyrococcus* spp. The single-step pathway using MGS (mannosylglycerate synthase) has only been found in *R. marinus*. The two-step pathway which utilizes MPGS (mannosylphosphoglycerate synthase) followed by MPGP (mannosylphosphoglycerate phosphatase) is also present *in R. marinus*. It is the only pathway found in *Pyrococcus* spp., *Aeropyrum pernix* and *Thermus thermophilus*.
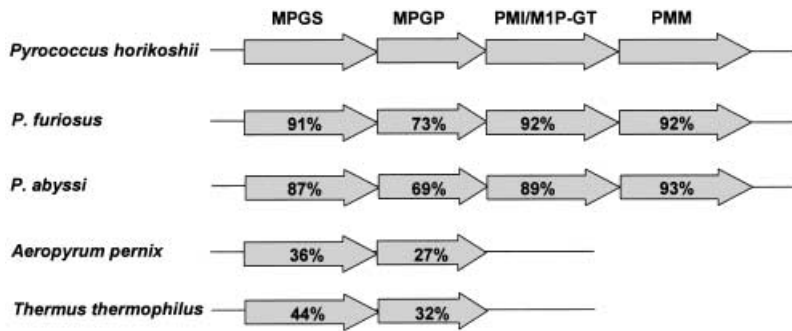
Fig. 4. Genomic organization of the mannosylglycerate operon in *Pyrococcus horikoshii*, *P. furiosus*, *P. abyssi*, *Aeropyrum pernix* and *Thermus thermophilus*. The percentage of amino acid identity between homologous sequences is indicated inside the arrows. MPGS, mannosylphosphoglycerate synthase; MPGP, mannosylphosphoglycerate phosphatase; PMI/M1P-GT, bifunctional phosphomannose isomerase/mannose-1-phosphate guanylyl transferase; PMM, phosphomannose mutase.

in several (hyper)thermophiles, instead of the branched pathway of *R. marinus,* probably imposes a lower flexibility on the regulation of MG synthesis in response to osmotic or thermal stress. However, the significance of the presence of two pathways in *R. marinus*, as well as the accumulation of the compatible solute mannosylglyceramide, which is unique to this organism, remains elusive.

Concluding remarks

Many answers in science give rise to questions and several questions have been raised from the studies of osmoadaptation in (hyper)thermophiles. We do not know how osmosensing is translated into the synthesis of compatible solutes, which genes are expressed upon salt upshock, what is the interplay between increased growth temperature and salt stress, and why these peculiar compounds are used for osmotic adjustment in microorganisms from hot environments, nor do we really know the function of some compatible solutes of (hyper)thermophiles. Much has been learned in recent years, though these are still basic facts about the osmotic relations of these organisms.

Research will proceed rapidly as the interest in organisms from extreme environments continues to grow and because complete genome sequencing of thermophiles and hyperthermophiles will increase the rate of identification of the genes involved in osmotic and thermal adjustment. In addition to the basic aspects of osmotic adaptation and thermal adaptation, compatible solutes are sought for industrial purposes and this aspect will also fuel research in this area.

Acknowledgements

References

Alfredsson, G.A., Kristjánsson, J.K., Hjörleifsdottir, S., and Stetter, K.O. (1988) *Rhodothermus marinus*, gen. nov., sp. nov., a thermophilic, halophilic bacterium from submarine hot springs in Iceland. *J Gen Microbiol* **134:** 299–306.

Anton, J., Oren, A., Benlloch, S., Rodriguez-Valera, R., Amann, R., and Rossello-Mora, R. (2002) *Salinibacter ruber* gen. nov., sp. nov., a novel, extremely halophilic member of the Bacteria from saltern crystallizer ponds. *Int J Syst Evol Microbiol* **52:** 485–491.

Argüelles, J.C. (2000) Physiological roles of trehalose in bacteria and yeast: a comparative analysis. *Arch Microbiol* **174:** 217–224.

Benaroudj, N., Hee Lee, H., and Goldberg, A.L. (2001) Trehalose accumulation during cellular stress protects cells and cellular proteins from damage by oxygen radicals. *J Biol Chem* **276:** 24261–24267.

Blöchl, E., Burggraf, S., Fiala, G., Lauerer, G., Huber, G., Huber, R., *et al.* (1995) Isolation, taxonomy and phylogeny of hyperthermophilic microorganisms. *World J Microbiol Biotechnol* **11:** 9–16.

Borges, N., Ramos, A., Raven, N.D.H., Sharp, R.J., and Santos, H. (2002) Comparative study on the thermostabilising effect of mannosylglycerate and other compatible solutes on model enzymes. *Extremophiles* **6:** 209–216.

Bouveng, H., Lindberg, B., and Wickberg, B. (1955) Low-molecular carbohydrates in algae. Structure of the glyceric acid mannoside from red algae. *Acta Chem Scand* **9:** 807–809.

Brown, A.D. (1976) Microbial water stress. *Bacteriol Rev* **40:** 803–846.

Brown, A.D. (1990) *Microbial Water Stress Physiology: Principles and Perspectives*. Chichester: John Wiley & Sons.

Cayol, J.-L., Ollivier, B., Patel, B.K.C., Prensier, G., Guezernnec, J., and Garcia, J.-L. (1994) Isolation and characterization of *Halothermothrix orenii* gen. nov., sp. nov., a halophilic, thermophilic, fermentative strictly anaerobic bacterium. *Int J Syst Bacteriol* **44:** 534–540.

Cayol, J.-L., Ducerf, S., Patel, B.K.C., Prensier, G., Garcia, J.-L., Thomas, P., and Ollivier, B. (2000) *Thermohalobacter berrensis* gen. nov., sp. nov., a thermophilic, strictly

halophilic bacterium from a solar saltern. *Int J Syst Evol Microbiol* **50:** 559–567.

Chen, L., Spiliotis, E.T., and Roberts, M.F. (1998) Biosynthesis of di-*myo*-inositol-1,1′ –phosphate, a novel osmolyte from hyperthermphilic archaea. *J Bacteriol* **180:** 281–300.

Ciulla, R.A., Burggraf, S., Stetter, K.O., and Roberts, M.F. (1994) Occurrence and role of di-*myo*-inositol-1,1′-phosphate in *Methanococcus igneus*. *Appl Environ Microbiol* **60:** 3660–3664.

da Costa, M.S., Santos, H., and Galinski, E.A. (1998) An overview of the role and diversity of compatible solutes in *Bacteria* and *Archaea*. *Adv Biochem Eng/Biotechnol* **61:** 118–153.

da Costa, M.S., Nobre, M.F., and Rainey, F.A. (2001) The genus *Thermus*. In *Bergey's Manual of Systematic Bacteriology.* Boone, D.R., and Castenholtz, R.W. (eds), Vol. 1, 2nd edn. New York: Springer, pp. 404–414.

Empadinhas, N., Marugg, J.D., Borges, N., Santos, H., and da Costa, M.S. (2001) Pathway for the synthesis of mannosylglycerate in the hyperthermophilic archaeon *Pyrococcus horikoshii*. *J Biol Chem* **276:** 43580–43588.

Galinski, E.A. (1995) Osmoadaptation in bacteria. *Adv Microb Physiol* **37:** 272–328.

Giæver, H.M., Styrvold, O.B., Kaasen, I., and Strøm, A.R. (1988) Biochemical and genetic characterization of osmoregulatory trehalose synthesis in *Escherichia coli*. *J Bacteriol* **170:** 2841–2849.

Gorkovenko, A., and Roberts, M.F. (1993) Cyclic 2,3-diphosphoglycerate as a component of a new branch in gluconeogenesis in *Methanobacterium thermoautotrophicum* delta H. *J Bacteriol* **175:** 4087–4095.

Hagemann, M., and Erdmann, N. (1994) Activation and pathway of glucosylglycerol synthesis in the cyanobacterium *Synechocystis* sp. PCC 6803. *Microbiol* **140:** 1427–1431.

Hartmann, M., Scohlten, J.C., Stoffers, P., and Wehner, F. (1998) Hydrographic structure of brine-filled deeps in the Red Sea – new results from the Shaban, Kebrit, Atlantis II and Discovery Deep. *Mar Geol* **144:** 311–330.

Horlacher, R., Xavier, K.B., Santos, H., DiRuggiero, J., and Boos, W. (1998) Archaeal binding protein-dependent ABC transporter: molecular and biochemical analysis of the trehalose/maltose transport system of the hyperthermophilic archaeon *Thermococcus litoralis*. *J Bacteriol* **180:** 680–689.

Karsten, U., Barrow, K.D., Mostaert, A.S., King, R.J., and West, J.A. (1994) ^{13}C- and ^{1}H- NMR studies on digeneaside in red alga *Caloglossa leprieurii*. A re-evaluation of its osmotic significance. *Plant Physiol Biochem* **32:** 669–676.

Koning, S.M., Albers, S.V., Konings, W.N., and Driessen, A.J. (2002) Sugar transport in (hyper) thermophilic archaea. *Res Microbiol* **153:** 61–67.

Lamosa, P., Martins, L.O., da Costa, M.S., and Santos, H. (1998) Effects of temperature, salinity, and medium composition on compatible solute accumulation by *Thermococcus* spp. *Appl Environ Microbiol* **64:** 3591–3598.

Lamosa, P., Burke, A., Peist, R., Huber, R., Liu, M.Y., Silva, G., *et al.* (2000) Thermostabilization of proteins by diglycerol phosphate, a new compatible solute from the hyperthermophile *Archaeoglobus fulgidus*. *Appl Environ Microbiol* **66:** 1974–1979.

Lechmacher, A., Vogt, A.-B., and Hensel, R. (1990) Biosynthesis of cyclic 2,3-diphosphoglycerate. Isolation and characterization of 2-phosphoglycerate kinase and cyclic 2,3-diphosphoglycerate synthetase from *Methanothermus fervidus*. *FEBS Lett* **272:** 94–98.

Martin, D.D., Ciulla, R.A., and Roberts, M.F. (1999) Osmoadaption in archaea. *Appl Environ Microbiol* **65:** 1815–1825.

Martins, L.O., and Santos, H. (1995) Accumulation of mannosylglycerate and di-*myo*-inositol-phosphate by *Pyrococcus furiosus*. response to salinity and temperature. *Appl Environ Microbiol* **61:** 3299–3303.

Martins, L.O., Carreto, L.S., da Costa, M.S., and Santos, H. (1996) New compatible solutes related to di-*myo*-inositol-phosphate in members of the order *Thermotogales*. *J Bacteriol* **178:** 5644–5651.

Martins, L.O., Huber, R., Huber, H., Stetter, K.O., da Costa, M.S., and Santos, H. (1997) Organic solutes in hyperthermophilic archaea. *Appl Environ Microbiol* **63:** 896–902.

Martins, L.O., Empadinhas, N., Marugg, J.D., Miguel, C., Ferreira, C., da Costa, M.S., and Santos, H. (1999) Biosynthesis of mannosylglycerate in the thermophilic bacterium *Rhodothermus marinus*. *J Biol Chem* **274:** 35407–35414.

Matussek, K., Moritz, P., Brunner, N., Eckerskorn, C., and Hensel, R. (1998) Cloning, sequencing, and expression of cyclic 2, 3-diphosphoglycerate synthetase, the key enzyme of cyclic 2, 3-diphosphoglycerate metabolism in *Methanothermus fervidus*. *J Bacteriol* **180:** 5997–6004.

Nicolaus, B., Gambacorta, A., Basso, A.L., Riccio, R., de Rosa, M., and Grant, W.D. (1988) Trehalose in Archaebacteria. *System Appl Microbiol* **10:** 215–217.

Nunes, O.C., Manaia, C.M., da Costa, M.S., and Santos, H. (1995) Compatible solutes in the thermophilic bacteria *Rhodothermus marinus* and '*Thermus thermophilus*'. *Appl Environ Microbiol* **61:** 2351–2357.

Oren, A. (1999) Bioenergetic aspects of halophilism. *Microbiol Mol Biol Rev* **63:** 334–348.

Oren, A., and Mana, L. (2002) Amino acid composition of bulk protein and salt relationships of selected enzymes of *Salinibacter ruber*, an extremely halophilic bacterium. *Extremophiles* **6:** 217–223.

Ramakrishnan, V., Verhagen, M.F.J.M., and Adams, M.W.W. (1997) Characterization of di-*myo*-inositol-1,1'-phosphate in the hyperthermophilic bacterium *Thermotoga maritima*. *Appl Environ Microbiol* **63:** 347–350.

Ramos, A., Raven, N.D.H., Sharp, R.J., Bartolucci, S., Rossi, M., Cannio, R., *et al.* (1997) Stabilization of enzymes against thermal stress and freeze-drying by mannosylglycerate. *Appl Environ Microbiol* **63:** 4020–4025.

Roeßler, M., and Müller, V. (2001) Osmoadaptation in bacteria and archaea: common principles and differences. *Environ Microbiol* **3:** 743–754.

Santos, H., and da Costa, M.S. (2001) Organic solutes from thermophiles and hyperthermophiles. *Meth Enzymol* **334:** 302–315.

Scholz, S., Sonnenbichler, J., Schäffer, W., and Hensel, R. (1992) Di-*myo*-inositol-1,1′-phosphate: a new inositol phosphate isolated from *Pyrococcus woesei*. *FEBS Lett* **306:** 239–242.

Scholz, S., Wolff, S., and Hensel, R. (1998) The biosynthesis pathway of di-*myo*-inositol-1,1′-phosphate in *Pyrococcus woesei*. *FEMS Microbiol Lett* **168:** 37–42.

Shima, S., Herault, D.A., Berkessel, A., and Thauer, R.K. (1998) Activation and thermostabilization effects of cyclic 2,3-diphosphoglycerate on enzymes from the hyperthermophilic *Methanopyrus kandleri*. *Arch Microbiol* **170:** 469–472.

Siderius, M., Van Wuytswinkel, O., Reijenga, K.A., Kelders, M., and Mager, W.H. (2000) The control of intracellular glycerol in *Saccharomyces cerevisiae* influences osmotic stress response and resistance to increased temperature. *Mol Microbiol* **36:** 1381–1390.

Silva, Z., Borges, N., Martins, L.O., Wait, R., da Costa, M.S., and Santos, H. (1999) Combined effect of the growth temperature and salinity of the medium on the accumulation of compatible solutes by *Rhodothermus marinus* and *Rhodothermus obamensis*. *Extremophiles* **3:** 163–172.

Simola, M., Hanninen, A.L., Stranius, S.M., and Makarow, M. (2000) Trehalose is required for conformational repair of heat-stress denatured proteins in yeast endoplasmic reticulum but not for maintenance of membrane traffic functions after severe heat stress. *Mol Microbiol* **37:** 42–53.

Singer, M.A., and Lindquist, S. (1998) Multiple effects of trehalose on protein folding in vivo and in vitro. *Mol Cell* **1:** 639–648.

Ventosa, A., Nieto, J.J., and Oren, A. (1998) Biology of aerobic moderately halophilic bacteria. *Microbiol Mol Biol Rev* **62:** 504–544.

Welsh, D.T. (2000) Ecological significance of compatible solute accumulation by micro-organisms: from single cells to global climate. *FEMS Microbiol Rev* **24:** 263–290.

Yokoyama, K., Araki, Y., and Ito, E. (1987) Biosynthesis of poly (galactosylglycerol phosphate) in *Bacillus coagulans*. *Eur J Biochem* **165:** 47–53.