Universidade de Aveiro Departamento de Biologia
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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada, realizada sob a orientação científica do Doutor António Carlos Matias Correia, Professor Catedrático do Departamento de Biologia da Universidade de Aveiro e co-orientação do Doutor Milton Simões da Costa, Professor Catedrático da Faculdade de Ciências e Tecnologias da Universidade de Coimbra.

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Dedico também à minha avó, Ana Ferreira, todo o carinho, fraternidade e boa disposição caracteristica. Saudade que fica e amor que permanece!

o júri

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palavras-chave *Rhodothermus marinus,* soluto compatível, manosilgliceramida, manosilglicerato

Resumo

Na presença de elevadas concentrações de sal no meio de cultura a bactéria halotermofílica *Rhodothermus marinus* acumula no interior das células compostos orgânicos de baixo peso molecular designados solutos compatíveis. Nestas condições e à temperatura de 65 ºC, este organismo acumula elevados níveis de manosilgliceramida (MGA) e manosilglicerato (MG) e níveis mais baixos de trealose, glutamato e glucose. O soluto manosilgliceramida é um derivado de manosilglicerato no qual o OH do grupo carboxílico é substituído por um grupo amino. Este soluto com carga neutra foi detectado pela primeira vez por RMN na bactéria *R. marinus* e, até ao momento, não há registo da sua presença ou acumulação em nenhum outro microorganismo. A forma como a bactéria *R. marinus* gere o seu metabolismo por forma a sintetizar este soluto permanece uma incógnita.

Em *R. marinus*, os dois genes localizados imediatamente a seguir aos dois genes envolvidos na biossíntese de MG codificam duas enzimas, uma identificada como uma "hypothetical protein" com um domínio pertencente à família Acetil transferases e a outra identificada como uma aminoácido desidrogenase. No presente trabalho estudou-se a possibilidade de a enzima aminoácido desidrogenase ser responsável pela libertação de um grupo amino a partir de um aminoácido e a possibilidade da "hypothetical protein" ser responsável pela transferência desse grupo amino para a molécula de manosilglicerato (molécula carregada negativamente) ou para o intermediário fosforilado, o manosil-3-fosfoglicerato (MPG), percursor de manosilglicerato. Realizaram-se ensaios enzimáticos usando extractos celulares de *R. marinus* e testaram-se diferentes substratos com o objectivo de detectar a síntese de MGA. O produto das reacções foi separado e analisado por cromatografia de camada fina (TLC). O gene que codifica a "hypothetical protein" foi clonado e expresso na estirpe *E. coli* BL21.

Simultaneamente, estudou-se a viabilidade de a estirpe CC-16 de *T. thermophilus*, uma estirpe naturalmente competente, ser um hospedeiro termofílico adequado para expressão dos genes responsáveis pela síntese de MG e, desta forma perspectivar também a sua utilização como hospedeiro para a sobreprodução do soluto raro, manosilgliceramida.

keywords

Rhodothermus marinus, compatible solute, mannosylglyceramide, mannosylglycerate

abstract

The halothermophilic bacterium *Rhodothermus marinus* has been described to accumulate intracellular solutes at high salt concentrations in the growth medium. In this condition at a temperature of 65ºC this organism accumulates high levels of mannosylglyceramide (MGA) and mannosylglycerate (MG) and low levels of trehalose, glutamate and glucose. Mannosylglyceramide, an ammonia derivative of mannosylglycerate (MG), is an uncharged compatible solute first detected by NMR and, thus far, was only found to accumulate in *R. marinus*. However, the orchestration of the metabolism of *R. marinus* for the biosynthesis of this solute remains unknown.

In *R. marinus*, the two genes immediately downstream the genes involved in MG biosynthesis encode two enzymes, one identified as a hypothetical protein with a motif belonging to acetyl transferases family and the other identified as an amino acid dehydrogenase. We investigated the possibility of the later enzyme be the responsible for the release of an amino group from an amino acid donor and the former for the transfer of amino group for MG (negative charge molecule)r for the mannosyl-3-phosphoglycerate (MPG) the phosphorylated precursor of MG. The enzyme assays to detect the production of MGA were carried out using cell extracts of *R. marinus*. Products were separated and analysed by thin layer chromatography (TLC). Moreover the gene encoding the hypothetical protein was cloned and expressed in *E. coli* BL21.

In parallel, we studied the viability of the genetic amenable *T. thermophilus* strain CC-16 be a suitable thermophilic host to express genes for the synthesis of MG, prospecting its utilization as a host for the overproduction of the rare solute mannosylglyceramide.

Table of Contents

List of Figures

List of Tables

Abbreviations

cBPG	cyclic-2,3-biphosphoglycerate
DGP	diglycerol phosphate
DIP	di-*myo*-inositol phosphate
GADPH	glyceraldehyde-3-phosphate dehydrogenase
GG	glucosylglycerate
GGG	glucosylglucosylglycerate
IPTG	isopropyl-β-D-thiogalactopyranoside
KCl	potassium chloride
LB	Luria-Bertani
NaCl	sodium Chloride
MG	α-manoosylglycerate
MGG	manosylglucosylglycerate
MGA	α-mannosylglyceramide
MGS	mannosylglycerate synthase
MGSD	fusion between *mpgS* and *mpgP* genes
MPG	α-mannosyl-3-phosphoglycerate
MPGP	mannosyl-3-phosphoglycerate phosphatase
MPGS	mannosyl-3-phosphoglycerate synthase
NAT	N-acetyl transferase
NMR	nuclear magnetic ressonance
O.D.	optical density
PAGE	polyacrylamide gel electrophoresis
3-PGA	D-3-phosphoglycerate
SDS	sodium dodecyl sulfate
TLC	thin layer chromatography

1 Introduction

1.1 Extremophiles and extreme environments

Extremophiles are classified on the basis of the particular extreme conditions in which they live. For example, thermophiles and hyperthermophiles live at high (above 75ºC) and very high temperatures (above 115ºC), respectively, psychrophiles live at very low temperatures, acidophiles and alcalophiles thrive at extreme of acidic or basic values of pH, respectively and halophiles that proper at the presence of high salt concentrations (5-30%, v/v) .

The most studied group among extremophiles are the thermophilic microorganisms. Thermophiles are divided into moderate thermophiles, extreme thermophiles and hyperthermophiles (Kristjansson 1991; Lasa and Berenguer 1993). Moderate thermophiles live at 50ºC sites, like hydrothermal areas. Unlike this group, extreme thermophiles are unable to survive below 50ºC due to thermophilic environment stability evolved. These microorganisms have an optimal growth rate above 70ºC that limit is strictly to prokaryotes, Bacteria and Archaea (Antranikian 2001; Rothschild and Mancinelli 2001). Microorganisms inhabiting at increased temperature levels (optimum growth > 85ºC) are called hyperthermophiles. Among this group, *Pyrolobus fumarii* is the most hyperthermophilic microorganism growing up to 113ºC (Blochl *et al.* 1997; Stetter 1999; Rothschild and Mancinelli 2001).

Thermophiles were isolated most from geothermal environments, like hot springs, artificial hot environments and from hydrothermal areas, like marine or saline hydrothermal vents. In marine hydrothermal environments water salinity can range from low salinity to that of seawater and microorganisms living in these environments are generally, halotolerant or slightly halophilic. The best studied examples are the thermophilic strains of *Thermus thermophilus* and *Rhodothermus marinus* (Nunes *et al.* 1995).

The increasing interest in thermophilic bacteria is due to their evolutionary importance as representatives of ancient lineages of life, unusual cell components and metabolism, and because they possess thermostable enzymes and other molecules that can be exploited for industrial purposes.

1.1.1 *Rhodothermus marinus*: thermophilic and slightly halophilic microorganism

The microorganism used in the present study was *R. marinus*, first isolated from marine hot springs (Figure 1) (Alfredsson 1988). *Rhodothermus marinus* sp. are thermophilic and slightly halophilic, growth optimally at 65-70ºC, 2% NaCl and pH 7, moreover they can survive at temperature ranging from 54ºC to 77ºC and at a salt concentration of 0.5% to over 6% NaCl (Bjornsdottir *et al.* 2006).

Strains of *R. marinus* are gram-negative cells, heterotrophic and restrict aerobic and can only grow in very limited zones in the hot springs, close to their openings, determined by temperature and salt concentration as well as of content of O_2 and organic material (Bjornsdottir *et al.* 2006). Since 1988, *R. marinus* strains have been isolated in similar habitats in geothermal environments distantly located: marine hot springs at Praia de Ribeira Quente (Nunes 1992) and Ferraria (Silva *et al.* 2000), on the island of São Miguel in the Azores, Portugal; in Stufe di Nerone, near Naples, Italy (Moreira *et al.* 1996) and on the island of Monserrat in the Caribbean Sea (Silva *et al.* 2000). These strains were also isolated from three additional geothermal sites in Iceland; coastal springs and a borehole effluent in Oxarfjordur, North East Iceland and borehole effluents from a powerplant at the Blue Lagoon and from a salt factory in Reykjanes, both in South West Iceland (Petursdottir *et al.* 2000).

In 1996, Sako and collaborators described a new species belonging to the genus *Rhodothermus*, isolated from shallow marine hydrothermal vents in Japan and classified it as *Rhodothermus obamensis*. However, "*R. obamensis"* was later reclassified as a *R. marinus* strain based on fatty acid analysis, DNA-DNA reassociation studies and 16S-rRNA gene sequence comparisons (Silva *et al.* 2000). Recently, a new species of *Rhodothermus* was isolated, characterized and classified as *Rhodothermus profundi* (type strain PRI 2902^T) by Marteinssona *et al.* (2010). Several morphological, physiological and chemotaxonomic characteristics distinguish *R. profundi* from *R. marinus*. Unlike *R. marinus*, this new species is non-pigmented, non-motile, grow between 55 and 80ºC in a maximum salt concentration about 5% in the growth medium (Marteinsson *et al.* 2010).

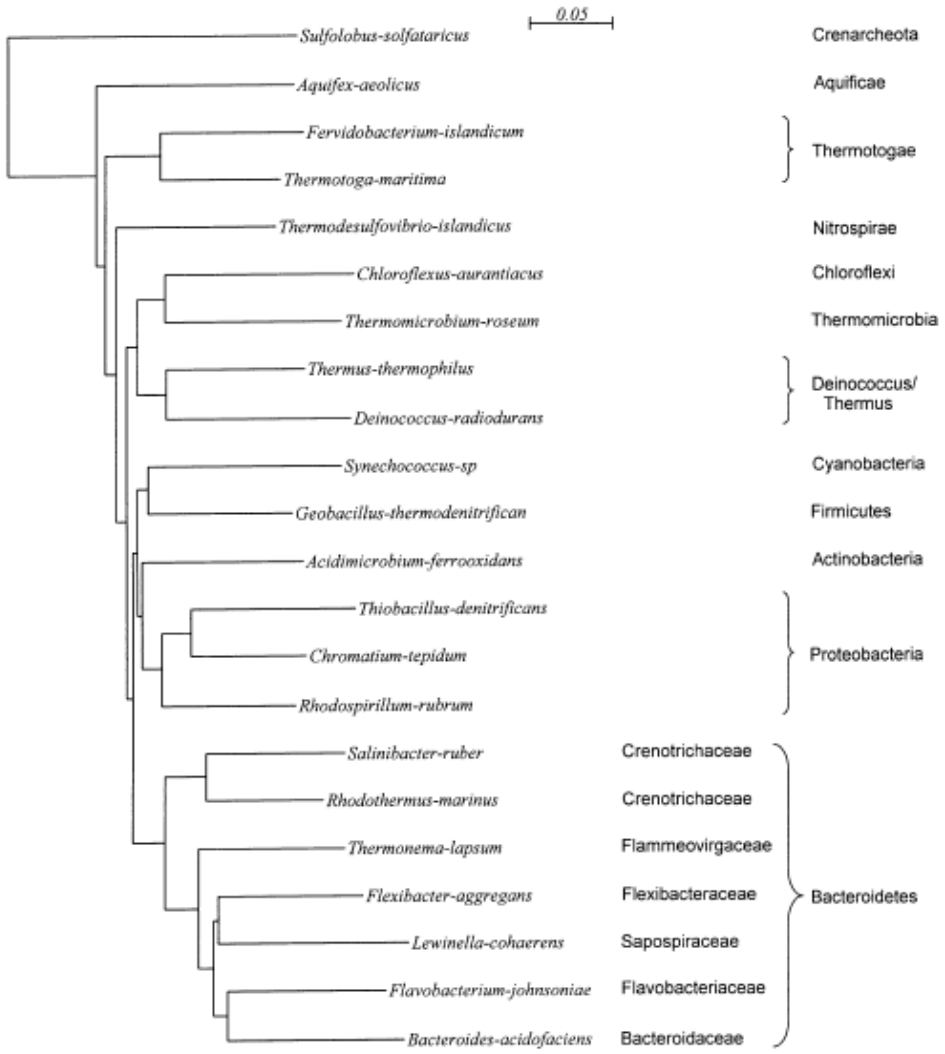

Figure 1. The phylogenetic position of *R. marinus* shows the relatedness within the phylum of *Bacteroidetes*. Evolutionary distances were computed from pair wise similarities using the correction of Jukes and Cantor. Distance tree was constructed by the neighbour joining algorithm. Figure from Bjornsdottir *et al.,* 2006.

1.2 Strategies for thermo- and osmo-adaptation

One of the most amazing properties of microorganisms is their ability to adapt to extreme environments, where pH, temperature, pressure and salt concentration values are higher or lower than what is considered standard for most living organisms (Lasa and Berenguer 1993).

In case of microorganisms living at high temperatures their macromolecules must be adapted, being more thermostable than those of mesophiles (Sterner and Liebl 2001). Some of these macromolecular adaptations have been identified, however many remain unknown. Besides, thermophiles are not only adapted to high temperature but also to additional extreme conditions. For example, microorganisms that are both halophilic and thermophilic, like *R. marinus,* inhabiting only in a very narrow zone in the hot springs (Bjornsdottir *et al.* 2006).

Organisms that can grow at high salt concentrations reach a positive internal turgor pressure across the cell membrane by two principal osmotic strategies (Empadinhas and da Costa 2008). The first consists in the accumulation of inorganic solutes (K^+, Na^+, Cl^-) to achieve osmotic equilibrium and has been called the "salt-in-the-cytoplasm". This form of osmoadaptation co-evolved with the structural modification of many cellular components, which are important for the functionality of cell metabolism in high ionic strength conditions. Until now, this strategy occurs in some extremely halophilic archaea like *Halobacteriaceae* and in the extremely halophilic bacterium *Salinibacter ruber* (Oren and Mana 2002).

The second mechanism is extensively used by bacterial halophiles and employs the accumulation of small organic molecules to counter balance the salt concentration in the environment and is known as "compatible solutes" (Santos and da Costa 2002; Lentzen and Schwarz 2006). Besides, the role of compatible solutes goes beyond osmotic adjustment alone, they also provide protection of cells and cell components from freezing, desiccation, high temperature and oxygen radicals. Also, in these organisms intracellular macromolecules have not undergone specific modifications and are, therefore, sensitive to high intracellular concentrations of salts and most organic solutes (da Costa *et al.* 1998).

The combination of both strategies, "salt-in-the-cytoplasm" and "compatible solute", has only been found in slightly and moderately halophilic methanogenic organisms. These organisms accumulate high levels of potassium together with neutral and anionic compatible solutes (Desmarais *et al.* 1997).

1.3 Compatible solutes: Role and Diversity

Compatible solutes can be accumulated to high levels by *de novo* synthesis or uptake from the environment (Poolman and Glaasker 1998). The uptake of compatible solutes is usually preferred to *de novo* synthesis because of lower energy costs. Most common compatible solutes are low-molecular-weight solutes, neutrally charged or zwitterionic and include amino acids and amino acid derivatives, sugars and sugar derivatives, polyols, betaines and ectoines (da Costa *et al.* 1998; Santos and da Costa 2002). Some are widespread among archaea, bacteria, yeast, filamentous fungus and algae and others are restricted to a few groups of microorganisms (da Costa *et al.* 1998; Santos and da Costa 2002).

Compatible solutes like trehalose, α-glutamate and proline are regularly found among bacteria. For example, the success of trehalose is due to single physical characteristics like chemical stability, non hygroscopic glass formation and the absence of internal hydrogen bond formation, which make this disaccharide capable to protect cells and biomolecules from environmental stress imposed by low water activity, heat, oxidation, desiccation or freezing (Arguelles 2000; Elbein *et al.* 2003; Liang *et al.* 2006). Trehalose is also frequently used by organism as a carbon source (Strom and Kaasen 1993; Horlacher and Boos 1997; Arguelles 2000). In yeast, trehalose can be used as a reserve compound. In insects, trehalose is the most

abundant sugar in the haemolymph (80-90%) and in thorax muscles, where it is consumed during flight (Richards *et al.* 2002; Elbein *et al.* 2003).

Ectoine and hydroxyectoine are compatible solutes only found in mesophilic bacteria (Roberts 2005). Ectoine is known as an osmoprotectant against increased external osmotic pressure and is the most abundant osmolyte of aerobic chemoheterotrophic bacteria (Martin *et al.* 1999; Santos and da Costa 2002; Roberts 2005). This compound has been found in all halophilic/ halotolerant *Proteobacteria* of the V-subdivision, in all representatives of the genus *Nocardiopsis*, in all Gram-positive cocci examined so far, in *Brevibacteria* and even in *Bacillus* species and *Sporosarcina halophila* (Galinski 1993).

Other compatible solutes like di-*myo*-inositol phosphate (DIP) are, until now, restricted to microorganisms living at extremely high temperatures (Figure 2) (Empadinhas and da Costa 2008). Di-*myo*-inositol phosphate was first found in *Pyrococcus woesi* and in *Methanococcus igneus* (Scholz *et al.* 1992; Ciulla *et al.* 1994). After that, it has been identified in hyperthermophilic bacteria (*Thermotoga* and *Aquifex* spp.) and archaea (Santos and da Costa 2002). This solute seems to be implicated in the protection of cellular components against heat denaturation, since it accumulates by organisms, primarily as a response to supraoptimal growth temperatures (Borges *et al.* 2006).

Mannosylglycerate (MG) is a sugar-derived compatible solute, usually found in thermophiles and rarely encountered in mesophiles (Figure 2). Mannosylglycerate was initially discovered in red algae of the order *Ceramiales* (Bouveng H. *et al.* 1955) and it has been lately found in the thermophilic bacteria *R. marinus* and *T. thermophilus,* in members of the genus *Rubrobacter*, which represent a lineage of the phylum *Actinobacteria*, in the crenarchaeotes *Aeropyrum pernix* and *Stetteria hydrogenophila*, in the euryarchaeotes *Archaeoglobus veneficus*, *Archaeoglobus profundus*, *Methanothermus fervidus* and in the three genera of the order Thermococcales, Thermococcus, Pyrococcus, and Palaeococcus (Martins and Santos 1995; Lamosa *et al.* 1998; Goncalves *et al.* 2003; Neves *et al.* 2005; H. Santos 2007).

Glucosylglycerate (GG) is a structural analogue to MG and was originally identified in the marine cyanobacterium *Agmenellum quadruplicatum* grown under nitrogen-limiting conditions (Kollman *et al.* 1979). This solute was initially thought to be restricted to mesophilic bacteria until the recently identification of GG in the thermophilic bacterium *Persephonella marina*, where it was suggested to act as a compatible solute under salt stress (Empadinhas and da Costa 2008). However, additional research is still essential to clarify the role of this compound as a true compatible solute.

Mannosylglyceramide (MGA) an ammonia derivative of MG found in *R. marinus* (Silva *et al.* 1999) and two recent identified solutes, which are variations of MG, mannosylglucosylglycerate (MGG) and glucosylglucosylglycerate (GGG), found in *Petrotoga myotherma* and *Petrotoga mobilis* (Santos *et al.* 2007; Fernandes *et al.* 2010) and in *Persephonella marina* (Santos *et al.* 2007), respectively, are very rare compounds (Figure 2). Mannosylglucosylglycerate, however, does not confer protection to *Petrotoga miotherma* against heat and oxidative stresses (Jorge *et al.* 2007).

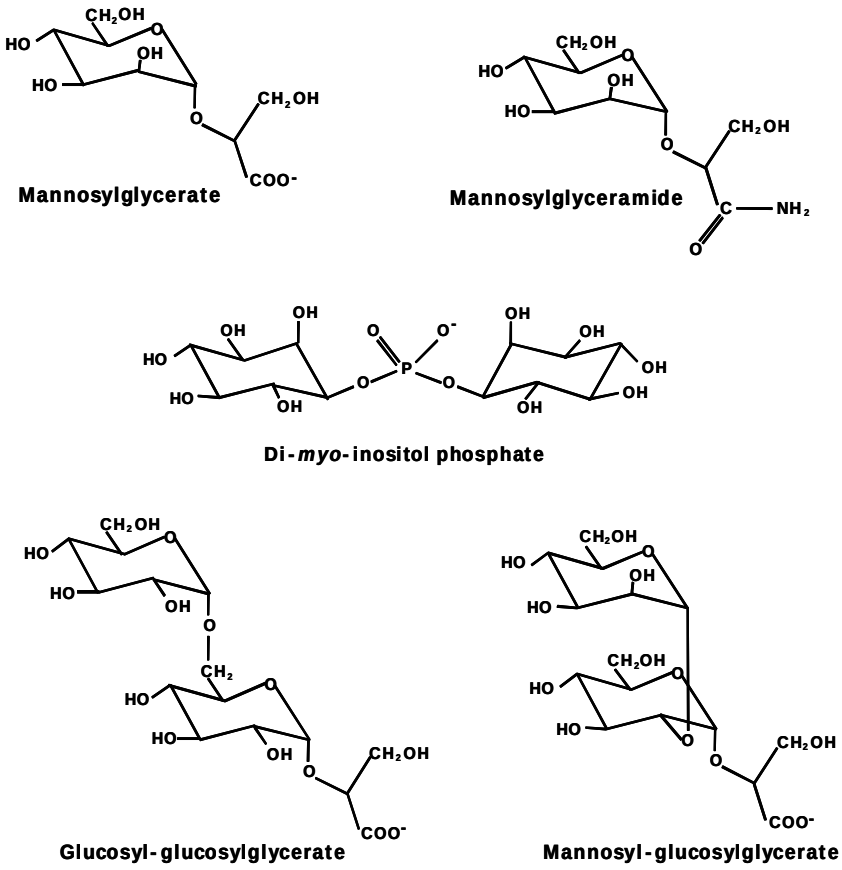

Figure 2. Structure representation of some compatible solutes from prokaryotes (Santos *et al.* 2007) .

1.3.1 Compatible solutes accumulated by *Rhodothermus marinus*

In 1995, Nunes and collaborators described in *R. marinus* two forms of MG, α-mannosylglycerate and β-mannosylglycerate. Subsequently, it was demonstrated by Nuclear magnetic resonance (NMR) that the initial identification of α-mannosylglycerate could not be correct because during the purification of this compound it did not bind to anionic exchange resins as expected for a negatively charged compound (Silva *et al*. 1999). So the two forms of MG initially identified in *R. marinus* were in fact two related compounds, α-mannosylglycerate and α-mannosylglyceramide.

In response to salt stress, at the optimum growth temperature, *R. marinus* accumulated intracellular solutes like mannosylglycerate (MG), mannosylglyceramide (MGA) and low levels of trehalose, glutamate and glucose. At the highest growth temperature, MG is the major compatible solute and MGA is not detected. Levels of MG in *R. marinus* depended on growth temperature since it increased with growth temperature at a given NaCl concentration in the range of 3-5% (Figure 3) (Martins *et al.* 1999; Silva *et al.* 1999). On the other hand, the MG accumulated under

moderate salt stress ridge to be replaced by the neutral derivative MGA, which become the dominant compatible solute (Silva *et al.* 1999).

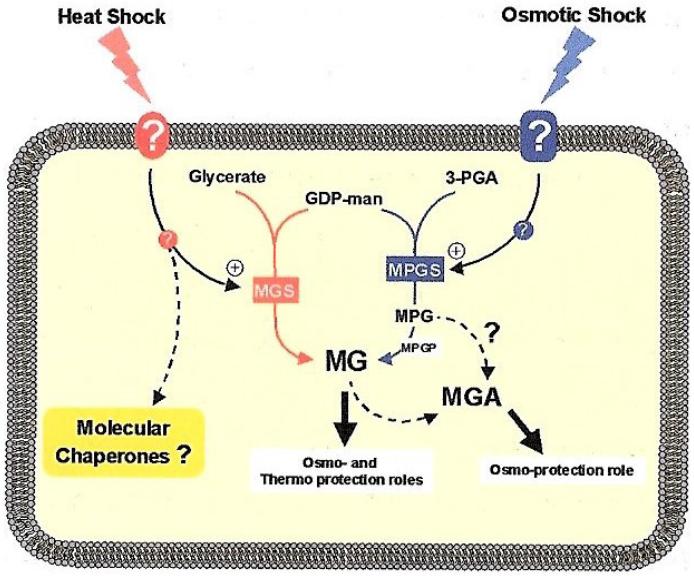

Figure 3. Current knowledge of osmotic and heat-stress responses in *R. marinus* (Borges, 2004).

1.4 Mannosylglycerate biosynthesis

The characterization of metabolic pathways for the synthesis of compatible solutes is crucial in order to understand the regulatory mechanisms involved in the adaptation of many organisms to salt or temperature ranges.

Mannosylglycerate biosynthesis was for the first time described in *R. marinus* and found to proceed via two alternative pathways (Martins *et al.* 1999). In one of them, called the single-step pathway, GDP-mannose is condensed with D-glycerate to produce MG in a reaction catalyzed by mannosylglycerate synthase (MGS). In the other, called two-step pathway, mannosyl-3-phosphoglycerate synthase (MPGS) catalyzed the conversion of GDP-mannose and D-3-phosphoglycerate into a phosphorilated intermediate, which is subsequently converted to MG by the action of a mannosyl-3-phosphoglycerate phosphatase (MPGP) (Martins *et al.* 1999; Borges *et al.* 2004). Thus far, *R. marinus* is the sole organism possessing the two pathways for the synthesis of MG (Figure 4).

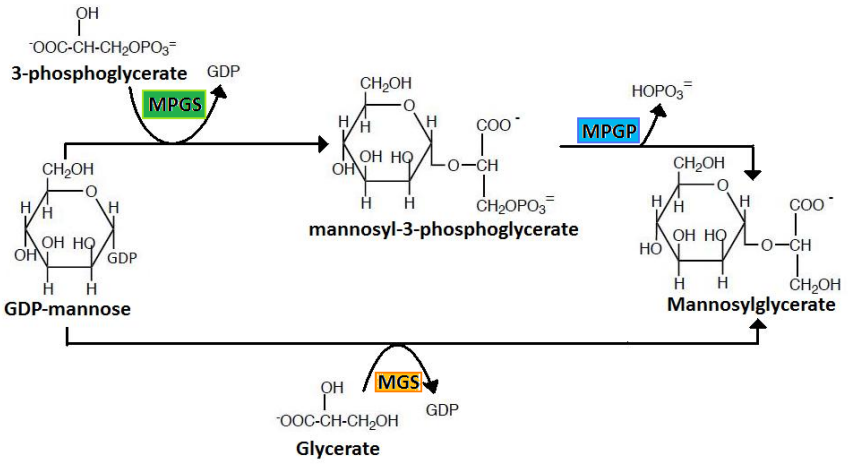

Figure 4. The two pathways for the synthesis of mannosylglycerate in *R. marinus* (Roberts 2005).

The single-step pathway is rare and has also been found and characterized in the mesophilic red algae *Caloglossa leprieurii* (Neves *et al.* 2005). On the other hand, the two-step pathway is common in prokaryotes, such as *Pyrococcus horikoshii*, *T. thermophilus*, *Palaeococcus ferrophilus* and *Thermococcus litoralis* (Figure 5) (2001; Empadinhas *et al.* 2003; Neves *et al.* 2005).In these organisms, the *mpgS* and *mpgP* genes are organized in a similar way, the *mpgP* gene is located immediately downstream of the *mpgS* gene in an operon-like structure (Figure 5). In contrast, in an uncultured Crenarchaeote the *mpgP* gene was not detected downstream of the *mpgS*, indicating that *mpgP* gene is located elsewhere in the chromosome or that the dephosphorylation of the intermediate MPG might be catalyzed by an unspecific phosphatase or that MPG could be a precursor for unknown macromolecules (Quaiser *et al.* 2002; Treusch *et al.* 2004) (Figure 5). Moreover, a bifunctional MGSD encoded by *mgsD* gene was found in the genome of the mesophilic bacterium *Dehalococcoides ethenogenes*. This gene is an unusual fusion between *mpgS* and *mpgP* genes combining the activities of both MPGS and MPGP (Figure 5) (Empadinhas *et al.* 2004).

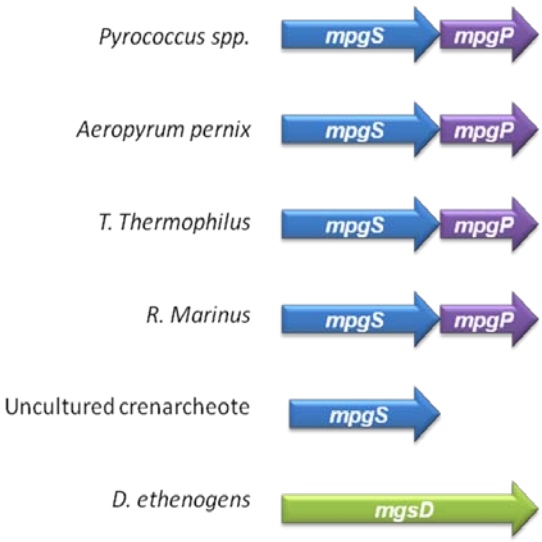

Figure 5. Schematic organization of mannosylglycerate synthesizing genes in prokaryotes.

1.4.1 Specialized roles of the two pathways for the synthesis of mannosylglycerate in *Rhodothermus marinus*

In 1999, Martins and collaborators detected that the MGS activity in cellular extracts of *R. marinus* was salt independent while the MPGS/MPGP system requires addition of NaCl or KCl to achieve full activity. The salt-dependence was already reported in the two-step pathway implicated in other compatible solutes biosynthesis such as trehalose and glucosylglycerol (Giaever *et al.* 1988; Hagemann 1994). Later, Borges and collaborators (2004) demonstrated that in *R. marinus* the two pathways for MG biosynthesis are regulated by salt and temperature, physical parameters that affect the intracellular MG concentrations. Levels of the enzyme from the single-step pathway (MGS) increased in response to heat stress, while induction of the synthesis of MPGS (the synthase of the two-step pathway) occurs when cells were exposed to osmotic stress. However, it was not established the existence of a salt-dependent control mechanism of gene expression (Borges *et al.* 2004).

1.5 Compatible solutes: biotechnological applications

1.5.1 Enzymes stabilizers and medical applications

For the recent years several novel low molecular weight solutes have been identified in hyper/thermophilic archaea and bacteria microorganisms but no occurrence were found in mesophilic species. For this reason there is a growing interest in their role as osmolytes and possible thermostabilizing agents that could be useful for the protection of enzymes, and other cell components.

For example, some methanogenic organisms accumulate cyclic-2,3-bisphosphoglycerate (cBPG), a compound which has a thermoprotectant role on glyceraldehyde-3-phosphate dehydrogenases (GADPH) (Ramos *et al.* 1997). Moreover it is known that cBPG is able to protect malate dehydrogenase and GADPH from *Methanothermus fervidus* and formyltransferase from *Methanopyrus kandleri* (Hensel and Konig 1988; Shima *et al.* 1998).

Mannosylglycerate has been shown to have a deep effect on thermoprotection and protection against freeze and dry of enzymes derived from different origins (Ramos *et al.* 1997). Comparative studies using ectoines, hydroxyectoine, DIP, diglycerol phosphate (DGP), MG and MGA on rabbit muscle lactate dehydrogenase showed that MG and hydroxyectoine were the most efficient stabilizers of this enzyme from heat inactivation (Borges *et al*. 2002).

Moreover, recent research for a potential tool in combating prion diseases demonstrated that ectoine and mannosylglyceramide inhibit aggregation of peptide fragment PrP106-126, preventing amyloid formation, while hydroxyectoine and MG hardly had any effect on the aggregation of this peptide. Moreover, ectoines and MGA reduced the PrP106-126 toxicity in human cells. This findings support that ectoine and mannosylglyceramide could be used as potential drug compounds against prion desease (Kanapathipillai *et al.* 2008).

1.5.2 *Thermus thermophilus* as a model organism for heterologous gene expression

Many studies suggest that protection of model enzymes from heat denaturation and freeze-drying are more effective by MG than other solutes (Ramos *et al.* 1997; Borges *et al.* 2002). Moreover, the production of this compatible solute appears to be suitable in *T. thermophilus* since all other thermophilic or hyperthermophilic microorganisms that produce this compatible solute grow very poorly for industrial production (Borges *et al.* 2002; Da Costa 2006).

Frequently, enzymes from thermophilic origin cannot be expressed in a conventional mesophilic hosts because of differences in specific processing and requirements for chaperones and other factors that, in addiction to high temperatures, may be needed for enzymes to fold correctly so their activity can be detected and their physiologic function determined. Strains of *T. thermophilus* are naturally competent organisms suitable for genetic manipulations and have been successfully used for the expression of genes from different sources, either for basic research or as cell factories for the overproduction of enzymes with industrial application (Park *et al.* 2004; Egorova and Antranikian 2005).

2 Objectives

This work aims to characterize the biosynthetic pathway of mannosylglyceramide the rare solute only found in *R. marinus*, through the identification of gene(s) and substrates involved and to get insights of a suitable bacterial system for the overproduction of the rare solute.

3 Material and methods

3.1 Strains, plasmids and culture conditions

The type strain of *R. marinus* (DSM 4252)was obtained from Deutsche Sammlung von Mikroorganismen und Zelkulturen, Germany. The strain was grown at 65ºC and pH 7.5 in Degryse medium 162 (DSMZ medium 630, (http://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium630.pdf) supplemented with 1% and 5% NaCl. Growth was evaluated by measuring the turbidity of the culture at 610 nm (OD_{610}). "*Rhodothermus obamensis*" (JCM 9785^T) was obtained from the Japanese Collection of Microorganisms, Japan.

Escherichia coli strains DH5α and BL-21 were used has hosts for the expression of "mga" gene using cloning vectors pET30a and pTRC99A (Figure 6). These strains were grown in Luria-Bertani (LB) medium, at 37ºC and pH 7. Kanamycin (30 µg/ml) and ampicillin (100 µg/ml) were added to the medium for selection of plasmids pET30a and pTRC99A, respectively.

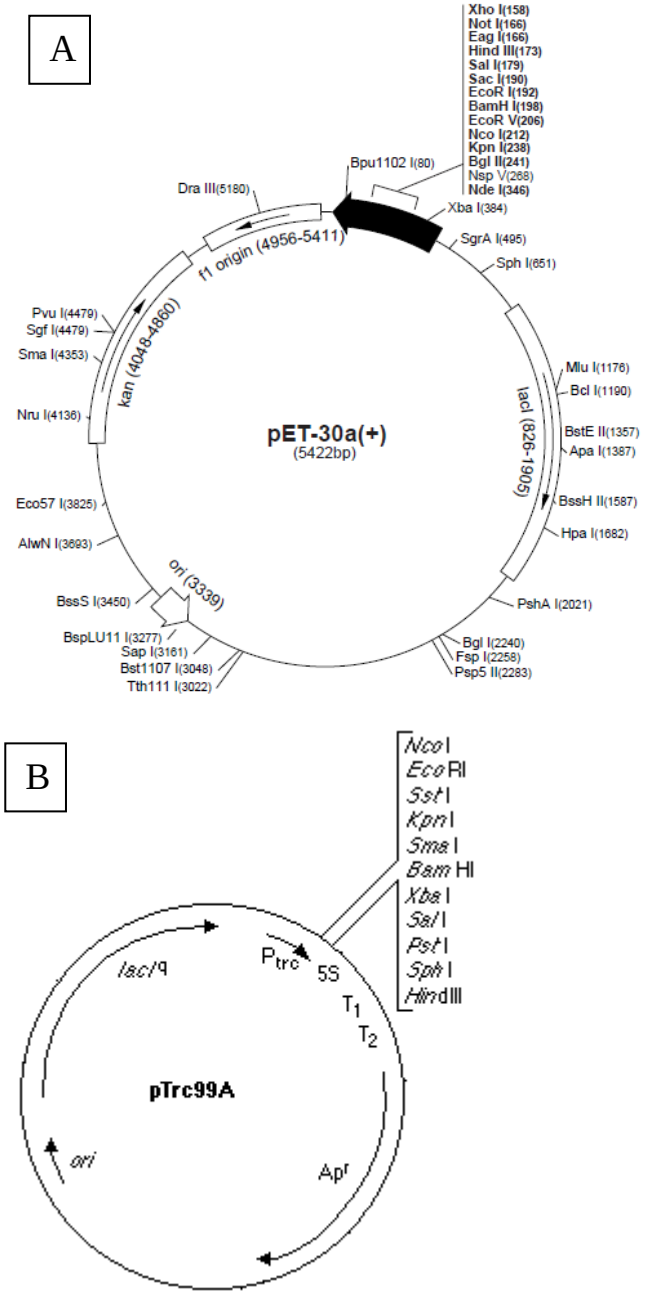

Figure 6. Cloning vectors pET-30a (A) and pTRC-99A (B) used for the expression of *"mga"* gene from *R. marinus.*

3.2 Extraction of intracellular organic solutes

Cells of *R. marinus* were grown at 65ºC in Degryse medium with 1% and 5% NaCl, harvested during the mid-exponential and stationary phases of growth (OD_{610}=0.4) by centrifugation (10 000 x g, 10min, 4ºC) and washed twice with a NaCl solution identical in concentration to that of the growth medium. Cell pellets were extracted with boiling 80% ethanol. The extraction was repeated and the combined extracts evaporated to dryness under negative

pressure; the residue was resuspended in water and chloroform, and centrifuged to remove lipid components (Silva *et al.* 1999).

The protein content of the cells was determined by the Bradford assay (Bradford 1976) after sonication of the cells, using an aliquot of the suspension before the extraction of compatible solutes.

3.3 Visualization of intracellular organic solutes by thin layer chromatography

The intracellular organic solutes were separated by thin layer chromatography (TLC) on Silica Gel 60 plates using different solvent systems: a) hexane, chloroform, acetic acid (7:2:1, v/v/v); b) chloroform, methanol, acetic acid, water (30:50:8:4, v/v/v/v); c) chloroform, methanol, ammonium solution at 25% (30:50:25, v/v/v), and visualized by spraying with α-naphthol sulfuric acid solution or ninhydrin solution followed by charring at 120ºC. Pure mannosylglycerate, trehalose, D-glutamate and glucose were used as standards.

3.4 Preparation of *Rhodothermus marinus* protein extracts

Cells were harvested during mid-exponential phase of growth (OD_{610}= 0.4) and centrifuged (13000 rpm, 5 min, 20ºC). The cell sediment was re-suspended in Tris-HCl (25 mM, pH 7.5) and in BTP buffer (25mM, pH 7.5), followed by disruption in a French-press (120 MPa) (Thermo Electron Corporation). Cell extracts were dialysed against 25mM Tris-HCl (pH 7.5) or 25 mM BTP (pH7.5) buffers to remove endogenous solutes and other low molecular weight compounds prior to measuring enzyme activities. The protein content was determined by the Bradford assay (Bradford 1976) using BSA as standard.

3.5 Enzyme assays

Table 1. Substrates of enzyme assays. Column A, possible amino group donors; column B, possible amino group acceptors.

A*		B*	Co-factors**
Amino acids			
D-alanine	L-histidine	MPG	$FeCl_3$ (1mM)
L-arginine	D-leucine	MG	$MgCl_2$ (5mM)
D-aspargine	L-lysine		NAD+ (5mM)
L-aspartate	D-methionine		NADP (5mM)
L-cysteine	L-ornithine		NaCl (100mM)
D-glycerate	L-proline		
Glycine	L-serine		
D-glutamine	L-valine		
L-glutamate			
Amonium Chloride			
Poliamines			
Spermine			
Spermidine			
1,3-diaminopropane			
Tryptone (20 mg/ml)			
Yeast extract (20 mg/ml)			
Casamino acids (20 mg/ml)			

* Substrates were used at a final concentration of 5 mM
** Final concentrations of co-factors used in enzyme assays

Enzyme assays with cells extracts of *R. marinus* were performed by combining each substrate represented in column A with substrates represented in column B of Table 1. Reactions were performed in BTP (50 mM, pH7.5) or Tris-HCl (50 mM, pH 7.5) buffers, at 60ºC during 45 minutes and stopped by cooled on ice. Co-factors indicated in Table 1 were also added to the enzymatic reactions. Control assays were carried out using the same conditions. Products were separated and visualized by TLC with appropriate standards as described above (1.3.).

3.6 DNA manipulation, cloning and analysis

The isolation of DNA from *R. marinus and "R. obamensis"* was performed as described by Nielsen *et al.* (1995). PCR amplifications of the hypothetical "*mga"* gene (800 bp) were performed with GC-RICH PCR system kit (Roche). The forward primer MGAS-Nco (5´-GACCATGGCACCGCGCATGTCCGG-3´) and the reverse primer MGAS-Hind (5´-TACAAGCTTTTAGGCTGCTCCGTTGCCG-3´) were designed based on hypothetical "*mga"* gene sequence of *R. marinus* (GenBank CP001807). The PCR mixtures were pre-incubated for 5 min at 95ºC and then subjected to 30 cycles denaturation at 95ºC for 1 min, annealing at 55ºC for 1 min and primer extension reaction was at 72ºC for 1 min. The extension reaction in the last cycle was

prolonged for 10 min. PCR product was visualized on 1% agarose gel electrophoresis and purified by band excision using a DNA purification Kit (Promega) and cloned into NcoI and HindIII restriction sites of plasmids pET30a and pTRC-99A to give pET30A-"mga" and pTRC99A-"mga", respectively. Transformations of *E. coli* DH5α and *E. coli* BL21 were carried out as described previously (Sambrook 1989). Ampicilin and kanamycin were added to the medium at a final concentration of 100 µg/ml and 30 µg/ml, respectively, for plasmid selection. The clones were sequenced by AGOWA GmbH (Berlin, Gemany).

3.7 Over expression of the hypothetical *"mga"* gene

Escherichia coli DH5α and BL21 containing pTRC99A-"mga" and pET30a-"mga", respectively, were grown until they reached a turbidity ($O.D_{610}$) of about 1.0, induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and grown further for 16 h at 37ºC. Culture aliquots of 1 and 2 ml were harvested by centrifugation. The culture aliquots were disrupted by sonication and centrifuged to remove cell debris. The active fractions were collected and concentrated in a 10-kDa-cutoff Centricon Ultracel YM-10.

The over expression of the recombinant enzyme was evaluated by SDS-PAGE.

3.8 *Thermus thermophilus* as thermophilic host for the over production of Mannosylglycerate

Thermus thermophilus strain CC-16 was previously transformed as described by Alarico et al. (2007) with plasmid pMK18 carrying the constitutive promoter of the *T. thermophilus* S-layer gene (*slpA*) followed by the *mgS* gene encoding the mannosylglycerate synthase (MGS) from the single step of MG synthesis in *R. marinus*. This strain was grown at 65ºC and pH 8.2 in *Thermus* medium (DSMZ 1033) (http://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium1033.pdf) without additional NaCl or supplemented with NaCl to a final concentrations of 1 to 3% (w/v). Growth of this strain was also performed with the addition of filter-sterilized D-glycerate at final concentration of 14 mM. Cultures were grown into 300 ml metal-capped Erlenmeyer flasks, containing 100 ml of medium, with an initial turbidity of 0.07 (OD_{610}) and were incubated in a reciprocal-water bath shaker (120 rpm).

4 Results and discussion

4.1 Mannosylglyceramide: searching for the key enzyme

Understanding the role of compatible solutes in osmo- and thermoadaptation is directly correlated with the study of the mechanisms of its uptake from external environment and/or by *de novo* synthesis. The biosynthesis of compatible solutes requires essentially the study of genes, enzymes and metabolic pathways related with provided precursors of these compatible solutes.

Mannosylglyceramide, structurally related to MG, has only been found in *R. marinus*. The closely related "*R. obamensis"* did not accumulate MGA. This inability of "*R. obamensis"* to accumulate MGA, unlike some strains of *R. marinus* provides important insights into the strategies developed by both *Rhodothermus* sp. to cope with salt stress (Silva *et al.* 1999).

We analysed the genome of *R. marinus*, specifically the genes surrounding the gene(s) involved in the two pathways for the synthesis of MG. Immediately downstream the *mpgS* and *mpgP* genes there are two genes in the opposite direction, identified as hypothetical protein (Rmar_0500) with a conserved motif belonging to family of Acetyltransferase_2 and as D-amino-acid dehydrogenase (Rmar_0499), respectively (http://www.genome.jp/kegg-bin/show_genomemap?ORG=rmr&ACCESSION=Rmar_0500) (Figure 7). Using "Promotor 2.0 Prediction Server" program (http://www.cbs.dtu.dk/services/Promoter/) we identified a hypothetical promoter region upstream the gene encoding the D-amino acid dehydrogenase as shown in Figure 7.

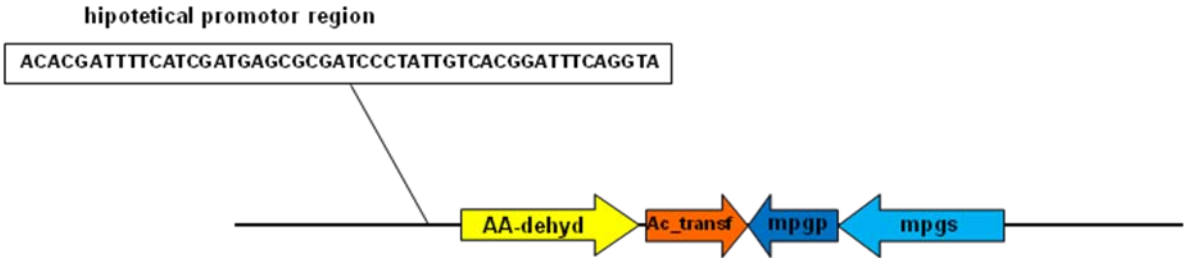

Figure 7. Schematic representation of downstream region of *mpgs/mpgp* genes in *R. marinus* genome. Genes are represented by arrows: AA-dehyd, D-amino acid dehydrogenase (Rmar_0499); Ac_transf, gene encoding for a hypothetical protein (Rmar_0500); *mpgp*, gene encoding the mannosyl-3-phosphoglycerate phosphatase (MPGP) and *mpgs*, gene encoding the mannosyl-3-phosphoglycerate synthase (MPGS).

The Rmar_0500 gene, from *R. marinus* contains 822 bp and code for a polypeptide with 273 amino acids. A homologue of this gene was amplified from "*R. obamensis*" genome, using the same pair of primes as used for Rmar_0500 gene. The two genes were sequenced and the encoding enzymes showed very high sequence homology (96% amino acid identity, by NCBI BLAST program) (Figure 8). Several homologues enzymes, although possess high sequence

homology show differences in substrate specificity. For example, an α-glucosidase from *T. thermophilus* strain HB27, with high activity to hydrolyse trehalose, shares 90% of amino acid identity with its homologue from *T. thermophilus* strain GK24, which did not hydrolyse trehalose and the highest activity is towards isomaltose (Alarico *et al.* 2008). We will further investigate if differences in amino acid identity of these two homologues enzymes will result in different specific activities. This will be achieved by expression and purification of recombinant enzymes from both strains and comparative activity studies, which could confirm or close down the involvement of Rmar_0500 gene in the synthesis of MGA in *R. marinus.*

```
Rh_mar          MAPRMSGTRQVATDEYFRFDVPGCYRTQAFQALLARHPEALQLYARHVEAQAHEPAYLQR 60
"Rh_oba"        MAPRMSGTRQVATDEHFRFDVPGCYRTQAFQALLARHPEALQLYARHVEAQAHMPAYLQR 60
                ***************:************************************ ******

Rh_mar          VRQLVPRLVRWLGAEVAADGRPGLCVQTSALLSRLLEELGIWNYVVAGGCVLSFVPADVR 120
"Rh_oba"        VRQLVPRLVRWLGEEVAADGRPGLCVQASVLLSRLLEELGIWNYMVTGGCVLSFVPADVR 120
                ************* *************:*.**************:*:*************

Rh_mar          PRVFYLFDLQPVEVPHAWVVAPPYDVIDLTLRQQRYPGPEGRRIPTQVLSCRAPQVTVQP 180
"Rh_oba"        PRVFYLFDLQPVEVPHAWVVAPPYDVIDLTLRQQRYPGPEGRRIPTQVLSCRAPQVTVQP 180
                ************************************************************

Rh_mar          EDVCTPALLQGLLLRGWTRETLLRRAFPEFWHFLKQFPARRVQTPTVSVTYIPARLLLPP 240
"Rh_oba"        EDVCTPALLQGLLLRGWARETLLRRAFPEFWHFLKQFPARRVQTPTVSVTYIPARLLLPP 240
                *****************:****************************************

Rh_mar          WHEAWERMPLINGKSFVQFRSEMTLVLSGNGAA 273
"Rh_oba"        WHEAWERMPLINGKSFVQFRSEMALVLSGNGAA 273
                ***********************:*********
```

Figure 8. Sequence alignment of deduced amino acid sequences of hypothetical protein (Rmar_0500) and its homologue from "*R. obamensis"*. The abbreviations are as follows: Rh_mar, *R. marinus* and "Rh_oba", "*R. obamensis"*.

4.2 Mannosylglyceramide: searching for the natural precursors

4.2.1 Qualitative method for detection

Rhodothermus marinus accumulate several compatible solutes in the presence of salt and heat stress. Mannosylglyceramide was only accumulated when the organism was growing in media containing above 3% to 5% of NaCl and until 75ºC. In these conditions, mannosylglyceramide is the major solute accumulated together with mannosylglycerate (Silva *et al.* 1999).

During the present study, it has become crucial to find a method to detect and identify the solute MGA. This was achieved by TLC using a solvent system consisting of chloroform/methanol/ammonia solution 25% (30/50/25, v/v/v) (Figure 9). Silica plates containing

the samples were stained with ninhydrin solution to reveal compounds with amine groups, like glutamate (data not shown), followed by spraying with α-naphthol sulfuric acid and charring at 120ºC to reveal glycosidic compounds. Mannosylglycerate, mannosylglyceramide and trehalose were the major solutes of *R. marinus* cellular extracts and were clearly separated and identified using this method (Figure 9).

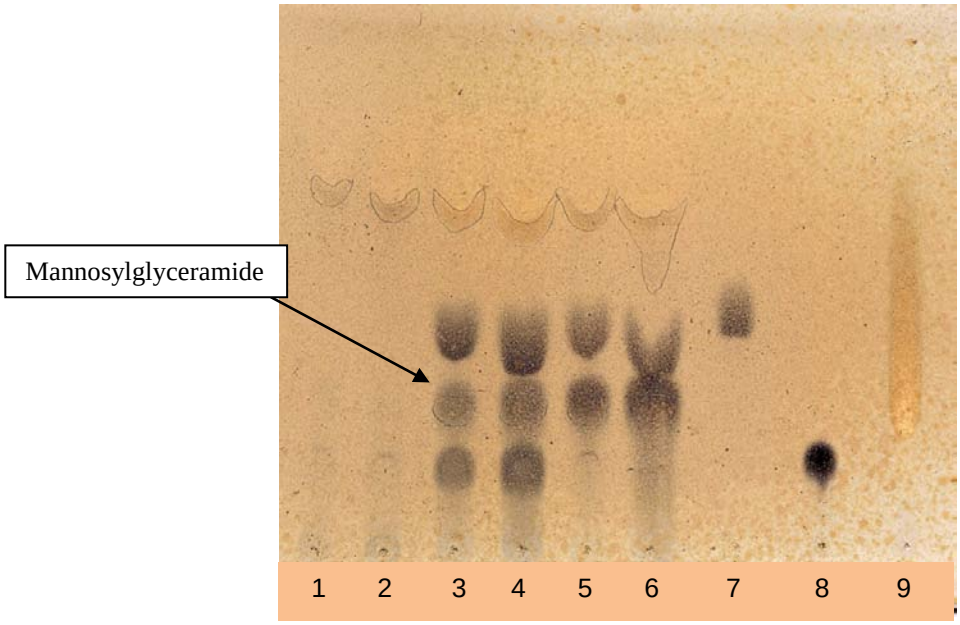

Figure 9. Thin-layer chromatography of *R. marinus* intracellular organic solutes using chloroform/methanol/ammonia solution 25% (30/50/25, v/v/v) as solvent system. Sample of intracellular organic solutes from *R. marinus* culture grown in Degryse medium with 1% NaCl and harvest at O.D.=0.4: 5 µl (lane 1) and 10 µl (lane 2) of sample. Sample of intracellular organic solutes from *R. marinus* culture grown in Degryse medium with 5% NaCl, harvest at O.D. = 0.4: 5 µl (lane 3) and 10 µl (lane 4) of sample, and harvest at O.D. =1.5: 5 µl (lane 5) and 10 µl (lane 6) of sample. Standards: mannosylglycerate (lane 7), trehalose (lane 8) and glutamate (lane 9).

4.2.2 Substrates tested in enzyme assays

Several enzyme assays with dialysed cellular extracts of *R. marinus* were performed at 60ºC, during 40 min combining amino acids, polyamines or other compounds as possible amino group donors with MG or MPG as possible amino group acceptors. TLC results are exhibit below. The efficient dialysis of cellular extracts was confirmed by TLC prior to perform the enzyme assays.

4.2.2.1 Amino acids

In *R. marinus*, gene Rmar_0499 identified as D-amino acid dehydrogenase is a NAD dependent enzyme. This type of enzymes is known to release amino group from amino acids. We tested some L- and D-amino acids (as possible amino group donors) together with MG or MPG in the reactions mixtures. Additionally, NAD^+ or NADPH was also used as co-factors but no activity concerning MGA synthesis was detected by TLC (Figure 10 and Figure 11).

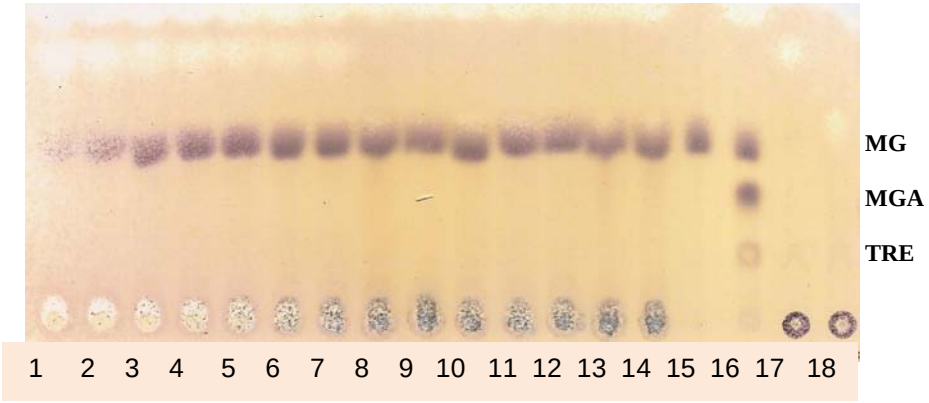

Figure 10. Thin-layer chromatography analysis of reaction assays products using several L- and D-amino acids. The enzyme assays were performed at 60ºC during 40 min in Tris buffer (pH 7.5) (lanes 1-7) or in BTP buffer (pH 7.5) (lanes 8-13) using MG and containing the amino acids: lanes (1 and 8) D-alanine; (2 and 9) D-aspargine; (3 and 10) glycine; (4 and 11) D-glutamine; (5 and 12) D-leucine; (6 and 13) D-metionine and (7 and 14) D-valine. Standards: mannosylglycerate (lane 15) and sample of intracellular organic solutes from *R. marinus* (lane 16): MG, mannosylglycerate; MGA, mannosylglyceramide and TRE, trehalose. Dialysed cellular extracts of *R. marinus* in Tris buffer (lane 17) and BTP buffer (lane 18).

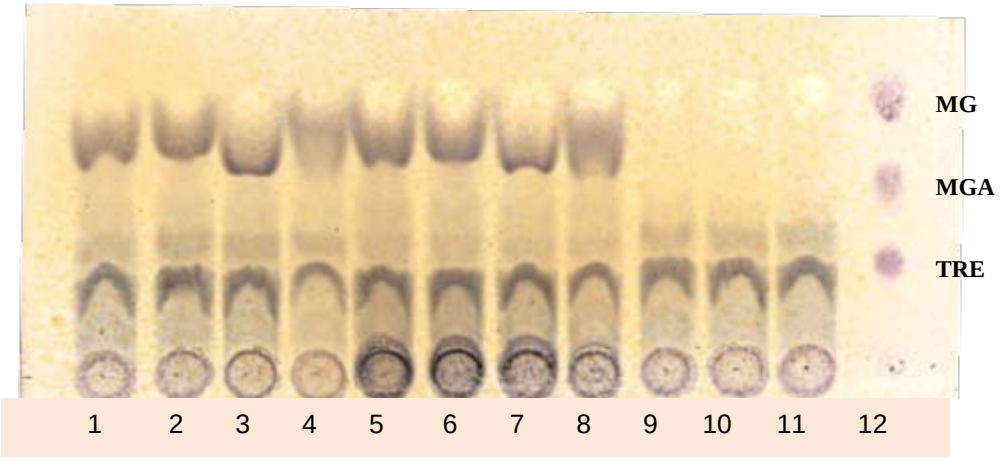

Figure 11. Thin-layer chromatography analysis of reaction assays products using several L-amino acids. The enzyme assays were performed in 15 mM BTP buffer (pH 7.5) at 60ºC, during 40 min using MG (lanes 1-4) or MPG (lanes 5-8) and the amino acids: lanes (1 and 5) L-arginine; (2 and 6) L-glutamine; (3 and 7) L-lysine and (4 and 8) L-ornithine. Controls: mixtures without MG or MPG and with L-arginine (lane 9), L-glutamine (lane 10) and L-lysine (lane 11). Standard (lane 12): sample of intracellular organic solutes from *R. marinus*: MG, mannosylglycerate; MGA, mannosylglyceramide and TRE, trehalose.

4.2.2.2 Tryptone, yeast extract and casamino acids

Some compounds used for microbiological growth have a variety of amino acids and some peptides in their composition. We performed enzyme reactions by testing some of that compounds, like tryptone, yeast extract and casamino acids combined with MG or MPG (Figure 12).

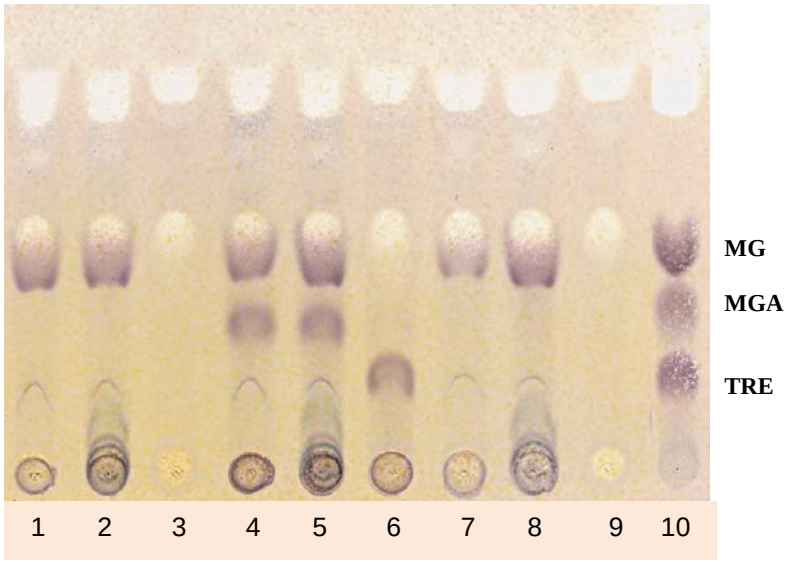

Figure 12. Thin-layer chromatography analysis of reaction assays products using tryptone, yeast extract and casamino acids. The enzyme assays were performed in 15 mM BTP buffer (pH 7.5) at 60ºC, during 40 min with MG (lane 1, 4 and 7) or MPG (lane 2, 5 and 8) and with tryptone (lane 1 and 2), yeast extract (lane 4 and 5) and casamino acids (lane 7 and 8). Controls: mixtures without MG or MPG and with tryptone (lane 3), yeast extract (lane 6) and casamino acids (lane 9). Standard (lane 10): sample of intracellular organic solutes from *R. marinus*: MG, mannosylglycerate; MGA, mannosylglyceramide and TRE, trehalose.

The TLC analysis revealed one spot in lane 4 and 5 with about the same solvent migration as MGA from the standard (lane 11). Complementary testes showed that those spots were not MGA but corresponded to glucose from the complete hydrolysis of trehalose present in yeast extract by enzyme(s) of the crude extract of *R. marinus*. In the control reaction (lane 6) without crude extract we only observed trehalose. In this solvent system, the standard glucose has approximately the same solvent migration as MGA (data not shown).

4.2.2.3 Polyamines

Physiological polyamines, like putrescine, spermidine and spermine are synthesized endogenously in the cells of all living organisms and their metabolic pathways are conserved, suggesting functions in transcriptional and translational regulation which affects growth, modulation of cell signalling, membrane stabilization and regulation of cell death (Kusano *et al.* 2008). Polyamines are also reported as ancient stress molecules as they are induced by different stresses, such as reactive oxygen species, heat and ultraviolet radiation (Rhee *et al.* 2007).

Some polyamines like spermidine, spermine and 1,3-diaminopropane, known to exist in *R. marinus* and precursors of unusual long and branched polyamines biosynthesis (Hamana *et al.* 1992) were also tested in the enzyme assays as possible amino group donors (Table 1, Material and Methods).

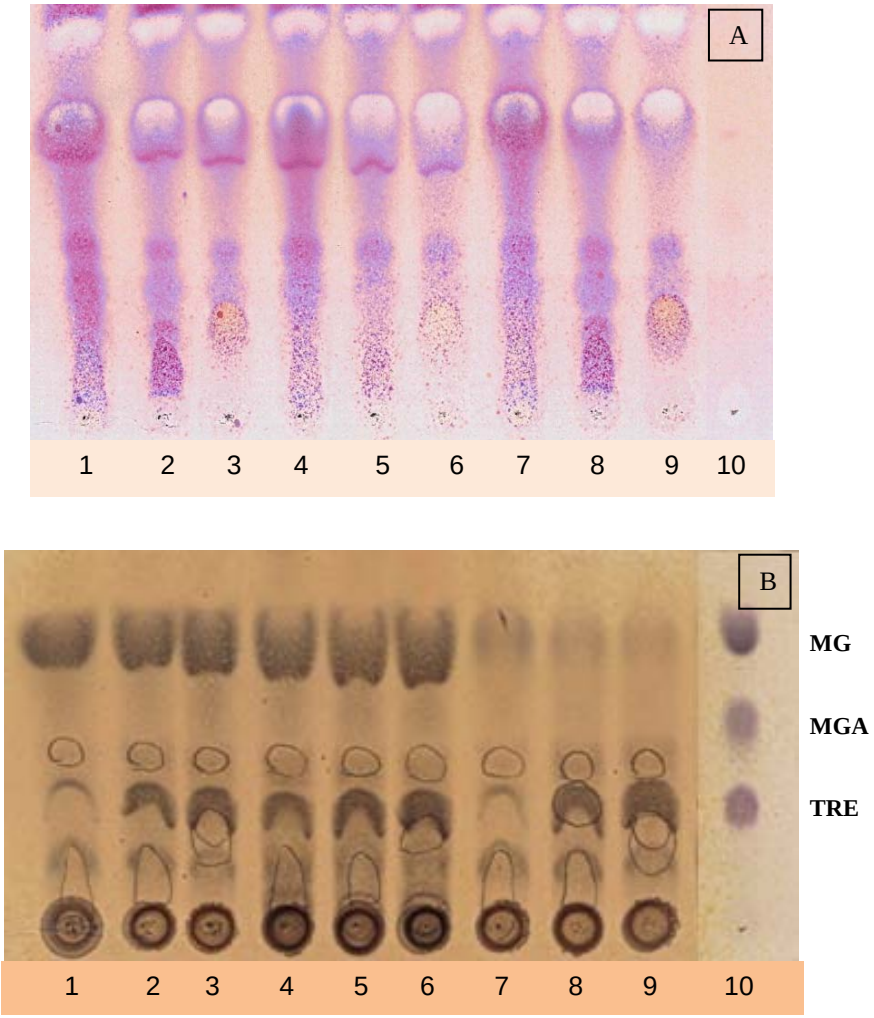

Figure 13. Thin-layer chromatography analysis of enzyme assays using polyamines. (**A**) Silica plate stained with ninhydrin and with (**B**) α-naphthol sulfuric acid. Enzyme assays with MG and polyamines: spermine (lane 1), spermidine (lane 2), 1,3 diaminopropane (lane 3); or with MPG and polyamines: spermine (lane 4), spermidine (lane 5) and 1,3-diaminopropane (lane 6). Controls: mixtures without MG or MPG and with spermine (lane 7), spermidine (lane 8) and 1.3-diaminopropane (lane 9). Standard (lane 10): sample of intracellular organic solutes from *R. marinus*: MG, mannosylglycerate; MGA, mannosylglyceramide and TRE, trehalose.

Although the utilization of a variety of substrates and co-factors, we were not able to attain to MGA synthesis, yet. We will further continue this study by testing different reaction conditions, such as different temperatures of incubation, different buffers, like potassium or sodium phosphate which are often used by amino acid dehydrogenases (Ali *et al.* 2003; Tanigawa *et al.* 2010). We will also test other substrates and co-factors such as NaCl and KCl that frequently increase the activity of enzymes in crude extracts (Martins *et al.* 1999). At this point, it is also crucial the purification of the hypothetical protein recombinant enzyme, which we suppose to be involved in the biosynthesis of MGA in order examine its activity.

4.3 Overexpression of putative *"mga"* gene

The putative "*mga*" gene encoding for the hypothetical protein was amplified from genomic DNA of *R. marinus*, cloned into expression vectors pET30a and pTREC99A and used to transform *E. coli* BL21 and *E. coli* DH5α, respectively.

SDS-PAGE analysis of cell extract from *E. coli* BL21 containing pET30a-"mga" clone grown with IPTG induction showed an extra band of a molecular mass of 31 kDa (Figure 14), which was not observed in non-induced crude extracts nor in cell extracts from *E. coli* DH5α containing pTREC99A-"mga" clone (data not shown) or in cell extracts from *E. coli* BL21 and DH5α with empty vectors used as controls.

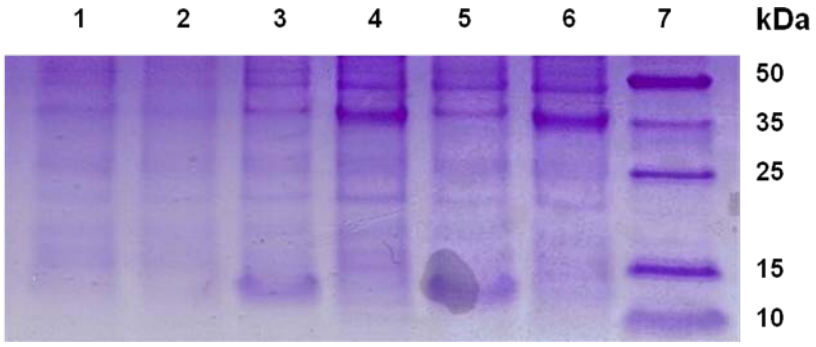

Figure 14. SDS-PAGE analysis of hypothetical protein overproduction in *E. coli* BL21. Crude extract from BL21 containing pET30a (lane 1) and BL21 containing pET30a-"mga" (lane 2) before induction with 0.5 mM of IPTG. 1 ml of crude extract from IPTG-induced BL21 containing pET30a (lane 3) and from IPTG-induced BL21 containing pET30a-"mga" (lane 4) and 2 ml of crude extract from IPTG-induced BL21 containing pET30a (lane 5) and from IPTG-induced BL21 containing pET30a-"mga" (lane 6). Molecular mass marker (lane 7).

We will further grow the selected *E. coli* BL21 containing pET30a-"mga" clone to purify the recombinant enzyme by using a nickel HisTrap column (high-affinity column). Enzyme assays similar to those mentioned above and with other possible precursors for MGA synthesis will be test using the purified recombinant enzyme alone or in the presence of dialyzed cellular extract of *R. marinus*, since it may contain co-factors and other molecules that can be essential or can potentiate the synthesis of this solute.

4.4 *Thermus thermophilus* as suitable organism for compatible solutes overproduction

Most *T. thermophilus* strains accumulate as major compatible solutes, MG and trehalose but there are some exceptions. For example, *Thermus thermophilus* CC-16 is a MG-negative naturally occurring strain that only accumulates trehalose (by *de novo synthesis* or by the uptake of this solute from the growth media) and is unable to grow up to 1% NaCl.

We have transformed strain CC-16 with a plasmid containing the "*mgs*" gene, from *R. marinus,* responsible for the synthesis of MG from GDP-mannose and D-glycerate in one single step. Strain CC-16 transformants became able to grow in media containing more than 1% up to 3% NaCl and to accumulate MG, however, only when D-glycerate was externally provided to the growth media. We concluded that D-glycerate is not a naturally occurring substrate in this strain and that MG is only synthesise when this compound is up taken from the growth medium.

To improve the utilization of strain CC-16 as a suitable organism, particularly, for the overproduction of MG we'll alternatively test the addiction to the growth media of glycerol and serine, which are precursors of D-glycerate in organisms like *Thermotoga maritime* (Yang *et al.* 2008), attempting the indirect production of MG since D-glycerate is an expensive commercial product.

If we succeed in the characterization of the biosynthetic pathway of MGA, we will also use this *T. thermophilus* strain to overproduce this unusual solute, thus far, only found in *R. marinus*.

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