Pathways for the Synthesis and Hydrolysis of some Compatible Solutes in the Bacterium *Rubrobacter xylanophilus* and in the Plant *Selaginella moellendorffii*

Characterization of some recombinant enzymes

Vias para a Síntese e Hidrólise de alguns Solutos Compatíveis na Bactéria *Rubrobacter xylanophilus* e na Planta *Selaginella moellendorffii*

Caracterização de algumas enzimas recombinantes

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“What we know is a drop.

What we do not know – an ocean.”

Isaac Newton

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ABSTRACT

Organisms that face osmotic stress have long been known to accumulate small organic molecules that do not perturb cell function, even when at high concentrations. Such molecules are typically called 'compatible' solutes and permit cells to adjust the turgor pressure required for metabolism and survival of organisms. Compatible solutes are fundamental molecules, accumulated or synthesized by cells, in the response several stress conditions and are viewed as general stress protectants.

Rubrobacter xylanophilus is known to accumulate a rare combination of compatible solutes, being trehalose and mannosylglycerate the major solutes and constitutively accumulated.

The present work describes the biochemical characterization of three enzymes involved in the synthesis of trehalose, the major accumulated compatible solute, in this thermophilic radiation-resistant actinobacterium. Although *Rubrobacter xylanophilus* possesses a total of four trehalose biosynthetic pathways, we functionally characterize two systems which we have shown to be active in cell extracts, under the conditions tested, namely trehalose-6-phosphate synthase/trehalose-6-phosphate phosphatase (Tps/Tpp) and the glycosyltransferring synthase (TreT).

Two of the described enzymes are involved in the most common pathway, the Tps/Tpp pathway, which has been reported in organisms of the three domains of life. This pathway involves two enzymatic steps; Tps catalyzes the transfer of glucose from GDP-glucose to glucose-6-phosphate (Glc6P), forming trehalose-6-phosphate (T6P) and GDP, while Tpp dephosphorylates T6P to trehalose and inorganic phosphate. Both enzymes showed high substrate specificity: Tps, for the combination of GDP-glucose and Glc6P, and Tpp for T6P. The thirdly characterized enzyme, designated TreT, coexists with Tps/Tpp pathway in *R. xylanophilus* and promotes the formation of trehalose from several nucleoside diphosphate (NDP)-glucoses and glucose. The favorite donor substrate was reported to be ADP-glucose, but only glucose served as an acceptor molecule. The proposed main function of the TreT enzyme in *R. xylanophilus* is to preferentially catalyze trehalose formation, although it is also able to reverse catalysis, hydrolyzing trehalose, but with much less efficiency.

The biosynthetic pathway for the other compatible solute mostly accumulated in this bacterium, mannosylglycerate, has been recently described by Empadinhas and co-workers (2011).

To withstand osmotic stress, plants accumulate and synthesize compatible solutes, which help to maintain the structure of enzymes, membranes and other cellular compounds. Mannosylglycerate and glucosylglycerate were not accumulated by the plant *Selaginella moellendorffii*, under the examined conditions, even though the genes coding for their synthesis and hydrolysis were identified in the plant's genome. The biosynthesis and hydrolysis of mannosyglycerate and glucosylglycerate in *S. moellendorffii* are described in this work. The *S. moellendorffii* mannosylglycerate synthase (MgS) catalyzes the synthesis of glycerate derivatives, mannosylglycerate and glucosylglycerate, when GDP-mannose or GDP-glucose were glucosyl donor substrates, respectively. Glycoside hydrolase, designated as mannosylglycerate hydrolase (MgH), is highly specific for the hydrolysis of both mannosylglycerate and glucosylglycerate.

This thesis unravels the identity of genes and enzymes involved in the synthesis and hydrolysis of the compatible solutes trehalose, mannosylglycerate and glucosylglycerate, in two phylogenetically unrelated organisms and furthers our understanding on how these important compounds are synthesized and hydrolyzed. The thesis results pave the way for future studies on the function and regulation of these molecules in the physiology of these organisms.

RESUMO

A adaptação a ambientes com salinidade elevada pressupõe, para muitos organismos, a acumulação de pequenas moléculas orgânicas, que não interferem com o metabolismo celular, mesmo quando a sua concentração é elevada.

Estas moléculas, designadas solutos "compatíveis", permitem que as células ajustem a sua pressão de turgescência, condição necessária ao metabolismo e à sobrevivência dos organismos. Os solutos compatíveis são moléculas fundamentais captadas do exterior ou sintetizadas pelas células em resposta a condições ambientais agressivas, sendo consideradas moléculas protectoras.

A bactéria *Rubrobacter xylanophilus* é conhecida por acumular uma rara combinação de solutos compatíveis, sendo a trealose e o manosilglicerato os principais (acumulados constitutivamente).

O presente trabalho descreve a caracterização bioquímica de três enzimas envolvidas na síntese do soluto compatível trealose nesta actinobactéria termofílica e extremamente resistente a radiações. Embora a bactéria *Rubrobacter xylanophilus* possua quatro vias de síntese para a trealose, apenas duas foram caracterizadas, por se ter demonstrado estarem funcionalmente activas em extratos celulares, nas condições testadas: a via trealose-6-fosfato sintetase/trealose-6-fosfato fosfatase (Tps/Tpp) e a via da enzima sintetase que transfere um grupo glucosil (TreT).

Duas das enzimas caracterizadas estão envolvidas na via Tps/Tpp, sendo esta a via mais comum, uma vez que existe em organismos dos três Domínios da Vida. A via Tps/Tpp inclui duas reacções enzimáticas catalisadas pelas enzimas Tps e Tpp. A enzima Tps catalisa a transferência de glucose de GDP-glucose para glucose-6-fosfato (Glc6P), formando trealose-6-fosfato (T6P) e GDP, enquanto a enzima Tpp desfosforila T6P, formando trealose e fosfato inorgânico. Ambas as enzimas mostraram ter uma elevada especificidade em relação aos substratos: a enzima Tps, relativamente à combinação GDP-glucose e Glc6P, e a enzima Tpp, relativamente à T6P. A terceira enzima caracterizada designa-se por TreT. Esta enzima co-existe com as enzimas da via Tps/Tpp na bactéria *R. xylanophilus* e promove a formação de

trealose a partir de vários nucleosídeos difosfato (NDP)-glucose (dador) e glucose (receptor). O substrato preferido, dador do grupo glucosil, foi descrito ser ADP-glucose; contudo, a glucose foi a única molécula receptora. A função da enzima TreT em *R. xylanophilus* é, preferencialmente, catalisar a formação de trealose, embora também seja capaz de a degradar, mas com uma eficiência muito menor.

A via biossintética para o soluto compatível manosilglicerato, também acumulado por esta bactéria, foi recentemente descrita por Empadinhas e colaboradores (2011).

Para lidar com o *stress* osmótico, as plantas também acumulam solutos compatíveis que ajudam a manter a estrutura das membranas celulares, das enzimas e de outros compostos. Os solutos manosilglicerato e glucosilglicerato não são acumulados por *Selaginella moellendorffii*, nas condições estudadas, embora os genes que codificam as enzimas para a sua síntese e hidrólise tenham sido identificados no genoma desta planta. As vias para a síntese e hidrólise de manosilglicerato e glucosilglicerato em *S. moellendorffii* são descritas neste trabalho. A sintetase de manosilglicerato (MgS) da planta *S. moellendorffii* catalisa a síntese dos derivados de glicerato, manosilglicerato e glucosilglicerato, quando a GDP-manose ou a GDP-glucose são substratos dadores do grupo glucosil, respectivamente. A hidrolase, designada por manosilglicerato hidrolase (MgH), é altamente específica para a hidrólise dos dois compostos manosilglicerato e glucosilglicerato.

Esta tese permitiu identificar os genes e caracterizar as respectivas enzimas recombinantes envolvidas na síntese e na hidrólise dos solutos compatíveis trealose, manosilglicerato e glucosilglicerato em dois organismos filogeneticamente não relacionados, possibilitando, assim, a compreensão de como estes compostos são sintetizados e hidrolisados. Os resultados deste trabalho permitem abrir caminho a futuros estudos sobre a regulação e a função das referidas moléculas na fisiologia destes organismos, com possíveis implicações importantes tanto a nível da investigação fundamental como da investigação biotecnológica.

THESIS CONTENTS

THESIS OUTLINE

One of the strategies to deal with osmotic stress, adopted by both prokaryotes and eukaryotes, is the accumulation of low-molecular-weight compounds, generally designated as compatible solutes. These diverse molecules can be taken from the environment or synthesized *de novo*.

In the last few years, knowledge on the metabolic pathways, especially for the synthesis of compatible solutes, has increased considerably. The study of compatible solutes catabolic pathways is also of vital importance, but very little information is available about the degradation of these molecules and the environmental and molecular regulatory conditions involved in this process.

The present work is an approach to the metabolic pathways for the synthesis and hydrolysis of three compatible solutes - trehalose, mannosylglycerate and glucosylglycerate - in the prokaryote, *Rubrobacter xylanophilus*, and in the eukaryote, *Selaginella moellendorffii*.

Chapter 1 is a brief description of compatible solutes, namely trehalose, mannosylglycerate and glucosylglycerate. Their distribution and biosynthetic pathways are introduced, including an analysis of the different genetic organizations of the genes coding for trehalose/mannosylglycerate/glucosylglycerate enzymes. In this chapter, the pathway multiplicity is also addressed. This chapter proceeds with a review about glycoside hydrolases, which serves as a basis for the discussion on a mannosylglycerate hydrolase characterized in chapter 3. A general description of the organisms used in this study is also presented in this chapter.

Chapter 2 describes two of the pathways for trehalose synthesis in the thermophilic bacterium *Rubrobacter xylanophilus*. In this chapter, in addition to the biochemical characterization of the most widespread trehalose biosynthetic pathway involving trehalose-6-phosphate synthase/phosphatase, the recently discovered single-step pathway, which involves a glycosyltransferring synthase, is also elucidated.

Chapter 3 describes the characterization of two different proteins, a glycosyltransferase and a glycoside hydrolase, encoded by the *mgS* and *mgH* genes, detected in the recently available

genome sequence of the lycophyte *Selaginella moellendorffii*. As in chapter 2, both genes were cloned and over-expressed in *Escherichia coli* and the resulting recombinant enzymes were characterized. Moreover, biochemical properties and kinetic parameters were examined in detail. Interestingly, these enzymes are implicated in the synthesis and hydrolysis of the same sugar-glycerate molecules, mannosylglycerate and glucosylglycerate.

Chapter 4 provides an overall discussion and integration of the results presented in this thesis.

ABBREVIATIONS

3-PGA	3-phosphoglycerate
aa	amino acid
ADP	adenosine diphosphate
ADP-glu	adenosine diphosphate-glucose
Arg	arginine (R)
Asn	asparagine (N)
Asp	aspartic acid (D)
BTP	2-[bis(hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol
CAPS	N-cyclohexyl-3-aminopropanesulfonic acid
CDP-glu	cytidine diphosphate-glucose
DIP	di-*myo*-inositol-phosphate
FPLC	fast protein liquid chromatography
Glc1P	glucose-1-phosphate
Glc6P	glucose-6-phosphate
GDP-glu	guanosine diphosphate-glucose
GG	glucosylglycerate
GGG	glucosylglucosylglycerate
GPG	glucosyl-3-phosphoglycerate
GpgP	glucosyl-3-phosphoglycerate phosphatase
GpgS	glucosyl-3-phosphoglycerate synthase
GT	glycosyltransferase
GT-B	glycosyltransferase – B fold
HAD	haloalcanoic acid dehalogenase
His	histidine (H)
IPTG	isopropyl-β-D-thiogalactopyranoside
MES	morpholineethanesulfonic acid
MG	mannosylglycerate
MGG	mannosylglucosylglycerate
MGP	mannosyl-3-phosphoglycerate
MgS	mannosylglycerate synthase
MpgP	mannosyl-3-phosphoglycerate phosphatase
MpgS	mannosyl-3-phosphoglycerate synthase
NDP-glu	nucleoside diphosphate-glucose
NMR	nuclear magnetic resonance
PCR	polymerase chain reaction
SDS-PAGE	sodium dodecyl sulphate-polyalcrylamide gel electrophoresis
Ser	serine (S)
T6P	trehalose-6-phosphate
TDP-glu	thymidine diphosphate-glucose
Thr	threonine (T)

TLC	thin layer chromatography
Tpp	trehalose-6-phosphate phosphatase
Tps	trehalose-6-phosphate synthase
TreP	trehalose phosphorylase
TreS	trehalose synthase
TreT	trehalose glycosyltransferring synthase
TreY	maltooligosyltrehalose synthase
TreZ	maltooligosyltrehalose trehalohydrolase
Tyr	tyrosine (Y)
UDP-glu	uridine diphosphate-glucose

CHAPTER 1

Introduction

CONTENTS

1. WATER AVAILABILITY

Water is crucial to life. Thermodynamically, the water accessible to cells is defined as water activity (a_w). Many organisms thrive in environments with low concentration of solutes, consequently high a_w, and most of them do not tolerate even slight decreases in this parameter. Others can grow in environments with extremely low a_w, such as concentrated salt or sugar solutions (Brown, 1976; da Costa *et al.*, 1998; Empadinhas and da Costa, 2006). Environments rich in solutes (for example, high-sugar foods) are dominated by xerophilic organisms, such as filamentous fungi and yeasts, some of which are able to grow at the lowest a_w value recorded so far: a_w 0.61. Habitats with a high concentration of salts, where water availability is also limited, are almost exclusively inhabited by prokaryotic microorganisms, particularly haloarchaea, capable of growing in saturated NaCl (a_w 0.75) (Grant, 2004). Salt marshes, bays, and estuaries are unique ecosystems characterized by frequent fluctuations in salinity. These fluctuations are also relevant in soils where drought, rain and freezing often change the osmolality within the environment (Miller and Wood, 1996). As a result of osmotic gradient, water permeates across the cytoplasmic membrane of the cells and could lead to their rupture. The levels of water stress tolerated by each organism vary significantly, and those with effective mechanisms of adaptation and able to adapt to osmotic challenges can survive under these environmental conditions (da Costa *et al.*, 1998).

Organisms exposed to a low a_w environment must possess mechanisms to avoid water loss by osmosis. At least a minimum of turgor pressure has to be maintained to allow cell survival and growth. The immediate physiological and genetic alterations that take place in the cell, as the level of environmental water changes, is frequently called osmoadaptation (Galinski and Truper, 1994; da Costa *et al.*, 1998; Yancey, 2005).

Two main mechanisms, reflecting different evolutionary strategies, have been developed by microorganisms for osmotic adjustment.

One strategy, known as the ‘salt-in-cytoplasm’ strategy, involves the intracellular accumulation of slightly higher amounts of KCl to counterbalance osmotic stress imposed by extracellular NaCl. This ability is restricted to halophilic prokaryotes (Kushner, 1978), mainly haloarchaea

and anaerobic bacteria of the order Haloanaerobiales. The red-pigmented and extremely halophilic bacterium *Salinibacter ruber*, isolated from salterns, is the only known representative of the aerobic bacteria that shares the strategy of KCl accumulation to cope with extreme salinities. It accumulates very high concentrations of Cl^- and K^+/Na^+, while other solutes were found only in minor concentrations (Anton *et al.*, 2002; Oren *et al.*, 2002).

The other strategy used by eukaryotes and most bacteria and archaea involves the synthesis or accumulation of organic molecules, designated as 'compatible solutes' (osmolytes), which have the aim of counterbalancing osmotic potential (Brown, 1976; da Costa *et al.*, 1998; Welsh, 2000; Oren *et al.*, 2002; Kunte, 2006). This last strategy does not require extensive structural adaptations and enables a rapid adaptation of the environmental osmotic changes (da Costa *et al.*, 1998; Empadinhas and da Costa, 2008a).

For plants, water deficit of soil, salinity, high and low temperature and nutrient imbalances are considered the main factors that limit their survival, growth and productivity (Bao *et al.*, 2009; Lee and Hwang, 2009). As in prokaryotes, two types of osmolytes, organic solutes (amino acids, glycine betaine, polyols, sugars, and other low molecular weight metabolites) and inorganic ions (Na^+, K^+, Ca^{2+} and Cl^-) play a key role in osmotic adjustment (Jakobsen *et al.*, 2007; Chen and Jiang, 2010).

Other photosynthetic organisms, mainly algae, can also experience osmotic stress and show similar responses. The basic mechanism of salt tolerance for algae has been found to be similar to that of vascular plants (Chapin, 1991). For example, the same compatible solutes (proline, glycerol and betaine) are accumulated by a unicellular green alga *Dunaliella salina* (order Chlorophyta) and vascular plants, under osmotic stress (Zhang *et al.*, 2002).

In the last years, genetic transformation techniques allowed the introduction of new pathways for the biosynthesis of various compatible solutes into plants, resulting in the production of transgenic plants with improved tolerance to stress. Considerable progress has been made in engineering the biosynthesis of compatible solutes in a variety of species, including some agriculturally important crops (McNeil *et al.*, 1999).

2. COMPATIBLE SOLUTES

Organic osmolytes are usually called compatible solutes based on the assumption that these solutes do not interact with macromolecules of cells in unfavorable ways; thus, they can be safely up- and downregulated with small impact on cellular functions (Brown and Simpson, 1972; Yancey *et al.*, 1982). On the other hand, inorganic ions, at high concentrations, normally attach to and destabilize proteins and nucleic acids (with the exception of haloarchaea and halobacteria).

The accumulation of compatible solutes, either by uptake from the medium or *de novo* synthesis, is a common response of microorganisms to osmotic stress, to counterbalance the external decrease in water availability and consequent decrease in internal turgor pressure.

The role of compatible solutes goes beyond osmotic adjustment alone, to the protection of the cells and cells components from a variety of different stress conditions like heat, freezing, desiccation, dehydration and oxygen radicals (Santos and da Costa, 2002; Elbein *et al.*, 2003; Yancey, 2005).

Compatible solutes are accumulated to high, often molar, concentrations in the cytoplasm and therefore represent substantial intracellular stocks of carbon and/or nitrogen, serving as intracellular reserves of nutrients and energy (Welsh, 2000).

There is a large variety of organic osmolytes, found in all domains of life. Some are widely distributed in nature, while others seem to be exclusive of specific groups of organisms. Interestingly, many organisms use a complex mixture of these compounds, for osmotic adaptation. Compatible solutes are generally polar and neutral (either zwitterionic or lacking charges) compounds. The most common compatible solutes of microorganisms include amino acids (glutamate, glycine, proline) and amino acid derivatives, sugars (trehalose, sucrose), sugar derivatives, and polyols (glycerol, inositol, mannitol, arabitol, sorbitol), betaines and ectoines.

Ectoine and hydroxyectoine are examples of compatible solutes found only in mesophilic bacteria (Santos and da Costa, 2002; Roberts, 2005). Sucrose is rarely synthesized by bacteria, with the exception of cyanobacteria, where it is synthesized in response to osmotic stress;

however, this sugar is the main osmoprotector in plants (Lunn, 2002; Lunn and MacRae, 2003). Polyols are commonly found in halotolerant/halophilic fungi, yeasts, algae and plants, being rare in prokaryotes (da Costa *et al.*, 1998). Few exceptions are known, including the bacteria *Zymomonas mobilis* and *Pseudomonas putida*, which accumulate sorbitol and mannitol, respectively (Loos *et al.*, 1994; Kets *et al.*, 1996). Among polyols, glucosylglycerol is a compatible solute primarily accumulated in cyanobacteria, like *Synechocystis* sp., in response to salt stress (Mikkat *et al.*, 1996; Roberts, 2005).

Negatively charged organic solutes like di-*myo*-inositol-phosphate (DIP), diglycerol phosphate (DGP), mannosylglycerate (MG), and its rare derivative mannosylglyceramide (MGA), have been identified in hyper/thermophilic bacteria and archaea and are almost exclusively restricted to them (Santos and da Costa, 2002; Santos *et al.*, 2007).

Di-*myo*-inositol-phosphate was the principal intracellular solute, accumulated in response to heat-stress, in *Thermococcus litoralis*, *Pyrococcus furiosus* and *Pyrococcus horikoshii* (Martins and Santos, 1995; Empadinhas *et al.*, 2001). In *Archaeoglobus fulgidus* DIP was accumulated in response to salinity and, more recently, this negatively charged solute was detected in the thermophilic bacterium *Rubrobacter xylanophilus*, where its concentration increased with rising growth temperature (Gonçalves *et al.*, 2003; Empadinhas *et al.*, 2007). Borges and co-workes (2010) verified that during the adaptation to thermal stress of a DIP-negative mutant of *Thermococcus kodakarensis*, DIP was replaced by aspartate. It seems that both negatively charged solutes (DIP and aspartate) may serve as K^+ counterions and may act as protectors of proteins against thermal denaturation (Neves *et al.*, 2005).

The compatible solute DGP is a singular solute only found in some strains of the genus *Archaeoglobus*, being accumulated in response to osmotic and thermal stresses (Lamosa *et al.*, 2000; Gonçalves *et al.*, 2003; Santos *et al.*, 2007). Lamosa and co-workers (2003) confirmed the protection granted by this molecule on *Desulfovibrio gigas* rubredoxin against heat denaturation *in vitro*.

The rare derivative of MG, mannosylglyceramide (MGA), has been found only in the thermophilic bacterium *Rhodothermus marinus*, where it was preferentially accumulated in response to salt stress. This bacterium also accumulates MG, not only due to NaCl

concentration, but also as a response to supra-optimal temperatures (Silva *et al.*, 1999; Empadinhas and da Costa, 2006).

Mannosylglucosylglycerate (MGG) is a rare solute identified in two thermophilic species of the order Thermotogales. In *Petrotoga miotherma*, MGG appears to participate in the adaptation of the organisms to sub-optimal growth salinities only, since its concentrations decreased during growth at supra-optimal salinities and temperatures, when proline and α-glutamate became the dominant compatible solutes (Jorge *et al.*, 2007). Curiously, in *Petrotoga mobilis*, MGG was detected at supra-optimal salinities and growth temperatures (Fernandes *et al.*, 2010).

Glucosylglucosylglycerate (GGG) was detected in *Mycobacterium smegmatis* where it was suspected to be one of the intermediates in the synthesis of the methylglucose lipopolysaccharide (MGLP) (Kamisango *et al.*, 1987). This MGLP, isolated from several *Nocardia* and *Mycobacterium* species, appears to modulate fatty acid synthesis and act as their carrier to the cell wall assembly. However, physiological evidence for this assumption remains to be experimentally confirmed (Mendes, 2011). More recently, Santos and co-workers (2007) found GGG in the phylogenetically unrelated bacterium *Persephonella marina*, which also accumulates GG. Both molecules, GG and its rare derivative GGG, are accumulated under osmotic stress (Santos *et al.*, 2007).

Recently, Yancey and co-workers (2002) found unusual osmolytes in deep-sea animals, such as trimethylamine *N*-oxide (TMAO) and scyllo-inositol. Free amino acids, polyols and betaine were also detected. Some of these compounds are probably involved in counterbalancing inhibitory effects of high hydrostatic pressure. Sulfur-based osmolytes, such as hypotaurine and thiotaurine, were found in vent and seep animals and seem more likely to protect cells as antioxidants (Yancey *et al.*, 2002). Another sulfur-based osmolyte, sulfotrehalose, was found to be the major organic solute accumulated by the halophilic archaea, *Natronococcus* sp. and several *Natronobacterium* species, for osmotic balance (Desmarais *et al.*, 1997).

2.1. Trehalose

Trehalose is a chemically inert natural sugar, formed by two glucose molecules bound via an α,α-1,1-glycosidic linkage (Fig. 1.1).

It is widely distributed, being encountered in eukaryotic (yeast, fungi, nematodes, insects, shrimps and plants) and prokaryotic organisms of the domains Bacteria and Archaea (Elbein *et al.*, 2003).

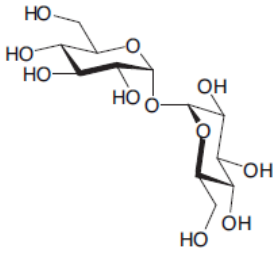

Figure 1.1. Struture of trehalose.

Different functions of trehalose may be associated with different organisms, but it is also possible to observe that trehalose has more than one function in the same organism.

Trehalose can protect proteins and cellular membranes from a variety of extreme environmental conditions, including desiccation, dehydration, heat or cold (Crowe *et al.*, 1998; Benaroudj *et al.*, 2001; Elbein *et al.*, 2003; Jain and Roy, 2009).

In plants, trehalose may control the growth and inflorescence blanching, and in yeasts, trehalose can function as a signaling or regulatory molecule (Elbein *et al.*, 2003; Rolland *et al.*, 2006; Satoh-Nagasawa *et al.*, 2006; Ramon *et al.*, 2007); trehalose is also known to play an important role in plant-microorganism interactions, being present in endomycorrhizae and ectomycorrhizae and in legume nodules (Iturriaga *et al.*, 2009).

In insects and fungi, trehalose serves as a source of energy or carbon, during flight (insects) or during germination of spores (fungi) (Elbein *et al.*, 2003).

In prokaryotes, trehalose is thought to serve as a source of carbon, being a structural component, or as a compatible solute in halophiles and cyanobacteria (Arguelles, 2000). Particularly, in mycobacteria, corynebacteria and nocardia, trehalose is an integral component of various glycolipids that are important cell wall structures (Daffe and Draper, 1998; Ryll *et al.*, 2001; Elbein *et al.*, 2003).

Trehalose is a molecule with several important applications in different fields, such as food, pharmaceutical and cosmetic industry. It is widely used as an alternative to sugar in industrial uses (sweetener, food stabilizer and as food additive). Trehalose protects against free radicals and UV radiation and its anti-aging effects makes this disaccharide a promising compound for cosmetics and for the pharmaceutical industry (Iturriaga *et al.*, 2009).

In medicine, trehalose is involved, for example, in the prevention of osteoporosis, it contributes to alleviate polyglutamine and polyalanine diseases, such as Huntington's disease, and it also inhibits the aggregation of the peptide β-amyloid and reduces its cytotoxicity, in Alzheimer's disease (Nishizaki *et al.*, 2000; Tanaka *et al.*, 2004; Liu and Ames, 2005). Trehalose protects fibroblasts and corneal epithelial cells in culture from death by desiccation (Eroglu *et al.*, 2000; Guo *et al.*, 2000; Matsuo, 2001). Furthermore, it has been shown that trehalose has many biotechnological applications. This disaccharide can be used as a stabilizer of thermolabile enzymes such as DNA polymerase, DNA ligase and restriction enzymes (Colaço *et al.*, 1992; Kaushik and Bhat, 2003). It improves microarray experiments and is also involved in the stabilization of biomaterials (Mascellani *et al.*, 2007; Teramoto *et al.*, 2008).

2.2. Mannosylglycerate

Mannosylglycerate (MG) is a carboxylate molecule, constituted by a mannose moiety linked to the C_2 of the glycerate (Fig. 1.2).

This compound is widely distributed in hyper/thermophilic bacteria and archaea, being detected, for example, in the thermophilic bacteria *Rhodothermus marinus*, *Thermus thermophilus* and *Rubrobacter xylanophilus*, in the hyperthermophilic archaea of the genera *Pyrococcus*, *Thermococcus*, *Palaeococcus*, *Aeropyrum*, and in some strains of the genus *Archaeoglobus* (Martins and Santos, 1995;

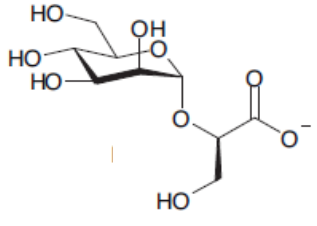

Figure 1.2. Struture of mannosylglycerate.

Nunes *et al.*, 1995; Lamosa *et al.*, 1998; Santos and da Costa, 2002; Neves *et al.*, 2005; Empadinhas *et al.*, 2007).

Low levels of this compound have been primarily encountered in the marine red alga *Polysiphonia fastigiata*. To date it has also been found in several species of the orders Ceramiales, Gelidiales and Gigartinales (Bouveng *et al.*, 1955; Karsten *et al.*, 2007).

MG has an important role in the osmoadaptation and possibly in thermal protection of several hyper/thermophilic bacteria and archaea. The level of MG increases primarily in response to osmotic stress, although in *Rhodothermus marinus* it also increases at supra-optimal growth temperatures, being implicated in the response to thermal stress (Borges *et al.*, 2004). The hyperthermophilic archaeon *Palaeococcus ferrophilus* showed a behavior resembling that observed in *Rhodothermus marinus*, since the accumulation of MG was detected under both salt and thermal stress conditions (Borges *et al.*, 2004; Neves *et al.*, 2005).

In *Rubrobacter xylanophilus*, the accumulation of MG seems to be constitutive, since osmotic stress, thermal stress and the composition of the environment had no effect on MG levels (Empadinhas *et al.*, 2007). The occurrence of MG has not yet been shown in mesophilic organisms, except in the case of red algae, where its role remains unknown (Santos and da Costa, 2002; Empadinhas and da Costa, 2008a, 2008b).

2.3. Glucosylglycerate

Glucosylglycerate (GG) is, like MG, a carboxylate solute, where mannose is replaced by glucose (Fig.1.3).

To date, the accumulation of GG has been restricted to several species of mesophilic archaea and bacteria. This compound was initially identified in the marine cyanobacterium *Agmenellum quadruplicatum* strain PCC7002, grown under nitrogen-limiting conditions. It has recently been shown that GG acts

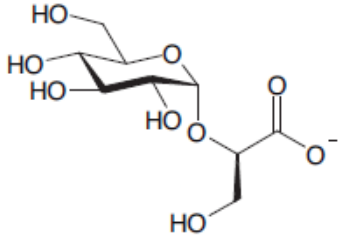

Figure 1.3. Struture of glucosylglycerate.

as a compatible solute, since this sugar-glycerate molecule is accumulated by the γ-proteobacterium *Erwinia chrysanthemi* grown under nitrogen starvation combined with osmotic stress (Kollman *et al.*, 1979; Goude *et al.*, 2004).

The thermophilic bacterium *Persephonella marina* also accumulates GG as a compatible solute since its concentration increases under salt stress (Costa *et al.*, 2007). Recently, it has been demonstrated that GG accumulates in *Streptomyces caelestis* and this bacterium is also able to excrete it into the growth medium (Pospisil *et al.*, 2007). In *Mycobacterium* sp., GG was found to be incorporated into the reducing end of a methylglucose lipopolysaccharide (MGLP) (Kamisango *et al.*, 1987). This MGLP seems to be unique to some members of the order Actinomycetales (Hunter *et al.*, 1979). GG was also identified in the polar head of a glycolipid in *Nocardia otitidiscaviarum* (Pommier and Michel, 1981; Tuffal *et al.*, 1998). This organism, which also synthesizes a MGLP, is phylogenetically related to mycobacteria (Pommier and Michel, 1986). However, the physiological role of the GG-containing glycolipid remains elusive.

3. BIOSYNTHESIS OF TREHALOSE, MANNOSYLGLYCERATE AND GLUCOSYLGLYCERATE

3.1. Biosynthetic pathways for trehalose

To date, five different biosynthetic pathways for trehalose have been described: the Tps/Tpp, the TreS, the TreY/TreZ, the TreT and the TreP pathway (Avonce *et al.*, 2006) (Fig. 1.4).

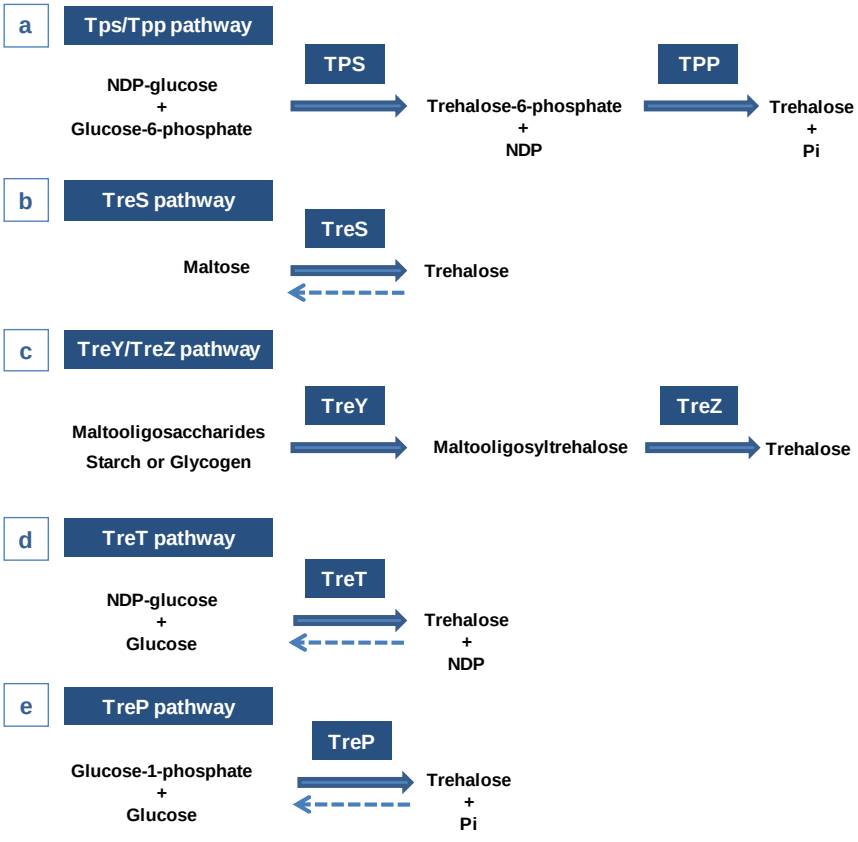

Figure 1.4. Pathways for trehalose biosynthesis present in a) archaea, bacteria, fungi, plants and arthropods; b) bacteria; c) archaea and bacteria; d) archaea and bacteria; e) bacteria and fungi. Abbreviations: Tps, trehalose-6-phosphate synthase; Tpp, trehalose-6-phosphate phosphatase; NDP, nucleoside diphosphate; Pi, free phosphate; TreS, trehalose synthase; TreY, maltooligosyltrehalose synthase; TreZ, maltooligosyltrehalose trehalohydrolase; TreT, glycosyltransferring synthase; TreP, trehalose phosphorylase.

3.1.1. Tps/Tpp pathway

The first pathway for trehalose synthesis, discovered 53 years ago (Cabib and Leloir, 1958), is the best known and most widely distributed (archaea, eubacteria, fungi, invertebrates, insects, and plants). It involves a trehalose-6-phosphate synthase (Tps) and a trehalose-6-phosphate phosphatase (Tpp). The Tps catalyzes the transfer of glucose from NDP-glucose to glucose-6-phosphate forming trehalose-6-phosphate (T6P) and NDP, while the Tpp dephosphorylates T6P, forming trehalose and inorganic phosphate. In *Escherichia coli*, Tps and Tpp enzymes are

encoded by *otsA* and *otsB* genes (osmoregulatory trehalose synthesis); their deletion originated osmotically sensitive mutants (Giaever *et al.*, 1988). The Tps/Tpp pathway is one of the pathways present in every prokaryotic strain known to date that possess multiple trehalose biosynthetic pathways (Freeman *et al.*, 2010). This pathway is the sole pathway in *Escherichia coli*, *Salmonella enterica* and in plants (Fernandez *et al.*, 2010). In the yeast *Saccharomyces cerevisiae*, trehalose is synthesized by an enzyme complex that is composed of four subunits: TPS1, TPS2, TSL1 and TPS3. The TPS1/TPS2 enzyme complex carries the catalytic activity for trehalose synthesis (encodes Tps and Tpp enzymes), while TSL1 and TPS3 may act in the regulation or structural stabilization of the TPS1/TPS2 complex (Reinders *et al.*, 1997). The existence of a multienzyme complex is not yet understood, but it may be related with a regulatory function on trehalose metabolism and/or on the interaction between trehalose metabolism, glycolysis and fermentation (Bell *et al.*, 1998; Noubhani *et al.*, 2000).

3.1.2. TreS pathway

The second biosynthetic pathway involves a trehalose synthase (TreS), encoded by the *treS* gene, which catalyses the intramolecular rearrangement of maltose, converting the α(1-4)-linkage into a α(1-1)- linkage of trehalose (Elbein *et al.*, 2003). This enzyme was first reported in *Pimelobacter* sp. (Nishimoto *et al.*, 1996) and has since been identified in several bacteria, namely, *Corynebacterium glutamicum*, *Mycobacterium smegmatis*, *Pseudomonas stutzeri*, *Propionibacterium freudenreichii*, *Arthrobacter aurescens* and in thermophilic archaea and bacteria (Tsusaki *et al.*, 1997; Koh *et al.*, 2003; Wolf *et al.*, 2003; Pan *et al.*, 2004; Lee *et al.*, 2005; Chen *et al.*, 2006; Cardoso *et al.*, 2007; Wu *et al.*, 2009). Recently, the TreS from *Mycobacterium smegmatis* has been found to convert glycogen into trehalose, via maltose (Pan *et al.*, 2008). The TreS enzyme also catalyzes the reverse reaction, i.e., trehalose to maltose, indicating that TreS could also be responsible for trehalose degradation, instead of trehalose synthesis (Koh *et al.*, 2003; Wolf *et al.*, 2003; Cardoso *et al.*, 2007).

3.1.3. TreY/ TreZ pathway

The third pathway for trehalose synthesis (TreY/TreZ) involves the conversion of maltooligosaccharides and starch into trehalose. This pathway has been elucidated in the hyperthermophilic crenarchaeon *Sulfolobus acidocaldarius* (Nakada *et al.*, 1996) and in the bacteria *Arthrobacter* sp., *Rhizobium* sp., *Brevibacterium helvolum*, *Mycobacterium tuberculosis* and, more recently, in *Corynebacterium glutamicum* and *Deinococcus radiodurans* (Maruta *et al.*, 1996a, 1996b; De Smet *et al.*, 2000; Kim *et al.*, 2000; Wolf *et al.*, 2003; Timmins *et al.*, 2005). These organisms synthesize trehalose in two enzymatic steps catalyzed by a maltooligosyltrehalose synthase (TreY), encoded by the *treY* gene, and a maltooligosyltrehalose trehalohydrolase (TreZ), encoded by the *treZ* gene. The first enzyme, TreY, rearranges the glucose moiety at the reducing end of a maltooligosaccharide or a glycogen chain by converting the α(1-4)- to an α(1-1)- linkage. After this step, the TreZ hydrolyzes the products of the TreY-catalyzed reaction (maltooligosyltrehalose) to produce free trehalose.

3.1.4. TreT pathway

A recently discovered biosynthetic pathway for trehalose was found in the hyperthermophilic archaea *Thermococcus litoralis*, *Pyrococcus horikoshii*, and *Thermoproteus tenax* (Qu *et al.*, 2004; Ryu *et al.*, 2005; Kouril *et al.*, 2008). Unlike the widely distributed Tps/Tpp pathway, the newly identified enzyme, trehalose glycosyltransferring synthase, designated as TreT, synthesizes trehalose from NDP-glucose and glucose, rather than from glucose-6-phosphate. This enzyme transfers the glucose moiety from NDP-glucose and joins it at position 1 of another glucose molecule to form trehalose, in one single-step. However, this enzyme can also catalyze the reverse reaction. In *T. litoralis*, the TreT enzyme is able to form ADP-glucose plus glucose from trehalose and ADP and in *P. horikoshii* trehalose is partially hydrolyzed (Qu *et al.*, 2004; Ryu *et al.*, 2005). Interestingly, the *T. tenax* TreT was showed to be unidirectional and active only in the reaction of trehalose synthesis. This enzyme did not catalyze trehalose degradation, being different from the other reversible TreTs (Kouril *et al.*, 2008). The recently

determined crystal structure of TreT from *P. horikoshii*, revealed that the conservation of essential residues and the high overall structural similarity of the N-terminal domain to that of Tps imply that the catalytic reaction of TreT, for trehalose synthesis, would follow an analogous mechanism to that of Tps (Woo *et al.*, 2010).

3.1.5. TreP pathway

Some authors also consider a fifth pathway for trehalose synthesis, which is restricted to fungi and a few bacteria, involving a trehalose phosphorylase (TreP) (Wannet *et al.*, 1998; Han *et al.*, 2003; Ren *et al.*, 2005; Schwarz *et al.*, 2007). In this pathway, trehalose is formed in the presence of glucose-1-phosphate (Glc1P) and glucose. This enzyme can also catalyze the reversible hydrolysis of trehalose, in the presence of inorganic phosphate; transferring a glucose molecule to a phosphate generates glucose-1-phosphate and releases the other glucose residue. There is uncertainty about the participation of TreP enzymes in the synthesis or degradation of trehalose, since the biosynthetic reactions have only been observed *in vitro* (Avonce *et al.*, 2006).

3.2. Genomic organization of trehalose synthesizing genes

There are several distinct genetic organizations related to trehalose synthesizing genes. With respect to *tps* and *tpp* genes, involved in the Tps/Tpp pathway, three different genomic organizations were found in prokaryotes: the *tps* and *tpp* genes are contiguous in an operon-like-structure; *tps* and *tpp* genes are distantly located in the genome; these two genes are fused and form a bifunctional enzyme (Fig. 1.5).

The first type of genetic organization was found in *Escherichia coli*, where *tps* and *tpp* constitute an operon in which *tpp* is proximal to the promoter, and *tps* is distal. The 3' end of the *tpp* coding region overlaps the 5' end of the *tps* coding region by 23 nucleotides (Kaasen *et al.*, 1992; Kaasen *et al.*, 1994). A similar genetic organization, where both genes are co-transcribed and thus, form an operon, is found in the archaeon *Thermoplasma acidophilum*

and in several strains of *Thermus thermophilus* (Alarico *et al.*, 2005). However, the *tps* gene in these organisms is proximal to the promoter, instead of *tpp*. In *T. thermophilus* RQ-1 three genes coding for two different trehalose biosynthetic pathways were found, namely *tps*, *tpp* and *treS*. These genes are organized in a cluster, with *tpp* immediately downstream *tps* and *treS* downstream *tpp* (Fig. 1.5). Although these genes are structurally linked, it was impossible to conclude that they were organized in an operon, since no obvious promoter could be identified, and transcriptional analysis was not performed (Silva *et al.*, 2003).

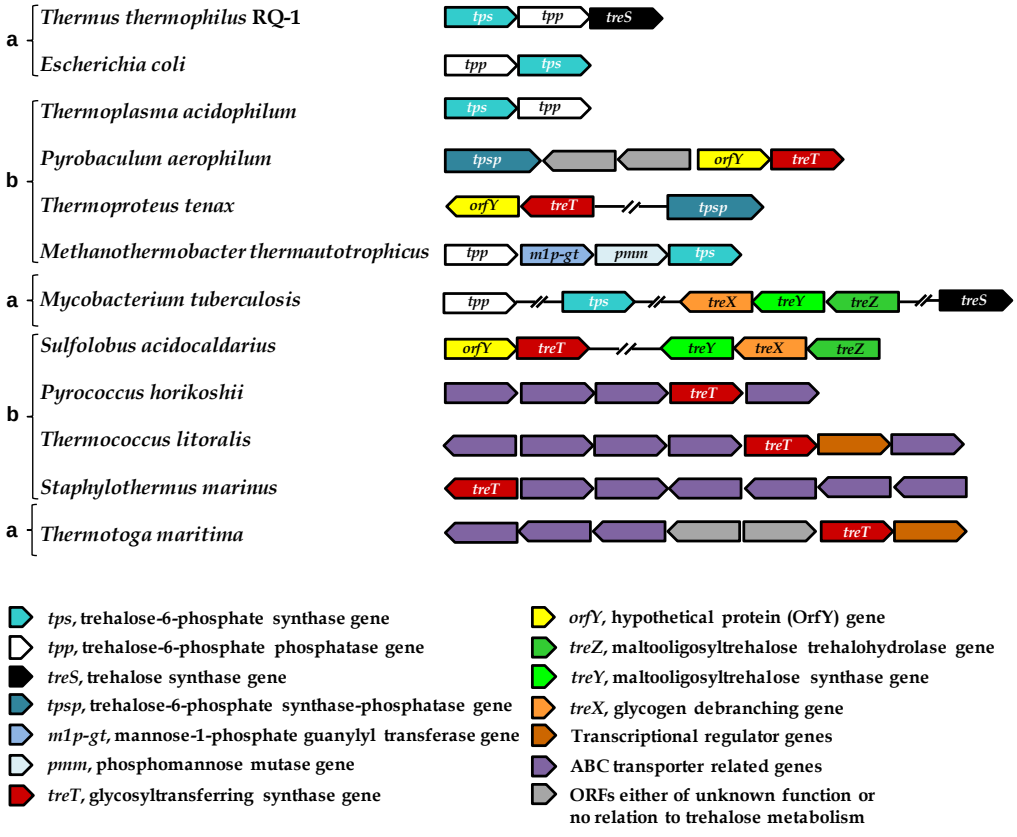

Figure 1.5. Organization of genes leading to the synthesis of trehalose in different organisms: a) bacteria and b) archaea. Genes, putative genes and their orientation are represented by arrows.

The second type of organization was found, for example, in *Mycobacterium tuberculosis* (De Smet *et al.*, 2000) and in the archaeon *Methanothermobacter thermautotrophicus*. In the latter, *tpp* and *tps* genes have two other genes in between them: one coding for a mannose-1-

phosphate guanylyl transferase and the other for a phosphomannose mutase, both putatively implicated in the synthesis of mannosylglycerate (Martins *et al.*, 1999) (Fig. 1.5).

The third genetic organization was described in the archaeon *Pyrobaculum aerophilum* and in *Thermoproteus tenax*. The reconstruction of the trehalose metabolism of *T. tenax* revealed the presence of the Tps/Tpp pathway (Siebers *et al.*, 2004), although only one gene (*tpsp*) was identified. This gene codes for a trehalose-6-phosphate synthase/phosphatase (TPSP), a fusion protein with a Tps domain in the N-terminal and a Tpp domain in the C-terminal (Zaparty, 2007). A similar two-domain structure had already been described for TPSP from plants, *Arabidopsis thaliana* and *Selaginella lepidophylla* (Blazquez *et al.*, 1998; Zentella *et al.*, 1999), and for the yeast *Saccharomyces cerevisiae* (Kaasen *et al.*, 1994).

Several mesophilic bacteria, belonging to the genera *Arthrobacter*, *Brevibacterium* or *Micrococcus*, as well as thermophilic archaea (members of the Sulfolobales order), synthesize trehalose from starch via the TreY/TreZ pathway (Kobayashi *et al.*, 1996; Di Lernia *et al.*, 1998; Gueguen *et al.*, 2001). In these organisms, the *treY* and *treZ* genes are contiguous and under the same promoter. In the thermophilic archaeon *Sulfolobus acidocaldarius* and in the bacterium *Mycobacterium tuberculosis*, another gene, *treX* (coding for a glycogen debranching enzyme), is incorporated in the operon structure involving *treY* and *treZ* genes (Maruta *et al.*, 1996c; De Smet *et al.*, 2000) (Fig. 1.5).

Three different situations relative to the *treT* genetic context were found in bacteria and archaea: organisms with a conserved organization of *treT* and *orfY* (a gene of unknown function) genes, organisms with a *treT* gene in a cluster comprising genes coding for a putative ABC transporter proteins and organisms which harbor the *treT* gene and genes coding for enzymes involved in alternative trehalose pathways (Kouril *et al.*, 2008) (Fig. 1.5).

The possible existence of a unidirectional TreT pathway in many Crenarchaeota, such as in members of the orders Thermoproteales and Sulfolobales, in the euryarchaeon *Methanosaeta thermophila* and, unexpectedly, in a few proteobacteria (*Syntrophus aciditrophicus*, *Syntrophobacter fumaroxidans*, *Pelobacter carbinolicus*), is supported by the conserved clustering of the *treT* homologs genes and the gene coding for OrfY (Kouril *et al.*, 2008) (Fig.

1.5). The organisms of this group also have genes involved in other trehalose pathways in their genomes, like Tps/Tpp or TreY/TreZ (Kouril *et al.*, 2008) (Fig. 1.5).

In contrast to the unidirectional catalysis of *Thermoproteus tenax* TreT enzyme, the previously studied reversible TreTs of the euryarchaeota *Thermococcus litoralis* and *Pyrococcus horikoshii* are part of a gene cluster harboring maltose/trehalose ABC transporter genes (Qu *et al.*, 2004; Ryu *et al.*, 2005) (Fig. 1.5).

In the crenarchaeotes *Staphylothermus marinus* and *Thermofilum pendens* and in some members of the order Thermotogales, the same genetic organization was observed, i.e., the TreT homolog is part of a predicted ABC transporter, suggesting reversible TreT activity in these archaea and bacteria. No genes encoding enzymes involved in alternative pathways for trehalose metabolism were identified in these organisms (Kouril *et al.*, 2008) (Fig. 1.5).

In respect to the *treT* gene, another genetic organization was found in the euryarchaeon *Methanoculleus marisnigri*, where three paralogous sequences were identified, and none of these *treT* homolog genes show clustering with genes coding for OrfY or ABC transporter proteins (Kouril *et al.*, 2008).

3.3. Trehalose hydrolysis or turnover

An enzyme that hydrolyses trehalose was first observed in *Aspergillus niger* (Bourquelot, 1893) and then in *Saccharomyces cerevesiae*, by Fischer, in 1895. Since then, the enzyme trehalase (α,α-trehalose-1-C-glucohydrolase) has been reported in several other organisms, including plants and animals (Elbein, 1974).

Several pathways for the hydrolysis of this disaccharide have been described in Bacteria and Eukarya domains, but the most common pathway includes trehalases. This enzyme (TreH) breaks down trehalose into two glucoses. This process has been found in all organisms that synthesize trehalose (Richards *et al.*, 2002), even when distinct forms of trehalase coexist, for example in yeasts (Jules *et al.*, 2008).

Interestingly, trehalose has never been found in mammals, even though trehalase has been found in significant amounts in the small intestine of several mammalian species (Richards *et al.*, 2002). The eukaryotic green alga *Euglena gracilis* has an enzyme which catalyses the reversible phosphorolysis of trehalose to glucose-1-phosphate and glucose, via the trehalose phosphorylase (TreP) (Maréchal and Belocopitow, 1972). In *Arabidopsis thaliana*, trehalase is encoded by a single gene, while a large diversity of Tps and Tpp genes exist in the plant's genome (Leyman *et al.*, 2001; Muller *et al.*, 2001; Lunn, 2007; Paul *et al.*, 2008).

In *Escherichia coli*, apart from the cytoplasmic trehalase (TreF), which was shown to be highly homologous to the periplasmic trehalase (TreA), an additional pathway for trehalose hydrolysis was detected, where T6P is converted into glucose and glucose-6-phosphate, by a phosphotrehalase (TreC) (Boos *et al.*, 1987; Gutierrez *et al.*, 1989; Rimmele and Boos, 1994; Horlacher *et al.*, 1996). On the other hand, limited biochemical information is available concerning pathways for trehalose degradation in Archaea, with the exception of the TreTs from *Thermococcus litoralis* and *Pyrococcus horikoshii* (Qu *et al.*, 2004; Ryu *et al.*, 2005).

3.4. Biosynthetic pathways for mannosylglycerate

3.4.1. Mannosylglycerate biosynthesis in bacteria and archaea

Two biosynthetic pathways for the synthesis of mannosylglycerate have been elucidated. In the single-step pathway, a mannosylglycerate synthase (MgS), encoded by the *mgS* gene, catalizes the transfer of the mannosyl moiety of GDP-mannose to D-glycerate, yielding mannosylglycerate (Fig. 1.6). This pathway has only been described in the thermophilic bacterium *Rhodothermus marinus* (Martins *et al.*, 1999). The characterization of the new and unique MgS enzyme led to the formation of the glycosyltransferase family 78 (GT78) (www.cazy.org). More recently, the three-dimensional structure of this enzyme was determined (Flint *et al.*, 2005).

In the two-step pathway, MG is synthesized via two sequential reactions, which are catalyzed by a mannosyl-3-phosphoglycerate synthase (MpgS), encoded by the *mpgS* gene, and a

mannosyl-3-phosphoglycerate phosphatase (MpgP), encoded by the *mpgP* gene. In the first reaction, the mannosyl moiety of GDP-mannose is transferred to D-3-phosphoglycerate (3-PGA) to form a phosphorylated intermediate, mannosyl-3-phosphoglycerate (MPG), which is then hydrolyzed to mannosylglycerate, in the second reaction.

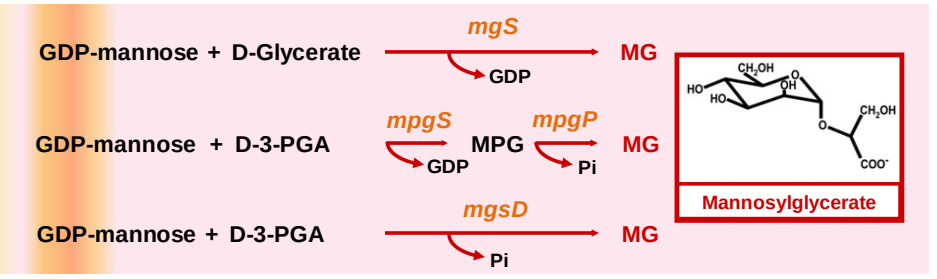

Figure 1.6. Pathways for mannosylglycerate biosynthesis. Abbreviations*: mgS*, mannosylglycerate synthase gene; GDP, guanosine diphosphate; D-3-PGA, D-3-phosphoglycerate; *mpgS*, mannosyl-3-phosphoglycerate synthase gene; *mpgP*, mannosyl-3-phosphoglycerate phosphatase gene; MPG, mannosyl-3-phosphoglycerate; Pi, free phosphate; *mgsD,* bifunctional mannosylglycerate synthase gene.

This two-step pathway was first detected in *R. marinus* by Martins and co-workers (1999) and later characterized by Borges and co-workers (2004). The use of two different acceptor molecules (D-glycerate and 3-PGA) combined with the differences in salt dependency (the reaction catalysed by MgS was salt-independent; while NaCl or KCl salts were required for full activity of the MpgS/MpgP system), led Martins and co-workers (1999) to suspect that two different pathways for MG synthesis were present in *R. marinus*. This thermophilic bacterium is, so far, the only known organism with two pathways for the synthesis of MG.

The two-step pathway for MG synthesis has been found in organisms known to accumulate MG, such as *Rhodothermus marinus*, most *Thermus thermophilus* strains, *Palaeococcus ferrophilus*, *Thermococcus litoralis*, *Aeropyrum pernix* and *Staphylothermus marinus* (Empadinhas *et al.*, 2001; Quaiser *et al.*, 2002; Empadinhas *et al.*, 2003; Neves *et al.*, 2005).

Rubrobacter xylanophilus is the only actinobacterium known to accumulate MG (Empadinhas *et al.*, 2007). The metabolic pathway for the synthesis of this molecule in this bacterium was recently studied by Empadinhas and co-workers (2011) and involves a highly divergent mannosyl-3-phosphoglycerate synthase (MpgS) without relevant sequence homology to

known mannosylphosphoglycerate synthases. This unique MpgS has an unparalleled dual MpgS and glucosyl-3-phosphoglycerate synthase (GpgS) activity, since it is capable of synthesizing mannosyl-3-phosphoglycerate (MPG) or glucosyl-3-phosphoglycerate (GPG), the precursors of MG and GG. The determination of MpgS crystal structure showed that the binding-site of this enzyme is modified to specifically select GDP-containing ligands, while the sugar-binding region is suited for accommodation of both glucose and mannose (Empadinhas *et al.*, 2011). The biochemical and kinetic properties and the crystallization studies of this enzyme led Empadinhas and co-workers (2011) to include it in the GT81 family of glycosyltransferases, which also includes GpgSs from mycobacteria (Empadinhas *et al.*, 2008; Empadinhas and da Costa, 2010).

The synthesis of MG or GG, by the *R. xylanophilus* MpgS enzyme, was observed in cells extracts, where unknown phosphatases desphosphorylated the intermediates MPG and GPG (Empadinhas *et al.*, 2011).

Another pathway for the synthesis of MG was found in the mesophilic bacterium *Dehalococcoides ethenogenes*. This gene comprises an MpgS and an MpgP in a single polypeptide. The expression of this gene (designated as *mgsD*), both in *Escherichia coli* and in the yeast *Saccharomyces cerevisiae*, showed that the recombinant enzyme, a bifunctional mannosylglycerate synthase (MgsD), synthesized MG *in vitro* and *in vivo*, arguing in favor of a similar function in the native organism (Empadinhas *et al.*, 2004). In *Dehalococcoides ethenogenes* MG seems to function as a true compatible solute in response to osmotic stress (Empadinhas and da Costa, 2010).

3.4.2. Mannosylglycerate genes in eukaryotes

The presence of two amino acid sequences identified in the red alga *Griffithsia japonica* (GenPep accession numbers AAM93991 and AAP80838), with high homology with the MgS protein of *Rhodothermus marinus*, suggests that the MG single-step pathway could also be present in organisms from the domain Eukarya (Borges *et al.*, 2004).

Recently, a gene coding for the MgS enzyme was found in the red alga *Caloglossa leprieurii* (Santos *et al.*, 2007) and an Expressed Sequence Tag (EST) was also identified in the red alga *Gracilaria changii* (Teo *et al.*, 2007) The acquisition of MG biosynthethic genes by organisms belonging to the order Rhodophyta possibly reflects endosymbiosis of a prokaryote carrying an *mgS* or *mpgS* gene (Martin *et al.*, 2003).

Fungal genomes of *Magnaporthe grisea*, *Neurospora crassa* and *Chaetomium globosum* revealed *mpgS*-like gene sequences (Empadinhas, 2004; Empadinhas and da Costa, 2008b).

The *mpgS* gene from *Magnaporthe grisea* was cloned and expressed in *Saccharomyces cerevisiae* and the MG accumulation by the transformed yeast was observed, which proved that the MpgS enzyme was functional (Empadinhas, 2004). Interestingly, the phosphatase gene usually associated with the *mpgS*, was not present in these fungi and the role of the phosphorylated compound MPG is, so far, unknown.

So far, MgS homologues have not been detected in the numerous prokaryotic genomes available. Interestingly, the genomes of two plants, the moss *Physcomitrella patens* and of the spikemoss *Selaginella moellendorffii* include full-length MgS homologues (Empadinhas and da Costa, 2010). It is probable that these organisms accumulate MG and that MG genetic tools could be inherited from algae from the order Rhodophyta (Stiller and Hall, 1997). Some red algae are known to accumulate MG and this accumulation also possibly occurs in mosses or plants, but its biological role, remains an enigma as well as the evolutionary scenario of this molecule in the domain Eukarya.

3.5. Genomic organization of mannosylglycerate synthesizing genes

The synthesis of MG proceeds via two different pathways in *Rhodothermus marinus*, while in *Thermus thermophilus* MG synthesis totally relies on the two-step pathway (Martins *et al.*, 1999; Empadinhas *et al.*, 2003; Borges *et al.*, 2004) (Fig. 1.7). In these thermophilic organisms, *mpgS* and *mpgP* genes are consecutive and under the same promoter. Remarkably, *T. thermophilus* strain CC-16 is an exception among other *T. thermophilus* strains and it can be considered a “naturally occurring variant” for the synthesis of MG, since *mpgS* and *mpgP* (or

mgS) genes are absent in its genome. However, strain CC-16 possesses genes involved in two pathways for the synthesis of trehalose (*tps, tpp and treS* genes), which allows it to grow in a medium containing up to 1.0% NaCl (Alarico *et al.*, 2005). The same type of organization is found for the homologous genes of the crenarcheote *Aeropyrum pernix*, although in *Staphylothermus marinus*, other hyperthermophilic crenarchaeote, the *mpgS* and *mpgP* are consecutive, but in opposite directions, under the control of individual promoters (Empadinhas *et al.*, 2003).

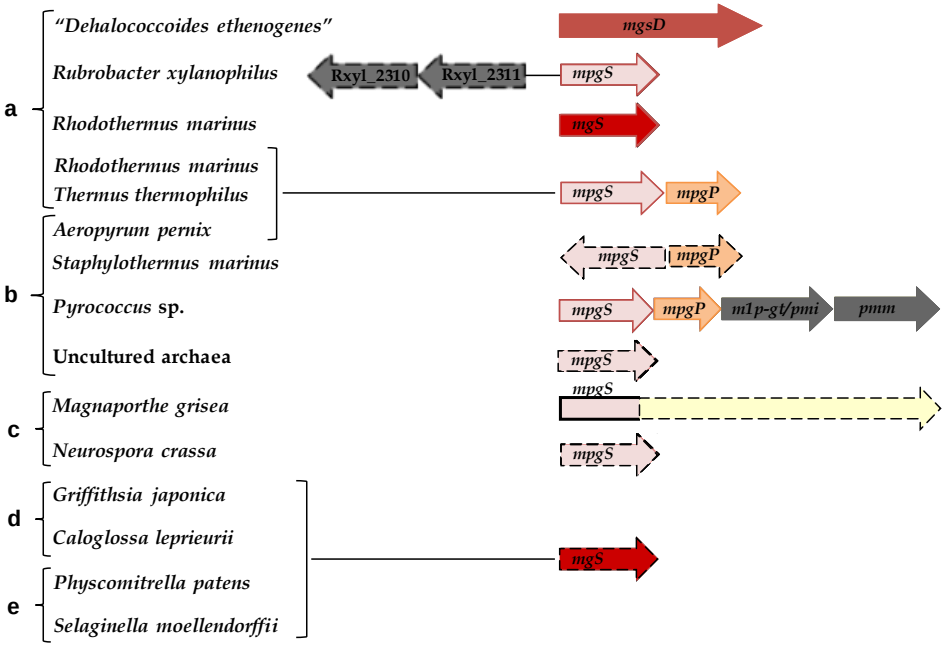

Figure 1.7. Organization of genes leading to the synthesis of MG in different organisms: a) bacteria; b) archaea; c) fungi; d) algae and e) plants. Pink, red and orange arrows represent genes committed to MG synthesis and their directions. Light grey arrows represent genes coding for enzymes involved in the production of MG precursors and grey arrows represent putative genes for a hydrolase (Rxyl_2310) and a 5′-nucleotidase (Rxyl_2311). The dashed arrows represent putative genes. Abbreviations: *mgS*, mannosylglycerate synthase gene; *mpgS*, mannosyl-3-phosphoglycerate synthase gene; *mpgP*, mannosyl-3-phosphoglycerate phosphatase gene; *m1p-gt/pmi*, bifunctional mannose 1-phosphate guanylyltransferase/phosphomannose isomerase gene; *pmm*, phosphomannose mutase gene; *mgsD*, bifunctional mannosylglycerate synthase gene.

In *Pyrococcus* sp. and in species of the order Thermococcales, *mpgS* and *mpgP* genes are sequentially arranged in the genome and organized in an operon-like structure that also

comprises two genes coding for enzymes involved in the synthesis of the precursor for MG enzyme, GDP-mannose (Empadinhas *et al.*, 2001; Neves *et al.*, 2005).

In the bacterium *Dehalococcoide ethenogenes*, *mpgS* and *mpgP* genes are fused and code for a bifunctional enzyme (Empadinhas *et al.*, 2004) (Fig. 1.7). This fused gene was not found in the closely related organism *Dehalogenimonas lykanthroporepellens* (Moe *et al.*, 2009). This fusion event between genes encoding enzymes that catalyze consecutive steps in a specific pathway is not unprecedented. Cyanobacteria also possess a unique gene for sucrose-phosphate synthase and sucrose-phosphate phosphatase (SPS/SPP) enzymes, implicated in sucrose synthesis (Lunn, 2002; Lunn and MacRae, 2003).

In mesophilic eukaryotes such as fungi *Magnaporthe grisea* and *Neurospora crassa*, a single *mpgS*-like gene was also identified (Empadinhas and da Costa, 2008b). In *M. grisea*, the *mpgS*-like sequence corresponds to the first of ten exons, encoding a 1718 amino acid protein, while in *N. crassa* it was a sole intronless gene. A typical phosphatase gene is absent near the putative *mpgS* gene (Fig. 1.7).

In the single-step pathway for MG synthesis in *Rhodothermus marinus*, the gene coding for the mannosylglycerate synthase (Rmar_1220) was isolated in the genome, not close to the other MG genes, *mpgS* and *mpgp* (Martins *et al.*, 1999; Borges *et al.*, 2004) (Fig. 1.7).

A single *mgS*-like gene was also found in the red algae *Griffithsia japonica* and *Caloglossa leprieurii* (Karsten *et al.*, 2003; Santos *et al.*, 2007). Recently, inspection of public genomic databases resulted in the identification of *mgS*-like genes in the plants *Physcomitrella patens* and *Selaginella moellendorffii*, suggesting the presence of the single-step pathway for MG synthesis in these early land plants (Empadinhas and da Costa, 2008b, 2010) (Fig. 1.7).

3.6. Biosynthetic pathways for glucosylglycerate

Two pathways for the synthesis of GG have been identified, as reported for MG. The first biosynthetic pathway (two-step reactions) was elucidated in the psychrotolerant archeon *Methanococcoides burtonii*, where the accumulation of GG remains to be demonstrated

(Costa *et al.*, 2006) and in the thermophilic bacterium *Persephonella marina* (Costa *et al.*, 2007). In this pathway the synthesis of GG involves two enzymes, a glucosyl-3-phosphoglycerate synthase (GpgS) and a glucosyl-3-phosphoglycerate phosphatase (GpgP), and a phosphorylated intermediate. The first enzyme, encoded by the *gpgS* gene, converts NDP-glucose and D-3-phosphoglycerate (3-PGA) into glucosyl-3-phosphoglycerate (GPG) and the second enzyme, encoded by the *gpgP* gene, desphosphorylates the intermediate, yielding free GG (Fig. 1.8).

An additional pathway for GG synthesis was later found in *Persephonella marina* (Fernandes *et al.*, 2007). In this pathway, NDP-glucose is condensed with D-glycerate to directly produce GG in a single glucosyl-transfer reaction, without the formation of the intermediate compound GPG. This reaction is catalyzed by a glucosylglycerate synthase (GgS), encoded by the *ggS* gene (Fig. 1.8). The thermophilic bacterium *Persephonella marina* is, so far, the only known organism to have two alternative pathways for the synthesis of GG, which probably allows a more flexible regulation of the GG pools by different stimuli.

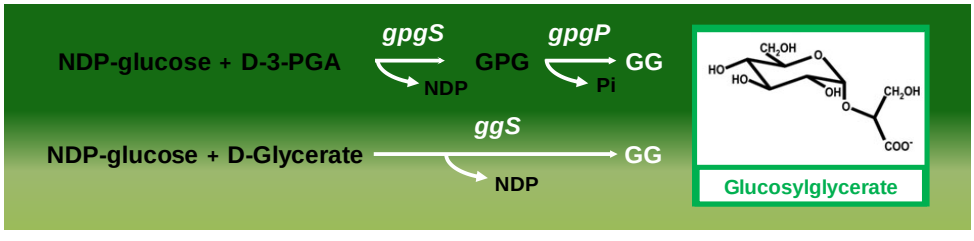

Figure 1.8. Pathways for glucosylglycerate biosynthesis. Abbreviations: *gpgS*, glucosyl-3-phosphoglycerate synthase gene; *gpgP*, glucosyl-3-phosphoglycerate phosphatase gene; GPG, glucosyl-3-phosphoglycerate; NDP, nucleoside diphosphate; Pi, free phosphate; *ggS*, glucosylglycerate synthase.

3.7. Genomic organization of glucosylglycerate synthesizing genes

The genes included in the two known pathways for GG synthesis, described above, are three: *gpgS*, *gpgP* and *ggS*. Different genetic organizations of glucosylglycerate synthesizing genes were identified in bacteria and archaea, which included isolated *gpgS* (without *gpgP*),

consecutive *gpgS* and *gpgP* (operon-like structure) and opposing *gpgS* and *gpgP*, either contiguous or separated (Fig. 1.9).

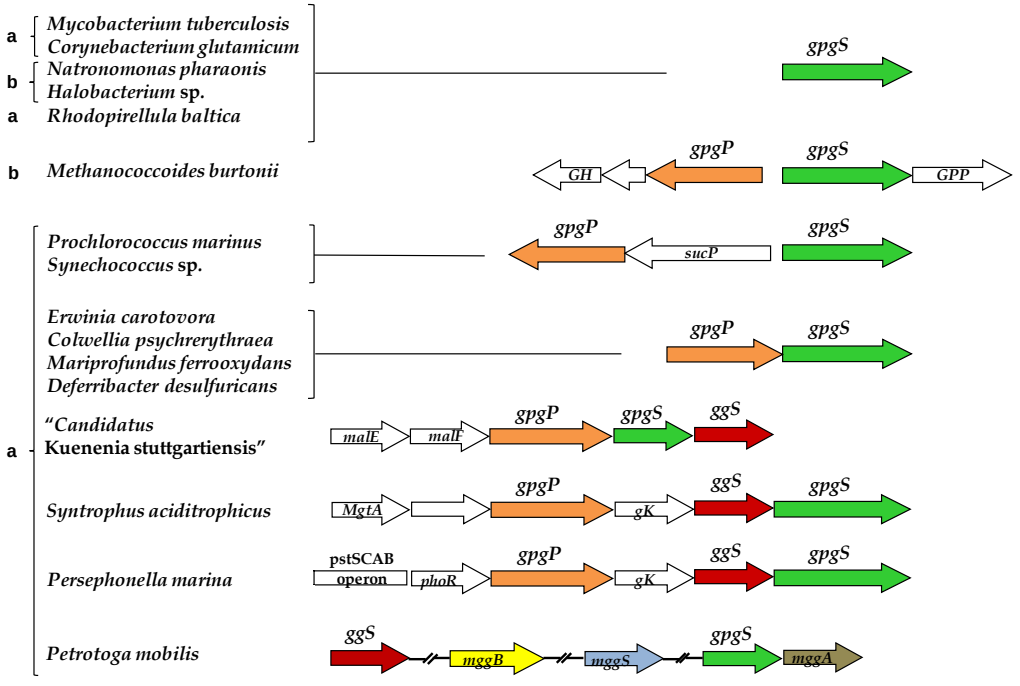

Figure 1.9. Organization of genes leading to the synthesis of GG in different organisms: a) bacteria; and b) archaea. Orange, green and red arrows represent genes committed to GG synthesis and their directions. Abbreviations: *gpgS*, gene coding for a glucosyl-3-phosphoglycerate synthase; *gpgP*, gene coding for a glucosyl-3-phosphoglycerate phosphatase; *ggS*, gene coding for a glucosylglycerate synthase; *GH*, gene coding for a putative glucosyl hydrolase; *GPP*, gene coding for a putative atypical UDP-glucose pyrophosphorylase; *sucP*, gene coding for a putative sucrose phosphorylase; pstSCAB operon, genes coding for a phosphatase uptake system, *phoR*, gene coding for a putative PhoR histidine kinase; *gK*, gene coding for a putative glycerate kinase/dehydrogenase, *MgtA*, gene coding for a cation-transporting ATPase; *malE*, gene coding for a putative trehalose/maltose binding protein, *malF*, gene coding for a putative trehalose/maltose transport protein; *mggB*, gene coding for a mannosylglucosyl-3-phosphoglycerate phosphatase; *mggS*, gene coding for a mannosylglucosylglycerate synthase; *mggA*, gene coding for a mannosylglucosyl-3-phosphoglycerate synthase.

In the actinobacteria *Mycobacterium tuberculosis* and *Corynebacterium glutamicum*, in some halobacteria like *Natronomonas pharaonis* and *Halobacterium salinarum*, and in the planctomycete *Rhodopirellula baltica*, isolated *gpgS* genes have been detected (Fig. 1.9). This single *gpgS*-like gene is the simplest structure detected both in bacteria and archaea and can

be considered the most primitive organization. Since free GG and GGG have been detected in small amounts in *Mycobacterium smegmatis* (Kamisango *et al.*, 1987) and considered to be the putative precursors for methylglucose lipopolysaccharide, it was proposed by Empadinhas and co-workers (2008) that the GPG formed by GpgS could be hydrolyzed into GG by one or more hydrolases or phosphatases.

Contiguous, but divergent oriented *gpgS* and *gpgP* genes were found only in the cold-adapted methanogenic archaeon *Methanococcoides burtonii*. These two genes are under the control of two unidirectional divergent promoters, located between them (Costa *et al.*, 2006) (Fig. 1.9).

Some cyanobacteria possess divergently oriented *gpgS* and *gpgP* genes, separated by a putative sucrose phosphorylase gene (Empadinhas and da Costa, 2010) (Fig. 1.9). In organisms from the phylum Proteobacteria, such as *Erwinia carotovora*, *Colwellia psychrerythraea, Mariprofundus ferrooxydans* and *Deferribacter desulfuricans*, *gpgS* and *gpgP* genes are contiguous and have the same orientation (Fig. 1.9).

In "*Candidatus* Kuenenia stuttgartiensis" *gpgS* and *gpgP* genes are also contiguous and with the same orientation, although belonging to a more complex operon-like genetic organization, which includes three other genes, coding for a trehalose/maltose transport system and for a putative glycosyltransferase (Fig. 1.9). In the deltaproteobacterium *Syntrophus aciditrophicus*, the *gpgS*/*gpgP* genetic structure involves a putative glycosyltransferase gene and a putative glycerate kinase/dehydrogenase gene between them (Fernandes *et al.*, 2007) (Fig. 1.9).

In the thermophilic bacterium *Persephonella marina*, the glucosylglycerate operon–like structure contains genes involved in two different pathways for GG synthesis, *gpgS, gpgP* and *ggS* (Costa *et al.*, 2007; Fernandes *et al.*, 2007). A putative glycerate kinase/dehydrogenase gene and a putative histidine kinase regulator gene (*phoR* - phosphate regulon sensor gene) are also present in this polycistronic operon-like structure (Fig. 1.9). The latter gene is probably implicated in the regulation of the operon for a high-affinity phosphate (Pi)-specific transport system. The *ggS* gene was initially identified as a putative glycosyltransferase, which turned out to catalyze the direct conversion of ADP-glucose and D-glycerate into GG (Fernandes *et al.*, 2007). Glycosylglycerate synthase (GgS) homologues were identified in the genomes of some members of the order Thermotogales, of organisms of the class

Deltaproteobacteria and in hyperthermophilic archaea.

The genome of the *Petrotoga mobilis* contains two genes coding for an "actinobacterial-type" GpgS and an unknown glycosyltransferase. This latter gene proved to be a GgS implicated in the single-step pathway for GG synthesis (Fernandes *et al.*, 2010). The GG formed in this organism is the intermediate in a pathway leading to mannosylglucosylglycerate (MGG), a new solute that was first detected in the slightly halophilic and thermophilic bacterium *Petrotoga miotherma* and that is restricted to *Petrotoga mobilis* and *Rhodopirellula baltica* (Jorge *et al.*, 2007; Fernandes *et al.*, 2010) (Costa *et al.*, unpublished results). In this organism, the *gpgS* gene, normally involved in the synthesis of GPG, seems to be involved in the synthesis of the GG-related solute MGG. The GPG, formed by the combination of UDP-glucose and 3-PGA, is then combined with GDP-mannose into mannosylglucosyl-3-phosphoglycerate (MGPG), which is dephosphorylated to MGG (Fernandes *et al.*, 2010).

The *ggS* gene of *Petrotoga mobilis* is not positioned in an operon-like structure and is not close to the *gpgS* gene, like in *Persephonella marina*, in *Syntrophus aciditrophicus* or in "*Candidatus* Kuenenia stuttgartiensis" (Fig. 1.9).

4. PATHWAY MULTIPLICITY

The implications of pathway multiplicity for the biosynthesis of compatible solutes are not completely understood, but irrefutably reflect one or several important physiological roles for those solutes (De Smet *et al.*, 2000).

The existence of two or more pathways for synthesis of a specific compatible solute in the same organism was first described in 1998, by Helling, who characterized the presence of two parallel pathways for the synthesis of glutamate in *Escherichia coli*. The main determinants of pathway choice in glutamate synthesis were substrate availability and/or modulation of enzyme activity (Helling, 1998).

Pathway multiplicity was also found for trehalose in several bacteria, such as *Mycobacterium tuberculosis*, *Thermus thermophilus* RQ-1, *Rhodobacter sphaeroides*, while in other organisms

trehalose biosynthesis relies on a single pathway, most commonly Tps/Tpp (De Smet *et al.*, 2000; Silva *et al.*, 2003; Makihara *et al.*, 2005).

The abundance and multilayered trehalose metabolism also in organisms belonging to the Archaea domain, point to an essential function of this molecule in physiology. However, so far virtually nothing is known about the regulation and the involvement of the pathways in specific cellular (stress) responses (Kouril *et al.*, 2008).

Two pathways for MG and GG synthesis were found and characterized in thermophilic bacteria *Rhodothermus marinus* and in *Persephonella marina*, respectively (Martins *et al.*, 1999; Borges *et al.*, 2004; Costa *et al.*, 2007; Fernandes *et al.*, 2007). *R. marinus*, as described above, has two alternative pathways for MG synthesis that could be implicated in different metabolic functions, synthesis or catabolism of MG. However, hydrolysis of MG was not observed when the characterization of the MgS enzyme was studied (Martins *et al.*, 1999).

Pathway multiplicity reflects a higher flexibility in the regulation of compatible solute pools upon different environmental stimuli. The existence of two pathways for MG synthesis in *R. marinus* is differently regulated, at the level of expression, to play specific roles in the adaptation to different types of stress conditions. The level of expression of MgS was selectively enhanced by thermal stress, whereas MpgS was up-regulated in response to salt stress (Borges *et al.*, 2004; Empadinhas and da Costa, 2008b).

The presence of two alternative pathways for GG in *Persephonella marina* could also mean that there is greater flexibility in the regulation of GG synthesis in response to stress (Costa *et al.*, 2007; Fernandes *et al.*, 2007). Furthermore, the presence in *P. marina* of an operon coding for a phosphate uptake system, upstream the genes for GG synthesis *gpgP*, *ggS* and *gpgS*, leads to the hypothesis that GgS synthesizes GG when the levels of phosphate are low, since there is no need to synthesize a phosphorylated intermediate in this single–step pathway (Costa *et al.*, 2007).

5. GLYCOSIDE HYDROLASES

Glycoside hydrolases (GH) are an extensive group of enzymes which hydrolyze the glycosidic bond between carbohydrates (two or more), or between a carbohydrate and a non-carbohydrate moiety.

The large diversity of carbohydrate structures is accompanied by an equal diversity of enzymes responsible for their hydrolysis (Warren, 1996; Bourne and Henrissat, 2001; Henrissat and Coutinho, 2001).

Several systems of classification exist for glycoside hydrolases, including those based on substrates or product specificities and stereochemical mechanisms. The simplest classification, expressed in EC numbers, is based on enzyme activity; in other words, it is based on the substrate used and the products formed. Sometimes, it is also based on the type of linkage forming the glycosidic bonds or the molecular mechanism of hydrolysis. This system is a straightforward means to classify glycoside hydrolases. However, the intrinsic problem of that classification is that it does not accommodate enzymes which act on several substrates, such as, for example, highly complex polysaccharides. This classification also fails to reflect the variety of 3D-structural features of these enzymes.

A few years ago, Henrissat (1991) proposed a classification of glycoside hydrolases in families, based on their amino acid sequence (http://afmb.cnrs-mrs.fr/CAZY/index.html).

Over the years, the number of families of glycoside hydrolases has constantly grown. These families can be further classified in “clans” of related families (GH-A to GH-M) in which the catalytic domain fold, the catalytic residues and the catalytic mechanism are conserved.

Glycoside hydrolases are particularly important in cell metabolism as they are either directly involved in the assimilation of glycoside compounds or play regulatory functions in controlling their levels in cells (Cantarel *et al.*, 2009).

6. ORGANISMS USED IN THIS STUDY

6.1. The Genus *Rubrobacter*

The first organisms of the genus *Rubrobacter* were isolated by Yoshinaka and co-workers in 1973 from water samples collected from hot springs, previously irradiated with gamma-radiation. These organisms are resistant to high levels of gamma-radiation, being more resistant than organisms of the genus *Deinococcus* (Asgarani *et al.*, 2000). These are Gram + microorganisms and slightly thermophilic, with an optimum temperature for growth of about 47°C (Yoshinaka *et al.*, 1973).

Initially these bacteria were included in the genus *Arthrobacter*. However, sequence analysis of 16S rRNA gene led to the clarification of their phylogenetic position, being included in a new genus, *Rubrobacter*, and designated as *Rubrobacter radiotolerans* (Suzuki *et al.*, 1988).

Some years later, a new species of the genus *Rubrobacter* - *Rubrobacter xylanophilus* was isolated (Carreto *et al.*, 1996) (Fig. 1.10). This organism was isolated from a hot runoff of a carpet factory, in the United Kingdom, and has an optimum growth temperature of 60°C.

Figure 1.10*Rubrobacter xylanophilus*.

Until 1999, only one strain of each species of *R. radiotolerans* and *R. xylanophilus* was known. Later, several isolates from both species were recovered, after gamma-irradiation of water samples from a hot spring in São Pedro do Sul (Central Portugal) (Ferreira *et al.*, 1999). More recently, another species of this genus, *Rubrobacter taiwanensis*, has been isolated from water (not irradiated) collected in hot springs in Taiwan (Chen *et al.*, 2004).

The three species of the genus *Rubrobacter* that represent the most ancient lineage of the phylum *Actinobacteria* are slightly halotolerant and have an optimal growth temperature range from 45°C to 60°C. All of these organisms are among the most resistant to gamma-radiation; however, the mechanisms involved in this process are still unknown (Chen *et al.*,

2004). Extreme radiation resistance is an inherent characteristic of the three species of the genus *Rubrobacter*, since it is also a feature of bacteria isolated from environments not subject to radiation. Chen and co-workers (2004) have suggested that the ability to resist to radiation damage could be acquired by an evolutionary process resulting from adaptation to environmental stress conditions, such as deserts or other arid environments (Mattimore and Battista, 1996). In fact, DNA sequences close to *Rubrobacter* spp. have been isolated from deserts, and new strains, representing novel species, have also been isolated from biodeteriorated monuments (Holmes *et al.*, 2000; Rainey *et al.*, 2005; Laiz *et al.*, 2009).

6.1.1. The compatible solutes of *Rubrobacter xylanophilus*

The bacterium *R. xylanophilus* accumulates a wide variety of organic solutes, including trehalose, mannosylglycerate, di-*myo*-inositol-phosphate (DIP), glycine-betaine, glutamate and a new phosphodiester compound, identified as di-*N*-acetyl-glucosamine phosphate, to cope with different stress conditions. Two major intracellular organic solutes, trehalose and MG, are accumulated in this poly-extremophile in all growth conditions examined (Empadinhas *et al.*, 2007).

Moreover, lower levels of the phosphodiester solutes DIP and di-*N*-acetyl-glucosamine phosphate were also detected in *R. xylanophilus* (Empadinhas *et al.*, 2007). DIP was considered to be an archetypal compatible solute of hyperthermophilic organisms, never having been detected in organisms with optimal growth temperatures below 80°C (Santos *et al.*, 2007). Di-*N*-acetyl-glucosamine phosphate, unlike all the polyol-derived phosphodiester solutes known to date, comprised a phosphate group linking two sugar moieties, in this case glucosamine. The adaptive success of *R. xylanophilus* to different environments may be related to the accumulation of this variety of compatible solutes.

6.2. The Genus *Selaginella*

Lycophytes are an ancient lineage of vascular plants that arose about 400 million years ago, during the Carboniferous period (Kenrick and Crane, 1997). Similarly to ferns, but not flowering plants, all lycophytes produce spores, not seeds (Banks, 2009). These plants do not

have true leaves (microphylls instead) and lateral roots. The only three extant orders of lycophytes include the Lycopodiales, the Selaginellales (the spikemosses) and the Isoetales. The Selaginellaceae family is comprised of a single genus, *Selaginella*, and approximately 700 species. Although *Selaginella* species occupy a wide range of habitats, from wet tropical forests to desert and alpine regions, most species occur in tropical and warm temperate climates.

Lycophytes are considered a key node of plant evolution, since they occupy an important phylogenetic position. These plants are included in a intermediate clade between nonvascular plants (the algae and bryophytes) and all other vascular plants, and this phylogenetic position will serve as a reference for bridging large-scale genome comparisons and also for research into comparative plant biochemistry and development (Weng *et al.*, 2005). At present, only one genus is recognized in the Selaginellaceae family, but this genus contains approximately 700 species that include temperate, tropical, frost-tolerant arctic, and drought-tolerant desert species.

Figure 1.11. *Selaginella moellendorffii*.

One of the species of this genus is the spikemoss *Selaginella moellendorffii* (Fig. 1.11). It is a small diploid plant that has the smallest genome size reported for a land plant species (~100 Mbp) (Wang *et al.*, 2005), about two-thirds that of *Arabidopsis thaliana* genome**.** The compact genome of this land plant will be a useful tool for annotating other genomes, and discovering new metabolic pathways, as well as proteins and their functions. Its genome, recently sequenced (August 2010) by the Department of Energy Joint Genome Institute (http://genome.jgi-psf.org/Selmo1/Selmo1.home.html), is a welcomed resource for researchers interested in testing theories of plant evolution, particularly those relating to the evolution of fundamental traits of vascular plants. Furthermore, *S. moellendorffii* genome sequence will help to define an ancient core of genes that are common to all vascular plants.

However, the most well known species of this genus is *Selaginella lepidophylla*, the 'resurrection' plant or rose of Jericho, a plant capable of surviving long periods of dehydration. Like *S. lepidophylla*, *S. tamariscina*, can also remain alive in a dry state and then restart growing when water becomes available again (Liu *et al.*, 2008). On the other hand, *Selaginella moellendorffii* does not display the anhydrobiotic capacity of *S. lepidophyll*a (Iturriaga *et al.*, 2006).

One of the strategies to protect cellular integrity in a desiccated state is the accumulation of soluble sugars. Trehalose is present in very low levels in most plants except in desiccation-tolerant plants, where this disaccharide is the key molecule to protect them against osmotic stress and especially drought (Avonce *et al.*, 2005; 2006). In *Selaginella lepidophylla*, an interchange between sucrose and trehalose accumulation occurs during dehydration. In the hydrated condition, trehalose was the major soluble sugar detected, while sucrose and glucose were minor solutes. On the other hand, in desiccated *S. lepidophylla*, a decrease in the trehalose content is accompanied by a three-fold increase of sucrose (Adams *et al.*, 1990). In *S. tamariscina*, a high level of disaccharides, mainly trehalose, is steadily maintained in both hydrated and desiccated states, but in seeds and vascular 'resurrection' plants the composition of sugar is adjusted during dehydration, as previously described for *S. lepidophylla* (Liu *et al.*, 2008).

CHAPTER 2

Metabolic pathways for trehalose synthesis in *Rubrobacter xylanophilus*

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CONTENTS

ABSTRACT

Trehalose is the primary organic solute in *Rubrobacter xylanophilus* under all conditions tested, including those for optimal growth. We detected genes of four different pathways for trehalose synthesis in the genome of this organism, namely, the trehalose-6-phosphate synthase (Tps)/trehalose-6-phosphate phosphatase (Tpp), TreS, TreY/TreZ, and TreT pathways. Moreover, *R. xylanophilus* is the only known member of the phylum *Actinobacteria* to harbor TreT. The Tps sequence is typically bacterial, but the Tpp sequence is closely related to eukaryotic counterparts. Both the Tps/Tpp and the TreT pathways were active in vivo, while the TreS and the TreY/TreZ pathways were not active under the growth conditions tested and appear not to contribute to the levels of trehalose observed. The genes from the active pathways were functionally expressed in *Escherichia coli*, and Tps was found to be highly specific for GDP-glucose, a rare feature among these enzymes. The trehalose-6-phosphate formed was specifically dephosphorylated to trehalose by Tpp. The recombinant TreT synthesized trehalose from different nucleoside diphosphate-glucose donors and glucose, but the activity in *R. xylanophilus* cell extracts was specific for ADP-glucose. The TreT could also catalyze trehalose hydrolysis in the presence of ADP, but with a very high K_m. Here, we functionally characterize two systems for the synthesis of trehalose in *R. xylanophilus*, a representative of an ancient lineage of the actinobacteria, and discuss a possible scenario for the exceptional occurrence of *treT* in this extremophilic bacterium.

INTRODUCTION

The disaccharide trehalose serves several roles in archaea, bacteria, yeast, fungi, plants, and invertebrates. However, trehalose is best known for its role as a universal protector molecule, protecting cells and biomolecules from stress imposed by low water activity, heat, oxidation, desiccation, and freezing (Elbein *et al.*, 2003). Five different pathways for the synthesis of trehalose have been examined (Avonce *et al.*, 2006; Paul *et al.*, 2008), the most common of which involves a trehalose-6-phosphate synthase (Tps) that converts nucleoside diphosphate (NDP)-glucose and glucose-6-phosphate (Glc6P) into trehalose-6-phosphate (T6P) that is then dephosphorylated to trehalose by a trehalose-6-phosphate phosphatase (Tpp) (Silva *et al.*, 2005). Other pathways for trehalose biosynthesis involve trehalose synthase (TreS) and the maltooligosyltrehalose synthase/ hydrolase (TreY/TreZ) system, which catalyze the conversion of maltose and maltooligosaccharides into trehalose, respectively (Nakada *et al.*, 1995). Additionally, the TreS from *Mycobacterium smegmatis* can also convert glycogen into trehalose via maltose (Pan *et al.*, 2008). A trehalose phosphorylase (TreP) can also catalyze trehalose synthesis in the presence of Glc1P and glucose in fungi and a few bacteria (Avonce *et al.*, 2006). A less common and recently discovered pathway involves a trehalose glycosyl transferring synthase (TreT) that converts NDP-glucose and glucose into trehalose. This enzyme has been examined only in the hyperthermophilic archaea *Thermococcus litoralis*, *Pyrococcus horikoshii*, and *Thermoproteus tenax* (Qu *et al.*, 2004; Ryu *et al.*, 2005; Kouril *et al.*, 2008). Many organisms have one or two, or less frequently three, pathways for trehalose synthesis (De Smet *et al.*, 2000; Wolf *et al.*, 2003). Mycobacteria and corynebacteria, for example, can accumulate trehalose or incorporate it into mycolic acids of the cell wall and generally possess three pathways for the synthesis of trehalose (Wolf *et al.*, 2003). In many organisms osmoregulated trehalose synthesis appears to involve Tps and Tpp; however, the accumulation of trehalose during osmotic adjustment in *Corynebacterium glutamicum*, which also possess TreS, is linked to the TreY/TreZ pathway (Wolf *et al.*, 2003). *Propionibacterium freudenreichii*, on the other hand, depends on the Tps/Tpp pathway for the synthesis of trehalose in response to osmotic, oxidative, and acid stresses, while TreS is involved in trehalose degradation (Cardoso *et al.*, 2007). *Rubrobacter xylanophilus* is a thermophilic, halotolerant, and extremely radiation- and desiccation-resistant bacterium that constitutively

accumulates trehalose as the major organic solute under optimal growth conditions and under salt and thermal stresses (Empadinhas *et al.*, 2007). In this work, we examined the synthesis of trehalose by Tps/Tpp and TreT, two active pathways for the synthesis of this disaccharide in *R. xylanophilus* cell extracts, and characterized the properties of the corresponding recombinant enzymes.

MATERIALS AND METHODS

Strains, culture conditions, and DNA isolation

Rubrobacter xylanophilus PRD-1^T (DSM 9941) was our laboratory strain. Cells were grown in *Thermus* medium without or with 2.5% NaCl at 60 or 67°C in a medium containing 1.0 g/liter tryptone and 1.0 g/liter yeast extract (Empadinhas *et al.*, 2007). The cultures were also grown in a medium containing 2.0 g/liter maltose as a single carbon source, 1 g/liter NH_4Cl, and 0.25 g/liter yeast extract. Chromosomal DNA was isolated as previously described (Rodrigues *et al.*, 2007). All chemicals were obtained from Sigma.

Enzyme assays for detection of trehalose synthesis in cell extracts

R. xylanophilus cells were recovered by centrifugation; extracts were obtained using a French pressure cell and dialyzed (20 mM Tris-HCl, pH 7.5). To provide evidence of the activities of the TreP, Tps/Tpp, TreT, TreS, and TreY/TreZ pathways in the cell extracts, the assays were performed as previously described, with slight modifications (Wannet *et al.*, 1998; De Smet *et al.*, 2000; Qu *et al.*, 2004; Cardoso *et al.*, 2007). The mixtures contained 15 µg of protein of *R. xylanophilus* extract (1 µg/µl) and the appropriate substrates in a final volume of 50 µl. The reactions were performed at 60°C for 30 min in 25 mM bis-Tris propane buffer at pH 7.0 with 4 mM $MgCl_2$ and then stopped on ice. Reaction products were visualized by thin-layer chromatography (TLC) on Silica Gel 60 plates (Merck) with a solvent system composed of chloroform/methanol/acetic acid/water (30:50:8:4, v/v/v/v) (Fernandes *et al.*, 2007).

TreP activity was examined with α- or β-Glc1P and glucose added to the cell extracts, and the reverse reaction was examined by the addition of trehalose and inorganic phosphate. For determination of the activity of Tps, the substrates were ADP-, GDP-, UDP-, or TDP-glucose and Glc6P. The T6P formed was dephosphorylated with 2 μg of the recombinant Tpp from *Thermus thermophilus* RQ-1 for trehalose detection on TLC (Silva *et al.*, 2005). Tpp activity was examined after incubation of extracts with T6P, the TreT activity was examined by the addition of NDP-glucose donors and glucose, and the TreS activity was examined with maltose or trehalose. For the examination of the TreY/TreZ pathway, the substrates maltotriose, maltoheptaose, starch, glycogen, and amylopectin were added. Sugars and sugar derivatives were added at a concentration of 10 mM and polysaccharides at 1% (wt/vol).

Detection of genes for trehalose synthesis in the *R. xylanophilus* genome and sequence analyses

BLAST searches were performed with the Tps (EC 2.4.1.15/EC 2.4.1.36), Tpp (EC 3.1.3.12), trehalose (maltose-converting) synthase (TreS) (EC 5.4.99.16), maltooligosyltrehalose synthase (TreY) (EC 5.4.99.15), maltooligosyltrehalose trehalohydrolase (TreZ) (EC 3.2.1.141), trehalose glycosyltransferring synthase (TreT), and trehalose phosphorylase (TreP) (EC 2.4.1.64/EC 2.4.1.231) sequences.

We analyzed the sequences of seven genes, including *treT*, for the levels of AGA/AGG (AGR) codons for arginine (Arg) (http://www.sysbio.muohio.edu/CodonO), which are used frequently in hyperthermophiles but rarely in mesophiles. We also determined the G+C ratio in each of these genes and compared it to that in the overall genome sequences to deduce the origins of these genes in the genome by possible lateral gene transfer events (Lawrence and Ochman, 1997; Eisen, 2000; Lobry and Necsulea, 2006). Genes *tps*, *tpp*, and *treT* were selected because they represent the two active pathways for the synthesis of trehalose in *R. xylanophilus* growing under the conditions described. We also selected gene *glnA*, encoding type I glutamine synthetase (EC 6.3.1.2, Rxyl_1125), because it is a housekeeping gene that has been proposed to be a good molecular clock (Kumada *et al.*, 1993), and the conserved ubiquitous gene *fusA* for elongation factor EF-G (EC 3.6.5.3, Rxyl_2158), which is an informational gene thus less likely to be laterally transferred (Hashimoto and Hasegawa, 1996; Jain *et al.*, 1999).

We also selected the *ino1* gene, for inositol-3-phosphate synthase (EC 5.5.1.4, Rxyl_1213), and the *dippS* gene (GenBank accession number EF523341, Rxyl_1212), both of which are involved in the synthesis of di-*myo*-inositol-phosphate (DIP), a compatible solute detected so far in hyperthermophilic prokaryotes and in *R. xylanophilus*, and thus likely to have been acquired from a hyperthermophile (Empadinhas *et al.*, 2007; Rodrigues *et al.*, 2007). For comparison, and because the G+C compositions of genomes might influence the synonymous codon usage (Lynn *et al.*, 2002), we analyzed the above-mentioned genes in organisms that possess *treT* and have different G+C genomic compositions (http://archaea.ucsc.edu/), different optimal growth temperatures, and different phylogenetic positions. We selected three hyperthermophiles: the euryarchaeon *Pyrococcus horikoshii* (41.9% G+C in the genome) because it also has the genes for DIP synthesis; the crenarchaeon *Pyrobaculum calidifontis* (57.2% G+C), which possesses *tps*/*tpp*; and the bacterium *Thermotoga maritima* (46.2% G+C), which contains the genes for DIP synthesis. We also analyzed the genes from mesophiles: the deltaproteobacterium *Myxococcus xanthus* (68.9% G+C), which, in addition to *treT*, also contains the *tps*/*tpp* genes; the methanogen *Methanoculleus marisnigri* (62.1% G+C), which contains three copies of *treT* and the *tps*/*tpp* pair; and the synthrophic deltaproteobacterium *Syntrophus aciditrophicus*, which contains the *tps*/*tpp* pair but has a moderate G+C content in the genome (51.5%).

Gene amplification and cloning

For sequence confirmation, and based on the draft genome sequence (www.jgi.doe.gov), several primers were designed to hybridize to the sequences immediately upstream and downstream from the putative *tps*, *tpp*, *treT*, *ino1*, *fusA*, and *glnA* genes. PCRs were carried out with the GC -RICH PCR system (Invitrogen). Products were cloned into pGEM-T Easy vector (Promega) with *Escherichia coli* DH5α as the host and sequenced (AGOWA GmbH, Berlin, Germany). For heterologous gene expression, the *tps*, *tpp*, and *treT* genes were amplified with the forward primers TPSNco, TPPNcoF, and TreTNde and reverse primers TPSXba, RubTPP2, and TreTBam, respectively (Table 2.1).

Table 2.1. Primers sequences used in this study.

Primer	Target sequence	Sequence (5'-3')[a]	Direction	Restriction enzyme
TPSNco	*tps* (Rxyl_2972)	GACC**ATG**GCGCAAAACGGCGG	Forward	NcoI
TPSXba		GCGTCTAGA**CTA**GGAGAAGGCCTTGCGGCG	Reverse	XbaI
TPPNcoF	*tpp* (Rxyl_2971)	AGCGCC**ATG**GAAACGGATAGGCCTTCG	Forward	NcoI
RubTPP2		GCGTCTAGA**CTA**GCGGGCCAGCTCCCG CAG	Reverse	XbaI
TreTNde	*treT* (Rxyl_2973)	GAATACGTCATATG**ATG**CTGCAGCGGGTGAAC	Forward	NdeI
TreTBam		AATGGATCC**TTA**CACGCCGAGAAGTTTAG	Reverse	BamHI
InoF	*ino1* (Rxyl_1213)	**ATG**GGCGAAAGGCGGCAGAG	Forward	-
InoR		**TCA**GCGGAGGGAGGAGGGG	Reverse	-
EFGF	*fusA* (Rxyl_2158)	**ATG**GCAGTACAGGTAGCGAAG	Forward	-
EFGR		**CTA**CCTCTCGGCTATCTTCTC	Reverse	-
GSIF	*glnA* (Rxyl_1125)	**ATG**AGCGAGATCACGCGCG	Forward	-
GSIR		**TTA**GAGGACCGGCAGGTAGC	Reverse	-

[a] The restriction enzyme recognition sequences are underlined; the start and stop codons are in bold.

Genes *tps* and *tpp* were cloned into the expression vector pET30b, leading to production of His-tagged recombinant proteins. However, *treT* was cloned into the expression vector pET11a, yielding a protein without a His tag. *E. coli* BL21(DE3) was used as the host (Novagen).

Gene expression and purification of recombinant enzymes

Cells carrying pET30b-*tps*, pET30b-*tpp*, or pET11a-*treT* were grown to an optical density at 610 nm of 1.0 at 37°C in LB medium (2 liters) containing kanamycin (30 µg/ml) or ampicillin (100 µg/ml) for selection, and IPTG (isopropyl-β-D-thiogalactopyranoside) (1 mM) was added to induce gene expression. Cells carrying $(His)_6$-Tps and $(His)_6$-Tpp were allowed to grow for ~ 6 h, harvested, and suspended in 20 mM sodium phosphate buffer with 0.5 M NaCl and 10 mM $MgCl_2$, DNase I (4 µg/ml), and a protease inhibitor cocktail (Roche). Cells carrying the recombinant TreT were allowed to grow for a further 10 h at 30°C, suspended in 20 mM Tris-

HCl at pH 7.6, and disrupted in a French pressure cell, and debris was removed by centrifugation. The extracts were heated for 10 min at 60°C, cooled, and centrifuged to remove host precipitated proteins. Supernatants with Tps and Tpp were supplemented with 20 mM imidazole and loaded onto a nickel HisTrap column equilibrated with 20 mM sodium phosphate buffer with 0.5 M NaCl and 20 mM imidazole at pH 7.4. His-tagged proteins were eluted at 5 ml/min with the same buffer containing 500 mM imidazole in a three-step gradient (30, 50, and 80%). His tags were removed by enterokinase digestion according to the supplier's instructions (Novagen). TreT was partially purified with two sequential Q-Sepharose fast-flow columns (Hi-Load 16/10) equilibrated with 20 mM Tris-HCl, pH 7.6. Elution was carried out with linear NaCl gradients (0.0 to 1.0 M). Active fractions were dialyzed, concentrated, and loaded onto a Superdex 200 column equilibrated with 50 mM Tris-HCl and 200 mM NaCl, pH 7.6. Fractions with active recombinant Tps, Tpp, or TreT were pooled, concentrated and dialyzed, and the purity and the molecular masses were estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and by gel filtration chromatography, as previously described (Fernandes *et al.*, 2007). The protein content of all samples was determined as previously described (Silva *et al.*, 2005).

Enzyme assays during purification of recombinant enzymes

The activities of Tps, Tpp, and TreT were examined with the assays for detection of trehalose synthesis in cell extracts. Tps activity was detected with GDP-glucose and Glc6P followed by 20 min of incubation with 2 U of alkaline phosphatase (Roche) at 37°C to release trehalose, which was visualized by TLC. Tpp activity was examined with T6P, and TreT activity was determined with ADP-glucose and glucose as previously described (Qu *et al.*, 2004; Silva *et al.*, 2005).

Tps characterization

The recombinant Tps activity was measured by the specific dephosphorylation of the T6P formed with the recombinant Tpp (2 μg) from *T. thermophilus* RQ-1 (Silva *et al.*, 2005). Reaction mixtures (100 μl) containing 0.4 μg Tps and 10 mM of each substrate were stopped

by the addition of 40 µl of 20% trichloroacetic acid, incubated on ice, centrifuged, and neutralized with 1 M NaOH, and the volume was adjusted to 200 µl with H_2O. A 50-µl aliquot was incubated with the *T. thermophilus* Tpp for 5 min at 70°C, the reaction was stopped on ice-ethanol, and the phosphate released was measured (Ames, 1966). The substrate specificity was examined with different sugar donors and acceptors (Silva *et al.*, 2005), the temperature profile was determined between 30 and 90°C, the thermal stabilities at 60 and 70°C were assessed in 25 mM bis-Tris-propane buffer at pH 7.0 and residual activity determined at 40°C, the pH dependence was determined between pH 4.0 and 9.0 (Silva *et al.*, 2005; Fernandes *et al.*, 2007), the effect of divalent cations was tested, and the V_{max} and K_m were determined with various substrate concentrations and calculated from the Michaelis-Menten equation (Silva *et al.*, 2005).

Tpp characterization

The Tpp activity was measured by quantification of the phosphate released from T6P (Ames, 1966). Reaction mixtures (50 µl) contained 50 ng of pure recombinant Tpp and 10 mM T6P. Substrate specificity was determined using several phosphorylated compounds (Silva *et al.*, 2005). Other parameters studied were those described above for Tps. The temperature profile was determined between 10 and 80°C. Thermal stability was evaluated in 25 mM Tris-HCl buffer (pH 7.5) containing 2 mM $MgCl_2$ and residual activity measured at 40°C. The pH dependence was determined between pH 5.0 and 10.5.

TreT characterization

The trehalose-forming activity of the recombinant TreT was examined by the quantification of NDP released from the condensation of NDP-glucose and glucose into trehalose. Reactions were initiated by the addition of TreT and stopped at different times by cooling on ice-ethanol, and the amount of NDP was determined (Fernandes *et al.*, 2007). The substrate specificity was examined with different sugar donors and acceptors, the temperature profile was determined between 20 and 80°C, the thermal stability was determined as described above, the pH

dependence was examined between pH 4.0 and 10.5, reactions in the presence of divalent cations or with EDTA (5 mM) were performed to examine cation dependence, and V_{max} and K_m were determined as previously described (Qu *et al.*, 2004). The reverse activity of TreT (trehalose hydrolysis in the presence of ADP) was measured with the glucose oxidase assay kit (Sigma). The substrates α,α-, α,β-, and β,β-trehalose alone or in the presence of ADP or ATP were also tested. The V_{max} and K_m values were determined with various concentrations of trehalose and ADP.

Nucleotide sequence accession numbers

A 4,020-bp sequence containing the *treT*, *tps*, and *tpp* genes from *R. xylanophilus* and additional 1,368-, 2,148-, and 1,332-bp sequences for the inositol-3-phosphate synthase (*ino1*), the elongation factor G (*fusA*), and the type I glutamine synthetase (*glnA*) genes from the same organism have been deposited in GenBank under accession numbers EU881704, EU881705, EU881706, and EU881707, respectively.

RESULTS

Detection of trehalose synthesis in cell extracts

From an array of experiments on cell extracts using several glucose donors and acceptors, the combination of GDP-glucose and Glc6P resulted in the synthesis of T6P via the Tps/Tpp pathway, which was partially dephosphorylated by a native Tpp activity (Fig. 2.1). Full dephosphorylation of the T6P formed was achieved only after the addition of a recombinant Tpp from *T. thermophilus* RQ-1 (not shown). The synthesis of trehalose also occurred with ADP-glucose and glucose via the TreT pathway (Fig. 2.1). We did not detect either TreS activity from maltose or the reverse reaction (not shown). We were also unable to detect trehalose synthesis from maltooligosaccharides or from α-1,4-linked polysaccharides via the TreY/TreZ pathway or trehalose phosphorylase (TreP) activity from Glc1P and glucose (Fig. 2.1).

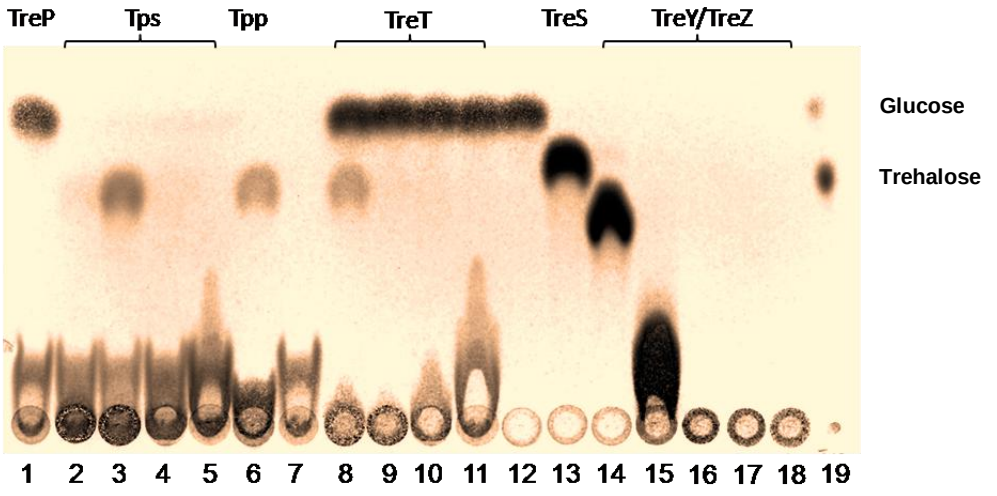

Figure 2.1. TLC analysis of reaction products obtained with *R. xylanophilus* cell extracts. Lane 1, TreP activity with Glc1P and glucose; lanes 2 to 5, Tps activity with Glc6P and ADP-glucose, GDP-glucose, UDP-glucose, or TDP-glucose, respectively (reactions dephosphorylated with the Tpp from *T. thermophilus* RQ-1); lanes 6 and 7, Tpp activity with T6P and Glc6P; lanes 8 to 11, TreT activity with glucose and ADP-glucose, GDP-glucose, UDP-glucose, or TDP-glucose, respectively; lane 12, cell extracts incubated with glucose; lane 13, TreS activity with maltose; lanes 14 to 18, TreY/TreZ activity with maltotriose, maltoheptaose, starch, glycogen, and amylopectin, respectively; lane 19, glucose and trehalose standards.

Identification of genes and genetic organization

BLAST searches led to the detection of genes of four putative pathways for the synthesis of trehalose in the *R .xylanophilus* genome, namely, the Tps/Tpp (Rxyl_2972/Rxyl_2971), TreT (Rxyl_2973), TreS (Rxyl_0315), and TreY/TreZ (Rxyl_0319/Rxyl_0318) pathways. Primer design for amplification of the region containing the *treT*, *tps*, and *tpp* genes was based on the draft genome sequence. However, our own sequences confirmed that the genome contains several errors in these genes (6 to 14% at the nucleotide level). A second putative *treT* gene (Rxyl_3082) encoding a protein with high amino acid identity (52%) to the product of the *treT* gene examined in this study (Rxyl_2973) was detected in the genome, but we could not amplify it, after several attempts, with multiple primers designed from the surrounding genes. Due to the errors detected in the draft genome sequence, we are not certain that this is an

authentic gene. To corroborate this view, the draft genome sequence also contains two copies of a putative *ino1* gene encoding inositol-3-phosphate synthase (Rxyl_0688 and Rxyl_1213) that share 100% identity (except in the nucleotides corresponding to the C-terminal 8 amino acids) but different genetic surroundings. In this case, we could amplify only one copy (Rxyl_1213) with primers based on the flanking genes and found that as much as 19% of the nucleotide sequence of this gene in the genome was incorrect. The *treT* and *tps* genes are sequentially arranged, with an intergenic region of 32 nucleotides (Fig. 2.2). A unique promoter sequence was detected upstream from *treT*. The *tpp* gene is in the opposite direction and under the control of a different promoter, which appears to be located upstream of the putative adjacent peptidase (ORF1) (Fig. 2.2). The TreS and TreY/TreZ pathways appear to be part of an operon-like structure with a single promoter and containing genes (Rxyl_0314 to Rxyl_0319) possibly involved in the synthesis/degradation of glycogen/maltooligosaccharides (not shown). A typical gene for TreP was not detected in the genome.

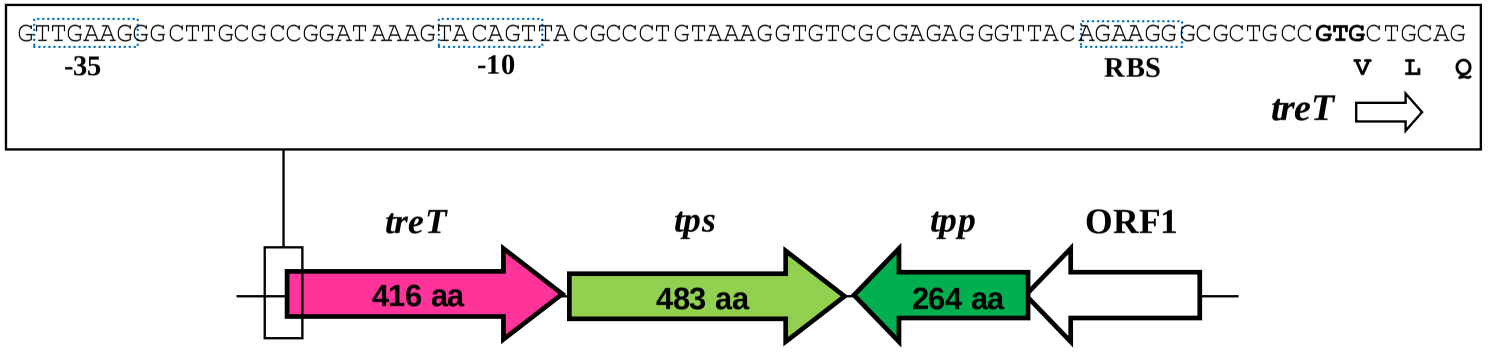

Figure 2.2. Genomic organization of the *R. xylanophilus tps*, *tpp* and *treT* genes. Arrows represent genes and their directions. The putative promoter elements (boxed) upstream of *treT* were detected with prokaryotic promoter prediction tools at http://bioinformatics.biol.rug.nl/websoftware/ppp/ppp_start.php and at www.fruitfly.org/seq_tools/promoter.html. RBS, putative ribosome binding site; *treT*, trehalose glycosyltransferring synthase gene; *tps*, Tps gene; *tpp*, Tpp gene; ORF1, putative peptidase; aa, amino acids.

Protein and nucleotide sequence analyses

Expression of the *R. xylanophilus tps*, *tpp* and *treT* genes in *E. coli* resulted in the high level production of recombinant His-tagged protein Tps and Tpp (Fig. 2.3). The *tps* gene has 1,452 bp coding for a polypeptide of 483 amino acids with a deduced molecular mass of 53.9 kDa (Fig 2.3 A), which was shown to be a dimer.

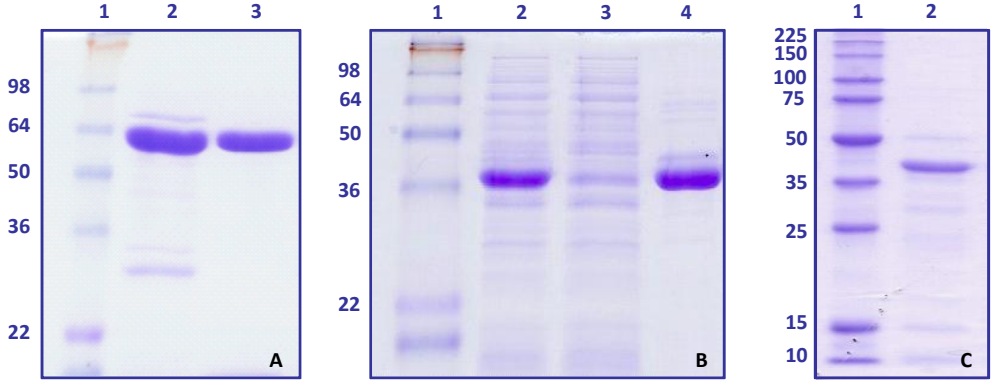

Figure 2.3. SDS-PAGE of the fractions obtained during the purification of the recombinant enzymes. A) Purification of recombinant Tps(His)$_6$: Lane 1- protein molecular weight markers (kDa); Lane 2 and 3- fraction eluted from the nickel HisTrap column; B) Purification of recombinant Tpp(His)$_6$: Lane 1- protein molecular weight markers (kDa); Lane 2- crude extract of cells, after denaturation (10 min at 60ºC); Lane 3- sample that did not bind to the nickel HisTrap column; Lane 4- fraction eluted from the nickel column; C) Purification of recombinant TreT: Lane 1- protein molecular weight markers (kDa); Lane 2- fraction eluted from the Superdex 200 column.

The Tps sequence has a high level of amino acid identity (>40%) with many bacterial Tps proteins, the archaeal Tps from *Methanothermobacter thermautotrophicus* (43%), and several sequences from fungi (30 to 35% amino acid identity) (Fig. 2.4).

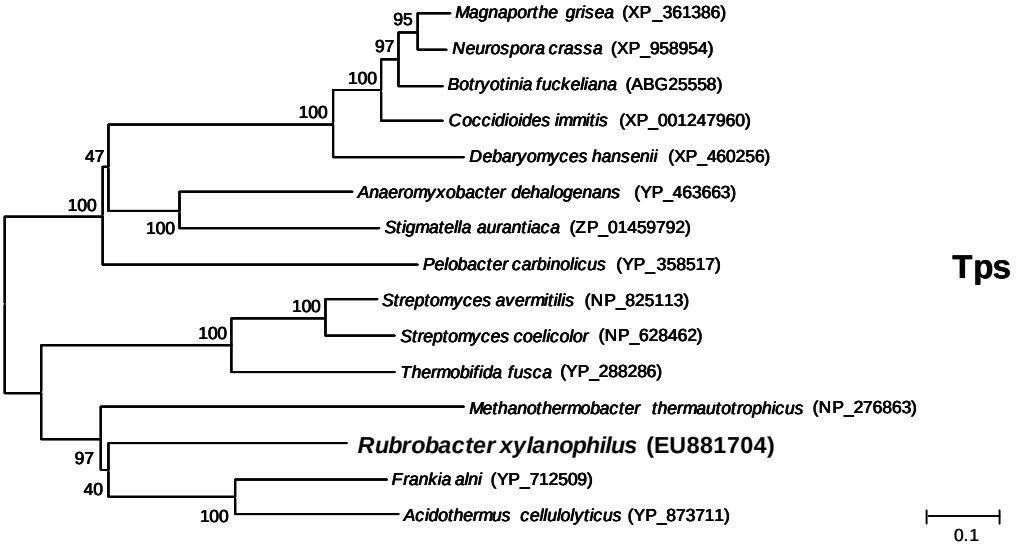

Figure 2.4. Unrooted phylogenetic tree based on available amino acid sequences of Tps homologues. The MEGA4 program was used for sequence alignments and to generate the phylogenetic trees (Tamura *et al.*, 2007). The bootstrap values and accession numbers for proteins are indicated. For *R. xylanophilus* sequences, GenBank accession numbers are indicated. Bar, 0.1 changes/site.

The *tpp* gene has 795 bp and codes for a polypeptide of 264 amino acids with 29.4 kDa (Fig. 2.3 B), which behaved as a monomer. The Tpp sequence had no close homologues, with its closest relatives being from the green nonsulfur bacterium *Herpetosiphon aurantiacus* (37%), the archaeon *M*. *thermautotrophicus* (37%), and several eukaryotic organisms, namely, insects of the genera *Drosophila*, *Culex* and *Anopheles* (36%). The Tpp proteins from the related actinobacteria of the genera *Streptomyces* and *Mycobacterium* share even lower overall amino acid identity (31 to 35%) with the enzyme from *R. xylanophilus* (Fig. 2.5).

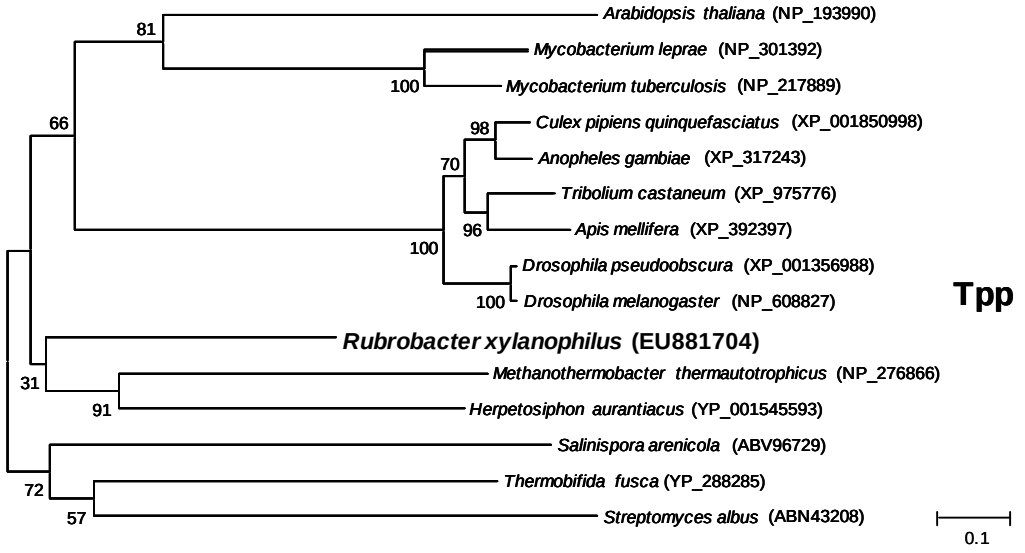

Figure 2.5. Unrooted phylogenetic tree based on available amino acid sequences of Tpp homologues. The MEGA4 program was used for sequence alignments and to generate the phylogenetic trees (Tamura *et al.*, 2007). The bootstrap values and accession numbers for proteins are indicated. For *R. xylanophilus* sequences, GenBank accession numbers are indicated. Bar, 0.1 changes/site.

The *treT* gene contains 1,251 bp coding for a 416-amino-acid polypeptide of 46.7 kDa (Fig. 2.3 C) that also behaved as a dimer. BLAST results indicate that TreT had a few archaeal homologues, mostly crenarchaeotes, but very few bacterial homologues. The phylogenetic tree shows that archaeal and bacterial TreT sequences do not form clearly distinct clusters (Fig. 2.6).

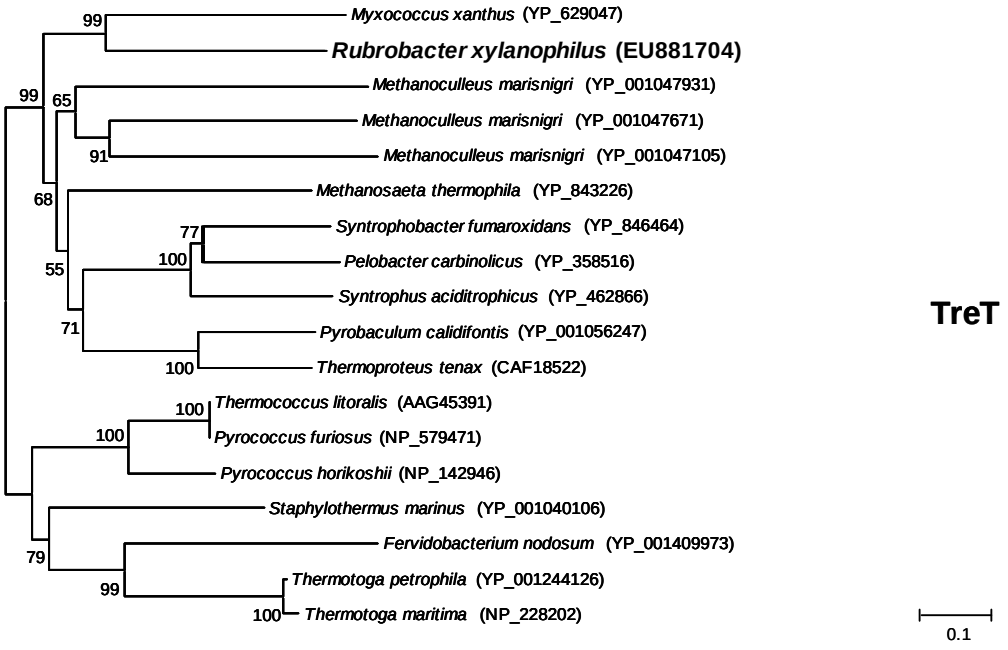

Figure 2.6. Unrooted phylogenetic tree based on available amino acid sequences of TreT homologues. The MEGA4 program was used for sequence alignments and to generate the phylogenetic trees (Tamura *et al.*, 2007). The bootstrap values and accession numbers for proteins are indicated. For *R. xylanophilus* sequences, GenBank accession numbers are indicated. Bar, 0.1 changes/site.

The levels of AGA and AGG (AGR) codons (preferred by hyperthermophiles and rare in mesophiles) in the *R. xylanophilus* genes *tps*, *treT*, *glnA*, *fusA*, and *dippS* are low (3.4 to 16%), although the frequency of the AGR codon in *tpp* is slightly higher (25%) (Fig. 2.7). On the other hand, the AGR codon level in the *ino1* gene (56.7%) is much higher, approaching those in the hyperthermophilic homologues. Moreover, five of the seven *R. xylanophilus* genes analyzed in this work, have a high G+C content (65.8 to 70.4%), similar to the experimentally determined 67.0% G+C in the *R. xylanophilus* chromosome (Carreto *et al.*, 1996). However, the G+C content of the *ino1* gene is only 61.1%, which, along with its atypical AGR codon content, suggests a foreign origin (Fig. 2.7). The G+C content in the *dippS* gene is also higher (73.2%) than the average *R. xylanophilus* genomic G+C content.

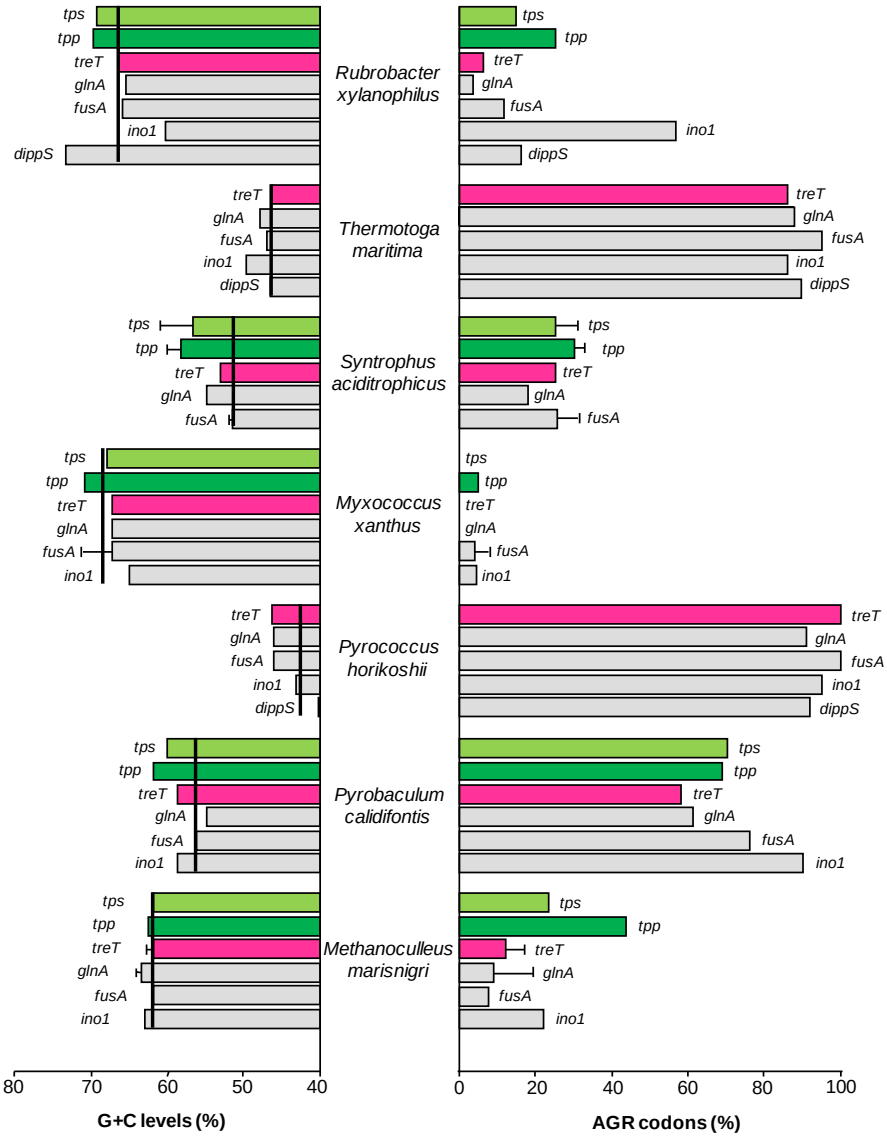

Figure 2.7. Analysis of G+C content and of Arg codons AGG and AGA (AGR) in the *tps*, *tpp*, *treT*, *glnA*, *fusA*, *ino1* and *dippS* genes in organisms harboring *treT* genes. *tps*, Tps gene; *tpp*, Tpp gene; *treT*, trehalose glycosyltransferring synthase gene; *ino1*, inositol-3-phosphate synthase gene; *glnA*, glutamine synthetase (type I) gene; *fusA*, elongation factor G gene; *dippS*, di-inositol-phosphate-phosphate synthase gene. *M. marisnigri* has two copies of *glnA* and three copies of *treT* (>42% amino acid identity). The *tps* and *tpp* genes from *P. calidifontis* and from *M. xanthus* are fused. *S. aciditrophicus* has fused *tps*/*tpp*, isolated *tps* and *tpp*, and three copies of *fusA*. *M. xanthus* has three copies of *fusA*. When multiple copies of the same gene are present in a genome, the average G+C content and AGR ratios are represented, as well as the corresponding standard deviations. Codon usage was determined with the CodonO software at http://www.sysbio.muohio.edu/CodonO. Vertical black lines indicate the overall genomic G+C level in each of the organisms examined (http://archaea.ucsc.edu/).

Properties of Tps and Tpp

Tps showed high substrate specificity for the combination of GDP-glucose and Glc6P. The enzyme was active at between 30 and 90°C, with maximum activity at around 60°C (Fig. 2.8 A).

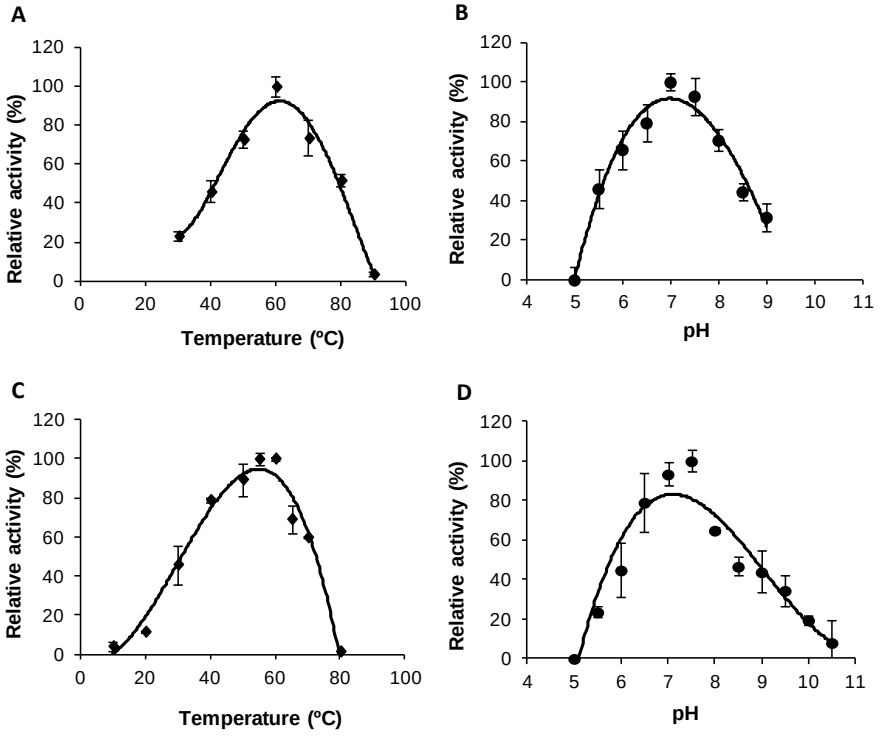

Figure 2.8. Temperature and pH dependence on the activity of the recombinant Tps (A, B) and Tpp (C,D) of *R. xylanophilus*.

Within the pH range examined, the activity of the enzyme Tps at 60°C was maximal at pH 7.0 (Fig. 2.8 B). Tps was active without cations, but maximum activation was obtained with 2 mM of Mg^{2+}. The specific activity of Tps without cations was 36% of maximal activity. The recombinant enzyme Tps exhibited Michaelis-Menten kinetics and K_m values of 0.7 ± 0.1 mM for GDP-glucose and 2.9 ± 1.2 mM for Glc6P. The V_{max} was 22 ± 6 µmol/min·mg protein (Fig. 2.9 A, B).

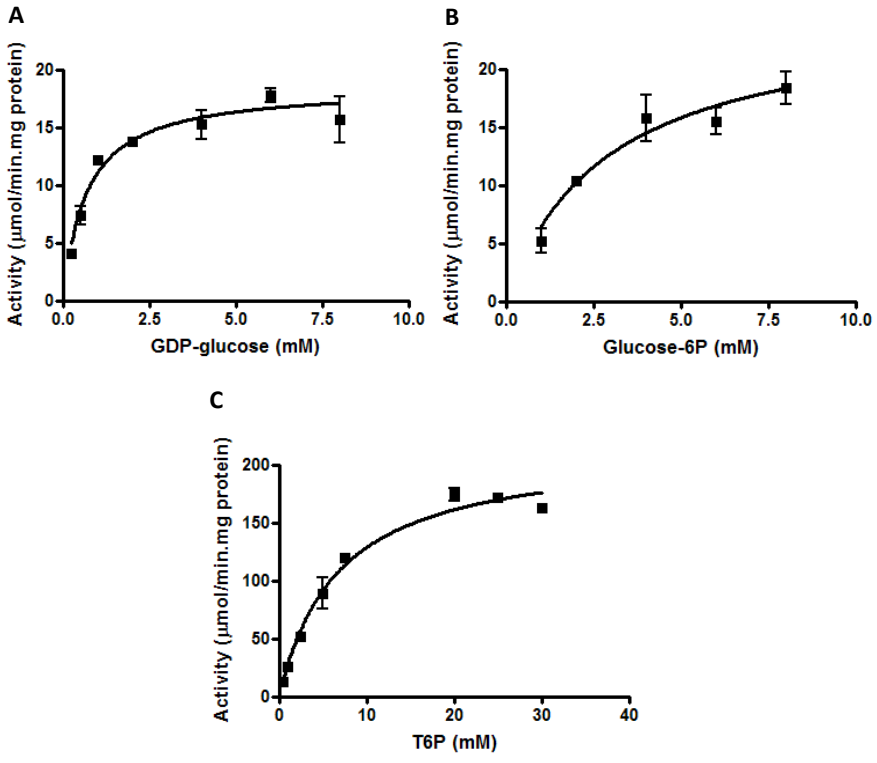

Figure 2.9. Rate dependence of recombinant enzymes: Tps, on GDP-glucose (A) and Glucose-6P concentrations (B); Tpp, on T6P (C).

The enzyme Tps had half-lives of 1.7 ± 0.2 h at 60°C and 0.7 ± 0.1 h at 70°C (Fig. 2.10 A).

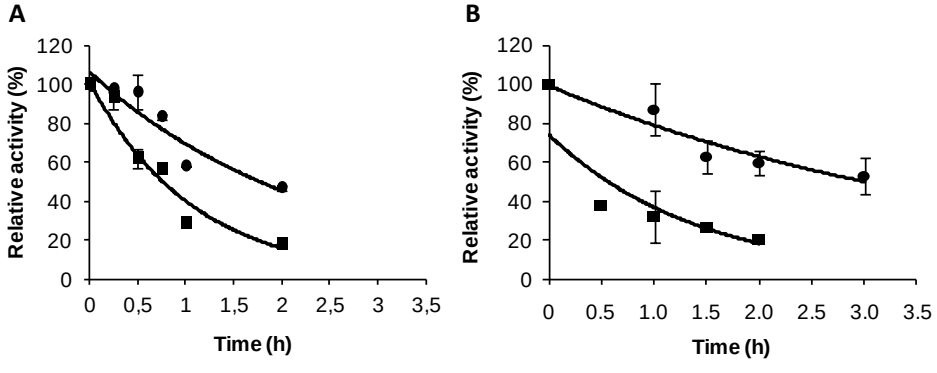

Figure 2.10. Thermostability of recombinant enzymes Tps(A) and Tpp (B) of *R. xylanophilus*, at 60ºC (●) and 70ºC (■).

Among the phosphorylated compounds tested, the recombinant Tpp was specific for T6P. Tpp had maximal activity at 60°C (Fig. 2.8 C), and no activity was detected at 80°C. The optimum pH for activity was around 7.5 (Fig. 2.8 D). The recombinant Tpp was dependent on divalent cations for activity, and 2 mM Mg^{2+} or Co^{2+} was required for maximal activity. Other cations tested, namely, Ni^{2+}, Ca^{2+}, and Fe^{3+}, did not stimulate enzyme activity. Tpp exhibited typical Michaelis-Menten kinetics, a K_m value for T6P of 6.1 $\pm$ 1.5 mM, and a V_{max} of 225 $\pm$ 18 µmol/min·mg protein (Fig. 2.9 C). The half-lives for thermal inactivation were 3.2 $\pm$ 0.9 h at 60°C and 1.0 $\pm$ 0.1 h at 70°C (Fig. 2.10 B).

Properties of TreT

Of the sugar donors examined, ADP-glucose (100.0%), GDP-glucose (48.2%), and UDP-glucose (32.7%) served as substrates for the recombinant TreT, but only glucose served as an acceptor. However, the TreT activity in cell extracts appeared to be specific for ADP-glucose and glucose (Fig. 2.1). Maximum activity was reached at 60°C, but the enzyme was active at between 20 and 80°C (Fig. 2.11 A).

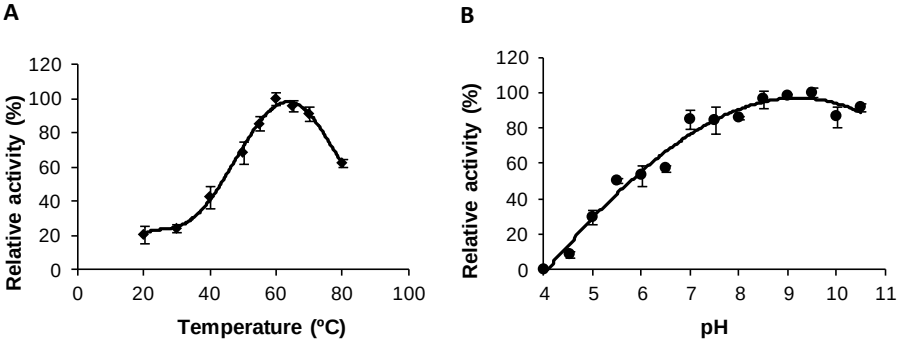

Figure 2.11. Temperature and pH dependence on the activity of the recombinant TreT (A, B) of *R. xylanophilus*.

Within the pH range examined, the activity of the enzyme remained nearly constant between pH 8.0 and 10.0 (Fig. 2.11 B). TreT was not dependent on divalent cations, but the activity was slightly enhanced by 2 mM Fe^{3+}, Mn^{2+}, Ca^{2+}, or Li^{+} and by 20 mM Mg^{2+}, Ca^{2+}, or Li^{+}. Curiously,

no activity was detected after the addition of 20 mM of Fe^{3+} and Mn^{2+}. Other cations tested, namely, Zn^{2+} and Ni^{2+}, gradually inhibited TreT activity at the concentrations tested (2 to 20 mM). TreT exhibited Michaelis-Menten kinetics: the K_m values for ADP-glucose and glucose were 0.8 ± 0.1 and 1.3 ± 0.2 mM, respectively, and the V_{max} was 37 ± 3 µmol/ min mg protein (Fig. 2.12 A, B). TreT could also catalyze the hydrolysis of trehalose, but 150 mM trehalose was required for a measurable reaction rate (5.0 ± 3.1 µmol/min mg). The high K_m values for trehalose (82 ± 18 mM) and for ADP (6.8 ± 0.8 mM) indicate that the formation of trehalose was highly favored (Fig. 2.12 C, D).

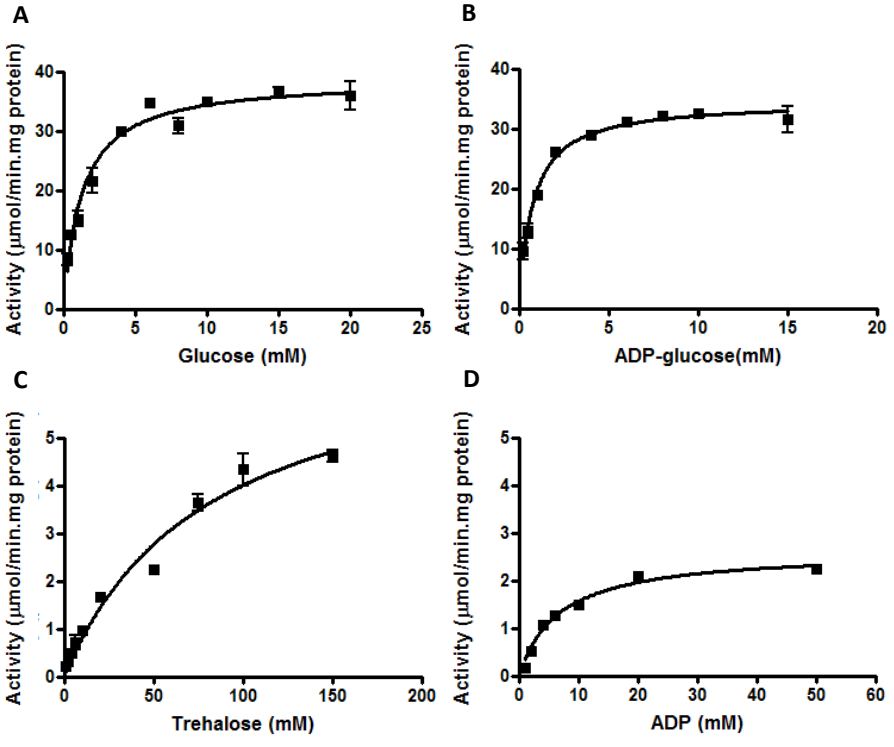

Figure 2.12. Rate dependence of recombinant enzyme TreT, on Glucose (A) and ADP-glucose (B); and on Trehalose (C) and ADP (D) concentrations (graphics C and D – TreT reverse reaction). Values for K_m and V_{max} were extrapolated from the figures shown above.

The half-lives for inactivation at 60°C and at 70°C were 309 ± 89 and 4.1 ± 0.3 h, respectively (Fig. 2.13).

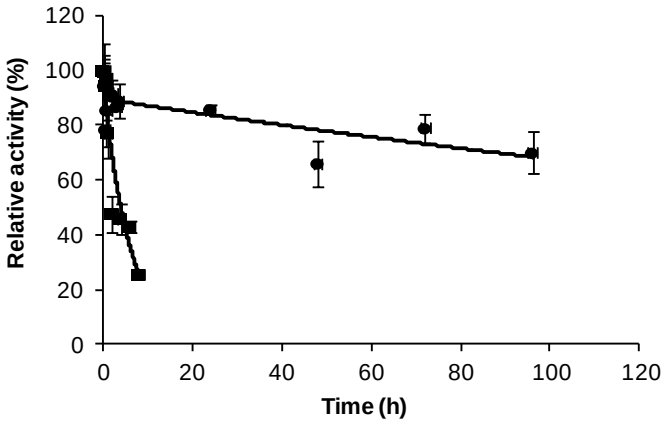

Figure 2.13. Thermostability of recombinant enzymes TreT of *R. xylanophilus*, at 60ºC (●) and 70ºC (■).

The following Table 2.2 summarizes the biochemical properties and kinetic parameters for the substrates of the three characterized enzymes involved in the synthesis and hydrolysis (for the case of TreT) of trehalose.

Table 2.2. Biochemical properties of the recombinant enzymes Tps, Tpp and TreT and kinetic parameters for the substrates involved in the synthesis of trehalose.

Property[a]	**Tps**	**Tpp**	**TreT**
Optimum temperature (ºC)	60	60	60
Optimum pH	7.0	7.5	8.0–10.0
Half-life (h) at:			
60ºC	1.7±0.2	3.2±0.9	309±89
70ºC	0.7±0.1	1.0±0.1	4.1±0.3
K_m (mM) for:			
GDP-glucose	0.7±0.1		
Glucose-6P	2.9±1.2		
Trehalose-6P		6.1±1.5	
ADP-glucose			0.8±0.1
Glucose			1.3±0.2
Trehalose			82±18[b]
ADP			6.8±0.8[b]
V_{max} (μmol/min.mg protein)	22±6	225±18	37±3
			5.0±3.1[b]
Mg^{2+} (mM) dependence[c]			
0	36±2	0	68±3
2	100	100	71±2
20	93±4	53±7	100

[a] Data are the mean values of at least three independent experiments; [b] Reverse reaction; [c] Expressed as percentage of maximum activity.

DISCUSSION

Prokaryotes have the highest diversity of pathways for trehalose biosynthesis, and all five known pathways can be found in the bacterial domain. While some of these organisms can have two or even three pathways for trehalose synthesis, most bacteria as well as plants and animals have only the Tps/Tpp pathway (Wolf *et al.*, 2003; Woodruff *et al.*, 2004; Avonce *et al.*, 2006). The TreS pathway is found almost exclusively in bacteria, and the TreT pathway is present mainly in hyperthermophilic bacteria and archaea (De Smet *et al.*, 2000; Qu *et al.*, 2004; Ryu *et al.*, 2005; Kouril *et al.*, 2008). Occasionally, more than one copy of those genes exists in the same organism: *Ralstonia solanacearum*, for example, has two *tps* genes; *Thermoanaerobacter tengcongensis* has two treP genes; and *Methanoculleus marisnigri* has three copies of *treT*. The genes of four pathways for trehalose synthesis were detected in the genome of *R. xylanophilus*: the Tps/Tpp and the TreT pathways, which we have shown to be functional in cell extracts, as well as the TreS and the TreY/TreZ pathways, which were not active under the conditions tested. This pathway multiplicity is unique among the organisms examined, strongly suggesting an essential role for this disaccharide in *R. xylanophilus* (Elbein *et al.*, 2003; Woodruff *et al.*, 2004). The sequential organization of *treT* and *tps* with an opposing *tpp* gene is also unique. Since our studies indicate that both TreT and Tps are active, this apparent redundancy could reflect maximization of substrate availability to ensure trehalose production. It also suggests that the expression of tpp is independently regulated, and our data indicate that the T6P formed is not fully dephosphorylated in *R. xylanophilus* cell extracts, leading to the hypothesis of an alternative role for the phosphorylated compound. In fact, T6P has been found to play important signaling roles in the regulation of sugar metabolism in *Saccharomyces cerevisiae*, by inhibiting hexokinase, and in *Arabidopsis thaliana*, where it activates ADP-glucose pyrophosphorylase (Paul *et al.*, 2008). T6P is also a precursor of complex structures found in actinobacteria, such as mycolic acids, although *R. xylanophilus* lacks these trehalose-containing structures (Carreto *et al.*, 1996; Takayama *et al.*, 2005).

The organization of the TreS and TreY/TreZ genes in a different and unique operon-like structure containing other genes for glycogen metabolism and the lack of activity in cell extracts from cultures grown on tryptone and yeast extract led us to hypothesize that these

genes may be expressed only when maltose or maltooligosaccharides are used as carbon sources, as is the case with the TreS from *Corynebacterium glutamicum* (Wolf *et al.*, 2003). However, we did not detect TreS activity when the organism was grown on maltose. It is also possible that these pathways may be activated by other environmental stimuli such as desiccation, which induces Tps/Tpp and TreS in *Bradyrhizobium japonicum* (Cytryn *et al.*, 2007).

The Tps from *Rubrobacter xylanophilus* preferred GDP-glucose as a glycosyl donor, unlike the majority of Tps proteins characterized so far, which utilize all natural glucose diphosphate nucleosides as donors for T6P synthesis or are specific for UDP-glucose (Giaever *et al.*, 1988; Elbein *et al.*, 2003; Silva *et al.*, 2005). Two classes of Tps proteins, reflecting their substrate preference, have been proposed, namely, the UDP-forming (EC 2.4.1.15) and the GDP-forming (EC 2.4.1.36) enzymes. The *R. xylanophilus* enzyme is of the second and rarer type. This Tps has maximum activity at 60°C, which slowly decreases to undetectable levels at around 90°C. While this is not common for the majority of enzymes, the glucosylglycerate synthase from *Persephonella marina* and the Tpp from *Thermus thermophilus* have similar profiles, as their activities gradually decrease at above the optimum temperature (Silva *et al.*, 2005; Fernandes *et al.*, 2007). It is possible that at higher temperatures they acquire more stable conformational changes (like the alpha-glucosidase from *Thermotoga maritima*, for example) which allow lower but stable activity for longer periods of time at temperatures above the optimum (Raasch *et al.*, 2000).

The Tpp from *R. xylanophilus* has high sequence similarity to eukaryal orthologues, mostly from insects, followed by some actinobacterial sequences. Similarly, some cyclases from *Mycobacterium tuberculosis* and the citrate synthase from *Geobacter sulfurreducens* are more similar to eukaryotic than to bacterial cyclases, and it has been suggested that the bacteria acquired these genes from eukaryotes (Ponting *et al.*, 1999; Bond *et al.*, 2005). Although we did not trace the origin of the *tpp* gene in *R. xylanophilus*, the high sequence identity with the insect homologues suggests gene transfers between these two domains. The Tpp from *R. xylanophilus* is, like the majority of Tpp proteins, highly specific for T6P (Elbein *et al.*, 2003). In fact, the dephosphorylation of compounds other than T6P is extremely rare, and only the enzymes from the insect *Phormia regina* and from *T. thermophilus* have also been shown to

use Glc6P at lower rates (Friedman and Alexander, 1971; Silva *et al.*, 2005). The TreT from *R. xylanophilus* catalyzes a reaction similar to that of the homofunctional enzymes from *Thermococcus litoralis* and *Pyrococcus horikoshii*, including the hydrolysis of trehalose with ADP as a cosubstrate (resembling the former), but it does not have the trehalase activity of the latter (Qu *et al.*, 2004; Ryu *et al.*, 2005). Interestingly, the TreT from *Thermoproteus tenax* could catalyze only the formation and not the hydrolysis of trehalose (Kouril *et al.*, 2008). The recombinant TreT from *R. xylanophilus* used ADP-glucose as well as other NDP-glucose donors, like the euryarchaeal enzymes but not the crenarchaeal TreT, which preferred UDP-glucose. However, the TreT activity in *R. xylanophilus* extracts appeared to be specific for ADP-glucose. Likewise, the Tps activity in *Propionibacterium freudenreichii* extracts is specific for ADP-glucose, unlike the recombinant enzyme, which used other NDP-glucose donors in addition to ADP-glucose (Cardoso *et al.*, 2007). This may be due to an interaction with an unknown regulatory protein present in cell extracts or to slight differences in folding between the native and recombinant proteins.

The presence of *treT* in the *R. xylanophilus* genome was surprising, since among all the actinobacterial genomes sequenced (>50), only that of this organism possesses a *treT* gene. With the exception of hyperthermophilic bacteria and a few deltaproteobacteria, *treT* is not found in most bacterial genomes. This gene is also present in a few euryarchaeal genomes but is frequent in those from hyperthermophilic crenarchaeotes (Qu *et al.*, 2004; Ryu *et al.*, 2005; Kouril *et al.*, 2008). Such a scattered distribution suggests that *treT* has been involved in lateral gene transfer events and that *R. xylanophilus* may have acquired it from hyperthermophiles (Eisen, 2000; Ragan, 2001). However, the *R. xylanophilus treT* AGR codon usage, the G+C composition, and the phylogenetic analysis failed to confirm this origin (Lobry and Necsulea, 2006). For example, and corroborating previous studies, our sequence analysis also supports the acquisition of the *R. xylanophilus ino1* gene from a hyperthermophile (Eisen, 2000; Nesbo *et al.*, 2001). However, the key gene (*dippS*) for the synthesis of the "hyperthermophilic" solute DIP in *R. xylanophilus* lacks a hyperthermophilic-type AGR codon usage. Although the distribution of *dippS* and *treT* suggests a hyperthermophilic origin for both, we could not detect the lateral transfer because hints of such an event seem to have been erased by amelioration to the recipient's genome (Lawrence and Ochman, 1997; Eisen, 2000; Lake *et al.*,

2007). On the other hand, the phylogenetic clustering of the *R. xylanophilus* TreT sequence with the TreT from *Myxococcus xanthus* and the apparent clustering with other deltaproteobacterial (syntroph) TreT sequences and with those from methanogens suggest an archaeal origin for the bacterial TreT proteins, possibly from methanogens that coexist with bacteria in some environments (Jackson *et al.*, 1999). This hypothetical methanogenic origin is further strengthened by the detection in the *R. xylanophilus* genome of the key gene for the synthesis of cyclic bisphosphoglycerate, an organic solute found only in methanogens (Matussek *et al.*, 1998). This gene is extremely rare, being found only in a few *Thermococcales* and methanogens and in *R. xylanophilus*, where it lacks the hyperthermophilic AGR-type codon usage (unpublished results). Altogether, these results seem to suggest a very ancient origin for *treT*. The ubiquity of intracellular trehalose and the unprecedented detection of four putative pathways for its synthesis in *R. xylanophilus*, a desiccation- and extremely gamma-radiation-resistant organism, encourage further research into the roles of this disaccharide in the metabolism and extreme phenotype of this ancient lineage of the actinobacteria.

ACKNOWLEDGMENTS

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CHAPTER 3

Synthesis and hydrolysis of MG and GG in *Selaginella moellendorffii*

This chapter will be the subject of a publication:

The first eukaryotic enzymes for synthesis and hydrolysis of mannosylglycerate and glucosylglycerate from the ancient plant *Selaginella moellendorffii*. In preparation.

CONTENTS

ABSTRACT

The compatible solute mannosylglycerate (MG) accumulates in some hyper/thermophilic bacteria and archaea coping mainly with salt stress while the related solute glucosylglycerate (GG) has been shown to play an important role in microbial adaptation to salt stress under nitrogen limitations. A rare mannosylglycerate synthase (MgS) gene detected in the genome of *Selaginella moellendorffii* was expressed in *E. coli* and the recombinant enzyme purified and characterized. A remarkable and unprecedented feature of this rare MgS was the ability to efficiently synthesize MG and GG alike, with maximal activity at 50°C, pH 8.0 and with Mg^{2+} as reaction enhancer. We have also identified a novel glycoside hydrolase gene in this plant's genome, which was functionally confirmed to be highly specific for the hydrolysis of MG and GG and named MG hydrolase (MgH), due to its homology with hyper/thermophilic bacterial MgHs. The recombinant enzyme was maximally active at 40ºC and at pH 6.0-6.5. The activity was independent of cations, but Mn^{2+} was a strong stimulator. Regardless of these efficient enzymatic resources we could not detect MG or GG in *S. moellendorffii* or in the extracts of five additional *Selaginella* species. Herein, we describe the properties of the first eukaryotic enzymes for the synthesis and hydrolysis of the compatible solutes MG and GG.

INTRODUCTION

The compatible solute mannosylglycerate (MG) accumulates in some hyper/thermophilic bacteria and archaea during adaptation to salt stress (Santos and da Costa, 2002; Neves *et al*., 2005; Empadinhas *et al*., 2007). This compatible solute was initially encountered in marine red algae, but its physiological role in these organisms has not been clearly elucidated (Karsten *et al*., 2003). Glucosylglycerate (GG) is a MG-related solute originally identified in actinobacterial polysaccharides and glycolipids and more recently as a free molecule in marine cyanobacteria and proteobacteria, where it plays a crucial role during adaptation to salt stress in nitrogen poor environments (Saier and Ballou, 1968; Pommier and Michel, 1981; Goude *et al*., 2004; Klahn *et al*., 2010).

The thermophilic bacterium *Rhodothermus marinus* can synthesize MG from GDP-mannose and D-glycerate using a rare mannosylglycerate synthase (MgS) or through the more common pathway involving a phosphorylated intermediate synthesized and dephosphorylated by a mannosyl-3-phosphoglycerate synthase (MpgS) and a mannosyl-3-phosphoglycerate phosphatase (MpgP) (Martins *et al*., 1999; Borges *et al*., 2004). Likewise, *Persephonella marina* can also synthesize GG via two similar alternative pathways involving a glucosylglycerate synthase (GgS) and glucosyl-3-phosphoglycerate synthase (GpgS) and glucosyl-3-phosphoglycerate phosphatase (GpgP) (Costa *et al*., 2007; Fernandes *et al*., 2007). The unique MgS was, until recently, restricted to *Rhodothermus marinus* but the detection of *mgS* sequences in the red algae *Griffithsia japonica*, *Caloglossa leprieurii* and *Gracilaria changii* indicates that MG is synthesized via the single-step pathway (Martins *et al*., 1999; Neves *et al*., 2005; Santos *et al*., 2007; Teo *et al*., 2007). Although MG or GG have never been reported in plants, the genomes of the moss *Physcomitrella patens* and of the spikemoss *Selaginella moellendorffii* also revealed full-length *mgS* homologues (Empadinhas and da Costa, 2010).

The plant S*elaginella moellendorffii* (Division Lycophyta) is a member of an ancient lineage of vascular plants that diverged from the seed plant lineage (Division Spermatophyta) soon after plants colonized terrestrial environments (Kenrick, 2003; Banks, 2009). Trehalose is present in very low levels in most plants except for the desiccation-tolerant plants known as 'resurrection' plants, namely *Selaginella lepidophylla* and *Selaginella tamariscina*, where

trehalose (or sucrose) is the key molecule against osmotic stress and especially drought (Adams *et al*., 1990; Iturriaga *et al*., 2006; Liu *et al*., 2008).

The recent identification and characterization of hydrolases specific for MG and GG in the bacteria *Thermus thermophilus* and *Rubrobacter radiotolerans* allowed us also to detect orthologs in *S. moellendorffii* genome (Alarico *et al.*, unpublished). Since there is very limited information about MG and GG catabolism, these eukaryotic *mgH* homologs represent, in addition to the *mgS* gene, powerful tools for comparative genomics, biochemical and physiological studies in the context of plants adaptation to stress conditions, namely drought (Weng *et al*., 2005). To examine the significance of *mgS* and *mgH* detection in *S. moellendorffii* and of the putative MG/GG biosynthesis and hydrolysis in this land plant, we analyzed the organic solute pools in different species of *Selaginella* and characterized the first eukaryotic enzymes for the synthesis and catabolism of MG and GG.

MATERIALS AND METHODS

Identification, sequence analysis, cloning and functional expression of *mgS* and *mgH* genes from S*elaginella moellendorffii*, strains and growth conditions

To identify the mannosylglycerate synthase gene (*mgS*) from S*elaginella moellendorffii*, the *Rhodothermus marinus* MgS sequence (GenPept accession number YP_003289793) was used as probe for BLAST searches at the National Center for Biotechnology Information (NCBI, http://blast.ncbi.nlm.nih.gov/Blast.cgi) database. BLAST analysis with the *Thermus thermophilus* HB27 (YP_004589) mannosylglycerate hydrolase (MgH) sequence (Alarico *et al*., unpublished) allowed us to identify two copies of the *mgH* gene in S*elaginella moellendorffii* genome.

To circumvent possible codon usage and context disparities between *S. moellendorffii* genes and the host for gene expression, both the *mgS* and *mgH* gene sequences from *S. moellendorffii* were optimized for *E. coli* expression and synthesized (GenScript Corp.). The *mgS* gene was cloned between the *Nco*I and *Hind*III sites and the *mgH* gene was cloned between the *Eco*RI and *Hind*III sites of the expression vector pET30a (Novagen) to allow the

translation of vector-encoded N-terminal His-tagged proteins. The constructs were sequenced to confirm the identity of the insert (Macrogen Europe, Netherlands) and transformed into *E. coli* BL21 (DE3).

The recombinant *E. coli* were grown to mid-exponential phase of growth (OD_{610nm}=0.8-1.0) in LB medium (pH 7.0) with kanamycin (30 µg/ml) at 30°C, in a shaker (180 rpm) with continuous aeration. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added at a final concentration of 1.0 mM (*mgS*) or 0.5 mM (*mgH*) to induce gene expression, the temperature was lowered to 25°C and cells carrying $(His)_6$-MgS and $(His)_6$-MgH were allowed to grow for additional 18 h. Cells were harvested by centrifugation (9000 × g, 10 min, 4°C) and suspended in 20 mM Bis-tris propane buffer (BTP) at pH 7.5 with 10 mM $MgCl_2$, DNase I (10 µg/ml), and a protease inhibitor cocktail (Roche). *E. coli* cells were disrupted in a French-press cell followed by centrifugation (15000 × g, 30 min, 4°C) to remove debris. The supernatants were filtered through a 0.22 µm-pore-size filter (Schleider & Schuell) and used for protein purification by fast protein liquid chromatography (FPLC).

Chemicals

Mannosylglycerate (MG), mannosylglyceramide (MGA) and glucosylglycerol were obtained from Bitop (Germany). Glucosylglycerate (GG) was chemically synthesized as described below. MGG was synthesized as previously described (Fernandes *et al*., 2010). GGG was kindly supplied by Helena Santos (ITQB, Oeiras). All other chemicals were obtained from Sigma-Aldrich. The chemical synthesis of glucosylglycerate was performed as previously described by Lourenço and co-workers (2009).

Preparation of cell-free extracts and enzyme assays for detection of MG and GG synthesis and hydrolysis

The activity of the recombinant MgS in extracts was detected in reaction mixtures (50 µl) containing 25 µl of cell-free extract, 5 mM (each) of GDP-mannose/GDP-glucose and D-glycerate, and 5 mM $MgCl_2$ in 25 mM BTP (pH 7.5), after incubation for 20 min, at 25°C. The

synthesis of MG or GG was monitored by thin-layer chromatography (TLC) with a solvent system composed of chloroform/methanol/acetic acid/water (30:50:8:4, v/v). MG and GG were visualized by spraying with α-naphthol-sulfuric acid solution followed by charring at 120ºC (Silva *et al*., 2003). MG, GG, mannose, glucose, GDP-mannose, GDP-glucose and guanosine were used as standards. *E. coli* cell-free extracts carrying an empty vector were used as negative controls.

The reaction mixture (50 µl) used to detect the activity of the recombinant MG hydrolase (MgH) in extracts and during purification contained 25 µl of sample, 5 mM MG or 5 mM GG in 25 mM morpholineethanesulfonic acid buffer (MES) at (pH 6.0). The mixture was incubated at 30°C for 30 min and cooled on ethanol-ice. Reaction products were loaded onto TLC plates and visualized as described above.

Purification of recombinant MgS and MgH from *S. moellendorffii*

E. coli cells carrying the recombinant MgS or MgH from *S. moellendorffii* were suspended in 20 mM sodium phosphate buffer, with 0.5 M NaCl and 20 mM imidazole (at pH 8.0 for MgS and pH 7.4 for MgH). The extract carrying the $(His)_6$-MgS was loaded onto a Ni-Sepharose high-performance column equilibrated with 20 mM sodium phosphate buffer with 0.5 M NaCl and 20 mM imidazole (pH 8.0). Elution was carried out with the same buffer containing 500 mM imidazole. The active fractions were loaded onto a Q-Sepharose column equilibrated with 20 mM BTP (pH 8.0) and elution was carried out with a linear NaCl gradient (0 to 0.5 M). The active fractions were pooled, concentrated by centrifugation in 30 kDa cutoff centricons (Amicon) and equilibrated with 20 mM BTP at pH 8.0. The $(His)_6$-MgH was purified to homogeneity in a single step with a Ni-Sepharose column. The active fractions were dialyzed against 50 mM BTP with 50 mM NaCl (pH 7.0). Protein content of all samples was evaluated by the Bradford assay (Bradford, 1976) and the purity was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Characterization of the recombinant MgS

The substrate specificity of the MgS was determined using GDP-, ADP- and UDP-mannose, GDP-, ADP-, UDP- and TDP-glucose, UDP-galactose, GDP-fucose, sucrose, glucose, D-frutose, L-fucose, D-mannose, glucose-1-phosphate, glucose-6-phosphate, mannose-1-phosphate, mannose-6-phosphate and frutose-6-phosphate as possible glucosyl donors; D-, L-, DL-glycerate, 3-phosphoglycerate (3-PGA), DL-lactic acid, glycolic acid, glycerol, D-frutose, glucose, α- and β-glucose-1-phosphate, glucose-6-phosphate, mannose, mannose-1-phosphate and mannose-6-phosphate were tested as possible acceptors. The reaction mixtures (50 µl) containing pure MgS (1.4 µg), 5 mM of each substrate, and 5 mM $MgCl_2$ in 25 mM BTP at pH 8.0, were incubated at 25°C for 20 min. The products were visualized by TLC, as described above. The reverse reaction was also performed using the substrates MG or GG with GDP (all at a final concentration of 5 mM) in 25 mM BTP (pH 8.0) with 5 mM $MgCl_2$ and 2.6 µg of pure MgS, and incubated at 50ºC, for 20 min. The synthesis of GDP-mannose or GDP-glucose, respectively, and glycerate was monitored by TLC with the solvent system acetic acid/ethyl acetate/water/ammonia 25% (6:6:2:1, v/v).

The MG/GG-forming activity was measured by the quantification of nucleoside diphosphate (GDP) released from the condensation of GDP-mannose or GDP-glucose and D-glycerate into MG or GG. Reactions were initiated by the addition of MgS and stopped at different times by cooling on ethanol-ice. The MgS was inactivated by the addition of 3 µl of 1N HCl, neutralized by 5 µl of Tris-HCl 500 mM (pH=9.3) and the reaction volume adjusted to 60 µl with H_2O. Controls were performed to account for possible substrates/products degradation following acid treatment. The amount of GDP released was determined at 340 nm as previously described (Fernandes *et al*., 2007; Nobre *et al*., 2008).

The temperature profile was determined between 10 and 80ºC with GDP-mannose and GDP-glucose, in 25 mM BTP at pH 8.0, with 5 mM $MgCl_2$; the effect of pH was determined at 50°C between pH 5.0 and 11.0 in MES (pH 5.0 to 6.5), BTP (pH 6.5 to 9.5) and *N*-cyclohexyl-3-aminopropanesulfonic acid (CAPS) (pH 10.0 to 11.0) buffers. Cation dependence was examined at 50°C in the presence of chloride salt of Mg^{2+}, Ca^{2+}, Ni^{2+}, Sr^{2+}, Co^{2+}, Mn^{2+} or Zn^{2+} (5 mM) and without cations or with EDTA (2 and 10 mM). The effect of NaCl (10, 50, 100 and 200 mM) and

KCl (50, 100, 250 and 500 mM) was also analyzed. The kinetic parameters K_m and V_{max} (determined from Lineweaver-Burk plots) for GDP-mannose, GDP-glucose and D-glycerate were examined at 25ºC and 50ºC, as described above. Reactions contained increasing concentrations of the appropriate substrate (0.1-20 mM) and 5 mM of the fixed substrate and were performed in 25 mM BTP at pH 8.0 with 5 mM $MgCl_2$ and 1.4 µg of MgS. All experiments were performed in triplicate.

Characterization of the recombinant MgH

Compounds tested as possible substrates were the disaccharides (α,α-, α,β-, and β,β-trehalose, sucrose, nigerose, turanose, maltose, cellobiose, leucrose, palatinose, gentiobiose, melibiose, α-1,2- and α-1,6-mannobiose), the trisaccharides (maltotriose, isomaltotriose, raffinose, panose), other maltoligosaccharides (maltotetraose, maltoheptaose), MG, mannosylglyceramide (MGA), GG, mannosylglucosylglycerate (MGG), glucosylglucosylglycerate (GGG) and glucosylglycerol. The reverse reaction (MG or GG synthesis) was tested with GDP-glucose, GDP-mannose, D-mannose, mannose-1P and -6P as possible donors and with D-glycerate, DL-glycerate and 3-PGA as possible acceptors. The reaction mixtures (50 µl) containing pure MgH (2.6 µg), 2.5 mM of each substrate in 25 mM MES buffer (pH 6.0), with or without $MnCl_2$ (10 mM), were incubated at 40ºC for 30 min. Products were visualized by TLC as described above with chloroform/methanol/acetic acid/water (15:25:4:2, v/v/v/v). Glucosylglycerol hydrolysis was examined with the Glucose Oxidase (GO) assay kit (Sigma) as described below.

Recombinant MgH activity was measured from the mannose released from MG, quantified at 340 nm from the amount of NADPH formed, with the K-MANGL 01/05 assay kit (Megazyme). The glucose released from GG was determined at 540 nm from the amount of oxidized o-Dianisidine formed with the Glucose Oxidase (GO) assay kit, according to the manufacturers' instructions. Standard reaction conditions (50 µl) contained 5 mM of MG or GG in 25 mM MES (pH 6.0) and 1.0 µg of pure recombinant MgH. The enzyme was inactivated in a boiling water bath (2 min) with appropriate controls and cooled on ethanol-ice.

The temperature profile with MG was determined between 20 and 60ºC in 25 mM MES at pH 6.0 and the effect of pH was determined at 40°C between pH 5.0 and 8.0 with citrate/Na_2HPO_4 (pH 5.0), MES (pH 5.5-6.5) and BTP (pH 7.0-8.0) buffers. Cation dependence and the effect of NaCl and KCl were examined at 40°C as described for the MgS. The K_m and V_{max} values were determined at 25ºC and 40ºC with various concentrations of GG and MG (1-75 mM) after incubation of mixtures for different periods (0-8 min) in 25 mM MES at pH 6.0, as described above. The molecular masses of the recombinant MgS and MgH were estimated at room temperature on a Superdex 200 as previously described (Nobre *et al*., 2008). All experiments were performed in triplicate.

Plant material, extraction and quantification of *Selaginella* spp. intracellular organic solutes by NMR

Five different species of *Selaginella*, namely *S. braunii*, *S. moellendorfii*, *S. sanguinolenta*, *S. stauntoniana* and *S. uncinata* were obtained from Plant Delights Nursery, Inc., USA, in hydrated state. The plant material obtained from the suppliers was processed after eight weeks storage under hydrated conditions, the dry weight of the samples was determined and the extraction of solutes was performed twice with boiling 80% ethanol. The extracts were freeze-dried and resuspended in ^2H_2O for ^1H-nuclear magnetic resonance (NMR) analysis, as previously described (Empadinhas *et al*., 2007). A second extraction of the compatible solutes from the *Selaginella* species was performed. The plants used were the same as before, but had been stored without watering, for two months, at room temperature.

Dried *Selaginella lepidophylla* was obtained from a supplier. The desiccated leaves were sampled by peeling off the external branches (completely brown). After hydration of the whole plant in water for around 16 hours, green leaves/branches were removed from the inner part of the plant and extracted.

For the extraction of organic solutes from all *Selaginella* spp., leaves were ground to a fine powder in liquid nitrogen with a mortar and pestle and the extraction was then executed, as described above.

RESULTS

Solutes accumulation on the *Selaginella* species

All *Selaginella* plants, in hydrated and dehydrated state, accumulated primarily trehalose and sucrose (Table 3.1). The results show that there is considerable variation in the accumulation of compatible solutes among the *Selaginella* species examined and that, in most cases, the fresh leaves have higher levels of compatible solutes than the dry leaves. Moreover, the levels of sucrose are generally lower than those of trehalose in the six *Selaginella* species (Table 3.1).

Mannosylglycerate and glucosylglycerate were not detected, by ^{1}H-NMR analysis, in any of the plants, under any of the conditions tested (Table 3.1).

Table 3.1. Accumulation of organic solutes by *Selaginella* spp., quantified by ^{1}H-NMR.

Plant species	Trehalose (mg/g plant)	Sucrose (mg/g plant)	Glucose (mg/g plant)	GB (mg/g plant)
S. moellendorffii	24.8 ± 2.5	37.1 ± 25.0	5.2	trace
S. moellendorffii*	9.4	5.6	0.9	0.1
S. uncinata	38.2 ± 9.7	23.3 ± 5.0	10.0	0.7
S. uncinata*	35.8	32.2	1.0	-
S. stauntoniana	7.0	8.9	6.7	0.4 ± 0.2
S. stauntoniana*	1.5	2.6	0.7	0.4
S. braunii	56.6 ± 10.1	12.2 ± 1.1	2.7	trace
S. braunii*	83.5	25.7	-	-
S. sanguinolenta	2.8	31.8	1.9	trace
S. sanguinolenta*	1.4	4.6	0.6	0.2
S. lepidophylla **(hydrated)**	18.3 ± 0.6	6.6 ± 0.1	9.0 ± 0.3	0.5 ± 0.0
S. lepidophylla **(dried)**	1.6 ± 0.1	0.6 ± 0.1	trace	trace

Abberviations: GB, Glycine Betaine. * After 2 months of dehydratation.

Identification of the gene coding for MgS and for MgH

BLAST searches with the MgS from *Rhodothermus marinus* allowed the detection of a sequence with 56% amino acid identity in the genome of *S. moellendorffii* (http://genome.jgi-psf.org/Selmo1/Selmo1.home.html). The MgS had 70% amino acid identity with a protein from *Physcomitrella patens* (XP_001764114) and 49% and 55% with two sequences from *Griffithsia japonica* (AAM93991 and AAP80838), respectively (Table 3.2).

Table 3.2. Amino acid sequence identities between MgS proteins (*Selaginella moellendorffii*, *Rhodothermus marinus* DSM 4252, *Griffithsia japonica*) and a putative glycosyltransferase (*Physcomitrella patens*).

Organisms	Accession number	Length (aa)	aa identity (%)
Plants			
Selaginella moellendorffii	XP_002978745	422	100
Physcomitrella patens	XP_001764114	429	70
Bacteria			
Rhodothermus marinus	YP_003290498	397	56
Red alga			
Griffithsia japonica	AAM93991	214	49
	AAP80838	119	55

Although the genome of the marine algae *Gracilaria changii* is not available, an expressed sequence tag (EST) coding for a putative MgS (DV967051) was identified (Teo *et al*., 2007). The MgS gene (*mgS*) was annotated as a putative glycosyltransferase belonging to the GT78 family on the carbohydrate-active enzyme database (CAZy, www.cazy.org) (Henrissat and Coutinho, 2001) with 1269 bp coding for a polypeptide of 422 amino acids with a calculated mass of 47.9 kDa, although the protein was shown to behave as a tetramer in solution, with a mass of about 217.2 $\pm$ 23.0 kDa.

BLAST searches with the MgH sequence from *Thermus thermophilus* HB27 (YP_004589) (Alarico *et al*., unpublished) led to the identification of two homologues (XP_002961898 and XP_002990235) in the genome of *Selaginella moellendorffii* with only 4 different amino acids

and 52% identity. The MgH had 60% amino acid identity with proteins from *Physcomitrella patens* subsp. *patens* (XP_001772747) and 54% and 51% identity with proteins from the bacterium *Meiothermus silvanus* (YP_003684093) and *Marinithermus hydrothermalis* (YP_004368038), respectively. Lower sequence identity (46%, 44% and 42%) was detected with proteins from the bacteria *Truepera radiovictrix* (YP_003704625) and *Rhodothermus marinus* (YP_003291046, YP_003289793), respectively (Table 3.3).

Table 3.3. Amino acid sequence identities between MgH proteins of *Selaginella moellendorffii* and homologues from different organisms.

Organism	Accession number	Length (aa)	aa identity (%)
Plants			
Selaginella moellendorffii	XP_002961898	488	100
	XP_002990235	488	99
Physcomitrella patens	XP_001772747	477	60
	XP_001785177	468	52
	XP_001761811	529	68
Bacteria			
Meiothermus silvanus	YP_003684093	423	54
Marinithermus hydrothermalis	YP_004368038	428	51
Thermus thermophilus HB27	YP_004589	415	52
Truepera radiovictrix	YP_003704625	429	46
Rhodothermus marinus	YP_003291046	444	44
	YP_003289793	452	42

The putative MgH gene (XP_002961898) had 1467 bp encoding a polypeptide of 488 amino acids with a calculated mass of 54.53 kDa, and belonged to the group of unclassified putative glycoside hydrolases in the corresponding database (www.cazy.org). Gel filtration experiments indicated that the recombinant MgH behaved as a monomeric protein with a molecular mass of 55.3 ± 1.6 kDa.

BLAST searches with known MpgSs (Cazy family GT55), GpgSs (GT81) and MpgPs/GpgPs (EC

3.1.3.70) for the two-step synthesis for MG or GG failed to detect any homologs in *S. moellendorfii* genome.

Cloning, functional expression and purification of the recombinant MgS and MgH

The native *mgS* and *mgH* gene sequences from the plant *Selaginella moellendorffii* are intronless, which facilitated their expression in *E. coli*. Activity assays carried out with *E. coli* cell extracts harboring the MgS gene led to the synthesis of MG and GG and extracts harboring MgH could hydrolyze both solutes while the control extracts from *E. coli* with empty vectors did not (results not shown). The purification of recombinant His-tagged proteins yielded pure proteins as judged by SDS-PAGE (Fig. 3.1). Both enzymes lacked detectable activity for the reverse reactions.

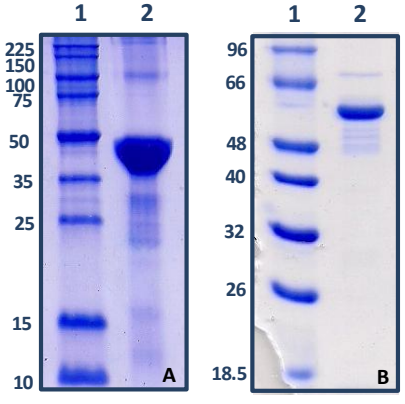

Figure 3.1. SDS-PAGE showing the recombinant mannosylglycerate synthase and hydrolase from *S. moellendorffii*. Lane 1(A and B) – Protein molecular weight markers (kDa); Lane 2 (A) Purified recombinant MgS(His)$_6$ and Lane 2 (B) Purified recombinant MgH(His)$_6$.

MgS properties

Among the substrates tested, the recombinant MgS synthesized MG and GG with high specificity from GDP-mannose/GDP-glucose and D-glycerate. ^{1}H-NMR spectra of the MgS reaction at 50ºC showed that the conversion of GDP-mannose/GDP-glucose and D-glycerate into MG/GG was complete while at 25ºC the substrates were not fully converted (Fig. 3.2).

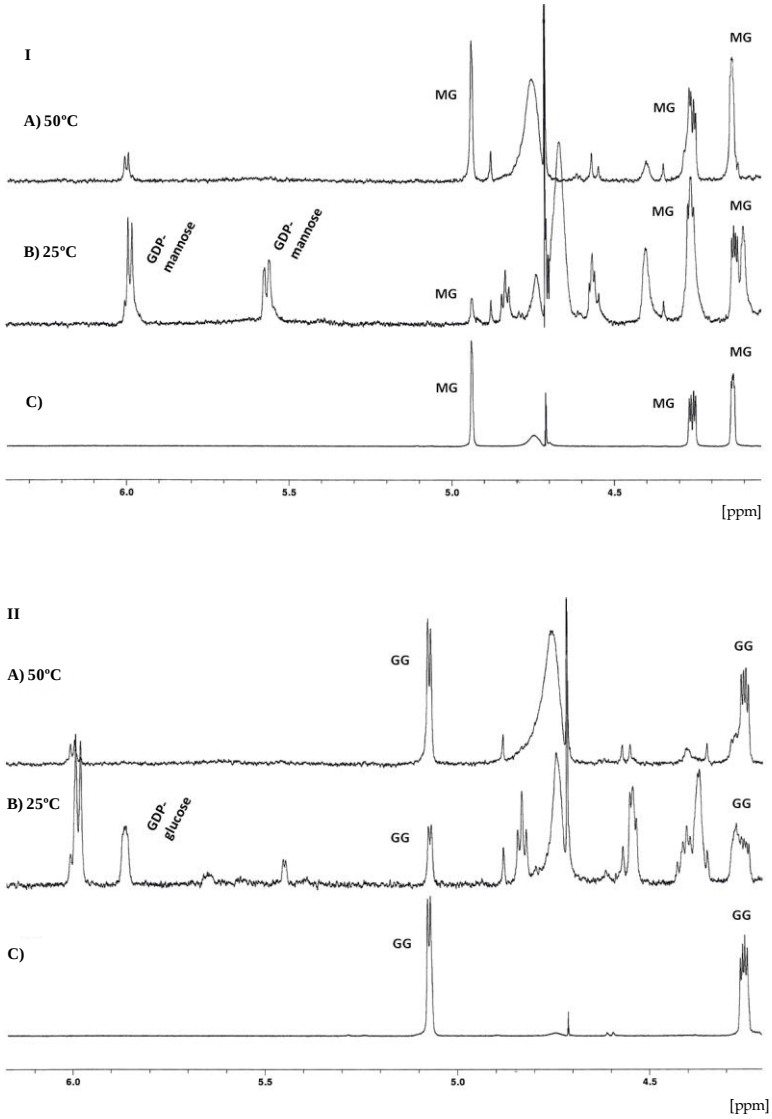

Figure 3.2. ^{1}H-NMR spectra of the MgS reaction at 50ºC and 25ºC. Reaction mixture contained 5 mM of each substrate GDP-mannose (I)/GDP-glucose (II) and D-glycerate, 5 mM $MgCl_2$ in 25 mM BTP (pH 8.0) and 2.7 µg of recombinant MgS. Reactions were incubated for 20 min, at 50ºC (A) and at 25°C (B). Standards (C): mannosylglycerate (I) and glucosylglycerate (II).

The MgS enzyme was active between 10 and 80°C, with maximum activity around 50ºC (Fig. 3.3 A, Table 3.4).

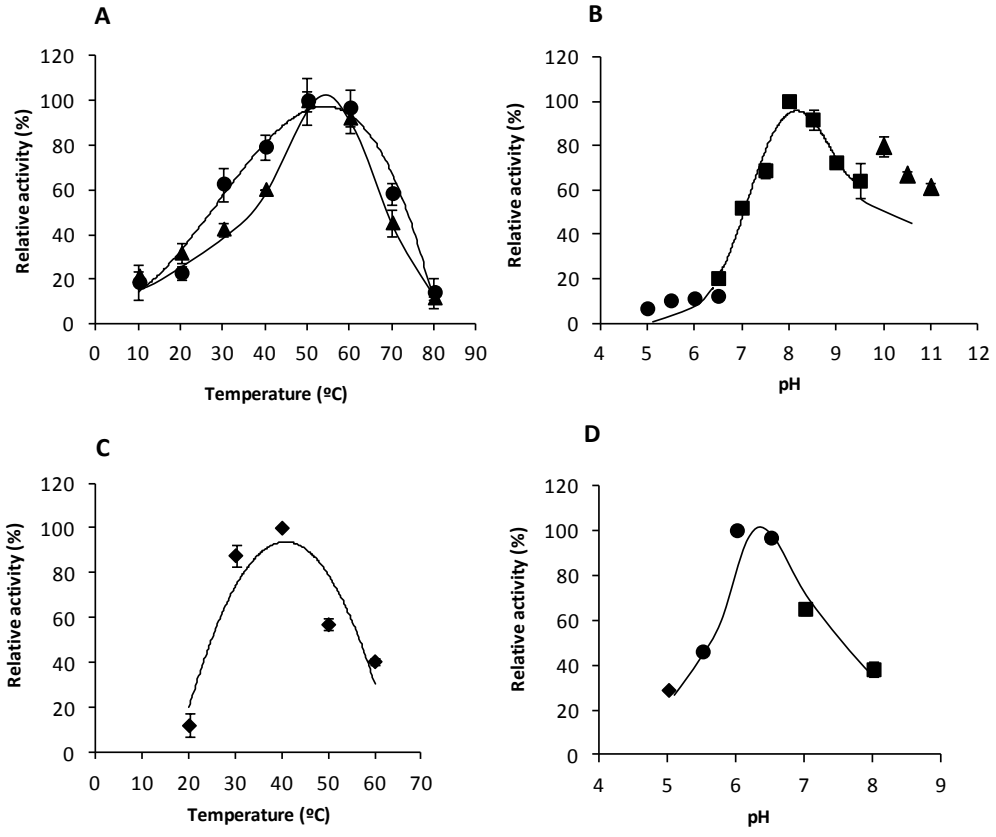

Figure 3.3. Temperature and pH dependence of recombinant MgS (A and B) and MgH (C and D) activity. A) MgS activity was determined at temperatures between 10 and 80°C, with GDP-mannose (•) and GDP-glucose (▲) as glucosyl donors; B) pH dependence was determined at pHs between 5.0 and 11.0 in the following buffers: MES for pH 5.0 to 6.5 (•), BTP for pH 6.5 to 9.5 (■) and CAPS for pH 10.0 to 11.0 (▲). C) MgH activity was determined at temperatures between 20 and 60°C, with MG as substrate (♦); D) pH dependence was determined at pHs between 5.0 and 7.0 in the following buffers: Citrato/Na_2HPO_4 for pH 5.0 (♦), MES for pH 5.5 to 6.5 (•) and BTP for pH 7.0 to 8.0 (■). The data are the means of three independent experiments. Error bars indicate SD.

Table 3.4. Biochemical properties of the MgS and MgH of *Selaginella moellendorffii*.

Property (Unit)		MgS	MgH
No. of amino acid residues		422	488
Calculated molecular mass (kDa)		47.9	54.5
Oligomerization		Tetramer	Monomer
Optimum temperature (ºC)		50	40
Optimum pH		8.0	6.0-6.5
Mg^{2+} / Mn^{2+}	**0 mM**	15.9 ± 1.3[b]	76.3 ± 0.7[c]
dependence[a]	**5 mM**	100[b]	82.6 ± 2.7[c]
	10 mM	95.8 ± 4.9[b]	100[c]

[a]Expressed as percentage of the maximum activity of the MgS (with GDP-mannose and D-glycerate) and of MgH (with MG); Mg^{2+} dependence for MgS and Mn^{2+} dependence for MgH. [b]Assays carried out at 50°C; [c]Assays carried out at 40ºC.

Among the sugar donors examined only GDP-glucose and GDP-mannose could be donors, both at 25ºC and 50ºC, with a slight preference for the former while D-glycerate was the only acceptor. Within the pH range examined the activity was maximal at pH 8.0, at 50°C (Fig. 3.2 B, Table 3.4). MgS was active without cations, but maximum activation was obtained with 5 mM of Mg^{2+} (Table 3.5).

Table 3.5. Effect of Mg^{2+} and Mn^{2+} concentrations on the activity of recombinant MgS and MgH from *Selaginella moellendorffii*.

	MgS	MgH
(mM)	**Mg^{2+} dependence[a]**	**Mn^{2+} dependence[b]**
0.0	15.9 ± 1.3	76.3 ± 0.7
0.5	45.2 ± 4.1	-
1.0	78.5 ± 3.0	-
2.5	-	76.4 ± 6.1
5.0	100.0	82.6 ± 2.7
10.0	95.8 ± 4.9	100.0
20.0	-	59.6 ± 7.5
50.0	87.4 ± 3.1	-

[a] Expressed as percentage of maximum activity of the MgS with GDP-mannose and D-glycerate; [b] Expressed as percentage of maximum activity of the MgH with MG.

Other cations (5 mM) inhibited MgS in the following order: Sr^{2+}<Ni^{2+}<Co^{2+}<Ca^{2+}<Mn^{2+}<Zn^{2+}. The presence of higher concentrations of NaCl and KCl also gradually inhibited MgS activity (results not shown). MgS was very stable on ice (> 2 months) and upon freeze/thawing, although the freeze/thawing cycles promoted enzyme precipitation. The MgS exhibited Michaelis-Menten kinetics and the K_m and V_{max} values for the substrates at 25ºC and at 50ºC are indicated in Table 3.6.

Table 3.6. Kinetic parameters for the substrates involved in the synthesis of MG and GG of recombinant MgS, determined at 25°C and 50ºC.

Fixed substrate (5 mM)	**Varied substrate**	V_{max} (µmol/min.mg protein)	K_m (mM)	V_{max}/K_m **ratio**
25ºC				
D-glycerate	**GDP-mannose**	2.6 ± 0.1	0.6 ± 0.1	4.3 ± 1.0
GDP-mannose	**D-glycerate**	2.6 ± 0.1	1.2 ± 0.3	2.2 ± 0.3
D-glycerate	**GDP-glucose**	2.0 ± 0.1	0.2 ± 0.1	10.0 ± 1.0
GDP-glucose	**D-glycerate**	2.2 ± 0.1	1.6 ± 0.4	1.4 ± 0.3
50ºC				
D-glycerate	**GDP-mannose**	9.5 ± 0.4	2.4 ± 0.4	4.0 ± 1.0
GDP-mannose	**D-glycerate**	10.9 ± 0.9	4.4 ± 0.7	2.5 ±1.3
D-glycerate	**GDP-glucose**	8.0 ± 0.3	1.0 ± 0.2	8.0 ± 1.5
GDP-glucose	**D-glycerate**	11.0 ± 1.1	3.4 ± 1.0	3.2 ± 1.1

To determine the kinetics parameters, GDP-mannose, GDP-glucose and D-glycerate were used up to 15-20 mM. The ability to efficiently synthesize both MG and GG was alike. Both at 25 and 50ºC the K_m values for GDP-glucose were slightly lower than those for GDP-mannose, reflecting a higher catalytic efficiency (V_{max}/K_m ratio) towards the synthesis of GG (Table 3.6).

At 25ºC the K_m for glycerate (with GDP-mannose) was slightly lower than with GDP-glucose and the catalytic efficiency towards glycerate (with GDP-mannose) was higher (Table 3.6). At 50ºC, the K_m for glycerate (with GDP-mannose) was slightly higher than that with GDP-glucose, indicating that MgS affinity towards glycerate with GDP-mannose was slightly lower (Table 3.6).

MgH properties

The MgH sequences had conserved domains found in the trehalase family (Pfam01204), but the activity towards trehalose isomers (α,α, α,β, and β,β) and for other oligosaccharides tested could not be detected (results not shown). The enzyme catalyzed the hydrolysis of MG and of GG, yielding mannose and glucose, respectively, and glycerate. The enzyme was active between 20 and 60°C, with maximum activity at around 40ºC (Fig. 3.3 C, Table 3.4). Within the pH range examined the activity of the enzyme was maximal at pH 6.0-6.5 (Fig. 3.3 D, Table 3.4). MgH was active without cations and the activity was not affected by the addition of EDTA (5 or 10 mM). Maximum activation was obtained with 10 mM of Mn^{2+} (Table 3.5). Other divalent cations could also activate the enzyme in the following order of efficiency: Mn^{2+}>Mg^{2+}>Ca^{2+} (5 mM) and Mn^{2+}>Ca^{2+}>Mg^{2+} (10 mM); the enzyme activity was inhibited by Co^{2+}, Sr^{2+}, Ni^{2+} and Zn^{2+} and by NaCl or KCl (results not shown). MgH was very stable on ice (>2 months) and upon freeze/thawing. The recombinant enzyme exhibited Michaelis-Menten kinetics at 40ºC and 25º and the K_m and V_{max} values for MG and GG are indicated in Table 3.7. Both at 40ºC and 25ºC, the enzyme had higher activity with MG than with GG, but the affinity towards GG was slightly higher. The catalytic efficiencies towards MG or GG at 25ºC were comparable, while at 40ºC, the catalytic efficiency towards MG was nearly three times higher (Table 3.7).

Table 3.7. Kinetic parameters for the substrates involved in the hydrolysis of MG and GG of recombinant MgH, determined at 25°C and 40ºC.

Varied substrate	V_{max} (µmol/min.mg protein)	K_m (mM)	V_{max}/K_m ratio
25ºC			
MG	13.6 ± 0.9	21.4 ± 4.0	0.6 ± 2.3
GG	2.3 ± 0.1	2.9 ± 0.5	0.8 ± 0.2
40ºC			
MG	40.5 ± 1.2	11.8 ± 1.1	3.4 ± 1.1
GG	7.3 ± 0.3	5.9 ± 0.8	1.2 ± 0.4

DISCUSSION

The detection of a mannosylglycerate synthase (MgS) gene in the *Selaginella moellendorffii* genome and the lack of studies on mannosylglycerate (MG) biosynthesis and accumulation in eukaryotes led us to study the enzymes involved in the synthesis of this solute in this plant. Common compatible solutes in plants include amino acids and derivatives (e.g. α-glutamate, proline, citrulline, ectoine and glycine betaine), polyols (glycerol, mannitol, inositol and pinitol) and mono-, di- and oligosaccharides (fructose, sucrose, trehalose and fructan), all of which highly water soluble (da Costa *et al.*, 1998; Roberts, 2000; Yancey, 2005; Chen and Jiang, 2010). The primary function of compatible solutes is to maintain cell turgor and, in the case of plants, to facilitate the uptake of water from soil. These compounds were also proposed to protect and stabilize membranes and/or proteins from stresses such as drought, high temperature and salinity, and can also act as free-radical scavengers (Chen and Murata, 2002; Wang *et al*., 2003).

Water is a key element to maintain phospholipids in a fluid phase into biological membranes and for the folding of many proteins. The loss of water, therefore, compromises cellular organization subjected to dehydration. Most anhydrobiotic organisms manage to survive periods of severe desiccation and preserve native cellular structures in the almost totally absence of water, presumably due to the accumulation of trehalose, which is sometimes called the carbohydrate of dormancy (Potts, 1994; da Costa *et al*., 1998; Iturriaga *et al*., 2009; Erkut *et al*., 2011).

The ancient vascular plants *Selaginella lepidophylla* and *Selaginella tamariscina* can also survive anhydrobiosis by accumulating trehalose or sucrose. An interchange between these solutes occurs during dehydration in *S. lepidophylla*, but not in *S. tamariscina*, where trehalose is always the dominant solute (Adams *et al*., 1990; Liu *et al*., 2008). The species of *Selaginella* studied in this work also accumulate trehalose and sucrose as principal organic solutes upon dehydration, along with lesser levels of glucose and traces of glycine betaine, but MG or GG were not detected under the conditions tested.

The accumulation and biosynthesis of MG or GG in microorganisms has been studied in detail

and, whereas MG is more frequently found in hyper/thermophilic bacteria and archaea, GG has been frequently encountered in mesophilic bacteria and archaea and rarely in thermophiles, with the exception of *Persephonella marina* (Empadinhas and da Costa, 2010).

Mannosylglycerate synthase (MgS) is an extremely rare enzyme found only in the thermophilic bacterium *Rhodothermus marinus*. The glycosyltransferase family GT78 was created to accommodate this "retaining" enzyme, which now also includes the MgS from *S. moellendorffii* (Martins *et al*., 1999; Fernandes *et al*., 2010). Unlike the MgS from *R. marinus*, which had a marked preference for GDP-mannose, the enzyme from *S. moellendorffii* used GDP-mannose and GDP-glucose to synthesize MG and GG with comparable efficiencies *in vitro* (Fig. 3.4) (Martins *et al*., 1999; Flint *et al*., 2005).

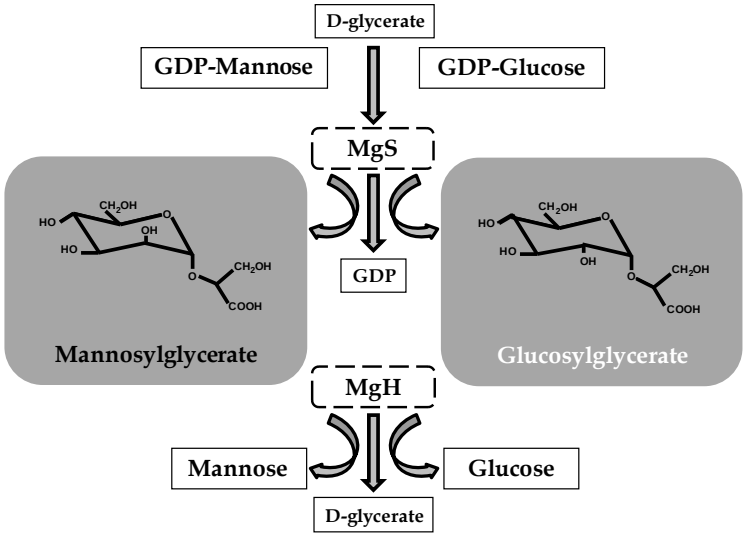

Figure 3.4. Proposed pathway for the synthesis and hydrolysis of MG and GG in the plant *Selaginella moellendorffii*. MgS, mannosylglycerate synthase; MgH, mannosylglycerate hydrolase; GDP, guanosine diphosphate. MgS catalyzes the direct glycosyl transfer of GDP-mannose and D-glycerate into MG and GDP-glucose and D-glycerate into MG. MgH catalyzes the hydrolysis of MG and GG into GDP-mannose and D-glycerate or GDP-glucose and D-glycerate, respectively.

This unprecedented substrate flexibility is shared by mannosyl-3-phosphoglycerate synthase (MpgS) from the thermophlic bacterium *Rubrobacter xylanophilus*, which can synthesize the

phosphorylated precursors for MG and GG *in vitro* while none of the known MpgSs or related GpgSs can synthesize both products (Empadinhas *et al*., 2011).

The presence of a functional MgS in *S. moellendorffii* was not corroborated by the accumulation of any of its possible low molecular weight products. *Petrotoga mobilis* has an active glucosylglycerate synthase (GgS) capable of producing GG but this solute has never been freely detected in the organism. Indeed, GG was the intermediate in a pathway leading to the synthesis of mannosylglucosylglycerate (MGG), which was accumulated during salt or thermal stress (Fernandes *et al*., 2010). Since MG- or GG-derived solutes have also not been detected in these plants, MgS may hypothetically, synthesize MG or GG as precursors for larger macromolecules as those found in actinobacteria, or accumulated under growth conditions not examined in this study.

The GgSs from *Persephonella marina* and *Petrotoga mobilis* could only synthesize GG from glucose donors and glycerate, although the nucleotide specificity was more flexible (Fernandes *et al*., 2007; Fernandes *et al*., 2010). Unlike the MgS from *R. marinus* and the GgSs from *Prs. marina* and *Ptg. mobilis*, which were strictly dependent on divalent cations and unaffected by NaCl or KCl, the plant MgS was independent of cations and was inhibited by NaCl or KCl. Interestingly, the affinities of the *S. moellendorffii* MgS for GDP-mannose and D-glycerate were significantly lower than those of the *R. marinus* MgS for the same substrates. This suggests a higher relative abundance of both precursors in the plant cells or that they are recruited by different pathways in *R. marinus*. In fact, the *R. marinus* MpgS uses GDP-mannose in an alternative pathway for MG (Borges *et al*., 2004).

The plant MgH was, like the MG hydrolases from *Thermus thermophilus* and *Rubrobacter radiotolerans*, able to efficiently hydrolyze MG and GG (Fig. 3.4) (Alarico *et al*., unpublished). This substrate ambiguity was also observed for a GDP-mannose mannosyl hydrolase from *E. coli*, which catalyzed the hydrolysis of GDP-mannose or GDP-glucose into mannose or glucose, respectively, and GDP (Legler *et al*., 2000). Unlike the bacterial MgHs that had a narrow pH range for activity with maximum at pH 4.0-4.5, the *S. moellendorffii* MgH was maximally active at pH 6.0-6.5. Although the catalytic efficiencies of the thermophilic MgHs towards MG or GG were comparable, the catalytic efficiency of the *S. moellendorffii* MgH was three-fold higher

towards MG than with GG at 40ºC. However, at physiological temperatures (25ºC), the efficiency is higher with GG (Alarico *et al*., unpublished). Further studies on the adaptation of *S. moellendorffii* to stress are required to explain this apparent paradox.

The distribution of the MgS gene in only a few groups of phylogenetically unrelated organisms (*Rhodothermus marinus*, *Griffithsia japonica*, *Caloglossa leprieurii, Gracilaria changii*, *Physcomitrella patens* and *Selaginella moellendorffii*) may indicate phenomena of lateral gene transfer (LGT) and endosymbiosis of a prokaryote carrying MgS (Eisen, 2000). The conservation of *mgS* from thermophilic bacteria to eukaryotes indicates selective advantage for the acquisition of MG biosynthetic genes by eukaryotes (Martin *et al*., 2003). Moreover, the optimal activity at high temperature (50ºC) and the preservation of about 50% of maximal activity at 70ºC also suggests a thermophilic origin for the plant MgS. However, due to the rarity of *mgS* homologs the evolution of this enzyme remains untraceable.

The presence of *mgH* genes in *S. moellendorffii* genome was also surprising since, with the exception of *P. patens*, they were absent from other eukaryotic lineages with sequenced genomes. This gene, unlike the *mgS*, was found in several bacterial lineages, some of which are known to synthesize MG or GG, and this distribution among bacteria indicates that the plant *mgH* may also have been acquired by LGT (Alarico *et al*., unpublished). Interestingly, the alphaproteobacteria *Rhizobium leguminosarum* and *Agrobacterium radiobacter*, known to establish intimate relations with plants, possess *mgH* homologs (YP_002973553 and YP_002542329, respectively) that could have transferred to plants (Knief *et al*., 2011).

In this work, we provide a comprehensive study of the biochemical and kinetic properties of two enzymes involved in the metabolism of the organic solutes MG and GG in an unexpected host, the plant *Selaginella moellendorffii*.

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CHAPTER 4

Discussion

CONTENTS

1. TREHALOSE

Trehalose is a ubiquitous disaccharide widely distributed in a large range of organisms and characterized most especially in insect, plant, and microbial cells. The peculiar properties of trehalose, known as an efficient osmolyte, have been known for many years. As described in the introduction of this thesis, trehalose is synthesized as a stress-responsive factor when cells are exposed to environmental stresses such as desiccation, heat, cold, oxidation to retain cellular integrity.

Trehalose biosynthetic pathways are distributed in all domains of life. From the five known pathways to synthesize trehalose, the Tps/Tpp is the most conserved one, since it exists in organisms of the three domains of life.

In Eukarya, the synthesis of the non-reducing disaccharide trehalose seems to rely only on the two-step pathway Tps/Tpp (Avonce *et al.*, 2006). This pathway, which involves a phosphorylated intermediate, is common to those of other compatible solutes such as sucrose, glucosylglycerol, galactosylglycerol and mannosylglycerate (Thomson, 1983; Curatti *et*

al., 1998; Empadinhas *et al.*, 2001; Hagemann *et al.*, 2001; Empadinhas *et al.*, 2003). In contrast to Eukarya domain, Bacteria and Archaea use additional and alternative pathways for trehalose synthesis. Interestingly, vertebrates have lost the capacity to synthesize trehalose; nevertheless can hydrolyze it to glucose, with trehalase.

Previously work by Empadinhas and co-workers (2007) on compatible solute accumulation in *Rubrobacter xylanophilus* and the report that trehalose, along with mannosylglycerate, were the major solutes accumulated, stimulated subsequent work in an attempt to look for the genes, metabolic pathways and enzymes involved in the synthesis of these principal organic solutes. The work presented in chapter 2 is about a unique combination of genetic systems for trehalose synthesis in *R. xylanophilus*.

2. TREHALOSE AND MANNOSYLGLYCERATE GENES IN *Rubrobacter xylanophilus*

In the genome of *Rubrobacter xylanophilus*, genes for four different trehalose pathways were detected, three of which were characterized with the corresponding recombinant enzymes, Tps, Tpp and TreT. Unlike the TreY/TreZ and the TreS pathways, the Tps/Tpp and TreT were active in cells extracts, under the conditions tested (chapter 1).

It has been previously shown that this thermophilic bacterium accumulates trehalose and mannosylglycerate constitutively which may have a synergistic effect on the adaptation of this organism to several stress conditions (Empadinhas *et al.*, 2007).

Some different genetic organizations for trehalose and mannosylglycerate genes have been found in bacteria and archaea. Curiously, in *R. xylanophilus*, the genes involved in the most common trehalose pathway are not organized in an operon-like structure, under the same promoter (chapter 2). A unique promoter sequence was detected upstream from *treT* gene and possibly the *tpp* promoter might be located upstream of the putative adjacent peptidase gene (ORF1) (Fig.4.1).

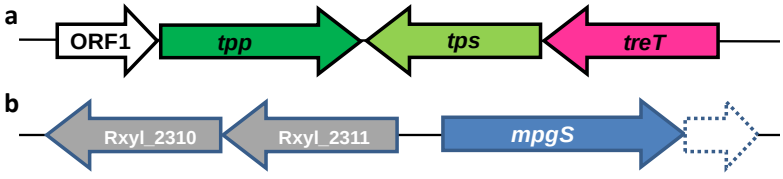

Figure 4.1. Organization of genes leading to the synthesis of trehalose (a) and mannosylglycerate (b) in *Rubrobacter xylanophilus*. Abbreviations: ORF1, putative peptidase gene; *tpp*, trehalose-6-phosphate phosphatase gene; *tps*, trehalose-6-phosphate synthase gene; *treT*, glycosyltransferring synthase gene; Rxyl_2310, putative hydrolase gene (HAD superfamily); Rxyl_2311, putative 5′-nucleotidase gene (EC 3.1.3.5); *mpgS*, mannosyl-3-phosphoglycerate synthase gene (chapter 2) (Empadinhas *et al.*, 2011).

The significance of operon structures for efficient regulation by co-transcription of functionally related proteins indicates that they are expected to be conserved in the course of evolution (Xie *et al.*, 2003). However, Itoh and co-workers (1999) have suggested that operon structures are evolutionary unstable, which might explain the heterogeneity found for trehalose genetic organizations. In *R. xylanophilus* the *tps* and *tpp* genes are sequentially arranged in the genome, although in opposite directions. The regulation of these genes was suggested to be independent, due the existence of two different and face-to-face promoters (chapter 2). This type of genetic organization probably allows a high degree of freedom of gene regulation, that could also occur in the case of divergent genes that are under the same promoter, since a putative regulatory protein may interact with the operator, between the divergent genes, to either activate/repress transcription in both or only one direction (Beck and Warren, 1988).

Interestingly, in several organisms, including *R. xylanophilus*, the TreT gene is clustered with genes involved in alternative pathways for trehalose synthesis. Some archaeal species of the genus *Pyrobaculum* harbor contiguous TreT and Tps/Tpp genes and species of the genus *Sulfolobus* have TreT genes in their genome close to TreY/TreZ genes. In the genome of some Delta-proteobacteria, like *Synthrophobacter fumaroxidans*, *Pelobacter carbinolicus* and *Synthrophus aciditrophicus*, trehalose genes for Tps/Tpp or TreY/TreZ pathways are also present and near the TreT gene (Kouril *et al.*, 2008).

The reversible TreTs of the Euryarchaeota *T. litoralis* and *P. horikoshii* are part of a gene cluster harboring the maltose/trehalose ABC transporter genes and this fact has supported the hypothesis that TreT was involved in trehalose degradation (Horlacher *et al.*, 1998). The

available genome sequence near the TreT gene from *R. xylanophilus* failed to reveal any genes coding for ABC transporter proteins, however we have found that TreT could also catalyze trehalose hydrolysis, but with a very high K_m and low rate (chapter 2).

In *R. xylanophilus* genome the *treS* (Rxyl_0315) and *treY*/*treZ* (Rxyl_0319/Rxyl_0318) genes appear to be part of a different and unique operon-like structure, with a single promoter, which contain genes for glycogen metabolism. Attempts to amplify *treS*, *treY* and *treZ* genes of *R. xylanophilus* were performed, but unfortunately we were not successful, due to the numerous errors detected in the draft genome sequence. These genes, glycogen-branching *(glgB)* (Rxyl_0316) gene and glycogen debranching *glgX* (Rxyl_0317) gene, are located immediately downstream the *treS* and could be involved in the synthesis/degradation of glycogen/maltooligosaccharides (chapter 2) (Mendes *et al.*, 2010).

The absence of activity of the TreS, TreY and TreZ enzymes in cell extracts from *R. xylanophilus* cultures, grown on tryptone and yeast extract, (chapter 2) led us to hypothesize that the respective genes could only be active when maltose or maltooligosaccharides were used as carbon sources. However, this was not verified when the organism was grown on maltose. This theory was supported by TreS of *Corynebacterium glutamicum* that was shown to be functionally active *in vivo* only when maltose was used as the carbon source (Wolf *et al.*, 2003).

The alternative trehalose pathways TreS and TreY/TreZ might be activated by other environmental stimuli, as for example in the nitrogen-fixing symbiotic bacterium *Bradyrhizobium japonicum*s where desiccation activates TreS and Tps/Tpp pathways (Cytryn *et al.*, 2007). In *Propionibacterium freudenreichii* the Tps/Tpp pathway is active in response to osmotic, oxidative, and acid stresses (Cardoso *et al.*, 2007). It may be also possible that the lack of activity observed in relation to TreS pathway could be due to its participation in trehalose catabolism, instead of its synthesis, as was proposed in *Propionibacterium freudenreichii* (Cardoso *et al.*, 2007).

Recently, Mendes and co-workers (2010) described the *R. xylanophilus treS* as a bifunctional gene (fused trehalose synthase/maltokinase gene). The *treS* from *R. xylanophilus* showed 61% and 29% of homology with the individual *treS* and *mak* genes from *Mycobacterium bovis*,

respectively (Mendes *et al.*, 2010). Future work is needed to confirm the real function(s) of the enzyme encoded by *treS* gene in *Rubrobacter xylanophilus*.

Different genomic organizations have also been reported for the genes involved in MG biosynthetic pathways: isolated genes, a structure with consecutive *mpgS*/*mpgP* or bifunctional *mpgS*/*mpgP* and a four-gene-operon-like organization (Empadinhas *et al.*, 2001; 2003; 2004). In *Rubrobacter xylanophilus*, the synthesis of MG in cells extracts was tracked from GDP-mannose and 3-phosphoglycerate, the substrates for the pathway involving a MpgS and a MpgP. The recombinant enzyme was characterized as a mannosyl-3-phosphoglycerate synthase (MpgS), with properties comparable to those of known MpgSs, but with a highly divergent sequence (Empadinhas *et al.*, 2011). MpgS has an unprecedented characteristic of synthesizing MPG and GPG *in vitro*, the precursors of MG and GG, respectively. Its crystal structure revealed a binding-site tailored to specifically select for GDP-containing ligands and a sugar-binding region suited for accommodating both glucose and mannose molecules (Empadinhas *et al.*, 2011).

MpgP gene was not detected near the MpgS gene in the genome of *R. xylanophilus* and the enzyme for the hydrolysis of the phosphorylated MG (MPG) and GG (GPG) remains unidentified.

Contiguous to the *mpgS* gene, but under a different promoter, two genes organized in an operon-like structure, were detected a putative hydrolase gene (HAD superfamily) and a putative 5′-nucleotidase gene (EC 3.1.3.5) (Fig. 4.1) (Empadinhas *et al.*, 2011), although the characterization of the respective enzymes is needed to confirm their possible implication on MPG/GPG dephosphorylation, *in vivo*.

3. TreT - GLYCOSYLTRANSFERRING SYNTHASE FROM *Rubrobacter xylanophilus*

R. xylanophilus is the only known member of the phylum Actinobacteria to harbor a TreT. The presence of *treT* gene in the *R. xylanophilus* genome was unexpected, since this gene was mainly found in hyperthermophilic archaea, in some hyperthermophilic bacteria and in a few deltaproteobacteria (Fig. 4.2).

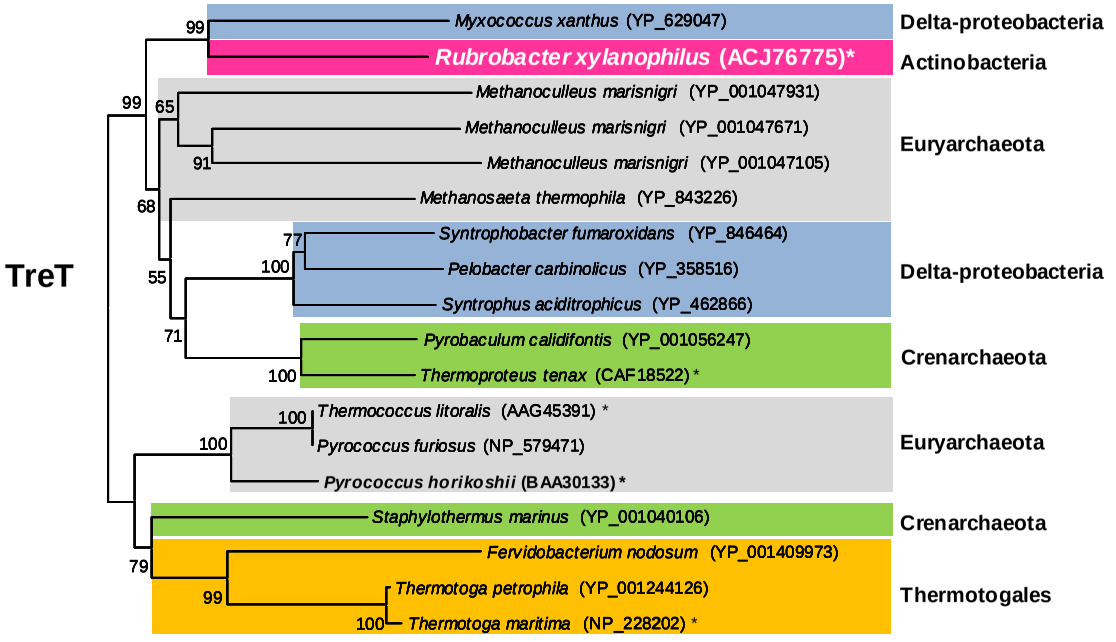

Figure 4.2. Unrooted phylogenetic tree on available amino acid sequences of TreT homologues. Asterisks indicate organisms harboring a functionally characterized TreT enzyme.

The biochemically characterized TreT from *Rubrobacter xylanophilus* shared significant levels of amino acid identity with the four trehalose-glycosyltransferring synthases characterized to date: 47%, 45% and 48% amino acids identity with archaeal TreTs from *Thermococcus litoralis*, *Pyrococcus horikoshii* and *Thermoproteus tenax*, respectively, and 45% with bacterial TreT from *Thermotoga maritima* (Fig. 4.3).

In *Pyrococcus horikoshii* TreT, the conservation of essential residues and the high structural similarity of the N-terminal domain to that of trehalose-6-phosphate synthase (Tps), suggests that the catalytic reaction of TreT would follow a parallel mechanism to that of Tps (Woo *et al.*, 2010). Comparison of the amino acid sequences of the five functionally characterized TreTs showed high levels of sequence conservation and the presence of some highly conserved motifs (Fig. 4.3).

The analysis of TreT crystal structure from *P. horikoshii*, showed a histidine cluster constituted by five residues (His45, His133, His182, His155 and His403), in the active site of the enzyme, which formed a cavity. These residues with arginine 153 (R153) and tyrosine 407 (Y407) are connected by a hydrogen-bond network bridged by putative water atoms at the center (Woo *et al.*, 2010).

Based on sequence homology to other glycosyltransferases the TreT from *Rubrobacter xylanophilus* (chapter 2) is expected to be included into the GT4 family, with a GT-B structural fold topology, like the TreT of *Pyrococcus horikishii* (Woo *et al.*, 2010), but further studies are needed to confirm this classification. So far, among trehalose-synthesizing enzymes, structural information is available only for Tps from *E. coli*, shown to belong to the glycosyltransferase-B fold family (Gibson *et al.*, 2002).

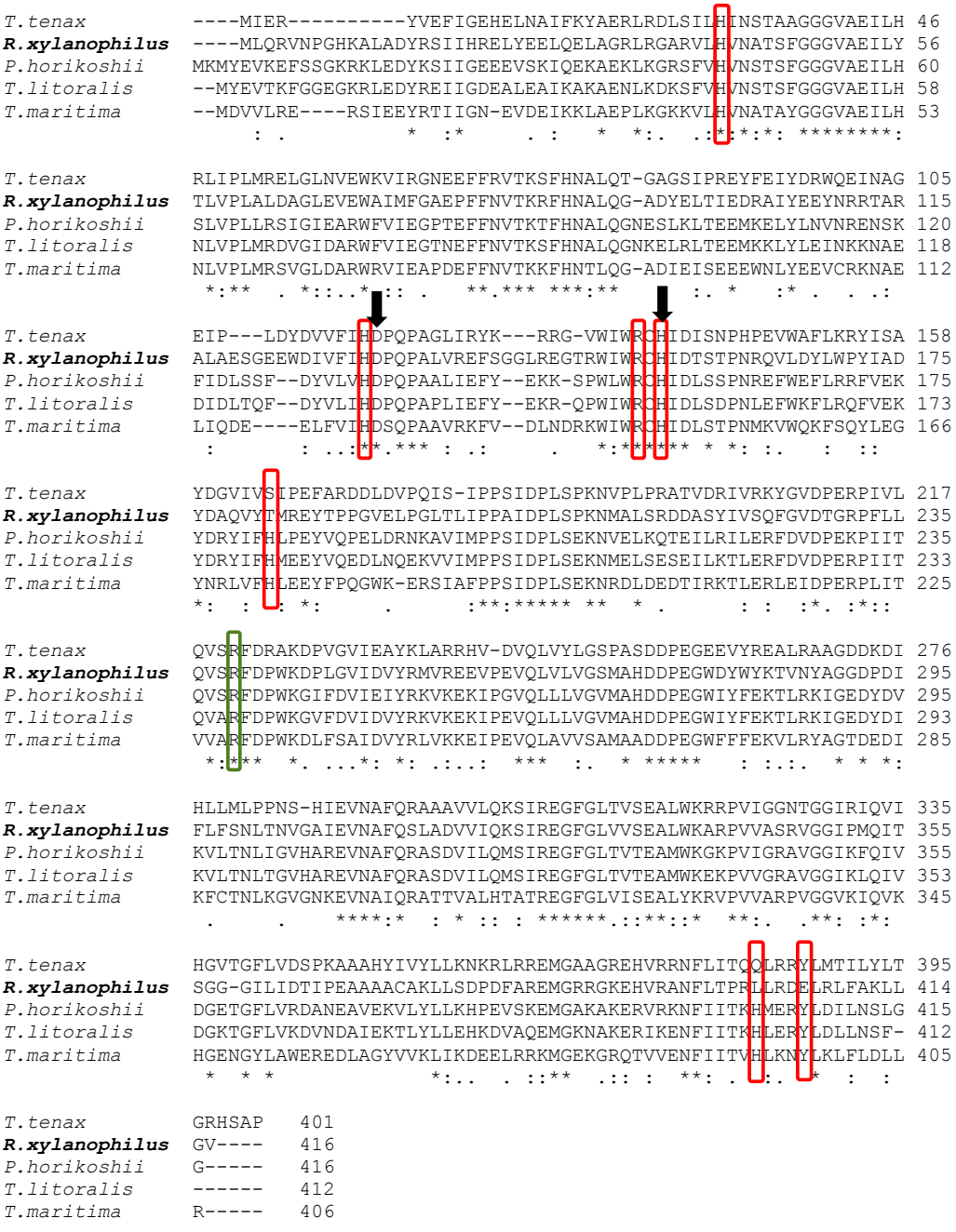

Figure 4.3. Sequence alignment among the five characterized TreTs. The accession numbers for these sequences are as follows: TreT from *Thermococcus litoralis* (AAG45391), TreT from *Pyrococcus horikoshii* (BAA30133), TreT from *Thermoproteus tenax* (CAF18522), TreT from *Thermotoga maritima* (NP_228202) and TreT from *Rubrobacter xylanophilus (*ACJ76775) (Qu *et al.*, 2004; Ryu *et al.*, 2005; Kouril *et al.*, 2008; Ryu *et al.*, 2010) (chapter 2). The Clustal X program was used for sequence alignment (Thompson *et al.*,

1997). Identical amino acids are indicated by '*' and conserved residues are indicated by ':' and '.'; gaps were introduced by Clustal program to optimize the alignment are indicated by '-'. In *P. horikoshii* TreT, the red boxes, indicate the residues that form a histidine cluster and the green box, indicate the residue (arginine 239 – R239), which binds to the phosphate moiety of the donor sugar nucleotide. The black arrows indicate the residues (aspartic acid 134 – D134 and histidine 155 – His155), which are essential residues of glycosyltransferase-B structural fold family (Lairson *et al.*, 2008; Woo *et al.*, 2010).

As mentioned in chapter 2, among the five characterized TreTs, only the TreT from *Thermoproteus tenax* is unidirectional, catalyzing only the formation of trehalose. On the other hand, TreTs from *Thermococcus litoralis*, *Pyrococcus horikoshii Rubrobacter xylanophilus* and *Thermotoga maritima* also catalyze the reversible reaction, being able to hydrolyze trehalose. The preferred function in trehalose degradation was first suggested by the TreT from *Thermococcus litoralis*, due to the organization of the encoding gene (*treT*) in a cluster comprising genes for the trehalose/maltose ABC transporter and due to the induction of TreT by maltose and trehalose in the growth medium (Xavier *et al.*, 1996; Qu *et al.*, 2004). The genetic context of the *treT* gene in a cluster harboring ABC transporter related genes was also described for *P. horikoshii* and *T. maritima*, where TreT catalysed the reversible reaction, i.e., the hydrolyses trehalose (Ryu *et al.*, 2005; Ryu *et al.*, 2010).

In respect to substrate specificities, the recombinant TreT from *R. xylanophilus* used ADP-glucose as well as other NDP-glucose donors, like the archaeal characterized enzymes and the bacterial TreT from *T. maritima*, but not the TreT from *T. tenax*, which used UDP-glucose with more efficiency (chapter 2) (Qu *et al.*, 2004; Ryu *et al.*, 2005; Kouril *et al.*, 2008; Ryu *et al.*, 2010; Ryu *et al.*, 2011).

TreT from the thermophilic bacterium *Thermotoga maritima* is able to employ other monosaccharides, such as mannose and fructose, as acceptors to produce disaccharide analogues of trehalos (Ryu *et al.*, 2010). This type of acceptor specificity has been similarly reported in TreT from *P. horikoshii*, which employed a galactose as an acceptor in the glucosyl transfer reaction with an NDP-glucose donor (Kim *et al.*, 2007). Both enzymes showed to use glucose as an acceptor substrate with higher preference then other monosaccharides. TreTs from *R. xylanophilus*, *T. litoralis* and *T. tenax* used only glucose as acceptor to synthesize trehalose (chapter 2) (Qu *et al.*, 2004; Kouril *et al.*, 2008).

In the reverse reaction catalyzed by TreT, i.e., for NDP-glucose synthesis, ADP was most preferred among NDPs used in all of the characterized TreT enzymes, with the exception of the TreT from *T. tenax*, which does not catalyze the reversible reaction.

Organisms from genera *Aquifex* and *Thermotoga* represents the deepest and shortest phylogenetic branches within the Bacteria domain (Stetter, 1996). So far, the presence of *treT* genes occurs in a restrict group of organisms including, as described before, hyperthermophilic archaea and bacteria, and a few deltaproteobacteria The recent characterization of the TreT enzyme from hyperthermophilic bacterium *Thermotoga maritima* reinforces the theory of lateral gene transference between archaea and bacteria (Nelson *et al.*, 1999; Worning *et al.*, 2000; Ryu *et al.*, 2010). In chapter 2, the exceptional occurrence of *treT* in *R. xylanophilus* was analyzed and was confirmed a very ancient origin for *treT* gene. Nevertheless, additional studies will have to be performed to corroborate with the hypothesis of the acquisition of this gene by lateral gene transference.

The participation of *R. xylanophilus* TreT on trehalose catabolism is an interesting alternative function, since it could reduce or increase the levels of trehalose and glucose, respectively, in the cell; however, trehalose formation is the favored enzymatic reaction catalyzed by this enzyme (chapter 2).

In *R. xylanophilus*, the co-existence of various pathways for trehalose metabolism points to an essential role of this molecule and raises questions about it physiological function and additionally about the regulation of alternative metabolic pathways.

Perhaps the trehalose accumulation under different stresses limits substrate availability selectively.

4. TREHALOSE AND MANNOSYLGLYCERATE, THEIR CORRELATION WITH DESICCATION TOLERANCE

Some phylogenetically unrelated organisms designated anhydrobiotic organisms (like, for example, nematodes, yeast, plants), can survive a desiccated state and for that purpose accumulate very high amounts of trehalose (as much as 20% of their dry weight) (Potts, 1994; Crowe *et al.*, 1997; Crowe, 2002). These organisms, some of which can tolerate almost total water losses, are able to support strong vacuum, high doses of ionizing and UV radiation, and

extreme temperatures without damage (such as mechanical and oxidative damage) (Crowe *et al.*, 1992).

Rubrobacter xylanophilus, an extremely gamma-radiation resistant bacterium, accumulates mainly trehalose and MG, under several growth conditions, as described previously. In nature, however, little is known about the factors that are involved in the regulation of trehalose and MG accumulation in this organism.

The extreme radiation resistance of *R. xylanophilus* is believed to be an unexpected consequence of the organism's adaptation to the more common physiological stress, desiccation (causing similar damage to DNA), since natural environments with extreme radiation levels do not exist in the biosphere (chapter 1) (Mattimore and Battista, 1996).

Some unrelated MG-accumulating organisms like *Pyrococcus furiosus* and *P. abyssi*, and the closely related species *Thermococcus gammatolerans* are also slightly radiation resistant (Gerard *et al.*, 2001; Jolivet *et al.*, 2003), which could lead us to speculate that radiation resistance is a result of an evolutionary process. There is no evidence, however, to link this notable ability to the accumulation of MG.

Nevertheless, desiccation resistance has been correlated, for example, with the accumulation of trehalose and sucrose by other organisms, predominantly plants (Potts, 1994). As was described in chapter 1, this disaccharide has many functions. Particularly, in plants, it plays an essential role in various stages of development, for example in the formation of the embryo and in flowering. Trehalose also appears to be involved in the regulation of carbon metabolism and photosynthesis and, recently, it has been discovered that this sugar plays a central role in plant-microorganism interactions (Elbein *et al.*, 2003; Iturriaga *et al.*, 2009). In response to desiccation stress, *Bradyrhizobium japonicum* trehalose genes, coding for Tps, Tpp and Tres, were significantly expressed and an elevated intracellular concentration of trehalose was verified (Cytryn *et al.*, 2007).

Some of these organisms tolerate desiccation due to an ability to protect vital components of their cellular machinery from damage, while others like *Deinococcus* spp., which are extremely resistant to ionizing radiation (IR), ultraviolet light (UV) and desiccation, have the an amazing ability to repair the damage quickly upon rehydration (Battista *et al.*, 1999; Shirkey *et al.*, 2003; Makarova *et al.*, 2007). Recent results of the comparative analysis have clarified and

substantially revised downward the number of uncharacterized genes that might be involved in DNA repair and contribute to desiccation and radiation resistance of *Deinococcus* species (Makarova *et al.*, 2007).

The responses to desiccation of the cyanobacterium *Nostoc commune* and of *D. radiodurans* are different despite their similar resistance to gamma-radiation (Battista *et al.*, 1999; Potts, 1999). *N. commune* has the ability to induce covalent modifications on the DNA molecule to protect it from degradation or oxidative damage and upon rehydration, these DNA modifications are removed and cells recover (Shirkey *et al.*, 2003).

The lack of studies on the mechanisms involved in *R. xylanophilus* radiation (or desiccation) resistance and on the regulation process of MG accumulation makes it difficult to explain the role of this molecule in this organism. On the contrary, the role of trehalose in anhydrobiotic organisms has been the subject of several studies (Iturriaga *et al.*, 2000; Hoekstra *et al.*, 2001; Lopez *et al.*, 2008).

Plants, throughout their life cycle, have to cope with multiple stresses that modify normal plant physiology, plant growth and development. Stresses are generally grouped in abiotic or biotic, the first being caused by physical or chemical environmental factors, such as cold, heat, salinity, drought, wind, oxidation or radiation, and the second caused by biological agents, such as bacteria, fungi, insects or herbivores (Mahajan and Tuteja, 2005).

The genus *Selaginella* contains some of the most drought tolerant plants known, including 'resurrection plants' such as *S. lepidophylla* and *S. tamariscina,* which can survive complete desiccation. Desiccation tolerance of *S. lepidophylla* has been attributed to the accumulation of high levels of trehalose. In other species of the genus *Selaginella* like *S. moellendorffii*, there is no evidence about the mechanisms that contribute to drought tolerance; however, one gene involved in the mannosylglycerate synthesis (*mgS*) was recently found in this species and could be implicated in desiccation tolerance (chapter 3). However, the accumulation of MG has not yet been correlated with drought tolerance in plants and, above all, there is no evidences about the accumulation of this molecule in vascular plants (chapter 3).

The moss *Physcomitrella patens* has been shown to tolerate abiotic stresses, including salinity, cold and severe desiccation (Wang *et al.*, 2009). Genetic analysis confirmed the existence of *mgS*-like sequence in *P. patens* (chapters 1 and 3), but no homogue sequences have been

identified in vascular plant species, including *Arabidopsis thaliana* and *Oryza sativa* (rice). It is possible that *P. patens* may accumulate MG as a trend acquired from red algae, where MG has been detected (Bouveng *et al.*, 1955). As described above for *R. xylanophilus*, the resistance to desiccation has not yet been associated with MG accumulation, so the possible accumulation of MG and its role in the bryophyte remains to be explained.

To our knowledge, there is no report about the presence of MpgSs in plants or algae, even though their presence in archaea, bacteria and several fungi has been confirmed.

5. MgS - MANNOSYLGLYCERATE SYNTHASE FROM *Selaginella moellendorffii*

The mannosylglycerate synthase (MgS) from thermophilic bacterium *Rhodothermus marinus* (Martins *et al.*, 1999) is a unique glycosyltransferase for which the new GT78 family was formed (CAZy - Carbohydrate-Active enzymes classification). Recently, the crystal structure of this enzyme was solved and a proposal to explain its unique mechanism of catalysis, considered to be at the border between the known inverting and retaining mechanisms, was put forward (Flint *et al.*, 2005). Interestingly, prokaryotic MgS shows broad substrate specificity with regard to sugar donor and also, to some extent, to the acceptor: GDP-mannose and D-glycerate are the preferred substrates, but GDP-glucose, GDP-fucose, UDP- mannose, and UDP-glucose can also be used as sugar donors, and D-lactate and glycolate, as the 3-carbon acceptors. In addition, the substrate specificity was undoubtedly dependent on temperature (Flint *et al.*, 2005).

Until now, this was the only enzyme that catalyzed MG synthesis in a single-step. In chapter 3, the characterization of the first eukaryotic recombinant MgS was elucidated. The *mgS* gene, coding for MgS, was found in the recently available genome of the land plant *Selaginella moellendorffii*.

Due to the substrate and product specificity, stereochemical mechanisms and the high amino acid homology with the MgS from *Rhodothermus marinus*, it seems acceptable to include this new eukaryotic MgS in the same glycosyltransferase family, GT78, although more studies should be performed, including the determination of three-dimensional structure by X-ray crystallography (MgS crystal structure from *S. moellendorffii* is in progress).

Eukaryotic MgS has the particularity of catalyzing efficiently the synthesis of both MG and GG, from GDP-mannose or GDP-glucose plus D-glycerate, *in vitro*, and this differential synthesis of MG or GG is not temperature dependent. The replacement of the sugar acceptor D-glycerate for 3-phosphoglycerate failed and the MG or GG synthesis was not observed via a phoshorylated intermediate (MPG or GPG), *in vitro* (chapter 3). In some cases, the presence of two alternative pathways for the synthesis of the same compatible solute also occurs (MG in *R. marinus*, GG in *Persephonella marina*, trehalose in *Mycobacterium* sp.) (De Smet *et al.*, 2000; Borges *et al.*, 2004; Costa *et al.*, 2007; Fernandes *et al.*, 2007), however in the lycophyte *S. moellendorffii* this was no an alternative MG pathway.

5.1. Biochemical characterization of MgSs

As described above, to date, there are only two functionally characterized MgSs, one from the thermophilic bacterium *Rhodothermus marinus* (Martins *et al.*, 1999) and, with this work, a new eukaryotic enzyme from the plant *Selaginella moellendorffii*. These two enzymes showed some distinct biochemical and kinetic properties summarized in Table 4.1 and analyzed in chapter 3. The most remarkable characteristic from the plant MgS, as addressed above, was the use of either GDP-mannose or GDP-glucose with comparable efficiency, for the synthesis of MG or GG products. This unprecedented dual MgS/GgS activity *in vitro* could be, to some extent, comparable to the MpgS from *Rubrobacter xylanophilus*, which synthesizes, *in vitro*, both MG and GG precursors (chapter 3) (Empadinhas *et al.*, 2011). This *in vitro* MgS/GgS activity, does not correlate with the accumulation of MG and GG, *in vivo*; *S. moellendorffii* possess the genetic resources but, unfortunately, the accumulation of these two related solutes in this plant and in other mesophiles awaits demonstration. On the other hand, red algae have *mgS*-like sequences and accumulate MG, althought no enzyme was funcionally caracterized, to date.

Table 4.1. Comparison of biochemical and kinetic properties of the MgSs of *Selaginella moellendorffii* and *Rhodothermus marinus*.

	Selaginella moellendorffii		*Rhodothermus marinus*
Property (Unit)	**MgS**		**MgS**[a]
No. of amino acid residues	422		397
Calculated molecular mass (kDa)	47.9		46.1
Optimum temperature (ºC)	50		85-90
Optimum pH	8.0		6.5
K_m (mM)			
GDP-man (+D-glyc)	2.4 ± 0.4[b]	0.6 ± 0.1[c]	0.2[d]
D-glyc (+GDP-man)	4.4 ± 0.7[b]	1.2 ± 0.3[c]	0.9[d]
GDP-glu (+D-glyc)	1.0 ± 0.2[b]	0.2 ± 0.1[c]	
D-glyc (+GDP-glu)	3.4 ± 1.0[b]	1.6 ± 0.4[c]	
V_{max} (µmol/min.mg protein)			
GDP-man (+D-glyc)	9.5 ± 0.4[b]	2.6 ± 0.1[c]	120[d]
D-glyc (+GDP-man)	10.9 ± 0.9[b]	2.6 ± 0.1[c]	
GDP-glu (+D-glyc)	8.0 ± 0.3[b]	2.0 ± 0.1[c]	
D-glyc (+GDP-glu)	11.0 ± 1.1[b]	2.2 ± 0.1[c]	
Mg^{2+} dependence (mM)[e]			
0	15.9 ± 1.3[b]		0[d]
5	100[b]		ND
50	ND		100[d]

[a] Adapted from Martins *et al.*, 1999, [b] Assays carried out at 50°C, [c] Assays carried out at 25°C, [d] Assays carried out at 90°C, [e] Expressed as percentage of the maximum activity of the MgS, with GDP-mannose and D-glycerate. Abbreviations: GDP-man, GDP-mannose; GDP-glu, GDP-glucose; D-glyc, D-glycerate; ND, not determined.

From a range of substrates tested for plant MgS (chapter 3), this glycosyltransferase was only specific for GDP-mannose/-glucose and D-glycerate (Table 4.1). A restricted group of glycosyltransferases also exhibit narrow substrate specificities; that is the case of Tps from *R. xylanophilus*, which was showed to be highly specific for GDP-glucose and glucose-6-phosphate (chapter 2). At 65ºC prokaryotic MgS showed broad substrate specificity, as

described above (Flint *et al.*, 2005). This broad substrate specificity at 65ºC may reflect an absolute requirement of MG and GG, at the optimum growth temperature.

With regards to the optimum temperature for activity, a large number of enzymes from mesophiles to hyperthermophiles are stable and active at temperatures considerably exceeding the upper growth limit of the producing organism (Zavodszky *et al.*, 1998; Sterner and Liebl, 2001). For *R. marinus* MgS, the temperature for maximal activity is around 85-90ºC, and for the plant MgS it is around 50ºC, 2-fold higher than physiological temperature of the plant.

The activity of eukaryotic MgS, although not dependent on the presence of Mg^{2+}, is enhanced by this cation. On the other hand, *R. marinus* MgS was strictly dependent on Mg^{2+} (Table 4.1). Many GT-B structural fold enzymes use metal ions, and TreTs from *Thermococcus litoralis* and *Thermoproteus tenax* showed Mg^{2+}-dependent activity (Qu *et al.*, 2004; Kouril *et al.*, 2008).

Comparison of the amino acid sequences of the functionally characterized MgSs and the putative homologue sequence from the moss *Physcomitrella patens* revealed high levels of sequence conservation and the presence of some highly conserved motifs (Fig. 4.4). A conserved D-X-D motif (or Asp-X-Asp, where X is any amino acid), highly conserved in the GT family and implicated in divalent metal-ion binding, which coordinates with the donor substrate (NDP-sugar molecule) (Wiggins and Munro, 1998; Charnock and Davies, 1999; Nielsen *et al.*, 2011), is present in the three sequences analyzed (Fig. 4.4, highlighted by a black box). This finding was not unexpected since the two functionally characterized MgSs are GDP-sugar using glycosyltransferases. In the case of the eukaryotic MgS, this enzyme is able to use efficiently GDP-mannose and GDP-glucose, in a temperature-independent manner, unlike the MgS from *Rhodothermus marinus* (chapter 3) (Flint *et al.*, 2005).

A conserved N-X-S/T (or Asn-X-Ser/Thr, where X is any amino acid other than proline) motif, showed to be common in glycosyltransferase sequences, was also detected (Fig. 4.4, highlighted by a gray box) (Tabish *et al.*, 2011). This consensus sequence of amino acids is associated to the site where the attachment of the carbohydrate, across a *N*-linked glycosylation occurs. The machinery involved in the *N*-linked glycosylation system of prokaryotic and eukaryotic organisms display significant similarities (Altschul *et al.*, 1990;

Weerapana and Imperiali, 2006) and this was supported by the presence of the N-X-S/T motif in MgSs, from a plant, *S. moellendorffii*, and a bacterium, *Rhodothermus marinus*.

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S.moellendorffii     MSLVCFPFKEEDVAVVVRNVECAAAHPRVSTVLCVGYSKGETWCAIEAKRPSIESSTGKR
P.patens             MSLVCFPFKEEDVAVVVGNIECAASHPRVAAILCVGYSQGETWHAIEAAKPRIERATGKE
R.marinus            MSLVVFPFKHEHPEVLLHNVRVAAAHPRVHEVLCIGYERDQTYEAVERAAPEISRATGTP
                     **** ****.*.  *:: *:. **:****  :**:**.:.:*: *:*   * *. :**.

S.moellendorffii     IILLQQKRIGVSLRSGKGDGMNTALAYFLDHTELKRIHFYDSDIVSFSADWITKAERQAD
P.patens             IFLVLQRRIGVSLRGGKGDGMNTALAFFLEKDVYPRLHFYDADIVSFSGEWISKAEKQAD
R.marinus            VSVRLQERLGT-LRPGKGDGMNTALRYFLEETQWERIHFYDADITSFGPDWITKAEEAAD
                     : :  *.*:*. ** ********** :**:.    *:****:**.**. :**:***. **

S.moellendorffii     LDFDVVRHYFPRSSTDAMITWFVTKIGFCLLWPKTVLPFIEQPLGGELLLTRKAAEALYT
P.patens             LDYDIVRHYFPRSSTDAMITWLVTKLGFAMLWPNSTLPFIEQPLGGELLLTKKAAEVLYG
R.marinus            FGYGLVRHYFPRASTDAMITWMITRTGFALLWPHTELSWIEQPLGGELLMRREVAAMLYE
                     :.:.:*******:********::*: **.:***:: *.:**********: ::.*  **

S.moellendorffii     DHRVRGQSDWGIDTLYTFIMVQKGLHLAEVYIPEGKVHALYSGLRDLRTMLVECFSAMQS
P.patens             DHRVRSQSDWGIDTLYTFVTAQAGLRLAEVYISEGKVHALYGGLRDLRTMLVECFSAVQS
R.marinus            DERVRRRSDWGIDTLYTFVTVQQGVSIYECYIPEGKAHRLYGGLDDLRTMLVECFAAIQS
                     *.*** :***********: .* *: : * **.***.* **.** **********:*:**

S.moellendorffii     LKDEAVPLN-EGTHRMEYTRPVPELVKQKVGYDVEKTLKLLRSNWTQGQRDLLQKHFDPA
P.patens             LRKEILPSEDGAVHCIEPAKAVPDLIKQKVGYDIEKTLKLLKSNWTPRQQELLHQHFDSA
R.marinus            LQHEVVG--QPAIHRQEHPHRVPVHIAERVGYDVEATLHRLMQHWTPRQVELLELFTTPV
                     *:.* :     . *  * .: **  : ::****:* **: * .:**  * :**. .  ..

S.moellendorffii     LAKGLLNASEWPTWGFADEEAWVAAYRTLLVHFEKGDEDWEELLFKIWVSRVLNHTMRHS
P.patens             LVKGLLNSAEWPCWSFADEDAWTSAYLKFLDHFEKGDSDWEELLFKVWVARVLNHTFKNV
R.marinus            REG-LRTCQRRPAFNFMDEMAWAATYHVLLEHFQPGDPDWEELLFKLWTTRVLNYTMTVA
                         * .. . * :.* ** **.::*  :* **: ** ********:*.:****:*:

S.moellendorffii     LRGYDAALDALRSLIWETQHQSAMLSKS-AAANHHIVSGHSASEALPRTAGQLREKRMDA
P.patens             MRGYDSALGALRDLVWDTRHQFAVKLKANSVPNHAIVSGHSAAEGLVTRAQSGRKKPKFE
R.marinus            LRGYDYAQQYLYRMLGRYRYQAALENGR----------GHPVPPRAALSTA---------
                     :**** *   *  ::   ::* *:              **...      :

S.moellendorffii     ACAV-----   422 aa
P.patens             VENLCTVQQ   429 aa
R.marinus            ---------   397 aa
```

Structure symbols:

- G - involved in binding GDP
- D - involved in binding divalent cation
- A - involved in binding acceptor
- M - involved in binding sugar

Figure 4.4. Sequence alignment among the two functionally characterized MgSs, from *Selaginella moellendorffii* and *Rhodothermus marinus*, and the putative MgS from *Physcomitrella patens* (acession numbers: XP_002978745, YP_003290498, XP_001764114) (Martins *et al.*, 1999) (chapter 3). The Clustal X program was used for sequence alignment (Thompson *et al.*, 1997). Conserved residues, strong and weak conservative substitutions are indicated by '*', ':' and '.', respectively. Gaps introduced by Clustal program to optimize the alignment are indicated by '-'. Structure symbols (Flint *et al.*, 2005) (Scheller *et al.*, 2010). Conserved DXD and N-X-S/T motifs, common to several glycosyltransferases, are highlighted by a black box and a gray box, respectively.

6. MgH - MANNOSYLGLYCERATE HYDROLASE FROM *Selaginella moellendorffii*

Over the last few years, some information has emerged from studies on the accumulation and biosynthesis of the two related compatible solutes, MG and GG, mainly in prokaryotes. At least two pathways have been identified for each solute in several bacteria and archaea and the corresponding genes and enzymes, as well as their properties, have been comprehensively described.

Interestingly, very little information of these compatible solutes in the Eukaryota is available (Empadinhas and da Costa, 2008b). The study of their catabolic pathways is also of extreme importance and virtually nothing is known about MG or GG catabolism nor the environmental regulatory conditions involved, in the three domains of life, Archaea, Eubacteria and Eukaryota.

In chaptes 3, besides the characterization of the mannosylglycerate synthase, the first eukaryotic enzymes involved in the catabolism of MG and GG have been biochemically studied. Curiously, the gene coding for this enzyme, the mannosylglycerate hydrolase (MgH), was detected in the genome of the lycophyte *S. moellendorffii*, which does not accumulate MG or GG, under the conditions examined (chapter 3). Oddly, in *Arabidopsis thaliana* there are eleven *tps* and ten *tpp* genes and paradoxically trehalose is almost undetectable in this plant (Leyman *et al.*, 2001; Vogel *et al.*, 2001).

BLAST searches at the NCBI database with MgH from *S. moellendorffii* yielded sequences with conserved domains (CD) found in the bacterial rhamnosidase superfamily (CD accession number cl01801) (Marchler-Bauer *et al.*, 2011)(Fig. 4.5).

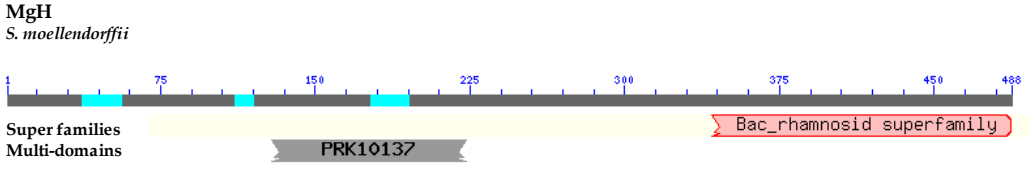

Figure 4.5. Schematic representation of the mannosylglycerate hydrolase (MgH) sequence from *S. moellendorffii* showing its putative conserved domains (CD).

In respect to the amino acid sequence of *S. moellendorffii* MgH, this hydrolytic enzyme has not been included into any of the existing glycoside hydrolase families and was listed as non-classified sequence in the corresponding database, despite showing conserved motifs found in members of glycoside hydrolase family 63 (mannosyl oligosaccharide glucosidase, EC 3.2.1.106) and of family 37 (trehalase, EC 3.2.1.28), in the Sanger database (http://pfam.sanger.ac.uk/family/). The same is true for the other two bacterial MgHs (Alarico *et al.*, unpublished).

6.1. Biochemical characterization of MgHs

Table 4.2 summarizes some biochemical and kinetic properties of the MgHs characterized so far, from the plant *S. moellendorffii* and from the two thermophilic bacteria *R. radiotolerans* RSPS-4 and *T. thermophilus* HB27.

The more significant feature of all three MgH was their ability to use MG and GG alike, as mentioned in chapter 3. The presence of cations was not required for activity of any of the MgHs, although 10 mM of Mn^{2+} had a strong effect on the stimulation of the plant MgH activity.

One interesting feature of the recombinant MgHs was their limited pH range for activity around pH 6.0, 4.0 and 4.5 (Table 4.2). Several intracellular enzymes involved in MG (or GG) synthesis demonstrate maximal activity around neutral pH, although they are also active at lower pH. The MpgP from the archaeon *Pyrococcus horikoshii* exhibits maximal activity in a pH range of 5.2 to 6.4, but it is still active at pH 3.7 (Empadinhas *et al.*, 2001). Moreover, the GpgS and Ggs from *Petrotoga mobilis* are still active at pH 5 and 4.5, respectively, although their maximal activity occurs at pH 7.0 (Fernandes *et al.*, 2010). The thermostable trehalase from *R. marinus* has maximal activity at pH 6.5, but retains about 50% of its activity at pH 4.5 (Jorge *et al.*, 2007). Since extracellular pH determines, to some extent, the intracellular pH (Rao *et al.*, 2001), it is possible that an environmental acid stress may lower the intracellular pH to activate MgH expression/activity with concomitant hydrolysis of endogenous MG/GG (probably synthesized by MgS). However, this remains to be experimentally confirmed.

Table 4.2. Comparison of biochemical and kinetic properties of the MgHs of *Selaginella moellendorffii*, *Rubrobacter radiotolerans* RSPS-4 and *Thermus thermophilus* HB27.

		Selaginella moellendorffii		*Rubrobacter radiotolerans*	*Thermus thermophilus*
Property (Unit)		**MgH**		**RradMGH**[a]	**TthMGH**[a]
No. of amino acid residues		488		442	415
Calculated molecular mass (kDa)		54.5		50.4	48.1
Optimum temperature (ºC)		40		55	70
Optimum pH		6.0		4.0	4.5
K_m **(mM)**					
	MG	11.8 ± 1.1[b]	21.4 ± 4.0[c]	6.4 ± 0.9[d]	2.6 ± 0.2[e]
	GG	5.9 ± 0.8[b]	2.9 ± 0.5[c]	4.2 ± 0.4[d]	3.7 ± 0.6[e]
V_{max} **(µmol/min.mg protein)**					
	MG	40.5 ± 1.2[b]	13.6 ± 0.9[c]	65.2 ± 4.3[d]	30.5 ± 0.8[e]
	GG	7.3 ± 0.3[b]	2.3 ± 0.1[c]	53.6 ± 2.3[d]	36.3 ± 2.2[e]
Mn^{2+} **dependence (mM)**[f]					
	0	76.3 ± 0.7[b]		[g]	[g]
	10	100[b]			
Half-life (h) at:					
	55ºC	ND		15.4 ± 0.5	ND
	60ºC	ND		6.8 ± 0.2	ND
	70ºC	ND		0.2 ± 0.0	16.1 ± 0.4
	80ºC	ND		ND	2.5 ± 0.2
	90ºC	ND		ND	0.2 ± 0.0

[a] Adapted from Alarico *et al.*, unpublished, [b] Assays carried out at 40°C, [c] Assays carried out at 25°C, [d] Assays carried out at 50°C, [e] Assays carried out at 70°C, [f] Expressed as percentage of the maximum activity of MgH with MG, [g] Cations were not required for activity. Abbreviations: MG, mannosylglycerate; GG, glucosylglycerate; ND, not determined.

The MgHs enzymes represent a new functional family for the specific hydrolysis of MG and GG. Comparison of the amino acid sequences of the functionally characterized MgHs revealed high levels of sequence conservation and the presence of some highly conserved motifs (Fig. 4.6). More studies, especially on the analysis of X-ray crystallography data, will help to

understand better which residues are involved in the recognition of MG or GG by these hydrolases. The determination of the crystal structure of the eukaryotic MgH is in progress.

```
S.moell     MAGPVRCLPPVVEATSIPHAPPVISKEVSEIVNNMLSVAIPAAAAASAQDQRFASQFRCG 60
T.therm     ------------------------------------------------------------
R.radio     ----------------------------------------------------MKEDEPAG 8

S.moell     PEFTTMKAQALEACRKILAENDQGGYTIPAKGLYPYQWNWDSALVSLGLAEMEEERAWEE 120
T.therm     -----MKA------VEVLQRNSRGAFTVPAHGLYPYQWLWDSAFIALGWTQVDWERAWQE 49
R.radio     PKVEDLIAQA----KMVLDFNWTGEYTRPGPRLYPHQWSWDSALIALGYARYAPDRAMRE 64
                 : *        :*  *  * :* *.  ***:** ****:::** :.   :** .*

S.moell     LDRLMS-AQWEDGMVPHIVFHKPSSTYFPGPEIWGSPDKPR-----NTTGITQPPVAAIS 174
T.therm     LLCLFDYGQGPDGMLPHIVFHEQSRDYFPGPDVWGREARAQP----ATSGITQPPVVATV 105
R.radio     LSHLFD-AQWKNGLLPQIVFNPDFAAYFPDASFWHADESPDAPTHLRTSGIVQPPVHATA 123
            *  *:. .*  :*::*:***:     ***....*     .       *:**.**** *

S.moell     VRRLLEEAKDKALALAMARKLFPKLLAWHRWFYRARDPEGTGLVATIHPWETGMDNSPAW 234
T.therm     VRYLYEKDPDRDRARSRARYLFPKLLAYHRWLYHARDPYRTGLVVIVHPWESGMDNSPAW 165
R.radio     VLALLRNAAEAPGVRSFCEKAFSRLVSWHDYLYRERDPGGENLVYIRHPWESGMDNSPMW 183
            *  * .:  :   . : ..  *.:*:::* ::*: ***   .**   ****:****** *

S.moell     DEALARVPID--DIPPYVRRDLGHVDAKMRPQKAEYDRYLTLLYRFRALDYDEAKLYYET 292
T.therm     DKPLSRVPVE--NLPPYERRDVKHVNPEERPRKEDYDRYLSLLYLFRRLEYDPRGIYRQS 223
R.radio     DAILESMFLYPSDIPSYKRADTHFVSSEDRPESAAYDRFAYLVKLFAERNYDEARIREDC 243
            *  *  : :   ::*.* * *  .*..: **..  ***:  *:  *   :**   :  :

S.moell     PFRVTDLCTNCILHKANEDLLWLAGATGACTDESEIRGWTARANVAFDTLFDVEAGLYRC 352
T.therm     PFKVVDVGFNAILQRANRDLYALAVLLQE--DPYEIEEWIVRGEVGLEALWDREAGFYFS 281
R.radio     PFLVQDVLFNSLLCRAERDLAELARTLGEEPSAFEARARKT-AEAINDKLWDGERGTYLG 302
            ** * *:  *.:* :*:.**  **       .  * .   . .:.  : *:* * * *

S.moell     KDQLTGQFLPAATSAGFLPLFAGVASGEKASAVARTLGR----WLDDVAYGIPSCDPRDP 408
T.therm     WDLVAGEPIAVKTSAGFLPLFAGTPHQGRASLLAQEAER----WGEKARYLLPSVDPTSP 337
R.radio     FDLVSGAHIKVLAAPNFVALYGEVPDRKRARAMLARLSSPSFSLTEGTGVPVTSYDRLGF 362
             * ::*  : . ::..*:.*:. ..   :*  :            : .   :.* *  .

S.moell     QFEALRYWRGPVWLIVNWMVSEGLKRYGYGELAQRVERDSYELVKNGGIFEYYCPLTGMG 468
T.therm     FFEPGRYWRGPVWINVNWMVAEGFRDYGFAALAARLKADALALMEREGFREYYDPLTGQG 397
R.radio     GFSSVRYWRGPVWVNIDWFLMHGLRRYGYEDEADRLREAIVRLCREEGFYEYFDPTTGMG 422
             *.. ********: ::*:: .*:: **:   * *:.     * .. *: **: * ** *

S.moell     AGGGCFSWTAAMCLAWLFKT 488
T.therm     RGGEGFSWSAALALFWTR-- 415
R.radio     HGSDLFSWTAALLLDVVLEG 442
             *.  ***:**: *
```

Figure 4.6. Sequence alignment among the three characterized MgHs. The accession numbers for these sequences are as follows: MgH from *Selaginella moellendorffii* (*S.moell*), XP_002990235, MgH from *Thermus thermophilus* (*T.therm*), YP_004589 and MgH from *Rubrobacter radiotolerans* (*R.radio*), JN704789 (chapter 3) (Alarico et al., unpublished). The Clustal X program was used for sequence alignment (Thompson *et al.*, 1997). Conserved residues, strong and weak conservative substitutions are indicated by '*', ':' and '.', respectively. Gaps were introduced by Clustal program to optimize the alignment are indicated by '-'.

7. FUTURE PERSPECTIVES

This work has provided some important contributions towards the biosynthetic and hydrolytic pathways of trehalose and mannosylglycerate/glucosylglycerate in two distinct organisms.

Trehalose and mannosylglycerate are indeed required for maintenance of cellular integrity in optimal growth conditions or even in response to external stress, in the extremely radiation-resistant bacterium *Rubrobacter xylanophilus.*

The disruption of trehalose/mannosylglycerate metabolisms could provide novel information concerning the specific role of each trehalose/mannosylglycerate synthesizing enzyme and to ascertain their importance in bacterial growth and survival. With this objective in mind, it will be of interest to exploit recent requirements in techniques for mutagenesis of actinobacteria (Luzhetskyy *et al.*, 2002; Siegl *et al.*, 2010) to construct strains with disruptions in trehalose and mannosylglycerate genes. While it might be anticipated that mutations will be compensated in part by complementary pathways, especially trehalose pathways, described in the present study, mutants might display unusual phenotypes under particular growth conditions which could provide insights into the basic function of trehalose/mannosylglycerate in *R. xylanophilus* physiology. For example, *Mycobacterium smegmatis* mutants defective in the three trehalose pathways are unable to grow unless trehalose is supplemented to the growth medium (Woodruff *et al.*, 2004). However, each pathway seems to have a specific role and hierarchy in closely related actinobacterial species (Tzvetkov *et al.*, 2003; Wolf *et al.*, 2003; Woodruff *et al.*, 2004; Murphy *et al.*, 2005). The study the physicochemical factors that regulate the synthesis and also the degradation of trehalose, mannosylglycerate/glucosylglycerate in different organisms, from prokaryotic to eukaryotic, by the examination of the relevance of compatible solute pools in the survival to specific stress conditions, will be another point of interest. With this aim, the identification of the regulatory genes and the evaluation of the connection between biosynthesis and hydrolysis of compatible solutes at transcriptional, post-transcriptional or post translational levels will be addressed in the near future.

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