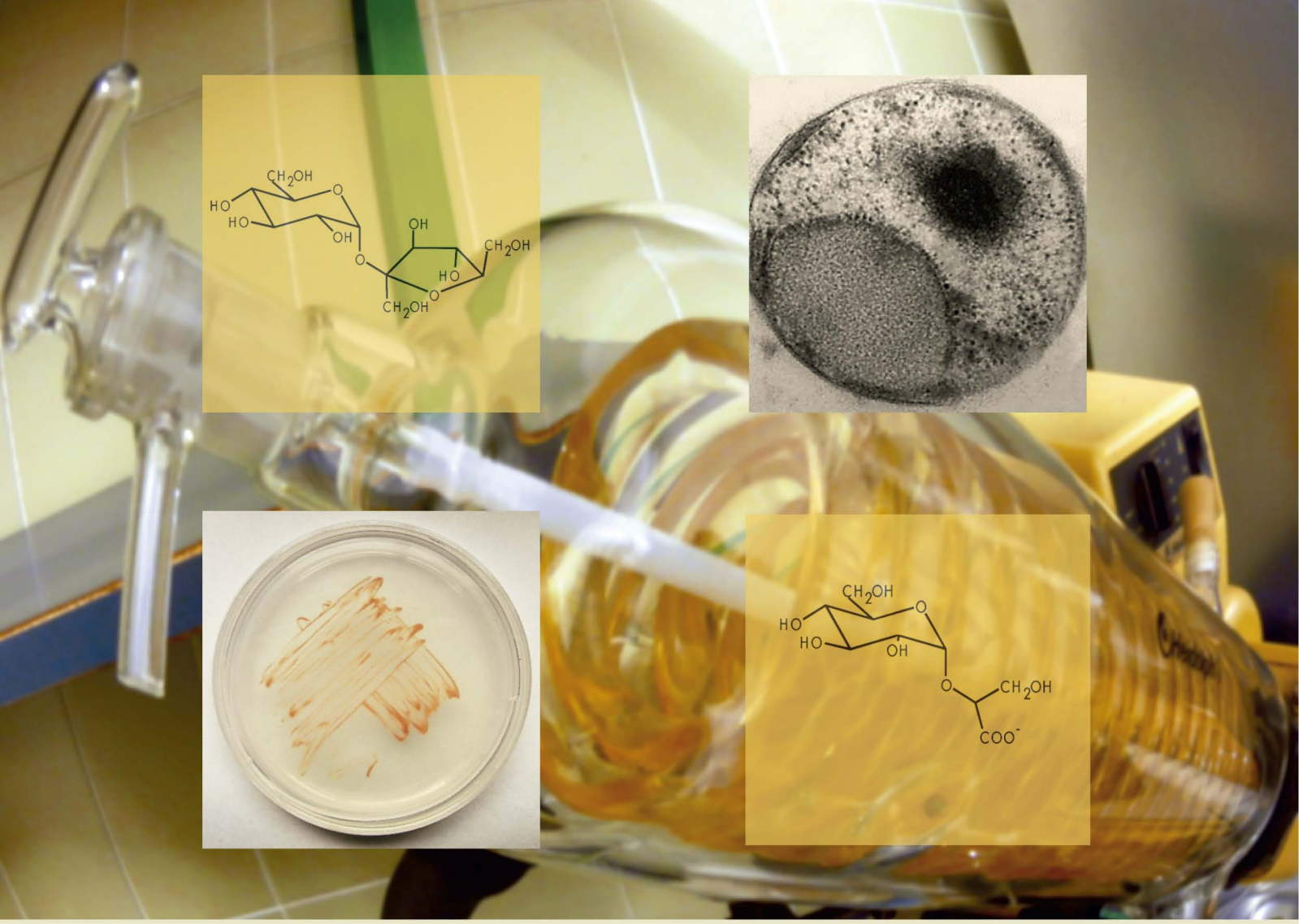

Ana Catarina Morouço Ferreira

THE OSMOADAPTATIVE STRATEGY OF TWO SLIGHTLY HALOPHILIC PLANCTOMYCETES SPECIES

Tese de Doutoramento em Biociências, especialização em Microbiologia, orientada pelo Professor Doutor Milton Simões da Costa e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra

Fevereiro 2016

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Universidade de Coimbra

Departamento de Ciências da Vida

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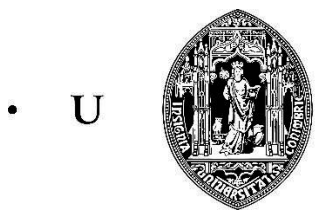
The osmoadaptative strategy of two slightly halophilic planctomycetes species

Ana Catarina Morouço Ferreira

Tese de Doutoramento apresentada ao Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia, Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biociências, especialização em Microbiologia, realizada sob a orientação científica do Professor Doutor Milton Simões da Costa (Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia, Universidade de Coimbra).



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Front cover: Photograph images related to the Planctomycetes unusual morphological characteristics and accumulation of compatible solutes that are presented in this thesis.
On the background, a rotary evaporator used during the extraction of compatible solutes from Planctomycetes. On the front a *Rubinisphaera brasiliensis* culture (bottom, left), transmission electron microscopy image of thin section of *Gimesia maris* showing internal compartmentalisation adapted from Lindsay and collaborators, 2001(top-right) and images of structural representation of sucrose (top, left) and glucosylglycerate (bottom, right).

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A Todos, Muito Obrigada!

"To remain indifferent to the challenges we face is indefensible.
If the goal is noble, whether or not it is realized within our lifetime is largely irrelevant.
What we must do therefore is to strive and persevere and never give up."

Dalai Lama XIV

ABSTRACT

Planctomycetes belong to a deep-branching group of *Bacteria* widely distributed in terrestrial and aquatic environments. The most intriguing features of the *Planctomycetes* are the multiplication by budding and the complex intracellular cell plan. Such unusual characteristics increased their relevance in areas of research such as, evolution, cell biology, ecology and genomics. The two mesophilic and heterotrophic representatives of the phylum *Planctomycetes* used in this study were cultured from aquatic environments with highly contrasting characteristics; *Gimesia maris* (formerly named *Planctomyces maris*) was isolated from the estuarine system Puget Sound (Washington) in the Pacific Ocean and *Rubinisphaera brasiliensis* (formerly named *Planctomyces brasiliensis*) was obtained from Lagoa Vermelha, a salt pit near Rio de Janeiro, Brazil. Considering that *G. maris* and *R. brasiliensis* are distinct aquatic microorganisms, both species possess biochemical strategies that enable them to thrive under such conditions.

The accumulation of organic molecules named compatible solutes is a common strategy among *Bacteria* to compensate lower extracellular water potential imposed by salt. The compatible solutes act as cell protectors without detrimental effects on normal cell metabolism. When present in the surrounding medium, compatible solutes can be taken up from the environment whereas their unavailability leads to *de novo* synthesis.

Besides the *Planctomycetes* peculiar phylogenetic position, complete genomes available and distribution in several environments, the physiological mechanisms present in these organisms for the adaptation to diverse ecosystems are still poorly understood. Thus, the intracellular compatible solutes content from the slightly halophilic *G. maris* and *R. brasiliensis* species was analysed in detail in response to stressful conditions.

For osmoadaptation both species accumulated α-glutamate, sucrose, ectoine and hydroxyectoine. In *G. maris,* α-glutamate remained the major solute at both sub-optimum and supra-optimum salinities, whereas in *R. brasiliensis* the levels of this amino acid were almost unchanged during growth at different salinities. Thus, the higher salt tolerance showed by *R. brasiliensis* could be related to the high ectoine concentration, which led to a much higher total compatible solutes content in the cells under supra-optimum salinities. In addition, sucrose up-regulation under nitrogen privation and during hyper-osmotic shock confirmed the osmoadaptation role of this disaccharide in *R. brasiliensis*. Surprisingly, sucrose became the major solute under nitrogen-limiting conditions suggesting that this disaccharide could be advantageous for cell revival after nitrogen reposition. In contrast, *G. maris* began to accumulate glucosylglycerate (GG) under

nitrogen-limiting conditions. Thus, the accumulation of compatible solutes was intensively studied in *G. maris* under different stress conditions but the accumulation of GG was not detected in the presence of a nitrogen source.

The identification of GG accumulation in *G. maris* specifically under nitrogen limiting conditions prompted us to analyse the transcript abundance of key genes known for the biosynthetic pathways of GG. Among them are a *gpgS* and a *gpgP* genes coding for a glucosyl-3-phosphoglycerate synthase and phosphatase, respectively, which are involved in the synthesis of GG by the common two-step pathway. Those genes are located in the *G. maris* genome as an operon-like structure along with a *spasE* gene coding for a putative sucrose phosphorylase (Spase), which we hypothesized to be involved in *G. maris* survival under nitrogen-limiting conditions. Via reverse transcription of total RNA from *G. maris* and subsequent qPCR analysis using suitable reference genes selected in this study, the transcript abundance of the biosynthetic genes was quantified in cells under hyper-osmotic shock or under nitrogen-limiting conditions. A clear dependence of the transcripts of *gpgS* and *spasE* genes to the shock treatment was shown with up-regulation under nitrogen-limiting conditions, suggesting a role for the putative Spase under those environmental conditions. The biosynthesis of GG under nitrogen-limiting conditions confirms the increase in *gpgS* transcript abundance and suggest that the synthesis of this glycerate-derivative is limited at first, by the transcriptional level of the *gpgS* gene. The production of GG and sucrose is therefore, mainly dependent on lower availability of a nitrogen source and subordinated to the osmolarity.

Key Words: Osmotic adaptation, Compatible solutes, real time quantitative PCR, *Gimesia maris, Rubinisphaera brasiliensis*

RESUMO

Os *Planctomycetes* constituem um grupo do domínio *Bacteria* de origem ancestral, amplamente distribuídos por ecossistemas terrestres e aquáticos. A multiplicação por gemulação e a estrutura intracelular complexa das células são características determinantes dos *Planctomycetes*. Estas características apresentam relevância crescente em vários domínios científicos, como a evolução, biologia celular, ecologia e genómica. Os dois organismos em estudo são espécies mesófilas, heterotróficas, representativas do filo *Planctomycetes*, isoladas de ambientes aquáticos com características distintas, uma vez que *Gimesia maris* (anteriormente denominada *Planctomyces maris*) foi isolada do estuário Puget Sound em Washington e banhado pelo Oceano Pacifico e *Rubinisphaera brasiliensis* (anteriormente denominada *Planctomyces brasiliensis*) foi obtida da Lagoa Vermelha, uma lagoa hipersalina próxima do Rio de Janeiro no Brasil. Considerando que *G. maris* e *R. brasiliensis* são espécies de ambientes aquáticos distintos, ambas possuem estratégias bioquímicas que lhes permitam prosperar nesses ambientes.

A acumulação de moléculas orgânicas designadas, solutos compatíveis é uma estratégia utilizada por diferentes organismos permitindo o equilíbrio osmótico das células. Os solutos compatíveis conferem proteção osmótica às células sem interferir com o metabolismo celular. Os solutos podem ser obtidos do exterior, quando presentes ou sintetizados pelas células em resposta a condições ambientais desfavoráveis.

Apesar da posição filogenética do filo *Planctomycetes*, da disponibilidade de genomas completos de alguns representantes deste filo e da distribuição por diferentes ecossistemas, pouco se sabe sobre os mecanismos fisiológicos presentes nestes organismos para a adaptação a diferentes condições ambientais. Desta maneira, a acumulação de solutos foi analisada em *G. maris* e *R. brasiliensis* durante a resposta a condições de agressão osmótica.

Em ambas as espécies, os solutos envolvidos na osmoadaptação são: α-glutamato, sacarose, ectoína e hidroxiectoína. Em *G. maris*, o α-glutamato permaneceu como soluto maioritário em condições de agressão sub-osmótica e supra-osmótica por outro lado, em *R. brasiliensis* os níveis deste aminoácido mantiveram-se quase inalterados em todas as salinidades testadas. A tolerância a salinidades mais elevadas demonstrada por *R. brasiliensis* estará relacionada com o aumento dos níveis de ectoína, que consequentemente aumentaram a concentração total de solutos nas células em condições de agressão supra-osmótica. Determinou-se ainda que a acumulação de sacarose é induzida pela ausência de azoto e em combinação com condições de agressão híper-

osmótica, confirmando a importância deste dissacárido na osmoadaptação de *R. brasiliensis*. Para além disso, a sacarose foi o soluto maioritário durante a ausência de azoto, podendo conferir proteção às células, quando a fonte de azoto for reposta, de modo a retomarem rapidamente o normal metabolismo celular. Por outro lado, *G. maris* acumula glucosilglicerato (GG) durante a ausência de azoto. Deste modo, a acumulação deste soluto foi estudada em *G. maris* submetido a diferentes salinidades e combinado com diferentes condições de azoto mas este organismo não acumula GG na presença de fonte de azoto.

A acumulação de GG por *G. maris* apenas na ausência de azoto levou-nos a analisar os níveis de transcrição de genes que codificam para a síntese de GG. Entre eles encontram-se os genes *gpgS* e *gpgP* que codificam para uma glucosil-3-fosfoglicerato sintetase e glucosil-3-fosfoglicerato fosfatase, respetivamente, que estão envolvidas na síntese de dois passos de GG em diferentes microrganismos. No genoma de *G. maris*, estes dois genes estão organizados numa estrutura semelhante a um operão juntamente com o gene *spasE* que codifica para uma provável sacarose fosforilase (Spase) e que possivelmente estará envolvida na sobrevivência de *G. maris* durante a ausência de azoto. Através da transcrição reversa do ARN total de *G. maris* e subsequente análise por qPCR usando genes de referência selecionados neste estudo, a abundância de transcritos da sequência codificante dos diferentes genes foi quantificada em células durante choque híper-osmótico ou durante a ausência de azoto. Demonstrou-se que a transcrição dos genes *gpgS* e *spasE* são dependentes do choque a que *G. maris* for exposto e que esta é induzida durante a ausência de azoto, sugerindo um papel importante da Spase em ambientes com baixa disponibilidade de azoto. A síntese de GG na ausência de azoto confirma a indução da transcrição do gene *gpgS* e sugere que a síntese de GG é limitada numa primeira fase pela abundância do transcrito do gene *gpgS*. A produção de GG e sacarose é por isso essencialmente dependente da baixa disponibilidade de uma fonte de azoto e subordinada pela osmolaridade.

Palavras-Chave: Adaptação osmótica, Solutos compatíveis, PCR quantitativo em tempo real, *Gimesia maris, Rubinisphaera brasiliensis*.

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ABBREVIATIONS

3-PGA	3-phosphoglycerate
ADP-glucose	adenosine diphosphate glucose
Cq	quantification cycle
DNA	deoxyribonucleic acid
DSMZ	Deutsche Sammlung von Mikroorganismen und Zelkulturen
EDTA	ethylenediaminetetraacetic acid
EctA	L-diaminobutyric acid acetyl transferase
EctB	L-diaminobutyric acid transaminase
EctC	ectoine synthase
EctD	ectoine hydroxylase
Frutose-6P	frutose-6-phosphate
GDP-mannose	guanosine diphosphate mannose
GDP-glucose	guanosine diphosphate glucose
GG	glucosylglycerate
GGG	glucosylglucosylglycerate
GgS	glucosylglycerate synthase
GPG	glucosyl-3-phosphoglycerate
GpgS/ GpgP	glucosyl-3-phosphoglycerate synthase/phosphatase
MG	mannosylglycerate
MGG	mannosylglucosylglycerate
MGP	mannosyl-3-phosphoglycerate
MgS	mannosylglycerate synthase
MpgS/MpgP	mannosyl-3-phosphoglycerate synthase/phosphatase
mRNA	messenger ribonucleic acid
NDP	nucleoside diphosphate
NDP-glucose	nucleoside diphosphate glucose
NDP-man	nucleoside diphosphate mannose
NMR	nuclear magnetic resonance
Pi	free phosphate
PCR	polymerase chain reaction
qPCR	quantitative real-time PCR
RNA	ribonucleic acid
rpoA	RNA polymerase, alfa subunit
rpnB	RNA subunit of ribonuclease P
rRNA	ribosomal ribonucleic acid
RT	room temperature
RT-PCR	reverse transcription PCR
Spase	sucrose phosphorylase
SpS/SpP	sucrose-6-phosphate synthase/phosphatase
Sucrose-6P	sucrose-6-phosphate
Susy	sucrose synthase
UDP-glucose	uridine diphosphoglucose

CHAPTER 1

INTRODUCTION

1.1 The Phylum *Planctomycetes*

1.1.1 Taxonomy, Historical Context and Evolution

The first member of the phylum, *Planctomyces bekefii,* was observed by Gimesi in 1924 and originally considered to be a fungus by the observation of rosette-forming cells resembling exospore formation of planktonic fungi **(Figure 1.1)** (Gimesi 1924). By then, other species discovered with similar morphological characteristics were crucial for the inclusion of the *Planctomycetes* group in the bacterial domain. The inclusion in the new order *Planctomycetales* and in the family *Planctomycetacea*, occurred in 1986 when Schmidt and Starr investigated two *P. bekefii* strains by 16S rRNA gene sequence analysis and by electron microscopy. Many uncultured species have now been obtained in pure culture unlike, *P. bekefii* (Schmidt and Starr 1978).

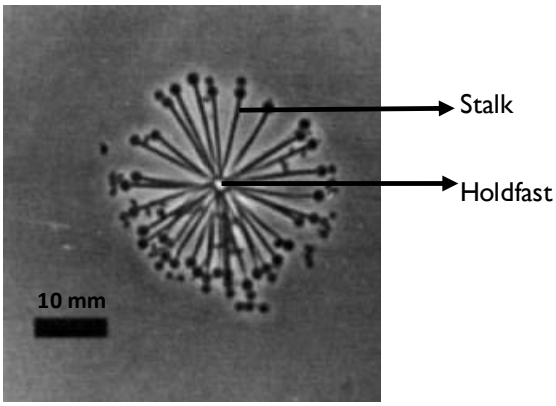


Figure 1.1. A phase contrast photomicrograph of the rosette form of *Planctomyces bekefii* from University Lake, St. Lucia, Australia.
Evidence of the holdfast supporting the cells-stalk junctions with the spherical cells in the top. Adapted from Ward and collaborators (2006).

Up to now, the phylum *Planctomycetes* comprises the classes *Phycisphaerae* and *Planctomycetia,* accompanied by an increase in the number of unclassified *Planctomycetes* (Schlesner and Stackebrandt 1986, Garrity and Holt 2001). Within the *Planctomycetia*, the family *Planctomycetaceae* includes most of the identified axenic cultures of the genera, *Planctomyces, Pirellula, Rhodopirellula, Blastopirellula, Gemmata, Schlesneria* and *Zavarzinella* (Bauld and Staley 1976, Franzmann and Skerman 1984, Hirsch and Müller 1985, Schlesner 1989, Schlesner *et al.* 2004, Kulichevskaya *et al.* 2007, Kulichevskaya *et al.*

2009). The order *"Candidatus Brocadiales"* also from the class *Planctomycetia* comprises only anammox bacteria that have not been obtained in pure culture (Schmid *et al.* 2000).

In a recent publication of the genome map of *Planctomyces brasilieinsis* the *Planctomyces* genera was re-classified due to discrepancy in phylogenetic and phenotypic characteristics within members of the genus. The axenic cultures *Plantomyces maris, Planctomyces limnophilus* and *Planctomyces brasiliensis* were accommodated in the genera *Gimesia, Planctopirus* and *Rubinisphaera*, respectively (Scheuner *et al.* 2014). In this work we will refer to *P. maris*, *P. limnophilus* and *P. brasiliensis* in separate genera as *Gimesia maris, Planctopyrus limnophilus* and *Rubinisphaera brasiliensis*, respectively.

The phylogenetic position of the *Planctomycetes* forming an independent and monophyletic branch of the domain Bacteria suggested the closest relationship to the last universal common ancestor (LUCA). However, this assumption is controversial (Brochier and Philippe 2002, Di Giulio 2003). The availability of the genome of *Gemmata obscuriglobus* revealed proteins homologous to eukaryotic membrane-coating proteins, in this case clathrins, which could be involved in the uptake of extracellular proteins through internal vesicles formation in *G. obscuriglobus* (Lonhienne *et al.* 2010). Those proteins mediate endocytosis in eukaryotes and could give clues about the relevance of Planctomycetes to the evolution of eukaryotes. Nevertheless, the genome of the "*Candidatus Kuenenia stuttgartiensis"*, an anammox bacterium, revealed a closest association with the genus *Chlamydiae*, an intracellular parasite, indicating a Gram-negative origin for the *Planctomycetes* (Staley *et al.* 2005, Strous *et al.* 2006).

Another assumption arises from the recently accommodation of the *Planctomycetes* in the PVC superphylum, which also accommodates the phyla *Verrucomicrobia, Chlamydiae and Lentisphaerae* **(Figure 1.2)** (Wagner and Horn 2006, Devos and Ward 2014). The unusual features shared by its members, the complex intracellular cell plan and the protein uptake by endocytosis may imply the earlier retention of eukaryotic characteristics from LUCA that were then diffused to the domains Bacteria, Archaea and Eukarya (Fuerst and Sagulenko 2011). However, a comparative genomic approach could not assume a link between the *Planctomycetes* and the *Eukarya* so the hypothesis of convergent evolution and lateral gene transfer (LGT) for the features of the PVC superphylum has been postulated (McInerney *et al.* 2011).

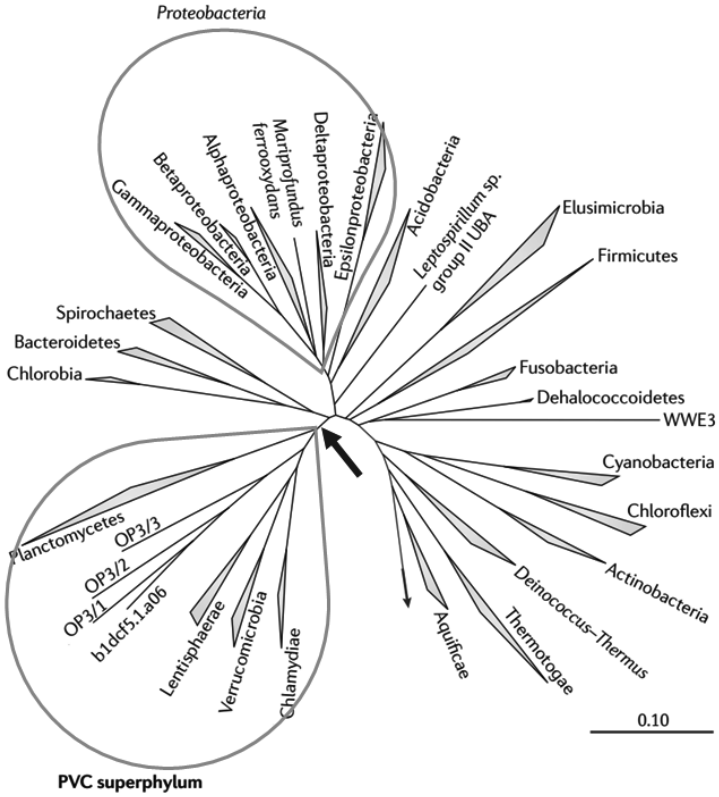


Figure 1.2. Phylogenetic relationship between *Planctomycetes* and other representatives of the *Planctomycetes-Verrucomicrobia-Chlamydiae* (PVC) superphylum based on 23S rRNA gene sequences.
An arrow (bold) indicates the monophyletic position of the phylum *Planctomycetes*. Bar indicates the 0.1 substitutions per nucleotide position. Adapted from Glöckner and collaborators (2010).

The available complete or draft genome sequences of *Planctomycetes* were, until recently, only obtained from a few pure culture representatives with *Rhodopirellula baltica* SH1 having the first complete genome published (Glöckner *et al.* 2003). Also, the available genome sequences extended to the anammox bacteria with 99% purity level, by the cultivation in bioreactors and subsequent metagenomic analysis (Strous *et al.* 2006). The *Planctomycetes* genomes are by far, the largest in size from the bacteria domain, ranging from 5.4 and 10.1 Mb (Guo *et al.* 2014). With 7325 ORF in the 7.1 Mb circular genome of *R. baltica*, only 32% have a predicted function being relatively higher in other species. In *Rubinisphaera brasiliensis*, *Gimesia maris* and *Planctopirus limnophilus* the proportion of genes with a predictable function increase from 41.1% and 57.6% (Guo *et al.* 2014). Moreover, a proteomic analysis in *R. baltica* confirmed the distinctive hypothetical proteins earlier found in the genome annotation project (Gade *et al.* 2005, Hieu *et al.* 2008). In certain environmental circumstances, the activation of proteins exclusive in *R. baltica* may contribute to cellular functions and metabolic pathways unique in *Planctomycetes*.

Bioinformatic analysis has been a prosperous approach to reveal genes for new secondary metabolites and antibiotics synthesis from these bacteria (Jeske *et al.* 2013).

1.1.2 Unique Morphological Aspects

The *Planctomycetes* and the other members of the PVC superphylum differ from the domain *Bacteria* in many respects (Wagner and Horn 2006). The *Planctomycetes* have a dimorphic life cycle and reproduce by budding with the exception of the branching class *Phycisphaerae* and the anammox bacteria, which divide by binary fission (Fukunaga *et al.* 2009). The life cycle of most *Planctomycetes* comprise adult sessile cells, during stationary growth phase and a motile daughter cell, during exponential growth phase, as proposed for *Rhodopirellula baltica* **(Figure 1.3)** (Schlesner *et al.* 2004). First, the adult sessile cell produce a holdfast to fix to a substrate or form rosettes. Then, the daughter cell formation occurs in the reproductive (non-holdfast) pole of the mother cell, followed by the development of a flagellum. The daughter cell detaches from the mother cell and during cells maturation it increases in size, loses its flagellum and becomes the mother cell for another generation of cell division (Gade *et al.* 2005, Ward *et al.* 2006).

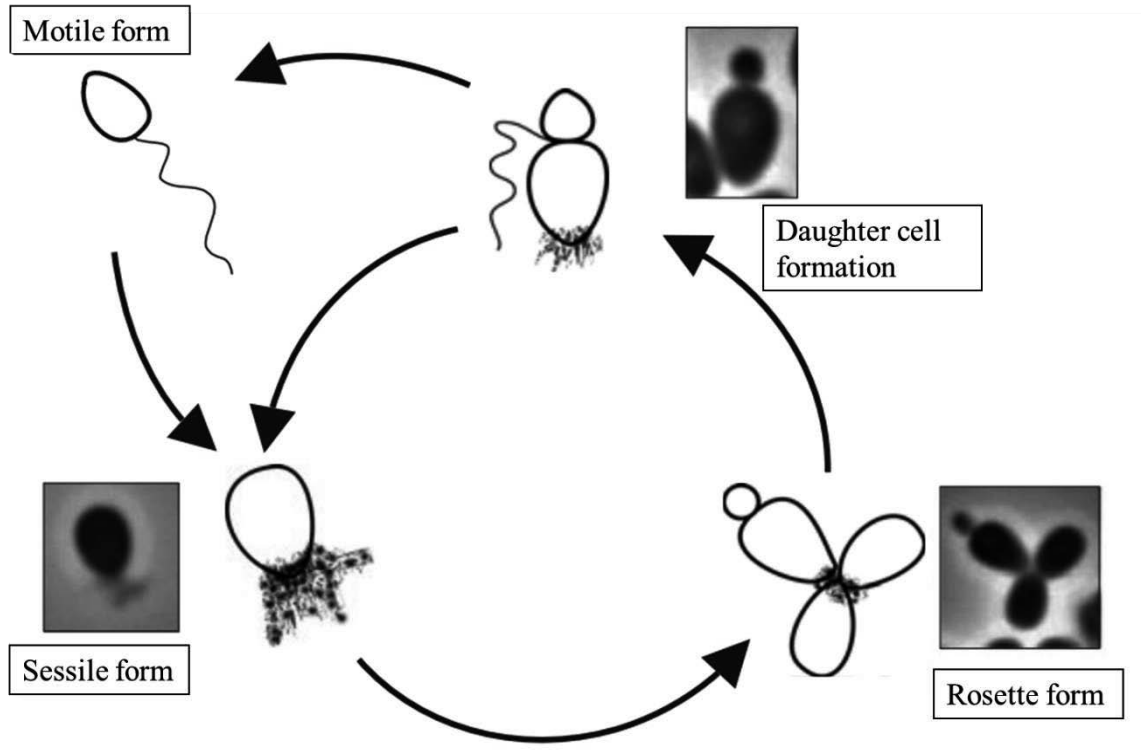


Figure 1.3. The life cycle of *Rhodopirellula baltica* SH1.
Adapted from Gade and collaborators (2005).

From pure culture, cell shape varies from spherical to ovoid and crateriform structures are present in the cell surface. All the members of the phylum *Planctomycetes* are Gram-negative and it was proposed that their cell wall lacks peptidoglycan. This assumption was based in the inability to find muramic acid and diaminopimelic acid in the wall structure of *Gimesia maris, Pirellula staleyi, Blastopirellula marina, G. obscuriglobus* and *Isosphaera pallida*. On the other hand, the cell envelope was thought to maintain cell structural integrity by a proteinaceous cell wall rich in proline and cysteine (Liesack *et al.* 1986, Giovanni *et al.* 1987). A recent study supports the typical Gram-negative organization of the planctomycetal cell envelope, since the characteristic constituents and features of peptidoglycan from Gram-negative bacteria were found in *Planctomycetes*. In addition, the cause for the naturally occurring strains of *Planctomycetes* resistant to peptidoglycan synthesizing inhibitors was proposed by their ability to produce β-lactamases. (Jeske *et al.* 2015). The resistance to β-lactamases and other antibiotics has been employed, to give selectivity to the isolation media during the isolation of *Planctomycetes* (Lage and Bondoso 2011).

However, the most intriguing characteristic of *Planctomycetes* is the cell compartmentalization in distinct regions. The compartmentalization of their cells occurs via internal membranes, which define cell regions that are bound by single bilayer membranes (Lindsay *et al.* 2001).

Each *Planctomycetes* has its own type of compartmentalization, but a basic cell plan defined by pirellulosome and paryphoplasm regions is shared by all species within the phylum **(Figure 1.4)** (Lindsay *et al.* 2001). The pirellulosome is an organelle bound by an internal membrane called the intracytoplasmic membrane (ICM), containing the nucleoid and all the DNA of the cell. The paryphoplasm is surrounded by the ICM, the cytoplasmic membrane and harbours part of the RNA (Lindsay *et al.* 1997).

The simplest compartmentalization is found in the genera *Pirellula, Blastopirellula, Rhodopirellula*, *Phycisphaera, Gimesia, Planctopirus, Rubinisphaera* and *Isosphaera,* however in the later species, the ICM invaginates so that a large inclusion occupies the central region of the cell (Fuerst 2005). In other species, like *G. obscuriglobus*, the nucleoid could be a structure analogous to the eukaryotic nucleus. A double membrane was proposed to enclose the nuclear body but recent findings suggest the involvement of invaginations of the cytoplasmic membrane (Santarella-Mellwig *et al.* 2013).

In the anammox *Planctomycetes*, genomic DNA is not located in a nuclear body, however, they have another organelle within the pirellulosome named anammoxosome that contains enzymes essential for the anaerobic ammonium oxidation (Strous *et al.* 2006, Van Niftrik and Jetten 2012).

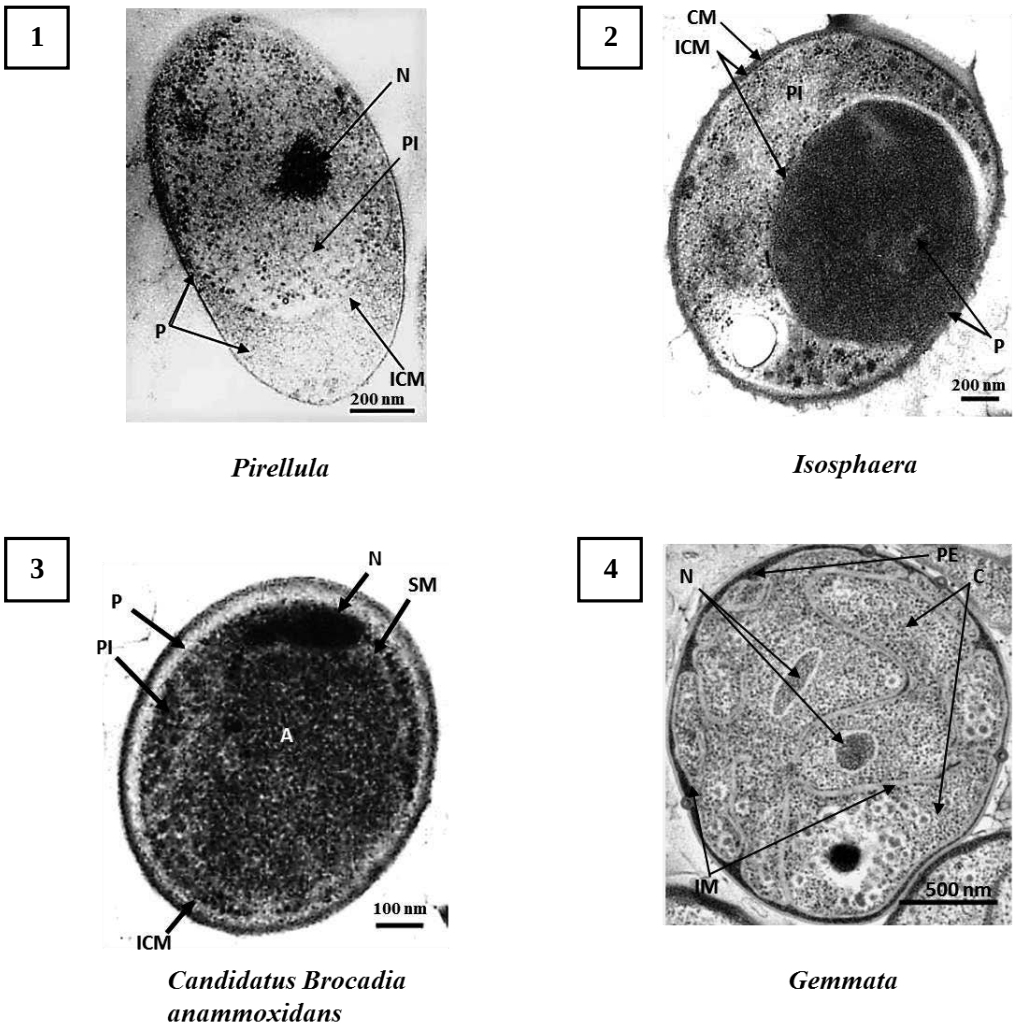


Figure 1.4. *Planctomycetes* complex cellular plan.
Thin section of compartmentalized *Pirellula sp., Isosphaera sp*., showing the pirellulosome (Pl) and the nucleoid (N) surrounded by the intracytoplasmic membrane (ICM) and separated from the paryphoplasm (P), which is surrounded by the ICM and the cytoplasmic membrane (CM), image **1** and **2**. Thin section of the "*candidatus Brocadia anammoxidans"* showing the nucleoid (N) and the anammoxosome (A) surrounded by a single membrane (SM); the paryphoplasm (P) and the nucleoid (N) are bounded by the intracytoplasmic membrane (ICM), image **3**. Thin section of *Gemmata obscuriglobus* showing the inner membrane (IM) invagination surrounding the cytoplasm space (C), the nucleoid (N) and the periplasm (PE) which is bounded by the cell wall, image **4**. Adapted from Lindsay and collaborators (2001) and Santarella-Mellwig and collaborators (2013).

1.1.3 Physiological Characteristics and Metabolic Features

Physiological properties and metabolic capabilities inferred from *Planctomycetes* derived typically from members in pure culture. Many of the *Planctomycetes* are chemoheterotrophic using carbohydrates as major carbon source. The sugar and sugar derivatives like glucose, fructose and xylose are the main carbon sources, but growth can be supported also by sugar alcohols like mannitol (Youssef and Elshahed 2014). As nitrogen sources, *Planctomycetes* prefer ammonia as well as N-acetylglucosamine, which is also a carbon source (Giovannoni *et al.* 1987, Schlesner *et al.* 2004).

Some species relies on oxygen for respiration (*G. maris, I. pallida* and *G. obscuriglobus*) while others are facultative anaerobes (*B. marina, R. brasiliensis, P. limnophilus* and *Schlesneria paludicola*) capable of nitrate reduction under anaerobic conditions (Ward *et al.* 2006). The anammox *Planctomycetes* are anaerobic and chemoautotrophic with the remarkable ability for anaerobic ammonia oxidation (Schmid *et al.* 2000).

In addition to the physiological characteristics presumed from axenic cultures, the available genome sequences increased metabolic information of *Planctomycetes*. For example, the C1 metabolism was known only in methanogenic bacteria and methylotrophic *Proteobacteria,* until the detection of C1 transfer genes in the genomes of *Planctomycetes* but undetected in the anammox "*Candidatus Kuenenia stuttgartiensis*" (Glöckner *et al.* 2003, Bauer *et al.* 2004). The involvement of Tetrahydromethanopterin-dependent C1 transfer in the growth of *Planctomycetes* on methane, methanol and derivatives rises their important role in marine carbon cycling (Kalyuzhnaya *et al.* 2005).

On the other hand, anammox *Planctomycetes* contributes to the nitrogen and carbon cycles by the anaerobic ammonia oxidation and apparently by carbon fixation through acetyl-coenzyme A pathway (Schouten *et al.* 2004). The anaerobic ammonium oxidation occurs in the anammoxosome by the oxidation of ammonia to nitrogen gas using nitrite or nitrate as electron acceptors. This unique metabolic activity enhanced the importance of anammox *Planctomycetes* in the nitrogen loss from marine environments, being responsible for about 50% of the total nitrogen turnover (Kuenen 2008). Beyond their important role in the nitrogen cycle, anammox bacteria can be used for ammonia-rich wastewater treatments (Güven *et al.* 2004, Cema *et al.* 2006).

The presence of genes encoding sulfatases in some members of the phylum *Planctomycetes* also suggests the production of extracellular sulfatases for the degradation of sulfated glycopolymers to use carbon as energy source (Woebken *et al.* 2007). In *R. baltica* strains the sulfatases expression profile depends on the life cycle, exposure to environmental stress conditions or available nutrients, supporting an important and specific role on their metabolism (Wecker *et al.* 2009, Wegner *et al.* 2013). Also the occurrence of higher number of sulfatase genes in fosmids and marine species, rather than freshwater *Planctomycetes* may explain the *Planctomycetes* found associated to macroalgae, microalgae and plants (Woebken *et al.* 2007), since marine *Planctomycetes* can degrade the sulfated polysaccharides present in the cell wall of marine algae (Popper *et al.* 2011).

The fundamental role of *Planctomycetes* on global climate must continue to be explored to better understand the relationship between physiological/metabolic features and their ecological relevance.

1.1.4 Ecological Relevance

The *Planctomycetes* are widely distributed in terrestrial and distinct aquatic environments, likewise sea water, brackish water and freshwater habitats. At first, members of the *Planctomycetes* were thought to occur only in aquatic environments, but then they were also reported in soil habitats using 16S rRNA gene libraries (Liesack and Stackebrandt 1992). Wang and collaborators demonstrated that *Gemmata*-like representatives, from soil habitats, can be isolated in pure culture from the same site as previously detected using 16S rRNA gene libraries (Wang *et al.* 2002). Also, *Planctomycetes* were found in thermophilic habitats, like *I. pallida,* a bacterium isolated from a hot spring, which can grow up to 55°C (Giovannoni *et al.* 1987). In the past years, the improvement of isolation methodologies have increase the characterization of new axenic species within the family *Planctomycetaceae* (Winkelmann and Harder 2009, Kulichevskaya *et al.* 2012, Lage and Bondoso 2012).

The introduction of pyrosequencing and metagenomic studies based on the 16S rRNA gene and also in specific probes developed recently, allowed the identification of high numbers and new lineages of *Planctomycetes* from distinct habitats (Gade *et al.* 2004, Martín-Cuadrado *et al.* 2007, Shu *et al.* 2011, Steven *et al.* 2011, Wang *et al.* 2012). From

acidic wetlands, termite guts, marine snow and associated with diatom blooms and eukaryotes (e. g. macroalgae and human gut) to harsh environments for *Planctomycetes* survival, like terrestrial and sea mud volcanoes, hydrothermal carbonate chimney, mangrove environments and uranium contaminated subsurface soil (Rath *et al.* 1998, Alain *et al.* 2006, Brazelton *et al.* 2006, Morris *et al.* 2006, Akob *et al.* 2007, Köhler *et al.* 2008, Liang *et al.* 2007, Santelli *et al.* 2008, Lage and Bondoso 2011, Ivanova and Dedysh 2012, Cayrou *et al.* 2013).

The ubiquity of *Planctomycetes* must derive from physiological mechanisms beneficial for the adaptation and survival to such diverse ecosystems. Recent studies begun to elucidate the impact of environmental changes in the transcriptome of *R. baltica* and synthesis of secondary metabolites for drug development directly from the available genome sequences (Wecker *et al.* 2009, Jeske *et al.* 2013). The exploration of mechanisms involved in adaptation to adverse environmental conditions, may give us clues about metabolic processes with biotechnological applications.

1.1.5 The Genus *Planctomyces*

Members of the genus *Planctomyces* were the first to be ascribed in the phylum Planctomycetes, such as *P. bekefii*, *Planctomyces guttaeformis* and *Planctomyces stranskae,* which are validly described but not yet obtained in pure culture (Starr and Shimdt 1989). The first report on the observation of a *Planctomyces* member in pure culture was in 1976 by Bauld and Staley. A marine bacterium named *Gimesia maris* (formerly named *Planctomyces maris*) was isolated from the estuarine system Puget Sound (Washington, USA), in the Pacific Ocean. Thereafter, the isolation of *Rubinisphaera brasiliensis* (formerly named *Planctomyces brasiliensis*) and *Planctopirus limnophilus* (formerly named *Planctomyces limnophilus*) from a saline pond of Lagoa Vermelha (Brazil) and a German freshwater lake, respectively increased the number of pure culture species within the genus *Planctomyces* (Hirsch and Müller 1985, Schlesner 1989).

Planctomyces spp. colonies can be pigmented or colourless and smooth to rough dry. Cells are spherical to ovoid or even bulbiform and range in size from 1 to 1.5 µm (Ward *et al.* 2006). They can attach to substrates by a holdfast located at the tip of a stalk and in natural habitats can form rosettes. As they reproduce by budding the mother cell is sessile and the buds are motile by one polar or subpolar flagellum (Ward 2010).

The main nitrogen source is ammonia and carbohydrates are the preferred carbon source. Within the pure culture species, R*. brasiliensis* is the only species capable of nitrate reduction, however, a dissimilatory reduction was reported for a *P. limnophilus* strain during the analysis of the complete genome (Labutti *et al.* 2010, Scheuner *et al.* 2014). The genomes of the three pure culture species are complete but the *G. maris* genome remains unpublished.

The optimal growth of the three species occur at 30°C on dilute media since growth is inhibited in rich media (Schlesner *et al.* 2004). As *R. brasiliensis* and *G. maris* were isolated from salt-rich environments growth depends on the presence of sea water. Optimum growth of *R. brasiliensis* occurs in 100% artificial sea water (ASW, corresponding to a salinity of 34.5‰) and *G. maris* optimum growth occurs in 50% ASW (Schlesner 1989, Schlesner *et al.* 2004). In addition, *R. brasiliensis* tolerates up to 300% ASW unlike *G. maris* which tolerates up to 150% ASW (Bauld and Staley 1976, Schlesner 1989). On the other hand, *P. limnophilus* is sensitive to high salt concentrations and grows in ASW concentrations up to 25% (Hirsch and Müller 1985).

Recently Jeske and collaborators (2013) improved the growth rate of *P. limnophilus* and performed a phenotypic analysis to search for bioactive compounds and compared to phenotypic responses of *R. baltica* under different carbon sources. This was the first analysis of the phenotypic response of mesophilic *Planctomycetes* to different environmental conditions.

1.2 Life in Extreme Environments

Water is a crucial element of life and its availability may affect growth or even survival of many organisms. Within the three domains of life, microorganisms are better adapted to fluctuations in the chemical and physical parameters of their surroundings, since several can thrive in environments considered extreme and inhospitable for most living organisms. This remarkable feature defines these microorganisms as extremophiles, which are classified based on the particular extreme conditions where they live **(Table 1.1)** (Rothschild and Mancinelli 2001, Pikuta *et al.* 2007). For example, halophiles live at hypertonic environments (5-30%, v/v) and NaCl is necessary, for example, for the stabilization of enzyme activity, cell walls and membranes. Other microorganisms are halotolerant and can also live in the presence of moderate salinity levels, without requiring saline conditions for growth (Ventosa *et al.* 1998).

Table 1.1. Classification and examples of extremophiles.
Adapted from Rothschild and Mancinelli (2001).

Environmental condition/ Type	Growth conditions	Representative microorganisms
Solute and Water activity		
Osmotolerants	Growth at different ranges of water activity or osmotic concentration	*Staphylococcus aureus, Shaccharomyces rouxii*
Halophile	Growth NaCl > 6%	*Halobacterium*
Temperature		
Psycrophile	Optimum Temperature < 15°C	*Polaromonas vacuolata*
Mesophiles	Optimum Temperature 20-45°C	*Escherichia coli, Rhodopirellula baltica*
Thermophiles	Optimum Temperature 60-80°C	*Thermus thermophilus, Isosphaera pallida*
Hyperthermophile	Optimum Temperature > 80°C	*Pyrolobus fumarii*
pH		
Acidophiles	Optimum pH between 0-5.5	*Singulisphaera rosea Zavarzinella formosa*
Neutrophiles	Optimum pH between 5.5-8.0	*Planctomyces, Rhodopirellula*
Alkalophile	Optimum pH >10	*Bacillus*
Pressure		
Barophilic	Pressure > 30 MPa	*Colwellia hadaliensis*
Radiation		
Radiation resistant		*Deinococcus radiodurans*

1.2.1 Water Availability, Osmoadaptation and Halophiles

The water availability has been expressed quantitatively by water activity (a_w), due to the need to measure the profound effect of osmotic concentration of a habitat on microorganisms. Most microorganisms are incapable of surviving in environments with low a_w, due to cell dehydration. Thus, the environmental distribution of microorganisms depends on the ability to adapt to environments with low a_w. Osmotolerant microorganisms increase internal solute concentration to thrive in such environments, e. g. hypersaline environments or concentrated sugar solutions (da Costa *et al.* 1998, Empadinhas and da Costa 2006). Some halophilic fungi can grow at high sugar concentrations and at values of a_w considered minimum for life (a_w= 0.605) (Grant 2004). Recently, halophilic prokaryotes, which can grow in saturated NaCl (a_w= 0.75) and eukaryotic microbes, present in saline substrates, were reported to grow almost at a_w of 0.611 and 0.632, respectively. These findings suggest that biological processes from the three domains of life can occur at lower a_w (Stevenson *et al.* 2015).

In Bacteria and Archaea the decrease in a_w is reached adding salt to the media in order to examine osmoadaptation (da Costa *et al.* 1998). Osmoadaptation is defined by the rapid physiological alterations taking place in the cell to cope with environmental water changes (Galinski 1995, Empadinhas and da Costa 2008). Two main strategies, reflecting different evolutionary mechanisms, have been developed by Bacteria and Archaea for osmotic adjustment, called the "salt-in-the-cytoplasm" and compatible solute strategy **(Figure 1.5)**.

The "salt-in-the-cytoplasm" involves the accumulation of inorganic solutes, such as K^+ and Cl^- to achieve osmotic equilibrium. This form of osmoadaptation coevolved with the structural modification of many cellular components, which are important for the functionality of cell metabolism in high ionic strength conditions (Galinski 1995, Muller and Oren 2003). This strategy occurs in some extremely halophilic archaea like *Halobacteriaceae* and in the extremely halophilic bacterium *Salinibacter ruber* (Oren *et al.* 2002).

The second mechanism is extensively used by eukaryotes and most bacteria and archaea, involving the accumulation of organic molecules to compensate the salt concentrations in the environment (Santos and da Costa 2002, Lentzen and Schwarz 2006).

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

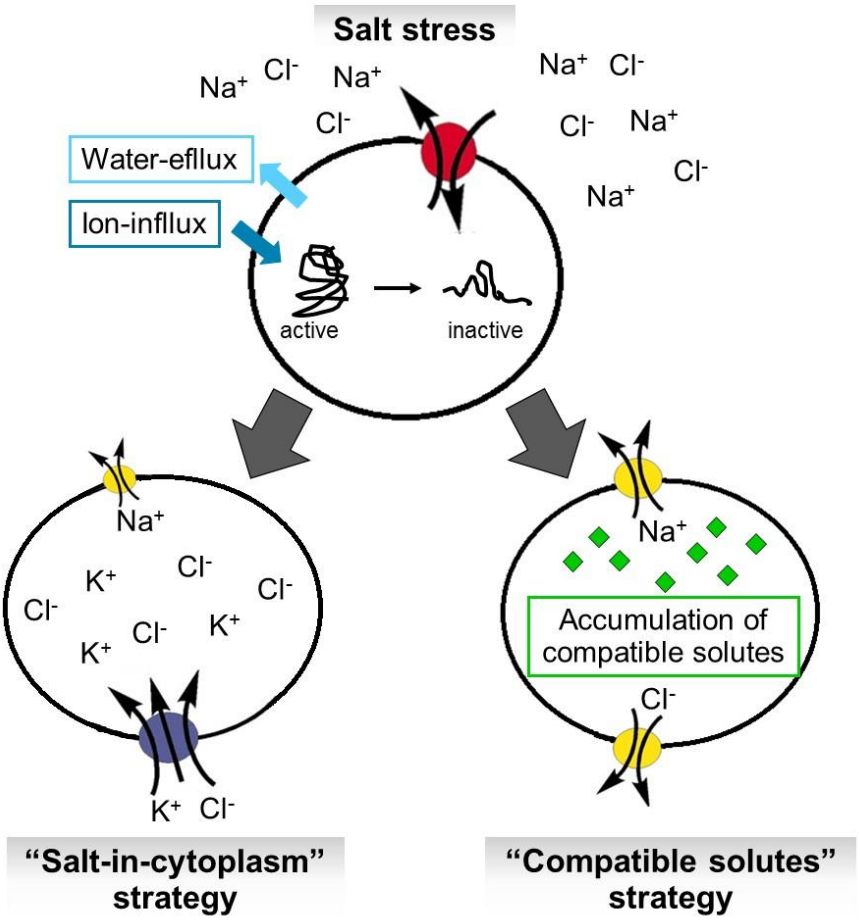


Figure 1.5. The two general osmoadaptation strategies.
In prokaryotes, hyperosmotic stress causes low water potential resulting in intracellular water loss, as well as accumulation of inorganic ions, which cause toxic effects on cellular metabolism (including denaturation of proteins). The osmotic equilibrium is achieved by the influx and accumulation of inorganic ions or by ion extrusion and simultaneously accumulation of compatible solutes. Adapted from Strange (2004) and Klähn and Hagemann (2011).

1.3 Compatible Solutes

Most organisms cope with low a_w by increasing the internal solute concentration. A solute which is accumulated in high concentrations and restores cell turgor pressure, without affecting normal cellular functions is designated a compatible solute (Brown 1976). These small and highly soluble organic molecules can be obtained through *de novo* synthesis or uptake from the environment (Poolman and Glaasker 1998). The uptake of compatible solutes is usually preferred to *de novo* synthesis because of lower energy costs. However, specific compatible solutes can be synthesized, because their absent in the environment or those that are present do not fulfil the organisms' specific requirements (Pfluger and Muller 2004).

Microorganisms which rely on the accumulation of these organic macromolecules have not undergone specific modifications and are, therefore sensitive to high intracellular concentrations of salts (da Costa *et al.* 1998). But the role of compatible solutes goes beyond the osmotic adjustment as they also provide protection to cells and cell components against freezing, desiccation, high temperature and oxygen radicals (Santos *et al.* 2007). Compatible solutes have also been shown to stabilize macromolecules *in vitro* and, in the near future, may be potential drugs for the development of new therapies, against protein misfolding diseases (Kanapathipillai *et al.* 2008, Jorge *et al.* 2011).

1.3.1 Role and Diversity of Compatible Solutes in Eukarya, Archaea and Bacteria

Most common compatible solutes are low-molecular-weight solutes, neutrally charged or zwitterionic and include amino acids and amino acid derivatives, sugars and sugar derivatives, polyols, phosphodiesters, betaines and ectoines **(Figure 1.6)** (da Costa *et al.* 1998, Santos and da Costa 2002). Some compatible solutes are widespread among archaea, bacteria, yeast, filamentous fungi and algae but others are restricted to a few groups of microorganisms (da Costa *et al.* 1998).

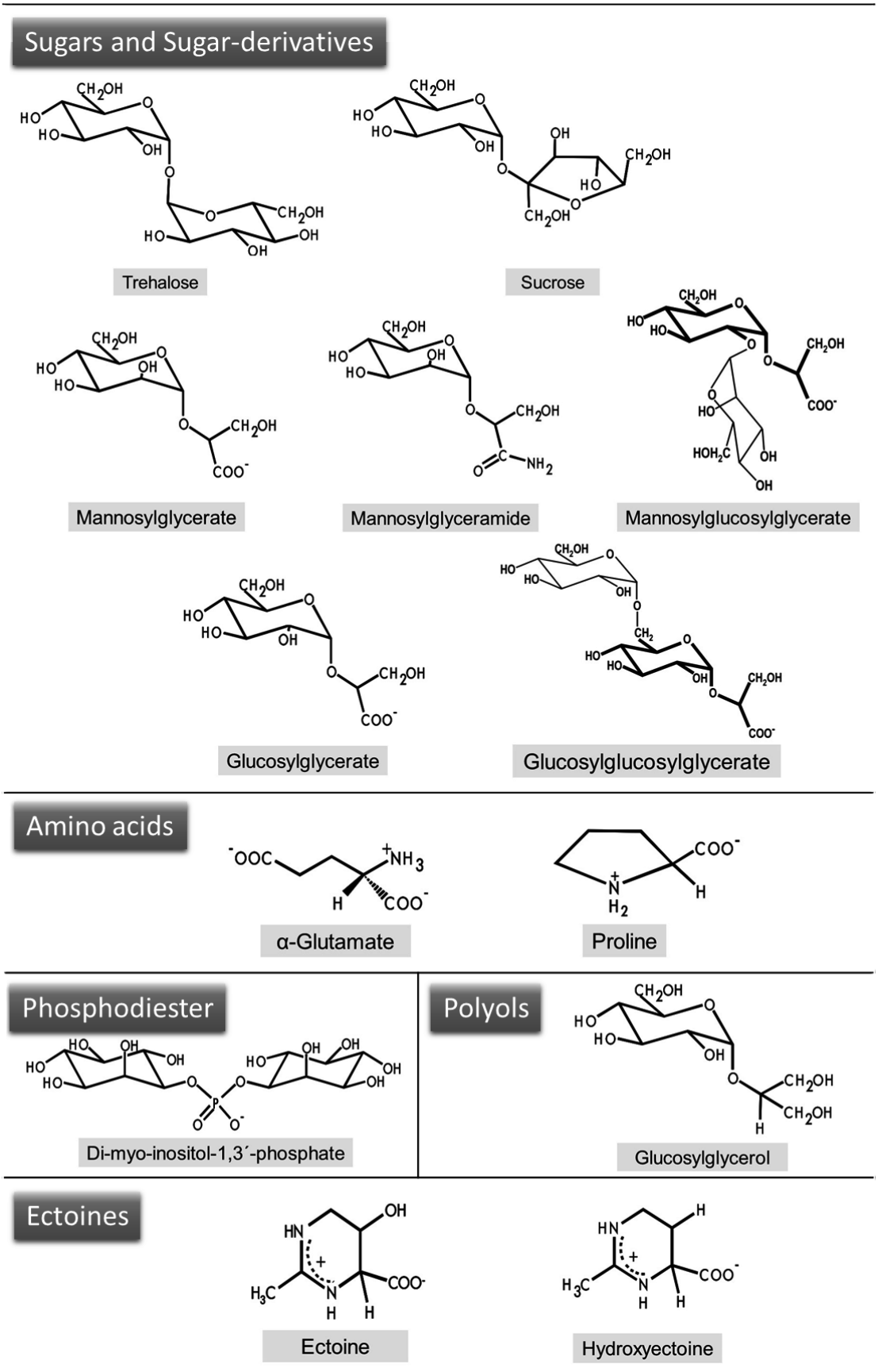


Figure 1.6. Structural representation of some compatible solutes classified according to their chemical structure.
Adapted from Santos and collaborators (2007) and Empadinhas and collaborators (2009).

Compatible solutes like trehalose, α-glutamate and proline are regularly found among bacteria. Trehalose is commonly used by microorganisms as a carbon source and as cellular protectant against stress conditions such as heat, oxidation, desiccation or freezing (Elbein *et al.* 2003, Liang *et al.* 2006). In eukaryotic microorganisms, this disaccharide can also act as a reserve compound, where in insects this disaccharide is abundant in the haemolymph and in thorax muscles to be consumed during flight (Richards *et al.* 2002, Elbein *et al.* 2003).

Compatible solutes such as sucrose are widespread in plants and found in some mesophilic bacteria. The cyanobacterium *Synechocystis sp*. in response to salt stress accumulates sucrose and also glucosylglycerol, a polyol derivative found in eukaryotes (e.g. algae, plants, yeast and fungi) but rarely found among prokaryotes (da Costa *et al.* 1998).

Other compatible solutes like ectoine and hydroxyectoine are found only in mesophilic bacteria (Roberts 2005). Ectoine is abundant within aerobic chemoheterotrophic bacteria and was identified in halophilic proteobacteria, in the genus *Nocardiopsis*, in Gram-negative representatives of the genus *Brevibacterium* and in *Bacillus* spp. (Galinski 1993, Martin *et al.* 1999, Santos and da Costa 2002).

Compatible solutes like di-myo-inositol phosphate (DIP) are, until now, restricted to hyperthermophiles (Empadinhas and da Costa 2008). This solute was found in archaea, such as in the hyperthermophile *Pyrococcus woesei* and in the methanogenic thermophile *Methanococcus igneus* (Scholz *et al.* 1992, Ciulla *et al.* 1994). The accumulation of DIP occurs as a response to supra-optimal growth temperatures suggesting a protection role of cellular components against heat denaturation (Borges *et al.* 2006).

Other compatible solutes like the sugar-derivative, mannosylglycerate (MG) found in thermophiles is rarely encountered in mesophiles and usually is accumulated in response to osmotic stress. Mannosylglyceramide (MGA) an ammonia derivative of MG found in *Rhodothermus marinus* together with two variations of MG, mannosylglucosylglycerate (MGG) and glucosylglucosylglycerate (GGG) found in *Petrotoga miotherma* and in *Persephonella marina*, respectively, are considered rare compounds (Jorge *et al.* 2007, Fernandes *et al.* 2010). In *R. marinus* high levels of MGA occurs in response to hyper-osmotic conditions during sub-optimal growth temperatures, unlike the large accumulation of MGG in *P. miotherma,* which occurs only in response to hypo-osmotic stress conditions (Silva *et al.* 1999, Jorge *et al.* 2007). On the other hand, a structure analogue to MG,

glucosylglycerate (GG) has been identified in mesophilic as well as thermophilic bacteria (Empadinhas and da Costa 2008).

1.3.2 Glutamate

In many bacteria, the high levels of K^+ are compensated by the accumulation of organic anions, mostly amino acids and amino acid derivatives, such as α-glutamate. At low salinity levels, the accumulation of α-glutamate balances the intracellular K^+ in a rapid response, however at high salt concentrations, negatively charged organic solutes are required to restore the internal turgor pressure (Roberts 2005, Empadinhas and da Costa 2008). With the exception of *Petrotoga miotherma,* which accumulates high levels of α-glutamate under high salinity levels and are almost undetected at low salt levels (Jorge *et al.* 2007). Additionally, a β-isomer of this solute has been detected together with the α-isomer in *Petrotoga mobilis* and in the hyperthermophilic archaeon *Aquifex pyrophilus* in a response to salt stress (Lamosa *et al.* 2006).

1.3.3 Sucrose

Like trehalose, sucrose is one of the most common naturally occurring disaccharides, composed of glucose and fructose. In plants, the osmotic pressure is maintained by the accumulation of high levels of sucrose in response to cold, drought or salt stress (Lunn 2008). Moreover, the function of sucrose in plants goes beyond the osmoregulation also acts as a storage reserve and at the transcriptional, translational and post-translational levels, regulating metabolic and developmental processes (Lunn and MacRae 2003, Rolland *et al.* 2006).

On the other hand, sucrose is rarely found in prokaryotes being synthesized only by cyanobacteria, some Proteobacteria and by methanotrophic bacteria, such as *Methylobacter alcaliphilus* strain 20Z and *Methylobacter modestohalophilus* strain 10S (Hagemann and Marin 1999, Khmelenina *et al.* 1999, Klähn and Hagemann 2011). Sucrose accumulation in cyanobacteria is favoured at low salinity levels but in two methanotrophic bacteria is favoured during hyper-osmotic stress (Klähn *et al.* 2010). However, some cyanobacteria such as the marine *Synechocystis* strains PCC6714 and PCC6803 also accumulate high levels of sucrose in response to desiccation and high temperatures (Warr *et al.* 1985, Desplats *et al.* 2005).

Recently, sucrose accumulation related to high salinity levels was found in *Rhodopirellula baltica* strain SH1, a marine bacterium of the phylum *Planctomycetes*, suggesting a more versatile role of sucrose accumulation in prokaryotes (D'Avó *et al.* 2013).

1.3.4 Mannosylglycerate

Mannosylglycerate was initially identified in a red alga of the order *Ceramiales,* serving as transitory solute until the beginning of mannitol accumulation (Bouveng 1955). This is one of the most widespread solutes in (hyper) thermophiles, present in members of Bacteria and Archaea belonging to distinct lineages (Santos *et al.* 2007). Within thermophiles, MG was found in different archaea such as the euryarchaeotes of the genus *Pyroccocus*, *Archaeglobus* and *Methanothermus;* the order *Thermococcales, Thermococcus, Pyrococcus Palaeococcus* and in the crenarchaeotes *Aeropyrum pernix* and *Stetteria hydrogenophila* (Martins and Santos 1995, Lamosa *et al.* 1998, Goncalves *et al.* 2003, Neves *et al.* 2005). Also, in thermophilic prokaryotes this organic solute was found in three bacterial species of unrelated lineages, *Rhodothermus marinus*, *Thermus thermophilus* and *Rubrobacter xylanophilus* (Nunes *et al.* 1995, Silva *et al.* 1999, Alarico *et al.* 2007).

In *R. marinus* the accumulation of MG and MGA occur in response to salt stress however, at the highest growth temperature, MG is the major compatible solute and MGA is undetected. The levels of MG in *R. marinus* are dependent on growth temperature. On the other hand, under moderate salt stress MG is replaced by the neutral derivative, MGA becoming the dominant compatible solute (Silva *et al.* 1999).

In addition, the exposure of *R. xylanophilus* to different stress conditions (e.g. thermal stress and salt stress) has slight or no effects on the accumulation of MG (Empadinhas *et al.* 2007). The accumulation of MG at non-stressing conditions and upon salt stress is exclusive to this actinobacteria suggesting a constitutive synthesis of this compatible solute.

1.3.5 Glucosylglycerate

Glucosylglycerate is a sugar-derivative solute, initially identified in the marine cyanobacterium *Synechococcus* sp. PCC7002 (formerly named *Agmenellum quadruplicatum*) as a response to nitrogen-limiting conditions (Kollman *et al.* 1979). The evidence of GG acting as a true compatible solute was later found by Goude and collaborators (2004), while studying the accumulation of compatible solutes in the phytopathogenic soil enterobacterium *Dickeya dadantii* strain 3937 (formerly named *Erwinia chrysanthemi*), under salt stress. In this bacterium, glutamine and α-glutamate were the main compatible solutes accumulated along with low levels of GG, under increased salinities. However, in salt-stress cells under nitrogen-limiting conditions, glutamine and α-glutamate concentrations decreased to almost undetectable levels and were replaced by increased concentrations of GG that became the main or sole compatible solute. These findings demonstrate that when nitrogen availability is low to sustain synthesis of amino acid compatible solutes, *D. dadantii* accumulates the nitrogen-free compatible solute, GG.

Recently, similar results were reported to the marine cyanobacterium *Prochlorococcus marinus* which do not synthesize glucosylglycerol, a solute common among cyanobacteria (Klähn and Hagemann 2011). On the other hand, this species accumulates increasing levels of GG in high salinity concentrations, under nitrogen depletion, suggesting a role for the negative charged solute in the replacement of α-glutamate as a counterion for K^+ (Klähn *et al.* 2010).

The accumulation of GG is scattered among mesophilic bacteria and archaea but until now *Persephonella marina* is the only known thermophilic bacterium accumulating GG. This member of the deep-branching order *Aquificales* with optimum growth at 70-73°C accumulates GG as a true compatible solute when grown under salt stress (Lamosa *et al.* 2013).

Glucosylglycerate was also identified in the extreme halophilic organism *Methanohalophilus portucalensis* strain FDF-1. This methanogenic archaeon constitutively accumulates α-glutamate, β-glutamine, glycine betaine, N-acetyl-β-lysine and low levels of GG during growth under optimum salinities and during salt stress. However, the

concentration of GG in this archaeon showed no response to fluctuations in salinity (Robertson *et al.* 1992).

In some bacteria like *Halomonas elongata*, the accumulation of GG occurs only in a salt-sensitive mutant strain, incapable of ectoine synthesis but able to produce the ectoine precursors (Cánovas *et al.* 1997b). In addition, this mutant accumulates Nγ-acetyldiaminobutyrate (NADA), the precursor of ectoine and hydroxyectoine. The accumulation of these compatible solutes is osmoregulated unlike the accumulation of GG, which decrease at high salinities. On the other hand, *H. elongata* wild-type achieved osmoadaptation accumulating ectoine, hydroxyectoine and glycine betaine (Cánovas *et al.* 1999).

The accumulation of GG has been found not only in free form (to fill specific nutritional constraints) but also as a component of polysaccharides and glycolipids. Free GG has been detected in low levels in some *Mycobacterium* sp. and proposed as a precursor for the synthesis of methylglucose lipopolysaccharide (MGLP), a complex polysaccharide implicated in fatty acid synthesis modulation and transport (Empadinhas *et al.* 2008, Pereira *et al.* 2008, Mendes *et al.* 2011, Mendes *et al.* 2012). The MGLP has been suggested as a distinctive feature of the genus *Mycobacterium* but was also found in the phylogenetic related species *Nocardia otitidiscaviarum*. In this member of the order *Actinomycetales*, GG was part of the polar head of a glycolipid. However, the physiological role of the GG-containing glycolipid remains elusive (Pommier and Michel 1981).

1.3.6 Ectoine and Hydroxyectoine

Ectoine and its derivative, hydroxyectoine are compatible solutes found only in mesophilic bacteria. The ability to synthesize ectoine is widespread among halophilic/halotolerant *Alpha-proteobacteria*, *Actinobacteria* and in some representatives of *Beta-, Gamma-, Epsilon-proteobacteria* and *Firmicutes* (Galinski 1993, Pastor *et al.* 2010). Unlike ectoine, hydroxyectoine is more common among Gram-positive halophilic and halotolerant bacteria including representatives of the genus *Nocardiopsis, Brevibacteria* and *Streptomyces,* however many ectoine-producing species also synthesize low amounts of hydroxyectoine (Severin and Galinski 1992, Frings *et al.* 1995).

The main function of ectoine and hydroxyectoine is to confer osmoprotection. However, the hydroxylated nature of hydroxyectoine seems to confer additional protective

role to heat stress. In the halophilic bacteria, *Halomonas elongata, Chromohalobacter salexigens* and *Streptomyces griseus* the accumulation of hydroxyectoine is related to osmoprotection and also to high growth temperatures (Pastor *et al.* 2010). Moreover, a *C. salexigens* mutant, affected in the production of the enzyme responsible for the synthesis of hydroxyectoine, is thermosensitive but not osmosensitive, confirming the thermoprotectant properties of hydroxyectoine (García-Estepa *et al.* 2006).

The function of ectoine as an osmoprotectant, against increased external osmostic pressure is well documented, as well as the extended function of hydroxyectoine to protect against heat stress (Martin *et al.* 1999, Santos and da Costa 2002, Roberts 2005). Apart from their protective role as compatible solutes, ectoine and hydroxyectoine are also distinguished by their ability to protect biomolecules from heat, freezing, desiccation and UV-radiation (Jebbar *et al.* 1992, Buenger and Driller 2004, Graf *et al.* 2008). The commercial availability of ectoine comes from its different biotechnological applications as enzyme stabilizer, in the cosmetic industry as enhancer of the moisturizing properties and also because of the benefits as a skin protector against UV-radiation (Oren 2010).

1.4 Biosynthesis of Compatible Solutes

The synthesis of sugar-derived compatible solutes, such as sucrose, trehalose, GG and MG have been elucidated for several organisms and involves a common strategy that generally proceeds in two-steps with the formation of a phosphorylated intermediate, combining the action of a synthase and a phosphatase (Empadinhas *et al.* 2001, Santos *et al.* 2007).

1.4.1 Sucrose Biosynthesis

The pathways for sucrose synthesis were first identified in plants and in cyanobacteria and have recently been elucidated in methanotrophic bacteria (But *et al.* 2015). Sucrose has essential functions in plants, having the ability to modulate the expression of genes involved in cell division and differentiation, including the control of flowering induction, differentiation of vascular tissue and seed development (Lunn and MacRae 2003). Furthermore, the synthesis of sucrose in marine and freshwater cyanobacteria has been related to osmotic stress response, helping to maintain osmotic balance and probably to stabilize proteins and to maintain membrane structure and function (Klähn *et al.* 2010).

In plants and cyanobacteria two biochemical pathways for the synthesis of sucrose have been investigated **(Figure 1.7)**. The more common two-step pathway involves the conversion of UDP-glucose and fructose-6-phosphate into sucrose-6-phosphate (Sucrose-6P) by the sucrose-6-phosphate synthase (SpS). This intermediate is then dephosphorylated by sucrose-6-phosphate phosphatase (SpP) to yield sucrose (Lunn 2002). Biochemical differences between cyanobacterial SpSs and the orthologous plant proteins includes the unspecific activity for UDP-glucose of cyanobacteria SpSs accepting also ADP-glucose, GDP-glucose and, to a minor extent, other sugar nucleotides as substrates (Porchia and Salerno 1996, Curatti *et al.* 1998). Also, the hydrolysis of Sucrose-6P in bacteria is an irreversible pathway leading to the efficient production of sucrose, even in the presence of low substrate concentrations (Lunn 2002).

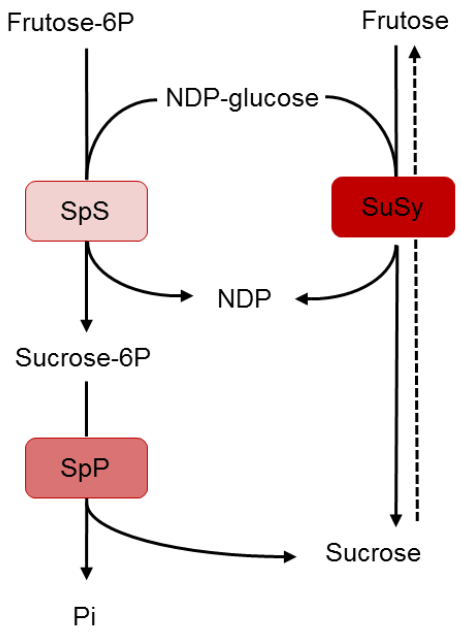


Figure 1.7. Pathways for sucrose synthesis.
The reactions represented are catalyzed by: SpS: sucrose-phosphate phosphatase, SpP: sucrose-phosphate phosphatase and SuSy: sucrose synthase. Adapted from Curatti and collaborators (2000) and Lunn (2002).

The available cyanobacterial genomes revealed that *spS* gene can be found in most cyanobacteria, supporting the prevalence of SpS enzymes for sucrose synthesis among cyanobacteria. Moreover, at least five families of *spS* genes are characterized in cyanobacteria and some plants possess multiple *spS* gene copies (Castleden *et al.* 2004). Generally, the genomes of photoautotrophic bacteria, containing *spS* gene also contain at

least one *spP* gene, which may be separated, contiguous, forming an operon-like structure or fused as a bifunctional gene **(Figure 1.8)**. Interestingly, genomes of *Prochlorococcus* spp. contain, at least, one *spS* gene but *spP* gene remains unknown (Lunn 2002, Scanlan *et al.* 2009). In this case, the predominant C-terminal SpP domain of the SpS protein may be biochemically active or a non-specific sugar phosphatase performs the second step to sucrose synthesis. Recently, the accumulation of high concentrations of sucrose, as a compatible solute, were found in different *Prochlorococcus marinus* strains indicating the presence of the ability for *de novo* synthesis of sucrose (Klähn *et al.* 2010).

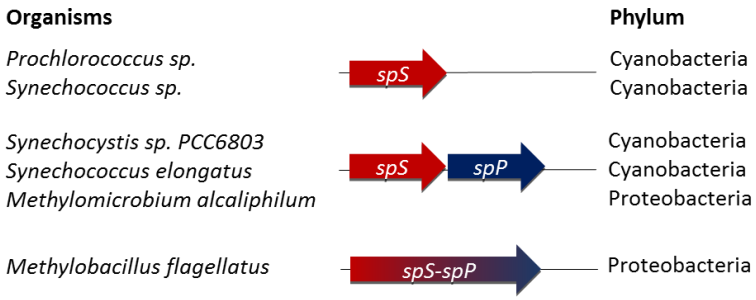


Figure 1.8. Organization of the genes leading to the synthesis of sucrose in different organisms. Arrows represents genes and their directions. Abbreviations: *spS*, sucrose-6-phosphate synthase gene; *spP*, sucrose-6-phosphate phosphatase gene; *spS-spP*, bifunctional sucrose synthase gene. Adapted from Lunn (2002).

The alternative, single step pathway, found in higher plants and in some filamentous cyanobacteria of the genus *Anabaena* involves the condensation of ADP-glucose and fructose into sucrose, by a reaction catalyzed by sucrose synthase (SuSy) (Curatti *et al.* 2000). Despite its name, the reaction catalyzed by SuSy is readily reversible but usually occurs into the direction of sucrose degradation (Lunn 2002). The role of SuSy pathway in cyanobacteria has been proposed in *Anabaena* sp. formerly named (*Nostoc* sp. strain 7120) as an important factor for N_2-fixation, producing the precursors for cell wall synthesis and for nitrogen fixation from sucrose (Cumino *et al.* 2007).

1.4.2 Mannosylglycerate Biosynthesis

The first pathway for mannosylglycerate biosynthesis was found in *Rhodothermus marinus,* consisting on a glucosyltransferase, designated mannosylglycerate synthase (MGS), that catalyzes the condensation of GDP-mannose and D-glycerate to produce MG (Martins *et al.* 1999). This single-step pathway is rare and has also been established for the mesophilic red algae *Caloglossa leprieurii* (Neves *et al.* 2005).

Conversely, the alternative two-step pathway, which includes a phosphorylated intermediate is common among prokaryotes, such as *Pyrococcus horikoshii*, *Thermus thermophilus*, *Palaeococcus ferrophilus* and *Thermococcus litoralis* **(Figure 1.9)** (Empadinhas *et al.* 2003, Neves *et al.* 2005). First, mannosyl-3-phosphoglycerate synthase (MpgS) catalyzes the conversion of GDP-mannose and D-3-phosphoglycerate into a phosphorylated intermediate. Then, mannosyl-3-phosphoglycerate (MPG) is dephosphorylated by mannosyl-3-phosphoglycerate phosphatase (MpgP) to yield MG (Martins *et al.* 1999, Borges *et al.* 2004).

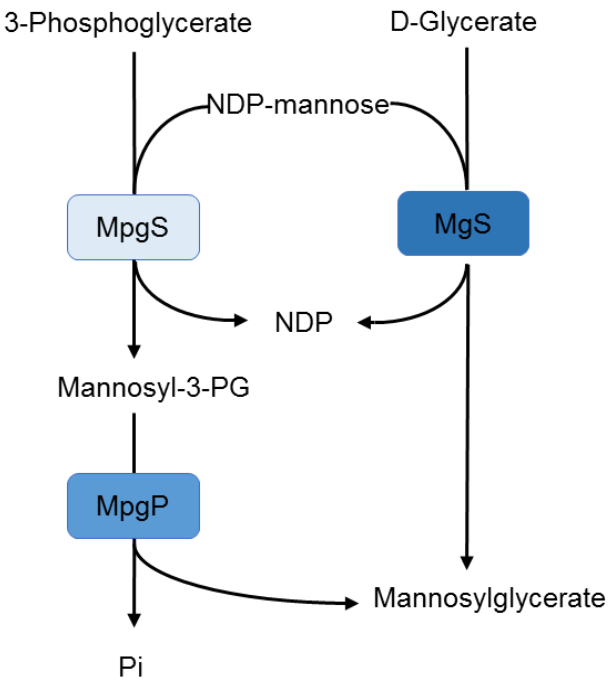


Figure 1.9. Pathways for mannosylglycerate synthesis.
The reactions are catalyzed by the represented enzymes: MpgS, mannosyl-phosphoglycerate synthase; MpgP, mannosyl-phosphoglycerate phosphatase and MgS, mannosylglycerate synthase. Adapted from Martins and collaborators (1999) and Borges and collaborators (2004).

Until now, the occurrence of both pathways for the synthesis of MG is restricted to *Rhodothermus marinus*. In the genome of *R. marinus* and *T. thermophilus* the genes encoding for the two-step pathway for MG synthesis, *mpgS* and *mpgP* genes, are organized in an operon-like structure **(Figure 1.10)**. Moreover, in an uncultured Crenarchaeote, the *mpgP* gene was not detected upstream of the *mpgS* gene, indicating that *mpgP* gene is located elsewhere in the chromosome or the dephosphorylation of the intermediate, MPG, may occur by a different phosphatase or the MPG may be a precursor for unknown macromolecules (Quaiser *et al.* 2002, Treusch *et al.* 2004). Also, a bifunctional mannosyl-3-phosphoglycerate synthase/phosphatase (MgsD), encoded by *mgsD* gene was found in the genome of the mesophilic bacterium *Dehalococcoides ethenogenes* **(Figure 1.10)**. The heterologous expression of *mgsD* gene in *Saccharomyces cerevisiae* led to the *in vivo* synthesis of MG, suggesting a role in the osmotic adaptation of *D. ethenogenes* (Empadinhas *et al.* 2004).

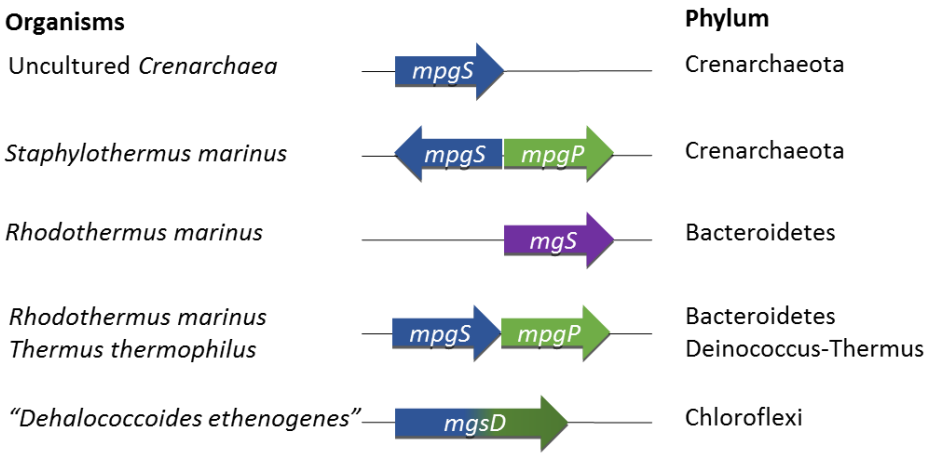


Figure 1.10. Organization of the genes leading to the synthesis of mannosylglycerate in different organisms. Arrows represents genes and their directions. Abbreviations: *mpgS*, mannosyl-3-phosphoglycerate synthase gene; *mpgP*, mannosyl-3-phosphoglycerate phosphatase gene; *mgS*, mannosylglycerate synthase gene; *mgsD*, bifunctional mannosylglycerate synthase gene. Adapted from Empadinhas and da Costa (2010).

1.4.3 Glucosylglycerate Biosynthesis

The genes involved in the biochemical pathway for GG synthesis were initially identified using the genes involved in the two-step pathway for MG synthesis (Costa *et al.* 2006, Costa *et al.* 2007). The screen for genes encoding MG synthesis, in the psychrotolerant archaeon *Methanococcoides burtonii,* revealed the presence of *mpgP* gene, however, *mpgS* gene was absent. Immediately upstream the gene encoding the hypothetical *mpgP*

gene, a gene coding for a glucosyltransferase with unknown function was detected and functionally characterized as GpgS. The GpgS enzyme catalyzes the conversion of GDP-glucose and D-3-phosphoglycerate to form the phosphorylated intermediate, glucosyl-3-phosphoglycerate (GPG). This intermediate is dephosphorylated to produce glucosylglycerate, by the biochemically characterized glucosyl-3-phosphoglycerate phosphatase (GpgP) encoded by the hypothetical *mpgP* gene **(Figure 1.11)**. Also, the *mpgP* gene product from *M. burtonii* was shown to catalyze the dephosphorylation of mannosyl-3-phosphoglycerate (MPG) to yield MG (Costa *et al.* 2006).

Within the two-step pathway for GG synthesis, the *gpgS* gene is widespread throughout most lineages of *Bacteria* and some *Archaea,* suggesting that GG biosynthesis is a disseminated phenomenon. However, the encoded GPG from the *gpgS* gene may participate in pathways for the synthesis of other molecules such as polysaccharides, and not be converted to GG (Empadinhas and da Costa 2010).

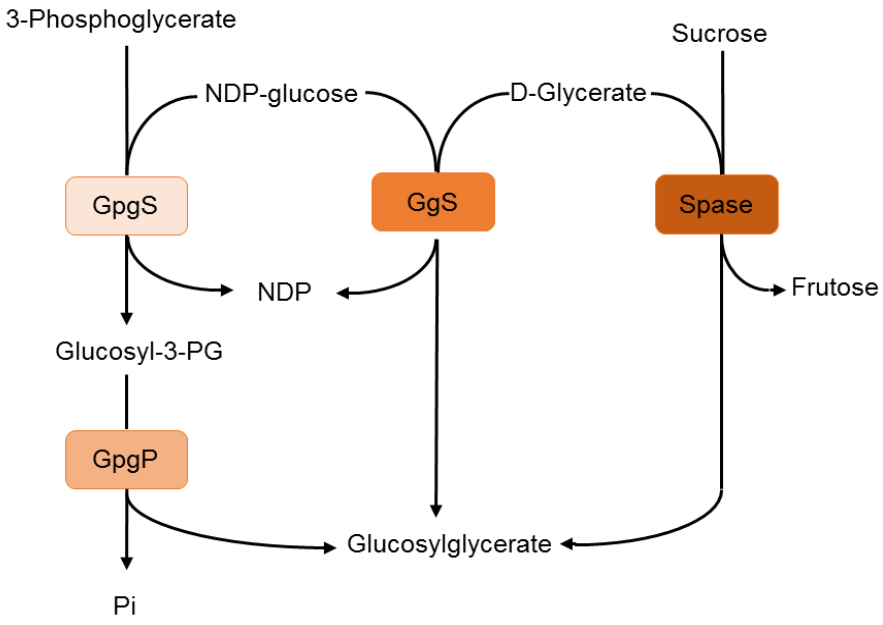


Figure 1.11. Pathways for glucosylglycerate synthesis.
The reactions are catalyzed by the representative enzymes: GpgS, glucosyl-phosphoglycerate synthase; GpgP, glucosyl-phosphoglycerate phosphatase; GgS, glucosylglycerate synthase; Spase, sucrose phosphorylase. Adapted from Costa and collaborators (2006) and Fernandes and collaborators (2007).

The increased number of available genomes led to the search for GG synthesizing genes, in other extreme microorganisms. In the thermophilic bacterium *Persephonela marina*, which accumulates GG, another biosynthetic pathway for GG synthesis was identified as well as the previous GpgS/GpgP pathway found in *M. burtonii* **(Figure 1.11)**. This pathway found only in *P. marina* involves a glucosylglycerate synthase (GgS) that catalyzes the synthesis of GG in one-step, by the direct conversion of ADP-glucose and D-glycerate (Costa *et al.* 2007, Fernandes *et al.* 2007). The presence of two alternative pathways for GG synthesis in *P. marina* suggests the ability for differential regulation between osmotic and thermal stress.

Most organisms possessing only the synthase and the phosphatase harbour the corresponding genes in the same direction and are contiguous **(Figure 1.12)**. In the *P. marina* genome the operon-like organization of *gpgS*, *gpgP* and *ggS* genes, indicates that their expression is under the control of the same promotor and suggests that regulation is likely to occur at the post-transcriptional level. In *M. burtonii* the *gpgS* and *gpgP* genes are contiguous but in opposite directions **(Figure 1.12)** (Empadinhas and da Costa 2006).

Furthermore, in the genomes of cyanobacteria as well as other microorganisms, a putative sucrose-phosphorylase gene (*spasE*) has been found contiguous or between the *gpgS* and *gpgP* genes (Empadinhas and da Costa 2010). Sucrose phosphorylase is a glycoside hydrolase which is known to catalyze the reversible phosphorolysis of sucrose into α-D-glucose-1-phosphate and D-fructose. Due to its promiscuous nature, Spase has also been used for the synthesis of α-glycosidic products by hydrolysis and transglycosylation reactions (Goedl *et al.* 2008, Sawangwan *et al.* 2009, Verhaeghe *et al.* 2014). In a recent study, the Spase enzyme from *Leuconostoc mesenteroides* was able to synthesize GG *in vitro* by the glycosylation of glycerate in the presence of sucrose (Sawangwan *et al.* 2009). These findings lead to the possibility of the involvement of these enzyme in GG synthesis *in vivo* from glycerate and sucrose, under specific nutritional requirements, which may represent a third pathway for GG synthesis.

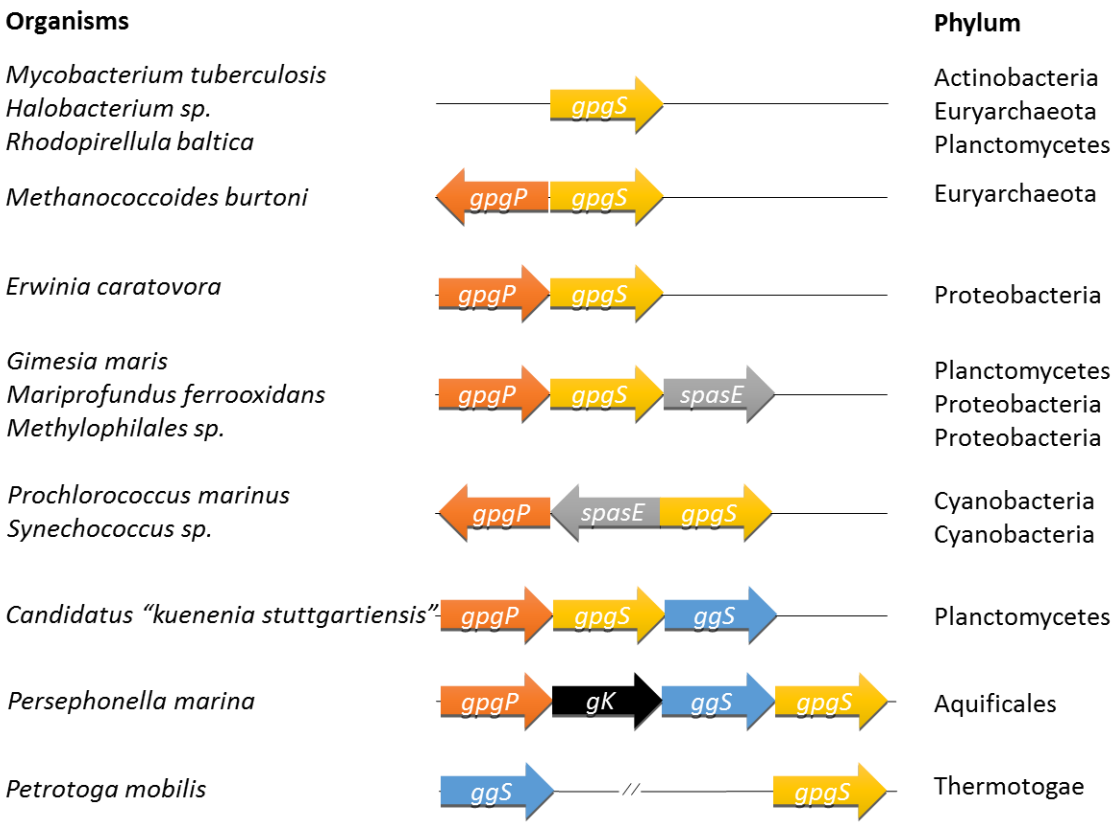


Figure 1.12. Organization of the genes leading to the synthesis of glucosylglycerate (GG) in different organisms.
Orange, yellow and blue arrows represent genes committed to GG synthesis and their directions. Abbreviations: *gpgS*, gene coding for a glucosyl-3-phosphoglycerate synthase; *gpgP*, gene coding for a glucosyl-3-phosphoglycerate phosphatase; *ggS*, gene coding for a glucosylglycerate synthase; *spasE*, gene coding for a putative sucrose phosphorylase; *gK*, gene coding for a putative glycerate kinase/dehydrogenase. Adapted from Empadinhas and da Costa (2010).

1.4.4 Ectoine and Hydroxyectoine Biosynthesis

Ectoine and hydroxyectoine are classified as N-acetylated amino acids and are the most widely produced compatible solutes in bacteria (Galinski *et al.* 1985). The biosynthesis of ectoine occurs in various Gram-positive and Gram-negative bacteria and the ectoine biosynthetic enzymes are encoded by an evolutionary highly conserved gene cluster, *ectABC* (Canovas *et al.* 1998, Bursy *et al.* 2008, Kuhlmann *et al.* 2008, Mustakhimov *et al.* 2010). The ectoine biosynthesis comprises three enzymatic reactions beginning with the conversion of L-aspartate-β-semialdehyde into L-diaminobutyric acid by L-diaminobutyric acid transaminase (EctB). Then, the L-diaminobutyric acid is acetylated to N-γ-acetyldiaminobutyric acid (NADA) by L-diaminobutyric acid acetyl transferase (EctA). In the final step, the condensation of NADA leads to the production of ectoine through the enzyme, ectoine synthase (EctC) (Peters *et al.* 1990). Some ectoine producers also

synthesize hydroxyectoine by the direct hydroxylation of ectoine to hydroxyectoine catalyzed by the additional enzyme, EctD **(Figure 1.13)** (Pastor *et al.* 2010).

The biosynthetic pathway of hydroxyectoine was biochemically established in the halophilic bacterium *Streptomyces chrysomallus* and the moderate halophilic bacterium *Chromohalobacter salexigens* (Prabhu *et al.* 2004, García-Estepa *et al.* 2006). In *C. salexigens* a second pathway for the synthesis of hydroxyectoine was proposed, based in the accumulation of high concentrations of hydroxyectoine in a salt-sensitive mutant of *C. salexigens*, affected in the ectoine synthase encoding gene (Cánovas *et al.* 1999). These results suggested that hydroxyectoine production may involve the hydroxylation of NADA and the subsequent conversion to hydroxyectoine, by a putative hydroxyectoine synthase.

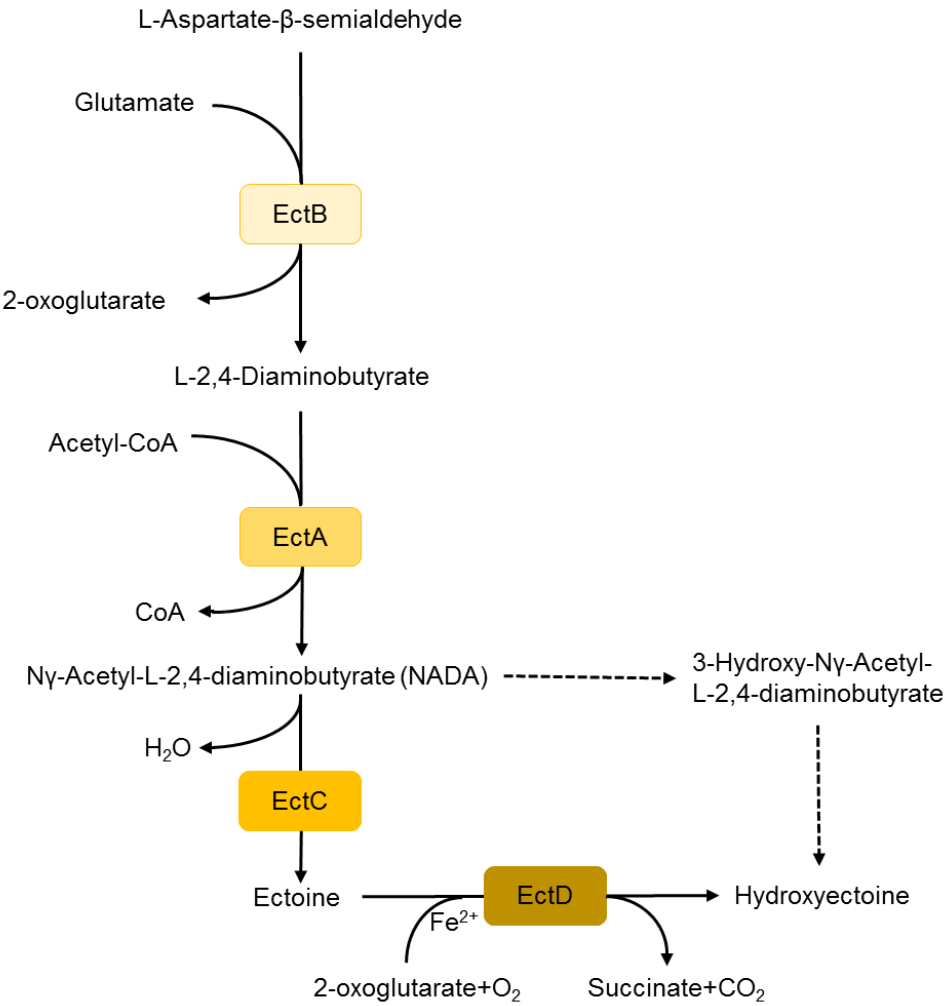


Figure 1.13. Pathways for ectoine and hydroxyectoine biosynthesis.
The reaction is catalyzed by the representative enzymes: EctA, L-diaminobutyric acid acetyl transferase; EctB, L-diaminobutyric acid transaminase; EctC, ectoine synthase; EctD, ectoine hydroxylase. Adapted from Pastor and collaborators (2010).

1.5 Molecular Tools for Gene Expression Analysis Involved in Compatible Solutes Biosynthetic Pathways

The gene expression process is highly complex and tightly regulated, allowing a cell to respond dynamically, both to environmental conditions and as intracellular changes. This mechanism acts as an 'on/off' switch, to control which genes are expressed in a cell, and as a 'volume control' that increases or decreases the level of expression of particular genes as necessary (Dupont *et al.* 2007).

Gene expression microarrays are still the method of choice for whole-genome experiments (Wecker *et al.* 2009, Wecker *et al.* 2010, Walther *et al.* 2010). However, qPCR (Real-Time quantitative Polymerase Chain Reaction) is often used to validate the relative expression of genes, previously identified by global gene expression assays, under different stress conditions (Canales *et al.* 2006). Such whole-genome experiments have been used to examine the effects of different environmental stress conditions, in the marine bacterium *R. baltica*, in the dissemilatory metal-reducing bacterium *Shewanella oneidensis*, in the opportunistic human pathogen *Pseudomonas aeruginosa*, in the sulfate-reducing bacterium *Desulfovibrio vulgaris*, in the phytopathogenic bacterium *Xylella fastidiosa*, in the marine cyanobacterium *Synechocystis* sp. and in the yeast *S. cerevisiae* (Kanesaki *et al.* 2002, Aspedon *et al.* 2006, Gao *et al.* 2004, Koide *et al.* 2006, , Mukhopadhyay *et al.* 2006, Wecker *et al.* 2009).

Microarrays are a powerful technique to unravel the entire set of deregulated genes in a specific condition, compared to a control condition. However, when one intends to study the differential expression of a known and specific subset of genes, qPCR is the most suitable technique (VanGuilder *et al.* 2008). Compared with conventional methods for the analysis of transcript abundance, such as Northern blotting or RNase protection assays, the qPCR has the advantage of high sensitive, specificity, reproducibility and broad dynamic range, making it one of the most widespread techniques in many areas of research (Bustin 2002, Bustin *et al.* 2005). Moreover, this technique allows for the identification of differentially expressed genes between treatments, stress conditions, development stages or genotypes.

However, several parameters need to be controlled in order to obtain accurate and reliable expression measurements. The most frequently used strategy to control such variations is relative normalization, where the expression of a target gene is measured with

respect to total RNA, rRNA or a stable expressed reference gene (VanGuilder *et al.* 2008). The use of rRNA genes and other housekeeping genes is advantageous due to the fact that the transcription is resistant to experimental conditions, making them suitable endogenous controls for single gene normalization (Huggett *et al.* 2005).

Various studies have been performed to identify the most effective reference genes for gene expression analysis by qPCR in prokaryotes in response to environmental stress conditions. These include (with the appropriate genes listed in parenthesis): the cyanobacteria *Synechocystis* sp., *Lingbya* sp. and *Nostoc* sp. grown under different light conditions or in nitrogen-limiting conditions (16S rRNA and *secA* genes), the nitrogen-fixing endophyte *Gluconacetobacter diazotrophicus* grown under different carbon sources (*rho*, 23S rRNA, and *rpoD*) and the acidophilic bacterium *Acidithiobacillus ferrooxidans* grown at different pH levels (*rpoC*, *era* and *alaS* genes) (Nieto *et al.* 2009, Galisa *et al.* 2012, Pinto *et al.* 2012).

Stress conditions are known to promote metabolism alterations in many organisms, with the production of compatible solutes, and at the molecular level, gene expression is also modified upon stress. Stress-inducible genes comprises genes involved in direct protection from stress, including the synthesis of osmoprotectants, detoxifying enzymes and transporters as well as genes encoding regulatory proteins such as transcription factors (Krasensky and Jonak 2012). In the ferrous iron-oxidizing bacterium *Acidithiobacillus ferroxidans,* genes related to iron transport were down-regulated during long-term exposure to heat stress suggesting that the iron processing capability was affected by long-term growth at 40°C (Ribeiro *et al.* 2012). Moreover, in the marine cyanobacterium *Synechocystis* sp. strain 6803 the expression of *spS* gene is transiently increased after salt shock, which corresponds to the accumulation of sucrose in this strain (Marin 2004). On the other hand, in the cyanobacterium *Nostoc punchiforme* the salt-induced synthesis of trehalose was confirmed and the synthesizing enzymes were activated, whereas trehalase activity was inhibited under salt stress. However, the mRNA levels of all trehalose synthesizing genes remained unchanged after exposure to environmental stress conditions. These results suggest that under salt-stress conditions the trehalose synthesis is preferred over the trehalose hydrolysis (Yoshida and Sakamoto 2009, Hagemann 2010).

In addition, different studies have been dedicated to the gene expression analysis to identify novel mechanisms and pathways in extremophiles, such as the ability to evolve and

thrive in extreme environments, as well as the identification of novel enzymes for use in biotechnological and bioremediation strategies (Cytryn *et al.* 2007, LeBlanc *et al.* 2008, Cusick *et al.* 2015).

CHAPTER 2

OBJECTIVES

In general, *Planctomycetes* are considered a slow growth group of *Bacteria*. Recently, the improvement of cultivation methods led to the reduction of the doubling time of *Planctopirus limnophilus*, *Gimesia maris*, *Gemmata obscuriglobus* and *Rhodopirellula baltica* (Schlesner *et al.* 2004, Jogler *et al.* 2011), enabling the search for the synthesis of secondary metabolites such as antimicrobial compounds and compatible solutes in *Planctomycetes* under different growth conditions (Jeske *et al.* 2013).

The behaviour of *Rhodopirellula baltica* under osmotic- and thermal-stress conditions has been examined for the diversity and pattern of the compatible solutes accumulated and by transcriptome analysis of both osmotic and thermal induced or repressed genes (Wecker *et al.* 2009; d'Avó *et al.* 2013). It was verified that the marine bacterium *R. baltica* responded to an increase in salinity by the exchange between the accumulation of trehalose (used for low-level osmotic adaptation) to sucrose. Although, the accumulation of mannosylglucosylglycerate (MGG) was by far the most intriguing feature since this is a very rare solute identified in two thermophilic microorganisms, *Petrotoga miotherma* and *Petrotoga mobilis* (Jorge *et al.* 2007, Fernandes *et al.* 2010). Moreover, MGG was proposed as a compatible solute in *R. baltica* since its synthesis is up-regulated by nitrogen depletion and osmotic stress (D'Avó *et al.* 2013). Understanding the mechanisms underlying the response of other *Planctomycetes* to several environmental pressures will contribute for the clarification of multi-tasked compatible solutes and novel biosynthetic pathways.

Aside from the planctomycetes´ particular phylogenetic position, the complete genomes available and distribution in several environments, the physiological mechanisms present in these organisms for the adaptation to diverse ecosystems are still poorly understood. Thus, the main objective of this thesis was to investigate the mechanisms underlying stress adaptation by the unusual and little studied group of *Bacteria* of the phylum *Planctomycetes*. From a detailed BLAST search, we detected homologous for biosynthetic pathways involved in the synthesis of compatible solutes revealing the existence of a broad and diverse distribution of putative biosynthetic pathways across the *Planctomycetes*. By the putative biosynthetic pathways obtained, we were able to foresee in advance the compatible solutes accumulated by two halophilic *Planctomycetes*.

Thus, this work comprised the following experimental aims:

1. To ascertain the selective accumulation of compatible solutes under different stress conditions, since previous results show that some compatible solutes accumulate during salt stress while the same organism accumulates other solutes under nutrient-limiting conditions. Therefore, we expected to find the same phenomenon operating in *Gimesia maris* and *Rubinisphaera brasiliensis;*

2. Determination of the time needed to observe changes in the levels of compatible solutes accumulated during different shock experiments by the analysis of the pool in compatible solutes from both species under nitrogen-limiting conditions over a period of 12 hours and compared to normal nitrogen conditions;

3. Design and validation of a qPCR method for the evaluation of the expression pattern of compatible solutes coding genes under different environmental stress conditions.

CHAPTER 3

MATERIAL AND METHODS

3.1 Bacterial Strains and Growth Conditions

The marine isolate *Gimesia maris* (DSM 8797^T) and the saline isolate *Rubinisphaera brasiliensis* (DSM 5305^T) were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany) and routinely grown at 30°C, in modified M13a medium (adapted from D´Avó and collaborators, 2013), designated PL medium, containing: 1.8 g of glucose; 1.0 g of ammonium sulfate ($(NH_4)_2SO_4$); 0.1 M of phosphate buffer (pH 7.5); 10 ml of vitamin solution and 20 ml of Hutner´s basal salts in one liter of full strength Artificial Sea Water (100% ASW). The vitamin solution contained per liter, 0.1 mg of vitamin B12; 2 mg of biotin; 5 mg of thiamine-HCl x 2 H_2O; 5 mg of Ca-pantothenate; 2 mg of folic acid; 5 mg of riboflavin; 5 mg of nicotinamide and 5 mg of p-aminobenzoic acid. Hutner´s salt solution contained per liter, 10 g of nitrilotriacetic acid; 29 g of $MgSO_4$ x 7 H_2O; 3.335 g of $CaCl_2$ x 2 H_2O; 12.67 mg of $(NH_4)6MoO_7O_{24}$ x 4 H_2O; 99 mg of $FeSO_4$ x 7 H_2O and 50 ml of "Metals 44" solution. The "metals 44" solution contained per liter, 250 mg of Na-EDTA; 1.095 g of $ZnSO_4$ x 7 H_2O; 500 mg of $FeSO_4$ x 7 H_2O; 154 mg of $MnSO_4$ x H_2O; 39.2 mg of $CuSO_4$ x 5 H_2O; 20.3 mg of $Co(NO_3)_2$ x 6 H_2O and 7.7 mg of $Na_2B_4O_7$ x 10 H_2O. The Artificial Sea Water (100%) contained per liter: 23.47 g of NaCl; 3.92 g of Na_2SO_4; 10.64 g of $MgCl_2$ x 6 H_2O; 1.10 g of $CaCl_2$; 192.0 mg of $NaHCO_3$; 664.0 mg of KCl; 96.0 mg of KBr; 26.0 mg of H_3BO_3; 24.0 mg of $SrCl_2$ and 3.0 mg of NaF.

Axenic cultures of *G. maris* and *R. brasiliensis* were routinely maintained in solid PL medium containing per liter: 0.025% peptone, 0.025% yeast extract, 0.1 M of Tris-HCl buffer (pH 7.5) and 1.5% agar. In addition, 0.1 mg/ml of ampicillin was added to both liquid and solid media to give selection for the growth of *G. maris* and *R. brasiliensis* (Cayrou *et al.* 2010).

To determine the basic growth parameters, the growth salinity range of *G. maris* and *R. brasiliensis* was examined at cells grown in 500 ml of PL medium containing ASW concentrations, varying from 18%, 25%, 50%, 100%, 150% and 200% or 18%, 25%, 100%, 200%, 300% and 350% (vol/vol), respectively. However, for the growth of *R. brasiliensis* cells, the phosphate buffer was added at a lower concentration (0.05 M, pH 7.5) to avoid mineral precipitation in ASW concentrations higher than 200%. The cells were incubated in a rotary air shaker at 130 rpm and growth was monitored by regularly measuring the

turbidity (optical density 610 nm; OD_{610}). Growth curves allowed the calculation of growth rate and doubling times.

The compatible solutes content was investigated in *G. maris* and *R. brasiliensis* submitted to distinct growth conditions, described below. All experiments were performed in triplicate. Cells were grown in Erlenmeyer flasks with 500 ml of PL medium with 0.1 mg/ml of ampicillin and with specific composition adjustments and incubated at 30°C in a rotary air shaker at 130 rpm. In all experiments cells were also incubated in PL medium (named N^+) for control purposes. After appropriate time intervals, cells were harvested by centrifugation (7 000 x g, 4°C, 15 minutes).

3.2 Determination of the Compatible Solute Pool During Growth in Different Salt Levels

The effect of salinity on the accumulation of compatible solutes by *G. maris* was examined in cells grown in PL medium (N^+ medium) containing ASW concentrations, varying from 25%, 50%, 100% and 150% (vol/vol). As *R. brasiliensis* tolerates higher salinity levels, the accumulation of compatible solutes was examined in cells grown in N^+ medium with ASW concentrations, varying from 25%, 100%, 200% and 300% (vol/vol). The cells of *G. maris* and *R. brasiliensis* grown in N^+ medium with 100% ASW (optimum growth conditions) were considered as control cells.

Cell growth was examined by measuring the turbidity at OD_{610} and harvested by centrifugation after 2 days (mid-exponential phase of growth, OD_{610}= 0.6 – 0.65), to be processed for extraction of compatible solutes, as described below.

3.3 Compatible Solute Pool During Different Shock Experiments

3.3.1 Prospecting the Accurate Conditions for the Shift in the Compatible Solutes Content Under Nitrogen-Limiting Conditions and Hyper-Osmotic Shock

To estimate the short-term response to different shock conditions by the shift in the compatible solutes content, *G. maris* and *R. brasiliensis* cells were pre-cultivated at 30°C in 500 ml of N^+ medium under optimum growth salinity (100% ASW) until initial-exponential phase (OD_{610}= 0.35), harvested by centrifugation (7 000 x g, 4°C, 10 minutes) for subsequent exposure to shock conditions described as followed:

a) **Nitrogen-depletion tests:** cells were re-suspended in an equal volume of nitrogen depletion in medium (without $(NH_4)_2SO_4$, named N^- medium) and under nitrogen standard conditions (1 g/l $(NH_4)_2SO_4$, named N^+ medium).

b) **Hyper-osmotic shock tests under nitrogen-limiting conditions:** cells were re-suspended in an equal volume of N^+ or N^- medium with ASW concentrations of 100% and 200%.

From both tests, cells were harvested after 5, 8 and 12 hours of exposure to each shock condition and processed for subsequent extraction of compatible solutes, as described below.

3.3.2 The Effect of Short-Term Exposure to Osmotic Shock in the Compatible Solutes Content During Nitrogen-Depleted Conditions

For the osmotic-shock experiments with *G. maris* and *R. brasiliensis,* cells were pre-cultivated at 30°C in 500 ml of N^+ medium until initial-exponential phase, harvested by centrifugation (7 000 x g, 4°C, 10 minutes) and re-suspended in an equal volume of N^+ or N^- medium containing different concentrations of ASW, described as followed:

a) *G. maris* cells were re-suspended in the above culture media containing, 18%, 50%, 100% and 200% ASW and incubated for 12 hours.

b) *R. brasiliensis* cells were re-suspended in the above culture media containing 18%, 100%, 200% and 350% ASW and incubated for 8 hours.

Cells from the two shock experiments were harvested by centrifugation (7 000 x g, 4°C, 15 minutes) and processed for subsequent extraction of compatible solutes, as described below.

3.4 Extraction of Intracellular Compatible Solutes

Cell pellets were washed twice in a mineral solution containing NaCl and $MgCl_2x6\ H_2O$ with identical concentrations to the growth medium and extracted twice with boiling 80% ethanol as described previously (D'Avó *et al.* 2013). The solvent from both supernatants was removed by rotary evaporation under negative pressure, to concentrate the organic solutes. To remove the lipid content, the residue was resuspended in distilled water and was extracted twice by adding a mixture of water-chloroform (2:1) and subsequent centrifugation (1 200 x g, 4°C, 30 minutes). The aqueous phase was freeze-dried and

suspended in $^{2}H_2O$ for nuclear magnetic resonance (NMR) analysis. An aliquot of the suspension was taken before the extraction of the organic solutes and disrupted by sonication, for subsequent quantification of total protein content determined by the Bradford assay (Bradford 1976).

3.5 Identification and Quantification of Intracellular Compatible Solutes Content by NMR

All spectra were typically acquired on a Bruker AVANCE III 500 spectrometer (Bruker, Rheinstetten, Germany). ^{1}H-NMR spectra were acquired at 500.13 MHz in a 5-mm inverse detection probe head with pre-saturation of the water signal. Chemical shifts are relative to 3-(trimethylsilyl)-1-propanesulfonic acid (sodium salt). For quantification purposes, a known amount of formate was added and a repetition delay of 60 seconds was used to ensure full relaxation of the signals (D'Avó *et al.* 2013).

3.6 Bacterial Shock Experiments Selected for Total RNA Extraction

Gene expression was analysed in *G. maris* cells pre-cultivated in 500 ml of PL medium until initial-exponential phase (OD_{610}=0.35), harvested by centrifugation (7 000 g, 4°C, 10 minutes) and re-suspended either in an equal volume of N^+ medium with ASW concentrations varying from 100% and 200% and as in an equal volume of N^- medium with 100% ASW. Cells were incubated for 12 hours at 30°C.

3.6.1 RNA Extraction

The total RNA was extracted from 50 ml of cells culture, collected from the shock tests described above. The samples collection procedure was adapted from Wecker and collaborators (2009). The cultures were collected and swirled for 30 seconds in an ethanol-dry ice bath to prevent shifts in the RNA profile. Cells were harvested by centrifugation at 3 000 x g (4°C) for 30 minutes, washed with 0.1 M of phosphate buffer and re-centrifuged. Cell pellets were shock-frozen in liquid nitrogen and stored at -80°C.

Total RNA extraction was performed with TripleXtractor reagent (GRiSP), a monophasic solution of guanidine isothiocyanate which prevents ribonuclease activity and maintains RNA integrity during cell lysis. The protocol used was adapted from Wegner and collaborators (2013). Cells were resuspended thoroughly in 1 ml of TripleXtractor reagent.

The suspension was incubated for 5 minutes at room temperature (RT) and centrifuged at 16 000 x g (4°C) for 10 minutes. For the phase separation, 200 µl of ice-cold chloroform were added to cells supernatants, thoroughly mixed by vortexing for 20 seconds and incubated for 10 minutes at RT. Another centrifugation was carried out at 16 000 x g (4°C) for 15 minutes resulting in the separation of an upper aqueous phase containing RNA, a lower organic phase and a white interphase, both containing most of the DNA and proteins. The upper aqueous solution was transferred to a new RNAse-free and sterile tube. The RNA precipitation was performed by the addition of 1 ml of isopropanol (100%), inverting the tubes several times and incubating for 1 hour at -20°C. Another centrifugation at 16 000 x g (4°C) for 30 minutes was carried out for the collection of the RNA pellet. The supernatants were carefully removed using a pipette and discarded. The pellets were washed twice in 75% ethanol and centrifuged at 9 000 x g (4°C) for 5 minutes. The ethanol was removed carefully using a pipette and discarded. The white pellet, almost invisible, was dried for 2 minutes on a speed vacuum dryer (Thermo Fisher) and dissolved in 100-150 µl RNAse-free water. RNA concentration was measured by NAnoDrop-1000 spectrophotometer (Thermo Scientific).

The total RNA was digested with DNAse I (Invitrogen) to remove trace amounts of genomic DNA. For 1 µg RNA, 2 µl of DNase I (1 U/µl) were added as well as 2 µl of 10x DNAse I Reaction Buffer (200 mM Tris-HCl, pH 8.4, 20 mM MgCl, 500 mM KCl) in a total reaction volume of 20 µl. Whenever necessary, the reaction was scaled up linearly. The tubes were incubated at RT for 40 minutes. Then, heat inactivation of DNAse I was performed by the addition of 1 µl of EDTA (25 mM) and subsequent incubation at 65 °C for 10 minutes. Phenolic extraction of total RNA was performed after adjusting sample volumes to 200 µl with RNAse-free water. To the RNA samples 1 volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added per 1 volume of sample. Tubes were shaken vigorously for 15 seconds and centrifuged at 12 000 x g (4°C) for 12 minutes. The upper aqueous phase was collected into new tubes and 150 µl of chloroform was added. Tubes were shaken and centrifuged as previously described. The upper aqueous phase containing RNA was collected, precipitated in 1 ml of absolute ethanol and 10 µl of sodium acetate (3M, pH 5.2) and incubated at -80°C, overnight. Then, the supernatants were removed, the pellets were washed in 75% ethanol and dried as described before. The RNA pellet was reconstituted in 15-20 µl RNase-free water. The quality and concentration of

total RNA was checked by 2% agarose gel electrophoresis and using NanoDrop-1000 spectrophotometer (Thermo Scientific). The absence of genomic DNA was confirmed by PCR reactions containing: 2 U of NZYTaq DNA polymerase (NZYTech), 1x NZYTaq Buffer, 2 mM of $MgCl_2$, 0.2 mM of each deoxyribonucleotide triphosphate (dNTP), 0.3 µM of each 16S rRNA primer **(Table 3.2)** and 85 ng/µl of total RNA. The PCR profile was: 2 minutes at 95°C followed by 35 cycles of 1 minute at 95°C, 1 minute at 60°C and 30 seconds at 72°C and a final extension at 72°C for 5 minutes. The PCR reactions were run on a 2% agarose gel electrophoresis.

3.6.2 Reverse Transcription

About 1 µg of DNAse-treated RNA was transcribed using RT-PCR Kit (GRISP) following the manufacturer´s instructions. A control PCR was performed using 1 µl of cDNA as template in the same reaction conditions and PCR program described before.

3.6.3 Blast Analysis, Primers and Probes Design

Homologous genes leading to the synthesis of glucosylglycerate (GG) via two-step pathway and an upstream gene classified as putative sucrose phosphorylase, previously identified in *G. maris* genome were chosen (Empadinhas and da Costa 2010). Additionally, to identify potential genes related to ectoine and sucrose synthesis we used the amino acid sequences of ectoine synthase (ectC) from *Halomonas elongata* and from *Sporosarcina pasteurii* (Accession number CAA09485.1 and Q9AP33.1, respectively) and the sucrose phosphate synthase (SpS) from *Synechocystis sp.* PCC 6803, *Synechococcus elongatus* and from *Prochlorococcus marinus* (Accession number WP_010874006.1, WP_011243042.1, WP_011824685.1, respectively) for BLAST search in the *G. maris* genome (NCBI, http://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences of the 5 genes, related to compatible solutes synthesis, were obtained from GenBank **(Table 3.1)**.

The primer pairs and the hydrolysis probe of each gene were designed using Primer Quest Software from Integrated DNA Technologies (IDT), considering an amplicon length between 80 and 200 bp, a melting temperature of 60°C and a primer length of 18 to 30 nucleotides. The primer pairs were analysed with OligoAnalyzer tool from IDT Technologies to identify hairpins formation, self-dimer and heterodimers. To assess specificity, the designed primer pairs and probe sequences were compared to *G. maris* genome in National

Center of Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) and a PCR reaction was performed using the designed primer pairs **(Table 3.2)** and chromosomal DNA as template, in the same reaction conditions and PCR program described before. The chromosomal DNA of *G. maris* cells grown at optimum growth conditions, was isolated as described by Costa and collaborators (2010).

The hydrolysis probes contained 6-carboxy-fluorescein (FAM) reporter dye at the 5´end, a lowa black FQ (lBFQ) quencher at the 3´end and the internal Zen quencher, which is placed internally distanced by 9 bases from the reporter dye on the 5´end. This shortened distance combined with the 3´ end quencher allows the detection of the PCR product with greater specificity and higher accuracy. Each PrimeTime qPCR assay composed of 2 primers and 1 probe were synthesized by IDT Technologies.

3.6.4 Analysis and Choice of Reference Genes

The candidate reference genes were selected by bibliographic search and transcription expression results from microarray data covering the expression of *R. baltica* SH1 cultures exposed to different stress conditions. Genes with relative transcript levels between 1.5 and -1.5, for salt shock experiments were selected using the GEO repository by the accession number GSE14075 (Barrett *et al.* 2007)**.** The sequences of the 3 selected genes were obtained from GenBank **(Table 3.1)**. The primer pairs and probe for each target gene were designed and synthesised as described above **(Table 3.2).**

The validation of the most stable reference genes was performed with two normalization algorithms: geNorm version 3.5 (Vandesompele *et al.* 2002) and NormFinder version 0.953 (Andersen *et al.* 2004). Briefly, the geNorm algorithm is based on a pairwise comparison approach and depends on the stability of each reference gene expression value (M), defined as the pair-wise comparison of this gene with all others. The NormFinder algorithm calculates the reference genes stability and perform an estimation of both, between different groups and inside one group. The most stable reference genes are top ranking by the lowest M value.

We also entered raw Cq values in the web-based RefFinder platform to compare the results from the two mathematical algorithms (**http://www.leonxie.com/referencegene.php**).

Table 3.1. Candidate reference genes and target genes evaluated in this study and corresponding accession number and location in *Gimesia maris* genome.

	Description	Accession number (nucleotide, locus tag)
Candidate reference genes		
16S rRNA	16S ribosomal RNA	ABCE01000043.1 (54659 to 57378; PM8797T_r19749)
rpoA	RNA polymerase, alfa subunit	ABCE01000002.1 (226204 to 227451; PM8797T_22163)
rnpB	RNA subunit of ribonuclease P	AF056384.1 (N/A)
Target genes		
gpgS	Glucosyl-3-phosphoglycerate synthase	ABCE01000012 (140386 to 141606; PM8797T_RS12080)
gpgP	Glucosyl-3-phosphoglycerate phosphatase	ABCE01000012 (139538 to 140341; PM8797T_16373)
spasE	Sucrose phosphorylase	ABCE01000012 (141611 to 143371; PM8797T_16383)
spS	Putative sucrose phosphate synthase	ABCE01000062.1 (3491 to 5719; PM8797T_30998)
ectC	Ectoine synthase	ABCE01000010.1 (16086 to 16481*; PM8797T_20403)

* Complement sequence.
N/A – Not available.

3.6.5 Quantitative Real Time PCR Conditions

The qPCR reactions were performed using the ABI Prism 7500 Real-Time PCR system (Applied Biosystems) on MicroAmp optical 96-well PCR plates sealed with MicroAmp optical adhesive film (Life Technologies). Each reaction with a total of 20 µl contained 200 nM forward primer, 200 nM reverse primer, 200 nM hydrolysis probe, 2 µl cDNA in 1x iTaq Universal Probes Supermix (BioRad). Primer pairs and hydrolysis probe sequences used in qPCR reactions are listed in **Table 3.2**. The reaction proceeded with denaturation step at 95°C for 30 seconds and amplification step of 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The relative efficiency and primer/probe specificity were acquired by standard curves using serial dilutions of cDNA. The reactions for qPCR were performed with three biological replicates and technical triplicates for cDNA and technical duplicates for reverse transcription negative control (without reverse transcriptase).

The relative quantities of each sample were calculated by REST software (Pfaffl *et al.* 2002) using gene-specific efficiency acquired from the standard curves and normalized to the mean Cq value. Expression data were imported to GraphPad QuickCalcs (GraphPad Software, Inc.) and the statistical significance of the results was assessed through the Student´s t test.

Table 3.2. Details of each primer and probe designed for the candidate reference genes and target genes.

	Amplicon lenght (bp)	Primer/ Probe	Sequence (5' - 3')	Tm (°C)	Amplification efficiency (%)
Candidate reference genes					
16S rRNA	153	Forward	AATCGCTAGTAATCGCAGGTCAGC	62	92.7
		Reverse	GTCCCAATCACGGAGTTCATCTTA	62	
		Probe	AAAGTCGCTGAGCTAACCCTTCGG	68	
rpoA	138	Forward	ATATTCATACCCTGGGTGATCTG	62	96.8
		Reverse	ATGCAGGTTCTGACCGATATG	62	
		Probe	CTGCTGGCTGGTAAGAACTTCGGT	68	
rnpB	149	Forward	GCCAGGTAAGGGTGAAAC	61	94.6
		Reverse	CGGAAGTCGTAACGGATC	62	
		Probe	CAAGACCAAGCAGGGAGCAGTTCT	68	
Target genes					
gpgS	154	Forward	ATTTATCCTGTCGCCAACCC	62	94.9
		Reverse	GATAATCCAGTGAGCCGAGTATTT	62	
	174	Probe	AGGTTATTATGCACGGGTCGCTGG	68	
gpgP		Forward	GCTGAGATCCTTTCGATCATTC	62	90.8
		Reverse	CCGAGTCCTGCCAAATTA	62	
		Probe	TGTACAGGCAATGACCCGGGAATT	68	
spasE	180	Forward	GGGTGACTGGACTCACATAAA	62	98.9
		Reverse	GGACGAACGACCTCAGATAAA	62	
		Probe	TCCAGCAGTTCAGACGAGGAGAGA	68	
spS/spP	156	Forward	GTCTGGAAGGCACGGTTAAA	62	95.1
		Reverse	GAGGCGAAGTAAACGAGAGATAG	62	
		Probe	ACGCATACCGCTGAAGGACAGATC	68	
ectC	146	Forward	CACGGAAACCGAAATCTGGTA	62	97.4
		Reverse	GGTGACGGTCGTGTTCATTTA	62	
		Probe	CGACATACGCCATCAAACCGGGAA	68	

Tm – Melting temperature

CHAPTER 4

RESULTS

4.1 Growth of *Planctomycetes* at Different Salt Concentrations

In a first series of experiments, two selected *Planctomycetes* isolated from salt-rich environments were compared for their ability to grow in media with different salinity levels. The highest growth rate was found for *Gimesia maris* and *Rubinisphaera brasiliensis* grown in medium containing 100% ASW and as halophiles, both species required at least 25% ASW in medium for growth. Salinity concentrations above or below 100% ASW (optimum salinity) in the medium resulted in the decrease in the growth rate at both sub-optimum and supra-optimum salinities confirming that both species are slightly halophilic organisms. Salt levels of about 150% ASW led to a large decrease in *G. maris* growth and this species was unable to grow in media containing more than 200% ASW. In contrast, *R. brasiliensis* had a higher tolerance to elevated salinities. A decline in growth was observed at supra-optimum salinities of 200% and was most notorious under the maximum salinity (300% ASW) in which *R. brasiliensis* grows.

4.2 The Effect of Salinity Levels on Organic Solute Pool of *Gimesia maris* and *Rubinisphaera brasiliensis*

The compatible solute content from *G. maris* and *R. brasiliensis* was first examined in cells grown in medium containing 100% ASW (optimum growth conditions) during exponential growth phase. Both species accumulated α-glutamate, sucrose and ectoine. Trace amounts of hydroxyectoine were also accumulated by *G. maris*. Additionally, a different pattern in the accumulation of each organic solute was found for both species; α-glutamate was the major solute along with smaller levels of sucrose, ectoine and hydroxyectoine accumulated by the marine isolate *G. maris*, whereas, the saline isolate *R. brasiliensis* accumulated α-glutamate and sucrose as major compatible solutes and small levels of ectoine **(Figure 4.1)**.

Since *G. maris* and *R. brasiliensis* showed a narrow and broad tolerance to differences in salinity levels, respectively, we examined the compatible solute pool from both species grown in medium containing different concentrations in mineral salts. Cells were grown in the presence of different ASW concentrations and collected during the exponential growth phase. In both species the total solute pool was dependent on salinity concentrations in the growth medium. In *G. maris* the total organic solutes was two times higher during

growth at supra-optimum salinities of 150%. Additionally, the two sub-optimum conditions examined containing 50% ASW and 25% ASW led to a 3- and 6 fold decrease in total organic solutes, respectively **(Figure 4.1).**

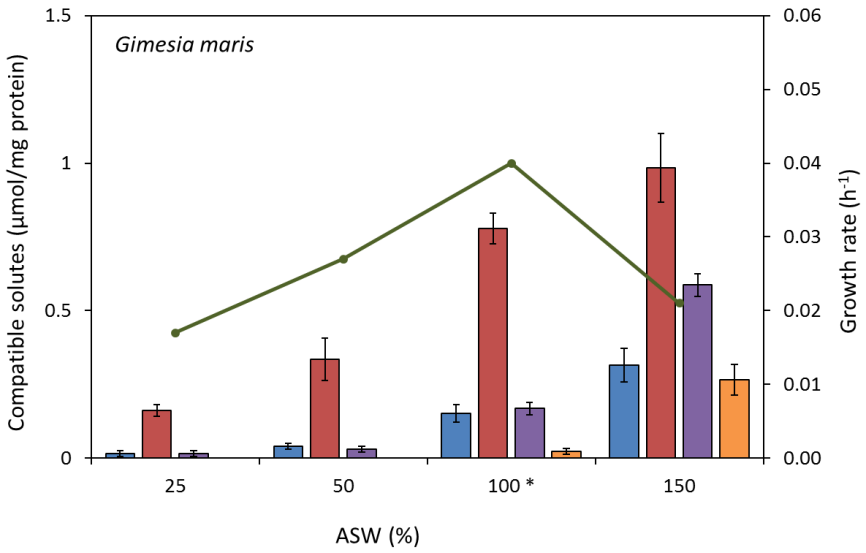
The response of *R. brasiliensis* to supra-optimum salinities was similar to *G. maris* since levels of total organic solutes showed 1.5 fold increase in 200% ASW. This trend was most noticeable at the highest salinity (300% ASW) examined for growth of *R. brasiliensis* since a 3 fold increase in total organic solutes was found over the levels under optimum growth conditions. The sub-optimum salinity containing 25% ASW led to a 3 fold decrease in total organic solutes **(Figure 4.2)**.

As expected, differences in salinity levels on growth medium had, not only affected the total concentration of organic solutes, but also affected the distribution and concentration of each compatible solute in both species.

In all salinities tested, α-glutamate was the main organic solute accumulated by *G. maris.* A linear increase in the accumulation of this solute was related to increasing salinity levels in the medium but it represented 80% of the total solutes in medium containing 50% ASW, 69% of the total solutes under optimum growth conditions and 46% of the total solutes at the maximum salinity in which *G. maris* grows **(Figure 4.1)**.

The increase in mineral salts to 150% led to a 3.5- and 11 fold increase in levels of ectoine and hydroxyectoine, respectively. However, ectoine represented 27% of total organic solutes and hydroxyectoine represented only 12% of total solutes. In addition, sucrose levels represented 15% of total solutes in the maximum salinity examined for growth of *G. maris* but were two times higher over those in optimum growth conditions.

The two sub-optimum conditions tested in *G. maris* led to a decrease in levels of ectoine and sucrose. Each intracellular solute represented only 10% of total organic solutes at medium containing 25% ASW and 50% ASW. It seems that the accumulation of α-glutamate is sufficient for *G. maris* to cope with low salinities **(Figure 4.1)**.



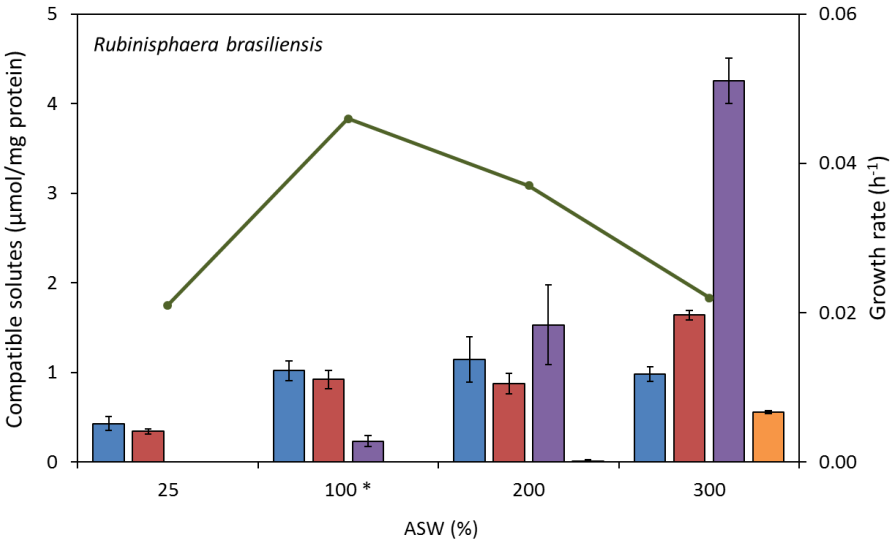
Solutes concentration (µmol/mg protein)	25%	50%	100%*	150%
Sucrose	0.02±0.01	0.04±0.01	0.15±0.03	0.31±0.08
Glutamate	0.16±0.02	0.34±0.07	0.78±0.05	0.98±0.12
Ectoine	0.02±0.01	0.03±0.01	0.17±0.02	0.59±0.04
Hydroxyectoine	n. d.	n. d.	0.02±0.01	0.27±0.05
Growth rate	0.017	0.027	0.040	0.021

Figure 4.1. Growth rate and compatible solutes content from *Gimesia maris* grown in N^+ medium containing 1 g/L ammonium sulfate during exponential growth phase (OD_{610}= 0.6 – 0.65) with different salinity concentrations.
Values are the means ± standard deviation of at least three measurements. n.d., not detected; *, Optimum growth conditions.

In *R. brasiliensis* α-glutamate and sucrose were the main compatible solutes at all salinities tested. In addition, at supra-optimum salinities ectoine became the major solute. The levels of α-glutamate were relatively constant under supra-optimum salinities of 200% ASW but showed 1.5 fold increase at the higher salinity (300% ASW) examined for growth whereas, levels of sucrose were found almost unchanged **(Figure 4.2)**.

Therefore, at supra-optimum salinities *R. brasiliensis* responded primarily with a 7.5 fold increase in ectoine levels, representing 43% of total solutes. This trend was most notable at the highest salinity examined for growth of *R. brasiliensis* since, levels of ectoine represented 57% of total solutes corresponding to a 8.5 fold increase over those at optimum growth conditions. The hydroxylated derivative of ectoine was primarily detected at 200% ASW and was associated with a lower response to supra-optimum salinities since hydroxyectoine showed 18 fold increase at 300% ASW over those present at 200% ASW and represented only 7.5% of total solutes **(Figure 4.2).**

The sub-optimum salinities of 25% ASW in *R. brasiliensis* led to the reduction in the accumulated organic solutes over those in optimum growth conditions since levels of sucrose and α-glutamate showed a 3 fold decrease. However, each represented around 45-50% of total solutes as observed in optimum growth conditions **(Figure 4.2)**. Possibly, *R. brasiliensis* accumulated both solutes to cope with salinities lower than the optimum growth conditions.



Solutes concentration (µmol/mg protein)	25%	100%*	200%	300%
Sucrose	0.43±0.08	1.02±0.11	1.14±0.25	0.98±0.08
Glutamate	0.34±0.03	0.92±0.10	0.87±0.12	1.64±0.05
Ectoine	n. d.	0.23±0.06	1.53±0.45	4.25±0.25
Hydroxyectoine	n. d.	n. d.	0.03±0.01	0.56±0.01
Growth rate	0.021	0.046	0.037	0.022

Figure 4.2. Growth rate and compatible solutes content from *Rubinisphaera brasiliensis* grown in N^+ medium containing 1 g/L ammonium sulfate during exponential growth phase (OD_{610}= 0.6 – 0.65) with different salinity concentrations.
Data are the means ± standard deviation of three measurements. n.d., not detected; *, Optimum growth conditions.

4.3 The Compatible Solute Pool from *Gimesia maris* and *Rubinisphaera brasiliensis* Under Different Shock Conditions

Previously, a salinity-dependent switch in the accumulated compatible solutes was revealed in *G. maris* and *R. brasiliensis*. Moreover, *G. maris* responded to high osmolarity accumulating higher concentrations of α-glutamate whereas, *R. brasiliensis* primarily increases ectoine levels. In order to test the osmotic roles of α-glutamate and ectoine in *G. maris* and *R. brasiliensis*, respectively, pre-cultivated cells under optimum growth conditions were exposed to hyper-osmotic shock. In addition, other stress conditions that limit cell growth such as nitrogen content were investigated alone or in combination to osmotic shock since it may influence the compatible solutes content. Therefore, both species were exposed for a limit period of time to different stress conditions to analyse the switch in compatible solutes diversity and concentration.

During the shock experiments, cultures were observed by light microscope and no growth was detectable. Moreover, shock experiments were investigated during the exposure of *G. maris* and *R. brasiliensis* for a maximum of 12 hours after the onset, since both species exhibited generation time of about 15-17 hours under optimum growth conditions.

4.3.1 The Effect of Different Exposure Time to Nitrogen-Limiting Conditions on the Compatible Solute Pool

Nitrogen is an essential element for microorganisms´ therefore, its low abundance in marine ecosystems limits their survival (Capone *et al.* 2008). Since some marine microorganisms were found to adapt to fluctuations in nitrogen concentrations by the accumulation of specific organic solutes, we also examined the role of nitrogen content of the medium on the levels of intracellular solutes in *G. maris* and *R. brasiliensis*. The compatible solute pool was first analysed in cells pre-cultivated under optimum growth conditions and then submitted to nitrogen depletion in the medium (ammonium sulfate was not added to the medium, N^- medium) as compared to cells under standard nitrogen conditions (1 g/L ammonium sulfate, N^+ medium) incubated for three time periods and up to 12 hours.

After 5 hours of incubation under N^- conditions, *G. maris* cells began to accumulate glucosylglycerate (GG), which was undetected under N^+ conditions **(Figure 4.3)**. In addition,

the intracellular levels of α-glutamate, sucrose and ectoine remained almost unchanged under both N^+ and N^- conditions.

Extending the incubation for 8 hours to N^- conditions, shifts in the intracellular solutes were found in *G. maris*. Under these conditions the levels of GG were higher over those present after 5 hours of incubation. This phenomenon was most noticeable after 12 hours of incubation under N^- conditions since, levels of GG increased from 0.19 μmol/mg of protein accumulated after 5 hours to 0.52 μmol/mg of protein accumulated after 12 hours of nitrogen depletion **(Figure 4.3)**. Moreover, the increased levels of GG by extending the exposure of *G. maris* to N^- conditions were accompanied by a decrease in α-glutamate. In contrast, extending the incubation of *G. maris* under N^+ conditions led to levels of α-glutamate two times higher over those present in cells incubated for only 5 hours of incubation. The α-glutamate present in cells incubated for 5 hours under N^+ conditions may be related to the levels of this amino acid present in the inoculum since cells were first pre-cultivated in N^+ conditions for 15 hours and then submitted to shock conditions for 5, 8 and 12 hours. Furthermore, levels of this amino acid remained almost unchanged between 8 and 12 hours of incubation under N^+ conditions. Surprisingly, the levels of sucrose increased by extending the incubation of *G. maris* to N^- conditions resulting in a 4.8 fold increase in sucrose levels after 12 hours of incubation over those present in cells incubated for 5 hours **(Figure 4.3).**

It is noteworthy that, the levels of ectoine were slightly lower under N^- conditions over those under N^+ conditions but were independent of the exposure time in short-term experiments, suggesting a constitutive accumulation of ectoine in N^+ conditions and the storage in cells under N^- conditions.

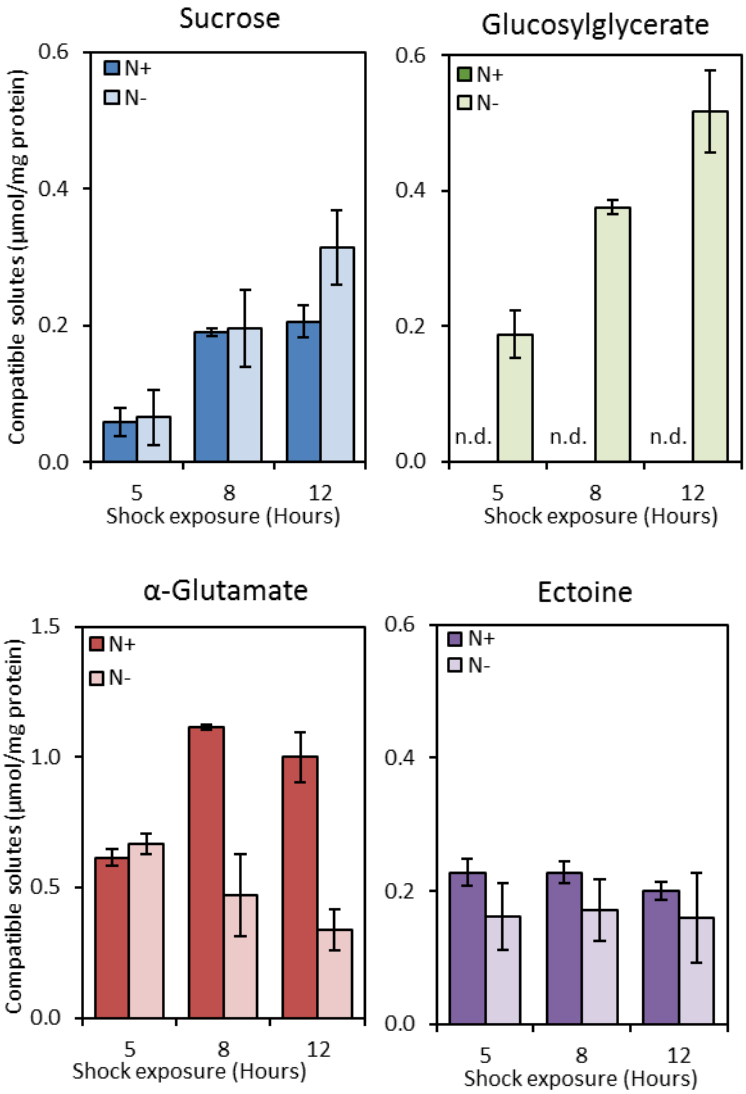


Figure 4.3. Influence of the exposure time period under nitrogen-limiting conditions (N^-) and standard nitrogen conditions (N^+) on the accumulation of sucrose, glucosylglycerate, ectoine and α-glutamate in *Gimesia maris*.
Cells were pre-cultivated in N^+ medium (containing 1 g/L ammonium sulfate) until initial growth phase (OD_{610} = 0.35). For shock experiments cells were subsequently transferred into N^+ medium or N^- medium (without added 1 g/L ammonium sulfate) for 5, 8 and 12 hours before harvest. Data are the means ± SD of three measurements. n.d. not detected.

As mentioned above, *G. maris* began to accumulate GG during low nitrogen growth conditions. In contrast, *R. brasiliensis* responded to the same conditions by a massive increase in sucrose levels. After 5 hours of incubation, slightly differences in levels of sucrose and α-glutamate were found in *R. brasiliensis* under N^+ and N^- conditions with sucrose levels two times higher in N^- conditions. This trend was prominent when the exposure to such conditions was extended to 8 hours **(Figure 4.4)**. Under the depletion of

nitrogen, sucrose levels showed a 4.6 fold increase over those present in the earlier 5 hours of incubation. Whereas, under N^+ conditions the concentration of this disaccharide were almost unchanged up to 8 hours of incubation and increased only slightly until 12 hours after the onset.

Additionally, the elevated sucrose levels after 8 hours of incubation in N^- conditions were accompanied by a depletion in intracellular α-glutamate. Levels of this amino acid in *R. brasiliensis* decreased from 1.16 µmol/mg of protein under N^+ conditions to 0.50 µmol/mg of protein under N^- conditions. Extending the exposure time to 12 hours in both nitrogen conditions had almost no effect on the α-glutamate levels **(Figure 4.4)**. As expected, the levels of ectoine from *R. brasiliensis* cells under N^- conditions were lower over N^+ conditions. However, ectoine levels were relatively constant extending the exposure time to both conditions to a maximum of 12 hours.

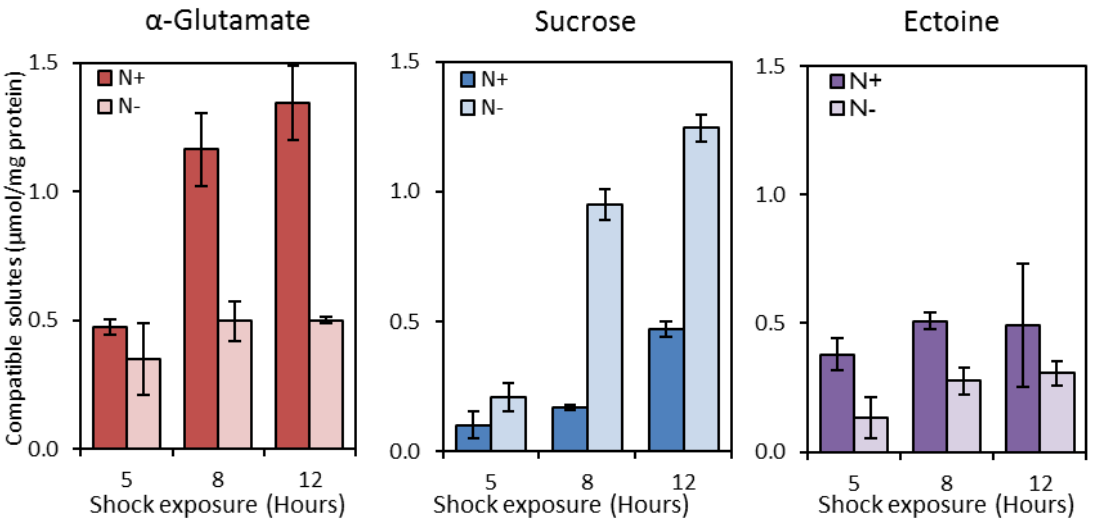


Figure 4.4. Influence of incubation time period under nitrogen-limiting conditions (N^-) and standard nitrogen conditions (N^+) in the accumulation of α-glutamate, sucrose and ectoine in *Rubinisphaera brasiliensis*.
Cells were pre-cultivated in N^+ medium (containing 1 g/L ammonium sulfate) until initial growth phase (OD_{610} = 0.35). For shock experiments cells were subsequently transferred into N^+ medium or N^- medium (without added 1 g/L ammonium sulfate) for 5, 8 and 12 hours before harvest. Data are the means ± SD of three measurements. n.d. not detected.

4.3.2 The Effect of Exposure Time to Hyper-Osmotic Shock in the Compatible Solute Pool Under Nitrogen-Limiting Conditions

In addition to fluctuations in nitrogen concentrations, the organism´s growth is also limited by salinity in marine environments. Therefore, we examined the shift on compatible solutes accumulated by *G. maris* and *R. brasiliensis* submitted to hyper-osmotic shock alone and in combination with nitrogen-limiting conditions (N^- condition).

First the accumulation of compatible solutes was analysed in pre-cultivated cells shocked in mineral salt concentrations of 200% ASW (hyper-osmotic condition) and compared to cells at 100% ASW (standard salt condition) over a period of 8 and 12 hours.

Both species, *G. maris* and *R. brasiliensis* increased the total amount of compatible solutes after 8 hours of incubation in medium containing 200% ASW. Nevertheless, extending the incubation time from 8 to 12 hours under hyper-osmotic conditions led to a slight increase in the levels of the total solutes of *G. maris* associated with a 2.4 fold increase in α-glutamate levels. On the other hand, the levels of total solutes remained relatively constant in *R. brasiliensis* with the extension of the incubation time to 12 hours under up-shock conditions.

The hyper-osmotic shock in *G. maris* led primarily to the increase in levels of sucrose and ectoine. The incubation of *G. maris* in 200% ASW for about 8 hours led to a 7 fold increase in levels of both sucrose and ectoine over those in standard conditions **(Figure 4.5A).**

Surprisingly, the levels of α-glutamate and hydroxyectoine from *G. maris* cells were dependent on the incubation time in hyper-osmotic shock since extending the incubation to 12 hours led to a 2.4 fold increase in α-glutamate levels and in a lesser extent an increase in hydroxyectoine levels. On the other hand, *R. brasiliensis* responded to hyper-osmotic shock by increasing the levels of ectoine but the levels of this solute along with the levels of sucrose and α-glutamate were not affected by the time exposure to such treatment **(Figure 4.6A).**

In additional experiments, pre-cultivated cells were shocked for 8 and 12 hours in medium containing 200% ASW and the nitrogen content was depleted. The incubation of *G. maris* under hyper-osmotic conditions combined with N-limiting conditions for 12 hours led to a decrease in total organic solutes whereas, in *R. brasiliensis* the decrease in total organic solutes was observed earlier, after 8 hours of exposure to the same conditions.

Likewise, the ratio of compatible solutes accumulated by both species was dependent on the time exposure to 200% ASW combined with N^- conditions. The exposure to high salinity levels of *G. maris* cells under N^- conditions led primarily to the accumulation of the negatively charged solute, GG which was accompanied by the decrease in intracellular levels of α-glutamate **(Figure 4.5B)**.

As mentioned above, an increase in levels of GG was observed when extending the exposure time of *G. maris* cells to N^- conditions. Additionally, levels of GG also increased in *G. maris* after 12 hours of incubation in 200% ASW under N^- conditions, from 0.28 µmol/mg of protein in the earlier 8 hours after the onset to 0.77 µmol/mg of protein after 12 hours of exposure. Moreover, the expected reduction in levels of ectoine and hydroxyectoine in *G. maris* was observed after 12 hours of exposure which led to a lower concentration of total solutes from *G. maris*.

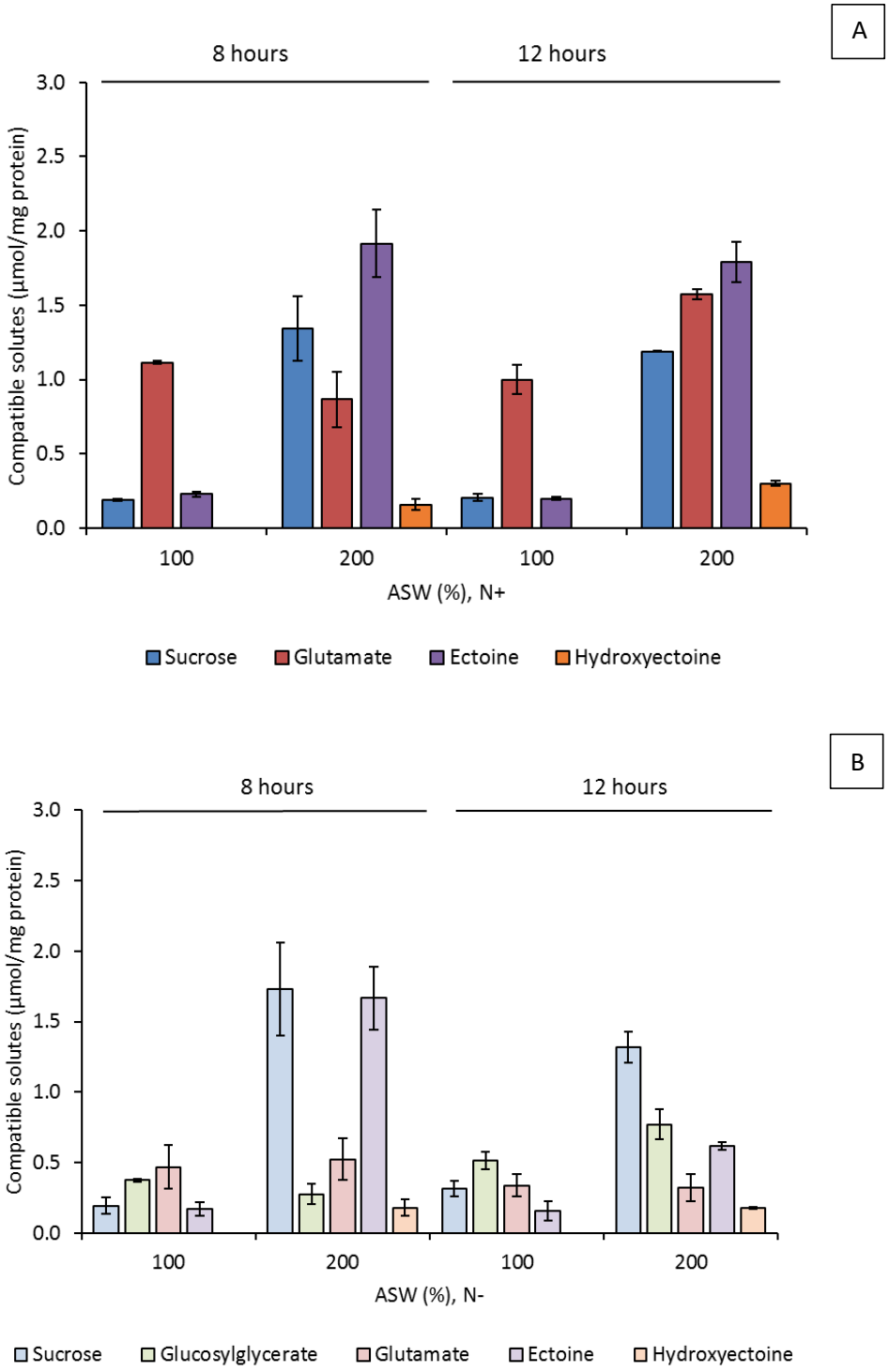


Figure 4.5. The compatible solutes content from *Gimesia maris* in hyper-osmotic shock conditions (200% ASW) under standard nitrogen concentrations (A, N^+) and nitrogen-limiting conditions (B, N^-) for 8 hours and 12 hours before harvest.

Cells were pre-cultivated in N^+ medium (containing 1 g/L ammonium sulfate) until initial growth phase (OD_{610} = 0.35). For shock experiments cells were subsequently transferred into N^+ medium or N^- medium (without added 1 g/L ammonium sulfate) with Artificial Sea Water (ASW) concentrations of 100% and 200% for 8 and 12 hours before harvest. Bars in light tone represent intracellular concentrations under N^- conditions. Data are the means ± SD of three measurements.

As mentioned elsewhere, *R. brasiliensis* responded primarily to N^- conditions by increasing sucrose levels. This phenomenon was prominent during the exposure of *R. brasiliensis* cells in 200% ASW under N^- conditions for 8 hours. Likewise, the increase in sucrose levels in salt-shocked cells after 8 hours under N^- conditions was accompanied by a decrease in levels of α-glutamate and ectoine over N^+ conditions **(Figure 4.6B).** After 12 hours of incubation of *R. brasiliensis* cells in hyper-osmotic shock under N^- conditions a decrease was observed in sucrose levels and to a lesser extent in ectoine and α-glutamate levels over cells incubated for 8 hours, prospecting the initial acclimation of cells to these shock conditions and further cellular death.

These results showed that shifts in compatible solutes of *R. brasiliensis* salt-shocked cells under N^+/N^- conditions occurred after 8 hours of incubation, whereas shifts in compatible solutes of *G. maris* salt-shocked cells under the same nitrogen concentrations were observed afterwards (after 12 hours).

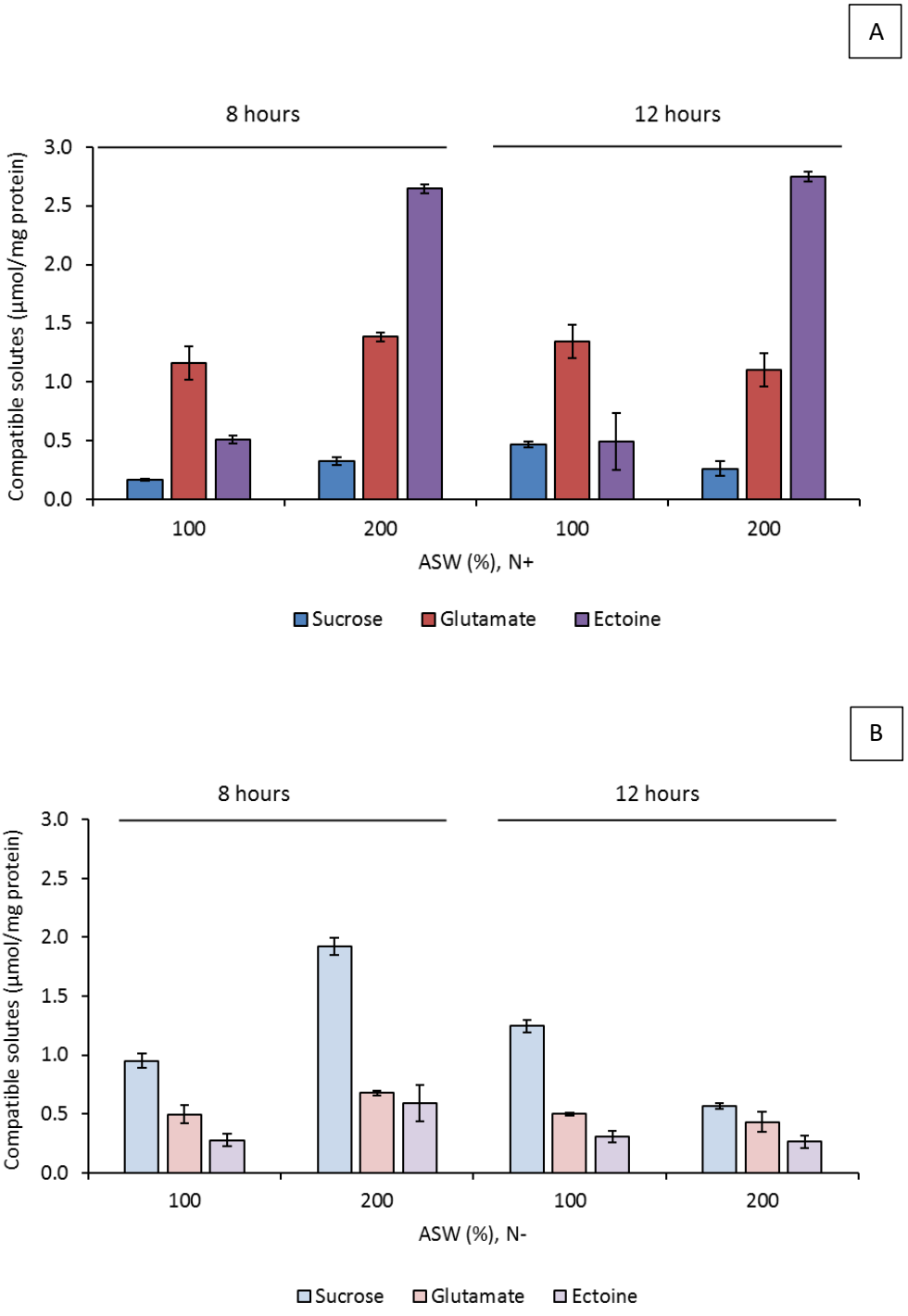


Figure 4.6. The compatible solutes content from *Rubinisphaera brasiliensis* in hyper-osmotic shock conditions (200% ASW) under standard nitrogen concentrations (A, N^+) and nitrogen-limiting conditions (B, N^-) for 8 hours and 12 hours before harvest.

Cells were pre-cultivated in N^+ medium (containing 1 g/L ammonium sulfate) until initial growth phase (OD_{610} = 0.35). For shock experiments cells were subsequently transferred into N^+ medium or N^- medium (without added 1 g/L ammonium sulfate) with Artificial Sea Water (ASW) concentrations of 100 % and 200 % for 8 and 12 hours before harvest. Bars in light tone represent intracellular concentrations under N^- conditions. Data are the means ± SD of three measurements.

4.3.3 The Effect of Short-Term Exposure to Osmotic Shock in the Compatible Solute Pool Under Nitrogen-Limiting Conditions

Previously, we determined the exposure time of 8 and 12 hours to specific stress conditions as the necessary to analyse shifts in intracellular solutes of *R. brasiliensis* and *G. maris*, respectively. In a set of experiments, pre-cultivated cells from both species were incubated for the respective incubation period in a broad range of mineral salts alone or in combination with N^- conditions that might influence the compatible solute pool (See material and methods for more detail).

During both N^+ and N^- conditions the total intracellular organic solutes from *G. maris* and *R. brasiliensis* increased when exposed to elevated salinities and caused a decrease in the intracellular levels of organic solutes in salinities lower than the standard salt conditions (100% ASW).

In *G. maris* the hyper-osmotic shock containing 200% ASW led to a 3 fold increase in total organic solutes over standard salinity conditions **(Figure 4.7A)**. As *R. brasiliensis* have a broad tolerance to mineral salts, the highest salinity examined was 350% ASW. The hyper-osmotic conditions of 200% ASW and 350% ASW led to 2 and 2.3 fold increase in total solutes, respectively over those in standard salt conditions **(Figure 4.8A).**

At the lowest salinity tested (18% ASW), the total organic solutes in *G. maris* and *R. brasiliensis* were very low or almost undetected under both N^+ and N^- conditions which probably resulted from the inability of both bacteria to grow in salinity levels less than 25% ASW.

Since *G. maris* cells had a lower range of growth at higher salinities over *R. brasiliensis* cells, the hypo-osmotic condition containing 50% ASW was also examined and led to 3 fold decrease in total organic solutes under N^+ conditions **(Figure 4.7A).** The decrease on total organic solutes in *G. maris* submitted to salinity levels lower than the optimum conditions was also reported earlier in salt loaded cells (see section 4.2 for more details).

Previously, an increase in intracellular levels of organic solutes was observed in both species in response to hyper-osmotic conditions of 200% ASW. Moreover, in *G. maris* the ratio of the accumulated organic solutes was dependent on the time period of incubation. In this section we described in more detail the switch in organic solutes accumulated by both species under different hyper-osmotic conditions.

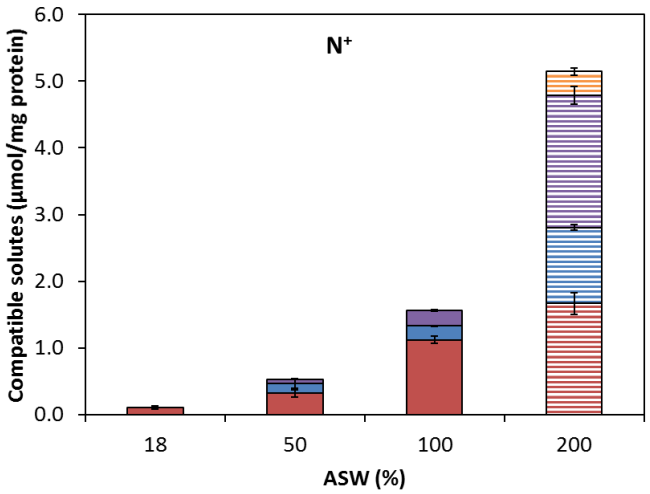
Under hyper-osmotic conditions of 200% ASW, *G. maris* and *R. brasiliensis* responded primarily by increasing levels of ectoine, although in *G. maris* intracellular ectoine represented 38% of total solutes whereas, in *R. brasiliensis* it represented 58% of total solutes. To a lesser extent *G. maris* also accumulated hydroxyectoine which was undetected under standard salt conditions (100% ASW). Likewise, sucrose levels represented 22% of total solutes showing a 6 fold increase in 200% ASW over those under 100% ASW. As expected, hyper-osmotic conditions in *G. maris* led to an increase in α-glutamate but represented only 32% of total solutes over those in standard salt conditions under which it represented 70% of total solutes **(Figure 4.7A).**

Surprisingly, *R. brasiliensis* accumulated similar concentrations of α-glutamate over standard salt conditions and the two hyper-osmotic conditions examined. However, the relative proportion of this amino acid decreased from 63% of total solutes in standard salt conditions to 32% of total solutes in 200% ASW and to 25% of total solutes in the highest salinity tested **(Figure 4.8A)**. This phenomenon was associated to the constitutive accumulation of α-glutamate in *R. brasiliensis* during short-term shock experiments.

Therefore, hyper-osmotic conditions of 200% ASW in *R. brasiliensis* led primarily to a 5 fold increase in the levels of ectoine over those in standard conditions. In a lesser extent levels of sucrose were two times higher and represented only 10% of total solutes. This trend was more pronounced in medium containing 350% ASW where the levels of sucrose increased from 0.19 µmol/mg of protein under standard salt conditions to 1.60 µmol/mg of protein in 350% ASW and represented 35% of total solutes. Unexpectedly, the increase in salinity to 350% ASW led to the accumulation of lower concentrations of ectoine over those in 200% ASW showing a 4.5 and 3.3 fold increase in 200% ASW and in 350% ASW, respectively over those in standard salt conditions.

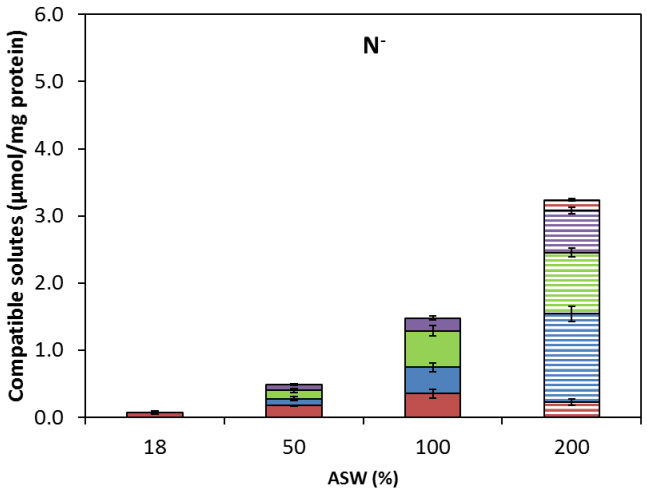
Moreover, ectoine levels in 200% ASW represented 58% of total solutes whereas in 350% ASW represented 40% of total solutes **(Figure 4.8A)**. That *R. brasiliensis* copes with salinities above the maximum used for growth combining the accumulation of sucrose and ectoine.

A



Solutes concentration (µmol/mg protein)	18%	50%	100%	200%
Hydroxyectoine	n. d.	n. d.	0.03±0.01	0.36±0.05
Ectoine	n. d.	0.06±0.01	0.23±0.01	1.98±0.14
Sucrose	n. d.	0.15±0.07	0.21±0.01	1.14±0.04
Glutamate	0.10±0.02	0.32±0.06	1.12±0.06	1.66±0.16

B



Solutes concentration (µmol/mg protein)	18%	50%	100%	200%
Hydroxyectoine	n. d.	n. d.	n. d.	0.15±0.02
Ectoine	n. d.	0.09±0.01	0.19±0.03	0.63±0.05
Glucosylglycerate	n. d.	0.12±0.03	0.54±0.08	0.91±0.07
Sucrose	n. d.	0.11±0.03	0.39±0.07	1.32±0.11
Glutamate	0.07±0.01	0.17±0.01	0.35±0.07	0.22±0.05

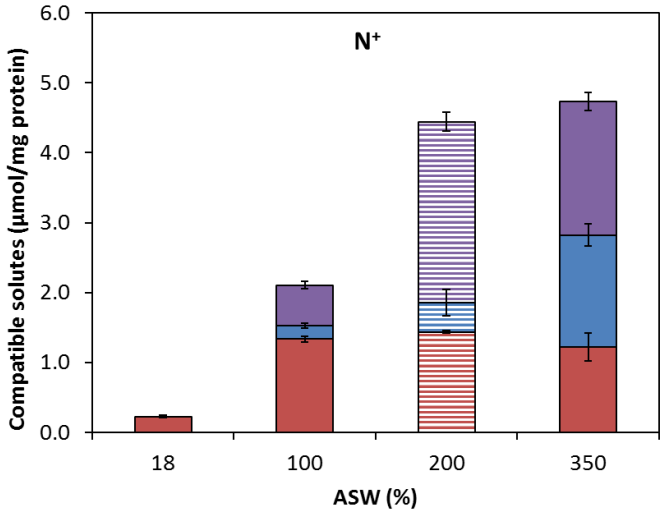
Figure 4.7. The effect of osmotic shock conditions in the compatible solutes content of *Gimesia maris* under standard nitrogen conditions (A, N^+) and nitrogen-limiting conditions (B, N^-) after 12 hours of exposure. Cells were pre-cultivated in N^+ medium (containing 1 g/L ammonium sulfate) until initial growth phase (OD_{610} = 0.35). For shock experiments cells were subsequently transferred into N^+ medium or N^- medium (without added 1 g/L ammonium sulfate) with Artificial Sea Water (ASW) concentrations of 18 %, 50 %, 100 % and 200 % for 12 hours before harvest. Data are the means ± SD of three measurements. Striped bars are replicates from previous shock experiments (see section 4.3.2 for more detail). n.d., not detected.

Nitrogen availability in the medium had a pronounced effect on the diversity and total concentration of compatible solutes. The exposure of *G. maris* and *R. brasiliensis* to N^- conditions under all salinities tested led to a decrease on the total solute concentration by around 10-15% over those under N^+ conditions. As mentioned previously, *G. maris* accumulated GG in N^- medium and also in combination with hyper-osmotic conditions (200% ASW) but remained undetected in N^+ conditions. The accumulation of GG occurred in *G. maris* in salinities above 50% ASW **(Figure 4.7B)**. A linear increase in GG levels was related to the elevation of mineral salts in the medium under low nitrogen conditions from 0.12 μmol/mg of protein in 50% ASW to 0.54 μmol/mg of protein in standard salt conditions (100% ASW) and to 0.91 μmol/mg of protein in 200% ASW. Besides, N^- conditions in *G. maris* led also to a 3- and 7.5 fold decrease in levels of α-glutamate under optimum salinity and 50% ASW, respectively.

Besides, under N^- conditions, *G. maris* responded to 50% ASW by decreasing the levels of ectoine. At the lowest salinity tested (18% ASW) *G. maris* as well as *R. brasiliensis* accumulated trace amounts of α-glutamate, which remained almost unchanged between N^+ and N^- conditions. Probably, low levels of this solute are sufficient to balance the cell´s turgor pressure during short-term exposure to sub-optimum salinities.

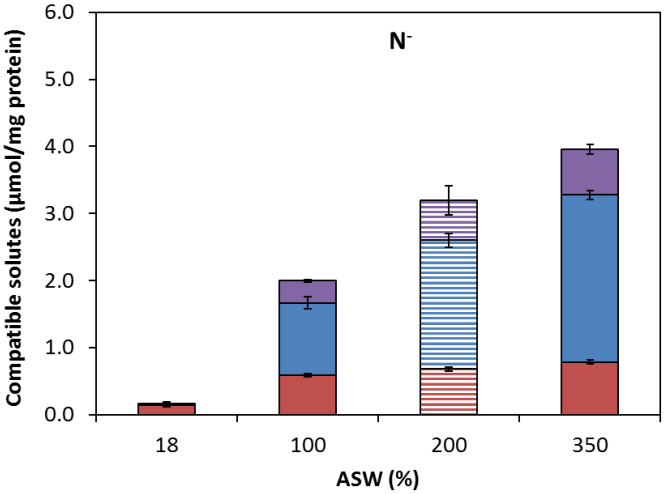
Under standard salt conditions, *R. brasiliensis* responded to N^- medium by a 5.5 fold increase in sucrose levels. Moreover, this disaccharide replaced α-glutamate as the major compatible solute since levels of sucrose represented 54% of total solutes. This trend was prominent in salt-shocked cells under N^- conditions since sucrose levels represented 64% of total solutes and showed a 5 fold increase for salinity concentrations of 200% ASW **(Figure 4.8B)**. Furthermore, mineral salt levels of 200% ASW and 350% ASW in *R. brasiliensis* under N^- conditions led to the decrease in levels of α-glutamate and ectoine over those in both hyper-osmotic conditions under N^+ medium and both nitrogen-containing solutes represented approximately 18% of total solutes. Surprisingly, sucrose became the major compatible solute when nitrogen was depleted from the medium at the two hyper-osmotic conditions examined and a linear increase in sucrose was found related to elevated salinity levels **(Figure 4.8B)**.

A



Solutes concentration (µmol/mg protein)	18%	100%	200%	350%
Ectoine	n.d.	0.58±0.05	2.59±0.14	1.91±0.13
Sucrose	n. d.	0.19±0.04	0.42±0.19	1.60±0.16
Glutamate	0.23±0.01	1.33±0.04	1.43±0.02	1.22±0.19

B



Solutes concentration (µmol/mg protein)	18%	100%	200%	350%
Ectoine	n. d.	0.33±0.02	0.59±0.22	0.68±0.07
Sucrose	0.02±0.01	1.08±0.09	1.92±0.10	2.49±0.07
Glutamate	0.14±0.02	0.59±0.02	0.68±0.03	0.78±0.03

Figure 4.8. The effect of osmotic shock conditions in the compatible solutes content of *Rubinisphaera brasiliensis* under standard nitrogen conditions (A, N^+) and nitrogen-limiting conditions (B, N^-) after 8 hours of exposure.

Cells were pre-cultivated in N^+ medium (containing 1 g/L ammonium sulfate) until initial growth phase (OD_{610} = 0.35). For shock experiments cells were subsequently transferred into N^+ medium or N^- medium (without added 1 g/L ammonium sulfate) with Artificial Sea Water (ASW) concentrations of 18 %, 100 %, 200 % and 350 %for 8 hours before harvest. Data are the means ± SD of three measurements. Striped bars are replicates from previous shock experiments (see section 4.3.2 for more detail). n.d., not detected.

4.4 Identification of Genes Related to Compatible Solutes Biosynthesis in *Gimesia maris*

Previously, the availability of a wide genome sequences from members of the phylum *Planctomycetes* helped to identify homologous genes involved in compatible solutes synthesis and to analyse the different distribution within genomes (Empadinhas and da Costa 2010, Jeske *et al.* 2013, Cunha *et al.* 2013). From the *Gimesia maris* genome sequence we gain insights into the genetic information on pathways involved in the synthesis of this organism´s compatible solutes. The synthesis of ectoine, sucrose and glucosylglycerate (GG) is supported by the identification of the following genes: *ectABC*, *spS*, *gpgS, gpgP* and *spasE,* respectively.

The putative cluster for GG biosynthesis included three genes encoding in this order, a GpgP (803 bp), a GpgS (1220 bp) and a Spase (1760 bp). All three genes have the same orientation and *gpgP* gene is separated from *gpgS* gene by only 30 bp. Downstream the *gpgP* gene is separated by 301 bp from an ORF of 3335 bp with opposite orientation than the gene *gpgP* and upstream the *spasE* gene is an ORF of 389 bp also in the opposite orientation of the three genes. The deduced proteins have no homologous amino acid sequences in the NCBI databases. Since both ORF sequences are apparently not in a functional relationship to the GG biosynthetic genes they were not investigated further **(Figure 4.9)**.

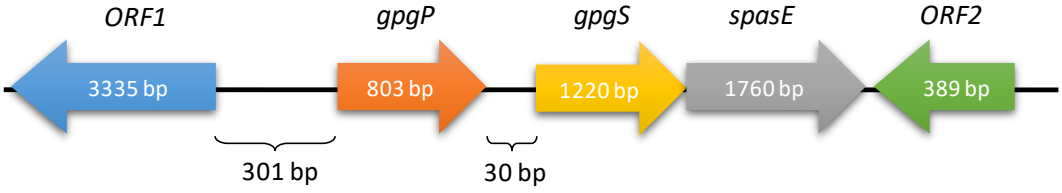


Figure 4.9. Genomic organization of *gpgS, gpgP* and *spasE* genes from *Gimesia maris*.
Arrows represent genes and their directions. *gpgS,* putative glucosyl-3-phosphoglycerate synthase; *gpgP,* putative glucosyl-3-phoshoglycerate phosphatase*; spasE*, putative sucrose phosphorylase; ORF 1 and ORF 2, Open Reading Frames (Adapted from Empadinhas and da Costa 2010).

The putative cluster *ectABC* for biosynthesis of ectoine included three genes encoding a putative *ectA*, a putative *ectB* and a putative *ectC*. Previous findings concluded that the L-ectoine synthase encoded by *ectC* gene is the key enzyme for ectoine synthesis (Cánovas *et al.* 1997a). In this matter, we selected the *ectC* (395 bp) gene for further analysis of transcript abundance.

In Bacteria the sucrose biosynthetic pathway described is composed of two enzymes, a SpS and a SpP encoded by a *spS* gene and a *spP* gene, respectively (Klähn and Hagemann 2011). In the genome of *G. maris* we found only a putative *spS* (2228 bp) gene and no putative *spP* was found.

Of the five genes chosen, *gpgS, gpgP*, *spasE, ectC* and *spS* we amplified fragments of about 145 to 200 bp from chromosome DNA before the total RNA isolation to be transcribed into cDNA. As observed in **Figure 4.10**, the size of each amplicon and the specificity were confirmed by gel electrophoresis. As a positive control, the amplification of a 16S rRNA fragment sequence was used. In addition, the transcribed cDNA was used as template in PCR with the same primers for the 16S rRNA fragment. As a negative control, the corresponding total RNA and cDNA-RT$^-$ (without reverse transcriptase) were used as templates to ensure the absence of contaminating DNA. As observed in **Figure 4.11**, PCR products were obtained only in the presence of cDNA and were used for downstream application for qPCR analysis.

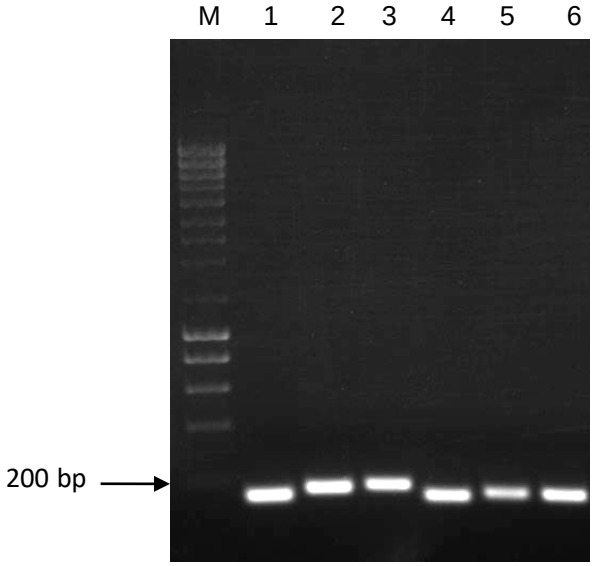


Figure 4.10. Electrophoresis gel showing PCR products that confirmed the primers specificity for DNA fragments from genes involved in compatible solute synthesis in *Gimesia maris*.
Lane M, DNA marker NZYDNA ladder III (NzyTech); lane 1, *gpgS* (154 bp); lane 2, *gpgP* (174 bp); lane 3, *spasE* (180 bp); lane 4, *ectC* (146 bp); lane 5, *spS* (156 bp); lane 6, 16S rRNA (153 bp).

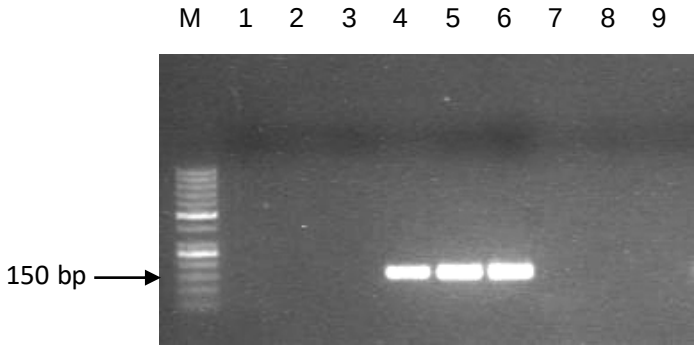


Figure 4.11. **Electrophoresis gel showing the amplification by PCR of a representative amplicon of 16S rRNA gene from: cDNA RT$^-$ (without reverse transcriptase; lanes 1, 2, 3); cDNA (lanes 4, 5, 6) and total RNA (lanes 7, 8, 9).**
The genetic material was extracted from *Gimesia maris* cells submitted for 12 hours to standard conditions (100% ASW, lanes 1, 4, 7), nitrogen limiting conditions (lanes 2, 5, 8) or hyper-osmotic conditions containing 200% ASW (lanes 3, 6, 9). Lane M, DNA marker GeneRuler ladder 50 bp (Fermentas^{TM}).

4.5 Properties of the Proteins Deduced from Encoding Genes

Previously, Empadinhas and da Costa (2010) found in *G. maris* genome a putative *gpgP* gene first annotated as *mpgP* gene. Many glucosyl-3-phosphoglycerate phosphatase proteins have been retrieved by the earlier mistaken identification as MpgP protein. This putative protein has a length of 267 amino acids and high homology for functional characterized GpgP. GpgS is a putative glucosyl-3-phosphoglycerate synthase annotated as a glucosyltransferase with an amino acid sequence of 406 amino acids. This protein shares 58% similarity and 40% identity with previous characterized GpgS from *R. baltica*. The Spase protein is annotated as a putative sucrose phosphorylase with a deduced length of 586 amino acids.

The SpS protein is 47% and 46% identical (64% and 63% similarity) to sucrose-phosphate synthase from *Synechocystis* sp. PCC6803 and *Synechococcus elongatus*, respectively. The EctC protein is annotated as a putative L-ectoine synthase and was previously found to be the key enzyme for the ectoine biosynthesis (Cánovas *et al.* 1997a). It has a length of 131 amino acids and the protein is 53% and 58% identical (69% and 74% similarity) to EctC from *Halomonas elongata* and *Sporosarcina pasteurii*, respectively.

4.6 The Effect of Imposed Nutrient Limitation and High Salinity on the expression of Compatible Solutes Biosynthetic Genes from *Gimesia maris*

The synthesis of GG was induced by nitrogen limitation in *G. maris* shocked-cells as shown above (section 4.3). In addition, *G. maris* cells synthesized higher concentrations of ectoine and sucrose under hyper-osmotic shock conditions containing 200% ASW and in a lesser extent in salt-loaded cells (see section 4.2 and section 4.3.3 for more detail). In this sense, *G. maris* cells were pre-cultivated in optimum growth medium and submitted for 12 hours to either hyper-osmotic conditions (200% ASW) or N-limiting conditions and total RNA was prepared to investigate the transcript abundance of candidate genes for GG, ectoine and sucrose biosynthesis. For more detail see material and methods.

RNA samples were extracted from a protocol optimized in this study and using the TripleXtractor reagent yielded RNA of high absorbance ratios at A260/280 and at A260/230, which in both cases were measured over a value of 2.

In general, total RNA is accepted as pure if the absorbance ratio of A260/280 range between 2.0-2.1 and the A260/230 ratio is close to 2.0. An absorbance A260/280 under 2.0 indicate acidic pH or the presence of impurities such as proteins or phenols in the eluted RNA. The ratio of A260/230 lower than 2.0 may indicate the presence of contaminants which absorb at 230 nm, such as polysaccharides or residual phenol/guanidine.

Moreover, total RNA was submitted to gel electrophoresis to access RNA integrity. The two rRNA moieties (16S and 23S) are present as distinctive bands and at the bottom of the gel a band is seen, comprising tRNA **(Figure 4.12)**.

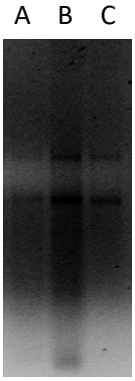


Figure 4.12. Gel electrophoresis of representative total RNA extracted from *Gimesia maris* cells incubated for 12 hours: A, in standard salt conditions of 100% ASW; B, under nitrogen-limiting conditions; C, under hyper-osmotic shock containing 200% ASW.

Therefore, it is assumed that analysis of the distinguished 16S and 23S summarize the stability of total RNA samples.

The total RNA was then reverse-transcribed into cDNA that was further used for qPCR analysis. We determined the relative transcript amounts compared to the amount of the RNA from cells shocked under standard conditions (100% ASW), which was normalized to one. In addition, 16S rRNA gene served as reference gene (internal normalizer). The expression values of three genes (16S rRNA, *rnpB* and *rpoA*) were analysed by geNorm and Normfinder to select the most stable reference gene in *G. maris* cells exposed for 12 hours to standard conditions, N-limiting conditions and hyper-osmotic shock containing 200% ASW. As observed in **Table 4.1**, 16S rRNA gene had the lowest M value for all shock experiments. By geNorm, 16S rRNA gene was top ranked followed by *rnpB* and *rpoA* genes whereas by NormFinder the 16S rRNA gene was followed by the less stable *rpoA* and *rnpB* genes in samples under N-limiting conditions or in hyper-osmotic shock conditions. Besides, the same results were observed in RefFinder platform for both algorithms using the raw Cq values.

Table 4.1. Ranking of the candidate reference genes according to their stability value (M) calculated by GeNorm and NormFinder using *Gimesia maris* total RNA.

Algorithm	Condition*	Ranking (less stable to more stable)		
		3	**2**	**1**
GeNorm	100% ASW, N^+	*rpoA*	*rnpB*	16S rRNA
M		0.114	0.076	0.064
	100% ASW, N^-	*rpoA*	*rnpB*	16S rRNA
M		0.227	0.122	0.119
	200% ASW, N^+	*rpoA*	*rnpB*	16S rRNA
M		0.185	0.145	0.110
NormFinder				
	100% ASW, N^+	*rpoA*	*rnpB*	16S rRNA
M		0.041	0.029	0.023
	100% ASW, N^-	*rnpB*	*rpoA*	16S rRNA
M		0.053	0.015	0.012
	200% ASW, N^+	*rnpB*	*rpoA*	16S rRNA
M		0.125	0.086	0.024

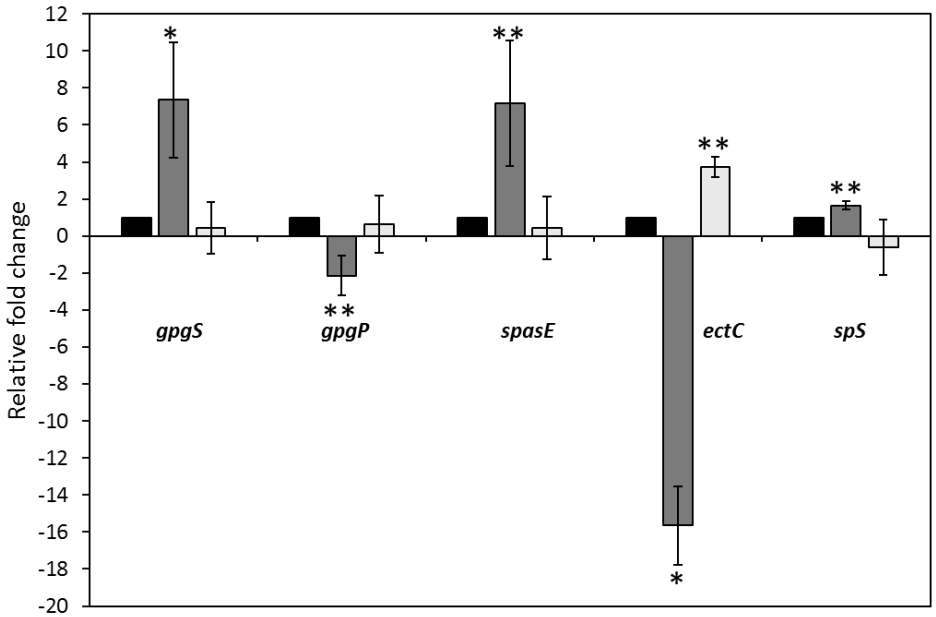
* 100% ASW, medium with full-strength of Artificial sea water (% ASW); 200% ASW; N^+, standard nitrogen conditions; N^-, nitrogen-limiting conditions.

By the transcripts expression profiles we observed the up-regulation of *gpgS* and *spasE* genes whereas the *gpgP* gene was slightly down-regulated under nitrogen limiting conditions **(Figure 4.13)**. The finding that *gpgS* and *spasE* genes may form a transcriptional unit and the similar transcript levels of both genes suggests the existence of a policystronic messenger. Moreover, the up-regulation of both *gpgS* and *spasE* genes was clearly dependent to nitrogen availability.

Moreover, the biosynthesis of GG under N-limiting conditions confirms the increase in gpgS transcript abundance. On the other hand, the expression of, gpgS, gpgP and spasE genes remained almost unchanged under hyper-osmotic conditions over those under control condition (100% ASW, N+ condition) **(Figure 4.13)**.

Furthermore, ectC gene was up-regulated during hyper-osmotic conditions and down-regulated under N-limiting conditions, which could be explained by the high biosynthesis of ectoine in G. maris cells after hyper-osmotic shock conditions and the depletion in ectoine levels under N-limiting conditions. In this sense these results suggest that ectC is involved in salt stress protection and transcript abundance is regulated by the imposed shock condition.

Unexpectedly, the expression of spS gene was not salt-dependent and was slightly up-regulated under N-limiting conditions **(Figure 4.13)**. Perhaps the regulation of sucrose synthesis occurs at a post-transcriptional level or by an uncharacterized biosynthetic pathway.



Shock conditions/genes	*gpgS*	*gpgP*	*spasE*	*ectC*	*spS*
■ 100% ASW, N+	1	1	1	1	1
■ 100% ASW, N-	7.35±3.12	-2.13±1.05	7.17±3.37	-15.66±2.12	1.64±0.23
□ 200% ASW	0.44±1.41	0.64±1.56	0.42±1.68	3.71±0.55	-0.62±1.50

Figure 4.13. Relative gene expression of five genes related to different compatible solute biosynthesis in *Gimesia maris*.
The genes expression identified from cells incubated for 12 hours under 100% ASW, N^- (without added 1 g/L ammonium sulfate) or 200% ASW (hyper-osmotic condition) was compared to the amount of the RNA from cells shocked under 100% ASW, N^+ (containing 1 g/L ammonium sulfate and named standard condition), which was normalized to one. The 16S rRNA gene served as internal normalizer. The data represent the mean ± standard deviation of three experiments. Statistical significance: * p-value<0.05 and ** p-value<0.01 when compared with the standard condition (100% ASW, N^+).

CHAPTER 5

DISCUSSION

Most members of the phylum *Planctomycetes* were isolated from aquatic habitats (Schlesner *et al.* 2004). The two species examined in this work were first ascribed in the genus *Planctomyces* and recently re-classified as *Gimesia maris* and *Rubinisphaera brasiliensis,* that were isolated from a marine ecosystem and a saline pit, respectively (Scheuner *et al.* 2014). Both species must cope with differences in total concentrations of inorganic ions, since salinity is an important environmental factor that affects growth of aquatic microorganisms. Likewise, these bacteria may encounter sudden fluctuations in the availability of water, oxygen and nutrients.

To adapt to a variety of different environmental conditions, aquatic microorganisms must sense and respond rapidly to changes in the external environment. Our laboratory has focused in the past, on the response mediated by the accumulation of compatible solutes of both thermophilic and mesophilic microorganisms in response to external osmotically active compounds. A previous study focused on the compatible solute accumulated by a member of the phylum *Planctomycetes*, namely *Rhodopirellula baltica* in response to differences in salinity, temperature and nitrogen content (D'Avó *et al.* 2013). This was the first report of the accumulation of compatible solutes within the *Planctomycetes*. In addition, *R. baltica* has been studied based on stress conditions that modulate the transcriptional profiling of genes coding for compatible solute synthesis, morphological changes and ion transport (Wecker *et al.* 2009, Jeske *et al.* 2013).

The work presented in this thesis was therefore planned to extend the knowledge about the adaptation of *Planctomycetes* to different stress conditions, by the accumulation of intracellular organic solutes. The expression of genes related to compatible solutes synthesis in specific environmental conditions complement the organic solute pool, based on the assumption that gene expression levels may influence the downstream production of organic solutes.

5.1 Osmoadaptation in *Rubinisphaera brasiliensis* and *Gimesia maris*

Planctomycetes are slow growing microorganisms with low density cultures and detectable colonies are produced only after 6-14 days (Strous *et al.* 1999, Ward *et al.* 2006). The species examined, *Gimesia maris* and *Rubinisphaera brasiliensis* have a slow growth since both exhibit generation times of about 15-17 hours. However, the alterations made to the growth medium composition were beneficial to increase bacterial density of both *G. maris* and *R. brasiliensis* that exhibited turbidity values ranging from OD_{610} = 0.9-1.1 during the stationary phase. In previous works, the maximum turbidity achieved by most members of the phylum *Planctomycetes* were about OD_{610} = 0.4-0.5 (Lage and Bondoso 2012). An exception arises from *R. baltica* that exhibit a shorter doubling time of about 10-14 hours (Rabus *et al.* 2002, D'Avó *et al.* 2013).

Regarding the tolerance to different salt levels in the medium, *G. maris* and *R. brasiliensis* require at least 25% ASW for growth, confirming the halophilic nature of both species. Likewise, our results corroborate previous findings of the ability of *R. brasiliensis* to grow in a broad range of salinities over other *Planctomycetes* isolated from salt-rich ecosystems (Scheuner *et al.* 2014). This halophilic bacterium grew in medium containing up to 300% ASW and at 350% ASW remained metabolically active as assessed by compatible solute accumulation. In contrast, *G. maris* grew in medium containing up to 150% ASW and tolerated up to 200% ASW. Similarly, *R. baltica* was previously shown to grow in ASW concentrations above 200% and remained metabolically active in medium containing 260% ASW. The growth of *R. baltica* in medium containing up to 200% ASW was associated to the accumulation of α-glutamate and sucrose rather than ectoine and hydroxyectoine that were absent from its organic solute pool (Wecker *et al.* 2009, D'Avó *et al.* 2013).

Moreover, differences in the compatible solute pool were found between the two *Planctomycetes* and were determined by NMR in cells acclimated to different salt concentrations. For osmoadaptation, *G. maris* and *R. brasiliensis* accumulate α-glutamate, sucrose, ectoine and hydroxyectoine. In *G. maris*, α-glutamate was the dominant compatible solute in all salinities tested and levels of this amino acid were dependent on the salinity concentration. Therefore, continuous growth at elevated salinity triggers an increase in the α-glutamate levels and to a lesser extent in the ectoine and hydroxyectoine

levels. In other Gram-negative bacteria, α-glutamate levels usually increase after exposure to elevated osmotic strength but in most cases, the combination of α-glutamate and other solutes is required at high salinities (da Costa *et al.* 1998, Poolman and Glaasker 1998, Empadinhas *et al.* 2009, Empadinhas and da Costa 2010).

On the other hand, α-glutamate levels remain almost unchanged during the rise of growth medium osmolarity in *R. brasiliensis*. Thus, ectoine and to a lesser extent, hydroxyectoine were the preferred compatible solutes to cope with elevated salinities. The accumulation of ectoine led to a much higher total compatible solute content on *R. brasiliensis* cells in comparison to *G. maris* cells. The higher salt tolerance showed by *R. brasiliensis* may be related to the higher intracellular concentration of total organic solutes.

Since *G. maris* was found to be more salt-sensitive species than *R. brasiliensis* and *R. baltica*, this suggest that high intracellular concentrations of ectoine and hydroxyectoine are a more effective osmoadaptative strategy among halophilic *Planctomycetes*. In contrast to *R. brasiliensis*, small levels of ectoine were found in *G. maris* under low osmolarity (25% ASW), which increased with the medium salinity. Therefore, the levels of ectoine are directly correlated to the salinity of the growth medium indicating that *G. maris* adjusts the intracellular ectoine pool to the degree of the imposed osmotic stress.

Ectoine was originally discovered as a compatible solute in the extremely halophilic phototrophic sulfobacterium *Ectothiorhodospira halochloris* (Galinski *et al.* 1985). This heterocyclic amino acid is widespread among taxonomically and physiologically diverse set of prokaryotes, could be found in some Eukaryotes such as plants, algae and its synthesis was not found in *Archaea* (da Costa *et al.* 1998, Roberts 2000). Furthermore, the production of low amounts of hydroxyectoine is often found in many ectoine-producing halophilic bacteria, such as the Gram-positive bacteria *Streptomyces griseus* and among Gram-negative bacteria of the family *Halomonadaceae*, namely *Halomonas elongata* and *Chromohalobacter salexigens* that accumulate ectoine and hydroxyectoine for osmoprotection and simultaneously, accumulate hydroxyectoine in response to heat stress (Malin and Lapidot 1996, García-Estepa *et al.* 2006).

There is evidence that most microorganisms exhibit a switch from one type of solutes to another after sudden changes in external osmolarity and/or extracellular nutrient availability, such as carbon and nitrogen sources (Galinski and Herzog 1990, Cánovas *et al.* 1999, Silva *et al.* 1999, Goude *et al.* 2004, Borges *et al.* 2004, Roberts 2005, Klähn *et al.*

2010, D'Avó *et al.* 2013). Therefore, the compatible solute pool from *G. maris* and *R. brasiliensis* was also studied in cells exposed for a short period to hyper- and hypo-osmotic conditions alone and in combination with differences in nitrogen content of the medium.

In *G. maris* sudden changes in the osmolarity of the medium to levels slightly higher than 150% ASW lead to an increase in total organic solutes associated with elevated levels of intracellular ectoine, sucrose and in a lesser extent α-glutamate. Our results show that α-glutamate under hyper-osmotic shock of 200% ASW is not sufficient for *G. maris* cells to compensate internal turgor pressure with the extracellular osmotic pressure. Therefore, ectoine and sucrose seem more efficient in *G. maris* under high salinity levels making ectoine the key organic solute for adaptation to up-shifts in osmolarity and not α-glutamate as shown in salt-adapted cells.

Nevertheless, hydroxyectoine is accumulated by *G. maris* in elevated salinities but remain rather low over ectoine levels. In *R. brasiliensis* hydroxyectoine is absent in salt-shocked cells but is accumulated by salt-acclimated cells. Perhaps, the accumulation of ectoine is sufficient for the earlier adaptation of *R. brasiliensis* to hyper-osmotic conditions over hydroxyectoine that previously demonstrated a less osmotic protectant role in halophilic bacterium, such as *C. salexigens* (García-Estepa *et al.* 2006).

The osmoprotectant role of ectoine was also found in *R. brasiliensis* under hyper-osmotic shock experiments above 200% ASW. However, under 350% ASW for a few hours after the onset of the shock, levels of sucrose were elevated along with levels of ectoine. This phenomenon come somewhat as a surprise since the combined accumulation of ectoine and sucrose as osmoprotectants had never been reported. Even in *R. brasiliensis* salt-adapted cells, the accumulation of sucrose was never associated with a response to elevated salinities. The hyper-osmotic shock experiments confirmed that sucrose plays a major role in *R. brasiliensis* survival during slight changes in salinities above 350% ASW with sucrose levels representing a 8.3 fold increase over the accumulated sucrose under optimum growth conditions.

Often, under high osmolarity, cyanobacteria cells produce glucosylglycerol as the main compatible solute and smaller amounts of sucrose and/or trehalose. An exception arises from cyanobacteria from the genus *Prochlorococcus* that do not synthesize glucosylglycerol. Instead, picoplanktonic *Prochlorococcus* strains accumulate glucosylglycerate (GG), a negatively charged solute, structurally close to glucosylglycerol.

During growth in high salinity this cyanobacterium accumulates sucrose as the major compatible solute and GG as a secondary organic solute. The previous findings of salt-induced accumulation of sucrose in *Prochlorococcus* strains refuted the notion that sucrose accumulation was restricted to freshwater cyanobacteria. Therefore, sucrose accumulation is unlikely involved in the restriction of freshwater cyanobacteria to low salinities, but rather the ability for internal inorganic ion export, which is a second prerequisite for fully cells salt acclimation (Klähn *et al.* 2010). Recently, sucrose was identified as a secondary solute under elevated saline conditions (200% ASW) in *R. baltica*, corroborating previous findings related to sucrose accumulation with the acclimation to high salinities (D'Avó *et al.* 2013).

The fact that, *R. brasiliensis* increased the levels of sucrose under hyper-osmotic conditions containing 350% ASW corroborates previous findings in relation to sucrose accumulation by the cells adjusted to high salinities. Also, the limit of salinity for the detection of this disaccharide increased for 350% ASW.

Trehalose has also been associated to salt-inducible compatible solutes for osmoadaptation in freshwater cyanobacteria and in *R. baltica* (Klähn and Hagemann 2011, D'Avó *et al.* 2013). However, neither *G. maris* nor *R. brasiliensis* seem to cope with low salinities by replacing sucrose with trehalose in the stress conditions examined so far.

Sudden changes in the nitrogen content of the medium were also associated with the synthesis of different compatible solutes in both species. In *G. maris* the accumulation of GG is associated primarily with the response to nitrogen limiting conditions, this being the first report of GG production by a member of the phylum *Planctomycetes*. At first, the ionic solute GG was believed to be produced only by halophilic organisms but it was rapidly observed among prokaryotes such as the halophilic bacteria, *Crhomoalobacter salexigens*, *Dickeya dadantii*, *Synechococcus sp.* PCC 7002, *Prochlorococcus marinus* strain SS1120 and NATL2A, *Streptomyces caelestis*, in the archaeon *Methanohalophilus portucalensis* and in the thermophilic bacterium, *Persephonella marina* (Kollman *et al.* 1979, Robertson *et al.* 1992, Cánovas *et al.* 1999, Goude *et al.* 2004, Klähn and Hagemann 2011, Lamosa *et al.* 2013). This thermophilic bacterium accumulates other solutes, along with GG, until now found only in (hyper)thermophilic organisms, namely the rare GG-derivative, glucosylglucosylglycerate (GGG), β-glutamate and di-myo-inositol phosphate (Lamosa *et al.* 2013).

However, the accumulation of GG acting as a compatible solute and related to nitrogen poor environments was initially reported in the enterobacterium *Dickeya dadantii* strain 3937 (Goude *et al.* 2004). This phenomenon was recently observed in *Synechococcus sp.* PCC 7002 and *Prochlorococcus marinus* strain SS1120; however the major solutes are glucosylglycerol and sucrose, respectively (Klähn *et al.* 2010).

In fact, the GG levels have been regularly found to be higher in organisms under nitrogen-limiting conditions, as was recently shown in two *Mycobacterium* species known to accumulate GG in the free form, namely *Mycobacterium smegmatis* and *Mycobacterium hassiacum*. The mycobacterial accumulation of GG occur only in nitrogen limiting conditions and its levels are unrelated to osmoadaptation (Behrends *et al.* 2012, Alarico *et al.* 2014). In *G. maris*, levels of GG increased proportionally with salinity levels always under nitrogen-limiting conditions. This phenomenon was also observed in other prokaryotes however, in most of them, low levels of this glycerate-derivative were also detected under optimum growth conditions (Goude *et al.* 2004, Empadinhas and da Costa 2008, Klähn *et al.* 2010). The accumulation of GG by *G. maris* presupposes a role in replacing α-glutamate as a counterion of K^+ ions in nitrogen poor environments since the accumulation of GG preceded lower levels of α-glutamate and became the main organic solute under optimum salinities for growth. Moreover, the linear increase of GG in salt-shocked cells under N^- conditions confirms the involvement of this glycerate-derivative as a true compatible solute in *G. maris*.

In *R. brasiliensis* the response to nitrogen privation by elevating sucrose levels come somewhat as a surprise. Probably, sucrose is related to cell protection and revival after nitrogen privation as observed for the GG involvement in the fitness of mycobacterium cells since a neutral solute, such as sucrose are unlikely involved in the balance of internal K^+ levels. In *Mycobacterium hassiacum* the accumulation of high levels of GG during low nitrogen conditions were shown advantageous for the subsequent hydrolysis to contribute to cell fitness, during the nitrogen restoration (Alarico *et al.* 2014).

It is noteworthy that the elevated levels of sucrose in nitrogen poor environments appears as a strategy present in both species *G. maris* and *R. brasiliensis*. Nevertheless, in *R. brasiliensis* sucrose became the main compatible solute whereas, in *G. maris* sucrose occurs as a secondary compatible solute. A different strategy was previously shown in *R. baltica* in nitrogen poor environments. This bacterium increases the levels of the rare

glycerate-derivative, mannosylglucosylglycerate (MGG) accompanied by the decrease in the levels of α-glutamate and sucrose (D'Avó *et al.* 2013). MGG is considered a rare compatible solute found only in two thermophilic organisms from the genus *Petrotoga*. In *Petrotoga miotherma* the accumulation of MGG occurs under low salt concentrations and it is replaced by α-glutamate and proline under salinities above the optimum for growth (Jorge *et al.* 2007). *Petrotoga mobilis* accumulates MGG as a major solute in response to high osmotic conditions and supra-optimum growth temperatures (Fernandes *et al.* 2010). The accumulation of MGG by *R. baltica* was the first ascribed in a mesophilic bacteria. In addition to the increase in the levels of MGG in *R. baltica* in response to nitrogen limitation, a linear increase of the accumulation of this solute was also found with increased medium salinity under nitrogen limiting conditions (D'Avó *et al.* 2013).

Our findings showed that the response to nitrogen depletion is species specific within halophilic *Planctomycetes* since the accumulation of GG and to a lesser extent sucrose were found in *G. maris*. In *R. brasiliensis* sucrose is clearly the preferred solute to cope with nitrogen low concentrations and *R. baltica* responded by increasing levels of the rare solute, MGG.

5.2 The dependence of the compatible solute pool on the length of incubation time during up-shock experiments

The switch in compatible solute content from *R. baltica* under different stress conditions and differences on the transcriptional level have already been reported in time series experiments (up to 5 hours) (Wecker *et al.* 2009). In short-term experiments, *G. maris* and *R. brasiliensis* differently sense environmental stress conditions.

To observe the switch in the compatible solute content, *R. brasiliensis* and *G. maris* needed an incubation period of 8 hours and 12 hours, respectively to shock conditions in short-term experiments, similar to the previously performed using *R. baltica.* Nevertheless, both species responded earlier (after 8 hours of incubation) to sudden changes in nitrogen concentrations in the medium or to hyper-osmotic shock conditions of 200% ASW. The response of *G. maris* to hyper-osmotic shock is primarily associated with increased levels of ectoine and sucrose. However, a slight increase in α-glutamate levels occurred by extending the exposure to 12 hours. The elevated concentration of this amino acid could be beneficial to replace ectoine and sucrose to balance external osmotic pressure as those

organic solutes may stabilize macromolecules during a prolonged exposure to hyper-osmotic shock. For example, ectoine that is a potent cell protectant, possibly being more efficient in stabilizing proteins during hyper-osmotic conditions over other solutes present (Lippert and Galinski 1992).

The ability of salt shocked-cells to sense N-deficient conditions was associated with the decrease in levels of the nitrogen-containing solutes, α-glutamate and ectoine. *G. maris* seems to respond slowly to hyper-osmotic shock conditions under nitrogen depletion in the medium (after 12 hours of incubation) whereas, *R. brasiliensis* respond to the same treatment after 8 hours of incubation.

Moreover, in *R. brasiliensis* the accumulation of sucrose increased in elevated salt levels under low nitrogen conditions. However, extending the exposure to those shock conditions from 8 to 12 hours resulted in the reduction of sucrose levels. This results show that sucrose is accumulated primarily in large quantities for immediate cellular recovery after a prolonged exposure to hyper-osmotic conditions under nitrogen depletion in medium.

This is the first report of the dependence of the response of *Planctomycetes* to the incubation period under different shock conditions and raises questions about how this unique bacteria sense external environmental stress conditions and regulates the response by accumulating compatible solutes. The answer could be in the high set of hypothetical proteins functionally uncharacterized and unique from *Planctomycetes* that may be involved in the external sensing of environmental stress conditions, regulation and new synthesizing pathways for compatible solutes (Wecker *et al.* 2009).

5.3 Transcript abundance dependent on shock conditions

Our findings show that *G. maris* accumulates GG under nitrogen limiting conditions but remain undetected under nitrogen standard conditions even with the compatible solute accumulation intensively studied under different salt conditions and nitrogen content. This led us to assess the expression pattern of genes likely to be involved in the synthesis of GG and also genes coding for other compatible solutes accumulated by *G. maris* during the exposure to hyper-osmotic conditions or nitrogen limiting conditions.

Genes involved in the synthesis of GG, ectoine and sucrose were found using characterized enzymes by searching homologous in *G. maris* genome. The genes involved in the synthesis of GG by the two-step pathway namely *gpgS* and *gpgP* are organized in *G.*

maris genome in an operon-like structure and are sufficient for the synthesis of GG (Empadinhas and da Costa 2010). The two step-pathway comprises a glucosyl-3-phosphoglycerate synthase (GpgS) that catalyzes the formation of glucosyl-3-phosphoglycerate (GPG) from NDP-glucose and D-3-phosphoglycerate and dephosphorylated by glucosyl-3-phosphoglycerate phosphatase (GpgP) to yield GG (Costa *et al.* 2006).

Moreover, in the *G. maris* genome upstream the *gpgS* gene was found a *spasE* gene, putatively coding for a sucrose phosphorylase (Spase). Often, a sucrose phosphorylase is found contiguous or between the *gpgS* and *gpgP* genes in the genomes of some cyanobacteria and other microorganisms. Recently, the Spase from *Leuconostoc mesenteroides* was able to synthesize GG *in vitro,* where glycerate and sucrose were the substrates for the direct production of GG (Sawangwan *et al.* 2009). This findings support the involvement of the Spase from *G. maris* in another pathway for GG synthesis from glycerate and sucrose, the latter being one of the principal compatible solutes in this planctomycete, according to the imposed external stress condition.

The presence of two pathways for the synthesis of GG had already been found in *Persephonella marina*. This thermophilic bacterium possess the GpgS/GpgP pathway and a single-step pathway that comprises the direct conversion of NDP-glucose and D-glycerate by a glucosylglycerate synthase (GgS) to yield GG (Fernandes *et al.* 2007).

The existence of two pathways for the synthesis of a compatible solute in the same organism is not unprecedented. Two different pathways for MG synthesis are present in the thermophilic bacterium *Rhodothermus marinus*. In the two step-pathway, GDP-mannose and D-3-phosphoglycerate are the substrates for a mannosyl-3-phosphoglycerate synthase (MpgS) leading to the formation of mannosyl-3-phosphoglycerate (MPG) and subsequent dephosphorylation by mannosyl-3-phosphoglycerate phosphatase (MpgP) to MG (Empadinhas *et al.* 2001). The other pathway involves the direct condensation of GDP-mannose and D-glycerate to MG via mannosylglycerate synthase (MgS) (Martins *et al.* 1999). The two pathways for MG synthesis are regulated by salt and temperature, physical parameters known to affect the intracellular concentration of MG. Levels of MpgS from the two step-pathway increased in response to osmotic stress, whereas MgS enzyme from the single-step pathway is induced when exposed to heat stress (Borges *et al.* 2004). However, the existence of a salt-dependent control mechanism of gene expression remain elusive.

The reason for the existence of two pathways for the synthesis of GG in *G. maris* remains unclear but we hypothesized that they are dependent to the environmental conditions, initially at the transcriptional level.

The transcript of *gpgS* and *spasE* genes behaved in the same manner in *G. maris* cells under the shock conditions tested, since under nitrogen limiting conditions both genes were up-regulated and hyper-osmotic shock of 200% ASW did not considerably affect the expression of both genes. Our results show that the synthesis of GG in *G. maris* may be dependent on the transcript levels of *gpgS* gene but not *gpgP* gene. Moreover, the *spasE* gene transcript abundance depends on nitrogen availability and could be involved in another pathway for GG synthesis in this organism. The involvement of the sucrose phosphorylase encoded by *spasE* gene in sucrose degradation is unlikely to occur during nitrogen privation since sucrose levels increase in *G. maris* cells under these shock conditions. Moreover, the putative *spS* gene coding for a sucrose phosphate synthase could be silent or the transcription not regulated by the cells under nitrogen privation, since transcript abundance remains relatively constant between control conditions (100% ASW) and the two shock conditions examined.

Additionally, the transcript abundance of *ectC* gene, coding for an ectoine synthase, and essential for the final step of ectoine synthesis depends on the imposed shock treatment, confirming the elevated synthesis of ectoine under hyper-osmotic shock conditions and the cease of ectoine synthesis under nitrogen-limiting conditions.

CHAPTER 6

CONCLUDING REMARKS

Gimesia maris and *Rubinisphaera brasiliensis* exposed to different salinity concentrations showed different tolerances. *Gimesia maris* grow in medium containing up to 150% ASW, *R. brasiliensis,* on the other hand grows in medium with up to 300% ASW and both species are dependent on at least 25% ASW for growth. Comparing the compatible solute pool from both species, at high salinity levels, we found a higher content of nitrogenous solutes with ectoine and α-glutamate content predominating. However, in *R. brasiliensis* the α-glutamate levels were relatively constant under low and high salinities. This shows that *G. maris* and *R. brasiliensis* are able to alter the intracellular organic solute content for osmoadaptation. Probably, the plasticity in compatible solutes may confer an advantage to *R. brasiliensis* under fluctuations in external environmental conditions.

In this study we showed that the ectoine levels were raised in *G. maris* and *R. brasiliensis* under hyper-osmotic conditions containing concentrations of mineral salts not tolerated for growth, suggesting that α-glutamate is not sufficient for osmoadaptation at elevated salinities. To a lesser extent sucrose levels were also high during hyper-osmotic shock and under nitrogen limiting conditions, which extended the limit of salinity levels of sucrose accumulation in bacteria (up to 350% ASW).

Among the compatible solutes of these organisms, the sugar-derivatives and glycerate-derivative compatible solutes were enriched in the two species under nitrogen depletion, while nitrogenous compounds, such as α-glutamate and ectoine were reduced. Both sucrose and GG were elevated in *G. maris* while in *R. brasiliensis*, GG is not present but the organism accumulates high levels of sucrose. This suggest that halophilic *Planctomycetes* species accumulate different compatible solutes to cope with the same stress conditions and the accumulation of nitrogen-free compatible solutes provides an advantage in nitrogen poor environments. The relative quantification of gene expression coding for compatible solutes synthesis in *G. maris* using a reference gene validated in this study, provided a valuable method to study the transcript abundance of genes during different stress conditions and to analyse new pathways for the synthesis of solutes.

An important contribution of this work is that it provides information about the behaviour of halophilic *Planctomycetes* under different environmental stress conditions and complements previous work focused on the osmotic response of *Rhodopirellula baltica.*

CHAPTER 7

FUTURE PERSPECTIVES

The work presented in this thesis expanded the previous knowledge on the osmotic stress response of members of the phylum *Planctomycetes* by elucidating the compatible solute pool and describing the transcript abundance of genes coding for specific compatible solutes synthesis under different stress conditions. Such findings raises questions for future work.

The accumulation of GG under certain environmental conditions associated with the high transcript abundance of *Spase* gene, makes *G. maris* a suitable candidate to study the implications of sucrose phosphorylase under nitrogen limiting conditions.

Moreover, the physiological role of sucrose in *G. maris* and *R. brasiliensis* seems unclear which could be revealed from the generation of strains deficient in the production of this disaccharide. The achievement of this goal could be provided by the recent advances in the development of genetic tools for the generation of chromosomal mutants (Schreier *et al.* 2012, Erbilgin *et al.* 2014) and the validation of 16S rRNA gene as reference gene (this study) for qPCR transcriptional analysis of gene expression. Such genetic tools will permit the access of different phenotypes under environmental stress conditions and will benefit the clarification of regulatory mechanisms of gene expression to control the solute pool in *G. maris* and *R. brasiliensis*.

In addition, the qPCR arrays could profile the expression of a panel of genes relevant to a pathway in order to complement the further characterization of enzymes involved in the synthesis of compatible solutes. Such as, the characterization of the enzymes putatively involved in the two biosynthetic pathways for the synthesis of GG analysed in *G. maris* as well as, in the sucrose biosynthetic pathway which remain unclear.

CHAPTER 8

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