José Manuel Rocha Pereira

Bachelor Degree in Molecular and Cellular Biology

Improvement of a photoautotrophic chassis robustness for Synthetic Biology applications

Dissertation to obtain the Master of Science Degree in Biotechnology

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Abstract

Cyanobacteria are photoautotrophic microorganisms with great potential for the biotechnological industry due to their low nutrient requirements, photosynthetic capacities and metabolic plasticity. In biotechnology, the energy sector is one of the main targets for their utilization, especially to produce the so called third generation biofuels, which are regarded as one of the best replacements for petroleum-based fuels. Although, several issues could be solved, others arise from the use of cyanobacteria, namely the need for high amounts of freshwater and contamination/predation by other microorganisms that affect cultivation efficiencies. The cultivation of cyanobacteria in seawater could solve this issue, since it has a very stable and rich chemical composition. Among cyanobacteria, the model microorganism *Synechocystis* sp. PCC 6803 is one of the most studied with its genome fully sequenced and genomic, transcriptomic and proteomic data available to better predict its phenotypic behaviors/characteristics. Despite suitable for genetic engineering and implementation as a microbial cell factory, *Synechocystis'* growth rate is negatively affected by increasing salinity levels. Therefore, it is important to improve. To achieve this, several strategies involving the constitutive overexpression of the native genes encoding the proteins involved in the production of the compatible solute glucosylglycerol were implemented, following synthetic biology principles. A preliminary transcription analysis of selected mutants revealed that the assembled synthetic devices are functional at the transcriptional level. However, under different salinities, the mutants did not show improved robustness to salinity in terms of growth, compared with the wild-type. Nevertheless, some mutants carrying synthetic devices appear to have a better physiological response under seawater's NaCl concentration than in 0% (w/v) NaCl.

Keywords:

Synechocystis, chassis, seawater, halotolerance, glucosylglycerol, synthetic biology

Resumo

As cianobactérias são microrganismos fotoautotróficos com elevado potencial na indústria biotecnológica, devido aos seus simples requisitos nutricionais, capacidade fotossintética e plasticidade metabólica. O sector da energia é considerado um dos principais alvos para a sua utilização, particularmente, na produção de biocombustíveis de terceira geração como substitutos dos combustíveis fósseis. Contudo, a utilização eficaz de cianobactérias apresenta alguns problemas como a necessidade de elevadas quantidades de água doce e contaminações/predação por outros microrganismos. Desta forma, o cultivo de cianobactérias utilizando água do mar pode ser uma das soluções, uma vez que esta possui uma composição química bastante rica e estável. Entre as cianobactérias, o microrganismo modelo *Synechocystis* sp. PCC 6803 é um dos mais estudados, tendo o seu genoma sido totalmente sequenciado e com informação ao nível da genómica, transcritómica e proteómica disponível para melhor prever determinados comportamentos fisiológicos. Apesar de ser geneticamente manipulável e útil em biotecnologia, a taxa de crescimento de *Synechocystis* é afetada negativamente por níveis elevados de salinidade. Assim, a halotolerância deste microrganismo necessita de ser melhorada. Para isso algumas estratégias, baseadas na sobre expressão constitutiva dos genes nativos de *Synechocystis* que codificam proteínas envolvidas na produção do soluto compatível glicosilglicerol, foram implementadas seguindo os princípios da biologia sintética. Uma análise dos mutantes obtidos revela funcionalidade dos módulos sintéticos ao nível transcricional. Contudo, analisando o crescimento dos mutantes de *Synechocystis*, em diferentes salinidades, verifica-se que estes não apresentam um melhoramento da robustez à salinididade comparado com a estirpe selvagem. No entanto, alguns mutantes com módulos sintéticos parecem responder melhor a uma concentração de NaCl idêntica à da água do mar, em vez de 0% NaCl.

Palavras-chave:

Synechocystis, chassi, água do mar, halotolerância, glicosilglicerol, biologia sintética

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List of abbreviations, acronymes and symbols

Amp - Ampicillin

Amp^R - Resistance to ampicillin

bp - Base pair

cDNA - Complementary DNA

Cm - Chloramphenicol

Cm^R - Resistance to chloramphenicol

dH_2O - Deionized water

DHAP - Dihydroxyacetone phosphate

DNA - Deoxyribonucleic acid

dNTP - Deoxyribonucleoside triphosphate

EDTA - Ethylenediaminetetraacetic acid

G3P - Glycerol-3-phosphate

gDNA - Genomic DNA

GG - Glucosylglycerol

GGA - Glucosylglicerate

GB - Glycine betaine

GGPP - Glucosylglycerol-phosphate phosphatase

GGPS - Glucosylglycerol-phosphate synthase

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer

Km - Kanamycin

Km^R - Resistance to kanamycin

LB - Lysogeny broth

mRNA - Messenger ribonucleic acid

OD - Optical density

ON - Overnight

ORF - Open reading frame

PBR - Photobioreactor

PCC - Pasteur culture collection

PCR - Polymerase chain reaction

RBS - Ribosome binding site

RNA - Ribonucleic acid

r.p.m. - Revolutions per min

RT - Room temperature

RT-qPCR - Reverse-transcriptase quantitative polymerase chain reaction

S.D. - Standard deviation

TAE - Buffer solution containing a mixture of Tris base, acetic acid and EDTA

UV - Ultraviolet

WF - Water footprint

wt - Wild-type

1. Introduction

1.1 A worldwide problem

According to the United Nations, the World's population is expected to grow from a current 7.3 to 9.5 billion people by 2050. From this total, the main rise will come from the urban population for 6.3 billion, where a two-fold increase is estimated[1]. Consequently, several issues arise to be solved as the demand for food, freshwater and energy increases[2]. The latter needs special attention, since its global demand is estimated, by the International Energy Agency (IEA), to be 46% more than the observed energy consumed in 2010 by 2035, within the current energy policies in vigor[3]. In the same way, it also reports the main energy supply would still come from fossil fuel for about 80% of the total need. As a result, the carbon dioxide (CO_2) emission would rise up by 46% leading to negative effects in the environment, including global temperature rise[3]. Additionally, fossil fuels are finite sources of energy with estimated exhaustion periods of 50-100 years and 100-200 years for oil/gas and coal, respectively, leading to a possible energy and economic insecurity as their demand rises. Particularly, due to limited alternatives to liquid transportation fuels from petroleum compared with electric power generation alternatives, such as wind, hydro and solar renewables[4].

1.2 Biofuels as an alternative to petroleum-based fuels

Biofuels are nearly carbon neutral renewable liquid fuels produced from biomass, which might be organic/biological waste or plant and microbial based[5,6]. Considered to be the renewable solution for transportation fuels, biofuels have shown a clear production increase over the last decade, where their production has increased by more than fivefold over it[7]. This is particularly true, due to their possible use in the current transportation infrastructure to some extent, apart from being a cleaner renewable energy supply[6,8,9].

Biofuels comprise three different types depending on their original feedstock.

1.2.1 First and second generation biofuels

First generation biofuels are the most abundant and derive from cultivated crop feedstock[4]. The main commercial available biofuels are bioethanol from microbial fermentation of sugar compounds and biodiesel from vegetable oil transesterification[4,8]. Despite great promise, however, several ethical and environmental issues arisen, namely, the competition with the food sector for arable land and food crops, such as sugarcane and corn[2,4,10]. Mainly with a growing world population. Additionally, soil degradation and desertification are among other drawbacks faced with first generation biofuels[2,6,11].

As an alternative, second generation biofuels emerged, since they don't impose such a problem regarding food security as first generation's[6,12]. These result primarily from the biochemical and thermochemical breakdown of the lignocellulosic material of plant's biomass

for microbial fermentation[13]. This accounts for the non-food and cheap portion of it, about 98.5% of the total global plant biomass[6]. Nevertheless, despite the cheap and abundant feedstock, the technology employed is still very costly. As a result, this type is non-commercially viable yet, being about two to three times more expensive than petroleum based fuels[14].

1.2.2 Third generation biofuels as the most promising alternative

Third generation biofuels are receiving special attention as a solution for the problems imposed by first generation's[15]. These are microalgae and cyanobacteria based, which are photoautotrophic microorganisms by performing photosynthesis[16]. The most striking advantage relies on microalgae/cyanobacteria requirement for lesser land, arable land is not necessary, because these microorganisms provide higher net energy yields and have higher growth rates than plant crops[16,17]. In this way, there would not exist a competition with the food sector. Despite these common advantages, between microalgae and cyanobacteria there are several differences that make the latter more suitable for complex manipulations and applications[18]. Hence, cyanobacteria are being pushed forward as an ideal organism for biotechnological applications in the bulk chemicals sector[19,20].

1.3 Cyanobacteria

Cyanobacteria compose a vast group of Gram-negative autotrophic prokaryotes capable of using photosynthesis to produce biomass[21,22]. Morphologically, they range remarkably from unicellular to colonial and filamentous with a varying size of up to two orders of magnitude[23]. Thereby, in conjunction with their diverse physiology, these bacteria are capable of withstand extreme environmental conditions, from high/low temperatures, pH and salinities[21]. Regarded as the first photosynthetic organisms originated on Earth, they are thought to be the main contributors, later with algae/plants, of the actual oxygenic atmosphere[19]. In addition, they are estimated to contribute to about 25% of the current global carbon fixation[24]. These bacteria can fix atmospheric CO_2, while harvesting solar electromagnetic radiation, in the visible range[25]. Photosynthetically, cyanobacteria have higher yields for solar energy conversion than algae and plants, for 10% against 5% and 1%, respectively[16]. Despite, being superior photosynthetic organisms, with chlorophyll a as the main photosynthetic pigment, cyanobacteria are also capable of grow photoheterotrophically or chemoheterotrophically[21]. The ability of some cyanobacteria to also perform atmospheric molecular nitrogen fixation shows their very diverse metabolic plasticity[21]. In this way, differentiating them again from plants regarding the need for minimal nutrients to thrive.

In the last decades, molecular biology, modification and characterization tools allowed for a deeper understanding of these microorganisms[26]. In fact, to date, there are at least 265 cyanobacterial genomes sequenced and annotated in online databases (Cyanobase; CyanoGEBA; Joint Genome Institute (JGI), Integrated microbial genome (IMG) – accessed in June 2015)[26]. Concomitantly, the genetic engineering and novel functions introduced to a broad

range of these organisms showed how suitable cyanobacteria are for the biotechnological industry[27,28].

1.3.1 Cyanobacteria in biotechnology

In the last years, cyanobacteria have been receiving a huge attention in the biotechnological sector. Mainly, due to their interesting photosynthetic features, biologically active compounds and their possible genetic manipulation to produce several chemicals of interest[27,29]. Currently, these prokaryotes are tested, with scaled-up processes being or already implemented, in a wide range of applications from biofuel, polyesters, fertilizers and commodity chemicals production to biorremediation[22,27–29]. Even though their multidisciplinary use is evident, cyanobacteria are principally seen as the future's most promising tool for biofuel production, as mentioned above. This is true, since they can be potentially used in an economically and environmentally effective sustainable way, in order to replace most of the current global use of fossil fuels[16]. Although, they share most of the advantages also associated with algae, several others arise which make them more suitable in the long run. Some of these are a higher photosynthetic efficiency, a simpler genetic background which makes it easier to genetically manipulate and the capacity for natural transformation[18].

Despite great promise, cyanobacteria, as well as algae, utilization in biotechnological applications still pose several challenges[29]. These comprise processes such as in cell disruption (mechanical, enzymatic or chemical) to access the intracellular biomolecules, harvesting and cultivation[16,19]. Cyanobacteria and algae are usually cultivated in open and closed ponds or in photobioreactors (PBRs)[16]. One of the biggest issues relates with the high water evaporation rates associated. Although the water evaporation is greatly addressed by a PBR system, its water footprint (WF), which is the freshwater use/expenditure in a certain activity, is still very high. This could still pose a huge pressure over the world's freshwater reserves, even though the employment of microalgae in biotechnology, notably in biodiesel production, is comparably less impactful than the use of most plant crops[30]. Such holds important meaning since for first generation biofuels, the WF can range considerably. Indeed, the WF to produce 1 Kg of biodiesel can range from an estimated 2168 to 15331 L with plant crops, such as sugar beet and sorghum, respectively. In contrast, for microalgae based biodiesel, it is estimated to be up to 3650 L depending of the cultivation system[30]. However, as an example, according to P. Gerbens-Leenes *et al*[31]*,* if all transportation fuels in Europe by 2030 were to be third generation based, according to the IEA projection for transportation fuels needs, then the Europe's blue WF (freshwater from surface and groundwater reserves) would increase up to four fold from the current value. As a result, the use of microalgae, as well as cyanobacteria, would still be very severe when the proper system and/or improvements are not employed.

The solutions thought to be ideal include the improvement of current technology to avoid water loss between all downstream processes, especially for cell cultivation and harvest[16]. However, the most promising ones include the use of wastewater and seawater to replace freshwater. The former allows for either microalgae growth for biodiesel production and

wastewater treatment by reducing its nutrient content. As a drawback, this is specially directed for wild-algae, i.e. microalgae that naturally inhabit these type of sewage waters[16,32]. As a result, if engineered model cyanobacteria to produce different chemicals were to be used, this could lead to competition with other adapted microorganisms. Additionally, wastewater has a diverse inconstant composition turning the cultivation of cyanobacteria unstable[16,33]. Seawater, in contrast, has a composition more constant and regular with a wide range of nutrients essential to cyanobacterial growth, except for phosphorous[16]. Its use is also estimated to reduce the biofuels production life-cycle need for freshwater by up to 90%[30]. In addition, its use could also prevent growth of more halointolerant competing and predator organisms that would affect cyanobacterial growth[19]. Remarkably, cyanobacteria can withstand a wide range of salinity (concentration of dissolved inorganic ions) levels. Nonetheless, the growth rate or even survival of some main genetically engineerable cyanobacteria are affected by high levels of osmotic stress[34]. Hence, it could be important to tackle this by implementing a synthetic biology approach in order to improve the robustness to salinity.

1.4 Synthetic Biology

Synthetic biology is a new field within biology originated in the 21st century[35]. It is characterized, fundamentally, as the rational design of new molecules and genetic/metabolic networks or the re-design of existing ones in ways not observable in nature (therefore synthetic). Additionally, through engineering principles and/or some modelling/predictive tools from systems biology, synthetic biology practitioners aim to understand and apply biology, to attain new functionalities and biologic systems, at levels not possible with genetic engineering itself[26,36–39].

Synthetic biology basilar foundations which contribute to biology's engineerability are standardization, abstraction and decoupling[36]. Standardization, as the name implies, refers to the use of globally accepted and reference standards in an interchangeable way. As for abstraction, biological parts also called Biobricks™, such as promoters, ribosome binding sites (RBS) and transcriptional terminators are used as building blocks which, through standardized measurements and consequent predicted behaviors for most of them, help manage biological complexity. These can be assembled into devices which will be transformed into a certain biological organism (chassis). As a result, more complex systems are formed in order to perform a desired function. Such represents a decoupling process where a complex and difficult problem is divided into smaller and simpler ones, which can be combined to possibly solve it[36,40].

Every day, new Biobricks™ are generated and uploaded into online open access databases, such as the Registry of Standard Biological Parts database, from the Biobrick's Foundation, with over 20,000 registered parts (http://parts.igem.org/; accessed in June 2015). These characterized parts can then be selected and assembled in various ways originating a new device to be tested. Moreover, these individual parts can be retrieved from a physical

plasmid (backbone) from the registry or a user itself can design a synthetic device, digitally, by accessing these parts deoxyribonucleic acid (DNA) sequence.

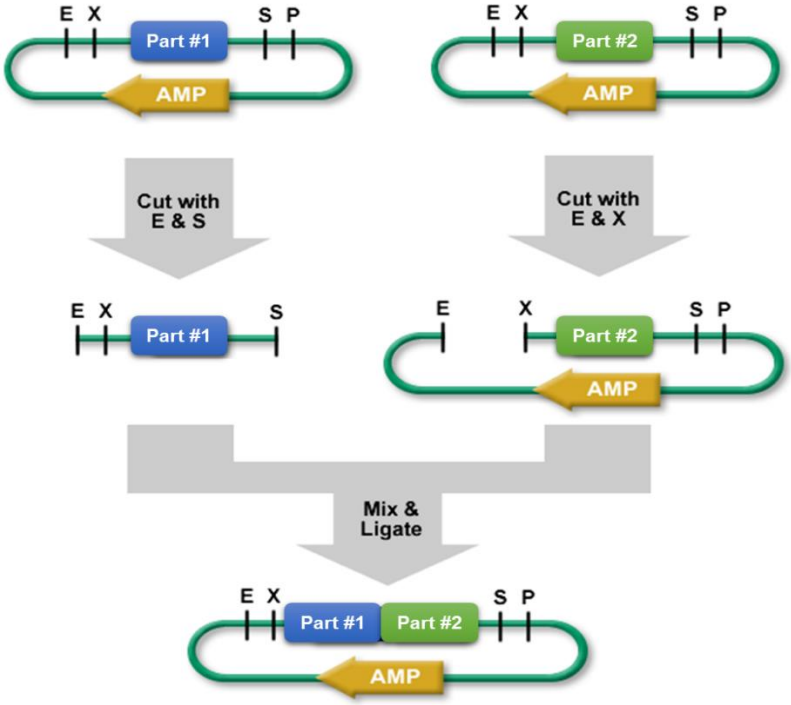

Figure 1.1 Biobrick Assembly Standard RFC[10] overview. Each part is flanked by a prefix (upstream) and suffix (downstream) with recognition sites for four different restriction enzymes (E – *EcoRI*, X – *XbaI*, S – *SpeI*, P – *PstI*) and are assembled through molecular cloning techniques into complexer devices. The feasability of this system relies on the compatibility and ligation of the S and X overhangs which forms a "scar" sequence between both parts without restoring any of these recognition sites. The resulting device is also flanked by both prefix and suffix allowing further assemblies until the final and desired synthetic device is obtained[41]. Adapted from http://parts.igem.org/Help:Standard_Assembly_%28zoom%29; accessed in June 2015.

The former is the most common way of access biological parts. Here, the usual cloning techniques in molecular biology are used in order to assemble the parts according to a standardized system, such as the Biobrick Assembly Standard RFC10. Accordingly, every biological part is preceded by a Biobrick prefix which has two restriction enzyme sites for *EcoRI* (E) and *XbaI* (X). Concomitantly, it is also followed by a Biobrick suffix which contains the restriction sites for *SpeI* (S) and *PstI* (P) (to note that biobricks cannot have any of these restriction sites in their sequence)[41]. In this way, different biological parts can be assembled by cleaving a vector containing "part #1" (donor) with E and S and a vector with E and X containing "part #2" (recipient) for upstream cloning, see Figure 1.1, or digesting the "part #1" containing vector (recipient) with S and P and the "part #2" containing vector (donor) with X and P for downstream cloning. This system takes advantage of the X and S compatible overhangs, whose ligation results in a "scar" sequence, without restoring any of these sites, between both parts. As a result, every time parts are assembled to form a device, it is always flanked by both prefix and suffix. Consequently, this system allows a fast and constant assembly process,

where several parts can be assembled in an intuitive mode[42]. Alternatively, a digitally designed device can be obtained by a DNA synthesis process, where the time consuming steps associated with DNA cloning techniques can be avoided. In fact, this practice is becoming more popular due to a continuous fall in DNA synthesis costs as seen for DNA sequencing years ago, despite its still relative high cost[35,36]. Altogether, these aspects in conjunction with a thriving community are pushing synthetic biology forward to be developed and implemented in a global, interactive and educational way.

1.4.1 Synthetic Biology of cyanobacteria

The main development regarding synthetic biology has been done, essentially, in heterotrophic bacteria. The majority of parts and synthetic devices created are targeted to the Gram-negative bacterium *Escherichia coli*, the Gram-positive bacterium *Bacillus subtilis* or the eukaryotic yeast *Saccharomyces cerevisiae* (http://parts.igem.org/Catalog#Browse_chassis; accessed in June 2015). Despite having some orthogonality, many of these parts do not have the same predicted behaviors in other hosts. This is true, notably, for cyanobacteria where many of the well characterized promoters and RBSs strengths are not the same as in *E. coli*, for example[26,43]. Consequently, cyanobacterial synthetic biology is still lagging behind compared with other chassis.

Despite lacking many functional well characterized parts, several efforts are being done in order to fill this gap concerning cyanobacterial engineering. Indeed, in the last five years many tools and parts have been created and tested, while others are currently being so[26,44,45]. At the same time, many established synthetic biology projects in cyanobacteria research contribute for its growth e.g., the Cyanofactory's European (http://www.cyanofactory.eu/) and Japanese (http://www.tuat.ac.jp/~cyano/) projects

The growth in cyanobacterial synthetic biology research is clearly derived from these bacteria capacities. As said before, the ability to thrive autotrophically, with a low nutrient requirement, in conjunction with the available molecular biology tools make them excellent chassis for biotechnological applications. As a result, cyanobacteria are being deeply studied, in order to fulfill its promise as the so called "green *E. coli*"[46].

1.5 Halotolerance in cyanobacteria

Cyanobacteria, just as other bacteria, are classified into three different groups according to their tolerance, i.e. halotolerance. Basically, these comprise freshwater (tolerance up to 3.5% (w/v) sodium chloride (NaCl)), moderately-halotolerant (tolerance up to ~10% (w/v) NaCl) and halophilic bacteria (tolerance up to 17.5% (w/v) NaCl), see Figure 1.2[34,47]. Normally, cyanobacteria thrive by maintaining a constant osmotic and ionic concentration, intracellularly, in their more hyperosmotic cytoplasm. Thereby achieved to regulate external water uptake and consequently maintaining an adjustable turgor pressure in order to grow[47]. When an external high salt concentration reaches bacteria, including cyanobacteria, two main problems arise to

be solved by the salt stressed microorganism. Firstly, the ion intracellular concentration rises, which can be toxic by disrupting the cell's metabolism, including photosynthesis[34,48]. Secondly, the water availability is reduced since a higher ionic concentration leads to less free water available[34,47]. Actually, less free water availability implies a lower enzymatic activity within the microorganism, possibly affecting its growth[49]. To face these issues, bacteria have developed two different strategies, namely, the "salt-in" and "salt-out" strategies to acclimate against high salt stressing conditions[34,47,50]. The "salt-in" strategy is characteristic of very halophilic bacteria, such as some archaea orders, it consists on a high inorganic ion uptake into the cell (up to ~22.5% (w/v), primarily KCl). Additionally, In order to resist high ionic stress, halophilic bacteria have also a proteome and consequently metabolism highly resistant to elevated ionic concentrations[47]. The "salt-out" strategy is the most widely used mechanism by bacteria to face osmotic stress. The objective is now to achieve a low ionic concentration within the cell, since enzymatic activity would be affected by higher levels of sodium, for example. To maintain an osmotic equilibrium, the cells synthesize small molecules called compatible solutes, which act as osmotic regulators. Compatible solutes allow the cell to adjust the osmotic concentration, while extruding small inorganic ions, mainly sodium. This way, bacteria acclimate and can recover their former state[34,47,50].

1.5.1 Compatible solutes

Compatible solutes are low-molecular mass organic molecules, usually with no charge, ranging from sugars to aminoacids and their derivatives. These are extremely useful compounds due to their osmotic and protective properties against dissecation and high/low temperatures, and the possibility of being biosynthesized in high amounts without having a negative effect on the cell's metabolism[34,47,50]. Interestingly, the type of compounds produced by different organisms is intrinsically correlated to the organisms' halotolerance group. For freshwater bacteria, the sugars sucrose and trehalose are the main ones. As for moderately halotolerant, these are glucosylglycerol (GG) and glucosylglicerate (GGA). While for halophilic bacteria, the main compatible solutes produced are glycine betaine (GB) and glutamate betaine, as shown in Figure 1.2.

Cyanobacteria, as autotrophic microorganisms synthesize their compatible solutes *de novo.* However, cyanobacteria possess transporters for compatible solutes uptake[47,51]. They use this mechanism to avoid a constant leakage of *de novo* synthesized compatible solutes, in order to prevent energy and carbon waste. In this way, the type of transporters encoded in a cyanobacterial genome is tightly related with the type of compatible solute they produce[34,47]. On the other hand, heterotrophic bacteria, preferentially, uptake external compatible solutes from the environment.

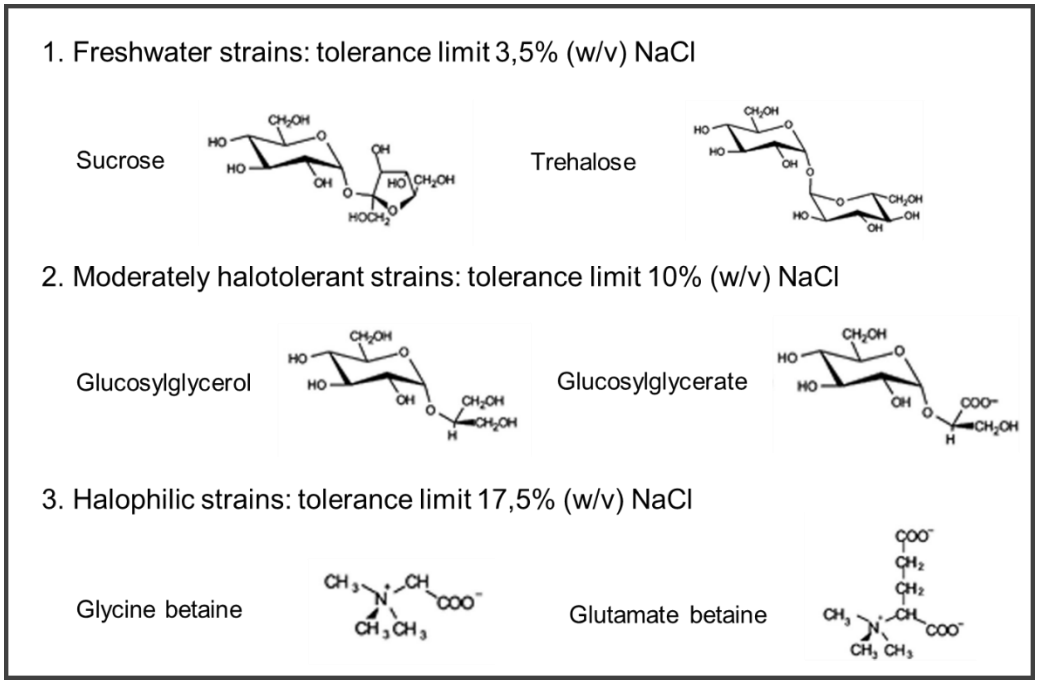

Figure 1.2. Representation of the three halotolerance groups of bacteria and their common compatible solutes. Additionally, NaCl tolerance limits for each group is shown, as well as the molecular structure of each compatible solute. Adapted from Hagemann (2011).

1.6 *Synechocystis* sp. PCC 6803 as a model organism

Among the vast group of cyanobacteria, the model freshwater cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) was the first photosynthetic organism to have its genome fully sequenced and annotated[52]. Additionally, the vast data available allowed the implementation of genome-wide metabolic models (e.g, i*Syn*811), which help to predict its cellular phenomena to some extent[53–55]. Besides, being a photosynthetic bacterium, as well as naturally transformable (homologous recombination), with other transformation techniques also applicable, such as electroporation, make it of high scientific and biotechnological interest[26]. *Synechocystis* has been deeply studied since its discovery. Thus allowed a better understanding of the many aspects surrounding photosynthesis, circadian rhythms and several other mechanisms from gene regulation to environmental stress. Some of these studies have been useful to research and understand other organisms, such as higher plants, due to its similarity with plant's chloroplasts[29].

Morphologically, this unicellular spherically shaped bacterium, as shown in Figure 1.3, is polyploid with about 12 copies of its 3.6 Mbase pair (bp) sized chromosome, as well as having seven different endogenous plasmids[26]. Physiologically, *Synechocystis* has a doubling time of 8 to 12 hours (h) when growing phototrophically on a minimum nutrient medium[26,29]. All these characteristics, despite some disadvantages, contributed to its acceptance as a model organism. Concomitantly, its photosynthetic capabilities are constantly used in order to test its viability in several biotechnological industries. As a result, nowadays, several applications regarding its utilization are being created with great promise and potential.

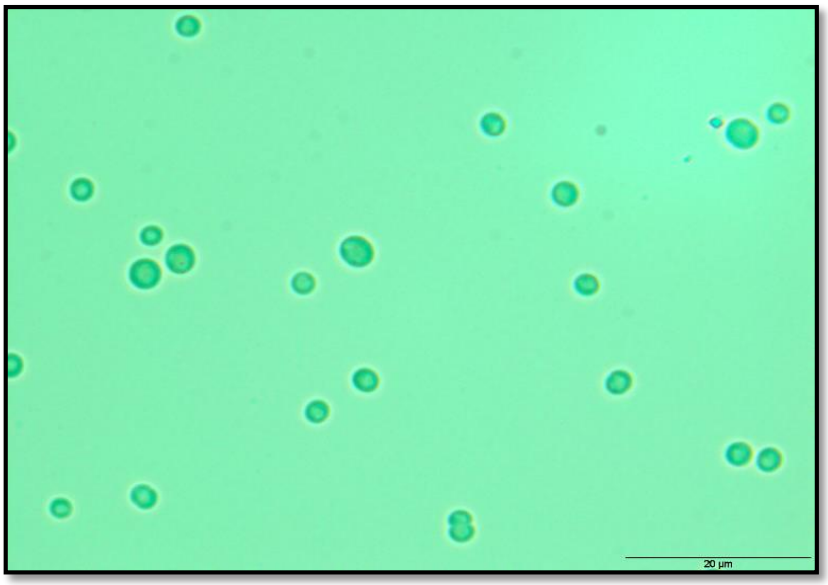

Figure 1.3. Microscopic view of *Synechocystis* sp. PCC 6803.

1.6.1 Glucosylglycerol in *Synechocystis* sp. PCC 6803

Sucrose and glucosylglycerol (GG) are the compatible solutes biosynthesized by the cyanobacterial microorganism *Synechocystis* sp. PCC 6803 naturally. Sucrose is utilized, mainly, under low osmotic concentrations. On the other hand, GG is of special interest since its responsible for *Synechocystis* tolerance to salinities up to 6% (w/v) NaCl, when not acclimated[47,56,57]. GG is produced in a two-step biosynthetic pathway where adenosine-5'-diphosphoglucose (ADP-glucose) and glycerol-3-phosphate (G3P) are the precursors, as shown in Figure 1.4. G3P originates from the biochemical transformation of dihydroxyacetone phosphate (DHAP) derived from the Calvin cycle, oxidative pentose phosphate and/or glycolysis pathways. The first biochemical reaction is catalyzed by the glucosylglycerol-phosphate synthase (GGPS), generating an intermediate called glucosylglycerol-phosphate (GGP) which is not protective against osmotic stress[58]. However, when dephosphorylated by the second step enzyme, glucosylglycerol-phosphate phosphatase (GGPP), the compatible solute GG is then obtained conferring its osmotic protective properties to allow *Synechocystis* survival at higher salinity levels[47,50].

These two enzymes present full activity only in a hyperosmotic medium[59]. For example, according to Hagemann *et al*[60], who tested the *in vitro* activity of the GGPS enzyme in a crude protein extract from *Synechocystis*, the maximum activity is achieved when in the presence of ~0.6% (w/v) NaCl, which is about one fifth of seawater's average NaCl concentration (~3% (w/v)). In fact, the regulatory mechanism, which keeps the low pool of enzyme available inactivated, is based in a sequence-independent binding of GGPS to nucleic acids that alter GGPS conformational structure, through electrostatic interactions[61].

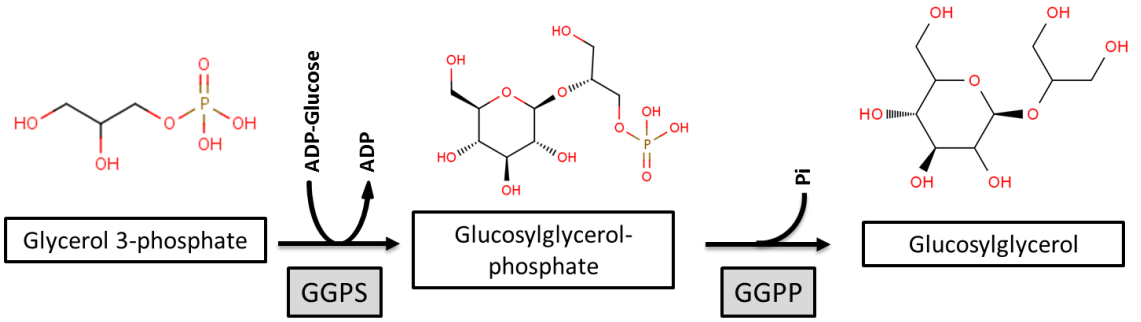

Figure 1.4 Schematic overview of the biosynthetic pathway of glucosylglycerol in *Synechocystis*. The first catalytic step is performed by glucosylglycerol-phosphate synthase (GGPS), where the intermediate glycerol-3-phosphate is formed. Afterwards, this intermediate is desphosphorylated by glucosylglycerol-phosphate phosphatase (GGPP) to form the final compatible solute glucosylglycerol. Molecular structures from chEBI and Chembase Databases.

Indeed, when growing in low-salt conditions, this cyanobacterium does not show any meaningful traces of GG, intracellularly. But, when a salt shock occurs, the GG synthesis is rapidly started due to these electrostatic interactions disturbance, without any lag-phase, while transmembrane transporters also extrude ions, mostly sodium[50]. Afterwards, when most of the toxic sodium is extruded, there is an upregulation of the genes encoding the GG production enzymes, depending on the salt concentration. At the same time, the cell's metabolism is restored and photosynthesis resumed to produce the necessary energy, as more GG is biosynthesized until a certain steady-state is reached up to 24h later[34,60,62]. Additionally, GGPS and GGPP were also tested in the presence of high levels of NaCl (up to 6% (w/v)) showing that the activity is maintained and how important this characteristic is physiologically[34]. This allows *Synechocystis*, when has its housekeeping proteins suddenly affected and inhibited by salt (NaCl), to recover faster from a bacteriostatic effect, as stated above. Despite being involved in the same biosynthetic pathway, both GGPS and GGPP are encoded by two genome far located genes, the *ggpS* (bp position 1948824 to 1947325 - *sll*1566, cyanobase) and *ggpP*/*stpA* (bp position 3041493 to 3042407 - *slr*0746, cyanobase), respectively[34,58,63]. Interestingly, some *Synechocystis ggpS* and *ggpP* knock-out mutants have been generated and studied. According to Marin *et al*[59] and Hagemann *et al*[58], mutants carrying these mutations were unable to grow on medium supplemented with more than 3.2% (w/v) NaCl, suffering a consequent cells lysis after salt shock. Both *ggpS* and *ggpP* transcription is salt regulated. Indeed, they are upregulated in higher salt conditions, although they are also transcribed, but at a lower extent, under isotonic conditions. Little is known about the regulation mechanism for *ggpP*. Nonetheless, for *ggpS*, the proposed regulation process involves the presence of a repressor protein (GgpR), encoded by a small gene (*ggpR*) which overlaps the promoter and transcriptional start point of *ggpS*. This repressor binds to the *ggpS* promotor, under low salt conditions, repressing its transcription, which is resumed after GgpR inactivation by NaCl[64]. All these interligated elements contribute for an efficient and fast acting system that allows cyanobacteria, in this case *Synechocystis*, to survive under harsh and unstable environments. Thereby, the understanding of most of the involved mechanisms in salt acclimation in cyanobacteria is desirable to design strategies

targeting an improvement in their robustness to salinity. This is particularly true to implement seawater in cyanobacteria cultivation, as mentioned before.

1.7 Objectives

The main goal of this study was to identify and implement strategies to improve *Synechocystis* halotolerance using a synthetic biology approach.

For this purpose we:

(I) Start by establishing *Synechocystis* tolerance limits to different salinity levels;
(II) Identify candidate genes to improve *Synechocystis* tolerance to salinity, in the particular case of this work the native ones: *ggpS* and *ggpP*;
(III) Design and assemble several synthetic devices with these genes, following synthetic biology standards;
(IV) Transform these synthetic devices into *Synechocystis* chassis (*Synechocystis* sp. PCC 6803 and its *ΔggpS* mutant) and characterize the resulting mutants at a growth and transcriptional levels.

2. Materials and methods

2.1 Bacterial strains and standard growth conditions

The cyanobacteria *Synechocystis* sp. PCC 6803 (obtained from the Pasteur Culture Collection of cyanobacteria, Paris, France) and a *ggpS* knock-out mutant (Δ*ggpS*) strains, were kept in BG11 medium[65] at 30 ºC and a 12 h light (25 µE m^{-2} sec^{-1}) /12 h dark regimen. Cosine-corrected irradiance was measured with a quantum meter (Dual Solar/Electric Quantum Meter, Spectrum Technologies, Inc.). When cultured in solid medium, BG11 supplemented with 1.5% (w/v) Difco® Agar Noble, 0.3% sodium thiosulfate and 10 mM TES–KOH buffer (pH 8.2) was used. The strains *E. coli* DH5α (Stratagene) and One Shot® TOP10 chemically competent *E. coli* (Invitrogen) were used for molecular cloning purposes and cultured at 37 ºC in selective Lysogeny Broth (LB)[66] medium. For solid medium, 1.5% (w/v) Bacteriological Agar was added. When necessary, BG11 and LB media were supplemented with the appropriate antibiotic, chloramphenicol (Cm, 10 or 25 µg mL^{-1}), ampicillin (Amp, 100 µg mL^{-1}), or kanamycin (Km, 50 µg mL^{-1} in LB; 25 to 500 µg mL^{-1} in BG11).

2.2 Synthetic devices assembly

In order to improve *Synechocystis* halotolerance, the coding sequences of the native *ggpS* and *ggpP* were amplified by PCR, from *Synechocystis* gDNA, and used in the assembly of the synthetic devices. The devices enable the constitutive overexpression of these genes and two sets of three different devices were designed. Two have *ggpS* or *ggpP*, while the third carries both genes with *ggpS* downstream of *ggpP.* The difference between the sets relies on the promoter used, see Table 2.2. One was assembled with the synthetic $P_{trc2.x.tetR}$ (medium strength), while the other with the synthetic $P_{trc.x.lacI}$ (high strength). The BioBrick™ RBS (BBa_B0030) was retrieved from the Registry of Standard Biological Parts (http://parts.igem.org/) in the pSB1A2 (Table 2.1). The cloning process was performed according to the Biobrick Assembly Standard RFC10 (Figure 1.4), as follows: for upstream assembly, (I) the vector containing the promoter was digested with *EcoRI* and *SpeI*: and the fragment was ligated to the recipient vector (pSB1A2) RBS digested with *EcoRI* and *XbaI*. For downstream assembly, (II) the coding sequences digested with *PstI* and *XbaI* were ligated to the recipient vector digested with *PstI* and *SpeI*. Finally, (III) all the synthetic devices, digested with *PstI* and *XbaI,* were cloned into the shuttle vector pSEVA351 (Table 2.1), cut with *PstI* and *SpeI,* with chloramphenicol as antibiotic marker for *Synechocystis* transformation, see Table 2.2.

Table 2.1. List of plasmids used in this work.

Plasmid	Resistance marker	Purpose/ Description	Source
pJ201:: $P_{trc2.x.tetR}$ / **pJ201:: $P_{trc.x.lacI}$**	Km^R	Plasmids with synthetic promoters	DNA 2.0, Inc.
pSB1A2	Amp^R	High-copy number BioBrick™ plasmid for *E. coli* cloning; Plasmid containing the BioBrick™ RBS B0030	Repository of standard biological parts (MIT)
pGEM-T	Amp^R	TA-cloning of PCR products	Promega
pGDggpS.KS	Amp^R/ Km^R	Plasmid used to generate DNA probe for Southern blot	Our lab (unpublished data)
pSEVA351	Cm^R	Replicative shuttle vector for *Synechocystis* transformation	SEVA-DB

The DNA digestions were carried out using the FastDigest™ Restriction Enzymes (ThermoScientific) according to the manufacturer's specifications. Additionally, the assembled plasmids were confirmed by restriction with the appropriate enzymes and/or PCR followed by DNA sequencing (STABVIDA).

Table 2.2. List of synthetic devices generated in this work.

Plasmid (backbone)	Synthetic devices
pSEVA351	$P_{trc2.x.tetR}$::RBS(B0030)::*ggpP*
	$P_{trc2.x.tetR}$::RBS(B0030)::*ggpS*
	$P_{trc2.x.tetR}$::RBS(B0030)::*ggpP*_$P_{trc2.x.tetR}$:: RBS(B0030)::*ggpS*
	$P_{trc.x.lacI}$:: RBS(B0030)::*ggpP*
	$P_{trc.x.lacI}$:: RBS(B0030)::*ggpS*
	$P_{trc.x.lacI}$:: RBS(B0030)::*ggpP*_$P_{trc.x.lacI}$:: RBS(B0030)::*ggpS*

2.3 Agarose gel electrophoresis

Nucleic acids electrophoresis analysis was performed in 1% (w/v) agarose (NZYTech) gels, with 1 x TAE buffer[67] supplemented with 0.5 µg mL^{-1} of ethidium bromide. Bands were visualized under ultra-violet (UV) light with a Gel Doc™ XR+ Imager (Bio-Rad). The GeneRuler™ DNA Ladder Mix (ThermoScientific) was used as molecular weight marker.

2.4 DNA purification and quantification

DNA purification from enzymatic reactions or gel was performed using the NZYGelpure kit (NZYTech), following the manufacturer's instructions. DNA was quantified using a Nanodrop ND-1000 (Nanodrop Technologies, Inc.).

2.5 Polymerase chain reaction (PCR)

The PCR assays were performed using the GoTaq® DNA polymerase (Promega) for confirmation purposes and the Phusion® High-Fidelity DNA polymerase (ThermoScientific) for ORF amplification from *Synechocystis* genomic DNA (gDNA), following the manufacturer's instructions. In each PCR reaction (20 µL), 1U of DNA polymerase was used and the magnesium chloride ($MgCl_2$) and deoxyribonucleoside triphosphate (dNTP) concentrations were 1.5 mM and 0.2 mM, respectively. As for oligonucleotides, see Table 2.3, the final concentration was 0.5 µM. For confirmation purposes, PCRs were performed in a thermocycler (Bio-Rad) using the following profile: 3 min denaturation step at 95 ºC; followed by 25 cycles of 30 sec at 95 ºC, 30 sec at annealing temperature (see Table 2.3. List of oligonucleotides used in this work.) and 72 ºC for extension (1 min for every Kbp of the target DNA was used); a final extension step at 72 ºC for 7 min. As for the reactions employing the Phusion® High-Fidelity DNA polymerase (ThermoScientific) the PCR profile was: 30 sec denaturation step at 98 ºC; followed by 35 cycles of 10 sec at 98 ºC, 30 sec at 60ºC and 45 sec at 72 ºC; a final extension step at 72 ºC for 7 min.

Table 2.3. List of oligonucleotides used in this work.

Primers	Sequence 5' → 3'	Ta (ºC)	Purpose
VF2	TGCCACCTGACGTCTAAGAA	50	Confirmation of constructs in pSB1A2; DNA sequencing
VR	ATTACCGCCTTTGAGTGAGC		
PS1	AGGGCGGCGGATTTGTCC	58	Confirmation of constructs in pSEVA351; DNA sequencing
PS2	GCGGCAACCGAGCGTTC		
ggpS.5O	GCTGGCTCGAGACCGTAGGGCAG	58	Southern blot DNA probe
ggpS.5I	GATTACAACCGGTTGTAATCACGGCTA		
BBa_ggpP.F	GTTTCTTCGAATTCGCGGCCGCTTCTAGATG GTATTACACCAACAACGTTTCTCC	60	*ggpP* ORF amplification
BBa_ggpP.R	GTTTCTTCCTGCAGCGGCCGCTACTAGTATT ATTACTGGGAAAAATGGACTCTTCG		
BBa_ggpS.F	GTTTCTTCGAATTCGCGGCCGCTTCTAGATG AACTCATCCCTTGTGATCCTTTAC	60	*ggpS* ORF amplification
BBa_ggpS.R	GTTTCTTCCTGCAGCGGCCGCTACTAGTATT ATTACATTTGGGGGGGCTCTCCCAGTACC		
ggpP.FI	ATTACAAACGGGCATTGAAGC	56	RT-qPCR
ggpP.RI	TGTCCGATTGTGATAGTAACG		
ggpS.FI	CGTGGGCACCAATCCGGCAAATATC	56	
ggpS.RI	GGTTAGTCAACACCGCATCGGGTAG		
rnpBF1	CGTTAGGATAGTGCCACAG	56	
rnpBR1	CGCTCTTACCGCACCTTTG		
S.petB1F	CCTTCGCCTCTGTCCAATAC	56	
S.petB1R	TAGCATTACACCCACAACCC		

Restriction enzyme recognition sites are underlined

2.5.1 Colony PCR

For confirmation of *Synechocystis* or *E. coli* transformation, a colony PCR was performed. Cells from each colony were transferred to 20 µL of deionized water (0.2 mL PCR tube) and incubated at 95 ºC for 5 min followed by a short spin. Finally, 2 µL of the supernatant were used in the PCR reaction, as described in section 2.5.

2.6 DNA ligation, *E. coli* DH5α transformation and plasmid DNA purification

DNA ligations were performed with the T4 DNA Ligase (ThermoScientific) according to the manufacturer's instructions. The vector:insert ratio used was 1:3 or 1:5 and the ligation reactions were incubated ON at 25 ºC. Ligations using the pGEM®-T-Easy vector (Promega) were carried out as described in the manufacturer's instructions.

The assembled plasmids were then transformed into chemically competent *E. coli* DH5α or One Shot® TOP10 chemically competent *E. coli* (Invitrogen) cells. For *E. coli* DH5α, 200 µL of cells were mixed with the DNA ligation and incubated on ice for 20 min. Afterwards, the mixture was heat shocked at 42 ºC for 90 sec in a water-bath, followed by an incubation on ice for 2 min. Then, 800 µL of LB medium were added to the cells that were left to recover for 45-90 min, in an orbital shaker at 37 ºC. As for the One Shot® TOP10 chemically competent *E. coli* (Invitrogen) the transformation process was performed according to the manufacturer's instructions. For both strains, 100 µL of the cell suspension were plated onto LB-agar supplemented with the appropriate antibiotic and then incubated ON at 37 ºC.

To isolate plasmid DNA, cells from isolated colonies were inoculated in 5 mL of LB medium supplemented with the appropriate antibiotic and incubated ON at 37 ºC with vigorous shaking (200 r.p.m). Plasmid DNA was prepared with the GenElute™ Plasmid miniprep Kit (Sigma) from 4 mL of culture and following the manufacturer's instructions.

2.7 Cyanobacterial DNA extraction

For confirmation of *Synechocystis* transformants by PCR, DNA extraction was performed using 2 mL of culture centrifuged at 14100 x*g* for 1 min and washed with 500 µL of dH_2O. Then, the cells were centrifuged again at 14100 *xg* for 1 min, resuspended in 150 µL of dH_2O and 1 µL of RNase solution (20 mg mL^{-1}, Sigma) and 0.1 g of 425-600 nm glass beads (acid washed, Sigma) were added. Cells were disrupted by two cycles of vigorous vortexing for 1 min followed by incubation on ice for 1 min. Finally, the cells were centrifuged at 14100 *xg* for 1 min and 100 µL of the supernatant was kept. For the PCR reactions, 5 µL of supernatant.

2.7.1 Phenol-Chloroform DNA extraction protocol

For *Synechocystis* transformants confirmation by Southern Blot, cyanobacterial gDNA was extracted using the phenol/chloroform method, according to Tamagnini *et al*[68]. Firstly, 30

mL of *Synechocystis* culture was centrifuged at 4190 *xg* for 10 min and resuspended in 2 mL of resuspension buffer (50 mM Tris-HCl, pH 8.0, with 10 mM EDTA). Then, 0.6 g of 425-600 nm glass beads (acid washed, Sigma), 25 µl of 10% (w/v) SDS, 250 µl of phenol (pH 7.0) and 250 µl chloroform (for a 1:1 (v/v) ratio) were added and cells were disrupted by five cycles of vigorous vortexing for 30 sec followed by incubation on ice for 1 min. The aqueous/organic phases were separated by centrifugation at 13000 *xg* for 10 min at 6 ºC and the upper aqueous phase was extracted twice with an equal volume of chloroform (500 µl). The DNA was precipitated with 1/10 volumes of 3 M sodium acetate (pH 5.2) and 2.5 volumes of ice cold 100% (v/v) ethanol at -20 °C for 1 hour. Afterwards, samples were centrifuged at 13000 *xg* for 20 min at 6 ºC. Then, the resulting pellet was washed with ice cold 70% (v/v) ethanol, dried, and resuspended in water and kept ON at 4 ºC for full hydration. Finally, for Southern blot only, 1 µL of RNase solution (20 mg mL^{-1}, Sigma) was added to samples for 1h at 37 ºC and the gDNA integrity checked by agarose gel electrophoresis.

2.8 Southern blot of the Δ*ggpS* knock-out mutants

The DNA probe (1223 bp) for the Southern blot assay was generated by PCR with the primers ggpS.5I and ggpS.5O (Table 2.3) covering the 5' flanking region of the *ggpS* gene using the pGDggpS.KS as template. Then, 300 ng of PCR product was labelled with digoxigenin using the DIG High Prime DNA Labelling kit (Roche Molecular Biochemicals). The DNA probe labelling and efficiency testing were performed according to the manufacturer's instructions.

The Southern blot was carried out using the *Synechocystis* strains gDNA (4 µg) that was digested with *Ava*II Fast-Digest® (ThermoScientific) for 45 min at 37 ºC, followed by an agarose gel electrophoresis. The remaining protocol was performed according to the DIG High Prime DNA Detection Starter kit (Roche Molecular Biochemicals) instructions. The final results were observed with a Chemi Doc™ XRS+ Imager (Bio-Rad).

2.9 *Synechocystis* transformation by electroporation

The transformation of the assembled plasmids into *Synechocystis* was performed by electroporation, based on the Chiaramonte *et al*[69] and Ludwig *et al*[70] optimization protocols. *Synechocystis* cultures of a wt and Δ*ggpS* strains were cultured at 25 ºC and continuous light regimen to an OD_{730}~0.5. Cells were harvested by centrifugation at 4190 *xg*, for 10 min and washed three times with 10 mL of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic (HEPES) acid buffer 1 mM, pH 7.5. The cells were then resuspended in 1 mL of HEPES and 60 µL of this suspension were mixed with 2 µg of plasmid DNA and electroporated with a Bio-Rad Gene Pulser™ (Bio-Rad), at a capacitance of 25 µF. The resistance used was 400 Ω for a constant time of 9 msec with an electric field of 12 kV cm^{-1}. Immediately after the electric pulse, the cells were transferred to 50 mL of fresh BG11 medium (100 mL Erlenmeyer flask) and incubated for 24 h at 25 ºC in a continuous light regimen (20 µE m^{-2} sec^{-1}). Next, the 50 mL of culture was centrifuged at 4190 *xg* for 10 min and resuspended in 500 µL BG11 medium. The cells were

spread onto Immobilon-NC membranes (0.45 µm pore size, 82 mm, Millipore) resting on solid BG11 petri-dishes supplemented with 10 µg mL^{-1} of chloramphenicol, at 25 ºC in a 16 h light / 8 h dark regimen. Colonies were observed after 1-2 weeks and were transferred to liquid BG11 medium with the same antibiotic concentration.

2.10 Halotolerance growth experiments

Pre-cultures of *Synechocystis* strains were grown in an orbital shaker at 150 r.p.m, at 30 ºC and under a 12 h light (25 µE m^{-2} sec^{-1}) / 12 h dark regimen, until an OD_{730} of ~2 was reached. When necessary the medium was supplemented with chloramphenicol (Cm, 10 µg mL^{-1}) and/or kanamycin (Km, 25 µg mL^{-1}). Then, the cultures were diluted, in fresh BG11 medium without antibiotic, to a final OD_{730}~0.5. Afterwards, 50 mL of the dilution were transferred to 100 mL Erlenmeyer flasks (previously sterilized) containing NaCl, providing the cultures with the following final NaCl concentrations: 0%, 3%, 5% and 7% (w/v). These cultures were maintained in the same conditions as the pre-culture and their growth was monitored measuring the OD_{730}, using a Shimadzu UVmini-1240 spectrophotometer. Each experiment was performed in duplicate and under aseptic conditions for 16 days.

2.11 Total RNA extraction and transcription analysis by quantitative real-time PCR (RT-qPCR)

Synechocystis cultures were prepared and cultured as described in section 2.10. Cells were grown until an OD_{730}~1 in 100 mL of BG11 medium (without antibiotic), in the presence or absence of NaCl: 0, 3 and 5% for wt; 0 and 3% for Δ*ggpS* mutant; 0 and 5% for the remaining mutants with synthetic devices. Cells were collected by centrifugation at 4190 *xg* for 10 min and the pellet was resuspended in 1 mL of fresh BG11 medium and transferred to screw-cap 2 mL tubes. Cells were centrifuged at 4190 *xg* and the pellet was resuspended in 500 µL of medium and 2 volumes (1 mL) of RNAprotect® Bacteria Reagent (Qiagen) was added and the mixture was vortexed for 5 sec, then incubated for 5 min at RT and centrifuged at 5000 *xg* for 10 min. The cell pellets were stored at -80 ºC.

For RNA extraction, the TRIzol® Reagent (Ambion) was used in combination with the Purelink™ RNA Mini Kit (Ambion). Briefly, the cells were disrupted in 1 mL TRIzol containing 0.2 g of 425-600 nm glass beads (acid washed, Sigma) using a FastPrep®-24 (MP Biomedicals) (2 × 60 sec at a setting of 4.0 m sec^{-1}), and the following extraction steps were performed according to the manufacturer's instructions. The RNA samples were treated with On-column PureLink® DNase for 1.5 hours at 25 ºC, following the manufacturer's instructions. RNA was quantified on a NanoDrop ND-1000 (NanoDrop Technologies, Inc.), the integrity/quality was checked using the Experion™ RNA StdSens Analysis Kit (Bio-Rad). The absence of gDNA contamination was determined by PCR, using specific primers for the *rnpB* reference gene (Table 2.3) and 80 ng of total RNA. The PCR products were analyzed by electrophoresis on a 1.5 % (w/v) agarose gel.

One µg of total RNA was used for cDNA synthesis using the iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad) in a final volume of 20 µL, using random primers and following the manufacturer's instructions. cDNA synthesis was confirmed by PCR with the *rnpB* primers, using 1 µL of cDNA.

For relative gene expression quantification, RT-qPCRs were performed for the *ggpP* (ggpP.RI and ggpP.FI primers), *ggpS* (ggpS.RI and ggpS.FI primers) and the reference genes *rnpB* and *petB* (Table 2.3)[71]. Five-fold standard dilutions of cDNA were made (1/5; 1/25; 1/125; 1/625) and used to check the relative efficiency and quality of the primers. The RT-qPCRs were carried out on iQ™ 96-well PCR plates covered with Optical Sealing Tape (Bio-Rad). The reaction mixtures were manually assembled and contained 0.25 µM of each primer, 10 µL of iQ™ SYBR® Green supermix (Bio-Rad) and 2 µL of template cDNA (dilution 1/25). The PCR profile was: 3 min at 95 ºC; followed by 35 cycles of 30 sec at 95 ºC, 30 sec at 56 ºC and 30 sec at 72 ºC. Negative controls (no template cDNA) were included and a melting curve analysis was performed in all assays. RT-qPCRs were performed with one biological replicate and technical triplicates/duplicates of each cDNA sample in the iCycler iQ™5 Real-Time PCR Detection System (Bio-Rad). The obtained data were analyzed using the iQ™5 Optical System Software v2.1 (Bio-Rad). Efficiency values were calculated and the Cq values for each data set were exported to a Microsoft Office Excel file, and imported into the qbasePLUS2 software (Biogazelle). The relative quantities of each sample were calculated using the gene-specific efficiency acquired from the dilution series and normalized to the mean Cq value.

3. Results and discussion

3.1 *Synechocystis* tolerance to salinity

The model photosynthetic cyanobacterium *Synechocystis* sp. PCC 6803 has huge potential to be used as a synthetic biology chassis. Due to its singular characteristics, this bacterium is being widely studied in order to fulfill its place in the biotechnology field. Indeed, many applications originated with its utilization ranging from bioremediation to biologically active biomolecules and biofuels production[22,29]. However, the downstream processes involved in cyanobacteria cultivation, as for microalgae, require large amounts of freshwater. Despite less severe than the plant crops water usage for biofuel production, the estimated water needs still pose risk to the World's freshwater reserves, if cyanobacteria utilization is to be intensive[31]. Therefore, the use of seawater in *Synechocystis* cultivation is one of the solutions to overcome this issue. Besides its abundance, it has a very stable chemical composition with almost all the nutrients essential for cyanobacterial growth[16]. Additionally, it would be also useful to avoid contamination from undesired organisms that could compete with or predate cyanobacteria, including *Synechocystis*[19].

Synechocystis is a moderately halotolerant bacterium. According to Pandhal *et al*[57] and Ferreira[56], this microorganism grows in salinities up to 6% (w/v) NaCl, which is about two-fold higher than seawater's NaCl concentration. However, even though it withstands such high salinities, its growth is severely affected with increasing levels of NaCl.

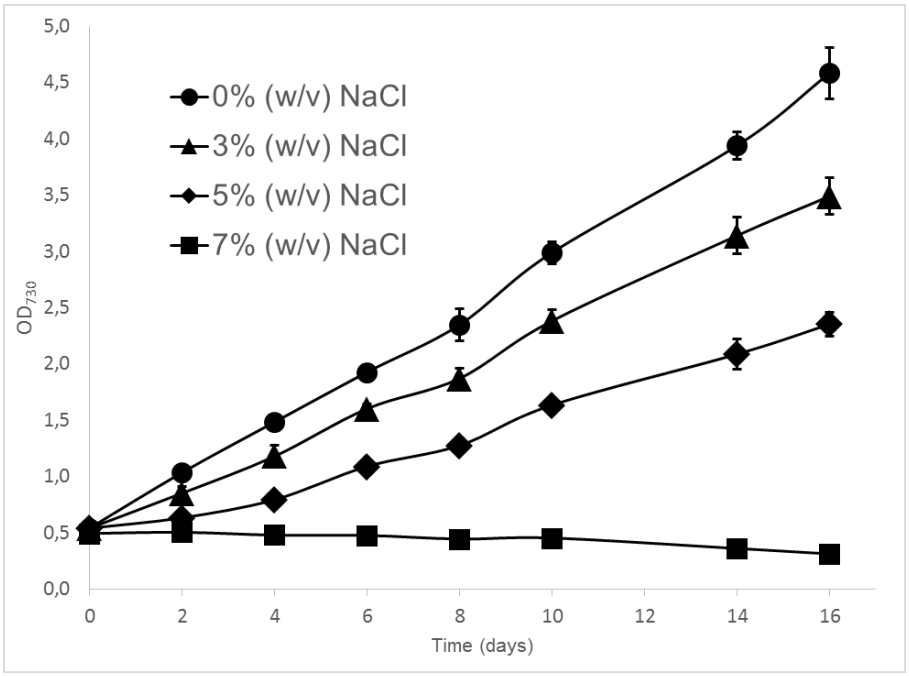

Figure 3.1 Salt stress effect on the growth of *Synechocystis* sp. PCC 6803 wild-type under different NaCl concentrations in BG11 medium. Cells were cultivated in a 12 h light (25 µE m^{-2} s^{-1})/ 12 h dark regimen at 30 ºC and 150 rpm. Data represents means ± SD from three independent experiments, except for the 7% NaCl condition with only two independent experiments..

As shown in Figure 3.1, a *Synechocystis* wild-type strain was cultured under 0, 3, 5 and 7% (w/v) NaCl for 16 days. From the results obtained, in 3% (w/v) NaCl there is a breakdown in growth of about 20%, while for the 5% condition it is around 50%, compared with the same strain growing in 0% NaCl. Finally, at 7%, the non-acclimated cells of *Synechocystis* are unable to grow and therefore end up dying after a few days. These results are in agreement with the ones obtained by Ferreira[56]. Other studies report a NaCl tolerance limit between 5.9 - 7% (w/v) which is within the range of the results obtained here[51,72]. This drawback makes *Synechocystis'* cultivation in seawater unattractive for the biotechnological industry. Therefore, strategies to improve its robustness to salinity are desirable.

3.2 Strategies to improve *Synechocystis* halotolerance

To date, some authors tested different strategies to improve the robustness of several organisms against salt stress. Some of these are essentially based in the transport or biosynthesis of osmotic regulators e.g. compatible solutes. As an example, Klähn *et al*[73] transformed the gene *ggpPS* from the heterotrophic bacterium *Azobacter vinelandii*, encoding a combined GG-phosphate synthase/phosphatase enzyme (GGPPS) for glucosylglycerol (GG) production, into *Arabidopsis thaliana.* Unlike the wild-type without GG production, three independent *Arabidopsis* lines of transformants had accumulation of high amounts of GG at different levels. Interestingly, the line with lower GG amount acquired tolerance against salt stress. The strains with higher GG concentrations showed a slow growth under control conditions and no improvement in halotolerance. A different approach was utilized by Waditee *et al*[74], in this case, the freshwater cyanobacterium *Synechococcus* sp. PCC 7942 was transformed with heterologous genes encoding: a Na^+/H^+ antiporter, a catalase, enzymes from the biosynthetic pathway of betaine (compatible solute) and/or a chaperone. Unexpectedly, only the strain expressing the Na^+/H^+ antiporter was able to grow in NaCl concentrations up to 3% (w/v) and seawater. In other studies, the same type of strategy actually had opposite results: the expression of a different Na^+/H^+ antiporter conferred Na^+ sensitivity, while the presence of betaine biosynthesis and catalase genes resulted in higher halotolerance for the same microorganism[75–77]. These results clearly show that an improvement in an organism halotolerance is achievable. However, the complexity and unpredictability of a biological system still poses difficulties in its engineering. Therefore, an iterative approach will be used. In this work, the strategies to improve *Synechocystis* robustness to salinity are based on the overexpression of the native genes involved in GG production.

3.2.1 Design and assembly of synthetic devices based on *Synechocystis'* native genes involved in GG production

Synechocystis acclimates salt stress by using the "salt-out" strategy which relies on the extrusion of toxic ions, while an osmotic balance with the external environment is assured by its compatible solutes sucrose and GG. The latter is essential for *Synechocystis* survival under

high salinities and is synthesized in two-steps by the GGP-synthase (GGPS) and the GGP-phosphatase (GGPP). These enzymes are encoded by the *ggpS* and *ggpP* genes, respectively, which are transcribed under a tight regulatory mechanism depending on the ionic intracellular concentrations[47,50].

To improve Synechocystis robustness to salinity, this work strategies rely on the constitutive overexpression of both *ggpS* and *ggpP* to increase the available pool of GGPS and/or GGPP enzymes and the intracellular concentration of GG to confer improved halotolerance. Additionally, codon optimization of both ORFs was not necessary and there was certainty in their functionality in this microorganism. In order to implement these strategies, synthetic devices employing these genes separately or together were designed. As shown in Figure 3.2, two different synthetic promoters were utilized, the $P_{trc2.x.tetR}$ and $P_{trc.x.lacI}$ with relative strengths 30 and 59 times higher than the reference promoter P_{rnpB}, respectively[78]. The RBS (B0030) was obtained from the Registry of Standard Biological Parts (http://parts.igem.org/Part:BBa_B0030) and no transcriptional terminator was added since there is one already present in the recipient vector[26]. As a result, two identical sets of synthetic devices in which only the promoter differs were assembled, in this work, for a total of six devices (Figure 3.2).

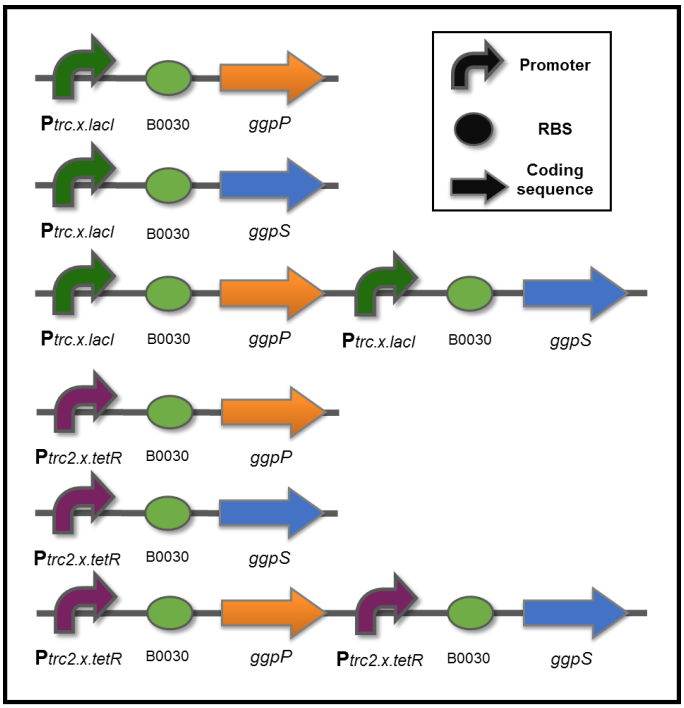

Figure 3.2 Schematic representation of the synthetic devices designed and generated in this work.

These devices were then cloned into the replicative pSEVA351 (SEVA-DB, http://seva.cnb.csic.es), to avoid the time consuming steps of homologous recombination and allow a faster transformation and assessment of the generated *Synechocystis* mutants. All the

assembled synthetic devices were confirmed by digestion with the appropriate restriction enzymes (Figure 3.3) and by DNA sequencing (see section 2.2).

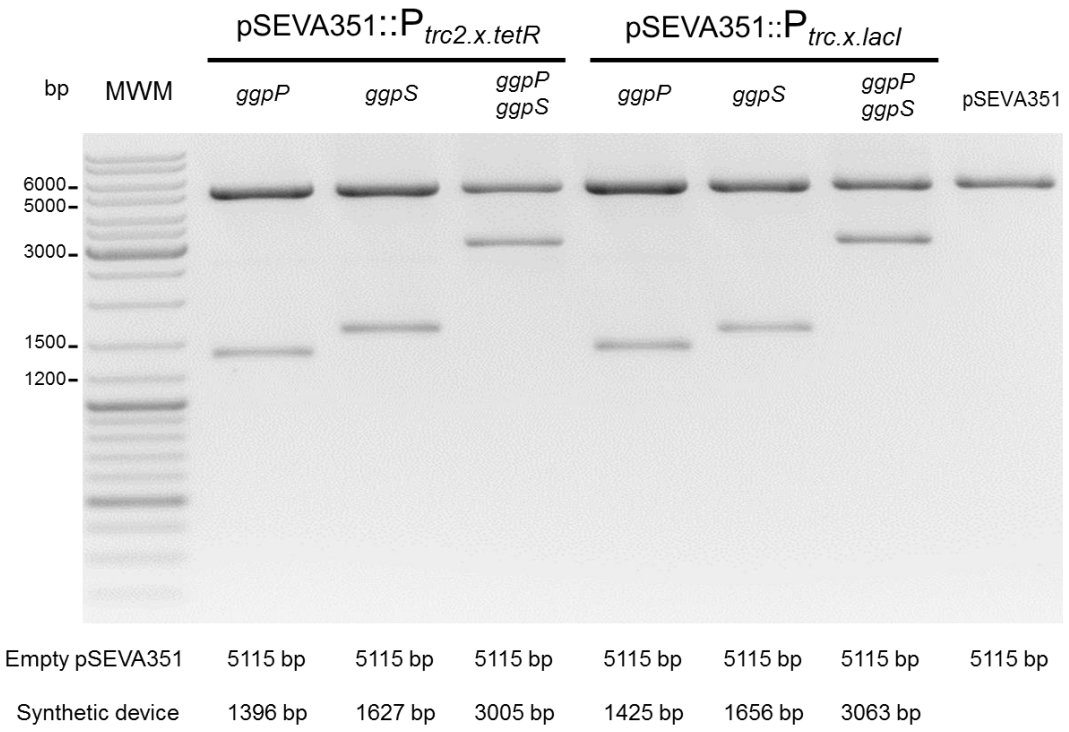

Figure 3.3 DNA electrophoresis of the plasmids with synthetic devices digested with *XbaI* and *PstI* confirming the correct assembly of the synthetic devices into pSEVA351. The expected sizes of the restriction fragments are shown below the figure. MWM – Molecular weight marker, Generuler™ DNA Ladder Mix (ThermoScientific).

3.2.2 Generation of mutants with the synthetic devices for GG production

The *Synechocystis* wild-type was transformed with the six synthetic devices depicted in Figure 3.2. Concomitantly, a *ggpS* knock-out mutant already available in our lab was also utilized to avoid the background influence from the *Synechocystis* natural GG production system. The *ggpS* knock-out mutant was transformed with the *ggpS* and *ggpP/ggpS* synthetic devices (four in total), but not with the ones carrying *ggpP* only, since without the first step enzyme (GGPS) no GG can be synthesized. It is important to notice that both the wt and the *ggpS* knock-out mutant were transformed with the empty pSEVA351, to be used as controls in characterization processes. Before transformation of the *ggpS* knock-out mutant, the full segregation of the Δ*ggpS*::Km and Δ*ggpS*::Km::SacB mutants was confirmed by Southern blot, see Figure 3.4.

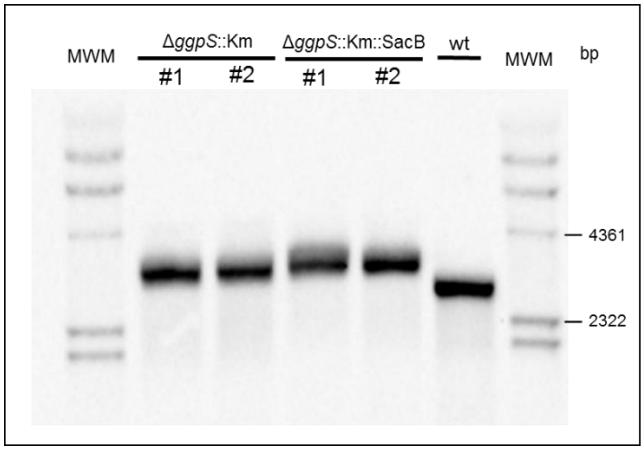

Figure 3.4 Confirmation of segregation of *Synechocystis ggpS* knock-out mutants by Southern blot. Genomic DNA was digested with *AvaII* and hybridized with a probe covering the 5' flanking region of the *ggpS* gene. Expected band size for the insertion *ggpS* knock-out mutants (3200 bp) and wt (2772 bp). MWM: Molecular weight marker, Lambda DNA/*HindIII* Marker, 2 (ThermoScientific)

All the mutants tested are fully segregated since no visible band with the same size of the wt is observed. The Δ*ggpS*::Km clone #1 (hereafter Δ*ggpS*) was selected to be transformed.

The transformation of both strains with the plasmids carrying the synthetic device was performed by electroporation. All the resulting transformants were confirmed by PCR and the positive ones selected for further rounds of characterization. In total, twelve different mutants were generated (Figure 3.5).and from these, at least, two clones of each were kept.

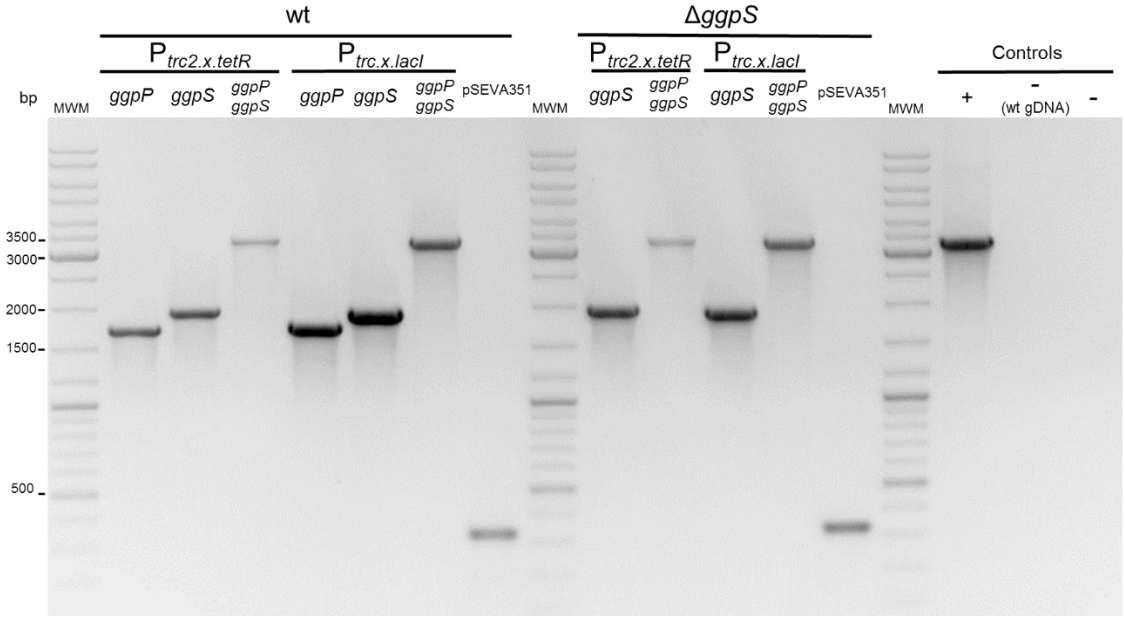

Figure 3.5 DNA electrophoresis of PCR products confirming the *Synechocystis* mutants carrying the pSEVA351 and pSEVA351 with synthetic devices specified by the type of promoter ($P_{trc2.x.tetR}$ and $P_{trc.x.lacI}$) and respective *ggpP* and/or *ggpS* ORFs. The PCR reactions were performed with the PS1/PS2 primer pair. The expected band size for the empty pSEVA351 is 321 bp and pSEVA351 with synthetic devices according to promoter type: $P_{trc2.x.tetR}$ (*ggpP* – 1673 bp ; *ggpS* – 1904 bp; *ggpP*+*ggpS* – 3256 bp) and $P_{trc.x.lacI}$ (*ggpP* – 1703 bp; *ggpS* – 1934 bp; *ggpP*+*ggpS* – 3316 bp). Controls: +: pSEVA351::$P_{trc.x.lacI}$.*ggpP*::$P_{trc.x.lacI}$.*ggpS* as template; -: (wt gDNA) - gDNA as template; -: No template. MWM – Molecular weight marker, Generuler™ DNA Ladder Mix (ThermoScientific).

3.3 Functional characterization of selected mutants carrying synthetic devices for GG production

The generated mutants were characterized at the physiological level by evaluating the mutants' growth under salt stress conditions, and at the transcriptional level by RT-qPCR (see Table 3.1).

Table 3.1 *Synechocystis* strains selected for further characterization at physiological and transcriptional levels.

***Synechocystis* strains**	**Growth**	**Transcription**
wt	X	X
wt pSEVA351	X	-
Δ*ggpS*	X	X
Δ*ggpS* pSEVA351::$P_{trc.x.lacI}$.*ggpS*	X	X
wt pSEVA351::$P_{trc.x.lacI}$.*ggpP*::$P_{trc.x.lacI}$.*ggpS*	X	X
wt pSEVA351::$P_{trc2.x.tetR}$.*ggpP*::$P_{trc2.x.tetR}$.*ggpS*	X	-

3.3.1 Growth analysis of *Synechocystis* mutants under different salinities

The overall phenotypic response from the selected *Synechocystis* mutants was verified by analyzing their growth at different NaCl concentrations (0, 3, 5 and 7% (w/v)) in BG11 medium. Growth curves of three independent experiments, for a 16 day time interval, were analyzed for six *Synechocystis* strains (Table 3.1).

As shown in Figure 3.6, there is no significant difference in growth between the wt and the mutant containing the empty pSEVA351, therefore the results from the mutants tested will be presented in each specific salt condition and compared with the wt.

0% (w/v) NaCl condition:

In 0% (w/v) NaCl, the Δ*ggpS* mutant has a similar growth to the wt, as expected, since GG is not essential in conditions in absence of salt[79]. The wt carrying the device with both *ggpP* and *ggpS* under regulation of the medium strength promoter ($P_{trc2.x.tetR}$) also presents similar growth to the wt. However, the complemented Δ*ggpS* and the wt with synthetic device with both genes under the regulation of the higher strength promoter ($P_{trc.x.lacI}$) show a breakdown in growth of about 35%. This decrease could be explained by the higher amount of GGPS which could negatively affect the microorganism due to this enzyme's regulatory mechanism. In isotonic conditions, GGPS activity is regulated by binding to nucleic acids, in a sequence-independent manner. Therefore, a higher pool of this enzyme could lead to associations with DNA sequences of essential genes, thus affecting growth[61].

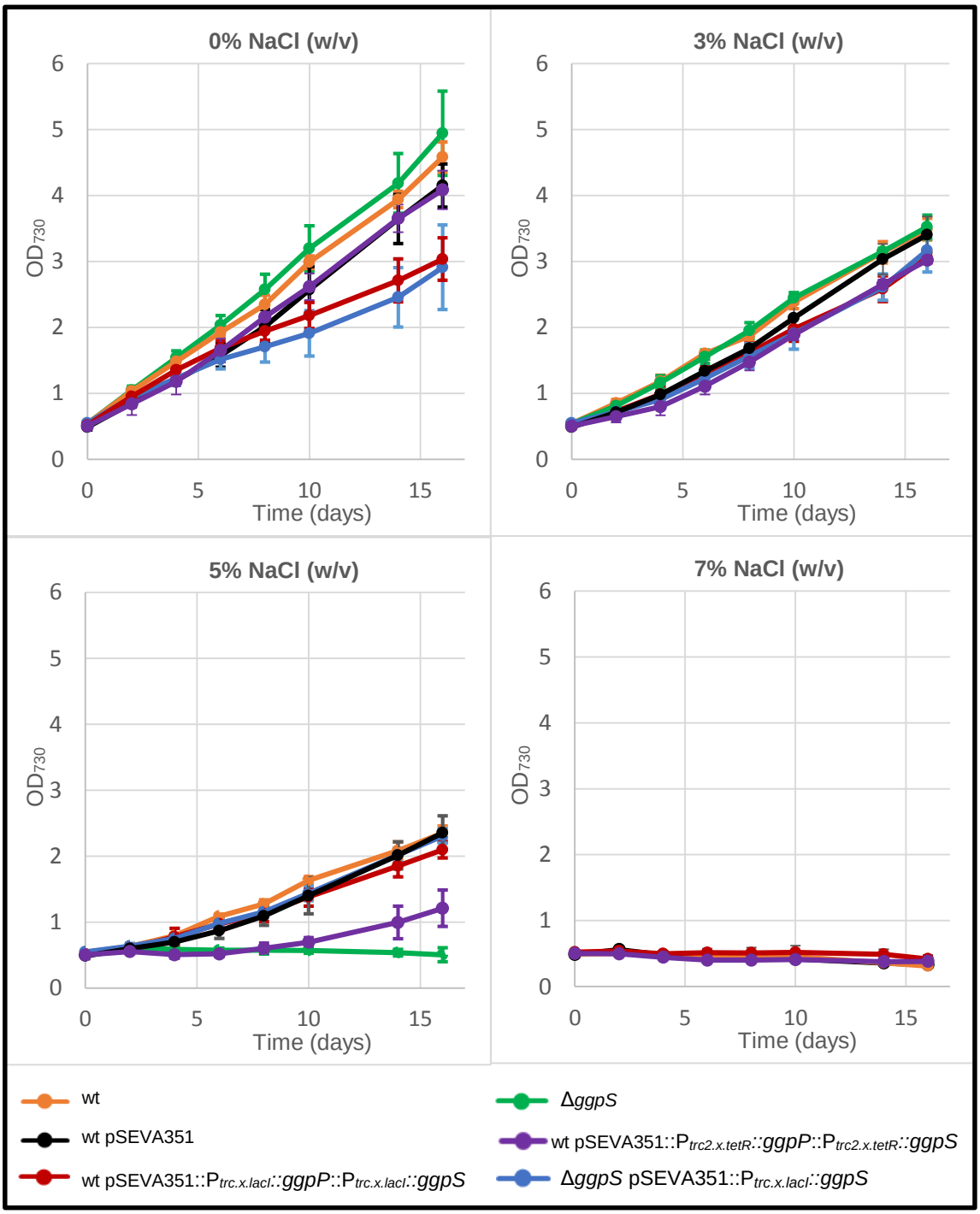

Figure 3.6 Salt stress effect on the growth of *Synechocystis* sp. PCC 6803 mutants under different NaCl concentrations (% w/v) in BG11 medium. The salt concentrations are 0, 3, 5 and 7% NaCl. Cells were cultivated in a 12 h light (25 µE m^{-2} s^{-1})/ 12 h dark regimen at 30 ºC and 150 rpm. Data represents means ± SD from three independent experiments, except for the 7% NaCl condition with only two independent experiments.

3% (w/v) NaCl condition:

In 3% (w/v) NaCl, the wt has a breakdown in its growth of about 20% compared with the 0% NaCl condition. This result is in line with the work from Ferreira[56] and can be explained by the several cellular processes affected and inhibited by ionic stress, especially photosynthesis[48]. As a result, lower energy levels are generated leading to diminished biomass production.

The Δ*ggpS* mutant is able to grow in 3% NaCl and has a similar growth pattern compared to the wt. Since, this mutant is unable to produce GG, the fact that it withstands such ionic stress might be related with an increase in sucrose content, which has already been described in the literature[79]. However, it was also reported that this higher sucrose concentration, shown to be about 10% of the normal GG concentration, is not sufficient to balance the external osmotic pressure, at 3% NaCl[34,59].

As for the complemented Δ*ggpS* and both the wt carrying the device with both *ggpP* and *ggpS* under regulation of the medium or high strength promoters, these mutants have a breakdown in growth of about 30-35% compared with the wt in 0% NaCl. Nonetheless, the complemented Δ*ggpS* and the wt carrying the device with both genes under regulation of the high strength promoter show a similar performance in 0 and 3% NaCl. According to the literature, the observed behaviors for these two mutants might have different causes. Indeed, their breakdown in growth in 0% (w/v), compared with the wt in 0% NaCl, could be related, essentially, with the regulatory mechanism of GGPS, as stated above, since the unbinding from nucleic acids occurs in NaCl concentrations of at least 0,6% NaCl[60,80]. While, their breakdown in growth in 3% (w/v), compared with the wt in 0% NaCl, might be associated with NaCl presence and disruption of essential processes.

5% (w/v) NaCl condition:

In 5% (w/v) NaCl, the wt has a breakdown in growth of about 50% compared with the 0% NaCl condition, and is in agreement with the results obtained by Ferreira[56]. The Δ*ggpS* mutant is unable to grow in 5% NaCl, which was expected since GG is essential for *Synechocystis* survival in this range of salt stress[34,47]. The complemented Δ*ggpS* mutant is capable of overcoming ionic stress and therefore grow in 5% (w/v) similarly to the wt. Therefore, the Δ*ggpS* mutant was successfully complemented with the synthetic device carrying *ggpS* under regulation of the stronger promoter ($P_{trc.x.lacI}$). In a similar way, Pade et al (2014) were also able to increase salt tolerance in a *ggpS* knock-out mutant by transforming it with a gene involved in the production of another compatible solute - isofloridoside (compatible solute)[81].

As for the wt carrying the synthetic device with *ggpP* and *ggpS* under regulation of the stronger promoter, its growth pattern is similar to the wt, in the same conditions.

Finally, the wt with both genes under regulation of the medium strength promoter ($P_{trc2.x.tetR}$) has a breakdown in growth of about 75% compared with the wt growing in 0% NaCl.

This is an intriguing result, since it would be expected to have at least the same behavior as the wt and the wt overexpressing both genes under the stronger promoter ($P_{trc.x.lacI}$). With the present results, the observed behavior for this mutant, at 5% NaCl, cannot be explained without additional data e.g., transcriptomic and/or proteomic variation between the tested strains under different NaCl concentrations.

7% (w/v) NaCl condition:

All the *Synechocystis* strains tested at 7% NaCl, namely the wt, the wt pSEVA351 and the two wt overexpressing both *ggpP* and *ggpS* under the medium ($P_{trc2.x.tetR}$) or high strength ($P_{trc.x.lacI}$) promoters, were unable to grow in this condition. To highlight that this concentration was reported to be bactericidal for unacclimated *Synechocystis* cells[56,57]. The inability to grow under such ionic stress results in rapid chlorosis, characterized by the chlorophyll a turnover and consequent photosynthesis impairment which leads to the microorganism death[57]. Nevertheless, it was observed that the chlorosis process is faster for the wt overexpressing both genes under the medium promoter, which can be probably related to its behavior in 5% NaCl. On the other hand, the wt with both genes under the stronger promoter, although being unable to grow, shows a slower chlorosis process. Actually, this mutant's culture remains green along the experiment, while the others turn yellow over time. Possibly, the ionic stress suffered by this mutant is not so severe to cause a bactericidal, but a prolonged bacteriostatic effect instead, due to the stronger promoter present in the synthetic device.

In summary, the tested mutants with synthetic devices did not show a clear improvement in their response against ionic stress. Indeed, their behavior reveals a similar or slower growth compared with the wt. However, the results obtained give relevant information: (I) the GG importance for *Synechocystis* growth at high NaCl concentrations (Δ*ggpS*); (II) the effectiveness of the synthetic device carrying *ggpS* in complementing the *ggpS* knock-out mutant showing that the device is in fact functional; (III) the different phenotypes of the mutants carrying the synthetic devices where only the promoter strength differs; (IV) the slower chlorosis in 7% NaCl for the wt overexpressing both genes under the stronger promoter indicating a possible increased response to high ionic stress; (V) the similar response of the complemented Δ*ggpS* and the wt carrying the device with the stronger promoter in 0 and 3% NaCl compared with to the wt in 0% NaCl and (VI) the fact that along the experiment, the wt maintains a breakdown in growth of about 20% between the 0 and 3% NaCl conditions, for example, while for the complemented Δ*ggpS* and the wt overexpressing both genes under the stronger promoter the breakdown in growth between the 0 and 3% NaCl conditions narrows over time (from about 20% at day 4 to 5% at day 10 on average).

3.3.2 Transcriptional analysis by quantitative real-time PCR (RT-qPCR) of the relative fold expression of *ggpS* and *ggpP* genes

The relative fold expression of *ggpS* and *ggpP* in the wt, the wt overexpressing *ggpP* and *ggpS* with the stronger promoter, Δ*ggpS* and complemented Δ*ggpS* were analyzed by RT-qPCR. These strains were cultured under different NaCl concentrations, in this way, the wt was tested for 0, 3 and 5% (w/v), the Δ*ggpS* for 0 and 3% (w/v) and both the complemented Δ*ggpS* and wt overexpressing *ggpP* and *ggpS* with the stronger promoter for 0 and 5% (w/v).

As shown in Figure 3.7, the wt cultivation under 3 and 5% NaCl leads to an increase of the relative fold expression of both *ggpS* and *ggpP* to levels about 7-9x higher than observed for the wt grown in 0% NaCl. The higher relative fold expression is in agreement with the literature, which indicates an upregulation of the transcription of these genes under ionic stress[63,82,83]. Though, according to this work, the relative fold expression of *ggpS* and *ggpP* in the wt does not change between 3 and 5% NaCl. However, according to Hagemann[62], the *ggpS* transcription rate is intrinsically proportional with the level of external salt stress. Therefore, further studies are required to clarify this situation.

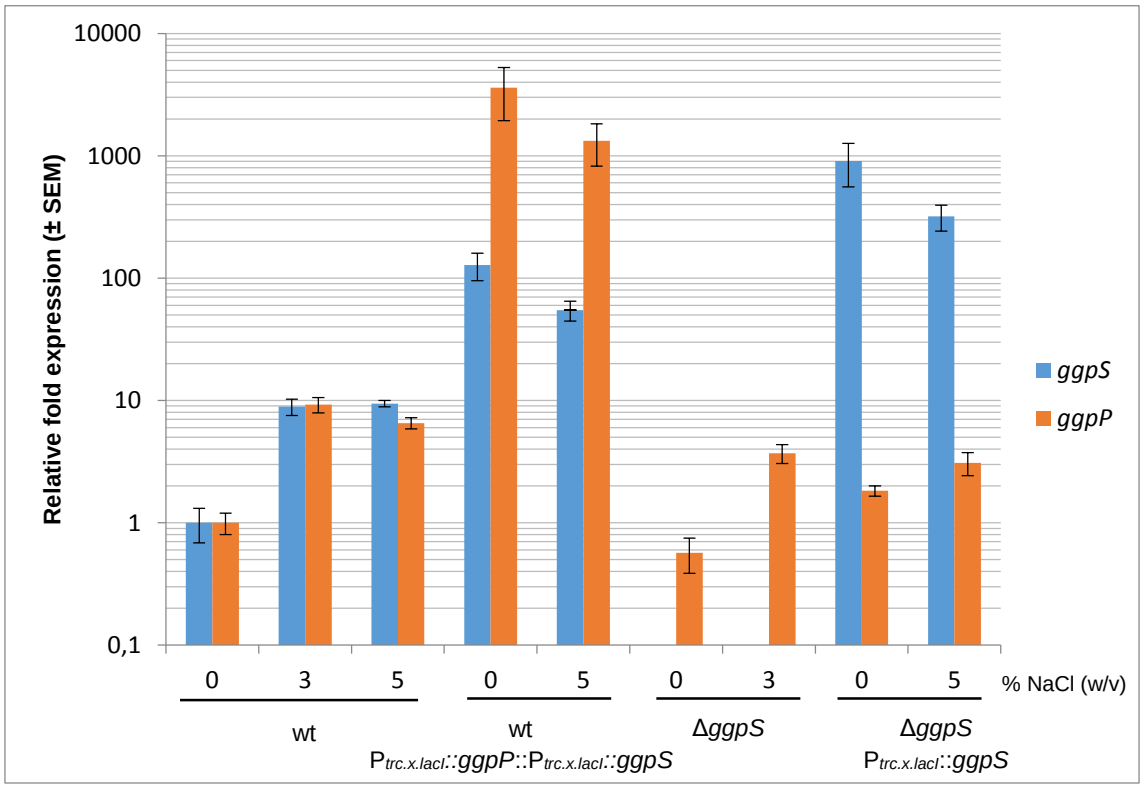

Figure 3.7 Transcriptional analysis by RT-qPCR of *ggpS* and *ggpP* transcripts for the wt, Δ*ggpS*, the complemented Δ*ggpS* and the wt carrying the synthetic device with both ggpP and ggpS under regulation of the stronger promoter under different % NaCl in BG11 medium. Cells were cultivated in a 12 h light (25 µE m^{-2} s^{-1})/ 12 h dark regimen at 30 ºC and collected for RNA extraction at an OD_{730} of ~1. The relative fold expression is normalized for the wt at 0% (w/v) NaCl. Data represents replicate means ± SEM from one experiment.

As for the wt overexpressing *ggpP* and *ggpS* with the stronger promoter, there is a 200-fold increase in *ggpP* transcript and a 7-9-fold increase in *ggpS*, compared with the wt in the same conditions. Therefore, the synthetic device is functional at the transcriptional level, however the difference in relative fold expression between both genes is significant. Indeed, *ggpP* is overexpressed about 20x more than *ggpS*, in this mutant. Possibly, it results from the synthetic device design, where the absence of a transcriptional terminator between both genes could lead to an impairment in the RNA polymerase transcription process from the second promoter. As an hypothesis, a RNA polymerase transcribing from the first promoter may interfere with the transcription process of the second one by limiting access of other RNA polymerases or stalling a RNA polymerase which started in it. Additionally, the relative fold expression for both *ggpP* and *ggpS* seems to be lower when in higher ionic concentrations, possibly resulting from a regulatory mechanism involved in mRNA degradation.

The Δ*ggpS* as expected does not shown any trace of *ggpS* transcript. Interestingly, there is still an increase in *ggpP* transcript in 3% NaCl compared to 0% NaCl, despite *ggpS* absence. This shows that *ggpP* transcription is independent of *ggpS*, probably related with the distant location of these genes in *Synechocystis* chromosome.

The complemented Δ*ggpS* mutant shows an overexpression of *ggpS*, to levels 90 and 30 fold higher than observed for the wt in 0 and 5% NaCl, respectively. Which is at least 5x higher than observed for the wt overexpressing both *ggpP* and *ggpS* with the stronger promoter. This higher transcription enforces the reason given for the expression of *ggpS* in the other synthetic device's mutant and rules out a problem with the promoter itself. Apart from it, the complementation of the Δ*ggpS* mutant is clearly shown.

In short, these results show that (I) there is an increase in the relative fold expression of both *ggpS* and *ggpP* in the wt background, under ionic stress conditions. Additionally, (II) the increased levels of *ggpP* transcription seem independent of *ggpS* expression. (III) As for the complementation of the Δ*ggpS* mutant, as seen in the growth analysis, it was confirmed by the *ggpS* overexpression. Finally, (IV) for the synthetic devices, the higher relative fold expression of selected genes indicate they are functional at transcriptional level.

4. Conclusion

In this study, strategies to improve the cyanobacterium *Synechocystis* sp. PCC 6803 robustness to salinity were implemented applying synthetic biology principles. For this, six different synthetic devices were assembled, carrying either or both the native *ggpP* and *ggpS* genes, that encode the proteins involved in glucosylglicerol synthesis in *Synechocystis*. Two sets of similar synthetic devices were created, differing only in the promoter used. The assembling process was successful and all the generated replicative plasmids transformed into a wild-type and *ggpS* knock-out strains. *Synechocystis* mutants overexpressing the native *ggpP* and/or *ggpS* genes were characterized at a transcriptional and physiological levels.

In summary, the synthetic devices are functional at the transcriptional level and the fact that the mutants carrying them with the stronger promoter show similar breakdown in growth in 0 and 3% NaCl compared to the wt in 0% NaCl is intriguing. Additionally, the fact that along the experiments, their growth breakdown between the 0 and 3% NaCl conditions narrows over time reveals potential of the strategies used. These could be improved, possibly, by combination with other mechanisms involved in halotolerance to assess an increase in robustness. Lastly, the present work allowed to deepen the understanding of this microorganism response to salt stress and reinforce the complexity and difficulty of engineering biological systems due to the dynamics and unpredictability, despite the several advances already made in the synthetic and systems biology fields. But, in the same way, this new output for the synthetic biology community, especially in cyanobacteria, will contribute to turn biology into a more predictable and engineerable field.

5. Future perspectives

In the present work, although the halotolerance was not improved, several other aspects of this work were positive and interesting to follow up. Indeed, as for future perspectives, (I) the mutants carrying synthetic devices will be characterized at the biochemical level. For this, GG quantification, based on nuclear magnetic resonance (NMR), will be performed in cell extracts from selected mutants cultured in different salinities, in order to evaluate if higher levels of GG are being produced. If not, (II) then a proteomic assay to determine GGPS and/or GGPP relative quantities, as well as identifying other possible targets related with salt stress is in consideration. But if it does, then it might mean that instead of GG, another compatible solute is necessary to improve *Synechocystis* robustness under high salt concentration for higher halotolerance. Therefore, (III) the design of synthetic devices carrying heterologous genes involved in for example betaine and ectoine synthesis could possibly overcome this situation. Additionally, (IV) an analysis by RNAseq of *Synechocystis* transcriptome is underway to evaluate how the mutants with synthetic devices respond to salt stress compared with the wt. Likewise, the same analysis will be performed for the *ΔggpS* mutant to see what changes occur in its transcriptome that could allow it to grow under NaCl concentrations considered bactericidal. (V) The effect of sucrose production in the *ΔggpS* mutant will be studied to understand how essential it is for this mutant growth in 3% NaCl. Therefore, a deletion in the *sps* gene, encoding for a protein involved in sucrose synthesis, will be added and the resulting mutant *ΔggpS*/*Δsps* tested at different salinities. Finally, (VI) acclimated cells of the mutants with synthetic devices will be tested in 7% NaCl.

6. References

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