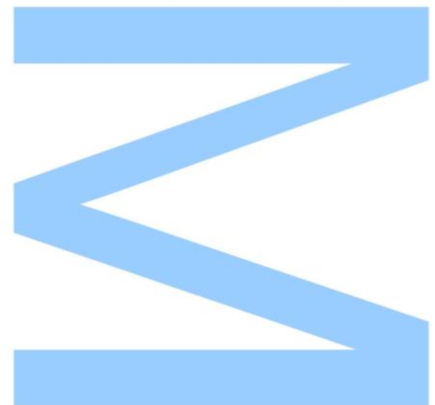
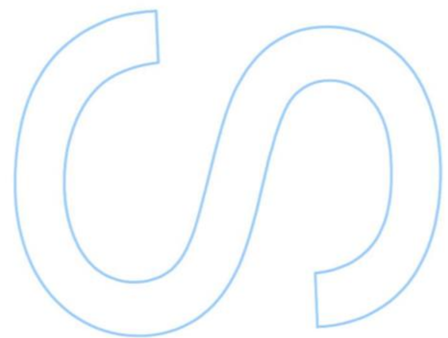
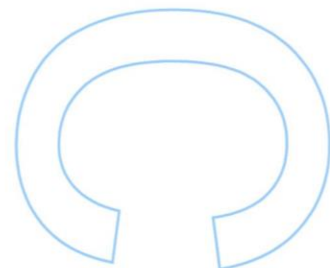
Improvement of *Synechocystis* sp. PCC 6803 halotolerance using a Synthetic Biology approach for the production of compatible solutes







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Todas as correções determinadas
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O Presidente do Júri,

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MSc

“The science of today

is the technology of tomorrow.”

Edward Teller

Agradecimentos

Este projeto resultou de um conjunto de desafios e provas de esforço, os quais não teria conseguido superar sem o apoio dado por várias pessoas, às quais deixo aqui uma nota de agradecimento.

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Abstract

Cyanobacteria are photoautotrophic microorganisms with simple nutritional requirements that can be used as solar-powered cell factories to produce compounds with industrial relevance through carbon-neutral synthesis. Among cyanobacteria, the biological model *Synechocystis* sp. PCC 6803 is the best characterized strain, since it was the first photosynthetic microorganism to have its genome completely sequenced. Moreover, the existence of several genomic, transcriptomic and proteomic data allowed the construction of metabolic models that enable the rational engineering of this organism, which is the basis for synthetic biology approaches. However, there are some drawbacks in using cyanobacteria for bioproduction, namely the high water demand cultivation and susceptibility to contamination/predation by other microorganisms. These obstacles could be overcome by implementing strategies for mass-cultivation in saline waters. However, *Synechocystis* growth is impaired by increasing salt concentrations and thus, the improvement of its halotolerance is required. For this purpose, a synthetic biology approach was used in this work to implement the heterologous production of compatible solutes in *Synechocystis*. Synthetic devices related to the production of betaine and ectoines were assembled and introduced in our chassis. The halotolerance of the mutants was assessed by monitoring the optical density of liquid cultures with different salt concentrations, transcription analysis and compatible solute quantification. The mutants carrying the devices were shown to survive to a salt concentration of 7% (w/v) NaCl, which is above the tolerance limit of non-acclimated cells from the wild-type strain. However, under these conditions, the growth of the mutants was impaired. Moreover, the preliminary results for the mutant carrying the betaine device in a replicative plasmid did not show transcription or betaine production. Another mutant was generated by integrating the betaine device in the *Synechocystis'* genome, aiming to achieve a more stable expression. While the device was confirmed at the transcriptional level, the mutant did not grow at 7% (w/v) NaCl. These results suggest that the introduction of these devices constitutes a metabolic burden to the cells, when exposed to salt stress. Therefore, the redesign of the devices and chassis engineering is required in order to optimize the performance of the mutants in high salinity conditions.

Keywords: *Synechocystis*; synthetic biology; halotolerance; betaine; ectoines; compatible solutes

Resumo

As cianobactérias são microrganismos fotoautotróficos com requisitos nutricionais simples que podem ser usadas como "fábricas celulares" solares para produzir compostos com relevância industrial através da síntese neutra em carbono. De entre as várias espécies de cianobactérias, o modelo biológico *Synechocystis* sp. PCC 6803 é a espécie mais bem caracterizada, tendo sido o primeiro organismo fotossintético a ter o seu genoma completamente sequenciado. Para além disso, a existência de bastantes dados de genómica, transcriptómica e proteómica permitiu a construção de modelos metabólicos que possibilitam a manipulação racional deste organismo, sendo este o principal pilar das abordagens de biologia sintética. Existem, no entanto, algumas desvantagens na utilização de cianobactérias para bioprodução, nomeadamente o elevado consumo de água potável e a susceptibilidade a contaminação/predação por outros microrganismos. Estes obstáculos poderiam ser ultrapassados através da implementação de estratégias para o cultivo em massa em água salgada. O crescimento de *Synechocystis*, porém, é comprometido na presença de sal, sendo por isso necessário aumentar a sua halotolerância. Com esta finalidade usou-se uma abordagem de biologia sintética para implementar a produção heteróloga de solutos compatíveis em *Synechocystis*. Foram construídos módulos sintéticos para a produção de betaína e de ectoínas, que foram introduzidos no chassi. Com o objetivo de caracterizar os mutantes gerados, testou-se a sua halotolerância através da monitorização da densidade ótica de culturas líquidas com diferentes concentrações de sal e realizou-se uma análise de transcrição e a quantificação dos solutos compatíveis. Os mutantes contendo os módulos sintéticos mostraram-se capazes de sobreviver com uma salinidade de 7% (p/v) NaCl, o que é superior ao limite de tolerância da estirpe selvagem. No entanto, nestas condições, o crescimento dos mutantes ficou bastante comprometido. Para além disso, não foi possível detetar transcrição dos genes nem produção de betaína. Foi também gerado um outro mutante em que o módulo sintético para a produção da betaína foi integrado no genoma de *Synechocystis*, com o objetivo de se obter uma expressão mais estável. Neste mutante foi confirmada a transcrição, mas não houve crescimento a 7% (p/v) NaCl. Estes resultados sugerem que a introdução destes módulos constitui um encargo metabólico demasiado grande para as células expostas a stress osmótico. Assim sendo, é necessário redesenhar-se os módulos sintéticos e fazer manipulação do chassi, de forma a optimizar a performance dos mutantes em condições de elevada salinidade.

Palavras-chave: *Synechocystis*; Biologia Sintética; halotolerância; betaína; ectoínas; solutos compatíveis

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List of abbreviations, acronyms and symbols

AhBet - synthetic gene cluster for betaine production, based on *A. halophytica* information.

Amp - ampicillin

Amp^R - ampicillin resistance

asK - aspartate kinase

Betaine - Glycine betaine

Cm - chloramphenicol

Cs(h)ect - synthetic gene cluster for ectoine and hydroxyectoine production, based on *C. salexigens* information.

EctA - L-diaminobutyric acid acetyltransferase

EctB - L-diaminobutyric acid transaminase

EctC - ectoine synthase

EctD - ectoine hydroxylase

GSMT - glycine-sarcosine-N-methyltransferase

GWB - gas wash bottle

Km - kanamycin

Km^R - kanamycin resistance

LB - lysogeny broth

Ma(h)ect - synthetic gene cluster for ectoine and hydroxyectoine production, based on *M. alcaliphilum* information.

NMR - nuclear magnetic resonance

O/N - overnight

PCR - polymerase chain reaction

RBS - ribosome binding site

rpm - rotations per minute

RT-PCR - reverse transcription polymerization chain reaction

SacB - gene that encodes for the enzyme levansucrase, which confers susceptibility to sucrose

SAH - S-adenosyl-L-homocysteine

SAM - S-adenosyl-methionine

SDMT - sarcosine-dimethyl-N-methyltransferase

SW+ - Seawater supplemented with sodium nitrate and dibasic potassium phosphate

SW_0 - Seawater

***Synechocystis* -** *Synechocystis* sp. PCC 6803

UV - ultraviolet

1 Introduction

1.1 Global crisis

Since the middle of the last century, global economy relies to a large extent on fossil carbon sources, with petroleum and natural gas serving as the main raw materials for the chemical industry, allowing the production of a wide range of useful consumer products (*e.g.* plastics, paints, dyes, adhesives) and contributing to a strong economic growth and an improvement of our quality of life.[1–4] However, the Petrochemical Age has also resulted in massive pollution and emission of greenhouse gases responsible for dramatic climate changes.[1] The non-renewable nature of fossil fuels coupled with the continuous growth of the world's population and the ever increasing demands for energy consumption and industrial expansion make our way of living unsustainable.[1,3–6] Biotechnology has shown a promising alternative for chemicals production, as the consequent rapid advances in genetic engineering are turning it into a more suitable approach for mass-production.[2,4,6–8]

1.2 Biotechnology - an alternative to fossil resources

During the last decades, biotechnology has developed powerful tools to increase the efficiency and specificity of microbial fermentation for chemical production.[1] Biotechnology makes use of recombinant microorganisms for the biosynthesis of several chemicals and biomaterials. Compared to chemical conversions, in which high temperatures and pressures are applied, the operating conditions for biological conversions are relatively simpler, reducing energy consumption, waste generation and use of organic solvents and lowering the volume of side products.[2,4,8,9] Additionally, biotechnology can be applied for selective biocatalysis, such as the production of optically pure L(+)- or D(-)-lactic acid, that cannot be achieved by conventional chemical synthesis.[8,10] Biotechnology can also be applied for the heterologous production. Contrarily to native hosts, which usually exhibit slow growth rates and fastidious nutrient requirements, these microbial heterologous expression systems can grow fast at high densities and, therefore, higher titers of the end-product can be obtained.[2,11–14] Bacterial hosts, such as *Escherichia coli*, bacilli (*e.g. Bacillus subtilis*) and lactic acid bacteria (*e.g. Lactobacillus lactis*), as well as filamentous fungi (*e.g. Aspergillus*

oryzae) and yeasts (*e.g. Saccharomyces cerevisiae*) are the main biological models.[11,15] Biotechnology has already established a solid position in producing high-value compounds.[6,7,11,16] *E. coli* has been used for the production of organic acids (*e.g.* succinic acid, lactic acid), alcohols (*e.g.* xylitol, mannitol, sorbitol, glycerol), antibiotics (*e.g.* valinomycin), amino acids (*e.g.* aspartic acid, glutamic acid), hormones (*e.g.* insulin), terpenoids (*e.g.* lycopene) as well as other chemicals.[8,10,14,15,17,18] Despite all these advantages, the production of chemicals using microbial fermentation has some limitations that can compromise bioproduction in large-scale. In general, the raw materials used as carbon source for bioproduction tend to be more expensive than fossil resources, accounting for more than 50% of the total costs. Besides, the energy costs for sterilization, fermenter aeration, stirring and occasional cooling are also considerable. Moreover, the bioproduction processes that rely on fermentation by heterotrophic microorganisms compete with the food sector for freshwater and biomass.[3–5,19] Therefore, bioproduction still has some obstacles to overcome in order to become the major process for the synthesis of chemicals.

1.2.1 Cyanobacteria in biotechnology

The use of photoautotrophic organisms, like cyanobacteria, would offer a better alternative for mass-production, given that carbohydrate feedstock costs would be eliminated.[20] Cyanobacteria, one of the largest and most important groups of bacteria on Earth, are photoautotrophic prokaryotes capable of performing oxygenic photosynthesis. They are capable of capturing and converting atmospheric carbon dioxide into organic compounds by using sunlight as energy source and water as electron donor.[21,22] Cyanobacteria display diverse morphology, including unicellular, filamentous and colonial forms. Some filamentous strains undergo cellular differentiation to develop heterocysts - cells specialized in N_2 fixation.[23] Cyanobacteria also occupy diverse aquatic and terrestrial habitats, even extremely harsh environments, where no other microalgae can be found.[23,24]

In the last decades, cyanobacteria have been increasingly studied because of their potential to be used as solar-powered cell factories for biotechnological applications. Their photoautotrophic metabolism, along with the availability of techniques for genetic manipulation and the capacity of some strains for natural transformation, makes them good candidates for bioproduction, as their utilization would allow the sustainable production of

numerous added-value organic compounds through carbon-neutral biosynthesis.[25–30] Besides, given the recent concerns on global warming and climate change, the application of cyanobacteria to biotechnological purposes constitutes a more eco-friendly approach for chemical production.[29] Cyanobacteria are also a source of bioactive metabolites with biotechnological and industrial relevance.[22,25,31] Currently, some bioindustrial processes already employ cyanobacteria and others are being implemented, namely the production of biofuels, biomaterials and fertilizers, as well as in bioremediation. Some strains are also being used as sources of protein, vitamins and antioxidants.[22,25,26,31] However, the susceptibility of cyanobacterial monocultures to contamination and predation by other microorganisms, and their high water consumption rates can difficult their application in biotechnological processes.[32,33] Besides, the usage of water in the operating systems and cleaning procedures of the reactors, as well as in the culture processing, is substantial.[33–35] The cultivation in large-scale would severely compete with the population's needs for freshwater, even though the use of cyanobacteria in bioindustrial applications results in a smaller impact than the use of most plant crops as raw-material for chemical/microbial processing.[35] To overcome this hurdle, the freshwater used for the cultivation of cyanobacteria could be replaced by other resources, like seawater, significantly reducing the consumption of freshwater and eliminating the need for nutrient supplementation, except for nitrates and phosphates.[35–37] Furthermore, the usage of seawater, either at its average salinity (3.5% (w/v) of salt) or at higher salt concentrations, could substantially minimize the risk of contamination by more halointolerant competing organisms.[32,38] Although cyanobacteria can tolerate a wide range of salinity levels, the growth rate or even survival of some genetically modified species is affected by the osmotic stress imposed by those environments. Therefore, the growth in environments with high salt concentrations could be resolved by using a synthetic biology approach to improve the salinity robustness.[39]

1.3 Synthetic Biology

Synthetic Biology is a new research field within the life sciences. Originated in the 21st century, it comprises the design and construction of new biological parts and systems, and the re-design of those observed in nature, in order to improve their functions. Through systems biology and synthetic biology approaches, scientists aim to better understand the genomic and metabolic networks of the cell and to use that information to generate

engineered biological systems capable of improved functionalities.[40–45] Synthetic Biology relies on three fundamental principles: decoupling, standardization and abstraction.[44] Decoupling is the notion of decomposing a complicated issue into many simpler problems that can be fixed independently, in such way that the results can be combined to resolve the entire problem.[44] Applying this notion to Biology allows the separation of complex biological systems into simpler genetic parts. One of the biggest efforts in designing such standardized biological parts was performed by Knight in 2003, with the creation of BioBricks™. The BioBricks are standardized biological parts, like promoters, ribosome binding sites and transcriptional terminators, with specific functions and predicted behaviours. These components can be assembled into devices and integrated in a chassis in order to perform a specific function.[46,47] These parts can then be modified through genetic engineering in order to present a more predictable behaviour when assembled into synthetic devices and implemented in a microbial host - the chassis.[41,46,48] Every day, new BioBricks are uploaded into different public databases, such as the Registry of Standard Biological Parts, from the BioBrick's Foundation, which has over 20,000 documented parts. These parts can be retrieved and assembled using the BioBrick assembly standard to build complex synthetic biological systems.[41,46,47,49,50] Conventional Molecular Biology cloning techniques are used to assemble these biological components according to the BioBrick Assembly Standard RFC10. As shown in Figure 1, each biological part contains a prefix, with restriction sites for the enzymes *Eco* RI (E) and *Xba* I (X), and a suffix, with restriction sites for the enzymes *Spe* I (S) and *Pst* I (P). It is important to note that the biological parts cannot have restriction sites for these four enzymes in their sequences. Cutting a plasmid that contains “part A” with E and S and a plasmid that contains “part B” with E and X originates two molecules that can be combined into a larger plasmid with both parts and the same restriction site configuration as the parent biological components.[46] This can only be achieved because the ligation of the overhangs generated by the *Xba I* and *Spe* I restriction enzymes forms a “scar” that is not recognisable by any restriction enzyme.

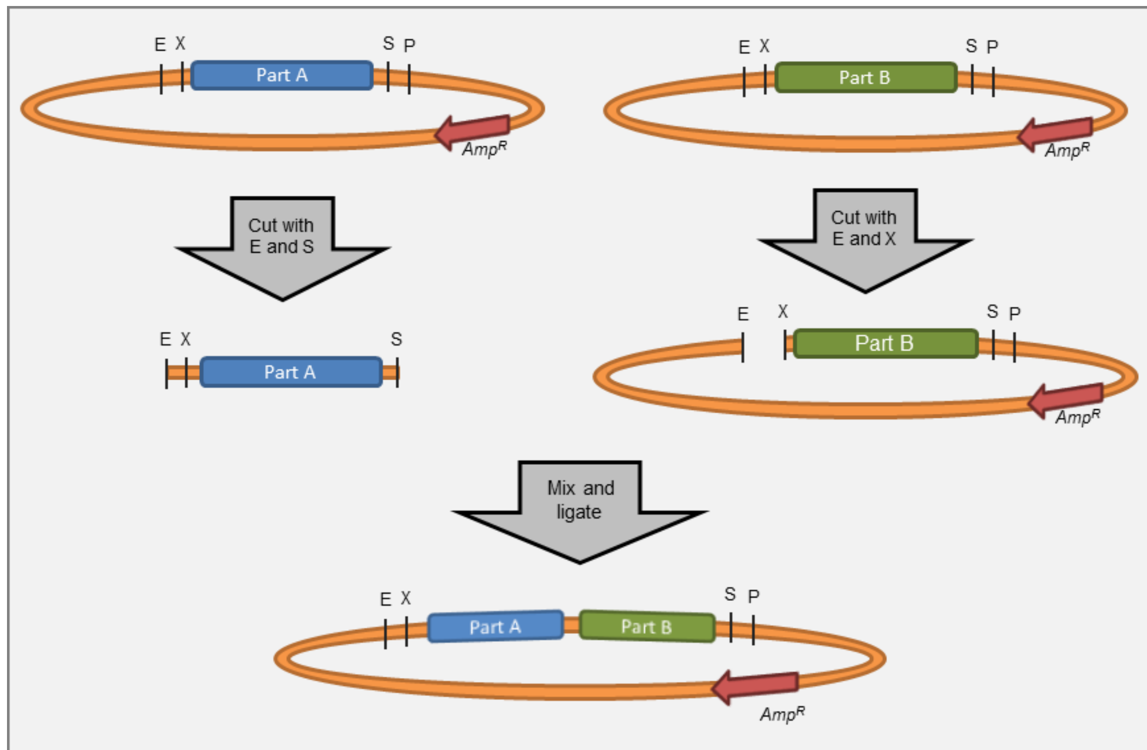


Figure 1 - Schematic representation of the Biobrick Assembly Standard RFC10. Each Biobrick^{TM} consists of a genetic element flanked by a prefix (upstream), with restriction sites for the enzymes *Eco*RI (E) and *Xba*I (X), and a suffix (downstream) with restriction sites for the enzymes *Spe*I (S) and *Pst*I (P). These components can be assembled into more complex devices using molecular cloning techniques. X and S generate compatible overhangs and the ligation of these two overhangs produces a scar that is not recognisable by any enzyme. Thus, the result of any combination of Biobrick components is a construct with the same restriction sites as the parent parts. Adapted from Knight (2003).[46]

Thus, this model of assembly of biological parts constitutes a standard system for the construction of synthetic devices, given that the combination of different BioBricks results in a larger molecule that is itself a BioBrick.[50] The third principle of synthetic biology is abstraction and can be applied in two ways: first, the information describing the functions of different biological parts needs to be hierarchically organised; second, the parts and devices that constitute the engineered biological systems need to be redesigned so they become easier to model and to combine.[44] Taking these principles in account, the assembly of the engineered biological systems could be performed in an analogous manner to electric circuits. That is, well-characterized, modular genetic components could be used to assemble a biological system that would translate to an outcome completely independent from other cellular processes.[41,43]

1.3.1 Cyanobacteria in synthetic biology

Most of the work in Synthetic Biology has been done in heterotrophic microorganisms. There is already a substantial amount of biological parts and synthetic devices developed for the Gram-negative bacterium *E. coli* and for the Gram-positive bacterium *B. subtilis*.[51,52] Despite showing some orthogonality, the performance of many available parts and devices differs significantly when applied in other hosts.[52] There is still a great disparity between the amount of work done with these chassis and the work done with cyanobacteria. Nonetheless, a lot of effort is being done in order to make the engineering of cyanobacteria easier and during the last years many tools and parts have been developed for these chassis, such as synthetic promoters (*e.g.*P_{trc1O}, P_{psbA2*}), ribosome binding sites (*e.g.* RBS*), reporter devices (*e.g.* *luxAB*), replicative vectors (*e.g.* pSEVA351) and integrative plasmids targeting cyanobacterial neutral sites - loci that can be disrupted without compromising the viability of the cell or causing any distinguishable phenotype.[28,52–57]

Among cyanobacteria, *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) is one of the most studied species as a photoautotrophic biological model for synthetic biology approaches.[26,28,52] *Synechocystis* was the first cyanobacterium to have its genome completely sequenced and annotated.[58] This unicellular cyanobacterium possesses 12 copies of its medium-size genome (3.6 Mbp), as well as seven endogenous plasmids.[59] Its competence for natural transformation (it has a high frequency of homologous recombination) allied to a set of available genetic tools and genome-scale metabolic models make this strain of great biotechnological interest.[26,28,52,55,60–62] All the previously mentioned features make *Synechocystis* a good candidate for biotechnological purposes. Using a synthetic biology approach, the genetic engineering of this strain would allow the creation of a more robust photoautotrophic chassis suitable for large-scale bioproduction.[26,29]

1.4 Response mechanisms to osmotic stress

High and changing salt concentrations represent major abiotic factors that limit the growth of microorganisms.[39] All organisms that inhabit high salt environments need to adjust their cytoplasm in order to maintain their intracellular content in osmotic equilibrium with the outside medium. Moreover, in environments of changing salinity there is also the need to osmotically adapt to fluctuations in the external salt concentration.[63] A non-adapted organism

exposed to an environment with higher salt concentration must increase its osmolarity in order to prevent water loss and, consequently, shrinkage and loss in turgor pressure.[38,39,64,65] Besides, the direct exposure to high concentrations of inorganic ions (especially Na^+) also leads to toxic effects on cellular metabolic processes.[38,39] Two different mechanisms were developed by halophilic and halotolerant prokaryotes for osmotic adaptation: the “salt-in” and the “salt-out” strategies.[64,65] Some specialized prokaryotes, like halophilic *Archaea* and some halophilic anaerobic *Bacteria*, rely on the salt-in strategy, accumulating inorganic ions to counteract the osmotic pressure exerted by the surrounding environment and assure water retention and turgor pressure. Usually, Na^+ is the dominant cation in the extracellular medium but cells accumulate K^+ in their cytoplasm, instead. These potassium and sodium concentration gradients over the cytoplasmic membrane are achieved at the expense of energy, via different ion pumps and other transport proteins. This approach requires special modifications in the enzymatic machinery for cells to withstand exposure to high ionic strength.[38,63] Moreover, the adaptability of cells to changing conditions is limited, as the enzymes and structural proteins are dependent on the continuous presence of high salt concentrations for activity and stability.[38,39,63,65] The other halophilic and halotolerant microorganisms make use of the salt-out strategy, accumulating organic compounds known as *compatible solutes*, to achieve an osmotic equilibrium with the extracellular environment.[39,63] Compatible solutes are polar, low-molecular-weight, highly water-soluble organic compounds that cells can accumulate, even in the molar concentration range, without disturbing the cellular metabolism. These solutes include sugars (*e.g.* trehalose and sucrose), polyols (*e.g.* glucosilglycerol), amino acids (*e.g.* proline and glutamate) or amino acid derivatives (*e.g.* betaines and ectoines). Most of them are uncharged or zwitterionic at physiological pH.[64,65] In the salt-out strategy, the low intracellular ionic concentrations are obtained by active extrusion and the osmotic equilibrium is provided by these organic solutes, either synthesized *de novo* or captured from the external medium.[65] Furthermore, this strategy allows the increase of the osmolarity inside the cells, without the accumulation of inorganic ions and thus there is no need for specific adaptations of proteins to work at high ionic strength.[63,65] Therefore, this halophiles are more adaptable, not only because they display tolerance to a wider range of salt concentrations, but also because they can grow at low salt conditions, given that the compatible solutes concentration inside the cells can be regulated.[64,65]

1.4.1 Halotolerance in cyanobacteria

Cyanobacteria can be found in different environments with a wide range of salinities, depending on the type of compatible solutes they accumulate. Freshwater cyanobacteria can tolerate up to 3.5% (w/v) of NaCl by accumulating sucrose and/or trehalose; moderately halotolerant bacteria can tolerate up to about 10% (w/v) NaCl by accumulation of glucosylglycerol; and halophilic bacteria can tolerate up to 17.5% (w/v) NaCl by accumulating glycine betaine or glutamate betaine to adjust their osmolarity.[39,63] The model cyanobacterium, *Synechocystis*, relies on the biosynthesis of sucrose and glucosylglycerol to maintain its osmotic pressure in hyperosmotic media. These two compatible solutes are responsible for the tolerance of this model strain to salinities up to 6% (w/v) NaCl.[66] However, *Synechocystis* suffers a substantial decrease in growth when exposed to these conditions.[67–69]

1.5 Improvement of compatible solute production

As discussed above, mass-cultivation of a phototrophic chassis for biotechnological purposes using seawater would constitute an attractive alternative, as freshwater is becoming a limiting resource in our planet.[38] For this purpose, the freshwater cyanobacterium *Synechocystis*, could be engineered to increase its halotolerance by increasing the production of native compatible solutes or the expression of genes related to the production of heterologous compatible solutes.[26,38,65]

1.5.1 Betaine and ectoines as chemical chaperones

It has been shown that extreme halophiles synthesize betaines or ectoines as compatible solutes to cope with the high ionic strength.[70,71] These molecules act as chemical chaperones, stabilizing the cells and their biochemical machinery and protecting them against the harsh conditions.[69] Their large hydration shells allow the stabilization of the water fraction that solvates the macromolecular structures due to the preferential exclusion from the immediate surface of the macromolecules, which favours their folded/native state.[64,72]

Glycine betaine (*N*,*N*,*N*-trimethylglycine; Figure 2), hereafter referred to as betaine, is one of the most common and most potent compatible solutes.[73–75]

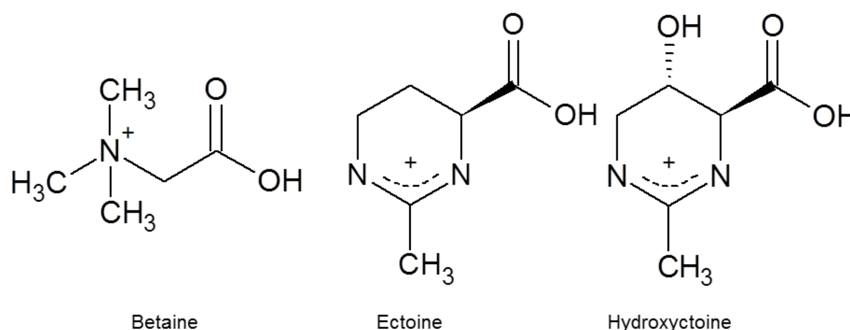


Figure 2 - Chemical structure of betaine and ectoines. The structures were designed using the ChemSketch 11.02 software from Advanced Chemistry Develompent, Inc. (ACD/labs).[76]

Betaine is a zwitterionic quaternary ammonium compound and a methyl derivative of the amino acid glycine.[75] Many halophilic microorganisms are capable of accumulating this compatible solute in concentrations well above 1 M.[73,77] However, *de novo* synthesis of betaine is not common, being confined to phototrophic eubacteria. The other organisms accumulate this compound by uptake, as it is released into the external environment either by the photoautotrophic microorganisms upon dilution stress, by decaying of plants and animals, or by the excretion fluids of mammals (*e.g.* urine).[72,78]

Aphanothece halophytica (originally called *Synechococcus* sp. PCC 7418) is a unique halotolerant cyanobacterium that can grow in extreme environments with salinities near NaCl saturation.[68,75,79] This cyanobacterium produces betaine as primary compatible solute.[70] Betaine is usually synthesized via a two-step oxidation of choline. However, *A. halophytica* produces this compound via a three-step methylation of glycine (Figure 3). This biosynthetic pathway involves two N-methyltransferases: glycine-sarcosine-N-methyltransferase (GSMT; EC 2.1.1.156) and sarcosine-dimethylglycine-N-methyltransferase (SDMT; EC 2.1.1.157). GSMT catalyses the methylation of glycine and sarcosine, while SDMT specifically catalyses the methylation of dimethylglycine to betaine. On each methylation reaction, one molecule of S-adenosyl methionine (SAM) is used as methyl group donor.[75,80] Several studies already showed that the heterologous expression of the genes that encode for the enzymes of this biosynthetic pathway results in betaine accumulation and improved osmotic tolerance.[73–75,81]

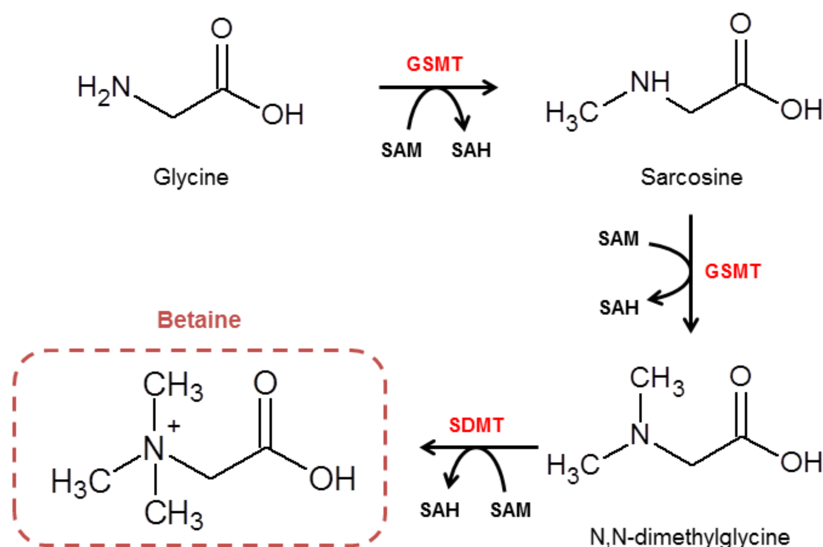


Figure 3 - Biosynthetic pathway of betaine in *Aphanothece halophytica*. GSMT - glycine-sarcosine-methyltransferase; SDMT - sarcosine-dimethylglycine-methyltransferase; SAM - S-adenosyl-methionine; SAH - S-adenosyl-homocysteine. Structures were designed using the ChemSketch version 11.02, from ACD/labs.[76]

Ectoine (1,4,5,6-tetrahydro-2-methyl-4-prymidinecarboxilic acid) and the hydroxylated derivative hydroxyectoine (1,4,5,6-tetrahydro-2-methyl-5-hydroxy-4-pyrimidinecarboxilic acid) can be defined as heterocyclic amino acids and as hydrogenated pyrimidine derivatives (Figure 2). Ectoines, like betaine, are among the most widely found compatible solutes.[69,82–87] Aside from their role in osmoadaptation, ectoines (especially hydroxyectoine) are also able to stabilize and protect proteins, DNA, membranes and whole cells against freezing, desiccation and high temperatures.[69,83,85,87] The biosynthetic pathway of ectoines is represented in Figure 4. It starts from L-aspartate-β-semialdehyde, an intermediate of the biosynthetic route of amino acids that result from the phosphorylation of L-aspartate by an aspartate kinase (asK). From this intermediate, the ectoine synthesis occurs in three steps: first, L-aspartate-β-semialdehyde is converted into L-diaminobutyric acid by the enzyme L-diaminobutyric acid transaminase (EctB; EC 2.6.1.76); then, the L-diaminobutyric acid is acetylated to N-γ-acetyldiaminobutyric acid by the enzyme L-diaminobutyric acid acetyltransferase (EctA; EC 2.3.1.178). Finally, the cyclic condensation of N-γ-acetyldiaminobutyric acid into ectoine is catalysed by the enzyme ectoine synthase (EctC; EC 4.2.1.108). Hydroxyectoine is synthesised via the reversible stereospecific hydroxylation of ectoine, catalysed by the enzyme ectoine hydroxylase (EctD; K10674).[69,82,88,89] The ectoine synthesis genes (*ectA*, *etcB* and *ectC*) are conserved among the ectoine-producing

bacteria and are usually arranged as an operon that, in most bacteria, also includes the *ectD* gene.

C*hromohalobacter salexigens* and *Methylomicrobium alcaliphilum* 20Z are the most studied halophilic microorganisms regarding ectoine and hydroxyectoine biosynthesis. *C. salexigens* is a halophilic γ-Proteobacterium that shows great versatility regarding salt tolerance. This species can grow in environments with NaCl concentrations ranging from 6 to 23% (w/v) NaCl, being its optimal growth condition at 8% (w/v) NaCl.[69,82,84] *M. alcaliphilum* 20Z is another halophilic bacterium that is adapted to grow in high-salinity ecosystems. This haloalkaliphilic metanotrophic species possesses the genes involved in the biosynthetic pathways of ectoines combined in a single cluster - *ectABC-asK-ectD*.[69,88,90,91]

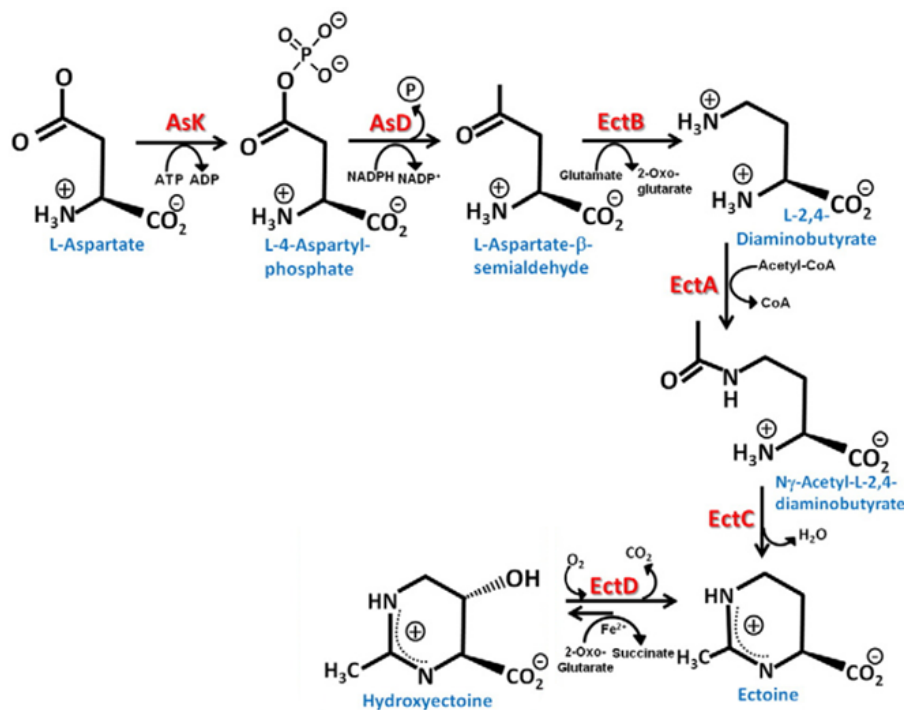


Figure 4 - Biosynthetic pathway of ectoines in *Chromohalobacter salexigens*. AsK - aspartate kinase; AsD - L-aspartate-β-semialdehyde dehydrogenase; EctB - L-diaminobutyric acid transaminase; EctA - L-diaminobutyric acid acetyltransferase; EctC - ectoine synthase; EctD - ectoine hydroxylase. Adapted from Pastor et al. (2010).[82]

1.5.2 Betaine and ectoines as high-value compounds

Betaine and ectoines are also relevant as added-value compounds. These compounds have shown applicability in molecular biology techniques, as well as in the nutraceutical, pharmaceutic and cosmetic industries.[84,87,92,93] Betaine was shown to improve DNA-polymerase-based assays (such as PCR amplification and DNA sequencing) and to promote the synthesis of longer cDNA products, due to its ability to stabilize the structure of nucleic acids.[92–94] Betaine has also medicinal relevance, playing an important physiological role as methyl donor, as well as nutritional relevance for livestock and aquaculture.[75,95] Ectoines have enormous commercial interest, due to their protective effect on biomolecules.[88,96] Studies have shown that ectoine has a very effective inhibitory effect on the genesis of amyloid fibres, being potential candidates for treating neurodegenerative diseases.[97] Ectoines are also of most importance to cosmeceutical industry. Their moisturizing properties are patented and several cosmetic products for prevention of skin damage have ectoines on their formulations.[96,98] Furthermore, ectoine has shown to protect the skin from cellular damage caused by exposure to ultraviolet (UV) radiation and sunscreens containing this compound are being tested.[99,100] But contrarily to betaine, they can only be produced via biotechnological processes.[88,96] Ectoines are produced via a large-scale, fermentation procedure called “bacterial milking”, where cyclic changes in the salt concentrations of the medium allow continuous ectoine production: first, the cells are exposed to salt stress, which leads to the production of ectoines; then, an hyposmotic stress is applied, forcing the cells to release the compatible solute to the medium; the ectoines are isolated from the medium and the cells are grown again under salt stress.[82,84,85]

1.6 Objectives

The main goal of this project is the construction of a photoautotrophic chassis, based on the unicellular cyanobacterium *Synechocystis* sp. PCC 6803, with increased robustness towards salinity, by the heterologous production of compatible solutes. For that purpose, we will:

i. assemble synthetic devices related the production of betaine, ectoine and hydroxyectoine;
ii. introduce the synthetic devices into *Synechocystis* wild-type;

iii. assess the halotolerance of the generated mutants, as well as their characterization at the transcription and compatible solute levels;

iv. generate a knockout mutant lacking the production of compatible solutes, to be used as a chassis for the implementation of heterologous pathways.

2 Materials and Methods

2.1 Bacterial strains and standard growth conditions

The cyanobacterium *Synechocystis* sp. PCC 6803 (obtained from the Pasteur Culture Collection of cyanobacteria, Paris, France) wild-type and mutant strains were kept in BG11 medium[101] at 30 ºC and a 12 h light (25 μE m^{-2} s^{-1}) / 12 h dark regimen. Cosine-corrected irradiance was measured with a quantum meter (Dual Solar/Electric Quantum Meter - Spectrum Technologies Inc., Aurora, IL, USA). When cultured in solid medium, BG11 supplemented with 1.5% (w/v) Difco® Agar Noble, 0.3% (w/v) sodium thiosulfate and 10 mM TES–KOH buffer (pH 8.2) was used. The strain *E. coli* XL1-Blue (Stratagene, La Jolla, CA, USA) was used for molecular cloning purposes and cultured at 37 ºC in selective Lysogeny Broth (LB) medium [102]. 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}; Sigma-Aldrich, St. Louis, MO, USA), ampicillin (Amp, 100 μg mL^{-1}; Sigma-Aldrich), or kanamycin (Km, 50 μg mL^{-1} in LB; 25 to 500 μg mL^{-1} in BG11; Sigma-Aldrich).

2.2 Synthetic devices assembly

The genes *gsmt* and *dmt* from *Aphanothece halophytica* were used as base for the heterologous production of betaine - hereafter this gene cluster is going to be referred as AhBet. A gene that encodes for an S-adenosylmethionine synthase (*metX*) was also included in this synthetic device, to prevent the shortage of the cofactor. For the heterologous production of ectoine and hydroxyectoine, two gene clusters were designed based on the *ectA*, *ectB*, *ectC* and *ectD* genes, either from *Chromohalobacter salexigens* or from *Methylomicrobium alcaliphilum* 20Z - the gene clusters were designated Cs(h)ect and Ma(h)ect, respectively. The Ma(h)ect device also contains a gene encoding for an aspartate kinase based on the *asK* gene present in the *ectABCD* operon of *M. alcaliphilum*. The open reading frames (ORFs) encoding proteins involved in the synthesis of compatible solutes were codon-optimized for *Synechocystis* using Gene Designer (DNA 2.0, Newark, CA, USA) and restriction sites incompatible with the RCF10 BioBrick standard were eliminated. Each ORF is preceded by the BioBrick™ RBS (BBa_B0030), retrieved from the Registry of

Standard Biological Parts[103]. These sequences were synthesized, and then assembled with two different constitutive promoters - P_{trc1O} and P_{psbA2*} (figure 5).

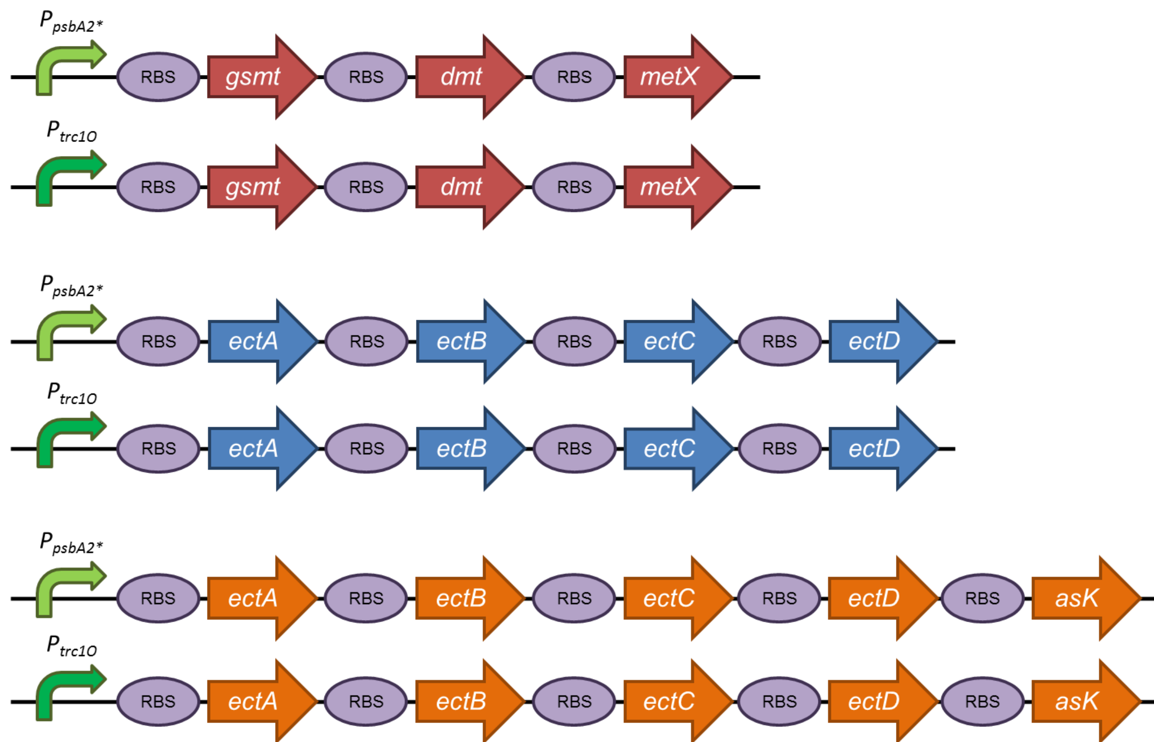


Figure 5 - Schematic representation of the synthetic devices carrying the genes for the production of betaine or ectoines. Red arrows represent ORFs related to the production of betaine, based on genes from *A. halophytica*; blue and orange arrows represent ORFs related to the production of ectoines, based on genes from *C. salexigens* and *M. alcaliphilum* 20Z, respectively; circles represent ribosome binding sites (RBS); curved arrows represent promoters.

The cloning process was performed according to the Biobrick Assembly Standard RFC10, as follows: (i) for upstream assembly, the vector containing the promoter was digested with *Eco*RI and *Spe*I: and the fragment was ligated to the recipient vector digested with *Eco*RI and *Xba*I; (ii) for downstream assembly, the coding sequences digested with *Pst*I and *Xba*I were ligated to the recipient vector digested with *Pst*I and *Spe*I (Table 1). The recipient vectors were treated with FastAP Thermosensitive Alkaline Phosphatase (Thermo Fisher Scientific, Madison, WI, USA) prior to the ligation reactions.

To transform *Synechocystis*, (iii) the synthetic devices, digested with *Pst*I and *Xba*I, were cloned either into the replicative vector pSEVA351 or the integrative vector pSN15K, cut with *Pst*I and *Spe*I, with chloramphenicol or kanamycin as antibiotic markers, respectively (Table 1).

Table 1 - List of plasmids used in this work.

Plasmid	Selection marker	Purpose / Description	Reference
pJ201_P_{psbA2*}	Km^R	Plasmid with P_{psbA2*} promoter	DNA 2.0
pSB1A2_P_{trc1O}	Amp^R	Plasmid with P_{trc1O} promoter	
pBSK_Ma(h)ect	Amp^R	Plasmid carrying the *ectABC-ectD-asK* gene cluster based on *M. alcaliphilum* genes	
pSB3K3_Cs(h)ect	Km^R	Plasmid carrying the *ectABC-ectD* gene cluster based on *C. salexigens* genes	
pSEVA351	Cm^R	Replicative vector for *Synechocystis* transformation	SEVA 2.0 [57]
pSEVA351_P_{trc1O}::AhBet	Cm^R	Synthetic device for betaine production	Our lab
pSN15K	Km^R	Integrative vector for *Synechocystis* transformation	
pGD*sps*	-	Generation of the *Synechocystis* double knockout mutant	
pGD*ggpS*.KS	Km^R and SacB		
pGD*ggpS*	-		

The DNA digestions were performed using the FastDigest™ Restriction Enzymes (Thermo Fisher Scientific) according to the manufacturer's specifications. Once assembled, the devices were confirmed by restriction with the appropriate enzymes and/or PCR, followed by DNA sequencing (STAB VIDA, Caparica, Portugal).

2.3 Agarose gel electrophoresis

Nucleic acids electrophoresis analysis was performed in 1% (w/v) agarose gels, with 1 x TAE buffer[104], stained with 0.5 µg mL^{-1} of ethidium bromide or 30 µL of Green Safe Premium (NZYTech, Lisbon, Portugal) per litre of gel. Bands were visualized under ultra-violet (UV) light with a Gel Doc™ XR+ Imager (Bio-Rad, Hercules, CA, USA). The GeneRuler™ DNA Ladder Mix (Thermo Fisher Scientific) was used as molecular weight marker.

2.4 DNA purification and quantification

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

2.5 Polymerase chain reaction (PCR)

The PCR assays were performed using the GoTaq® DNA polymerase (Promega, Madison, WI, USA) or Phusion® High-Fidelity DNA polymerase (Thermo Fisher Scientific), following the manufacturer's instructions. In each PCR reaction (20 µL), the magnesium chloride ($MgCl_2$) and deoxyribonucleoside triphosphate (dNTP) concentrations were 1.5 mM and 0.2 mM, respectively. The oligonucleotides (Table 2) were used at a final concentration of 0.5 µM. For the reactions using GoTaq DNA Polymerase, 1U of enzyme was used and the PCRs were performed in a thermocycler (Bio-Rad) using the following profile: 3 minute denaturation step at 95 ºC; followed by 30 cycles of 30 seconds at 95 ºC, 30 seconds at the annealing temperature and an extension step at 72 ºC (1 minute for every kbp of the target DNA); a final extension step at 72 ºC for 5 minutes. As for the reactions using the Phusion DNA polymerase, 0.4 U of enzyme were used and the PCR profile was: 30 seconds denaturation step at 98 ºC; followed by 30 cycles of 10 seconds at 98 ºC, 30 seconds at the annealing temperature and 15 seconds per kbp at 72 ºC; a final extension step at 72 ºC for 7 minutes. The annealing temperatures were calculated using the Tm Calculator Thermo Fisher Scientific Web Tool (ThermoFisher Scientific) and the information provided in Table 2.[105]

2.5.1 Confirmation of transformants by colony PCR

For confirmation of *Synechocystis* or *E. coli* transformation, colony PCR was performed. Cells from each colony were transferred to 20 µL of water (molecular biology grade; Sigma-Aldrich) and incubated at 95 ºC for 10 minutes followed by 2 minutes of centrifugation at 14100 x *g*. Finally, 2 µL of the supernatant were used in the PCR reaction. For confirmation of *Synechocystis* mutants in liquid culture, DNA extraction was performed using 2 mL of culture centrifuged at 14100 x *g* for 2 minutes and washed with 500 µL of dH_2O. Then, the cells were centrifuged again at 14100 x *g* for 1 minute, resuspended in 150 µL of dH_2O and 1 µL of RNase solution (20 mg mL^{-1}, Sigma-Aldrich) and 0.1 g of 425-600 nm glass beads (acid washed, Sigma-Aldrich) were added. Cells were disrupted by two cycles of vigorous vortexing for 1 minute followed by incubation on ice for 1 minute. Finally, the cells were centrifuged at 14100 x g for 2 minutes and 100 µL of the supernatant was kept. PCR reactions were performed using 4 µL of supernatant as template.

Table 2 - List of primers used in this project.

Primer Name	Sequence 5' → 3'	Tm (ºC) Phusion	Tm (ºC) Taq	Purpose
PUC_F	AGGGTTTTCCCAGTCACGAC	65.6	59.1	Confirmation of the synthetic devices and transformants
PUC_R	ACACAGGAAACAGCTATGAC	57.3	53.2	
pJ201_Fwd	CTCGAAAATAATAAAGGGAAAATCAG	63.0	51.3	
pJ201_Rev2	CGAACTCAGAAGTGAAACGCCG	70.2	60.5	
VF2	TGCCACCTGACGTCTAAGAA	63.9	57.3	
PS1	AGGGCGGCGGATTTGTCC	71.9	63.7	
PS2	GCGGCAACCGAGCGTTC	70.9	63.2	
Ahbet_RI	CCGGCTTCAATTAGTCG	59.9	51.8	
Cs_hect_RI	GCCACAAGAAATAGGTATCGGGC	68.4	60.2	
Ma_hect_RI	GCTTTCAGCATACGAGTGGC	65.2	58.5	
N15.3O	CTAAACTTACGGCATTGGCATCAACGGGAG	76.4	65.0	
G-metX Fi	CCCAAGTTTCCATCCTGTACG	-	57.2	RT-PCR
G-metX Ri	GGTCTACCTTGGTGGGATCC	-	59.4	
SD-metX Fi	TGAGACACCGGAGCTAATGC	-	53.9	
SD-metX Ri	AAGCGGGTCTTGTCAGTAGGC	-	61.5	
SD-DMT Fi	TGCTAAGCGGGTACTAGATGC	-	58.4	
SD-DMT Ri	CCTTCGTCCAAGATCAAATCG	-	55.1	
SD-GSMT Fi	ATTTATGATGCCTCCGTGCG	-	57.1	
SD-GSMT Ri	GCTTCTTCCATCACTTTCCGC	-	58.7	
rnpBF1	CGTTAGGATAGTGCCACAG	-	53.8	
rnpBR1	CGCTCTTACCGCACCTTTG	-	58.4	
sps.5o	CGCCGCTCGAGGCAATGAATTGGGCGGTGGAATAG	-	69.6	Generation / confirmation of the double knockout mutant
sps.3o	AAGGTTTCTCGCCACAATAGGTCAGGCTGGCATAG	-	66.2	
ggpS_Fi	CGTGGGCACCAATCCGGCAAATATC	-	65.1	
ggpS_Ri	GGTTAGTCAACACCGCATCGGGTAG	-	63.6	
ggpS.5i	GATTACAACCGGTTGTAATCACGGCTA	69.8	-	
ggpS.5o	GCTGGCTCGACACCGTAGGGCAG	76.6	-	
SacBint_Fwd	TTGACGGTGACGGAAAAACG	-	57.6	
SacBint_Rev	CGGAAGAATGATGTGCTTTTGCC	-	59.0	
KmR_Rev	CAAACCCGGGCGATTTACTTTTCGACCTC	-	65.7	

2.6 DNA ligation and *E. coli* XL1-Blue transformation

DNA ligations were performed with the T4 DNA Ligase (Thermo Fisher Scientific) according to the manufacturer's instructions. The vector to insert ratio used was 1 : 3 and the ligation reactions were incubated O/N at 22 ºC. The enzyme was then thermally inactivated by incubation at 65 ºC for 10 minutes. The assembled plasmids were then transformed into chemically competent *E. coli* XL1-Blue. Aliquots of 100 μL of cells were thawed on ice prior

to the transformation, then mixed with 10 µL of DNA ligation and incubated on ice for 30 minutes. Afterwards, the mixture was submitted to a heat shock of 90 seconds at 42 ºC, in a water-bath, followed by incubation on ice for 2 minutes. Then, 900 µL of pre-heated LB medium were added to the cells and were left to recover for one hour at 37 ºC, in an orbital shaker. 100 µL of the cell suspension were plated onto LB-agar supplemented with the appropriate antibiotic and then incubated O/N at 37 ºC.

2.7 Plasmid DNA purification

To isolate plasmid DNA, cells from isolated colonies were inoculated in 5 mL of LB medium supplemented with the appropriate antibiotic and incubated O/N at 37 ºC with shaking (150 rpm). The plasmid DNA was then extracted from the cell cultures using the NZYTech Miniprep kit (NZYTech), following the manufacturer's instructions.

2.8 Phenol-Chloroform genomic DNA extraction protocol

For *Synechocystis* transformants confirmation by PCR and Southern Blot, cyanobacterial gDNA was extracted using the phenol/chloroform method, according to Tamagnini *et al.*.[106] Firstly, 50 mL of *Synechocystis* culture were centrifuged at 4190 *x g* for 10 minutes 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-Aldrich), 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 2 cycles of vigorous vortexing for 1 minute followed by incubation on ice for 1 minute. The aqueous/organic phases were separated by centrifugation at 13000 *x g* for 10 minutes 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 at least 1 hour. Afterwards, samples were centrifuged at 13000 x *g* for 20 minutes at 6 ºC. Then, the resulting pellet was washed with ice cold 70% (v/v) ethanol, dried, and resuspended in water (molecular biology grade; Sigma-Aldrich). For Southern Blot, 1 µL of RNase solution (20 mg mL^{-1}, Sigma-Aldrich) was added to samples and incubated for 1 hour at 37 ºC and the gDNA integrity checked by agarose gel electrophoresis.

2.9 *Synechocystis* transformation by electroporation

The introduction of the replicative plasmids carrying the synthetic devices into *Synechocystis* was performed by electroporation, based on the Chiaramonte *et al* [107] and Ludwig *et al* [108] protocols. *Synechocystis* cultures were cultured at 30 ºC and continuous light regimen to an OD_{730} ≈ 0.5. Cells were harvested by centrifugation at 4190 *x g*, for 10 minutes and washed three times with 10 mL of HEPES buffer 1 mM, pH 7.5. The cells were then resuspended in 1 mL of HEPES buffer. From this suspension, 60 µL were mixed with 1 µg of plasmid DNA and electroporated with a Bio-Rad Gene Pulser™ (Bio-Rad), at a capacitance of 25 µF, 400 Ω resistance for 9 milliseconds time constant with an electric field of 12 $kVcm^{-1}$. Immediately after the electric pulse, the cells were transferred to 400 µL of fresh BG11 medium (1.5 mL microcentrifuge tubes) and spread onto Immobilon-NC membranes (0.45 µm pore size, 82 mm - Merck Millipore, Billerica, MA, USA) resting on solid BG11 plates that were incubated at 25 ºC in a 16 h low light / 8 h dark regimen for 24 hours. Then, the membranes were transferred to BG11 agar supplemented with 10 µg mL^{-1} of chloramphenicol and placed at 25 ºC under a 16 h light / 8 h dark regimen. Colonies were observed after 1 to 2 weeks and were transferred to BG11 plates with the same antibiotic concentration. After 1 week of incubation, the colonies were transferred to BG11 agar containing 20 µg mL^{-1} of chloramphenicol and subsequently transferred to liquid BG11 medium.

2.10 Natural transformation of *Synechocystis*

2.10.1 Insertion of P_{trc1O}::AhBet into *Synechocystis* chromosome

The synthetic device P_{trc1O}::AhBet was introduced into the chromosome of *Synechocystis* by natural transformation, using a method described by Pinto *et al.* (2012).[53] *Synechocystis* cultures were inoculated in liquid BG11 medium to an initial OD_{730} ≈ 0.2 and grown at 30 ºC under a 12 h light (25 µE m^{-2} s^{-1}) / 12 h dark regimen, to an OD_{730} ≈ 0.5. The cells were harvested by centrifugation at 4190 x *g* for 10 minutes and resuspended in BG11 medium to a final OD_{730} ≈ 2.5. Aliquots of 100, 200 and 500 µL of cell suspension were mixed with pSN15K P_{trc1O}::AhBet plasmid (table 1) to a final concentration of 20 µg mL^{-1} and incubated for 5 hours, in light (25 µE m^{-2} s^{-1}) at 25 ºC. The aliquots were then spread onto Immobilon-NC membranes (0.45 µm pore size, 82 mm; Merck Millipore) resting on solid BG11 plates, at 25 ºC in a 16 h low light / 8 h dark regimen. After 24 hours of incubation, the membranes

were transferred to selective plates containing kanamycin at 10 µg mL^{-1}. For complete segregation, the kanamycin-resistant colonies were grown at increasing antibiotic concentrations (up to 100 µg mL^{-1}) and then transferred to liquid medium, in which, kanamycin concentrations up to 400 µg mL^{-1} were used.

2.10.2 Generation of the *Synechocystis* Δ*sps*Δ*ggpS*.KS mutant

A *Synechocystis* knockout mutant that lacks the genes encoding for the first enzyme involved in sucrose (*sps*) and in glucosylglycerol (*ggpS*) biosynthetic pathways was generated using the procedure for natural transformation described in section 2.10.1. *Synechocystis* Δ*sps* clones containing the KS selection cassette (*Synechocystis* Δ*sps*.KS) were already available in the lab. Upon confirmation of full segregation by colony PCR, *Synechocystis* Δ*sps*.KS cultures were grown in BG11 medium at 25 ºC, under a 12 h light (25 µE m^{-2} s^{-1}) / 12 h dark regimen until an OD_{730} ≈ 0.5 was reached. Cells were concentrated to an OD_{730} ≈ 2.5 and aliquots of 100, 200 and 500 µL were incubated with the pGD*sps* plasmid (see table 2) at a final concentration of 20 µg mL^{-1} for 5 hours, in light at 25 ºC with shaking. The cell suspensions were then spread onto Immobilon-NC membranes (0.45 µm pore size, 82 mm; Merck Millipore) resting on BG11 agar plates and grown for 48 hours at 25 ºC under 16 h low light / 8 h dark conditions. The membranes were transferred to BG11 agar plates containing 5% (w/v) sucrose. Transformants were obtained after 1 to 2 weeks. For complete segregation, the resistant colonies were grown in BG agar plates containing 10% (w/v) sucrose and later transferred to liquid medium. The colonies were screened for kanamycin (Km) sensitivity and full segregation was confirmed by colony PCR and by Southern blot. Subsequently, the Δ*sps* mutant was used for the generation of the double knockout mutant. Cells were transformed using the same procedure with the plasmid pGD*ggpS*.KS (table 2), to replace the gene by the selection cassette, and the mutants were selected in solid BG11 medium containing 10 µg mL^{-1} Km. The segregation process was achieved by growing the resistant transformants in increasing concentrations of Km (up to 100 µg mL^{-1}) and then in liquid medium.

2.11 Southern Blot

The DNA probe for the Southern Blot was already available in the laboratory. It was generated by PCR with the primers ggpS.5I and ggpS.5O (Table 2) covering the 5' flanking region of the *ggpS* gene, using the pGD*ggpS*.KS as template, and labelled with digoxigenin using the DIG High Prime DNA Labelling kit (Roche, Basel, Switzerland). The Southern blot was carried out using 4 µg gDNA of *Synechocystis* wild-type and Δ*sps* mutant strains digested with *Ava*II Fast-Digest® (Thermo Fisher Scientific) for 45 minutes 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) instructions. The final results were observed with a Chemi Doc™ XRS+ Imager (Bio-Rad).

2.12 Halotolerance experiments

2.12.1 Growth in 100 mL Erlenmeyer flasks

Pre-cultures of the *Synechocystis* mutants, as well as the wild-type strain, were grown at 30 ºC and under a 12 h light (25 µE m^{-2} s^{-1}) / 12 h dark regimen in an orbital shaker at 150 rpm, until an OD_{730} ≈ 2 was reached. When necessary the medium was supplemented with chloramphenicol (Cm, 10 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-cultures and their growth was monitored measuring the OD_{730} for 16 days, using a Shimadzu UV mini-1240 spectrophotometer (Shimadzu, Kyoto, Japan). For each experiment, at least three biological and two technical replicates were performed.

2.12.2 Growth in Seawater

Synechocystis cultures were grown in seawater, hereafter called SW_0 medium, previously filtered with a 0.2 µm filter and autoclaved. A supplemented version of SW_0, hereafter called SW+, was prepared by adding sodium nitrate and potassium hydrogen phosphate to the final

concentrations of 4.4 mM and 5.7 mM, respectively. Pre-cultures of *Synechocystis* wild-type and mutant strains were grown as described in section 2.12.1. Then, the cell cultures were centrifuged at 4190 x *g* at room temperature for 10 minutes and the pellets were washed with 1 mL of medium (either SW_0 or SW+) and centrifuged again to remove the remaining BG11 medium. The cells were then resuspended in medium (SW_0 or SW+) to a final OD_{730} ≈ 0.5. 25 mL of culture were transferred to 50 mL Erlenmeyer flasks and grown in the same conditions as stated in section 2.12.1. The OD_{730} was measured every 4 days, for 16 days.

2.12.3 Gas Wash Bottles

Pre-cultures of the *Synechocystis* mutants, as well as the wild-type strain, were grown at 30 ºC and under a 12 h light (65 µE m^{-2} s^{-1}) / 12 h dark regimen with a water-saturated air flux of 1 L min^{-1} (the flux was adjusted using an AALBORG model P single tube flowmeter - AALBORG, Orangeburg, NY, USA), until an OD_{730} ≈ 2 was reached. When necessary the medium was supplemented with chloramphenicol (Cm, 10 µg mL^{-1}). The cultures were then diluted in fresh BG11 medium without antibiotic, to a final OD_{730} ≈ 0.2 or OD_{730} ≈ 0.5. Afterwards, 250 mL of the dilution were transferred to 500 mL glass gas wash bottles (previously sterilized) containing NaCl, providing the cultures with the following final NaCl concentrations: 0 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} for 10 days.

2.13 Quantification of compatible solutes by NMR

The production of compatible solutes was assessed by nuclear magnetic resonance (NMR).[109] Pre-cultures of the *Synechocystis* mutants, as well as the wild-type strain, were grown at 30 ºC and under a 12 h light (25 µE m^{-2} s^{-1}) / 12 h dark regimen in an orbital shaker at 150 rpm, until an OD_{730} ≈ 2 was reached. When necessary the medium was supplemented with chloramphenicol (Cm, 10 µg mL^{-1}). Then, the cultures were diluted, in fresh BG11 medium without antibiotic, to a final OD_{730} ≈ 0.5. The cultures were grown in 100 mL Erlenmeyer flasks at 0 and 3% (w/v) NaCl. These cultures were maintained in the same conditions as the pre-culture until and OD_{730} ≈ 1 was reached. 500 mL were then centrifuged at 4190 x *g* for 10 minutes and the supernatant was discarded. The cells were washed with ice cold water with the same salt concentration and centrifuged at 4190 x *g* for 10 minutes.

The supernatant was discarded and the pellets were resuspended in 20 mL of ice cold water and 1 mL was reserved for total protein quantification. The cell suspensions were centrifuged at 4190 x *g*, at 4 ºC, for 10 minutes, the supernatants were discarded and the pellets were stored at -20 ºC. The quantifications were performed in collaboration with CERMAX from the ITQB NOVA (Lisbon, Portugal).

2.14 Determination of photosynthetic pigments

The content on chlorophylls and carotenes was determined in the generated mutants that carry synthetic devices, as well as in the wild-type strain, according to Meeks and Castenholz (1974) [110]. Cultures of each strain were diluted to an $OD_{730} \approx 0.5$ and grown at 25 ºC with shaking (150 rpm), under a 12 h (25 µE m^{-2} s^{-1}) light / 12 h dark regimen until an $OD_{730} \approx 1.5$ was reached. Four millilitres of culture were centrifuged at 14,100 x *g* for 2 minutes and the pellets washed once with dH_2O. The cells were resuspended in 1 mL of 90% (v/v) methanol, briefly vortexed and incubated O/N at 4 ºC, protected from the light. Afterwards, samples were centrifuged at 14,100 x *g* for 2 minutes and 750 µL of the supernatant were used for the spectrophotometric assays. The content on chlorophylls and carotenes was also determined for the cultures grown in seawater. For that, 1 mL of culture was used instead. The absorption spectra were obtained by measuring the absorbance between 700 to 300 nm against a 90% (w/v) methanol blank, using Shimadzu UV-2401PC spectrophotometer (Shimadzu). Technical duplicates and two biological replicates were performed.

2.15 Total RNA extraction and transcription analysis by reverse transcription PCR (RT-PCR)

Synechocystis cultures were prepared and cultivated as described in section 2.12.1. Cell cultures were grown for 10 and 20 days in 50 mL of BG11 medium (without antibiotic), in the presence or absence of NaCl: 0% (w/v) for wt; 0 and 7% (w/v) for the mutants with the synthetic device. The equivalent of 100 mL of a culture at $OD_{730} \approx 1$ was collected for RNA extraction, cells were centrifuged at 4190 x *g* for 10 minutes and the pellet was resuspended in 1 mL of fresh BG11 medium and transferred to screw-cap 2 mL tubes. Cells were centrifuged at 14100 x *g* for 2 minutes and the pellets were resuspended in 500 µL of medium and 1 mL of RNAprotect® Bacteria Reagent (QIAGEN, Valencia, CA, USA). The

mixture was vortexed for 5 seconds, then incubated for 5 minutes at RT and centrifuged at 5000 x *g* for 10 minutes. The cell pellets were stored at -80 ºC.

The RNA extraction was performed using TRIzol® Reagent (Thermo Fisher Scientific) in combination with the Purelink™ RNA Mini Kit (Thermo Fisher Scientific). Briefly, 1 mL TRIzol and 0.2 g of 425-600 nm glass beads (acid washed, Sigma) were added to the samples and the cells were mechanically disrupted using a FastPrep®-24 (MP Biomedicals LLC) (2 × 60 seconds at a setting of 4.0 $m\ s^{-1}$). The subsequent 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 room temperature, following the manufacturer's instructions. RNA was quantified on a NanoDrop ND-1000 (Thermo Fisher Scientific) and its integrity was checked using the Experion™ RNA StdSens Analysis Kit (Bio-Rad). A PCR was performed to confirm the absence of gDNA contamination, using specific primers for the *rnpB* reference gene (Table 2) and 100 ng of total RNA. The PCR products were analyzed by electrophoresis on a 1.5 % (w/v) agarose gel.

One or half microgram of total RNA were used for cDNA synthesis using the iScript™ Reverse Transcription Supermix for RT-PCR (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 2 µL of cDNA.

2.16 Statistical Analysis

The statistical analysis was assessed using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA, USA; www.graphpad.com). Growth rates were determined by linear regression of the exponential phase of the growth curves and the statistical analysis was performed using the unpaired, two-tailed *t*-test.

3 Results

3.1 Construction of the synthetic devices

In this work, a synthetic biology approach was applied to improve the halotolerance of *Synechocystis* by implementing the heterologous production of the strong compatible solutes betaine and ectoines. The gene cluster designed for betaine production - designated as AhBet - is comprised by the genes required for the methylation of glycine into betaine (*dmt* and *gsmt*), as well as a copy of the *Synechocystis* native SAM synthase (*metX*) to prevent shortage of the cofactor. The gene clusters designed for the production of ectoine and hydroxyectoine were based on the genes related to the conversion of L-aspartyl-phosphate into ectoine (*ectA, ectB* and *ectC*) and its subsequent hydroxylation to hydroxyectoine (*ectD*) from the halophilic microorganisms *C. salexigens* and *M. alcaliphilum* 20Z - the clusters were designated Cs(h)ect and Ma(h)ect, respectively. A gene that encodes for an aspartate kinase (*asK*) was also included in Ma(h)ect gene cluster to evaluate the possible need to increase the pool of the precursor. Two different promoters - P_{trc1O} and P_{psbA2*} - were used in the assembly of the synthetic devices. The P_{trc1O} promoter is a constitutive promoter 78-fold stronger than the cyanobacterial reference promoter P_{rnpB}, while the P_{psbA2*} promoter is a constitutive promoter 7-fold stronger than the P_{rnpB} (unpublished work).[27,54] The use of these two promoters would allow the evaluation of the device fine-tuning, assessing which device expression level is more adequate for the heterologous production of these compounds without compromising the cellular viability. However, cloning gene clusters into the vector carrying the P_{psbA2*} promoter failed in all attempts. Therefore, the construction of the synthetic devices with the P_{psbA2*} promoter was not pursued and only the synthetic devices with the P_{trc1O} promoter were considered for this work.

3.1.1 Assembly of the synthetic devices

The P_{trc1O}::AhBet synthetic device was already assembled and introduced into the pSEVA351 replicative vector prior to this work. For a more stable expression/integration of the genes related to betaine production in *Synechocystis*, the P_{trc1O}::AhBet device was transferred to the pSN15K integrative vector targeting *Synechocystis* chromosomal neutral

site N15.[54] The plasmid was transformed in *E. coli* and confirmed by restriction analysis with *Xba*I and DNA sequencing.

The synthetic devices used for the heterologous production of ectoines were constructed by introducing the P_{trc1O} promoter into the vectors that carry the Ma(h)ect and Cs(h)ect gene clusters (pBSK and pSB3K3, respectively), digested and dephosphorylated (see section 2.2). The resulting plasmids were cloned in *E.coli* and the assembly of the promoter with the gene clusters was confirmed by PCR. As shown in Figure 6A, the P_{trc1O}::Ma(h)ect sample had an amplicon with 500 bp, which is bigger than the one obtained for the plasmid without the P_{trc1O} promoter (426 bp). In Figure 6B, it can be seen that the fragment amplified in the P_{trc1O}::Cs(h)ect sample was bigger than the one amplified in the reaction using the cluster plasmid template, thus confirming the insertion of the promoter.

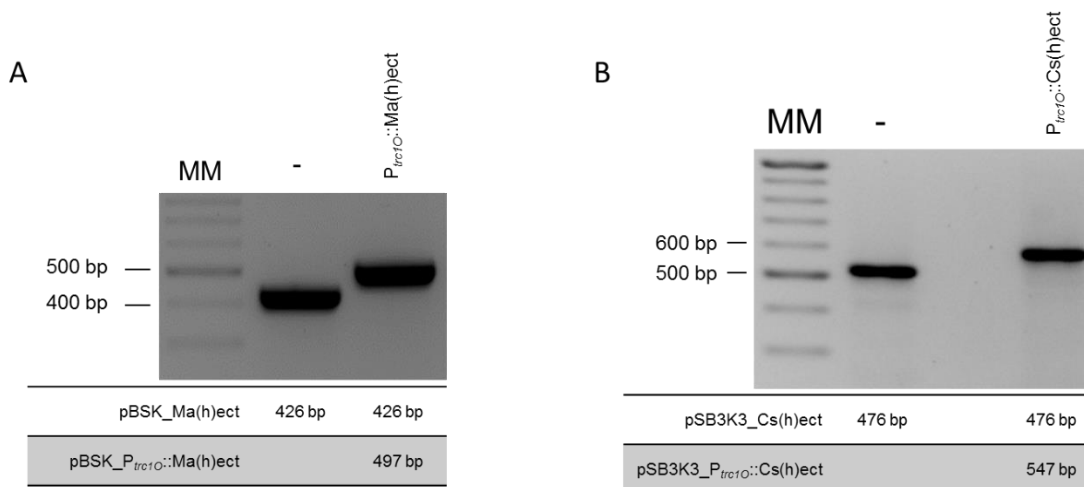


Figure 6 - Confirmation of the P_{trc1O}::Ma(h)ect (A) and P_{trc1O}::Cs(h)ect (B) constructs by PCR. The reaction A was performed using the primers pUC_F and Ma-h-ect_Ri (see table 2) and the pBSK_Ma(h)ect plasmid DNA was used as negative control (-). The reaction B was performed using the primers VF2 and Cs-h-ect_Ri (see table 2) and the pSB3K3_Cs(h)ect plasmid DNA was used as negative control (-). MM - molecular weight marker.

Given these results, the samples were sent for DNA sequencing and the results confirmed the correct assembly of the devices. The P_{trc1O}::Ma(h)ect and the P_{trc1O}::Cs(h)ect devices were transferred to the pSEVA351 replicative vector and subsequently cloned in *E. coli.* The transformants were screened by colony PCR, and none of the colonies from the transformation with pSEVA351 P_{trc1O}::Cs(h)ect have shown the presence of the device. Therefore, only the P_{trc1O}::Ma(h)ect device was considered for the heterologous production of ectoines in *Synechocystis.* The pSEVA351 P_{trc1O}::Ma(h)ect plasmid was confirmed by restriction analysis with *Xba*I and DNA sequencing.

3.2 Generation and characterization of a *Synechocystis* mutant carrying the P_{trc1O}::AhBet synthetic device

3.2.1 Confirmation of the introduction of P_{trc1O}::AhBet device in the chassis

The P_{trc1O}::AhBet synthetic device was incorporated in the chassis by electroporation via the pSEVA351 replicative vector. The transformants were screened by colony PCR (Figure 7).

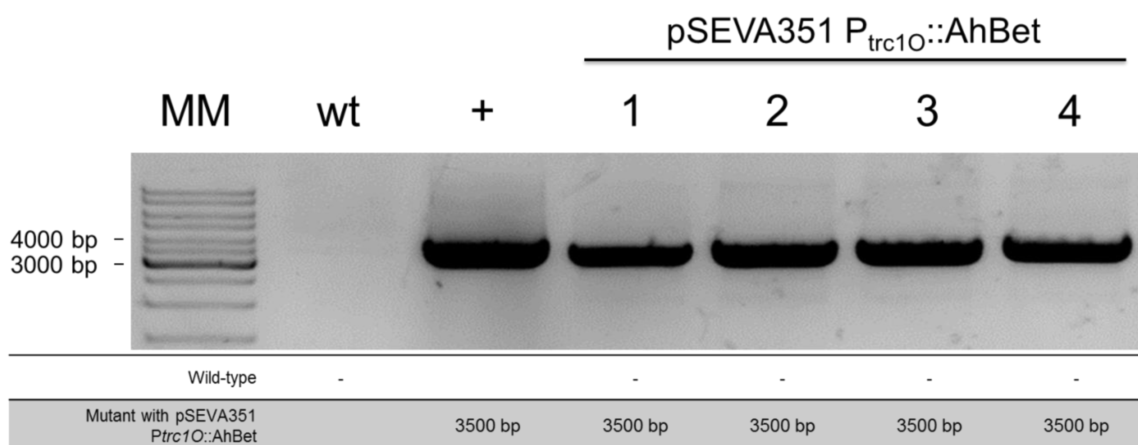


Figure 7 - Confirmation of the *Synechocystis* mutant carrying the pSEVA351 P_{trc1O}::AhBet by colony PCR. The reaction was performed using the primers PS1 and PS2 (see table 2). Genomic DNA from the wild-type was used as negative control (wt) and pSEVA351_P_{trc1O}::AhBet plasmid DNA was used as positive control (+). MM - molecular weight marker.

A 3.5 kbp fragment was obtained in all the clones, which was consistent with the size of the synthetic device. The four clones were used for the characterization studies of this mutant. However, it was not possible to confirm the presence of the plasmid by colony PCR at the end of the experiments. Therefore, the plasmid was retrieved from *Synechocystis* and cloned back again in *E. coli,* subsequently isolated from the transformants and the presence of the device was confirmed by restriction analysis.

3.2.2 Halotolerance of the mutant harbouring the pSEVA351 P_{trc1O}::AhBet

Once the presence of the synthetic device was confirmed in the *Synechocystis* cultures, the tolerance the mutant carrying the pSEVA351 P_{trc1O}::AhBet to salinity was assessed by growing it in BG11 medium supplemented with different NaCl concentrations, under autotrophic conditions. The effects of increasing salt concentrations on the growth of the mutant are shown in Figure 8.

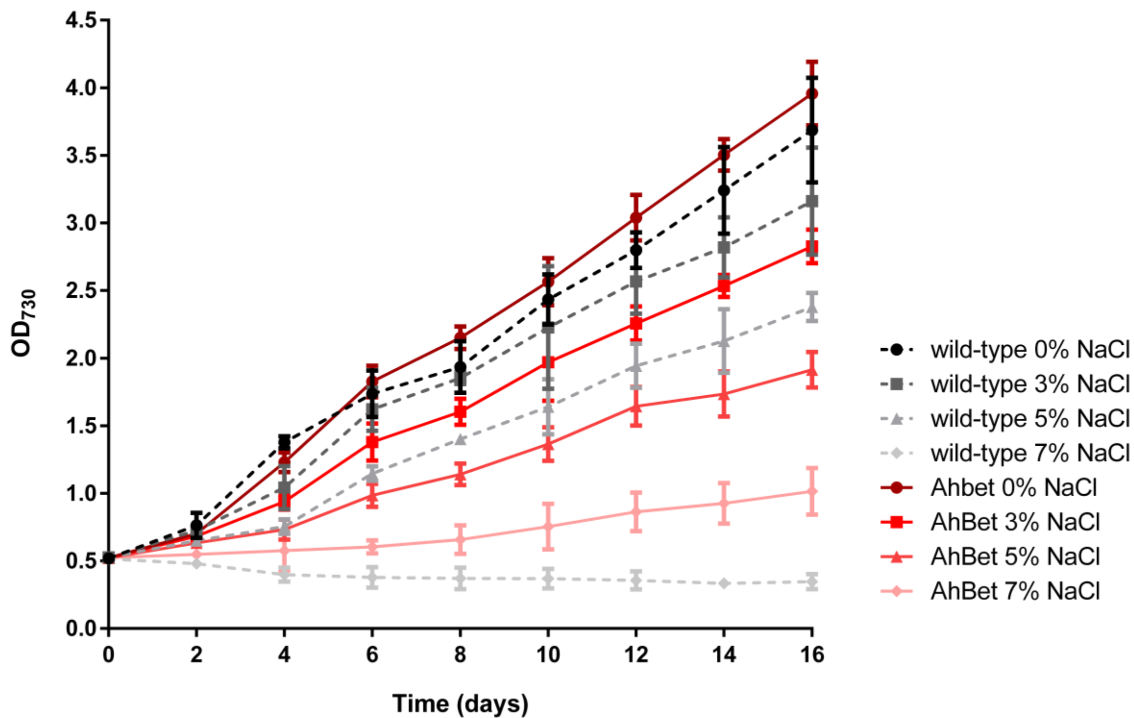


Figure 8 - Growth of *Synechocystis* mutant carrying the pSEVA351 P_{trc1O}::AhBet at different salt concentrations. Cultures were grown in BG11 medium supplemented with 0, 3, 5 and 7% (w/v) NaCl, at 30 ºC and 12 h light (25 $\mu E\ m^{-2}\ s^{-1}$) / 12 h dark regimen in an orbital shaker at 150 rpm. The OD_{730} of the cultures was measured every 2 days for 16 days. Mean ± SD obtained from three technical replicates and two biological replicates.

It was observed that the growth rate of the mutant grown at 0% (w/v) NaCl is similar to the wild-type strain. However, increasing the salt concentration of the medium negatively affects the growth of the mutant strain. Furthermore, it seems that the negative effects of salt concentration on growth are more pronounced for the mutant than the wild-type strain, as the mutant presented slightly lower growth rates than the wild-type strain when grown at 3 and 5% (w/v) NaCl. It can be observed that the growth of the mutant suffers a 40% decrease

when grown at 3% (w/v) NaCl ($p < 0.0001$) and a 64% decrease when grown at 5% (w/v) NaCl ($p < 0.0001$). Nonetheless, this mutant was able to grow at 7% (w/v) NaCl ($p < 0.0001$), which is above the tolerance limit of the wild-type strain (Figure 9).

Figure 9 - Liquid cultures of *Synechocystis* wild type (left) and mutant carrying the betaine synthetic device (right) in BG11 medium supplemented with 7% (w/v) NaCl after 16 days of exposure.

3.2.3 Growth in gas wash bottles

A scale-up approach was performed to grow higher volumes of culture to obtain enough biomass for the subsequent analyses in a shorter period of time. The P_{trc1O}::AhBet mutant and the wild-type strains were grown in 250 mL of BG11 medium at an initial $OD_{730} \approx 0.2$ or 0.5, in gas wash bottles with an air flow of 1 L min^{-1}, at 30 ºC and under a 12 h light (65 µE m^{-2} s^{-1}) / 12 h dark. As expected, there was a significant increase in the growth rate of the cultures when grown in gas wash bottles (GWBs) (Figure 10).

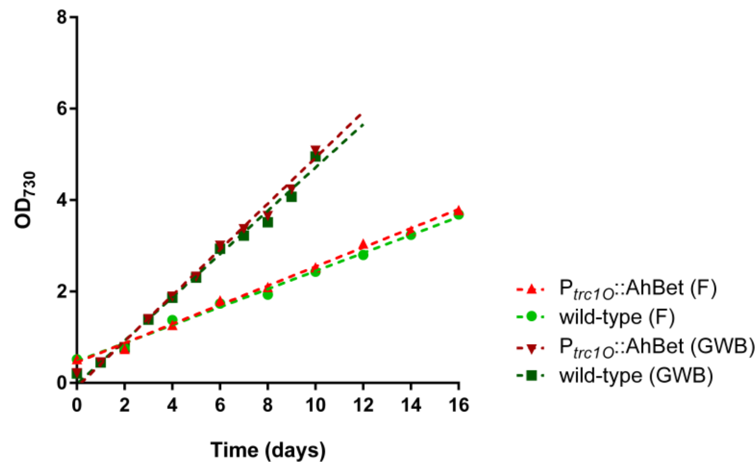


Figure 10 - Growth of *Synechocystis* wild-type and P_{trc1O}::AhBet strains at 0% (w/v) NaCl in 100 mL Erlenmeyer flasks (F) and in gas wash bottles (GWB). Cultures were grown in 250 mL of BG11 medium without NaCl, at 30 ºC and in a 12 h light (65 µE m^{-2} s^{-1}) / 12 h dark regimen, in GWBs with an air flow of 1 L min^{-1}. The OD_{730} of the cultures was measured every day for 10 days.

Cultures of wild-type and P_{trc1O}::AhBet mutant strains were also grown in medium with 7% (w/v) NaCl, under the abovementioned conditions. In contrast to what was seen in the growth experiments performed in Erlenmeyer flasks, the betaine-producing mutant was not able to grow at this salt concentration (Figure 11). However, it is important to note that, compared with the wild-type, the culture of the mutant was greener (Figure 12 A) and presented cellular aggregation, when grown at 7% (w/v) NaCl (Figure 12 B and C).

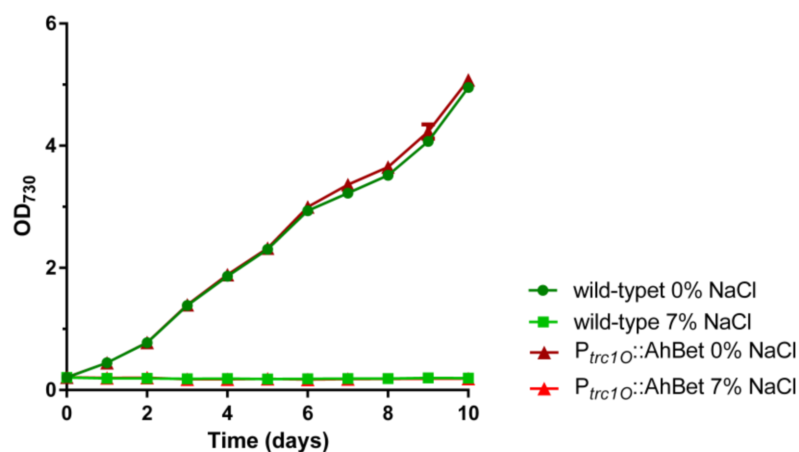


Figure 11 - Growth of *Synechocystis* wild-type and mutant carrying the pSEVA351 P_{trc1O}::AhBet strains at 0 and 7% (w/v) NaCl in gas wash bottles (GWB). Cultures were grown in 250 mL of BG11 with 0 and 7% (w/v) NaCl, at 30 ºC and in a 12 h light (65 µE m^{-2} s^{-1}) / 12 h dark regimen, in GWBs with an air flow of 1 L min^{-1}. The OD_{730} of the cultures was measured every day for 10 days.

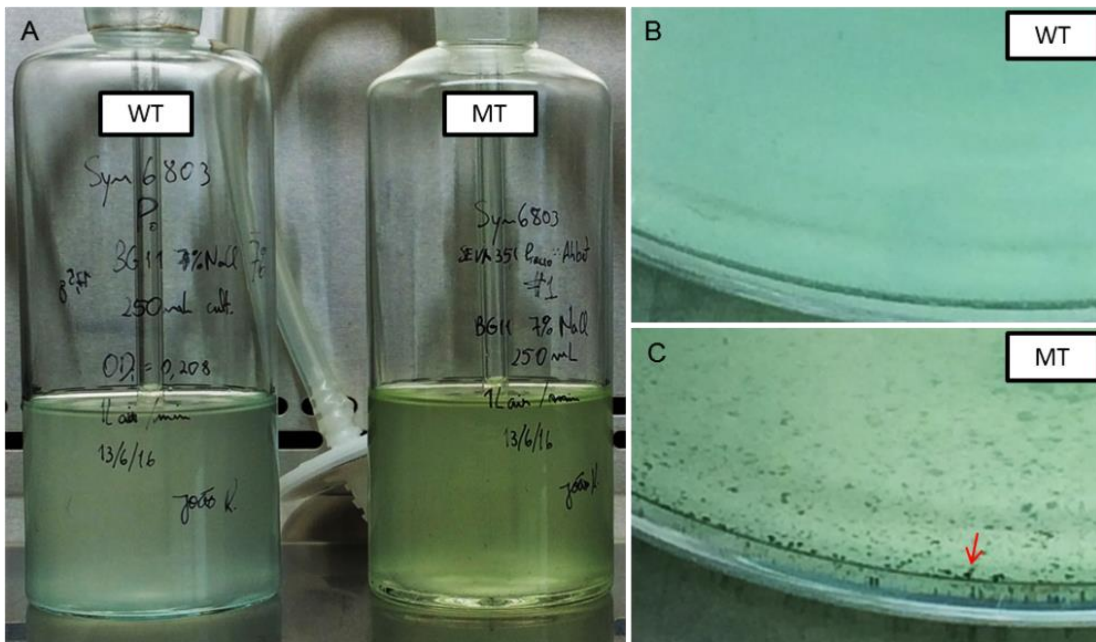


Figure 12 - Liquid cultures of *Synechocystis* wild-type (left, WT) and mutant harbouring the pSEVA351 P_{trc1O}::AhBet (right, MT), grown in gas wash bottle systems and BG11 7% (w/v) NaCl. Detail of the wild-type liquid culture (B, WT), showing the absence of aggregates. Detail of the mutant liquid culture (C, MT), evidencing cellular aggregates (red arrow). Cultures were grown in 250 mL of BG11 with 7% (w/v) NaCl, at 30 ºC and in a 12 h light (65 µE m^{-2} s^{-1}) / 12 h dark regimen, with an air flow of 1 L min^{-1}.

3.2.4 Betaine and glucosylglycerol quantification by NMR

A preliminary assessment of the production of compatible solutes by NMR was performed for the wild-type and the mutant carrying the P_{trc1O}::AhBet device grown in absence and presence of salt (3% (w/v) NaCl). This salt concentration was chosen because (i) the growth impairment is not so pronounced when compared to the growth at higher salt concentrations, so it was possible to cultivate the strains in a short period of time and (ii) it has already been shown that the heterologous production of betaine in *Anabaena* sp. PCC 7120 occurs even at 0% (w/v) NaCl and that these levels increase with the salt concentration.[74] However, in the preliminary analysis, betaine was not detected and the levels of glucosylglycerol were similar in the mutant and the wild-type grown in 3% (w/v) NaCl (data not shown).

3.2.5 Transcription analysis of the genes of the synthetic device in the mutant carrying the pSEVA351 P_{trc1O}::AhBet

The transcription of the genes related to the betaine biosynthetic pathway - *gsmt* and *dmt* - was assessed by reverse transcription PCR (RT-PCR). RNA was extracted from the wild-type strain grown for 10 days at 0% (w/v) NaCl, and from the mutant carrying the device grown for 10 days at 0 and 7% (w/v) NaCl. The synthesis of cDNA was confirmed by PCR using the primers for the *rnpB* reference gene, and a 189 bp fragment was obtained in all samples, which is consistent with the expected size of the amplicon (Figure 13).

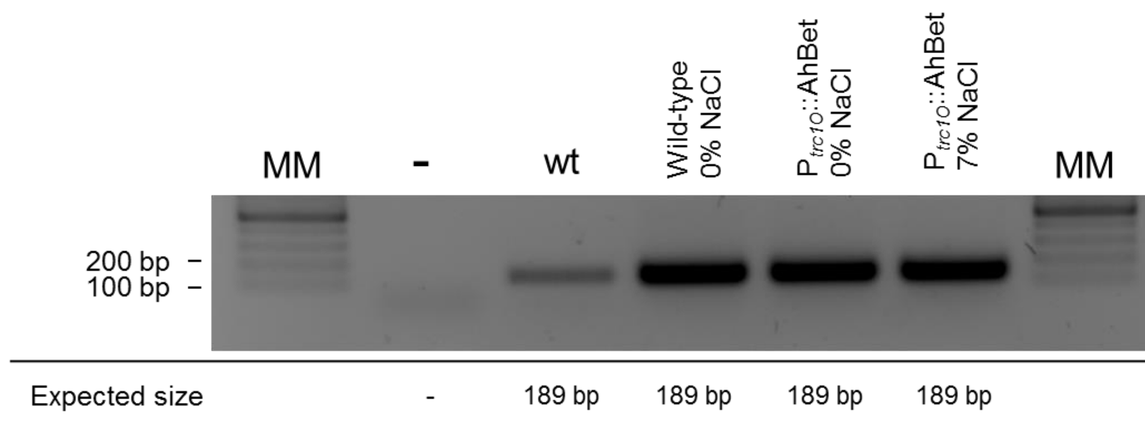


Figure 13 - Confirmation of cDNA synthesis from RNA extracted from the cultures with 10 days. The PCR was performed using a set of primers specific for the *rnpB* reference gene (see table 2). pSEVA351 P_{trc1O}::AhBet plasmid DNA was used as negative control (-) and wild-type genomic DNA was used as positive control (wt). MM - molecular weight marker.

However, when the primers specific for the *gsmt* and *dmt* genes were used no amplification was detected in any of the P_{trc1O}::AhBet cDNA samples, even when the amount of cDNA template was doubled, suggesting that the synthetic device was not being transcribed (Figure 14).

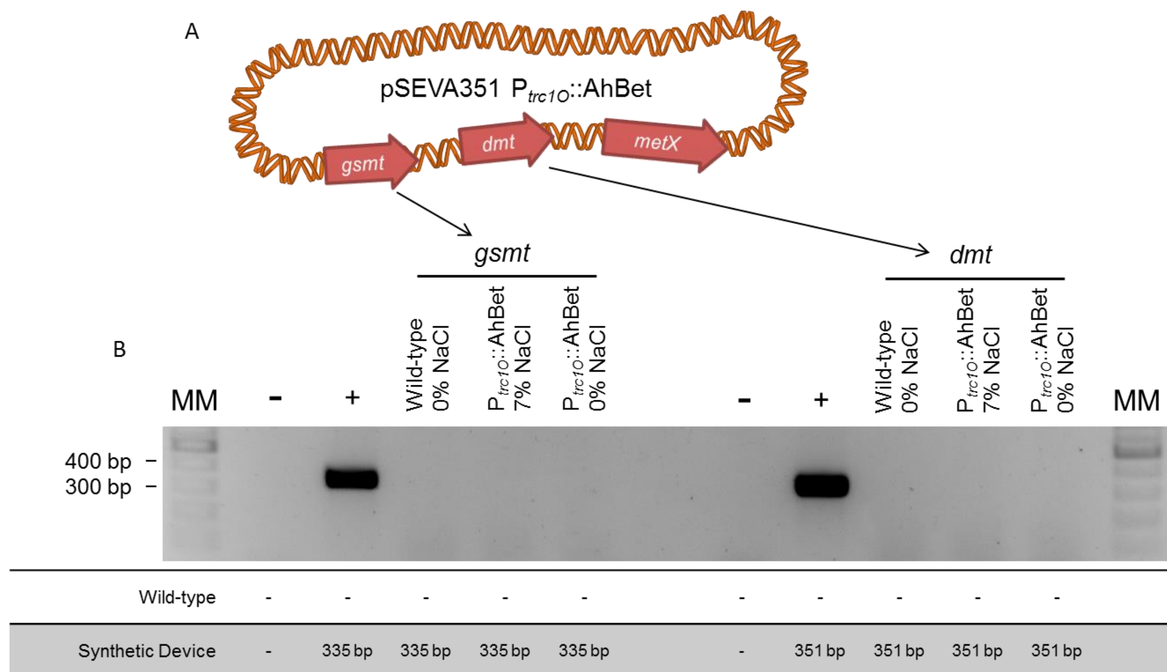


Figure 14 - Schematic representation of the pSEVA351 P_{trc1O}::AhBet plasmid (A) and transcription analysis of *gsmt* and *dmt* by RT-PCR of *Synechocystis* wild-type and pSEVA351 P_{trc1O}::AhBet mutant grown at 0% (w/v) NaCl for 10 days and pSEVA351 P_{trc1O}::AhBet mutant grown at 7% (w/v) NaCl for 10 days (B). pSEVA351_P_{trc1O}::AhBet plasmid DNA (+) was used as positive control. A negative control of the reaction (-) was also performed using ddH_2O as template. MM - molecular weight marker. Image adapted from Servier Medical Art PowerPoint image bank (Servier SAS, Suresnes, France).[111]

3.2.6 Confirmation of the integration of P_{trc1O}::AhBet device into *Synechocystis* genome

In order to have a more stable expression of the genes related to betaine production, the P_{trc1O}::AhBet synthetic device was introduced in the neutral site N15 of *Synechocystis* by natural transformation, using the pSN15K integrative vector.[54] The transformants were screened for the presence of the synthetic device by PCR. As shown in Figure 15, the amplification of a 2400 bp band was observed for the clones 1 and 16, which is consistent with the integration of the device. Thus, these two clones were used for further analyses.

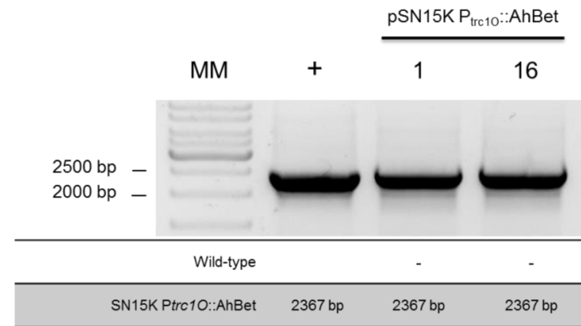


Figure 15 - Confirmation of the *Synechocystis* pSN15K P_{trc1O}::AhBet mutant by colony PCR. The reaction was performed using the primers N15.3O and Ahbet_RI (see table 2). Plasmid DNA was used as positive control (+). MM - molecular weight marker.

3.2.7 Halotolerance of the *Synechocystis* SN15K P_{trc1O}::AhBet mutant

The halotolerance of the SN15K P_{trc1O}::AhBet mutant was assessed. The mutant was grown in liquid BG11 medium at 0 and 7% (w/v) salt concentration and the results are shown in Figure 16. The mutant and the wild-type strain exhibited similar growth rates in the absence of salt. However, when grown in medium supplemented with 7% (w/v) NaCl, no growth was observed.

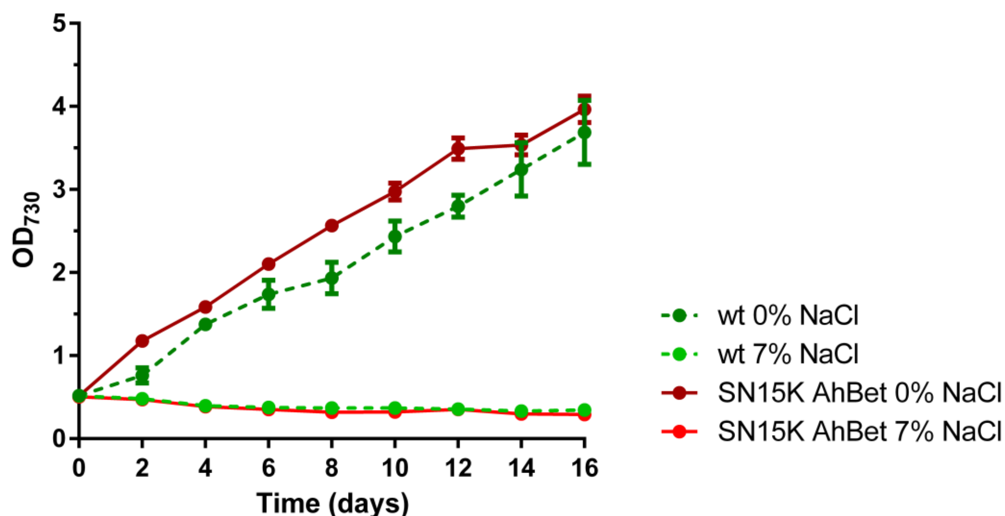


Figure 16 - Growth of *Synechocystis* SN15K P_{trc1O}::AhBet mutant at 0 and 7% (w/v) NaCl. Cultures were grown at 30 ºC and 12 h light (25 µE m^{-2} s^{-1}) / 12 h dark regimen in an orbital shaker at 150 rpm. The OD_{730} of the cultures was measured every 2 days for 16 days. Mean ± SD; n ≥ 3.

3.2.8 Transcription analysis of the *gsmt, dmt* and *metX* genes in SN15K P_{trc1O}::AhBet mutant

The transcription of the genes from the synthetic device was also evaluated for the SN15K P_{trc1O}::AhBet mutant. RNA was extracted from a culture of this mutant grown at 0% (w/v) NaCl for 10 days. The synthesis of cDNA was confirmed by PCR, using the *rnpB* primers, and a 189 bp fragment was obtained in all samples, that is consistent with the size of the *rnpB* amplicon (Figure 17).

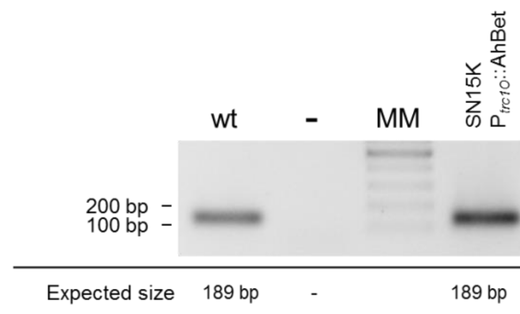
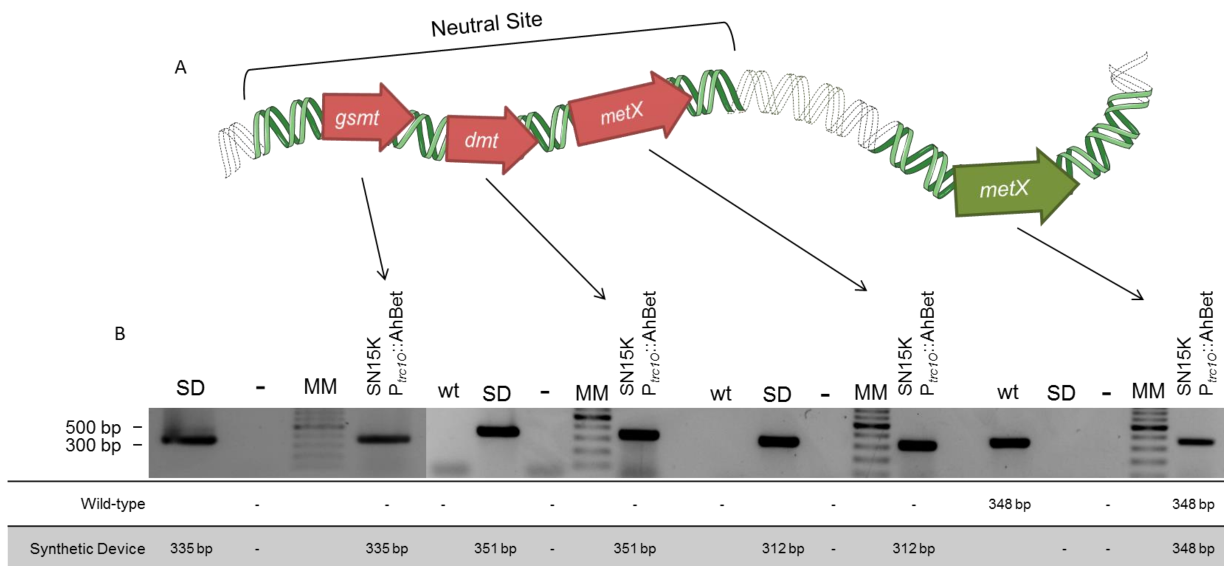


Figure 17 - Confirmation of cDNA synthesis from mRNA of SN15K P_{trc1O}::AhBet grown at 0% (w/v) NaCl for 10 days. The PCR was performed using a set of primers specific for the *rnpB* reference gene (see table 2). Plasmid DNA was used as negative control (-) and wild-type genomic DNA was used as positive control (wt). MM - molecular weight marker.

The transcription of the genes from the synthetic device - *gsmt, dmt* and *metX* - was assessed using specific primers (see table 2). As *Synechocystis* possesses a native *metX* gene, its transcription was also evaluated. The presence of a 300 to 350 bp amplicon was observed in the reactions using the primers for the *gsmt, dmt* and *metX* genes and the cDNA sample from the SN15K P_{trc1O}::AhBet mutant, confirming the transcription of the synthetic device (Figure 18). Moreover, the amplification of a 350 bp fragment was also observed when the primers specific for the native copy of *metX* were used, which is consistent with the size of the *metX* amplicon.



	SD	-	MM	SN15K P_{trc1O}::AhBet	wt	SD	-	MM	SN15K P_{trc1O}::AhBet	wt	SD	-	MM	SN15K P_{trc1O}::AhBet	wt	SD	-	MM	SN15K P_{trc1O}::AhBet
Wild-type		-		-	-		-		-	-		-		-	348 bp		-		348 bp
Synthetic Device	335 bp	-		335 bp		351 bp	-		351 bp		312 bp	-		312 bp		-	-		348 bp

Figure 18 - Schematic representation of a portion of the *Synechocystis* genome including the inserted synthetic device in the neutral site (A) and confirmation of the transcription of *gsmt*, *dmt* and *metX* in *Synechocystis* SN15K P_{trc1O}::AhBet mutant by RT-PCR (B). Genomic DNA of the wild-type strain (wt) and pSN15K_P_{trc1O}::AhBet plasmid DNA (SD) were used as controls. A negative control of the reaction (-) was also performed using ddH_2O as template. MM - molecular weight marker. Image adapted from Servier Medical Art PowerPoint image bank (Servier SAS).[111]

3.3 Generation and characterization of a *Synechocystis* mutant carrying the P_{trc1O}::Ma(h)ect synthetic device - preliminary results

3.3.1 Confirmation of the P_{trc1O}::Ma(h)ect mutant

The P_{trc1O}::Ma(h)ect synthetic device was introduced in *Synechocystis* wild-type strain by electroporation via the pSEVA351 replicative vector. The transformants were transferred to liquid medium and then confirmed by PCR. All clones, expect clone 3.4, exhibited a 600 bp amplicon, consistent with the presence of the synthetic device (Figure 19).

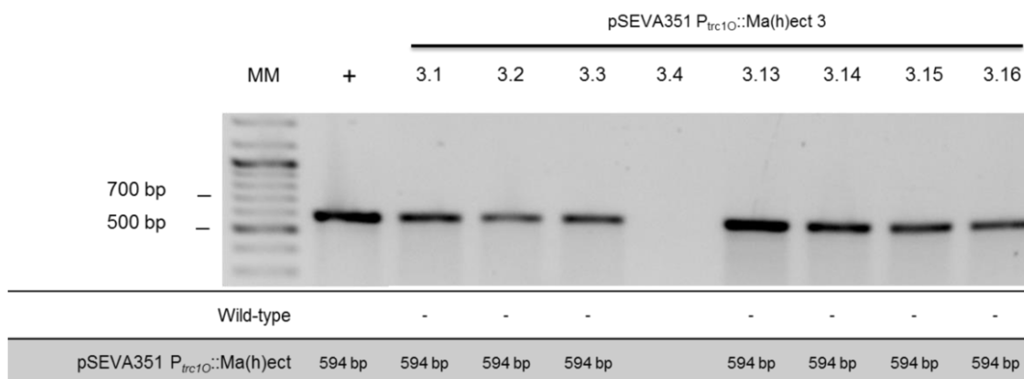


Figure 19 - Confirmation of the *Synechocystis* pSEVA351 P_{trc1O}::Ma(h)ect mutant by PCR. Eight clones were screened for the presence of synthetic device using the primers PS1 and Ma-h-ect_Ri (see table 2). Plasmid DNA was used as positive control (+). MM - molecular weight marker.

Two clones - 3.3 and 3.16 - were chosen for a preliminary phenotypic characterization of the P_{trc1O}::Ma(h)ect *Synechocystis* mutant. These mutants were grown in BG11 medium with 0 and 7% (w/v) NaCl. The results are shown in Figure 20.

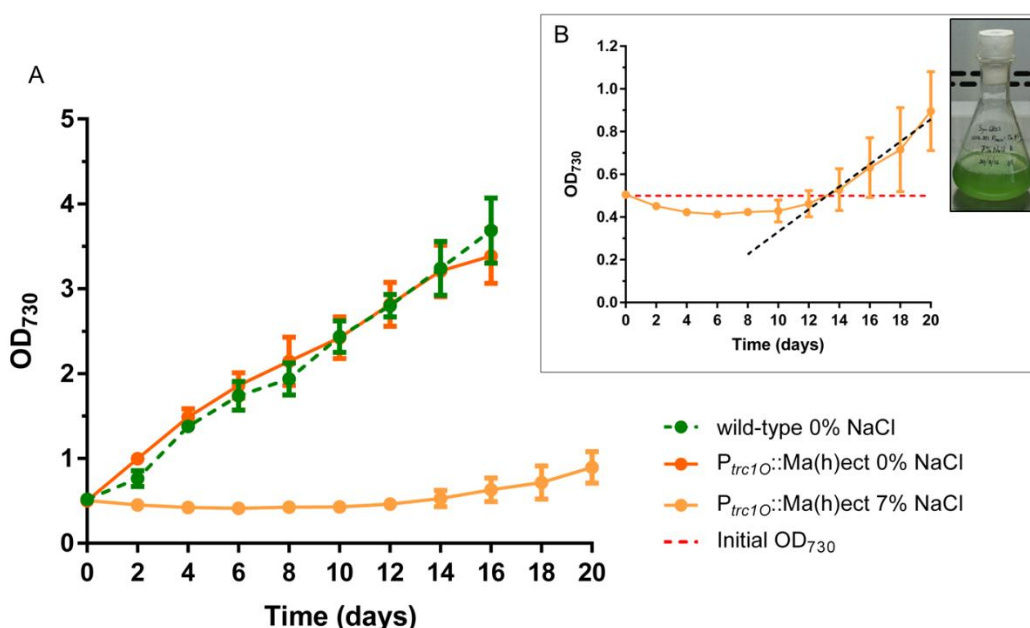


Figure 20 - Growth of *Synechocystis* pSEVA351 P_{trc1O}::Ma(h)ect mutant at different salt concentrations (A) and detail of the growth of the mutant at 7% (w/v) NaCl (B). Cultures were grown in BG11 medium supplemented with 0 and 7% (w/v) NaCl, at 30 ºC and 12 h light (25 μE m^{-2} s^{-1}) / 12 h dark regimen in an orbital shaker at 150 rpm. The OD_{730} of the cultures grown at 0% (w/v) NaCl was measured every 2 days for 16 days. The OD_{730} of the cultures grown at 7% (w/v) NaCl were measured every 2 days for 20 days. The Mean ± SD; n = 3.

In the absence of salt, it was observed that the mutant and the wild-type grow at similar rates. When grown at 7% (w/v) NaCl, the growth of the mutant was inhibited during the first days, however, the cultures maintained a green colour (Figure 20B). Therefore, the halotolerance of this mutant was assessed for a more prolonged period of time and after 12 days the growth was resumed (Figure 20B).

3.4 Growth on seawater - a proof of concept

Synechocystis wild-type strain and the mutants P_{trc1O}::AhBet and P_{trc1O}::Ma(h)ect were grown in seawater with or without nitrogen and phosphorus supplementation (SW+ and SW_0, respectively). The preliminary results show that, in SW_0 medium, the cultures turned yellow (Figure 21A) and did not show a significant growth (Figure 22).

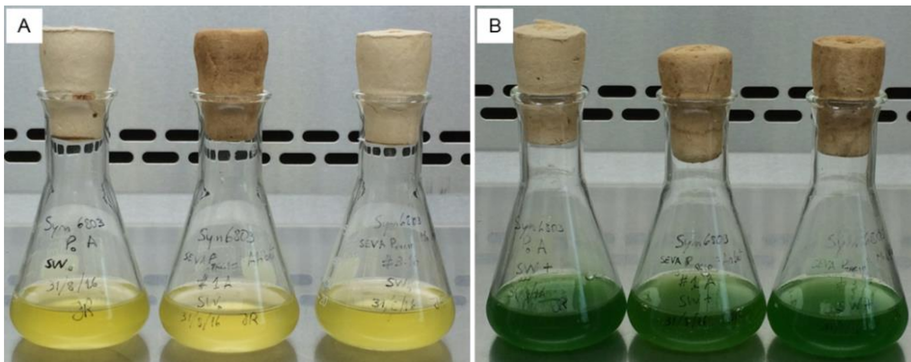


Figure 21 - Liquid cultures of *Synechocystis* wild-type (left), P_{trc1O}::AhBet (middle) and P_{trc1O}::Ma(h)ect (right) in SW_0 (A) and in SW+ (B) after 16 days of exposure.

On the contrary, the strains inoculated in SW+ medium presented the typical green colour (Figures 21B). Besides, no differences were observed in the growth of the wild-type at SW+ and at BG11 with 3% (w/v) NaCl. The same was observed for the mutant carrying the P_{trc1O}::AhBet synthetic device (Figure 22).

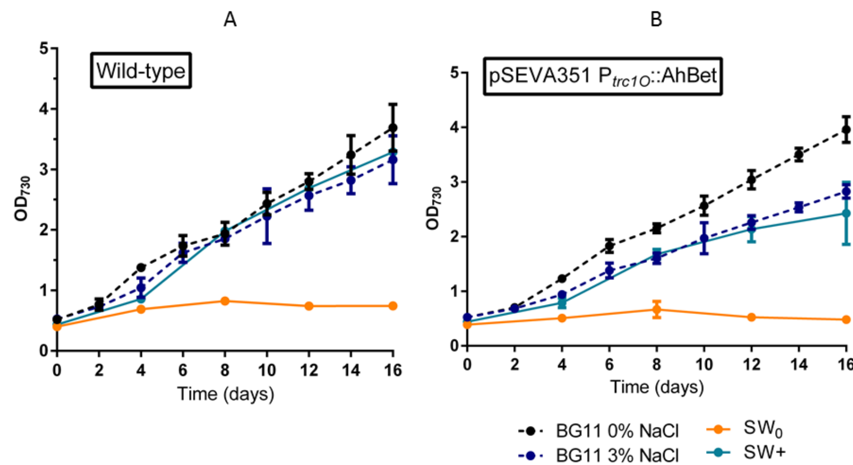


Figure 22 - Growth curve of *Synechocystis* wild-type (A) and P_{trc1O}::AhBet mutant (B) and in BG11, in SW_0 and in SW+. Cultures were grown at 30 ºC and 12 h light (25 µE m^{-2} s^{-1}) / 12 h dark regimen in an orbital shaker at 150 rpm. The OD_{730} of the cultures in BG11 was measured every 2 days and the cultures grown in seawater were measured every 4 days for 16 days. Mean ± SD; n ≥ 3.

Chlorophylls and carotenoids are two classes of pigments that are present in cyanobacteria and are crucial for photosynthesis.[112] Since environmental conditions can influence the pigment composition and since salt stress can inhibit the repair mechanisms of the photosystems, the content of chlorophylls and carotenoids was assessed.[113,114] The preliminary results presented in Figure 23 showed that the mutants have slightly lower amounts of these photosynthetic pigments. However, it is important to note that the P_{trc1O}::Ma(h)ect mutant possesses less pigments than the other strains even when grown in BG11 medium. Therefore, further analyses are required to confirm these changes in pigment composition.

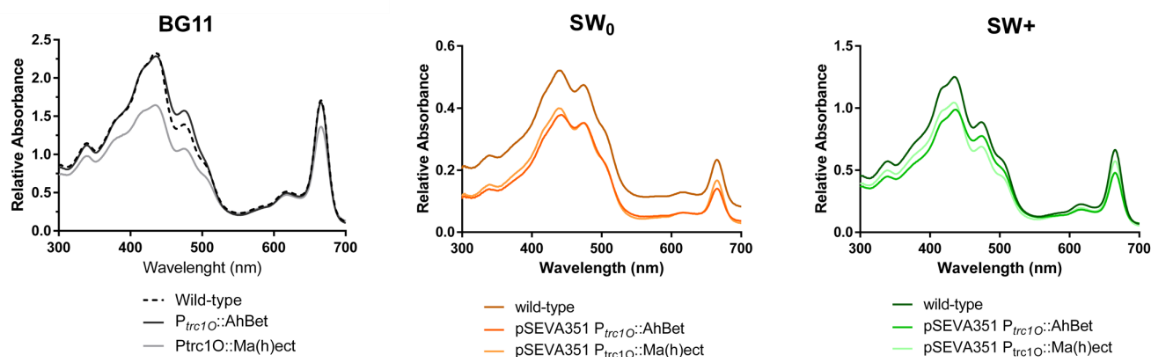


Figure 23 - Absorption spectra of chlorophylls and carotenoids from the *Synechocystis* wild-type and the P_{trc1O}::AhBet and P_{trc1O}::Ma(h)ect mutants when grown in BG11 (left), SW_0 (middle) and SW+ (right).

3.5 Generation of the *Synechocystis* Δ*sps*Δ*ggpS* knockout mutant

To evaluate the effect of heterologous production of compatible solutes in *Synechocystis* without the influence of the native compatible solutes, sucrose and glucosylglycerol, the genes that encode for the first enzyme of each biosynthetic pathways (*sps* and *ggpS*, respectively) were deleted by homologous recombination. Figure 24 represents the approach used to remove the *sps* gene.

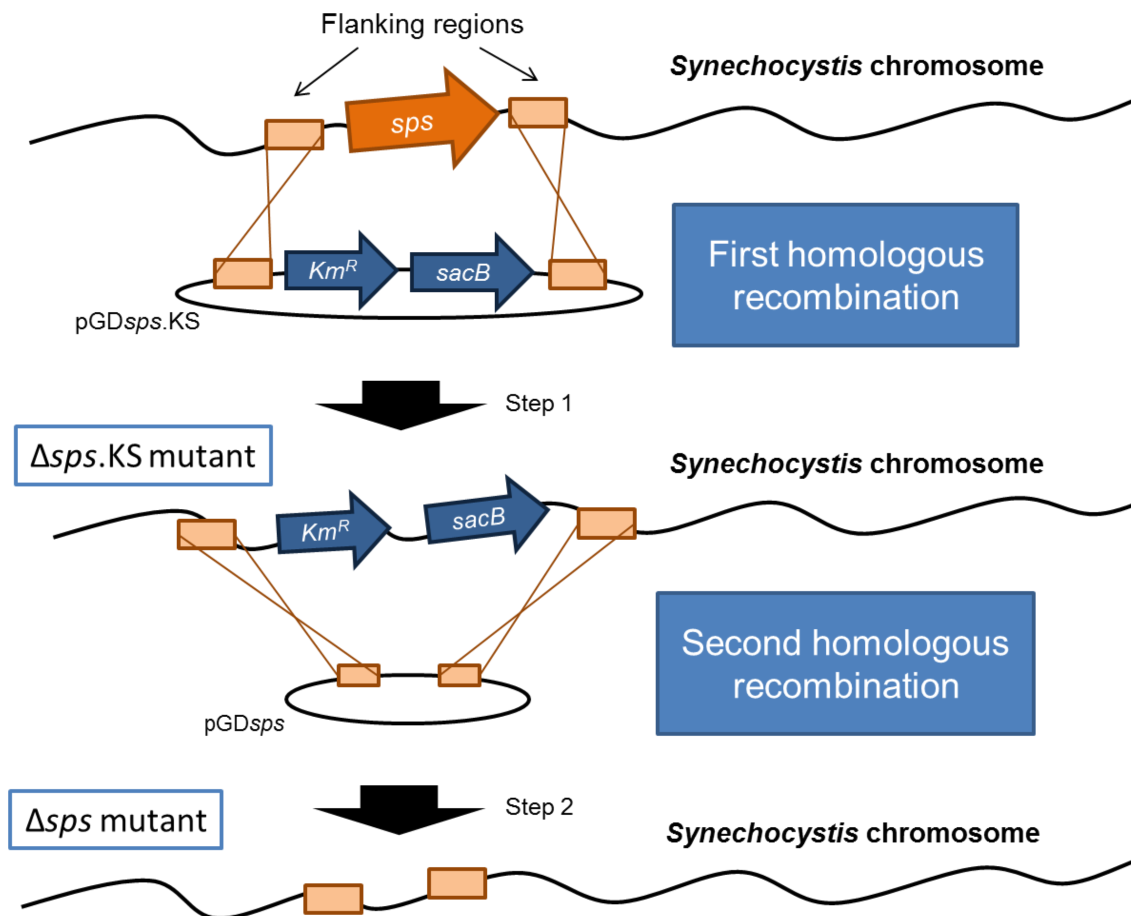


Figure 24 - Schematic representation of the deletion of the *sps* gene by double homologous recombination event. First the gene was replaced by a selection cassette that confers resistance to kanamycin and susceptibility to sucrose (step 1). The transformants are segregated in kanamycin. The selection cassette is then removed by a second homologous recombination (step 2) and the generated mutants are segregated in sucrose. *sps* - gene encoding for the sucrose-phosphate synthase; Km^R - gene conferring Kanamycin resistance; *sacB* - gene conferring susceptibility to sucrose. Adapted from Pinto *et al.* (2012).[53]

The generation of the Δ*sps* knockout mutant was confirmed by PCR to ensure the elimination of the KS selection cassette. In the first PCR, primers that amplify the flanking regions of the *sps* gene were used and a 1464 bp fragment was amplified in all samples, which is

consistent with the size of the flanking regions and the absence of the cassette (Figure 25A). To further confirm these results, a second PCR was performed using primers that bind specifically to the KS selection cassette. When the SacBint_Rev and KmR_Rev primers were used, no amplification was detected, confirming the absence of the selection cassette (Figure 25B).

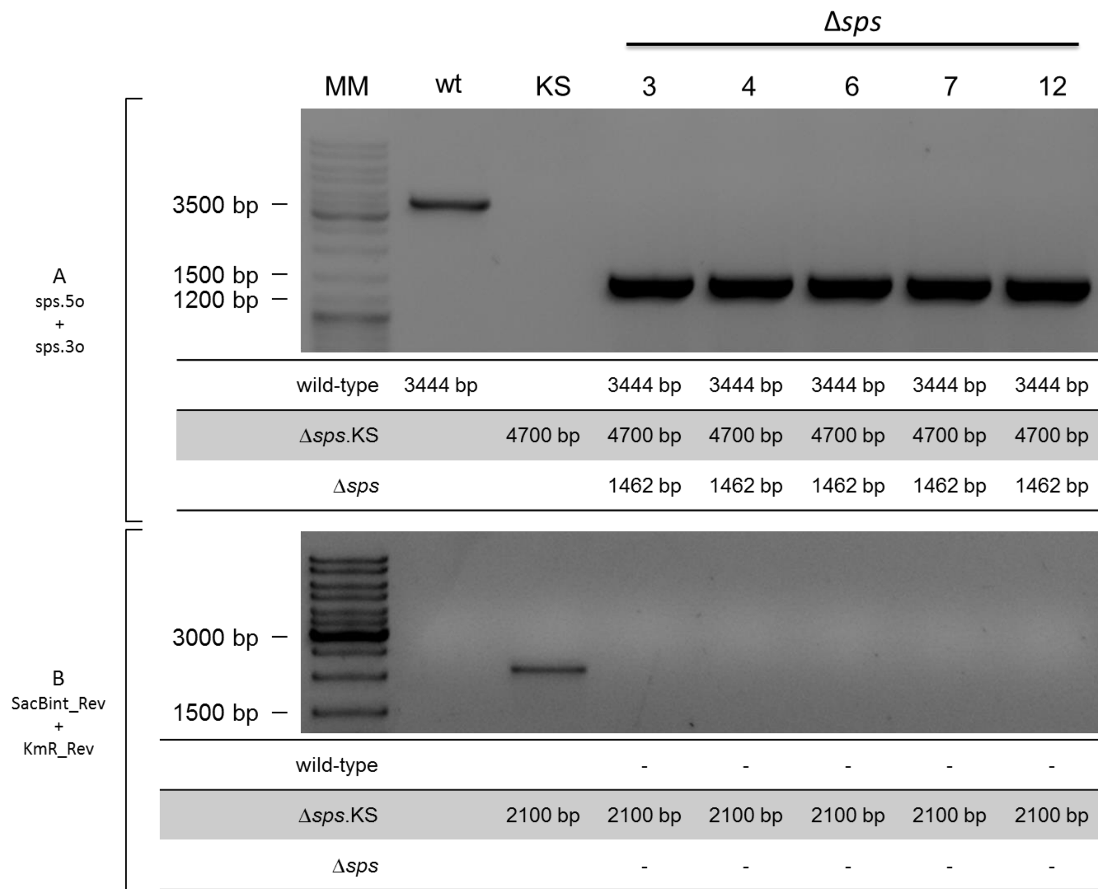
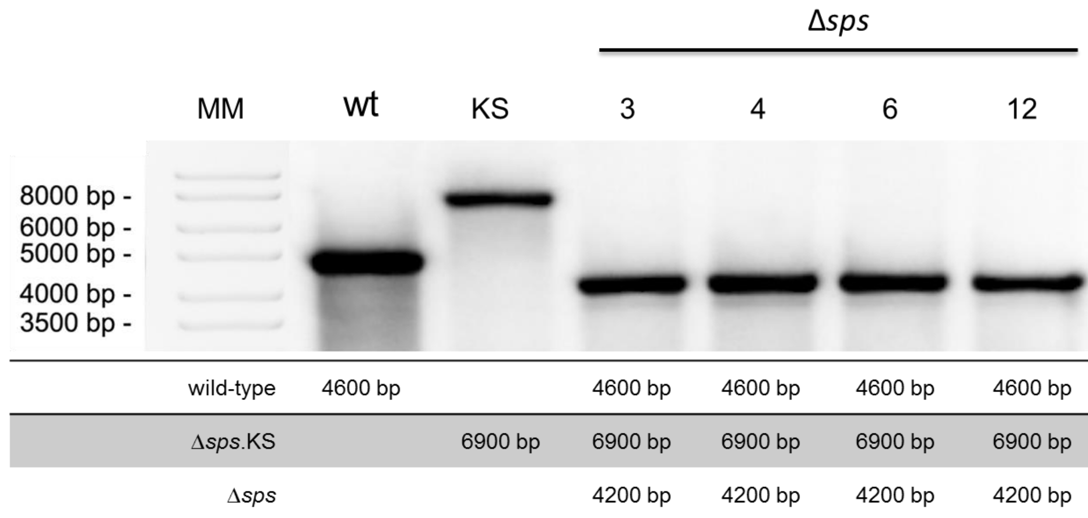


Figure 25 - Confirmation of full segregation of the *Synechocystis* Δ*sps* mutant by PCR. Two reactions were performed: one using the primers that bind to the flanking regions of the *sps* gene (A); a second one using the primers that amplify the KS selection cassette (B) (see table 2). Genomic DNA of the wild-type strains (wt) and pGD*sps*.KS plasmid DNA (KS) were used as controls. Genomic DNA of the clones was used as template for the reactions.

A Southern Blot was also performed for the clones (clones 3, 4, 6 and 12) (Figure 26). A 4 kbp band was observed in all samples, confirming the full segregation of the knockout mutant.



	MM	wt	KS	3	4	6	12
wild-type		4600 bp		4600 bp	4600 bp	4600 bp	4600 bp
Δ*sps*.KS			6900 bp	6900 bp	6900 bp	6900 bp	6900 bp
Δ*sps*				4200 bp	4200 bp	4200 bp	4200 bp

Figure 26 - Confirmation of the segregation of *Synechocystis* Δ*sps* knockout mutant by Southern blot. Genomic DNA was digested with *Mun*I and hybridized with a probe covering the 3' flanking region of the *sps* gene. *Synechocystis* wild type (wt), *Synechocystis* mutant Δ*sps*.KS carrying the selection cassette (KS), and four clones of *Synechocystis* knockout mutants - Δ*sps* - without the selection cassette (Δ*sps* 3, Δ*sps* 4, Δ*sps* 6 and Δ*sps* 12, respectively). MM - molecular weight marker.

The clones 3 and 6 of *Synechocystis* Δ*sps* mutant were selected for the deletion of the *ggpS* gene, using the same strategy used for the removal of the *sps* (Figure 24). Four Km-resistant transformants (3.3, 3.6, 6.3 and 6.6) were screened by PCR, to assess if they were fully segregated. A 250 bp fragment was amplified in all samples when using the primers specific for the selection cassette, confirming the presence of the cassette (Figure 27A). However, the amplification of a 590 bp fragment in the samples of the clones 3.3, 6.3 and 6.6 when using the primers specific for the *ggpS* gene indicated that the replacement of the gene by the cassette was incomplete (Figure 27B).

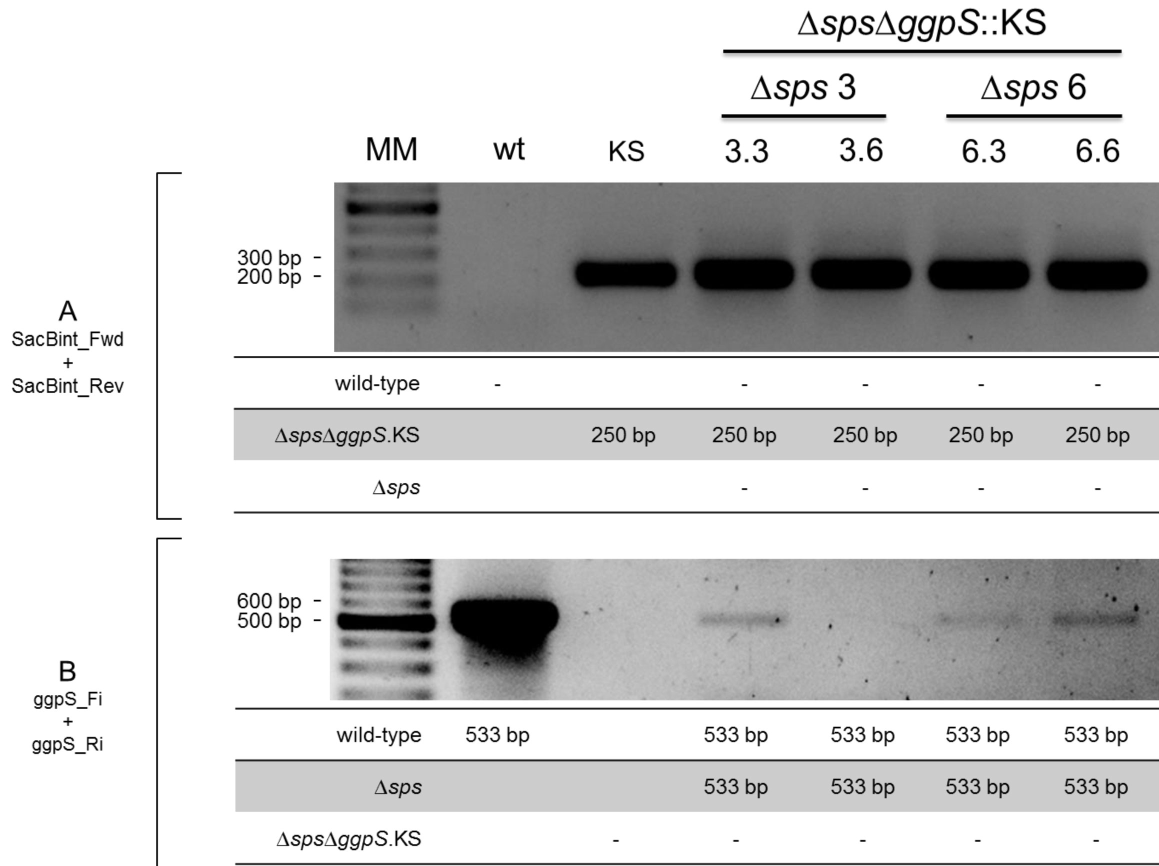


Figure 27 - Screening of the *Synechocystis* Δ*sps*Δ*ggpS*.KS by PCR. Two PCR reactions were performed, using genomic DNA as template: one was carried out using the primers SacBint_Rev and KmR_Rev, specific for the KS selection cassette (A); and another one using the primers ggpS_Fi and ggpS_Ri, specific for the *ggpS* gene (B). *Synechocystis* wild-type genomic DNA (wt) and pGD*ggpS*.KS plasmid DNA (KS) were used as controls. MM - molecular weight marker.

Only the clone 3.6 was completely segregated. Therefore, the clones were transferred to liquid BG11 medium supplemented with higher concentrations of Km, to force the replacement of all the copies of the *ggpS* gene by the cassette. The segregation process is ongoing.

4 Discussion and future perspectives

The unicellular model cyanobacterium *Synechocystis* sp. PCC 6803 has the potential to be used as a cell factory in many biotechnological applications for the production of added-value compounds.[25,26,29] For that purpose, it is necessary to grow this cyanobacterium in large scale bioreactors. The mass-cultivation in salt water would allow the utilization of an abundant hydric resource that does not compete with our needs for freshwater and minimizes the possibility of contamination with other microorganisms.[37] It was previously demonstrated by our research group that *Synechocystis* can withstand salt concentrations up to 6% (w/v) NaCl.[115] However, despite being able to grow in media with approximately the same salt concentration as the average salinity of the seawater, the growth of *Synechocystis* is impaired and the salt stress causes the inhibition of the repairing mechanisms of the photosystems.[113] One strategy to increase the performance of this strain is to increase the production of native compatible solutes, namely glucosylglycerol. Such approach was already implemented in this group, by overexpressing the genes encoding the enzymes involved in glucosylglycerol biosynthesis.[116] However, no significant improvement in the growth of the chassis was observed under different salinities.[116] It is important to note that there is a correlation between the compatible solutes produced by the cells and their tolerance limit and, thus, increasing the production of the native solute does not lead to an increase of the halotolerance of the chassis.[65,81] Therefore, the present work focused on the implementation of pathways leading to the heterologous production of the more potent compatible solutes betaine and ectoines. Besides their stronger effects on protecting cells against osmotic stress, betaine and ectoines are also added-value compounds [82,93], so their production in a photoautotrophic chassis would also have relevance for the industry.

Regarding the *Synechocystis* mutant harbouring the betaine device, pSEVA351 P_{trc1O}::AhBet, it was observed that the introduction of the synthetic device did not change the growth rates of the chassis when grown at 0% (w/v) NaCl. Furthermore, the cells were able to grow when exposed to 7% (w/v) NaCl, which is above the tolerance limit of the wild-type strain. However, it was not possible to relate this phenotype with the transcription of the genes comprising the device. Moreover, a preliminary assessment of compatible solute production was also performed and no betaine was detected in the mutant when grown at 3% (w/v) NaCl. On the other hand, glugosylglycerol was present in levels similar to the wild-type, which suggests that the growth of the mutant at 7% (w/v) NaCl is related with the

presence of the synthetic device. Previous work on heterologous expression of the genes of *A. halophytica* related to betaine production in *Synechococcus* sp. PCC 7942 and *Anabaena* sp. PCC 7120 demonstrated the production of betaine even in the absence of NaCl.[74,117] However, the same behaviour was not observed in our mutant. One possibility for these unexpected results might be related to the strength of the promoter used in the assembly of the device. The synthetic device possesses a strong constitutive promoter and it was introduced in *Synechocystis* using a low copy number plasmid (it is estimated that, in normal conditions, about 10 plasmid copies are present per cell[118]). As betaine is a potent compatible solute[74], perhaps the concentration required for the survival of the mutant is very low and, therefore, the need to maintain a low amount of this compatible solute could only be achieved by reducing the number of plasmid copies. This would difficult the confirmation of the presence of the device in the mutant. Nonetheless, the transcription of the genes and the production of compatible solutes needs to be further assessed. Waditee *et al.* (2012) also showed that the betaine accumulation increased with the salt concentration, demonstrating that this biosynthetic pathway is regulated by the ionic strength. Therefore, the quantification of compatible solutes should be assessed for higher salt concentrations, particularly 7% (w/v) NaCl. It is important to note that in the abovementioned works, a two-step strategy is used: (i) the cultures are grown in the absence of salt to obtain significant biomass and (ii) the cultures are subjected to salt stress and then the production of betaine increases.

For a more stable expression of the betaine device, another mutant was generated by integrating it in the genome of *Synechocystis*. In this case, both the presence and the transcription of the device were confirmed but the mutant did not present the phenotype previously observed. Therefore, it seems that the heterologous expression of the synthetic device constitutes a metabolic burden to the cells. As the device is integrated in the genome, the cells cannot regulate the expression by altering the number of copies of the device and, thus, the burden is probably more pronounced, comparing with the mutant harbouring the device in a replicative vector.

The growth of the wild-type and the mutant carrying the pSEVA351 P_{trc1O}::AhBet was also performed in gas wash bottles as a faster growth cultivation method. In these conditions, the gas exchanges are higher, allowing the cells to perform photosynthesis at higher rates and, consequently, grow faster. However, growth at 7% (w/v) NaCl was not observed. The salt stress has negative effects on the photosynthetic machinery.[113] Therefore, the cells may not be able to produce the compatible solutes due to the additional metabolic burden related to

the repair of the damages caused by the presence of salt. In accordance with the predictions of the genome scale metabolic model of *Synechocystis*[55], the betaine does not pose significant energy losses, but can interfere with the pentose phosphate pathway, which is related with base pair synthesis and DNA replication. The external supplementation with glycine, the precursor of the biosynthesis of betaine, could be a strategy to prevent the eventual shortage of this amino acid, alleviating the metabolic burden in the mutant harbouring the pSEVA351 P_{trc1O}::AhBet and improving its performance at high salt concentrations. Waditee *et al.* (2012) showed that supplementing *A. halophytica* with glycine resulted in higher yields of betaine. However, in *Anabaena* sp. PCC 7120, growth inhibition was observed upon supplementation with this amino acid.[74] The lack of betaine may also be related to the accumulation of one of the intermediates, due to possible differences in enzymatic activities and/or different amounts of each enzyme from this pathway.

Regarding the *Synechocystis* mutant harbouring the ectoine device, pSEVA P_{trc1O}::Ma(h)ect, the cultures do not grow but keep viable when exposed to a 7% (w/v) NaCl concentration. Moreover, it seems that the cultures have an acclimation phase during the first 10 days, before resuming growth. This preliminary characterization suggests that the metabolic burden imposed by the synthesis of these compatible solutes is more severe than the one observed for betaine. According to the predictions of the genome scale metabolic model of *Synechocystis*[55], the precursor of ectoines is obtained from oxaloacetate, so their production in high titters would drain a central metabolite from the tricarboxylic acid cycle, contributing to great energy loss to the cells. Supplementation with aspartate would constitute a strategy that could increase the production of ectoines without great interference with the TCA cycle. However, to confirm this phenotype, further tests are needed. Besides the characterization of the growth of this mutant at different salt concentrations, transcription analysis should also be performed, to confirm the expression of the genes, as well as the quantification of ectoines by NMR or HPLC.[91,119]

Another method to assess the performance of the mutants would be the determination of their content on photosynthetic pigments. As previously mentioned, salt stress has inhibitory effects on the photosystems repair mechanisms. Hence this assessment would allow the evaluation of the extent on the photosystems damage. Preliminary tests were performed for the strains, grown in BG11 liquid medium in the absence of salt, and the results showed that the mutant that carries the betaine-device does not exhibit differences in pigment content comparing to the wild-type, while a slightly lower amount of chlorophylls and carotenoids was

observed for the mutant harbouring the ectoine-device. These results might be due to the more pronounced metabolic burden associated with the production of ectoines. Therefore, these analyses should be confirmed and further assessments should be conducted in the presence of salt (3 and 7% (w/v) NaCl) and at different time points to evaluate changes on pigments over time. Oxygen evolution is another good technique to evaluate the photosynthetic efficiency of these mutants under salt stress. The activity of oxygen evolution of the photosystem II is known to decrease almost linearly with increasing salt concentration.[120] Therefore, the production of oxygen would allow the analysis of the effectiveness of these mutants to grow in saline waters.

Considering all the previously mentioned results, the introduction of these synthetic devices seems to be a promising approach to increase the resistance of *Synechocystis* to salinity. Nevertheless, its expression is too strong and it constitutes a metabolic burden to the cells when exposed to salt stress. Therefore, the redesign of the synthetic device is an approach worth exploring. This could be achieved by the utilization of a weaker promoter, similar to P_{psbA2*}. Besides the modification of the devices, the performance of *Synechocystis* mutants could also be improved through chassis engineering. One possibility is the elimination of the biosynthetic pathways of the native compatible solutes, sucrose and glucosylglycerol. It is important to note that the mutants were generated using the *Synechocystis* wild-type strain as chassis, so they produce the native compatible solutes, which consumes energy. This approach is already being addressed by the generation of the Δ*sps*Δ*ggpS* double knockout mutant that will serve as chassis for the implementation of these synthetic devices. Another possibility is the overexpression of key enzymes for the production of the precursors of betaine and ectoines. Waditee *et al.* (2007) has shown that overexpressing 3-phosphoglycerate dehydrogenase from *A. halophytica* in *E. coli* led to an increase in the serine levels. Because serine and glycine are interconvertible, the glycine levels were increased, and an increase in betaine production was observed.[74,121]

Finally, preliminary tests on the performance of the *Synechocystis'* strains grown in seawater were also conducted. The main objective of this work was the generation of a chassis with increased robustness towards salinity and thus, these tests functioned as a proof of concept for the usage of seawater for mass-cultivation of *Synechocystis*. The results showed that all the strains are capable of growing in seawater, but only when supplemented with nitrogen and phosphorous sources. Nitrogen and phosphorus are two macronutrients that are essential for DNA replication, ATP production and protein synthesis. Besides, nitrogen

starvation leads to changes in the central carbon metabolism of cyanobacteria, due to the redirection of carbon to the synthesis of storage compounds and to a loss in phycobiliproteins, thus interfering with photosynthesis.[26,122–124] The amount of photosynthetic pigments of the mutants is significantly lower than when grown in BG11 medium, which is probably related to the presence of salt, as well as the lower availability of nitrogen and phosphorus. Nonetheless, more tests are required to better characterize the performance of the chassis when grown in seawater.

In conclusion, it was demonstrated that the introduction of the P_{trc1O}::AhBet synthetic device in *Synechocystis* allowed the mutant to grow at salt concentrations above the halotolerance limit of the wild-type strain. However, it was not possible to relate this phenotype with neither the transcription of the genes comprising the device nor the production of the compatible solute. These results suggest that the insertion of the synthetic device in *Synechocystis* poses a high metabolic burden to the cells when exposed to salt stress. The phenotype observed for the mutant that carries the P_{trc1O}::Ma(h)ect device when grown at 7% (w/v) NaCl is also indicative of metabolic burden. Therefore, this could be alleviated by the redesign of the devices. Chassis engineering constitutes another viable strategy to reduce the metabolic burden. It could be addressed by eliminating biosynthetic pathways of the native compatible solutes and/or to overexpress key enzymes involved in the synthesis of the precursors of the heterologous compatible solutes.

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6 Supplementary data

6.1 Poster communication

J. Rodrigues, C. C. Pacheco, P. Tamagnini. Improvement of *Synechocystis* sp. PCC 6803 halotolerance using a Synthetic Biology approach for the production of compatible solutes. IJUP 2016, 9th edition - Porto, Portugal.

Improvement of *Synechocystis* sp. PCC 6803 halotolerance using a Synthetic Biology approach for the production of compatible solutes

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ENCONTRO INVESTIGAÇÃO JOVEM

Introduction

Cyanobacteria are photoautotrophic prokaryotes that due to their minimal nutritional requirements can be used in many biotechnological applications to produce added-value compounds. Among cyanobacteria, *Synechocystis* sp. PCC 6803 is the best characterized strain and its medium-size genome is completely sequenced and annotated[1].

In order to grow cyanobacteria in large-scale bioreactors it is necessary to use large amounts of freshwater. Moreover, contamination and predation by other microorganisms can compromise the growth of the cyanobacterial cultures and consequently the yield of the desired compound. Therefore, the use of seawater instead of freshwater could turn the production in outdoors bioreactors more feasible[2].

This study focuses on the construction of photoautotrophic chassis based on the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 with increased robustness towards salinity. This will be achieved by introduction of synthetic devices for the production of compatible solutes.

Methods

Synechocystis sp. PCC 6803 mutants capable of synthesizing heterologous compatible solutes

Synechocystis sp. PCC 6803 wild type strain and a double knockout mutant lacking the genes *sps* and *ggpS* (involved in the production of the native solutes sucrose and glucosylglycerol, respectively) will be used as chassis. This will allow us to evaluate the heterologous production of compatible solutes with and without the native osmolytes. The double knockout mutant will be generated by double homologous recombination followed by segregation in BG11 medium with proper antibiotics (Pinto *et al.* - 2012)[3].

Synthetic devices for the production of compatible osmolytes were based on genes from three different halophilic microorganisms. The genes were codon optimized for *Synechocystis* sp. PCC 6803 and the devices were designed according to the BioBrick™ assembly standard RFC10[4]. The devices will be organized in operons and the transcription will be regulated by one of two synthetic promoters, with different relative strengths. The synthetic devices will be then introduced into *the* chassis by electroporation.

Halotolerance growth experiments

Liquid cultures of the *Synechocystis* mutants will be grown at 30 °C with shaking at 150 rpm and under a 12 h light (25 µE m^{-2} s^{-1}) / 12 h dark regimen, in media with different NaCl concentrations. The growth will be evaluated by measuring the optical density at 730 nm. *Synechocystis* wild type and the double knockout mutant will be used as controls.

The compatible solute production will also be measured in extracts, obtained from the cultures at different time points, using the KI-I_2 spectrophotometric assay[5] and by Nuclear Magnetic Resonance (NMR).

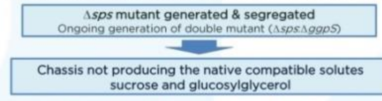
Results

1. Generation of the double knockout mutant (Δ*sps*:Δ*ggpS*)

Confirmation of the deletion of the *sps* gene by Southern blot using a probe that hybridizes with the 3' flanking region of the gene.

	Expected size (bp)
wt	4600
Δsps::KS	7000
Δsps	4000

Fig. 1 - Confirmation of the segregation of *Synechocystis sps* knockout mutant by Southern blot. Genomic DNA was digested with *Mun*I and hybridized with a probe covering the 3' flanking region of the *sps* gene. *Synechocystis* sp. PCC 6803 wild type (wt), *Synechocystis* sp. PCC 6803 mutant Δsps::KS carrying the selection cassette (KS), and four clones of *Synechocystis* sp. PCC 6803 knockout mutants - Δsps - without the selection cassette (Δsps 1, Δsps 2, Δsps 3 and Δsps 4). GeneRuler DNA Ladder (ThermoFisher) was used as molecular marker.



Δ*sps* mutant generated & segregated
Ongoing generation of double mutant (Δ*sps*:Δ*ggpS*)

Chassis not producing the native compatible solutes sucrose and glucosylglycerol

2. Construction of the synthetic device for the production of an heterologous compatible solute

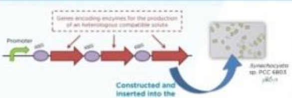


Fig. 4 - Schematic representation of the synthetic device (CS1) that was introduced into one of our chassis - *Synechocystis* sp. PCC 6803 wild type - for the production of an heterologous compatible solute. The device was constructed based on a halophilic cyanobacterium and codon optimized for *Synechocystis*.

3. Confirmation of the integration of the synthetic device CS1 into the chassis

Confirmation by Colony PCR of two clones using primers specific for the synthetic device

	Expected size (bp)
Syn PCC 6803 (wt)	No amplification
pDNA with synthetic device (+)	3500
Syn PCC 6803 mutant (A)	3500

Fig. 3 - Colony PCR on *Synechocystis* sp.PCC 6803 wild type (wt) and *Synechocystis* mutant carrying the synthetic device CS1 (clones A1 and A2). The PCR was performed using specific primers that amplify the synthetic device portion. A negative control (-) was performed and plasmid DNA containing the synthetic device was used as positive control (+). The PCR products were run in a 1% (w/v) agarose gel 1x TAE buffer and stained with Green Safe Premium (NZY tech). GeneRuler Ladder Mix (ThermoFisher) was used as molecular marker (MM).

A *Synechocystis* mutant carrying the synthetic device CS1 was obtained

4. Characterization of the mutant carrying the synthetic device CS1 and functionality of the device

Fig. 4 - Liquid cultures of *Synechocystis* sp. PCC 6803 wild type (left) and mutant synthetic device CS1 (right) in BG11 medium supplemented with 7% (w/v) NaCl

The mutant with the synthetic device CS1 can grow at high salt concentrations while the wild type cannot

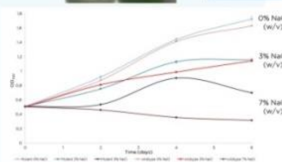


Fig. 5 - Characterization of *Synechocystis* sp. PCC 6803 wild type (red) and mutant carrying the synthetic device CS1 (blue) in BG11 without NaCl or supplemented with different concentrations of NaCl. The cultures were grown at 30 °C with shaking at 150 rpm and under a 12 h light (25 µE m^{-2} s^{-1}) / 12 h dark regimen.

Conclusion: Preliminary results show an increased robustness towards salinity of our strain containing the synthetic device

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