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Construct generation for the production of the heterologous compatible solute glycine betaine using *Synechocystis* sp. PCC 6803 as photoautotrophic chassis

Rui André Simões Martins
Mestrado Integrado em Bioengenharia, ramo de Biotecnologia Molecular, Faculdade de Engenharia da Universidade do Porto

Orientador
Professora Doutora Paula Tamagnini
Professora Associada da Faculdade de Ciências da Universidade do Porto; Líder de grupo no i3S- Instituto de Investigação e Inovação em Saúde, Instituto de Biologia Molecular e Celular, Universidade do Porto

Co-orientador
Doutora Catarina Pacheco
Investigadora Auxiliar no i3S - Instituto de Investigação e Inovação em Saúde, Instituto de Biologia Molecular e Celular, Universidade do Porto

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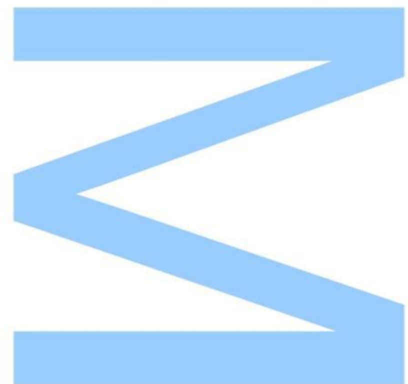

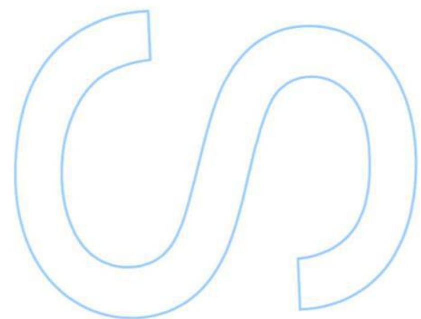

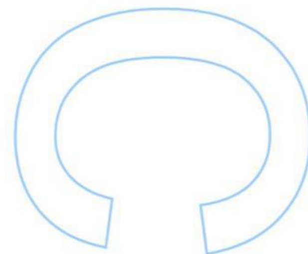

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Construct generation for the production of the heterologous compatible solute glycine betaine using *Synechocystis* sp. PCC 6803 as photoautotrophic chassis

Abstract

Cyanobacteria are a large group of prokaryotic photosynthetic organisms that are present in an enormous variety of habitats and show different morphologies. Their capacity to perform photosynthesis brought a lot of attention for their research and development as biofactories due to the low investment needed for its maintenance, for example in feedstock costs and metabolic requirements - water, CO_2, sunlight and minerals - compared to other bacteria used in industry for the synthesis of bio-compounds. *Synechocystis* sp. PCC 6803 is one of the best studied cyanobacteria, its genome is completely sequenced, it is amenable to genetic manipulation, it is possible to predict the system's behavior due to existing metabolic flux models and therefore, it is considered ideal chassis for the synthesis of heterologous compounds. Although in the future many compounds with economic interest will be produced by these bacteria in large bioreactors, the use of cyanobacteria for bioproduction presents a drawback, the amount of water needed for cultivation is high, bringing up the problem of freshwater consumption. The volume of water needed for synthesis of small quantities of products is unbearable from an environmental point of view and a strategy is needed to overcome this issue. Seawater is an efficient alternative, but *Synechocystis*' growth is affected in these conditions as the salinities are above the ideal for this organism. Glycine betaine is a compatible solute that could be used to increase *Synechocystis*' halotolerance, but the synthesis of this solute is not natural in this bacterium and needs to be genetically engineered. A synthetic device for the synthesis of the compatible solute glycine betaine, including the genes coding for glycine/sarcosine-N-methyltransferase (GSMT) and dimethylglycine-N-methyltransferase (DMT) - Ahbet cluster -, was previously designed and assembled in our lab. In this module, each open reading frame (ORF) is preceded by the ribosome binding site (RBS) B0030 and the module is under the control of the strong constitutive promoter P_{trc1O}. The characterization of *Synechocystis* harboring this device suggested that it needed to be fine-tuned by changing the regulatory elements. Therefore, in this work, the P_{trc1O} promoter was replaced by other synthetic promoters with lower strengths and that were previously characterized by the research group. Two synthetic devices meant for the synthesis of betaine in *Synechocystis* were obtained by reassembling betaine cluster with the promoters $P_{trc.x.lacI}$ and P_{J23101}.

Construct generation for the production of the heterologous compatible solute glycine betaine using *Synechocystis* sp. PCC 6803 as photoautotrophic chassis

Resumo

As cianobactérias são um vasto grupo de organismos fotossintéticos procarióticos que existem numa enorme variedade de habitats e possuem diferentes morfologias. A capacidade de fazerem fotossíntese trouxe imensa atenção para a sua pesquisa e desenvolvimento como “fábricas biológicas” devido ao baixo investimento necessário na sua manutenção, como por exemplo em custos metabólicos e de matéria prima – água, CO_2, luz solar e minerais – comparado a outras bactérias usadas em indústria para a síntese de compostos biológicos. *Synechocystis* sp. PCC 6803 é uma das cianobactérias mais bem estudadas, o seu genoma está completamente sequenciado, pode ser manipulada geneticamente, é possível prever o comportamento do sistema devido à existência de modelos de fluxo metabólico e, portanto, é considerada um chassis ideal para a síntese de compostos heterólogos. Apesar de que no futuro muitos compostos com interesse económico vão ser produzidos por estas bactérias em bio-reatores de grandes dimensões, o uso de cianobactérias para produção de compostos biológicos apresenta uma desvantagem, a grande quantidade de água potável necessária para o seu cultivo. O volume de água necessário para a síntese de baixas quantidades de produtos é intolerável de um ponto de vista ambiental e é necessária uma estratégia para ultrapassar este problema. A água do mar é uma opção, mas o crescimento de *Synechocystis* é afetado nestas condições uma vez que os níveis de salinidade são superiores aos ideais para este organismo. A glicina betaína é um soluto compatível que poderia ser usado para aumentar a halotolerância de *Synechocystis,* mas a síntese deste soluto não é natural nesta bactéria e precisa de ser inserida por meios de engenharia genética. Um módulo sintético para a síntese deste soluto compatível, que inclui os genes que codificam as enzimas glicina/sarcosina-N-metiltransferase (GSMT) e dimetilglicina-N-metiltransferase (DMT) - operão Ahbet -, foi previamente desenhado no nosso laboratório. Neste módulo, cada sequência codificante (ORF) é precedida por um local de ligação ribossomal (RBS) B0030 e o módulo está sob controlo do promotor constitutivo P_{trc1O}. A caracterização de *Synechocystis* com este conjunto de genes revelou que havia necessidade de alterar os elementos regulatórios. Assim sendo, neste trabalho, o promotor P_{trc1O} foi substituído por outros promotores sintéticos com forças inferiores e que foram previamente caracterizados pelo grupo. Dois módulos sintéticos tendo em vista a síntese de betaína em *Synechocystis* foram obtidos pela junção do operão de genes que codificam

as proteínas envolvidas na produção de betaína com os promotores $P_{trc.x.lacI}$ e P_{J23101}.

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List of abbreviations

AhBet - synthetic gene cluster for betaine production

Amp - ampicillin

Amp^R - ampicillin resistance

Cm - chloramphenicol

Cm^R - chloramphenicol resistance

GB - Glycine Betaine

GG - glucosylglycerol

GGA - glucosylglycerate

GSMT - glycine-sarcosine-N-methyltransferase

Km - kanamycin

Km^R - kanamycin resistance

LB - lysogeny broth

RBS - ribosome binding site

rpm - revolutions per minute

RT-PCR - reverse transcription polymerase chain reaction

SAH - S-adenosyl-L-homocysteine

SAM - S-adenosyl-methionine

Suc - Sucrose

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

Tre - Trehalose

Construct generation for the production of the heterologous compatible solute glycine betaine using *Synechocystis* sp. PCC 6803 as photoautotrophic chassis

Introduction

Cyanobacteria and compatible solutes

Cyanobacteria have been carving their path on earth for more than 3000 million years (Schopf, 1993). As photosynthetic organisms, they colonized every light-exposed environment, from desert rocks to freshwater and marine ecosystems (Pade & Hagemann, 2014). These prokaryotes are gram-negative and exist in unicellular, colonial or filamentous morphologies (Oren, 2014).

Different environments are associated with different stress factors, which can be physical and/or chemical parameters. Temperature, pH levels, gamma radiation doses, osmotic activity and hydrostatic pressure are all examples of parameters that can affect an organism's capability to survive. Strains that are able to resist extreme levels of a parameter are considered extremophiles (da Costa, Santos, & Galinski, 1998). One parameter that has a big influence in the adaptation of organisms, and therefore cyanobacteria, to the environment is total salinity. Total salinity contemplates the concentration of dissolved ions in a determined compartment or system, and it varies in total value as well as in diversity of ions. Together with water availability, these factors have a considerable importance in an organism's ability to grow and survive (Pade & Hagemann, 2014).

A decrease of extracellular water levels may cause the cell to lose water to the environment to achieve equilibrium in inorganic ions concentrations. Loss of water leads to a decrease in cell volume and turgor pressure and to an increase in internal ion concentrations, leading to possible damage in the cell's metabolism or even to its stop, denaturation of proteins and interference with other macromolecules, with some ions being able to exert direct toxic effect in the cell. This shortage of water availability and its effects in the cell can be achieved not only if water is loss but also if solute levels present in the environment increase. Failing to adapt to these circumstances on the long run may result in death (Pade & Hagemann, 2014). Some strategies were developed through time to deal with the situations mentioned above. "Salt-in" and "salt-out" strategies are normally employed by microorganisms. "Salt-in" consists in the accumulation of inorganic salts inside the cell when external salt concentrations are too high, with the purpose of exceeding it to create inward water tendency. "Salt-out" is a strategy that spends energy to expel

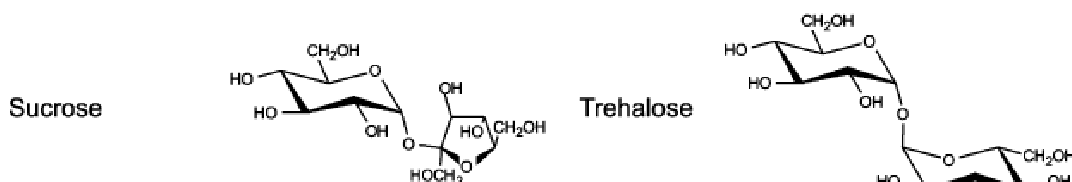
salt from the cell to avoid protein aggregation. Instead of accumulating inorganic ions, cyanobacteria, as well as most of microorganisms, use the "salt-out" strategy and accumulate compatible solutes (Hagemann, 2013).

Compatible solutes are a functional group of small, highly soluble, low-weight organic molecules that can be accumulated inside cells in high quantities, helping them deal with environmental stress without interfering in their metabolism (da Costa et al., 1998; Hagemann, 2013; Pade & Hagemann, 2014). These compounds include sugars, polyols and amino acids and their derivatives, that can have not only stress-counter effects but also stabilization effects (Ducat, Way, & Silver, 2011; Shivanand & Mugeraya, 2011).

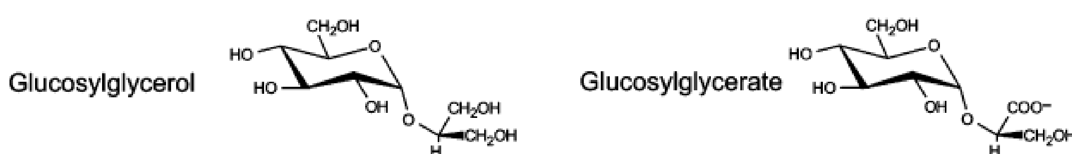
In cyanobacteria, different salinities lead to different compatible solutes being synthesized. For the evaluation of salinity levels, only NaCl will be considered as it is the most abundant salt in water environments. Some compatible solutes are more commonly produced in low salt concentrations and others in medium and high salt concentrations. In freshwater environments, organisms are adapted to low ionic strength and cyanobacteria from these habitats produce mainly sucrose (Suc) and trehalose (Tre) (Fig. 1) to deal with concentrations up to 3,5% NaCl (≈ 600 mM). Many cyanobacteria from marine environments portray moderate salt tolerance and the main compatible solutes produced are glucosylglycerol (GG) and less often glucosylglycerate (GGA) (Fig. 1), to resist salinities up to 10% NaCl, about 1,7 M. Finally, cyanobacteria from hypersaline environments manifest high salt tolerance, up to 17,5% NaCl (about 3,0 M) producing mainly glycine betaine (GB). High and moderate salt tolerance strains are also capable of accumulating other compatible solutes at lower NaCl concentrations, like Suc, Tre and GG (Fig. 1) (Klähn & Hagemann, 2011). The effect compatible solutes exert on molecules inside the cell can be explained by the water exclusion model, where is stated that they alter the water structure without any direct binding to macromolecules, but promoting free water to head for their surface, therefore maintaining hydration, preventing denaturation and allowing polypeptide chains to properly fold. These characteristics suggest multiple applications for compatible solutes and therefore a high commercial demand (Pade & Hagemann, 2014; Shivanand & Mugeraya, 2011).

This work will be focused in the heterologous synthesis of the compatible solute glycine betaine in *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*). In general, betaines have shown therapeutic potential in a variety of areas. They have the capacity to reduce side effects of anti-inflammatory compounds, treat initial stages of cirrhosis and lower hypothesis of heart attacks and strokes due to their anticoagulant properties (Shivanand & Mugeraya, 2011).

1. Fresh water strains: tolerance limit 0.6 M NaCl



2. Moderately halotolerant strains: tolerance limit 1.7 M NaCl

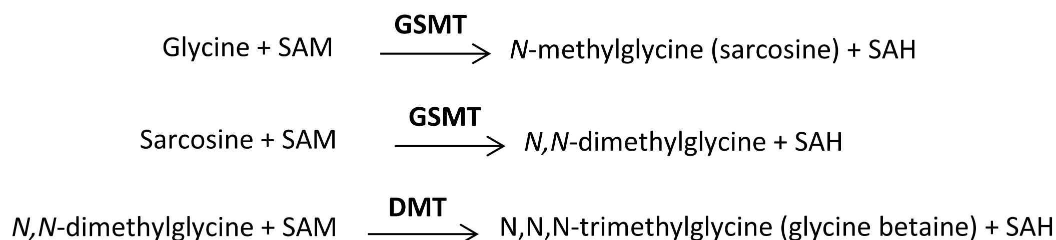


3. Halophilic strains: tolerance limit 3.0 M NaCl

Figure 1 – Different compatible solutes produced by cyanobacteria according to different salt concentrations, withdrawn from (Hagemann, 2011), with permission from Oxford University Press.

Glycine betaine (GB, N,N,N-trimethylglycine) was discovered in the 19th century and is considered the original betaine. It is a quaternary ammonium compound (Empadinhas & Da Costa, 2008; Hagemann, 2011). and is frequently produced by halophilic and halotolerant cyanobacteria and taken up by many other organisms that use it to cope with salt stress (Empadinhas & Da Costa, 2008). Synthesis of this solute in the majority of organisms can be achieved by oxidation of its precursor choline in a two-step pathway, first choline dehydrogenase catalyzes the formation of betaine aldehyde and then betaine aldehyde dehydrogenase the synthesis of glycine betaine (Empadinhas & Da Costa, 2008; Hagemann, 2013). Although the previous pathway is very common, another route examined in the cyanobacterium *Aphanothece halophytica* is characterized by a direct methylation of glycine, a mechanism that seems to be preferably used by

all cyanobacteria accumulating GB. This pathway consists in reactions carried out by the enzymes glycine/sarcosine-N-methyltransferase (GSMT) and dimethylglycine-N-methyltransferase (DMT) in order to methylate glycine with the contribution of a methyl donor S-adenosyl-methionine (SAM) that is converted to S-adenosylhomocysteine (SAH) (Hagemann, 2013; Sibani et al., 2002).



The methylation pathway is more dominant when it comes to the synthesis of GB in cyanobacteria, as glycine is not a limiting factor and is available, whereas the choline oxidative pathway seems to be inactive and is only activated when its precursor choline is present in the environment (Hagemann, 2013).

Several studies have already stated diverse applications of glycine betaine. This compound conferred protection of recombinant fusion proteins (Barth et al., 2000) and anti-inflammatory effects in superficial inflammatory lesions (Nitikhunkasem, Khaiat, & Hopkins, 2002). It protects from oxidative stress and works also has a methyl donor, being able to increase membrane fluidity and absorption of nutrients through alterations in membrane lipids. These characteristics could be employed in anti-ageing products (“Betaine – Trimethyl Glycine: A Review,” 2010; Kampf, Cupp, & Kleinfeld, 2006). Glycine betaine has been identified as a compatible solute in normal human keratinocytes, brain and liver and has effect on cell volume regulation and function. Its topical application in the oral mucosa has also reduced skin-irritating effects of sodium lauryl sulphate and shows a possible application in toothpastes as well as other cosmetics, for example shampoos as it improves hair strength, elasticity and reduces brittleness (“Betaine – Trimethyl Glycine: A Review,” 2010; Soderling, Le Bell, Kirstila, & Tenovuo, 1998; Warskulat, Reinen, Grether-Beck, Krutmann, & Häussinger, 2004). All these capacities make glycine betaine very interesting and appealing at industrial levels. It would be very important the existence of industrial processes for their synthesis that came out cheap and beneficial for the environment.

A lot of processes carried out by microorganisms in an industrial level hold an inconvenient, the feedstock costs, where a big percentage of the investment is made in carbohydrates meant to be metabolized by the organisms used, to obtain valuable molecules such as amino acids or vitamins. Cyanobacteria may have the conditions to reduce this costs with two big advantages arising; since they are photosynthetic organisms the feedstock investment would be inexistent – instead, the metabolic requirements would be low, as many cyanobacteria only require water, CO_2, sunlight and a small percentage of minerals - and it could be beneficial for the environment the sequestration of CO_2 from the atmosphere in the process. If these are not enough reasons for their use in bio-industry, it must be said that due to their short doubling time and natural transformability, they are an optimum choice for genetic engineering. These organisms hold great prospects for the future and are an investment for a sustainable path in the history of mankind (Ducat et al., 2011; Hagemann, 2011; Shivanand & Mugeraya, 2011). Despite the advantages there is an inconvenient, which is the need of freshwater to culture cyanobacteria. The use of this basic resource should already be considered today, it would be a great impact if it was also used for culturing cyanobacteria. If genetic engineered for high salt resistance, these microorganisms could use saline and hypersaline wastewaters, for example industrial waste waters. Instead of freshwater being spent, this approach would stimulate a process of recycling (Ducat et al., 2011).

Cyanobacteria can be used for production of different types of biotechnological products, not only compatible solutes. They can be used for synthesis of exopolysaccharides, bioplastics and biofuel for example (Shivanand & Mugeraya, 2011). Employing synthetic biology techniques and strategies and therefore genetically manipulate these organisms, compounds can be obtained from cyanobacteria in the final form. This approach was performed before, with the synthesis of isobutanol, ethylene and hydrogen in *Synechococcus elongatus* PCC 7942 (Al-Haj, Lui, Abed, Gomaa, & Purton, 2016; Atsumi, Higashide, & Liao, 2009; Sakai, Ogawa, Matsuoka, & Fukuda, 1997; Zhou, Zhang, Zhang, Li, & Ma, 2012) and ethanol, isoprene and acetone in *Synechocystis* sp. PCC 6803 (Al-Haj et al., 2016; Lindberg, Park, & Melis, 2010; Liu & Curtiss, 2009; Zhou et al., 2012).

Synthetic Biology

Synthetic Biology is a field that combines biology and engineering to create new biological sequences and systems, and to re-arrange existing organisms' machinery to provide them new functions that may be of economic or environmental interest. With a small difference to genetic engineering, where individual genes are transferred between cells, synthetic biology implicates the creation of new parts of DNA and the detailed study of the sequences to improve the efficiency of synthesis of the final product. Industries will have savings in time, cost and complexity of production of certain products, therefore increasing job and economic growth opportunities ("http://syntheticbiology.org/"; Science Communication Unit, UWE, 2016).

When restriction enzymes were discovered in the 1970s, the idea that genes could be edited and arranged with other gene sequences rapidly emerged. Although the initial ideas were creating disease-resistant crops or human insulin, novel applications arose, such as the production of fuels and pharmaceuticals (Science Communication Unit, UWE, 2016).

The synthesis of new compounds is nowadays much easier than it was with the advent of synthetic biology, due to the standardization of the DNA parts and the assembling process, known as the BioBrick standard. This development was proposed by Tom Knight in 2003 (Knight, 2003) and the DNA parts, sequences coding for specific biologic functions which can be promoters, ribosome binding sites or terminators for example, are standardized because they ensure compatibility among them, and the researcher can decide on how to join them. This standard assembly process divides a plasmid in two, the plasmid backbone and the required DNA part. Each part is flanked by a prefix and a suffix, which contain restriction enzymes sites. These restriction sites allow the digestion and ligation with other parts, ensuring that the final and resulting part will have a prefix and suffix as in the beginning of the process. (Knight, 2007; "Registry of Standard Biological Parts"). For the RFC10 Standard, which is the most commonly used, the prefix has *EcoRI* (E) and *XbaI* (X) restriction sites, and the suffix *SpeI* (S) and *PstI* (P) restriction sites. The ligation of two parts leaves a scar between them. *EcoRI* digested sites only ligate with fragment ends that were digested with *EcoRI*, the same happens with *PstI* digested sites, they only ligate with other *PstI* digested ends. Although, *XbaI* overhangs can ligate *SpeI* digested fragments. This is the base for the interchangeability and standardization, where part A that is meant to be

inserted upstream of part B simply needs to be digested with *EcoRI* and *SpeI*, while the part B should be digested with *EcoRI* and *XbaI*, followed by ligation (Fig. 2). If part A is supposed to be inserted downstream of the part B, then it should be digested with *XbaI* and *PstI*, while part B should be digested with *SpeI* and *PstI*.

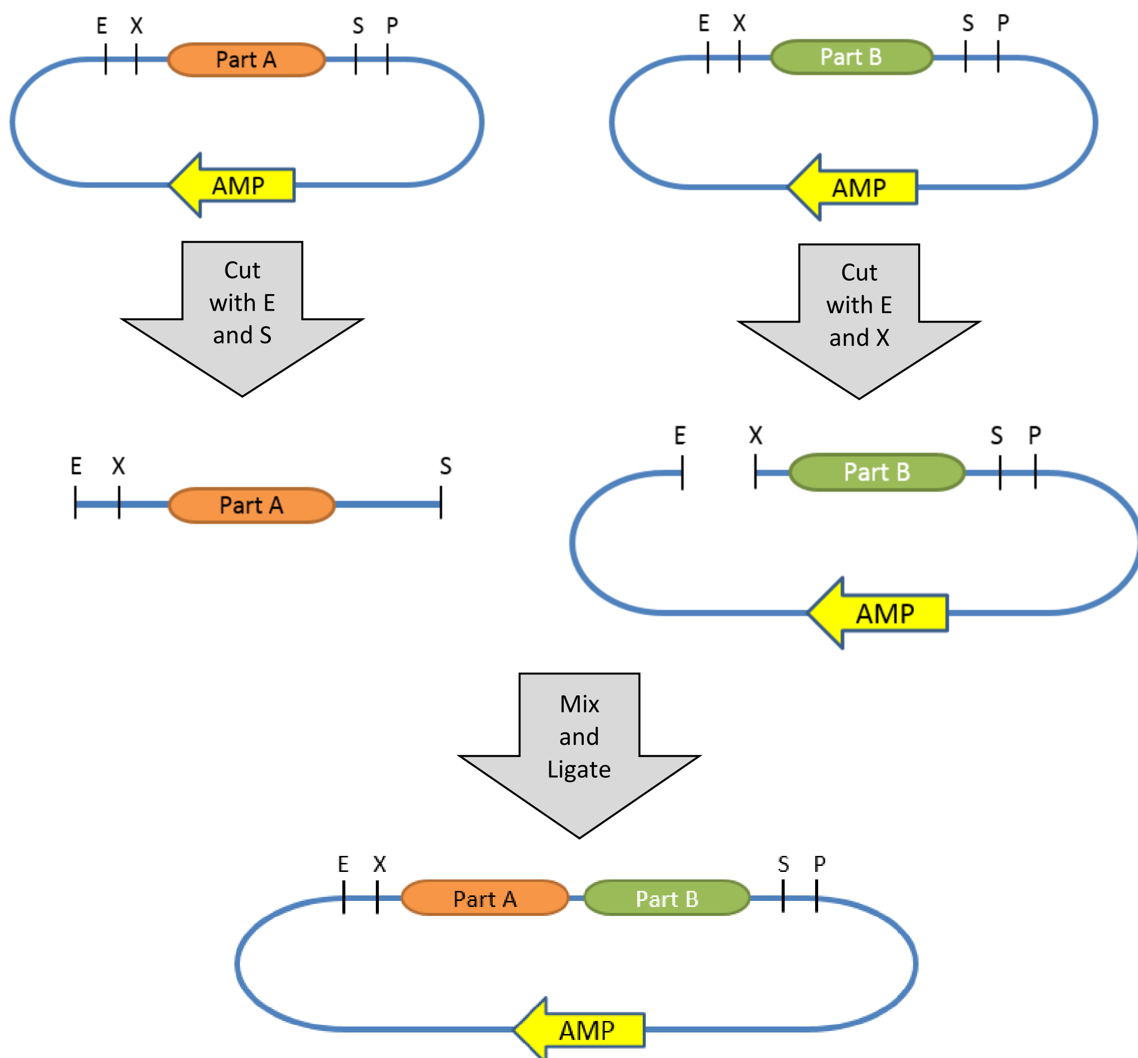


Figure 2 – Schematic representation of the BioBrick Standard Assembly Process RFC10. Each new BioBrick™ is initially received with a pSB1A2 backbone. Each part is flanked by a prefix upstream composed of E and X restriction sites and a suffix downstream composed of S and P restriction sites. E and P overhangs only ligate with other E and P overhangs, respectively, while X and S overhangs can ligate between them, resulting in a scar that is not digested by any other enzyme. Any ligation performed results in the connected parts flanked by the same prefix and suffix as in the beginning of the process.

As in software development the program needs hardware to run, the resulting plasmid or vector, containing the DNA parts A and B, also needs a

chassis, the host cell, where it can be introduced and originate a new phenotype.

Synechocystis sp. PCC 6803 as a chassis

A lot of effort has been employed throughout the last few years to develop genetic engineering strategies for cyanobacterial strains, with *Synechocystis* being one of the most studied (Al-Haj et al., 2016). Its genome was completely sequenced in 1996 (T. Kaneko et al., 1996) – about 12 copies of a single chromosome, four large plasmids and three small - and since then it became very well characterized and eventually considered a model organism, ideal to be used as a chassis for heterologous compatible solute synthesis (Heidorn et al., 2011; Takakazu Kaneko et al., 2003).

Synechocystis is a unicellular freshwater strain and naturally transformable cyanobacterium. It synthesizes GG as main compatible solute and Suc in lower amounts. Due to Suc high importance in essential metabolism of the cell, its accumulation is generally limited to low salt concentrations. The accumulation of Suc, as well as Tre, is considered to be more necessary as general cell defenses than as specific protectants for higher salt concentrations (Klähn & Hagemann, 2011).

BG11 is the most used medium to culture cyanobacteria. It can be adapted according to needs of each strain. An important consideration is that *Synechocystis* does not fix nitrogen and therefore it must be provided in the medium, which normally is in the form of sodium nitrate (Heidorn et al., 2011; Takakazu Kaneko & Tabata, 1997; Mikkat, Effmert, & Hagemann, 1997).

Optimal growth conditions for *Synechocystis* cells can be obtained at high pH, between 9 and 11, and around 33 °C. If the medium is buffered with CAPS to sustain higher values of pH, with an initial pH of 11 and 33 °C, a maximum value of growth of 1.8 day^{-1} is obtained (Lopo et al., 2012).

Objectives:

The main objective of this work was to introduce a cluster of genes involved in the synthesis of the compatible solute glycine betaine in the photoautotrophic chassis *Synechocystis* sp. PCC 6803. This compatible solute should increase the tolerance of the cyanobacterium to high salinities. In previous work performed in the group, the betaine cluster was assembled with the strong constitutive promoter P_{trc1O}. The characterization of *Synechocystis* harboring the cluster suggested that the regulatory elements needed to be replaced. To achieve this, we proposed to:

(i) replace gene regulatory elements: the P_{trc1O} promoter by 3 promoters with inferior strengths;
(ii) transfer the reassembled modules to replicative plasmids, such as pSEVA, to introduce them in *Synechocystis;*
(iii) characterize the mutants in terms of growth/halotolerance, transcript(s) levels (RT-PCR) and if possible betaine production.

Materials and methods

Organisms and standard growth conditions

E. coli XL1-Blue cells were cultivated in Lysogeny-Broth (LB) medium supplemented with the appropriate antibiotic, kanamycin (Km, 50 µg mL^{-1}), ampicillin (Amp, 100 µg mL^{-1}) or chloramphenicol (Cm, 34 µg mL^{-1}). For solid medium, 1.5% (w/v) Bacteriological Agar was added to LB.

DNA constructions – parts assembly

In previous work of the group, the genes coding for enzymes involved in the synthesis of glycine betaine, GSMT, DMT and an additional gene, *metX*, coding for S-adenosyl methionine synthetase, were synthesized as a cluster (Ahbet cluster). This cluster was assembled with three constitutive synthetic promoters $P_{trc.x.lacI}$, $P_{trc2.x.tetR}$ and P_{J23101} (part: BBa_J23101, http://parts.igem.org) already available in the group. The assemblies attempted in this work used parts in the BioBrick format and were performed according to the standard assembly protocol.

Each vector containing a promoter was digested with *SpeI* and *PstI* since the purpose was to insert the betaine cluster downstream the promoter. The pBSK_Ahbet plasmid was first digested with *DraI* and then with *XbaI* and *PstI* to isolate the betaine cluster more efficiently. As an alternative approach, the insertion of the promoter $P_{trc2.x.tetR}$ upstream of the Ahbet cluster was attempted. pJ201_$P_{trc2.x.tetR}$ plasmid was digested with *EcoRI* and *SpeI* and the isolated promoter was cloned into pBSK_Ahbet plasmid digested with *EcoRI* and *XbaI*. The correct constructions obtained were digested with *XbaI* and *PstI* enzymes to isolate the segments Promoter::Ahbet. The isolated fragments were cloned into pSEVA251, pSEVA351 replicative vectors and pSN15K integrative vector digested with *XbaI* and *PstI* for subsequent transformation in *Synechocystis.* All DNA assemblies were confirmed by restriction analysis followed by sequencing (STAB VIDA).

DNA purification and quantification

DNA purification from gel or from enzymatic reactions was performed using NZYGelpure kit (Nzytech, Lda.), according to the manufacturer's protocol. DNA concentrations were determined using a Nanodrop ND-1000 (Nanodrop Technologies, Inc.).

Agarose gel electrophoresis

Nucleic acid enzymatic digestion results were separated by electrophoresis in 1% (w/v) agarose (Sigma®) gels, in 1x TAE buffer (Bio-Rad). The GeneRuler™ DNA Ladder 1kb (Thermo Scientific®) was used as molecular weight marker.

DNA ligation

DNA ligation reactions (10 µL) were made with 1 U T4 DNA ligase (Thermo Scientific®), according to manufacturers' instructions. Alternatively, ligations using the pSEVA vectors were also performed using the T4 DNA Ligase provided with the pGEM®-T Easy Vector Systems (Promega®), according to the instructions.

E. coli XL1-Blue transformation and plasmid preparation

Plasmids were transformed in chemically competent *E. coli* XL1-Blue cells. Briefly, 100 µL of cells were mixed with 10 µL of ligation reaction and incubated on ice for 20-30 min. Then the mixture was heat shocked at 42 ºC for 90 secs in a water-bath, and placed back on ice for 2 min. 900 µL of LB medium were added to the mixture and the cells were left to recover in an orbital shaker at 37 ºC for 60 min, or 90 min if Cm was used. 100 µL of the cell suspension were plated onto LB-agar supplemented with the appropriate antibiotic to select the transformants. The remaining volume was centrifuged at 13000 *g* for 1 min., the supernatant was removed and the cells were resuspended and plated in the same medium, as previous. The plates were incubated overnight at 37 ºC and single isolated colonies were inoculated in 5 mL LB medium with proper antibiotic and grown overnight at 37 ºC with shaking (200 r.p.m.). Plasmid DNA was isolated using the NZYMiniprep kit (Nzytech, Lda.) starting with 4 mL of culture and following protocol instructions from the manufacturer.

Results and discussion

Assembly of different devices for heterologous synthesis of glycine betaine in *Synechocystis* sp. PCC 6803

Glycine betaine is a solute that confers resistance to high salinities and has gained interest throughout the years due to its protective properties and variety of applications. Its use as a salt protectant for cyanobacteria to be used as cell factories is being researched, to allow them to grow in saline water instead of freshwater.

Previously, in the research group, a module for the synthesis of glycine betaine (Ahbet) was assembled with the strong constitutive promoter P_{trc1O} - relative activity of 83 compared to P_{rnpB}, the promoter from the Ribonuclease P gene of *Synechocystis* -, inserted in the replicative vector pSEVA351 and introduced into the unicellular cyanobacterium *Synechocystis* 6803 (Rodrigues, 2016). The characterization of the generated mutant revealed an improved phenotype concerning NaCl tolerance but also suggested that there was a need to fine tune the regulation of this module. Therefore, this work started by the isolation of the Ahbet gene cluster from a pBSK vector (Fig. 3) in order to assemble it with three other promoters - $P_{trc.x.lacI}$, $P_{trc2.x.tetR}$ and P_{J23101} - weaker than P_{trc1O}, with relative forces of 59, 30 (Ferreira, 2014) and 5 (Camsund, Heidorn, & Lindblad, 2014) respectively, and compared to the P_{rnpB} promoter.

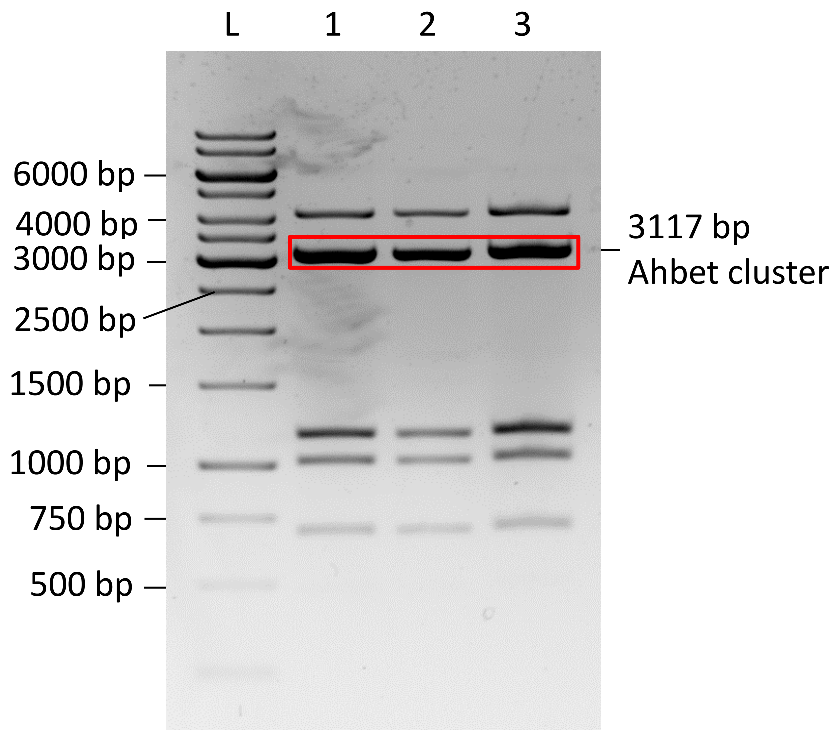


Figure 3 - Agarose gel electrophoresis of DNA fragments resulting from the digestion of the plasmid containing the betaine module (**1, 2, 3**), **pBSK_Ahbet**, for the isolation of the cluster of genes involved in the synthesis of glycine betaine and subsequent assembly with different regulatory elements. The digestion was performed with *DraI*, followed by *XbaI* and *PstI*. The upper band corresponds to the supercoiled undigested plasmid. The **Ahbet** cluster fragment (3117 bp) is highlight in red, and the lower bands correspond to fragments of the digested plasmid. **L** – GeneRuler^{TM} 1 kb ladder.

From the three promoters used, only the P_{J23101} promoter is a stable constitutive promoter both in *E. coli* (Anderson, 2006; Kosuri et al., 2013) and in *Synechocystis* (Camsund et al., 2014). The other two promoters, $P_{trc.x.lacI}$ and $P_{trc2.x.tetR}$ have only been characterized in cyanobacteria in previous work of the group (Ferreira, 2014). $P_{trc.x.lacI}$ is based on the P_{trc} promoter (Brosius, Erfle, & Storella, 1985), which is a hybrid promoter constituted by the consensus -35 sequence from the *E. coli*'s tryptophan operon promoter, P_{trp}, and the P_{lacUV5} region that includes the -10 box and the *lacO* operator*;* with the core promoter boxes separated by 17 bp (Brosius et al., 1985). $P_{trc.x.lacI}$ contains two LacI (P_{lac} repressor) binding operators - DNA regions where regulatory proteins bind, repressing DNA transcription -, the *lacOid* upstream and the *lacO* downstream of the core promoter (Fig. 4). The *lacOid* operator sequence is symmetric and has high affinity to the LacI repressor. $P_{trc2.x.tetR}$ is also based on the P_{trc} promoter but instead of the *lacO* operator harbors a modified *tetO* operator sequence (Fig. 4), downstream of the consensus regions. The modified *tetO* inhibits the binding of the repressor TetR in *E. coli*, making this promoter not repressible even in the presence of the TetR protein (Krueger, Scholz, Wisshak, & Hillen, 2007)**.**

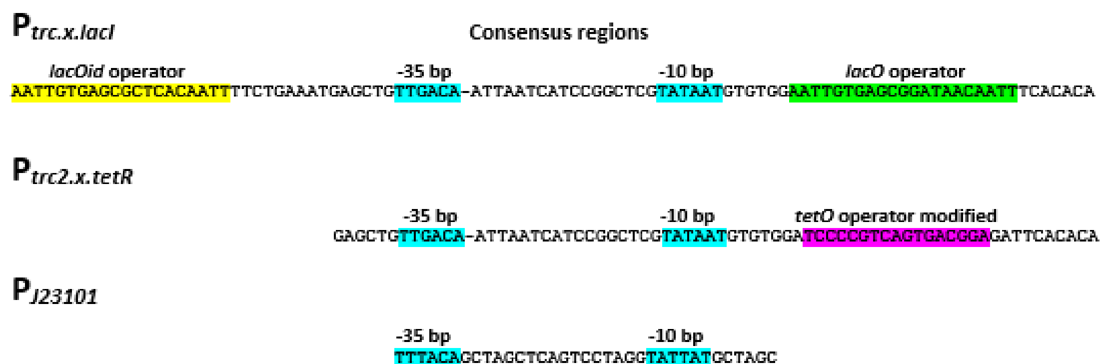


Figure 4 – Sequences of the synthetic promoters used in this work. The operators and -35 and -10 boxes are highlighted. Yellow: *lacOid* operator. Blue: -35 bp and -10 bp consensus regions. Green: *lacO* operator. Pink: modified *tetO* operator.

The devices' assembly started with the Ahbet cluster insertion downstream of the $P_{trc.x.lacI}$ promoter, already present in the pJ201 plasmid, originating the plasmid pJ201_$P_{trc.x.lacI}$::Ahbet. The assembly of the device was confirmed by restriction analysis with *NotI* enzyme (Fig. 5, lane 3), and was subsequently confirmed by sequencing.

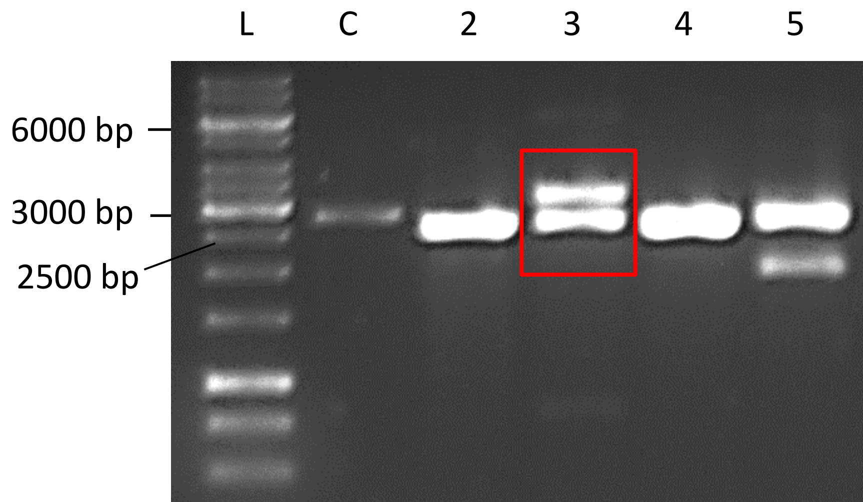


Figure 5 - Agarose gel electrophoresis of the DNA fragments originated by the digestion of the plasmid **pJ201 putatively containing the $P_{trc.x.lacI}$::Ahbet** with *NotI* (2-5). The **$P_{trc.x.lacI}$::Ahbet** module is present in clone 3 highlighted in red (bands with the expected size: 2651 bp and 3221 bp). **L –** GeneRuler™ 1 kb ladder. **C - Control, pJ201_$P_{trc.x.lacI}$** plasmid digested with *SpeI* and *PstI*.

Attempts to introduce the cluster downstream of the $P_{trc2.x.tetR}$ promoter were not successful. Alternatively, the assembly of the $P_{trc2.x.tetR}$ promoter upstream of the Ahbet cluster was also attempted but was also unsuccessful. This promoter, $P_{trc2.x.tetR}$, was only characterized in *Synechocystis,* no characterization was done in *E. coli*. There is a possibility that this promoter together with the RBS B0030, which is known to be strong in *E. coli* (Heidorn et al., 2011), could originate a synergic effect resulting in some type of toxicity to the cells harboring the construct. Concerning the assembly of the third device, the Ahbet cluster was cloned downstream of the P_{J23101} promoter, present in the pSB1A2 plasmid, originating the construct pSB1A2_P_{J23101}::Ahbet (Fig. 6). Restriction analysis of the plasmid DNA, obtained from different transformants, revealed the presence of several constructs correctly assembled (Fig. 6, lanes highlighted with red box). The DNA obtained from clone 10 was further confirmed by sequencing.

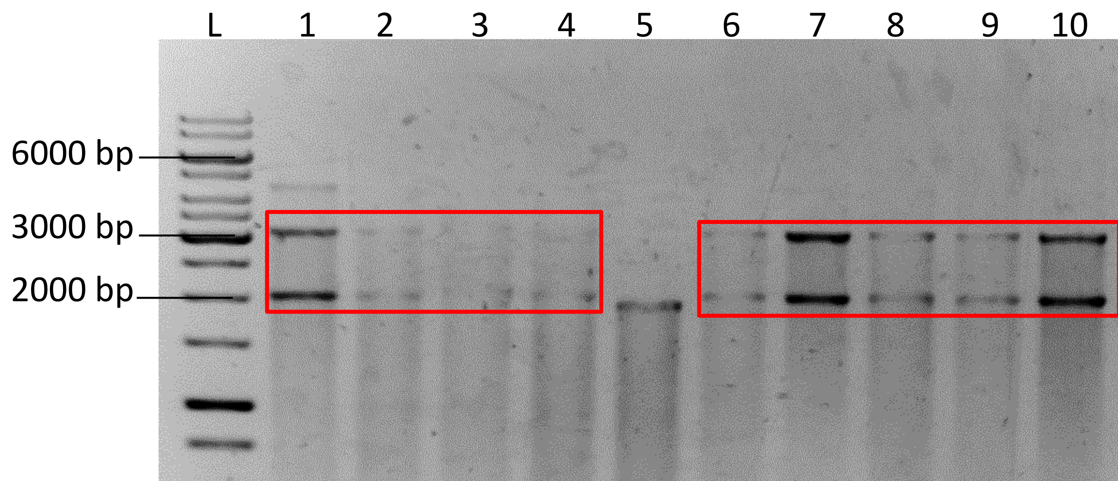


Figure 6 - Agarose gel electrophoresis of the DNA fragments resulting from the digestion of the plasmid **pSB1A2_P*J23101*::Ahbet** putatively containing the promoter P*J23101* assembled with the betaine module (**1 to 10).** The digestion was performed with *NotI.* The fragments highlighted in red correspond to the expected sizes (2055 bp and 3158 bp). The upper band in lane 1 represents undigested plasmid and lane 5 depicts a false positive clone; **L –** GeneRuler™ 1 kb ladder.

With two of the devices for the synthesis of betaine correctly assembled (P*trc.x.lacI*::Ahbet and P*J23101*::Ahbet), the subsequent step was to isolate these modules and clone them into the pSEVA vector, which is able to be transformed into *Synechocystis* (Fig. 7).

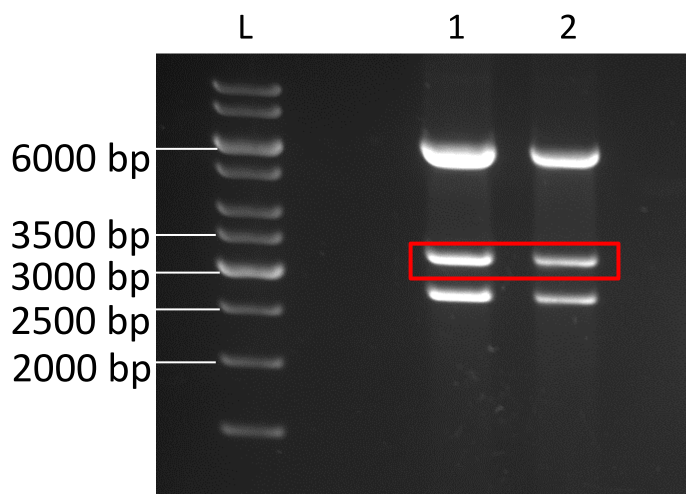


Figure 7 - Agarose gel electrophoresis of DNA fragments originated by the digestion of the plasmid **pJ201_P*trc.x.lacI*::Ahbet (1, 2),** for the isolation of the fragment **P*trc.x.lacI*::Ahbet**. The digestion was performed with *XbaI* and *PstI*. The upper band corresponds to undigested plasmid. The **P*trc.x.lacI*::Ahbet** fragment (3223 bp) is highlighted in red, and the lowest band corresponds to the plasmid backbone (2649 bp). **L –** GeneRuler™ 1 kb ladder.

Isolation of the fragment P_{J23101}::Ahbet was not successful. The DNA obtained from the digestion of the pSB1A2_P_{J23101}::Ahbet plasmid was successively low, not allowing its isolation. Considering the limited time available for the assembly of the constructs, we proceeded with the insertion of the $P_{trc.x.lacI}$::Ahbet fragment in the pSEVA251 plasmid. Similarly to the P_{J23101}::Ahbet, the transference of the $P_{trc.x.lacI}$::Ahbet fragment to the pSEVA251 plasmid was unsuccessful. The ligation between the $P_{trc.x.lacI}$::Ahbet fragment and the pSEVA251 plasmid digested with *XbaI* and *PstI* was tried several times*.* Throughout the process*,* different ratios vector-insert were chosen, namely 1:3 and 1:5, without differences in the resulting number of colonies or in the constructs, with all of them representing false positives.

Thermo Scientific T4 DNA ligase was the enzyme mostly used during the work, with the ligation incubations being carried out at room temperature according to manufacturer's instructions. Taking in consideration the frequent false positive results obtained, another T4 DNA ligase (pGEM®-T Easy Vector kit, Promega®) was used because of its activity at lower temperatures, with overnight incubations carried out at 4-8 °C. The reduced temperature slows the molecules' movements and enhances the chances of ligation between the vector and the insert. The use of this protocol with an alternative ligase did not lead to any positive results.

Other factors could contribute to the unsuccessful ligation of the promoter cluster fragments to the pSEVA vector, namely, the presence of contaminants or agarose in the purified DNA fragments that can influence the activity of the T4 ligase (Matsumura, 2015). The ligase buffer also has an impact in the enzyme activity due to the sensitivity of the buffer's ATP to freeze/thaw cycles. For this reason, the ligase buffer was replaced several times, with no different results.

The percentage of vector re-ligation is a factor that is often considered for efficiency purposes. The double digestion of the pSEVA251 plasmid with *XbaI* and *PstI* results in incompatible sticky ends that are treated with alkaline phosphatase, no plasmid re-ligation should occur, but the dephosphorylation process may not be completely effective and re-ligation might take place. As control, a ligation was performed without insert. If the number of colonies resulting from the transformation with this control ligation was similar to the number of colonies resulting from the transformation of ligation with insert, this would suggest a problem with the insert since only vector re-ligation was occurring. However, the results showed a higher number of colonies in the transformation of the ligation

with insert than in the control. This suggested that only a small percentage of re-ligation was occurring, but further analysis proved all colonies to be false positives. Considering these results, the use of an additional selecting agent could improve the isolation of correct transformants. As glycine betaine provides resistance to high salinities, an experience was set up to determine if NaCl could be used as an additional selecting agent. Mutants harboring the constructs pJ201_$P_{trc.x.lacI}$::Ahbet and pSB1A2_P_{J23101}::Ahbet, and the wild type *E. coli* XL1-Blue were grown in LB medium with NaCl levels varying from 3% to 7% (w/v). Mutants only survived the same salt concentrations as the control, up to 4%, although was expected that glycine betaine could allow them to resist higher salinity levels. Reason may be that the P_{J23101} promoter strength in *E. coli* is insufficient to synthesize enough betaine so the cell can survive in such amounts of salt. However, in the case of the $P_{trc.x.lacI}$ promoter, whose strength is not defined in *E. coli*, one can only assume that if that strength resembles the strength reported in *Synechocystis*, it may be that the cells are metabolically overcharged and consequentially their growth is compromised. Unfortunately, these results show that NaCl cannot be used as a selecting agent and that the betaine module may not be functional in *E. coli.*

In an attempt to circumvent the problems in transferring the betaine devices to pSEVA251 (Km^R), different plasmid backbones were used: the pSEVA351 (Cm^R) replicative plasmid (Silva-Rocha et al., 2013) and the pSN15K (Km^R) integrative plasmid (Pinto et al., 2015). All the cloning steps followed were the same as for the pSEVA251 plasmid but still no positive results were obtained.

Conclusion and Future Perspectives

In conclusion, this work allowed the assembly of the glycine betaine module with two promoters, $P_{trc.x.lacI}$ and P_{J23101}, and the isolation of the fragment $P_{trc.x.lacI}$::Ahbet for further insertion in a pSEVA vector.

In the future, the characterization of the promoters $P_{trc.x.lacI}$ and $P_{trc2.x.tetR}$ - as well as other promoters that may be of value - in *E. coli,* would be very important for a better understanding of the mechanisms inherent to possible problems that arise during cloning procedures, as well as for significant conclusions about the functionality of the Ahbet module in *E. coli*. The finalization of the objectives proposed for this study would also be valuable to conclude what are the ideal regulatory segments for the synthesis of glycine betaine in *Synechocystis.*

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