Exploring The Possibilities and Challenges of enhancing Salinity tolerance in synechocystis sp. PCC 6803 by directed evolution.

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ABSTRACT

Cyanobacteria are incredibly versatile microorganisms which have numerous applications in biotechnology. However, mining cyanobacteria for diversity and using them as a chassis has proven difficult due to the limited number of fully annotated genomes available. In the present study, we aim to generate a strain of *Synechocystis sp.* PCC 6803 with increased salt tolerance through directed evolution. Glucosylglycerol phosphate synthase is the key enzyme in the pathway for glucosyl glycerol, an osmolyte which confers salt tolerance in Synechocystis sp. PCC 6803. This pathway can be reconstituted in *Escherichia coli* allowing a rapid acceleration in the timespan necessary to perform directed evolution. The native *Synechocystis* gene *ggps* was extracted from the genome and error prone PCR was performed to generate a mutant library and This library was assembled into a level 1 construct. An assay was designed utilising the Edinburgh genome foundry automated robotic laboratory allowing for large high throughput selection.

Key Words: error prone PCR, salt tolerance, glucosylglycerol

1. INTRODUCTION

Salt tolerant microorganisms are used heavily in industry. Their ability to survive at high salt concentrations makes them ideal for use in wastewater treatment, food fermentation, increasing crop salt tolerance and cosmetic production. Mining microbial genomes for salt tolerance is therefore important as it can expand the range of their biotechnological applications (Kirsch, Klähn and Hagemann, 2019).

Photosynthetic microorganisms such as cyanobacteria present a great opportunity in the race to develop sustainable energy resources. At present, cyanobacteria are attracting interest in biotechnology research areas such as biophotovoltaics and biofuels. If this technology is to be successful and we are to use cyanobacteria as a chassis in industry, it would be incredibly beneficial to have salt tolerant strains that can undertake the requirements of large scale industrial processes. For example, salt tolerance may be beneficial in biophotovoltaics where higher concentrations of salt in the medium can generate a greater power output (McCormick et., al. 2011). However, modular metabolic engineering processes for cyanobacteria are lagging behind significantly in comparison to bacterial, yeast and mammalian systems. Few cyanobacteria strains have fully annotated genomes (Shih et al., 2013), which is a problem for synthetic biology approaches that require a good understanding of how parts will function in the context of a larger system (Santos-Merino, Singh and Ducat, 2019). Due to the limited number of well annotated cyanobacterial genomes, it is difficult to find a suitable chassis that can sustain modular cloning techniques. Hence, it is beneficial to explore random mutagenesis of salt tolerant genes in model cyanobacteria.

Synechocystis sp. PC 6803 (hereafter referred to as *Synechocystis*) is a moderately halotolerant strain of cyanobacteria that produces the compatible solute glucosylglycerol (GG) in response to osmotic stress (Marin et al., 1998). Salt activated GG biosynthesis relies on *stpA* and *ggpS* that encode GG phosphate phosphatase (GGPP) and GG phosphate synthase (GGPS) respectively. In the first step of the pathway, GGPs converts ADP-glucose and glycerol-3-phosphate into ADP and GG-phosphate. GGPP then dephosphorylates GG-phosphate to GG (Hagemann and Erdmann, 1994)(Figure 1).

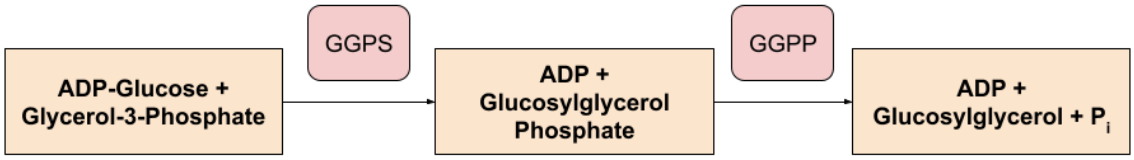

Fig. 1) Biosynthesis pathway of GG. Enzymes: GGPS - glucosylglycerol phosphate synthase, GGPP - glucosylglycerol phosphate phosphatase.

ggps and *stpa* have both been shown to function in *Escherichia coli* separately (Hagemann et al., 1997)(Marin et al., 1998). Due to the difficulties and long process in transforming synechocystis it was decided to reconstitute the glucosylglycerol pathway in *Escherichia coli.*

The aim of this study was to randomly mutagenize *ggpS*, the key gene in GG synthesis (Marin et al., 1998), and screen the mutant *E.coli* libraries on a salt gradient. Bacterial growth was used as an indicator of improved salt tolerance.

2. RESULTS

2.1 Salt growth curve of BL21 (DE3) cells

To determine the salt tolerance of the wild type *Escherichia coli* BL21 (DE3), a salt growth curve was performed (Fig. 2). The E.coli which attained the highest OD_{600} in this experiment was in 1% salt LB media. However the initial OD_{600} of these cultures was significantly higher than all others. Nevertheless, the results indicate a significant impact on growth after the concentration of salt in the LB media surpassed 3% (w/v) demonstrated by significant increases in doubling times commencing after 3% salt concentration (table 1). The *E.coli* which grew in LB media containing 0.5% salt grew comparatively quickly for the first 5 hours demonstrating a more rapid growth rate in the first 5 hours than *E.coli* grown at 1% and 1.5%, with a doubling time of 1.05 hours in comparison to 1.21 for the 1% salt culture and 1.09 for 1.5%. However, the E.coli grown in 0.5% salt, which is LB without additional salt, saw a decline between hours 5 and 6, not seen in the other lower salt cultures. However decline before recovery is shown in the 3.5% culture at hour 5.

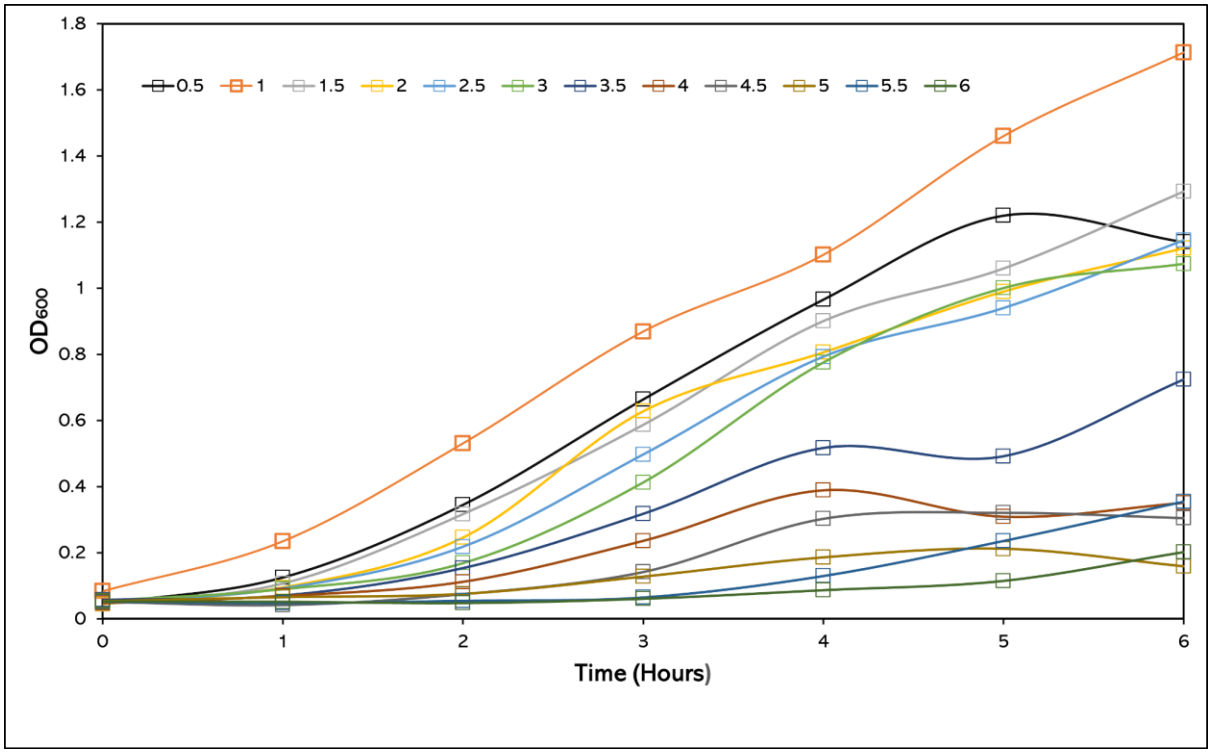

Fig. 2) 6 hour Growth Curve of BL21 (DE3) Cells In LB Media of Varying Salt Concentrations. LB media containing various salt concentrations were inoculated with BL21 (DE3) Overnight culture and the OD_{600} was measured every hour to measure the growth of the *Escherichia coli*.

Table. 1) Doubling Times of Salty E.coli Cultures at 6 hours. Doubling times were calculated using the formula $Dt = hours/ \log_2(final\ OD_{600}/ Initial\ OD_{600})$

Lysogeny Broth Salt Concentration (%)	Doubling Time at 6 Hours (Hours)
0.5	1.29
1.0	1.38
1.5	1.23
2.0	1.26
2.5	1.32
3	1.40
3.5	1.65
4	2.05
4.5	2.35
5	3.85
5.5	2.27
6	3.41

2.2 stpA Level T

Due to being unsuccessful in purifying extracted *stpa* in sufficient concentrations, a gene block was designed with restriction sites and overhangs to be inserted into a level T reaction (Supplementary material section 2.1). A level T reaction was done and transformed into TOP10 cells. The cells were spread on plates for blue/white selection. White colonies were picked for colony PCR (Figure 3). The results show the expected band sizes across the white colonies, suggesting the stpA level T construct was present.

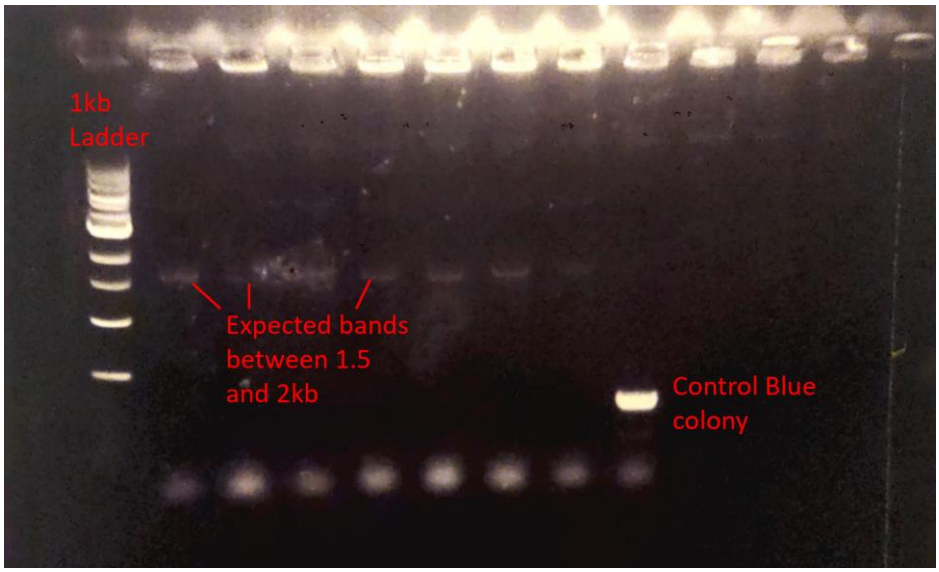

Figure. 3) Imaged Agarose gel electrophoresis. Colony PCR was performed generating bands at the expected positions between 1.5 and 2kb.

2.3 Mutant library generation

Error prone PCR (epCPR) of the level 1 Cyanogate assembly was used to generate a *ggpS* mutant library. As the epPCR reaction was unsuccessful after multiple attempts, it was hypothesised that the plCH47732 level 1 acceptor vector or alternatively the PTRC10 promoter or pC0.082 terminator in level 0 was incorrect, inhibiting the reaction. A Q5 PCR reaction was performed using level 0, level 1 and purified ggpS as templates, and reactions with all templates but the level 1 assembly were successful (Fig. 4).

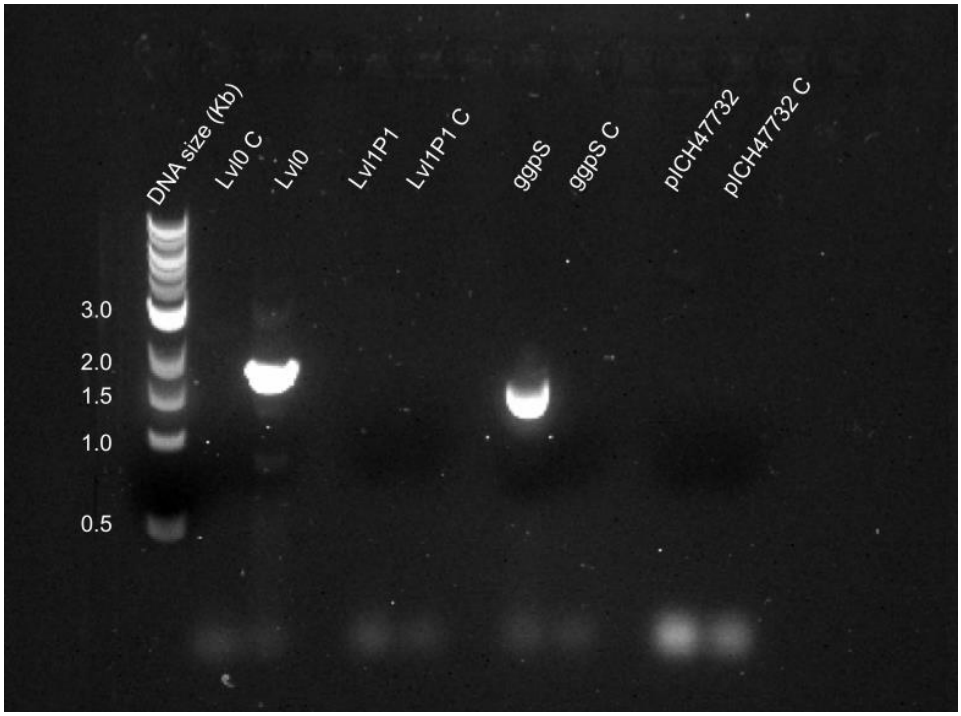

Figure. 4) Agarose gel electrophoresis image: Q5 PCR of *ggpS* from *ggpS*, lvl0 and lvl1P1 templates, and Q5 of pICH47732 backbone from lvl1 template.

The level 1 construct was digested and ran on 1% agarose gel in gel electrophoresis (Figure 5). The results demonstrate that the full level 1 assembly is larger than expected. The expected size of the assembly is 6096bp. However, the band shows the assembly being approximately 7000 to 8000 base pairs, larger than expected .

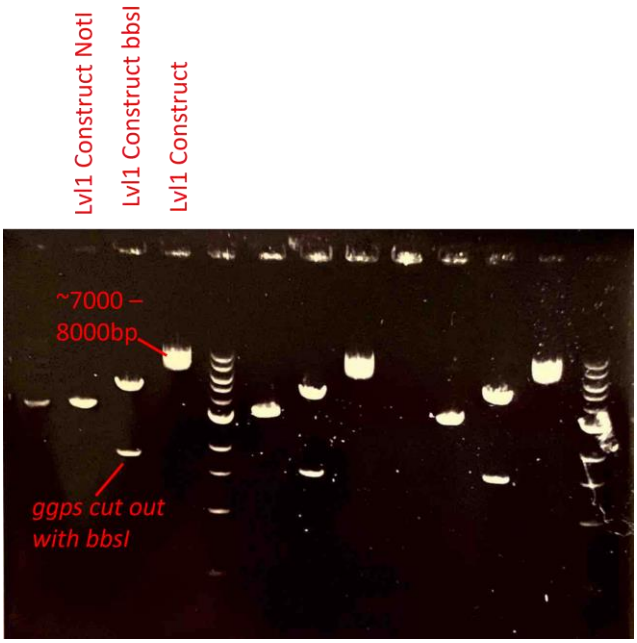

Figure. 5) Agarose Gel Electrophoresis image of undigested Level 1 construct and digested with NotI and BbsI. Plasmid miniprep was digested with NotI and bbsI and run on 1% agarose gel in gel electrophoresis and imaged. Repeats can be found on the right.

To circumvent the plCH47732 backbone, epPCR of *ggpS* was performed again using the level 0 assembly as a template and NEB Taq polymerase (section 3.3). The resulting library was digested with BbsI, creating 1.5kb double stranded linear DNA fragments with 4 base pair overhangs compatible with JUMP assembly. The *ggpS* library was then assembled into a pJUMP29-1A acceptor vector with a constitutive PJ23100 promoter, pET ribosome binding site and L3SAP51 terminator (section 3.4) .

The JUMP level 1 assembly was transformed into TOP10 and BL21 (DE3) cells. Cells were plated on IPTG + Xgal + kanamycin and white colonies were selected for colony PCR (section 3.2.5) to check that they had acquired the *ggpS* inserts. The lanes for the positive control and 19 of the selected white colonies were blank, indicating an error in the protocol (figure 5).

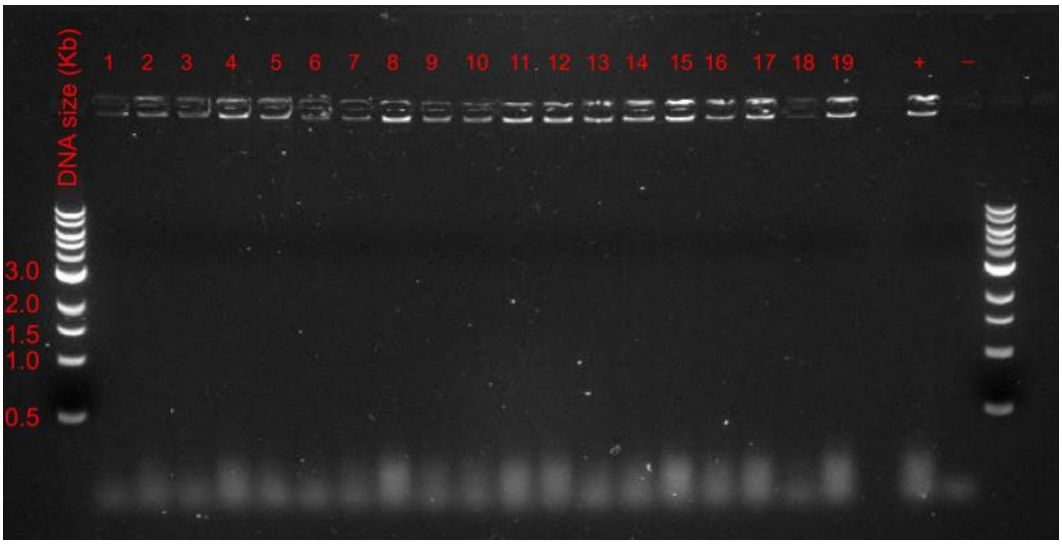

Fig. 5) Agarose gel electrophoresis image of Colony PCR. 19 white TOP10 colonies post-transformation with level 1 JUMP assembly plasmids, positive control (+) = blue colony containing *lacZ* gene, negative control (-) = DNA replaced with water.

3. DISCUSSION

We were unable to transform BL21 (DE3) cells with a *ggps* mutant library. This was due to problems faced during cloning and epPCR, taking time.The issues surrounding error prone PCR were likely a consequence of primer mis-annealing. In-silico results showed no reason for primer mis-annealing to take place, suggesting that the extra DNA in the plasmid is a likely cause. This could come from the backbone or the cyanogate promoter and terminator parts. To troubleshoot the epPCR, we also ran experiments with NEB 10mM dNTPs instead of the custom mix (section 3.3). We found the custom dNTP mix led to unsuccessful epPCR reactions. This could be a problem for mutant library preparation as an imbalance in dNTP concentrations leads to more errors. As our purified epPCR reaction was not sent for sequencing in time, we cannot confirm that a mutant library was generated.

In order to complete our mutant library assembly we switched to JUMP modular cloning system, utilising a verified new acceptor vector and parts. However, we observed no white colony growth on BL21 (DE3) plates, but white colonies were found with TOP10.

As the positive control for the colony PCR of mutant library transformation into TOP10 cells was blank, we cannot be certain that the cells were lacking the correct insert. This may be

because the PCR extension time was set for a 1.8kb fragment, which accounted for the 1.5kb of *ggpS* and 0.3kb of flanking regions on pJUMP29-1A but not the promoter, ribosome binding site and terminator.

Upon successful transformation and miniprep, the next steps would be to send these plasmids to a lab capable of high levels of automation such as the Edinburgh Genome Foundry. A high throughput assay was developed and agreed upon in anticipation of this to be used in future experiments. Competent *E.coli* BL21 (DE3) would be transformed with level T stpA and handed to the Edinburgh Genome Foundry. Wherein, these cells would be made competent and transformed with the *ggps* mutant library in the level 1 JUMP vector while some cells will be transformed with a non-mutant library and some with pJUMP29-1A vector. Two plating methods would be used: Plating onto petri dishes of varying salt concentrations, and plating onto a Q-tray with a salt gradient. The preliminary experiments outlined indicate plating between 2%- 4% would allow for the greatest chance of finding beneficial mutations. Surviving colonies would be picked and inoculated into 1.4 mL LB with antibiotics, and incubated at 37 °C, 1000 rpm. cultures would then be normalised to OD_{600} = 0.05 in 3 different salt concentrations in up to 16 96 well plates, glycerol stocks of these would be prepared and allowed to grow for 24 hours.

This would reveal candidates for running on a BioLector to produce growth curves to analyse any increased tolerance to high salt conditions. Highly automated robotic labs provide the opportunity to have much higher throughput assays than could be done by hand.

4. Materials and Methods

4.1.1 Chemicals and materials

Primers were designed using Benchling and synthesised by Integrated DNA Technologies (IDT). These were:

Primer		Sequence (5'--> 3')
Genomic Forward	GF	CAGTGAAGACATAATGAATTCATCCCTTGTGATCC
Genomic Reverse	GR	CAGTGAAGACATAAGCATTGGCGATCGCCTACAT
Level 0 Forward	L0F	TTGAGTGAGCTGATACCGCT
Level 0 Reverse	L0R	GTCTCATGAGCGGATACATATTTGAATG
Level 1 Forward	L1F	GAACCCTGTGGTTGGCATGCACATAC
Level 1 Reverse	L1R	CTGGTGGCAGGATATATTGTGGTG
Level T Forward	LTF	TAGTGAGTGGGTTGCGCTC
Level T Reverse	LTR	GTTACCACCGCTGCGTTC

Enzymes, buffers and dNTPs were purchased from New England Biolabs (NEB) except GoTaq G2 DNA polymerase which was purchased from Promega. The QIAprep Spin miniprep kits and QI-Aquick gel extraction kit were purchased from Qiagen. The cyanogate parts were

provided by the Alistair McCormick lab (University of Edinburgh), while the JUMP assembly parts were kindly provided by the Chris French Laboratory (University of Edinburgh). The *stpa* gene block was synthesised by TWIST Bioscience.

4.1.2 preparation of NaCl Lysogeny Broth and BL21 Growth Curve

34% (w/v) sterile saline stock solution was prepared using double distilled water and NaCl. This stock solution was then added to LB media, creating media of increasing salt concentrations in 50ml falcon tubes (Table 2). The final volume of the media was 29 mL which was then inoculated with 1mL of E.coli in LB media.

Table 2. Volumes of 34% Saline solution, LB media and E.coli Inoculum for preparation of NaCl LB media E.coli growth curve.

Concentration of NaCl in LB media (w/v)	Volume of 34% Saline Solution (mL)	Volume of LB media (mL)	Volume of BL21 in LB
0.5%	0	29	1
1%	0.441	28.559	1
1.5%	0.882	28.118	1
2%	1.323	27.677	1
2.5%	1.764	27.236	1
3%	2.205	26.795	1
3.5%	2.646	26.354	1
4%	3.087	25.913	1
4.5%	3.528	25.472	1
5%	3.969	25.031	1
5.5%	4.380	24.590	1
6%	4.821	24.149	1

The cultures were then incubated at 37°C at 250rpm and a 1ml sample was taken every hour where the Optical Density at 600 nm was measured. For samples whose optical density was significantly greater than 1, a 10 fold dilution was performed, in order to maintain the linear relationship between the optical density from the spectrophotometer and the concentration of the *E.coli* BL21 (DE3) culture.

4.1.3 preparation of Top10 and BL21 Competent cells

Chemically competent Top10 and BL21 cells were made using the calcium chloride method. Overnight cµlture was used to inoculate 100mL of LB and incubated at 37°C, 200rpm until OD_{600} = 0.4 - 0.6. Then transferred to two 50mL falcon tubes, left on ice for 30 minutes and

centrifuged at 4000g for 5 minutes at 4°C. The supernatant was drained and pellet resuspended in 25mL 0.1 M $MgCl_2$ followed by incubation on ice for 30 minutes and centrifugation at 4000g for 5 minutes at 4°C. The supernatant was drained and pellet resuspended in 25mL ice cold 0.1M CaCl, incubated on ice for 30 minutes and centrifuged at 4000g for 5 minutes at 4°C. The supernatant was then drained and the pellet resuspended in 1.25 ml ice cold CaCl2/Glycerol solution (1.7 ml 0.1 M CaCl2, 0.3 ml 100 % glycerol). 100µL aliquots were made and flash frozen on dry ice before storage at -80°C.

4.2.1 PCR extraction of ggpS from *Synechocystis sp. PCC 6803* Genome

200µL of synechocystis was centrifuged in a microcentrifuge at 13,000 rpm for one minute. The supernatant was drained and the pellet resuspended in 200µL ddH_2O. 10µL of this was then diluted in 90µL ddH_2O for a 10x dilution to create a stock and 20µL of this was transferred to a PCR tube and frozen at -20°C for 10 minutes. The Thermocycler was set to an initial denaturation of 98°C for 30 seconds followed by 35 cycles of 98°C for 10 seconds, 66°C for 30 seconds and 72°C for 30 seconds before a final extension at 72°C for 2 minutes and held at 4°C. The 25µL reaction contained 0.25µL Q5 Polymerase (New England Biolabs) wit 5µL 5x Q5 buffer and 0.5µL of 10mM dNTP mix with 0.5uM of designed Forward (GF) and Reverse Primers (GR), 10µL of lysed synechocystis and 6.75µL water. The PCR product was then run on 1% Agarose gel and extracted using the QI-Aquick gel extraction kit (Qiagen).

4.2.2 Cyanogate Level 0 Assembly

The Cyanogate molecular cloning method was used initially to construct our assemblies. The extracted *ggps* gene was inserted into the level 0 backbone PICH41308 (Vasudevan et al., 2019). The reaction mix contained 100 ng of PICH41308 backbone, 200 ng *ggps* insert, 2µL 10x T4 ligase buffer, 1µL bbsI-HF, 1µL T4 ligase-HF in a 20µL reaction. The assembly reaction was carried out with 15 cycles 10 minutes at 37°C, 10 minutes at 16°C followed by a cycle at 37°C for 20 minutes, one at 65°C for 10 minutes and held at 16°C.

4.2.3 Cyanogate Level 1 Assembly

The assembly was conducted in a thermocycler with 15 cycles 10 minutes at 37°C, 10 minutes at 16°C followed by a cycle at 37°C for 20 minutes, one at 65°C for 10 minutes and held at 16°C. The reaction mix contained: 200 ng of the insert DNA, ggpS, PTRC10 promoter and pC0.082 terminator in level 0 with 100 ng of pICH47732 acceptor vector, 2µL of T4 Ligase Buffer (New England Biolabs), 2µL Bovine Serum Albumin, 1µL BsaI restriction enzyme, 1 µL T4 DNA ligase and filling to 20µL with ddH2O (Vasudevan et al., 2019)

4.2.4 Cyanogate stpA Level T Assembly

The stpA gene with promoters and terminators with cutsites and overhangs was synthesised by TWIST Bioscience. The assembly was conducted in a thermocycler with 15 cycles 10 minutes at 37°C, 10 minutes at 16°C followed by a cycle at 37°C for 20 minutes, one at 65°C for 10 minutes and held at 16°C. The reaction mix contained: 200 ng of the insert DNA, stpA gene block and End linker in level 1 with 100ng of pSEVA421 acceptor vector 2µL of T4 Ligase Buffer (New England Biolabs), 2µL Bovine Serum Albumin, 1µL BbsI restriction enzyme, 1 µL T4 DNA ligase and filling to 20µL with ddH2O (Vasudevan et al., 2019)

4.2.5 Plasmid Transformations

50µL aliquots of Competent TOP10 or BL21 cells were incubated on ice for 30 mins with 5µL of Assembly reaction mix. The cells were heat shocked at 42°C for 30 seconds and returned to ice for 2 minutes. 450µL of SOC media was added and the cells incubated at 37°C for 1 hour. 50µL of culture was then spread on an LB plate with IPTG, XGAL and correct antibiotic. Spectinomycin for level T and level 0, Ampicillin for cyanogate level 1, kanamycin for JUMP level 1 with all constructs containing an IPTG inducible *lacz* reporter gene for blue/white selection . Remaining cells were spun at 6000rpm for 1 minute in a microcentrifuge. The supernatant was removed leaving 50µL in which the pellet was resuspended, then spread on a second LB agar plate and incubated overnight at 37°C.

4.2.6 Confirmation of Plasmid Assembly and Transformation

Successful assembly and transformation was verified by blue/white selection, colony PCR and plasmid digests with gel electrophoresis. White colonies indicate the presence of the lacZ gene in the transformed E.coli. White colonies were picked and resuspended in 50µL ddH_20 in a PCR tube. 40µL from the PCR tube was taken and inoculated in 1mL LB. Remaining 10mL was frozen at -20°C for 10 minutes to lyse the cells for template DNA. the 20µL reaction mix contained 5µL 5x Green GoTaq reaction buffer 0.2mM dNTP mix (New England Biolabs), 1µM forward and reverse primers and 0.125µL GoTaq G2 DNA polymerase (Promega). The Initial denaturation step was 95°C for 2 minutes with 35 cycles of 95°C for 1 minute, annealing at 42-65°C (Primer dependent e.g. L1F/L1R, L0F/L0R) extension at 72°C for 2 minutes (1Min/Kb) with a final extension of 72°C for 5 minutes and hold at 4°C. The reaction was then run on 1% Agarose gel electrophoresis and colonies with correct bands were miniprepped using the QIAprep Spin Miniprep Kit. The miniprep would then be digested using 1µL of miniprep, 1µL of appropriate cutting enzyme, 2µL of appropriate buffer and filled to 20µL with ddH_2O. This would then be run on agarose gel to look for expected band sizes.

4.3 Error Prone PCR

To generate a mutant library of ggpS, error prone PCR was used using Taq DNA Polymerase with thermopol buffer (New England Biolabs). Specifically designed primers were made for ggpS in Cyanogate level 1, however due to primer mis-annealing these primers were replaced with the primers GF and GR. Furthermore NEB dNTPs (10mM dATP, 10mM dCTP,10mM dTTP,10mM dGTP) were used due to unsuccessful use of custom mix (3.5mM dATP, 4mM dCTP,13.5mM dTTP,6mM dGTP) which would have further increased mutation rate. The reaction mix contained 5uL 10x NEB Taq DNA polymerase Thermopol buffer, 0.2mM GF forward primer, 0.2mM GR reverse primer, 0.5µL Taq Polymerase (New England Biolabs), 50ng level 0 *ggps* template, 2.95mM $MgCl_2$ and 0.1mM $MnCl_2$ to increase the mutation rate of the Taq polymerase. Filling to 50µL with ddH_2O. The Thermocycler steps were an initial denaturation at 95°C for 1 minute, 25 Cycles of 30 seconds at 95°C, 45 seconds at 53°C and 90 seconds at 68°C, a final elongation at 68°C for 5 minutes and held at 4°C. The PCR product was then purified using the Monarch DNA gel extraction kit (New England Biolabs) and stored at -20°C.

4.4 JUMP Level 1 Assembly

Due to the primer mis-annealing, the cyanogate level 1 backbone could not be used to generate the final plasmid mutant library. Consequently the decision was made to move out of cyanogate and into a level 1 JUMP vector. The reaction mix contained 20 fmol/µL of all DNA parts including pJUMP29-1A acceptor vector, PJ23100 Promoter in level 0, L3SAP51 terminator in level 0, pET Ribosome Binding Site in level 0 and purified *ggps* insert. As well as 0.25µL NEB T4 ligase, 1µL NEB BsaI Restriction enzyme, 2µL T4 Ligase buffer and fill to 20µL with dd$H_2$0. The thermocycler steps were 1 cycle at 37°C for 15 minutes then 25 cycles at 37°C for 3 minutes and 16°C for 3 minutes. Then 1 cycle of 37°C for 15 minutes, 50°C for 5 minutes and 80°C for 5 minutes before a further 25 cycles of 37°C for 3 minutes and 16°C for 3 minutes and 1 cycle of 37°C for 15 minutes, 50°C for 5 minutes and 80°C for 5 minutes and finally held at 4°C.

5. Author Contributions

M.W., K.L., G.D. performed experiments . M.W., K.L. Designed experiments and wrote manuscript. C.F., N.L. and J.S. gave advice on experimental design and troubleshooting.

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7. Conflicts of Interests

The authors declare no conflicts of interest.

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