CHARACTRISATION OF AMINO ACID BIOSYNTHETIC PATHWAYS IN SYNECHOCYSTIS SP. PCC 6803

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Thesis submitted for the degree of R1C760102 – Biomolecular Science Msc (Res), School of Biology, University of East Anglia

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Abstract

Cyanobacteria are one of the most important phyla of organisms on the planet, accounting for 20-30% of global carbon fixation[1]. Cyanobacteria have a wide array of potential applications in biotechnology. Key to unlocking that potential is gaining a deeper understanding of fundamental biological processes in cyanobacteria, most notably in model species such as *Synechocystis* sp. PCC 6803 (*Synechocystis*). Much of our current understanding of this model organism's central metabolism is based solely on identifying genes with homologs characterised in model heterotrophs. This includes amino acid biosynthesis, which includes several uncharacterised pathways that could be unique to cyanobacteria. A comprehensive understanding of amino acid biosynthesis will enhance their industrial utilisation. To characterise genes encoding enzymes involved in amino acid biosynthesis, this study aims to generate a novel method for generating auxotrophic mutants in *Synechocystis*. Multiple approaches were trialled. Initially mutants were generated using increasing concentrations of kanamycin, eliminating *slr0036* as an essential gene. I then attempted to segregate genes by supplementing media with single amino acids. This approach proved unsuccessful, likely due to the absence of importers or insufficient transport of amino acids into the cytosol. I then attempted to leverage the OppA oligopeptide importer, to import short chain oligopeptides into the cell where they could then be catabolised into their constituent amino acids. This method for compensating for the loss of function of the gene, allowed for successful generation of the *slr1312* and *slr2035* auxotrophic mutants. I tried to allow for import of single amino acids by introducing high affinity *Escherichia coli* (*E. coli*) K12 amino acid importers into Synechocystis, however due to time constraints I was unable to finish this. This study provides an invaluable technique for characterisation of genes encoding proteins involved in amino acid biosynthesis and identification of novel enzymes involved in novel cyanobacterial pathways.

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1. Introduction

1.1 Cyanobacteria

Cyanobacteria constitute an ancient and diverse phylum of prokaryotic organisms that have shaped the Earths ecosystems for billions of years. They are found in most ecosystems, contributing 20-30% of global carbon fixation[1]. Cyanobacteria are capable of conducting oxygenic photosynthesis, utilising the energy primarily for carbon fixation. Photosynthesis primarily occurs in thylakoid membranes[2], an array of flattened, stacked, interconnected membranes covered in light absorbing complexes termed phycobilisomes. These increase the available surface area for photosynthesis to take place. Cyanobacteria also contain carboxysomes, polyhedral microcompartments which play a vital role in carbon fixation via the 1,5-bisphospate carboxylase/oxygenase (RuBisCO) enzyme[3]. Many cyanobacteria are also naturally competent and can freely take up exogeneous DNA from the environment[4].

1.2 Biotechnology applications

Climate change poses a difficult challenge for the global community to overcome. It is driving the need for greater research into more renewable and sustainable solutions to some of mankind's more carbon intensive industries. One technology which promises to help reduce our reliance on CO_2 intensive industries is the use of cyanobacteria for absorption of CO_2 and biosynthesis of products that would otherwise add to our carbon footprint. Cyanobacteria could be used to produce a vast range of products, from biofuels and bioplastics to high end food and pharmaceutical products[5].

Biofuels are a promising area of research as the fuel could be used in existing engines. This would help to reduce the economic impact of moving to renewables as existing vehicles and infrastructure could use the fuel with potentially less contribution to climate change [6]. This would be especially useful for airlines as there is currently no viable alternative to jet fuel, as modern batteries lack the energy density needed to sustain long flights [7]. The use of cyanobacteria to produce biofuels would be more sustainable than other biofuel production methods, such as the use of sugar cane, as cyanobacteria can be grown much faster, with minimal nutrients, on non-arable land, without the use of fresh water in photobioreactors or raceway ponds[5].

Amino acids are precursors to many compounds desired by industry[8]. They can be used in the production of many dyes, suncreams, pharmaceuticals and much more[9][10]. Redirecting carbon flux via genetic manipulation is key to improving viability of this organisms use in industry. Currently this area of industry, like many others attempting to use cyanobacteria, are struggling with

commercialisation. Production of various compounds at scale, is proving extremely difficult. This is not just the case for biofuels but many other industries such as production of suncream and moisturiser derivatives. Skin care products produced via cyanobacteria have been produced in the lab, but production at scale is proving difficult [9]. Increased understanding of cyanobacterial central metabolism will allow for manipulation of the genome to more efficiently redirect carbon flux towards production of desired compounds.

1.3 *Synechocystis* sp. PCC 6803

The model organism, *Synechocystis*, is a freshwater autotroph which is naturally competent and can therefore take up free exogenous DNA from its environment [11]. This makes it a good candidate for genetic modification as the cells can more easily be altered to incorporate new genes and plasmids or knockout genes of interest. This is important as significant modification to the cyanobacterial genome will be needed for biosynthesis of products to become economically viable. *Synechocystis* has multiple copies of its genome [12], which can make it challenging to fully segregate a mutant.

Synechocystis is the most widely studied cyanobacterium [13]. Despite this most genes have been assigned function based on homologs characterised in *E. coli* or other heterotrophs [14]. However, many cyanobacterial proteins differ in terms of substrates while other pathways are specific to cyanobacteria.

1.4 Amino acid biosynthesis

Our current understanding of amino acid biosynthesis in cyanobacteria is based solely on homology with heterotrophs [13] (Figure 1). The majority of pathways have genes with high conservation to those characterised in *E. coli*. However, some *Synechocystis* pathways, such as those synthesising methionine, tyrosine and phenylalanine are not fully characterised due to differences with *E. coli*. Identification of these genes depends on either generating auxotrophic mutants or enzyme assays of likely candidates, which can be challenging to identify via bioinformatics [15][16].

Figure 1: Amino acid biosynthesis in *Synechocystis*. Amino acids are highlighted, adapted from (Mills, McCormick, & Lea-Smith, 2020). The 20 naturally occurring amino acids are highlighted in red and amino acids that are incorporated into peptidoglycan are highlighted in blue. Auxotropic mutants generated in this project have been underlined in red and partially segregated mutants generated in this project are underlined in green.

1.5 Amino acid and oligopeptide importers

A range of amino acid importer proteins have been characterised in *Synechocystis* [17]. The BgtA/BgtB complex transports arginine, histidine, lysine and glutamine (figure 2). The NatABCDE complex transports alanine, glycine, leucine, proline, serine and histidine, and possibly L-cystine [18]. Glutamate is transported via GtrABC and GltS . It is unknown whether transporters for the other ten amino acids

are present. In addition, the OppA system transporter, involved in import of peptides generated via peptidoglycan recycling, may also import amino acids or small peptides [14] (Figure 3).

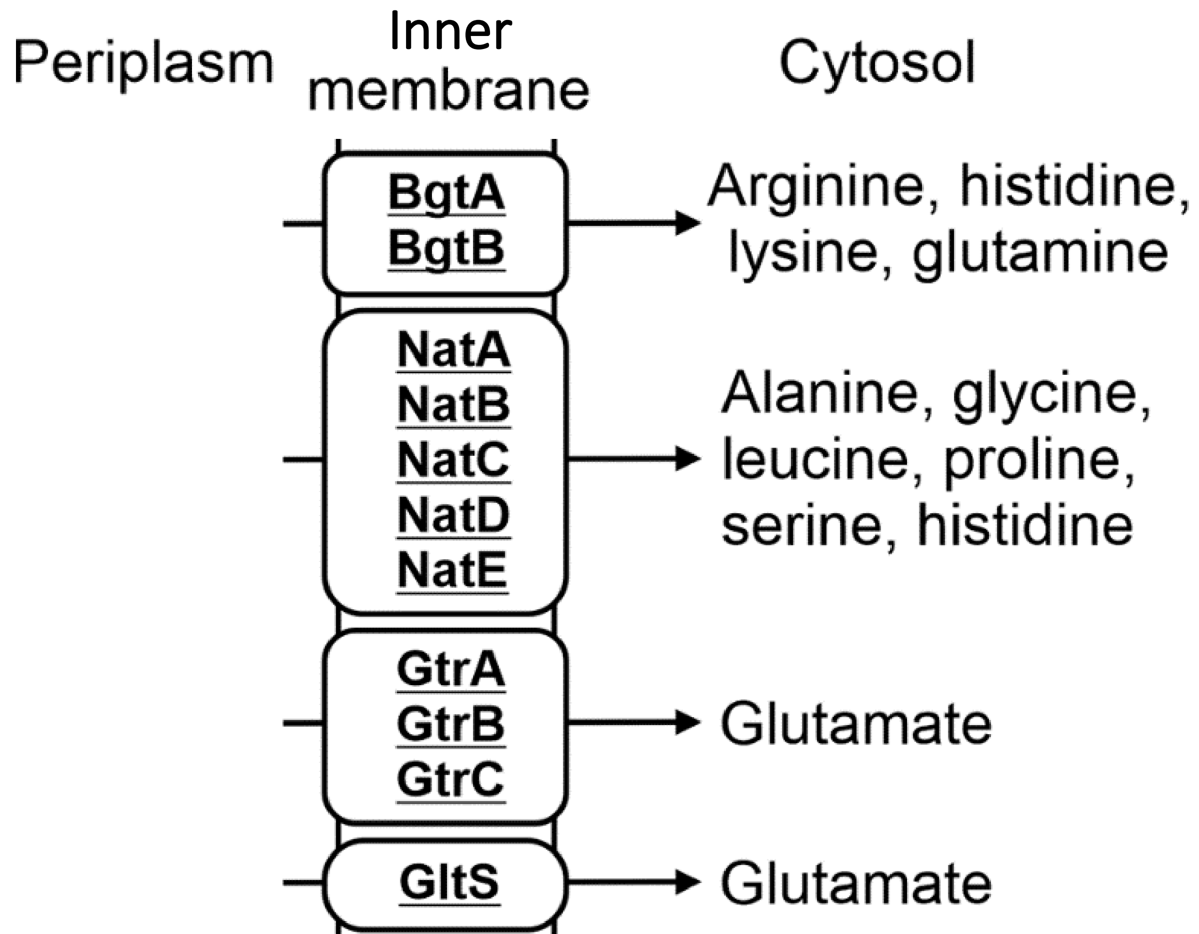


Figure 2: Transporters involved in amino acid biosynthesis. Adapted from (Mills, McCormick, & Lea-Smith, 2020).

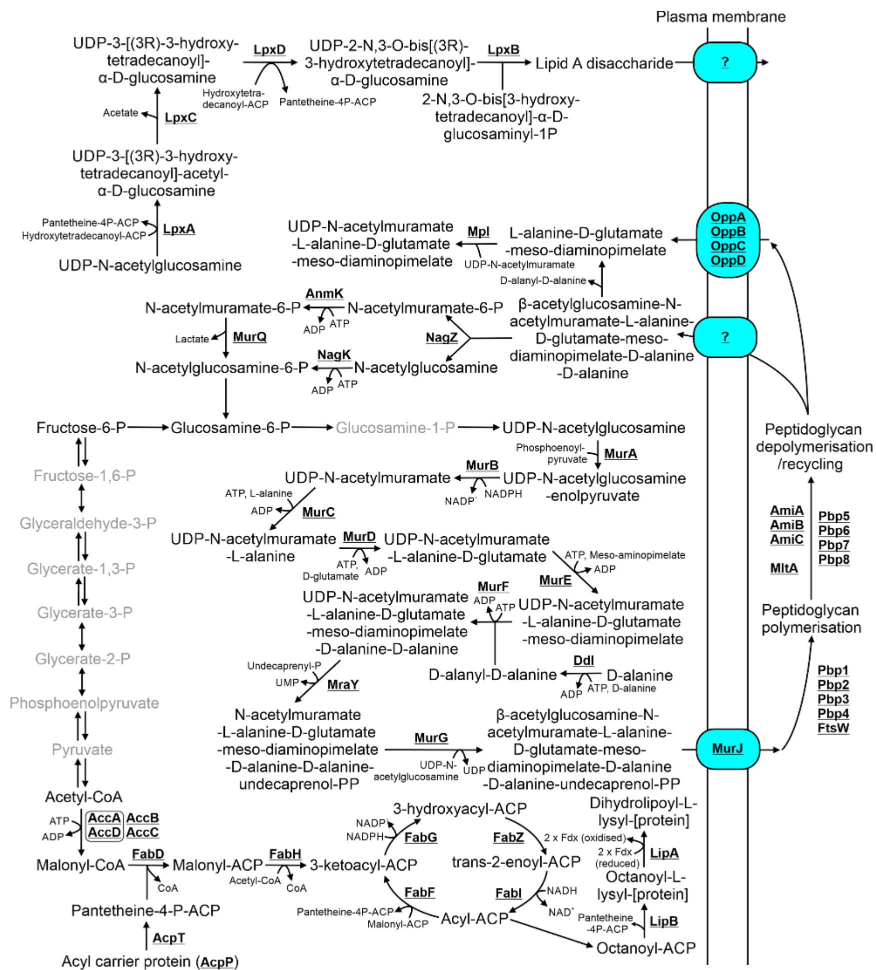


Figure 3: Peptidoglycan recycling showing the Opp transporter involved in peptide import. Adapted from (Mills, McCormick, & Lea-Smith, 2020).

1.6 Utilising Cyanosource to understand cyanobacterial amino acid biosynthesis

The cyanosouce project is a collaboration between the University of East Anglia, the Earlham Biofoundry and the University of Edinburgh, which aims to produce a mutant library for every gene in *Synechocystis* and make available the plasmids required to generate them. This project takes advantage of those cyanosource plasmids to rapidly generate mutants in a range of amino acid biosynthesis pathways. This project seeks to further cyanosouce by determining which of the genes in *Synechocystis* are essential for life and which are not. Amino acid auxotrophic mutants would then be available as part of a mutant library for other groups to use in their own research.

1.7 Aims

Improving our understanding of central metabolism in *Synechocystis* is key to utilising this species and other cyanobacteria in industry. The primary aim of this project is the development of a novel method for generating auxotrophic mutants in biosynthetic pathways in *Synechocystis*. Deletion of genes encoding putative amino acid biosynthesis enzymes was achieved using plasmids sourced through the cyanosouce project. Plasmids allowed for deletion of genes involved in amino acid biosynthesis. A range of techniques were used to try and generate these mutants including using increasing concentrations of kanamycin, supplementing media with single amino acids and compensating for loss of function of the gene with an oligopeptide mixture, N-Z amine. The main obstacles with this project are overcoming the lack of high affinity amino acid importers present in the cell surface membrane of *Synechocystis,* and knocking out the gene of interest on every copy of the chromosome. As a secondary aim, I attempted to express high affinity *E. coli* K12 amino acid importers using their natural promotors and terminators.

2. Materials and methods

2.1 BGII media

BGII medium was prepared according to Lea-Smith, Vasudevan, & Howe, 2016. Appendix table 1 shows the components used.

2.2 Transformation of premade plasmids into *Synechocystis*

Plasmids were generated by the CyanoSource project, containing a CodA/Kan cassette between an up and down flank, complementary to the regions flanking the gene targeted for deletion. These parts were integrated into a pUC19 plasmid. These plasmids were then transformed into *Synechocystis* according to Lea-Smith, Vasudeva, Howe 2016[19].

A fresh wild-type Synechocystis colony was used to inoculate 30-50 mL of liquid BGII medium. Cultures were then grown for 2-3 days to OD_{750nm} = 0.2 to 0.6, at 30°C. 1-2 mL of media was then centrifuged at 2,300 x g for 5 minutes. The supernatant was then discarded, and the pellet washed with fresh BGII liquid medium to a final volume of 100 µl. ~1 µg of pUC19 was then added to the cells and mixed by gently tapping. Tubes were then laid horizontally in the incubator for 4-6 hours at 30°C. Aliquots of 20 and 80 µl of the culture/plasmid mixture were then plated onto separate BGII plates without antibiotics. Plates were then incubated for ~24 hours at 30°C. 2.5-3 mL of 0.6% agar solution containing 100 µg/mL of kanamycin was then cooled to ~42°C and added to the top of the agar. Plates

were then incubated at 30°C until colonies were visible, which took approximately 7 days. Individual colonies were then plated on BGII + Kanamycin, 30 µg/mL agar plates split into 6 sections with separate colonies on each section.

2.3 Verification of mutants

Mutants were verified using PCR and gel electrophoresis. Primers designed for amplification of flanking regions during the construction of plasmids by CyanoSource were used for the verification of mutants (Appendix table 2). Template DNA was obtained by adding cells collected from plates using a toothpick, which was added to 50 µL of dH_2O. This would then be mixed using the toothpick until the solution was a homogeneous green colour.

PCR components per 25 µL reaction were set up as follows, 1.5 µL buffer containing 25 mM $MgCl_2$, 1.5 µL 1.25 mM DNTPs (0.075 mM final concentration), 0.2 µL 100% DMSO, 0.25 µL sigma polymerase (1 unit/mL), 1.5 µL 50 µM forward primer (3 µM final concentration), 1.5 µL 50 µM reverse primer (3 µM final concentration), 1 µL of template DNA and 17.55 µL distilled water. Components were then mixed gently and placed into a thermocycler. The following amplification protocol was used: 30 cycles of denaturation, annealing and extension, with the following temperatures and times. Initial denaturation, 30 seconds at 98°C. Denaturation, 10 seconds at 98°C. Annealing, 10 seconds at 60°C. Extension, 4 minutes at 72°C. Final extension, 7 minutes at 72°C. Reactions were then held at 12°C.

2.4 Generation of segregated mutants

Transformants were streaked out on BGII containing increasing concentrations of kanamycin in order to generate segregated mutants. These concentrations were 100, 200 and 300 µg/mL of kanamycin. Appropriate amino acids were added to plates at either 5 µg/mL or 50 µg/mL. The peptide N-Z amine was added at concentrations of 300 µg/mL, 600 µg/mL, 1mg/mL and 6 mg/mL. The amino acids in N-Z amine are listed in appendix table 3.

2.5 Production of single amino acid importer plasmids

Primers for amplifying *E. coli* K12 amino acid importers were designed using Primer3 to include the gene(s) and natural promoter and terminator (Appendix table 4). PCR products were generated using AccuTaq polymerase and if this was unsuccessful, Q5 polymerase. Components for AccuTaq PCR reactions were per 50 µL reaction: 5 µL AccuTaq buffer, 2.5 µL of 1.25 mM DNTPs (75 µM final concentration), 5 µL template DNA, 1 µL 100% DMSO, 1 µL 50 µM forward primer (1 µM final

concentration), 1 μL 50 μM reverse primer (1 μM final concentration), 34 μL Milli-Q water, 0.5 μL AccuTaq polymerase (1 unit/mL). The following amplification protocol was used: Initial denaturation, 30 seconds at 98°C; Denaturation, 15 seconds at 94°C; Annealing, 20 seconds at 65°C; Extension, 4 minutes at 68°C; Final extension, 6 minutes at 72°C. Components for Q5 PCR reactions per 50 μL reaction were: 0.25 μL Q5 polymerase (1 unit/mL), 5 μl 5 x Q5 buffer, 5 x Enhancer, 0.5 μL 1.25 mM DNTPs (12.5 μM final concentration), 10.75 μL Milli-Q water, 25 μL template DNA, 1.25 μL 50 μM forward primer (1.25 μM final concentration), 1.25 μL 50 μM reverse primer (1.25 μM final concentration) and 1 μL 100 % DMSO. The following amplification protocol was used: Initial denaturation, 30 seconds at 98°C; Denaturation, 10 seconds at 98°C; Annealing, 30 seconds at 62°C; Extension, 4 minutes at 72°C; Final extension, 2 minutes at 72°C.

Inserts were cloned into the shuttle plasmid pCAT.011 via restriction enzyme digest and ligation. Appendix table 5 shows the restriction enzyme for assembly. Assembly and ligation components per 25 μL reaction were: 1 μL pCAT.011 plasmid, 10 μL generated insert, 2 μL ligation buffer 10x, 1 μL BpiI, 1 μL ligase, 2.5 μL Milli-Q water and 2 μL Bsa 10x. Components then underwent assembly and ligation in a single step. Step 1: 5 minutes at 37°C; step 2: 5 minutes at 16°C. Steps 1 and 2 were then repeated 34 times. After 34 cycles, Step 3: 5 minutes at 60°C; step 4: 5 minutes at 12°C. The assembly product was then transformed into *E. coli* DH5α using the method outlined in appendix 6. Transformants were plated on LB media containing 100 μg/mL of spectinomycin and 40 μg/mL of X-Gal. Plates were incubated overnight at 37°C and white cells were then selected for verification. This was done via PCR and restriction enzyme digestions on the purified plasmids. Correct plasmids were then purified using a GeneClean® III kit according to the manufacturer's instructions.

3. Results

3.1 Generation of partially segregated mutants using kanamycin

I first tried to generate mutants of genes encoding enzymes in amino acid biosynthesis pathways by plating partially segregated mutants on increasing concentrations of kanamycin. This selects for cells with the antibiotic resistance cassette inserted into all chromosomes, if the gene is non-essential. Initially, *slr1133*, encoding argH, which catalyses the last reaction of the arginine biosynthesis pathway, was targeted. Despite re-streaking the mutant on plates containing 300 μg/mL of kanamycin, the strain was only partially segregated, as evident by the presence of bands corresponding to the wild-type and knockout profile (Figure 4).

However, two genes, *slr0036* and *sll1683*, were knocked out via this method (Figure 5 and 6, respectively). *slr0036* encodes for a putative AspC, which catalyses the reversible conversion of 2-oxoglutarate and L-aspartate to L-glutamate and oxaloacetate. An additional AspC candidate encoded by *sll0402* is also present in *Synechocystis* [14], and the non-essentially of *slr0036* suggests this may be the main or only enzyme catalysing this reaction. *sll1683* encodes for the lysine decarboxylase (cad) catalysing conversion of L-lysine to cadaverine. Non-essentially of this gene suggests cadaverine is not required for *Synechocystis* viability.

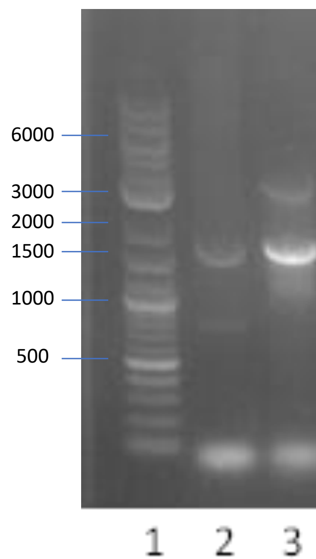
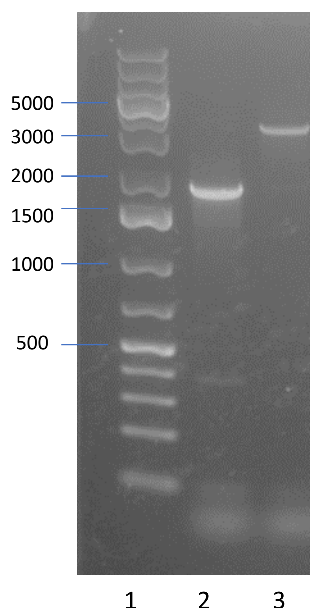


Figure 4: Generation of a partially segregated *Δslr1133* mutant. Mutants grown on BGII media containing 300 μg/mL of kanamycin were tested. Primers flanking the region targeted for deletion



were used for amplification. Expected band size in brackets. Lane 1: NEB 1 kb plus DNA ladder; Lane 2: wild-type (1653 bp), lane 3: Δ*slr1133* mutant (2949 bp).

Figure 5: Generation of a fully segregated *Δsll1683* mutant. Mutants were generated on BGII media containing 300 µg/mL of kanamycin. Primers flanking the region targeted for deletion were used for amplification. Expected band size in brackets. Lane 1: GeneRuler 1 kb plus DNA ladder; Lane 2: wild-type *sll1683* (2510 bp); Lane 3: *Δsll1683* (3819 bp).

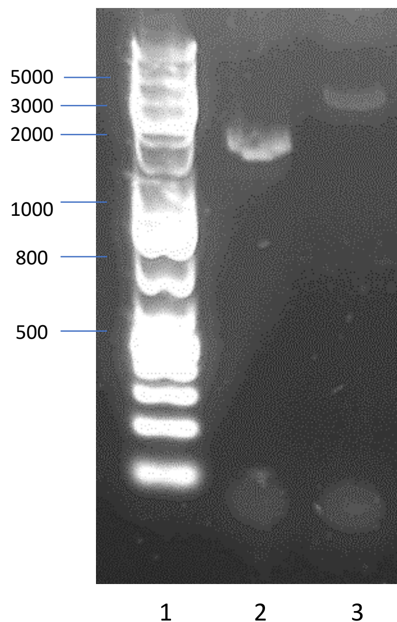


Figure 6: Generation of a fully segregated *Δslr0036* mutant. Mutants were generated on BGII media containing 300 µg/mL of kanamycin. Primers flanking the region targeted for deletion were used for amplification. Expected band size in brackets. Lane 1: GeneRuler 1 kbp plus DNA ladder; Lane 2: wild-type (2374 bp); Lane 3: *Δslr0036* (3963 bp).

3.2 Producing partially and fully segregated mutants using single amino acids

Next, I tried to generate auxotrophic mutants by segregating strains on media supplemented with the appropriate amino acid(s). Two concentrations were tested, 5 µg/mL and 50 µg/mL. *Synechocystis* grew on either of these concentrations for the following amino acids: proline, tryptophan, aspartate, serine, arginine, threonine and tyrosine. However, lysine proved to be toxic to wild-type *Synechocystis*, and inhibited growth on plates containing even 5 µg/mL. Supplementation with amino acids did not result in production of any fully segregated auxotrophic mutants for any of the genes investigated.

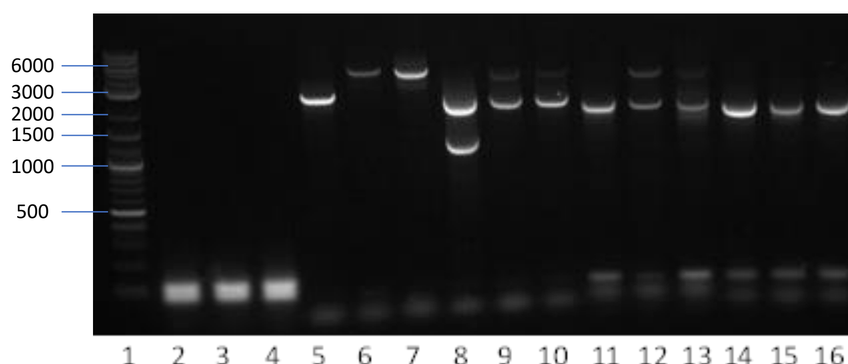


Figure 7: Generation of mutants on medium containing single amino acids and kanamycin 300 µg/ml. Primers used were designed to flank the region targeted for deletion. Mutants were generated on BGII media containing a single peptide concentration of 5 and 50 µg/mL and 300 µg/mL of kanamycin. Expected band size in brackets. Lane 1: NEB 1 kb plus DNA ladder; lane 2, 3, 4: failed amplification of wild-type and mutant *sll1883*; lane 5: wild-type *slr0036* (2374 bp); lane 6: *Δslr0036* 5 µg/mL Aspartate (3963 bp); Lane 7: *Δslr0036* 50 µg/mL Aspartate (3963 bp); lane 8: wild-type *sll0402* (2229 bp) with off target sequence (~1400 bp); Lane 9: *sll0402* partial knockout 5 µg/mL Aspartate (3818 bp); lane 10: *sll0402* partial knockout 50 µg/mL Aspartate (3818 bp); lane 11: wild-type *slr2035* (2047 bp); Lane 12: *slr2035* partial knockout 5 µg/mL Proline (3694 bp); Lane 13: *slr2035* partial knockout 50 µg/mL Proline (3694); lane 14: wild-type *sll1662* (1947 bp); Lane 15: *sll1662* partial knockout 5 µg/mL Phenylalanine (3814 bp); Lane 16: *sll1662* partial knockout 50 µg/mL Phenylalanine (3814 bp).

Table 1: List of genes targeted for deletion. Fully segregated genes have been highlighted in yellow.

Gene number	Gene name	Amino acid biosynthesis pathway
slr0055	*TrpG*	Tryptophan
sll1883	*ArgJ*	Arginine
sll0109	*AroH*	Tyrosine and Phenylalanine
sll1662	*PheA*	Phenylalanine
slr2081	*TyrA*	Tyrosine
slr0738	*TrpE*	Tryptophan
sll0900	*HisG*	Histidine
sll1908	*SerA*	Serine
slr1348	*CysE*	Cystine and Alanine
slr0550	*DapA*	Lysine
sll1058	*DapB*	Lysine

sll0455	*ThrA*	Threonine
slr2072	*IlvA*	Isoleucine
slr2035	*ProB*	Proline
sll0402	*AspC*	Aspartate
slr0036	*AspC*	Aspartate
sll1683	*Cad*	Cadaverine
slr1133	*ArgH*	Arginine

3.3 Generation of segregated mutants by culturing strains on medium supplemented with an oligopeptide mixture

I first determined whether *Synechocystis* could importing peptides found in N-Z-amine. To do this *Synechocystis* was cultured on medium with various concentrations of N-Z-amine in the presence of $NaNO_3$ or absence of any other nitrogen sources (Figure 8). *Synechocystis* demonstrated growth on all plates which was robust on concentrations higher than 600 µg/ml, showing that a robust system of peptide import is present. For future work we used plates with a concentration of 1 mg/mL N-Z-amine.

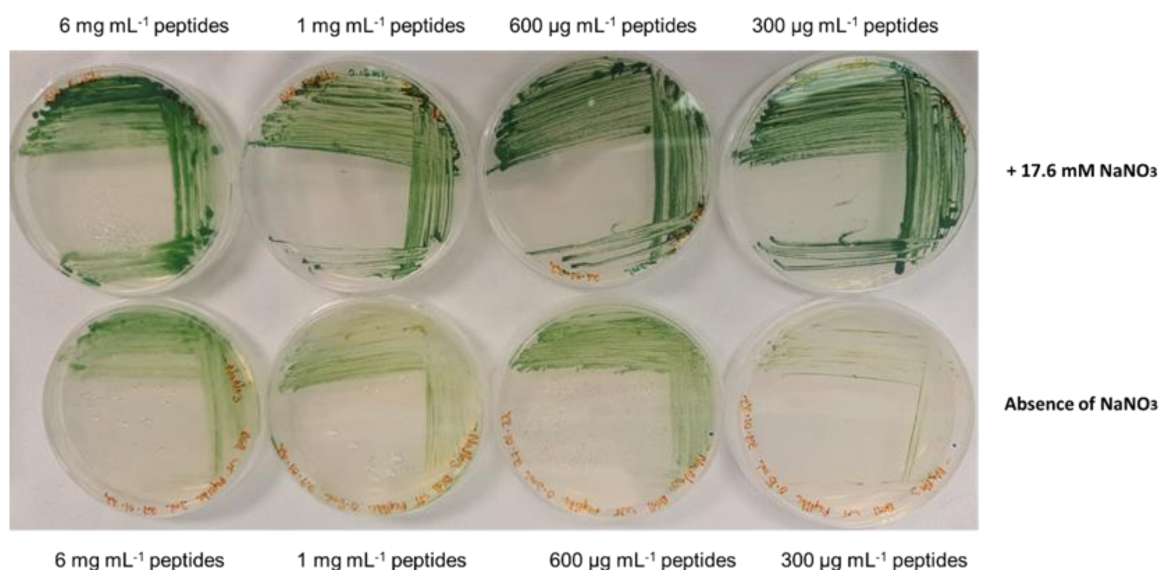


Figure 8: Growth of *Synechocystis* on BG11 medium supplemented with N-Z-amine at different concentrations. The top row of plates were grown with sodium nitrate while the bottom row of plates were grown in the absence of sodium nitrate. Concentrations of N-Z-amine used in each plate are shown at the top.

I next tried segregating mutants on medium supplemented with 1 mg/mL N-Z-amine. This method resulted in generation of two auxotrophic mutants in two separate pathways: *slr2035* encoding for ProB, the first enzyme in the proline biosynthesis pathway, and *sll1883*, encoding for ArgJ, the first enzyme in the arginine biosynthesis pathway (Figure 9).

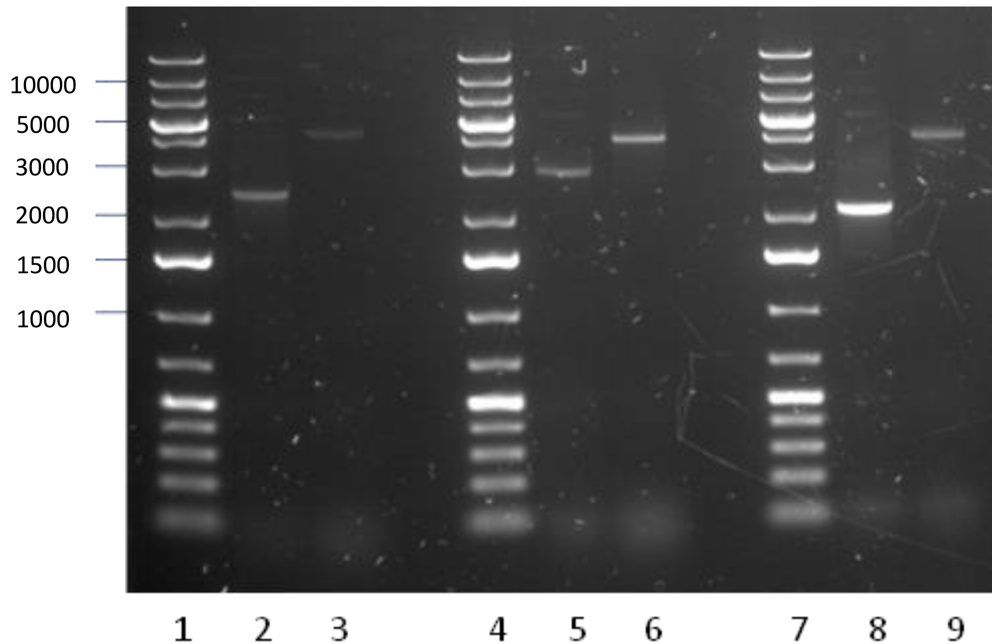


Figure 9: Generation of fully segregated mutants, *Δslr*0036, *Δslr*1312 and *Δslr*2035. Primers used were designed to flank the region targeted for deletion. Mutants were generated on BGII media containing an oligopeptide mixture without sodium nitrate. Expected band size in brackets. Lane 1, 4, 7: GeneRuler 1 kbp plus DNA ladder; Lane 2: wild-type *slr0036* (2374 bp); lane 3: *Δslr0036* (3963 bp); lane 5: wild-type *slr1312* (2812 bp); lane 6: *Δslr1312* (3684 bp); lane 8: wild-type *slr2035* (2047 bp); lane 9: *Δslr2035* (3694 bp).

Although two genes were fully segregated using this method (figure 9 and 10), many other genes remained partially segregated (Figure 11). These include *sll1683* (figure 11), *slr0738* and *sll1883* (figure 10). Many mutants lack a PCR product corresponding to the knockout profile, including *slr0702, sll1908, sll1662, slr1312, slr0055* and *sll0900.* Although a segregated *sll1883* knockout was obtained (Figure 11), the plate became contaminated and had to be discarded and I lacked time to replicate this (Figure 10).

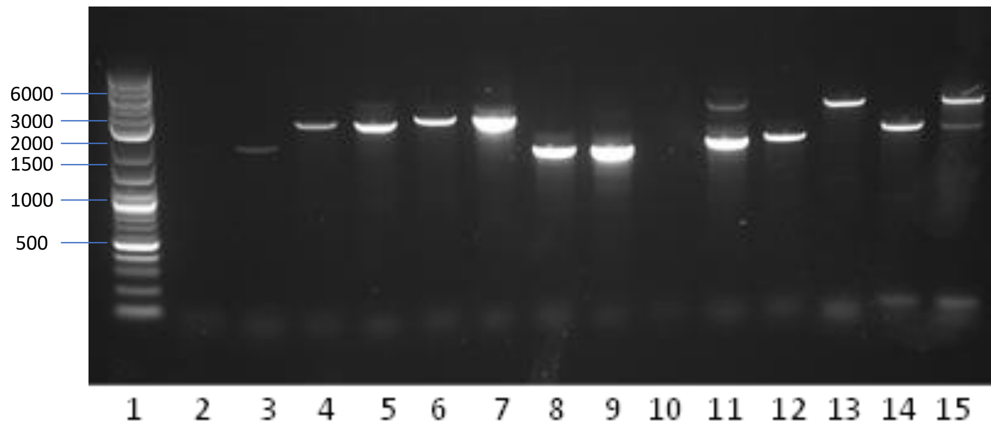


Figure 10: Generation of partially segregated mutants using an oligopeptide mixture and kanamycin 300 µg/ml. Primers used were designed to flank the region targeted for deletion. Mutants were generated on BGII media containing an oligopeptide mixture and 300 µg/mL of kanamycin in the BGII media. Expected band size in brackets. Lane 1: NEB 1 kb plus DNA ladder; lane 2: wild-type *sll1058* (1685 bp); lane 3: mutant *sll1058* (3699 bp); lane 4: wild-type *slr0738* (2508 bp); lane 5: mutant *slr0738* (3777 bp); lane 6: wild-type *sll1908* (2724 bp); lane 7: mutant *sll1908* (3821 bp); lane 8: wild-type *sll0900* (1727 bp); lane 7: mutant *sll0900* (3855 bp); lane 10: wild-type *sll1662* (1947 bp); lane 11: mutant *sll1662* (3814 bp); lane 12: wild-type *slr2035* (2047 bp); lane 13: mutant slr2035 (3694 bp); lane 14: wild-type *sll1883* (2420 bp); lane 15: mutant *sll1883* (3919 bp).

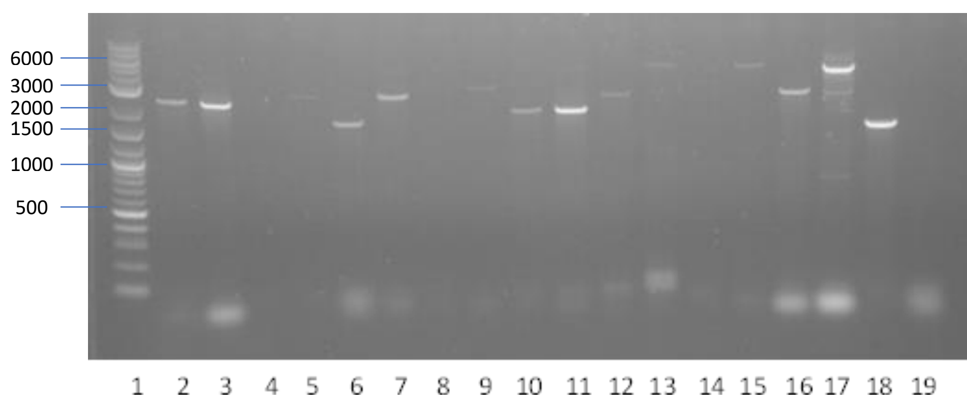
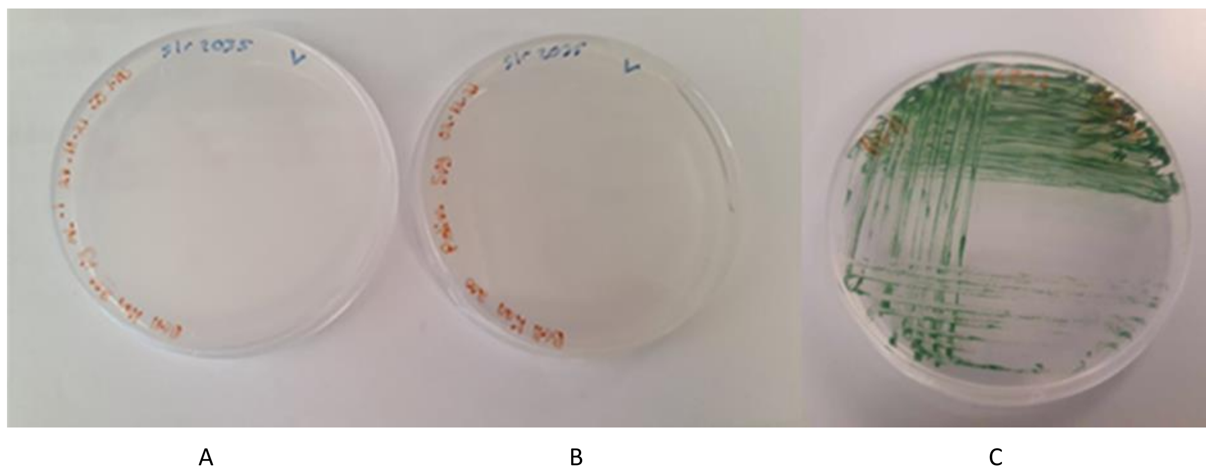


Figure 11: Generation of partially segregated mutants using an oligopeptide mixture and kanamycin 300 µg/ml. Primers used were designed to flank the region targeted for deletion. Mutants were generated on BGII media containing an oligopeptide mixture and 300 µg/mL of kanamycin in the BGII media. Expected band size in brackets. Lane 1: NEB 1 kb plus DNA ladder; lane 2: wild-type *sll0402* (2229 bp); lane 3: mutant *sll0402* (3818 bp); lane 4: unsuccessful generation of wild-type *slr1058* PCR product; lane 5: wild-type *slr0702* (2508 bp); lane 6: mutant *slr1058* (3699 bp); lane 7: mutant *slr0702*

(3777 bp); lane 8: wild-type *sll1908* (2724 bp); lane 9: mutant *sll1908* (3821 bp); lane 10: wild-type *sll1662* (1947 bp); lane 11: mutant *sll1662* (3814 bp); lane 12: wild-type *sll1883* (2420 bp); lane 13: mutant *sll1883* (3919 bp); lane 14: wild-type *slr1312* (2812 bp); lane 15: mutant *slr1312* (3684 bp); lane 16: wild-type *sll1683* (2510 bp); lane 17: mutant *sll1683* (3819 bp); lane 18: wild-type *slr0055* (1462 bp); lane 19: mutant *slr0055* (3815 bp).

3.4 Confirmation that *slr2035* is an essential gene in the absence of proline

To confirm whether these are auxotrophic mutants we streaked out the *Δslr2035* on N-Z-amine free BG11 plates without and with 5 µg/mL proline (figure 12). The auxotrophic mutants were unable to grow on these plates, suggesting that the cells either lack importers for proline or they demonstrate insufficient activity to compensate for loss of the gene.



A B C

Figure 12: *Slr2035* fully segregated mutants are unable to grow in the absence of the oligopeptide mixture. The strain was streaked on BGII medium containing 200 µg/mL of kanamycin (Plate A) or BGII media containing 200 µg/mL of kanamycin and 5 µg/mL of proline (Plate B). Plate C shows *slr2035* growing on BGII media supplemented with N-Z-amine and 200 µg/mL of kanamycin, without sodium nitrate.

3.5 Generation of importer gene plasmids

In conjunction with trialling generation of auxotrophic mutants on different supplemented medium, we attempted to generate recombinant *Synechocystis* strains overexpressing *E. coli* amino acid importers (Appendix table 6). This may allow generation of auxotrophic mutants on medium supplemented with individual amino acids. Inserts for importers gene plasmids, GlnQPH, CycA, GltP,

YaaJ, DcuB, CadB and ProP were generated using AccuTaq polymerase, whereas BrnQ, GlnQPH, LysP, MetINQ, TdcC, Ansp and arcD were generated using Q5 polymerase.

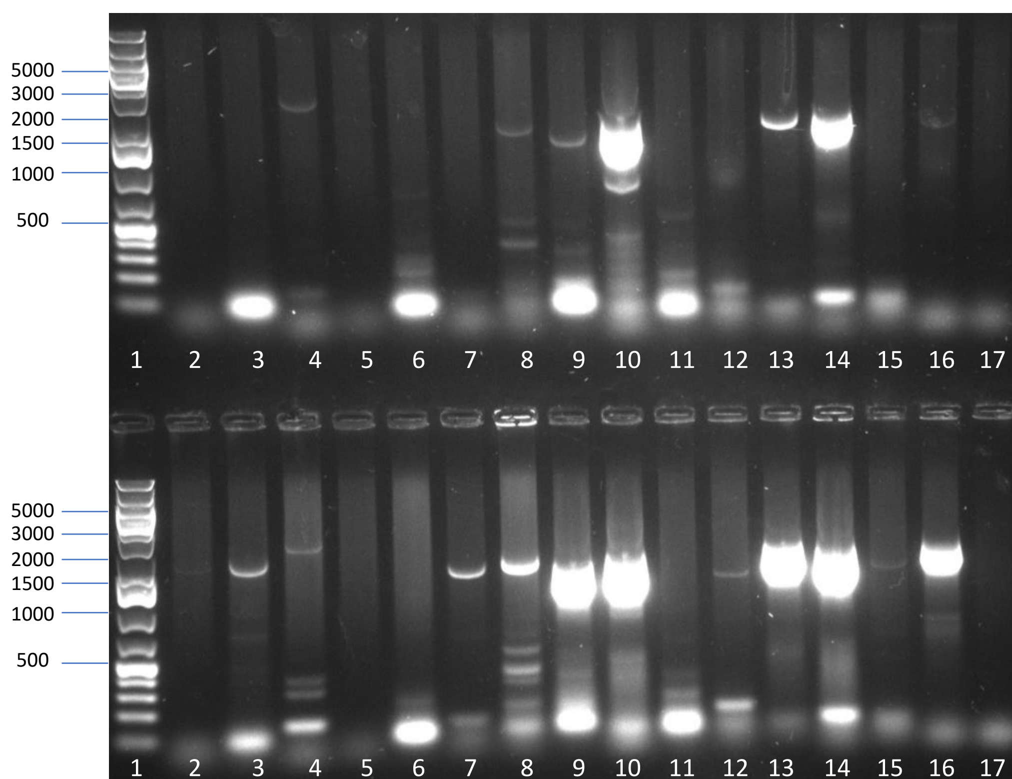


Figure 13: Generation of importer inserts using AccuTaq. Primers used were designed to flank the importer gene and its natural promoter and terminator. PCR was performed on wild-type E. coli K12. Expected band size in brackets. The top row was generated at an annealing temperature of 60°C and the bottom row was generated at 55°C. Lane 1: GeneRuler 1 kb plus DNA ladder; lane 2: BrnQ (1795 bp); lane 3: AroP (1941 bp); lane 4: GlnQPH (2503 bp); lane 5: HisJQPM (3482 bp); lane 6: LysP (1943 bp); lane 7: CadB (1618 bp); lane 8: CycA (1764 bp); lane 9: GltP (1588 bp); lane 10: YaaJ (1742 bp);

lane 11: MetINQ (2774 bp); lane 12: TdcC (1404 bp); lane 13: DcuB (1905 bp); lane 14: AnsP (1873 bp); lane 15: ArcD (1609 bp); lane 16: ProP (1899 bp); lane 17: TcyP (1511 bp).

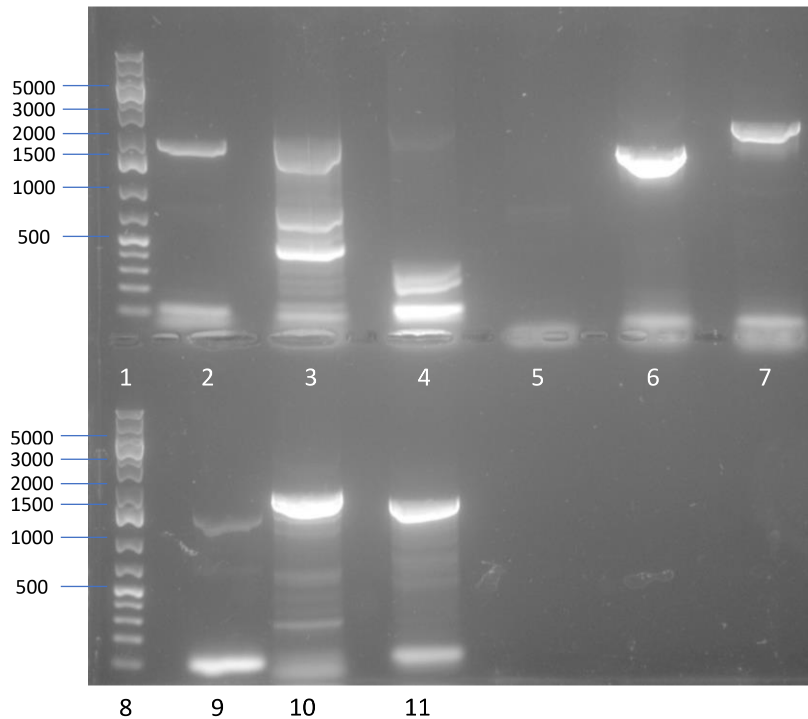


Figure 14: Generation of importer inserts using Q5. Primers used were designed to flank the importer gene and its natural promoter and terminator. PCR was performed on wild-type E. coli K12. Expected band size in brackets. Lane 1, 8: GeneRuler 1 kb plus DNA ladder; lane 2: BrnQ (1795 bp); lane 3: AroP (1941 bp); lane 4: GlnQPH (2506 bp); lane 5: failed HisJQPM (3482 bp); lane 6: LsyP (1943 bp); lane 7: MetINQ (2774 bp); lane 9: TdcC (1404 bp); lane 10: AnsP (1873 bp); lane 11: ArcD (1609 bp).

I was unable to show insertion of the importer gene into the plasmid via restriction enzyme digest due to the low plasmid copy number of pCAT .011 (Appendix table 5). Instead, PCR was performed on the purified plasmid using the primers designed to amplify the importer gene. I was unable to transform these plasmids into *Synechocystis* due to time constraints. However, all plasmids were generated except for those with the HisJQPM and TcyP inserts.

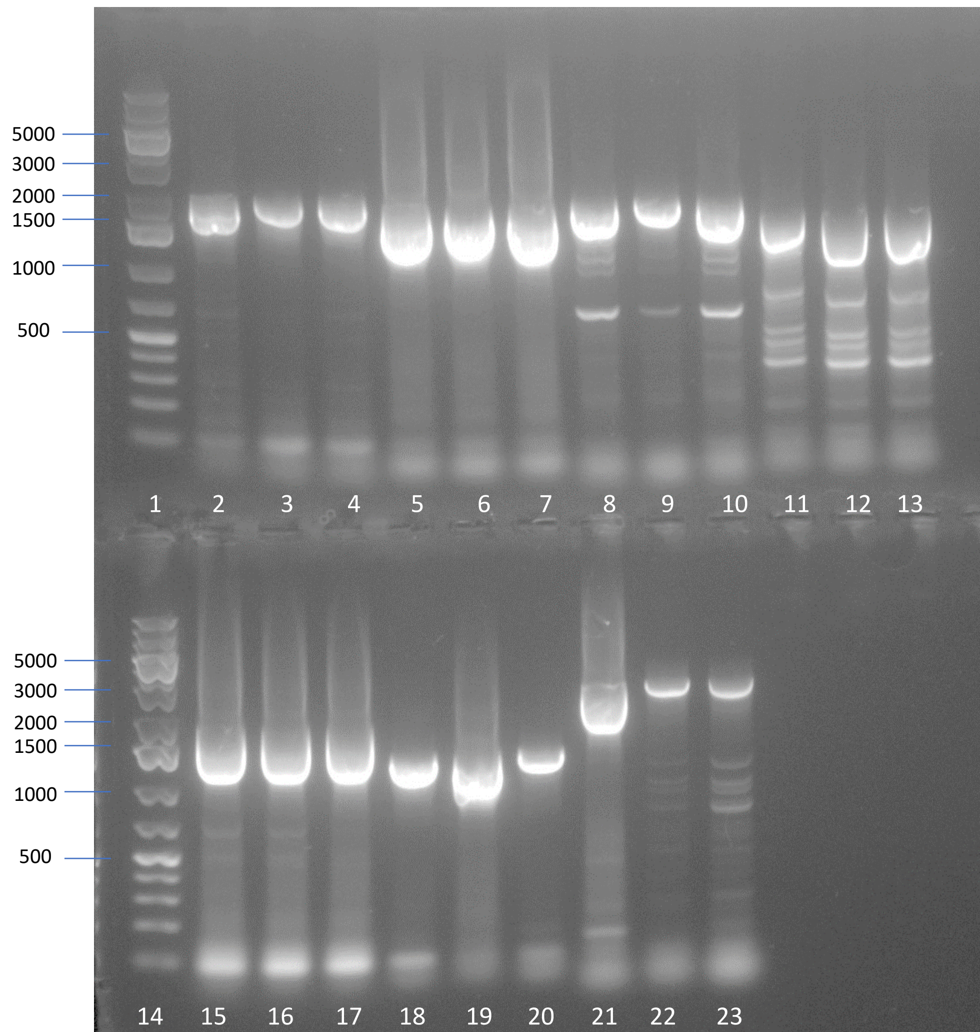


Figure 15: Verification of successful generation of plasmids containing importer genes. Primers used were designed to flank the importer gene and its natural promoter and terminator. PCR was performed on purified plasmids. Expected band size in brackets. Lane 1: GeneRuler 1 kb plus DNA ladder; lane 2, 3, 4: LysP (1943 bp); lane 5, 6, 7: BrnQ (1795 bp); lane 8, 9, 10: AnsP (1873 bp); lane 11, 12, 13: ArcD (1609 bp); lane 15 ,16, 17: AroP (1941 bp); lane 18, 19, 20: TdcC (1404 bp); lane 21, 22, 23: MetINQ (2774 bp).

4. Discussion

In this study to the best of my knowledge produced the first amino acid auxotrophic mutant in *Synechocystis*. Auxotrophic mutants were generated of genes encoding enzymes in two separate pathways, suggesting that this method could be used to generate further mutants in some of the other pathways. I have also showed that *Synechocystis* can grow on an oligopeptide mixture as its soul source of nitrogen, suggesting that oligopeptide importers are present in the plasma membrane. Although this method was successful in generating auxotrophic mutants in two separate pathways, it was not successfully used to generate auxotrophic mutants for 15 other genes. A possible reason for this issue is the relative abundance of certain amino acids in the N-Z amine mixture may have been too low to compensate for loss of function of the gene. Another possibility is the unknown peptidases in *Synechocystis* may have only cleaved certain peptides and released only a subset of amino acids in sufficient quantities. Future studies should utilise other peptide mixtures, which may contain and release higher amounts of other amino acids.

An additional outcome of this study was generation of a cadaverine deficient mutant. Although this gene is not involved with amino acid biosynthesis, it may be lead to lysine toxicity, which should be tested in the future. The function of cadaverine has not been determined in cyanobacteria and future studies are required to investigate this, with phenotypic studies of this mutant a first step.

I was also able to generate plasmids expressing *E. coli* amino importers. While I was unable to transform any plasmids into *Synechocystis* due to time issues, any mutants which demonstrate expression of the importers may be useful for generating auxotrophic mutants in pathways for which supplementation with peptides is insufficient. However, the importer will have to be inserted into the plasma membrane in the correct orientation.

Overall, this study successfully pioneered a novel method for generating auxotrophic mutants in cyanobacteria. Due to time constraints, I was unable to replicate my results in more amino acid biosynthesis pathways. Future studies should focus particularly on trying to generate auxotrophic mutants of the known genes in the methionine, tyrosine and phenylalanine pathways. Combined with bioinformatics study, primarily comparative genomics, this method could then be used to identify the unknown genes in these pathways.

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6. Appendix

Appendix table 1: components for BG11 growth media in accordance with Lea-Smith, Vasudevan, & Howe, 2016[19].

Stock solution recipes	
Chemical	Amount (g)
100x BG11 (per L)	
$NaNO_2$	149.6
$MgSO_4.7H_2O$	7.49
$CaCl_2.2H_2O$	3.6
Citric acid	0.6
Add 1.12 mL 0.25 M Na_2EDTA, pH 8.0	
0.25 M Na2EDTA, pH 8.0 (per 100 ml)	
Na_2EDTA	9.3
Trace elements (per 100 ml)	
H_3BO_3	0.286
$MnCl_2.4H_2O$	0.181
$MnCl_2.4H_2O$	0.022
$Na_2MoO_2.2H_2O$	0.039
$CuSO_4.5H_2O$	0.008
$Co(NO_3)_2.6H_2O$	0.005

Appendix table 2: primers used for verification of mutants. Primers generated by CyanoSource, originally used to generate the plasmids. Lower case letters are restriction enzyme digest sites used in plasmid construction by CyanoSource.

sll0109_LF	atcgaGGTCTCAGGAGTCACCACTAAGGCATTGTCC
sll0109_RR	gctcaGGTCTCTAGCGGGCTAACTTGCCCGTTTTT
sll0402_LF	atcgaGGTCTCAGGAGAACAAACAGCGCATCATGGG
sll0402_RR	gctcaGGTCTCTAGCGaaagcggacaggggagaaag
sll0900_LF	atcgaGGTCTCAGGAGCGGGGTAAAACTGTGATTGG
sll0900_RR	gctcaGGTCTCTAGCGGGACACGGTGCAGGAAAAA
sll1662_LF	atcgaGGTCTCAGGAGAAGGAAACCAAACAGGGGAA
sll1662_RR	gctcaGGTCTCTAGCGGTTGCCCAATTCTCCCAAC
sll1683_LF	atcgaGGTCTCAGGAGCCAAAATGGCGATCGGACTG
sll1683_RR	gctcaGGTCTCTAGCGTTTGCAGATAAATGGTAAA
sll1883_LF	atcgaGGTCTCAGGAGaacagtacaccgatgccctg
sll1883_RR	gctcaGGTCTCTAGCGGCCTTCTTTCTGTAGGGCT
slr0036_LF	atcgaGGTCTCAGGAGCCGACTGTAAATGCTCTTTG
slr0036_RR	gctcaGGTCTCTAGCGCAACCCTTGTCCCCCATACC
slr0055_LF	atcgaGGTCTCAGGAGGGTGCTAAAAAGCCGTTGAG
slr0055_RR	gctcaGGTCTCTAGCGgtcgatgtcccggtcgtaaa
slr0550_LF	atcgaGGTCTCAGGAGGAGGGAAAAATTCCCCAAGC
slr0550_RR	gctcaGGTCTCTAGCGGCAAAAACGGGGCTAAGCAA
slr0608_LF	atcgaGGTCTCAGGAGgaaggcgcagtggaattgtc
slr0608_RR	gctcaGGTCTCTAGCGaaaaagtcagggccacaggt
slr0657_LF	atcgaGGTCTCAGGAGccatggagcaggcagaaattg
slr0657_RR	gctcaGGTCTCTAGCGATGTGAAGAAGTCCCGCCTG
slr0738_LF	atcgaGGTCTCAGGAGgcactggtggacttctctcc
slr0738_RR	gctcaGGTCTCTAGCGAACGCATAGGCTAGCAGTCC
slr0827_LF	atcgaGGTCTCAGGAGcgtaaacgcctttctcccct
slr0827_RR	gctcaGGTCTCTAGCGAATGGCCACTGGTTGTGTT
slr1312_LF	atcgaGGTCTCAGGAGAACCTACAACATCGTTGCCG
slr1312_RR	gctcaGGTCTCTAGCGACAGAGCGGCGGTAAAGTAA
slr2035_LF	atcgaGGTCTCAGGAGtctgtcttctttgtcggggc
slr2035_RR	gctcaGGTCTCTAGCGGCAAGCTATACGCCAGTGA

slr2072_LF	atcgaGGTCTCAGGAGTTTACCAAGCGGACTTCCAG
slr2072_RR	gctcaGGTCTCTAGCGGAGGGGCAACTTCTTCGGC
slr2081_LF	atcgaGGTCTCAGGAGtccccagaacgattgagtgc
slr2081_RR	gctcaGGTCTCTAGCGgtccctggcaccaatcatca
sll0455_LF	atcgaGGTCTCAGGAGATGCTTCCTTCGCTGTCCAA
sll0455_RR	gctcaGGTCTCTAGCGAATGAGAATGGCGCAGTCCA
slr0186_LF	atcgaGGTCTCAGGAGAATAGAACCAACGGTGCGAC
slr0186_RR	gctcaGGTCTCTAGCGgagaccaaatgttcatccaag
slr1348_LF	atcgaGGTCTCAGGAGCACGGCTGGATTTATCGCAT
slr1348_RR	gctcaGGTCTCTAGCGCGGATTGCGTAGCCAGTAA
slr0091_LF	atcgaGGTCTCAGGAGctgtcaatacgccccattctg
slr0091_RR	gctcaGGTCTCTAGCGGCTCCGGCTATGTAACTAGAATTGGT
sll1349_LF	atcgaGGTCTCAGGAGGGTTGGGTAGGAAAAAACGG
sll1349_RR	gctcaGGTCTCTAGCGTGCTGAATGGTCAAATCCT
slr0458_LF	atcgaGGTCTCAGGAGGGGAATCAGCCGGAATTAGA
slr0458_RR	gctcaGGTCTCTAGCGgggtcagccaatctaaacacaa
sll0404_LF	atcgaGGTCTCAGGAGGGCTTTTCAAGCACGCTCTT
sll0404_RR	gctcaGGTCTCTAGCGcgatcgccaaaggtacaggt

Appendix table 3: concentration of amino acids in N-Z amine. The average MW is approximately 250 Da. The MW distribution is 54% at 100-200 Da, 36% at 200-500 Da, and 9% at 500-1000 Da. (average MW of a single amino acid is 110Da)

Amino Acid	Amount (mg/g)
Alaine	30
Arginine	31
Asparagine	67
Cytosine	3
Glutamate	186
Glycine	19
Histidine	22
Isoleucine	44
Leucine	75
Lysine	68
Methionine	27
Phenylalanine	40
Proline	88
Serine	51
Threonine	42
Tryptophan	10
Tyrosine	28
Valine	59

Appendix table 4: nucleotide sequence for importer genes

Primer Name	Nucleotide Sequence
MetNIQ_For	GACTAATGATGCCGAACTTAACGCC
MetNIQ_Rev	GACTTGGAGCCATTTACTGACTGCG
TdcC_For	GACTATCAGTTTACCCAGCGACACT

TdcC_Rev	GATCGAACCACTTTGCGTCCATGAC
Ansp_For	GACTAGAGGAACACTGTACCCACGAS
Ansp_Rev	GACTTTGCTGGCTGGATGTACTTCA
DcuB_For	GACTACTCGGATAAGTCGGCAGGAT
DcuB_Rev	GACTTCGGTGCGCATATGTCTGAAA
BrnQ_For	GACTGAAGACTTGCCTTATTGCCATAAGCCA
BrnQ_Rev	GACTGAAGACCCTCCCATCAACACAATAAAAA
ArcD_For	GACTATGGTGGAAATGATGGGACCA
ArcD_Rev	GACTTATTTGCACGCAGATGTTGGT
ProP_For	GACTAGGGCGTGGTGGTGTTAATTT
ProP_Rev	GACTTCGCCCTGTTCCAGTTTATCG
TcyP_left	GACTAAGCTTTGCCTTTGAAATATAAGAGACCAG
TcyP_right	GACTTCTAGATCCCAGAATTAATGGTGTGCC
AroP_left	GACTGAAGACCCTCCCATCAACACAATAAAAA
AroP_right	GACTGAAGACCCTCCCCCACCTTGCCGAAGTC
GltP_left	GACTAAGCTTGCCTCCTGCCATAAACTCGC
GltP_right	GACTTCTAGATCCCTCGCATCAGGCATTCA
BrnQ_left	GACTGAAGACTTGCCTTATTGCCATAAGCCA
BrnQ_right	GACTGAAGACCCTCCCATCAACACAATAAAAA
Yaaj_left	GACTAAGCTTTGCCAGTCCTTGCAGGAAATTT
Yaaj_right	GACTTCTAGATCCCGATGATATCACGACGCAA
cadb _left	GACTAAGCTTTGCCTCCAGGTAAAAAAGG
cadb _right	GACTTCTAGATCCATCAACTTGCGATCC
GlnQPH_left	GACTGAAGACTTTGCCTCCCTTTCGGGTGAAA
GlnQPH_right	GACTGAAGACCCTCCCTGTCACGCAGGG
HisJQPM_left	GACTGAAGACTTTGCCTTCCGTCACCCCTCA
HisJQPM_right	GACTGAAGACCCTCCCCTGGGAAGCTGTACC
CycA_left	GACTAAGCTTTGCCATTCTGTTCCCCTCGAAC
CycA_right	GACTTCTAGATCCCGGCATTGCGCCATC
LysP_left	GACTGAAGACTTTGCCTTATCGCTCACATCTT
LysP_right	GACTGGCTTTGCCTTTTCGCTCACATCTT

Appendix table 5: nanodrop measurements of the concentration of nucleic acids found in plasmid purification from importer genes found in *E. coli*.

Gene	Sample	Concentration of nucleotides ng/ml
YaaJ	1	30
	2	41
GltP	1	32.9
	2	26.9
	3	21.3
ProP	1	35.6

	2	14.8
	3	46.2
CycA	1	46.7
	2	51.1
	3	72.2
AroP	1	29.2
	2	25.8
	3	34.2
MetINQ	1	13.9
	2	26.7
	3	50
LysP	1	32
	2	26.4
	3	16.3
BrnQ	1	24.2
	2	23.7
	3	7.6
AnsP	1	19.1
	2	30.3
	3	30.7
ArcD	1	23.5
	2	21.2
	3	14.3
TdcC	1	11.2
	2	15.6
	3	12.4

Appendix table 6: list of high affinity importers found in *E. coli* K12[20], all genes used in this project have been highlighted and a list is also available in appendix 5.

Amino acid	**ATP-type**	**Ion-driven**
Alanine		YaaJ, CycA
Arginine	ArgT-HisQPM, ArtJIMQP	ArcD
Asparagine		AnsP
Aspartic acid	GltIKJL	DcuB, GltP
Cystine	TcyJLN	TcyP
Glycine		YaaJ, CycA
Glutamine	GlnQPH	
Glutamic acid	GltIKJL	GltP, GltS, GadC
Histidine	HisJQPM	
Isoleucine	LivFGHMKJ	BrnQ
Leucine	LivFGHMKJ	BrnQ
Lysine	ArgT-HisQPM	LysP, CadB
Methionine	MetINQ	

Phenylalanine	LivFGHMK	PheP, AroP
Proline		PutP, ProP
Serine		TdcC, SdaC, SstT
Threonine		TdcC, SstT
Tryptophan		Mtr, AroP
Tyrosine		TyrP, AroP
Valine	LivFGHMKJ	BrnQ
Cysteine		

Appendix table 7: list of importer genes from *E. coli* DH5α with expected band sizes and restriction enzyme cut sites added.

Gene	Band size	Restriction enzyme cut sites
BrnQ	1795	BPiI
AroP	1941	BPiI
GlnQPH	2506	BPiI
HisJQPM	3482	BPiI
LysP	1943	BPiI
CadB	1618	HINDIII + XbaI
ArcD	1609	BPiI
CycA	1764	HINDIII + XbaI
GltP	1588	HINDIII + XbaI
TcyP	1511	HINDIII + XbaI
Yaaj	1742	HINDIII + XbaI
MetINQ	2774	BPiI
TDcC	1404	BPiI
ProP	1899	HINDIII + XbaI
DcuB	1905	XbaI + EcoRI
AnsP	1873	BPiI

Nickie's Quick Competent Cells

• Pick single colony from plate
• Set up overnight culture
• Inoculate 10 mL LB with 100 μL 0/N culture
• Incubate for 3 h shaking
• Spin down cells
• Resuspend in 10 mL ice cold 100 mM $MgCl_2$
• Leave on ice for 5 min
• Spin down cells
• Resuspend in 1 mL ice cold 100 mM
•Leave on ice for at least 30 min prior to use or (for higher transformation efficiency) leave overnight
•Cells are competent for 1 or two days (on 2 day competence will be lower)
• This method is not suitable for freezing the cells!

Transformation

Pre-chill Eppendorf tube on ice
• Add 1 μL plasmid
• Add 50 μL competent cells

- Gently mix
- Leave on ice for 30 min
- Heat shock for 2 min in 42°C water bath
- Transfer back to ice for 2 min
- Add 500 µL LB Incubate for at least 1 h at 37°C
- Spread 55 µL on plate labelled 10%
- Spin down the rest and remove most of the supernatant
- Gently Resuspend the pellet and spread the remaining cell suspension on a plate labelled 90%
- Incubate at 37°C overnight

Appendix figure 1: transformation of *E. coli* DH5