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Characterisation of Amino Acid Biosynthetic Pathways in *Synechocystis* sp. PCC 6803 via Analysis of Auxotrophic Mutants

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Declaration:

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Table of Contents:

Acknowledgements……5

Abstract ……6

List of figures……7

1. **Introduction** ……..……8

1.1 Cyanobacteria. ……..……8

1.2 Biotechnology applications of cyanobacteria..……..……8

1.3 *Synechocystis* sp. PCC 6803……....……...9

1.4 Amino acid biosynthesis in *Synechocystis* sp. PCC 6803……..……...10

1.5 Characterisation of amino acid biosynthesis via generation and analysis of auxotrophic mutants…….……12

1.6 Project aims…….…..14

2. **Materials and Methods** ……15

2.1 Preparation of BG11 media and cultivation of *Synechocystis*……...15

2.2 Transformation of plasmid DNA into *Synechocystis*..……..…15

2.3 Generation of *Synechocystis* mutants cultured on BG11 medium supplemented with single amino acids…….……...16

2.4 Generation of *Synechocystis* mutants cultured on terrific broth nutrient rich media……..……..……..16

2.5 Generation of *Synechocystis* mutants cultured on brain heart infusion nutrient rich media……...……...17

2.6 Generation of *Synechocystis* mutants cultivated on a nitrogen deficient media supplemented with oligopeptides ……..……..……17

2.7 Construction of plasmids for expression of high affinity amino acid Transporters……..……17

3. **Results** ……19

3.1 Multiple genes encoding putative amino acid biosynthesis are essential in *Synechocystis*……...19

3.2 Lysine is toxic to *Synechocystis* at concentrations of 5 µg mL^{-1}……..……21

3.3 *Synechocystis* failed to grow on brain heart infusion or terrific broth nutrient rich media……..22

3.4 *Synechocystis* can harvest peptides as a nutrient source on nitrogen deficient media……..……23

3.5 Generation of auxotrophic mutants on BG11 media supplemented with an oligopeptide mix……..……..…….26

3.6 *E. coli* K-12 amino acid importers were transformed into *Synechocystis* ..…..28

4. **Discussion** ……36

4.1 Generation of auxotrophic mutants on oligopeptide supplemented media....36

4.2 Lysine as a media additive is toxic to *Synechocystis*…….……..…..36

4.3 Future directions…….……..37

5. **References** ……37

6. **Appendix**……..40

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ABSTRACT

Cyanobacteria are an important phylum which play a key role in ecological processes such as nitrogen and carbon fixation, in addition to being a potential renewable platform for production of high value chemical precursors. Many stains of cyanobacteria also produce notable natural products such as herbicides, anti-microbial, and anti-fungal compounds. To utilise cyanobacteria more effectively, greater understanding of central metabolism would aid engineering strategies for redirection of metabolic flux to secondary metabolites. Even in well studied cyanobacteria such as S*ynechocystis* sp. PCC 6803 (*Synechocystis*), many genes involved in the biosynthesis of amino acids, precursors for a range of chemicals and natural products, remain uncharacterised. In this study I attempted to characterise genes encoding for amino acid biosynthetic enzymes via generation of auxotrophic mutants, a process whereby a target gene is deleted while mutants are cultivated on media containing the metabolite synthesised by the deleted target pathway. An initial approach at generating auxotrophic mutants on BG11 media supplemented exclusively with a single amino acid yielded only partially segregated mutants. This suggests that native *Synechocystis* amino acid transporters were either absent, or inefficient at importing amino acids to compensate for loss of gene function. A second approach successfully obtained auxotrophic mutants in multiple amino acid biosynthesis pathways by using BG11 media supplemented with an oligopeptide mix. This is the first study in which an auxotrophic mutant was generated in *Synechocystis* using these methods. This thesis establishes and discusses methods of generating auxotrophic mutants to provide insight for further characterisation of proteins involved in amino acid biosynthesis.

LIST OF FIGURES

Figure 1: Current understanding of the pathways involved in amino acid biosynthesis in *Synechocystis* ……11

Figure 2: Transporters that have been identified in *Synechocystis* including several mediating amino acid importation……13

Figure 3: Assembled plasmid for target gene knockout……...15

Figure 4a: Example agarose gel to illustrate how mutant generation was verified using PCR……20

Figure 4: Generation of a partially segregated *slr*0036, *sll*0402, and *slr*2035 mutants in *Synechocystis* cultured on BG11 media supplemented with single peptides……..21

Figure 5: Inhibition of *Synechocystis* growth due to lysine toxicity……22

Figure 6: A *Synechocystis* mutant (*slr*0036) failed to be cultured on TB (Left) and BHI (right) nutrient rich media……23

Figure 7: Wild-type *Synechocystis* cultivated on BG11 -N^0 media supplemented with an oligopeptide mix (1 mg mL-1)……24

Figure 8: A *Synechocystis* mutant (*slr*1133) cultivated on BG11 -N^0 media supplemented with an oligopeptide mix (1 mg mL^{-1})……25

Figure 9: Media conditions effect growth of wild-type *Synechocystis*……26

Figure 10: Generation of fully segregated deletion mutants of *slr*0036, *slr*1312 and *slr*2035……27

Figure 11: Amino acid transporters expressed in *E. coli* K-12……..29

Figure 12: Generation of inserts except LysP, MetNIQ, and HisJQPM using AcuTaq© high-fidelity polymerase……...31

Figure 13: Generation of remaining inserts except HisJQPM using Q5© high-fidelity polymerase……....32

Figure 14: PCR verifies generation of inserts……..33

Figure 15: PCR verifies ligation of LysP inserts into pCAT.011……..….34

Figure 16: PCR verifies ligation of Ansp and MetNIQ inserts into pCAT.011……35

1. INTRODUCTION

1.1 Cyanobacteria.

Cyanobacteria are a diverse phylum of prokaryotes characterised by their ability to undergo oxygenic photosynthesis [1], and their contribution to 20 – 30 % of global carbon fixation [2]. Cyanobacteria are also the primary nitrogen fixing organisms in open marine ecosystems [3]. Whilst aspects of cyanobacterial physiology are conserved with other prokaryotes, cyanobacteria are defined by cellular apparatus that are involved in photosynthesis and DNA uptake. The key photosynthetic organelles of cyanobacteria are the thylakoid membranes and carboxysomes. Thylakoid membranes provide a large surface area to accommodate photosynthetic pigments and complexes [4], whilst carboxysomes contain carbonic anhydrase and arrays of RuBP-carboxylase which is required for efficient carbon fixation. Cyanobacteria, like many other prokaryotes have cell surface appendages such as pili. Whilst pili are often used in locomotion and surface adhesion, in cyanobacterial strains they also facilitate natural transformation via the uptake of naked exogenous DNA [5]. Like many photosynthetic organisms, cyanobacteria are polyploid. This polyploid trait is presented in the strain of interest relevant to this study, S*ynechocystis* sp. PCC 6803 (*Synechocystis*) and consequently, the number of chromosomes throughout the growth and division of *Synechocystis* is variable depending on growth conditions and the phase of growth of the cell [6] [7]. The polyploid trait is relevant to this study as having multiple copies of a chromosome, hence multiple copies of a gene can potentially complicate genetic manipulation. An example of this, is for target gene deletion, the gene copy must be knocked out in every single chromosome copy otherwise over multiple generations the target gene will still be present, and a mutant cell can revert to its wild-type genotype, undoing attempts of genetic manipulation.

1.2 Biotechnology applications of cyanobacteria.

The conversion of water and carbon dioxide into biomass and other valuable secondary metabolites using energy derived from photosynthesis makes cyanobacterial strains potential platforms for producing high value chemicals, including cosmetics, dyes, herbicides, and pharmaceuticals [8]. There is great potential for capitalising on the value of cyanobacteria derived products, with pigments alone having a market value of $1.5 billion in 2019 [9]. It is highly likely that chloroplasts descended from a symbiotic event involving cyanobacteria and a non-photosynthetic host [10], as such, certain physiological and biochemical traits are conserved in higher photosynthetic organisms. This potentially makes cyanobacteria excellent

platforms for production of plant derived compounds. In addition, cyanobacteria produce many natural products, such as herbicides, and medically and industrially relevant compounds including fungicides, bactericides, biofuels, and pigments [8]. However, commercial production of many of these compounds will require synthetic biology approaches to direct metabolic flux to synthesising precursors to increase yields. An increase in global demand for biotechnology derived products including dyes, biofuels, pharmaceuticals, nutrient and food supplements poses a global issue. To meet this demand arable land has been used to produce these products for example sugar cane for biodiesel. The use of arable land to produce biotechnological derived products competes with the increasing need for crop production to meet the requirements of a growing global population. Cyanobacteria may therefore prove to be an incredibly useful phylum to produce products as they can be cultivated on non-arable land using wastewater thereby not competing with crops for resources.

1.3 S*ynechocystis* sp. PCC 6803.

Synechocystis is the most widely studied cyanobacterial strain and was the first fully sequenced phototrophic organism [11]. *Synechocystis* is a strain of freshwater, unicellular, Gram-negative bacteria, which incorporate a series of internal membrane-bound thylakoid membranes and carboxysomes. Structurally *Synechocystis* is encompassed by three main layers, the peptidoglycan layer, an outer membrane, and a protein sheath (S-layer).

Synechocystis has been used to investigate metabolic and photosynthetic processes conserved in other cyanobacteria, higher algae, and plants [12][13]. Whilst *Synechocystis* lacks the extensive suite of cloning tools available to *Escherichia coli* (*E. coli*), recent advancements have made modular cloning techniques available, including a Golden Gate based assembly method [14]. These recent advancements have permitted mutant libraries such as CyanoSource to be constructed [15]. This library will consist of approximately 3,500 deletion mutants targeting nearly every gene in the *Synechocystis* genome. These mutant libraries facilitate understanding of gene function and allow for rapid plasmid development using standard vector parts for use in synthetic biology applications via generation of unmarked knockouts and strains with multiple deletion mutations. Decades of research, development of powerful modular cloning systems, the complete genome sequencing, and having the most genes characterised out of any cyanobacterial strain makes *Synechocystis* an ideal candidate as a cyanobacterial model organism. The ability to re-streak *Synechocystis* indefinitely, is also incredibly valuable to this study which requires repeated streaking of *Synechocystis* cells over several plates with different conditions.

1.4 Amino acid biosynthesis in S*ynechocystis* sp. PCC 6803.

Despite the commercial and environmental significance of cyanobacteria, many aspects of central metabolism, including amino acid biosynthesis, are poorly understood, with several pathways uncharacterised in *Synechocystis* [1]*.* Amino acids are the precursors for many natural and chemical products, therefore better understanding of these pathways may aid future synthetic biology approaches. Our understanding of amino acid biosynthesis in *Synechocystis* is primarily derived from identification of homologues characterised in *E. coli* and other better studied heterotrophic bacteria [1] (Figure 1). Despite this, there is limited characterisation of several key amino acid biosynthesis genes that are not encoded by homologues found in *E. coli*. Some notable differences between *E. coli* and *Synechocystis* are in the methionine biosynthesis pathway. *Synechocystis* does not encode homologues to MetA, MetB, or MetC, the first three enzymes in the *E. coli* pathway. This suggests a novel methionine biosynthesis pathway is present in *Synechocystis*. The biosynthesis of phenylalanine and tryptophan in *Synechocystis* is also dissimilar to *E. coli*. *Synechocystis* synthesises phenylalanine and tryptophan from prephenate by a partially characterised pathway whilst *E. coli* synthesises these amino acids from L-arogenate (L-pretyrosine) [16]. The *Synechocystis* arginine biosynthesis pathway is similar to *E. coli* however *Synechocystis* does not encode homologues to ArgA and ArgE and instead utilises an alternative ornithine acetyltransferase that encodes both the first and fifth stages in arginine biosynthesis. Lysine biosynthesis also varies in *E. coli* compared to *Synechocystis, E. coli* requires three enzymes, DapC, DapD, and DapE, to synthesis lysine from 2-3-4-5 tetrahydrodipicolinate whereas in *Synechocystis* these three enzymes are replaced by DapL.

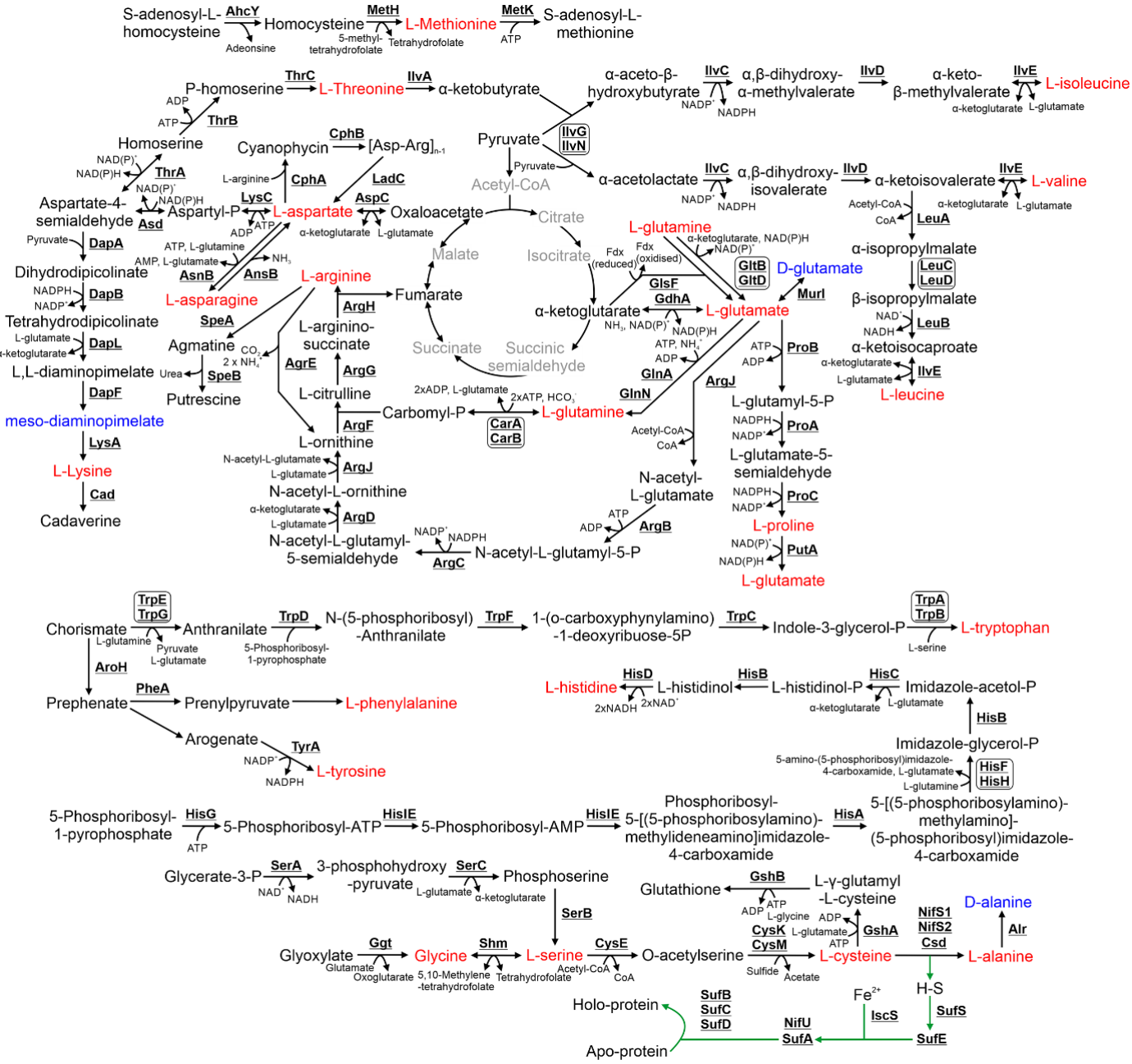

Figure 1: Current understanding of the pathways involved in amino acid biosynthesis in *Synechocystis* [1]. The 20 L-amino acids are highlighted in red and two D-amino acids are highlighted in blue.

1.5 Characterisation of amino acid biosynthesis via generation and analysis of auxotrophic mutants.

In *E. coli*, enzymes involved in amino acid biosynthesis have been characterised via analysis of auxotrophic mutants [17]. To generate an auxotrophic mutant, a gene encoding an enzyme catalysing the synthesis of an amino acid is targeted for deletion. The deleted mutant will then be grown on media supplemented with the appropriate amino acid to compensate for the loss of gene function [18]. This method of characterisation is dependent on utilising strains with appropriately expressed amino acid importers to permit compensation via uptake of peptides from media. To date no auxotrophic mutants have been generated in *Synechocystis* using this method.

Multiple transporters in *Synechocystis* have been identified [19] [1] (Figure 2). *Synechocystis* contains a basic amino acid transporter (L-alanine, glycine, L-leucine, L-proline, L-serine, and L-histidine) and glutamine permease composed of two subunits, BgtA and BgtB, encoded by *slr*1735 and *sll*1270 respectively [20]. L-arginine and L-lysine are imported via a neutral ABC type amino acid transporter which consists of five sub-units, natABCDE. Transporters have not been identified for the other amino acids.

Recycling of the peptidoglycan layer in *Synechocystis* leads to production of oligopeptides which are imported via a general oligopeptide transporter called Opp [21]. Importation of oligopeptides could then be cleaved by peptidases to obtain single amino acids, acting as an alternate source to compensate for loss of gene function.

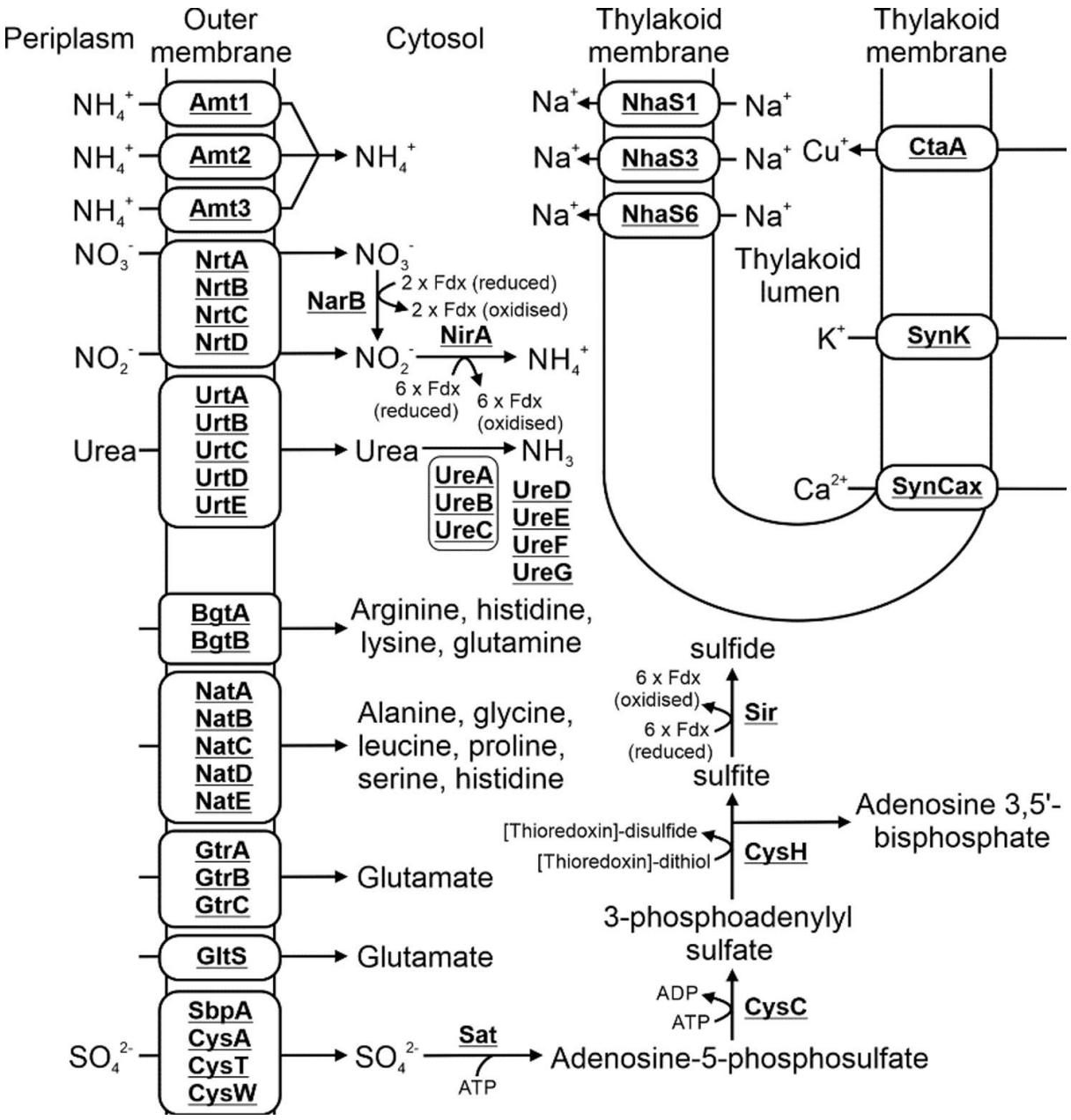

Figure 2: Transporters that have been identified in *Synechocystis* including several mediating amino acid importation. Adapted from Mills *et al.,* 2019 [1].

1.6 Project aims.

This thesis will focus on improving understanding of amino acid biosynthesis in *Synechocystis*. A critical issue in utilising *Synechocystis* and other cyanobacterial species for synthetic biology applications is the lack of understanding of key metabolic pathways. Current understanding of pathways, specifically amino acid biosynthetic pathways is limited, with a lack of suitable homologues of other prokaryotes for comparative genomics, and difficulty producing auxotrophic mutants for characterisation of genes. This has left gaps in our understanding of *Synechocystis* amino acid biosynthesis, specifically in the methionine, tyrosine, and phenylalanine pathways.

The primary aim of this research will be the development and optimisation of a method for generating auxotrophic mutants in *Synechocystis* via the deletion of genes encoding putative amino acid biosynthetic enzymes. I utilised plasmids assembled through the CyanoSource project [22] to accelerate generation of deletion mutants targeting a range of genes encoding putative enzymes in different amino acid pathways. Mutants were cultured on media supplemented with individual amino acids, nutrient rich media, and media supplemented with a broad-spectrum oligopeptide mix (N-Z-amine-A), in an attempt to generate fully segregated mutants suitable for further characterisation studies.

A key issue surrounding the generation of auxotrophic mutants in *Synechocystis* is the lack of efficient amino acid transporters to import peptides in sufficient quantities to compensate for loss of biosynthetic gene function. A secondary aim of this research will be the overexpression of high affinity *E. coli* K-12 transporters into *Synechocystis* as a method to obtain auxotrophic mutants. This method is hoped to facilitate generation of auxotrophic mutants and compensate for the lack of native high affinity amino acids transporters in *Synechocystis*. This excludes the amino acids L-alanine, glycine, L-leucine, L-proline, L-serine, L-histidine, glutamine, L-arginine, and L-lysine as these transporters are present in wild-type *Synechocystis.*

2. MATERIALS AND METHODS

2.1 Preparation of BG11 medium and cultivation of *Synechocystis*.

BG11 medium was prepared according to Castenholtz, 1988 [23]. *Synechocystis* was cultivated on agar plates or in liquid culture in an AlgaeTron AG300 photobioreactor (PSI) at 30 °C under a constant 50 µmol photons m^{-2} s^{-1} warm white light illumination photoperiod. Liquid cultures were shaken at 160 rpm. All further photobioreactor growth was under these light conditions.

2.2 Transformation of plasmid DNA into *Synechocystis*.

Plasmids were obtained premade from the CyanoSource library. Each plasmid was designed to produce a marked deletion mutant of a single specific gene. The basic structure of each plasmid is shown in Figure 3.

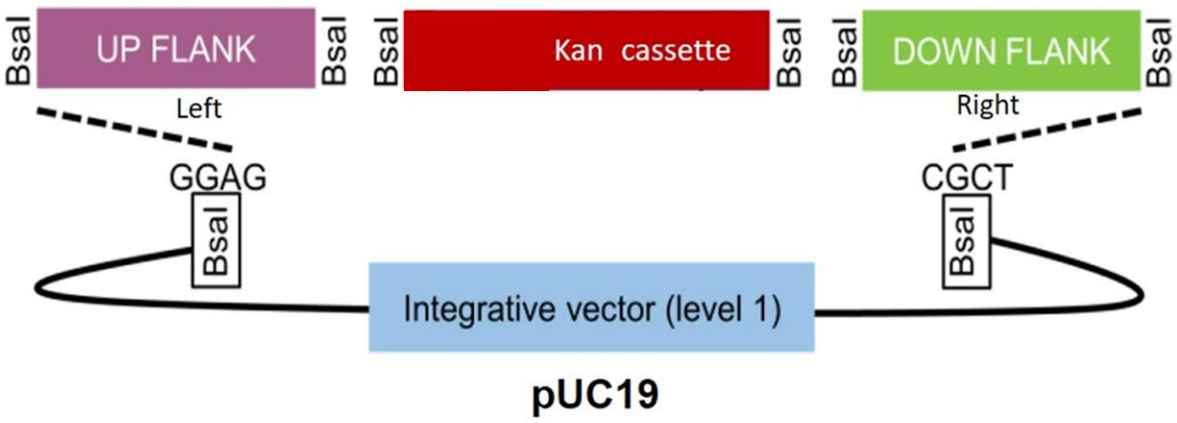

Figure 3: Assembled plasmid for target gene knockout. Overhangs created by BsaI digestion are used to integrate the respective flanks to form the final vector. Flank regions are complimentary to the chromosome adjacent to the region to be deleted, intersected with a KanR gene encoding for kanamycin resistance for positive selection.

Wild-type *Synechocystis* cells from existing plates were inoculated into 30 – 50 mL of BG11 liquid medium. This culture was grown for 2 – 3 days to OD_{750nm} = 0.2 - 0.6. A sample of 1 – 2 mL of the culture was centrifuged at 2,300 x g for 5 minutes. The supernatant was discarded, and the pellet resuspended with gentle pipetting in liquid BG11 medium. *Synechocystis* cells were resuspended in BG11 medium to a final volume of 100 µL in a 15 mL round-bottomed tube. 1 µL of plasmid DNA was added and mixed with gentle tapping. Samples were incubated

at 30 °C for 4 – 6 hours. Aliquots of 20 µL and 80 µL were spread onto separate BG11 plates without kanamycin. After 24 hours, 2.5 – 3 mL of 0.6 % agar solution containing 0.5 mg mL^{-1} kanamycin sulfate was added on top of the agar and spread edge to edge. Plates were then incubated at 30 °C for approximately 7 days until colonies were visible. Individual colonies from each plate were streaked on BG11 + kanamycin (30 µg mL^{-1}) plates using the blunt end of a toothpick.

2.3 Generation of *Synechocystis* mutants cultured on BG11 medium supplemented with single amino acids.

Single amino acids (all obtained from Sigma) were filter sterilised (0.22 µm pore) and dissolved in either dH_2O or 1 M HCl according to manufacturer's instructions. Individual amino acids were added to BG11 plates at concentrations of 5 and 50 µg mL^{-1}. Cells were sequentially streaked and cultured on BG11 plates with gradually increasing concentrations of kanamycin at 100, 200, and 300 µg mL^{-1}. After 3 - 5 days growth, single colonies were picked using the blunt end of a toothpick and added to 25 µL of dH_2O. These cells were agitated using a bench vortex to extract cellular DNA which was used in a PCR reaction. Primers for this reaction bind to regions flanking the gene to be deleted. All primers used in this study are listed in the appendix (Supplementary table 1).

A PCR was set up to a total volume of 25 µL, concentrations of reagents used are indicated in brackets, containing 2.5 µL Sigma buffer (25 mM), 0.5 µL dNTPs (1.25 mM), 0.2 µL 100% DMSO, 0.25 µL Sigma GoTaq© (1 unit mL^{-1}), 1.5 µL of both forward and reverse primers (50 µM), 1 µL of extracted *Synechocystis* DNA and 17.55 µL of dH_2O. The PCR protocol consisted of an initial denaturation stage at 94 °C for 1 min, followed by 30 cycles of 94 °C for 1 min, 60 °C for 1 min, 72 °C for 4 min with a final extension cycle of 72 °C for 5 min. 12 µL of PCR product and 6 µL Gene ruler 1 KB plus DNA ladder (Thermo Fisher) were ran on a 1 % agarose (w/v) gel, with 1 µL of Gelgreen (Sigma) stain per 6 µL of ladder and sample, and 3.5 µL of Gelred (Sigma) per 100 mL of agarose. Gels were run at 400 A and 100 V for 60 mins. Gels were viewed and imaged under UV light to validate mutants.

2.4 Generation of *Synechocystis* mutants cultured on terrific broth nutrient rich media.

Terrific broth (TB) media was prepared by first making a phosphate stock, by adding 2.3 g of KH_2PO_4 and 16.4 g of K_2HPO_4 to 100 mL dH_20, followed by filter sterilization (0.22 µm pore). Then 900 mL of dH_2O was added to 24 g of yeast extract, 20 g of tryptone, and 4 mL of glycerol

and sterilized by autoclaving at 15 psi (1.05 Kg cm $^{2\ -1}$). Once cooled 100 mL of prepared phosphate buffer was added, and inverted to mix.

2.5 Generation of *Synechocystis* mutants cultured on brain heart infusion nutrient rich media.

Brain heart infusion (BHI) media was prepared by mixing 500 mL of dH_2O, 18.5 g of BHI powder, and 7.5 g of agar, followed by inverting to mix, and autoclaving (according to conditions described in section 2.4).

2.6 Generation of *Synechocystis* mutants cultured on a nitrogen deficient media supplemented with oligopeptides.

Nitrogen deficient BG11 media (BG11 -N^0) was made according to Castenholtz, 1988 [23] except that $NaNO_3$ was removed from the 100 x BG11 solution. An oligopeptide mix was made by adding 2.5 g of N-Z-Amine-A (Sigma) into 50 mL of dH_2O and stirred whilst heating until dissolved. The oligopeptide mix was filter sterilised (0.22 µm pore) and added to obtain BG11 -N^0 plates with an oligopeptide concentration of 300 µg mL^{-1}, 600 µg mL^{-1}, 1 mg mL^{-1} and 6 mg mL^{-1}. Wild-type *Synechocystis* cells were streaked on BG11 -N^0 plates with varying peptide concentrations to determine the optimal concentration for growth. Mutants generated in section 2.2 were then streaked on BG11 and BG11 -N^0 plates supplemented with a concentration of 1 mg mL^{-1} oligopeptides and with progressively increasing concentrations of kanamycin of 100, 200, and 300 µg mL^{-1}. Mutants were verified for segregation according to PCR and gel electrophoresis protocols outlined in section 2.3.

2.7 Construction of plasmids for expression of high affinity amino acid transporters.

Regions in the *E. coli* K-12 genome encoding high affinity amino acid transporters were located using the NCBI genome database. Primers were designed via Primer3 to be 18 – 30 bp long with a Tm of 58 – 62 °C [22]. Primers were also designed to amplify open reading frames (ORF) with native promoter and terminator regions present (Table S1). Transcriptional sites described in Mitschke *et al.,* 2011 [24] were consulted to ensure non-coding RNA was not amplified, this is a necessary precaution as it is possible non-coding RNA can contain ORFs that may be translated into unwanted peptides.

Inserts were generated via PCR in a total volume of 50 µL containing 5.0 µL AccuTaq© buffer (Sigma), 2.5 µL DNTP's (1.25 mM), 1 µL 100% DMSO, 0.5 µL AccuTaq© LA DNA high fidelity polymerase (Sigma) (1 unit mL^{-1}), 1 µL of both forward and reverse primers (50 µM), 5 µL of

template *E. coli* K-12 DNA and 34 µL of dH_2O. Amplification was performed with an initial denaturation step at 98 °C for 30 secs, followed by 30 cycles of 94 °C for 15 secs, 65 °C for 20 secs, 68 °C for 20 min, and a final extension step at 68 °C for 10 min. This protocol was repeated with a Q5© high-fidelity polymerase (NEB) according to the manufacturer's instructions to increase likelihood of successful generation of all inserts. Products were verified via gel electrophoresis according to the protocol described in section 2.3, inserts were excised from agarose gels under UV light using a scalpel and then cleaned using a GeneClean® III kit (Thermo Fisher) according to the manufacturer's instructions. Purified inserts were eluted into 14 µL of MilliQ water and stored at - 20°C.

Plasmid DNA was purified from *E. coli* DH5α using a Wizard III© purification kit (Promega) according to the manufacturer instructions. One pot restriction-ligation steps were carried out on ice in a PCR tube. The reaction contained 2 µL T4 DNA ligase buffer 10X (NEB), 1 µL T4 ligase (NEB), 37.5 ng of purified digested insert, 50 ng of purified pCAT.011 backbone vector and MillQ water to a total volume of 20 µL. Reagents were gently pipetted to mix and incubated at room temperature for 10 mins for cohesive ends.

For transformation steps, competent *E. coli* cells were prepared according to Lea-Smith *et al,.* 2016 [25]. For blue-white screening, 25 µL of competent cells were streaked on LB plates containing 100 µg mL^{-1} spectinomycin and 40 µg mL^{-1} X-Gal. Spectinomycin selection was used for all the *E. coli* K12 transporter plasmids. LB plates were incubated overnight at 37 °C. White colonies were selected and verified to confirm whether they contained the correct plasmid according to the PCR protocol described in section 2.3, using the primers for amplification of the cassettes expressing transporters. If the product was the correct size, then the plasmid was purified using a Wizard III© purification kit (Promega) according to manufacturer's instructions and stored at - 20°C. Assembled plasmids were transformed into *Synechocystis* according to the protocol in section 2.2.

3. RESULTS

3.1 Multiple genes encoding putative amino acid biosynthesis are essential in *Synechocystis*.

Initially, I attempted to generate auxotrophic mutants in *Synechocystis* by culturing partially segregated mutants on BG11 media supplemented with single amino acids (Figure 4). In theory, these conditions could compensate for deletion of genes encoding enzymes in the corresponding biosynthetic pathway. This also requires a relevant transporter being expressed and having sufficient activity to import the amino acid from the media.

To verify the generation of a knockout mutant, PCR was used amplify DNA from both wild-type and mutant *Synechocystis*. The bp size of the wild-type *Synechocystis* DNA, plus the suicide plasmid, minus the length of target gene to be deleted was calculated, If the sum of this expected value aligned with the gel electrophoresis band provided by a known DNA hyper ladder it is strong evidence that the target gene was deleted. Mutants bands were observed to verify if full segregation had occurred. If multiple bands were presented full segregation had not been achieved.

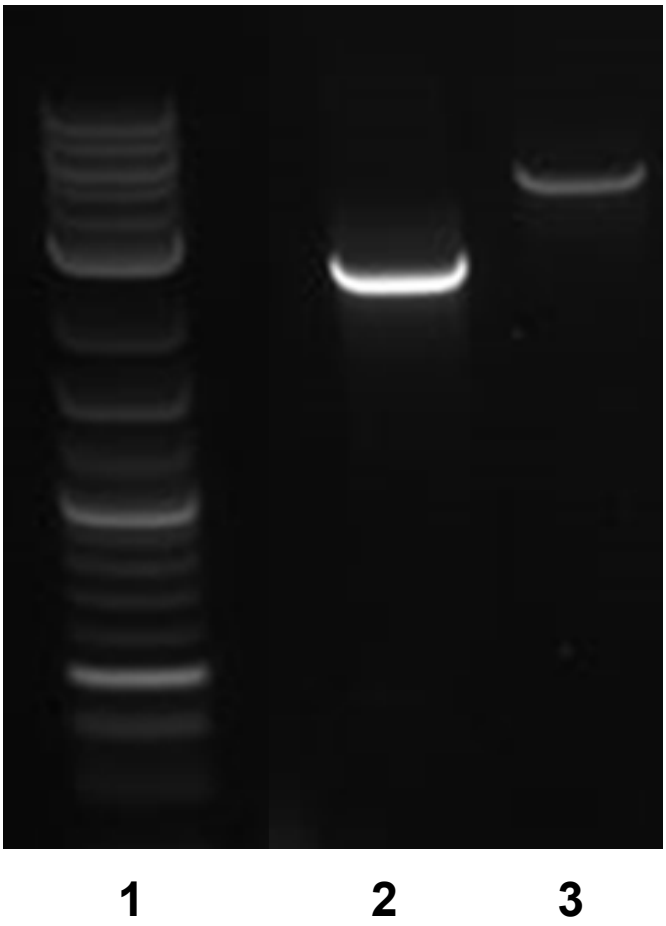

Figure 4a: Example agarose gel to illustrate how mutant generation was verified using PCR. Lane 1: 1 kb DNA hyperladder, Lane 2: example wild-type *Synechocystis* band, Lane 3: example *Synechocystis* knockout mutant.

I was only able to obtain partially segregated mutants of the genes *slr*0036, *sll*0402, and *slr*2035 using this method (Figure 4). The knockout banding observed in lanes 6 and 7 may suggest that *slr*0036 could be fully segregated. The retention of wild-type banding observed in lanes 9 and 10, may indicate that there was too little selection pressure or insufficient activity of native amino acid transporters to ensure the complete knockout of all gene copies of *sll*0402 and *slr*2035. The multiple banding in Lane 8 with a wild-type band and an unknown band observed may be explained by off-target PCR amplification as a result of inefficient primer design. A partially segregated mutant is generated when selective pressure provided by kanamycin supplementation in the media, permits deletion of target genes from a chromosome via a recombination event involving the suicide plasmid, however the selective pressure is not great enough to permit total deletion of all gene copies from all chromosome copies. As these mutants could be partially segregated it is highly likely that there was insufficient activity of transporters to import amino acids in enough quantity to compensate for the total loss of gene

function. The concentration of peptides in the media may have also be insufficient to permit full segregation.

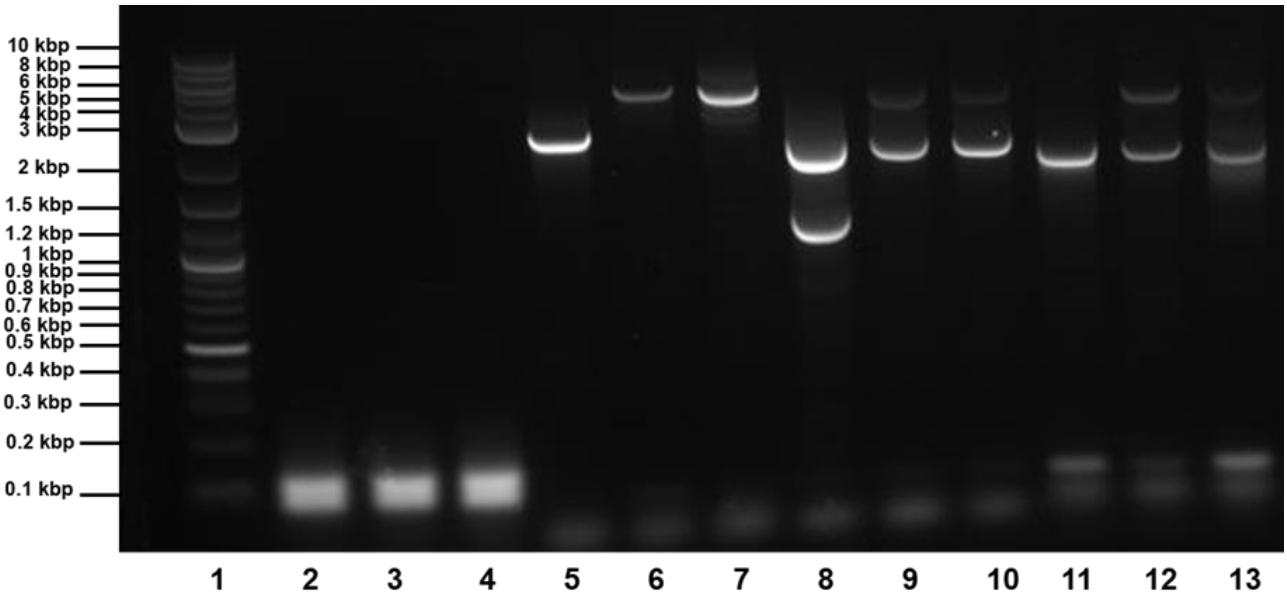

Figure 4: Generation of a partially segregated *slr*0036, *sll*0402, and *slr*2035 mutants in *Synechocystis* cultured on BG11 media supplemented with single peptides. Partial segregation was confirmed using primers flanking the region targeted for deletion. Mutants were cultured with single peptide concentrations of 5 and 50 µg mL^{-1}. Expected size in brackets. Lane 1: 1 kb DNA hyperladder, lane 2, 3, and 4: failed amplification of wild-type *sll*1883 and mutant *sll*1883, lane 5: wild-type *slr*0036 (2374 bp), lane 6: *slr*0036 knockout 5 µg mL^{-1} Asp (3963 bp), lane 7: *slr*0036 knockout 50 µg mL^{-1} Asp (3963 bp), lane 8: wild-type *sll*0402 (2229 bp), lane 9: *sll*0402 partial knockout 5 µg mL^{-1} Asp (3818 bp), lane 10: *sll*0402 partial knockout 50 µg mL^{-1} Asp (3818 bp), lane 11: wild-type *slr*2035 (2047 bp), lane 12: *slr*2035 partial knockout 5 µg mL^{-1} Pro (3684 bp), lane 13: *slr*2035 partial knockout 50 µg mL^{-1} Pro (3684 bp).

3.2 Lysine is toxic to *Synechocystis* at concentrations of 5 µg mL^{-1}.

Whilst trying to generate auxotrophic mutants I cultured *Synechocystis* on BG11 media supplemented with single amino acids at concentrations of 5 and 50 µg mL^{-1}. However, it was observed that *Synechocystis* was unable to grow on plates containing lysine at concentrations higher than 5 µg mL^{-1}. Further experiments will be required to determine the lowest concentration needed for inhibition of growth, but a previous study suggests that as little as

0.6 µg mL^{-1} of lysine is sufficient for inhibition [26]. Therefore, we did not attempt to generate auxotrophic mutants of genes in the lysine biosynthesis pathway via this method.

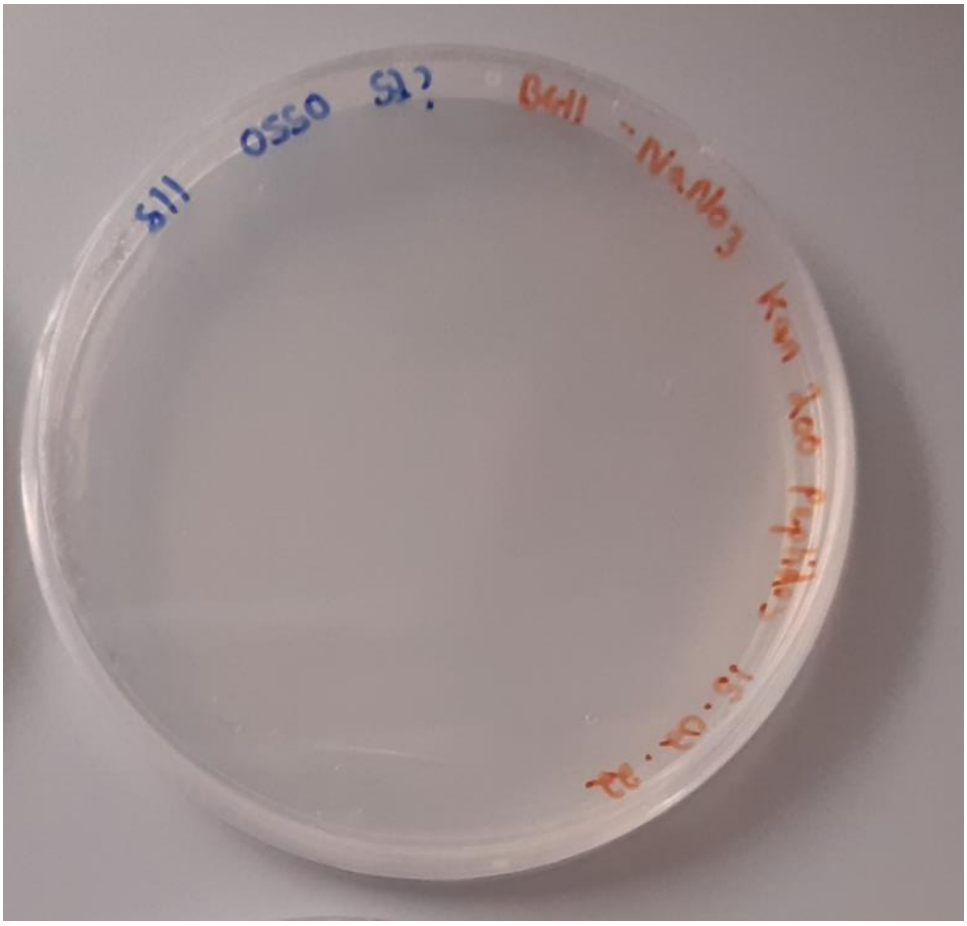

Figure 5: Inhibition of *Synechocystis* growth due to lysine toxicity. A mutant encoding for deletion of *sll*0550, a gene in the lysine biosynthesis pathway. This mutant could not be cultivated on media containing 5 µg mL^{-1} lysine.

3.3 *Synechocystis* failed to grow on brain heart infusion or terrific broth nutrient rich media.

The next method trialled to obtain auxotrophic mutants was by segregating on enriched media. Nutrient rich TB and BHI media were both used to culture mutants. These medias contain a broad range of nitrogen sources including peptides that could in theory be imported into *Synechocystis* to compensate for loss of gene function encoding proteins in amino acid biosynthetic pathways. However, both wild-type *Synechocystis* and partially segregated mutants were unable to grow at all on either medium (Figure 6).

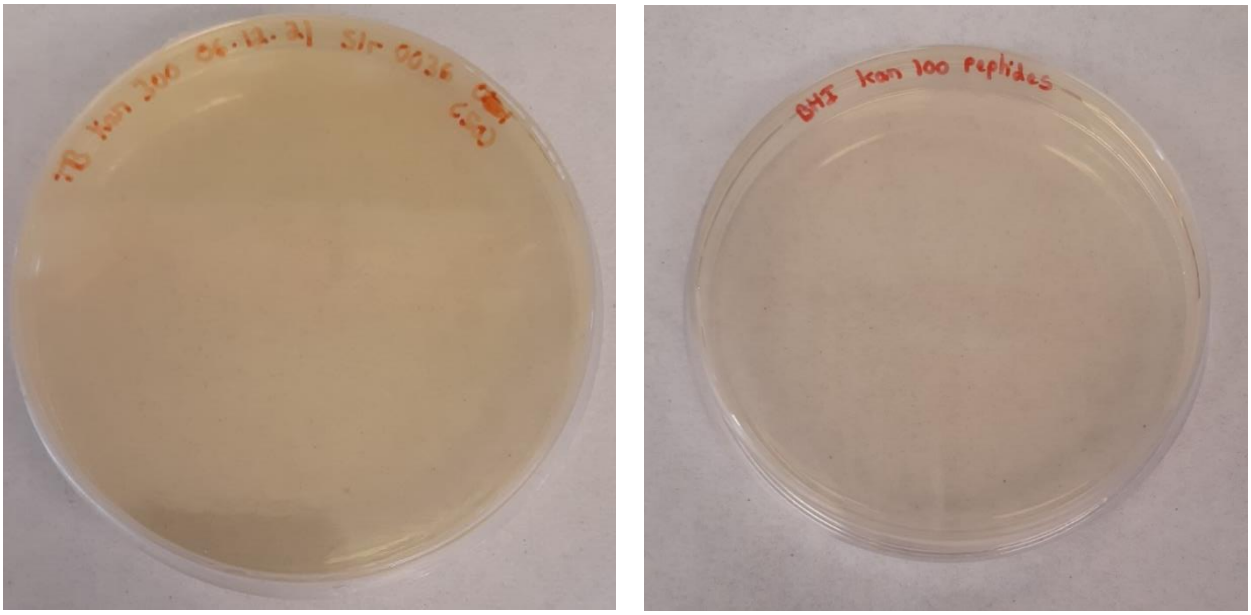

Figure 6: A *Synechocystis* mutant (*slr*0036) failed to be cultured on TB (Left) and BHI (right) nutrient rich media. *Synechocystis* was unable to be cultured using this media with either wild-type cells or any of the mutants generated.

3.4 *Synechocystis* can harvest peptides as a nutrient source on nitrogen deficient media.

I next tried using an oligopeptide mixture, N-Z-amine-A (Sigma) as an amino acid source to compensate for loss of gene function and culture auxotrophic mutants. I first tested whether wild-type *Synechocystis* could grow on plates with N-Z-amine-A as the sole N source (Figure 7) As conditions with no inorganic nitrogen present could potentially facilitate importation of peptides in greater quantities. Following success of culturing wild-type *Synechocystis* on BG11 -N^0 media (Figure 7), I next cultured generated mutants on BG11 -N^0 media (Figure 8). This demonstrated that *Synechocystis* could import enough oligopeptides to sustain both its nitrogen and peptide requirements by cleaving oligopeptides into individual amino acids. For future cultivation of mutants, I trialled various oligopeptide concentrations to determine optimal growth conditions of mutants on both BG11 +N^0 and BG11 -N^0 media (Figure 9). It was demonstrated that oligopeptide concentrations ranging from 300 µg mL^{-1} to 6 mg mL^{-1} could sustain both N and peptide requirements for mutant growth (Figure 9). An optimal concertation of 1 mg mL^{-1} was used for future cultivation of *Synechocystis* mutants.

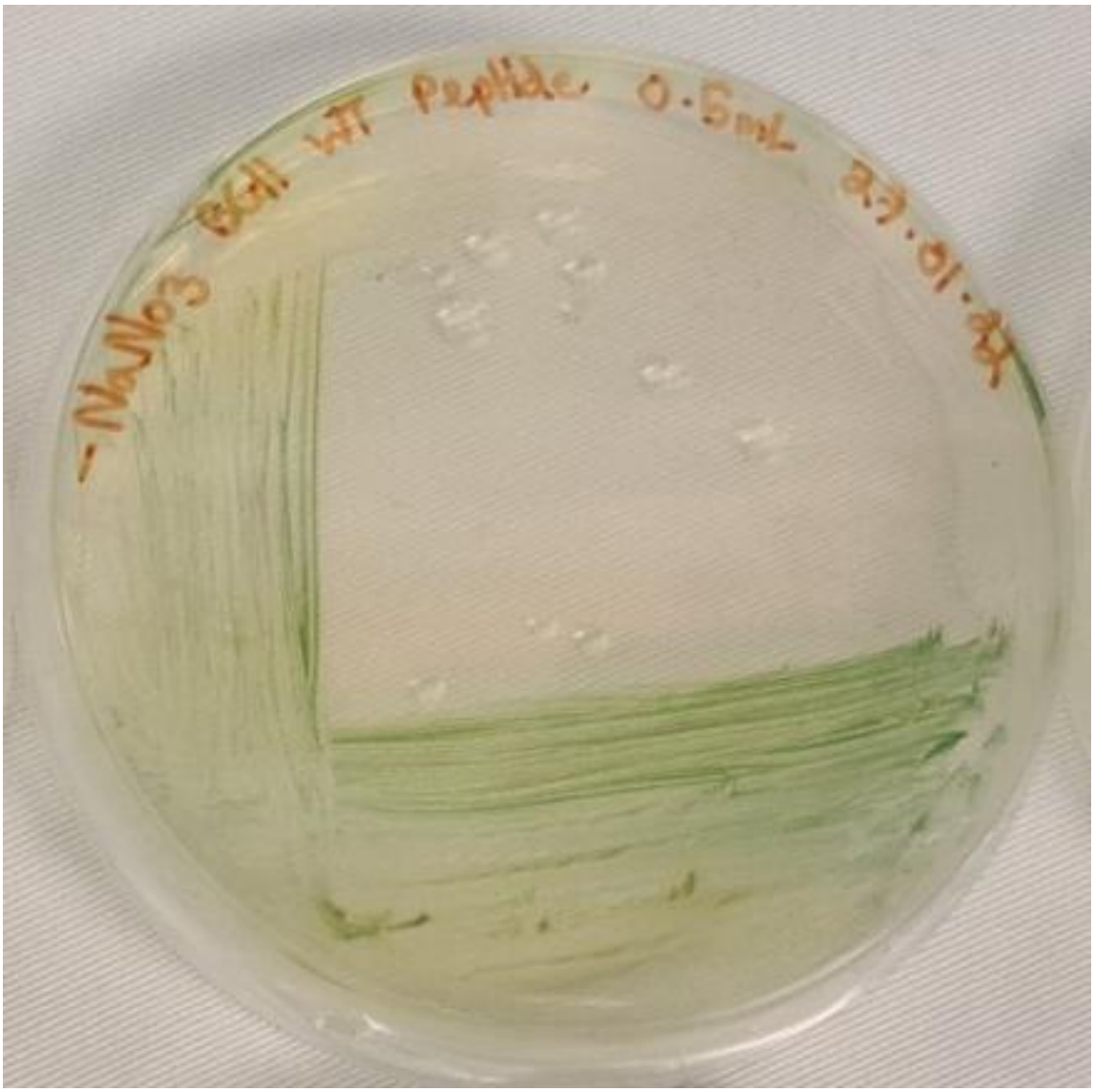

Figure 7: Wild-type *Synechocystis* cultivated on BG11 -N^0 media supplemented with an oligopeptide mix (1 mg mL^{-1}). *Synechocystis* could be cultivated despite the absence of inorganic nitrogen in media.

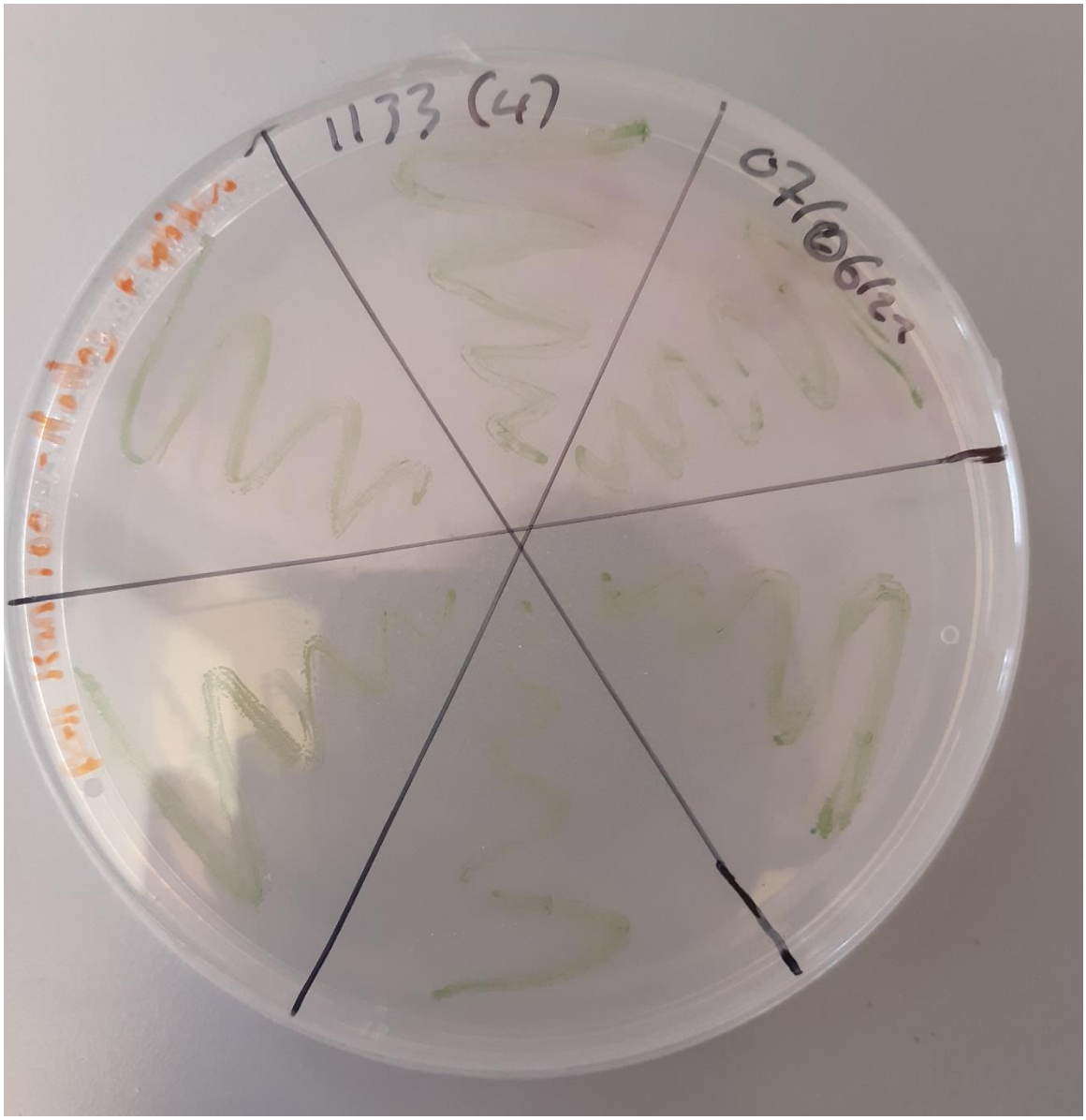

Figure 8: A *Synechocystis* mutant (*slr*1133) cultivated on BG11 -N^0 media supplemented with an oligopeptide mix (1 mg mL^{-1}). *Synechocystis* mutants could grow using oligopeptides as both a N source and a peptide source.

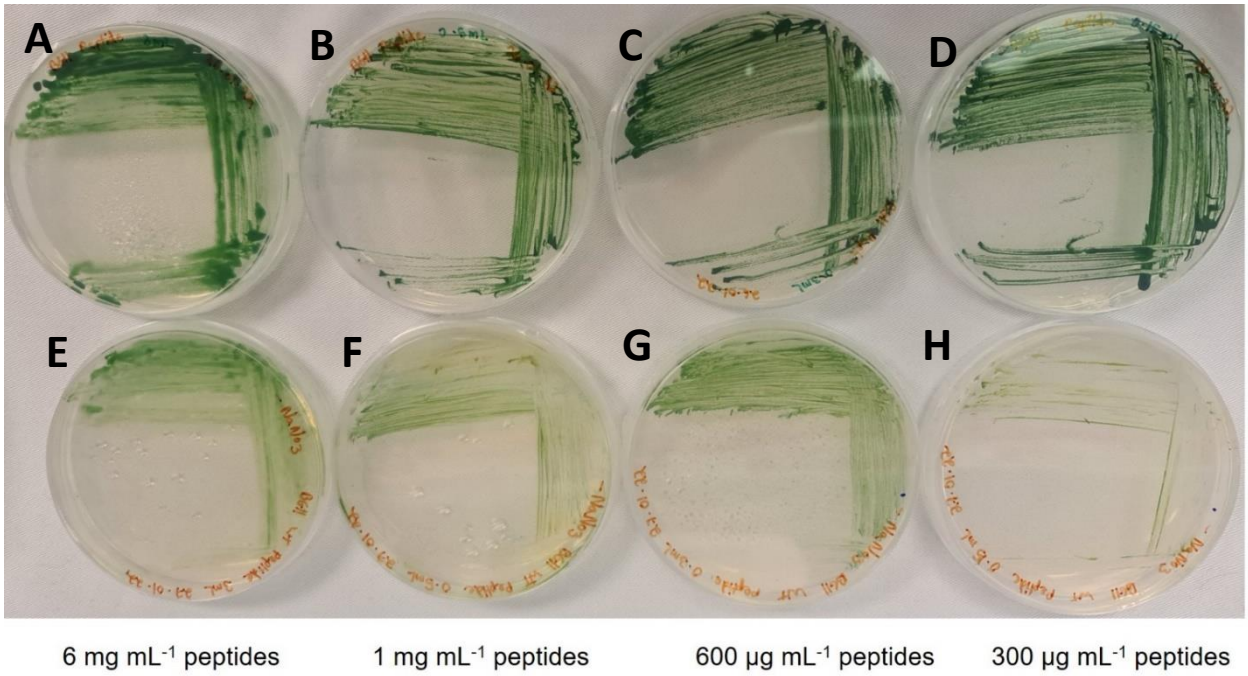

Figure 9: Media conditions effect growth of wild-type *Synechocystis*. Top row wild-type cells cultured on $+N^0$ (17.6 mM $NaNO_3$) media with varying concentrations of N-Z-Amine-A oligopeptide mix. From left to right. A: 6 mg mL $^{-1}$, B: 1 mg mL $^{-1}$, C: 600 µg mL $^{-1}$, D: 300 µg mL $^{-1}$. Bottom row wild-type *Synechocystis* cultured on BG11 $-N^0$ media with varying concentrations of N-Z-Amine-A oligopeptide mix. From left to right. E: 6 mg mL $^{-1}$, F: 1 mg mL $^{-1}$, G: 600 µg mL $^{-1}$, H: 300 µg mL $^{-1}$.

3.5 Generation of auxotrophic mutants on BG11 media supplemented with an oligopeptide mix.

I next tried to generate auxotrophic mutants by culturing strains on BG11 medium supplemented with a N-Z-amine-A (Sigma) oligopeptide mix. This oligopeptide mix contains peptides of varying concentrations (Supplementary Figure 1). In theory, these peptides should be imported into the cell and cleaved by peptidases into individual amino acids that could compensate for loss of the gene in the corresponding biosynthetic pathway. Strains were cultured on progressively increasing concentrations of kanamycin of 100, 200, and 300 µg mL^{-1}. At a concentration of 300 µg mL^{-1} fully segregated mutants of the following genes were generated, *slr*0036, *slr*1312, and *slr*2035 which encode for the synthesis of an aspartate aminotransferase, arginine decarboxylase, and a glutamate kinase respectively (Figure 10). These genes are part of the aspartate, arginine, and glutamic acid biosynthesis pathways,

respectively, which suggests that this method can be used to generate auxotrophic mutants in various amino acid biosynthesis pathways.

*Slr*0036 which encodes AspC, a putative aspartate aminotransferase, is one of two potential enzymes catalysing synthesis of aspartate from oxaloacetate.

*Slr*1312 encodes SpeA, an arginine decarboxylase involved in the arginine to putrescine decarboxylation pathway. Putrescine and other polyamines play an important role in regulating protein function and certain potassium ion channels [27].

*Slr*2035 encodes for proB, catalysing the first step in the proline biosynthesis pathway. This result suggests that other genes involved in the proline biosynthetic pathway (*sll*0373 and *sll*0055) could also be deleted by this method. Despite this success, I was unable to generate auxotrophic mutants in any other amino acid biosynthesis pathways using this method. A likely explanation for this was that certain peptides were of low quantity in the oligopeptide mix and couldn't compensate for the loss of gene function.

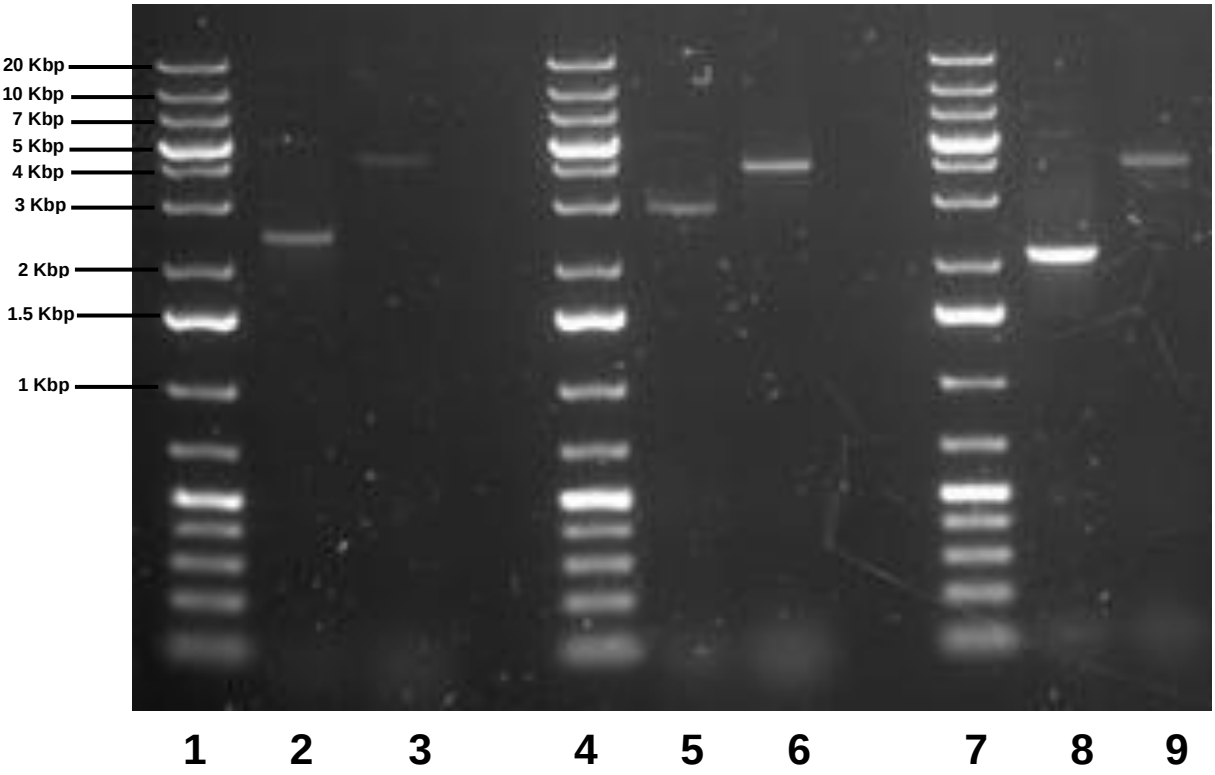

Figure 10: Generation of fully segregated deletion mutants of *slr*0036, *slr*1312 and *slr*2035. Segregation was confirmed using primers flanking the region targeted for deletion. Expected size in brackets. Lane 1, 4, 7: generuler 1 kbp plus DNA ladder. Lane 2: wild-type *slr*0036 control (2374 bp), lane 3: *slr*0036 knockout (3963 bp); lane 5: wild-type *slr*1312 control

(2812 bp), lane 6: *slr*1312 knockout (3684 bp), lane 8: wild-type *slr*2035 control (2047 bp), lane 9: *slr*2035 knockout (3694 bp).

3.6 *E. coli* K-12 amino acid importers were transformed into *Synechocystis*

Although auxotrophic mutants have been generated in some biosynthetic pathways, many mutants could not be fully segregated. An alternate method for generating auxotrophic mutants via the overexpression of *E. coli* K-12 high affinity peptide importers that are absent in *Synechocystis* [28][1] was trialled but could not be completed by the end of my research period. When possible, transporters importing multiple amino acids and with as few subunits as possible were selected for overexpression to reduce assembly complexity.

Amino Acid	**Importer Category**	
	ABC Type	Ion Driven
Alanine		**YaaJ**, **CycA**
Arginine	ArgT-HisQPM**,** ArtJIMQP	**ArcD**
Methionine	**MetNIQ**	
Cytosine	TcyJLN	**TcyP**
Threonine		**TdcC,** SstT
Glycine		**YaaJ**, CycA
Asparagine		**AnsP**
Aspartate	GltIKJL	**DcuB**, **GltP**
Glutamine	**GlnQPH**	
Isoleucine	LivFGHMKJ	**BrnQ**
Valine	LivFGHMKJ	**BrnQ**

Phenylalanine	LivFGHMKJ	**AroP**
Histidine	**HisJQPM**	
Lysine	ArgT-HisQPM	**LysP**, **CadB**
Isoleucine	LivFGHMKJ	**BrnQ**
Leucine	LivFGHMKJ	**BrnQ**
Proline		PutP, **ProP**
Serine		**TdcC,** SdaC, SstT
Threonine		**TdcC,** SstT
Tryptophan		**AroP,** Mtr
Tyrosine		**AroP,** TryP

Figure 11: Amino acid transporters expressed in *E. coli* K-12. Transporters to be overexpressed in *Synechocystis* are highlighted in bold. Adapted from Zhou and Imlay, 2020 [28].

Inserts for native upstream and downstream regions encoding amino acid transporters from *E coli* K-12 were first generated using an high fidelity AcuTaq© (Sigma) polymerase, along with primers designed to flank these encoding regions for witha relevant type IIS restriction endonuclease recognition site. Inserts were generated at variable annealing temperatures of 55 and 60 °C (Figure 12). This method generated inserts for all transporters except for HisJQPM, LysP, and MetNIQ (Figure 12). To generate inserts for the remaining transporters I used an alternative high fidelity Q5© polymerase (NEB) along with primers used in the AcuTaq© protocol. This method generated inserts for the remaining LysP and MetNIQ transporters but not HisJQPM (Figure 13). It is possible that the insert size for HisJQPM was too large compromising the efficiency of the Q5© polymerase. Once inserts had been generated DNA was excised from gels, cleaned, and purified to be ligated into a pCAT.011 vector, a small amount of this DNA was used to verify insert generation (Figure 14). A one pot T4 (NEB) digestion/ligation reaction was used to assemble inserts into a pCAT.011

plasmid to permit replication in *Synechocystis*. Initially, only LysP was successfully ligated (Figure 15). T4 digestion/ligation has a high success rate, therefore errors may have been made assembling the ligation mix. Ligations were repeated, and AnsP and MetNIQ were successfully ligated (Figure 16). Whilst inserts were verified as correctly generated (Figure 14), I ran out of time to successfully transform and express these transporters into wild-type *Synechocystis* for further characterisation experiments.

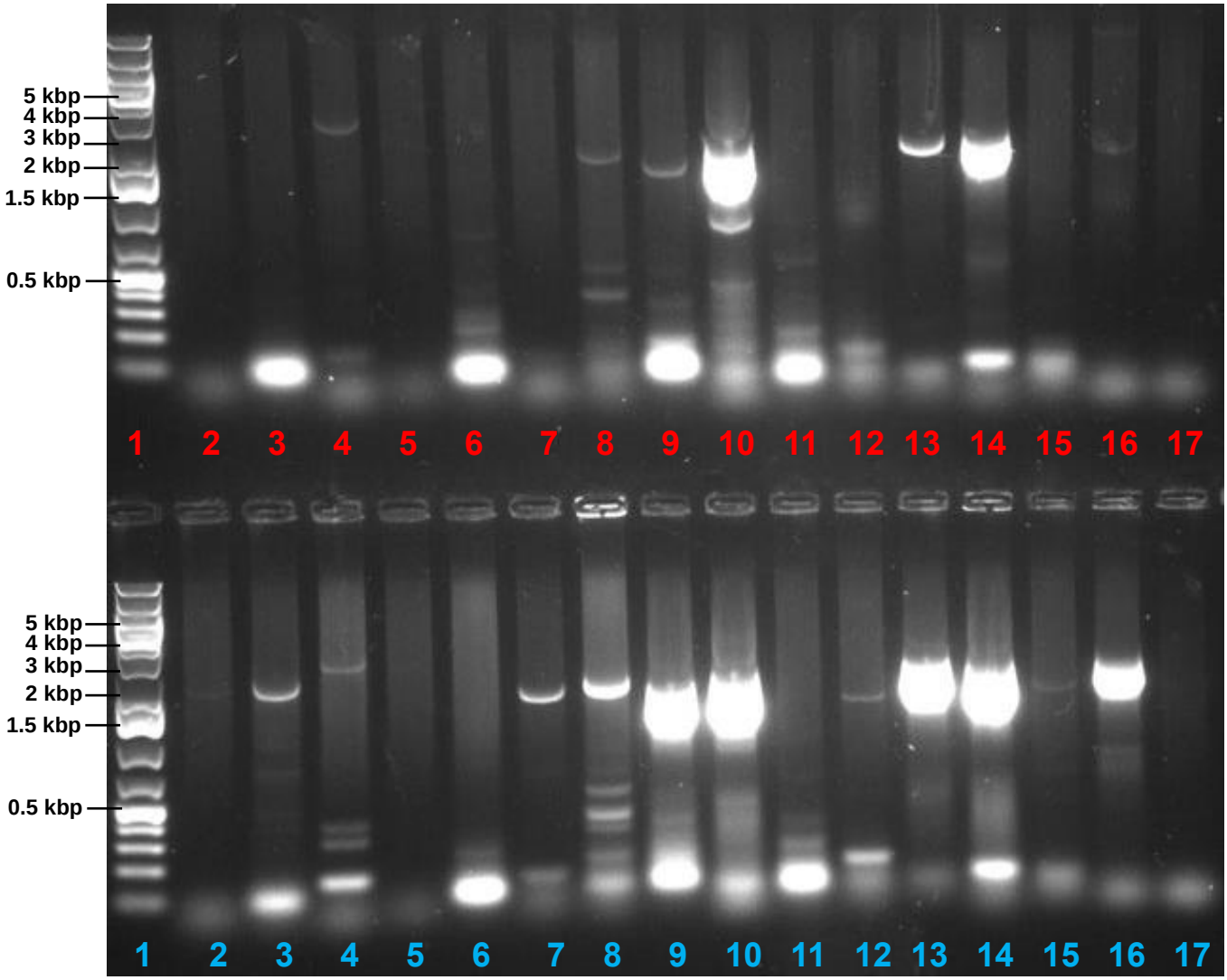

Figure 12: Generation of inserts except LysP, MetNIQ, and HisJQPM using AcuTaq© high-fidelity polymerase. Insert generation was confirmed using primers flanking the transporter targeted for expression. A temperature gradient of 55 °C to 60 °C was used to increase chances of successful insert generation. Red numbers indicate inserts generated at 60 °C blue numbers indicate inserts generated at 55 °C. Expected band size in brackets. Lane 1: generuler 1kbp plus DNA ladder, lane 2: BrnQ (1795 bp), lane 3: AroP (1941 bp), lane 4: GlnQPH (2506 bp), lane 5: HisJQPM (3482 bp), lane 6: Lysp (1952 bp), lane 7: CadB (1618 bp), lane 8: CycA (1764 bp), lane 9: GltP (1588 bp), lane 10: YaaJ (1742 bp), lane 11: MetNIQ (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 (1889 bp), Lane 17; TcyP (1511 bp).

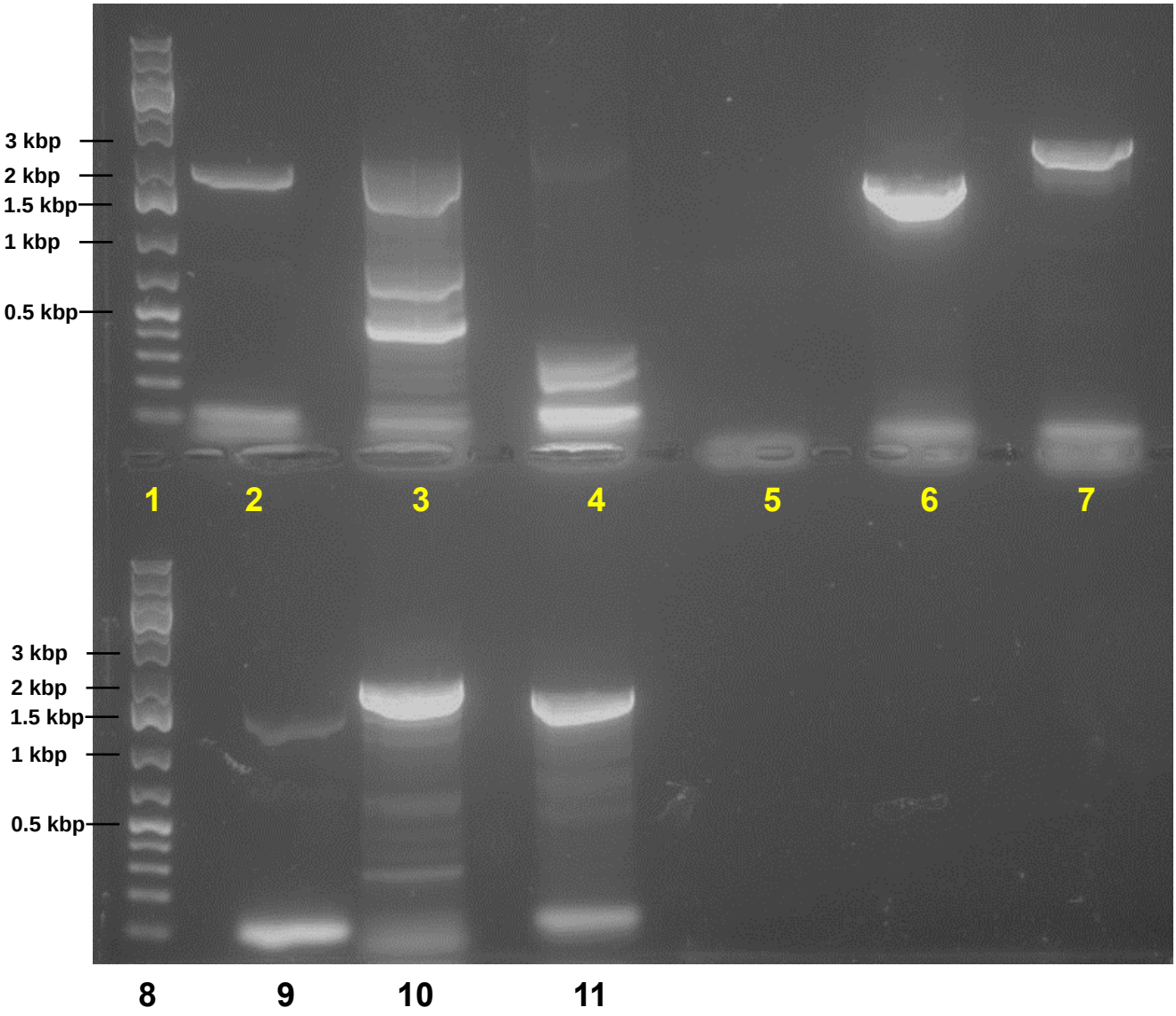

Figure 13: Generation of remaining inserts except HisJQPM using Q5© high-fidelity polymerase. Insert generation was confirmed using primers flanking the transporter targeted for expression. Expected band size in brackets. Lane 1 and 8: generuler 1kbp plus DNA ladder, lane 2: BrnQ (1795 bp), lane 3: AroP (1941 bp), lane 4: GlnQPH (2506 bp), lane 5: HisJQPM (3482 bp), lane 6: LysP (1952 bp), lane 7: MetNIQ (2774 bp), lane 9: tdcC (1404 bp), lane 10: AnsP (1873 bp), lane 11: ArcD (1609 bp).

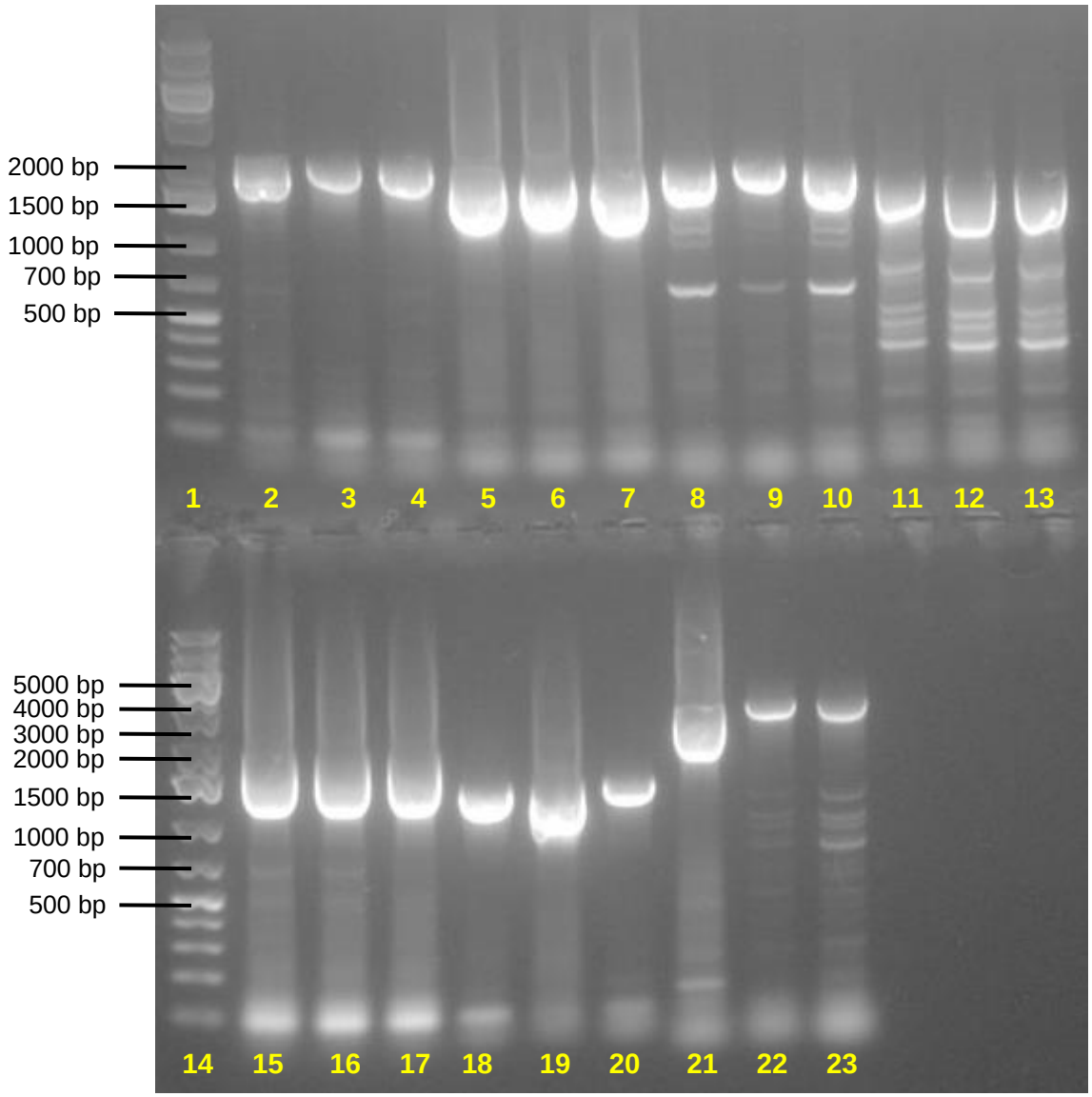

Figure 14: PCR verifies generation of inserts. Ligation was confirmed using primers flanking the transporter targeted for expression. Expected band sizes in brackets. Lane 1 and 14: generuler 1kbp plus DNA ladder, lane 2, 3, and 4: LysP (1943 bp), lane 5, 6, and 7: BrnQ (1795 bp), lane 8, 9, and 10: AnsP (1873 bp), lane 11, 12, and 13: (1609 bp), lane 15, 16, and 17: AroP (1941 bp), lane 18, 19, and 20: TdcC (1404 bp), lane 21, 22, and 23: MetNIQ (2774 bp). Amplification of off target sequences is seen clearly in lanes 22 and 23. This amplification may be a result of sub-optimal primer designs resulting in a too low TM. A low TM could lead to the formation of intermolecular hairpins which could bind to non-targeted regions for amplification. Consequently, these regions will also be amplified, yielding results observed in lanes 22 and 23

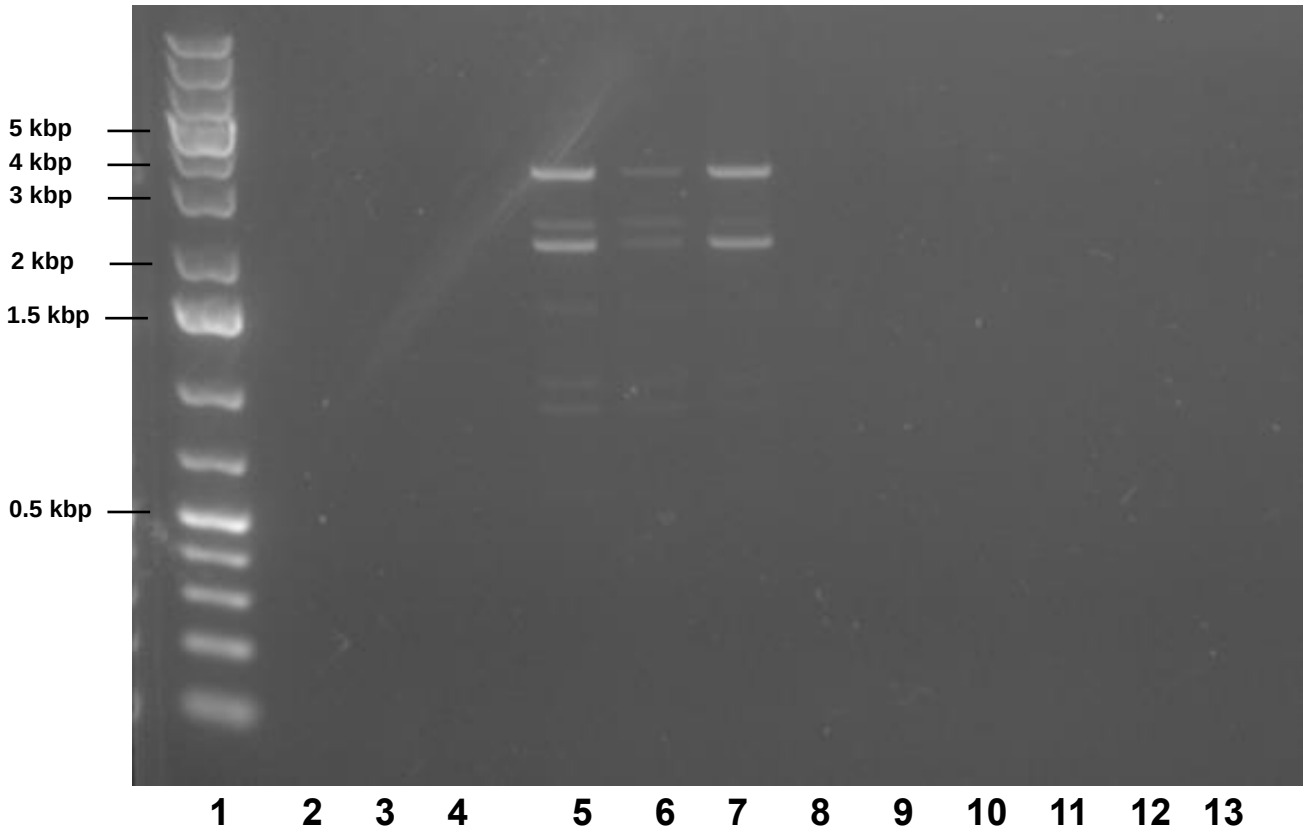

Figure 15: PCR verifies ligation of LysP inserts into pCAT.011. Ligation was confirmed using primers flanking the transporter targeted for expression. Each ligation/digestion reaction was repeated three times to increase the chance of success. Expected band sizes in brackets. Lane 1: generuler 1kbp plus DNA ladder, lane 2, 3, and 4: BrnQ (3361 bp), lane 5, 6, and 7: LysP (3107 bp), lane 8, 9, and 10: AnsP (3802 bp), lane 11, 12, and 13: ArcD (2700 bp). Similar to Figure 14:, sub-optimal primer design may have yielded off target amplification.

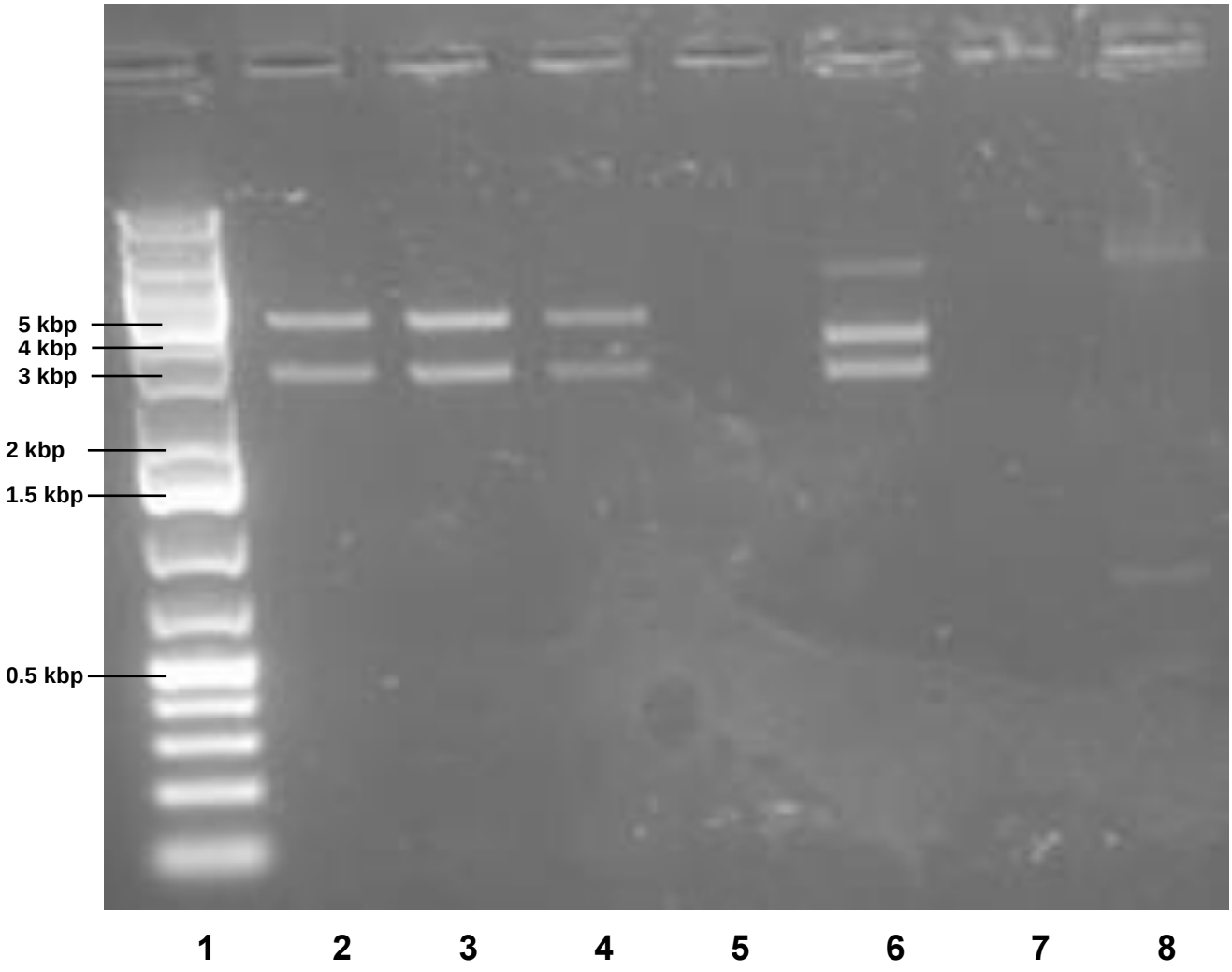

Figure 16: PCR verifies ligation of Ansp and MetNIQ inserts into pCAT.011. Ligation was confirmed using primers flanking the transporter targeted for expression. Expected band size in brackets. Lane 1: generuler 1kbp plus DNA ladder, lane 2, 3, and 4 AnsP (3802 bp), lane 5: empty, lane 6, 7, and 8: MetNIQ (5378 bp).

4. DISCUSSION

4.1 Generation of auxotrophic mutants on oligopeptide supplemented media.

Multiple methods were trialled to generate auxotrophic mutants in *Synechocystis*. Of these, using BG11 media supplemented with an oligopeptide mixture was the only strategy that permitted full segregation of genes. A potential explanation to why this approach was successful was that oligopeptides could be imported into *Synechocystis* mutants at higher quantities compared single amino acids, and hence compensate for full loss of gene function. Oligopeptide importers are involved in recycling peptidoglycan pentapeptides, an extremely active process in the cell likely involving constant and high import of oligopeptides for subsequent assembly into newly synthesised polymers, this active process may have provided the conditions for increased uptake hence full segregation [29]. However, this method was not able to permit the cultivation of knockout mutants of certain amino acid biosynthetic pathways, which could be due to some amino acids being poorly represented in this oligopeptide mix and not provide the conditions necessary to compensate for loss of gene function. Concentrations of cytosine, glycine, and tryptophan were low in N-Z-Amine-A mix; hence an oligopeptide mix rich in these amino acids could potentially permit the full segregation of genes in these biosynthetic pathways.

Another possibility is the lack of peptidases to cleave oligopeptides into single amino acids in sufficient quantities to compensate for loss of the gene function prevented full segregation. Trialling other oligopeptide mixtures may by a viable strategy for generating mutants in other pathways, although overexpression of amino acid transporters from other bacterial species will likely be required as strategy for future mutant generation and further characterisation studies.

4.2 Lysine as a media additive is toxic to *Synechocystis.*

Synechocystis was unable to grow on media supplemented with lysine. It is possible that an excess of lysine leads to formation of toxic compounds that cause lysis or partial permeation of cell membranes [30]. However, another potential explanation for cell death is that excess lysine is decarboxylated into cadaverine which is possibly toxic to cells at high concentrations. In *E. coli*, overexpression of CadBA occurs under high lysine conditions [31] and CadBA regulates lysine decarboxylation [32]. The process of lysine decarboxylation produces weak malic and lactic acids which may be deleterious to the cell [32]. It is possible that this weak acid accumulation result in inhibition of *Synechocystis* growth. Lysine could also inhibit

peptidoglycan synthesis which would compromise photosynthetic apparatus and membrane integrity [33].

4.3 Future directions

Generation of auxotrophic mutants in *Synechocystis* is the first step in characterising amino acid biosynthetic pathways. Basic metabolic understanding of *Synechocystis* will facilitate redirection of metabolic flux to producing high value chemical precursors in greater quantities. In particular, a system for generating auxotrophic mutants for genes encoding proteins in the methionine, tyrosine and phenylalanine biosynthetic pathways will be essential for identifying unknown biosynthetic pathways and should be a major focus of future research. Current attempts at generating auxotrophic mutants for the purpose of characterising amino acid biosynthesis in *Synechocystis* have been limited. Methods typically employ a suicide plasmid-based system which whilst somewhat successful, fail to generate auxotrophic mutants for a large range of amino acid biosynthetic pathways. Research could be further moved forward by expanding on the methods described in this thesis, in particular, the expression of high affinity transporters from other prokaryotic species may have the most potential for the future generation of auxotrophic mutants.

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6. APPENDIX

Product Description
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.

The approximate amino acid content of this product is as follows:

Amino Acid	Amount (mg/g)
Ala	30
Arg	31
Asp	67
Cys	3
Glu	186
Gly	19
His	22
Ile	44
Leu	75
Lys	68
Met	27
Phe	40
Pro	88
Ser	51
Thr	42
Trp	10
Tyr	28
Val	59

The total amino acid content is approximately 890 mg/g.

Supplementary Figure 1: N-Z-Amine-A Oligopeptide mix. Amounts of each amino acid per g of oligopeptide mix is shown.

Supplementary Table 1. Primers sequences used in this study. Function of primer indicted in far right column

Oligo name	Primer sequence (5' to 3')	Function
*sll*0109_LF	ATCGAGGTCTCAGGAGTCACCACTAAGGCATTGTCC	Confirmation of target gene deletion
*sll*0109_LR	AGCTCGGTCTCTTCATCCTCAACCTCCTCGTTAATTGC	Confirmation of target gene deletion
*sll*0109_RF	TGCACGGTCTCAGTAAAGATTGACCAAGGCTTGACC	Confirmation of target gene deletion
*sll*0109_RR	GCTCAGGTCTCTAGCGGGCTAACTTGCCCGTTTTT	Confirmation of target gene deletion
*sll*0402_LF	ATCGAGGTCTCAGGAGAACAAACAGCGCATCATGGG	Confirmation of target gene deletion
*sll*0402_LR	AGCTCGGTCTCTTCATAATCAAAACATCCCTGGGGTA	Confirmation of target gene deletion
*sll*0402_RF	TGCACGGTCTCAGTAAGCAACCGTCCAACCCGTT	Confirmation of target gene deletion
*sll*0402_RR	GCTCAGGTCTCTAGCGAAAGCGGACAGGGGAGAAAG	Confirmation of target gene deletion
*sll*0900_LF	ATCGAGGTCTCAGGAGCGGGGTAAAACTGTGATTGG	Confirmation of target gene deletion
*sll*0900_LR	AGCTCGGTCTCTTCATAACAATTTAAGGTTGACCCGACTG	Confirmation of target gene deletion
*sll*0900_RF	TGCACGGTCTCAGTAATCTTCTTGGTTAGGCCAAAGTAAAC	Confirmation of target gene deletion
*sll*0900_RR	GCTCAGGTCTCTAGCGGGACACGGTGCAGGAAAAA	Confirmation of target gene deletion
*sll*1662_LF	ATCGAGGTCTCAGGAGAAGGAAACCAAACAGGGGAA	Confirmation of target gene deletion
*sll*1662_LR	AGCTCGGTCTCTTCATAAATTCTCCCCAGGAAAGCTG	Confirmation of target gene deletion
*sll*1662_RF	TGCACGGTCTCAGTAATTCCTAACCCTAGCCCTTA	Confirmation of target gene deletion

*sll*1662_RR	GCTCAGGTCTCTAGCGGTTGCCCAATTCTCCCAAC	Confirmation of target gene deletion
*sll*1683_LF	ATCGAGGTCTCAGGAGCCAAAATGGCGATCGGACTG	Confirmation of target gene deletion
*sll*1683_LR	AGCTCGGTCTCTTCATTACAAATCAGGAAATACCGTTTTTACCG	Confirmation of target gene deletion
*sll*1683_RF	TGCACGGTCTCAGTAAACCAAAATTAATAAGTTGAAAATAATTTGTG	Confirmation of target gene deletion
*sll*1683_RR	GCTCAGGTCTCTAGCGTTTGCAGATAAATGGTAAA	Confirmation of target gene deletion
*sll*1883_LF	ATCGAGGTCTCAGGAGAACAGTACACCGATGCCCTG	Confirmation of target gene deletion
*sll*1883_LR	AGCTCGGTCTCTTCATTCTCAAACTCAGAGGCCAATCA	Confirmation of target gene deletion
*sll*1883_RF	TGCACGGTCTCAGTAATCTGTTGGGTCGCTGGGATAAAAT	Confirmation of target gene deletion
*sll*1883_RR	GCTCAGGTCTCTAGCGGCCTTCTTTCTGTAGGGCT	Confirmation of target gene deletion
*slr*0036_LF	ATCGAGGTCTCAGGAGCCGACTGTAAATGCTCTTTG	Confirmation of target gene deletion
*slr*0036_LR	AGCTCGGTCTCTTCATTGTGAATCTGAACCTGTGAATTTAAGG	Confirmation of target gene deletion
*slr*0036_RF	TGCACGGTCTCAGTAATTTAGTTCAGGTTTAAGGATTATTCTCG	Confirmation of target gene deletion
*slr*0036_RR	GCTCAGGTCTCTAGCGCAACCCTTGTCCCCCATACC	Confirmation of target gene deletion
*slr*0055_LF	ATCGAGGTCTCAGGAGGGTGCTAAAAAGCCGTTGAG	Confirmation of target gene deletion
*slr*0055_LR	AGCTCGGTCTCTTCATCTTCAGATCTTCGGCTACGG	Confirmation of target gene deletion
*slr*0055_RF	TGCACGGTCTCAGTAACATGTGCAGAGTGTGCAATTCC	Confirmation of target gene deletion
*slr*0055_RR	GCTCAGGTCTCTAGCGGTCGATGTCCCGGTCGTAAA	Confirmation of target gene deletion
*slr*0550_LF	ATCGAGGTCTCAGGAGGAGGGAAAAATTCCCCAAGC	Confirmation of target gene deletion

*slr*0550_LR	AGCTCGGTCTCTTCATAGACTATCTCAACGAAAAAGGGCA	Confirmation of target gene deletion
*slr*0550_RF	TGCACGGTCTCAGTAAGGTAAAGAACTTGGGGATAAATATTCG	Confirmation of target gene deletion
*slr*0550_RR	GCTCAGGTCTCTAGCGGCAAAAACGGGGCTAAGCAA	Confirmation of target gene deletion
*slr*0608_LF	ATCGAGGTCTCAGGAGGAAGGCGCAGTGGAATTGTC	Confirmation of target gene deletion
*slr*0608_LR	AGCTCGGTCTCTTCATCCCTGGTCGTTGTAGCGAAT	Confirmation of target gene deletion
*slr*0608_RF	TGCACGGTCTCAGTAATTAACGGTGCCCAGCCTC	Confirmation of target gene deletion
*slr*0608_RR	GCTCAGGTCTCTAGCGAAAAAGTCAGGGCCACAGGT	Confirmation of target gene deletion
*slr*0657_LF	ATCGAGGTCTCAGGAGCCATGGAGCAGGCAGAAATTG	Confirmation of target gene deletion
*slr*0657_LR	AGCTCGGTCTCTTCATGGTGATTTCGGGATAGGGC	Confirmation of target gene deletion
*slr*0657_RF	TGCACGGTCTCAGTAAATCCTACTTTTCAAACACTC	Confirmation of target gene deletion
*slr*0657_RR	GCTCAGGTCTCTAGCGATGTGAAGAAGTCCCGCCTG	Confirmation of target gene deletion
*slr*0738_LF	ATCGAGGTCTCAGGAGGCACTGGTGGACTTCTCTCC	Confirmation of target gene deletion
*slr*0738_LR	AGCTCGGTCTCTTCATGGGCGAGTTCAGTGAAGTGA	Confirmation of target gene deletion
*slr*0738_RF	TGCACGGTCTCAGTAAGTTTCTTCCATCACCCCTAC	Confirmation of target gene deletion
*slr*0738_RR	GCTCAGGTCTCTAGCGAACGCATAGGCTAGCAGTCC	Confirmation of target gene deletion
*slr*0827_LF	ATCGAGGTCTCAGGAGCGTAAACGCCTTTCTCCCCT	Confirmation of target gene deletion
*slr*0827_LR	AGCTCGGTCTCTTCATAAAGTTTACTCCTCTGCACCC	Confirmation of target gene deletion
*slr*0827_RF	TGCACGGTCTCAGTAATCAGCCATGGTGAATCCC	Confirmation of target gene deletion

*slr*0827_RR	GCTCAGGTCTCTAGCGAATGGCCACTGGTTGTGTT	Confirmation of target gene deletion
*slr*1312_LF	ATCGAGGTCTCAGGAGAACCTACAACATCGTTGCCG	Confirmation of target gene deletion
*slr*1312_LR	AGCTCGGTCTCTTCATAATTCCTCTATCGAAGATGGATTAATTC	Confirmation of target gene deletion
*slr*1312_RF	TGCACGGTCTCAGTAAGCCTTGAGCAATGGACAAAT	Confirmation of target gene deletion
*slr*1312_RR	GCTCAGGTCTCTAGCGACAGAGCGGCGGTAAAGTAA	Confirmation of target gene deletion
*slr*2035_LF	ATCGAGGTCTCAGGAGTCTGTCTTCTTTGTCGGGGC	Confirmation of target gene deletion
*slr*2035_LR	AGCTCGGTCTCTTCATGGTTGCATTGCCATTGTCATC	Confirmation of target gene deletion
*slr*2035_RF	TGCACGGTCTCAGTAAAAGAGTCTGAAGACAGACAAATG	Confirmation of target gene deletion
*slr*2035_RR	GCTCAGGTCTCTAGCGGCAAGCTATACGCCAGTGA	Confirmation of target gene deletion
*slr*2072_LF	ATCGAGGTCTCAGGAGTTTACCAAGCGGACTTCCAG	Confirmation of target gene deletion
*slr*2072_LR	AGCTCGGTCTCTTCATGGAAAACGAGAGCAAAAACGGTA	Confirmation of target gene deletion
*slr*2072_RF	TGCACGGTCTCAGTAATTTTTTGCCCCATTCGTGCTA	Confirmation of target gene deletion
*slr*2072_RR	GCTCAGGTCTCTAGCGGAGGGGCAACTTCTTCGGC	Confirmation of target gene deletion
*slr*2081_LF	ATCGAGGTCTCAGGAGTCCCCAGAACGATTGAGTGC	Confirmation of target gene deletion
*slr*2081_LR	AGCTCGGTCTCTTCATTGCCGAGAAACCCCAATCAA	Confirmation of target gene deletion
*slr*2081_RF	TGCACGGTCTCAGTAAATGGCCTGAACTCCATCGTC	Confirmation of target gene deletion
*slr*2081_RR	GCTCAGGTCTCTAGCGGTCCCTGGCACCAATCATCA	Confirmation of target gene deletion
*sll*0455_LF	ATCGAGGTCTCAGGAGATGCTTCCTTCGCTGTCCAA	Confirmation of target gene deletion

*sll*0455_LR	AGCTCGGTCTCTTCATAATTTTCCTCCCGTTGCCT	Confirmation of target gene deletion
*sll*0455_RF	TGCACGGTCTCAGTAAAGCCAATGTTGATTGCAAAAC	Confirmation of target gene deletion
*sll*0455_RR	GCTCAGGTCTCTAGCGAATGAGAATGGCGCAGTCCA	Confirmation of target gene deletion
*slr*0186_LF	ATCGAGGTCTCAGGAGAATAGAACCAACGGTGCGAC	Confirmation of target gene deletion
*slr*0186_LR	AGCTCGGTCTCTTCATAATAAACGCACCTTTTCAAGGGT	Confirmation of target gene deletion
*slr*0186_RF	TGCACGGTCTCAGTAATGGCCCGGTGACAGTTA	Confirmation of target gene deletion
*slr*0186_RR	GCTCAGGTCTCTAGCGGAGACCAAATGTTCATCCAAG	Confirmation of target gene deletion
*slr*1348_LF	ATCGAGGTCTCAGGAGCACGGCTGGATTTATCGCAT	Confirmation of target gene deletion
*slr*1348_LR	AGCTCGGTCTCTTCATCCGGTCAATTCCTACCTAAAATTG	Confirmation of target gene deletion
*slr*1348_RF	TGCACGGTCTCAGTAATATTCTCCCGGTAGCCATAAC	Confirmation of target gene deletion
*slr*1348_RR	GCTCAGGTCTCTAGCGCGGATTGCGTAGCCAGTAA	Confirmation of target gene deletion
*slr*0091_LF	ATCGAGGTCTCAGGAGCTGTCAATACGCCCCATTCTG	Confirmation of target gene deletion
*slr*0091_LR	AGCTCGGTCTCTTCATAATGTTAATTATGCTTTAGTAACGCAC	Confirmation of target gene deletion
*slr*0091_RF	TGCACGGTCTCAGTAAGATTATTCATGGCCGACCGT	Confirmation of target gene deletion
*slr*0091_RR	GCTCAGGTCTCTAGCGGCTCCGGCTATGTAACTAGAATTGGT	Confirmation of target gene deletion
*sll*1349_LF	ATCGAGGTCTCAGGAGGGTTGGGTAGGAAAAAACGG	Confirmation of target gene deletion
*sll*1349_LR	AGCTCGGTCTCTTCATGGTGAGGAGGATGGGCCAAATA	Confirmation of target gene deletion
*sll*1349_RF	TGCACGGTCTCAGTAAGAATTGCCCTTGTCCTGAATAGTTT	Confirmation of target gene deletion

*sll*1349_RR	GCTCAGGTCTCTAGCGTGCTGAATGGTCAAATCCT	Confirmation of target gene deletion
*slr*0458_LF	ATCGAGGTCTCAGGAGGGGAATCAGCCGGAATTAGA	Confirmation of target gene deletion
*slr*0458_LR	AGCTCGGTCTCTTCATGTTAAGATGAGGGTAACAATTATTTGATTC	Confirmation of target gene deletion
*slr*0458_RF	TGCACGGTCTCAGTAACTGAGTATGGCTATGGTTCCCT	Confirmation of target gene deletion
*slr*0458_RR	GCTCAGGTCTCTAGCGGGGTCAGCCAATCTAAACACAA	Confirmation of target gene deletion
*sll*0404_LF	ATCGAGGTCTCAGGAGGGCTTTTCAAGCACGCTCTT	Confirmation of target gene deletion
*sll*0404_LR	AGCTCGGTCTCTTCATGGTGGGGAAGGGAAAAG	Confirmation of target gene deletion
*sll*0404_RF	TGCACGGTCTCAGTAAGACAATAATCTCCCATGGCTC	Confirmation of target gene deletion
*sll*0404_RR	GCTCAGGTCTCTAGCGCGATCGCCAAAGGTACAGGT	Confirmation of target gene deletion
BrnQ_F	GACTGAAGACTTGCCTTATTGCCATAAGCCA	Expression of amino acid transporter
BrnQ_R	GACTGAAGACCCTCCCATCAACACAATAAAAA	Expression of amino acid transporter
AroP_F	GACTGAAGACCCTCCCATCAACACAATAAAAA	Expression of amino acid transporter
Arop_R	GACTGAAGACCCTCCCCCACCTTGCCGAAGTC	Expression of amino acid transporter
GlnQPH_F	GACTGAAGACTTTGCCTCCCTTTCGGGTGAAA	Expression of amino acid transporter
GlnQPH_R	GACTGAAGACCCTCCCTGTCACGCAGGG	Expression of amino acid transporter
HisJQPM_F	GACTGAAGACTTTGCCTTCCGTCACCCCTCA	Expression of amino acid transporter
HisJQPM_R	GACTGAAGACCCTCCCCTGGGAAGCTGTACC	Expression of amino acid transporter
LysP_F	GACTGAAGACTTTGCCTTATCGCTCACATCTT	Expression of amino acid transporter

LysP_R	GACTGGCTTTGCCTTTTCGCTCACATCTT	Expression of amino acid transporter
CadB_F	GACTAAGCTTTGCCTCCAGGTAAAAAAGG	Expression of amino acid transporter
CadB_R	GACTTCTAGATCCATCAACTTGCGATCC	Expression of amino acid transporter
CycA_F	GACTAAGCTTTGCCATTCTGTTCCCCTCGAAC	Expression of amino acid transporter
CycA_R	GACTTCTAGATCCCGGCATTGCGCCATC	Expression of amino acid transporter
GltP_F	GACTAAGCTTGCCTCCTGCCATAAACTCGC	Expression of amino acid transporter
GltP_R	GACTTCTAGATCCCTCGCATCAGGCATTCA	Expression of amino acid transporter
TcyP_F	GACTAAGCTTTGCCTTTGAAATATAAGAGACCAG	Expression of amino acid transporter
TcyP_R	GACTTCTAGATCCCAGAATTAATGGTGTGCC	Expression of amino acid transporter
YaaJ_F	GACTAAGCTTTGCCAGTCCTTGCAGGAAATTT	Expression of amino acid transporter
YaaJ_R		Expression of amino acid transporter
MetNIQ_F	GACTAATGATGCCGAACTTAACGCC	Expression of amino acid transporter
MetNIQ_R	GACTTGGAGCCATTTACTGACTGCG	Expression of amino acid transporter
TdcC_F	GACTATCAGTTTACCCAGCGACACT	Expression of amino acid transporter
TdcC_R	GATCGAACCACTTTGCGTCCATGAC	Expression of amino acid transporter
DcuB_F	GACTACTCGGATAAGTCGGCAGGAT	Expression of amino acid transporter
DcuB_R	GACTTCGGTGCGCATATGTCTGAAA	Expression of amino acid transporter
Ansp_F	GACTAGAGGAACACTGTACCCACGAS	Expression of amino acid transporter

Ansp_R	GACTTTGCTGGCTGGATGTACTTCA	Expression of amino acid transporter
ArcD_F	GACTATGGTGGAAATGATGGGACCA	Expression of amino acid transporter
ArcD_R	GACTTATTTGCACGCAGATGTTGGT	Expression of amino acid transporter
ProP_F	GACTAGGGCGTGGTGGTGTTAATTT	Expression of amino acid transporter
ProP_R	GACTTCGCCCTGTTCCAGTTTATCG	Expression of amino acid transporter

Supplementary Table 2: Plasmids used in this study.

Plasmid	**Plasmid Design**	**Function**
pUC19: *slr*0036 + KanR	pUC19 + 3' *slr*0036 flank + 5' *slr*0036 flank + KanR cassette	Removal of *slr*0036 from *Synechocystis* chromosomes via recombination event
pUC19: *slr*1312 + KanR	pUC19+ 3' slr1312 flank + 5' *slr*1312 flank + KanR cassette	Removal of *slr*1312 from *Synechocystis* chromosomes via recombination event
pUC19: *slr*2035 + KanR	pUC19 + 3' *slr*2035 flank + 5' *slr*2035 flank + KanR cassette	Removal of *slr*2035 from *Synechocystis* chromosomes via recombination event
pCAT.011: BrnQ + KanR	pCAT.011 + BrnQ Insert + KanR cassette	Introduction of *E. coli* K12 BrnQ transporter into *Synechocystis*
pCAT.011: AroP + KanR	pCAT.011 + AroP Insert + KanR cassette	Introduction of *E. coli* K12 AroP transporter into *Synechocystis*
pCAT.011: GlnQPH + KanR	pCAT.011 + GlnQPH Insert + KanR cassette	Introduction of *E. coli* K12 GlnQPH transporter into *Synechocystis*
pCAT.011: HisJQPM + KanR	pCAT.011 + HisJQPM Insert + KanR cassette	Introduction of *E. coli* K12 HisJQPM transporter into *Synechocystis*
pCAT.011: LysP + KanR	pCAT.011 + LysP insert + KanR cassette	Introduction of *E. coli* K12 LysP transporter into *Synechocystis*
pCAT.011: CadB + KanR	pCAT.011 + CadB Insert + KanR cassette	Introduction of *E. coli* K12 CadB transporter into *Synechocystis*

pCAT.011: CycA + KanR	pCAT.011 + CycA Insert + KanR cassette	Introduction of *E. coli* K12 CycA transporter into *Synechocystis*
pCAT.011: GltP + KanR	pCAT.011 + GltP Insert + KanR cassette	Introduction of *E. coli* K12 GltP transporter into *Synechocystis*
pCAT.011: YaaJ + KanR	pCAT.011 + YaaJ Insert + KanR cassette	Introduction of *E. coli* K12 YaaJ transporter into *Synechocystis*
pCAT.011: MetNIQ + KanR	pCAT.011 + MetNIQ Insert + KanR cassette	Introduction of *E. coli* K12 MetNIQ transporter into *Synechocystis*
pCAT.011: TdcC + KanR	pCAT.011 + TdcC Insert + KanR cassette	Introduction of *E. coli* K12 TdcC transporter into *Synechocystis*
pCAT.011: DcuB + KanR	pCAT.011 + DcuB Insert + KanR cassette	Introduction of *E. coli* K12 DcuB transporter into *Synechocystis*
pCAT.011: AnsP + KanR	pCAT.011 + AnsP Insert + KanR cassette	Introduction of *E. coli* K12 AnsP transporter into *Synechocystis*
pCAT.011: ArcD + KanR	pCAT.011 + ArcD Insert + KanR cassette	Introduction of *E. coli* K12 ArcD transporter into *Synechocystis*
pCAT.011: ProP + KanR	pCAT.011 + ProP Insert + KanR cassette	Introduction of *E. coli* K12 ProP transporter into *Synechocystis*
pCAT.011: TcyP + KanR	pCAT.011 + TcyP Insert + KanR cassette	Introduction of *E. coli* K12 TcyP transporter into *Synechocystis*