Growth phase-specific promoters of cyanobacteria for synthetic biology applications

Thesis submitted for the degree of Doctor of Philosophy

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ABSTRACT

Cyanobacteria are a diverse group of photosynthetic gram-negative bacteria able to tap into virtually unlimited solar energy supplies and convert it into chemical energy and biomass using minimal inputs: sunlight, water, carbon dioxide and simple mineral nutrients. In recent years, cyanobacteria have attracted great interest as a cost-effective and energetically sustainable chassis for a wide array of applications in biotechnology and synthetic biology. However, molecular tools to engineer cyanobacteria are relatively limited. In particular, promoters to drive gene expression in a precise and controlled manner require development.

In this study, I have developed an approach to identify and characterise auto-inducible promoters that are activated in high density cultures of cyanobacteria to avoid interference of transgene expression with culture growth. To assess the strength and specificity of candidate promoters, an *in vivo* GFP-based assay was established to assess promoter activity in growing cultures of *Synechococcus* spp. PCC 7002. For this purpose, a vector backbone was developed which is compatible with the largest library of standardised biological components available to date, BioBricks. In addition, a library of synthetic ribosome binding sites was developed to increase the range of detectable transcriptional activity. This heterologous GFP reporter system was able to reproduce the native growth phase-specific promoter activity from a different species of cyanobacteria.

In order to identify growth phase-specific promoters, I first identified five nutrient-limited conditions that specifically limited growth of *Synechocystis* spp. PCC 6803: nitrate, phosphate, sulphate, magnesium and potassium. These conditions provided a range of backgrounds in which I could investigate transcriptional responses to changes in growth where cyanobacteria entered stationary phase for different reasons. Quantitative PCR analysis of published genes revealed that reported growth phase-specific expression patterns were not robust across different conditions. RNA sequencing was therefore performed to compare gene expression profiles across nutrient conditions and growth phases to identify novel co-regulated gene clusters. Of particular interest were a large group of genes that showed robust up-regulation during late exponential phase across all of the conditions tested.

In summary, this study addresses one of the key limitations in cyanobacterial engineering: robust promoters to tightly regulate gene expression. I have developed tools in order to use standard biological components and to characterise promoter behaviour in cyanobacteria. Genes with robust growth phase-specific expression profiles have been identified and analysis of upstream regions can now be performed to determine growth phase-specific motifs and promoters. This work lays important foundations to develop essential, robust tools for the rational design of cyanobacteria.

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For Ma and Pa

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AUTHOR'S DECLARATION

I declare that this dissertation is the result of my own work except where explicit reference is made to the contribution of others. This thesis has not been submitted for any other degree at the University of Glasgow or elsewhere.

Mary Ann Madsen

ABBREVIATIONS

2CS	Two-component signal transduction system
bp	Base pairs
EDTA	Ethylenediaminetetraacetic acid
IPTG	Isopropyl ß-D-1-thiogalactopyranoside
K	Potassium
Mg	Magnesium
N	Nitrate
P	Phosphate
RBS	Ribosome binding site
RNAP	RNA polymerase
rpm	Revolutions per minute
S	Sulphate
SDS	Sodium dodecyl sulphate
TIR	Translation initiation rate
TRIS	Tris(hydroxymethyl)aminomethane

Chapter 1. Introduction

1.1 The need for alternative technology

Since the industrial revolution, modern civilisation has relied heavily on fossil fuels. They provide us with up to 85 % of our current energy consumption (Crabtree and Lewis, 2007) thus enabling us to travel, heat our homes and browse our smartphones. These reserves are finite, however, and supplies are rapidly diminishing in spite of an ever-growing demand for oil-derived products. Depletion of current oil reserves is predicted by 2040, gas by 2042 and coal by 2112 (Shafiee and Topal, 2009). Furthermore, these reserves are becoming increasingly difficult to extract and refine resulting in increasing costs (Laherrere, 2005). Not only are fossil fuels inherently unsustainable given their limited supply but their combustion has detrimental effects on the environment including the release of pollutants and greenhouse gases contributing to climate change, primarily carbon dioxide (CO_2) (Stocker et al., 2013). Overall, there is an immediate need to shift from petroleum-based industries to sustainable and eco-friendly technologies.

Two major changes are currently underway with respect to energy supplies. In transportation, petroleum-derived fuel is being replaced with electricity. In electricity production, fossils fuels are being replaced by renewable energy sources from wind, sun and biomass. The most abundant energy source is the sun; a single hour of solar irradiation provides the equivalent of an entire year's energy consumption worldwide (Crabtree and Lewis, 2007). Photosynthetic organisms such as plants, algae and cyanobacteria are able to capture and convert solar energy into chemical energy and biomass. This involves the extraction of electrons from water, the reduction of atmospheric CO_2 into organic compounds and the release of oxygen. In this way they can tap virtually unlimited energy supplies to drive potentially carbon negative processes.

An interesting application for photosynthetic energy capture is biotechnology. The biological production of pharmaceutical and commodity products currently

employs primarily heterotrophic bacteria (e.g. *E. coli*) or mammalian cells (e.g. CHO cells) as hosts, all of which rely on external energy supplies in the form of organic carbon compounds such as sugars or amino acids. Replacing traditional microbial hosts with photosynthetic organisms seems a logical step towards sustainability of biotechnology (Abed et al., 2009). Cyanobacteria are particularly attractive hosts due to their rapid growth (compared to plants) and ease of transformation (compared to algae). Indeed, cyanobacteria have shown early signs of promise for diverse applications including biodesalination (Minas et al., 2014, Amezaga et al., 2014), bioremediation (Vijayakumar, 2012, Subashchandrabose et al., 2013), production of biofuels (Nozzi et al., 2013, Parmar et al., 2011), bioplastics (Ruffing, 2011, Yu et al., 2013) and other commodity chemicals (Ducat et al., 2011). However, production titres and rates are still unable to compete with heterotrophic fermentation (Yu et al., 2013). Substantial development is still required, particularly the optimisation of molecular tools to engineer cyanobacteria.

1.2 Cyanobacteria as solar-powered biofactories

Cyanobacteria (formerly “blue green algae”) were the first organisms to evolve oxygenic photosynthesis and responsible for the oxygenation of the earth's atmosphere more than two billion years ago (Olson, 2006). Now, they have the potential to change the world again in the form of solar-powered cell factories. Cyanobacteria have numerous advantages for biotechnology and synthetic biology applications. First and foremost, photosynthesis enables the generation of large amounts of biomass from solar energy, water and mineral nutrients. Furthermore, they convert an estimated 25 Giga tonnes of CO_2 per year into energy dense biomass (Waterbury et al., 1979). Besides the benefits of oxygenic photosynthesis and carbon capture, cyanobacteria are an incredibly diverse group of bacteria in terms of ecology, morphology and metabolism thus providing a wide and varied platform from which to begin engineering. Being prokaryotes, cyanobacteria grow relatively quickly and are relatively simple biological systems with small genomes that are amenable to genetic engineering. These attributes established cyanobacteria as key models for core processes in

photosynthetic organisms and as a result, there is a wealth of knowledge surrounding the core biology of cyanobacteria to inform the rational design of these organisms.

1.2.1 Central carbon metabolism of cyanobacteria

The primary advantage of cyanobacteria over heterotrophic systems for biotechnology is their ability to perform oxygenic photosynthesis. This essentially occurs in two phases: 1) the conversion of light energy into chemical energy as ATP and NADPH and 2) carbon capture and assimilation into organic compounds. These two phases occur in the thylakoids, which form distinct structures in the cytoplasm of cyanobacterial cells as shown in Figure 1-1. The thylakoid membrane has a distinct protein composition from the plasma membrane (Pisareva et al., 2011) and it contains the protein machinery for cellular respiration alongside the photosynthetic machinery. Thus, photosynthesis and cellular respiration are closely interlinked as they share common components, and the spatial organisation of components involved in these core metabolic pathways is both versatile and flexible. Figure 1-2 presents an overview of the central carbon metabolism and interesting production pathways for bioengineering, which will be discussed in closer detail in the following sections.

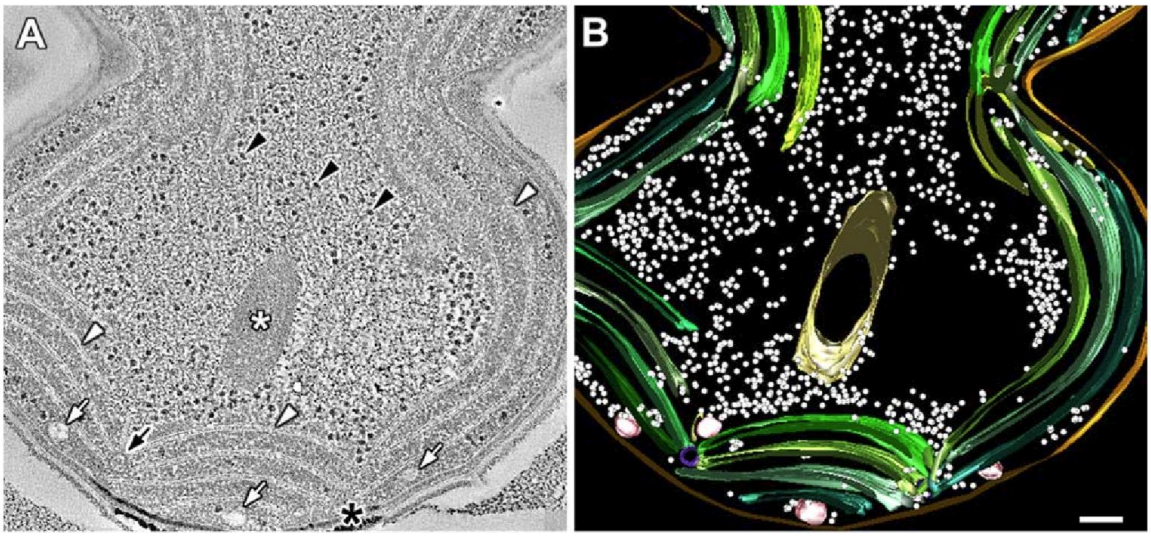

Figure 1-1. Subcellular organisation of *Synechocystis* spp. PCC 6803.

(A) Electron tomographic slice of a dividing *Synechocystis* spp. PCC 6803 cell. Thylakoid membrane pairs (white arrowheads) surround the periphery of the cell and converge at thylakoid centres (black arrow) adjacent to the cytoplasmic membrane. Carboxysome (white asterisk) and ribosomes (black arrowheads) are found in the central cytoplasm. **(B)** 3D model showing a 100 nm thick section of thylakoid membrane pairs (green), thylakoid centre (blue), carboxysome (yellow), ribosomes (white) and cytoplasmic membrane (brown). Scale bar is 50 nm. Figure adapted from van de Meene et al., 2006.

Figure 1-2. Overview of central carbon metabolism of cyanobacteria and associated bioproduction pathways *(overleaf)*

Photosynthetic and respiration electron transport chains in the thylakoid membrane generate chemical energy in the form of ATP and NADPH. Inorganic carbon is assimilated by Rubisco via the Calvin-Benson Cycle, intermediates of which can be redirected towards the production of diverse outputs indicated in black boxes. Figure from Knoop and Steuer, 2015.

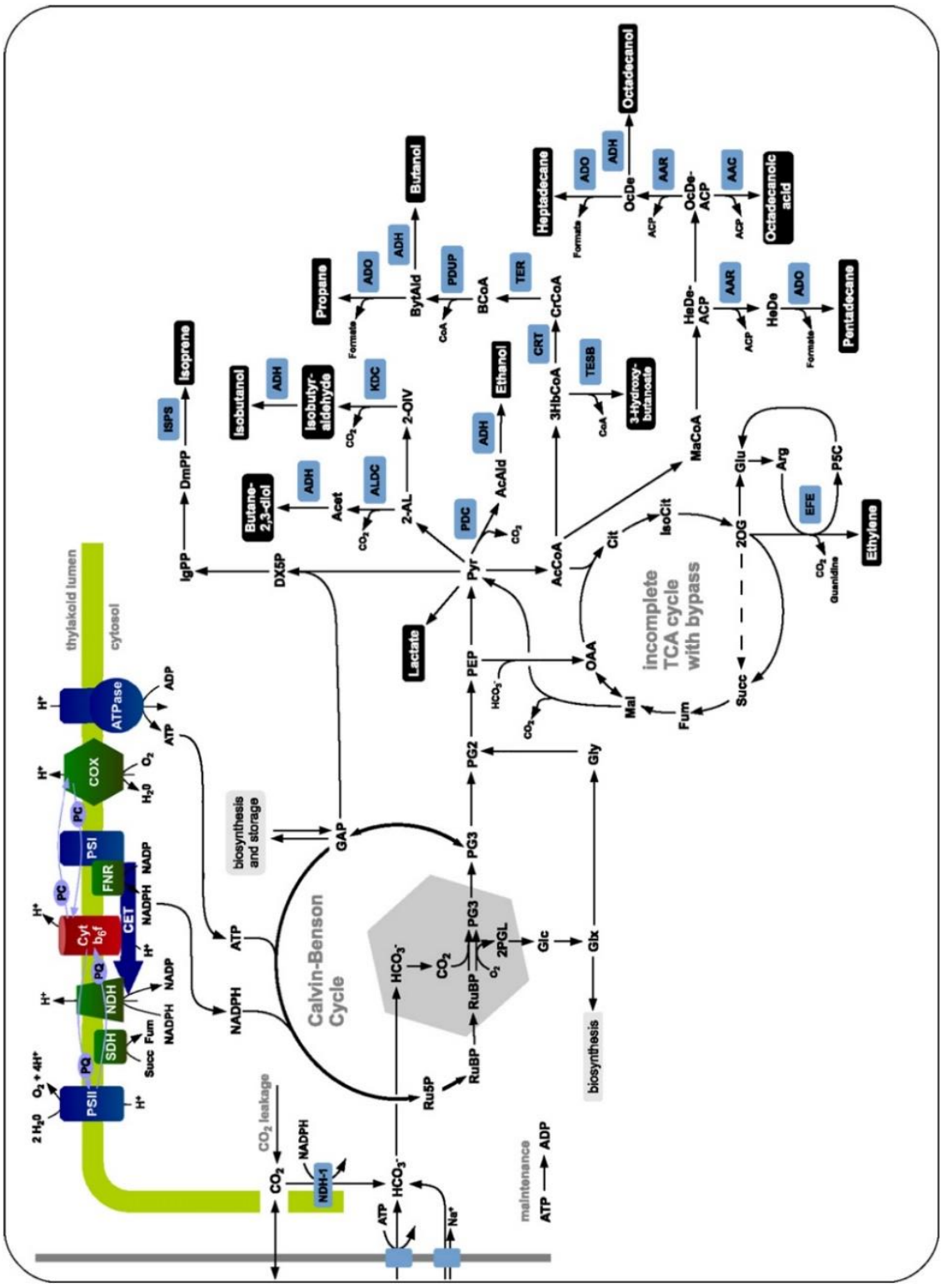

Figure 1-2. Overview of central carbon metabolism of cyanobacteria and associated bioproduction pathways

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1.2.1.1 Photosynthetic conversion of solar into chemical energy

Of great significance for practical applications is the fact that cyanobacteria have a superior photosynthetic capacity compared with other photoautotrophs. This is in part due to the presence of unique antenna complexes called phycobilisomes which are not found in plants or algae (Grossman et al., 1993). The pigments present in these light-harvesting structures expand the absorption spectrum of visible light cyanobacteria can utilise compared with other photoautotrophs. This translates into an enhanced conversion of solar energy into biomass: 10% in cyanobacteria compared to 5% in algae and 1% in plants (Parmar et al., 2011, Posten and Schaub, 2009, Hase et al., 2000).

Cyanobacteria are the widely accepted predecessors of plants and algal chloroplasts (Martin and Kowallik, 1999) and the photosynthetic electron transport chain is essentially identical in these organisms. Light energy is captured by chromophores within pigmented proteins and transferred to the photosynthetic reaction centres of two photosystems, PSII and PSI, which function in series to transfer electrons from water to NADPH and build an electrochemical gradient across the thylakoid membrane to drive ATP synthesis (Shevela et al., 2013). First, light energy is transferred to P680, the PSII primary donor comprised of two chlorophyll dimers, which catalyses the extraction of electrons from water ($H_2O \rightarrow O_2 + 4H^+ + 4e^-$) and transfer to a mobile plastoquinone (PQ). The doubly reduced PQ molecule then binds two protons and leaves the PSII binding pocket as PQH_2. The electrons are transferred to the large redox protein complex cytochrome b_6f (cyt b_6f) which mediates electron transfer between the two photosystems and pumps protons across the membrane. The electrons are carried from cyt b_6f to PSI by the lumen soluble redox carrier, plastocyanin (PC). Under copper-depleted conditions, PC may be replaced by cytochrome c_6 (cyt c_6, also known as cyt c_{533}). In PSI, electrons are reenergized by a second reaction centre, P700, and NADPH production is catalysed by flavoprotein ferredoxin-NADP+ reductase (FNR).

Photosynthetic electron transport induces the accumulation of protons in the thylakoid lumen thus building a proton electrochemical potential difference across the thylakoid membrane to drive ATP synthesis. First, four protons are

generated during the oxidation of two water molecules in the thylakoid lumen. Then when electrons are shuttled via PQ to cyt b_6f, protons are taken from the cytoplasmic side of the thylakoid membrane to balance the charge of PQ and released to the lumen by cyt b_6f (Shevela et al., 2013). This proton motive force drives the phosphorylation of ADP and P_i to produce ATP by ATP synthase.

Alternatively, two cyclic photosynthetic electron transport chains exist, each associated with one of the two photosystems. One chain utilises the reaction centre of PSI to energise electrons, which are transferred from the cytoplasmic side of PSI to cyt b_6f thus creating a loop between PSI and cyt b_6f (Mullineaux, 2014). The net result is the light-driven translocation of protons to the lumen generating a proton motive force for ATP synthesis. An alternative cyclic chain utilises PSII with a similar net production of ATP. In this case electrons are extracted during water oxidation and transferred to a terminal oxidase which catalyses the reduction of O_2 to produce water and is commonly referred to as the "water-water cycle".

1.2.1.2 Carbon assimilation

The dominant pathway for carbon fixation is the Calvin-Benson cycle, or reductive pentose phosphate pathway, and is the primary pathway targeted for biofuel production. The Calvin-Benson cycle uses the reducing power of NADPH and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) to assimilate inorganic carbon (Sharkey and Welse, 2012). To alleviate inefficiencies in carboxylase activity arising from its ability to accept oxygen as a substrate, cyanobacteria have acquired mechanisms to concentrate carbon into Rubisco-containing organelles called carboxysomes (Ogren, 1984, Ogren, 2003). Rubisco combines CO_2 with the sugar phosphate ribulose-1,5-bisphosphate (RuBP) which spontaneously splits into two molecules of 3-phosphoglyceric acid (PG3). This is a key metabolite for many pathways of industrial value. PG3 conversion via the glycolysis pathway (phosphoglyceromutase, enolase and pyruvate kinase) produces pyruvate, a precursor for numerous biofuel candidates including ethanol and ethylene (Dienst et al., 2014, Jindou et al., 2014). In the Calvin-Benson cycle, PG3 is converted by two enzymes, phosphoglycerate kinase and

G3P dehydrogenase, to produce glyceraldehyde-3-phosphate (G3P), which can also be re-directed into the production of another potential biofuel, isoprene (Lindberg et al., 2010). In cyanobacteria, G3P is converted into dihydroxyacetone phosphate (DHAP) by triose phosphate isomerase and subsequently converted into pentose phosphate by a bifunctional fructose-1,6-bisphosphatase/sedoheptulose-1,7-bisphosphatase (FBPase/SBPase) not found in plants (Tamoi et al., 1996). Finally, pentose phosphate is phosphorylated to regenerate RuBP substrate for Rubisco.

1.2.1.3 Cellular respiration

For many decades, the existence of respiration in cyanobacteria was an open question and its physiological relevance a big debate. The role of cellular respiration in cyanobacteria still remains unclear and less characterised than photosynthesis. Respiration contributes very little (just 10-20 %) ATP to the overall metabolism of cyanobacterial cells under standard physiological conditions (Peschek et al., 2004). However, it may play an important role for survival in stress conditions by maintaining ATP levels under reduced photosynthetic function. Respiratory electron transport occurs alongside photosynthetic electron transport in the thylakoid membrane and in the plasma membrane. The first step of respiration is typically catalysed by the NAD(P)H dehydrogenase enzyme in bacteria. This results in the oxidation of a reduced pyridine nucleotide (NADH or NADPH) and the transfer of two electrons to a plastoquinone electron carrier. The NDH-1 enzyme complex in cyanobacteria is very similar to the 14-subunit complex of *E. coli* although the genes for three subunits involved in substrate binding seem to be missing in the cyanobacterial genome (Vermaas, 2001). Controversy surrounds the role of NDH-1 in cyanobacteria; it has been proposed as the primary source of electrons to the PQ pool but mutagenesis studies suggest that succinate dehydrogenase (SDH) may be more important in respiratory electron transport (Cooley and Vermaas, 2001, Ogawa and Mi, 2007). The latter is surprising because no gene encoding the enzyme for succinate synthesis, 2-oxoglutarate dehydrogenase, has been identified in the cyanobacterial genome. Nevertheless, accumulation of greater amounts of succinate compared to 2-oxoglutarate in *sdh* mutants suggests the

presence of a modified tricarboxylic acid (TCA; also referred to as citric acid or Krebs) cycle in cyanobacteria. Electrons transferred to the PQ pool are carried through the cytochrome b_6f complex and plastoquinone/cytochrome c_6 pool to a terminal cytochrome aa_3-type cytochrome-c oxidase which transfers electrons to the final acceptor, O_2.

1.2.1.4 Regulation of central metabolism in cyanobacteria

The presence of both photosynthetic and respiratory electron transport chains in the same membrane requires tight regulation of electron flow and numerous mechanisms have been proposed to maintain redox balance in cyanobacteria. Firstly, immediate adjustment of light-harvesting capacity has been observed from seconds to minutes following changes in light intensity. For instance, the Orange Carotenoid-Binding protein (OCP) responds to strong blue light by quenching excitation energy at the phycobilisomes surrounding PSI and PSII (Kirilovsky, 2007). Secondly, supercomplexes have been suggested to steer electron flow by physically linking components of the electron transport chain. Indeed, “respirasome” complexes have been described for mitochondrial respiration and there is evidence for physical interaction between cyanobacterial photosynthetic components such as the FNR and phycobilisome in PCC 6803 (van Thor et al., 1999, Korn et al., 2009, Lapuente-Brun et al., 2013). Thirdly, there is evidence for the segregation of components into functional islands within the thylakoid membrane. Fluorescence Recovery after Photobleaching (FRAP) has demonstrated low mobility of chlorophyll-containing proteins in the thylakoid membranes of *Synechococcus* spp. PCC 7942 (Mullineaux and Sarcina, 2002). Furthermore, electron microscopy showed an asymmetrical distribution of photosystem reaction centres in this organism suggesting localisation of linear and cyclic photosynthetic electron transport chains to different regions of the thylakoid membrane (Sherman et al., 1994). Finally, regulation at the transcriptional level has also been demonstrated to control expression of photosynthetic and respiratory machinery (Singh et al., 2009). Persistence of electron transfer components in the thylakoid membrane following down-regulation in gene expression suggests that changes in gene expression levels may be a longer-term adaptation strategy. In summary, the

added complexity of two distinct energy production processes which share common components in the same membrane requires extra levels of regulation for rapid and efficient maintenance of cellular redox status. While this adds complexity to cyanobacteria as a biotechnology chassis, it also supports the remarkably versatile and flexible metabolism of phototrophic prokaryotes.

1.2.2 Diversity of cyanobacteria

Cyanobacteria represent the largest and most diverse group of photosynthetic gram-negative prokaryotes ranging from simple unicellular organisms to complex filaments and aggregates (Stanier and Cohen-Bazire, 1977). They are able to thrive in diverse and extreme habitats of varying light intensity, temperature, salinity, pH and water availability (Waterbury, 2006) and can therefore be applied in a wide range of settings for site-specific applications such as bioremediation or large-scale controlled conditions for bioproduction. Importantly, cyanobacteria are capable of high density growth in varied water sources ranging from fresh to hypersaline water and even wastewater (Abed et al., 2009). As a result, they are able to utilise otherwise unproductive land and do not compete for limited arable land or freshwater resources.

Cyanobacteria are tremendously metabolically versatile and can switch between different modes of metabolism in response to changing conditions (Stal, 1995). All cyanobacteria carry out oxygenic photosynthesis, however some species of cyanobacteria such as *Oscillatoria* spp. can switch to anoxygenic photosynthesis using hydrogen sulphide as an electron donor (Cohen et al., 1986) or, in the case of *Aphanocapsa* spp. PCC 6714, switch to heterotrophic growth on organic carbon sources (Rippka, 1972). Other species can differentiate into specialised such as heterocysts for nitrogen fixation, e.g. *Nostoc* spp. (Stal, 2008) or akinetes for resistant spores, e.g. *Nostocales* spp. (Kaplan-Levy et al., 2010). Of interest for bioproduction is the variety of valuable products naturally synthesised by cyanobacteria including bioactive compounds (Singh et al., 2005), biodegradable plastics (Stal, 1992), biofuels (Parmar et al., 2011) and biofertilisers (Vaishampayan et al., 2001). Cyanobacteria use a range of transport proteins to remove nutrients, metals and pesticides, which are useful targets for improving

bioextraction (Vijayakumar, 2012). Furthermore, the ability of cyanobacteria to degrade or neutralise persistent organic pollutants such as polycyclic aromatic hydrocarbons has applications in bioremediation (Subashchandrabose et al., 2013). The enormous diversity of cyanobacteria enables engineers to select strains particularly suited for specific applications.

1.2.3 Advantages of prokaryotic systems

Cyanobacteria are gram-negative prokaryotes and therefore relatively simple biological systems to bioengineer. Firstly, they are single cell organisms with a large surface to volume ratio resulting in high metabolic rates, growth rates and short generation times (Rai and Padh, 2001). Moreover, cyanobacteria lack the membrane-bound mitochondria and nucleus of eukaryotes. Thirdly, cyanobacterial genomes are relatively small, typically consisting of a single chromosome and smaller self-replicating plasmids which are more compact and feature less redundancy than eukaryotic genomes. The small size of cyanobacterial genomes results in relatively low costs for genomic and transcriptomic analyses. The genome sequences of over 100 different cyanobacteria are available in the online database CyanoBase (Nakao et al., 2010). Overall, cyanobacteria are relatively simple biological systems with low redundancy and thus low potential for interference of engineered systems by endogenous regulatory processes. They therefore have many advantages over other photosynthetic systems such as plants or algae (see Table 1-1).

Table 1-1. Genomes of phototrophic model organisms

Organism	Genome size (Mbp)[a]	Number of genes	Reference
Freshwater cyanobacterium *Synechocystis* spp. PCC 6803	4	3,725	Kaneko et al., 1996
Algae *Chlamydomonas reinhardtii*	121	15,143	Merchant et al., 2007
Plant *Arabidopsis thaliana*	125	25,498	The Arabidopsis Genome Initiative, 2000

[a] Mbp = mega base pairs

1.2.4 Genetic manipulation of cyanobacteria

Cyanobacteria are relatively easy to genetically manipulate by natural transformation, electroporation and conjugation using autonomous or genome-integrating vectors (Koksharova and Wolk, 2002, Wilde and Dienst, 2011). Many species are naturally competent and natural transformation is generally the preferred method to introduce transgenes to cyanobacteria. Natural transformation involves the direct uptake and incorporation of foreign DNA from the environment into their genome. However, the process of DNA uptake is still poorly understood. Type IV pili, multifunctional filaments which protrude from the outer membranes of bacteria, are fundamental to the natural competence of cyanobacteria (Yoshihara et al., 2001, Yoshihara et al., 2002) although their exact role in DNA uptake remains to be elucidated. Exogenous DNA must be double-stranded for binding and uptake to cyanobacteria (Essich et al., 1990). It is converted to single-stranded DNA by a calcium-dependent nuclease located in the plasma membrane for transfer across the cell envelope (Barten and Lill, 1995). Once inside the cell, the exogenous DNA is stably integrated into the host chromosomal or plasmid DNA by homologous recombination.

Self-replicating plasmids can also be introduced to cyanobacteria via conjugation or electroporation. Tri-parental conjugation is the second most common approach to transform cyanobacteria (Huang et al., 2010). It involves the transfer of plasmid DNA from a “cargo” strain of *E. coli* to cyanobacteria with the aid of a “helper” *E. coli* strain expressing the required conjugation machinery. Tri-parental conjugation is a relatively time-consuming approach compared with natural transformation, however, as it requires additional steps to prepare the *E. coli* strains and select for transformed cyanobacteria. Alternatively, whole plasmids can be introduced into cyanobacteria via electroporation, which involves the application of an electrical field to increase membrane permeability. This approach is rarely used, however, as there are concerns of mutagenic effects observed in some strains of cyanobacteria (Bruns et al., 1989, Mühlenhoff and Chauvat, 1996).

1.2.5 Unique challenges for cyanobacterial engineering

There are a number of special considerations to take into account when engineering cyanobacteria. First and foremost, cyanobacteria represent an incredibly diverse phylum of bacteria and this diversity has been observed at the level of the genome sequence. Comparative genomic studies have demonstrated considerable variation across multiple aspects including size (1.4 to 11.6 Mbp), GC content (31-63%), number of protein coding sequences (1214-8446) and coding nucleotide proportions (52-94 %; Larsson et al., 2011, Shih et al, 2013). Furthermore, the sequence of the smallest cyanobacterial genome is not present in that of the largest genome. This variation poses challenges for the development of universal molecular tools to engineer cyanobacteria. Secondly, cyanobacteria have the added complexity of photosynthesis-related pathways (compared to heterotrophic prokaryotes) occurring at the same site (in the thylakoid membranes) as respiration-related pathways (compared to photoautotrophic eukaryotes). As a result, these two processes share many common factors resulting in substantial crosstalk between photosynthesis and cellular respiration (Mullineaux, 2014). Meticulous regulation and coordination of the different electron transport chains is required to maintain the redox status of the cells. Secondly, cyanobacteria are the only known prokaryotes to have a circadian clock, which coordinates metabolic and behavioural responses with daily fluctuations in light and temperature (Mackey et al., 2011). Thirdly, cyanobacteria have evolved effective defence mechanisms against foreign DNA in the form of restriction modification systems, physical barriers such as extracellular nucleases, and functional barriers determined by the ability of foreign DNA to replicate and be expressed in cyanobacteria (Stucken et al., 2013). Finally, cyanobacteria carry multiple genome copies per cell. Copy numbers range from three in *Synechococcus* spp. PCC 7942 to 142 in *Synechocystis* spp. PCC 6803 subst. GT (Griese et al., 2011). For these reasons, the development of cyanobacteria for practical applications has struggled to match that of alternative hosts. However, while cyanobacteria are relatively challenging prokaryotes to engineer, it is important to remember that biological engineering is a great challenge in and of itself.

1.3 Synthetic biology approaches to simplify biological complexity

Although the fundamental laws of physics and chemistry apply to biological processes, the predictability of experimental manipulation in biology lags far behind that of other disciplines such as physics, chemistry and engineering. The main problem is that biological systems are immensely complex, inherently stochastic and highly dynamic: they grow and die, adapt and evolve. The burgeoning new field of synthetic biology attempts to overcome this complexity by applying traditional engineering approaches to biological problems. This includes the use of computational modelling to inform the rational design of biological processes and the use of standardised parts and devices to enhance predictability of behaviour of engineered systems. Synthetic biology relies on four main principles, which distinguish it from other disciplines such as systems biology or biotechnology: decoupling, abstraction and modularity, standardisation and modelling.

1.3.1 Decoupling, abstraction and modularity

Synthetic biology attempts to reduce biological complexity by decoupling, or breaking down a complicated problem into many simpler problems which can each be dealt with independently. One way to approach the problem of biological complexity is to separate a process into different levels of increasing complexity using an abstraction hierarchy as illustrated in Figure 1-3 (Endy, 2005). At the most basic level of the hierarchy is the DNA sequence, which encodes genetic components, or “parts”, such as promoters and protein coding sequences. Parts can be combined into devices with a defined function, devices combined to form circuits and so forth. In this way, biological complexity is subdivided into smaller, more manageable chunks. Bioengineers can assemble, optimise and characterise modules at any level of the hierarchy without concern for other levels and later combine them to form a functioning whole. For this to

succeed, parts must be modular in the sense that they are discrete, reusable and well-defined.

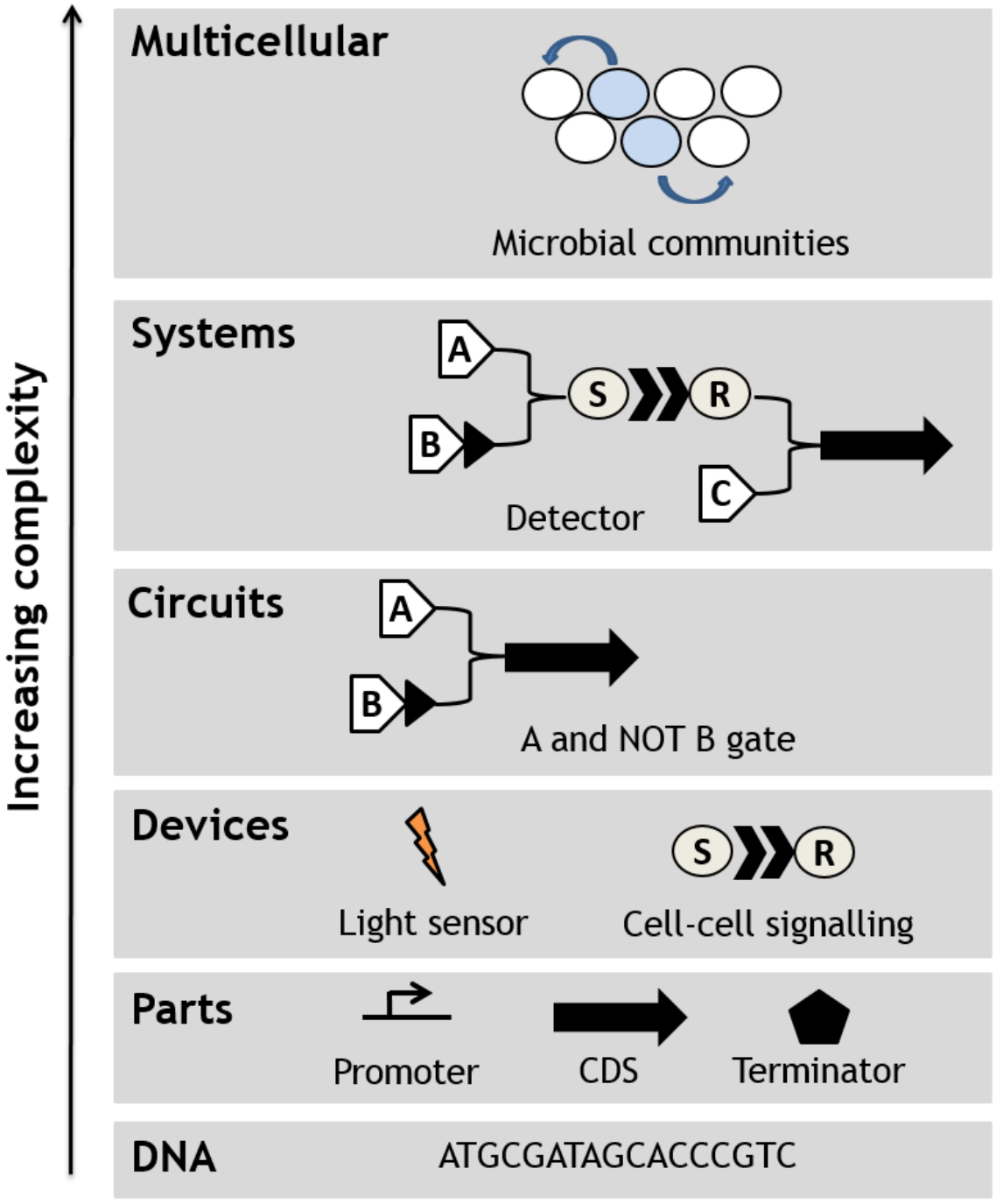


Figure 1-3. The abstraction hierarchy

Separation of biological complexity into different levels of increasing complexity. Adapted from Federici et al., 2013.

1.3.2 Standardised components for rapid assembly

Successful bioengineering relies on the availability of pre-defined, reliable materials. One of the aims of synthetic biology is to generate a catalogue of biological parts available in a standardised format for rapid assembly. In 2003, a standard design was proposed for the assembly of biological parts, termed BioBricks (Knight, 2003). This format consists of BioBrick parts flanked by unique restriction enzyme recognition sites with EcoRI and XbaI directly upstream of the BioBrick (in the "BioBrick prefix") and SpeI and PstI directly downstream (in the "BioBrick suffix"; Figure 1-4A). Directed construction of multiple parts can be performed using the distinct fragments generated by restriction digestion with different combinations of the four sites flanking the BioBrick. Importantly, restriction digestion of XbaI and SpeI sites creates compatible overhangs, which can recombine to form a "scar," which is not recognized by any restriction enzyme (Figure 1-4B). BioBricks are thus idempotent in nature as the products generated by the assembly of two BioBrick components maintain the characteristics of the individual components and are subject to the same rules for further assembly.

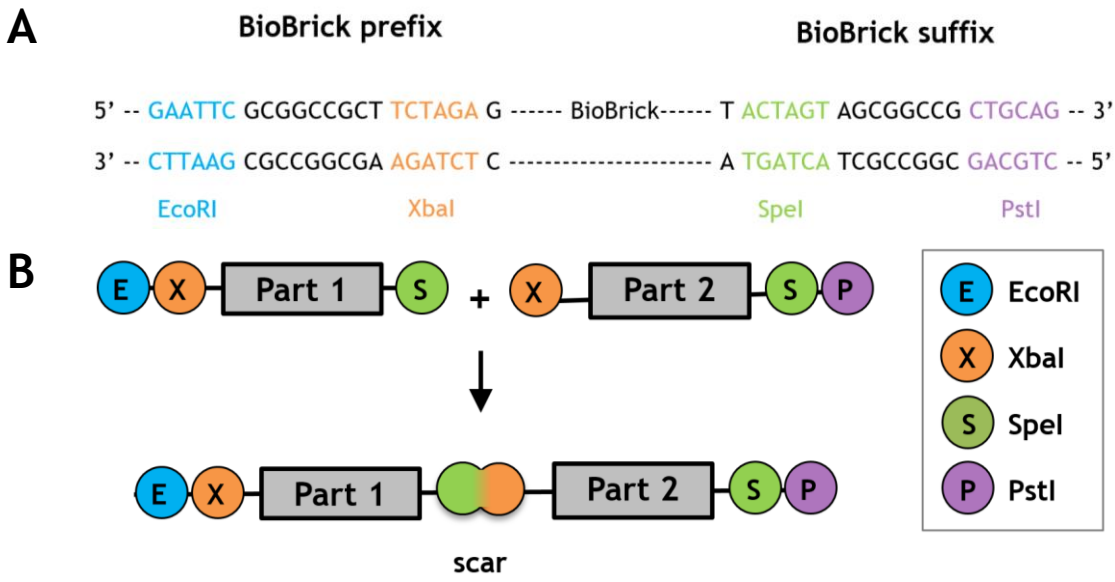


Figure 1-4. BioBrick part design and assembly

(A) Standardised BioBrick parts are flanked by unique restriction sites: EcoRI and XbaI in the prefix and SpeI and PstI in the suffix. **(B)** SpeI and XbaI sites recombine to form a scar upon which neither restriction enzyme can act upon.

Numerous registries of standard biological parts have since emerged, including the Synthetic Biology Engineering Resource Centre (SynBERC) Registry, the Joint BioEnergy Institute Public Registry (JBEI-ICE Public) and the BIOFAB: International Open Facility Advancing Biotechnology to name a few. Due to the idempotent nature of BioBricks, the Registry of Standard Biological Parts (www.parts.igem.org; last accessed 06/09/2015) remains the largest and most popular collection of genetic components (Kahl and Endy, 2013). Most of these parts have been developed in and for *E. coli* however other chassis listed in the registry include yeast, bacteriophage T7, and *Bacillus subtilis.* More recently, focus has turned to cyanobacteria but currently available BioBricks have not been effective in cyanobacteria including promoters (Huang et al., 2010) and ribosome binding sites (Markley et al., 2014). Development and characterisation of suitable parts for cyanobacteria is required before modules can be developed at higher levels of the hierarchy.

1.3.3 Component characterisation for predictable behaviour

Successful bioengineering also relies on parts with robust behaviour. This requires thorough characterisation of parts in a manner in which the character of one part can be directly compared with that of another. A strong promoter in one laboratory may be considered weak in another. Reference standards have therefore been introduced as “yardsticks”. For instance, a standardised promoter assay was developed in *E. coli* in which promoter activity was normalised to the activity of a standard reference promoter to yield relative promoter units (Kelly et al., 2009). Assessment of the same promoter library across different laboratories demonstrated that this normalisation strategy reduced variation between test conditions and measurement instruments. Upon selection of suitable reference standards and thorough characterisation of individual parts, scientists will be able to rationally select the best-suited components for the desired process. The more thorough the characterisation and the more robust the part, the greater the chance of the process behaving as intended.

1.3.4 Computational modelling

The construction of novel biological devices using standardised components may give the misleading impression of an oversimplified “Plug and Play” strategy. Indeed, it does enable the construction of increasingly complex modules by non-specialists; the international Genetically Engineered Machine (iGEM) student competition has successfully served as a workbench as well as a flagship for synthetic biology (Vilanova and Porcar, 2014). However, careful consideration is required when designing modules to ensure that when combined, components retain the desired behaviour. Mathematical modelling and the application of Biological Computer Aided Design (BioCAD) are important tools in the rational design of biological organisms. Simpler software tools have been developed to predict transcription and translation initiation rates and to design plasmid vectors using libraries of standardised parts (Salis et al., 2009, Ellis et al., 2009, Taton et al., 2014). The aim is to eventually use high-level software integrating genomic, transcriptomic and metabolic data to inform the design of novel biological systems. Before this can be achieved in cyanobacteria, however, it is important to start at the beginning.

1.4 Taking control of gene expression

Control over gene expression is key for regulating processes in biological systems. The first step of gene expression is the initiation of transcription at promoters in the DNA, typically directly upstream of the gene, which are specifically recognised by the RNA polymerase holoenzyme (Browning and Busby, 2004). Promoters are therefore the first design consideration for temporal and spatial regulation of gene expression. They can be used as a biological switch to trigger a biotechnological process in response to a given stimulus e.g. environmental changes or supplements. Libraries of well-characterised, standardised promoters spanning a wide range of transcriptional activity are desirable for optimisation of engineered biological systems. Promoters directly influence the amount of messenger RNA (mRNA) transcript available for translation into functional protein. Strong promoters can thus compensate for

mRNA instability, poor translation efficiency and unstable or inefficient products. Consequently, this is the first point of optimisation in metabolic engineering as enzymes within a given pathway may have differing efficiencies. Promoters of varying strengths can be used to modulate the amounts of different components within a pathway to alleviate bottlenecks within a multistep process. Several constitutive, inducible and repressible promoters have already been developed for cyanobacteria and are listed in Table 1-2 (Heidorn et al., 2011, Wang et al., 2012, Berla et al., 2013). However, their use to date has highlighted the need for further improvements.

Limitation of product titre due to insufficient transgene expression has been illustrated by L-lactic acid production in PCC 6803 (Angermayr and Hellingwerf, 2013). The gene encoding lactate dehydrogenase (LDH) from *Lactococcus lactis* was placed under the control of three different promoters in PCC 6803. Initial titre was very low but could be increased by using a stronger promoter. Furthermore, a linear relationship was observed between intracellular enzyme concentration and rate of product formation. Hence, promoter strength was the limiting factor. The strongest promoter used in this study was P_{trc}, which resulted in the production of 20 mmol L-lactic acid L^{-1} in 4 weeks.

Stronger promoters have since been identified such as the “super strong” *cpcBA* promoter of PCC 6803 (Zhou et al., 2014). The endogenous *cpcBA* promoter drives the expression of phycocyanin, a pigment-protein complex present in the phycobilisome and the most abundant soluble protein in cyanobacteria (Grossman et al., 1993). This promoter was recently used to enhance production of ß-phellandrene, a monoterpene of commercial value in medical, cosmetic and cleaning industries as well as a potential fuel. Indeed, P_{cpcBA}-driven expression of heterologous ß-phellandrene synthase resulted in the highest product titre reported for cyanobacteria to date: 260 µg per g dry cell weight

Table 1-2. Selected promoters used in *Synechocystis* spp. PCC 6803 and *Synechococcus* spp. PCC 7002

Promoter	Source	Description	Expression hosts	References
P_{rbcL}	*Synechocystis* spp. PCC 6803	Constitutive	*Synechocystis* spp. PCC 6803	Huang et al., 2010
P_{rnpB}	*Synechocystis* spp. PCC 6803	Constitutive	*Synechocystis* spp. PCC 6803	Huang et al., 2010 Angermayr and Hellingwerf, 2013
P_{psbA}	*Amaranthus hyridus*	Constitutive	*Synechococcus* spp. PCC 7002	Jacobsen and Frigaard, 2014
P_{psbA1}	*Synechococcus* spp. PCC 7942	Light-inducible	*Synechococcus* spp. PCC 7002	Ruffing, 2014
P_{psbA2}	*Synechocystis* spp. PCC 6803	Light-inducible	*Synechocystis* spp. PCC 6803	Angermayr and Hellingwerf, 2013 Lindberg et al., 2010
P_{nirA}	*Synechocystis* spp. PCC 6803	Nitrate-inducible	*Synechocystis* spp. PCC 6803	Ivanikova et al., 2005
P_{isiAB}	*Synechococcus* spp. PCC 7002	Iron-inducible	*Synechococcus* spp. PCC 7002	Boyanapalli et al., 2007
P_{petE}	*Synechocystis* spp. PCC 6803	Copper-inducible	*Synechocystis* spp. PCC 6803	Oliveira and Lindblad, 2008
P_{coaT}	*Synechocystis* spp. PCC 6803	Cobalt-inducible	*Synechocystis* spp. PCC 6803	Blasi, 2012
P_{cpcBA} P_{cpc560}	*Synechocystis* spp. PCC 6803	“Super strong ”; Most active during early-mid exponential phase	*Synechocystis* spp. PCC 6803 *Synechococcus* spp. PCC 7002	Formighieri and Melis, 2014b Zhou et al., 2014 Xu et al., 2011
P_{trc}	*Escherichia coli*	Constitutive in the absence of the *lac* repressor IPTG-inducible in the presence of the *lac* repressor	*Synechocystis* spp. PCC 6803	Huang et al., 2010 Camsund et al., 2014 Angermayr and Hellingwerf, 2013

(Formighieri and Melis, 2014b). However, this also fails to compare with product titres achieved in *E. coli*: 25 mg per g dry cell weight (Formighieri and Melis, 2014a). Although the gene encoding ß-phellandrene synthase was driven by the endogenous phycocyanin promoter, protein levels did not accumulate to similar levels as native phycocyanin subunits from untransformed PCC 6803. Further characterization of the *cpcBA* promoter will be required to explain the discrepancies between expression levels of downstream genes.

1.4.1 The RNA polymerase holoenzyme

Transcription is catalysed by the RNA polymerase (RNAP) holoenzyme, which is comprised of a detachable sigma (σ) factor bound to the core enzyme (Figure 1-5). The core enzyme is responsible for transcriptional elongation of mRNA transcript whereas the σ-factor is responsible for promoter recognition and binding during transcription initiation. The RNAP core enzyme is comprised of five subunits, ßß'$\alpha_2\omega$, and is highly conserved across kingdoms (Sweetser et al., 1987). The ß and ß' subunits form the active site of the enzyme as a crab-claw structure which binds both the DNA template and the nascent RNA molecule (Korzheva et al., 2000). The two identical α subunits are comprised of two domains joined by a flexible linker (Blatter et al., 1994). The amino-terminal domain (αNTD) is involved in the assembly of the ß and ß' subunits while the carboxy-terminal domain (αCTD) has DNA-binding activity. The fifth subunit, ω, is not essential for viability or transcriptional elongation, however it is also conserved across kingdoms and a chaperone role in ß' assembly has been suggested (Hampsey, 2001).

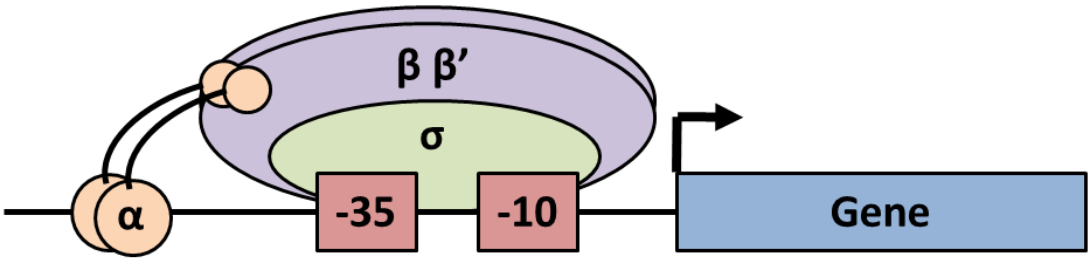


Figure 1-5. Structure of the RNA polymerase holoenzyme

see overleaf for the complete figure legend

Figure 1-5. Structure of the RNA polymerase holoenzyme

The RNA polymerase holoenzyme consists of five subunits, ββ'$\alpha_2\omega$, and a detachable sigma (σ) factor. It is the sigma factor that specifically recognizes promoter elements (e.g. -10 and -35 elements) directly upstream of the gene.

In cyanobacteria, the core RNAP is similar to that of *E. coli* except the ß' subunit is split in two parts: the ß' and γ subunits correspond to the carboxy- and amino-terminal domains of the *E. coli* ß' subunit respectively (Schneider and Hasekorn, 1988, Xie et al., 1989). The effect of this split is unknown; however, it may contribute to the slower yet more precise transcription performed by the cyanobacterial RNAP compared to *E. coli* (Imashimizu et al., 2011).

1.4.2 Sigma factors

While the core RNA polymerase enzyme is responsible for transcription elongation, it is the detachable σ-factor, which is important for promoter recognition and transcription initiation (Ishihama, 2000). Usually, bacteria contain multiple σ-factors and this is a primary point of response to changing environmental conditions. The ability of σ-factors to recognise specific sequences in promoter elements enables coordinate regulation of large sets of genes, or gene clusters, in response to a given stimulus. Thus, functional modulation of RNA polymerase by σ-factor replacement, or "sigma switching," results in global changes in gene expression patterns via promoter selectivity.

Most prokaryotes, including *E. coli,* have two evolutionary independent families of sigma factors, σ^{70} and σ^{56}. All σ-factors in cyanobacteria belong to the σ^{70} family, however, and they can be divided into three broad categories (Gruber and Gross, 2003). Group 1 σ-factors, also known as the primary σ-factors, are essential for viability and maintain the expression of housekeeping genes. Group 2 σ-factors are structurally very similar to group 1. While group 2 factors are not essential for growth, they provide a mechanism for environmental adaptation. Multiple variants of group 2 σ-factors are found in cyanobacteria, typically between three and seven. In the freshwater model cyanobacterium *Synechocystis* spp. PCC 6803 (hereafter PCC 6803), these factors are typically

induced under stress conditions such as nitrogen limitation or under oxidative stress (Osanai et al., 2008). Group 3 σ-factors are an alternative type and differ structurally from the other two groups. In PCC 6803, these factors are involved in specific stress survival regulons such as flagellar biosynthesis, heat shock response and sporulation.

While cyanobacteria are extremely diverse and sigma factor composition differs between species, they do share several characteristics. Imamura and Assayama (Imamura and Asayama, 2009) assessed the phylogenetic relationship among σ-factors across six species of cyanobacteria and found that certain clusters were conserved while others likely evolved in a species-dependent manner. Consistent between all species was the presence of a unique group 1 σ-factor closely related to the *E. coli* primary σ-factor, RpoD. Group 2 σ-factors were also relatively consistent between species and consisted of four clusters: B, C, D and E. One σ-factor was present in each cluster with the exception of the heterocyst-forming cyanobacteria *Anabaena* spp. PCC 7120, which encoded multiple B-type σ-factors. Group 3 σ-factors also typically consisted of four clusters: F, G, H and I, however this group was more divergent resulting in clusters lacking in a few of the species, in particular H- and I-type σ-factors.

1.4.3 Promoter structure

Sigma factors recognise specific nucleotide sequences, or motifs, in the upstream promoter regions of genes. In bacteria, promoters typically consist of two conserved 6 base pair DNA sequences centred approximately 10 and 35 nucleotides upstream of the transcription start site as illustrated in Figure 1-6 (Browning and Busby, 2004). These two elements are designated the -10 TATA box and the -35 hexamer respectively. Occasionally, additional enhancer motif elements may be present further upstream of these two conserved regions. Three types of promoter have been described for cyanobacteria (Imamura and Asayama, 2009). Type 1 promoters possess both the -10 and -35 hexamers (consensus sequences TATAAT and TTGACA respectively) and are typically recognised by the group 1 σ-factor. Under certain conditions, group 2 σ-factors are induced and may replace the group 1 σ-factor in the RNAP holoenzyme to

drive transcription from type 1 promoters more efficiently. For instance, the light-induced up-regulation of *psbA* genes encoding a photosystem II reaction centre protein results in replacement of the primary σ-factor by the group 2 SigD factor in PCC 6803 (Imamura et al., 2003b). Type 2 promoters possess a -10 hexamer and upstream enhancer motifs; however the -35 consensus hexamer is not present. Examples of these types of promoters are limited as is the knowledge surrounding their regulation by σ-factors, however it has been suggested that multiple group 2 σ-factors may cooperate with the primary σ-factor to initiate transcription from these promoters (Imamura et al., 2006). Finally, type 3 promoters are distinct from the other two types of promoters and the group 3 σ-factor, SigF, has been shown to regulate a handful of genes driven by this type of promoter (Asayama and Imamura, 2008). Little else is known about type 3 promoters and it has yet to be clarified whether other group 3 σ-factors are involved in the regulation of transcription from these promoters.

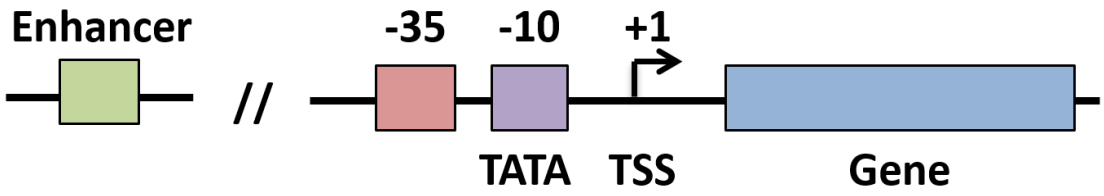


Figure 1-6. Structure of a typical bacterial promoter

Two conserved regions occur 10 and 35 nucleotides upstream of the transcription start site (TSS). Additional enhancer elements may be present further upstream.

1.4.4 Transcription factors

Transcription factors are important regulators coupling gene expression to environmental signals in bacteria. In *E. coli*, approximately 5 % of the total gene count encodes transcription factors (Browning and Busby, 2004). In contrast to sigma factors, transcription factors are not core components of the RNAP holoenzyme. Instead, it is likely that transcription factors bind specific sequences in the DNA independently of the RNAP in order to modulate the binding of RNAP (Balleza et al., 2009). In this way, transcription factors function as regulatory switches to either repress or activate transcription as global

regulators of large gene clusters or by targeting individual genes. Negative regulators repress transcription by binding close to the core promoter elements and creating a steric hindrance to prevent RNAP binding. The classic example is the *lac* repressor, which inhibits transcription of the *lacZYA* operon of *E. coli* in the absence of lactose (Gilbert and Müller-Hill, 1966). It binds to the major groove of the primary *lac* operator (*lacO1*) overlapping the transcription start site via a helix-turn-helix motif (Lewis, 2005). In this way, it prevents transcription of the encoded lactose metabolism genes and saves precious cellular resources in the absence of substrate. By contrast, positive regulators bind to regions outside of the promoter, typically upstream of the -35 element, to aid the recruitment and stabilisation of RNAP in order to activate transcription. In the example of the *lac* operon, cyclic AMP receptor proteins (CRPs; also referred to as catabolite activator proteins, CAPs) undergo a conformational change upon cAMP binding which promotes CRP binding adjacent to the *lac* promoter and subsequent transcription of the *lac* operon (Busby and Ebright, 1999).

Transcription factor activity is regulated by three main mechanisms. Firstly, DNA-binding affinity can be modulated by small ligands whose concentrations fluctuate in response to changing environmental conditions. For instance, the presence of allolactose (a product of lactose) activates the expression of genes involved in lactose metabolism by inhibiting DNA binding ability of the *lac* repressor (Müller-Hill, 1996). Isopropyl ß-D-1-thiogalactopyranoside (IPTG) is an analog of allolactose commonly used to activate expression of promoters containing a *lac* operator such as P_{trc}. Secondly, covalent modification can also alter the DNA-binding affinity of transcription factors. Common examples are two-component signal transduction systems consisting of a sensor kinase, which, upon detection of specific external stimuli, phosphorylates and activates a response regulator, which often contains a DNA-binding domain (Parkinson and Kofoid, 1992). Thirdly, the activity of some transcription factors is regulated by their intracellular concentration determined either at the transcript or protein level. Finally, transcription factor activity can also be regulated by sequestration by regulatory proteins. In this way, bacteria are able to monitor external conditions and adjust accordingly.

1.4.5 Limitations of available expression systems for cyanobacteria

In an ideal system for synthetic biology, the host organism, or chassis, should have minimal interaction with the engineered system and merely provide the machinery and resources necessary for the engineered process. Orthologous systems have been developed which are comprised of components which have evolved outside of the chassis and act independently of endogenous processes (An and Chin, 2011, Camsund and Lindblad, 2014). This prevents undesirable interaction between transcriptional regulatory elements and host factors thus reducing potential interference from host regulatory networks. Efforts have been made to optimize and implement orthologous expression systems commonly used in prokaryotes, however they have had limited success in cyanobacteria. For instance, the most common orthologous approach to regulate gene expression in bacteria is based on the *lacO-lacI* system from *E. coli*. This typically involves IPTG induction of gene expression from the chimeric P_{trc} promoter from *E. coli* consisting of the -35 sequence (TGAC) of P_{trp} and the -10 sequence (TATAA) from P_{lac} separated by a 17 base pair spacer containing a *lacO* operator sequence (Amann et al., 1988). This is a relatively strong promoter in PCC 6803; however, repression is poor under non-induced conditions (Huang et al., 2010). In an attempt to enhance non-induced silencing, a library of P_{trc}-derived promoters was generated by varying *lacO* operator sequence, location and spacing relative to the -35 and -10 elements (Camsund et al., 2014). Despite these efforts, repression under non-induced conditions remained poor. However, placement of *lacO* downstream of the -10 element in $P_{trc10\text{-}prox}$ resulted in greatly enhanced expression, so this promoter can serve as a strong constitutive promoter for cyanobacteria.

Another inducible gene expression system which has undergone substantial development for application in cyanobacteria is based upon the Tet repressor (TetR) from bacteriophage lambda. In this system, gene expression is repressed by the binding of TetR to its cognate DNA sequence *tetO* within the P_{tetR} promoter (Bertram and Hillen, 2008). Addition of the inducer anhydrotetracycline (aTc) inhibits the DNA binding ability of TetR and thus

activates transcription from P_{tetR}. In *E. coli*, TetR has a very high affinity for *tetO* over non-specific DNA sequences resulting in a wide regulatory window from one molecule of mRNA per three cells of *E. coli* under non-induced conditions to 5000-fold induction in the presence of aTc (Lutz and Bujard, 1997). Initially, very low P_{tetR} activity was detected using a GFP-based promoter assay in PCC 6803 (Huang et al., 2010). Conversion of the -10 element to the PCC 6803 consensus sequence (TATAAT) only served to further reduce promoter activity (Huang and Lindblad, 2013). Systematic alteration of nucleotide sequences between the -10 element and transcriptional start site resulted in substantial increases in promoter strength of up to 467-fold induction by promoter L15 in the presence of aTc. However, poor repression was still observed under non-induced conditions. Modification of TetR expression to improve protein stability resulted in slightly improved repression at the expense of weaker induced promoter strength. Despite extensive modification, this orthogonal expression system continues to suffer from leaky repressed activity and a relatively narrow range of regulation.

The most effective promoters to regulate gene expression in cyanobacteria are currently native cyanobacterial promoters. These originate from a handful of model species and have mostly been characterised in their native settings (Heidorn et al., 2011, Wang et al., 2012, Berla et al., 2013). While these tools have been critical for unravelling the core biology of cyanobacteria, they were originally developed to study specific processes in a limited number of species of cyanobacteria and are not necessarily suitable for practical applications. For instance, a large number of light-inducible promoters have been developed during extensive research into photosynthesis and circadian rhythm in cyanobacteria. One example is the promoter of the photosystem II D1 polypeptide (*psbA2*). P_{psbA2} is considered a relatively strong promoter in PCC 6803 and is routinely used to control heterologous processes such as lactate and monoterpene production (Eriksson et al., 2000, Angermayr and Hellingwerf, 2013, Formighieri and Melis, 2014b). Light-responsive promoters are not ideal regulators for gene expression however, as high-density cultures experience a steep gradient of light intensity throughout the culture. This results in zones where light intensity and therefore promoter activity becomes limiting.

Similarly, extensive nutrient limitation studies in cyanobacteria have identified a variety of nutrient-regulated promoters. These promoters are generally not ideal for biotechnology as their regulation relies on factors essential for growth. For instance, nutrient-inducible promoters such as the nitrate-inducible promoter of ferredoxin-nitrite reductase (*nirA*) necessarily couples biomass accumulation with heterologous protein synthesis, which may result in growth inhibition and therefore reduced biomass and hence product yield (Ivanikova et al., 2005). Alternatively, nutrient-repressible promoters such as the promoter of the sodium-dependent bicarbonate transporter (*sbtA*) regulated by inorganic carbon availability rely on the depletion of nutrients, which may be required for product synthesis (Wang et al., 2004). More recently, metal-inducible promoters regulated by ions such as copper, zinc, nickel, cobalt and cadmium have been developed and optimal conditions established to prevent disturbances in metabolism (Peca et al., 2008, Blasi, 2012). While these promoters are highly efficient in terms of both repression during non-induced conditions and ranges of regulation, these approaches are not suitable for large-scale industrial processes due to high costs and environmental concerns. Furthermore, the sensitivity of the promoters means that all culture media would have to be of extremely high purity, incurring further costs and challenges to the biomanufacturing process.

1.5 Stationary phase growth as a manufacturing platform

Practical applications typically require large biovolumes to maximise the effect of the engineered process, for instance in bioproduction, biodesalination, bioremediation or bioextraction. Computational analysis of stoichiometric constraints of cyanobacterial metabolism indicates trade-offs between biomass production and product synthesis (Knoop and Steuer, 2015). Bacterial cultures grown in batch culture divide exponentially until one or more essential factors in the environment becomes limiting. At this point, cells stop dividing and the culture reaches a steady state cell density, which is nominally described as the stationary phase of growth (Llorens et al., 2010). Promoters specifically active during this growth phase would be particularly useful as transgene induction at this stage would a) maximize the biovolume to perform the desired process, b)

avoid trade-offs between biomass production and product synthesis and c) minimise time of exposure to toxic compounds arising from the engineered system.

Over the last couple of years, a number of studies aimed at producing commodity chemicals in cyanobacteria report the highest production titres at the onset of stationary phase growth. This includes the production of isoprene and isopropanol in *S. elongatus* spp. PCC 7942 (Gao et al., 2015; Hirokawa et al., 2015), methanol in marine cyanobacteria including *Synechococcus* spp. 8102 and *Prochlorococcus marinus* (Mincer and Aicher, 2016), and biopolymers in *Spirulina* (Martins et al., 2014). These observations, paired with the predicted trade-off between biomass generation and product synthesis, indicate that the separation of growth and production phases can enhance the productivity of cyanobacteria cell factories (Knoop and Steuer, 2015). Hirokawa et al. developed a stationary phase production system in PCC 7942. In this system, the growth phase occurred under light and aerobic conditions and cultures were then transferred to dark and anaerobic conditions for the isopropanol production phase (Hirokawa et al., 2015). While this approach product yield in this experimental set up, it is not suitable for processes requiring light and/or oxygen. Alternative approaches should therefore be considered for a stationary phase production platform.

Promoters that specifically respond to changes in growth phase, particularly the transition from exponential to stationary phase, would be invaluable to the development of stationary phase production platforms. Notably, they can serve as auto-inducible switches regulated by endogenous cues thus avoiding the need for supplements and enhancing the sustainability of the system. Stationary phase-specific promoters have been developed for other bacteria including *E. coli*, *Pseudomonas putida* and *Bacillus subtilis* (Miksch and Dobrowolski, 1995, Cases et al., 1996, Schumann, 2007). These systems rely on “sigma switching” whereby the RNA polymerase holoenzyme alters its promoter specificity by replacing the primary sigma factor with alternative sigma factors. Promoters regulated in this manner have been identified in cyanobacteria, such as the nitrogen-regulated promoter PglnB-54,-53, which is activated by RNA polymerase sigma factor SigC during stationary phase growth in PCC 6803 (Asayama et al.,

2004). As discussed in the previous section, nutrient-regulated gene expression is not ideal, particularly when it depends on the depletion of a key macronutrient such as nitrogen. Efforts have been made towards unravelling late exponential phase and stationary phase-specific gene expression and its regulation, however more general late growth regulators and promoters are still lacking for cyanobacteria.

1.6 Objectives

The main aim of this PhD project was to develop robust promoters, which can act as a genetic switch to activate gene expression in high-density cultures of cyanobacteria.

The first objective was to develop an assay to characterise promoter activity in growing cultures of cyanobacteria. For applications in synthetic biology, it was important to develop tools compatible with current registries of standard biological parts. This involved the development of a BioBrick-compatible plasmid vector, selection of a suitable reporter and the development of a library of ribosomal binding sites to detect a wide range of transcriptional activity.

The second objective was to identify genes specifically induced during late exponential or early stationary phase in order to identify growth phase-specific promoters. To ensure that identified genes and promoters were robust, it was important to identify a range of conditions that resulted in transition from exponential to stationary phase for different reasons, i.e. limitation by different factors. This provided a variety of backgrounds to study gene expression patterns. Expression profiles of published growth phase-specific genes were then examined by quantitative RT-PCR (qPCR) and novel growth phase-specific gene clusters were identified by RNA sequencing (RNAseq).

Chapter 2. Materials and methods

All reagents used for media preparation were purchased from Sigma-Aldrich (Dorset, England), Thermo Fisher Scientific (Loughborough, UK) or VWR (Lutterworth, UK). Custom-made primers were purchased from Invitrogen (Life Technologies, Darmstadt, Germany). All restriction enzymes were purchased from New England Biolabs (Hitchin, UK).

2.1 Standard cyanobacteria methods

2.1.1 Strains of cyanobacteria

2.1.1.1 Synechococcus spp. PCC 7002

Synechococcus spp. PCC 7002 (hereafter PCC 7002) was a kind gift from Prof. John Golbeck, Pennsylvania State University, PA, USA. It was grown photoautotrophically in Medium A+ (Stevens and Porter, 1980) as described in *Section 3.2.1*. In brief, cyanobacteria were selected as single colonies from plates and inoculated to 20 ml media supplemented with spectinomycin (50 µg ml^{-1}) in glass culture tubes (VWR, Lutterworth, UK). Cultures selected for further growth analysis were inoculated to 150 ml media in 250 ml Erlenmeyer flasks (VWR, UK). Cultures were grown in long day (LD; photoperiod 16/8 h light/dark and light intensity 120 µmol m^{-2} s^{-1}) at 30 °C. Cultures were rapidly sparged with ambient air from an aquarium pump (AP4 Interpet Aqua Air Pump, 123 Aquatics, Westerham, UK) humidified with distilled water through a Büchner flask (VWR, UK) as shown in Figure 3-1.

2.1.1.2 Synechocystis spp. PCC 6803

Synechocystis spp. PCC 6803 (hereafter PCC 6803) was a kind gift from Prof. Linda Lawton, Robert Gordon University, Aberdeen, UK. It was grown photoautotrophically in BG11 growth medium (Stanier et al., 1971) as described in *Section 4.2.1*. In brief, 150 ml liquid cultures were grown in 250 ml Dreschel

flasks (VWR, Lutterworth, UK) maintained in water baths equilibrated at 30 °C in short day (SD; photoperiod 12/12 h light/dark). Cultures were sparged with ambient air with 5 flasks per aquarium pump outlet (AP4 Interpet Aqua Air Pump, 123 Aquatics, Westerham, UK) humidified with distilled water through a Büchner flask (VWR, UK) as shown in Figure 4-1. Nutrient-limited media are described in *Section 4.2.3*.

2.1.2 Growth of cyanobacteria on agar plates

To prepare solid media for plates, double strength growth media (BG11 for PCC 6803 or Medium A+ for PCC 7002) and 2 % agar were prepared and autoclaved separately at 121 °C for 20 min. The solutions were allowed to cool to 40 °C before being combined 1:1 (volume) and dispensed into petri dishes. Plates were sealed with parafilm and grown in LD at 30 °C. Single green colonies appeared after 7-10 days. Plates were then transferred to SD at 23 °C with a light intensity of approximately 50 μmol m^{-2} s^{-1} for longer-term maintenance.

2.1.3 Long-term storage of cyanobacteria

2 ml of mid-exponential cyanobacterial cultures (OD_{730} 1 to 3) were harvested in a 2 ml Eppendorf tube by centrifugation at 7000 rpm for 2 min and the supernatant removed. Under low light intensity, cells were gently resuspended in 1 ml of half strength media with 8 % DMSO by pipetting. The suspension was transferred to a 2 ml Nalgene® cryogenic vial (VWR, Lutterworth, UK) and a further 1 ml of half strength media with 8 % DMSO was added to the suspension. The cryogenic vials were transferred to a Mr. Frosty freezing container (VWR, UK) containing 100 % isopropanol equilibrated at 4 °C and stored at -80 °C for a minimum of 6 h before transfer to a cardboard box stored at -80 °C.

Frozen stocks of cyanobacterial cells were thawed in the dark or under low light conditions. Cryogenic vials were rapidly thawed in distilled water equilibrated to 30 °C. The cells were gently mixed by inversion and subjected to two rounds of centrifugation at 5000 rpm for 1.5 min followed by resuspension in 2 ml fresh media by inversion. The 2 ml cell suspension was transferred to a sterile glass

culture tube wrapped in aluminium foil and allowed recover overnight in the dark. Cells were then cultured as described in *Section 2.1.1*.

2.1.4 Monitoring growth

2.1.4.1 Optical density

Optical density of cyanobacteria was measured at 730 nm (OD_{730}) in a Lambda 45 UV/VIS Spectrophotometer (PerkinElmer, Cambridge, UK). Unless otherwise stated, liquid cultures of cyanobacteria were diluted in fresh media to the linear range of the spectrophotometer (OD_{730} 0.05 to 1).

2.1.4.2 Cell counting

Liquid cultures of cyanobacteria were diluted in fresh media to OD_{730} 0.1 to 0.6. 1 ml of diluted culture was added to a glass Sedgewick Rafter counting chamber (Thermo Fisher Scientific, Loughborough, UK). The counting chamber was placed on the stage of an Axiovert 200 microscope (ZEISS, Jena, Germany) with a 40x/0.6 objective (ZEISS, Germany) and allowed to settle for 10 min before image capture. Imaging was performed using an AxioCam HRc digital camera (ZEISS, Germany) and AxioVision LE software (ZEISS Vision GmbH, version 3.0.6 SPR) where 10 random 512 x 512 fields of the slide were acquired. Image analysis was performed using Image J software v. 1.49 (Rasband and Bright, 1995) as follows. Scales were set using a reticule-containing image by translating measured distance into pixels (100 µm = 632 pixels). Grey scale images of cells were inverted and the threshold set from 0 to 90. Noise reduction was performed using the erosion operation “Open” to remove isolated pixels followed by watershed segmentation to separate adjacent cells. Finally, the number of cells was determined by analyzing particles with a minimum size of 0.2 µm.

2.2 Analysis of gene expression levels

Expression levels of individual genes and entire transcriptomes were analysed by quantitative real-time PCR (qPCR) and RNA sequencing (RNAseq) respectively. Different approaches were used to purify total RNA and prepare complementary DNA (cDNA) libraries for these two techniques and a summary of the workflows is illustrated in Figure 2-1.

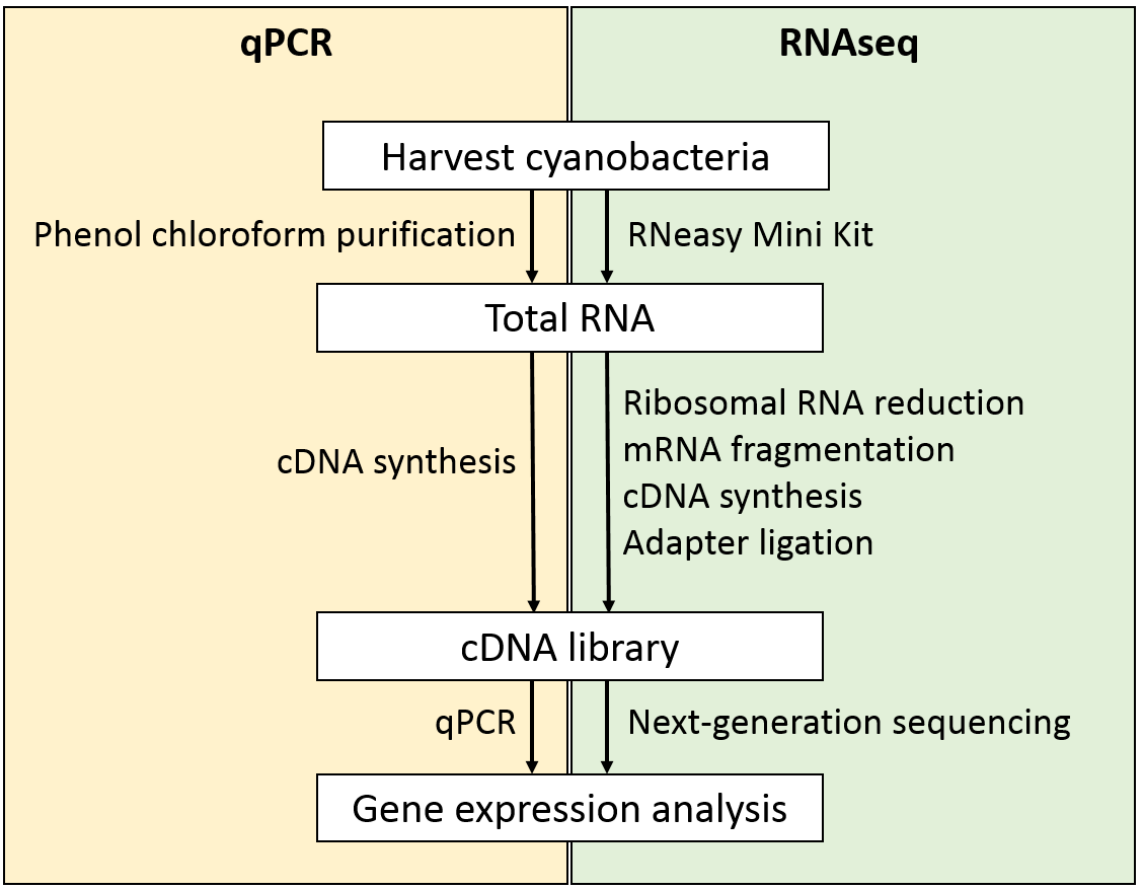


Figure 2-1. Workflow of gene expression analyses

Approaches used to purify total RNA and prepare complementary DNA (cDNA) libraries for gene expression analysis. For RNA sequencing, messenger RNA (mRNA) fragmentation, cDNA synthesis, adapter ligation and sequencing were performed at the Glasgow Polyomics Facility.

2.2.1 Quantitative real-time Polymerase Chain Reaction (qPCR)

2.2.1.1 RNA purification for qPCR

Total RNA was extracted from cell pellets weighing approximately 30 mg. Pellets were resuspended in 250 µl TE buffer (10 mM Tris-HCl, pH 8 with 1 mM EDTA). 0.5 g of 0.5-mm-diameter glass beads, 25 µl 10 % SDS, 250 µl phenol equilibrated with 0.1 M sodium citrate, pH 4.3 and 250 µl chloroform were added. Cells were disrupted using a QIAGEN TissueLyser (QIAGEN, Manchester, UK) with three cycles of 30 Hz for 1 min with 2 min of rest on ice between runs. Liquid phases were then separated by centrifugation at 13,200 rpm for 15 m and the upper aqueous phase was extracted twice with an equal volume of chloroform. RNA was precipitated with 0.1 volume of 10 M LiCl and 2.5 volumes of ice-cold 100 % ethanol overnight. The RNA was washed in 750 µl of 70 % ethanol, vacuum-dried for 10 min in a Concentrator 5301 (Eppendorf, Hamburg, Germany) and resuspended in TE buffer.

2.2.1.2 Reverse-Transcriptase Polymerase Chain Reaction

Genomic DNA was removed from samples containing 1 µg of total RNA with RQ1 RNase-free DNaseI (Promega, Southampton, UK) following the manufacturer's instructions. Reverse transcription of RNA to produce cDNA was performed using the First Strand cDNA Synthesis Kit and random primers following manufacturer's instructions (Thermo Fisher Scientific, Loughborough, UK). cDNA was diluted 1:10 and stored at -20 °C.

2.2.1.3 Generation of standards for qPCR

PCR amplification of standards was performed as described in *Section 2.3.2* (High-precision PCR conditions) using cDNA as template and primers designed for qPCR. PCR products were separated by agarose gel electrophoresis, purified with the QIAquick Gel Extraction Kit (QIAGEN, Manchester, UK) and DNA concentration was determined by spectrophotometry (BioPhotometer plus 6132, Eppendorf, Hamburg, Germany). Purified PCR product concentrations were

adjusted to 1 pg μl^{-1} and ten-fold serial dilutions were performed to produce standard curves for each qPCR run.

2.2.1.4 Quantitative Real-Time Polymerase Chain Reaction (qPCR)

qPCR was performed in a StepOnePlus Real-Time PCR System (Life Technologies, Darmstadt, Germany). Primers listed in Table 2-1 were designed to amplify 100-250 bp products with an annealing temperature of 60 °C using the web-based Primer3 software tool (Koressaar and Remm, 2007, Untergasser et al., 2012). Primers were reconstituted in water to 100 µM and interrogated using Brilliant III Ultra Fast SYBR Green QPCR Master Mix (Agilent, Santa Clara, CA, USA). The reaction setup is described in Table 2-2 and the cycler conditions in Table 2-3. Optimal reference genes were selected using geNorm software (Vandesomepele et al., 2009). Standard curves were included in all the qPCR runs using serial dilutions of gel purified RT-PCR products (see *Section 2.2.2.2*). Negative controls (no template water controls) were included and a melting curve analysis was performed in all assays. qPCRs were performed with minimum three biological replicates of each condition.

Table 2-1. Primers for quantitative RT-PCR

Gene	Primer name	Sequence (5' to 3')	Amplicon (bp)
adk	adk-6803Q-F	CAATCGGCTGTTGGTTTACAC	128
	adk-6803Q-R	CCAATTTTTCCAGGTTGGTGG	
cpcB	6803Q-cpcB-F	CTACACCAGCCGTCGTATGG	101
	6803Q-cpcB-R	CTTCTAGAACGGAAGCGTCG	
isiA	isiA-6803Q-F	CTCTTTTCCGGGGAAGCCAT	136
	isiA-6803Q-R	TTAATTGCCAAAGGCGGGCC	
petB	petB-6803Q-F	CGTCACCACTGTCACCTTTG	126
	petB-6803Q-R	GTCACCAATTGATCCCCAACC	
psbA1	psbA1-6803Q-F	CCTGTGGTCACGGTTCTGTT	152
	psbA1-6803Q-R	TGCCATCAATATCCACCGGG	
pta	pta-6803Q-F	GGCCCTGAATAGCTATGACC	113
	pta-6803Q-R	CGCCATAACGCTGTTCCAG	
rnpB	rnpB-6803Q-F2	GTGAGGACAGTGCCACAGAA	148
	rnpB-6803Q-R2	GATACTGCTGGTGCGCTCTT	
rpoA	rpoA-6803Q-F	AAAGCTACACCGATCAGCCC	146
	rpoA-6803Q-R	CAGTTTGGCCCCTTCTGCTA	
rrn16Sa	rrn16Sa-6803Q-F	AGCGTCCGTAGGTGGTTATG	108
	rrn16Sa-6803Q-R	GGGAATTCCTGCTACCCCTA	
sigA	sigA-6803Q-F	CCCACCGAGGAAGAAATCG	123
	sigA-6803Q-R	GCGGGAGTCCTCTTCTTTC	
sigC	sigC-6803Q-F	GCAGACTCCCAGGATTTGG	124
	sigC-6803Q-R	GCACCTCTTCATCTCGCTTC	
sll7077	sll7077-6803Q-F	GATCGACCTACACCGCTATG	120
	sll7077-6803Q-R	CAAGGGTTAGGATTGCGGTC	
slr9003	slr9003-6803Q-F	CCTATCCCAATGGGTGAATCAA	131
	slr9003-6803Q-R	CCATTTTTGAGATCGCTTCCGT	

Table 2-2. Quantitative RT-PCR reaction setup

Component	Stock concentration	Volume per reaction (µl)
cDNA	1:10	5
Forward primer	10 µM	0.4
Reverse primer	10 µM	0.4
Brilliant III SYBR Mastermix	2 X	10
dH_2O		4.2
Total volume		**20**

Table 2-3. Quantitative RT-PCR thermocycler conditions

Step	Temperature (°C)	Time (s)
1. Initial denaturation	95	180
2. Denaturation	95	10
3. Annealing and extension	60 (*plate read)*	20
Repeat cycles 2-3, 40 times		
4. Melt curve	95	60
	55	30
	95	30

2.2.2 RNA sequencing

2.2.2.1 RNA purification for RNA sequencing

Total RNA was extracted from cell pellets weighing approximately 30 mg with an RNeasy Mini Kit (QIAGEN, Manchester, UK) following the manufacturer's instructions. Messenger RNA (mRNA) enrichment was achieved by removing 16S and 23S ribosomal RNAs (rRNAs) using the MICROBExpress Kit as recommended by the supplier (Ambion, Life Technologies, Darmstadt, Germany). In brief, samples were combined with magnetic beads attached to complementary rRNA

molecules. The beads were separated from the samples using a magnet and enriched RNA containing mRNA, tRNA, 5S rRNA and other small RNAs was recovered. Two rounds of rRNA reduction was required to sufficiently enrich mRNAs as determined using Agilent® 2100 Bianalyzer™ (Agilent, Santa Clara, CA, USA; Figure 2-2).

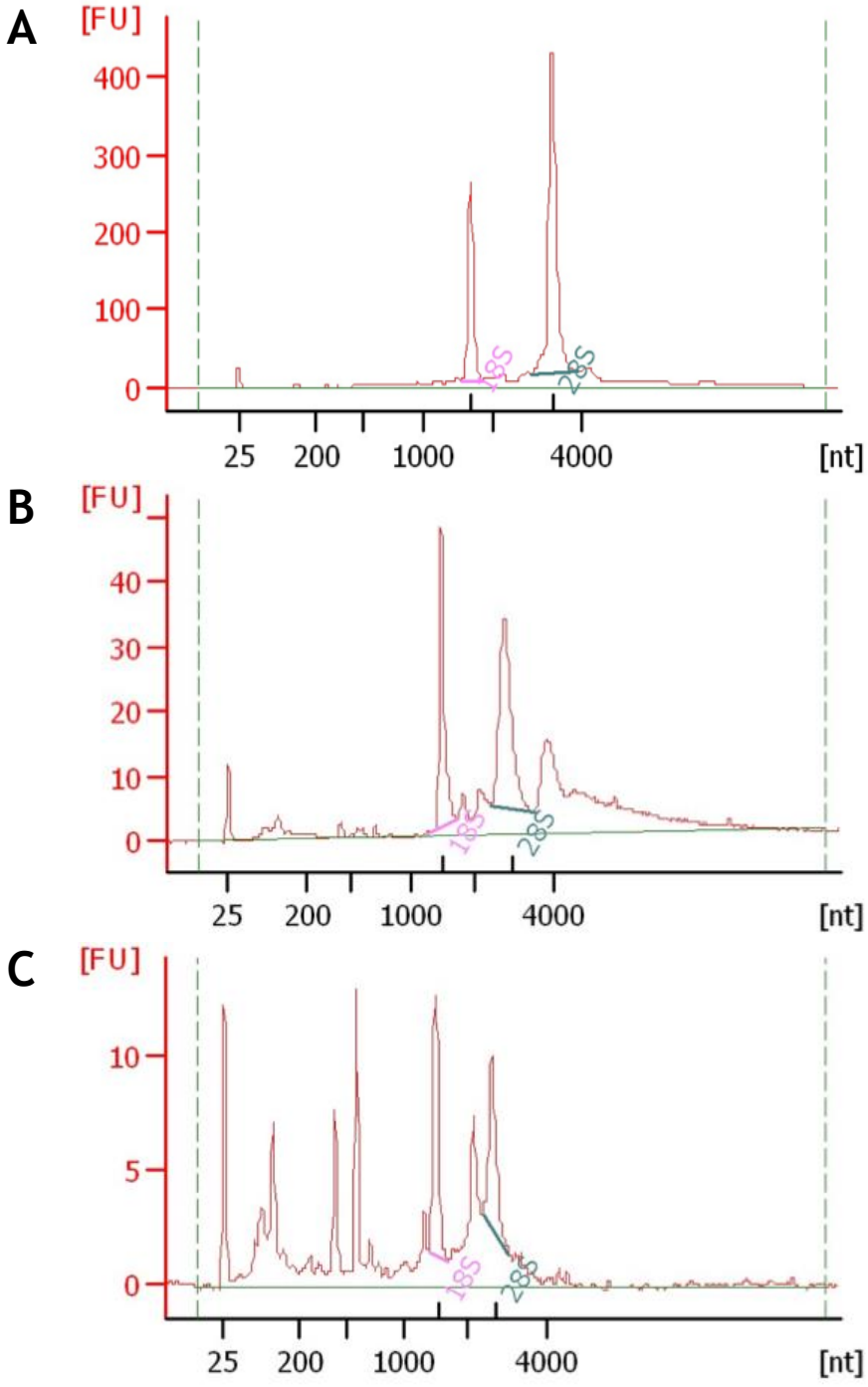


Figure 2-2. Electropherograms of RNA samples following ribosomal RNA reduction

See overleaf for the complete figure legend

Figure 2-2. Electropherograms of RNA ssamples following ribosomal RNA reduction

Electropherograms of RNA harvested from PCC 6803 showing fluorescence intensities (FU) of RNA molecules with differing nucleotide (nt) lengths. **(A)** Total RNA purified with RNeasy Mini Kit. Messenger RNA enrichment following **(B)** one and **(C)** two rounds of ribosomal RNA reduction.

2.2.2.2 RNA quality control

RNA quantity was determined by spectrophotometry (BioPhotometer plus 6132, Eppendorf, Hamburg, Germany). RNA quality was checked by agarose gel electrophoresis. Quality of samples for RNA sequencing was further tested using Agilent® 2100 Bianalyzer™ (Agilent, Santa Clara, CA, USA).

2.2.2.3 RNA sequencing

Enriched mRNA samples (see *Section 2.2.1.2*) were processed at the Glasgow Polyomics for cDNA library preparation and next-generation sequencing. In brief, samples were prepared for sequencing using the TruSeq Stranded mRNA Library Prep Kit as recommended by the supplier (Illumina, San Diego, CA, USA). This involved fragmentation of RNA followed by cDNA synthesis and adapter ligation. cDNA libraries were sequenced using the Illumina MiSeq System (Illumina, USA). Reads were processed and mapped to the PCC 6803 genome as described in Table 6-1.

2.2.3 Statistical analysis

Cuffdiff software was used to calculate expression levels and test statistical significance of observed changes between samples (Trapnell et al., 2012). Multi-dimensional scaling and clustering analyses of the RNA sequencing data was performed using the CummeRbund visualization package for Cuffdiff sequencing data (Trapnell et al., 2012). This package was run using RStudio software v. 3. R scripts to perform multi-dimensional scaling and clustering analyses can be found in Appendices I and II respectively.

2.3 Generation of transgenic material

2.3.1 Isolation of genomic DNA from cyanobacteria

50 ml cyanobacterial culture was harvested during mid-exponential phase growth (approximately OD_{730} 0.3), washed twice and resuspended in 400 µl SSC Buffer (0.15M NaCl, 0.015 M sodium citrate, 0.01M EDTA and 0.5 M sucrose, pH 8.0). After 30 min incubation at 60 °C, lysozyme was added to a final concentration of 2 mg ml^{-1} and incubated for a further 60 min at 37 °C. SDS was added to a final concentration of 0.3 % and incubated at 45 °C for 15 min. SDS was added to a final concentration of 1 % and Proteinase K to final concentration of 100 µg ml^{-1} and incubated overnight at 50 °C. Cells were centrifuged at 13,200 rpm for 10 min and the supernatant was transferred to a fresh 2 ml tube. One volume of chloroform-isoamylalcohol (24:1 volume) was added, mixed by vortexing for 30 s, centrifuged at 13,200 rpm for 1 min and the supernatant was recovered to a new tube. This step was repeated until debris was no longer visible at the interphase. One volume of isopropanol was added, mixed by inversion and centrifuged at 13,200 rpm for 20 min at 4 °C. The supernatant was removed, the genomic DNA pellet was washed with 500 µl of 70 % ethanol and centrifuged at 13,200 rpm for 5 min at 4 °C. The supernatant was removed and the pellet was vacuum-dried for 10 min in a Concentrator 5301 (Eppendorf, Hamburg, Germany). The genomic DNA pellet was resuspended in 100 µl distilled water and stored at -20 °C.

2.3.2 High-precision Polymerase Chain Reaction conditions

All PCR reactions were performed in a peqSTAR 96 universal gradient thermocycler (Peqlab, Erlangen, Germany). The reaction setup is described in Table 2-4 and the cycler conditions in Table 2-5. Primers to amplify promoters from PCC 6803 genomic DNA are presented in Table 2-6. Primers to generate the RBS-GFP library described in Section 3.2.5 are presented in Table 2-7 using the BioBrick plasmid pSB1A2-E0240 carrying the coding sequence for GFPmut3b (kind gift from Prof. Susan Rosser, University of Edinburgh, Edinburgh, UK) as

template. Annealing temperatures were determined with the web-based Northwestern Oligonucleotide Properties Calculator (Kibbe, 2007). Generally, annealing temperatures were set at 3 °C below the salt adjusted melting temperature calculated by the programme. Extension time was chosen according to fragment length: 15 s per kilobase using Phusion High-Fidelity DNA Polymerase (New England Biolabs, Hitchin, UK).

Table 2-4. High-precision PCR reaction setup

Component	Stock concentration	Volume per reaction (µl)
DNA template	50 ng μl^{-1}	2
Forward primer	10 µM	2.5
Reverse primer	10 µM	2.5
dNTPs	10 mM	1
DMSO	100 %	1.5
Phusion® HF Reaction Buffer	5 X	10
Phusion® HF DNA Polymerase	2 units μl^{-1}	0.5
dH_2O		30
Total volume		**50**

Table 2-5. High-precision PCR thermocycler conditions

Step	Temperature (°C)	Time (s)
1. Initial denaturation	95	120
2. Denaturation	95	30
3. Annealing	*Variable*	30
4. Extension	72	*Variable (15 s per kb)*
Repeat cycles 2-4, 35 times		
5. Final extension	72	300

Table 2-6. Primers to amplify promoters from PCC 6803

Promoter	Primer name	Sequence (5' to 3')	Amplicon (bp)
P_{cpcBA}	7002-PcpcBA-F	GTTATAAAATAAACTTAACAAATCTATACCCA CCTGTAGAG	594
	7002-PcpcBA-R	CATGGAATTAATCTCCTACTTGACTTTATGAG TTG	
P_{cpcBA2}	6803-PcpcBA-F2	TTCGTTATAAAATAAACTTAACAAATCTATAC	594
	6803-PcpcBA-R2	GGAATTAATCTCCTACTTGACTTTATG	
P_{rbcL}	6803-PrbcL-F	ATTCTAATTAGAAAGTCCAAAAATTG	235
	6803-PrbcL-R	AAACATTGAATAGCCTAGCTTTCTC	
P_{rnpB}	PrnpB-F	TTCAATGCGGTCCAATACCTCC	208
	PrnpB-R	TTTTTCTAGTGTGCCATTGATTAGAGC	

Table 2-7. Primers to generate RBS-GFP library

Primer name	Sequence (5' to 3')[a]	Amplicon (bp)
RBS6-GFP-F	gaattcgcggccgcttctagagAATATCCGATCATATACACCAC AGGCATAACAAGGatgcgtaaaggagaagaacttttc	935
RBS7-GFP-F	gaattcgcggccgcttctagagTAAAATTACAAAACACTTTTTA GCTAGGACACATatgcgtaaaggagaagaacttttc	934
RBS8-GFP-F	gaattcgcggccgcttctagagACACCTATAAGATCCTAGGGA TAAAAAGATATCatgcgtaaaggagaagaacttttc	934
RBS9-GFP-F	gaattcgcggccgcttctagagAGAATTACATATCGCACAAAG GTATATAAAAatgcgtaaaggagaagaacttttc	934
Suffix-R[b]	tactagtagcggccgctgcag	

[a] BioBrick prefix sequence is underlined, RBS is in uppercase and start of GFP coding sequence is in lowercase

[b] Reverse primer for PCR amplification of each member in the RBS-GFP library

2.3.3 Standard cloning techniques

2.3.3.1 Subcloning of PCR products

When PCR products were to be used for downstream cloning, products were separated on an agarose gel and purified with the QIAquick Gel Extraction Kit

(QIAGEN, Manchester, UK). A single adenine nucleotide was added to the 3' ends of the purified PCR fragments using GoTaq® DNA polymerase (Promega, Southampton, UK). The reaction setup is described in Table 2-8 and the cycler conditions in Table 2-9. The adenylated PCR fragments were then cloned into the pGEM-T® Easy (Promega, UK) vector according to the manufacturer's instructions.

Table 2-8. Adenylation of 3' ends of purified PCR fragments reaction setup

Component	Stock concentration	Volume per reaction (µl)
Purified PCR product	*Variable*	10.8
Colourless GoTaq® Reaction Buffer	5 X	3
dATP	10 mM	0.2
GoTaq® DNA Polymerase	2 units μl^{-1}	1
Total volume		**15**

Table 2-9. Adenylation of 3' ends of purified PCR fragments thermocycler conditions

Step	Temperature (°C)	Time (min)
1. Denaturation	95	3
2. Adenylation	70	30

2.3.3.2 Restriction digests

Restriction digestion reaction mixtures were set up as described in Table 2-10 and incubated for 1 h at 37 °C. Restriction products were separated by agarose gel electrophoresis and appropriate DNA bands were purified with the QIAquick Gel Extraction Kit (QIAGEN, Manchester, UK).

Table 2-10. Restriction digestion reaction setup

Component	Stock concentration	Volume per reaction (µl)
Plasmid DNA	500 ng μl^{-1}	26
Restriction enzyme(s)	20 units μl^{-1}	0.5
NEBuffer	10 X	3
dH_2O		*up to 30 µl*
Total volume		**30**

2.3.3.3 Annealing of synthetic oligonucleotides

Short fragments of synthetic double-stranded DNA were used to introduce BioBrick restriction sites to pAQ1Ex. Complementary primers listed in Table 2-11 were designed to generate restriction site overhangs for insertion to digested plasmid as illustrated in Figure 2-3. Forward and reverse primers were combined in water to a final concentration of 10 µM, boiled for 5 min at 95 °C and allowed to gradually cool to room temperature. Annealed primers were immediately used in a ligation reaction.

Table 2-11. Primers to generate synthetic double-stranded DNA

Primer name	Sequence (5' to 3')[a]	5' site	3' site
pAQ-BBPre-F	AATTCGCGGCCGCTTCTAGAGGC	EcoRI	NotI
pAQ-BBPre-R	GGCCGCCTCTAGAAGCGGCCGC		
pAQ-BBSuf-F	TATGTACTAGTAGCGGCCGCTGCAGG	NdeI	BamHI
pAQ-BBSuf-R	GATCCCTGCAGCGGCCGCTACTAGTAC A		

[a] Inserted sequences are underlined

2.3.3.4 Ligation reactions

Vector DNA concentration was adjusted to 50 ng μl^{-1} and Equation 1 was used to determine the appropriate amount of Insert DNA for an optimal 3:1 molar ratio of vector to insert for the ligation reaction. Ligations were performed with T4 DNA Ligase (New England Biolabs, Hitchin, UK) as described in Table 2-12.

Ligation reactions were incubated at 16 °C overnight. *E. coli* were subsequently transformed with the total ligation reaction mixture.

Equation 1

$$\frac{\text{ng of Vector x kb size of Insert}}{\text{kb size of Vector}} \text{ x 3:1 molar ratio = ng of Insert}$$

Table 2-12. Ligation reaction setup

Component	Stock concentration	Volume per reaction (µl)
Vector DNA	50 ng μl^{-1}	1
Insert DNA	*Variable*	*Variable*
T4 DNA Ligase Buffer	10 X	2
T4 DNA Ligase	400 units μl^{-1}	1
dH_2O		*Up to 20 µl*
Total volume		**20**

2.3.3.4 Site-directed mutagenesis (SDM)

Two overlapping primers for site-directed mutagenesis (Table 2-13) were designed using SDM-Assist software (Karnik et al., 2013). The high-fidelity PCR reaction was set up in two individual reactions, one for each primer. Following 10 rounds of thermal cycling, the two reaction mixtures were combined and subjected to a further 15 rounds of thermal cycling. Prior to transformation of *E. coli*, the PCR products were digested with DpnI, an enzyme that degrades methylated DNA thus selectively removing the original plasmid template. The total PCR reaction mixture was combined with 2 µl (approximately 4000 units) of DpnI (New England Biolabs, Hitchin, UK) at 37 °C overnight. *E. coli* was subsequently transformed with 5 µl of this reaction mixture.

Table 2-13. Primers for site-directed mutagenesis

Primer name	Sequence (5' to 3')[a]
pAQ1b-XSDM-F	GGTTTTTTGCTCGAGCCGAACGCAGCG
pAQ1b-XSDM-R	GCGTTCGGCTCGAGCAAAAAACCCGTC

[a] Substituted sequences are underlined

2.3.4 Transformation of Escherichia coli

2.3.4.1 Generation of chemically competent Escherichia coli

E. coli cells (TOP10 strain) were streaked for single colonies and cultured overnight in 5 ml LB broth supplemented with 50 µg ml^{-1} streptomycin in a shaker at 37 °C and 180 rpm. 0.5 ml of the liquid overnight culture was added to 20 ml fresh LB broth supplemented with streptomycin and incubated for a further 90 min at 37 °C and 180 rpm. The cells were harvested at OD_{600} 0.4 by centrifugation at 10,000 rpm for 1 min at 4 °C. The supernatant was discarded and the cell pellet was resuspended in 10 ml ice cold 50 mM $CaCl_2$. The cells were pelleted by centrifugation at 10,000 rpm for 1 min at 4 °C, the supernatant was discarded and the pellet was resuspended in 1 ml ice cold 50 mM $CaCl_2$. 1 ml of glycerol was added and the mixture was divided into 75 µl aliquots and stored at -80 °C.

2.3.4.2 Transformation of Escherichia coli

75 µl aliquots of chemically competent *E. coli* cells were thawed on ice. 1 µl of plasmid DNA or ligation reaction mixture was added and incubated on ice for 30 min. The mixture was incubated in a water bath equilibrated at 42 °C for 45 s and returned to ice for a further 2 min. 230 µl of sterile LB medium was added and the mixture was incubated in a shaker at 37 °C and 180 rpm for 90 min. The whole suspension was plated on LB plates containing an appropriate antibiotic (see Table 2-14) and incubated at 37 °C overnight. Single colonies were picked and re-cultivated overnight in liquid LB medium containing appropriate

antibiotics in a shaker at 37 °C and 180 rpm. These cultures were used for plasmid DNA purification.

Table 2-14. Antibiotic concentrations for Escherichia coli

Antibiotic	Final concentration (µg ml^{-1})
Ampicillin	100
Chloramphenicol	30
Kanamycin	50
Streptomycin	50

2.3.5 Plasmid DNA purification from Escherichia coli

E. coli cells from a liquid overnight culture were pelleted in a 2 ml tube by centrifugation for 1 min at 13,200 rpm. The supernatant was discarded and the procedure was repeated until all the cells were pelleted. The bacterial pellet was resuspended in 400 µl of Miniprep Solution 1 (Table 2-15) by vortexing. 400 µl of Miniprep Solution 2 (Table 2-15) was added and the sample was immediately mixed by inversion of the tube. After 3 min incubation at room temperature, 400 µl of Miniprep Solution 3 (Table 2-15) was added to the sample, mixed by inversion and centrifuged for 10 min at 13,200 rpm. 1000 µl of the supernatant was transferred to a fresh 2 ml tube, 1000 µl of chloroform-isoamylalcohol (24:1 volume) was added and the sample was vortexed for 30 s before centrifugation for 1 min at 13,200 rpm. The upper phase was recovered to a fresh 2 ml tube and chloroform purification was repeated once more. An equal volume of isopropanol was then added to the recovered upper phase, mixed by inversion and centrifuged at 13,200 rpm for 20 min at 4 °C. The supernatant was removed, the plasmid DNA pellet was washed with 750 µl 70 % ethanol and centrifuged at 13,200 rpm for 5 min at 4 °C. The supernatant was removed and the pellet was vacuum-dried for 10 min in a Concentrator 5301 (Eppendorf, Hamburg, Germany). The plasmid DNA pellet was resuspended in 100 µl of distilled water and stored at -20 °C. Plasmid DNA concentration was quantified using a BioPhotometer plus 6132 (Eppendorf, Hamburg, Germany).

Table 2-15. Solutions for plasmid DNA purification

	Reagent	Concentration
Miniprep Solution 1	Tris-HCl	50 mM
	EDTA	10 mM
	RNase A	10 µg ml^{-1}
	Lysozyme	0.01 %
	pH adjusted to 8.0	
Miniprep Solution 2	NaOH	0.2 M
	SDS	1 %
Miniprep Solution 3	Potassium acetate	3 M
	pH adjusted to 5.5 with acetic acid	

2.3.6 Plasmid verification in Escherichia coli

To verify constructs, plasmid DNA was digested in restriction digest reactions set up as described in Table 2-16 and incubated for 1 h at 37 °C. The restriction products were separated by agarose gel electrophoresis and DNA band patterns were compared to *in silico* patterns generated with Vector NTI software (Life Technologies, Darmstadt, Germany).

Table 2-16. Reaction digestion test reaction setup

Component	Stock concentration	Volume per reaction (µl)
DNA template	500 ng $µl^{-1}$	2
Restriction enzyme(s)	20 units $µl^{-1}$	0.1
NEBuffer	10 X	1
dH_2O		*up to 10 µl*
Total volume		**10**

Construct sequences were also verified by sequencing at an external facility (GATC, Konstanz, Germany). Sequencing primers used are listed in Table 2-17. Sequencing reactions usually produced high quality reads of approximately 700 – 1200 base pairs.

Table 2-17. Primers for sequencing

Primer name	Sequence (5' to 3')
VF2	TGCCACCTGACGTCTAAGAA
VR	ATTACCGCCTTTGAGTGAGC
M13-FP	TGTAAAACGACGGCCAGT
M13-RP	CAGGAAACAGCTATGACC
pAQ1-seq-F	CACATGAGAATTTGTCCAG
pAQ1BB-seq-R3	GTGGGTCGATGTTTGATG

2.3.7 Natural transformation of cyanobacteria

PCC 7002 was grown to OD_{730} 1-1.5 under standard conditions (see *Section 2.1.1.2*). 1.5 ml cells were combined with 1 µg DNA and incubated for 24 h in LD at 30 °C with very slow sparging with humidified ambient air. Cells were pelleted at 4000 rpm for 1 min and resuspended in 100 µl Medium A+, spread on Medium A+ plates containing spectinomycin (50 µg ml^{-1}) and incubated under standard conditions (see *Section 2.1.2*).

2.3.8 PCR verification of transformed cyanobacteria

Single colonies were cultured in 20 ml liquid Medium A+ supplemented with 25 µg ml^{-1} spectinomycin for 4 days under standard conditions. 400 µl culture was pelleted by centrifugation at 13,200 rpm for 3 min and the supernatant was discarded. The pellet was resuspended in 200 µl of TE buffer supplemented with 1 % Triton 100X. Samples were boiled for 3.5 min at 95 °C, an equal volume of chloroform was added, the mixture was vortexed for 30 s and the upper aqueous phase was extracted twice. 1 µl of the purified aqueous phase was immediately

used as a template for PCR reactions using GoTaq® DNA Polymerase (Promega, Southampton, UK) detailed in Table 2-18 and the thermal cycling conditions are described in Table 2-19. Primers typically consisted of the forward primer used to amplify the promoter from PCC 6803 and a reverse primer consisting of the complemented BioBrick suffix (Suffix-R; 5'-TACTAGTAGCGGCCGCTGCAG-3').

Table 2-18. PCR verification of cyanobacterial transformants reaction setup

Component	Stock concentration	Volume per reaction (µl)
Template	*Variable*	1
Forward primer	10 mM	0.2
Reverse primer	10 mM	0.2
Green GoTaq® Flexi Buffer	5 X	2
$MgCl_2$	25 mM	0.6
dNTPs	10 mM	0.1
GoTaq® DNA Polymerase	5 units μl^{-1}	0.1
dH_2O		5.8
Total volume		**10**

Table 2-19. PCR verification of cyanobacterial transformants thermocycler conditions

Step	Temperature (˚C)	Time (s)
1. Initial denaturation	95	120
2. Denaturation	95	30
3. Annealing	*Variable*	30
4. Extension	72	*Variable (60 s per kb)*
Repeat cycles 2-4, 35 times		
5. Final extension	72	300

2.4 Quantification of reporter gene activity

Fluorescent reporter activity was quantified mid-way through the day cycle, i.e. 8 h after the lights turn on in LD. Optical density of cyanobacterial cultures was measured at 730 nm (OD_{730}) in a Lambda 45 UV/VIS Spectrophotometer (PerkinElmer, Cambridge, UK). The optical density of the cultures was adjusted to OD_{730} 0.3 in fresh media and fluorescence was measured using a LS 55 Luminescence Spectrophotometer (PerkinElmer, Cambridge, UK) using the excitation and emission wavelengths described in Table 3-1.

Chapter 3. Developing a standardised promoter assay

3.1 Introduction

Synechococcus spp. PCC 7002 (formerly "Agmenellum quadruplicatum PR-6"; hereafter PCC 7002) is an attractive chassis for practical applications. It has achieved the highest growth rates (Minas et al., 2014) as well as the highest glycogen production rates (Aikawa et al., 2014) reported for cyanobacteria in the literature to date. In addition, PCC 7002 is a unicellular marine cyanobacterium able to grow across a wide range of salinities (Batterton and Van Baalen, 1971) and thus does not compete for either limited fresh water or arable land resources. It is tolerant of extreme high light (Nomura et al., 2006b), capable of photohetertrophy (Lambert and Stevens, 1986), easily naturally transformed (Stevens and Porter, 1980, Frigaard et al., 2004) and the genome is fully sequenced (http://genome.microbedb.jp/cyanobase/SYNPCC7002; last accessed 20/09/2015). Compared to other model species of cyanobacteria, there are relatively few tools available to engineer PCC 7002. Promoters to drive gene expression in this organism are particularly limited (Heidorn et al., 2011, Berla et al., 2013, Wang et al., 2012).

Orthologous expression systems are desirable to control engineered processes as they prevent interference from host transcriptional machinery. However, inducible systems from *E. coli* and bacteriophages have proven relatively inefficient in cyanobacteria (Huang et al., 2010, Huang and Lindblad, 2013, Camsund et al., 2014, Zhou et al., 2014). These differences in promoter efficiency have been attributed to differences in RNA polymerase, such as the split ß' subunit which occurs in two parts in cyanobacteria as γ and ß' (Schneider and Hasekorn, 1988, Xie et al., 1989). Additionally, the absence of σ^{54}-family sigma factors in cyanobacteria as well as differences between existing σ^{70} factors further impact promoter efficiency. Heterologous expression systems derived from more closely-related organisms (i.e. other species of

cyanobacteria) in which RNA polymerase core enzyme is conserved may therefore be more successful. Transcriptional isolation of expression constructs driven by heterologous cyanobacterial promoters may possible given the variation of sigma factors responsible for promoter recognition across cyanobacterial species (Imamura and Asayama, 2009). To date, the vast majority of promoters have been characterised in their native settings, most commonly the freshwater model *Synechocystis* spp. PCC 6803 (hereafter PCC 6803). Given the enormous diversity of cyanobacteria as well as the variation in transcriptional machinery, it is important to confirm that the native promoter behaviour is maintained in heterologous systems.

Characterisation of biological components is a key enabling principle in synthetic biology. The relative strength and behaviour of promoters are important considerations in the rational design of engineered systems. Quantitative approaches to characterise promoter activity and compatibility with widely available standardised biological parts enables rapid assembly and optimisation of rationally designed gene expression cassettes. A standardised promoter assay was originally developed in *E. coli* using BioBrick parts (Kelly et al., 2009). Promoters of interest were cloned upstream of a green fluorescent protein (GFP) reporter in a plasmid vector and promoter activity was measured in transformed *E. coli* cultures based on the fluorescence intensities of GFP. Furthermore, promoter activity was measured relative to the activity of a standard reference promoter in relative promoter units. This promoter assay has been adapted for application in PCC 6803 (Huang et al., 2010). In this case, promoter-GFP modules were introduced to PCC 6803 on a broad-host-range plasmid vector, pPMQAK1, and promoter activity was measured relative to the promoter of RNase P subunit B (P_{rnpB}) from PCC 6803. Standardised approaches to characterise promoter activity have not been described for other species of cyanobacteria.

3.1.1 Chapter aim

This work aimed to adapt the standardised BioBrick promoter assay for application in *Synechococcus* spp. PCC 7002. Several factors must be considered when translating such approaches between biological systems. First, a plasmid vector was developed to introduce BioBrick promoter-reporter modules to PCC 7002. Second, a reporter protein was required to accurately quantify promoter activity. Third, a library of ribosome binding sites was generated to drive stable translation rates of reporter protein and detect a wide range of transcriptional activity. Finally, the activity of published promoters from PCC 6803 were assessed using the promoter assay in PCC 7002. This assay will enable thorough and accurate characterisation of promoter activity for predictable engineering of PCC 7002 using characterised promoters in an “off-the-shelf” manner.

3.2 Results

3.2.1 PCC 7002 culture conditions

The Laboratory for Plant Physiology and Biophysics had not previously cultured cyanobacteria, so a system for cultivating cyanobacteria required development. The overall aim was to develop a relatively simple system using common laboratory materials that could be easily reproduced in other laboratories. *Synechococcus* spp. PCC 7002 is a marine cyanobacterium and the chemically defined A+ Media was selected for its cultivation. The optimal temperature for growth of PCC 7002 is 38 °C however it is able to grow from a range of 29-43 °C (Stanier et al., 1971, Van Baalen, 1962). An ambient temperature of 30 °C was selected to allow the ability to culture alongside other species of cyanobacteria, particularly *Synechocystis* spp. PCC 6803 which has an optimal temperature of 30 °C (Sheng et al., 2011). PCC 7002 growth rates remain unchanged across a range of light intensities from 100 to 700 μE m^{-2} s^{-1} (Nomura et al., 2006a), so cultures were illuminated with a moderate intensity of 120 μE m^{-2} s^{-1}. In order to maximise growth rates whilst maintaining normal circadian rhythm, cultures were illuminated with a 16/8 h day/night photoperiod. Growth rates increase with aeration of increasing CO_2 concentrations of up to 4 % (Aikawa et al., 2014) however to minimise costs, cultures were sparged with ambient air (0.04 % CO_2) using an aquarium pump. In addition, sparging provided continuous agitation of the cultures. In order to prevent evaporation of the cultures and concentration of media components, the air was humidified with distilled water by sparging through a Büchner flask (Figure 3-1A). Rapid screening of promoters was performed in 20 ml cultures maintained in glass culture tubes aerated using a rubber stopper through which air was directed to the bottom of the culture using a Pasteur pipette (Figure 3-1B). Analysis of promoter activity throughout culture growth required larger cultures of 150 ml maintained in Erlenmeyer flasks aerated in a similar manner (Figure 3-1C). The developed growth system proved to be functional for producing reproducible fast growth of PCC 7002 (see later Figure 3-9A).

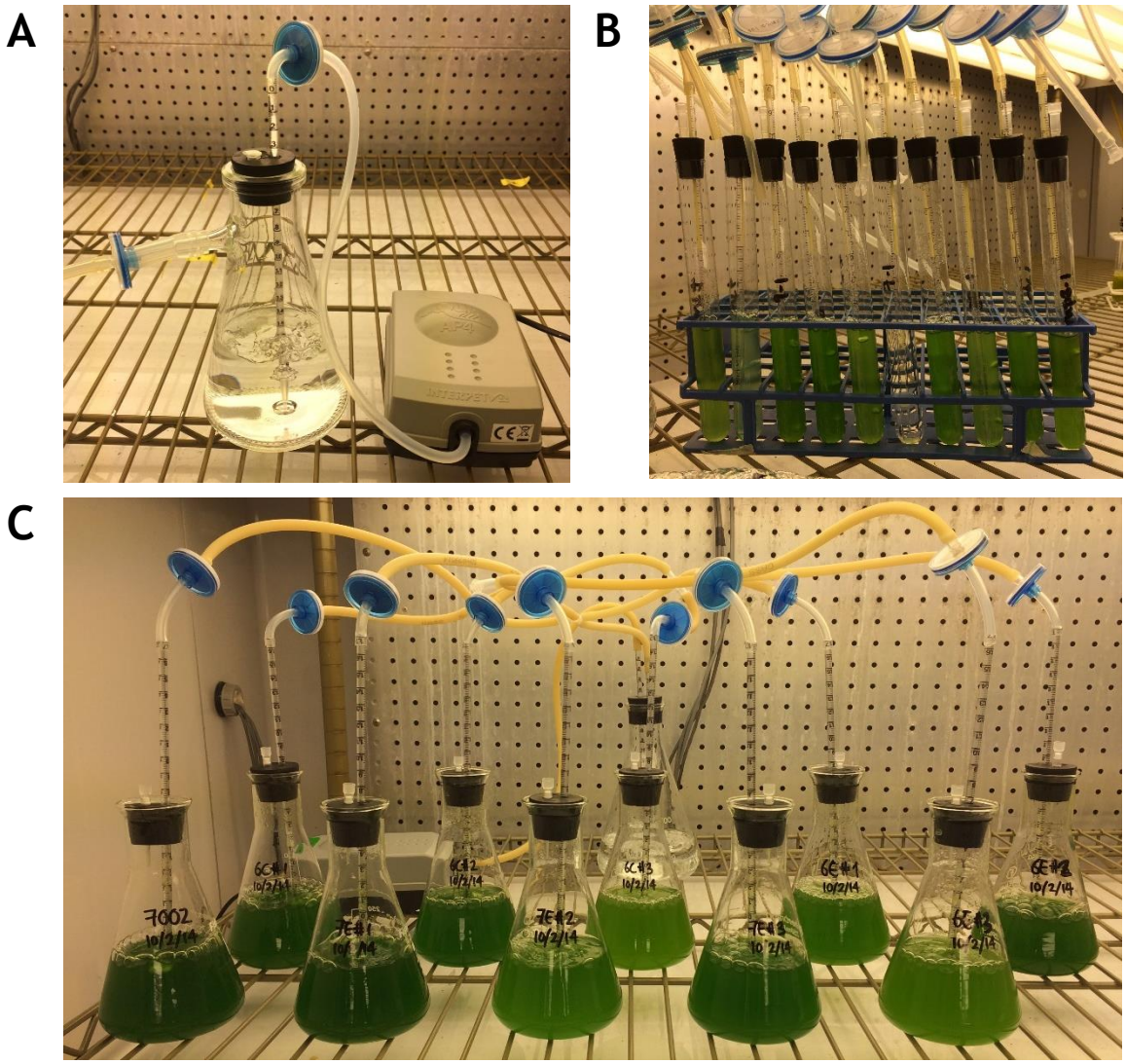


Figure 3-1. Culture conditions of *Synechococcus* spp. PCC 7002

PCC 7002 were cultivated in chemically defined A+ Media and maintained in a 30 °C growth chamber. The cultures were illuminated with a 16/ 8 h day/night photoperiod at 120 µE m^{-2} s^{-1}. Cultures were sparged with ambient air from an aquarium pump and humidified with distilled water through a Büchner flask **(A)**. Rapid screening was performed in 20 ml cultures **(B)** and longer-term growth experiments in 150 ml cultures **(C)**.

3.2.2 Plasmid vector for PCC 7002 transformation

Transformation protocols are established for PCC 7002 (Xu et al., 2011, Frigaard et al., 2004) and a variety of plasmid vectors have been developed for stable transgene integration in this strain (Begemann et al., 2013, Ruffing, 2014, Jacobsen and Frigaard, 2014, Xu et al., 2011). However, none of these vectors are compatible with standardised biological components for synthetic biology applications. It was therefore important to develop a vector compatible with available registries of standardised parts for rapid assembly. To date, the largest and most widely used library is the Registry of Standard Biological Parts comprised of BioBricks (Knight, 2003, Shetty et al., 2008, Kahl and Endy, 2013). These standardised components are flanked by unique restriction sites that reside in prefix and suffix sequences either side of the biological part (see Figure 1-4).

A well-described expression system with a published protocol was selected for development (Xu et al., 2011) and was received as a kind gift from Prof. Donald Bryant, Pennsylvania State University, PA, USA. This system utilises a suicide vector, pAQ1Ex (Figure 3-2A), which carries an f1 phage replication origin only active in *E. coli*. Two regions, Flank A and Flank B, occur either side of the cloning site for stable integration of transgenes within a neutral site on the pAQ1 plasmid, native to PCC 7002, by homologous recombination. The cloning site carries the phycocyanin promoter (P_{cpcBA}) from PCC 6803, a gene encoding enhanced yellow fluorescence protein (eYFP) and a selectable marker gene conferring spectinomycin resistance (*aadA*). Unique restriction sites flank each element within the cloning site: EcoRI and NcoI flank the promoter, NdeI and BamHI flank eYFP and XbaI and SalI flank the marker gene.

In order to be compatible with the Registry of Standard Biological Parts, the signature BioBrick prefix and suffix sequences were introduced directly upstream and downstream of the transgene insertion site respectively. These elements were synthesised by annealing synthetic oligonucleotides to generate double-stranded DNA flanked by restriction site overhangs for insertion to pAQ1Ex (see *Section 2.3.3.3)*. The prefix was inserted between EcoRI and NcoI and the suffix

between NdeI and BamHI restriction sites. This resulted in the presence of EcoRI and XbaI upstream of the cloning site and SpeI and PstI downstream.

The BioBrick standard requires domestication of standardised parts, i.e. the removal of recognition sequences for the restriction enzymes involved in assembly occurring outwith the prefix and suffix sequences. One restriction site, XbaI, was present upstream of the selectable *aadA* marker gene, so site-directed mutagenesis was performed to convert this restriction site to an XhoI site and the resulting vector backbone was designated pAQ1BB. The constitutive promoter P_{cpcBA} was cloned upstream of the BioBrick part BBa_E0040 encoding green fluorescent protein (GFP) using the new BioBrick restriction sites to generate pAQ1BB_P_{cpcBA}_GFP (Figure 3-2B). Successful construction was verified by restriction digestion (Figure 3-2C) and sequencing. The complete nucleotide sequence of pAQ1BB_P_{cpcBA}_GFP can be found in Appendix III.

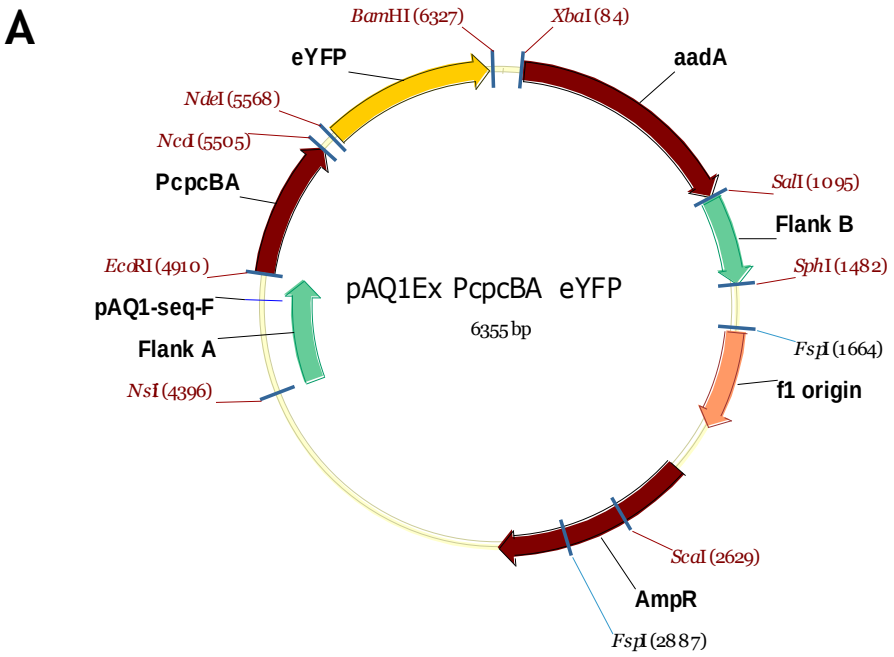
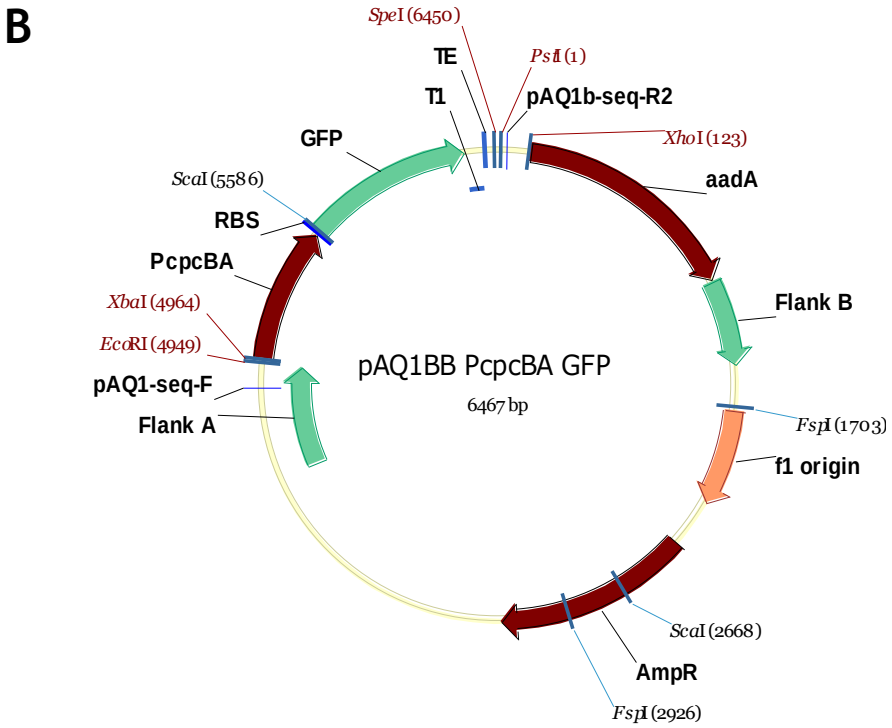


Figure 3-2. Plasmid vectors for transformation of *Synechococcus* spp. PCC 7002

see overleaf for the rest of the figure and the complete figure legend



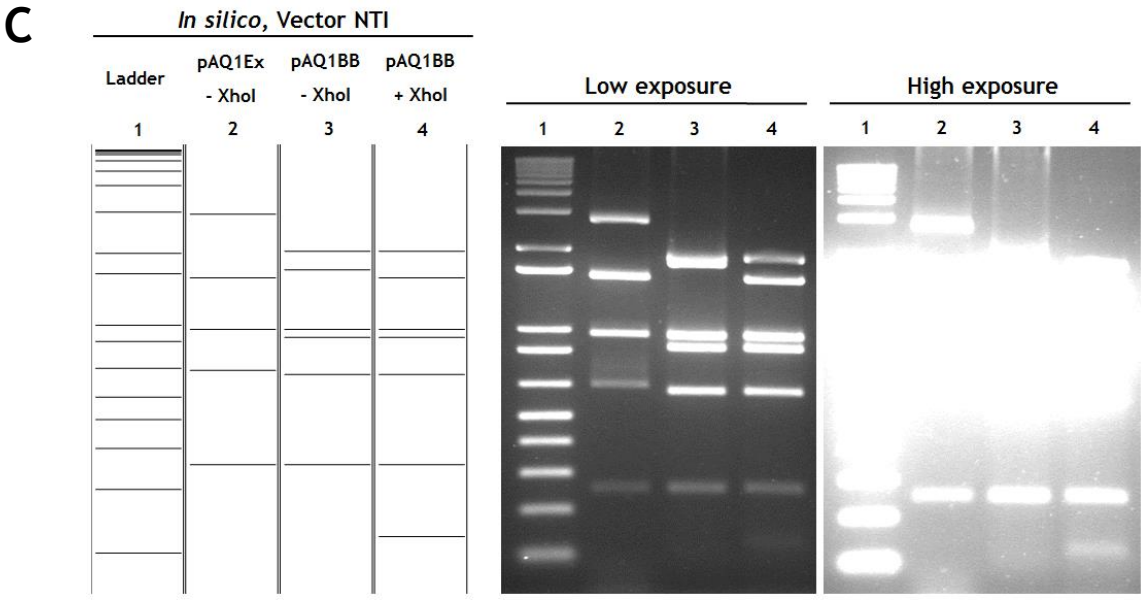


Figure 3-2. Plasmid vectors for transformation of *Synechococcus* spp. PCC 7002

(continued from previous page)

Plasmid maps of **(A)** pAQ1Ex_P_{cpcBA}_eYFP and **(B)** pAQ1BB_P_{cpcBA}_GFP. **(C)** Plasmids were verified by restriction digestion with FspI, PstI, ScaI and XbaI with or without XhoI (indicated). *In silico* restriction digest banding patterns were predicted using Vector NTI software and low and high exposures of the same DNA agarose gel are presented. 1 kb Plus DNA ladder in Lane 1. pAQ1Ex_P_{cpcBA}_eYFP in Lane 2. pAQ1BB_P_{cpcBA}_GFP in Lanes 3 and 4.

3.2.3 Reporters to monitor promoter activity

The identification of the green fluorescent protein from the jellyfish *Aequorea victoria* revolutionised studies in molecular biology (Tsien, 1998). Fluorescent proteins absorb light at one wavelength, transform this energy and emit a photon at a second wavelength of lower energy state. The non-invasive nature of fluorescent reporters enables rapid, real-time monitoring and quantification of varied activities in living cells. Two fluorescent reporter proteins were assessed in PCC 7002: green fluorescent protein (GFP) and enhanced yellow fluorescent protein (eYFP). Expression of these genes was driven by the phycocyanin promoter from PCC 6803, P_{cpcBA}, and constructs were introduced to PCC 7002 by natural transformation using the vectors described in *Section 3.2.2*. The fluorescent activity of each reporter was detected in transformed cultures diluted to the same optical density to normalise for differences in cell number and comparisons of excitation and emission spectra are presented for eYFP and GFP in Figure 3-3. eYFP signal saturated using standardised detection settings so alternative wavelengths were used to measure eYFP fluorescence (Table 3-1). By contrast, GFP detected using standardised settings generated a relatively weak signal. While both of these reporters are readily detected in transformed cultures of PCC 7002, the signal obtained by the reporters relative to the detection limit of the spectrophotometer provides narrow windows in which promoter strength can be compared (Figure 3-4).

GFP was selected for further development based on the following reasoning. Firstly, GFP is a standardised BioBrick part flanked by the BioBrick prefix and suffix for rapid assembly. Secondly, none of the signature restriction sites are present in the gene coding sequence in contrast to the gene encoding eYFP in which a PstI site is present. Thirdly, this is an unstable variant of GFP that has a LVA degradation tag for rapid and accurate detection of changes in promoter activity.

Table 3-1. Detection settings for fluorescent reporters

Reporter	Excitation (nm)	Emission (nm)
GFP	480	514
eYFP (optimal settings)	514	527
eYFP (this study)	460	514

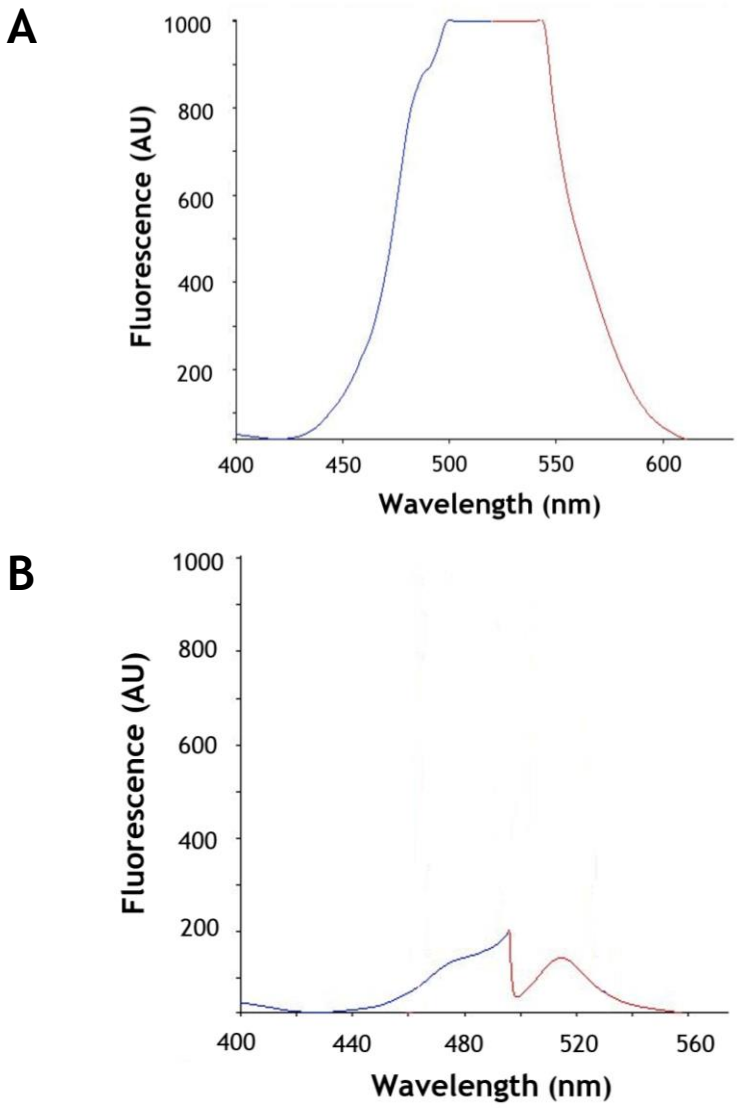


Figure 3-3. Fluorescent reporter excitation and emission spectra

PCC 7002 were transformed with eYFP or GFP driven by the phycocyanin promoter, P_{cpcBA}. Liquid cultures were grown to approximately OD_{730} 1 and then diluted to OD_{730} 0.3 to normalise for differences in cell number. Excitation (blue) and emission (red) spectra are presented for **(A)** eYFP and **(B)** GFP in PCC 7002 background.

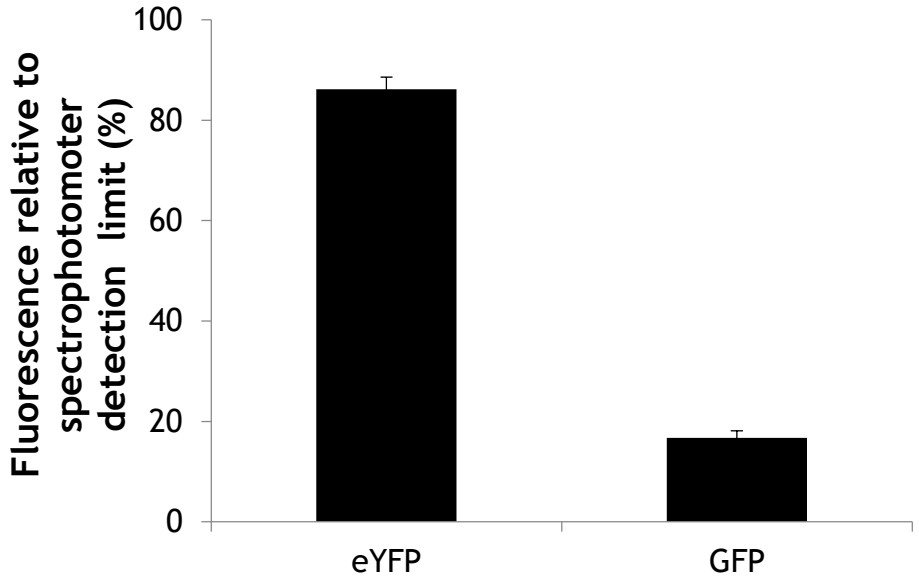


Figure 3-4. Relative reporter activity in PCC 7002

PCC 7002 transformed with eYFP or GFP driven by the phycocyanin promoter, P_{cpcBA}. Liquid cultures were grown to approximately OD_{730} 1 and then diluted to OD_{730} 0.3 to normalise for differences in cell number. Fluorescence was detected in PCC 7002 background using standardised settings for GFP and modified settings for eYFP (Table 3-1) and is presented as a percentage relative to the detection limit of the fluorescent spectrophotometer.

3.2.4 Ribosome binding sites for translation initiation

A large difference in fluorescence signal strength was observed for the two reporters assessed in the previous section although the transcription of the genes encoding these reporters was driven by the same promoter, P_{cpcBA}. One possible explanation is the difference in ribosome binding sites (RBSs) located directly upstream of the reporter coding sequences. The RBS is involved in translation initiation during protein synthesis and could therefore have a direct effect on reporter signal. RBS efficiency depends on multiple factors including secondary mRNA structure influenced by flanking nucleotide sequences, the Shine-Dalgarno (SD) sequence complementary to the 16S ribosomal RNA, spacing between the SD and start codon, as well as the sequence of the start codon itself (Reeve et al., 2014). *In silico* translation rate calculators estimate translation initiation rates

(TIRs) based on these factors and a number of tools have been developed for this purpose including the Salis Lab RBS Calculator (Salis et al., 2009), RBS designer (Na and Lee, 2010) and UTR designer (Seo et al., 2013). All three tools yield high accuracy levels in *E. coli*. The Salis RBS calculator was selected for this study, as it is the most frequently updated model (Reeve et al., 2014). Analysis of promoter-reporter modules revealed two sites at which translation initiation may occur: an ATG start codon at the 3' end of the P_{cpcBA} promoter and at the start codon at the 5' end of the reporter coding sequences (Figure 3-5). Overall, predicted TIRs are higher in the eYFP module compared with the GFP module reflecting the difference observed in signal generated by the two reporters. It is important to note that, particularly in the case of eYFP, translation initiation is predicted to occur from the promoter.

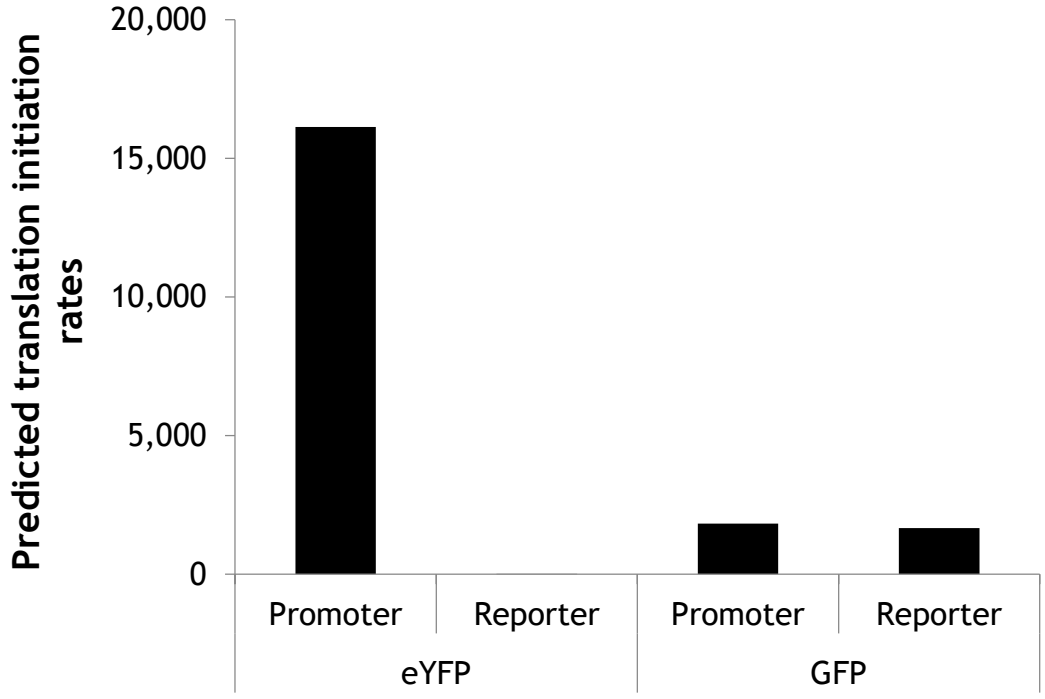


Figure 3-5. Predicted translation initiation rates for promoter-reporter modules

Bar charts show *in silico* translation initiation rates predicted using the Salis Lab RBS calculator at the 3' end of the P_{cpcBA} promoter and the 5' end of the gene encoding either eYFP or GFP.

It is important to separate transcriptional and translational activation and furthermore maintain translation at constant levels in order to accurately quantify changes in transcriptional activity. Therefore, test promoters should not contain a start codon from which translation initiation may occur. Furthermore, RBSs must be developed to drive constant translation rates of the reporter irrespective of the upstream promoter sequence. In order to address the first issue, the P_{cpcBA} promoter was PCR amplified using primers to exclude the 3' ATG start codon and this new promoter was designated P_{cpcBA2}. The next step was to design a library of synthetic RBSs spanning a wide range of TIRs so the activity of weak promoters could be detected using strong RBSs and vice versa. A RBS library (Table 3-2) was forward engineered using the web-based Salis Lab RBS calculator. This RBS calculator requires four pieces of information in order to generate the synthetic RBS sequences. First, the DNA sequences occurring either side of the RBS must be specified. The sequence occurring directly upstream of the RBS is designated the "pre-sequence". In order to maintain stable translation rates irrespective of the upstream sequence, the pre-sequence was defined as the mixed XbaI/SpeI sequence, TACTAGAG, generated by ligation of promoter-reporter modules. Second, the DNA sequence occurring directly downstream of the RBS is designated the "protein coding sequence", which in this case was the full length GFP coding sequence spanning from the ATG start codon to the TAA stop codon. Third, the target TIR must be specified by selecting a value between 0 to 100,000 where larger values represent higher TIRs. Finally, the reference organism must be specified, in this case *Synechococcus* sp. PCC 7002. The output is the synthetic RBS sequence as well as predicted translation initiation rates at each potential translation initiation start site in the entire sequence spanning from the start of the pre-sequence to the end of the protein coding sequence. To check whether upstream promoter sequences affected predicted translation rates, *in silico* TIRs were determined for the library of synthetic RBSs as well as the RBS present in the standard GFP BioBrick (BBa_0030) using three different upstream sequences: the XbaI/SpeI mixed site used to design the synthetic RBSs and two promoters from PCC 6803, P_{cpcBA2} and the RNase P subunit B promoter (P_{rnpB}) (Figure 3-6). The maximum predicted TIR that could be achieved was 10,435 using RBS9 and target TIRs of other members in the RBS library were

determined relative to this value. While predicted TIRs varied for the BioBrick RBS BBa_0030, TIRs were stable for the synthetic RBS library irrespective of the upstream sequence.

Table 3-2. Synthetic ribosome binding site sequences for GFP

RBS	Sequence	Predicted TIR
RBS6	AATATCCGATCATATACACCACAGGCATAACAAGG	4265
RBS7	TAAAATTACAAAACACTTTTTAGCTAGGACACAT	6360
RBS8	ACACCTATAAGATCCTAGGGATAAAAAGATATC	7189
RBS9	AGAATTACATATCGCACAAAGGTATATAAAA	10435

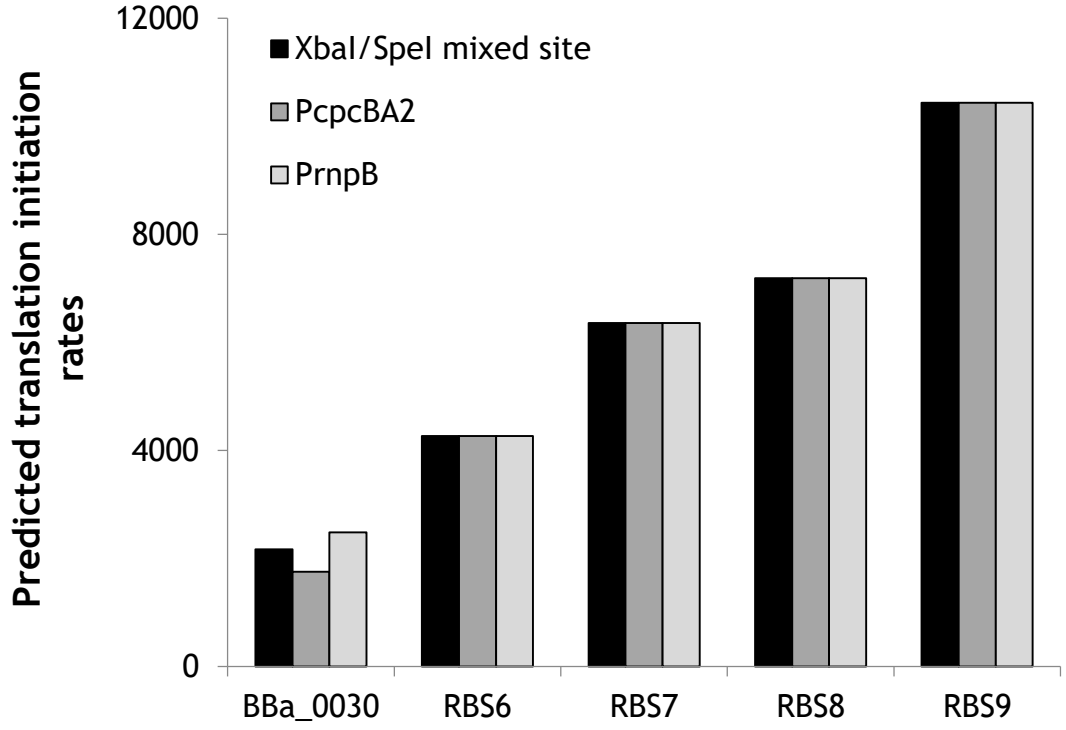


Figure 3-6. Predicted translation initiation rates for the synthetic RBS library

Bar charts show *in silico* translation initation rates predicted using the Salis Lab RBS Calculator for GFP with different upstream ribosome binding sites (along the x-axis). TIR was compared for different upstream sequences: the XbaI/SpeI mixed site generated during ligation of promoter-reporter modules and two promoters from PCC 6803.

While *in silico* modelling is an important tool for rational design, biological systems are highly complex and may not behave as predicted. It was therefore necessary to test the synthetic RBS library *in vivo*. Each member of the library was introduced directly upstream of the ATG start of the GFP reporter gene by PCR using primers containing the desired RBS sequence. PCR products were subcloned to a pGEM-T Easy Vector and confirmed by sequencing to generate a RBS-GFP library. As an example, the sequence of RBS7-GFP can be found in Appendix IV. In order to assess the activity of the synthetic RBSs, each member of the RBS-GFP library was cloned downstream of the P_{cpcBA2} promoter and reporter activity was determined in transformed cultures of PCC 7002 (Figure 3-7). Measured fluorescence did not always reproduce the predicted TIRs, for instance RBS6 and RBS8 resulted in relatively low reporter signal. RBS7 generated a slightly stronger signal compared with the BioBrick RBS BBa_0030 and RBS9 generated a very strong signal approximately 3-fold higher than RBS7. These RBS-GFP modules cover a wide range of translation rates that can serve as a useful tool to characterise promoters spanning a wide range of transcriptional activity.

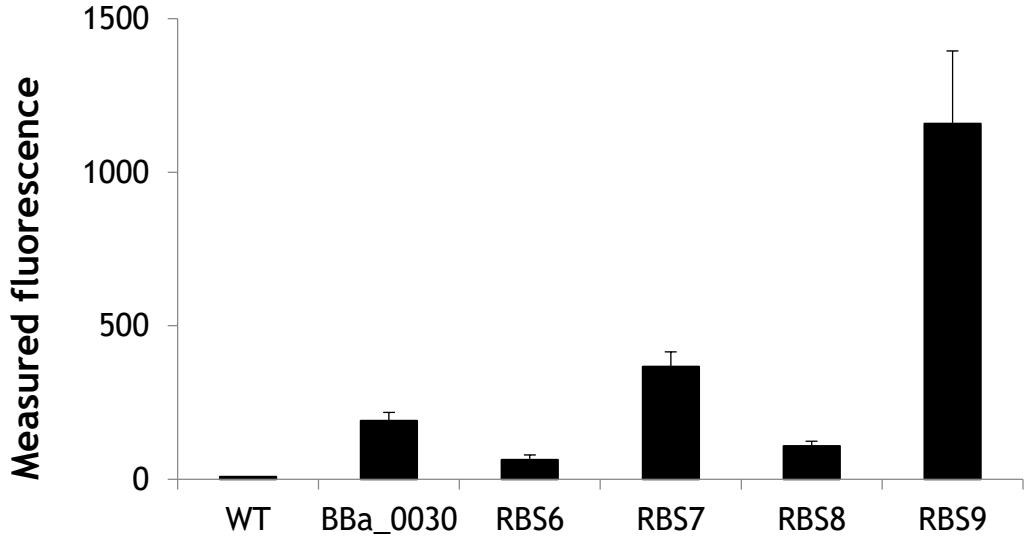


Figure 3-7. Synthetic ribosome binding sites to drive translation initiation of GFP

PCC 7002 were transformed with reporter modules carrying different synthetic ribosome binding sites to drive translation of GFP. The expression of all modules was driven by the phycocyanin promoter P_{cpcBA2}. Three transformed colonies were selected and grown to OD_{730} 1, diluted to OD_{730} 0.3 and fluorescence was detected using standardised settings in PCC 7002 background. Wild-type PCC 7002 (WT) were included as a negative GFP control.

3.2.5 Testing the fluorescence assay with published promoters

To test the fluorescence assay, three published promoters were selected from PCC 6803 (Table 3-3). Each candidate promoter was PCR amplified from PCC 6803 genomic DNA, subcloned to and sequenced in the pGEM T-Easy vector prior to insertion directly upstream of the RBS7-GFP reporter in pAQ1BB. Fluorescence was measured in transformed cultures of PCC 7002 (Figure 3-8). The phycocyanin promoter (P_{cpcBA2}) was the only candidate to generate a signal above the background of wild-type PCC 7002. Given that P_{cpcBA} has been described as a "super strong promoter" (Zhou et al., 2014), it may be that P_{rnpB} and P_{rbcL} are too weak to generate a signal using RBS7-GFP.

Table 3-3. Published promoters from *Synechocystis* spp. PCC 6803

Promoter	Description	Position[a]	Reference
P_{cpcBA2}	Phycocyanin operon promoter	-591 to 0	Xu et al., 2011
P_{rnpB}	RNase P subunit B promoter	-200 to 0	Huang et al., 2010
P_{rbcL}	RuBisCO large subunit promoter	-252 to -18	Huang et al., 2010

[a] Relative to the start codon of the coding sequence where the first nucleotide is +1

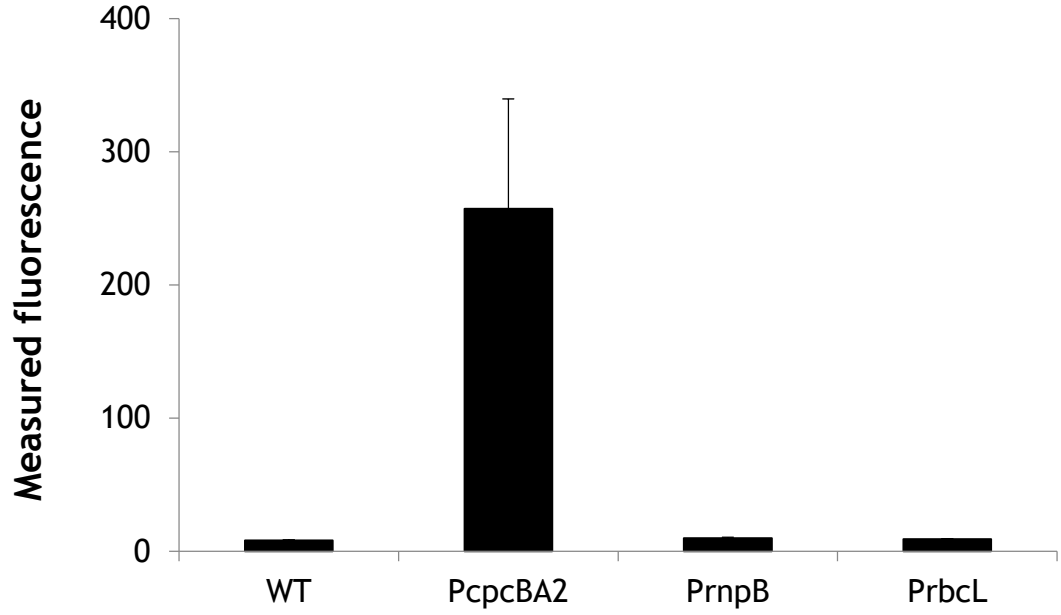


Figure 3-8. Testing published promoters from *Synechocystis* spp. PCC 6803

see overleaf for the complete figure legend

Figure 3-8. GFP assay of published promoters from *Synechocystis* spp. PCC 6803

PCC 7002 were transformed with GFP driven by three different published promoters from PCC 6803: the phycocyanin promoter (P_{cpcBA2}), Rnase P subunit B promoter (P_{rnpB}) and RuBisCO large subunit promoter (P_{rbcL}). Three transformed colonies were selected and grown OD_{730} 1, diluted to OD_{730} 0.3 and fluorescence was detected using standardised settings in PCC 7002 background. Wild type PCC 7002 (WT) were included as a negative GFP control.

The activity of P_{cpcBA2} was assessed throughout culture growth of PCC 7002. The highest fluorescence signal was obtained during early growth from days 4 to 10 followed by a decline in fluorescence as the cultures entered late growth (Figure 3-9A). As expression of the fluorescent reporter is under the control of P_{cpcBA2}, this suggests that this promoter is most active during early growth. This promoter activity profile agrees with previous reports (Xu et al., 2011). To determine whether this profile reflects native transcriptional activity of P_{cpcBA2} in the endogenous strain from which the promoter was isolated, PCC 6803, transcript levels of the gene under the control of this promoter, *cpcB*, was assessed by quantitative real-time PCR (qPCR) throughout culture growth. Indeed, *cpcB* transcript levels are highest during early growth followed by a decline as the cultures enter late growth (Figure 3-9B). Thus, the same pattern of promoter activity was detected by qPCR and GFP activity, indicating that the developed fluorescence assay is reliable.

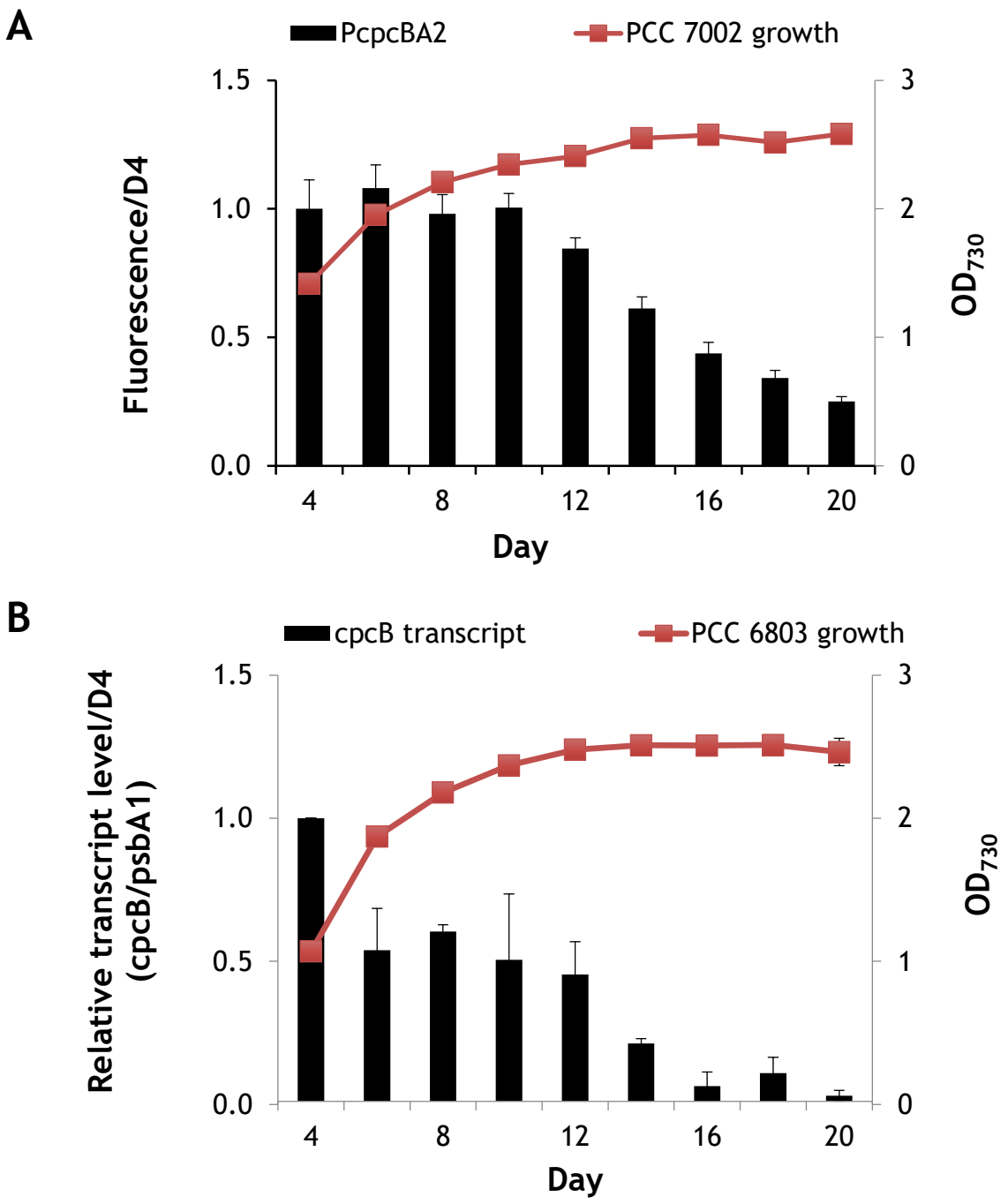


Figure 3-9. Phycocyanin promoter activity in endogenous and heterologous systems

(A) PCC 7002 were transformed with RBS7-GFP under the control of P_{cpcBA2}. Three transformed colonies were selected and fluorescence was measured throughout culture growth. Samples were diluted to OD_{730} 0.3 and fluorescence was detected using standardised settings. **(B)** Transcript levels of *cpcB* driven by the *cpcBA* promoter were measured by qPCR and normalized to the reference gene *psbA1* in PCC 6803. Growth curves are shown in red as means ± S.E.M. of 3 independent cultures. Black bars represent means ± S.E.M. of 3 independent cultures scaled to day 4.

3.3 Discussion

The rational design of biological organisms relies on robust tools that behave in a reproducible manner. Thorough characterisation of individual components enhances the accuracy of predicted function when assembled into modules that are more complex. The tools currently available for cyanobacteria are not ideal for industrial applications and it is essential to begin optimisation at the most basic level, namely the first step of gene expression: transcription initiation at the promoter. Assays have been developed to characterise promoter behaviour in a growing number of chassis organisms including the freshwater cyanobacterium PCC 6803 (Kelly et al., 2009, Huang et al., 2010). In this chapter, an assay was established to characterise promoter activity in the marine cyanobacterium PCC 7002 using components that are compatible with the largest library of standardised biological parts available to date (BioBricks). A plasmid vector for homologous recombination, pAQ1BB, was developed to include key restriction sites required for the assembly of BioBrick parts and furthermore these restriction sites were removed from other sites within the vector. A fluorescent reporter, GFP, was selected to quantify promoter activity in PCC 7002 and synthetic ribosome binding sites (RBSs) were developed to drive a wide range of translation rates in order to detect a wide range of transcriptional activity. I showed that the developed GFP reporter system in PCC 7002 was able to reproduce the native growth phase-specific promoter activity determined by qPCR in PCC 6803. Using RBS7, the assay was not sensitive enough to produce a signal with weaker promoters. Stronger RBSs such as RBS9 should be used to follow the activity of these promoters.

3.3.1 Further vector development

3.3.1.1 Alternative plasmid vectors

The assay developed in this chapter utilises a plasmid vector for homologous recombination to introduce transgenes into a native plasmid in PCC 7002. This system could be subject to "gene dosage" variation as a) recombination may not occur with all copies of the native pAQ1 plasmid in every cell and b) the native plasmid may alter its copy number under different conditions. Copy number of bacterial plasmids is regulated independently of the bacterial chromosome (del Solar and Espinosa, 2000). The target site for integration of promoter-reporter modules is pAQ1, an endogenous plasmid, which maintains the same copy number as the chromosome throughout growth under standard conditions in PCC 7002 (Xu et al., 2011). Nevertheless, exact copy numbers per cell and across different growth conditions may vary and thus result in potential differences in gene dosage, thus decoupling reporter signal from promoter activity.

Self-replicating plasmids maintained as autonomous replicons independent of the host's genetic material may provide a solution to this problem as these plasmids typically maintain stable copy numbers within cells. Moreover, use of autonomous vectors avoids the lengthy selection process for homozygous transformants. The promoter assay developed for PCC 6803 utilises an autonomous plasmid vector, pPMQAK1, which is compatible with standardised BioBrick parts (Huang et al., 2010). This vector carries a broad-host-range replication origin derived from the RSF1010 plasmid (Meyer, 2009) and maintains a low copy number of approximately 10 copies per cell in PCC 6803 (Marraccini et al., 1993).

There are no reports on the maintenance of autonomously replicating plasmids in PCC 7002; however, this replicon is active in diverse strains of cyanobacteria including *Nostoc* spp. PCC 7120, *N. punctiforme*, and *Synechococcus* sp. PCC 7942 to name a few (Sode et al., 1992, Mermet-Bouvier et al., 1993). Indeed, pPMQAK1 was evaluated for this promoter assay and successful transformation PCC 7002 was confirmed by PCR (data not shown). Nevertheless, this vector was not pursued due to very low yield of DNA following purification of restriction

digests and thus very low cloning efficiency in *E. coli*. While the low copy number of this plasmid can in part explain these observations, a recent study has pinned this inefficiency to the origin of transfer, *oriT* (Taton et al., 2014). RSF1010 carries a mobilisation element, which encodes three proteins including MobA, which nicks one of the DNA strands at *oriT* for transfer across the conjugation pore during horizontal gene transfer between bacteria. As a result, the alkaline lysis step during plasmid purification produces single-stranded DNA, which are useless for cloning. Furthermore, the complex formed between MobA and the 5' end of the cleaved strand interferes during restriction digestion and other enzymatic reactions. New variants of the RSF1010 replicon have since been developed in which proteins encoded in the mobilisation element have been inactivated resulting in higher cloning efficiency (Taton et al., 2014).

3.3.1.2 Additional features of BioBrick vectors

The plasmid vector developed in this chapter carries the necessary restriction sites for assembly of BioBrick parts. Furthermore, domestication of the plasmid was performed to remove these restriction sites from other locations within the plasmid. While this work was critical for compatibility with standardised BioBrick parts, further development is required to meet the standard design for BioBrick vectors (Shetty et al., 2008). The next step would be to genetically isolate modules within the cloning site by introducing forward and reverse transcriptional terminator sequences either side of the cloning site as well as translational stop codons. This will prevent run-through of transcription and/or translation from areas outwith the cloning site thus preventing aberrant transgene product formation. Finally, the introduction of BioBrick verification primer binding sites will enable sequence confirmation using standardised primers.

3.3.1.3 Alternative cloning approaches for gene assembly

The BioBrick design was rapidly embraced by the synthetic biology community due to its idempotent nature (Knight, 2003). That is to say, products generated by the assembly of two BioBrick components maintain the characteristics of the individual components and thus are subject to the same rules for further

assembly (see Figure 1-4B). This arises from the formation of an eight base pair scar generated upon the ligation of XbaI and SpeI restriction sites, which neither enzyme can recognise. This can be problematic particularly in the assembly of fusion proteins as this scar encodes an in-frame stop codon. Furthermore, the BioBrick design relies on traditional cloning techniques that are limited by 1) the restriction sites available for assembly and 2) the number of components that can be assembled simultaneously, namely two. Next-generation cloning techniques are rapidly emerging which promise high precision, rapid assembly of multiple components in single reactions. These include methods based on end-homology such as Gibson isothermal assembly (Gibson et al., 2009), Type IIS restriction enzymes such as MoClo (Weber et al., 2011), and site-specific recombination such as Gateway (Hartley et al., 2000) or serine integrase recombinational assembly (Colloms et al., 2014). Adoption of such techniques will significantly reduce time and effort for gene assembly.

3.3.1.4 Cell-free approaches to assess transcriptional activity

In vivo approaches for part characterisation are important to assess activity in the context of living cells however this can be a time-consuming and laborious task when screening large libraries. Alternatively, *in vitro* cell-free approaches can shorten the process from days to hours and enable high-throughput library generation and screening using automated methods such as liquid handling robots and microfluidics (Gulati et al., 2009). Crude extract cell-free systems from *E. coli* have already shown promise for cost-effective protein synthesis (Hodgman and Jewett, 2012) and both transcription and translation are comparable between *in vivo* and *in vitro* reactions (Chappell et al., 2013). Cyanobacterial cell extracts have previously been used to investigate effects of biologically active compounds such as antibiotics and toxins (Pietsch et al., 2001, Guedes et al., 2011, Carvalho et al., 2013) and could be optimised for *in vitro* part characterisation.

3.3.2 Transcriptional tools for reliable engineering

Modelling is a key enabling principle in synthetic biology and a number of software tools have been developed to design and test DNA parts *in silico*. In this study, I assessed the utility of the Salis Lab RBS calculator (Salis et al., 2009) to design synthetic ribosome binding sites and predict translation initiation rates for GFP in PCC 7002. There are currently no reports of use of the Salis Lab RBS calculator for PCC 7002 so this study is the first demonstration of the application of this tool for the rational design of PCC 7002. It enabled the generation of a library of synthetic RBSs to drive a wide range of translational activation of GFP reporter protein in PCC 7002. While this is a powerful tool to control gene expression levels, fluorescence measured in transformed cultures of cyanobacteria did not always correlate with *in silico* translation initiation rates predicted by the RBS calculator. The disconnect observed between predicted TIR and measured fluorescence may be due to the frequent absence of Shine-Dalgarno sequences in the 5' untranslated regions of cyanobacterial genes (Ma et al., 2002). Just 26 % of genes in cyanobacteria have a SD sequence located at the optimal position. This suggests alternative mechanisms for ribosomal recognition and binding of mRNA transcripts during translation initiation in cyanobacteria. Indeed, in the absence of SD sequences, ribosomal protein S1 provides ribosome-messenger recognition via AU-rich sequences in the 5' untranslated regions of the gene (Tzareva et al., 1994). *In silico* RBS calculators are not yet advanced enough to accommodate for such mechanisms resulting in inaccuracies in predicted translation initiation rates.

The disconnect between predicted and actual translation rates demonstrated in this study highlights the importance of verifying computational models *in vivo* and furthermore selecting appropriate tools for predictable outputs. Ribosome binding sites are important for translation of gene products however this may not be best the point of optimisation to modulate rates of gene product formation. One of the drawbacks is the highly context (i.e. sequence) dependent efficiency of ribosome binding sites (Reeve et al., 2014). Quantification of gene product formation under the control of different ribosome binding sites would require relatively labour-intensive and time-consuming approaches. By contrast,

promoters are more robust regulators of gene expression and the transcriptional activity of promoters is relatively easily quantified using assays such as the one described in this chapter. Transcription initiation rates are less influenced by surrounding sequences so promoters behave in a more predictable manner. Libraries of promoters spanning a wide range of transcriptional activity are therefore a more useful tool for the design and optimisation multistep processes.

3.3.3 Reference promoters for comparable measures of promoter activity

The original proposal for promoter characterisation by Kelly et al. was to quantify promoter activity in Relative Promoter Units (RPUs) by normalising promoter activity to a stable reference promoter (Kelly et al., 2009). While this generates units which are more comparable across different laboratories, this approach requires access to the selected reference promoters. Furthermore, the selected promoters may not in fact be suitable references for particular experimental setups as promoter activity may vary under different conditions. Instead, more recent promoter analyses have simply been presented as fluorescence normalised to optical density (Huang et al., 2010, Camsund et al., 2014, Markley et al., 2014). Variation will arise from the use of different instruments so the measurement of one or more reference promoters under standard conditions will aid the interpretation of promoter strength. The P_{cpcBA2} promoter assessed in this chapter is not a suitable candidate reference promoter due to changes in activity observed at different times during growth. Constitutive promoters from PCC 6803 such as P_{rnpB} and P_{rbcL} may be better candidate reference promoters however transcriptional activity could not be detected using RBS7-GFP. Given that P_{cpcBA} has previously been described as a "super strong" promoter, it may be that P_{rnpB} and P_{rbcL} are indeed active in PCC 7002 however stronger RBSs such as RBS9 are required to detect their activity.

3.3.4 Heterologous promoters to control gene expression in cyanobacteria

Previous attempts to engineer orthogonal transcriptional systems to drive gene expression in cyanobacteria have had limited success (Huang et al., 2010, Huang and Lindblad, 2013, Camsund et al., 2014, Zhou et al., 2014). These systems were derived from bacteria whose transcriptional machinery, such as the RNA polymerase core enzyme, differed from that of cyanobacteria, however (Schneider and Hasekorn, 1988, Xie et al., 1989). By contrast, this study aimed to develop transcriptional systems using promoters from more closely related organisms, i.e. other species of cyanobacteria. Given that the composition of the RNA polymerase core enzyme is conserved across the major cyanobacteria subgroups, heterologous expression systems developed using this approach may show improved function.

Promoters from PCC 6803 are routinely used to control transgene expression in other species of cyanobacteria including *Synechococcus* spp. PCC 7942 and *Anabaena* spp. PCC 7120 (Heidorn et al., 2011, Wang et al., 2012, Berla et al., 2013). In this study, I used promoters from PCC 6803 to drive the expression of GFP in *Synechococcus* spp. PCC 7002. Cyanobacteria are an extremely diverse phylum, and there is of course the possibility that promoters from PCC 6803 will not function in PCC 7002. There are many similarities between the two strains. They are both members of the taxonomic subsection I Chroococcales as they are spherical unicellular organisms that reproduce by binary fission (Rippka et al., 1979). Neither of these strains are capable of nitrogen fixation, forming spores or symbiotic relationships with other organisms (Larsson et al., 2011). Furthermore, while they naturally inhabit different environments (PCC 6803 in freshwater and PCC 7002 in seawater), both strains are able to grow across a wide range of salinities (Minas et al., 2014). The genomes of these two organisms are of a similar size (3.96 and 3.41 Mbp in PCC 6803 and PCC 7002 respectively), however sequences differ substantially with 33 % similarity as determined by nucleotide alignment of the two genome sequences. Furthermore, differences in gene regulation have been previously observed for salt-induced synthesis and accumulation of the osmolyte glucosylglycerol (Engelbrecht et al., 1999).

It was therefore important to confirm that promoters from PCC 6803 retained their behaviour in PCC 7002. Indeed, this is the first study to illustrate that the growth-phase-specific behaviour of a promoter from PCC 6803 retains the same activation pattern in PCC 7002. Furthermore, this pattern was observed using two different techniques (qPCR and a fluorescence assay) confirming that the design of the GFP-based promoter assay accurately reflects promoter activity. This is a highly valuable tool to identify and characterise critical tools that are of much need for the engineering of cyanobacteria.

Chapter 4. Modulating growth kinetics in cyanobacteria

4.1 Introduction

In the previous chapter, an assay was established to characterise promoter activity in the marine cyanobacterium *Synechococcus* spp. PCC 7002. The aim is now to identify promoters to control gene expression in this chassis from a different species of cyanobacteria, the freshwater *Synechocystis* spp. PCC 6803 (hereafter PCC 6803). Inducible promoters are the most popular approach to regulate gene expression in bacteria due to tight regulation and the ability to control the timing of gene expression. This typically involves the addition of small molecules and heavy metals, which are costly and often toxic and therefore not suitable for large-scale processes in industrial applications (Heidorn et al., 2011, Wang et al., 2012, Berla et al., 2013). Alternatively, auto-inducible systems regulated by endogenous cues can serve as a more sustainable approach to control gene expression. In bacteria, promoters that respond to changes in growth are a common tool, particularly promoters activated once cultures cease growth and division (Miksch and Dobrowolski, 1995, Cases et al., 1996, Schumann, 2007). In order to identify promoters which respond specifically to growth phases of cyanobacterial cultures, the first step is to determine factors that affect culture growth.

In contrast to heterotrophic bacteria that require organic carbon sources, cyanobacteria have relatively simple input requirements: light, carbon dioxide, water and mineral nutrients. The most common limiting factor in both freshwater and marine ecosystems is the mineral nutrient supply (Vitousek and Howarth, 1991) and indeed numerous studies have demonstrated that nutrient availability is a key growth determinant in cyanobacterial cultures (Hirani et al., 2001, Richaud et al., 2001, Kim et al., 2011, Krasikov et al., 2012, Fuszard et al., 2013). Nitrogen, phosphorus and sulphur are regarded as macronutrients as they are major components in essential biomolecules, most notably nucleic acids

and proteins. Cyanobacteria have evolved mechanisms to overcome macronutrient limitation in the wild by a) enhancing nutrient uptake and accessibility and b) releasing intracellular nutrient reserves, most commonly by degrading light-harvesting phycobilisome complexes to release nitrogen and sulphur stores (Grossman et al., 1993, Grossman et al., 1994, Schwarz and Forchhammer, 2005). These mechanisms enable the maintenance of growth; however, depletion of internal reserves will eventually occur in batch cultures. As a result, macronutrient deprivation, i.e. complete removal of the macronutrient, in PCC 6803 results in a short period of exponential growth followed by growth arrest at a significantly lower density compared to cultures grown under replete nutrient conditions (Krasikov et al., 2012, Richaud et al., 2001, Hirani et al., 2001). Potassium and magnesium, considered macronutrients in plant nutrition, are also essential for growth of cyanobacteria (Grusak, 2001, Wolk, 1973). They constitute the two major intracellular cations present in bacterial cells and deprivation of these nutrients impairs growth of heterocystous, filament-forming cyanobacteria (Utkilen, 1982, Alahari and Apte, 1998). The effects of limited supplies of these and other nutrients have yet to be described in other cyanobacteria, including PCC 6803.

Micronutrients are required at lower concentrations but are still essential to maintain proper metabolism e.g. as cofactors. Typically, cyanobacteria cope with micronutrient deficiency by simply reducing cellular demand for that nutrient (Grossman et al., 1994). For instance, iron and copper are two key micronutrients given their important roles in electron transfer proteins for photosynthesis. In order to reduce cellular demand, cyanobacteria replace the electron transfer proteins containing these metal ions with alternative proteins that do not contain them. Thus, the iron-containing protein ferredoxin is replaced by flavodoxin, which does not contain iron. Similarly, plastocyanin is replaced with cytochrome c_{553} under copper limiting conditions. In this way, cyanobacteria are able to reduce cellular demand for these metal ions without significant loss of photosynthetic function (Sandmann et al., 1990). The effects of other micronutrient deficiencies have not been studied in much detail in cyanobacteria.

4.1.1 Chapter aim

A great number of studies have focused on the short-term shock responses to nutrient deprivation at physiological, transcriptomic and proteomic levels (Suzuki et al., 2004, Krasikov et al., 2012, Kopf et al., 2014, Zhang et al., 2008, Osanai et al., 2006, Fuszard et al., 2013, Richaud et al., 2001, Hirani et al., 2001). This typically involved transfer of cultures during exponential phase growth from replete (hereafter "standard") media to deficient media in which the particular nutrient was completely removed. By contrast, this study focused on the effects of nutrient limitation on culture growth, where "limitation" refers to a poor supply of a given nutrient as opposed to complete elimination of that nutrient (typically 12.5 % relative to the standard concentration). The aim of this work was to identify conditions that result in transition from exponential to stationary phase for different reasons, i.e. limitation by different factors. This enabled later identification of genes that respond to the growth transition irrespective of the particular limiting factor. The hope was that this approach would enable the identification of robust growth phase-specific promoters. Nutrient limitation was studied as opposed to complete deprivation to allow for enough growth to provide sufficient RNA material for future gene expression analysis and further mimic the gradual decline in nutrient availability that cultures experience in batch cultures.

4.2 Results

4.2.1 Synechocystis spp. PCC 6803 culture conditions

A tightly controlled system was required for reproducible growth of PCC 6803 as samples were to be collected for subsequent transcriptomic analyses. Firstly, stable temperatures were achieved in controlled water baths (Figure 4-1) equilibrated at the optimal temperature of 30 ° C (Sheng et al., 2011). Cultures were illuminated with Sylvania Cool White fluorescent bulbs with a 12 hour day/night photoperiod. The exact light intensity within the water baths could not be determined however a schematic diagram illustrating the layout of the water bath with flanking lights is presented in Figure 4-1A. Secondly, stable aeration rates of cyanobacteria cultures were achieved in Dreschel flasks (Figure 4-1B) through which ambient air (0.04 % CO_2) humidified with distilled water was delivered into the culture from an aquarium pump via a Büchner flask. In addition, sparging provided continuous agitation of the cultures.

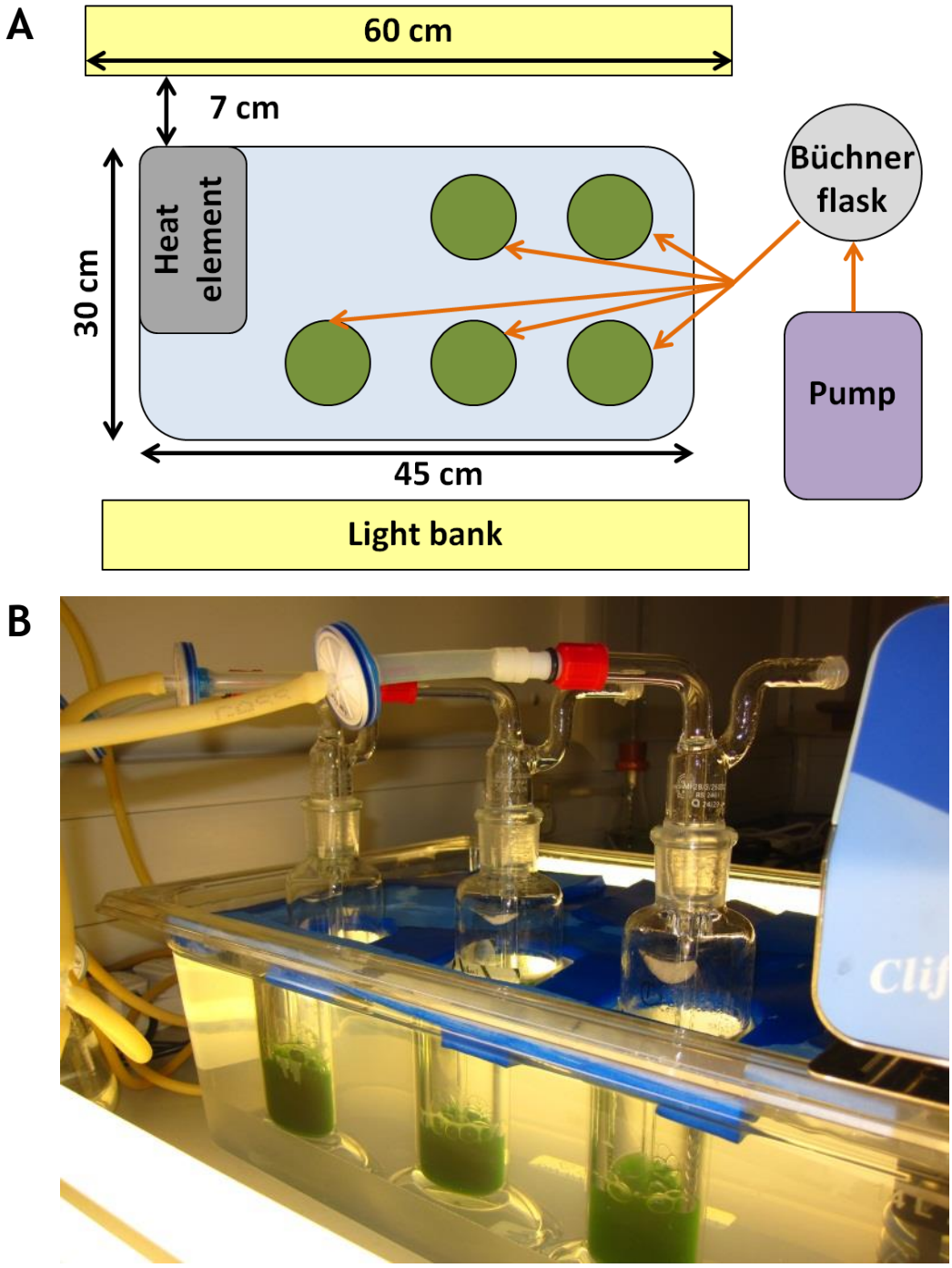


Figure 4-1. Culture conditions of *Synechococcus* spp. PCC 6803

(A) Aerial view diagram and **(B)** side view photograph of PCC 6803 cultivated in Dreschel flasks maintained in 14 L water baths equilibrated at 30 °C. The cultures were illuminated with a 12 h photoperiod by light banks (yellow rectangles; **A**) consisting of two Sylvania Cool White fluorescent light bulbs. Cultures were sparged with ambient air (0.04 % CO_2) from an aquarium pump and humidified with distilled water through a Büchner flask. Orange arrows show direction of air flow. Black arrows show distances.

4.2.2 Monitoring growth of cyanobacteria

Growth of cyanobacteria can be monitored using a number of different techniques including quantification of optical density, cell number and pigment concentration. Pigment concentration was not a suitable method for this study as loss of pigment is expected as a result of phycobilisome degradation in response to various nutrient limitations including nitrogen, phosphorus and sulphur (Grossman et al., 1994). Optical density (OD) is the most common approach to monitor growth of cyanobacteria, typically at a wavelength of 730 nm (OD_{730}) as this lies outside of the absorption spectrum of cyanobacterial pigments (Blankenship et al., 2011). OD is a measure of turbidity, which can arise from a number of factors including cell number and cell size. It was therefore important to investigate the relationship between OD and cell number. PCC 6803 was grown to late exponential phase and diluted in fresh BG11 media to produce a range of densities from OD_{730} 0.01 to 0.6. Optical density was determined for each sample and 1 ml of sample was added to a Sedgewick Rafter counting chamber. Images were captured using a bright field microscope (Figure 4-2A) and cell number was determined by ImageJ analysis as described in *Section 2.1.4.2*. A tight linear relationship was observed between cell number and optical density (R^2=0.9975; Figure 4-2B). OD accurately reflected cell number and was a relatively fast and easy approach, so this method was selected to quantify changes in culture density of PCC 6803.

Initially, I was not aware of the importance of measuring optical density within the linear range of the spectrophotometer (OD_{730} 0.01 to 1.0). Early experiments therefore do not reflect accurate measurements of culture density (indicated in the figure legends) however conditions limiting culture density could still be identified and were subsequently studied more accurately.

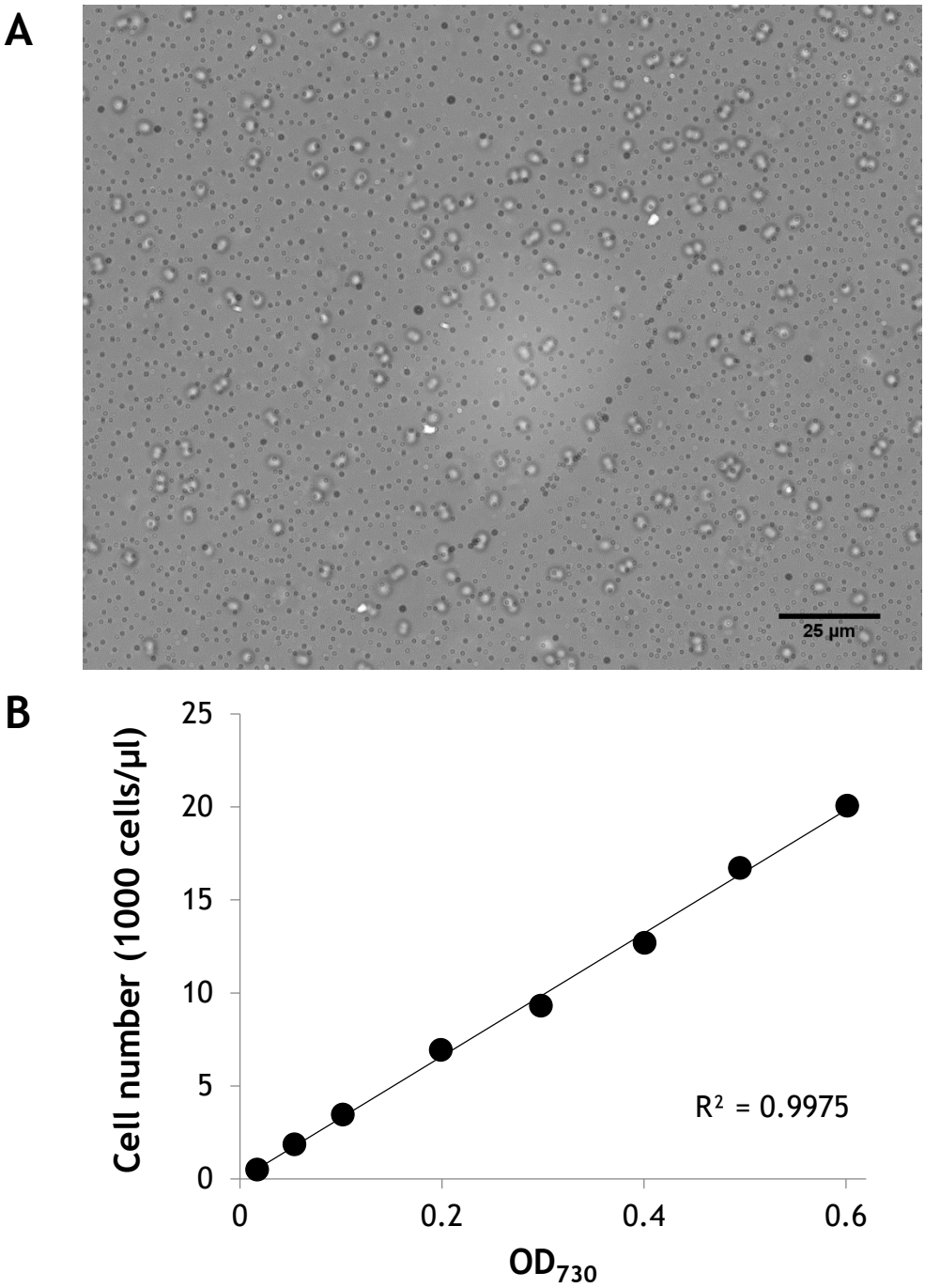


Figure 4-2. Comparison of optical density and cell number for PCC 6803

PCC 6803 were grown to late exponential phase under standard conditions. Dilutions were performed from a single culture to produce samples with densities ranging from OD_{730} 0.01 to 0.6. Cell number was determined by ImageJ analysis of bright field microscope images **(A)** of cells in a known volume using a Sedgewick Rafter counting chamber. **(B)** Cell number was plotted against optical density at 730 nm (OD_{730}) and a linear regression line fitted with the coefficient of determination (R^2) presented on the graph. Ten images were analysed for each data point and presented as means ± S.E.M. Note that error bars are smaller than symbol size.

4.2.3 Mineral nutrient availability affects growth of PCC 6803

Nutrient availability is a major factor affecting growth of cyanobacteria, in particular macronutrients that are key components in essential biomolecules (Schwarz and Forchhammer, 2005). Altering nutrient levels in the media may therefore serve as a tool to modulate growth kinetics of cyanobacterial cultures. In order to investigate the effect of nutrient availability in PCC 6803, growth was monitored in different dilutions of the standard media, BG11, ranging from 12.5 % to 100 % in which all of the nutrients were provided at the indicated concentration relative to the standard media (Figure 4-3). Growth kinetics were largely unaffected during the early stages of growth including the transition from lag phase to early exponential phase. The exception is 100 % BG11 in which reduced airflow resulted in slower exponential growth. Importantly, the maximum density achieved by the cultures correlated with nutrient supply: cultures grown in the presence of lower nutrient concentrations ceased growth at lower densities. Furthermore, reducing nutrient supplies to 12.5% compared to standard concentrations resulted in a marked decrease in final culture density while still generating sufficient cell material for downstream analyses, so this concentration was taken forward as the “limited” nutrient concentration.

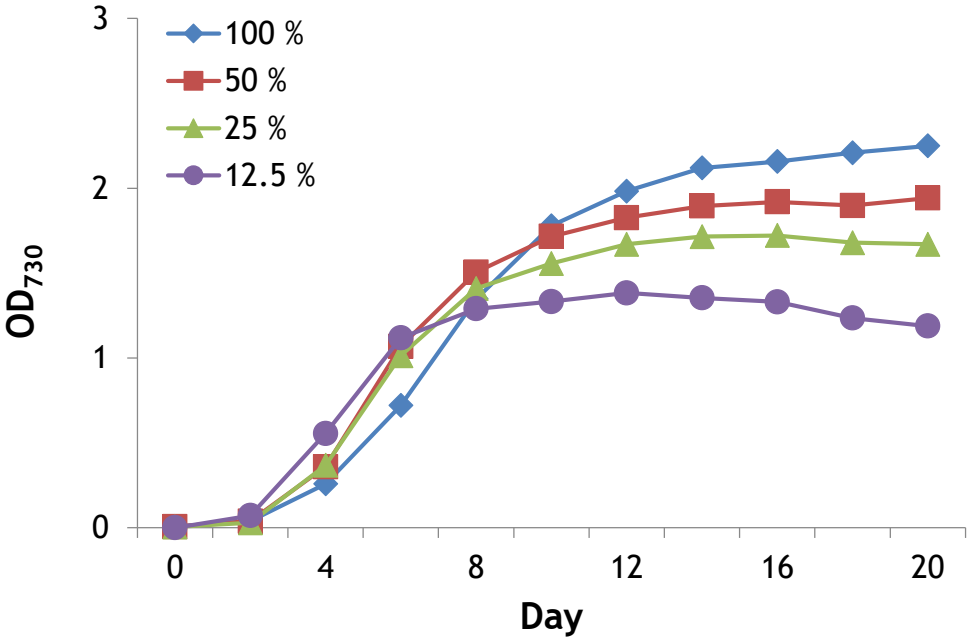


Figure 4-3. Effects of reducing nutrient supplies on growth of PCC 6803

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Figure 4-3. Effects of reducing nutrient supplies on growth of PCC 6803

Growth curves show optical density at 730 nm (OD_{730}) over time of PCC 6803 cultivated under varying nutrient concentrations. Percentages indicate total nutrient levels relative to standard BG11 media. Data points represent values from single cultures. N.B. Optical density was not measured in the linear range of the spectrophotometer.

In order to identify the particular components limiting culture growth, a screen was performed to assess the effects when individual nutrients were supplied at the limited concentration (12.5 %) compared to the standard concentration (100 %). The concentration of individual nutrients under standard and limited conditions is detailed in Table 4-1. Due to spatial and material constraints, growth under nutrient limited conditions was assesssed in cultures grown in Erlenmeyer flasks as previously described for *Synechococcus* spp. PCC 7002 (see *Section 3.2.1* and Figure 3-1C). Culture aeration rates were not constant in this experimental setup resulting in variation in CO_2 availability and therefore growth rates. Apparent differences in growth rate will therefore not be discussed. Three conditions resulted in a reduction of final culture density compared with standard media: BG11-$NaNO_3$, BG11-K_2HPO_4 and BG11-$MgSO_4$ (Figure 4-4). These conditions were taken forward for closer investigation and will hereafter be referred to as BG11-N, BG11-KP and BG11-MgS respectively.

Table 4-1. BG11 media composition for *Synechocystis* spp. PCC 6803

Salt	Standard media (100 %)	Limited media (12.5 %)
$NaNO_3$	17.6 mM	2.2 mM
K_2HPO_4	230 µM	28.75 µM
$MgSO_4.7H_2O$	304 µM	38 µM
$CaCl_2.2H_2O$	245 µM	30.6 µM
Na_2CO_3	189 µM	23.6 µM
Citric acid	286 µM	35.75 µM
$FeSO_4.7H_2O$	21.6 µM	2.7 µM
EDTA (disodium)	2.7 µM	0.34 µM

Trace elements were supplied at constant levels:
H_3BO_3, 46.3 µM; $MnCl_2.4H_2O$, 9.15 µM; $ZnSO_4.7H_2O$, 0.77 µM; $Na_2MoO_4.2H_2O$, 1.61 µM; $CuSO_4.5H_2O$, 0.32 µM; $Co(NO_3)_2.6H_2O$, 0.17 µM.

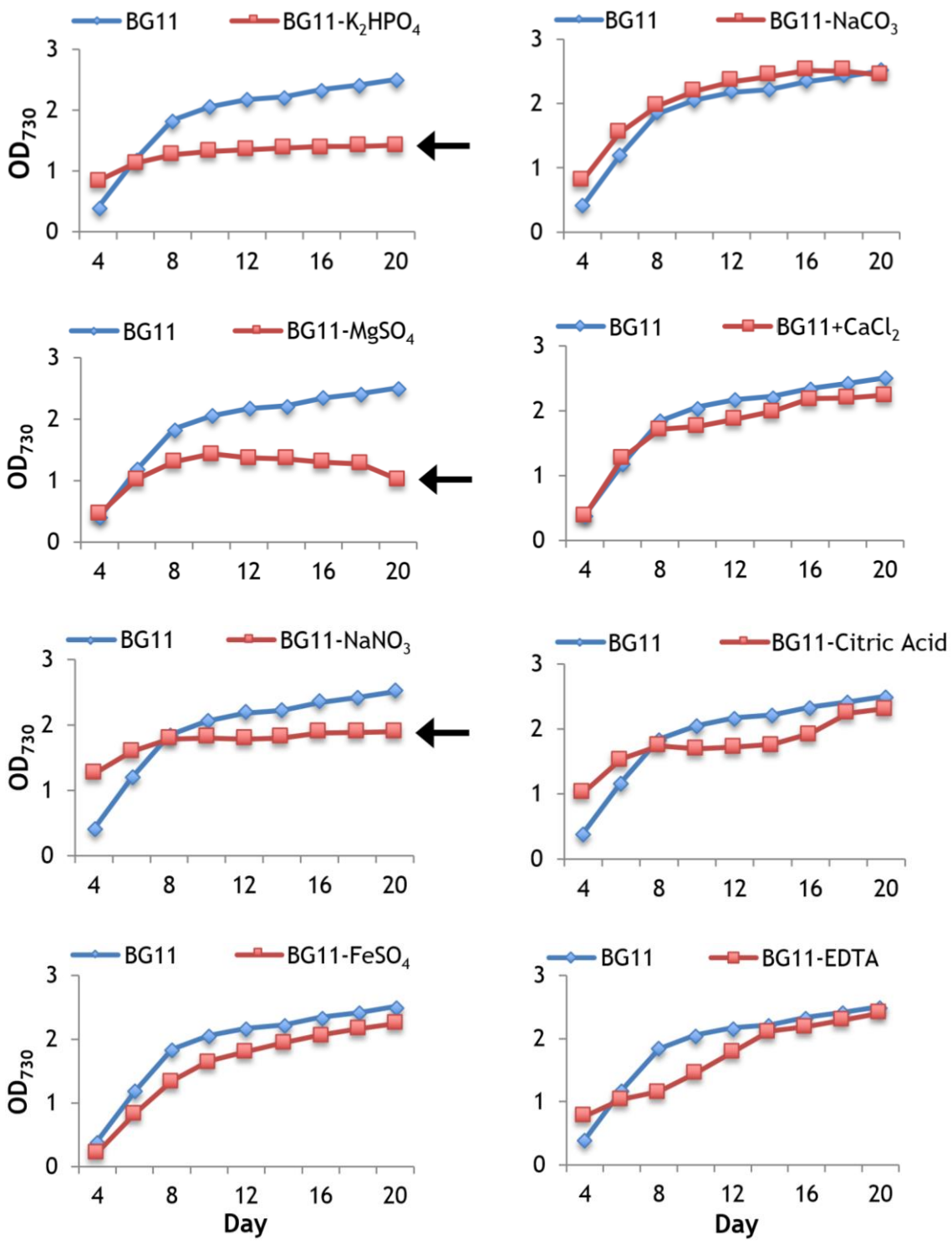

Figure 4-4. Screen for growth effects of nutrient limitation in PCC 6803

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Figure 4-4. Screen for growth effects of nutrient limitation in PCC 6803

PCC 6803 was cultivated in Erlenmeyer flasks maintained in a growth chamber with temperatures fluctuating between 25-45 °C. Cultures were illuminated over a 12 h day/night photoperiod with a light intensity of 120 µmol m^{-2} s^{-1} and sparged with humidified ambient air (0.04 % CO_2). Optical density at 730 nm (OD_{730}) was monitored over time. Blue lines show growth in standard nutrient conditions (BG11) and red lines show growth under nutrient limited conditions in which the indicated nutrient was supplied at 12.5% relative to the standard concentration. Black arrows indicate nutrient conditions taken forward for further analysis. N.B. Optical density was not measured in the linear range of the spectrophotometer.

Given the variability of this experimental setup, it was important to confirm these conditions did indeed result in premature growth arrest and furthermore accurately characterise differences in growth within the linear range of the spectrophotometer. Growth was therefore assessed in the controlled setting of stably aerated Dreschel flasks maintained in temperature controlled water baths as described in *Section 4.2.1*. All three nutrient-limited conditions identified in the screen (BG11-N, BG11-KP and BG11-MgS) did indeed result in premature growth arrest and a marked decrease in final culture density compared to growth in standard media (Figure 4-5A). A linear regression line of best fit was applied to each growth curve to determine the days during which cultures underwent exponential growth and the slope of this line was used to determine growth rates during this phase (Figure 4-5B-E). Both BG11-N and BG11-KP showed a slight increase in exponential growth rates whereas BG11-MgS showed a marked increase during this phase. All three conditions resulted in a pronounced reduction in the maximum density achieved at the onset of stationary phase compared to standard conditions. Larger decreases in maximum culture density were observed in BG11-N and BG11-KP than in BG11-MgS. Furthermore, BG11-N and BG11-MgS were not able to maintain stationary phase and a rapid decline in culture density was observed shortly after maximum density was achieved in these conditions.

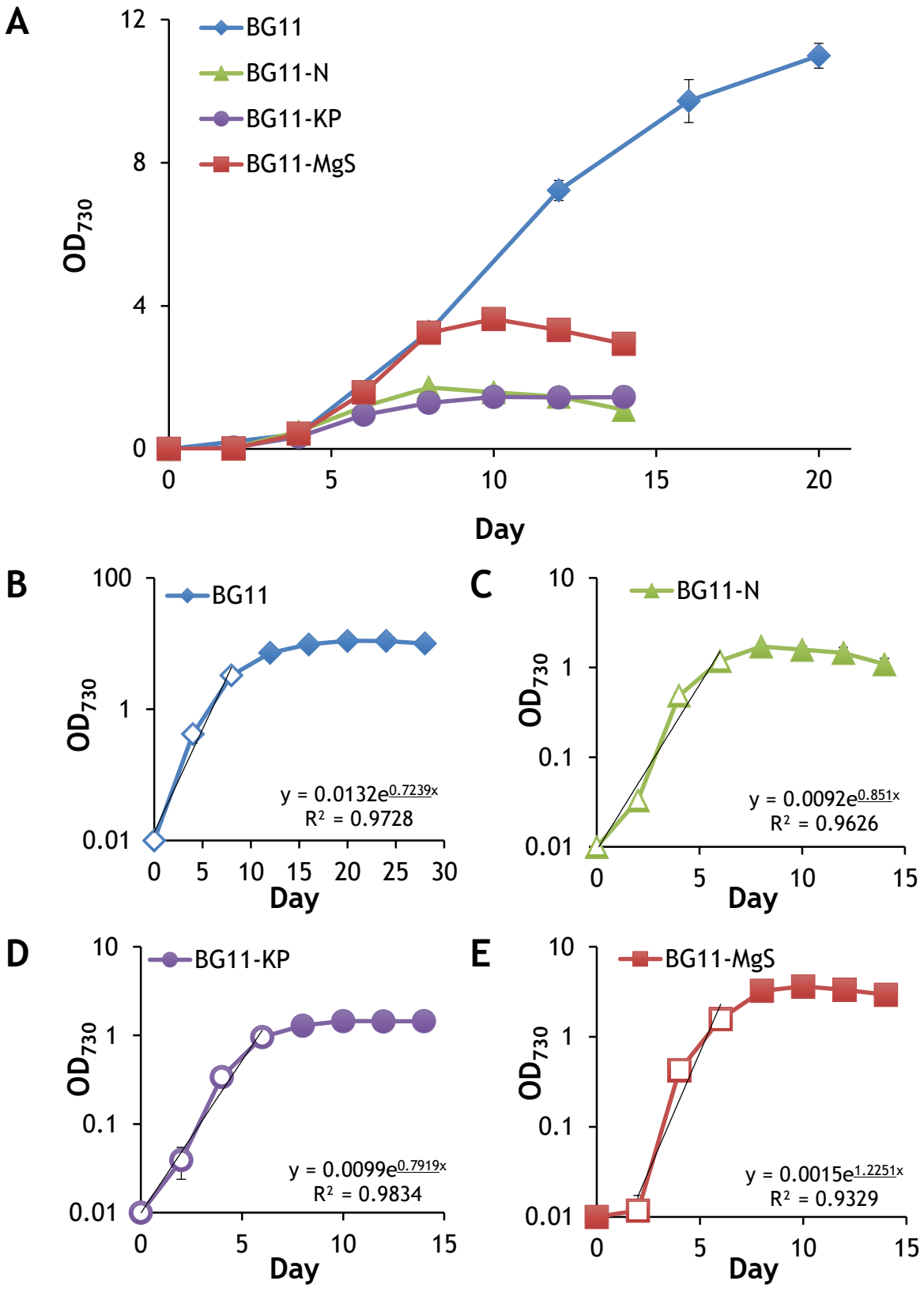

Figure 4-5. Growth of PCC 6803 in BG11, BG11-N, BG11-KP and BG11-MgS

see overleaf for the complete figure legend

Figure 4-5. Growth of PCC 6803 in BG11, BG11-N, BG11-KP and BG11-MgS

(A) Growth curves show optical density at 730 nm (OD_{730}) of PCC 6803 cultivated under standard (BG11) conditions, sodium nitrate limitation (BG11), di-potassium phosphate limitation (BG11-KP), or magnesium sulphate limitation (BG11-MgS) in which limited nutrients were supplied at 12.5 % relative to standard concentration. **(B-E)** Semi-logarithmic plots of growth under each nutrient condition. An exponential regression line was fitted to points during exponential growth (indicated with internal white boxes). The equation of the line and the coefficient of determination (R^2) derived from exponential growth points are presented on the graph. Growth rates were determined by the slope of the line (underlined). Data are presented as means ± S.E.M of three independent cultures. Note error bars are often smaller than symbol size.

4.2.4 Teasing apart effects of single nutrient limitations

BG11 media supplies macronutrients (nitrogen, phosphorus and sulphur) in chemical forms easily assimilated by cyanobacteria (nitrate, phosphate and sulphate respectively). These nutrients are co-supplied with a counter ion as an electrically neutral salt. Potassium (K) and magnesium (Mg) are supplied with phosphate (P) and sulphate (S) as K_2HPO_4 (KP) and $MgSO_4$ (MgS) respectively. Both of these cations are essential for growth of cyanobacteria (Wolk, 1973) and deficiency results in impaired growth in filamentous cyanobacteria (Utkilen, 1982, Alahari and Apte, 1998). It was therefore important to investigate the specific effects of macronutrient and counter ion for each condition identified in the previous section. In the case of $NaNO_3$, sodium (Na) is provided as a counter ion for nitrate (N) as well as various other components in BG11 (Table 4-1). The growth effects observed in BG11 were therefore attributed to N limitation and the effect of Na limitation was not investigated.

Severe growth inhibitions have been reported for PCC 6803 grown under P-limitation with and without the replacement of K (Hirani et al., 2001, Fuszard et al., 2013). It was therefore hypothesised that P was the main contributing factor to the growth inhibitions observed in BG11-KP. However, KP is the sole source of both K and P in BG11 media (Table 4-1). It was therefore important to determine the specific effects of each component on PCC 6803 growth. First, the effects of P-limitation were investigated by replacing K with KCl of the same osmolarity.

PCC 6803 growth in BG11-P resulted in a slight increase in exponential growth rates similar to difference observed in BG11-KP (Figure 4-6A and B). Surprisingly, while cultures grown in BG11-P also showed a marked decrease in final culture density compared to standard BG11, it was a much higher density (more than triple) than in BG11-KP. Furthermore, cultures grown in BG11-P were able to sustain exponential growth for a longer period (up to day 14) compared to BG11-KP (up to day 8). These findings suggested that P was not in fact the main factor inhibiting growth in BG11-KP. The effects of K-limitation were therefore investigated by replacing P with NaH_2PO_4 of the same osmolarity. PCC 6803 grown in BG11-K showed a severe decrease in final culture density similar to cultures grown in BG11-KP (Figure 4-6A and C). Furthermore, maximum density was achieved in both BG11-K and BG11-KP by day 10 in contrast to BG11-P where growth ceased on day 18. A marked increase in exponential growth rate was observed in BG11-K compared with BG11-P and BG11-KP. Interestingly, BG11-K cultures maintained maximum culture density for an extended period of time (8 days) following the cessation of exponential growth.

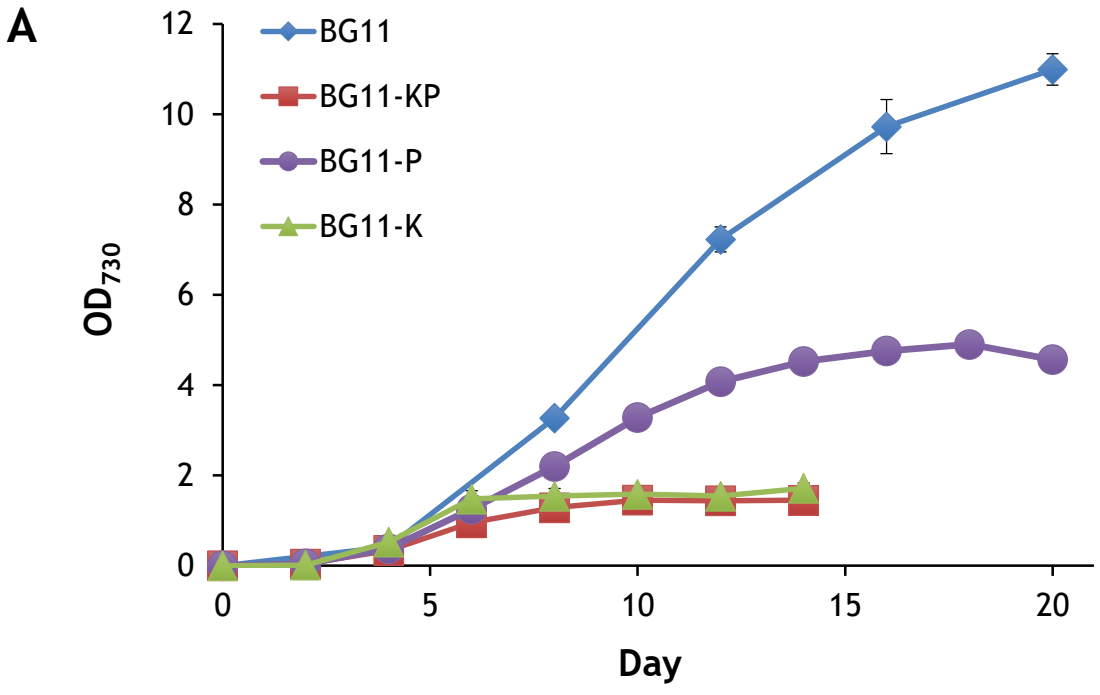

Figure 4-6. Growth of PCC 6803 in BG11-P and BG11-K

see overleaf for the complete figure and figure legend

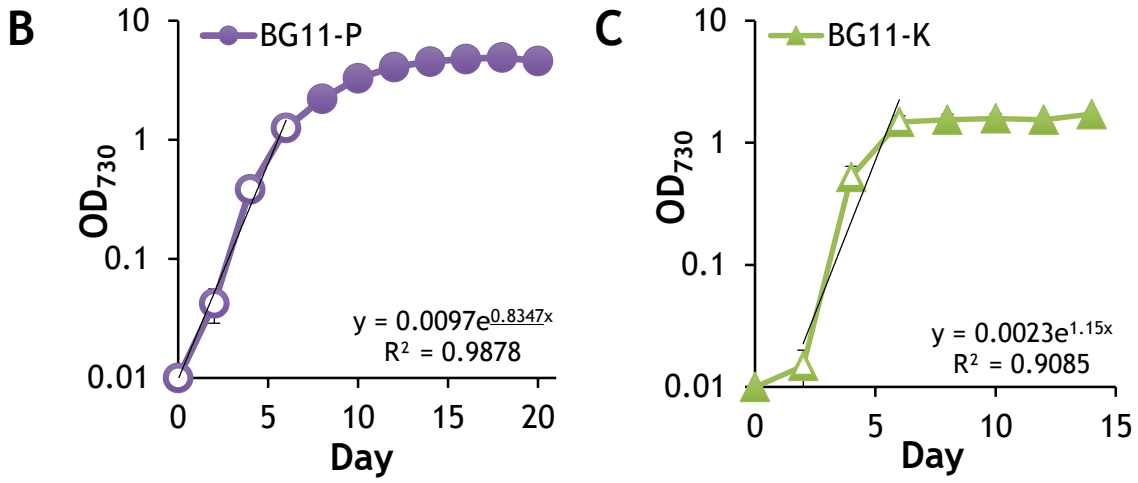

Figure 4-6. Growth of PCC 6803 in BG11-P and BG11-K

(continued from previous page)

(A) Growth curve presenting optical density at 730 nm (OD_{730}) of PCC 6803 cultivated under standard (BG11) conditions or the indicated nutrient limitation in which the nutrient is supplied at 12.5 % relative to standard concentration. **(B-C)** Semi-logarithmic plots of growth under each nutrient condition. An exponential regression line was fitted to points during exponential growth (indicated with internal white boxes). The equation of the line and the coefficient of determination (R^2) derived from exponential growth points are presented on the graph. Growth rates were determined by the slope of the line (underlined). Data are presented as averages ± S.E.M of three independent cultures. Note error bars are often smaller than symbol size.

Sulphur is supplied as sulphate (S) in four separate components in BG11 media and the relative contribution of each component to total S concentration in BG11 is presented in Table 4-2. $MgSO_4$ (MgS) provides the greatest proportion of sulphate to the media (93.8 %) and, as demonstrated in *Section 4.2.3*, growth in BG11-MgS results in a marked decrease in final culture density compared with growth in standard media (Figure 4-5A and D). This agrees with a previous report of severe growth inhibition in S-deprived cultures of PCC 6803 in which the same component, MgS, was completely eliminated from BG11 (Richaud et al., 2001). This study did not replace Mg in the media despite MgS being the sole source of magnesium (Mg) in BG11. As Mg is known to affect cell division in bacteria (Groisman et al., 2013), it was important to determine the specific effects of S- and Mg-limitation on the growth of PCC 6803. Growth under S- and Mg-limitation was assessed independently by replacing the counter ion with the same osmolarity of MgCl or Na_2SO_4 respectively. BG11-S showed a marked increase in

growth rate during the exponential phase and a slightly larger increase was observed in BG11-Mg compared to cultures grown in standard BG11 (Figure 4-7). Both conditions achieved maximum culture density at the end of exponential growth followed by a rapid decline in density (Figure 4-7B and C), similar to that observed in BG11-MgS (see Figure 4-5E).

Table 4-2. Sulphate in BG11 media

Compound	Final concentration of SO_4	Percentage of total SO_4
$MgSO_4$	118 µM	93.8 %
$FeSO_4$	7.46 µM	6 %
$ZnSO_4$	0.26 µM	0.2 %
$CuSO_4$	0.12 µM	0.01 %

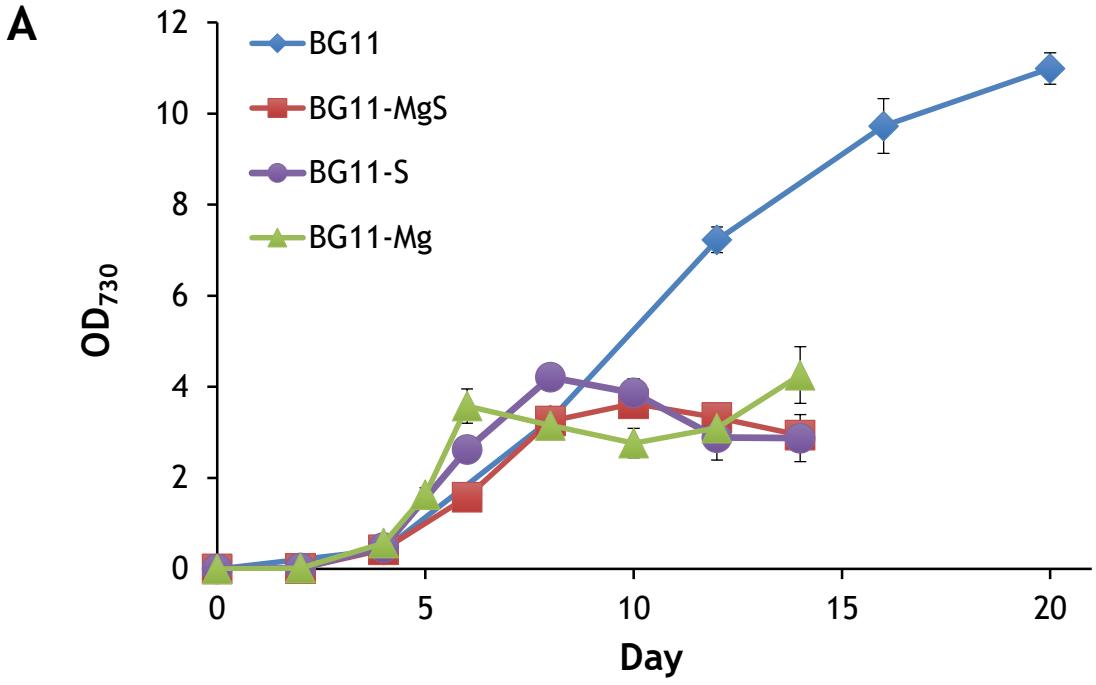

Figure 4-7. Growth of PCC 6803 in BG11-S and BG11-Mg

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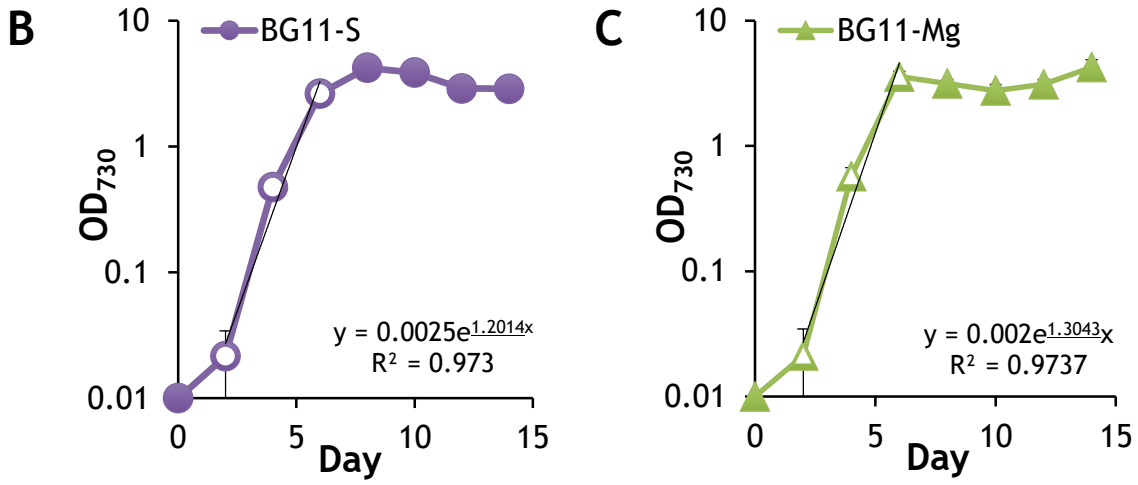

Figure 4-7. Growth of PCC 6803 in BG11-S and BG11-Mg

(continued from the previous page)

(A) Growth curve presenting optical density at 730 nm (OD_{730}) of PCC 6803 cultivated under standard (BG11) conditions or the indicated nutrient limitation in which the nutrient is supplied at 12.5 % relative to standard concentration. **(B-C)** Semi-logarithmic plots of growth under each nutrient condition. An exponential regression line was fitted to points during exponential growth (indicated with internal white boxes). The equation of the line and the coefficient of determination (R^2) derived from exponential growth points are presented on the graph. Growth rates were determined by the slope of the line (underlined). Data are presented as averages ± S.E.M of three independent cultures. Note error bars are often smaller than symbol size.

4.3 Discussion

4.3.1 Nutrient availability as a means to modulate growth kinetics in cyanobacteria

Cyanobacteria are complex organisms, which have evolved to cope with ever-changing environments prone to nutrient deprivation. A detailed understanding of the mineral nutrients affecting growth of cyanobacteria is important for the development of large-scale processes and provides a tool with which to investigate growth phase-specific responses. Nutrient deprivation studies in cyanobacteria have primarily focused on the effects of nitrate (N), phosphate (P), sulphate (S), iron (Fe) and carbon (C). Effects of these nutrients have been studied in isolation focusing on responses to nutrient deprivation, i.e. total elimination of the nutrient. Furthermore, these studies have often neglected to consider the effects of counter ions supplied with these nutrients, notably potassium (K) and magnesium (Mg) (Richaud et al., 2001, Krasikov et al., 2012, Fuszard et al., 2013). By contrast, I have compared the effects of poor supplies of individual components in the media on culture growth. The concentration for nutrient limitation was selected by characterising growth under a range of dilutions of BG11 media and a concentration was selected which resulted in a marked decrease in final density whilst still generating enough cell material for subsequent analyses. Specific components limiting growth within BG11 media were then identified and the effects of macronutrients and counter ions within these components characterised. This study utilised a single concentration to represent nutrient limitation and indeed a range of concentrations representing varying degrees of limitation as well as interactions between nutrients could be explored. Nevertheless, the obtained results are informative for the ultimate purpose to identify growth phase-specific regulators as we now have a range of backgrounds in which cultures of cyanobacteria transition from exponential into stationary phase for different reasons. Chapter 6 will describe the results obtained by sequencing RNA of cells harvested at early and late exponential phase across all of the nutrient-limited conditions identified in this chapter.

In agreement with the literature, the availability of N, P and S directly affects growth of PCC 6803 (Hirani et al., 2001, Richaud et al., 2001, Kim et al., 2011, Krasikov et al., 2012, Fuszard et al., 2013). This study is the first report of growth inhibition in response to potassium (K) and magnesium (Mg) limitation in PCC 6803. Both K and Mg are considered macronutrients for plant nutrition (Grusak, 2001) and indeed my results demonstrate that they are essential for cyanobacterial nutrition as well. In total, seven conditions were identified in which cultures cease growth in response to poor nutrient supplies and these findings are summarized in Table 4-3. These include two double nutrient-limited conditions (dipotassium phosphate, KP; magnesium sulphate, MgS) and five single nutrient-limited conditions (N, P, S, K, Mg). While effects on exponential growth rates as well as final culture density in different nutrient-limited conditions varied, none of the nutrient limited conditions inhibited exponential growth rates and all conditions resulted in premature growth arrest compared to standard nutrient conditions.

Table 4-3. Summary of growth effects in different nutrient conditions

Condition	Growth rate		Maximum density (OD_{730})	
	divisions day^{-1}	% rel to BG11	Mean ± S.E.M.	% rel to BG11
BG11	0.7239		11.00 ± 0.34	
BG11-KP	0.792	109.4	1.45 ± 0.01	13.2
BG11-MgS	1.225	169.2	3.63 ± 0.06	33.0
BG11-N	0.851	117.5	1.72 ± 0.08	15.6
BG11-P	0.835	117.2	4.91 ± 0.20	44.6
BG11-S	1.201	166.0	4.21 ± 0.25	38.3
BG11-Mg	1.304	180.1	3.15 ± 0.24	28.7
BG11-K	1.15	158.9	1.58 ± 0.15	14.4

4.3.2 Nitrate supply is a key consideration for process design of cyanofactories

Of the nutrients that were examined in this study, nitrate (N) availability had the most pronounced effect on final culture density compared with the other single nutrient-limited conditions. Given the relatively high N supply in BG11 (Table 4-1), this suggests that this is the most important factor for PCC 6803 growth. This finding agrees with the literature as nitrogen availability is the most limiting factor for primary production in nature, and photosynthetic organisms require more nitrogen than other nutrients (Vitousek and Howarth, 1991). In this study, I observed an abrupt transition from exponential growth into the death phase in which culture density rapidly declined. This was somewhat surprising given that cyanobacteria have evolved mechanisms to deal with N stress, notably the degradation of N-rich phycobilisomes (Grossman et al., 1993, Grossman et al., 1994, Schwarz and Forchhammer, 2005). One might therefore hypothesise that these stores would help sustain stationary phase cultures. Instead, my findings suggest that internal N stores are already consumed during the exponential phase in order to maintain exponential growth. This hypothesis is supported by the relatively pale colour of exponentially growing cultures in BG11-N compared to BG11 (data not shown), a common observation under N deprivation. The rapid decline in culture density following exponential growth suggests that cyanobacteria are not in fact able to cope with N deprivation. Assessment of pigment concentration throughout growth of PCC 6803 in BG11-N compared to BG11 could give insight to how cyanobacteria manage N supplies and inform process design of cyanobacterial cell factories as this can have direct impacts on both biomass generation and protein synthesis.

4.3.3 Cyanobacteria sense and respond to P-limitation before supplies run out

Phosphate (P) starvation has been reported to severely inhibit growth of PCC 6803 (Hirani et al., 2001, Fuszard et al., 2013). Even upon replacement of K, complete elimination of P resulted in a final density of just 10 % compared to cultures grown in the presence of P (Hirani et al., 2001). While poor P supplies also inhibited growth in my experiments, the effect was less severe than previously reported. Cells cultivated in BG11-P were able to maintain growth for an extended period (up to day 18) compared with other nutrient-limited conditions (up to day 6-10). While BG11-P produced a final culture density comparable to that of BG11-S and BG11-Mg, relatively slow exponential growth rates enabled the prolonged growth phase (Table 4-3). It is important to note that this is not due to a decrease in BG11-P growth rates but rather the marked increase in BG11-Mg and BG11-S growth rates.

External P levels may influence growth rates and cyanobacteria do in fact sense and respond to phosphate levels via the SphS-SphR two-component signal transduction system (Hirani et al., 2001). These systems allow bacteria to monitor external conditions and adjust to changing environments accordingly (Parkinson and Kofoid, 1992). In PCC 6803, the histidine kinase P sensor SphS monitors external P levels via an extended N-terminal region spanning the cell membrane (Burut-Archanai et al., 2009). Upon detection of limited P supplies, a histidine residue is phosphorylated in the carboxy-terminal transmitter motif. The phosphoryl group is transferred to a specific aspartate residue on the amino-terminal receiver motif of the cognate response regulator SphR. A DNA-binding domain located in the C-terminus of the response regulator induces P-responsive genes such as the phosphate-specific transport systems Pst1 and Pst2. Given the reduction in growth rate observed under P-limitation in both this study as well a previous report (Hirani et al., 2001), genes involved in growth are likely targets of SphR or downstream regulators. These genes could be interesting targets to enhance biomass production rates and furthermore serve as markers to indicate diminishing supplies of a key component for product synthesis.

4.3.4 Potassium maintains turgor pressure and shape of bacterial cells

Potassium (K) is the major intracellular cation in bacteria. While K is not incorporated into chemical compounds, it serves important roles including maintenance of osmotic pressure and pH, enzyme activation and as a second messenger to stimulate compatible solute accumulation (Epstein, 2003). Notably, osmotic pressure creates cellular hydrostatic forces known as turgor pressure, which is essential for growth and determination of the shape of bacterial cells. As K is not metabolised, there are no intracellular reserves from which to scavenge K to increase turgor pressure in growing cells providing a possible explanation for the abrupt halt in growth observed under K-limitation (Figure 4-6C).

K is also important to maintain intracellular pH and, while the mechanism of pH homeostasis is not clearly understood in cyanobacteria, lack of control of intracellular pH results in growth limitation in the heterocystous, filament-forming cyanobacterium *Anabaena* spp. 7120 (Giraldez-Ruiz et al., 1997). In this case, calcium (Ca) supplies under low pH conditions were used to modulate intracellular pH. In my nutrient limitation screen, Ca-limitation (as $CaCl_2$; Figure 4-4) had no noticeable effects on growth, however accurate quantification in a more controlled setting may reveal subtle growth effects. Nevertheless, imbalances in pH homeostasis arising from K-limitation may be a contributing factor to the growth effects observed in this study.

Growth inhibition following transfer to K-deficient media has also been observed in the filamentous cyanobacterium *Anabaena torulosa* (Alahari and Apte, 1998). K-starved cells were able to recover growth upon resupply of K. The actual cause of growth arrest was not determined, however decreases in both photosynthetic function and protein synthesis were observed following K deprivation. These may be general responses arising from growth arrest, however, as cyanobacteria shut down anabolic pathways and enter a “stand-by” metabolic mode (Schwarz and Forchhammer, 2005).

Interestingly, K does not appear to be important to maintain stationary phase in PCC 6803. BG11-K was the only single nutrient-limited condition in which maximum culture density was steadily maintained for an extended period following the cessation of exponential growth. Furthermore, the double-nutrient limited condition BG11-KP showed a similar effect. By contrast, all other conditions resulted in a (often immediately) rapid decline in culture density once exponential growth has stopped. These findings suggest that while K essential for growth, it is not necessary for stationary phase viability. This opens an interesting possibility for industrial applications, as K supply can be a means to determine culture density and thus control timing of entry to stationary phase.

In summary, K is important to support biomass production by maintaining turgor pressure in growing cells and has potential as a tool to control time of entry into the production phase before essential nutrients are exhausted in batch cultures.

4.3.5 Magnesium is a key factor coordinating cell growth and metabolism

Magnesium (Mg) is the second most abundant cation in bacteria after potassium and plays a critical role in membrane stabilisation, ribosomes and as a cofactor in enzymatic reactions such as DNA replication and transcription. Furthermore, in photosynthetic organisms, Mg is a core component of the most abundant pigment, chlorophyll, and has been proposed as the dominant counterion to conserve the pH gradient and transmembrane potential of thylakoids in plants (Barber et al., 1974). In mammalian cells, Mg is the key factor coordinating cell growth and metabolism. It acts as a sensor for cell size and regulates cell division by timing the transition between cell cycle phases, notably entry to S phase during which DNA replication occurs (Wolf and Cittadini, 1999). A general feature of Mg-limitation in bacteria is the enlargement of cells that may be the result of continued growth in spite of the inability to progress through the cell cycle (Groisman et al., 2013). In agreement, the most pronounced effect of Mg-limitation in the filamentous cyanobacterium *A. nidulans* was increased cell size resulting from continued DNA, protein and cell mass synthesis despite inhibited cell division (Utkilen, 1982). This was attributed to an inhibition in cell wall

lipoprotein synthesis in which Mg plays an important role to neutralise the negative charge of phosphate residues.

Similar to phosphate, bacteria are able to sense intra- and extracellular Mg levels using a two-component system. The PhoP/PhoQ system of *Salmonella enterica* was the first signal transduction system to be identified and characterised in which an ion, namely Mg, acts as a first messenger (Garcia Véscovi et al., 1996). In this case, Mg ions form a metal bridge between negative residues in the periplasmic domain of PhoQ and the phospholipid head groups of the cytoplasmic membrane. Absence of Mg destabilised PhoQ resulting in PhoP phosphorylation and subsequent regulation of Mg-responsive genes. Mg sensing systems have not been described for cyanobacteria, and my results suggest that PCC 6803 are not able to detect and respond to reduced Mg availability early in culture growth. Given that PCC 6803 are not able to maintain high culture density during stationary phase once Mg supplies have run out, it will be important to ensure sufficient supplies of Mg are provided both during the growth and production phases of cyanobacterial cell factories.

4.3.6 Quantification of growth in cyanobacteria

There are numerous approaches to quantify growth of cyanobacterial cultures. For my experiments, I selected optical density, as this was a relatively fast and easy method to assess changes in growth. Importantly for synthetic biology applications, this can be measured using standard laboratory equipment and is relatively comparable across laboratories. Nevertheless, this may not always be the best approach. While a linear relationship was observed between optical density and cell number from a single culture grown under standard conditions, the relationship between these two variables may not remain constant under different conditions. This is due to the fact that optical density reflects a number of factors including cell number, cell size and particulate matter in the media, which may arise in applications such as bioproduction. Optical density may therefore increase even if cell numbers remain the same. This may be the case in BG11-Mg, for instance, where cell elongation is a common response in bacteria (Groisman et al., 2013). Nevertheless, optical density is a good measure

for biomass as it reflects the combined information of both cell size and number within a culture. This is perhaps the most interesting parameter for practical applications and aids in our purpose to identify promoters specifically active once maximum biomass has been achieved.

In summary, seven different nutrient conditions (two double nutrient-limited and five single nutrient-limited conditions) have been identified in which PCC 6803 ceases growth in response to different components in the media. Indeed, mineral nutrients are just one factor that may affect culture growth and one could have assessed growth effects under variable light intensity, carbon availability as well as heterotrophic conditions. For interrogating growth-phase specific expression, however, these conditions will suffice. Few reports describe late growth of cyanobacterial cultures, so nutrient limitation can provide a useful tool to dissect the various growth phases in cyanobacteria. Importantly, by identifying common responses across a range of conditions in which cyanobacteria have ceased growth, we can be more confident that these responses are indeed a result of changes in growth as opposed to particular stresses. Chapter 5 will investigate expression patterns of published growth phase-specific genes by qPCR and Chapter 6 will identify co-regulated gene clusters by RNA sequencing using the conditions established in this chapter.

Chapter 5. Testing published growth phase-specific genes

5.1 Introduction

Promoters are important regulatory elements for high-level gene expression and recombinant protein production. As discussed in *Section 4.1*, growth phase-specific promoters are a particularly attractive tool to regulate gene expression as they do not require the addition of costly and often toxic supplements to induce or repress expression but rather respond to endogenous cues induced by changes in growth. For this purpose, it is useful to divide growth into two distinct phases. First, the exponential growth phase during which cells are actively growing and dividing (hereafter "early growth"). Then as environmental factors becoming limiting during late exponential and stationary phase growth, cells eventually cease growth (hereafter "late growth"). The late growth phase is typically the optimal time for engineered processes to occur, as this is when maximum biomass has been achieved. Promoters specifically active during late culture growth are desirable as they avoid interference of transgene expression with culture growth. A key feature of promoters for industrial applications is tight regulation and efficient silencing during non-induced conditions. Furthermore, promoters should be robust in the sense that they are specifically activated by the desired stimulus, in this case growth phase. Thus late growth-specific promoters should not be active during early culture growth and be robustly activated during late growth irrespective of the particular factor which is limiting further growth.

Growth phase-specific expression systems have been developed for prokaryotes including *E. coli*, *Bacillus subtilis* and *Pseudomonas putida* to name a few (Miksch and Dobrowolski, 1995, Cases et al., 1996, Schumann, 2007). These systems typically rely on "sigma switching" whereby the RNA polymerase holoenzyme alters its promoter specificity in response to environmental stimuli by replacing the primary sigma factor with alternative sigma factors. In this way,

bacteria can alter gene expression over a wide range of different functions to enable acclimation to changing environmental conditions. Thus, promoters specifically recognized by alternative sigma factors involved in the regulation of growth phase-specific gene expression are a valuable tool for regulating large-scale processes. In *E. coli*, late growth-specific expression is regulated by the alternative sigma factor, RpoS (σ^S) (Hengge-Aronis, 2002). Intracellular levels of σ^S increase as cells cease growth prior to entry to stationary phase and this factor subsequently induces the expression of some 30 genes upon entry to stationary phase (Jishage and Ishihama, 1995). Based on the consensus sequence of promoters regulated by σ^S, libraries of synthetic promoters for late growth-specific induction have been constructed and optimized for *E. coli* (Miksch et al., 2005).

As usual, cyanobacteria are lagging behind its prokaryotic peers. Efforts have been made towards unravelling late growth-specific gene expression and its regulation, however growth-phase specific promoters are still lacking. A few alternative sigma factors have suggested roles in regulating late growth-specific expression, however a general late growth regulator remains to be identified (Osanai et al., 2008, Imamura and Asayama, 2009). Furthermore, target promoters of factors implicated in regulating late growth gene expression have not yet been identified. Microarray analyses have revealed genes that follow late growth-specific expression patterns. However, these studies compared changes in gene expression across a limited number of conditions. First, late exponential phase-specific genes were identified in two genotypes, wild type and *ΔsigB*, which have virtually identical growth kinetics (Foster et al., 2007). Second, stationary phase-specific genes were identified using two conditions, autotrophic and mixotrophic cultures, with very different growth kinetics (Berla and Pakrasi, 2012). These studies can inform the identification of late growth-specific promoters and indeed candidates have been suggested. However, validation is required to confirm the robustness of the growth-specific expression patterns under a range of conditions.

5.1.1 Chapter aim

This work aimed to assess the robustness of candidate genes exhibiting late growth-specific expression patterns identified in previous microarray studies (Berla and Pakrasi, 2012, Foster et al., 2007). The previous chapter identified a range of backgrounds in which cultures of cyanobacteria ceased growth for different reasons, thus providing additional conditions with which to explore transcriptional changes in response to growth. Transcript levels of the candidate genes were measured by quantitative real-time PCR (qPCR) analysis throughout culture growth in different conditions including standard nutrient conditions (BG11) and double nutrient-limited conditions, 12.5 % $MgSO_4$ (BG11-MgS) and 12.5 % K_2HPO_4 (BG11-KP). Genome-wide oscillations in transcription occur because of circadian rhythm and in PCC 6803 up to 9 % of genes oscillate in expression during a 24-hour period (Kucho et al., 2005). In order to avoid circadian oscillations, all samples were harvested midway through the 12 hour day period, i.e. 6 hours after the lights turned on. Phenol-chloroform extraction was performed to isolate RNA from frozen cell pellets and cDNA template was generated using a random priming strategy. The identification of genes that are robustly regulated by changes in growth can serve to a) identify candidate growth phase-specific promoters and b) serve as markers to indicate growth phase of cells harvested at different times during growth.

5.2 Results

5.2.1 Identification of a suitable reference gene for qPCR analysis

Expression of candidate growth phase-specific genes was assessed by measuring transcript levels in cultures grown under different nutrient conditions by quantitative real-time PCR (qPCR). This technique enables accurate quantification of expression levels of a limited number of genes in different samples. However, to account for variation during sample preparation and quantification, the values obtained for the test genes need to be normalized to a constitutive reference gene in the same sample. The expression levels of common reference genes may vary considerably depending on the samples and experimental conditions (Thellin et al., 1999, Dheda et al., 2004). It was therefore necessary to identify a suitable reference gene that was stably expressed throughout growth of cyanobacterial cultures under the different conditions. geNorm software was used to assess the stability of a selection of candidate reference genes. This algorithm determines the pairwise variation of each gene with all other genes based on their crossing threshold (C_t) values across a representative set of samples (Vandesomepele et al., 2002). Genes with the greatest variation are sequentially excluded until two genes with the most stable expression levels are identified.

Table 5-1 presents the candidate reference genes interrogated in nine samples consisting of PCC 6803 cells harvested at three different times during growth (days 4, 8 and 12) under three different nutrient conditions (BG11, BG11-MgS and BG11-KP). The list includes commonly used reference genes for normalisation of qPCR data in PCC 6803 and the primary sigma factor SigA, which maintains constant expression levels irrespective of growth phase in PCC 6803 (Imamura et al., 2003b). Reference genes for *Bacillus anthracis* were also interrogated. Reference genes for *E. coli* (*cysG, hcaT, idnT* and *ihfB*) (Zhou et al., 2011) were also considered but homologs for these genes could not be identified in PCC 6803 using the Basic Local Alignment Search Tool (BLAST).

geNorm identified *psbA1* and *adk* as the best reference genes for this particular experiment (Figure 5-1). As *psbA1* is the most commonly reported reference gene for qPCR studies in PCC 6803, this gene was selected as reference in the subsequent experiments.

Table 5-1. Candidate reference genes for qPCR in PCC 6803

Gene	Description	Organism	Reference
rrn16Sa	16S ribosomal RNA	PCC 6803	Pinto et al., 2012
rnpB	RNA subunit of ribonuclease P	PCC 6803	Pinto et al., 2012
petB	Cytochrome b_6	PCC 6803	Pinto et al., 2012
rpoA	RNA polymerase, alpha subunit	PCC 6803	Pinto et al., 2012, Lemeille et al., 2005
psbA1	Photosystem II D1 protein	PCC 6803	Foster et al., 2007
sigA	Primary sigma factor	PCC 6803	Imamura et al., 2003b
pta	Phosphotransacetylase	*B. anthracis*	Reiter et al., 2011
adk	Adenylate kinase	*B. anthracis*	Reiter et al., 2011

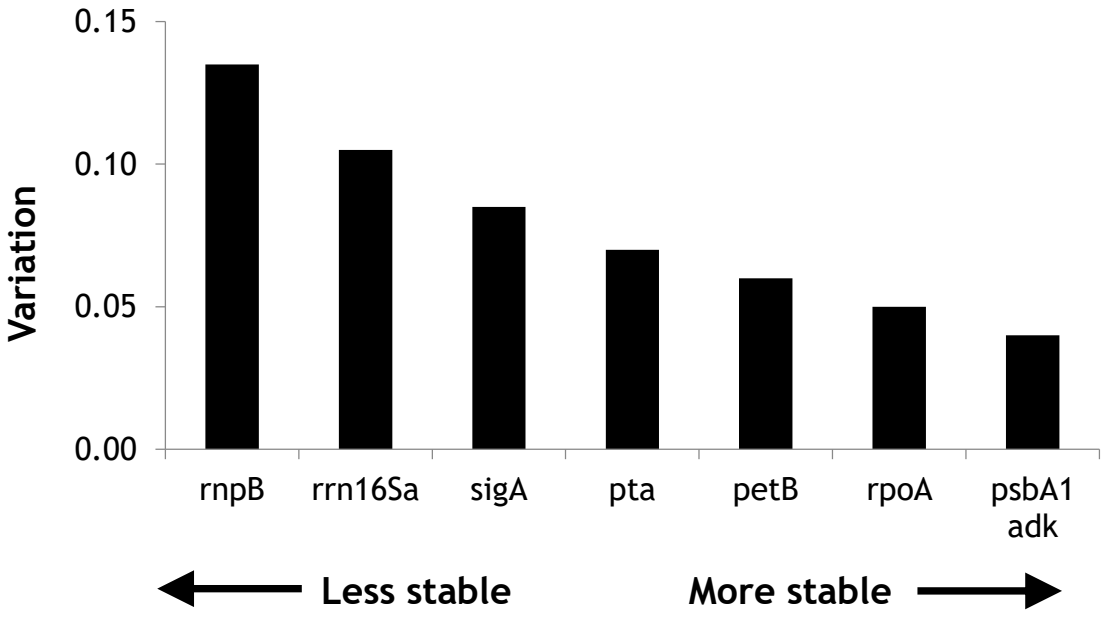

Figure 5-1. Stability of candidate reference genes throughout growth of PCC 6803

Variation of C_t values of candidate reference genes determined by qPCR and geNorm analysis in nine samples consisting of PCC 6803 harvested at 3 different times during growth (days 4, 8 and 12) under three different nutrient conditions (BG11, BG11-MgS and BG11-KP).

5.2.2 Expression profiles of sigma factors implicated in regulation of late growth gene expression

In many species of bacteria, sigma factors are key components in the regulation of late growth-specific gene expression by altering promoter specificity of the RNA polymerase holoenzyme (Miksch and Dobrowolski, 1995, Cases et al., 1996, Schumann, 2007). Three alternative sigma factors are implicated in regulating late growth-specific expression in PCC 6803. SigC regulates stress-related regulons important for stationary phase survival (Asayama et al., 2004). A more general role for SigC in stationary phase-specific gene regulation remains to be demonstrated, however. SigB, on the other hand, is implicated in general regulation of late exponential phase-specific gene expression (Foster et al., 2007). Gene inactivation of *sigB* modulated differential gene expression in PCC 6803 during the transition from exponential to stationary phase growth including the down-regulation of *sigC* transcript. Furthermore, this study demonstrated a potential role of SigH in the regulation of late growth-specific gene expression. Regulation of sigma factors has been demonstrated at the transcript level by qPCR analysis in PCC 6803 sigma mutants (Lemeille et al., 2005). Thus, sigma factors involved in regulating late growth gene expression may serve as useful indicators that cultures are preparing to enter stationary phase growth and as tools to identify target promoter sequences during this transition.

The group 2 factor SigC is important for survival during stationary phase growth in PCC 6803 (Asayama et al., 2004). Gene disruption of *sigC* results in a significant reduction in cell viability during the stationary phase compared with wild-type cells as demonstrated by viable cell counts on BG11 plates, trypan blue staining and visualization of lysed cells by transmission electron microscopy. SigC regulates stationary phase-specific expression of nitrogen-related genes in response to nitrogen status (Asayama et al., 2004) and mediates stress responses such as heat acclimation (Tuominen et al., 2008).

Up-regulation of *sigC* expression during the late exponential phase and again during the late stationary phase has been demonstrated by primer extension, qPCR and microarray analyses (Lemeille et al., 2005, Imamura et al., 2003b, Foster et al., 2007). This reported expression pattern was not observed in cultures of PCC 6803 grown in standard BG11 in our laboratory, however (Figure 5-2A). *sigC* transcript levels oscillated throughout growth with peaks in expression occurring on different days across three independent experiments (Figure 5-2B). Furthermore, there was no significant difference in expression levels when comparing transcript levels to that of day 4 as determined by Student's t-tests.

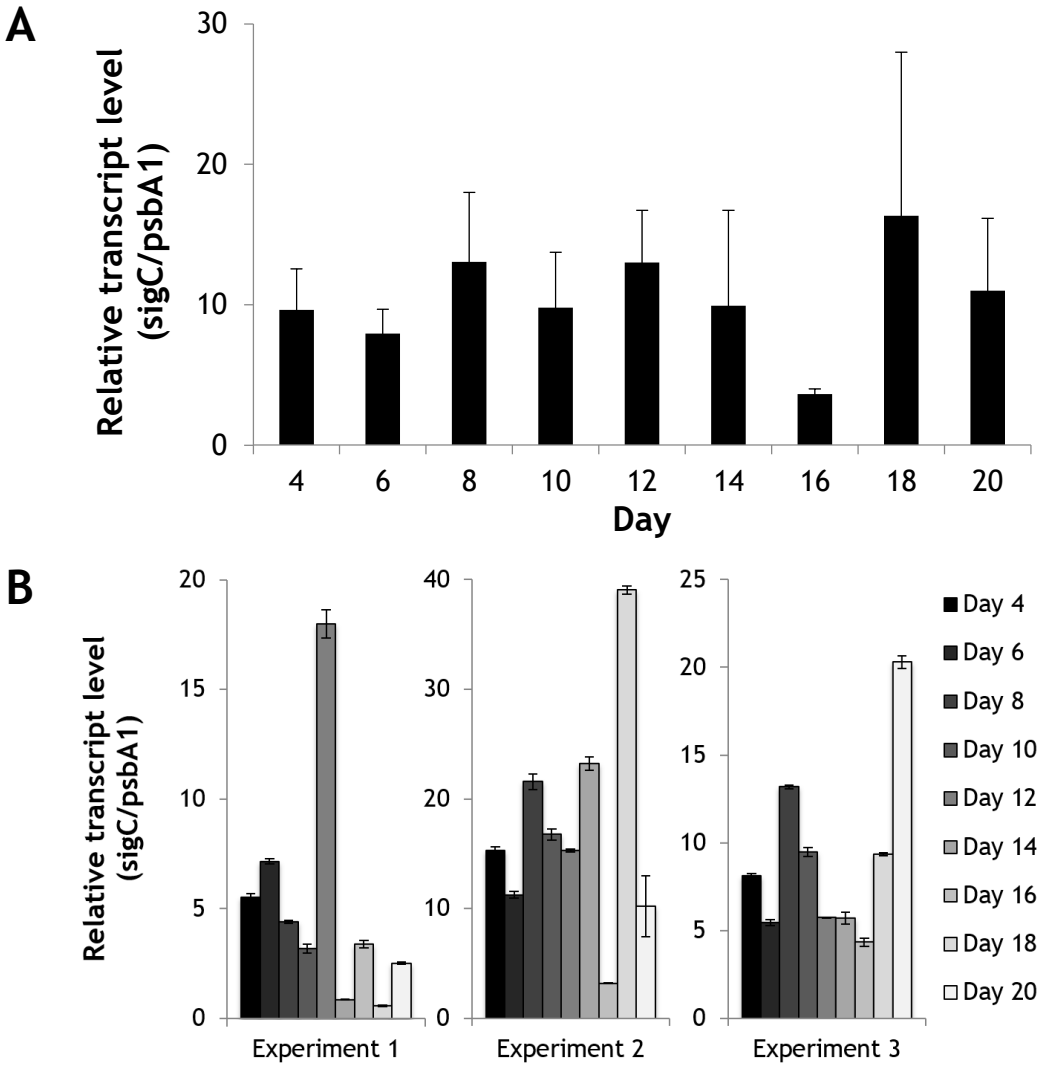

Figure 5-2. Relative transcript levels of *sigC* in BG11

see overleaf for the full figure legend

Figure 5-2. Relative transcript levels of *sigC* in BG11

RNA was isolated throughout growth of PCC 6803 under standard nutrient conditions. Transcript levels were normalised to the reference gene *psbA1* and are presented as means ± S.E.M. of **(A)** three independent experiments and **(B)** technical replicates within each experiment.

SigH belongs to the alternative group 3 sigma factors of which little is currently known (Imamura and Asayama, 2009). Northern blot analyses demonstrate a 30-fold increase in *sigH* transcript following heat stress. This factor is not involved in the immediate response to heat stress however, as heat shock response genes such as *groEL* are induced prior to SigH (Huckauf et al., 2000). While SigH function remains unclear, a role in regulating late growth gene expression has been suggested (Foster et al., 2007). Transcript analyses support a role during late growth as primer extension, qPCR and microarray analyses have demonstrated up-regulation of *sigH* transcript in late exponential and stationary phase cultures of PCC 6803 (Imamura et al., 2003b, Lemeille et al., 2005, Foster et al., 2007). In fact, *sigH* showed the greatest response in a microarray study comparing gene expression in late against early exponential growth (11-fold up-regulation). Despite multiple reports of *sigH* induction during late growth, this expression pattern was not observed across three independent cultivations of PCC 6803 grown in standard BG11 in our laboratory (Figure 5-3). *sigH* transcript levels oscillated throughout growth and highly variable up-regulation was observed at the last time point, day 20, with 1.5- to 7-fold up-regulation compared to day 4. The difference was not statistically significant as determined by Student's t-tests.

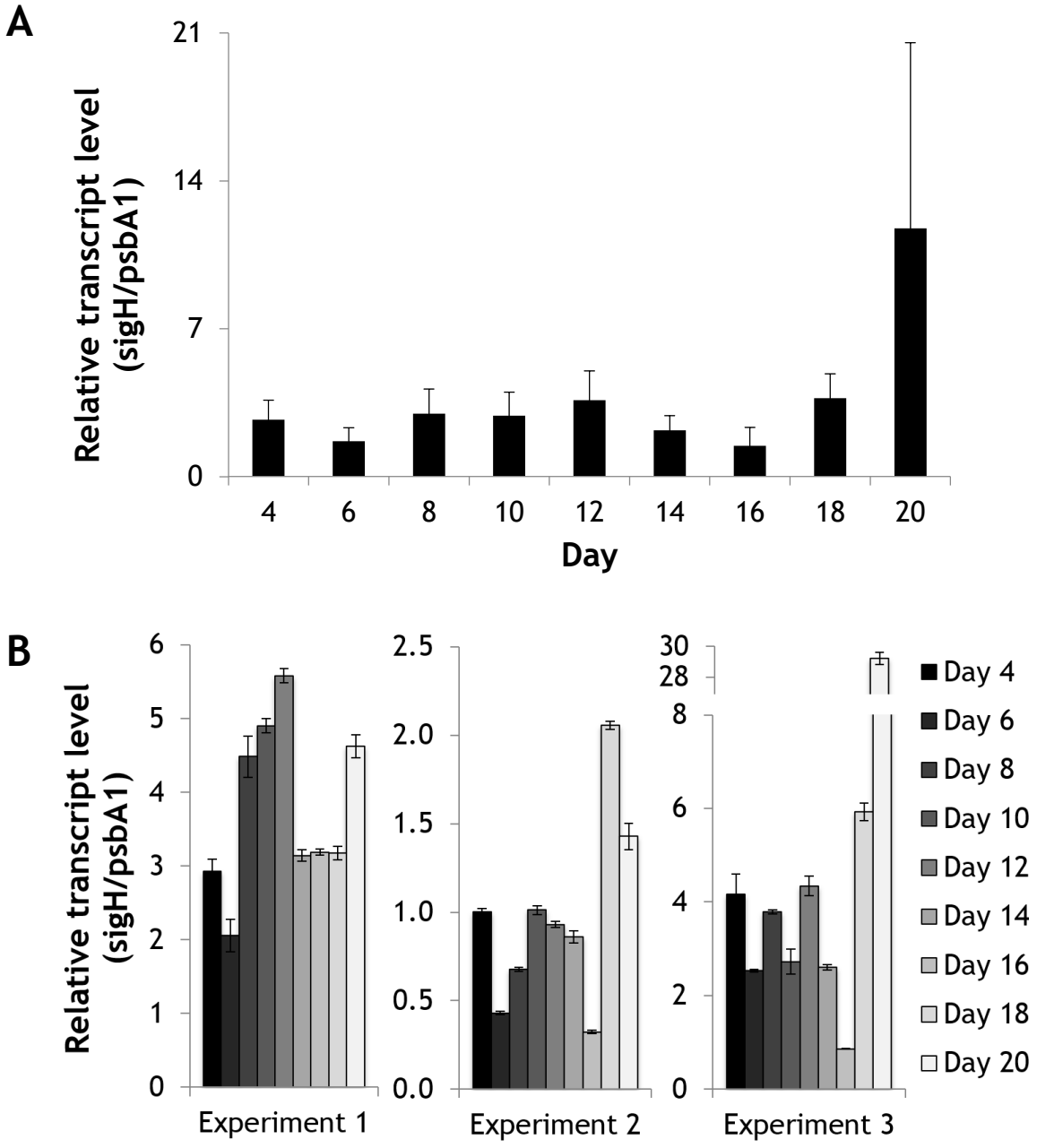

Figure 5-3. Relative transcript levels of *sigH* in BG11

RNA was isolated throughout growth of PCC 6803 under standard nutrient conditions. Transcript levels were normalised to the reference gene *psbA1* and are presented as means ± S.E.M. of **(A)** three independent experiments and **(B)** technical replicates within each experiment.

5.2.3 Expression profile of the iron-stress-inducible gene isiA

The gene encoding the iron-stress-inducible chlorophyll-binding protein IsiA was identified as a late growth-specific gene through microarray analysis of two genotypes, wild type and *ΔsigB* PCC 6803 (Foster et al., 2007). This gene was significantly up-regulated during late exponential growth with 8.7-fold increase in expression levels compared with mid-exponential cultures. Importantly, northern blot analysis demonstrated a high specificity for *isiA* expression during the late exponential phase as little to no transcript was observed in early exponential or stationary phase cells. PCC 6803 grown under standard conditions in our laboratory reproduced the late growth phase-specific expression pattern. Significant up-regulation was observed on day 8 ($p=0.029$) followed by a decrease in *isiA* transcript levels later in culture growth (Figure 5-4A). While variation was observed in transcript levels relative to the reference gene, the expression pattern was highly reproducible across experiments and specific to this point in growth under standard conditions (Figure 5-4B).

As suggested by the name, iron-stress-inducible expression of *isiA* is well described across cyanobacteria including PCC 6803 (Laudenbach et al., 1988, Leonhardt and Straus, 1992, Vinnemeier et al., 1998). It was therefore necessary to determine whether *isiA* expression was induced in response to changes in growth as opposed to the concentration of iron in the media. Gene expression was interrogated in cultures grown under $MgSO_4$ limitation (BG11-MgS) where cells prematurely cease growth compared to standard conditions (refer to Figure 4-5E and 4-5B respectively). As a result, changes in growth phase occur before cells experience iron depletion in BG11-MgS. The growth phase-specific expression profile of *isiA* was lost in cultures grown in BG11-MgS and overall *isiA* transcript levels were much lower than those observed in cultures grown in standard BG11 (Figure 5-5). A gradual increase in *isiA* transcripts was observed as cultures cease growth on day 8 (approximately 7-fold compared to day 5). Elevated transcript levels were maintained throughout late growth until day 12 at which point further up-regulation was observed (approximately 15-fold increase compared to day 5). No significant difference in expression levels

compared to day 5 was detected using Student's t-tests due to the variation observed across the three experiments (Figure 5-5B).

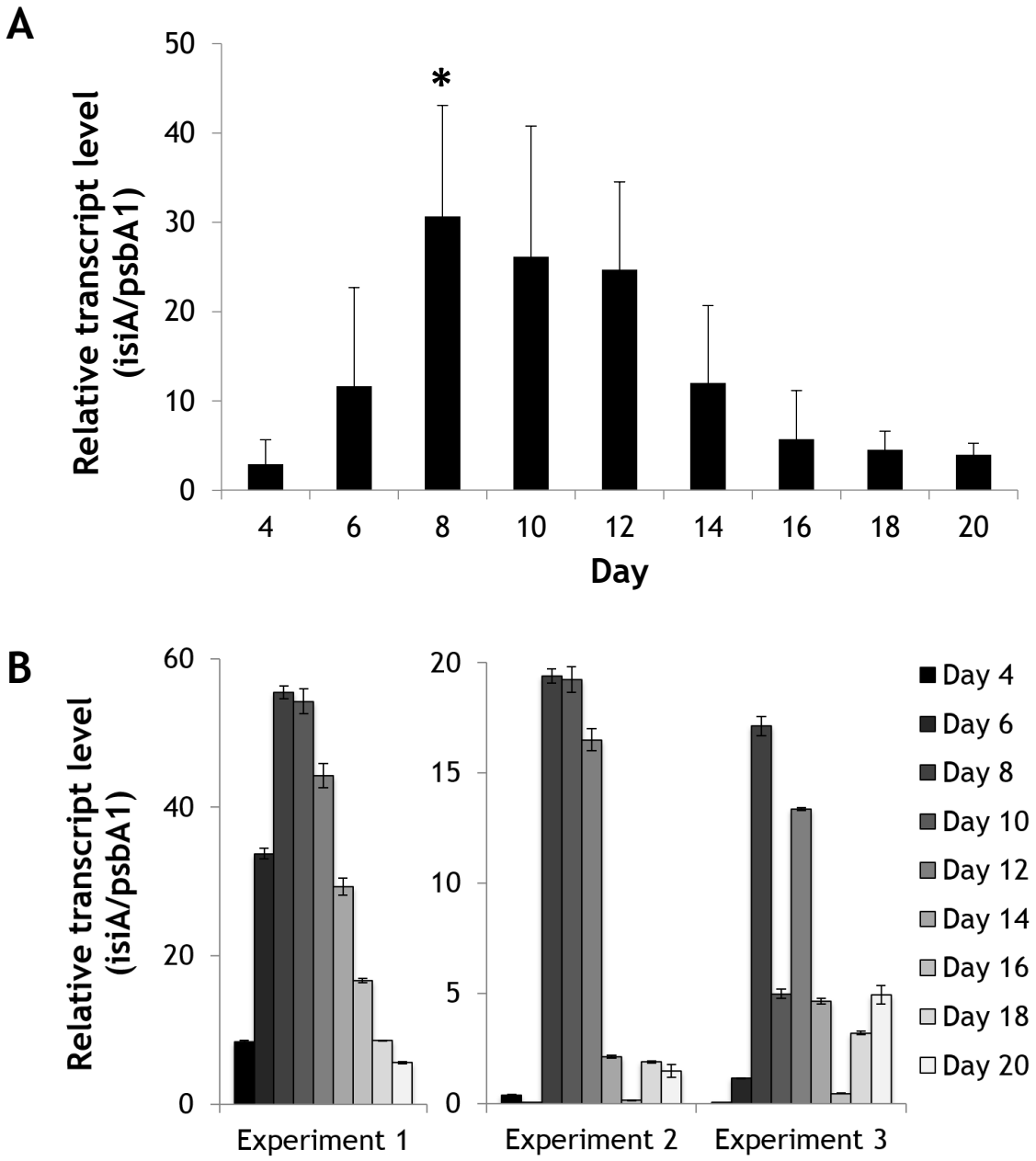

Figure 5-4. Relative transcript levels of *isiA* in BG11

RNA was isolated throughout growth of PCC 6803 under standard nutrient conditions. Transcript levels were normalised to the reference gene *psbA1* and are presented as means ± S.E.M. of **(A)** three independent experiments and **(B)** technical replicates within each experiment. Asterisk indicates a significant difference compared to expression on day 4 ($p<0.05$) determined by Student's t-test.

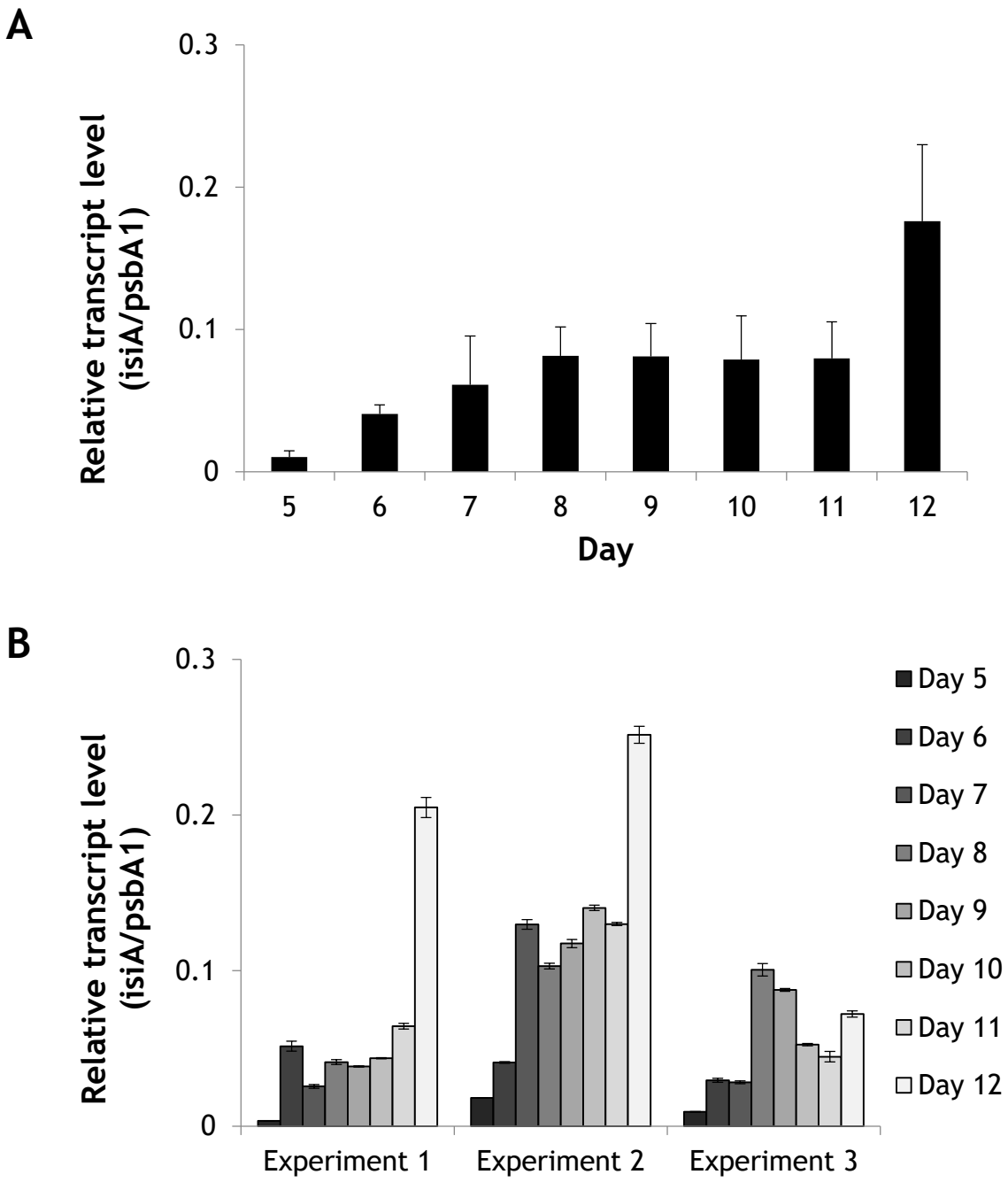

Figure 5-5. Relative transcript levels of *isiA* in BG11-MgS

RNA was isolated throughout growth of PCC 6803 in the presence of 12.5 % $MgSO_4$ relative to the standard concentration (BG11-MgS). Transcript levels were normalised to the reference gene *psbA1* and are presented as means ± S.E.M. of **(A)** three independent experiments and **(B**) technical replicates within each experiment.

In summary, the expression pattern of *isiA* under nutrient limitation does not agree with the late exponential phase-specific expression pattern suggested by Foster et al. Given that the expression levels of *isiA* are known to correlate with iron availability and do not show a consistent growth-specific expression pattern across our test conditions, this gene was not taken forward for further analysis.

5.2.4 Expression profiles of plasmid-encoded genes

The induction of plasmid-encoded genes during late growth was demonstrated by microarray analysis comparing gene expression in exponential and stationary phase cultures of PCC 6803 (Berla and Pakrasi, 2012). Plasmid copy number often increases during stationary phase growth, however, so it was necessary to ensure that increased levels of transcription were a result of promoter activity as opposed to gene dosage. Candidate genes were therefore selected from plasmids that maintain stable copy numbers irrespective of growth phase. Berla and Pakrasi assessed the potential utility of promoters by calculating a stationary phase promoter score (SPPS) based on the following equation:

$$\text{SPPS} = \log_2(\text{fold change}) + \log_2(\text{normalised expression})$$

The genes with the highest SPPS were *sll7077*, which resides on the large pSysA plasmid, and *slr9003* on the small pCC5.2 plasmid. Under my experimental settings, *sll7077* expression was variable and little up-regulation was observed during late growth across three independent cultivations of PCC 6803 in standard BG11 (Figure 5-6). On the other hand, *slr9003* showed a slightly more consistent up-regulation during later time points although Student's t-tests failed to identify any significant differences in expression levels compared to day 4 due to large variation across the three experiments (Figure 5-7). Up-regulation was also observed during earlier time points at which point cultures were still in exponential growth. To determine whether late growth up-regulation of *slr9003* was the result of changes in growth, transcript levels were measured in the double nutrient-limited condition BG11-MgS. In this condition, significant up-regulation was observed during late growth on days 8 ($p=0.012$) and 12 ($p=0.017$; Figure 5-8A). The expression pattern was also more consistent across the three

replicates in this condition (Figure 5-8B). Similarly, cultures grown in the double nutrient-limited condition BG11-KP exhibited significant up-regulation on day 8 compared to day 4 ($p=0.019$), however the magnitude of up-regulation was less than that observed under the other nutrient conditions (Figure 5-9). Furthermore, in contrast to the other nutrient conditions, *slr9003* induction is primarily observed at earlier time points and elevated transcript levels are not maintained later in growth in BG11-KP.

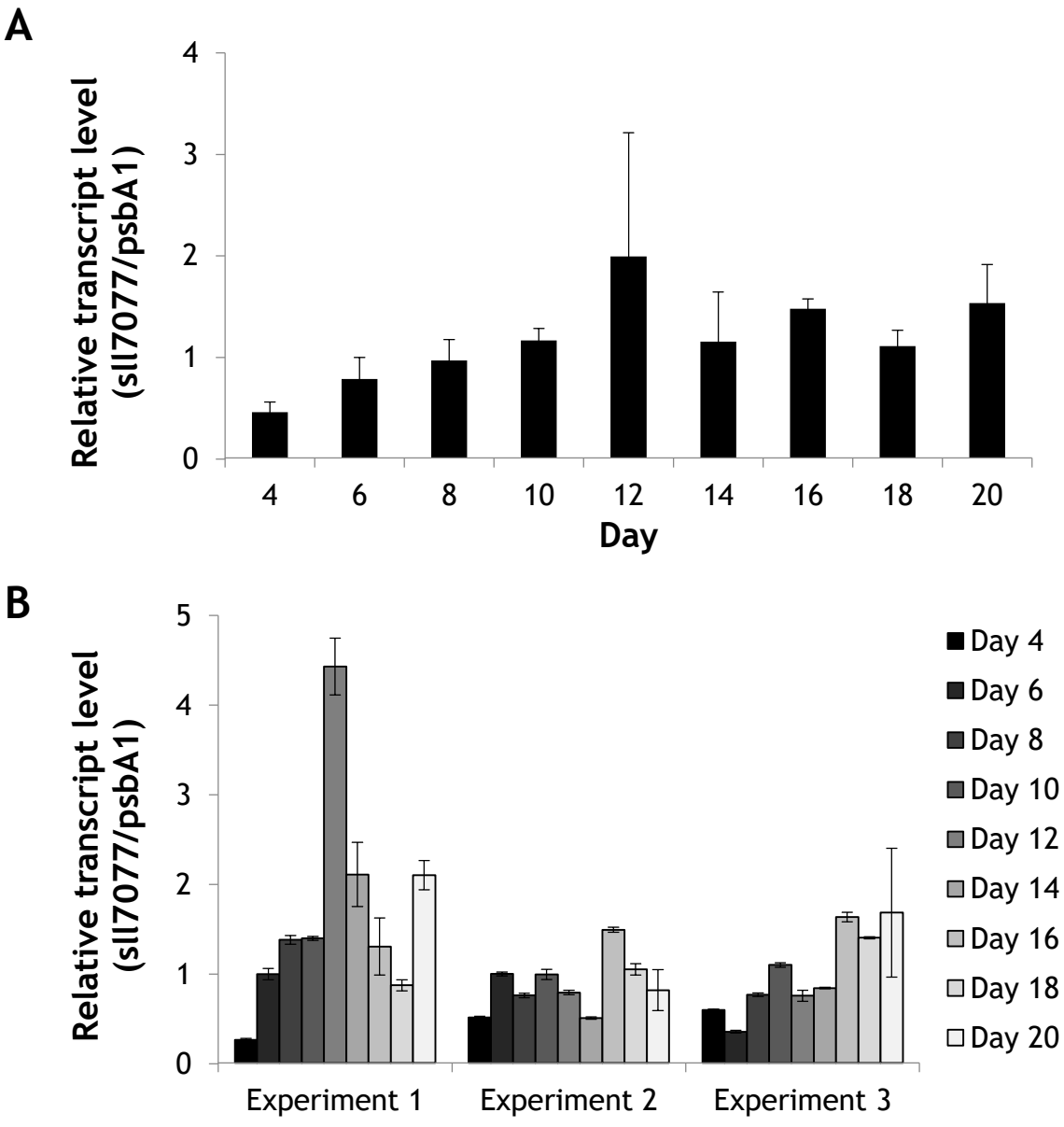

Figure 5-6. Relative transcript levels of *sll7077* in BG11

RNA was isolated throughout growth of PCC 6803 under standard nutrient conditions. Transcript levels were normalised to the reference gene *psbA1* and are presented as means ± S.E.M. of **(A)** three independent experiments and **(B)** technical replicates within each experiment.

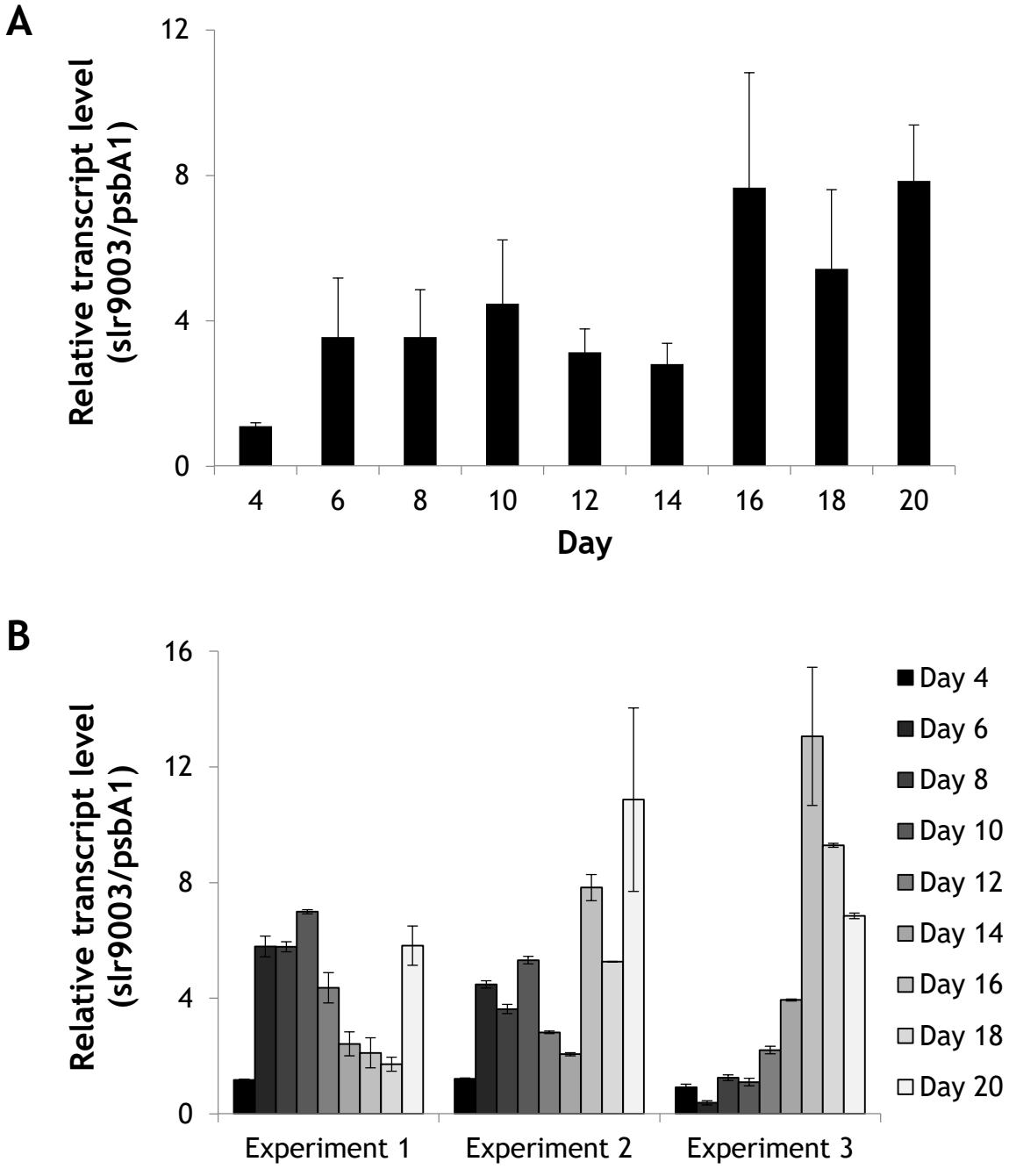

Figure 5-7. Relative transcript levels of *slr9003* in BG11

RNA was isolated throughout growth of PCC 6803 under standard nutrient conditions. Transcript levels were normalised to the reference gene *psbA1* and are presented as means ± S.E.M. of **(A)** three independent experiments and **(B)** technical replicates within each experiment.

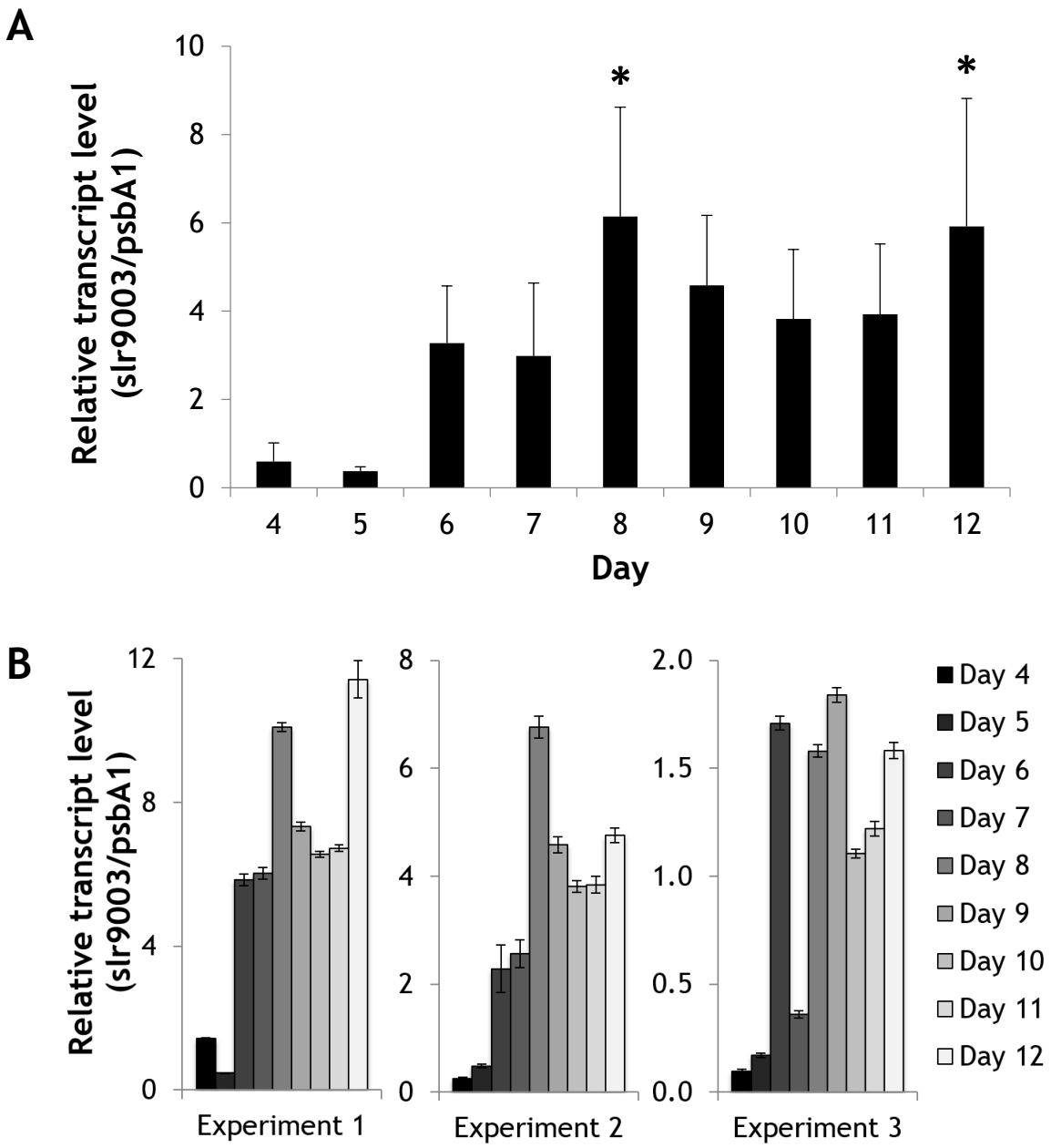

Figure 5-8. Relative transcript levels of *slr9003* in BG11-MgS

RNA was isolated throughout growth of PCC 6803 in the presence of 12.5 % $MgSO_4$ relative to the standard concentration. Transcript levels were normalised to the reference gene *psbA1* and are presented as means ± S.E.M. of **(A)** three independent experiments and **(B**) technical replicates within each experiment. Asterisk indicates a significant difference compared to expression on day 4 ($p<0.05$) determined by Student's t-test.

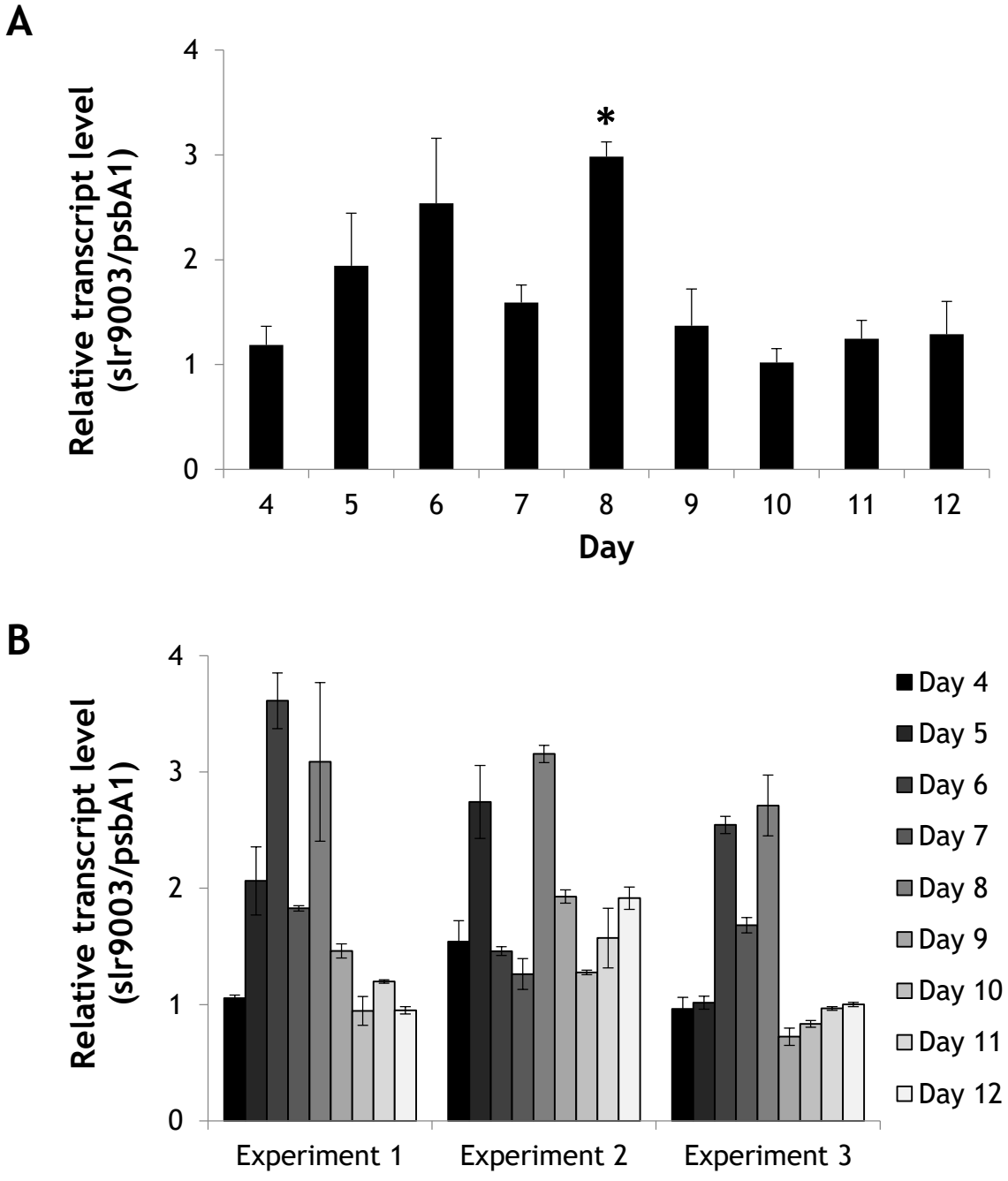

Figure 5-9. Relative transcript levels of *slr9003* in BG11-KP

RNA was isolated throughout growth of PCC 6803 in the presence of 12.5 % K_2HPO_4 relative to the standard concentration. Transcript levels were normalised to the reference gene *psbA1* and are presented as means ± S.E.M. of **(A)** three independent experiments and **(B**) technical replicates within each experiment. Asterisk indicates a significant difference compared to expression on day 4 ($p<0.05$) determined by Student's t-test.

5.3 Discussion

A key characteristic of inducible promoters for synthetic biology applications is the robust activation of gene expression in response to a specific stimulus. Ideally, these promoters should maintain this specific behaviour irrespective of the context in which they are applied. This enables predictable engineering at higher levels of the abstraction hierarchy without concern for lower levels (refer to Figure 1-3). Late growth-specific promoters that are activated as cultures cease cell growth are desirable for large-scale processes as they respond to endogenous signals and thus do not require the addition of supplements. Such promoters have yet to be identified in PCC 6803. The first step towards the identification of growth phase-specific promoters is the identification of growth phase-specific genes. Sigma factors are important regulators of growth phase-specific gene expression in bacteria and two sigma factors, SigC and SigH, have been implicated in the regulation of stationary phase-specific expression in PCC 6803 (Asayama et al., 2004, Foster et al., 2007). Transcript levels of the genes encoding these sigma factors oscillated throughout culture growth and were not suitable indicators of growth phase in PCC 6803. I therefore turned my attention to genes with published growth-specific expression profiles. Closer investigation of selected genes showed that these profiles were not robust across all of the conditions tested suggesting a response to factors other than changes in growth. The genes investigated in this chapter were not robust indicators of growth phase in PCC 6803 and the promoters of these genes were not ideal candidates for synthetic biology. Chapter 6 will describe the results obtained by RNA sequencing to identify gene clusters robustly induced during late growth across all of the single nutrient-limited conditions described in Chapter 4.

5.3.1 Enhancing the resolution of growth phase-specific gene expression profiles

This study has highlighted the importance of interrogating gene expression a) at numerous time points throughout culture growth and, more importantly, b) in conditions with different growth kinetics. By measuring transcript levels regularly throughout culture growth, a clearer picture of expression patterns can be ascertained. Previous studies compared expression levels at a maximum of three time points representing different growth phases: early to mid-exponential, late exponential and stationary phase growth. As a result, consistent oscillations in gene expression may be interpreted as growth-phase specific patterns. This was evident in the expression levels of sigma factor genes *sigC* and *sigH* that fluctuated throughout growth under standard conditions in our laboratory (Figure 5-2 and 5-3, respectively). Thus, by interrogating samples at numerous time points throughout culture growth, the studies in this chapter have enhanced the resolution of gene expression patterns. This elucidated a large variation across experimental replicates for the majority of genes investigated in this study as peaks in expression occurred at different magnitudes on different days. These expression patterns therefore do not meet the robustness criterion and the tested genes are not ideal candidates for promoters in synthetic biology.

5.3.2 Ensuring the robustness of growth phase-specific gene expression profiles

Another limitation of previous studies is the use of just two conditions to interrogate transcriptomic responses to changes in growth. Foster et al. compared early and late exponential growth in wild type and Δ*sigB* PCC 6803. However, both of these genotypes exhibited virtually identical growth kinetics under standard conditions (Foster et al., 2007, Singh et al., 2006). As a result, genes selected from this study (*sigH* and *isiA*) exhibited the least robust growth-specific expression patterns of the genes tested in this chapter. In spite of highly reproducible induction of *isiA* transcript specifically during late exponential

growth under standard conditions (Figure 5-4), this expression pattern was lost in cultures which prematurely ceased growth in response to nutrient limitation (BG11-MgS; Figure 5-5) arguing against growth phase-specific regulation of *isiA* transcript. There are numerous reports of the iron-dependent regulation of the operon in which this gene resides, *isiAB* (Laudenbach et al., 1988, Leonhardt and Straus, 1992, Burnap et al., 1993, Vinnemeier et al., 1998). The promoter of this operon has also been used as a bioreporter for iron availability in PCC 7002 (Boyanapalli et al., 2007). Given the late induction of *isiA* expression under nutrient limitation, this suggests nutrient-dependent as opposed to growth phase-dependent regulation.

By contrast, Berla and Pakrasi identified genes commonly up-regulated during stationary phase growth in two conditions with very different growth kinetics by using mixotrophic cultures which ceased growth at a much lower density compared with cultures grown under standard conditions (Berla et al., 2013). Indeed, the gene that exhibited the most robust growth phase-specific expression pattern of those tested in this chapter was identified from this study (*slr9003*). This reinforces the importance of confirming results across a range of conditions modulating the process of interest, in this case the transition into stationary phase growth of cyanobacteria. To this end, I utilised two double nutrient-limited conditions, BG11-MgS and BG11-KP, to confirm expression patterns observed under standard nutrient conditions. Confidence in positive results increases with every additional condition, and assessment of growth-phase specific genes would ideally utilise all of the conditions identified in Chapter 4.

5.3.3 Sigma factor activity is not modulated at the transcript level

It is the sigma factor within the RNA polymerase holoenzyme that confers the specificity of promoter recognition and binding. “Sigma switching” modulates the transcription of large subsets of genes to coordinate responses to changing environmental conditions (Imamura and Asayama, 2009). Sigma factors implicated in regulating growth phase-specific gene expression can serve as a useful tool for the identification of genes and promoters that respond to changes

in growth. Firstly, transcriptome analyses comparing stationary phase expression in wild type and knockout strains would reveal potential targets of the sigma factor of interest. Analyses of upstream regions of these targets may then elucidate important motifs required for stationary phase-specific expression. Secondly, chromatin immunoprecipitation studies could be performed to identify the DNA sequences with which the sigma factor directly interacts. Finally, sigma factor transcript and/or protein levels may serve as a marker for entry to stationary phase growth and thus inform time point selection for –omics studies investigating growth-phase specific processes.

In contrast to previously reported expression profiles, I observed oscillations in *sigC* and *sigH* transcripts encoding two sigma factors implicated in the regulation of stationary phase gene expression. A closer review of the literature revealed that sigma factor transcript and protein levels do not necessarily correlate. For instance, SigC protein levels remained constant during the light-to-dark transition despite a decrease in *sigC* transcript (Imamura et al., 2003a, Tuominen et al., 2003). In comparison, SigH protein was not detectable under heat stress despite a 30-fold increase in *sigH* transcript (Huckauf et al., 2000, Imamura et al., 2003b). In fact, none of the group 3 sigma factor proteins were detectable under standard conditions despite the presence of their transcripts. This suggests that sigma factor regulation does not occur at the transcript level but instead in the form of translational attenuation or protein instability. Such post-transcriptional regulatory mechanisms may be beneficial for the rapid response to stress stimuli.

Given that transcript levels do not serve as an indicator for sigma factor activity, it is still possible that sigma factors are involved in the regulation of stationary phase-specific gene expression. If indeed this is the case, it is expected that specific motifs will be present in the promoters of genes that respond to changes in growth. Chapter 6 will describe the results obtained by RNA sequencing comparing gene expression in late versus early cultures and an approach to identify growth-phase specific motifs. This should include motifs required for recognition and binding of sigma factors and other transcription factors involved in regulating gene expression in response to growth.

5.3.4 $P_{slr9003}$, a potential auto-inducible promoter

Of the genes assessed in this chapter, the plasmid-encoded gene *slr9003* exhibited the most consistent up-regulation during late growth across three different nutrient conditions with varied growth kinetics (Figure 5-7, 5-8 and 5-9). While *slr9003* expression was consistently induced during late growth, however, expression was still observed during early growth in all conditions. The promoter of this gene is therefore not an ideal candidate for practical applications as a key characteristic of inducible promoters is efficient silencing under non-induced conditions. This is particularly important for growth phase-specific promoters that aim to minimise biosynthetic demand and potential toxicity during the active growth phase. Moreover, elevated transcript levels were not always maintained following induction, in particular BG11-KP (Figure 5-9). While it would be ideal to identify promoters that remain active throughout late growth, promoters with transient growth phase-specific activity can still be useful to activate self-perpetuating systems such as a circular T7 transcriptional system.

A more detailed analysis of *slr9003* could be performed including determination of expression patterns across single nutrient-limited conditions by qPCR and promoter activity using the assay established in Chapter 3. However, due to time constraints and the intent to identify more robust candidates using RNA sequencing in Chapter 6, this gene was not taken forward for further analysis. Optimisation of this promoter would be required to a) optimise specificity to enhance repression during early growth and b) maintain promoter activity post-induction during late growth across different conditions. To achieve this, DNA elements necessary for late growth-specific activation should be determined by first identifying the shortest fragment of the *slr9003* promoter required for late growth-specific activation and second altering nucleotide sequences within this fragment to identify motifs required to maintain this specificity. Once the necessary DNA elements have been established, spacing as well as the nucleotide sequences occurring between these elements could be modified to enhance promoter strength and specificity. Furthermore, reducing the size of this

promoter to the bare minimum would ease the construction of larger multipart modules.

5.3.5 Expanding the growth phase-specific promoter library for cyanobacteria

This chapter identified a potential candidate late growth-specific promoter for cyanobacteria. While this is useful for synthetic biology applications, it is important to develop a library of promoters spanning a wide range of transcriptional activity. Moreover, substantial development is still required to optimise the specificity of the candidate promoter identified in this chapter. As mentioned in the previous section, classical promoter analyses may be performed to identify the DNA elements, or motifs, required for growth phase-specific activation. This is a laborious and time-consuming task, however, and alternative approaches may enable more rapid identification of growth phase-specific DNA motifs. One example is performing a large transcriptomic study comparing gene expression across growth phases and conditions modulating growth kinetics to identify subsets of genes specifically induced at different times of growth. Upon identification of growth phase-specific gene sets, analyses of upstream regions of these genes can identify motifs overrepresented in the promoters of these genes compared to the rest of the genome. This type of analysis would identify all of the DNA motifs responsible for growth phase-specific promoter activity and would not be limited by the study of one particular promoter. Thus, growth phase-specific promoter libraries can be developed which span a wide range of transcriptional activity and specificity, providing a diverse toolkit for biological engineering of cyanobacteria. The next chapter will describe such an approach in more detail and present preliminary results.

Chapter 6. Identifying novel growth phase-specific genes

6.1 Introduction

The previous chapter demonstrated the importance of investigating changes in response to growth across a range of conditions affecting growth kinetics. Genes following growth phase-specific expression patterns were selected from previous microarray studies (Foster et al., 2007, Berla and Pakrasi, 2012) and assessed in nutrient-limited conditions established in Chapter 4. While growth phase-specific patterns were typically observed under standard conditions, this was not always maintained when cultures prematurely ceased growth in response to specific nutrient limitations. To identify robust growth phase-specific promoters, it is therefore necessary to study global changes in gene expression across a variety of conditions in which cultures have stopped growing for different reasons.

The majority of transcriptomic studies in cyanobacteria have used microarray analyses. In this case, samples are added to a chip on which DNA probes are immobilized and transcript levels within the samples are determined as hybridization intensities. More recently, RNA sequencing (RNAseq) has emerged as a powerful method to analyse differential gene expression (Wang et al., 2009). This approach does not require any knowledge of transcript sequence and thus results in unbiased transcript detection. Furthermore, it enables the detection of a greater dynamic range of expression levels due to very low background signal and no upper limit for quantification. Combining transcriptomic analyses with a range of conditions altering growth kinetics provides a powerful approach to identify genes (and promoters) specifically regulated by changes in growth.

Cyanobacteria have multiple approaches to regulate gene expression (see *Introduction 1.3*). These include two-component signal transduction systems, RNA polymerase sigma factors, and transcription factors to name a few. These mechanisms of regulation typically rely on the recognition and binding of

specific nucleotide sequences, or motifs, in the DNA. Alternative sigma factors have been implicated in the regulation of gene expression during late growth (Asayama et al., 2004, Foster et al., 2007), however regulatory networks in living organisms are highly complex and a myriad of factors may be involved. Clustering analysis can identify subsets of co-regulated genes that may be regulated by common factors. The upstream regions of these genes can then be analysed to discover over-represented motifs that are likely targets of these factors. For this purpose, it is important to define a "foreground" in which enriched elements are to be identified and a relevant "background" to which the foreground should be compared. Promoters containing identified motifs can then be assessed for growth phase-specific activity.

6.1.1 Chapter aims

This work aimed to investigate global changes in gene expression arising from changes in growth in cultures of *Synechocystis* spp. PCC 6803 across six nutrient conditions established in Chapter 4. RNA sequencing (RNAseq) was performed to determine transcript levels and differences in expression levels were detected using statistical tools. For motif discovery, a suitable background was defined by identifying the full set of genes that are actively expressed in PCC 6803 in at least one of the conditions tested. Genes with growth phase-specific expression patterns were identified and clustering analysis performed to identify co-regulated sets of genes. This will generate lists of genes with interesting expression profiles that will serve as the foreground for analysis of upstream regions and motif discovery.

6.2 Results

6.2.1 Experimental design

Synechocystis spp. PCC 6803 was grown under six different nutrient conditions described in Chapter 4: standard (BG11), nitrate-limited (BG11-N), phosphate-limited (BG11-P), sulphate-limited (BG11-S), magnesium-limited (BG11-M) and potassium-limited (BG11-K) media where limited nutrients were provided at 12.5 % compared to the standard concentration. To compare transcriptional profiles, RNA samples were extracted from two time points representing early and late growth. Day 4 was selected as the early growth time point as this was the point at which all cultures had entered the exponential phase of growth. Day 8 and Day 16 were selected as the late growth time point in nutrient-limited and standard conditions respectively as this was the point where cultures transitioned from exponential to stationary phase growth.

Genome-wide oscillations in transcription occur as a result of circadian rhythm, and in PCC 6803 up to 9 % of genes oscillate in expression during a 24 hour period (Kucho et al., 2005). In order to normalise for the effects of transcriptional oscillation, all samples were harvested midway through the 12 hour day period, i.e. 6 hours after the lights turned on. Total RNA was isolated and subjected to two rounds of ribosomal RNA depletion prior to cDNA library synthesis (see Figure 2-2) and next-generation RNA sequencing using the Illumina MiSeq system. RNAseq and initial data analysis was performed at the *Glasgow Polyomics* facility in the University of Glasgow (summarized in Table 6-1). The reference genome selected for the alignment of RNAseq reads was the original PCC 6803 genome sequence published on Cyanobase, a dedicated genome database for cyanobacteria (Kaneko et al., 1996, Nakamura et al., 1998, Nakao et al., 2010). While this genome does not include the sequences of the three smaller plasmids pCA2.4, pCB2.4 and pCC5.2, it remains the only sequence available for wild-type PCC 6803. As a result, the nine genes encoded on these three plasmids were not represented in our dataset.

A total of 39 RNA samples (three time points in standard media, two time points in five nutrient-limited media, three replications) were sequenced. A total of 640,131,273 reads were obtained with average reads of 16.4 million reads per sample and reads had an average length of 75 base pairs. 546,270,878 reads (85.3 %) were mapped to the genome with a tolerance of a 2 base pair mismatch. The number of reads mapped to each coding sequence was calculated and normalised for gene length (number of fragments mapped per kilobase of gene) and library depth (total number of aligned reads in the experiment). This normalisation strategy generated values as fragments per kilobase of gene per million reads mapped (FPKM).

Table 6-1. Workflow of RNAseq analysis

Input	Activity	Software	Output	File	Reference
RNA	Sequencing	Illumina MiSeq	Reads	Fastq	
Reads	Align to PCC 6803 genome	TopHat	Aligned reads	.bam	Trapnell et al., 2012
Aligned reads	Normalisation to gene length and total reads	Cuffdiff	FPKM	.txt	Trapnell et al., 2012

6.2.2 Multi-dimensional scaling

Multi-dimensional scaling (MDS) is a method that reduces the dimensionality of a dataset and visualises the level of similarity between individual samples within the dataset (Mugavin, 2008). This is achieved by converting a set of similarities based on genome-wide FPKM values into a set of points where the distances reflect the relative similarities. MDS was performed using the CummeRbund visualisation package for Cuffdiff sequencing data (Trapnell et al., 2012). Generally, good replication was observed within each sample (Figure 6-1). The early samples harvested at the start of the exponential phase before cultures experienced nutrient limitation clustered closely together with the exception of BG11-N. In fact, there was clear separation of the BG11-N, both early and late samples, from the rest of the dataset suggesting a distinct growth pattern in this condition. Day 8 samples grown in BG11 clustered with day 4 samples in the same condition reflecting the fact that at this time point BG11 cultures were still growing exponentially. This showed that genome wide transcript levels do indeed provide a good indicator of growth phase irrespective of the actual culture density. The late samples clearly separated from all the early samples. Furthermore, they separated according to condition. These findings are in accordance with the fact that at this time point, culture growth slowed down due to specific different nutrient limitations. Relatively large variation was observed in the late cultures grown in standard BG11, reflecting the larger variation in the corresponding culture growth curves (compare Figure 4-6B).

Figure 6-1. Multi-dimensional scaling of RNAseq dataset *(overleaf)*

The distances between points reflect the relative similarities between samples based on genome wide transcript levels. The different colours correspond to nutrient condition. Labels indicate the nutrient condition (SM: standard BG11 media, KL: potassium limitation, PL: phosphate limitation, ML: magnesium limitation, SL: sulphate limitation, NL: nitrate limitation), time point (4: day 4 corresponding to early growth, 8: day 8 corresponding to late growth in nutrient-limited conditions, 16: day 16 corresponding to late growth in standard media), and replicate number.

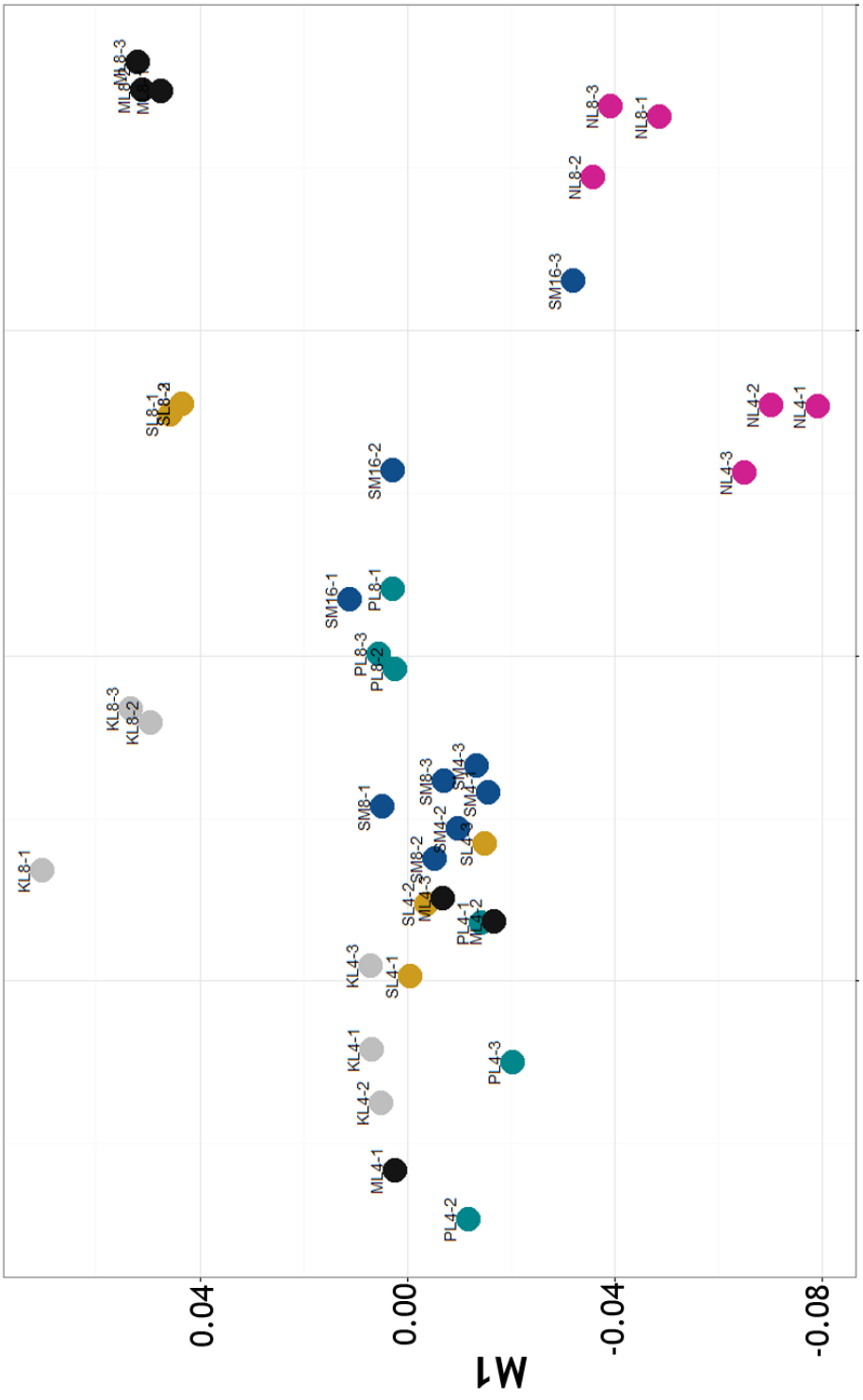

Figure 6-1. Multi-dimensional scaling of RNAseq dataset

see previous page for the complete figure legend

6.2.3 Selecting a cut-off and defining a background of expressed genes

The ultimate aim of this work was to identify overrepresented motifs in the upstream regions (i.e. the promoters) of genes that follow a desired expression pattern. It was necessary to define a relevant reference, or "background", to which these upstream regions should be compared as organisms may differ in terms of GC content and codon usage (Yu et al., 2012). For this purpose, I determined the full set of genes that showed expression in at least one of the conditions tested, thereby avoiding any bias that might be generated by genes that are permanently silenced or specifically expressed under conditions not applied here. To select a suitable cut-off for what is considered "expressed", FPKM values for each gene in a given sample were plotted on a logarithmic scale (Figure 6-2). In this example (BG11 day 4 replicate 1), 160 genes had a FPKM of 0 followed by a steep incline until sigmoidal distribution began at FPKM 2. Similar curves were obtained for other samples. Thus, genes were considered "expressed" if they showed a FPKM value of at least 2 in at least one condition. This resulted in the exclusion of 77 genes, which can be found in Appendix V. With the exception of 4 genes encoding ribosomal RNAs, all of the genes excluded from the background list encoded putative, hypothetical and unknown proteins or transposons. The remaining 3318 genes comprised the background list of expressed genes. Upstream regions of differing lengths (300, 600 and 1000 base pairs) have been extracted for these genes for future analysis.

The next step was to define the test sets, or "foreground". These are the upstream regions of genes with distinct expression patterns to be identified in the following sections.

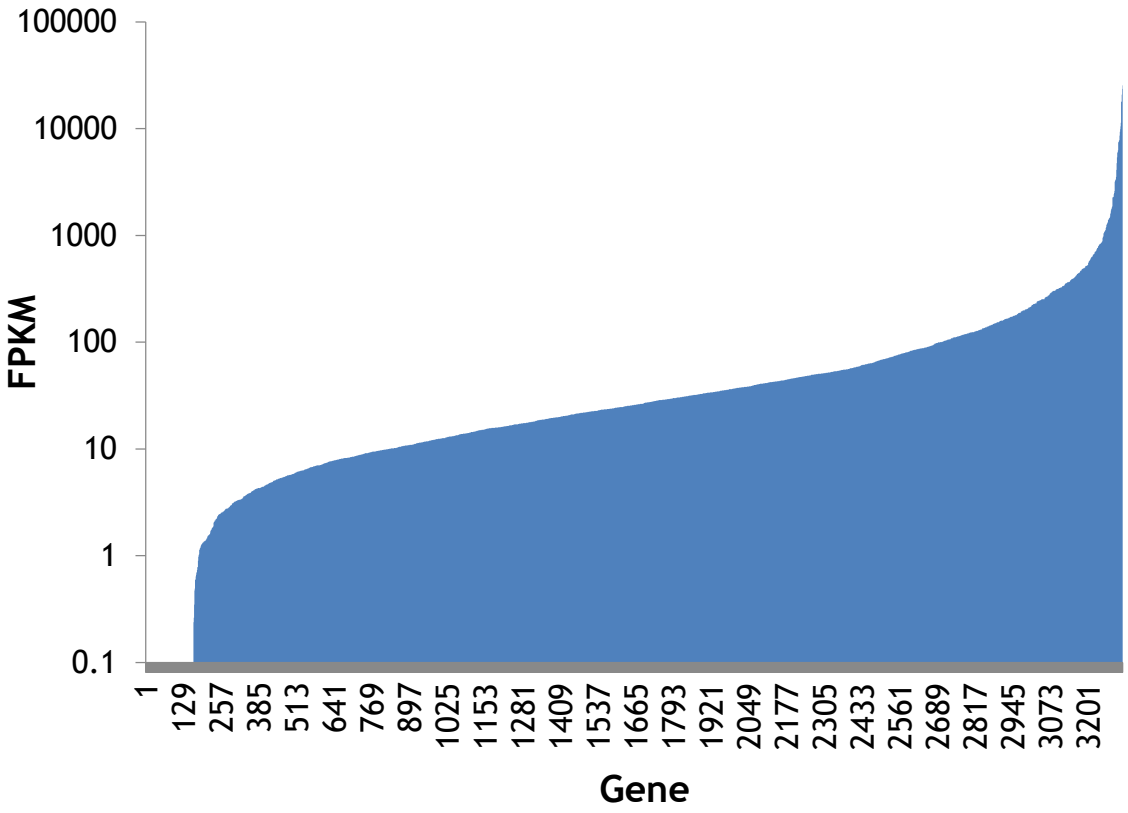

Figure 6-2. FPKM values for each gene within a single sample

Normalized read counts (FPKM) plotted for each gene within a single sample (BG11 day 4 replicate 1) on a logarithmic scale.

6.2.4 Overview of genes commonly regulated during late growth

Auto-inducible promoters that respond to changes in growth can serve as a sustainable tool for large-scale processes. To avoid negative effects of transgene expression on culture growth, promoters that become active once the cultures have reached high cell density are of particular interest. In order to identify genes commonly regulated in response to changes in growth, Student's t-tests were performed to determine significance of differences in transcript levels between early and late cultures within each condition. Subsets of genes with significant differential expression were then selected by p value ($p<0.05$). Of interest were the genes that are commonly up- or down-regulated in order to identify growth-specific responses irrespective of growth condition. 321 genes were identified which are differentially expressed between early and late time

points across all conditions. These differentially expressed genes were divided into functional categories according to the Cyanobase designation (http://genome.kazusa.or.jp/cyanobase/; last accessed 14/09/2015) and the number of genes in each functional category is presented in Table 6-2. The most prominent response was the down-regulation of 188 genes during late growth, primarily with functions related to photosynthesis, respiration and translation. A smaller set of genes were consistently up-regulated during late growth. Some of the up-regulated genes were involved in regulatory functions and transport. However, the vast majority (55.2 %) encoded hypothetical or unknown proteins. The following sections will describe commonly up- and down-regulated genes in closer detail.

6.2.5 Genes commonly up-regulated during late growth

Promoters induced during late growth are desirable for processes requiring a large biomass such as bioproduction, bioremediation or biodesalination. These promoters should have a robust activation pattern where they are reproducibly induced during late growth irrespective of the particular conditions in which the cyanobacteria were grown. Genes commonly up-regulated during late growth are therefore useful for identifying late growth-specific promoters. In total, 87 genes were significantly up-regulated during late growth across all six conditions (Appendix VI). Clusters of co-regulated genes were identified by *k*-means analysis of transcript changes throughout growth across the nutrient conditions. Assignment of commonly up-regulated genes into 5 clusters (designated UP1-UP5; Figure 6-3) efficiently separated different response profiles and additional clusters only separated single genes with similar expression profiles. For instance, UP5 represents a gene with particularly large differences between early and late time points in BG11 compared to other conditions, particularly BG11-P. UP1 to UP4 showed smaller but consistent differences early and late time points, however differences in the magnitude of response differed. For instance, UP4 showed relatively larger responses in late cultures compared to UP1. Clustering analysis did not reveal any remarkable co-regulation of genes with similar function. This may be a result of the large proportion of

hypothetical genes. One example, *sll0528*, was strongly induced during late growth across all conditions (Figure 6-4).

Table 6-2. Functional categories of genes differentially expressed at late versus early time points in all growth conditions

General pathways[a]	Number of genes	Up[b]	Down[c]
Amino acid biosynthesis	97	1	6
Biosynthesis of cofactors, prosthetic groups and carriers	124	1	8
Cell envelope	67	0	3
Cellular processes	76	1	3
Central intermediary metabolism	31	1	0
DNA replication, restriction, recombination and repair	60	1	0
Energy metabolism	132	4	5
Fatty acid, phospholipid and sterol metabolism	39	0	4
Hypothetical	1,076	43	37
Other categories	306	8	15
Photosynthesis and respiration	141	4	38
Purines, pyrimidines, nucleosides, and nucleotides	41	1	3
Regulatory functions	146	8	4
Transcription	30	0	1
Translation	168	3	22
Transport and binding proteins	196	6	7
Unknown	474	5	32
Total number	3,165[a]	87	188

[a] based on Kazusa annotation prior to May 2002

[b] for individual genes see Appendix VI

[c] for individual genes see Appendix VII

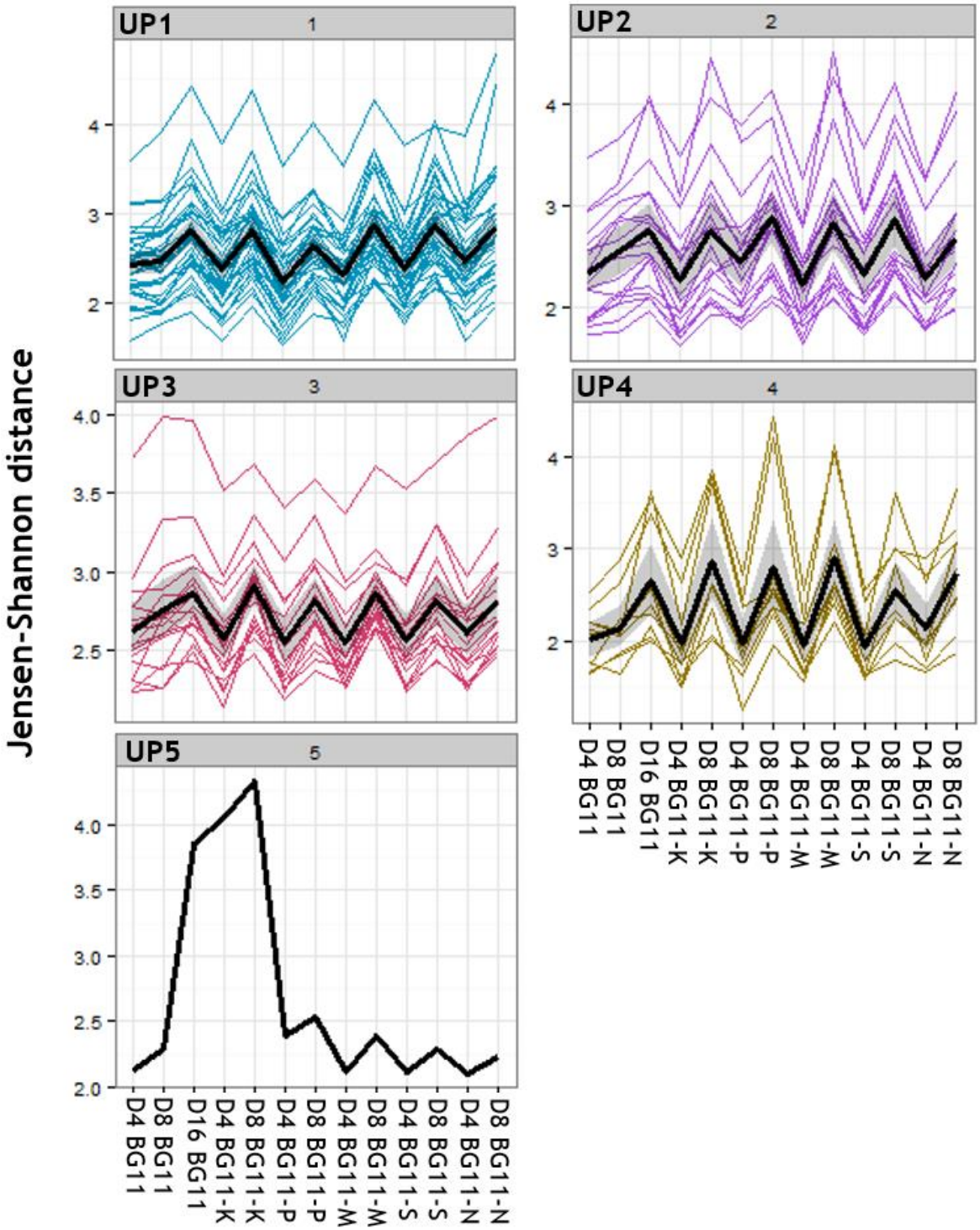

Figure 6-3. Expression profiles of genes up-regulated during late growth

see overleaf for the complete figure legend

Figure 6-3. Expression profiles of genes up-regulated during late growth in all conditions

Data shown were obtained by RNAseq analysis of RNA from PCC 6803 during different times during growth (days 4, 8 and 16) in six conditions (standard media, BG11; potassium-limitation, BG11-K; phosphate-limitation, BG11-P; magnesium-limitation, BG11-M; sulphate-limitation, BG11-S; nitrate-limitation, BG11-N). Based on significant differences at $p < 0.05$, 83 genes commonly up-regulated during late growth across conditions were identified and subjected to *k*-means clustering based on Jensen-Shannon divergence. Each graph shows the mean transcriptional response profile (bold black line) of the genes in a particular cluster. Clusters were designated UP1-UP5 as indicated at the top left of each graph.

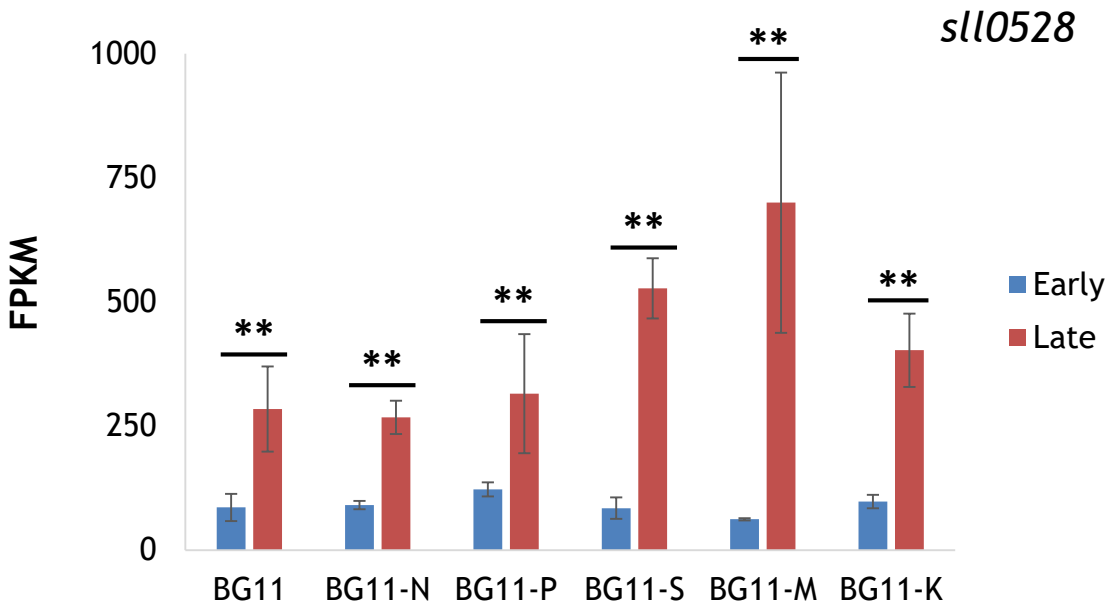

Figure 6-4. *sll0528* encoding a protein of unknown function up-regulated during late growth

Bar charts show normalized read counts quantified by RNAseq of *sll0528* encoding a hypothetical protein. RNA was harvested from PCC 6803 in six conditions (standard media, BG11; potassium-limitation, BG11-K; phosphate-limitation, BG11-P; magnesium-limitation, BG11-M; sulphate-limitation, BG11-S; nitrate-limitation, BG11-N) at two time points representing early growth (Day 4 in all conditions) and late growth (Day 8 in nutrient-limited conditions, Day 16 in standard conditions). Data are presented as means ± S.E.M. of three biological replicates. Student's t-tests were performed to determine statistical significance of the difference observed in late versus early expression levels within each condition (** $p<0.001$).

The remaining genes encoded proteins involved in a variety of functions including transport, regulatory functions and stress response. One gene, the cell death suppressor protein Lls1 homolog encoded by *slr1747* (Figure 6-5), may play an important role in stationary phase survival. Many genes were involved in sensory functions and signal transduction. For instance, transcripts involved in the synthesis of the key second messenger, cyclic adenosine monophosphate (cAMP), were consistently induced across all conditions (Figure 6-6). Interestingly, 5 genes involved in two-component signal transduction systems were also consistently up-regulated and these genes were distributed across 4 of the 5 clusters. For instance, *slr0484* which encodes the two-component sensor histidine kinase Hik23 is presented in Figure 6-7A. The cluster lacking a two-component system, UP3, instead included a gene encoding the translation initiation factor InfC (Figure 6-7B) which is induced under many other stresses including osmotic, iron, heat and cold stress (Mikami et al., 2002, Singh et al., 2003, Inaba et al., 2003).

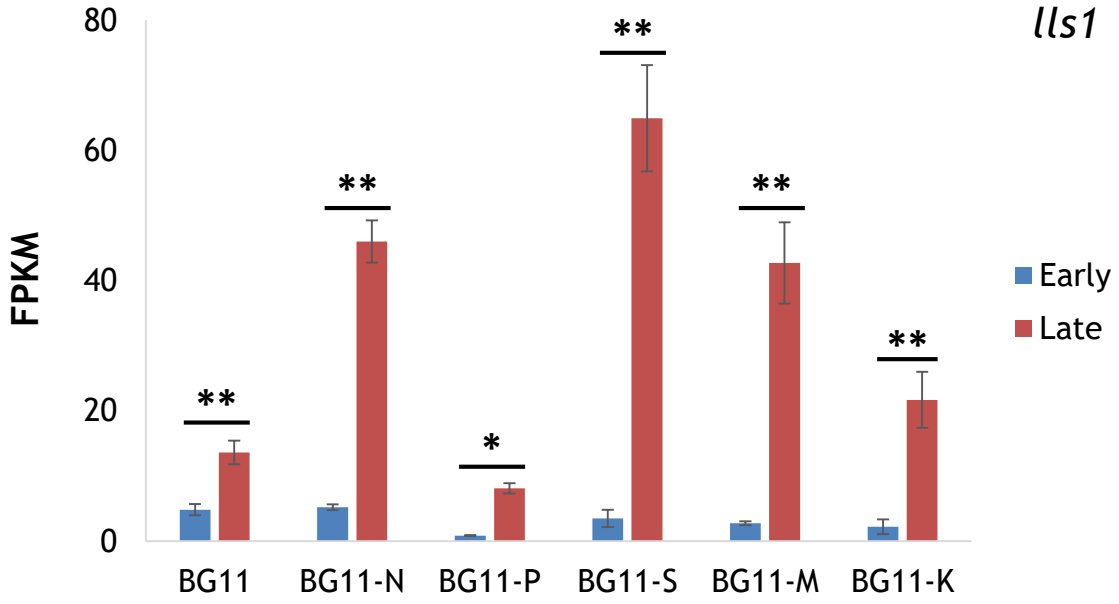

Figure 6-5. Cell death suppressor homolog gene up-regulated during late growth

see overleaf for the complete figure legend

Figure 6-5. Cell death suppressor homolog gene up-regulated during late

Bar charts show normalized read counts quantified by RNAseq of *slr1747* encoding a cell death suppressor protein Lls1 homolog. RNA was harvested from PCC 6803 in six conditions (standard media, BG11; potassium-limitation, BG11-K; phosphate-limitation, BG11-P; magnesium-limitation, BG11-M; sulphate-limitation, BG11-S; nitrate-limitation, BG11-N) at two time points representing early growth (Day 4 in all conditions) and late growth (Day 8 in nutrient-limited conditions, Day 16 in standard conditions). Data are presented as means ± S.E.M. of three biological replicates. Student's t-tests were performed to determine statistical significance of the difference observed in late versus early expression levels within each condition (* p<0.05; ** p<0.001).

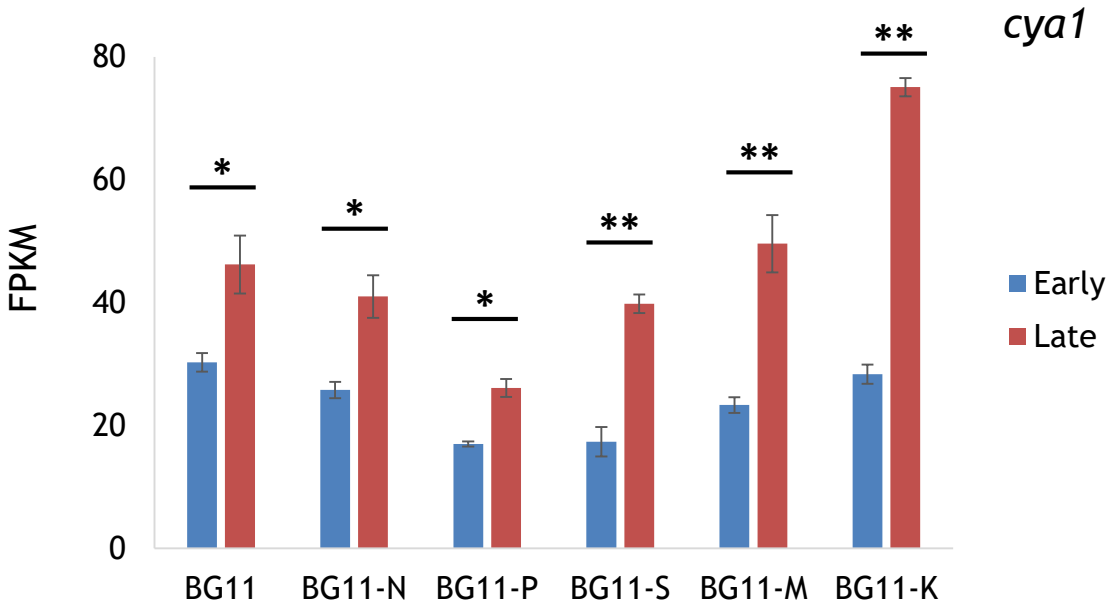

Figure 6-6. Adenylate cyclase up-regulated during late growth

Bar charts show normalized read counts quantified by RNAseq of *slr1991 (cya1)* encoding adenylate cyclase involved in cAMP production. RNA was harvested from PCC 6803 in six conditions (standard media, BG11; potassium-limitation, BG11-K; phosphate-limitation, BG11-P; magnesium-limitation, BG11-M; sulphate-limitation, BG11-S; nitrate-limitation, BG11-N) at two time points representing early growth (Day 4 in all conditions) and late growth (Day 8 in nutrient-limited conditions, Day 16 in standard conditions). Data are presented as means ± S.E.M. of three biological replicates. Student's t-tests were performed to determine statistical significance of the difference observed in late versus early expression levels within each condition (* p<0.05; ** p<0.001).

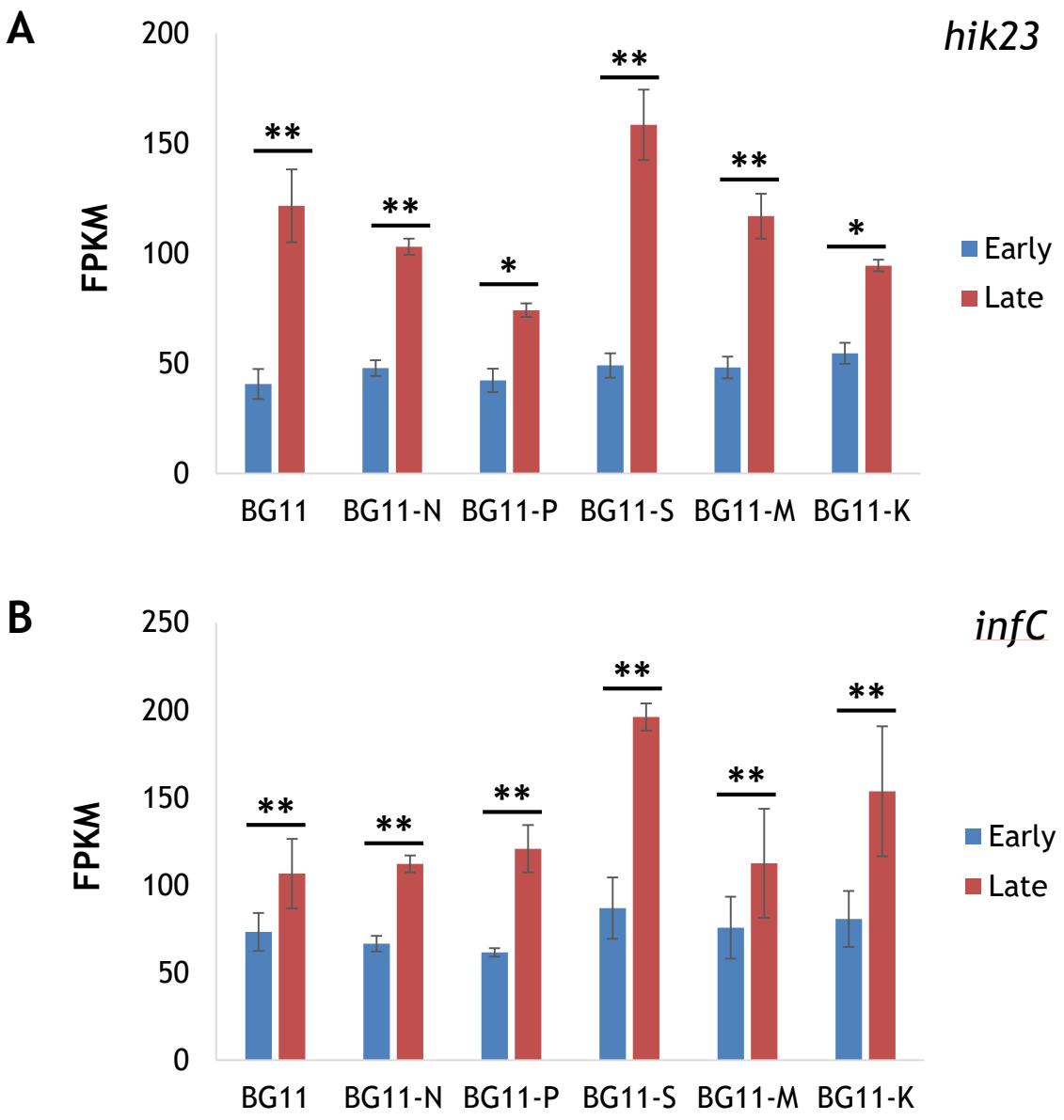

Figure 6-7. Genes involved in gene expression up-regulated during late growth

Bar charts show normalized read counts quantified by RNAseq of **(A)** *slr1324* encoding a histidine kinase, Hik23, and **(B**) *slr0974* encoding elongation initiation factor IF-3, InfC. RNA was harvested from PCC 6803 in six conditions (standard media, BG11; potassium-limitation, BG11-K; phosphate-limitation, BG11-P; magnesium-limitation, BG11-M; sulphate-limitation, BG11-S; nitrate-limitation, BG11-N) at two time points representing early growth (Day 4 in all conditions) and late growth (Day 8 in nutrient-limited conditions, Day 16 in standard conditions). Data are presented as means ± S.E.M. of three biological replicates. Student's t-tests were performed to determine statistical significance of the difference observed in late versus early expression levels within each condition (* $p<0.05$; ** $p<0.001$).

Outside of transcription and translation, genes with other sensory/regulatory functions were also consistently up-regulated during late growth. For instance, *pta* encoding a protein involved in acetyl phosphate (AcP) synthesis, which has been proposed as a global sensor of physiological state in cyanobacteria (Morrison et al., 2005) was consistently up-regulated during late growth (Figure 6-8). Transcripts for type-2 NADH dehydrogenases (Figure 6-9), which are proposed redox sensors to monitor both membrane (plastiquinone) and soluble (NADH) fractions (Howitt et al., 1999) were also up-regulated. Of great relevance for bioenergy production in cyanobacteria, increases were also observed in *hypA1* and *hypD* transcripts (Figures 6-10) which encode important assembly proteins required for the maturation of hydrogenase enzyme (Hoffmann et al., 2006).

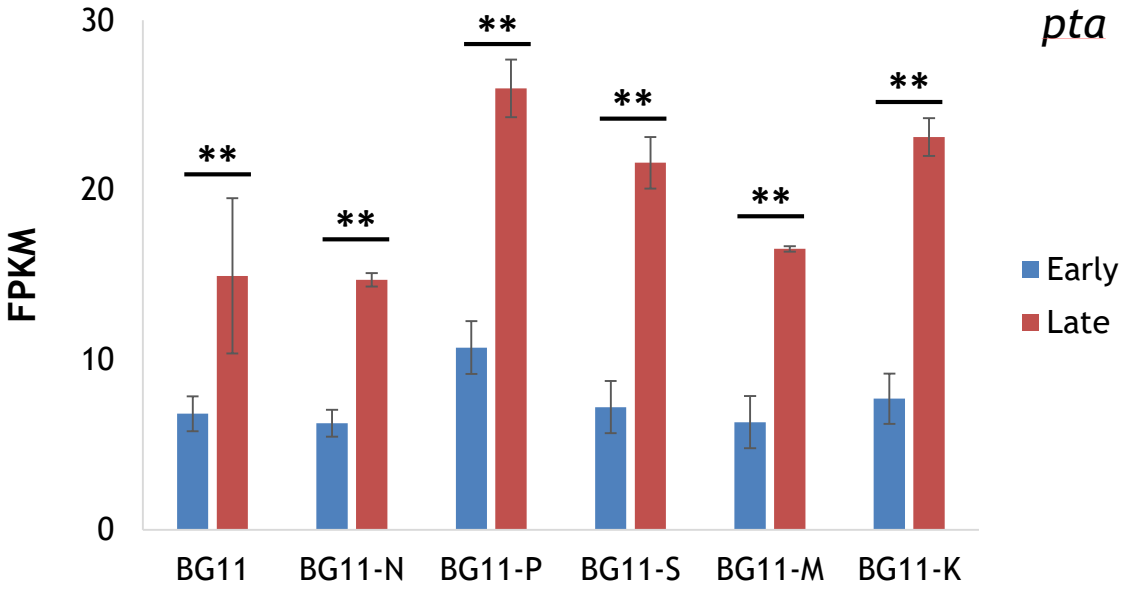

Figure 6-8. Phosphotransacetylase gene up-regulated during late growth

Bar charts show normalized read counts quantified by RNAseq of *slr2132 (pta)* encoding phosphotransacetylase. RNA was harvested from PCC 6803 in six conditions (standard media, BG11; potassium-limitation, BG11-K; phosphate-limitation, BG11-P; magnesium-limitation, BG11-M; sulphate-limitation, BG11-S; nitrate-limitation, BG11-N) at two time points representing early growth (Day 4 in all conditions) and late growth (Day 8 in nutrient-limited conditions, Day 16 in standard conditions). Data are presented as means ± S.E.M. of three biological replicates. Student's t-tests were performed to determine statistical significance of the difference observed in late versus early expression levels within each condition (* $p<0.05$; ** $p<0.001$).

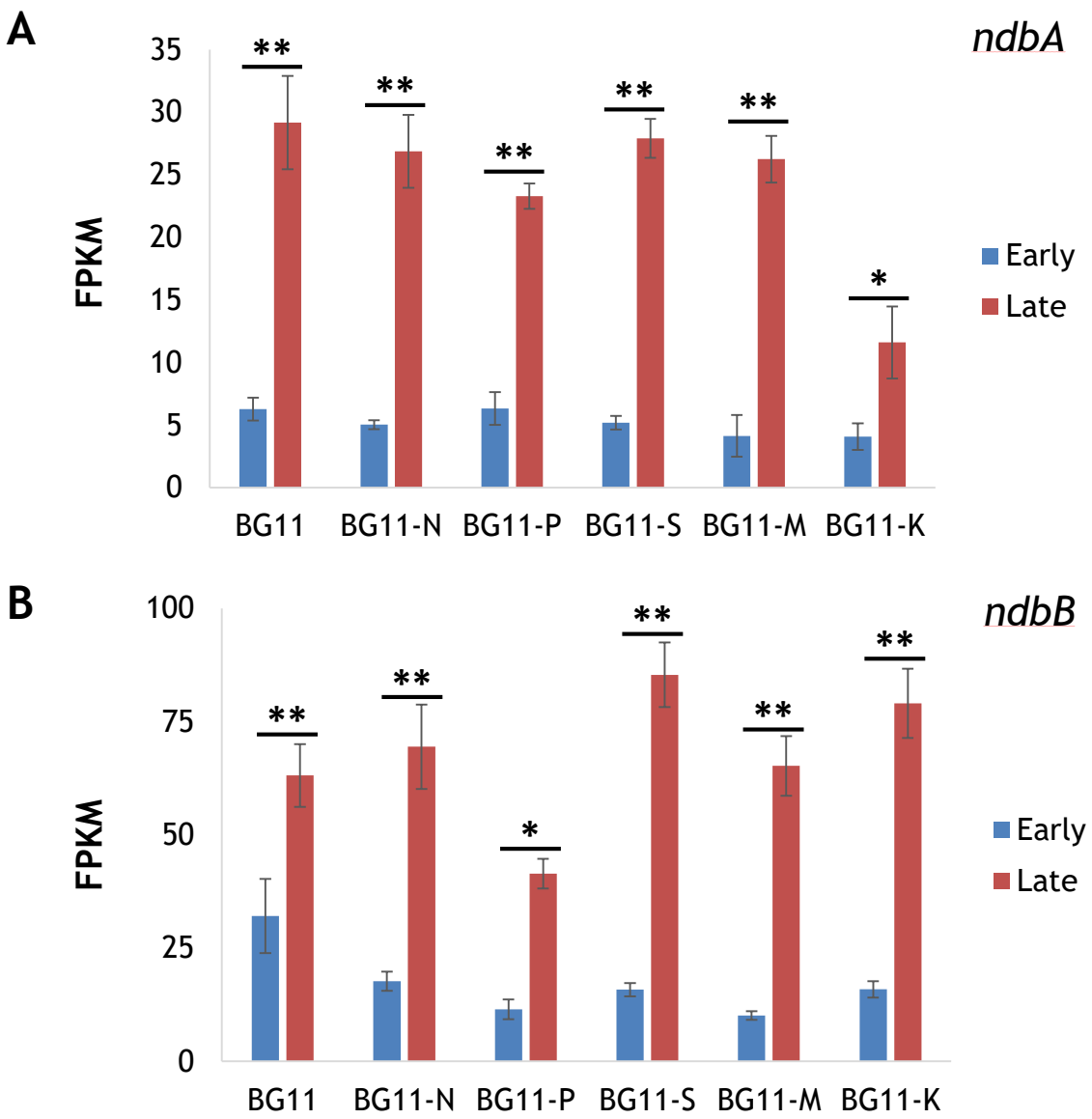

Figure 6-9. Type-2 NADH dehydrogenase genes up-regulated during late growth

Bar charts show normalized read counts quantified by RNAseq of **(A)** *slr0851 (ndbA)* and **(B)** *slr1743 (ndbB)* encoding a type 2 NADH dehydrogenase. RNA was harvested from PCC 6803 in six conditions (standard media, BG11; potassium-limitation, BG11-K; phosphate-limitation, BG11-P; magnesium-limitation, BG11-M; sulphate-limitation, BG11-S; nitrate-limitation, BG11-N) at two time points representing early growth (Day 4 in all conditions) and late growth (Day 8 in nutrient-limited conditions, Day 16 in standard conditions). Data are presented as means ± S.E.M. of three biological replicates. Student's t-tests were performed to determine statistical significance of the difference observed in late versus early expression levels within each condition (* $p<0.05$; ** $p<0.001$).

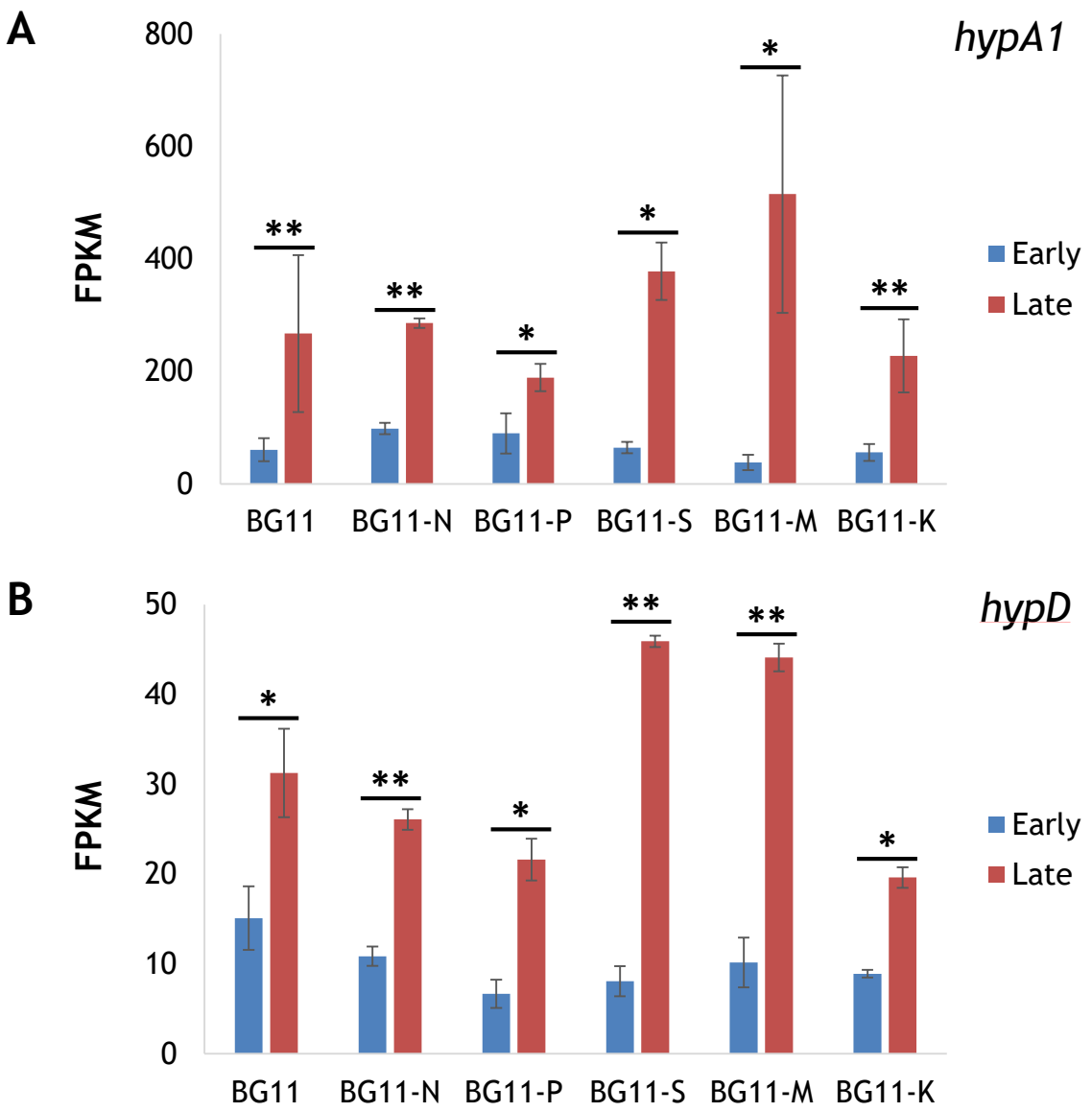

Figure 6-10. Putative hydrogen production-related genes up-regulated during late growth

Bar charts show normalized read counts quantified by RNAseq of **(A)** *slr1675* (*hypA1*) and **(B)** *slr1498* (*hypD*) encoding putative hydrogenase expression/formation proteins. RNA was harvested from PCC 6803 in six conditions (standard media, BG11; potassium-limitation, BG11-K; phosphate-limitation, BG11-P; magnesium-limitation, BG11-M; sulphate-limitation, BG11-S; nitrate-limitation, BG11-N) at two time points representing early growth (Day 4 in all conditions) and late growth (Day 8 in nutrient-limited conditions, Day 16 in standard conditions). Data are presented as means ± S.E.M. of three biological replicates. Student's t-tests were performed to determine statistical significance of the difference observed in late versus early expression levels within each condition (* $p<0.05$; ** $p<0.001$).

6.2.6 Genes commonly down-regulated during late growth

Promoters repressed during late growth can be a useful tool to modulate the expression levels of genes necessary for the growth phase but unnecessary or undesirable for the production phase. These types of promoters can thus be used to redirect metabolic flux towards desired pathways once cells have ceased growth and division. Similar to the identification of commonly up-regulated genes in the previous section, genes consistently down-regulated during late growth in all the conditions were selected by p value ($p<0.05$). In total, 188 genes were significantly down-regulated during late growth across all six conditions (Appendix VII). A relatively small proportion of these genes encoded hypothetical proteins (38 genes, 20.2 %), reflecting the intensive dissection of stress responses in PCC 6803 over the past decades.

Loss of pigmentation associated with phycobilisome degradation is a common response to light and nutrient limitation in cyanobacteria (Grossman et al., 1994) and indeed the strongest and most consistent response across the six conditions was the decrease in transcript levels of genes involved in biosynthesis of the major pigment, chlorophyll (Figure 6-11). Furthermore, large changes in transcript levels were observed for genes encoding components of the photosynthetic apparatus including phycobilisomes (both pigments and core components, Figure 6-12), photosystems (Figure 6-13) and the soluble electron carrier ferredoxin (Figure 6-14). Large changes were also observed in transcript levels of genes encoding ATP synthase (e.g. *atpA*, Figure 6-15) and translational machinery (e.g. ribosomal proteins and tRNA synthetases, Figure 6-16). Collectively, these data support the general consensus of a shift towards "stand-by" energy metabolism during late growth in bacteria (Schwarz and Forchhammer, 2005).

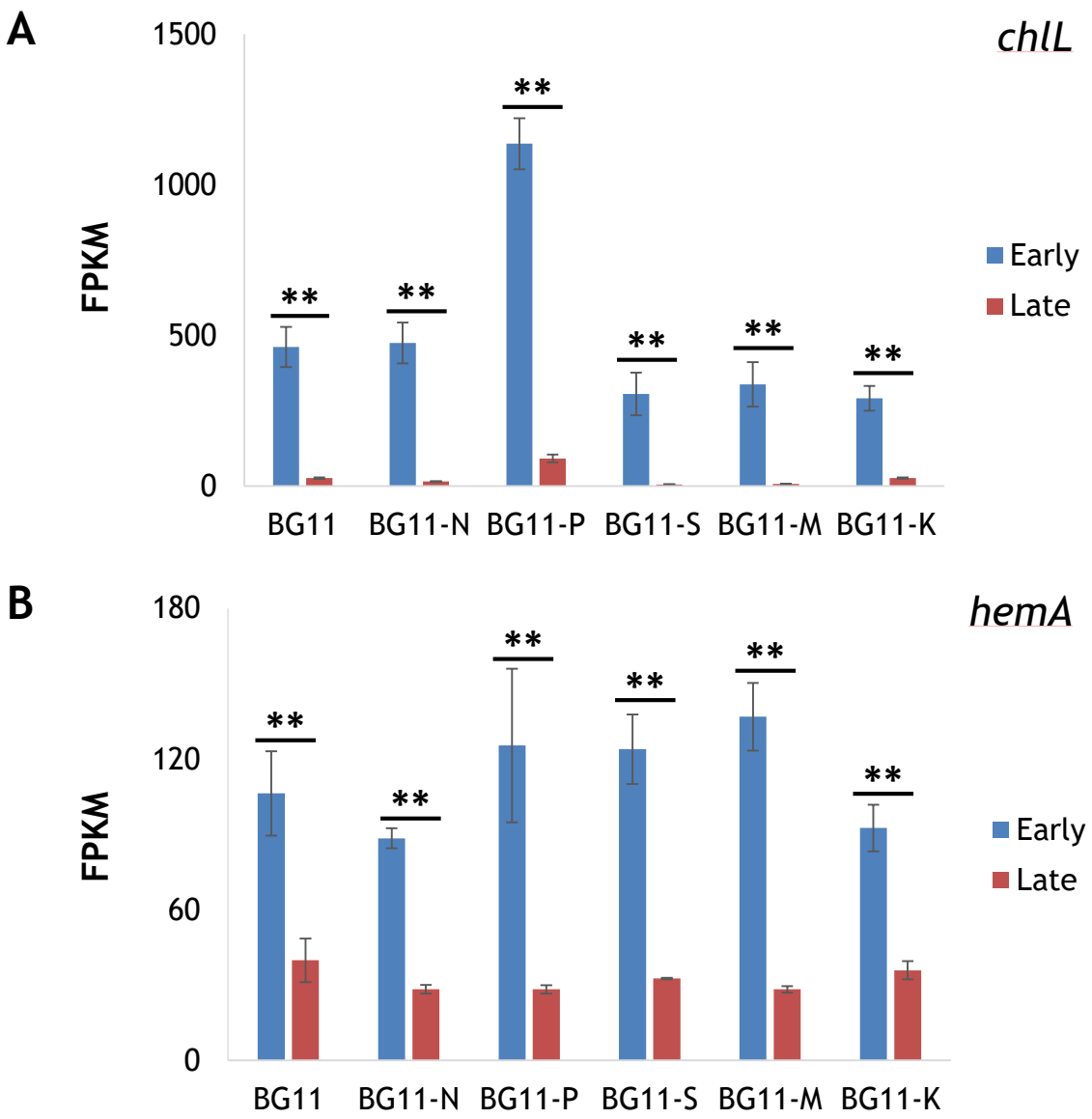

Figure 6-11. Chlorophyll biosynthesis genes down-regulated during late growth

Bar charts show normalized read counts quantified by RNAseq of **(A)** *slr0749* encoding the light-independent protochlorophyllide reductase iron protein subunit ChlL and **(B**) *slr1808 (hemA)* encoding tRNA-Gln reductase. RNA was harvested from PCC 6803 in six conditions (standard media, BG11; potassium-limitation, BG11-K; phosphate-limitation, BG11-P; magnesium-limitation, BG11-M; sulphate-limitation, BG11-S; nitrate-limitation, BG11-N) at two time points representing early growth (Day 4 in all conditions) and late growth (Day 8 in nutrient-limited conditions, Day 16 in standard conditions). Data are presented as means ± S.E.M. of three biological replicates. Student's t-tests were performed to determine statistical significance of the difference observed in late versus early expression levels within each condition (* $p<0.05$; ** $p<0.001$).

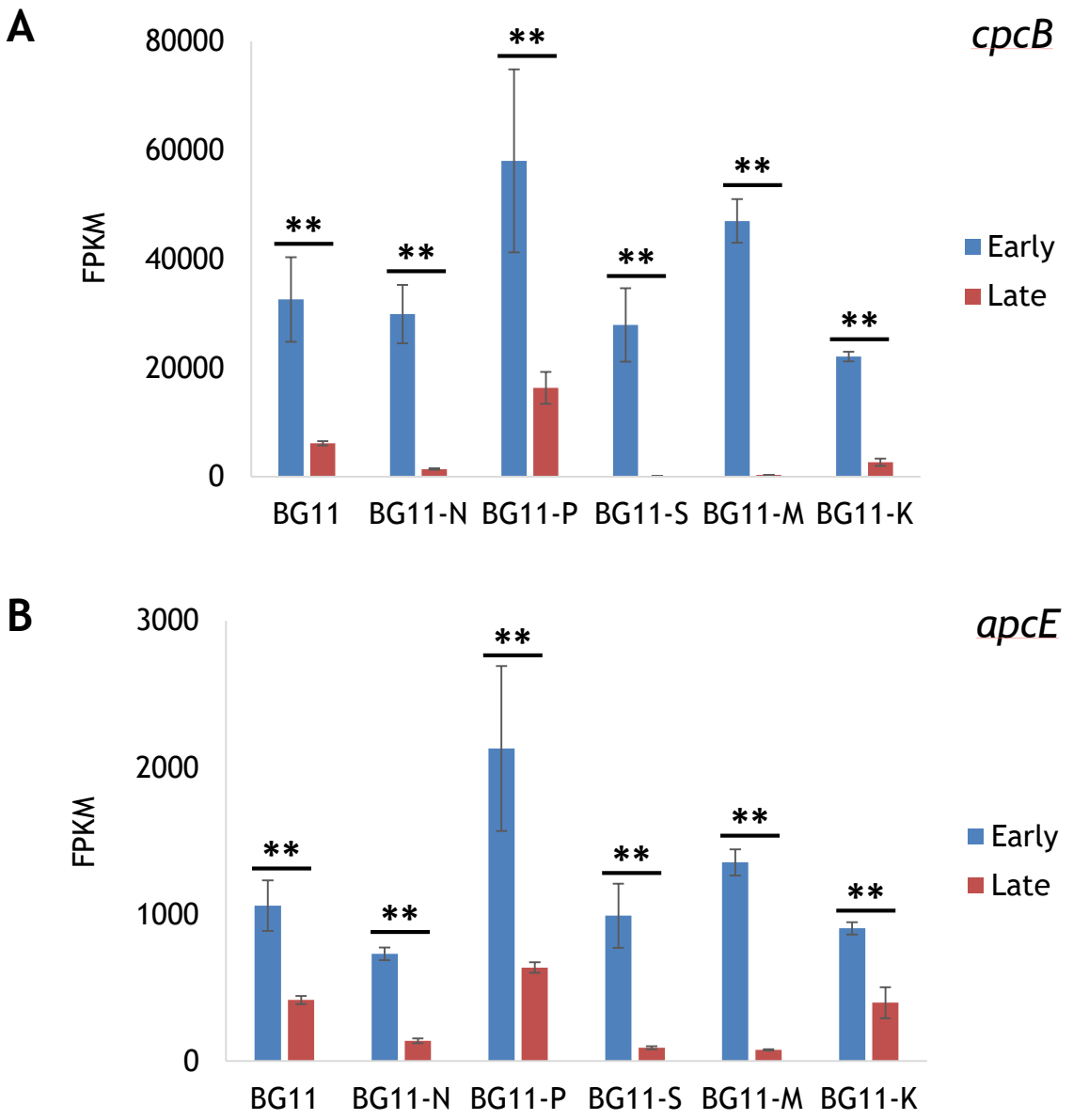

Figure 6-12. Phycobilisome genes down-regulated during late growth

Bar charts show normalized read counts quantified by RNAseq of **(A**) *sll1577* encoding phycocyanin beta subunit CpcB and **(B)** *slr0335* encoding phycobilisome core-membrane linker polypeptide ApcE. RNA was harvested from PCC 6803 in six conditions (standard media, BG11; potassium-limitation, BG11-K; phosphate-limitation, BG11-P; magnesium-limitation, BG11-M; sulphate-limitation, BG11-S; nitrate-limitation, BG11-N) at two time points representing early growth (Day 4 in all conditions) and late growth (Day 8 in nutrient-limited conditions, Day 16 in standard conditions). Data are presented as means ± S.E.M. of three biological replicates. Student's t-tests were performed to determine statistical significance of the difference observed in late versus early expression levels within each condition (* $p<0.05$; ** $p<0.001$).

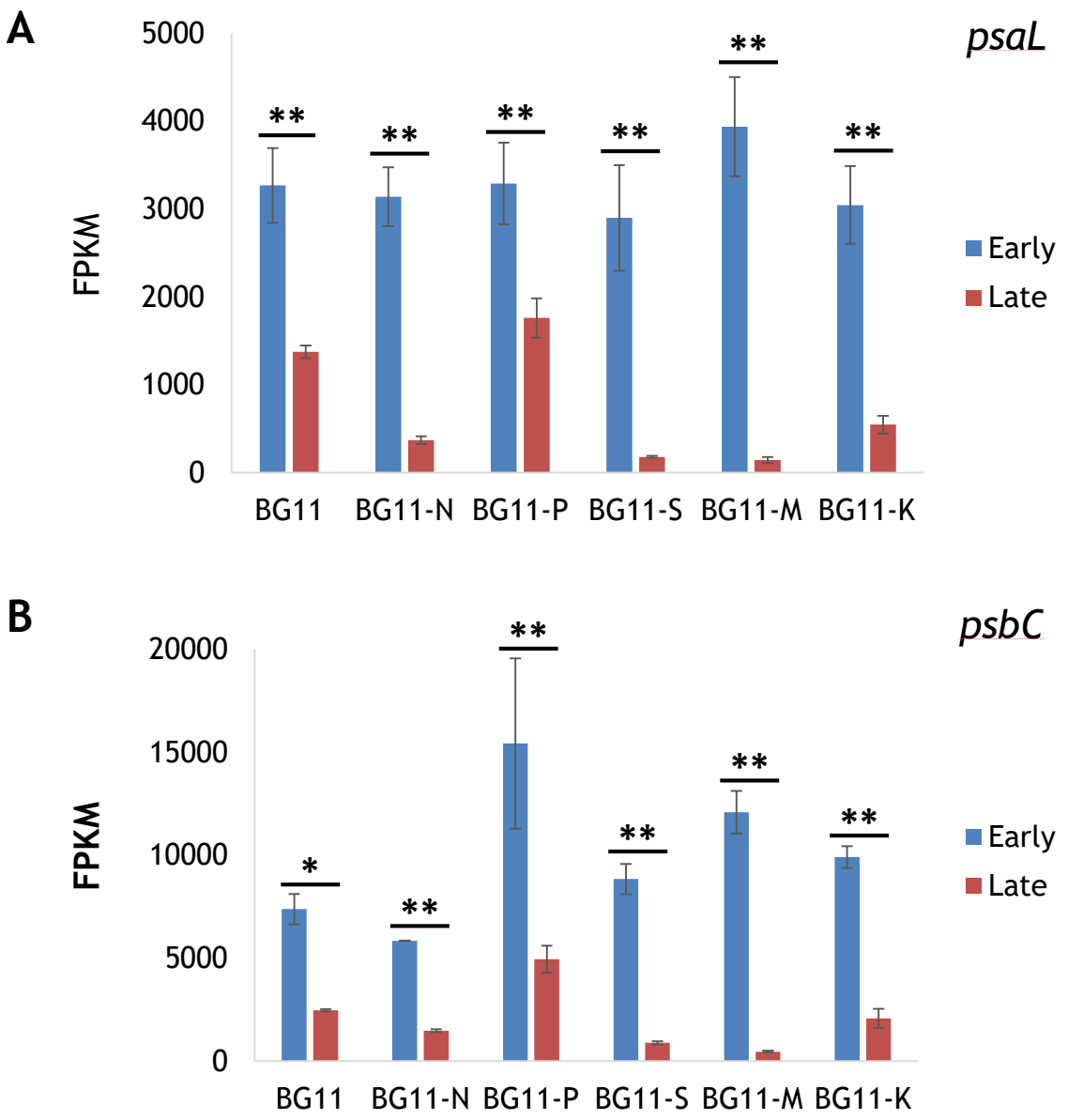

Figure 6-13. Photosystem genes down-regulated during late growth

Bar charts show normalized read counts quantified by RNAseq of **(A**) *slr1655* encoding photosystem I subunit XI, PsaL and **(B)** *sll0851* encoding photosystem II CP43 protein, PsbC. RNA was harvested from PCC 6803 in six conditions (standard media, BG11; potassium-limitation, BG11-K; phosphate-limitation, BG11-P; magnesium-limitation, BG11-M; sulphate-limitation, BG11-S; nitrate-limitation, BG11-N) at two time points representing early growth (Day 4 in all conditions) and late growth (Day 8 in nutrient-limited conditions, Day 16 in standard conditions). Data are presented as means ± S.E.M. of three biological replicates. Student's t-tests were performed to determine statistical significance of the difference observed in late versus early expression levels within each condition (* $p<0.05$; ** $p<0.001$).

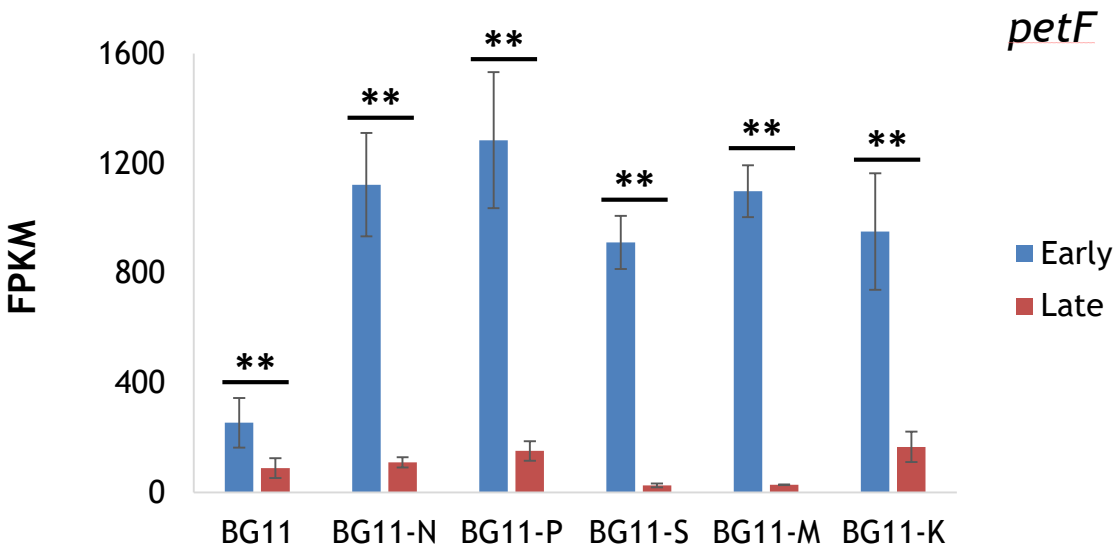

Figure 6-14. Photosynthetic electron transport down-regulation during late growth

Bar charts show normalized read counts quantified by RNAseq of *slr0150* (*petF)* encoding the soluble electron carrier ferredoxin. RNA was harvested from PCC 6803 in six conditions (standard media, BG11; potassium-limitation, BG11-K; phosphate-limitation, BG11-P; magnesium-limitation, BG11-M; sulphate-limitation, BG11-S; nitrate-limitation, BG11-N) at two time points representing early growth (Day 4 in all conditions) and late growth (Day 8 in nutrient-limited conditions, Day 16 in standard conditions). Data are presented as means ± S.E.M. of three biological replicates. Student's t-tests were performed to determine statistical significance of the difference observed in late versus early expression levels within each condition (* $p<0.05$; ** $p<0.001$).

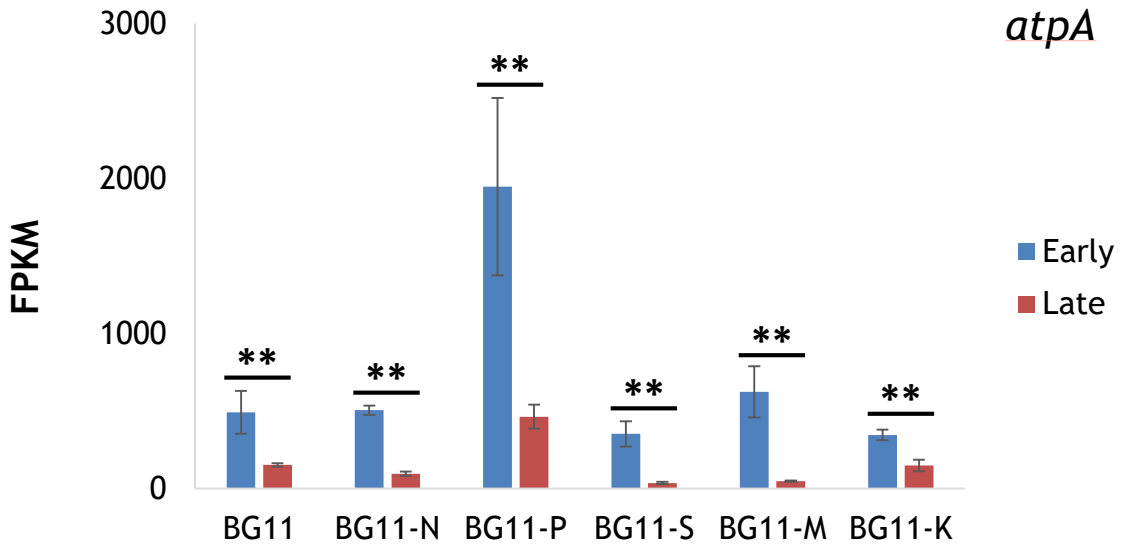

Figure 6-15. ATP synthase down-regulation during late growth

Bar charts show normalized read counts quantified by RNAseq of *sll1326* encoding the ATP synthase alpha chain AtpA. RNA was harvested from PCC 6803 in six conditions (standard media, BG11; potassium-limitation, BG11-K; phosphate-limitation, BG11-P; magnesium-limitation, BG11-M; sulphate-limitation, BG11-S; nitrate-limitation, BG11-N) at two time points representing early growth (Day 4 in all conditions) and late growth (Day 8 in nutrient-limited conditions, Day 16 in standard conditions). Data are presented as means ± S.E.M. of three biological replicates. Student's t-tests were performed to determine statistical significance of the difference observed in late versus early expression levels within each condition (* $p<0.05$; ** $p<0.001$).

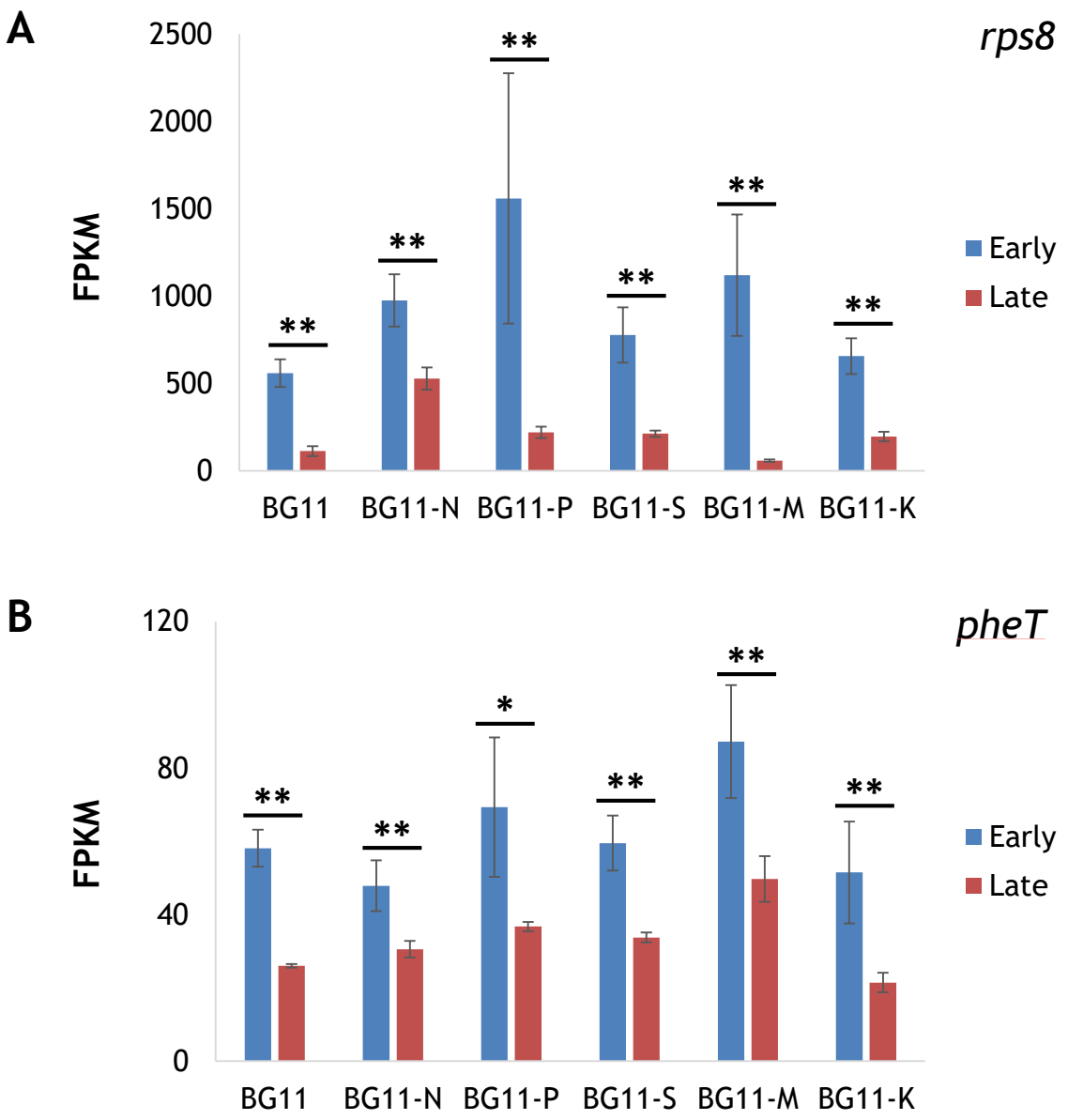

Figure 6-16. Translation machinery genes down-regulated during late growth

Bar charts show normalized read counts quantified by RNAseq of **(A)** *sll1809* encoding a 30S ribosomal protein, Rps8 and **(B)** *sll1553* encoding a phenylalanyl-tRNA synthetase, PheT. RNA was harvested from PCC 6803 in six conditions (standard media, BG11; potassium-limitation, BG11-K; phosphate-limitation, BG11-P; magnesium-limitation, BG11-M; sulphate-limitation, BG11-S; nitrate-limitation, BG11-N) at two time points representing early growth (Day 4 in all conditions) and late growth (Day 8 in nutrient-limited conditions, Day 16 in standard conditions). Data are presented as means ± S.E.M. of three biological replicates. Student's t-tests were performed to determine statistical significance of the difference observed in late versus early expression levels within each condition (* $p<0.05$; ** $p<0.001$).

Clusters of co-regulated genes were identified by *k*-means analysis of transcript changes throughout growth across the nutrient conditions. Assignment of commonly down-regulated genes into 8 clusters (designated DOWN1-8; Figure 6-17) efficiently separated different response profiles and additional clusters simply separated expression profiles of single genes. Genes involved in similar functions tended to be co-regulated as they tend to be organized in operons in bacteria (Table 6-4). For instance, ribosomal proteins in close proximity to one another on the cyanobacterial chromosome dominated cluster DOWN1. Similarly, photosynthesis-related genes and components of ATP synthase featured in clusters DOWN5 and DOWN6 respectively. Clusters also clearly separated genes according to differences in the magnitude of response observed between early and late cultures across conditions, i.e. relatively larger changes were observed in some media compared to others. For instance, genes in DOWN8 showed particularly large differences between early and late time points in BG11 compared to other conditions, particularly BG11-K and BG11-P. Genes in DOWN3 and DOWN5 showed larger differences in BG11-Mg and BG11-S than in BG11-P. Genes in DOWN7 showed larger differences in BG11-K, BG11-M and BG11-S compared with BG11-P. Genes in cluster DOWN4 showed relatively small but consistent changes between early and late samples in all conditions tested.

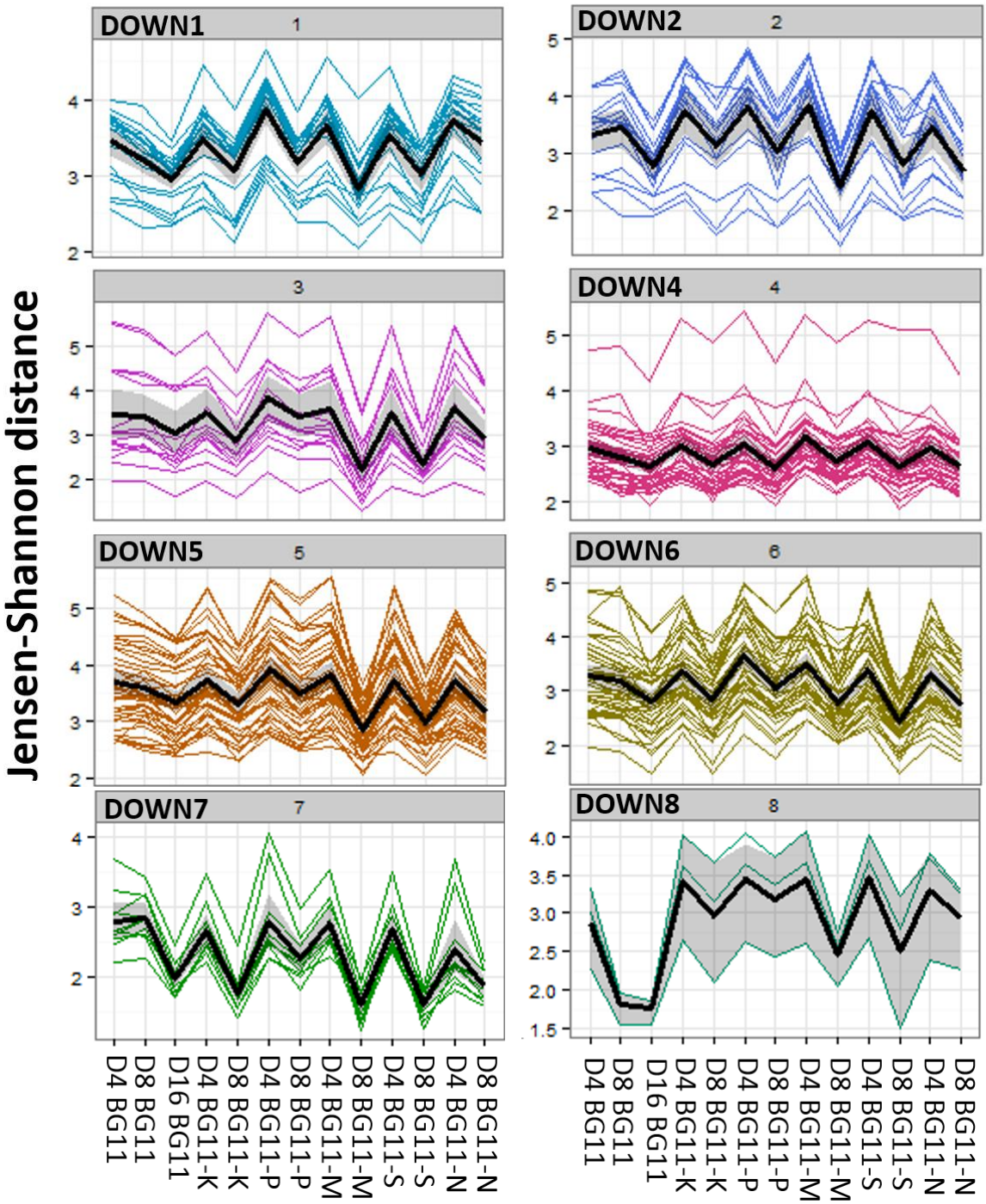

Figure 6-17. Expression profiles of genes commonly down-regulated during late growth

Data shown were obtained by RNAseq analysis of RNA from PCC 6803 during different times during growth (days 4, 8 and 16) in six conditions (standard media, BG11; potassium-limitation, BG11-K; phosphate-limitation, BG11-P; magnesium-limitation, BG11-M; sulphate-limitation, BG11-S; nitrate-limitation, BG11-N). Based on significant differences at $p < 0.05$, 83 genes commonly down-regulated during late growth across conditions were identified and subjected to k-means clustering based on Jensen-Shannon divergence. Each graph shows the mean transcriptional response profile (bold black line) of the genes in a particular cluster.

6.3 Discussion

The identification of genes robustly regulated by changes in growth are the first step towards identifying growth phase-specific promoters. Until now, growth phase-specific gene expression has been compared in a limited number of conditions that did not necessarily affect growth kinetics (Foster et al., 2007, Berla and Pakrasi, 2012). By contrast, I have studied the changes in gene expression across six conditions with vastly differing growth kinetics. This enabled the identification of genes that are consistently up- or down-regulated during late growth of PCC 6803 irrespective of the growth limiting factor. These sets of co-regulated genes were further subdivided into clusters showing similar expression profiles which may be regulated by common factors. It should be noted that the *k*-means clustering analysis pre-defines the number of clusters. The optimal cluster number is determined in a minimum function but it always constitutes a compromise. This accounts for the fact that some genes do not fit well with the average cluster profile (and should be eliminated) while some of the clusters are very similar (and should be combined). Differences between the obtained clusters were due to relatively larger changes in some media compared to others. For example, genes in DOWN8 showed particularly large differences between late and early time points in BG11, while genes in DOWN3 and DOWN5 showed larger differences in BG11-Mg and BG11-S than in BG11-P. Genes in cluster DOWN4 showed relatively small but consistent changes between early and late samples in all conditions tested.

Many additional analyses can be performed on this large dataset to identify genes with many other interesting expression patterns (e.g. genes which respond to specific nutrient limitations) however these questions were outwith the scope of this project. Furthermore, additional validation of the RNAseq results will need to be performed using alternative approaches such as qPCR to confirm the observed gene expression profiles. Nevertheless, I have identified large sets of genes that are co-regulated in response to growth and can be taken forward for analysis of upstream regions. Furthermore, I have defined a relevant background of expressed genes in order to identify enrichment within selected gene clusters.

6.3.1 Cyanobacteria switch to "standby" mode during late growth

In nature, conditions are seldom able to maintain constant growth and as a result, cyanobacteria have evolved to cope with continuous cycles of growth and starvation. During prolonged starvation, many bacteria transition into non-proliferative states commonly referred to as stationary phase growth. In the laboratory, stationary phase is sooner or later reached in batch cultures. In cyanobacterial research, few studies have focused on stationary phase itself but rather responses to stress which result in the cessation of growth, most commonly nutrient limitation. While there are specific responses for particular nutrients, a number of general responses occur under any starvation condition including the cessation of cell division and significant changes in cell morphology and metabolism (Schwarz and Forchhammer, 2005). In particular, a general decrease in anabolic and increase in catabolic processes is commonly observed during acclimation.

A well-studied phenomenon is the degradation of light-harvesting phycobilisomes (PBSs). These pigmented structures are rich in sulfur and nitrogen and degradation may provide amino acids for the synthesis of proteins important for acclimation (Grossman et al., 1993). Indeed, my results demonstrated both a strong decrease in transcripts encoding phycobilisome components (Figure 6-13) as well as an increase in *nblA* transcript involved in phycobilisome degradation during late growth in all conditions. As PBSs serve as antenna complexes for photosynthesis, PBS degradation also reduces energy transfer to the chlorophyll molecules and thus the potential formation of reactive oxygen species (ROS) and subsequent damage to cellular components including DNA, RNA and proteins. This down-regulation of electron transfer is important given the general decrease in anabolic processes and thus decrease in reoxidation of the final electron acceptors. Long-term N-starvation studies in *Synechococcus* spp. PCC 7942 have revealed dramatic decreases in the activity of photosystems I and II (0.1 % compared to actively growing cells) as well as slow turnover of proteins involved in photosynthesis but not translational machinery (Sauer et al., 2001). In agreement, I observed significant down-regulation of transcripts encoding subunits of photosystems, ATP synthase and ribosomes suggesting this a typical

response during late growth in cyanobacteria. Furthermore, the strongest response I observed during late growth was the down-regulation of transcripts involved in chlorophyll biosynthesis, the major pigment present in the photosystems. Overall, there is a shift towards low metabolism during late growth in cyanobacteria indicating a transition into “standby” mode until more favourable conditions arise.

6.3.2 Factors with positive effects on survival

Negative mechanisms to reduce toxicity and cellular demand to enhance survival during late growth are well described for cyanobacteria, particularly the reduced activity of photosynthesis as discussed in the previous section. By contrast, positive mechanisms that enhance survival during late growth are lacking in the dataset. This may be due to a general lack of knowledge of such mechanisms in cyanobacteria, which is reflected in the fact that the majority of genes up-regulated during late growth in all conditions had no functional annotation. One gene, *slr1747*, is a homolog of the cell death suppressor protein Lls1 (lethal leaf spot 1). This gene is highly conserved in plants and is important for limiting the spread of cell death in leaves (Gray et al., 1999). In maize, the encoded protein contains two structural motifs that are highly conserved in bacterial phenolic dioxygenases. Phenolic compounds become highly reactive under oxidative conditions induced by stress, generating ROS and phenolic free radicals and have been proposed as mediators of cell death in plants (Appel, 1993). These compounds are produced as secondary metabolites in cyanobacteria and the common induction of the Lls1 homolog during late growth suggests a similar role in cyanobacteria.

6.3.2.1 Natural shifts towards energy production during late growth

Hydrogenase enzymes have attracted a lot of interest in recent years due to their ability to convert solar power into hydrogen energy. PCC 6803 possesses the bidirectional Hox enzyme belonging to the [NiFe] class of hydrogenases that catalyse both hydrogen oxidation and proton reduction (Khanna 2015). Despite their popularity, the precise physiological role of hydrogenases is still under

debate. Their activity has been linked to photosynthesis, acting as a valve to dissipate excess electrons generated during the light reactions of photosynthesis (Appel et al., 2000). Biosynthesis of this enzyme requires the help of several additional proteins including HypA1 and HypD which incorporate nickel (Ni) and iron (Fe) respectively into the active centre of the enzyme (Hoffmann et al., 2006). Both *hypA1* and *hypD* transcripts were consistently induced in my study suggesting a role for hydrogenase activity during late growth. Indeed, the dissipation of excess reductant resulting from reduced metabolism may play an important role during late growth. This has great implications for the applications of high-density cyanobacterial cultures in the generation of renewable fuels.

6.3.3 Signalling cascades are important for late growth survival in bacteria

Stationary phase survival, and in fact stress survival in general, relies heavily on signalling cascades to regulate gene expression and thus coordinate a diverse array of metabolic activity in bacteria (Llorens et al., 2010). Indeed, I observed a large increase in transcripts related to both signalling and gene regulation during late growth in PCC 6803. This included the induction of genes involved in two-component signal transduction systems as well as the production of intracellular signaling molecules cyclic AMP and acetyl phosphate.

6.3.3.1 Two-component signal transduction systems

Two-component signal transduction systems (2CSs) are a common approach to monitor external conditions and adjust accordingly, typically by influencing gene expression (Parkinson and Kofoid, 1992). Six genes involved 2SCs were commonly regulated (five up and one down) during late growth across all conditions. The sole histidine kinase that was consistently down-regulated during late growth in this study was *hik31*. A virtually identical copy of this gene resides on the plasmid pSYSX (*slr6041*), however the plasmid copy of this gene did not respond in the same manner to changes in growth. Chromosomal *hik31* expression during active growth followed by down-regulation during late growth has previously

been demonstrated by RT-PCR (Nagarajan et al., 2012). This system is implicated in the negative control of autotrophic events and glucose metabolism and a general role in the regulation of growth and cellular homeostasis has been suggested (Kahlon et al., 2006, Summerfield et al., 2011). Microarray analyses comparing gene expression in wild type and *Δhik31*-inactivated mutants in response to low-oxygen revealed the negative regulation of a large set of genes associated with growth, most notably photosynthesis, ATP synthesis and translation. While I observed common down-regulation of genes within these same functional categories, the negative regulation mechanism previously proposed as a result of phenotypic and microarray analyses argue against gene regulation by Hik31 in response to changes in growth.

Hik34, a histidine kinase sensor unique to cyanobacteria, is implicated in the regulation of gene expression under various stresses including salt, heat and oxidative stress (Marin et al., 2003, Suzuki et al., 2005, Kanesaki et al., 2007). Under heat stress, Hik34 has a proposed role as a negative regulator since inactivation of *hik34* results in enhanced expression of heat shock genes and thus enhanced thermotolerance (Suzuki et al., 2005). Aside from heat shock proteins that are not commonly regulated during late growth according to our data, Hik34 also negatively regulates the expression of photosynthesis-related genes including *hemA*, *cpcC*, and *atpF,* which encode the tRNA-Gln reductase responsible for catalysing the first step of chlorophyll biosynthesis, the phycocyanin linker protein and a subunit of ATPase respectively. These genes are commonly down-regulated during late growth in my dataset and may be targets of Hik34.

The roles of the remaining 2CSs commonly induced during late growth in this study (*hik23, hik26, sll5060* and *slr1594*) are still undefined. Neither stimuli inducing their expression nor potential targets have been described. 2CS studies in cyanobacteria typically involve gene inactivation followed by phenotypic and transcriptomic analysis of mutants under various stress conditions. This is the first study to demonstrate the induction of these 2CSs. Previous nutrient-limitation studies failed to identify these 2CSs, however this may be due to their focus on shock responses to complete elimination of the nutrient of interest

(Hirani et al., 2001, Richaud et al., 2001, Krasikov et al., 2012, Zhang et al., 2008, Suzuki et al., 2004). By contrast, I studied changes in response to gradual nutrient depletion that evidently induce a different response regulated by a different set of factors. This suggests important roles for these uncharacterized 2CSs in the gradual acclimation to nutrient depletion.

6.3.3.2 Cellular cAMP

Adenylate cyclase catalyses the conversion of ATP to cylic AMP (cAMP), a secondary messenger which plays important roles in the regulation of various biological activities including enzyme activity and gene expression in both prokaryotes and eukaryotes. Transcript levels of *cya1,* which encodes adenylate cyclase, was consistently induced during late growth in this study, suggesting up-regulation of cAMP production. In cyanobacteria, cellular cAMP levels vary in response to environmental factors including light, pH and nitrogen availability (Cann, 2004). This influences the activity of cAMP-receptor proteins (CRPs; also referred to as catabolite activator proteins, CAPs) which act as global transcriptional regulatory proteins via binding to specific motifs in the promoter regions of target genes. One such protein, SYCRP1, is implicated in twitching motility observed in response to blue light illumination in PCC 6803. Perception of blue light (450 nm) results in marked increases in cellular cAMP levels which promotes SYCRP1 binding to promoters of genes encoding type IV pili which form hair-like filaments extending from the outer membrane of cyanobacteria (Ohmori and Okamoto, 2004). Interestingly, increases in cAMP secretion during stationary phase growth have been reported for the filamentous cyanobacterium *Anabaena flos-aquae* (Francko and Wetzel, 1981), however the same was not observed in stationary phase cultures of PCC 6803 (Sakamoto et al., 1991). There was no evidence of up-regulation of cAMP-receptor proteins during late growth in my dataset, however only two are annotated in the PCC 6803 genome to date.

6.3.3.3 Acetyl phosphate, a global sensor of physiological state

Phosphotransacetylase catalyses the production of acetyl phosphate which has been suggested as a global sensor of physiological state in cyanobacteria (Morrison et al., 2005). Acetyl phosphate levels fluctuate in response to growth

phase, carbon source, pH and temperature. I observed consistent up-regulation of the gene encoding phosphotransacetylase, *pta*, during late growth suggesting increased acetyl phosphate production during this phase. This can have direct impacts on transcription as acetyl phosphate may act as an alternative phosphodonor for response regulators in 2CSs. Indeed, several bacterial response regulators are activated by acetyl phosphate *in vitro* (McCleary and Stock, 1994) and a recent study in *B. subtilis* has demonstrated *in vivo* enhancement of Rre phosphorylation in the presence of elevated acetyl phosphate levels (Cairns et al., 2015). This may serve as an alternative mechanism to activate 2CSs and thus coordinate gene expression when ATP levels are likely reduced given low transcription of ATP synthase subunits during late growth.

6.3.4 Promoters regulated in response to changes in growth

Bacteria have numerous mechanisms to regulate gene expression including mechanisms that do not involve direct contact with promoters of genes. For instance, changes in DNA topology, particularly DNA supercoiling, play an important role in mediating responses to environmental stress in bacteria (Dorman, 1996). The induction of a large number of uncharacterized regulatory factors in this study gives hope for the identification of growth phase-specific promoters. Indeed, regulatory mechanisms can be subject to signal cascades involving multiple factors and do not necessarily depend on direct interaction with DNA. For instance, Hik31 regulation of glucose metabolism appears to be post-transcriptional given the lack of change observed in the transcriptome of *hik31*-inactivated mutants (Kahlon et al., 2006). However, the majority of 2CSs mediate a direct transcriptional response via binding of the response regulator to the promoter regions of genes (Los et al., 2010). Given that four uncharacterised genes encoding components of 2CSs were reproducibly induced during late growth across multiple conditions, it is likely that growth phase-specific promoters do indeed exist in cyanobacteria. Furthermore, it is likely that motifs specifically recognized by these 2CSs are present in promoters important for growth-specific regulation.

Motif discovery algorithms have been developed to identify specific nucleotide sequences overrepresented in one set of DNA sequences as compared with another. The work described in this chapter provides the experimental dataset to enable such analysis in future. I have defined a background set of expressed genes and identified foreground sets of genes that are robustly co-regulated in response to culture growth phase. As multiple factors may be regulating expression in response to growth, I performed clustering analysis to identify subsets of co-regulated genes to enrich for potential factor-specific motifs. It is important to note that bacterial genes tend to be organised in operons in which multiple genes are transcribed from a single promoter. Visualisation of where the RNAseq reads map to the cyanobacterial genome will inform on operon structure and thus the selection of promoters to be analysed. Motifs have previously been identified in nitrate- and phosphate-responsive genes (Suzuki et al., 2004, Luque et al., 1994). These can serve as useful tools to aid in the selection of motif discovery algorithm as well as optimisation of the length of upstream sequences to be included in the analysis.

6.3.5 Specific nutrient-responsive genes and promoters

For practical applications, the dataset presented in this chapter provides a goldmine of information of global transcriptomic responses to changes in growth across a variety of conditions. This data can give indications of changes in metabolism and thus flux arising from specific nutrient limitations, thus enabling the tailoring of media and conditions to enhance flux towards particular processes. This information can be further expanded by incorporating more conditions including light intensity, carbon availability as well as combined effects of multiple nutrient limitations to name a few. Specific nutrient responses can also be identified and analysis of gene clusters and upstream sequences may provide more auto-inducible regulatory elements to the growing molecular toolbox for cyanobacteria. While it is generally not ideal to regulate processes using factors necessary for growth, this can still serve as a sustainable approach to regulate gene expression and further complement processes in custom media tailored to enhance metabolic flux. This approach can be applied to identify other nutrient-responsive promoters that do not have such inhibitory

effects on growth. Finally, this is the first study of response to cation (Mg and K) limitation in PCC 6803, and it is now possible to glean both general as well as specific responses to cation stress. This will add another level of understanding to this already thoroughly studied model organism, thus enhancing the rational design of this system.

Chapter 7. General discussion

Cyanobacteria are an attractive chassis for practical applications due to their ability to convert solar energy into chemical energy and biomass. While cyanobacteria have shown early signs of promise for a wide array of applications, they still do not present a real alternative to heterotrophic systems. The recurring limitation is the lack of tightly regulated promoters to control gene expression in this organism at a desired point of time during the process (Angermayr and Hellingwerf, 2013). A synthetic biology approach can help tackle this problem using well-defined, standardised genetic tools for rapid assembly of rationally designed gene expression cassettes to enhance productivity. In bacteria, promoters that are specifically active in high-density cultures are desirable as they prevent interference of transgene expression during active culture growth. Furthermore, auto-inducible promoters regulated by endogenous cues arising from changes in growth phase avoid the need for costly and often toxic supplements. I therefore set out to identify promoters specifically activated in high-density cultures of cyanobacteria. A summary of this work is presented in Table 7-1 and will be discussed in closer detail in the following sections.

Table 7-1. Overview of the work and future development *(overleaf)*

Abbreviations : PCC 7002, *Synechococcus* spp. PCC 7002; RBS, ribosome binding site; GFP, green fluorescent protein; N, nitrate; P, phosphate; S, sulphate; K, potassium; Mg, magnesium; PCC 6803, *Synechocystis* spp. PCC 6803; BG11-K, potassium-limited BG11 media; BG11-Mg, magnesium-limited BG11 media.

Table 7.1 Overview of the work and future developments

Task	Advantages	Disadvantages	Potential applications	Future improvements
Expression vector pAQ1BB	• BioBrick compatible	• PCC 7002 specific • Classical cloning strategy • Homologous recombination	• Promoter characterization • Expression of BioBrick devices in PCC 7002	• Next-generation cloning • Genetic isolation of modules in cloning site • Standardised sequencing primer binding sites • Alternative landing pads for homologous recombination
Strong promoter P_{cpcBA2}	• BioBrick compatible • Removal of translation start codon • Prevent aberrant protein formation and waste of cellular resources	• Highest activity during exponential growth phase • Relatively long sequence	• Drive high level expression in early culture growth	• Determine essential elements and reduce promoter length • Develop promoters specifically active during late exponential or stationary phase
RBS-GFP library	• Wide range of translation initiation • BioBrick compatible • Validation of Salis Lab RBS calculator for PCC 7002	• PCC 7002 specific • RBSs specific for GFP	• Detect wide range of transcriptional activity • Verify transgene integration in landing pads/generation of knockout strains	• Further expand range and increase resolution of translation initiation • Develop libraries for other reporters
Growth conditions	• Highly reproducible growth • Defined composition for nutrient limited conditions: N, P, S, K, Mg • Two novel conditions: BG11-K, BG11-Mg • K as a tool to control cell density	• Variable air flow • Light intensity unknown • Mineral nutrient limitation only • Possibly PCC 6803 specific	• Characterise exponential to stationary phase transition • Determine nutrient-specific responses	• Air flow regulators • Temperature controlled chambers • Expand range of conditions
Gene expression analysis	• High resolution expression profiles (timepoints, conditions) • Early growth-specific gene clusters • Late growth-specific gene clusters • Transcriptomes of two novel conditions sequenced: BG11-K, BG11-Mg	• Only two time points for RNAseq • Transcriptome only; does not capture post-transcriptional regulation	• Determine general response during exponential to stationary phase transition • Determine specific responses to nutrient limitation	• Further expand time points and conditions for RNAseq • Validate RNAseq results by qPCR • Analyse upstream regions of gene clusters to identify overrepresented motifs • Assess activity of promoters containing motifs

7.1 Standardised molecular tools for cyanobacteria

7.1.1 Further development of the pAQ1BB plasmid vector

To assess the strength and specificity of candidate promoters, I established a GFP-based assay to quantify promoter activity *in vivo* throughout culture growth of the marine cyanobacterium *Synechococcus* spp. PCC 7002. The components of this assay were developed in accordance with the BioBrick design for compatibility with the Registry of Standardised Parts. Importantly, a plasmid vector, pAQ1BB, was developed to include unique key restriction sites required for BioBrick assembly. Additional features should be incorporated to genetically isolate modules within the cloning site including forward and reverse transcriptional terminators and translational stop codons to prevent aberrant transgene product formation. Furthermore, standardised sequencing primer binding sites will improve sequencing quality to confirm correct assembly. BioBrick verification primers are not commonly available at sequencing facilities such as GATC (Konstanz, Germany) so I instead propose using universal primers such as the M13 forward and reverse primers.

7.1.2 Developing landing pads for stable transgene integration

The pAQ1BB vector facilitates stable integration of BioBrick modules into the PCC 7002 genome by homologous recombination, which sadly restricts its application to this particular species of cyanobacteria. Broad host range autonomous vectors could provide a more general tool for cyanobacteria and have indeed been recently developed (Taton et al., 2014). These vectors do have their limitations however as they require constant selective pressure and typically maintain a low copy number in bacteria. Instead, pAQ1BB can be modified by replacing the nucleotide sequences involved in recombination using the unique flanking restriction sites for transformation of other organisms. A library of plasmid vectors targeting different regions, or “landing pads”, in the genomes of different species of cyanobacteria would be a highly valuable tool. For instance, numerous modules could be introduced into a single organism at

neutral sites where deletion or interruption has no phenotypic affect. BioBricks of flanking sequences for homologous recombination to a neutral site in an annotated open reading frame with no known function (*slr0168*) have already been developed for PCC 6803 (BBa_K142000 and BBa_K142001). Alternatively, genes encoding key metabolic enzymes may serve as landing pads for simultaneous redirection of metabolic flux and introduction of heterologous modules.

7.1.3 Speeding up part assembly

Arguments can be made against the selection of the BioBrick design as it relies on classical cloning techniques, which are limited by the restriction sites available for assembly, and the number of components that can be simultaneously assembled, namely two. Indeed, it is essential to adopt next-generation cloning technologies, which enable high precision, rapid assembly of multiple components in single reactions. Paired with software tools to design plasmid vectors, this will drastically reduce time and effort required to generate complex expression systems. Golden Gate cloning is a particularly attractive method which utilizes Type IIS restriction enzymes to cut DNA outside of their recognition sites enabling scarless fusion when necessary (Weber et al., 2011). A Golden Gate cloning toolbox has recently been developed plants (Engler et al., 2014), however since its introduction, Golden Gate cloning has failed to replace BioBrick assembly as the favoured approach by the synthetic biology community (Kahl and Endy, 2013). Despite its drawbacks, the plasmid developed in this work single-handedly opens up an enormous catalog of molecular tools for the bioengineering of PCC 7002, notably protein domains and coding sequences of diverse function such as biosynthesis, post-translational modification and membrane transport.

7.1.4 Designing ribosome binding sites to enhance product synthesis

Currently available parts may require some optimisation to improve efficiency in PCC 7002 such as enhancing translation initiation via the ribosome binding site (RBS). A number of *in silico* translation rate calculators have been developed in recent years and I have validated the utility of the Salis Lab RBS Calculator for PCC 7002. This calculator, although not perfect, is a reasonably accurate tool to reverse engineer RBSs of defined translation initiation rates in this chassis. It enabled the generation of a library of synthetic ribosome binding sites to drive a range of translation rates for GFP. Not only can this library be used to detect a wide range of transcriptional activity, but it can also be a valuable tool to verify successful integration of modules to novel landing pads or assess the effects of DNA topology on gene transcription. The Salis Lab RBS Calculator did have its inaccuracies, however, as gene product formation did not always agree with predicted translation initiation rates. Other RBS calculators have been developed and could prove more accurate for this cyanobacterium. Nevertheless, RBS efficiency is highly context (i.e. sequence) dependent so synthetic RBSs would need to be designed and verified for each construct. In summary, RBS calculators are useful tools to optimise gene production formation, but they are not ideal features with which to modulate gene expression levels.

7.1.5 Promoter design and characterisation

Promoters are robust regulators of gene expression and therefore ideal components for regulating processes in biological systems. Furthermore, the transcriptional activity of promoters is easily characterised using assays such as the one I have developed thus enhancing the predictability of the function of multipart modules. Few characterised promoters are known to regulate gene expression in PCC 7002. One such promoter is P_{cpcBA} of PCC 6803, which has been described as a “super strong” regulator in PCC 7002 (Xu et al., 2011, Zhou et al., 2014). This promoter was selected to optimize the promoter assay. Variable reporter signal under the control of P_{cpcBA} and subsequent *in silico* RBS analysis identified undesirable translation initiation from the 3’ end of the promoter

sequence. I therefore optimised this promoter by removing the 3' start codon and further introducing flanking BioBrick restriction sites to generate P_{cpcBA2}. This was important to prevent a) translation initiation from a part intended to regulate transcriptional activation thus enhancing the prediction of multipart module function, b) aberrant protein formation and c) waste of cellular resources. However, some promoters do contain essential DNA elements downstream of the transcriptional start site. For instance, the *secA* promoter of PCC 6803 contains two important regulatory elements located within the *secA* protein coding sequence required to maintain its light-regulated expression (Mazouni et al., 1998). While promoters containing transcriptional start sites are not ideal, aberrant protein formation can be avoided by introducing a stop codon between the promoter and RBS sequences. Assessment of P_{cpcBA2} in PCC 7002 revealed high activity during early growth followed by a decline during late growth. This promoter is therefore not ideal for regulating processes requiring large amounts of biomass. Nevertheless, the pattern of activity observed in the heterologous reporter system reproduced native transcriptional activity in the freshwater cyanobacterium *Synechocystis* spp. PCC 6803. The promoter assay is therefore a valuable new tool for the characterization of promoters in PCC 7002. Furthermore, activity of a *Synechocystis* promoter in *Synechococcus* is promising as the use of heterologous promoters could reduce the risk of crosstalk with the native transcriptional system of this cyanobacterium.

7.2 Identification of late growth-specific promoters

7.2.1 Establishing conditions to study the transition from exponential to stationary phase

To identify robust late growth-specific promoters from PCC 6803, growth conditions had to be established in order to interrogate changes in gene expression across a range of backgrounds where cultures have entered stationary phase for different reasons. A controlled system was established for growth of PCC 6803 that is easily reproducible in other laboratories. There are, however, potential sources of variation between laboratories and setups that may affect

exponential growth rates. Firstly, ambient air exposure may vary subtly between different settings and airflow may vary depending on the aquarium pump. Gas tanks of defined composition fitted with airflow regulators will ensure more reproducible growth across laboratories. Secondly, light intensity experienced by cultures could not be determined due to the experimental set up in water baths. Alternatively, the use of temperature-controlled chambers can allow for direct measurement of light intensity. Despite these limitations, the established growth system allowed reproducible growth of PCC 6803.

Five nutrients were identified which specifically limited growth: nitrate (N), phosphate (P), sulphate (S), magnesium (Mg) and potassium (K). Notably, this is the first report of growth inhibition in response to limited Mg and K supplies in PCC 6803. Given that these nutrients constitute essential components for biomolecules, turgor pressure and the regulation of cell division, it is expected that these conditions will similarly limit growth in other species of cyanobacteria, but this will need to be confirmed. I selected a nutrient concentration that limited culture growth while still generating enough cell material for analysis. Ideally, growth should be characterised for a range of concentrations and varying combinations of limiting nutrients. However, the chosen growth conditions were sufficient to identify late growth-phase regulated genes. Instead, including additional conditions would increase confident in the robustness of responses observed and the regulators involved. For instance, growth under variable light intensity and carbon availability as well as heterotrophic conditions should be assessed. The effects of other mineral nutrients such as calcium (Ca) and iron (Fe) could also be assessed.

7.2.2 Enhancing resolution of transcript analysis to identify robust gene expression profiles

Comparison of transcript levels at regular intervals throughout growth enabled the generation of high-resolution expression profiles of individual genes. Genes following growth phase-specific expression patterns were selected from published microarray studies and transcript levels were measured by quantitative RT-PCR (qPCR). Growth phase-specific expression profiles were

typically reproducible under standard nutrient conditions. However, standard nutrient profiles were often distorted under double nutrient-limited conditions. These findings highlighted the importance of assessing expression profiles under a range of conditions. One gene, *slr9003*, showed a relatively robust growth phase-specific expression pattern across the three conditions tested. Nevertheless, the promoter of this gene was not an ideal candidate to activate late growth-specific expression since a) some induction in gene expression was observed during early growth and b) elevated transcript levels were not always maintained during late growth. Thus, substantial optimisation would be required to silence the promoter under non-induced conditions (early growth) and to maintain activity during induced conditions (late growth).

Sigma factors implicated in the regulation of growth phase-specific gene expression were assessed as potential markers for entry into stationary phase growth. qPCR analysis of cultures grown under standard nutrient conditions revealed highly variable, oscillating patterns despite numerous reports of late exponential and stationary phase induction. Previous studies compared expression levels across three time points representing early to mid-exponential, late exponential and stationary phase growth. By contrast, I compared expression levels across ten time points spanning culture growth. The enhanced resolution of my analysis revealed fluctuations of gene expression over time and thus tested robust growth phase-specific gene expression more stringently. The oscillations in transcript levels suggests post-transcriptional mechanisms to regulate sigma factor function, so these genes are not suitable indicators of growth phase in PCC 6803.

7.2.3 Identifying novel growth phase-specific genes

Comparison between early and late time points across the established conditions provided an opportunity to identify genes with growth phase-specific responses independent of the actual growth conditions. Following RNA sequencing, a large number of co-regulated genes were identified. In agreement with the general observation that stationary phase cultures enter a “standby” metabolic mode, the most prominent response was the down-regulation of photosynthesis,

respiration and translation during the late exponential phase across all conditions. By contrast, the majority of genes up-regulated during this phase encode uncharacterised proteins suggesting a great number of physiological functions relating to stationary phase growth and survival which remain to be unravelled. Interestingly, a number of genes involved in two-component signal transduction systems were also up-regulated during the late exponential phase across all conditions. These genes may encode important regulators of gene expression during stationary phase growth, suggesting the presence of specific DNA motifs in the promoters of genes induced during this phase. To enhance motif discovery, genes were subdivided into clusters of similar expression profiles. Furthermore, a background list of expressed genes was defined to provide a relevant reference to which the upstream regions of these gene clusters should be compared.

With the ultimate aim of identifying growth phase-specific promoters, I focused my analysis on genes, which were consistently up- or down-regulated across all of the conditions. Many additional analyses can and should be performed on this large dataset to identify genes with other interesting expression patterns. For instance, genes could be identified that respond to specific nutrient limitations that have not been investigated before, namely K and Mg limitation. Furthermore, the data can be mined to identify nutrient-specific as well as general changes in metabolism during late exponential phase. However, these questions were outwith the scope of this project. For the identification of growth phase-specific genes and promoters, it would be useful to analyse additional time points, particularly during the stationary phase, to increase confidence in growth phase-specific expression patterns and confirm maintained activation during the stationary phase. In the absence of these additional time points, it is essential to analyse the expression profiles of selected genes using the qPCR approach discussed in the previous section. This will both validate the RNA sequencing results and confirm growth phase-specific expression profiles for individual genes.

7.2.4 RNA sequencing versus microarray analysis

Shortly after the genome sequence of PCC 6803 was made available in 1996, a microarray platform, denoted cyanoCHIP, was commercialised by Takara Bio Inc. (Kaneko et al., 1996). This powerful tool provided a comprehensive view of transcriptional activity and more than 700 microarray experiments have since provided valuable information on gene regulation across numerous environmental conditions and genetic backgrounds in this organism (Hernández-Prieto et al., 2014). Microarray expression analyses are based on the hybridisation of cDNA to probes immobilised on a solid surface. Originally, probes consisted of double stranded PCR fragments of C-terminal 1 kilobase protein coding genes (Hirani et al., 2001). This has the distinct disadvantage of requiring prior knowledge of gene and transcript sequences and importantly does not differentiate between sense and anti-sense RNAs. By contrast, next generation sequencing of RNA (RNAseq) enables unbiased strand-specific transcript detection. Expression profiling using RNAseq has revealed the existence of non-protein coding transcripts (ncRNAs) occurring outside of protein coding genes which could not be detected in microarrays. Surprisingly, ncRNAs make up a substantial part of the transcriptome, in particular antisense RNAs, and account for nearly two thirds of the transcriptome of PCC 6803 grown under standard conditions (Mitschke et al., 2011). Additionally, RNAseq enables the determination of transcription unit architecture, notably transcriptional start sites, which are invaluable for promoter analyses such as the one intended for this study. Thus, RNAseq was the most appropriate method to sample the transcriptome of PCC 6803 in this study.

7.2.5 Gene expression levels in single cells versus bulk cell populations

Gene expression is an intrinsically stochastic process arising from random biomolecular interactions. As a result, gene expression levels can vary drastically between individual cells within an isogenic population and thus affect the chances of survival under stress conditions (Raj and van Oudenaarden, 2008). Indeed, a recent RNAseq study demonstrated that variation in single cell gene

expression levels increases over time following nitrogen starvation in PCC 6803 (Wang et al., 2015). Given that my RNAseq study has focused on the responses of bulk cell populations to stress under different nutrient conditions over time, it is expected that individual cells within these samples may show different transcript profiles. The purpose of my study was to identify genes that reproducibly respond to changes in growth phase, however. It is known that variation in gene expression levels decreases as cell population size increases, particularly for lowly expressed genes (Piras and Selvarajoo, 2015). It was therefore appropriate to assess average expression levels across a large population, as opposed to individual cells, in order to identify robust candidate promoters.

7.2.6 The importance of silencing during non-induced conditions

Inducible promoters are extremely useful tools for industrial applications due to two key characteristics. Firstly, efficient silencing during non-induced conditions prevents undesirable interactions with other stages within the process. Secondly, robust activation in response to a specific stimulus enables precise timing of each stage in a multi-step process. While a library of promoters spanning a wide range of transcriptional activation would be ideal for metabolic engineering, it is not a necessity. For instance, weak promoters can be used to drive high expression when present in high copy numbers or combined with strong ribosome binding sites and stable gene products. Promoters with high transcriptional activity under induced conditions are therefore not essential for successful engineering. By contrast, efficient silencing during non-induced conditions is vadtly more important. Leaky expression results in misallocation of precious cellular resources and potential undesirable interactions. In the case of stationary phase-specific gene expression, inefficient silencing during exponential phase can result in trade-offs between biomass production and product biosynthesis and therefore negative effects on growth rates, amount of biomass and final product titres. Thus, absence of expression during the exponential phase is more important than high expression in stationary phase for a growth phase-regulated manufacturing platform.

7.2.7 Future work: Motif discovery and promoter identification

I identified a large number of genes with potential function as transcription factors during late exponential growth of cyanobacterial cultures. This suggests the presence of promoters that are specifically recognized by these factors. Motif discovery algorithms such as SeAMotE (Agostini et al., 2014) should be applied to identify specific motifs overrepresented in the upstream regions of the co-regulated gene clusters as compared with the background set of expressed genes. For this purpose, it is necessary to optimise the length of the sequence as well as the position relative to the transcriptional start site (TSS). Sequence length is important as sequences that are too long may generate false positives whereas sequences which are too short may not identify any motifs at all. Initially, upstream regions of differing lengths (300, 600 and 1000 base pairs) have been extracted and are currently being analysed. While promoter elements typically occur upstream of the TSS, they may also occur within the gene itself such as the light-inducible *secA* promoter described in *Section 7.1.5* (Mazouni et al., 1998). It may therefore be necessary to include sequences extending into the protein coding sequence. Furthermore, the defined gene clusters may require further refinement to exclude genes co-transcribed as polycistronic mRNA molecules from a single promoter.

Upon successful identification of overrepresented motifs, it will be necessary to characterise promoters containing these motifs *in vivo*. Naturally occurring promoters containing these motifs should be assessed first. In this case, promoters from PCC 6803 should be cloned upstream of RBS9-GFP in the pAQ1BB vector to ensure detection of transcriptional activity. As in the case of motif discovery, promoter length will again need to be optimised for the promoter assay to ensure the maintenance of growth phase-specific activation. Once a suitable length has been determined, classical promoter analyses can be performed to determine the minimum sequence required to maintain this activation pattern. Furthermore, essential elements and spacing between elements can be determined and optimised. This can inform the design of synthetic promoters, which may reduce the risk of crosstalk with native transcriptional systems.

The promoter assay in this study was established for PCC 7002. By contrast, the genes following growth phase-specific expression patterns were identified in PCC 6803. There is a risk that promoters identified from PCC 6803 may not function in a heterologous system, let alone maintain growth phase-specific activation patterns. If this is the case, activity of growth phase-specific promoters will need to be verified and characterised in PCC 6803 using appropriate vector backbones and can still serve as useful tools for the bioengineering of PCC 6803. I have however shown that the growth phase-specific activity of a promoter from PCC 6803 was maintained in PCC 7002. This promoter drives the expression of a photosynthesis-related gene involved in a process commonly down-regulated in response to multiple stresses across cyanobacteria and may be regulated by a factor common across bacteria. Similarly, transition to stationary phase growth is a common response to growth limitation in cyanobacteria. It is therefore possible that common factors are involved in regulating transcription during this phase and that growth phase-specific promoter identified in one species of cyanobacteria may prove useful tools in other species.

7.3 A proposed design for a cyanobacterial cell factory

The marked down-regulation of photosynthesis and ATP synthesis-related genes in high density cultures of cyanobacteria indicates that light may not be a suitable energy source in late exponential phase. Many species of cyanobacteria are capable of switching between photoautotrophic and heterotrophic growth (Rippka et al., 1979), so a system can be conceived in which the biomass is generated during a photoautotrophic growth phase whereas product synthesis is carried out in a subsequent heterotrophic phase as proposed in Figure 7-1. While photosynthesis may not be a suitable driver during the production phase, it is still highly advantageous during the growth phase as it minimises inputs and thus costs required to generate large amounts of biomass compared with fully heterotrophic systems. Growth phase-specific promoters can complement this system by activating the engineered process once the biomass has been achieved. Furthermore, timing the switch to heterotrophic production can potentially be controlled by modulating potassium (K) supplies. Implementing

such a system would require optimisation to time the addition of organic carbon sources and confirmation that growth phase-specific promoters maintain their characteristics under these changing conditions. Overall, a mixotrophic cell factory will still offer great advantages over heterotrophic and traditional production methods.

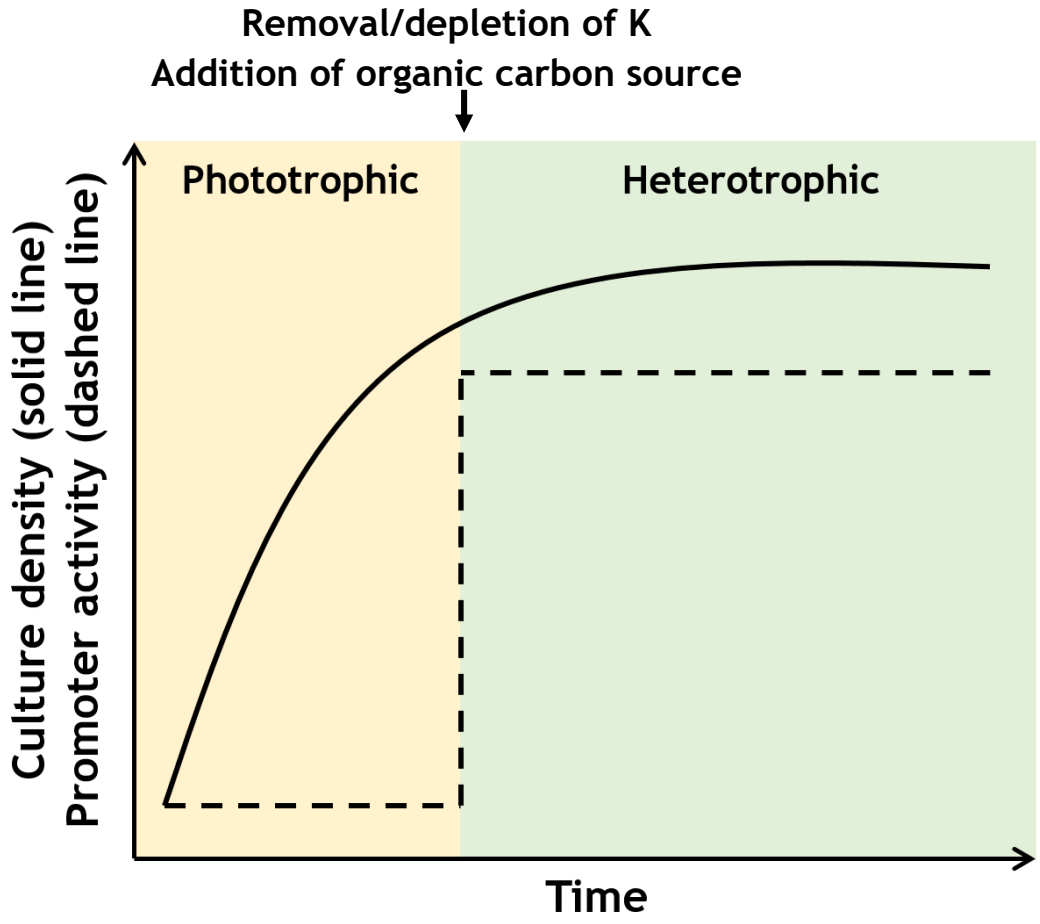

Figure 7-1. Proposed design for a mixotrophic cyanofactory

Cyanobacterial cell factories may be divided into two distinct phases: the growth phase under photoautotrophic conditions in which light drives biomass generation (yellow box) and the production phase under heterotrophic conditions (green box). Potassium (K) supply can be used to control timing of the latter phase. Growth phase-specific promoters active during the production phase can drive the engineered process.

7.4 Process selection and design

Once a cell factory system has been established, a process must be selected, designed, implemented and optimised. At this stage, computation modelling is an important tool to rapidly test possible modifications and assess their effects on metabolism and thus product synthesis. There is a wealth of experimental data available for the freshwater model cyanobacterium PCC 6803. However, it can be difficult to integrate information from studies performed under differing conditions using differing techniques. In this current age of "big data" in which large amounts of information are continuously generated by 'omics studies, normalization strategies are rapidly developing. Biologists are teaming up with programmers to develop more advanced and sophisticated software. Indeed, comprehensive metabolic models have been developed for PCC 6803 and inform novel compound discovery, identification of bottlenecks in engineered pathways and knockout strategies to enhance production of desired metabolites (Knoop et al., 2013, Nogales et al., 2014, Maarleveld et al., 2014). While this study investigated global changes in the transcriptome, it spans a large number of conditions and can give indications of changes in metabolism and thus flux arising from changes in growth as well as under specific nutrient limitations. For instance, up-regulation of genes involved in hydrogenase biosynthesis indicate a propensity for hydrogen energy production during high-density growth. Closer examination of the dataset may highlight many other processes as promising candidates under any or all of the conditions examined in this study. Furthermore, this dataset can be mined to inform media composition to enhance flux towards desired processes. Countless other conditions can be examined and every few months a new transcriptome is revealed under yet another condition. While extensive work is still required to validate metabolic models, integration of this dataset into existing models will enhance a fundamental tool for the engineering of cyanofactories.

7.5 Outlook

Like most of my fellow PhD candidates, I had to finish my project with many open ends, most notably the identification of growth phase-specific motifs and promoters. This work does, however, lay a solid foundation to enable the identification and characterization of these auto-inducible regulators that have great potential for a huge diversity of applications in cyanobacteria. They can serve as genetic switches to activate the degradation of toxic pollutants, extraction of valuable chemicals from wastewater and production of a wide array of valuable compounds for medicine, nutrition and energy. Furthermore, this work also provides a goldmine to data to inform the rational design of cyanobacteria.

More generally, the rational design of biological organisms using synthetic biology approaches shows great promise to enhance biological engineering. Standardised biological parts are abundant and diverse. Part characterisation is an ongoing project across different organisms and conditions. Computational models are being developed, refined and validated. Indeed, this discipline is still in its infancy and the wider community needs to come to a consensus on standardised approaches such as next-generation assembly methods. However, a concerted effort is underway to establish and validate this booming field. Given the rate at which synthetic biology has advanced in the last decade, there is hope to replace the global demand for fossil fuels before their limited supplies are exhausted with technologies that leave smaller, and maybe even positive, footprints behind.

REFERENCES

Abed, R. M., Dobretsov, S. & Sudesh, K. 2009. Applications of cyanobacteria in biotechnology. *Journal of Applied Microbiology,* 106**,** 1-12.

Agostini, F., Cirillo, D., Ponti, R. D. & Tartaglia, G. G. 2014. SeAMotE: a method for high-throughput motif discovery in nucleic acid sequences. *BMC Genomics,* 15**,** 925.

Aikawa, S., Nishida, A., Ho, S. H., Chang, J. S., Hasunuma, T. & Kondo, A. 2014. Glycogen production for biofuels by the euryhaline cyanobacteria Synechococcus sp. strain PCC 7002 from an oceanic environment. *Biotechnology for Biofuels,* 7**,** 88.

Alahari, A. & Apte, S. K. 1998. Pleitropic effects of potassim deficiency in a heterocystous, nitrogen-fixing cyanobacterium, Anabaena torulosa. *Microbiology,* 144**,** 1557-1563.

Amann, E., Ochs, B. & Abel, K. J. 1988. Tightly regulated tac promoter vectors useful for the expression of unfused and fused proteins in Escherichia coli. *Gene,* 69**,** 301-315.

Amezaga, J., Amtmann, A., Biggs, C. A., Bond, T., Gandy, C. J., Honsbein, A., Karunakaran, E., Lawton, L., Madsen, M. A., Minas, K. & Templeton, M. R. 2014. Biodesalination: A case study for applications of photosynthetic bacteria in water treatment. *Plant Physiology,* 164**,** 1661-1676.

An, W. & Chin, J. W. 2011. Orthogonal gene expression in Escherichia coli. *Methods in Enzymology,* 497**,** 115-134.

Angermayr, S. A. & Hellingwerf, K. J. 2013. On the use of metabolic control analysis in the optimization of cyanobacterial biosolar cell factories. *Journal of Physical Chemistry B,* 117**,** 11169-11175.

Appel, H. M. 1993. Phenolics in ecological interactions: The importance of oxidation. *Journal of Chemical Ecology,* 19**,** 1521-1552.

Appel, J., Phunpruch, S., Steinmüller, K. & Schulz, R. 2000. The bidirectional hydrogenase of Synechocystis sp. PCC 6803 works as an electron valve during photosynthesis. *Archives in Microbiology,* 173**,** 333-338.

Asayama, M. & Imamura, S. 2008. Stringent promoter recognition and autoregulation by the group 3 sigma-factor SigF in the cyanobacterium Synechocystis sp. strain PCC 6803. *Nucleic Acids Research,* 36**,** 5297-5305.

Asayama, M., Imamura, S., Yoshihara, S., Miyazaki, A., Yoshida, N., Sazuka, T., Kaneko, T., Ohara, O., Tabata, S., Osanai, T., Tanaka, K., Takahashi, H. & Shirai, M. 2004. SigC, the group 2 sigma factor of RNA polymerase, contributes to the late-stage gene expression and nitrogen promoter recognition in the cyanobacterium Synechocystis sp. strain PCC 6803. *Bioscience Biotechnology and Biochemistry,* 68**,** 477-487.

Balleza, E., López-Bojorquez, L. N., Martínez-Antonio, A., Resendis-Antonio, R., Lozada-Chávez, I., Balderas-Martínez, Y. I., Encarnación, S. & Collado-

Vides, J. 2009. Regulation by transcription factors in bacteria: beyond description. *FEMS Microbiology Reviews,* 33, 133-151.

Barber, J., Mills, J. & Nicolson, J. 1974. Studies with cation specific ionophores show that within the intact chloroplast Mg++ acts as the main exchange cation for H+ pumping. *FEBS Letters,* 49, 106-110.

Barten, R. & Lill, H. 1995. DNA-uptake in the naturally competent cyanobacterium, Synechocystis sp. PCC 6803. *FEMS Microbiology Letters,* 129, 83-87.

Batterton, J. C. J. & Van Baalen, C. 1971. Growth responses of blue-green algae to sodium chloride concentration. *Archiv für Mikrobiologie,* 76, 151-165.

Begemann, M. B., Zess, E. K., Walters, E. M., Schmitt, E. F., Markley, A. L. & Pfleger, B. F. 2013. An organic acid based counter selection system for cyanobacteria. *PLOS ONE,* 8, e76594.

Berla, B. M. & Pakrasi, H. B. 2012. Upregulation of plasmid genes during stationary phase in Synechocystis sp. strain PCC 6803, a cyanobacterium. *Applied and Environmental Microbiology,* 78, 5448-5451.

Berla, B. M., Saha, R., Immethun, C. M., Maranas, C. D., Moon, T. S. & Pakrasi, H. B. 2013. Synthetic biology of cyanobacteria: unique challenges and opportunities. *Frontiers in Microbiology,* 4, 246.

Bertram, R. & Hillen, W. 2008. The application of Tet repressor in prokaryotic gene regulation and expression. *Microbial Biotechnology,* 1, 2-16.

Blankenship, R. E., Tiede, D. M., Barber, J., Brudvig, G. W., Fleming, G., Ghirardi, M., Gunner, M. R., Junge, W., Kramer, D. M., Melis, A., Moore, T. A., Moser, C. C., Nocera, D. G., Nozik, A. J., Ort, D. R., Parson, W. W., Prince, R. C. & Sayre, R. T. 2011. Comparing photosynthetic and photovoltaic efficiencies and recognizing the potential for improvement. *Science,* 332, 805-809.

Blasi, B. 2012. Characterization of Stress Responses of Heavy Metal and Metalloid Inducible Promoters in Synechocystis PCC6803. *Journal of Microbiology and Biotechnology,* 22, 166-169.

Blatter, E. E., Ross, W., Tang, H., Gourse, R. L. & Ebright, R. H. 1994. Domain organization of RNA polymerase alpha subunit: C-terminal 85 amino acids constitute a domain capable of dimerization and DNA binding. *Cell,* 78, 889-896.

Boyanapalli, R., Bullerjahn, G. S., Pohl, C., Croot, P. L., Boyd, P. W. & Mckay, R. M. 2007. Luminescent whole-cell cyanobacterial bioreporter for measuring Fe availability in diverse marine environments. *Applied and Environmental Microbiology,* 73, 1019-1024.

Browning, D. F. & Busby, S. J. 2004. The regulation of bacterial transcription initiation. *Nature Reviews Microbiology,* 2, 57-65.

Bruns, B. U., Briggs, W. R. & Grossman, A. R. 1989. Molecular characterization of phycobilisome regulatory mutants of Fremyella diplosiphon. *Journal of Bacteriology,* 171, 901-908.

Burnap, R. L., Troyan, T. & Sherman, L. A. 1993. The highly abundant chlorophyll-protein complex of iron-deficient Synechococcus sp. PCC7942 (CP43') is encoded by the isiA gene. *Plant Physiology,* 103**,** 893-902.

Burut-Archanai, S., Incharoensakdi, A. & Eaton-Rye, J. J. 2009. The extended N-terminal region of SphS is required for detection of external phosphate levels in Synechocystis sp. PCC 6803. *Biochemical and Biophysical Research Communications,* 378**,** 383-388.

Busby, S. & Ebright, R. H. 1999. Transcription activation by catabolite activator protein (CAP). *Journal of Molecular Biology,* 293**,** 199-213.

Cairns, L. S., Martyn, J. E., Bromley, K. & Stanley-Wall, N. R. 2015. An alternate route to phosphorylating DegU of Bacillus subtilis using acetyl phosphate. *BMC Microbiology,* 15**,** 78-90.

Camsund, D., Heidorn, T. & Lindblad, P. 2014. Design and analysis of LacI-repressed promoters and DNA-looping in a cyanobacterium. *Journal of Biological Engineering,* 8**,** 4.

Camsund, D. & Lindblad, P. 2014. Engineered transcriptional systems for cyanobacterial biotechnology. *Frontiers in Bioengineering and Biotechnology,* 2**,** 40.

Cann, M. J. 2004. Signalling through cyclic nucleotide monophosphates in cyanobacteria. *New Phytologist,* 161**,** 23-34.

Carvalho, L. R., Costa-Neves, A., Conserva, G. A. A., Brunetti, R. L., Hentschke, G. S., Malone, C. F. S., Torres, L. M. B., Sant'Anna, C. L. & Rangel, M. 2013. Biologically active compounds from cyanobacteria extracts: In vivo and in vitro aspects. *Brazilian Journal of Parmacognosy,* 23, 3, 471-480.

Cases, I., De Lorenzo, V. & Pérez-Martín, J. 1996. Involvement of sigma 54 in exponential silencing of the Pseudomonas putida TOL plasmid Pu promoter. *Molecular Microbiology,* 19**,** 7-17.

Chappell, J., Jensen, K. & Freemont, P. S. 2013. Validation of an entirely in vitro approach for rapid prototyping of DNA regulatory elements for synthetic biology. *Nucleic Acids Research,* 41**,** 3471-3481.

Cohen, Y., Jørgensen, B. B., Revsbech, N. P. & Poplawski, R. 1986. Adaptation to hydrogen sulfide of oxygenic and anoxygenic photosynthesis among cyanobacteria. *Applied and Environmental Microbiology,* 51**,** 398-407.

Colloms, S. D., Merrick, C. A., Olorunniji, F. J., Stark, W. M., Smith, M. C., Osbourn, A., Keasling, J. D. & Rosser, S. J. 2014. Rapid metabolic pathway assembly and modification using serine integrase site-specific recombination. *Nucleic Acids Research,* 42**,** e23.

Cooley, J. W. & Vermaas, W. F. 2001. Succinate dehydrogenase and other respiratory pathways in thylakoid membranes of Synechocystis sp. strain PCC 6803: Capacity comparisons and physiological function. *Journal of Bacteriology,* 183**,** 4251-4281.

Crabtree, G. W. & Lewis, N. S. 2007. Solar energy conversion. *Physics Today,* 60**,** 37-42.

Del Solar, G. & Espinosa, M. 2000. Plasmid copy number control: an ever-growing story. *Molecular Microbiology,* 37**,** 492-500.

Dheda, K., Huggett, J. F., Bustin, S. A., Johnson, M. A., Rook, G. & Zumla, A. 2004. Validation of housekeeping genes for normalizing RNA expression in real-time PCR. *Biotechniques,* 37**,** 112-4, 116, 118-119.

Dienst, D., Georg, J., Abts, T., Jakorew, L., Kuchmina, E., Börner, T., Wilde, A., Dühring, U., Enke, H. & Hess, W. R. 2014. Transcriptomic response to prolonged ethanol production in the cyanobacterium Synechocystis sp. PCC6803. *Biotechnology and Bioengineering,* 7**,** 21.

Dorman, C. J. 1996. Flexible response: DNA supercoiling, transcription and bacterial adaptation to environmental stress. *Trends in Microbiology,* 4**,** 214-216.

Ducat, D. C., Way, J. C. & Silver, P. A. 2011. Engineering cyanobacteria to generate high-value products. *Trends in Biotechnology,* 29**,** 95-103.

Ellis, T., Wang, X. & Collins, J. J. 2009. Diversity-based, model-guided construction of synthetic gene networks with predicted functions. *Nature Biotechnology,* 27**,** 465-471.

Endy, D. 2005. Foundations for engineering biology. *Nature,* 438**,** 449-453.

Engelbrecht, F., Marin, K. & Hagemann, M. 1999. Expression of the ggpS gene, involved in osmolyte synthesis in the marine cyanobacterium Synechococcus sp. strain PCC 7002, revealed regulatory differences between this strain and the freshwater strain Synechocystis sp. strain PCC 6803. *Applied and Environmental Microbiology,* 65, 11, 4822-4829.

Engler, C., Youles, M., Gruetzner, R., Ehnert, T., Werner, S., Jones, J. D. G., Patron, N. J. & Marillonnet, S. 2014. A Golden Gate modular cloning toolbox for plants. *ACS Synthetic Biology,* 3**,** 839-843.

Epstein, W. 2003. The roles and regulation of potassium in bacteria. *Progress in Nucliec Acid Research and Molecular Biology,* 75**,** 293-320.

Eriksson, J., Salih, G. F., Ghebramedhin, H. & Jansson, C. 2000. Deletion mutagenesis of the 5' psbA2 region in Synechocystis 6803: Identification of a putative cis element involved in photoregulation. *Molecular Cell Biology Research Communications,* 3**,** 292-298.

Essich, E., Stevens, S. E. J. & Porter, R. D. 1990. Chromosomal transformation in the cyanobacterium Agmenellum quadruplicatum. *Journal of Bacteriology,* 172**,** 1916-1922.

Federici, F., Rudge, T. J., Pollak, B., Haseloff, J. & Gutiérrez, R. A. 2013. Synthetic Biology: Opportunities for Chilean bioindustry and education. *Biological Research,* 46**,** 383-393.

Formighieri, C. & Melis, A. 2014a. Carbon partitioning to the terpenoid biosynthetic pathway enables heterologous ß-phellandrene production in Escherichia coli cultures. *Archives of Microbiology,* 196**,** 853-861.

Formighieri, C. & Melis, A. 2014b. Regulation of ß-phellandrene synthase gene expression, recombinant protein accumulation, and monoterpene hydrocarbons production in Synechocystis transformants. *Planta,* 240.

Foster, J. S., Singh, A. K., Rothschild, L. J. & Sherman, L. A. 2007. Growth-phase dependent differential gene expression in Synechocystis sp. strain PCC 6803 and regulation by a group 2 sigma factor. *Archives in Microbiology,* 187**,** 265-279.

Francko, D. & Wetzel, R. 1981. Dynamics of cellular and extracellular cAMP in Anabaena fos-aquae (cyanophyta): Intrinsic culture variability and correlation with metabolic variables. *Journal of Phycology,* 17**,** 129-134.

Frigaard, N. U., Sakuragi, Y. & Bryant, D. A. 2004. Gene inactivation in the cyanobacterium Synechococcus sp. PCC 7002 and the green sulfur bacterium Chlorobium tepidum using in vitro-made DNA constructs and natural transformation. *Methods in Molecular Biology,* 274**,** 325-340.

Fuszard, M. A., Ow, S. Y., Gan, C. S., Noirel, J., Ternan, N. G., Mcmullan, G., Biggs, C. A., Reardon, K. F. & Wright, P. C. 2013. The quantitative proteomic response of Synechocystis sp. PCC6803 to phosphate acclimation. *Aquatatic Biosystems,* 9**,** 5.

Gao, X., Gao, F., Liu, D., Zhang, H., Nie, X. & Yang, C. 2016. Engineering the methylerythritol phosphate pathway in cyanobacteria for photosynthetic isoprene production from CO_2. *Energy & Environmental Science,* Advance article.

Garcia Véscovi, E., Soncini, F. C. & Groisman, E. A. 1996. Mg2+ as an extracellular signal: environmental regulation of Salmonella virulence. *Cell,* 84**,** 165-174.

Gibson, D. G., Young, L., Chuang, R. Y., Venter, J. C., Hutchison, C. A., 3rd & Smith, H. O. 2009. Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nature Methods,* 6**,** 343-345.

Gilbert, W. & Müller-Hill, B. 1966. Isolation of the lac repressor. *Proceedings of the National Academy of Sciences,* 56**,** 1891-1898.

Giraldez-Ruiz, N., Mateo, P., Bonilla, I. & Fernandez-Piñas, F. 1997. The relationship between intracellular pH, growth characteristics and calcium in the cyanobacterium Anabaena sp. strain PCC7120 explosed to low pH. *New Phytologist,* 137**,** 599-605.

Gray, J., Close, P. S., Briggs, S. P. & Johal, G. S. 1999. A novel suppressor of cell death in plants encoded by the Lls1 gene of maize. *Cell,* 89**,** 25-31.

Griese, M., Lange, C. & Soppa, J. 2011. Ploidy in cyanobacteria. *FEMS Microbiology Lett,* 323**,** 124-131.

Groisman, E. A., Hollands, K., Kriner, M. A., Lee, E. J., Park, S. Y. & Pontes, M. H. 2013. Bacterial Mg2+ homeostasis, transport, and virulence. *Annual Review of Genetics,* 47**,** 625-646.

Grossman, A. R., Schaefer, M. R., Chiang, G. G. & Collier, J. L. 1993. The phycobilisome, a light-harvesting complex responsive to environmental conditions. *Microbiology Reviews,* 57**,** 725-749.

Grossman, A. R., Schaefer, M. R., Chiang, G. G. & Collier, J. L. 1994. *The responses of cyanobacteria to environmental conditions: Light and nutrients,* The Netherlands, Kluwer Academic Publishers.

Gruber, T. M. & Gross, C. A. 2003. Multiple sigma subunits and the partitioning of bacterial transcription space. *Annual Review of Microbiology,* 47**,** 441-466.

Grusak, M. A. 2001. Plant macro- and micronutrient minterals. *Encyclopedia of Life Sciences.* Chichester, England: John Wiley & Sons, Ltd.

Guedes, A. C., Barbosa, C. R., Amaro, H. M., Pereira, C. I. & Malcata, F. X. 2011. Microalgal and cyanobacterial cell extracts for use as natural antibacterial additives against food pathogens. *International Journal of Food Science & Technology,* 46, 4, 862-870.

Gulati, S., Rouilly, V., Niu, X., Chappell, J., Kitney, R. I., Edel, J. B., Freemont, P. S. & Demello, A. J. 2009. Opportunities for microfluidic technologies in synthetic biology. *Journal of the Royal Society Interface,* 6 Suppl 4**,** S493-506.

Hampsey, M. 2001. Omega meets its match. *Trends in Genetics,* 17**,** 190-191.

Hartley, J. L., Temple, G. F. & Brasch, M. A. 2000. DNA cloning using in vitro site-specific recombination. *Genome Research,* 10**,** 1788-1795.

Hase, R., Oikawa, H., Sasao, C., Morita, M. & Watanabe, Y. 2000. Photosynthetic production of microalgal biomass in a raceway system under greenhouse conditions in Sendai city. *Journal of Bioscience and Bioengineering,* 89**,** 157-163.

Heidorn, T., Camsund, D., Huang, H. H., Lindberg, P., Oliveira, P., Stensjo, K. & Lindblad, P. 2011. Synthetic biology in cyanobacteria engineering and analyzing novel functions. *Methods in Enzymology,* 497**,** 539-579.

Hengge-Aronis, R. 2002. Stationary phase gene regulation: what makes an Escherichia coli promoter sigmaS-selective? *Current Opinion in Microbiology,* 5**,** 591-595.

Hernández-Prieto, M. A., Semeniuk, T. A. & Futschik, M. E. 2014. Toward a systems-level understanding of gene regulatory, protein interaction, and metabolics network in cyanobacteria. *Frontiers in Genetics,* 5, 191.

Hirani, T. A., Suzuki, I., Murata, N., Hayashi, H. & Eaton-Rye, J. J. 2001. Characterization of a two-component signal transduction system involved in the induction of alkaline phosphatase under phosphate-limiting conditions in Synechocystis sp. PCC 6803. *Plant Molecular Biology,* 45**,** 133-144.

Hirokawa, Y., Suzuki, I. & Hanai, T. 2015. Optimization of isopropanol production by engineered cyanobacteria with a synthetic metabolic pathway. *Journal of Bioscience and Bioengineering,* 119, 5, 585-590.

Hoffmann, D., Gutekunst, K., Klissenbauer, M., Schulz-Friedrich, R. & Appel, J. 2006. Mutagenesis of hydrogenase accessory genes of Synechocystis sp. PCC 6803. Additional homologus of hypA and hypB are not active in hydrogenase maturation. *FEBS Journal,* 273**,** 4516-4527.

Howitt, C. A., Udall, P. K. & Vermaas, W. F. 1999. Type 2 NADH dehydrogenases in the cyanobacterium Synechocystis sp. strain PCC 6803 are involved in

regulation rather than respiration. *Journal of Bacteriology,* 181, 3994-4003.

Huang, H. H., Camsund, D., Lindblad, P. & Heidorn, T. 2010. Design and characterization of molecular tools for a Synthetic Biology approach towards developing cyanobacterial biotechnology. *Nucleic Acids Research,* 38, 2577-2593.

Huang, H. H. & Lindblad, P. 2013. Wide-dynamic-range promoters engineered for cyanobacteria. *Journal of Biological Engineering,* 7, 10-20.

Huckauf, J., Nomura, C., Forchhammer, K. & Hagemann, M. 2000. Stress responses of Synechocystis sp. strain PCC 6803 mutants impaired in genes encoding putative alternative sigma factors. *Microbiology,* 146, 2877-2889.

Imamura, S. & Asayama, M. 2009. Sigma factors for cyanobacterial transcription. *Gene Regulation and Systems Biology,* 3, 65-87.

Imamura, S., Asayama, M., Takahashi, H., Tanaka, K., Takahashi, H. & Shirai, M. 2003a. Antagonistic dark/light-induced SigB/SigD, group 2 sigma factors, expression through redox potential and their roles in cyanobacteria. *FEBS Letters,* 554, 357-362.

Imamura, S., Tanaka, K., Shirai, M. & Asayama, M. 2006. Growth phase-dependent activation of nitrogen-related genes by a control network of group 1 and group 2 sigma factors in a cyanobacterium. *The Journal of Biological Chemistry,* 281, 2668-2675.

Imamura, S., Yoshihara, S., Nakano, S., Shiozaki, N., Yamada, A., Tanaka, K., Takahashi, H., Asayama, M. & Shirai, M. 2003b. Purification, characterization, and gene expression of all sigma factors of RNA polymerase in a cyanobacterium. *Journal of Molecular Biology,* 325, 857-872.

Imashimizu, M., Tanaka, K. & Shimamoto, N. 2011. Comparative study of cyanobacterial and E. coli RNA polymerases: Misincorporation, abortive transcription, and dependence on divalent cations. *Genetics Research International,* 2011, 572689.

Inaba, M., Suzuki, I., Szalontai, B., Kanesaki, Y., Los, D. A., Hayashi, H. & Murata, N. 2003. Gene-engineered rigidification of membrane lipids enhances the cold inducibility of gene expression in synechocystis. *The Journal of Biological Chemistry,* 278, 12191-12198.

Ishihama, A. 2000. Functional modulation of Escherichia coli RNA polymerase. *Annual Review of Microbiology,* 54.

Ivanikova, N. V., Mckay, R. M. & Bullerjahn, G. S. 2005. Construction and characterization of a cyanobacterial bioreporter capable of assessing nitrate assimilatory capacity in freshwaters. *Limnology and Oceanography: Methods,* 3, 86-93.

Jacobsen, J. H. & Frigaard, N. U. 2014. Engineering of photosynthetic mannitol biosynthesis from CO_2 in a cyanobacterium. *Metabolic Engineering,* 21, 60-70.

Jindou, S., Ito, Y., Mito, N., Uematsu, K., Hosoda, A. & Tamura, H. 2014. Engineered platform for bioethylene production by a cyanobacterium expressing a chimeric complex of plant enzymes. *ACS Synthetic Biology,* 3, 487-496.

Jishage, M. & Ishihama, A. 1995. Regulation of RNA polymerase sigma subunit synthesis in Escherichia coli: Intracellular levels of sigma 70 and sigma 38. *Journal of Bacteriology,* 177, 6832-6835.

Kahl, L. J. & Endy, D. 2013. A survey of enabling technologes in synthetic biology. *Journal of Biological Engineering,* 7, 13.

Kahlon, S., Beeri, K., Ohkawa, H., Hihara, Y., Murik, O., Suzuki, I., Ogawa, T. & Kaplan, A. 2006. A putative sensor kinase, Hik31, is involved in the response of Synechocystis sp. strain PCC 6803 to the presence of glucose. *Microbiology,* 152, 647-655.

Kaneko, T., Sato, S., Kotani, H., Tanaka, A., Asamizu, E., Nakamura, Y., Miyajima, N., Hirosawa, M., Sugiura, M., Sasamoto, S., Kimura, T., Hosouchi, T., Matsuno, A., Muraki, A., Nakazaki, N., Naruo, K., Okumura, S., Shimpo, S., Takeuchi, C., Wada, T., Watanabe, A., Yamada, M., Yasuda, M. & Tabata, S. 1996. Sequence analysis of the genome of the unicellular cyanobacterium Synechocystis sp. strain PCC6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions. *DNA Research,* 3, 109-136.

Kanesaki, Y., Yamamoto, H., Paithoonrangsarid, K., Shoumskaya, M., Suzuki, I., Hayashi, H. & Murata, N. 2007. Histidine kinases play important roles in the perception and signal transduction of hydrogen peroxide in the cyanobacterium, Synechocystis sp. PCC 6803. *The Plant Journal,* 49, 313-324.

Kaplan-Levy, R. N., Hadas, O., Summers, M. L., Rücker, J. & Sukenik, A. 2010. *Akinetes: Dormant cells of cyanobacteria,* Springer-Verlag Berlin Heidelberg.

Karnik, A., Karnik, R. & Grefen, C. 2013. SDM-Assist software to design site-directed mutagenesis primers introducing "silent" restriction sites. *BMC Bioinformatics,* 14, 105.

Kelly, J. R., Rubin, A. J., Davis, J. H., Ajo-Franklin, C. M., Cumbers, J., Czar, M. J., De Mora, K., Glieberman, A. L., Monie, D. D. & Endy, D. 2009. Measuring the activity of BioBrick promoters using an in vivo reference standard. *Journal of Biological Engineering,* 3, 4.

Kibbe, W. A. 2007. OligoCalc: an online oligonucleotide properties calculator. *Nucleic Acids Research,* 35, W43-46.

Kim, H. W., Vannela, R., Zhou, C. & Rittmann, B. E. 2011. Nutrient acquisition and limitation for the photoautotrophic growth of Synechocystis sp. PCC6803 as a renewable biomass source. *Biotechnology and Bioengineering,* 108, 277-285.

Kirilovsky, D. 2007. Photoprotection in cyanobacteria: The orange carotenoid protein (OCP)-related non-photochemical-quenching mechanism. *Photosynthesis Research,* 93, 7-16.

Knight, T. 2003. *Idempotent vector design for standard assembly of biobricks* [Online]. MIT Artificial Intelligence Laboratory. Available: http://hdl.handle.net/1721.1/21168 [Accessed 27/11/2014 2011].

Knoop, H., Grundel, M., Zilliges, Y., Lehmann, R., Hoffmann, S., Lockau, W. & Steuer, R. 2013. Flux balance analysis of cyanobacterial metabolism: the metabolic network of Synechocystis sp. PCC 6803. *PLoS Compututational Biology,* 9**,** e1003081.

Knoop, H. & Steuer, R. 2015. A computational analysis of stoichiometric constraints and trade-offs in cyanobacterial biofuel production. *Frontiers in Bioengineering and Biotechnology,* 3**,** 47.

Koksharova, O. A. & Wolk, C. P. 2002. Genetic tools for cyanobacteria. *Applied Microbiology and Biotechnology,* 58**,** 123-137.

Kopf, M., Klahn, S., Scholz, I., Matthiessen, J. K., Hess, W. R. & Voss, B. 2014. Comparative Analysis of the Primary Transcriptome of Synechocystis sp. PCC 6803. *DNA Research,* 21**,** 527-539.

Koressaar, T. & Remm, M. 2007. Enhancements and modifications of primer design program Primer3. *Bioinformatics,* 23**,** 1289-1291.

Korn, A., Ajlani, G., Lagoutte, B., Gall, A. & Sétif, P. 2009. Ferredoxin:NADP+ oxidoreductase association with phycocyanin modulates its properties. *The Journal of Biological Chemistry,* 284**,** 31789-31797.

Korzheva, N., Mustaev, A., Kozlov, M., Malhotra, A., Nikiforov, V., Goldfarb, A. & Darst, S. A. 2000. A structural model of transcription elongation. *Science,* 289**,** 619-625.

Krasikov, V., Aguirre Von Wobeser, E., Dekker, H. L., Huisman, J. & Matthijs, H. C. 2012. Time-series resolution of gradual nitrogen starvation and its impact on photosynthesis in the cyanobacterium Synechocystis PCC 6803. *Physiologia Plantarum,* 145**,** 426-439.

Kucho, K., Okamoto, K., Tsuchiya, Y., Nomura, S., Nango, M., Kanehisa, M. & Ishiura, M. 2005. Global analysis of circadian expression in the cyanobacterium Synechocystis sp. strain PCC 6803. *Journal of Bacteriology,* 187**,** 2190-2199.

Laherrere, J. 2005. Forecasting production from discovery. *In ASPO*.

Lambert, D. H. & Stevens, S. E. 1986. Photoheterotrophic growth of Agmenellum quadruplicatum PR-6. *Journal of Bacteriology,* 165**,** 654-656.

Lapuente-Brun, E., Moreno-Loshuertos, R., Acín-Pérez, R., Latorre-Pellicer, A., Colás, C., Blasa, E., Perales-Clemente, E., Quirós, P. M., Calvo, E., Rodríguez-Hernández, M. A., Navas, P., Cruz, R., Carracedo, Á., López-Otín, C., Pérez-Martos, A., Fernández-Vizarra, E. & Enríquez, J. A. 2013. Supercomplex assembly determines electron flux in the mitochondrial electron transport chain. *Science,* 340**,** 1567-1570.

Larsson, J., Nylander, J. A. A. & Bergman, B. 2011. Genome fluctuations in cyanobacteria reflect evolutionary, developmental and adaptive traits. *BMC Evolutionary Biology*, 11:187.

Laudenbach, D. E., Reith, M. E. & Straus, N. A. 1988. Isolation, sequence analysis, and transcriptional studies of the flavodoxin gene from Anacystis nidulans R2. *Journal of Bacteriology,* 170**,** 258-265.

Lemeille, S., Geiselmann, J. & Latifi, A. 2005. Crosstalk regulation among group 2-sigma factors in Synechocystis PCC6803. *BMC Microbiology,* 5**,** 18.

Leonhardt, K. & Straus, N. A. 1992. An iron stress operon involved in photosynthetic electron transport in the marine cyanobacterium Synechococcus sp. PCC 7002. *Journal of General Microbiology,* 138**,** 1613-1621.

Lewis, M. 2005. The lac repressor. *Comptes Rendus Biologies,* 328**,** 521-548.

Lindberg, P., Park, S. & Melis, A. 2010. Engineering a platform for photosynthetic isoprene production in cyanobacteria, using Synechocystis as the model organism. *Metabolic Engineering,* 12**,** 70-79.

Llorens, J. M. N., Tormo, A. & Martinez-García, E. 2010. Stationary phase in gram-negative bacteria. *FEMS Microbiology Reviews,* 34**,** 476-495.

Los, D. A., Zorina, A., Sinetova, M., Kryazhov, S., Mironov, K. & Zinchenko, V. V. 2010. Stress sensors and signal transducers in cyanobacteria. *Sensors (Basel),* 10**,** 2386-2415.

Luque, I., Flores, E. & Herrero, A. 1994. Molecular mechanism for the operation of nitrogen control in cyanobacteria. *EMBO Journal,* 13**,** 2862-2869.

Lutz, R. & Bujard, H. 1997. Independent and tight regulation of transcriptional units in Escherichia coli via the LacR/O, the TetR/O and AraC/I_1-I_2 regulatory elements. *Nucleic Acids Research,* 25**,** 1203-1210.

Ma, J., Campbell, A. & Karlin, S. 2002. Correlations between Shine-Dalgarno sequences and gene features such as predicted expression levels and operon structures. *Journal of Bacteriology,* 184**,** 5733-5745.

Maarleveld, T. R., Boele, J., Bruggeman, F. J. & Teusink, B. 2014. A data integration and visualisation resource for the metabolic network of Synechocystis sp. PCC 6803. *Plant Physiology,* 164**,** 1111-1121.

Mackey, S. R., Golden, S. S. & Ditty, J. L. 2011. The itty-bitty time machine genetics of the cyanobacterial circadian clock. *Advances in Genetics,* 74**,** 13-53.

Marin, K., Suzuki, I., Yamaguchi, K., Ribbeck, K., Yamamoto, H., Kanesaki, Y., Hagemann, M. & Murata, N. 2003. Identification of histidine kinases that act as sensors in the perception of salt stress in Synechocystis sp. PCC 6803. *Proceedings of the National Academy of Sciences,* 100**,** 9061-9066.

Markley, A. L., Begemann, M. B., Clarke, R. E., Gordon, G. C. & Pfleger, B. F. 2014. Synthetic Biology Toolbox for Controlling Gene Expression in the Cyanobacterium Synechococcus sp. strain PCC 7002. *ACS Synthetic Biol*ogy, 4, 5, 595-603.

Marraccini, P., Bulteau, S., Cassier-Chauvat, C., Mermet-Bouvier, P. & Chauvat, F. 1993. A conjugative plasmid vector for promoter analysis in several cyanobacteria of the genera Synechococcus and Synechocystis. *Plant Molecular Biology,* 23**,** 905-909.

Martin, W. & Kowallik, K. 1999. Annotated English translation of Mereschkowsky's 1905 paper 'Über Natur und Ursprung der Chromatophoren imPflanzenreiche'. *European Journal Phycology,* 34**,** 287-295.

Martins, R. G., Gonçalves, I. S., de Morais, M. G. & Costa, J. A. V. 2014. Bioprocess engineering aspects of biopolymer production by the cyanobacterium Spirulina strain LEB 18. *International Journal of Polymer Science,* Article ID 895237.

Mazouni, K., Bulteau, S., Cassier-Chauvat, C. & Chauvat, F. 1998. Promoter element spacing controls basal expression and light inducibility of the cyanobacterial secA gene. *Molecular Microbiology,* 30**,** 1113-1122.

Mccleary, W. R. & Stock, J. B. 1994. Acetyl phosphate and the activation of two-component response regulators. *The Journal of Biological Chemistry,* 269**,** 31567-31572.

Merchant, S. S., Prochnik, S. E., Vallon, O., Harris, E. H., Karpowicz, S. J., Witman, G. B., Terry, A., Salamov, A., Fritz-Laylin, L. K., Marechal-Drouard, L., Marshall, W. F., Qu, L. H., Nelson, D. R., Sanderfoot, A. A., Spalding, M. H., Kapitonov, V. V., Ren, Q., Ferris, P., Lindquist, E., Shapiro, H., Lucas, S. M., Grimwood, J., Schmutz, J., Cardol, P., Cerutti, H., Chanfreau, G., Chen, C. L., Cognat, V., Croft, M. T., Dent, R., Dutcher, S., Fernandez, E., Fukuzawa, H., Gonzalez-Ballester, D., Gonzalez-Halphen, D., Hallmann, A., Hanikenne, M., Hippler, M., Inwood, W., Jabbari, K., Kalanon, M., Kuras, R., Lefebvre, P. A., Lemaire, S. D., Lobanov, A. V., Lohr, M., Manuell, A., Meier, I., Mets, L., Mittag, M., Mittelmeier, T., Moroney, J. V., Moseley, J., Napoli, C., Nedelcu, A. M., Niyogi, K., Novoselov, S. V., Paulsen, I. T., Pazour, G., Purton, S., Ral, J. P., Riano-Pachon, D. M., Riekhof, W., Rymarquis, L., Schroda, M., Stern, D., Umen, J., Willows, R., Wilson, N., Zimmer, S. L., Allmer, J., Balk, J., Bisova, K., Chen, C. J., Elias, M., Gendler, K., Hauser, C., Lamb, M. R., Ledford, H., Long, J. C., Minagawa, J., Page, M. D., Pan, J., Pootakham, W., Roje, S., Rose, A., Stahlberg, E., Terauchi, A. M., Yang, P., Ball, S., Bowler, C., Dieckmann, C. L., Gladyshev, V. N., Green, P., Jorgensen, R., Mayfield, S., Mueller-Roeber, B., Rajamani, S., Sayre, R. T., Brokstein, P., et al. 2007. The Chlamydomonas genome reveals the evolution of key animal and plant functions. *Science,* 318**,** 245-250.

Mermet-Bouvier, P., Cassier-Chauvat, C., Marraccini, P. & Chauvat, F. 1993. Transfer and replication of RSF1010-derived plasmids in several cyanobacteria of the genera *Synechocystis* and *Synechococcus*. *Current Microbiology,* 27**,** 323-327.

Meyer, R. 2009. Replication and conjugative mobilization of broad host-range IncQ plasmids. *Plasmid,* 62**,** 57-70.

Mikami, K., Kanesaki, Y., Suzuki, I. & Murata, N. 2002. The histidine kinase Hik33 perceives osmotic stress and cold stress in Synechocystis sp PCC 6803. *Molecular Microbiology,* 46**,** 905-915.

Miksch, G., Bettenworth, F., Friehs, K., Flaschel, E., Saalbach, A., Twellmann, T. & Nattkemper, T. W. 2005. Libraries of synthetic stationary-phase and

stress promoters as a tool for fine-tuning of expression of recombinant proteins in Escherichia coli. *Journal of Biotechnology,* 120**,** 25-37.

Miksch, G. & Dobrowolski, P. 1995. Growth phase-dependent induction of stationary-phase promoters of Escherichia coli in different gram-negative bacteria. *Journal of Bacteriology,* 177**,** 5374-5378.

Minas, K., Karunakaran, E., Bond, T., Gandy, C., Honsbein, A., Madsen, M., Amezaga, J., Amtmann, A., Templeton, M. R., Biggs, C. A. & Lawton, L. 2014. Biodesalination: an emerging technology for targeted removal of Na+and Cl−from seawater by cyanobacteria. *Desalination and Water Treatment***,** 1-22.

Mincer, T. J. & Aicher, A. C. 2016. Methanol production by a broad phylogenetic array of marine phytoplankton. *PLOS One,* 11, 3, e0150820.

Mitschke, J., Georg, J., Scholz, I., Sharma, C. M., Dienst, D., Bantscheff, J., Voß, B. Steglich, C., Wilde, A., Vogel, J. & Hess, W. R. 2011. An experimentally anchored map of transcriptional start sites in the model cyanobacterium Synechocystis sp. PCC6803. *Proceedings of the National Academy of Sciences,* 108, 5, 2124-2129.

Morrison, S. S., Mullineaux, C. W. & Ashby, M. K. 2005. The influence of acetyl phosphate on DspA signalling in the cyanobacterium Synechocystis sp. PCC6803. *BMC Microbiology,* 5**,** 47.

Mugavin, M. E. 2008. Multidimensional scaling: a brief overview. *Nursing Research,* 57**,** 64-68.

Mühlenhoff, U. & Chauvat, F. 1996. Gene transfer and manipulation in the thermophilic cyanobacterium Synechococcus elongatus. *Molecular Genetics and Genomics,* 252**,** 93-100.

Müller-Hill, B. 1996. *The Lac Operon: A short history of a genetic paradigm,* New York, Walter de Gruyter.

Mullineaux, C. W. 2014. Co-existence of photosynthetic and respiratory activities in cyanobacterial thylakoid membranes. *Biochimica et Biophysica Acta,* 1837**,** 503-511.

Mullineaux, C. W. & Sarcina, M. 2002. Probing the dynamics of photosynthetic membranes with fluorescence recovery after photobleaching. *Trends in Plant Science,* 7**,** 237-240.

Na, D. & Lee, D. 2010. RBSDesigner: software for designing synthetic ribosome binding sites that yields a desired level of protein expression. *Bioinformatics,* 26**,** 2633-2634.

Nagarajan, S., Sherman, D. M., Shaw, I. & Sherman, L. A. 2012. Functions of the duplicated hik31 operons in central metabolism and responses to light, dark, and carbon sources in Synechocystis sp. strain PCC 6803. *Journal of Bacteriology,* 194**,** 448-459.

Nakamura, Y., Kaneko, T., Hirosawa, M., Miyajima, N. & Tabata, S. 1998. CyanoBase, a www database containing the complete nucleotide sequence of the genome of Synechocystis sp. strain PCC6803. *Nucleic Acids Research,* 26**,** 63-67.

Nakao, M., Okamoto, S., Kohara, M., Fujishiro, T., Fujisawa, T., Sato, S., Tabata, S., Kaneko, T. & Nakamura, Y. 2010. CyanoBase: the cyanobacteria genome database update 2010. *Nucleic Acids Research,* 38, D379-381.

Nogales, J., Gudmundsson, S., Knight, E. M., Palsson, B. O. & Thiele, I. 2014. Detailing the optimality of photosynthesis in cyanobacteria through systems biology analysis. *Proceedings of the National Academy of Sciences,* 109, 2678-2683.

Nomura, C. T., Persson, S., Shen, G., Inoue-Sakamoto, K. & Bryant, D. A. 2006a. Characterization of two cytochrome oxidase operons in the marine cyanobacterium Synechococcus sp. PCC 7002: inactivation of ctaDI affects the PS I:PS II ratio. *Photosynthesis Research,* 87, 215-228.

Nomura, C. T., Sakamoto, T. & Bryant, D. A. 2006b. Roles for heme-copper oxidases in extreme high-light and oxidative stress response in the cyanobacterium Synechococcus sp. PCC 7002. *Archives in Microbiology,* 185, 471-479.

Nozzi, N. E., Oliver, J. W. & Atsumi, S. 2013. Cyanobacteria as a platform for biofuel production. *Frontiers in Bioengineering and Biotechnology,* 1, 7.

Ogawa, T. & Mi, H. 2007. Cyanobacterial NADPH dehydrogenase complexes. *Photosynthesis Research,* 93, 69-77.

Ogren, W. L. 1984. Photorespiration: Pathways, regulation and modification. *Annual Review of Plant Physiology,* 35, 415-442.

Ogren, W. L. 2003. Affixing the O to Rubisco: Discovering the source of photorespiratory glycolate and its regulation. *Photosynthesis Research,* 76, 53-63.

Ohmori, M. & Okamoto, K. 2004. Photoresponsive cAMP signal transduction in cyanobacteria. *Photochemical & Photobiological Sciences,* 3, 503-511.

Oliveira, P. & Lindblad, P. 2008. An AbrB-like protein regulates the expression of the bidirectional hydrogenase in Synechocystis sp. strain PCC 6803. *Journal of Bacteriology,* 190, 1011-1019.

Olson, J. M. 2006. Photosynthesis in the Archean era. *Photosynthesis Research,* 88, 109-17.

Osanai, T., Ikeuchi, M. & Tanaka, K. 2008. Group 2 sigma factors in cyanobacteria. *Physiologia Plantarum,* 133, 490-506.

Osanai, T., Imamura, S., Asayama, M., Shirai, M., Suzuki, I., Murata, N. & Tanaka, K. 2006. Nitrogen induction of sugar catabolic gene expression in Synechocystis sp. PCC 6803. *DNA Research,* 13, 185-195.

Parkinson, J. S. & Kofoid, E. C. 1992. Communication modules in bacterial signaling proteins. *Annual Review of Genetics,* 26, 71-112.

Parmar, A., Singh, N. K., Pandey, A., Gnansounou, E. & Madamwar, D. 2011. Cyanobacteria and microalgae: a positive prospect for biofuels. *Bioresource Technology,* 102, 10163-10172.

Peca, L., Kós, P. B., Máté, Z., Farsang, A. & Vass, I. 2008. Construction of bioluminescent cyanobacterial reporter strains for detection of nickel, cobalt and zinc. *FEMS Microbiology Letters,* 289**,** 258-264.

Peschek, G. A., Obinger, C. & Paumann, M. 2004. The respiratory chain of blue-green algae (cyanobacteria). *Physiologia Plantarum,* 120**,** 358-369.

Pietsch, C., Wiegand, C., Amé, M. V., Nicklisch, A., Wunderlin, D. & Pflugmacher, S. 2001. The effects of a cyanobacterial crude extract on different aquatic organisms: Evidence for cyanobacterial toxin modulating factors. *Environmental Toxicology,* 16, 6, 535-542.

Pinto, F., Pacheco, C. C., Ferreira, D., Moradas-Ferreira, P. & Tamagnini, P. 2012. Selection of suitable reference genes for RT-qPCR analyses in cyanobacteria. *PLOS ONE,* 7**,** e34983.

Piras, V. & Selvarajoo, K. 2015. The reduction of gene expression variability from single cells to populations follows simple statistical laws. *Genomics,* 105, 137-144.

Pisareva, T., Kwon, J., Oh, J., Kim, S., Ge, C., Wieslander, A., Choi, J. S. & Norling, B. 2011. Model for membrane organization and protein sorting in the cyanobacterium Synechocystis sp. PCC 6803 inferred from proteomics and multivariate sequence analysis. *Journal of Proteome Research,* 10**,** 3617-3631.

Posten, C. & Schaub, G. 2009. Microalgae and terrestrial biomass as source for fuels--a process view. *Journal of Biotechnology,* 142**,** 64-69.

Rai, M. & Padh, H. 2001. Expression systems for production of heterologous proteins. *Current Science,* 80**,** 1121-1128.

Raj, A. & van Oudenaarden, A. 2008. Nature, nurture, or chance: stochastic gene expression and its consequences. *Cell,* 135, 2, 216-226.

Rasband, W. S. & Bright, D. S. 1995. NIH Image: A public domain image processing program for the Macintosh. *Microbeam Analysis,* 4**,** 137-149.

Reeve, B., Hargest, T., Gilbert, C. & Ellis, T. 2014. Predicting translation initiation rates for designing synthetic biology. *Frontiers in Bioengineering and Biotechnology,* 2**,** 1.

Reiter, L., Kolsto, A. B. & Piehler, A. P. 2011. Reference genes for quantitative, reverse-transcription PCR in Bacillus cereus group strains throughout the bacterial life cycle. *Journal of Microbiology Methods,* 86**,** 210-217.

Richaud, C., Zabulon, G., Joder, A. & Thomas, J. C. 2001. Nitrogen or sulfur starvation differentially affects phycobilisome degradation and expression of the nblA gene in Synechocystis strain PCC 6803. *Journal of Bacteriology,* 183**,** 2989-2994.

Rippka, R. 1972. Photoheterotrophy and chemoheterotrophy among unicellular blue-green algae. *Archiv für Mikrobiologie,* 87**,** 93-98.

Rippka, R., Deruelles, J., Waterbury, J. B., Herdman, M. & Stanier, R. Y. 1979. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *Microbiology Reviews,* 111**,** 1-61.

Ruffing, A. M. 2011. Engineered cyanobacteria: Teaching an old bug new tricks. *Bioengineered Bugs,* 2**,** 136-149.

Ruffing, A. M. 2014. Improved free fatty acid production in cyanobacteria with Synechococcus sp. PCC 7002 as host. *Frontiers in Bioengineering and Biotechnology,* 2**,** 17.

Sakamoto, T., Murata, N. & Ohmori, M. 1991. The concentration of cyclic AMP and adenylate cyclase activity in cyanobacteria. *Plant and Cell Physiology,* 32**,** 581-484.

Salis, H. M., Mirsky, E. A. & Voigt, C. A. 2009. Automated design of synthetic ribosome binding sites to control protein expression. *Nature Biotechnology,* 27**,** 946-950.

Sandmann, G., Peleato, M. L., Fillat, M. F., Lázaro, M. C. & Gómez-Moreno, C. 1990. Consequences of the iron-dependent formation of ferredoxin and flavodoxin on photosynthesis and nitrogen fixation on Anabaena strains. *Photosynthesis Research,* 26**,** 119-125.

Sauer, J., Schreiber, U., Schmid, R., Völker, U. & Forchhammer, K. 2001. Nitrogen starvation-induced chlorosis in Synechococcus PCC 7942. Low-level photosynthesis as a mechanism of long-term survival. *Plant Physiology,* 126**,** 233-243.

Schneider, G. J. & Hasekorn, R. 1988. RNA polymerase subunit homology among cyanobacteria, other eubacteria and archaebacteria. *Journal of Bacteriology,* 170**,** 4136-4140.

Schumann, W. 2007. Production of recombinant proteins in Bacillus subtilis. *Advances in Applied Microbiology,* 62**,** 137-189.

Schwarz, R. & Forchhammer, K. 2005. Acclimation of unicellular cyanobacteria to macronutrient deficiency: emergence of a complex network of cellular responses. *Microbiology,* 151**,** 2503-2514.

Seo, S. W., Yang, J. S., Kim, I., Yang, J., Min, B. E., Kim, S. & Jung, G. Y. 2013. Predictive design of mRNA translation initiation region to control prokaryotic translation efficiency. *Metabolic Engineering,* 15**,** 67-74.

Shafiee, S. & Topal, E. 2009. When will fossil fuel reserves be diminished? *Energy Policy,* 37**,** 181-189.

Sharkey, T. D. & Welse, S. E. 2012. *Autotrophic carbon dioxide fixation*, Springer Science + Business Media B. V.

Sheng, J., Kim, H. W., Badalamenti, J. P., Zhou, C., Sridharakrishnan, S., Krajmalnik-Brown, R., Rittmann, B. E. & Vannela, R. 2011. Effects of temperature shifts on growth rate and lipid characteristics of Synechocystis sp. PCC6803 in a bench-top photobioreactor. *Bioresource Technology,* 102**,** 11218-11225.

Sherman, D. M., Troyan, T. A. & Sherman, L. A. 1994. Localization of membrane proteins in the cyanobacterium Synechococcus sp. PCC7942 (radial asymmetry in the photosynthetic complexes). *Plant Physiology,* 106**,** 251-262.

Shetty, R. P., Endy, D. & Knight, T. F., Jr. 2008. Engineering BioBrick vectors from BioBrick parts. *Journal of Biological Engineering,* 2**,** 5-16.

Shevela, D., Pishchalnikov, R., Eichacker, L. A. & Govindjee 2013. *Oxygenic photosynthesis in cyanobacteria*, CRC Press.

Shih, P. M., Wu, D., Latifi, A., Axen, S. D., Fewer, D. P., Talla, E., Calteau, A., Cai, F., Tandeau de Marsac, N., Rippka, R., Herman, M., Sivonen, K., Coursin, T., Laurent, T., Goodwin, L., Nolan, M., Davenport, K. W., Hans, C. S., Rubin, E. M., Eisen, J. A., Woyke, T., Gugger, M. & Kerfeld, C. A. 2013. Improving the coverage of the cyanobacterial phylum using diversity-driven genome sequencing. *Proceedings of the National Academy of Sciences,* 110, 3, 1053-1058.

Singh, A. K., Bhattacharyya-Pakrasi, M., Elvitigala, T., Ghosh, B., Aurora, R. & Pakrasi, H. B. 2009. A systems-level analysis of the effects of light quality on the metabolism of a cyanobacterium. *Plant Physiology,* 151**,** 1596-1608.

Singh, A. K., Mcintyre, L. M. & Sherman, L. A. 2003. Microarray analysis of the genome-wide response to iron deficiency and iron reconstitution in the cyanobacterium Synechocystis sp. PCC 6803. *Plant Physiology,* 132**,** 1825-1839.

Singh, A. K., Summerfield, T. C. & Sherman, L. A. 2006. The heat shock response in the cyanobacterium Synechocystis sp. Strain PCC 6803 and regulation of gene expression by HrcA and SigB. *Archives in Microbiology,* 186**,** 273-286.

Singh, S., Kate, B. N. & Banerjee, U. C. 2005. Bioactive compounds from cyanobacteria and microalgae: An overview. *Critical Reviews in Biotechnology,* 25**,** 73-95.

Sode, K., Tatara, M., Takeyama, H., Burgess, J. G. & Matsunaga, T. 1992. Conjugative gene transfer in marine cyanobacteria: Synechococcus sp., Synechocystis sp. and Pseudanabaena sp. *Applied Microbiology Biotechnology,* 37**,** 369-373.

Stal, L. J. 1992. Poly(hydroxyalkanoate) in cyanobacteria: An overview. *FEMS Microbiology Letters,* 103**,** 169-180.

Stal, L. J. 1995. Physiological ecology of cyanobacteria in microbial mats and other communities. *New Phytologist,* 131**,** 1-32.

Stal, L. J. 2008. Nitrogen Fixation in Cyanobacteria.

Stanier, R. Y. & Cohen-Bazire, G. 1977. Phototrophic prokaryotes: the cyanobacteria. *Annual Review of Microbiology,* 31**,** 225-274.

Stanier, R. Y., Kunisawa, R., Mandel, M. & Cohen-Bazire, G. 1971. Purification and properties of unicellular blue-green algae (order Chroococcales). *Bacteriological Reviews,* 35**,** 171-205.

Stevens, S. E. & Porter, R. D. 1980. Transformation in Agmenellum quadruplicatum. *Proceedings of the National Academy of Sciences,* 77**,** 6052-6056.

Stocker, T. F., Qin, D., Plattner, G.-K., Tignor, M., Allen, S. K., Boschung, J., Nauels, A., Xia, Y., Bex, V. & Midgley, B. 2013. Climage Change 2013: The

Physical Science Basis. *Cambridge, United Kingdom and New York, USA: Cambridge University Press*.

Stucken, K., Koch, R. & Dagan, T. 2013. Cyanobacterial defense mechanisms against foreign DNA transfer and their impact on genetic engineering. *Biological Research,* 46**,** 373-382.

Subashchandrabose, S. R., Ramakrishnan, B., Megharaj, M., Venkateswarlu, K. & Naidu, R. 2013. Mixotrophic cyanobacteria and microalgae as distinctive biological agents for organic pollutant degradation. *Environment International,* 51**,** 59-72.

Summerfield, T. C., Nagarajan, S. & Sherman, L. A. 2011. Gene expression under low-oxygen conditions in the cyanobacterium Synechocystis sp. PCC 6803 demonstrates Hik31-dependent and -independent responses. *Microbiology,* 157**,** 301-312.

Suzuki, I., Kanesaki, Y., Hayashi, H., Hall, J. J., Simon, W. J., Slabas, A. R. & Murata, N. 2005. The histidine kinase Hik34 is involved in thermotolerance by regulating the expression of heat shock genes in Synechocystis. *Plant Physiology,* 138**,** 1409-1421.

Suzuki, S., Ferjani, A., Suzuki, I. & Murata, N. 2004. The SphS-SphR two component system is the exclusive sensor for the induction of gene expression in response to phosphate limitation in synechocystis. *The Journal of Biological Chemistry,* 279**,** 13234-13240.

Sweetser, D., Nonet, M. & Young, R. A. 1987. Prokaryotic and eukaryotic RNA polymerases have homologous core subunits. *Proceedings of the National Academy of Sciences,* 84**,** 1192-1196.

Tamoi, M., Ishikawa, T., Takeda, T. & Shigeoka, S. 1996. Molecular characterization and resistance to hydrogen peroxide of two fructose-1,6-bisphosphatases from Synechococcus PCC 7942. *Archives of Biochemistry and Biophysics,* 334**,** 27-36.

Taton, A., Unglaub, F., Wright, N. E., Zeng, W. Y., Paz-Yepes, J., Brahamsha, B., Palenik, B., Peterson, T. C., Haerizadeh, F., Golden, S. S. & Golden, J. W. 2014. Broad-host-range vector system for synthetic biology and biotechnology in cyanobacteria. *Nucleic Acids Research,* 42**,** e136.

The Arabidopsis Genome Initiative. 2000. Analysis of the genome sequence of the flowering plant Arabidopsis thaliana. *Nature,* 408**,** 796-815.

Thellin, O., Zorzi, W., Lakaye, B., De Borman, B., Coumans, B., Hennen, G., Grisar, T., Igout, A. & Heinen, E. 1999. Housekeeping genes as internal standards: use and limits. *Journal of Biotechnology,* 75**,** 291-295.

Trapnell, C., Roberts, A., Goff, L., Pertea, G., Kim, D., Kelley, D. R., Pimentel, H., Salzberg, S. L., Rinn, J. L. & Pachter, L. 2012. Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nature Protocols,* 7**,** 562-578.

Tsien, R. Y. 1998. The green fluorescent protein. *Annual Review of Biochemistry,* 67**,** 509-544.

Tuominen, I., Pollari, M., Aguirre Von Wobeser, E., Tyystjärvi, E., Ibelings, B. W., Matthijs, H. C. & Tyystjärvi, T. 2008. Sigma factor SigC is required for heat acclimation of the cyanobacterium Synechocystis sp. strain PCC 6803. *FEBS Letters,* 582**,** 346-350.

Tuominen, I., Tyystjärvi, E. & Tyystjärvi, T. 2003. Expression of primary sigma factor (PSF) and PSF-like sigma factors in the cyanobacterium Synechocystis sp. strain PCC 6803. *Journal of Bacteriology,* 185**,** 1116-1119.

Tzareva, N. V., Makhno, V. I. & Boni, I. V. 1994. Ribosome-messenger recognition in the absence of the Shine-Dalgarno interactions. *FEBS Letters,* 337**,** 189-194.

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B. C., Remm, M. & Rozen, S. G. 2012. Primer3--new capabilities and interfaces. *Nucleic Acids Research,* 40**,** e115.

Utkilen, H. C. 1982. Magnesium-limited growth of the cyanobacterium Anacystis nidulans. *Journal of General Microbiology,* 128**,** 1849-1862.

Vaishampayan, A., Sinha, R. P., Hader, D.-P., Dey, T., Gupta, A. K., Bhan, U. & Rao, A. L. 2001. Cyanobacterial biofertilizers in rice agriculture. *The Botanical Review,* 67**,** 453-516.

Van Baalen, C. 1962. Studies on marine blue-green algae. *Botanica Marina,* 4**,** 129-139.

Van De Meene, A. M., Hohmann-Marriott, M. F., Vermaas, W. F. & Roberson, R. W. 2006. The three-dimensional structure of the cyanobacterium Synechocystis sp. PCC 6803. *Archives of Microbiology,* 184**,** 259-270.

Van Thor, J. J., Gruters, O. W., Matthijs, H. C. & Hellingwerf, K. J. 1999. Localization and function of ferredoxin:NADP(+) reductase bound to the phycobilisomes of Synechocystis. *EMBO Journal,* 18**,** 4128-4136.

Vandesomepele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. & Speleman, F. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology,* 3**,** RESEARCH0034.

Vandesomepele, J., Kubista, M. & Pfaffl, M. W. 2009. Reference gene validation software for improved normalization. *In:* LOGAN, J., EDWARDS, K. & SAUNDERS, N. (eds.) *Real-Time PCR: Current technology and applications.* Caister Academic Press.

Vermaas, W. F. 2001. *Photosynthesis and Respiration in Cyanobacteria*, Macmillan Publishers Ltd, Nature Publishing Group.

Vijayakumar, S. 2012. Potential Applications of Cyanobacteria in Industrial Effluents-A Review. *Journal of Bioremediation and Biodegradation,* 03.

Vilanova, C. & Porcar, M. 2014. iGEM 2.0--refoundations for engineering biology. *Nature Biotechnology,* 32**,** 420-424.

Vinnemeier, J., Kunert, A. & Hagemann, M. 1998. Transcriptional analysis of the isiAB operon in salt-stressed cells of the cyanobacterium Synechocystis sp. PCC 6803. *FEMS Microbiology Letters,* 169**,** 323-330.

Vitousek, P. M. & Howarth, R. W. 1991. Nitrogen limitation on land and in the sea: How can it occur? *Biogeochemistry,* 13**,** 87-115.

Wang, B., Wang, J., Zhang, W. & Meldrum, D. R. 2012. Application of synthetic biology in cyanobacteria and algae. *Frontiers in Microbiology,* 3**,** 344.

Wang, H.-L., Postier, B. L. & Burnap, R. L. 2004. Alterations in global patterns of gene expression in Synechocystis sp. PCC 6803 in response to inorganic carbon limitation and the inactivation of ndhR, a LysR family regulator. *The Journal of Biological Chemistry,* 279**,** 5739-5751.

Wang, J., Chen, L., Chen, Z. & Zhang, W. 2015. RNA-seq based transcriptomic analysis of single bacterial cells. *Integrative Biology*, 7, 1466-1476.

Wang, Z., Gerstein, M. & Snyder, M. 2009. RNA-Seq: a revolutionary tool for transcriptomics. *Nature Reviews Genetics,* 10**,** 57-63.

Waterbury, J. B. 2006. The Cyanobacteria—Isolation, Purification and Identification. 1053-1073.

Waterbury, J. B., Watson, S. W., Guillard, R. R. L. & Brand, L. E. 1979. Widespread occurrence of a unicellular, marine, planktonic, cyanobacterium. *Nature,* 277**,** 293-294.

Weber, E., Engler, C., Gruetzner, R., Werner, S. & Marillonnet, S. 2011. A Modular Cloning System for Standardized Assembly of Multigene Constructs. *PLOS ONE,* 6**,** e16765.

Wilde, A. & Dienst, D. 2011. Tools for Genetic Manipulation of Cyanobacteria. 685-703.

Wolf, F. I. & Cittadini, A. 1999. Magnesium in cell proliferation and differentiation. *Frontiers in Bioscience,* 4**,** D607-617.

Wolk, C. P. 1973. Physiology and cytological chemistry of blue-green algae. *Bacteriological Reviews,* 37**,** 32-101.

Xie, W. Q., Jäger, K. & Potts, M. 1989. Cyanobacterial RNA polymerase genes rpoC1 and rpoC2 correspond to rpoC of Escherichia coli. *Joural of Bacteriology,* 171**,** 1967-1973.

Xu, Y., Alvey, R. M., Byrne, P. O., Graham, J. E., Shen, G. & Bryant, D. A. 2011. Expression of genes in cyanobacteria: adaptation of endogenous plasmids as platforms for high-level gene expression in Synechococcus sp. PCC 7002. *Methods in Molecular Biology,* 684**,** 273-293.

Yoshihara, S., Geng, X. & Ikeuchi, M. 2002. pilG gene cluster and split pilL genes involved in pilus biogenesis, motility and genetic transformation in the cyanobacterium Synechocystis sp. PCC 6803. *Plant and Cell Physiology,* 43**,** 513-521.

Yoshihara, S., Geng, X., Okamoto, S., Yura, K., Murata, T., Go, M., Ohmori, M. & Ikeuchi, M. 2001. Mutational analysis of genes involved in pilus structure, motility and transformation competency in the unicellular motile cyanobacterium Synechocystis sp. PCC 6803. *Plant and Cell Physiology,* 42**,** 63-73.

Yu, T., Li, J., Yang, Y., Qi, L., Chen, B., Zhao, F., Bao, Q. & Wu, J. 2012. Codon usage patterns and adaptive evolution of marine unicellular cyanobacteria Synechococcus and Prochlorococcus. *Molecular Phylogenetics and Evolution,* 62**,** 206-213.

Yu, Y., You, L., Liu, D., Hollinshead, W., Tang, Y. J. & Zhang, F. 2013. Development of Synechocystis sp. PCC 6803 as a phototrophic cell factory. *Marine Drugs,* 11**,** 2894-2916.

Zhang, Z., Pendse, N. D., Phillips, K. N., Cotner, J. B. & Khodursky, A. 2008. Gene expression patterns of sulfur starvation in Synechocystis sp. PCC 6803. *BMC Genomics,* 9**,** 344.

Zhou, J., Zhang, H., Meng, H., Zhu, Y., Bao, G., Zhang, Y., Li, Y. & Ma, Y. 2014. Discovery of a super-strong promoter enables efficient production of heterologous proteins in cyanobacteria. *Scientific Reports,* 4**,** 4500.

Zhou, K., Zhou, L., Lim, Q., Zou, R., Stephanopoulos, G. & Too, H. P. 2011. Novel reference genes for quantifying transcriptional responses of Escherichia coli to protein overexpression by quantitative PCR. *BMC Molecular Biology,* 12**,** 18.

APPENDIX I

R script to perform multi-dimensional scaling

(Author: Dr. Graham Hamilton, *Glasgow Polyomics*, University of Glasgow)

```
# SET YOUR WORKING DIRECTORY
library(cummeRbund)
# Load the Cufflinks data
cuff<-readCufflinks('cuffdiff_all_conditions_28May')

# List the replicates
replicates(cuff)

# Change the rep.list to the replicates required for the PCA plot.
rep.list<-
c("StandardMedia4Days_0","StandardMedia4Days_1","StandardMedia4Days_2","Sta
ndardMedia8Days_0","StandardMedia8Days_1","StandardMedia8Days_2")

# Give the samples a sort name for the MDS plot
labs<-c('SM4-1','SM4-2','SM4-3','SM8-1','SM8-2','SM8-3')

# Colours for the sample circles
cols <- c(rep("aquamarine",3),rep("aquamarine4",3))

fpkms<-repFpkmMatrix(genes(cuff),fullnames=FALSE,rep.list)

# Run this for log transformations
pseudocount<-1.0
fpkms<-log10(fpkms+pseudocount)

dist<-JSdist(makeprobs(fpkms))

fit<-cmdscale(dist,eig=TRUE, k=2)
res<-
data.frame(names=rownames(fit$points),M1=fit$points[,1],M2=fit$points[,2])
plot<-ggplot(res)

plot<-plot + geom_point(aes(x=M1,y=M2),color=cols,size=10) +
geom_text(aes(x=M1,y=M2,label=labs,hjust=0.5, vjust=-1)) + theme_bw()
plot
```

APPENDIX II

R script to perform clustering analysis

(Author: Dr. Graham Hamilton, *Glasgow Polyomics*, University of Glasgow)

```
# SET YOUR WORKING DIRECTORY
library(cummeRbund)
# Load the Cufflinks data
cuff<-readCufflinks('cuffdiff_all_conditions_28May')

# Read in list of genes, change file name. File should be an XLOC id per line.
myGeneIDs<-
read.table("Background_Expressed_genes.txt",sep="\t",header=FALSE)
# Get XLOC ids from read in table
XLOCs<-myGeneIDs[,'V1']

genes<-getGenes(cuff,XLOCs)

# Cluster the genes using the Jensen-Shannon distance and K means
clusters<-30
cluster.distances<-csCluster(genes,k=clusters)

# Plot the clusters
clust.plot<-csClusterPlot(cluster.distances)
clust.plot

# Write the names of the genes in the clusters to files
i <- 1
while(i <= clusters){
  num <- (i)
  file_name <- "clusters"
  file_type <- "txt"
  file_name <- paste(file_name, num, sep="_", collapse = NULL)
  file_name <- paste(file_name, file_type, sep=".", collapse = NULL)
  clust_ids <- names(cluster.distances$clustering)[cluster.distances$clustering ==
(i)]
  write.table(clust_ids,file_name, sep='\t',row.names=F,col.names=F,quote=F)
  i <- i + 1
}
```

APPENDIX III

Complete nucleotide sequence of the pAQ1BB_PcpcBA_GFP vector.
See Figure 3-2B for the plasmid map.

Location	Feature
1..22	BioBrick prefix
23..616	Phycocyanin promoter of PCC 6803
625..637	BBa_0030 – medium strength RBS
644..1363	BBa_E0040 – GFP coding region
1372..1451	BBa_B0010 – terminator T1 from E. coli rrnB
1461..1500	BBa_B0012 – terminator TE from coliphage T7
1501..1521	BioBrick suffix
1642..1647	XhoI restriction site
1649..2647	*aadA* gene conferring spectinomycin resistance
2659..3035	Flank B for homologous recombination
3243..3698	Phage f1 origin of replication
3881..4791	Beta-lactamase (Ampr) coding region
5956..6467	Flank A for homologous recombination

```
   1 GAATTCGCGGCCGCTTCTAGAGGTTATAAAATAAACTTAACAAATCTATA
  51 CCCACCTGTAGAGAAGAGTCCCTGAATATCAAAATGGTGGGATAAAAAGC
 101 TCAAAAAGGAAAGTAGGCTGTGGTTCCCTAGGCAACAGTCTTCCCTACCC
 151 CACTGGAAACTAAAAAAACGAGAAAAGTTCGCACCGAACATCAATTGCAT
 201 AATTTTAGCCCTAAAACATAAGCTGAACGAAACTGGTTGTCTTCCCTTCC
 251 CAATCCAGGACAATCTGAGAATCCCCTGCAACATTACTTAACAAAAAAGC
 301 AGGAATAAAATTAACAAGATGTAACAGACATAAGTCCCATCACCGTTGTA
 351 TAAAGTTAACTGTGGGATTGCAAAAGCATTCAAGCCTAGGCGCTGAGCTG
```

401 TTTGAGCATCCCGGTGGCCCTTGTCGCTGCCTCCGTGTTTCTCCCTGGAT
451 TTATTTAGGTAATATCTCTCATAAATCCCCGGGTAGTTAACGAAAGTTAA
501 TGGAGATCAGTAACAATAACTCTAGGGTCATTACTTTGGACTCCCTCAGT
551 TTATCCGGGGGAATTGTGTTTAAGAAAATCCCAACTCATAAAGTCAAGTA
601 GGAGATTAATTCCATGTACTAGAGTCACACAGGAAAGTACTAGATGCGTA
651 AAGGAGAAGAACTTTTCACTGGAGTTGTCCCAATTCTTGTTGAATTAGAT
701 GGTGATGTTAATGGGCACAAATTTTCTGTCAGTGGAGAGGGTGAAGGTGA
751 TGCAACATACGGAAAACTTACCCTTAAATTTATTTGCACTACTGGAAAAC
801 TACCTGTTCCATGGCCAACACTTGTCACTACTTTCGGTTATGGTGTTCAA
851 TGCTTTGCGAGATACCCAGATCATATGAAACAGCATGACTTTTTCAAGAG
901 TGCCATGCCCGAAGGTTATGTACAGGAAAGAACTATATTTTTCAAAGATG
951 ACGGGAACTACAAGACACGTGCTGAAGTCAAGTTTGAAGGTGATACCCTT
1001 GTTAATAGAATCGAGTTAAAAGGTATTGATTTTAAAGAAGATGGAAACAT
1051 TCTTGGACACAAATTGGAATACAACTATAACTCACACAATGTATACATCA
1101 TGGCAGACAAACAAAAGAATGGAATCAAAGTTAACTTCAAAATTAGACAC
1151 AACATTGAAGATGGAAGCGTTCAACTAGCAGACCATTATCAACAAAATAC
1201 TCCAATTGGCGATGGCCCTGTCCTTTTACCAGACAACCATTACCTGTCCA
1251 CACAATCTGCCCTTTCGAAAGATCCCAACGAAAAGAGAGACCACATGGTC
1301 CTTCTTGAGTTTGTAACAGCTGCTGGGATTACACATGGCATGGATGAACT
1351 ATACAAATAATAATACTAGAGCCAGGCATCAAATAAAACGAAAGGCTCAG
1401 TCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCT
1451 CTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATA
1501 TACTAGTAGCGGCCGCTGCAGTTAATTAAGGATCCGGCTGCTAACAAAGC
1551 CCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCAT
1601 AACCCCTTGGGGCCTCTAAACGGGTCTTGACGGGTTTTTTGCTCGAGCCG
1651 AACGCAGCGGTGGTAACGGCGCAGTGGCGGTTTTCATGGCTTGTTATGAC
1701 TGTTTTTTTGGGGTACAGTCTATGCCTCGGGCATCCAAGCAGCAAGCGCG
1751 TTACGCCGTGGGTCGATGTTTGATGTTATGGAGCAGCAACGATGTTACGC
1801 AGCAGGGCAGTCGCCCTAAAACAAAGTTAAACATCATGAGGGAAGCGGTG
1851 ATCGCCGAAGTATCGACTCAACTATCAGAGGTAGTTGGCGTCATCGAGCG
1901 CCATCTCGAACCGACGTTGCTGGCCGTACATTTGTACGGCTCCGCAGTGG
1951 ATGGCGGCCTGAAGCCACACAGTGATATTGATTTGCTGGTTACGGTGACC
2001 GTAAGGCTTGATGAAACAACGCGGCGAGCTTTGATCAACGACCTTTTGGA

2051 AACTTCGGCTTCCCCTGGAGAGAGCGAGATTCTCCGCGCTGTAGAAGTCA
2101 CCATTGTTGTGCACGACGACATCATTCCGTGGCGTTATCCAGCTAAGCGC
2151 GAACTGCAATTTGGAGAATGGCAGCGCAATGACATTCTTGCAGGTATCTT
2201 CGAGCCAGCCACGATCGACATTGATCTGGCTATCTTGCTGACAAAAGCAA
2251 GAGAACATAGCGTTGCCTTGGTAGGTCCAGCGGCGGAGGAACTCTTTGAT
2301 CCGGTTCCTGAACAGGATCTATTTGAGGCGCTAAATGAAACCTTAACGCT
2351 ATGGAACTCGCCGCCCGACTGGGCTGGCGATGAGCGAAATGTAGTGCTTA
2401 CGTTGTCCCGCATTTGGTACAGCGCAGTAACCGGCAAAATCGCGCCGAAG
2451 GATGTCGCTGCCGACTGGGCAATGGAGCGCCTGCCGGCCCAGTATCAGCC
2501 CGTCATACTTGAAGCTAGACAGGCTTATCTTGGACAAGAAGAAGATCGCT
2551 TGGCCTCGCGCGCAGATCAGTTGGAAGAATTTGTCCACTACGTGAAAGGC
2601 GAGATCACCAAGGTAGTCGGCAAATAATGTCTAACAATTCGCTCGACTCT
2651 AGGTCGACGCCTCCTGAATAAATCTATTTATACAGGGGTTGGACACGGCC
2701 CCTAATTTTGCTTGGTCACGCTGTAACCAATGAGCAAAGACCTTTTCGCG
2751 CTGATCGTTAGGGGCGATCGCCTCATAGACCCGCTGACGGTTATCCCGCG
2801 ATCCAAAGCGCCCCTTGTATTCCAATTTCCGGTCAAGCTTGCCTAGGAGC
2851 GCCTGAGCAATTTCGAGCGGGCTCATTTTGTCGGTGATCGTTCTGCCAAG
2901 AATCCACTTGATCGGCTTGGCATACCGCAAACAATTTTCCTTGAACCCTT
2951 CAAGGCTCGCCCCGGTGAATGTCACCCCTGGTTTTAAGAACTGATGGATG
3001 TTGAGTAGCTCTAACAGGTGAATCTTTGGTGAGAGGCATGCGACGTCGGG
3051 CCCAATTCGCCCTATAGTGAGTCGTATTACAATTCACTGGCCGTCGTTTT
3101 ACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTG
3151 CAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACC
3201 GATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGACGCGCCC
3251 TGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGAC
3301 CGCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTCGCTTTCTTCCCTT
3351 CCTTTCTCGCCACGTTCGCCGGCTTTCCCCGTCAAGCTCTAAATCGGGGG
3401 CTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAAAAA
3451 ACTTGATTAGGGTGATGGTTCACGTAGTGGGCCATCGCCCTGATAGACGG
3501 TTTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTGGACTCTTG
3551 TTCCAAACTGGAACAACACTCAACCCTATCTCGGTCTATTCTTTTGATTT
3601 ATAAGGGATTTTGCCGATTTCGGCCTATTGGTTAAAAAATGAGCTGATTT
3651 AACAAAAATTTAACGCGAATTTTAACAAAATATTAACGCTTACAATTTCC

3701 TGATGCGGTATTTTCTCCTTACGCATCTGTGCGGTATTTCACACCGCATC
3751 AGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTT
3801 TCTAAATACATTCAAATATGTATCCGCTCATGAGACAATAACCCTGATAA
3851 ATGCTTCAATAATATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCG
3901 TGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTC
3951 ACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCA
4001 CGAGTGGGTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAG
4051 TTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGTTCTGC
4101 TATGTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAGAGCAACTCGGT
4151 CGCCGCATACACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCAC
4201 AGAAAAGCATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTG
4251 CCATAACCATGAGTGATAACACTGCGGCCAACTTACTTCTGACAACGATC
4301 GGAGGACCGAAGGAGCTAACCGCTTTTTTGCACAACATGGGGGATCATGT
4351 AACTCGCCTTGATCGTTGGGAACCGGAGCTGAATGAAGCCATACCAAACG
4401 ACGAGCGTGACACCACGATGCCTGTAGCAATGGCAACAACGTTGCGCAAA
4451 CTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGA
4501 CTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTC
4551 CGGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGGTCT
4601 CGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGT
4651 AGTTATCTACACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGAC
4701 AGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGGTAACTGTCAGAC
4751 CAAGTTTACTCATATATACTTTAGATTGATTTAAAACTTCATTTTTAATT
4801 TAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGACCAAAATCC
4851 CTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATC
4901 AAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCA
4951 AACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTTGCCGGATCAAGAGC
5001 TACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCA
5051 AATACTGTTCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTC
5101 TGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTG
5151 CTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAG
5201 TTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGTGCACACA
5251 GCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTG
5301 AGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTAT

5351 CCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGG
5401 GGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGAC
5451 TTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAA
5501 AACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTT
5551 TGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGTA
5601 TTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGACCGAG
5651 CGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCCAATACGCAAACC
5701 GCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAGCTGGCACGACAGGT
5751 TTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAG
5801 CTCACTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTAT
5851 GTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTATG
5901 ACCATGATTACGCCAAGCTATTTAGGTGACACTATAGAATACTCAAGCTA
5951 TGCATGGGGTTTTCTCGTGTTTAGGCAGCATCTTGATCCGCCTCATCATT
6001 GTTATAGACTTCGAGCAAATCTCGTAAAACAAGCTCCCGGATATAGTCGC
6051 TGAAATTTAAGCCTTGCTCATTGGCTTTTTTAGTAGCCGCGCTGTACATG
6101 AGTGGGGGAAAACGAACCGCGCTCGCTGATTTTAAATTCTTATTCGGTCT
6151 AGTCGTCATGGGATCGCCTAAGAAAGTCTCTATCATTTTACAGTATCCAA
6201 AAGATTTGACACCCCCATTCATGGTGGTATTTTTTCTTTTCCTTTTCCCC
6251 ATAGCACTGTGGCTAGCAATAAAGCTATGGGCGATCCCTACATTTATTCT
6301 GTAGCACCAACGCTACAGCCCCTTAATTTCCTGTGGATAAACAGTGTGGA
6351 AATTGAGAAGAACACATGAGAATTTGTCCAGTTTTATTTTGATGGTTATT
6401 TTTTGCGGTTGCTTTTTAAGGGAATTGTGCGTGTGGTTTCCAGTCCCCAT
6451 CTGTGCATAAGAGAAAG

APPENDIX IV

Nucleotide sequence of RBS7-GFP.

Location	Feature
1..22	BioBrick prefix
23..56	Synthetic ribosome binding site RBS7
57..776	BBa_E0040 – GFP coding region
785..864	BBa_B0010 – terminator T1 from E. coli rrnB
874..913	BBa_B0012 – terminator TE from coliphage T7
914..934	BioBrick suffix

```
   1 GAATTCGCGGCCGCTTCTAGAGTAAAATTACAAAACACTTTTTAGCTAGG
  51 ACACATATGCGTAAAGGAGAAGAACTTTTCACTGGAGTTGTCCCAATTCT
 101 TGTTGAATTAGATGGTGATGTTAATGGGCACAAATTTTCTGTCAGTGGAG
 151 AGGGTGAAGGTGATGCAACATACGGAAAACTTACCCTTAAATTTATTTGC
 201 ACTACTGGAAAACTACCTGTTCCATGGCCAACACTTGTCACTACTTTCGG
 251 TTATGGTGTTCAATGCTTTGCGAGATACCCAGATCATATGAAACAGCATG
 301 ACTTTTTCAAGAGTGCCATGCCCGAAGGTTATGTACAGGAAAGAACTATA
 351 TTTTTCAAAGATGACGGGAACTACAAGACACGTGCTGAAGTCAAGTTTGA
 401 AGGTGATACCCTTGTTAATAGAATCGAGTTAAAAGGTATTGATTTTAAAG
 451 AAGATGGAAACATTCTTGGACACAAATTGGAATACAACTATAACTCACAC
 501 AAGATGGAAACATTCTTGGACACAAATTGGAATACAACTATAACTCACAC
 551 CAAAATTAGACACAACATTGAAGATGGAAGCGTTCAACTAGCAGACCATT
 601 ATCAACAAAATACTCCAATTGGCGATGGCCCTGTCCTTTTACCAGACAAC
 651 CATTACCTGTCCACACAATCTGCCCTTTCGAAAGATCCCAACGAAAAGAG
 701 AGACCACATGGTCCTTCTTGAGTTTGTAACAGCTGCTGGGATTACACATG
 751 GCATGGATGAACTATACAAATAATAATACTAGAGCCAGGCATCAAATAAA
 801 ACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGT
 851 CGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCT
 901 TTCTGCGTTTATATACTAGTAGCGGCCGCTGCAG
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APPENDIX V

Complete list of genes excluded from the “background” list of expressed genes.

Gene ID	Gene product
rrn16Sa	16S rRNA; 16s ribosomal RNA
rrn16Sb	16S rRNA; 16s ribosomal RNA
rrn23Sa	23S rRNA; 23S ribosomal RNA
rrn23Sb	23S rRNA; 23S ribosomal RNA
sll6017	hypothetical protein
sll6076	hypothetical protein
slr6029	hypothetical protein
slr6088	hypothetical protein
slr6025	probable antirestriction protein
slr6084	probable antirestriction protein
slr6011	probable nuclease
slr6070	probable nuclease
sll1397	putative transposase [ISY100a: 52234 - 53180]
slr1357	putative transposase [ISY100c: 1098251 - 1099197]
sll1998	putative transposase [ISY100d: 1623697 - 1624643]
slr0230	putative transposase [ISY100f: 2534034 - 2534980]
slr0704	putative transposase [ISY100g: 3097363 - 3098309]
sll0431	putative transposase [ISY100h: 3512289 - 3513235]
sll0699	putative transposase [ISY100i: 123475 - 124420]
slr1715	putative transposase [ISY100m: 1463383 - 1464328]
sll1256	putative transposase [ISY100p: 1725405 - 1726350]
sll1436	putative transposase [ISY100q: 1901359 - 1902304]
slr1936	putative transposase [ISY100r: 2235489 - 2236434]
sll0200	putative transposase [ISY100s: 2524547 - 2525492]
smr0002	putative transposase [ISY100v: 3095975 - 3096319, join 3097194 - 3097362, join 3098314 - 3098743]
ssr1175	putative transposase [ISY100v: 3095975 - 3096319, join 3097194 - 3097362, join 3098314 - 3098743]
ssr1176	putative transposase [ISY100v: 3095975 - 3096319, join 3097194 - 3097362, join 3098314 - 3098743]
sll1780	putative transposase [ISY203b: 1200306 - 1201479]
sll1255	putative transposase [ISY203c: 1728942 - 1730115]
sll1560	putative transposase [ISY203d: 1970882 - 1972055]
sll1474	putative transposase [ISY203g: 3400332 - 3401505]

sll1997	putative transposase [ISY203h: 1623060 - 1623693, join 1624643 - 1625182]
sll1999	putative transposase [ISY203h: 1623060 - 1623693, join 1624643 - 1625182]
sll0317	putative transposase [ISY203i: 2443391 - 2443924, join 2444874 - 2445513]
sll1860	putative transposase [ISY523d: 2226601 - 2227471]
slr0350	putative transposase [ISY523e: 2441031 - 2441901]
sll0012	putative transposase [ISY523f: 2482725 - 2483595]
sll0677	putative transposase [ISY523h: 3093889 - 3094759]
sll5131	transposase
slr6105	transposase
slr7008	transposase
slr7104	transposase
slr8045	transposase
sll6010	unknown protein
sll6069	unknown protein
slr6005	unknown protein
slr6007	unknown protein
slr6008	unknown protein
slr6009	unknown protein
slr6012	unknown protein
slr6016	unknown protein
slr6021	unknown protein
slr6022	unknown protein
slr6028	unknown protein
slr6064	unknown protein
slr6066	unknown protein
slr6067	unknown protein
slr6068	unknown protein
slr6071	unknown protein
slr6075	unknown protein
slr6080	unknown protein
slr6081	unknown protein
slr6087	unknown protein
ssl6018	unknown protein
ssl6023	unknown protein
ssl6077	unknown protein
ssl6082	unknown protein
ssr6019	unknown protein
ssr6020	unknown protein
ssr6024	unknown protein

ssr6026	unknown protein
ssr6027	unknown protein
ssr6078	unknown protein
ssr6079	unknown protein
ssr6083	unknown protein
ssr6085	unknown protein
ssr6086	unknown protein

APPENDIX VI

Complete list of genes up-regulated across late samples in all conditions.

Cluster	Gene ID	Gene symbol	Gene product	Functional category[a]
UP1	slr1844	uvrA	excinuclease ABC subunit A	DNA replication, restriction, modification, recombination and repair
UP1	sll1154		putative antibiotic efflux protein	Drug and analog sensitivity
UP1	slr1498	hypD	putative hydrogenase expression/formation protein HypD	Hydrogenase
UP1	slr1675	hypA1	putative hydrogenase expression/formation protein HypA1	Hydrogenase
UP1	sll0036		hypothetical protein	Hypothetical
UP1	sll0141		hypothetical protein	Hypothetical
UP1	sll0183		hypothetical protein	Hypothetical
UP1	sll0335		hypothetical protein	Hypothetical
UP1	sll0549		hypothetical protein	Hypothetical
UP1	sll0926		hypothetical protein	Hypothetical
UP1	sll1049		hypothetical protein	Hypothetical
UP1	sll1274		hypothetical protein	Hypothetical
UP1	sll1355		hypothetical protein	Hypothetical
UP1	sll1541		hypothetical protein	Hypothetical
UP1	sll1620		hypothetical protein	Hypothetical
UP1	sll1659		hypothetical protein	Hypothetical
UP1	sll1769		hypothetical protein	Hypothetical
UP1	slr0326		hypothetical protein	Hypothetical
UP1	slr0594		hypothetical protein	Hypothetical
UP1	slr0957		hypothetical protein	Hypothetical
UP1	slr1464		hypothetical protein	Hypothetical
UP1	slr1657		hypothetical protein	Hypothetical
UP1	ssl1762		hypothetical protein	Hypothetical
UP1	slr1743	ndbB	type 2 NADH dehydrogenase NdbB	NADH dehydrogenase
UP1	sll0217		flavoprotein	Other
UP1	slr0078		putative 6-pyruvoyl tetrahydrobiopterin synthase	Other
UP1	slr1192		probable alcohol dehydrogenase	Other

UP1	ssl0452	nblA1	phycobilisome degradation protein NblA	Phycobilisome
UP1	slr0328		low molecular weight phosphotyrosine protein phosphatase	Protein modification and translation factors
UP1	slr0484	hik26	two-component sensor histidine kinase	Regulatory functions
UP1	slr0741		transcriptional regulator	Regulatory functions
UP1	slr1324	hik23	two-component hybrid sensor and regulator	Regulatory functions
UP1	slr1738		transcription regulator Fur family	Regulatory functions
UP1	slr0853	rimI	ribosomal-protein-alanine acetyltransferase	Ribosomal proteins: synthesis and modification
UP1	sll1481		ABC-transporter membrane fusion protein	Transport and binding proteins
UP1	sll1623		ABC transporter ATP-binding protein	Transport and binding proteins
UP1	slr0529	ggtB	glucosylglycerol transport system substrate-binding protein	Transport and binding proteins
UP1	sll1942		unknown protein	Unknown
UP1	slr1809		unknown protein	Unknown
UP2	sll0261		hypothetical protein	Hypothetical
UP2	sll0514		hypothetical protein	Hypothetical
UP2	sll0528		hypothetical protein	Hypothetical
UP2	sll1884		hypothetical protein	Hypothetical
UP2	sll1895		hypothetical protein	Hypothetical
UP2	slr0959		hypothetical protein	Hypothetical
UP2	slr1119		hypothetical protein	Hypothetical
UP2	slr1413		hypothetical protein	Hypothetical
UP2	slr1593		hypothetical protein	Hypothetical
UP2	slr1674		hypothetical protein	Hypothetical
UP2	ssl1633	hliC, scpB	high light-inducible polypeptide HliC, CAB/ELIP/HLIP superfamily	Hypothetical
UP2	slr0851	ndbA	type 2 NADH dehydrogenase	NADH dehydrogenase
UP2	sll0398		deoxyguanosinetriphosphate triphosphohydrolase	Purine ribonucleotide biosynthesis
UP2	slr2132		phosphotransacetylase	Pyruvate and acetyl-CoA metabolism
UP2	slr1285	hik34	two-component sensor histidine kinase	Regulatory functions
UP2	slr1594		two-component response regulator PatA subfamily	Regulatory functions
UP2	sll0045	spsA	sucrose phosphate synthase	Sugars

UP2	sll1040		unknown protein	Unknown
UP2	slr0960		unknown protein	Unknown
UP3	sll1498		carbamoyl-phosphate synthase small chain	Amino acids and amines
UP3	slr0051	ecaB	periplasmic beta-type carbonic anhydrase	CO2 fixation
UP3	slr0618	cobQ	cobyric acid synthase	Cobalamin, heme, phycobilin and porphyrin
UP3	sll0002		penicillin-binding protein	Drug and analog sensitivity
UP3	sll0584	ycf36	hypothetical protein YCF36	Hypothetical
UP3	sll0661	ycf35	hypothetical protein YCF35	Hypothetical
UP3	sll1618		hypothetical protein	Hypothetical
UP3	sll1697		hypothetical protein	Hypothetical
UP3	slr1799		hypothetical protein	Hypothetical
UP3	slr5082		hypothetical protein	Hypothetical
UP3	slr5118		hypothetical protein	Hypothetical
UP3	slr5119		hypothetical protein	Hypothetical
UP3	sll1540		dolichyl-phosphate-mannose synthase	Polysaccharides and glycoproteins
UP3	slr0974	infC	initiation factor IF-3	Protein modification and translation factors
UP3	slr1991	cya1	adenylate cyclase	Regulatory functions
UP3	sll1496		mannose-1-phosphate guanyltransferase	Sugars
UP3	slr1635		putative transposase [ISY203e: 2048410 - 2049583]	Transposon-related functions
UP3	sll0252		unknown protein	Unknown
UP4	slr1747		cell death suppressor protein Lls1 homolog	Cell killing
UP4	sll0330		sepiapterine reductase	Fatty acid, phospholipid and sterol metabolism
UP4	sll1158		hypothetical protein	Hypothetical
UP4	sll1675		hypothetical protein	Hypothetical
UP4	sll6052		hypothetical protein	Hypothetical
UP4	sll6055		hypothetical protein	Hypothetical
UP4	slr0292		hypothetical protein	Hypothetical
UP4	sll5060		two-component hybrid sensor & regulator	Regulatory functions
UP4	slr0096		low affinity sulfate transporter	Transport and binding proteins
UP4	slr2131		RND multidrug efflux transporter	Transport and binding proteins
UP5	slr1729	kdpB	potassium-transporting P-type ATPase B chain	Transport and binding proteins

[a] based on Kazusa annotation prior to May 2002

APPENDIX VII

Complete list of genes down-regulated across late samples in all conditions.

Cluster	Gene ID	Gene symbol	Gene product	Functional category[a]
DOWN1	sll1323	atpG	ATP synthase subunit b' of CF(0)	ATP synthase
DOWN1	slr0967		hypothetical protein	Hypothetical
DOWN1	sll0939		hypothetical protein	Hypothetical
DOWN1	sll0576		putative sugar-nucleotide epimerase/dehydratase	Other
DOWN1	ssl3437	rps17	30S ribosomal protein S17	Ribosomal proteins: synthesis and modification
DOWN1	ssl3432	rps19	30S ribosomal protein S19	Ribosomal proteins: synthesis and modification
DOWN1	sll1812	rps5	30S ribosomal protein S5	Ribosomal proteins: synthesis and modification
DOWN1	sll1809	rps8	30S ribosomal protein S8	Ribosomal proteins: synthesis and modification
DOWN1	sll1806	rpl14	50S ribosomal protein L14	Ribosomal proteins: synthesis and modification
DOWN1	sll1813	rpl15	50S ribosomal protein L15	Ribosomal proteins: synthesis and modification
DOWN1	sll1805	rpl16	50S ribosomal protein L16	Ribosomal proteins: synthesis and modification
DOWN1	sll1811	rpl18	50S ribosomal protein L18	Ribosomal proteins: synthesis and modification
DOWN1	sll1807	rpl24	50S ribosomal protein L24	Ribosomal proteins: synthesis and modification
DOWN1	ssl3436	rpl29	50S ribosomal protein L29	Ribosomal proteins: synthesis and modification
DOWN1	sll1808	rpl5	50S ribosomal protein L5	Ribosomal proteins: synthesis and modification
DOWN1	sll1810	rpl6	50S ribosomal protein L6	Ribosomal proteins: synthesis and modification
DOWN1	slr0875	mscL	large-conductance mechanosensitive channel	Transport and binding proteins
DOWN1	slr0841		periplasmic protein, function unknown	Unknown
DOWN1	ssr1853		unknown protein	Unknown
DOWN1	ssr0693		unknown protein	Unknown
DOWN1	sll0733		unknown protein	Unknown

DOWN2	sll0947	lrtA	light repressed protein A homolog	Adaptations and atypical conditions
DOWN2	sll0573	arcC	carbamate kinase	Amino acids and amines
DOWN2	slr0244		hypothetical protein	Hypothetical
DOWN2	slr0888		hypothetical protein	Hypothetical
DOWN2	slr0144		hypothetical protein	Hypothetical
DOWN2	slr0146		hypothetical protein	Hypothetical
DOWN2	slr0149		hypothetical protein	Hypothetical
DOWN2	sll0253		hypothetical protein	Hypothetical
DOWN2	sll0735		hypothetical protein	Hypothetical
DOWN2	slr1667		hypothetical protein (target gene of sycrp1)	Hypothetical
DOWN2	sll0830	fus	elongation factor EF-G	Protein modification and translation factors
DOWN2	slr0301		phosphoenolpyruvate synthase	Pyruvate and acetyl-CoA metabolism
DOWN2	slr0150	petF, fdx	ferredoxin, petF-like protein	Soluble electron carriers
DOWN2	sll0741		pyruvate flavodoxin oxidoreductase	Soluble electron carriers
DOWN2	slr0727		unknown protein	Unknown
DOWN2	sll1241		unknown protein	Unknown
DOWN3	slr2052		hypothetical protein	Hypothetical
DOWN3	sll1783		hypothetical protein	Hypothetical
DOWN3	sll1305		probable hydrolase	Other
DOWN3	slr0737	psaD	photosystem I subunit II	Photosystem I
DOWN3	ssr3383	apcC	phycobilisome small core linker polypeptide	Phycobilisome
DOWN3	ssl3093	cpcD	phycobilisome small rod linker polypeptide	Phycobilisome
DOWN3	sll1578	cpcA	phycocyanin alpha subunit	Phycobilisome
DOWN3	sll1577	cpcB	phycocyanin beta subunit	Phycobilisome
DOWN3	sll0771	glcP, gtr	glucose transport protein	Transport and binding proteins
DOWN3	sll1307		periplasmic protein, function unknown	Unknown
DOWN3	sll1306		periplasmic protein, function unknown	Unknown
DOWN3	slr1243		unknown protein	Unknown
DOWN3	sll1304		unknown protein	Unknown
DOWN3	sll0780		unknown protein	Unknown
DOWN3	slr1410		periplasmic WD-repeat protein	WD repeat proteins

DOWN4	slr0220	glyS	glycyl-tRNA synthetase beta chain	Aminoacyl tRNA synthases and tRNA modification
DOWN4	slr1550	lysS	lysyl-tRNA synthetase	Aminoacyl tRNA synthases and tRNA modification
DOWN4	sll1553	pheT	phenylalanyl-tRNA synthetase	Aminoacyl tRNA synthases and tRNA modification
DOWN4	slr1808	hemA	transfer RNA-Gln reductase	Aminoacyl tRNA synthases and tRNA modification
DOWN4	sll1363	ilvC	ketol-acid reductoisomerase	Branched chain family
DOWN4	slr2072	ilvA	L-threonine deaminase	Branched chain family
DOWN4	slr0783	tpi	triosephosphate isomerase	CO2 fixation
DOWN4	slr0067		MRP protein homolog	Drug and analog sensitivity
DOWN4	sll1910	zam	protein conferring resistance to acetazolamide Zam	Drug and analog sensitivity
DOWN4	sll0053	accC	biotin carboxylase	Fatty acid, phospholipid and sterol metablism
DOWN4	slr1369	cdsA	phosphatidate cytidylyltransferase	Fatty acid, phospholipid and sterol metablism
DOWN4	slr1020	sqdB	sulfolipid biosynthesis protein SqdB	Fatty acid, phospholipid and sterol metablism
DOWN4	slr0426	folE	GTP cyclohydrolase I	Folic acid
DOWN4	slr2135	hupE	hydrogenase accessory protein HupE	Hydrogenase
DOWN4	ssl1911	gifA	glutamine synthetase inactivating factor IF7	Hypothetical
DOWN4	slr1098		hypothetical protein	Hypothetical
DOWN4	slr0695		hypothetical protein	Hypothetical
DOWN4	ssr1528		hypothetical protein	Hypothetical
DOWN4	sll1654		hypothetical protein	Hypothetical
DOWN4	sll0360		hypothetical protein	Hypothetical
DOWN4	sll0577		hypothetical protein	Hypothetical
DOWN4	slr0245		Histone deacetylase family protein	Other
DOWN4	slr0825		probable peptidase	Other
DOWN4	sll0629	psaK2	alternative photosystem I reaction center subunit X	Photosystem I
DOWN4	sll1418	psbP2	photosystem II oxygen-evolving complex 23K protein PsbP homolog	Photosystem II
DOWN4	sll1281	psbZ, ycf9	photosystem II PsbZ protein	Photosystem II
DOWN4	slr0774	secD	protein-export membrane protein SecD	Protein and peptide secretion
DOWN4	sll0145	frr, rrf	ribosome releasing factor	Protein modification and translation factors

DOWN4	slr1722		inosine-5'-monophosphate dehydrogenase	Purine ribonucleotide biosynthesis
DOWN4	slr1164		ribonucleotide reductase subunit alpha	Purine ribonucleotide biosynthesis
DOWN4	slr1096		dihydrolipoamide dehydrogenase	Pyruvate dehydrogenase
DOWN4	sll0998	ycf30	LysR family transcriptional regulator	Regulatory functions
DOWN4	sll1584		ferredoxin like protein	Soluble electron carriers
DOWN4	slr0583		similar to GDP-fucose synthetase	Surface polysaccharides, lipopolysaccharides and antigens
DOWN4	sll0146	natC	Integral membrane protein of the ABC-type, Nat permease for neutral amino acids	Transport and binding proteins
DOWN4	sll1835		periplasmic protein, function unknown	Unknown
DOWN4	slr1928	pilA5	type 4 pilin-like protein	Unknown
DOWN4	sll1239		unknown protein	Unknown
DOWN5	slr1329	atpB	ATP synthase beta subunit	ATP synthase
DOWN5	sll1325	atpD	ATP synthase delta chain of CF(1)	ATP synthase
DOWN5	sll1533	pilT2	twitching mobility protein	Chemotaxis
DOWN5	sll1091	chlP	geranylgeranyl hydrogenase	Cobalamin, heme, phycobilin and porphyrin
DOWN5	sll1184	ho1	heme oxygenase	Cobalamin, heme, phycobilin and porphyrin
DOWN5	slr1331		periplasmic processing protease	Degradation of proteins, peptides and glycopeptides
DOWN5	sll1502	gltB	NADH-dependent glutamate synthase large subunit	Glutamate family / Nitrogen assimilation
DOWN5	slr0680		hypothetical protein	Hypothetical
DOWN5	slr1438		hypothetical protein	Hypothetical
DOWN5	slr0889		hypothetical protein	Hypothetical
DOWN5	slr0575		hypothetical protein	Hypothetical
DOWN5	sll1390		hypothetical protein	Hypothetical
DOWN5	sll0822		hypothetical protein	Hypothetical
DOWN5	slr2032	ycf23	hypothetical protein YCF23	Hypothetical
DOWN5	slr2049	ycf58	hypothetical protein YCF58	Hypothetical
DOWN5	slr2094	fbpI	fructose-1,6-/sedoheptulose-1,7-bisphosphatase	Other
DOWN5	slr1834	psaA	P700 apoprotein subunit Ia	Photosystem I
DOWN5	slr1835	psaB	P700 apoprotein subunit Ib	Photosystem I
DOWN5	sll0819	psaF	photosystem I reaction center subunit III precursor (PSI-F),	Photosystem I

			plastocyanin (cyt c553) docking protein	
DOWN5	ssr2831	psaE	photosystem I subunit IV	Photosystem I
DOWN5	ssl0563	psaC	photosystem I subunit VII	Photosystem I
DOWN5	slr1655	psaL	photosystem I subunit XI	Photosystem I
DOWN5	sll0258	psbV	cytochrome c550	Photosystem II
DOWN5	slr0906	psbB	photosystem II core light harvesting protein	Photosystem II
DOWN5	sll0851	psbC	photosystem II CP43 protein	Photosystem II
DOWN5	slr2067	apcA	allophycocyanin alpha subunit	Phycobilisome
DOWN5	slr1986	apcB	allophycocyanin beta subunit	Phycobilisome
DOWN5	slr1459	apcF	phycobilisome core component	Phycobilisome
DOWN5	slr0335	apcE	phycobilisome core-membrane linker polypeptide	Phycobilisome
DOWN5	slr2051	cpcG1	phycobilisome rod-core linker polypeptide	Phycobilisome
DOWN5	sll1814	secY	preprotein translocase SecY subunit	Protein and peptide secretion
DOWN5	sll1099	tufA	elongation factor Tu	Protein modification and translation factors
DOWN5	sll1815	adk	adenylate kinase	Purine ribonucleotide biosynthesis
DOWN5	sll1286		transcriptional regulator	Regulatory functions
DOWN5	slr1545	sigG	RNA polymerase ECF-type (group 3) sigma-E factor	RNA synthesis, modification and DNA transcription
DOWN5	sll1568	pgl1	fibrillin	Surface structures
DOWN5	slr1890		bacterioferritin	Transport and binding proteins
DOWN5	sll1089		periplasmic protein, function unknown	Unknown
DOWN5	slr1232		unknown protein	Unknown
DOWN5	slr1437		unknown protein	Unknown
DOWN5	sll1665		unknown protein	Unknown
DOWN5	sll1583		unknown protein	Unknown
DOWN5	sll0022		unknown protein	Unknown
DOWN5	sll0376		unknown protein	Unknown
DOWN5	sll1491		periplasmic WD-repeat protein	WD repeat proteins
DOWN6	sll1713	hisC	histidinol-phosphate aminotransferase	Aromatic amino acid family
DOWN6	sll1322	atpI	ATP synthase A chain of CF(0)	ATP synthase
DOWN6	sll1326	atpA	ATP synthase alpha chain	ATP synthase
DOWN6	ssl2615	atpH	ATP synthase C chain of CF(0)	ATP synthase
DOWN6	slr1330	atpE	ATP synthase epsilon chain of CF(1)	ATP synthase

DOWN6	sll1327	atpC	ATP synthase gamma chain	ATP synthase
DOWN6	slr0452	ilvD	dihydroxyacid dehydratase	Branched chain family
DOWN6	sll0254		probable phytoene dehydrogenase Rieske iron-sulfur component	Carotenoid
DOWN6	slr0012	rbcS	ribulose bisphosphate carboxylase small subunit	CO2 fixation
DOWN6	sll1185	hemF	coproporphyrinogen III oxidase, aerobic (oxygen-dependent)	Cobalamin, heme, phycobilin and porphyrin
DOWN6	slr0506	por	light-dependent NADPH-protochlorophyllide oxidoreductase	Cobalamin, heme, phycobilin and porphyrin
DOWN6	sll1655		similar to biotin [acetyl-CoA-carboxylase] ligase	Fatty acid, phospholipid and sterol metablism
DOWN6	slr0710	gdhA	glutamate dehydrogenase (NADP+)	Glutamate family / Nitrogen assimilation
DOWN6	sll1196	pfkA	phosphofructokinase	Glycolysis
DOWN6	slr0869		hypothetical protein	Hypothetical
DOWN6	slr1506		hypothetical protein	Hypothetical
DOWN6	slr1634		hypothetical protein	Hypothetical
DOWN6	sll1638		hypothetical protein	Hypothetical
DOWN6	sll1925		hypothetical protein	Hypothetical
DOWN6	sll0359		hypothetical protein	Hypothetical
DOWN6	sll0147		hypothetical protein	Hypothetical
DOWN6	sll0051		hypothetical protein	Hypothetical
DOWN6	sll7069		hypothetical protein	Hypothetical
DOWN6	slr0011	rbcX	possible Rubisco chaperonin	Hypothetical
DOWN6	sll0772		probable porin; major outer membrane protein	Membranes, lipoproteins and porins
DOWN6	sll1712		DNA binding protein HU	Nucleoproteins
DOWN6	slr1540		mRNA-binding protein	Nucleoproteins
DOWN6	slr1761	ytfC	FKBP-type peptidyl-prolyl cis-trans isomerase, periplasmic protein	Other
DOWN6	sll1534		probable glycosyltransferase	Other
DOWN6	sll1308		probable oxidoreductase	Other
DOWN6	sll0427	psbO	photosystem II manganese-stabilizing polypeptide	Photosystem II
DOWN6	sll1580	cpcC1	phycobilisome rod linker polypeptide	Phycobilisome
DOWN6	sll1579	cpcC2	phycobilisome rod linker polypeptide	Phycobilisome
DOWN6	sll0542		acetyl-coenzyme A synthetase	Pyruvate and acetyl-CoA metabolism

DOWN6	sll1626		LexA repressor	Regulatory functions
DOWN6	sll1087		similar to sodium/glucose cotransporter	Transport and binding proteins
DOWN6	slr2057		water channel protein	Transport and binding proteins
DOWN6	slr0708		periplasmic protein, function unknown	Unknown
DOWN6	slr0151		unknown protein	Unknown
DOWN6	sll0630		unknown protein	Unknown
DOWN7	slr0749	chlL	light-independent protochlorophyllide reductase iron protein subunit ChlL	Cobalamin, heme, phycobilin and porphyrin
DOWN7	slr0750	chlN	light-independent protochlorophyllide reductase subunit ChlN	Cobalamin, heme, phycobilin and porphyrin
DOWN7	sll1429		unknown protein	Unknown
DOWN7	sll7062		unknown protein	Unknown
DOWN7	sll7063		unknown protein	Unknown
DOWN7	sll7064		unknown protein	Unknown
DOWN7	sll7066		unknown protein	Unknown
DOWN7	sll7067		unknown protein	Unknown
DOWN7	sll7085		unknown protein	Unknown
DOWN7	sll7087		unknown protein	Unknown
DOWN8	sll0788		hypothetical protein	Hypothetical
DOWN8	sll0789		two-component response regulator OmpR subfamily	Regulatory functions
DOWN8	slr6043	copA	probable cation efflux system protein, czcA homolog	Transport and binding proteins

[a] based on Kazusa annotation prior to May 2002