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Investigation of Pilin Function in the Cyanobacterium *Synechocystis* sp. PCC 6803

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Abstract

The aim of this project was to investigate the involvement of a specific operon from *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis* 6803) in iron acquisition. The operon selected consists of the four genes, *slr1928, slr1929, slr1930* and *slr1931 (slr1928-31),* all hypothesized to encode pilin-like proteins (Yoshihara *et al*., 2001). Bacterial pili are demonstrated to function as so-called nanowires, through which the organism may donate electrons to extracellular acceptors (Gorby *et al*., 2006). In this way pili might contribute to iron uptake by facilitating reduction of ferric iron. Pilin-like proteins related to the operon *slr1928-31* appear to contribute to iron uptake (Gorby *et al*., 2006; Singh *et al*., 2003), leading to the hypothesis that the operon *slr1928-31* may possess similar functions.

In order to determine the potential functions of *slr1928-31*, a mutant strain lacking the operon was produced. Assessment of differences between cultures where the operon was present (wild type, WT) and absent (mutant strain, Δ *slr1928-31*-Kan) was performed by testing growth of both strains under various conditions. Different iron sources (ferric ammonium citrate - Fac, iron (II, III) oxide - FeO, ferric chloride hexahydrate - FeCl, goethite - Goe and potassium hexacyanoferrate (III) - Hexacyan) were supplied to the medium to test their utility in the presence or absence of the operon *slr1928-31.* Different nutrient supplements (glucose) or treatments with stress-inducing chemicals (DCMU or NaCl) were tested to induce different modes of growth. The effects of the mutation were also examined by two spectroscopic methods, (I) 77 K fluorescence spectroscopy and (II) whole-cell room temperature absorption spectroscopy.

The growth assay showed few differences in growth between WT and Δ *slr1928-31*-Kan cultures on media without NaCl but generally better growth for the mutant strain on media with NaCl (except for the DCMU condition). The results from both whole cell absorption spectroscopy and 77 K fluorescence spectroscopy corresponded with these results: The main differences between WT and Δ *slr1928-31*-Kan strains were seen on medium with NaCl or with NaCl and glucose, for which WT cultures on FeCl under the former condition and WT cultures on Fac, FeCl or Hexacyan under the latter condition had too little growth for spectroscopic analysis. Otherwise the differences occurred mainly between the iron sources. The most clear indications of stress were observed for samples grown on Goe, suggesting that this was the most challenging iron source to utilize. Additionally, the relative availability of the iron sources tested seemed to change with addition of NaCl to the medium, possibly inducing precipitation of iron from the water-soluble sources and consequently better growth on water-insoluble iron sources in the presence of NaCl.

No function of the *slr1928-31* gene products in iron acquisition was detected in this study. Whether or not this operon has a minor or indirect role in iron uptake under some circumstances remains to be established. These pilin-like proteins could also have a regulatory function, possibly initiated in response to iron stress.

According to the results of this work, the mutant strain Δ *slr1928-31*-Kan appears to be less stressed than the WT strain under some high-NaCl conditions. This outcome suggests that the operon *slr1928-31* might be involved in salt-sensory mechanisms and/or responses to stress such as high NaCl concentrations in the growth medium. Growth under stress may serve as a framework in which the function of pilin-like proteins encoded by the operon *slr1928-31* should be further investigated.

Sammendrag

Hensikten med dette prosjektet var å undersøke om et bestemt operon fra *Synechocystis* 6803 er involvert i bakteriens jernopptak. Det utvalgte operonet består av 4 gener som koder for pilin-liknende proteiner: *slr1928, slr1929, slr1930* and *slr1931 (slr1928-31)* (Yoshihara et al., 2001). Det er tidligere vist at pili kan fungere som en såkalt nanowire, og at bakterien gjennom disse kan avgi elektroner til eksterne akseptorer (Gorby *et al*., 2006). På denne måten kan pili bidra til jernopptak ved å fremme reduksjon av treverdig jern. Det er mulig at genproduktene fra operonet *slr1928-31* kan bidra til dette, ettersom en potensiell rolle i jernopptak er blitt demonstrert for andre beslektede pilin-liknende proteiner (Gorby *et al*., 2006; Singh *et al*., 2003).

For å avgjøre hvorvidt genproduktene fra operon *slr1928-31* har noen funksjon i opptak av jern hos *Synechocystis* 6803 ble det konstruert en bakteriestamme uten det aktuelle operonet, Δ *slr1928-31*-Kan. Forskjeller mellom villtype (WT) og mutant ble vurdert ved hjelp av et vekstforsøk. Ulike jernkilder (Fac, FeO, FeCl, Goe og Hexacyan) ble benyttet for å avgjøre hvorvidt det er forskjeller i hvordan de to stammene nyttiggjør seg ulike jernforbindelser. Forskjellige næringskilder (glukose) og stressfremkallende behandlinger (DCMU eller NaCl) ble testet for å indusere ulike vekstformer. Effecten av mutasjonen ble også undersøkt ved bruk av to ulike spektroskopiske metoder: (I) 77 K fluorescens spektroskopi og (II) whole-cell absorpsjonsspektroskopi.

Vekstoforsøket viste få ulikeheter mellom kulturer av WT og Δ *slr1928-31*-Kan dyrket i medier uten NaCl. For kulturer dyrket i medier med NaCl ble det observert generelt bedre vekst for den mutante stammen, med unntak av medium med DCMU. Whole cell absorpsjonsspektra viste også liknende trender: De mest markante forskjellene mellom stammene ble observert på medium med NaCl eller NaCl og glukose, hvor WT-kulturer på FeCl for den første betingelsen og WT-kulturer på Fac, FeCl eller Hexacyan for den siste betingelsen, ikke hadde tilstrekkelig vekst for spektroskopiske analyser.

De øvrige forskjellene var mellom de ulike jernkildene, hvor vekst på Goe totalt sett virket mest utfordrende. De samme trendene ble observert i 77 K fluorescens-spektra. I tillegg tydet resultatene på at den relative tilgjengeligheten av jernforbindelsene endret seg ved tilsetning av NaCl til mediet. Jern fra de vannløselige forbindelsene ble muligens felt ut, slik at veksten på de uløselige forbindelsene ble forbedret.

I denne studien ble det ikke identifisert noen funksjon i jernopptak for genproduktene fra operon *slr1928-31*. Det kreves ytterligere forskning for å stadfeste om operonet kan være involvert i opptak

av jern under bestemte forhold som ikke ble testet her. De pilin-liknende proteinene kodet av operon *slr1928-31* kan ha en regulatorisk funksjon, muligens igangsatt som respons på jern-relatert stress.

Basert på de oppnådde resultatene ser det ut til at stammen Δ *slr1928-31*-Kan blir mindre påvirket av stressfaktorer, eksempelvis NaCl, i sitt vekstmiljø. Operon *slr1928-31* kan være involvert i mekanismer for å detektere saltinnhold i mediet og/eller responser på stress fremkalt av f.eks. NaCl. Vekst under stress kan fungere som rammeverk for videre studier av operon *slr1928-31*.

Preface

This Master of Science thesis is a product of my years as a student in the 5-year biotechnology program at the Norwegian University of Science and Technology. The work of this project was carried out in the period August 2013 through June 2014, and delivered to the Department of Biotechnology the 12th of August 2014. My main supervisor was associate professor Martin F. Hohmann-Marriott.

There are several people whose never-receding support and encouragement have been vital in the completion of my thesis work. First of all, I would like to direct a great thanks to my supervisor Martin Hohmann-Marriott, for his enthusiasm, patience and the knowledge he willingly shares. Martin kindly created a thesis project based on my interests in molecular genetics and microbiology, and his optimism was a motivating factor throughout the project.

I am also very grateful for all help and advice from my laboratory supervisor, PhD candidate Jacob Lamb. Through his patient guidance, I have learnt a lot about different procedures in the lab. He generously spared his time to help out and always gave thorough answers to all my questions. Jake's sense of humor and positive attitude was helping me keep up the spirits all through of my master's work.

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I would like to direct a warm thanks to my parents, Rolf and Eva, and my siblings Sunniva and Benjamin, who have backed me up and given me the motivation for my studies. A special thanks to my class mates and good friends, Birgit Drejer Ringstad and Axana Chernyavskaya, with whom I've shared good times and frustrations throughout our study years. Last, but not least, a deep thanks to my dear Audun. I would never been where I am now without his ever-lasting positivity and loving care, for which I am forever grateful.

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Abbreviations and symbols

A_0	Primary electron acceptor of PS I
ADP	Adenosine 5’ diphosphate
Amp	Ampicillin
ATP	Adenosine 5’ triphosphate
Chl *a*	Chlorophyll *a*
Chlor	Chloramphenicol
Cl^-	Chloride ion
CO_2	Carbon dioxide
Cyt b_6f	Cytochrome b_6f
DCMU	3-(3,4-Dichlorophenyl)-1,1-dimethylurea
DNA	Deoxyribonucleic acid
e-	Electron
EC	Extracellular
E. coli	*Escherichia coli*
ETC	Electron transport chain
Fac	Ferric ammonium citrate
Fe	Iron
Fe^{2+}	Ferrous iron
Fe^{3+}	Ferric iron
FeCl	Ferric chloride hexahydrate
FeO	Iron (II, III) oxide
Goe	Goethite

H^+	Proton
H_2O	Dihydrogen oxide /water
H_2S	Hydrogen sulfide
Hexocyan	Potassium hexacyanoferrate (III)
Kan	Kanamycin
Kb	Kilobase
LHC	Light harvesting complex
Mb	Megabases
Mm	Millimeters
Na^+	Sodium ion
NaCl	Sodium chloride
NAD^+	Nicotinamide adenine dinucleotide, oxidized
NADH	Nicotinamide adenine dinucleotide, reduced
$NADP^+$	Nicotinamide adeninde dinucleotide phosphate, oxidized
NADPH	Nicotinamide adeninde dinucleotide phosphate, reduced
Nm	Nanometers
O_2	Molecular oxygen
PC	Plastocyanin
PCC	Pasteur culture collection
Pheo	Pheophytin
P_i	Inorganic phosphate
PM	Plasma membrane
PMF	Proton-motive force

PQ	Plastoquinone
PQH_2	Plastoquinol
PS I	Photosystem I
PS II	Photosystem II
Q_A	Plastoquinone bound to PS II
Q_B	Plastoquinone bound to PS II
RNA	Ribonucleic acid
Spec	Spectrinomycin
Synechocystis 6803	*Synechocystis* sp. PCC 6803
µm	Micrometers
WT	Wild type

1. Introduction

1. 1: Cyanobacteria

1.1.1: Cyanobacteria

Unicellular organisms, such as bacteria, may live under many different conditions (Madigan, 2000). A bacterial phylum, which is found across a broad range of habitats, is cyanobacteria (Büdel, 2011). Cyanobacteria are prokaryotes, although they are often misleadingly referred to as blue-green algae. These bacteria can adapt to various environments and survive very high or low temperatures, light intensities, oxygen levels, hydration levels, salinities or even multiple of these challenging conditions in one habitat. Fossil findings as old as 2500 billion years indicate that these bacteria are of ancient origin. There are also even older fossils of putative cyanobacteria, dating 3500 billion years back (Schaechter *et al*., 2009).

Cyanobacteria derive their energy primarily from oxygenic photosynthesis, although some species may utilize other mechanisms to fulfill their energy requirements (Kaplan *et al*., 2008; Eiler, 2006): some are mixotrophs, which combine different strategies such as photosynthesis for energy and organic carbon sources for biosynthesis. Some may employ photoheterotrophic means of growth, depending solely on energy from light and carbon from organic compounds. Others display growth exclusively by heterotrophy, using various organic sources to obtain both energy and carbon.

The ability of cyanobacteria to live either aerobically or anaerobically has led to a widely accepted hypothesis concerning the development of today's oxygen-rich atmosphere. Oxygen-producing photosynthesis most likely evolved in cyanobacteria several billion years ago and resulted in an initial rise of molecular oxygen (O_2). Over the years, the net O_2 production from microbial photosynthesis resulted in a stable atmospheric level, enabling evolution of aerobic life forms (Buick, 2008). O_2 produced by cyanobacteria and eukaryotic photosynthesis is balanced by respiration and decomposition of organic matter (Kasting and Siefert, 2002).

1.1.2: Synechocystis *sp. PCC 6803*

The freshwater strain *Synechocystis* sp. PCC 6803 (*Synechocystis* 6803 hereafter), is a striking example of the adaptability of cyanobacteria to variable growth conditions as it may fulfill its energy requirements by any of the three previously mentioned strategies (Yu *et al*., 2013). Additionally, its genome was the first of all phototrophic organisms and the fourth overall to be fully sequenced (Ikeuchi and Tabata, 2001). According to Cyanobase, the genome of *Synechocystis* 6803 consists of around 3.9 megabases (mb), distributed between a large circular chromosome and

7 smaller circular plasmids. In total, 3725 genes have been identified, 3317 of which are found on the chromosome. The genome of a variety of laboratory strains has recently been sequenced (Kanesaki *et al*., 2012).

1.1.3: Synechocystis *6803 as a model organism*

Synechocystis 6803 is an ideal experimental organism for several reasons (Vermaas, 1996). Its capability for both photoautotrophic and heterotrophic growth makes it suited for studies of disruptions in the respiratory and photosynthetic machineries. Examples of this are deletions of photosystems I or II (PS I or PS II), both resulting in photoheterotrophic growth, where electrons (e^-) are either used in respiration or shuttled into cyclic transport, respectively. *Synechocystis* 6803 does also possess innate competence, and is readily transformed with exogenous DNA. This ability, together with its completely sequenced genome and cyanobacterial databases facilitates studies within molecular genetics (Yu *et al*., 2013; Ikeuchi and Tabata, 2001; Vermaas, 1996). Vermaas also reports that *Synechocystis* 6803 is ideal for studies in molecular genetics due to its ability to take up foreign DNA and integrate it into its genome by homologous recombination: Genes may be selectively eliminated, disrupted or substituted by transformation with a suitable construct (Vermaas, 1996). Another feature is the presence of electrically conductive appendages (pili) on the bacterium surface. These nanowires may potentially be used for microbial fuel cells in carbon-neutral production of electricity in the future (Madiraju *et al*., 2012).

1.2: Photosynthesis and respiration

1.2.1: Oxygenic photosynthesis

Photosynthesis is a process in which light energy is converted into chemical energy. Various organisms such as plants, algae and many bacterial species, use photosynthesis as their main means of obtaining the energy required to sustain life (Shevela *et al*., 2013). Photosynthetic organisms use energy from sunlight to reduce carbon dioxide (CO_2) into energy-rich carbohydrates (Hall and Rao, 1999). Most of these species carry out oxygenic photosynthesis where water (H_2O) is oxidized to complete the redox reaction, releasing O_2 as a product. Other photosynthetic bacteria produce their energy by anoxygenic photosynthesis, where another chemical compound than H_2O, such as hydrogen sulfide (H_2S), is oxidized and no oxygen is formed (Shevela *et al*., 2013).

Following oxygenic photosynthesis, the carbohydrates and O_2 produced are used by the phototrophs themselves or other organisms to drive cellular respiration (section 1.2.3). During this process, the stored energy is released and utilized for production of adenosine 5' triphosphate (ATP). This molecule is the main energy carrier of living cells and required for the organism's growth and

maintenance. ATP is synthesized by the enzyme ATP synthase, driven by a gradient created by proton build-up in the thylakoid lumen. In addition, the reducing agent nicotinamide adenine dinucleotide phosphate (NADPH) is produced and utilized in the Calvin cycle. Thus, photosynthesis is the basis for energy production in all organisms (Alberts *et al*., 2007; Hall and Rao, 1999). The industrial sector also depends on photosynthetic products in another way, as the energy we utilize in fossil fuels originates from this process (Hall and Rao, 1999). Photosynthesis is a possible target for development of environmentally friendly and sustainable energy sources based on solar radiation (Shevela *et al*., 2013).

1.2.2: The photosynthetic process – components and reactions

Photosynthesis is comprised by a complex set of reactions. The main points as well as the details have been extensively described in different variations, such as by Alberts and colleagues (2007) and Blankenship (2014). In *Synechocystis* 6803 the photosynthetic machinery is located in the cytoplasm within a system of membranes called thylakoids. In plants and algae, thylakoids are found within an organelle (the chloroplast), a relic of the engulfment of a cyanobacterium-like organism in ancient times. Due to this endosymbiosis, all photosynthetic organisms possess, within their thylakoid membranes, the same basic units for the photosynthetic reactions. The conversion of light energy into chemical energy depends on two large complexes called photosystems I and II (PS I and II). Each of these includes complexes of proteins and pigment molecules, forming the two main components of the photosystems: An antenna complex, comprising several light-harvesting complexes (LHC), and a reaction center. These two constitute a system to capture sunlight and convert the energy from photons into a form that is usable by the cell. The two photosystems work in concert to carry out a set of reactions, ultimately producing ATP and NADPH. These small molecules are further used as energy source and reducing power in a process called carbon fixation, where CO_2 is converted into organic carbon (carbohydrates) through a sequential set of reactions termed the Calvin Benson cycle.

The first step in the whole process from light energy into chemical energy is the absorption of light by pigment molecules in the PS II antenna complex. The capture of photons initiates the whole photosynthetic process. Energy from the photons is passed between adjacent pigment molecules via resonance energy transfer, eventually reaching a pair of special chlorophyll molecules located in the reaction center. By absorbing this energy, chlorophyll is excited and yields an unstable high-energy e^-. The e- can be donated to a sequence of electron acceptors, while the oxidized chlorophyll receives an electron. Electrons may also return to a lower orbital by generating heat or fluorescence, which can be an efficient mechanism for preventing oxidative damage from excessive light.

The pigment content of *Synechocystis* 6803 has been described by different research groups such as Colyer and colleagues (Colyer *et al*., 2005). The main light-harvesting pigments of PS II antenna in *Synechocystis* 6803 are phycobiliproteins bound together into large structures called phycobilisomes. In contrast, the main light-harvesting pigment of PS I is chlorophyll *a* (Chl *a*). The main pigment of the reaction centers in both PSII and in PSI is also Chl *a*, which is the only type of chlorophyll present in *Synechocystis* 6803. The photosystems also possess carotenoids, pigments collecting photons of wavelengths not covered by chlorophyll. An additional function of carotenoids is to protect against the effects of excess light.

In order to harness the energy, the electron that has been extracted from the special set of chlorophylls is immediately donated to an e^- acceptor: Pheophytin (Pheo) in PSII and the primary electron acceptor A_0 in PS I. In PS II the e^- is passed along to a pair of e^- acceptors called plastoquinones, first to Q_A and then to Q_B, both bound to PS II. Q_B binds protons to form the complex plastoquinol (PQH_2), which then detaches from PS II to become part of the plastoquinone (PQ) pool. PQH_2 is the first mobile carrier in an electron transport chain (ETC), where the stored energy is released in a stepwise and controlled fashion. The e^- is passed along the chain of gradually stronger e^- acceptors. A transmembrane complex called cytochrome b_6f (Cyt b_6f) docks to PQH_2 and the next mobile e^- carrier plastocyanin (PC), and facilitates transfer of electrons to PC. The last e^- acceptor in the ETC is the PS I reaction center, whose light-oxidized chlorophylls receive an e^- from the ETC. Electron $^-$ transfer in the ETC, energy is used to pump protons (H^+) against their gradient across the thylakoid membrane. These H^+ are generated by oxidation of H_2O, and the oxidation PQH_2 by the cytochrome b_6f complex. A proton-motive force (PMF) is created across the membrane, driving ATP synthesis as H^+ flow back across the membrane through ATP synthase. The light-catalyzed oxidation of H_2O also yields an e^- that restores the excited chlorophyll in PS II before the next round of light-driven reactions.

The chlorophyll in the PS I reaction center is also excited by incoming light but the generated e^- is stabilized at a higher energy level than that of photosystem II. This results in sufficient energy for NADPH production. The excited e^- is passed via the carrier ferredoxin to $NADP^+$, where the enzyme ferredoxin-NADP reductase catalyzes the e^- transfer. The whole photosynthetic process is summarized in a simplified manner in figure 1.1.

When the cell needs more ATP, it may shunt e^- back to Cyt b_6f instead of onward to the oxidized form of NADPH ($NADP^+$). Thus, the energy is used instead to enhance the PMF driving ATP synthesis, without producing any NADPH or O_2. This mode of e^- transport is called cyclic

photophosphorylation, or cyclic e^- transfer. Under certain stress conditions the cell may also utilize cyclic transport of electrons. One example is the PS II shut-down and photosynthetic inhibition by the herbicide 3-(3,4-Dichlorophenyl)-1,1-dimethylurea DCMU (Thomas *et al.*, 2001). Another example is processes initiated for cell acclimation to high external concentrations of sodium chloride (NaCl) (Hagemann, 2011).

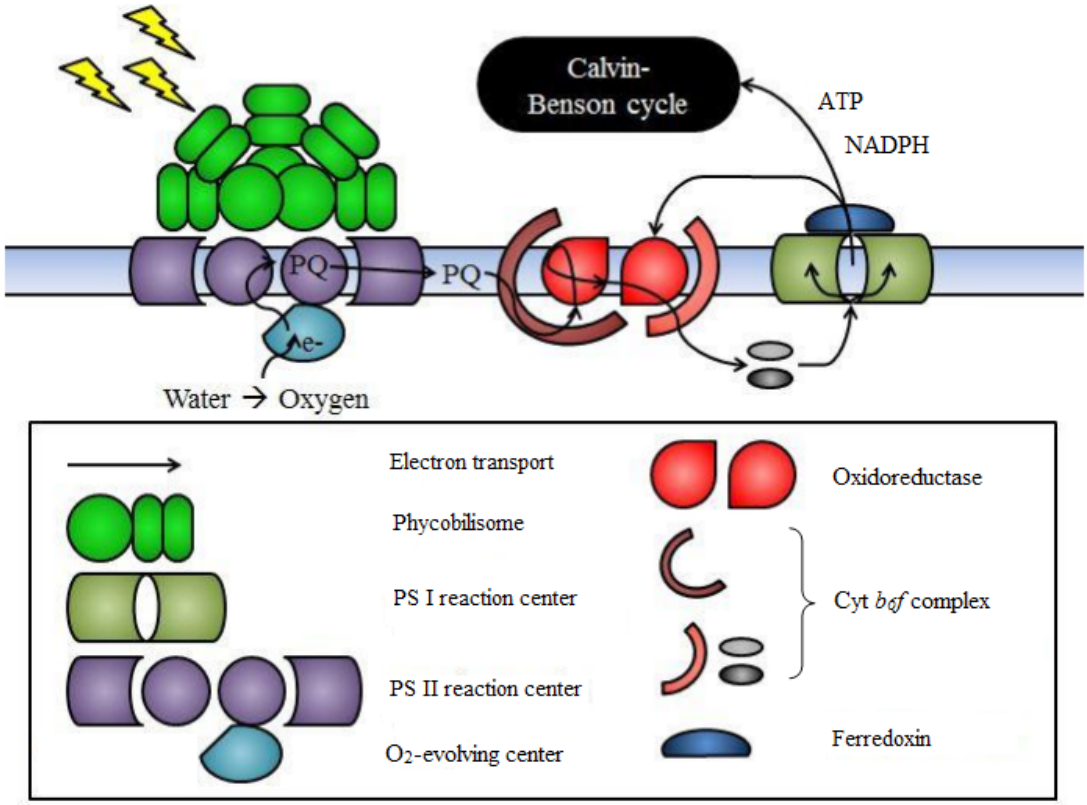

Figure 1.1: *A simplified illustration showing the components of the photosynthetic machinery in* Synechocystis *6803. Light (depicted as thunderbolts) is absorbed primarily by phycobilisomes, whereupon energy is transferred to chlorophyll. Electrons (e^-) in a special pair of chlorophylls extracted and passed along a chain of e^- acceptors including plastoquinones (PQ) and the cytochrome (Cyt)* b_6f *complex. Ultimately, ATP and NADPH are produced and utilized in the Calvin-Benson cycle for production of carbohydrates. (Original figure by Jacob J. Lamb, modified with his permission).*

1.2.3: Aerobic respiration

As described in sections 1.2.1 and 1.2.2, carbohydrates as well as O_2 are produced during photosynthesis in plants, algae and cyanobacteria. Most eukaryotes and also prokaryotes may

liberate this stored energy by oxygen-dependent respiration, oxidizing carbohydrates to produce ATP (Hall and Rao, 1999). Alberts and colleagues have given a simplified summary of the process (Alberts *et al*., 2007). Hydrolysis of ATP is an energetically favorable reaction, thus ideal to drive energy-requiring processes in the cell. In eukaryotic organisms, the process takes place in the mitochondria. In prokaryotes, a similar set of reactions occur in cytosol and the plasma membrane. The general reaction, which is completed through multiple steps, is often written as the following equation:

$C_6H_{12}O_6 + 6O_2 + 6H_2O + 38\ ADP + 38\ P_i \rightarrow 6CO_2 + 12\ H_2O + 38\ ATP$

Carbohydrates, generally written as $C_6H_{12}O_6$, are oxidized to form CO_2, coupled to the reduction of O_2 to form H_2O. As the carbohydrates are oxidized in a step-wise fashion, e^- are transferred from carriers such as nicotinamide adenine dinucleotide (NADH), via several e^- transport protein complexes in an ETC, until reaching O_2 as the ultimate e^- acceptor. When an e^- reaches O_2, the negative charge is neutralized by H^+, resulting in the production of H_2O. As for photosynthesis, energy is released in each step as e^- are transferred to increasingly stronger electron-acceptors along the ETC. This energy is utilized to pump H^+ against their gradient across the inner mitochondrial (eukaryotes) or cytoplasmic membrane (bacteria), creating an electrochemical gradient in the opposite direction that may drive ATP synthesis from adenosine 5' diphosphate (ADP) and inorganic phosphate (P_i).

1.2.4: Anaerobic respiration

Bacteria may not always have O_2 available as an e^- acceptor in respiration, yet they produce ATP by this e^- transfer within respiration. Under anoxic conditions, alternative acceptors such as nitrate or sulfate may be utilized as final e- acceptors of the ETC (Alberts *et al*., 2007). Vargas and colleagues have recently demonstrated another alternative in bacteria possessing a specific type of pili termed type IV pili (Vargas *et al*., 2013): by attachment to ferric oxides in their environment, bacteria such as *Geobacter* may use these compounds as e^- acceptors by donating e^- through the pili. Both Vargas' group as well as Gorby and colleagues have demonstrated that type IV pili may function as nanowires for e^- transport from internal ETC in both photosynthesis and respiration, donating e^- to extracellular iron complexes (Gorby *et al*., 2006; Vargas *et al*., 2013). Once reduced, iron in the form of Fe^{2+} is readily available for uptake by bacterial cells (Singh *et al*., 2003). Transport of e^- through pili may contribute to solving two important problems: (I) Disposal of excess e^- and (II) iron acquisition. A more thorough description of type IV pili, their function as nanowires and the challenges of iron acquisition in *Synechocystis* 6803 is found in sections 1.3 and 1.4.

1.3: Bacterial pili and nanowires

1.3.1: Type IV pili

Pili are appendages found on the surface of many bacterial cells. These extensions aid the bacteria in a variety of functions, such as adhesion and biofilm formation. Different types of pili exist, of which the most common are type IV pili. These pili display some functions that distinguish them from other pilus types, such as mediation of bacterial motility and DNA uptake (Proft and Baker, 2009). For instance, type IV pili enable *Synechocystis* 6803 to employ phototactic movement. They also contribute to the natural competence of *Synechocystis* 6803 cells (Bhaya *et al*., 2000).

Bhaya and colleagues have described the general structure of type IV pili (Bhaya *et al*., 2000): Thin and flexible filaments consisting of hundreds to thousands of protein subunits called pilins. A certain pilus type is usually composed of a single type of pilin, and the subunits are bound together in homopolymers. Multiple pili may aggregate to form bundles at the cell poles. Each pilus is about 6-8 nanometers (nm) in diameter, and lengths varying from a few micrometers (μm) up to 2.5 millimeters (mm). *Synechocystis* 6803 has pili of two morphologically distinct types, referred to as thick and thin pili. The former are demonstrated to be structurally analogous to type IV pili. Thick pili are found to be involved in transformation and motility. To date, thin pili have unknown function.

1.3.2: Nanowires and extracellular electron donation

When O_2 or other soluble e^- acceptors are scarce in the environment, bacteria may donate e^- from the ETC to extracellular electron acceptors, such as insoluble metal oxides (Gorby *et al*., 2006). Away to utilize e^-, described more closely in section 1.4, is for reduction of iron from insoluble ferric form (Fe^{3+}) into soluble and available ferrous iron (Fe^{2+}), an important mechanism for bacterial iron acquisition (Crossley *et al*., 2007). An example involving both of these demands is reduction of iron in order to solubilize it for uptake (Andrews *et al*., 2003), or as an alternative e^- acceptor (Gorby *et al*., 2006). In nature, iron is predominantly found in ferric oxyhydroxides (Ferreira and Straus, 1996). Iron from such complexes may be accessed by transferring e^- so as to reduce the metal. Experiments by Gorby and colleagues have shown that pili may act as bacterial nanowires, through which e^- can be transferred and donated to ferric oxides in the extra cellular environment (Gorby *et al*., 2006). According to experiments conducted by Vargas and colleagues, pili also appear to be involved in attachment to ferric oxides, a prerequisite for reduction. Their studies have demonstrated that pili must be conductive in order to function in extracellular long-range e^- transport. Additionally, they have identified a specific cytochrome associated with pili as

an essential component in the reduction of Fe^{3+} (Vargas *et al*., 2013). The structure of nanowires and a potential interface with the respiratory and photosynthetic electron transport in *Synechocystis* 6803 is depicted in figure 1.2.

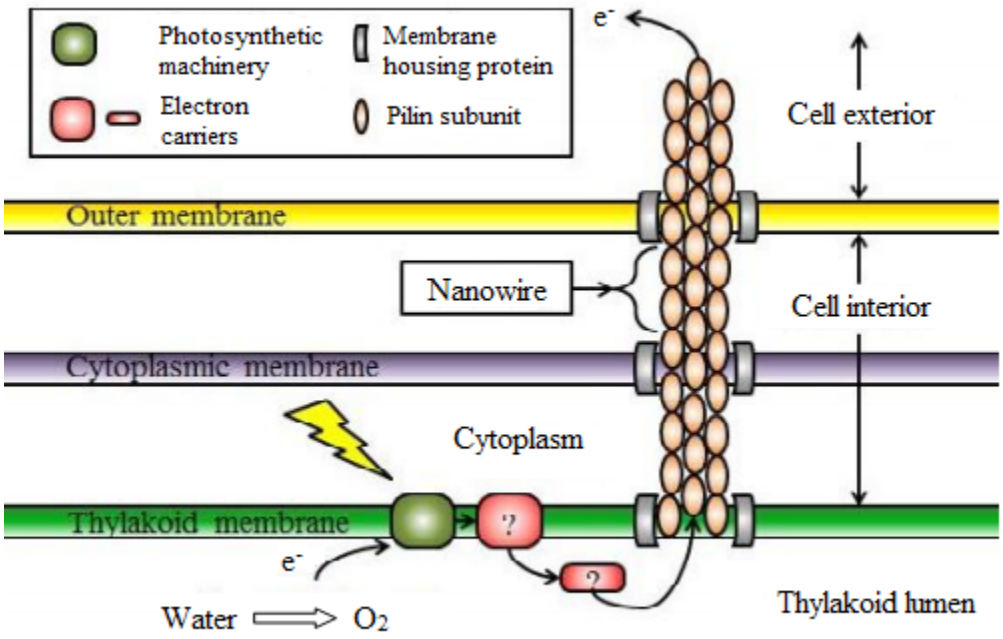

Figure 1.2: *A simplified illustration showing how pili may function as nanowires in the cyanobacterium* Synechocystis *6803. Presumably, electrons (e^-) from the photosynthetic process may be donated to e^- acceptors in the environment through pili. Membrane-associated proteins may connect the pilus subunits (pilins) to the different membranes of the bacterial cells, and e^- carriers that are yet unidentified may couple pili to the photosynthetic machinery. (Original figure by Jacob J. Lamb, modified with his permission).*

Gorby and colleagues have demonstrated that conductive pili in *Synechocystis* 6803 are produced in response to e^- acceptor limitations, and also that their production is facilitated by limited access to CO_2. One hypothesis is that *Synechocystis* 6803 uses these nanowires to discard excess e^- that would otherwise be used for fixing CO_2 under non-limiting conditions (Gorby *et al*., 2006). As pili possibly contribute to iron reduction, they may help bacteria access iron. This project aims to investigate the link between iron acquisition and specific pilins by deleting their respective genes.

1.3.3: Pilin-encoding genes

The natural transformability and the sequenced genome of *Synechocystis* 6803 make it an ideal choice for research in molecular genetics (Vermaas, 1996). Experiments have been conducted on the various pili features and functions such as motility, some based on genetic deletion of one or

more pilin-encoding genes. A set of such genes have been identified in *Synechocystis* 6803 that are potential homologs to pilin-encoding genes in bacteria with type IV pili (Bhaya *et al*., 2000). A specific operon containing the four putative pilin-encoding genes *slr1928, 1929, 1930* and *1931 (slr1928-31)* (Yoshihara et al., 2001) is the focus of this study.

1.4: Nutrient acquisition and stress

1.4.1: Iron acquisition in bacteria

In order to sustain growth and development, living organisms require adequate amounts of certain elements, such as iron (Singh *et al*., 2003). This metal is essential to most life forms (Andrews *et al*., 2003) and is involved in many vital processes such as metabolism, e^- transport, and enzymatic activity (Ferreira and Straus, 1996; Messenger and Barclay, 1983). All phototrophic organisms need iron for maintenance of their photosynthetic machinery. *Synechocystis* 6803 and other cyanobacteria demand exceptionally high quantities of this metal compared to prokaryotes in general, as well as other phototrophs (Kranzler *et al*., 2013). Iron is found in ample amounts in nature, being the fourth most abundant element in the Earth's crust (Andrews *et al*., 2003) but the forms of iron directly accessible to bacteria are scarce (Ferreira and Straus, 1996). The major factor limiting the bioavailability of iron is the oxygen-rich atmosphere on Earth, rapidly oxidizing iron from the ferrous form Fe^{2+} into the ferric form Fe^{3+} (Neilands, 1995; Singh *et al*., 2003). At physiological pH, the former state is soluble and thus readily available to bacteria, whereas the oxidized state precipitates to form insoluble oxyhydroxide complexes (Kranzler *et al*., 2013; Singh *et al*., 2003). As a consequence, the concentration of Fe^{3+} in aqueous environments is below a billionth of the concentration needed by most bacteria for optimal growth. Another issue is the potential toxicity of the free radicals produced when iron interacts with reactive oxygen species. Bacteria have developed different strategies to overcome these challenges, enabling them to acquire iron from the environment as well as protecting the cell from damage by free radicals (Andrews *et al*., 2003).

1.4.2: Mechanisms of iron acquisition in cyanobacteria

Cyanobacteria utilize one or both of two different mechanisms to scavenge iron from their surroundings (Kranzler *et al*., 2013). Both of these approaches aim to achieve bioavailable iron by improving its solubility (Andrews *et al*., 2003), either by secretion of iron-chelating agents or by reduction of Fe^{3+} (Kranzler *et al*., 2013). Employing the first strategy, bacteria cope with iron-restrictive conditions by synthesizing and secreting siderophores. These are chelating compounds binding to Fe^{3+} with high affinity, competing with the hydroxyl ion to make complexes (Andrews *et*

al., 2003; Neilands, 1995). Bacteria have evolved a system of receptors and transport proteins, through which complexes of iron and siderophores are internalized (Ferreira and Straus, 1996). The other prime strategy is based on reduction of Fe^{3+} by plasma membrane (PM) bound enzyme complexes (Katoh *et al*., 2001). *Synechocystis* 6803 has no known siderophore biosynthesis, nor any known transport of exogenous siderophores; however, uptake of iron from exogenous siderophore complexes as well as acquisition of iron from both organic and inorganic sources has been demonstrated by Kranzler and colleagues (Kranzler *et al*., 2011). They found that both uptake mechanisms involve Fe^{3+} reduction. As described in section 1.3, *Synechocystis* 6803 may donate e^- through their pili, as first demonstrated by Gorby and colleagues for *Synechocystis* as well as for other bacterial species such as *Shewanella oneidensis* MR-1 (Gorby *et al*., 2006). Iron oxides may thus function both as a source of Fe^{2+} as well as a terminal e^- acceptor for the photosynthetic or respiratory ETCs.

1.4.3: Iron in photosynthesis

About 2.2-2.3 billion years ago the atmosphere was anaerobic and accordingly, the reduced form of iron was much more prevalent than today. The ferrous iron-dependent photosynthesis that is performed by the present time's iron-oxidizing phototrophic organisms is likely to have evolved during this era, as iron was a readily available metal at the time. Iron is also important in other ways, such as for incorporation into proteins as prosthetic groups (Andrews *et al*., 2003). Paradoxically, the O_2 produced in photosynthesis led to the development of an aerobic environment, destabilizing Fe^{2+} and favoring oxidation into Fe^{3+} (Kranzler *et al*., 2013).. This fact may pose a problem for today's cyanobacteria, as they normally require 22-23 iron atoms for functional photosynthetic machinery. Iron plays a significant part in the photosynthetic ETC as well as the membrane-bound complexes of the light reaction (Ferreira and Straus, 1996). Iron-dependent processes were unproblematic prior to oxygenation of the atmosphere, but the utility diminished as aerobic conditions prevailed (Andrews *et al*., 2003). As indicated in the previous sections, life still depends heavily on iron and bacteria have developed adaptions to maintain iron homeostasis.

1.4.4: Iron stress

A variety of effects have been observed in response to iron deficiency in cyanobacteria, especially in their photosynthetic apparatus. Overall photosynthetic activity is decreased (Liu *et al*., 2006). Different effects of limited iron availability have been described, for instance by Ferreira and Straus (1996): cyanobacteria show decreases in pigmentation and in the iron-containing complexes of the ETC when grown in iron-deficient media. Another response in iron-stressed cells is the replacement of iron-dependent protein in favor of proteins without this requirement. A notable example is the

replacement of the mobile electron carrier ferredoxin, which contains iron-sulfur groups, with flavodoxin. Cyanobacteria may also alter their light harvesting complexes, for instance by synthesizing an alternative chlorophyll-binding protein in that surrounds PS I (IsiA). Flavodoxin and IsiA are encoded by two adjacent genes, *isiB* and *isiA*, respectively (Falk *et al*., 1995). IsiA forms a ring of 18 IsiA copies around the PS I reaction center and binds up to 50 % of Chl *a* under iron stress (Liu *et al*., 2006). Expression of IsiA is also induced during other stressful conditions, such as salt stress. This protein may thus be regarded as a general indicator of stress in cyanobacteria (Ivanov *et al*., 2006). The number of thylakoids is also reduced under iron-limited conditions (Liu *et al*., 2006). The mechanisms initiated to counter iron deficiency are reversible, and cells can return back to normal mode of action within 24 hours (Singh *et al*., 2003).

1.4.5: Stress induced by NaCl

Cyanobacteria have adapted to life in a wide variety of environments, including highly saline conditions. Despite some species being sensitive to salt, others show a moderate or even high degree of halotolerance (Pandhal *et al*., 2008). *Synechocystis* 6803 is an example of a moderately halotolerant cyanobacterium and may grow in NaCl concentrations up to 1.2 M (Hagemann, 2011; Huang *et al*., 2006). Nevertheless, excessive amounts of salts such as NaCl create challenging conditions for growth. In order to survive in saline environments, cyanobacteria have developed special acclimatization processes to cope with salt stress (Hagemann, 2011).

Hagemann has described salt acclimation in cyanobacteria in some detail (2011): The main process by which living cells obtain water is osmosis. High concentrations of salts such as NaCl disable this mechanism and the cells may even lose water to the environment. Decreased internal water content also reduces the cell's turgor pressure, which is needed for growth. Another problem is the increase of inorganic ions inside the cell, which may have toxic effect by inhibiting metabolic activities. All cyanobacteria may counter these effects by commencing a specific binate coping strategy. Part one is the synthesis of compatible solutes, small organic compounds with high water solubility. They function to equalize the internal and external osmotic potential, thus enabling the cell to take up water. The compatible solutes are so named because they will not influence the cell's metabolism in a negative way, even at high concentrations. Most bacteria acquire these solutes from their environment, whereas cyanobacteria usually employ *de novo* synthesis. However, *Synechocystis* 6803 will take up external solutes if such compounds are present in their environment. Part two of the acclimation system has evolved to cope with the salt entering the cell when the concentration is higher externally than internally. The countering-mechanism put into action is active excretion of toxic inorganic ions, mainly sodium (Na^+) and chloride (Cl^-). Together, these two strategies help the

cells into a salt-acclimatized state where concentrations of inorganic ions are low and concentrations of compatible solutes are high. Usually, a reduction is observed in growth rate and photosynthetic activity in salt-acclimatized cells compared to normal cells. Photosynthesis provides the energy required to achieve acclimatization; however, some notable changes take place in the photosynthetic machinery during the adaption process. As import of compatible solutes and export of ions requires energy, the cells need to produce more ATP to meet this demand. Consequently, cyclic e^- transport around PS I is activated for an increased ATP synthesis. In this process, PS II is not needed to generate e^-. The e^- generated by PSII may instead be donated to the terminal acceptor in the respiratory ETC, as both systems are located in the thylakoid membrane. Thus, stability of PS II is ensured along with a larger ATP supply by PS I cyclic transport activity.

1.4.6: Spectroscopic methods and stress indicators

Limiting iron in the growth medium may impose stress on *Synechocystis* 6803 cells, resulting in changes that can be detected by spectroscopic methods such as whole cell room temperature absorption spectroscopy (hereafter whole cell absorption) and 77 K fluorescence spectroscopy (hereafter 77 K).

Whole cell absorption is a method for measuring light absorption by different photosynthetic pigments: Chlorophylls, carotenoids and phycobilins. Chlorophylls (mainly Chl *a* in *Synechocystis* 6803) have two absorption maxima: One around 435 nm and one around 680 nm. Different carotenoids present in cells of *Synechocystis* 6803 result in an absorption maximum around 480 nm, and phycobilins result in an absorption maximum around 625 nm. The amplitudes at these wavelengths indicate the relative amounts of each pigment type present in the cultures (Lamb *et al*., 2014).

The level of stress in *Synechocystis* 6803 cultures can be assessed by calculating the absorption ratios between different pigments: The relative amount of carotenoids to chlorophylls (predominantly Chl *a*) is determined by the 435 nm/680 nm absorption ratio, whereas the relative amount of phycobilins to Chl *a* is determined by 625 nm/680 nm absorption ratio (Lamb *et al*., 2014). The size of the amplitudes are determined by the level of stress experienced by the cells, and their adaption by utilizing one type of pigment more extensively than the others. In iron-stressed *Synechocystis* 6803 cells, the amount of Chl *a* and phycobilins will decrease, and the amount of carotenoids increase, as compared to normal conditions (Andrizhiyevskaya *et al*., 2002; Ivanov, *et al*., 2000; Ivanov *et al*., 2007). Another indication of stress is a blue shift of 5-6 nm for the red Chl *a* absorption peaks (Ivanov *et al*., 2000; Liu *et al*., 2006).

77 K is a method for measuring fluorescence emission from PS I and PS II, in addition to proteins and complexes only present under stress conditions. The fluorescence maxima for CP 43 and CP 47, the core antenna proteins of PS II, are at 685 nm and 695 nm, respectively. The fluorescence maximum for PS I is 725 nm. The relative fluorescence from each photosystem can be determined by calculating the (685+695)/725 nm fluorescence ratio (Lamb *et al*., 2014).

Under non-stress conditions, the fluorescence from PS II will be low relative to that from PS I (Ivanov *et al*., 2006), yielding a low (685+695)/725 nm ratio. The fluorescence pattern will be altered under stress conditions such as an iron-limited or high-NaCl environment, mainly due to stress-induced synthesis of a chlorophyll-binding protein, IsiA (Ivanov *et al*., 2006). This protein is similar to CP43 (core antenna protein of PS II), and will give a high fluorescence peak in the same area as PS II, around 685 nm (Andrizhiyevskaya *et al*., 2002). To distinguish the fluorescence contribution by IsiA from that of PS II, the 695 nm/685 nm fluorescence ratio was calculated. A low value indicates more IsiA and hence stress in the bacteria (Lamb *et al*., 2014). A blue shift in the fluorescence peak for PS I may also be observed for iron-deficient cells, partly due to the complex of IsiA and PS I, and partly due to other structural alterations in PS I in order to acclimatize to the iron-limited conditions (Liu *et al*., 2006). The lower or higher fluorescence from PS II is associated with so-called state 1-state 2 transitions, which take place in order to adjust light energy distribution between the photosystems according to changes in the environment (Mullineaux & Emlyn-Jones, 2004). Non-stressed cells and cells driving cyclic e^- transport are normally in state 2, where fluorescence from PS II is low, whereas iron stress is associated with state I, where fluorescence from PS II is increased relative to fluorescence from PS I ((Ivanov *et al*., 2006).

Sudhir and colleagues have previously used spectroscopic techniques to identify effects of salt stress on the photosynthetic machinery in cyanobacteria (Sudhir, *et al*., 2005). They found similar effects to those seen under iron deficiency, such as blue shift in the red peak for Chl *a*. Whole-cell absorption and 77 K thus provide methods to visualize stress signs in cells of *Synechocystis* 6803 both under iron deficiency and an excess of NaCl.

1.5: Project aims

The aim of this project was to investigate the involvement of a specific operon from *Synechocystis* 6803 in iron acquisition. The operon selected consists of the four genes, *slr1928, slr1929, slr1930* and slr*1931 (slr1928-31),* hypothesized to encode pilin-like proteins (Yoshihara *et al*., 2001). The motivation for investigating *slr1928-31* was based on the work of Singh and colleagues, demonstrating an increased transcription of genes similar to this operon upon chelation of iron in

the medium (Singh *et al*., 2003). As iron is trapped in complexes and becomes less accessible, bacteria have to increase their efforts in iron acquisition. If pilins were involved in the process, a consequence of iron chelation would be an up-regulation of pilin-encoding genes, providing more pilins for participation in iron uptake. Pili have been shown to possess 'nanowire' functions, whereby the organism may donate electrons to extracellular acceptors (Gorby *et al*., 2006). These observations have led to speculations on a potential role of pili in iron reduction prior to iron uptake. This function has so far not been investigated in cyanobacteria.

In order to determine the potential functions of *slr1928-31*, a *Synechocystis* 6803 mutant strain lacking the operon will be produced. A knock-out vector will be constructed and the operon will be deleted by homologous recombination with this vector. An antibiotic resistance cassette from the vector will replace the operon in the genome of *Synechocystis* 6803, as means of selection. Assessment of differences between cells where the operon is present (WT) and absent (mutant strain Δ *slr1928-31*-Kan) will be performed by testing growth of both strains under various conditions. Different iron sources, nutrient supplements or treatments with stress-inducing chemicals will be tested for a comprehensive evaluation. The effects of the mutant will also be examined by two spectroscopic methods, (I) 77 K fluorescence spectroscopy and (II) whole-cell room temperature absorption spectroscopy, to visualize changes in photosystem activity and pigment composition, respectively.

The background for this study is a central challenge of our time: Developing new technologies to meet an increasing demand for energy as well as protecting the environment. It may be possible that pili in *Synechocystis* 6803 can be utilized for the transfer of e^- to electrodes, thereby creating a photovoltaic cell. The possibilities of utilizing microbiology and molecular biology for generation of renewable and sustainable energy are growing, for example in solar energy based on microbial fuel cells (Madiraju *et al*., 2012). Increasing the knowledge about *Synechocystis* 6803 nanowires is important in this respect, considering its potentials in the future.

2.: Materials and methods

2.1: General techniques

The laboratory work of this project was carried out using standard sterile techniques of microbiology. All work with bacterial cells was done in a ventilation hood. Media and other solutions were made with distilled deionized water and autoclaved for 20 minutes at 120°C. Liquid media were stored at room temperature. Agar plates were stored at 4 °C. DNA samples, as well as buffers and enzymes, were kept at -20°C for storage. Enzymes were kept in glycerol to remain liquid. All antibiotics used are dissolved in water and stored at 4°C, except Chloramphenicol and also DCMU, which are dissolved in ethanol and methanol, respectively, and stored in freezer at -20°C. Centrifugations of small volumes were done by micro-centrifuge at 13,000 rpm and large volumes in a table-top centrifuge at 2000 g.

2.2: *Synechocystis* 6803 growth and culturing

Wild type (hereafter WT) cells of *Synechocystis* 6803 were grown on BG-11 agar plates and re-plated regularly for maintenance. After the construction of a knock-out strain, mutant cells were grown on BG-11 agar plates containing the appropriate antibiotic and maintained by frequent re-plating. The mutant strain was also stored in BG-11 medium containing 15 % glycerol at -80°C. Liquid cultures of *Synechocystis* 6803 were set up in sterile Erlenmeyer flasks. The cultures were prepared in a ventilation hood while using a Bunsen burner for optimal sterile conditions. Cells were transferred to a volume of 250 mL of BG-11 and mixed until complete dissolution was obtained. Both plates and cultures were kept at 30 °C in continuous illumination at 30 $\mu E.m^{-2}.s^{-1}$. Liquid cultures were also grown with continuous oxygen supply provided by aquarium pumps. To avoid dehydration of the cells the air was bubbled through distilled, deionized water (ddH2O).

2.3: Media and agar plates

2.3.1: LB medium for growth of Escherichia coli

For experiments involving *Escherichia coli* (*E. coli*) cultivation, LB medium was used. The medium components and concentrations used for LB medium are given in table 2.3.1.1. The supplements, treatments or antibiotics used when appropriate, and their respective concentrations, are given in tables 2.3.1.2 and 2.3.1.3. The medium was prepared by adding all components to water during constant magnet stirring. Water was added first to avoid complex formation of medium ingredients. After addition of all components, water was added up to desired volume. Whenever appropriate, additional chemicals were added after autoclaving. For agar plates, 25 µL of medium was used.

Table 2.3.1.1*: Standard recipe for E. coli growth medium, LB (100 mL)*

Component	**Amount**
Tryptone	1 g
NaCl	1 g
Yeast extract	0.5 g
H_2O	100 mL
Total volume of medium	100 mL

Table 2.3.1.2*: Additional supplements to LB medium, solid only*

Component	**Mass per 100 mL**
Agar	2 g

Table 2.3.1.3*: Additional supplements to LB medium, liquid or solid*

Antibiotic	**Stock (mg/ML)**	**Final (µg/ML)**	**Dilution factor**	**Solvent**	**Storage (°C)**
Ampicillin (Amp)	50	50	1000	ddH_2O	4
Kanamycin (Kan)	50	50	1000	ddH_2O	4

2.3.2: BG-11 medium for growth of Synechocystis *6803*

For experiments involving *Synechocystis* 6803 cultivation, BG-11 medium was used. The composition of stock solutions used for BG-11 medium is given in table 2.3.2.1. The medium components and concentrations used for BG-11 medium are given in table 2.3.2.2. The supplements, treatments or antibiotics used when appropriate, and their respective concentrations, are given in tables 2.3.2.3 and 2.3.2.4. Alternative iron sources used for growth experiments in this project is given in table 2.3.2.5. All media were prepared by adding all components to water during constant magnet stirring. Water was added first to avoid complex formation of medium ingredients. After addition of all components, water was added up to desired volume. Whenever appropriate, additional chemicals were added after autoclaving. For agar plates, 25 µL of medium was used. For liquid cultures, 250 mL of medium was used.

***Table 2.3.2.1**: Composition of stock solutions for BG11 medium*

Stock solution	**Component**	**Concentration**
100x BG-11	$NaNO_3$	149.60 g/L
	$MgSO_4 \cdot 7H_2O$	7.49 g/L
	$CaCl_2 \cdot 2H_2O$	3.60 g/L
	Citric acid	0.60 g/L
	NaEDTA, 0.25 M, pH 8.0	1.12 mL/L
	Trace minerals	100.00 mL/L
	ddH_2O	Up to 1 L
Trace minerals	H_3BO_3	2.860 g/L
	$MnCl_2 \cdot 4H_2O$	1.810 g/L
	$ZnSO_4 \cdot 7H_2O$	0.222 g/L
	$Na_2MoO_4 \cdot 2H_2O$	0.390 g/L
	$CuSO_4 \cdot 5H_2O$	0.079 g/L
	$Co(NO_3)_2 \cdot 6H_2O$	0.0494 g/L
	ddH_2O	Up to 1 L
1000x	Ferric ammonium citrate	0.60 g/100 mL
1000x	Na_2CO_3	2.00 g/100 mL
1000x	K_2HPO_4	3.05 g/100 mL
100x	TES-NaOH, 1 M, pH 8.2	10 mL/L

***Table 2.3.2.2**: Standard recipe for Synechocystis 6803 growth medium, BG-11 (100 mL)*

Component	**Volume**
100x BG-11	1 mL
Ferric ammonium citrate	100 µL
Na_2CO_3	100 µL
K_2HPO_4	100 µL
TES NaOH buffer ph 8.2	1 mL
H_2O	Up to 100 mL
Total volume of medium	100 mL

***Table 2.3.2.3**: Additional supplements to BG-11 medium, solid only*

Component	**Mass per 100 mL (g)**
Bacteriological agar	1.5
Sodium thiosulfate	0.3

***Table 2.3.2.4:** Additional supplements to BG-11 medium, liquid or solid*

Component	**Stock**	**Final**	**Dilution factor**	**Solvent**
Glucose	1 M	5 mM	200	ddH_2O
DCMU	20 mM	20 µM	2000	Methanol
Ampicillin (Amp)	50	25 mg/mL	2000	ddH_2O
Kanamycin (Kan)	50	25 mg/mL	2000	ddH_2O

Table 2.3.2.5*: Iron sources substituting ferric ammonium citrate in BG-11 media used for growth experiments*

Iron substitute	**Concentration (µM)**
Ferric chloride hexahydrate	23
Goethite	23
Iron (II, III) oxide	23
Potassium hexocyanoferrate (III)	23

Table 2.3.2.6: *Mass and concentration of NaCl added to BG-11 medium.*

Component	**Mass (g)**	**Concentration (mM)**
NaCl	4.14	350

2.4: Primers

2.4.1: Primer design

The polycistronic operon consisting of the four genes *slr1928, slr1929, slr1930* and *slr1931 (slr 1928-31*) was selected for deletion studies and further characterization of the mutant phenotype. A knock-out vector was constructed by ligation of a Litmus28i backbone from New England Biolabs (hereafter Lit), 500-800 bp left and right flanking regions of the operon to be deleted (hereafter LF and RF, respectively) and a cassette conferring resistance to Kanamycin (hereafter Kan^R). Lit contains the necessary elements for transformation and replication of the plasmid. These fragments were ligated together by Gibson assembly. Upon transformation, the endogenous gene was exchanged for the exogenous one in the bacterial genome.

In order to amplify the desired DNA fragments by PCR (section 2.5), primers were designed according to some basic principles. Adjacent regions to each fragment were searched manually to find DNA sequences of about 20-25 bp. In order to be ideal primers, the chosen sequences had a GC content above 50 %, as well as starting and ending with a G or C, giving strong binding due to 3 hydrogen bonds. In addition, the base sequence should be mixed, i.e. repeats should be avoided. Based on these initial observations, applicability of selected sequences was tested using IDT oligo-

analyzer. Output from this program gave information about melting temperature and probability for hairpin structure formation. The melting temperature for the sequence is correlated with GC content, and should ideally be 55-60 °C. This value should be about the same for each primer in a pair. The melting temperature for hairpin formation should preferably be below 30-35 °C. As primers were designed from single-stranded DNA and the complementary form was needed for the reverse primers, the primers were flipped and the base sequence changed into the complementary one, resulting in the reverse complement form. This was performed using the program Reverse Complement from bioinformatics.org. A map of the construct was obtained by using the program Vector Editor. The primers were made as to facilitate plasmid production during Gibson assembly. They were designed with 15 bp long complementary overhangs on each end in order to aid formation of a circular vector. This is done by adding the reverse complement of the first 15 bases of the primer for sequence 1 to the start of the primer for sequence 2, to which sequence 1 will be connected in the construct. The primers were also designed with a cut site at each end to enable easy exchange of the antibiotic cassette, such as Kanamycin for Chloramphenicol. The cut site can be added at the end of the complementary overhang sequence (included in the 15 bases) and will be the same for both of the adjacent primers. Primers were designed so as to not disrupt any genes that are not targets of the knock-out. The Lit backbone was amplified using M13 forward and M13 reverse primers with overhangs complementary to RF and LF, respectively.

2.4.2: Primer sequences

slr1928-31_LF_fw	tttcccagtcacgacGGTACGGAGGATCAGGACAAG
slr1928-31_LF_rv	caacgtggcggccgcGGTGAAACCCTGCTGAGCTG
slr1928-31_Kan_fw	tttcaccgcggccgcCACGTTGTGTCTCAAAATCTC
slr1928-31_Kan_rv	cactggtgcggccgcTACAACCAATTAACCAATTCTG
slr1928-31_RF_fw	ggttgtagcggccgcACCAGTGGAGGTTGTACAATTA
slr1928-31_RF_rv	aacagctatgaccatgCTTGAGTGTGGCTTCCATTTC
slr1928-31_m13_fw	gaagccacactcaagCATGGTCATAGCTGTTTCC
slr1928-31_m13_rv	ctgatcctccgtaccGTCGTGACTGGGAAAACC

2.5: Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) was used for amplification of DNA fragments to be used as components of the knock-out vector, as well as amplification of the final construct for verification.

PCR was also used to analyze segregation of genomes in mutant strains. The two variants are described in separate sections as cloning PCR and colony PCR, respectively.

2.5.1: Cloning PCR

The standard reaction volumes used was either 10 or 50 µL. PCR was always run in two or more parallels for each sample. WT *Synechocystis* 6803 cells were used as template for the flanking regions. Litmus backbone and Kanamycin resistance cassette were amplified from pre-existing constructs. Prior to PCR, the primers were diluted to make a concentration of 10 µM. The constituents of the master mix used for all reactions, exemplified for 100 µL, are given in table 2.5.1.1. The enzyme was always added last. The volume to be used was 9.5 µL master mix in 10 µL reactions and 44 µL master mix in 50 µL reactions.

Table 2.5.1.1*: The composition of standard master mix for a total volume of 100 µL*

Component	**Volume (µL)**
5x Phusion buffer	20
dNTP	5
H_2O	74
Phusion DNA polymerase	1
Total volume	100

Cloning PCR was performed as follows:

1. Initial denaturation of DNA, 98 °C for 10 min to disrupt WT cells used as template

2. 14 cycles of 98°C for 7 s, annealing at 62°C (-1°C per cycle) for 20 s, extension at 72°C for 30 s/kb

3. 16 cycles of 98°C for 7 s, annealing at 62°C for 20 s, extension at 72°C for 30 s/kb

4. Extension at 72°C for 5 min

The PCR products were analyzed by agarose gel electrophoresis, as described in section 2.6. The PCR samples were cleaned using a PCR purification kit, described in section 2.7.

2.5.2: Colony PCR

Colony PCR was performed using the same components as cloning PCR (section 2.5.1), except that the reactions were always performed in 10 µL total volumes, and templates were prepared by mixing one single colony of *Synechocystis* 6803 with 10 µL of water.

Colony PCR was performed as follows:

1. Initial denaturation of DNA, 95 °C for 10 min to disrupt WT cells used as template

2. 14 cycles of 94°C for 30 s, annealing at 62°C (-1°C per cycle) for 1 min, extension at 72°C for 1 min/kb

3. 16 cycles of 94°C for 30 s, annealing at 62°C for 1 min, extension at 72°C for 1 min/kb

4. Extension at 72°C for 5 min

The PCR products were analyzed by agarose gel electrophoresis, as described in section 2.6. The PCR samples were cleaned using a PCR purification kit, described in section 2.7.

2.6: Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate PCR products and other DNA fragments according to size, in order to verify the presence of a desired fragment. Prior to loading, samples were mixed with 10× loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% glycerol). Standard volumes used were 1 µL sample, 1 µL dye and 3 µL water for cloning PCR and the whole sample plus 1 µL dye for colony PCR and enzymatic digests (section 2.8). The gel was made from a TAE buffer solution containing 0.8 % agarose and 30 µL GelGreen. The procedure was performed at 75 V and 400 A for 1 hour at room temperature. Exposure to UV light generated fluorescence from GelGreen bound to DNA, thereby visualizing the obtained DNA fragments. GelDoc was utilized to image each gel after visualization.

2.7: PCR clean-up

This procedure was performed using a QIAQuick PCR purification kit (250). If all parallel samples contained the correct PCR product, as verified by gel, the parallels for the same product were cleaned as one. If the samples produced an odd number, an extra tube without DNA was used as counter-balance during centrifuge steps. 5 volumes of binding buffer PB was added to each samples and mixed by vortex. The same volume as the sample and PB combined was added to the blank. The mix was added to a spin column and centrifuged for 1 minute. The flow-through was discarded

and 750 µL buffer PE was added to wash. The samples were centrifuged for another minute and flow-through discarded. The samples were centrifuged 1 minute and flow-thorugh again discarded. 30 µL of buffer EB or sterile H_2O was added to elute DNA. The samples were left 1-5 minutes before centrifuge in 1 minute. The amount of DNA in the resulting solution was measured by NanoDrop spectrophotometer, giving the concentration as ng DNA per µL.

2.8: Enzymatic digest

For amplification of Lit and Kan^R, plasmids including the desired sequences were used as templates for PCR. These plasmid templates had to be eliminated before further use. The restriction enzyme DpnI was ideal to use in this respect as it only cuts methylated DNA. Plasmid templates included methyl groups, whereas PCR products did not. Thus, only unwanted templates were degraded. The set-up was as follows: 1 µL DpnI and 3 µL Cut Smart buffer was mixed well with the PCR samples on ice, and left for 1-3 hours at 37 °C. The samples were then cleaned by PCR clean-up kit to remove buffer, primers and other reaction components (section 2.7) and verified on gel (section 2.6). Prior simulation of restriction pattern shows expected bands for gel electrophoresis. Digested and clean samples were then ready to use for plasmid assembly and transformation.

2.9: Gibson assembly

This procedure was performed according to a Gibson Assembly® protocol from New England Biolabs (NEB), utilizing a NEB Gibson Assembly® master mix. DNA fragments obtained in large quantities by PCR can be ligated together to form a plasmid functioning as a knock-out vector. The amount to be used for each fragment was 0.2 picomoles, except for Lit, where 100 ng was used. The number of picomoles in the DNA samples and the volumes to be used for the reaction was calculated as shown in section 2.x. The lengths of all DNA fragments are given in table 2.9.1. The reaction was set up on ice. The fragments were added in the order they will be found in the plasmid, starting with the backbone. Water was added up to a total volume of 10 µL. The DNA fragments were then mixed with 10 µL Gibson assembly master mix. The reaction was run for 1 hour at 50 °C.

***Table 2.9.1**: Lengths of fragments used to produce Δ slr1928-31-Kan plasmid*

DNA fragment	Length in base pairs
Lit	2642
Left flank	844
Kan^R	974
Right flank	442

2.10: Transformation of *E. coli*

Transformation of *E. coli* was performed in order to obtain high concentrations of a desired plasmid. Frozen aliquots of *E. coli* DH5-alpha competent cells were thawed on ice for approximately 10 minutes. The whole Gibson assembly mix was added to the cells. All transformations were carried out using 3 controls: 2 negative for the PCR products obtained from plasmid templates, i.e. Lit and Kan^R, and one positive using the plasmid pUC19. For the former, the same volumes as used in Gibson were added to the cells. The negative controls for Lit and Kan^R were performed to verify that the plasmid templates were degraded during digest, and the pUC19 was performed to test if the competent cells were functional. After addition of DNA, the cells were left on ice for 30 minutes. Then a 2 minute heat-shocked was performed, using a 37 degreee water bath, and the cells were put back on ice for 3 minutes. The whole solution was then added to 2 mL LB medium, and grown for 90 minutes in a 37°C shaking incubator. Samples of the cultures were then plated out in two different concentrations – 50 µL directly as diluted sample and 50 µL after centrifugation of 1 mL and re-suspension in 200 µL as the concentrated sample. The concentrated samples were spun down in Eppendorf tubes in a micro-centrifuge for 1 minute, and the supernatant was discarded. Cells were spread out on plates contained 100 µL of the appropriate antibiotic per 100 mL medium. The plates were inverted and left overnight in a 37°C incubator. After overnight growth, the plates were put in the fridge to avoid overgrowth.

2.11: Liquid cultures of *E. coli*

In order to isolate plasmid from transformed *E. coli* colonies, liquid cultures from selected colonies were prepared and grown overnight. During the course of this work, this step was carried out by picking 4 colonies from each plate. The selected colonies were isolated, clear and close to the center of the plate, as these were most likely to contain the desired plasmid. Each selected colony was picked up by a pipette and transferred to LB medium, leaving the pipette tip in. The volumes of LB

medium used were 2 mL if standard 12-18 hours, or 4-5 mL if longer growth time. The appropriate antibiotic was added with a dilution factor of 1000. The liquid cultures were left overnight in a 37°C shaking incubator. A negative control was also performed, leaving a tube without bacteria overnight. For comparison, the cultures with bacteria should have an opaque, cloudy appearance whereas the standard LB control should still be transparent and clear.

2.12: Plasmid isolation and verification

2.12.1: Isolation of plasmid from E. coli

This procedure was performed using the centrifugation protocol and solutions from Wizard® Plus SV Minipreps DNA Purification System (Promega). Isolation of plasmid was performed separately for each overnight culture. One mL of culture was spun down, supernatant discarded and another mL was spun down before re-suspension of pellet in 250 µL Re-suspension solution. 250 µL Cell lysis solution was added and the tube inverted to mix. 10 µL alkaline protease solution was added, inversion to mix and incubation 5 minutes at room temperature. 350 µL neutralizing solution was added and the mix centrifuged for 10 minutes. The lysate was then decanted into a spin column and centrifuged 1 minute. Flow-through was discarded and 750 µL wash solution was added before centrifuging another minute. Flow-through was discarded, and wash step repeated with 250 µL wash solution. The mix was centrifuged 2 minutes without adding any new solution. 50 µL nuclease-free H_2O was added and the sample centrifuged for 1 minute to elute. The concentration was measured by NanoDrop and the result was verified by performing a digest of the samples (section 2.12.2) followed by gel electrophoresis for 1 hour 20 minutes.

2.12.2: Verification of plasmid

Enzymatic digest was used to verify that the correct plasmid was made during Gibson assembly (section 2.9). The reaction was performed after plasmid isolation by mixing 1 µL of plasmid sample, 1 µL buffer, 0.5 µL of the enzyme Hind III and H_2O up to 10 µL total volume. The reaction was run at 37 °C for 1 hour, and run on gel for verification. The expected banding pattern was simulated with Vector Editor prior to gel electrophoresis.

2.13: Sequencing

Sequencing was performed by GATC Biotech. Two samples for sequencing were prepared by calculating a volume containing 500 ng of plasmid DNA and adding H_2O up to 5 µL. One sample had 5 µL forward primer and the other the same volume of reverse primer. The resulting sequence was aligned with the expected sequence using the web-based alignment tool provided by NCBI.

2.14: Transformation of *Synechocystis* 6803

Synechocystis 6803 WT cultures were prepared as described in section 2.2. After growth for approximately 3 days, these starter cultures were used to set up overnight cultures. OD of *Synechocystis* 6803 cultures were measured at 730 nm. The ideal OD for transformation is between 0.35 and 0.6. 2x 50 mL of WT cultures were spun down for 8 minutes at 3000g and the cells were re-suspended in BG11 to make a final OD of 2.5. Plasmid DNA was added to 0.5 mL of culture with OD 2.5. Another tube with the same volume of cells but without DNA was used as control. The samples were incubated for 6 hours at 30 °C under constant illumination at 30 $\mu E.m^{-2}.s^{-1}$. The tubes were shaken gently halfway through the incubation period. 200 µL of each sample was plated out onto sterile nitrocellulose filters on BG11 agar plates with glucose. The surface of the plates were dried completely, sealed and incubated overnight at 30 °C and constant illumination at 30 $\mu E.m^{-2}.s^{-1}$. The filters were transferred to new BG-11 agar plates containing the appropriate antibiotic in addition to glucose. These plates were further incubated for approximately 2 weeks until colonies appeared. 4 colonies were picked and re-streaked regularly for complete segregation, verified by colony PCR (section 2.5.2).

2.15: Growth assay

OD of cultures was measured prior to experiments. 1 mL BG-11 was used as blank, and for culture OD diluted samples with 200 µL cells into 800 µL BG-11 was used for accurate measurement. 50 mL each of WT and mutant cultures were centrifuged in Falcon tubes for 8 minutes at 3000 g. Supernatant was discarded in hood to keep the cultures sterile. The pellet was re-suspended in 10 mL sterile H_2O and centrifuged again for 8 minutes. After this step, the pellet was re-suspended in H_2O to get an OD of 1. A dilution series was made by repeatedly adding 100 µL sample into 900 µL water. The new solutions had OD 0.1 and 0.01. 3x 5 µL of each OD was spotted onto the plates, both WT and mutant on the same plate for each condition. The surface of the plates were dried completely, sealed and incubated at 30 °C under constant illumination at 30 $\mu E.m^{-2}.s^{-1}$. For a 10-dayperiod, images were taken of each plate. The first 3 days included 2 daily photos, morning and afternoon, whereas for the remaining days a single photo was taken each day at approximately every 24^{th} hour. Raw files of the images were used to measure the density of each spot compared to each other as well as the agar background. Based on these values, growth curves were constructed.

Growth experiments on *Synechocystis* 6803 mutants were conducted using BG-11 with different iron sources and salt concentrations. The iron sources with their respective abbreviations used throughout this thesis are given in table 2.15.1.

Table 2.15.1: *The iron sources used in BG-11 medium, with their respective abbreviations.*

Compound	**Abbreviation**
Ferric ammonium citrate	Fac
Ferric chloride hexahydrate	FeCl
Goethite	Goe
Iron (II, III) oxide	FeO
Potassium hexacyanoferrate (III)	Hexacyan

The cells were grown under 6 different conditions: No supplements, glucose-supplement, glucose supplement and DCMU, NaCl, NaCl with glucose supplement and NaCl with glucose supplement and DCMU. Glucose was added for mixotrophic growth. DCMU was added to inhibit PS II and thus photosynthesis, stimulating cyclic electron transport. NaCl was added to induce stress and initiate salt acclimation processes, also stimulating cyclic electron transport.

2.16: 77 K fluorescence spectroscopy

Cultures of *Synechocystis* 6803 were grown as described in section 2.15. After a growth period of 10 days, cells were collected from the plates and added to BG-11 medium to yield an OD of 1 at 750 nm. Samples were transferred to capillary tubes and frozen in liquid nitrogen to reach a temperature of 77 K required for the spectroscopic measurements. Fluorescence emission from the samples was measured within the wavelength interval from 650 nm to 800 nm, using a custom-built fluorescence spectrometer. The spectra were normalized around the main peak for PS I fluorescence at 725 nm.

2.17: Whole cell room temperature absorption spectroscopy

Samples with an OD of 1 at 750 nm were prepared as described in section 2.16. Samples were transferred to glass cuvettes and kept at room temperature. Absorption for all samples was measured within the wavelength interval from 350 nm to 750 nm at a scan speed of 300 nm per minute, using a Hitachi U-3010 Spectrophotometer. The spectra were normalized around the main red peak for Chl *a*, at 680 nm.

2.18: Calculations

Calculation of picomoles of DNA in solution:

$$\#\text{ of picomoles} = \frac{\text{ng of DNA} * 1000}{\#\text{ of bp} * 650}$$

The amount used was 0.2 picomoles of each DNA fragment, except for vector backbone (100 ng). The amount of DNA in ng is obtained from NanoDrop measurements. The fragment length in bp is obtained from Vector Editor.

3. Results

The aim of this project was to investigate the involvement of a specific operon from *Synechocystis* 6803 in iron acquisition. The operon selected consists of the four genes, *slr1928, slr1929, slr1930* and slr*1931* (*slr1928-31*), all hypothesized to encode pilin-like proteins (Yoshihara *et al*., 2001). Other related pilin-like proteins appear to contribute to iron uptake (Gorby *et al*., 2006; Singh *et al*., 2003), leading to the hypothesis that genes encoded by the operon *slr1928-31* may possess similar functions.

In order to determine the potential functions of the operon *slr1928-31* gene products, a mutant strain lacking the operon was produced via genetic deletion. Assessment of differences between cultures where the operon was present (WT) and absent (mutant strain Δ *slr1928-31*-Kan) was performed by testing growth of both strains under various conditions. Different iron sources, nutrient supplements or treatments with stress-inducing chemicals were tested. The effects of the deletion were also examined by two spectroscopic methods, (I) 77 K fluorescence spectroscopy and (II) whole cell room temperature absorption spectroscopy.

3.1: Plasmid constructs for genetic deletion

3.1.1: Design of deletion vectors

A deletion vector was designed as described in section 2.4. A map of the plasmid designed for this experiment is shown in figure 3.1.1.1. The plasmid included a backbone (pLitmus 29i) containing the necessary genetic elements for plasmid replication as well as an Ampicillin resistance cassette (*Amp*^{R}). The other vector components were the left and right flanking regions of the operon to be deleted (LF and RF) and a kanamycin resistance cassette (*Kan*^{R}).

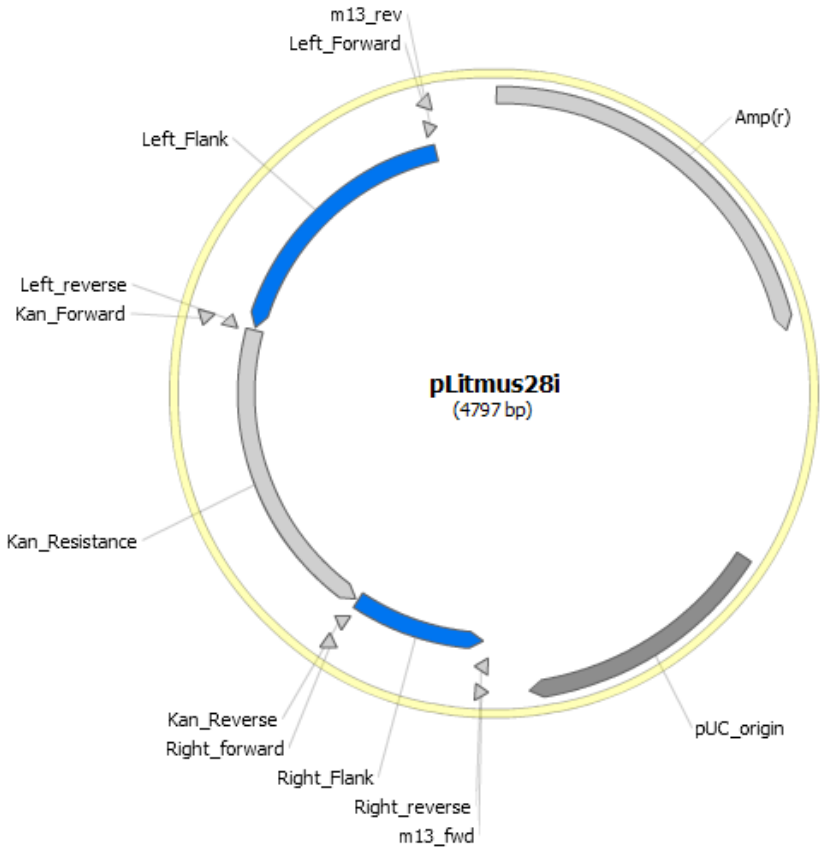

Figure 3.1.1.1: *Plasmid construct for deletion of the operon* slr1928-31 *in* Synechocystis *6803. The vector backbone comes from the plasmid pLitmus28i (NEB) with an Ampicillin resistance cassette (*Amp^R*) and an origin of replication (pUC origin). The vector was constructed as to allow replacement of the operon* slr1928-31 *with a kanamycin resistance cassette. The Kanamycin resistance cassette (*Kan^R*) was integrated into the genome of* Synechocystis *6803 by homologous recombination, replacing the operon* slr1928-31. *The antibiotic resistance cassette was introduced in order to easily isolate the mutant strain by growth on selective medium containing Kan. The primers used for amplification of each DNA fragment in the vector is also indicated: M13 forward (fwd) and reverse (rev) for Lit, Left fwd and rev for LF, Kan fwd and rev for* Kan^R *and Right fwd and rev for RF.*

3.1.2: Verification of vector constituents

Each of the DNA fragments forming the final deletion vector; Lit, LF, Kan^R and RF; were amplified in duplicates by PCR as described in section 2.5. The sizes of the different fragments were known from the plasmid map and are given in table 3.1.2.1.

Table 3.1.2.1: *Sizes in base pairs of each constituent in the deletion vector for the operon* slr1928-31: *Left and right flanking regions (LF and RF), antibiotic resistance cassette* (Kan^R) *and Litmus backbone (Lit).*

DNA fragment	**Size of fragment (bp)**
Left flank (LF)	844
Kan^R	974
Right flank (Rf)	442
Lit	2642

All DNA fragments to be included in the deletion vector for operon *slr1928-31* were verified by agarose gel electrophoresis, as described in section 2.6. Figures 3.1.2.1 and 3.1.2.2 show pictures of the gels verifying the DNA fragments used to construct the deletion vector, Kan^R in the former figure and Lit, LF and RF in the latter.

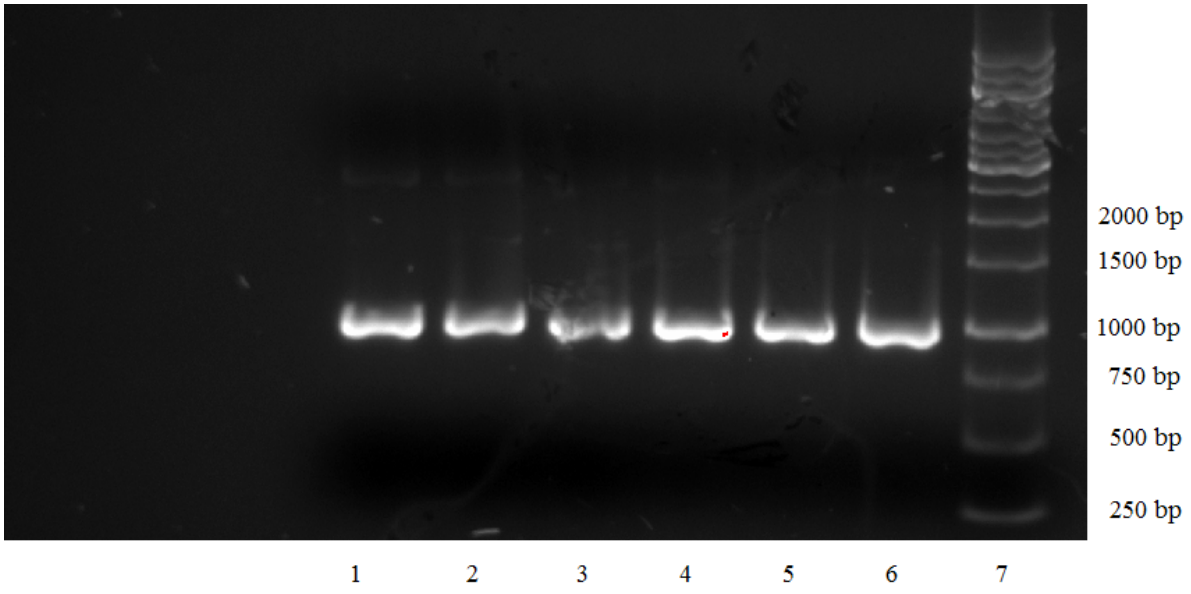

Figure 3.1.2.1*: Resulting banding pattern from agarose gel electrophoresis of six parallel* Kan^R *PCR samples. Wells 1-6 contain* Kan^R *PCR products from six parallel reactions. Well 7 contains GeneRuler™ 1 kb DNA ladder (Thermo Scientific), with band sizes in base pairs (bp) up to 2 kb given to the right.*

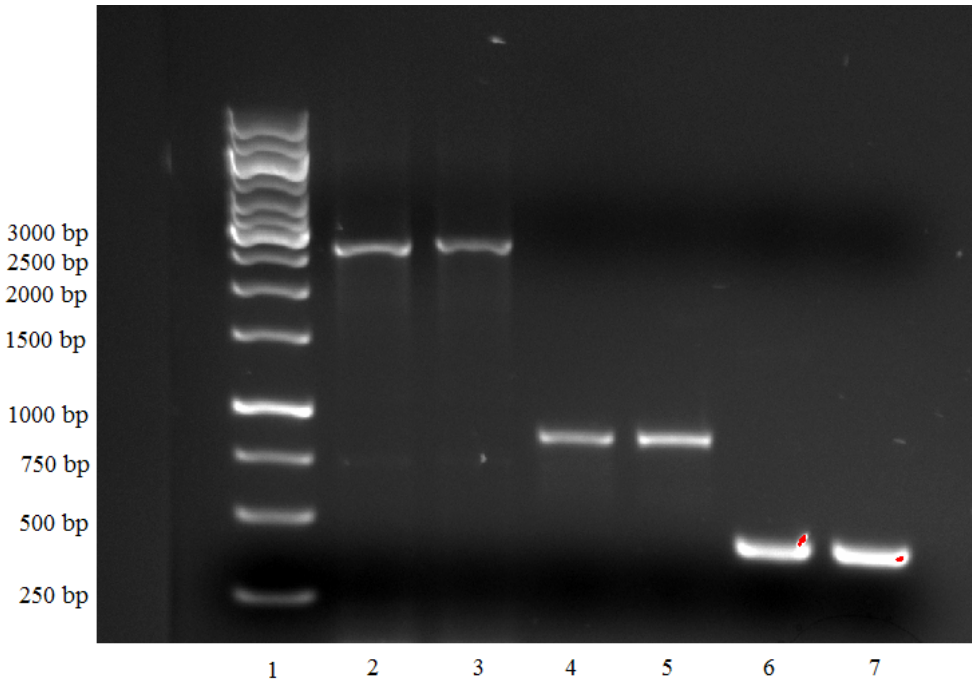

Figure 3.1.2.2*: Resulting banding pattern from agarose gel electrophoresis of two parallel reactions each for Lit, LF and RF PCR products. Well 1 contains GeneRuler™ 1 kb DNA ladder (Thermo Scientific), with band sizes in base pairs (bp) up to 3 kb given to the left. Wells 2 and 3 contain Lit PCR products. Well 4 and 5 contains LF PCR products. Wells 6 and 7 contains RF PCR products.*

After PCR clean-up (section 2.7), the concentration of each fragment was measured spectroscopically using a Nano Drop instrument. Table 3.1.2.2 shows the concentrations of the fragments used to construct the deletion vector for operon *slr1928-31*.

Table 3.1.2.2: *Concentrations in ng/µL of each constituent in the deletion vector for the operon* slr1928-31: *Left and right flanking regions (LF and RF), antibiotic resistance cassette* (Kan^R) *and Litmus backbone (Lit).*

DNA fragment	**Concentration in ng/ µL**
Left flank (LF)	71
Kan^R	323
Right flank (Rf)	74
Lit	88

3.1.3: Verification of deletion vector assembly

The vector constituents Lit, LF, *Kan*^{R} and RF were ligated into a complete plasmid by Gibson assembly, as described in section 2.9. The vector assembly was verified by PCR amplification (6 reactions) followed by agarose gel electrophoresis. The gel confirming the presence of the desired plasmid is shown in figure 3.1.3.1. Reactions 1-5 resulted in clear bands of about 5 kb, whereas reaction 6 did not result in any band of this size. Reaction 1 gave the clearest band. The plasmid from this reaction was selected for transformation into *Synechocystis* 6803 after verification by sequencing (performed by GATC Biotech).

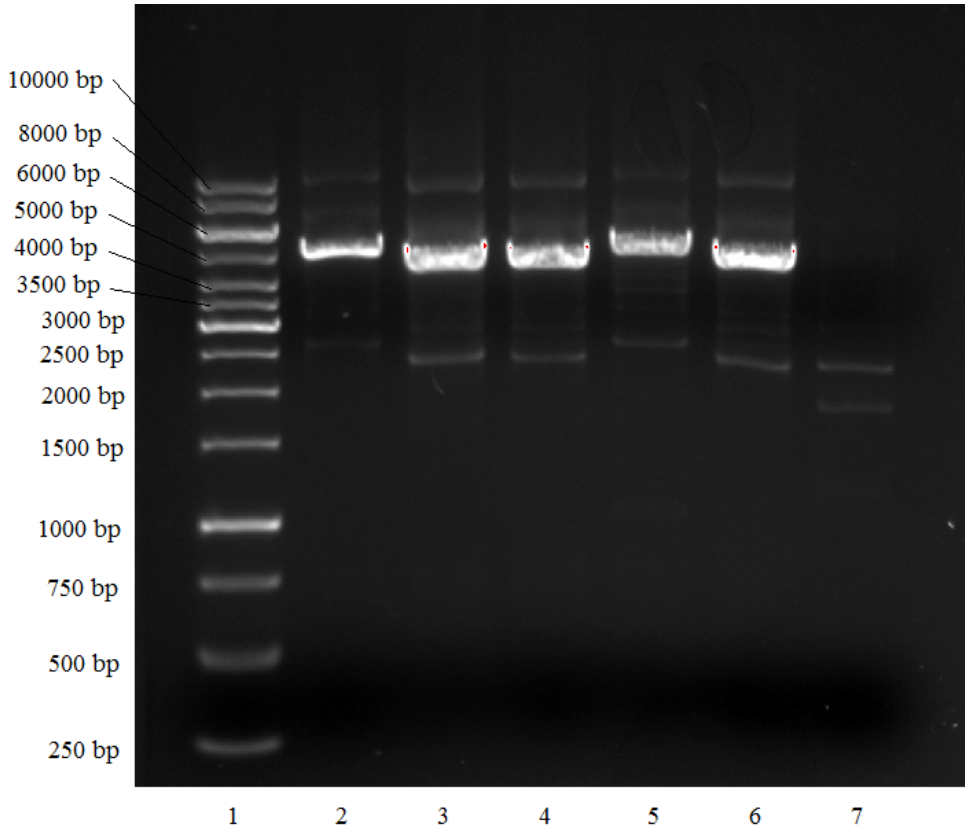

Figure 3.1.3.1: *Resulting banding pattern from agarose gel electrophoresis of six parallel Gibson assembly PCR samples. Well 1 contains GeneRuler*^{TM} *1 kb DNA ladder (Thermo Scientific), with band sizes in base pairs (bp) given to the left. Wells 2-7 contain Gibson assembly PCR products from six parallel reactions.*

3.2: Genetic deletion

3.2.1: Transformation of Synechocystis *6803 with deletion vector*

Cultures of *Synechocystis* 6803 were transformed as described in section 2.14. The cultures were initially grown on non-selective medium, thereafter on medium containing Kan. Cells growing on the selective medium were assumed to be successfully transformed with the deletion vector, and thus to have lost the operon *slr1928-31*.

3.2.2: Verification of the mutant strain Δ slr1928-31-*Kan*

Colony PCR followed by gel electrophoresis was performed in order to fully confirm that the mutant strain *Δ slr1928-31*-Kan indeed contained the intended insert and was fully segregated. The gel confirming the presence of the desired insert is shown in figure 3.2.2.1. The plasmid was then isolated and sequenced, confirming that the intended insert was present.

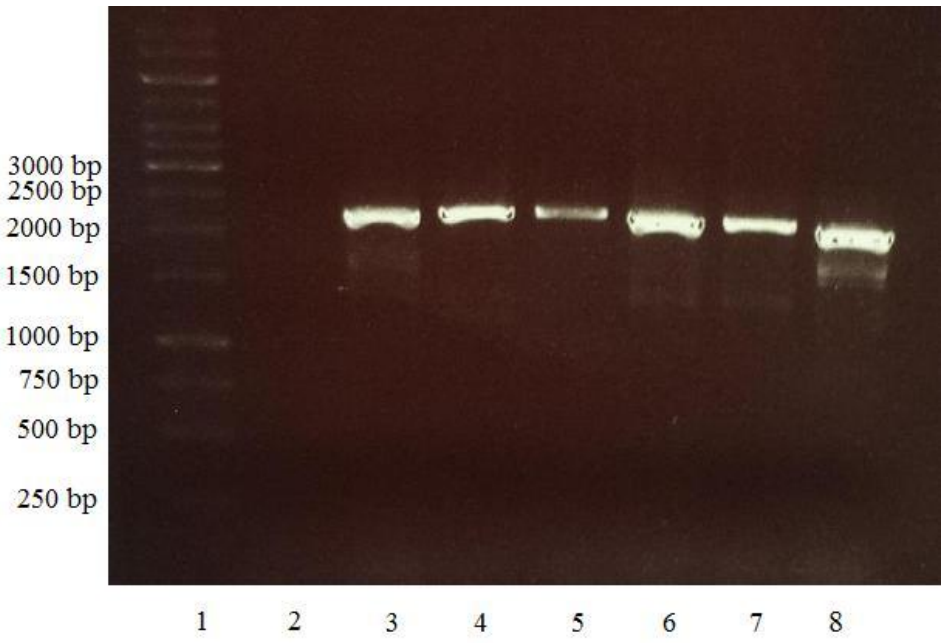

Figure 3.2.2.1*: Resulting banding pattern from agarose gel electrophoresis of six parallel colony PCR samples from cultures of* Synechocystis *6803 transformed with the deletion vector for the operon* slr1928-31. *Well 1 contains GeneRuler™ 1 kb DNA ladder (Thermo Scientific), with band sizes in base pairs (bp) up to 3 kb given to the left. Well 2 is empty. Wells 3-8 contain colony PCR products from six parallel reactions.*

3.3: Growth assay

3.3.1: Preliminary analysis of growth and phenotype on different iron sources

A preliminary growth experiment was performed to identify potential growth differences between WT *Synechocystis* 6803 and the mutant strain *Δ slr1928-31*-Kan on BG-11 media with one of 5 different iron sources: Ferric ammonium citrate (Fac), ferric chloride hexahydrate (FeCl), goethite (Goe), iron (II, III) oxide (FeO) and potassium hexacyanoferrate (III) (Hexacyan). The abbreviations given in brackets are created for use in this thesis for the sake of convenience. Each plate had 3 colonies with an OD 730 nm of 1.0, 0.1 and 0.01 for both WT and mutant cultures. Colonies were compared visually within and between the two strains, but few visible changes in phenotype between WT and *Δ slr1928-31*-Kan were observed for any OD. Yet a more thorough analysis of the mutant strain is needed in order to detect subtle pigment changes or variations in growth that do not visibly alter the phenotype. The in-depth analysis involved a growth curve analysis for the preliminary experiment, as well as a large-scale growth experiment where growth was examined under 30 different conditions.

3.3.2: Large-scale analysis of growth and phenotype under different growth conditions

A large-scale growth experiment was performed to identify potential growth differences between WT *Synechocystis* 6803 and the mutant strain *Δ slr1928-31*-Kan. Cultures were grown on BG-11 media supplied with one of 5 different iron sources: Fac, FeCl, FeO, Goe and Hexacyan. For each of these, 6 different conditions were tested: (I) Media without glucose, DCMU or NaCl (II) media with glucose, (III) media with glucose and DCMU, (IV) media with NaCl, (V) media with NaCl and glucose, and (VI) media with NaCl, glucose and DCMU. This yielded a total number of 30 growth conditions. Each plate had 3 colonies with an OD 730 nm of 1.0, 0.1 and 0.01 for both WT and mutant cultures. Visual inspection indicated some differences between the WT and *Δ slr1928-31*-Kan strains. Further investigation was performed employing three different methods: Growth curve analysis, whole cell absorption spectroscopy and 77K fluorescence spectroscopy. For all methods, WT and mutant colonies were compared on each plate. Only small changes were observed from these comparisons. However, more variation was obtained when comparing the strains across different iron sources but the same supplements. Growth curve analysis is described in sections 3.3.3-3.3.8. The results are described for the plots with cultures of OD 1.0 at 730 nm in this section, and for OD 0.1 and 0.01 in the appendix. The analysis of growth levels is based on the cell density calculated for given points of time during the experiment. Whole-cell absorption spectral analysis is described in section 3.4. The 77K technique is described in section 3.5. Plate-wise comparisons are described in the appendix

3.3.3: Media without glucose, DCMU or NaCl

The growth curves for WT and *Δ slr1928-31*-Kan cultures on BG-11 medium without glucose, DCMU or NaCl are shown in figures 3.3.3.1 and 3.3.3.2, respectively. For both strains the highest cell density/growth was observed on media supplied with FeO, followed by Goe, after about 125 hours of growth. The growth was only somewhat higher than that on the remaining three iron sources. The growth was similar for Fac, FeCl and Hexacyan for both strains. The levels of growth were similar throughout the whole growth period. In many cases, the standard error for different conditions overlapped to some extent, indicating that the differences between them were not significant.

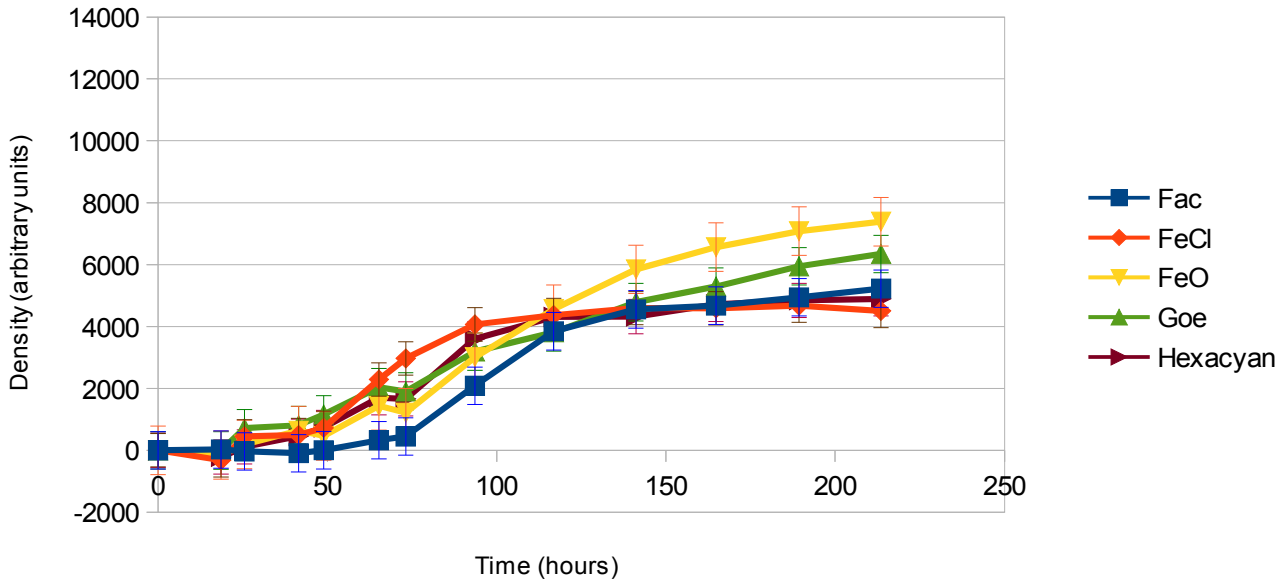

Figure 3.3.3.1: *Cultures of WT* Synechocystis *6803 with an OD of 1.0 inoculated on solid BG-11 media with different iron sources, without glucose, DCMU or NaCl added.*

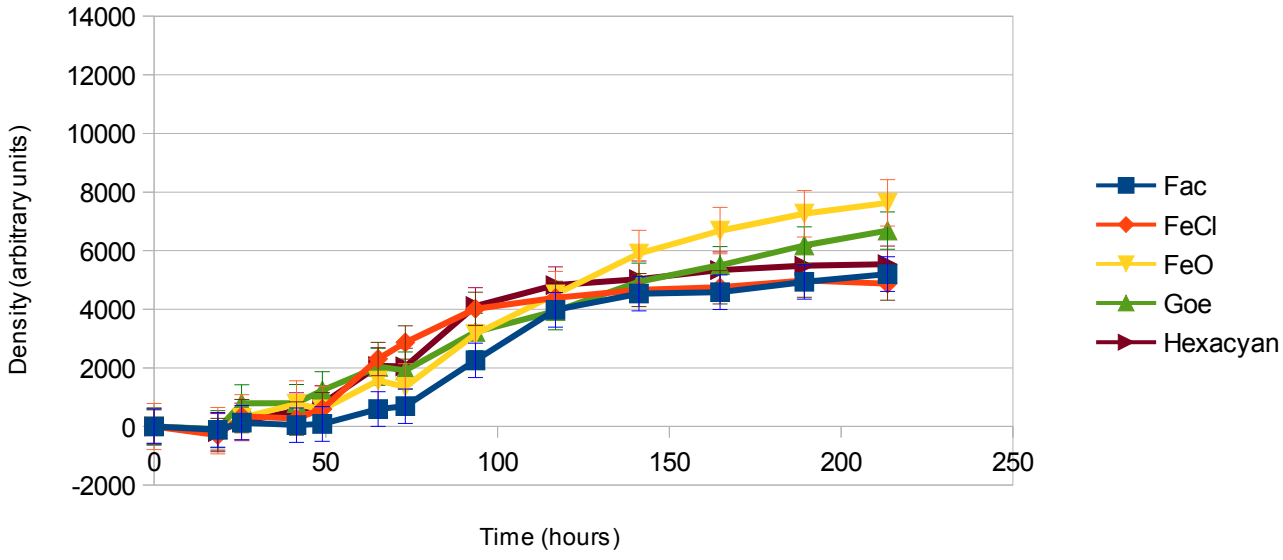

Figure 3.3.3.2: *Cultures of mutant strain* Δ slr1928-31-*Kan with an OD of 1.0 inoculated on solid BG-11 media with different iron sources, without glucose, DCMU or NaCl added.*

3.3.4: Media with glucose

The growth curves for WT and *Δ slr1928-31*-Kan cultures on BG-11 medium with glucose are shown in figures 3.3.4.1 and 3.3.4.2, respectively. The levels of growth for WT cultures were similar to those for *Δ slr1928-31*-Kan cultures on all iron sources: Fac and Hexacyan samples have higher levels of growth than FeCl, FeO and Goe after about 100 hours of growth. For WT cultures, the growth on Fac and Hexacyan is very similar. For the mutant strain *Δ slr1928-31*-Kan the growth observed on Hexacyan is slightly higher than that on Fac. In both cases the growth on FeCl, FeO and Goe is similar, although for *Δ slr1928-31*-Kan the growth is slightly lower on Goe. The error bars in the growth curves for Fac and Hexacyan *Δ slr1928-31*-Kan samples are only overlapping with each other after about 100 hours of growth, indicating that they are significantly different prior to this point of time. For WT cultures, the error bars in the growth curves for Fac and Hexacyan are slightly overlapping with the other iron sources as well as each other, implying that none of these iron sources result in a significant change in growth. For both strains, the error bars in the growth curves for FeCl, FeO and Goe are overlapping, indicating that the differences in growth between these iron sources were not significant.

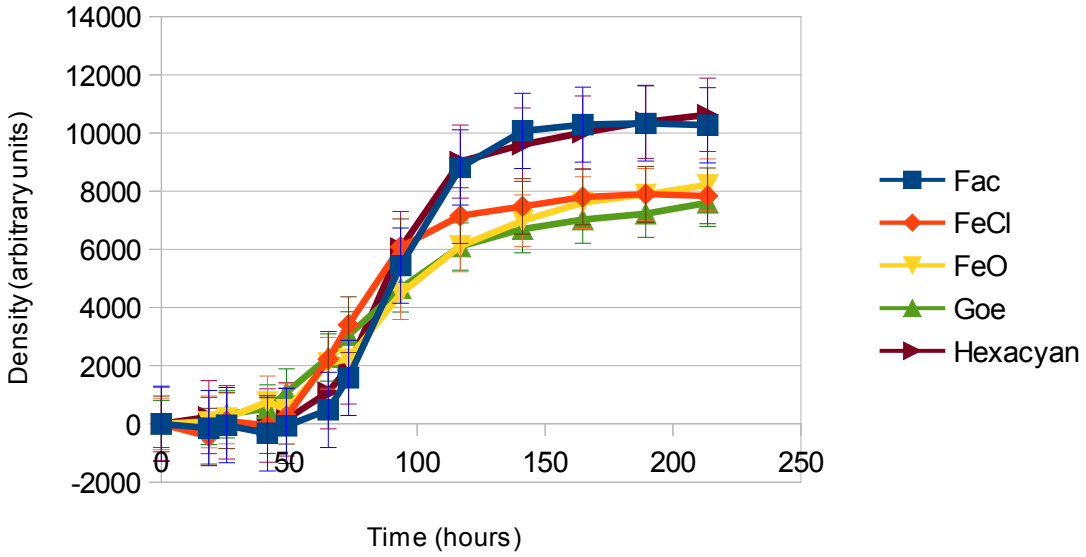

Figure 3.3.4.1: *Cultures of WT* Synechocystis *6803 with an OD of 1.0 inoculated on solid BG-11 media with different iron sources, with glucose added.*

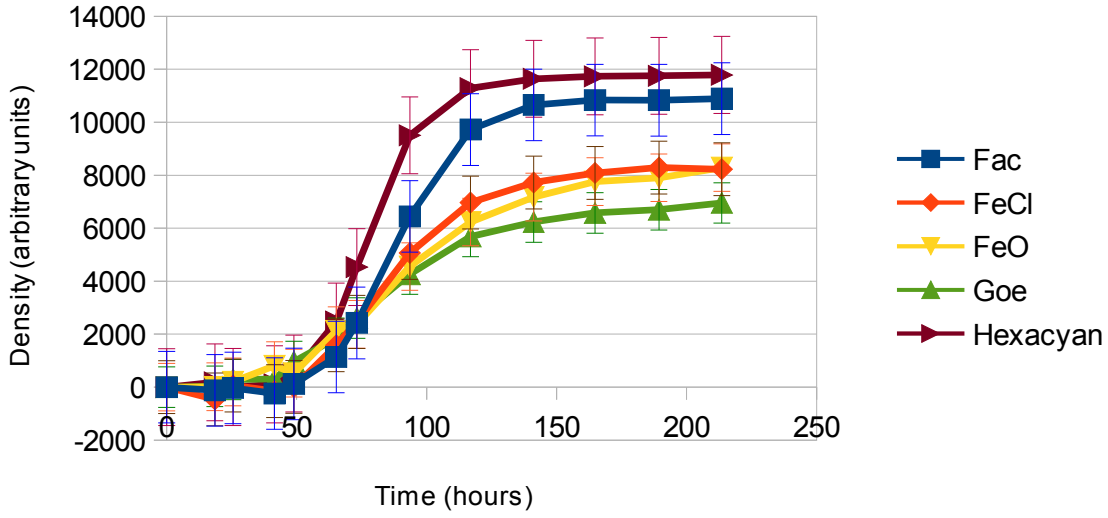

Figure 3.3.4.2: *Cultures of mutant strain* Δ slr1928-31-*Kan with an OD of 1.0 inoculated on solid BG-11 media with different iron sources, with glucose added.*

3.3.5: Media with glucose and DCMU

The growth curves for WT and *Δ slr1928-31*-Kan cultures on BG-11 medium with glucose and DCMU are shown in figures 3.3.5.1 and 3.3.5.2, respectively. The growth for WT cultures was similar to that for *Δ slr1928-31*-Kan cultures on all iron sources: A substantially higher level of growth was observed for Fac, Hexacyan and FeCl than for FeO and Goe after about 150 hours of growth. Almost equal levels of growth were observed on Fac, Hexacyan and FeCl, with overlapping

error bars indicating no significant difference. Growth on FeO was also very similar to that on Goe, with overlapping error bars indicating no significant difference.

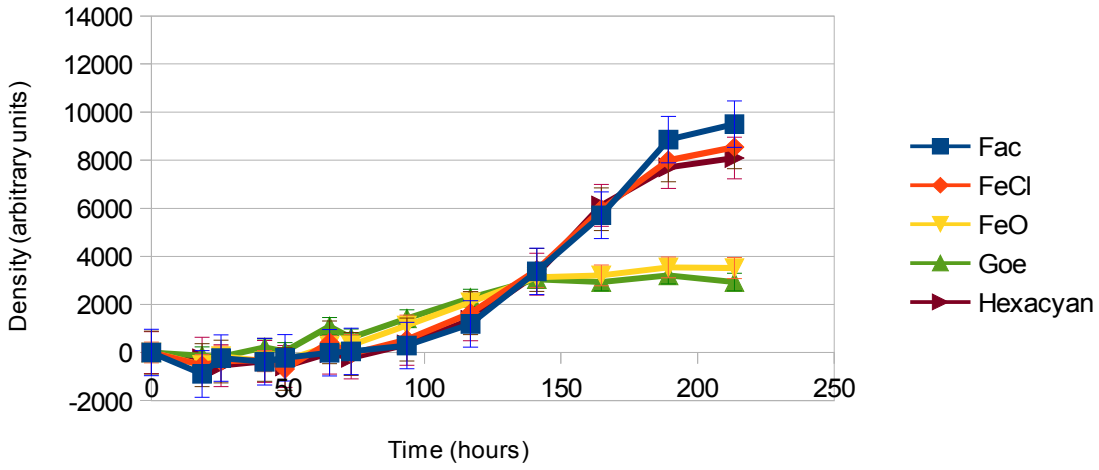

Figure 3.3.5.1: *Cultures of WT* Synechocystis *6803 with an OD of 1.0 inoculated on solid BG-11 media with different iron sources, with glucose and DCMU added.*

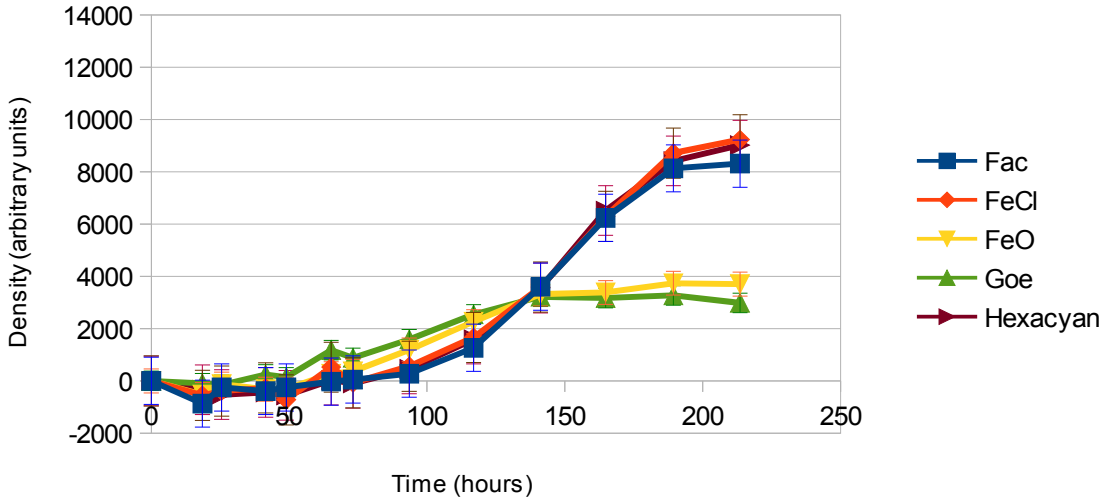

Figure 3.3.5.2: *Cultures of mutant strain* Δ slr1928-31-*Kan with an OD of 1.0 inoculated on solid BG-11 media with different iron sources, with glucose and DCMU added.*

3.3.6: Media with NaCl

The growth curves for WT and *Δ slr1928-31*-Kan cultures on BG-11 medium with 350 mM NaCl are shown in figures 3.3.6.1 and 3.3.6.2, respectively. For both strains, the highest cell growth was observed for Goe, Hexacyan and FeO after about 150 hours of growth. For WT cultures, Fac and FeCl gave poor growth. For *Δ slr1928-31*-Kan cultures, Fac and FeCl had somewhat lower growth than the other three iron sources. Growth on these iron sources seemed to be delayed but reached the same levels at the last time point. The error bars are overlapping for FeO, Goe and Hexacyan for

both strains, indicating no significant difference in growth on these iron sources. For *Δ slr1928-31*-Kan culture growth curves, the error bars for these three iron sources are also overlapping with Fac and FeCl at the last time point, indicating a significant difference before the maximum cell density is reached.

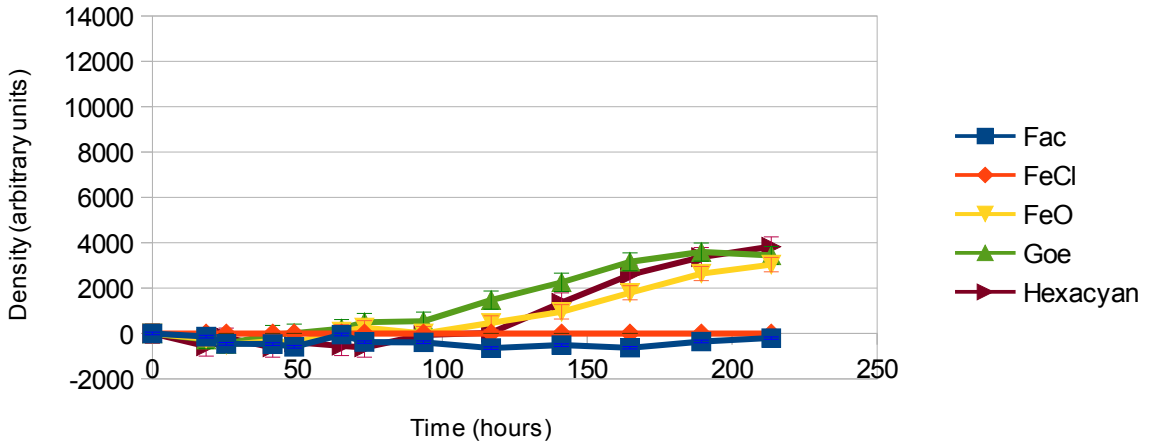

Figure 3.3.6.1: *Cultures of WT* Synechocystis *6803 with an OD of 1.0 inoculated on solid BG-11 media with different iron sources, with 350 mM NaCl added.*

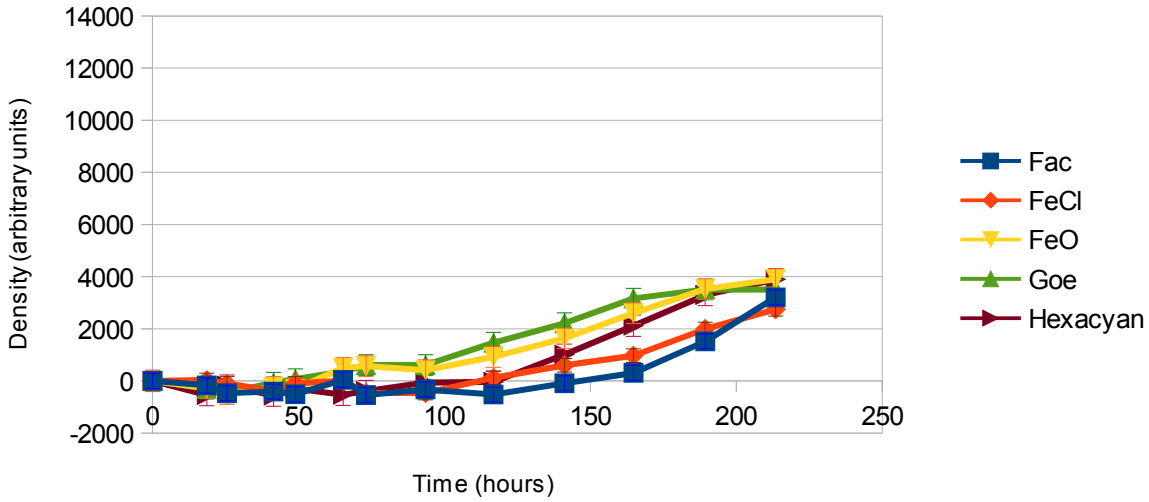

Figure 3.3.6.1: *Cultures of mutant strain* Δ slr1928-31-*Kan with an OD of 1.0 inoculated on solid BG-11 media with different iron sources, with 350 mM NaCl added.*

3.3.7: Media with NaCl and glucose

The growth curves for WT and *Δ slr1928-31*-Kan cultures on BG-11 medium with 350 mM NaCl and glucose are shown in figures 3.3.7.1 and 3.3.7.2, respectively. For both strains the highest cell growth was observed for Goe, followed by FeO. The error bars between these two were not overlapping after about 150 hours of growth, indicating that they are significantly different after this point of time. For WT cultures no growth was observed on Fac, FeCl or Hexacyan. These latter two samples are not included in figure 3.3.1.7 as their density was too low to be measured. The growth for *Δ slr1928-31*-Kan cultures on Fac was only somewhat lower than for FeO, with overlapping error bars for the last two time points indicating no significant difference. The growth on Hexacyan

was somewhat lower than on Fac, and no overlapping error bars for the last two time points indicated that the difference was significant after about 175 hours of growth.

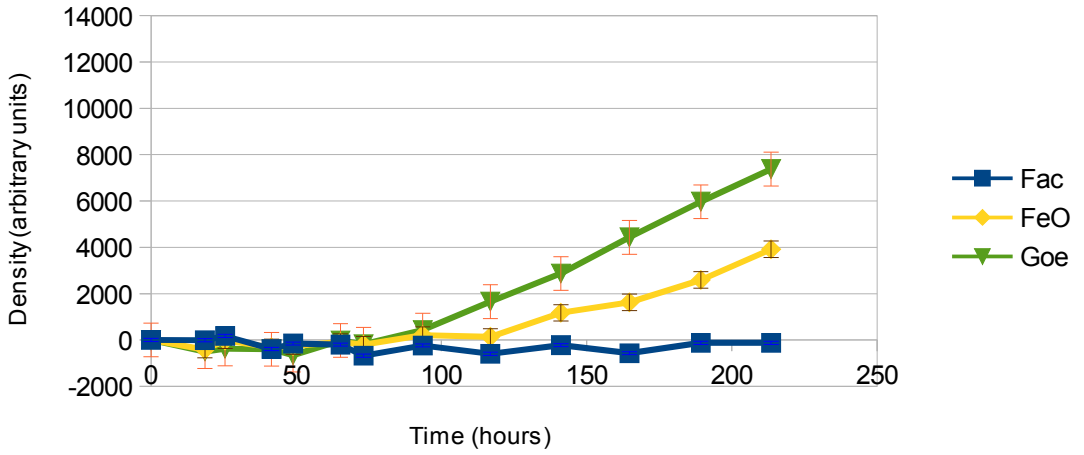

Figure 3.3.7.1: *Cultures of WT* Synechocystis *6803 with an OD of 1.0 inoculated on solid BG-11 media with different iron sources, with 350 mM NaCl and glucose added. The cell densities of cultures grown on FeCl or Hexacyan were too low to be plotted.*

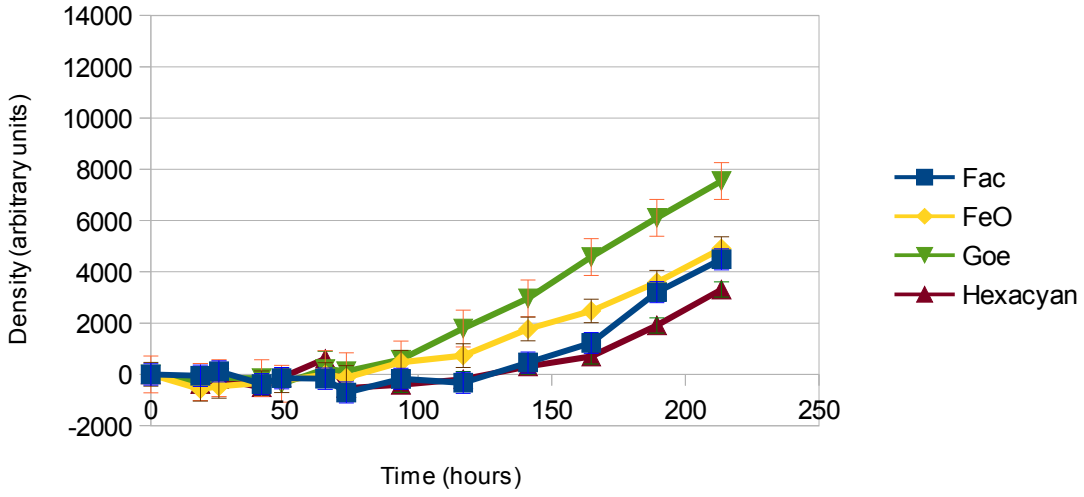

Figure 3.3.7.2: *Cultures of mutant strain* Δ slr1928-31-*Kan with an OD of 1.0 inoculated on solid BG-11 media with different iron sources, with 350 mM NaCl and glucose added. The cell density of the culture grown on FeCl was too low to be plotted.*

3.3.8: Media with NaCl, glucose and DCMU

The growth curves for WT and *Δ slr1928-31*-Kan cultures on BG-11 medium with NaCl, glucose and DCMU are shown in figures 3.3.8.1 and 3.3.8.2, respectively. For both strains growth was only observed on FeO and Goe. The growth on media with other iron sources yielded cell densities too low to be plotted. The growth on FeO and Goe is similar for both strains. After about 150 hours the

growth levels are higher on FeO than Goe. The error bars are not overlapping, indicating that the samples are significantly different in growth after this point of time.

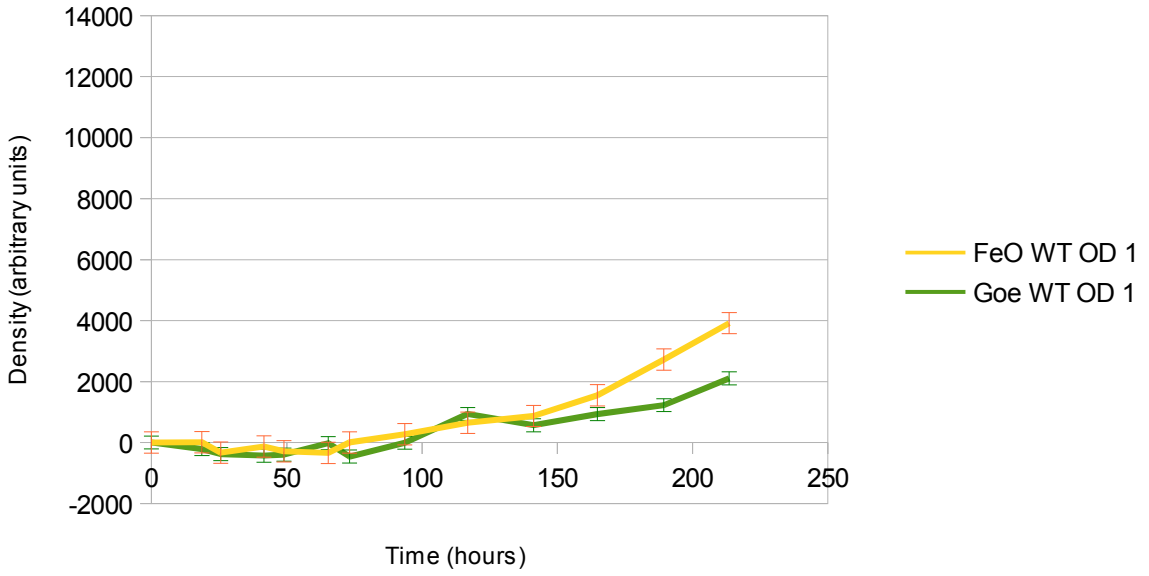

Figure 3.3.8.1: *Cultures of WT* Synechocystis *6803 with an OD of 1.0 inoculated on solid BG-11 media with different iron sources, with 350 mM NaCl, glucose and DCMU added. The cell densities of cultures grown on Fac, FeCl or Hexacyan were too low to be plotted.*

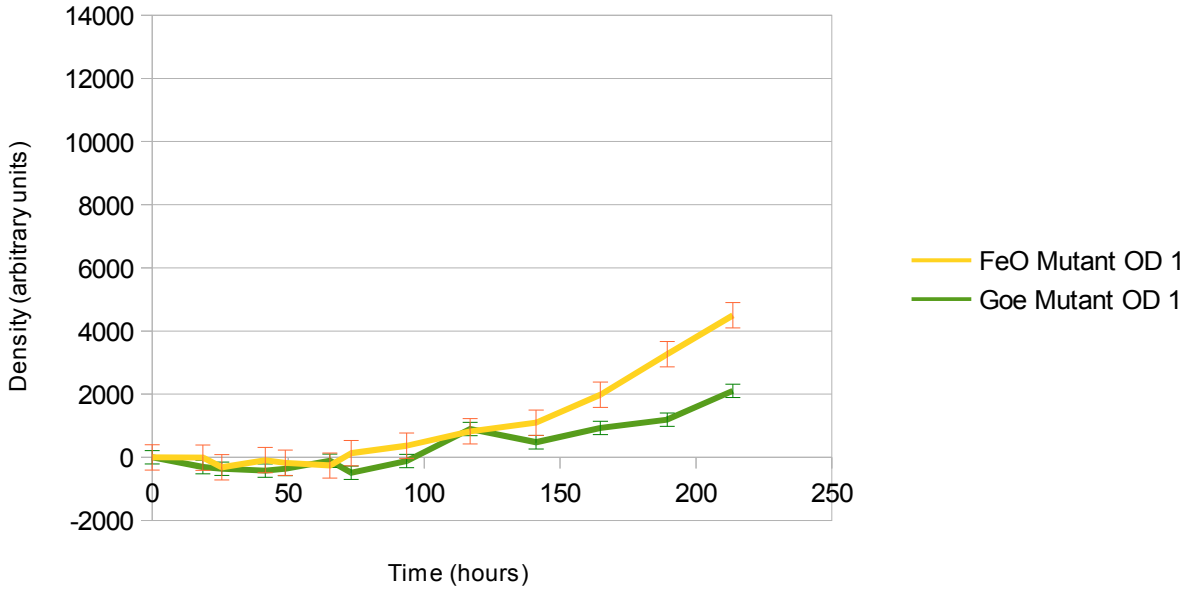

Figure 3.3.8.2: *Cultures of mutant strain* Δ slr1928-31-*Kan with an OD of 1.0 inoculated on solid BG-11 media with different iron sources, with 350 mM NaCl, glucose and DCMU added. The cell densities of cultures grown on Fac, FeCl or Hexacyan were too low to be plotted.*3.4: Whole cell absorption spectroscopy

This method was used to identify absorption of light by photosynthetic pigments in a selected area of wavelengths, here 350 nm to 750 nm. Chlorophylls have two absorption maxima: One around 435 nm and one around 680 nm. Different carotenoids present in cells of *Synechocystis* 6803 result in an absorption maximum around 480 nm, and phycobilins result in an absorption maximum around 625 nm. The amplitudes at these wavelengths indicate the relative amounts of each pigment type present in the cultures (Lamb *et al*., 2014).

The level of stress in *Synechocystis* 6803 cultures can be assessed by calculating the absorption ratios between different pigments: The relative amount of carotenoids to chlorophylls (predominantly Chl *a*) is determined by the 435 nm/680 nm absorption ratio, whereas the relative amount of phycobilins to Chl *a* is determined by 625 nm/680 nm absorption ratio (Lamb *et al*., 2014). Cultures of *Synechocystis* 6803 will normally have less phycobilins than Chl *a*, and more carotenoids than Chl *a*, under stressful growth conditions (Andrizhiyevskaya *et al*., 2002; Ivanov, *et al*., 2000; Ivanov *et al*., 2007). The spectra are all normalized to baseline and around the 680 nm peak. The carotenoid/Chl *a* ratios and the phycobilin/Chl *a* ratios for all samples are given in table 3.4.1 and 3.4.2, respectively.

Table 3.4.1: *Ratios between absorption by carotenoids (maximum 435 nm) and chlorophylls (maximum 680 nm) from whole cell absorption spectra for cultures of* Synechocystis *6803 WT and the mutant strain* Δ slr1928-31-*Kan under different conditions. The topmost row lists the different iron sources used, and which strain (WT = wild type, mut = mutant) the ratio was calculated for. The leftmost column lists the different growth media. The blank cells indicate that no spectra were obtained for these samples.*

	Fac WT	**Fac mut**	**FeCl WT**	**FeCl mut**	**Goe WT**	**Goe mut**	**Hexacyan WT**	**Hexacyan mut**
Without additives	1.51	1.56	2.33	2.24	2.05	2.17	2.06	1.98
Glucose	1.62	1.60	2.03	1.97	1.90	2.00	1.94	1.83
Glucose + DCMU	1.58	1.57	1.71	1.69	2.11	2.08	1.62	1.62

Table 3.4.2: *Ratios between absorption by phycobilins (maximum 625 nm) and chlorophylls (maximum 680 nm) from whole cell absorption spectra for cultures of* Synechocystis *6803 WT and the mutant strain* Δ slr1928-31-*Kan under different conditions. The topmost row lists the different iron sources used, and which strain (WT = wild type, mut = mutant) the ratio was calculated for. The leftmost column lists the different growth media. The blank cells indicate that no spectra were obtained for these samples.*

	Fac WT	**Fac mut**	**FeCl WT**	**FeCl mut**	**Goe WT**	**Goe mut**	**Hexacyan WT**	**Hexacyan mut**
Without additives	0.94	0.96	1.10	1.06	1.02	1.02	1.06	1.02
Glucose	0.94	0.96	0.97	1.01	1.10	1.07	1.27	1.32
Glucose + DCMU	1.02	1.04	1.07	1.06	1.14	1.11	1.09	1.09

3.4.1: Media without glucose, DCMU or NaCl

Figure 3.4.1.1 shows the whole cell absorption spectra for samples of *Synechocystis* 6803 WT and *Δ slr1928-31*-Kan cultures grown on BG-11 medium without glucose, DCMU or NaCl. The absorption spectra showed considerable variation below 550 nm and less variations for longer wavelengths. Around the peaks at 435 nm and 480 nm, absorption was highest for samples of cultures grown on FeCl, followed by Goe and Hexacyan. Cultures grown on the latter two iron sources showed only slightly lower absorption around 435 nm, but a larger difference at 480 nm. Samples of cultures grown on Fac had markedly lower absorption around both peaks. These results indicated that the FeCl samples had the highest content of both Chl *a* and carotenoids under this condition. Samples grown on Hexacyan and Goe had about the same amounts of these pigments, whereas samples grown on Fac had the lowest content of both pigments. Around 625 nm, the absorption was very similar for all samples, suggesting similar amounts of phycobilins for all samples. Around the 680 nm peak, absorption was somewhat higher and shifted towards blue wavelengths for Goe samples, indicating stress on this iron source. Only small differences were observed between WT and *Δ slr1928-31*-Kan cultures on the same iron source, indicating that no real difference in pigment contents existed between the strains.

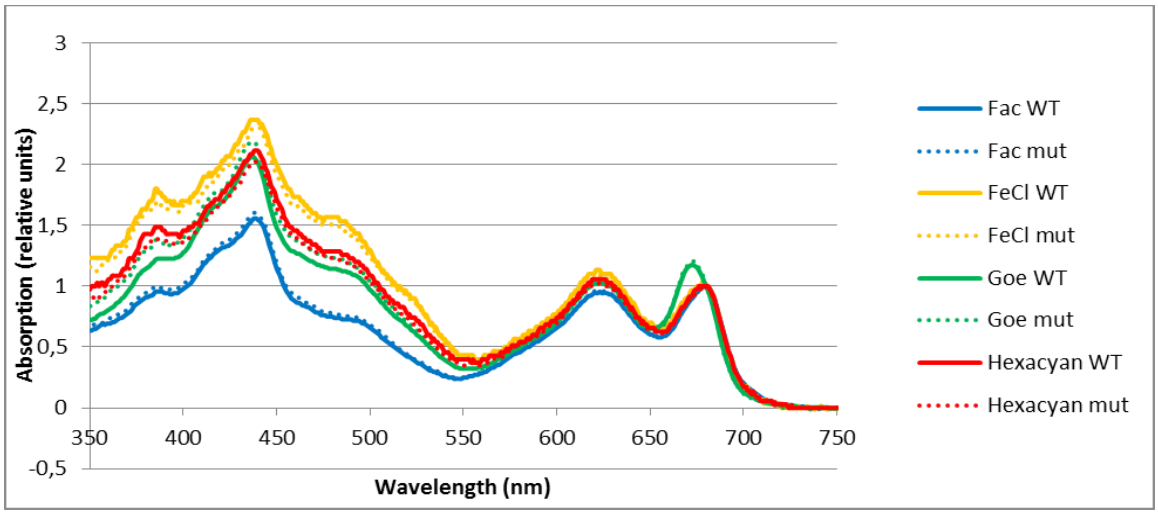

Figure 3.4.1.1: *Whole cell room temperature absorption spectra for* Synechocystis *6803 WT and* Δ slr1928-31-*Kan (mut) cultures on media with different iron sources, without glucose, DCMU or NaCl. The spectra are normalized around 680 nm.*

3.4.2: Media with glucose

Figure 3.4.2.1 shows whole cell absorption spectra of *Synechocystis* 6803 WT and *Δ slr1928-31*-Kan cultures grown on BG-11 medium with glucose added. Absorption spectra for WT and *Δ slr1928-31*-Kan cultures on media with glucose showed some variations over the whole range of wavelengths. Around 435 nm the absorption levels were variable, with FeCl yielding the highest peak, followed by Hexacyan, Goe and Fac. Hence, the amount of Chl *a* was similar in all samples, although noticeably lower for Fac. Around the 480 nm peak, the absorption for Hexacyan samples was very similar to that of FeCl samples, and absorption for Goe samples was similar to that of Fac samples. Samples grown on the former two iron sources had higher absorption than the latter two, indicating that Hexacyan and FeCl samples had more carotenoids than had Fac and Goe samples. Around 625 nm, all samples show close to identical peaks except a markedly higher peak for Hexacyan samples indicating higher phycobilin contents. Around the 680 nm peak, the absorption for Goe samples was higher and shifted somewhat towards blue wavelengths as compared to the remaining samples, which all gave nearly identical absorption patterns. This implies that Goe samples had more Chl *a* and apparently higher levels of stress as compared to the other iron sources. Only marginal differences were observed between WT and *Δ slr1928-31*-Kan cultures on the same iron source, indicating that no real difference in pigment contents existed between the strains.

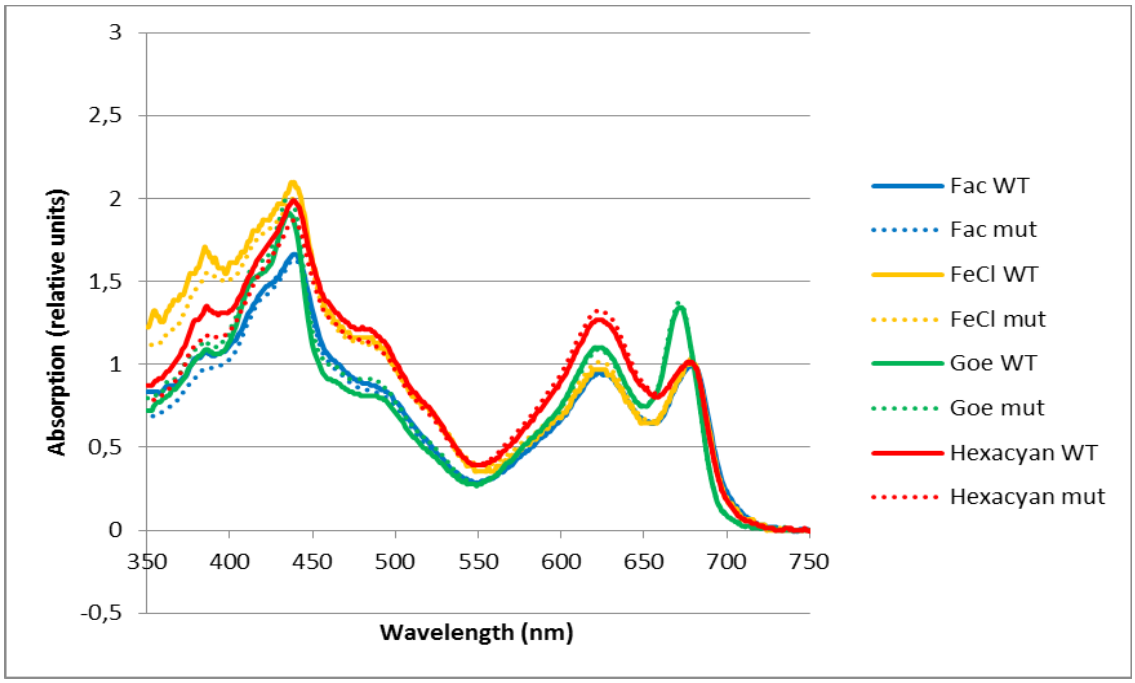

Figure 3.4.2.1: *Whole cell room temperature absorption spectra for* Synechocystis *6803 WT and* Δ slr1928-31-*Kan (mut) cultures on media with different iron sources and glucose added. The spectra are normalized around 680 nm.*

3.4.3: Media with glucose and DCMU

Figure 3.4.3.1 shows whole cell absorption spectra of *Synechocystis* 6803 WT and *Δ slr1928-31*-Kan cultures grown on BG-11 medium with glucose and DCMU added. All samples, except cultures grown on Goe, show very similar levels of absorption over the whole range of wavelengths. Samples of cultures grown on Goe have markedly higher absorption levels as well as blue-ward shifts in the peaks around 435 nm and 680 nm. The absorption is also somewhat higher than the remaining samples around 480 nm, but very similar to the other samples around 625 nm. These results suggest that all samples have similar pigment contents, except Goe samples which have more Chl *a* and seemingly higher stress levels. Only marginal differences were observed between WT and *Δ slr1928-31*-Kan cultures on the same iron source, indicating that no real difference in pigment contents existed between the strains.

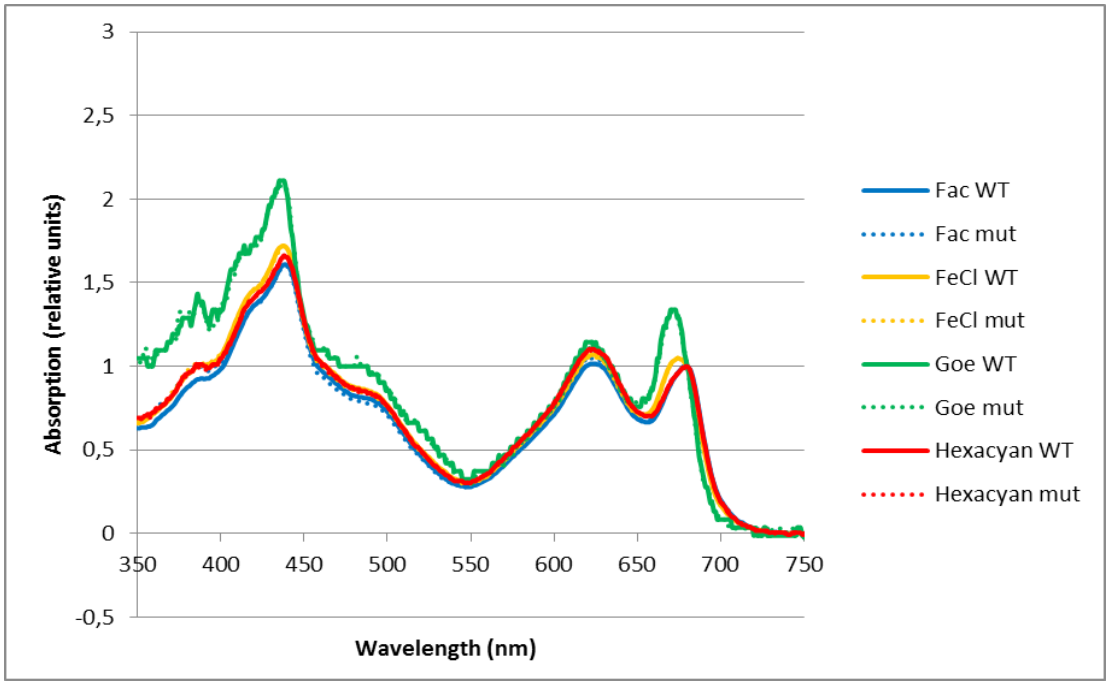

Figure 3.4.3.1: *Whole cell room temperature absorption spectra for* Synechocystis *6803 WT and* Δ slr1928-31-*Kan (mut) cultures on media with different iron sources, with glucose and DCMU added. The spectra are normalized around 680 nm.*

3.4.4: Media with NaCl

Figure 3.4.4.1 shows whole cell absorption spectra of *Synechocystis* 6803 WT and *Δ slr1928-31*-Kan cultures grown on BG-11 medium with 350 mM NaCl added. Spectra were only obtained for WT culture on Hexacyan, and *Δ slr1928-31*-Kan cultures on Fac and FeCl, as there was little growth to generate samples for spectroscopic measurements for the remaining samples. The absorption around all peaks (except at 625 nm), and thus also Chl *a* and carotenoid contents, were highest for *Δ slr1928-31*-Kan cultures grown on FeCl, followed by WT culture on Hexacyan and last mutant culture on Fac. Around 625 nm, the absorption for *Δ slr1928-31*-Kan culture on FeCl and WT culture on Hexacyan was very similar, but both were higher than for the *Δ slr1928-31*-Kan culture on Fac. For the peaks at 435 nm and 480 nm the difference in absorption levels was substantial, whereas the differences were smaller around 625 nm and 680 nm. This result indicates that the amounts of Chl a and carotenoids are much higher in the mutant strain grown on FeCl whereas the phycobilin contents are more similar in all samples. Around 680 nm, the *Δ slr1928-31*-Kan culture on FeCl showed a slight shift to shorter wavelengths as compared to standard conditions, indicating that this culture experienced some stress.

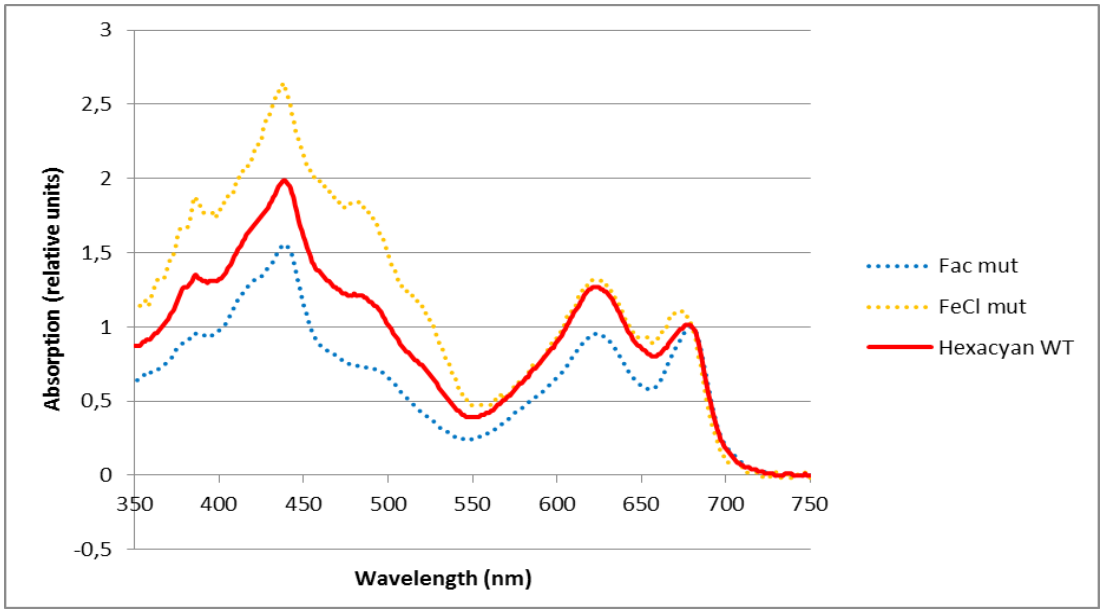

Figure 3.4.4:1 *Whole cell room temperature absorption spectra for* Synechocystis *6803 WT and* Δ slr1928-31-*Kan (mut) cultures on with different iron sources, with 350 mM NaCl added. The spectra are normalized around 680 nm. Absorption spectra for samples of WT cultures grown on Fac, FeCl and Goe, as well as* Δ slr1928-31-*Kan cultures grown on Goe and Hexacyan, were not attained due to poor growth.*

3.4.5: Media with NaCl and glucose

Figure 3.4.5.1 shows whole cell absorption spectra of *Synechocystis* 6803 WT and *Δ slr1928-31*-Kan cultures grown on BG-11 medium with glucose and 350 mM NaCl added. Spectra were only obtained for *Δ slr1928-31*-Kan cultures on Fac, Hexacyan and Goe, and WT culture on Goe, as the other cultures did not yield enough material for spectral characterization.

Around 435 nm, the absorption levels were very similar for all samples except for the *Δ slr1928-31*-Kan culture on Hexacyan, which had somewhat higher absorption. These results implied that the mutant strain on Hexacyan had more Chl *a*. Around 480 nm, absorption was highest for the *Δ slr1928-31*-Kan culture on Hexacyan, followed by the *Δ slr1928-31*-Kan culture on Fac with a somewhat lower absorption level. Both strains grown on Goe had nearly identical absorption levels, markedly lower than for the mutant strain on Hexacyan or Fac. The *Δ slr1928-31*-Kan cultures on Fac and Hexacyan thus appeared to have more carotenoids than both Goe samples. Around 625 nm and 680 nm, both strain grown on Goe had nearly identical absorption, markedly higher than the absorption for the *Δ slr1928-31*-Kan cultures on Hexacyan and Fac. Hence, both strains on Goe had more phycobilins than the *Δ slr1928-31*-Kan cultures on Fac and Hexacyan. The two latter samples

also had very similar absorption, suggesting that their phycobilin contents were about the same. A slight blue-ward shift in the red chlorophyll peak was observed for the Goe samples.

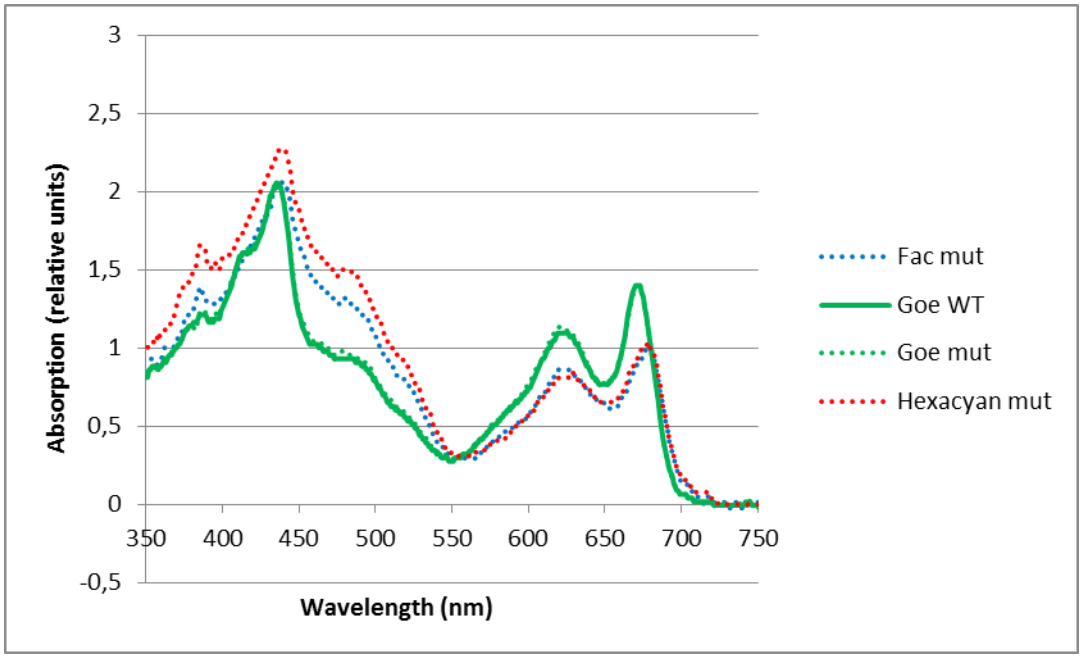

Figure 3.4.5.1: *Whole cell room temperature absorption spectra for* Synechocystis *6803 WT and* Δ slr1928-31-*Kan (mut) cultures on media with different iron sources, with glucose and 350 mM NaCl added. The spectra are normalized around 680 nm. Absorption spectra for samples of WT cultures grown on Fac, FeCl and Hexacyan, as well as* Δ slr1928-31-*Kan cultures grown on FeCl, were not attained due to poor growth.*

3.4.5: Media with NaCl, glucose and DCMU

No spectra were obtained for these samples due to poor growth and consequently insufficient cell material for spectral characterization.

3.5: 77 K fluorescence spectroscopy

This method was used to measure fluorescence emission from PS I and II in the area of wavelengths from 650 nm up to 800 nm. The fluorescence maxima for CP 43 and CP 47, the core antenna proteins of PS II, are at 685 nm and 695 nm, respectively. Their levels of fluorescence are usually very similar. The fluorescence maximum for PS I is 725 nm. The relative fluorescence from each photosystem can be determined by calculating the (685+695)/725 nm fluorescence ratio. Under non-stress conditions, the fluorescence from PS II will be low relative to that from PS I, yielding a low (685+695)/725 nm ratio. Under stress conditions, an alternative chlorophyll-binding protein called IsiA will be expressed in *Synechocystis* 6803 (Ivanov), yielding a high fluorescence peak

around 685 nm (Lamb *et al*., 2014). To distinguish the fluorescence contribution by IsiA from that of PS II, the 695 nm/685 nm fluorescence ratio was calculated. A low value indicates more IsiA and hence stress in the bacteria. The spectra are all normalized to baseline and around the 725 nm peak. The PS II/PS I ratios and the IsiA/PS II ratios for all samples are given in table 3.5.1 and 3.5.2, respectively.

Table 3.5.1: *Ratios between absorption by PS II (maximum 685 nm and 695 nm nm) and PS I (maximum 725 nm) from 77 K fluorescence spectra for cultures of* Synechocystis *6803 WT and the mutant strain* Δ slr1928-31-*Kan under different conditions. The topmost row lists the different iron sources used, and which strain (WT = wild type, mut = mutant) the ratio was calculated for. The leftmost column lists the different growth media. The blank cells indicate that no spectra were obtained for these samples.*

	Fac WT	**Fac mut**	**FeCl WT**	**FeCl mut**	**Goe WT**	**Goe mut**	**Hexacyan WT**	**Hexacyan mut**
Without additives	0.23	0.24	0.47	0.35	4.60	4.74	0.37	0.36
Glucose	0.23	0.18	0.47	0.35	3.43	3.89	0.66	0.55
Glucose + DCMU	0.28	0.30	0.44	0.42	4.60	4.74	0.38	0.40

Table 3.5.2: *Ratios between absorption by IsiA (maximum 685 nm) and PS II (maximum 695 nm) from 77 K fluorescence spectra for cultures of Synechocystis 6803 WT and the mutant strain Δ slr1928-31-Kan under different conditions. The topmost row lists the different iron sources used, and which strain (WT = wild type, mut = mutant) the ratio was calculated for. The leftmost column lists the different growth media. The blank cells indicate that no spectra were obtained for these samples.*

	Fac WT	**Fac mut**	**FeCl WT**	**FeCl mut**	**Goe WT**	**Goe mut**	**Hexacyan WT**	**Hexacyan mut**
Without additives	1.44	1.46	0.76	0.70	0.56	0.49	1.08	1.08
Glucose	1.19	1.37	0.76	0.70	0.46	0.44	0.84	0.90
Glucose + DCMU	1.40	1.34	1.01	1.01	0.56	0.49	1.15	1.11

3.5.1: Media without glucose, DCMU or NaCl

Figure 3.5.1.1 shows fluorescence spectra at 77 K for samples of *Synechocystis* 6803 WT and *Δ slr1928-31*-Kan cultures grown on BG-11 medium without glucose, DCMU or NaCl. Around 685 nm, both strains on Goe had very high peaks, whereas the remaining samples were very similar to each other and barely yielded any peaks at all in this area. The high peak observed for the Goe samples implied that the stress-induced chlorophyll-binding protein IsiA was present in the Goe samples.

The PS II/PS I fluorescence ratios were substantially higher for both strains on Goe than for the remaining samples, indicating an increase in PS II fluorescence associated with stress. The PS II/IsiA ratios were markedly lower for both strains on Goe than for the remaining samples, indicating that stress induced expression of IsiA in these cultures. The low fluorescence peaks for PS II and the low ratio values for PS II/PS I for the remaining samples are characteristic for non-stress conditions. Nevertheless, the ratio values for PS II/IsiA absorption are highest for both cultures on Fac, somewhat lower for both strains on Hexacyan than those for the respective Fac samples, even lower for both strain on FeCl, and lowest for both cultures on Goe. This indicated that the stress levels and thus the amounts of IsiA were lowest in the Fac samples, increased for Hexacyan and FeCl, and the highest in the Goe samples.

Around 725 nm, a slight blue-ward shift was observed for both strains on Goe, also suggesting presence of stress for cultures grown on this iron source. Only marginal differences were observed between WT and *Δ slr1928-31*-Kan cultures on the same iron source, suggesting that there is no real difference between the strains under this condition, except for a slightly higher peak at 685 nm for

slr1928-31-Kan samples than WT samples on Goe. This result indicated that the mutant strain experienced slightly higher levels of stress.

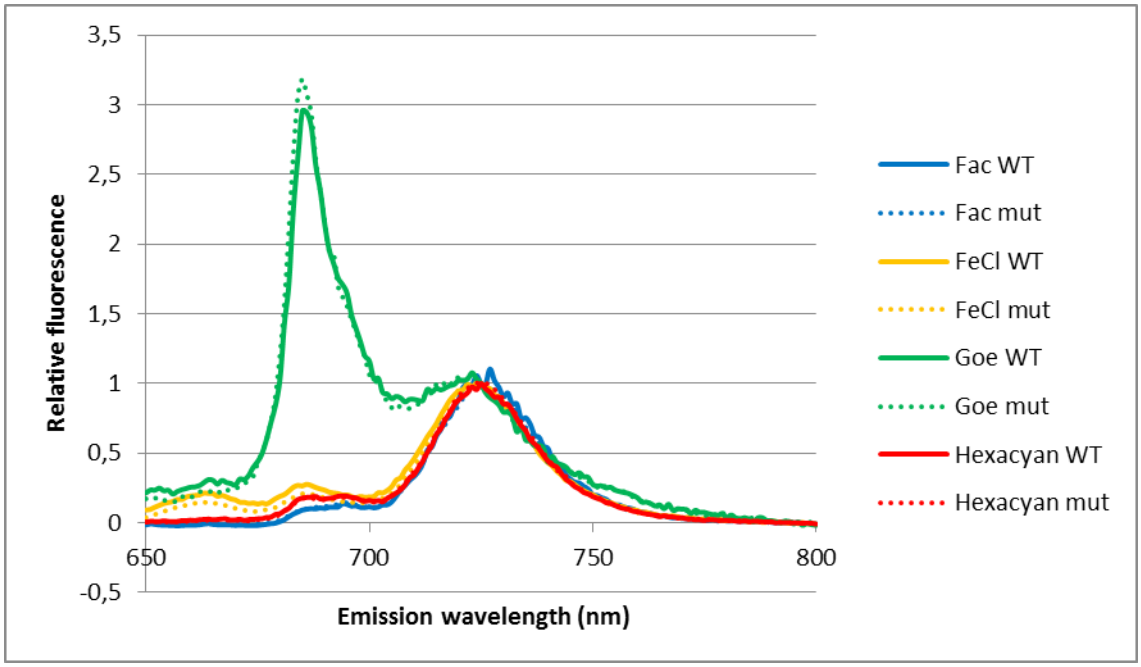

Figure 3.5.1.1: *77K fluorescence spectra for* Synechocystis *6803 WT and* Δ slr1928-31-*Kan (mut) cultures on media with different iron sources, without glucose, DCMU, NaCl. The spectra are normalized around 725 nm.*

3.5.2: Media with glucose

Figure 3.5.2.1 shows fluorescence spectra at 77 K for samples of *Synechocystis* 6803 WT and *Δ slr1928-31*-Kan cultures grown on BG-11 medium with glucose added. Around 685 nm, both strains on Goe had very high peaks, whereas the remaining samples were very similar to each other and barely yielded any peaks at all in this area. The high peak observed for the Goe samples implied that the stress-induced chlorophyll-binding protein IsiA was present in the Goe samples.

The PS II/PS I fluorescence ratios were substantially higher for both strains on Goe than for the remaining samples, indicating an increase in PS II fluorescence associated with stress. The PS II/IsiA ratios were markedly lower for both strains on Goe than for the remaining samples, indicating that stress induced expression of IsiA in these cultures. The low fluorescence peaks for PS II and the low ratio values for PS II/PS I for the remaining samples are characteristic for non-stress conditions. Nevertheless, the ratio values for PS II/IsiA absorption are highest for both cultures on Fac, somewhat lower for both strains on Hexacyan than those for the respective Fac samples, even lower for both strain on FeCl, and lowest for both cultures on Goe. This indicated

that the stress levels and thus the amounts of IsiA were lowest in the Fac samples, increased for Hexacyan and FeCl, and the highest in the Goe samples.

Around 725 nm, a slight blue-ward shift was observed for both strains on Goe, also suggesting the presence of stress for cultures grown on this iron source. Only marginal differences were observed between WT and *Δ slr1928-31*-Kan cultures on the same iron source, except for a higher peak at 685 nm for *slr1928-31*-Kan samples than WT samples on Goe. This result indicated that the mutant strain experienced slightly higher levels of stress.

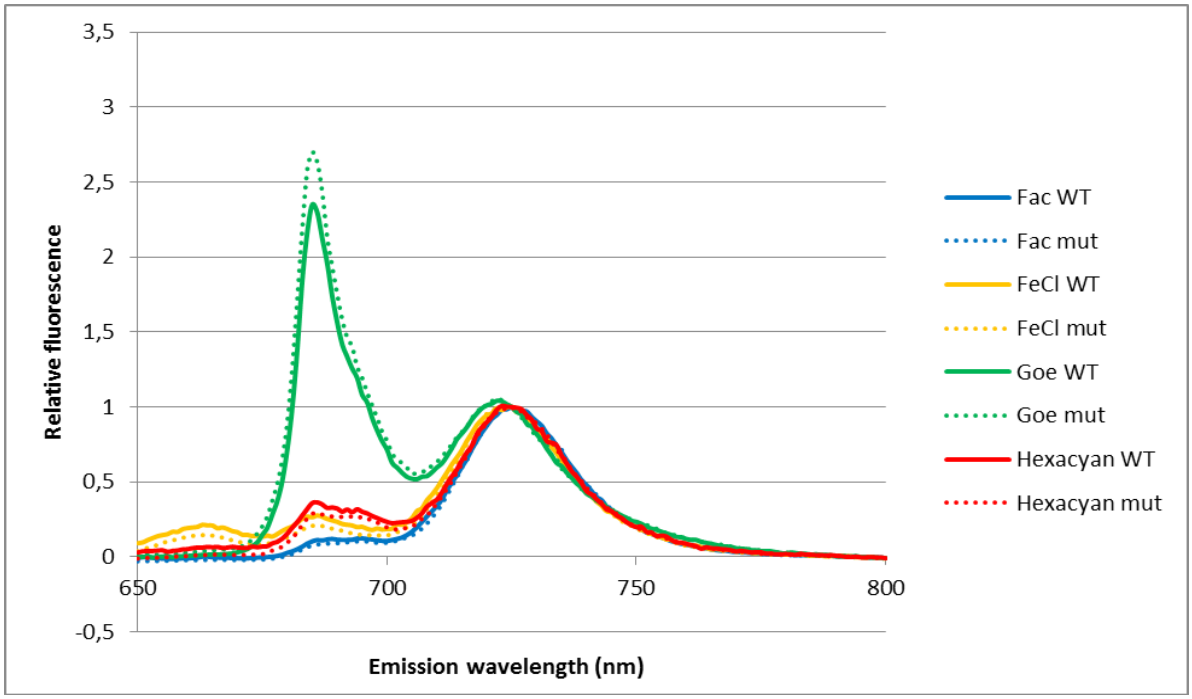

Figure 3.5.2.1: *77K fluorescence spectra for* Synechocystis *6803 WT and* Δ slr1928-31-*Kan (mut) cultures on media with different iron sources, with glucose added. The spectra are normalized around 725 nm.*

3.5.3: Media with glucose and DCMU

Figure 3.5.3.1 shows fluorescence spectra at 77 K for samples of *Synechocystis* 6803 WT and *Δ slr1928-31*-Kan cultures grown on BG-11 medium with glucose and DCMU added. Around 685 nm, the Goe samples yielded very high peaks, whereas the remaining samples were very similar and barely yielded any peak at all in this area. The high peak observed for the Goe samples implied that the stress-induced chlorophyll-binding protein IsiA was present in the Goe samples.

The PS II/PS I fluorescence ratios were substantially higher for both strains on Goe than for the remaining samples, indicating an increase in PS II fluorescence associated with stress. The PS

II/IsiA ratios were markedly lower for both strains on Goe than for the remaining samples, indicating that stress induced expression of IsiA in these cultures. The low fluorescence peaks for PS II, the low ratio values for PS II/PS I and the high ratio values for PS II/IsiA for the remaining samples are characteristic for non-stress conditions.

Around 725 nm, a slight blue-ward shift was observed for Goe samples, suggesting the presence of stress for cultures grown on this iron source. Only marginal differences were observed between WT and *Δ slr1928-31*-Kan cultures on the same iron source, except for a marginally higher peak at 685 nm for *slr1928-31*-Kan samples than WT samples on Goe. This result indicated that the mutant strain experienced slightly higher levels of stress.

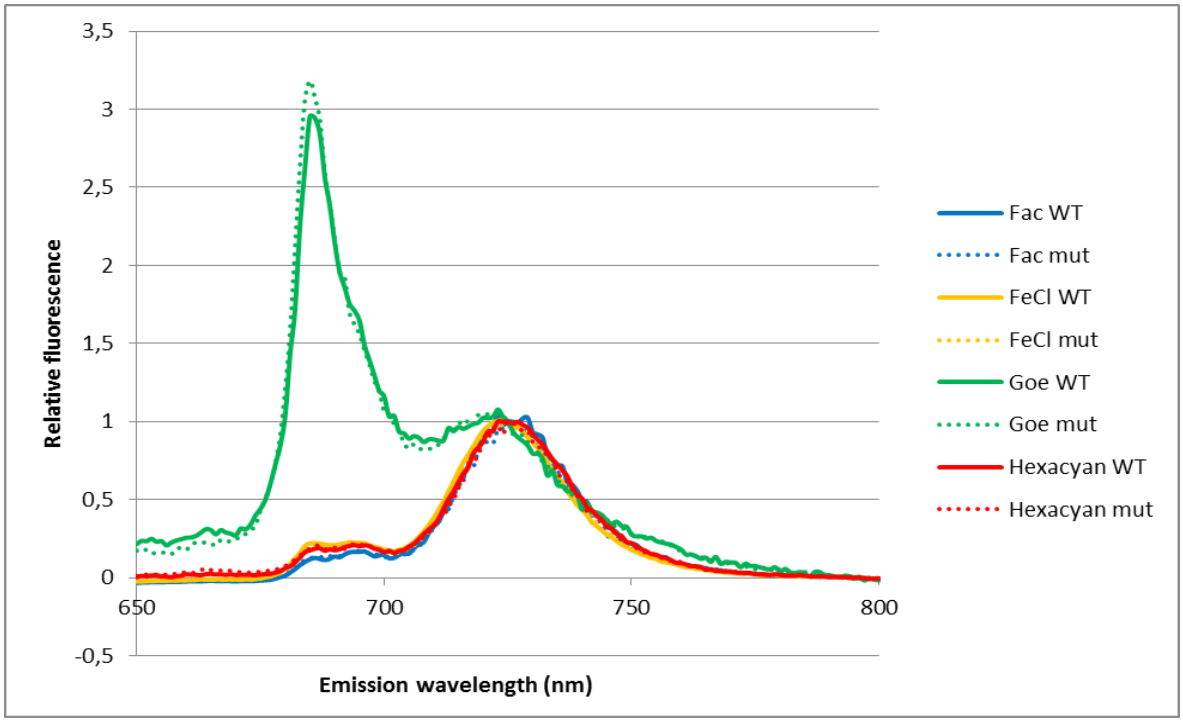

Figure 3.5.3.1: *77K fluorescence spectra for* Synechocystis *6803 WT and* Δ slr1928-31-*Kan (mut) cultures on media with different iron sources, with glucose and DCMU added. The spectra are normalized around 725 nm.*

3.5.4.: Media with NaCl

Figure 3.5.4.1 shows fluorescence spectra at 77 K for samples of *Synechocystis* 6803 WT and *Δ slr1928-31*-Kan cultures grown on BG-11 medium with 350 mM NaCl added. No spectrum was obtained for FeCl WT samples as there was too little growth to generate samples for spectroscopic measurements. For samples of both strains grown on Fac, as well as WT sample on Hexacyan and

Δ slr1928-31-Kan samples on FeCl or Goe, smooth spectra with clearly outlined peaks were attained.

Around 685 nm, the fluorescence for both strains grown on Hexacyan was quite similar to that of both strains grown on Fac. Markedly higher fluorescence peaks was observed for the mutant strain on FeCl or Goe. These observations indicated that the latter two cultures experienced some stress whereas cultures of both strains on Fac and Hexacyan did not. The WT culture on Goe yielded an irregular spectrum with large variations in peak discernibility due to too low sample volume. Hence, the fluorescence from this sample was less usable for evaluating the utility of Goe under the present condition.

The PS II/PS I fluorescence ratios also supported what was found by visual spectrum inspection: A notable increase in the PS II/PS I ratio was observed for the *Δ slr1928-31*-Kan culture on FeCl, and both strains on Goe, corresponding with the expected increase in PS II fluorescence during stress. The low fluorescence peaks for PS II and the low ratio values for PS II/PS I for the remaining samples are characteristic for non-stress conditions.

The PS II/IsiA ratios were very similar for all samples, also indicated by the lower peak for the Goe samples around 685 nm under this condition than for the Goe samples on media without NaCl. Apparently IsiA is not expressed to a great extent in either strain under this condition but the small sample volumes of the Goe samples might potentially have affected the result for this culture so that the effect could not be detected.

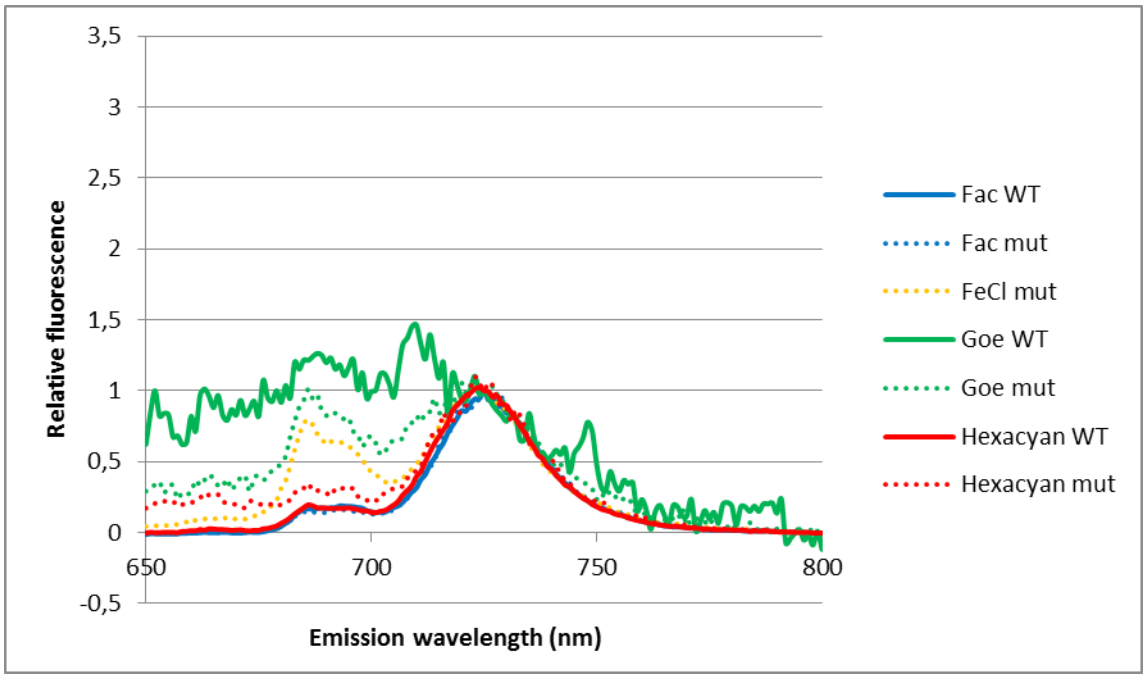

Figure 3.5.4.1: *77K fluorescence spectra for* Synechocystis *6803 WT and* Δ slr1928-31-*Kan (mut) cultures on media with different iron sources, with 350 mM NaCl added. The spectra are normalized around 725 nm.*

3.5.5: Media with glucose and NaCl

Figure 3.5.5.1 shows fluorescence spectra at 77 K for samples of *Synechocystis* 6803 WT and *Δ slr1928-31*-Kan cultures grown on BG-11 medium with glucose and 350 mM NaCl added. Spectra were not attained for WT cultures on Fac, FeCl or Hexacyan due to too little growth to generate samples for spectroscopic measurements.

Around 685 nm, the Goe samples yielded very high peaks, whereas Fac and Hexacyan samples were very similar and barely yielded any peak at all in this area. The peak for the *slr1928-31*-Kan culture on FeCl was higher than the latter two samples, substantially lower than Goe, and with an irregular curve due to a small sample volume. The high peak observed for the Goe samples implied that the stress-induced chlorophyll-binding protein IsiA was present.

The PS II/PS I fluorescence ratios were substantially higher for both strains on Goe than for the remaining samples, indicating an increase in PS II fluorescence associated with stress. A notable increase in the PS II/PS I ratio was also observed for the *Δ slr1928-31*-Kan culture on FeCl, corresponding with the higher peak in this area as compared to this strain on Fac or Hexacyan, and indicating stress in this FeCl culture.

The PS II/IsiA ratio for the *Δ slr1928-31*-Kan culture FeCl was similar to that of *Δ slr1928-31*-Kan culture on Fac. The mutant strain grown on Hexacyan gave a somewhat higher ratio value but all of these three samples were still quite similar. The low fluorescence peaks for PS II, the low ratio values for PS II/PS I and the high ratio values for PS II/IsiA for the *Δ slr1928-31*-Kan cultures on Fac or Hexacyan are characteristic for non-stress conditions. The PS II/IsiA ratios were markedly lower for both strains on Goe than for the remaining samples, indicating that stress induced expression of IsiA in the Goe cultures. The slightly irregular curve for the *Δ slr1928-31*-Kan culture on FeCl could be due to a small sample volume that might potentially have affected the result.

A somewhat higher peak was observed for *Δ slr1928-31*-Kan samples than for WT samples on Goe at this wavelength. For the remaining iron sources, only the WT cultures had sufficient growth for spectroscopic analysis. Around 720 nm, a slight blue-ward shift was observed for both strains on Goe and the mutant strain on FeCl, another indication that these cultures experience stress.

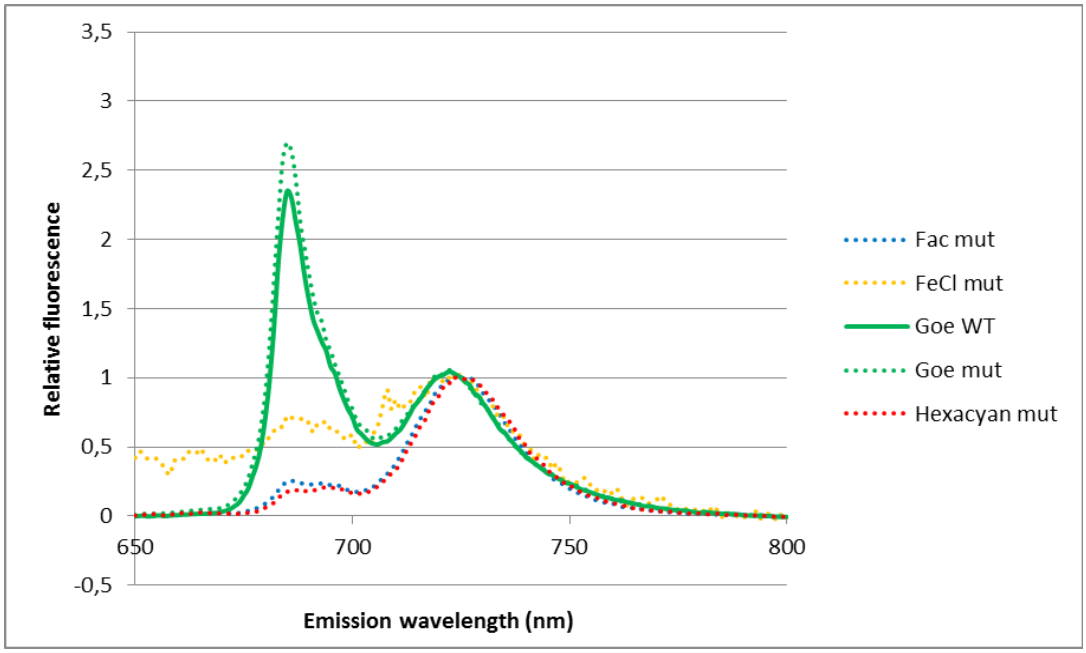

Figure 3.5.5.1: *77K fluorescence spectra for* Synechocystis *6803 WT and* Δ slr1928-31-*Kan (mut) cultures on media with different iron sources, with glucose and 350 mM NaCl added. The spectra are normalized around 725 nm.*

3.5.6: Media with glucose, DCMU and NaCl

No spectra were attained for these samples due to poor growth and consequently insufficient cell material for spectral characterization.

3.6: Important findings

There results suggested some interesting dissimilarities between the WT strain and the mutant strain *Δ slr1928-31*-Kan. The most prominent difference appeared when the cultures were grown on BG-11 media containing NaCl or NaCl and glucose. On some iron sources under this condition the mutant strain seemingly had better growth as compared to the WT. This was an unexpected result: The mutant strain lacks the operon *slr1928-31*, which is assumed to be involved in iron acquisition, and its growth would thus be expected to diminish. Possible explanations to this finding are given in the discussion section.

Another noticeable result was the apparent reversal of which iron sources were the more favorable on media containing NaCl, NaCl and glucose, or NaCl with glucose and DCMU. Under these conditions the water-insoluble iron sources FeO and Goe yielded better growth in most cases. This was in contrast to media without NaCl, on which the water-soluble iron sources Fac, FeCl and Hexacyan were preferred. Possible explanations to these results are given in the discussion section.

The results from the spectroscopic analysis suggested that stress is present in the cultures under some conditions. A very clear indication is the high peak around 685 nm in the 77 K spectra, associated with the stress-induced synthesis of IsiA. Slight shifts in the red Chl *a* peak in whole cell absorption spectra also implied stress under some conditions. Possible explanations for this are given in the discussion section. The absolute amounts of the pigments as indicated by the whole cell absorption spectra will not be included in the discussion as the relative amounts indicated by the absorption ratios for carotenoids/Chl *a* and phycobilins/Chl *a* give more valuable information about pigment changes.

4. Discussion

This project aimed to investigate the function of pilins in bacterial iron acquisition. A specific polycistronic operon in the cyanobacterium *Synechocystis* 6803 was selected for this study, consisting of the four genes *slr1928*, *slr1929*, *slr1930* and *slr1931* (*slr1928-31*). These genes all probably encode pilin-like proteins (Yoshihara *et al*., 2001). The motivation for investigating *slr1928-31* was based on the work of Singh and colleagues, demonstrating an increased transcription of genes similar to this operon upon chelation of iron in the medium (Singh et al, 2003). Furthermore recent research (Lamb et al., 2014) indicate that the pilin encoded by the gene *sll1694* is required for accessing iron locked in iron oxides he potential functions of the pilin-like genes *slr1928-31* were determined by deletion of the operon, followed by comparisons of WT and the mutant strain Δ *slr1928-31*-Kan through growth assays and spectroscopic analysis of pigment changes.

4.1: Growth assays comparing WT with the mutant strain Δ *slr1928-31* – Kan

4.1.1: Expected outcomes

In order to examine the role of the operon *slr1928-31* in iron acquisition, growth of WT and Δ *slr1928-31*-Kan cultures of *Synechocystis* 6803 were assessed on BG-11 media supplied with one of five different iron sources, and six different growth conditions for each iron source. Glucose was provided as a nutrient supplement, DCMU was added to inhibit PS II and NaCl was added to induce salt stress. The test conditions selected for this experiment allowed studies of different modes to obtain energy: (photoautotrophy, heterotrophy and cyclic electron transport) for a comprehensive growth analysis. Singh and colleagues (2005) have demonstrated potential functions of pilins related to those encoded by the operon *slr1928-31*. A possible involvement in iron acquisition was found, indicated by an up-regulation of pilin-encoding genes when the bacteria need to increase their efforts in iron uptake. Hence, the Δ *slr1928-31*-Kan strain was deficient in proteins that might normally aid the bacterium in iron acquisition. Pilins are possibly also involved in stress responses (Singh *et al*., 2005). Based on these findings, the expected outcome of the experiment was better growth for WT than for the Δ *slr1928-31*-Kan strain. The differences in growth were anticipated to be smaller under non-stress conditions and more prominent when iron acquisition is challenging or under other stressful conditions.

4.1.2: Utility of different iron sources under various growth conditions for Synechocystis 6803

4.1.2.1: Media without glucose, DCMU and NaCl

When grown on BG-11 media without glucose, DCMU and NaCl, growth of WT cultures was very similar to that of Δ *slr1928-31*-Kan cultures on all iron sources tested. The small differences observed between the strains under this condition did not appear to be significant according to the overlapping standard error bars in the growth curves. Cultures of *Synechocystis* 6803 WT seemed able to utilize all the provided iron sources equally. This also held true for cultures of the mutant strain Δ *slr1928-31*-Kan.

According to the results obtained, WT cultures seemed to gain iron in sufficient amounts for effective photosynthetic e^- transport and growth on all iron sources tested. The outcome was in line with prior expectations on media supplied with Fac, as BG-11 is the general growth medium used when culturing cyanobacteria, supporting normal photoautotrophic growth (Stanier, R. Y *et al*., 1971). The iron sources FeCl and Hexacyan are water-soluble (Science Lab MSDS, Sigma-Aldrich MSDS) and therefore quite easily accessible. Therefore, similar growth levels for WT cultures on these iron sources as compared to Fac could be expected. Cyanobacteria such as *Synechocystis* 6803 possess mechanisms to acquire iron from insoluble complexes. In line with this, cultures of *Synechocystis* 6803 WT seemed able to obtain iron from the less accessible, water-insoluble iron sources FeO (Sigma-Aldrich MSDS) or Goe (Pestell MSDS).

As the pilins encoded by the deleted operon *slr1928-31* were hypothesized to function in iron acquisition, the Δ *slr1928-31*-Kan cultures were anticipated to acquire less iron than WT cultures, and consequently grow more slowly due to less effective photosynthesis. Unexpectedly, the growth levels for cultures of the strain Δ *slr1928-31*-Kan were very similar to those for WT cultures on all iron sources tested. These observations indicated that the gene products from operon *slr1928-31* were not essential for iron acquisition under photoautotrophic – non stressed growth conditions. Consequently, the cells were able to fulfil their iron requirements by acquiring iron from any of the sources tested. The pilins encoded by this operon have more minor or indirect functions or roles in iron uptake under other growth conditions, or in stress responses as suggested by other research results (Singh *et al*., 2005).

4.1.2.2: Media with glucose

When grown on BG-11 media supplemented with glucose, growth of WT cultures was very similar to that of Δ *slr1928-31*-Kan cultures on all iron sources tested. Cultures of both strains grown on media with one of the iron sources FeCl, FeO or Goe showed similar growth to that on glucose-free

media. According to the overlapping error bars, the slight differences in growth between these iron sources were not statistically significant for either strain. In contrast, WT and Δ *slr1928-31*-Kan cultures grown on one of the iron sources Fac or Hexacyan showed significantly better growth than the other three iron sources, indicated by the standard error found in the respective growth curves. This trend was very clear for the Δ *slr1928-31*-Kan strain, for which the error bars of Fac and Hexacyan samples were not overlapping with those for the remaining three iron sources. The error bars for WT samples grown on Fac and Hexacyan were only slightly overlapping with the remaining iron sources. In addition, the trends were otherwise very similar to those observed for the mutant and therefore assumed to be the same. The results indicated that *slr1928-31* gene products were not involved in essential processes of iron acquisition under mixotrophic growth conditions.

Improved growth under this condition was possible because the cultures could use glucose as a carbon source in addition to CO_2. In order to utilize glucose to enhance growth, the cells also depended on maintaining the iron supply for photosynthesis and electron transport. A possibility here is that whenever the cultures could obtain iron for photosynthesis in ample amounts, they could utilize the glucose for additional energy and consequently increased growth. Therefore, a likely explanation for the increased growth on Fac and Hexacyan could be that these iron sources were soluble in water and the cultures could readily obtain adequate amounts of iron to drive photosynthesis and additionally utilize glucose. FeCl is also easily soluble but the chloride contents of this medium might challenge the bacteria to some extent so that the glucose added was not sufficient to improve growth. Iron from the insoluble compounds Goe and FeO is less accessible than in the other iron sources, and the cells put more effort into keeping up the level of growth when acquiring iron from these compounds.

4.1.2.3: Media with glucose and DCMU

When grown on BG-11 media containing both glucose and DCMU, growth of WT cultures was very similar to that of Δ *slr1928-31*-Kan cultures on all iron sources tested. Cultures of both strains grown on media containing glucose, DCMU and one of the iron sources Fac, FeCl and Hexacyan, showed similar growth to the respective samples on non-glucose and non-DCMU media. There were no significant differences in growth between samples on these iron sources. In contrast, cultures grown on FeO or Goe showed reduced growth as compared to the former three iron sources. The standard error found in the growth curves for these samples indicated that the difference was significant.

A possible mechanism by which *Synechocystis* 6803 may reduce ferric iron for uptake is e^- donation from PS II to external acceptors through pili. When DCMU is added to the growth medium, e^- flow from PS II is inhibited and cannot be utilized for iron acquisition. The reduced growth on the least accessible iron sources FeO and Goe corresponded with this, as a larger effort is required to acquire iron from these compounds as compared to those soluble in water. Fewer e^- available will limit the reduction of FeO and Goe prior to uptake, consequently slowing down the bacterial growth. Cultures of both strains were able to obtain adequate amounts of iron from the most accessible sources, Fac, FeCl and Hexacyan, as iron acquisition from these compounds required less reducing power.

The Δ *slr1928-31*-Kan strain showed similar growth to the WT strain on media containing glucose and DCMU for all the iron sources tested. This observation indicated that the operon *slr1928-31* was not involved in any essential processes for iron acquisition under this growth condition.

4.1.2.4: Media with NaCl

On BG-11 media with high levels of NaCl, some notable differences were observed between WT and Δ *slr1928-31*-Kan strains in that the latter strain showed better growth than WT on media supplied with the iron sources Fac or FeCl. The standard error found in the growth curves for the Δ *slr1928-31*-Kan strain on the various iron sources indicated that the difference in growth on media supplied with Fac or FeCl compared to the remaining iron sources was only statistically significant within certain time intervals of the growth period. Within these time intervals, no growth was observed for WT on media supplied with Fac or FeCl.

Significantly higher levels of growth for Δ *slr1928-31*-Kan cultures than WT cultures indicated that the operon *slr1928-31* may be involved in the cell's ability to sense salt content in its environment and initiate the appropriate counter-acting responses. The operon is functioning normally in WT cultures, resulting in reduced growth. The deletion of the operon *slr1928-31* would impair the sensory system so that the Δ *slr1928-31*-Kan cultures could continue their growth without initiating energy-requiring salt stress responses. Stress responses in bacteria have evolved so that the cells can adjust to and survive challenging conditions. A mutation causing defective responses to stress would be expected to have some negative effects for the bacterium. The growth period of 10 days in this experiment appeared to be insufficient in order to observe the detrimental effects that NaCl eventually will bring about. In future experiments on the function of the operon *slr1928-31* the growth period should be longer in order to detect these effects.

The growth of the Δ *slr1928-31*-Kan strain was low on media with NaCl for all iron sources tested. Reduced growth under conditions of high NaCl content as compared to conditions without NaCl indicated that the mutant cultures are still experiencing salt stress despite their seemingly reduced sensitivity. The growth was significantly better for Δ *slr1928-31*-Kan than for WT on Fac and FeCl, suggesting that the concentration of 350 mM NaCl did not seem to be sufficient to induce processes for salt acclimation in these less sensitive cultures. Comparisons with WT indicated that stress responses were initiated normally on FeO, Goe and Hexacyan, as the growth of both strains was similar on these iron sources.

On media without NaCl, the water-soluble iron sources did not appear to induce any stress on the cultures, whereas the less accessible compounds seemed more challenging. An apparent reversal of these characteristics was observed in presence of NaCl, as cultures of both strains showed better growth on FeO and Goe as compared to Fac and FeCl, and the same growth levels as Hexacyan, under this condition. A possible explanation might be that NaCl could affect the complex-forming properties of the iron compounds, thus altering the access to them. Iron from the water-soluble compounds Fac, FeCl and Hexacyan might precipitate under high-NaCl conditions and become less accessible for ferric iron reduction, whereas iron from oxide complexes (FeO and Goe) could be more readily accessed by this mechanism.

4.1.2.5: Media with NaCl and glucose

On BG-11 media with NaCl and glucose, some notable differences were observed between WT and Δ *slr1928-31*-Kan cultures. The mutant strain showed growth on Fac and Hexacyan, whereas WT cultures did not grow on these iron sources. No growth was observed for either strain on FeCl. On FeO and Goe, the growth was similar for both strains. The differences in growth between the strains on media with glucose, NaCl and one of the iron sources Fac or Hexacyan were statistically significant according to the standard error found in their respective growth curves. These results suggested that the concentration of 350 mM NaCl was insufficient to induce processes for salt acclimation in the less sensitive Δ *slr1928-31*-Kan cultures. Comparisons with WT cultures indicated that stress responses were initiated normally on FeO, Goe and FeCl, as the growth levels for both strains were similar on these iron sources.

The observations of better growth on media with NaCl and glucose supplied with either FeO or Goe as compared to Fac, FeCl or Hexacyan, corresponded with the results for glucose-free media with NaCl. Nevertheless, there were some differences between these conditions. Cultures of Δ*slr1928-31*-Kan had some growth on glucose-free medium supplied with FeCl, but no growth on this iron

source on glucose-supplemented media. Cultures of WT had some growth on glucose-free medium supplied with Hexacyan, but no growth on this iron source on glucose-supplemented media. These results might possible be due to altered solubility or complex-forming characteristics of these iron sources when glucose is added to a medium with NaCl.

4.1.2.6: Media with NaCl, glucose and DCMU

When grown on BG-11 media with NaCl, glucose and DCMU, growth of WT cultures was very similar to that of Δ*slr1928-31*-Kan cultures on all iron sources tested. No growth was observed for either strain on media supplied with Fac, FeCl or Hexacyan. Growth was observed for both strains on media supplied with FeO and Goe, suggesting that the cultures could acquire iron from these sources. This observation was in agreement with what was observed for media with NaCl and media with NaCl and glucose, for which a possible precipitation of the normally water-soluble iron sources reduced their availability. Despite low levels of growth on both iron sources, growth on FeO was significantly better than on Goe for both strains. This corresponds with the fact that Goe is a more complex iron source. The low levels of growth implied that the growth conditions were challenging for both strains. As DCMU inhibited photosynthesis and possibly limited iron reduction, and NaCl induced stress in the cultures, a reduction in growth under the given condition was expected.

As the differences in growth between WT and Δ*slr1928-31*-Kan cultures on media with NaCl, glucose and DCMU were minimal, the stress levels under this condition seemed to override the effects of decreased stress sensitivity in the mutant. Hence, the stress levels may have been sufficient to initiate a stress response also in Δ*slr1928-31*-Kan samples.

4.2: Whole cell room temperature absorption spectroscopy

Whole cell absorption spectroscopy was performed to detect differences in pigment content between WT and the mutant strain Δ*slr1928-31*-Kan. The mutant strain was expected to show some impairment in utilization of iron and a higher propensity to exhibit signs of stress. Additionally, the presence of NaCl in the medium as well as provision of water-insoluble iron sources are factors expected to induce stress. Under stress conditions, cultures of *Synechocystis* 6803 will normally have less phycobilins than Chl *a*, and more carotenoids than Chl *a*. The relative amounts of each pigment can be determined by calculating the ratios between their absorption peaks, 435 nm/680 nm for carotenoids/Chl *a*, and 625 nm/680 nm for phycobilins/Chl *a* (Lamb *et al*., 2014). The Chl *a* peak around 680 nm tends to shift towards shorter wavelengths, i.e. the blue part of spectrum, under stress conditions. This shift is often associated with the presence of the stress induced light-

harvesting complex. The absolute amounts of the pigments as indicated by the spectra are not discussed here as the relative amounts indicated by the absorption ratios for carotenoids/Chl *a* and phycobilins/Chl *a* give more valuable information about pigment changes. Culture samples grown on media supplied with FeO were not included in these measurements as its properties were similar to those of Goe.

It was expected to obtain spectra for all samples for which growth had been observed. For media with NaCl, spectra were only attained for WT cultures on Hexacyan, and Δ*slr1928-31*-Kan cultures on Fac or FeCl. For media with NaCl and glucose, spectra were only attained for WT cultures on Goe, and Δ*slr1928-31*-Kan cultures on Fac, Goe or Hexacyan. For media with NaCl, glucose and DCMU, no spectra were attained. Growth was also observed for WT cultures on NaCl and Goe, Δ*slr1928-31*-Kan cultures on NaCl and Goe or Hexacyan, and both strains on NaCl with DCMU on Goe. A possible explanation of why spectra were only attained for some samples was that low growth for all samples on media containing NaCl yielded small volumes in order to achieve an OD of 1. As the whole cell absorption method required a minimum sample volume to measure absorption, small variations in volume may have incidentally enabled or prevented the measurement.

4.2.1: Carotenoid/Chl a ratios

In this section the relative increase or decrease in carotenoids as compared to Chl *a* under different conditions is discussed. The exact ratio values are given in section 3.x.

4.2.1.1: Differences between WT and the mutant strain Δ *slr1928-31*-Kan

The differences in carotenoids/Chl *a* absorption ratios between cultures of *Synechocystis* 6803 WT and the mutant strain Δ*slr1928-31*-Kan were only marginal for all media without NaCl. The differences in ratio values ranged from 0.004 up to 0.12. These numbers are very small, implying that there is no real difference in carotenoid to Chl *a* levels between the strains on media without NaCl. This corresponded with the growth assay results, which indicated that there were no significant differences in growth between the strains.

For all media with NaCl or NaCl and glucose, except for Hexacyan on NaCl and Goe on NaCl and glucose, spectra and consequently carotenoid/Chl *a* absorption ratios were only attained for Δ*slr1928-31*-Kan samples. This corresponded with the outcome of the growth assay for samples for which growth was observed only for Δ*slr1928-31*-Kan cultures: On Fac or FeCl with NaCl, and on Fac and Hexacyan on glucose-supplemented media with NaCl. The samples for which growth was observed but no spectra attained, both strains showed similar growth levels under the same

conditions. Therefore, no differences would be expected in carotenoid/Chl *a* ratios for these particular samples had spectra been attained.

4.2.1.2: Differences between the different test conditions

Based on the absorption ratios of carotenoids and Chl *a,* there were some differences between the growth conditions tested. This was in line with prior expectations as the iron sources had varying degrees of availability and the different additives made the conditions either more favorable (glucose) or more challenging (DCMU and NaCl) for bacterial growth. The carotenoid/Chl *a* absorption ratio for WT culture on glucose-free, DCMU-free and non-NaCl medium supplied with Fac was assumed to be a standard to which the ratios under other conditions could be compared.

4.2.1.3: Media without glucose, DCMU or NaCl

The difference in carotenoid/Chl *a* absorption ratios between Fac and the remaining iron sources was approximately 0.5 or more for all samples under the standard condition, with Fac ratios as the lowest values. These results suggest that Fac was the most suitable iron source for *Synechocystis* 6803 cultures, in accordance with this being the iron source of choice for cyanobacterial growth. Despite Fac being the preferred iron source, the other iron compounds were also expected to be readily utilized under this growth condition as no particular stress-inducing factors were present. This was supported by similar growth on all iron sources. Nevertheless, the cells appeared to experience some stress according to the high carotenoid/Chl *a* absorption ratios. As indications of stress was only observed in whole cell absorption spectra but not in the growth assay, the stress induced by providing another and probably less accessible iron source than Fac appeared to be too subtle to affect growth. This observation corresponded with the non-stress conditions for growth that likely made the varying degree of availability of the iron sources less weighty.

4.2.1.4: Media with glucose

On media with glucose Fac samples gave lower carotenoid/Chl *a* absorption ratios than samples on the other iron sources. This result was in line with the anticipated outcome as Fac was expected to be the most ideal iron source. The ratios were similar for the remaining iron sources FeCl, Goe and Hexacyan. Based on the growth assay, Fac and Hexacyan samples could be expected to have about the same ratio values as their growth levels were very similar. The higher carotenoid/Chl *a* absorption ratios for Hexacyan samples indicated that Fac was a more suitable iron source than Hexacyan, but that the stress induced by providing Hexacyan instead of Fac was too subtle to affect growth. The same held true for the other iron sources FeCl and Goe. These observations

corresponded with the non-stress conditions for growth that likely made the varying degree of availability of the iron sources less weighty.

4.2.1.5: Media with glucose and DCMU

On media with glucose and DCMU the highest carotenoid/Chl *a* absorption ratios were observed for the Goe samples, followed by the FeCl samples, and last the Hexacyan samples only slightly higher than the Fac samples. These results corresponded with the expected increase in stress levels as the iron sources became less accessible: Goe was least accessible, followed by FeCl, Hexacyan, and last Fac as the most readily available source of iron. The ratio values are lower for this condition than for media with no additives and media with glucose for samples with FeCl or Hexacyan as iron source. These results were in conflict with prior expectations as DCMU-containing media could be regarded as more challenging for growth and more likely to induce stress. A possible explanation might be that pigment changes were less prominent signs of stress when photosynthesis was inhibited. A higher level of stress may be required in order for detection by this method.

4.2.1.6: Media with NaCl

On media with NaCl, the carotenoid/Chl *a* absorption ratio for the Δ*slr1928-31*-Kan sample on Fac had the same ratio value as the standard, indicating that the cultures were not stressed. The ratio was substantially higher for the FeCl sample than those on Fac or Hexacyan, implying that this condition induced a greater level of stress. This result was in line with the poor growth observed on FeCl but conflicted with the poor growth on Fac and the assumption that the Δ*slr1928-31*-Kan strain is less sensitive to stress. Perhaps a certain level of stress was required for the mutant to sense it, and an even higher level in order to initiate the appropriate responses. Following this assumption, the mutant cultures were sensing stress on medium with FeCl but not medium with Fac as FeCl was likely to be more challenging to grow on. In both cases the stress levels were apparently insufficient for the less sensitive mutant strain to respond properly. The WT sample on Hexacyan had about the same ratio value as for samples with this iron source on media without additives or with glucose supplement. This result was expected as this culture was able to acclimatize to NaCl-induced stress.

4.2.1.7: Media with NaCl and glucose

On media with NaCl and glucose, the carotenoid/Chl *a* absorption ratios were similar for all samples: Both strains on Goe, and Δ*slr1928-31*-Kan samples on Fac or Hexacyan. The latter sample had a slightly higher ratio than the other samples. This outcome is in accordance with the growth levels for Goe and Hexacyan samples, as both Goe samples had similar growth whereas the Δ*slr1928-31*-Kan strain on Hexacyan grew more poorly and therefore presumably experienced

more stress. The Δ*slr1928-31*-Kan cultures showed less growth on Fac than on Goe under this condition. Nevertheless, these samples had the same carotenoid/Chl *a* absorption ratio, indicating that they had the same level of stress. The culture on Fac was assumed to be unable to initiate normal stress responses whereas the culture on Goe was assumed to respond normally. It is possible that the Δ*slr1928-31*-Kan strain on Goe was acclimatizing and its stress levels decreasing. The stress levels sensed by the mutant strain might have increased more slowly than for WT, so that this culture seemed less stressed at the time. If the measurements had been carried out after a longer period of growth, the effect of the mutation could have been more profound in that the damage of the cell became extensive before the stress response system was initiated. The lower levels of growth on Fac as compared to Goe could have been due to the relative availability of the iron sources and was apparently not affecting the stress levels.

4.2.2: Phycobilin/Chl a ratios

In this section the relative increase or decrease in phycobilins as compared to Chl *a* under different conditions is discussed. The exact ratio values are given in section 3.x.

4.2.2.1: Differences between WT and the mutant strain Δ *slr1928-31*-Kan

The differences in phycobilin/Chl *a* absorption ratios between cultures of *Synechocystis* 6803 WT and the mutant strain Δ*slr1928-31*-Kan were only marginal for all media without NaCl. The differences in ratio values ranged from 0 up to 0.05. These numbers are very small, implying that there is no real difference in phycobilin to Chl *a* levels between the strains on media without NaCl. This corresponded with the growth assay results, which indicated that there were no significant differences in growth between the strains.

For all media with NaCl or NaCl and glucose, except for Hexacyan on NaCl and Goe on NaCl and glucose, spectra and consequently carotenoid/Chl *a* absorption ratios were only attained for Δ*slr1928-31*-Kan samples. This corresponded with the outcome of the growth assay for samples for which growth was observed only for Δ*slr1928-31*-Kan cultures: On Fac or FeCl with NaCl, and on Fac and Hexacyan on glucose-supplemented media with NaCl. The samples for which growth was observed but no spectra attained, both strains showed similar growth levels under the same conditions. Therefore, no differences would be expected in carotenoid/Chl *a* absorption ratios for these particular samples had spectra been attained.

4.2.2.2: Differences between the different test conditions

Based on the absorption ratios of phycobilins and Chl *a,* there were some differences between the growth conditions tested. This was in line with prior expectations as the iron sources had varying degrees of availability and the different additives made the conditions either more favorable (glucose) or more challenging (DCMU and NaCl) for bacterial growth. The phycobilin/Chl *a* absorption ratio for WT culture on glucose-free, DCMU-free and non-NaCl medium supplied with Fac was assumed to be a standard to which the ratios under other conditions could be compared.

4.2.2.3: Media without glucose, DCMU or NaCl

The difference in phycobilin/Chl *a* absorption ratios between Fac and the remaining iron sources on media without glucose, DCMU or NaCl were less prominent than the differences found in carotenoid/Chl *a* absorption ratios. The Fac samples had the lowest ratio values but the largest difference was only about 0.16 (when compared to the WT culture on FeCl), which can be considered small. Hence, the samples were not really different, as was expected due to absence of any stress-inducing factors and also supported by similar growth on all iron sources.

4.2.2.4: Media with glucose

On media with glucose, Fac samples gave lower phycobilin/Chl *a* absorption ratios than samples on the other iron sources. The ratios were however only marginally higher for FeCl samples, and slightly higher for Goe samples. Therefore, these samples probably had about the same levels of stress. These observations corresponded with the non-stress conditions for growth that likely made the varying degree of availability of the iron sources less weighty. A larger difference was observed between Fac and Hexacyan samples. Based on the growth assay, Hexacyan samples could also be expected to have about the same ratio values as Fac due to their similar growth levels. The higher phycobilin/Chl *a* absorption ratios for Hexacyan samples indicated that the stress levels were lower in these samples than those grown on Fac. In contrast, the carotenoid/Chl *a* absorption ratios for Fac samples had the lower value, which indicates low stress levels. These conflicting results do not give any basis for pointing out one of the iron sources as the more ideal under this condition.

4.2.2.5: Media with glucose and DCMU

On media with glucose and DCMU, phycobilin/Chl *a* absorption ratios were similar for all samples, with 0.12 as the largest difference. The ratio values are higher for all samples under this condition than for the standard sample. This was conflicting with prior expectations as DCMU-containing media could be regarded as more challenging for growth and more likely to induce stress. A possible explanation might be that pigment changes were less prominent signs of stress when

photosynthesis was inhibited. A higher level of stress may be required in order for detection by this method.

4.2.2.6: Media with NaCl

On media with NaCl, the phycobilin/Chl *a* absorption ratios were similar for Δ*slr1928-31*-Kan cultures on FeCl and WT cultures on Hexacyan. Both of these had higher ratios than Δ*slr1928-31*-Kan cultures on Fac. The latter sample had the same ratio value as the standard. Based on the results from the growth assay it was assumed that the stress response in the mutant strain on Fac or FeCl was defective. For WT cultures on Hexacyan however the response is functioning normally. Accordingly, the latter culture is sensing stress in a normal fashion whereas the other two cultures are not. It is possible that the WT strain on Hexacyan was acclimating and its stress levels decreasing. The stress levels sensed by the mutant strain on Fac or FeCl might have increased more slowly than for WT, so that these cultures seemed less stressed. If the measurements had been carried out after a longer period of growth, the effect of the mutation could have been more profound in that the damage of the cell became extensive before the stress response system was initiated. The reason why cultures on FeCl appeared to be less stressed than those on Fac remains unclear. The somewhat lower levels of growth on FeCl compared to Hexacyan could have been due to the relative availability of these iron sources and were apparently not affecting the stress levels as the ratios were the same.

4.2.2.7: Media with NaCl and glucose

On media with NaCl and glucose, phycobilin/Chl *a* absorption ratios were higher for both strains on Goe than Δ*slr1928-31*-Kan samples on Fac and Hexacyan. The former two samples had very similar ratios, as had the latter two samples. The latter two samples also had reduced ratio values as compared to the standard, whereas ratios for Goe samples were increased. According to these observations, Δ*slr1928-31*-Kan cultures on Fac and Hexacyan had higher levels of stress than the standard whereas Δ*slr1928-31*-Kan cultures on Goe were less stressed. This outcome corresponded with the improved growth on Goe and poor growth on Fac and Hexacyan under this condition, as compared to their respective samples on standard BG-11 supplied with this iron source.

4.3: 77 K fluorescence spectroscopy

The analysis method 77K fluorescence spectroscopy was performed to detect differences in Chl *a* emissions from PS I and PS II between WT and the mutant strain Δ*slr1928-31*-Kan. Expression of the stress-induced chlorophyll-binding protein IsiA may also be visualized by this method. Under stress conditions, a high peak associated with IsiA will normally appear at 685 nm in 77K emission

spectra. The PS I peak around 720 nm also tends to shift towards shorter wavelengths, i.e. the blue part of spectrum. The shifted peak is associated with a complex formed by IsiA and PS I. The Δ*slr1928-31*-Kan strain was expected to show a higher level of stress. Cell samples grown on media supplied with FeO were not included in these measurements as its properties were similar to those of Goe. Under non-stress conditions, the fluorescence from PS II will be low relative to that from PS I, indicated by the ratio between their respective absorption maxima: 685 nm and 695 nm for PS II, and 725 nm for PS I. Under stress conditions, IsiA will be expressed and yield a high fluorescence peak around 685 nm. The ratio between the specific fluorescence maxima of PS II and IsiA could be used to determine the relative amounts of these components (Lamb *et al*., 2014).

4.3.1: Media without glucose, DCMU or NaCl

Fluorescence spectra at 77 K for samples of *Synechocystis* 6803 WT and *Δ slr1928-31*-Kan cultures grown on BG-11 medium without glucose, DCMU or NaCl showed similar spectra for cultures grown on Fac, FeCl and Hexacyan. Around 685 nm, both strains on Goe had very high peaks, whereas the remaining samples were very similar to each other and barely yielded any peaks at all in this area. The high peak observed for the Goe samples implied stress-induced expression of the chlorophyll-binding protein Isi in the Goe samples. This observation corresponded with prior expectations as Goe was the most complex iron source tested. It was therefore harder to utilize than the remaining iron compounds and consequently more likely to induce stress in the *Synechocystis* 6803 cultures.

An increased stress level was also indicated by the fluorescence ratios: The PS II/PS I ratios were substantially higher for both strains on Goe than for the remaining samples, indicating an increase in PS II fluorescence that is associated with stress. The PS II/IsiA ratios were markedly lower for both strains on Goe than for the remaining samples, indicating that stress induced expression of IsiA in these cultures. Again, these results were expected due to the complexity and lower availability of Goe as compared to the remaining iron sources.

The low fluorescence peaks for PS II and the low ratio values for PS II/PS I for the remaining samples are characteristic for non-stress conditions. This could be expected as this growth condition did not include any particular stress-inducing factors. However, the low availability of Goe seemed to be sufficient to induce some stress. Around 720 nm, a slight blue-ward shift was observed for both strains on Goe, also suggesting presence of stress for cultures grown on this iron source.

The ratio values for PS II/IsiA absorption are highest for both cultures on Fac, somewhat lower for both strains on Hexacyan than those for the respective Fac samples, even lower for both strain on

FeCl, and lowest for both cultures on Goe. The stress levels and thus the amounts of IsiA thus seemed lowest in the Fac samples, increased for Hexacyan and FeCl, and the highest in the Goe samples. This result corresponded with the expected increase in stress with decreasing availability of the iron sources. As no increase was seen for PS II fluorescence, the stress levels were presumably not very high and the cultures could still grow normally.

Only marginal differences were observed between WT and *Δ slr1928-31*-Kan cultures on the same iron source, suggesting that there is no real difference between the strains under this condition. One exception was for the slightly higher peak at 685 nm for *Δ slr1928-31*-Kan samples than WT samples on Goe. This result indicated that the mutant strain experienced slightly higher levels of stress, which was expected due to the presumably less effective iron acquisition or utilization of the *Δ slr1928-31*-Kan cultures than WT cultures.

4.3.2: Media with glucose

Fluorescence spectra at 77 K for samples of *Synechocystis* 6803 WT and *Δ slr1928-31*-Kan cultures grown on BG-11 medium with glucose showed similar spectra for cultures grown on Fac, FeCl and Hexacyan. Around 685 nm, both strains on Goe had very high peaks, whereas the remaining samples were very similar to each other and barely yielded any peaks at all in this area. The high peak observed for the Goe samples implied stress-induced expression of the chlorophyll-binding protein Isi in the Goe samples. This observation corresponded with prior expectations as Goe was the most complex iron source tested. It was therefore harder to utilize than the remaining iron compounds and consequently more likely to induce stress in the *Synechocystis* 6803 cultures.

An increased stress level was also indicated by the fluorescence ratios: The PS II/PS I ratios were substantially higher for both strains on Goe than for the remaining samples, indicating an increase in PS II fluorescence that is associated with stress. The PS II/IsiA ratios were markedly lower for both strains on Goe than for the remaining samples, indicating that stress induced expression of IsiA in these cultures. Again, these results were expected due to the complexity and lower availability of Goe as compared to the remaining iron sources.

The low fluorescence peaks for PS II and the low ratio values for PS II/PS I for the remaining samples are characteristic for non-stress conditions. This could be expected as this growth condition did not include any particular stress-inducing factors. However, the low availability of Goe seemed to be sufficient to induce some stress. Around 720 nm, a slight blue-ward shift was observed for both strains on Goe, also suggesting presence of stress for cultures grown on this iron source.

The ratio values for PS II/IsiA absorption are highest for both cultures on Fac, somewhat lower for both strains on Hexacyan than those for the respective Fac samples, even lower for both strain on FeCl, and lowest for both cultures on Goe. The stress levels and thus the amounts of IsiA thus seemed lowest in the Fac samples, increased for Hexacyan and FeCl, and the highest in the Goe samples. This result corresponded with the expected increase in stress with decreasing availability of the iron sources. As no increase was seen for PS II fluorescence, the stress levels were presumably not very high and the cultures could still grow normally.

Only marginal differences were observed between WT and *Δ slr1928-31*-Kan cultures on the same iron source, suggesting that there is no real difference between the strains under this condition. One exception was for the slightly higher peak at 685 nm for *Δ slr1928-31*-Kan samples than WT samples on Goe. This result indicated that the mutant strain experienced slightly higher levels of stress, which was expected due to the presumably less effective iron acquisition or utilization of the *Δ slr1928-31*-Kan cultures than WT cultures.

4.3.3: Media with glucose and DCMU

Fluorescence spectra at 77 K for samples of *Synechocystis* 6803 WT and *Δ slr1928-31*-Kan cultures grown on BG-11 medium with glucose and DCMU showed similar spectra for cultures grown on Fac, FeCl and Hexacyan. Around 685 nm, both strains on Goe had very high peaks, whereas the remaining samples were very similar to each other and barely yielded any peaks at all in this area. The high peak observed for the Goe samples implied stress-induced expression of the chlorophyll-binding protein Isi in the Goe samples. This observation corresponded with prior expectations as Goe was the most complex iron source tested. It was therefore harder to utilize than the remaining iron compounds and consequently more likely to induce stress in the *Synechocystis* 6803 cultures.

An increased stress level was also indicated by the fluorescence ratios: The PS II/PS I ratios were substantially higher for both strains on Goe than for the remaining samples, indicating an increase in PS II fluorescence that is associated with stress. The PS II/IsiA ratios were markedly lower for both strains on Goe than for the remaining samples, indicating that stress induced expression of IsiA in these cultures. Again, these results were expected due to the complexity and lower availability of Goe as compared to the remaining iron sources.

The low fluorescence peaks for PS II, the low ratio values for PS II/PS I and the high ratio values for PS II/IsiA for the remaining samples are characteristic for non-stress conditions. This could be expected as this growth condition did not include any particular stress-inducing factors. However, the low availability of Goe seemed to be sufficient to induce some stress. Around 720 nm, a slight

blue-ward shift was observed for both strains on Goe, also suggesting presence of stress for cultures grown on this iron source.

Only marginal differences were observed between WT and *Δ slr1928-31*-Kan cultures on the same iron source, suggesting that there is no real difference between the strains under this condition. One exception was for the slightly higher peak at 685 nm for *Δ slr1928-31*-Kan cultures than WT cultures on Goe. This result indicated that the *Δ slr1928-31*-Kan strain experienced slightly higher levels of stress, which was expected due to the presumably less effective iron acquisition or utilization of the *Δ slr1928-31*-Kan cultures than WT cultures.

4.3.4: Media with NaCl

Fluorescence spectra at 77 K for samples of *Synechocystis* 6803 WT and *Δ slr1928-31*-Kan cultures grown on BG-11 medium with NaCl showed similar spectra for cultures grown on Fac and Hexacyan. For samples of both strains grown on Fac, as well as WT sample on Hexacyan and *Δ slr1928-31*-Kan samples on FeCl or Goe, smooth spectra with clearly outlined peaks were attained. This indicated that their cell densities were sufficient for fluorescence detection. No spectrum was obtained for the FeCl WT sample, corresponding with the lack of growth for this sample and consequently too little growth to generate samples for spectroscopic measurements. No growth was observed for the WT culture on Fac under this condition but a spectrum was attained. This indicated that more cell material was present in the Fac sample as compared to the FeCl sample, sufficient for a measurement to be performed. According to these results, Fac appeared a better iron source than FeCl also under conditions in which iron might precipitate from both of these compounds, suggesting that Fac precipitates into more readily accessible complexes than does FeCl.

Around 685 nm, the fluorescence for both strains grown on Hexacyan was quite similar to that of both strains grown on Fac. Markedly higher fluorescence peaks was observed for the *Δ slr1928-31*-Kan strain on FeCl or Goe. These observations indicated that the latter two cultures experienced some stress whereas cultures of both strains on Fac and Hexacyan did not. The apparent differences in stress levels do not seem to be related to growth, as lower or higher growth levels do not correspond with presence or absence of stress. In the mutant strain the absence of stress correspond with the supply of the most accessible iron sources, Fac or Hexacyan. Also, no stress was present in the WT culture on Hexacyan whereas the Fac sample did not yield sufficient cell density to attain a spectrum. No meaningful spectra were attained for WT on any other iron source so the stress levels present in these cultures are hard to determine. For the *Δ slr1928-31*-Kan strain however, the stress levels can be assessed from the spectra and fluorescence ratios. A possible explanation for the

differences in stress levels could be that the cultures in which stress is absent were able to initiate stress responses and acclimatize, whereas the stressed cultures were not. These findings supported the hypothesized defects in stress responses when the operon *slr1928-31* is missing, delaying the acclimation and requiring a higher stress level to trigger the sensory mechanisms. This also corresponded with the assumption that Fac and Hexacyan were less accessible in the presence of NaCl, and increased the stress to a level sufficient for the cells to initiate a response. The stress observed for the *Δ slr1928-31*-Kan samples on FeCl or Goe was likely caused by NaCl and not by iron deficiency. The WT culture on Goe yielded an irregular spectrum with large variations in peak discernibility due to too low sample volume. Hence, the fluorescence here was less usable and the actual stress levels in this culture were hard to evaluate.

The PS II/PS I fluorescence ratios also supported what was found by visual spectrum inspection: A notable increase in the PS II/PS I ratio was observed for the *Δ slr1928-31*-Kan culture on FeCl and Goe, corresponding with the expected increase in PS II fluorescence during stress. The low fluorescence peaks for PS II and the low ratio values for PS II/PS I for both strains on Hexacyan and Fac are characteristic for non-stress conditions.

The PS II/IsiA ratios were very similar for all samples, also indicated by the lower peak for the Goe samples around 685 nm under this condition than for the Goe samples on media without NaCl. Apparently IsiA was not expressed to a great extent in either strain under this condition, suggesting that the levels of stress were only moderate and not sufficient to induce IsiA expression.

4.3.5: Media with NaCl and glucose

Fluorescence spectra at 77 K for samples of *Synechocystis* 6803 WT and *Δ slr1928-31*-Kan cultures grown on BG-11 medium with NaCl and glucose showed similar spectra for *Δ slr1928-31*-Kan cultures grown on Fac and Hexacyan. For these two samples as well as both strains on Goe, smooth spectra with clearly outlined peaks were attained. This indicated that their cell densities were sufficient for fluorescence detection. Spectra were not obtained for WT cultures on Fac, FeCl or Hexacyan, corresponding with the lack of growth for these samples and consequently too little growth to generate samples for spectroscopic measurements. No growth was observed for the *Δ slr1928-31*-Kan culture on FeCl under this condition but a spectrum was attained. This indicated that sufficient cell material was present in this sample.

Around 685 nm, the Goe samples yielded very high peaks, whereas Fac and Hexacyan samples were very similar and barely yielded any peak at all in this area. The high peak observed for the Goe samples implied that the stress-induced chlorophyll-binding protein IsiA was present. The PS

II/PS I fluorescence ratios were substantially higher for both strains on Goe than for the remaining samples, indicating an increase in PS II fluorescence associated with stress. As very similar spectra were seen for both strains on Goe, the stress responses seemed to be normal in the mutant. Additionally, the better growth on Goe as compared to the remaining iron sources on medium with NaCl and glucose is in conflict with the much higher stress levels for the cultures on this iron source. According to the conflicting results more research is required to establish the relation of growth and stress on various iron sources on media containing NaCl.

A notable increase in the PS II/PS I ratio was also observed for the *Δ slr1928-31*-Kan culture on FeCl, corresponding with the higher peak in this area as compared to this strain on Fac or Hexacyan, and indicating stress in this FeCl culture. However, the curve for the mutant culture on FeCl was irregular, probably due to a small sample volume, and the information from this spectrum should be considered less reliable.

The PS II/IsiA ratios for the *Δ slr1928-31*-Kan cultures on FeCl and Hexacyan were similar, and both were higher than the PS II/IsiA ratios for both strains on Goe. The slightly irregular curve for the *Δ slr1928-31*-Kan culture on FeCl could be due to a small sample volume that might potentially have affected the result, and is thus omitted here. The results indicated that the Goe cultures experienced stress whereas the *Δ slr1928-31*-Kan cultures on Fac and Hexacyan did not. This corresponded with the relative availability of the iron sources, as Fac and Hexacyan are usually more readily available than Goe. However, the results were in conflict with the better growth on Goe and presumed precipitation of Fac and Hexacyan under this condition. Accordingly, more research is required to establish the relation of growth and stress on various iron sources on media containing NaCl.

A somewhat higher peak was observed for *Δ slr1928-31*-Kan samples than for WT samples on Goe at this wavelength. This result indicated that the mutant strain experienced slightly higher levels of stress, which was expected given that the mutant seemed to initiate stress responses normally under this condition but probably somewhat less effectively than WT.

For the remaining iron sources, only the WT cultures had sufficient growth for spectroscopic analysis. Around 720 nm, a slight blue-ward shift was observed for both strains on Goe and the mutant strain on FeCl, another indication that these cultures experience stress.

4.4: Further work

This research project has identified a potential role in stress responses of the pilin-like proteins encoded by the operon *slr1928-31*. An effect of the lack of the pilins encoded by this operon was observed under conditions with high NaCl concentrations, where Δ*slr1928-31*-Kan cultures seemingly experienced less stress than WT cultures. The Δ*slr1928-31*-Kan strain is assumed to be less sensitive to high NaCl concentrations, a trait accompanied by continued growth in spite of NaCl in the medium. A defect in NaCl-sensing mechanisms may prevent the normal stress response, in which cultures are acclimatized to high NaCl concentrations. Other studies support this assessment, as they indicate that other pilin-like proteins in *Synechocystis* 6803 most likely have a function in stress responses (Singh et al, 2005). These experiments suggest that growth under stress may serve as a framework in which the function of the operon *slr1928-31* gene products should be investigated. In this regard, other stress conditions may also be considered for further studies of the operon *slr1928-31,* such as manganese (Mn) depletion and oxidative stress.

According to the results of these experiments, the pilin-like proteins encoded by the operon *slr1928-31* did not appear to be directly involved in iron acquisition. Still, these pilins might possess some more indirect function in iron uptake or utilization, possibly in connection with their suggested role in stress responses. The up-regulation of some pilin-encoding genes when iron becomes less accessible might be explained by a potential regulatory role of the pilins they encode, initiated in response to iron limitations. These pilins might in turn contribute to regulation of pilins or other proteins in order to increase the efforts in iron acquisition. Hence, the pilins encoded by the operon *slr1928-31* could have a function in counter-acting iron limitations by enhancing the synthesis of other pilins in order to obtain more iron from less accessible iron sources. Due to time constraints only a single growth assay was completed. Performing the growth experiment several times could give a firmer indication as to what functions these pilins could have. There also exist other pilins in *Synechocystis* 6803 that could be interesting targets in a similar deletion study. One such gene is *slr1120,* a potentially pil-related gene whose product appears to be an enzyme involved in motility as well as being essential for normal growth of *Synechocystis* 6803 (Yoshihara *et al*., 2001).

In a wider perspective, research on *Synechocystis* 6803 may prove valuable in the future. The field of renewable energy is growing, and development of sustainable technology requires more knowledge and new approaches. An example where electrically conductive pili come into play is in microbial fuel cells, producing electricity from renewable energy sources (Reguera, G. *et al*., 2006). Reguera and colleagues demonstrated that *Geobacter sulfurreducens* can donate e^- through pili to a

fuel cell electrode serving as an external e^- acceptor. Microbiology and molecular genetics could be promising areas in this regard, by utilizing the abundance of metabolic functions that exist in bacteria as well as the possibilities of genetic manipulation for specific purposes.

5. Conclusions

The aim of this project was to investigate how the gene products of a specific operon from *Synechocystis* 6803 were involved in iron acquisition. The operon selected consisted of the four genes, *slr1928, slr1929, slr1930* and slr*1931 (slr1928-31),* all hypothesized to encode pilin-like proteins (Yoshihara *et al*., 2001). Based on earlier research, other pilin-like proteins in *Synechocystis* 6803 are hypothesized to have a role in iron reduction and uptake (Gorby *et al*., 2006; Singh *et al*., 2003).

The growth assay showed few differences in growth between WT and Δ *slr1928-31*-Kan cultures on media without NaCl but generally better growth for the mutant strain on media with NaCl and NaCl with glucose. Based on these findings, the operon *slr1928-31* did not appear to have any direct function in iron acquisition but rather a function in salt sensory mechanisms and/or salt acclimation. Presumably, eliminating this operon reduced the cell's sensitivity to stress induced by NaCl so that the cultures continued to grow despite the increasing NaCl concentrations. This implied that a higher level of stress was required for the cells to initiate the appropriate responses. The stress levels were probably sufficient to initiate a stress response in Δ *slr1928-31*-Kan samples on medium with NaCl, glucose and DCMU but not on media with NaCl only or with NaCl and glucose. These results corresponded with those of whole cell absorption spectroscopy and 77 K fluorescence spectroscopy: The main differences between WT and Δ *slr1928-31*-Kan strains in the absorption spectra were seen in medium with NaCl or with both NaCl and glucose, for which WT cultures on FeCl for the former condition and WT on Fac, FeCl and Hexacyan on the latter condition, had insufficient cell densities for spectroscopic analysis. Otherwise the differences occurred mainly between the iron sources, suggesting that Goe was the least accessible iron source under most conditions. In addition, the relative availability of the iron sources tested seemed to be reversed with addition of NaCl to the medium. Iron from the water-soluble sources was possibly precipitated, resulting in better growth on the water-insoluble compounds in the presence of NaCl.

It has previously been demonstrated that the operon *slr1928-31* is not among the secreted proteins in *Synechocystis* 6803 (Sergeyenko and Los, 2000) and thus not found in the extracellular pilus structure. As these pilins are more likely part of the internal pilus machinery, they may have a role in coupling the pili to other cellular functions, such as stress sensing in this case. One possibility is that these pilins have a regulatory role, initiated in response to stress such as iron limitation or high NaCl concentrations. The gene products of this operon could be involved in regulating e^- donation through pili, possibly linking disposal of e^- to salt acclimation, and contribute in a system initiating

cyclic electron transport to drive salt stress responses. Deletion of the operon *slr1928-31* thus disrupts the process, interfering with the ability to sense salt and/or initiate the stress response.

In summary, the results of this study suggest that the pilin-like proteins encoded by the operon *slr1928-31* might be involved in sensory mechanisms and/or responses to stress, such as high NaCl concentrations in the growth medium. They could also have a regulatory role in iron acquisition, possibly initiated in response to iron limitations or other stress factors. These pilins might in turn contribute to regulation of other pilins or proteins in order to increase the efforts in iron acquisition. These experiments suggest that growth under stress may serve as a framework in which the function of the operon *slr1928-31* gene products should be further investigated. These results are only indications as to what functions this operon possesses under a limited set of conditions. The actual involvement of the operon *slr1928-31* gene products in iron acquisition or stress responses remain to be established through more research on this matter.

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